Polyethylene bio-degradation by caterpillars of the wax moth *Galleria mellonella*

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In brief

In this study, experimental evidence for the biodegradation of polyethylene (PE) by larvae of the wax moth *Galleria mellonella* is presented. As biodegradation occurs very rapidly, the discovery lays the basis for the development of biotechnological applications that could play a pivotal role in management of plastic waste.

Plastics are synthetic polymers derived from fossil oil and largely resistant to biodegradation. Polyethylene (PE) and polypropylene (PP) represent ~92% of total plastic production. PE is largely utilized in packaging, representing ~40% of total demand for plastic products (www.plasticseurope.org) with over a trillion plastic bags used every year [1]. Plastic production has increased exponentially in the past 50 years (Figure 1A). In the 27 EU countries plus Norway and Switzerland up to 38% of plastic is discarded in landfills, with the rest utilized for recycling (26%) and energy recovery (36%) via combustion (www.plasticseurope.org), carrying a heavy environmental impact. Therefore, new solutions for plastic degradation are urgently needed. We report the fast bio-degradation of PE by larvae of the wax moth *Galleria mellonella*, producing ethylene glycol.

PE comprises a linear backbone of carbon atoms (Figure 1B), which is resistant to degradation. Although PE is believed not to be susceptible to bio-degradation, a few attempts have been made, as PE is the most common packaging plastic. Slow

(>weeks/months) PE biodegradation has been observed, given appropriate conditions. For example, modest degradation of PE was observed after nitric acid treatment and incubation for 3 months in a liquid culture of the fungus *Penicillium simplicissimum* [2]. Slow PE degradation was also recorded after 4 to 7 months exposure to the bacterium *Nocardia asteroides* [3]. In both cases, infrared spectroscopy (FTIR) analysis of treated samples revealed formation of an absorbance peak around 3300 cm^{-1}, a signature for ethylene glycol, confirming PE degradation. More recently, Yang et al. reported bacterial degradation of PE over several weeks [4]. However, no production of ethylene glycol from the biodegradation was described. The authors reported that PE biodegradation depended on the activity of microorganisms present in the gut of the larvae of the Indian mealmoth *Plodia interpunctella* (two bacterial strains, *Bacillus sp*. YP1 and *Enterobacter asburiae* YT1). Faster biodegradation (~0.13 mg cm^{-2} day^{-1}) of another plastic, poly(ethylene terephthalate) (PET) by a microbial consortium including a newly isolated bacterium, *Ideonella sakaiensis*, was described recently [5]. Although PET is a resistant material, one might expect its biodegradation to be easier than PE, as PET has a polyester backbone and can be hydrolysed. We report here the fast biodegradation of PE by the wax worm, the caterpillar larva of the wax moth *Galleria mellonella* of the snout moth (Pyralidae) family of Lepidoptera.

When a PE film was left in direct contact with wax worms, holes started to appear after 40 minutes, with an estimated 2.2±1.2 holes per worm per hour (Supplementary Table 1a). Figures 1C, D show the result of leaving ~100 wax worms in contact with a commercial PE shopping bag for ~12 hours, which caused a mass loss of 92 mg. To exclude the possibility that mechanical action of the masticatory system was solely responsible for the observed PE breakdown, worm homogenate was smeared on and left in contact with PE films. Gravimetric analysis of the treated samples confirmed a significant mass loss of 13% PE over 14 hours treatment (one-way ANOVA $p = 0.029$) compared to the untreated samples (Figure 1E and Supplementary Tables 1b and 1c). This corresponds to an average degradation rate of 0.23 mg cm^{-2} h^{-1}, which is markedly higher than the rate of PET biodegradation by a microbial consortium recently reported [5].

To test if the PE polymer was chemically degraded by contact with the worm homogenate, we carried out FTIR analysis. When the FTIR probe was pointed on untreated samples, the spectroscopic results confirmed the identity of the PE film, with

peaks at 2921 and 2852 cm^{-1} being the classical signatures of PE (Figure 1F, black line). However, when the probe was pointed on sample smeared with worm homogenate, an additional peak at ~3350 cm^{-1} was seen (Figure 1F, red line). This FTIR peak corresponds to the one previously described as the ethylene glycol signature (also compare Figure 1G with Figure 4b in [4]) [3] [6]. In addition, a peak at 1700 cm^{-1} appeared in the treated sample, which is the classical signature of the carbonyl bond (Figure 1G, red line). The ethylene glycol signature was also seen when the probe was pointed close to holes in PE caused by intact worms, but not when the probe was pointed at a distance (Supplementary Figure 1A-C).

The formation of products after treatment with wax worm extract was also characterised by HPLC-MS, covering a m/z range from 100 to 600 (Figure 1H and Supplementary Figure 1E). Figure 1H shows the spectra for untreated PE (top, black) and the treated PE (bottom, red). In the samples treated with the wax worm extract three new peaks appeared at the lower end of the m/z region (110.0, 122.9 and 170.0). The chemical identity of these lighter fractions was not confirmed but their presence supports the hypothesis of PE degradation by the wax worm homogenate.

To analyse further the effect of wax worm homogenate on the PE surface, Atomic Force Microscopy (AFM) was performed (Figure 1I, J). After treatment with homogenate, we observed an obvious change in the topography of the PE surface (Figure 1J), corresponding to a significant (one-way ANOVA $p = 0.005$) +140% increase in surface roughness (Supplementary Figure 1D and Supplementary Table 1d). These results indicate that the physical contact of the wax worm homogenate with the PE surface modified the integrity of the polymer surface.

What allows the wax worm to degrade a chemical bond not generally susceptible to bio-degradation? The answer may lie in the ecology of the wax worm itself. They feed on beeswax, and their natural niche is the honeycomb; the moth lays its eggs inside the beehive, where the worms grow to their pupa stage, eating beeswax [7]. Beeswax is composed of a highly diverse mixture of lipid compounds, including alkanes, alkenes, fatty acids and esters [8]. The most frequent hydrocarbon bond is the CH_2-CH_2, as in PE (Figure 1B). Although the molecular details of wax biodegradation require further investigation, it seems likely that the C-C single bond of these aliphatic compounds is one of the targets of digestion. The appearance of holes when PE films are left in direct

contact with wax worms, and the FTIR analysis of degraded PE, indicate chemical breakdown of the PE, including breakage of C-C bonds. It is not clear whether the hydrocarbon-digesting activity of *G. mellonella* derives from the organism itself, or on enzymatic activities derived from its intestinal flora [7], as with PE digestion by *Plodia interpunctella* [4]. Further investigation is also required to determine if related species have the capacity for PE degradation, and to analyse its molecular basis including the detailed nature of the products. Nevertheless, given the fast rate of biodegradation reported here, these findings have potential for significant biotechnological applications.

Figure Legends

Figure 1. A. The black line represents the increase (in millions of tons) in plastic production worldwide in the past 50 years (http://discardstudies.com, accessed: 4th February 2016). Inset: Pie chart shows the diffusion of plastics classified by polymer type (PE, polyethylene; PP, Polypropylene; PVC, Polyvinyl Chloride; PET, Polyethylene Terephthalate; PS, Polystyrene; PUR, polyurethane). **B.** Chemical formulae of polyethylene (PE), ethylene glycol and palmitic acid ester of myricyl alcohol, one of the multiple compounds that constitute beeswax. **C.** Plastic bag after exposure to ~100 wax worm for 12 hours. **D**. Magnification of the area indicated in C. **E**. Gravimetric analysis of homogenate-treated versus untreated PE, showing a reduction (13%) of mass per unit of area in the former. **F** and **G.** FTIR analysis of the homogenate-treated and control PE films. **H.** Mass spectroscopy analysis of homogenate-treated and control PE. In the sample treated with the wax worm extract three new peaks at lighter m/z appear (110.0, 122.9 and 170.0). **I, J.** Atomic Force Microscopy on homogenate-treated (J) and untreated (I) PE film (representative examples of 3 topographic maps each).

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Figure

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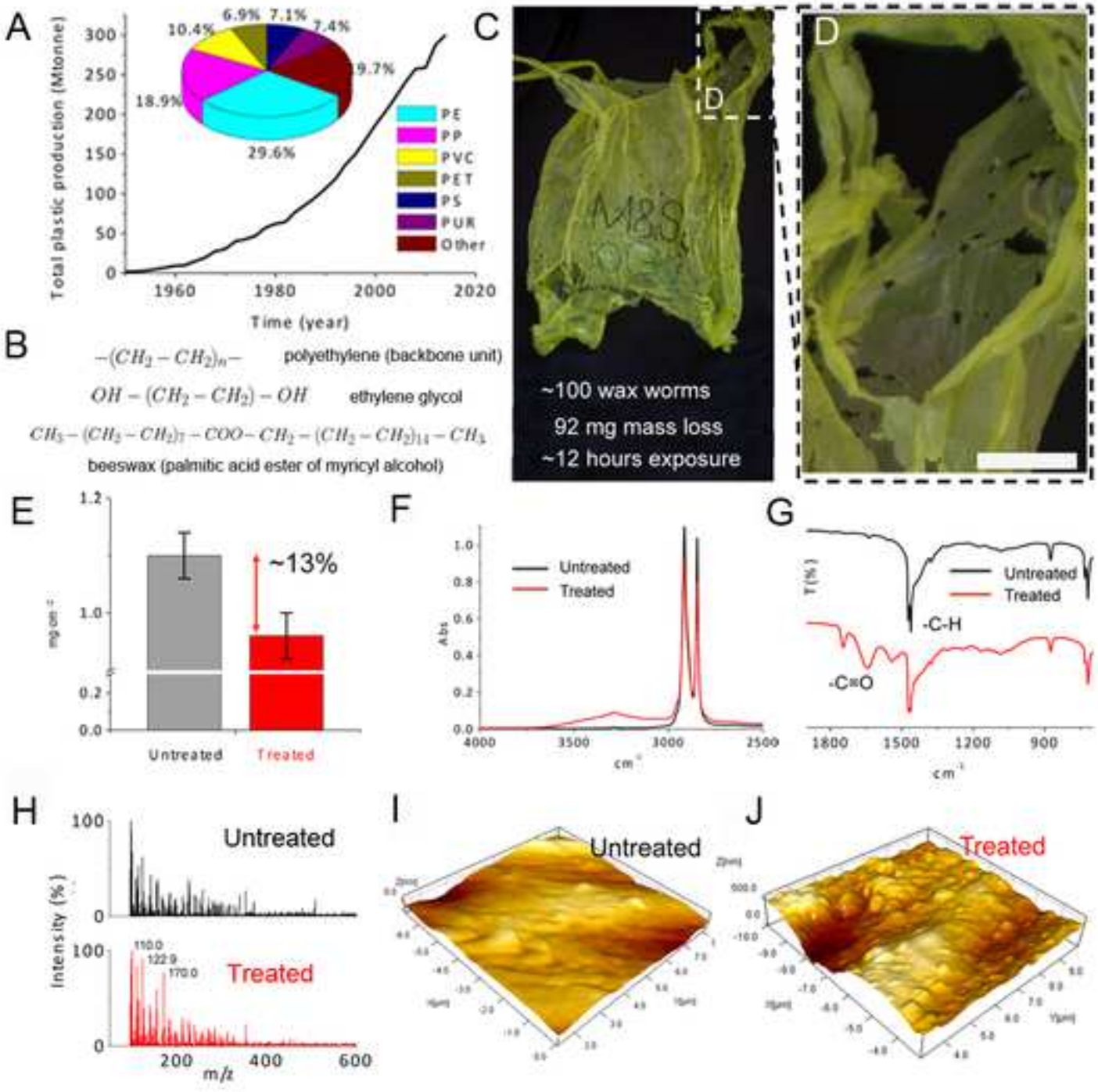

Supplemental Information: Polyethylene bio-degradation by caterpillars of the wax moth *Galleria mellonella*

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Supplemental Experimental Procedures

Samples of wax worms. Two sources of wax worms of the moth *G. mellonella* were used: environmentally bred worms from the Spanish countryside (Cantabria), and commercially bred worms from Hobby Zoo Pinto shop (Spain).

Sample of PE. PE was sourced from commercially available PE plastic bags (Marks and Spencer, 2015).

Preparation of the wax worm homogenate. The crude wax worm extract was made by homogenising fresh worms in a mortar at low temperature (0-4 °C). The resulting paste was then smeared on the surface of a film of PE and left in contact for a certain amount of time as detailed in the appropriate experimental section. The thickness of the smeared paste was about 0.5 cm.

Biodegradation of a commercial PE shopping bag. The results shown in figure 1C and 1D were obtained as follows. ~100 wax worms were left in contact with a commercial PE shopping bag. The bag was weighed initially (2730 mg); after incubation worms were picked off the bag, the bag was cleaned with deionized water, carefully dried, and then finally re-weighed (2638 mg).

Gravimetric analysis of treated PE samples. The results shown in figure 1D were obtained as follows. The crude wax worm homogenate was made as described above. The resulting paste was smeared on the surface of several films of PE and left in contact for 2 hours at room temperature. Then, the paste was gently removed and replaced with a fresh layer of wax worm homogenate. The routine was repeated 7 times for a total of 14 hours. The samples were cleaned with deionized water and carefully dried, and finally weighed. Untreated sample of PE underwent the same protocol of washing and drying. The mass per unit area was determined before and after treatment.

FTIR analysis. The results shown in figure 1F and 1G were obtained as follows. The crude wax worm homogenate was made as described above. The homogenate was smeared on the surface of several films of PE and left in contact for 2 hours at room temperature. The samples were cleaned with deionized water and carefully dried.

Untreated sample of PE underwent the same protocol of washing and drying. Films that had been treated with homogenate and un-treated controls were analysed by ATR FTIR to characterise the results of breakdown. A iS50 ATR apparatus (Thermo Scientific, USA) was used. The samples were placed face down on the ATR crystal and scanned between 700 to 4000 cm^{-1}. For each sample, the background was corrected and four spectra were taken and averaged.

HPLC-MS analysis. The results shown in figure 1H were obtained as follows. The crude wax worm homogenate was made as described above. The homogenate was smeared on the surface of several films of PE and left in contact for 24 hours at room temperature. The samples were carefully cleaned with deionized water and dried. Untreated sample of PE underwent the same protocol of washing and drying. Both the treated and untreated PE samples were analysed by HPLC-MS (Waters ZQ mass spectrometer with a Waters 2795 HPLC). The samples were submerged in acetonitrile and sonicated for around 1 minute. Then, the PE was removed and the solvent evaporated using a vacuum. The soluble products were then dissolved in 1 ml of fresh acetonitrile, which was then transferred to a microcentrifuge tube and spun down for 2 minutes. The supernatants of the untreated and treated samples were then placed in HPLC vials and run via LCMS. The chromatograms shown in the Supplementary Figure 3A, B and C display the total ion current (TIC) *versus* the elution time for the solvent alone (acetonitrile) untreated and treated samples respectively. An increase in these indicates an increase in current at the mass spectrometer detector as will be observed when a compound elutes from the column. The difference between the traces, untreated and treated is the peak observed at 5.75 minutes. This peak is only observed in the treated sample. The untreated sample has a TIC that is essentially identical to the solvent alone (acetonitrile). The mass spectra reported in the figure 1H are derived from the fractions eluted at 5.75 minutes for the untreated and treated samples.

Atomic Force Analysis (AFM). The results shown in figure 1I and 1J were obtained as follows. The crude wax worm homogenate was made as described above. The homogenate was smeared on the surface of several films of PE and left in contact for 2 hours at room temperature. The samples were cleaned with deionized water and carefully dried. Untreated sample of PE were subjected to the same protocol of washing and drying. Both the treated and untreated samples were analysed by a commercial AFM system (Anasys Instruments, USA). Samples were scanned with a line rate between 0.1-0.3 Hz in contact mode with a silicon cantilever (AppNano) having a

nominal radius of 10 nm and spring constant of 0.5 N/m. Images were acquired with at least a resolution of 500 × 500 pixels per image. The AFM images were processed using Scanning Probe Image Processor (SPIP)-6.3.4. The morphology maps were first flattened, then their roughness was evaluated by SPIP. The roughness of the different areas, for a total of 75 um^2, was averaged to compare the control and treated samples. All measurements were performed at room temperature.

Statistical validation. One-way analysis of variance (ANOVA) was used to determine whether there were any significant differences between the means of independent (unrelated) groups of data. When the p-value was greater than 0.05 there was no statistically significant difference between group means**.** The complete results obtained from the ANOVA tests run in this study are shown in the Supplementary Table 1. The results were calculated by using online software available at:

http://www.danielsoper.com/statcalc3/calc.aspx?id=43

(Accessed: 6th February 2016).

Given the mean, standard deviation, and (n) in each group, p value is calculated by an ANOVA.

SS: sums of squares;

df: degrees of freedom;

MS: mean squares;

F and p-values.

Author Contributions

P. B., F. B. and C. J. H. designed the experiments, P.B. and F.B. conducted the experiments, P.B., F.B., and C.J.H. wrote the paper.

Supplemental Figure 1

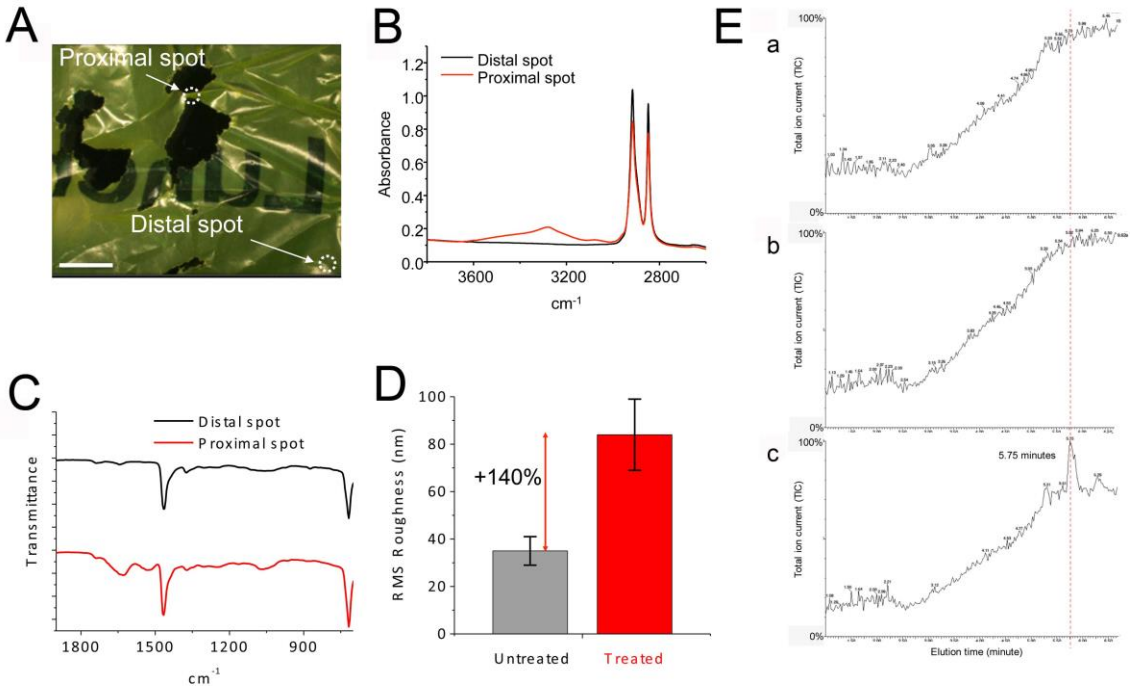

Figure S1. A. PE degraded film (holes) after exposure to the wax worm. Scale bar: 5mm. **B** and **C.** FTIR analysis of the PE film. **D.** AFM of homogenate-treated and control PE. The histogram represents distinct measurements (n=3 mean ± standard error) of treated (red column) and untreated (grey column) PE film. Treated PE showed an increase of roughness calculated as % of treated sample. **E.** Chromatograms for the total ion current (TIC) *versus* the elution time for the solvent alone (acetonitrile) (a) untreated (b) and treated (c) samples.

Supplemental Table 1

a)

# worms	# holes	time(h)	hole worm^{-1} h^{-1}
1	6	4	1.50
1	15	24	0.63
1	3	3	1.00
1	3	3	1.00
1	3	3	1.00
1	10	4	2.50
1	20	4	5.00
1	10	4	2.50
1	2	2	1.00
1	9	5	1.80
1	16	5	3.20
1	9	3	3.00
1	13	9	1.44
1	15	5	3.00
6	140	48	2.92
		Average	**2.10**
		St.dv.	***1.20***

Quantitative estimation of hole formation when a PE film was left in direct contact with worms. Individual (or in one case, several) worms were left in contact with the film, and the number of holes counted at different time points. Each line in the table represents a separate experiment.

b)

	(before)	(after)		Before t.	After t.	Delta
	mg	mg	cm^2	mg cm^{-2}	mg cm^{-2}	mg cm^{-2}
1	3.93	3.36	4	0.982	0.839	0.143
2	3.70	3.55	4	0.926	0.887	0.039
3	4.52	3.62	4	1.129	0.905	0.224
4	4.66	3.80	4	1.164	0.950	0.214
5	4.44	3.79	4	1.110	0.948	0.162
6	3.58	3.41	3	1.193	1.136	0.058
7	3.49	3.17	3	1.164	1.058	0.107
			Average	**1.096**	**0.960**	**0.135**
			St.er.	***0.038***	***0.039***	***0.027***
			St.dv.	***0.102***	***0.103***	***0.072***

Gravimetric analysis carried out as described in material and methods.

c)

	SS	Df	MS	F	p
Between:	0.065	1	0.065	6.162	**0.029**
Within:	0.126	12	0.011		
Total:	0.191	13			

Statistical validation of the gravimetric analysis carried out as described in material and methods.

d)

	SS	Df	MS	F	p
Between:	3,645.7	1	3,645.7	30.07	**0.005**
Within:	485.03	4	121.26		
Total:	4,130.8	5			

Atomic Force Microscopy was carried out on 3 separate independent experiments as described in material and methods.