# Interaction between marine benthic bacteria and plastic/compostable carrier bags: settlement, alteration, and degradation processes

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## Abstract

Plastic production and pollution have increased dramatically since the last 60 years. As plastic products are produced to be very resistant against biological degradation, they remain in the marine environment for decades and cause numerous harms to marine organisms. As alternative to plastic, compostable and bioplastic materials were developed. As bacteria are extremely abundant in the ocean, their potential contribution to biological degradation of plastic is of great interest. So far, to our knowledge, the settlement on plastic and its biological degradation by marine benthic bacteria is virtually unknown. The present study focuses on the settlement and biological degradation of aerobic and anaerobic benthic bacteria on a polyethylene (PE) and compostable carrier bag material. As the degradation process of plastic can be accelerated by UV-light, bags with and without prior exposure to UV-radiation were compared with regards to bacterial settlement under laboratory conditions. In a first experiment, slurries from natural oxic and anoxic sediments from Eckernförde Bay were incubated with pieces of PE and compostable bags. A second incubation experiment, using PE and compostable material, was carried out with pure cultures of *Alcanivorax borkumensis*, representative for the aerobic bacteria, and the anaerobic bacterium *Desulfovibrio marinus*. Analyses included epifluorescence microscopy for cell quantification on the bags and for the investigation of biological degradation weight-loss, scanning electron microscopy (SEM) and RAMAN spectroscopy. The experiment including slurries showed that cell densities were statistically significantly ($F = 17.33$, $p = 0.0001$) higher on compostable bags than on PE bags. Bags not exposed to UV-light had significantly ($F = 37.613$, $p = <.0001$) higher cell densities than UV-treated bags. Furthermore, aerobic bacteria settled in significantly ($F = 106.127$, $p = <.0001$) higher densities on the bags than anaerobic bacteria. Weight-loss analyses, SEM images and Raman spectroscopy of both bag materials showed no signs of microbial degradation. In the second experiment, *D. marinus* settled in multi-layer biofilms on both bag types while *A. borkumensis* showed considerably lower cell densities. Also after incubation with pure cultures biological degradation could not be observed. The compostable bags might have been higher colonized due to higher surface

roughness and, assumingly, due to stronger physicochemical interactions between bag and cell surfaces. Anaerobic bacteria in the slurry experiment might have settled on bags in lower densities due to a lower metabolic rate than aerobic bacteria. UV-exposure of the bags did not accelerate bacterial settlement and biological degradation as the exposure duration might have been too short. *D. marinus* showed a fast biofilm formation, assumingly due to strong physicochemical interactions between bags and *D. marinus*, leading to a faster initial attachment. In conclusion, no biological degradation was observed in the two experiments. Even the, supposedly 100 % biodegradable, compostable bags did not show any sign of biodegradation. Also compostable bag material might therefore remain in the marine system for a long time, potentially causing similar harms as PE material. It can also be assumed that plastic debris will remain longer in anoxic marine environments than in oxic conditions due to slower bacterial activity.

# Content

# List of Tables

# List of Figures

# 1 Introduction

The annual plastic production increased from 1.7 million tons in 1950 to 288 million tons in 2012 globally (PlasticsEurope, 2013). Polyethylene (PE) is the most used synthetic polymer with a yearly production of approximately 140 million tons (Sivan, 2011). Generally, plastic consists of synthetic organic polymers which derived from petroleum and is produced to resist aging and biological degradation (Rios, Moore, & Jones, 2007). Plastic debris reaches the marine environment through sea-based and land-based sources (Cheshire et al., 2009) due to improper waste management, accidental loss and by natural catastrophes. Of all marine debris, 60 to 80 % consists of plastic (Gregory & Ryan, 1997). Marine pollution has been observed in remote regions, such as Antarctica (Gregory et al., 1984) and the ocean gyres (Moore et al., 2001). Most of the PE, which accumulates on shorelines, ocean surfaces and seabeds is in the form of plastic films such as carrier bags (Barnes et al., 2009).

Marine organisms can be harmed by plastic debris due to ingestion and entanglement (Moore, 2008). Moreover, macroplastic ( >5 mm; Moore, 2008) undergoes fragmentation and forms microplastic ( <5 mm; Moore, 2008) (Ng & Obbard, 2006). Microplastic can be taken up by zooplankton (Cole et al., 2013), filter feeders and transferred to predatory organisms up the food chain (Farrell & Nelson, 2013). Due to plastic sustainability in the environment and threat to organisms, alternatives to synthetic polymers are developed and tested (O'Brine & Thompson, 2010). Different kinds of plastic alternatives can be found on the market, such as natural plastics produced by microorganisms, plastics with polymer blends, such as starch and photo-biodegradable plastics (Shah et al., 2008).

In marine habitats, microbes are extremely abundant and capable of decomposing complex organic matter. Hence, the question arises if microbial degradation of plastic debris is generally possible and might represent an important process in marine environments. Several studies already stated the biological degradation of synthetic plastics by microorganisms such as *Pseudomonas* sp. B2 (Artham & Doble, 2009), *Bacillus cereus*, *B. sphericus* (Sudhakar et al., 2007a) and *Clostridium* spp. (Sudhakar et al., 2007b). So far, most studies focused on pelagic bacteria and degradation of plastic in

the upper ocean layer. Supposedly, plastic waste, such as PE carrier bags, reaches the ocean; it is buoyant due to its hydrophobic surface and low density and will therefore float on the ocean surface for a long time (Fig. 1). On the ocean surface, plastic is exposed to UV-radiation, oxygen and heat. UV-radiation initiates photo-oxidative degradation and leads to a decreasing molecular weight (Andrady, 2011). Furthermore, thermal degradation through high temperatures can cause molecular scission (Shah et al., 2008). The abiotic degradation of plastic can therefore change the physical and chemical properties of polymers.
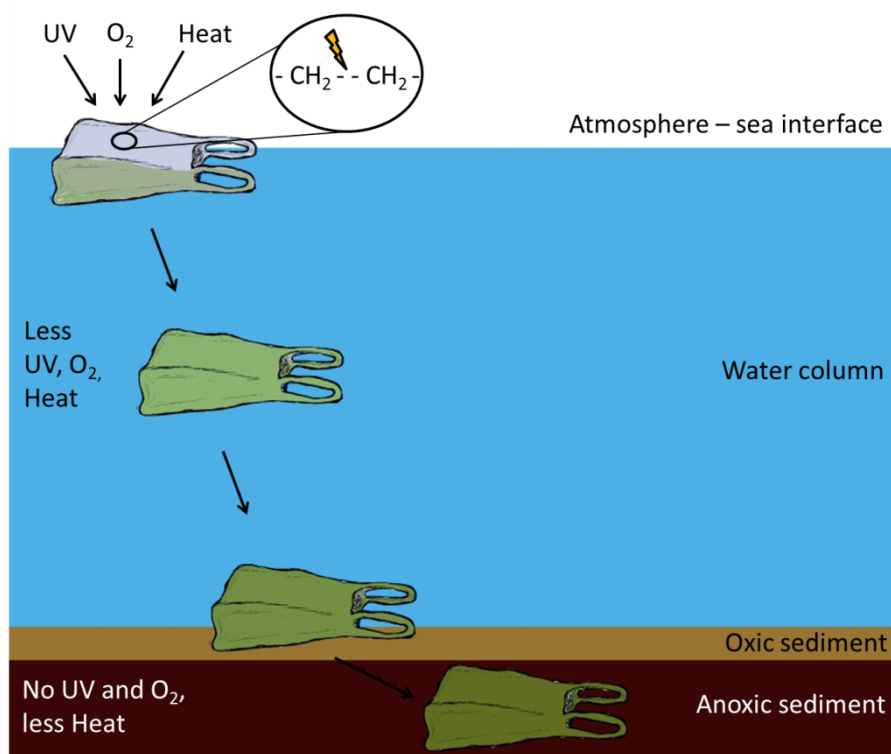


**Figure 1.** The path of a carrier bag from the ocean surface to the sediment. Due to abiotic degradation (by UV-radiation, oxygen and heat), and the settlement of marine organisms, the plastic material sinks to the ocean floor.

Furthermore, biofilm formation on the plastic surface will take place as it is exposed to seawater. A biofilm is a microbial assemblage encased by mainly polysaccharide material and irreversibly attached to a surface (Donlan, 2002). Subsequent to the biofilm, marine flora and fauna will colonize the surface as well (Muthukumar et al., 2011). Muthukumar et al. (2011) state that initial fouling on surfaces is directly proportional to the surface hydrophobicity and turns the surface hydrophilic with time. Biofouling therefore

increases hydrophilicity and weight of plastic materials. After approximately three weeks of floating on the ocean surface, PE bags start to sink below the seawater- air interface and become neutral buoyant (Lobelle & Cunliffe, 2011). Sooner or later the increasing hydrophilicity and weight will lead to the sinking of plastic material into the water column. Not only the change of plastic surface leads to sinking, also wind-mixing causes vertical distribution of plastic (Kukulka et al., 2012). Eventually, plastic sinks to the ocean floor. As oxygen concentration decreases and light vanishes with depth, it is likely that longevity of plastic increases (Barnes et al., 2009).

Most plastic monitoring studies focused on the ocean surfaces, beaches and shallow sea floors (NRC, 2009). Although little is known about the dimension of plastic pollution in areas less accessible, such as sea floors >30 m (Watters et al., 2010), Barnes et al. (2009) state that plastic debris has been found on the sea floor of every oceans. For example, Koutsodendris et al. (2008) showed a mean marine litter distribution of 72–437 Items/$km^2$ on the sea floor at four Greek Gulfs of which 56 % consisted of plastic litter. Galgani et al. (1996) found debris ranging from 0 to 78 pieces per $ha^{-1}$ on the continental slope and bathyal plain of the northwestern Mediterranean Sea. More than 70 % of the total debris consisted of plastic bags. On the ocean floor, plastic material remains in the oxic sediment layer first but due to sedimentation, it can be buried into the anoxic sediment layer. It is unknown if degradation of plastic is even more reduced in the anoxic sediment layer due to the lack of oxygen and light. As microorganisms in the sediment largely control the carbon sequestration and nitrogen conversion (Wu et al., 2008) and are therefore important drivers of the global biogeochemical cycles (Strom, 2008), it is essential to investigate their possible contribution to plastic biological degradation.

So far, the only data about benthic bacterial settlement and degradation of plastic debris was published by Kumar et al. (2007) and Tosin et al. (2012). Kumar et al. investigated the bacterial colonization on plastic carrier bags exposed to mangrove soil. Tosin and colleagues tested the mechanical and biological degradation of a PE and a biodegradable carrier bag in a simulation of the eulittoral and sandy sublittoral zone. Mangrove soil, as well as sediment in the eulittoral and sublittoral zone does not represent the condition of marine sediment dominating most of the ocean floor. Typical marine sediment is not as productive and exposed to sunlight as mangrove soil, neither is

it exposed to wave turbulences as in the eulittoral zone. The sublittoral zone is indeed covered by seawater, however, the experiment by Tosin et al. (2012) was conducted between the water-sediment interface and not within the sediment. So far the bacterial settlement and degradation of plastic in the anoxic sediment is unknown (Harrison, Sapp, & Schratzberger, 2009; Tosin et al., 2012). This information is important in order to clarify the fate of buried plastic in typical marine sediment.

The present study focused on the settling pattern of benthic aerobic and anaerobic bacteria on plastic and the possible use of plastic as a carbon source. The study examined the fate of a PE and a compostable carrier bag (as alternative to plastic) in the marine sediment. This study also included a comparison between carrier bags, which were either exposed or not exposed to UV-radiation prior to microbial settlement experiments in order to discover favored settling grounds. In order to answer the questions formulated below, two laboratory experiments were conducted. The first experiment was conducted with sediment from Eckernförde Bay as natural environment and the second experiment concentrated on a selected aerobic and anaerobic bacteria culture. This is, to our knowledge, the first study analyzing the fate of carrier bags in oxic and anoxic marine sediments.

The following study questions are aligned:

1. Do marine benthic bacteria settle in different densities on compostable and PE bags?
2. Do marine benthic aerobic and anaerobic bacteria settle in different densities on the bags?
3. Do marine benthic bacteria settle in different densities on UV-treated and untreated bags?
4. Does biological degradation of the bags take place?

## 2 Material and Methods

### 2.1 Preparations of carrier bags

Commercially available Polyethylene (PE) carrier bags and compostable bags offered by the company "SWIRL" were used (Fig. 2A). The compostable bags were stated to be 100 % biodegradable and consisted of > 50 % biodegradable Polyester and > 20 % corn starch (personal correspondence with Swirl) and met the compostability requirements EN 13432 according to DIN CERTCO.

For the laboratory experiments, the bags were cut into 1 x 2 cm square-shaped pieces and fixed to a short stainless steel wire. The wire was bend to a bail on one side for better sample transfer and additional sample weight to create negative buoyancy (Fig. 2B).
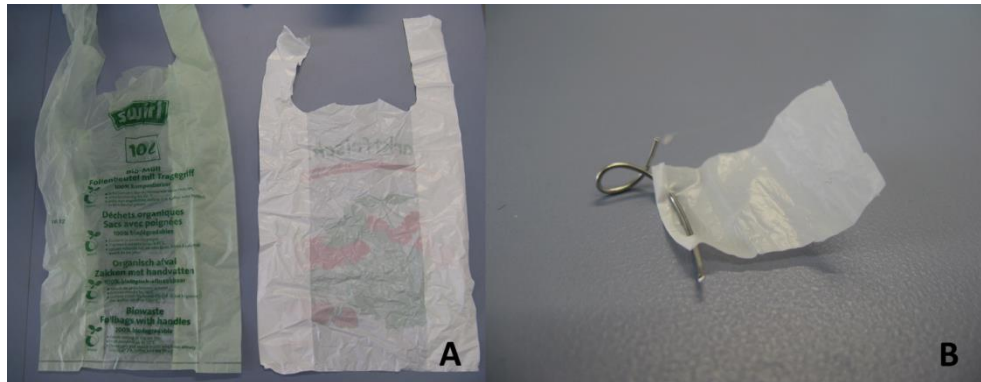


**Figure 2. A.** The two carrier bags used in this experiment; left: compostable bag; right: PE bag. **B.** 1 x 2 cm square-shaped bag sample fixed to wire.

The bag samples fixed to the wire were sterilized with 70 % ethanol for two minutes, washed with ultrapure water Type 1 ISO 3696 for two minutes and then placed into autoclaved 200 ml serum vials.

## 2.2 Preparations 1.Experiment with sediment slurries

### 2.2.1 Treatments

For the first experiment, the chosen bags were either UV-treated (as described in section 2.2.2) or untreated before the start of the experiment. Four different sample types were generated: UV-treated PE sample, untreated PE sample, UV-treated compostable sample and untreated compostable sample. Each sample type was placed in oxic and anoxic sediment slurries (as described in section 2.2.3) and kept under oxic/anoxic condition during the experiment. A scheme of all treatments is presented in Fig. 3.
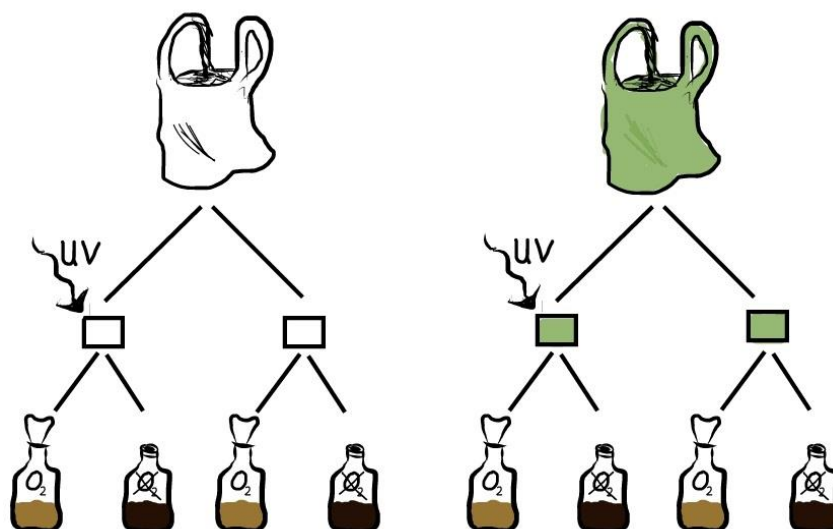


**Figure 3.** Scheme of treatments for 1.experiment with sediment slurries. PE and compostable bags were cut into pieces and either UV-treated (UV) or untreated. The four generated sample types were placed in oxic sediment slurry (light brown sediment) and anoxic sediment slurry (dark brown sediment).

Before the start of the experiment, six equally treated bag samples (preparation as described in section 2.1) were placed into one autoclaved 200 ml serum vial, resulting in eight different set ups (Fig. 3). The oxic treatments were closed with autoclaved cotton plugs in order to allow gas exchange with the atmosphere and to maintain oxic conditions. Furthermore, the cotton plug was covered with aluminum foil to avoid dust

settling on the plug and bottle opening (Fig. 4A). For the anoxic treatments, the vials were closed with butyl rubber stoppers and crimped to prevent gas exchange (Fig. 4B). Afterwards, the anoxic treatments were gassed with $N_2/CO_2$ (80/20) for fifteen minutes to achieve anoxic conditions. All treatments were filled with either 60 ml oxic or anoxic sediment slurry (as described in section 2.2.3). The transfer of anoxic slurry into the vials took place inside the Innovative Technology Inert Lab glovebox.

Each treatment was replicated three times and handled identically. Both oxic and anoxic treatments were incubated at 10 °C in the dark.
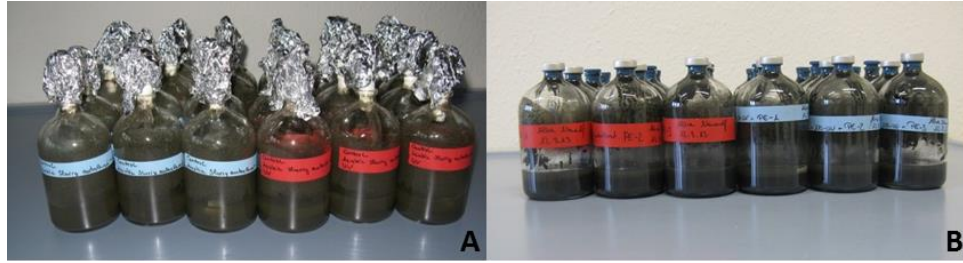


**Figure 4.** Treatments (including controls) of the first experiment. **A.** Oxic treatments. **B.** Anoxic treatments.

### 2.2.2 UV-treatment

In order to investigate the influence of UV-radiated carrier bags on bacterial settlement, the bags were UV-treated before the start of the experiment. For this purpose the duration time of a carrier bag on the ocean surface was estimated to be at least two weeks. Therefore, a UV-treatment, simulating the received solar energy of two weeks, was chosen. The value for the average daily UV-dose in Germany was extracted from the annual report of the Bundesamt für Strahlenschutz. Approximately 3000 J/m$^2$ was the average daily dose of erythema effective radiation in July 2009 and used in this experiment. For the pretreatment, a UV-lamp (VL – 260 G) with a wavelength of 254 nm and the energy of 1400 µWatt/cm$^2$ was used. In order to transfer the given values to a two week irradiation dose using the described UV-lamp, the following equation was used:

$$s = \frac{J}{W}$$

*J* is the abbreviation for Joule, *W* for Watt and *s* for time in seconds. The bags were irradiated for 49 minutes for a two week UV-dose. In order to recognize the UV radiated side of the samples, the right corner of the wire-fixed sample side was cut off.

### 2.2.3 Sediment slurry preparation

Sediment cores were taken with a mini multi corer (MUC) on board of the research vessel "Littorina" at the long-term monitoring station "Boknis Eck" (54°31.2' N, 10°02.5' E) located in the Eckernförde Bay in February 2013.

**Oxic sediment slurry**

For the oxic sediment slurries, the lighter parts of the sediment cores (0.5 to 1 cm sediment depth) were removed and transferred into sterilized duran glass bottles. The bottles were closed with cotton plugs and cooled at 5 °C until further use. A seasalt medium was chosen in order to prepare the slurries. Seasalt was added to a 1 l duran glass bottle filled with 1 l ultrapure water. As Kiel Bay possesses a salinity of around 18, the salinity of the medium was adjusted to that value by adding the seasalt and checking the salinity with a refractometer. The pH was adjusted to 8.3. The bottle was closed with a screw cap and autoclaved. Afterwards 250 ml of medium and 250 ml oxygenized sediment were filled into sterilized 1 l duran glass bottles. Two bottles of oxic slurries were prepared. The bottles were closed with a cotton plug and covered with aluminum foil (for the same reason as mentioned in 2.2.1).

**Anoxic sediment slurry**

For the anoxic slurries the darker, hydrogen sulfide-smelling sediment from 5-10 cm sediment depth was removed from the MUC core liner and transferred into sterilized duran glass bottles. The bottles were closed with butyl stoppers and screw caps and kept at 5 °C until further use. In order to prepare the anoxic sediment slurries, the *196. Modified Desulfobacter postgatei* medium (medium recipe by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ)) was selected. The ingredients for the 196. Modified medium are listed in table 1. Magnesium and

calcium concentrations were adjusted to seawater quality (table 1) and lactate was not added due to organic rich sediment. Solution A was boiled for a few minutes, gassed with $N_2/CO_2$ (80/20) for 15 minutes and autoclaved. Solution C and F were gassed with $N_2/CO_2$ (80/20), solution F was autoclaved whereas solution B, C, D and E were sterile filtered. The vessels of the gassed solutions were always closed with either rubber stoppers and screw caps or rupper stoppers and crimped in order to maintain anoxic conditions. Solution B − F were added to solution A via injection in the sequence as indicated. The pH was adjusted to 7.1 − 7.4 either using HCl for reducing or NaOH for raising the pH.

**Table 1** Ingredients for one liter 196. Modified medium (DSMZ).

| Solution A | |
| --- | --- |
| $Na_2SO_4$ | 3.00 g |
| $KH_2PO_4$ | 0.20 g |
| $NH_4Cl$ | 0.30 g |
| NaCl | 21.00 g |
| $MgCl_2$ x 6 $H_2O$ | 10.83 g |
| KCl | 0.50 g |
| $CaCl_2$ x 2 $H_2O$ | 1.53 g |
| Resazurine | 0.50 ml |
| Distilled water | 870.00 ml |
| **Solution B** | |
| Trace element solution SL-10 (see medium 320 in DSMZ) | 1.00 ml |
| **Solution C** | |
| $NaHCO_3$ | 5.00 g |
| Distilled water | 100.00 ml |
| **Solution D** | |
| Na -Lactate | 2.50 g |
| Distilled water | 10.00 ml |
| **Solution E** | |
| Vitamine solution | 10.00 ml |
| **Solution F** | |
| $Na_2S$ x 6 $H_2O$ | 0.40 g |
| Ultrapure water | 10.00 ml |

In order to maintain anoxic conditions in both medium and sediment during slurry mixing, the slurry preparation was conducted inside the glovebox. Two sterilized 1 l duran glass bottles were each filled with 250 ml medium and 250 ml sediment. The bottles were closed with butyl stoppers and screw caps.

Oxic and anoxic sediment slurries were stored at 5 °C before the start of the experiment. At the beginning of the experiment, 60 ml of either oxic or anoxic sediment slurry were transferred into prepared serum vials (as described in section 2.2.1). As two bottles of slurry for each slurry type were prepared, slurry of both bottles was transferred into each vial in order to have homogenous slurries in each treatment.

### 2.2.4 Controls

Controls with sterilized sediment were prepared for all treatments to discriminate between effects appearing on bag samples caused by live and dead bacteria in the slurries. Therefore, 1 l of each oxic and anoxic sediment slurry stored in 1 l duran glass bottles was autoclaved at 121 °C for 35 minutes to kill all living cells. Treatments with autoclaved sediment were prepared using the same treatment scheme as shown in Fig. 3.

**Table 2** Overview of controls used for experiment 1.

| Control | Purpose | Analyses |
|---|---|---|
| **1.Control: autoclaved sediment slurries** | Compare bag samples incubated in slurry with living bacteria with bag samples incubated in slurry with dead bacteria | Weighing of bag sample, scanning electron microscopy (SEM), RAMAN spectroscopy (section 2.5.2, 2.5.4 and 2.5.5) |
| **2.Control: no bag samples** | Compare bacterial turnover activity without bag samples and with bag samples | Total alkalinity (TA) and Total sulfide (TS) (section 2.5.3) |

Furthermore, to investigate whether the bag materials have an influence on the bacterial activity, active sediment slurries without bag pieces were prepared. Table 2 summarizes the selected controls and for which analyses they were included. The controls were treated the same way as explained in section 2.2.1. From now on, slurry with living bacteria will be defined as active slurry and slurry with dead bacteria as inactive slurry.

## *2.2.5 Sampling pattern*

As treatments (including controls) sum up to a high amount of vials, the experiment was started, sampled and ended over two days. Hence, oxic and anoxic treatments were sampled on two respective days. Bag pieces of the oxic treatments were sampled after 7, 21, 50 and 98 days of incubation. The anoxic treatments were sampled after 7, 21, 49 and 99 days after experiment start. Total alkalinity (TA) and total sulfide (TS) measurements of the anoxic treatments were conducted after 1, 8, 22, 50 and 98 days of incubation. The experiment ended the same day as the last sampling event.

## 2.3 Preparations 2.Experiment with bacteria cultures

### 2.3.1 Treatments

In this experiment selected bacteria cultures were used. PE- and compostable bag samples were not UV-treated as in the first experiment. The two sample types (PE sample and compostable sample) were placed in oxic and anoxic media with aerobic and anaerobic bacteria cultures. The treatments were kept under oxic/anoxic condition during the experiment. This experiment consisted of four different treatments (Fig. 5).
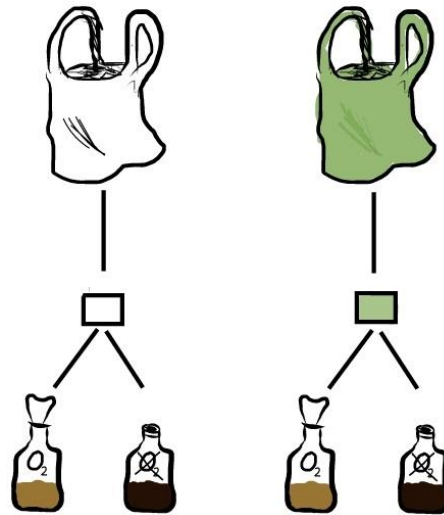


**Figure 5.** Scheme of Treatments for 2.experiment with bacteria cultures. PE and compostable bags were cut into pieces and placed untreated in oxic medium with aerobic bacteria culture (oxic conditions, O2) and anoxic medium with anaerobic bacteria culture (anoxic conditions, $O_2$ crossed out).

The same preparations as explained in section 2.1 and 2.2.1 took place. However, all treatments were filled with either 60 ml oxic or anoxic medium and bacteria culture (see culture volume added to treatments in section 2.3.2) (Fig. 6). Each treatment was replicated three times and handled identically. The treatments were incubated at 20 °C and kept in the dark.
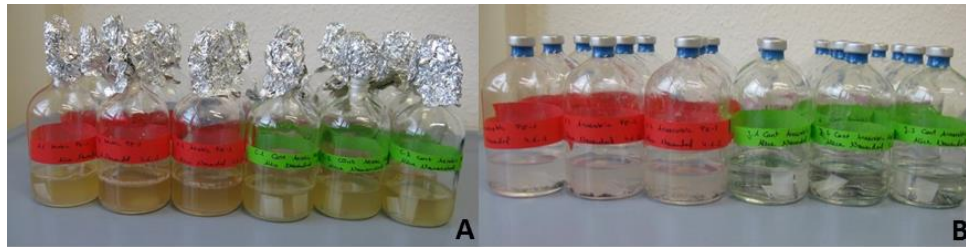
**Figure 6.** Treatments (including controls) of the second experiment. A. Oxic treatments. B. Anoxic treatments.

### 2.3.2 Bacteria cultures and Media

**Aerobic culture and medium**

Pure cultures of the aerobic bacteria *Alcanivorax borkumensis* strain SK 2 were chosen as representatives for the aerobic culture. This rod-shaped marine y-Proteobacterium has been reported as a cosmopolitan species (Golyshin et al., 2003). The *514. Bacto marine broth* medium recipe by the Leibniz Institute German Collection of Microorganisms and Cell Cultures (DSMZ) was used for culturing *Alcanivorax borkumensis* in the laboratory (table 3). All ingredients were directly added to one liter of ultrapure water except for Fe (III) citrate which was boiled separately in 15 ml ultrapure water until dissolution. The Fe (III) citrate was cooled to room temperature and added to the medium. Two times one liter of medium was prepared and autoclaved. The medium had a pH of 7.5 and 7.6, respectively. At the start of the experiment, 60 ml of medium and 50 µl of the active culture (see calculation of culture volume in section 2.3.3) were added to the prepared vials (as described in section 2.3.1) using a pipette and a needle. Subsequently, the serum vials were closed with cotton plugs and aluminum foil.

**Anaerobic culture and medium**

*Desulfovibrio marinus* was chosen as representative for an anaerobic bacteria culture. *Desulfovibrio marinus* is a strictly anaerobic sulfate-reducing bacterium occurring in single vibrio shapes and in chains (Dhia Thabet et al., 2007). *Desulfovibrio marinus* was kept in *196. Modified desulfobacter postgatei* medium (DSMZ) (table 1). Two liters of medium were prepared the same way as explained in section 2.2.3 and adjusted to a pH

of 7.1 and 7.3, respectively. At the start of the experiment, 60 ml of medium and 1 ml of culture (see calculation of culture volume in section 2.3.3) were added to the prepared vials (as described in section 2.3.1) by injecting with a needle through the rubber stoppers.

**Table 3** Ingredients for one liter 514. Bacto marine broth medium.

| | |
|---|---|
| Meat peptone | 5.00 g |
| Bacto yeast extract | 1.00 g |
| Fe (III) citrate | 0.10 g |
| NaCl | 19.45 g |
| $MgCl_2$ (anhydrous) | 5.90 g |
| $Na_2SO_4$ | 3.24 g |
| $CaCl_2$ x $2H_2O$ | 2.39 g |
| KCl | 0.55 g |
| $NaHCO_3$ | 0.16 g |
| KBr | 0.08 g |
| $SrCl_2$ x 6 $H_2O$ | 57.00 mg |
| $H_3BO_3$ | 22.00 mg |
| Na-silicate 36% | 2.90 µl |
| NaF | 2.40 mg |
| $(NH_4)NO_3$ | 1.60 mg |
| $Na_2HPO_4$ x 2 $H_2O$ | 10.00 mg |

### 2.3.3 Counting of cell concentration

The two bacteria species cultured in the laboratory presumably contained different cell densities in the culture vessels. Therefore, the cell concentration had to be investigated in order to inject approximately the same amount of cells of both cultures to the treatments at the beginning of the experiment. 20 µl of each culture was mixed with 5 ml Phosphate Buffered Saline (PBS) in the filtering column and filtered on Whatman 0.2 µm Nuclepore filters. The filters were stained with 4′,6-Diamidin-2-phenylindol (DAPI; 1 µl DAPI / 1ml ultrapure water) for ten minutes in the dark. Afterwards, filters were washed in PBS for five minutes, dried and placed on an object slide in the dark. One droplet of Citifluor® was applied on each filter in order to avoid rapid photobleaching of the dye. A

cover slip was placed on top of the filters. The object slides containing the filters were stored at -20 °C until the counting took place. Counting was carried out using a Leitz Aristoplan epifluorescence microscope and filter cube A4. For the cell number enumeration, either 70 grids (equalling 0.847 mm$^2$) or 800 cells were counted.

*A. borkumensis* had a 20.5 times higher cell number than the anaerobic culture *D. marinus*. Therefore the injection volume was 50 µl for the Alcanivorax and 1 ml for the Desulfovibrio culture at the start of the experiment.

In order to investigate the initial and final cell concentration in the treatments right after start and end of the experiment, a volume of 0.5 ml medium was removed from each bottle. The final sampling took place five days after the experiment had ended but assuming that the cells reached its stationary phase, a considerable change of cell density during the five days was not expected.

In order to fix the samples until filtration, cell staining and cell counting took place, cells were killed using 1.5 ml 4 % Formaldehyde-PBS. The samples were mixed and stored in the fridge for two hours. Afterwards, the mixture was centrifuged for ten minutes at 10000 g with an Eppendorf 5424 Centrifuge at room temperature. The supernatant was carefully removed and the remaining pellet was washed with 1.5 ml PBS, mixed and centrifuged. The washing process was repeated for three times before adding a 1.5 ml PBS-Ethanol solution (1:1). The fixed samples were stored at -20 °C until the evaluation of the cell densities took place. For the investigation of the cell concentrations, the frozen, fixed samples were thawed and re-suspended. The same method mentioned in the previous paragraph was applied but only a sample volume of 100 µl was filtered instead.

This method was also applied for the cell-free control to check for possible contamination of other cells and for the control treatment without bag samples to monitor cell culture growth (see section 2.3.4 for control explanation). For the cell-free control, only the replicate with the highest contamination, investigated with the live/dead stain method (see section 2.5.6 for description) was used. This method was not applied to the control treatment with dead cells to check for contamination of living cells. As the control was injected with dead bacteria and DAPI stains living and dead cells, it is therefore not possible to discriminate living from dead cells and check for contamination.

## 2.3.4 Controls

For the second experiment, controls were used in order to verify whether appearing effects in the treatments were caused by living bacteria, dead bacteria or medium. Table 4 lists all three controls chosen for this experiment.

The first control was chosen to identify the influence of dead bacteria on the bag samples. For the preparation of the control, a 4 % Formaldehyde-PBS-solution was used to kill all living cells. 6 ml of *D. marinus* and 1 ml of *A. borkumensis* were removed from the culturing vessels. The removed aliquot of *D. marinus* culture was divided into three centrifuge tubes (2 ml into each tube) which were filled with 6 ml of 4 % Formaldehyde-PBS-solution. The removed volume of *A. borkumensis* was added to 3 ml 4 % Formaldehyde-PBS-solution also in a centrifuge tube. All samples were thoroughly mixed and stored at 7 °C in the dark. After two hours, all cells were assumed to be dead and the samples had to be washed in order to clear the solutions off formaldehyde. The mixture was centrifuged for ten minutes at 4400 g with an Eppendorf Centrifuge 5804R. The supernatant was carefully removed and 1 ml of oxic medium for the aerobic culture and 6 ml of anoxic medium for the anaerobic culture were added to the pallet to wash the cells. Again, the solutions were mixed and centrifuged.

**Table 4** Overview of controls used for experiment 2.

| Control | Purpose | Analyses |
|---|---|---|
| **1. Control: dead bacteria** | Compare bag samples incubated with dead bacteria cultures and bag samples incubated with living bacteria cultures | Weighing of bag sample, scanning electron microscopy (SEM), RAMAN (section 2.5.2, 2.5.4 and 2.5.5) |
| **2. Control: no bacteria** | Compare bag samples incubated only with medium with bag samples incubated with bacteria cultures | Weighing of bag sample, SEM, RAMAN (section 2.5.1, 2.5.4 and 2.5.5) |
| **3. Control: no bag samples** | Compare bacterial turnover activity without bag samples and with bag samples | TA and TS (section 2.5.3) |

The washing step was repeated three times. In the end, medium was filled up to the original sampling volume and mixed. 50 µl of dead *A. borkumensis* culture and 1 ml of

dead *D. marinus* culture (see section 2.3.3 for volume explanation) were injected into prepared serum vials explained in section 2.3.1. From now on, cultures with living bacteria will be defined as active culture and cultures with dead bacteria as inactive culture.

To investigate the influence of the oxic and anoxic medium on the bag samples, a second control was utilized. Accordingly, same preparations as mentioned in section 2.3.1 took place only that the bacteria cultures were not added.

For the two controls the same treatment scheme as shown in Fig. 5 was used. Furthermore, to identify whether the bag samples have an influence on the bacterial turnover activity, a third control including only medium and bacteria was prepared. All treatments were treated the same way.

### 2.3.5 Sampling pattern

Both oxic and anoxic treatments (including controls) were sampled 2, 6, 13 and 29 days after beginning of the experiment. Bag pieces of the first and second control were only removed at the first and last sampling event. TA and TS measurements were conducted one day after start of the experiment/every sampling event and were only measured for anoxic treatments. The experiment was finished after 29 days.

### 2.4 Sampling

For both experiments, one bag sample of each treatment was removed at each of the sampling events mentioned in sections 2.2.5 and 2.3.5. In order to avoid oxygenation of the anoxic treatments, sampling of those took place inside the glovebox with an oxygen level < 1 ppm.

To remove one bag sample, a sterile inoculating loop, reformed to a hook in order to reach the bag sample on the bail of the wire, was used. The bag pieces were removed from the wire and carefully washed in PBS in order to remove sediment/medium and non-attached bacteria. The bag samples removed inside the glovebox were washed and placed in a sterile petri dish. After all three replicates of one treatment were sampled; the

removed bag pieces were transferred outside the glovebox for further processing. All bag samples were cut into four equal parts with a sterilized scalpel for cell-count, Scanning Electron Microscopy (SEM) (with and without biofilm) and RAMAN spectroscopy. The cell-count sample was stained immediately after sampling (for further description see section 2.5.1) and the sample for SEM with biofilm was placed into an Eppendorf cap. For the SEM without biofilm and RAMAN spectroscopy, the divided pieces were rinsed with ultrapure water, placed on a paper towel and carefully wiped over the sample. Rinsing and paper-drying was conducted for three times in order to remove the biofilm. The procedure ended with a last time rinsing in order to avoid fibrous material on the sample. The samples were placed into separate Eppendorf caps. The Eppendorf caps with SEM- (with/without biofilm) and RAMAN- samples were frozen at -20 °C until further use.

## 2.5 Analyses

### 2.5.1 Cell density

Each bag sample was stained with 50 µl DAPI and processed according to the protocol used for filters in section 2.3.3. The counting was carried out at the Leitz Aristoplan epifluorescence microscope and filter cube A4. For the cell number enumeration, either 70 grids (equals 0.847 mm$^2$) or 800 cells were counted. With the area of the grids counted and the evaluated cell number, it was possible to calculate the number of cells per cm$^2$.

### 2.5.2 Weighing of bag pieces

Before and after the experiment, one bag sample (without wire) of each treatment was weighted with an electron microbalance Sartorius M3P (accuracy ± 0.001 mg). Therefore the bag pieces for this analysis had to be labeled in order to distinguish them from the other bag samples in the treatments for the second weighing event. All bag samples had four corners, except for bag samples of the UV-treatments in the first experiment, of which one corner was cut off on the wire side. Therefore, one corner of the wire-less side

of the bag pieces for this analysis was cut off before the first weighing was conducted. At the end of the experiments the same sample piece was removed, rinsed with ultrapure water, placed on a paper towel and carefully wiped with the towel. Rinsing and paper-drying was conducted for three times in order to remove the biofilm. The procedure ended with a last rinsing in order to avoid fibrous material on the sample. Subsequently, each sample was placed in a sterile petri dish, closed with parafilm and kept at 20 °C in the dark to dry. The second weighing followed right after the samples were completely dry.

### 2.5.3 Total sulfide (TS) and total alkalinity (TA)

TS and TA were measured for the anoxic treatments one day after experimental start and every sampling event.

**TS measurement**

For the determination of sulfide concentration, the Cline Method (Cline, 1969) was applied. This method is very accurate in low sulfide concentrations ranging from $0 - 50$ µmol/l. As TS in the treatments could be higher than the range just mentioned, the samples were diluted by a factor of either 10 or 20, depending on the concentration measured at the previous sampling event.

For the Cline method, two solutions had to be prepared: an anoxic NaCl solution and a zinc-acetate-gelatine solution. The first solution was made by adding 36 g of NaCl into 1 l of ultrapure water. The vessel was closed with a butyl plug and a screw cap. The solution was then gassed with $N_2$ for one hour by injecting one needle connected to the $N_2$ hose and one needle releasing gas. For the second solution 25 ml of the prepared anoxic NaCl solution was warmed up in a flask and 50 mg of gelatine was added and dissolved before adding 261 mg of zinc acetate. The flask was closed in order to reduce gas exchange during cooling down to room temperature. Meanwhile, 6 ml scintillation vials were filled with 4.25 ml anoxic NaCl solution for a dilution factor of 10 (4.5 ml for a dilution factor of 20) and with 0.25 ml zinc-acetat-gelatine solution.

The sampling of the supernatant of the anoxic treatments was carried out with a needle and a $N_2$ flushed syringe. Supernatant for TS but also for TA measurements was removed

at the same time. For TS and a dilution factor of 10, 500 µl of supernatant (for a dilution factor of 20, 250 µl of supernatant) was sampled. For TA 100 µl was removed and filled into Eppendorf caps. The sample for the sulfide measurement was filled into the scintillation vials with zinc-acetat-gelatine solution and mixed immediately. Sulfide and zinc-acetat-gelatine solution form a complex whereas sulfide becomes insoluble. Lastly, 10 µl of Fe-catalysor and 10 µl of color reagent were added, mixed and rested for one hour until photospectroscopy was conducted. Fe-catalysor accelerates the binding process of sulfide and zinc-acetat-gelatine solution and the color reagent stains the produced complex blue. The absorption by the sulfide/gelatine-acetate complex was determined with the Shimadzu UVmini-1240 UV-VIS spectrometer at a wavelength of 670 nm. The total sulfide concentration was calculated with the received absorption value and the cline calibration equation, also including the dilution factor.

**TA measurement**

The sampled supernatants were measured right after sampling took place. As the second experiment contained bacterial cultures spread in the medium, it was not possible to sample supernatants as done for the first experiment where supernatant was on top of the sediment layer. Therefore, the sampled medium/bacteria mixture of the second experiment was centrifuged at 10000 g for 10 minutes at 4 °C in order to avoid cells manipulating TA measurements. The 876 Dosimat Plus Metrohm device was used to measure the consumed amount of 0.01 M HCl by the titrated sample in order to calculate TA. For this, 20 µl of pH indicator, 50 µl of the sample and approximately 1 ml of ultrapure water were pipetted into the Pavlova vessel. A hose fixed to the vessel supplied $N_2$ and mixed the solution throughout the titration. Then 0.01 M HCl was manually added to the solution in the vessel in 20 µl units. HCl adds $H^+$ to the solution and the pH indicator turns pink once the $H^+$ consumption capacity is exhausted. The titration was stopped when the color of the solution turned to a stable pink. The consumed amount of HCl was noted and used for calculating the TA of the sample.

Before titrating the samples, IAPSO (International Association for the Physical Sciences of the Oceans) Standard Seawater was titrated three times the same way as just described. The sample volume of the IAPSO Standard Seawater for the calibration of TA

was 500 μl. The following equation was used in order to calculate the total alkalinity of the IAPSO:

$$TAcal\ (mmol/l) = 1000 * 1 * average\ of\ 3\ IAPSO\ titrations(ml) * \frac{conc.HCl\ (mol/l)}{sample\ vol.(ml)}$$

With the *TAcal* value the correction factor *F* was calculated as followed:

$$F = \frac{2.325\ mmol/l\ (theoretical\ value)}{TAcal\ (actual\ value)}$$

Afterwards, the consumed amount of HCl by the titrated sample volume of 50 μl was used to calculate the TA value with the last equation given.

$$TA\ (mmol/l) = 1000 * F * Volume\ HCl\ (ml) * \frac{conc.HCl\ (mol/l)}{sample\ vol.(ml)}$$

## *2.5.4 Scanning Electron Microscopy (SEM)*

**Preparation of bag samples with biofilm**

Bag samples with biofilm of each treatment of the first and second experiment from the last sampling event were chosen for SEM analyses. Due to a high number of treatments, only one replicate of each treatment was analyzed. The replicate with the highest cell number in the biofilm (investigated with the method described in section 2.5.1) was selected in order to have a higher probability of imaging a biofilm on the bag samples. For an ideal preservation of the biofilm on the bag samples, critical point drying was applied before SEM.

First of all, the bag samples, which were stored in Eppendorf caps, were thawed and ethanol (99.5%) was added. After 15 minutes, one to two bag samples were placed into one sinter cap and closed with a sinter lid. A total of six caps were transferred into the little vessel of the critical point dryer, filled with ethanol (99.5%) and closed. The vessel was placed into a chamber of the E 3000 Series Critical Point Drying apparatus which was then filled with carbon dioxide ($CO_2$) in order to displace the ethanol and drain the liquid and gas out of the chamber. This flushing process was conducted for five times. Afterwards, the critical point was adjusted to 32 °C and 80 bar. At this point there is no defined phase and liquid, caught in the organic material of the biofilm, will be removed without evaporation taking place, which would risk to damaging the biofilm.

Lastly, the bag samples had to be fixed for SEM analysis whereas an adhesive film was placed on an aluminum stub and the bag sample was fixed on top of it. A conductibility paste (LeitC after Göcke) was applied on each corner of the bag samples in order to enable conductibility to the ground. Moreover, the bag samples, fixed on the stubs, were sputter coated (Leica EM SCD 500) with gold/palladium of 10 nm thickness.

**Preparation of bag samples without biofilm**

Bag samples of each treatment (including controls) of the first and second experiment from the last sampling event were chosen for SEM analysis. This time, the biofilm was rinsed right after the sampling event as describes in section 2.4. For SEM analyses, only one replicate of each treatment was selected. The same replicates, which were chosen for imaging the biofilm were selected (see one paragraph before). For the controls, the replicates with the least cell contamination via live/dead analysis were selected. First of all, the bag samples which were stored in Eppendorf caps were thawed, air dried and placed on aluminum stub with adhesive foil. The conductibility paste was applied on each corner of the bag samples and then sputter coated (Leica EM SCD 500) with gold/palladium of 10 nm thickness.

**SEM Analysis**

The Hitachi S-4800 scanning electron microscope was used to image biofilms and surfaces of the bag samples. For this, one stub was fixed to a transfer pole, which was

pushed into a chamber and transported through a gate chamber system as the main chamber is always under vacuum. The main chamber was equipped with a stub table where the transferred stub was fixed on. Images were taken in the warm-modus, 3 kV and a lower SE-detector.

### 2.5.5 RAMAN spectroscopy

In order to detect changes of the bag materials due to biological degradation, confocal RAMAN spectroscopy was applied after the experiment had ended. This method was only applied for the first experiment. UV untreated PE and compostable bags incubated in oxic sediment slurry were chosen. As controls, the non-incubated PE and compostable bag as well as bag samples incubated in inactive oxic sediment slurry were selected. At the last sampling event the incubated bag samples were removed and fixed as described in section 2.4. Before RAMAN spectroscopy, the bag pieces were thawed in petri dishes and after drying fixed on object slides with tape. The Horiba Jobin Yvon HR 800 spectrometer with a 473 nm laser was used for spectroscopic analyses. An even area on the bag sample was selected in order to keep the bag sample in focus during the measurement. A defined grid was scanned in which every few μm a measurement was taken. For each measuring point one spectrum was obtained. Afterwards, the spectra were compared to check for spatial heterogeneity. Additional single point measurements were made to substantiate observations from Raman mapping. To compare the spectra of the controls and the incubated bag samples, a Gauss-Lorentz profile was fit to selected Raman peaks using the LABspec software. Peak parameters considered were RAMAN shift, amplitude and full width at half maximum amplitude (FWHM).

### 2.5.6 Live/Dead Staining

In order to test whether the controls of both experiments were contaminated with bacteria during incubation and sampling, live/dead staining was applied. For this purpose the Live/Dead BacLight® Bacterial Viability Kit was used. This kit consists of SYTO 9 which labels intact membranes and stains in fluorescent green and propidium iodine, which labels damaged membranes and shows a red fluorescence. A 1:1 mixture of

live/dead working solution and the applicable medium (depending on type of control) was prepared. 50 µl of working solution were then applied on to each control bag sample. After fifteen minutes of incubation in the dark at room temperature, the samples were shortly washed in PBS, dried and placed on a glass slide. Before placing the cover slip, one droplet of BacLight® mounting oil was added on top of the sample. The samples were analyzed with the same microscope mentioned in section 2.5.1 with the filter cube I3 right after staining. Either 50 grids (equals 0.605 mm$^2$) or 800 dead and alive cells were counted.

### 2.5.7 Statistical and graphical analyses

The statistical analysis was conducted using the software R (R Core Team, 2013), including the packages nlme and lattice (Pinheiro et al., 2013; Sarkar, 2008). The experiments had a multi-factorial design with repeated measurements. For the analysis of variance of the cell density results in the first experiment, a linear mixed model was applied in order to include all factors (bag (PE and compostable bag), environment (oxic and anoxic slurry), UV (UV-treated and untreated bags) and time). As replicates were repeatedly measured during the experiment, the test power of the model could be increased artificially. Therefore, the replicates were specified as random factor in the linear mixed model. Furthermore, the cell density as response was log-transformed in order to fulfill the assumptions, homogeneity of variances and normal distribution of the residuals, of the model. For the other responses and the second experiment, only specific data and factors were chosen to run statistical analyses. Therefore, a less complex, linear model was chosen for the analysis of variance. For significant results variance homogeneity and normality were proven graphically (Zuur et al., 2009). Tukey HSD post-hoc test was used to identify the direction of the effect if the analysis of variance revealed significant effects. The graphs were generated with the ggplot function using the packages ggplot2, scale, grid Extra and plyr (Wickham, 2009; Wickham, 2012; Auguie, 2012; Wickham, 2011).

# 3 Results

## 3.1 1.Experiment with sediment slurries

### 3.1.1 Cell density

The temporal biofilm development on the carrier bags in the first experiment is summarized in Fig. 7. The cell densities on the bags were significantly influenced by time (F = 43.38, p < 0.0001; table 5). Tukey HSD post-hoc test revealed that cell density increased significantly from day 7 to the last respective sampling day for UV-treated, untreated compostable bags incubated in oxic sediment slurry as well as the untreated compostable bags in anoxic slurry (p = 0.01; p = 0.0006; p = 0.03, respectively).
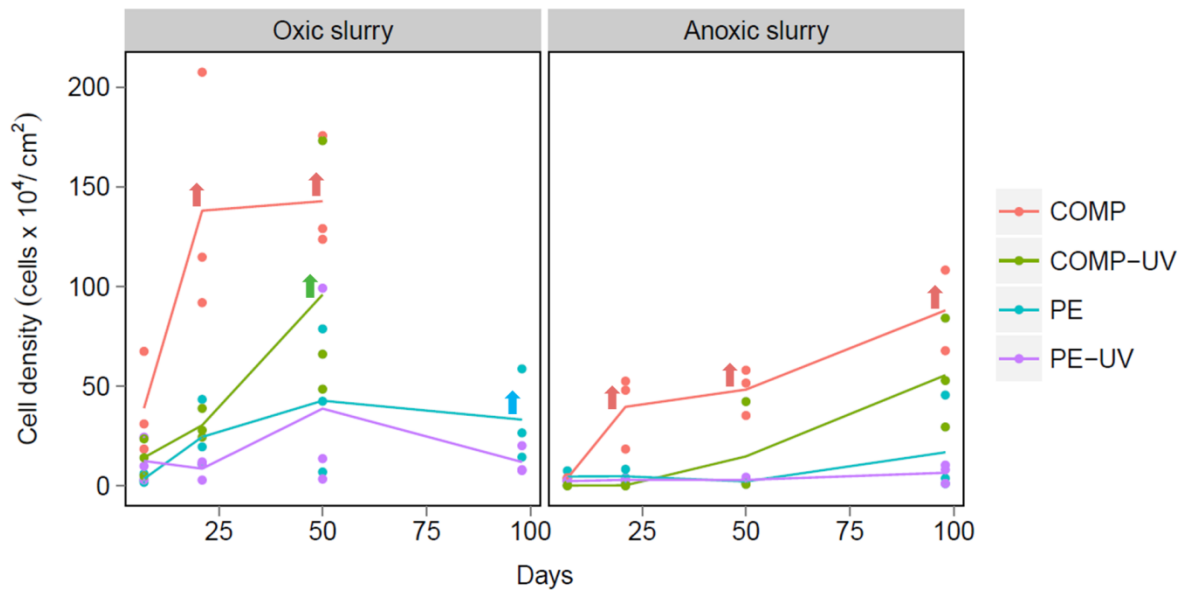


**Figure 7**. Temporal development of cell densities on PE and compostable bags in the different treatments. The arrow indicates a multi-layer biofilm on the bag sample in the treatment. Values are missing for the UV-treated and untreated compostable bag incubated in oxic slurry at the last sampling as the biofilm was too voluminous for reliable cell quantification. Single replicates (points) with calculated mean (line). COMP = compostable bag; PE = PE bag; COMP-UV/PE-UV = UV-treated bags.

All other treatments, except for the UV-treated PE bag (PE-UV) in oxic conditions, showed an increasing trend. Several data points were designated with arrows to indicate a

patchy multi-layer biofilm in the treatment at that sampling event. This signifies that counting of cells was still possible at several regions but the overall cell number is expected to be higher as spots with a thick biofilm were avoided (e.g. PE bag in oxic sediment slurry at the last sampling point). On UV-treated and untreated compostable bags in the oxic sediment slurry counting was not possible at the last sampling event due to a multi-layer biofilm (Fig. 8B).
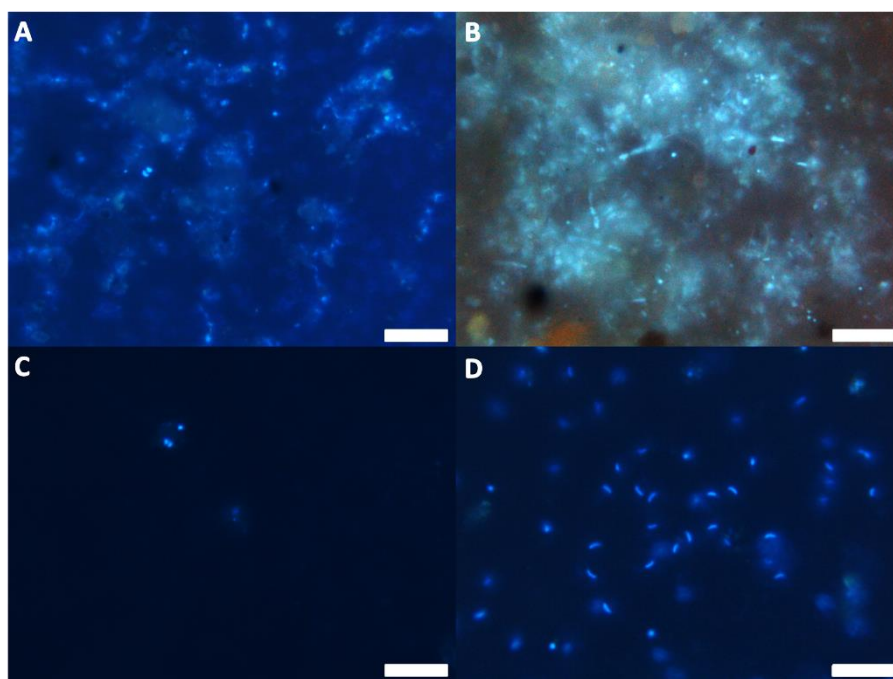


**Figure 8. A.-B.** Biofilm on compostable bag incubated in oxic sediment slurry. **A.** After 7 days of incubation. **B.** After 98 days of incubation. **C.-D.** Biofilm on PE bag incubated in anoxic sediment slurry. **C.** After 7 days of incubation. **D.** After 99 days of incubation DAPI stained cells, epifluorescence microscopy. Scale bars = 10 µm.

Organizing the maximum mean cell number of each treatment starting with the highest value, the following sequence can be examined for both oxic and anoxic environments: COMP > COMP-UV > PE > PE-UV. The maximum mean cell number of $1.43 \times 10^6$ cells/cm$^2$ was evaluated on the compostable biofilm in the oxic slurry at the third sampling event. Additionally, the highest colonized single replicate was found in the same treatment at the second sampling event with $2.07 \times 10^6$ cells/cm$^2$.

Comparing the respective treatment levels at a distinct sampling event, cell densities were always higher under oxic than under anoxic conditions. The treatments COMP and COMP-UV in both oxic and anoxic slurry showed a steep increase of cells in the biofilms. A stagnation phase was not reached at the end of the experiment. Cells counted on the PE bag in the oxic treatment did either increase or stagnate over time (Fig. 7) due to the multi-layer biofilm, it cannot be evaluated if the cell number increased a lot or not.

On the other hand, the cell density on the PE bag in the anoxic environment slightly increased towards the end of the experiment (Fig.7, 8C-D). Moreover, the cell number on the UV-treated PE bag in the oxic environment decreased. Examining the spread of the three replicates of the UV-treated PE bag in oxic slurry after 50 days of incubation, only one replicate had an outstanding higher cell number compared to the other replicates at that sampling event but also at the next sampling event.
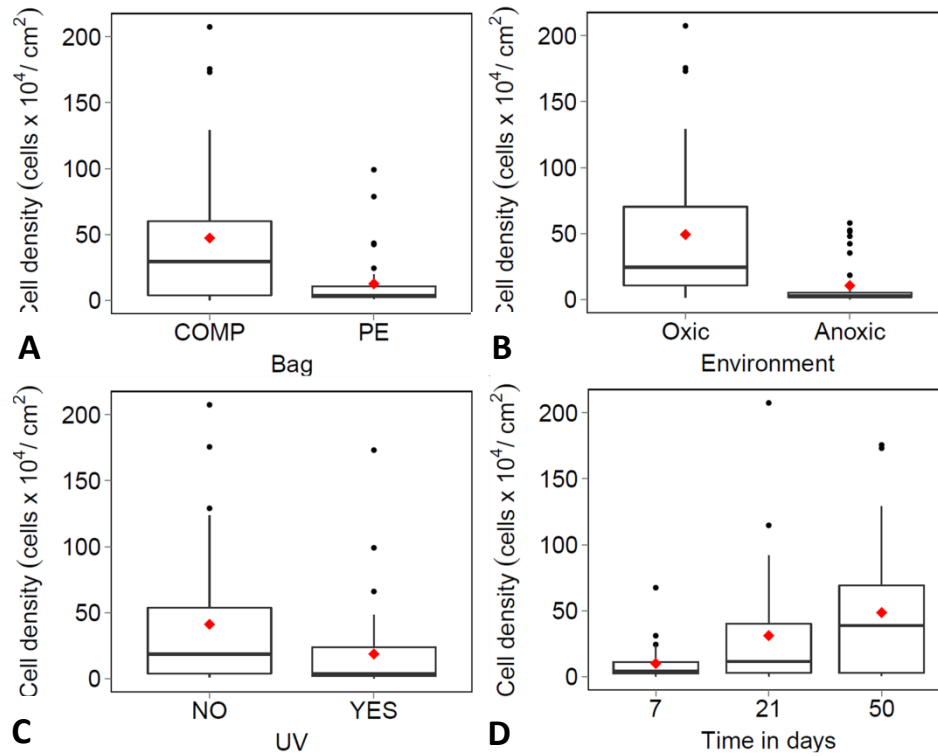


**Figure 9.** Boxplot for each factor against the cell numbers of every treatment. **A.** Factor bag: compostable and PE bag. **B.** Factor environment: oxic and anoxic slurry. **C.** Factor UV: No = no UV-treatment, Yes = UV-treatment. **D.** Factor time: sampling after 7, 21 and 50 days of incubation. Measurements after 50 days of incubation were not taken into account due to missing values. Black line in boxplot indicates the median and red diamond the mean of the data.

Further information can be obtained by analyzing the four factors: bag, environment, UV and time seperately with all evaluated cell densities (Fig. 9). A strong significant

difference (F = 17.33, p = 0.0001) was observed comparing both bag types, at which the mean cell number detected on compostable bags was 3.7 times higher than on PE bags. An even stronger effect was detected between oxic and anoxic treatments (F = 106.12, p < 0.0001), bag samples incubated in oxic sediment slurry were on average 4.6 times higher populated than the bags in anoxic treatments. Moreover, untreated bags were on average 2.2 times stronger colonized than UV-treated bags (F = 37.61, p < 0.0001). Examining the temporal colonization of all bag samples, the cells in the biofilms increased with time (F = 16.49, p < 0.0001) whereas the strongest increase was found between 7 and 50 days after experimental start (Fig. 9D).

Two factor combinations are illustrated in Fig. 10. Examining the factor combination bag and environment (Fig. 10A), a significant interactive effect was found (F = 12.84, p = 0.0008). The compostable bags always showed a higher cell density than the PE bags irrespective of the environment. The mean cell density on the compostable bags was five times higher in the anoxic sediment slurry and four times higher in the oxic sediment slurry in contrast to the PE bags. Moreover, bags incubated in oxic slurry were higher populated than bags in anoxic treatments. For instance, the compostable bags were four times and the PE bags six times more colonized in the oxic treatment than in the anoxic treatment. The difference in cell densities of the PE bags in oxic slurry compared to the anoxic slurry is even higher than shown in the single factor environment analysis (Fig. 9B).

The factor combination bag and UV (Fig. 10B) showed a significant interactive effect as well (F = 26.97, p < 0.0001 ). Higher cell colonization was detected on treated and untreated compostable bags than on treated and untreated PE bags. The untreated compostable bag was on average five times higher populated than the untreated PE bag and the UV-treated compostable bag two times higher than the treated PE bag. Furthermore, UV-treated bags were on average less colonized than untreated bags. Whereas untreated compostable samples had a three times higher cell density than the UV-treated compostable samples and the untreated PE bag only had a slightly higher cell density than the UV-treated PE bag.

Opposing the factors UV and environment (Fig. 10C), a significant interactive effect was detected (F = 9.45 , p = 0.004). Bags incubated in oxic sediment slurry had a higher

cell density compared to bags in anoxic slurry irrespective to the UV treatment. In oxic slurry, the cell density on untreated bags was four times and on UV-treated bags eight times higher than on untreated/UV-treated bags in the anoxic sediment slurry. UV-treated bags in both environments had on average lower cell densities than untreated bags.
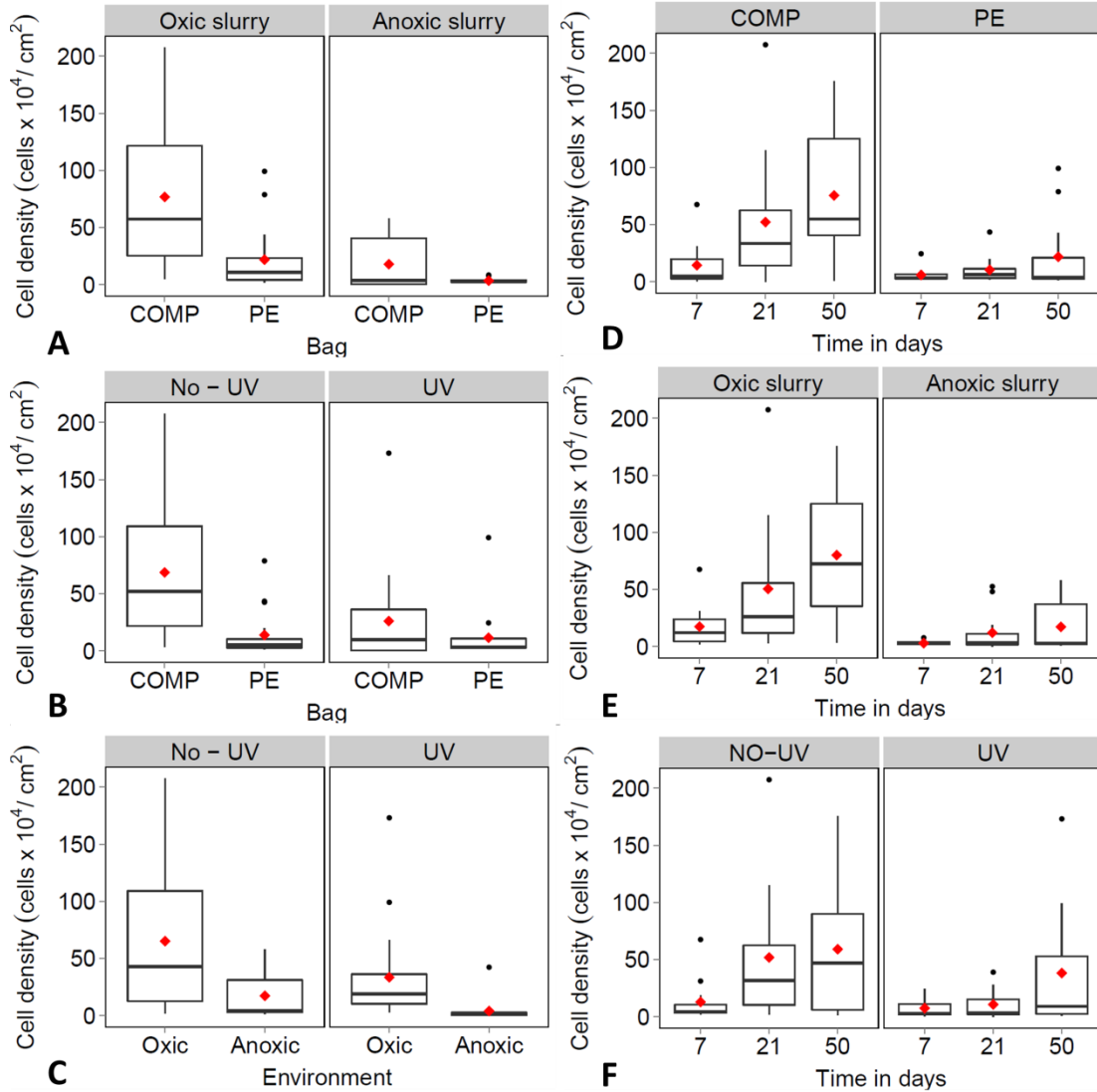


**Figure 10.** Boxplots of two factors against the cell densities of every treatment. **A.** Bags and environment. **B.** Bags and UV. **C.** Environment and UV. **D.** Time and bags. **E.** Time and environment. **F.** Time and UV. **Factor levels: Bag:** COMP and PE; **Environment:** oxic and anoxic slurry; **UV:** UV- and no treatment; **Time:** 7, 21 and 50 days incubation. Measurements after 50 days of incubation were not taken into account due to missing values. Black line in boxplot indicates the median and red diamond the mean of the data.

The factor combinations bag – time and UV – time had significant interactive effects on the cell densities (F = 5.45, p = 0.008; F = 3.49, p = 0.04, respectively). The cell density over time was higher on untreated bags and compostable bags than on UV-treated bags, PE bags (Fig. 10D,F).

Lastly, the boxplots (Fig. 10A-C) indicate a higher variation of cell density on untreated bags, on compostable bags and in oxic slurry compared to UV-treated bags, PE bags and in the anoxic treatment. This also can be observed in the temporal development of the cell densities (Fig 10D-F). Significant interactive effects were also identified for the factor combinations bag : environment : UV and bag : environment : time (F = 9.06 , p = 0.004; F = 3.85, p = 0.03). Detailed statistical results are shown in table 6.

**Table 5** Analysis of Variance table of linear model testing effects of time on cell densities after 7 days (7d) and 50/ 98 days (50d, 98d) of incubation. *Df* = degrees of freedom; *Sum Sq* = sums of squares; *Mean Sq* = mean squares and *F-value* = Fischer statistic.

| Effect combination | Df | Sum Sq | Mean Sq | F-value | p-value |
|---|---|---|---|---|---|
| **7d : 50/98d** | 1 | $2.40 \times 10^{12}$ | $2.40 \times 10^{12}$ | 43.38 | < 0.0001 |

**Table 6** Analysis of Variance table of the 4-factorial linear mixed model testing effects of factors on the cell density. Excluding the last sampling event due to missing values. COMP = compostable bag; PE = PE bag. Df = degrees of freedom; *F-value* = Fischer statistic.

| Effect combination | numDF | denDF | F-value | p-value |
|---|---|---|---|---|
| Bag (PE : COMP) | 1 | 46 | 17.33 | 0.0001 |
| Environment (Oxic : Anoxic) | 1 | 46 | 106.13 | < 0.0001 |
| UV (UV : no UV) | 1 | 46 | 37.61 | < 0.0001 |
| Time (7 : 21 : 50 days) | 2 | 46 | 16.49 | < 0.0001 |
| Bag : Environment | 1 | 46 | 12.84 | 0.0008 |
| Bag : UV | 1 | 46 | 26.97 | < 0.0001 |
| Environment : UV | 1 | 46 | 9.45 | 0.004 |
| Bag : Time | 2 | 46 | 5.45 | 0.008 |
| Environment : Time | 2 | 46 | 0.37 | 0.69 |
| UV : Time | 2 | 46 | 3.49 | 0.04 |
| Bag : Environment : UV | 1 | 46 | 9.06 | 0.004 |
| Bag : Environment : Time | 2 | 46 | 3.85 | 0.03 |
| Bag : UV : Time | 2 | 46 | 0.64 | 0.53 |
| Environment : UV : Time | 2 | 46 | 0.67 | 0.51 |
| Bag : Environment : UV : Time | 2 | 46 | 2.37 | 0.11 |

### 3.1.2 Analysis of cell contamination of control samples with Live/Dead Staining

Cells attached on bag samples were observed in almost every control treatment with inactive sediment slurries (Fig. 11). Whereas all PE bag pieces were contaminated with living and dead cells, only live cells were found on compostable bags. The overall contamination on PE bag samples with $3900 \pm 3134$ cells/cm$^2$ was much higher in contrast to the compostable bag pieces with $455 \pm 719$ cells/cm$^2$. Moreover, every oxic treatment was contaminated with cells however only the PE bags and one compostable replicate was observed with cells in the anoxic experiments. Additionally, cell densities on PE bags in the oxic treatments were on average 2.4 and on compostable bags 32 times higher in contrast to PE/compostable bags in anoxic conditions..
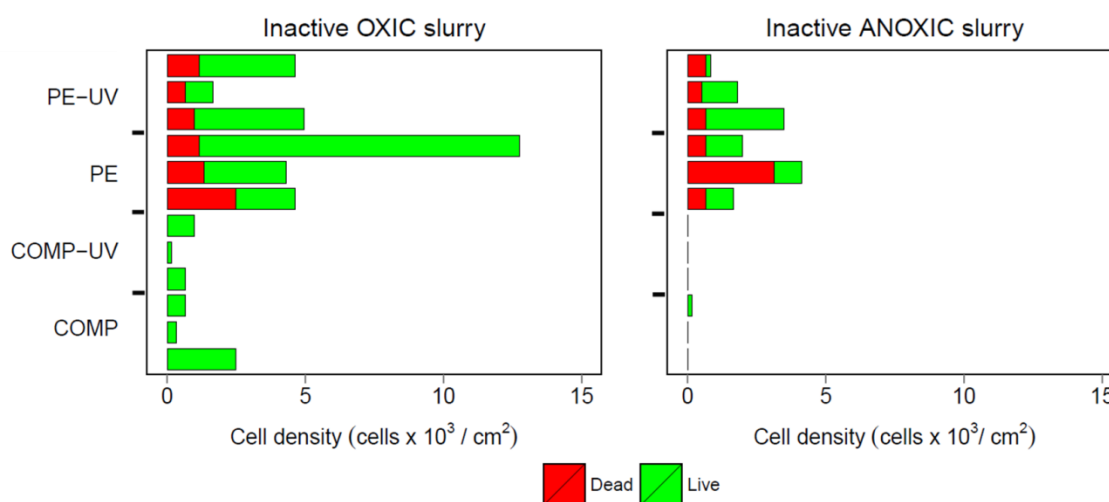


**Figure 11.** Cell densities of living and dead bacteria detected on bag samples in the control treatments with inactive sediment slurries at the end of the experiment. Left: Oxic slurry, right: Anoxic slurry. All three replicates of each treatment are shown. No cell contamination is represented by a line. COMP = compostable bag; PE = PE bag; COMP-UV/PE-UV = UV-treated bag.

The highest cell concentration found in the oxic experiment consisted of 91 % living cells and was 3.1 times higher in total cell number than the highest contaminated bag in the anoxic treatment containing 76 % of dead cells. Both of the just mentioned bags were untreated PE bags. Comparing contamination of cells on untreated with UV-treated bags (eg. PE-UV in oxic with PE in oxic condition) a slightly higher cell density was detected on the untreated bags.

### 3.1.3 Weighing of bag pieces

The weighing results pointed out that bag samples treated with active sediment slurries had a similar weight loss compared to bags in control treatments with inactive slurries after 98 days of incubation (Fig. 12. The weight loss of all compostable bags in the control treatments was slightly lower with a range of 0.4 – 1.5 % in contrast to all compostable bags in the active slurries 0.69 – 2.5 %.
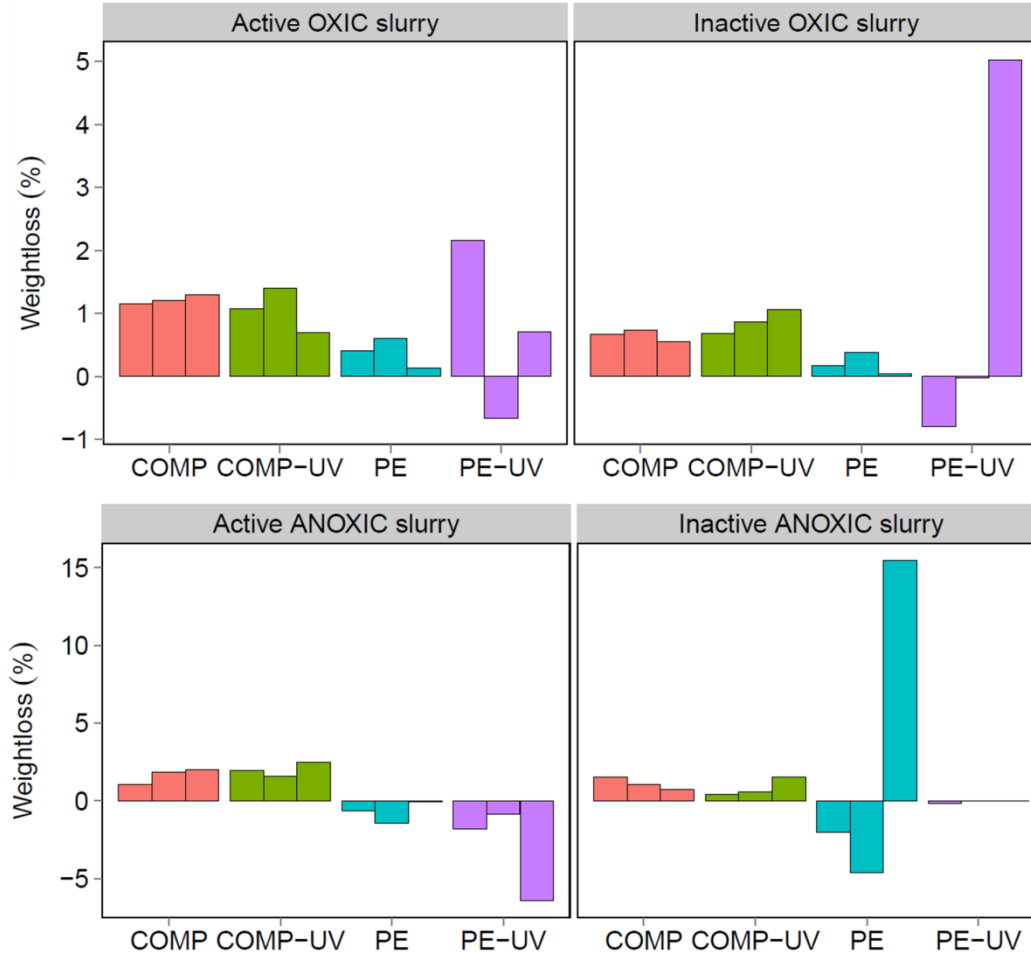


**Figure 12**. Bar charts giving the weight loss (positive values) or weight gain (negative values) in % of bag samples incubated in oxic/anoxic active sediment slurries (left) and oxic/anoxic inactive sediment slurries (right) after the experiment was ended. All three replicates are listed for each treatment. COMP = compostable bag; PE = PE bag; COMP-UV/PE-UV = UV-treated bag.

PE bag samples not only lost weight during the experiment but also gained weight. PE samples in the control treatment had a higher weight loss and a wider range with -4.6 – 15.5 % in comparison to the PE treatment in active slurry (-6.4 – 2.2 %). The highest

weight loss of 15.5 % was found in the anoxic control treatment with untreated PE bag samples. Combining all PE samples, the weight change ranged from -6.4 to 15.5 %  and for all compostable bag samples from 0.4 to 2.5 %.  Altough the two highest weight losses were found in PE treatments, the majority of PE replicates showed a lower percental loss than the compostable bags. Additionally, compostable bags in active anoxic slurry lost slightly more weight with 1.1 – 2.5 % than compostable bags incubated in oxic slurry with a weight loss of 0.7 – 1.4 %. All PE bags incubated in active anoxic slurry gained weight whereas almost all PE samples in active oxic slurry lost weight. Furthermore, the weight loss in anoxic treatments of each PE and compostable bag showed a higher variation than in the oxic treatments. UV-treated and untreated bags did not show any trend.

**Table 7** Analysis of Variance table of linear model testing effects of active and inactive slurries on bag weight loss. *Df* = degrees of freedom; *Sum Sq* = sums of squares; *Mean Sq* = mean squares and *F-value* = Fischer statistic.

| Effect combination | Df | Sum Sq | Mean Sq | F-value | p-value |
|---|---|---|---|---|---|
| **Oxic** **Active : Inactive slurry** | 7 | 3.50 | 0.50 | 0.33 | 0.93 |
| **Anoxic** **Active : Inactive slurry** | 7 | 72.69 | 10.38 | 0.64 | 0.72 |

### 3.1.4 Temporal development of TA and TS in anoxic sediment slurries

TA increased over time in all anoxic treatments (Fig. 13). TA increased significantly for the anoxic treatments with active slurry (including the control without bag samples) and with inactive slurry from the first day to the last day of incubation (F = 723.77, p < 0.0001; F = 68.15, p < 0.0001, respectively). Tukey HSD post-hoc test revealed significant differences of TA in all active (COMP p = 0.00; COMP-UV p = 0.00; PE p = 0.00; PE-UV p = 0.00 and control p < 0.0001) and all inactive slurry treatments, except for the treatment with UV-treated compostable bags (COMP p = 0.01; COMP-UV p = 0.08; PE p= 0.01 and PE-UV p = 0.003), from the first day to the last day of incubation.

In the active slurry treatments, TA started with 41.6 ± 0.7 meq / L and increased to 51.0 ± 2.1 meq / L at the end of the experiment. Control treatments with inactive sediment slurry started with concentrations 17 ± 0.9 meq / L and finished with 24.5 ± 2.9 meq / L.
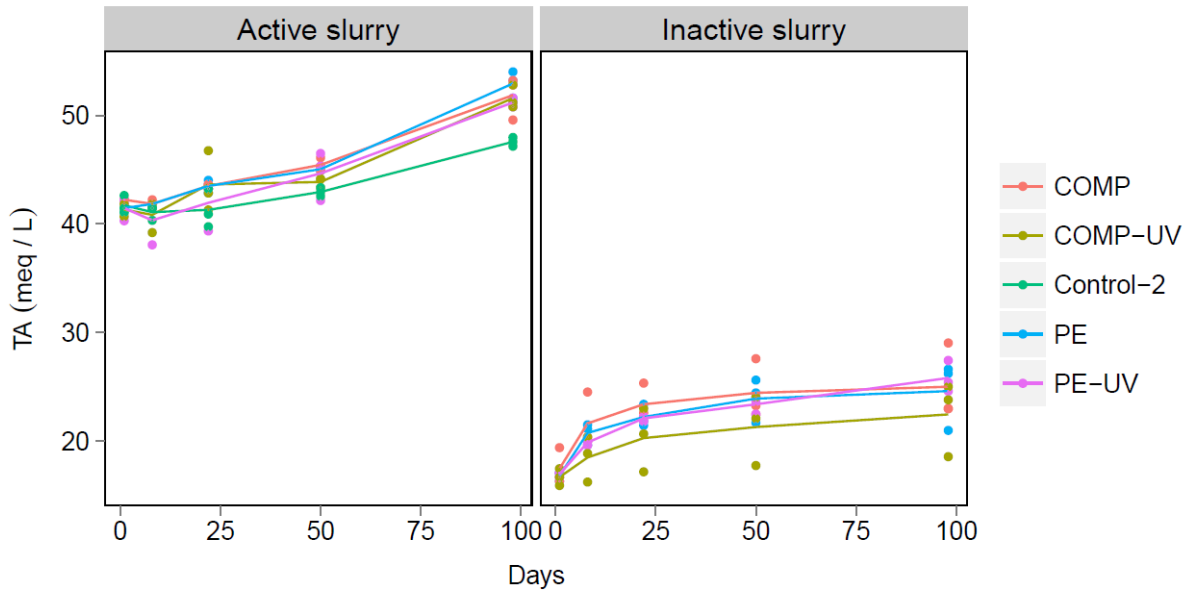


**Figure 13**. TA development of anoxic active sediment slurry treatments (including control without bag samples) (left) and anoxic control treatments with inactive sediment slurry (right) over time. Single replicates (points) with calculated mean (line). COMP = treatment with compostable bag; PE = treatment with PE bag; COMP-UV/PE-UV = treatment with UV-treated bag; Control-2 = control treatments without bag samples.

Comparing the ratios of the highest TA value from the active with the inactive treatments at the start and end of the experiment, the TA of the active treatments was 2.3 times

higher in the beginning and 1.9 times higher at the final measurement. All treatments with active slurry followed the same trend of a steady increase over time whereas the maximum TA was not reached when the experiment was ended. Although TA values of all active treatments seemed to be close together, a significant difference was observed at the last sampling event (F = 9.01, p = 0.002). Tukey HSD post-hoc test indicated significant differences between the control without bag samples (Fig. 13, Control-2) and COMP-UV, COMP, PE-UV, and PE (p = 0.01; p= 0.008; p = 0.02 and p = 0.002, respectively).

In the control treatments with inactive slurry, the increasing trend for TA started with a steeper rise in the beginning and a more damped increase close to the following stagnation. During this stagnation at each sampling event, TA of one replicate of the compostable treatment was slightly higher than in the other treatments; one replicate of the UV-treated compostable bag treatment on the other hand was considerably lower than all the other treatments at each measurement.
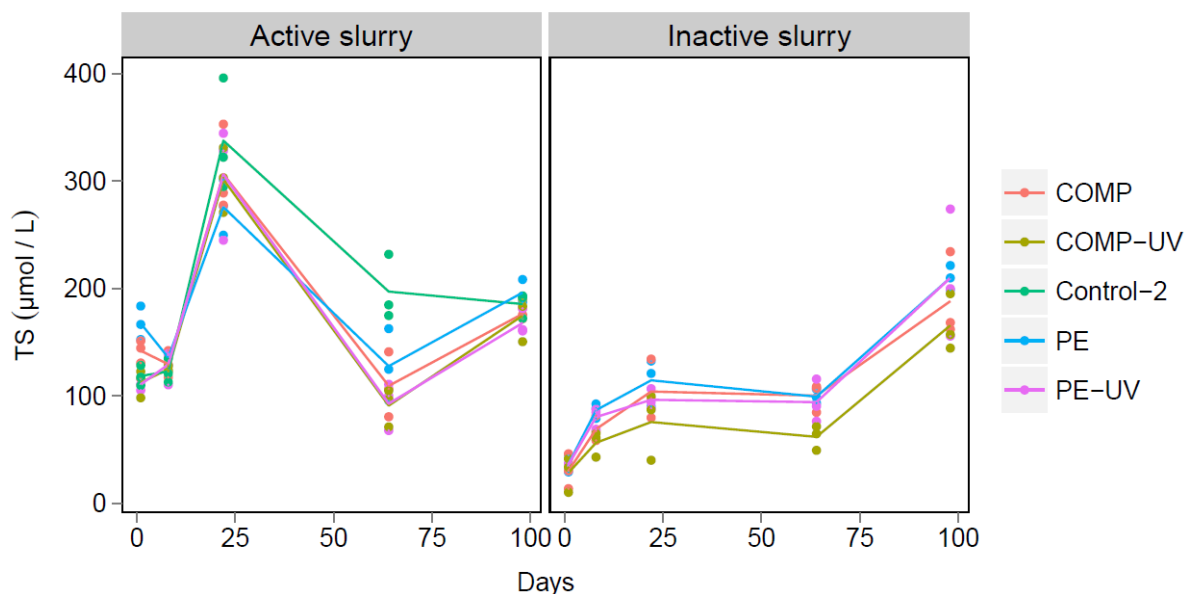


**Figure 14**. TS development of active sediment slurry treatments (including control without bag samples) (left) and control treatments with inactive sediment slurry (right) over time. Single replicates (points) with calculated mean (line). COMP = treatment with compostable bag; PE = treatment with PE bag; COMP-UV/PE-UV = treatment with UV-treated bag; Control-2 = control treatments without bag samples.

The total sulfide (TS) trend over time fluctuated for both active and inactive sediment slurry experiments (Fig. 14). All active slurry treatments fluctuated the same way. First,

TS slightly decreased followed by the highest peak at the third measurement with $305.6 \pm$ 40.9 µmol / L. Subsequently, a steep decrease followed by a slight increase was observed. A significant difference was detected for the TS concentrations in the active slurry after 64 days of incubation (F = 7.56, p = 0.005). The Tukey HSD post-hoc test revealed a significant difference of TS concentrations between the control treatment (without bag samples) and COMP-UV, COMP, PE-UV and PE (p = 0.002; p = 0.0007; p = 0.005 and p < 0.0001). The inactive slurry treatments on the other hand, started with increasing TS concentrations followed by a slight decrease /stagnation phase and ended in a peak of $193.5 \pm 38.2$ µmol / L. TS fluctuation was higher in the active slurry treatments than in the inactive sediment treatments. In contrast, active slurry treatments showed a higher peak compared to the inactive slurry experiments but at the end of the experiment both slurry treatments had similar TS concentrations.

Comparing TA and TS of the active slurry experiments, TA concentrations showed a steady increase, while TS exhibited considerable fluctuations over time. The active slurry treatments COMP, COMP-UV, PE and PE-UV did not vary from each other but the control without bag samples had a lower TA at the 3., 4. and 5. sampling event and a higher TS at the 3. and 4. measument than the others. The TA and TS of the control treatments with inactiv slurry showed a more or less steady increase, with TA almost reaching a stagnation phase and TS still increasing towards the end of the experiment. More detailed statisitcal information are given in table 8.

**Table 8** Analysis of variance table  testing effects of anoxic active/inactive slurries and time on TA and TS. *Df* = degrees of freedom; *Sum Sq* = sums of squares; *Mean Sq* = mean squares and *F-value* = Fischer statistic.

| *Effect combination* | *Df* | *Sum Sq* | *Mean Sq* | *F-value* | *p-value* |
|---|---|---|---|---|---|
| **TA**<br>**Active sediment slurry**<br>**1 days : 98 days** | 1 | 662.5 | 662.5 | 723.77 | < 0.0001 |
| **TA**<br>**Inactive sediment slurry**<br>**1 days : 98 days** | 1 | 338.1 | 338.1 | 68.15 | < 0.0001 |
| **TS**<br>**Active sediment slurry :**<br>**2.Control at 64 days** | 4 | 22895 | 5724 | 7.56 | 0.005 |

### 3.1.5 Scanning electron microscopy (SEM) imaging

SEM images of the PE and compostable bag prior any treatment or incubation are presented in Fig. 15. The compostable bag was characterized by a diverse surface structure. Mostly, an uneven, hilly surface with numerous bumps, including smooth regions was observed (Fig. 15A-B). The PE surface structure was very smooth which by zooming in showed interlaced filaments in some areas (Fig. 15C-D). The SEM analyses of UV-treated PE and compostable bags did not indicate any changes in the surface structure compared to bag surfaces not exposed to UV-light.
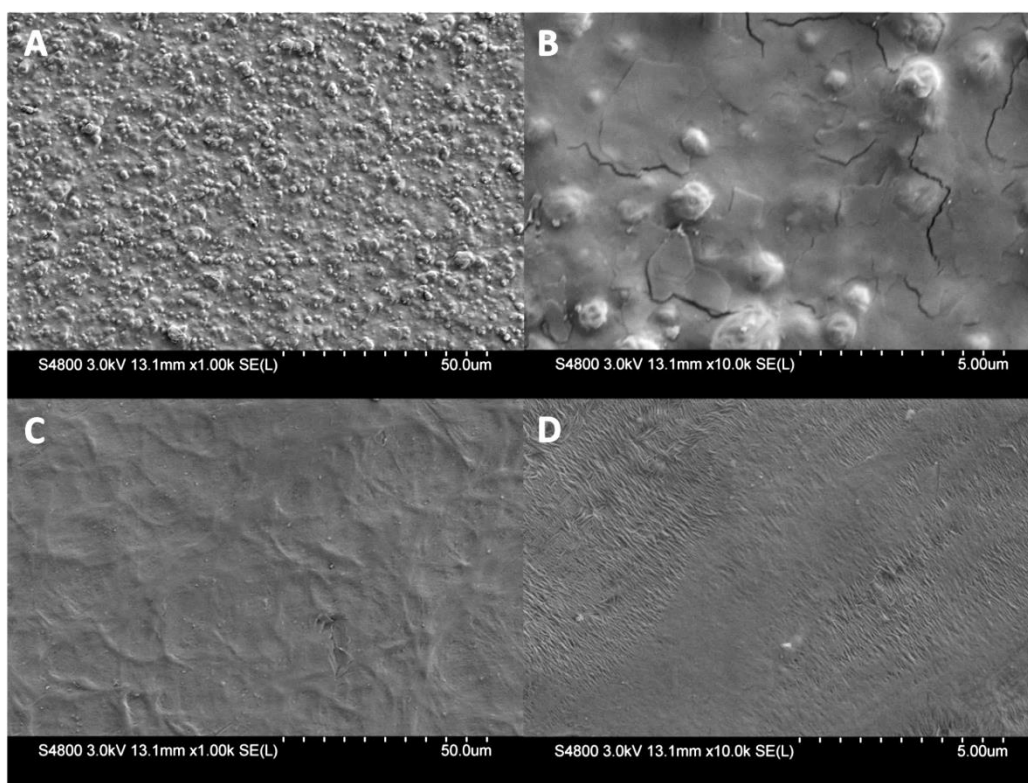


**Figure 15.** SEM images of bag surfaces. **A.** Surface structure of the compostable carrier bag. **B.** Close-up of the compostable carrier bag. **C.** Surface structure of the PE bag. **D.** Close-up of the PE bag.

Bacteria cells were present in small and high accumulations, but also as single cells in the different treatments after an incubation time of 98 days. Certain locations of the compostable bags were often colonized by numerous microbes (Fig. 16A), at which cells not only expanded horizontally over the bag surface but also piled up to clusters. Numerous cells showed several emerged filaments reaching to other neighboring cells

(Fig. 16B). As seen in Fig. 16C, the biofilm on the compostable bag was concentrated with sediment particles at some areas which made the detection of bacterial cells impossible. Microbes on PE bag surfaces were predominantly present in small groups located in depressions and uneven areas (Fig. 16D).
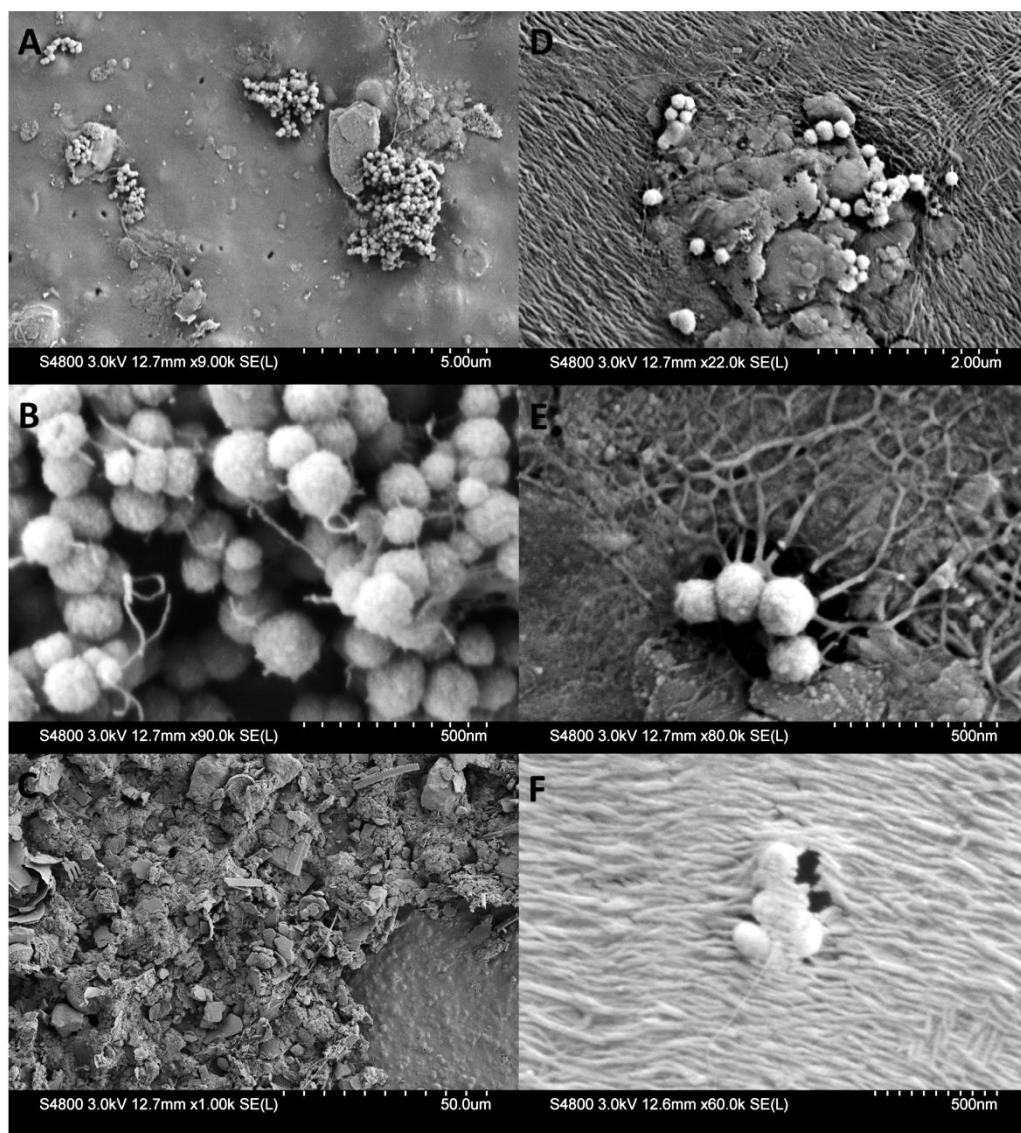


**Figure 16.** SEM images of biofilms after 98 days of incubation. **A.-C.** Compostable bag incubated in oxic sediment slurry. **A.** Several bacteria accumulations. **B.** Close up of bacteria accumulation with filaments. **C.** Sediment particles cover the surface. **D.-F.** PE bags incubated with oxic sediment slurry. **D.** Bacteria settling in an uneven location. **E.** Close up of bacteria attaching onto the surface with filaments. **F.** Bacteria settling into the material.

Image E in Fig. 16 showed a small group of bacteria on top of a hole with numerous filaments reaching from the cells to the surface. The filaments were diverged into several branches and formed a net over the bag surface. Furthermore, cells within the bag material were also detected (Fig. 16F).

Bag surface imaging with SEM after removal of bacteria did not show any clear sign of surface destruction by bacteria. Partly, the biofilms were not completely removed and sediment particles and microbes still present. SEM imaging of the surface structure of the control treatments with inactive sediment slurries did not indicate any changes. However, single bacteria cells were obtained.

### *3.1.6 RAMAN spectroscopy*

The RAMAN spectra of the PE and compostable bags are shown in Fig. 17. Comparing the three spectra of the PE bag (no incubation, control incubation and incubation) little differences were observed due to different signal to noise ratios. The signal to noise ratio was higher for the spectra of the non-incubated PE bag and therefore did not fluctuate as much as the other two spectra. The peak positions and FWHM´s of the spectra were identical and did not vary from each other. The only difference was observed at the slope of the last peak. Whereas the non-incubated and incubated PE samples had one shoulder, the control incubated PE sample had two shoulders.
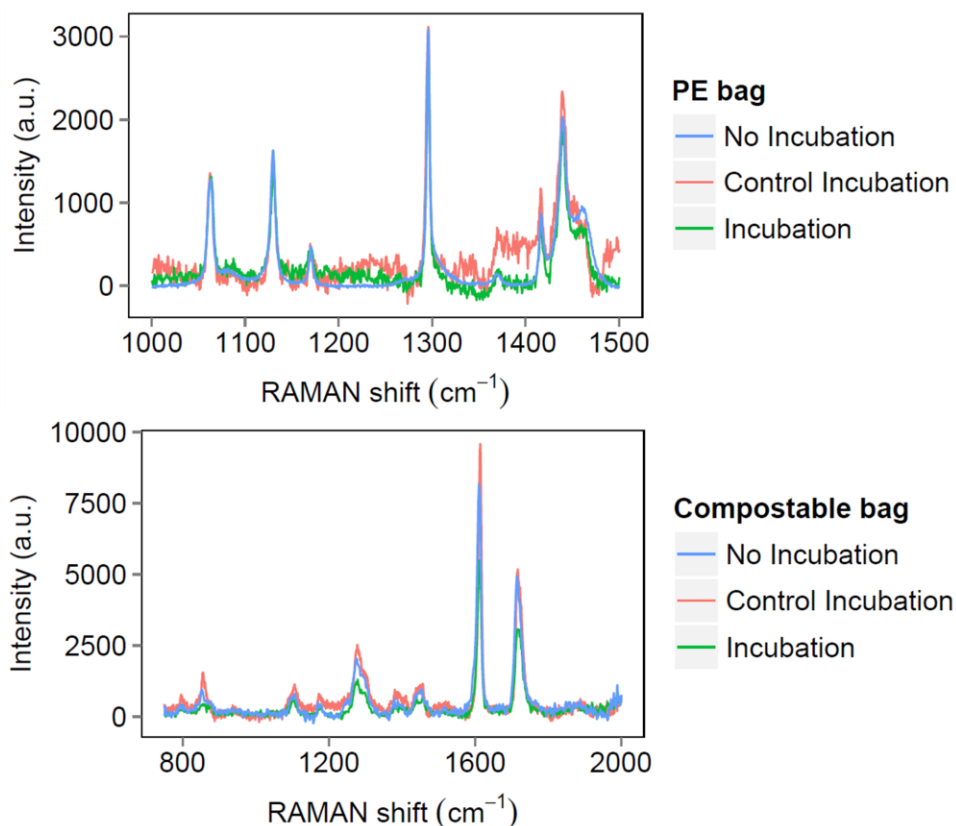


**Figure 17.** RAMAN spectra of PE (top) and compostable (below) bags. The following bag samples were analyzed: bag sample not incubated (no incubation), bag samples incubated in inactive oxic slurry (control incubation) and bag samples incubated in active oxic slurry (incubation).

Comparing the three spectra of the compostable bag (no incubation, control incubation and incubation) only little differences were observed due to different signal to noise ratios of each spectrum. FWHM values of the highest peak at 1611 $cm^{-1}$ of the point

measurements for each of the three profile fits fluctuated. The bag incubated in active slurry had a FWHM of 15 cm$^{-1}$, the non-incubated bag 13 cm$^{-1}$ and the bag sample incubated in inactive slurry 9 cm$^{-1}$. In order to check whether the bag incubated in active slurry had a higher FWHM due to material alteration, the FWHM values of the highest peak at 1611 cm$^{-1}$ of the map measurements were observed and compared to the FWHM of the non-incubated and control incubated bags. The FWHM values of the map measurements of the incubated bag fluctuated around the FWHM´s of the non-incubated and control incubated bags.

The spectra of the PE and compostable bags incubated in active slurry did not vary from the spectra of the PE/compostable non-incubated bags and bags incubated in inactive slurry.

## 3.2 2.Experiment with bacteria cultures

### 3.2.1 Cell density

The temporal development of *Alcanivorax borkumensis* and *Desulfovibrio marinus* cultures settling on PE and compostable bags is demonstrated in Fig. 18 and 19. Counting of *A. borkumensis* cells on the different bags was conducted at every sampling event whereas bags incubated with *D. marinus* only at the first measurement event. Afterwards, both bags were overgrown by multilayers of *D. marinus* cells and made quantification impossible (Fig. 19D). Comparing the mean cell density of *A. borkumensis* on both bag types to the mean density of *D. marinus* on both bags after two days of incubation, the cell number was 12.5 times higher of *D. marinus*.
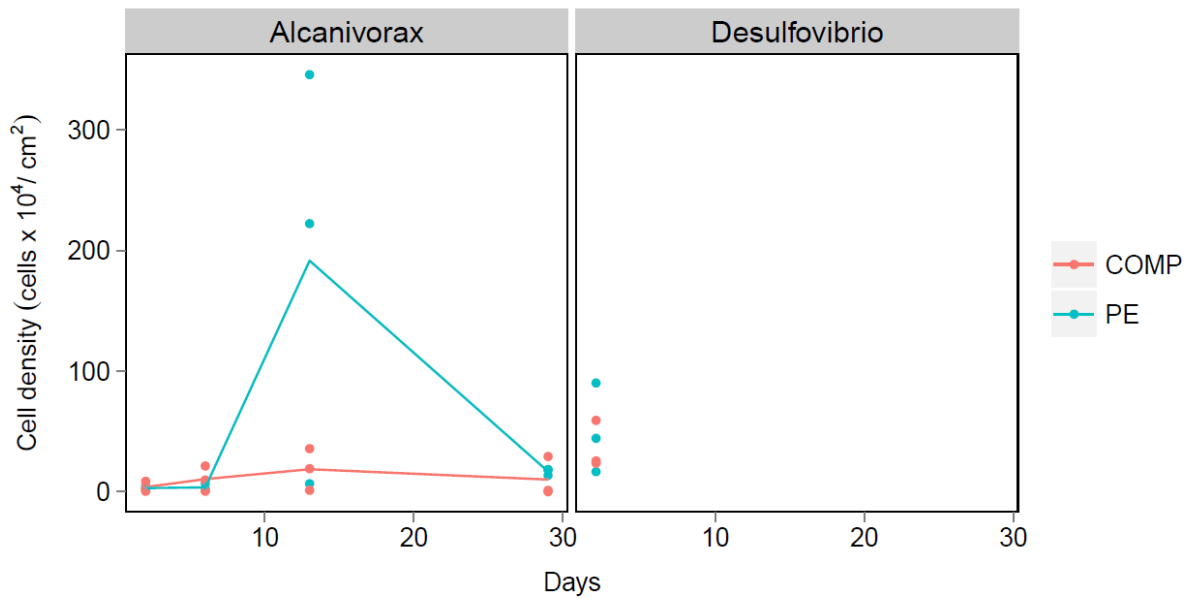


**Figure 18.** Temporal development of cell density on PE and compostable bags in the different treatments. Values are missing for bags treated with Desulfovibrio marinus after two days of incubation as cell-layers were too voluminous for reliable cell quantification. Single replicates (points) with calculated mean (line). PE = PE bag; COMP = compostable bag.

In the oxic experiment, the cell density of *A. borkumensis* on both bags was similar during the first six days of incubation (Fig. 18). Afterwards, the cell numbers on two PE replicates increased enormously by 50 and 168 times, respectively. On the other hand, the

population on the compostable samples remained steady and only increased by five times at half time and decreased by a factor of two at the end of the experiment. At the end of the experiment, the cell number of *A. borkumensis* on PE bags dropped down to the same level as found on compostable bags. However, counting the cell numbers on the PE samples at the last sampling event was not completely ideal as the dye failed and stained cells not as visible as usual. Though, counting of *D. marinus* cells was not possible on both bag types after the second day of incubation, the cell density on the bags was by far higher than of *A. borkumensis*. Therefore, cell density of the anaerobic culture was superior to the aerobic culture.
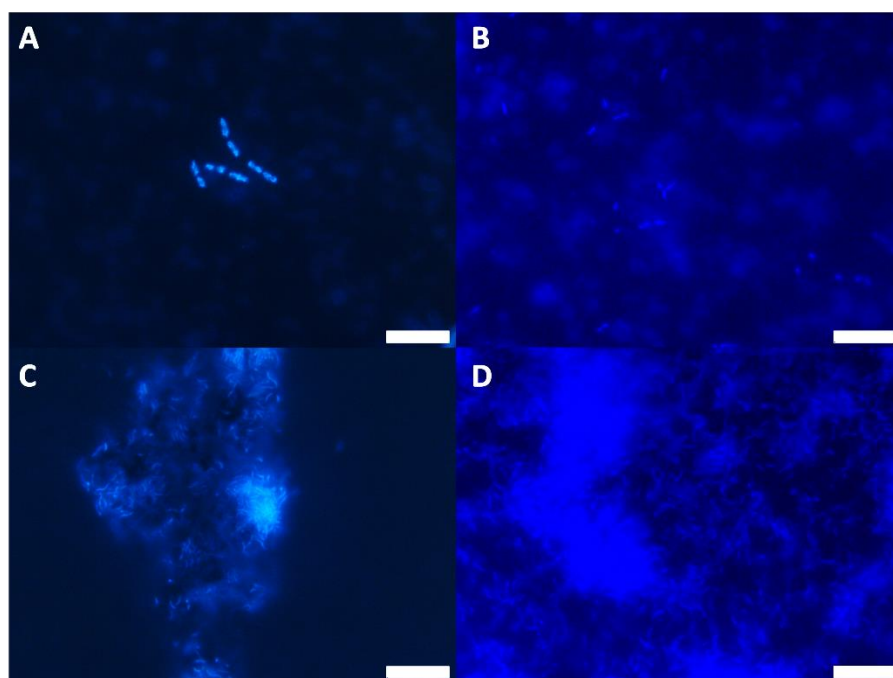


**Figure 19. A.-B.** *Alcanivorax borkumensis* on PE bag. **A.** After 2 days of incubation. **B.** After 29 days of incubation **C.-D.** *Desulfovibrio marinus* on compostable bag. **C.** After 2 days of incubation. **D.** After 29 days of incubation. DAPI stained cells, epifluorescence microscopy. Scale bars = 10 µm.

### 3.2.2 Analysis of cell contamination of control samples with Live/Dead Staining

Cell contamination on bag samples was detected in every control treatment with inactive/no cells except for seven compostable bags from both controls and oxic/anoxic condition (Fig. 21). Dead and live cells were only observed on PE bags (Fig. 21B) whereas compostable bags only contained living cells if contaminated. First of all, the highest contamination was found on a PE replicate incubated in cell-free oxic medium with a cell density of 4.2 x $10^6$ cells/cm$^2$ and 88 % of living cells (Fig. 21A; Fig. 20, left PE-2). Cell contamination in the oxic control treatments was much higher compared to the anoxic treatments.
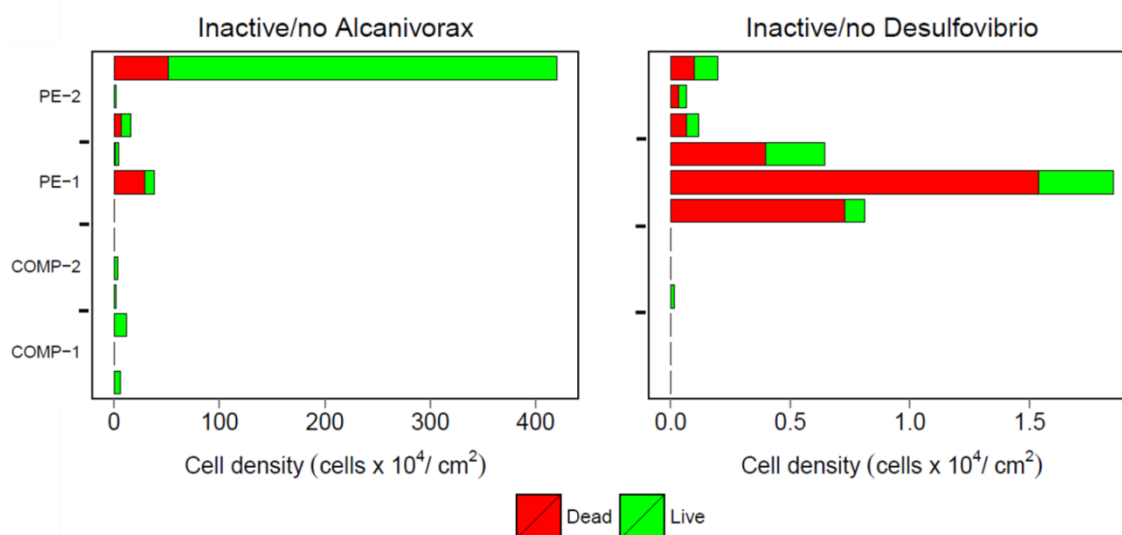


**Figure 20.** Cell densities of living and dead bacteria detected on bag samples in the control treatments with inactive/cell-free *Alcanivorax borkumensis* (left) and inactive/cell-free *Desulfovibrio marinus* (right) at the end of the experiment. All three replicates of each treatment are shown. Note different scaling of x-axis. COMP-1/PE-1 = bag incubated with inactive cells; COMP-2/PE-2 = bag incubated with cell-free medium.

The highest cell number in the anoxic treatments was observed on a PE sample incubated with inactive *D. marinus* with approximately 18.500 cells/cm$^2$ (Fig. 20, right PE-1). Contamination on compostable bags in the oxic treatments ranged from 15.041 to 57.851 cells/cm$^2$ and were therefore much higher than the latter (cannot be seen in Fig. 20 due to different x-axis scaling). Comparing the degree of contamination on PE bags with compostable bags, PE bags had higher cell densities in each oxic and anoxic condition

but compostable bags in oxic conditions had a higher cell density than PE bags in anoxic control treatments.
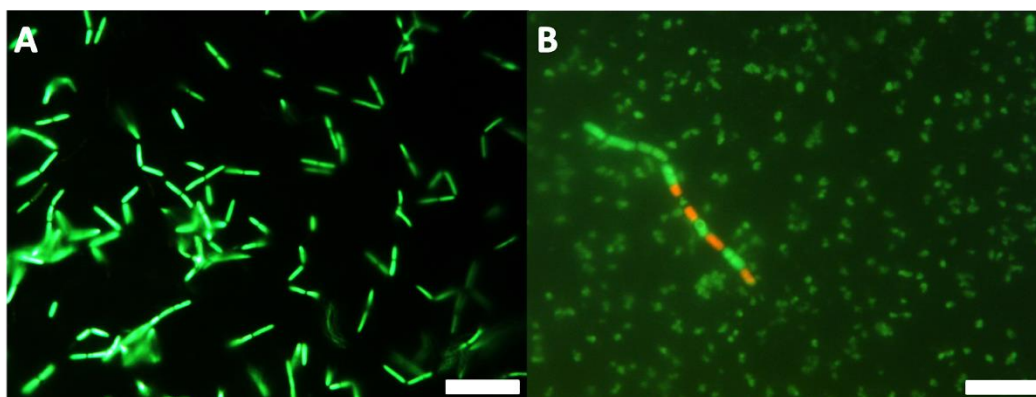


**Figure 21. A.** Living cells (green) on PE bag incubated in oxic medium; **B.** Living (green) and dead (red) cells on PE bag incubated in inactive Alcanivorax culture. Live and dead staining, epifluorescence microscopy. Scale bars = 10 µm.

### 3.2.3 Weighing of bag pieces

Bag samples incubated with active cultures, inactive cultures and cell-free media showed weight loss (Fig. 22). The presence of active and inactive/no cells significantly influenced the weight loss of the compostable bags incubated in the anoxic treatments (F = 29.13, p = 0.0008). The Tukey HSD post-hoc test revealed significant differences between compostable bags incubated with active *D. marinus* (3.4 ± 0.03 %) and inactive *D. marinus* (3.6 ± 0.05 %; p = 0.001) as well with no cells (3.6 ± 0.03 %; p = 0.001). Hence, the control treatments had a higher weight loss compared to the treatement with active *D. marinus*.
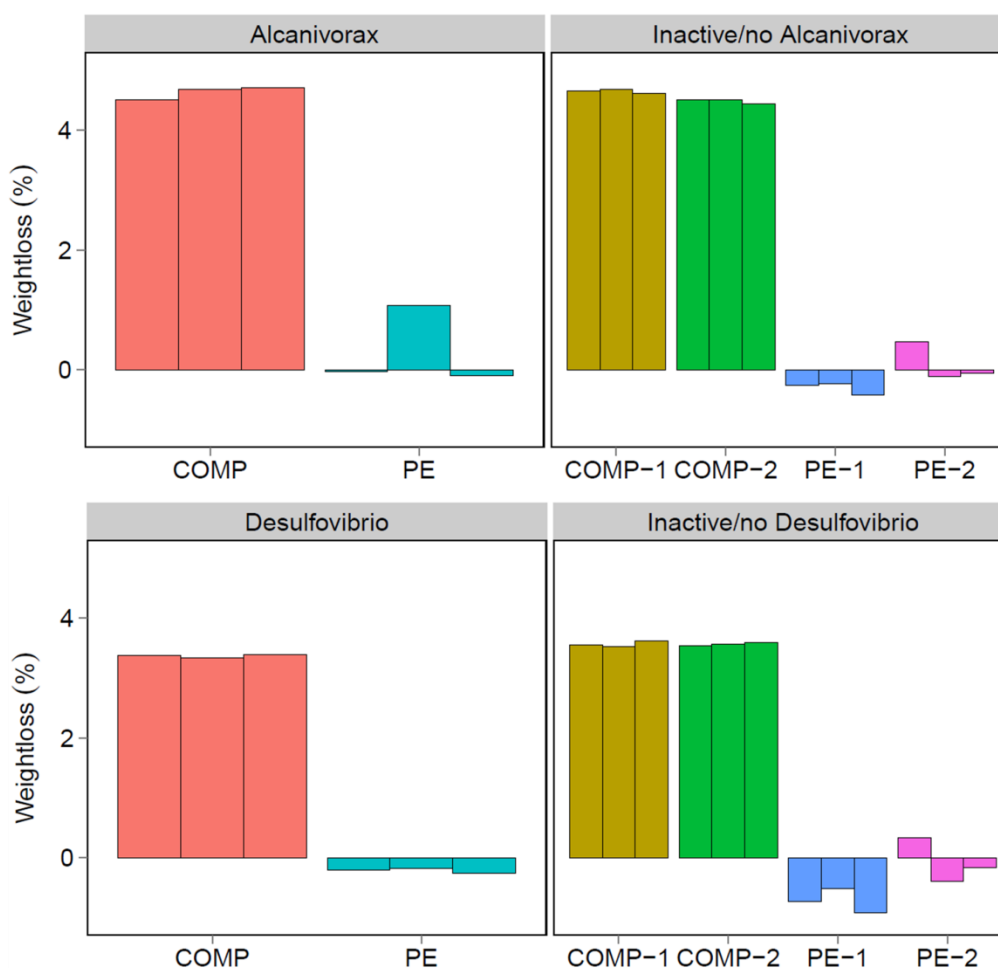


**Figure 22.** Bar charts giving the weight loss (positive values) or weight gain (negative values) in % of bag samples incubated with active Alcanivorax/Desulfovibrio cultures (left), inactive Alcanivorax/Desulfovibrio cultures and cell-free controls (right) after the experiment had ended. All three replicates are listed for each treatment; COMP-1/PE-1 = bag incubated in inactive culture; COMP-2/PE-2 = bag incubated in cell-free medium.

Almost all PE samples gained weight, except for one replicate incubated with active *A. borkumensis*, with cell-free *A. borkumensis* and with cell-free *D. marinus*. All PE samples incubated with active bacteria experienced a -0.3 ± 1.1 % change and PE samples incubated with inactive bacteria and no cells a -0.9 ± 0.5 % change in weight.

The highest weightloss (4.71 %) was detected for the compostable bag incubated in active *A. borkumensis*. Furthermore, all compostable samples had a higher weight loss compared to PE bags. Comparing the weight loss between bags incubated with Alcanivorax and Desulfovibrio cultures, the latter lost less weight. The compostable bag samples incubated with *A. borkumensis* lost 1.4 times more weight in contrast to the compostable samples incubated with *D. marinus* ($F = 358.1$, $p < 0.0001$).

Examining the bags in the control treatments with inactive Alcanivorax/Desulfovibrio culture and no bacteria, a small difference can be seen within PE samples (Fig. 22, right). All replicates of PE treatments incubated with dead bacteria gained weight and incubated with no bacteria, one replicate lost weight.

**Table 9** Analysis of Variance table of linear model testing effects of *A. borkumensis* and *D. marinus* on compostable bag weight loss and of active and inactive/no cells *D. marinus* on compostable bag weight loss. *Df* = degrees of freedom; *Sum Sq* = sums of squares; *Mean Sq* = mean squares and *F-value* = Fischer statistic.

| Effect combination | Df | Sum Sq | Mean Sq | F-value | p-value |
|---|---|---|---|---|---|
| **COMP** **Alcanivorax : Desulfovibrio** | 1 | 2.39 | 2.39 | 358.1 | < 0.0001 |
| **COMP** **Desulfovibrio: Desulfovibrio controls** | 2 | 0.08 | 0.04 | 29.13 | 0.0008 |

## 3.2.4 Temporal development of TA and TS in experiments with Desulfovibrio marinus

The TA concentrations from the start to the end of the experiment increased highly significant for the treatments with active and inactive/no Desulfovibrio cells (F = 1412.69, p < 0.0001; F = 186.62, p < 0.0001, respectively) (Fig. 23). All treatments started off with a TA ranging from 56 – 62 meq/L. However the active treatments experienced a steep increase up to 88 meq/L after the second sampling event and stagnated on that level till the end of the experiment.

TA concentrations of the control treatments with inactive/no cells slightly increased with a mean value from 60 to 62 meq/L over the time but stagnated towards the end. Comparing the mean TA value of all treatments of the active with inactive/no cell treatments at the last sampling, treatments with active *D. marinus* reached a 1.4 times higher TA concentration. Furthermore, TA did not vary within treatments incubated in active *D. marinus* as well as treatments within inactive/no cell experiment.
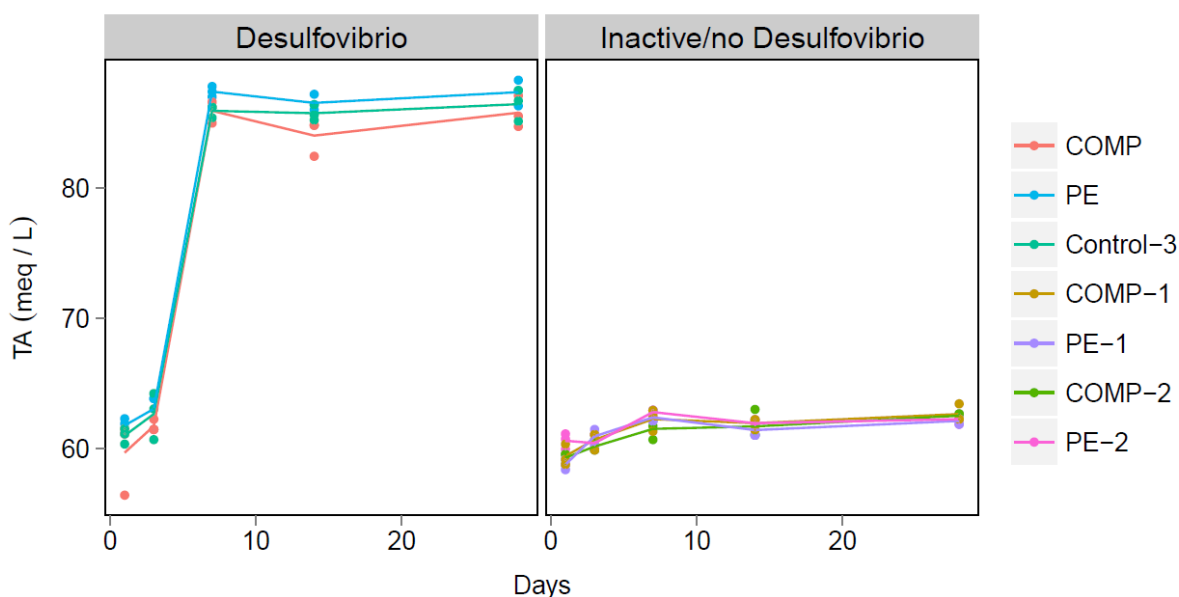


**Figure 23.** TA development of active *D. marinus* treatments (including control without bag samples) (left) and control treatments with inactive/no *D. marinus* (right) over time. Single replicates (points) with calculated mean (line). COMP-1/PE-1 = control treatment with bag incubated with inactive cells; COMP-2/PE-2 = control treatment with bag incubated with cell-free medium; Control-3 = control treatment without bag samples.

The TS concentrations decreased significantly over time for the treatments with active *D. marinus* (F = 199.51, p < 0. 0001) whereas TS of inactive/no cell culture only decreased very little over time and stagnated towards the end of the experiment (Fig. 24). Both active and inactive/no cells treatments started with TS concentrations in a close range but at the end the inactive/no cells controls had a 9 times higher TS level. The difference in TS of inactive/no cells controls over time was highly significant (F = 16.62, p < 0.0001). One replicate of the control treatments (COMP-1) followed the same temporal TS development as the other control treatments, yet with a much lower concentration.
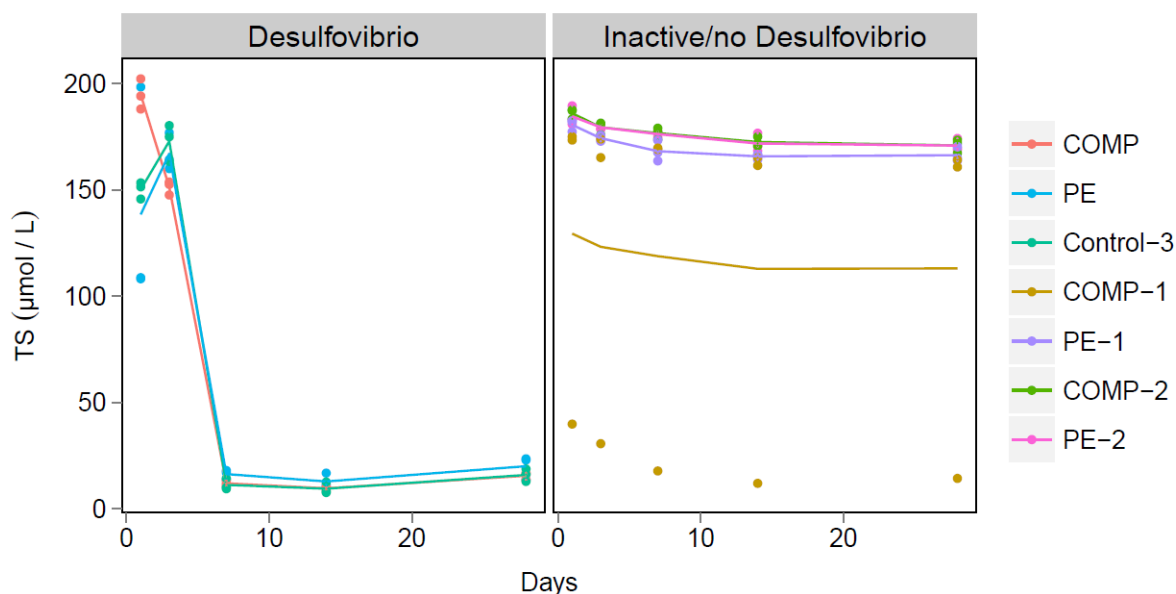


**Figure 24.** TS development of active *D. marinus* treatments (including control without bag samples) (left) and control treatments with inactive/no *D. marinus* (right) over time. Single replicates (points) with calculated mean (line). COMP-1/PE-1 = control treatment with bag incubated with inactive cells; COMP-2/PE-2 = control treatment with bag incubated with cell-free medium; Control-3 = control treatment without bag samples.

The individual treatments of the active culture experiments followed the same temporal pattern. The only observed difference occurred within the first two sampling events. The TS concentration of the compostable bag treatment was higher than the PE and control treatment without bag samples (Control-3). For the latter two, TS increased after the first day of incubation and dropped after three days of incubation similar to TS in the compostable treatment.

Comparing TA with TS data, the TA concentrations steeply increased while TS values steeply decreased for the active culture treatments after the second measurement event. Afterwards, both concentrations stagnated throughout the incubation time. The same process, just much lower, was observed for the control treatments with inactive/no cell culture; during the slight increase of TA, TS decreased a little.

**Table 10** Analysis of Variance table of linear model testing effects of active, inactive/no cells of *D. marinus* and time on TA and TS. *Df* = degrees of freedom; *Sum Sq* = sums of squares; *Mean Sq* = mean squares and *F-value* = Fischer statistic.

| Effect combination | Response | Df | Sum Sq | Mean Sq | F-value | p-value |
|---|---|---|---|---|---|---|
| **1 day : 29 days** | TA | 1 | 2970.66 | 2970.66 | 1412.69 | $< 0.0001$ |
| **Active Desulfovibrio** | TS | 1 | 93384 | 93384 | 199.51 | $< 0.0001$ |
| **1 day : 29 days** | TA | 1 | 49.19 | 49.19 | 186.62 | $< 0.0001$ |
| **Inactive/no Desulfovibrio** | | | | | | |
| **Active : Inactive/no Desulfovibrio 29 days** | TS | 6 | 105445 | 17574.2 | 16.62 | $< 0.0001$ |

### 3.2.5 Scanning electron microscopy (SEM) imaging

Cells of *Alcanivorax borkumensis* and *Desulfovibrio marinus* were detected on both types of bags (Fig. 25), except that no *A. borkumensis* cells were seen on the examined PE replicate. Small groups of *A. borkumensis* cells were scattered on the compostable bag sample (Fig. 25A and B). Cells of *Desulfovibrio marinus* on the contrary covered large surface areas of both bag types (Fig. 25C-E).
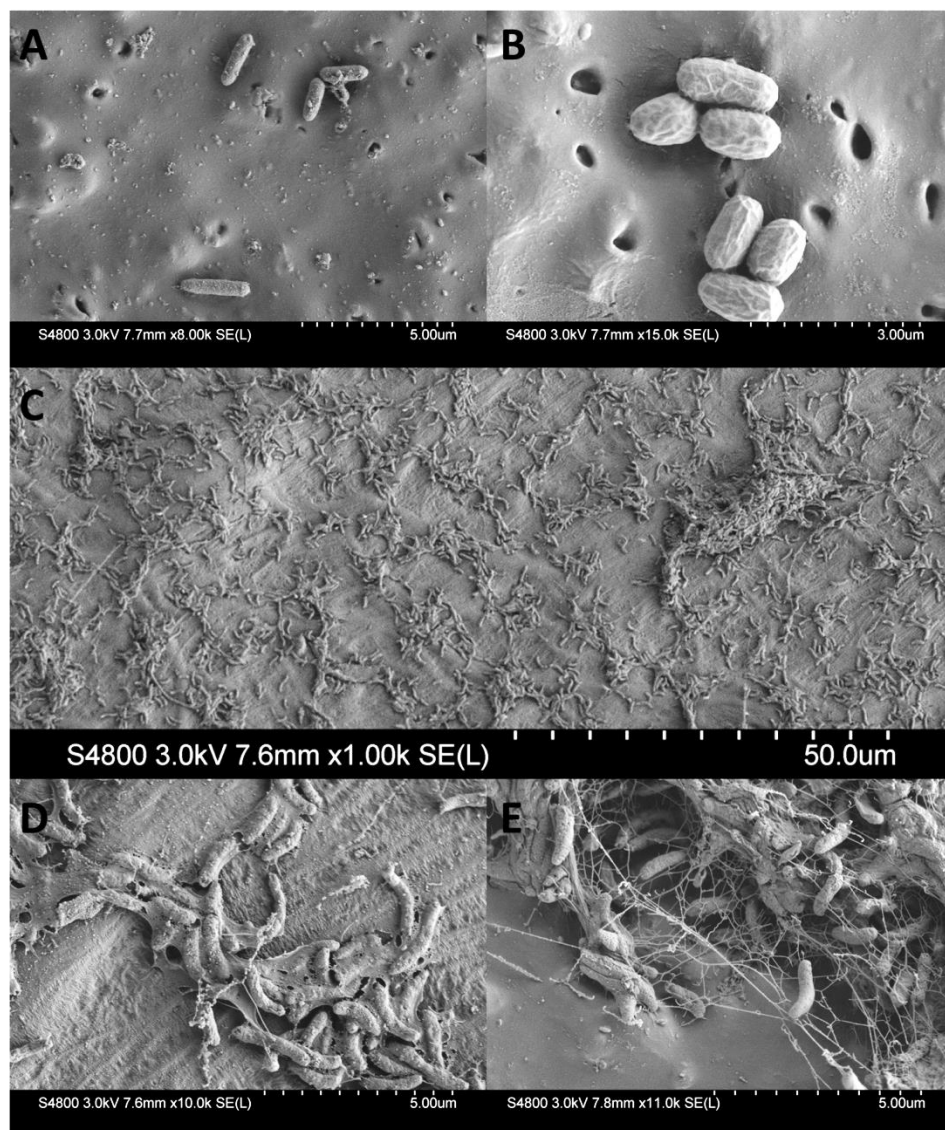


**Figure 25.** SEM images of biofilms on the two bag types after 29 days of incubation. **A+B.** Compostable bag incubated with *Alcanivorax borkumensis*. **C+D.** PE bag incubated with *Desulfovibrio marinus*. **C.** Cells covering the whole PE surface. **D.** Close-up of bacterial accumulation. **E.** Compostable bag with *Desulfovibrio marinus* cell cluster.

Mostly, a single cell layer covered the surface, whereas patches of bag material were still visible. Cells also formed clusters (Fig. 24C and E) and were embedded in biofilm components (Fig. 24D and E).

When removing the biofilm from the bags, no traces of microbial destruction were observed on any sample. The surface structure of the bags incubated in control treatments with inactive cells and without cells did not show any difference compared to the surfaces shown in Fig. 15.

### 3.2.6 Cell concentration in the liquid phases of the treatments

The temporal development of the cell concentration in the liquid phase of the different treatments is shown in Fig. 26. The cell concentration of aerobic and anaerobic cultures increased in each treatment from the start to the end of the experiment. At the start of the experiment the mean of *D. marinus* cell concentrations was 2.6 times higher than the mean of *A. borkumensis* cell concentrations. At the end of the experiment the treatments incubated with *A. borkumensis* had a 1.4 times higher cell concentration than *D. marinus*.
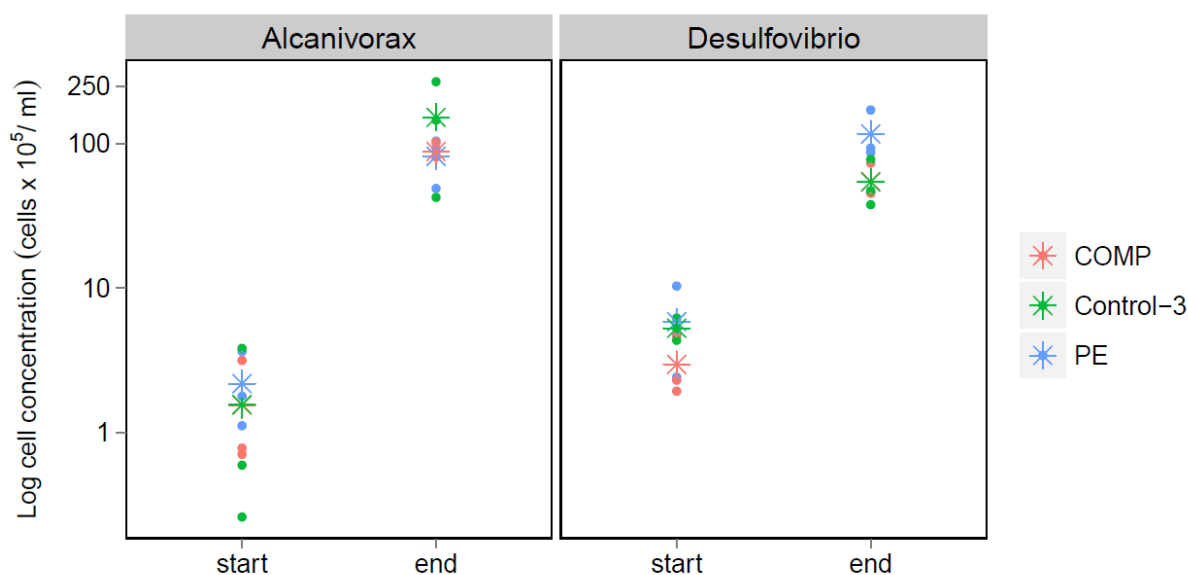


**Figure 26.** Temporal development of cell concentration in the liquid phase of each *Alcanivorax borkumensis* (left) and *Desulfovibrio marinus* (right) treatment. Single replicates (points) and mean (star). COMP = treatment with compostable bag; PE = treatment with PE bag; Control-3 = control treatment without bag samples.

The control treatments with cell-free media were tested for cell contamination in the liquid phase (Fig. 27). At the start of the experiment, no cells were counted in all treatments. But at the end of the experiment 4.1 x $10^6$ (PE treatment) and 4.8 x $10^6$ cells/ml (COMP treatment) were observed in the oxic control and for the anoxic controls 2.6 x $10^4$ (PE treatment) and 5.2 x $10^4$ cells/ml (COMP treatment). On average, the oxic control treatments were 115 times more contaminated with cells than the anoxic controls.
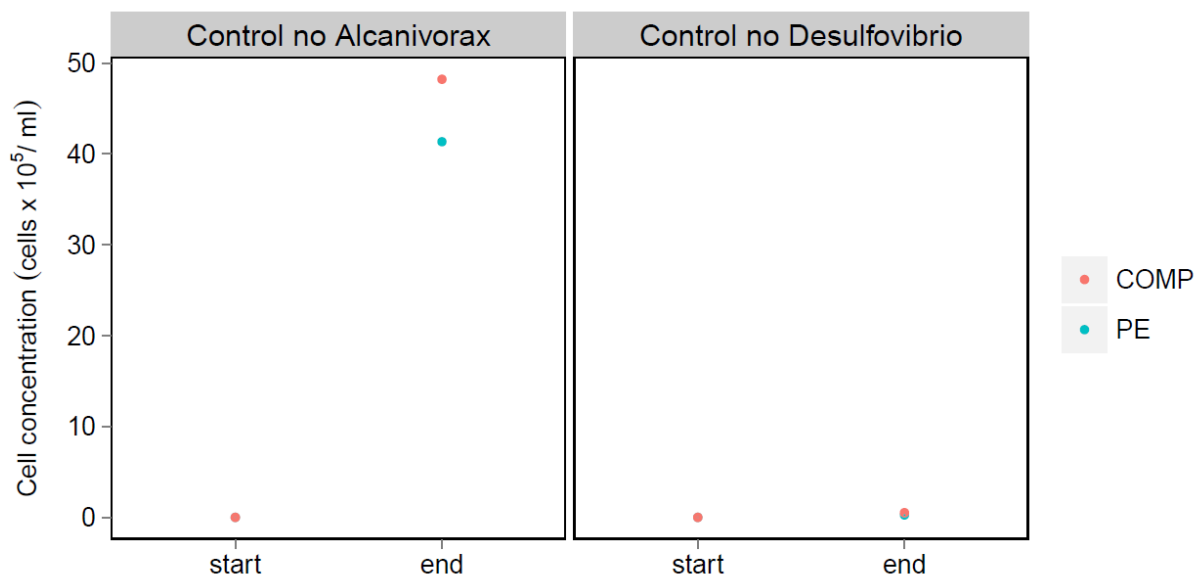


**Figure 27.** Temporal development of cell contamination of the control treatments without cells of *Alcanivorax borkumensis* (left) and *Desulfovibrio marinus* (right) showing one replicate.

## 4 Discussion

### *4.1 Biofilm formation*

In order to discuss the bacterial settlement on the bags in detail for both experiments, it is necessary to exemplify biofilm formation first. Biofilm formation comes in two phases, the docking and locking stage. The initial attachment (docking stage) of microorganisms to surfaces is affected by many variables and is therefore a complex procedure (Donlan, 2002). The attachment can depend on the surface of the substratum, hydrodynamics, characteristics of aqueous medium and properties of the cell.

To start with, the surface of the substratum is important as attachment can depend on the surface structure and its physicochemical properties (Donlan, 2002). Attachment can increase with surface roughness (Characklis et al., 1990) as well as with increasing hydrophobic surface property (Bendinger et al., 1993; Fletcher & Loeb, 1979; Oliveira et al., 2001). As no standardized method for measuring surface hydrophobicity exists, contradictory results have been stated at times (Donlan, 2002). Linear velocity has an influence on the thickness of the hydrodynamic boundary layer on top of the surface (Lawrence et al.,1987). Therefore, low velocity increases the thickness of the boundary layer and can hinder the cell reaching the surface. Furthermore, the pH, nutrient level, temperature and ionic strength of an aqueous medium may play an important role in the attaching rate as well (Donlan, 2002). For example, the ionic strength can have an influence on repulsive forces between microbes and surfaces (Fletcher, 1988). The bacterial surface properties are a further dependent factor for attachment. For example, hydrophobic interactions tend to increase with an increasing nonpolar surface by one or both surfaces. Rosenberg et al. (1986) also state that negatively charged bacteria can have hydrophobic surface compounds, which can contribute to cell surface hydrophobicity (Donlan, 2002). It is also vital whether bacterial cells are motile or non-motile as motility is an advantage to overcome repulsive forces (Korber et al., 1989).

All of the above mentioned factors play an important role in whether bacterial attachment takes place and whether the second phase of biofilm formation will be initiated. The attachment of bacteria to a surface stimulates the production of

extracellular polysaccharides (EPS) (Vandevivere et al., 1993) and leads to the locking phase.

## 4.2  1. Experiment with sediment slurries

**Discussing cell density**

Summarizing the cell count results, the cell density was significant higher on compostable and on untreated bags compared to polyethylene (PE) and UV-treated bags. Aerobic bacteria settled in significantly higher densities on the bag surfaces than anaerobic bacteria. On all compostable bags, cell densities increased gradually during the experiment and did not reach a stagnation phase towards the end of the experiment. The cell numbers on PE bags increased slightly over time but seemed to stagnate towards the experimental end.

First of all, the settlement of bacteria on PE and compostable bags will be discussed. One explanation why more bacteria were found on compostable bags could be the bag surface structure. Due to its numerous hills, the compostable bag had a rough surface which decreases shear forces and increases surface area and therefore could lead to the superior bacterial attachment. Although the PE surface structure exhibited interlaced filament which offer attaching opportunities as well, the surface structure of the compostable bag seemed to have a higher roughness. The compostable bags were stated to be 100 % biodegradable and consisted of > 50 % biodegradable polyester and >20 % cornstarch (personal correspondence with Swirl). As we only know approximately 70 % of the composition of the compostable bag, only assumptions can be made about physical and chemical properties of the bag surface. It is possible that the compostable bag is more hydrophobic than the PE bag, which would lead to stronger physicochemical interactions between the surface and bacteria.

Furthermore, it is possible that certain parameters in the slurries, such as ingredients from the media or nutrients in the sediment had different effects on the bag surfaces resulting in different physicochemical properties and interactions with bacteria. Discussing the properties of the cells in the different slurries and their possible influence on surface attachment is not possible, as the bacterial communities were not identified.

The fact that aerobic bacteria colonized both types of bags in higher densities than the anaerobic bacteria might be attributed to their different metabolism. Aerobic respiration has a higher energy release compared to anaerobic respiration and can therefore lead to a higher bacterial activity and cell division rate. SEM images of bags incubated in oxic slurry exhibited cell colonies which supports the assumption of higher bacterial activity and cell division as a result of their metabolic differences. However, as the initial cell concentrations in oxic and anoxic slurries were not examined, it is unknown if both slurries had similar cell concentrations at the start of the experiment. Therefore, it is unknown if different settling patterns of aerobic and anaerobic bacteria on the bags occurred due to different metabolic rates or due to different initial cell concentrations in the slurries.

The UV-treatment of bags resulted in lower cell densities compared to the untreated bags. UV-radiation accelerates the leaching of monomers and additives (Yang et al., 2011), for example antioxidants or antiozonants, which stabilize polymers. These substances might have changed the chemical and physical properties of the bag surfaces, which could have led to less bacterial settlement. It is expected that the compostable bags do not contain such additives but UV-radiation might have changes the electrostatic or hydrophobic properties of the surface. The UV-effect should be discussed cautiously, as the used UV source emits light at 254 nm and therefore falls into the range of UV-C radiation. UV-C radiation does not reach the earth surface and is even more energetic than UV-A and B radiation. Hence the treated bags were not exposed to exactly natural conditions. As UV-C radiation is more energetic than UV-A and B, it was expected that alteration of the bags would be strong. However, no optical sign of alteration due to UV-treatment of the bag surfaces was observed from the SEM images.

**Discussing bacterial turnover activity in anoxic treatments**

The bacterial turnover activity was only monitored for the anoxic treatments as no suitable method could be applied for the oxic treatments. As the oxic treatments depended on gas exchange for the whole experimental duration, the system had to be open and respiration measurements were therefore not possible. Total alkalinity (TA) and total sulfide (TS) production is an indicator for anaerobic bacterial activity. Analyzing the bacterial turnover activity in the anoxic treatments using TA and TS measurements, no stagnation towards the end of the experiment was detected. The bacterial turnover activity still increased as no limitation of resources was possibly reached and cell growth was still unhindered at the end of the experiment. The control treatment without bag samples had a slightly lower TA towards the end of the experiment and slightly higher TS than the treatments with bag samples. As both TA and TS are indicators for bacterial turnover activities, it is questionable whether the activity of the control treatment increased or decreased. It is possible that the turnover activity decreased as seen in the TA value and TS dissolved from iron sulfide.

Concluding that the bacterial turnover activities were almost identical for the active anoxic slurry treatments indicates that approximately the same amounts of bacteria within the anoxic slurries were added to each anoxic treatment at the start of the experiment. It also cleared out the possibility that PE bags might be less colonized than compostable bags because of fewer bacteria in the slurry. Therefore, the statement can be confirmed that bacteria settled less on PE bags than on compostable bags. This can only be stated for the anoxic treatments. TS values fluctuated over time for all treatments with active slurries suggesting that sulfide production but also sulfide precipitation took place.

The control treatments with inactive sediment slurry had a slight TA increase which could be due to dissolution of carbonate material from the sediment or due to contamination with carbonate-producing cells. Observing the cell density on bags of the anoxic control treatments, the degree of contamination varied between the replicates and treatments. The variation of the cell concentrations of the controls was not seen in the TA and TS results.

**Discussing biological degradation of bags**

In order to detect whether biological degradation of the bags took place or not, weight loss data, SEM images and RAMAN spectra needed to be analyzed.

All compostable and almost all PE bags lost minor weight. One PE-UV replicate incubated in oxic slurry showed possible biological degradation as it lost more than double of the weight than the other PE samples. But comparing the weight loss with the loss of one PE-UV sample incubated in the oxic control slurry, the loss was even higher for the control and therefore it is unlikely that the weight loss can be explained by biological degradation. As UV-radiation accelerates the leaching of monomers and additives, as already mentioned, this might be a reason for the occurring weight loss. But as all the other UV-treated PE bags gained weight, it is more likely that the detected weight loss was a random effect. All in all, no conspicuous weight loss of the bags was observed when comparing the bags with the control bags. The occurring weight loss might be caused by dissolving substances of the bags when exposed to the slurries.

Considering the SEM images, the bag surface structures did not show any alteration by bacterial activity. Comparing SEM images of the present study with SEM images published by Zettler et al. (2013), their collected plastic particles from the North Atlantic pelagic water showed bacteria embedded in pits whereas bacteria and pit shapes were identical. Here, this was not the case. For RAMAN spectroscopy, the PE and compostable bag incubated in oxic slurry were chosen as they contained the highest cell densities and assumingly had the highest chances to be biological degraded. However, the RAMAN spectra of the incubated bags indicated no change in chemical composition.

As no conspicuous weight loss, no alteration of bag surfaces on SEM images and no change in RAMAN spectra were detected, it is concluded that no biological degradation of either PE or compostable carrier bags took place during the experiment. For the UV-treated bags, it was expected that UV radiation initiates and accelerates the biological degradation process. Albertsson et al. (1987) stated that UV-light and oxidation produces carbonyl groups which are attacked by microorganisms and degrade the shorter segments of the PE chains to carbon dioxide. But this was not the case in the present study and leads to the assumption, that 14 days of UV-exposure might not be sufficient for photo-oxidative reactions on the bag surfaces. Furthermore, O'Brine & Thompson (2010) stated

that polymer chain scission, due to oxidation, requires further degradation to become bio-available.

As inert surfaces accumulate nutrients and serve as favorable settling ground for microbes (ZoBell, 1943) it is possible that bacteria first lived of the nutrients and carbon from the slurry accumulated in the biofilm before degrading surface materials. It is possible that incubation time was too short for a limitation of carbon to occur and for biological degradation to be initiated. In oligotrophic oceans the concentration of nutrients on plastic debris might play a significant role for microbial activity in the upper layer (Zettler et al., 2013) and a following limitation in the biofilm could lead to biological degradation of plastic as nutrients are also limited in the surrounding seawater. A study by Tosin et al. (2012) revealed a strongly inhibited degradation of a PE and biodegradable bag in an aquarium simulating eutrophic water compared to when oligrotrophic water was used. Suggestions had been made, that either the microbial physiology, the microbial community or the water quality had changed resulting a reduced degradation. For this experiment, sediment from Boknis Eck, Eckernförde Bay, was removed during spring. During this season, spring blooms lead to a high sedimentation rate of organic matter and more than 50 % of the total primary production is sedimented (Smetacek et al., 1984). Furthermore, sediment in Boknis Eck is an efficient recycler of inorganic nutrients (Bange et al., 2011). Hence, the sediment used in the experiment is assumed to be rich in carbon and nutrients, it is therefore questionable if biological degradation of either PE or compostable bags would take place when enough carbon is available. Although EPS production by bacteria lead to an immobility of bacterial cells, complex channel systems in the matrix might facilitate the exchange opportunity of nutrients and gases from the surrounding sediment to the inner biofilm (Robinson et al., 1984). Hence, it can be assumed that biofilms in our experiments were supplied with nutrients and carbon as they were surrounded by nutrient- and carbon-rich sediments.

So far the only study monitoring the degradation of plastic on and in the sea floor was done by Tosin et al. (2012) and Kumar et al. (2007). Tosin and colleagues tested the mechanical and biological degradation of a PE and a biodegradable (company Mater-Bi) carrier bag in laboratory experiments, simulating the eulittoral and sandy sublittoral zone.

The biodegradable bag disappeared after 9 months of incubation in the eulittoral zone and 68.9 % of the bag was biological degraded in the sandy sublittoral zone after 236 days. The PE bag was fully intact and visible after incubation in the eulittoral zone simulation for 9 months. Due to the fact, that the eulittoral zone is influenced by tides, it can be assumed that the degradation of the biodegradable bag was predominantly by mechanical forces. Kumar et al. (2007) published a 5 % weight loss of polyethylene carrier bags which were exposed to aerobic heterotrophic bacteria in mangrove soil for eight weeks. As no controls were used in order to determine whether the 5 % weight loss occurred due to bacteria or not, the result should be discussed cautiously.

As these studies focused on different coastal substrata than the sediment experiment in this study, it is difficult to compare and draw conclusions.

## 4.3  2. Experiment with bacteria cultures

**Discussing cell density**

Observing the cell density data of the performed culture experiments, the anaerobic *Desulfovibrio marinus* culture colonized both bags much denser than the aerobic *Alcanivorax borkumensis* cells. Evaluating the cell concentration in the liquid phases of the treatments at the start and end of the experiment showed that both cultures had approximately the same cell concentration at both sampling events. This indicates that the superior colonization of *D. marinus* on the bags was not due to the injection of a higher cell concentration at the start of the experiment but due to a stronger settling rate. As the biofilms of *D. marinus* were too voluminous on the bag samples for reliable cell quantification from the second sampling event on, it was not possible to distinguish whether the species settled preferably on the compostable or PE bag. However, estimations at the last sampling event revealed a denser biofilm on the compostable bags, which again could be explained by the surface roughness of the bag.

The temporal *A. borkumensis* cell density on the compostable bag only increased a little during the experiment and stagnated towards the end, whereas on the PE bag, the cell density steeply increased. *Alcanivorax borkumensis* belongs to the group of hydrocarbon-degrading bacteria (Yakimov et al., 1998), and moreover appears predominantly in oil-polluted regions (Harayama et al., 1999). Schneiker et al. (2006) stated that the genome of *A. borkumensis* contains many determinants of exopolysaccharides and pili genes, which are involved in biofilm formation on oil-water interfaces. Therefore it was expected that *A. borkumensis* has a much higher settling rate on both bags compared to *D. marinus*.

   As this did not occur, it is possible that the cell surface properties of *D. marinus* caused stronger interactions between the bag surfaces and the cells. It is also possible that the media changed the physicochemical properties of the bag surfaces. As each species was incubated in a species-specific medium, it is possible that the medium of *A. borkumensis* changed the bag surface properties and inhibited bacterial settlement. For *D. marinus* it could have been the opposite reaction. Substances in the anoxic medium might

have increased the hydrophobicity of the bag surfaces and led to an enhanced attachment of *D. marinus.* As attachment of *A. borkumensis* was higher on PE bags than on compostable bags, it is assumed that this is due to promoted physicochemical interactions between the PE bag and cells rather than the surface structure.

Examining the cell contamination of the controls without *A. borkumensis*, the PE bag and the liquid phase of this treatment were highly contaminated with cells. On the other hand, evaluating the compostable bag from the same control, the bag was much less contaminated whereas the liquid phase was even higher contaminated than the PE treatment just mentioned. Once again, the PE bag was superiorlxy colonized compared to compostable bag in an oxic treatment (here, control treatment). It is possible that substances in the oxic medium promote the attachment of cells on the PE bag while inhibiting the settlement on compostable bags.

**Discussing bacterial turnover activity of anoxic treatments**

TA and TS data of *Desulfovibrio marinus* treatments showed clear signs of correlation. Whereas TA steeply increased after the second sampling, TS steeply decreased. This was unexpected; as TA and TS are both indicators for bacterial turnover activity and should therefore both positively correlate. The high TA levels indicated a strong bacterial turnover activity due to high cell concentrations. This observation also correlated with the high cell density on the bag samples and cell concentration in the treatments. TA stagnation was reached after 7 days of incubation and did not change until the end of the experiment, indicating that a possible stagnation of cell growth was reached. A possible explanation for the low TS concentrations could be continuous sulfide precipitation as iron sulfide.  TA and TS progress for the control treatment without bag samples was identical to the other treatments just mentioned and hence signifies that bag samples had no influence on the bacterial turnover activity.

The control treatments with inactive/no *D. marinus* did not show any variation within the treatments in both TA and TS except for one replicate of the control treatment with compostable samples incubated in inactive cells. At each sampling point TS of that replicate was much lower than of the other controls which could be due to a preparation artifact at the start of the experiment. The living and dead stain indicated cell

contamination varying between the anoxic control treatments, but no variation was seen in the TA and TS values. Therefore the control treatments should not falsify the comparison to the experiments with active cultures.

**Discussing biological degradation of bags**

In order to discuss biological degradation of both bag types, weight loss data and SEM images needed to be analyzed.

Comparing the weight loss data, the only statistically significant difference was detected between compostable bags incubated with active and inactive/no *D. marinus* culture. However, the effect size was fractional with a loss of 3.4 ± 0.03 % in the active *D. marinus*, 3.6 ± 0.05 % and 3.6 ± 0.03 % in the control treatments. Furthermore, the compostable bags in the control treatment lost slightly more weight than the bag incubated with active *D. marinus* cells; therefore weight loss due to biological degradation is unlikely. The occurring weight loss of the bags might be due to dissolving substances when exposed to liquid. Moreover, almost all PE samples gained weight during incubation time, which might be due to maceration of the material. But as all samples were completely dried for several days before the second weighing took place, this explanation seems unlikely.

SEM images showed different bacterial attachment comparing both cultures. Whereas *A. borkumensis* produced slightly visible filaments, *D. marinus* produced EPS in which cells were embedded in. *D. marinus* seems to show a faster biofilm formation than *A. borkumensis* during our experiments. Although *D. marinus* produced an EPS rich biofilm, no optical signs of bacterial alteration of the bag surfaces were observed on SEM images when removing the biofilm.

Summarizing that no weight loss and no bag surface alteration due to bacterial activity were observed signifies that no biological degradation took place. Again, this finding can be explained by the short incubation time, and bacteria using the bags as substrate rather than carbon source. As the media used for the experiment equaled optimal culturing conditions for both bacteria cultures, both species possibly lived of the nutrients and carbon delivered by the medium rather of the bags.

## *4.4 Discussing the fate of biodegradable products in the marine environment*

As already mentioned, the compostable bag consisted of $> 50$ % biodegradable polyester and >20 % 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). In both experiments no biodegradation of the compostable bags were detected after 28 days (culture experiment) and three months (sediment slurry experiment) of incubation.

Accinelli et al. (2012) stated a similar finding by exposing a compostable carrier bag produced by the company Mater-Bi (100 % biodegradable and compostable thermoplast) to soil, compost, marsh and seawater for three months. The carrier bags incubated in soil and compost had a weight loss of 37 % and 43 %, respectively, whereas the bags incubated in marsh and seawater only lost approximately 1.5 % of their weight. In this study, the highest weight loss of a compostable sample was 2.5 % after three months of incubation with anoxic slurry and 4.7 % after 28 days of incubated with *Alcanivorax borkumensis* culture. Here, weight loss of the compostable bags was higher than of the Mater-Bi carrier bag incubated in seawater. However, weight loss did not occur due to biodegradation, but was rather caused by dissolving substances when exposed to liquid. Hence, it was stated that higher biodegradation of bioplastic in compost and soil took place due to more bacteria and fungi able to degrade bioplastic than in the aquatic systems (Accinelli et al., 2012).

Not only 100 % biodegradable materials have been developed as alternative to plastic, but also plastic mixed with degradable compounds such as additives initiating oxidative chain scission or biodegradable compounds for instance starch blends. O'Brine & Thompson (2010) deployed a Mater-Bi bag (consisted of corn starch, vegetable oils and compostable esters), two oxo-biodegradable plastics (consist of Totally Degradable Plastics Additives (TDPA™)) 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 after 40 weeks of incubation. Though the Mater-Bi bag fully disappeared, it is not proven that microbial biodegradation was the cause for the fast degradation as the study was conducted in the field and several other

factors (currents, waves, macrofauna) need to be considered. The oxo-biodegradable plastics on the other hand, only showed minor degradation. Rutkowska et al. (2002) tested the biodegradation of polyethylene films containing starch. After 20 months of incubation in the Baltic Sea only minor microbial degradation was observed, which was explained by low water temperature and low solar radiation.

Summarizing the present and mentioned studies, degradable plastic bags and biodegradable bags were not all degraded after a short period of time and might therefore remain in the marine system for a prolonged time, potentially causing similar harms as PE material. Degradable and biodegradable bags are rapidly degraded in certain disposal facilities and hence are good when correctly disposed.

# 5 Conclusion

Comparing both experiments, different results were obtained. Experiments with natural sediment slurries showed a clear pattern of higher cell densities on compostable bags than on PE bags and higher colonization rates by aerobic bacteria compared to anaerobic bacteria. The culture experiment on the other hand revealed different bag settling preferences between the cultures and higher cell densities on the bags by the anaerobic culture. The sediment experiment is of avail when working on study questions involving the whole bacterial community in marine sediment. The culture experiment in contrast focuses on species-specific study questions. Here, it became clear that biofilm formation is a complex species- and material-specific process and depends on several factors.

In order to gain more knowledge about the specific settlement of *Alcanivorax borkumensis* and *Desulfovibrio marinus* on plastic surfaces, several more parameters need to be involved, such as bacteria and substratum surface properties. For future studies several of the applied methods require improvements. For instance, cell quantification using epifluorescence microscopy. Cell counting was restricted to the top layer of the biofilm and is therefore only applicable for early biofilm formation and slow growing cultures. For more precise results, cell densities need to be quantified in multi-layer biofilms as well. Additionally, the bacterial turnover activity was only measured for anoxic treatments and not for oxic treatments. However, it is essential to monitor and compare the bacterial turnover activity in both environments in order to detect possible differences in the activity of bacterial communities within one environment and between both environments.

The lack of biodegradation of a standard PE bag and a biodegradable bag by benthic aerobic and anaerobic bacteria after an incubation time of three months exhibits that carrier bags will not only remain for a long time close to the ocean surface (Rutkowska et al., 2002) but also at and in the ocean floor. For future studies a longer incubation time is of great interest in order to discover the possible beginning of biological degradation processes. A possibility to enhance biological degradation of plastic would be the limitation of nutrients and carbon in both sediment and culture experiments. This would

not reflect natural conditions but would clarify whether benthic bacteria can degrade plastic or not. Additionally, genetic analyses would increase the understanding of natural bacterial communities growing on plastic surfaces and would distinguish whether the microbial communities on plastic bags differ from those present in the adjacent sediment. As plastic pollution occurs in all oceans, it is of great interest if benthic bacterial communities settling on plastic vary from each other at different locations inducing different rates of PE degradation. As plastic travels for a long time on ocean surfaces before settling to the ocean floor, it is likely that the initial attached biofilm is taken down to the ocean floor and into the sediment. It would be interesting to find out whether the initial biofilm formed at the surface remains on the bag in the oxic sediment layer and if it will be exchanged by anaerobic bacteria when reaching the anoxic sediment layer.

In conclusion, the present study was the first study focusing on bacterial settlement and biological degradation of a PE and compostable bag in the marine sediment. The compostable bag was not biological degraded after three months and it will most likely remain in marine sediment for a long time, potentially causing similar harms as PE material. It can also be assumed that plastic debris will remain longer in anoxic marine sediment than in oxic sediment due to slower bacterial activity. Plastic on earth exists for over 50 years and concerns about environmental problems in the marine system started round about 10 years ago. Hence, this is a young topic in science with numerous study questions unanswered.

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## Declaration on Oath

I hereby declare that I have completed the present master thesis "Interaction between marine benthic bacteria and plastic/compostable carrier bags: settlement, alteration, and degradation processes" on my own without using others than the stated sources and auxiliary means. The electronic version corresponds to this written version.

I assure that this thesis has not been submitted at other places to achieve the master's degree (M.Sc.). I agree, that a copy of this thesis will be made available in the library of the Christian-Albrechts-University, Kiel.

Alice Nauendorf

Kiel, 13th December, 2013

# Appendix

## Supplemental material
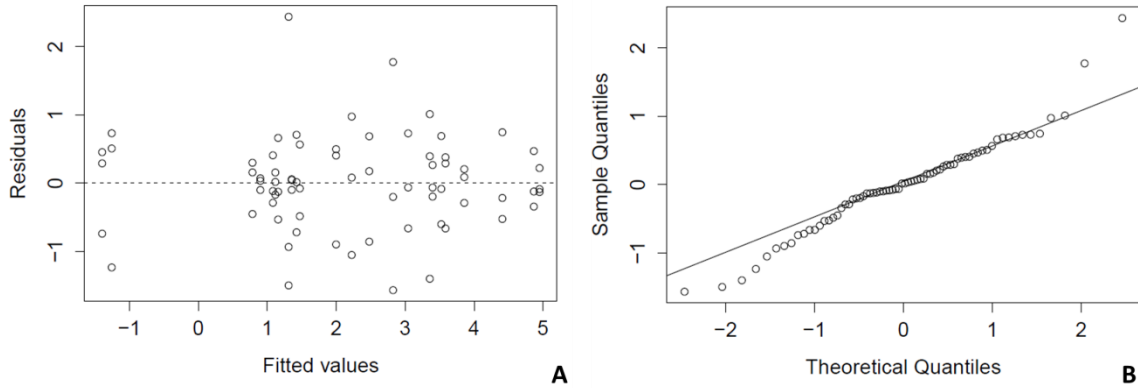


**Figure A1. Slurry experiment.** *Cell density of all treatments.* Graphical evaluation of **A.** homogeneity and **B.** normality after fitting the linear mixed model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
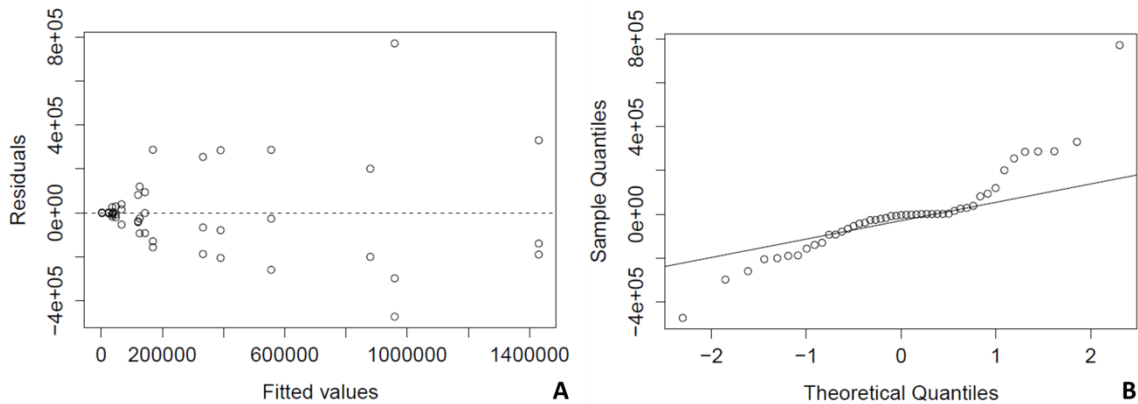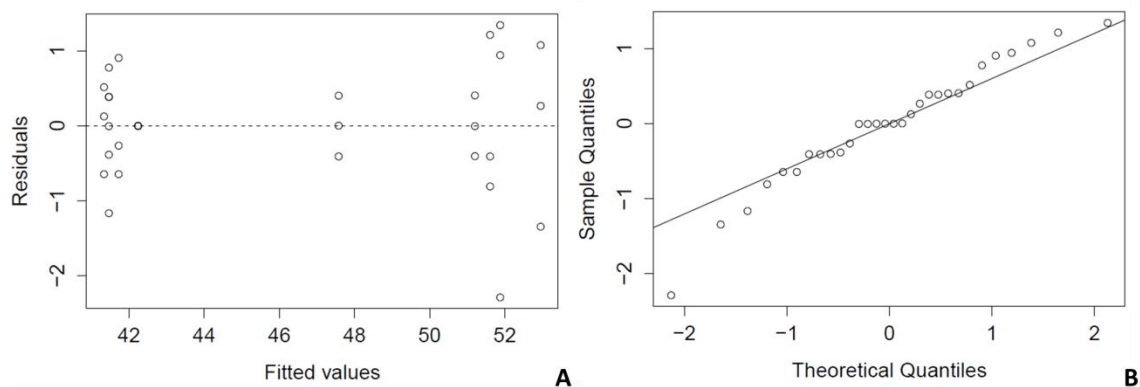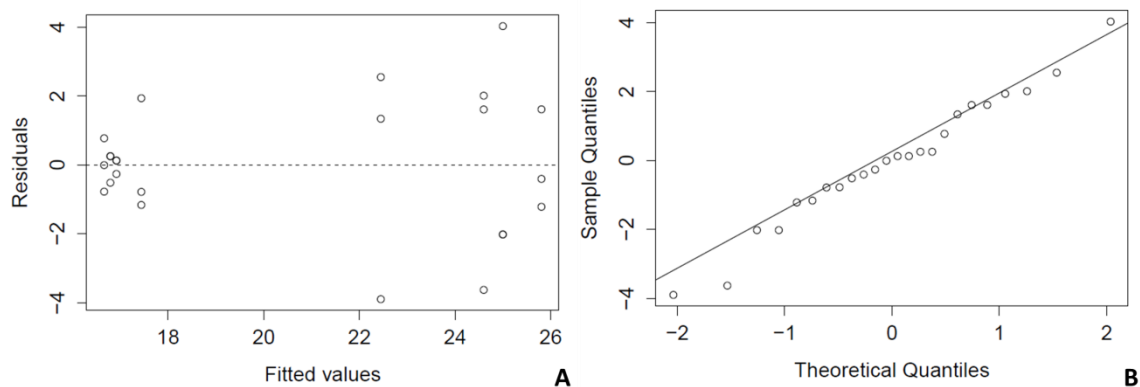


**Figure A2. Slurry experiment.** *Cell density of all treatments at start and end of experiment.* Graphical evaluation of **A.** homogeneity and **B.** normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed with a slight pattern but fulfill the assumption (B). **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
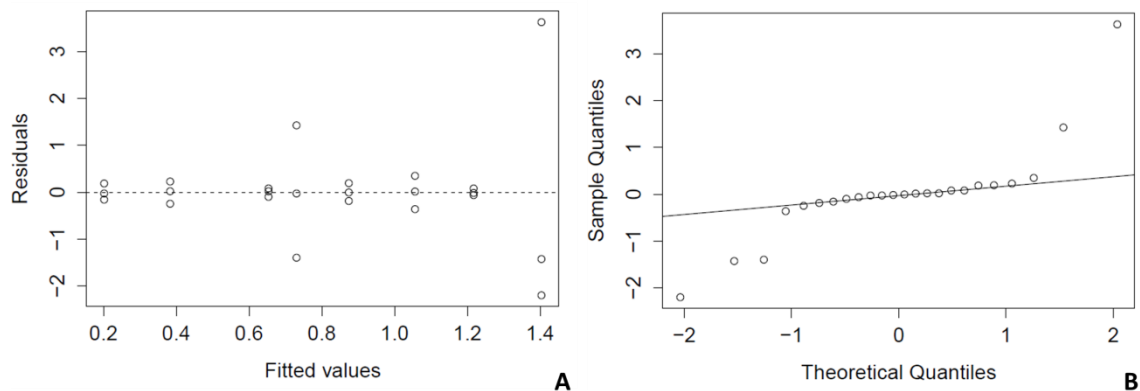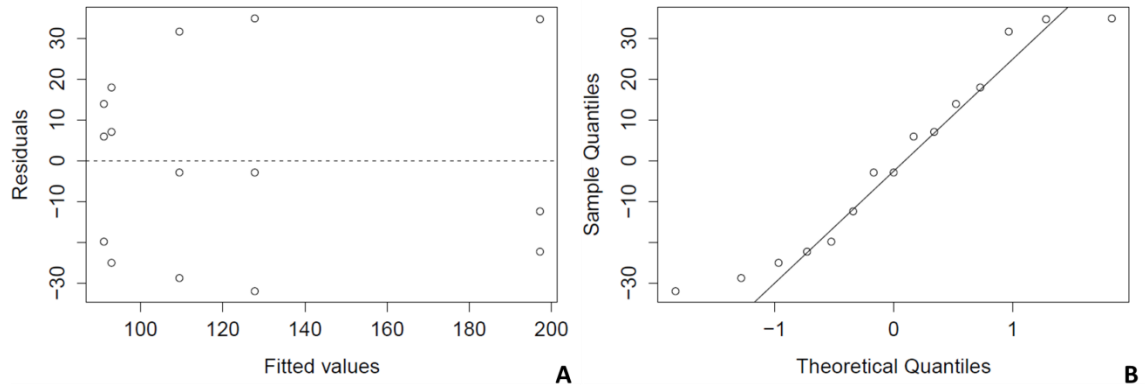
**Figure A3. Slurry experiment.** *Weight loss of oxic treatments.* Graphical evaluation of **A.** homogeneity and **B.** normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
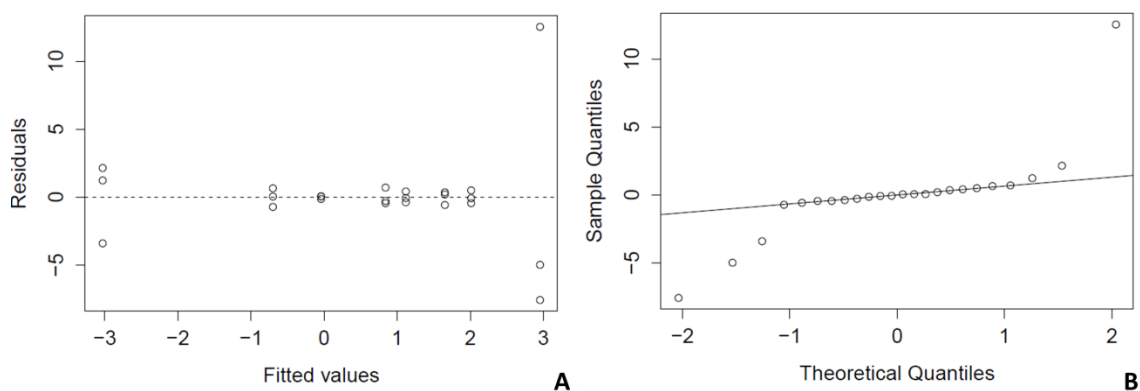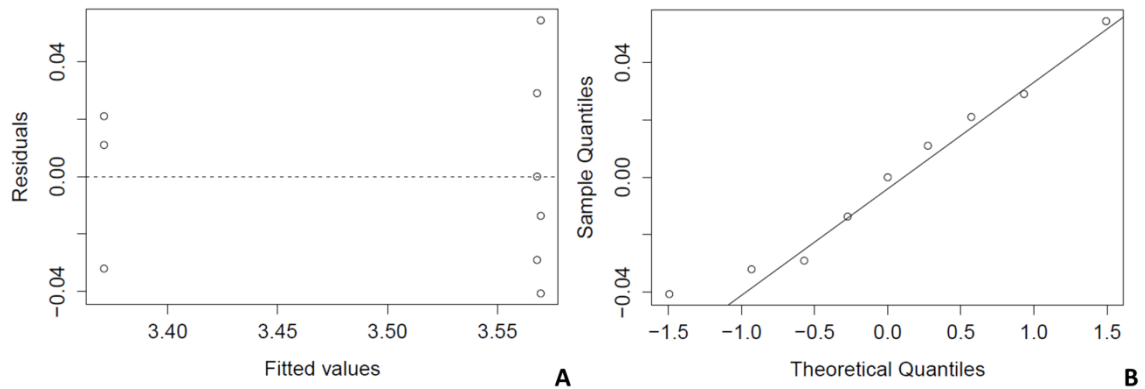


**Figure A4. Slurry experiment.** *Weight loss of anoxic treatments.* Graphical evaluation of **A.** homogeneity and **B.** normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
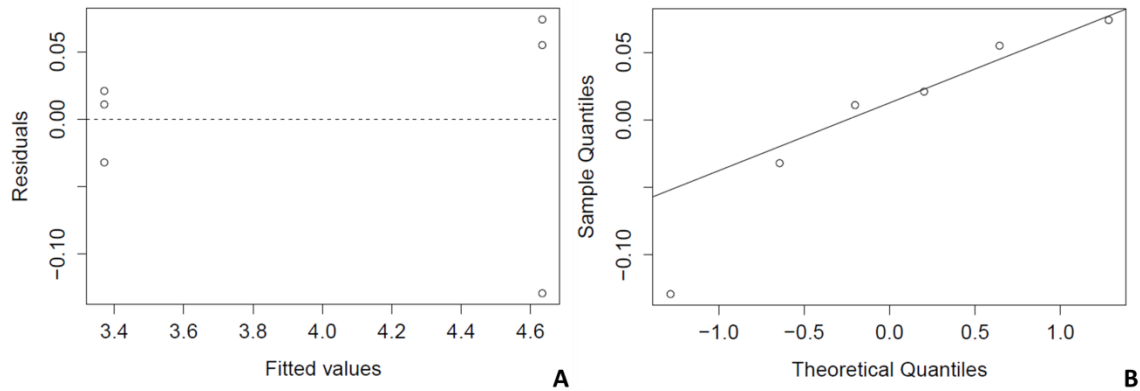
**Figure A5. Slurry experiment.** *TA (from first and last sampling) of active anoxic treatments.* Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.



**Figure A6. Slurry experiment.** *TA (from first and last sampling) of inactive anoxic treatments.* Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
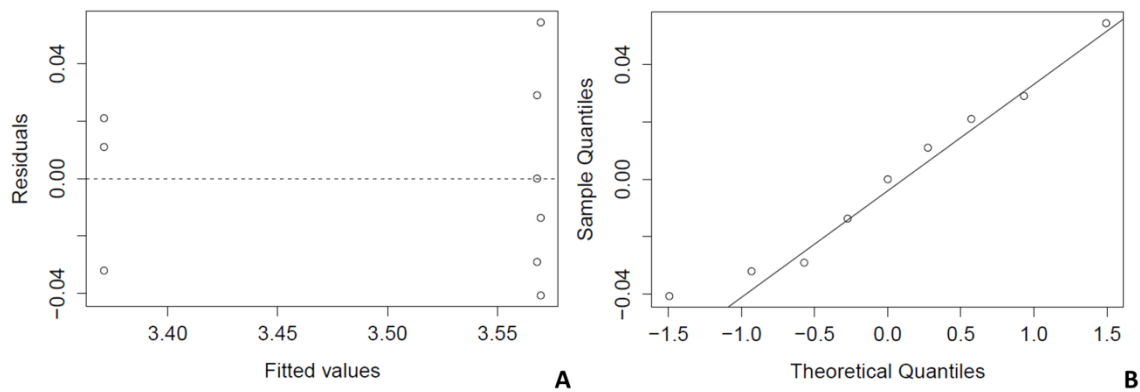
**Figure A7. Slurry experiment.** *TA (from 64 days of incubation) of active anoxic treatments.* Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
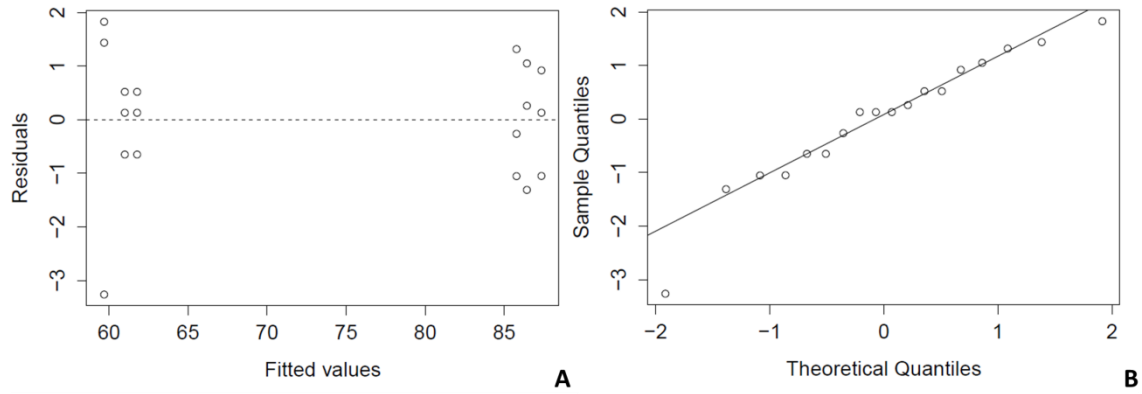


**Figure A8. Culture experiment.** *Weight loss of compostable bags incubated in anoxic treatments.* Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.

**Figure A9. Culture experiment.** *Weight loss of compostable bags incubated with A. borkumensis and D. marinus.* Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.



**Figure A10. Culture experiment.** *Weight loss of compostable bags incubated with active and inactive/no cells  D. marinus.* Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
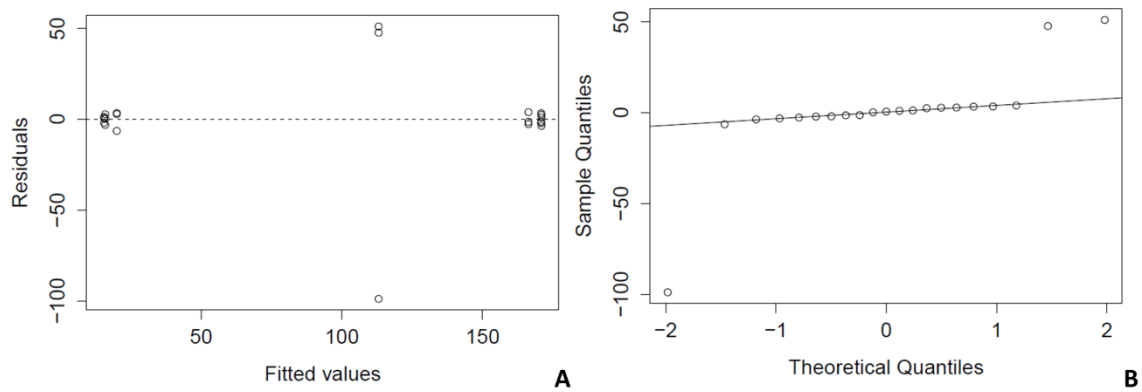
**Figure A11. Culture experiment.** *TA of active D. marinus at the start and end of the experiment.* Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
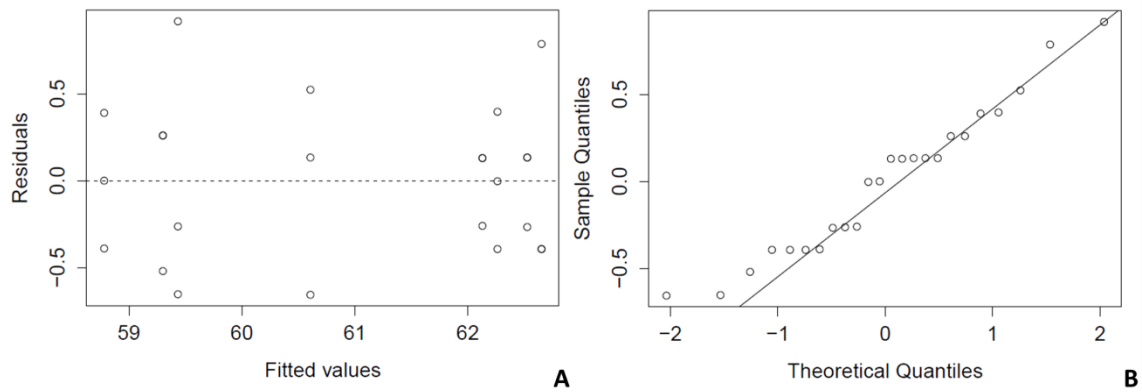


**Figure A12. Culture experiment.** *TA of inactive D. marinus at the start and end of the experiment.* Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well represented by circles, indicating normality.
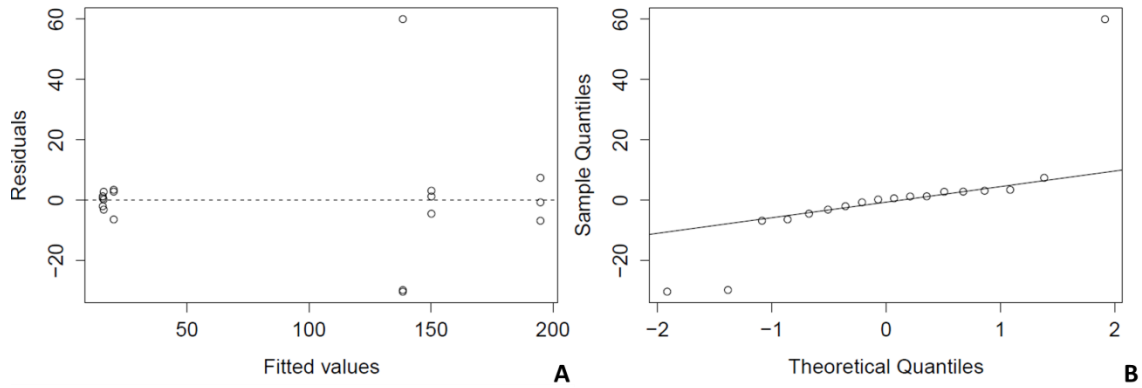
**Figure A13. Culture experiment.** *TS of active D. marinus at the start and end of the experiment.*
Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized
residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-
Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well
represented by circles, indicating normality.



**Figure A14. Culture experiment.** *TS of active and inactive D. marinus at the end of the experiment.*
Graphical evaluation of **A**. homogeneity and **B**. normality after fitting the linear model. **A.** Standardized
residuals versus fitted values. Residuals are distributed without any pattern, indicating homogeneity. **B.** Q-
Q-plots. Expected normal distribution (line) versus the observed distribution (circles). The line is well
represented by circles, indicating normality.