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What happens to biodegradable plastics in soil and compost?

Development and comparison of methods

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Miljø og naturressurser med fordypning i jord og miljø

Abstract

Plastics in terrestrial ecosystems negatively affect their functioning by altering physical properties and disturbing soil microorganisms. The same could be true for biodegradable plastics entering nature through incomplete degradation in composting plants, and their subsequent application to soil in fertilizer substrate. So far, no standard analysis protocol for biodegradable plastic degradation exist. This Master's thesis has focused on developing methods for the analysis of biodegradable plastic degradation in a compost matrix and lays a foundation which later research can be built upon.

Fenton's reagent and hydrogen peroxide were tested as a sample up-concentrating pre-treatment of an organic matter matrix containing biodegradable microplastics. The degradation of four different biodegradable plastics in nylon bags in a compost tumbler and a compost oven incubation were assessed. Samples for pH and phospholipid fatty acids (PLFA) of different treatments were collected to compare their development and interchangeability. Fenton's reagent was the better suited up-concentrating pre-treatment for samples with some uncertainty remaining. Assessing the biodegradable plastic degradation indicated an incomplete process in home composts and (Norwegian) composting plants. pH values coarsely reflected the composting conditions and suggested interchangeability of most treatments. Analysis of pH together with PLFA results would have been optimal, but could not be accomplished as the COVID-19 epidemic hindered the PLFA analysis. While some uncertainties in the developed methods remain, it can be concluded that a basis for establishing biodegradable plastic degradation analysis was created. Subsequent research should continue their development to assess whether biodegradable plastic remains from composting plants contribute to the accumulation of plastics in terrestrial ecosystems.

Hva skjer med bionedbrytbar plast i jord og kompost?

Utvikling og sammenligning av metoder

Sammendrag

Plast i terrestriske økosystemer kan negativt påvirke økosystemenes funksjon ved å forandre fysiske egenskaper og forstyrre mikroorganismer i jord. Det samme kan gjelde for bionedbrytbar plast som kommer inn i naturen på grunn av ufullstendig nedbrytning i komposteringsanlegg og påfølgende tilføring til jord gjennom gjødsel. Det finnes ingen standardmetoder for å analysere degradering av bionedbrytbar plast ennå. Denne masteroppgaven har fokusert på å utvikle analysemetoder for bionedbrytbar plast i kompost og utgjør en basis som videre forskning kan bygge på.

Fentons reagens og hydrogenperoksid ble tested som forberedelsesmetode for organisk materiale som inneholder bionedbrytbar mikroplast. Degraderingen av fire forskjellige bionedbrytbare plasttyper i nylonposer plassert i en roterbar kompost og i en kompostinkubasjon i en ovn ble undersøkt. Videre ble det tatt kompostprøver for fosfolipidanalyse (PLFA-analyse) fra behandlingene (roterende kompost, nylonposer i en roterende kompost, 1.5L inkubasjon i en ovn), og pH ble målt for å sammenligne deres utvikling og kompatibilitet. Fentons reagens var den best egnete forberedelsesmetoden for prøver, men resultatet var fortsatt noe upresist. Undersøkelsen av nedbrytningen av bionedbrytbar plast tydet på at nedbrytningen i hagekomposter og i (norske) komposteringsanlegg er ufullstendig. pH-verdier er indikatorer for komposteringsforholdene. og resultatene tyder på at de fleste behandlingsmetodene kan byttes ut med hverandre. Analyse av pH-verdiene sammen med PLFA-resultatene ville vært optimalt, men ble forhindret på grunn av at COVID-19-epidemien gjorde PLFA-analysen umulig. Selv om noen usikkerheter i metodeutviklingen eksisterer, kan man slå fast at det ble etablert en basis for å utvikle standardmetoder for analyse av bionedbrytbar plast. Videre forskning burde bygge videre på dette arbeidet for å undersøke om bionedbrytbare plastrester fra komposteringsanlegg bidrar til akkumulering av plast i terrestriske økosystemer.

Preface

The basis for this research originally came from my passion for combating the pollution of nature with plastics and uncovering the consequences of it. As this topic recently got more media and research attention, I got the possibility to help developing methods for the analysis of biodegradable plastics to assess their impact on the environment - and that I am thankful for.

I could not have done this without the support of my supervisors Åsgeir R. Almås (NMBU), Erik Joner (NIBIO), Claire Coutris (NIBIO) and especially Pierre-Adrien Rivier (NIBIO), who I could cooperate with and who instructed and helped me over the course of almost one year. Also NIBIOs project *DGRADE - Constraints to degradation of biodegradable plastics in terrestrial systems*, which is managed by Claire Coutris, gave me the opportunity to work with this topic. Furthermore, I would like to thank Miljøringens studiestipend who monetarily supported me, though the COVID-19 pandemic rendered planned sample analysis in the german *Bundesanstalt für Materialforschung und -prüfung* fruitless. Lastly, thanks to Johanna Sætherø Steen, Isabell B. Seeger and my supervisors who have helped with proof-reading.

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Ås, May 27, 2020, Andreas Brilke

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List of Abbreviations

C:N ratio carbon to nitrogen ratio

CM chicken manure

compost A homogenised mixture of 90% horse manure and 10% chicken manure by dry weight

CPLA crystallised polylactic acid

GC-MS gas chromatography–mass spectrometry

HM horse manure

LCA Life Cycle Assessment

PA polyamide

PET polyethylene terephthalate

PLA polylactic acid

PLFA phospholipid fatty acids

1 Introduction

1.1 Biodegradable plastics - current situation and challenges

Plastics provide solutions for problems which are hardly solvable using other materials. Lightweight, moldable, easy and cheap to produce, their use extends from medical appliances and technology to packaging, agricultural mulch films and many others. Due to high demand, 359 million tonnes of plastics were produced globally in 2018 (PlasticsEurope, 2019). Varying plastic products can have vastly different lifespans. While some products are used for years, others are disposed of after a single use. This way, 6300 million metric tonnes (Mt) of the total 8300 Mt plastics ever produced have become waste by 2015 (Geyer et al., 2017). 79% of this waste is either stored in landfills or have entered nature (Geyer et al., 2017). As degradation in nature at best is slow, if not incomplete, the plastic materials accumulate. To counter this issue, a new plastic type has been explored since the 1960's (Philip et al., 2007). Biodegradable and compostable plastics are designed for complete degradation under certain conditions. However, certifications, standards, labels and terms such as *bioplastics* still cause widespread confusion about a product's properties. Bioplastics can be made from biological materials (e.g. maize) and be biodegradable (e.g. polylactic acid) or durable (e.g. Bio-PE) (Spierling et al., 2019). Whether plastics are made of biological materials or fossil fuels does not give an indication about their biodegradability, as this is determined by the plastic's chemical structure and the environmental conditions (Hann et al., 2019). Also, some plastic products are falsely labelled as biodegradable (Harding et al., 2017). This confusion may cause the (accidental) addition of plastics to compost, soil and other environments where degradation might not occur at all (Fotopoulou and Karapanagioti, 2017).

While transparency efforts for the correct handling of biodegradable plastics are made by standardising composting methods, some uncertainty about their fate still remains. For example, testing conditions for industrial biodegradable plastic composting according to the European standard EN 13432 (6-month biodegradation period) are not met by actual conditions in most Norwegian plants (3-6 weeks) (Hann et al., 2019). Digestate from these is used as fertilizer on agricultural fields, which poses a way for plastics to enter the soil (Hann et al., 2019). Industrial composting plants in other countries may have the same problem. Also sewage sludge and digestate from biogas plants are used as agricultural fertilizer, posing an additional source of microplastics (<5mm diameter) (He et al., 2018; Chae and An, 2018). Corradini et al. (2019) have shown that microplastics accumulate in soil, by applying sewage sludge. Resting on the soil surface, microplastics can be mixed into the soil matrix by bioturbation, anthropogenic soil management and precipitation (Rillig et al., 2017; Steinmetz et al., 2016; Zubris and Richards, 2005). Remains of biodegradable plastics in soil improver, a product of composting plants, may face the same fate.

As Rillig (2012) pointed out, high concentrations of microplastics in the soil may affect its performance and biodiversity. Plastic particles may affect pore space, bulk density, water holding capacity, hydraulic conductivity and others (de Souza Machado et al., 2018). These alterations affect microbial activity and therefore biophysical soil properties, which dictate many terrestrial ecosystem processes

(de Souza Machado et al., 2018). These findings already apply to plastic dry weight concentrations of ≤2% of total soil weight, while even higher concentrations (≤7%) are found at highly contaminated places (Fuller and Gautam, 2016). While plastics in soil have the potential to improve some soil properties, negative effects generally are more severe (de Souza Machado et al., 2018). For example, increased rootability in clay soils, due to plastic particles partly loosening its compaction, is not necessarily accompanied by higher overall porosity. In fact, de Souza Machado et al. (2018) suggest widespread plastic pollution in soil to be a long-term anthropogenic effect and driver of global change in terrestrial ecosystems. How much biodegradable plastics contribute to this needs more research.

Without fitting conditions for biodegradation, biodegradable and compostable plastics can also persist and accumulate in soil (Narancic and O'Connor, 2019). Replacing conventional agricultural mulch films with biodegradable ones is becoming more popular. Farmers may choose ploughing down biodegradable agricultural mulch film, which incorporates it into the soil matrix, while collecting and sending it to a composting plant instead would be optimal. However, this may defeat the point of convenience of use and ultimately might lead to pollution of soils with fragmented agricultural mulch. UV radiation and high temperatures in the top soil may transform it to microplastics (Horton et al., 2017). Given the different climatic factors imposed by geographical location, it is unlikely to find degradation conditions in the soil at all places. Thus, improper treatment of biodegradable plastics may lead to more plastics in soil and nature in general (Narancic and O'Connor, 2019).

Due to the above mentioned problems, bioplastics may represent a threat to soils, but they also hold future opportunities. On the one hand, its downsides, like confusion about how and where it can degrade, may pose an additional source of plastics in nature. On the other hand, biodegradable and compostable plastics are at an early stage of development and thus have room for improvement, while conventional plastics stem from a matured production technology (Gironi and Piemonte, 2011). Yet, biodegradable plastics possess more sustainable production profiles in aspects like green house gas emissions, low energy use and fossil fuel consumption (Rudnik, 2019). A state of the art Life Cycle Assessment (LCA), described in ISO 14040:2006 (International Organization for Standardization, 2006), is used to determine environmental impact (Rudnik, 2019). Biodegradable plastics have the disadvantage that they are not reused or recylced and thus are not part of a circular economy at present (Spierling et al., 2019). However, Spierling et al. (2019) proposed possibilities of including biodegradable plastics in a circular economy while also highlighting the difficulties of measuring the economical circularity of a product. Still, a better LCA performance of biodegradable and compostable plastics in the future is likely, which could make them more sustainable than conventional plastics (Rudnik, 2019). This, combined with a rising global annual production of biodegradable plastics from 450 tonnes in 1990 to 880.000 tonnes in 2017 (European Bioplastics e.V., 2017), makes evaluating their environmental impact a crucial task. A further rise in the production volume of biodegradable and compostable plastics is expected, as prices get increasingly competitive to conventional plastics (e.g. 1kg polylactic acid (PLA) cost >20 USD in 1998 and 1.80 USD in 2016) (Rudnik, 2019). Consequently, biodegradable plastics will likely be more abundant in many products, which makes knowledge about their end-of-life state essential for environmental assessments. Standardised

analytical methods for biodegradable plactis are not yet established.

1.2 Composting theory

As the potential harm of biodegradable and compostable plastics lies in the incomplete degradation in composting plants and their subsequent introduction to nature, background information on the composting process is needed to put the results from this study in a context. ISO 17088 of the International Organization for Standardization (2012) defines composting as:

> *"The autothermic and thermophilic biological decomposition of biowaste (organic waste) in the presence of oxygen and under controlled conditions by the action of micro-, and macro-organisms in order to produce compost."*

In the composting process, microorganisms such as fungi and bacteria break down organic materials in the presence of oxygen to compost, CO_2, water and heat (Rudnik, 2019). The right amount of organic matter (energy for microorganisms), water and oxygen as well as the right temperature are crucial to successful composting (Rudnik, 2019). Microbes utilize extracellular enzymes for the biodegradation of biodegradable plastics as a source of energy under starvation and in absence of microbial nutrients (Bano et al., 2017). While many different methods of composting of organic materials exist, three of them are basic, centralised types (The Compost Council of Canada, 2019): i) the in-vessel method, ii) the aerated static pile method and iii) the windrow method. In this thesis, a simple version of the in-vessel method (i) was used. Usually in-vessel method composters possess control systems, which monitor the biological activity by measuring temperature, relative CO_2/O_2 concentrations and automatically turn the compost to allow for aeration (Rudnik, 2019). The compost tumbler used in this thesis did not have any sensors but temperature was automatically recorded by digital temperature loggers and turning was done manually.

If a material will completely biodegrade (i.e. mineralise) in a certain environment depends on the presence of the right microorganisms, its crystallinity, the presence of additives (for plastics), temperature, moisture and the pH (Mohee and Unmar, 2007). This means that a biodegradable plastic may degrade in one environment but not another (Tuomela, 2002). Therefore, it is crucial to assess the behaviour of different biodegradable plastics in a range of environments and determine whether they show complete biodegradation and under what conditions (Narancic and O'Connor, 2019).

1.2.1 The three phases of composting

The composting process consists of three phases, whose duration is dictated by organic matter composition and efficiency of the process, which can be determined by oxygen consumption (Tuomela, 2002). The three phases are (Tuomela, 2002):

1. *Mesophilic phase* (20 - 45°C): The first phase of composting can last between a few hours and several days. Mesophilic bacteria and fungi degrade soluble and easily degradable organic matter like starch, lipids and monosaccharides. In this process, organic acids are produced,

lowering the pH to around 5 - 5.5. The temperature steadily rises due to the heat released by the exothermic degradation reaction. Degradation of proteins causes the release of ammonia, which brings the pH back up to 8 - 9. With rising temperature, the growth rate of mesophilic microorganisms slows down as their tolerance to the heat decreases. At 45°C, their growth completely stops, and they get replaced by thermophilic microbes (Cheng and Zhen, 1987).

2. *Thermophilic phase* (40 - 75°C): This phase can last from a few days to several months. As thermophilic fungi and bacteria take over, the degradation rate of organic matter increases. In this phase, the dominant microbial population is of the genus *Bacillus*. Should the temperature exceed 55°C - 60°C, the activity and diversity of microorganisms decreases significantly. Because temperatures of 65°C and above kill many forms of microbes, which limits decomposition, many compost managers use aeration and mixing or turning to keep temperatures lower. After reaching peak temperature, the pH stabilizes to around 7.

3. *Cooling and maturation phase*: When all easily degradable carbon sources have been consumed, microbial activity decreases and the compost starts cooling down. By doing so, the compost stabilizes. Mesophilic bacteria and fungi reappear and the maturation phase follows. Microbial composition of the reappeared microorganisms differ from the mesophilic phase, as actinonmycetes often grow extensively in this phase. Some protists and a wide range of microorganisms are usually present. While biological processes at this phase are slow, the compost further humifies and becomes mature.

1.2.2 Microorganisms present during composting

During each of the three phases, different microorganism communites dominate (Tuomela, 2002; Diaz et al., 2005; Cheng and Zhen, 1987). While fungi, actinomycetes and unicellular bacteria form the majority of a compost's microorganisms, viruses, protozoa and macroorganisms make up the minority (Diaz et al., 2005). Most bacteria are heterotrophic, and denitrifying, nitrogen-fixing bacteria as well as hydrogen-oxidising and sulfur-oxidising bacteria are present. Actinomycetes oftentimes show extensive growth during the cooling and maturation phase (Tuomela, 2002). Fungi grow in all composts and at all heat levels, but may seem to temporarily disappear around the peak heat. Moreover, small numbers of anaerobic bacteria have been found in compost environments. Especially during the thermophilic phase, anaerobic microenvironments may be created by the rapid consumption of oxygen in composting processes, so that denitrifying bacteria are engaged and produce nitrate. During the maturation phase, protists and a wide range of macroorganisms may appear in the compost.

1.2.3 Composting conditions

Carbon and nitrogen are crucial factors for composting, as one of them usually is a limiting factor (Richard, 1996). For microorganisms, carbon is an energy source and a small fraction gets incorporated into their cells (Tuomela, 2002). Nitrogen is critical for microbial population growth, because it is a key constituent of proteins, which form over 50% of the bacterias cell dry weight (Tuomela, 2002). While a carbon to nitrogen ratio (C:N ratio) of 25 is optimal, too little nitrogen leads to small

Table 1: Acceptable and ideal condition ranges for aerobic composting according to Cooperband (2002).

Conditions	**Acceptable**	**Ideal**
C:N ratios of combined feedstocks	20:1 to 40:1	25 to 35:1
Moisture content	40 - 65%	45 - 60%
Available oxygen concentration	>5%	>10%
pH	5.5 - 9.0	6.5 - 8.0
Temperature	43°C - 66°C	54°C - 60°C

microbial communities, while too much results in the lost of excess nitrogen as ammonia or other mobile species (Tuomela, 2002). Moisture management relies on microbial activity and oxygen supply (Richard, 1996). Moisture has another key role in composting, as most decomposition occurs in thin liquid films on the surfaces particles (Tuomela, 2002). Too much moisture will, however, fill up pore spaces, limit oxygen transport and thus create anoxic conditions (Tuomela, 2002).

Oxygen and temperature both fluctuate with microbial activity, which consumes oxygen and produces heat in the degradation process (Richard, 1996). Low oxygen levels lead to the growth of anaerobic microorganisms, which can be detected by the accompanied odorous compounds (Tuomela, 2002). Aeration of the compost resupplies oxygen and removes excess heat. Municipal composting systems in Norway mostly are aerated static piles or windrow composts. At temperatures between 45°C and 59°C, degradation is highest due to a reduction in microbial diversity, where only the most efficient ones for that temperature are still present (Richard, 1996). Automated composting systems attempt to keep the temperature between 55°C and 60°C to compromise between reaction rate, pathogen reduction and odour generation (Tuomela, 2002). Recently, (Xu et al., 2019) suggested improving biodegradation in the thermophilic phase of composting by the addition of a certain microbial consortium, which may change composting efficiency in the future.

1.3 Aims and objectives

This Master's thesis focuses on developing analytical methods for biodegradable and compostable plastic degradation. Plastics in soil has been given little attention by researchers until Rillig (2012) raised the issue. While a lot of research went into the topic of marine plastic waste, their terrestrial counterpart remains relatively little studied, which leaves knowledge gaps regarding their distribution, fate and environmental impact (Horton et al., 2017; Chae and An, 2018; de Souza Machado et al., 2018; He et al., 2018; Guo et al., 2020). Both qualitative analytical methods and the quantitative analysis (amounts of plastics in the terrestrial environment) need to be improved to correctly evaluate the situation. Here, analytical methods were developed and compared to each other.

- To assess the effect and validity of chemical digestion on a compost-biodegradable plastic mixture, an established method for sample up-concentration of conventional microplastics in an organic matter matrix was tested on biodegradable plastics. The goal was to examine whether

the treatments removed more organic matter than biodegradable plastic and thus if they were a valid method for up-concentration.

- An in-compost tumbler incubation method for biodegradable plastics was developed to assess weight loss during biodegradation without loosing plastic particles in the organic matter. This method may indicate the completeness of degradation in composting plants.
- Oven incubations in 50 mL falcon tubes and 1.5L glass tubs were used to assess composting on a small scale. Easier plastic particle recovery and controlling composting conditions instead of letting them follow natural processes was the goal. In glass tubs, controlled conditions were used to examine degradation times at different temperatures and temperature thresholds for degradation.
- Environmental conditions (pH, PLFA) between different treatments for biodegradation were compared to analyse the treatments interchangeability. Thereby, the validity of scaling down composting experiments to laboratory size was explored. This could increase the efficiency of experiments and give more precisely controlled composting conditions.

Being able to predict the fate of biodegradable plastics and recognize similarities and differences to conventional ones is essential for researching them. Assessing complete degradation was beyond the scope of our experiments as the needed technology currently is state of the art and necessary equipment was unavailable, so that accounting for e.g. nanoplastics (<1µm - 10µm) is not feasible. In this thesis, the goal was to contribute finding apt methods for analysing if biodegradable plastics degraded under real life conditions or if they posed a similar environmental threat as conventional plastics. A base for analytical methods was developed, which may help future researchers refining it to a sophisticated analysis method.

2 Materials

2.1 Compost mixture

As the original experimental design included an earthworm (*Dendrobaena veneta*) feeding experiment, a compost blend which maximised their intake of food had to be found. The earthworm experiments were later omitted, as biodegradable plastic degradation in compost was the more promising experiment. After testing different compositions of horse manure (HM) and chicken manure (CM), a 90/10 ratio based on dry weight was found to be an optimum for worm growth. It also lacked mold forming on it, as was the case with several other mixtures like 80/20, 70/30 and down to 50/50. Mold was interpreted as a possible interference with the worms feeding behaviour, and might also affect the compost's bacteria. Worm growth was determined by exposing earthworms to different compost blends in a 1.5 liter glass tub for two weeks and measuring their weight every 7 days (see Figure A1). We chose HM and CM because they made a good living ground for earthworms, bacteria and fungi. The blend allowed for both a cold and a warm compost, depending on the batch and addition of liquids. Therefore, it fulfilled all criteria of a suitable compost blend and will henceforth simply be referred to as *compost*.

Furthermore, as these are natural materials, their properties vary between different batches and sources. Calculations (see chapter 3.1) were redone for each new batch of horse manure and chicken manure used to ensure consistency of the mixture. However, the material's water content was measured, but still varied between different experiments due to evaporation and necessary addition of urine as a nitrogen source. In oven incubations, the weight loss due to water evaporation was measured and counteracted by adding water until the original weight was reached. In compost tumblers, it was squeezed by hand and the water content based on liquids flowing out was evaluated. Thus, some uncertainty still remained. Not all composts in tumblers started equally fast and a less than optimal water content seems be one of the explanatory variables for that.

2.2 Microplastics particle size

Three different sizes of plastics were used for the three different scales of incubation experiments. The smallest experiment was carried out in 50mL falcon tubes with compost and microplastic particles inside. The size of the plastic particles was between 63 and 500µm in maximum diameter. This size was chosen because the worms had to be able to eat it in the initial experimental design. It would have assessed the degradation happening by gut bacteria in worms. A microplastic particle size of 1×1mm was initially intended, but did not get eaten by the worms in pretests. A medium sized incubation experiment was carried out in 1.5L glass boxes (Coline glass food container, Clas Ohlson, Sweden) filled with compost and biodegradable plastics. First, the particle size was 1×1cm but got adapted to 2×2cm later, as it was difficult and time consuming to recover the smaller plastic particles when they were crumbled up by degradation effects. Lastly, a big scale incubation experiment was conducted in a 135L compost tumbler chamber (JK270, Jora Composters, Sweden). It had biodegradable plastic sheets of 10×10cm size as crumbled-up balls inside of non-biodegradable nylon bags that

were 12×12cm in size (see chapter 2.4).

2.3 Biodegradable plastics

The four different products made of biodegradable plastic originally were chosen to be an industrially compostable food waste bag, two garden compost food waste bags and biodegradable plastic knives. Later on, one of the two garden compost food waste bags (*OK compost home white*) was replaced by biodegradable agricultural mulch as it opened up for analysing another product class of biodegradable plastics. The two food waste bags, *OK compost home white* and *OK compost home green*, were assumed to have very similar if not equal properties, thus diminishing the importance to analyse both. Given that the four products chosen were meant to degrade under a variety of different conditions and were also designed to fulfill distinct purposes, a multifaceted insight in biodegradable plastics could be gained.

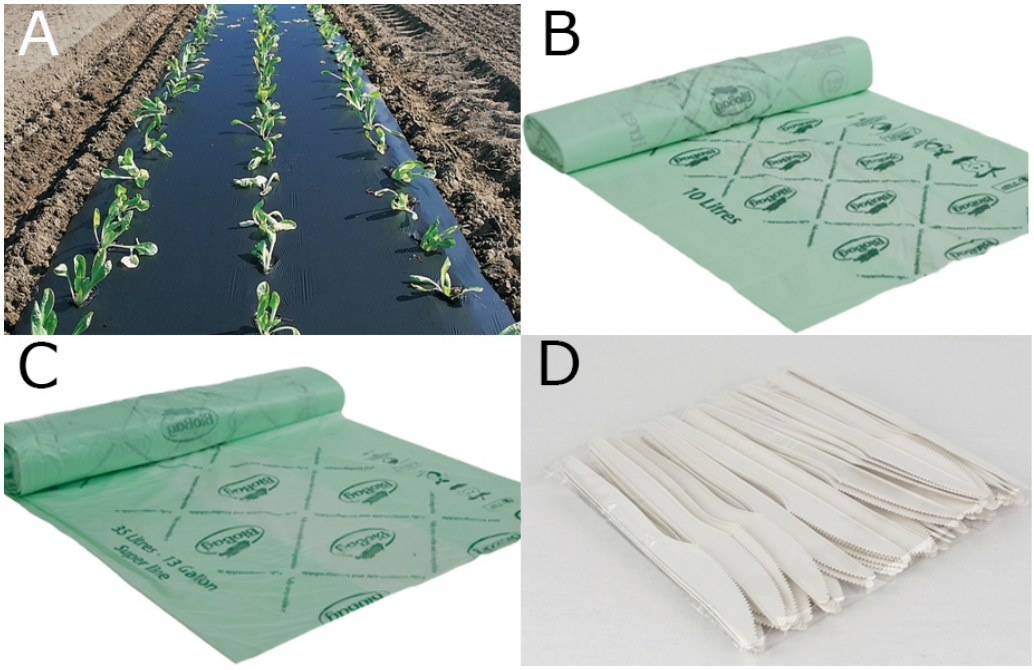

Figure 1: The biodegradable plastic products used: Biodegradable mulch film (A), *OK compost home* (B), *OK compost industrial* (C) and biodegradable knives (D). (Image sources: servicio.no & biobagworld.com)

2.3.1 Mater-Bi

Mater-Bi is a biodegradable and compostable bioplastic produced by the company Novamont (Italy). It consists of starches, cellulose and vegetable oils and is biodegradable and compostable in accordance with the European standard EN 13432 (European Bioplastics e.V., 2015) and the US standard ASTDM 6400 (ASTM International, 2019; Novamont S.p.A., 2020). Since both refer to industrial composting and given the location of the project that this thesis is a part of, the focus will lie on the European standard. The biodegradation of *Mater-Bi* is guaranteed for industrial composting, domestic composting and for biodegradation in soil, depending on the certification given (Novamont S.p.A.,

2017). However, not all of these certifications are rooted in the EN 13432 standard but rather in those of TÜV Austria, which not necessarily live up to the EN testing standards. TÜV Austria is a certification body which is authorized by European Bioplastics, the association that represents interests of the bioplastics industry in Europe (Rudnik, 2019).

Labels	Reference Standard	Test Conditions (if different from reference standard)	Test Threshold
	EN 13432		90% in 6 months[3]
	EN 13432	Ambient temperature (20°C – 30°C)	90% in 12 months
	ISO 17556[1]		90% in 2 years[4]

Notes:

1. *This is the test method for aerobic biodegradability of plastics in soil.*
3. *Test threshold the same as EN 13432*
4. *Test threshold the same as EN 17033*

Figure 2: TÜV Austria certifications for industrial and home composting, as well as biodegradation in soil. It should be noted that *OK compost HOME* and *OK biodegradable SOIL* products both are partly made of *Mater-Bi* but got TÜV Austria certifications that exceed the EN 13432 standard for industrial composting. Labels such as the *compostable logo*, on the right side of TÜV Austria INDUSTRIAL, are designed to address the confusion of whether or not a product is truly compostable by giving it credibility (Rudnik, 2019). Edited from (Hann et al., 2019).

2.3.2 EN 13432

The European standard EN 13432 "*Packaging - Requirements for packaging recoverable through composting and biodegradation*" defines four minimum characteristics a material must have to be considered industrially compostable (European Bioplastics e.V., 2015):

1. *Biodegradability*: The materials capability to be converted into CO_2 by microorganisms. According to the laboratory test method EN 14046, at least 90% biodegradation must have occured in under six months.

2. *Disintegration during biological treatment*: Fragmentation has to occur and initial material may not be visible in the final compost. The pilot composting test EN 14045 assures this by composting the initial material with biowaste for three months, after which less than 10% of the materials original mass may be left.

3. *Heavy metals*: Their concentration must be below given maximum levels which ensure no reduction of agricultural value and no ecotoxicological effects on the growth of plants.

4. *Lack of negative consequences for the composting process*

Industrial composting is composed of an active phase with temperatures between 50°C and 60°C as well as a curing phase, where the compost matures at temperatures below 40°C (European Bioplastics e.V., 2015). A variety of different composting technologies are practically in use, varying in length of both active and curing phase (European Bioplastics e.V., 2015).

2.3.3 Biodegradable agricultural mulch film

In modern agriculture, mulch films are a popular application as they can increase crop yield and improve their quality while also diminishing the need for irrigation and the use of pesticides (European Bioplastics e.V., 2018). Biodegradable agricultural plastic mulch is meant to replace non-biodegradable plastic mulch films and as such is a drop-in plastic product. If it would properly degrade in the soil or could be collected and composted, plastic pollution of agricultural soils could be reduced.

The biodegradable mulch film used is produced by the company BioBag under the name *BioAgri*. According to the producer, *BioAgri* is biodegradable and compostable in agreement with EN 13432. Furthermore, ISO 17556:2019 was used to test the aerobic biodegradation under optimal conditions in soil over a period of two years for a constant temperature between 20°C and 28°C. This is done by measuring the actual biological oxygen demand and compare it to the theoretical oxygen demand needed for total biodegradation of the material (International Organization for Standardization, 2019).

According to the European standard EN 17033:2018, a biodegradable mulch film also has to fulfill the following requirements among others (Hayes and Flury, 2018):

- *Constituents*: Given concentration limits for heavy metals, no hazardous substances of very high concern (<0.1%) and loss on ignition at 550°C (≥60%).

- *Biodegradation*: ≥90% of the mulch's carbon has to be converted to CO_2 within 2 years under ambient soil conditions (specified by ISO 17556:2019 in above paragraph).
- *Ecotoxicity*: No acute ecotoxicity to plants (≥90% of germination rate and plant growth compared to mulch free soil), no acute ecotoxicity to invertebrates and microorganisms (nitrification should be ≥80% of biodegradable mulch free soil)

According to the producer, the mulch film is biodegradable in soil with an average lifespan of 1 to 24 months, depending on climate and temperatures (BioBag International AS, 2020a). It got the *OK biodegradable SOIL* certification presented in Figure 2. The longevity partly depends on the thickness of the mulch film. Here, first 15µm and later 35µm thickness were used. This is due to the decision in NIBIOs biodegradable plastics project that the 35µm mulch film is more sturdy and thus more likely to be recovered from the fields to be composted, opposed to degrading in the soil. Theoretically, 90% or more of the biodegradable plastics should be degraded in soil after two years time (Hann et al., 2019).

2.3.4 OK compost home food waste bag

Biodegradable food waste bags certified with *OK compost home* are designed to be composted in a common garden compost. They are produced by BioBag under the name *BioBag* (product) and are meant to compost according to the European standard EN 13432 and US standard ASTM D6400 (BioBag International AS, 2020b). Furthermore, the certification agency TÜV Austria certified these bags with *OK compost home*, meaning that they will compost at conditions which usually are found in homely garden composts (TÜV Austria, 2020). Biodegradation at 20°C to 30°C and a time period of 12 months with a degradation of 90% or more is specified by TÜV Austria (Hann et al., 2019). This is not supported by a European or US standard, but rather exceeds their claims in terms of easy biodegradability and is solely certified by TÜV Austria.

2.3.5 OK compost industrial food waste bag

OK compost industrial food waste bags were designed to be composted in industrial composting facilities. They are produced by BioBag under the name *BioBag* (product) and got the TÜV Austria certification *OK compost industrial* as well as the seedling logo that guarantees biodegradation according to EN 13432 (see Figure 2 next to the *OK compost industrial* logo). Also, the TÜV Austria certification is referring to the European standard EN 13432 (TÜV Austria, 2020).

2.3.6 Biodegradable knives

The biodegradable knives are sold by Servicio AS (Norway) as *kniv komposterbar, CPLA, 16cm* (article number: 26095) and are made of crystallised polylactic acid (CPLA). CPLA is more heat resistant than conventional PLA so that it can be used in contact with hot foods and drinks, like in a disposable coffee cup. Both, PLA and CPLA are safe for contact with food, are bio-based (e.g. from sugar cane, corn), biodegradable and certified with EN 13432 (European Bioplastics e.V., 2015).

2.4 Nylon bags

Meshed nylon bags were used in the compost tumbler incubation experiment (chapter 3.6). Their purpose was to keep plastic fragments of samples in one place inside the bags. Plastic fragments may be formed during the incubation experiment due to degradation of the biodegradable plastic by bacteria and fungi. Nylon bags did not show any signs of degradation in pretests and were therefore assumed to safely hold all the plastic particles from one sample together. This way, the weight of the nylon bags content could be compared before and after conducting the experiment to assess weight change.

However, depending on the size of each opening in the mesh (mesh size), some particles might be transported out of the bags. To evaluate whether this was the case, a pretest was conducted. The pretest incubation had a mesh size of 20×20µm of PETEX mesh (polyethylene terephthalate (PET)), SEFAR, Switzerland). A part of the content of one bag was put in 25mL water in a falcon tube and put in a overhead shaker (Reax 2, Heidolph, Germany) overnight. The purpose was to loosen particles from one another and lead to greater explanatory power of this pretest. Loosened particles are more likely to slip through the mesh and all other scenarios would have less plastic passing through it. Next day, the sample was filtered through a 50µm and a 100µm NITEX mesh (polyamide 6.6 (PA), SEFAR, Switzerland). The change of materials from PET to PA6.6 was due to the greater resistance to hydrolysis and especially abrasion, which were thought to be critical properties for bags to endure the experiment. Both PET and NITEX are non-biodegradable materials. If any plastic or compost particles that could not move out of the 20µm mesh would pass through the bigger ones, it would also be possible to have mass loss by the same process during the experiment. Mass loss would falsify the weight loss data, which would mistakenly be attributed to the degradation of the biodegradable plastics. However, neither plastic particles nor particulate organic matter (particles >0.45µm) could be seen upon inspecting the filtered samples under a light microscope (MZ8 stereomicroscope, Leica, Germany), whose theoretical minimum resolution lies at 0.2µm but probably does not come close to this level of detail due to imperfections of the glass in lenses and is more likely to lie between 1µm and 5µm (The University of Waikato, 2012). Mass loss due to plastic fragment transport out of the nylon bags was therefore ruled out for the materials and timespan used in this experiment, but it cannot be ruled out that plastic particles smaller than 0.2µm were not detected.

As the degradation in the pretest for the 20µm mesh size bags was quite slow, one bag with each 50µm mesh and 100µm mesh containing compost and biodegradable plastics was added. The hypothesis was that larger mesh sizes allowed more microorganisms to pass through and thus lead to stronger degradation inside the bags. They remained inside the compost tumbler for 7 days during the optimal incubation temperature for degradation (see Figure 7). Degradation was the highest for 100µm, then 50µm and lastly 20µm mesh size (see Figure A2). This pretest, regarding mesh size openings and degradation speed, was done visually as its outcome was obvious enough to see with the eyes (see Figure A2). Since 20µm still had the lowest degradation of the three sizes, the mesh seemed to hinder the degradation process to a certain degree. To optimise the degradation but also applying a precautionary principle towards matter loss, the 50µm mesh size was chosen for the experiment.

Sheets of 12×24cm were cut out from the roll of 50µm mesh, folded to a 12×12cm size and closed with a high temperature bag sealer (bag sealer, Packer, England). This was done with at least two seals on each side to improve bag stability and reduce the risk of bags opening during the composting process. Furthermore, the seals were used to mark them in a way that each individual bag could be identified. This was done by having three instead of two seals on one or several sides and additionally, some bags had seals over the corners.

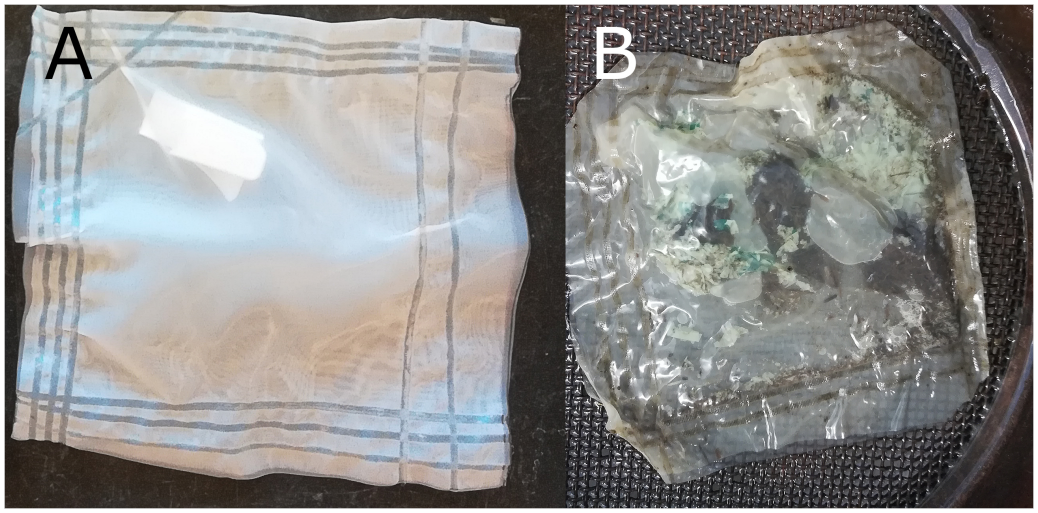

Figure 3: Examples of nylon bags before (A) and after (B) incubation. Fragmentation of the biodegradable plastic in picture B can be seen. A shows the seams used for identification of the bags.

2.5 Hydrogen peroxide and Fenton's reagent

In the falcon tubes, samples of compost and biodegradable microplastics were incubated. For further analysis of these, it would be optimal to increase the ratio of microplastic to compost so that it was easier to detect the microplastic. Effectively, this increased the signal-to-noise ratio of the sample. One way to do this is by chemically digesting the organic matter so that the relative abundance of plastic rises. Hurley et al. (2018) compared four different chemicals and their effect on samples which included organic matter and non-biodegradable plastic. Noting that there could be a difference in how much the chemicals affect the biodegradable plastics in contrast to non-biodegradable plastics, two digesting chemicals stood out.

One of them was Fenton's reagent. It was mixed in accordance to Hurley et al. (2018). A 1:1 volume ratio of hydrogen peroxide and a catalyst were mixed to get this reagent. The catalyst was made up of 20g iron(II)sulfate ($FeSO_4 \cdot 7H_2O$) per 1L Milli-Q® (deionised) water. Fenton's reagent was found to be an effective way of reducing organic matter content of the sample without affecting the non-biodegradable plastics majorly (Hurley et al., 2018). Its ferrous iron (Fe^{2+}) catalyses the decomposition of hydrogen peroxide which creates hydroxyl and hydroperoxyl radicals in the following reaction (Fenton, 1894):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + HO^- \tag{1}$$

Hydrogen peroxide (33% (v/v) H_2O_2) was used for comparison to the Fenton's reagent in terms of

increasing the biodegradable plastics to compost ratio.

2.6 Chemicals used in phospholipid fatty acid (PLFA) analysis

The phospholipid fatty acid (PLFA) analysis explained in the appendix (p.54), the following chemicals were used: potassium hydroxide (KOH), chloroform ($CHCl_3$), methanol (MeOH), acetone (C_3H_6O), Milli-Q® water, Dinitrogen (N_2) for sample evaporation and citric acid monohydrate ($C_6H_8O_7$) for the citrate buffer. Also the following containers were used: 50mL teflon tubes, 50mL Kimax tubes and 15mL Kimax tubes. More details about the procedure can be found in NIBIOs instructions by Norli (2017) .

3 Methods

As this master's thesis approaches a relatively new topic, it was mostly not possible to follow established routines for experiments. Rather, new methods had to be developed. That includes pretests, follow-up adaptations, experimenting with possible solutions and dealing with some uncertainty. To emphasis the work done and to document preliminary results, they will be mentioned in the appropriate context in the course of this chapter.

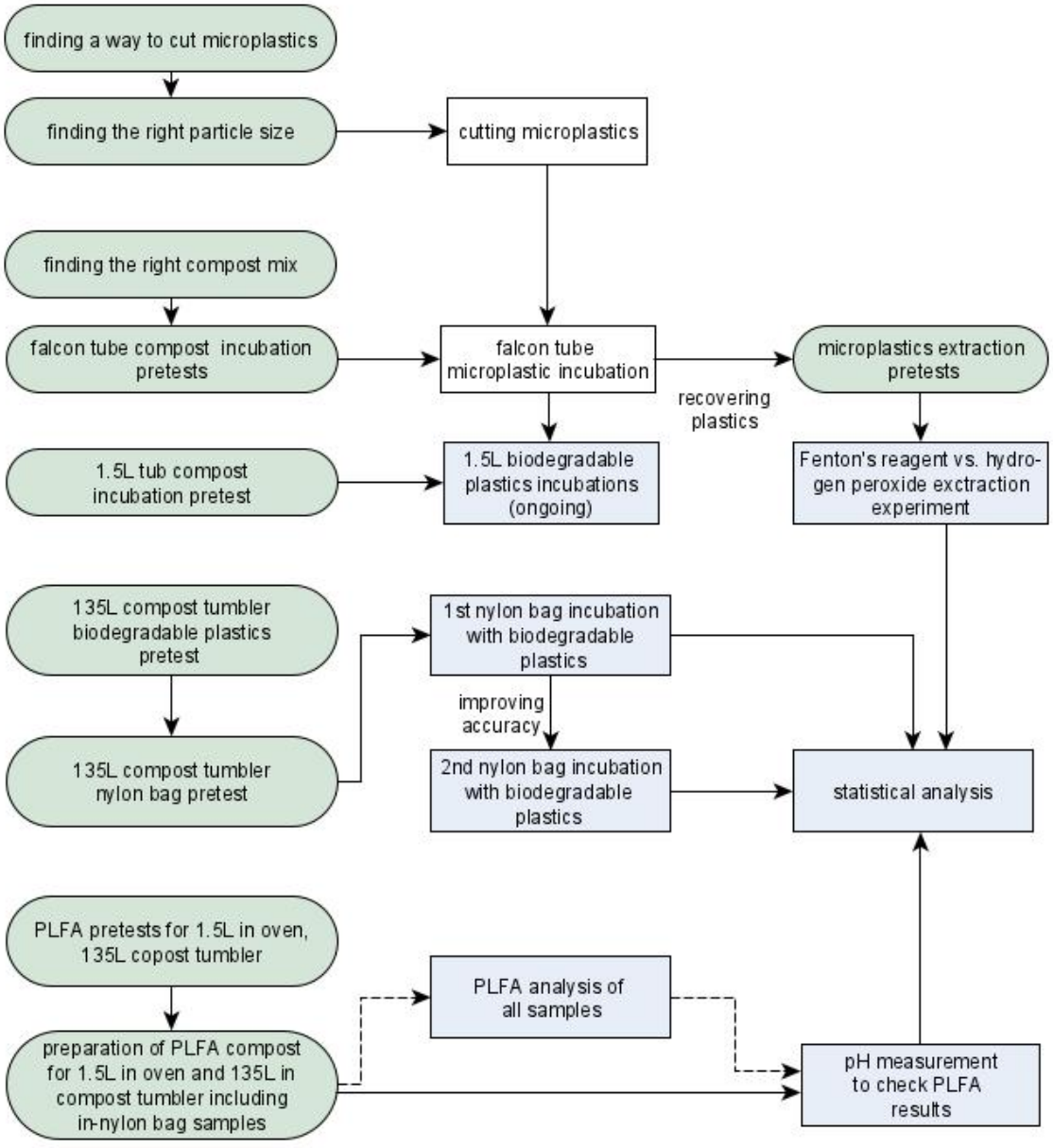

Figure 4: Flowchart of methods in chronological order from top to bottom. Round green boxes indicate pretests, squared uncoloured boxes are experiments that did not directly yield results and blue boxes have given results.

3.1 Calculating sample properties

To achieve accurate results for water content, water holding capacity and dry matter content, the correct formulas have to be applied. The standard literature used for this at the soil department at

NIBIO Ås is *Manual of Soil Analysis* by Margesin and Schinner (2005). For reasons of coherence these formulas were used here as well. These calculations were done in Microsoft® Excel® for Office 365 (2018).

3.1.1 Water content

Measuring the soil water content is crucial to achieving the right level of moisture for a warm compost to work as intended. It should be moist enough for composting to occur, but not wet enough for anoxic conditions to arise. These would change the type of reactions that occur and compromise optimal degradation (Cooperband, 2002). Furthermore, the water content is needed in the early stage of the PLFA analysis (see chapter 3.7).

To calculate the water content of a sample, first the empty container is weighed. Then the moist sample is placed in the container, weighed again and placed in a heating cabinet until completely dry. That state is reached when no further weight loss occurs over time. Normally this drying process happens at 105°C but a more optimal temperature for organic matter is stated to be at 50°C, due to volatilisation and possible partly degradation of organic compounds (Margesin and Schinner, 2005). Consequently, 50°C was used here. Finally, the dried sample is cooled down in a desiccator to avoid moisture from the air to build up on it, and weighed again. This test was always done with multiple samples (3 or 5) to achieve higher accuracy. Also, standard deviation was calculated to get a better overview of the data's spreading. The formula used for the water content is:

$$\text{water content (\%)} = \frac{\text{container plus moist sample (g) - container plus dried sample (g)}}{\text{container plus dried sample (g) - empty container (g)}} \times 100 \quad (2)$$

3.1.2 Water-holding capacity (WHC)

Since most aerobic processes of microbial transformation happen at 40 - 60% of the maximum water-holding capacity, it is important to measure this characteristic of the substrate in the case of composting (Margesin and Schinner, 2005). To get this number, a cylindrical tube with a cloth as the water outlet on the bottom side was filled with compost. This was placed in water so that the substrate level was lower than the surrounding water level and left overnight. The next day it was taken out and allowed to naturally drain through gravity. When the weight loss flattened out, the cylinder was emptied in another pre-weighed container, weighed and put in a heating cabinet to dry. After weighing the dried compost, the water-holding capacity in weight percent of the dry matter of the sample was achieved by the following formula:

$$\text{WHC (\%)} = \frac{\text{container plus drained sample (g) - container plus dried sample (g)}}{\text{container plus dried sample (g) - empty container (g)}} \times 100 \quad (3)$$

3.1.3 Dry mass content (w_{dm})

For mixing a compost blend based on the dry mass content of its components, it is crucial to have calculated the dry mass content of the materials. In this case it was calculated for the mechanically

homogenised chicken manure and each of the batches of manually homogenised horse manure. This way, a 90% HM/10% CM mix based on the dry weight was converted to the moist mass needed to achieve such a ratio. The formula used was:

$$w_{dm} = \frac{\text{container plus dried sample (g) - empty container (g)}}{\text{container plus moist sample (g) - empty container (g)}} \times 100 \quad (4)$$

3.2 Creating microplastics

Microplastics particles of the initial materials mentioned in chapter 2.3 were needed for the falcon tube incubation experiment. First, particles with a size of around 1×1mm were cut using a scalpel. Although time consuming, this worked well for cutting this size, though the created particles were too big for the earthworms to eat. Subsequently, various attempts in creating smaller particles in a less time consuming way were tested on these bags. Cryogenic grinding in a mixer mill (MM300, Retsch, Germany) after exposure to liquid nitrogen only yielded oatmeal sized flakes. Blending in a knife mill (GM200, Retsch, Germany) after direct exposure to liquid nitrogen, or blending in a usual kitchen blender had no effect at all. Finally, cutting fine strips of around 1mm thickness with a scalpel and ruler, placing them in a glass and thoroughly cutting them with a sharp pair of scissors for around 10 minutes created some small particles. While most particles still were too big, sieving to a size of 63 - 500µm yielded the wanted particle size. As the biodegradable knives (see chapter 2.3.6) were less elastic, blending them in the Retsch GM200 instead resulted in medium to small particles, which could be sieved to 63 - 500µm in diameter.

3.3 Up-concentrating compost samples by chemical digestion

For the falcon tube incubation experiment, biodegradable microplastics and compost were mixed. To further work with these samples, in theory it would be ideal to only analyse the plastic itself and none of the compost - for example for STA-FTIR analysis. To reduce the compost to plastic ratio several sample treatments were tested.

A density based approach to separate the materials by floatation in water did not work as the plastic particles seemed to behave in the same way as the compost. Also a water pulsation bath did not change the outcome. Another possible method was considered to be chemical digestion by hydrogen peroxide or Fenton's reagent (see chapter 2.5). Since these only were tested on non-biodegradable plastics by Hurley et al. (2018), a pretest was conducted to compare their effect on the biodegradable plastics. The goal was to assess whether the treatments would remove more organic matter than plastic (up-concentration of the sample). Each of the plastic types mentioned in chapters 2.3.3 to 2.3.6 as well as the horse manure and chicken manure used for creating the compost and the compost itself were exposed to the two digesting agents. The plastic's particle size was 1×1cm to easily recover them. For every material tested, five replicates were used to improve result accuracy and to allow for calculating the standard deviation. The first pretest with Fenton's reagent showed that a non-cooled reaction with organic matter, especially the chicken manure, lead to strong foaming which transported parts of the sample out of the container and thus falsified the matter loss result. Subsequently, all further

experiments with Fenton's reagent were cooled in an ice bath and conducted with less organic matter per sample which diminished the foaming. Hydrogen peroxide samples did not have to be cooled, as little foaming was present.

Every sample was enclosed by a 100mL glass container, which was placed under a fume hood. Then, 20mL of hydrogen peroxide or Fenton's reagent were added. Each material was weighed before the experiment started (T0), exposed to the respective chemicals for two hours, rinsed with tap water, dried at 50°C until no further weight loss occurred, cooled down in a desiccator and weighed again. As the organic matter used was moist, its pre-experiment dry weight had to be calculated with Equation 4 on page 17 afterwards. Plastic particles could be recovered by pouring the organic matter free samples over a sieve with 500µm openings. They were dried in an oven at 50°C, cooled down in a desiccator and weighed after removing their static electricity using a Universal AntiStatic Kit (Mettler Toledo, USA). The organic matter samples were meticulously transferred from the glasses to filter papers inside a funnel. Inside these, the residual compost material was rinsed with water from a spray bottle to wash out the remaining digesting agent. These folded 120mm diameter filter papers were dried, cooled down in a desiccator and weighed beforehand, as their weight had to be subtracted from the final measurement. After air drying for around 10 minutes the samples could be transferred to the heating cabinet to be dried. Finally, the matter loss due to the exposure to the chemical used could be calculated by subtracting the post-experiment dry weight from the pre-experiment dry weight.

3.4 Oven incubation in 50mL falcon tubes

Incubating biodegradable microplastics in a compost matrix inside falcon tubes required a lot of attention to details. Both adding water and its evaporation could quickly alter the conditions to be too dry or too wet and thus anoxic. This had to be avoided to create and maintain optimal conditions for degradation to occur. The goal of testing composting on such a small scale was the easier recovery of microplastic particles. To see whether it could be a valid replacement to warm the compost in a laboratory oven instead of utilising natural processes in a large volume compost, was another objective. The falcon tube incubation was the first incubation in this project and delivered a base for later experiments on a different scale.

To allow for a larger contact area of compost and air inside the falcon tubes, they were lying on their sides rather than standing upright. Holes of 1mm diameter drilled in the upward facing side were used for oxygen supply. To avoid contamination, the plastic remnants of the falcon tube material were removed. A pretest with only tap water inside the falcon tubes indicated the right number of holes needed for ideal ventilation. Triplicate incubations at 60°C over one weekend with 3, 6, 10 and 20 holes showed that 6 was the optimal amount. A following pretest used homogenised field moist compost so that only a few drops of liquid ran out when squeezed by hand. It compared water loss with 3 or 6 holes in the falcon tube, both in standing and lying position and supplied with either 3, 4 or 5mL water. Each treatment was conducted in triplicates. The treatment with 11g wet weight compost, 6 holes and 5mL water seemed most promising but not optimal. To further improve the experimental setup, a third pretest was conducted. It compared 6 and 9 holes at 60°C and 3, 4 or 5mL water added

over the course of one weekend - all falcon tubes lying on their side. 9 holes and additional 4mL of water was concluded to be optimal incubation conditions.

As a precautionary measure, the final incubation had 3 replicates for each 2, 3 and 4mL water added per plastic type. This test was conducted on the following materials: *OK compost home white*, *OK compost home green* and biodegradable knives. *OK compost industrial* and agricultural plastic were not yet available in the laboratory at the time. In addition, one control sample (T0) was kept, making it a total of 10 falcon tubes per plastic type tested. Each sample contained 11.29g of fresh compost (2.22g dry mass) and 0.025g of biodegradable plastic (1% of dry weight of compost). The plastic had the static electricity removed before weighing, as it could have a relatively big effect on its weight.

Finally, the incubation was carried out over 20 days. Each work day, the samples were weighed and refilled with water to reach the initial weight and therefore maintain constant moisture. To not dry out samples during weekends and to simulate more natural temperature fluctuations, the following temperature pattern was applied by the heating cabinet: Mondays (50°C), Tuesdays to Thursdays (60°C), Fridays to Mondays (40°C). Lastly, the samples were stored for later analysis.

3.5 Oven incubation in 1.5L glass containers

Different degradation times of a material at different temperatures and a threshold in terms of temperature were the key questions of this experiment. Similar to the falcon tubes, the middle scale incubation relied on the heating cabinet to heat up the samples. The point of this scale of composting experiments was to visually examine degradation process of the biodegradable plastics at different temperatures. Furthermore, microbial communities in the compost of these 1.5L glass containers were compared to those of a 135L compost tumbler (see chapter 3.7). For visual examination, 9 pieces of 1×1cm (later 2×2cm) of each biodegradable plastic were placed together in a 1.5L glass box filled with 1L compost. The glass containers had 20 holes of 1mm diameter in the lid to allow for air flow and prevent anoxic conditions. Four plastic types were tested: agricultural mulch film, *OK compost home* (green), *OK compost industrial* and biodegradable knives (corresponding to chapters 2.3.3 to 2.3.6).

The four containers were placed in the heating cabinet together for 20 days at each of the following temperatures: 35°C, 45°C, 55°C and 65°C. Four incubations of 20 days with four freshly prepared containers holding one plastic type each were conducted in the oven. Each incubation contained fresh compost and biodegradable plastics. Additionally, one set of samples was kept at room temperature (20 ± 1°C) for over 130 days. Every second day, the containers were weighed and the initial weight/moisture restored by evenly adding water to them. At the same time, 1 to 3 plastic particles per glass tub were carefully removed with tweezers and inspected through a magnifying glass. It was noted down how they looked, so that the onset of fragmenting or degradation could be expressed in days for every temperature.

3.6 Compost tumbler incubation

The compost tumbler-sized experiment used nylon bags to be able to recover plastic pieces after the incubation rather than losing them in the compost. A pre-experiment where plastic fragments (10×10cm) were directly placed in the compost tumbler without mesh bags proved that recovery was difficult and mass balance impossible to obtain. A duration of 20 days was planned for this experiment, as it is the approximate length of the active phase of Norwegian composting plants (Hann et al., 2019). The compost mix received additional nitrogen in the form of urine due to a slow start of the microbial processes. A pretest was executed to determine if the nylon bags would withstand the harsh composting conditions and if the mesh opening would be big enough to allow for the bacteria to pass through (see chapter 2.4).

Compost tumbler incubations contained 100L of compost (see chapter 2.1) in 135L compost tumbler chambers (in-vessel composting), whose temperature was recorded with loggers (Em50, Decagon Devices, USA) and which were turned at least every second day to add oxygen to the compost. Two compost tumbler incubations were executed to strengthen results. Both used 50µm mesh bags and the same biodegradable plastics. The first incubation lasted for 29 days with a sampling time point after 15 days. The second incubation was designed to challenge previous results. It also had one treatment which contained some grams of compost and biodegradable plastics inside the bag. This allowed to assess plastic degradation in direct contact with compost while being able to fully recover plastic particles. Its incubation had samplings at 15, 45 and 90 days, where samples were taken out from each separate compost tumbler chamber. This experiment was designed for the biodegradable plastics project of NIBIO and its time frame exceeds the deadline of this thesis, so that only the 15 day timepoint can be included.

Finally, the calculation of weight loss compared the weight of the nylon bags after incubation to the weight prior to the incubation. For weighing the materials after the experiment, they were cleaned with water to remove additional weight. The before and after incubation samples were dried in an oven at 50°C until no further weight loss occurred, cooled down in a desiccator to avoid weight gain by condensation and then weighed. The before materials were also weighed separately so that the weight of each nylon bag and each of its components was known. This was important to be able to subtract the weight of the nylon bag and compost after incubation. This way, only the weight change of the plastics would be left to compare. To achieve this, empty bags and bags only containing compost were added. Their change in weight could then be subtracted from the nylon bag after incubation and weight loss analysis corrected for the nylon bag and compost was made possible.

3.7 Analysis of phospholipid fatty acids (PLFA)

The analysis of phospholipid fatty acids (PLFA) was conducted following NIBIOs manual by Hans Ragnar Norli (Norli, 2017). Since the last step of the analysis was hindered by the COVID-19 pandemic and consequently no data are available, the steps are not explained here but outlined in the appendix (p.54).

3.8 pH measurement of PLFA samples

Because microbial communities go hand in hand with the pH (Tuomela, 2002), it can indicate differences in these, for different treatments. Thus, results from the PLFA analysis could be strengthened or questioned.Before each batch was measured, the pH electrode was calibrated (Orion 8172BNWP, Thermo scientific, USA). compost samples had been air dried for at least 12 hours, moistened with distilled water with at least 5 times the volume of the sample, shaken in an end-over-end shaker for 5 minutes and left to sediment for at least 2 hours but not more than 24 hours. Finally, the pH was measured 3 times per sample so that the average and standard deviation could give an accurate result for each sample.

3.9 Statistical analysis

A statistical analysis of the data was conducted to assess whether treatments significantly vary from one another. First, result graphs for chemical digestion (chapter 4.1), compost tumbler incubation (chapter 4.3) and pH measurements for PLFA samples (chapter 4.4) were made in Microsoft® Excel® for Office 365 (2018).

Samples were generally paired, so that each plastic type was analysed for each of the treatments. pH values stem from the same initial compost, but received different treatments, either an oven or compost tumbler incubation. This way, two treatments or groups of treatments could be compared. Often the assumption of a t-test of normal distribution was not fulfilled. As the population size (N) of many samples was small (2 to 5) a t-test was seen as too unreliable since its assumptions were difficult to fulfill with such a small population size. Instead, the non-parametric Mann-Whitney test was used to test if there was a statistically significant difference between groups which were connected by a dependent variable, like pH. For all tests, the significance level was set to 0.05. The assumption of a non-normal distribution was tested by the Anderson-Darling test in the computer program MiniTab (Minitab 18.1 Statistical Software, 2017). If the data were normally distributed, the Mann-Whitney test was still valid in case of the shape of distributions being the same or similar (Laerd statistics, 2018). MiniTab was also used to test for some of the samples' homogeneity of variances, an assumption that needs to be fulfilled for conducting a t-test and to conduct the Mann-Whitney tests.

4 Results

4.1 Upconcentrating compost samples by chemical digestion

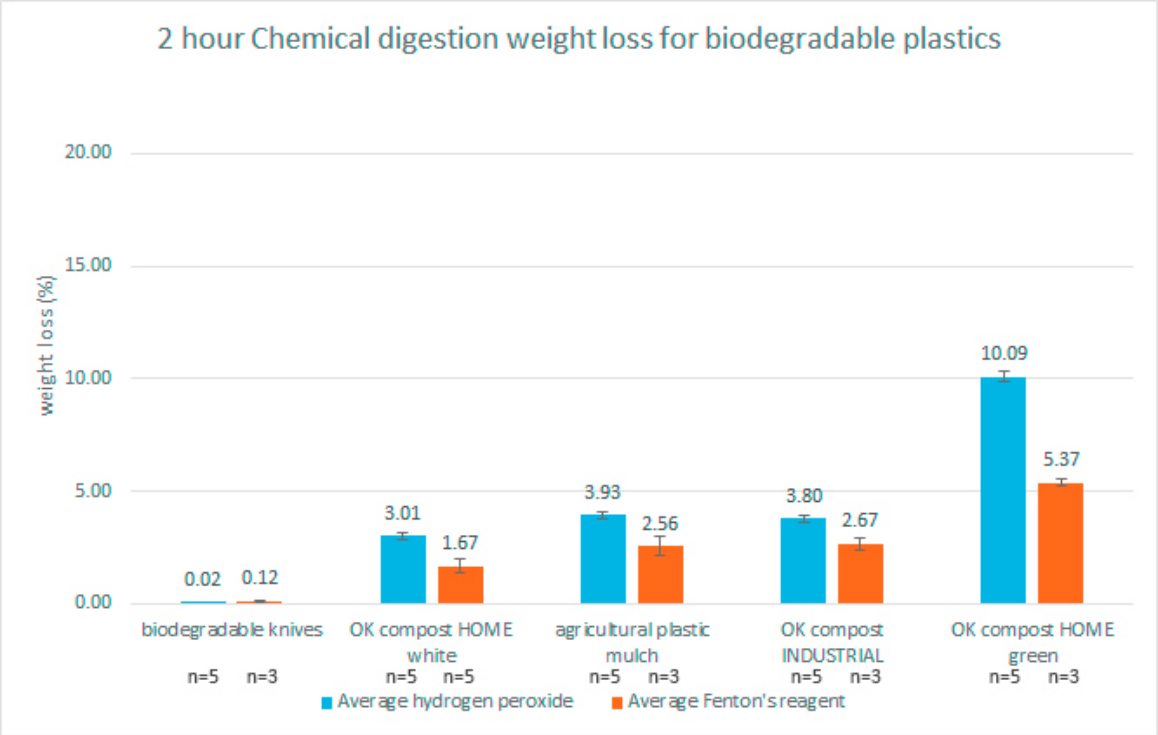

Figure 5: Average weight loss of biodegradable plastics after a 2 hour exposure to either hydrogen peroxide or Fenton's reagent, including number of samples and standard deviation. The n for Fenton's reagent is not consistent because the experiment was repeated for selected materials to improve the quality of results. The scale is choosen to be easily comparable to Figure 6.

For biodegradable knives, the median weight loss after a 2 hour exposure to Fenton's reagent was significantly higher than after exposure to hydrogen peroxide (0.02% and 0.11%, $p<0.05$, Mann-Whitney test, Figure 5). For *OK compost home white*, the median weight loss after a 2 hour exposure to hydrogen peroxide was significantly higher than after exposure to Fenton's reagent (3.05% and 1.89%, $p<0.05$, Mann-Whitney test, Figure 5). For agricultural plastic mulch, the median weight loss after a 2 hour exposure to hydrogen peroxide was significantly higher than after exposure to Fenton's reagent (3.96% and 1.33%, $p<0.05$, Mann-Whitney test, Figure 5). For *OK compost industrial*, the median weight loss after a 2 hour exposure to hydrogen peroxide was significantly higher than after exposure to Fenton's reagent (3.80% and 2.16%, $p<0.05$, Mann-Whitney test, Figure 5). For *OK compost home green*, the median weight loss after a 2 hour exposure to hydrogen peroxide was significantly higher than after exposure to Fenton's reagent (10.03% and 4.35%, $p<0.05$, Mann-Whitney test, Figure 5). The difference in weight loss between *OK compost home white* and *green* indicates that the materials possess different properties, contrary to previous believe when *OK compost home white* was replaced by the agricultural plastic mulch.

For the compost mixture displayed in Figure 6, the median weight loss after a 2 hour exposure to hydrogen peroxide was not significantly higher than after exposure to Fenton's reagent (14.26% and 12.40%, $p=0.676$, Mann-Whitney test, Figure 6). For horse manure, the median weight loss after a

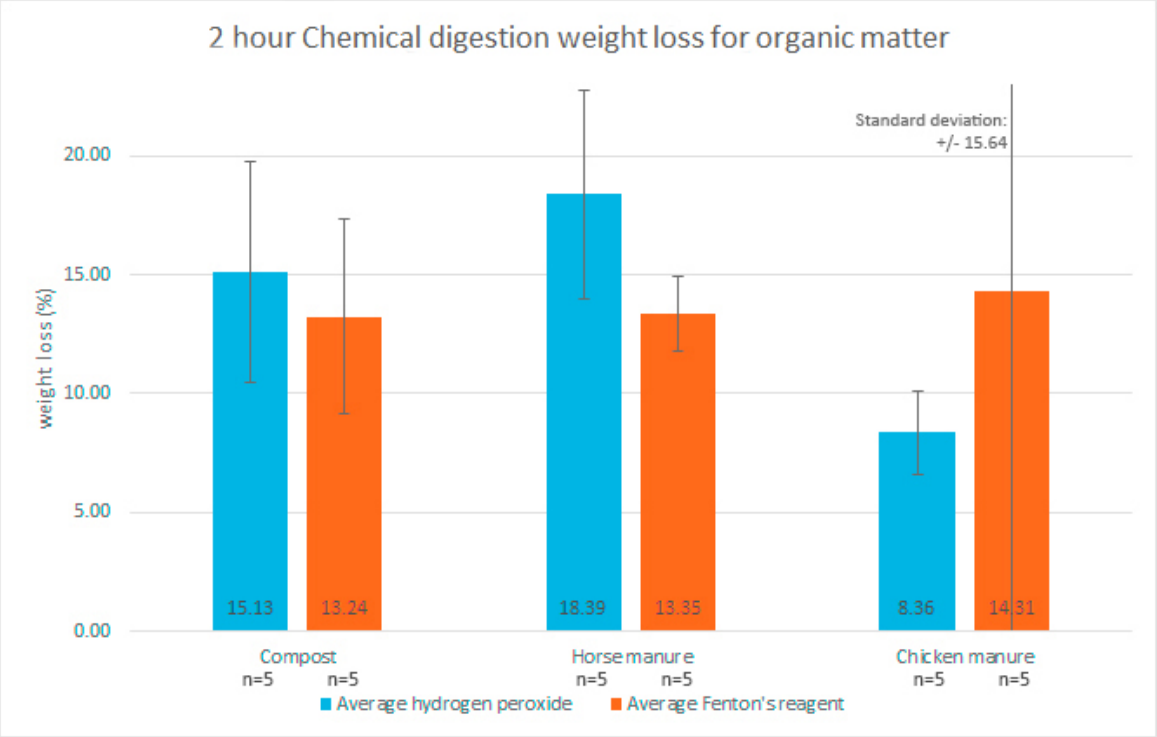

Figure 6: Average weight loss of organic matter after a 2 hour exposure to either hydrogen peroxide or Fenton's reagent, including number of samples and standard deviation. The standard deviation for chicken manure treated with Fenton's reagent is noticeably higher than the others, supposedly due to heavy foaming that transported matter out of the filter papers that were weighed to gain these results.

2 hour exposure to hydrogen peroxide was not significantly higher than after exposure to Fenton's reagent (18.39% and 13.27%, p=0.095, Mann-Whitney test, Figure 6). For chicken manure, the median weight loss after a 2 hour exposure to Fenton's reagent was not significantly higher than after exposure to hydrogen peroxide (11.43% and 8.69%, p=0.531, Mann-Whitney test, Figure 6). Chicken manure was consistently foaming when treated with Fenton's reagent, which has led to a high standard deviation. Weight loss for organic matter samples were consistently higher than for biodegradable plastics. Raw data for this experiment can be seen in appendix Figure A7 and A8.

4.2 Oven incubation

These tests relied on qualitative observations rather than measurements. Therefore, these results are merely indications of trends rather than statistically significant results. These experiments have shown that high temperatures seem to be key for the fragmentation and degradation of biodegradable plastics in a compost matrix. In the 20 day time frame of the experiment, most biodegradable plastics only started to fragment and degrade at 55°C (see Figure 7). *OK compost home* is an exception to this, as it already showed this effect at 45°C and fragmented after 116 days at room temperature. This indicates that it is the material which most easily degrades, which supports the manufacturers claim in chapter 2.3.4. It is not definitive proof, however, but rather shows a trend.

Biodegradable knives made of CPLA did not fragment, but developed a film resembling mold. This was connected with the knives becoming slimy and brittle and therefore interpreted as degradation.

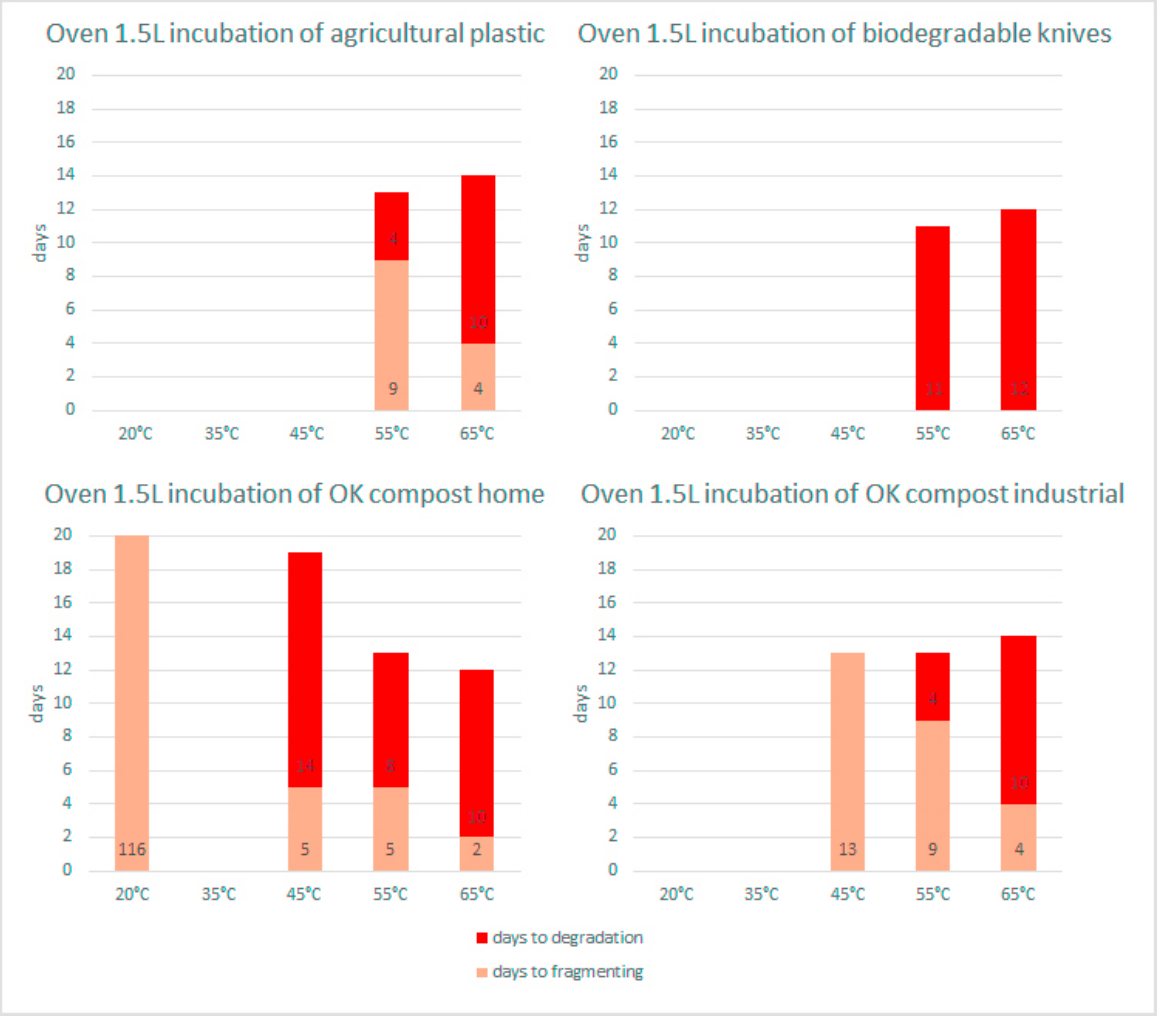

Figure 7: Days to fragmentation and degradation of biodegradable plastics in an oven at indicated temperatures and sorted by materials. Absence of data refers to the process not being observed rather than missing data. The numbers in each category indicate the number of days that passed until it reached the next state. For example, agricultural plastic at 55°C began to fragment after 9 days and has shown first signs of degradation 4 days later. Note that *OK compost home* is the only material that fragmented in the room temperature incubation and exceeds the scale of the graph.

Agricultural plastic mulch (15µm thickness) and *OK compost industrial* showed equal reaction times at 55°C and 65°C. Also, *OK compost home* displayed a similar pattern at these temperatures. Even though the CPLA knives did not fragment, their degradation also set in after roughly the same time as for all other biodegradable plastic samples.

4.3 Compost tumbler incubation

The weight loss of samples shown in Figure 8 and 9 including the nylon bags' weight (green columns) are consistently lower than the samples corrected for nylon bag weight change (blue columns). This is also true for the second incubation experiment, whose data is displayed in Figure 10. This emphasizes the importance of correcting the weight change of the nylon bag, as the biofilm which forms on the bag during composting changes the nylon bags' weight and not correcting for it would lead to an underestimation of the weight loss of biodegradable plastics. Here, correction for compost weight

change led to an apparent weight gain of of the biodegradable plastics. Statistically comparing the first (Figure 8) and second (Figure 10) incubation weight change of biodegradable plastics yields the following results: For agricultural plastic mulch, the median weight loss after the incubation was significantly different for the first (15 day, Figure 8) and second (14 day, Figure 10) incubation (10.83% and 5.35%, $p<0.05$, Mann-Whitney test). For *OK compost home*, the median weight loss after the incubation was significantly different for the first (15 day) and second (14 day) incubation (25.95% and 9.54%, $p<0.05$, Mann-Whitney test). For *OK compost industrial*, the median weight loss after the incubation was not significantly different for the first (15 day) and second (14 day) incubation (8.26% and 1.37%, $p=0.27$, Mann-Whitney test). Finally, for *OK compost home*, the median weight loss after the incubation was significantly different for the first (15 day) and second (14 day) incubation (1.49% and 0.17%, $p<0.05$, Mann-Whitney test).

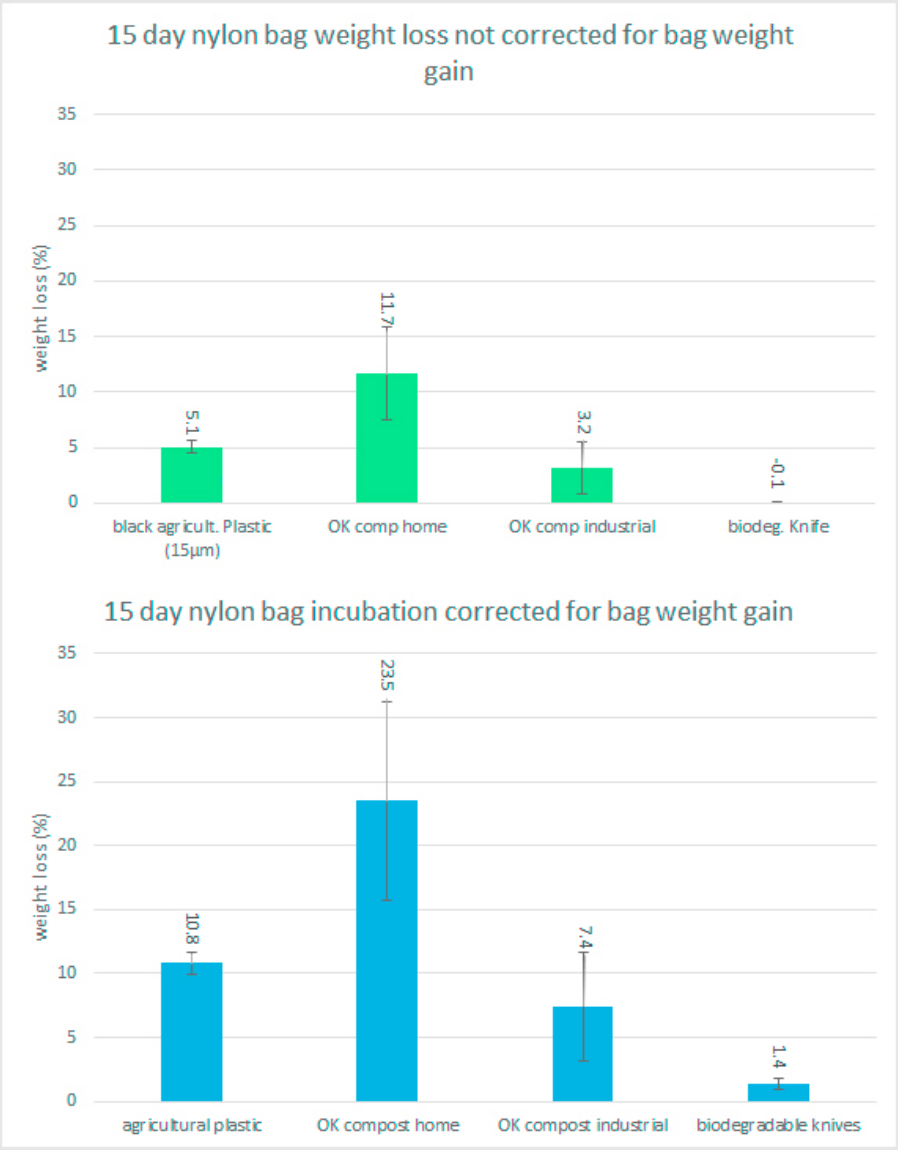

Figure 8: Weight loss of the four biodegradable plastics inside of 50µm mesh size nylon bags after 15 days incubation. The scale is chosen to be easily comparable to the 29 day degradation in Figure 9. Empty bags used for nylon bag weight change correction had n=5 with an average weight change of +2.1% and a standard deviation of 0.5. Raw data can be seen in Table A3 and A6.

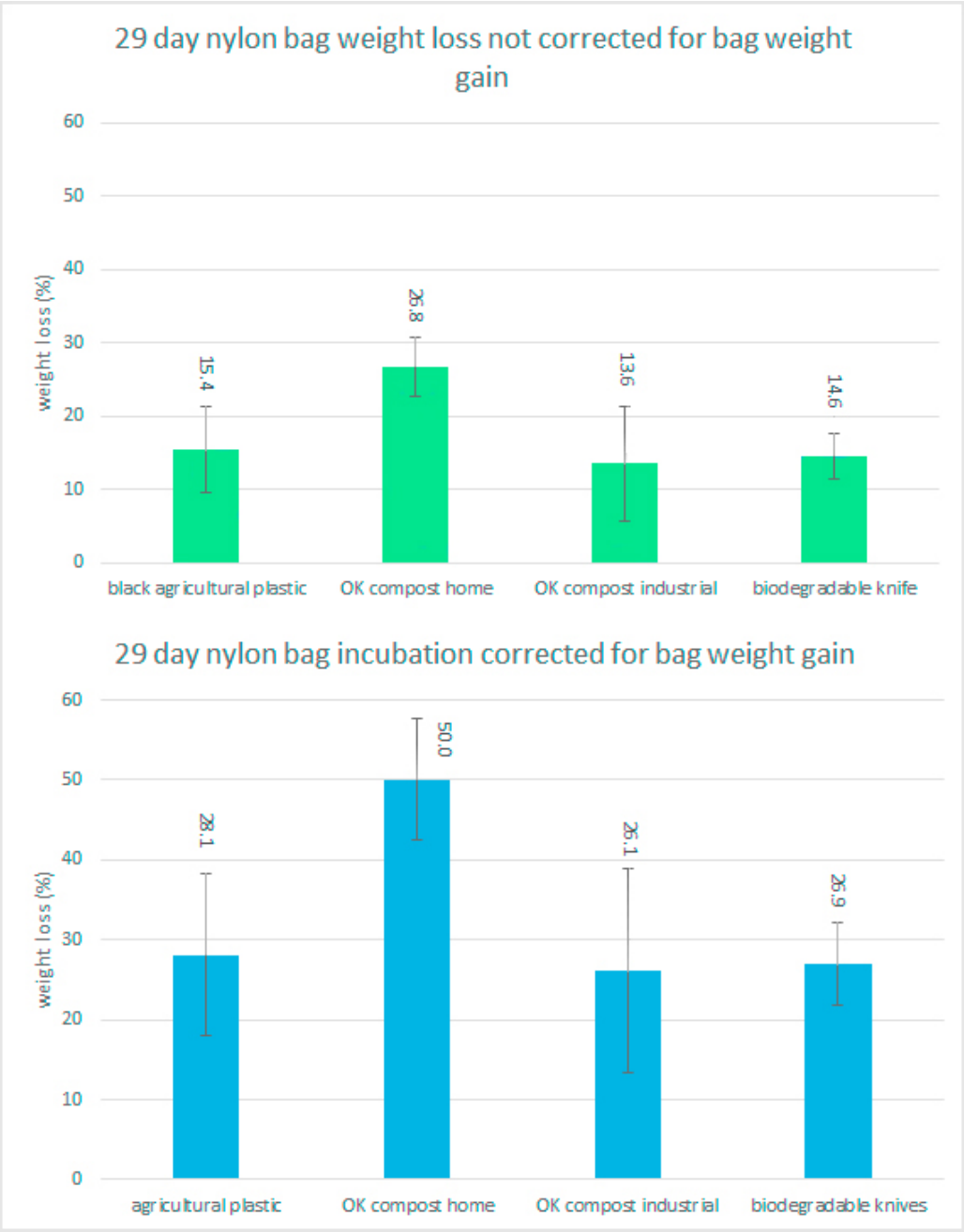

Figure 9: Weight loss of the four biodegradable plastics inside of the 50µm mesh size nylon bags after 29 days incubation. Empty bags used for nylon bag weight change correction had n=5 with an average weight change of +2.7% and a standard deviation of 0.6. Raw data can be seen in Table A4.

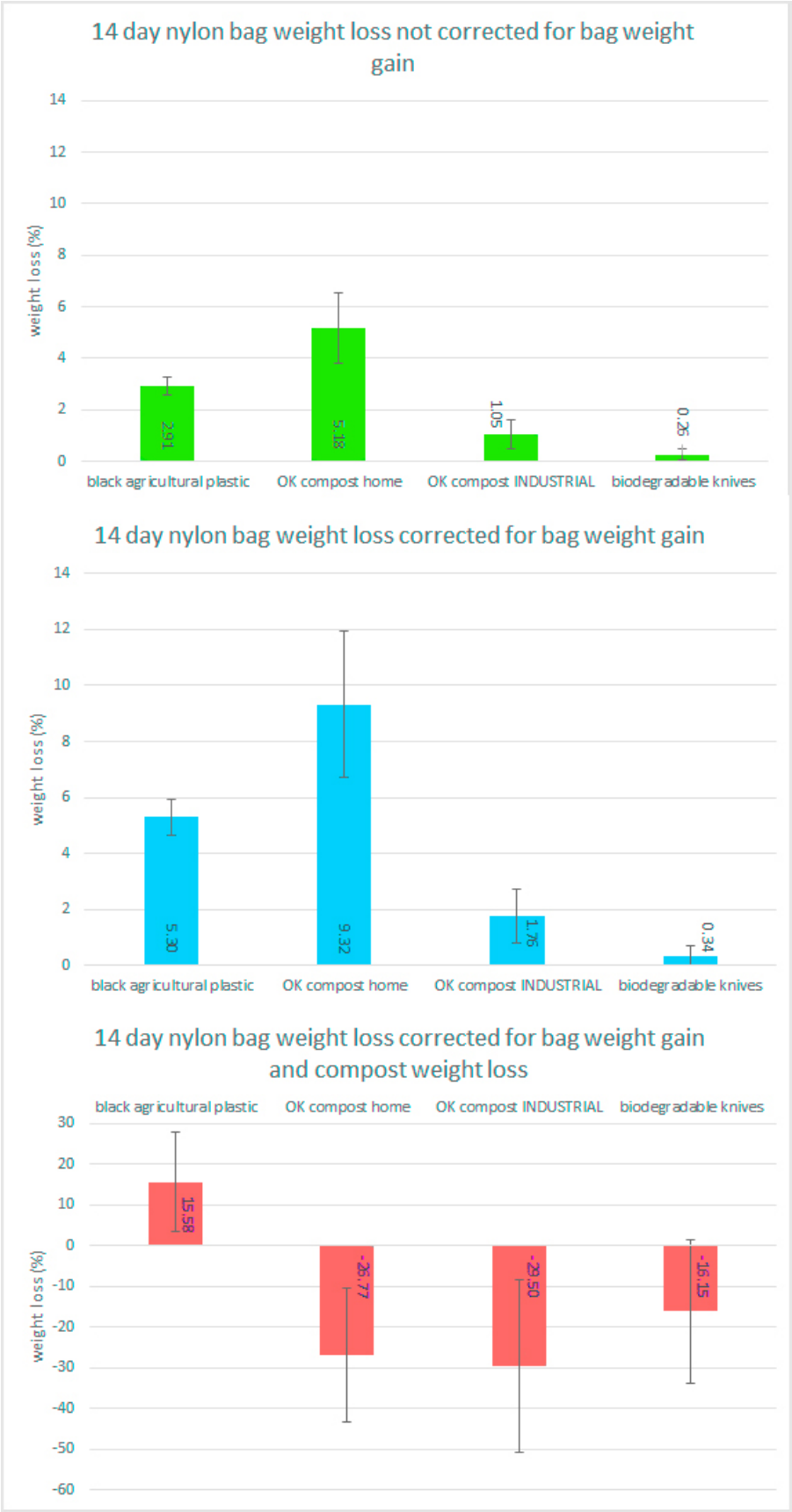

Figure 10: Weight loss of the four biodegradable plastics inside of the 50µm mesh size nylon bags after 14 days incubation. Empty bags used for nylon bag weight change correction had n=5 with an average weight change of -0.2% and a standard deviation of 0.5. The compost used for weight change correction had n=5 with an average weight change of -30.8% and a standard deviation of 3.1. Raw data can be seen in Table A5.

4.4 pH measurement of PLFA samples

4.4.1 Comparing compost pH inside nylon bags with different mesh sizes in tumblers

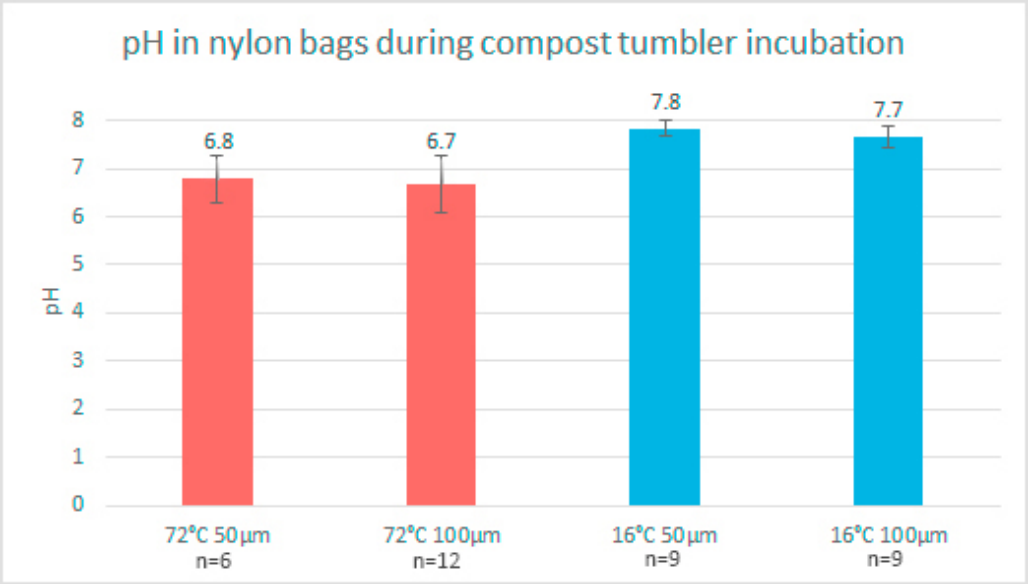

Figure 11: pH values for 50µm and 100µm at 72°C in red and 16°C in blue. Each column is made of triplicate measurements of several replicate samples whose exact values can be seen in appendix Table A2. Average values are stated above columns and individual standard deviations are indicated by error bars. The temperature development for this incubation can be seen in appendix Figure A11.

These results refer to the data displayed in Figure 11, where 72°C samples have been taken 1 day after starting the compost and 16°C 6 days after starting. Since the number of samples was small, and none of the groups had a normal distribution (Anderson-Darling $p<0.05$) the Mann-Whitney test was used for statistical analysis. At 72°C, the pH value for the 50µm mesh size nylon bags was not significantly higher than for the 100µm bags (medians of 6.79 and 6.36, $p>0.05$, Mann-Whitney test). Also at 16°C, the pH values for the 50µm mesh size nylon bags was not significantly higher than for the 100µm bags (medians of 7.75 and 7.68, $p>0.05$, Mann-Whitney test). When comparing temperatures a different image emerges. For 50µm mesh size, the pH value at 16°C was significantly higher than at 72°C (medians of 7.75 and 6.79, $p<0.05$, Mann-Whitney test). Also for 100µm mesh size, the pH value at 16°C was significantly higher than at 72°C (medians of 7.68 and 6.39, $p<0.05$, Mann-Whitney test).

4.4.2 Comparing compost pH inside nylon bags and outside (bulk compost) in tumblers

These results refer to the data displayed in Figure 12. Because most samples are not normally distributed (Anderson-Darling $p<0.05$) and number of samples of the oven sample groups are relatively small, the analysis was conducted via the Mann-Whitney test. At the peak temperature of 72°C, the pH value of the compost tumbler was not significantly higher than inside the 50µm nylon bags (medians of 7.21 and 6.40, $p>0.05$, Mann-Whitney test). During the cooling phase, at 16°C, the pH value of the 50µm nylon bags was not significantly higher than inside the compost tumbler (medians of 7.75 and 7.52, $p>0.05$, Mann-Whitney test).

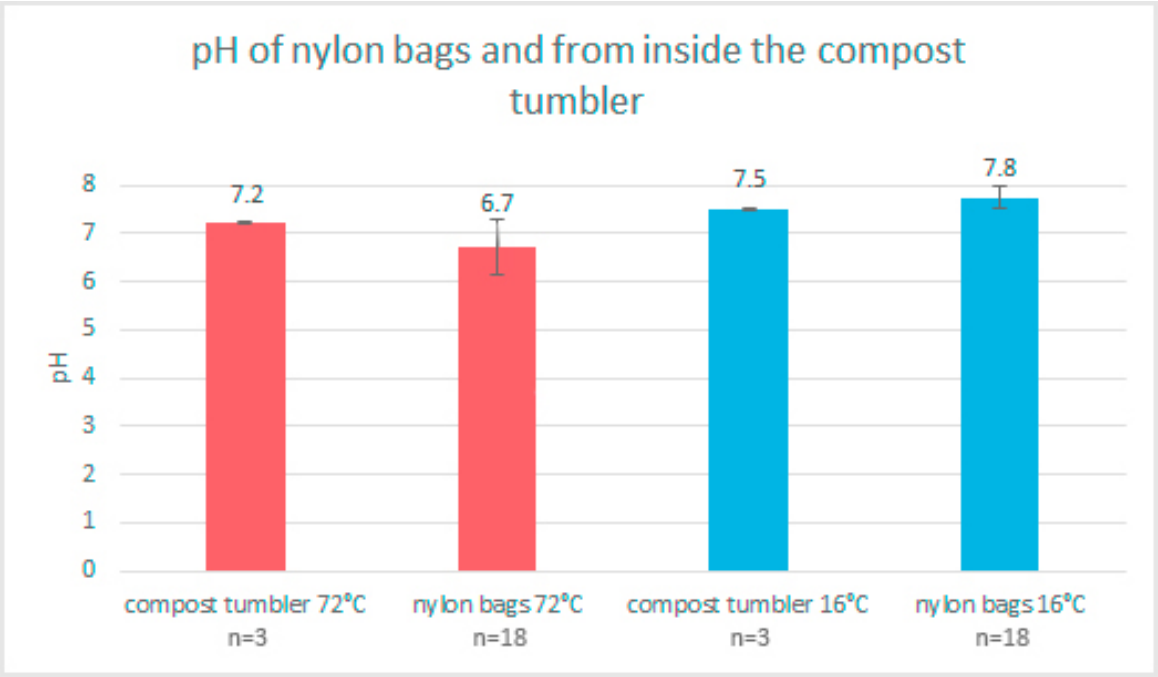

Figure 12: The number above the column states the average value and the error bars indicate the standard deviation. For raw data see Table A2 and temperature curve in Figure A11.

4.4.3 Comparing compost pH in compost tumblers and oven incubations

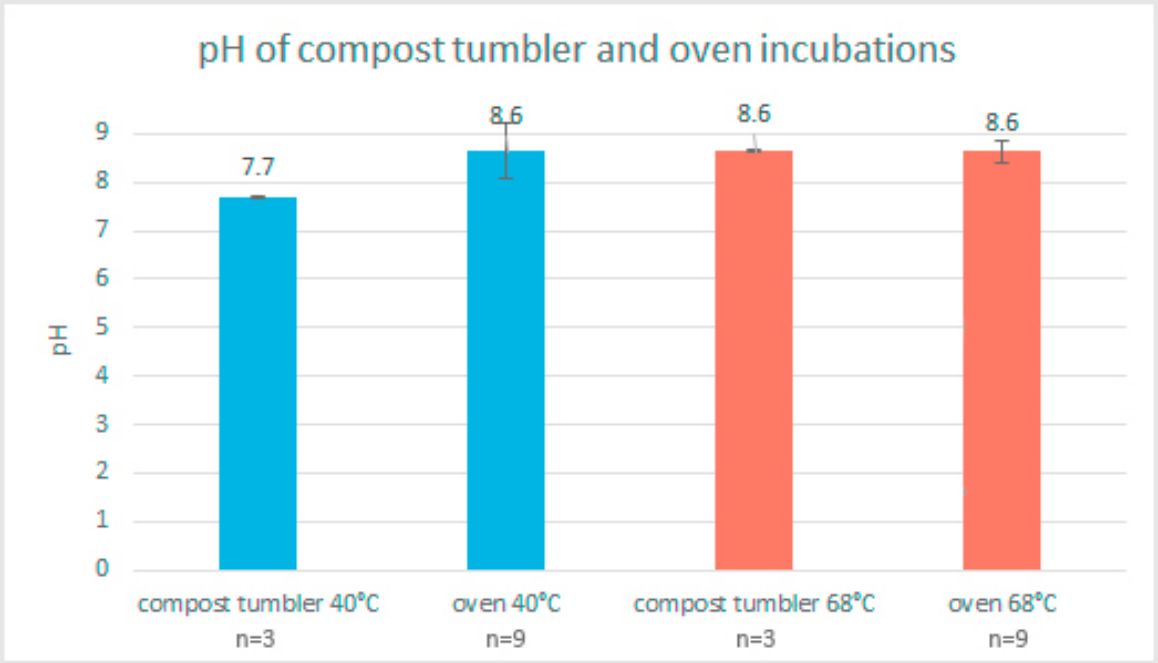

Figure 13: Compost pH during the first compost incubation in compost tumblers and 1.5L oven incubations. Average values are shown above the column, and the error bars indicate standard deviation. The number of samples between treatments vary due to a combination of 3 replicate samples from inside the oven (for raw data see appendix Table A1). These data are from the first compost incubation, which had a different compost batch than the second compost incubation in Figure 14. The temperature development for this incubation can be seen in appendix Figure A9.

Here, the compost pH between compost tumbler and 1.5L oven incubations is compared. Different time points and therefore different temperatures were sampled in order to see similarities between

composting processes at two different scales. Two different compost tumbler batches, one started on the 28.01. (Figure 13) and one on the 27.02.2020 (Figure 14), were used for comparison. These results refer to the data displayed in Figure 13. Even though the data for oven samples at 40°C and 68°C are normally distributed (Anderson-Darling $p>0.05$) the number of samples for tumbler samples is too small for getting reliable results from a t-test or reliably proving its assumptions. Therefore, the non-parametric alternative, the Mann-Whitney test, was used for data analysis. At 40°C, the pH value of the oven incubation was significantly higher than inside compost tumbler (medians of 8.65 and 7.85, $p<0.05$, Mann-Whitney test). At 68°C, the pH value of the oven incubation was not significantly higher than inside compost tumbler (medians of 8.67 and 8.64, $p>0.05$, Mann-Whitney test).

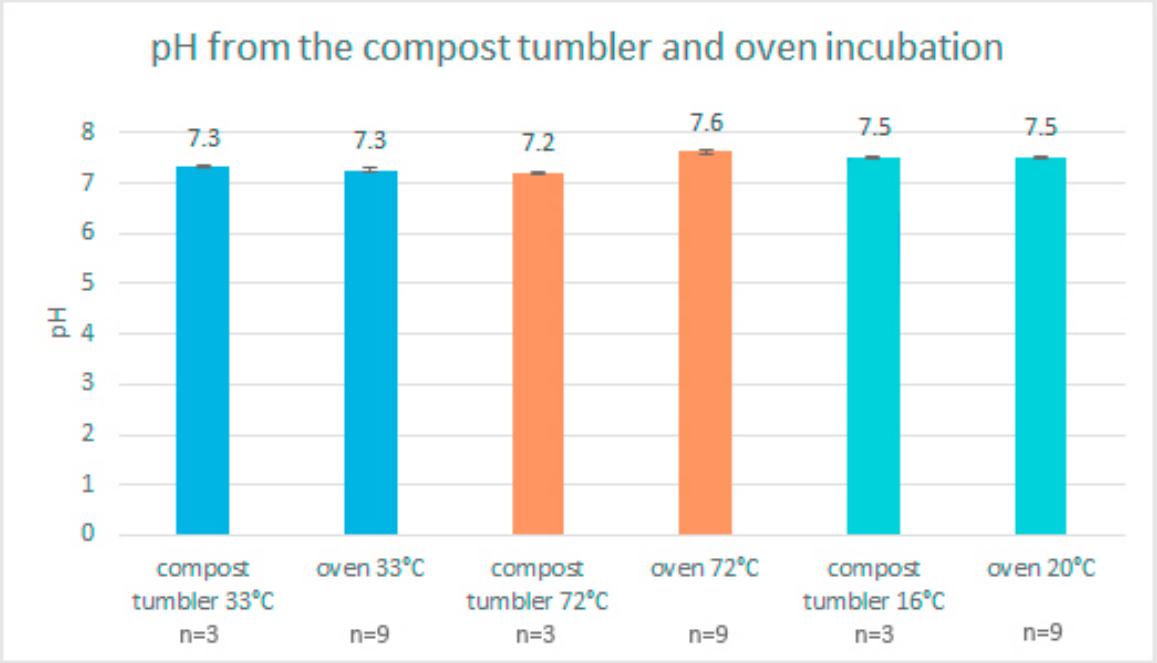

Figure 14: Compost pH during the second compost incubation in copost tumblers and 1.5L oven incubations. Average values are shown above the column, and the error bars indicate standard deviation. The number of samples varies due to the same reason as in Figure 14. The temperature difference between 16°C and 20°C is due to the assumption that at the sinking temperature there would be little difference between these two temperatures and due to complications to get the oven sample to 16°C rather then to room temperature. The temperature development for this incubation can be seen in appendix Figure A11.

These results refer to the data displayed in Figure 14. Due to the non-normal distribution of all oven samples and the small number of samples in general these samples were compared with the Mann-Whitney test. In the mesophilic phase at 33°C, the pH value of the compost tumbler was not significantly higher than inside the oven incubation (medians of 7.32 and 7.24, $p>0.05$, Mann-Whitney test). During the thermophilic phase at 72°C, the pH value of the oven incubation was significantly higher than inside the compost tumbler (medians of 7.65 and 7.21, $p<0.05$, Mann-Whitney test). In the cooling phase at 16/20°C, the pH value of the compost tumbler was not significantly higher than inside the oven incubation (medians of 7.52 and 7.40, $p>0.05$, Mann-Whitney test).

5 Discussion

5.1 Upconcentrating compost samples by chemical digestion

For all biodegradable plastic types, a statistically significant difference between the weight loss after being exposed to hydrogen peroxide or Fenton's reagent was found. Biodegradable knives were more affected by Fenton's reagent while it led to less weight loss for all other materials. Either Fenton's reagent is less aggressive towards these plastic types, or it adds small crystals to the plastic's surface which adds weight and thus simulates a lower weight loss, while the actual degradation of the plastics remains unknown. Since the question could not be answered in this thesis, more focused research is still needed. Some suspended particles appeared in the catalyst solution of Fenton's reagent after some days, which raised suspicion towards the possibility of crystallisation on sample surfaces as well. Though Tagg et al. (2017) did not find indications of Fenton's reagent affecting polymer chemistry, their results are not necessarily applicable here. They had a ten minute reaction time instead of two hours and experimented on non-biodegradable plastics. Their findings should consequently not be transferred to biodegradable plastics, so that this new evaluation of the method was necessary. Additionally, both chemical digestions led to limited weight reduction of the compost while already partly degrading the biodegradable plastics. This shows that up-concentrating biodegradable plastic-organic matter samples is more challenging than doing so for conventional plastics.

Comparing weight loss of organic matter to that of plastic shows two distinct differences. Firstly, the average organic matter weight loss is higher than the plastic's for both treatments. Consequently, in mixtures of compost and biodegradable plastics both treatments will lead to an increased ratio of plastics to organic matter. The signal to noise ratio for sample analysis rises. Secondly, the organic matter standard deviation is higher than the plastic's. This probably is partly due to a more complex matrix which is more unevenly exposed to the treatment chemicals than the plastic particles. Moreover, foaming occurred for organic matter samples, which in the case of chicken manure and Fenton's reagent, transported matter out of the filter paper it was later weighed in.

Dividing the average weight loss of organic matter by the one of biodegradable plastics reveals a weight loss ratio. Since this averages biodegradable plastics with different uses and different weight loss percentages, as well as organic matter with a quite high standard deviation, this ratio can only show a trend rather than an accurate result. For hydrogen peroxide this ratio is 3.3 and for Fenton's reagent it is 5.5. For the examined materials, Fenton's reagent consequently removes more organic matter per weight unit biodegradable plastics removed. It therefore seems to be the better choice for the up-concentration of biodegradable plastics in a compost organic matter matrix compared to hydrogen peroxide. This finding is consistent with that of Hurley et al. (2018), who compared the effect of these two treatments, amongst others, on non-biodegradable plastics. However, due to the stated sources of uncertainty, this result should be followed up by dedicated research which has the resources to eliminate remaining concerns. Furthermore, even if the Fenton's reagent is more favourable, none of these treatments are ideal for the study of biodegradable plastic materials since there up-concentration effect is limited and also affects the biodegradable plastics themselves. Accu-

rate analysis of the amount of biodegradable plastics in an organic matter matrix is a key element in assessing whether biodegradable plastics contribute to (micro)plastics pollution. As Fotopoulou and Karapanagioti (2017) pointed out, biodegradable plastics will not necessarily degrade in soil and thus can accumulate where it affects functioning of terrestrial ecosystems (de Souza Machado et al., 2018). Accurate analysis is also in the interest of the law by a precautionary principle (føre-var prinsippet) which, for Norway, can be found in the constitution (Grunnloven) §112 and the nature diversity act (naturmangfoldloven) §9. Consequently, this thesis' results implicate that Fenton's reagent is a fitting pre-treatment for sample analysis regarding this problem. Due to mentioned uncertainties, further research is needed to strengthen these findings.

5.2 Oven incubation

Since home composting conditions are milder than those in industrial composting plants, degradation will take more time. Here, the active composting phase was assumed to last around 20 days, but it is questionable if the most effective temperatures for degradation would be reached in a garden compost without proper management. None of the materials were degrading at all between 20°C and 45°C for the time frame of 20 days, which was expected with regards to the products use and necessary properties for it. Only the *OK compost home* bag made for a common garden compost showed signs of fragmentation after 116 days at a constant 20°C. For most real world climatic conditions, this is a temperature which will not be maintained for such a long contiguous period. In fact, especially the northern countries will most likely accumulate only a few days per year with such conditions, so that the necessary days for fragmentation would accumulate over the course of many years. The producers claim of compostability may not hold true because of the prolonged degradation time of *OK compost home*, which can negatively affect nature. Degradation may or may not follow long time after the fragmentation, as there is a chance that the necessary conditions needed for this are not met (Mohee and Unmar, 2007). Missing degradation could lead to the accumulation of biodegradable (micro)plastics in compost and soil over time with negative effects for the terrestrial ecosystem involved (de Souza Machado et al., 2018).

For the other three materials tested, the slow or missing degradation holds true on a even bigger timescale, which could cause a more persistent and intense accumulation of plastics in nature. It is also questionable whether biodegradable plastics that were certified by EN 13432 to completely biodegrade in 6 months will degrade in the 3 to 6 weeks, which are the common time for the active phase in Norwegian composting plants (Hann et al., 2019). While degradation for some plastic particles was observed in this experiment, it remains unclear how long it takes for material to completely degrade. Assessing this is currently achieved by state of the art technology and was not feasible for this thesis with the accessible equipment. However, revealing an indication of incomplete degradation in (Norwegian) composting plants and garden composts was achieved.

While this experiment only reveals a trend, it suggests that biodegradable plastics may pass through the Norwegian composting plants. This implies biodegradable microplastics accumulation in addition to the already present pollution by conventional plastics. It also shows the importance of regarding

biodegradable plastics as a tool for minimising plastic pollution, instead of being the solution itself. Since the end product from composting plants is often used as soil improver, plastics could spread to a variety of environments, besides the already mentioned agricultural fields. These results should be seen as preliminary and suggest further research in this field. A case study on a Norwegian composting plants would be a logical follow up research and may be combined with further research in sample pre-treatment (see chapter 5.1).

5.3 Compost tumbler incubation

The results suggest that incubating biodegradable plastics in nylon bags with 50µm mesh openings is a viable method for accessing weight loss. Correcting the total weight loss for the weight change of nylon bags consistently revealed similar trends with higher weight loss percentages of the biodegradable plastics. Doing so also increases the uncertainty of results as the standard deviation of nylon bags and biodegradable plastic weight loss is paired. However, using nylon bags for incubation presents the possibility to easily assess the degradation of biodegradable plastics of all chemical structures in their early to middle phase of degradation. The manufacturer of *OK compost home* bags claim that it can degrade under less ideal conditions than these defined by EN 13432. While this test cannot prove the companies claims, it does consistently show a more advanced degradation compared to the other materials. Therefore, this method has the potential to discover such degradation trends in a relatively undemanding process. If this method got refined in future research, it could serve as a tool for the analysis of biodegradable plastics degradation, also in cases where funding for more sophisticated equipment is unavailable. Analysing late-stage degradation currently is state of the art technology and was not possible due to the restriction of accessibility. Consequently, this method may indicate the degradation speed in composting plants or hint at an incomplete degradation without delivering the most accurate result possible. This method could help adapt composting conditions so that complete degradation is more likely to occur which in turn could save the environment and especially terrestrial ecosystems from permanent harm, as indicated by de Souza Machado et al. (2018). However, further refinement of the method is needed.

As seen in the pretest to this experiment, a bigger mesh size leads to a higher degradation rate. How much a certain mesh size slows down the process and the biggest mesh size for maximum degradation without plastic particle loss is a challenge for later research. Moreover, statistically comparing the first and second incubation of biodegradable plastics showed significant differences even though the experimental setup was identical. This stresses the importance of composting conditions on the degradation, as they were not directly controlled and developed at least slightly differently. The examination of degradation consequently is only valid for the conditions tested, as alterations in conditions may lead to significantly different results. Compost was added to some nylon bags to allow direct contact to biodegradable plastics, as an attempt to eliminate the mesh size complication altogether. This rendered results useless (see figure 10). The high standard deviation of weight loss of compost inside the nylon bags led to imprecise corrections for their weight change. As a result, most plastic types were suggested to have gained weight, which could be ruled out. This might be due to the fact

that compost inside the bags tended to form clumps whose outside was partly covered with the plastics inside the bags, which protected from degradation. It could also have other reasons like compression of the compost inside the nylon bags by the weight of the surrounding compost. Curiously, plastic particles touching other plastic particles inside the nylon bags did not have similar effects on weight loss.

All in all, more research is needed to perfect this method in terms of finding the best suited mesh size and possibly a way to successfully incorporate compost into the bags. However, this method delivers an easy way to assess biodegradable plastic degradation in compost by terms of weight loss. This could be beneficial to detect trends in degradation rate and likeliness of complete degradation and may also be accessible with less available funding.

5.4 pH measurement of PLFA samples

As Tuomela (2002); Diaz et al. (2005); Cheng and Zhen (1987) have shown, different microorganisms and pH values are present at different stages of the composting process. Consequently, the pH can be used as a supporting indicator to PLFA to assess what microbes are present at what phases of the composting process (see chapter 1.2.1). The pH is not a precise indicator of microbial communities, but composts with similar pH at similar temperatures are more likely to host comparable microbial communities. If no statistically significant difference in pH between treatments could be found, it would indicate that treatments may be interchangeable. This had to be confirmed by using PLFA samples in addition. Composting experiments could thus be scaled down to reduce needed labor and time. pH results should be used together with PLFA analysis, if possible.

5.4.1 Comparing pH between nylon bags of different mesh sizes

The results show no significant difference in the nylon bag pH of 50µm and 100µm mesh size bags at 16°C and 72°C. This indicates equal microbial populations inside the bags, and thus similar degradation properties, which suggests that treatments might be interchangeable. Moreover, pH values between peak (72°C) and sinking temperature (16°C) show a significant difference. Consequently, significant variances in pH are dictated by temperature and not the mesh size, in this case. Nylon bag compost meets the theoretically anticipated pH for the respective composting phases and temperatures stated in Chapter 1.2.1. This indicates that the incubation of biodegradable plastics inside nylon bags with 50µm and 100µm may yield similar results, and seem to possess good composting properties. However, as the 100µm showed a faster degradation rate in the pretest, it seems to allow more efficient exchange with the compost matrix it is placed in. While this indicates a higher degradation rate, a more thorough examination would be favourable to rule out drawbacks like particulate matter loss. Though this was not found in the pretest, a more thorough examination is advised for future research. It could also be relevant to find the maximum mesh size where no particulate biodegradable plastic is leaked out, to maximise the degradation and come closer to a rate of composting without nylon bags.

5.4.2 Comparing nylon bag and compost tumbler pH

The data suggests that conditions in nylon bags and directly in the compost tumbler are comparable. For 16°C and 72°C, no significant difference in pH was detected. This means that the degradation of biodegradable plastics (analysed in chapter 4.3) is representative of the one done directly in a compost matrix. Therefore, nylon bags with 50µm mesh display a method for the incubation of biodegradable plastics in compost without loosing the samples in the organic matter. Still, the slower degradation rate at smaller mesh sizes should be taken into account. Moreover, it should be pointed out that a high standard deviation for nylon bags at 72°C and low population size of compost tumbler samples present some uncertainty. To further examine these results, I propose a repetition with a bigger population sizes. The methodological choices were limited by the time available for this thesis. I can conclude that nylon bags pose a viable method of assessing biodegradable plastic degradation in compost, while also acknowledging that the degradation rate seems to be influenced by mesh size.

5.4.3 Comparing compost tumbler and oven incubation pH

Based on pH alone, it is difficult to conclude that the degradation in an oven is comparable to that in a compost tumbler. This result is important to verify the suitability for the compost incubation to be scaled down to a size apt for laboratory experiments rather than experimentation in outdoor compost tumblers. Two out of five comparisons show a significant difference in pH. Based on this, a trend towards compatibility can be shown but not confirmed. Two key differences between treatments are that the compost tumbler was aerated by turning, while the oven incubation got stirred and that composting in a compost tumbler is a self-warming process, while oven incubations were etxternally heated up. Furthermore, the oven was mostly set to the last compost tumbler sampling temperature, so that it experienced the temperature change (e.g. from 33°C to 72°C) much more rapidly than the tumbler, where natural processes dictated the pace. It is also plausible that the relative moisture changes in the smaller oven samples were stronger than in the compost tumblers. Even though the 1.5L glass boxes were watered regularly, as to remain their original weight and therefore water content, a faster change in moisture might have influenced oven pH results. As chapter 1.2.1 describes, pH changes go along with microbial activity which is directly influenced by moisture. Here, results from PLFA analysis would be helpful to give a more refined answer to whether these treatments are comparable. The methodological choices were constrained by the availability of equipment. In future research, the PLFA data should be included in this assessment and potentially a repetition with more similar equipment could be conducted (e.g. a miniature compost tumbler heated in an oven or a more refined glass tub incubation). Based on currently available data, the compatibility of treatments can be neither confirmed nor denied.

6 Conclusion

This Master's thesis aimed to develop methods for analysing biodegradable plastic degradation. With the right methods, researchers are able to evaluate whether biodegradable plastics contribute to the accumulation of (micro)plastics in the terrestrial environment. Based on my findings, most methods tested looked promising, even if some require further refining research. Up-concentration of biodegradable plastic-compost samples by chemical digestion was found to be most effective with Fenton's reagent, but also degraded the biodegradable plastic for both chemicals tested and consequently is not an optimal solution. Incubating biodegradable plastics in nylon pouches in compost yielded an easy to execute, though not perfect, method of examining the degradation of biodegradable plastics by lost weight, without losing particles. It also stressed the importance of the specific composting conditions for the degradation. The 1.5L oven incubation showed the effect of temperature on degradation which raised suspicion about the incomplete degradation of biodegradable plastics in composting plants and garden composts. Comparing compost pH of different treatments at the same stage of composting indicated the possibility of scaling down composting experiments to a laboratory scale, though PLFA data was not available so that this finding could not be further assessed. Furthermore, composting plastics in garden composts was concluded to take longer in northern climates than it was foreseen by the producer. Therefore, unwanted pollution with plastics may occur due to the removal from compost before total degradation was reached.

Since the research of biodegradable plastics in terrestrial ecosystems is a field that arose quite recently, many questions remain yet unanswered and no standard protocol for their analysis exists. The specific degradation conditions and materials used in each compost incubation in this thesis may limit the generalisability to a certain degree. Limited time and the COVID-19 pandemic restricted the advances made in the development of methods. Still, the approaches for analysing biodegradable plastic degradation demonstrated in this thesis provided new insights into its trends, processes and promising methods of analysis. Moreover, some analytical methods were found to be unfit. This thesis could contribute in finding a base for creating a standard protocol of biodegradable plastic degradation analysis. To better understand the possibilities and limitations of the developed techniques, further research is needed. By doing so, the degradation reached in (Norwegian) composting plants and whether the resulting soil improver may be a source of microplastics in soil, may be assessed. Since plastic pollution was found to negatively affect terrestrial ecosystems and might be a driver of global change (de Souza Machado et al., 2018; Rillig, 2012), this thesis helped in determining if biodegradable plastics currently contribute to this problem.

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Appendix

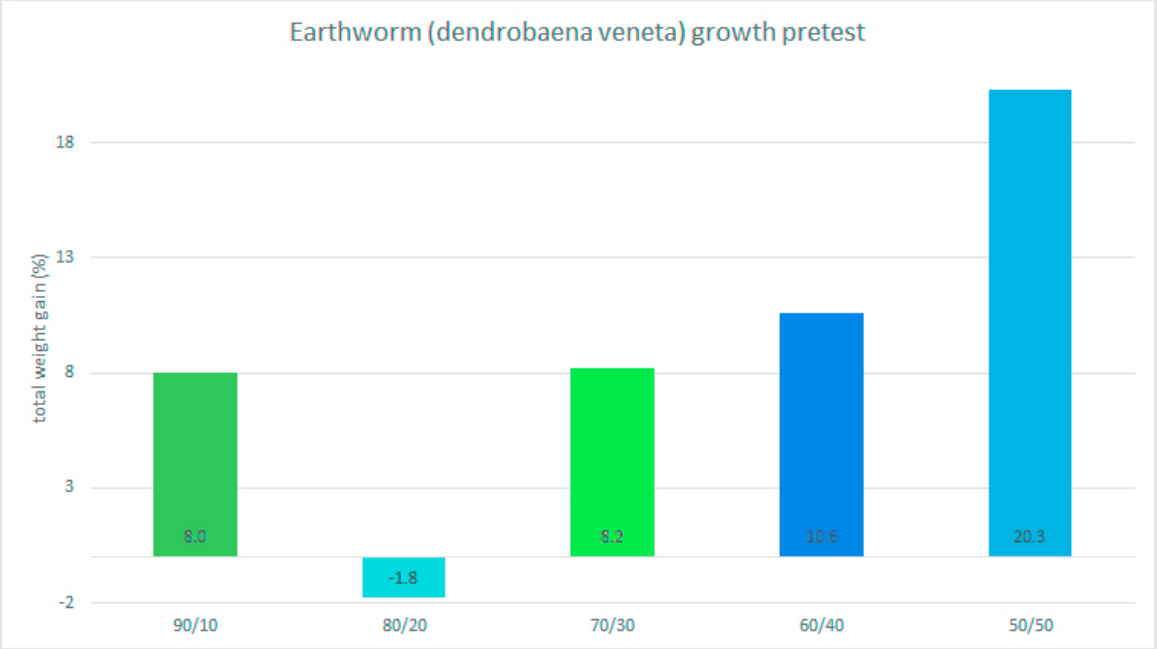


Figure A1: Average growth of 10 earthworms (*Dendrobaena veneta*) in different horse manure/chicken manure ratio blends. For example, 90/10 is 90% horse manure and 10% chicken manure based on their dry weight. Only the 90/10 and 80/20 blend did not develop mold on top. Consequently, a 90/10 ratio was choosen as the compost material to work with.

Table A1: pH measurements for the PLFA samples from the first incubation experiment. All numbers have been rounded to the second decimal number by the rounding function of Microsoft® Excel® for Office 365 (2018).

Treatment	ID	pH 1	pH 2	pH 3	Average	Standard deviation
T0	T0	8.8	8.76	8.73	8.8	0.03
Compost tumbler	40°C	7.24	7.85	7.97	7.7	0.32
	68°C	8.64	8.65	8.64	8.6	0.00
Oven incubation	40°C I	8.26	8.47	8.44	8.4	0.09
	40°C II	8.67	8.65	8.65	8.7	0.01
	40°C III	8.87	8.86	8.83	8.9	0.02
	68°C I	8.67	8.67	8.65	8.7	0.01
	68°C II	8.71	8.7	8.73	8.7	0.01
	68°C III	8.6	8.54	8.53	8.6	0.03

Table A2: pH measurements for the PLFA samples from the second incubation experiment. For the nylon bag treatments, ID 1002 refers to sample number two of 100µm nylon bag smaples and 503 to sample number three of 50µm samples. pH 1-3 refers to the three pH measurements for each sample. All numbers have been rounded to the second decimal number by the rounding function of Microsoft® Excel® for Office 365 (2018).

Treatment	ID	pH 1	pH 2	pH 3	Average	Standard deviation
T0	Horse manure T0	7.73	7.72	7.72	7.7	0
	Chicken manure T0	7.01	7	7	7	0
	Compost T0	7.18	7.2	7.21	7.2	0.01
Compost tumbler	33°C (up)	7.31	7.32	7.34	7.3	0.01
	72°C (peak)	7.2	7.21	7.23	7.2	0.01
	16°C (down)	7.5	7.52	7.52	7.5	0.01
Nylon bags	72°C 1002	6.24	6.26	6.27	6.3	0.01
	72°C 1004	6.49	6.49	6.49	6.5	0
	72°C 1006	7.69	7.72	7.72	7.7	0.01
	72°C 1007	6.25	6.27	6.28	6.3	0.01
	72°C 503	7.27	7.29	7.28	7.3	0.01
	72°C 505	6.31	6.3	6.31	6.3	0
	16°C 504	7.75	7.74	7.76	7.8	0.01
	16°C 507	7.68	7.68	7.68	7.7	0
	16°C 508	8.05	8.09	8.08	8.1	0.02
	16°C 1001	7.34	7.39	7.4	7.4	0.03
	16°C 1003	7.92	7.96	7.97	8	0.02
	16°C 1005	7.64	7.69	7.68	7.7	0.02
Oven incubation	30°C I	7.26	7.26	7.27	7.3	0
	30°C II	7.48	7.47	7.48	7.5	0
	30°C III	7.25	7.24	7.26	7.3	0.01
	33°C I	7.32	7.34	7.34	7.3	0.01
	33°C II	7.24	7.24	7.23	7.2	0
	33°C III	7.22	7.22	7.21	7.2	0
	72°C I	7.63	7.67	7.65	7.7	0.02
	72°C II	7.68	7.66	7.69	7.7	0.01
	72°C III	7.54	7.54	7.54	7.5	0
	20°C I	7.36	7.35	7.35	7.4	0
	20°C II	7.37	7.4	7.4	7.4	0.01
	20°C III	7.49	7.52	7.53	7.5	0.02

Table A3: Weight of materials used in the first nylon bag incubation in a 135L compost tumbler chamber and its weight after 15 days. The associated temperature curve is displayed in figure A9. Each row of a material displays one replicate.

	bag (g)	plastics (g)	total (g)	incubated (g)
black	0.866	1.085	1.951	1.854
agricultural	0.862	1.059	1.921	1.838
plastics	0.843	1.064	1.907	1.807
(15µm)	0.859	1.09	1.949	1.836
OK	0.827	0.982	1.809	1.717
compost	0.834	0.909	1.743	1.552
home	0.816	0.984	1.8	1.532
	0.832	0.972	1.804	1.519
OK	0.849	1.108	1.957	1.837
compost	0.824	1	1.824	1.761
industrial	0.84	1.152	1.992	2.001
	0.839	0.982	1.821	1.755
biodegrad.	0.856	1.048	1.904	1.906
knives	0.853	1.176	2.029	2.025
	0.843	1.16	2.003	2.012
	0.852	1.149	2.001	2.002
empty	0.82			0.834
bags	0.83			0.846
	0.833			0.859
	0.809			0.822
	0.821			0.838

Table A4: Weight of materials used in the first nylon bag incubation in a 135L compost tumbler chamber and its weight after 29 days. The associated temperature curve is displayed in figure A9. Each row of a material displays one replicate.

	bag (g)	plastics (g)	total (g)	incubated (g)
black	0.774	1.123	1.897	1.516
agricultural	0.763	1.167	1.93	1.754
plastics	0.789	1.092	1.881	1.691
(15µm)	0.784	1.098	1.882	1.46
OK	0.79	0.97	1.76	1.226
compost	0.792	1.041	1.833	1.466
home	0.796	0.982	1.778	1.286
	0.788	1.046	1.834	1.298
OK	0.826	0.981	1.807	1.522
compost	0.806	0.963	1.769	1.668
industrial	0.783	1.152	1.935	1.443
	0.79	0.989	1.779	1.644
biodegrad.	0.781	1.145	1.926	1.58
knives	0.651	1.01	1.661	1.397
	0.775	0.957	1.732	1.472
	0.761	1.029	1.79	1.621
empty	0.77			0.793
bags	0.743			0.768
	0.761			0.775
	0.767			0.792
	0.745			0.76

Table A5: Weight of materials used in the second nylon bag incubation in a 135L compost tumbler chamber and its weight after 14 days. The associated temperature curve is displayed in figure A10. Each row of a material displays one replicate.

	bag (g)	plastics (g)	compost wet(g)	compost dry (g)	total (g)	incubated (g)
compost +	0.774	0.847	10.896	3.712	5.333	3.796
black	0.761	1.006	9.654	3.289	5.056	3.781
agricultural	0.768	0.938	11.327	3.859	5.565	4.282
plastics	0.778	0.908	9.583	3.265	4.951	3.724
(35µm)	0.89	0.947	11.507	3.920	5.757	4.178
compost	0.895	1.068	11.153	3.800	5.763	5.072
+						
OK	0.741	1	10.788	3.675	5.416	4.355
compost	0.753	0.944	10.256	3.494	5.191	4.272
home	0.69	1.056	9.186	3.130	4.876	3.956
	0.915	0.928	13.275	4.523	6.366	4.929
compost	0.891	1.053	12.925	4.404	6.348	4.975
+						
OK	0.776	1.04	9.826	3.348	5.164	4.469
compost	0.729	1.062	11.255	3.835	5.626	4.462
industrial	0.778	1.061	10.498	3.577	5.416	4.874
	0.769	1.056	11.543	3.933	5.758	4.534
compost	0.758	0.951	14.112	4.808	6.517	4.939
+						
biodegrad.	0.801	1.033	10.602	3.612	5.446	4.655
knives	0.795	0.961	12.693	4.325	6.081	4.755
	0.718	1.091	10.136	3.453	5.262	3.969
	0.823	1.096	10.607	3.614	5.533	4.55
empty	0.834				0.834	0.834
bags	0.859				0.859	0.866
	0.841				0.841	0.836
	0.871				0.871	0.867
	0.825				0.825	0.82
compost	0.845		10.884	3.708	4.553	3.396
	0.836		12.385	4.220	5.056	3.478
	0.819		12.14	4.136	4.955	3.383
	0.817		13.008	4.432	5.249	3.764
	0.824		10.408	3.546	4.370	3.256

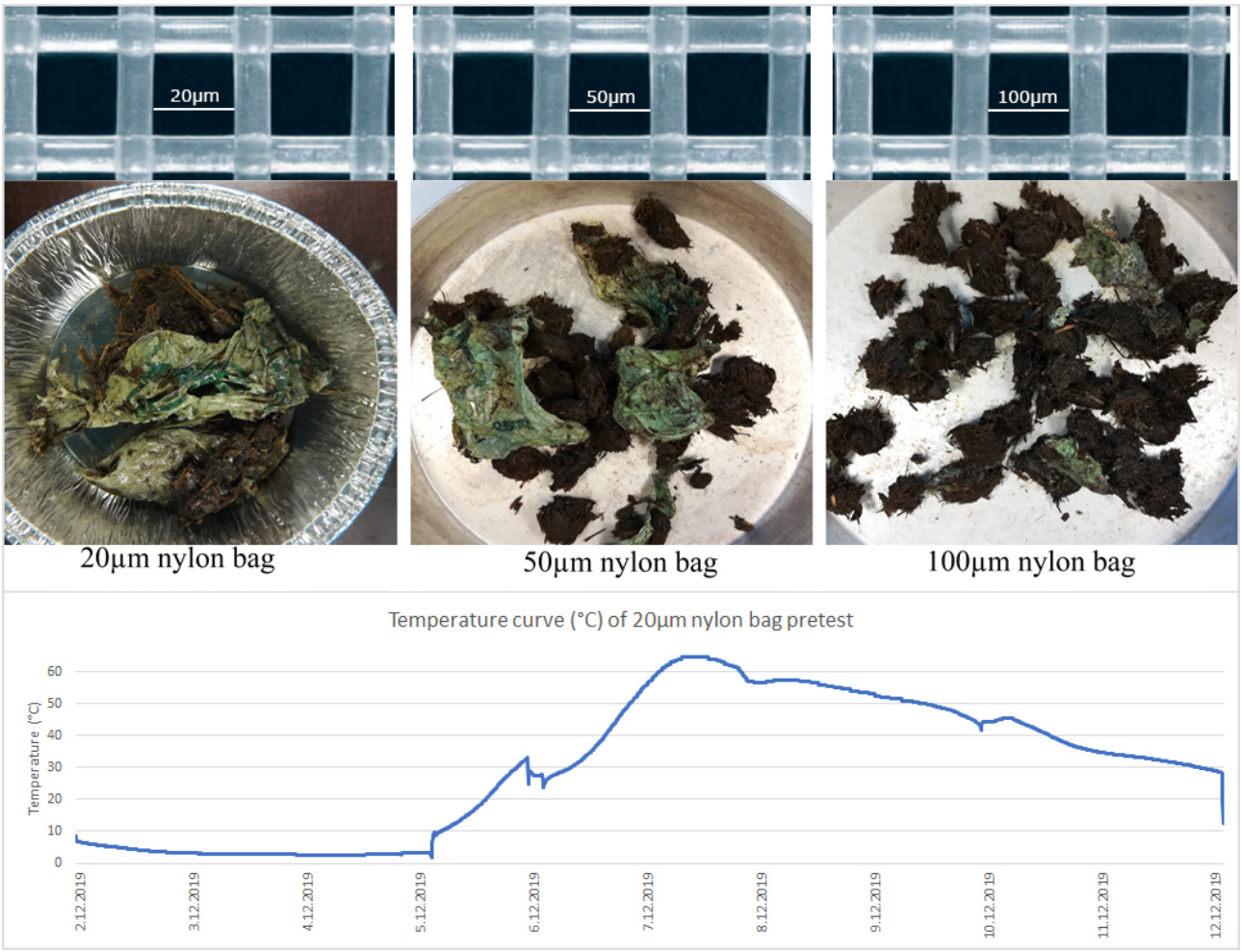


Figure A2: Content of the different bags after pre-test incubation, including the temperature development of the compost. Sudden jumps in temperature can be explained by opening the compost tumbler to turn it and renew oxygen supply. On the 5.12.2019 nitrogen in the form of urine has been added to help the microbial processes. The nylon mesh picture at the top is not to scale between different mesh sizes but serves to clarify mesh size differences.

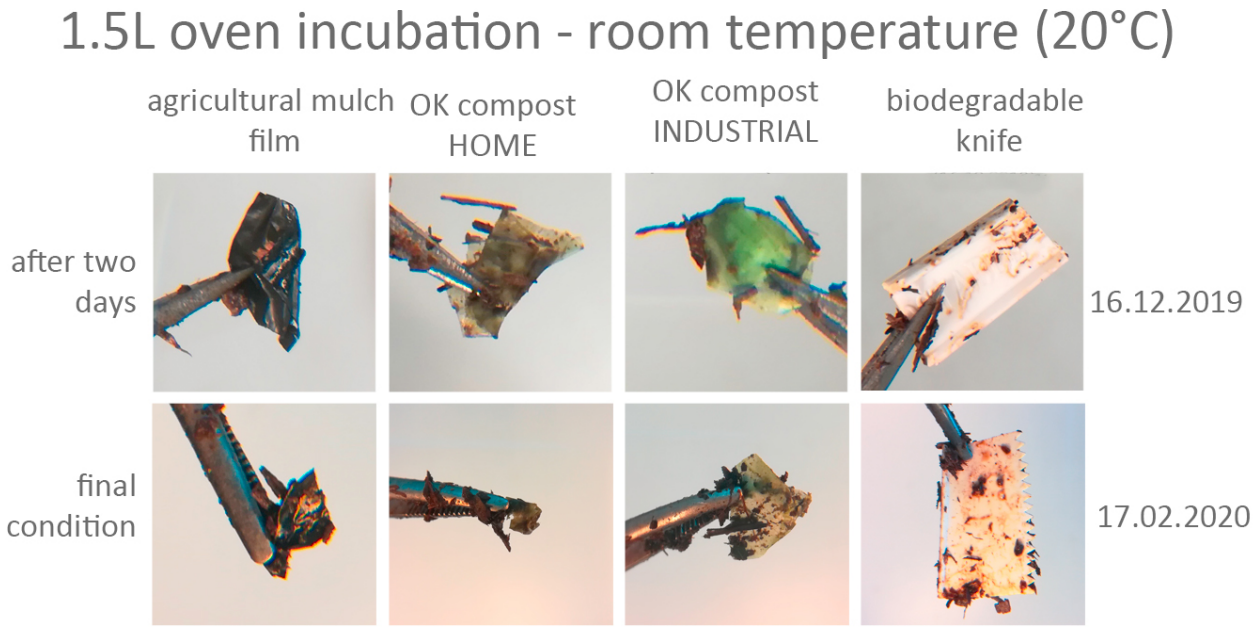


Figure A3: Visual examination of the 1.5L incubation at room temperature. Though the OK compost HOME sample started fragmenting after 116 days of incubation, the other plastics remained intact. It should be noticed that these samples had been examined oftentimes over the course of several months which added a certain physical stress.

Figure A4: Visual examination of the 1.5L incubation at 35°C. None of the biodegradable plastics have started fragmenting or degrading in the period of 20 days.

Figure A5: Visual examination of the 1.5L incubation at 45°C. OK compost HOME was fragmenting after 5 days and degrading after 19 days. OK compost INDUSTRIAL started fragmenting after 13 days. The agricultural mulch film and the biodegradable knife remained in original condition in the course of 20 days.

Figure A6: Visual examination of the 1.5L incubation at 55°C. None of the biodegradable plastics have started fragmenting or degrading in the period of 20 days.

Figure A7: Visual examination of the 1.5L incubation at 65°C. OK compost HOME started fragmenting after 2 days, followed by OK compost INDUSTRIAL and the agricultural mulch film after 4 days. The biodegradable knife did not fragment, but started to degrade after 12 days, together with OK compost home.

Table A6: Weight of materials used in the second nylon bag incubation in a 135L compost tumbler chamber and its weight after 14 days. The associated temperature curve is displayed in figure A10. Each row of a material displays one replicate.

	bag (g)	plastics (g)	total (g)	incubated (g)	weight loss (%)	Average	Standard deviation
black agricultural plastics (35µm)	0.796	0.981	1.777	1.724	2.983	2.910	0.357
	0.795	0.929	1.724	1.665	3.422		
	0.837	1.001	1.838	1.783	2.992		
	0.851	0.966		1.775	2.312		
	0.872	0.888	1.76	1.71	2.841		
OK compost home	0.825	1.097	1.922	1.85	3.746	5.184	1.366
	0.834	0.923	1.757	1.697	3.415		
	0.897	0.96	1.857	1.732	6.731		
	0.846	1.064		1.787	6.440		
	0.822	1.128	1.95	1.841	5.590		
OK compost industrial	0.829	1.101	1.93	1.903	1.399	1.050	0.551
	0.879	0.983	1.862	1.847	0.806		
	0.793	0.996	1.789	1.754	1.956		
	0.766	0.996		1.753	0.511		
	0.88	1.019	1.899	1.888	0.579		
biodeg. knives	0.813	1.046	1.859	1.857	0.108	0.263	0.200
	0.788	1.002	1.79	1.786	0.223		
	0.813	0.943	1.756	1.753	0.171		
	0.923	0.987		1.907	0.157		
	0.804	1.023	1.827	1.815	0.657		
empty bags	0.834			0.834	0.000	0.169	0.542
	0.859			0.866	-0.815		
	0.841			0.836	0.595		
	0.871			0.867	0.459		
	0.825			0.82	0.606		

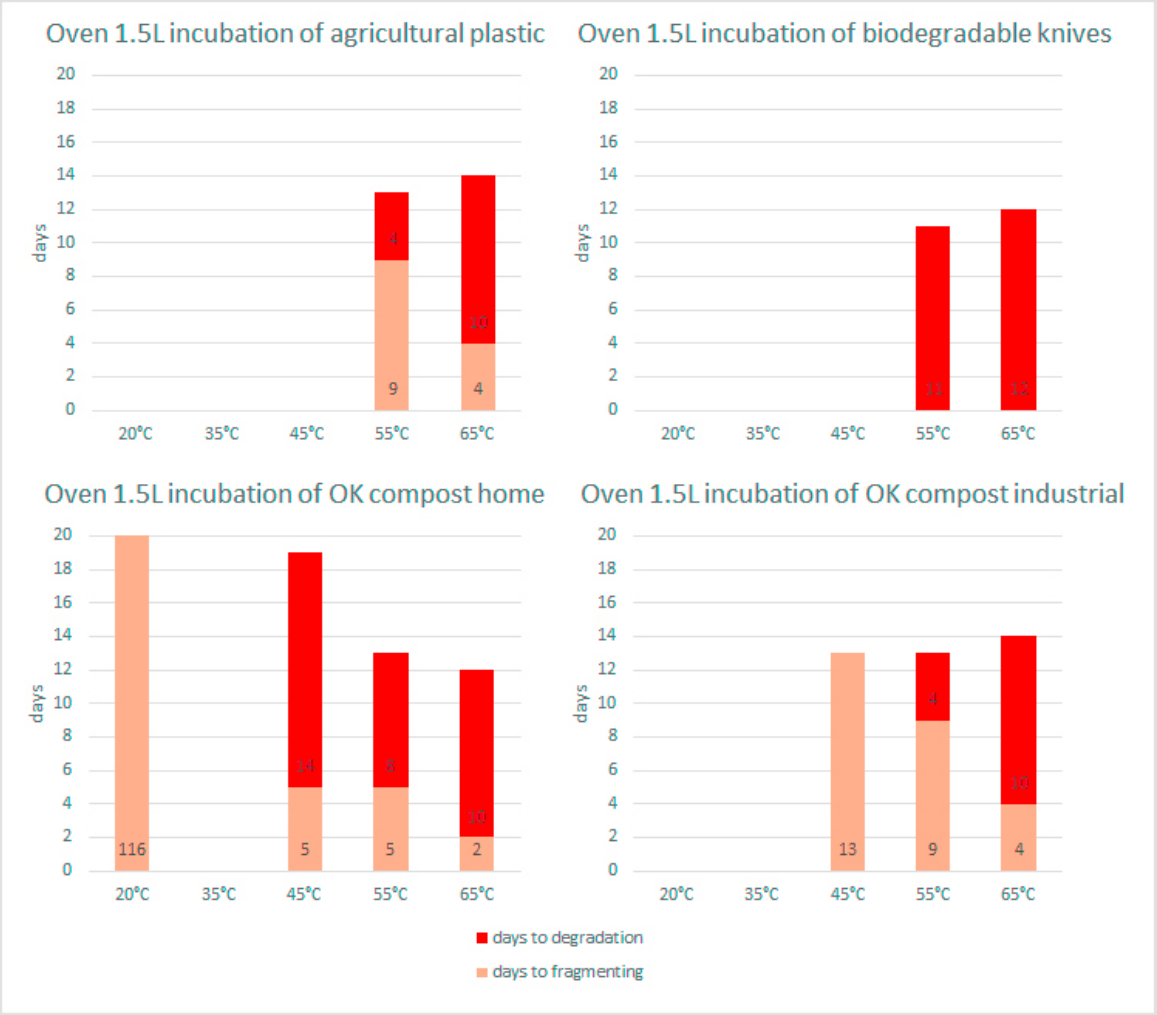


Figure A8: Days to fragmentation and degradation of biodegradable plastics in an oven at indicated temperatures and sorted by temperature. Zeros refer to the process not being observed rather than missing data. The numbers in each category indicate the number of days that passed until it reached the next state. For example, agricultural plastic at 55°C began to fragment after 9 days and has shown first signs of degradation 4 days later. Note that room temperature is on a different scale than other temperatures.

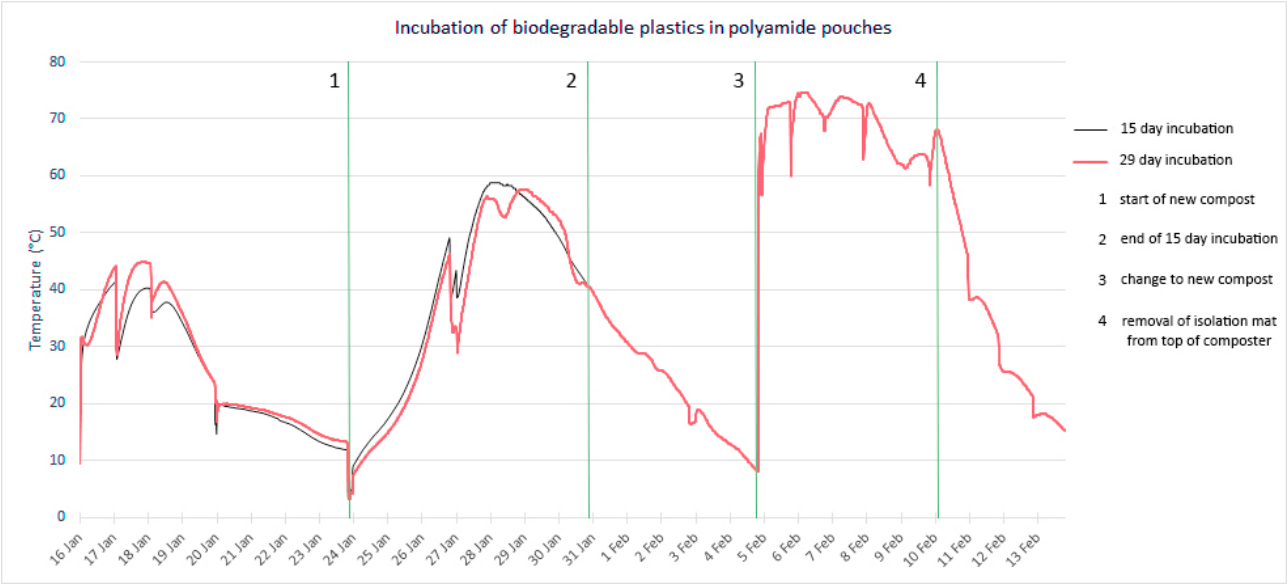


Figure A9: Temperature curve for the first incubation in nylon bags. Each timepoint (15/29 days) was incubated in a separate 135L chamber in the compost tumbler. Its temperature was recorded by two loggers whose average is displayed here. Sudden changes in temperature can be explained by the need to open the tumblers to take out the loggers and turn the compost tumblers to allow for aeration of the compost to prevent anoxic conditions which would alter microbial processes. On the fifth of February, nylon bags were transferred to a freshly started compost to expose the biodegradable plastics to a second thermic phase of the compost.

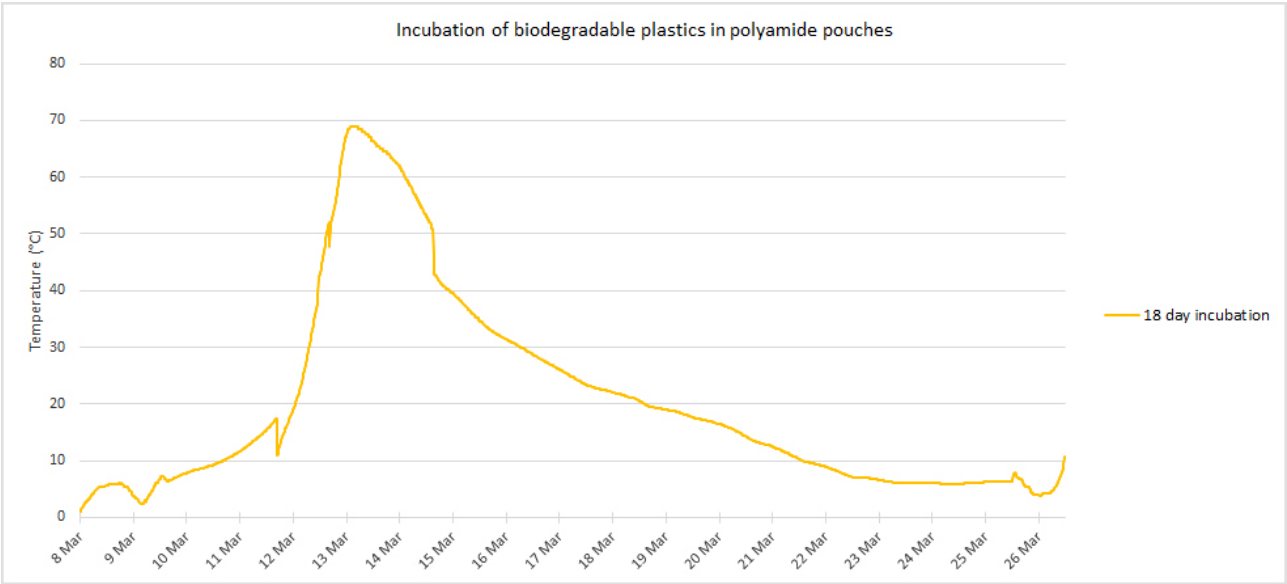


Figure A10: Temperature curve for the second incubation of biodegradable plastics in nylon bags. The 18 day long incubation was conducted in a separate 135L chamber in a compost tumbler. Its temperature has been recorded by one logger. Since the incubation for all three time points of the second experiment started simultaneously this chamber has been chosen for sample extraction because it retained a high temperature the longest.

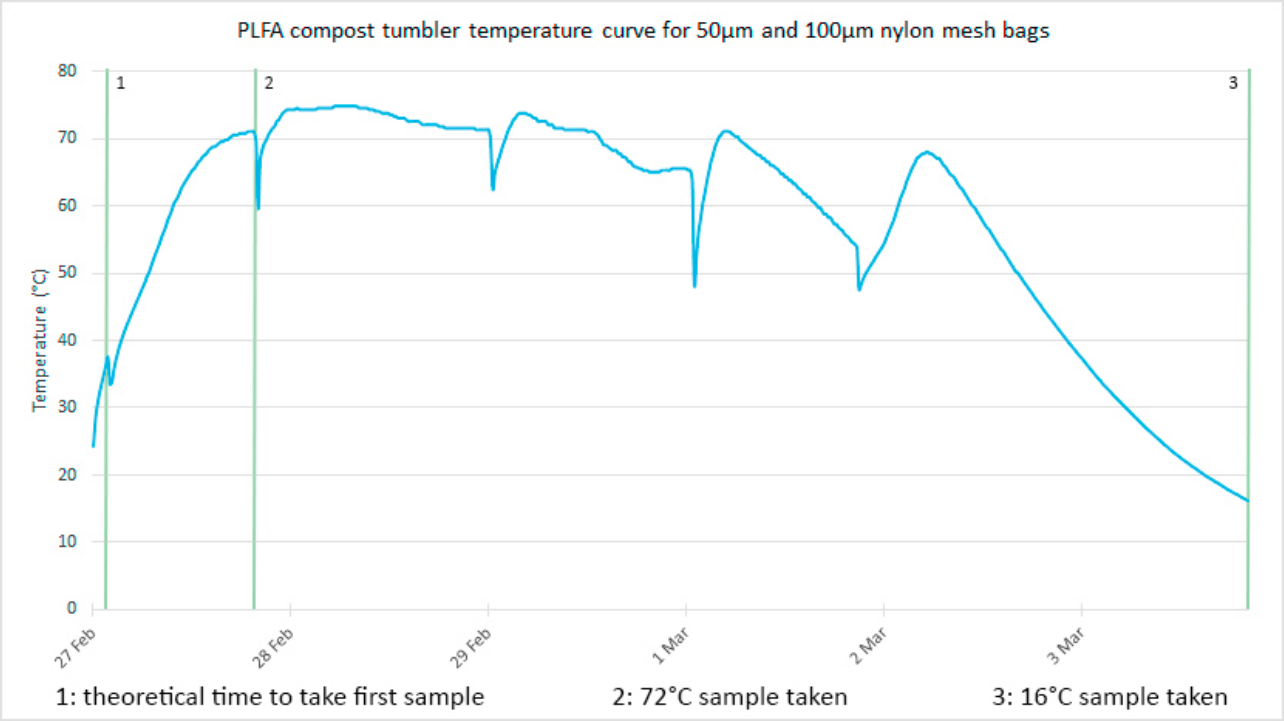


Figure A11: Temperature curve for second PLFA incubation and pH samples. Green lines indicate points in time where samples (theoretically) have been taken. Each of these are placed in one of the three phases of composting.

Table A7: Raw data for sample pre-treatment with Fenton's reagent. The material states whether values are in gram or milligram. Each row indicates one replicate. For compost only total weight indicates a replicate, while HM (horse manure) and CM (chicken manure) indicate its mixture. Biodegradable knives only has three replicates, because values from a pretest were found to be accurate enough while other materials were tested again. The dry weight was calculated from wet weight with formula 4 on page 17. The dry mass content was 87.7% (CM), 29.5% (HM) and 35.3% (compost).

	compost mix	initial dry weight	treated dry weight (g)	filter dry weight (g)	weight loss (%)
biodegradable knives (mg)		162.061	161.876		0.114
		374.296	373.892		0.108
		545.559	544.889		0.123
agricultural mulch film (mg)		1.567	1.516		3.255
		1.368	1.338		2.193
		1.612	1.57		2.605
		1.579	1.546		2.090
		1.463	1.424		2.666
OK compost home white (mg)		2.945	2.889		1.902
		1.566	1.535		1.980
		1.689	1.657		1.895
		1.711	1.689		1.286
		2.042	2.016		1.273
OK compost home green (mg)		7.456	7.064		5.258
		6.661	6.292		5.540
		7.475	7.073		5.378
		8.141	7.692		5.515
		7.778	7.375		5.181
OK compost industrial (mg)		8.123	7.916		2.548
		6.749	6.577		2.549
		8.398	8.128		3.215
		7.227	7.049		2.463
		7.517	7.323		2.581
Chicken manure (g)		1.829		1.044	-4.322
		1.805		1.043	11.426
		1.792		1.023	6.925
		1.795		1.02	14.695
		1.788		1.019	42.832
Horse manure (g)		1.245		1.023	15.291
		1.225		1.027	14.701
		1.194		1.047	13.266
		1.130		1.044	12.650
		1.237		1.051	10.846
Compost (g)	HM	1.085			
	CM	0.119			
	total	1.205	2.089	1.041	13.011
	HM	1.076			
	CM	0.117			
	total	1.192	2.117	1.052	10.683
	HM	1.075			
	CM	0.117			
	total	1.192	1.97	1.028	20.960
	HM	1.045			
	CM	0.121			
	total	1.166	2.083	1.024	9.164
	HM	1.063			
	CM	0.118			
	total	1.181	2.061	1.026	12.398

Table A8: Raw data for sample pre-treatment with hydrogen peroxide. The material states whether values are in gram or milligram. Each row indicates one replicate. For compost only total weight indicates a replicate, while HM (horse manure) and CM (chicken manure) indicate its mixture. The dry weight was calculated from wet weight with formula 4 on page 17. The dry mass content was 87.7% (CM), 29.5% (HM) and 35.3% (compost).

	compost mix	initial dry weight	treated dry weight (g)	filter dry weight (g)	weight loss (%)
biodegradable knives (mg)		663.311	663.103		0.031
		702.68	702.498		0.026
		603.501	603.353		0.025
		589.412	589.291		0.021
		587.823	587.72		0.018
agricultural mulch film (mg)		1.74	1.668		4.138
		1.337	1.284		3.964
		1.408	1.35		4.119
		1.275	1.227		3.765
		1.577	1.519		3.678
OK compost home white (mg)		1.959	1.899		3.063
		2.101	2.037		3.046
		2.269	2.206		2.777
		1.923	1.862		3.172
		2.014	1.954		2.979
OK compost home green (mg)		1.711	1.542		9.877
		1.435	1.291		10.035
		1.528	1.367		10.537
		1.899	1.708		10.058
		1.476	1.329		9.959
OK compost industrial (mg)		2.49	2.394		3.855
		3.54	3.411		3.644
		2.25	2.168		3.644
		2.326	2.232		4.041
		2.577	2.479		3.803
Chicken manure (g)		0.789	1.73	0.981	5.127
		0.789	1.694	0.972	8.547
		0.877	1.772	0.971	8.686
		0.877	1.781	0.984	9.142
		0.877	1.774	0.987	10.282
Horse manure (g)		0.678	1.554	1.01	19.725
		0.648	1.528	0.999	18.390
		0.619	1.537	0.99	11.594
		0.737	1.596	0.985	17.051
		0.707	1.506	0.977	25.191
Compost (g)	HM	1.064			
	CM	0.118			
	total	1.183	2.008	0.994	14.260
	HM	1.034			
	CM	0.118			
	total	1.151	2.041	0.99	8.699
	HM	1.052			
	CM	0.123			
	total	1.175	2.028	0.998	12.337
	HM	1.085			
	CM	0.117			
	total	1.202	1.919	0.981	21.971
	HM	1.050			
	CM	0.124			
	total	1.174	1.951	0.993	18.383

Analysis of phospholipid fatty acids (PLFA)

Since decomposition in a compost mainly occurs due to bacteria and fungi (Tuomela, 2002), analysing their communities can indicate the state of the compost in terms of biodegradation (Klamer and Bååth, 1998). It also allows for the comparison of the development of two compost microorganism communities. Here, they originated from the same mixture and then either developed in a compost tumbler (see chapter 3.6) or an incubation in a 1.5L glass tub in an oven (see chapter 3.5). A PLFA analysis makes use of the fact that different parts of a microbial community are made up of divergent fatty acid compositions (Klamer and Bååth, 1998). This method does not allow for the detection of single organisms or specific groups of these, but rather gives an indication of major changes in community composition of microbes and fungi (Klamer and Bååth, 1998).

The PLFA analysis consists of four steps (Norli, 2017):

1. Extraction of the phospholipid fatty acids from the sample: Lipids were extracted with a one-phase mixture. Then, 2 phases are created: a $CHCl_3$-phase containing lipids and one containing water, methanol and water soluble substances.

2. Fractionation of lipid classes with soild phase extraction: The lipids were seperated into different classes with increasing polarity - neutral lipids, glycolipids and polar lipids (phospholipids).

3. Conversion of phospholipid fatty acids methylesters with transesterification: The fatty acids bonded to phospholipids were separated and transferred to methyl esters, which could be analysed by gas chromatography-mass spectrometry (GC-MS).

4. Instrumental analysis by GC-MS.

To be able to compare the different treatments, the following experiment design was chosen. One batch of compost was used for all samples to ensure the same conditions at time T0. Then, samples were taken from:

- *Inside the compost tumbler*: The temperature of the compost was monitored with two data loggers per compost. Sampling points were during rising temperature (40°C), at its peak (68°C), during sinking temperature (30°C) and after it has completely cooled down to outside temperatures (-0.5°C).

- *Inside 1.5L glass boxes in the oven*: Here, the oven temperature was set to imitate the sampling temperatures of the compost tumbler. After one hour at that temperature, triplicate samples from the 3 boxes in the oven were taken.

To further strengthen results and account for the conditions inside of the nylon bags that were inside the compost tumbler as well, a second PLFA compost experiment was conducted. For this, a new T0 compost was made. While the experiment design was the same with the addition of triplicate samples of nylon bags with a 50µm and 100µm, sampling temperatures were 33°C (rising temperature), 72°C (peak) and 16°C (cooled down). One exception was that no nylon bag samples could be taken at 33°C,

due to the fact that the compost was heating up too quickly and thus not giving the nylon bags enough time to properly adapt.

Samples for each temperature for the first PLFA compost included: 1 sample from the compost tumbler and 3 samples from the oven.

Samples for each temperature for the second PLFA compost included: 1 sample from the compost tumbler, 3 nylon bag samples with a 50µm mesh, 3 nylon bag samples with a 100µm mesh and 3 samples from the oven.

As the PLFA analysis was done in batches of maximum 17 samples, the following steps were repeated 4 times to include all samples. The method is split into blocks (”days”) as one block occupies approximately one working day. According to Norli (2017) the PLFA analysis steps were:

Day 1 - Extraction:

1. Put 2g of a representative, wet sample in a 50mL teflon tube.
2. Add citrate buffer (0.15 M, pH 4.0) so that the soil water content plus buffer add up to 2.0mL - requires water content calculation for the sample (see chapter 3.1.1).
3. Prepare blank samples with 2.5mL $CHCl_3$, 5.0mL MeOH and 2.0mL citrate buffer.
4. Add 2.5mL $CHCl_3$ and 5.0mL MeOH to all other samples. Vortex these until the contents are homogenous. Leave them for at least 2h at room temperature while they extract.
5. Centrifuge them for 5 minutes at 2500 rpm and 4°C.
6. Transfer 6mL of the top layer that does not include particulate matter to a 50mL kimax tube with a pipette.
7. Add 3.2mL $CHCl_3$ and 3.2mL citrate buffer to the extracted part to split its phases. Vortex for 1 minute and let the phases separate over night in complete darkness.

Day 2 - Lipid fractionation by solid phase extraction:

1. Transfer 3.0mL of the bottom layer lipid extract into a small glass tube that fits a nitrogen (N_2) evaporator. Evaporate the sample under a stream of nitrogen to avoid oxidation.
2. Dissolve the dried sample in 100µl $CHCl_3$ and vortex for 30 seconds.
3. Activate the filter columns by eluting with 5mL $CHCl_3$. These are placed over a small, vacuum chamber and have small kimex tubes placed under them to catch liquids.
4. Apply the vortexed sample on one filter column each. Apply 100µl $CHCl_3$ two more times after which the sample is shortly vortexed and applied on the same filter column again. Each sample should receive $CHCl_3$ three times and be applied on the columns three times.
5. Add 1.5mL $CHCl_3$ to each filter column to elute the neutral lipids.

6. Exchange the small kimex tubes under the filter columns with clean ones and elute the phospholipids with 1.5mL MeOH.

7. Apply 100µl of the internal standard in the kimax tubes and 100µl of external standard into one extra tube. Total tubes now are: 17 samples, 3 blanks, 1 external standard.

8. Evaporate in the nitrogen evaporator at 40°C.

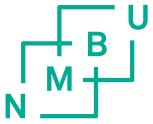
Day 3 - Methanolysis (Transesterification):

1. Add 200µl BCl_3-methanol reagent to the evaporated samples, seal the tubes and heat them at 60°C for 20 minutes.

2. Let them cool to room temperature, add 1.0mL Milli-Q® water and 1.0mL hexane, then vortex for 30 seconds.

3. Allow the layers to settle, transfer at least 200µl to a gas chromatography vial with an insert. The sample is ready for analysis.

Finally, gas chromatography–mass spectrometry analysis of the prepared samples was conducted by a third party in NIBIO Ås.



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