RESUILS AND DISCUSSION

III-1. Physical and Characterizations of the Studied Samples

III-1-1. Mechanical analysis

The data of mechanical analysis of the target area, first location (Cairo-Alex Road), second location (Toushky), and third location (Dikirnis, El-Mansoura) were studied where the particle size fraction is a primary physical property. The data are shown in Table 6. It is clear from the data that:

1-Coarse sand 1.12 – 7.04% except samples no.12 and 13 (46.74 and 51.93% respectively).

2- Fine sand 26.98 - 39.05% except sample no.13 (12.94%).

3- Clay 30.12 - 39.52% except samples no.12 and 13 (8.28 and 17.81% respectively).

4- Silt 17.72 - 32.51% except sample no.12 (8.72 %).

The studied samples consist mainly of fine sand, clay, and silt since they were the predominant size fraction for almost all the samples except sample no.12 (Toushky) at the second location and sample no.13 (Dikirnis, El-Mansoura) at the third location, where the coarse sand is the predominate size and representing 46.74% and 51.93% respectively, whereas fine sand came the second fraction for sample no.12 only (36.26), while clay and silt takes place by approach ratio to complete the soil composition fraction (8.28 and 8.72% respectively). The obtained results illustrate that most samples have approximate values for road surface and side surface, also, the subsurface samples show similar values. The coarse sand represented the predominant size fraction in sample no.12 and 13 to approach nearly fifty percent of the soil composition. From Table 6, we can see that the main constituent of the soil distributed through the fine sand, clay, and silt which approach percentage along the road surface, side surface, and road depth except samples no.12 and 13. Referring to the obtained results, we can find that the highest values of coarse sand for all the studied samples are find in the second and third locations (samples no.12 and 13) which have values equal to 46.74 and 51.93%, respectively in spit of the completely difference between their environments. This is due to that they have Loamy sand and Sandy loam texture class, respectively; whereas, all the other samples are Loam or Clay loam, where coarse sand ranging from 1.12% (sample no.7) to 7.04 % (sample no.8).

Table 6: Mechanical analysis of the samples.

Sample number	C. Sand, %	F. Sand, %	Clay, %	Silt, %	Texture class
1	2.37	31.21	35.09	31.33	Clay loam
2	1.74	33.68	38.56	26.02	Loam
3	5.16	31.05	39.52	24.27	Loam
4	4.47	39.05	35.34	21.14	Loam
5	3.67	32.19	33.42	30.72	Clay loam
6	4.95	28.71	35.11	31.23	Clay loam
7	1.12	35.19	33.19	30.50	Clay loam
8	7.04	26.98	34.26	31.72	Clay loam
9	3.06	37.45	30.12	29.37	Clay loam
10	4.54	30.50	32.45	32.51	Clay loam
11	6.12	30.40	32.14	31.34	Clay loam
12	46.74	36.26	8.28	8.72	Loamy sand
13	51.93	12.94	17.81	17.32	Sandy loam
14	6.19	32.14	36.52	25.15	Loam
15	1.82	34.49	32.19	31.50	Clay loam
16	2.77	32.51	34.19	30.53	Clay loam
17	3.56	31.62	33.47	31.35	Clay loam

C. Sand: Coarse sand, F. Sand: Fine sand.

III-1-2. Textural class of the studied soil

Textural is physically called soil separates and it is an important soil characteristic. Summing up the size fraction distribution in the soil of the three locations we found that the texture tends to be Loam and Clay loam in all the tested soil samples as they have approach values for their fractions: C-sand, F-sand, clay, and silt except samples no.12 and 13, which represent a Loamy sand and Sandy loam textural class, respectively, this is due to the highest value of coarse sand for both. Fig. 8 shows the textural class for each sample in the studied locations represented in the triangle composition of textural class of the soil used by the United States Soil Survey. Coarse textured soil exhibits higher bulk density values than do finer textures soils where sand and Sandy loam soil may vary in bulk density from 1.2 to 1.8 g/cc. This is confirmed with the data obtained in our result for the bulk density of samples no.12 and 13 as listed in Table 7.

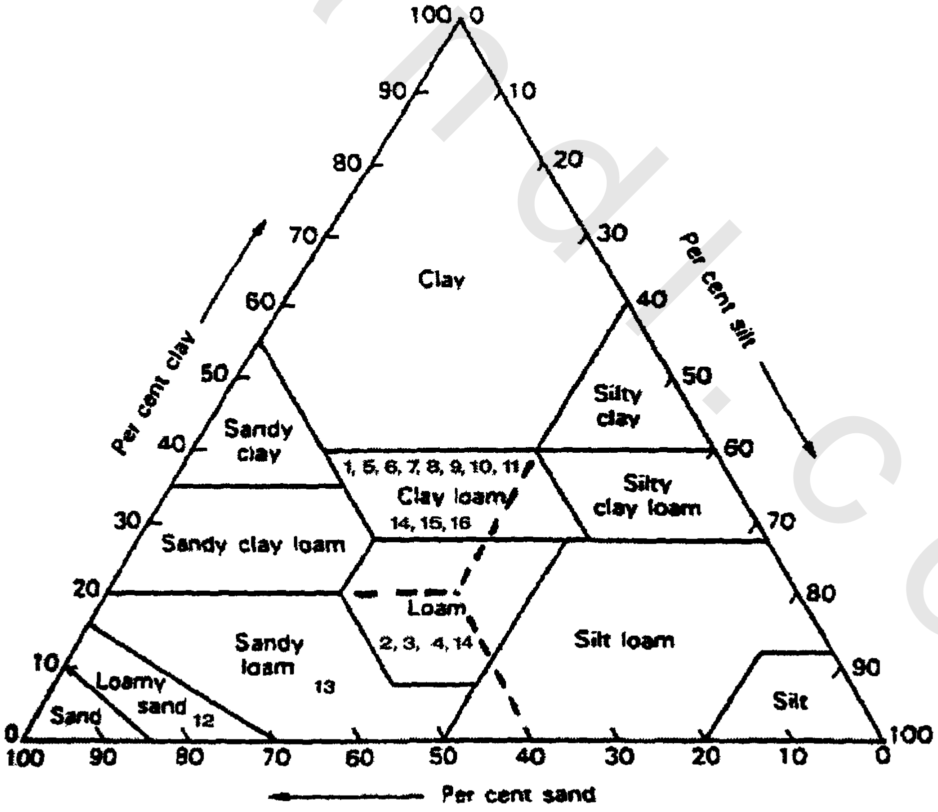


Fig. 8: The compositions of texture classes of the studied samples.

III-1-3. Moisture content and loss upon ignition

Water, a renewable resource, belongs to hydrological cycle. It continuously moves at the earth's surface and evaporates from the earth into atmosphere. After reaching the atmosphere, water is moved by the wind until it finally falls to the ground again as some kind of precipitation. In the cycle, part of this water on the ground is evaporated directly from the soil, or runs off the surface into streams, lakes, swamps, and oceans. Another part is adsorbed by the soil and this part representing the moisture of the soil, or percolated through the soil to reach the ground water and springs.

The data tabulated in Table 7 show that in the first location the moisture content of the samples ranging from 12.25% in sample no.11 to 24.58% in sample no.1, and this variation is depending on some factors, the most important are the rate of evaporation, the soil texture and amount of rains or the irrigation. By making correlation of the moisture content of the samples of second and third stages (20 and 30 km), we find out that the moisture content of the surface samples of the two stages (samples no.3, 4, 7, and 8) have moisture content values equal to (16.20%, 15.73%, 13.99%, and 14.47%) respectively, which are lower than the values of the depth samples (samples no.5, 6, 9, and 10) with moisture content values equal to (18.03%, 18.06%, 23.91%, and 23.13%) respectively, of the same location. This decreasing in the moisture content from the surface to the depth samples can be attributed to the difference in the rate of evaporation **(Coleman and Hendrix, 1949)**, since this rate is higher in the surface than in the depth, and also it can be explained by that part of this water is percolated through soil to the ground water, but it can't be explained by the difference in the texture, since all the samples have only two textures (Loam and Clay loam). The samples of the first stage (samples no.1 and 2) at 10 km have close moisture content values (24.58% and 22.25%), and it is higher than that of the other samples in this location, this is attributed to that the location was lately irrigated.

A sharp decrease was observed for the second location (Toushky), where the moisture content registed the lowest value (1.5%) in all the studied samples, this backs to the texture of this location (Loamy sand) which have a value nearly approach to fifty percent from the soil composition as coarse sand leading to high permeable soil **(Struthers et al., 2007)** where water downward to the ground water. Also, this can be explained by the higher rate of evaporation than the other locations due to the high temperature of this area.

In the third location, sample no.13 has moisture content value equals to 16.32%, and also the other samples of this location have moisture content values ranging from 16.53 in sample no.14 to 18.25 in sample no.17 and it is almost the same range of the surface samples of second and third stages in the first location. In general, we can say that the moisture content registed a notice decrease for the side surface with increasing the distance (10-30 km), since 2 > 4 > 8 in moisture content values. Also road surface samples decreased with the same parameter (distance) along the first location since 1 > 3 > 7 in moisture content values.

The results of loss upon ignition (Table 7) illustrate that all the investigated samples have an approximate similar values except samples no.12 and 13 which tend to show somewhat the lowest and highest values, respectively comparing to the whole values of loss upon ignition of all samples which ranging from 10.52 (sample no.12) to 16.22 (sample no.13). This backs to that the total hydrocarbon registed the lowest value for sample no.12 and highest value for sample no.13 compared to the all studied samples.

III-1-4. Bulk density

Bulk densities are important factors in soil studies. A soil in excellent conditions for plant growth should has a bulk density that is not low for adequate support, and also not to high for proper porosity and aeratation. Most mineral soils have bulk densities between 1.0 and 2.0 g/cc where soil of bulk density values (1-1.5 g/cc) generally indicate favorable physical condition for soil plant growth.

The bulk density of the tested samples ranging from (1.11 to 1.27 g/cc) for samples no.3 and no.13, respectively as listed in Table 7. We can notice that the surface samples (samples no.1, 2, 3, 4, 7, and 8) at the first location are almost lower in their bulk density values from the subsurface samples (samples no.5, 6, and

10) except sample no.9. The increase in the bulk density of the samples by depth is due to the higher content of the organic matter in the surface samples, and as the organic matter content increases the bulk density of the surface decreases because the volume of the samples which is supposed to be occupied by the soil particles is then occupied by the organic matter particles which are lighter in weight, so decrease the bulk density values **(Jullien and Francois, 2006).** Also, compaction is another factor leading to increase the bulk density of the samples where it is increase by depth and its effect makes the soil more compacted and leads to decrease in their air volume, which in turn leading to the increase in the sample weight, so as a result, increase in the bulk density values.

Table 7: Moisture content, loss upon ignition and bulk density of the studied samples.

Sample number	Moisture content,%	Loss upon ignition,%	Bulk density (g/cc)
1	24.58	11.97	1.12
2	22.25	12.11	1.14
3	16.20	11.24	1.11
4	15.73	11.79	1.16
5	18.03	12.38	1.18
6	18.06	12.34	1.19
7	13.99	12.54	1.15
8	14.47	13.37	1.15
9	23.91	12.78	1.14
10	23.13	12.85	1.20
11	12.25	13.46	1.21
12	1.5	10.52	1.22
13	16.32	16.22	1.27
14	16.53	13.45	1.21
15	17.52	12.58	1.15
16	17.65	11.62	1.18
17	18.25	11.82	1.16

III-2. Chemical and Characterization of the Studied Samples

III-2-1. Organic matter

Organic amendments are currently used in agricultural soils to improve their physical and chemical properties because soil degradation in Mediterranean countries is a serious problem in natural and agricultural environments. Degrading processes can reduce the quality of the soil physical, chemical and biological properties, and decrease the soil's capacity to produce biomass. Soil degradation mainly involves destruction of soil structure. Many of these processes are due to losses of soil organic matter by intensive agricultural practices, and sometimes are related to a high content of sodium in the soil due to irrigation with highly saline water, as sodium, which can restrict soil particle aggregation. Low organic matter content commonly occurs in many abandoned agricultural soils of Mediterranean area, and this is clear from the low content of the organic matter of the samples used in this study.

By looking to the results listed in Table 8 we can find that in the first location at 10 km stage, sample no.1 registed the highest value of organic matter for all the studied samples (3.4 %) also at 20 km stage in the same location samples no.3 and 4 contain high content of organic matter (2.4% and 2.6%) than the depth samples of the same area (samples no.5 and no.6) which contain (1.4% and 1.2%), respectively. This increase may be due to that the organic matter is derived from living organisms which, partly decomposed as well as decomposed plant and animal residue besides the biosolid amendments **(Garcý´a-Orenes et al., 2005)** where all these components are usually higher in the surface soils than the subsurface or depth ones **(Oades, 1993)**. At the third stage (30km), it was found that this area as general contains a lower content of organic matter (1.5%, 0.8%, 0.9% and 1.3% respectively) for samples no 7, 8, 9 and 10, if it compared to the other stages. Also, sample no.11 contains 1.8% organic matter since it is almost far about only 2 km from the third stage. While sample no.12 contains slightly higher content of organic matter (1.9%) and this is may be due that it is a young area (second location-Toushky) and needed a larger amounts of amendment that may be added to that location to increase its fertility and to improve soil physical and chemical properties. The third location (Dikirnis) shows somewhere a high value of organic matter this may be referred to that this location was contaminant by crude oil, rich with hydrocarbons that leading to increase the organic matter content. So, we can say that the values of the organic matter content show clearly that the locations of the present study are poor in organic matter. Moreover, the surface samples in all locations showed relatively higher values than the subsurface samples.

Table 8: Some chemical properties of the studied samples.

Sample number	CEC (meq/100g soil)	pH	EC (dc/m)	O.M (%)
1	22.0	8.24	2.86	3.4
2	31.0	8.33	2.52	1.9
3	32.5	7.92	1.55	2.4
4	26.0	7.75	1.85	2.6
5	23.1	7.33	1.70	1.4
6	25.7	7.35	2.40	1.2
7	27.4	7.38	0.98	1.5
8	31.8	7.41	0.77	0.8
9	24.3	7.55	0.86	0.9
10	31.8	7.58	1.24	1.3
11	12.2	7.98	1.34	1.8
12	24.0	7.05	2.15	1.9
13	19.8	7.12	0.68	2.9
14	28.0	7.24	1.83	2.2
15	30.5	7.72	1.52	1.60
16	32.1	7.62	1.65	2.0
17	31.7	7.48	1.71	1.8

CEC: Cation exchange capacity, EC: Electrical conductivity, O.M: Organic matter.

III-2-2. Electrical conductivity

Three pathways of current flow contribute the electrical conductivity of the soils, they are: a) Liquid phase pathway via dissolved solid contained in the soil occupying the large pore, b) Solid-liquid phase pathway primarily via exchangeable cation associated with clay minerals and c) Solid phase pathway via soil particles that are in direct and continuous contact with one another. Because of these three pathways of conductance the electrical conductivity measurement is influenced by some soil physical and chemical properties like soil salinity, saturation percentage, water content, and bulk density.

Looking to the obtained results of the electrical conductivity of the samples under this study (Table 8), it is clear that in the first location the electrical conductivity is inversely proportional with the distance along the three stages of this location through the road surface, side surface, road depth, and side depth where 2.86 > 1.55 > 0.98 dc/m along 10, 20, and 30 km, respectively for the road surface of samples no.1, 3, and 7. Also, in the side surface samples 2, 4, and 8 we find that 2.52 > 1.85 > 0.77 dc/m along 10, 20, and 30 km. The same trends are obvious for road depth samples 5 and 9 where 1.70 > 0.86 dc/m and latterly side depth samples 6 and 10 where 2.40 > 1.70 dc/m. Making a spot light on the electrical conductivity of the all studied samples we can find that it is affected by the concentrations of the soluble cations and anions as shown in Table 9 **(Corwin and Lesch, 2005)**. The EC is increasing by the increasing in the concentration of the soluble cations and anions and vise versa. For example, in sample no.13 the concentrations of (Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-, HCO_3^- and SO_4^{-2}) were (2.6, 0.7, 2.0, 3.0, 1.3, 5.0 and 2.0 meq/L), respectively; this sample has an EC value equals 0.68 dc/m, and this value represents the lowest value for the soluble anions and cations in side, and the lowest value of EC in the other side. While in sample no.1 the concentrations of (Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-, HCO_3^-, and SO_4^{-2}) were (17.6, 0.9, 7.0, 5.4, 13.6, 5.0, and 12.3 meq/L), respectively, it has an EC value equal 2.86 dc/m, which represents the highest values for the soluble anions and cations, and the maximum value of EC in the studied samples. Also in sample no.5, when the concentrations of (Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-, HCO_3^-, and SO_4^{-2}), were decreased to (14.2, 0.8, 1.0, 1.2, 9.2, 2.1, and 5.9 meq/L), respectively, the EC value decreased also from 2.86 to 1.70 dc/m.

The results also shown that there is no significance effect of the depth on the EC relative to the other samples and there is no sharp difference between the surface and the depth samples. Temperature is another factor influencing EC where electrolytic conductivity increases by a rate of approximately 1.9 % per °C increase in temperature **(U.S. Salinity Laboratory Staff, 1954)** which explains the jump of EC value of sample no.11 (protected area) since the sample was collected from a greenhouse. So customarily, EC is expressed as a reference temperature therefore soil EC has become one of the most reliable and frequently used for purpose of comparison and measurements to characterize field variability for application to precision agriculture due to its ease of measurement and reliability, also, soil salinity is quantified in term of the total concentration of soluble salt and can be indicated by EC **(Corwin and Lesch, 2005)**.

III-2- 3. Soil pH

Soil pH influences plant growth by its effect on activity of beneficial microorganisms. Bacteria that decomposed soil organic matter, releasing nitrogen and other nutrients values for plant use, are hindered by strong acidity. Fungi usually tolerate strong acidity better that do other microbes. Because of the above the soil pH may be the most important soil chemical property **(Jackson, 1969)**.

The importance of soil pH is arrives from the fact that it is the main factor controlling the solubility of heavy metal in soils. It has been proved that each unit of increase in the pH results in approximately a 2-fold decrease of concentration of metal such as Zn, Ni, and Cd in the soil solution **(Bolan and Duraisamy, 2003)**.

Contour maps of soluble Cd, Cu, As, Pb, and Zn show that the concentrations of these metals in the acid zone (pH 2-5) were much higher than those found in natural zone (pH 6-8) **(Burgos et al., 2006)**.

The obtained data from Table 8 show that the pH values of the first location are ranging between 7.33 for sample no.5 and 8.33 for sample no.2. According to Table 3, samples no.1, 2, 3 and 11 are described as moderately alkaline. Samples no.4, 8, 9, and 10 are described as slightly alkaline, while samples no.5, 6, and 7 are described as neutral. The sample of second location (no.12) is described as neutral, also the samples no.13 and 14 (third location) are described as neutral, while samples no. 15, 16, and 17 are described as slightly alkaline. So, we can conclude that there is a slightly heterogeneity of pH in the studied samples, since acidic water accumulated in surface bubbles could have contributed to the heterogeneity of pH and trace elements distribution **(Bolan and Duraisamy, 2003).**

III-2-4. Cation exchange capacity

The cation exchange capacity is the quantitative measurement of all cation adsorbed on the surface of the soil colloids. Adsorption and cation exchange are of great practical significance in soil physics, soil chemistry, soil fertility, nutrient in soil, and nutrient uptake by plant.

The dependence of CEC in cation exchange reaction, in a particular exchangeable H^+, the proton H^+ is present as -OH group on the surface plains of the minerals as in the case of kaolinite and it is then called *exposed hydroxyl group.* These -OH groups dissociate their proton at high soil pH values, they are the sources of variable negative charge. So, as the clay content increases, the exposed hydroxide group increases, the negative charge increases, which in turn leading to increase in the CEC **(Tan, 2003).**

The obtained data (Table 8) of the cation exchange capacity of the first location decreasing from road to side road samples in the first and second stages (10 and 20 km) while the reverse trend is shown in the third stage. On the other side, the cation exchange capacity registed high value for the road depth than that the side depth samples (no.5 and 6) at 20 km and samples no.9 and 10 at 30 km stage. The second location (Toushky), has the lowest CEC (12.2 meq/100 g soil) comparing to all the studied samples. The samples of the third location have values approach to those of the first location except sample no.13 which has lightly smaller value. The CEC values at the third location show that the soil surface samples, almost, have high values, where the highest one is found for sample no.16 (32.1 meq/100 g soil).

Regarding to the obtained results (Tables 6 and 8) we can find that CEC values are closely tie with the clay content where sample no.3 has the highest value of both CEC and clay percentage, also, the lowest value of CEC possesses the lowest percentage of clay content. The correlation between CEC and clay content along all the studied samples is shown in Fig. 9.

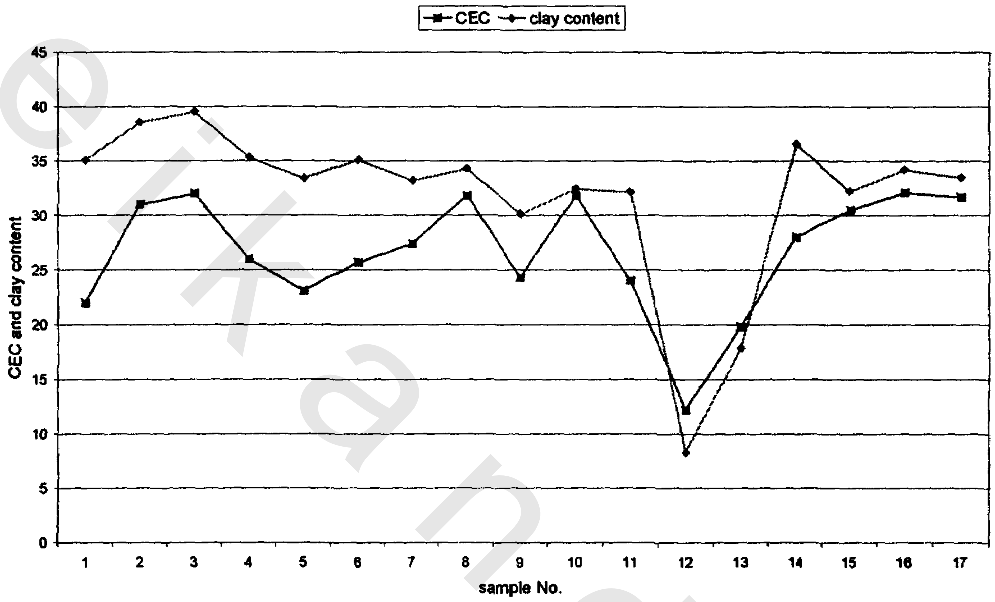


Fig. 9: Relation between CEC and clay content of the samples.

III-2-5. Soluble cations and anions

The concentration of salts in the soil profile is increased as water is removed from the soil via evaporation and transpiration. Probably the most important principle to be understood is that soluble salts in soil are transported by water. Soluble salts increase or decrease in the plant root zone, depending upon whether or not the net downward movement of salt is less or greater than the net salt input from the irrigation water source. The salt balance in the soil, as affected by the quantity and quality of the irrigation water and the effectiveness of drainage and leaching, are the key to managing saline or alkaline soils with irrigation practices. The most important soluble cations and anions in soil are (e.g., Na^+, K^+, Mg^{+2}, Ca^{+2}, Cl^-, HCO_3^-, and SO_4^{-2}).

Sodium is not considered an essential nutrient for plant growth. In the fertilizer industry it is called as *soda.* The dissolution of Na attributed to hydrolysis reaction, which is considered as a weathering reaction. The chemical reaction can be represented as follow:

$$NaAlSi_3O_8 + H_2O \longrightarrow HAlSi_3O_8 + NaOH$$

The data in Table 9 represent the soluble cations and anions of the studied samples. The results show that at the first location, the soluble Na in the samples ranging from 18.2 meq/L, in sample no.6, to 1.9 meq/L, in sample no.10. The samples of 10 and 20 km stages (samples no.1, 2, 3, 4, 5, and 6) are generally characterized by higher content of soluble Na than the samples of the 30 km stage (no.7, 8, 9, and 10) which are characterized by lower content of soluble Na. So, we can conclude that the samples of 10 and 20 km stages are more saline than the samples of 30 km where Na represents the dominate cation, Ca and Mg came in the second rank while K takes the last situation. These results are confirmed by the values of the EC, which was higher in 10 and 20 km stage samples than the samples of 30 km stage. It is also evident that the depth has no essential effect on the soluble Na since that there is no sharp differences or essential trend between the surface and depth samples.

In general, high soluble Na contents are very harmful to the physical condition of the soil, besides, sodium tends to disperse the soil colloids, a dispersed soil is puddle when wet and hard when dry, which is leading to decreasing in the pore spaces **(Deenik and Yost, 2006).**

Potassium is one of the most essential macronutrient elements in the plant growth. In the fertilizer industry, this element is, also, called *soda.* The dissolution of K from aluminum silicates is attributed to hydrolysis reaction, which is a weathering reaction. The chemical reaction of *soda* can be represented as follow:

$$KAlSi_3O_8 + H_2O \longrightarrow HAlSi_3O_8 + KOH$$

The KOH dissolves in water and releases potassium in the form of K^+. The concentration of soluble potassium in all the studied samples ranging from 0.3 to 1.0 meq/L, except for samples no.11 and 12 which contain a higher values of soluble K^+ (2.1and1.4 meq/L) respectively. And this was expected, since sample no.12 from a young area and it is needed more potassium and also for sample no.11 which is followed to a protected area and it is expected to contain more potassium that was added to the soil as a fertilizer.

Consequently, the significance of potassium in the soil is explained by its adsorption by plant from the soluble potassium forms and also the exchangeable forms. It is believed that their contents are controlled by certain equilibrium between soluble K, exchangeable K, and fixed K. Most of these potassium forms are taken up in large amounts by the growing plants and it is perhaps the most abundant cation in the plant tissues.

Magnesium is another macronutrient that is present in the soils mostly in the inorganic form. The primary minerals containing Mg are dolomite, Mg silicate and Mg phosphates. Dolomite $CaMg(CO_3)_2$, which is a major constituent of dolomitic limestone and is considered the most common source of Mg in soils. The dissolution of dolomitic limestone is affected by CO_2 in the soil water, where the chemical reaction can be represented as follow:

$$CaMg(CO_3)_2 + H_2CO_3 \longrightarrow Ca^{2+} + Mg^{2+} + 4HCO_3^-$$

The represented data show that the values of the soluble Mg^{2+} in the soil are ranging from 1.0 to 7.0 meq/L, in samples no.3 and/or 5 and 1, respectively. The samples of the 10 km stage (samples no.1 and 2) are characterized by a higher content of soluble Mg^{2+} (7.0 and 6.2 meq/L), as in the case of the other soluble cations Na^+ and Ca^{+2} this may be in the first stage that Mg^{2+} was released in soil by high amount **(Tan, 1994)**. The samples of the 20 km stage show no difference in the soluble Mg^{2+} with depth since the values of the soluble Mg^{2+} of samples no.3 and 5 have the same value of the soluble Mg^{2+} (1 meq/L) and also there is no significance difference of the samples no.4 and 6, they have almost the same value of the soluble Mg^{2+} (3.2 and 3 meq/L), respectively.

Also, the samples of the 30km stage show no significance differences in their values except sample no.10, which is characterized by low content of soluble Mg^{2+} (1.1 meq/L) as in the case of the other cations of these samples area. As whole, magnesium displays different trends as regards to the studied locations.

Calcium is found in soil in inorganic form, which represent the most significant form, though scientifically all the organic matter contains Ca. So, the organic calcium is considered of minor importance in soils, and the major source of Ca in soil is considered to be of inorganic, such as the primary minerals in limestone and basic rocks. The primary mineral sources of Ca are calcite, aragonite and dolomite where calcite is the major constituent of limestone, calcareous marls and calcareous sandstone.

Calcium carbonate is insoluble in water, but will dissolve in water containing CO_2, a process called carbonation, which is a weathering process, where the chemical reaction can be represented as follow:

$$H_2O + CO_2 \longrightarrow H_2CO_3$$

$$H_2CO_3 + CaCO_3 \longrightarrow Ca(HCO_3)_2$$

The calcium bicarbonate is soluble in water, and release the Ca^{2+} ion which is an important plant nutrient.

The soluble Ca^{2+} in the present samples, are ranging from 5.6 to 1.2 meq/L, in samples no.2 and 5, respectively. The samples of the first stage (10 km) is characterized by a high content of the soluble Ca^{2+} (5.4 and 5.6 meq/L) for samples no.1 and 2 and this results are confirmed by the pH values of this two samples since they have the highest pH values 8.24 and 8.33, respectively; this may be explained as a result of the addition of Ca^{2+} sources in order to increase the pH of the soil **(Tan, 1994)**. The samples of the 30 km stage are characterized by that the surface samples (no.7 and 8) have higher values of soluble Ca^{2+} (3.6 and 2.8 meq/L) than the subsurface samples of the same stage (no.9 and 10), which have lower values of soluble Ca^{2+} (2.5 and 2.3 meq/L). This is also found in the road samples of the 20 km (second stage) in which the surface sample (no.3) has a value of 3.2 which is higher than the value of the subsurface sample (no.5) which has a value equal 1.2 meq/L, this is not found in side surface sample of the same location (sample no.4). For the second location, calcium comes in the second order in the soluble cations constituent, as well as in the third location, where calcium varied in different values of their samples but still in the second cations order.

Table 9: Soluble cations and Soluble anions, meq/L.

Sample number	Soluble cations				Soluble anions		
	Na^+	K^+	Mg^{+2}	Ca^{+2}	Cl^-	HCO_3^-	SO_4^{-2}
1	17.6	0.9	7.0	5.4	13.6	5.0	12.3
2	15.3	0.6	6.2	5.6	12.4	4.8	6.2
3	10.3	1.0	1.0	3.2	5.0	6.2	1.0
4	10.4	0.9	3.2	4.0	7.2	6.5	3.2
5	14.2	0.8	1.0	1.2	9.2	2.1	5.9
6	18.2	0.3	3.0	4.7	18.6	3.3	3.0
7	3.1	0.8	2.3	3.6	1.8	3.2	2.3
8	2.1	0.8	2.0	2.8	1.3	4.3	2.0
9	3.2	0.8	2.1	2.5	1.2	3.8	2.1
10	1.9	0.5	1.1	2.3	1.0	2.8	1.1
11	4.3	2.1	2.0	4.1	7.2	2.8	2.0
12	6.2	1.4	3.3	2.5	3.8	5.2	3.3
13	2.6	0.7	2.0	3.0	1.3	5.0	2.0
14	14.3	0.8	3.7	4.7	12.4	4.6	3.7
15	10.3	1.0	1.1	3.2	5.0	6.2	1.0
16	10.4	0.9	3.2	4.0	7.2	6.5	3.2
17	14.2	0.8	1.5	1.8	9.2	2.1	1.5

III-2-6. Heavy metals

The obtained data of the studied heavy metals are included in Table 10 where Fe represents major element and the other elements Mn, Zn, Cd, Co, Ni, Pb, Cr, and Cu, represent the minor minerals of soil.

III-2-6-1. Iron

Iron represents the predominant heavy metal in our studied locations samples. It is an important element in the soil, and it is also one of the most abundant elements in the soil. The most important minerals of iron in the soils are *hematite*, Fe_2O_3, and *magnetite*, Fe_3O_4. The hydrated form of *hematite* is often called *limonite*, $2Fe_2O_3.3H_2O$. *Hematite* is red in color and its presence gives the soil its red color, and this is explaining the slightly red color of the first location (samples no.1 to 11), which contain Fe with concentration ranging from 4200.9ppm in sample no.11 to 4324.8ppm in sample no.9. While the highly redish color of sample no.13 (third location) was characterized by a very high content of Fe (25320.0ppm) in all the studied samples.

The data shows that there is no significance trend of the Fe in the road and side road samples, in the first location, where iron concentration has not sharp increase or decrease and it was fluctuated between road, side road, and depth along 32 km. Also, we can notice that at 10 km the road surface sample (no.1) contains higher content of Fe (4296.1ppm) than the side surface (sample no.2), which contains 4278.6ppm of Fe, this is not found in the samples at 20 km stage, since the road surface sample (no.3) contains 4278.6ppm, which is lower than the Fe content of the side surface sample (no.4), which is equal 4284.6ppm. At 30 km stage, we can notice that there no difference between the road and side surface samples (no.7 and 8) that the Fe content of the two samples is almost equal (4310.8 and 4309.8ppm), respectively. On the other side, the protected area is registed the lowest concentration for the first location and also for Fe concentration for the tested location samples. Subsurface samples show no significance trend, since the road depth sample at 20 km (sample no.5) contains iron concentration (4257.9ppm) lower than that of the side depth sample no.6 which contains (4305.0ppm), while the reverse was found in the samples of the third location, since the road depth sample no.9

contains (4324.8ppm) which is higher in Fe content than the side depth sample no.10 which contains (4283.9ppm). At the second location the Fe content equals to 4232.4ppm, while at the third location, iron content decrease drastically along 50 km ranging from 25320.0 to 4276.0ppm in sample no.13 (highly polluted area) and sample no.17 (fare away the polluted area), respectively. This may be due to the contaminance of soil in this region by sludge and crude oil which help in the presence of anaerobic and aerobic condition which favorite the releasing of more Fe^{2+} and Fe^{3+} in the soil **(Caravaca and Rolda'n, 2003).**

III-2-6-2. Manganese

Mn represents the second order of heavy metal concentration in the studied samples. The Mn concentrations of the samples have a general trend, that the road surface samples in the first location have lower values than the side surface, side depth, and road depth samples along the location distance. In the first stage (10 km), we find that the road surface sample (no.1) has a value equals to 337.1ppm, which is lower than the value of the side surface sample (no.2) which has a value equals to (349.5ppm). In the second stage (20 km), the road surface sample represents lower values of Mn content than the side surface and road depth, side depth samples where sample no.3 has Mn value equals to 334.2ppm, which is lower than the value of sample no.4 (side surface), which has a value equals to 359.4ppm; also, the sample no.5 (road depth) and sample no.6 (side depth) which have 718.5 and 767.0ppm, respectively. Sample no.7 in the third stage (30 km) has the same trend, where the road surface sample has a value equals to 473.0ppm, which is lower than the value of the samples no.8 (582.1ppm), no.9 (582.3ppm) and no.10 (592.7ppm) for side surface, road depth, and side depth, respectively.

In other word, the samples of first location are characterized by the increasing in the concentrations of Mn with depth, since the surface samples in the road and side samples (no.3 and 4) have values lower than the values of the depth samples (no.5 and 6). Also the road and the side samples in the third stage of the same location (7 and 8) have lower values than the depth samples (no.9 and 10). From all above we can conclude that the Mn concentration is increasing by getting away from the road and also increasing by depth.

On the other side, Mn content in the second location represents the lowest value for all the studied samples (198.3ppm), while Mn content in the third location varied in a narrow range and it ranged between 437.0ppm and 420.0ppm for samples no.15 and17 respectively, except sample no.13 which has the highest value in this location. This may be due to that sample no.13 locates at the center of pollution present in this area. In general, we can say that the fluctuation of Mn in the different locations may back to different factors that affecting the Mn concentration in the soil such as sorption of Mn onto soils which can be facilitated by several mechanisms as the oxidation of Mn to higher valence oxides and/or precipitation of insoluble compounds in soils subjected to wetting and drying, besides, absorption into the crystal lattice of clay minerals, and adsorption on ex-change sites. In calcareous soils, chemisorption onto $CaCO_3$ and following precipitation of $MnCO_3$ may play an important role, adding to the presence of chelating agents is not able to form stable Mn complexes in soils because Fe or Ca can substitute for Mn.

III-2-6-3. Zinc

Adsorption of Zn^{2+} on soil is often coupled with proton release from the mineral surface as metal ions are adsorbed as:

$$\text{-SOH} + Zn^{2+} \rightleftharpoons \text{>SO-}Zn^{+} + H^{+}$$

Zinc is so closed with the pH of soil. During the adsorption experiments pH decreased ≡1.0 unit. This decrease in pH is indicative of the deprotonation process of mineral surface when H^+ ions are expelled form the surface as metal ions. The zinc is characterized by that it under neutral to acidic conditions, the organic ligands bind to surface metal atoms, forming active surface complexes, analogous to aqueous metal–organic complexation. At higher pH, the dissociation of organic acids increases, the adsorption of anions decreases, and therefore, the concentration of ligand increases, competing with soil surfaces for Zn **(Bradl, 2004).**

The increase in the pH when Zn is adsorbed and the variations in Zn(II) adsorption with ionic strength suggest that Zn exhibits a mix of inner and outer-sphere adsorption in the soil. This specific or inner-sphere adsorption is due to the strong irreversible bonding between metals and functional surface groups bound into the structure of a solid phase such as Fe and Mn oxides or organic matter. The outer-sphere or electrostatic adsorption is a reversible process that takes place on the clay fraction on the soil surface.

The results of Zn concentration in the studied samples relieve that the road surface and road depth are higher in their values than the side surface samples in the first two stages (10 and 20 km). Since in the first stage (10 km) the road surface sample (no.1) has a value equals to 93.2ppm, which is higher than the side surface value of the same stage (sample no.2), which has a value equals to 87.9ppm. Also in the second stage (20 km), the road surface samples and road depth (no.3 and 5) contain values equal to 93.6ppm and 96.5ppm, respectively, which are higher than the values of the side surface samples (no.4 and 6) which have a value equal to (80.4 and 45.5ppm), respectively.

In the third stage (30 km), we can notice that the reverse trend was found, that the samples of the road surface and road depth samples have a lower values than the side surface and side depth samples, since the road samples (no.7 and 9) have values equal 75.6 and 80.8ppm, respectively, which are lower than the values of the side samples (no.8 and 10) which contain 90.8 and 96.6ppm, respectively.

Whereas sample no.12 (second location) has a low value of Zn 41.4ppm and this is may be due to that it is a young soil and has a very low rate of contamination and the reverse is found in sample no.13 in the third location, which has the highest value of Zn between all the studied samples (580ppm); this is due to that it is a highly contaminated area. Also, sample no.11 in the first location has a somewhat high value of Zn (161.2ppm) this could be as a result of the addition of higher rate of fertilizers to that location **(Bradl, 2004).**

III-2-6-4. Cadmium

The occurrence of cadmium in natural soils is largely influenced by the amount of cadmium in the parent rock. The average cadmium concentration in soils derived from igneous rocks is reported to be in the range from <0.10 - 0.30ppm, while soils derived from sedimentary rocks contain 0.30 - 11ppm Cd **(Lorenz et al., 1997).** Adsorption is the main operating mechanism of Cd reaction at low concentrations with soils. Adsorption is a function of pH in case of Loamy sand and Sandy loam soils. The sorption capacity of the soil increases approximately three times per unit increase in pH. In addition to adsorption, precipitation can play an important role in controlling Cd levels in soils **(Lorenz et al., 1997).**

In general, Cd solubility in soils decreased as pH increased reaches the lowest values for calcareous soils (pH 8.4). The precipitation of $CdCO_3$ occurs in sandy soils with low CEC, low content in organic matter, and alkaline pH at high Cd concentrations. Precipitation occurs in general at higher Cd^{2+} activities while ion exchange predominates at lower Cd^{2+} activities. Studies of behavior of Cd^{2+} in the presence of $CaCO_3$ showed that initial chemisorption of Cd^{2+} on $CaCO_3$ was very rapid, while $CdCO_3$ precipitation.

The data show that the cadmium is not detected at the first and second location, which indicating the absence of cadmium sources along these locations along these areas. But in the third location trace values of Cd were detected in samples no.13 and 14 only (0.12ppm and 0.08ppm, respectively), which indicating the presence of source of contamination affected sample no.13. The presence of this trace Cd value along all the studied samples may be due to the highly pollution of this location with crud oil, and as we going far from this source the level decreased in sample no.14 and not detected in other samples of this location along 50 km.

III-2-6-5. Cobalt

Cobalt was found to accumulate in hydrous oxides of Fe and Mn in soils. It was also found that Co adsorption by certain soils was increased by removal of Fe, which is believed to expose clay mineral surfaces that were more reactive than previously exposed

Fe oxide surfaces. Co sorption capacity of soils was found to highly correlate with Co content and soil surface area and to be a lesser extent with Mn and clay contents and pH. Almost, all of the Co in soils could be accounted for by the presence of Mn minerals, which indicating that these minerals can be an important sink for Co in soil as found in our present study (sample no.13). Sorption of Co by Fe and Mn oxides as a function of pH has significant Co sorption only at pH values above 6.0. Two forms of bound Co have been identified, the first form, which is characterized as being slowly dissociable, seems to be bound in a monolayer by chemisorption and would exchange with Zn^{2+}, Cu^{2+}, or other ions, the second form of Co is not dissociable and is believed to either enter the crystal lattice or become occluded in the precipitates of another phase **(Rossini and Fernández, 2007)**.

The results of cobalt content of the first location indicating no significance difference between the road and side samples, since the samples no.1 and 2 at 10 km (first stage) have a cobalt content values equal to 20.4 and 21.9ppm, respectively, while the cobalt content in the second stage decreased from road to side samples, since the samples no.3 and 4 have cobalt content values equal to 28.8 and 27.4ppm, respectively. But, the reverse was found in the samples of the third stage (30 km) that the cobalt content increased from road to side samples, since the samples no.7 and 8 have cobalt content values equal to 26.3 and 30.1ppm, respectively. So, the cobalt content has no significance trend from road to side.

By studying the depth effect along the second stage, we can find out that the cobalt content is decreasing by depth (samples 5 and 6) and this behavior is achieved through the depth in the third stage only in sample no.9. The surface samples of the second and third stages (samples no.3 and 4, and 7) have cobalt contents equal to 28.8, 27.4, and 26.3ppm, respectively, and these values decreased in their corresponding depth samples (samples no.5, 6, and 9) to 25.5, 25.9, and 24.9ppm, respectively. While the reverse was found in sample no.8 (30.1ppm) and its corresponding depth sample (sample no.10) which contains 21.7ppm. The cobalt content of the protected area in the same location (sample no.11) has the approach value of the other samples. In the second location, cobalt content (8.10ppm) shows the lowest content against all the studied samples. The third station shows undetectable difference in the cobalt content between their samples, since the values ranging from 19.8ppm to 24.0ppm for samples no.13 and 15 respectively, which is indicating the same rate of contamination by cobalt along this location.

III-2-6-6. Nickel

Nickel is required to maintain health in animals. A small amount of nickel is probably essential for humans, although a lack of nickel has not been found to affect the health of humans. Exposure of the general population to nickel and its compounds results from breathing air, ingesting drinking water and food that contain nickel and its compounds. The most common adverse health effect of nickel in humans is an allergic reaction. Some people are sensitive to nickel when jewelry or other things containing it are in direct contact with the skin. Once a person is sensitized to nickel, further contact with it will produce a reaction. The most common reaction is a skin rash at the site of contact.

The U.S. Department of Health and Human Services has determined that nickel and certain nickel compounds may reasonably be anticipated to be carcinogenic. Cancers of the lung and nasal sinuses have resulted when workers breathed dust containing high levels of nickel compounds while working in nickel refineries or nickel processing plants.

Our data of the studied samples show that the values of nickel content in the surface samples of the first stage are 44.3 to 46.5ppm for samples no.1 and 2. So, it shows no significance difference from road to side. While that of the second stage (at 20km) were 48.8 and 33.0ppm for samples no.3 and 4, it decreased from the road to side samples. Also the same trend was found in the samples of the third stage, since the Ni content decreased from 19.05ppm for sample no.7 to 12.06ppm for sample no.8.

By studying the depth effect in this location (the first), the obtained data of Ni in the surface samples was 48.8, 33.0, 19.05, and 12.06ppm for samples no.3, 4, 7, and 8, respectively. The values of their equivalent depth samples were 15.06, 16.47, 16.65, and 16.05ppm for samples no.5, 6, 9, and 10, respectively. By correlating these data, we can find out that Ni concentrations are decreasing by depth except for samples no.8 and no.10. This increase in Ni concentration by depth in samples no.8 and 10 may be attributed to the fact that metal profiles in polluted soils usually penetrate a little below the 10-cm region even after many years **(Smith, et al., 1999).** The Ni content in the second location (sample no.12) represents the lowest value in all the studied samples (1.8ppm) as in the case of cobalt and manganese. In the third location, it ranged between 23.8ppm and 40.0ppm for samples no. 13 and 14 respectively. On the other side, results obtained from the spectrophotometric analysis of the "diagnostic" trace heavy metals (nickel and vanadium) showed that there were no significant differences in the amounts of nickel in the subsurface polluted soils when compared with the unpolluted reference (control) soils **(Leo, and Samuel, 2005)**

III-2-6-7. Lead

Lead in the environment is strongly absorbed by sediments and soil particles, and is therefore largely unavailable to plants and animals. Many of the inorganic salts of lead (lead oxides and sulfides) are not readily soluble in water and are sequestered in sediments. In aquatic systems, uptake is influenced by various environmental factors such as temperature, salinity, pH, and the presence of organic matter **(Schock, 1990).** The chemistry of Pb in soils is affected by three main factors: first, specific adsorption to various solid phases, second precipitation of sparingly soluble or highly stable compounds, and third, formation of relatively stable complexes or chelates that result from interaction with soil organic matter.

In the first location, lead is not detected in all the samples of this area except the samples no.7, 8, 9, and 10, which contain Pb content equal to 4.25, 4.05, 4.29, and 4.35ppm and these samples are representing the samples of the third stage (at 30 km). In the second location, lead not detected in this area since it is representing a young soil and the traffic accumulation is absence. In the third location, lead is detected only in samples no.13 and 14 and not detected in the other samples of this area.

These differences between our studied location soils in Pb content, presence or absence of Pb, suggest that content on the soil was the result of accumulation process from traffic sources during the past years suggesting that they have a common origin associated with traffic it could be the consequence of a long deposition process during the past when leaded gasoline was used especially that location is near the bus station, and the main source of Pb is the vehicular traffic, sludge application and the use of fertilizer **(Rossini and Fernández, 2007)**.

Damage to the nervous system and gastrointestinal symptoms are the main signs of lead poisoning, lead also interferes with the formation of red blood cells, leading to anemia. Lead is especially toxic to the growing brain and can affect the behavioral development of young, even at low concentrations. For example, in polluted cities, fumes from cars burning leaded gasoline have probably caused air concentrations high enough to affect children's development.

III-2-6-8. Chromium

Adsorption and precipitation behavior of Cr in soils is controlled by a variety of factors such as redox potential, oxidation state, pH, soil minerals, competing ions, complexing agents, and others. The mainly stable oxidation states of chromium in the environment are Cr(III) and Cr(VI). The most important among these factors are the hydrolysis of Cr(III) and Cr(VI), redox reactions of Cr(III) and Cr(VI), and adsorption/desorption and precipitation of Cr(VI) **(Bhide et al., 1996)**.

By looking to the results, we can find out that the Cr concentrations decreased as we going away from the road, in both surface and depth samples and this is clear from the results. In the second stage (20 km), the road samples in both the surface and depth (samples no.3 and 5) have Cr concentrations equal to 50.7 and 51.7ppm, which were higher than the side samples in both the surface and depth (samples no.4 and 6) have Cr concentrations equal to 45.6 and 46.4ppm, respectively. Also, we can find the same case in the samples of the third stage (30 km), the road samples in both the surface and depth (samples no.7 and 9) which have Cr concentrations equal to 52.8 and 57.0ppm, which were higher than the side samples in both the surface and depth (samples no.8 and 10) have Cr concentrations equal to 50.7 and 48.6ppm, respectively. The reverse was found in the samples of the first stage (10 km), since the road sample (no.1) contains Cr concentration equals to (32.9ppm), and it is lower than the concentration of Cr in the side sample (no.2), which is equals to 40.5ppm. Sample no.12 (Toushky) in the second location contains the lowest value of Cr concentration through all the studied samples (24.4ppm), this may be referred to that this area is still young and affected by low contaminations rate. Samples no.11 and 13 contain Cr concentrations similar to the other samples in spite of they comes from protected and highly contaminant area, respectively, but they have the same pH value which leads to increase the adsorption of Cr.

In general, Cr(VI) is more toxic and more mobile than Cr(III) **(Bhide et al., 1996).** Cr(III) is rapidly and specifically adsorbed by Fe and Mn oxides and clay minerals, with about 90% of added being adsorbed within 24 h. Adsorption increases with increasing pH and content of soil organic matter while it decreases in the presence of competing cations or dissolved organic ligands in the solution, while Cr(VI) species are adsorbed by a variety of soil phases with hydroxyl groups on their surfaces such as Fe, Mn, and Al oxides. The adsorption increases with decreasing pH due to the protonation of the hydroxyl groups. Cr(VI) is more toxic and generally more mobile than Cr(III) **(Bartlett and James, 1988).** Since under alkaline to slightly acidic conditions Cr(VI) compounds, i.e., CrO_4^{2-}, $HCrO_4^-$, and $Cr_2O_7^{2-}$, are not strongly absorbed by many soils, they can be very mobile in surface environments. On the other hand, under these conditions Cr(III) readily precipitates as $Cr(OH)_3$ or as the solid solution $Fe_xCr_{1-x}(OH)_3$. It can also be chelated by organic molecules that are adsorbed to mineral surfaces.

III-2-6-9. Copper

Cu shows a strong affinity for soil organic matter so that the organic-fraction Cu is high compared to that for other metals even though the absolute amounts are low. The most important sinks for Cu in soils are Fe and Mn oxides, soil organic matter, sulfides and carbonates while clay minerals and phosphates are of lesser importance. Adsorption maxima among soil constituents decrease in the order Mn oxide > organic matter > Fe oxide > clay mineral. Specific adsorption seems to play a more important role than nonspecific adsorption (i.e., cation exchange).

The maximum value of copper has been found by the sample no.7 (57.6ppm) at the first location, 30 km stage; also, a significance trend is obvious for the samples of this stage. The results indicating that there is a decreasing in the copper concentration in the samples with depth and by getting away from the road, since the road surface sample of this stage (samples no.7) has value equals to 57.6ppm, which is greater than samples no.8, 9, and 10 (49.7, 53.4 and 42.1) respectively. Also the samples of the second stage show the same trend, since the road surface samples (no.3 and 4) have values equal to 53.5 and 42.7ppm, and these values decreased by depth to 42.7 and 41.8ppm in samples no.5 and 6, respectively. Copper not detected in the second location (sample no.12), since it belongs to young soil and not contaminated yet, but it was recorded in the third location (sample no.13) by a value of copper concentration within the range of all the studied samples (42.4ppm), and also the rest of the samples of this location.

In general, all the studied locations have, almost, approach values of copper content where Cu can be introduced to soils by Cu-containing pesticides; sorption of Cu onto soil by organic matter associated with the clay fraction of the soil. Also, Cu in soil solution exists primarily in a form of complexes with soluble organics, besides complexation, by organic matter in the form of humic and fulvic acids, is an effective mechanism of Cu retention in soils. It has been shown that Cu is most extensively complexed by humic materials in comparison to other metals.

Table 10: Heavy metals concentrations of the studied samples, ppm.

Sample number	Fe	Mn	Zn	Cd	Co	Ni	Pb	Cr	Cu
1	4296.1	337.1	93.2	nil	20.4	44.3	nil	32.9	50.7
2	4278.6	349.5	87.9	nil	21.9	46.5	nil	40.5	52.8
3	4278.6	334.2	93.6	nil	28.8	48.8	nil	50.7	53.5
4	4284.6	359.4	80.4	nil	27.4	33.0	nil	45.6	43.5
5	4257.9	718.5	96.5	nil	25.5	15.06	nil	51.7	42.7
6	4305.0	767.0	45.5	nil	25.9	16.47	nil	46.4	41.8
7	4310.8	473.0	75.6	nil	26.3	19.05	4.25	52.8	57.6
8	4309.8	582.1	90.8	nil	30.1	12.06	4.05	50.7	49.7
9	4324.8	582.3	80.8	nil	24.9	16.65	4.29	57.0	53.4
10	4283.9	592.7	96.6	nil	21.7	16.05	4.35	48.6	42.1
11	4200.9	537.1	161.2	nil	20.7	14.85	nil	51.2	56.4
12	4232.4	198.3	41.4	nil	8.10	1.8	nil	24.4	nil
13	25320.0	742.5	580.0	0.12	19.8	23.8	6.06	54.3	42.4
14	5320.0	432.0	85.0	0.08	21.0	40.0	nil	45.5	38.5
15	4285.0	437.0	89.0	nil	24.0	35.0	nil	47.3	43.0
16	4265.0	425.0	82.0	nil	22.0	39.0	nil	50.1	40.0
17	4276.0	420.0	86.0	nil	21.0	35.0	nil	49.2	41.0

III-3. Evaluation of Petroleum Hydrocarbon Contaminations in the Studied Samples

III-3-1.Total petroleum hydrocarbons content (TPH) study of the collected soil samples

The concentrations of TPH in soil samples give more information about the levels of oil pollution in the studied sites. It was reported that soils are classified into three groups according to their total petroleum hydrocarbon (TPH) level: I) 1 to 4ppm in unpolluted soil. II) 5 - 100ppm for moderate polluted soils. III) up to 12000ppm in highly polluted soils **(El-Tokhi and Mostafa, 2001).**

In the first location: Data obtained from Table 11 show that the concentrations of the TPH content values for the three investigated road surface samples (samples no.1, 3 and 7) are 760, 647 and 820ppm dry weight, respectively, while the concentration of the TPH content values for the their side samples (samples no.2, 4 and 8) are 790, 680 and 910ppm dry weight, respectively. Correlating the data obtained from surface samples with their corresponding side samples, it is clear that the TPH content values of surface samples on the road are less than that for 1km aside the road. The TPH of sample no.11 was 930ppm, although it supposed to be a protected area and the TPH expected to be less than that value.

By studying the depth effect for each surface sample and its corresponding sample taken 30cm depth. Samples no.3 and 5 with TPH equal to 647and 800ppm respectively, Samples no.4 and 6 with TPH equal to 680 and 790ppm respectively, samples no.7 and 9 with TPH equal to 820 and 840ppm respectively. It was observed that the surface samples have TPH content values lower than their corresponding depth samples except for samples no.8 and 10 with TPH equal to 910 and 860ppm, respectively.

It was reported by **(Atlas, 1981 and Leahy and Cowell, 1990)** that the terrestrial oil spills are characterized primarily by vertical movement of the oil into the soil rather than the horizontal spreading associated with slick formation which might explain the incensement of TPH with depth. But for samples no.8 and 10, their TPH may indicate recent oil input.

From all the above we can conclude that TPH content of the samples of the first location is increasing by getting away from the road and also increasing with depth, except for samples no.8 and 10. Also based on the previous classification, the investigated soils are higher than the alarming level and are considered to be in the class of highly polluted group.

In the second location: Quantitative determination of TPH in sample no.12 is nil giving an indication that no TPH contamination is observed in this location (Toushky), hence it is a virgin area and representing a young soil and almost there is no sources of contamination, based on the previous classification, the investigated soil is classified as unpolluted soil **(El-Tokhi and Mostafa, 2001).**

In the third location: The TPH values of the third location have values ranging between 62,120ppm dry weight and 780ppm dry weight. The highest value was observed for sample no.13 and it is higher by about 40 fold than the next sample (sample no.14). The obtained high value for this sample is logic, hence it is taken from oil field production and it can be classified as a very highly contaminated sample. Data obtained shows that TPH content values are deeply decreased as going away from this site to reach 1560ppm dry weight for sample no.14, then it continues decreasing by more getting away from the source of contamination till reaches its lowest value at sample no.17 (780ppm dry weight) and it is almost the same range of the surface samples of the first location. Also based on the previous classification, the investigated soils are higher than the alarming level and are considered to be in the class of highly polluted group.

The results obtained indicate that hydrocarbons are associated with soils in amounts likely to affect the soil in one way or another **(Wu et al., 2003).**

Table 11: Total petroleum hydrocarbons content (TPH) of the studied samples, ppm.

Sample number	TPH, ppm dry weight	Sample number	TPH, ppm dry weight
1	760	10	860
2	790	11	930
3	647	12	nil
4	680	13	62120
5	800	14	1560
6	790	15	960
7	820	16	790
8	910	17	780
9	840	---	---

III-3-2. Gas chromatographic analysis

Gas chromatograms of the hydrocarbons contaminants are characterized by two general features: resolved compounds and an unresolved complex mixture (UCM). The latter is generally considered to be a mixture of many structurally complex isomers and homologues of branched and cyclic hydrocarbons that cannot be resolved by capillary GC columns **(Bouloubassi and Saliot, 1993).** It has been shown that the UCM consists primarily of linear carbon chains connected at branch points, which result in *"T-shaped"* molecules **(Gough and Rowland, 1990).** These molecules are resistant to biodegradation. In general, the presence of a UCM is considered to be associated with degraded or weathered petroleum residues **(Farrington and Tripp, 1977 and Le Dreau et al., 1997).**

For the first location: The GC spatial pattern distribution of the extracted oil of surface soil samples (samples no.1, 3, 7 and 11) as shown in Fig.10 reveal that the origin of contamination is mainly petrogenic for samples no.1 and 7, while samples no.3 and

11, reveal that the origin of contamination is mixed petrogenic with biogenic due to the predominance of n-C_{25} **(Jeng and Huh, 2006).** They are characterized by a broad spectrum of regularly spaced normal alkanes hydrocarbons peaks, protruding over an unresolved envelope comprising the unresolved complex mixture (UCM). Based on the previous concept that each type of hydrocarbon pollutants has its own feature (fingerprint), gas chromatographic fingerprint of the studied samples reveal that the type of contamination is mainly petroleum hydrocarbons and / or a combination of terrestrial plant waxes and petroleum sources. Weathering causes considerable changes in the chemical and physical properties of the incident pollutant. The extent and rate of weathering is controlled by a number of conditions and natural processes such as type of the spilled oil, environmental conditions and microbiological activities. Weathering is largely caused by evaporation and loss can be up to 70 and 40% of the volume of light and medium crudes respectively. For heavy or residual oils, the losses are only about 5-10 %f the volume. The range of n-paraffins observed by gas chromatography, shown in Table 12 is a measure of the amount of low boiling materials lost by weathering. Samples no.1 and 3 have the same initial carbon number, n-C_{16}, while the initial carbon number for the samples no.7 and 11 was n-C_{17} and n-C_{22} respectively. This results show that low molecular weight n-alkanes up to n-C_{16} and n-C_{17} were lost due to the effect of vaporization weathering and dissolution (natural weathering), photo-oxidation, phytodegradition and bioremedition) confirming a low weathering rate of the spilled hydrocarbons. The final carbon number is different in the four samples, it was n-C_{39}, n-C_{44}, n-C_{37} and n-C_{38} for samples no.1, 3, 7 and 11 respectively as shown in Table 12.

Sample no.1 shows a well defined and broad UCM with a predominate peak at C_{20}, this might indicate contamination with mid range distillates; kerosene, solar, jet fuel and diesel products, which are typically products in a relative broad carbon range (C_6-C_{26}) and the UCM is a well defined **(Wang and Fingas, 2003)** and this could be explained by the agriculture activity in these areas. Sample no.7 shows a well defined UCM signal of the higher molecular weight region which may indicates the presence of lubricating oil as a source of contamination **(Tolosa et al., 2004)**.

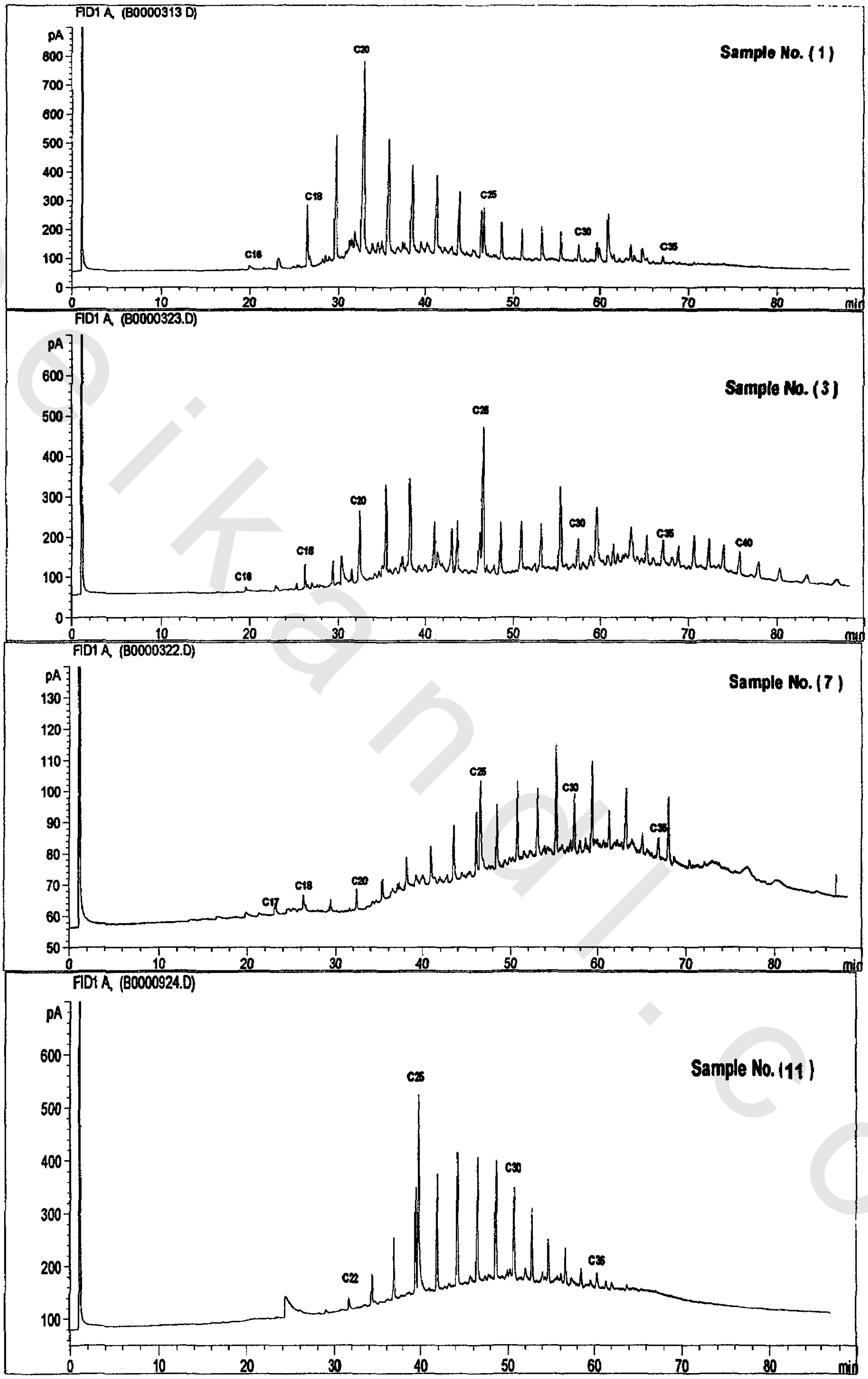


Fig. 10: Gas chromatograms of the road surface samples of the first location.

The gas chromatographic fingerprint for the side samples of the same location (samples no.2, 4 and 8), shows that the origin of contamination is also mainly petrogenic and / or terrestrial higher plant waxes **(Kaplan and Kitts, 2004)**. They have different initial carbon number as shown in Table 12, they are n-C_{20}, n-C_{14} and n-C_{18} respectively. Also the final carbon number for the samples was different, since it was n-C_{43}, n-C_{44}, and n-C_{40} for samples no.2, 4 and 8 respectively.

Sample no.4 displays some kind of unusual biomodel distribution on n-C_{16}- n-C_{25} and n-C_{26}- n-C_{44}, which indicates a mixed sources of hydrocarbons mid oil distillates and terrestrial plant waxes **(Méndez et al., 2001 and Wang and Fingas, 2003). Mederios and Bicego, (2004)** reported that the predominance of peaks > n-C_{25} indicating an input from terrestrial higher plant waxes. The presence of a well defined UCM in the high molecular weight region in sample no.8 may indicate the presence of lubricating oil as a source of contamination **(Tolosa et al., 2004)**.

By studying the depth effect in this location, the GC spatial pattern distribution of the extracted oil of the surface samples (samples no.3, 4, 7 and 8) as shown in Fig.10 and 11 reveals that the origin of contamination is mainly petrogenic with biogenic. While their corresponding depth samples (samples no.5, 6, 9 and 10) are characterized by that the origin of contamination is mixed petrogenic with biogenic. They are characterized as shown in Fig.12 by the predominance of n-C_{25}, which is representing 7.84%, 56.75%, 17.59% and 15.71% of the total n-alkanes area for samples no.5, 6, 9 and 10 respectively. Samples no.6, 9 and 10 have the same initial carbon number (n-C_{16}) as shown in Table 12, and this is indicating the same degree of weathering, which was higher than the degree of weathering for sample no.5 with initial carbon number n-C_{14}.

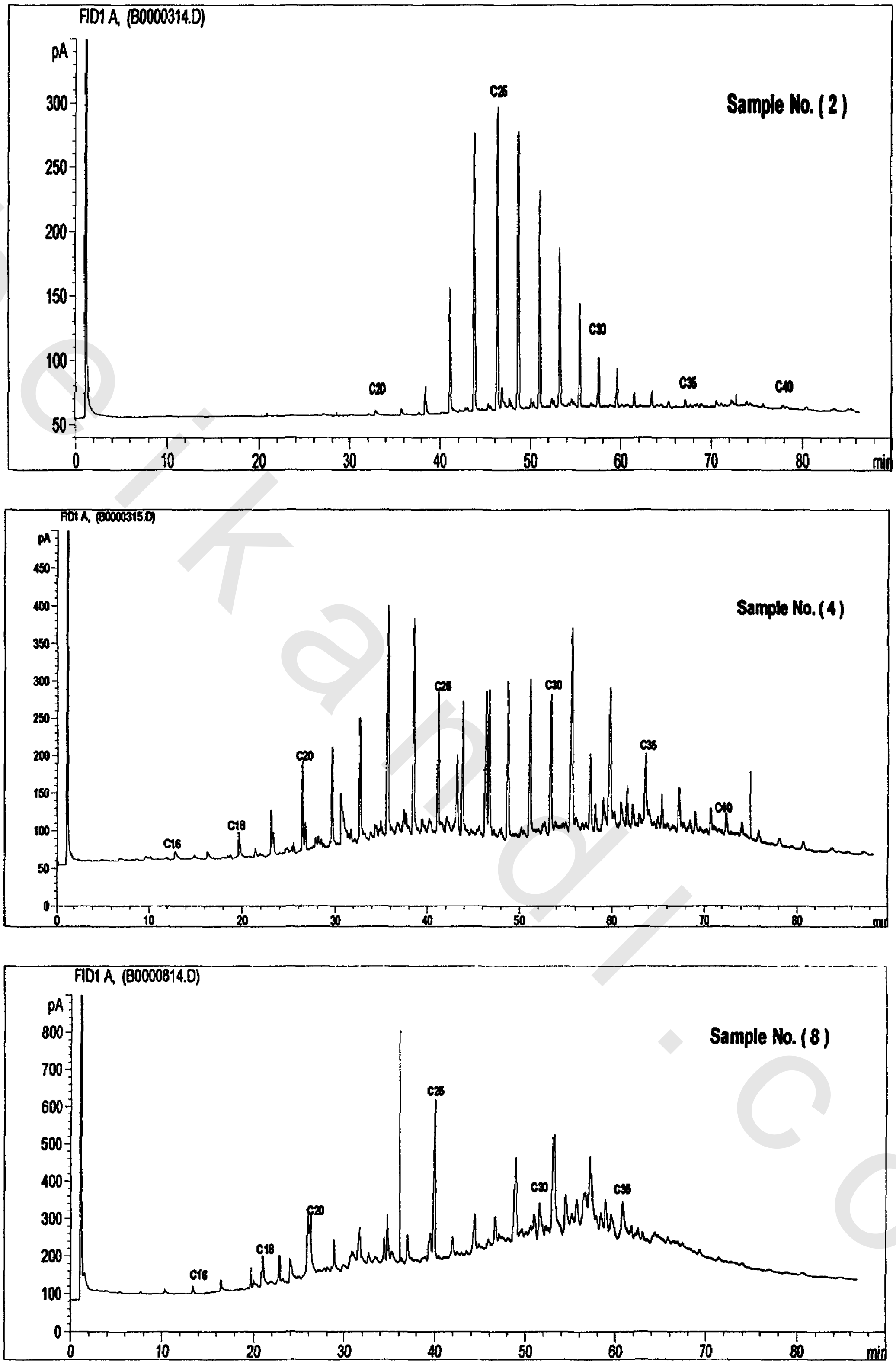


Fig.11: Gas chromatograms of the side samples of the first location.

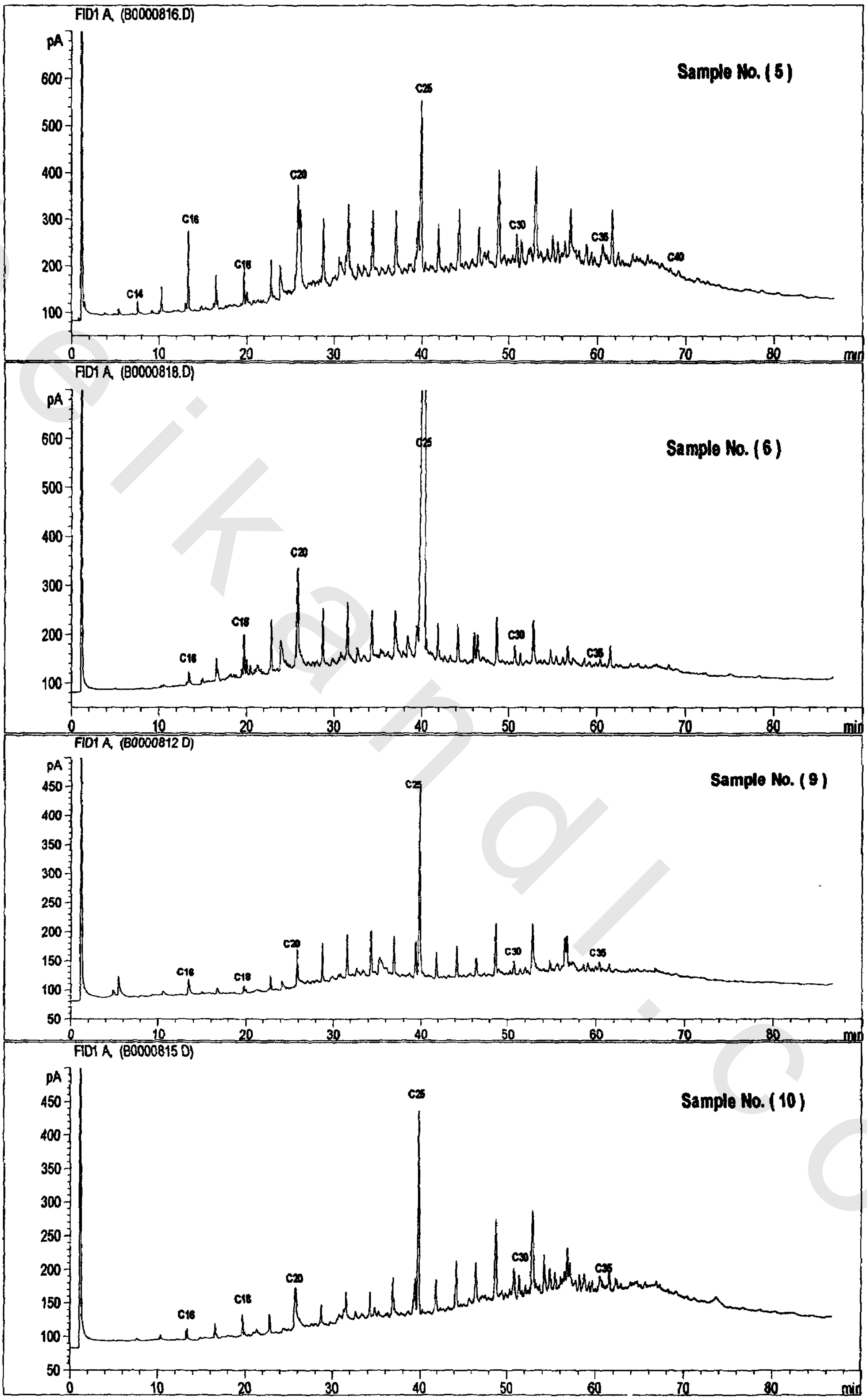


Fig. 12: Gas chromatograms of the depth samples of the first location.

In the second location: As expected, gas chromatographic profile of the second location (sample no.12) shows neither resolved peaks nor unresolved (complex mixture), confirming that there is no hydrocarbons contaminations.

In the third location: According to their gas chromatographic profile, samples of the third location can be divided into two categories. The 1st category includes sample no.13 which has the characteristic feature of petroleum origin. The 2nd category includes samples no.14, 15, 16, and 17, they have the characteristic feature of mixed petrogenic with biogenic.

The GC chromatographic profile of sample no.13 shows regularly spaced n-alkanes peaks ranged from n-C_{11} – n-C_{31} superimposed on the UCM with the presence of pristine (Pr) and phytane (Ph), indicating that the source of contamination is mainly petroleum hydrocarbons **(Wang et al., 1998).** The components up to n-C_{11} were lost (< n-C_{11}) due to the natural weathering process mainly; evaporation and dissolution **(Kaplan and Kitts, 2004)** consuming recent oil input and / or a slow weathering rate of the spilled oil. The presence of low weathered profile gives an indication of continuous petroleum inputs **(El-Tokhi and Mostafa 2001).** This is logic as this sample was taken from an oil field.

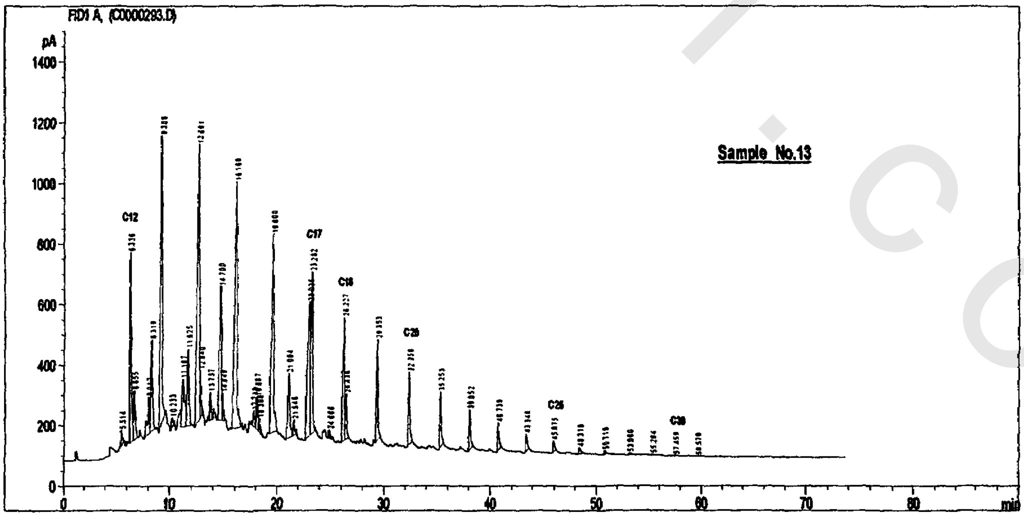
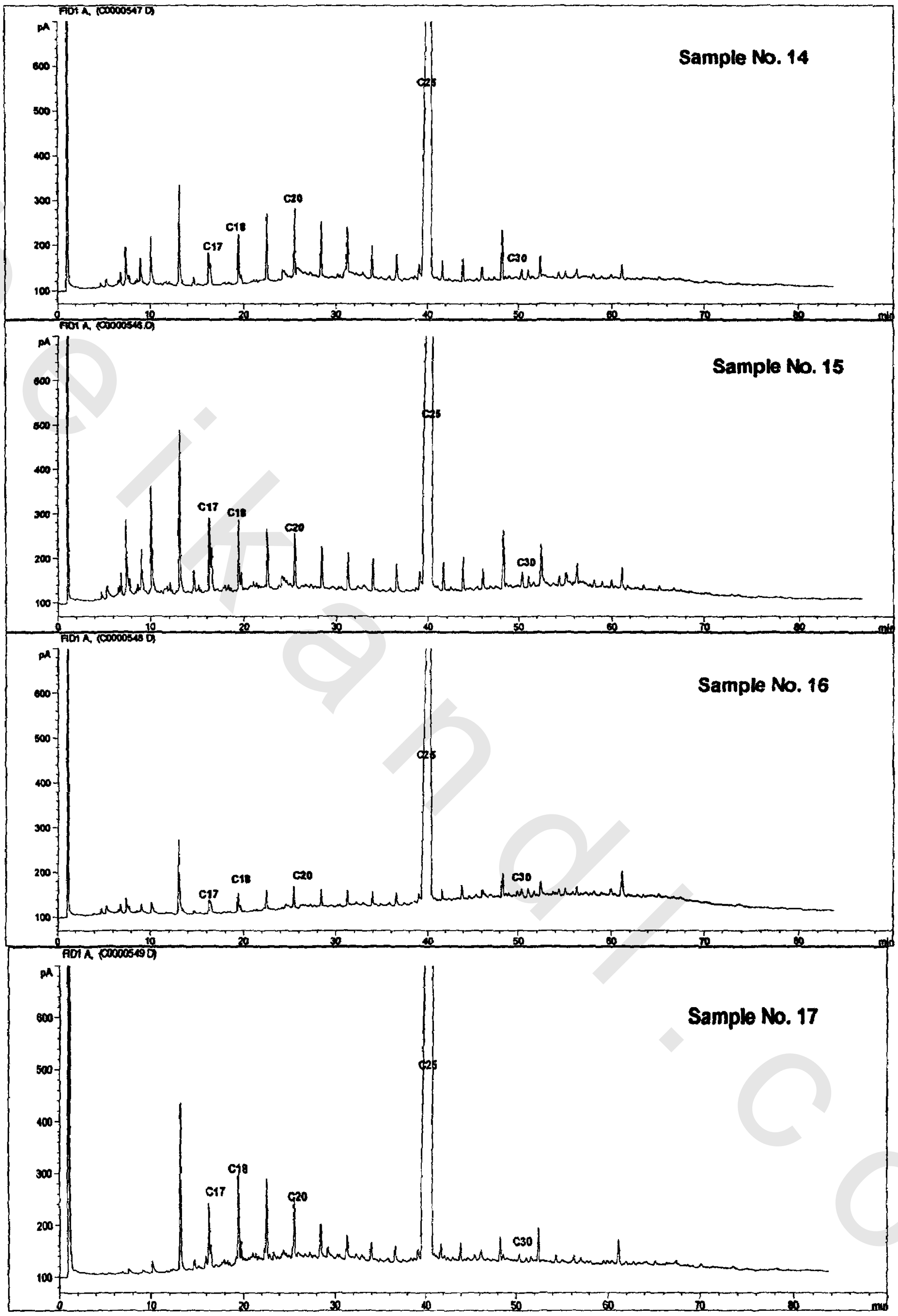


Fig. 13: Gas chromatograms of the samples of the third location.



Cont. Fig. 13: Gas chromatograms of the samples of the third location.

The presence of Ph and UCM in all the 2nd category samples indicating petrognic hydrocarbons. The significance increase of n-C_{25} idicating biogenic hydrocarbons **(De-Oteyza and Grimalt, 2006).** The predominace of such peak disturbs the distribution pattern and all the 2nd category samples are characterized by highly weathered profile, and as shown in Fig.13 the n-C_{25}, which is representing 68.86%, 63.04%, 78.49% and 78.22% of the total n-alkanes area for samples no.14, 15, 16 and 17, respectively. While the samples of the 2nd category have initial carbon numbers, n-C_{13} for samples no.14 and 15, and n-C_{14} for samples no.16 and 17 as shown in Fig.13 and Table 12.

Several investigators used other parameters depending on gas chromatographic analysis for identification of the degree of weathering, mainly biodegredation **(Barakat et al., 2002).** Also the origin of hydrocarbons can also be confirmed by a set of parameters and ratios that are useful in distinguishing the contribution of biogenic hydrocarbons among these are:

III-3-2-1. The carbon preference index (CPI)

This index is a measure of the abundance of odd-to-even numbered n-alkanes in a given sample. Petroleum origin contamination characteristically have CPI values around (1.0). CPI was used to differentiate between petrogenic and biogenic origins **(Méndez et al., 2001)**

For the first location: the values obtained for the surface samples are listed in Table 12 indicating that the CPI was 1.32, 0.73, 1.30, 0.81 and for samples no.1, 3, 7 and 11 respectively. The values higher than 1 (samples no 1 and 7) is an indication for mainly petrogenic origin, while the values less than 1 (sample no.3 and 11) is an indication for mixed petrogenic with biogenic, and this is confirming the gas chromatirapgic profile data. While the values for the aside samples were 0.99, 1.35 and 2.11 for samples no.2, 4 and 8, respectively. These data for samples no.2 and 4 around 1 and this indication for mainly petrogenic origin but CPI value for sample no.8 is higher than 1 indicating weathering with biogenic addition.

By studying the depth effect, we found that the values for CPI were 1.32, 4.18, 2.09 and 1.56 for samples no.5, 6, 9 and 10. These data indicating also for mainly petrogenic origin in samples no.5 and 10 since they are around 1. But for samples no.6 and 9 mixed biogenic and petrogenic origin. Samples no.1 and 5 have the same CPI value which may indicate the same oil pollution origin.

For the third location: the values obtained for the surface samples are listed in Table12 indicating that the CPI values were 0.99, 7.03, 6.04, 9.98 and 8.64 for samples no.13, 14, 15, 16 and 17 respectively. As the reverse of the samples of the first location, all these values were higher than 1 except sample no.13 and this indicating high biogenic addition and confirming the gas chromatirapgic profile data.

III-3-2-2. The normal alkane- to- isoprenoid ratio

This ratio ranges between 1.53 to18.03 and it is used as an index for the degree of weathering (mainly biodegradation) processes. Based on the concept that normal alkanes are usually degraded more rapidly than isoprenoid alkanes, this ratio decreases as weathering proceeds. The chromatograms show that the samples have undergone different vaporization weathering degrees. Sample no.17 shows the highest value for the n. / iso alkanes, which an indication that this sample is slightly weathered, because as the ratio increase, the degree of weathering decrease **(Wang et al., 1995).**

III-3-2-3. The UCM / TRP

The ratio of unresolved complex mixture (UCM) to the total resolved peaks (TRP) is an important parameter, indicative of chronic / degraded petroleum contamination. Losses of n-alkanes and increase in UCM (complex mixture of unseparated alicylic, naphthenic and aromatic hydrocarbons) are indicative of weathering.

The gas chromatographic profile shows resolved peaks with different initial and final carbon numbers superimposed on the prominent UCM.

For the first location: Data obtained from Table 12 shows that the percentage of the UCM is ranged between 47.60 and 89.13% while the percentage of the total resolved peaks ranged between 52.40 and 10.87%. So these samples were subjected to different degrees of weathering. The (UCM) / (TRP) ranges between 0.91 to 8.21. The highest UCM/ TRP value was found for sample no.7 which was characterized by well defined UCM signal in the high molecular weight region is an indicative of chronic and very highly weathered sample and the source of contamination is may be lubricating oil.

The n-paraffin distribution of the oil extracted from the soil shows the absence or decrease in some low molecular weight n-alkanes as result of natural weathering process mainly; evaporation and dissolution **(Kaplan and Kitts, 2004).**

For the third location: Data obtained from Table 12 shows that the percentage of the UCM ranged between 50.02% and 64.31%, and the TRP ranged between 35.69% and 49.98%, but the (UCM) / (TRP) ranged between 1.0% to 1.8%. It is clear from the table that there is no significance difference in the percentage of the UCM in the samples of this location except for sample no.16, which give an indication that the samples were almost subjected to the same degree of weathering.

Concerning weathering effect, the soil samples can be classified into three groups. The first group includes sample no.7, with UCM / TRP equals to 8.12 and it can be classified as very highly weathered sample, the second group includes samples no.5, 8, 9, 10 and 11, which could described as weathered samples. While the rest samples can be described as slightly weathered **(Méndez et al., 2001).**

Table 12: Diagnostic parameters of the studied samples.

Sample number	Initial n-C_n	Finial n-C_n	CPI	n. / iso alkanes	UCM,%	TRP,%	UCM/ TRP
1	C_{16}	C_{39}	1.32	2.93	63.15	36.85	1.71
2	C_{20}	C_{43}	0.99	11.42	47.60	52.40	0.91
3	C_{16}	C_{44}	0.73	3.99	74.85	25.15	2.98
4	C_{14}	C_{44}	1.35	2.88	68.03	31.97	2.31
5	C_{14}	C_{40}	1.32	1.97	80.34	19.66	4.09
6	C_{16}	C_{39}	4.18	6.25	72.19	27.81	2.60
7	C_{17}	C_{37}	1.30	2.25	89.13	10.87	8.21
8	C_{18}	C_{40}	2.11	1.95	81.44	18.56	4.39
9	C_{16}	C_{36}	2.09	1.91	80.16	19.48	4.16
10	C_{16}	C_{38}	1.56	3.60	84.44	15.56	5.43
11	C_{22}	C_{38}	0.81	6.93	82.51	17.49	4.72
12	---	---	---	---	---	---	---
13	C_{11}	C_{31}	0.99	1.53	53.49	46.51	1.15
14	C_{13}	C_{35}	7.03	12.25	52.05	47.95	1.09
15	C_{13}	C_{40}	6.04	8.72	53.97	46.03	1.17
16	C_{14}	C_{40}	9.98	15.32	64.31	35.69	1.80
17	C_{14}	C_{41}	8.64	18.03	50.02	49.98	1.00

III-4. Method for Treatment of Petroleum Contaminated Soil

III-4-1. Characterization of the collected polluted soil

Development of a bioremediation protocol for optimum biodegradation conditions requires a complete site characterization to define the physicochemical characteristics of the site, distribution and type of contaminants and indigenous microbial community. In most field studies, enhancing biodegradation of petroleum hydrocarbons depends on the specific microbial population present. The composition of the microbial population is affected by the environmental conditions and the composition of the hydrocarbons **(Admon et al., 2001 and Bento et al., 2005).** Soils from various sources and locations exhibit differences in both rate and extent of the biodegradation of contaminants. Though at present little details are known about the influence of soil type on biodegradation kinetics, it is likely that the highly sorptive surfaces of some clay and organic matter fractions limit the bioavailability of petroleum hydrocarbons to soil microorganisms **(Gogoi et al., 2003).** Given the high levels of contaminant concentration in the field, it is expected that there should be significant levels of bioavailable contaminants. In the case of intensely weathered soils where the contaminants have had time to migrate into the micropores, which are less accessible to microbial attack, generally bioavailability of hydrocarbons declines. In the case of such intensely weathered soils, the kinetics are not limited by the number of hydrocarbon degraders or the intrinsic petroleum hydrocarbon biodegradability, but rather by mass transport (desorption, diffusion and convection) phenomena. Also the soil chemistry is equally important in developing a biodegradation potential for contaminated soil. Results of different physicochemical characterizations analyses of the collected polluted soil are listed in Table 13.

Data analysis showed that the collected soil used in this study had 17.81% clay, 64.87% sand and 17.32% slit with a Sandy loam texture. Its pH was 7.12 and total organic carbon was 42,902ppm, nitrogen and phosphorus content was 0.38 and 0.12 (%w/w), respectively. Soil dry mass (dm) as 83.68% and the moisture content was 16.32%.

Table 13: Physical and chemical characterization of the soil sample.

Parameters	Value
Physico-chemical characteristics	
Soil type	
Course Sand%	51.93
Fine Sand%	12.94
Clay%	17.81
Slit%	17.32
Soil Texture	Sandy loam
Moisture%	16.32
pH	7.12
Salinity (mg/kg)	76.15
Electrical conductivity (dS/m)	0.68
Bulk Density (g/cm^3)	1.27
TPH (ppm)	62,120
TOC (ppm)	42,902
Total N (%, w/w)	0.38
Total P (%, w/w)	0.12
Total K (%, w/w)	0.93
Bacterial viable cells (CFU/g dry soil)	$9x10^7$
Soluble anions (meq/l)	----
HCO_3^-	5
Cl^-	1.3
SO_4^{2-}	0.5
Soluble cations (meq/l)	
Na^+	2.6
K^+	0.70
Ca^{2+}	3.0
Mg^+	2.0
Metal concentrations (ppm)	
Fe	25,320
Mn	742.5
Zn	580
Cd	0.12
Co	19.8
Ni	23.8
Pd	6.06
Cr	19.0
Cu	42.4
V	38.4
V/Ni	1.61

Analysis of total petroleum hydrocarbons (TPH) from PCS revealed that it is polluted with TPH of 62,120ppm dry weight. According to **Duarte et al., (2001),** the levels of oil in low-polluted soil averaged 710ppm soil and those in the high-polluted soils averaged 5,500ppm soil. Based on this classification, the obtained value of the collected PCS was extremely above the alarming level and can be considered very highly polluted by oil. This high value may be due to the continuous supply of petroleum inputs in this area.

The GC chromatographic profile, Fig. 17 (zero-time) shows regularly spaced n-alkane peaks ranged from (n-C_{10}-n-C_{42}) superimposed on an unresolved complex mixture (UCM) containing naphthenes and aromatics indicating that the source of contamination is mainly petroleum hydrocarbons. The light components up to n-C_{10} were lost due to the natural weathering process mainly vaporization **(Kaplan and Kitts, 2004).** The presence of low weathered profile, the relatively slightly high UCM value 51.29% with total paraffins of 48.71%, Pr/Ph ratio of 3.45 and carbon preference index (CPI) value of 1.04 gave an indication of recent and/or continuous petroleum inputs in this area, confirming the natural weathering and chronic pollution and the CPI $\approx$ 1 indicating no biogenic addition **(El-Tokhi and Moustafa, 2001 and Wang et al., 2001).**

The sample is characterized by a high level of saturated hydrocarbon content as shown in Fig. 17 (zero-time), that may be attributed to the sandy matrix of the sample, which poorly adsorbs asphaltenes and resins **(Gogi et al., 2003).**

The collected soil sample is characterized by high HM concentration. Table 13, which may be attributed to the high level of petroleum contamination. The metal contaminants in petroleum oil contaminated sites are usually; iron, nickel, vanadium, manganese, zinc, copper, lead and chromium **(Benka-Coker and Ekundayo, 1998).** The V/Ni ratio was 1.61 which is considered to be within the finger print ratio (1-3) characterizing the Egyptian crude oil as reported by **Moustafa, (1990).**

The soil microbial properties are perhaps the vital factors determining natural biodegradability. The total viable count on LB agar plates directly after collection showed a good microbial population of 9×10^7 but with low biodiversity only two bacterial strains were identified. These are Gram positive *Brevibacterium otitidis* N1 and Gram positive *Cellulomonas hominis* N2. The presence of heavy metals and high oil content in the PCS over a long period of time could have affected the diversity of the microbial communities. Similar results were reported by **Chen et al., (1997) and Lors and Mossmann, (2005)** and they attributed low biodiversity to the nature of pollution in the soil, which includes high concentration of TPH and metals. This mixed contamination could well exert selective effects on the specific microflora, leading to inhibition of the most sensitive species to the benefit of the resistant ones that can adapt themselves to such an environment. According to **Rahman et al., (2003)** low biodiversity may be because of the use of sandy soil with low nutrients and microflora. According to **Huesemann, (1994),** high hydrocarbon levels are associated with varying degrees of inhibitory effects on soil microbes for example oxygen and/or nutrients limitations. Not only this but the terrestrial oil spills are characterized primarily by vertical movement of the oil into the soil rather that of the horizontal spreading associated with slick formation. Infiltration of oil into the soil might prevent evaporative loses of volatile hydrocarbons, which are toxic to microorganisms **(Leahy and Colwell, 1990).** This PCS is also characterized as mentioned above by high concentrations of heavy metals as listed in Table 13 which according to **Oliveira and Pampulha, (2006)** affect the number and diversity of culturable indigenous microorganisms in the polluted soil.

It is apparent as listed in Table 13 that concentrations of inorganic nutrients, i.e. N of 380mg/kg, P of 120mg/kg and potassium of 230mg/kg are low which according to **Gogoi et al., (2003)** could limit the metabolism of the indigenous microorganisms capable of degrading hydrocarbons in the soil environment. Application of extraneous nutrients is required for developing a feasible bioremediation method in this area.

III-4-2. Enrichment and isolation of heavy metals tolerable bacteria

Studying the effect of several metals in combination is desirable to simulate the situation in nature, since a single metal seldom dominates. Luria Bertani (LB) medium is a complete one used to enrich and flourish the endogenous microbial community in the collected PCS. It was amended with a mixture of equal concentrations of HM Cu/Mn/Ni/Zn (0-1000mg/l) to provide a nutrient rich environment for enriching and isolating HM tolerable microorganisms from the collected PCS **(Raja et al., 2006).**

Although nitrogen and phosphorus are frequently the limiting nutrients with respect to microbial activity, phosphate salts were not added so to avoid precipitation of different metal ions amended to LB for isolating HM tolerable microorganisms from the collected PCS **(Riis et al., 2002).**

Table 14 shows the total viable count, diversity and identification of the bacterial isolates obtained and their tolerance to amended HM mixtures. Results demonstrate that metal-tolerant bacteria can be readily isolated from environments containing elevated levels of toxic metals. Some have adapted and some are endemic to their environment, while the environmental conditions may have selected for others.

After enrichment in LB enrichment medium the total viable count increased to be $6x10^9$ and the biodiversity of the culturable microorganisms increased. Use of fresh enrichment LB medium was reported to stimulate growth of indigenous soil bacteria **(Ueno et al., 2006).**

Generally the TCFU and diversity decrease with the increase of HM concentrations. Similar observations were reported by **Sokhn et al., (2001)** and they attributed this as over time and increase in HM concentrations, only organisms able to tolerate or adapt to the toxicity of HM probably persisted.

The isolated microorganisms comprised mainly of six Gram-positive bacteria with distinct morphologies, they were identified to be *Brevibacterium otitidis* N1, *Cellulomonas hominis*

N2, *Clavibacter agropyri* N3, *Geobacillus thermoglucosidasius* N4, *Micrococcus luteus* N5 and *Pediococcus pentosaceus* N6 and one Gram negative bacterium, *Sphingomonas macrogoltabidus* N7.

Table 14: Living cell counts (CFU/g) and diversity of isolates before and after enrichment in LB amended with different concentrations of Heavy metals (Cu/Mn/Ni/Zn) mixture.

CFU/g soil before enrichment			**Number of diversity**	**Identification of bacterial isolates**
$9x10^7$			2	*Brevibacterium otitidis* N1 *Cellulomonas hominis* N2
Heavy metals concentration (mg/l)	**CFU/g soil after enrichment**	**CFU related to the reference %**	**Number of diversity**	**Identification of bacterial isolates**
zero	$6x10^9$	---	7	*Brevibacterium otitidis* N1 *Cellulomonas hominis* N2 *Clavibacter agropyri* N3 *Geobacillus thermoglucosidasius* N4 *Micrococcus luteus* N5 *Pediococcus pentosaceus* N6 *Sphingomonas macrogoltabidus* N7
50	$4x10^9$	66.67	7	*Brevibacterium otitidis* N1 *Cellulomonas hominis* N2 *Clavibacter agropyri* N3 *Geobacillus thermoglucosidasius* N4

Cont. Table 14: Living cell counts (CFU/g) and diversity of isolates before and after enrichment in LB amended with different concentrations of Heavy metals (Cu/Mn/Ni/Zn) mixture.

Heavy metals concentration (mg/l)	CFU/g soil after enrichment	CFU related to the reference %	Number of diversity	Identification of bacterial isolates
50	4x10^9	66.67	7	*Micrococcus luteus* N5 *Pediococcus pentosaceus* N6 *Sphingomons macrogoltabidus* N7
100	2x10^9	33.33	4	*Brevibacterium otitidis* N1 *Cellulomonas hominis* N2 *Clavibacter agropyri* N3 *Sphingomons macrogoltabidus* N7
200	5x10^8	8.33	2	*Brevibacterium otitidis* N1 *Cellulomonas hominis* N2
300	4x10^8	6.67	2	*Brevibacterium otitidis* N1 *Cellulomonas hominis* N2
400	4x10^8	6.67	1	*Cellulomonas hominis* N2
500	2x10^5	0.003	1	*Cellulomonas hominis* N2
600	---	---	---	---
700	---	---	---	---
800	---	---	---	---
900	---	---	---	---
1000	---	---	---	---

Microbial populations that consist of strains that belong to various genera have been detected in petroleum-contaminated soil or water **(Ghazali et al., 2005).** It is generally believed that bacterial strains resistant to the xenobiotic pollutant and capable of degrading it effectively can be found in contaminated sites **(Koutny et al., 2003).**

Brevibacterium species, *Clavibacter* species, *Geobacillus* species, *Micrococcus* species, *Pediococcus* species and *Sphingomonas* species are from the hydrocarbon utilizing bacteria that mostly reported as indigenous hydrocarbon utilizing bacteria in oily soil highly contaminated soil with PAHs and heavy metals **(Odokuma and Dickson, 2003, Chaillan et al., 2004, Lors and Mossmann, 2005, Meintanis et al., 2006 and Poli et al., 2006)**

Results of this work showed that heavy metal resistance among bacteria is widespread. The strains isolated by enrichment procedures with elevated levels of Cu/Ni/Mn/Zn mixture showed an extreme tolerance to the tested heavy metals but with different efficiencies. The seven bacterial isolates tolerated up to 50mg/l HM with no significant change in TCFU. Four bacterial isolates tolerated up to 100mg/l HM; *Brevibacterium otitidis* N1, *Cellulomonas hominis* N2, *Clavibacter agropyri* N3 and *Sphingomonas macrogoltabidus* N7. Two bacterial isolates tolerated up to 300mg/l; *Brevibacterium otitidis* N1, *Cellulomonas hominis* N2. But only one bacterial isolate tolerated up to 500mg/l HM; *Cellulomonas hominis* N2.

It was obvious from the results listed in Table 14 that the tolerance of microorganisms to HM in broth medium is much lower than in the soil as they tolerated up to 500mg/l HM although the collected PCS is characterized by higher HM concentration, as listed in Table 13. Similar observation was reported by **Edgehill (1996)** and attributed this as much of the HM in the soil is apparently unavailable to indigenous microorganisms as a result of precipitation or adsorption.

The high tolerance of *Brevibacterium otitidis* N1, *Cellulomonas hominis* N2 to heavy metals explains their predominance in the collected PCS.

Friis and Myers-Keith, (1986) have previously reported that reduction in growth is mainly because of the interaction between the metal cations along with phosphate, carboxyl, hydroxyl and amino-groups of the cell surface. **Castro-Silva et al., (2003)** reported that HM resistance is a widespread attribute among microorganisms isolated from mining environments. According to **Bopp et al., (1983),** studies demonstrated that bacteria isolated from polluted sites are 40 to 200 times more resistant to HM than related strains isolated from unpolluted sites. **Hurst et al., (1997)** also considered that the presence of high concentrations of HM is associated with an increase in the number of microorganisms resistant to them. In addition to these results, it is incorrect to affirm that the sensitivity to the metals in the environment will be the same as in the laboratory tests. **Raja et al., (2006)** have reported that under conditions of imposed stress, metal resistance in microorganisms possibly helps them to adapt spontaneously by mutation and natural selection.

The high incidence of heavy metal resistance detected in this work indicates the potential of these microorganisms as bioremediation agents.

III-4-3. Selection of phenanthrene (phe) degrading bacteria

The seven isolated bacteria were tested for their ability to degrade Phe, as model compound of PAHs present in crude oil. They showed different growth capabilities on Phe, and biodegradation efficiencies, as listed in Table 15. This can be summarized in the following order: *Cellulomonas hominis* N2 > *Brevibacterium otitidis* N1 > *Sphingomonas macrogoltabidus* N7 > *Micrococcus luteus* N5 > *Clavibacter agropyri* N3 ≈ Pediococcus *pentosaceus* N6, while *Geobacillus thermoglucosidasius* N4 showed no growth or biodegradation capabilities.

In contaminated sites diverse groups of indigenous microorganisms capable of utilizing and degrading contaminants such as PAHs might have been present **(Guo et al., 2005).** Other studies showed that previous exposure and acclimatization of microbes to PAHs in soils would enhance their biodegradation rates. The biodegradation potentials of strains isolated from hydrocarbon-contaminated environments are as active as or even higher than those originating from non-contaminated soils **(Guo et al., 2005).**

Table 15: Growth and biodegradation efficiency (%BD) of different bacterial isolates on 500ppm phenanthrene.

Bacterial isolates	I/I_0	pH	%BD
***Brevibacterium otitidis* N1**	10.75	6.55	49
***Cellulomonas hominis* N2**	13.24	6.35	98
***Clavibacter agropyri* N3**	1.50	6.98	10
***Geobacillus thermoglucosidasius* N4**	1.08	7.00	----
***Micrococcus luteus* N5**	1.60	6.95	15
***Pediococcus pentosaceus* N6**	1.52	6.98	9.5
***Sphingomonas macrogoltabidus* N7**	2.91	6.92	20

The recorded decrease in pH is an indication for the biodegradation of Phe, and may have been due to the formation of acidic intermediates and metabolites **(Aislabie et al., 2006 and Marin et al., 2006).**

The presence of HM tolerable and Phe degrading microorganisms in oily soil is consistent with earlier studies **(Sokhn et al., 2001 and Wong et al., 2005).**

Results listed in Tables 14 and 15 show the high tolerance of *Cellulomonas hominis* N2 to heavy metals associated with the PCS and its high biodegradation capabilities. That is why it was chosen for further studies; biosurfactant production and the impact of heavy metals on its biodegradation capabilities.

III-4-4. Biosurfactant production and emulsification index

Bacterial isolates that display substantial potential for production of biosurfactant enhance bioremediation of hydrocarbons in oily soil **(Bento et al., 2005).**

Cellulomonas hominis N2 showed good growth on hexadecane as a soul source of carbon and energy with TCFU of

$1x10^{10}$ with complete degradation of the added hexadecane after 7 days of incubation starting with inoculum of $2.5x10^{5}$ and the surface tension was reduced by 30% using uninoculated flask as the reference surface tension.

Emulsification index is a method extensively used to identify and quantify biosurfactants in microbial cultures. *Cellulomonas hominis* N2 showed good emulsification index of 18%.

Biosurfactants are non-toxic, non-hazardous, biodegradable, environmentally friendly amphiphilic compounds that reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids or of a fluid and a soil and increase the surface areas of insoluble compounds leading to increased mobility, bioavailability and subsequent biodegradation **(Banat et al., 2000).** They are produced by many bacterial strains that can degrade or transform the components of petroleum products **(Rahman et al., 2003).**

III-4-5. Impact of heavy metals on growth of *Cellulomonas hominis* N2 on phenanthrene

Significant variations in the growth patterns were observed for each of the HM used in the study individually and as a mixture of HM ions.

Distinct patterns of HM *Cellulomonas hominis* N2 resistance were vindicated at Table 16 and Fig. 14.

Growth of strain N2 in the presence of HM mixture Cu/Mn/Ni/Zn were consistently lower than that of the reference flask without HM supplementation. Similar observations were reported by **Kumar et al., (1992), and Raja et al., (2006).**

Generally there was a decrease in TCFU with the increase in HM concentrations, though the growth was not particularly affected at low concentration. In flasks containing 5mg/l of individual HM there was an increase of TCFU than the reference flasks without amending HM in the following order Cu 270%, Mn

250%, Zn 233% and Ni 220%. But flasks containing 5mg/l of Cu/Mn/Ni/Zn mixture there was a significant decrease in TCFU to reach 35% of that in the reference flask, indicating its highest toxicity relative to the presence of each HM alone in the culture medium.

This was in agreement with other recent studies showing that Ni at low concentrations was non toxic and actually enhanced growth and metabolic activity of aerobic bacteria **(Amor et al., 2001).**

At concentration of 10mg/l the growth was generally reduced related to the reference in the following order Cu 10%, Cu/Mn/Ni/Zn mixture 15%, Mn 25, Zn 53.33% and Ni 66.67%. Indicating order of toxicity of Cu > Cu/Mn/Ni/Zn mixture > Mn > Zn > Ni at 10mg/l HM

At concentration of 25mg/l the growth was generally reduced from the reference in the following order Ni 6.67%, Cu 9%, Cu/Mn/Ni/Zn mixture 10%, Mn and Zn are nearly having the same effect of 20% and 19.33%, respectively. Indicating order of toxicity of Ni > Cu > Cu/Mn/Ni/Zn mixture > Mn ≈ Zn at 25mg/l HM.

At concentration of 50mg/l the growth was generally reduced from the reference in the following order Cu/Mn/Ni/Zn mixture and Cu are nearly having the same effect of 1% and 1.2%, respectively, Ni and Mn having the same effect of 10% and Zn 11.33%. Indicating order of toxicity of Cu/Mn/Ni/Zn mixture ≈ Cu > Mn = Ni > Zn at 50mg/l HM.

At concentration of 100mg/l the growth was generally reduced from the reference in the following order Cu/Mn/Ni/Zn mixture 0.005%, Ni and Cu are nearly having the same effect of 0.43% and 0.6%, respectively, Mn 5% and Zn 6.67%. Indicating order of toxicity of Cu/Mn/Ni/Zn mixture > Ni ≈ Cu > Mn ≈ Zn at 100mg/l HM.

At concentration of 250mg/l the growth was generally reduced from the reference in the following order Cu/Mn/Ni/Zn mixture 0.0005% where complete inhibition occurred, Mn 5% and

Zn showed the same effect as 50mg/l of 6.67%. Indicating order of toxicity of Cu/Mn/Ni/Zn mixture >> Mn > Zn at 250mg/l HM.

At concentration of 500mg/l the growth was generally reduced from the reference in the following order Cu/Mn/Ni/Zn mixture 0.0001% where complete inhibition occurred and decreased than the start of the experiment but still there were some colonies of N2 survived. Mn showed the same effect as 250mg/l of 5% and Zn 4%. Indicating order of toxicity of Cu/Mn/Ni/Zn mixture >> Mn > Zn at 250mg/l HM.

At concentration of 750mg/l the growth was generally reduced from the reference in the following order Cu/Mn/Ni/Zn mixture showed the same effect as 500mg/l of 0.0001% where complete inhibition occurred and decreased than the start of the experiment but still there were some colonies of N2 survived. Zn showed high toxicity effect with sharp decrease of 0.4% then Mn 3%. Indicating order of toxicity of Cu/Mn/Ni/Zn mixture >> Zn >> Mn at 750mg/l HM.

Finally, at concentration of 1000mg/l the growth was generally reduced from the reference in the following order Cu/Mn/Ni/Zn mixture showed the same effect as 500mg/l of 0.0001% where complete inhibition occurred and further decreased than the start of the experiment but still there were some colonies of N2 survived. Zn showed reduction of 0.007% where complete inhibition occurred, while Mn showed high toxicity effect with sharp decrease of 0.01% and complete inhibition occurred. Indicating order of toxicity of Cu/Mn/Ni/Zn mixture >> Zn > Mn at 1000mg/l HM.

In this study the degree of growth in response to metal ions varied with the metal and the concentration of each heavy metal ion supplementation in the medium and being individually used or supplemented as mixture. Similar observations were reported by **Raja et al., (2006).**

Sokhn et al. (2001), Riis et al., (2002) and Wong et al., (2005) have reported that heavy metals, Cu, Mn, Ni and Zn are essential for bacteria in trace amounts, high concentration are known to be toxic. However, tolerance and adaptation of microorganisms to HM are common phenomenon and the presence of tolerant bacteria in polluted environments has frequently been observed.

Friis and Myers-Keith, (1986) have previously reported the reduction in growth is mainly because of the interaction between the metal cations and the cell surface along with phosphate, carboxyl, hydroxyl and amino groups.

Microbes showing resistance to Ni have generally been isolated and characterized from anthropogenically polluted sites **(Raja et al., 2006).**

Table 16: Effect of heavy metals on growth (TCFU/ml) and biodegradation efficiency (%BD) of *Cellulomonas hominis* N2 on 500ppm phenenthrene.

Applied metals	Concentration (mg/l)	CFU/ml Zero time	CFU/ml 7days	CFU related to the reference %	%BD
Cu/Mn/Ni/Zn mixture	0	$1.4x10^5$	$2x10^9$	---	96.8
	5		$7x10^8$	35	77.2
	10		$3x10^8$	15	74.2
	25		$2x10^8$	10	74
	50		$2x10^7$	1	69.4
	100		$1x10^5$	0.005	20
	250		$1x10^4$	0.0005	15
	500		$2x10^3$	0.0001	13
	750		$2x10^3$	0.0001	8
	1000		$1x10^3$	0.00005	7.6
Mn	0	$2.1x10^5$	$2x10^9$	---	96
	5		$5x10^9$	250	90.2
	10		$5x10^8$	25	86.6
	25		$5x10^8$	25	85.6
	50		$2x10^8$	10	83.6
	100		$1x10^8$	5	80.5
	250		$1x10^8$	5	75.5
	500		$1x10^8$	5	69.8
	750		$6x10^7$	3	49.4
	1000		$2x10^5$	0.01	40.8

Cont. Table 16: Effect of heavy metals on growth (TCFU/ml) and biodegradation efficiency (%BD) of *Cellulomonas hominis* N2 on 500ppm phenenthrene.

Applied metals	Concentration (mg/l)	CFU/ml Zero time	CFU/ml 7days	CFU related to the reference %	%BD
Zn	0	$2.5x10^{5}$	$1.5x10^{9}$	---	97.5
	5		$3.5x10^{9}$	233.33	89
	10		$8x10^{8}$	53.33	87.4
	25		$2.9x10^{8}$	19.33	86.6
	50		$1.7x10^{8}$	11.33	82
	100		$1x10^{8}$	6.67	80.8
	250		$1x10^{8}$	6.67	79.4
	500		$6x10^{7}$	4	76.7
	750		$6x10^{6}$	0.4	57.80
	1000		$1x10^{5}$	0.007	30.4
Cu	0	$1.6x10^{5}$	$1x10^{9}$	---	97.2
	5		$2.7x10^{9}$	270	92.8
	10		$1x10^{8}$	10	79
	25		$9x10^{7}$	9	78
	50		$1.2x10^{7}$	1.2	73.6
	100		$6x10^{6}$	0.6	60
Ni	0	$2x10^{5}$	$3x10^{9}$	---	96.5
	5		$6.6x10^{9}$	220	88.6
	10		$2x10^{9}$	66.67	88.8
	25		$2x10^{8}$	6.67	84.6
	50		$3x10^{8}$	10	83.2
	100		$1.3x10^{7}$	0.43	80

III-4-6. Impact of heavy metals on biodegradation of phenanthrene

Distinct patterns of HM impact on biodegradation of Phe by *Cellulomonas hominis* N2 and its resistance to HM are shown at Table 16 and Fig. 15.

These results show high biodegradation efficiency of *Cellulomonas hominis* N2 on Phe in BSM free of HM, reaching an average value of 96.8%.

Significant variations in the Phe biodegradation patterns in presence of each of the HM used in the study individually or as a mixture of HM ions were observed.

Generally biodegradation efficiencies of strain N2 in the presence of HM Cu, Mn, Ni and Zn supplemented individually or as mixture were consistently lower than that of the reference flask without HM supplementation. The effect of Cu/Mn/Ni/Zn mixture was generally higher than individual HM. In all cases this shows the inhibitory effect of HM on biodegradation of Phe even at low concentrations of HM.

Heavy metals are known to inhibit the degradation of xenobiotics **(Siunova et al., 2007).**

At concentration of 5mg/l HM, biodegradation of 500mg/l Phe was in the following order Cu 92.8%, Mn 90.2%, Zn and Ni nearly reached the same value 89 and 88.6, respectively. The lowest biodegradation efficiency obtained in flasks contained HM mixture 77.2%, indicating toxicity impact of 5mg/l HM on biodegradation of Phe in the following order HM mixture > Ni ≈ Zn > Mn >Cu.

At concentration of 10mg/l HM, biodegradation of Phe was in the following order Ni 88.8%, Zn 87.4%, Mn 86.6%, and Cu 79%. The lowest biodegradation efficiency obtained in flasks contained HM mixture 74.2%, indicating toxicity impact in the following order HM mixture > Cu > Mn > Zn > Ni.

At concentration of 25mg/l HM, biodegradation of Phe was in the following order Zn 86.6%, Ni 84.6%, Mn 85.6%, and Cu 78%. The lowest biodegradation efficiency obtained in flasks contained HM mixture 74% which was nearly the same as that obtained in 10mg/l, indicating toxicity impact in the following order HM mixture > Cu > Mn > Ni > Zn.

At concentration of 50mg/l HM, biodegradation of Phe was in the following order Mn and Ni nearly reached to the same value

83.6% and 83.2%, respectively, Zn 82%, and Cu 73.6%. The lowest biodegradation efficiency obtained in flasks contained HM mixture 69.4%, indicating toxicity impact in the following order HM mixture > Cu > Zn > Ni ≈ Mn.

At concentration of 100mg/l HM, biodegradation of Phe was in the following order Zn, Mn and Ni nearly reached the same value 80.8%, 80.5% and 80%, respectively, and Cu biodegradation efficiency was highly reduced to reach 60%. The lowest biodegradation efficiency obtained in flasks contained HM mixture which was sharply decreased to 20%, indicating toxicity impact in the following order HM mixture >> Cu > Ni ≈ Mn ≈ Zn.

At concentration of 250mg/l HM, biodegradation of Phe was in the following order Zn 79.4% and Mn 75.5%. The lowest biodegradation efficiency obtained in flasks contained HM mixture which was sharply decreased to reach 15%, indicating toxicity impact in the following order HM mixture >> Mn > Zn.

At concentration of 500mg/l HM, biodegradation of Phe was in the following order Zn 76.7% and Mn 69.8%. The lowest biodegradation efficiency obtained in flasks contained HM mixture which was furtherly decreased to reach 13%. Indicating toxicity impact in the order HM mixture >> Mn > Zn.

At concentration of 750mg/l HM, biodegradation of Phe was in the following order Zn 57.8% and Mn 49.4%. The lowest biodegradation efficiency obtained in flasks contained HM mixture which was sharply decreased to reach 8%. Indicating toxicity impact in the following order HM mixture >> Mn > Zn.

At concentration of 1000mg/l HM, biodegradation of Phe was furtherly decreased in the following order Mn 40.8% and Zn 30.4%. The lowest biodegradation efficiency obtained in flasks contained Cu/Mn/Ni/Zn mixture which was decreased to reach 7.6%, this was nearly the same as that obtained in 750mg/l. Results indicating toxicity impact in the following order HM mixture >> Zn> Mn.

HM are often added at low concentrations in the form of trace mineral solutions in microbial cultures to maintain or enhance microbial growth. Cu, Mn, Ni and Zn are also components of several bacterial enzymes. It would therefore not be surprising to observe variable growth efficiencies and substrate removal capabilities with varying HM concentrations, which could either, present a favorable effect or an inhibitory effect depending on their concentrations.

In the present work, the HM added to the medium only at inhibitory concentrations for biodegrading enzymes. No enhancements of degradation capacities than that of the reference flasks free from HM were observed even at low concentrations tested, although enhancement of growth occurred in BSM supplemented with 5mg/l of individual HM tested, as listed in Table 16.

According to **Sokhn et al., (2001),** this can be explained as high levels of HM are directly toxic to enzymes involved in the degradation of toxic intermediates formed from the degradation pathway of Phe. The outcome is incomplete mineralization of Phe and the presumed accumulation of its toxic metabolites and consequently decreases in microbial growth and biodegradation efficiencies was occurred.

It was obvious from the results that *Cellulomonas hominis* N2 showed good biodegradation potentials in absence and presence of HM with various extends according to the HM ions, its concentration and being supplemented alone or as mixture. This can be attributed to its isolation form highly HM and PCS (article in press), which made N2 adapted to those sever conditions. This may be also due to the ability of *Cellulomonas hominis* N2 to produce biosurfactant, where the presence of biosurfactant generally reduces HM toxicity and enhances biodegradation of hydrocarbons **(Sandrin et al., 2000).**

Other investigators reported biodegradation of different hydrocarbons and xenobiotics in presence of HM. **Siunova et al., (2007)** reported degradation of 98% of naphthalene in presence of 100μM Ni using *Pseudomonas chloraphis* PCL1391.

Sokhn et al., (2001), reported that Cu up to 70ppm has little effect on microbial activites during Phe degradation. However, elevated concentrations (700 and 7000ppm) showed marked reduction in microbial activities during the degradation of Phe.

Collorad et al., (1994) reported that *Alcaligenes eutrophus* degraded polychlorinated biphenyls and 2,4-dichlorophenoxyacetic acid effectively in presence of Ni and Zn.

Sandrin and Marier, (2003) reported that Ni in a concentration of 200µM is known to inhibit aerobic biodegradation of naphthalene, biphenyl, xylene and a number of other xenobiotics using *Alcaligenes* and *Pseudomonas* strains.

Riis et al., (2002) reported that Zn up to 262mg/l showed biodegradation of diesel fuel using certain microbial community and Cu showed the highest toxicity to the community used, and reported that degradation process in liquid culture with bacteria revealed significant inhibition by heavy metals.

Edgehill, (1996) reported that in presence of Cu at concentrations of 2 and 8mg/l *Arthrobacter* strain ATCC 33790 did not remove 89-124mg/l of pentachlorophenol.

According to **Amor et al., (2001),** HM inhibit microorganisms by blocking essential functional groups or interfering with essential metal ions incorporation of biological molecules. In some cases microorganisms are resistant to some HM through different possible mechanisms.

According to **Atagana, (2006),** presence of Cu is very inhibitory to the degradation of PAHs. This could be due to the toxicity of Cu^+ to microbial cells as these ions may directly interact with the physiological functions of the cells and possibly the membrane development in the cells. Enzyme activities, which are very important in the microbial degradation of organic substrates, could also be hindered by the presence of copper in the medium. This may explain the reduction of biodegradation efficiency in flasks amended with Cu than that amended with Ni, Mn or Zn, as listed in Table 13. However the degradation in presence of Cu^{2+} occurred due to the utilization of the strongly adapted *Cellulomonas hominis* N2 previously isolated from highly HM and PCS (article in press).

Chen et al., (1997) reported the potential toxic effect of high level of metals on microbial metabolism of petroleum hydrocarbons even though the microorganisms have been previously exposed to heavy metals accumulated over the years.

Effect of HM and PAHs together on growth and biodegradation efficiencies is complex as they both affect eachother.

Lipophilic compounds such as PAHs have a narcotic mode of toxic action and may interact with lipophilic components of cytoplasmitic membranes of bacteria, thus affecting their permeability and structure. Hence in the presence of HM and PAHs together, the penetration of HM may be more easily into microbial cells and affect their functions **(Sikkema et al., 1995, Gogolev and Wilke, 1997 and Shen et al., 2006).** This clearly confirms our results.

The order of toxicities obtained from the effect of HM on the bacterial growth somewhat differs from that obtained from the effect on biodegradation efficiencies. This clearly indicates that the biodegradation efficiencies are not linearly related to growth rates. There must be other factors to be involved; the enzymatic activities of the microorganisms in the tested media may be an important one of these factors. Therefore the bacterial growth is not a limiting factor in their biodegradation efficiency but the types of the enzymes involved in biodegradation process and degrees of their activities have also an important roll.

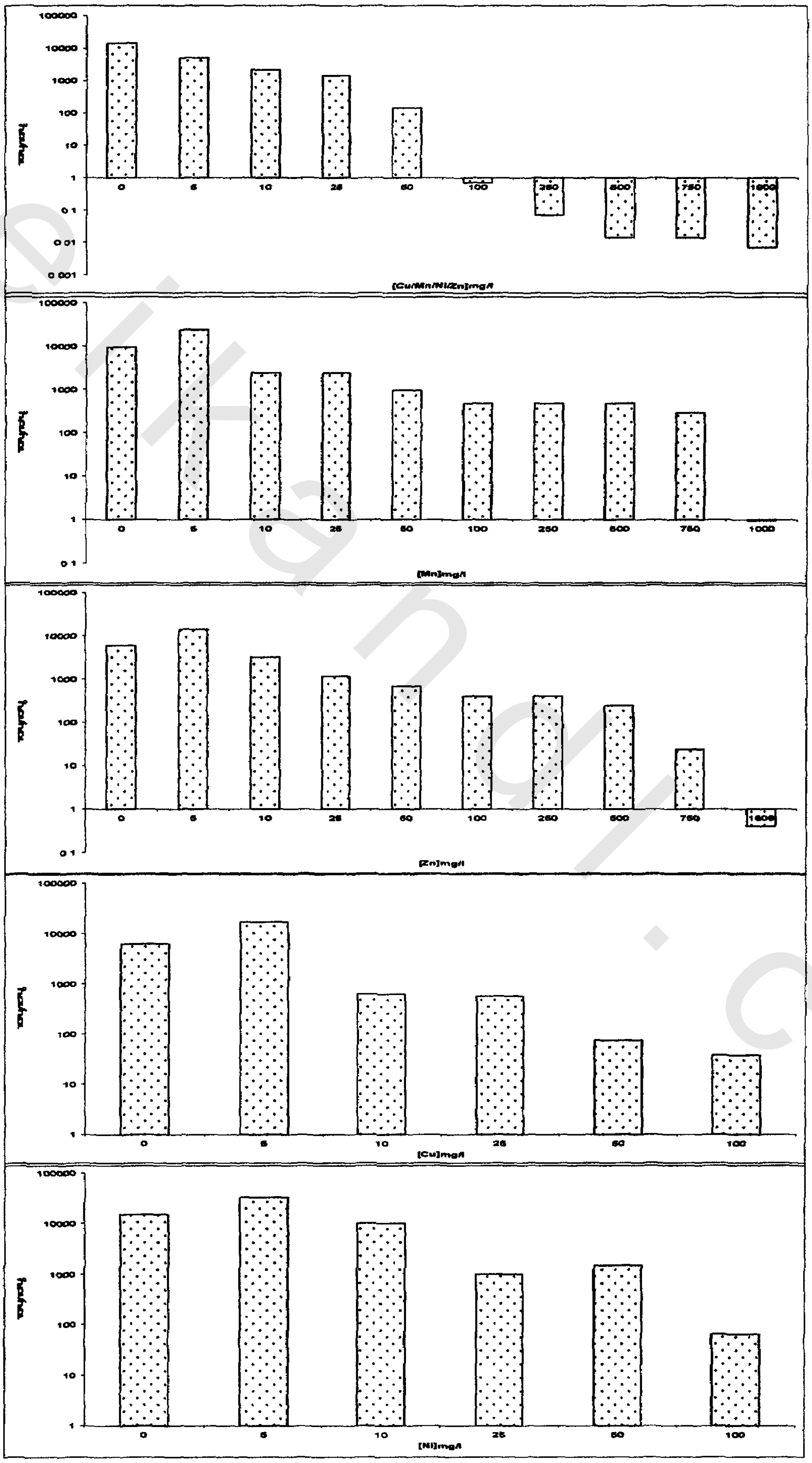

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Fig. 14: Impact of HM on growth of *Cellulomonas hominis* N2 on Phe.

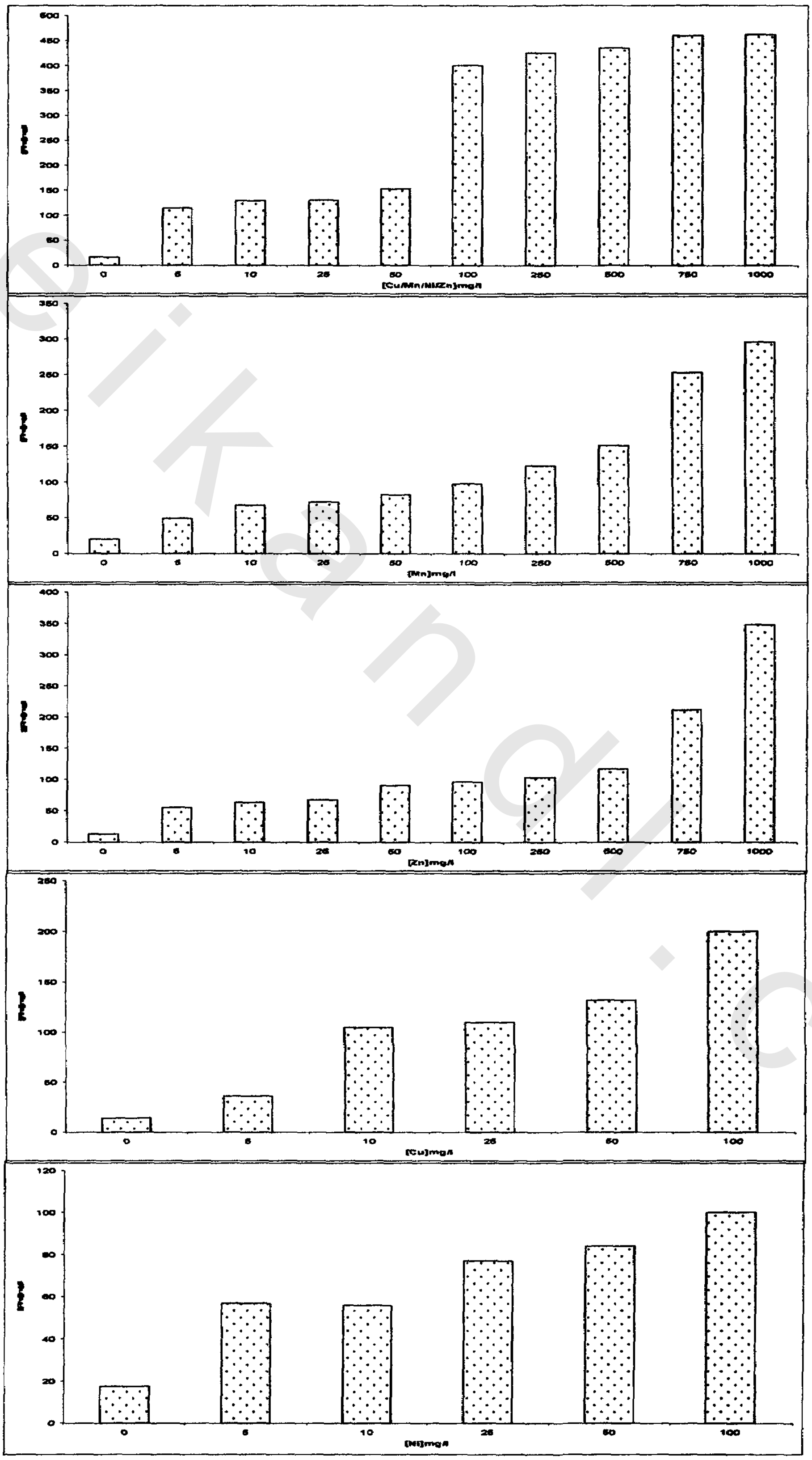

Fig. 15: Impact of HM on biodegradation of Phe.

III-4-7. Biostimulation of petroleum contaminated soil (PCS)

The soil microcosm used in this study corresponded to a feasibility study carried out prior to field implementation of bioremediation technology.

Biodegradation experiments were constructed with bulk soil without sieving. Thereby, degradation could take place at "realistic" condition with pores of different sizes being present. Sterilized soil was not used in this study to mimic the realistic environmental field as much as it could be and because sterilization also can alter considerably the sorption properties of organic matter and then the out come of hydrocarbon biodegradation experiments **(Amellal et al., 2001).** One important characteristic of bioremediation is that it is carried out in non-sterile open environments that contain a variety of organisms **(Watanabe, 2001).**

Mrayyan and Battikhi (2005) reported that contaminated soils that have intrinsically low N and P will require nutrients addition to allow sufficient increase in biomass for environmentally significant hydrocarbon degradation to occur. That was why $(NH_4)_2SO_4$ and K_2HPO_4 were added to the collected PCS for biostimulation process.

Abbondanzi et al., (2006) reported that, chemical analysis and microbial counts are used in the assessment of contaminated sediments and soils and in monitoring the effectiveness of remediation processes.

III-4-7-1. Effect of biostimulation on TPH

The most direct way to measure bioremediation efficiency is to monitor hydrocarbon disappearance rates **(Margesin and Shinner, 2001).**

Fig.16 shows the trend of TPH reduction during biostimulation process. Biostimulation microcosm showed a good reduction of TPH reaching 80% after 35 days of incubation. It was evident from the results listed in Table 17, that indigenous microorganisms responsible for petroleum hydrocarbon degradation presented an adaptation period of (0-10 days) during which no destruction of the pollutant was markedly evident. It was reported that stimulation of pollutant degradation rates may not appear until days or weeks after nutrients are added **(Johnson and Scow, 1999).**

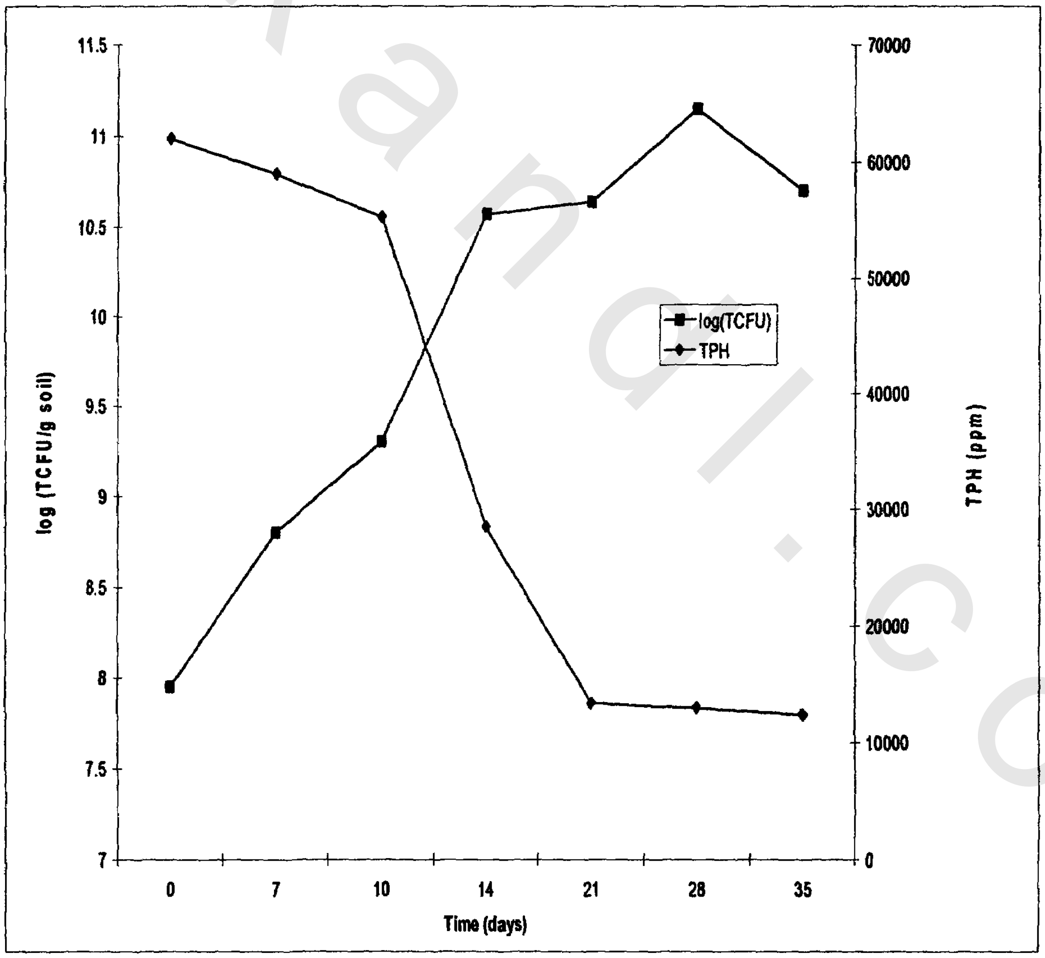

Fig. 16: Effect of biostimulation on growth of indigenous bacteria and TPH of PCS.

Table 17: Biostimulation effect on PCS.

Incubation Period (days)	TCFU/g soil	pH	TPH (wt%)	%BD	Moisture content (wt%)
Zero	$9x10^7$	7.12	6.2	---	38.06
7	$6.3x10^8$	7.10	5.9	4.84	38.06
10	$2x10^9$	7.6	5.53	10.81	38.06
14	$3.7x10^{10}$	7.23	2.85	54.03	38.25
21	$4.4x10^{10}$	7.30	1.34	78.39	38.37
28	$1.4x10^{11}$	7.24	1.30	79.03	40.86
35	$5x10^{10}$	7.13	1.24	80.00	40.90

Biostimulation with addition of inorganic nutrients or fertilizers has long been used as a strategy to enhance the biodegradation rate of contaminants in nutrient limited environments **(Alexander, 1999, Bachoon et al., 2001, Mishra et al., 2004 and Milles et al., 2004).**

Chemical and physical properties of soil have a reflective influence on aeration, nutrient availability, water retention and consequently on biological activity. Some of the important properties are the particle size, the chemical composition, porosity, moisture content, the aeration status, cation exchange capacity and the organic fraction. In particular, particle size affects the surface chemistry of soils and the size of the pores. Thus, the most advantageous pore structure is one in which water is retained but a considerable fraction of the pores remain packed with air. This probably explains the observed high biodegradation in this study because the collected PCS has a greater percentage of sand and a lower percentage of clay. It could also be due to the high organic carbon content of the soil (42,902mg/kg) and the fairly high pH value (7.12).

In –ve control microcosm without addition of nutrients (natural attenuation), showed no significant decrease in TPH concentration reaching 10% after 35 days of incubution period, which shows that the indigenous population can biodegrade the petroleum hydrocarbons in the oily soil but the process is very slow. **Margesin and Shinner, (2001)** reported that natural attenuation process was slower, but nevertheless it was effective over a longer period of time. According to **Alexander (1999),** the natural microbial community, especially in chronic hydrocarbon-contaminated sites usually degrades oil hydrocarbons if suitable conditions are present. Reclamation of crude oil contaminated soil by microorganisms is a well documented process. Indigenous microorganisms utilize the TPH of crude oil as a source of C and energy and break them down to simpler non toxic compounds **(Mishra et al., 2004).** Previous studies have reported that indigenous microbial communities could have a considerable potential to remedy the oil-contaminated soils **(Yu et al., 2005).**

In this study the inorganic forms of NO_3^- and NH_4^+ of soil were not determined, and the total nitrogen was 380mg/kg of soil. Accordingly, the microbial populations of microcosms without nutrients (natural attenuation) had to use part of total nitrogen sources, probably through slow transformation to assimilable forms and its immediate incorporation to their biomass, and as nitrogen acquired by fixation. In fertilized microcosms, the immediate availability of assimilable forms was translated into rapid use of TPH, this may explain this phenomenon. Similar observations were reported by **Sabate et al., (2004)** and attributed also to the availability of nutrients.

Reaching only 80% biodegradation percentage reflecting the treatment difficulty of a weathered soil contaminated with a high crude oil concentration. Similar results were reported by **Trindade et al., (2005).**

Presence of HM such as zinc, copper, cadmium, nickel, chromium and manganese at different concentrations have been reported to have impending effects on the degradative efficiencies as it inhibited the enzyme activities of soil microorganisms **(Baldrian et al., 2000, Wong et al., 2005 and Atagana, 2006).**

The presence of HM in the soil has been reported to inhibit the enzyme activities during biological degradation of hydrocarbons **(Wong et al., 2005).** It could also be due to the microorganisms attacking the metal ions and as a result were not degrading the hydrocarbons effectively **(Alexander, 1999).**

As described in other studies, the degradation pattern of organic chemicals in soil usually shows a rapid initial phase of descent followed by a period of little or no change in concentration. This kinetics is known as the *"hockey slick"* phenomenon **(Alexander, 1999).** A depletion of nutrients, a descent of microbial populations, lower bioavailability and higher recalcitrance of residual contaminants explain this kind of dynamic **(Sabate et al., 2004).** In our case, as shown in Fig. 16 the additional supplementation of nutrients (every 14 days) did not decrease the effect of this kinetics. Similar observations were reported by **Sabate et al., (2004)** and attributed to the accumulation of toxic intermediate metabolites that caused the lack of complete contaminates biodegradation in soil.

Although there was a good microbial population by the end of the experiment (35 days), the residual TPH in the microcosm was 12,400mg/kg. Similar observations were reported by **Nocentini et al., (2000).** An explanation can be that the residual fraction of contamination at the end of the test was hardly bioavailable, not available and/or not biodegradable.

Margesin and Schinner (1998) reported that between 10-30% of the initial soil pollution remains in soil after bioremediation techniques have been applied. Complete hydrocarbon reduction cannot occur due to their low bioavailability, especially after the labile compounds are being used by the microorganisms and the accumulation of recalcitrant compounds occurred.

According to **Uraizee et al., (1997) and Trindade et al., (2005),** crude oil components are differentially degraded; branched alkanes are more resistant to microbial degradation than n-alkanes due to their molecular structure. Moreover, the biologically inert fraction, as asphaltenes, also affects the degradation of crude oil in soils because they inhibit the transport of the biodegradable fraction decreasing the rate and extent of biodegradation. The more recalcitrant fraction of contamination seems to be degraded with a rate that is about an order of magnitude lower than the one observed for other components. As a consequence, after an initial period of biological treatment, the hydrocarbon concentration tends to stabilize at a value that can be called residual concentration **(Margsin et al., 2000 and Nocentini et al., 2000).**

III-4-7-2. Bacterial growth and biodiversity during biostimulation process

It is desirable to monitor the fate of indigenous microorganisms in order to prove its contribution to pollutant degradation and to assess its influence on the ecosystem **(Watanabe, 2001).**

Immediate increase in the bacterial populations, as listed in Table 17 and illustrated in Fig. 16 indicated that the indigenous bacteria are well adjusted to their environment. According to **Bento et al., (2005),** this rapid growth could ensure rapid degradation of the pollutant. However, **Brown et al., (1991)** reported that the increase in total bacterial numbers couldn't be unambiguously associated with enhanced degradation, as it was related to increases in the diversity of oil degrading population and with increase in mineralization rates for specific hydrocarbons. This might explain the presence of two bacterial strains *Brevibacterium otitidis* N1 and *Cellulomonas hominis* N2 in the microcosm with the abundance of N2 all over the incubation period. No increase in TCFU was recorded in the negative control microcosm throughout the incubation period, but bacterial isolates N1 and N2 were the only culturable microorganisms found and the abundant was N2.

The increase in bacterial population may be due to the production of biosurfactant which induced desorption of hydrocarbons from soil to the aqueous phase of soil slurries leading to increased microbial mineralization, either by increasing hydrocarbon solubility or by increasing the contact surface with hydrophobic compounds and may lead also to increase in microbial population **(Moran et al., 2000, Rahman et al., 2003, Maier, 2003, Mukherjie and Das, 2005 and Das and Mukherjie, 2007).**

Biosurfactant secreted by bacteria are more effective than chemical surfactants in enhancing the solubility and biodegradation of petroleum hydrocarbons including PAH **(Cybulski et al., 2003, Wong et al., 2005 and Das and Mukherjie, 2007).**

In the present study *Cellulomomnas hominis* N2 was found to be efficient biosurfactant producer which offered the advantage of a continuous supply of natural, non toxic and biodegradable surfactants at low cost for solubilizing the hydrophobic oil hydrocarbons prior to biodegradation. **Bento et al., (2005)** reported that bacteria that produce emulsifiers or biosurfactants may have a selective advantage because they can counter the increased viscosity and decreased H_2O solubility of the hydrocarbons, so increase the biodegradation process. According to **Straube et al., (1999)**, light oil theoretically stimulate the production of surfactant and act as co-solvent, increasing the bioavailability of hydrophobic contaminants by helping to desorb them from soil particles. Consequently, the biosurfactant production increases the accessibility of petroleum hydrocarbons to soil bacteria, enhancing the biodegradation process. The biodegradation of light fractions was very obvious during biostimulation process as shown in the GC chromatograms of the oil extracted from PCS under study Fig. 17.

It has been reported previously that bioremediation is negligible if the population of hydrocarbon-degrading microorganisms is less than 10^5 CFU/g in soil **(Mishra et al., 2001).** This might explain the good biodegradation of TPH obtained in biostimulation microcosm as the total viable count at the start was high $9x10^7$.

Compared to metal toxicity in soils, toxicity in broth media is clearly higher. Similar observation was reported by **Gogolev and Wilke, (1997),** this is mainly due to the metals being less available to bacteria in soil because of their adsorption to humic substances, oxides and clay minerals.

Increase in bacterial population leads to the rapid assimilation of the available nutrients, thus depleting the nutrients reserves. Consequently, the objective of readdition of nutrients every 14 days to soil microcosm was to restore the availability of essential nutrients. Several researchers have recently described an increase in microbial activity and rate of biodegradation following addition of inorganic nutrients **(Radwan et al., 2000 and Rahman et al., 2003).**

III-4-7-3. Changes in pH during biostimulation process

The trend of changes in pH allover the incubation period was recorded and listed in Table 17. The recorded pH is nearly around neutral value, which might have enhanced the biodegradation process **(Alexander, 1994).**

The decrease recorded may have been due to the formation of low molecular weight organic acids during the degradation of the carbon compounds **(Aislabie et al., 2006 and Marin et al., 2006). Rahman et al., (2003)** recorded the increase in pH and attributed this to the release of byproducts during hydrocarbon degradation.

Soil pH is often found to have the largest effect owing to its strong effects on solubility and speciation of metals both in soil as a whole and particularly in the soil solution. Thus, each unit decrease in pH results in approximately two fold increases in the concentration of metals **(Aislabie et al., 2006).** The pH recorded around neutrality with an average of 7.25 might have limited the adverse effect of HM. pH values > 7.0 decrease the HM solubility drastically **(Riis et al., 2002).**

III-4-7-4. Change in moisture content during biostimulation process

Moisture content and aeration were established as key factors **(Sabate et al., 2006).** Table 17 shows the trend of change in moisture content allover the incubation period. The initial drop in soil moisture content during the first 10 days of incubation was expected because in heavily polluted soils, oil coating the surface of soil particles makes soil more hydrophobic and water droplets adhere to the hydrophobic layer formed, this prevents wetting of the inner parts of the soil aggregates and reduce the water holding capacity of the soil **(Dibble and Bartha, 1979 and Odokuma and Dickson, 2003).** This may also explain the increase of moisture content with the decrease in TPH of the PCS during the biostimulation process as listed in Table 17. Fluctuation in moisture content was due to soil drying and evaporation of H_2O, and as the soil sample was radiated through the introduction of nutrients, continuous watering and tilling, moisture content increased.

Water was added day after day to keep the moisture content in all microcosms with an average value of approximately 40%. In soils, water contents of between 20-70% capacities are generally optimal for microbial activity **(Morgan and Watkinson, 1989).** The high sand component of the used PCS as listed in Table 13 might have allowed adequate water retention and aeration, both conditions of which are necessary for the adequate microbial growth in the soil. The presence of moisture in the soil might have also enhanced the solublization of the soluble hydrocarbons which consequently enhanced the biodegradation process **(Atagana, 2006).**

Wet conditions may limit oxygen availability; hence, aeration may be required to enhance aerobic hydrocarbon biodegradation. Each microcosm was mixed day after day to ensure good aeration condition.

Periodic tilling has commonly been employed in soil bioremediation studies to ensure adequate supply of oxygen to the microorganisms for faster degradation **(Mishra et al., 2001 and Atagana, 2006).**

III-4-7-5. Effect of biostimulation on different oil components

GC/FID analyses of the oil extracted from the PCS before biostimulation (Zero time) and during prescribed time intervals of biostimulation process are depicted in Fig. 17. GC analysis showed that alkanes' level was significantly reduced and all the components of the complex hydrocarbons mixture were consumed to a different extent and with different depletion rates after biostimulation treatment, as listed in Table 18. There was no significant loss observed in the aliphatic hydrocarbons of the negative control microcosm after 35 days of incubation except in the relatively light fraction C_{10}-C_{13}, where there was a loss of approximately 8% this may be due to the natural weathering process; vaporization and dissolution **(Kaplan and Kitts, 2004)** and /or biodegradation **(Wang et al., 2001).**

TRP/UCM ratio increased during the first two weeks of treatment, and then decreased to the end of incubation period, 5 weeks. This indicates that the biodegradation rate of UCM (aromatics, naphthenes and alicyclic hydrocarbons) during the first two weeks was higher than that of TRP (linear and branched alkanes). This was very obvious in % BD of UCM and TRP where they reached 58.81% and 49%, respectively during the first two weeks. The biodegradation rate of UCM became slightly lower than that of TRP to the end of incubation period with %BD of 79.25% and 80.79%, respectively at 35 days of incubation. As generally the sequence of hydrocarbon degradation is as follows: alkanes fraction biodegraded extensively followed by aromatic fractions, then NSO and asphaltenes fractions biodegraded the least **(Mishra et al., 2004).**

Ratios of n-C_{17}/Pr, n-C_{18}/Ph and Pr/Ph, and CPI values were calculated over the prescribed time intervals and recorded in Table 18, n-C_{17}/Pr and n-C_{18}/Ph ratios have been used as indicator of biological degradation. They showed continuous decrease allover the incubation period indicating that the biodegradation of n-alkanes was higher than that of iso-alkanes. This was very obvious in % BD of n-C_{17} and n-C_{18}, which reached 95.11% and 92.61%, respectively and % BD of Pr and Ph, which reached 74.22% and 73.30%, respectively at the end of incubation period, 35 days. Pr/Ph ratio fluctuated throughout the incubation period and didn't follow a certain trend. Similar observations were reported by **Bachoon et al., (2001).**

Braddock et al., (1999) reported that the loss of linear alkanes was more strongly affected by nutrient addition than branched alkanes. CPI value remained nearly the same ≈ 1 allover the incubation period indicating no biogenic addition **(Wang et al., 2001).**

These results suggest that the conditions in the biostimulation microcosm with nutrients and aeration are conductive to increased microbial growth and metabolism of petroleum hydrocarbon utilizes.

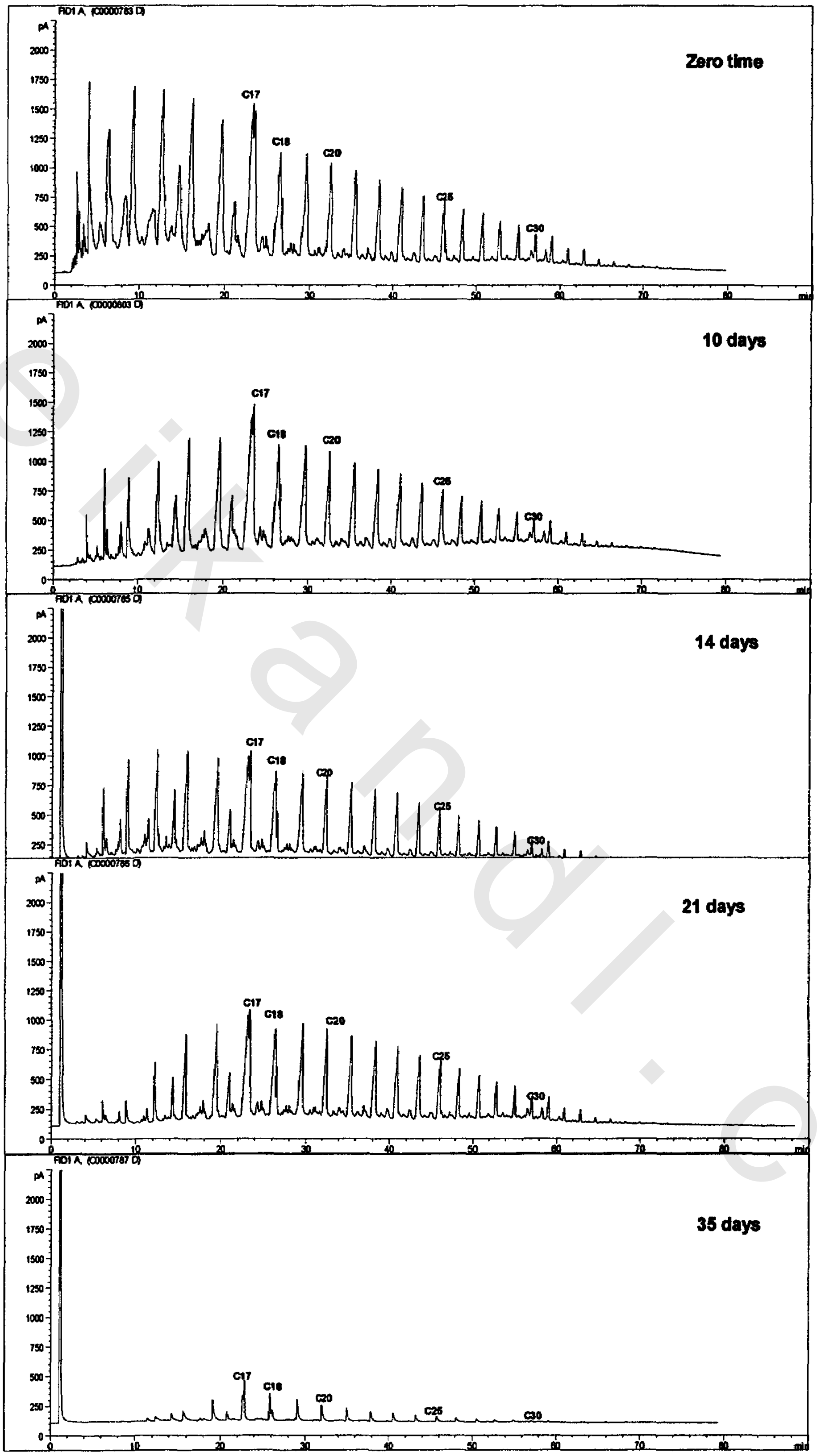

Fig. 17: GC chromatogram of oil extracted from PCS before and after biostimulation.

Table 18: Biostimulation effect on hydrocarbon distribution of PCS.

Parameters	Incubation period (days)				
	zero	10	14	21	35
C_o-C_{fin}	C_{10}-C_{42}	C_{11}-C_{42}	C_{11}-C_{42}	C_{11}-C_{39}	C_{13}-C_{38}
TRP/UCM	0.95	1.12	1.17	0.88	0.85
n-C_{17}/Pr	3.16	2.29	2.18	2.52	0.60
n-C_{18}/Ph	6.41	5.02	4.89	4.87	1.79
Pr/Ph	3.45	3.33	3.18	3.33	3.08
CPI	1.04	1.10	1.11	1.10	0.99
%TRP	48.71	52.75	53.87	46.81	45.91
%UCM	51.29	47.25	46.13	53.19	54.09
%BD of TRP	----	3.31	49	79.36	80.79
%BD of UCM	----	17.92	58.81	77.36	79.25
%BD of n-C_{17}	----	27.5	47.59	50.99	95.11
%BD of n-C_{18}	----	21.68	37.37	45.53	92.61
%BD of Pr	----	----	23.89	32.32	74.22
%BD of Ph	----	----	17.23	29.83	73.30