# Assessment on Phytoremediation of Crude Oil Polluted Soils with *Achillea millefolium* and Total Petroleum Hydrocarbons Removal Efficiency

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*The study presents experimental data on phytostabilization/ phytoremediation of 5.57% total petroleum hydrocarbons contaminated soils, using plants of the Achillea millefolium species. Studies have been conducted on pots placed in outdoors in three experimental variants in the absence/presence of an additional treatment: 1. Contaminated soil; 2. Contaminated soil treated with fertilizer agent, stabilized sewage sludge; 3. Contaminated soil treated with fertilizer and amendment based on indigenous volcanic tuff with clinoptilolite. After five months of growth the plant roots have formed a strong twinned network throughout the vegetation soils of volume pots. The content reduction of the total petroleum products in the contaminated soil was 45.4% and 65.4% for the variant of contaminated soil treated with fertilizer agents, anaerobically stabilized sewage sludge from the municipal treatment plant in the absence/presence of the amendment with indigenous volcanic tuff. Soil polluted with petroleum hydrocarbons does not reduce the total content of phenolics and antioxidant capacity of Achillea millefolium crop; rather it can be boosted when the soil is treated with stabilized sewage sludge and indigenous volcanic tuff.*

*Keywords: total petroleum hydrocarbons, phytoremediation, sewage sludge, volcanic tuff, Achillea millefolium*

Currently the concept of using plants to remedy soils contaminated with inorganic or organic pollutants is based on observations regarding the faster reduction of pollution in vegetated areas than on polluted soils devoid of vegetation [1÷4]. On the other hand the correlation between current agricultural technology and the phytoremediation potential of a contaminated soil is not well determined, the most advanced agricultural techniques can not increase the speed and extent of the phytoremediation processes. Plants are primarily affected by the most persistent pollution compounds that can cause disturbances in the rhizosphere system, the acquisition of micro and macronutrients [5÷7]. A number of components of crude oil *i.e.* aromatic hydrocarbons are the most dangerous and recalcitrant, all the while stimulating pathogenic microbial potential. Polycyclic aromatic hydrocarbons, however, have a tendency to adsorb on the soil particles and they remain non-biodegradable in these forms a large period of time [8÷10]. From these soil particles the aromatic compounds may not be translocation in plants in bioavailability forms. Vegetation installed on total petroleum hydrocarbons (TPH) polluted soil can take over and carry pollutant compounds according to their physico-chemical characteristics [11÷14]. It was found that a polluted soil can be invaded by certain plants, weeds, specific to a particular geographical location. Weeds are gradually replacing the selected cultivated crops because pollution is reduced. The efficiency of the phytoremediation process depends on the age of the polluted landfill and on the plant species selected to receive the phytoremediation strategy. The age of the pollutants and the pollution type are limiting factors for the biodegradability of these compounds [15÷16].

*Achillea millefolium L.* (Asteraceae), known as yarrow is very common in mountain meadows, pathways, crop fields and home gardens and has been used as medicine by many cultures for over 3000 years and today appears in the national Pharmacopoeias of countries such as Germany, Czech Republic, France, Switzerland, Britain and North America [17÷19]. Its infusion, alcohol extract or decoct is widely used as a remedy to treat digestive problems, diabetes, hepato-biliary diseases, skin and mucosa inflammations and amenorrhea, and also consumed for its antitumor, antimicrobial, anti-inflammatory and antioxidant properties, among others [20]. Powerful antioxidant properties of this plant are associated with the presence of flavonoids such as apigenin, luteolin and rutin and casticin (vitexicarpin) matricin and other proazulenic sesquiterpene lactones explain the anti-inflammatory properties [21÷22].

The purpose of this study is to obtain data on the effect of vegetation on yarrow crop in reducing TPH content in the soil. More, the study includes relations between TPH pollution and different parts of the plant and as well, about the antioxidant activity of plants methanolic extract.

## Experimental part

### *Materials and methods*

#### Soils experimental variant

The soil used in this experiment was taken from a collection point and the temporary storage of crude oil coming from several probe extraction operations from wells. The soil poses recent and continuous crude oil pollution, since the initial degree of soil pollution was at high level, 17%. For these studies the high polluted soil was mixed with unpolluted soil. Unpolluted soil, blank variant,

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was taken from an agricultural area located near the polluted site. The mixture was prepared as follows: polluted and unpolluted soils were cleaned of plant debris, stones etc. Soils were dried and shredded. They were sieved through sieves with mesh the size of 10 mm for polluted soils and 1mm for unpolluted soils. The mixture contains two parts unpolluted soil and one part polluted soil. The studies were carried out in three experimental variants: polluted soil with an amount of 55.45±0.25/kg D.M. total petroleum hydrocarbons (TPH), untreated and fertilized with sewage sludge anaerobically stabilized in the absence/presence of an amendment indigenous volcanic tuff. The resulting mixture was used as such or fertilized with anaerobic stabilized sewage sludge [23] in an amount of 5 $t \cdot ha^{-1}$ in the absence/presence of an inorganic amendment [24÷25], modified indigenous volcanic tuff name *Aln Tuf*. [27], which was prepared in ECOIND laboratory. The amount of inorganic amendment used was 2.0 $t \cdot ha^{-1}$.

Plant characteristics

The plant selected for the phytoremediation study is *Achillea millefolium* species, known commonly as yarrow. The plant is part of the *Asteraceae* family, an herbaceous perennial plant with hairy leaves and white or rosy flowers. It grows in plains or in subalpine regions. It originates from Europe and West Asia. The plant was picked up together with a layer of unpolluted soil of 2 cm in which the rhizosphere was occupied with a dense network of root fibers.

Soil physico-chemical characteristics determination

The soil samples were analyzed to know physical and chemical properties by standard methods: *p*H was analyzed by glass electrode using 1:2.5 soil: distilled water ratio after two hours equilibration (ISO 10390:2005) with Thermo Orion pH meter, organic carbon was analyzed by standard titration method with Mohr solution, (STAS 7184/21-82), total nitrogen by modified Kjeldahl digestion (R ISO 11261:2000). In order to determine the variation of TPH in soils, their concentration is periodically determined, in the top layer of the vegetation pot (2 cm depth). The soil is dried and ground through a 5 mesh sieve. To determine the TPH from the soils an analysis is performed periodically of the concentration [8], in the upper level:1) 0.5-1.0g of dry soil are weighed (M), then add 5g $Na_2SO_4$ anhydrous and 25mL solvent, $CCl_4$ (Fluka Analytical); 2) 30 min stirring at 50 rotations/min and then filtered; 3) the glass and filter paper (Whatman No. 4 paper) are washed with solvent $CCl_4$, which is added to the filtrate; 4) the filtrate is evaporated on water bath; 5) the residue is dissolved in $CCl_4$, then passed through the chromatographic column filled with aluminium oxide. The elute was collected in a tarred capsule, $m_1$[g]; 6) $CCl_4$ is evaporated at room temperature and weighed at constant mass, $m_2$ [g]; 7) the same is done for the control from 28 mL $CCl_4$ ($m_3$ – mass of capsule without control residue [g], $m_4$ – mass of capsule with control residue [g]); 8). Calculating TPH: TPH [$g \cdot kg^{-1}$] $=1000 \cdot [(m_2 - m_1) - (m_4 - m_3)] \cdot M^{-1}$

Nutrient characteristics determination

The sewage sludge samples was analyzed to know physical and chemical properties: *p*H was analyzed by glass electrode (SR EN ISO 10523-2012), using Thermo Orion pH meter, D.M/humidity was analyzed by SR ISO11465:1998, organic carbon was analyzed by standard titration method with Mohr solution, nitrogen by ASTM D 5373-08 and SR ISO 10694-98, STAS 398-92 and phosphorus by STAS 12205-84 (with Specord 205 Spectrophotometer, Analytic Jena, at 830nm). Triplicate determination was made.

Herb crops in pots

Experiments are carried out in vegetation pots equipped with 6.5 kg soil. The experimental variants used were: blank normal soil (symbol M), soil polluted with TPH (symbol P), the variant with polluted soil fertilized with anaerobically stabilized sewage sludge (symbol P+B) and the variant with polluted soil fertilized and treated with modified volcanic indigenous tuff (P+B+T symbol). For the experiments the plants are put together with a layer of the original soil of 2-3 cm in pots already prepared. Each variant was studied in three replicates. The obtained cultures for the experimental variants were periodically watered and monitored.

Extracts obtaining

The aerial parts (leaves, stalks, and stems) sample of *Achillea millefolium* plants were harvested at full flowering stage in 2013, summer. *Achillea millefolium* was air-dried at room temperature for 7 days, and then the dry plant was cut and pulverized. The dried, powdered plant material (5g) was macerated for 7 to 14 days using p.a. methanol (50 mL) as solvent and subsequently the extract was filtered through a Whatman No. 4 paper. The extracts from *Achillea millefolium* grown in pots were named: $E_M$ (plants grown in normal soil, unpolluted), $E_{P+B}$ (plants grown on polluted soil fertilized with anaerobically stabilized sewage sludge) $E_{P+B+T}$ (plants grown on polluted soil fertilized and treated with modified volcanic indigenous tuff).

Antioxidant activity: radical scavengers assay

Detection of radical scavengers' activity was made by DPPH• (1,1-Diphenyl-2-picrylhydrazyl, 0.5 mM) assay in triplicate. Quercetin, $C_{15}H_{10}O_7$, [2-(3,4-dihydroxyphenyl)-3,5,7- trihydroxy-4H-chromen-4-one], p.a. Fluka, was used as positive standards. In tubes was inserted 0.2 mL of diluted sample, 0.8 mL of methanol and 2 mL of DPPH•, 0.5 mM. The solution was allowed to stand for 30 min, and the absorbance was read at 517 nm using a spectrometer (T90-UV-VIS Spectrometer PG Instruments Ltd). The free radical-scavenging activity was calculated according to the equation: $\%\ inhibition = 100 \cdot (A_{DPPH\bullet} - A_S)/A_{DPPH\bullet}$, where: $A_S$ is the absorbance of the solution containing the sample at 517 nm, and $A_{DPPH\bullet}$ is the absorbance of the DPPH• solution [29].

Total Phenol Assay

Total phenolic contents were examined as GAE (gallic acid), expressed as GAE $mg \cdot g^{-1}$ D.M. 0.5 mL of *Achillea millefolium* extracts were transferred to glass tubes, to which 2.5 mL Folin-Ciocalteu reagent (diluted 1:10) was subsequently added and incubated at room temperature for 10 min. 2 mL of sodium bicarbonate ($75\ mg \cdot mL^{-1}$) was added to the mixture and it was incubated for 5 min at $50^0$C and then cooled, and its absorbance was measured at 760 nm using a spectrometer (T90-UV-VIS Spectrometer PG Instruments Ltd). The sample absorbance was compared to gallic acid absorption. A prior calibration curve of gallic acid was obtained [30].

**Results and discussions**

*Soil and nutrients characteristics*

In table 1 are shown the characteristics of unpolluted and polluted soil variants, untreated and treated which were used in the experimental study block.

| No. | Soil type | *Physical-chemical characteristics | | | | | |
|---|---|---|---|---|---|---|---|
| | | pH | Organic carbon [%] | Total petroleum hydrocarbon [%] | Total N [%] | C:N ratio | C:N Recommended ratio [26] |
| 1 | Blank - unpolluted soil | 6.8 | 1.30 | 0.001 | 0.06 | 22 | 10÷15 |
| 2 | Polluted soil | 7.4 | 19.2 | 5.57 | 0.12 | 160 | 10÷15 |

*Triplicate determination was made.

**Table 1**
PHYSICAL AND CHEMICAL CHARACTERISTICS OF POLLUTED AND UNPOLLUTED SOIL USED IN THE PHYTOREMEDIATION EXPERIMENTAL STUDY

| No. | Fertilizer agent | Physical-chemical characteristics | | | | |
|---|---|---|---|---|---|---|
| | | pH | Humidity [%] | Organic matter [%] | Total N [%] | Total P [g·kg$^{-1}$ D.M.] |
| 1 | Sewage sludge | 8.5 | 91.5 | 59.78 | 1.138 | 1.107±0.050 |

**Table 2**
CONTENT OF NUTRIENTS IN THE USED FERTILIZER AGENT

From table 1, it can be seen that the ratio of carbon: nitrogen in polluted soils is C:N = 160.

This ratio is 10-15 higher than the agricultural limits for plant crops [26]. To improve it, it is recommended the addition of either organic fertilizer containing high amounts of nitrogen or mixtures of organic fertilizer suitable inorganic amendments, so that chosen treatment can stimulate the growth of plants [16, 23].

In table 2 are shown the characteristics of the used fertilizer agent, stabilized sewage sludge.

The inorganic amendment based on indigenous modified tuff Tuf Aln was prepared in the ECOIND Laboratory according to the ECOIND patent [27].

*Assessment of phytoremediation, growth performance of Achillea millefolium and total petroleum hydrocarbons removal efficiency*

In this study we followed the resilience of *Achillea millefolium* plant seedlings taken from a normal meadow, regarding the experimental soil variants and the degree of reduction of the TPH content in the soil for a study period of 4 months of growth. Furthermore, plant species probably gradually adapted to the presence of recalcitrant pollutants in the soil in the same way as in the other variants [7÷9]. In table 3 are presented the characteristics of plants cultivated on the experimental polluted soil variants respectively polluted soils fertilized in the absence/ presence of the modified indigenous volcanic tuff amendment vs. characteristics of plants cultivated the blank variant, M.

Three vegetative cycles were followed: first, from 1March to June; second, from 2 July to August, and third from 3 September to October. After each vegetative cycle, the inflorescences were harvested. It must be mentioned that the plants produce new blossoms after each inflorescence harvesting. In the first complete cycle of vegetation which was held in March-June, the plants placed on the polluted and treated soil developed through this cycle similarly to the plants on the unpolluted soil. From the data presented in table 3 it is observed: an increase in the plant height from the first cycle than the ones grown on the second cycle of vegetation, July-August. The treatment of polluted soil with fertilizer agent and amendment determined a vegetative cycle of plants similarly with the one observed on the unpolluted soil.

Also, from table 3 it can be seen that plants of *Achillea millefolium* grown in polluted soils presents signs of suffering in the upper parts of tissue. Plants do not grow or bloom. In the third vegetative cycle, in the period September-October, plants presented biometric parameters similar to those of second cycle, in similar experimental variants. In table 4 are presented the characteristics of the flowers belonging to the plants grown on the experimental variations of polluted soils, respectively polluted soils fertilized in the presence/absence of a modified indigenous volcanic tuff amendment. It is noted that plants grown on polluted, untreated soil, did not produced flowers in the first vegetative cycle, but they have flourished in the second and third vegetative cycle. The inflorescences that are harvested by the first growth cycle of the fertilized and amended soils have the size in the range of 1.8÷4.0 cm. These flowers were smaller than those collected from the blank experimental variants.

From the data presented in table 4 it is observed: an increase in the inflorescences from the first cycle than the ones grown on the second and third cycles of vegetation. The sizes of inflorescences off second and third vegetative cycles were small than those harvested flowers from similar variations of vegetation cycle.

| No | Date | Experimental variant/plant height [cm] (minimum ÷ maxim value interval and *mean values) | | | |
|---|---|---|---|---|---|
| | | M | P | P+B | P+B+T |
| Vegetative cycle 1March÷June (four months) | | | | | |
| 1 | 05.03.2013 | 3÷7 *5.5 | 1÷2 *1.3 | 3÷7 *4.6 | 3÷7 *5.9 |
| 2 | 25.05.2013 | 8÷12 *9.6 | 3÷4 *2.7 | 6÷14 *9.6 | 6÷16 *11.4 |
| 3 | 25.06.2013 | 20÷22 *21.5 | 3÷6 *4.3 | 16÷18 *17.3 | 18÷24 *22.1 |
| Vegetative cycle 2 July÷August (six months) | | | | | |
| 1 | 10.07.2013 | 3÷7 *5.7 | 3÷7 *5.0 | 3÷7 *5.3 | 3÷7 *5.5 |
| 2 | 30.08.2013 | 10÷12 *11.5 | 3÷8 *6.5 | 10÷15 *13.2 | 10÷15 *14.1 |
| Vegetative cycle 3 September÷October (eight months) | | | | | |
| 1 | 30.10.2013 | 8÷10 *8.3 | 5÷7.5 *6.8 | 8÷12 *10.1 | 10÷13 *12.5 |

**Table 3**
HEIGHT VARIATION IN THE PLANTS FROM THE *ACHILLEA MILLEFOLIUM* SPECIES GROWN IN THE EXPERIMENTAL VARIATIONS ON POLLUTED SOILS IN THE ABSENCE/PRESENCE OF A MODIFIED INDIGENOUS TUFF.

| No | Date/ Stage | Experimental variants/inflorescence diameter [cm] (minimum — maxim value interval and *mean values) | | | |
|---|---|---|---|---|---|
| | | M | P | P+B | P+B+T |
| Vegetative cycle 1 | | | | | |
| 1 | The first flowering | 2.0÷4.0 *3.2 | Plants do not bloom | 1.5÷2.0 *1.6 | 1.8÷4.0 *3.5 |
| Vegetative cycle 2 | | | | | |
| 2 | The second flowering | 2.0 | 1÷1.5 *1.2 | 1.0÷1.5 *1.2 | 1.0÷2.5 *1.8 |
| Vegetative cycle 3 | | | | | |
| 3 | The third flowering | 2.2÷3.1 *2.5 | 0,8÷1.0 *0.9 | 1.1÷1.4 *1.2 | 2.5÷2.9 *2.7 |

**Table 4**
CHARACTERISTICS OF PLANT INFLORESCENCE OF THE *ACHILLEA MILLEFOLIUM* SPECIES CULTIVATED ON THE EXPERIMENTAL VARIANTS (MINIMUM, MAXIM AND MEAN VALUES)

| No | Experimental variants | TPH content in the soils, [g·kg$^{-1}$ D.M.] | | | |
|---|---|---|---|---|---|
| | | Inițial | I Cycle | II Cycle | III Cycle |
| 1 | P | 55.71±1.53 | 53.30±1.48 | 51.65±1.22 | 45.53±1.45 |
| 2 | P+B | 55.31±1.53 | 46.57±1.43 | 30.16±1.13 | 23.57±0.91 |
| 3 | P+B+T | 55.21±1.53 | 40.89±1.41 | 19.07±0.76 | 16.92±0.72 |

**Table 5**
QUANTITIES OF TPH DETERMINED IN THE CULTIVATED SOIL OF THE STUDIED EXPERIMENTAL VARIANTS, AND THE EFFICIENCIES REDUCTION IN THE TREATED SOIl

In table 5 it is shown the quantity of TPH determined periodically in the cultivated soil of the studied experimental variants and in figure 1 it is shown the efficiency of the pollution reduction.

Depth of soil sampling for the analysis of petroleum products was 2-5 cm. From the table 5 it is observed the gradual reduction of TPH in the cultivated soils treated with organic fertilizer in the absence/presence of an amendment as modified indigenous tuff. The amount of TPH in the upper layer of rhizosphere gradually decreases according to the alternation of plant phenophases of development and according to the treatment applied to polluted soil. So, for example, TPH in contaminated soils, decreased from 55.71 g·kg$^{-1}$ D.M to 45.53 g·kg$^{-1}$ D.M. during the 8 months of vegetation. The treatment of contaminated soil with an appropriate fertilizing agent (the second experimental variant) resulted in plant growth due to the increased metabolism and reduction of TPH from 55.31 g·kg$^{-1}$ D.M to 23.57 g·kg$^{-1}$ D.M.

The treatment of contaminated soil with selected fertilizing agent and volcanic tuff determined a fast development of the plants in the soil and the TPH reduction from 55.21 g·kg$^{-1}$ D.M to 16.92 g·kg$^{-1}$ D.M. Crops of the *Achillea millefolium* species have consumed through own metabolic processes the bio available TPH compounds, or have induced in the soil derivate compounds of TPH. So after four months of growing, the crude oil content in the top layers of fertilized soil decreased by 21.4 and 45.4% after six months of growing plants of *Achillea millefolium* species. After four months of yarrow growing, the TPH content in the top layers of treated with sewage sludge mixed with modified indigenous volcanic tuff decreased by 26.0 respectively 65.4% after six months of phytoremediation process.

Furthermore after eight months of growing, the TPH content in the top layers of treated with sewage sludge in absence/presence modified indigenous volcanic tuff decreased by 57.4 respectively 69.4% of phyto-remediation process. Efficiency of the TPH, soil pollution removal with *Achillea millefolium* in three vegetative cycles is presented in figure 1.

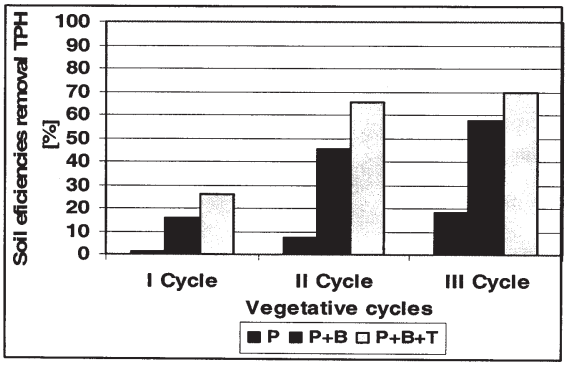


Fig. 1. Efficiency of the TPH, soil pollution removal

*Antioxidant activity of Achillea millefolium extracts*

Antioxidant activity extracts from aerial parts of yarrow, expressed as DPPH• inhibition ratio (%), was followed during 2 weeks. The results can be seen in figure 2

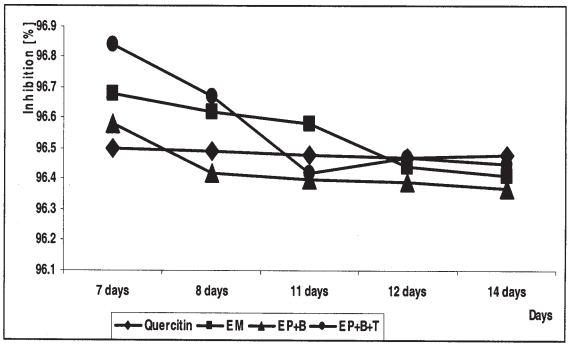


Fig. 2. Comparison of antioxidative activities expressed as capability of scavenging of free DPPH• radical (inhibition ratio, %)

compared to quercetin (1mg / mL), a natural flavonol which is one of the most powerful natural antioxidants.

It is known that the methanolic yarrow extract demonstrated a high antioxidant activity that may be due to the presence of phenolic substances and indeed we found that the DPPH• radical scavenging capacity of yarrow is unexpectedly high, more than 96%. Surprisingly, the three extracts ($E_M$, $E_{P+B}$, $E_{P+B+T}$) have, immediately after obtaining (7 days of maceration) an ability to capture the free DPPH• radical higher than quercetin. The antioxidant capacity decrease slightly in the next 7 days with 0.28% ($E_M$), 0.22% ($E_{P+B}$) and 0.40% ($E_{P+B+T}$) respectively, while in the case of quercetin remains relatively constant. Even after a week from extraction, the antioxidant activity is remarkable, slightly more pronounced for plants grown on soil fertilized and treated with volcanic tuff, but we can say that no remarkable differences between the control plant and grown on polluted soils were observed .

| No | Sample | Total phenol contents, (gallic acid equivalent) of *Achillea millefolium* methanolic extracts in aerial parts of plant, [mg·g$^{-1}$] |
|---|---|---|
| 1 | $E_M$ | *51,31 |
| 2 | $E_{P+B}$ | *53,79 |
| 3 | $E_{P+B+T}$ | *60,23 |

. *The average value of three determinations

**Table 6**
TOTAL PHENOLS CONTENTS (GALLIC ACID EQUIVALENT) IN YARROW EXTRACTS IN AERIAL TISSUES

*Total phenolic compounds in yarrow extracts*

Crude extracts of yarrow herb is rich in flavonoids and polyphenolics like chlorogenic acid, luteolin 7-O-glucoside, rutin, luteolin and other compounds. All phenolic compounds are determinate with Folin Ciocalteu assay, as total phenols. The levels of total phenols are presented in table 6 were expressed as dry herb (mg GAE ·g$^{-1}$, D.M.).

Generally, in medicinal plants, it has been observed similarities or close correlation between the profiles of the antioxidant capacity and of the total phenol contents. Total phenol content of the three extracts is slightly different and can be compared in an order that correspond to antioxidant activity: $E_{P+B+T} > E_{P+B} > E_M$. It looks like a soil polluted with oil products favors the synthesis of phenolic compounds in plant.

## Conclusions

The *Achillea millefolium* culture used for phyto-remediation strategy TPH polluted soil requires minimal agricultural works so that this technology becomes accessible and economical. Installing a ground cover with species of plants of *Achillea millefolium* species on soils polluted with 5.54% TPH has been achieved by prior treatment with fertilizer based on anaerobically stabilized sewage sludge in the absence/presence of inorganic amendments as modified indigenous volcanic tuff. Plants grown in the treated experimental variants were developed similarly to plants grown on normal soils. These plants have vegetative cycles similar to those grown on blank normal soils. The efficiencies reduction of the total petroleum hydrocarbons over a eight -month growing season that went through three cycles vegetative plants was up to 57.4% for the phytoremediation of polluted and fertilized topsoil and to 69.4% for the phytoremediation of polluted, fertilized and amendment with modified volcanic indigenous tuff. Soil polluted with petroleum hydrocarbons does not reduce the total content of phenols in the aerial parts of *Achillea millefollium* crop and therefore the antioxidant capacity; rather it can be boosted when the soil is treated with stabilized sewage sludge and indigenous volcanic tuff.

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