



Article

# Microbial Degradation of Plastic in Aqueous Solutions Demonstrated by CO<sub>2</sub> Evolution and Quantification

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Received: 10 January 2020; Accepted: 9 February 2020; Published: 11 February 2020



**Abstract:** The environmental accumulation of plastics worldwide is a consequence of the durability of the material. Alternative polymers, marketed as biodegradable, present a potential solution to mitigate their ecological damage. However, understanding of biodegradability has been hindered by a lack of reproducible testing methods. We developed a novel method to evaluate the biodegradability of plastic samples based on the monitoring of bacterial respiration in aqueous media via the quantification of CO<sub>2</sub> produced, where the only carbon source available is from the polymer. *Rhodococcus rhodochrous* and *Alcanivorax borkumensis* were used as model organisms for soil and marine systems, respectively. Our results demonstrate that this approach is reproducible and can be used with a variety of plastics, allowing comparison of the relative biodegradability of the different materials. In the case of low-density polyethylene, the study demonstrated a clear correlation between the molecular weight of the sample and CO<sub>2</sub> released, taken as a measure of biodegradability.

**Keywords:** plastic; biodegradation; *Rhodococcus rhodochrous*; *Alcanivorax borkumensis*; polyethylene; bioplastic; molecular weight

## 1. Introduction

Plastic is a versatile material. It is waterproof, strong, easily shaped and can be made rigid or flexible. Furthermore, it is relatively cheap to manufacture, making it widely available across the world. However, the inherent stability and durability of this material has resulted in widespread accumulation of plastics in both terrestrial and aquatic environments [1]. Indeed, evidence of floating plastic debris can be found from the equator to polar ice caps [2] and has led to the current global crisis [3–5]. There is considerable debate about possible solutions [6]. One key focus is the development and use of plastic alternatives, involving new degradable polymers such as hydrodegradable [7], compostable [8], biocomposite [9,10], bioplastic [11] and pro-oxidant-additive-containing (PAC)/oxo-degradable plastics [12]. Opinion is greatly divided over the environmental impact and the biodegradability of these materials [13]. This is largely due to the methods employed to measure biodegradation, where biodegradation is commonly evidenced by either the loss of plastic mass or the monitoring of microbial respiration, with both approaches using samples that have been buried in soil [14–16]. However, soil composition varies globally; thus, the relevance and reproducibility of these tests have been questioned [8,14,17,18]. Additionally, these methods are not suitable for studying plastic decomposition in open or aquatic environments, where oxygen and nutrient concentrations, temperature and microbial ecology differ significantly [17,19]. A number of alternative approaches have been reported in the

literature [20–23] but have not been widely applied. To this end, there is an urgent need for a relevant, reliable and versatile procedure for the measurement of biodegradability in aqueous conditions, and this has been the aim of this study.

Here, we present a standardised and reproducible bioassay to evaluate the biodegradation of plastic in a defined minimal media, based on the detection and quantification of CO<sub>2</sub>, produced as a result of bacterial respiration, using gas chromatography (GC). We studied the biodegradability of different samples of low-density polyethylene (LDPE), starch-based compostable and oxo-degradable plastic using the soil bacterium *Rhodococcus rhodochrous* [20,24,25]. The PAC or oxo-biodegradable plastics are polyolefins blended with additives that stimulate the cleavage of polymer chains under oxidative conditions, a feature expected to lead to improved biodegradability [26]. Artificially aged samples of plastic were used to investigate how these parameters impacted biodegradability. Sampling of LDPE and oxo-LDPE aged for different lengths of time allowed the study of the relationship between polymer molecular mass and degradation properties. In addition, the research included the evaluation of the activity of the marine bacterium *Alcanivorax borkumensis* [27,28] on a sample of bioplastic to demonstrate the wider applicability of this method. The data obtained provide evidence of the suitability of this technique for the comparative monitoring and the robust evaluation of the environmental impact of plastic.

## 2. Results

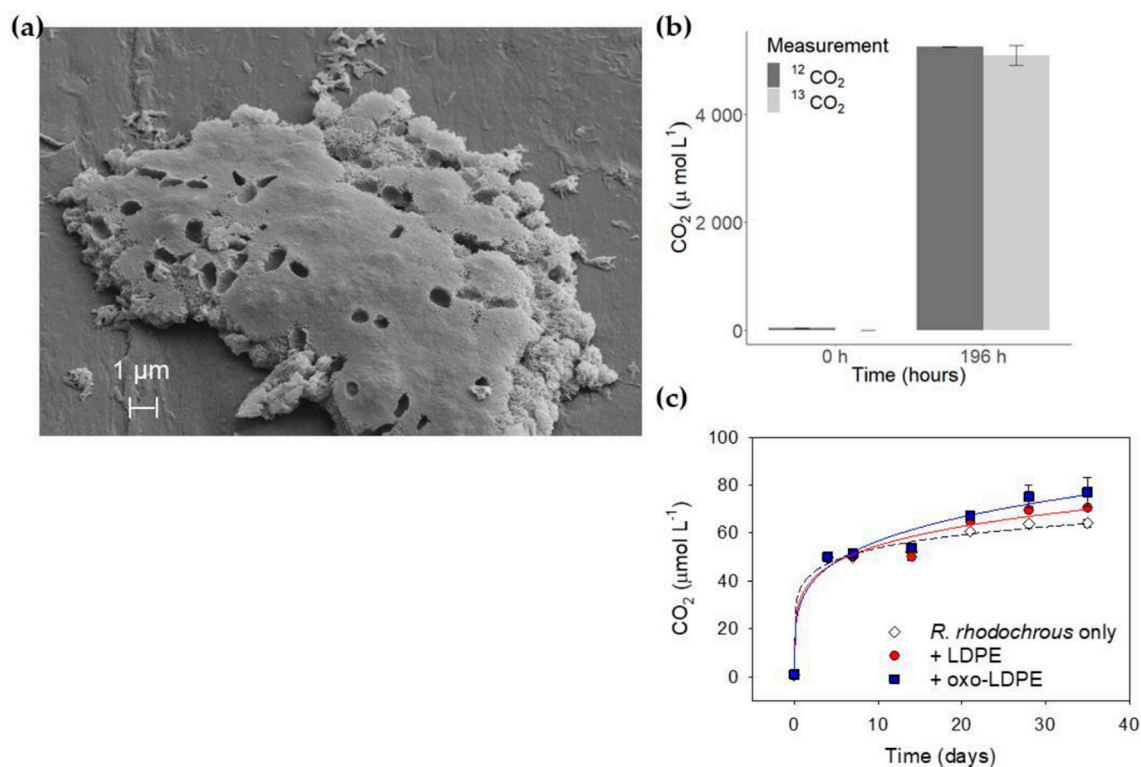
### 2.1. Assessment of Microbial Growth

We initially assessed microbial growth on plastic, employing the soil bacterium *R. rhodochrous* as a model organism (Table S1) with previously reported methods [20,22,23,29,30]. LDPE was chosen as the target plastic, as it is one of the most common polymers used for packaging and dominates the composition of plastic waste on the sea surface [31]. Scanning electron microscopy (SEM) and SYBR green staining of *R. rhodochrous* grown on solid media with LDPE as the sole carbon source showed microbial colonisation of the plastic surface within 20 days (Figure S1a). Efforts to dislodge cells from the film with multiple water washes, in order to estimate bacterial growth by cell counts, proved unsuccessful, as bacteria were observed still attached to the film. More aggressive methods such as acid, detergent or bleach washes also failed to remove bacteria completely (Figure 1a and Figure S1). It is unlikely that this robust adhesion was specific to polymer type as there was no significant difference between the number of bacterial cells per mm<sup>2</sup> when grown on either LDPE or oxo-LDPE ( $p = 0.6$ , Figure S1d). This contradicts previous suggestions that additives, such as those found in oxo-LDPE, may alter the hydrophobicity of the polymer surface, thus influencing microbial attachment [24,25]. Biochemical assays supported the hypothesis of irreversible adhesion, as evidenced by a lack of measurable growth on LDPE in comparison to glucose (Figure S2). Consequently, any assay requiring the removal of biomass or a bacterial suspension would be fundamentally inaccurate [23]. It is noteworthy that substantial interference by samples of heat-treated plastic were observed, in the absence of any microbe, in both the luciferase (ATP/ADP) and AlamarBlue assays [32,33].

### 2.2. Measurement of Growth by Monitoring CO<sub>2</sub> Production

The use of gas chromatography for the detection and quantification of CO<sub>2</sub> produced by bacterial respiration has been used consistently in environmental science [34,35]. However, the high-throughput GC approach has not been explored as a tool for the evaluation of polymer biodegradation. We modified the method to detect CO<sub>2</sub> evolved from a well-defined aqueous media containing a single microbial culture and a single type of polymer as the only carbon source. Thus, the produced CO<sub>2</sub> can be directly linked to the mineralisation of the supplied carbon source via bacterial respiration. To confirm the validity of the assay, growth of the soil bacterium *R. rhodochrous* was carried out on <sup>13</sup>C-glucose [36]. The data (Figure 1b) showed that the concentration of CO<sub>2</sub> after 196 h was consistent with that produced as <sup>13</sup>CO<sub>2</sub>, confirming the suitability of our methodology ( $p = 0.98$ ). At the point at which the stationary

phase of growth was achieved, 32% of the available carbon had been released as CO<sub>2</sub>, with the majority of carbon likely assimilated into biomass [37,38].



**Figure 1.** Microbial growth and measurement of biological degradation of polymer films. (a) *Rhodococcus rhodochrous* grown on low-density polyethylene (LDPE) underwent water washes and acid washing prior to visualisation by SEM (scale bar 1 µm). Large deposits of dead cells were clearly still visible after this treatment, revealing the challenges in the removal of bacterial films. (b) *R. rhodochrous* grown on <sup>13</sup>C-glucose. There was no difference between measurement of CO<sub>2</sub> (dark grey) or <sup>13</sup>CO<sub>2</sub> (light grey) after 196 h ( $n = 3, p = 0.98$ ), directly linking carbon uptake with respiration. (c) Measurement of CO<sub>2</sub> production by *R. rhodochrous* in minimal media (open diamond) on unaged LDPE (red circle) or unaged oxo-LDPE (blue square) revealed no significant effect due to the presence or type of plastic ( $n = 5, p = 0.99$ ).

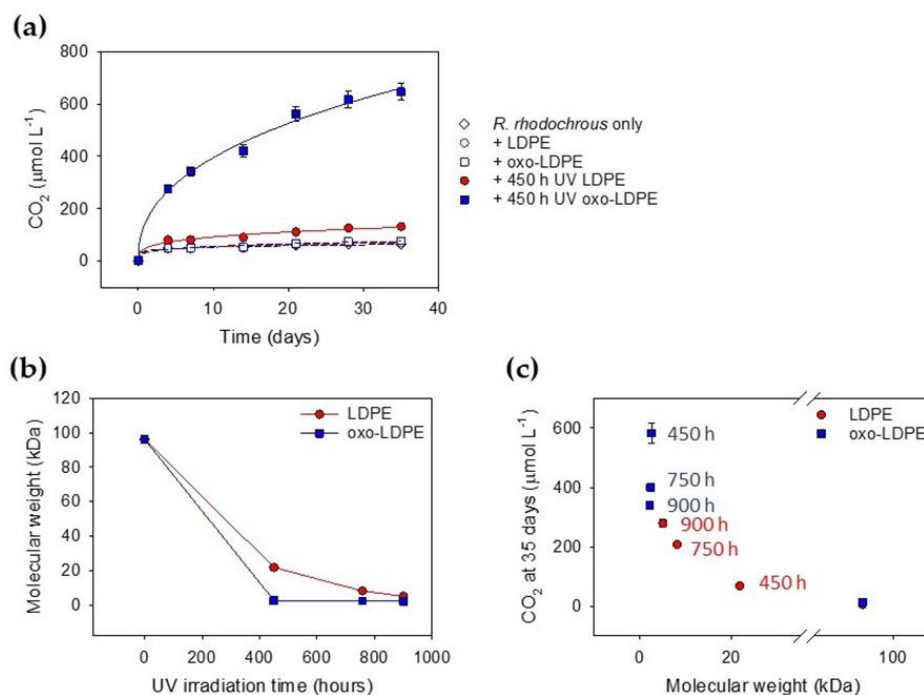
The detection of evolved CO<sub>2</sub> to monitor respiration, and thus growth, of *R. rhodochrous* on samples of unaged LDPE and oxo-LDPE was carried out over 35 days (Figure 1c). All values of CO<sub>2</sub> presented were corrected for the spontaneous release of CO<sub>2</sub> for control experiments (Figure S3). It should be noted that no fragmentation of plastic samples was observed. Within the initial days, ~0.05 mM CO<sub>2</sub> was released from all cultures, most likely due to carry over from the starter culture that was saturated with highly soluble CO<sub>2</sub>. Thereafter, stationary phase was achieved, with no significant difference between *R. rhodochrous* grown with no carbon source and the bacteria grown on unaged LDPE or oxo-LDPE ( $p = 1$ ). Sample acidification, from the release of CO<sub>2</sub>, was ruled out as an explanation for the limited growth, as the pH of the media remained unchanged during the incubation. The available concentrations of phosphate (24 mM) and nitrogen (5.6 mM) in the starting media were too high to limit the production of 0.05 mM CO<sub>2</sub>. Thus, the data suggest that bioavailable carbon was the limiting factor in microbial growth and that unaged LDPE and oxo-LDPE showed limited evidence of biodegradation.

### 2.3. Effect of UV Aging on Biodegradation

Polymers found in the environment are subject to chemical changes as a result of heat, light and water [39]. Artificial aging by sustained UV exposure is commonly used to simulate environmental damage, as it allows reproducible samples to be generated [40]. We investigated the role of artificial UV aging in the biodegradability of LDPE and oxo-LDPE using samples of films irradiated for 450, 758 and 900 h, in accordance with international standards (ASTM 5208 Cycle C). The films, unaged and aged, were incubated with *R. rhodochrous* for 35 days and CO<sub>2</sub> was measured over time. As expected, a clear link between UV aging and biodegradation was observed, which was further enhanced in the presence of the oxo-additive. Indeed, the biodegradation of oxo-LDPE (+450 h UV) was 90-fold greater than that of LDPE (+450 h UV) and 45-fold greater than that of unaged oxo-LDPE after 35 days (Figure 2a, Table S2). However, biodegradation of LDPE (+450 h UV) was only 3-fold greater than that of unaged LDPE ( $p = 0.26$ ), demonstrating the impact of the additive. Longer UV exposure of oxo-LDPE samples, for 758 and 900 h, showed a decreasing trend in CO<sub>2</sub> evolution compared with that observed after 450 h. Indeed, less CO<sub>2</sub> was released between 450 and 758 h, though there was no significant difference between CO<sub>2</sub> evolved after lengthier irradiation (+758 h UV and +900 h UV  $p = 0.67$ ) (Figure S4, Table S2). These results are somewhat unexpected and suggest that chemical changes to the irradiated materials influence the carbon bioavailability. Conversely, biodegradation of LDPE continued to increase with irradiation time, such that the samples of both LDPE and oxo-LDPE that had undergone 900 h of exposure generated similar quantities of CO<sub>2</sub> ( $p = 0.59$ ). This demonstrates that lengthier UV exposure is necessary to facilitate biodegradation of conventional LDPE, while, in contrast, it impairs the biodegradation of oxo-LDPE.

### 2.4. Influence of Molecular Weight on Biodegradation

UV irradiation of both LDPE and oxo-LDPE polymers is known to result in changes in the chemical structure of the matrix, as a result of oxidation [41,42]. To understand the influence of UV aging on biodegradation, changes in polymer molecular weight as a function of irradiation were measured (Figure 2b). Here, there was a marked difference in the time required for the polymer samples to reach a molecular mass of <3 kDa. Oxo-LDPE achieved this reduction within 450 h of irradiation, whereas LDPE required double the exposure time (900 h) to reach the same mass. When the different samples were incubated for 35 days with *R. rhodochrous* and CO<sub>2</sub> was measured, a direct correlation between CO<sub>2</sub> produced and molecular mass of the polymer sample was observed (Figure 2c). The greatest concentration of CO<sub>2</sub> was generated by microbial action on oxo-LDPE after 450 h, when the molecular mass reached <3 kDa. This is consistent with the theoretical proposal that only polymers of less than 5 kDa are bioavailable [43]. The data appear to suggest that UV-irradiated samples of oxo-LDPE are more biodegradable than equivalently aged conventional LDPE, as evidenced by the higher concentration of CO<sub>2</sub> released. This correlated with the samples of oxo-LDPE reaching a lower molecular mass in a shorter period of time. The experiments allow comparison between different types of artificially aged polymers, although these findings would need to be validated with naturally aged samples.



**Figure 2.** Artificial UV aging accelerated the rate of biodegradation of oxo-LDPE compared with LDPE as a result of changes in molecular weight. (a) CO<sub>2</sub> production from *R. rhodochrous* ( $n = 5$ ) grown with no supplied carbon (open black diamond) on unaged LDPE (open red circle) and unaged oxo-LDPE (open blue square) compared to 450 h UV-irradiated plastic of both LDPE (solid red circles) or oxo-LDPE (solid blue squares). UV exposure of oxo-LDPE significantly affected CO<sub>2</sub> production ( $p < 0.001$ ), while there was only a small increasing trend in CO<sub>2</sub> production between UV-treated LDPE and unaged plastics ( $p = 0.26$ ). (b) The molecular weight (kDa) of LDPE (red open circles) and oxo-LDPE (blue solid squares), as measured by gel permeation chromatography, compared to UV exposure time ( $n = 3$ ). (c) The concentration of CO<sub>2</sub> measured at 35 days for *R. rhodochrous* grown on LDPE (red) or oxo-LDPE (blue) after UV irradiation ( $n = 5$ , error bars represent standard error) compared to the molecular weight of the sample revealed a clear link between molecular weight and CO<sub>2</sub> production. The length of UV irradiation exposure is cited alongside each data point.

### 2.5. Starch-Based Plastic

“Compostable” plastics are often derived from biomass [44]. Here, the polymers contain carbonyl or ester functional groups within the backbone, making them particularly prone to hydrolysis [21,45]. These plastics are designed to biodegrade under composting conditions (i.e., a soil-based, warm environment). *R. rhodochrous* is a bacterium found in soil and may play a role in the decomposition of plastic in the natural environment; thus, a sample of commercially available starch-based compostable plastic was also assayed (Figure S5, Table S3). The data show that *R. rhodochrous* released 2.5 times more CO<sub>2</sub> when grown on a compostable plastic than on LDPE but 5.5 times less than on oxo-LDPE after 450 h of UV irradiation. The ability to utilise compostable plastic as a substrate is likely related to the composition of the polymer and is consistent with more recent findings that suggest compostable plastics may be degraded more readily than all other forms of plastic [21].

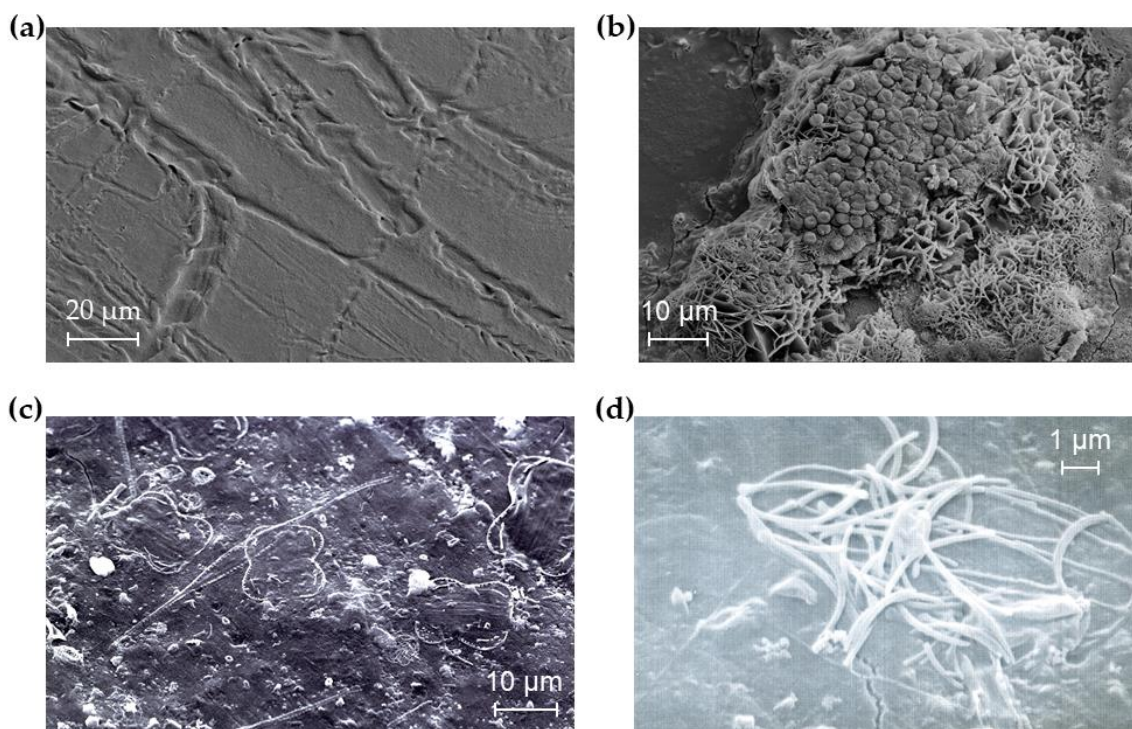
### 2.6. Biodegradation in Water

Artificial aging of plastic is routinely applied and accepted practice (ASTM 5208), as it allows accelerated decomposition and the preparation of reproducible samples. However, it is not entirely representative of environmental weathering [39,46,47]. To this end, prior to testing, samples of LDPE and oxo-LDPE were surface-weathered in sea water for 82 days, undergoing natural variations in sunlight and UV intensity. SEM revealed evidence of damage on both polymer films due to

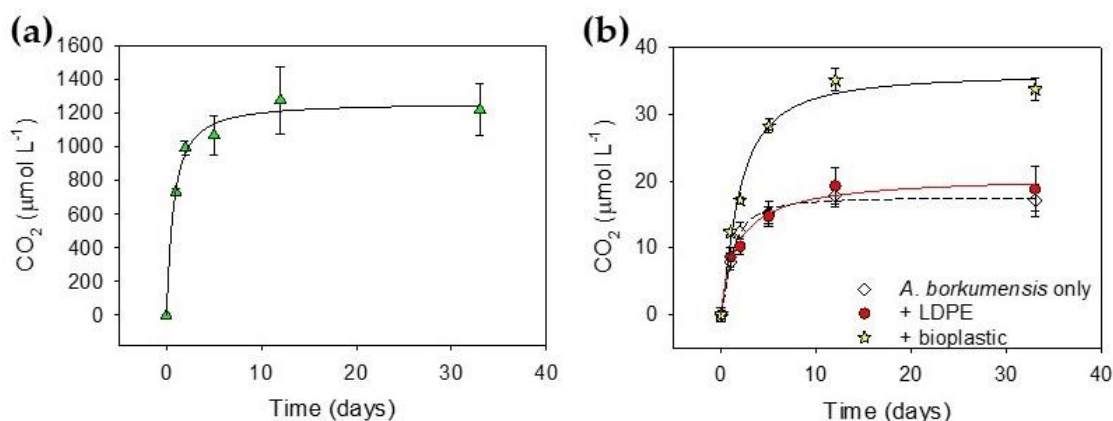


microbial activity (Figure 3a) and the presence of microorganisms still attached to the film (Figure 3c,d). Additionally, extensive calcium phosphate deposits were identified (Figure 3b), which could dissolve during incubation. Alterations in the calcium concentration could affect bacterial growth and behaviour, especially on biofilm-forming species, as changes in calcium and phosphorous concentrations have been shown to affect the planktonic state and biofilm thickness [48,49]. Consequently, it was difficult to ensure consistent sampling of environmentally weathered samples. Until a method that allows complete removal of bacteria without altering the polymer, as is the case with autoclaving/heating, a large sample size is going to be critical when assessing the biodegradation of environmental samples.

There is increased interest in the microbial composition of aquatic environments and, more specifically, microbes that play a role in the degradation of plastic in the sea [50]. To this end, we used our bioassay with a model organism from the marine environment, *Alcanivorax borkumensis* [51,52]. The growth media was altered to accommodate the higher salt concentration required to cultivate *A. borkumensis* since it was unable to grow in the same media used for *R. rhodochrous*. Incubation of *A. borkumensis* with pyruvate as a natural carbon substrate generated a typical growth curve (Figure 4a). Stationary phase was achieved after 10 days and *A. borkumensis* had respired 26% of the available carbon (Figure 4a).



**Figure 3.** Microbial growth on plastic in environmental samples may influence testing. Oxo-LDPE was suspended on the surface in sea water tanks for 82 days. The plastic was washed and visualised by SEM. (a) Tracks consistent with hyphal damage were observed (scale bar 20 μm). (b) Salt deposits were observed (scale bar 10 μm). (c) Evidence of crystallised microbes on the plastic surface (scale bar 10 μm). (d) Residual microbial presence was identified (scale bar 1 μm).



**Figure 4.** Monitoring respiration of *Alcanivorax borkumensis* when grown on a variety of substrates. (a) *A. borkumensis* was grown in liquid culture with pyruvate as the sole carbon source and CO<sub>2</sub> was measured over time ( $n = 4$ , error bars represent standard error). (b) *A. borkumensis* was grown in liquid culture with no carbon source (open diamonds), LDPE (red circles) or with a bioplastic (yellow star) and CO<sub>2</sub> production was measured over time by GC. *A. borkumensis* evolved twice as much carbon when grown on bioplastic compared with bacteria or LDPE alone ( $n = 5$ ,  $p = 0.0006$ ).

### 2.7. Monitoring of *A. Borkumensis* Growth on Plastics

*A. borkumensis* has been shown to be capable of growth on larger alkanes of up to C38 [51]; however, its ability to metabolise polyethylene has not been investigated. *A. borkumensis* was grown on LDPE and CO<sub>2</sub> production was monitored. Here, as for *R. rhodochrous*, there was an initial release of CO<sub>2</sub> prior to a plateau in CO<sub>2</sub> production. There was no significant difference between CO<sub>2</sub> generated by *A. borkumensis* on LDPE and the sample without plastic ( $p = 0.88$ ) (Figure 4b), revealing that *A. borkumensis* is unable to utilise LDPE as a substrate.

“Bioplastics” can refer to polymer composites [14] and are marketed as a biodegradable alternative to LDPE. *A. borkumensis* was grown in liquid culture with a polymer composite, and CO<sub>2</sub> production was monitored over time. Interestingly, there was only a 2-fold increase in CO<sub>2</sub> evolved from *A. borkumensis* in the presence of the polymer film compared with *A. borkumensis* alone ( $p = 0.0006$ ) (Figure 4b). Over the incubation, insufficient CO<sub>2</sub> was released to suggest that any nutrient, other than carbon, had become limiting. The bioplastic was more biodegradable than LDPE but 40 times less than pyruvate.

## 3. Discussion

To date, assessing the biodegradability of plastic has relied upon either composting approaches (i.e., burying samples in soil), which have shown limitations in reproducibility [14,15], or samples being suspended in the sea, where samples risk being lost prior to recovery for quantification [21]. Our method allows the reliable assessment of plastic biodegradability via the quantification of CO<sub>2</sub> produced in aqueous media as a direct result of microbial metabolism. We have demonstrated that different bacterial strains can be employed to evaluate polymer degradation. The method is reliable, reproducible and allows microbial activity to be monitored either without interfering with the cell culture or trying to remove adhered cells, reducing the risk of contamination or disrupting biofilms. The method was adapted to accommodate the different nutrient requirements for each species and thus could be adapted to imitate a specific aquatic environment. As the microbiological ecosystems of terrestrial, freshwater and marine habitats are inherently different, this approach will allow the assessment of the role of individual bacterial strains or communities in plastic biodegradation.

The key role that abiotic degradation plays in the biological decomposition of plastic is highlighted by our experiments on artificially aged samples of LDPE and oxo-LDPE. The method presented here allowed the measurement of biodegradability irrespective of the chemistry of the polymer. Our data

demonstrate a dependence between the biodegradability and molecular mass of plastic samples. The aged oxo-LDPE samples form fragments of lower mass in a shorter period of time and this results in a significantly higher amount of CO<sub>2</sub> detected, which can be taken as a measure of biodegradability. The wider applicability of the method was demonstrated by testing compostable plastic and bioplastics and showing that these are more readily degraded than untreated LDPE samples. This is the first example of work where aging, chemical structure of plastic and biodegradability have been connected. The method provides a robust and reproducible approach for comparing different types of polymers and evaluating the effect of environmental and/or artificial aging.

## 4. Materials and Methods

### 4.1. Cell Culture

*Rhodococcus rhodochrous* (ATCC-29672, also termed *Rhodococcus ruber*) and *Alcanivorax borkumensis* (ATCC-700651) were cultured in media as recommended (i.e., tryptone soya broth (Oxoid, Hampshire, UK) and marine broth (BD Difco, Berkshire, UK), respectively). *R. rhodochrous* was grown at 27 °C, shaking at 120 rpm and *A. borkumensis* was grown at 30 °C, shaking at 120 rpm. Bacteria were handled in a class II biosafety cabinet throughout. Prior to incubation with plastic, each bacterium was grown overnight in minimal media (Tables S4 and S5; prepared using chemicals largely purchased from Fisher Scientific (Leicestershire, UK)) supplemented with 1% glucose or pyruvate for *R. rhodochrous* and *A. borkumensis*, respectively. The bacteria were pelleted by centrifugation at 4000 rpm for 5 min and washed in fresh minimal media to remove any residual carbon.

### 4.2. Plastic Preparation

Full details of all the plastic used here can be found in Table S3. The commercially prepared plastic film was cut to the required size, washed with 70% ethanol and allowed to dry overnight in a microbiological safety cabinet prior to use in each assay.

### 4.3. Preparation of Bacteria on Minimal Media Plates

Plates containing minimal media with no carbon source were prepared. Bacterial suspensions in the exponential phase were prepared and diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.1 in minimal media prior to plating. Four sterile plastic pieces of 1 cm<sup>2</sup> were placed on top of the cultures. The plates were allowed to dry before sealing with parafilm, inversion and incubation at 30 °C.

### 4.4. Scanning Electron Microscopy

Plastic samples in liquid medium were rinsed briefly in deionised water, air-dried and placed on double-sided self-adhesive carbon discs on aluminium stubs. Plastic samples grown with bacteria on agar plates were carefully lifted from the plate and placed directly on double-sided self-adhesive carbon discs on aluminium stubs. The mounted samples were coated with approximately 7.5 nm of gold in a Cressington 108 auto sputter coater, the thickness being controlled with a Cressington MTM 10 thickness monitor (Watford, UK). Coated samples were examined and imaged using secondary electrons in either a Zeiss Sigma Gemini scanning electron microscope, with a field emission electron gun at an acceleration voltage of 10 kV (Jena, Germany), or in a JEOL JSM 6480LV scanning electron microscope at an acceleration voltage of 30 kV (MA, USA).

### 4.5. Preparation of Bacteria for the Plastic CO<sub>2</sub> Bioassay

A starter culture was prepared in liquid media and grown overnight at 30 °C. Prior to measuring cell density, sterile glass beads were added to the overnight culture and mixed to disperse any clumps of bacterial cells. The solution was diluted in minimal media to achieve an OD<sub>600</sub> of 0.2, as per standard methodology. A 0.4 mL volume was applied to each autoclaved container, 0.5 mg of sterile plastic added where required, the tubes were sealed and the cultures were incubated at 27 °C, shaking at



120 rpm throughout. Five replicates of each test condition were prepared in 12 mL gas-tight vials, with butyl rubber septa seals (Exetainers, Labco, Ceredigion, UK), for the measurement of CO<sub>2</sub> production. The tubes remained sealed during the length of the experiment to prevent contamination.

#### 4.6. CO<sub>2</sub> Analysis

At various time points throughout the assays, the samples were removed from the incubator for GC analysis and returned to the incubator as soon as possible. A 50 µL sample was withdrawn from the headspace using a gas-tight syringe in an autosampler (Multipurpose Sampler MPS2, Gerstel GmbH, Mülheim an der Ruhr, Germany) and injected into a gas chromatograph fitted with a flame ionising detector (GC-FID) and hot nickel catalyst (Agilent Technologies UK Limited, Cheshire, UK) to reduce the CO<sub>2</sub> to CH<sub>4</sub>. Conditions were as follows: column at 30 °C, detector at 375 °C and catalyst at 385 °C. CO<sub>2</sub> was separated using a stainless-steel column (length 6' × 1/2ø) packed with Poropak (Q 80/100) and with a hydrogen/air mix (7%/93% zero grade, BOC, London, UK) as the carrier gas (430 mL min<sup>-1</sup>). Headspace concentrations of CO<sub>2</sub> as CH<sub>4</sub> were calculated from peak areas using an electronic integrator. The concentration of CO<sub>2</sub> was calibrated against a known standard (3700 ppm) (BOC, London, UK) and air.

#### 4.7. <sup>13</sup>C Glucose Analysis

Samples (as above supplemented with 1% <sup>13</sup>C glucose (Cambridge Isotope Laboratories, MA, USA)) were incubated in 3.5 mL gas-tight vials (Exetainers, Labco, Ceredigion, UK). Gas samples (30 µL) were taken from the headspace of the <sup>13</sup>C glucose and control incubation vials and injected into 3.5 mL exetainers. These were preinjected with CO<sub>2</sub> to act as a carrier for the sample, giving a final concentration of 1100 ppm. A 500 µL gas sample was withdrawn from the headspace using a gas-tight syringe in an autosampler (Multipurpose Sampler MPS2, Gerstel GmbH, Mülheim an der Ruhr, Germany) and injected into a flash elemental analyser (1112 Series, Thermo, Bremen, Germany), interfaced with a continuous flow isotope ratio mass spectrometer (Sercon 20/22, Crewe, UK). CO<sub>2</sub> was separated from other gases on a GC Poropak column (PoraPLOT Q, Agilent Technologies UK Limited, Cheshire, UK). Carbon isotope calibration utilised the international standard for carbon, Ref. 8542, sucrose, -10.47‰ δ<sup>13</sup>C versus Vienna-PeeDee Belemnite (VPDB), National Institutes of Standards and Technology.

#### 4.8. Data Fitting

There was a significant difference between the concentration of CO<sub>2</sub> released from the media over the course of the experiment ( $p < 0.01$ ). Therefore, averages of CO<sub>2</sub> produced at each time point for the media alone or media containing the relevant plastic were subtracted as a baseline. ANOVA and post hoc Tukey tests were performed using the statistical package R (version 3.5.1). Statistics were performed on all data points and the final time point.

The authors considered the application of the Monod equation to quantify respiration rates [37]. However, it was not possible, in this instance, to accurately define the concentration of carbon substrate due to the distribution of macromolecular weight.

#### 4.9. Gel Permeation Chromatography

GPC was performed by Smithers Rapra and Smithers Pira Ltd. (Shropshire, UK).

#### 4.10. ATP Assay

Three replicates of each test condition were prepared in 3 mL vials. The reactions were incubated at 27 °C, shaking at 120 rpm. At each time point, 0.2 mL of extractant B/S was added to the sample and mixed well. The ATP assay was carried out using the BioThema ATP Biomass kit (BioThema, Handen,

Sweden), following the manufacturer's instructions, using a multiwell plate reader (BMG LABTECH, Ortenberg, Germany).

**Supplementary Materials:** Supplementary materials can be found at <http://www.mdpi.com/1422-0067/21/4/1176/s1>.

**Author Contributions:** Conceptualisation, R.-S.R. and I.A.S.; methodology, R.-S.R., K.H.R. and I.A.S.; software, R.-S.R., K.H.R. and I.A.S.; validation, R.-S.R., K.H.R., C.A.H. and I.A.S.; formal analysis, R.-S.R., K.H.R., E.J.L., C.A.H. and I.A.S.; investigation, R.-S.R., K.H.R., E.J.L. and I.A.S.; resources, R.-S.R. and I.A.S.; data curation, R.-S.R., K.H.R., E.J.L., C.A.H. and I.A.S.; writing—original draft preparation, R.-S.R., C.A.H. and M.R.; writing—review and editing, R.-S.R., K.H.R. and M.R.; visualisation, R.-S.R., M.R. and I.A.S.; supervision, R.-S.R. and C.A.H.; project administration, R.-S.R.; funding acquisition, R.-S.R. and I.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** R.-S.R., E.J.L., C.A.H. M.R. and I.A.S. were supported by QMUL. K.H.R. was partially funded by Symphony Environmental Ltd.

**Acknowledgments:** We acknowledge the skill and expertise of R. Angold for the SEM. We thank M. Trimmer for expertise regarding GC. We acknowledge B. Huseklepp and Z. Malik for their contribution to Figure S1 and C. Economou for technical support. R.R. is grateful to E.R.G. Main and S. Wilson for stimulating discussions and advice.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript or in the decision to publish the results.

## Abbreviations

GC	Gas chromatography
LDPE	Low-density polyethylene
SEM	Scanning electron microscopy

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