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MICROBIAL COLONIZATION OF PLASTIC BAGS IN THE LABORATORY AND FIELD CONDITIONS

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Abstract
Plastic bags, produced in a large number and directed after usage to the environment, are extremely permanent in nature. Due to the fact that they are harmful to the environment it would be advisable to find a biological way of their decomposition. Some of the materials are known to be biodegradable but the period of their degradation vary and depends on the environmental factors. That is why in this study two types of plastic bags: biodegradable and oxydegradable were used in both, field and lab experiment to confirm their susceptibility towards microbial colonization and in the next step – their biodegradation. During 6 month experiment the number of bacteria, fungi and actinobacteria present on the plastic surface were estimated with plating method. Also the bacterial changeability and the level of their biodiversity in the soil samples were analyzed using PCR-DGGE.

Streszczenie
Torby plastikowe, produkowane w znacznych ilościach i wyrzucane po zużyciu są niezwykle trwałym materiałem. Z tego względu są one uznawane za wyjątkowo szkodliwe dla środowiska i warto poszukać możliwości ich biologicznego rozkładu. Część tych materiałów plastikowych jest znana jako biodegradowalne, jednak czas ich rozkładu w środowisku różni się w zależności od czynników środowiskowych, które na nie działają. Z tego względu w eksperymencie wykorzystano dwa typy toreb plastikowych: biodegradowanej i oksydegradowalnej, których inkubację prowadzono w warunkach laboratoryjnych i terenowych w celu potwierdzenia ich podatności na mikrobiologiczną kolonizację, a co za tym idzie i na możliwości biodegradacyjne. W eksperymencie trwającym 6 miesięcy oznaczano liczebność bakterii, grzybów i promieniowców kolonizujących powierzchnie plastikowe metodą płytkową. Zmienność bakteryjna w próbkach gleby była dodatkowo oznaczana metodą PCR-DGGE.

Keywords: **Biodegradable and oxydegradable plastic bags decomposition; Microbial activity; PCR-DGGE biodiversity analysis.**

1. INTRODUCTION

Pollution is an any factor influencing ecosystem balance and the disappearance of at least one species. Such pollution can be chemical, biological or physical [1]. Plastic materials, including plastic bags, are considered to be pollution due to their durability in the environment for many years after disposal [2, 3]. There is often a misunderstanding between polymer chemists and microbiologists because for chemists degradation means the loss of mechanical or other physical properties, while for microbiologists it is a degradation to compounds harmless for the environment [4]. It should be also mentioned that to some

point of its decomposition plastic foils are considered to be "macropollutants". The main danger of their presence in the environment is to be swallowed by wild animals or cover materials in the landfills or soil preventing from microbial access and decomposition [4].

Nowadays in Poland there are 20 times more plastics produced than 50 years ago. Over 80% of plastic materials is directed to the landfills, 8% is burnt and only 7% recycled. The problem arises mainly due to the rising level of plastics production together with a lack of the well working system for collecting and recycling them [5].

Oxydegradable plastics are produced as by-products of the crude oil refrainment. The method of the production is based on minute amount of the supplementation added to the plastics to change its structure. These supplements are usually TDPA (Totally Degradable Plastic Additive) or d2w (degradable to water), added in order to accelerate polyethylene decomposition. The crucial point of the technology is that the plastic decompose already in the moment of its production and it could be accelerated by heat, sun light or pressure. Plastics can be consumed by microorganisms in the moment when their structure is decomposed to the level lower than 40 000 Da [5].

The biodegradable polymers are sensitive to microbial enzymes. For biopolymers production substrates such as polylactide (PLA), sugar, starch, vegetable oils, or chemically modified cellulose are used. They are usually obtained from maize, potatoes, wood, cereal or sugar cane [6]. In case of this experiment starch biopolymer and polyethylene with TDPA bags were used.

Biodegradation begins when the polymeric chain is shortened and the level of polymerization is decreased. In the end of the process simple chemical compounds, such as biomass, water and gases (carbon dioxide or methane) are produced. Polymer decomposition scheme presents Figure 1.

The removal of the pollutants from the environment using microorganisms is called bioremediation. Bioremediation can be performed as [7]:

- natural bioremediation – (also called bioattenuation) based on regular monitoring of the pollution concentration degraded by autochthonous microorganisms;
- biostimulation – the most common, based on stimulation of growth and activity of naturally occurring microorganisms by adding oxygen or necessary feeding elements;

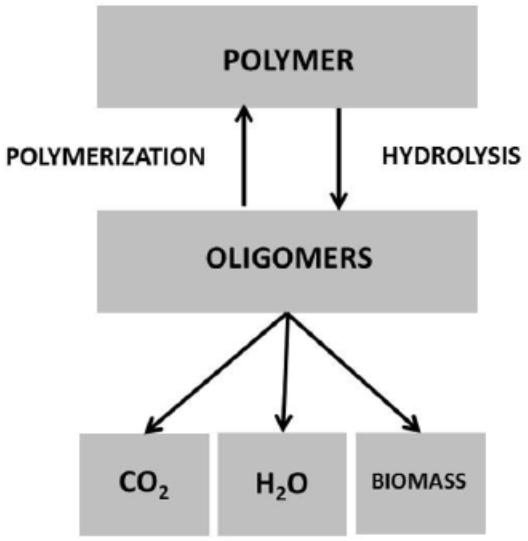

Figure 1.
Polymer decomposition scheme [5]

- bioaugmentation – adding a particular sort of bacteria, also genetically modified, able to degrade the pollution.

In the soil, the part of the environment where the biodegradation of plastics is performed, 3 groups of microorganisms according to their size can be distinguished: mesofauna (invertebrates between 0.1 mm and 2 mm in size, which live in the soil or in a leaf litter layer on the soil surface, typical members of this group are nematodes, mites, springtails (*Collembola*), proturans and paurapods), macrofauna (over 1 cm, such as myriapods, centipedes, millipedes, slugs, snails, fly larvae, beetles, beetle larvae, and spiders) and the most important from the biodegradation point of view – microorganisms (bacteria, fungi and actinobacteria). Their physiological activity is the basis of many soil processes such as: decomposition of organic matter, nitrogen, phosphorus and sulphur changes. Their activity depends on the temperature and physio-chemical parameters [8].

The aim of this work was to compare the level of microbial colonization of two sorts of plastic material – biodegradable and oxydegradable, in laboratory and field conditions. The experiment was performed for 6 months. The estimation of bacteria, fungi and actinobacteria number was performed using plating method after 1, 2, 3 and 6 months of the incubation in soil environment and on the plastic surfaces. To estimate bacteria biodiversity changes PCR-DGGE analysis of soil samples was performed.

2. MATERIALS AND METHODS

2.1. Experiment settings

The experiment lasted 6 months. Two types of plastic bags (biodegradable made of starch and oxydegradable made of polyethylene with TDPA) were cut to 5 × 5 cm squares and dug in the soil (5 cm under its surface) in the laboratory and the field conditions. For lab experiment constant humidity (30%), temperature (22±3°C) and pH (pH=7.0) were maintained. In case of field experiment pH, temperature and humidity were fluctuating and they were measured during the sampling period – after 1, 2, 3 and 6 months.

2.2. Plating method for microbial enumeration

The number of bacteria, fungi and actinobacteria was estimated using plating method on the plastic square surface and in the soil sample. The soil suspension in both cases was diluted to 10^{-6} in sterile 0.9% NaCl solution. For bacterial and fungal/actinobacterial analysis agar broth (BTL, Łódź) and Czapek agar media (BTL, Łódź) were used, respectively. Each dilution was inoculated on the plates in triplicate. The plates were incubated in 20°C: agar broth medium for 24-48 h, Czapek agar medium 7 and 14 days for fungi and actinobacteria, respectively. The microbiological analysis for soil was performed in the beginning of the experiment and after 1, 2, 3 and 6 months, on the plastic squares after 1, 2, 3 and 6 months.

2.3. Bacterial DNA isolation and PCR-DGGE analysis

Soil samples collected after 1, 3 and 6 months of the experiment were stored at -20°C until DNA isolation. Total genomic DNA was extracted from 0.2 g of the soil samples with mechanical method. The samples washed 3 times with 1 × PBS buffer (Sigma) were disintegrated with bead beating (Roth, Germany) in lysis buffer containing Tris-HCl 100 mM, EDTA 100 mM and NaCl 1.5 M (pH = 8.0). The samples were incubated for 20 minutes in 1400 rpm and 200 µl 10% SDS was added. After 30 minutes of incubation in 65°C samples were centrifuged twice at 13 000 rpm and placed on spin filters (A&A Biotechnology). DNA attached to the filter was washed twice with 70% ethanol solution (A&A Biotechnology). The amount of DNA was measured spectrophotometrically using Qubit (Invitrogen) and stored at -20°C until PCR amplification.

In this study partial 16S rRNA gene amplification was performed using primers 338F with GC clamp and 518R, which amplified a partial (ca. 180 bp) 16S rRNA gene fragment of all the bacteria [9]. PCR procedure was described previously [10]. PCR amplification for fungi and actinobacteria was also performed but with no positive results obtained.

The DGGE of the PCR products underwent electrophoretic separation in the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (8% 37:1 acrylamide-bisacrylamide, Fluka) with a gradient of 30-60% denaturant was prepared according to the manufacturer's instruction. The gel was run for 6 h at 70 V in a 1 × TAE buffer at a constant temperature of 60°C. The gel was stained with SYBR Gold (1:10 000, Invitrogen) in MiliQ water for 30 minutes and distained in MiliQ water for 40 min, then visualized under UV light and photographed using Quantity One 1D (BioRad). The analysis of DGGE fingerprints was performed using a Quantity One 1D software (BioRad). Bacterial biodiversity was estimated on the basis of densitometric measurements and Shannon biodiversity index.

3. RESULTS

The microbial enumeration was performed after 24-48 h on agar broth for bacteria and after 7 and 14 days on Czapek agar for fungi and actinobacteria, respectively. The microbiological analysis for soil was performed in the beginning of the experiment and after 1, 2, 3 and 6 months, on the plastic squares of two types of plastic bags, biodegradable and oxydegradable after 1, 2, 3 and 6 months. The results of the soil microbial analysis under the laboratory and field conditions are presented in Figure 2 and 3. Bacteria number in the laboratory conditions decreased during the experiment, while for field conditions decreased till 3rd month of the experiment, and increased slightly in the end of the experiment. In case of fungi their number increased in the laboratory condition, while in the field conditions fungi number was slightly lower in the beginning of the experiment, than relatively constant (Figure 3a). Actinobacteria number increased in the field conditions till 3rd month of the experiment, but decreased drastically in the end of the experiment (Figure 3b). In the laboratory conditions actinobacteria number seems to be constant. Figures 4 and 5 present the results of microbial enumeration on biodegradable and oxydegradable plastic bags surface after 1, 2, 3

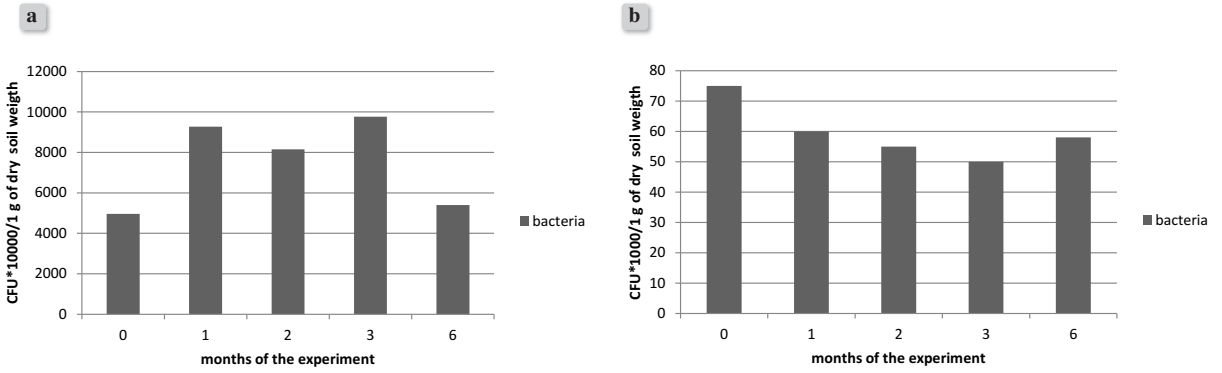

Figure 2.
Bacteria enumeration using plating method for soil during the field (a) and laboratory (b) experiment

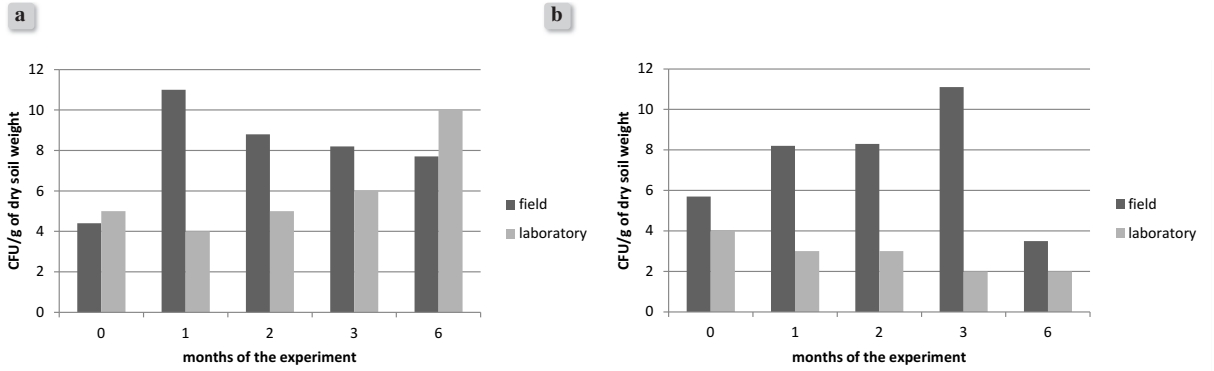

Figure 3.
The comparison of the fungi (a) and actinobacteria (b) enumeration using plating method for soil samples in the laboratory and field conditions during the experiment

and 6 months of the field and lab experiment. As it can be seen biodegradable bag's surface (Figure 4) is colonized mainly by bacteria and their number is relatively stable. Fungi and actinobacteria level decreases in the laboratory conditions. Oxydegradable foil surface (Figure 5) is also colonized mainly by bacteria, while the level of fungi is higher for laboratory conditions and actinobacteria number is comparable for both environments (except the first month of the lab experiment).

To estimate biodiversity of bacteria and their changeability during the experiment PCR-DGGE analysis was undertaken. The result of the electrophoretic resolution underwent densitometric analysis. The diagram constructed on the basis of the bacterial fingerprints is presented at Figure 6a. On the basis of densitomentric analysis Shannon biodiversity index was calculated and presented in Figure 6b. To estimate biodiversity of fungi and actinobacteria in the soil samples PCR amplification with these groups specific primers were also performed but with no positive results. It could be explained by too low concentration of fungi and actinobacteria DNA isolated from the soil samples.

4. DISCUSSION

The number of soil microorganisms in the laboratory conditions (Figure 2b) is far lower than in the one in the field samples (Figure 2b). It is probably caused by the source of the material. Laboratory soil was dedicated to home plants, probably partially disinfected or sterilized. The number of bacteria decreased gradually during the experiment, probably because of the soil impoverishment. Bacterial number in the field during the experiment was the lowest in the beginning and the end of the experiment started in the end of January and the first, the second and the third months were March, April and May, respectively. As

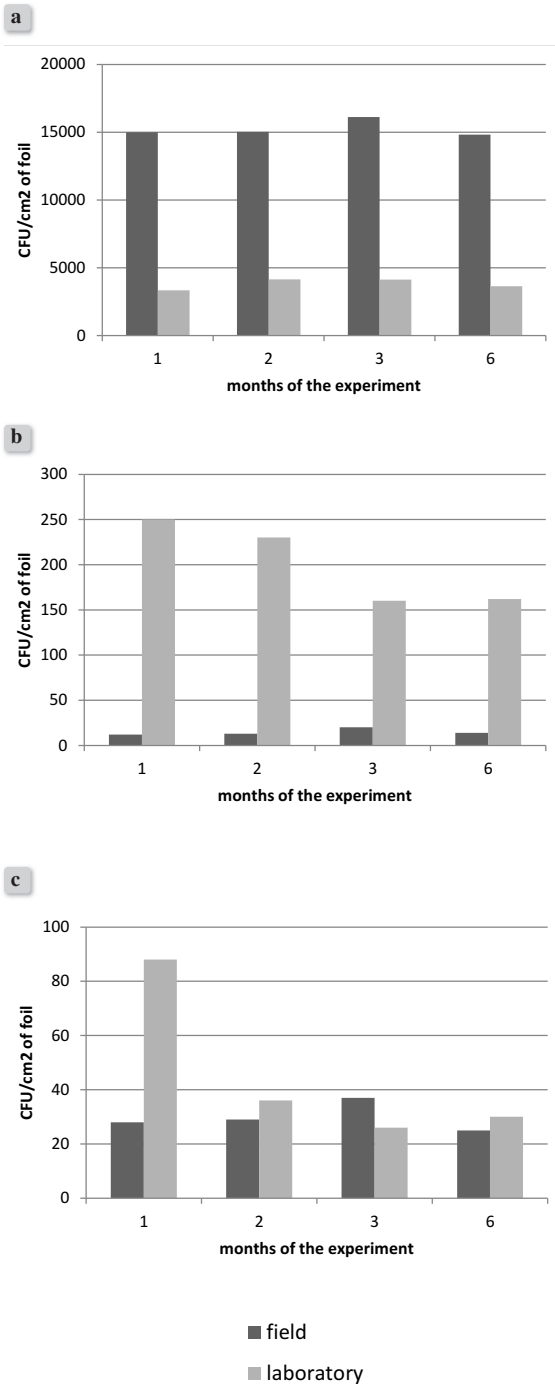

Figure 4.
Bacteria (a), fungi (b) and actinobacteria (c) enumeration using plating method for biodegradable foil in the laboratory and field conditions during the experiment

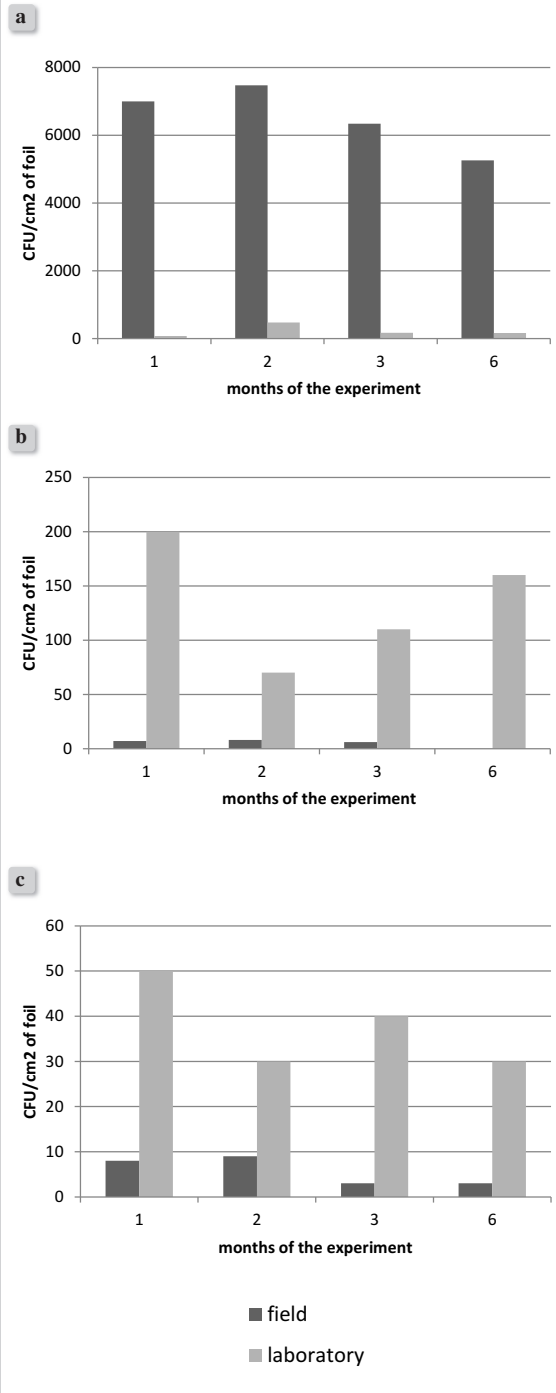

Figure 5.
Bacteria (a), fungi (b) and actinobacteria (c) enumeration using plating method for oxydegradable foil in the laboratory and field conditions during the experimentfield conditions during the experiment

it was stated in the bibliography the highest bacterial number is in spring and autumn [11]. These data corresponds with DGGE analysis, where the number of the bands in the fingerprints (Figure 6a), as well as biodiversity index calculated on the basis of DGGE fingerprints (Figure 6b) also increased gradually for

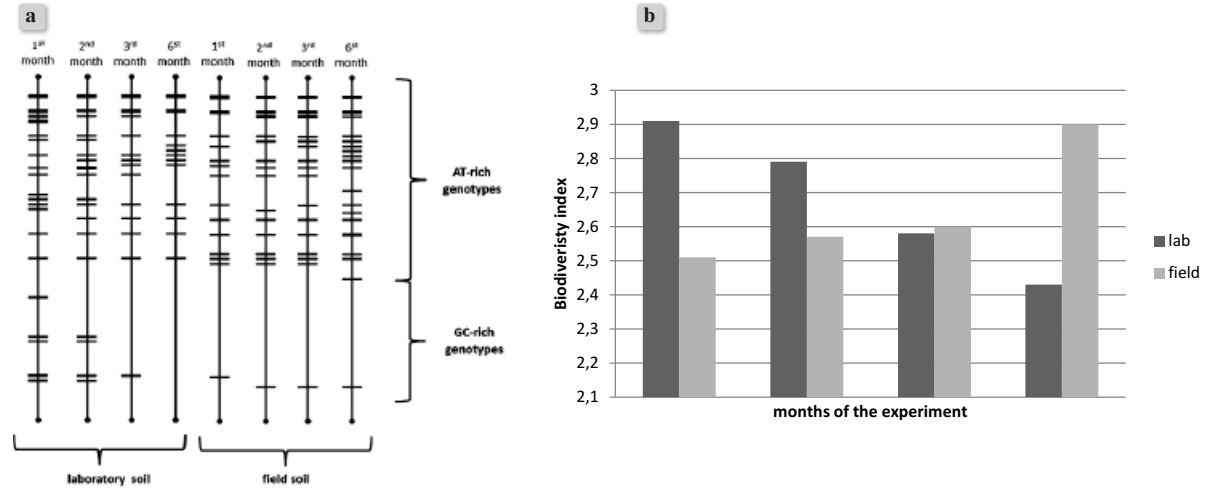

Figure 6.
PCR-DGGE bacterial fingerprints analysis in the soil samples in which foil biodegradation was performed for the field and laboratory conditions; a) PCR-DGGE diagrams presenting bacterial fingerprints obtained for soil samples in 1st, 2nd, 3rd and 6th month of the experiment; b) Shannon biodiversity index changes in 1st, 2nd, 3rd and 6th month of the experiment calculated on the basis of PCR-DGGE bacterial fingerprints

the field experiment, while for laboratory soil bacterial diversity level was decreasing during the experiment, probably due to the soil impoverishment. As it can be seen in Figure 6a bacterial changeability is linked mainly with the GC-rich genotypes, appearing and disappearing in the lower part of the gel, while in the upper part of the DGGE fingerprints AT-rich genotypes' group seems to be relatively constant. These results point that the fertility of the soil during vegetation season is linked with the biodiversity of bacteria responsible for the mineralization processes. It is also the time of allochtonic bacteria income, which was not observed in the laboratory experiment. Bacteria number and diversity changes during year seasons in the field, while laboratory soil bacterial biodiversity decreases gradually.

The number of fungi in the field soil increased drastically in the first month of the experiment, but their number gradually decreased from 2nd to 6th month of the experiment. It could be explained with the spore presence in the material, whose level was higher in winter, but in the beginning of spring they germinated in the field and due to the favorable vegetation conditions only vegetative fungi forms were collected for lab experiment. In case of laboratory soil the level of fungi was increasing gradually, also probably because of the spore presence and their gradual germination in the material. In unfavorable conditions (the soil was getting poor during the experiment) vegetative forms could produce fresh spores germinating gradually in the soil.

The number of actinobacteria in the field soil was increasing gradually during the spring time. This group of microorganisms is responsible for soil fertility and their number increases in the growing season. In the end of the experiment the weather conditions were unsuitable (hot, rainless season) for actinobacterial growth and their number dropped drastically. The number of these microorganisms in the laboratory soil was estimated at comparable levels, slightly decreasing during total length of the experiment. It could be explained with the experiment settings where no vegetation was planted in the soil and no organic material except foil serving as feed medium was present. Not only the type of the food source, but also pH was not optimal for actinobacteria and fungi. In case of actinobacteria it should be more alkaline while for fungi, more acidic.

Biodegradation of foil should be preceded by its surface colonization. As it was suspected the biodegradable plastic was colonized with microorganisms in the higher level (Figures 4) than oxydegradable (Figure 5).

The number of bacteria in case of the biodegradable foil was at the level of 15 000 and 4 500 CFU per 1 cm^2 of foil, while on oxydegradable – 7 000 and 100 per 1 cm^2 of foil, in the field and the lab experiment, respectively. The number of bacteria colonizing biodegradable plastic is higher in the field soil than the lab soil probably because of the initial bacterial number present in the environment of the experiments, but interestingly, the level of bacteria is stable

during total length of the experiment in both cases (Figure 4a). In case of oxydegradable foil bacteria number seems to decrease during time (Figure 5a).

Number of fungi colonizing foils is slightly higher on biodegradable one in the beginning of the experiment (Figure 4b and 5b) in both environments. In lab soil fungi number decreases during the experiment for biodegradable foil, but in the field conditions their number does not change drastically. The fungi number colonizing oxydegradable foil first decreases, than, from the 2nd month of the experiment, increases in laboratory conditions, while in the field fungi colonizing this foil disappear in the end of the experiment.

For both soils the actinobacteria number was the highest in the beginning of the experiment in lab conditions while in the field conditions the level of actinobacteria was fluctuating slightly and it was comparable during total length of the experiment. It seems reasonable to suspect that actinobacteria does not possess proper (or flexible enough) enzymatic apparatus than bacteria thus they are not able to degrade such polymeric compounds.

These results confirmed the previous studies [12] where the most active group of microorganisms during biodegradation was bacteria. It was also previously stated [5] that the oxydegradable foil should be first depolymerized with physical factors to the level accessible for microorganisms' colonization.

Biodegradable foil seems to colonize easier with bacteria in the field conditions while the other two groups of microorganisms are not present on its surface in a large number. Also in the laboratory conditions the number of bacteria on the foil surface is higher. Nonetheless, it should be also underline that the number of bacteria during the experiment is relatively stable which can lead to the conclusion that the foil surface possessed several ecological microniches for bacterial colonization, but these bacteria are not necessarily responsible for biodegradable foil decomposition.

In case of oxydegradable foil fungi seems to be colonizing their surface far better than bacteria and actinobacteria, but only in the laboratory conditions. In the field conditions all three microbial groups number decrease during the experiment. Such situation can be explained with the spore presence in the lab soil, growing and sporulating again in the relatively unfavorable conditions (no vegetation and pH = 7.0). Oxydegradable foil seems to be resistant towards microbial colonization and physical conditions can enhance their degradation.

5. CONCLUSIONS

On the basis of the study performed it could be stated that oxydegradable foil was colonized far worse than biodegradable one with bacteria and fungi. Actinobacteria seems not to be a relevant group of microorganisms biodegrading that sort of the material, probably because of the lack of the proper enzymes. The results revealed that in the laboratory conditions only fungi possess potential for oxydegradable foil colonization. In case of biodegradable foil mainly bacteria are colonizing its surface. However, their stable number during total length of the experiment suggests that bacteria colonized biodegradable foil surface but they are probably not responsible for their biodegradation. Field conditions didn't fasten foil biodegradation (the foil surface was not damaged in both experiments) but it seems to be reasonable to suspect that non-sporulating microorganisms colonize foil surface faster in the field conditions, while sporulation enables easier foil colonization in the laboratory experiment.

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