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REPORT

Degradation, Biodegradation and toxicity of Oxo-biodegradable Plastics in the oceans

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This report present the methodology and the results obtained using ^{13}C-oxo polyethylene to study biodegradation of polymer by a single strain in the framework of OXOMAR project. It will be format and publish in review publication journal:
Use of stable isotope to follow the biodegradation of Polyethylene.

Experiments
Biotic and abiotic degradation approaches were associated to uncover the fate of oxo-biodegradable plastics. For this purpose, an oxo-biodegradable polymer, was produced from polyethylene labelled with 100% ^{13}C stable isotopes. The formulation was aged and characterized to compare with conventional ^{12}C oxo-polymer. Biodegradation tests were conducted on ^{13}C-oxo polyethylene to emphasize the use of oxo polymer as a carbon source by microorganisms, confirm the mineralization process and the incorporation of ^{3}C labelled carbon into biomass and CO_2..

PART A: Ageing and characterization of ^{12}C and ^{13}C Polyethylene material.

Tested material
The material samples were transparent LDPE films, 100 μm thick, provided by Symphony environmental. The films composition was a commercial formulation containing an iron photo-inducer supplying radicals through a photo-redox process and an organometallic type manganese thermo-inducer, catalysing the primary hydroperoxide. The oxidation state of the catalysts was Mn^{2+} and Fe^{3+} and the ligand was stearate. Labelled ^{13}C-LDPE and conventional ^{12}C-LDPE powders were used to generated the final ^{13}C-Oxo-LDPE and ^{12}C-Oxo-LDPE samples.

Ageing treatment
The ^{13}C-Oxo-LDPE and ^{12}C-Oxo-LDPE samples were exposed over 2000 h in the accelerated photo-ageing unit ($\lambda \geq 300$ nm, temperature of the exposed surface was set at 60 ± 1 ºC). The monitoring of the oxidation extent was carried out by transmission FTIR spectrophotometry. The oxidized samples were analysed by thermal analysis (DSC), polarized light microscopy and size exclusion chromatography (SEC) to determine modification in composition and structure.
In order to assess the impact of preparation steps (extrusion, ageing) and compare the characteristics of different material, the same analysis were done on initial ^{13}C and ^{12}C polyethylene powder and ^{13}C and ^{12}C from extruded material.

Oligomers production:
The extraction of degradation products from the ^{13}C and ^{12}C oxidized films was performed at room temperature using a rotary shaker. Briefly, 5 mg of samples were extracted in glass vials for 7 days in 1ml water. The supernatant containing extracted oligomers was separated from the solid film residue by centrifugation. The oligomers solution were analysed directly after extraction by NMR spectroscopy, Mass spectrometry and the total organic carbon content was determined.

Results - Characterization of solid samples

Infra red spectroscopy

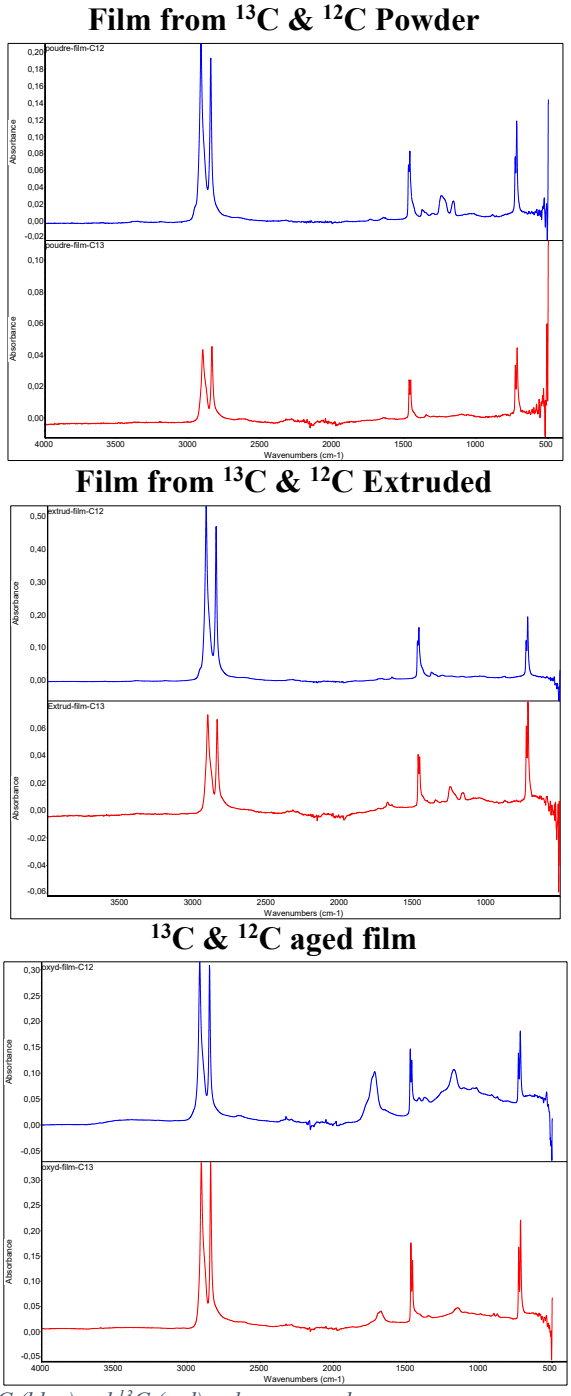


Figure 1 – Infra red spectra of ^{12}C (blue)and ^{13}C (red) polymer samples

To evaluate the impact of aging on the chemical composition of the films, the progression of oxidation was determined through the accumulation of carboxylic acid groups detected by FTIR spectrophotometry (Figure 1). No sign of oxidation were recorded on unaged powder and extruded film. As expected, the signals of several functional groups can be observed in the carbonyl region (1715 cm^{-1}) of the IR spectrum, confirming the variation of oxidation of only ^{12}C and ^{13}C aged samples. The level of oxidation recorded in ^{13}C aged samples is much lower than the one in ^{12}C aged samples. However, spontaneous fragmentation of both type of film can be observed after photo-ageing.

DSC analysis

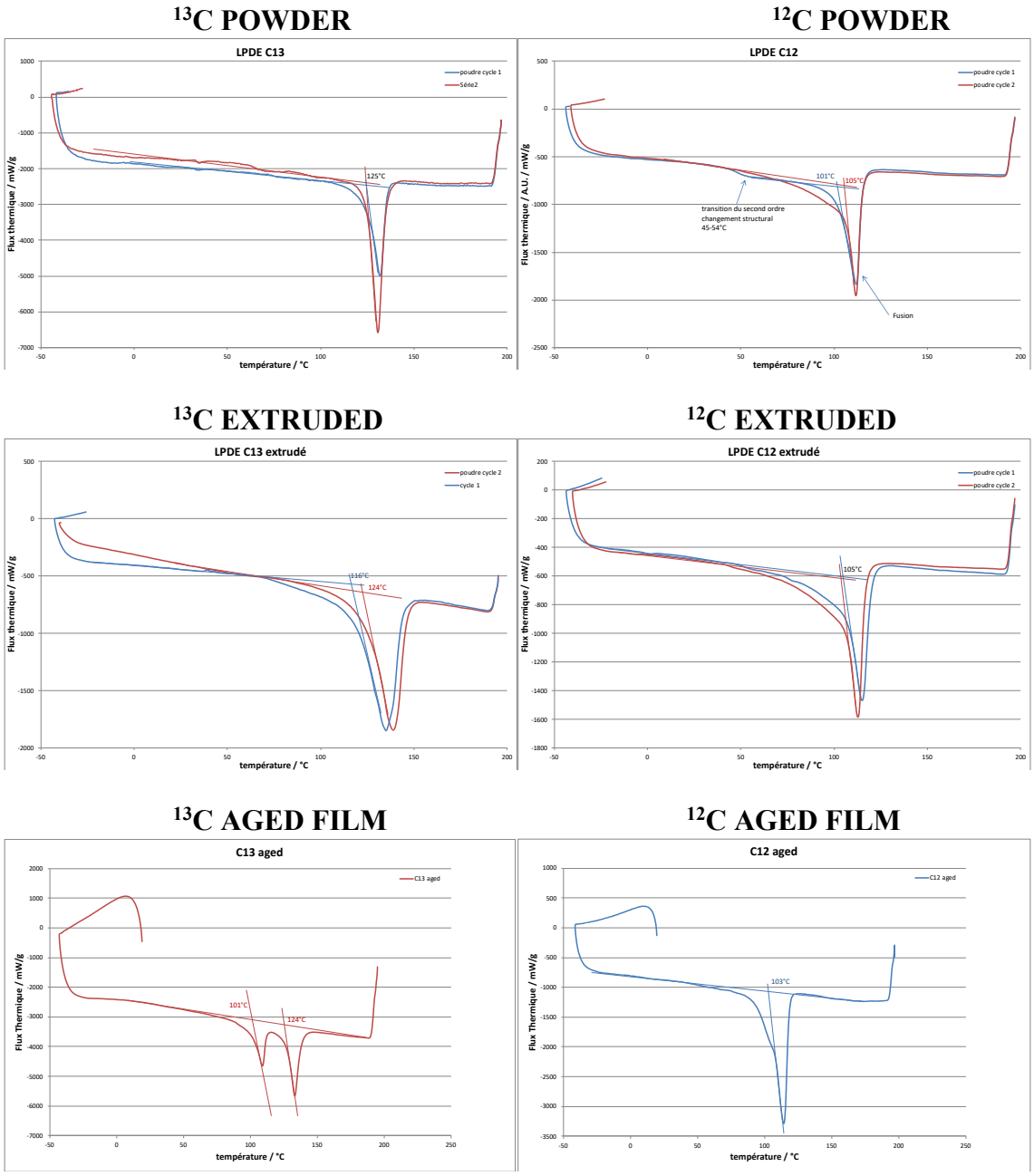
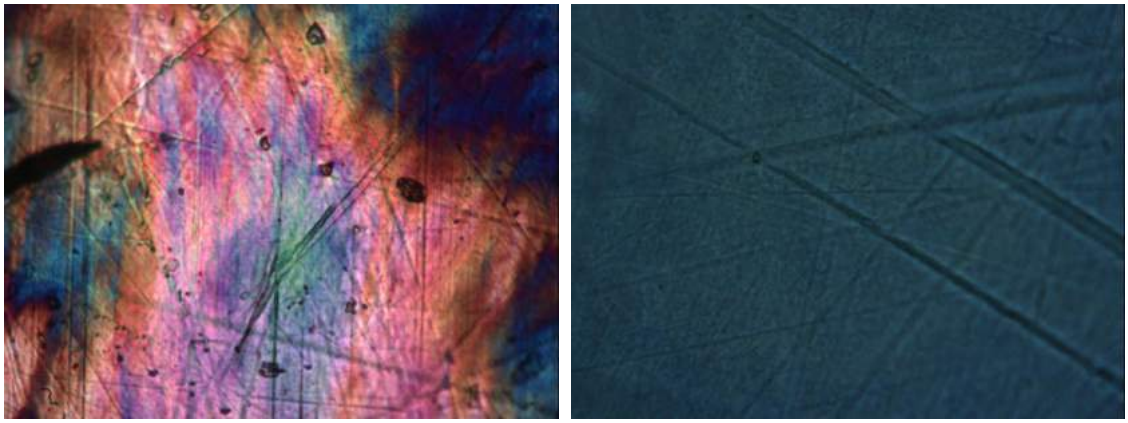


Figure 2 – DSC curve of ^{12}C and ^{13}C polymer samples

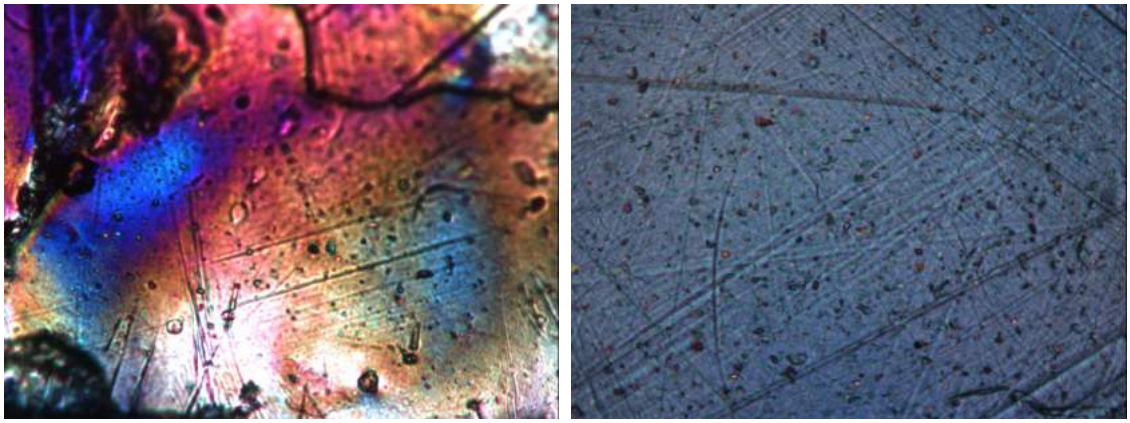
From DSC analysis, different melting temperature can be observed between ^{12}C (103 °C) and ^{13}C samples (125 °C) indicating different morphology of the polymers (Figure 2). The melting temperature value is steady around 103 °C for all the ^{12}C samples type (powder, extruded, aged). The melting temperature value is steady around 125 °C for ^{13}C powder and extruded samples but an extra peak appear on ^{13}C aged sample at 103°C. This might indicate the blend of 2 polymer sizes after ageing

Polarized Light microscopy

Film from ^{13}C Powder **Film from ^{12}C Powder**



Film from ^{13}C Extruded **Film from ^{12}C Extruded**



^{13}C aged film **^{12}C aged films**

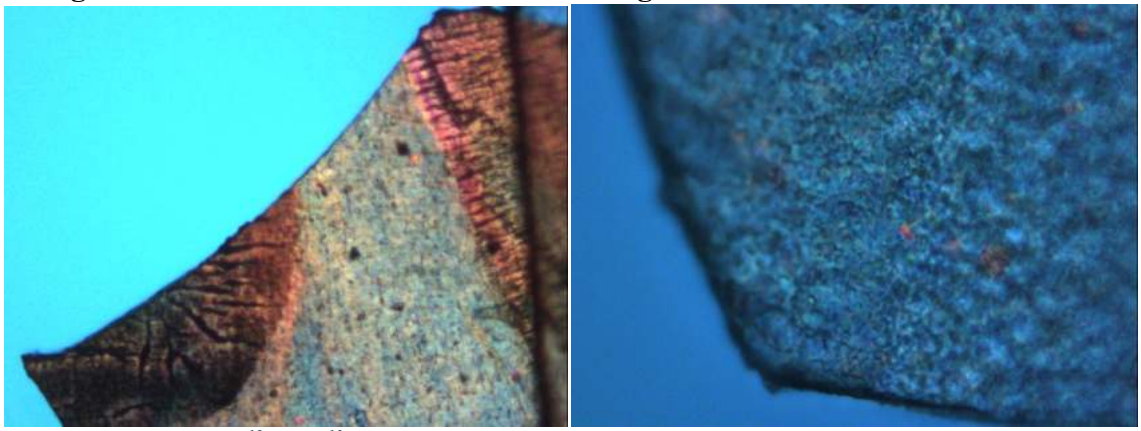


Figure 3 Observation of ^{12}C and ^{13}C samples under polarized light microscope

Observation under polarized light microscope clearly indicated differences in composition between ^{12}C and ^{13}C samples, characterized by the presence of multiple color zone in ^{13}C polymers (Figure 3). These colour zone must reflect different diffraction of light and could be linked to different level of crystallinity.

Size exclusion chromatography

The determination of average and number molecular weight showed that carbon chain length of unaged ^{13}C polyethylene was bigger (313 200 Da) than the one in unaged ^{12}C polyethylene (274 200 Da) (Table 1). This indicate that initial material used to produce oxo formulation was different. The extrusion process slightly decrease the average molecular weight of the ^{12}C polymers and slightly increase the average molecular weight of the ^{13}C polymers. The process has more impact on the number molecular weight of the ^{13}C polymer and reduce by half the original value. This value remain higher than ^{12}C polymer value. As expected after photodegradation, the average and number molecular weight of both polymer decrease drastically to drop down to a low value of 3100 Da for ^{12}C sample and 16500 Da for ^{13}C sample. As observed with FTIR analysis the ^{13}C sample has not reach the same level of oxidation and fragmentation as the ^{12}C sample.

Samples	**Mw**	**Mn**	**Ip**
^{12}C Powder	274200	28000	9,8
^{12}C Powder-extruded	250200	24130	10,4
^{12}C Powder-Aged	3100	1800	1,7
^{13}C Powder	313200	83100	3,8
^{13}C Powder-extruded	322900	41600	7,8
^{13}C Powder-Aged	16500	4500	3,7

Table 1 Determination of average and number molecular weight of ^{12}C and ^{13}C samples

^{1}H NMR spectroscopy

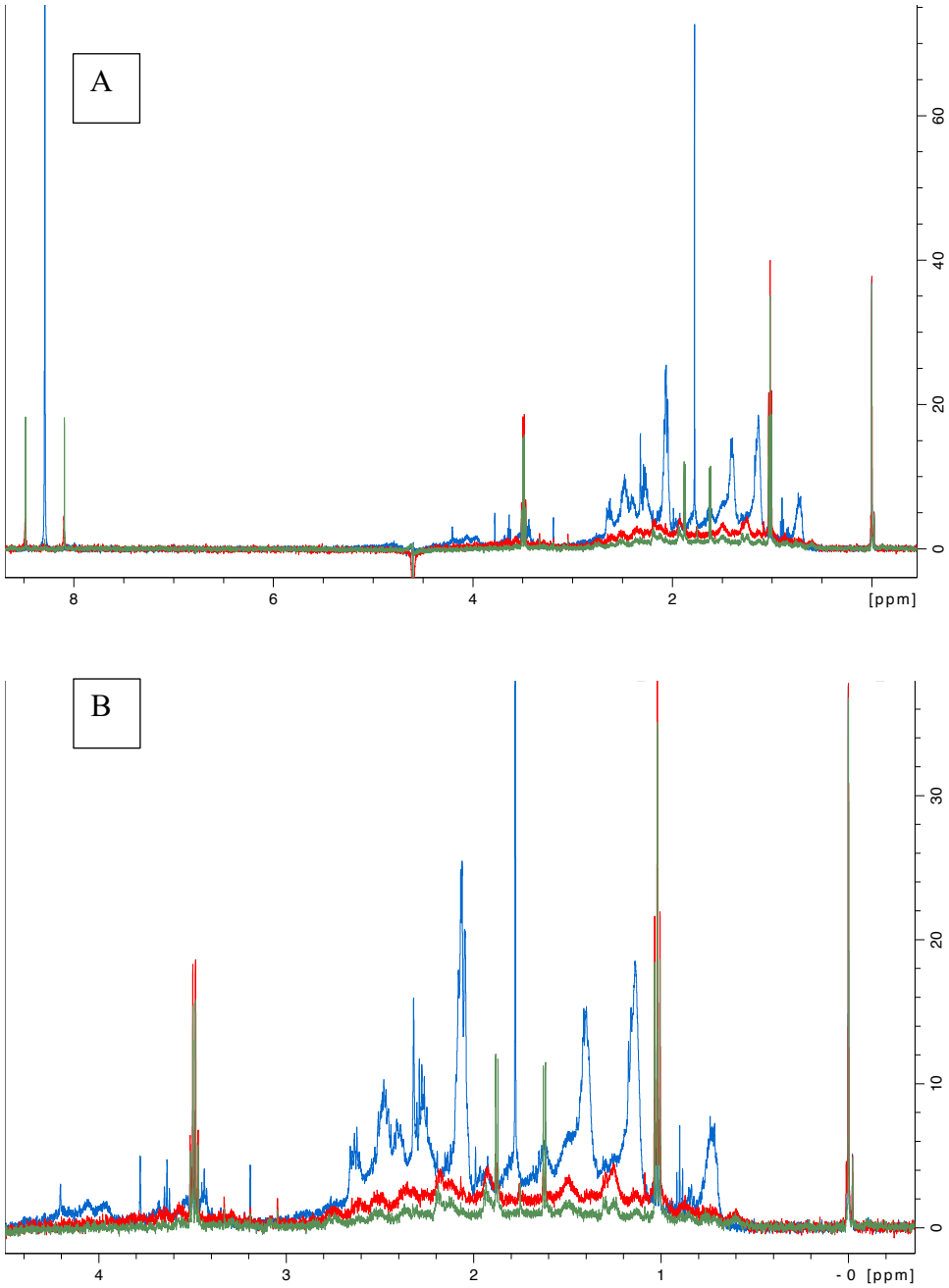


Figure 4 ***: ^{1}H NMR spectra of oligomers a:(0-8,5 ppm) _ b: zoom in (0-4,5 ppm)*** *Blue : ^{12}c aged film_oligomers red: ^{13}c aged film batch #1_oligomers green: ^{13}c aged film batch #2-oligomers*

The ^{1}H NMR spectra of oxidized oligomers extracted with water for 7 days is presented in Figure 4. As described in literature, the signals consist in several large peaks, corresponding to the superposition of individual peaks, located within the proton chemical shift range of ∂=0.7-3.0 ppm. The overall signal was divided into two sections corresponding to i) the chemical shifts of protons close to an alkane functions (CH_3 (0.7-1.1 ppm) and CH_2 (1.1-1.4 ppm)) named as “aliphatic protons” and ii) the chemical shifts of protons next to an oxidized functions (CH_3 (1.7-2.0 ppm), CH_2 (1.4-1.7 and 2.0-2.3 ppm) and CH (2.3-3.0 ppm)) named as “oxidized protons” (Eyheraguibel et al., 2017). The relative intensity of the signals was determined by

integrating the area under the curve and compared it to the integral of internal standard component ($Tspd_4$). The extraction from ^{12}C oxidized film (blue spectrum) provides more oligomers than the ones from ^{13}C oxidized film (red and green spectra). Two batches of ^{13}C oxidized film are presented. The batch #1 and #2 provide respectively 60 % and 70% less oligomers than ^{12}C oligomers (Table 2). This confirm again a lower level of oxidation for ^{13}C samples.

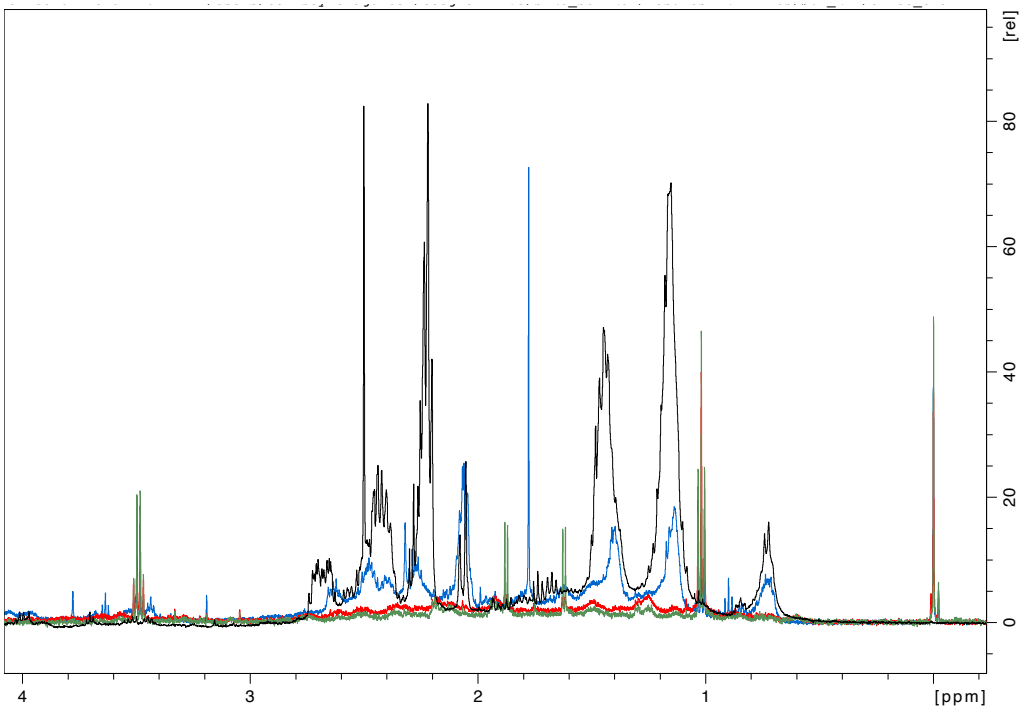


Figure 5 - ***^{1}H NMR spectra of oligomers (0-4 ppm)*** *blue : ^{12}C aged film_oligomers;red: ^{13}C aged film batch #1_oligomers;green: ^{13}C aged film batch #2-oligomers;* ***black:*** *other ^{12}C aged film_oligomers from oxomar project*

These results were compare with other formulation previously used in OXOMAR project and aged by photooxidation in SEPAP 12/24 and thermo oxidation in oven. The extraction from other ^{12}C oxidized film (black spectrum) provides two times more oligomers than the ^{12}C oligomers generated through UV_B oxidation- blue spectra (Fig 5; Table 2).

Sample Water Extract	Integral 4.0 – 0.4	Relative Integral
^{12}C oligomers	360 458 953	100 %
^{13}C batch #1_oligomers	160 354 676	44,4 %
^{13}C batch #2-oligomers	113 117 488	31,4 %
other ^{12}C oligomers in OXOMAR	-	201 %

Table 2 - ***Comparison of the amount of oligomers extracted with water from ^{12}C and ^{13}C oxidized films***

Mass spectrometry

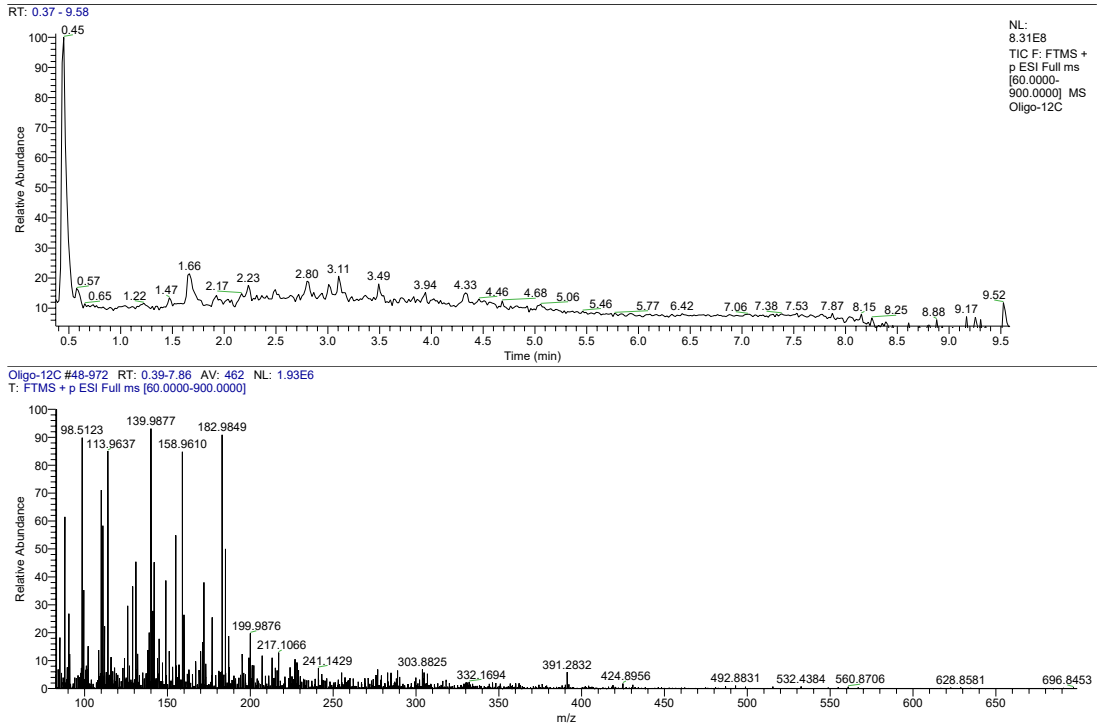


Figure 6 - Chromatogram and mass spectrum of ^{12}c aged film_oligomers

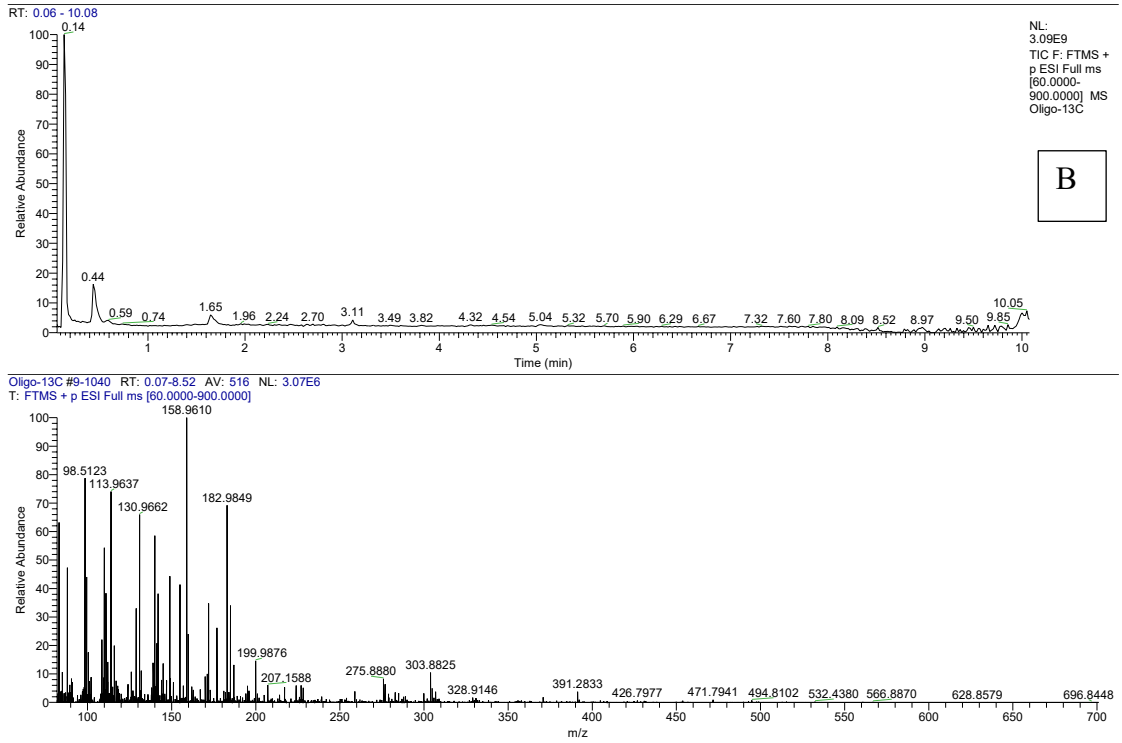


Figure 7 -Chromatogram and mass spectrum of ^{13}c aged film_oligomers- batch 2

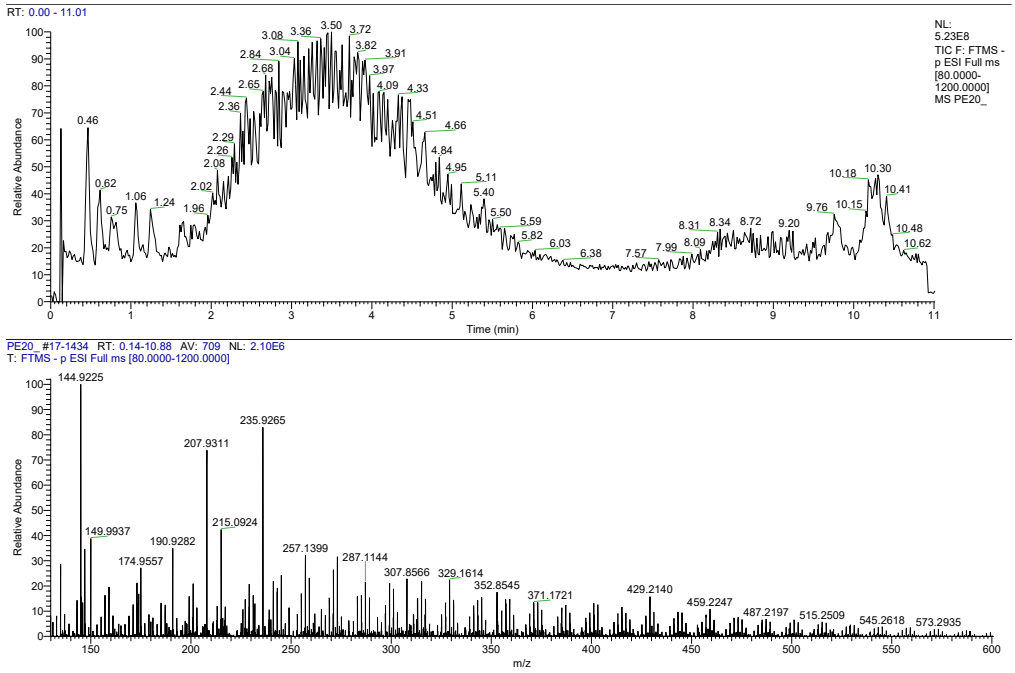


Figure 8 - Chromatogram and mass spectrum of other ^{12}c aged film_oligomers from oxomar

7 days of extraction usually provide complex mixtures of oligomers characterized by a broadband ion mass spectrum with hundreds of peaks in a mass range between 100-850 Da , as presented in Figure 8 showing a wide chromatographic peak and the mass spectrum of oligomer extract from other formulation previously used in OXOMAR project. A high number of peaks (1772 compounds) is generally detected in these non-labelled samples (Table 3).On the contrary, few peaks can be observed in the chromatograms of ^{12}C oligomers (Figure 6) and even less peaks are present in the chromatograms of ^{13}C oligomers (Figure 7). The same results are observed in the mass spectrum where the number of peaks (corresponding to compounds) is greater in ^{12}C oligomers (1046 compounds detected) than in ^{13}C oligomers (688 compounds detected) but way below usual sample (1772 compounds). This lead to the conclusion that in this study, both ^{12}C and ^{13}C oligomers extracted from ^{12}C and ^{13}C corresponding polymers are produced in much lower quantity.

Sample	Number of peak above 10^5 intensity
^{12}C_oligomers	1046
^{13}C_b2_oligomers	688
other_ ^{12}C _oligomers	1772

Table 3- Comparison of the amount of oligomers extracted with chloroform from ^{12}C

Total Organic Carbon

The measurement of total organic carbon from ^{12}C and ^{13}C oligomers solution indicated the same tendency as other measured parameters. A higher carbon content in oligomers solution was recorded in the extract from ^{12}C oxidized film. The level of carbon was about 10 time lower in the extracted from ^{13}C oxidized film (batch 1 and 2).

	TOC (mg/L)	RELATIVE
^{12}C aged film_oligomers	406,6	100%
^{12}C aged film_oligomers	394,4	96%
^{13}C aged film batch #1_oligomers	54,0	13,3%
^{13}C aged film batch #1_oligomers	56,1	13,8%
^{13}C aged film batch #2-oligomers	41,8	10,3%
^{13}C aged film batch #2-oligomers	44,3	10,9%

Table 4 - Comparison of the amount of oligomers extracted with chloroform from ^{12}C

PART B: Biodegradation of ^{12}C and ^{13}C Polyethylene material.

Biodegradation experiment were conducted on ^{13}C-oxo polyethylene to emphasize the use of oxo polymer as a carbon source by microorganisms, confirm the mineralization process and the incorporation of ^{13}C labelled carbon into biomass and CO_2..

1.1. Biodegradation experiment

The biodegradation of oxidized ^{12}C and ^{13}C PE films was studied using a pure bacterial strain: *Rhodococcus rhodochrous* (ATCC 29672) purchased from American Type Culture Collection. PE films were incubated in a medium containing only necessary growth supporting mineral ions and where the tested material was the only source of carbon. Biodegradation tests were conducted, in 3.5 ml gas-tight glass vials (Exetainers Labco UK) containing 5mg of PE sample in 1 ml of minimum media inoculated with 10^5 cells per ml. An abiotic control containing no cells and a biotic control with no polymer were also prepared. 3 replicates of each condition were tested. The cultures were kept at 27 ºC with gentle shaking. Biodegradation kinetics were monitored by following CO_2 evolution during 58 days of incubation. 50 µl samples were collected directly from the head spaces of each vials using a gas tight syringe and injected in GC/MS to determine CO_2 concentration. Mass spectrum were recorded in Single Ion Mode (SIM) to provide better sensitivity and selectivity. Retention time and mass to charge ratio were used to distinguish $^{12}CO_2$ (m/z = 44) and $^{13}CO_2$ (m/z = 45). The incorporation of ^{13}C into the emitted CO_2 was determined from mass spectrometry data using Data analysis software ® (Thermo scientific). The degradation of oligomers was monitored by ^{1}H NMR and LC-MS. The growth of bacteria was determined by following the ATP content at the end of experiment using ATP Biomass Kit HS by Biothema (Sweden) and following recommendation of the provider.

4 conditions were tested

1- Pure strain of *Rhodococcus rhodochrous* (10e5 cells/ml) incubated with PE_^{12}C
2- Pure strain of *Rhodococcus rhodochrous* (10e5 cells/ml) incubated avec PE_^{13}C
3- Pure strain of *Rhodococcus rhodochrous* (10e5 cells/ml) incubated: Biotic Control
4- PE_^{13}C alone : Abiotic Control

The cell growth was monitored by following the evolution of CO_2 in the head space of culture. A slight increase of CO_2 was recorded in the biotic control sample, during the first 20 day of culture, indicating a weak respiration and activity of the cells (Figure 9). The level of CO_2 remain steady for the rest of experiment. This can be explained as a quick adaptation response to stressful conditions (no carbon source) involving cell autophagy processes to adjust cellular biomass and use of storage compound (starch, protein) to survive.

On the contrary, cells growing on both ^{13}C and ^{12}C oxidized polyethylene films show an increase of CO_2 concentration over the 58 days of culture, confirming the cellular activity and respiration on this type of substrate. A quick increase operated during the first 10 days of cultivation as free oligomers are available in the medium. A second step occurred between 15 and 58 days of cultivation, with a slower growth and a limited production of CO_2. The reduction of easily accessible compounds slows down the activity of the cells and therefore the respiration. No significant differences can be recorded between the two type of carbon uses indicating that the *Rhodococcus* strain was able to grow similarly on ^{13}C and ^{12}C.

One should notice that abiotic control sample, containing only ^{13}C oxidized polyethylene films in water, show also the release of CO_2 directly from the polymer film to the head space of the vial. This confirms that photo-oxidation processes can produce volatile degradation products

but the amount of abiotic CO_2 is much lower than the one coming from the respiration of the cells

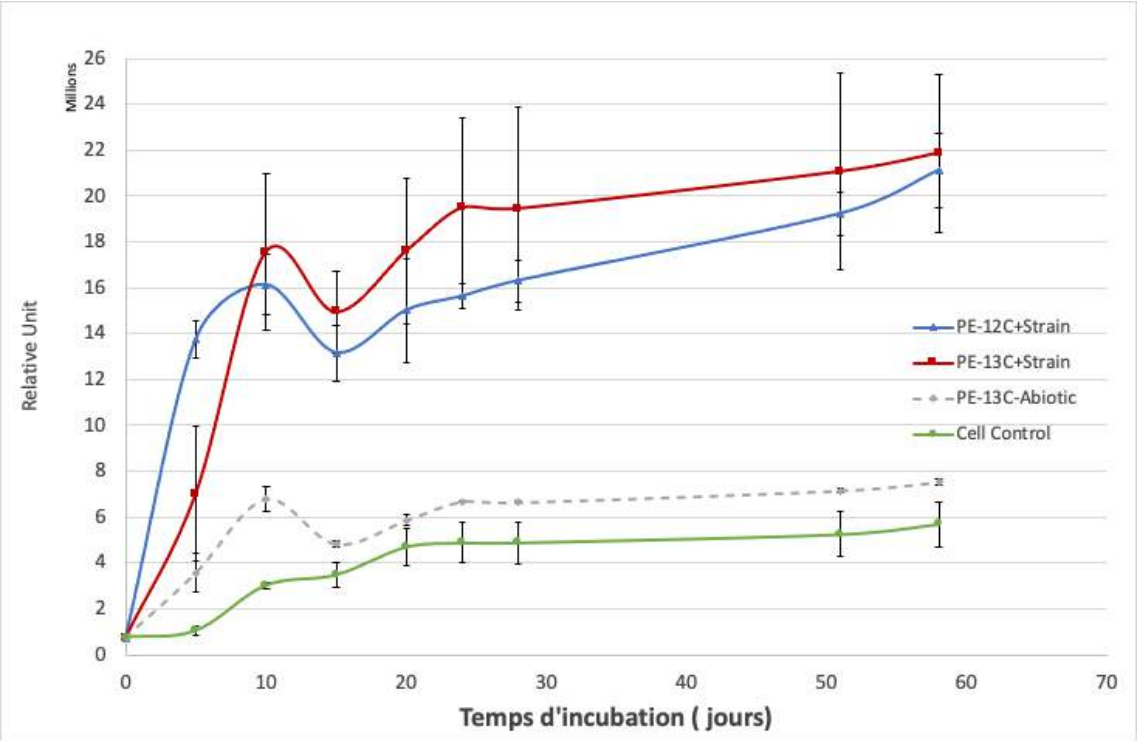


Figure 9 - Evolution of CO2 concentration

At the end of experiment, the growth of bacteria was monitored by ATP measurement on the whole final sample (planktonic cells and biofilm). The increase of ATP content confirmed that a large amount of cells has grown on both PE_^{12}C and PE_^{13}C polymer (Figure 11). The ATP concentration was respectively 8 time and 10 time higher than for cell control growing without polymer.

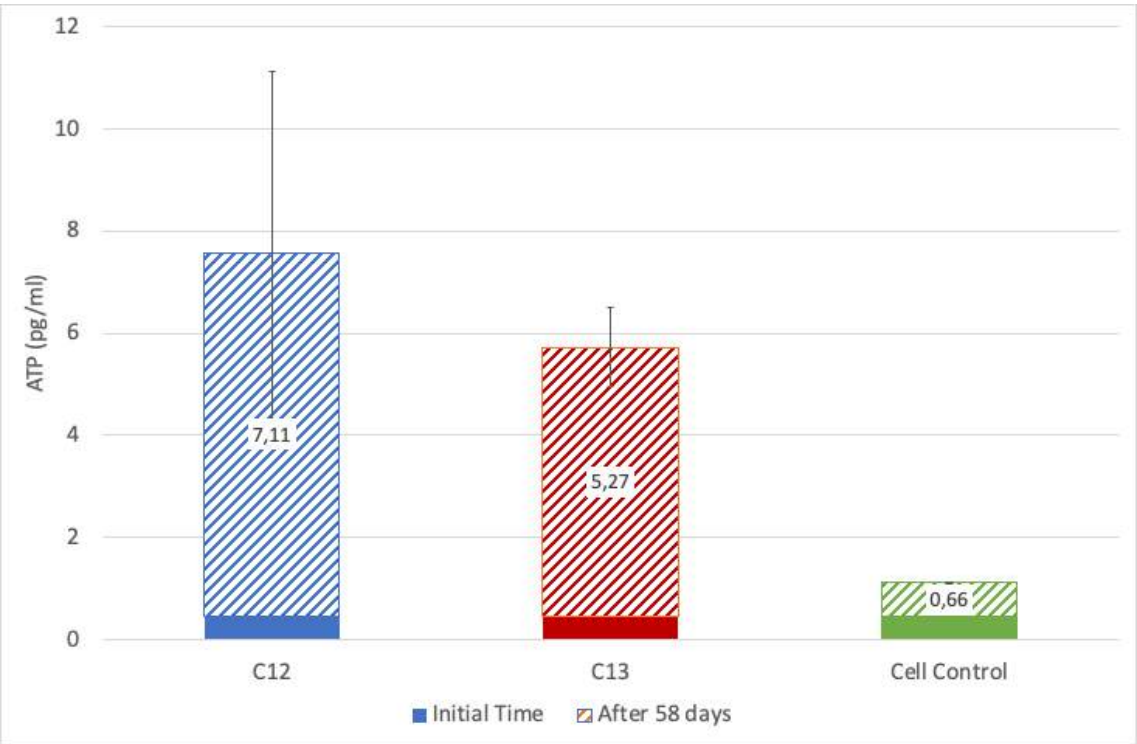


Figure 10 -ATP concentration after 58 days of incubation.

While gas chromatography analysis measures the amount of CO2 gas released over time (Figure 11 ; upper chromatogram), the mass spectrometry data allow the monitoring of the isotopic composition of CO_2 (Figure 11 ; lower spectra). For each sample collected during biodegradation experimentation, isotopic composition was determined by following $^{12}CO_2$ (m/z = 44 =1 carbon atom (12) + 2 oxygen atom (2x16) and $^{13}CO_2$ (m/z = 45=1 carbon atom (13) + 2 oxygen atom (2x16) released. Figure 11 shows the isotopic composition of CO_2 gas released by cells growing on ^{12}C oxidized polyethylene films. It reveals that 99% of this CO_2 is composed by ^{12}C (so not labelled) and only 1% of this CO_2 is labelled with a ^{13}C carbon ; this correspond to the natural ^{13}C enrichment of CO_2 as in atmospheric CO_2. The natural ^{13}C enrichment of the ^{12}C polyethylene (1%) did not change this ratio.

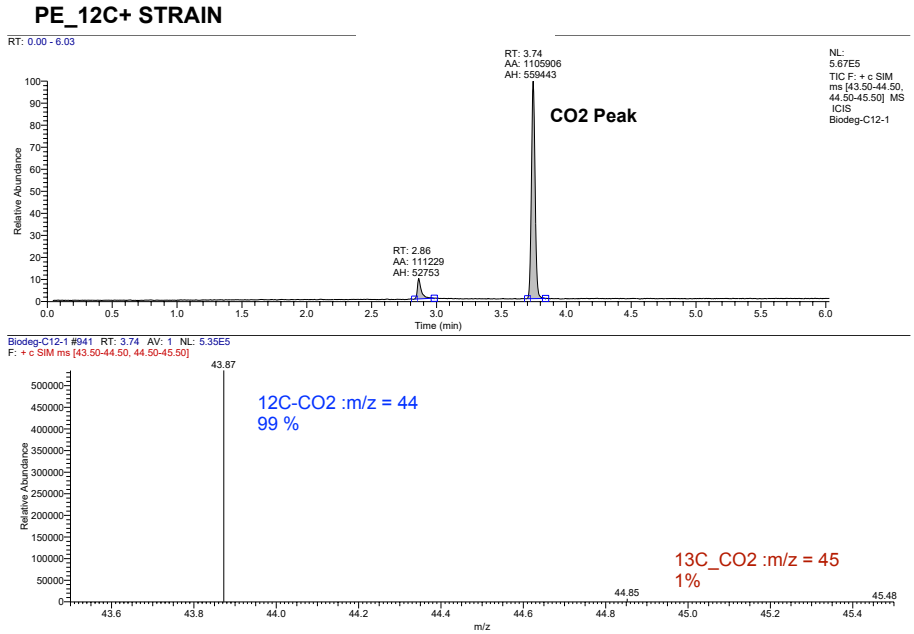


Figure 11 – Monitoring of $^{12}CO_2$ (m/z = 44) and $^{13}CO_2$ (m/z = 45) by GC/MS using Single Ion Mode - CO_2 from cells grown on ^{12}C oxidized polyethylene films

Figure 12 shows the isotopic composition of CO_2 gas released by cells growing on ^{13}C oxidized polyethylene films. It reveals a huge increase of $^{13}CO_2$ (m/z = 45). At the end of experiment, 52% of this CO_2 is composed by ^{12}C (not labelled) while 48% of this CO_2 is labelled with a ^{13}C carbon ; this result clearly indicates the incorporation of ^{13}C from ^{13}C polyethylene into the respirated CO_2. This enrichment reflect the mineralization of the labelled polymer through respiration and confirm the biodegradation of oxo- polyethylene.

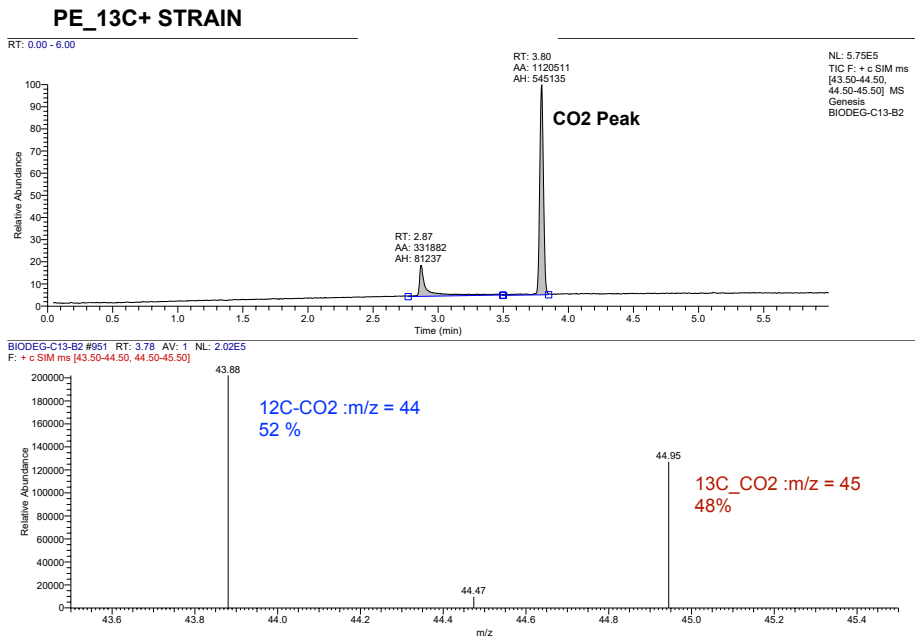


Figure 12 - Monitoring of $^{12}CO_2$ (m/z = 44) and $^{13}CO_2$ (m/z = 45) by GC/MS using Single Ion Mode ; CO_2 from cells grown on ^{13}C oxidized polyethylene films

The incorporation of ^{13}C into the CO_2 started from the beginning of the incubation and increase during the whole experiment (Figure 13). This confirms the use of ^{13}C polyethylene as a carbon source by Rhodococcus strain all along the experiment. After 7 Days, 27% of expired CO_2 was already labelled. The incorporation of ^{13}C carbon kept increase up to 48 % in 58 days (Figure 14). Simultaneously, the level of $^{13}CO_2$ remained very low (Figure 13), when cells grows on ^{12}C oxidized polyethylene films as well as in the biotic control, respecting in both case the natural enrichment value around 1 % (Figure 14).

The level of $^{13}CO_2$ emitted by abiotic control also increased with time but to lower extend compare to $^{13}CO_2$ produce by the strain respiration. The normalization of the data could be considered by subtracting the abiotic values to the one obtained from cell respiration.

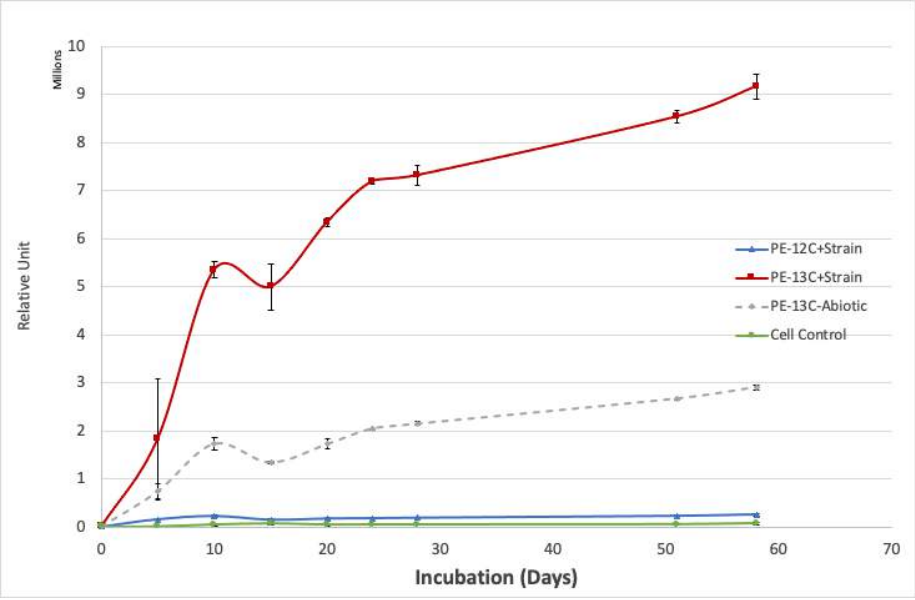


Figure 13- Evolution of $^{13}CO2$ concentration

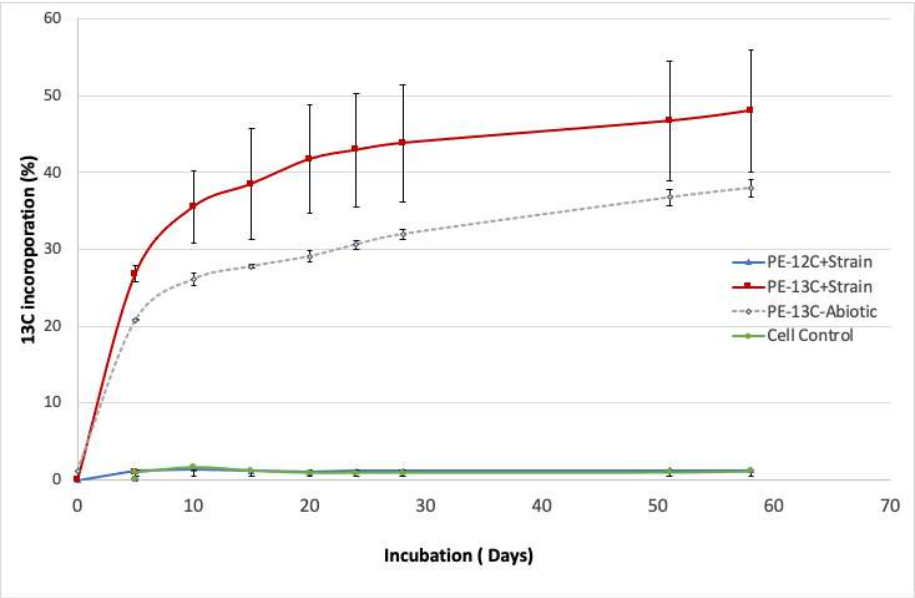


Figure 14 - Incorporation of 13Carbone into Expired CO2

To complete these results of the experimentation, supplementary analysis were conducted to assess the incorporation of ^{13}Carbone into biomass. Both GM/MS Pyrolysis and CP-MAS NMR analysis were performed on cell pellets but amount of cells was too small to provide results.

Conclusion:

The biodegradation of ^{13}C-Oxo-LDPE and ^{12}C-Oxo-LDPE showed positive results, as the Rhodococcus bacterium was able to growth on both materials. The use of ^{13}C labelled polymers certainly confirms the biodegradation and the ultimate mineralization of such material. A substantial incorporation of ^{13}C from polymer was recorded in the CO_2 produced by the bacteria and collected during experimentation.