

**NEUTRALIZATION OF OIL SPILLS USING ECOLOGICAL SORBENT  
GLAUCONITE MODIFIED BY BIOSURFACTANTS  
OF *PSEUDOMONAS* SPP. PS-17**

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**Abstract**

The purpose of the present study was to investigate possible methods to enhance the rate of biodegradation of oil sludge from soil contamination by the petroleum products using glauconite modified by biosurfactants *Pseudomonas* spp. PS-17, thus reducing the time usually required for bioremediation. Enhancement of biodegradation was achieved through bioaugmentation and biostimulation. Contaminated soil samples were treated with ecological sorbent glauconite modified by biosurfactants *Pseudomonas* spp. PS-17. Maximum degradation was achieved after the 7 weeks of treatment. Measures against oil spill (gasoline, diesel fuel, motor oil) on parking areas, stations and highways could be carry out through the formation on the area perimeter the engineering chemical barriers with glauconite modified by biosurfactant *Pseudomonas* spp. PS-17 (10 g biosorbent per 1000 g glauconite). Application rate of glauconite modified with biosorbent is depending on the total oil content and range within 2.2-220 kg per m<sup>2</sup>, respectively and depend of a minor and extreme pollution.

**Key words:** glauconite, biosurfactants, biodegradation, *Pseudomonas* sp. PS-17, oil spill, pollution

**INTRODUCTION**

The release of contaminants, such as petroleum and petroleum byproducts, into the environment is one of the main causes of global pollution and has become a focus of great concern both in industrialized and developing countries due to the broad environmental distribution in soil, groundwater, and air (de Cássia et al. 2014). The contamination sources are diverse: accidents during fuel transportation by ships and trucks; leakage from underground storage tanks that are subject to corrosion, such as gas stations; oil extraction and processing operations; and inadequate release of waste generated by industries that use oil byproducts in the production of plastics, solvents, pharmaceuticals,

and cosmetics (de Cássia et al. 2014). Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants (Das and Chandran 2011). Currently accepted disposal methods of incineration or burial in insecure landfills can become prohibitively expensive when amounts of contaminants are large. Mechanical and chemical methods generally used to remove hydrocarbons from contaminated sites have limited effectiveness and can be expensive. Bioremediation is the promising technology for the treatment of these contaminated sites since it is cost-effective and will lead to complete mineralization. Bioremediation functions basically on biodegradation, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms. Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants (Das and Chandran 2011).

Although petrochemical plants and oil refineries are beneficial to society, they produce a large amount of hazardous waste. Moreover, oil spills during exploration, transportation, and refining, have caused serious environmental problems (de Cássia et al. 2014). Half the world's oil production (around three billion tons/year) is transported by ship and hydrocarbon contamination levels in different marine ecosystems have increased due to accidents. The major hydrocarbon source in oceans comes from routine operations of ship washing, natural oil leakage on the sea bed, and accidents during oil exploration and transportation (de Cássia et al. 2014).

The remediation of contaminated sites can be achieved by physicochemical or biological methods (de Cássia et al. 2014). Conventional physicochemical methods can rapidly remove the majority of spilled oil, but, in most cases, removal simply transfers contaminants from one environmental medium to another and can even produce toxic by-products. Moreover, crude oil cannot be completely cleaned up with physicochemical methods. Thus, more attention is being given to biological alternatives (Malik and Ahmed 2012, Lin et al. 2014, de Cássia et al. 2014). Microbial degradation is the major and ultimate natural mechanism by which one can clean up the petroleum hydrocarbon pollutants from the environment (Das and Chandran 2011). The recognition of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reported by Jones et al. (1983). They studied the extensive biodegradation of alkyl aromatics in marine sediments which occurred prior to detectable biodegradation of n-alkane profile of the crude oil and the microorganisms, namely, *Arthrobacter*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas*, and *Rhodococcus* were found to be involved for alkylaromatic degradation. Nine bacterial strains, namely, *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus subtilis*, *Bacillus* sp., *Alcaligenes* sp., *Acinetobacter lwoffii*, *Flavobacterium* sp., *Micrococcus roseus*, and *Corynebacterium* sp. were isolated from the polluted stream which could degrade crude oil (Das and Chandran 2011).

Strains belong to the genus *Pseudomonas* are among the best known biosurfactant producers. Since biological surface-active compounds are considered to be very useful in improving the bioavailability of hydrocarbon pollutants in soil, the ability of biosurfactant production in combination with the capacity to degrade hydrocarbons makes pseudomonads one of the most useful tools in bioremediation of petroleum-polluted soils (Pacwa-Płociniczak et al. 2014).

The success of oil spill bioremediation depends on one's ability to establish and maintain conditions that favor enhanced oil biodegradation rates in the contaminated

environment. Various factors influence the rate of oil biodegradation. One important requirement is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present and that the pH is between 6 and 9. The physical and chemical characteristics of the oil and oil surface area are also important determinants of bioremediation success. There are two main approaches to oil spill bioremediation: (a) bioaugmentation, in which known oil-degrading bacteria are added to supplement the existing microbial population, and (b) biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting cosubstrates (Das and Chandran 2011).

Natural ecological sorbent glauconite was proposed for neutralization of contaminated objects (Manenko and Khopyak 2001, Manenko et al. 2007, 2009, 2010, Khopyak et al. 2010, 2014a). Glauconite is a greenish mineral of the mica group, a hydrous silicate of potassium, iron, aluminum, and magnesium, found in sedimentary rocks as an accessory mineral. When in quantities over about 50%, the rock is no longer a glauconitic sandstone or mudstone, it is called a greensand. These rocks have been used historically as fertilizers, water softeners, and artist's pigments. Greensand is a naturally occurring mineral mined from ocean deposits from a sedimentary rock known as "Glauconite". It is often an olive-green colored sandstone rock found in layers in many sedimentary rock formations. Greensand forms in anoxic (without oxygen) marine environments that are rich in organic detritus and low in sedimentary inputs. Some greensands contain marine fossils (i.e. New Jersey Greensand). Greensand has been found in deposits all over the world (Abudelgawad et al. 1975, El-Amamy et al. 1982, Rabenhorst and Fanning 1989). Published research on green minerals in sedimentary rocks reveals a lack of consistency in use of mineralogic terms. Terms such as "glauconite", "greensands", "glaucony", or "glauconitic minerals", are used frequently in the literature, and confusion persists. Glauconite, strictly speaking, is a series name for dioctahedral, interlayer-deficient micas with compositions of  $K_{0.8}R^{3+}_{1.33}R^{2+}_{0.67}Al_{0.13}(Si_{3.87})O_{10}(OH)_2$ . The term also has been employed as a common field term for small, rounded, green to black pellets and grains (Fig. 1). Field identification often was confined to morphological appearance and color (Harding et al. 2014).



A



B

Fig. 1. Framed and powdered forms of glauconite

Source: glaukos.ru access on 28/05/2015

Glauconite is also a promising material during purification of underground waters in the dynamic mode since it features the whole complex of unique properties thanks to the features of the crystalline structure and high efficiency when purifying water of hardness salts, compounds of iron, manganese, heavy metals, and radionuclides, organic compounds, etc. Goncharuk et al. (2008), based on the conducted research, recommend glauconite sand modified by aluminum salts as a promising adsorbent for defluoridation water. For ensuring a high degree of purification and high filtration capacity the use of glauconite sand (even a pulverized fraction) may be successful when treating underground water in a dynamic mode for removing the excess content of fluoride. In addition, it is known that the natural form of the sorbent – glauconite – is sufficiently effective for water conditioning also in terms of heavy metals including iron, manganese, which are characteristic components of underground waters, which makes it possible to use a mixed medium of the natural and modified forms of the mineral for obtaining water of high quality for drinking purposes, while glauconite properties – the buffering of the sorbate solution – is conducive to preserve the pH values of the initial water (Goncharuk et al. 2008).

The problem of soil contamination by the petroleum products during their pipeline transportation and use at petrol stations, parking lots, streets and courtyards residential development does not lose its relevance today. To combat possible oil spills suggested to use ecological sorbent glauconite and biosurfactants *Pseudomonas* spp. PS-17. Hygienic assessment of Technology Regulations that apply the process of absorption (sorption) and disposal (solubilization, emulsification) of oil (gasoline, diesel fuel, motor oil) using glauconite modified by biosurfactants *Pseudomonas* spp. PS-17 (Manenko et al. 2007, Manenko and Khopyak 2001) was the purpose of our study.

## MATERIALS AND METHODS

Production schedules of neutralization potential spills of petroleum products (diesel, gasoline, motor oil) with ecological sorbent glauconite modified biosurfactants in parking areas, petrol stations and various roads were developed and based on these regulations: “The system of technological documentation in food industry. Technological documents. The requirements of the content and design, 18-3.402-82”; “Typical construction and specifications ISO 1.5-93”; “State standardization system of Ukraine. General requirements for the construction, presentation design and content of standards”, and “State Specifications 1.3-93. State standardization system of Ukraine. The procedure for the development and construction, presentation, execution, coordination, approval, registration marks and technical specifications”.

The materials for the Specifications, the primary toxicological hygienic passports of modified and natural glauconite (Artemenko 2004, Manenko and Khopyak 2001), Report to the protocol N1/2004 “Sanitary and hygienic evaluation of a documents for the use of biological preparation *Pseudomonas* spp. PS-17 production in Ukraine”

(Manenko and Khopyak 2004), toxicological passport of the bioreagent culture *Pseudomonas* spp. PS-17 (Grin and Govorunova 1991) were used in our assessments. Effectiveness of the glauconite with bioreagent *Pseudomonas* spp. PS-17 regarding oil products was assessed using UV microscopy and by reducing the degree of oil concentration in the soil (Methods for determination of petroleum products in the soil; Determination of the concentration of the oil in the soil by infrared spectrophotometry).

## RESULTS AND DISCUSSION

Ecological sorbent “natural glauconite” by the physical properties, chemical and mineral composition have to correspond to the requirements specified in the tables 1-3.

Table 1

The physical properties of the glauconite

Indicators	Reference value
Appearance	crystalline powder
Color	gray-green
The natural humidity, W [%]	12.0
The density of particles, [g/cm <sup>3</sup> ]	2.63
The average density at natural humidity, [g/cm <sup>3</sup> ]	1.9
Static exchange capacity, not less [mEq/g]	9.71
Mass fraction of potassium in terms of K <sub>2</sub> O, not less [%]	1.6
Mass fraction of magnesium in terms of MgO, at least [%]	0.35
Mass fraction of moisture, not more [%]	2.0
Class of the size, not more [mm]	1.0
Mass fraction of glauconite, not less [%]	20.0

Name of substance (by Geneva nomenclature) – glauconite silicate, iron and magnesium – seladonit, iron and magnesium aluminum silicate – scolite. The chemical formula of glauconite is  $K(Fe^{3+}, Fe^{2+}, Mg, Al)(OH)_2(AlSiO_{10}) \cdot nH_2O$ . The composition of glauconite also includes P, Ca and a wide range of trace elements. Features of the structure provide a large active surface area (96-140 m<sup>2</sup>/g) and high cation exchange capacity (26-41 mEq/100 g). The contents of major oxides in glauconite (%): K<sub>2</sub>O – 4.0 ÷ 6.4; P<sub>2</sub>O<sub>5</sub> – 1.3 ÷ 2.4; CaO – 2.0 ÷ 5.0; MgO – 0.5 ÷ 1.5; SiO<sub>2</sub> – 75.5 ÷ 92.0; Al<sub>2</sub>O<sub>3</sub> – 1.5 ÷ 6.5; Fe<sub>2</sub>O<sub>3</sub> – 1.5 ÷ 6.5 (Manenko and Khopyak 2001, Manenko et al. 2007, 2009, 2010, Khopyak et al. 2014a, b). Distinguished from zeolite, glauconite

has a rather stratified structure than framed. However, the intramolecular forces are not balanced by interaction with the chemical ions found in the vesicle of any of such strata. These forces can interact with the chemical ions contained in the solutions or in the air. It results in their accumulation on the active surfaces of the wafers that compose a crystal. The active surface area increases sufficiently and this is the main distinctive feature between the glauconite and the zeolite.

Glauconite is powder of green color at temperature 18-25°C, odorless, not volatile, not soluble in fats and water (Fig. 1). Calculable  $CL_{50}$  (*concentratio letalis*) is 14,000 mg/m<sup>3</sup>. Concentration of glauconite in chambers for poisoning (600 mg/m<sup>3</sup>) not caused death of animals. The clinical signs of acute inhalation action are characterized the irritation of respiratory tracts. Acute inhalation threshold was  $Lim_{ac} = 400$  mg/m<sup>3</sup>.  $DL_{50}$  for glauconite is about 5,000 mg/kg. A threshold dose is 2.27 mg/kg. A maximally not active dose is 0.746 mg/kg. Local resorbing actions during application on a skin and an eye absents;  $DL_{50} > 2,000$  mg/kg. Ability to sensibilization of organism, allergenic, mutagenic, carcinogenic, embryotoxic, gonadotoxic and teratogenic effects were absent. Glauconite was suggested to IV class of toxicity in inhalation influence, parenteral and percutaneous infusions. Maximum Allowable Concentrations (MAC) of glauconite dust in working zone is 6 mg/m<sup>3</sup> (IV class of toxicity). Glauconite not form toxic connections in an air environment, not burn. The expressed absorptive properties of glauconite and possibility of water release from chlororganic, phosphororganic and synthetic piretroidic pesticides were determined (Manenko et al. 2007, 2009, 2010).

In our study, the chemical and mineral compositions of the glauconite were studied and presented in Tables 2 and 3.

Table 2

The chemical composition of the glauconite (%)

SiO <sub>2</sub>	TiO <sub>2</sub>	Al <sub>2</sub> O <sub>3</sub>	Fe <sub>2</sub> O <sub>3</sub>	FeO	MnO	CaO	MgO	K <sub>2</sub> O	Na <sub>2</sub> O	P <sub>2</sub> O <sub>5</sub>	H <sub>2</sub> O
53.99	0.14	7.31	14.18	0.54	0.02	4.43	2.56	4.51	0.14	2.25	3.21

Table 3

The mineral composition of the glauconite (%)

Glauconite	Quartz	Montmorillonite	Other minerals
to 70	to 40	to 20	to 10

The basic culture medium consists (g/L): NaNO<sub>3</sub> – 3; K<sub>2</sub>HPO<sub>4</sub> – 2; KH<sub>2</sub>PO<sub>4</sub> – 1.2; MgSO<sub>4</sub> – 0.5; sodium citrate – 5; carbon source (glycerin, oils, glucose, alcohols) – 10 ÷ 30. According to the physical and chemical characteristics, basic components of biosurfactants *Pseudomonas* spp. PS-17 have to correspond to the requirements

specified in the Tables 4 and 5. Class of compounds – a set of surface biosurfactants (mono- and dirhamnolipids), alginate polymer nature (poliuronid block structure, a linear copolymer manuronic acid with 5-epimer  $\alpha$ -hyaluronic acid).

Table 4

Physical and chemical characteristics of biological surfactants *Pseudomonas* spp. PS-17

Indicators	Reference value
The appearance	viscous liquid
Color	light green or light brown
Smell	specific
Mass fraction of dry matter, not less [%]	1.9
Mass fraction of active ingredient content consisting in rhamnolipids free rhamnose, not less [%]	0.5

Table 5

The main components of a biological product biosurfactant *Pseudomonas* spp. PS-17

Components	Contents (g/L)
Mono- and dirhamnolipids (surfactants)	3-7
Biopolymer alginate (a polysaccharide, M = 300,000-400,000)	1.5-3
Salts (NaNO <sub>3</sub> , KH <sub>2</sub> PO <sub>4</sub> , K <sub>2</sub> HPO <sub>4</sub> )	footprints
Fatty acid	5.3
Rhamnose	1-2
Amino acids, peptides	1-3

Composition of the surfactants (bioreagents: surfactants mono- and dirhamnolipids – 5.0-10.0 g/dm<sup>3</sup>; biopolymer alginate (M = 400.000 polysaccharide) – 1.5-3.0 g/dm<sup>3</sup>; biomass – 2-3 g/dm<sup>3</sup>; salts: NaNO<sub>3</sub> – 0.5-1.0 g/dm<sup>3</sup>; K<sub>2</sub>HPO<sub>4</sub>, MgSO<sub>4</sub>, FeSO<sub>4</sub>, CaCl<sub>2</sub>, residual amounts of peptides, polysaccharides – 2-3 g/dm<sup>3</sup>; fluorescent pigment – 0.2-0.4 g/dm<sup>3</sup>; enzymes (lidasa, catalase, urease, oxidase), amino acids, rhamnose, fatty acids, rhamnolipid alginate) form a natural culture fluid composition – surface-active Biocomplex (10-12 g/dm<sup>3</sup>). Product of industrial manufacture is in 10, 50, and 200-liter plastic canisters. Storage and transportation of the drug carried out at temperature from -20°C to +30°C. Warranty period of storage is 2 months (2-3 days before the use) at a temperature of 15°C to 30°C and 6 months at a temperature from -20°C to +15°C. Biological culture in the form of a liquid, and in combination with microorganisms-destructors pollution and with natural ecosorbent glauconite used for cleaning of soils from oil products through their solubilization (emulsification). The product is ecologically safe; it is included in the ecological

chain metabolism of organic biodegradable substances in environmental objects (final products are sugar, water, carbon dioxide).

Measures to combat possible oil spills shall be carried out through the creation of engineering and geochemical barriers (Fedoryshyn et al. 2000) on the perimeter of gas stations and parking lots, i.e. sorption-filtrating geochemical engineering barrier providing capture contaminants without delay for filtration of water (barrier shall consist of three layers by 10 cm thick and fine sand with glauconite by ratio (%) 50:50; 70:30; 80:20 in each layer respectively); sorption-filtrating geochemical engineering barrier, which collects pollution without skipping water at the same time (barrier consists with the 15 sm glauconite layer and sand at ratio (%) 80:20); geochemical engineering barrier of membrane type with 100% content of glauconite; sorbent density is less than 0.8, filtration rate is less than 0.01 m per day, thickness is 30 cm, coefficient of porosity (in fractions of units) is less than 0.8. Formation around industrial sewage water tank and storage tanks for storm water engineering and geochemical barriers with colloidal type consists with 100% glauconite in those sorbent density is more than 1.0, filtration rate is more than 1.0 m per day, thickness – 30 cm, voids ratio (in fractions of a unit) is more than 1.0. Modifying of glauconite in engineering and geochemical barriers is carried out during the formation of barriers by adding to glauconite the biosurfactants in ratio 10 g liquid for 1000 g of the glauconite.

For disposal of surface asphalt (or concrete) of auto parking lot and motorway during oil pollution, rules for glauconite using are following: under small petroleum contamination (at MAC 1 mg/kg oil) – 2.2 kg per 1 m<sup>2</sup>; in the case of significant outpouring (10 MAC) – 22 kg per 1 m<sup>2</sup>; in the case of high pollution (100 MAC) – 220 kg per 1 m<sup>2</sup>; while extreme contamination (> 100 MAC) – more than 220 kg per 1 m<sup>2</sup>. After holding sorption during 12-24 hours, ecological sorbent is removed and taken to the landfill solid waste. The remnants of the adsorbed glauconite with oil products must be sweep away to tanks with storm (rain) water, and biosurfactants *Pseudomonas* spp. PS-17 in the amount of 100 ml to 1 liter of wastewater (or 100 L/m<sup>3</sup> of wastewater) must be added. Exposure during 7 weeks causes to solubilization (emulsification) of petroleum products, which absorbed the remnants glauconite and *Pseudomonas* spp. PS-17 and degrades to sugars, carbon dioxide and water. In further, the sediment glauconite must be pumped to the tank and drained to the sewer pit delivering wastewater to treatment plants.

Example of action the sorbent glauconite with biosurfactants *Pseudomonas* spp. PS-17 illustrated by ultraviolet microscopy and schedule (Figs 2 and 3). Glauconite is a part of glauconitolite, the mineral with layered structure. The basis of its structure are octahedral and tetrahedral silicon and oxygen octahedral aluminosilicates in o-hydroxy mesh, when combined, form a structural layer. The layers superimposed one on one form the multistory packages. Glauconite possesses a three-layer package relations between packets carried by the presence of potassium cations. Distance between packets is 1 nm. Feature micromorphology of glauconite is so-called “tunnel structure” that contribute to its high porosity, large active specific surface, high cation exchange capacity. Due to isomorphous exchange is possible not only isomorphous (chemical) sorption, but the physical sorption (between the structural layers

and around them) and sorption of large cations. In addition to high sorption capacity, the glauconite also can reduce the mutagenic effects of contaminated soil and water. As shown in Fig. 2, petroleum products in the presence of glauconite modified by bioreagent *Pseudomonas* spp. PS-17 were solubilized very quickly and absorbed by biosorbent. In Fig. 2 is presented the decrease of petroleum products concentration from 5,000 mg/kg to 0 during 7 weeks after treatment with glauconite modified by biosurfactant *Pseudomonas* spp. PS-17.

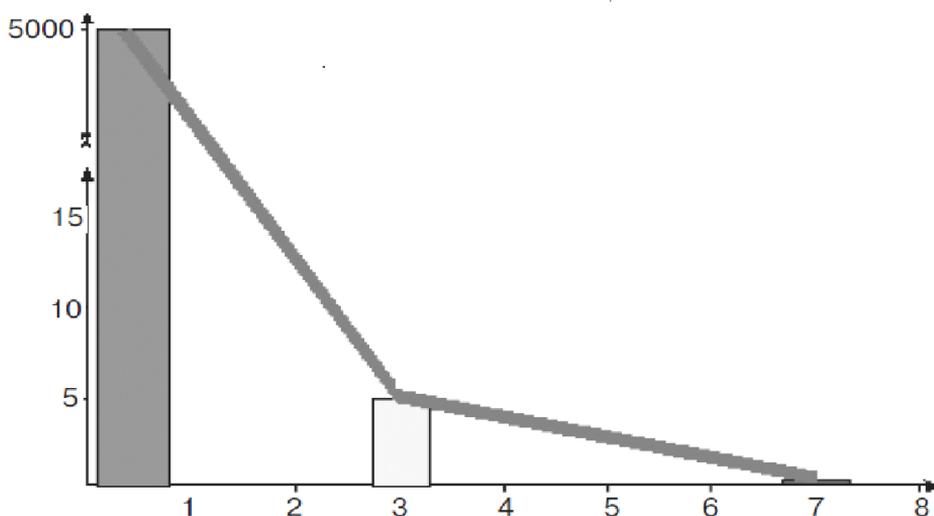


Fig. 2. Dynamics of reduce the concentration of petroleum products during 7 weeks after treatment with glauconite modified by bioreagent *Pseudomonas* spp. PS-17

Axis X – time of exposure, weeks; axis Y – concentration of petroleum products after treatment with glauconite modified by bioreagent *Pseudomonas* spp. PS-17, mg/kg

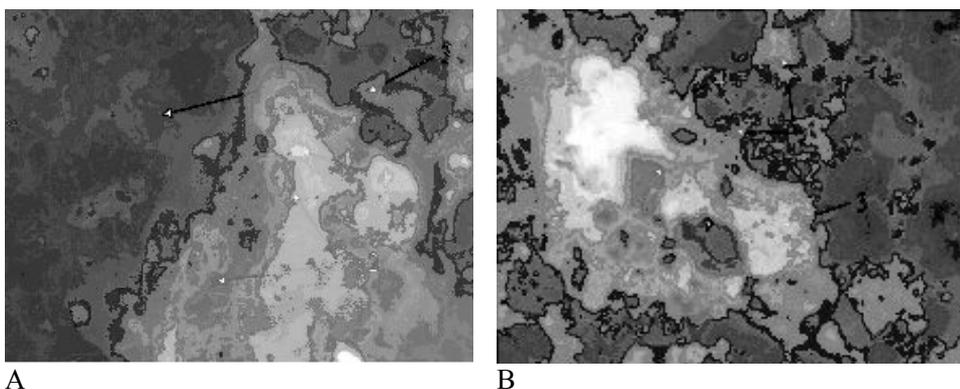


Fig. 3. UV microscopic images before (A) and after (B) sorption and neutralization of petroleum products by glauconite modified by bioreagent *Pseudomonas* spp. PS-17

Our previous study reveals that glauconite raw (greensand) from Khmelnytsky deposit in Ukraine has a high adsorption capacity concerning to mercury ion. The maximum adsorption capacity of glauconite is achieved after 1.5 hours of contact with a Hg(II) solution. Adsorption capacity of glauconite is increase significantly with increasing mercury concentration in solution. It has been shown that the efficiency of Hg(II) adsorption on glauconite is practically unchanged in solutions with pH 5-7. Our study is an inclination for the using of glauconite as engineering and geochemical barriers to protect uncontaminated soils, groundwater, and to prevent long-term contamination of soil surface by residual amounts of mercury. High adsorption capacity of glauconite to Hg(II) in  $\mu\text{g}$  level gives reason to propose this natural adsorbent for purification of anthropogenic-exposed soil and waste water, as well as for wastewater treatment at the enterprises with mercury use in technological processes. We believe that future studies is promising to analyze the adsorption properties of thermo- and acid-modified (treated with polyelectrolyte) glauconite that lead to a significant increase its absorption capacity (Khopyak et al. 2014a).

We also demonstrated the possibility of using of natural absorptive agent glauconite for neutralization of substandard medicines in the pharmaceutical company "Lvivdialik" (Lviv, Ukraine). Substandard medicines of II class of hazard (gentamicin sulfate, thiamine chloride, Yonoglyuk) were inactivated by mixing with glauconite. After neutralization boiled mixture by glauconite, total hazard index of drugs mixture was increased to 10.7, indicating a high adsorption capacity of glauconite. The proposed method of neutralization of substandard medicines by mixture with glauconite can be effectively complement to the methods of neutralization of substandard drugs (Khopyak et al. 2014b).

Large amounts of crude oil entering the marine environment, groundwater and soil can cause significant harm to resident organisms. Petroleum is a hydrophobic hydrocarbon with negative effects on the structural and functional properties of cell membranes in living organisms, offering considerable risk of contamination in both marine and terrestrial ecosystems. When in contact with water, oil and its byproducts spread and form a thin layer on the surface that prevents gas exchange between the air and water, blocking the passage of sunlight and impeding the respiration and photosynthesis process. Thus, hydrocarbon waste impacts phytoplankton communities, causing a fundamental breakdown of the food chain. The potential threat to human health posed by hydrocarbons is linked to the physical and chemical properties of these compounds, which are absorbed by the skin and quickly spread through the organism if ingested or inhaled (de Cássia et al. 2014).

A number of microorganisms, such as fungi, yeasts, and bacteria, feed on substances that are immiscible in water, producing and using a surface-active substance (biosurfactant) (Sobrinho et al. 2013). Among bacteria, the genus *Pseudomonas* is known for its capacity to produce extensive quantities of glycolipids. These biosurfactants are classified as rhamnolipids (de Cássia et al. 2014). Pseudomonads are the best known bacteria capable of utilizing hydrocarbons as carbon and energy sources and producing biosurfactants (Rahman et al. 2003, Cameotra and Singh 2008, Pornsunthorntaweew et al. 2008). Among Pseudomonads, *P. aeruginosa* is widely studied for the production of glycolipid type biosurfactants. However, glycolipid type biosurfactants are also reported from some other species like *P. putida* and *P. chlororaphis*.

Biosurfactants increase the oil surface area and that amount of oil is actually available for bacteria to utilize it. Biosurfactants can act as emulsifying agents by decreasing the surface tension and forming micelles. The microdroplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded (Das and Chandran 2011).

The most common role of biosurfactants is to enhance the dispersal of contaminants in the aqueous phase and increase the bioavailability of the hydrophobic substrate to microorganisms, with subsequent removal of such pollutants through biodegradation (de Cássia et al. 2014). In the petroleum industry, biosurfactants have been applied effectively for the exploration of heavy oil, offering advantages over their synthetic counterparts throughout the entire petroleum processing chain (extraction, transportation and storage) (de Cássia et al. 2014). Biosurfactants are used in microbial-enhanced oil recovery, the cleaning of contaminated vessels and to facilitate the transportation of heavy crude oil by pipeline (Tabatabaee et al. 2005). Different groups of biosurfactants exhibit diverse properties and display a variety of physiological functions in producer microorganisms; these include enhancing the solubility of hydrophobic/water-insoluble compound, heavy metal binding, bacterial pathogenesis, cell adhesion and aggregation, quorum sensing and biofilm formation (Luna et al. 2011). Numerous examples demonstrate the potential application of biosurfactants in environmental decontamination. The results of Pacwa-Łłociniczak et al. (2014) indicated that the *Pseudomonas* sp. P-1 strain and/or biosurfactant produced by this strain have the potential to be used in bioremediation of hydrocarbon-contaminated soils. The *Pseudomonas* sp. P-1 strain, isolated from heavily petroleum hydrocarbon-contaminated soil, was investigated for its capability to degrade hydrocarbons and produce a biosurfactant (Pacwa-Łłociniczak et al. 2014). The strain degraded crude oil, fractions A5 and P3 of crude oil, and hexadecane (27, 39, 27 and 13% of hydrocarbons added to culture medium were degraded, respectively) but had no ability to degrade phenanthrene. Therefore, the *Pseudomonas* sp. P-1 strain has the ability to degrade various hydrocarbons (hexadecane, crude oil and fractions A5 and P3 of crude oil), and is efficient in rhamnolipid production and hydrocarbon emulsification. The features of the P-1 strain make it promising agent for cleaning up environments contaminated with petroleum compounds (Pacwa-Łłociniczak et al. 2014).

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions (Das and Chandran 2011). The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate, pyruvate. Sugars required for various biosynthesis and growth are synthesized by gluconeogenesis. The degradation of petroleum hydrocarbons can be mediated by specific enzyme system. Other mechanisms involved are (1) attachment of microbial cells to the substrates and (2) production of biosurfactants (Das and Chandran 2011).

As highly reduced hydrocarbons are abundant in the environment, enzymes that catalyze the terminal or subterminal oxygenation of alkanes are relatively easy to find. A number of these enzymes have been biochemically characterized in detail, because the potential of alkane hydroxylases to catalyze high added-value reactions is widely recognized. Nevertheless, the industrial application of these enzymes is restricted owing to the complex biochemistry, challenging process requirements, and the limited number of cloned and expressed enzymes. Rational and evolutionary engineering approaches have started to yield more robust and versatile enzyme systems, broadening the alkane oxygenase portfolio. In addition, metagenomic approaches provide access to many novel alkane oxygenase sequences (Díaz et al. 2002). Alkane hydroxylases play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds. Environmental studies demonstrate the abundance of alkane degraders and have led to the identification of many new species, including some that are (near)-obligate alkanotrophs (van Beilen and Funhoff 2007). The n-alkane-assimilating yeast *Candida maltosa* contains several structurally related cytochromes P450 encoded by the CYP52 multi-gene family, which are inducible by various long-chain hydrocarbons and fatty acids and which are responsible for the initial hydroxylation steps in the metabolism of these substrates (Zimmer et al. 1996).

Members of the genus *Pseudomonas*, due to the presence of a complex enzymatic system, show a wide variety of metabolic and physiological properties (Pacwa-Łóćiniczak et al. 2014). They are also the most predominant group of microorganisms that degrade xenobiotic compounds. The phylogenetic analysis of P-1 revealed that close relatives of this strain are strains belonging to *P. aeruginosa*. It has been reported that various bacteria from genus *Pseudomonas*, including *P. aeruginosa* strains, inhabit oil-contaminated soils and can break down more than 100 different organic compounds (Glick et al. 1994, Hong et al. 2005, Saikia et al. 2012). They are the best known bacteria capable of utilizing a number of aliphatic and aromatic hydrocarbons as carbon and energy sources (Das and Chandran 2011, Kadali et al. 2012, Puškárová et al. 2013).

Alisi et al. (2009) assessed the feasibility of bioremediation of a soil, containing heavy metals and spiked with diesel oil (DO), through a bioaugmentation strategy based on the use of a microbial formula tailored with selected native strains. The soil originated from the metallurgic area of Bagnoli (Naples, Italy). The formula, named ENEA-LAM, combines ten bacterial strains selected for multiple resistance to heavy metals among the native microbial community. The application of this microbial formula allowed to obtain, in the presence of heavy metals, the complete degradation of n-C(12-20), the total disappearance of phenantrene, a 60% reduction of isoprenoids and an overall reduction of about 75% of the total diesel hydrocarbons in 42 days. Concurrently with the increase of metabolic activity at community level and the microbial load, the gradual abatement of the ecotoxicity was observed. The ENEA-LAM strains survived and some minor native strains, undetectable in the soil at the beginning of the experiment, developed. Such a bioaugmentation approach allows the newly established microbial community to strike a balance between the introduced and the naturally present microorganisms. The results of Alisi et al. (2009) indicated that the use of a tailored microbial formula may efficiently facilitate and

speed up the bioremediation of matrices co-contaminated with hydrocarbons and heavy metals.

The influence of medium constituents on the production of biosurfactants by *Candida tropicalis* cultivated in waste frying oil was investigated by Batista et al. (2010). The combined effect of yeast extract on surface tension reduction, biosurfactant yield, emulsification activity, and biomass were studied. Emulsification indices against lubrication and automobile waste oil of approximately 65 to 95% were observed. The crude biosurfactant produced in the medium removed approximately 78 to 97% of the petroleum and motor oil adsorbed in sand samples (Batista et al. 2010). Gusmão et al. (2010) investigated the application of a crude biosurfactant produced by *Candida glabrata* UCP1002 in a soil-water-hydrophobic contaminant system and found a removal rate as high as 92.6%. Luna et al. (2011) evaluated a new biosurfactant, denominated Lunasan, produced by *Candida sphaerica* UCP 0995. This biosurfactant removed 95% of motor oil adsorbed to sand, demonstrating considerable potential for use in bioremediation processes (Gusmão et al. 2010).

The capability of a cell bound biosurfactant produced by *Lactobacillus pentosus*, to accelerate the bioremediation of a hydrocarbon-contaminated soil, was compared with a synthetic anionic surfactant (sodium dodecyl sulphate SDS) (Moldes et al. 2013). Treatment of soil for 15 days with the biosurfactant produced by *L. pentosus* led to a 65.1% reduction in the hydrocarbon concentration, whereas SDS reduced the octane concentration to 37.2% compared with a 2.2% reduction in the soil contaminated with octane in absence of biosurfactant used as control. Besides, after 30 days of incubation soil with SDS or biosurfactant gave percentages of bioremediation around 90% in both cases. Moldes et al. (2013) concluded that biosurfactant produced by *L. pentosus* accelerates the bioremediation of octane-contaminated soil by improving the solubilisation of octane in the water phase of soil, achieving even better results than those reached with SDS after 15-day treatment. It was observed that the presence of biosurfactant from *L. pentosus* accelerated the biodegradation of octane in soil (Moldes et al. 2011). After 15 days of treatment, biosurfactants from *L. pentosus* reduced the concentration of octane in the soil to 58.6 and 62.8%, for soil charged with 700 and 70,000 mg/kg of hydrocarbon, respectively, whereas after 30 days of treatment, 76% of octane in soil was biodegraded in both cases. In the absence of biosurfactant and after 15 days of incubation, only 1.2 and 24% of octane was biodegraded in soil charged with 700 and 70,000 mg/kg of octane, respectively. Thus, the use of biosurfactants from *L. pentosus*, as part of a well-designed bioremediation process, can provide mechanisms to mobilize the target contaminants from the soil surface to make them more available to the microbial population (Moldes et al. 2011).

Moreover, immobilized cells have been used and studied for the bioremediation of numerous toxic chemicals. Immobilization not only simplifies separation and recovery of immobilized cells but also makes the application reusable which reduces the overall cost (Das and Chandran 2011). Immobilized hydrocarbon-degrading bacteria represent a promising application in the bioremediation of hydrocarbon-contaminated areas. Rahman et al. (2006) examined the capacity of immobilized bacteria to degrade petroleum hydrocarbons. A mixture of hydrocarbon-degrading bacterial strains was immobilized in alginate and incubated in crude oil-contaminated artificial sea-

water. The biodegradation capacity of the microorganisms was not compromised by the immobilization following a 30-day incubation period. Removal of n-alkanes was similar in immobilized cells and control cells. To test reusability, the immobilized bacteria were incubated for sequential increments of 30 days. No decline in biodegradation capacity of the immobilized consortium of bacterial cells was noted over its repeated use (Rahman et al. 2006). Cunningham et al. (2004) used polyvinyl alcohol (PVA) cryogelation as an entrapment matrix and microorganisms indigenous to the site. They constructed laboratory biopiles to compare immobilised bioaugmentation with liquid culture bioaugmentation and biostimulation. Immobilised systems were found to be the most successful in terms of percentage removal of diesel after 32 days.

Low cost of the sorbent raw materials, regardless of the degree of enrichment, and fast cleaning process could make this low-cost technology, despite location of technological equipment in contaminated areas. Ministry of Health in Ukraine allowed wide use and concluded that glauconite modified by bioreagent *Pseudomonas* spp. PS-17 is not threat to human health and has no danger to the environment.

## CONCLUSIONS

1. Glauconite at temperature 18-25°C is powder of green color, odorless, not volatile, not soluble in fats and water. Calculable  $CL_{50}$  is 14,000 mg/m<sup>3</sup>. Concentration of glauconite in chambers for poisoning (600 mg/m<sup>3</sup>) not caused death of animals. The clinical signs of acute inhalation action are characterized the irritation of respiratory tracts. Acute inhalation threshold was  $Lim_{ac} = 400$  mg/m<sup>3</sup>.  $DL_{50}$  for glauconite is about 5,000 mg/kg. A threshold dose is 2.27 mg/kg. A maximally not active dose is 0.746 mg/kg. Local resorbing actions during application on a skin and an eye absents;  $DL_{50} > 2,000$  mg/kg. Ability to sensibilization of organism, allergenic, mutagenic, carcinogenic, embryotoxic, gonadotoxic and teratogenic effects were absent. Glauconite was suggested to IV class of toxicity under inhalation influence, parenteral and percutaneous infusions. MAC of glauconite dust in working zone is 6 mg/m<sup>3</sup> (IV class of toxicity). Glauconite not form toxic connections in an air environment, not burn. The expressed absorptive properties of glauconite and possibility of water release from chlororganic, phosphororganic and synthetic piretroidic pesticides were determined (Manenko et al. 2007, 2009, 2010). Bioreagent *Pseudomonas* spp. PS-17 with glauconite is also safe, biodegrades to the sugar, water and carbon dioxide.
2. The decrease of petroleum products concentration from 5,000 mg per kg to 0 during 7 weeks after treatment with glauconite modified by biosurfactant *Pseudomonas* spp. PS-17 was observed in our study. Measures to Combat Potential spills of petroleum products (gasoline, diesel fuel, motor oil) on parking areas, stations and highways could be carry out through the formation on the area perimeter the engineering chemical barriers with glauconite modified by biosurfactant *Pseudomonas* spp. PS-17 (10 g biosorbent per 1000 g glauconite). Application rate of glauconite modified with biosorbent depending on the total oil content and range within 2,2-220 kg per m<sup>2</sup>, respectively and depend of a minor and extreme (emer-

gency) pollution. Glauconite with biosurfactant *Pseudomonas* spp. PS-17 is added in ratio 100 L per m<sup>3</sup> to tanks with storm (rain) water. After 7-10 days, sewage water can be drained to the sewer collection that delivers wastewater to treatment plants.

3. Parking and stations must be equipped with emergency reserve of biosorbent and biosurfactant *Pseudomonas* spp. PS-17. In the case of surface soil contamination, it would be necessary to conduct working with cleaning according to the standards of the glauconite modified by *Pseudomonas* spp. PS-17.

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NEUTRALIZACJA WYCIEKÓW ROPY Z WYKORZYSTANIEM  
EKOLOGICZNEGO SORBENTU GLAUKONITU ZMODYFIKOWANEGO  
PRZEZ BIOSURFAKTANTY *PSEUDOMONAS* SPP. PS-17

**Streszczenie**

Celem niniejszej pracy było zbadanie efektywności wykorzystania glaukonitu zmodyfikowanego przez biosurfaktanty *Pseudomonas* spp. PS-17 w celu przyśpieszenia biodegradacji produktów naftowych z zanieczyszczonej gleby, co zwykle zmniejsza czas i wymagania biorekultywacji. Wzmocnienie biodegradacji produktów naftowych zostało osiągnięte poprzez bioaugmentację i biostymulację. Zanieczyszczone próbki gleby poddano działaniu ekologicznego sorbentu glaukonitu zmodyfikowanego przez biosurfaktanty *Pseudomonas* spp. PS-17. Maksymalny rozkład produktów naftowych uzyskano po 7 tygodniach ekspozycji z glaukonitem. Ochronę przed wyciekami produktów naftowych (benzyna, olej napędowy, olej silnikowy) na obszarach zabudowanych, stacjach postojowych i autostradach można zorganizować poprzez utworzenie na terenie obwodu bariery inżynierii chemicznej z glaukonitem zmodyfikowanym przez biosurfaktanty *Pseudomonas* spp. PS-17 (10 g na 1000 g biosorbentu glaukonitu). Zastosowanie glaukonitu zmodyfikowanego przez biosorbent *Pseudomonas* spp. PS-17 zależy od całkowitej zawartości produktów naftowych na zanieczyszczonym terenie (2,2-220 kg na m<sup>2</sup>) i jest determinowane jego stopniem zanieczyszczenia.