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KERATINOLYTIC FUNGI IN AN ACIDIC PETROLEUM WASTE LAGOON AT A PETROLEUM REFINERY

GRZYBY KERATYNOLITYCZNE W LAGUNIE KWAŚNYCH ODPADÓW RAFINERYJNYCH NA TERENIE RAFINERII ROPY NAFTOWEJ

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The incidence of keratinolytic fungi in an acidic petroleum waste lagoon (before bioremediation) at a petroleum refinery situated within a highly populated area was examined. High concentrations of petroleum hydrocarbons (aliphatics and PAHs) made the growth of keratinolytic fungi in clay and litter collected from the lagoon impossible. The natural self-purification process considerably decreased the hydrocarbon contamination, increased the pH and caused the abundant growth of Trichophyton ajelloi in organic soil that contained the root-adjacent material from the grass growing in green oases at the lagoon. Ecological and epidemiological aspects of the data were discussed.

INTRODUCTION

The contamination of soils and aquifers with petroleum hydrocarbons and their derivatives is an important environmental problem in many countries all over the world, including Poland. This contamination often occurs in areas of petroleum refineries, coking plants, military and transport bases, fuel basins and refilling stations. Factors affecting the contamination include evaporation of volatile compounds, dissolution, adsorption, emulsifying and photochemical and microbiological degradation. The contamination of soil and other habitats with petroleum hydrocarbons considerably changes the qualitative and quantitative compositions and the activities of indigenous microorganisms [8, 9]. Our preliminary experiments (unpublished data) showed that this contamination also altered the composition of keratinolytic fungi in soil. These fungi are able to degrade keratinous substrata provided to the environment by animals, including humans. Therefore, keratinolytic fungi are extremely abundant at highly populated areas and sites penetrated by animals [4]. Due to the pathogenic or potentially pathogenic properties of keratinolytic fungi, studies of these microorganisms are also of epidemiological significance. Areas rich in keratinous substrata and keratinolytic fungi are often contaminated with petroleum hydrocarbons and their derivatives. It is, therefore, of interest to recognize the influence of these compounds on keratinolytic fungal populations. This paper addresses the incidence of keratinolytic fungi in an acidic petroleum waste lagoon (before bioremediation) at a Polish petroleum refinery situated within a highly populated area.

MATERIAL AND METHODS

There were three acidic petroleum waste lagoons (I-III) at the refinery in Czechowice-Dziedzice [21]. This paper concerns the second (middle) lagoon before bioremediation. The litter containing wastes from the acidic refining (with H_2SO_4) of petroleum and clay heavily contaminated with wasted oils from the refinery's area were disposed in this lagoon for more than one hundred years. Most of the lagoon's surface was devoid of plants. However, there were several small green oases of shrubs and grass on this surface. The materials collected from the lagoon were the following: litter, clay (named clay 2) and organic soil. The first two materials were taken directly from the lagoon's surface, while shaking the roots of the grass growing in the green oases allowed to collect the organic soil. The control sample was clay 1, which was not contaminated with petroleum hydrocarbons and collected from the bottom of a dried pond. This pond was situated at the distance of ca. 3 kilometers from the lagoons' area. The bottom of the pond was devoid of plants. Each sample was collected from 5 places of the lagoon or the pond from the depth of up to 20 cm. Subsamples from each place had ca. 1 kg so that the total weight of each sample was ca. 5 kg. Samples were first cleaned from stones and plant remains, then crumbled and thoroughly mixed. These mixed samples were examined for keratinolytic fungi and physico-chemical parameters.

The mixed samples were surveyed for keratinolytic fungi using the standard hair baiting technique [20]. Each sample was distributed into forty Petri dishes. Forty grams of the examined material were put into each Petri dish and the dishes were covered with 400 mg of defatted, cut into 1-cm long pieces and autoclaved children's hair. The dishes were incubated for four months in the dark at 23 ^{o}C. The moisture of the materials in the dishes was kept at the level of 25–30%. At two-week intervals, the dishes were examined for growth of keratinolytic fungi. The pieces of hair that had been attacked by these microorganisms were observed under light microscopy and inoculated on 1:10 *Sabouraud* + salts medium (TK medium) [11] with chloramphenicol (100 mg/l) and cycloheximide (500 mg/l). Pure fungal cultures were identified according to their macro-and micromorphological characteristics, with the aid of selected taxonomic monographs [2, 5, 10]. The general rule was that the presence of a given species on hair bait confirmed by its growth on TK medium meant the isolation of one strain of this species from a Petri dish. The test for keratinolytic activity of isolated strains was that of *Ulfig et al.* [14].

Each mixed sample was examined for the following physico-chemical parameters: particle size distribution by *Bouyoucosa-Casagrande* aerometric method in *Prószyński's* modification [PN-R-04032]; humidity by gravimetric method [6]; pH in H_2O by electrometric method (PN ISO 10390); conductivity by conductometric method (PN ISO 11265); total organic carbon (C_{TOT}) by Tiurin method (PN-91/Z-15005); total nitrogen (N_{TOT}) by *Kjeldahl* method (ISO 11261); total sulfur (S_{TOT}) by *Eschka* method (PN ISO 334); S-SO_4 by gravimetric method (ISO 11048); available phosphorus (P_2O_5) by *Egner-Riehm* method (PN-R-04023); available potassium (K_2O) by *Egner-Riehm* method (PN-R-04022); total petroleum hydrocarbons (TPH, non-polar aliphatic hydrocarbons) by infrared spectrometry (FT-IR) with extraction in CCl_4; total petroleum organic carbon (TPOC, polar and non-polar aliphatic compounds) by infrared spectrometry (FT-IR) with extraction in CCl_4 (PB-07, PB-10, PN-82/C-04565.01, EPA 3620, EPA 8440, PN-V-04007); and polycyclic aromatic hydrocarbons (PAHs) by HPLC with fluorescence detection (PB-06, PB-09, ISO/FDIS 13877). The mixed samples from the lagoon were also examined for the total organic matter content extractable by petroleum ether (petroleum ether extract = PEE; PN-75/C-04573.10), heavy metals (Fe, Mn, Ni, Cr, Zn, Cu, Pb, Cd, Co, and Hg) by ICP and AAS atomic spectrometry methods (EPA 6010B), and BTEXs (benzene, toluene, ethylobenzene, m+p-xylene, o-xylene, 1,2,5-trimethylobenzene, 1,2,4-trimethylobenzene, and naphthalene) by GC/MS method [12]. However, the concentrations of heavy metals and BTEXs were low and, therefore, are not presented in the paper.

RESULTS

The occurrence of keratinolytic fungi in the samples from the lagoon and in the control sample (clay 1) is presented in Table I. Among 160 Petri dishes examined, 80 (50%) were positive for the above-mentioned microorganisms. Altogether, 104 fungal strains belonging to three species were isolated. Good growth of keratinolytic fungi was observed in clay 1. The mycelium and macroconidia of *Trichophyton ajelloi* predominantly covered the hair bait in all Petri dishes with clay 1. The "colonies" of *Aphanoascus durus,* or only of its anamorph *Chrysosporium* sp., and *Chrysosporium* anamorph of *Arthroderma curreyi* were noticed in nine and seven Petri dishes, respectively. Abundant growth of *Trichophyton ajelloi* (monoculture) was observed in all Petri dishes with organic soil from the lagoon. No keratinolytic fungi were detected in litter and clay 2.

Physico-chemical characteristics of the samples are presented in Table II. The samples clay 1, clay 2 and organic soil belonged to the granulometric (mechanical) group loamy silt, whereas the litter was classified to the sandy loam by USDA. The terminology of these granulometric groups by PTG was different. The humidity range of the samples was rather narrow (19,7–27,2%). The reaction (pH) of clay 1 and organic soil was nearly neutral, while the reaction of litter and clay 2 was acidic. The highest conductivity was observed in clay 1 and the lowest was measured in clay 2. The lowest C_{TOT} was noticed in clay 1 and the highest was in the litter. The C_{TOT} and N_{TOT} values of the samples from the lagoon were much higher than the C_{TOT} and N_{TOT} values for clay 1. The C:N ratios were generally high. The highest C:N ratio was in the litter, while the lowest C:N ratio was calculated for clay 1. The C:N ratios for the samples from the lagoon were significantly higher than the C:N ratio for clay 1. Also, the S_{TOT} values for the samples from the lagoon were much higher than the S_{TOT} value for clay 1. Similar was the distribution of $S\text{-}SO_4$ concentrations. The highest concentration of available phosphorus (P_2O_5) was noticed in clay 2. The P_2O_5 concentrations were much lower in other samples. The highest concentration of available potassium (K_2O) was observed in organic soil and the lowest was measured in clay 2. The TPH concentration in clay 1 was under the detection limit of the method, while the TPH concentrations were high in the samples from the lagoon. In the lagoon, the highest TPH concentration was noticed in litter and the lowest was in organic soil. Similar were the distributions of TPOC and PEE values. The concentration of total PAHs was low in clay 1. The concentrations of these compounds were much higher in the lagoon; the highest was in clay 2 and the lowest in organic soil.

Table I.

Table II.

Table II cd.

DISCUSSION

The nearly neutral reaction (pH), high C:N ratio, low nitrogen, sulfur and available phosphorus contents along with high potassium content characterized the clayey soil from the bottom of the dried pond (control). The localization of the pond in the vicinity of a human population, as a source of keratinous remnants, along with the above-mentioned factors resulted in the good growth of keratinolytic fungi in the control soil. However, the qualitative composition of these microorganisms was rather restricted. *Trichophyton ajelloi* prevailed on hair bait, with only several strains of *Aphanoascus durus* and *Chrysosporium* anamorph of *Arthroderma curreyi* observed. The geophilic dermatophyte *Trichophyton ajelloi* is considered as a species favoring high humidity and acidophilic reaction as well as irrespective to organic carbon content [3, 4]. The latest studies [18, 19] confirmed the higrophilic nature of this fungus and its independence of C_{TOT}. However, the above-quoted studies also showed that the incidence of *Trichophyton ajelloi* was rather associated with low total nitrogen and sulfur contents than with acidic reaction. The present data appear to confirm the association of this dermatophyte with low total nitrogen and sulfur content. Little is known on the ecology of *Aphanoascus durus*. This species abundantly occurred in the ash heap ground considerably influenced by a municipal landfill site [13]. However, no correlation between its frequencies and physico-chemical and microbiological characteristics of soils was found [16]. The third species, *Arthroderma curreyi*, is considered as acidophilic. Studies of keratinolytic fungi in the surrounding of a municipal landfill site [16] and in sewage sludge [18] univocally corroborated the acidophilic nature of this species. The studies also showed that the occurrence of *Arthroderma curreyi* was associated with high concentrations of available potassium and total nitrogen. The results obtained in this study only confirmed the association of this fungus with available potassium.

The data indicate that a remarkable part of the lagoon's content was the clay (silty medium loam by PTG or loamy silt by USDA) typical of the examined area of Czechowice-Dziedzice. However, the lagoon's physico-chemical conditions were different from those observed in the control clay, which was caused mainly by high quantities of petroleum hydrocarbons and their derivatives together with acidic reaction derived from sulfuric acid. The contaminants were predominantly non-polar aliphatic hydrocarbons and polar derivatives extractable in CCl_4 and petroleum ether. Most of the contaminants were liquid hydrocarbons (heavy oils), black solid compounds such as asphaltenes and resins, and PAHs. The concentrations of toxic BTEXs were negligible. There is little information on the influence of petroleum hydrocarbons on keratinolytic fungi in available literature. On the basis of general data [1] and our preliminary reports [15, 17] it can be assumed that the high concentrations of petroleum hydrocarbons made the growth of keratinolytic fungi in clay 2 and litter from the lagoon impossible. Shaking the roots of the grass growing in the lagoon's green oases allowed to collect the sample of organic soil so that this sample included the root-adjacent material. There are a number of microorganisms able to degrade hydrocarbons in the root-adjacent soil, including the rhizosphere [7]. It is believed, therefore, that due to the long-term activity of these microorganisms the concentrations of hydrocarbon contaminants were much lower in organic soil than in other materials from the lagoon. The relatively low concentrations of hydrocarbons enabled the abundant growth of *Trichophyton ajelloi*. The other species characteristic for the contaminant-free control clay was however eliminated in the natural process of soil self-purification from petroleum contaminants. It is possible that the strains of *Trichophyton ajelloi* adapted to the extreme lagoon's conditions participated in this process [17].

Trichophyton ajelloi has been reported to be a causative agent of cutaneous mycoses in humans [5]. However, these cases are very rare. Therefore, it can be generally considered that the keratinolytic fungi from the lagoon and its surrounding did not pose a threat to public health. It was observed, however, that petroleum contaminants supported the growth of potentially pathogenic species, *Microsporum gypseum* in particular in other types of soils (unpublished data). This finding illustrates possible epidemiological problems that may arise from the contamination of the environment with petroleum hydrocarbons.

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Summary

The study was to examine the incidence of keratinolytic fungi in the lagoon with acidic petroleum wastes (before bioremediation) at a petroleum refinery situated within a highly populated area. High concentrations of petroleum hydrocarbons (non-polar and polar aliphatics and PAHs) made the growth of keratinolytic fungi in clay and litter from the lagoon impossible. The natural self-purification process considerably decreased the hydrocarbon contamination, increased the pH and caused the abundant growth of *Trichophyton ajelloi* in organic soil. This soil included the root-adjacent material from the grass growing in several green oases at the lagoon. It is possible that the strains of *Trichophyton ajelloi* adapted to the extreme lagoon's conditions actively participated in the soil self-purification process. The fungal species isolated in the present study did not pose a threat to public health. However, petroleum contaminants may support the growth of potential pathogens, for example *Microsporum gypseum*, in other types of soils.

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Streszczenie

Celem pracy było przebadanie grzybów keratynolitycznych w lagunie kwaśnych odpadów rafineryjnych (przed bioremediacją) w obrębie rafinerii ropy naftowej znajdującej się na terenie gęsto zaludnionym. Wysokie stężenia weglowodorów ropopochodnych (nie polarnych i polarnych związków alifatycznych oraz WWA) uniemożliwiły wzrost badanych grzybów w glinie i odpadach z laguny. Naturalny proces samooczyszczania znacznie obniżył zawartość węglowodorów, podwyższył pH i spowodował obfity wzrost *Trichophyton ajelloi* w glebie organicznej. Gleba ta zawierała materiał pochodzący ze strefy korzeniowej traw rosnących w kilku zielonych oazach na obrzeżu laguny. Nie można wykluczyć, że szczepy *Trichophyton ajelloi* zaadaptowane do skrajnych warunków w lagunie brały aktywny udział w procesie usuwania zanieczyszczeń ropopochodnych. Gatunki grzybów wyizolowane w niniejszej pracy nie stwarzały istotnego zagrożenia

epidemiologicznego. Zanieczyszczenia ropopochodne mogą jednak sprzyjać wzrostowi potencjalnych patogenów, np. *Microsporum gypseum* w innych typach gleb.

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