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Abstract

Biodegrability of high density polyethylene film (HDPE) and low density polyethylene film (LDPE) both containing a balance of antioxidants and pro-oxidants was studied with defined microbial strains particularly with *Rhodococcus rhodochrous* and *Nocardia asteroides* in mineral medium. After an abiotic pre-treatment consisting of photooxidation and thermo-oxidation corresponding to about 3 years of outdoor weathering the samples were inoculated, incubated up to 200 days and during the period their metabolic activities were followed by measuring adenosine triphosphate content. Simultaneously the cultures were also monitored by optical microscopy and FTIR spectroscopy. The first initial phase of fast growth caused by the presence of low molecular extractable compounds was followed by a long period of stabilized metabolic activity suggesting that microorganisms continued to gain energy from the substrate but evidently at a much slower rate. Complementary analysis performed at the end of incubation revealed that during the experiment time biodegradation processes probably affected surface layer of materials only.

Keywords: Adenosine triphosphate; Polyethylene; Biodegradation; Pro-oxidant; Photooxidation; Thermo-oxidation

1. Introduction

Traditionally the biodegradability of pro-oxidant activated PE has been evaluated in complex media like soil, waste water sludge or compost. The approach brings several advantages like availability of very diverse microbial inoculum or close relation to the real conditions in the nature and/or in waste treatment processes. Recently two studies presented quantitative indications of the biodegradability of PE film containing a pro-oxidant additive. Chiellini et al. [1] followed incubation of previously thermally exposed film in soil or in compost conditions and reported 50–60% and 80% mineralization, respectively, after approximately one and a half year of incubation. Jakubowicz [2] claims even 60% mineralization during only 6-month incubation.

Although such experiments are relevant to natural conditions, the chemical and biological complexities of the system make it difficult to gain a better insight into detailed mechanisms of biodegradation. For a better understanding of the process fundamentals the key task is to define more controlled experimental conditions i.e. experiments with identified microbial strains in a medium formulated from defined chemical compounds. Establishing such controlled conditions was the key effort throughout this study; this approach appeared to be complementary to previous works using complex media.

During previous experiments [3,4] bacterial and fungal strains promising for oxidized PE degradation were selected and it was shown that at least some of them form dense biofilm. Scanning electron microscopy also revealed signs of

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bioerosion on the material surface. Some of the strains were utilized also in the present work.

To follow growth and development of microbial cultures the method of determination of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) was adopted. ATP is the key molecule of all living cell energetic metabolism. Its level reflects metabolic activity of a culture. Moreover the adenylates can be extracted and determined from entire sample, which means simultaneously from both the biofilm on the material surface and the surrounding liquid medium, the assay is extremely sensitive and the response linear across several orders of magnitude.

2. Materials and methods

2.1. Tested material

The material samples were transparent HDPE film 20 μm thick and transparent LDPE film 60 μm thick. Both films contained iron photo-inducer, different from the Scott/Gilead compound (Schulman – Bornen, Belgium) supplying radicals through a photo-redox process and an organometallic type thermo-inducer (EPI, Vancouver, Canada) catalysing the primary hydroperoxide decomposition. Both additives were present in both films. To balance the prodegradant activity of the photo- and thermo-inducer during the first year of storage and use under indoor conditions phenolic antioxidants were used in the blends. Due to the added antioxidants the thermal-induction period was longer than 400 h at 60 °C in dark.

2.2. Abiotic treatment

The samples were exposed for 120 h in the accelerated photoageing chamber SEPAP 12.24 [3,5] ($\lambda \geq 300$ nm, temperature of the exposed surface was set at 60 ± 1 °C). After the exposure in SEPAP the samples were considered sterile and treated in a sterile way. The first 20 h of exposure corresponded to the photochemical induction period (total photo-transformation of phenolic antioxidants into inactive compounds). During the additional 100 h fast photooxidation proceeded and at the end of the period the absorbance measured at 1715 cm^{-1} should be equal or higher to $x/100$, where x was the film thickness in microns. At this level of oxidation spontaneous fragmentation of the film should be observed. For the examined PE films 100 h of exposure in SEPAP 12.24 corresponds to 3 months weathering under European outdoor conditions in the period from March to October.

Exposure in SEPAP was followed by 300 h of exposure in an aerated oven at 60 °C. Based on the activation energy determined when thermo-oxidation proceeded without restraint (after the total consumption of phenolic antioxidants) 300 h of thermo-oxidation at 60 °C was equivalent to 2–3 years of thermo-oxidation at room temperature in the dark (particles buried in the soil or particles not exposed to sunlight).

It should be emphasized that instead of exposure in SEPAP 12.24 under dry conditions the abiotic pre-treatment also included 120 h exposure in SEPAP 12.24 H where the films were exposed submerged under 2 mm water layer with the controlled temperature (60 ± 1 °C) where the constant concentration of dissolved oxygen is assured by re-oxygenation in the external circuit. The UV light ($\lambda \geq 300$ nm) passes through the thin water film without any intensity decrease. The experiment was designed to check if release of any water extractable compounds from the material tested could influence its photo-oxidisability and thermo-oxidisability in the surface layer in the aqueous environment.

To obtain more uniform and more convenient particle size the exposed film, already highly fragmented and very fragile was passed through a metallic screen with hole diameters of about 1 mm.

In the actual phase of thermo-oxidation, the apparent activation energy was determined to be 100 kJ/mol, whereas in the phase corresponding to the thermal-induction period during which phenolic antioxidant counteracted the influence of the pro-oxidant additive, the apparent activation energy was only 40 kJ/mol.

2.3. Strains of bacteria and fungi

Rhodococcus rhodochrous ATC 29672, *Aspergillus flavus* ATCC 26873, *Mortierella alpina* ATCC 36965, *Cladosporium cladosporoides* ATCC 20251 were purchased from American Type Culture Collection; *Nocardia asteroides* LAB 911 was previously isolated in our laboratory [4].

2.4. Medium and conditions of the cultivation

The mineral medium utilized throughout the study had the following composition: 3.8 g $Na_2HPO_4 \cdot 12H_2O$, 1.8 g KH_2PO_4, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.03 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.5 g NaCl, 0.3 g NH_4Cl and 1 ml of trace element solution per liter. The trace element solution contained 0.20 g $MnSO_4$, 0.029 g H_3BO_3, 0.022 g $ZnSO_4 \cdot 7H_2O$, 1.0 g Na_2MoO_4, traces of $Co(NO_3)_2$, and traces of $CuSO_4$ dissolved in 500 ml of water.

Thimerasol at final concentration 0.01% (w/w) was added into abiotic control cultivations as a growth inhibitor.

In general incubations for microscopy, GPC and spectroscopy observation were done in closed 100 ml glass flasks with 10 ml of media, incubation for ATP level determination in 4 ml closed glass vials with 0.4 ml of media. In both cases the head-spaces were sufficiently large to provide the cultures with oxygen; moreover the flasks and vials were opened monthly so that the head-space air could be refreshed. Gas-tight sealing of the vessels was necessary to prevent water evaporation during the long incubation. The cultures were kept at 27 °C with gentle shaking. PE substrate concentrations were about 4 mg/ml for flask cultures and about 5 mg/ml for cultures in vials.

2.5. Transmission and ATR-FTIR

Small fragments whose section could be as small as 1000 μm^2 were analysed by micro-FTIR spectrophotometry,

the combination of FTIR spectrophotometry and IR microscopy (NEXUS, Thermo Nicolet). The IR beam entering the spectrophotometer was focused on a predefined 1000 μm^2 zone of the small fragment and a micro-FTIR spectrum was recorded in the transmission mode with a resolution of $\pm 2\ cm^{-1}$ and absorbance defined with ± 0.001 precision. The optical path was determined using the main absorption band of HDPE or LDPE at 1468 and 1465 cm^{-1}, respectively. μATR-FTIR spectra were recorded on a Nicolet 800 FTIR spectrometer connected to NIC-PLAN IR microscope.

2.6. Size exclusion chromatography (SEC)

The whole cultures were dried in vacuum, polymer was dissolved in a defined volume of added 1,3,5-trichlorobenzene at 160 °C to obtain 0.1% solution and filtered. Santonox R phenolic antioxidant of 250 ppm was present in the solvent to prevent the oxidation of the sample by oxygen. Molecular weight distributions were determined with high temperature GPC system Waters 150C ALC/GPC. Solutions of polystyrene molecular weight standards were used for the calibration.

2.7. ATP and ADP assays

ATP Biomass Kit HS by Biothema (Sweden) was used for ATP determination. For each determination ATP from the entire culture in a 4 ml vial was extracted. At least two vials were analysed for each time point. ADP was determined after transformation of ADP to ATP directly in the luminometer cuvette. Reaction mixture contained 30 μl of the sample extract, 240 μl of diluent B from the ATP kit, amended with 20 mM KCl and 2 mM $MgSO_4$, and 10 μl of solution containing 0.1 M phosphoenolpyruvate and 5 mg/ml protein kinase in 0.05 M Tris−acetate buffer pH 7.2 [6]. The mixture was incubated for 45 min at 35 °C, equilibrated to the lab temperature for 15 min and then the light producing reaction was started by the addition of 60 μl ATP reagent HS (Biothema) and reconstituted with 2.5 ml of water. A blank experiment was done simultaneously to correct results for the background signal of the reagents.

2.8. Microscopy techniques

PE film fragments were separated from the suspension by carefully pipetting out the water phase. For the optical microscopy microorganisms on the PE film fragments were initially fixed with fixative solution containing formaldehyde and ruthenium red and then stained with safranine.

Samples for scanning electron microscopy were prepared with the procedure described in Ref. [4].

2.9. NMR spectroscopy

NMR spectra were recorded after filtration of the media through 0.2 μm porosity filter.

NMR samples were prepared as follows: supernatants (540 μl) produced from biodegradation tests were supplemented with 60 μl of a 5 mM solution of $TSPd_4$ (sodium tetra deuterated trimethylsilyl propionate, Eurisotop) in D_2O (Eurisotop). D_2O was used for locking and shimming while $TSPd_4$ constituted a reference for chemical shifts (0 ppm) and quantification. ^1H NMR spectra were recorded at 500.13 MHz on a Bruker Avance 500 spectrometer at 21 °C with 5 mm-diameter tubes containing 600 μl of sample. One hundred and twenty-eight scans were collected (90° pulse, 4.67 s acquisition time, 3.0 s relaxation delay, 7002.80 Hz SW, 65536 data points). Water signal was eliminated by pre-saturation. No filter was applied before Fourier transformation but a baseline correction was performed on spectra before integration with Bruker software. Under these conditions, the limit of quantification is in the range of 0.05 mM.

3. Results

3.1. Abiotic treatment

The following procedure was aimed to prepare samples corresponding to the material after weathering during the predefined time period in outdoor conditions (see Section 2.2). After this period by the action of the pro-oxidants, light and heat the material should be substantially chemically transformed and thus more susceptible to a microbial attack.

To obtain samples required for the experiments with microorganisms large areas of HDPE or LDPE film were exposed in SEPAP 12.24 photoageing unit. The unit was originally designed for the exposure of small sized samples. In case of HDPE samples with larger surface some problems arose due to mechanical perturbation caused by the air circulation necessary to maintain the sample temperature at 60 °C and spontaneous fragmentation of the sample was observed prematurely i.e. before the oxidation extent corresponding to an absorption increase at 1712 cm^{-1} was equal to $x/100$, where x is the thickness of the film in microns (see Section 2.2). Indeed a PE film usually lost 50% of its mechanical properties when the oxidation extent corresponding to an absorbance increase at 1712 cm^{-1} equals to $x/1000$, so it was not surprising that the air circulation could provoke cracking when the oxidation extent equaled only $0.4x/100$ after 70 h of exposure in SEPAP 12.24.

The fragments of photo-oxidized film were transferred to a sterile box and submitted to thermo-oxidation at 60 °C during 528 h. At the end of the abiotic treatment the absorbance increase at 1712 cm^{-1} determined by micro-FTIR spectroscopy was $3x/100$.

During the abiotic oxidation the molecular weight of the polymer decreased dramatically (Table 1). Surprisingly also narrowing of the molecular weight distribution curve was observed (Fig. 1).

As will be described in detail later, some substances can be released from the oxidized plastic to the water phase. Motivated by concern about whether the pro-oxidant additives could be washed out of the material in humid or aquatic environment an experiment was carried out where the samples were exposed submersed in water in the photoageing unit SEPAP

Table 1
Average molecular weight of the polymer samples before and after the abiotic treatment (see Section 3.1)

Sample	M_W	M_N	I_P
HDPE initial	288 000	34 000	8.5
HDPE after the abiotic treatment	12 800	5200	2.5
LDPE initial	24 200	4800	5.0
LDPE after the abiotic treatment	16 500	5300	3.1

M_W, weight average molecular weight; M_N, number average molecular weight; I_P, polydispersity index.

12.24 H. Our observation then proved that the course of the photooxidation was identical as for the dry conditions and thus the additives were not leached out in a significant extent.

3.2. Metabolic activity of cells during incubation with the oxidized PE

3.2.1. Evolution of ATP content

All of the following described experiments were done in medium containing only necessary growth supporting mineral ions and where the tested material was present as the only source of carbon and energy so that no objection could be raised that the microorganisms were profiting from other media component than the tested material.

Because in the previous studies [3,4] the bacterial strains of *R. rhodochrous* and *N. asteroides* were found to be the most active in terms of biofilm formation on the PE surface, the most detailed attention was paid to the experiments with those microorganisms.

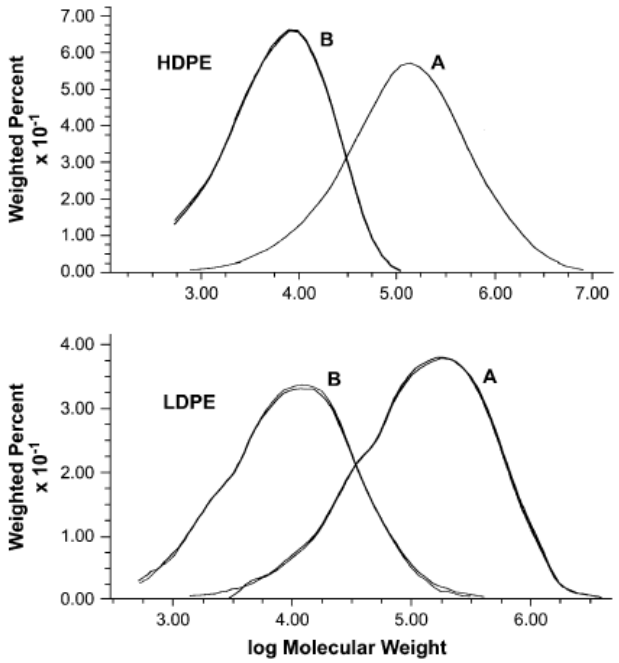

Fig. 1. Molecular weight distribution curves of the polyethylene films. (A) Initial material; (B) after the abiotic treatment (two curves of two parallel samples are displayed for each case).

With the aim of following the evolution of the microorganism culture and its metabolic activity, ATP content was determined in given time intervals. In the case of oxidized HDPE film and *R. rhodochrous* (Fig. 2) the initial phase after the contact with the oxidized PE film and the bacteria was characterized by a fast growth that peaked after about 3 or 4 days of incubation. The observed behaviour was provoked by the availability of the easily metabolisable, probably lower molecular weight substances, present in the treated PE film and/or extracted from the film to the aqueous medium. Then the ATP content dropped as a consequence of the above described "easy" substrate depletion. After approximately 50 days the ATP content, so the corresponding metabolic activity, stabilized itself and maintained virtually the same level up to the end of the observation i.e. up to more than 6 months. The situation was slightly different for the oxidized LDPE film. Here the initial growth was not so intense probably indicating a lower amount of substances being extracted from the material which could be eventually explained by the three times greater thickness of the film compared to HDPE and the corresponding smaller active surface. Nevertheless the ATP content remained at a level similar to the HDPE film.

To prove that the extracted matter really was responsible for the initial fast growth and to find out if the oxidized HDPE without water extractible substances still support bacterial growth another experiment was prepared. The sterile mineral medium and the oxidized HDPE film were combined in a ratio identical as for the previous incubation and shaken 24 h at 27 °C. At the end of this period the solid and the liquid were separated by filtration through a 0.2 μm filter. The solid part was dried in vacuum at the laboratory temperature and cultures were prepared containing the same content of the material as described before for the oxidized HDPE or LDPE films. The liquid part was inoculated and 0.4 ml volumes were distributed into vials. The metabolic activity in the cultures with the solid residue after the extraction peaked and then reached a steady level similar to the complete HDPE film. This suggested that the material still contained some part of the rapidly

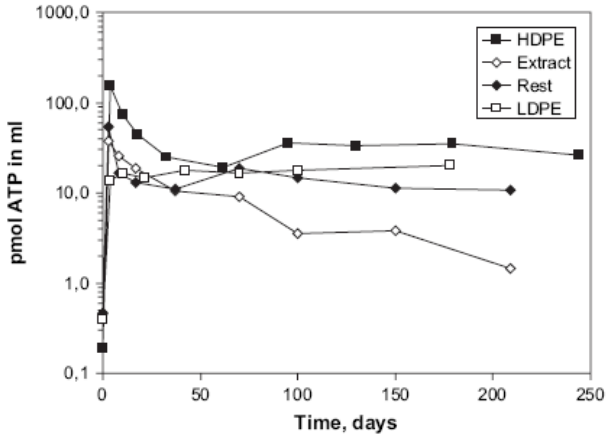

Fig. 2. Evolution of ATP content in *R. rhodochrous* cultures with oxidized PE films and control culture with the aqueous extract from the oxidized HDPE.

metabolisable substances and after their consumption the culture stabilized its ATP content at a level not so far from the complete oxidized LPDE or HDPE films. In contrast to the above observations the cultures with the extract after the same initial growth and the consumption of extracted substances entered into a phase of a slow steady decline of the metabolic activity.

The ATP level curve of the extract could thus be used as some kind of reference demonstrating that the observed stable phase for the other curves was the consequence of a certain level of ongoing substrate supply from the oxidized polymer film to microorganisms.

In the case of *N. asteroides* culture after the initial growth the ATP level remained stable at a similar level for both oxidized HDPE and LDPE films (Fig. 3). Similarly, as in the case described above, the incubations with the HDPE film extract were also followed showing very slow but steady decrease in ATP level. It should be noted that the observation period was shorter than for *R. rhodochrous*.

3.2.2. ADP/ATP ratio at the end of the cultivation

The metabolic state of a microbial population can be better characterized with a knowledge of the other adenylate nucleotide contents. For the selected samples ADP contents were determined in addition to ATP. The ADP/ATP ratio can be considered as a measure of the cell's energetic state. To reach exponential growth cells must be rich in energy and that is why in this type of population most of the adenine nucleotide pool could be found in the form of ATP and the respective ADP/ATP ratio is low. The fact is well documented in the first line of Table 2 for the culture in rich complete Trypcase Soja medium at the end of the exponential growth phase (ADP/ATP = 0.25). Similar values could also be found for cultures with the oxidized HDPE and LDPE as the only substrate in mineral medium in the early phase of incubation (4 days).

Cells with a limited energetic supply cannot keep the ADP/ATP ratio so low, hence all of the population cannot grow exponentially, but the cells are still alive with the working metabolic machinery. It is also possible that in the bulk population some subpopulation of dividing cells still exist. The more the energy inputs are limited, the more the cells are starving and at some point they are no longer able to keep their metabolic functions and their structural integrity and enter the irreversible process of senescence. Those differences in cell energetic status are reflected also in the ADP/ATP ratio. For the cultures with the oxidized HDPE and LDPE films and the rest after the oxidized HDPE film extraction described earlier the values of the ratio were virtually in the same range (ADP/ATP about 2.2) whereas cultures with the extractable portion only gave clearly higher number documenting higher level of the energy deprivation (ADP/ATP about 6.0).

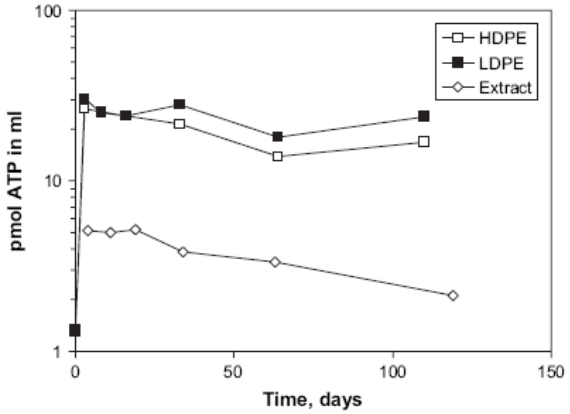

Fig. 3. Evolution of ATP content in *N. asteroides* cultures with oxidized PE films and control culture with the aqueous extract from the oxidized HDPE.

Table 2
ADP/ATP ratio for *R. rhodochrous* cultures during and at the end of the observation period

Substrate	Incubation (days)	ADP/ATP
TS medium	4	0.25 (0.02)
Oxidized HDPE	4	0.14 (0.03)
Oxidized LDPE	4	0.43 (0.09)
Oxidized HDPE	250	2.2 (0.21)
Oxidized LDPE	185	2.2 (0.31)
HDPE rest after the extraction	200	2.5 (0.01)
HDPE extract (control)	200	6.0 (0.11)

TS medium, complete rich medium; standard deviations of the values are in brackets, $n \geq 2$.

Similar analysis was done for the *N. asteroides* cultures (Table 3). Again the ADP/ATP values were lower for the cultures with tested materials than for the cultures with the same extract only but the difference was not so pronounced. The observation can be explained by shorter incubation period (120 days instead of 200 days).

The values obtained showed that the cell populations in the presence of the oxidized PE samples were in better energetic state than the control cultures with the extract only. It suggests that the cell after the initial period of exponential growth continued to extract substances from the oxidized PE and gained some energy by their metabolism. But it is also obvious that the values even lower than those of the control incubation were still rather high suggesting that the cells had only limited energetic supply and that the energy generation from the tested material is slow and difficult.

3.3. Consumption of extractable molecules

The presumption that some substances can be extracted from the oxidized PE film by an aqueous medium and subsequently consumed by bacteria was tested with the help of

Table 3
ADP/ATP ratio for *N. asteroides* cultures at the end of the observation period

Substrate	Incubation (days)	ADP/ATP
Oxidized HDPE	120	2.26 (0.09)
Oxidized LDPE	120	1.75 (0.19)
HDPE extract (control)	120	3.51 (0.30)

Standard deviations of the values are in brackets, $n \geq 2$.

NMR spectroscopy. The aqueous extract from oxidized HDPE was prepared by the procedure described in the preceding section. ^{1}H NMR spectra showed the presence of significant amounts of organic substances (Fig. 4). On the whole the same organic species were observed in oxidized HDPE and LDPE extracts with chemical shifts ranging between 0.8 and 3.7 ppm (Fig. 4a and b). These chemical shifts are compatible with substituted or unsubstituted CH and CH_2 groups, showing that these compounds are derivatives of short oxidized PE fragments but possibly also of the additive component (e.g. stearate). More precisely the two very well defined signals at $\delta = 3.67$ and 1.20 ppm correspond to ethanol (denoted 1). The presence of formate can also be noticed on both spectra resonating at $\delta = 8.46$ ppm (not shown). Ethanol and formate are oxidation end-products of polyethylene. Although the extracted compounds are the same from the two types of polymers, two main differences can be observed: (i) integration of the different signals relative to that of the internal reference ($Tspd_4$) clearly showed that the amount of organic compounds extracted from LDPE (Fig. 4a) was 50% lower than that of HDPE (Fig. 4b), this is consistent with the ATP titration results and explicable by the thickness difference between HDPE and LDPE samples; (ii) the concentrations of ethanol and formate are rather constant in both samples while the other compounds (denoted 2) are in much higher amounts in HPDE sample. Fig. 4c and d clearly demonstrates complete assimilation of the extracted species from both polymers after 4 days of incubation with *R. rhodochrous*. These results are consistent with the low ADP/ATP ratio measured after 4 days of incubation; the rapid initial growth of microorganisms was favoured by these easily metabolisable compounds extracted from the polymers. Also they showed that in the later phases of incubation no substrate was present in the cultures grown on extract from oxidized HDPE films which were used as references for the experiment with ATP determination (see Section 3.2.1) thus there was virtually no difference between this type of reference and an eventual reference consisting of pre-cultivated microbial cells in medium without substrate.

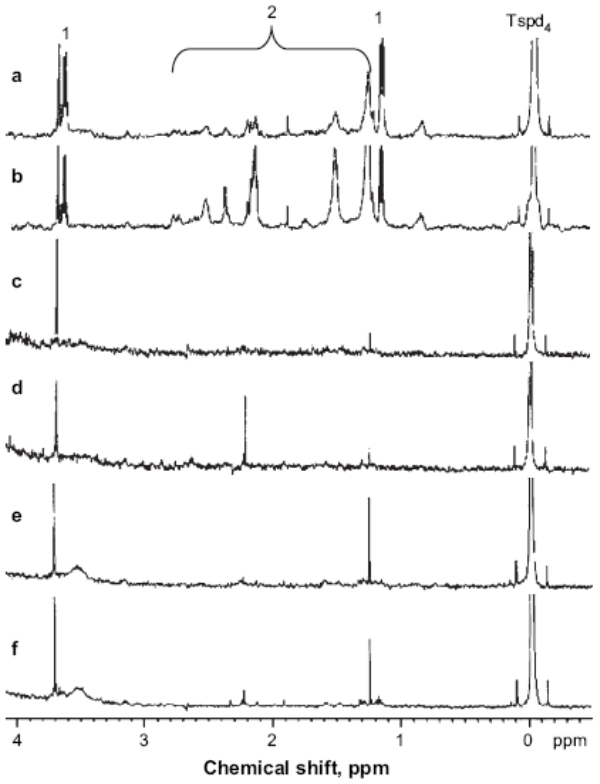

Fig. 4. ^{1}H NMR spectra of the oxidized HDPE and LDPE water phase extractable fractions and their consumption after incubation with *R. rhodochrous*. (a) Oxidized LDPE extractable fraction; (b) oxidized HDPE extractable fraction; (c) oxidized HDPE extractable fraction after 4 days incubation with *R. rhodochrous*; (d) oxidized LDPE extractable fraction after 4 days incubation with *R. rhodochrous*; (e) non-oxidized HDPE extractable fraction; (f) non-oxidized HDPE extractable fraction after 4 days incubation with *R. rhodochrous*, 1: ethanol, 2: unknown organic compounds extracted from the polymers.

Finally Fig. 4e shows that no organic compounds are extracted from non-oxidized HDPE, so no leaching of the additives occurs in the absence of light or heat exposure. This result is consistent with the efficiency of abiotic treatment of PE in water (see Section 3.1) that indirectly proved this absence of leaching. As expected, no change in NMR spectrum was observed when non-oxidized HDPE was incubated with the *Rhodococcus* strain (Fig. 4f).

3.4. Biofilm formation

The surfaces of the film were observed by optical microscopy and scanning electron microscopy (SEM).

Because the tested PE films were transparent it was possible to use also transmission optical microscopy to observe the microorganisms on their surface. Application of this classical technique for the plastic film biodegradation study was quite original and brought the advantage of the fast and immediate monitoring of the film surface. More detailed microimages were obtained with SEM at the end of the incubation period.

Some of the tested microorganisms demonstrated their capacity to form biofilm on the surface of the oxidized LDPE and HDPE films tested. Bacteria *R. rhodochrous* and *N. asteroides* and fungus *M. alpina* were the most active (Figs. 5 and 6). Especially *N. asteroides* formed very dense filamentous mycelium on the surface. The biofilm development was the most progressive during the first 30 days of incubation. After this period no marked changes were identified by optical microscopy. Biofilm on the surface of the oxidized LDPE film was more dense and compact whereas on the oxidized HDPE film rather only islands of cells could be found indicating that probably important part of the population persists in the suspension form in the medium.

Contrary to previous studies [3,4] no clear signs of bioerosion could be found on the surface of microorganism exposed films. However, it must also be noted that the attempts to wash the biofilm out of the material [4] before the SEM sample

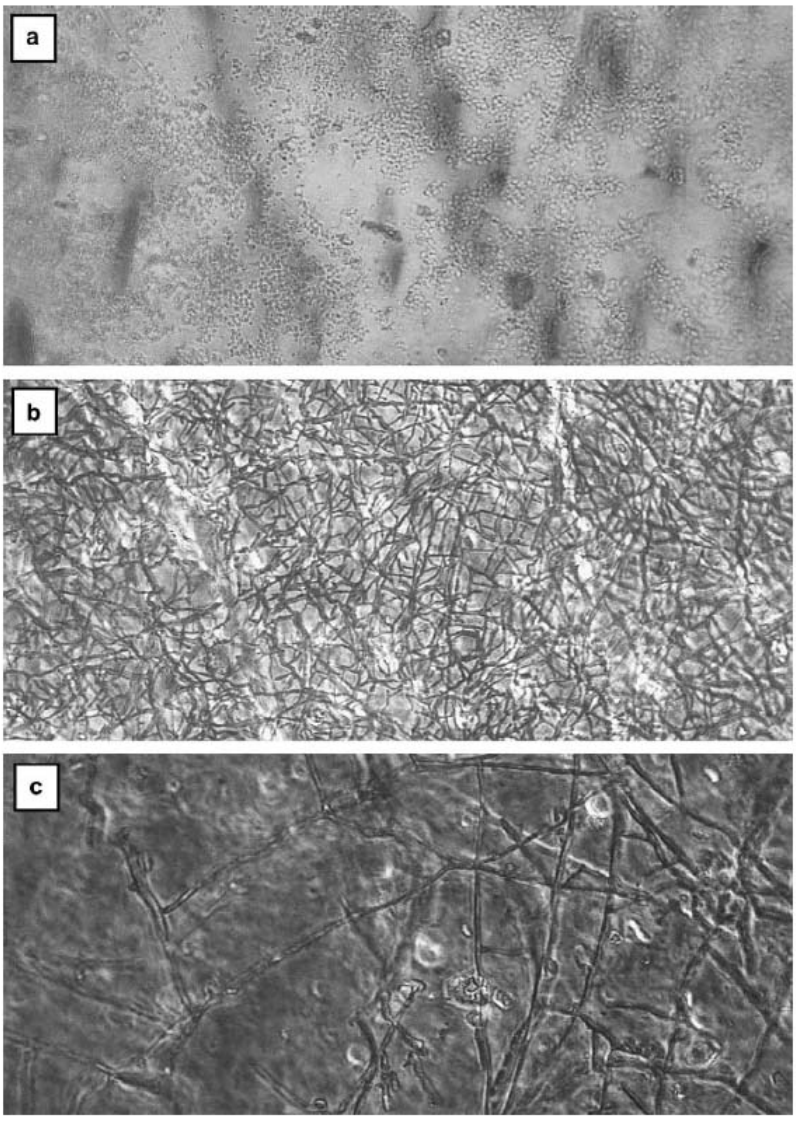

Fig. 5. Optical microscopy observation of the microorganisms on the oxidized LDPE film surface. Magnification cca. 100×; (a) *R. rhodochrous* (95 days of incubation); (b) *N. asteroides* (88 days of incubation); (c) *M. alpina* (70 days of incubation).

preparation were quite unsuccessful and that some bioerosion traces could stay hidden under the biofilm.

Existence of the biofilm was independently proved by μATR-FTIR spectroscopy in the cases of *R. rhodochrous* and *N. asteroides* on the oxidized LDPE film (Fig. 7). The bands at 1653 cm^{-1} and the nearby bands on its right can be assigned to protein material, the broad bands peaking at 1133 and 993 cm^{-1} showed the presence of polysaccharides.

3.5. SEC analysis

To test if the PE film degradation occurs in a significant part of the polymer volume the molecular weight distributions were compared between the material samples incubated in the presence of microorganisms and the samples incubated abiotically under identical conditions. The longest incubation was 203 days in the case of the oxidized HDPE with *R. rhodochrous* and 141 days in the case of the oxidized LDPE with *R. rhodochrous*. With *M. alpina*, *A. flavus*, *C. cladosporoides* and *N. asteroides* the periods of incubation were from 114 to 133 days. For both of the two materials the analysis did not reveal any significant difference in M_w distribution between the biotic sample and the abiotic control (data not shown). The observations suggest that the microbial attack was only superficial probably involving chain end carboxylic acids and that the microorganisms during the experiment period were not able to perturb the whole material volume.

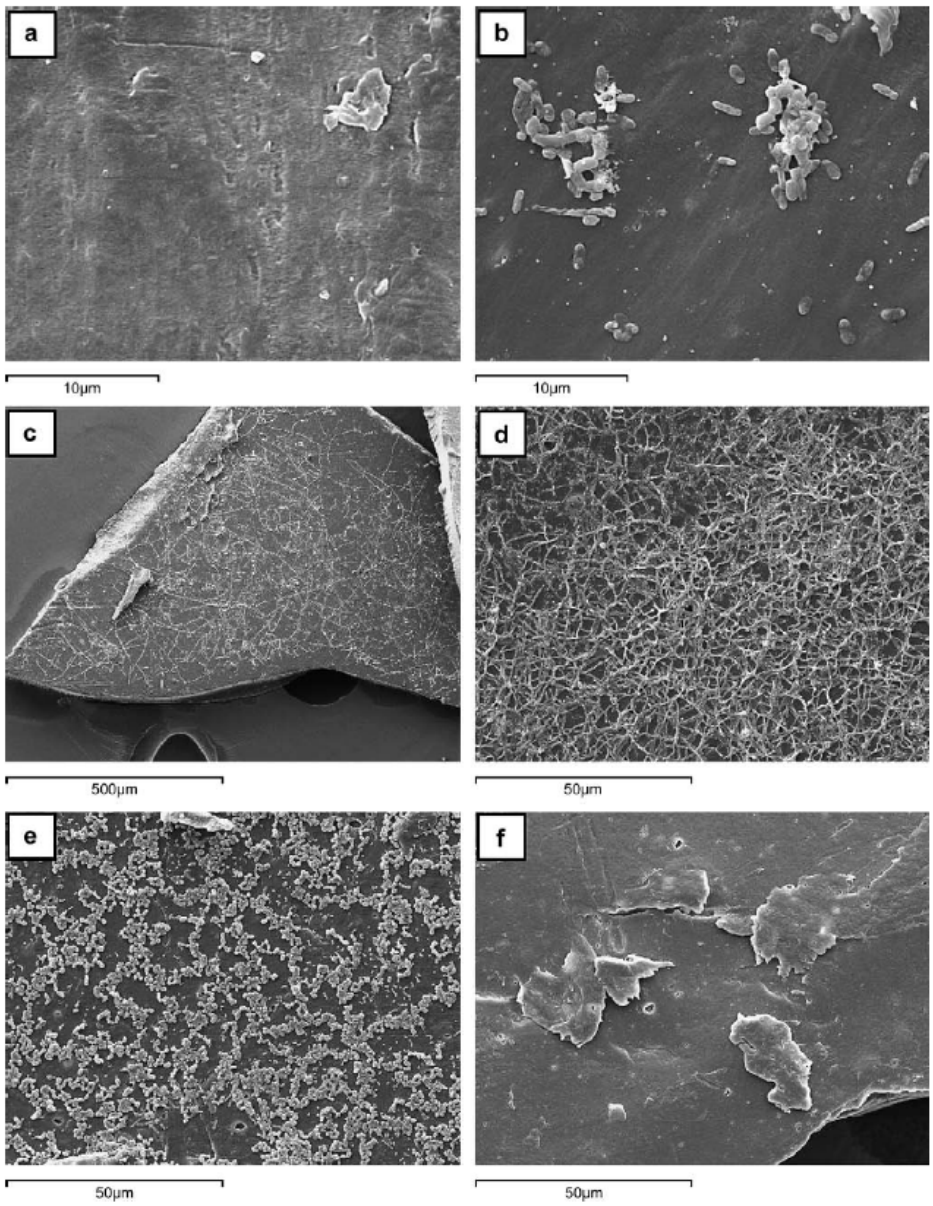

Fig. 6. SEM images of the tested materials after approximately 100 days of incubation with the designed microorganisms and the control samples incubated without microorganism (abiotic). (a) Oxidized HDPE abiotic; (b) oxidized HDPE + *R. rhodochrous*; (c) oxidized LDPE + *M. alpina*; (d) oxidized LDPE + *N. asteroides*; (e) oxidized LDPE + *R. rhodochrous*; (f) oxidized LDPE abiotic.

4. Discussion

The tested PE films represent a new branch of polymer materials with time programmed mechanical properties. The fine balance of antioxidant and pro-oxidant contents guarantees that after the preset period of service life relatively fast abiotic oxidation begins. As a consequence the material loses its mechanical properties and disintegrates into small fragments. These features provide a potential solution to the problems of ''visual pollution'' by plastic litter and its macro-toxicity in the environment that are constantly in the centre of public attention. On the molecular level the abiotic oxidation results in dramatic reduction in molecular weight, introduction of polar groups and increase in hydrophilicity. Evidently the oxidation renders the material much more vulnerable to microbial attack.

In accordance with other authors [7,1] we observed that the early stage of biodegradation was characterized by fast growth probably mainly supported by the consumption of low molecular weight compounds extracted into the water phase. The compounds were probably the low molecular weight degradation product of polyethylene chains, most often terminated with carboxylic groups, already observed in other studies [8,9]. The fast initial phase was followed finally by

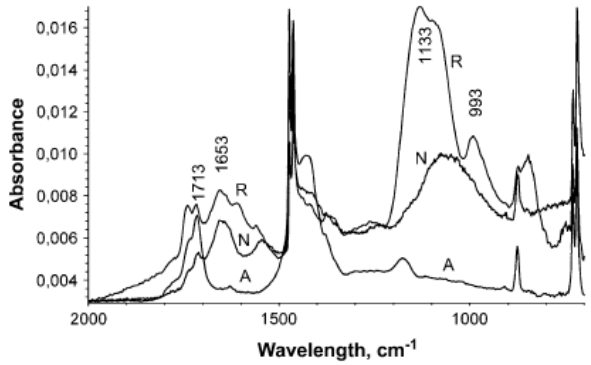

Fig. 7. ATR-FTIR spectra of the biofilm covered oxidized LDPE film. (A) Abiotic control; (N) *N. asteroides* culture (65 days of incubation); (R) *R. rhodochrous* culture (82 days of incubation).

stabilization of metabolic activity, which kept virtually the same level during many months. The data suggest that microorganisms continued to gain energy from the material hence the biodegradation was still going on even at some slow rate. However, it was not possible to make even a rough estimation of the process extent and eventually the time necessary for the ultimate biodegradation of the sample under applied laboratory conditions. It seems also that within the experiment period the observed microbial action was limited to the surface layer of the materials.

Some of the microbial strains tested in the study were able to form biofilm on the material surface, particularly *R. rhodochrous* and closely related *N. asteroides*. The same strains formed biofilm also on another pro-oxidant activated polyethylene film, as reported in a previous study [4]. Recently another *Rhodococcus* strain was isolated from soil and its capacity to form biofilm on similar type of material was demonstrated [10]. This group of bacteria, highly abundant in natural environments like soil, was identified as very active towards the higher molecular weight hydrocarbons [11]. One of the crucial features making them able to metabolise these water insoluble compounds could be the production of bio-surfactants [12] that render their surface more hydrophobic, so they can adhere to the hydrophilic surface of substrate, and in the same time mobilize substrate molecules and increase their accessibility for enzymes.

For the first time to our knowledge the adenine nucleotide determination was utilized for monitoring of material biodegradation. The method well known to biologists can produce interesting data about the energetic status of microorganisms, complementary to the classic methods in biodegradation studies.

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