

# *A mechanistic understanding of polyethylene biodegradation by the marine bacterium Alcanivorax*

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## Research Paper

# A mechanistic understanding of polyethylene biodegradation by the marine bacterium *Alcanivorax*

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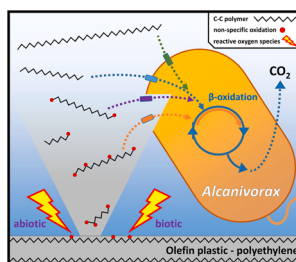
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## HIGHLIGHTS

- *Alcanivorax* isolated from plastic marine debris degrades low density polyethylene (LDPE).
- Pristine and weathered PE can be assimilated by the marine biodegrader.
- Proteomics revealed a high specialisation in uptaking and metabolising PE degradation products.
- Polymer oxidation and scission may occur via reactive oxygen species generated by the bacterium.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Polyethylene (PE) is one of the most recalcitrant carbon-based synthetic materials produced and, currently, the most ubiquitous plastic pollutant found in nature. Over time, combined abiotic and biotic processes are thought to eventually breakdown PE. Despite limited evidence of biological PE degradation and speculation that hydrocarbon-degrading bacteria found within the plastisphere is an indication of biodegradation, there is no clear mechanistic understanding of the process. Here, using high-throughput proteomics, we investigated the molecular processes that take place in the hydrocarbon-degrading marine bacterium *Alcanivorax* sp. 24 when grown in the presence of low density PE (LDPE). As well as efficiently utilising and assimilating the leachate of weathered LDPE, the bacterium was able to reduce the molecular weight distribution ( $M_w$  from 122 to 83 kg/mol) and overall mass of pristine LDPE films (0.9 % after 34 days of incubation). Most interestingly, *Alcanivorax* acquired the isotopic signature of the pristine plastic and induced an extensive array of metabolic pathways for aliphatic compound degradation. Presumably, the primary biodegradation of LDPE by *Alcanivorax* sp. 24 is possible via the production of extracellular reactive oxygen species as observed both by the material's surface oxidation and the measurement of superoxide in the culture with LDPE. Our findings confirm that hydrocarbon-biodegrading bacteria within the plastisphere may in fact have a role in degrading PE.

## 1. Introduction

Societal dependence on plastics has led to the global production of over 350 million tonnes of these materials *per annum*, with polyethylene (PE) being the predominant polymer produced, *i.e.* 29.8 % (PlasticsEurope, 2021). The properties of PE, such as high strength-to-weight ratio, stiffness, toughness, ductility, corrosion resistance and durability, has made it the preferred material in a wide variety of applications (Andrady and Neal, 2009), but also an environmental problem due to its recalcitrance (Barnes et al., 2009). Despite its resistance, environmental weathering is known to fragment PE into micro and nano-plastics – a phenomenon considered to expand the ecological risk of these materials (Baudrimont et al., 2020; Kögel et al., 2020; Roweczyk et al., 2020). It has been estimated that millions of tonnes of plastics annually enter the oceans accumulating as plastic marine debris (Jambeck et al., 2015; Lebreton et al., 2017). PE has high transportability in water due to its low density and, hence, this polymer has become one of the most abundant and ubiquitous synthetic materials in the environment, especially on shorelines and surface seawaters worldwide (Bond et al., 2018; Wright et al., 2020).

A considerable amount of research has been carried out on PE degradation, *e.g.* by insect larvae (Pivato et al., 2022; Yang et al., 2014; Wang et al., 2022) or in marine ecosystems (Gilan et al., 2004; Oberbeckmann and Labrenz, 2020; Sivan, 2011), all with mixed results. While selected studies have reported some level of PE biodegradation by marine microbial isolates or consortia (Harshvardhan and Jha, 2013; Paço et al., 2017; Syranidou et al., 2019), the underlying molecular mechanisms of such processes have remained controversial (Wright et al., 2020). Researchers do agree however, that PE biodegradation is a relatively slow process but can be accelerated by abiotic weathering of the material, *e.g.* through ultraviolet (UV) radiation and temperature (Albertsson and Karlsson, 1990; Andrady, 2011; Oberbeckmann and Labrenz, 2020; Restrepo-Flórez et al., 2014). In fact, photooxidation and thermooxidation of PE have been shown to generate long-chain mono and dicarboxylic acids, ketoacids, fatty acids, aldehydes and alkanes (Gewert et al., 2018; Singh and Sharma, 2008). Such compounds are more accessible to bacteria than PE and, hence, can be more rapidly utilised (Albertsson et al., 1995; Romera-Castillo et al., 2018). In this case, though, biodegradation *via* assimilating low molecular weight compounds derived from the abiotic degradation of plastic constitutes a secondary process (Gewert et al., 2015; Zheng et al., 2005), while primary direct biological degradation of pristine PE plastics (*i.e.* polymer chain scission) remains unproven.

Members of the obligate hydrocarbonoclastic bacteria, such as *Alcanivorax* (Yakimov et al., 2019), are frequently suggested as biodegraders of marine plastic debris (Dussud et al., 2018; Carter et al., 2016; Zettler et al., 2013). Their ability to metabolise hydrocarbons (*e.g.* short- and long-chain alkanes *via* the  $\beta$ -oxidation metabolic pathway

(Schneiker et al., 2006)) together with their recurring presence within plastispheres has led to the hypothesis that they are capable of degrading plastics such as PE in natural environments.

We set out to test this hypothesis by using an *Alcanivorax* strain isolated from plastic marine debris (Zadjelovic et al., 2020a), and that exhibited rapid growth in the presence of weathered low density PE (LDPE) as the sole carbon supply. Using a high-throughput proteomic analysis, we present a comprehensive mechanistic understanding of how this marine microbe assimilates weathered LDPE. Most interestingly, we observed how the bacterium acquired the isotopic signature of the plastic, physicochemically modified the material and showed an induction of metabolic pathways involved in aliphatic-compound degradation when grown in the presence of both weathered and pristine LDPE. This indicated the ability of the bacterium for primary biodegradation of pristine plastic and infer that it is accomplished by non-specific polymer oxidation *via* the production of extracellular reactive oxygen species.

## 2. Methods

## 2.1. Media, substrates and bacterial growth conditions

Degradation assays were carried out using basal Bushnell-Haas mineral medium (Bushnell and Haas, 1941) supplemented with 30 g L<sup>-1</sup> NaCl and 1 mL L<sup>-1</sup> trace metal solution used in artificial seawater (Wyman et al., 1985). It is important to note that this media contains no carbon-based buffering agent which has proven problematic with some obligate hydrocarbonoclastic bacteria as these can be used for cell growth. From hereafter this medium is referred to as “mineral media”. Substrates used as the carbon source for microbial growth were: pristine and weathered LDPE pellets (~2–3 mm, Sigma-Aldrich®) and films (IAEA-CH-7, International Atomic Energy Agency, IAEA; used because of its isotopic homogeneity and known  $\delta^{13}\text{C}$  value allowing carbon stable isotope ratio analysis), the three alkanes hexadecane (C16), pentacosane (C25) and pentacontane (C50), as well as sodium succinate and the 16-carbon length dicarboxylic acid thapsic acid (all obtained from Sigma-Aldrich®; Supplementary Table 1). Mineral medium was adjusted to pH 7.0 and autoclaved for sterilisation (at 121 °C for 15 min). LDPE films and pellets were autoclaved separately at 100 °C for 1 h to avoid melting of the plastic (Harshvardhan and Jha, 2013). Aqueous solution of succinate and hexadecane were both filter-sterilised prior to adding to the mineral medium (succinate using 0.2  $\mu\text{m}$  Minisart® NML hydrophilic filters, Sartorius; and hexadecane using 0.2  $\mu\text{m}$  Millex®-FG PTFE hydrophobic filters, Merck). Other carbon sources (*i.e.* alkanes C25, C50) were aseptically transferred to the cultures before inoculating. Substrates were added at a final concentration of 3 g L<sup>-1</sup> (*i.e.* for thapsic acid and alkanes C25 and C50), 5 g L<sup>-1</sup> (*i.e.* for succinate) and 10 g L<sup>-1</sup> (*i.e.* for hexadecane, and pristine and weathered LDPE).

*Alcanivorax* sp. 24 (GenBank SAMN11041869) was previously isolated from marine plastic debris and described as capable of degrading a wide array of polyesters (Zadjelovic et al., 2020a, 2020b). The bacterium was routinely cultured on marine agar (BD Difco™). Mid-stationary-grown cells on solid media (30 °C for 48 h) were harvested by collecting loopfuls of cells and washed in mineral media (containing no organic carbon) prior to inoculating the experimental treatments, applying a 1:50 dilution (OD<sub>600</sub> of ~0.01). Cultures were performed in 50 mL glass Erlenmeyer flasks containing 30 mL of media and corresponding substrate. Flasks were incubated in the dark at 30 °C with orbital shaking at 200 rpm. Growth was monitored by optical density measurements at 600 nm (OD<sub>600</sub>) using a cuvette spectrophotometer (Jenway 7305).

## 2.2. Physicochemical characterisation of thermo-weathered and *Alcanivorax*-incubated LDPE

Thermooxidative weathering was performed as previously described (Bonhomme et al., 2003). Briefly, LDPE pellets and films were kept in glass beakers in an oven set at 80 °C for 6 months at standard atmospheric pressure. Transmission Fourier-transform infrared (FTIR) spectroscopy (PerkinElmer® Spectrum™ GX) was used to monitor the oxidation state of pristine and weathered LDPE. Spectra were taken by averaging 32 scans in the range of 600–4000 wavenumbers cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup> and intervals of 1.0 cm<sup>-1</sup>. The carbonyl index was used as a measure of PE oxidation. For this, the absorbance intensity of the carbonyl peak at 1712 cm<sup>-1</sup> was normalised to the intensity of the control region 2030 cm<sup>-1</sup>, which is not affected by oxidation (Satoto et al., 1997).

Additionally, Attenuated Total Reflection-FTIR (ATR-FTIR, Nicolet™ iN10 MX Infrared Imaging Microscope) was used to analyse the surface oxidation of PE films incubated in the presence of *Alcanivorax*. Samples were placed on gold coated microscopy slides (Thermo Fisher Scientific) and spectra were collected at low pressure mode using a Germanium tip ATR. The detector was cooled using liquid nitrogen. The collection point-aperture was of 150 μm width and 150 μm height. Spectra were taken averaging 16 scans within the range 675–4000 wavenumber cm<sup>-1</sup>. Collection times were 3 s using default spectral resolution. The background was collected prior to every sample.

Gel permeation chromatography/size exclusion chromatography (GPC/SEC) was applied in order to evidence variations in the molecular weight (M<sub>w</sub>) distribution of the PE. Pristine and weathered LDPE film samples were prepared in nominal concentrations of 5 mg mL<sup>-1</sup> in 1,2,4-trichlorobenzene (TCB). These were placed in a SP260VS high-temperature dissolution device at 140 °C for 18 h. Using the same instrument, samples were filtered through 10 μm stainless steel frits before injection. Samples were run on an Agilent PL220 instrument equipped with differential refractive index (DRI), viscometry (VS) and dual-angle light scatter (LS 90 + 15) detectors. The system was equipped with 2 × PLgel Olexis columns (300 × 7.5 mm) and a 10 μm guard column. The mobile phase was trichlorobenzene (TCB) with 250 ppm butylated hydroxytoluene (BHT) additive. Samples were run at 1 mL min<sup>-1</sup> at 160 °C. Polystyrene standards (Agilent EasiVials) were used to create a third-order calibration between 3,187,000 and 580 g/mol. Experimental M<sub>w</sub>, molar mass (M<sub>n</sub>) and dispersity (D) values of pristine and weathered PE were determined by conventional calibration using Agilent GPC/SEC software.

## 2.3. Total organic carbon analysis (TOC)

Organic carbon released by pristine and weathered LDPE was measured using a TOC-L nutrient analyser equipped with an ASI-L autosampler (Shimadzu). Samples measured were obtained from supernatants of 1 g of pristine and weathered LDPE pellets washed in 100 mL of mineral media. Carbon content of media containing 0.5 % w/v sodium succinate was also measured. Mineral media and Milli-Q water

were measured as controls.

## 2.4. Determining polymer mass variation

In order to evaluate the effect of *Alcanivorax* sp. 24 on the total mass variation of the material, sections of 3 cm<sup>2</sup> of pristine and weathered LDPE films were sanitised with 70 % ethanol, dried overnight in a 60 °C oven and masses were recorded using a four-digit analytical scale (Sartorius CP124S ≤ ± 0.1 mg). Incubations were setup in 50 mL glass flasks with a working volume of 30 mL of sterile mineral media to which plastic sections were aseptically transferred to. *Alcanivorax* cells were washed in mineral media and added reaching a t<sub>0</sub> OD<sub>600 nm</sub> of ~0.1. Flasks where no cells or equivalent autoclaved dead cells, were included to control for non-biological mass fluctuations. Five replicates of each condition were incubated for 34 days (30 °C with shaking at 200 rpm). Evaporation of the cultures was corrected along the experiment by the addition of sterile Milli-Q water. After the 34-day incubation, plastics were retrieved from the glass flask and rinse using sterile Milli-Q water. Plastics were then immersed overnight in hydrogen peroxide 30 % v/v (H<sub>2</sub>O<sub>2</sub>; Sigma-Aldrich®) at 60 °C in order to remove all organic matter (biofilms) attached to the plastic films (Erni-Cassola et al., 2017). The remaining H<sub>2</sub>O<sub>2</sub> was removed by rinsing LDPE films with sterile Milli-Q water. Samples were dried overnight at 60 °C and masses were recorded using the same analytical scale.

## 2.5. Stable isotope analysis

Bulk <sup>13</sup>C/<sup>12</sup>C isotopic content in samples was determined as previously performed (Eley et al., 2018). Briefly, the mean δ<sup>13</sup>C values were measured using a Delta XP ThermoFisher isotope-ratio mass spectrometer interfaced with a Costech elemental analyser. The analysed samples were both cell pellets and IAEA-CH-7 PE films (δ<sup>13</sup>C value of -32.15 ± 0.05‰, International Atomic Energy Agency, Austria).

## 2.6. Sample preparation for proteomics

Cells from 20 mL cultures containing the substrates succinate, alkanes (hexadecane, pentacosane or pentacontane), thapsic acid and pristine or weathered LDPE films (isotope reference material IAEA-CH-7), were harvested after 7 days of incubation by centrifugation (3220g, 15 min at 4 °C). Cell pellets were frozen on dry ice and stored at -20 °C until further analysis. Cells from PE biofilms were recovered by sonication (i.e. sonication bath, 3 cycles of 10 min) using mineral media for resuspension and pelleted by centrifugation. All cell pellets were resuspended in 300 μL of 1 × LDS loading buffer (Invitrogen™) supplemented with 1 % β-mercaptoethanol, followed by three cycles of thorough vortexing and 5 min incubations at 95 °C. 30 μL of each sample was run for a short migration on a NuPAGE™ 10 % Bis-Tris precast polyacrylamide gel (1.0 mm, 10-well, Invitrogen™) as previously done (Zadjelovic et al., 2020b). Gel bands containing the entire proteome were cut (approximately 1 × 0.5 cm) for further in-gel protein digestion (Chhun et al., 2021). Trypsin digestion and peptide recovery was carried out as previously described (Christie-Oleza and Armengaud, 2010; Shevchenko et al., 2007).

## 2.7. Shotgun proteomic analysis

NanoLC-ESI-MS/MS analysis was performed using an UltiMate 3000 RSLCnano system (Dionex-LC Packings) coupled to an Orbitrap fusion mass spectrometer (Thermo Scientific). A 120 min LC separation on a 25 cm column and mass spectral acquisition was performed using the settings and parameters previously described (Christie-Oleza et al., 2015). RAW mass spectral files were processed using the default settings and the function of matching between runs in MaxQuant version 1.5.5.1 (Cox and Mann, 2008). Peptides were identified using the coding domain sequence database of *Alcanivorax* sp. 24 (Zadjelovic et al.,



2020a) and proteins were quantified by label-free quantification (LFQ). Protein group LFQ intensities generated by MaxQuant were further analysed using Perseus version 1.5.6.0 (Tyanova et al., 2016) for further signal normalisation (*i.e.* protein signals were normalised to protein size and then to total sample signal) and comparative proteomic analysis. Polypeptides were considered valid when detected in at least all three replicates of one condition. Cut-off parameters for comparative analyses used a false discovery rate (FDR) of 0.01 and  $S_0$  (minimal fold change) set to 2.

The RAW mass spectrometry data and additional proteomic information have been deposited to the ProteomeXChange Consortium via PRIDE; available with the identifier PXD028724.

## 2.8. Reactive oxygen species (ROS) analysis

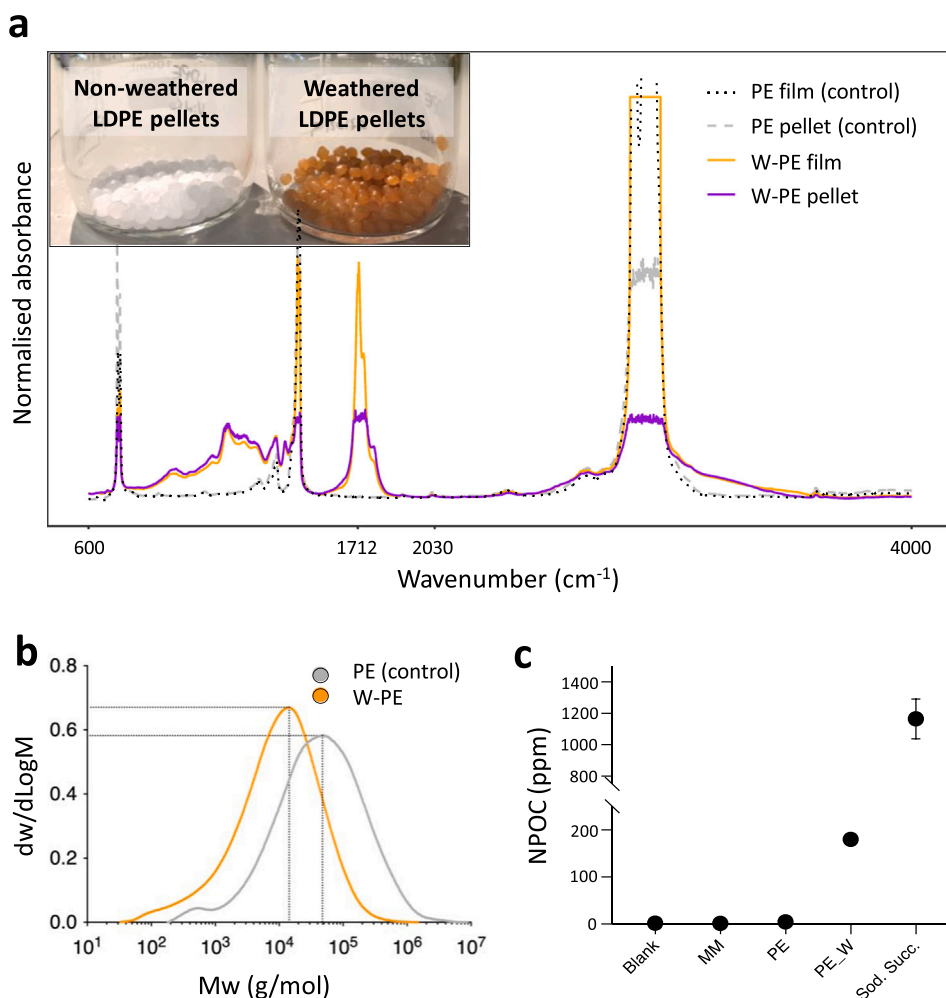
Lucigenin (4 mM working solution prepared in esterised Milli-Q water; Sigma-Aldrich®) was used as the ROS reporter. Washed cells were resuspended in mineral media to an  $OD_{600}$  of 0.01. Aliquots of 10 mL of the cell suspension were dispensed in 50 mL conical Falcon tubes containing 100  $\mu$ L of the Lucigenin working solution and the different carbon sources: four pristine or weathered LDPE pellets, 0.3 % (v/v) of filter-sterilised C16, or 0.1 % (w/v) of sodium succinate. An additional condition was included where no source of carbon was added. All conditions had their corresponding negative controls (*i.e.* where the cell suspension was replaced by the same volume of sterile mineral medium) and were all performed in triplicates. Cultures were incubated for 14 days at 30 °C with orbital shaking at 180 rpm. At each timepoint, 1 mL of culture was harvested for  $OD_{600}$  measurement to monitor cell growth.

The 1 mL was further centrifuged at 13,000 rpm for 1 min to pellet the cells, and supernatants were preserved at -20 °C for subsequent fluorescence measurement (*i.e.* to monitor superoxide production in the culture). Fluorescence was measured with a fluorescence spectrometer (Varian Cary Eclipse Fluorescence Spectrometer, Agilent) at 390/440 nm  $\lambda_{ex} / \lambda_{em}$ . While non-oxidised Lucigenin has a maximum  $\lambda_{ex} / \lambda_{em}$  at 370/505 nm and is heavily quenched by chloride, oxidised Lucigenin produces a well-differentiated and non-quenched maximum fluorescence at 390/440 nm  $\lambda_{ex} / \lambda_{em}$ . Fluorescence measurements were performed using 400  $\mu$ L of supernatant from each sample in a white 96-well plate. A standard curve was generated doing a dilution series of  $KO_2$  (Sigma-Aldrich) in Lucigenin-containing mineral medium. Superoxide production in the cultures was normalised by growth (*i.e.*  $OD_{600}$ ).

## 3. Results

### 3.1. Abiotic weathering of PE

After six months at 80 °C in the dark, LDPE materials displayed visual thermooxidative weathering as the initial white-opaque pellets turned yellow/orange (Fig. 1a). The cumulative oxidative effect of temperature on the plastic was confirmed *via* Fourier transform infrared (FTIR) spectroscopy (Bonhomme et al., 2003; Hakkarainen and Albertsson, 2004). Weathered LDPE films and pellets revealed a strong increase in carbonyl groups (*i.e.* -C=O; absorption peak in the 1712  $cm^{-1}$  region; Fig. 1a), whereas control plastics kept at room temperature remained unaltered. Thermooxidation also led to a decrease in the molecular weight of the polymers within the LDPE (Fig. 1b). The number average-



**Fig. 1.** Abiotic weathering and carbon leaching from pristine and weathered PE. (a) FTIR spectra from pristine and weathered (thermooxidised at 80 °C for 6 months; W-PE) LDPE film and pellet. The characteristic carbonyl groups of PE weathering absorb at 1712  $cm^{-1}$ . The inserted image shows pristine and weathered LDPE pellets. (b) Molecular weight distribution within pristine and weathered LDPE pellets measured by GPC/SEC. (c) Dissolved organic carbon analysis showing the Non-Purgeable Organic Carbon (NPOC) present in Milli-Q water (Blank), mineral media (Media), and mineral media to which pristine LDPE and weathered (W-PE) pellets were added (*i.e.* 1 g in 100 mL of medium). Mineral media containing the labile carbon source routinely used to grow *Alcanivorax* (*i.e.* sodium succinate at 0.5 % w/v) was also analysed. Points and error bars represent the average values and standard deviation from three independent replicates.

( $M_n$ ), the weight average- ( $M_w$ ), and the size average molecular weight ( $M_z$ ) were reduced by 76.4 %, 80.4 %, and 84.4 %, respectively (Table 1), indicating a strong depolymerisation by weathering. Polymeric oxidation is thought to cause chain scissions. Polymer weathering is a multifactorial process and, as previously shown (Bond et al., 2018; Gewert et al., 2018), different factors such as UV- and thermooxidation render variable arrays of substances. Due to practical reasons, here we used thermal weathering of PE.

Most interestingly, the weathering of PE generated a considerable amount of leached dissolved organic carbon from the material which was not observed from the pristine plastic (Fig. 1c). We observed on average 140 times more organic carbon coming from weathered LDPE pellets (176.4 ppm) than from an equal amount of pristine material (1.3 ppm), i.e. when adding 1 g of LDPE pellets to 100 mL of mineral medium. This means that  $\sim 2$  % of the weathered LDPE pellets leached from the plastic in the form of dissolved organic carbon becoming available for microbial assimilation. Hence, the weathered PE treatments contained 0.02 % w/v of readily available PE in a dissolved form.

### 3.2. Growth and isotopic signature of *Alcanivorax* when incubated in the presence of pristine and weathered PE

Growth of *Alcanivorax* sp. 24 was monitored in the presence of succinate, pristine and weathered PE (i.e. 1 g of LDPE pellets in 100 mL of mineral medium), as well as in the absence of a source of carbon (Fig. 2a). Surprisingly, the  $\sim 180$  ppm of carbon leached from the weathered PE (data inferred from Fig. 1c) induced an initial higher growth rate than the labile substrate succinate (i.e. day 2; Fig. 2b). Nevertheless, *Alcanivorax* sp. 24 achieved an expected higher cell yield over time as a consequence of the 1200 ppm of carbon available in the succinate condition. Growth with no carbon or with pristine LDPE pellets was negligible (Fig. 2b), the latter expected by the recalcitrance and low accessibility of PE to the bacterium.

*Alcanivorax* sp. 24 grown in the presence of both pristine and weathered PE acquired the isotopic signature of the plastic (Fig. 2c), confirming not only that the strain was able to assimilate the leachate from weathered PE but also to acquire carbon from pristine PE. The isotopic signature ( $\delta^{13}\text{C}$ ) of *Alcanivorax* was obtained from cultures incubated seven days with pristine and weathered IAEA-CH-7 PE films (i.e. a material checked for its isotopic homogeneity with a  $\delta^{13}\text{C}$  value of  $-32.15 \pm 0.05\text{‰}$ ). Cells incubated with succinate and no carbon source were included as controls. Fossil fuel-based materials such as PE are known for being  $^{13}\text{C}$  depleted and, hence, provide  $\delta^{13}\text{C}$  values under  $-30\text{‰}$  whereas succinate, obtained from C4 plants, has a much higher  $^{13}\text{C}$  content with  $\delta^{13}\text{C}$  values ranging around  $-13\text{‰}$  (Berto et al., 2017). The isotopic value of *Alcanivorax* grown with pristine and weathered PE ( $\delta^{13}\text{C}$  of  $-33.63 \pm 0.15\text{‰}$  and  $-31.92 \pm 0.47\text{‰}$ , respectively) was almost identical to that obtained for the standard PE material (i.e.  $-32.15 \pm 0.05\text{‰}$ ) indicating that the cells had acquired the isotopic  $^{13}\text{C}:^{12}\text{C}$  ratio of the plastic (Fig. 2c). As expected, the  $\delta^{13}\text{C}$  signature of *Alcanivorax* grown in the presence of succinate was less negative ( $-16\text{‰}$ ). The starved cells retrieved from cultures with no carbon added seemed to conserve the isotopic ratio from the substrate they had originally been

**Table 1**

Polymer molecular weight distribution of pristine and weathered LDPE films incubated 34 days in the presence and absence of *Alcanivorax* sp. 24, and determined by GPC.

	$M_n$ (g/mol)	$M_w$ (g/mol)	$M_z$ (g/mol)
PE control <sup>a</sup>	9200	122,900	681,200
PE+ <i>Alcanivorax</i>	7400	83,500	376,600
W-PE control <sup>a</sup>	2200	24,100	106,800
W-PE+ <i>Alcanivorax</i>	1000	20,600	76,500

<sup>a</sup> Control films were incubated and processed in exactly the same way as those incubated with the bacterium.

grown on, i.e. the peptone and yeast extract-based medium marine broth with  $\delta^{13}\text{C}$  of around  $-24\text{‰}$  (Abraham et al., 1998). In this context, the isotopic composition of small organisms reflects the  $\delta^{13}\text{C}$  of their nutrition source (Haubert et al., 2005).

### 3.3. *Alcanivorax* is able to decrease the mass and the polymer's molecular weight distribution of pristine LDPE

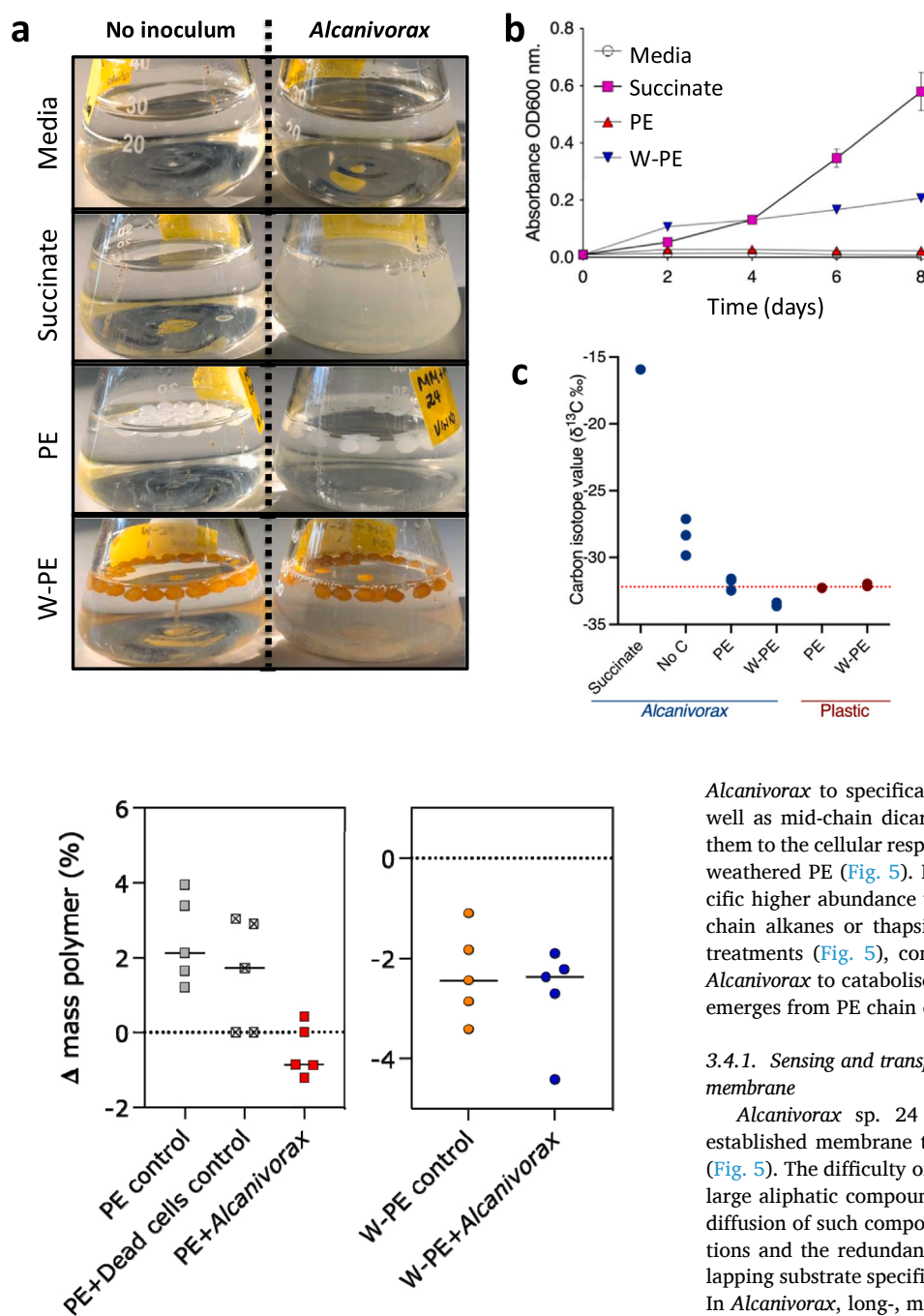
Given the unexpected observation of *Alcanivorax* acquiring the isotopic signature of the pristine PE, we further investigated whether the bacterium could actually physicochemically modify the plastic. Weathered and non-weathered LDPE films were incubated in *Alcanivorax* sp. 24 cultures for 34 days after which mass variation (Fig. 3) and overall polymer molecular weight was determined (Table 1). Interestingly, *Alcanivorax* produced  $\sim 0.9$  % median mass reduction of pristine LDPE films whereas, curiously, controls (i.e. non inoculated and dead cell controls) showed a median mass gain of  $\sim 2.1$  % and  $\sim 1.7$  %, respectively (Fig. 3). Mass gain by submerged plastics is not unexpected, and could be explained by the swelling hydration and/or salt deposition within the material (Andrady, 2017; Echeverria et al., 2020; Marqués-Calvo et al., 2006). A median mass loss of  $\sim 2.4$  % was observed for weathered LDPE films, independently of the presence of *Alcanivorax* (Fig. 3). This mass loss was in line with the value calculated from the dissolved organic matter released from weathered PE (i.e.  $\sim 2$  %), which is presumably in the form of readily-available oligomers for microbial biodegradation.

*Alcanivorax* sp. 24 decreased the polymer weight distribution of pristine LDPE (Table 1). The presence of the bacterium reduced the  $M_w$  of pristine LDPE from 122,900 to 83,500 g/mol and of weathered LDPE from 24,100 to 20,600 g/mol. The respective reduction of  $M_n$ ,  $M_w$  and  $M_z$  was of 20.1 %, 32.0 % and 44.7 % for pristine LDPE and 55.0 %, 14.7 % and 28.4 % for weathered LDPE. This broad depolymerisation is yet another indication that *Alcanivorax* sp. 24 is capable of physicochemically modifying both pristine and weathered LDPE. Therefore, the molecular mechanisms involved in primary PE biodegradation deserved further investigation.

### 3.4. Response of *Alcanivorax* to the presence of PE via shotgun proteomic profiling

A proteomic analysis was performed to determine the molecular response of *Alcanivorax* sp. 24 to the presence of pristine and weathered LDPE films. The analysis included planktonic cells and biofilms attached to the PE, the latter expected to provide a stronger signal due to direct contact with the substrate. This comprehensive proteomic analysis was complemented with that of *Alcanivorax* sp. 24 grown on medium- and long-chain alkanes (i.e. hexadecane, C16; pentacosane, C25; and pentacosane, C50) as well as on short- and medium-chain dicarboxylic acids (i.e. succinate, 4 C; and thapsic acid, 16 C), all substrates that can potentially derive from PE weathering (Gewert et al., 2018). Hakkarainen and Albertsson (Hakkarainen and Albertsson, 2004) identified more than 200 different aliphatic degradation products from abiotic PE oxidation, and Eyheraguibel and co-workers (Eyheraguibel et al., 2017) recently described 1320 highly oxidised oligomers, i.e. alkanes, alkenes, ketones, aldehydes, alcohols, carboxylic and dicarboxylic acids, lactones, keto-acids and esters. Amongst these aliphatic substrates, carboxylic acids are the most stable and, therefore, tend to accumulate during extended periods of weathering, whereas other products such as aldehydes, ketones and alcohols are further oxidised to carboxylic acids (Hakkarainen and Albertsson, 2004).

The proteomic analysis allowed the confident detection of 2671 proteins (Supplementary Table 2), representing 63 % of the proteins encoded by this strain (Zadjelovic et al., 2020a). The overall comparison between conditions revealed a gradient from labile to recalcitrant substrates, i.e. succinate being the most labile substrate followed by alkanes C16 and C25, and thapsic acid (Fig. 4). Amongst the more recalcitrant



**Fig. 2.** Growth of *Alcanivorax* sp. 24 on pristine and weathered PE. Culture images (a) and growth curves (b) of *Alcanivorax* sp. 24 incubated with no source of carbon (Media, negative growth control), succinate (positive growth control), as well as pristine and weathered LDPE (W-PE) pellets. *Alcanivorax* cells grown with succinate and with no carbon source (No C) were also measured. Culture images were taken from one of three culture replicates after eight days of incubation. Non-inoculated flasks of each treatment were included as controls. Points and error bars represent the average values and standard deviation of three independent culture replicates. (c) Isotopic signature of *Alcanivorax* sp. 24 grown in the presence of pristine (PE) and weathered (W-PE) IAEA-CH-7 PE films. The red dotted line indicates the  $\delta^{13}\text{C}$  value from the plastic  $-32.15 \pm 0.05\%$ . The measurements obtained from three independent biological replicates per condition are shown. The thermooxidative weathering did not modify the isotopic composition of the LDPE films.

**Fig. 3.** LDPE film mass variation in the presence and absence of *Alcanivorax* sp. 24 after 34 days. Controls also included flasks where an equivalent amount of dead *Alcanivorax* cells were added. Positive values indicate mass gains whereas negative values indicate mass losses. Horizontal solid black lines indicate median values from five independent replicates.

substrates for *Alcanivorax*, we observed pristine PE and long alkane C50, followed by weathered PE (where the proteomes of planktonic- and biofilm-grown cells grouped very closely). A stronger differentiation between planktonic- and biofilm-grown cells was observed with pristine PE as a carbon source, suggesting that cells directly attached to the PE may have more access to this recalcitrant substrate (Fig. 4). Hence, the accessibility of PE as a source of carbon for heterotrophic bacterial cells seems to be strongly mediated by PE thermooxidation, as well as by the adhesion of *Alcanivorax* to the material's surface.

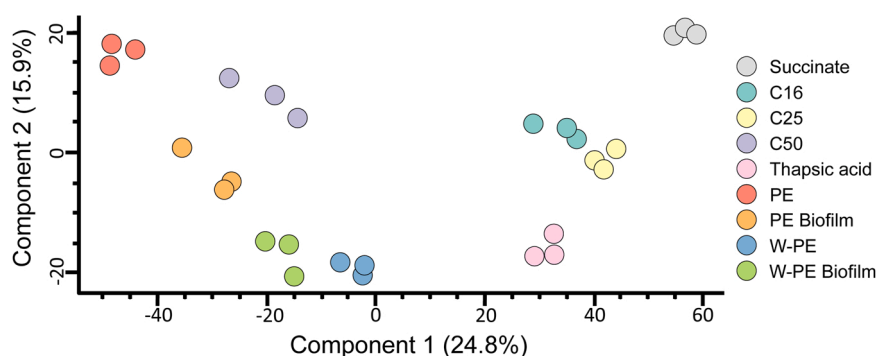
Comparative proteomics between all treatments vs growth on succinate (control) allowed us to identify the cellular processes used by

*Alcanivorax* to specifically catabolise mid- and long-chain alkanes, as well as mid-chain dicarboxylic acids (*i.e.* thapsic acid), and compare them to the cellular response of the strain when grown with pristine and weathered PE (Fig. 5). Interestingly, while some proteins showed specific higher abundance when grown in the presence of mid- and long-chain alkanes or thapsic acid, these were all upregulated in the PE treatments (Fig. 5), confirming the metabolic versatility encoded by *Alcanivorax* to catabolise the complex mix of aliphatic compounds that emerges from PE chain oxidation and scission.

#### 3.4.1. Sensing and transport of aliphatic substrates across the outer membrane

*Alcanivorax* sp. 24 showed a strong increase in several well-established membrane transporters for large hydrophobic compounds (Fig. 5). The difficulty of ascertaining the exact importation pathway of large aliphatic compounds has traditionally been attributed to passive diffusion of such compounds when supplied in high enough concentrations and the redundancy of outer membrane transporters with overlapping substrate specificity (Hong et al., 2006; van Beilen et al., 1992). In *Alcanivorax*, long-, medium- and short-chain alkanes are believed to be transported across the outer membrane by the long-chain fatty acid transporters OmpT-1, OmpT-2 and OmpT-3, respectively (Hearn et al., 2009; van den Berg, 2005; Wang and Shao, 2014). Here, while OmpT-1 (ALC24\_3306) and OmpT-2 (ALC24\_4227) showed a significant increase in the presence of alkanes C50 and C25, respectively ( $30 \times$  and  $13 \times$ ), both outer membrane transporters were also upregulated in all PE conditions. Interestingly, these transporters were not increased in the presence of thapsic acid suggesting a specific induction by the presence of alkanes (Fig. 5 and Supplementary Table 2). On the other hand, OmpT-3 (ALC24\_3619), thought to transport shorter chain aliphatic compounds, was not as tightly regulated and was significantly upregulated in all treatments when compared to succinate (being highest in presence of alkane C25, PE biofilm and both weathered PE fractions, *i.e.*  $>150 \times$ ; and lowest in presence of thapsic acid, *i.e.*  $8 \times$ ). Furthermore, we hypothesise that the co-regulated hypothetical lipoproteins encoded next to each OmpT, *i.e.* ALC24\_3307, ALC24\_3618 and ALC24\_3985 (the latter not found next to OmpT-2 presumably because the genome is not





**Fig. 4.** Principal component analysis of the proteomic data generated from *Alcanivorax* sp. 24 grown under different treatments. Substrates used were the dicarboxylic acids succinate and thapsic acid; the alkanes hexadecane (C16), pentacosane (C25) and pentacontane (C50); and planktonic- and biofilm-grown cells on pristine (PE) and weathered PE films (W-PE). Individual biological replicates are shown ( $n = 3$ ).

closed), may also play a role in the transport of the aliphatic compounds (Fig. 5). These large lipoproteins share high homology with AlkR, previously proposed as a transcriptional activator in *Alcanivorax dieselolei* (Wang and Shao, 2014) despite containing no domain for such a role.

Together with the three OmpT transporters, our proteomic analysis highlighted a strong increase of four outer membrane TonB dependent channels, all with a salient and significant increase in all conditions vs. the succinate treatment (7–2400  $\times$ ; indicated as TBDT in Fig. 5), as well as the porin ALC24\_0252 (10–150  $\times$ ), the latter being more specific to conditions in which alkanes were present (Supplementary Table 2). Together with the outer membrane channels, *Alcanivorax* sp. 24 also produced four TonB-dependent channel proteins with homology to OmpS, which was previously suggested as an outer membrane protein and the first signal transmitter for sensing alkanes outside the cell (Wang and Shao, 2014). While the OmpS-like proteins with highest homology to that described in *Alcanivorax dieselolei* (Wang and Shao, 2014) were generally upregulated in all conditions (i.e. ALC24\_0920, E-value = 0, 3–14  $\times$ ; and ALC24\_4208, E-value =  $10^{-156}$ , 25–140  $\times$ ), the OmpS-like protein ALC24\_3720 was overall downregulated when compared to the succinate treatment (E-value =  $10^{-79}$ , 9–1373  $\times$ ), suggesting a refined system for sensing aliphatic-like substrates in *Alcanivorax* sp. 24. The role these OmpS-like outer membrane proteins play in the uptake and sensing of aliphatic compounds remains unclear and certainly constitutes a key target for future analyses.

### 3.4.2. Transport of aliphatic substrates across the inner membrane

While alkanes are believed to require hydroxylation before being transported through the inner membrane (see the *alkane oxidation* section below), the transport of oxidised aliphatic compounds may be facilitated by active membrane transporters (Rosa et al., 2018). Our proteomic analysis revealed, on the one hand, two inner membrane transporters that were specifically upregulated under the alkane treatments (i.e. the TRAP-like transporters ALC24\_2561–2563 and ALC24\_3963–3964) and, on the other hand, two transporters specifically upregulated in the thapsic acid treatment (i.e. the TRAP transporter ALC24\_2734–2735 and ABC transporter ALC24\_3113–3115). All four transporters were upregulated in PE treatments (Fig. 5), although those specific for thapsic acid were only significantly upregulated in the presence of weathered PE suggesting that thermooxidation of LDPE generates medium-chain-length dicarboxylic acids which are not as abundantly produced during the possible biodegradation of pristine LDPE. This observation is strengthened by enzymes from the  $\beta$ -oxidation pathway that were specifically upregulated in presence of thapsic acid and weathered PE treatments only (Fig. 5 and further discussed below).

### 3.4.3. Alkane oxidation

Hydroxylation is the first step for alkane assimilation. Short- and medium-chain alkanes are believed to be terminally hydroxylated by the

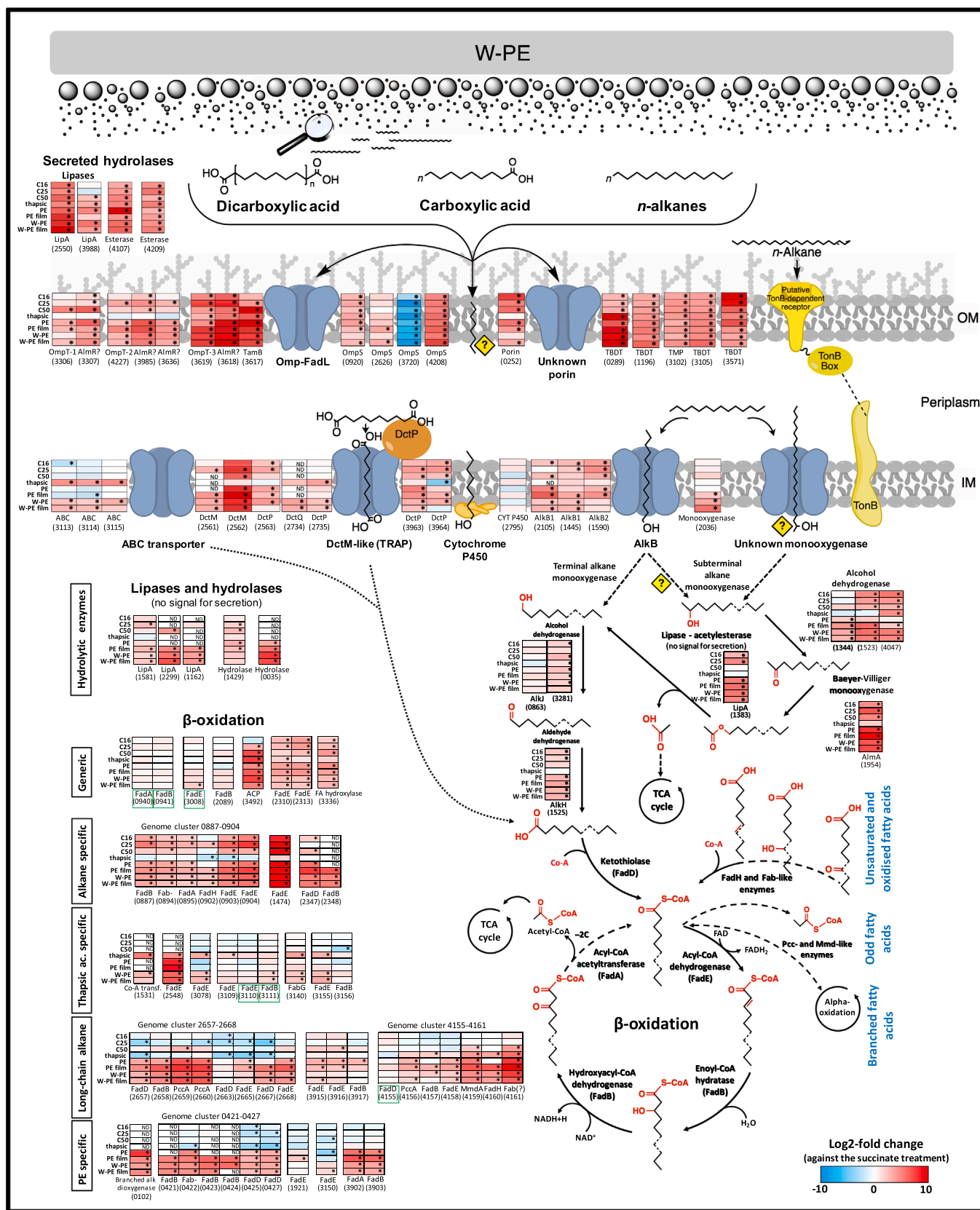
alkane monooxygenase AlkB (Rojo, 2009). *Alcanivorax* sp. 24 encodes three AlkB enzymes (ALC24\_1445, ALC24\_1590 and ALC24\_2105), the expression of which were significantly increased in most of the alkane as well as in the PE treatments, even in the presence of pristine PE (Fig. 5 and Supplementary Table 2). The assimilation of long-chain alkanes (e.g. C50) is currently unclear although it has been hypothesised that this may be accomplished after substrate subterminal hydroxylation. Subterminally hydroxylated alkanes are then presumably converted into an ester by the Baeyer-Villiger monooxygenase AlmA (ALC24\_1954) (Minerdi et al., 2012) and further hydrolysed by the esterase ALC24\_1383 as proposed in Fig. 5. Interestingly, AlmA showed the strongest increases in all PE treatments (i.e. over 100–700  $\times$  when compared to the succinate treatment), possibly as a consequence of the generation of long-chain alkanes from PE. As expected, an increased abundance of AlmA and AlkB enzymes was not observed in the thapsic acid treatment suggesting these pathways are tightly regulated by the presence of alkanes in *Alcanivorax* sp. 24.

### 3.4.4. Model proposed for fatty acid degradation

Hydroxylated alkanes are further oxidised into fatty acids and funnelled into the  $\beta$ -oxidation pathway for degradation (Fig. 5). While the  $\beta$ -oxidation pathway is straightforward when dealing with canonical linear fatty acids, specialised fatty acid-degrading microbes tend to encode numerous enzymes for similar steps of the pathway (Cole et al., 1998; Kang et al., 2010). Such enzymatic redundancy may come as a consequence of the high complexity of aliphatic compounds that can be encountered (e.g. long-chain, branched-chain, unsaturated or subterminally-oxidised fatty acids) and, here, the pool of substrates derived from LDPE degradation may well be a good example. Fig. 5 shows our attempt to assign the large pool of fatty acid degrading enzymes to certain groups of substrates: medium and large alkanes funnelled in as fatty acids, as well as medium-size dicarboxylic acids. Interestingly, these enzymes were all upregulated in the PE treatments as well as an extra set of enzymes which were only upregulated in the presence of the plastic (Fig. 5) and which we believe are involved in degrading more complex aliphatic compounds, e.g. unsaturated and subterminally oxidised as well as branched-, odd- and large- fatty acids. Hence, products derived from PE degradation are ultimately converted into acetate and funnelled into the TCA cycle for carbon assimilation and energy generation.

### 3.4.5. Primary PE chain scission

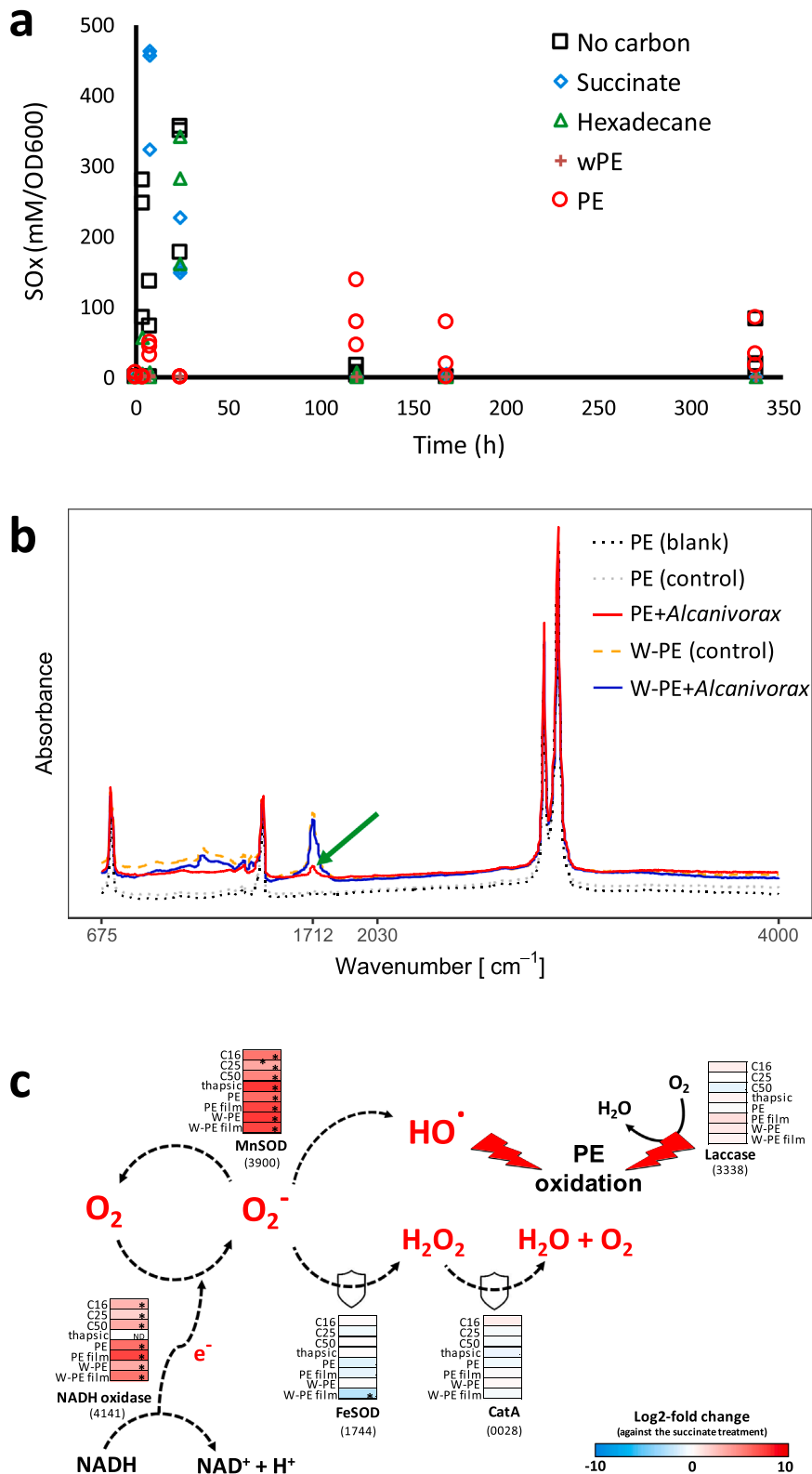
Unlike short- and medium-chain length alkanes, the biological incorporation of long- and extra-long-chain alkanes, such as those in PE, seems unrealistic and polymeric chain scissions are required for transportation across biological membranes. While the C-C bonds that form the PE backbone chains are highly stable and non-reactive, PE oxidation will generate weaker sites for enzymatic chain scission. The presence of



**Fig. 5.** Proteomics-informed model of the metabolic pathways used by *Alcanivorax* sp. 24 to uptake and degrade aliphatic substrates. The log<sub>2</sub> fold change of each condition *i.e.* alkanes: hexadecane (C16), pentacosane (C25) and pentacontane (C50); dicarboxylic acid (thapsic); and planktonic- and biofilm-grown cells on pristine (PE) and weathered PE films (W-PE) compared to the succinate treatment are indicated. Scale shows log<sub>2</sub> fold change from - 10 (blue) to 10 (red). Asterisks indicate where differences were significant (Student's t-test *p* < 0.05). Numbers in brackets indicate the ALC24<sub>1</sub> locus number. Highly abundant proteins (>0.1 %) are highlighted with green squares. OM: outer membrane; IM: inner membrane.

aliphatic compounds induced a varied upregulation of lipase and hydrolase-like enzymes in *Alcanivorax* sp. 24. This increase in abundance was mostly pronounced in the PE treatments (Fig. 5 and Supplementary Table 2). Although most hydrolases (i.e. ALC24\_0035, 1429, 1581, 2299 and 4209) were predicted to be cytoplasmic and, hence, are likely to have a role in the biodegradation of imported oligomers, enzymes ALC24\_1162, 2550 and 3988 are presumably secreted to the

periplasm or outer membrane where they could hydrolyse oxidised PE chains, reducing their molecular weight and facilitating their biological assimilation (Fig. 5).



**Fig. 6.** ROS production in *Alcanivorax* sp. 24 cultures and LDPE film surface oxidation. (a) Superoxide radicals produced over two weeks in *Alcanivorax* sp. 24 cultures were detected using lucigenin and measured by fluorescence. Substrates used were succinate, hexadecane (C16), LDPE (PE) and weathered LDPE (W-PE). *Alcanivorax* sp. 24 grown in the media with no source of carbon and energy (media) was included as a control. Fluorescence was subtracted from the background signal from controls and normalised by culture cell density (i.e. OD<sub>600</sub>). Markers represent the signal of three independent biological replicates. (b) ATR-FTIR spectra of pristine and weathered LDPE film surfaces incubated with and without (controls) *Alcanivorax* sp. 24. The carbonyl peak at 1712 cm<sup>-1</sup> was used as indicative of oxidation. The green arrow highlights the oxidation peak produced by the bacterium on the pristine LDPE film surface. PE blank correspond to the pristine plastic prior incubation and processing. (c) Schematic representation inferred from the proteomic analysis of enzymes involved in catalysing ROS and their potential role in the biological oxidation of PE. The comparative proteomics information is represented as in Fig. 5. Oxygen (O<sub>2</sub>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide (O<sub>2</sub><sup>-</sup>) and hydroxyl radicals (HO<sup>•</sup>), as well as superoxide dismutase (SOD) and catalase enzymes (CatA) are represented.

### 3.5. *Alcanivorax* generates extracellular reactive oxygen species (ROS) that oxidises LDPE film surfaces

While the availability of oligomeric aliphatic compounds was obvious in thermooxidised PE, the open question that remained was how *Alcanivorax* sp. 24 drove primary polymer scission and obtained carbon from pristine PE as indicated by i) the isotopic signature acquired by the bacteria in presence of the plastic, ii) the overall mass reduction and broad depolymerisation of both pristine and weathered LDPE materials, and iii) the bacterial proteomic pattern that suggested a clear induction of catabolic pathways involved in both alkane and dicarboxylic-like substrates degradation. Marine microorganisms are well-known for producing extracellular ROS, particularly superoxide (Hansel and Diaz, 2021; Sutherland et al., 2019, 2020), which we believe is the main pathway used by microbes to initiate PE chain scission and degradation. Hence, we measured the production of extracellular ROS by *Alcanivorax* sp. 24 when grown in the presence of different substrates over 14 days (Fig. 6a). Interestingly, while a peak in superoxide production was observed in most conditions during the initial 24 h of incubation, pristine LDPE induced a lower but much prolonged production of ROS by *Alcanivorax* throughout the two weeks of incubation (Fig. 6a). Incubations with weathered PE showed negligible levels of superoxide production which could be due to the lower production of ROS by the bacterium or by a possible quenching effect of the plastic leachate *i.e.* rapidly reacting with the superoxide and not allowing it to oxidise the lucigenin reporter.

The detection of an oxidation peak in pristine LDPE films incubated with *Alcanivorax* (green arrow on Fig. 6b) is evidence that the ROS produced by the bacterium may in fact influence the polymer's physicochemistry. This oxidation peak occurred at the same wavenumber where thermal oxidation abiotically induces the formation of carbonyl groups. Noteworthy, this modification was detected by ATR-FTIR which collects spectra from the material's surface and not from the polymer bulk as done by standard FTIR.

While extracellular ROS have been almost ubiquitously detected across the tree of life, the mechanisms involved in their production have only scarcely been investigated in bacteria (Hansel and Diaz, 2021). Both the heme peroxidase from the marine *Roseobacter* isolate (*i.e.* the only characterised superoxide-generating enzyme described to date in a marine bacterium (Andeer et al., 2015)) and the NADPH oxidase (NOX) from the fungi *Aspergillus nidulans* (*i.e.* an oxidoreductase able to transfer electrons from NADPH to oxygen generating superoxide (Lara-Ortiz et al., 2003)) were used to search the genome of *Alcanivorax* sp. 24 with no encoded homologue found. The comparative proteomic dataset flagged several oxidoreductases that could ultimately generate the observed ROS, such as the NADH oxidase ALC24\_4141 (up to  $174 \times$  more abundant in the biofilm attached to pristine PE; Fig. 6c), or the laccase ALC24\_3338 and the cytochrome P450 ALC24\_2795, these latter two enzymes not showing a differential detection between conditions (Fig. 6c). The proteomic analysis also revealed a strong increase of the Mn-containing superoxide dismutase (SOD) ALC24\_3900 in all conditions when compared to the succinate treatment ( $13\text{--}213 \times$ ; Fig. 6c), in contrast to the Fe-containing SOD ALC24\_1744 and catalase ALC24\_0028, which showed a similarly high abundance across all conditions. The upregulation of the Mn-SOD may come as a consequence of an increase in superoxide radicals generated indirectly by the intrinsic metabolic activity of *Alcanivorax* sp. 24 when grown on aliphatic compounds or by an intentional generation of such oxidative species to oxidise recalcitrant substrates making them more accessible. A Mn-containing SOD produced by the bacterium *Sphingobacterium* sp. T2 was identified as a lignin-oxidising enzyme through the production of hydroxyl radicals (Rashid et al., 2015), a highly reactive oxidant which could be involved in PE oxidation as modelled in Fig. 6c.

## 4. Discussion

Here we show how the hydrocarbon-degrading marine bacterium *Alcanivorax* sp. 24 can degrade and assimilate carbon from both weathered and pristine LDPE. Although measurable bacterial growth was only visible when incubated in the presence of weathered LDPE –*i.e.* because of the relatively large amounts of readily-available sub-products leached from this material– *Alcanivorax* acquired the isotopic carbon signature of the plastic even when incubated with pristine LDPE. This finding highlights how traditional techniques for measuring bacterial growth on such recalcitrant materials are not well suited for monitoring plastic biodegradation (Wright et al., 2020). The acquisition of the isotopic carbon signature of the plastic by the bacterium is currently the best –if not the only– valid option to monitor plastic biodegradation (Boschker and Middelburg, 2002; Zumstein et al., 2019). Given the unanticipated isotopic signature of *Alcanivorax* with pristine PE, we further explored other evidence of plastic degradation such as material weight loss and reduction in polymeric molecular weight, both measurements indicating an effect of the bacterium on the material's physicochemical properties. The strong decrease in the polymer's molecular weight as a consequence of chain scissions would cause oligomer leaching as well as the embrittlement of the material, nevertheless, material fragmentation and release of nano-fragments was not tested.

Previous studies had shown microbial growth on weathered PE by assimilating oxidised oligomers, such as the soil bacterium *Arthrobacter paraffineus* which assimilated most of the products derived from weathered PE (Albertsson et al., 1998), *Rhodococcus rhodochrous* consuming 95% of these PE-derived substrates (Eyheraguibel et al., 2017), or the stimulation of marine microbial communities in response to PE leachates (Romera-Castillo et al., 2018). Nevertheless, the metabolic pathways involved in PE substrate assimilation were only explored on weathered short-chain PE (Gravouil et al., 2017), but never when exposed to high molecular weight PE materials. Our proteomic analysis provided a mechanistic understanding for the biodegradation of PE highlighting the metabolic complexity involved in such a process, which requires well-adapted microbes capable of processing the large array of mixed aliphatic substrates that derive from PE degradation (Gewert et al., 2018; Walsh et al., 2021). PE does not simply depolymerise into its composing monomers during degradation (Gewert et al., 2015). Rather, the non-specific scission of PE chains –be it biotic or abiotic– provides a large array of different aliphatic substrates, *i.e.* alkanes, alkenes, ketones, aldehydes, alcohols, carboxylates, lactones, keto-acids and esters, all with a range of different sizes as well as additional in-chain oxidations and ramifications (Bond et al., 2018; Gewert et al., 2018; Hakkarainen and Albertsson, 2004). *Alcanivorax* sp. 24, as expected from a member of the hydrocarbonoclastic group (Yakimov et al., 2019), displayed a large fraction of its encoded potential when grown in the presence of both pristine and weathered LDPE (Fig. 5). Interestingly, as well as expressing the substrate-specific catabolic pathways, *i.e.* those observed when grown on medium- and long-chain alkanes and dicarboxylates, *Alcanivorax* also upregulated a large array of apparently-redundant enzymes involved in the oxidation of aliphatic-like substrates and eventual depolymerisation *via* the  $\beta$ -oxidation pathway as previously suggested in *Rhodococcus ruber* (Gravouil et al., 2017). In addition to the enzymes required for substrate breakdown, *Alcanivorax* sp. 24 produced an interesting array of membrane transporters. These were both specific transporters for medium- and large-chain alkanes (Moreno and Rojo, 2017; Wang and Shao, 2014), as well as for dicarboxylates (Fischer et al., 2015; Kelly and Thomas, 2001) (Fig. 5), again highlighting the ability of the bacterium to process the complex mix of substrates derived from the breakdown of PE. When grown with pristine PE, which requires of primary depolymerisation processes by the bacterium, it was more advantageous for *Alcanivorax* to grow in direct contact with the material due to its recalcitrance and low availability of released oligomers (Fig. 4).

While it is accepted that abiotic weathering of plastics initiates



polymer chain scission (Andrady, 2011, 2017) – and demonstrated here by the observed reduction in  $M_n$ ,  $M_w$  and  $M_z$  after LDPE weathering, the involvement of biological processes in primary PE breakdown is currently unclear. The long C-C backbone chains of PE are distinct from polyesters, the latter containing hydrolysable functional groups for polymer depolymerisation (e.g. PETases for PET biodegradation (Yoshida et al., 2016)). The claims of ‘secreted’ enzymes –e.g. alkane monooxygenases AlkB or engineered cytochromes– being capable of depolymerising the extremely long and inert polymer chains directly on the plastic’s surface (Yeom et al., 2021), is debatable. Nevertheless, we think the biological production of extracellular ROS may well be involved in primary PE breakdown by generating unspecific oxidations of the PE chains as occurs during abiotic weathering. The biological oxidation of plastic surfaces has indeed previously been reported (Ghatge et al., 2020; Inderthal et al., 2021; Mohanan et al., 2020) as shown in Fig. 6b, and yet the mechanism that drives such process remains unknown. Marine bacteria have been reported to produce extracellular superoxide almost ubiquitously (Hansel and Diaz, 2021; Sutherland et al., 2019) and, here, we show that *Alcanivorax* is not an exception, i.e. with a sustained production of ROS in presence of pristine LDPE. Similarly, a higher ROS generation and expression of antioxidant enzymes was observed in the hydrocarbon-degrader *Rhodococcus erythropolis* when grown in the presence of such substrates (Sazykin et al., 2019). Hence, *Alcanivorax* may carry out the primary breakdown of PE –i.e. observed by the surface oxidation of pristine LDPE (Fig. 6b) and LDPE chain scission observed by the material’s broad depolymerisation when incubated with the bacterium (Table 1)– through the production of extracellular ROS. Based on our proteomic analysis, we were able to identify an array of enzymes that could be responsible for such a process. The NADH oxidase (ALC24\_4141) upregulated in all treatments when compared to succinate could generate superoxide as suggested for NOX enzymes in marine microorganisms (Hansel and Diaz, 2021). The proteomics dataset also revealed other possible ROS-generating enzymes: the laccase ALC24\_3338 (Beloqui et al., 2006; Gravouil et al., 2017; Santo et al., 2013; Wei et al., 2010) and the cytochrome P450 ALC24\_2795 (Lewis, 2002), although neither protein was differentially abundant between conditions. Laccases –secreted enzymes traditionally associated to lignin degradation by fungi (Youn et al., 1995)– have also been related to numerous other biodegradation processes, including PE (Santo et al., 2013). Cytochromes P450, as well as carrying out well-defined metabolic processes in the cell, can generate ROS when uncoupled from their enzymatic reaction (Veith and Moorthy, 2018) and, hence, show great promise in PE biodegradation as previously suggested (Yeom et al., 2021). In contrast, the Mn-containing SOD represents a significantly understudied mechanism, which was reported to generate highly reactive hydroxyl radicals in a lignin-degrading bacterium (Rashid et al., 2015), and of which the homologous enzyme in *Alcanivorax* ALC24\_3900 was highly upregulated in all PE treatments (Fig. 6c). Further research will elucidate if generation of ROS constitutes a central part of a mechanism by which microbes can gain direct access to the carbon locked within recalcitrant and inert polymers such as PE.

Hydrocarbon-degrading bacteria found within the plastisphere have been repeatedly related to the degradation of plastics (Denaro et al., 2020; Nauendorf et al., 2016; Roager and Sonnenschein, 2019), in particular polyolefins such as PE (Delacuvellerie et al., 2019; Dussud et al., 2018; Syranidou et al., 2019), without applying a robust enough battery of experiments to support such claims. While we show that the hydrocarbon-degrading bacterium *Alcanivorax* sp. 24 has the metabolic potential to biodegrade most leachates released from PE degradation, as also shown previously on heavily oxidised and short-chain PE (Eyheraguibel et al., 2017; Gravouil et al., 2017), we also prove it can initiate primary biodegradation of pristine LDPE via non-specific surface oxidations. While further taxonomical identification of this strain is needed, it is to be tested whether other *Alcanivorax* and obligate hydrocarbonoclastic bacteria are capable of such efficient primary PE

degradation and use of PE leachates. Furthermore, the remaining challenge is proving whether the observed biodegradation occurs in complex biofilms and if these microbes are capable of initiating chain scission of pristine PE under natural conditions, as well as testing other PE-based materials – i.e. consumer PE, liner low density PE and high density PE.

## 5. Conclusion

Together, our data provide key new insights into PE biodegradation. *Alcanivorax* sp. 24 expressed an impressive metabolic potential for catabolising the complex mix of aliphatic substrates generated from PE degradation, and this was observed when the bacterium was exposed to both weathered and pristine PE. The induction of such metabolic pathways as well as the acquisition of the isotopic signature of the plastic suggests that the bacterium can efficiently use the small amounts of substrates that leach from this material or, itself, can initiate chain scission of pristine PE i.e. as shown by the mass loss and broad depolymerisation of the pristine and weathered LDPE materials tested. Primary chain scission of pristine PE is likely to occur via the production of extracellular ROS and non-specific in-chain oxidation of high molecular weight PE polymers, a hypothesis that will gain weight within the next few years. It is also important to highlight that the experiments presented here were performed in pure cultures under optimal laboratory conditions. Further work is required to confirm that hydrocarbon-degrading bacteria are able to perform such tasks in complex multi-species biofilms where competitors and predators abound. Furthermore, the availability of other more readily-available substrates, such as the photosynthate or even alkanes produced by phototrophic microbes (Christie-Oleza et al., 2017; Love et al., 2021), could repress the metabolic pathways involved in PE degradation by the heterotrophic community. Complex microbial consortia could also accelerate PE chain scission via the presence of more efficient ROS-producing microbes (Hansel and Diaz, 2021), enhancing the availability of aliphatic substrates for hydrocarbon-degrading bacteria.

## Credit authorship contribution statement

The experimental work was performed by V.Z. with the supervision of J.A.C.-O. TOC and FTIR analysis were carried out with the assistance of G.E.-C. GPC experiments were performed by D.L. Y.E. and S.B. performed the isotope analysis. ROS detection experiments were carried out by T.O.-V. This research had the input of E.M.H.W., P.N.G., C.D., and M. I.G. The manuscript was written by V.Z and J.A.C.-O, and reviewed by all authors.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Additional information

Complementary information can be found at the publisher's website. [Supplementary Table 1](#). Substrate characteristics and chemical structures.

[Supplementary Table 2](#). Proteomic quantification of *Alcanivorax* sp. 24 incubated with different substrates and comparative analysis with the reference substrate succinate.

#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2022.129278](https://doi.org/10.1016/j.jhazmat.2022.129278).

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