BIODEGRADATION OF POLYHYDROXYBUTYRATE PLASTIC:

A COMPARISON OF AMERICAN AND INTERNATIONAL STANDARD TEST METHODS

USING SOILS FROM WESTERN WASHINGTON

by

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ABSTRACT

Biodegradation of Polyhydroxybutyrate Plastic:
A Comparison of American and International Standard Test Methods Using
Soils from Western Washington

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Plastic is a ubiquitous pollution in the environment with more than 350 million tons of new material produced each year. While the majority of plastics are petroleum based polyolefin plastics, biodegradable plastics are becoming more common. However, there is no single standard for determining legally or chemically what constitutes a biodegradable plastic. Biodegradation is highly challenging to effectively simulate in a lab. Additionally, countries and even states have different requirements for test methods used to define biodegradability. This can cause confusion over whether or not a plastic qualifies as biodegradable in different countries or states. This work compared two standard test methods for examining plastic degradation in soil, ASTM D5988 and ISO 17556. ASTM D5988, Washington State's legal standard for degradable plastic, used acidic soils gathered from three locations in western Washington. ISO 17556 used soil artificially constructed according to the method. These tests compared degradation of polyhydroxybutyrate (PHB) granules. Notable, both the PHB plastic and the cellulose reference material degraded at different rates in each test. This work demonstrated the need for examining the differences in standard methods used to quantify plastic degradation and the pitfall of using ASTM D5988 with acidic soils local to Washington that fall outside the allowed pH range of the test.

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Literature Review

Introduction

It is a truth universally acknowledged that a plastic once produced will exist forever in want of a final resting place. The majority of plastics—moldable, artificial, organic polymers—that have been manufactured still exist in the same form as on production day (Canopoli et al., 2020; Shah et al., 2008). The most common conventional plastics in use, polyolefins, are composed of nonaromatic carbon and hydrogen atoms typically derived from a non-renewable resource, often crude oil (Ghanta et al., 2014; Fotopoulou et al., 2019). Polyolefins are highly resistant to microbial attack and are a durable material (Shah et al., 2008). Conventional polyolefins are a cheap, strong, and versatile material (Goel et al., 2021). These characteristics are why plastic use is so prevalent. Polyolefins are also problematic as they do not readily degrade over time. Consequently, there is a growing movement to replace conventional plastics with those that do degrade (Vasil, 2019). Plastics that readily degrade are termed biodegradable plastics. The issue lies in how biodegradability is defined. Not all biodegradable plastics meet the same definition. Biomes with diverse biota, precipitation, temperature, and other factors will interact differently with plastics, and a nominally biodegradable plastic may or may not break down (Shah et al., 2008). Additionally, biodegradability testing occurs primarily in situ under laboratory conditions, and even in the best of cases, a given plastic would not react identically to how it would degrade in the natural environment (Lucas et al., 2008). Different simulations approximate different environmental conditions. However, the same simulations may produce different results across plastic types or within specific environments. Real life degradation is highly challenging to effectively simulate in a lab.

Tests that simulate environmental degradation are useful estimates of plastic biodegradability and are most useful if compared against one another or run with consistency. Despite this, both testing and labeling for legal purposes vary considerably by locality (Nyholm, 1991; Filiciotto et al., 2021). Some countries and even some US states do not have any standard methodology for what constitutes biodegradable plastic (Canada Recycled Content, 2023). Among localities that do mandate certain tests, each locality has different requirements (Wash. Rev. Code, 2022). Companies are penalized in most places for lying about their products, but without a consistent test regimen, how can a manufacturer confirm whether their product meets the biodegradability requirements for a broad market? A potential solution to the problem of plastics falls flat because of a lack of universally acknowledged standards regarding how to measure biodegradability.

This review will explore the differences in conventional fossil-fuel derived plastics, bioplastics, and biodegradable plastics, focusing on how they are manufactured and how they decompose. To explain the difference in plastic types, first this review will look at conventional plastics. This includes plastics such as polyethylene, produced from petroleum. This review will examine the scale of plastic production and their broad usage in the modern world followed by typical methods of production and the processes by which conventional plastics degrade after disposal. The same examination will follow for biodegradable plastics and will include a discussion of nomenclature regarding how biodegradability is defined. The review will then address plastic labeling conventions and international laws regarding plastic decomposition with emphasis on how countries base their laws on standards from different sources. Finally, the types of methods for simulating degradation of plastic in natural environments will be explored.

Conventional Plastics

Scale of Plastics

Plastics are among the most widely manufactured and widely used materials produced in modern industry (Goel et al., 2021). Since the mid 20th century, plastics have been substituted for prior materials to the point where replacing them in modern life has become extremely difficult (Shah et al., 2008). In 2015 alone, global plastic production exceeded 350 million tons with an annual growth in production of 8.4% (Rajan et al., 2017). By 2018, this number had reached nearly 400 million tons of plastic worldwide (Goel et al., 2021). Plastic has since continued to skyrocket to record high levels (Cho et al., 2021). Polyolefins, nonaromatic (chemical structures that do not contain rings) purely hydrocarbon polymers, are the most common plastic (Fotopoulou et al., 2019). Polyolefins include polyethylene and polypropylene which together comprised nearly 50% of global plastic production in 2018 (Goel et al., 2021). There are many problems associated with the prolific use and production of plastics especially environmentally persistent polyolefins (Canopoli et al., 2020). However, denouncing plastic entirely would ignore the massive gains in quality of life and broad access to products that must be attributed, at least in part, to the use of cheap, reliable plastics (Pei et al., 2011; Rajan et al., 2017). Plastics of a variety of types and origins are used in everything from milk containers to house construction, electronics, and artificial joints (Shah et al., 2008). In packaging, plastics have replaced cellulose-based products including paper and cardboard because of the superior physical properties that plastic exhibits (Canopoli et al., 2020).
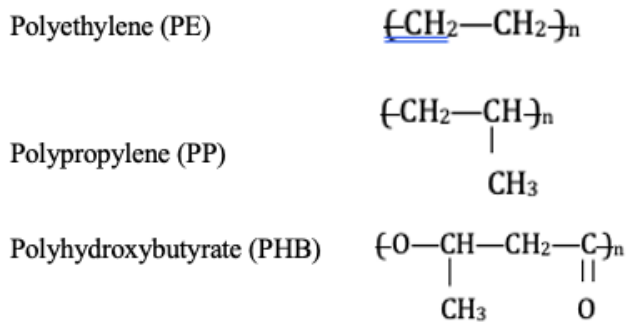
Uses and Production

The same characteristics that make plastic useful ensure that it lingers for many years after its usefulness has worn out. Polyolefin utility frequently stems from the mechanical

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properties of durability and chemical stability, but the result is a group of materials that are highly resistant to alteration by the natural environment (Fotopoulou et al., 2019; Shah et al., 2008). Neither polyethylene (PE) nor polypropylene (PP) degrade readily into biologically accessible materials (Fotopoulou et al., 2019). Studies have shown degradation rates of common polyolefins that would require decades to completely degrade the original material (Canopoli et al., 2020; Roy et al., 2014). Polyethylene, for example, shows only minimal degradation after ten years in a landfill (Canopoli et al., 2020). The processes that cause plastic polymers to degrade do so by compromising the structural integrity of the material (Hakkarainen et al., 2004). This process occurs regardless whether the plastic has reached the end of its utility, and degradation is therefore not ideal until after disposal of the plastic. Of course, plastic is not a single chemical, and each plastic interacts differently with its environment. Conventional plastics, which here refers to plastics that do not readily degrade, are an immense group that unfortunately comprise the vast majority of plastics currently being produced (Vasil, 2019).

As a category, plastics are synthetic organic polymers that can be molded or extruded into various shapes (Shah et al., 2008; Akhlaq et al., 2022). Hundreds of chemical configurations exist under the broad umbrella of plastic materials. Polyolefins PE and PP are very common plastics. Additional plastic formulations include polystyrene, polyurethane, polyethylene terephthalate, polyvinyl chloride, acrylics, and others (Goel et al., 2021). Each plastic has its own slight variations in physical and mechanical properties that make it suited for specific applications in industry and daily life (Shah et al., 2008). Each polymer likewise has a unique chemical structure (Figure 1). Many plastic polymers have a carbon backbone with unique functional groups producing their unique chemical properties.

Figure 1

Chemical structures of PE, PP, and PHB

Polyethylene (PE) $\leftarrow CH_2 - CH_2 \rightarrow_n$

Polypropylene (PP) $\leftarrow CH_2 - CH \rightarrow_n$
$\qquad\qquad\qquad\qquad\qquad | $
$\qquad\qquad\qquad\qquad\qquad CH_3$

Polyhydroxybutyrate (PHB) $\leftarrow O - CH - CH_2 - C \rightarrow_n$
$\qquad\qquad\qquad\qquad\qquad\quad | \qquad\qquad ||$
$\qquad\qquad\qquad\qquad\quad CH_3 \qquad\quad O$

Note. (Nagarkar et al., 2019; Shah et al., 2008) These are the monomer units of two conventional and one biodegradable plastic. PE and PP (conventional) have entirely carbon-based backbones, while PHB (biodegradable) has an oxygen heteroatom in the direct polymer chain in addition to a carbonyl functional group.

Both PE and PP have historically, and still commonly today, been produced from materials extracted from fossil fuels like coal, petroleum, and natural gas (Ghanta et al., 2014). Thus, not only do these common plastics not easily degrade, but they rely on nonrenewable resources (Akhlaq et al., 2022). They are, however, cheap to produce, and therefore easy to replace after a single use and disposal (Cho et al., 2021; Ghanta et al., 2014). Regarding their presence in the environment, disposal is where the true problem of plastic arises.

Degradation and Decomposition

As PE and PP are the polymers most common in landfills (Canopoli et al., 2020) their propensity to break down predicts how much plastic pollution will remain in the environment. Other conventional plastics such as polystyrene and polyvinyl chloride also end up in landfills and remain there virtually unchanged (Shah et al., 2008). The high molecular weight of long chain PE and PP polymers and the lack of non-carbon functional groups make these plastics

resistant to microbial attack (Cho et al., 2021; Fotopoulou et al., 2019). Before polyolefins can be degraded by biological mechanisms, they must first undergo a certain amount of abiotic degradation, the depolymerization of plastic by non-biological mechanisms (Lucas et al., 2008). This includes oxo-degradation, photo-degradation, and thermo-degradation (Fotopoulou et al., 2019). Purely mechanical shearing of plastic is not considered a type of degradation as this customarily leads only to microplastic pieces not true molecular decomposition (Akhlaq et al., 2022; Goel et al., 2021).

Chemical degradation—most commonly oxidative cleavage of the polymer chains—and photodegradation are the most common causes of non-biological degradation. Oxo-degradation involves the incorporation of oxygen atoms in the plastic polymer by breaking C-C bonds with different conventional plastics being more or less susceptible to C-C bond breakage (Fotopoulou et al., 2019). In a landfill study, Canopoli et al. (2020) used carbonyl index, an indication of amount of C=O bonds in the sample where more oxygen bonds equal a greater index value, to examine PE and PP using FTIR spectroscopy. The increased presence of oxygen bonds over time demonstrated that buried PP is slowly oxidatively cleaved. Fresh PP, PP buried for less than 10 yrs., and greater than 10 yrs. had respective indexes of 0.76, 1.34, and 1.78. After ten years PP had only a little more than twice the carbonyl bonds of fresh PP, suggestive of a very slow degradation mechanism. Other chemical decomposition can occur in specific cases. For example, metal catalysts aluminum and iron are known to decompose PE and PP during pyrolysis (Canopoli et al., 2020). This is a process precipitated artificially, not a process that occurs naturally in the environment.

Photo-degradation is a specific form of oxo-degradation that involves ultraviolet radiation from light and an oxygen molecule forming a hydroperoxide followed by free radicals which

leads to scission of a C-C bond (Canopoli et al., 2020). Fotopoulou et al. (2019). This causes

smaller PE molecules to form along with a new ketone, ester, or carboxylic acid. Exposing PE

and PP to sunlight is known to seriously weaken PP material despite PP's resistance to

photodegradation at moderate temperatures. During one experiment, PP began to fragment after

2 months of exposure to natural sunlight (Fotopoulou et al., 2019). Nevertheless, this amounts to

a very slow degradative process unlike comparatively rapid biodegradation. Photodegradation of

PE and PP is due entirely to photon absorbing impurities (Canopoli et al., 2020; Fotopoulou et

al., 2019). Consequently, the propensity of PE to photodegrade can be improved by a

deliberately including UV-absorbing groups along the polymer backbone (Hakkarainen et al.,

2004). Perfectly formed PE and PP will in theory not degrade under UV light. Degradation of

this type is cumulative because as more surface functional groups form due to C-C cleavage, the

material has more capacity to absorb light. For PE and PP these impurities are typically carbonyl

$C=O$ groups formed either spontaneously during polymerization or as a result of oxo-

degradation. Photodegradation occurring due to sunlight is the most effective natural abiotic

process that decomposes polymers (Lucas et al., 2008).

Both oxo-degradation and photo-oxidation can be catalyzed by increased temperatures

and thermo-degradation, i.e. bond scission due to heating (Canopoli et al., 2020; Fotopoulou et

al., 2019; Lucas et al., 2008). Primarily, abiotic decomposition is important for polyolefin

plastics like PE because extremely limited biotic decomposition occurs (Roy et al., 2014).

Thermo-degradation by itself can in some cases degrade polymers directly. Just as UV solar

radiation can break chemical bonds, heat can do the same in some conventional plastics

(Fotopoulou et al., 2019; Frostling et al., 1984). An experiment that heated PE to 60˚C in an air

oven for a period of time showed that after 120 days about 4.5% of the PE sample had been

oxidatively separated from the primary sample (Roy et al., 2014). This temperature is not high enough to cause melting in PE. While melting is an additional thermal process, it is not degradation in the sense of breaking polymeric bonds within a long-chain molecule (Lucas et al., 2008).

Conventional plastics proliferate in the environment and in landfills for centuries, but over long periods of time, chemical degradation will eventually cause them to break into small enough pieces that they become insignificant (Canopoli et al., 2020). Lengthy degradation time is the argument against conventional plastic. Break down over centuries does not constitute appreciable degradability on a human timescale (Wagland et al., 2009).

Biodegradable Plastic

Nomenclature

A major source of confusion in plastics has to do with attempts to greenwash plastic products or deceptively market them as less harmful to the environment. There is a widespread lack of understanding about the difference between a bioplastic and biobased plastic; and between degradable, biodegradable, and compostable plastics (Vasil, 2019). This leaves room for exploiting a concerned but poorly informed public (Goel et al., 2021). A bioplastic can be either biobased, biodegradable, or both. Bioplastic is a catch-all term and should not be interpreted as definitely indicative of production from renewable resources or decomposition after disposal (Song et al., 2009). A biobased plastic is not necessarily biodegradable, but the source material is at least partially derived from biomass such as soy or sugarcane (United States, Department of Ecology State of Washington). The overall percent mass contributed from renewable sources can be as little as 25% (Vasil, 2019). By this definition, polyethylene can be a bioplastic if a small percentage of the ethylene monomer is produced from dehydrogenation of plant-based ethanol,

typically produced from corn (Goel et al., 2021). Confusion over terminology has led to a ban in Washington State on marketing plastic consumables as biodegradable unless supported by concrete evidence (Vasil, 2019; Wash. Rev. Code, 2022). There is a codified standard for quantifying bio-based carbon content, but the language surrounding plastic marketing remains largely unregulated (Narayan, 2014). This allows companies and individuals to exploit nebulous language to mislead consumers who want sustainable options and do not understand the vagaries of bioplastics.

Uses and Production

The ultimate fate of a plastic, whether it degrades or not, is not indicative of the source material's origin. Biodegradable plastics can be produced from fossil fuels just as conventional plastics frequently are, or they may have a different source (Amann et al., 2012, Nagarkar et al., 2019). Currently, many biodegradable plastics are sourced from crude oil (Amann et al., 2012). If a biodegradable plastic is derived from biomass, the source material can vary from corn-based ethanol to synthesis from sustainable agricultural or dairy waste used as feedstock for microbial fermentation (Akhlaq et al., 2022, Pachekoski et al., 2013). Production from biomass includes bacteria and fungi as well as plants (Pei et al., 2011). Switching from traditional fossil-fuels to a renewable and CO_2-neutral production process would greatly reduce the carbon footprint of many plastics currently marketed as a greener alternative (Amann et al., 2021). Even before considering degradation, this low carbon-cost production from renewable biomass would be a significant step towards making plastics more sustainable.

Currently, the use of bioplastics is quite limited in comparison to conventional plastics. According to Vasil (2019), plant-based plastics constitute just 1% of the more than 360 million tons produced each year. While more than one hundred biodegradable formulations of plastic

were available in the late 2000s, only a few hundred thousand tons were produced because large scale production was prohibitively expensive (Pei et al., 2011). However, the market is rapidly expanding as consumers and regulators push industries towards more sustainable products (Vasil, 2019). With increased demand, there is growing need for understanding of how biodegradable plastic behave under different conditions after they are discarded.

Degradation and Decomposition

The primary characteristic that differentiates biodegradable from conventional plastic is the decomposition that occurs to the plastic after disposal. Many biodegradable plastics can degrade abiotically, but they degrade biotically rapidly enough that the slow process of abiotic degradation is typically insignificant (Akhlaq et al., 2022). Still, biodegradable plastics do experience degradation from abiotic factors, often more so than conventional plastic due to the presence of non-carbon functional groups that exist in the chemical structure of the plastic (see Figure 1). Abiotic degradation can and often does precede a biological component with a synergistic effect. By itself however, abiotic degradation is driven by non-biological factors.

Biotic degradation, commonly referred to as biodegradability, requires a major component of biological activity separate or in addition to abiotic factors (Goel et al., 2021; Lucas et al., 2008). While one definition of biotic degradation demands that microorganisms utilize a polymer as their primary substrate and grow directly on the material (Nyholm, 1991), it can also refer to microbes or other microorganisms breaking down polymers with extra- or intracellular enzymes. Microbial enzymatic breakdown is the main biotic avenue by which polymers are biodegraded (Akhlaq et al., 2022; Amann et al., 2012). Enzymes can fragment the polymers into pieces small enough to be biologically accessible. These pieces are then consumed and incorporated in biological material (Akhlaq et al., 2022). In order to be considered truly

biodegradable there must be a minimum level of mineralization, conversion of a plastic to CO_2, methane, and water (Goel et al., 2021; Roy et al., 2014). Otherwise, it is difficult to claim that the polymer has been assimilated and incorporated in the biomass of an organism which should be the final criteria for complete biodegradation.

Timing of degradation is crucial to understanding a material's true biodegradability. Relative time of decomposition must be considered as there is a significant difference between a material that is completely converted to CO_2 in a few weeks versus a few years (Wagland et al., 2009). While testing guidelines (Table 1) have been provided by the Organization for Economic Cooperation and Development (OECD), these are not recognized worldwide (Filiciotto et al., 2021). Nevertheless, the guidelines are useful as a means of categorizing more clearly and precisely how biodegradable a material is. A plastic that is inherently biodegradable in an environment is vastly more biodegradable than a plastic that is ultimately biodegradable (see Table 1.1), but without the qualifier, both plastics may be classified similarly because they are both considered biodegradable.

Table 1

Biodegradation Classifications by Extent and Timeframe of Conversion to CO_2

Biodegradable Extent	Minimum (%) Degradation	Maximum Test Duration	Analytical Method
Inherently Biodegradable	70	14 days	DOC or BOD analysis
Readily Biodegradable	60	28 days	CO_2 evolved or O_2 demand
Ultimately Biodegradable	90	6 months (marine); 24 months (soil, marine sediment)	CO_2 evolved or O_2 demand

Note. (Filiciotto et al., 2021) Analysis of degradation can use dissolved organic carbon (DOC), biological oxygen demand (BOD), capture of evolved CO_2, or O_2 demand compared to theoretical.

Plastic shape and form are also highly relevant to speed of decomposition. Plastics of lower molecular mass are typically easier for microbes to decompose due to easier access of plastic degrading intracellular enzymes (Ong et al., 2017). Plastic film will degrade at a different rate compared to pellets of the same material due to different surface areas (Fernandes et al., 2020). Structurally, surface morphology and crystallinity strongly influence the degradability of a specific plastic polymer (Ong et al., 2017).

Environmental Variability

Another common misconception behind biodegradation is that plastic should decompose in the environment. This references the environment as if it is a single location or biome. The reality is more complex. Even with an identical material, the biodegradation of a plastic will look different depending on the surrounding microbiome (Al-Khattaf et al., 2022; Cho et al., 2021). It also depends on humidity, oxygen availability, temperature, and a multitude of other factors (Lott et al., 2020). Oxygen availability in particular can be highly influential to the rate at which the plastic degrades (Ong et al., 2017). The final disposal location of a plastic, whether marine or terrestrial, will not have identical conditions to another location and will likely cause the plastic to behave differently than under laboratory conditions as well (Al-Salem et al., 2019; ASTM, 2018a). Qualifying biodegradability should also be environment specific. A plastic that is ultimately degradable in soil, may or may not be inherently biodegradable in seawater (Filiciotto et al., 2021). Even among biodegradable plastic, if conditions are unsuitable, degradation can take two years or longer (Akhlaq et al., 2022; Fernandes et al., 2020).

Approximating environmental conditions in lab settings is important as an environmental proxy. However experimentally, demonstrating that a material will degrade via biotic mechanisms outside lab conditions is crucial for an accurate determination of a polymer's biodegradable potential. Lott et al. (2020) designed an experiment in the Mediterranean Sea to bridge a gap in standard test methods. Previously tests had only existed for the sea surface and the shoreline such as ASTM D7991 which looks at plastic degradation in marine sediment (ASTM, 2022). Using a test frame to contain the samples, one plastic with demonstrated biodegradability and one conventional plastic, Lott placed samples in benthic (sea floor) and pelagic (open ocean) locations in the Mediterranean Sea and monitored disintegration as a proxy for biodegradation. The test showed a variety of results including the varying scale of degradation of the benthic versus pelagic locations and the difficulties of maintaining tests in the open environment where the apparatus could be colonized by animals and algae (Lott et al., 2020). The differing environments were crucial to the degree of plastic degradation, a fact that is frequently not taken into account when a plastic is described as biodegradable.

Compostable Plastics

Compostability and biodegradability are frequently conflated and can cause confusion for many consumers. These terms are not identical despite regularly being used interchangeably (Vasil, 2019). Compostability, while a useful subset of biotic degradation, is associated with a specific range of environmental conditions and complete mineralization—metabolic conversion of polymers to CO_2, methane, water, and trace elements if any (Roy et al., 2014). Composting uses a mixed microbial community under aerobic conditions to rapidly convert heterogenous organic material into soil (Song et al., 2009). It is human driven and controlled, not spontaneous in the environment (Goel et al., 2021). According to the Washington State Department of

Ecology, for a material to be compostable, in a controlled composting test no more than 10% of the original dry mass should remain after 84 days.

There is also a significant difference between industrially compostable and domestic or home compostable plastics which is largely, though not solely, temperature dependent. Industrially compostable products require a temperature of 55°C to break down, a temperature that home composting does not achieve (Vasil, 2019). In practice, unregulated domestic compost rarely reaches more than a few degrees more than ambient temperatures and typically does not exceed 30°C at any point (Song et al., 2009). In the United States compostable certification only refers to professionally managed composting facilities. Europe has a separate home composting certification (United States, Department of Ecology State of Washington). Not all biodegradable plastics meet the standards for compostability, and because compostability is artificial, the majority of plastics that are released or escape into the environment are not subjected to composting conditions. It is important for environmentally conscientious consumers to understand that unless they are disposing of compostable plastic materials in an industrial compost stream, the plastic will end life in a landfill and not degrade much more significantly than conventional plastics.

Polyhydroxybutyrate (PHB)

Because biodegradability testing is so variable, in order to compare tests, it is necessary to have a reliable, well quantified material to use across the methods. A common bioplastic promoted as a biodegradable alternative to PE is polyhydroxybutyrate (hereafter PHB). PHB is a polyester (Table 1) from a group of biodegradable plastics called polyhydroxyalkanoates (PHA) (Shah et al., 2008). PHB is the most studied plastic from the PHA family (Fernandes et al., 2020). Originally discovered in 1925, interest in PHB has grown in recent years due to its similar

or superior physical, mechanical, and chemical properties compared to PE and PP (Akhlaq et al., 2022; Pachekoski et al., 2013). Plastic bags, bottles, and disposable diapers can all be produced from PHB as well as from conventional plastic polymers (Shah et al., 2008). While PHB can be synthetically polymerized using metal catalysts or genetically modified plants, the most efficient and therefore preferred method is biosynthesis from sustainable biomass (agricultural, dairy, or forest waste) as feedstock for microbial fermentation by various bacterial strains (Akhlaq et al., 2022, Pachekoski et al., 2013). PHB is synthesized intracellularly and extracted via enzymatic digestion, although this digestion can require organic solvents such as acetone and chloroform which are not environmentally friendly (Akhlaq et al., 2022; Rajan et al., 2017). Bacterial fermentation, among other methods, can contribute to a reduced carbon footprint during initial PHB production (Goel et al., 2021). Even before considering degradation, this low carbon-cost production from biological processes means that PHB is potentially a more renewable alternative to the majority of conventionally produced plastics.

At the end of PHBs life cycle, this plastic undergoes both abiotic and biotic degradation. PHB experiences a heat catalyzed abiotic degradation through an E1cB elimination reaction where the removal of a hydrogen from the molecule causes the polymeric chain to break (Akhlaq et al., 2022). The presence of the ester groups also makes PHB vulnerable to hydrolysis, cleavage using a water molecule (Akhlaq et al., 2022). UV rays from sunlight are sufficient to cause PHB to fragment into pieces of low enough molecular weight for biological attack which accelerates the degradation process (Altaee et al., 2016; Lucas et al., 2008).

Because PHB is synthesized by bacteria, PHB depolymerase enzyme exists ubiquitously in the environment unlike enzymes required to break down many synthetic plastics (Akhlaq et al., 2022). Seawater, soil, and compost all cause degradation of PHB, and in a given

environment, microbes capable of degrading PHB are likely between 0.5-9.6% of total colonies (Rajan et al., 2017). Microbes capable of degrading PHB bacteria from genera *Aspergillus fumigatus, Variovorax paradoxus, Pseudomonas, Bacillus,* and *Xanthomonas* (Fernandes et al., 2020; Ong et al., 2017). However, some studies suggest that while many bacteria are capable of degrading PHB intra-cellularly, there are fewer bacterial strains that produce enzymes that degrade this plastic outside the cell walls. PHB must enter a bacteria cell before most PHB depolymerase enzymes can attack it (Cho et al., 2021). Fungi including *Penicillium, Paecilomyces, Acremonium, Verticillium,* and *Zygosporium* have also proven capable of PHA degradation. Relative to bacteria, fungi have more PHA degradation potential because their PHA-depolymerases are more mobile (Fernandes et al., 2020).

Of the environments where PHB is known to degrade, soil is usually considered the most important (Al-Khattaf et al., 2022; Altaee et al., 2016). For this reason, PHB is a convenient plastic to use in laboratory testing. As an environmental proxy, soil is easy to obtain. While terrestrial environments differ, the presence of soil is ubiquitous.

Plastic Labeling and Regulations

Language regarding biodegradability differs in large part because the requirements for labeling plastic are unclear. The regulations can be quite vague and often differ between countries, states, and even cities. This causes difficulties for consumers and for plastic producers. If labeling standards are unclear, the local regulations may not specify a test that should be used to certify biodegradability. Even more confusing, testing also differs country to country. Knowing if one test will meet multiple countries' standards could streamline a companies' production of a biodegradable product for international use. However, currently there is little information on how standard biodegradability tests compare to each other.

Labeling Requirements: The United States of America and Washington State

Plastic labeling is a complex and frequently misleading process. In places without strict requirements, a claim to compostability may be only a claim. The product may never biodegrade (Mojo, 2010). Alternatively, a plastic cup may be labeled compostable and genuinely be compostable. Consumers frequently interpret this as a less wasteful alternative to a standard plastic cup, and many people purchase compostable labeled cups for this purchase in an attempt to be more sustainable (Vasil, 2019). However, the reality is that in a landfill where the vast majority of such items end up, they do not decompose significantly more quickly than conventional plastic (Song et al., 2009). Furthermore, while the cups may be compostable, they are not home compostable. If placed in a kitchen compost pile, they would not break down. Industrial composting facilities are required, but most products do not make this readily apparent (Mojo, 2010). This is problematic for companies that want to minimize their waste stream and reduce their carbon footprint, and it is a challenge for concerned consumers. Even with targeted education, a study in Germany in 2001 found that only 82% of the studied population could identify a compostable logo (Song et al., 2009). Labels for biodegradability outside composting are usually even less clear and vary greatly by region and country.

In the United States, the Federal Trade Commission (FTC) releases Green Guides with recommendations for environmental marketing claims. These Guides are not binding, nor do they supersede federal or local laws, although the FTC can take action if a marketer makes a deceptive, misleading, or inaccurate environmental claim. Overstatement of environmental characteristics and misrepresentation of environmental benefits are prohibited. As a hypothetical example, a marketer claiming a 50% increase in sustainable material use in the product is deceptive if the material increased from 2% to 3% because the overall benefit is negligible.

Certifications and endorsements do not preclude a company from ensuring that their product meets the standards required by law. Regarding biodegradability, a marketer must have "reliable scientific evidence that the entire item will break down and return to nature within a reasonable short period of time after customary disposal" (Federal Trade Commission, 2012). However, the Green Guide has no discussion of what constitutes reliable scientific evidence or the time period that is acceptable. The Guide provides examples of unacceptable claims, but without specific definitions, it is unclear precisely how companies should meet these guidelines. Policies can shift how companies approach production potentially pushing them towards more sustainable alternatives (Filiciotto et al., 2021). Without clear instructions, some marketers may choose to forgo environmental labeling entirely to avoid possible litigation. This could cause companies to miss out on a consumer market for those who choose products that do report environmental benefits.

Localities may have more or less specific laws than the US federal government. The State of Washington bans the use of biodegradable marketing unless the product meets certain standards (Vasil, 2019). The Washington State Department of Ecology mandates decomposition percentages for compostable labeling and has definitions for bioplastics. Marketing products using terms like degradable is prohibited without meeting specific American Society for Testing and Materials specifications (Wash. Rev. Code, 2022). The Washington Statute references the Federal Trade Commission's Green Guide and provides additional highly specific language for how products must meet requirements of certifications. Ultimately, the state requirements for Washington are more restrictive than federal requirements. This is not true for many US states.

International Regulations: Canada and the European Union (EU)

Labeling and marketing regulations for biodegradable plastic differ outside the United States as well. Countries frequently lack consistent labeling for biodegradable materials or any legal requirement for testing. While the United Nations (UN) sustainable development goals include finding renewable resources for plastic production, no guidelines have been provided for classifying biodegradable material (Otarashvili, n.d.) The International Standards Organization (ISO) is supported by the United Nations Standards Coordinating Committee though many nations choose to develop and use their own standard tests (Filiciotto et al., 2021). Some countries that have a compostability standard also prohibit 'biodegradable' terminology because they do not have a biodegradable legal standard separate from compostable (Canada Recycled Content, 2023). For example, while Canada allows the use of multiple test methods in order to determine whether a product meets legal requirements, other countries like the USA require the use of a specific standardized method (Canada Recycled Content, 2023; Wash. Rev. Code, 2022). Standard tests are not certifications, but they may constitute evidence needed to obtain a certification depending on the country.

In Canada, the Canadian Environmental Protection Act Registry recognizes the difficulty that consumers face when confronted with words that imply plastic items will fragment, decompose, or otherwise be harmlessly incorporated in the environment. Regulated parties are not allowed to use terminology like biodegradable because of the potentially misleading nature. Compostable items must be accredited by a third party and shown to disintegrate at least 90% in a composting facility. The difficulty with this approach is that two of the proposed standards are from American Society for Testing and Materials (ASTM), which itself does not provide

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accreditation. Precise labeling of all products in now required with clear language about composting being industrial rather than home scale (Canada Recycled Content, 2023).

In the EU, as recently as 2020, it was a goal of the European Commission to "develop and harmonize standards that take into account material and product categories, as well as anaerobic digestion and the various environments that waste will end up in" as part of their circular plastic economy (European Commission, 2020). Historically, only an activated sludge simulation test was accepted as a test for ready biodegradability (Nyholm, 1991). The European Commission also notes specifically that for the purposes of regulating biodegradable plastic, the definition of biodegradation must specify the extent of biodegradation over a pre-defined timeframe. A claim for biodegradation must additionally stipulate the specific open environment in which biodegradation is being considered. One of the Commission's recommendations is for the adoption of a definition of biodegradability as a system rather than a unique property so that material properties and specific environmental conditions can be taken into account (Directorate-General, 2017). Oxo-degradable products that fragment into micro-plastics were banned by the EU in 2019, although this did not account for products that use oxo-degradable properties to enhance biodegradability (Filiciotto et al., 2021).

Different requirements are imposed for plastics based on aerobic composting and anaerobic digestion (Directorate- General, 2017). The standard typically referenced by the European Commission is EN 13432 which determines suitability of all packaging materials for use in commercial composting. Other standards include ASTM D6400 and the German DIN V 54900, both of which are considered less strict because they only require 60% (90% for copolymers) biodegradation in 6 months rather than 90% (Directorate- General, 2017). It must be noted that a standard is not a certification, much less a regulation. While the standard may

have less stringent requirements, if it is an accepted standard, then regulation may allow for a material that only meets this less strict requirement.

In summary, both Canada and the European Union have their own legal definitions concerning biodegradability, which are distinct from the definitions used in the United States. This means that there is little continuity for product labeling. Different tests are used to measure biodegradation, and passing one test does not equate to passing another.

Standard Organizations and Tests

Many regulations across countries reference standards that are produced by private companies (Canada Recycled Content, 2023; Directorate-General, 2017; Wash. Rev. Code, 2022). These standard tests are available online for a moderate fee and are updated regularly (Filiciotto et al., 2021). The most common standards organizations are ASTM International (American Society for Testing and Materials), the International Standard Organization (ISO), and the European Committee for Standardization (abbreviated CEN with standards names abbreviated EN). Each organization produces their own method for examining similar material characteristics, including specifically for biodegradable plastics (Table 1.2). Some countries also have internal standard test methods or organizations such as the German Institute for Standardization (DIN) and the Japanese Industrial Standards Committee (JISC) in Japan (Krzan et al., 2006; Shah et al., 2008).

Table 2

Standard Tests for Examining Plastic Biodegradability and Compostability

Organization and Test Number	Standard Title
ASTM D5988	Standard Test Method for Determining Aerobic Biodegradation of Plastic Materials in Soil
ASTM D7991-22	Determining Aerobic Biodegradation of Plastic Buried in Sandy Marine Sediment under Controlled Laboratory Conditions

ASTM D5526-18	Standard Test Method for Determining Anaerobic Biodegradation of Plastic under Accelerated Landfill Conditions
ASTM D6400	Standard Specification for Labeling of Plastics Designed to be Aerobically Composted in Municipal or Industrial Facilities
ISO 17556	Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolve
EN 13432	Packaging - Requirements for packaging recoverable through composting and biodegradation - Test scheme and evaluation criteria for the final acceptance of packaging

These tests, while useful as a consistent method for categorizing materials, each give rise to their own slightly different methods of biodegradability (Filiciotto et al., 2021). Some standards have pass/fail requirements, and some do not (Narayan, 2014). Even standards that do have requirements like ASTM D5988 and ASTM D7991 which demand 90% material assimilation in two years or less have long timelines and no method to differentiate plastics that take the full two years or only a few months. Having a universal understanding of inherent versus ultimate degradability (Table 1) would make the interpretation of these standards clearer. There has also been little direct comparison between standards from different organizations even though laws may reference multiple standard tests at the same time (Filiciotto et al., 2021; Wash. Rev. Code, 2022). This can add complication and financial difficulties for companies because they may be forced to conduct multiple tests to determine if their product meets requirements in multiple countries.

Methods for Simulating Biodegradation

For many reasons, it is not advisable to use the open environment to test the biodegradability of a material. There is the risk of escape, contamination, and confounding variables, but simulating an environment in a lab is also quite difficult (Filiciotto et al., 2021).

There are many types of environments, and while lab tests must be replicable, few environments are precisely the same all the time. Therefore, it must be emphasized that in situ testing is a proxy and claims of performance are necessarily limited and must be qualified (ASTM, 2018a). It is possible to simulate a variety of environments and measure degradation using a variety of techniques (ASTM, 2018a; ASTM, 2018b).

Table 3

Degradation Methods by Material and Assay

Degradation Method	Environmental proxy material	Assay type
ASTM D5988	Surface soil from three disparate locations	CO_2 Evolution absorbed by KOH
ASTM D7991-22	Marine beach sediment from a tidal zone	CO_2 Evolution absorbed by KOH
ASTM D5526-18	Pretreated municipal-solid-waste and anaerobic inoculum	CO_2 and CH_4 Evolution measured by gas chromatograph
ISO 17556	Surface soil or constructed standard soil from sand, compost, and bentonite clay	CO_2 evolution absorbed by KOH or biological oxygen demand

Standardized experiments in the field beyond controlled laboratory settings have been limited, despite their importance for verification of biodegradability in the open environment (Lott et al., 2020). In the lab, CO_2 production can be tracked to estimate the plastic material consumed and respired by microbes, biological oxygen demand (BOD) can be measured, and weight loss of the plastic before and after the experiment can be calculated (ISO 17556, 2019). These measurements are extremely difficult to obtain outside the lab, leading to challenges with verifying lab results in open environment tests.

Soil

Two commonly used methods from examining plastic degradation in the environment are ASTM D5988 *Standard Test Method for Determining Aerobic Biodegradation of Plastic*

Materials in Soil and ISO 17556 *Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved.* The use of the methods can be quite similar, or they can differ considerably in setup. In the case of both of these tests, a plastic sample can be placed in a highly characterized soil obtained from the local environment. The ASTM test requires sealed containers with limited available oxygen that must be opened at least every week (ASTM, 2018a). ISO 17556 can use this setup, or a CO_2 free gas-line setup can be used to prevent oxygen from being a limiting reagent. For both tests the CO_2 evolved from the soil and the plastic is captured using as CO_2 absorbing solution such as potassium hydroxide. The evolved CO_2 is compared to the CO_2 produced from soil blanks that contain no plastic. This is used to determine the amount of plastic that had been mineralized by the microbes in the soils while accounting for background soil respiration. Both of these tests allow for soil amendments such as nitrogen to prevent limiting reagents. This is useful to limit test duration, but this does not represent a natural environment where nitrogen may be the limiting reagent. Likewise, these tests have a specified pH range that the soil must meet. Natural environments may have a soil pH outside this range, so altering the pH for the test does not represent the true outcome for the plastic in the environment where the soil was collected (ASTM, 2018a; ISO 17556, 2019). Understanding the difference in these methods could allow companies to conduct a single test that is acceptable to multiple regulatory bodies, but there has been little direct comparison between these methods.

Other Environments

Plastic can end up in any environment not only in soil. Other methods beyond ASTM 5988 and ISO 17556 are necessary to simulate environments such as marine locations and landfill. While ISO have not produced a test for marine conditions, ASTM

D7991-22 *Determining Aerobic Biodegradation of Plastic Buried in Sandy Marine Sediment under Controlled Laboratory Conditions* is a proxy method for plastic degradation on tidal beaches where plastic waste often accumulates. The method is similar to ASTM 5988 in that the plastic is placed in marine sediment in a sealed container and evolved CO_2 is captured. ASTM also has a standard method for approximating degradation in a landfill, a manmade environment but one where a huge quantity of plastic collects after disposal. ASTM D5526-18 *Standard Test Method for Determining Anaerobic Biodegradation of Plastic under Accelerated Landfill Conditions* can be used as a proxy to examine how plastic materials decompose without the necessity of retrieving old plastics from landfills (ASTM, 2018b).

Research Gap

Many studies have used methods certified by ASTM and ISO, but few if any studies have directly compared them. Many countries have requirements for testing plastics before labeling them biodegradable (Krzan et al., 2006). These requirements, however, are not consistent between countries and the direct comparison of standards has largely not been examined (Shah et al., 2008, Lott et al., 2020, Fernandes et al., 2021). The similarity of the tests used by different governments and businesses to determine biodegradability is unknown. Are these standards consistent enough with each other to similarly separate inherently biodegradable plastics from ultimately biodegradable plastics (Al-Salem et al., 2019)? If the tests are inconsistent, the classification 'biodegradable' may not be comparable country to country. This causes economic disincentives for companies that may have demonstrated a material is biodegradable by the regulations of one countries, only for them to have to demonstrate the same thing using a different test in order to market the product elsewhere. In order to streamline the marketing of biodegradable plastic, and therefore to increase available biodegradable plastics available to

consumers, the similarity or differences between standard methods for determining

biodegradability must be understood.

Conclusion

       Lack of consistency in testing requirements and labeling is a source of confusion for consumers and a source of legal and economic complications for industry. With little established comparison data for how similar testing standards are for biodegradable plastics, there is little basis for consistent comparison of new or old biodegradable plastic materials (Shah et al., 2008). If the results of two tests were known to be highly similar, a company wishing to market a product as biodegradable in two locations that do not use the same legal metric, would only have to run one of the test and cut down on their overhead. Unfortunately, screening results for this type of test have historically resulted in highly variable outcomes (Nyholm, 1991). A consistent set of standard tests would speed the advancement of a product to market, and more biodegradable plastics could be made available.

       From a consumer perspective, if a plastic degrades more under certain test conditions, it is important to know which test more closely mimics the natural environment to understand if the plastic actually naturally degrades, or if it has been greenwashed. If many products are misleading, even consumers with good intentions in purchasing biodegradable plastic will lose confidence and perhaps stop attempting to mitigate their plastic consumption with plastics that actual degrade in the natural environment rather than conventional plastic. Ultimately, this returns to the broad problem of plastic waste. Large quantities of plastic waste linger for long periods of time, and right now, there are few consistent methods for exploring alternatives that do not have the same long term consequences.

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Introduction

Plastics are among the most abundantly manufactured materials in the 21st century with production in 2015 exceeding 350 million tons (Rajan et al., 2017). As a category they are synthetic, organic polymers (Shah et al., 2008) that are often highly resistant to chemical and physical alteration by the natural environment (Fotopoulou et al., 2019). Likewise few plastics readily break down in landfills leading to persistent plastic pollution in many ecosystems (Canopoli et al., 2020; Goel et al., 2021). With rising concerns about plastic pollution, common polyolefins—plastics such as polyethylene (PE) and polypropylene (PP) comprised of only nonaromatic carbon and hydrogen atoms—are becoming less desirable, and alternative bioplastics are slowly increasing in popularity despite frequently having a higher production cost (Pei et al., 2011; Vasil, 2019). One category of plastics that are widely acknowledged as more biodegradable than conventional polyolefins are polyhydroxyalkanoates plastics, or PHAs (Fernandes et al., 2020). Polyhydroxyalkanoates are derived from microbial fermentation rather than fossil fuels and have similar mechanical properties to PE and PP making them a suitable substitute (Akhlaq et al., 2022) However, PHA end-of-life stage involves far more rapid chemical decomposition than polyolefin plastics (Rajan et al., 2017).

Despite the high prevalence of plastic use and the general understanding that some plastics degrade faster than others, there is no legal consensus for how to determine which plastics do not persist in the environment. States, municipalities, and countries use different standard methods and language for describing what constitutes biodegradability (Filiciotto et al., 2021). Plastic degradation in the open environment is difficult to quantify, so laboratory tests to determine speed and extent of degradation are crucial to accurately identifying readily biodegradable plastic (Lott et al., 2020). Due to the inconsistencies in which method is required, tests are not consistent

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between countries. Additionally, because there has been little direct comparative testing, it is difficult to determine if a material that is considered biodegradable in one country is or would be considered biodegradable in another.

It is unclear whether the standard tests used by different governments and businesses to determine biodegradability for consumer labeling are consistent enough to separate plastics that readily degrade from those that do not (Al-Salem et al., 2019). Additionally, when the tests are inconsistent between localities, the classification "biodegradable" may not be comparable (Filiciotto et al., 2021). A few private organizations produce standard methods for testing, and some countries such as Germany and Japan also produce their own protocols (Filiciotto et al., 2021; Krzan et al., 2006). In the United States, the American Society for Testing and Materials (ASTM) produces standard methods to examine plastic degradation under a variety of conditions. Another frequently referenced organization is the International Standards Organization (ISO). Many studies have examined biodegradable plastics using ASTM or ISO methods, but few studies if any have directly compared them (Al-Salem et al., 2019; Fernandes et al., 2020; Shah et al., 2008). Without consistent testing guidelines, plastic regulators, producers, and consumers cannot determine if a plastic that is labeled as biodegradable will actually degrade in the environment or not.

This study compares standard method ASTM 5988-18 and ISO 17556 for examining aerobic biodegradation of plastic in soil. The soil utilized for ASTM 5988-18 was collected from in Washington State and therefore the test is a proxy for whether the selected plastic with degrade in certain Washington environments. Polyhydroxybutyrate (PHB), a well-studied biodegradable PHA, is used in both tests to determine if the results of degradation (or lack thereof) are replicable across different environments with potentially highly variable microbial ecosystems.

Methods and Materials

For both methods ASTM D5988 and ISO 17556, the introduced plastic was polyhydroxybutyrate biopolymer granules from Goodfellow. The granules were ≤ 5 mm particle size or less with a molecular weight of 500 kg/mol. The plastic to soil ratio in each reaction chamber was normalized in both tests to 1 g of plastic per 200 g of soil. Theoretical grams of CO_2 from the PHB were calculated using its chemical formula and was 2.046 g CO_2 per gram of PHB. A cellulose filter paper was used as a positive control with a theoretical CO_2 evolution of 1.628 g CO_2 per gram of filter paper.

ASTM D5988

Natural soil was collected according to ASTM D5988 procedures from three locations in Thurston and Grays Harbor counties in western Washington (see Table 4). The top soil was dug to a maximum depth of 15 cm. The soil from each location was kept in individual buckets and stored a 4˚C refrigerator. The buckets were not completely sealed in order to both retain moisture and prevent an anaerobic environment. The three soils were sieved to < 2 mm, combined in equal parts by weight in a clean bucket, and mixed by hand with a trowel and then shaken to homogenize the soil mixture as much as possible. The mixed soil was then stored at 4˚C until use less than 30 days later. The mixture was sampled as necessary for characterization tests.

Table 4

Locations for ASTM soil sampling

Soil ID and GPS Location	MM (46.9510365, -123.8016560)	KS (46.8073625, -123.0638034)	CP (47.0294432, -122.9041316)
Collection Date	11/22/2023	11/26/2023	11/26/2023
Biome	Old logging track: Red alder, sword fern, wood sorrel,	Agricultural field: pasture grasses, *Trifollum spp.*,	Forest: English ivy, big leaf maple, sword fern, moss.

|	*Kinbergia spp.* (moss)	*Geranium spp.*, *Asteraceae spp.*	
USGS soil data	Ilwaco silt loam and Mopang silt loam	Spanaway gravelly sandy loam	Dystric Xerochrepts
Date of use	12/17/2023	12/17/2023	12/17/2023

The soil moisture and soil ash content were determined in accordance with ASTM D 2974-8 (1993). Initial percent moisture was 29.64%. At the conclusion of the test the average percent moisture of all soil samples was 29.96% with $\sigma = 0.02$. Moisture content of the soil was maintained between 80 to 100% throughout the test using deionized water. Initial ash content was 13.5% with $s = 0.195$, and final ash content was an average of 14.0% with $s = 2.13$ which suggested more variability in organic material after the test incubation. The moisture holding capacity (MHC), determined according to ASTM D425 (2017), was 21.98%. Due to a lack of centrifuge crucibles, the centrifuge moisture test was performed in perforated falcon tubes layered in a second larger tube. The soil carbon to nitrogen ratio determination used a CHN analyzer equivalent to a Kjeldahl digestion (Gautam et al., 2023) to demonstrate that the initial C:N ratio was about 14:1 which is within acceptable test limits. Nitrogen was not amended. The soil pH after air drying initially measured 5.68 ± 0.05 according the calibration specifications of the Orion pH meter and Ross pH probe. This pH was outside the 6 to 8 pH range used for ASTM D5988. The method explained that a low pH may contain an atypical microbial community. However, as acidic soils were representative of soils in Washington State (National, 2024), this soil was deemed acceptable as a proxy for degradation of this plastic in the local environment. In order to avoid potential microbial perturbation a naturally acidic soil, the pH was not adjusted. Final average pH of the soil at the test conclusion was 5.34 ± 0.05.

The experimental set-up used nineteen, gallon sized glass jars as reactor vessels for this test (see Figure 2). Ten sample jars had plastic and soil. Three blank (control) jars had soil only.

Three reference jars had soil and finally cut cellulose filter paper for a positive control. The remaining three technical control jars were empty to account for possible leakage and atmospheric CO_2 within the jar. The weights of the vessels, their lids, and soil and sample contents were routinely recorded to track moisture loss. Water was added on days 24 and 65 to return all jars to their original weight. All jars with soil received approximately 200 g of refrigerated soil mixture. All jars with sample (plastic or filter paper) received approximately 1000 mg of sample. Each vessel contained a 100 mL beaker with 50 mL of deionized water to act as a humidifier, and all jars had a 150 mL beaker with 20 mL of precisely standardized potassium hydroxide (KOH) of approximately 0.5 M to captured evolved CO_2.

Figure 2

ASTM Mason Jar Set
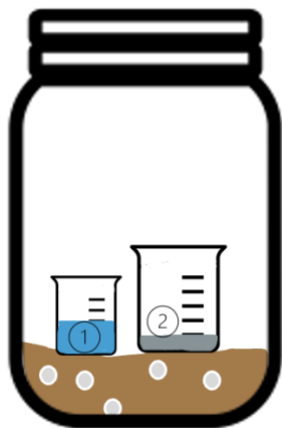
Note. Up 1 Water to act as an internal humidifier. 2 Potassium hydroxide (20 mL) titrated and replaced weekly. The material to be degraded (plastic or cellulose reference shown as gray circles) was mixed into the soil.

ASTM D5988 calls for a perforated plate to keep to beakers above the soil, but this equipment was not available. One water and one KOH containing beaker were each placed on

the surface of the soil gently to avoid compaction. The jars were sealed tightly and placed in a dark environmental chamber at 25˚C ± 1˚C for 72 days. This was the longest time possible due to outside constraints. At appropriate intervals between 3 and 7 days, the vessels were removed from the incubator, the KOH solution removed for titration, and the vessel allowed to exchange air with the atmosphere for between 15 and 30 minutes. A new KOH solution of known concentration in a clean beaker was placed gently into the vessel following which the vessel was resealed and returned to the incubator.

ISO 17556

Testing procedures consistent with standard method ISO 17556 were followed to determine the ultimate aerobic biodegradability of plastic materials in a standard (constructed) soil by measuring the amount of carbon dioxide evolved.

Using the ISO recipe, a standard soil was constructed using 70 mesh industrial quartz sand from Teton Supply Co., bentonite clay from Molivera Organics, a mix of KS and MM natural soil (see Table 2), and mature compost from wood debris and vegetable and food scraps as microbial inoculum. The compost was sieved to < 2 mm particle seize prior to addition. Based on CHN analysis, the carbon to nitrogen ratio prior to chemical amendment was about 17:1 although the overall abundance of both carbon and nitrogen was about 3 to 5 times lower in the standard soil compared to the ASTM soil. The standard soil was then amended per ISO protocol with potassium dihydrogen phosphate, magnesium sulfate, sodium nitrate, urea, and ammonium chloride by weight ratio. Total water holding capacity was measured using the same adapted centrifuge technique as ASTM D 5988, and enough water was added to bring the moisture content to 50% of the soil's total water holding capacity. Organic matter and pH of the standard soil were tested. The soil pH was 8.61±0.05 which was outside the specified range ISO 17556.

Basic soils have the potential to retain more evolved CO_2. However, while pH adjustment was allowed by this method, no technique for adjustment was provided. For the same reason as with ASTM D5988, no pH adjustment was attempted. Soil was stored in a mostly closed bucket in a refrigerator at $4\pm1°C$ until ready for use.

Sixteen gas flow set-ups were prepared using an aquarium pump, Erlenmeyer flasks, flexible PVC tubing, glass rods, and rubber stoppers as shown in Figure 3. The pump pushed air through five stoppered flasks at a rate of a few milliliters per minute (1). Because no gas regulator was available, some variation in gas flow could not be prevented. Two 500 mL Erlenmeyer flasks (2) were filled with approximately 450 mL of 10M KOH to scrub atmospheric carbon dioxide. The third 500 mL flask in sequence (3) contained the soil and sample material. Three blanks contained only soil as a control. Three positive reference flasks contained soil and finely cut cellulose filter paper. The remaining ten flasks contained soil and the plastic sample. All sample flasks were filled 200 g of soil, and the test and reference flasks received 1000 mg of the appropriate test material that was then mixed thoroughly with the soil. The soil in all flasks was gently compressed. The last two flasks (150 mL Erlenmeyer) in sequence (4) contained 30 mL each of 0.5 M KOH to collect evolved CO_2.

Figure 3

Gas-line set up for ISO 17556

Note. Pump (1) pushes atmospheric air through two 500mL flasks of potassium hydroxide (2) to remove CO_2. The CO_2 free gas then enters the reaction flask with soil (3) and plastic or reference material. Evolved CO_2 is captured in ~0.5 M potassium hydroxide (4) and remaining gases are vented.

The input gas flow tubing extended into the solution or soil to ensure that CO_2 was correctly removed from the air, the soil conditions were aerobic, and the evolved CO_2 was captured. The complete gas line and flask set ups were placed in a dark incubator at $25 \pm 1 °C$ for 72 days.

The flasks containing soil were periodically weighed when moisture content changed soil appearance for ISO (approximately every three weeks) though this proved difficult to see. Vessels that lost more than 5 g of weigh had deionized water added to bring the mass back up to the original starting mass. Deionized water was added on days 19, 39, and 58 to return the jars to

their original weight. Two ISO samples did dry out completely at one point during the test and were excluded from the data due to depleted KOH volume and likely microbial perturbation. At appropriate intervals, the first KOH containing flask in sequence was removed from the incubator for titration. The second KOH vessel was moved forward in sequence, and a new flask of KOH solution was added in the fifth position.

Titration and Carbon Dioxide Evolution Determination

At intervals of three then every seven days as indicated by amount of KOH consumed, evolved CO_2 was determined by manual titration of KOH solutions. Using approximately 0.3 M hydrochloric acid (HCl), 5.00 mL of KOH from each sample was titrated to a phenolphthalein end point. The HCl concentration was determined each day using KOH titrated from fresh potassium hydrogen phthalate solution and verified using freshly made sodium carbonate solution. The percent difference between these calibrating titrations did not exceed 2.91%. During each sampling, one blank, one positive reference, one technical control, and one sample were randomly selected to be titrated in triplicate for quality assurance.

Assuming that oxygen is not a limiting factor, twelve grams of carbon in the sample was equivalent to forty-four grams of evolved CO2. Total theoretical CO_2 in grams for each sample and reference was calculated using stoichiometry

$$ThCO_2 = \frac{44}{12} \times W \times Y \qquad (1)$$

where W was the percent of carbon of the material and Y was the mass in grams of the material in each vessel. For the PHB plastic samples C=55.8% while for the cellulose reference C=44.4%. To account for soil respiration, the net evolution of CO_2 was calculated by subtracting the carbon dioxide produced by the blank

$$Z_n = Z_b - Z_t \qquad (2)$$

where Z_n was the mL of HCl required for titration of CO_2 generated solely from the test material; Z_b was the average mL of HCl used to titrate the soil vessels without material (blanks); Z_t was mL of HCl used to titrate each vessel with soil and test material. Evolved CO_2 in milligrams from the test material was then calculated

$$ECO_2 = \frac{N \times Z_n \times 44}{2} \tag{3}$$

where N was the precise normality of the HCl determined that day. The percentage of theoretical CO_2 evolved was given as

$$\%E = \frac{(ECO_2 \times 0.001)}{ThCO_2} \times 100 \tag{4}$$

The standard error of percentage of biodegradation was

$$S_e = \sqrt{\frac{s_{test}^2}{n_1} + \frac{s_{blank}^2}{n_2}} \times \frac{100}{C_i} \tag{5}$$

where n_1 and n_2 are the number of test and blank vessels respectively; s was the standard deviation of total CO_2 produced; C_i was the initial mg of carbon added to the reaction vessel.

SEM and z-stacker

Three studs were produced with pristine plastic and three studs each for samples from the ISO 17556 and ASTM D5988 with a high, medium, and low degradation sample. A Leica M205 FCA microscope was used to take color pictures with multiple focal planes. The studs were then sputter coated with approximately 5 nm of gold and palladium coating and placed in a JOEL scanning electron microscope for further surface imaging.

Results and Discussion

The average cumulative biodegradation of the PHB samples, represented by the percent of theoretical carbon dioxide evolution, was less than 3% for both ASTM D5998 (ASTM Sample see Figure 4) and ISO 17556 (ISO Sample Figure 4). For ASTM D5998, the average cumulative percent CO_2 evolved was 0.36% (s = 0.55). For the test ISO 17556, the average cumulative percent of CO_2 evolved was 2.29% (s = 0.949). Relative to each other, ASTM D5998 and ISO 17556 had statistically different amounts of biodegradation using Welches t-test with a p-value of 0.0005 and 10.704 degrees of freedom.

Figure 4

Evolved Carbon Dioxide from Plastic
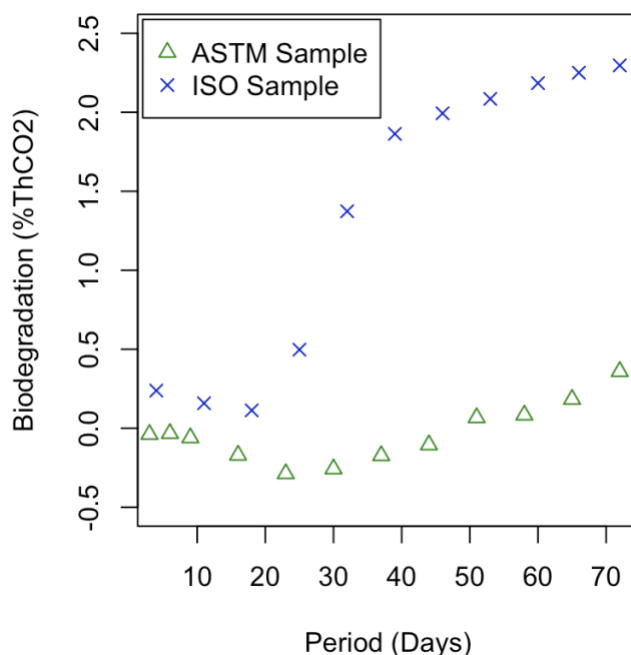
Note. Only the ISO test showed significant plastic degradation. ASTM plastic had a longer lag phase before degradation appeared to begin.

Over the course of the 72 day test, the cumulative carbon dioxide evolution from ASTM D5998 across the ten samples was not significantly different from zero (p=0.07027, df=9) with a

standard error, s_e of 1.964. This suggests that for this ratio of 1000 mg of plastic to 200 g of soil

under ASTM D5998 conditions, this method of carbon dioxide monitoring is not sensitive

enough to quantify the amount of plastic that did degrade. Limited degradation occurred on some

samples as observed by the JEOL SEM micrographs (see Figure 6).

The cellulose filter paper reference (Figure 5 ASTM Ref) showed the most degradation

of any material with an average of 16.24% (s=4.28) degradation between three samples.

Extrapolating from the rate of degradation, at six months the cellulose reference likely would not

have reached 70% degradation, which is the minimum requirement for ASTM D5998. If the

reference does not degrade sufficiently, the test overall would be considered invalid. This test did

not reach six months.

Figure 5

**Evolved Carbon Dioxide over Test Duration**.
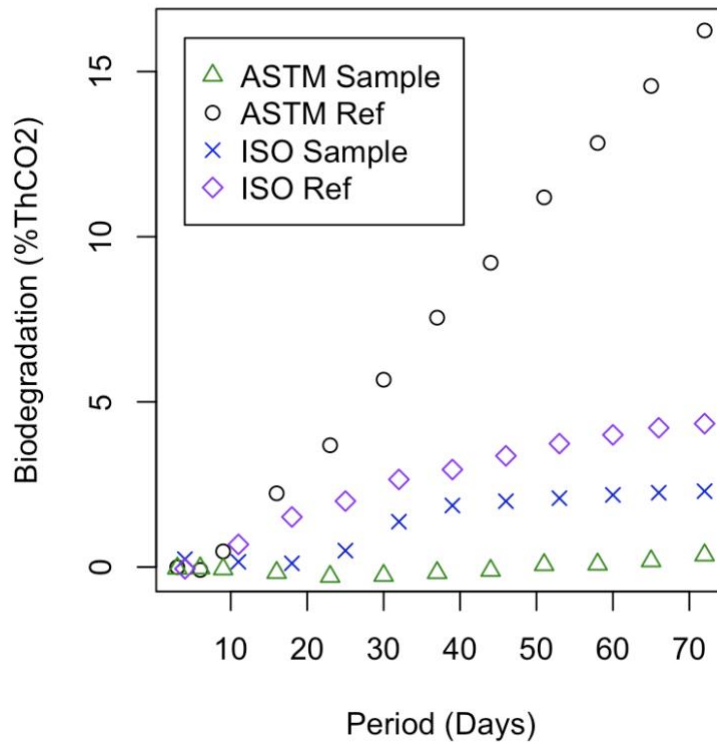
Note. ASTM sample plastic (ASTM Sample) and ISO sample plastic (ISO Sample) degraded very little over 72 days. ASTM cellulose reference (ASTM Ref) degraded significantly more than the ISO cellulose reference (ISO Ref).

For ISO 17556, the cumulative carbon evolution was significantly different from zero ($p = 0.0003$, df=7). While degradation was still low at 2.29%, the s_e value of 0.184 was much smaller. Either the different method of collecting CO_2 via gas-line or the slightly greater degradation was sufficient to allow for quantifying the evolved CO_2 for this method. However, the ISO cellulose reference degraded considerably less than the same material in the ASTM test with a final average cumulative degradation of 4.34% ($s = 0.362$). It is unlikely that this reference would reach minimum degradation (60% in six months for ISO 17556) to demonstrate test validity. Given the higher degradation of the same material in the other test, the cellulose filter was likely not the primary issue. The higher pH of the ISO soil, 8.61 compared to 5.68 in the ASTM soil, could have trapped a greater quantity of CO_2, or the increased gas-exchange that resulted in more variable moisture and drier over all conditions could have negatively affected the cellulose degradation. It is also possible that the microbes in the ISO 17556 soil were less capable of breaking about cellulose than the microbes from the ASTM D5998 soil. Both soils came from compost and biomes respectively where woody debris was heavily present (see Table 4). Therefore, cellulose degrading bacteria would have had material in the soils from which they could have originated.

Neither test reached a stage to determine validity, and the PHB pellets degraded very little in both tests. This indicates that future experiments using similar soils could benefit from longer incubation times and the use of a different reference material.

Figure 6

JEOL Scanning Electron Microscope Images of Plastic.
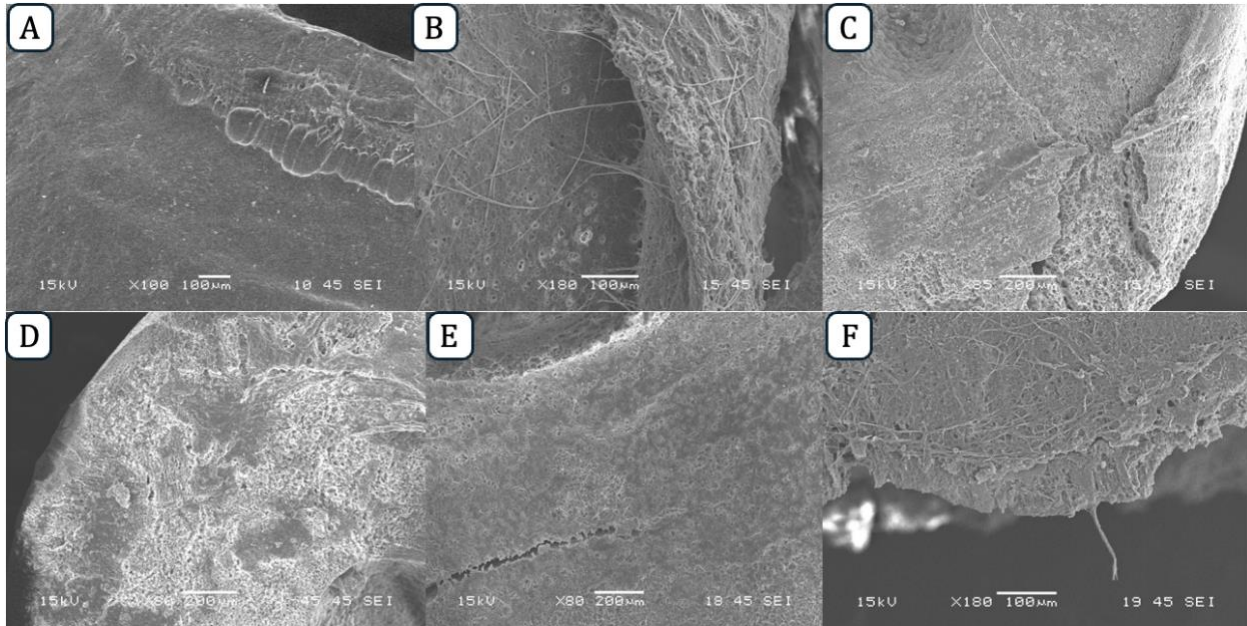
Note. (A) Virgin plastic, (B,C,D) ASTM degraded plastic, and (E,F) ISO degraded plastic.

      Qualitatively the ISO plastics showed more growth of an unknown fungus directly on the

PHB pellets than the ASTM, with only one ASTM sample having strands visible at 180x

magnification in the SEM. The ISO test had multiple samples with fungus growing on the

plastic, one of which (Figure 7) was visible to the naked eye.

Figure 7

ISO Sample with Visible Fungal Growth.
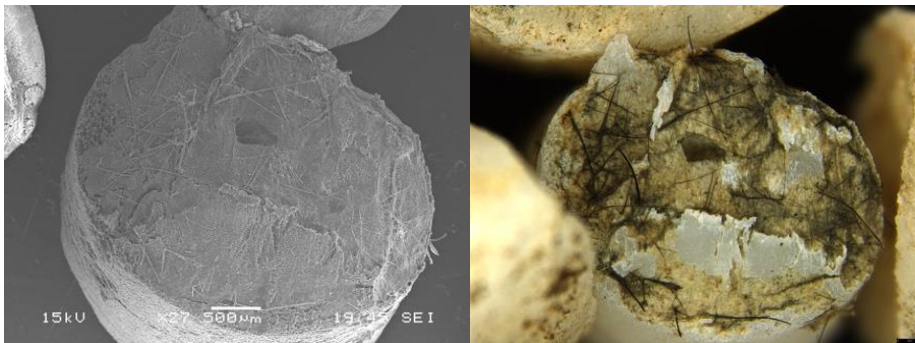
Note. Scanning Electron Microscope image (left) and Leica M205 FCA Microscope image (right).

In addition to test mediums, there were significant differences between the test pH, oxygen availability, and water content of the tests. This is also true of open environments. As a proxy for Pacific Northwest environments, the ASTM test with soils gathered from Washington State did not demonstrate enough biodegradation in the reference material to guarantee a valid test. However, these soils are appropriate for acidic soils local to the region. Given the amount of plastic that degraded, this formulation of PHB was not highly biodegradable in PNW soil. Additionally, for Washington soils, the ISO test did not have comparable plastic or reference degradation.

Conclusion

Plastic biodegradation in the environment is not an easily quantifiable concept. Every environment has unique characteristics that may impede or encourage the degradation of a specific plastic. As a proxy for the environment, it has not yet been definitively demonstrated the ASTM and ISO standard methods for examining the biodegradation of plastic produce comparable or disparate results. Depending on the specific local environment, the outcome of these tests may vary. It is a considerable legal hurdle to define biodegradability when a test run on a plastic in one environment may not apply to the same plastic under different conditions.

Even regarding the degradation of the reference material, cellulose, the two different methods exhibited different behavior when using western Washington soils. As for PHB degradation, one test showed statistically significant degradation while the other did not. That said, neither test reached end point nor produced viable results from reference materials. The significantly different degradation of the cellulose reference itself suggests that more comprehensive testing is required to effectively determine to what extent these two tests are directly comparable.

Context and Significance

The result of this test illustrated the need for comparative tests between different standards. If two tests were known to be highly similar, a company wishing to market a product as biodegradable in two locations that do not use the same legal metric, would only have to run one test and cut down on costs. Inconsistent testing methods causes companies that market more environmentally sustainable plastics to potentially face financial hurdles due to the difficulties in demonstrating the biodegradability of their product. They may be required to run multiple complex, time consuming tests to demonstrate the same chemical property in multiple countries. Additional testing requirements can add to the expense of biodegradable plastics. This burden can be passed onto consumers or may simply decrease the availability of eco-friendly products (Vasil, 2019). Consistent standard testing would make advancing a product to market quicker and easier so more biodegradable plastics could be made available. From a consumer perspective, if a plastic degrades more under certain test conditions, it is important to know which test more closely mimics the natural environment to understand if the plastic is actually naturally degrades or if it has been greenwashed.

The results of this experiment also demonstrate that an environment is not a singular entity. Washington State uses ASTM D5998 as a standard for plastic degradation. However, using local Washington soils, the reference did not degrade sufficiently to demonstrate validity in either test. Likewise, the PHB plastic did not degrade significantly or quickly. In this experiment ASTM D5998 had fewer alterations to the soil than the constructed ISO 17556 soil. By that metric ASTM D5998 more closely mimics the local natural environment. Washington soils are commonly acidic (National, 2024), and the ISO 17556 constructed soil had a basic pH. This

underscores to synthetic nature of ISO 17556 which may not effectively simulate realistic plastic degradation in this specific environment.

While as much care as possible was taken in obtaining the PHB pellets, this plastic is also a factor. PHB is known to degrade from a variety of experiments (Al-Khattaf et al.,2022; Fernandes et al., 2020; Rajan et al., 2017). Importantly, only one formulation of PHB was used in this study with molecular weight, surface area, and density consistent across the test samples. Any of these three factors could significantly impact degradation rate (Altaee et al., 2016). This particular formulation of PHB may be less biodegradable than others, and as such would not have been a suitable plastic to use as a comparison for the standard methods. Without a specific plastic sample that has been demonstrated previously to degrade under these specific test conditions, it is not possible to rule out the plastic itself as a cause of incomparable tests.

This experiment clearly demonstrated that ISO 17556 degraded PHB with a positive slope. ASTM D5988 degraded so little that the cumulative evolved CO_2 was not significantly different from zero. However, neither test reached a sufficient degradation point in the cellulose reference to conclusively show test validity. In order to validate ASTM D5988, after six months the cellulose reference must have degraded at least 70% (ASTM, 2018a). Likewise, to validate ISO 17556, after six months the reference must be more than 60% degraded (ISO 17556, 2019). Neither test ran for that duration, nor did either reference reach even half of the required degradation level. While it might be possible to extrapolate from the initial degradation phase, from these results, it cannot be determined if after six months either test would be valid.

The cause of the PHB degrading significantly less under ASTM D5988 conditions compared to the plastic in the ISO 17556 test is unclear. However, the cause of this difference could be due to a variety of variable conditions within the tests, the pH, the moisture content, or

others. Nevertheless, from this experiment, there was evidence that these two tests may not be comparable. This was evident as well from the more obvious difference in the degradation of the cellulose reference. It should also be noted that for the ISO test, there are two options in constructing the soil mixture. As clearly shown by the variability in pH with the two soils used in this experiment, various soil mixtures can easily produce varied chemical and physical parameters. Soil microbial community structure and function can be heavily influenced by pH (Fernandes et al., 2020), and PHB degradation is highly influenced by the microbial community that is present. It is therefore reasonable to ask how the variation in soil composition could impact plastic degradation tests. Future work could address these issues by examining PHB degradation in identical soils with artificially altered pH and naturally occurring soils of similar pH. Ultimately, in order to fully compare these tests, it would be necessary to run this experiment through the six month conclusion to demonstrate whether or not the reference material would degrade enough to validate the tests. Without this validation, it is possible to say that these tests under these conditions did not produce statistically similar PHB degradation but not whether this is a conclusive result.

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Appendices

<u>The first entry in this appendix is the methods written up at the beginning of the project before the experiment was initiated.</u>

SOP Soil Sampling, Characterization, and Plastic Degradation

Introduction

I am comparing standard ASTM and ISO methods for aerobic degradation of plastic in soil. Up to three methods will be used or potentially only two based on external time constraints. All three methods involved burying biodegradable plastic in soils (of various types) and collecting the CO_2 produced using a CO_2 absorbing medium. Weight loss before and after degradation will also be measured. The concept behind my experiment is to determine if the different 'standard' methods comparably degrade the same plastic.

Cleaning Protocol

Unless otherwise specified all materials are hand washed using tap water and lab soap then thoroughly rinsed with tap water and either wiped with a clean paper towel or allowed to air dry. After titration, beakers with remaining untitrated KOH are emptied into collection containers for neutralization. The beakers are run through a lab dishwasher at 70˚C to remove residual chemicals. Flasks are triple rinsed with DI water between old and new KOH but not run through a dishwasher due to insufficient numbers to take them out of circulation.

Equipment List Soil Collection & Moisture Determination

Top-loading balance (capable of at least 2 kg weight)
Refrigerator
Analytical balance
2 mm sieve
5 buckets + lids
Trowel
Metal tray (dissection with no wax approx. 6x9 inches)
High speed blender (glass)
3 Evaporating dish (at least 100mL volume)
Desiccator with tray and desiccant (must hold three evaporating dishes)

Soil Collection and Storage and Laboratory Soil Mixture Preparation
1. Collect three soils from different locations/ biomes, and note the date, plant types, soil depth, and site history if possible. Keep in plastic buckets with mostly closed lid to maintain moisture as much as possible without creating an anaerobic environment.
2. Keep soils at 4˚C until ready to introduce plastic. Use within one month of collection.
3. Sieve all three soils to less than 2mm. Larger material may be discarded (see Waste Section).

4. In a new clean bucket (see Cleaning Procedures), make lab mixture of soil by combining equal parts by weight from each of three sieved soil samples.
   a. Determine soil with least amount sieved and add that much of the other two soils (may need to split between two buckets). At least 10kg of laboratory mixture soil is required. Sieve more if necessary.
   b. Mix lab soil mixture thoroughly with trowel- this is the soil to be characterized – continue to refrigerate until use with plastic.

Moisture Determination of Laboratory Soil

1. Mix the lab soil thoroughly and select an approximately 300 g representative sample.
   a. Determine the weight of the metal pan (**P**)
   b. Using a trowel, scoop lab soil from the lab soil bucket. Determine the mass of this sample (**W**) and spread evenly on the flat pan.
   c. Leave the soil uncovered on the counter to allow the sample to come to moisture equilibrium with room air (at least 24 hr.).
   d. Stir occasionally to maintain maximum air exposure of the entire sample.
   e. When the mass of the sample reaches a constant value (**D**), calculate the moisture removed during air drying as a percentage of the original mass. **M= [W-(D-P)/W] *100%**
2. Divide air-dried sample in half. Grind half of the air-dried sample for 1 to 2 min in a high-speed blender. Use the ground portion for the ash tests. Save the other half of the unground portion for the centrifuge moisture test. Save in the desiccator.
3. Label porcelain dishes and tin foil.
4. Record to the nearest 0.01 g the mass of a high-silica or porcelain evaporating dish fitted with a heavy-duty aluminum foil cover. The dish shall have at least a 100 mL capacity.
5. Determine the amount, in grams, of air-dried sample equivalent to 50 g of wet sample, as follows: Equivalent Sample Mass, g =**50.0 – [(50 X M)/100**] where: *M* =moisture removed in air drying, %.
   a. Weigh the ground sample into porcelain dish to the nearest 0.01 g the equivalent of 50 g of test specimen before drying. Keep remaining ground sample in desiccator for later use.
6. Thoroughly mix the ground sample and place the ground specimen in the evaporating dish. The thickness of sample in container should not exceed 3 cm.
7. Cover immediately with the aluminum foil and record mass to the nearest 0.01 g.
8. Remove aluminum foil and place in oven.
9. Dry uncovered for at least 16 h at 105°C
   a. Remove from the oven, cover with aluminum foil tightly, cool in a desiccator for approximately 20 minutes, and record the mass **B**.
   b. Dry again for 1 hr. Remove and cool to room temperature. If there is no change in mass (from Step a) then proceed to Step 10. If the mass does change, dry again for 1 hr., cool, and weigh until mass is stable.
10. Calculate the moisture content as follows: **Moisture Content, % = [(W - *B*) X 100]/Wet** where: *Wet* = mass of the wet test specimen, g, and *B* = mass of the oven-dried specimen.
11. Save the ground, oven dried sample in desiccator for use in further tests.

Standard Soil Preparation
1. Using purchased components and chemicals mix 2kg of standard soil
    a. Industrial quartz sand = 1400 g, kaolinite clay = 200 g, natural soil from laboratory soil mixture = 320 g, aerated mature compost = 80 g
        i. Compost must be homogenous, at least 2-3 months mature if not a year, and free from large inert objects (glass, rocks, etc.)
    b. Mix all together thoroughly and store at 4°C until use.
2. Determine total water holding capacity using the centrifuge method below.
3. Dissolve these salts in DI water: Potassium dihydrogen phosphate = 0.4g, magnesium sulfate = 0.2g, sodium nitrate = 0.8g, urea = 0.4g, ammonium chloride = 0.8g
    a. Add the dissolved salts and water to soil mixture and mix thoroughly. Add any additional water necessary to bring soil to about 50% of moisture holding capacity.

Equipment list CHN
This equipment is available in Lab 1, Room 2035, The Evergreen State College

Tin capsules
Tiny Spatula
Tweezers

CHN samples
1. Weigh a precise amount of soil (between 0.2 and 0.4 g) into tin capsule according to the directions with the CHN balance.
    a. Fold capsule carefully so no sharp edges are left. Drop to check if folding has created a puncture.
    b. Weigh and record precise weight of capsule and location in container it will be stored. Record which soil it was too.
2. Repeat so each of the three soil types (lab, marine, standard) have three tins full.
3. Let technician know you are ready to run the CHN so he can schedule a time to help get the samples on and run the instrument
4. Determine the C:N ratio for each of the soils

Equipment List of Soil pH
*italics indicates equipment from previous list

pH meter and probe
Top loading balance
Kim wipes
DI water bottle
100 mL Beaker (slurry)
150 mL Beaker (rinse)
Buffer solutions 4, 7, 10
Stir Plate
Stir Bar

Soil pH

1. Use air dried soil from Moisture Determination of Laboratory Soil for pH of lab soil. Use standard soil before water addition for the standard soil. Marine sediment does not require pH testing.
2. Turn on pH meter and allow to warm up for 30 minutes.
3. Make a 5:1 distilled water to soil slurry with 50 mL of water and 10g of soil in a beaker.
4. Stir the slurry with stir bar while calibrating meter.
5. Calibrate pH probe with three buffers 4.7.10 – expected range in pH 6-8.
6. Record pH of soil slurry

Equipment List Ash content
Italics indicates item from previous equipment list

Muffle furnace
Analytical balance
Three crucibles
Aluminum foil
Sharpie
Desiccator with tray and desiccant
Tongs
Face shield
Lab coat
Heat resistant gloves

Ash and Organic Matter Content

1. Using a pencil label the outside of three porcelain crucibles.
2. Cover each crucible with sharpie labeled aluminum foil (so the same cover always goes to the same crucible) and determine the mass of each crucible with foil.
3. Place about 30 g of the oven-dried ground soil from the moisture determination in a crucible and record the precise mass.
4. Remove the aluminum foil and place the crucible in a cold muffle furnace. Set to 440˚C and allow to warm up to temperature in the furnace and hold for 90 minutes.
5. Wearing heat gloves, lab coat, and face shield remove the crucibles carefully with tongs, cover with foil, and immediately place in a desiccator to cool for 30 mins before weighing.
   a. Repeat step 4 and 5 and weigh again.
   b. If masses are within 0.1g, discontinue heat and power down muffle.
   c. If masses are different, repeat steps 4 and 5 until the most recent two masses are within acceptable limits.
6. Calculate ash content where Ash Content % ={(Final mass x 100)/starting dry mass}
7. Calculate organic matter: OM % = 100.0 – Ash content %

Equipment List Centrifuge Moisture Content
Italics indicate items on previous lists

Centrifuge (capable of 1000 times force of gravity)

$N= \sqrt{(RCF/0.0000111rm)}$ where N= rpm, RCF=relative centrifugal force (1000), r = radius of rotation of center of gravity of the test specimen, m=mass of the body

Gooch crucible 25mL capacity (6) – crucibles with little holes in the bottom

Babcock Trunnion Cups (4) with caps– to support crucible in centrifuge

Filter paper (4) (medium speed, high wet strength) to go in bottom of crucible

Analytical balance

Oven

Desiccator and tray (no desiccant)

Desiccator and tray (with desiccant)

Metal tins

Centrifuge Moisture Equivalent

1. Conduct this test using air-dried laboratory soil (not oven dried) and standard soil prior to adding water.
2. Number the crucibles using sharpie on the outside. If crucibles are significantly different masses, arrange them so they are paired by approximately equal weight on either side of the centrifuge.
3. Place filter papers in the bottom of the crucibles then wet thoroughly with DI water.
4. For each of the two soils, weigh two five-gram samples into labeled crucibles. Do not pack the soil and spread evenly in the bottom of the crucible.
5. Place crucibles in beakers with DI water in the bottom. The water should be at least 5mm higher than the soil but not so high that it overflows into the crucible.
    a. Allow soil to take up water until saturated, at least 8 hrs. or overnight. Saturation is evident by free water on the surface of the soil.
6. Place crucibles in humidifying container (desiccator that has water in the lower half).
    a. Leave crucible to drain for at least 12 hrs.
    b. Pour off any water standing on surface of samples when they are removed from this humidifier.
7. Place crucibles in centrifuge cups with weight evenly distributed across centrifuge.
8. Bring centrifuge to required speed to achieve 1000 times the force of gravity within 5 minutes with five successive equal incremental steps lasting 1 minute.
    a. Centrifuge for 1 hour.
    b. Allow centrifuge to come to rest with as little braking as possible but do not exceed 5 minutes.
9. Have four weighed and labeled metal tins and aluminum foil covers ready when centrifuge stops.
10. Immediately after centrifuging, transfer soil to metals tins as quickly as possible. It is not necessary to remove all the soil from the crucible.
11. Weigh the soil and metal tin with aluminum foil cover, then remove cover and place in 110˚C oven.
    a. Bake for 90 minutes, remove from oven, cover with foil, place in desiccator until cool and weigh.

b. Return to oven without aluminum cover for 1 hour, remove, cool, and weigh. If masses are within 0.05g test is complete. If masses are different, repeat this step until last two masses are within range.
12. Calculate water content % = (Mass of water / Mass of solid particles) *100 or ((Initiate mass- Baked Mass)/Baked Mass)*100
13. Use the average of the two samples of each soil.

Equipment list for glass set up

Glass scoring tool
Ethanol (for lubricating glass insertion)
Glass tubes
Leather gloves

Glass Scoring

1. Wear protective double dip or leather gloves, close toed shoes, and goggles.
2. Determine the glass tubing width desired by testing inserting tubes with ethanol as a lubricant. Spray the outside of the tube with ethanol and holding close to the end of the tube to prevent snapping insert the tube into a hole in the stopper.
   a. Tube should be as snug as possible when ethanol dries without cracking the rubber.
3. To cut the glass to size, make a score in the side of the glass tube and snap at that point. Beware of flying glass.
4. Make templates for each of the types of flasks used in Method 3. Using a piece of paper or cardboard, mark the bottom of the stopper and mark the end point of the two glass tubes.
   a. For flasks where one glass tube must be submerged and the other not submerged, end points are X and X_1
   b. For flask with soil where one tube must be beneath the soil, the end lengths of the glass tubing are Y and Y_1

Equipment list for Chemical Production
Italics indicate equipment from previous lists

Analytical balance
Stir bar
Stir plate
Weigh paper or boats
Scoopula
2000mL volumetric flask
100mL volumetric flask
Buret (50mL)
Gloves
Goggles
Lab coat

Volumetric pipet 10 mL
Pipet bulb
100mL graduated cylinder
100mL beaker
1000mL Plastic PE bottle (2)

Mixing Chemicals

1. To make a precisely known concentration of KHP for titration of NaOH, precisely weigh about 2g of KHP. This is create ~0.98M KHP solution.
    a. Add KHP to 100mL volumetric flask and fill flask to line with DI water.
    b. Add stir bar and let spin until KHP is fully dissolved.
    c. Calculate concentration of KHP = X g KHP *(1/204.22g mol-1)*1/0.1L
    d. Store extra KHP in a closed glass bottle for later use.
2. To make two liters of 10M KOH (sixteen liters are needed), dissolve 1,112.2g of solid KOH pellets in 2000mL of DI water. For this solution precision isn't important so there is no need to titrate.
    a. Weigh pellets and add to a 2L volumetric flask.
    b. Fill flask to line with DI water then add stir bar.
    c. Allow to stir in fume hood until all of the KOH has dissolved. This may take some time and may make the flask quite warm.
3. To make two liters of 0.5N KOH (1.43L needed to fill all CO_2 absorbing containers), dissolve 56.11g of solid KOH pellets in 2000mL of DI water.
    a. Weigh pellets and add to a 2L volumetric flask.
    b. Fill flask to line with DI water then add stir bar.
    c. Allow to stir in fume hood until all of the KOH has dissolved.
    d. To find to precise concentration of the KOH, use KHP solution to titrate. Using a 10mL volumetric pipet, place 10mL of KHP solution in a beaker. Add a drop of phenolphthalein.
    e. Fill a buret with at least 10mL of 0.5N KOH solution and record the precise volume.
    f. Slowly add KOH while swirling the beaker until the faintest pink color appears. Stop adding KOH immediately and record the volume of KOH used.
        i. Repeat steps e and f twice to have three volumes of KOH. Take the average volume and calculate the precise concentration of KOH = __M KHP *0.0100L KHP *(1 mol KOH/1 mol KHP)*(1/ ___mL avg. vol KOH)
    g. Add KOH to flask quickly to avoid exposure to air. Store extra KOH in a tightly closed plastic bottle. It must be titrated again immediately before use if not used immediately.
4. To make 2 L of 0.3N HCl starting from more concentrated HCl use this equation:
    $C_1V_1 = C_2V_2$ so from 12M HCL the initial volume is 50mL = (2000mL *0.3N)/12M
    a. Measure in a graduated cylinder 50mL of 12M HCL
    b. Fill a 2 L volumetric flask with at least 1000 mL of DI water and in fume hood add with 50 mL of HCl. Swirl to mix and fill to line of flask with DI water.

c. Titrate with known concentration of KOH. Add 10 mL of KOH to beaker using a volumetric pipet and add a drop of phenolphthalein indicator. Fill a buret with at least 20 mL of HCl and record the precise volume in the buret.

d. Slowly add HCl to KOH while swirling the beaker until the faintest pink color appears. Stop adding HCl immediately and record the volume of HCL used.

    i. Repeat steps c and d twice and take the average of the three volumes. Calculate the precise concentration of the HCL = 0.5 N KOH * 0.01 L KOH*(1 mol HCl/ 1 mol KOH)*(1/__avg vol HCl used)

e. Store HCl in closed glass bottle. It does not need to be titrated with every used, but it should be checked periodically, every two weeks or if calculations are changing noticeably.

Equipment list for Methods 1 and 2

Toploading balance
150 ml beaker (50)
100 ml beaker (40)
40 gallon mason jars
Environmental chamber
Trowel
Cellulose filter paper (12)

Method 1 – ASTM 5988 (Laboratory Soil)

1. Prepare the following number of gallon mason jars:
   a. Three technical controls with no material of any kind added.
   b. Three blanks with only sediment.
   c. Three with reference material in the form of a cellulose filter paper.
   d. As many as possible up to ten test jars with plastic added (depends on getting replacement jars for ones broken in transit)
   e. Weigh, fill, and add beakers to jars according to standard method before placing in 25˚C environmental chamber.
2. For the first three weeks sample twice a week according to titration protocol below. After the first three weeks sample once a week for 72 days or until a CO_2 plateau is reached.
3. Collect any remaining material with a fine sieve and rinse with DI water to remove sediment, allow to air dry completely, and record the final mass if the material is sufficiently clean.
4. Calculate the CO_2 produced.

Equipment list for Method 2

Top loading balance
Analytical balance
Scoopula
Weigh paper
500mL Erlenmeyer flask (48)

100 mL Erlenmeyer flask (35)
Glass tubing (160 pieces)
Plastic tubing
Aquarium pump
Environmental chamber

Method 2 – ISO 17556 (Standard Soil)

1. Prepare the following number of flasks:
    a. Three blanks with only standard soil.
    b. Three with reference material in the form of a cellulose filter paper.
    c. Up to ten flasks (depending on number that can be obtained) with plastic.
2. For each flask listed above, label two 500ML flasks 10M KOH and two 100mL flasks labeled 0.5 KOH
    a. Weigh, fill, and add material to flasks according to ISO standard method.
    b. In an environmental chamber set to 25˚C, link in sequence the five necessary flasks using prepared stoppers with glass tubing and flexible gas impermeable tubing.
    c. Using an aquarium pump, aerate the sequence of flasks starting from the 10M KOH at a rate of several milliliters per minute (amount should be equal across setups but precise amount is not essential).
3. Follow the ISO titration protocol below. Sample as often as necessary to prevent the second barium hydroxide flask in sequence from forming precipitate up to every other day.
4. If CO_2 production noticeably changes due to lack of moisture or the soil visibly changes color, remove the soil flask from the sequence, weigh and add enough DI water to return to its original weight.
5. On March 5th sample one final time all three flasks containing 0.5M KOH
6. Assuming no plateau has been reached, extract the remaining test material.
7. Collect and rinse any remaining plastic material with DI water to remove soil, allow to air dry completely, and record the final mass.
8. Calculate the CO_2 produced.

Equipment List for Titration

50 mL buret
White piece of paper

ASTM Titration (Methods 1 and 2)

1. Set up a 50mL buret filled with 0.3N HCl. Record starting volume.
2. Make 2L of 0.5M KOH according to Mixing Chemical section. Keep capped until use.
3. Remove one random mason jar from the environmental chamber (don't spill the solution in the beakers) and open the lid. Use a computer random number generator to do a random 1-19 order and sample in that order.
    a. Record the time you opened the jar. Start a 15minute timer.

     b. Remove any beakers from the jar and record the mass of the jar. If the mass has changed more then 5g, add water to the soil or sediment to return the weight to the original mass.

4. Place a drop of phenolphthalein in the KOH beaker. It should be pink. If it is not you may have exceeded to capacity of the KOH to absorb CO_2. Titrate anyways but make a note.
5. Slowly add HCl to the KOH swirling the KOH beaker. As soon as all of the phenolphthalein color is gone (liquid is completely colorless against a white background) immediately stop adding HCl. Record the final HCL volume for that sample.
6. If necessary add more HCL and record the new initial volume for the next sample.
7. If 15 minutes has passed, fill a new 150mL beaker with measured 20mL (soil) or 30mL (marine) of fresh KOH. If not, wait at least fifteen minutes then proceed with this step. Jar must be open for between 15-60 minutes.
8. Place the beaker in the jar, return the water beaker if necessary (Method 1 soil), and tightly cap the jar.
9. Return the jar to the environmental chamber.
10. Repeat until all the jars have been titrated.
11. See waste section for titrated liquid disposal then wash glassware.

ISO Titration

1. Set up a 50mL buret filled with 0.3N HCl. Record starting volume.
2. Make 2L of 0.5M KOH according to Mixing Chemical section. Keep capped when not being poured.
3. Measure 30mL of fresh KOH and pour in to a clean labeled 100mL Erlenmeyer flask.
4. Using a computer random number generator, randomly select set up 1-16.
     a. As quickly as possible without spilling or breaking the glass tubing, remove the flask of 0.5M KOH closest to the sample from the set-up.
     b. Immediately move the remaining flask over to the closer position to the sample flask. Place the new flask in the second position.
     c. Press all stoppers down securely and make sure the glass tubing is submerged.
5. Record the time and date the flask was removed from the set up.
6. Place a drop of phenolphthalein in the KOH flask. It should be pink. If it is not you may have exceeded to capacity of the KOH to absorb CO_2. Titrate anyways and make a note.
7. Slowly add HCl to the KOH swirling the KOH flask. As soon as all of the phenolphthalein color is gone (liquid is completely colorless against a white background) immediately stop adding HCl. Record the final HCL volume for that sample.
8. If necessary add more HCL to the buret and record the new initial volume for the next sample.
9. Repeat until all set-ups have been sampled.
10. See waste section for titrated liquid disposal.

Characterization of Soil at the end
1. Repeat pH, moisture, and ash tests of the soil when the CO_2 collection is finished.

Retrieving plastic for weight loss determination

1. Using the 2mm sieve and a 0.1mm sieve, each PHB sample will be physically sorted from the soil as no density separation could be determined. The plastic will be rinsed thoroughly with DI water with as much soil material removed as possible without scrubbing or soap.
2. Clean dry plastic will not be weighed due to the uncertainty of retrieving all remaining undegraded plastic using the sieving method or retrieval.

SEM prep for new plastic/ degraded plastic
1. Dry PHB material from three sample vials from both tests and from the pristine material will be carefully placed on a metal stud with double sided conductive tape.
2. Samples may be photographed using the Z-stacker Leico M205 FCA microscope.
3. Using the sputter coater, all samples with receive an approximately 5mm coating of gold and palladium according the instrument instructions before being placed in the SEM.

Waste

Soil waste:
1. After sieving extra or too large soil or marine sediment will go in a bucket to be poured out at a location where it was sampled.
2. After the experiment once the plastic is removed, soil and sediment will be returned to original locations. The standard soil (Method 3) that was produced can do in a compost bin or outside with a landowners permission.

Chemicals:
1. Unused solid chemicals can be returned to Jenna.
2. KHP, KOH, and HCl will be stored until the end of the experiment at which point they may be returned to Jenna for neutralization and poured down the drain.
3. Phenolphthalein in neutral titrated solutions is dilute enough to go down the drain, so titrated solutions can be washed down the sink.
4. Polyhydroxybutyrate is safe for trash disposal at any point and certainly once the test is complete.

R Script

```
#Master script to use for Plastic titration data
hist(ISO_removed$Total_ECO2,main="ISO Evolved CO2 Totals",xlab="Milligrams of
CO2",col="green")
hist(ASTM_S$Total_ECO2,main="ASTM Evolved CO2 Totals",xlab="Milligrams of
CO2",col="purple")
summary(ISO_removed)
summary(ASTM_S)

#ASTM descriptive stats
sd(ASTM_S$Total_ECO2)
mean(ASTM_S$Total_ECO2)
shapiro.test(ASTM_S$Total_ECO2)

sd(ASTM_S$Percent_ThCO2)
mean(ASTM_S$Percent_ThCO2)
shapiro.test(ASTM_S$Percent_ThCO2)
t.test(ASTM_S$Percent_ThCO2)

#ISO descriptive stats
sd(ISO_removed$Total_ECO2)
mean(ISO_removed$Total_ECO2)
shapiro.test(ISO_removed$Total_ECO2)

sd(ISO_removed$Percent_ThCO2)
mean(ISO_removed$Percent_ThCO2)
t.test(ISO_removed$Percent_ThCO2)

#Comparisons
t.test(ASTM_S$Percent_ThCO2,ISO_removed$Percent_ThCO2)
plot(ASTM_Rplot$Day,ASTM_Rplot$RefCO2,main="CO2 production by day",xlab="Period
(Days)",ylab="Biodegradation (%ThCO2)",pch=1)
points(ASTM_Rplot$Day,ASTM_Rplot$PlasticCO2,pch=2,col="green4")
points(ISO_Rplot$Day,ISO_Rplot$Plastic,pch=4,col="blue3")
points(ISO_Rplot$Day,ISO_Rplot$Ref,pch=5,col="purple2")
legend(x="topleft",inset=0.05,legend=c("ASTM Sample ","ASTM Ref","ISO Sample","ISO
Ref"),pch=c(2,1,4,5),col=c("green4","black","blue2","purple2"))

#zoom of plastics
plot(ISO_Rplot$Day,ISO_Rplot$Plastic,main="Plastic evolution of CO2 by day",xlab="Period
(Days)",ylab="Biodegradation (%ThCO2)",ylim=c(-0.5,2.5),pch=4,col="blue3")
points(ASTM_Rplot$Day,ASTM_Rplot$PlasticCO2,pch=2,col="green4")
legend(x="topleft",inset=0.01,legend=c("ASTM Sample","ISO
Sample"),pch=c(2,4),col=c("green4","blue3"))
```