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Biodegradability of Cellulose Diacetate in Aqueous Environments

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Abstract

Cellulose acetate with a degree of substitution (DS) of 2.5, commonly referred to as cellulose diacetate, has been discussed as an important source of microplastic in the environment, especially since it is used to produce cigarette filters. According to EU Single-Use Plastics Directive tobacco products are one of the ten most found SUP products in beach litter by number. However, at present only very few biodegradation studies with natural microbial communities in aqueous media have been reported. In the present study aqueous aerobic biodegradation simulation tests were performed on commercial materials according to international standards (ASTM D6691, ISO 14851 and ISO 19679) to address this bias. Cellulose diacetate proved to be biodegradable or showed strong indication to be non-persistent in freshwater ($>90\%$ relative biodegradation after 100 days at 21 °C), seawater ($>90\%$ after 142 days at 30 °C) and seawater/sediment interface ($>70\%$ after 360 days at 25 °C) under defined laboratory conditions. In freshwater, biodegradation of cellulose diacetate was characterized by a prolonged lag phase (75 days), followed by $>90\%$ relative biodegradation in a short time frame (25 days). This indicates that an abiotic degradation or hydrolysis to reduce the DS is not a pre-requisite to initiate the biodegradation of cellulose diacetate. In addition, it was found that the lag phase can be significantly shortened (from 75 to 5 days) by using pre-adapted microorganisms. In contrast to what could have been expected from literature our present study demonstrates that microorganisms can adapt to a DS as high as 2.5 and metabolize the material. This underlines the importance of studies with natural communities of microorganisms to get a more realistic idea of the persistence of a polymer material.

Keywords Cellulose acetate · Persistence · Freshwater · Marine · Litter · Microplastic · Biodegradation simulation test

Introduction

With an annual production volume of around 1,000,000 t [1, 2], cellulose acetate is one of the most widely produced biobased polymers. The material is used for a variety of applications, like cigarette filters, textiles & apparels, LCD & photographic films, tapes & labels, and extrusion & molding. The cigarette filter application is the leading segment, accounting for over 80% of the market share, and is expected

to grow further especially in developing regions [3]. Due to its abundance and the frequent littering of cigarette filters (tobacco products are by number one of the ten most found Single-Use Plastic (SUP) products in beach litter according to EU Single-Use Plastics Directive [4]), it is often assumed by international governmental agencies, advocacy and media groups that cellulose acetate based materials persist in the oceans for up to a decade [5] and will be transformed into smallest particles only by mechanical impact (e.g. wave action) and/or physical factors (e.g. UV radiation). According to the World Health Organization an estimated 4.5 trillion cigarette butts are thrown away each year worldwide [6]. A recent study by Belzagui et al. [7] found that smoked cigarette butts detach approximately 100 small microfibers (<0.2 mm) per day by mechanical agitation in water and translated this to about 0.3 million tons of potential microfibers that might be annually reaching aquatic environments from this source. As a result, cellulose acetate is described as a main source of persistent "microplastic" in waterways and marine environments, although it has been known for more

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than 50 years that cellulose acetate can biodegrade in aqueous environments, since this has a decisive influence on the performance of reverse osmosis membranes [8]. Scientific studies on the biodegradation behavior of cellulose acetate are still surprisingly rare compared to the importance in the discussion of the consequences of littering for the environment. Our study aims to fill some of the knowledge gaps about the biodegradability of cellulose acetate.

The basis of cellulose acetate is the natural polymer cellulose which is derivatized by partial acetylation of the polymer chain. The resulting acetate side groups are common in nature for polysaccharides [9], but not with as high a proportion as in commercial cellulose acetate materials.

In the case of a completely acetylated cellulose, one refers to a degree of substitution (DS) of 3, i.e. all three hydroxyl groups of a glucose subunit are replaced by acetyl groups. Generally, the term cellulose acetate includes materials with different degrees of substitution. Commercially, the most important are cellulose acetates with a DS of about 2.5 (so-called "cellulose diacetates" (CDA), about 95% market share [1]) and a DS of about 2.9 ("cellulose triacetates"). The distribution of the acetate groups over the glucose units follows to a certain extent a randomized pattern [10]. An exemplary illustration of a section of the molecular structure is given in Fig. 1.

The DS not only has an important influence on the material properties but also influences the biodegradation of a cellulose acetate material. In contrast to many other polymeric materials, the biodegradation of cellulose acetate requires the participation of at least two types of enzymes, namely esterases and cellulases [11]. First, a certain amount of acetylation must be reversed by acetyl esterase enzymes. Next, cellulase enzymes can break down the cellulose backbone. However, it is not a prerequisite that the acetylation level of the material is first significantly lowered before complete breakdown by biodegradation can begin. Deacetylation and cellulose degradation can occur almost simultaneously and could be hardly detectable macroscopically by a change in the degree of acetylation [12, 13].

Based on studies with isolated enzymes or microorganisms it is mostly concluded that microorganisms cannot metabolize cellulose acetate with higher DS, especially with DS > 2. For example, Haske-Cornelius et al. [14] reported

that esterases were not able to deacetylate cellulose acetates with a DS higher than 1.8, while Takeda et al. [15] found that neither regioselectively nor randomly substituted CA with DS ≥ 2 was susceptible to enzymatic degradation by cellulase from *Toricoderma reesei*. Other studies have also demonstrated decreasing biodegradation rate as function of increasing DS [16, 17], but there are exceptions. A study by Sakai et al. [18] showed that two bacteria strains of Neisseria sicca could degrade CA with a DS of 2.3. The authors explained this as a combined action of esterases deacetylating CA, followed by cellulases cleaving the cellulose backbone into smaller fragments. Several other authors have also suggested that a suitable combination of enzymes can significantly promote the degradation rate of cellulose acetate [13, 19]. Although studies with isolated enzymes or microorganisms can be valuable for elucidating mechanistic processes [20], they cannot simply be extrapolated to natural environments where several degradation mechanisms (e.g., hydrolysis, oxidation, biodegradation, fragmentation) may proceed simultaneously.

The biodegradation of non-water-soluble polymer materials in general is a heterogeneous and more complex process [20, 21]. Firstly, microorganisms must colonize the surface of the material to form a biofilm. This biofilm can be described as a complex form of microbial life characterized by a high degree of interaction between different types of organisms (e.g., bacteria, protozoa, fungi) [22]. After an adaption period, the material can be enzymatically attacked from this biofilm and finally metabolized. During these steps there will be interaction and competition between the microorganisms. Simulation tests using a natural inoculum, as they are described in international standards (e.g., ISO 14851, ISO 19679), are a compromise between artificial lab environment and practical field conditions. These tests combine the complex environment with defined conditions concerning, e.g., temperature and pH [21]. A first drawback of such simulation tests compared to real field conditions is the uncertainty to have a representative microbial composition at the beginning and during the test. For example, the microbial composition of sewage sludge used as inoculum in the ISO 14851 simulation test is usually unknown and may vary from one test to the next, even if it comes from a specific source, as the microbiology in a wastewater

Fig. 1 Example of a possible section from a cellulose diacetate polymer (structural formula) with the cellulose backbone (black) and acetate side groups (red)
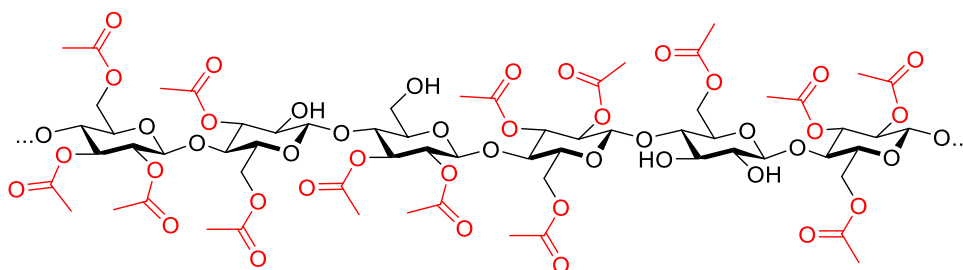
treatment plant is also variable and influenced by many factors [23, 24]. In the worst case, suitable microorganisms may be absent entirely, or may be decimated by inter- or intra-specific interactions (e.g., competition, predations) [24]. The second drawback of simulation tests is the lack of natural exchange and colonization between populations. Possible solutions for this problem are periodic re-inoculations with small amounts of fresh inoculum or the use of pre-conditioned or pre-adapted inoculum from the start or during the test. Chiellini et al. [25] and Julinova et al. [26] found that the ultimate biological fate of polyvinyl alcohol (PVOH) depends largely upon the environment it reaches. High levels of biodegradation were observed in aqueous environments that contained acclimated bacterial species often associated with PVOH-contaminated waste water and sewage sludge. As the biodegradation of cellulose acetate is a complex process involving two types of enzymes, we expect that cellulose acetate would also benefit from the use of pre-adapted inoculum.

From studies with a mixed natural inoculum derived from activated sludge, it has been shown that cellulose diacetate with a DS of up to 2.5 can biodegrade in aqueous aerobic simulation tests [12] and in in-vitro experiments with radiolabeled polymer [27]. Studies in marine environment are still limited but have also gained interest in recent years. For example, Allen et al. [28] conducted a study on various biodegradable polymers and found only slow biodegradation for cellulose diacetate using selected microorganisms rather than a natural mixture of microorganisms. The seawater was pre-adapted to various polymers, but not to cellulose acetate. Gerritse et al. [29] included cigarette filters as one of several plastic objects in a laboratory seawater microcosm and found that the fragmentation rate of cigarette filters (the paper wrapping not taken into account) was much higher than for standard plastic items and even superior to paper coffee cups. However, a conclusion on the proportion of actual mineralization due to biodegradation could not/was not given. By exposing cellulose diacetate films, fabrics and foams to a continuous flow of seawater at 20 °C the test materials were rapidly degraded within a few months, thus proving the possible metabolic degradation of cellulose diacetate by microorganisms [5].

The goal of the present study was to investigate the biodegradation behavior of cellulose diacetate "microplastic" in aerobic aqueous simulation tests according to international standard methods. These test methods are widely used and applied to different polymeric materials, although it is important to keep in mind the limitations described above, especially with respect to the composition of the microbial community. Nevertheless, they are probably the best compromise to evaluate the persistence of "microplastic" under semi-natural conditions. Outdoor tests in natural environment can hardly be carried out with "microplastic" particles

or fibers since this requires local fixation and the ability to clearly distinguish between biological degradation and physical degradation. In standard methods, like ISO 14851, ASTM D6691 and ISO 19679, the former takes place via monitoring of the oxygen consumption and/or carbon dioxide evolution, which can be assigned unambiguously to biodegradation. To simulate "microplastics", cellulose diacetate granules or fibers were milled prior to the biodegradation test. Currently, there is no binding ISO definition of when a particle is to be classified as a "microplastic", resulting in a host of different definitions [30]. But there is a proposal from the European Chemicals Agency (ECHA) that refers to man-made solid polymer materials, including modified natural polymers, with a particle size of ≥ 100 nm and ≤ 5 mm or a fiber length of ≥ 300 nm and ≤ 15 mm, in which the lower size limit is due to analytical and technical constraints and considered as temporary [31].

With this study, we aimed at providing an overview on how cellulose diacetate degrades in aqueous environments using the currently available standards for determination of the ultimate aerobic biodegradability of plastic materials. The biodegradation of cellulose diacetate was evaluated in freshwater, seawater and seawater/sediment interface over a period of multiple months. For simplicity the term freshwater was used throughout this paper to refer to the ISO 14851 simulation test with wastewater treatment sludge. In total, five biodegradation tests were performed: three in freshwater (ISO 14851 A, B and C), one in seawater (ASTM D6691) and one in seawater/sediment interface (ISO 19679). All cellulose diacetates used in the tests described below are commercially produced standard products. We hypothesized that (i) cellulose diacetate is not persistent in aqueous environment, that (ii) abiotic hydrolysis of cellulose diacetate is not necessary as a separate first step before biodegradation can occur, and that (iii) pre-adaptation of the microbial community can significantly accelerate the biodegradation of cellulose diacetate.

Materials and methods

Materials

As cellulose diacetate test materials either fibers or granules of commercial origin were used. The materials were provided by Cerdia Produktions GmbH, except for one fiber material in the seawater test (CDA fiber 8 dpf B) coming from an unknown but different manufacturer. Unlike the granules, cellulose diacetate fibers underwent an additional manufacturing step in which the granules were dissolved in acetone and subsequently dry spun. The fibers used for the tests contain about 0.4% by weight of

titanium dioxide like the majority of fibers currently used for cigarette filters and a small amount of a water dispersible spin finish on the surface (< 1% by weight) from the spinning process.

In addition, in cigarette filters the fibers usually contain about 8% by weight of triacetin (glycerol triacetate), which is sprayed onto the fibers during filter manufacture to make the fiber surface locally sticky for a short time, binding the fibers together and thereby increasing filter hardness. For the purpose of our study, only fibers that did not contain triacetin were used, as triacetin will be extracted by water in a short period of time and is known to be readily biodegradable in aqueous environment [32].

The biodegradation tests were performed on "microplastics" after cryogenic milling with liquid nitrogen. Cryogenic milling keeps the influence with regard to a chemical change of the surface as small as possible. By the milling the length of the fibers was reduced to a maximum of a few millimeters with most of the fibers shorter than 500 µm. The fibers used differed in terms of their linear mass density, which is specified in accordance with the international practice in denier per filament (dpf with denier = g/9000 m fiber length) [33]. The fibers in the tests had a dpf of 1.5 or 8 (see Table 1) and all featured the usual trilobal cross-section. The corresponding circumference of the fibers' cross-section with 1.5 and 8 dpf differs by a factor of about 2.3. The particles obtained by milling of the granules with a sieve insert of 125 µm were additionally screened through a 125 µm sieve and the coarse particles discarded.

As reference materials native microcrystalline cellulose powder for thin layer chromatography (Avicel, Merck Art. Nr. 2331) and a cryogenically milled non-additivated, low-density polyethylene film (Lupolen 2420 K, LyondellBasell) were used. The cellulose powder had a particle size of less than 160 µm. The low-density polyethylene (LDPE) film had a thickness of 50 µm and was milled through a screen of 1 mm. The fraction < 1 mm was used in the test.

The reference and test materials were analyzed for total solids (TS), volatile or organic solids (VS) and total organic carbon content (TOC). The total solids or dry matter was measured by drying the sample at 105 °C until a constant weight was reached. The volatile solids or organic matter was determined by heating the dried sample at 550 °C for at least 4 h. The total organic carbon content of the reference and test items was determined using a high temperature (950–1200 °C) combustion method. The formed CO_2 is measured with IR detection using a Skalar PrimacsSNC-100 analyzer and SNAcces software. The theoretical oxygen demand (ThOD) was calculated using the formula listed in Annex A of ISO 14851 (2019) and based on elemental CHNO analysis according to DIN 51732 [34]. The results are summarized in Table 1.

Table 1 Overview of analyses on the reference and test materials

Material	TS (%)	VS (% on TS)	TOC (%)	ThOD (mg g^{-1})
ISO 14851 A				
Cellulose powder	96.4	100.0	42.5	1149
CDA fiber 1.5 dpf	95.7	99.6	47.5	1272
ISO 14851 B				
Cellulose powder	97.3	99.2	43.6	1135
CDA particle	100.0	98.4	49.1	1263
ISO 14851 C				
Cellulose powder	97.4	99.7	41.8	1125
CDA fiber 1.5 dpf	97.3	99.5	45.4	1277
ASTM D6691				
Cellulose powder	97.0	100.0	42.7	1212
CDA fiber 8 dpf A	97.6	99.5	47.4	1239
CDA fiber 8 dpf B	97.9	99.5	47.1	1224
ISO 19679				
Cellulose powder	98.3	99.8	42.7	–
LDPE film	99.9	99.9	84.6	–
CDA particle	100.0	98.4	49.1	–
CDA fiber 8 dpf A	97.6	99.5	47.4	–

Simulation Test Methods

In this study three test methods were used to determine the degree and rate of biodegradation of cellulose diacetate: (1) The international standard ISO 14851 ("Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium—Method by measuring the oxygen demand in a closed respirometer") [35] was selected to simulate freshwater environment, while (2) the American standard ASTM D6691 ("Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in the Marine Environment by a Defined Microbial Consortium or Natural Sea Water Inoculum") [36] and (3) the international standard ISO 19679 ("Plastics—Determination of aerobic biodegradation of non-floating plastic materials in a seawater/sediment interface—Method by analysis of evolved carbon dioxide") [37] were used to simulate marine environment. ISO 14851 and ASTM D6691 are designed for testing plastic materials in the water column (also known as the "pelagic" zone in marine environment), while ISO 19679 focuses on non-floating plastic materials that settle on marine sandy sediment at the interface between seawater and

the seafloor ("benthic" environment). The ISO 14851 and ASTM D6691 tests are continuously mixed using a magnetic inductive stirrer and stir bars to keep the reference item, test item and growing biomass into suspension throughout the test. The ISO 19679 test is a static test without agitation to avoid burial of the plastic in the sediment. A short description of the individual test methods is given hereafter.

ISO 14851

The inoculum for the freshwater tests was a mixture of activated sludge collected from two to three sewage-treatment plants (see supplementary information, SI Table 1), all located in Belgium and treating domestic and/or industrial wastewater. After filtration of the sludges over an 80 µm sieve, mixing in equal parts (1:1 or 1:1:1 ratio), decantation of the supernatant and replacement with mineral medium, the final sludge inoculum was obtained. This inoculum was actively aerated for a couple of hours at room temperature. The mineral medium was prepared by adding 1 ml of the following stock solutions ($CaCl_2 \cdot 2H_2O$ (36.4 g l^{-1}), $FeCl_3 \cdot 6H_2O$ (0.25 g l^{-1}) and $MgSO_4 \cdot 7H_2O$ (22.5 g l^{-1})) and 10 ml of phosphate buffer solution (8.5 g l^{-1} KH_2PO_4, 21.75 g l^{-1} K_2HPO_4, 33.4 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$ and 0.5 g l^{-1} NH_4Cl) per liter of distilled water.

A set of 500 ml reactors (Fig. 2) was filled with 245 g of mineral medium and 5 g of inoculum in order to obtain a test medium with a concentration of approximately 30 mg suspended solids per liter. The sludge was sampled from the vortex to transfer a homogeneous sample to the different reactors. Next, 25 mg of reference or test material was added to the bottles, except for the control reactors which contained only 250 g of test medium and are used to measure the background activity. After the reactors were filled, a CO_2 absorber (NaOH pellet or KOH solution) was added to the rubber carriers, the bottles were closed air-tight with OxiTop®-C heads and were incubated on inductive stirrers in a thermostatic cabinet at 21 ± 1 °C in the dark. A visual presentation of the test set-up is given in Fig. 2. In one study 4% (= 10 g) of pre-exposed test medium from previous simulation tests was added in addition to the sludge inoculum to investigate whether it is possible to shorten the lag phase of cellulose diacetate. The simulation tests that produced the pre-exposed medium were performed in the exact same manner as the aforementioned ISO 14851 procedure and high levels of biodegradation were achieved for non-commercial cellulose diacetate based materials: > 90% absolute biodegradation after 98 days in one test and after 28 days in the other test, confirming the presence of CDA-degrading microorganisms. The two pre-exposed test mediums were mixed in a 1:1 ratio.

The test medium (= mineral medium + sludge inoculum) was analyzed for total suspended solids (TSS), volatile suspended solids (VSS), pH, NH_4^+–N and NO_x^-–N at the start of the experiment, see supplementary information (SI Table 2). At the end of the test, the presence of nitrite or nitrate was examined by means of analytical test strips (Nitrate Test, Merck Art. Nr. 1.10020). If nitrite or nitrate are present, the concentration of NH_4^+–N, NO_3^-–N and/or NO_2^-–N was determined by a discrete analyzer system and spectrophotometric detection. Additionally, the pH was measured in every reactor. The results are given in the supplementary information (SI Table 4).

During the aerobic biodegradation of organic materials in an aqueous medium, oxygen is consumed, and carbon is converted to CO_2. A CO_2 absorber traps the CO_2 released and

Fig. 2 Example of a test reactor for evaluation of biodegradation in freshwater/seawater (left) and seawater/sediment interface (right)

the induced pressure-drop is directly related to the consumed oxygen and hence to the biodegradation of the test item. At regular time intervals, before the absorption capacity of the CO_2 absorber is exceeded, the absorber is removed, and the amount of CO_2 evolved is titrimetrically measured. At the time of removal of the CO_2 absorber the vessels are opened and air in the headspace is refreshed.

The amount of oxygen consumption in the reactors was measured at regular interval (every 3–4 h) using WTW OxiTop®-C for BOD measurement and Achat OC 2.03 software. The specific biochemical oxygen demand of the test material (BOD_S, in mg g^{-1} of test material) was calculated as the difference between the oxygen consumption in the test reactor and the control reactor, divided by the concentration of the test material. The biodegradation was calculated as the ratio of the specific biochemical oxygen demand to the theoretical oxygen demand (ThOD, in mg g^{-1} of test material). For each test, the mean absolute biodegradation, the standard deviation and the relative biodegradation (i.e. the biodegradation of the test material relative to the reference material) were calculated using Microsoft Excel 365.

Additionally, the amount of CO_2 captured was determined by titration of the NaOH pellet or KOH solution with hydrochloric acid using a Metrohm 888 Titrando and tiamo™ 2.5 software. The percentage of biodegradation was calculated by dividing the cumulative net CO_2 production of the test item (test reactor minus control reactor, in mg) by the theoretical amount of carbon dioxide evolved ($ThCO_2$, in mg) and multiplying by 100. The $ThCO_2$ was calculated from the mass (in mg) of test material introduced into the test reactor, multiplied by the total organic carbon content (TOC, in %) of the test material and corrected for the molar mass of carbon dioxide and carbon.

ASTM D6691

The inoculum was derived from natural seawater collected from the North Sea (Belgian coast). The seawater is pumped up by Farys North Sea Pool (Blankenberge) from the low-water line using a seawater pipeline under the beach and is stored in a buffer tank. In the lab inorganic nutrients were added to the seawater in a concentration of 0.05 g l^{-1} NH_4Cl and 0.1 g l^{-1} KH_2PO_4, after which the seawater was pre-incubated for 7 days at 30 °C and sieved on an 80 µm screen prior to use. At the start of the experiment, each 500 ml reactor (see Fig. 2) was filled with the same amount of enriched seawater (250 g). The reference and test item (60 mg) were added directly to the reactors. After filling of the reactors, KOH solution was added to the rubber carriers and the reactors were closed with OxiTop®-C heads and put on an inductive stirrer. The reactors were incubated at a constant temperature of 30 ± 1 °C in the dark. The seawater was analyzed for total solids (TS), volatile solids (VS), pH,

NH_4^+–N and NO_x^-–N at the start of the experiment, see supplementary information (SI Table 3). The analyses at end are identical to ISO 14851.

The way in which O_2 and CO_2 were measured, and biodegradation calculated was identical to ISO 14851.

ISO 19679

Sediment and seawater were collected from the North Sea (Belgian coast) at low tide. In the lab, the sediment was drained to remove excess water and the seawater was enriched with nutrients (NH_4Cl (0.05 g l^{-1}) and KH_2PO_4 (0.1 g l^{-1})). A preliminary oxidation was applied to the sediment to decrease the organic matter content and the background respiration by flushing the sediment and seawater with air for 7 days at 25 ± 2 °C. Subsequently, a set of 250 ml reactors was filled with 30 g of wet sediment and 70 g of enriched seawater, with a seawater/sediment volume ratio between 3:1 and 5:1 and a sediment layer of about 0.3 to 0.5 cm (see Fig. 2). Potassium hydroxide solution was added to the absorber compartment, after which the reactors were closed airtight and incubated at a constant temperature of 25 ± 2 °C. At regular intervals (once per week) the amount of CO_2 produced was determined by titration of the KOH solution. After 3 weeks of pre-conditioning the diverging reactors were removed to obtain a set of reactors with a lower, more similar endogenous respiration. To these reactors 20 mg of reference or test item was added, except for the control reactors. Fresh potassium hydroxide solution was added, and the reactors were closed and incubated at 25 ± 2 °C in the dark. At that moment the actual biodegradation experiment was started. The seawater and sediment were analyzed for total solids (TS), volatile solids (VS), pH, total nitrogen (TN) and total organic carbon (TOC) at the start of the experiment, see supplementary information (SI Table 3).

The way in which CO_2 was measured and biodegradation calculated was identical to ISO 14851. Oxygen consumption was not determined as ISO 19679 is a CO_2 based methodology.

Scanning Electron Microscope (SEM)

Milled cellulose diacetate samples were fixed to the sample holder by double sided adhesive carbon discs, and sputter coated (Edwards Sputter Coater S150B) with gold. The images were taken with a Zeiss EVO MA15 SEM in high vacuum mode using an SE1 detector, 20 kV voltage and a magnification of 75×.

Figure 3 show SEM images of test materials CDA fiber 8 dpf A and CDA fiber 8 dpf B. There is no apparent difference between the two materials from different producers. The length of the fibers was mostly in the range of some hundred micrometers.

Fig. 3 SEM images of the cellulose diacetate (CDA) fiber materials used in the biodegradation test in marine water according to ASTM D6691 (A left, B right)
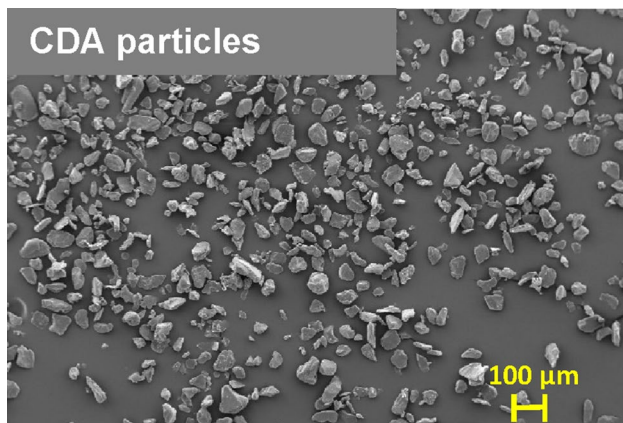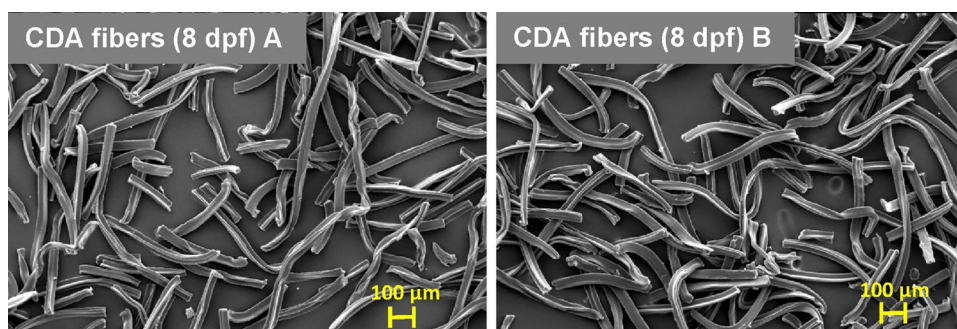
Fig. 4 SEM image of the cellulose diacetate (CDA) particle material

A SEM image of the cellulose diacetate particles is depicted in Fig. 4.

Results and Discussion

The validity requirement for the reference items was fulfilled in all tests. Positive reference item cellulose reached more than 60% biodegradation at the end of the test in freshwater (as defined by ISO14851), more than 70% at the end of the test in seawater (as defined by ASTM D6691) and more than 60% after 180 days in seawater/sediment interface (as defined by ISO 19679). In addition, the percentage of biodegradation of negative reference LDPE remained below 10% in the ISO 19679 test (Table 2).

Table 2 Absolute (abs) biodegradation, standard deviation (sd) and relative (rel) biodegradation in % based on O_2 consumption and/or CO_2 production

| Material	# Repl.	Time (days)	% of mineralization					
			Based on O_2 consumption			Based on CO_2 production		
			abs (%)	sd (%)	rel (%)	abs (%)	sd (%)	rel (%)
ISO 14851 A								
Cellulose powder	2	118	82.5	7.0	100.0	74.0	5.9	100.0
CDA fiber 1.5 dpf	2	118	72.3	11.6	87.7	68.8	8.7	93.0
ISO 14851 B								
Cellulose powder	3	40	84.2	1.8	100.0	–	–	–
CDA particle	3	40	21.8	39.5	25.9	–	–	–
ISO 14851 C								
Cellulose powder	3	40	89.9	0.5	100.0	–	–	–
CDA fiber 1.5 dpf	3	40	72.3	2.4	80.4	–	–	–
ASTM D6691								
Cellulose powder	2	252	84.3	0.2	100.0	81.7	0.0	100.0
CDA fiber 8 dpf A	2	252	97.7	8.9	115.9	83.9	3.6	102.8
CDA fiber 8 dpf B	2	252	96.3	3.3	114.2	83.7	9.6	102.5
ISO 19679								
Cellulose powder	2	360	–	–	–	83.5	5.3	100.0
LDPE film	2	360	–	–	–	4.3	0.3	5.2
CDA particle	2	360	–	–	–	61.0	31.6	73.1
CDA fiber 8 dpf A	2	360	–	–	–	32.1	6.7	38.5

Tests in Freshwater According to ISO 14851

Biodegradation in freshwater was tested according to the standard ISO 14851 ("Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium - Method by measuring the oxygen demand in a closed respirometer"). The test method is similar to the OECD method 301 F, which was developed to screen chemicals for ready biodegradability in an aerobic aqueous medium [38]. The main difference between the two methods is the use of a fast-biodegrading polymer as a reference material in case of the ISO test. According to ISO 14851 and OECD 301 F the biodegradation is determined by measuring the oxygen demand in a closed respirometer. The consumption of oxygen is determined from the change in pressure in the bottles, since evolved carbon dioxide is absorbed, for example by potassium hydroxide solution. Alternatively, the quantity of oxygen required to maintain constant gas volume in the respirometer bottle can be measured. The water in the bottles contains defined minerals (e.g., $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, phosphate buffer) and a small amount of inoculum (e.g., bacteria, protozoa), which is usually derived from activated sludge from a wastewater treatment plant. Activated sludge is used since other sources were found to give higher scattering of results [38]. An influence by UV light is excluded in this test, as it is performed under exclusion of light. This is necessary to avoid the growth of photo-autotrophic organisms (for example, algal blooms or mixotrophic protozoa) that absorb CO_2 and release O_2, which can interfere with the biodegradation measurements [39].

Figure 5 shows the biodegradation process of cellulose diacetate microfibers compared to cellulose powder in the first freshwater experiment (ISO 14851 A) using sludge inoculum without any pre-adaptation to the test material.

In the case of the cellulose diacetate fibers, a long lag phase of approximately 75 days is notable compared to cellulose, which is probably due to the adaptation of the deacetylating microorganisms to the fiber substrate [35] and biofilm formation [40], respectively. The majority of the degradation then took place in a time frame similar to that of cellulose. After 118 days the cellulose diacetate fibers reached an absolute biodegradation of 72.3% ± 11.6%. A similar observation was made by Gu et al. [41] in a simulated thermophilic compost environment. After a lag phase of 25 days, the rate of degradation rapidly increased resulting in a mineralization of 77.6% after 60 days for cellulose acetate powder with a DS of 2.5. This is a strong indication of a degradation mechanism consisting of the simultaneous action of deacetylating and cellulose-degrading enzymes, which is in agreement with former reports of only minor changes in DS during the biodegradation process of cellulose acetates [12, 13]. In other words, the DS of the remaining material is not a suitable indication for the progress of biodegradation.

This illustrates that, contrary to what is often claimed, abiotic hydrolysis of cellulose diacetate to a significantly lower DS is not necessary as separate first step before biodegradation can occur. Otherwise, a significant biodegradation or O_2 consumption, respectively, would already have occurred before the strong increase in biodegradation due to a metabolism of the cleaved acetate groups. For example, a reduction of the DS from 2.5 to 2.1 would translate to a release of 16% of the acetyl groups or 7.3% of the total carbon content. Acetates or acetic acid are known to be readily biodegradable, so sodium acetate is used as a reference compound in OECD 301 tests [38]. The apparent absence of significant abiotic hydrolysis is supported by studies on hydrolysis rates of cellulose diacetate in water as

Fig. 5 Biodegradation of cellulose diacetate (CDA) fibers and cellulose powder reference in freshwater according to the ISO 14851 method, based on O_2 consumption. Mean biodegradation (lines) and standard deviation (shaded area).
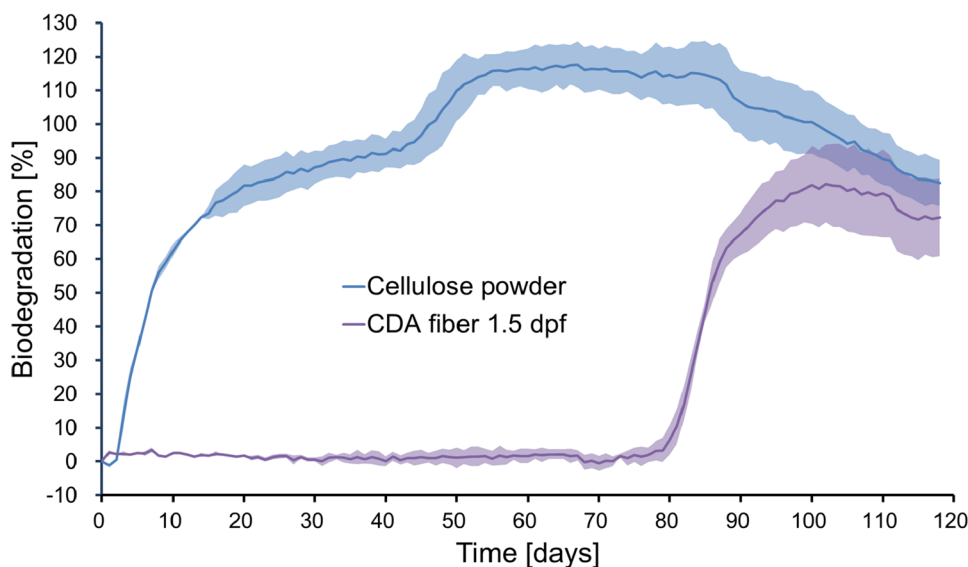
a function of pH and temperature, which were carried out by Vos et al. [42] several decades ago, since cellulose diacetate is an important material for membranes in separation technology and hydrolysis would significantly alter the properties. Although porous membranes of cellulose diacetate were found to be most stable in water at a pH around 5, the expected hydrolysis under ISO 14851 conditions with a pH of 7 and a temperature of 21 °C would result in the release of less than 2% of the acetyl groups or less than 1% of the total carbon content after 80 days. The pH-values measured in our test were 7.1 at start (SI Table 2) and 6.5 in the cellulose diacetate reactors at the end (118 days) (SI Table 4). The decrease in pH is mainly caused by nitrification due to the release of hydrogen ions (H^+) when ammonia is oxidized to nitrite [43].

Even if one assumes an abiotic hydrolysis happening without metabolism and only at the surface to have locally a higher decrease in DS, this could not explain the fast progress of biodegradation, since after biodegradation of the surface layers the remaining material would require again a similar length of time for abiotic hydrolysis.

The course of the CDA degradation curve in Fig. 5 also demonstrates that modeling the half-life ($t_{0.5}$) or disappearance time (DT50) of biodegradation is not a suitable approach for assessing the environmental persistence of materials with a longer lag phase, like cellulose acetate. An aerobic degradation rate could also be misleading, especially if it was calculated based on only partial biodegradation (e.g., 50%), as it does not reflect the actual course of biodegradation. After 80 days, the measured biodegradation in this test was still below 10%, whereas an absolute degradation of 50% was already reached after 85 days, and 80% after 97 days.

The biodegradation expressed in % refers to the theoretical oxygen demand calculated on the basis of the elemental composition of the material. An evaluation based on the absolute percentage values must take into account, that a complete degradation (100%) is usually not measured in a biodegradation test, since part of the metabolized carbon also serves the growth of the microorganisms or the build-up of biomass, respectively, which will not result in O_2 consumption or CO_2 release. Percentage values above 100% as observed for the cellulose reference in this test (Table 2) can be explained by nitrification (see Supplementary Information SI Table 4). Ammonium salts and nitrogen-containing test compounds can be oxidized to nitrite or nitrate during the incubation period of a biodegradation test. This additional oxygen consumption, which is not linked to biodegradation of the reference or test material, can lead to an overestimation of the biodegradation and is visible in the cellulose chart (Fig. 5) as the secondary acceleration between 40 and 55 days of testing. In the control reactors the nitrification process occurred at a later stage and not simultaneously in

all replicates, which resulted in the slowly decreasing biodegradation trend from 85 days onwards.

Nevertheless, after about 100 days the cellulose diacetate fibers were metabolized to an extent similar to the cellulose reference. This is generally interpreted as that the material is fully biodegradable. In most cases, a somewhat lower percentage compared to the rapidly degrading reference is also accepted. When evaluating biodegradation curves, it cannot be simply assumed that the respective proportions of carbon content converted to CO_2 or biomass at a certain level of metabolism are the same for different materials [44].

Also the evolution of CO_2 was measured in parallel, although at longer intervals. The measurement confirmed the result of the O_2 consumption according to ISO 14851. The corresponding curve can be found in the supplementary information (SI Fig. 1).

The complexity of the processes involved in this type of biodegradation test at the microbial level is also demonstrated by the fact that there can be high variability between replicates within a test set where both the initial and the experimental conditions are the same, i.e., also the same inoculum is added. Figure 6 shows the course of biodegradation of 3 replicates of the same cellulose diacetate particles material over a period of 40 days in the second freshwater experiment (ISO 14851 B), using sludge inoculum without any pre-adaptation to the test material. The particles are commercial cellulose diacetate grind to a size of less than 125 μm. Despite the same conditions, one of the replicates already started to biodegrade after 16 days, whereas the others displayed a prolonged lag phase. A possible explanation for this phenomenon is the change in the microbial composition after the start of the test due to biofilm formation and maturation [45]. However, a characterization of the microbial community was not carried out within our study and will be a subject for future studies. An effect of pH seems unlikely since at the end of the test a pH of 7.2 was measured in all reactors.

Values below 0% can be explained by a lower O_2 consumption in the cellulose diacetate reactors opposed to the control reactors, causing a negative net signal and thus a negative biodegradation. In this case the negative trend is limited and should be attributed to natural variation in the background activity rather than a toxic effect of the cellulose diacetate particles on the microbial population.

Test in Freshwater According to ISO 14851 with Pre-exposed Water

The adaptation of a microbial community to a given substrate is a natural phenomenon, known for example from resistancy to antibiotics [46] or heavy metals [47]. To check how cellulose diacetate behaves if already at the beginning of the test a microbial community potentially more positive

Fig. 6 Variation in the biodegradation course of three replicates (R1, R2, R3) of the same cellulose diacetate (CDA) particles in the same test in freshwater according to the ISO 14851 method, based on O_2 consumption
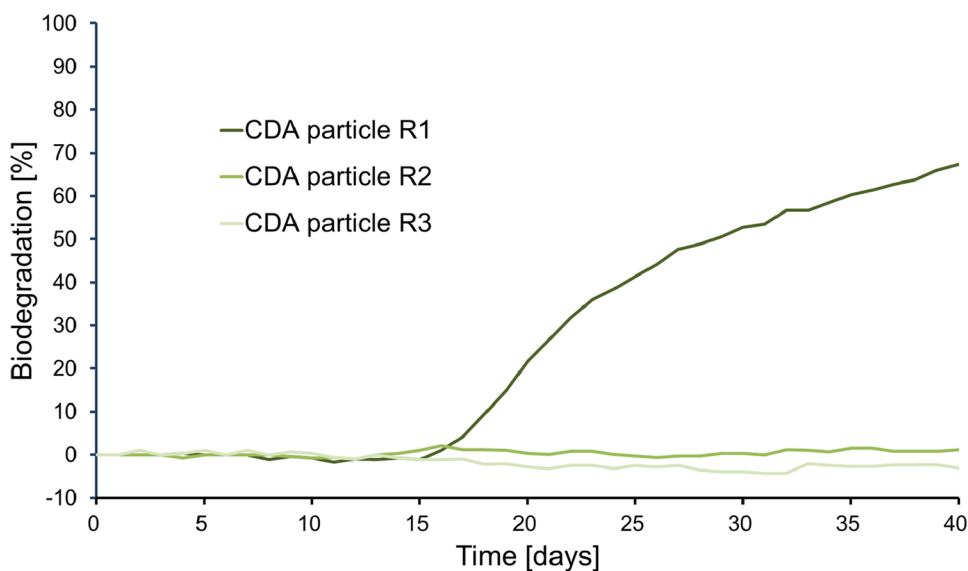
for the degradation of cellulose acetate is present, a small amount of test medium (4%) from previous biodegradation tests on cellulose acetate was added to the test medium at start in our third freshwater experiment (ISO 14851 C). Since cellulose acetate was degraded in the former tests, that water should contain a somewhat larger number of the microorganisms relevant for or, respectively, adapted to the degradation of cellulose acetate than in case of only fresh activated sludge as inoculum.

As can be seen in Fig. 7, the duration of the lag phase was significantly reduced to less than 7 days for the cellulose diacetate fibers, which is only slightly longer than that of the cellulose reference, and 50% biodegradation was reached already after 24 days. This result not only demonstrates the influence of the microbial composition at the beginning, it

also underlines that the observed degradation of cellulose diacetate is indeed a biological process and does not rely on an abiotic chemical hydrolsis step.

Moreover, our test results demonstrate that naturally occuring microbial communities can adapt to cellulose diacetate.

Test in Seawater According to ASTM D6691

The temperature used in the marine biodegradation simulation test is 30 ± 2 °C. This corresponds to the usual maximum temperatures for water at the sea surface [48]. Under natural conditions, biodegradation of plastic particles will generally occur at much lower temperatures. The temperature at the upper end is a compromise, since under

Fig. 7 Biodegradation of cellulose diacetate (CDA) fibers and cellulose powder reference in freshwater according to the ISO 14851 method with addition of 4% of water from a previous biodegradation test, based on O_2 consumption. Mean biodegradation (lines) and standard deviation (shaded area)
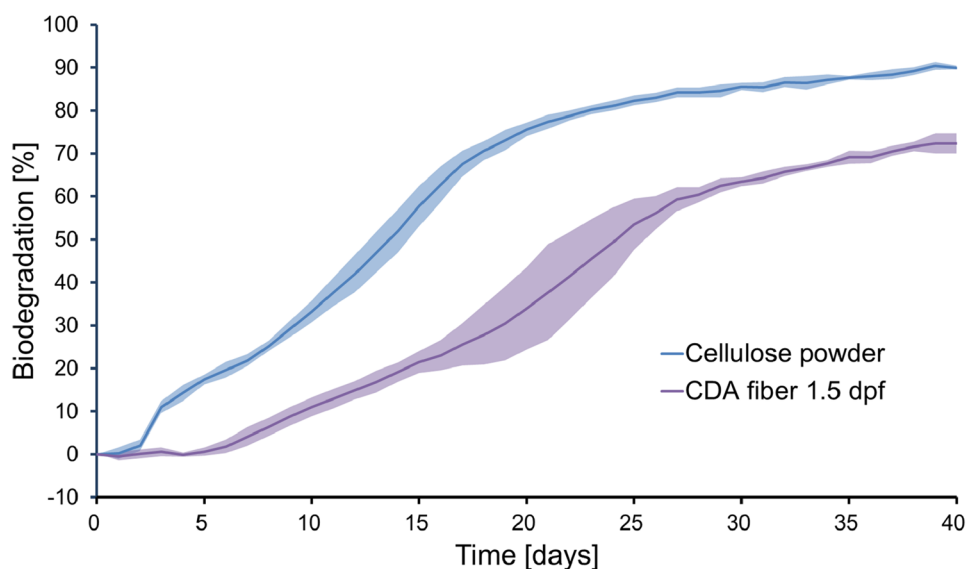
cold conditions it would take several years for a test result to be available, even for biodegradable natural substances [49, 50]. To extrapolate biodegradability to lower temperatures, there must be no phase transition of the polymer or a significant change in its solubility between the accelerated test temperature and real-life conditions. In the case of cellulose diacetate, such changes do not occur in the relevant temperature ranges.

As can be seen from Fig. 8, the cellulose diacetate fibers clearly biodegrade in seawater. Like in the test according to ISO 14851, a lag phase could be observed at the beginning, before a clear and more or less steady increase in biodegradation occurred.

A look at the individual replicates in Fig. 9 shows that the apparent difference between the fiber samples A and B is only caused by a single replicate of sample B that shows an initial steep increase immediately after the adaptation phase. Due to the small number of replicates, it is not possible to state whether this accelerated biodegradation is a large exception. Since the other replicate of sample B behaves comparably to both replicas of sample A, the observed difference in biodegradation behavior is probably not due to an intrinsic difference of the two commercial materials. However, no conclusive explanation was found for why such a significant difference between replicates was observed. Increasing the number of microorganisms in the seawater and enriching the flora might help to reduce the variability

Fig. 8 Biodegradation of cellulose diacetate (CDA) fiber samples and cellulose powder reference in seawater according to the ASTM D6691 method, based on O_2 consumption. Mean biodegradation (lines) and standard deviation (shaded area)
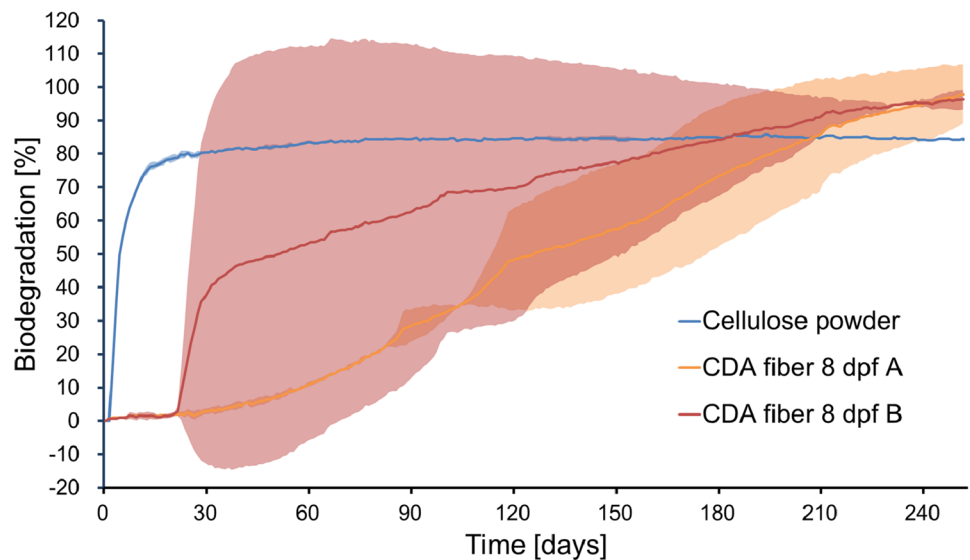
Fig. 9 Variation in the biodegradation course of the single replicates (R) of the same cellulose diacetate (CDA) fiber materials A and B in the same test in seawater according to the ASTM D6691 method, based on O_2 consumption
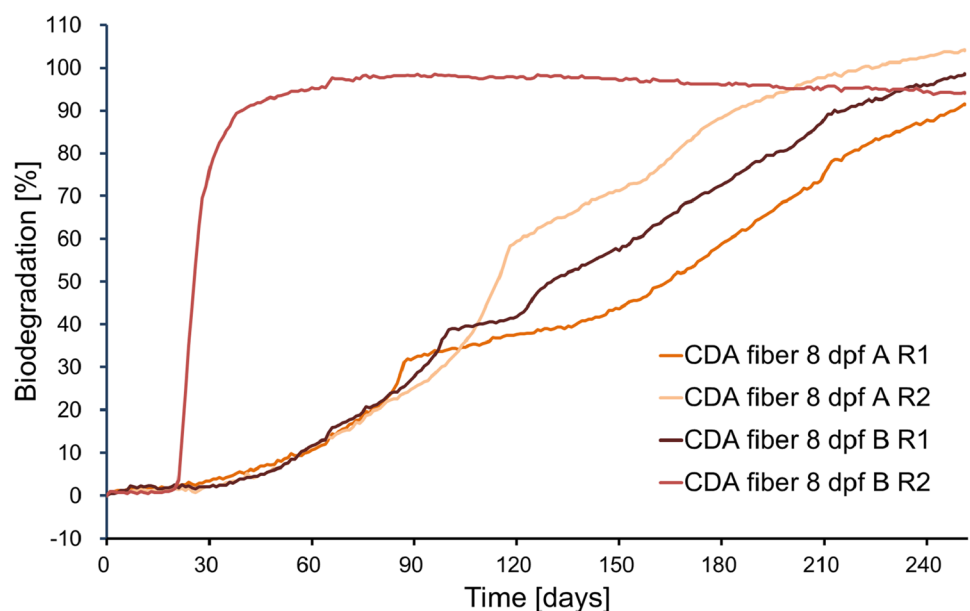
between replicates. Currently, the ISO Technical Committee for Environmental aspects ISO/TC 61/SC 14 is working on a new standard (ISO/AWI 18957) which suggest different options for increasing the activity in the seawater, for example, addition of an organic nutrient source (like peptone or yeast extract), mixing of seawater from different locations (marine and estuarine), concentration of microorganisms through filtration or sonication [51].

In contrast to the test in freshwater, a contribution of abiotic hydrolysis is more likely in seawater because of the higher pH of the environment which helps to remove acetyl groups ($-COCH_3$) [52]. The pH of seawater has a range of 7.4 to 9.6 [53] and is commonly about 8.1 [54], especially for surface seawater. The seawater sampled from the North Sea had a pH of 8.0, which dropped to 7.6 after addition of the nutrients. pH was not corrected because we know from experience that the effect is only temporarily. At the end of the test (252 days) a pH of 8.2 ± 0.0 was measured in the control reactors. The cellulose diacetate samples were in the range of 7.3–7.4, with only the faster degrading replicate of sample B having a final pH of 8.6. Potentially a lower pH caused by nitrification (see SI Table 4) prevented faster degradation in most replicates. However, the evolution of the pH-value in the individual samples during the test period were not measured within this study.

Based on the study with porous cellulose acetate membranes by Vos et al. [42] the abiotic hydrolysis rate in water at pH 7.6/30 °C would be estimated to be around 200 times higher than at pH 7.0/21 °C, and the estimated release of acetate groups in the range of 5% (2–3% of total carbon) during a lag phase of 20 days. Even though study results from examinations in water cannot simply be applied to seawater, an influence of abiotic hydrolysis is at least conceivable already at an early stage of the biodegradation process.

It can be assumed that such hydrolysis is initially limited to the surface, since the fibers are not a porous material. There, this could support the enzymatical attack by lowering the steric hurdles for deacetylation enzymes, although this could not explain the faster progressing biodegradation of one replicate in Fig. 9. Abiotic hydrolysis could also indirectly promote biodegradation, since it makes the surface more hydrophilic and thus generally creates better conditions for the formation of a biofilm [55, 56].

The test results reveal that cellulose acetate is not persistent in seawater under standard laboratory conditions (30 °C, 0.05 g l^{-1} NH$_4$Cl and 0.1 g l^{-1} KH$_2$PO$_4$). This is in line with the findings of Mazzotta et al. [5] in a continuous flow mesocosm using filtered natural seawater at 20 °C. Further research at even lower temperatures with environmentally relevant nutrient concentrations could help to further estimate the fate of microparticles under more common natural conditions, but this would probably lead to significantly longer test durations and would not solve the problem that

the isolated microbial diversity changes adversely with regard to the degradation potential. However, such studies could provide additional evidence, especially if a comparison is made to different natural materials under the same conditions.

The graphs of the respective CO_2 evolution are provided with the supplementary information (SI Fig. 2 and SI Fig. 3).

Test in a Seawater/Sediment Interface According to ISO 19679

ISO 19679 ("Plastics—Determination of aerobic biodegradation of non-floating plastic materials in a seawater/sediment interface—Method by analysis of evolved carbon dioxide") is one of the more recently published standards for evaluation of biodegradation in marine environment. The scope is the aerobic biodegradation of plastic materials when settled on marine sandy sediment at the interface between seawater and the seafloor. The sinking of a material can occur directly due to its density, but for materials with a lower density than the surrounding seawater, sedimentation can occur after a certain time period as a result of biofouling [57] or other transformation processes [58]. For cellulose acetate the seawater/sediment interface is an important environment to look at. The density of cellulose diacetate (1.31 g cm^{-3}) is significantly higher than the density of seawater. This results in predominantly vertical transport of the material in the marine environment, i.e., rather rapid sinking to the bottom. As has been found repeatedly in beach clean-ups, cellulose acetate filter butts are one of the most commonly found litter item on beaches or shore areas, where many people are present [59–61]. This is also likely to be a major potential entry route of cellulose acetate into the marine environment. Literature indicates that cigarette filters made of cellulose acetate float only for a certain time until they become sufficiently soaked with water and sink [59, 62], i.e. cellulose acetate materials will not be transported over long ranges by rafting and can be expected to be deposited on the seafloor not far from the disposal site. This is also supported by the fact that in less populated coastal areas pollution by cigarette butts does not play a significant role, while items made of other plastics are sometimes washed ashore in large numbers [59]. Nevertheless, transportation of cellulose acetate microplastics cannot be excluded completely.

With 25 ± 2 °C also this test was performed at a much higher temperature than generally found in natural environments. The benthic zone temperature depends upon the benthic zone depth. It ranges from warmer temperature at shallow depth due to proximity to the water surface and may further drop to 0–2 °C at the most extreme depths of the abyssal zone [63]. Like in case of tests described before the containers were shielded from light.

As it can be seen in Fig. 10, there is again a more pronounced lag phase observed for the cellulose acetate samples compared to cellulose. The 3-to-5-month lag phase is followed by a steady increase of the percentage of biodegraded material and like in open sea conditions simulated by ASTM D6691, cellulose acetate also clearly biodegrades in the seawater/sediment conditions as simulated by ISO 19679.

The slightly different behavior of the milled cellulose acetate granules compared to the milled fibers is mainly based on the somewhat faster degradation of one replicate in case of the granules. Thus, it is not possible to deduce from this test result an influence of the physical form of the microparticle or of the presence of spinfinish on the fiber surface. Looking at the maximum diameter, this is higher in the case of the ground cellulose acetate granules with up to 125 µm than in the case of the fibers, which in turn have a greater length.

The overall test duration allowed by the standard is 2 years. Although the test presented here has only been running for one year and maximum degradation has not yet been reached, there is a strong indication that cellulose acetate is not persistent under marine aerobic conditions.

Conclusions

In biodegradation tests performed according to established standards, cellulose diacetate with the most common degree of substitution (DS 2.5) proved to be biodegradable or showed strong indication of non-persistence under laboratory simulation test conditions (i.e. test medium, temperature, pH) in aqueous environments, including freshwater, seawater and seawater/sediment interface. The conditions described in the standards do not always correspond to the optimum conditions for the maximum degree of biodegradation to occur, but they are designed to determine the potential biodegradability of plastic materials or give an indication of their biodegradability in natural environments.

Our results confirm observations made in previous scientific studies. However, especially when evaluating degradation in freshwater according to ISO 14851, the potential problem of concluding about biodegradability or persistence from behavior within a short test period becomes apparent. Degradation does not always occur at a constant rate but can happen within a short period of time after a longer adaptation period. The use of pre-adapted microorganisms proved to be very effective in shortening the lag phase and reducing the variability between replicates.

The results also show that microorganisms can adapt to a degree of substitution of 2.5 and metabolize the cellulose acetate material. Respective microorganisms could be present in both freshwater and seawater, and preceding abiotic hydrolysis to a significantly lower DS is not a prerequisite for biodegradation.

Despite all the shortcomings in transferring results of the simulation tests to actual behavior in the natural environment, the observed degradation behavior also indicates a reasonable rate of biodegradation compared to natural materials, although slower than the very fast metabolized microcrystalline cellulose powder often used as a reference material.

The observed degradation behavior cannot be fully explained by what is published so far on the potential biodegradation mechanisms of cellulose diacetate. This also shows how crucial it is to better understand the complex processes involved in the degradation of polymeric materials to better assess their actual potential impact on the

Fig. 10 Biodegradation of cellulose diacetate (CDA) particles and fibers and cellulose and LDPE powder reference in seawater/sediment interface according to the ISO 19679 method, based on CO_2 production. Mean biodegradation (lines) and standard deviation (shaded area)
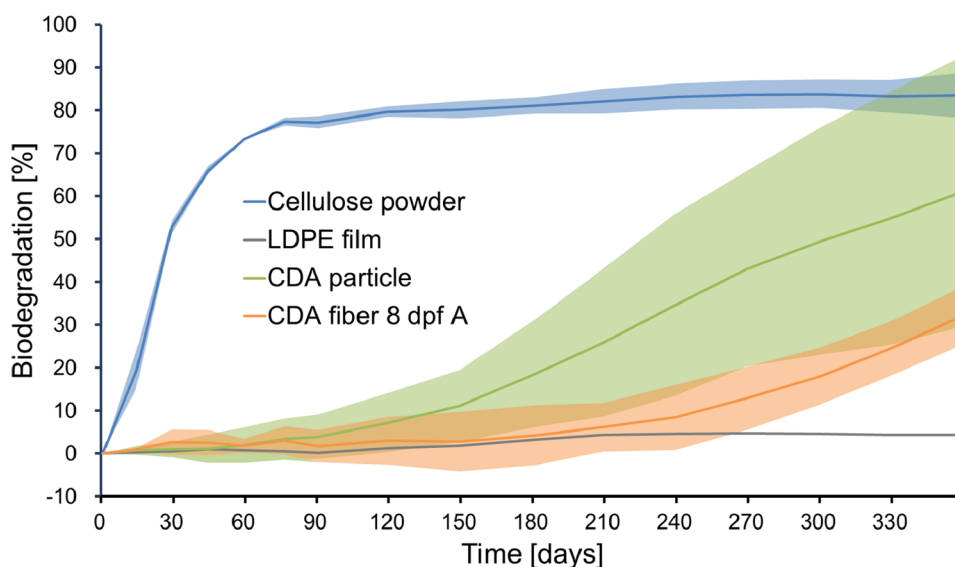
environment. More detailed studies in test settings similar to the international standards could be of interest in this respect.

Like in case of all materials, biodegradability and non-persistence do not alter the acute negative impact of irresponsible littering, but it minimizes the long-term effect. This is especially relevant for cellulose acetate, as it is associated with cigarette filters in particular. However, cellulose acetate as a biobased biodegradable material whose production furthermore is not in competition with food production, can offer an alternative to reduce the environmental impact by (micro)plastic, also with its standard DS 2.5 acetylation level.

Declarations

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