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Microbial colonization and degradation of polyethylene and biodegradable plastic bags in temperate fine-grained organic-rich marine sediments

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ABSTRACT

To date, the longevity of plastic litter at the sea floor is poorly constrained. The present study compares colonization and biodegradation of plastic bags by aerobic and anaerobic benthic microbes in temperate fine-grained organic-rich marine sediments. Samples of polyethylene and biodegradable plastic carrier bags were incubated in natural oxic and anoxic sediments from Eckernförde Bay (Western Baltic Sea) for 98 days. Analyses included (1) microbial colonization rates on the bags, (2) examination of the surface structure, wettability, and chemistry, and (3) mass loss of the samples during incubation. On average, biodegradable plastic bags were colonized five times higher by aerobic and eight times higher by anaerobic microbes than polyethylene bags. Both types of bags showed no sign of biodegradation during this study. Therefore, marine sediment in temperate coastal zones may represent a long-term sink for plastic litter and also supposedly compostable material.

1. Introduction

The production of plastic material has constantly increased over the past 50 years (PlasticsEurope, 2013) and contributes to the resulting pollution of marine environments (Derraik, 2002). Polyethylene (PE) is a major component of plastic waste found in oceans (Morét-Ferguson et al., 2010; Zettler et al., 2013), on shorelines, at the ocean surface, and on the sea floor, mostly in the form of carrier bags (Barnes et al., 2009).

Marine plastic pollution may harm marine organisms via ingestion or entanglement, and may favor the dispersal of invasive species (Gregory, 2009). Additionally, the release of hazardous chemicals, including additives (components of plastic) and the accumulation of hydrophobic toxins (adsorbed onto plastic from surrounding sea water) were reported (Teuten et al., 2009). Due to the longevity of plastic in the environment and the ensuing long-term threat to organisms, alternatives to these synthetic polymers were developed and tested (O'Brine and Thompson, 2010). Different types of degradable plastics are commercially available, such as natural plastics produced by microorganisms, or plastics with polymer blends, such as starch and photo-biodegradable plastics (Shah et al., 2008). To date, studies focusing on different types of degradable plastics, marine environments and locations have been conducted by Accinelli et al. (2012); Andrady et al. (1993), Rutkowska et al. (2002) and Tosin et al. (2012). The mentioned studies obtained conflicting results and it remains unclear whether degradable plastics are less harmful.

Microbes are ubiquitously abundant in the marine environment, capable of decomposing complex organic matter. Hence, the question arises whether microbial degradation of plastic litter is possible and whether it has the capacity to counteract the gradual accumulation of plastics in marine environments. So far, most studies on the microbiological colonization and degradation of plastic are restricted to the upper ocean layer. Zettler et al. (2013) described a diverse microbial community growing on plastic material from North Atlantic surface water, which differed from the bacterial composition of the surrounding water. Pits in the plastic surface of the same size and shape as that of bacteria were interpreted as possible features of biodegradation. In another study, polyethylene (PE) incubated for 20 months in 2 m water depth in the Baltic Sea showed no biodegradation (Rutkowska et al., 2002).

The initial positive buoyancy and the hydrophobicity of PE may be altered by UV–radiation, oxidation, high temperatures (Andrady, 2011, Shah et al., 2008), and biofilm formation (Muthukumar et al., 2011). After approximately three weeks of floating at the ocean surface,

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PE bags start to sink below the seawater–air interface (Lobelle and Cunliffe, 2011). Adhesion of more particles onto the PE surfaces (Gregory, 2009) and wind-induced downwelling cause bags to sink further, until eventually they settle onto the seafloor (Kukulka et al., 2012). As light decreases with depth, it is likely that the rate of abiotic plastic degradation decreases in deep waters (Barnes et al., 2009). Although little is known about the dimension of plastic pollution of the seabed at depths >30 m (Watters et al., 2010), plastic debris have been found on the seafloor of every ocean (Barnes et al., 2009). Galgani et al. (1996) found up to 78 litter items/ha on the continental slope and bathyal plain of the northwestern Mediterranean Sea. Here, more than 70% of the total debris consisted of plastic bags. Once on the ocean floor, plastic material is buried into the seabed by ongoing sedimentation and/or bioturbation. During burial it passes the thin oxic sediment surface layer before reaching the anoxic sediment below. It is unknown how degradation rates of plastic in sediments are affected by the lack of oxygen and light. As microorganisms in the sediment largely control carbon sequestration and nitrogen conversion (Wu et al., 2008) and therefore play an important role in marine biogeochemical cycles (Strom, 2008), it is crucial to investigate their possible contribution to the biological degradation of deposited plastic.

To our knowledge, the only data available on benthic microbial settlement and degradation of plastic debris were published by Kumar et al. (2007) and Tosin et al. (2012). Kumar et al. (2007) investigated plastic degradation in mangrove soil. Tosin et al. (2012) tested plastic degradation in a simulation of the eulittoral and sublittoral zone. However, tropical mangrove soils and coarse-grained sandy sediments represent the ocean floor only to a limited extent (Fütterer, 2000). So far the microbial colonization and degradation of plastic in temperate, fine-grained sediments, which are more common at continental shelves, is unknown. However, this information is essential to estimate the fate of buried plastic in wide regions of the seafloor.

The present study focuses on the fate of PE and compostable carrier bags in oxic and anoxic coastal fine-grained, organic-rich sediment. A laboratory experiment was conducted to examine the colonization intensity of aerobic and anaerobic microorganism on the two plastic bags, the microbial alteration of the bag surfaces, and their mass changes.

2. Material and methods

2.1. Preparations of carrier bag materials

Commercially available polyethylene (PE) carrier bags and biodegradable bags produced by Melitta Europa GmbH & Co. KG were used. The putatively biodegradable bags ("COMP" hereafter) consist of >50% biodegradable polyester, >20% corn starch and additional, undisclosed components (personal correspondence with manufacturer). These bags meet the standards for compostability EN 13432 according to DIN CERTCO. From both bag types, 1 × 2 cm-samples were prepared and fixed to a short stainless steel wire to provide negative buoyancy. The plastic samples were sterilized with 70% ethanol for 2 min and washed with ultra-purified water for 2 min before the experiment was started.

2.2. Study site and sediment sampling

Sediment cores were obtained in February 2013 with a miniaturized *mu*lti *c*orer (MUC) onboard the research vessel "Littorina" at the long-term monitoring station "Boknis Eck" (54°31.2′ N, 10°02.5′ E, 28 m water depth) located in the Eckernförde Bay, Baltic Sea (Germany). These sediments are rich in organic carbon (5–6 wt.%) with an annual sedimentation rate of 1.4 mm yr^{-1} (Whiticar, 2002). Oxygen penetration depth into the sediment is around 0.1–0.2 cm (Preisler et al., 2007). Microbial cell numbers in the surface sediment layer (0–1 cm) range between 1.7 and 7.2 × 10^9 cells/g dry weight with the highest numbers found during winter and early spring compared to cell numbers in fall (Meyer-Reil, 1983). Deeper sediment layers (~25 cm) feature cell numbers of ca. 1.3 × 10^8 cells cm^{-3} (Treude et al., 2005). More details of Eckernförde Bay are summarized in Table 1.

2.3. Sediment slurry preparation

After MUC sampling, sediment cores were sub-sampled immediately on board for the preparation of oxic and anoxic sediment–seawater mixtures (slurries).

2.3.1. Oxic sediment slurry.

For the oxic slurries, the light colored top (0–1 cm sediment depth) of the MUC cores was transferred into autoclaved one liter Duran® glass bottles, which were closed with autoclaved cotton plugs and cooled at 5 °C until slurry preparation was conducted (one month later). Sea salt medium was chosen in order to prepare the slurries. Salinity and pH of the medium were adjusted to the Kiel Bight values of around 18 and 8.3, respectively. Afterwards the medium was autoclaved. Sediment and sea salt medium were mixed in a 1:1 ratio (vol/vol).

2.3.2. Anoxic sediment slurry.

For the anoxic slurries, the black, reduced (sulfidic smell) sediment from 5 to 10 cm sediment depth of the MUC samples was transferred headspace-free into autoclaved one liter Duran® glass bottles. The bottles were closed with autoclaved butyl stoppers and screw caps, and were kept at 5 °C until slurry preparation (after one month). In order to prepare the anoxic sediment slurries, the DSMZ (German Collection of Microorganisms and Cell Cultures) 196 modified *Desulfobacter postgatei* medium was used. Magnesium and calcium concentrations were adjusted to match seawater ($MgCl_2(6H_2O)$ 10.83 g l^{-1}; $CaCl_2(2H_2O)$ 1.53 g l^{-1}). No additional carbon source was added. Salinity was adjusted to Baltic Sea value. In order to maintain anoxic conditions in both medium and sediment during slurry mixing, the slurry preparation was conducted inside a glovebox (N_2 gas) with an oxygen level <1 ppm. For slurry preparation, sediment and medium were combined in a 1:1 ratio (vol/vol). All sediment slurries were stored at 5 °C prior to the experiment start.

Table 1
Characteristics of Eckernförde Bay.

Parameter	Data
Water depth	28 m
Carbon content in sediment	5–6 wt.% (Whiticar, 2002)
Sedimentation rate	1.4 mm yr^{-1} (Whiticar, 2002)
Porosity	0.88 (Preisler et al., 2007) Up to 0.92 (Treude et al., 2005)
O_2 penetration depth	0.1–0.2 cm (Preisler et al., 2007)
Temperature max. at 25 m	11–13 °C October (Lennartz et al., 2014) 1–2 °C March (Lennartz et al., 2014)
Oxygen concentration max. at 25 m	290–350 μmol l^{-1} February, March (Lennartz et al., 2014)
Oxygen concentration min. at 25 m	0–20 μmol l^{-1} September, October (Lennartz et al., 2014)
Microbial cell number in sediment	1.7 × 10^9 and 7.2 × 10^9 cells per g of dry weight, 0–1 cm sediment depth (Meyer-Reil, 1983) 1.2 × 10^8 to 1.4 × 10^8 cells cm^{-3}, 24–26 cm sediment depth (Treude et al., 2005)
Sulfate reduction rate	4–5.4 mmol SO_4^{2-} m^{-2} d^{-1} (integrated over 0–25 cm sediment depth) (Treude et al., 2005) 32.4 nmol SO_4^{2-} cm^{-2} d^{-1} (integrated 0–18 cm sediment depth) (Bertics et al., 2013)

2.3.3. Control sediment slurry.

Controls with sterilized sediment were prepared for all treatments to discriminate between biological and non-biological effects on plastic bags. Control slurries were autoclaved at 121 °C for 20 min to terminate microbial activity. In the following, slurries containing live microorganisms are called "active slurry", whereas sterilized slurries are called "inactive slurry".

2.4. Treatment preparation

Six sterilized replicate samples of each bag type were placed into one autoclaved 200 ml serum vial. The oxic treatments were closed with autoclaved cotton plugs, in order to allow gas exchange with the atmosphere and to maintain oxic conditions. For the anoxic treatments, the vials were closed with autoclaved butyl rubber stoppers and crimped to prevent gas exchange. Afterwards, the anoxic treatments were flushed with N_2/CO_2 (80 vol%/20 vol%) for 15 min to achieve anoxic conditions. All treatments were filled with 60 ml of either oxic or anoxic sediment slurry (see Section 2.3). The transfer of anoxic sediment slurry into the vials was realized inside a glovebox ($O_2 < 1$ ppm).

Each treatment was prepared in triplicates. Incubation was kept at 10 °C in the dark. One replicate bag sample of the oxic treatments was collected at days 7, 21, 50, and 98 after experiment start. Replicate bag samples of anoxic treatments were each removed after 7, 21, 49 and 99 days.

2.5. Bag sampling

One pseudo-replicate bag sample of each treatment was removed on each sampling day (see Section 2.4). In order to avoid oxygen contamination, sampling of anoxic treatments took place inside a glovebox ($O_2 < 1$ ppm).

After removal of wires, the bag samples were carefully washed in phosphate buffered saline (PBS), in order to remove sediment and non-attached microorganisms. All bag samples were cut into four equal pieces with a sterilized scalpel for cell-count analyses, Scanning Electron Microscopy (SEM), and RAMAN spectroscopy of the surfaces. Cell-count samples were stained immediately after sampling (see Section 2.6.1). Samples for SEM of biofilms were frozen at −20 °C. Samples used for SEM and Raman spectroscopy of the bag surface were rinsed with ultra-purified water, placed on a paper towel and carefully wiped to remove the biofilm as much as possible without damaging the bag surface. Subsequently, the samples were rinsed with ultra-purified water, and frozen at −20 °C until further use.

2.6. Sample analyses

2.6.1. Cell density on the bag pieces

The bag samples were stained with 50 μl 4′,6-diamidin-2-phenylindol (DAPI; working solution 1 μg/ml) and handled as described in Krause et al. (2014). Cell counting was carried out under UV–light using a Leitz Aristoplan epifluorescence microscope. For the cell enumeration, either 70 grids (equaling 0.847 mm^2) or minimum 800 cells were counted, depending on which was obtained first. Cell density was expressed as cells cm^{-2}.

In order to test the possible contamination of the inactive sediment slurry incubations, live/dead staining was applied. For this purpose the Live/Dead BacLight® Bacterial Viability Kit and protocol (https://tools.thermofisher.com/content/sfs/manuals/mp07007.pdf) of Molecular Probes, Inc. was used. The samples were analyzed with the same microscope mentioned in this section with the filter cube I3 right after staining. Either 50 grids (equals 0.605 mm^2) or a minimum of 800 dead and alive cells were counted.

2.6.2. Determination of bag pieces mass loss

Before the experiment, one bag sample (without wire) of each treatment was weighted with an electron microbalance Sartorius M3P (accuracy ±0.001 mg). After experiment termination, i.e., after 98 (oxic treatment) and 99 (anoxic treatment) days of incubation, the pieces were rinsed with ultra-purified water, dried and weighted again. Mass change (expressed in %) was calculated by subtracting mass measured at the end of the experiment from the mass at the start of the experiment.

2.6.3. Biofilm and surface imaging

For scanning electron microscopy (SEM) of biofilms, bag samples were dried using the critical point drying method.

Samples were sputter coated with gold/palladium (10 nm thickness) and imaged using a Hitachi S-4800 scanning electron microscope was used. Images of the biofilms and surfaces of the bag samples were obtained at 3 kV of accelerating voltage by using a lower SE-detector.

2.6.4. Raman analyses

To identify microbially-induced chemical alteration of the bag surfaces, the longest incubated PE and COMP samples (98 days oxic, 99 days anoxic conditions) were analyzed using confocal Raman spectroscopy. The analyses were carried out with a LabRAM HR800 (Horiba Jobin Yvon GmbH, Bensheim, Germany) spectrometer, equipped with a 473 nm laser and a 600 grooves/mm diffraction grating. The detector entrance slit was opened to 100 μ width. Material of COMP samples was excited at a power of 3 mW over 3 integration cycles of 10 s duration each. PE samples were probed using 6 mW at the sample and 6 integration cycles of 5 s each. The incident beams were focused onto the samples through a 100× magnification objective. The confocal hole was fully opened to 1100 μm yielding an axial resolution of 4.6 μm. A Si-waver was used before and after the measurements to calibrate the instrument. Multiple points were analyzed of each sample to check for spatial heterogeneity. Sample alteration was determined based on inter-comparison of Raman shifts and full width at half maximum intensities (FWHM) of characteristic Raman peaks. The profile parameters were determined by applying a profile fitting routine using LabSpec 5.0 (Horiba Jobin Yvon GmbH, Bensheim, Germany).

2.6.5. Wettability measurements

Contact angles of double-distilled water (surface tension = 72.1 mN m^{-1}, dispersion component = 19.9 mN m^{-1}, polar component = 52.2 mN m^{-1} (Busscher et al., 1984)) on incubated bag samples and pristine bag samples were measured by using a high-speed optical contact angle-measuring device OCAH 200 (DataPhysics Instruments GmbH, Filderstadt, Germany) according to the sessile drop method. 1 μl drops were applied on the samples and circle or ellipse fitting were used for evaluation of apparent contact angles. On each surface, the contact angles of 3–5 drops were measured.

2.6.6. Statistical analyses

The statistical analysis of the obtained results was conducted using the software R (R Core Team, 2013). For comparing the biofilm development on the bag surfaces, weight change and surface wettability of the different treatments, the non-parametric Wilcoxon rank sum test was used. The non-parametric test was chosen, as the assumptions (normal distribution and homogeneity of variances) for a parametric test were not fulfilled. If the test indicated significant differences, the effect size was obtained. The graphs were generated with the ggplot function of R.

3. Results

The biofilm development derived from pseudo-replicate bag samples is shown in Fig. 1. At the start of the experiment no cell counting was conducted as samples were sterilized (see Section 2.1) and expected to be cell-free. Several replicates of the COMP samples revealed a

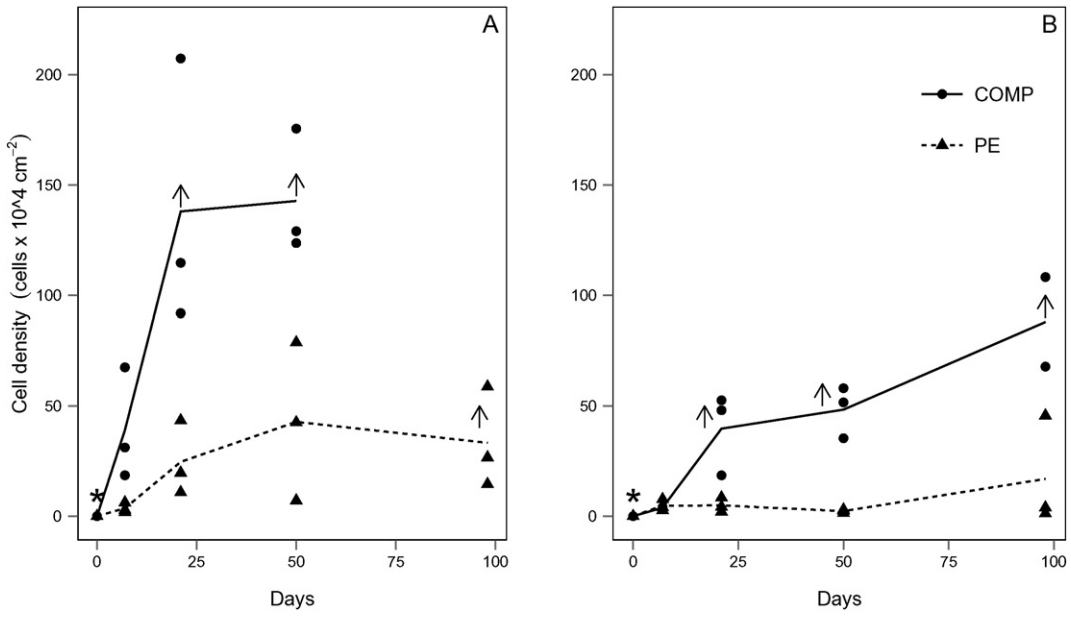

Fig. 1. Temporal development of cell densities on PE and COMP bags incubated in (A) oxic and (B) anoxic sediment slurry. At time zero (stars) cell numbers were expected to be zero as samples were sterilized (see Section 2.1). Arrows indicate multi-layer biofilms on the bag sample. Values are missing for the last sampling event of the COMP bag incubated in oxic slurry, as the biofilm was too voluminous for reliable cell quantification. Single replicates are indicated by dots/triangles with calculated mean (line). COMP = biodegradable bag; PE = polyethylene bag.

patchy multi-cell-layer biofilm. Even though cell counting was possible in less colonized regions of the biofilms, total cell numbers are expected to be underestimated, because in regions with multi-cell-layer biofilm cells could not be quantified. On all COMP bags incubated in oxic sediment slurry as well as on one COMP bag incubated in anoxic slurry, cell counting was impossible for the last sampling event (98 days) due to a widespread multilayered biofilm. Cell numbers for these bags are expected to be higher compared to the earlier stages. The microbial cell density on the COMP bag increased from 0 to 1.43×10^6 (SD $\pm$ 2.33×10^5) cells cm^{-2} (from days 0 to 50) in oxic and from 0 to 8.81×10^5 ($\pm 2.02 \times 10^5$ SD) cells cm^{-2} (from day 0 to day 99) in anoxic sediment slurries. Up to incubation day 50, the colonization rate on the COMP bag was roughly 28,000 cells day^{-1} in oxic and 9700 cells day^{-1} in anoxic sediment slurry. Cell numbers on the PE bag increased from 0 to 3.33×10^5 ($\pm 1.87 \times 10^5$ SD) cells cm^{-2} in oxic and from 0 to 1.69×10^5 ($\pm 2.03 \times 10^5$ SD) cells cm^{-2} (from days 0 to 98/99) in anoxic sediment slurries. The colonization rate on the PE bag was roughly 8600 cells day^{-1} in oxic and 460 cells day^{-1} in anoxic sediment slurry (data points up to incubation days 49 and 50, respectively, were included for colonization rate calculations for comparability to COMP data).

Comparing cell densities from oxically and anoxically incubated COMP bags showed that aerobic microbes settled in significantly higher densities than anaerobic (Wilcoxon rank sum test $W = 10$, $p = 0.006$, $r = -0.65$; only data until day 49/50 were included in the statistical analyses due to multi-cell-layer biofilm at day 98). Similar results were obtained for the PE bag, where aerobic microbes settled in significantly higher densities than anaerobic ones (Wilcoxon rank sum test $W = 29$, $p = 0.01$, $r = -0.51$). Furthermore, the COMP bag showed a five times higher abundance of aerobic and eight times higher abundance of anaerobic microbes compared to the PE bag (Wilcoxon rank sum test $W = 73$, $p = 0.003$, $r = -0.71$; $W = 71$, $p = 0.006$, $r = -0.65$, respectively; only data until day 49/50 were included in the statistical analyses due to multi-cell-layer biofilm at day 98).

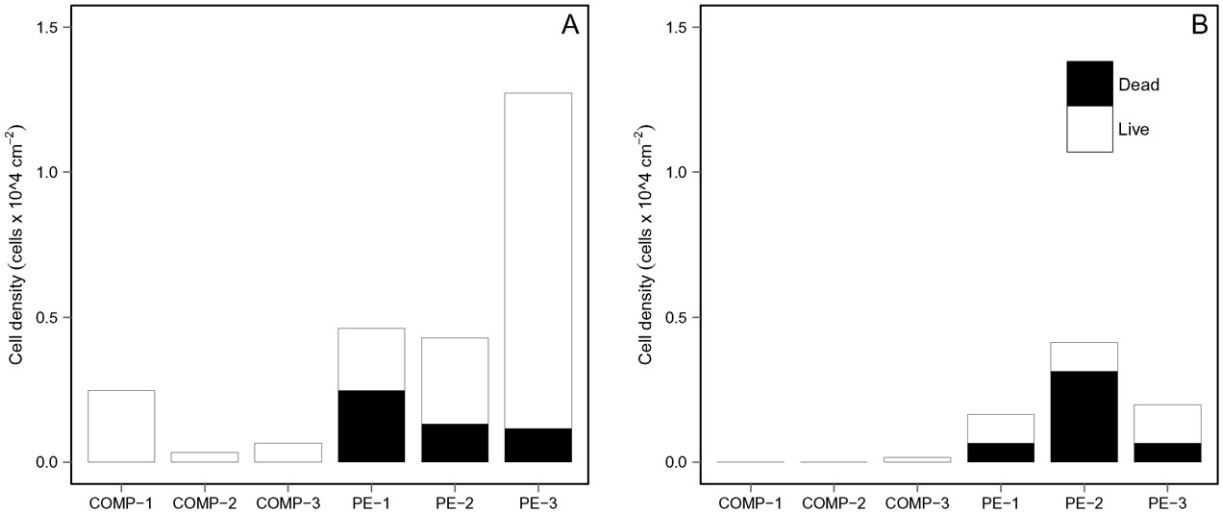

Fig. 2. Cell densities of living and dead microbes detected on bag samples incubated with inactive (A) oxic and (B) anoxic sediment slurries at the end of the experiment. All three replicates of each treatment are represented (COMP-1 — COMP-3; PE-1 — PE-3). No cell contamination is represented by a line. COMP = biodegradable bag; PE = polyethylene bag.

Bag samples incubated in inactive oxic and anoxic sediment slurries showed cell contaminations towards the end of the experiment (Fig. 2). In order to detect the degree of contamination, cell density on the bag samples incubated in active sediment slurries was compared with the cell density found on the bag samples incubated in inactive sediment slurries. On average, PE bag samples were 46 and 65 times more colonized in active oxic and anoxic sediment slurry after 99 days, respectively, than in inactive oxic and anoxic sediment slurry. COMP bag samples were 1×10^3 and 9×10^3 times more colonized in active oxic and anoxic sediment slurry after 49/50 days, respectively, than in inactive oxic and anoxic sediment slurry.

COMP bag samples incubated in both oxic and anoxic environments and PE bag samples incubated in oxic environments lost mass during the experiment (Fig. 3). COMP bag samples on average lost more mass [−1.2 (±0.06 SD) % (in oxic conditions) and −1.6 (±0.41 SD) % (in anoxic conditions)] during the experiment than PE bag samples [−0.4 (±0.19 SD) % (in oxic conditions) and +0.7 (±0.56) % (in anoxic conditions)]. Comparing the mass loss data of PE and COMP samples from active sediment slurries with those from inactive sediment slurries no significant difference was detected for all treatments (Wilcoxon rank sum test (active sediment slurry: inactive sediment slurry): COMP oxic $W = 9$, $p = 0.1$; COMP anoxic $W = 8$, $p = 0.2$; PE oxic $W = 2$, $p = 0.4$; PE anoxic $W = 3$, $p = 0.7$). Moreover, mass gain was observed for PE samples treated with anoxic active and inactive sediment slurries. Comparing COMP bags incubated in oxic and anoxic sediment slurries (both active and inactive), showed that bags treated with anoxic sediment slurries lost significantly more mass (Wilcoxon rank sum test $W = 32$, $p = 0.03$, $r = -0.54$).

SEM images of the pristine PE and COMP samples are presented in Fig. 4. The COMP bag was characterized by a diverse surface structure. Mostly, an uneven, hilly surface with numerous bumps covered the surface (Fig. 4A). At higher magnification (×10 k) smooth regions between the blisters as well as discontinuous, angular cracks were revealed (Fig. 4B). The PE surface structure was rather smooth (Fig. 4C), even at higher magnification, revealing a few uneven regions and interlaced filaments in some areas (Fig. 4D). After an incubation time of 98 days, several areas of the COMP bag surfaces were colonized by microbes (Fig. 5A). These colonies not only expanded horizontally over the bag surface but also piled up to clusters with multiple cell layers. Filaments extended from some cells to others (Fig. 5B). In some areas, the COMP surface was densely covered with sediment particles, prohibiting the detection of microbial cells (Fig. 5C). Microbes on PE bag surfaces were predominantly present in small groups located in depressions and uneven areas (Fig. 5D). Fig. 5E shows a small group of bacteria above a depression with numerous filaments entrenching the cells with the bag surface. The filaments branched out to form a web over the bag surface. Cells were also detected to coalesce with the bag material (Fig. 5F).

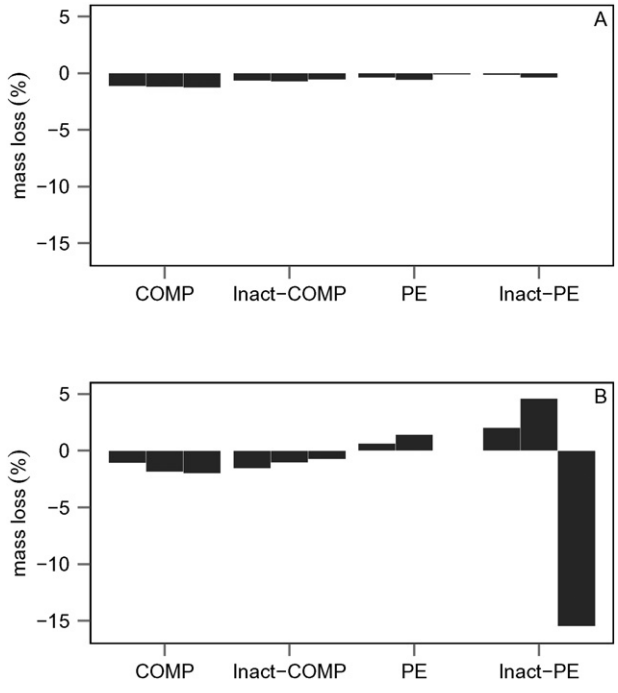

Fig. 3. Mass loss (negative values) or mass gain (positive values) in % of the bag sample weights after the experiment was ended. During the experiment, samples were incubated in (A) oxic and (B) anoxic sediment slurries. All three replicates are listed for each treatment. COMP = biodegradable bag incubated in active sediment slurry; PE = polyethylene bag incubated in active sediment slurry; Inact-COMP/Inact-PE = bag incubated in an inactive sediment slurry.

COMP and PE bag surface imaging with SEM after removal of microorganisms did not show any signs of surface alteration. Some biofilms and sediment particles remained on the surface due to incomplete removal. SEM imaging of the surface structure (both COMP and PE) of the treatments with inactive sediment slurries did not indicate any surface alteration. Only very few single microbial cells were observed.

Raman spectra of PE and COMP samples (Figs. 6, 7) exhibited autofluorescence, leading to elevated backgrounds at higher Raman shifts. The effect was more pronounced in COMP samples, likely due to excitation of fluorescent impurities. Spectra of incubated samples exhibited additional fluorescence around 2500 cm^{-1}. Although variable in strength, the pattern of this additional contribution occurred on both materials and was insensitive to incubation conditions. The additional contribution and the absence of background maxima around 2500 cm^{-1} in pristine samples indicated fluorescent remains of presumably sediment particles or/and cells on the samples, despite cleaning prior to Raman analysis. The COMP bags were composed of a polyester-starch blend and an additional component, the identity of which was not disclosed by the manufacturer. The polyester was readily identified as poly(butylene adipate co-terephthalate) (PBAT) by the stretching vibration of the carbonyl group at 1716 cm^{-1}, the vibrations of the benzene component and the C—H stretching therein at 1614 and 3080 cm^{-1}, respectively, as well as the broad band peaking at 2927 cm^{-1}, indicating C—H stretching of various alkyl groups. Although the presence of a starch component, indicated by an intense and well-defined band in the region 475–485 cm^{-1} (Kizil et al., 2002), could not be verified in the present study, it is not ruled out. The apparent reason for the absence of starch bands in the present spectra is a poor signal-to-noise ratio due to strong fluorescence introduced by an excitation line at 473 nm. However, the identified components as well as their Raman peak positions closely matched those of the biodegradable materials studied by Nobrega et al. (2012), who characterized the degradation of blends of the aforementioned polyester with varying proportions of glycerol and starch. The PE bags showed Raman spectra typical of this compound. Three additional peaks at 141, 443 and 605 cm^{-1} were not assignable to PE and indicated the presence of the TiO_2 polymorphs Anatase (141 cm^{-1}) and Rutile (443, 605 cm^{-1}) (Parker and Siegel, 1990). The TiO_2 was detected in the pristine as well as in the incubated PE samples but not in the COMP samples. A possible contamination of the samples with TiO_2 from the sediment slurries is therefore ruled out. On the other hand, both compounds are known to enhance antibacterial and mechanical properties of polymers such as PE, while also changing their photochemical stability (e.g. Thomas et al., 2013; Nguyen et al., 2013). Hence, admixture of TiO_2 during the manufacturing of the investigated PE seems plausible.

Comparing the Raman peaks of spectra of differently treated samples (pristine, inactive sediment, active sediment) the most obvious change was an increased fluorescence in incubated samples. Peak widths and intensities of selected Raman peak profiles are shown in Tables 2 and 3. There was considerable variation in peak intensities of both materials, but no correlation to the method of incubation. However, the spectrum of the pristine PE sample featured peaks at 1084 and

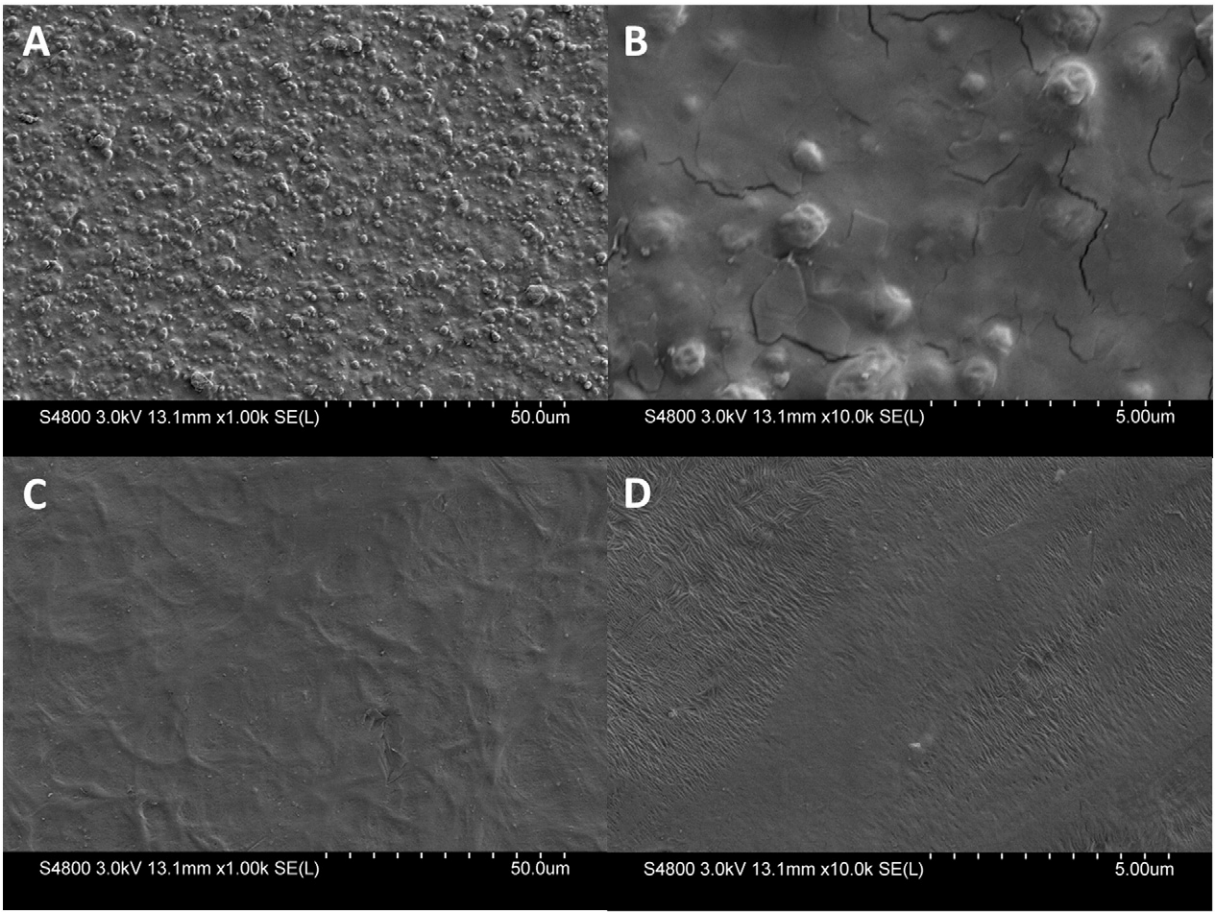

Fig. 4. SEM images of pristine bag surfaces (without treatment). (A) Surface topography of COMP bag. (B) Close-up of COMP bag. (C) Surface topography of PE bag. (D) Close-up of PE bag.

1367 cm^{-1}. These bands were assigned to amorphous PE (Furukawa et al., 2006) and were too weak to yield stable quantitative fitting parameters in spectra of the incubated material, suggesting a slight decrease of the amorphous PE component due to the incubation. However, similarly to the intensities, a systematic variation of peak widths is absent throughout the test series, indicating that neither microbial activity nor the redox regime had a major effect on the degradation of PE samples. Although both TiO_2 modifications were rarely detected in one spectrum, Raman probing of different locations on each PE sample revealed the presence of both rutile and anatase throughout the PE test series. Rutile was the more abundant phase, exceeding anatase by a factor of 42, regardless of incubation conditions.

As both bag types featured rusty stains after incubation in oxic sediment slurries, we decided to obtain Raman spectra from these regions as well. Only spectra of stainless parts are shown in Fig. 6 as no visible difference was detected (Tables 2, 3).

Contact angle (CA) measurement results of pristine and incubated PE and COMP bags are given in Fig. 8. CAs of the pristine PE and COMP bags were significantly different (Wilcoxon rank sum test $W = 0$, $p = 0.008$, $r = -0.84$) from each other. The pristine PE bag showed hydrophobic properties with a CA of 104° (±4° SD), whereas the pristine COMP bag had a more hydrophilic surface with a mean CA of 88 (±5° SD) (Fig. 9). Both bag types treated with active sediment slurries were more hydrophilic at the end of the experiment than the pristine bag samples. The CAs of the PE samples treated with active oxic and anoxic sediment decreased both by 25° after 98 (oxic) and 99 (anoxic) days of incubation. The CAs of COMP samples incubated in active oxic sediment decreased by 16° after 98 days of incubation; those of COMP samples incubated in anoxic sediment by 15°. A significant difference between the CAs of bags treated with either active or inactive sediment was found only for PE bags incubated in anoxic sediment slurries (Wilcoxon rank sum test $W = 16$, $p = 0.03$, $r = -0.77$). Moreover, CAs measured on the PE bag incubated in active oxic sediment slurry, had large deviations between the replicates. The same PE sample featured a brown rust stain on the surface (see above). CAs measured on that stained area were lower than those obtained on stainless parts of the same sample and led to the large deviation.

4. Discussion

In the present study benthic microbes settled in higher densities on the biodegradable (COMP) bag than on the polyethylene (PE) plastic bag. Moreover, aerobic microbes colonized both bag surfaces in higher densities than anaerobic microbes. On all COMP bags, microbial cell densities increased and did not reach a stationary phase by the end of the experiment. Final obtained cell density values represent only a fraction of present cells due to the development of a multi cell layer biofilm. Cell numbers on PE bags increased slightly over time but seemed to stagnate towards the experimental end.

Initial biofilm formation on surfaces depends on several factors. Attachment can increase with surface roughness (Characklis et al., 1990) as well as with hydrophobic surface property (Bendinger et al., 1993; Fletcher and Loeb, 1979; Oliveira et al., 2001). COMP bag had indeed a visually rougher surface structure than the PE bag, but showed more hydrophilic surface properties (CA: 88° for COMP; 104° for PE). A contact angle of 88° equals a surface energy of ca. 36 to 34 mN m^{-1} and 104° equals less than 34 mN m^{-1}. The optimal surface energy for bacterial colonization corresponds to 31–43 mN m^{-1} (Becker and Wahl, 1991). Both bag types fall in that range and became more hydrophilic when submerged in either active or inactive sediment

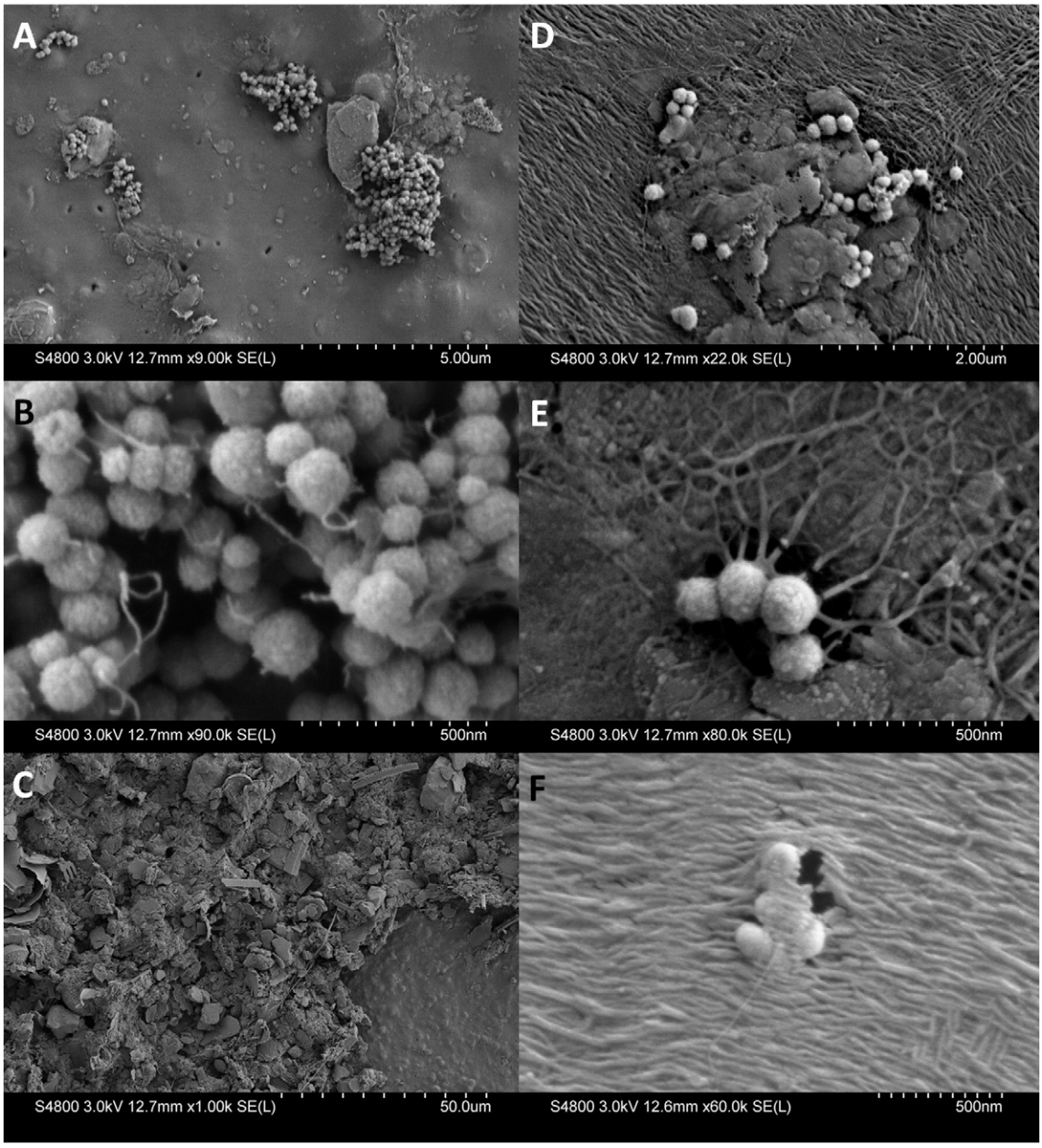

Fig. 5. SEM images of biofilms on the bags after 98 (oxic) and 99 (anoxic) days of incubation. (A–C) COMP bag incubated in oxic sediment slurry. (A) Microbial accumulations. (B) Close-up of microbial accumulation with filaments. (C) Sediment particles covering the bag surface. (D, E) PE bags incubated with oxic sediment slurry.(D) Microbes settling in an uneven location. (E) Close-up of microbes attaching onto the surface with filaments. (F) PE bag incubated in anoxic sediment slurry with microbes penetrating into the material. COMP = biodegradable bag; PE = polyethylene bag.

slurries, indicating that components of the sediment slurries/adsorption of macromolecules changed physicochemical properties of the bag surface. We therefore assume that surface wettability was probably of minor importance for bacterial attachment compared to the surface roughness. According to Donlan (2002), bacterial attachment does not only depend on properties of the substratum but also on the bulk liquid and sediment properties as well as on cell surface properties. Hence, it is difficult to conclude whether the COMP bag was colonized in higher rates due to its surface roughness or due to other factors. Also, the identity of the microbes settling and growing on the bags was not studied and therefore their relative contribution to the community establishments was not resolved. Furthermore, Raman spectroscopy results indicated the presence of TiO_2 in PE samples. TiO_2 is commonly used as an effective photocatalytic bactericide (Bonetta et al., 2013), suitable to inhibit or slow down microbial colonization.

The fact that aerobic microbes colonized both types of bags faster than anaerobic might be attributable to their different metabolisms. Aerobic respiration results in higher energy gain compared to anaerobic and can therefore lead to a higher bacterial activity and cell division rate (Jørgensen, 2000). However, as the initial cell concentrations in oxic and anoxic sediment slurries were not examined, it is unknown if both sediments had similar cell concentrations at the start of the experiment. Comparing cell numbers determined in Eckernförde Bay sediment by Meyer-Reil (1983) and Treude et al. (2005), cell density is approximately one order of magnitude higher in 0–1 cm than in 24–25 cm sediment depth (see Table 1).

Mass loss, surface wettability changes, and shifts in surface chemical composition of the bags were monitored in order to detect possible biodegradation by aerobic and anaerobic benthic microorganisms during incubation. PE samples incubated in anoxic active sediment slurry

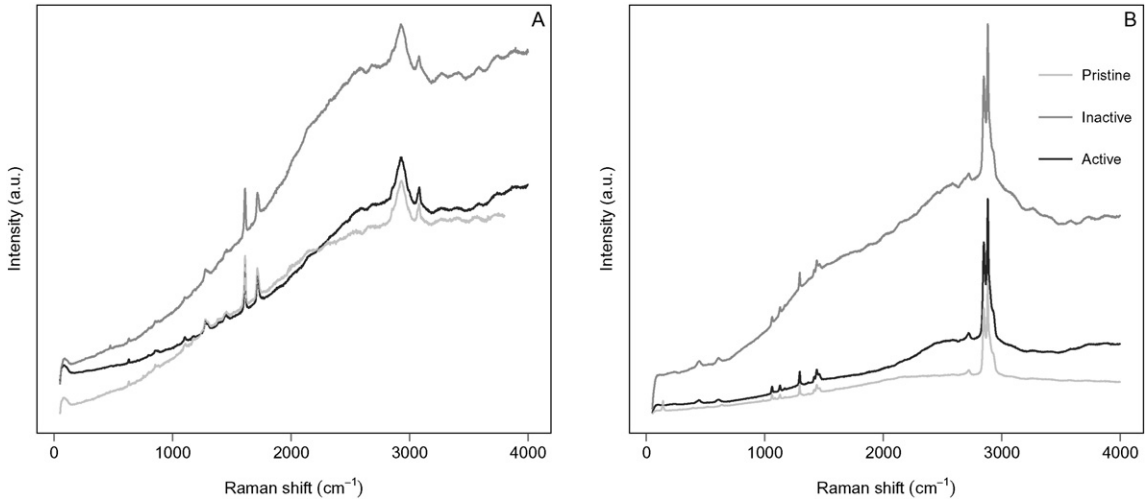

Fig. 6. Raman spectra of (A) COMP bags and (B) PE bags incubated in oxic sediment slurries. The following bag samples were analyzed: bag sample without treatment (*pristine*), bag samples incubated in inactive slurry (*inactive*) and bag samples incubated in active slurry (*active*). COMP = biodegradable bag; PE = polyethylene bag.

were found to be significantly more hydrophilic than PE samples in anoxic inactive sediment slurry. This effect might be due to the adsorption of molecules, bacterial settlement or the release of extracellular polymeric substances (EPS) rather than biodegradation as no further alterations were detected. Other studies on the degradation on plastic in sediments revealed different results. Tosin and colleagues tested the mechanical and biological degradation of a PE and a Mater-Bi carrier bag in laboratory experiments with oxic sand, simulating the eulittoral (physical degradation) and sublittoral zone (biological degradation). The biodegradable bag visually disappeared after 9 months in the eulittoral simulation and was degraded by 68.9% after 236 days in the sublittoral simulation. The PE bag was still fully intact after the 9-months in the eulittoral zone simulation. The sand used in the study was obtained directly below the water line and was possibly coarser compared to the fine-grained sediment of Eckernförde Bay. As the authors did not use any tidal simulation, no physical stress led to the disappearance of the biodegradable bag. It is possible that the obtained sand contained a higher concentration of aerobic bacteria and that the experimental temperature (room temperature) led to a higher metabolic rate and accelerated degradation than in our experiment (10 °C). Another explanation for degradation could be the mixture of substances of the Mater-Bi bag leading to an easier biodegradation compared to the ingredients of the COMP bag used in this study.

Kumar et al. (2007) reported a 5% mass loss of PE carrier bags, which were exposed to mangrove soil in a laboratory study for eight weeks. As no sterile controls were used, potential biodegradation should be discussed cautiously. However, organic-rich mangrove soil should have a higher potential for biodegradation of plastic compared to temperate sediments as microbes are expected to be metabolically more active at higher temperatures.

COMP bags used in this study consists of >50% biodegradable polyester and >20% biodegradable cornstarch. Monomers in polyesters are bonded via ester linkages and as numerous kinds of esters appear in nature, several organisms are able to degrade ester materials (Shimao, 2001). We therefore expected to detect more indications of biodegradation of the COMP bag after three months of incubation in active sediment slurries. Accinelli et al. (2012) exposed a biodegradable Mater-Bi carrier bag to soil, compost, marsh and seawater for three months. The carrier bags incubated in soil and compost had a mass loss of 37% and 43%, respectively, whereas the bags incubated in marsh and seawater

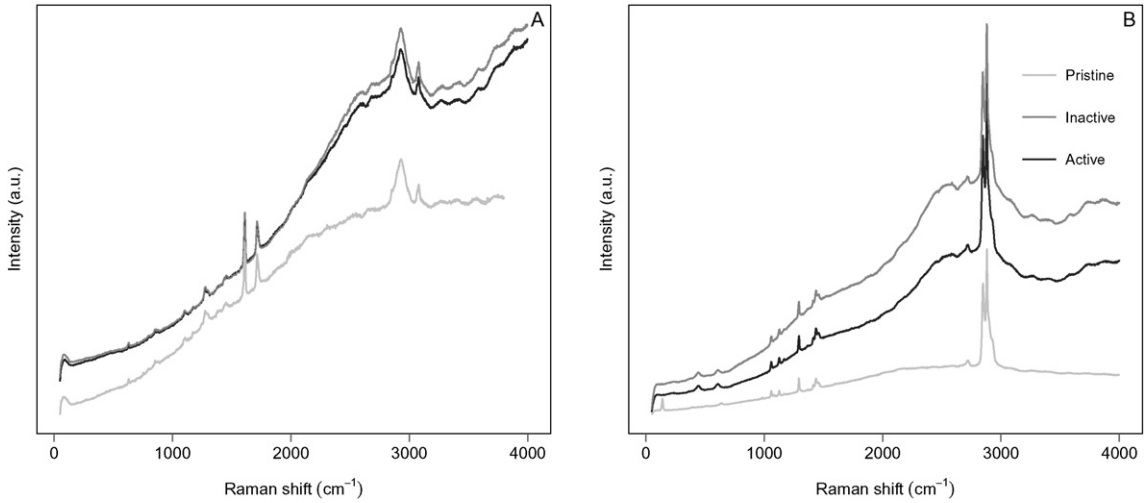

Fig. 7. Raman spectra of (A) COMP bags and (B) PE bags incubated in anoxic sediment slurries. The following bag samples were analyzed: bag sample without treatment (*pristine*), bag samples incubated in inactive slurry (*inactive*) and bag samples incubated in active slurry (*active*). COMP = biodegradable bag; PE = polyethylene bag.

Table 2
Peak widths (FWHM), and peak areas of the benzene (1610(±0.4) cm^{-1}) and C═O stretching (1717(±0.1) cm^{-1}) modes in the Raman spectra of COMP bag samples: pristine bag, bag incubated in anoxic active and inactive sediment slurry, bag incubated in oxic active (one profile of a stainless part and one profile of a rusty stain) and inactive sediment slurry.

	Pristine	Anoxic active	Oxic active clean	Oxic active rusty	Anoxic inactive	Oxic inactive
FWHM (cm^{-1})						
Benzene	14.0	13.4	13.8	14.8	13.6	14.1
C═O stretch	21.9	21.5	21.4	23.0	21.3	22.1
Area (a.u.)						
Benzene	68,168.9	63,692.7	71,038.2	55,351.9	72,470.8	32,633.1
C═O stretch	58,503.2	61,145.7	69,142.5	54,811.2	70,005	34,881.7

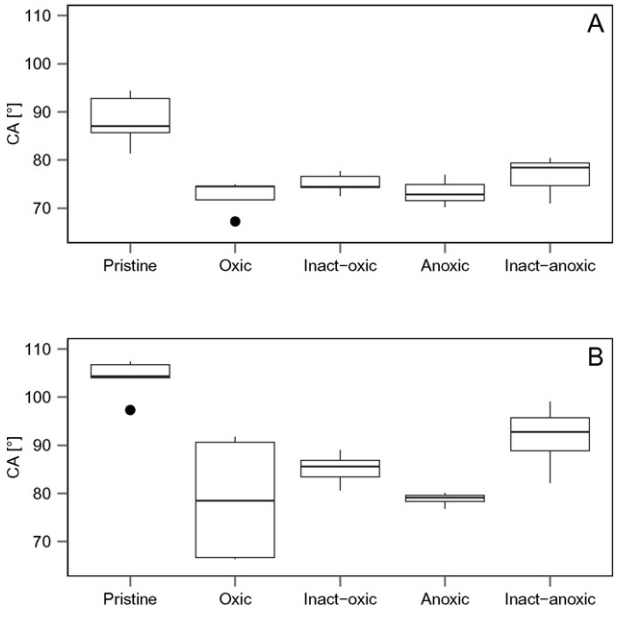

Fig. 8. Boxplot of contact angles (CA) measured on (A) COMP and (B) PE bags at the end of experiments with different treatments. Pristine = non-treated bag; Oxic = bag incubated in active oxic sediment slurry; Anoxic = bag incubated in active anoxic sediment slurry; Inact-oxic = bag incubated in oxic inactive sediment slurry; Inact-anoxic = bag incubated in anoxic inactive sediment slurry. COMP = biodegradable bag; PE = polyethylene bag. Box = interquartile range; whiskers = 25th and 75th percentiles; dots = outlier.

lost only approximately 1.5% of their weight. In our study, abiotic mass loss of the COMP bag was in a similar range to the Mater-Bi carrier bag incubated in seawater. Accinelli et al. (2012) discovered that the high rate of biodegradation of bioplastic in compost and soil was due to the higher amount of bacteria and fungi capable of degrading bioplastic compared to aquatic systems. However, different results were obtained by O'Brine and Thompson (2010), when deploying a Mater-Bi bag, two oxo-biodegradable plastics, and a standard PE bag near the sea surface for 40 weeks. After 16 to 24 weeks of incubation, the Mater-Bi bag disappeared from their test rig whereas 98% of the other plastics remained even after 40 weeks of incubation. Though the Mater-Bi bag fully disappeared, it is not proven that microbial degradation was the cause for its fast disappearance, as the study was conducted in the field and numerous other factors (currents, waves, fouling, etc.) need to be considered. Why in our study some bag samples slightly lost and others gained weight is currently unclear, but cannot be explained by microbial degradation as we observed no statistical difference between bags incubated in active versus inactive slurries.

The lack of biodegradation of the plastic and biodegradable material in the present study could be explained by: (A) an alternative carbon source in the sediment and (B) a lack of possible abiotic degradation initiators. As to hypothesis (A), the sediment at Boknis Eck is an efficient recycler of inorganic nutrients (Bange et al., 2011) and rich in organic carbon (5–6 wt.%) (Whiticar, 2002). Possibly, microbes prefer smaller carbon chains available in the surrounding sediment over those longer carbon chains of PE or COMP bags. Presumably, incubation time was too short for a limitation of carbon to occur in the sediment used in this experiment and for microbes to switch to the plastic bags as an alternative carbon source. Nevertheless, in nature, sediments are expected to reach carbon limitation when buried into the deep biosphere (Parkes et al., 2005). Plastic, recently introduced to human life style, will probably take thousands of years until reaching the deep biosphere and carbon limiting conditions when disposed into the ocean.

As to hypothesis (B), several abiotic factors cause oxidation of plastic surfaces and the formation of carbonyl groups, which can be utilized by microorganisms (Gilan (Orr) et al., 2004; Sudhakar et al., 2007). As marine plastic does not enter the ocean floor directly, but instead enters it via the land or via the ocean surface pathway, plastic is often exposed to conditioning abiotic factors. In this study, no pretreatment simulating

Table 3
Raman shifts (RS), peak widths (FWHM), and peak areas of characteristic vibrational modes in the spectra of the following PE bag samples: pristine bag, bag incubated in anoxic active and inactive sediment slurry, bag incubated in oxic active (one profile of a stainless part and one profile of a rusty stain) and inactive sediment slurry.

	RS/cm^{-1}	σ RS/cm^{-1}	Pristine	Anoxic active	Oxic active clean	Oxic active rusty	Anoxic inactive	Oxic inactive
FWHM (cm^{-1})								
CH_2 bending	1459.4	0.9	19.8	20	20	20	19.8	20
CH_2 bending	1437.2	0.3	15.7	15.5	16.4	16.1	15.9	14.2
CH_2 bending	1414.5	0.7	9.0	12.2	7.0	10.4	9.4	9.1
CH_3 wagging	1366.9	1.6	8.0	–	–	–	–	–
CH_2 twisting	1293.1	0.5	7.9	10.6	9.9	11.0	10.9	9.4
CH_2 rocking	1168.0	0.8	12.3	9.5	8.7	11.5	10.3	8.2
C—C stretching	1126.7	0.4	10.4	11.6	12.0	12.4	12.4	10.9
C—C stretching	1085.8	2.9	10.1	–	–	–	–	–
C—C stretching	1059.7	0.4	10.1	11.7	11.0	12.5	11.8	11.2
Area (a.u.)								
CH_2 bending	1459.1	0.9	8293.4	15,717.3	12,403.3	8315.5	12,592.1	13,566.5
CH_2 bending	1437.2	0.3	16,117.4	14,619.8	19,795.7	13,066.7	20,655.2	16,176.7
CH_2 bending	1414.8	0.7	2036.1	4327.2	1937.3	2038.8	2307.0	3705.7
CH_3 wagging	1369.0	5.7	897.7	–	–	–	–	–
CH_2 twisting	1293.1	0.5	11,881.9	14,499.1	14,441.7	11,296.7	15,161.2	19,814.5
CH_2 rocking	1168.1	0.8	746.8	1227.1	860.8	827.9	898.6	1290.2
C—C stretching	1126.7	0.4	4433.3	5385.3	4718.8	3724.7	4833.7	5673.3
C—C stretching	1080.9	1.7	1686.9	–	–	–	–	–
C—C stretching	1059.8	0.3	5509.8	6277.9	5068.7	4285.7	5666.1	6717.5

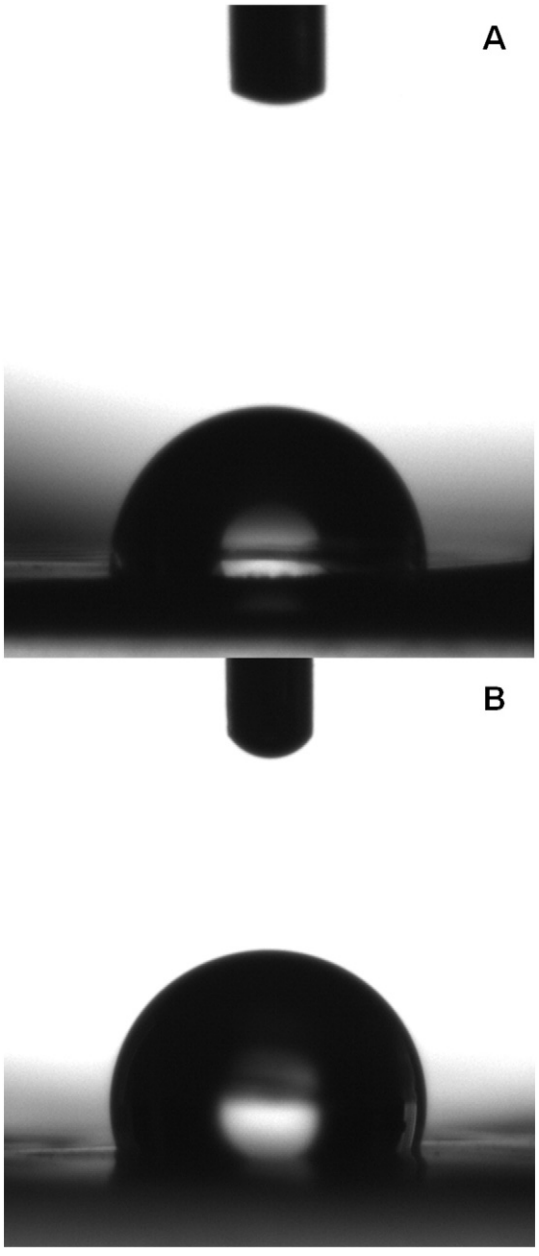

Fig. 9. Droplet of water on non-treated pristine (A) COMP bag and (B) PE bag for contact angle measurements. COMP = biodegradable bag; PE = polyethylene bag.

the exposure to e.g. sunlight, waves or temperature changes was included. Therefore, biodegradation might have been inhibited as carbon chains were too long to be degraded directly by bacterial activity and not broken down by e.g. photo−/thermal oxidation. Moreover, the lack of UV–radiation and diminished oxygen in the sediment may result in the reduction of both abiotic degradation and breakdown of polymers into smaller carbon fractions, which in turn could then be utilized by other microbes.

5. Conclusion

In our study no signs of biodegradation were detected when polyethylene and biodegradable plastic bags were exposed to marine sedimentary conditions for 100 days. Therefore, plastic waste is likely to accumulate in the deep sediment layers, which might ultimately act as a long-term plastic sink. Though previous studies presented data on partial degradation of biodegradable plastic products in the marine system, there are no reports on a complete biodegradation. We therefore emphasize that the development of plastic products that are degradable by natural marine aerobic and anaerobic microbial communities over short time scales, are necessary to avoid accumulation of plastic waste in the ocean. Further research is required to obtain more information on bacterial communities growing and living from different types of anthropogenic biodegradable substances.

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