Biodegradable Plastics in the Marine Environment: Enzymatic Degradation and Impact on Invertebrates

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Dissertation

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Summary

The pollution of the environment by synthetic plastics is a perpetual problem that poses a challenge to mankind. Conventional plastic is hardly degradable in the environment and thus accumulates in a wide variety of ecosystems. To counteract the increasing pollution from persistent plastics, the development of biodegradable materials is seen as a promising alternative. However, with growing production and utilization of biodegradable products, these plastics reach into the marine environment in the same way as conventional plastics. However, there is limited information about their degradability under marine environmental conditions. Low degradation rates could lead to long residence times of these plastics in the ocean, potentially allowing marine organisms to interact with biodegradable plastics, or fragments thereof. Small fragments can be ingested by many marine organisms, though little research has been done on the effects of biodegradable plastics on marine life. Furthermore, it is unclear whether ingested biodegradable microplastics could be degraded by digestive enzymes and what the consequences would be for organisms who interact with these materials. Therefore, the objective of this thesis is to investigate the enzymatic degradability of biodegradable plastics under marine conditions and by digestive fluids of marine invertebrates, and to evaluate the effects biodegradable plastic could have on marine invertebrates. Five different biobased biodegradable plastic compounds, issued from the European Horizon 2020 Project ´Bioplastics Europe´, were exemplarily used as research subjects. These compounds are based on different common biodegradable polymers, including polylactic acid (PLA), polybutylene succinate (PBS), poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) and polybutylene adipate terephthalate (PBAT).

Microplastics from these five compounds showed little to no enzymatic degradation when exposed to different hydrolytic enzymes at environmentally relevant temperatures under seawater conditions. Temperature seemed to have a strong influence on the degradation of some of the materials, as the hydrolysis increased with rising temperatures. After six months of incubation in estuarine mud and seawater under controlled conditions, plastic-bars from four of the five compounds showed no signs of degradation. Only one compound based on PHBV showed a net mass loss and alterations on the plastic surface. Degradation under environmentally relevant conditions seems to proceed very slowly, indicating only a marginal degradability of biodegradable plastics in the marine environment.

The uptake of fluorescence-dyed biodegradable microplastics could be observed in several marine invertebrates of different trophic levels and sizes in this study. Since biodegradable

plastics are generally known to be enzymatically degradable by several enzymes, they might be hydrolyzed by marine invertebrates following ingestion. The gastric fluid of the edible crab *Cancer pagurus* and the iimerican lobster *Homarus americanus* were capable of hydrolyzing microplastics of a PLA/PBAT-based compound *in-vitro*. Different carboxylesterases in the gastric fluid were identified as enzymes responsible for the observed degradation. After feeding experiments with the rockpool shrimp *Palaemon elegans*, elevated carboxylesterase activities could be analyzed in the midgut gland of shrimp fed with the same PLA/PBAT-based compound. These results indicate, that ingested biodegradable microplastics might be enzymatically degraded by digestive enzymes from crustaceans.

The uptake and interaction with microplastics can also be associated with several implications for organisms. Especially ingested biodegradable plastics have the potential to release harmful substances during enzymatic cleavage. This can lead to the induction of several cellular reactions in the organisms, such as the production of reactive oxygen species, resulting in oxidative stress. Known for counteracting the formation of reactive oxygen species is the enzyme superoxide dismutase. However, no elevated superoxide dismutase activities were found in *P. elegans* fed with microplastics of biodegradable and conventional origin. When exposing rotifers and *Artemia* nauplii to leachates of the five biodegradable compounds, one of the materials based on PHBV induced a high mortality in both organisms. Further tests identified toxic effects on these organisms to originate from the additives in the compound, rather than the base polymer. Most biodegradable plastic products have a high number of chemical substances in their formulations, to improve their physical properties for applications. These results demonstrate, that biodegradable plastics in the environment can pose a threat to marine organisms if they contain toxic chemicals in their formulations.

Overall, this study provides important insights into the degradation of biodegradable plastics in the marine environment and by digestive enzymes of marine invertebrates, as well as the implications for organisms related to the interaction with biodegradable plastics. Biodegradable plastics tested in this study show only poor degradability in the marine environment. Microplastics of biodegradable plastics might persist in the marine environment, being available to marine invertebrates. Organisms of different size ranges and trophic levels are able to ingest biodegradable microplastics in the same way as conventional microplastics. Once ingested, biodegradable microplastics might be hydrolyzed when exposed to digestive enzymes. Marine invertebrates can be affected by biodegradable plastic through leaching of toxic chemicals in the plastic`s formulation.

Zusammenfassung

Die Verschmutzung der Umwelt durch synthetische Kunststoffe ist ein beständiges Problem, welches die Menschheit vor eine immer größer werdende Herausforderung stellt. Konventionelles Plastik ist in der Umwelt kaum abbaubar und reichert sich so in verschiedensten Ökosystemen an. Um dieser zunehmenden Verschmutzung durch Plastik entgegenzuwirken, wird die Entwicklung von biologisch abbaubaren Kunststoffen als eine vielversprechende Alternative gesehen. Mit zunehmender Produktion und Verfügbarkeit dieser Materialien, steigt jedoch die Wahrscheinlichkeit, dass auch diese Materialien unkontrolliert in die Umwelt gelangen. Obwohl diese Kunststoffe unter bestimmten Bedingungen als bioabbaubar gelten, ist unklar ob dies auch unter suboptimalen Bedingungen, wie z.B. im Ozean, gegeben ist. Geringe Abbauraten könnten ebenfalls zu langen Verweilzeiten des Plastiks im Meer führen, wodurch Meeresorganismen potenziell mit diesen Kunststoffen, oder Fragmenten davon, interagieren können. Kleine Fragmente können von vielen Meeresorganismen aufgenommen werden, wobei die Effekte von bioabbaubarem Plastik auf Meereslebewesen kaum erforscht sind. Zudem ist unklar, ob diese Kunststoffe nach einer Aufnahme durch Verdauungsenzyme abgebaut werden könnten. Ziel dieser Arbeit ist es daher, die Abbaubarkeit bioabbaubarer Kunststoffe unter marinen Bedingungen und durch Verdauungsflüssigkeiten mariner Invertebraten zu bestimmen, sowie die Auswirkungen dieser Kunststoffe in Kontakt mit marinen Wirbellosen zu untersuchen. In dieser Studie wurden exemplarisch fünf verschiedene, biobasiert und biologisch abbaubare Kunststoffe verwendet, die aus dem Horizon 2020 Projekt „Bioplastics Europe“ stammen. Diese Materialien basieren auf gängigen biologisch abbaubaren Polymeren, darunter Polylactide (PLA), Polybutylensuccinat (PBS), Poly(hydroxybutansäure-co-hydroxyvaleriansäure) (PHBV) und Polybutylenadipat-Terephthalat (PBAT).

In-vitro Abbauversuche mit verschiedenen hydrolytischen Enzymen und Mikropartikeln der ausgewählten Kunststoffe zeigten wenig bis keinen enzymatischen Abbau bei umweltrelevanten Temperaturen unter Meerwasserbedingungen. Die Temperatur zeigte einen starken Einfluss auf den enzymatischen Abbau einiger der Kunststoffe, da die Hydrolyse mit steigender Temperatur zunahm. Nach einer sechsmonatigen Inkubation in Meeressediment und Meerwasser unter kontrollierten Bedingungen zeigten die Prüflinge von vier der fünf Kunststoffe keinerlei Anzeichen eines Abbaus. Nur Prüflinge des Kunststoffs auf PHBV-Basis wiesen einen Massenverlust und Oberflächenerosion auf, was auf einen geringen aber vorhandenen Abbau schließen lässt. Allgemein scheint der Abbau unter umweltrelevanten

Bedingungen im Ozean sehr langsam zu verlaufen, was auf eine nur geringe Abbaubarkeit bioabbaubarer Kunststoffe, sollten diese in die Meere gelangen, hinweist.

Verweilen bioabbaubare Kunststoffe in den Meeren, können diese durch physikalische und chemische Stressoren verwittert werden und Mikroplastik bilden. Mikroplastik von diesen Materialien kann wiederum von Meeresorganismen aufgenommen werden. Die Aufnahme von bioabbaubarem Mikroplastik, welches fluoreszierend gefärbt wurde, konnte in dieser Studie mit mehreren wirbellosen Meeresorganismen verschiedener trophischer Ebenen und Größen beobachtet werden. Eine Aufnahme dieses Mikroplastiks bedeutet, dass diese im Verdauungstrakt den Verdauungsenzymen ausgesetzt sein und dadurch abgebaut werden könnten. Die Magenflüssigkeiten des Taschenkrebses *Cancer pagurus* und des Amerikanischen Hummers *Homarus americanus* waren in der Lage, Mikroplastik eines PLA/PBAT-basierten Kunststoffes zu hydrolysieren. Analysen der Magenflüssigkeit von *C. pagurus* ergaben, dass verschiedene Carboxylesterasen höchstwahrscheinlich für den beobachteten Abbau verantwortlich sind. Zudem konnten nach Fütterungsversuchen mit der Kleinen Felsengarnele *Palaemon elegans* erhöhte Carboxylesterase-Aktivitäten in der Mitteldarmdrüse der Garnelen analysiert werden, die mit dem gleichen PLA/PBAT-basierten Mikroplastik gefüttert wurden. Diese Ergebnisse deuten darauf hin, dass aufgenommenes bioabbaubares Mikroplastik durch Verdauungsenzyme von Krustentieren enzymatisch abgebaut werden könnte.

Mikroplastik kann zudem negative Auswirkungen auf die Gesundheit der Organismen haben, die Mikroplastik aufnehmen. Insbesondere bioabbaubare Kunststoffpartikel, die im Magen von Organismen enzymatisch gespalten werden, haben das Potenzial, schädliche Substanzen freizusetzen. Dies kann zu verschiedenen zellulären Reaktionen in den Organismen führen, wie z. B. der Produktion reaktiver Sauerstoffspezies, die zu oxidativem Stress führen. Um der Bildung reaktiver Sauerstoffspezies entgegenzuwirken, bilden Organismen im Rahmen einer Immunantwort z.B. das Enzym Superoxiddismutase. Bei *P. elegans*, die in dieser Studie mit konventionellem und bioabbaubarem Mikroplastik gefüttert wurden, konnten jedoch keine erhöhten Superoxiddismutase-Aktivitäten festgestellt werden. Bei Rädertierchen und Artemien-Nauplien, die man im Rahmen dieser Arbeit in Medien aussetzte, in denen zuvor die fünf bioabbaubaren Kunststoffe inkubiert wurden, führte dies bei einem der Materialien auf PHBV-Basis zu einer hohen Sterblichkeit. Weitere Tests ergaben, dass der toxische Effekt auf diese Organismen nicht vom Grundpolymer PHBV induziert wurde, sondern wahrscheinlicher von den Additiven in der Formulierung des Plastiks herrührt. Die meisten bioabbaubaren Kunststoffe beinhalten eine große Anzahl chemischer Substanzen, um ihre physikalischen

Eigenschaften für verschiedenste Anwendungen zu verbessern. Diese Ergebnisse legen nahe, dass bioabbaubare Kunststoffe in der Umwelt eine Bedrohung für Meeresorganismen darstellen können, wenn toxische Chemikalien in ihren Formulierungen aus dem Material austreten.

Im Gesamten liefert diese Studie wichtige Erkenntnisse über den Abbau bioabbaubarer Kunststoffe unter marinen Bedingungen und durch Verdauungsenzymen von Krustentieren, sowie über die Auswirkungen von bioabbaubarem Mikroplastik auf marine wirbellose Organismen. Die in dieser Studie getesteten bioabbaubaren Plastiks sind in den Ozeanen nur schlecht abbaubar. Mikropartikel dieser Kunststoffe können in den Ozeanen für längere Zeit verweilen und mit Meerestieren interagieren. Organismen verschiedener Größen und trophischer Ebenen können bioabbaubares Mikroplastik auf die gleiche Weise aufnehmen wie herkömmliches Mikroplastik. Nach der Aufnahme durch Organismen kann bioabbaubares Mikroplastik abgebaut werden, wenn es Verdauungsenzymen ausgesetzt wird. Marine Organismen können durch toxische Substanzen, die den Kunststoffen entweichen, geschädigt werden.

List of abbreviations

± SD	± standard deviation
ABM	Arctic Biomaterials
AMF	agricultural mulch films
ASTM	American Society for Testing and Materials
BPE	BioPlastics Europe
BPI	Biodegradable Products Institute
C	Cutlery
CAA	Clean Air Act
CAT	Catalase
CH_4	Methane
CO_2	carbon dioxide
DIN	Deutsches Institut für Normung
DMSO	dimethyl sulfoxide
DANN	desoxyribonucleic acid
ECHA	European Chemicals Agency
EDTA	ethylenediaminetetraacetic acid
EU	European Union
GPx	glutathione peroxidase
GR	glutathione reductase
H_2O	water
HA	hydroxyalkanoate
HB	3-hydroxybutyric acid
HDPE	high-density polyethylene
HV	hydroxyvaleric acid
IFBB	Institute for bioplastics and biocomposites
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LDPE	low-density polyethylene
MUF	4-Methyl-umbelliferone
NaCl	sodium chloride
OECD	Organisation for Economic Co-operation and Development
O_2	oxygen

PBAT	polybutylene adipate terephtalate
PBS	polybutylene succinate
PE	polyethylene
PET	polyethylene terephtalate
PHA	polyhydroxyalkanoate
PHBV	poly(hydroxybutyrate-co-valerate)
PLA	polylactic acid
PP	polypropylene
ppt	parts per thousand
PS	polystyrene
PTFE	polytetrafluoroethylene
PVC	polyvinyl chloride
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
ROS	reactive oxygen species
RP	rigid packaging
SA	succinic acid
SDS PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SOD	superoxide dismutase
SP	soft packaging
T	toys
UV	ultraviolet
XOD	xanthine oxidase

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1 Scientific Background

1.1 Environmental plastic pollution

Humans are massively affecting the biosphere of the earth. The tremendous impact that humanity exerted on the planet over the last centuries and decades has framed this recent geological era "Anthropocene". The Anthropocene is characterized by the anthropogenic changes, that have shaped the earth's environment and altered its conditions when compared to the previous Holocene (Crutzen, 2006). One of these changes, which has a strong impact on terrestrial and aquatic ecosystems, is the introduction of harmful contaminants into the environment. Anthropogenic environmental pollution is progressively increasing since the early days of humankind (Hill, 2020). However, the environmental pollution alarmingly increased in the last centuries with the beginning of the industrial revolution. Along with the growing human population and prospering economy, environmental pollution escalated as well and is still growing with no prospect of mitigation (Landrigan et al., 2018). Industrial emissions, poor sanitation infrastructure, and inefficient waste managements are just a few of many examples of sources of pollution (Briggs, 2003). In total, this leads to serious global problems, affecting biodiversity, ecosystems, and, finally, human health (Hill, 2020). In the last decades, environmental awareness rose and efforts were made to mitigate the impacts of pollution to the environment. An example is the implementation of the Clean Air Act in the United States 1970 to regulate the emissions of hazardous air pollutants, or the REACH regulation for **R**egistration, **E**valuation, **A**uthorization and Restriction of **Ch**emicals, implemented in Europe in 2007 to regulate chemical substances (CAA, 1970; REACH, 2006). However, pollution remains a major problem. The ongoing technological advancement resulted in the introduction and liberation of new contaminants, which, in turn, require solutions to protect the earth's ecosystems. One of these new pollutants that have emerged in the last century are plastics.

Plastics are made from synthetic polymers, that are usually derived from raw material such as crude oil. The two main processes to synthesize these polymers are polymerization and polycondensation, where monomers are linked together to form polymer chains. The word plastic derives from the Greek *πλαστικός* (*plastikos*), which means "capable of being molded into various forms" (Online Etymology Dictionary, 2022). As this description already implies, plastics or products made from plastic are extremely versatile and can be processed to be used in a wide range of applications. Due to their advantageous properties, such as durability, light weight and flexibility, plastics can be found in almost every sector and are thought to be indispensable for our daily routine in modern society. The applications for plastic products are

manyfold, but from most plastics produced, 36 % are used in the packaging industry, followed by the construction industry with 16 % and the textile industry with 12 %. Other sectors, where plastics are frequently used are as consumer products (10 %) or in electronics (4 %) (Geyer et al., 2017). Starting from the middle of the 20th century, plastic production increased exponentially up to present, only interrupted by a slight decrease due to the oil crisis in 1973, by the financial crisis in 2008 and the Covid-19 pandemic impact on global economic consumption in 2020. Nonetheless, in 2020 about 367 million metric tons of plastic were produced worldwide (Plastics Europe, 2021). This sheer mass of produced plastics poses a great challenge to its end-of-life management, leading to an uncontrolled release of plastic into the environment.

Due to the growth of the human population and the ongoing industrial development in many countries, combined with the lavish and unsustainable use of plastic products, plastic waste is generated in excess, overwhelming existing plastic waste management systems. Only 9 % of the global plastic waste is recycled, whereas about half of all plastic waste ends up in landfills. About 22 % of the global plastic waste completely evades a controlled waste management, finding its way into the environment and eventually into the ocean (OECD, 2022a, 2022b). The majority of marine plastic pollution emerges from land and reaches the ocean indirectly through a variety of pathways. Plastic debris is transported by wind, via natural waterways or sewage systems, and surface runoff (Lebreton et al., 2017). Most of the plastic debris in the ocean originates from coastal cities with high population densities (Jambeck et al., 2015). Additionally, plastic is directly released into the ocean intentionally or unintentionally, in most of the cases by industrial offshore activities including shipping and commercial fishing (Andrady, 2011). In 2015, it was estimated that between 4.8 to 12.7 million tons of plastic are discharged into the ocean (Jambeck et al., 2015). With respect to the increased plastic production, Wayman and Niemann (2021) assumed that there might have been up to 14.5 million tons of plastic discharged into the ocean in 2018. If the current pace in development of plastic production and waste management is maintained, Lau et al. (2020) predict a 2.6-fold increase for the amount of plastic entering the oceans by 2040. The durability of plastic products, which is desired during use, leads to a long-term accumulation of persistent plastics in the environment, especially in the ocean.

Plastic in the oceans is moved by wind, currents, and marine organisms even to the most remote places (Yi and Kannan, 2016; Forsberg et al., 2020). Spreading across the globe, plastic debris can be found everywhere from densely populated regions to the most remote and isolated areas

in the Arctic (Peng et al., 2018). Its distribution within different marine ecosystems and in the water column also depends on the plastic properties, such as shape and density, which affect its buoyancy (Kooi et al., 2016). Negatively buoyant plastics such as polyvinyl chloride (PVC) and polyethylene terephthalate (PET) sink to the ocean floor, while positively buoyant plastics such high-density polyethylene (HDPE), low-density polyethylene (LDPE) or polypropylene (PP) float at the water surface (Reisser, 2015). Wind patterns and ocean currents form vast offshore areas, such as the Great Pacific Garbage Patch, where plastic litter and microplastics accumulate at considerable concentrations (Leal Filho et al., 2021) (**Figure 1.1**).

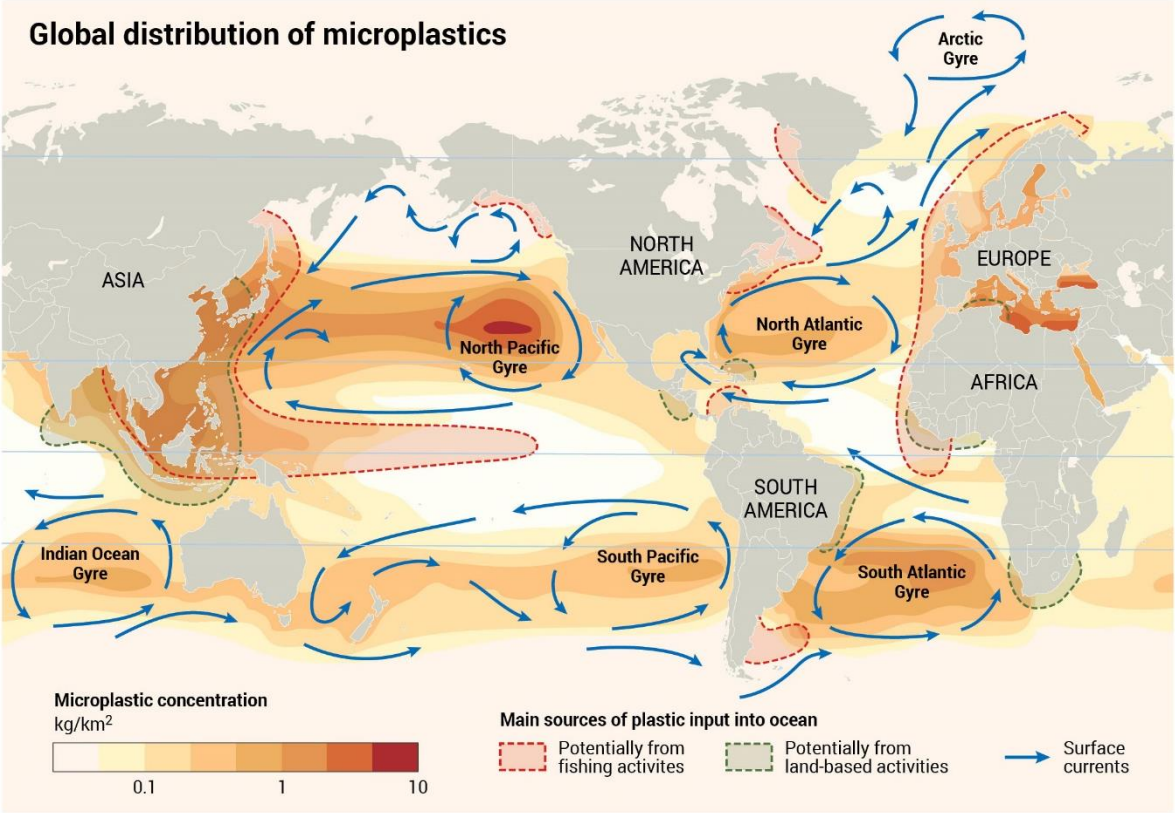

Figure 1.1: Global distribution of microplastics, with ocean surface currents and potential input sources. Taken from https://www.grida.no/resources/13339 (visited at 11.10.2022), created by Riccardo Pravettoni and Philippe Rekacewicz. Sources from Geyer et al. (2015), Jambeck et al. (2015), Van Sebille et al. (2015), Law (2017) and Lebreton et al. (2017).

Besides plastic debris of macroscopic size, there is also a growing concern about the distribution and accumulation of microplastics in the environment. Although the term microplastic is not clearly defined, there is a wide consensus in the scientific literature about microplastics being plastic particles smaller than 5 mm (Hidalgo-Ruz et al., 2012). Based on their origin, microplastics can be categorized into two groups: primary and secondary microplastics. Primary microplastics are produced with a size already smaller than 5 mm, with the intention of being applied in specific fields, such as raw materials for plastic production (plastic pellets),

or microbeads in abrasive cleaners or cosmetic products (GESAMP, 2015). Secondary microplastics derive from larger plastic items, which are mechanically or chemically fragmented during ageing (GESAMP, 2015). A detailed description of the fragmentation and degradation of plastic to micro- and nanoplastic (< 0.1µm, Koelmans et al., 2015) in the ocean can be found in section 1.3. Microplastics are widespread in the oceans because they are easily dispersed due to their small size and low weight. High concentrations of microplastics have been found in deep sea sediments, seafloor beds and even in Arctic Sea ice (Ling et al., 2017; Peeken et al. 2018; Cunningham et al., 2020). In some locations, the temporal dynamics of microplastics depositions in oceanic and coastal sediments correlate with the development of the global plastic production (Brandon et al., 2019; Chen et al., 2020; Martin et al., 2020).
Plastic waste in the macro- and micrometer size pollutes terrestrial and aquatic habitats, leading to serious ecological and socio-economic problems (Wright and Kelly, 2017; Beaumont et al., 2019). The ocean as the largest ecosystem provides key services to humans, including carbon storage, cultural benefits, and provisions in form of pharmaceutical components, mineral resources, or food for billions of people (Sandifer and Sutton-Grier, 2014). With increasing plastic pollution of the ocean, these services are at risk, which in turn affects the respective sectors and industries with millions of jobs connected to them. In the fishing industry, for example, plastic contaminants in fish and ghost fishing through discarded plastic fishing gear can lead to high expenses (Al-Masroori et al., 2004; Gilardi et al., 2010; Thushari and Senevirathna, 2020). Concerning the tourism sector of a region, plastic contamination on beaches and in coastal areas reduces the aesthetical value and can lead to a lower number of visiting tourists (Thushari and Senevirathna, 2020). Furthermore, plastic pollution in the ocean has several ecological effects and has been identified as one of the top threats for biota (Gray, 1997). The impacts of plastics on ecosystems and organisms are still under research, but there is already an extensive list of severe consequences that plastic has on a variety of species (Kurtela and Antolović, 2019; Bucci et al., 2020).

Ingestion of plastic is one of the most common interactions of plastic pollution and marine life (Kühn, 2015). Microplastics are easily mistaken for food by various marine organisms and are ingested deliberately (Jovanović, 2017; Ory et a., 2017) or passively (Sussarellu et al., 2016; Li et al., 2022). The size of plastics in microparticle range makes them available to an even wider range of organisms compared to macroscopic plastic litter, particularly to organisms of lower trophic levels (Botterell et al., 2019). Uptake of microplastics can depend on a variety of factors, such as size, shape, color, density of the particles (Wright et al., 2013; Ivar do Sul and Costa, 2014), and the formation of biofilm on the plastic surface (Fabra et al., 2021). Moreover, the

susceptibility of an organism to ingest microplastics is strongly dependent on the mode of feeding (Campbell et al., 2017; Mizraji et al., 2017). Generally, organisms with non-selective feeding habits, such as suspension and deposit feeders (Van Cauwenberghe et al., 2014; Sussarellu et al., 2016) most likely ingest plastic particles (Lusher et al., 2016). Predatory and detritivorous animals are also considered to take up microplastic passively with the prey (Murray and Cowie, 2011; Devriese et al., 2015). Microplastics have been found in fish, mussels, crustaceans and other organisms (Van Cauwenberghe et al., 2015; Cau et al., 2019; Garrido Gamarro et al., 2020). Besides the uptake of macroplastic litter by fauna and the obvious resulting impacts such as false satiation, blocking of the digestive tract, and buoyancy issues (Mrosovsky et al., 2009; Boerger et al., 2013; Cole et al., 2015), the impacts of microplastics after uptake might be more inconspicuous, but not less severe (Wright et al., 2013). The biological effects associated with an incidental or intentional uptake of microplastics have been investigated in various laboratory studies. A variety of species showed effects ranging from reduced food consumption, reduced fecundity, developmental delays, and reduced growth rate (Wright et al., 2013; Cole et al., 2014; Jeong et al., 2017) to increased mortality and susceptibility for hepatic stress. The induction of oxidative stress markers was repeatedly reported as a sensitive indicator of cellular stress responses (Browne et al., 2013; Lee et al., 2013; Rochman et al., 2013a; Mazurais et al., 2015).

To turn a plastic into a final product, the matrices of plastic products contain chemicals that facilitate processing and enhance the performance of the product. These additives are present in almost every plastic. They vary in their chemical properties and concentrations depending on the polymer, its procession, and its intended application (OECD, 2004). Commonly used additives are ultra-violet (UV)-stabilizers, hydrocarbons, antioxidants, plasticizers, lubricants, flame-retardants, and many others (Mascia, 1974). Furthermore, plastics may contain non-intentionally inserted chemicals that are side or breakdown products, which are introduced during the production process or formed during the degradation of plastic (Muncke, 2009). These substances may leach out of the plastic material, affecting organisms that ingest or otherwise come in contact with them (Kim et al., 2006; Lithner et al., 2009; Capolupo et al., 2020). Leaching of chemicals from plastics has been reported for additives, unpolymerized residual monomers, and degradation-, reaction- and transformation products that are not or only weakly bound in the polymer chain (Sheftel, 2000; OECD, 2004). Releasing rates of leachates differ depending on the type of polymer and the additives, and might be higher in aged and weathered plastics than in newly produced ones (Koelmans et al., 2014; Rochman et al., 2013b).

Moreover, leaching may be facilitated in the presence of gastrointestinal fluids with high amounts of surfactants (Koelmans et al., 2013; Endo et al., 2013).

The concerns regarding the ongoing plastic pollution of our environments and the growing public awareness of this issue, has forced decision makers and authorities to take remedial action (Pettipas et al. 2016; Bergmann et al. 2017). Measures to prevent excess plastic waste generation and to integrate environmentally sound management of plastic waste include bans on disposable items and improved recycling strategies (European Commission, 2021; VerpackG, 2017). Besides legislative measures to mitigate plastic discharge into the environment, also technical innovations, for example specialized ocean cleanup systems for plastics are brought forward (Sainte-Rose et al., 2016). In recent years, there has also been an increasing interest in the development of so-called "bioplastics" (Shen et al., 2020; Filiciotto and Rothenberg, 2021).

1.2 Biobased and biodegradable plastics

"Bioplastics" is a collective term that is not only used for biodegradable plastics, but also for plastic that are biobased or possess both of these properties. This, however, leads to ambiguities since biodegradable or biobased properties impact the life cycle of a plastic material in very different ways.

Around 99% of the modern plastic products are synthetic polymers based on fossil resources. However, the first man-made polymers were based on modified natural materials such as starch and glucose (IFBB, 2018). Since the 1950s, these materials have been replaced almost entirely by petrochemical materials. Nonetheless, several biobased materials prevailed and have been steadily developed over the last 30 years (IFBB, 2018). The difference between a conventional and a biobased plastic is the replacement of petroleum or other fossil resources for the raw materials by feedstock based on renewable biomass. To be considered a biobased plastic, the polymeric material of the product must at least partially derive from biomass (Brockhaus et al., 2016), provided that biomass refers to non-fossilized and biodegradable organic materials from plants, animals or microorganisms (DIN EN 16575:2014-10). The currently most common biomass resources for this purpose are carbohydrate-rich products and byproducts from agricultural sectors, especially grain, starch, sugar cane, and sugar beet (Endres, 2017). There are several ways of obtaining a biobased plastic, by either modification of natural polymers, synthesis of biobased monomers and subsequent polymerization, or the production of polymeric material directly in microorganisms or plants (Storz and Vorlop, 2013). Research

and development in waste re-utilization and biopolymer synthesis also enable agricultural and food wastes, as well as byproducts from industrial processes to be used as renewable resources (Tsang et al., 2019; Chan et al., 2021; Saratale et al. 2021; de Souza Vandenberghe et al., 2021). Compared to fossil-based plastics, biobased plastics produced in this way can have a lower carbon-footprint and contribute to a more sustainable plastic life cycle (Zheng and Suh, 2019).

Regarding the biodegradability of a plastic, the decisive factor is not the feedstock, but its chemical structure (Endres, 2017). Although a plastic material does not need to be biobased to be biodegradable, both properties are incorporated more and more often into the same products, to achieve a maximum sustainability of the materials. The monomers of biodegradable polymers are usually connected by glycosidic-, peptide-, and ester-bonds, that can be degraded by different enzymes (Rosato et al., 2022). A plastic is considered biodegradable if it meets certain criteria regarding its biodegradability, which are defined by several institutions and organizations (e.g. OECD, ASTM and ISO). These standards enable an established assessment of the degree of biodegradability under different conditions. Common test methods to determine the biodegradability of a material involve measurements of CO_2 production (OECD 301 B, ISO 14852, ASTM D5864-11), decrease of dissolved organic carbon (OECD 301 A, DIN EN ISO 7827) and oxygen consumption (ISO 9408). These tests are usually conducted with test material in a mineral medium and an inoculum, under aerobic conditions. The tests are carried out for defined periods of time, in which the degree of DOC removal, CO_2 production or oxygen uptake is measured and checked for levels to be passed to approve biodegradability (OECD, 1992). Depending on the threshold levels and the conditions at which a material turns out to be biodegradable, institutions such as TÜV AUSTRIA Belgium, DIN CERTCO, or BPI (Biodegradable Products Institute) issue certifications for these products. In the following, biodegradable polymers are described, which are common in modern plastic products. These polymers consist of monomers obtained from either fossil fuels or biomass and determine the properties of the plastic.

Polylactic acid (PLA)

Polylactic acid belongs to the family of aliphatic polyesters. It is a thermoplastic, derived from renewable resources and is therefore considered biobased. PLA is typically manufactured through ring opening condensation of lactide, a dimer of lactic acid, or directly through polycondensation of lactic acid (Yu et al., 2009; Penczek et al., 2003) (**Figure 1.2**). Raw materials for the synthesis of PLA can derive from agricultural byproducts and feedstocks such

as corn starch or sugarcane (Adsul et al., 2007; Singvi et al., 2010). PLA is probably the most popular and widely used synthetic biobased polymer because it is very cost-effective in production and has exceptional properties compared to other biobased polymers. In 2019, the annual production capacity of PLA was approximately 290,000 tons worldwide (Fortune Business Insights, 2021). Pure PLA is rather brittle and crystallizes slowly. Therefore, it is often modified and blended with other polymers before being processed (Olabisi & Adewale, 2016). In its processed form, PLA exhibits similar mechanical and physical properties as polyethylene (PE), polypropylene (PP), and polystyrene (PS). Therefore, PLA finds its main application in packaging (Karkhanis & Matuana, 2019). PLA is also considered biodegradable and compostable, since the ester groups in the polymer backbone can be hydrolyzed by enzymatic activity. Several hydrolytic enzymes, such as proteases from a variety of microbes and other organisms, are able to degrade PLA (Williams 1981; Lim et al., 2005; Kawai, 2010).

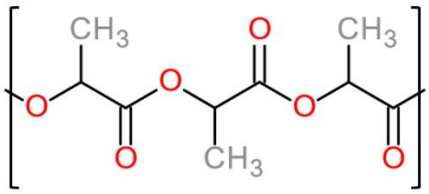

Figure 1.2: Structural formula of polylactic acid (PLA).

Polybutylene succinate (PBS)

Another common biodegradable aliphatic polyester is PBS (**Figure 1.3**), a thermoplastic with a strong increase in production since the early 1990s. With good chemical and heat resistance, toughness, and excellent mechanical properties similar to polypropylene, PBS is used in a variety of applications such as foams, mulch films or packaging (Zeng et al., 2009; Bi et al., 2018). PBS is synthesized by polycondensation of succinic acid and 1.4-butandiol. These raw materials are typically obtained from non-renewable resources (Xu and Guo, 2010). However, both materials can also be obtained biotechnologically through microbial fermentation of starch and sugar (Patel et al., 2018). The production of PBS is more expensive than that of conventional plastics such as polystyrene (PS) or polyethylene terephthalate (PET), and also more costly than the production of PLA. Nonetheless, it has a good moldability, making it easy to process and it allows to reduce the addition of plasticizers. To increase its density and flexibility for various purposes, PBS is often blended with a copolymer (Yu et al., 2011). PBS can be degraded by microorganisms from soil, freshwater, and marine environments, usually

through enzymatic hydrolysis by lipase or cutinase (Lee et al. 2008; Ding et al. 2012; Hu et al. 2016).

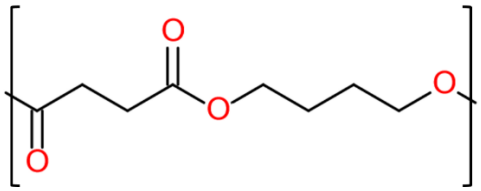

Figure 1.3: Structural formula of polybutylene succinate (PBS).

Poly(3-hydoxybutyrate-co-3-hydroxyvalerate) (PHBV)

PHBV is copolymerized from 3-hydroxybutanoic acid and 3-hydroypentanoic acid (**Figure 1.4**) and belongs to the polyhydroxyalkanoates (PHAs), a family of linear aliphatic polyesters composed of hydroxyalkanoate (HA) units (Li et al., 2016). Unlike other plastics that are chemically synthesized, PHAs can be obtained through bacterial fermentation by *Pseudomonas*, *Ralstonia,* and *Cupriavidus* strains (Oliveira et al., 2007; Mao et al., 2013; Wang et al., 2013). These microorganisms produce and accumulate PHA granules intracellularly, when they are deficient in trace elements and in excess of carbon (Lee, 1996). For the cultivation of these bacteria, various carbon-rich feedstocks from agricultural and food wastes can be used, contributing to a sustainable production process (Nikodinovic-Runic et al., 2013). PHAs show faster biodegradation than other biodegradable plastics (e.g. PLA), and their biodegradability properties have been demonstrated in compost, water, and soil (Mergaert et al., 1993; Luo et al., 2003). PHBV, in particular, has outstanding biodegradability and biocompatibility properties within the family of PHAs. Besides others, it is used for packaging, cosmetics, medical and hygiene materials (Rivera-Briso & Serrano-Aroca 2018). However, despite its promising characteristics, its use remains below expectations due to its high production costs (Li et al., 2016). The production costs of PHAs are three to four times higher than that of conventional petroleum-based plastics, preventing their market expansion (Kourmentza et al. 2017). Thus, research is focusing on optimizing the PHA production process in terms of effectivity and cost reduction (Kunasundari & Sudesh 2011; Wang et al., 2017).

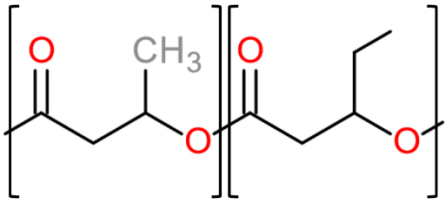

Figure 1.4: Structural formula of poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV).

Polybutylene adipate terephthalate (PBAT)

PBAT is an aromatic aliphatic polyester, synthesized by polycondensation from 1.4-butandiol with adipic and terephthalic acid (**Figure 1.5**). Currently, the reactants are primarily produced from fossil-derived raw materials (Ray, 2013). Here, the combination of an aliphatic component (adipic acid) with an aromatic component (terephthalic acid) results in a biodegradable polyester with good mechanical properties and thermal stability. PBAT shows similar properties as low-density polyethylene (LDPE) and has a higher flexibility than other biodegradable plastics such as PLA (Bordes et al., 2009; Nagarajan et al., 2013). However, products made from pure PBAT are lacking acceptable properties for many consumer products. PBAT is ideal for blending. Low-cost materials like starch and PLA are often added to improve its mechanical properties, while maintaining its biodegradability. PBAT-based products are used as packaging, cutlery, mulch films, and others (Jian et al., 2020). By introducing aliphatic components into the aromatic polyester chain, the PBAT material shows an increased hydrolytic susceptibility. PBAT is considered biodegradable and fulfills the criteria for complete compostability according to the definition by several international institutions (BPI, DIN CERTCO). It shows good biodegradability under elevated temperatures (Jian et al., 2020).

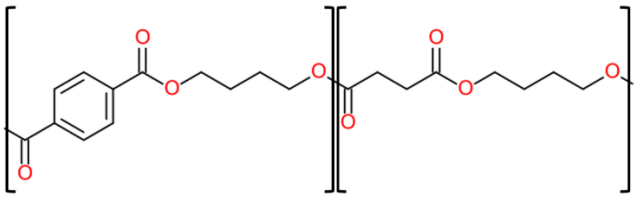

Figure 1.5: Structural formula of polybutylene adipate terephthalate (PBAT).

Particularly in recent years, the interest in plastics that are biobased or biodegradable has risen significantly, leading to a rapid increase in the production of these materials. Their global production is estimated to grow from around 2.23 million tons in 2022 to roughly 6.3 million tons in 2027 (European Bioplastics, 2022). This is mainly due to the ecological concerns emerging with the massive production of conventional petrochemical plastics and the concomitant limitation of fossil resources. Under the premise of enhanced sustainability, biodegradable plastics are advertised and marketed as a promising measure to counteract environmental plastic pollution. However, along with the increasing demand for biodegradable plastics, their utility and integration were more and more questioned (Yates and Barlow, 2013). The degradability of many "biodegradable"-labeled materials is under debate, since an effective degradation often can only occur under specific conditions, for example in industrial composting plants with high temperatures under anoxic conditions (Briassoulis and Dejean, 2010). Moreover, consumers may see less necessity in paying attention to a proper disposal of these materials, because of their "biodegradable" status (Taufik et al., 2020). Therefore, the likelihood of these materials entering the environment is the same as for conventional plastics, if not even higher. Microplastics originating from biodegradable plastics based on PLA and PBAT, the two most commonly used biodegradable polymers, have already been found in environmental samples from marine sediments (Okoffo et al., 2022). If and how fast these materials are degraded in natural environments such as the ocean, where the conditions for a rapid biodegradation seem to be rather unfavorable, is mostly unclear (Wang et al., 2021a).

1.3 Degradation of plastics

Plastics that are released into the environment are exposed to specific conditions that prevail on site. These conditions are decisive for whether a plastic is degraded and how rapidly it is degraded (Quecholac-Piña et al., 2020). In addition to abiotic factors such as pH, temperature, and UV radiation, biotic factors such as substrate availability, nutrients, and occurrence of polymer-degrading microbes are defining the complex process of biodegradation (Karamanlioglu et al., 2017). Besides the exposure conditions, there is a variety of other factors that also greatly affect biodegradability of a plastic (**Figure 1.6).** These are chemical properties (molecular weight, chemical structure), physical properties (crystallinity and crystal structure, melting point, glass transition temperature), but also the surface conditions (hydrophilicity, hydrophobicity, surface area) of the plastic (Tokiwa et al., 2009).

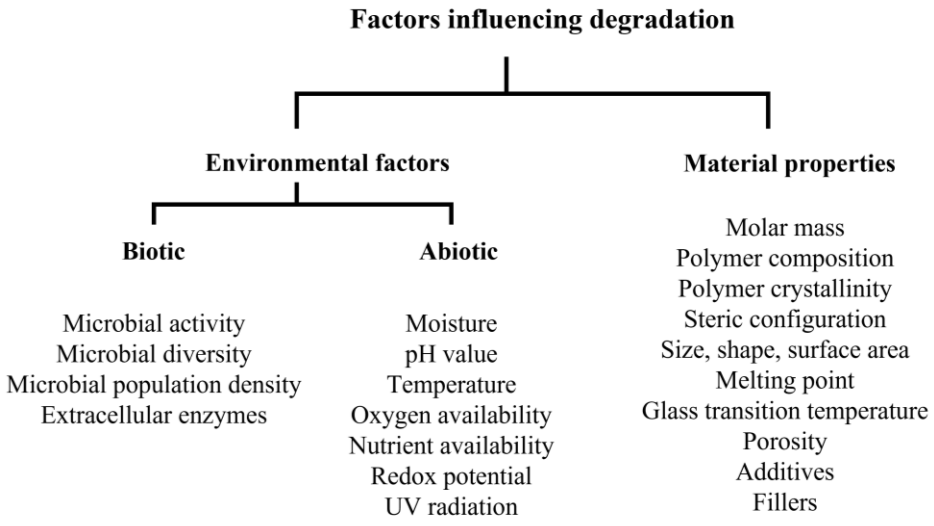

Figure 1.6: Factors influencing the degradation of plastics in the environment. Modified after Kliem et al. (2020).

The degradation of plastics in the environment is typically initiated by abiotic physical and chemical factors (Andrady, 2011). Sunlight and UV radiation, temperature fluctuations, mechanical abrasion, and the availability of water and oxygen facilitate the degradation (Chamas et al., 2020). Cracking and embrittling of the structure due to physical stress leads to the continuous fragmentation of larger plastic items to smaller particles in the micro- and nanometer range (**Figure 1.7**) (Andrady, 2017). In the presence of O_2 and H_2O, oxidation and hydrolysis of polymer chains can change the molecular structure of the plastics creating short chain degradation intermediates.

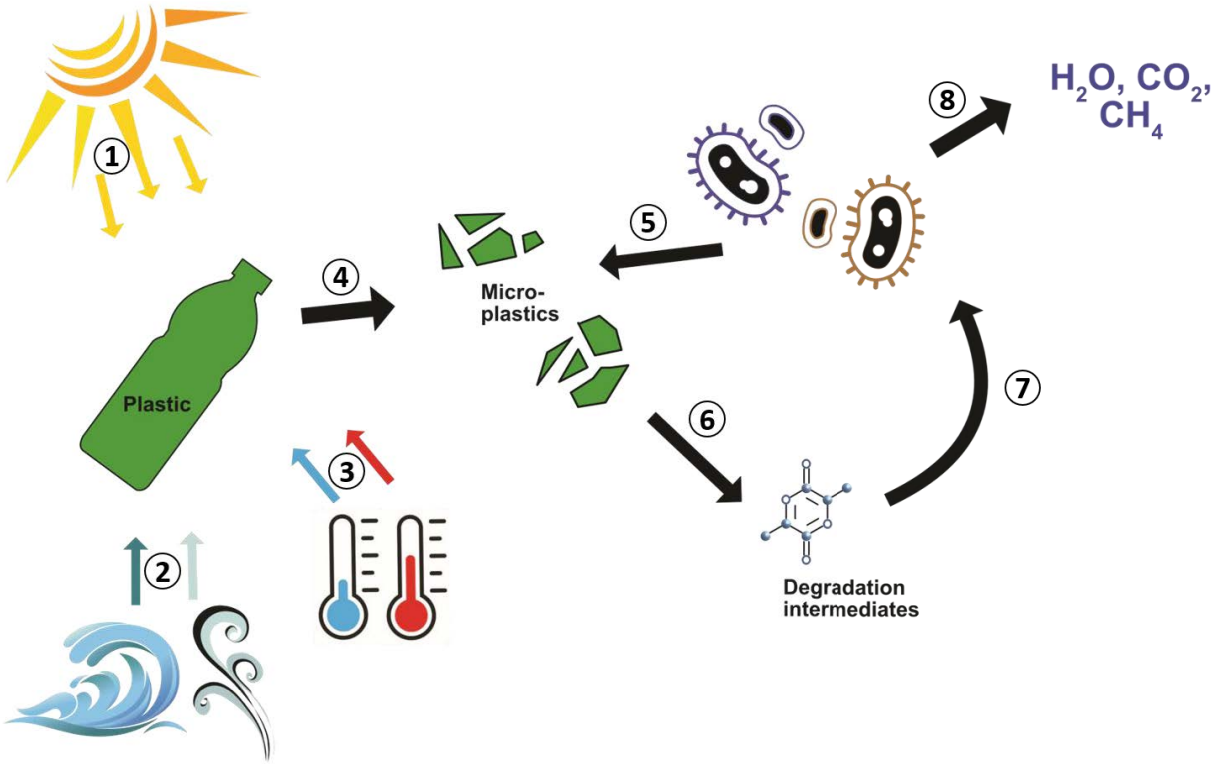

Figure 1.7: Degradation mechanisms of plastics in the marine environment. Abiotic factors such as UV radiation (1), mechanical forcing by waves and wind (2) and temperature fluctuations (3) fragment larger plastic items to microplastics (4). Microorganisms colonize the microplastics and secrete extracellular enzymes (5) that attach to the surface and catalyze the hydrolysis (6) of the polymer chain to degradation intermediates. These intermediates are assimilated by the microorganisms (7) and are finally metabolized to water, carbon dioxide or methane (8).

Simultaneously to degradation by abiotic factors, biotic degradation takes place. However, the microbial and enzymatic biodegradation is favored by a larger accessibility of the surface area of the plastic, which is typically increasing with decreasing particle size (Herzog et al., 2006). Therefore, biotic degradation is more effective after preceding fragmentation by abiotic factors. Microorganisms that colonize the surface of the plastic release extracellular enzymes, that attach to the surface of the polymer. Most enzymes that are capable of degrading plastics belong to the family of hydrolases. They catalyze the breakdown of chemical bonds of their substrate by the insertion of a water molecule (Gricajeva et al., 2022). Once attached on the surface, they catalyze the cleavage of long carbon chains of the polymer to low molecular weight oligomers, dimers, and monomers (Mueller 2006; Yamamoto-Tamura et al., 2015). Generally, the hydrolysis is considered the rate-limiting step in the biodegradation of plastics (Husárová et al., 2014; Wang et al., 2021b). Subsequently, the generated smaller subunits are assimilated by microorganisms and degraded to the end-products CO_2, H_2O, or CH_4 (Amobonye et al., 2021). In recent years, several enzymes from microorganisms have been identified that are capable of

degrading different types of plastics (**Table 1.1**). Such enzymes are released from microorganisms in marine sediments, seawater and other environments (Boetius 1995; Debashish et al., 2005; Patel et al., 2018). However, most of these enzymes were tested under optimal conditions for enzymatic activity, namely elevated temperatures and optimal pH-buffered incubation media. There is a lack of information as to whether the same enzymes would also be able to hydrolyze a plastic under non-optimal conditions, specifically in the marine environment. Furthermore, formulations of plastic products might include components that significantly affect the biodegradability (Emadian et al., 2017).

Table 1.1: Enzymes that are known to degrade common biobased biodegradable polymers

Polymer	Enzyme	Reference
PLA	Protease	Lim et al., 2005
	Proteinase K	Williams, 1981
	Lipase	Fukuzaki et al., 1989
	Pronase	Williams, 1981
	Bromelain	Williams, 1981
	α-Chymotrypsin	Lim et al., 2005
	Trypsin	Lim et al., 2005
	Elastase	Lim et al., 2005
PBS	Lipase	Lee et al., 2008; Ding et al., 2012, Zumstein et al., 2016
	Cutinase	Hu et al., 2016
	Proteinase K	Lim et al., 2005
	Cholesterol Esterase	Tserki et al., 2006
PHBV	Depolymerase	Timmins et al., 1997
	Cutinase	Gamerith et al., 2017
PBAT	Lipase B	Kanwal et al., 2022
	Esterase	Wallace et al., 2017

Breakdown of larger plastic items and biodegradation in the environment are largely attributed to abiotic factors and microorganisms. However, several recent studies suggest that macro- and megafauna is also contributing to the process of biofragmentation and degradation of plastics (So et al. 2022). It has been shown, that macrofauna can rapidly break down plastics by accidentally biting into and ingesting macroplastic debris or microparticles. Bite marks and tooth punctures on plastic pieces indicate mistaking of plastic with prey and, thus, fragmentation (Carson et al., 2013; Eriksen et al., 2016; Po et al., 2020). There is also evidence that fragmentation of plastic occurs in the digestive tracts of various organisms as the consequence of internal maceration (Huerta Lwanga et al., 2017; Porter et al., 2019, Lo et al., 2022). In crustaceans, for example, plastics are fragmented by the action of the gastric mill in

the stomach (Dawson et al., 2018; Saborowski et al., 2019; Cau et al., 2020; Mateos-Cárdenas et al., 2020). Depending on the mode of feeding, organisms, especially those representing the benthic fauna, are known to ingest plastics and microplastics (Bour et al., 2018). Fragmentation by macrofauna can happen in any habitat where consumers are present, and is a much faster process than fragmentation through weathering, as it is not dependent on UV radiation or microbial communities. The increase in surface-to-volume ratio of plastic by biofragmentation leads to an enhanced bioavailability and an increased surface for enzymatic attack (Wright et al., 2013). However, while there are some studies reporting chemical alteration of plastic in the gut of terrestrial organisms (Brandon et al., 2018; Peng et al., 2019, Lou et al., 2020; Peng et al., 2020; Wang et al., 2020b), studies about the degradation of plastics by digestive enzymes from marine macrofauna are scarce. However, enzymatic degradation by digestive fluids might play a bigger role for novel plastics with improved biodegradability. Higher crustaceans, for example, are known to have a broad range of highly active digestive enzymes that are capable of breaking down ester, peptide and glycosidic bonds (Saborowski, 2015; Vogt 2021).

1.4 Biodegradable plastics as alternative

With the goal of replacing conventional persistent plastics by materials that can be degraded by microorganisms and fungi, biodegradable plastics seem to be a viable alternative. Nevertheless, some obstacles are in the way of a complete replacement of conventional plastics by biodegradable plastics. There are economic and technical reasons why biodegradable plastics would not yet be able to cover all application sectors where conventional plastics are used. Production of novel biodegradable polymers such as PHAs is much more costly than their conventional counterparts (Li et al., 2016). However, the oil price is continuously increasing while the production costs of some biodegradable plastics slowly decline, narrowing this gap in production costs (Gill, 2014). As for the performance of biodegradable plastics, much research is done to refine the properties of biodegradable polymers, opening even more applications for these novel materials. However, performance is still lacking in terms of physical properties for most products, when compared to conventional polymers (Iwata, 2015). Therefore, they rely on blending with other polymers or the addition of various other additives (Khan et al., 2016). This is one of the reasons why the implementation of biodegradable plastics also raised environmental concerns.

Although biodegradable plastics are conceived as nontoxic alternatives to petroleum-based plastics, there are studies reporting toxic effects on biota, that originate from chemicals leaching

out of biodegradable plastics (Zimmermann et al., 2020a; Quade et al., 2022; Uribe-Echeverría and Beiras, 2022). Zimmermann et al. (2020b) showed that the majority of biobased and biodegradable plastics contain a high number of various chemicals and toxic compounds. Some additives, such as plasticizers, have been shown to be lethal for crustaceans and mollusks at very low concentrations in the µg/L and ng/L range (Oehlmann et al. 2009). Furthermore, mesocosm studies indicated a connection between toxicity and degradation of biodegradable plastics (Quade et al., 2022). There is limited information about the products formed during plastic degradation and their potential implications (Karlsson and Albertsson, 2004). However, it is suspected that the degradation processes of plastics alter their toxicity (Jahnke et al., 2017; Ouyang et al., 2021). While there is a multitude of studies investigating the effects of microparticles from conventional plastics, research on microparticles from biodegradable plastics and their impacts on marine organisms are scarce. The chemical properties of biodegradable plastics make them susceptible to hydrolysis by a variety of enzymes, raising the question of the consequences after ingestion by animal consumers. Breaking down ingested plastics mechanically and chemically through the activity of digestive enzymes could result in the release of harmful degradation products (Degli-Innocenti et al., 2001). Although there are studies classifying the leachate exposure after ingestion of conventional microplastic as negligible for fish (Koelmans et al., 2014), the leaching of substances might be higher from plastics with enhanced biodegradability. Uribe-Echeverría and Beiras (2022) reported acute toxicity of leachates from a biodegradable plastic on sea urchin larvae from *Paracentrotus lividus*. There are only few impact studies with biodegradable microplastics and marine invertebrates, e.g. by Green (2016) and Green et al. (2016) with the marine lugworm *Arenicola marina* and the oyster *Ostrea edulis* under uncontrolled field conditions. In *A. marina*, exposition to microplastics reduced biological activity independent of conventional or biodegradable plastic. For *O. edulis*, the presence of PLA particles increased respiration rates of the oysters. Particularly, there is a lack of data regarding ingestion and toxicity of biodegradable microplastics by marine organisms under controlled laboratory conditions.

To gain a better understanding of the potential use and limitations, but also the impacts of biobased biodegradable plastics, the EU Horizon 2020 project “Bioplastics Europe” (BPE) was launched in October 2019. BPE is an interdisciplinary project with the goal of supporting the EU-Plastic Strategy and promoting a circular plastic economy, by providing sustainable strategies and solutions for biobased biodegradable plastics. The framework of this project includes the design of innovative products, developing health, safety standards and end-of-life solutions, and assessing the environmental and economic product life cycles. The goal is to

develop business models to efficiently reuse and recycle biobased biodegradable plastics, while ensuring safety for environment and society (Bio-Plastics Europe, 2022). For this reason, five plastic compounds were exemplarily provided by manufacturers involved in this project for experimental and analytical data collection. These compounds are based on different polymers that are commonly used in biodegradable plastic products (see section 1.2). The intended applications of the end products from these compounds range from rigid and soft packaging, to agricultural mulch films, toys, and cutlery. The work conducted in this thesis is directly connected to the work package 5 embedded in the BPE project, where pre-normative research is performed on the degradability of these compounds as well as on the potential impacts of these biodegradable biobased plastics and their chemicals released during degradation.

1.5 Objectives

The present thesis aims at investigating the degradability of biobased biodegradable plastics under marine conditions and their potential impacts on marine organisms. This study will provide further insight into the enzymatic degradation of biodegradable plastics under marine conditions, investigating five selected compounds. The uptake of biodegradable microparticles by several marine invertebrates, which may encounter microplastics in the environment, is examined. A subsequent screening with invertebrates exposed to microplastics and plastic leachates should clarify whether the biodegradable plastics release toxic chemicals. In addition, it will be investigated if ingested biodegradable microparticles are hydrolyzed by digestive enzymes from crustaceans, with a focus on the edible crab *Cancer pagurus*. The information generated in this work will thus be beneficial for the further development towards maximum sustainable biodegradable biobased compounds.

1.6 Research questions

Considering the background summarized in the previous sections, several research questions were defined to contribute to the overall objective of this thesis. These questions are targeted at biodegradable plastics in general, but will be exemplarily investigated using the five plastic compounds provided within the BPE project.

Research question I

How is the (enzymatic) degradability of biodegradable plastics affected by marine environmental conditions?

To address this question, a novel pH-Stat assay was developed to rapidly screen the *in-vitro* enzymatic degradability of the different compounds in artificial seawater. Different hydrolytic enzymes were deployed, that have an equivalent specificity to enzymes synthesized by marine microorganisms. The hydrolysis of the compounds by these enzymes was measured at relevant seawater temperatures and compared to conventional synthetic and natural polymers. Furthermore, samples of the compounds were incubated in estuarine mud and seawater under controlled conditions to determine the degradation under quasi-natural conditions.

Research question II

Are gastric enzymes in the stomach of marine invertebrates capable of hydrolyzing ingested microplastics originating from biodegradable plastics?

To investigate the hydrolysis of the plastic compounds by digestive enzymes, gastric fluid from the edible crab *Cancer pagurus* and the american lobster *Homarus americanus* were withdrawn and applied in *in-vitro* assays. Gastric enzymes of the edible crab were separated by several analytical methods to further identify and characterize those enzymes that are capable of hydrolyzing biodegradable plastic. Furthermore, rockpool shrimp *Palaemon elegans* were fed with microparticles from the plastic compounds and digestive enzyme activities in the midgut gland of the shrimp are measured.

Research question III

Do biodegradable plastics exhibit toxic effects on marine invertebrates of different trophic levels?

To assess the potential toxicity of the plastic compounds on marine invertebrates, two different approaches were pursued. First, an ISO standardized mortality test with the rotifer *Brachionus plicatilis* was performed with leachates from the five different compounds. This procedure was then extended and applied to nauplii of the brine shrimp *Artemia persimilis*. For comparison, virgin polymers used in the plastic compounds and common additives were subjected to the same test procedure. Secondly, microparticles from conventional plastics and the project plastics were fed to rockpool shrimp *Palaemon elegans* to investigate the induction of oxidative stress at different time points after feeding.

2 Material and methods

This chapter summarizes the materials used and methods applied in this thesis. Methods which were already described in detail in the publications or manuscripts, are addressed only briefly. Experimental approaches and information, which were not included in the publications, are outlined in detail in the following sections.

Plastic compounds

The plastic materials used in this study were produced within the framework of the Horizon 2020 EU-project "Bio-Plastics Europe (BPE)". The materials were provided by the project partners Arctic Biomaterials OY (ABM, Tampere, Finland) and Natureplast SAS (Ifs, France). Five biobased biodegradable compounds, that differ in their chemical composition and potential application, were selected for the present study (**Table 2.1**). The detailed composition of polymers and additives used in the compounds is confidential and was not disclosed by the manufacturers.

Table 2.1: Specification of the compounds used for this thesis (taken from Publication II).

Designation	Polymer type	Producer	Application
BPE-C-PLA	PLA/PBS	ABM	Cutlery
BPE-RP-PLA	PLA/PBS	ABM	Rigid packaging
BPE-AMF-PLA	PLA/PBAT	Natureplast	Agricultural mulch films
BPE-SP-PBS	PBS	Natureplast	Soft packaging
BPE-T-PHBV	PHBV	Natureplast	Toys

Preparation of microparticles by cryogenic milling

The experimental assessment of plastics in degradation and exposition assays is facilitated by the use of microparticles. Therefore, the plastics compounds examined in this thesis were ground to a fine powder by cryogenic milling (6775 Freezer/Mill, Spex SamplePrep). Detailed information on the grinding procedure and processing of the plastics is given in **Publication II**. In this thesis, I used microplastics smaller than 200 µm, which were isolated from the ground material by sieving through a gaze with a mesh size of 200 µm. This size fraction was used because previous investigations of stomach contents of shrimp have shown, that the majority of ingested particles is smaller than 200 µm (Korez et al., 2020).

2.1 Degradation tests under marine conditions

To obtain a comprehensive understanding of the biodegradability of the five plastic compounds, several degradation experiments under different conditions were carried out. These include *in-*

vitro assays on the enzymatic hydrolysis of the polymers as well as the degradation of plastic items in estuarine mud and seawater under controlled laboratory conditions.

2.1.1 *In-vitro* enzymatic degradation

Many common test methods for assessing the degradation rates of plastics in the environment are laborious and time consuming. In contrast, enzymatic *in-vitro* tests are non-elaborate and deliver results in a short period of time. However, they are mostly conducted under quite artificial environmental conditions. To overcome this lack of transferability of results and effort, I introduced an improved pH-Stat titration assay to quantify the enzymatic hydrolysis of synthetic and natural polymers in artificial seawater.

pH-Stat titration

The rate of enzymatic hydrolysis of the plastic compounds was measured by pH-Stat titration as described in detail by Miksch et al. (2021) (**Publication I**). Briefly, the principle of the pH-Stat titration is based on the release of carboxylic groups by enzymatic cleaving of the polymer, resulting in an acidification of the medium during degradation of the plastics and a continuous counter-titration with a base. The hydrolysis rate of a plastic is then calculated from the base added over time. Microparticle (< 200 µm) suspensions of the plastic compounds were incubated with different commercially available hydrolases, which are similar to hydrolases found in marine microorganisms (**Table 2.2**). Measurements were conducted in artificial seawater (salinity: 32 ppt) and at environmentally relevant temperatures between 4 and 30 °C.

Table 2.2: Specification of commercially available enzymes used in this thesis (taken from Publication II).

Enzyme	Source	Enzyme activity		Distributor	Cat. No.
Esterase	*Bacillus subtilis*	10	$U \cdot mg^{-1}$	Sigma	96667
Lipase	*Candida antarctica*	9	$U \cdot mg^{-1}$	Sigma	62288
Protease	*Bacillus licheniformis*	2.4	$AU \cdot mg^{-1}$	Sigma	P4860

Plate clearing assay

Plate clearing assays with plastic compounds were performed as a visual semi-quantitative measure of enzymatic polymer degradation. Detailed information about the the assay is given in **Publication II**. Briefly, plastic emulsions were prepared by dissolving the materials in dichloromethane and subsequent mixing with water. The dichloromethane was then evaporated and agarose was added to the aqueous suspension. To initiate polymerization, the suspension was heated and gently shaken until the agarose dissolved. The suspension was poured into a

petri dish. Hydrolytic enzymes were then added onto the surface of the plate. Degradation of the plastics was indicated by the formation of clear halos around the spotted enzymes.

2.1.2 Degradation under controlled conditions

To compare the *in-vitro* enzymatic degradation rates of the BPE-compounds with the degradation in marine environments, specimens of the plastic compounds were incubated in estuarine mud and seawater under controlled conditions.

Incubation in natural estuarine mud

Natural estuarine mud for degradation experiments was collected from the Weser estuary off Bremerhaven (53°32'21.3" N 8°34'33.2" E) in November 2020. The intertidal sampling area was accessible during low tide (**Figure 2.1**). Sediment of the upper 20 cm was taken with a spade and transferred into 20-L buckets. For the transport to the lab, the mud was covered with a thin layer of estuarine water to prevent drying.

Four dumb-bell shaped bars of each compound were incubated for six months in the natural untreated mud from the Weser estuary. The mud was evenly distributed in a box (0.55 x 0.35 x 0.3 m, length, width, height) to form a layer of 15 cm. The plastic-bars were placed on top of the layer with sufficient distance (5 cm) between each other. Subsequently, the bars were covered by another layer of mud of about 10 cm. About 1 L of deionized water was sprayed on the mud surface every two weeks to prevent the mud from drying. Redox potential, temperature, and pH were monitored at approximately 10 cm depth every week.

Incubation in autoclaved estuarine mud

To examine whether degradation occurs due to biotic or abiotic hydrolysis, material samples of approximately 1 cm² were cut out from untreated BPE-T-PHBV-bars and incubated in natural and autoclaved mud from the Weser estuary. Glass jars, water, mud, and all other tools used in preparation of this experiment were autoclaved at 121°C for 40 minutes. Approximately 350 g of natural or autoclaved mud was placed in each of six glass jars (0.75 l volume) and autoclaved water was added to form a thin layer of 1 cm on top. Three samples of BPE-T-PHBV were added to each glass jar and were buried approximately 5 cm into the mud, with approximately 4 cm distance between each other. One glass jar with three plastic samples was examined each month for up to six months.

Figure 2.1: (a) Satellite image of the Weser estuary (taken from https://www.copernicus.eu/en/media/image-day-gallery/weser-river-estuary-and-bremerhaven-port-germany). The sample site at the eastern shore of the river near Bremerhaven, Germany, is depicted with red crosshairs (53°32'21.3" N 8°34'33.2" E). (b) Close-up of the sampling site. The spot is only accessible during low tide.

Incubation in seawater

Four dumb-bell shaped bars of each of the BPE materials were incubated for six months in a basin (0.6 x 0.4 x 0.4 m, length, width, height) with natural seawater that was connected to a recirculating flow-through system with a total volume of 160 L. The bars were fixed and tightened with nylon fishing line within a rack, to avoid contact between the bars and between the bars and the tank. The basin with the rack was placed in a temperature-controlled room at 15 °C and a light/dark cycle of 12:12 hours. The salinity of the water was monitored with a refractometer (Atago, Tokyo, Japan) every two weeks and adjusted to 35 ppt by adding deionized water, if necessary. The pH, temperature, and redox potential of the seawater were monitored every two weeks with a pH meter (pH 3110, Xylem Analytics, Weilheim, Germany)

connected to a SenTix® 41 pH electrode (Xylem Analytics, Weilheim, Germany) or a double pore redox electrode (Hamilton AG, Waengi, Switzerland). Concentrations of ammonium, nitrite, and nitrate were measured monthly in the laboratory of the Zentrum für Aquakultur-forschung of the Alfred Wegener Institute in Bremerhaven with an automated chemical analyzer (QuAAtro39 AutoAnalyzer, SEAL Analytical GmbH, Germany).

Analysis of degradation parameters

After incubation for up to six months in seawater and in mud, the plastic-bars and T-PHBV samples were removed, carefully rinsed with deionized water, dried, and stored separately in sealed freezer bags. Plastic-bars and T-PHBV samples were dried at 40 °C for 24 hours and weighed to identify mass changes of the samples after incubation. Samples of approximately 1 cm^2 were cut out from one bar of each plastic compound, glued on SEM-stubs with conductive carbon pads (Plano GmbH Wetzlar, Germany) and sputter-coated with gold/palladium. The same procedure was applied to the monthly T-PHBV samples taken from the natural and autoclaved mud. Scanning electron micrographs (SEM) of the surfaces were taken with a FEI Quanta 200 device. Plastic-bars which were not used for SEM analyses were used to identify the changes in mechanical properties after incubation. Maximum tensile strength of the incubated and untreated (control) plastic-bars were tested at the Laboratory of Material Science at the University of Applied Sciences in Bremerhaven with a servo-hydraulic testing machine Zwick HC25 (Zwick/Roell, Germany) following DIN EN ISO 527-1. Briefly, the plastic-bars were clamped at both ends into the servo-hydraulic testing machine and tension was applied by pulling on both ends until the bars reached their breaking point.

2.1.4 Enzyme screening of estuarine mud

To identify enzymatic activity in the sediment that might play a role in the degradation of the plastic-bars, sediment samples were collected in May 2022 from the same site where the mud for the incubation experiments was taken. Samples were taken with a small shovel from the surface. Additional samples of fine and coarse sediment, respectively, were taken at 10 cm depth. The sediment was collected in glass jars (0.75 L) and immediately transported to the lab for analysis.

Api®Zym strips (BioMerieux, Marcy l'Etoile, France) were used for semi-quantitative identification of enzyme activities in the mud samples. The Api®Zym system is made of microcupules containing substrates for 19 different enzymes. After addition of enzyme extracts, the respective substrate in the microcupules is metabolized. After incubation, the metabolic end products are detected through colored reactions. Extracts of the mud were prepared after Patel

et al. (2018) with slight modifications. Twenty grams of the mud were weighed into a 250-mL glass beaker and filled up with 0.9 % saline solution to the 100-mL mark. The suspension was then stirred in the glass beaker for one hour at room temperature before it was filtered through Whatman filter paper (GE Healthcare UK Limited, Little Chalfont, United Kingdom, Cat. No. 10334553). Additionally, extracts were filtered through a syringe filter to remove remaining microparticles. 65 µL of the double-filtered extracts were then dispensed into each of the 20 microcupules of the Api®Zym strip. The strips were then covered and incubated at room temperature. After 24 hours, ZYM A and ZYM B reagents were added to the microcupules to induce the color reaction. The results of a positive reaction were graded as low, medium or high according to the intensity of the color.

2.2 Plastic hydrolysis by gastric fluids and analyses of digestive enzymes

In addition to the enzymatic degradation of the BPE-materials by hydrolytic enzymes from microorganisms in the environment, their hydrolysis by gastric fluid from marine decapod crustaceans was investigated.

2.2.1 Extraction of gastric fluid and *in-vitro* degradation assays

Gastric fluid from live crabs (*Cancer pagurus*) and lobsters (*Homarus americanus*) was used to test the degradability of plastics by digestive enzymes. Detailed information about the isolation of gastric fluid from adult crabs is given in **Publication III (Manuscript 1)**. Gastric fluid from lobster was withdrawn in the same way. The crabs were collected by beam trawling with the research vessel FK Uthörn in the North Sea south of the island of Helgoland, Germany. The live adult lobster was purchased from a local seafood merchant in Bremerhaven, Germany. Crabs and lobster were transferred to the laboratories in Bremerhaven where they were maintained in separate flow-through aquaria (60 and 180 L) within a recirculating seawater system (500 L). Body mass (wet weight) and carapace width of the crabs ranged from 300 to 1100 g and 12.5 to 18 cm, respectively. Body mass of the lobster was 1420 g, with a body length of 28.5 cm (rostrum to telson). The animals were fed with frozen fish and shrimp three times a week. Gastric fluids of up to 2 mL were extracted 48 hours after feeding by introducing a flexible plastic tube connected to a syringe through the esophagus into the gastric chamber of the animals (**Figure 2.2**). Gastric fluids were then slowly aspirated, transferred to reaction tubes, and centrifuged. The supernatant was transferred into new tubes and frozen at -80 °C for further use.

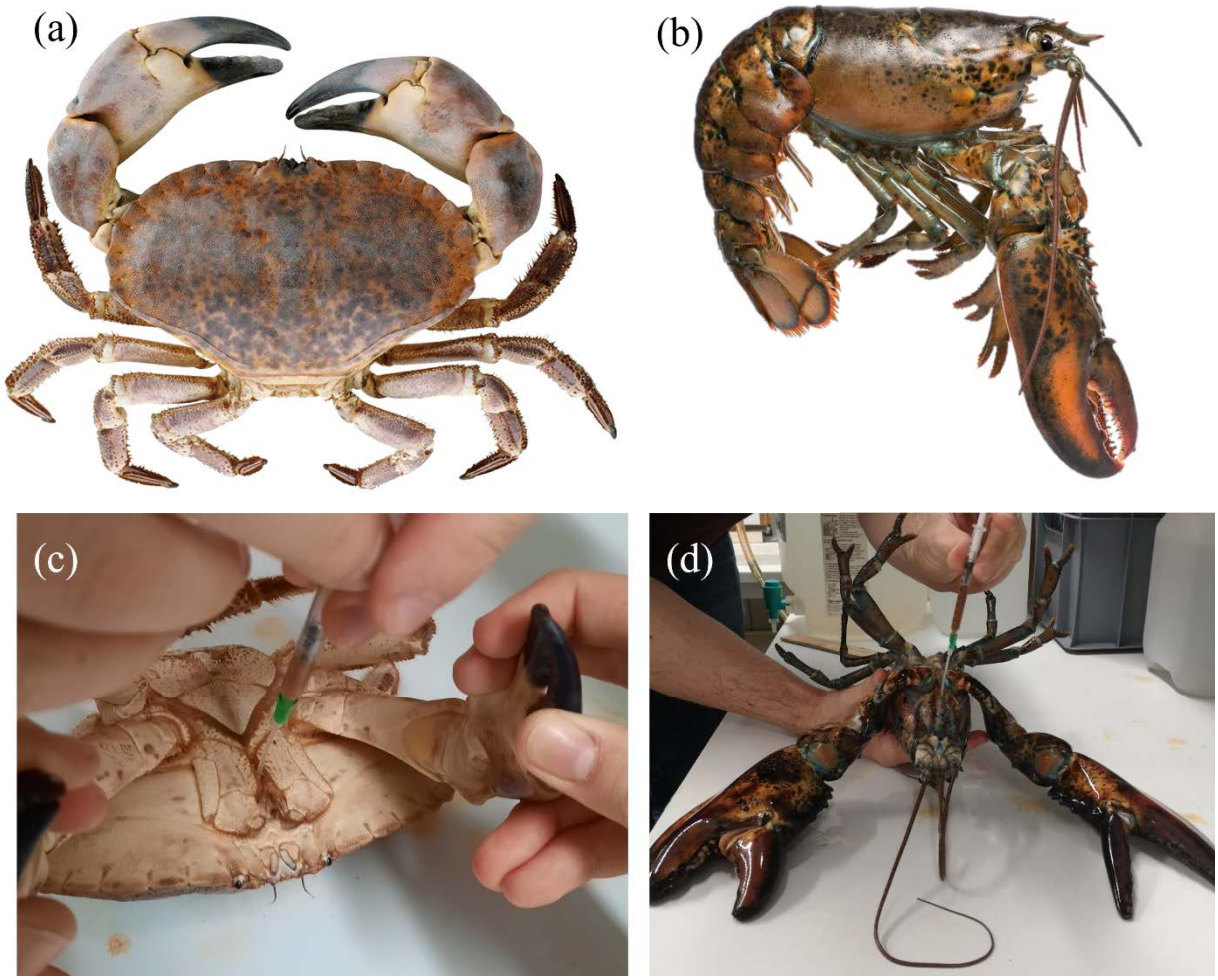

Figure 2.2: Dorsal view of *Cancer pagurus* (a) and lateral view of *Homarus americanus* (b). Extraction of gastric fluid from the gastric chamber with a plastic tube and syringe from *C. pagurus* (c) and *H. americanus* (d). Picture (a) and (b) are taken from https://www.wir-fischen.sh/produkte/detailansicht/taschenkrebs/ and https://adobe.ly/3GtGb05, respectively.

For the pH-Stat titration assay, the same approach based on **Publication I** was used, albeit with gastric fluid from *C. pagurus* and *H. americanus* instead of commercially available enzymes. A detailed description of the procedure is given in **Publication III (Manuscript 1)**. Briefly, aliquots of gastric fluid were slowly thawed on ice and incubated with microparticle suspensions of the five BPE-materials. The addition of base to counteract the acidification of the suspension by the release of carboxylic degradation products was recorded and used to calculate the hydrolysis rate. Enzyme blanks of the gastric fluids without microplastics were measured and subtracted from the hydrolysis rate.

2.2.2 Protein separation and enzyme activities

Proteins (enzymes) from the gastric fluids of *C. pagurus* were separated by different methods, to obtain an overview of the individual enzymes that would potentially be able to hydrolyze the

BPE-materials. First, the enzymes in the gastric fluid were separated by anion exchange chromatography into different fractions according to their change. The obtained fractions were then analyzed for carboxylesterase activities. Carboxylesterases are hydrolytic enzymes that are widely distributed in nature and are known to catalyze the hydrolysis of carboxylic esters. Because of their substrate specificity, this group might be a promising candidate to also hydrolyze ester bonds of biodegradable polymers. Fractions with high activities were identified and used for further separation by gel electrophoresis, where the single proteins in a fraction were separated according to their molecular mass. In the following sections, these single steps are described briefly. A detailed description is given in **Publication III (Manuscript 1)**.

Anion exchange chromatography

The proteins (enzymes) in the gastric fluid of *C. pagurus* were separated according to their charge. The gastric fluid samples were first desalted and rebuffered with a gel filtration column, before they were separated by anionic exchange chromatography with a BioRad NGC system equipped with an anion exchange column (BioRad, UNO Q6R). Elution of the proteins was conducted by increasing the NaCl concentration of the elution buffer from 0 to 1 $mol \cdot L^{-1}$, resulting in a separation of the gastric fluid into 65 fractions of 1 mL each.

Carboxylesterase activity assay

The activity of carboxylesterases in the gastric fluid of *C. pagurus* and in the separated fractions of the gastric fluid were assayed with fluorogenic substrates based on 4-methylumbelliferone (MUF) derivatives of fatty acid esters (MUF-butyrate, MUF-heptanoate, MUF-oleate). MUF derivatives were dissolved in dimethyl sulfoxide (DMSO) and diluted in Britton-Robinson buffer at pH 5 to 9 with pH steps of 1. The stock solutions of the MUF derivatives were pipetted into a 96-well microplate and samples of gastric fluid or gastric fluid fractions were added. The increase in fluorescence was measured with a microplate reader and compared with standard curves of 4-methylumbelliferone. Fractions with high carboxylesterase activities were pooled, concentrated and subjected to pH-Stat titration as described above, to investigate their hydrolytic potential on the BPE-materials.

Gel electrophoresis (SDS-PAGE)

Selected fractions of the gastric fluid were separated according to their mass by native SDS-PAGE. Samples of the fractions were mixed with sample buffer (SDS and bromophenol blue) and loaded onto polyacrylamide gels. A molecular weight marker was added to the leftmost and rightmost lane of each gel. After electrophoretic separation, the gels were washed in

demineralized water and buffer. Subsequently, the gels were incubated in an aqueous solution of the fluorogenic substrates (MUF-derivatives) to visualize protein bands that hydrolyze fatty acid esters. After another washing cycle with demineralized water and buffer, the gels were Coomassie-stained overnight.

2.3 Uptake of biodegradable microplastics by aquatic invertebrates

The uptake of microparticles from conventional plastics is documented for a multitude of aquatic organisms (Cole et al., 2013). However, to clarify whether aquatic invertebrates also ingest microparticles from biodegradable plastics, the uptake of fluorescence-dyed microplastics from the five BPE-compounds was investigated for invertebrates of different sizes and trophic levels.

Microparticles of the ground BPE-compounds were stained with Nile Red after Shim et al. (2016). Briefly, 500 µg Nile Red (Sigma Cat. No. 72485) was dissolved in 100 mL ethanol (99.8 %). 50 mg of ground biodegradable plastics (< 200 µm) were added to 5 mL of the Nile Red solution. After incubation for 24 hours in a closed 20-mL glass vial, the ethanol was evaporated with a nitrogen gas stream under a fume hood. The stained particles were then suspended in 2 mL deionized water, transferred to a reaction tube, vortexed for 10 seconds, and subsequently centrifuged at 10,000 g for 5 min. The supernatant was discarded and the reaction tube was refilled to 2 mL with deionized water. This step was repeated twice to clean the particles of ethanol or dye remains. The microparticles were then used to feed zooplanktonic organisms and decapod crustaceans.

2.3.1 Uptake by zooplankton

Water flea, *Daphnia pulex*, adult brine shrimp, *Artemia franciscana,* and opossum shrimp, *Mysis* spec.*,* were purchased from Mrutzek Meeresaquaristik GmbH, Ritterhude, Germany. The rotifer *Brachionus plicatilis* and nauplii of the brine shrimp *Artemia persimilis* were hatched from cysts provided by Microbiotests (Gent, Belgium, order code TK22) and Black Label (REBIE-Onlineshop, Bielefeld, Germany), respectively. *Artemia* nauplii used were 72 h and *B. plicatilis* used were 24 h old.

Daphnia pulex and adult *A. franciscana* were maintained in 600-mL glass beakers filled with 400 mL tap water and 400 mL tap water with 9.1 g *Artemia* salt (Artemio Sal, JBL, Neuhofen, Germany), respectively. *Mysis* spec. were kept in an aquarium (11 × 17 × 11 cm) filled with 2 L of brackish water prepared from deionized water and 2 % sea salt (Seequasal, Münster, Germany). Adult *Artemia* and *Mysis* spec. were fed with algae food flakes (NovoVert, JBL,

Neuhofen, Germany), whereas *D. pulex* were fed with microalgae. Individuals of all three species were maintained at 10 ± 1 °C and a 12:12 h light:dark cycle with continuous aeration. Rotifers were kept in artificial seawater (**Table 2.4**) in 42-well cell-culture plates at 25 °C in darkness. *Artemia* nauplii were raised in saline water (23 % salinity) which was prepared with tap water and *Artemia* salt (Artemio Sal, JBL, Neuhofen, Germany).

During the experiments, the nauplii were kept individually in 24-well plates at 25 °C under continuous illumination. To each well, either 20 µL of 100 $mg \cdot mL^{-1}$ green fluorescent microbeads (G1000, 9.9 µm diameter, Fluoro-Max™, ThermoFisher) or 30 µL of 25 $mg \cdot mL^{-1}$ Nile Red stained microplastic suspensions were added. In a second approach, *Mysis* spec. and adult *Artemia franciscana* were fed with food flakes (NovoVert, Neuhofen, Germany), which were coated with microbeads or microplastics. Food flakes of 5 mg were spiked with 10 µL of green fluorescent microbeads (100 $mg \cdot mL^{-1}$) or 15 µL of Nile Red-dyed microplastic (25 $mg \cdot mL^{-1}$), before they were placed in the wells with the animals. After one to eight hours of exposure, the animals were transferred onto microscope slides and were observed under a fluorescence microscope (Nikon Instruments, SMZ25) with TRITC (530-560 nm excitation, 590-600 nm emission) and FITC (460-500 nm excitation, 510-560 nm emission) fluorescence filters.

2.3.2 Uptake by decapod crustaceans

Common ditch shrimp *Palaemon varians* were purchased from Mrutzek Meeresaquaristik GmbH, Ritterhude, Germany. The shrimp were kept in an aquarium (11 × 17 × 11 cm) filled with 2 L of brackish water prepared with deionized water and 2 % sea salt (Seequasal, Münster, Germany). *Palaemon varians* were maintained at 10 ± 1 °C and a 12:12 h light:dark cycle and were fed with food flakes (NovoVert, JBL, Neuhofen, Germany). Half of the water in the aquaria was exchanged every week by new water. Each vessel was equipped with continuous aeration.

Adult specimens of the rockpool shrimp *Palaemon elegans* were collected in August 2022 in the Swedish Gullmarsfjorden at Fiskebäckskil near Kristineberg Marine Research Station (58°14’52.3”N 11°26’48.9”E, **Figure 2.3**) by fishing with landing nets. The water temperature in the bay where the shrimp were collected was around 19 °C. Collected shrimp were transported in thermos-containers to a temperature-controlled room of 15 °C and a 15:9 h light:dark cycle. The animals were first kept in the thermos-containers, to let them gradually acclimatize to the lower temperature. After 24 hours, they were transferred into two tanks (60 L each) with a continuous flow-through of cooled surface water of 15 °C from the

Gullmarsfjorden. Not more than 40 animals were simultaneously maintained in each tank. After further 48 hours, a total of 20 randomly selected animals were transferred from the tanks to single glass jars (0.75 L) filled with 500 mL of the same water. Each glass jar was continuously aerated.

Figure 2.3: (a) Satellite image of the Gullmarsfjorden near Skaftö Island (picture taken from Mapcarta). The sample site in the bay next to Kristineberg Marine Research Station is depicted with red crosshairs. (b) Close-up of the sampling site (58°14'52.3"N 11°26'48.9"E).

Palaemon varians were exposed to either 100 mg·mL^{-1} green microbeads or 30 µL of 25 mg·mL^{-1} Nile Red stained microplastics, and also fed with food flakes spiked with the microbeads and -particles as described previously. After one to eight hours of exposure, the animals were transferred onto microscope slides and inspected under a fluorescence microscope to detect the fluorescent particles (Nikon Instruments, SMZ25) with TRITC (530-560 nm excitation, 590-600 nm emission) and FITC (460-500 nm excitation, 510-560 nm emission) fluorescence filters.

Palaemon elegans were fed with 5 mg of food flakes (NovoVert, JBL, Neuhofen, Germany), which were coated one day before feeding with 2.5 mg of fluorescence-dyed microplastic particles (BPE-AMF-PLA, BPE-T-PHBV, and LPDE) and 100 µL of deionized water to aggregate the particles to the food flake. The processed food was dried over night at room temperature. Food flakes without particles were used as control. After 8 hours of access to the

food items, the shrimp were removed from the jars and dissected. The stomach was removed and frozen at -20 °C for further analyses. To extract microplastics from the tissue, the stomachs were homogenized with a mortar in a reaction tube and a density separation using sodium iodide solution (1.8 $kg \cdot L^{-1}$) was conducted. The solution was then filtered through a Polycarbonate Track-Etched black disk membrane (PCTE) with 0.2 µm pore size and 25 mm diameter (GVS, Maine, USA) and the remaining microplastics were counted under a fluorescence microscope (LEITZ DMRBE, 301-371.011, Leica, Wetzlar, Germany). Because of the high numbers of microplastics, ten random pictures of the filter at 5x magnification were made using the microscope camera (Axiocam 705 color, ZEISS, Jena, Germany). The average count of microplastics on the image areas (1.79 mm^2) was then extrapolated to the filter area covered by the samples (314.16 mm^2).

2.4 Toxicity experiments with marine invertebrates

Toxicity tests with marine invertebrates were conducted to screen for toxicity of chemicals potentially leaching out of the BPE-compounds. Organisms were exposed to leachates of the BPE-compounds and to commercial additives, which are commonly used in the production of biodegradable plastics. In the following, a short overview is given about the exposure conditions, the test organisms, and the test procedures. For a more detailed description refer to **Publication IV (Manuscript 2).** Furthermore, the effects of biodegradable microplastic ingestion were compared with those of conventional microplastic ingestion. Therefore, *P. elegans* were fed with microplastics based on LDPE, PHBV and PLA/PBAT. At different time points after microplastic ingestion, the antioxidant response in the midgut gland of the shrimp were analyzed. Furthermore, digestive enzyme activities in the midgut glands were detected.

2.4.1 Leachate exposure experiments

For preparation of the plastic leachate media, milled particles (< 200 µm) of the five BPE-compounds were chosen because of their high surface/volume ratio, which enhances leaching. Fifty mg of microplastic particles of each compound were placed in a 15-mL glass test tube. Ten mL of liquid (either rotifer medium or tap water, see Section 2.3.1) were added to the test tube. Pure medium or tap water without microplastics was used as control. The test tubes were closed with Teflon sealed screws and placed in a rotating mixer for 24 hours at 20 ± 1°C. The resulting media were filtered to remove residual particles. For the *Artemia* medium, *Artemia* salt (Artemio Sal, JBL, Neuhofen, Germany) was added after filtering to avoid depletion of

algae nutrients. The respective leachate-containing medium was then used for toxicological tests with rotifers or *Artemia* nauplii.

BPE-compounds that showed enhanced toxicity to any of the test organisms, were subsequently tested for the toxicity of their base polymers. PHBV pellets were kindly provided by Natureplast (Ifs, France) and were fragmented by cryogenic milling in the same way as the corresponding plastic blends. Suspensions of polymer particles and medium were prepared as described for the plastic leachate media with the BPE-compounds. The medium was used for toxicological tests with rotifers and *Artemia* nauplii.

Five commonly used additives were selected to evaluate their toxicity for marine organisms (**Table 2.3**). The additives were kindly provided by ArcticBiomaterials (ABM, Tampere, Finland) and are commonly used in the production of biodegradable plastics. Additives were filled into test tubes and liquid (either rotifer medium or tap water, see 2.3.1) was added. For the control, only medium or tap water without additive was used. The test tubes were placed in a rotating mixer and processed as described for the plastic leachate media. The respective medium with the dissolved additive was then used for toxicological tests with rotifers and *Artemia* nauplii.

Table 2.3: Specifications of the additives used for toxicity testing.

	Benzophenone	CT-L03	Elvaloy™ PTW	Exolit® AP 422	Glycerol triacetate
Application	UV stabilizer	Melt strength enhancer	Impact modifier	Flame retardant	Plasticizer
Producer	Sigma	Polyvel Inc.	Dow Chemical	Clariant	Sigma
Cat. no.	B9300	-	-	-	1.08238
State/form	solid/powder	solid/pellet	solid/pellet	solid/powder	liquid
Concentration	0.1 $mg \cdot mL^{-1}$	10 $mg \cdot mL^{-1}$	100 $mg \cdot mL^{-1}$	1 $mg \cdot mL^{-1}$	4.3 $\mu l \cdot mL^{-1}$

Mortality test with Brachionus plicatilis (ISO 19820)

Toxicity tests with the rotifer *B. plicatilis* were carried out with the commercial test kit RotoxKit M (Microbiotests, Gent, Belgium, order code TK22). Standard seawater was prepared as per the instructions of the test kit (**Table 2.4**). Hatching of the rotifer cysts, which are included in the test kit, was initiated in a hatching well by placing the cysts in 20 ppt standard seawater and incubation for 24 to 26 hours at 25 ± 1 °C and continuous illumination.

Toxicity tests were carried out in test plates with 6 rinsing wells and 36 test wells. Hatched rotifers were first transferred to each rinsing well filled with either additive solution, plastic or

base polymer leachates or control medium. After one hour in the rinsing wells, rotifers were individually transferred into test wells filled with test medium. Thereafter, the rotifers were incubated at 25 °C in darkness and the survivors were counted after 24 and 48 hours under a dissection microscope to calculate the mortality. Rotifers were considered dead if they did not display any movement after five seconds of observation.

Table 2.4: Salts used for preparation of 1L standard seawater for *Brachionus plicatilis* medium.

Salt	Concentration ($g \cdot L^{-1}$) or *($mg \cdot L^{-1}$)
NaCl	26.4
KCl	0.84
$CaCl_2 \cdot 2H_2O$	1.67
$MgCl_2 \cdot 6H_2O$	4.6
$MgSO_4 \cdot 7H_2O$	5.58
$NaHCO_3$	0.17
H_3BO3	30 *

Mortality test with Artemia persimilis nauplii

Cysts of *A. persimilis* (Black Label) were obtained from REBIE-Onlineshop (Bielefeld, Germany). Standard seawater of 23 ppt salinity was prepared from tap water and specific *Artemia* salt with microalgae (Artemio Sal, JBL, Neuhofen, Germany). Brine shrimp cysts were added to standard seawater and were incubated at 25 °C for 48 hours at continuous aeration and illumination.

The mortality tests were performed in 20-well cell-culture plates. Hatched brine shrimp larvae were first transferred to the rinsing wells filled with test medium. After 1 hour, *Artemia* nauplii from the rinsing wells were placed in each of the three test wells filled with test medium or control medium. The larvae were incubated in darkness for 24 hours at 25 °C. Thereafter, the mortality of the nauplii was determined. The *Artemia*-nauplii were considered dead if they did not move during ten seconds of observation.

2.4.2 Microplastic exposure experiment

Adult specimen of the rockpool shrimp *Palaemon elegans* from the Swedish Gullmarsfjorden were collected and maintained as described in section 2.3.2. After 48 hours in the aquaria, a total of 28 randomly selected animals were transferred from the tanks to single aerated glass jars. Animals were again fed with 5 mg of food flakes (NovoVert, JBL, Neuhofen, Germany), which were coated one day prior to feeding with 2.5 mg of microplastic particles (BPE-AMF-PLA, BPE-T-PHBV, LPDE) and 100 µL of deionized water. Food flakes without particles were used as control. Half of the water in the glass jars was exchanged 24 hours after each feeding.

For the test of acute exposition, shrimp were sampled after 4, 24, and 48 h after one single feeding event. For the test of chronic medium-term exposition, shrimp were sampled after 12 days. The shrimp were fed every other day with the coated food flakes.

During sampling, the body length and weight of the shrimp were measured. The midgut gland of the shrimp was dissected, weighed, transferred into cryovials, and immediately shock-frozen in liquid nitrogen. Thereafter, the samples were stored at -80 °C. The samples were transported from Kristineberg Station to the Alfred Wegener Institute in Bremerhaven (Germany) in dry shippers (CX100, Taylor Wharton) and stored again at -80 °C. The frozen tissues were slowly thawed on ice immediately before enzyme analyses. The individual midgut glands were transferred into 2-mL reaction tubes and homogenized in Tris-HCl buffer (20 $mmol \cdot L^{-1}$, pH 7.6) in a ratio of 1:10 (w:v) with a conical micro-pestle. The homogenized samples were then centrifuged for 10 min at 14,000 g and 4 °C. The supernatants were split and transferred into each of two new 1.5-mL reaction tubes. One half of each supernatant was mixed 1:1 (v:v) with Tris-HCl buffer as above but supplemented with 2 $mmol \cdot L^{-1}$ EDTA as recommended as extraction buffer for the assay of superoxide dismutase. The other half of the supernatant was frozen at 80 °C for later analysis of digestive enzyme activities.

Oxidative stress response (superoxide dismutase, SOD)

The measurements of the activity of antioxidant enzymes such as superoxide dismutase (SOD) can indicate if an organism suffers from oxidative stress. The superoxide dismutase activity was measured after Livingstone et al. (1992), modified by Saborowski et al. (2022). The assay is based on two coupled reactions. Firstly, the xanthine oxidase (XOD) activity is adjusted prior to routine analyses. The XOD provides superoxide radicals (O_2^-) that reduce cytochrome c. 0.882 mL of SOD assay buffer (100 mL K_2HPO_4 (43 $mmol \cdot L^{-1}$)) and 50 mL EDTA (0.1 $mmol \cdot L^{-1}$, pH 7.68), 100 µL of cytochrome c, 10 µL of xanthine and 8 µL of XOD were mixed in a cuvette. The absorbance was measured in a photometer (Specord 200 Plus, Analytik Jena, Jena, Germany) at 550 nm for three minutes at room temperature. XOD activity was adjusted to obtain an increase of absorption of 0.02 per minute. Deionized water was used as blank.

Secondly, the superoxide dismutase (SOD) activity is measured by the inhibition of cytochrome c reduction. The SOD within the sample, converts the superoxide radicals to hydrogen peroxide and molecular oxygen. 50 % inhibition of cytochrome c reduction corresponds to one unit of SOD activity. For the measurements, 0.872 mL of SOD assay buffer, 100 µL of cytochrome c, 10 µL of xanthine, 8 µL of XOD and 10 µL of the sample were mixed in a cuvette. The absorbance was recorded with a photometer (Specord 200 Plus, Analytik Jena, Jena, Germany)

at 550 nm for three minutes at room temperature. Deionized water was used as a blank. The amount of sample was adjusted to obtain 50 % inhibition. The SOD activity was then calculated from the slopes of the XOD and SOD reaction rates as reported in the Supporting Information of Saborowski et al. (2022).

Analysis of digestive enzyme activities

Activities of digestive enzymes were measured as described in detail in **Publication III (Manuscript 1)**. 4-methylumbelliferone (MUF) derivatives were used as fluorogenic substrates. MUF-butyrate, MUF-heptanoate and MUF-oleate were dissolved in dimethyl sulfoxide (DMSO, Sigma Cat. No. 276855) to obtain a 5 mM substrate solution. 1 mL of the substrate solution were diluted with 49 mL of 50 mmol·L^{-1} Tris-HCl buffer (pH 7.0), resulting in stock solutions of 0.1 mmol·L^{-1} fluorogenic substrate and 2 % DMSO. Extracts of the midgut glands (samples) were diluted with Tris-HCl buffer (50 mmol·L^{-1}, pH 7.0) in a ratio of 1:10 (v:v). 290 mL of the stock solution was then pipetted into the wells of a 96-well microplate and 10 µL of sample was added to each well. The plate was placed in a microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Corporation, USA) and shaken for 10 seconds before measurement at 355 nm excitation and 460 nm emission. The increase in fluorescence was measured for 5 minutes in 10 intervals of 30 seconds at room temperature. The results were recorded by Ascent Software for Fluoroskan Ascent FL. Standard curves were prepared with 0-35 µM 4-methylumbelliferone (MUF, Sigma Cat. No. M1381).

3 Publications and manuscripts

3.1 Contributions

This chapter contains the publications and manuscripts, which were prepared in the frame of my thesis and are part of this dissertation. For each publication and manuscript, an overview of the individual author contributions is provided. My contribution to the total workload of these works is described in percentage.

Publication I

pH-Stat Titration: A Rapid Assay for Enzymatic Degradability of Bio-Based Polymers

Lukas Miksch, Lars Gutow, Reinhard Saborowski

L.M.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-Original draft preparation. **L.G**.: Conceptualization, Methodology, Formal analysis, Writing-Review & Editing, Supervision. **R.S**.: Conceptualization, Methodology, Formal analysis, Writing-Review and Editing, Supervision.

My contribution in % of the total workload:

Conceptualization and Experimental Design:	**ca. 80%**
Experimental work and acquisition of data:	**ca. 100%**
Data analysis and interpretation of the data:	**ca. 70%**
Preparation of figures and tables:	**ca. 60%**
Drafting of manuscript:	**ca. 80%**

Publication II

Bioplastics in the Sea: Rapid In-Vitro Evaluation of Degradability and Persistence at Natural Temperatures

Lukas Miksch, Matthias Köck, Lars Gutow, Reinhard Saborowski

LM: Conceptualization, Investigation, Methodology, Writing – original draft. **MK:** NMR methodology, Writing – Review and editing. **LG:** Conceptualization, Funding acquisition,

Project administration, Writing – Review and editing. **RS:** Conceptualization, Funding acquisition, Supervision, Writing – Review and editing.

My contribution in % of the total workload:

Conceptualization and Experimental Design:	**ca. 80%**
Experimental work and acquisition of data:	**ca. 80%**
Data analysis and interpretation of the data:	**ca. 80%**
Preparation of figures and tables:	**ca. 60%**
Drafting of manuscript:	**ca. 80%**

Publication III (Manuscript 1)

Gastric carboxylesterases of Cancer pagurus (Crustacea, Decapoda) hydrolyze biodegradable plastics

Lukas Miksch, Lars Gutow, Reinhard Saborowski

LM: Conceptualization, Investigation, Methodology, Writing – original draft. **LG:** Conceptualization, Funding acquisition, Project administration, Writing – Review and editing. **RS:** Conceptualization, Funding acquisition, Supervision, Writing – Review and editing.

My contribution in % of the total workload:

Conceptualization and Experimental Design:	**ca. 80%**
Experimental work and acquisition of data:	**ca. 100%**
Data analysis and interpretation of the data:	**ca. 80%**
Preparation of figures and tables:	**ca. 90%**
Drafting of manuscript:	**ca. 80%**

Publication IV (Manuscript II)

Toxicity of bioplastics and bioplastic additives on *Brachionus plicatilis* (Rotifera) and *Artemia persimilis* (Crustacea, Brachiopoda)

Lukas Miksch, Ann-Christin Scheer, Lars Gutow, Reinhard Saborowski

LM: Conceptualization, Investigation, Data curation, Formal analysis, Validation, Supervision, Writing - original draft, Writing - review and editing. **ACS**: Methodology, Data curation, Validation, Writing - review and editing. **LG**: Conceptualization, Funding acquisition, Supervision, Formal analysis, Data curation, Writing - review and editing. **RS**: Conceptualization, Funding acquisition, Supervision, Data curation, Writing - review and editing.

My contribution in % of the total workload:

Conceptualization and Experimental Design:	**ca. 80%**
Experimental work and acquisition of data:	**ca. 20%**
Data analysis and interpretation of the data:	**ca. 70%**
Preparation of figures and tables:	**ca. 70%**
Drafting of manuscript:	**ca. 80%**

3.2 Publication I

pH-Stat Titration: A Rapid Assay for Enzymatic Degradability of Bio-based Polymers

Lukas Miksch, Lars Gutow, Reinhard Saborowski

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pH-Stat Titration: A Rapid Assay for Enzymatic Degradability of Bio-Based Polymers

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Abstract: Bio-based polymers have been suggested as one possible opportunity to counteract the progressive accumulation of microplastics in the environments. The gradual substitution of conventional plastics by bio-based polymers bears a variety of novel materials. The application of bioplastics is determined by their stability and bio-degradability, respectively. With the increasing implementation of bio-based plastics, there is also a demand for rapid and non-elaborate methods to determine their bio-degradability. Here, we propose an improved pH Stat titration assay optimized for bio-based polymers under environmental conditions and controlled temperature. Exemplarily, suspensions of poly(lactic acid) (PLA) and poly(butylene succinate) (PBS) microparticles were incubated with proteolytic and lipolytic enzymes. The rate of hydrolysis, as determined by counter-titration with a diluted base (NaOH), was recorded for two hours. PLA was hydrolyzed by proteolytic enzymes but not by lipase. PBS, in contrast, showed higher hydrolysis rates with lipase than with proteases. The thermal profile of PLA hydrolysis by protease showed an exponential increase from 4 to 30 °C with a temperature quotient Q_{10} of 5.6. The activation energy was 110 kJ·mol^{-1}. pH-Stat titration proved to be a rapid, sensitive, and reliable procedure supplementing established methods of determining the bio-degradability of polymers under environmental conditions.

Keywords: polymer degradation; microparticles; PLA; PBS; enzymes; specificity; thermal profile; activation energy

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1. Introduction

As an attempt to substitute petroleum-based plastics, policy makers and producers are increasingly promoting the innovation and development of greener so-called "bioplastics" [1]. The term bioplastic is used for plastic material that is either bio-based, biodegradable, or both. The implementation of bio-based polymers as basis for the production of plastic goods is a promising alternative, as common reduce, reuse, and recycle strategies are insufficient to counteract the extensive accumulation of plastic waste in the environment [2]. With the perspective of having sustainably produced, biocompatible, and biodegradable materials, the development of bioplastics especially from waste and renewable resources has become popular [3]. Advances in waste reutilization [4,5] and in the synthesis technology of biopolymers make it possible to convert agricultural and food waste into usable bio-based materials. Natural polymers like poly(lactic acid) (PLA), polyhydroxyalkanoate (PHA), and polyhydroxybutyrate (PHB) have been isolated through different processes from by-products of milk, sugar, biodiesel, and other industrial processes [3,6]. The effective utilization of waste flows, low carbon footprint, and the biodegradability make bioplastics a sustainable option to replace conventional plastics.

Enzymatic biodegradation of synthetic polymers is, basically, a similar process as the degradation of natural polymers, such as cellulose or chitin [7]. Extracellular microbial enzymes attach to the surface of the polymer and form enzyme–substrate complexes. Accordingly, degradation rates are highest in irregular micro-particles with a high surface:volume ratio. These evolve in the environment through fragmentation of larger items

by physicochemical and mechanical forcing. Depending on the substrate, specific enzymes hydrolyze the polymer chain and release oligo- and monomers. These degradation products may be finally assimilated and metabolized to water and carbon dioxide [8]. The enzymatic degradation is strongly influenced by environmental factors, such as pH and temperature [9,10], but also by the nature of the polymer [11,12]. Two common biodegradable polymers in commercial use are poly(lactic acid) (PLA) and poly(butylene succinate) (PBS). Both polymers are aliphatic polyesters and exhibit advantageous physical properties resembling those of polypropylene (PP) or low-density polyethylene (LDPE). Their chemical structure consists of monomers linked by ester bonds. Different hydrolytic enzymes, particularly esterases, are able to cleave these linkages. PLA was hydrolyzed in-vitro by serine proteases like proteinase K and subtilisin [13], whereas PBS was primarily hydrolyzed by lipase and cutinase [14,15].

Various standard tests and methods are available for the analytical assessment of the biodegradability of bioplastics [16]. These comprise the carbon dioxide forming test (Organisation for Economic Co-operation and Development, OECD 301b), differential scanning calorimetry (International Organization for Standardization, ISO 11357), weight-loss measurements (ISO 13432), or Fourier transform infrared spectroscopy [17]. Most of these methods are time consuming, laborious, or depend on expensive equipment. Accordingly, there is a demand for effective and accurate procedures that allow for the rapid analysis of the biodegradability of bioplastics under controlled conditions. A promising method is pH-Stat titration. It is based on maintaining a constant pH by the controlled addition of a diluted acid or base in a system where a pH affecting reaction takes place [18]. The hydrolysis of polyesters forms carboxyl groups, which reduce the pH in the solution. The quantity of added base to maintain a constant pH is thus a direct measure of the rate of hydrolysis of the bioplastic and, therefore, of its biodegradability. Although there is no uniform approach described, pH Stat titration has been used in several studies to assay the enzymatic degradation of aliphatic polyesters [19–24]. Most of these studies were conducted under elevated temperatures near enzyme optimum (Table 1). As the oceans are a potential sink of bio-based plastics, it is also of high relevance how natural environmental factors affect the process of degradation. Therefore, investigations of the hydrolysis of biopolymers under realistic environmental conditions are required.

Table 1. Procedural parameters of reported polymer degradation experiments by pH Stat titration.

Polymer (Substrate)	Enzyme (Source)	Temperature	Medium	Period	Reference
Poly-(trimethylene succinate)	Lipase [a]	37 °C	0.9% NaCl	10 h	[19]
Several model polyesters	Lipase [b,c,d,e,f] Hydrolase [g] α-Chrymotrypsin [h] Subtilisin [i] Esterase [j]	25–50 °C	0.9% NaCl	15 min–20 h	[20]
Several model polyesters	Lipase [b,e,k,l,m,n,o] Hydrolase [p] Proteinase K	40 °C	0.9% NaCl	15 min	[21]
Poly(3-hydroxybutyrate) (PHB)	PHB depolymerases	37 °C	1 mmol·L^{-1} Tris-HCl	20 min	[22]
Poly(ethylene terephtalate) (PET)	Cutinase [q,r,s]	30–90 °C	1 mmol·L^{-1} Tris-HCl with 10% glycerol	15 min	[23]
Poly(vinyl acetate) (PVC)	Cutinase [q,r,s]	40 °C, 50 °C, 70 °C	1 mmol·L^{-1} Tris-HCl with 10% glycerol	1–192 h	[24]

[a] Rhizopus delemar, [b] Pseudomonas spp., [c] Chromobacterium viscosum, [d] Rhizomucor miehei, [e] Candida cylindracea, [f] wheat germ, [g] Thermonospora fusca, [h] bovine pancreas, [i] Bacillus subtilis, [j] porcine liver, [k] Aspergillus oryzae, [l] Mucor miehei, [m] porcine pancreas, [n] Pseudomonas cepacia, [o] Pseudomonas fluorescens, [p] Thermobifida fusca, [q] Humilica insolens, [r] Fusarium solani, [s] Pseudomonas mendocrina.

In this study, we adapt the pH-Stat titration method for routine analysis to determine the biodegradability of bio-based plastics under relevant environmental conditions with support of specific proteolytic and lipolytic enzymes. Parameters for an improved performance and reliability of the method were tested and procedural difficulties identified.

2. Materials and Methods

2.1. Chemicals

Proteinase K from *Tritirachium album* (19133) was purchased from Qiagen (Hilden Germany) and the NaOH-standard solution (5564732) from Omnilab (Bremen, Germany). All other chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA): poly(L-lactide) (PLA, 765112), poly(1,4-butylene succinate) (PBS, 448028), protease from *Bacillus licheniformis* (P4860), and lipase from *Candida antarctica* (62288) (Supporting Information, Supplementary Tables S1 and S2).

2.2. Titration Device

We used the automatic titrator TitroLine® 7000 (SI Analytics GmbH, Mainz, Germany) for all pH-Stat applications. The titration unit was equipped with a 20-mL exchangeable head, a magnetic stirrer (TM 235), and a pH-electrode model A 162 2M DIN ID. The reaction vial was a 20-mL glass vial. The opening of the vial (17 mm) was too narrow to insert the electrode and the titration tip. Therefore, the titration tip was equipped with a 1 mm diameter polytetrafluoroethylene (PTFE)-tube. The reaction vial was placed in a custom-made thermostat jacket (Supporting Information, Figure S1). A circulation thermostat (e.g., Lauda, Lauda-Königshofen, Germany) connected to the thermostat jacket maintained a constant temperature in the reaction vial.

2.3. pH-Stat Titration

The titration system was set by default on the following parameters: mode: pH-Stat; endpoint: 8.2; step size: 0.001; measuring interval: 1 min; total time: 150 min; dosing speed: 1.5%; stirring speed: 1200 rpm. The pH was kept constant at 8.2 by titration of NaOH-solution. The concentrations ranged from 5 to 10 mmol·L^{-1}, depending on the velocity of the reaction after the enzyme was added. Substrate (biopolymer) suspensions (3 mg·mL^{-1}) were prepared in a solution of 3.4% sodium chloride in water (referred to as artificial seawater). PLA suspensions were prepared with a commercial product with an average M_n of 10,000. Aggregates of polymer particles were thoroughly crushed with a spatula before the suspension was sonicated in an ultrasonic bath for 8 min. Preliminary experiments showed that 3 mg·mL^{-1} was the highest concentration to ensure homogenous suspension of the particles and, thus, highest saturation. PBS granules were ground with a cryogenic mill (SPEX SamplePrep, 6775 Freezer/Mill) and sieved for fractions smaller than 200 µm. The suspensions were stirred in a glass beaker at 800 rpm for 16 h before aliquots of 10 mL were subjected to pH-Stat titration. Enzyme solutions (10–80 µL) were added to the reaction vial with a 100-µL microsyringe (Model 710 N, Hamilton Bonaduz AG, Bonaduz, Switzerland). The increase of volume due to titrant supply was always less than 1 mL. The electrode was calibrated every day before use. Routine measurements was carried out in triplicate.

2.4. Operational Parameters

To strengthen the quality standards, we separated the pH-Stat titration procedure into specific phases and separated unspecific background reactions from the true hydrolysis of the bioplastic material. To determine the optimal duration of the measurement, incubation experiments with PLA and protease from *Bacillus licheniformis* were run for up to 120 min. A linear regression was fitted to describe the amount of titrant (NaOH) added as a function of time. The coefficient of determination (R^2) of the NaOH consumption curve was progressively calculated from the start to identify the period after which the coefficient of determination of the linear regression was consistently above $R^2 = 0.99$.

2.5. Enzyme Specificity and Enzyme Concentration

Hydrolysis rates of PLA and PBS were determined with protease from *Bacillus licheniformis*, Proteinase K from *Tritirachium album*, and lipase from *Candida antarctica*. The hydrolysis rates were analyzed separately for PLA and PBS. Additionally, hydrolysis of PLA was assayed at five different protease concentrations (three replicates each) of up to 80 µL, which equals 30 to 240 mAU (Anson Units). Additionally, the dependency of the hydrolysis rate on the enzyme concentration was described by the following non-linear regression model:

$$f_{(x)} = a + (b - a) \cdot (1 - e^{(-c \cdot x)}) \quad (1)$$

with a being $f_{(x)}$ when x (concentration) is zero. b is the asymptote to which the curve approaches, which is the maximum reaction velocity. c is the rate constant of the curve.

2.6. Thermal Profiles

The temperature controlling system, consisting of a circulation cooler and a thermostat jacket, allowed maintaining constant and environmentally relevant temperatures during pH-Stat titration. Ten mL of a PLA-suspension (3 mg·mL^{-1}) were incubated with 40 µL protease solution (120 mAU). The hydrolysis of PLA was measured at temperatures between 4 and 30 °C. The Q_{10} temperature coefficient was calculated as:

$$Q_{10} = \left(\frac{V_2}{V_1}\right)^{\frac{10}{T_2 - T_1}} \quad (2)$$

where V_1 and V_2 represent the reaction velocities at the temperatures T_1 and T_2 in °C or K.

The apparent activation energy (E_A) of the reaction was calculated from the slope of the Arrhenius plot:

$$\ln V = -E_A \frac{1}{RT} \quad (3)$$

where V represents the reaction velocity, R is the universal gas constant, and T the temperature in K.

2.7. Statistics

For each of the two polymers (PLA and PHB), the hydrolysis rate was compared between the three enzymes (protease, Proteinase K, and lipase) by a 1-factorial analysis of variance (ANOVA) followed by Tukey's HSD (honestly significant difference) test. Prior to the ANOVA, the data were tested for heteroscedasticity by Levene's test. Similarly, the hydrolysis rates of PLA at five different protease quantities (30, 60, 120, 180, and 240 mAU) were compared by a 1-factorial ANOVA with subsequent Tukey's HSD test for pairwise comparisons. The significance level of all statistical analyses was $\alpha = 0.05$. Analyses and graphs were done with the program GraphPad Prism version 7.05 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3. Results & Discussion

3.1. Duration and Sequence of pH-Stat Titration

Compared to natural substrates such as proteins, carbohydrates, or lipids, the enzymatic hydrolysis of bioplastics is slow. This is particularly due to the physical characteristics of the bioplastic particles. They are solid and do not dissolve in water. Only the surface of the particles is accessible to enzymatic action. Accordingly, the accurate determination of the in-vitro degradability of bioplastics requires a sensitive and reliable procedure.

The pH-Stat titration assay, as shown for the hydrolysis of PLA by a protease, follows a characteristic temporal profile of pH changes in the reaction vial and titrant (NaOH) supply, which can be separated into four distinct phases (Figure 1).

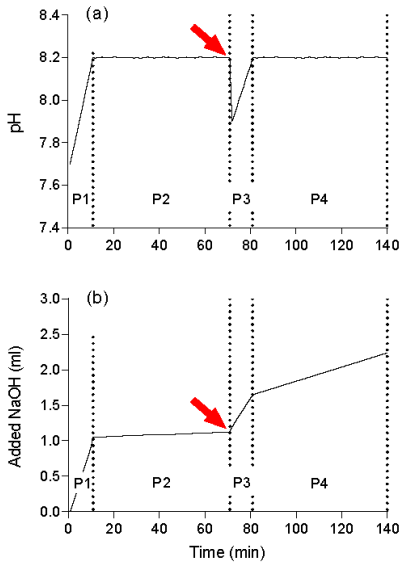

Figure 1. Typical course of (**a**) pH and (**b**) NaOH supply over time in a pH-Stat titration assay of a poly(lactic acid) (PLA) suspension as substrate and a PLA degrading enzyme. Phase 1 (P1): adjustment of the pH to 8.2. Phase 2 (P2): supply of low amounts of titrant to counterbalance an unspecific pH decrease before enzyme addition (substrate blank). Phase 3 (P3): re-adjustment of the pH to 8.2 after enzyme addition (indicated by an arrow). Phase 4 (P4): titration after enzyme addition to counterbalance a pH decrease of substrate hydrolysis (reaction) and an unspecific pH drop (blank).

During Phase 1, the automatic titration system adjusts the pH of the suspension to the starting point of 8.2. Subsequently, the adjusted pH is monitored for 60 min (Phase 2) for conspicuous variations, which may reveal technical errors. During Phase 2, minor quantities of NaOH are continuously added because unspecific reactions in the suspension result in a slight decrease in pH (substrate blank). The addition of the slightly acidic enzyme solution induces a drastic pH drop in the suspension. After re-adjustment to pH 8.2 (Phase 3), NaOH is continuously added to counterbalance the decrease in pH in response to the formation of carboxyl groups from the enzymatic degradation of the substrate (Phase 4). Accordingly, the slope of the curve in Phase 4 is proportional to the hydrolysis rate plus the unspecific reactions of the substrate blank (Phase 2).

To evaluate the rates of unspecific reactions (substrate blank), we tested four substrate (PLA) concentrations (0, 1.5, 3, and 6 $g \cdot L^{-1}$) and five enzyme volumes of 10, 20, 40, 60, and 80 µL protease in 10 mL artificial seawater, corresponding to enzyme activities of 30, 60, 120, 180, and 240 mAU, respectively. The hydrolysis rate of the substrate blank (Phase 2) ranged between 8 and 26% of the enzyme-catalyzed reaction (Phase 4) for all enzyme concentrations. The blank reaction without enzyme did not correlate with the substrate concentration.

The unspecific reaction may be due to autolysis of biopolymers [25] or diffusion of atmospheric CO_2 into the reaction mixture and dissociation to carbonic acid. We tried to minimize CO_2 diffusion by overlaying the reaction mixture with an inert gas, such as nitrogen or helium. This, however, caused variations in temperature of the reaction solution and was not further considered. Another option to stabilize the initial pH is to use a buffer for suspension [22–24]. However, as the hydrolysis rates at environmental temperatures are expected to be rather low, a buffer could mask potential hydrolysis rates. Furthermore, a buffer does not represent natural conditions. Instead, we used seawater

and corrected the slope of Phase 4 for the substrate blank (slope of Phase 2) to obtain the actual enzyme-catalyzed reaction V:

$$V = c \cdot \left[\left(\frac{\delta v_4}{\delta t_4} \right) - \left(\frac{\delta v_2}{\delta t_2} \right) \right] \cdot 10^6 \quad (4)$$

where c is the concentration of the added NaOH solution (mmol·L^{-1}), v_2 and v_4 the volumes (mL) of NaOH added in phase 2 and 4, and t_2 and t_4 the duration (min) of the phases 2 and 4. The conversion factor 10^6 was used to express the reaction velocity as nmol·min^{-1} under the given conditions. Additionally, the hydrolysis rate of the enzyme alone without substrate in artificial seawater (enzyme blank) was measured and subtracted from the hydrolysis rate of enzyme with substrate, to correct for possible autocatalytic activity of the enzyme solution.

3.2. *Titrant Leakage*

After the initial adjustment of the pH in Phase 1, further increases in pH beyond the default value of 8.2 occurred during Phase 2 before the enzyme solution was added. This increase in pH was due to leakage of titrant from the tip of the titration device. To evaluate the magnitude of this leakage effect, the increase of the pH over time was analyzed by linear regression. The slope of the linear regression was tested for deviation from zero by an F-test. The increase in pH was more distinct when higher concentrated NaOH solutions were used (Figure 2). In artificial seawater, the increase was always statistically significant (i.e., slope of linear regression always significantly >0) and accounted for 0.01 pH units per hour with 5 mmol·L^{-1} NaOH and 0.02 to 0.06 pH units per hour at higher NaOH concentrations of 10 to 50 mmol·L^{-1}. Upon request, the manufacturer of the titration device confirmed that small amounts of NaOH titrant may constantly diffuse from the titration tip into the reaction solution and raise the pH.

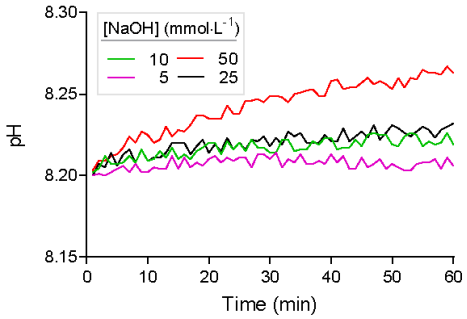

Figure 2. pH drift in artificial seawater over time with different concentrations of NaOH titrant due to diffusion from the titration tip.

To minimize leakage, tips with different opening diameters were tested. However, NaOH diffused from the original tips of the manufacturer, as well as from custom-made tips. Similarly, reducing the opening diameter of the tip below 0.5 mm did not stop leakage of NaOH. To minimize this source of error, the NaOH titrant has to be adjusted to a concentration that does not significantly affect the measurement of the enzymatic activity. Simultaneously, the concentration of the NaOH has to be high enough to avoid excess addition of the titrant that may exceed the capacity of the reaction vial. The use of a buffer would also partially eliminate this increase in pH, but was not considered for reasons described above.

When determining the leakage effect in PLA suspensions of 3 g·L^{-1}, the increase of pH was lower than in artificial seawater (Figure 3). This is probably due to the unspecific acidifying reactions in the PLA suspension as observed in phase 2 of the titration profile

(see Section 3.1). This unspecific reaction is stronger in PLA suspensions than in artificial seawater and therefore counterbalances the pH increase due to NaOH leakage. The pH increase in PLA-suspension of 3 mg·mL^{-1} was statistically significant for NaOH concentrations of 25 mmol·L^{-1} ($F_{1,58}$ = 21.96, p < 0.01) and 50 mmol·L^{-1} ($F_{1,58}$ = 66.27, p < 0.01). At lower NaOH concentrations of 10 and 5 mmol·L^{-1}, the pH of the PLA-suspension dropped below 8.2 because of the unspecific reaction described above (3.1). Here, the titration system added small amounts of NaOH to keep the pH at 8.2. Therefore, NaOH concentrations of 10 mmol·L^{-1} ($F_{1,58}$ = 0.01, p = 0.91) and 5 mmol·L^{-1} ($F_{1,58}$ = 0.82, p = 0.37) showed no significant change of pH in the reaction vial.

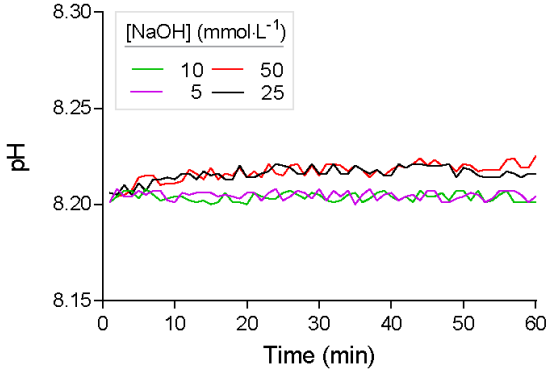

Figure 3. pH drift in substrate (PLA) suspension (3 mg·mL^{-1}) in artificial seawater over time with different concentrations of NaOH titrant. Endpoint of the titration was set to 8.2. Titrant was added at NaOH concentrations of 5 and 10 mmol·L^{-1} (dashed lines) to counterbalance the pH drop (see main text).

3.3. Time Frame of pH-Stat Measurement

With fast reactions due to high temperature (22 °C), the coefficient of determination reached R^2 = 0.99 on average after 17 min in phase 4 of the pH-Stat titration assay. At lower temperatures (12 °C) and slower reactions, R^2 = 0.99 was reached on average after 50 min (Figure 4).

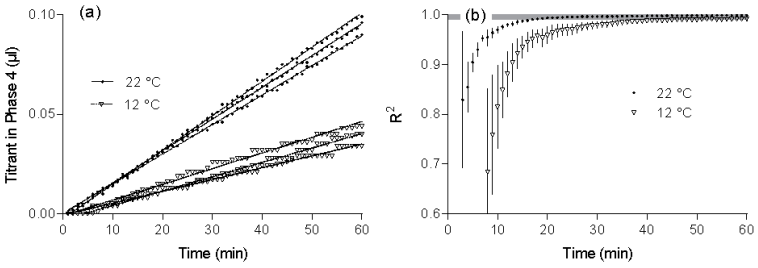

Figure 4. (**a**) Addition of titrant (0.01 mol·L^{-1} NaOH solution) during the 60 min lasting hydrolysis of PLA (3 g·L^{-1}) by protease from *Bacillus licheniformis* (2.4 AU·mL^{-1}). (**b**) Progressive approximation of the coefficient of variation to R^2 = 0.99 (indicated by a grey bar).

Previous studies measuring hydrolysis rates at high temperatures had time frames ranging from 15 min (initial slope) up to several hours (Table 1). From the course and the coefficients of determination of the NaOH consumption curves (Figure 4) we concluded, that 20-min titration duration may be sufficient for fast reactions, whereas slow reactions showed strong pH variations after 30 min. Longer measurement periods of 60 to 120 min further increase the accuracy of the assay. However, depending on the enzyme used

and the experimental conditions (temperature, pH) extended exposure times may be disadvantageous for less stable enzymes.

Therefore, prior testing of the enzyme activities under the specific assay conditions is recommended for measurements over longer time periods. Additionally, substrate limitation as a result of continuous substrate conversion will lead to a decrease in hydrolysis rate over time. Therefore, considering time constraints, throughput efficiency, and enzyme stability, we chose 60 min as an appropriate period for the pH-Stat assay of PLA hydrolysis in our study.

3.4. Effect of Enzyme Concentration

The hydrolysis rate varied significantly with the amount of added enzyme (ANOVA: $F_{4,9} = 12.71$, $p < 0.01$–Figure 5). The average reaction velocity continuously increased from 8.0 ± 0.5 nmol·min^{-1} at 30 mAU to 17.9 ± 1.4 nmol·min^{-1} at 120 mAU. No statistically significant increase in reaction velocity was observed at volumes above 120 mAU. The non-linear regression model explained 85% of the variation and identified the maximum reaction velocity at 18.9 nmol·min^{-1}. The calculated maximum velocity is within the range of the 95% confidence intervals of the reaction velocities at added enzyme amounts of 120–240 mAU.

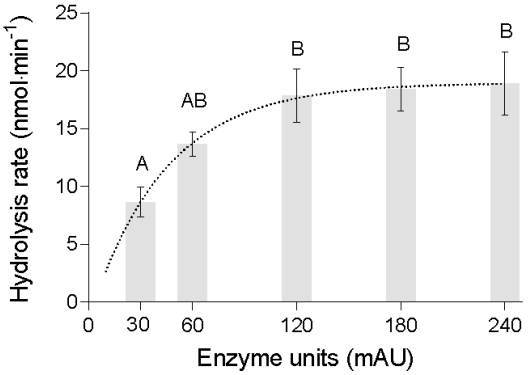

Figure 5. Rate of PLA hydrolysis (3 mg·mL^{-1}) at different quantities (Anson Units) of protease from *Bacillus licheniformis* at 22 °C and pH 8.2 (means ± SD, n = 2–3).

The stagnation of the hydrolysis rate of PLA at higher volumes of the protease solution may be due to the limitation of accessible substrate at higher enzyme concentrations. The surface area of the polymer to which the enzymes can attach and hydrolyze the substrate is limited. If all accessible sites on the polymer are occupied, the cleavage and therefore the hydrolysis rate stagnates even if enzyme is added [26]. Similar results were reported for the hydrolysis of poly-(trimethylene succinate) films by lipase from *Rhizopus delemar* in pH-Stat degradation experiments [19].

3.5. Enzyme Specificity

A set of enzymes was selected to examine their hydrolytic potential for PLA and PBS at 22 °C and pH 8.2. Both polymers were incubated with 100 µL lipase from *Candida antarctica* (18 U), 50 µL proteinase K from *Tritirachium album* (30 mAU), and 10 µL protease from *Bacillus licheniformis* (30 mAU), respectively. The hydrolytic activity for PLA varied significantly between the enzymes (ANOVA: $F_{(2,6)} = 115.8$, $p < 0.0001$–Figure 6a). PLA was efficiently degraded by the proteinase K from *Tritirachium album* with a hydrolysis rate of 13.7 ± 1.0 nmol·min^{-1} and by the protease from *Bacillus licheniformis* at 8.3 ± 1.0 nmol·min^{-1}. The lipase from *Candida antarctica* showed almost no hydrolytic activity with 0.5 ± 0.6 nmol·min^{-1}. Accordingly, both proteases were capable of degrading PLA, whereas the lipase was ineffective in PLA degradation. Efficient degradation of PLA

by serine proteases has repeatedly been described [13,27,28]. However, the hydrolytic potential of most lipases is limited to aliphatic polyesters with low melting temperature and a lack of optically active carbon [29], which does not apply to PLA.

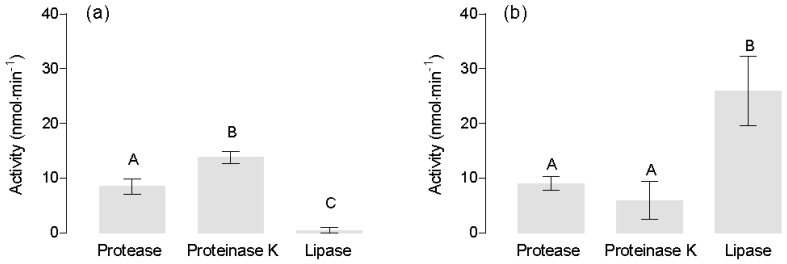

Figure 6. Enzymatic degradation of (**a**) PLA and (**b**) poly(butylene succinate) (PBS) at 22 °C and pH 8.2 (mean ± SD, $n = 3$). Different letters indicate statistically significant differences (Tukey multiple comparison test).

The hydrolytic activity for PBS also varied between the enzymes (ANOVA: $F_{(2,6)} = 19.33$, $p = 0.0024$–Figure 6b). Lipase from *C. antarctica* efficiently degraded PBS with a hydrolysis rate of 25.9 ± 5.3 nmol·min^{-1}. The hydrolysis rates of protease from *B. licheniformis* and the proteinase from *T. album* were significantly lower at 9.1 ± 1.1 and 6.0 ± 1.2 nmol·min^{-1}, respectively, although a potential of proteinase K for degrading PBS has been confirmed previously [22]. PBS is more efficiently hydrolyzed by lipases than proteases because of the conformational similarity of the polymer to tri- and diglycerides and the strong adsorption of lipase to the surface of PBS [30].

The degradation rates obtained by pH-Stat titration were verified with an alternative method. We chose a slightly modified fluorescent assay [31], which is based on the reaction of a fluorophore with liberated carboxyl groups of the PLA. The fluorescent method confirmed that protease from *B. licheniformis* and proteinase K from *T. album* were able to hydrolyze PLA (Supporting Information, Figure S2). However, the fluorescent assay is specific with regard to the polymer type and not applicable to measure the degradation products of PBS. It is also less sensitive than the pH-Stat method. Accordingly, the pH-Stat titration method is applicable to a wider range of polymers and performs better in in-vitro assays on small polymer quantities.

3.6. Thermal Profiles

Temperature controlled pH-Stat titration allows for investigating biodegradability of polymers within a wide range of environmentally relevant temperatures from 4 °C to above 30 °C. However, adjusting the temperature of the reaction mixture below 4 °C was challenging because of heat generation by the stirring unit, which is necessary to dispense the titrated NaOH in the reaction vial efficiently.

The hydrolysis rate of PLA by *B. licheniformis* protease strongly increased with temperature and followed first-order kinetics (Figure 7a). Activities ranged from 1.4 ± 0.2 nmol·min^{-1} at 4 °C to 66.0 ± 10.2 nmol·min^{-1} at 30 °C. The temperature coefficient Q_{10} of 5.6 (calculated over the entire temperature range from 4 °C to 30 °C) reveals that a rise in temperature by 10 °C induces a 5.6-fold increase in hydrolytic activity.

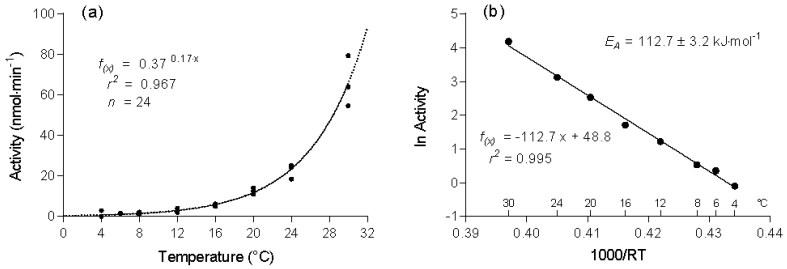

Figure 7. (**a**) Hydrolysis rates of PLA at different temperatures. (**b**) Arrhenius-plot calculated from data of the temperature profile of PLA hydrolysis.

Degradation of PLA by protease (*B. licheniformis*) proceeded more slowly at environmentally relevant seawater temperatures of up to 20 °C compared to seawater temperatures above 25 to 30 °C. Such elevated temperatures occur only in tropical waters [32,33] or in exposed rock pools warmed by solar radiation during low tide [34,35]. On the ground, highest temperatures appear at the surface and depend strongly on irradiation, soil humidity, and topography. At beaches, surface temperatures increase with distance from the shoreline and may exceed 50 °C on dry sand [36,37] but rapidly cool down already a few decimeters below the surface. Accordingly, temperature may become a limiting factor in the decomposition of biodegradable plastics in natural habitats [38]. Consequently, industrial composting processes typically utilize temperatures of 50 to 65 °C to accelerate the degradation process [16,39,40].

The Arrhenius-plot showed a strong linear relation between the ln of the reaction velocity and the reciprocal of the absolute temperature (Figure 7b). The apparent activation energy (E_A) of the hydrolysis of PLA by *B. licheniformis* protease was 112.7 ± 3.2 kJ mol^{-1}. The activation energy of a chemical reaction is a proxy for the velocity of the reaction. The lower the activation energy, the easier and faster a reaction can proceed.

Thermodynamic data on the hydrolysis of biodegradable polymers at environmentally relevant temperatures are lacking whereas data on thermal degradation at high temperatures of up to several hundred degree Celsius are available. For example, the activation energy of the thermogravimetric degradation of corn starch-based plastic accounts for 120–140 kJ·mol^{-1} [41]. The activation energy of the thermodegradation of hemp-poly lactic acid composites was about 160 kJ·mol^{-1} [42]. Those authors suggested that a high binding energy of the polymers improves the thermal stability of the compound, as it requires a higher activation energy to induce degradation.

Activation energies of thermogravimetric decomposition of natural substrates or their derivatives are in the same range. The E_A of cellulose from different plants are between 150 and 200 kJ·mol^{-1} [43] whereas the apparent E_A of the thermogravimetric decomposition of chitin and chitosan account for 125 and 169 kJ·mol^{-1}, respectively [44].

The E_A of enzymatic degradation of natural polymers is distinctly lower. Cellulolytic activity in soil showed activation energies of 22–28 kJ·mol^{-1} [45]. The E_A of the degradation of cellulose films by cellulose from sac fungus *Trichoderma reesei* was 37 kJ·mol^{-1} [46]. Similarly, the hydrolysis of swollen chitin by chitinase from *Trichoderma harzianum* showed an E_A of 70 and 80 kJ·mol^{-1} [47] and from *Paenibacillus* sp. of 19 kJ·mol^{-1} [48]. Chitinases of the psychrophilic Antarctic bacterium *Arthrobacter* sp. showed E_A of 62–63 kJ·mol^{-1} when hydrolyzing soluble chitin [49].

The capability of our method to establish thermal profiles rapidly and to deduce thermodynamic parameters allows studying material characteristics for various purposes. These may comprise industrial or medical applications, degradation kinetics, and effects of weathering. These results may be important to evaluate the degradability of bio-based polymers under conditions of the natural environment as well as industrial composting facilities.

3.7. Limitations

Despite many advantages, the pH-Stat titration method as described here has also limitations.

(1) Buffering Capacity of Plastic Suspensions

The pH of a polymer suspension depends on the properties of the liquid (water) and the polymer used. This intrinsic pH of the suspension was usually around 8.0 in our experiments with seawater and PLA/PBS. In phase 1 of each titration assay, the pH of the suspension was adjusted to 8.2, at which the hydrolytic reaction was observed.

Seawater or freshwater has a negligible buffer capacity. This has the advantage that even the lowest hydrolysis rates are detectable. However, due to the negligible buffering capacity, we observed that the adjusted pH moved towards the original intrinsic pH of the suspension with time. This may be due to the interaction of the plastic particles or leaching additives with the water.

If the intrinsic pH is lower than the adjusted pH, a decrease in pH appears in phase 2 (substrate blank) and is compensated by the addition of sodium hydroxide. If the intrinsic pH is higher than the adjusted pH, no sodium hydroxide will be added when the pH is increasing and no net change will be measured. This may mask a potential reaction and lead to an underestimation of the hydrolysis rate. To ensure the most accurate measurement of the hydrolysis rate, the adjusted pH should always be slightly higher than the intrinsic pH of the polymer suspension.

(2) Particle Size and Surface Area

The enzymatic reaction takes place in a suspension of very small particles. These particles (powder) have to be prepared first by e.g., cryo-milling and sieving. The enzymes act at the surface of the particles, which, however, cannot be defined in small mg-amounts of sample. Surface characteristics may vary between plastic types.

4. Conclusions

pH-Stat titration is a valuable tool for investigating the biodegradability of polymers, which may ideally complement already existing testing methods or even substitute some of them. When screening bio-based and other polymers for their degradability by various enzymes, the pH-Stat titration provides rapid and reliable results within only a few hours instead of weeks or months. To ensure a smooth and accurate performance of this method under environmental conditions, the tuning of the parameters to the chosen conditions and selected test materials is necessary.

The precise measurement of pH and base consumption, which reflect the formation of carboxyl groups through hydrolysis of the polymer, allows for a high sensitivity of the method and dealing with extremely small polymer quantities. For PLA and PBS microplastics we were able to demonstrate differential enzymatic degradability by proteolytic enzymes and lipase, respectively, and to describe the temperature dependencies of hydrolysis rates. This method is also suitable to monitor the enzymatic biodegradability of any other biopolymer, including future inventions facing the challenge to reduce environmental pollution by persistent plastic materials. No buffer solutions or other chemical reagents, which may interfere with the hydrolytic reaction, are required in the reaction solution to keep the pH constant during the measurement. Accordingly, degradation processes in natural media, such as freshwater or seawater, can be studied accurately. This makes this method an excellent tool for studying polymer degradation under different environmentally relevant conditions in terms of temperature, salinity, or pH. Additionally, the resulting degradation products, i.e., oligomers and monomers, can be isolated and subjected to detailed chemical analysis for studying each step in the process of polymer decomposition, which is also beneficial in the development of innovative future materials.

Supplementary Materials: The following Supplementary Materials are available online at https://www.mdpi.com/2073-4360/13/6/860/s1, Figure S1: Technical illustration of the thermal jacket, Figure S2: Fluorometric assay of PLA degradation products, Table S1: Enzyme specifications, Table S2: Polymer specifications

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3.3 Publication II

Bioplastics in the Sea: Rapid In-Vitro Evaluation of Degradability and Persistence at Natural Temperatures

Lukas Miksch, Matthias Köck, Lars Gutow, Reinhard Saborowski

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Bioplastics in the Sea: Rapid *In-Vitro* Evaluation of Degradability and Persistence at Natural Temperatures

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The progressive substitution of petroleum-based polymers, such as polyethylene, polyvinylchloride, or polyethylene terephtalate, by so-called bioplastics facilitated the development and production of many new materials. The continuously refined properties of bioplastic compounds and their blends enable various applications. With growing production and utilization of bioplastic products, these materials are increasingly discarded into the environment. Although many of these materials are labeled biodegradable, there is limited information about their degradability under environmental conditions. We tested the enzymatic degradability of five bioplastic compounds with the rapid pH-Stat titration assay at environmentally relevant seawater temperatures between 5 and 30°C and pH 8.2. These plastics, issued from the European Horizon 2020 Project ´Bioplastics Europe´, are based on polylactic acid (PLA), polybutylene succinate (PBS), and poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV). Suspensions of microparticles (< 200 µm) were incubated with each of the three hydrolytic enzymes, protease, lipase, and esterase. A PLA-based compound blended with polybutylene adipate terephthalate (PBAT) showed the highest hydrolysis rate of 30 nmol·min^{-1} when incubated with lipase at 30°C. All other materials showed low hydrolysis rates of less than 10 nmol·min^{-1}. Below 20°C, hydrolysis almost ceased. Plate clearing assays with the same enzymes at 37°C and pH 5 and pH 8, respectively, largely confirmed the results of the pH-Stat titration assays. Our findings indicate that there is a potential degradation of most of the materials with at least one of these hydrolytic enzymes. Nonetheless, the rate of enzymatic degradation under environmentally relevant conditions is low, which indicates only a marginal degradability of bioplastics in the marine environment.

Keywords: enzymatic degradation, hydrolysis, bio-based, polymers, enzymes, plastics

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1 INTRODUCTION

Petroleum-derived plastic materials (petro-plastics) shape the daily life of humans and have become indispensable in modern industrialized societies. For decades, the use of plastics is continuously increasing, with an annual global production of about 360 million tons by 2018 (PlasticsEurope, 2019). Excessive use and poorly controlled disposal caused a massive discard of persistent petro-plastics into the environment and a pollution of terrestrial and aquatic ecosystems worldwide

(Borrelle et al., 2020). Annually, an estimated amount of about 4-12 million tons of plastic debris ends up in the oceans (Jambeck et al., 2015), where it may remain for centuries before it degrades (Worm et al., 2017). Marine plastic debris covers a wide size range from large abandoned fishing nets to the smallest micro- and nanoparticles (Lebreton et al., 2018). Plastic debris may interfere with marine biota, often causing deleterious effects on organisms and ecosystems (do Sul and Costa, 2014; Gall and Thompson, 2015; Kühn et al., 2015).

To reduce the consumption of fossil resources and to promote the degradability of plastics, extensive research effort is being made to replace conventional petroleum-based polymers with so-called bioplastics. The term bioplastics refers to synthetic polymers derived from renewable biomass sources (Vert et al., 2012), but is also frequently used for biodegradable polymers and materials that combine both of these properties, therefore covering a wide spectrum of different polymers (e.g., Dilshad et al., 2021; Muneer et al., 2021). The majority of bioplastics are aliphatic polyesters composed of monomers linked by ester bonds. Common polymers used as the basis for bioplastics are polylactic acid (PLA), polybutylene succinate (PBS), and poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV) (Garlotta, 2001; Luo and Netravali, 2003; Xu and Guo, 2010; Ferreira et al., 2019; RameshKumar et al., 2020).

Depending on their application, biodegradable plastics have to combine a wide range of properties with the requirements of biodegradability standards. The chemical and mechanical characteristics of such materials can be substantially improved or adjusted by blending with additives and fillers (Angelini et al., 2015; Gug and Sobkowicz, 2016; Shafqat et al., 2021). Currently, bioplastics represent only one percent of the global annual plastic production. However, the demand for bio-based and biodegradable plastics is rapidly growing. Due to the continuous improvement of the compounds and the diversification of applications, the annual bioplastic production will prospectively grow from 2.1 million tons in 2020 to 2.9 million tons in 2025 (European Bioplastics e.V., 2021).

Synthetic biopolymers are degraded by hydrolytic cleavage of their ester bonds, catalyzed by microbial enzymes (Singh and Sharma, 2008). However, only 60% of the produced bioplastics are currently considered biodegradable (European Bioplastics e.V, 2021). Moreover, many of these biodegradable materials are sufficiently degraded only under specific conditions, such as high temperatures and in industrial composting facilities (e.g., Bátori et al., 2018; Ruggero et al., 2019; Folino et al., 2020). In the environment, the degradation rate of most biodegradable plastics seems to be low (Wang et al., 2021). Accordingly, improved biodegradability of customized bioplastics in the environment demands a sound understanding of their enzymatic degradation under realistic conditions.

A variety of hydrolytic enzymes from microbes or other organisms are capable of degrading bioplastics (Meereboer et al., 2020; Ali et al., 2021; Polman et al., 2021). Such enzymes, comprising various peptidases and esterases, are present in the environment, including seawater and marine sediments (Arnosti et al., 2014; Liu and Liu, 2018; Patel et al., 2019). Hydrolases, namely proteinase K, protease, esterase, and lipase, have been used in *in vitro* assays on the enzymatic degradability of bioplastics (Miksch et al., 2021; Richert and Dąbrowska, 2021). The pH-Stat titration is a rapid assay, which is based on maintaining the pH during a pH-affecting reaction. The hydrolysis of polyesters forms carboxyl end groups, which reduce the pH in the surrounding medium. Accordingly, the pH-Stat detects the drop in pH and adds an alkaline solution. Based on the amount of the added solution over a certain period of time, the degradation rate can be calculated. According to previous studies, we chose for the present investigation a proteolytic enzyme (protease), capable of hydrolyzing peptide and ester bonds, as well as an esterase and a lipase, which hydrolyze short-chain and long-chain esters, respectively. Although the selected enzymes do not derive from a marine microorganism, they represent important enzymes in the class of hydrolases, which are prevalent in almost all organisms and possess a rather broad substrate specificity. Moreover, the chosen enzymes are readily available commercially, and their specificity is equivalent to enzymes synthesized by marine fungi and bacteria.

In the frame of the EU Horizon 2020 project Bioplastics Europe (BPE) (Bioplastics Europe, 2022), we investigated the enzymatic degradability of selected customized bioplastics in seawater by rapid *in vitro* methods. Five bioplastic compounds, which were designed for different applications, were provided by project partners. These materials were tested for degradation at environmentally relevant seawater temperatures and their hydrolysis rates were compared with the conventional petroleum-based polymer poly(methyl methacrylate) (PMMA) and a biogenic structural protein (collagen).

2 MATERIALS AND METHODS

2.1 Chemicals

NaOH standard solution (cat. no. 5564732) was purchased from Omnilab (Bremen, Germany). Agarose (cat. no. 16500) was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). All other chemicals and enzymes (**Table 1**) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

TABLE 1 | Specifications of enzymes used in this study.

Enzyme	Source	Enzyme activity	Distributor	Cat. no.
Protease	*Bacillus licheniformis*	2.4 AU·mg^{-1}	Sigma	P4860
Lipase	*Candida antarctica*	9 U·mg^{-1}	Sigma	62288
Esterase	*Bacillus subtilis*	10 U·mg^{-1}	Sigma	96667

2.2 Polymers

Bioplastic compounds, in the following referred to as BPE-materials, were provided by Arctic Biomaterials OY (ABM, Tampere, Finland) and NaturePlast SAS (Ifs, France). The materials were supplied as granules (3 x 3 mm, 25 mg). The designations of the materials are composed of the abbreviations of the project, the intended use of the application, and the predominant base polymer (**Table 2A**). Petroleum-based PMMA was manufactured by Kunststoff- und Farben-Gesellschaft mbH (KFG, Biebesheim, Germany) and supplied as powder with particles < 200 µm (**Table 2B**). The structural protein collagen was purchased from Sigma (**Table 2B**). The material was cryo-milled as described under 2.3. and the size fraction < 200 µm was analyzed.

2.3 Preparation of Microparticles

Granules of the bioplastic compounds were ground in a cryogenic mill (SPEX SamplePrep, 6775 Freezer/Mill). The grinding program included 15 min of pre-cooling before the initiation of the grinding process. Grinding was performed in four to eight cycles, with 1 to 2 min of agitation and 1 to 2 min of cooling within each cycle. Grinding was performed with 15 impacts per second (cps). Pre-cooling was essential to achieve sufficient brittleness of the material to avoid agglutination of melting particles. One gram of material was processed per grinding operation. The ground material was sieved for fractions smaller than 200 µm. The yield of the < 200 µm fraction varied depending on the polymer type from about 20% to 60% of the total ground material.

2.4 ^{1}H- and ^{13}C-NMR Spectroscopy

The composition of the bioplastic compounds was verified by NMR spectroscopy. Prior to the NMR analysis, the materials were solubilized in deuterated chloroform ($CDCl_3$). The NMR spectra were recorded on a Bruker Neo spectrometer (equipped with a 1.7 mm cryo probe) at 300 K operating at 600 MHz (^{1}H) and 125 MHz (^{13}C). For each sample, a one-dimensional (1D) ^{1}H-NMR spectrum and a two-dimensional (2D) ^{1}H,^{13}C-HSQC spectrum were acquired. The 1D ^{1}H-NMR spectra were acquired with a 30° pulse, 128 scans, and 65536 data points (acquisition time of 3.6 s, relaxation delay of 1.0 s). The 2D ^{1}H,^{13}C-HSQC spectra were run with 16 scans and 2048 data points in F_2 and 256 data points in F_1 (acquisition time of 113 ms, relaxation delay of 1.5 s).

2.5 pH-Stat Titration

The rate of enzymatic hydrolysis of the bioplastics was measured by pH-Stat titration using a TitroLine® 7000 titrator (SI Analytics GmbH, Mainz, Germany). The titration unit was equipped with a 20-mL exchangeable head and a 1 mm diameter PTFE tube as a titration tip. The unit was connected to a magnetic stirrer (TM 235), a pH-electrode model A 162 2M DIN ID, and a circulation thermostat (Lauda, Lauda-Königshofen, Germany). The reaction vial was a 20-mL glass vial placed in a custom-made thermostat jacket to maintain a constant temperature (Miksch et al., 2021).

pH-Stat titration was performed after Miksch et al. (2021) whilst the rate of acidification due to the hydrolysis of the bioplastic was determined by counter-titration of a base. Briefly, suspensions of bioplastic microparticles (3 mg·ml^{-1}) were prepared in a solution of 3.2% sea salt (Seequasal, Münster, Germany) in deionized water (referred to as artificial seawater, ASW). The suspensions were first stirred in a glass beaker with a magnetic stirring bar at 800 rpm for 16 h before aliquots of 10 mL were subjected to pH-Stat titration. Ten to 100 µL of enzyme solution of commercial lipase, protease, and esterase (**Table 2**) were added to the reaction vial with a 100-µL micro-syringe (Model 710 N, Hamilton Bonaduz AG, Bonaduz, Switzerland). The pH was kept constant at 8.2 by titration of 10 mmol·L^{-1} NaOH solution. The addition of NaOH solution after enzyme addition was recorded every minute for 60 min. Each measurement involved an initial recording of the addition of NaOH solution for 60 min without enzyme, to correct for the effects of atmospheric CO_2 and polymer autolysis. For each replicate at all temperatures an additional enzyme blank was measured, where the hydrolysis rate of the enzyme in seawater without substrate was determined, to correct for the autocatalytic activity of the enzyme. The hydrolysis of the five bioplastics and the petroleum-based polymer PMMA was assayed at 5, 10, 15, 20, 25, and 30°C. The electrode was calibrated each day before use. Routine measurements were carried out in triplicate.

To compare the enzymatic degradation of the bioplastics with that of a natural polymer, hydrolysis of non-soluble collagen by each of the three enzymes was measured at 20°C using the same methodology.

2.6 Plate Clearing Assay

Plate clearing assays were performed as an alternative measure of enzymatic polymer degradation. Polymer emulsions and

TABLE 2 | Specifications of polymers used in this study.

A Specifications of BPE materials.

Designation	Base polymer	Producer	Application	Biodegradability
BPE-C-PLA	PLA/PBS	ABM	Cutlery	No information
BPE-RP-PLA	PLA/PBS	ABM	Rigid Packaging	Manufactured from compostable plastic (EN 13432)
BPE-AMF-PLA	PLA/PBAT	NaturePlast	Agricultural	No information mulch films
BPE-SP-PBS	PBS	NaturePlast	Soft packaging	Industrially compostable (NF EN 13432:2000)
BPE-T-PHBV	PHBV	NaturePlast	Toys	Industrially compostable (ASTM D6400)
B Specification of PMMA and collagen, used for comparison.				
PMMA	PMMA	KFG	Industries	Not biodegradable
Collagen	Protein	Sigma (C 9879)	Biogenic	Biodegradable

polymer agar plates were prepared after Uchida et al. (2000); Urbanek et al. (2020) with slight modifications. Briefly, 0.4 g of plastic particles were dissolved in 10 mL of dichloromethane by stirring at 28°C. After dissolution, 400 µl of Triton-X and 20 mL of distilled water were added. The mixture was sonicated for 2 to 4 min with a Sonopuls ultrasonic homogenizer GM 2070.2 (Bandelin, Berlin, Germany). Next, the dichloromethane was evaporated by stirring under vacuum for 1 h. The remaining emulsion was diluted with 60 mL of distilled water and 800 mg of agarose (Thermo Fisher, cat. no. 16500) was added. After heating the mixture in a microwave oven for 60 s at 600 W, the pH was adjusted to 5 and 8, respectively, by adding 0.1 mol·L^{-1} NaOH. About 20 ml of this solution was poured into a Petri dish and allowed to cool and polymerize. The enzymes lipase, esterase, and protease (10 µL each) were applied onto the surface of the gel with the help of small ceramic wells (diameter: 10 mm, height: 8 mm) and incubated for 24 h at 37°C. The formation of clear zones (halos) around the wells was interpreted as enzymatic degradation of the polymer. The diameter of the clear zones was used as a measure for the amount of polymer degraded by the enzyme. This plate clearing assay could not be performed on BPE-AMF-PLA, because it was not possible to create an opaque emulsion of this plastic.

2.7 Statistics

Statistical analysis and graphs were done with the program GraphPad Prism version 7.05 for Windows, GraphPad Software, La Jolla, CA, USA, https://www.graphpad.com. The temperature dependency of the hydrolysis rates of the tested polymers by the enzymes was described by non-linear regression models. The temperature dependency of the hydrolysis was described either by a Boltzmann sigmoidal model (Eq. 1) or by an exponential model (Eq. 2):

$$f_{(x)} = a + \frac{(b - a)}{1 + \exp\left(\frac{c-x}{d}\right)} \quad \text{Eq. 1}$$

with a = minimum of the fit, b = maximum of the fit, c = point of inflection (50% level of the function), and d = slope.

$$f_{(x)} = a \cdot e^{(bx)} \quad \text{Eq. 2}$$

with a = hydrolysis rate at a temperature of 0°C, and b = rate constant.

3 RESULTS

3.1 ^{1}H- and ^{13}C-NMR Spectra

The ^{1}H-NMR spectra of BPE-C-PLA and BPE-RP-PLA clearly indicate a mixture of PLA (^{1}H signals: CH and CH_3) and PBS (^{1}H signals: 3 x CH_2) with ratios of about 2.4:1 and 2.6:1, respectively (**Figures 1A, B**). BPE-AMF-PLA is a mixture of PLA and PBAT (seven ^{1}H signals) in a ratio of about 30:1. (**Figure 1C**). BPE-SP-PBS shows the indicative ^{1}H signals (three methylene groups) for PBS (**Figure 1D**). BPE-T-PHBV shows the characteristic signals for poly-(hydroxybutyrate-co-hydroxyvalerate)-copolymer (PHBV) with the HB (three ^{1}H signals) and HV (four ^{1}H signals) subunits and traces of PLA (**Figure 1E**). Detailed ^{1}H- and ^{13}C-NMR results are listed in the following.

BPE-C-PLA (**Figure 1A**): NMR data (600 MHz, $CDCl_3$): ^{1}H, δ5.13 (q, 1H, PLA-C*H*), 4.08 (br, 4H, PBS-O-CH_2), 2.59 (br, 4H, PBS-CO-CH_2), 1.67 (br, 4H, PBS-CH_2), 1.54 (d, 3H, PLA-CH_3); ^{13}C, δ69.1 (PLA-CH), 64.3 (PBS-O-CH_2), 29.1 (PBS-CO-CH_2), 25.3 (PBS-CH_2), 16.6 (PLA-CH_3), n.d. (CO).

BPE-RP-PLA (**Figure 1B**): NMR data (600 MHz, $CDCl_3$): ^{1}H, δ5.12 (q, 1H, PLA-C*H*), 4.07 (br, 4H, PBS-O-CH_2), 2.58 (br, 4H, PBS-CO-CH_2), 1.66 (br, 4H, PBS-CH_2), 1.54 (d, 3H, PLA-CH_3); ^{13}C, δ68.9 (PLA-CH), 64.1 (PBS-O-CH_2), 29.0 (PBS-CO-CH_2), 25.2 (PBS-CH_2), 16.6 (PLA-CH_3), n.d. (CO).

BPE-AMF-PLA (**Figure 1C**): NMR data (600 MHz, $CDCl_3$): ^{1}H, δ8.05 (s, 4H, PBAT-ar*CH*), 5.13 (q, 1H, PLA-C*H*), 4.38 (br, 4H, PBAT-O-CH_2), 4.08 (br, 4H, PBAT-O-CH_2), 2.29 (br, 4H, PBAT-CO-CH_2), 1.93 (br, 4H, PBAT-O-CH_2-CH_2), 1.79 (br, 4H, PBAT-CO-CH_2-CH_2), 1.63 (br, 4H, PBAT-CO-CH_2-CH_2), 1.54 (d, 3H, PLA-CH_3); ^{13}C, δ68.9 (PLA-CH), 64.8 (PBAT-O-CH_2), 63.8 (PBAT-O-CH_2), 33.8 (PBAT-CO-CH_2), 25.4 (PBAT-O-CH_2-CH_2), 25.3 (PBAT-O-CH_2-CH_2), 24.3 (PBAT-CO-CH_2-CH_2), 16.6 (PLA-CH_3), n.d. (CO).

BPE-SP-PBS (**Figure 1D**): NMR data (600 MHz, $CDCl_3$): ^{1}H, δ4.07 (br, 4H, O-CH_2), 2.58 (br, 4H, CO-CH_2), 1.66 (br, 4H, CH_2); ^{13}C, δ64.1 (O-CH_2), 29.0 (CO-CH_2), 25.2 (CH_2), n.d. (CO).

BPE-T-PHBV (**Figure 1E**): NMR data (600 MHz, $CDCl_3$): ^{1}H, δ5.22 (h, 2 x 1H, HB-C*H*+HV-C*H*), 2.58/2.43 (2 x d, 2 x 2H, HB-CH_2), 2.56/2.45 (2 x d, 2 x 2H, HV-CH_2), 1.24 (d, 3H, HB-CH_3), 1.22 (m, 2H, HV-EtCH_2), 0.85 (q, 3H, HV-EtCH_3); ^{13}C, δ67.7 (HB-C*H*+HV-C*H*), 40.9 (HB-CH_2), 40.6 (HV-CH_2), 29.6 (HV-EtCH_2), 19.7 (HB-CH_3) n.d. (CO).

3.2 pH-Stat Titration

3.2.1 BPE-Materials

The hydrolysis rates of BPE materials varied with the applied enzymes and temperature.

Hydrolysis of BPE-C-PLA incubated with protease increased exponentially with a temperature to 9.0 ± 2.2 nmol·min^{-1} at 30°C (**Figure 2A**). Hydrolysis by lipase and esterase was only detectable at 30°C at rates of 2.6 ± 0.4 nmol·min^{-1} and 2.4 ± 1.2 nmol·min^{-1}, respectively.

BPE-RP-PLA displayed a sigmoidal increase of hydrolysis rate with temperature when incubated with protease (**Figure 2B**). The highest rate was 4.6 ± 1.4 nmol·min^{-1} at 30°C. No hydrolysis (< 0.1 nmol·min^{-1}) of this polymer was observed with lipase and esterase at either temperature.

BPE-AMF-PLA showed the highest hydrolysis rate when incubated with lipase (**Figure 2C**). The hydrolysis rate increased exponentially with temperature from 4.7 ± 0.5 nmol·min^{-1} at 5°C to 28.5 ± 3.7 nmol·min^{-1} at 30°C. The same polymer was only poorly hydrolyzed by protease and esterase. Almost no hydrolytic activity was observed with these enzymes below 20°C. Above 20°C, the hydrolysis rate was still low with maximum activities of 2.5 ± 1.1 nmol·min^{-1} for protease and 2.6 ± 0.5 nmol·min^{-1} for esterase.

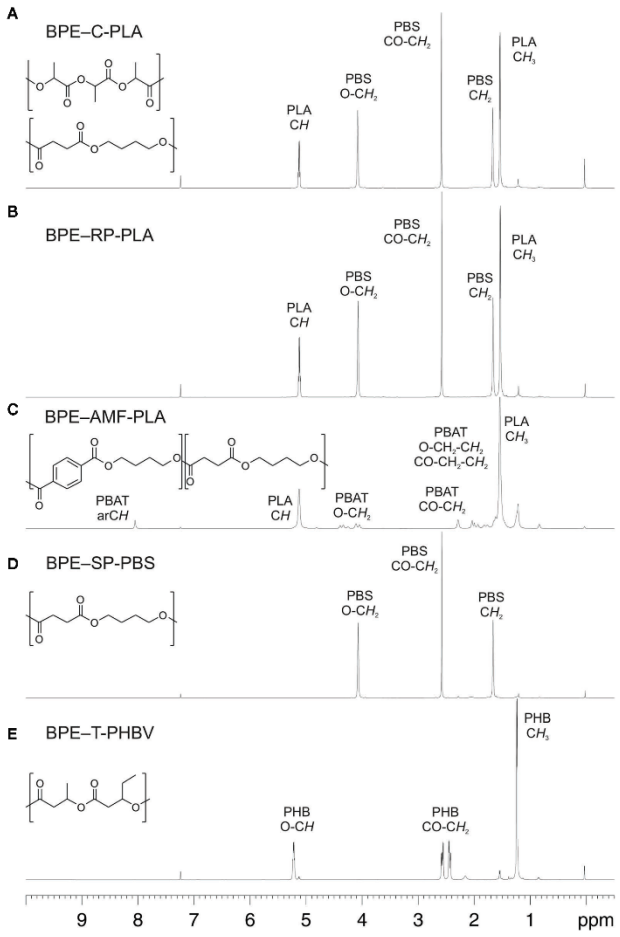

FIGURE 1 | ^{1}H-NMR spectra of the investigated BPE materials (structures shown on the left): **(A)** BPE-C-PLA, **(B)** BPE-RP-PLA, **(C)** BPE-AMF-PLA, **(D)** BPE-SP-PBS, and **(E)** BPE-T-PHBV. For all spectra the region between 0.5 and 10 ppm is shown. The BPE peaks are assigned.

BPE-SP-PBS was hydrolyzed by all three enzymes (**Figure 2D**). The hydrolytic activity was low and showed a sigmoidal increase with temperature. Accordingly, the highest activities were measured at 30°C at rates of 3.9 ± 0.2 nmol·min^{-1} for protease, 5.0 ± 0.2 nmol·min^{-1} for lipase, and 4.3 ± 0.5 nmol·min^{-1} for esterase.

BPE-T-PHBV showed very low or no hydrolysis when incubated with the three enzymes (**Figure 2E**). The hydrolytic activity slightly increased at 30°C to 1.4 ± 0.7 nmol·min^{-1} for protease, 0.3 ± 0.3 nmol·min^{-1} for lipase, and 1.1 ± 0.9 nmol·min^{-1} for esterase.

3.2.2 PMMA and Collagen

The petroleum-based PMMA was hydrolyzed by lipase at a very low rate, which varied only slightly with temperature (**Figure 3**). The highest hydrolysis rate of 0.7 ± 0.6 nmol·min^{-1} was measured at 20°C. Hydrolysis by protease and esterase occurred only at 20°C at very low rates of 0.2 ± 0.4 nmol·min^{-1} and < 0.01 nmol·min^{-1}, respectively.

Hydrolysis of non-soluble collagen was highest when incubated with protease (**Figure 4**). At 20°C the hydrolysis rate was 67.7 ± 5.7 nmol·min^{-1}, which was about six times higher than the highest hydrolysis rate of BPE-AMF-PLA at the same temperature (i.e., 11.4 ± 2.2 nmol·min^{-1}, **Figure 2C**). Hydrolysis of collagen by lipase was very low at 0.7 ± 0.1 nmol·min^{-1}. Almost no hydrolytic activity (< 0.1 nmol·min^{-1}) was measured with esterase (**Figure 4**).

3.3 Plate Clearing Assay

Meaningful plate clearing assays were performed with BPE-C-PLA, BPE-RP-PLA, and BPE-SP-PBS. No opaque suspension could be prepared with BPE-AMF-PLA, thus obstructing the

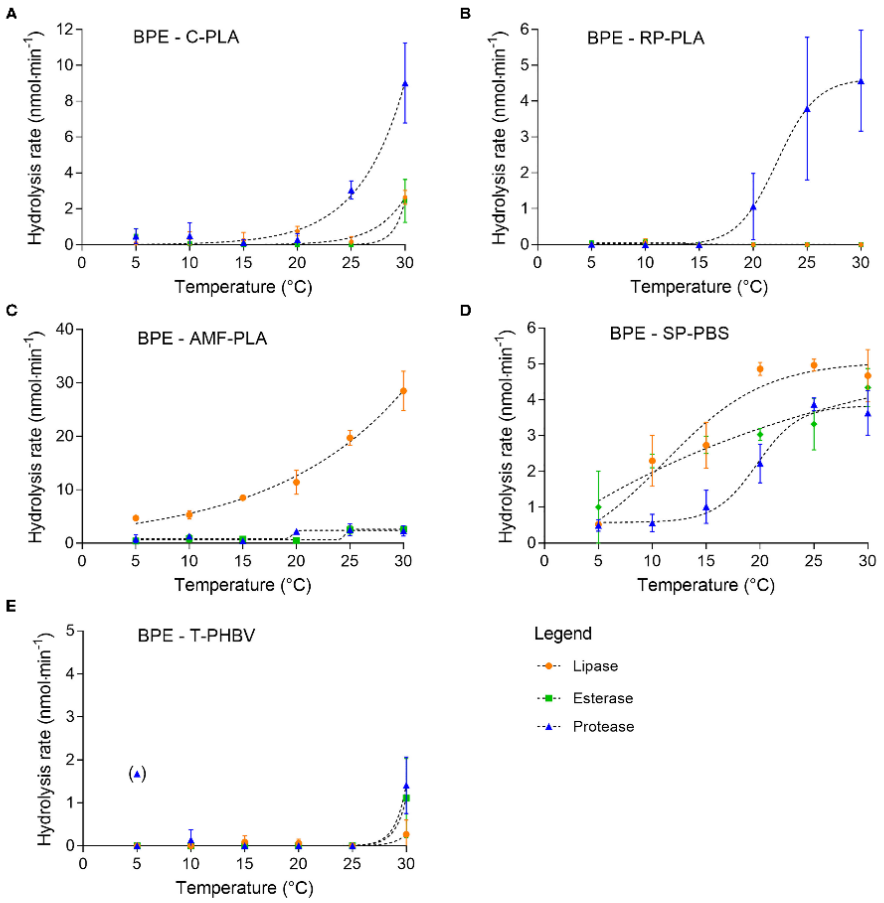

FIGURE 2 | Hydrolytic degradation of the five BPE materials by lipase, esterase, and protease **(A–E)** measured by pH-stat degradation at temperatures between 5°C and 30°C (means ± SD, n = 3).

plate clearing assays. The control (H_2O) showed no clear zones (**Table 3** and **Figure 5**).

BPE-C-PLA: all three enzymes, protease, lipase, and esterase developed clear zones on plates of BPE-C-PLA at pH 8 (**Table 3**). The diameter of the clear zone was the largest for protease. At pH 5, a clear zone developed only for esterase.

BPE-RP-PLA: protease and lipase formed clear zones at pH 8 with the largest zone with protease, suggesting the highest hydrolytic activity for this enzyme (**Figure 5** and **Table 3**). At pH 5, a clear zone was observed only for lipase with the same diameter as at pH 8.

BPE-SP-PBS: large clear zones were formed by all three enzymes at pH 8. At pH 5, clear zones were visible only for lipase and esterase but not for protease (**Figure 5** and **Table 3**). The diameters of the clear zones were substantially smaller at pH 5 than at pH 8.

BPE-T-PHBV: no indication of enzymatic activity was detected at pH 8 and pH 5.

4 DISCUSSION

The five bioplastics consisted mainly of PLA, PBS, PBAT, and PHBV, as confirmed by NMR spectroscopy. The bioplastic materials showed different compositions and ratios of these basic materials, which, apparently, affected their degradability. The enzymatic degradability was demonstrated with rapid *in vitro* assays. pH-Stat titration and plate clearing assays showed mostly low or no degradation at environmental water temperatures. Our results indicate a poor biodegradability of these bioplastics in marine environments.

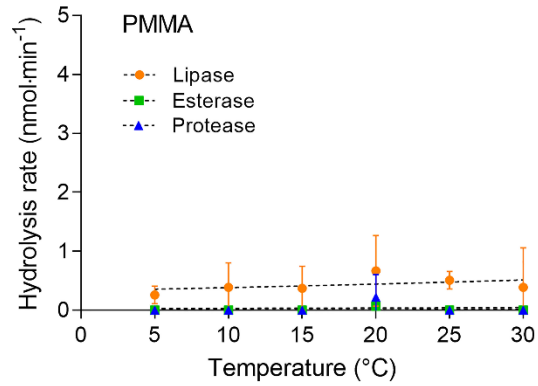

FIGURE 3 | Hydrolytic degradation of PMMA measured by pH-stat titration at temperatures between 5°C and 30°C (means ± SD, n = 3).

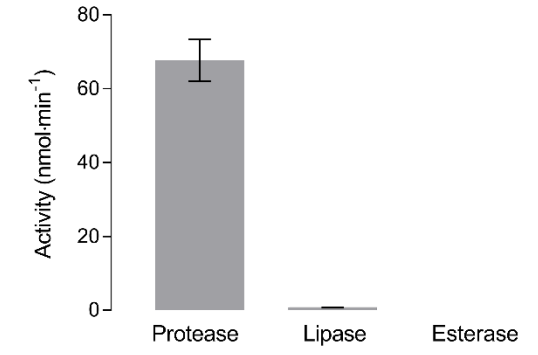

FIGURE 4 | Hydrolysis of non-soluble collagen by protease, lipase, and esterase measured by pH-stat titration at 20°C (means ± SD, n = 3).

4.1 pH-Stat Titration

Hydrolysis rates were highest for BPE-AMF-PLA, BPE-C-PLA, and BPE-RP-PLA, although with different enzymes. These materials are based on PLA, which is the major component, accounting for at least 50% of each blend. Pure PLA is efficiently degraded by protease from *Bacillus licheniformis* as measured by pH-stat titration (Miksch et al., 2021). However, of the three materials containing PLA as base polymer, only BPE-C-PLA and BPE-RP-PLA showed a noticeable degradability with protease.

BPE-C-PLA and BPE-RP-PLA are blends of PLA and PBS with ratios of 2.5:1 and 2.6:1, respectively. PLA is well degradable by protease from *B. licheniformis* and other serine proteases (Oda et al., 2000; Lim et al., 2005), whereas PBS polymers are preferably hydrolyzed by lipase (Rizzarelli et al., 2004; Ding et al., 2012). Accordingly, considerable hydrolytic activity was present when these materials were incubated with protease, while the substantially lower hydrolysis rate when incubated with lipase may indicate the degradation of the PBS fraction of the material.

BPE-AMF-PLA is a blend of PLA and PBAT. It was most efficiently hydrolyzed by lipase from *Candida antarctica*. PLA cannot be hydrolyzed by lipase (Jarerat and Tokiwa, 2003) whereas PBAT is well degraded by lipase from, e.g., *Pseudomonas cepacia*, *Candida cylindracea* (Herrera et al., 2002), and *Pelosinus fermentans* (Biundo et al., 2016). Therefore, the hydrolytic activity of the lipase on BPE-AMF-PLA likely displays the degradation of the PBAT component of the material rather than PLA. Surprisingly, BPE-AMF-PLA was not degraded by protease although the material is composed of PLA by 70 to 80%, according to producer information. Apparently, the PLA was not accessible for the protease or the enzyme may have been inhibited by compounds of the bioplastic blend.

PBS polymers are hydrolyzed by lipase (Rizzarelli et al., 2004; Ding et al., 2012). Among the three PBS-containing materials, only BPE-SP-PBS was significantly degraded by lipase, but also by protease and esterase. Materials sharing the same base polymer may differ in their degradability depending on the associated copolymers or additives. Differences in physical properties, such as molecular weight, crystallinity, or hydrophilic/hydrophobic properties may affect the enzymatic degradation (Singh and Sharma, 2008; Tokiwa et al., 2009) and, thus, determine the degradability of bioplastics.

BPE-T-PHBV was the least degradable of all bioplastic polymers tested herein. A slight increase in hydrolysis was observed with all enzymes though at very low rates and only at the highest temperature of 30°C. According to the course of the degradation curve, an exponential increase may occur above 30°C. Degradation of PHBV in seawater has been observed previously (Rutkowska et al., 2008). However, the catalytic properties were attributed to specific PHBV-depolymerases (Li et al., 2007). Our results indicate that peptidases and esterases

TABLE 3 | Diameter (mm) of clear zones formed around the area of enzyme application for lipase, esterase, and protease on plates of the BPE-polymers at pH 5 and pH 8.

	BPE-C-PLA		BPE-RP-PLA		BPE-AMF-PLA		BPE-SP-PBS		BPE-T-PHBV	
	pH 5	pH 8	pH 5	pH 8	pH 5	pH 8	pH 5	pH 8	pH 5	pH 8
H_2O (control)	0	0	0	0	n.d.	n.d.	0	0	0	0
Lipase	16	16	16	15	n.d.	n.d.	17	24	0	0
Esterase	0	16	0	0	n.d.	n.d.	17	24	0	0
Protease	0	32	0	26	n.d.	n.d.	0	46	0	0

n.d., not determined.

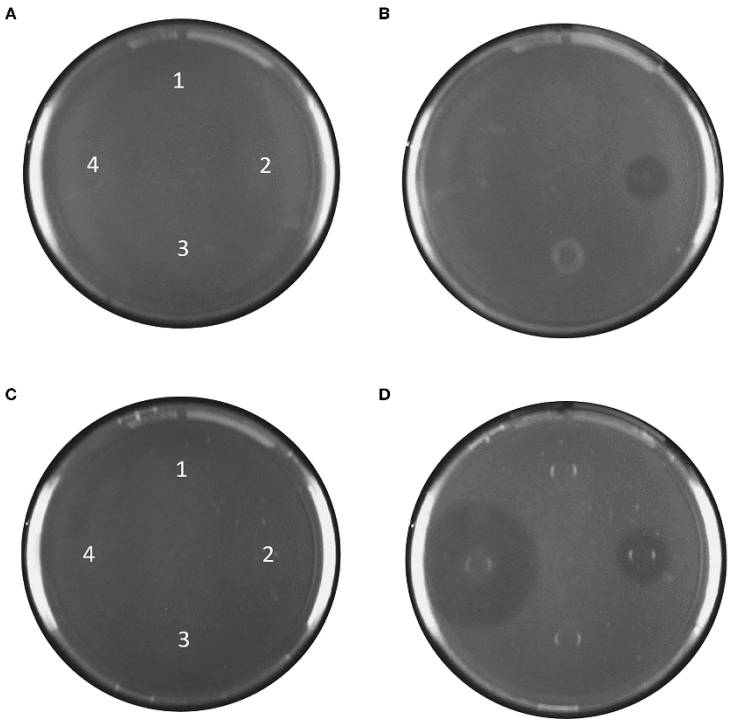

FIGURE 5 | Agar plates with BPE-RP-PLA at pH 5, (**A**) before incubation and (**B**) after 24 h at 37°C. BPE-RP-PLA plates at pH 8, (**C**) before incubation and (**D**) after 24 h at 37°C. Blank and samples applied at the indicated spots: 1) Deionized water, 2) lipase, 3) esterase, and 4) protease. After 24 h at 37°C, clear zones are visible for lipase at pH 5 (**B**) and for lipase and protease at pH 8 (**D**). The contrast of the images was increased by 25% to emphasize the clear zones.

play little to no role in the degradation of PHBV, but their contribution to degradation might increase at higher temperatures. However, their relevance in the degradation of PHBV in natural aquatic environments remains to be quantified.

Depending on the composition of the material, pH-Stat titration may underestimate the degradability of the synthetic polymer. During hydrolysis, alkaline degradation products may be formed, which can affect the pH. This would counteract the formation of degradation products with carboxyl end groups, where the acidifying effect is used as a measure of degradability. Alkaline leachates would increase the pH during the blank measurement of the material (Miksch et al., 2021). This effect was observed for BPE-C-PLA and BPE-RP-PLA but not for the other bio-based polymers (data not shown). The use of alkaline additives was confirmed by the manufacturer. The unspecific shift in pH was quantified by the blank measurement without enzyme and can be subtracted from the total rate. However, an increasing alkalization during the progressive enzymatic degradation of the material cannot be excluded.

4.2 Thermal Profiles

Enzymatic hydrolysis of the bioplastics increased with temperature. Hydrolysis rates were low below 20°C and highest at 25°C and 30°C. The thermal profiles of BPE-C-PLA and BPE-AMF-PLA showed an exponential increase whereas the profiles of BPE-RP-PLA and BPE-SP-PBS were best described by a sigmoidal regression model. The exponential increase displays the progressive rise in enzyme activity with temperature, which may continue up to the optimum of the enzymes at 40 to 60°C (Distributor Information, Sigma Aldrich). At higher temperatures, enzyme activity will cease due to progressive thermal denaturation of the enzymes. Consequently, the curve will flatten and then follow a sigmoidal course as well with an inflection point well above 30°C. Additionally, hydrolysis is also favored when the incubation temperature approaches the melting point of the material due to the higher accessibility of the substrate to the enzyme (He et al., 2001; Marten et al., 2005; Herzog et al., 2006).

The sigmoidal course of the thermal profiles of BPE-RP-PLA and BPE-SP-PBS at temperatures clearly below the thermal optimum of the enzymes must be attributed to the properties of the material rather than the catalytic properties of the enzymes. Depending on the composition of the material, only a limited fraction of the degradable component may be accessible to the enzyme. Hydrolysis may be limited by the surface availability of the degradable component so that increasing

temperature cannot further accelerate the hydrolysis rate. This may particularly affect the hydrolysis of blends and of polymers with amorphous and crystalline fractions, as the amorphous fraction is degraded faster than the crystalline fraction (Göpferich, 1996).

Additionally, the enzymatic activity may be inhibited by chemical additives, which may leach from the hydrolyzed material (Stloukal et al., 2015). To improve the properties and facilitate the processing of plastics, substances such as plasticizers, impact modifiers, or fillers are added during the production process (Jacobsen and Fritz, 1999; Liu et al., 2013; Wiesinger et al., 2021). The broad and diverse application of plastics requires a wide spectrum of additives. Unfortunately, the additives in the bioplastics used in this study were not specified by the manufacturer. The NMR spectra show various small signals in addition to the signals of the base polymers, indicating the presence of additives.

4.3 Comparison With PMMA and Collagen

To better estimate the enzymatic degradability of the bioplastics, we compared their hydrolysis rates with those of the conventional petroleum-based polymer PMMA and the biogenic polymer collagen. PMMA is a synthetic polymer primarily made from crude oil. Due to its favorable properties, such as low weight and high mechanical strength and toughness, PMMA is used in a wide range of applications. It is a very stable and persistent polymer, with no susceptibility to hydrolytic degradation (Boudaoud et al., 2018) and almost no biodegradation in the environment (Kaplan et al., 1979; Smith et al., 1987). Accordingly, PMMA showed among the lowest enzymatic hydrolysis of all materials tested in our experiment. However, it was in a similar range as the hydrolysis of BPE-RP-PLA by lipase and esterase and BPE-T-PHBV by all three enzymes. These results indicate that below 20°C the enzymatic degradation of most bioplastics is as inefficient as the degradation of conventional plastic such as PMMA.

Collagens are the main structural proteins in skin, bones, tendons, and cartilage of vertebrates and invertebrates (Prockop and Kivirikko, 1995) and, thus, highly abundant in the marine environment (Silva et al., 2014). Collagens are virtually insoluble in seawater (Wolf et al., 2006) and very stable due to their dense triple-helix protein structure (Shoulders and Raines, 2009). Although much is known about the rapid degradation of collagen in tissue (McAnulty and Laurent, 1987; Liao and Cui, 2004), studies about the degradation rates of collagens in seawater are rare. Collagens are hydrolyzed by collagenolytic proteases (Ran et al., 2013). Only a few studies addressed collagenolytic enzymes in marine bacteria (Merkel et al., 1975; Kurata et al., 2007; Zhao et al., 2008). Our experiment showed a substantial degradation of collagen by the protease but not by the esterase and the lipase. The hydrolysis rate of collagen was six to 14 times higher than the highest rates for the bioplastic compounds (BPE-AMF-PLA and BPE-SP-PBS both hydrolyzed by lipase at 20°C), and more than 60 times higher than the rates for all other bioplastic compounds. Accordingly, the enzymatic degradation of bioplastics is low compared to the degradation of natural biogenic collagen.

4.4 Plate Clearing Assay

The plate clearing assays largely confirmed the results of pH-Stat titration, albeit with some deviations. Significant hydrolysis rates as detected by pH-Stat titration were confirmed by clear zones on the agar plates at pH 8. Clear zones were larger at pH 8 than at pH 5 for almost all enzymes, confirming favorable conditions for the chosen enzymes at pH values of natural seawater. Lipase from *Candida antarctica* and esterase from *Bacillus subtilis* have a pH optima in the neutral to lower alkaline range (Kaiser et al., 2006; Eom et al., 2013), and the serine protease from *Bacillus licheniformis* in the alkaline range (Sareen and Mishra, 2008). The diameter of the clear zones did not consistently correlate with the hydrolysis rates measured by pH-Stat titration. The diameter of the clear zones ranged from 13 to 46 mm indicating considerable degradation, whereas the corresponding pH-Stat hydrolysis rates were mostly low. Additionally, the plate clearing assay indicated a considerable degradation of BPE-RP-PLA by lipase, which was not detected by the pH-Stat titration. The differences may be explained by the higher temperatures and the longer incubation time of the plate clearing assays. For example, no enzymatic degradation of BPE-T-PHBV was observed up to 25°C but distinctly increased at 30°C. A further exponential increase at higher temperatures is likely. Additionally, the dissolution and subsequent re-crystallization of the plastics in the plate clearing assay may have changed the structural properties of the polymer thereby potentially affecting the degradability of the material. Further studies are needed to confirm the suitability of plate clearing assay for the verification of pH-Stat titration results.

4.5 Environmental Relevance

Degradation of bioplastics in the environment requires the presence of the appropriate enzymes or mixtures thereof produced by microorganisms. Besides temperature, salinity, pH, and UV radiation (Voinova et al., 2008), also depth, oxygen, and organic substrate availability determine the microbial community in marine habitats and their catalytic potential (Chen et al., 2019; Tobias-Hünefeldt et al., 2019). Our *in vitro* experiments proved the general enzymatic degradability of the tested materials by single enzymes, but we did not test the combined effects of enzymes co-occurring in the environment. Mixtures of several enzymes showed synergistic effects on the degradation of natural materials (Spagnuolo et al., 1997; Murashima et al., 2003). A combination of various enzyme classes may show different effects on the hydrolysis of the materials as well. However, the presence of highly active proteases in such mixtures may be adverse as they may degrade other enzymes like lipases and esterase (Drouault et al., 2000) and reduce their catalytic potential.

Three of the tested materials are labeled as industrially compostable (**Table 2**) at the specific conditions in industrial composting plants of 50 - 60°C [European Bioplastics e.V. (EUBP), 2015]. However, under natural seawater conditions below 20°C, the enzymatic degradation is slow and elevated temperatures of up to 30°C occur only in surface waters of tropical regions (Deser et al., 2010). Plastic debris is not restricted to the surface, where the temperature is usually the highest, but also disperses throughout the water column and down to the seafloor (Kershaw et al., 2015) where temperatures are low. Since the density of the five tested bioplastics is between 1.2 and 1.5

$g \cdot cm^{-3}$, (Manufacturer Information) these materials likely sink to the ocean floor and are exposed to low temperatures, where they degrade only slowly or not at all.

5 CONCLUSION

Degradation rates of the tested bioplastics were low at environmentally relevant temperatures. Accordingly, uncontrolled release of these materials into the environment would inevitably lead to an accumulation in sensitive ecosystems similar to conventional, petroleum-based plastics. To counteract the pollution of the marine environment with putative biodegradable plastics, more effort in research and development is needed to design polymers that are easier to degrade at low environmental temperatures of the oceans.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the online repository PANGAEA (https://doi.pangaea.de/10.1594/PANGAEA.943844).

AUTHOR CONTRIBUTIONS

LM: Conceptualization, Investigation, Methodology, Writing – original draft. MK: NMR methodology, Writing – Review and editing. LG: Conceptualization, Funding acquisition, Project administration, Writing – Review and editing. RS: Conceptualization, Funding acquisition, Supervision, Writing – Review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.4 Publication III (Manuscript 1)

Gastric carboxylesterases of *Cancer pagurus* (Crustacea, Decapoda) hydrolyze biodegradable plastics

Lukas Miksch, Lars Gutow, Reinhard Saborowski

Will be submitted to Environmental Science and Technology

Gastric carboxylesterases of *Cancer pagurus* (Crustacea, Decapoda) hydrolyze biodegradable plastics

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Environmental pollution by plastics is a perpetual problem that poses a great challenge to humanity. A promising strategy to counteract the progressing pollution can involve the replacement of persistent synthetic plastics by biodegradable materials. Polylactic acid (PLA), polybutylene succinate (PBS), and polyhydroxyalkanoates (PHAs) are the most common ones among a variety of polymers that are processed in biodegradable materials. These compounds are enzymatically degradable by various hydrolytic enzymes. Biodegradable plastics and their fragments can reach the environment in the same way as conventional plastics. Therefore, they are accessible to terrestrial, freshwater, and marine biota. Once ingested by marine organisms, the mixture of highly active enzymes in their digestive tracts may break down biodegradable compounds and promote degradation. We incubated microparticles of five different biodegradable plastics *in-vitro* with the gastric fluid of the Edible crab *Cancer pagurus* and evaluated the hydrolysis rates by pH Stat titration. A material based on PLA and polybutylene adipate terephtalate (PBAT) showed the highest degradation rate. The enzymes in the gastric fluid of crabs were separated by anion exchange chromatography. Fractions with carboxylesterase activity were identified using fluorescent C4-, C7-, and C18-methylumbelliferyl (MUF)-derivatives and combined to yield three separate pools. These pools with high carboxylesterase activity did also hydrolyze the PLA/PBAT plastic blend. Enzymes with high activity on C4- and C7-MUF-derivatives showed apparent molecular masses of 40-45 kDa as determined by native gel electrophoresis (SDS-PAGE). Our study shows, that digestive carboxylesterases in the gastric fluid of *C. pagurus* showed a high potential for hydrolyzing certain biodegradable plastics. Since esterases are common in the digestive tract of organisms, it seems likely, that the ability to hydrolyze biodegradable plastics by invertebrates is widespread. However, the fate of released degradation products and their biochemical and physiological consequences are yet unknown and, therefore, deserve intensive research.

Key words: biodegradable plastics, emerging pollutants, crustaceans, Decapoda, enzymes, enzymatic degradation.

1. Introduction

Plastics are synthetic polymers that are omnipresent in the daily life of humans due to their variety of advantageous properties. In 2020, 367 million tons of plastics were produced worldwide (Statista, 2022). High production and excessive use of plastic products challenges communal life and environmental care with an unprecedented amount of persistent waste. Unmanaged and mismanaged plastic waste enters the environment and accumulates in terrestrial and aquatic ecosystems (Li et al., 2016). Conventional plastics are highly resistant to degradation, but will eventually break down to smaller fragments through UV exposure and mechanical forcing by wind and wave action (Barnes et al., 2009). This continuous fragmentation leads to the generation of tiny particles, referred to as microplastics at a size smaller than 5 mm (Andrady, 2017). Meanwhile, microplastics are ubiquitous in all ecosystems (Alimi et al. 2018). Due to their small size, they can be ingested by a variety of organisms and induce deleterious effects (Guzzetti et al., 2017; Galloway et al., 2017; Pirsaheb et al., 2020).

To counteract environmental pollution by persistent synthetic polymers, the interest in the development of biodegradable plastics has increased. A plastic is considered biodegradable when it meets certain degradation standards defined by international institutions such as OECD, ASTM, and DIN-Certco. Biodegradable materials can be based on fossil resources, such as the polymer polybutylene adipate terephthalate (PBAT), but can also derive from extant biomass. Common polymers that are both bio-based and biodegradable are polylactic acid (PLA), polybutylene succinate (PBS), and polyhydroxy-alkanoates (PHAs).

Irrespective of the raw material, the biodegradation process of synthetic biodegradable polymers is similar to that of natural polymers such as cellulose or chitin (Polman et al., 2021). Hydrolytic enzymes, which are capable of cleaving the polymer chains, play a key role in this process (Sing and Sharma, 2008). In the marine environment, extracellular enzymes are released by microbes and other organisms that form biofilms on the surface of the plastic (Martin et al., 2005). These hydrolytic enzymes comprise a variety of different peptidases and esterases, some of which have been demonstrated to hydrolyze biodegradable plastics *in-vitro* (e.g. Ali et al., 2021; Polman et al., 2021; Miksch et al., 2021). Although some biodegradable plastics are degraded by microbial enzymes, degradation rates of most of these materials are very low under marine conditions (Wang et al. 2021; Miksch et al., 2022). As a result, microplastics originating from biodegradable materials may also accumulate in the ocean potentially constituting a new form of emerging pollutants (Okoffo et al., 2022).

Progressive mechanical and chemical fragmentation of bioplastic items may contribute to the pool of microplastics in the environment. Due to their size and their widespread distribution in the water column, microplastics are easily mistaken for food by various marine organisms (Galloway et al., 2017; Guo and Wang, 2019). Besides others, microplastics were found in fishes (Jabeen et al., 2017), mollusks (Scanes et al., 2019), and crustaceans (Martinelli et al., 2021). Higher crustaceans possess a well-defined digestive system with a voluminous stomach, which contains a variety of highly active digestive enzymes derived from the midgut gland, *syn.* hepatopancreas (Vogt, 2021). The digestive enzymes are

mainly composed of hydrolases, which are able to break down ester bonds, peptide bonds, and glycosidic bonds (Saborowski, 2015). Upon ingestion, conventional and biodegradable microparticles are exposed to these enzymes. Accordingly, we address the question if enzymes in the digestive tract of crabs are able to hydrolyze biodegradable plastics.

In this study, we investigated the potential of the Edible crab *Cancer pagurus* (Decapoda, Brachyura) to hydrolyze biodegradable plastics. We chose this species as a model organism because it is large enough to provide repeatedly up to two mL of gastric fluid by gastroscopy. Isolated gastric fluid was incubated *in-vitro* with different biodegradable plastics under controlled conditions. Enzymatic hydrolysis of the polymers was determined by pH-Stat titration (Miksch et al., 2021). Enzymes were separated and characterized by anion-exchange chromatography and native gel electrophoresis (SDS-PAGE).

2. Materials and Methods

2.1. Chemicals

The NaOH-standard solution (5564732) for pH Stat titration was purchased from Omnilab (Bremen, Germany). 4-methylumbelliferone derivatives and all other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Plastics

The test materials (Table S1, Supplementary Material) were provided by Arctic Biomaterials OY (ABM, Tampere, Finland) and NaturePlast SAS (Ifs, France). The materials are based on common bio-based and biodegradable polymers. The materials were supplied as granules (3 × 3 mm, 25 mg), which were ground to a fine powder (< 200 µm) with a cryogenic mill (SPEX SamplePrep, 6775 Freezer/Mill) as described by Miksch et al. (2022). A conventional plastic (PMMA) was manufactured and provided by Kunststoff- und Farben-Gesellschaft GmbH (KFG, Biebesheim, Germany) and supplied as powder with particles < 200 µm.

2.3. Origin and maintenance of crabs

Adult crabs (*Cancer pagurus* Linnaeus, 1758) were collected by beam trawling with the research vessel FK Uthörn in the North Sea near Helgoland, Germany (54°09'54.7N 7°52'06.2E). Six animals, two females without eggs and four males, were immediately after catch transferred to 50-L tanks with running seawater and shipped to the Alfred Wegener Institute in Bremerhaven (Germany). The crabs weighted 300 to 1100 grams. In the laboratory, the animals were kept individually in 50-L aquaria, which were integrated in a 500-L recirculation seawater system. The temperature was 15 ± 1 °C and the light:dark cycle 12:12 hours. The crabs were fed three times per week with frozen shrimp or fish. Half of the seawater in the recirculation system was exchanged every other week. Water parameters (T, pH, NH_4, NO_2^-, and NO_3^-) were monitored once a week.

2.3. Isolation of the gastric fluid

Gastric fluids of crabs were extracted every two weeks. The extraction was done by inserting a small PTFE tube (1 mm inner diameter) connected to a syringe (1 mL) through the esophagus into the gastric chamber of the crab. Digestive fluids were then slowly aspirated. Sampling was performed 48 hours after feeding. The gastric fluid was transferred into 2-mL reaction tubes and centrifuged for 10 min at 20,000 g and 4 °C to remove suspended solids. The supernatant was aliquoted into portions of 100 or 500 µL and stored at -80 °C until further use.

2.4. Enzyme screening and protein concentration of the gastric fluid

ApiZym strips (BioMerieux, Marcy l'Etoile, France) were used for the identification of a set of 19 hydrolytic enzymes in the gastric fluid. 65 µL of the gastric fluid were dispensed into each of the microcupules of the ApiZym strip. The strips were placed in the supplied tray, covered with the lid, and incubated in the dark at room temperature. After 24 hours, ZYM A and ZYM B reagents were added to the microcupules to initiate the chemical color reaction. The results were visually compared with a control (no color development) and ranked by numbers from low intensity (1) to high intensity (5) and intermediates according to the strength of the color. No visible color development was graded as 0. The protein concentration in the gastric fluid was determined after Bradford (1976) by using bovine serum albumin as reference.

2.4. pH-Stat titration

pH-Stat titration was carried out with the automatic titrator TitroLine 7000 (SI Analytics GmbH, Mainz, Germany). The titration unit was equipped with a 20-mL exchangeable head and a 1 mm diameter PTFE-tube as titration tip. The unit was connected to a magnetic stirrer (TM 235), a pH-electrode model A 162 2M DIN ID, and a circulation thermostat (Lauda, Lauda-Königshofen, Germany). The reaction vial was a 20-mL glass vial placed in a custom-made thermostat jacket to maintain constant temperature (Miksch et al., 2021).

pH-Stat titration was performed after Miksch et al. (2021). Briefly, suspensions of polymer micro-particles (3 mg·mL^{-1}) were prepared in a solution of 32 ppt sea salt (Seequasal, Münster, Germany) in deionized water (referred to as artificial seawater, ASW). The suspensions were stirred in a glass beaker with a magnetic stirring bar at 800 rpm for 16 hours before aliquots of 10 mL were subjected to pH stat titration. Eighty µL of gastric fluid or enzyme solution was added to the reaction vial with a 100-µL micro-syringe (Model 710 N, Hamilton Bonaduz AG, Bonaduz, Switzerland). The pH was kept constant at 8.2 by titration of 10 mmol·L^{-1} NaOH-solution. Addition of NaOH-solution was recorded every minute for 60 minutes before and after enzyme addition. The hydrolysis of the polymers was measured at 15 °C and 30 °C. The electrode was calibrated every day before use. Routine measurements were carried out in triplicate.

2.5. Fractionation of gastric fluid

Gastric fluids were first desalted and rebuffered over Sephadex™ NAP-5 gel filtration columns (Amersham Biosciences, Uppsala, Sweden) and diluted (1:2 v/v) with 0.1 mol·L^{-1} Tris-HCl buffer (pH 7.5). Thereafter, 1 mL of the sample was injected into the LC-system (NGC Chromatography System, BioRad) equipped with an anion exchange column (UNO Q1 R, BioRad). The proteins in the gastric fluids were eluted by increasing the concentration of NaCl from 0 to 1 mol·L^{-1} in the buffer (0.1 mol·L^{-1} Tris-HCl, pH 7.5) with a flow rate of 1 mL·min^{-1}. The absorbance of the eluate was detected at 280

nm. The gastric fluids were separated into 65 fractions of 1 mL each, which were immediately monitored for esterase enzyme activity in a 96-well microplate. Fractions with high enzyme activity were frozen and stored at -80 °C for further usage. In order to determine whether the potential to hydrolyze biodegradable plastic was maintained after anionic exchange separation, fractions with high carboxylesterase activity were pooled and subjected to ultrafiltration (Vivaspin 15R, Sartorius, Göttingen, Germany). Twelve mL of enzyme-containing fractions were spun at 3000 g and 4 °C for 60 minutes, reducing the volume to about 300 µL. The concentrated enzyme solutions were tested for their potential of hydrolyzing plastics by pH Stat titration as described above.

2.6. Enzyme activity assay

Esterase and lipase activities in each of the 65 gastric fluid fractions were assayed with fluorogenic substrates immediately after chromatographic separation. The hydrolysis of fatty acid esters and the resulting increase in fluorescence was interpreted as esterase or lipase activity (Prim et al., 2003; Knotz et al., 2006). 4-methylumbelliferone derivatives from esters of fatty acids (MUF-butyrate, MUF-heptanoate, MUF-oleate) were dissolved in dimethyl sulfoxide (DMSO) and then diluted with 40 mmol·L^{-1} Britton-Robinson buffer, pH 5 to pH 9 (Britton and Robinson, 1931) to obtain stock solutions with 0.1 mmol·L^{-1} substrate concentration and 2 % DMSO. 250 µL of the stock solution was pipetted into a 96-well microplate and 50 µL of sample was added to each well. The plate was shaken for 10 seconds in a microplate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific Corporation, USA) before measuring the fluorescence in 20 intervals of 15 seconds over 5 minutes at room temperature (λ_{ex} 355 nm, λ_{em} 460 nm). The results were recorded by the Ascent Software for Fluoroskan Ascent FL. Standard curves with 0-35 µmol·L^{-1} 4-methylumbelliferone (MUF, Sigma cat.no M1381) were prepared at pH 5 to pH 9, as the fluorescence signal varies with pH.

To determine the effect of SDS on enzymatic activity, the same stock solutions with MUF-derivatives dissolved in DMSO were prepared, but only with Britton-Robinson buffer at pH 7. 0.12 % SDS was then added to the stock solution of each substrate (MUF-butyrate, MUF-heptanoate, MUF-oleate) and vortexed. 250 µL of the stock solution was pipetted into a 96-well plate and 50 µL of sample was added, to obtain a final concentration of 0.1 % SDS in each well. The measurement of fluorescence was then carried out in the same way as described above.

2.7. SDS-PAGE

Proteins in selected fractions of the gastric fluids were separated by native SDS-PAGE (12 % acryl-amide) in a vertical Hoefer SE 250 electrophoresis device. Fractions were diluted 1:2 with sample buffer, which contained 2% SDS and bromophenol blue. The samples were not treated with mercaptoethanol nor were they heated. Polyacrylamide gels were loaded with 10 µL of sample per lane and 2.5 µL of molecular weight markers (Page Ruler Plus Prestained Protein Ladder, Thermo Fisher Scientific, cat.

no. 26619) in each leftmost and rightmost lane. Electrophoresis was conducted at a constant current of 15 mA per gel and a maximum voltage of 300 V at 4 °C.

After electrophoretic separation, the gels were first washed in demineralized water for 10 minutes and then in 50 mmol·L^{-1} Tris-HCl buffer (pH 7) for 10 minutes. Thereafter, the gels were incubated in 100 mL of fluorogenic substrate solution (0.1 mmol·L^{-1}) for another 10 minutes. The gels were photographed under a BioRad Gel DOC EZ Imager (BioRad, Hercules, California) to identify active protein bands that hydrolyzed the substrates. The gels were then washed again for 10 minutes in demineralized water, before Coomassie-staining overnight and subsequent de-staining with an aqueous solution of ethanol and phosphoric acid. Blue-dyed protein bands were identified and compared with fluorogenic protein bands.

2.10. Statistics

Statistical analysis and graphs were done with the software GraphPad Prism version 7.05 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The significance level of all statistical analyses was $\alpha = 0.05$. The hydrolysis rates of the different compounds by the gastric fluid were compared by a 1-factorial analysis of variance (ANOVA) (n = 3). Prior to the ANOVA, the data were tested for heteroscedasticity by Brown-Forsythe test. The carboxylesterase activities of the fractions with and without addition of SDS were compared with a paired t-test.

3. Results

3.1. The gastric fluid of *Cancer pagurus*

Depending on the size of the individual crabs, between 200 µL and 1 mL of gastric fluid could be obtained from each crab. The fluid showed an intense color, varying from bright orange to dark olive brown. The gastric fluid was slightly acidic with pH values ranging from 6.0 to 6.4. The protein content measured by Bradford method accounted on average for 8.77 % ± 0.0. The enzyme screening with ApiZym® showed intensive color development and thus high activity for 16 of the 19 examined enzymes (Table 1). The only enzymes with low intensity were cysteine arylamidase, valine arylamidase, and α-chymotrypsin.

Table 1: Semi-quantitative assay of enzyme activities in the gastric fluid of *Cancer pagurus* ranked 0 - 5 according to color intensity in ApiZym® strips.

Enzyme	Intensity	Enzyme	Intensity
Control	0	Acid phosphatase	5
Alkaline Phosphatase	5	Naphtol-AS-BI-phosphatase	5
Esterase (C4)	4	α-Galactosidase	3
Esterase/Lipase (C8)	4	β-Galactosidase	5
Lipase (C14)	4	β-Glucuronidase	5
Leucine arylamidase	5	α-Glucosidase	5
Valine arylamidase	1	β- Glucosidase	5
Cysteine arylamidase	0	N-acetyl-β-D-glucosaminidase	5
Trypsin	5	α-Mannosidase	4
A-Chymotrypsin	1	α-Fucosidase	4

3.2. Hydrolysis of plastics by the gastric fluid

pH-Stat titration of plastics with the gastric fluid of *C. pagurus* showed significantly different hydrolysis rates (ANOVA: $F_{2,6} = 78.55$, $p < 0.01$). The conventional petroleum-based plastic PMMA showed no degradation. The same was observed for the biodegradable plastics BPE-C-PLA and BPE-RP-PLA. BPE-SP-PBS and BPE-T-PHBV showed low hydrolysis rates of 0.26 ± 0.25 nmol·min^{-1} and 0.72 ± 0.63 nmol·min^{-1}, respectively. BPE-AMF-PLA showed the highest hydrolysis rate with 8.01 ± 1.32 nmol·min^{-1} (Figure 1).

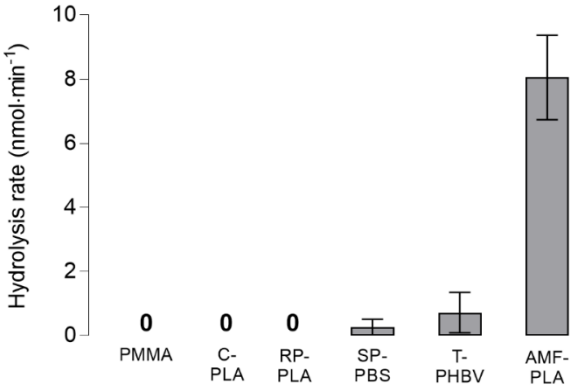

Figure 1: Hydrolytic degradation of five bio-based plastics and one petroleum-based plastic by the gastric fluid of *Cancer pagurus* measured by pH-Stat titration at 15°C, pH 8.2 and 3.2 ppt salinity (means ± SD, n =3). A zero indicates no measured hydrolysis.

3.2 Chromatographic separation of gastric fluids

Anion exchange chromatography resulted in a fractionation of the gastric fluid into 65 separate fractions of 1 mL each. The best separation was achieved by using a low gradient with a NaCl-increase of 0 - 0.6 mol·L^{-1} over the first 40 min. The remaining proteins on the column were eluted with a higher NaCl concentration of up to 1 mol·L^{-1}. The chromatogram of the gastric fluid showed six distinct protein peaks. Most of the absorbance at 280 nm was measured between fractions 25 and 55, where five of the highest absorbance peaks were located. The highest absorbance was measured in fraction 36. High absorbance was also present in fractions 46/47, 50/51, and 63 (Figure 2a).

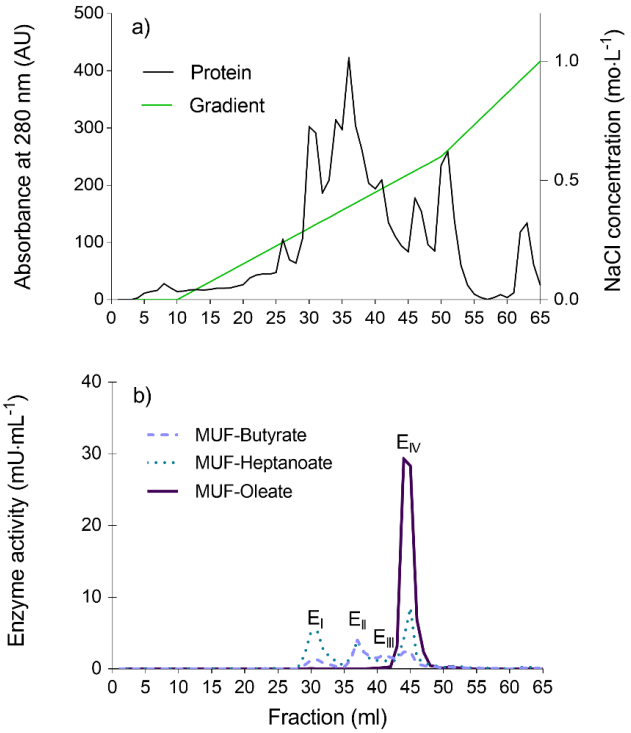

Figure 2: a) LC chromatogram of gastric fluids from *Cancer pagurus*. The x-axis shows the consecutive fractions of 1 mL each at a flow rate of 1 mL·min^{-1}. The left y-axis shows the absorbance at 280 nm (protein) in arbitrary units (AU). The green line represents the steadily increasing NaCl-concentration in the buffer over time (right y-axis). b) Enzyme activity (mU·mL^{-1}) of esterases and lipases of each fraction measured as hydrolysis of the specific fluorogenic substrates at pH 7. Four maxima with elevated esterase/lipase activity ($E_{(I)}$ – $E_{(IV)}$) were identified.

3.3 Enzyme Activities

3.3.1 Screening for carboxylesterases

Enzymes in the separated fractions of the gastric fluid were capable of hydrolyzing short-chain, medium-chain, and long-chain carboxylesters (Figure 2b). MUF-butyrate (short-chain, C4) was mainly hydrolyzed by fractions 29 to 51. The activity peaked in fractions 30 (1.21 mU·mL^{-1}), 37 (4.06 mU·mL^{-1}), 41 (2.0 mU·mL^{-1}), and 45 (2.43 mU·mL^{-1}). Hydrolysis of MUF-heptanoate (medium-chain, C7) appeared in the same fractions as hydrolysis of MUF-butyrate, but with different intensities. Fraction 45 showed the highest activity of 8.56 mU·mL^{-1}, followed by fraction 30 (5.18 mU·mL^{-1}) and fraction 37 (3.92 mU·mL^{-1}). The lowest activity peak appeared in fraction 41 with 1.15 mU·mL^{-1}. Hydrolysis of MUF-oleate (long-chain, C18) showed only one distinct activity peak reaching over fractions 44 and 45 (29.3 and 28.3 mU·mL^{-1}, respectively). The fractions showing carboxylesterase activity peaks are denoted as $E_{(I)}$ to $E_{(IV)}$ as displayed in Figure 2b.

3.3.2 pH profiles

Carboxylesterase activities of $E_{(I)}$ to $E_{(IV)}$ showed specific pH profiles between pH 5 and 9.

$E_{(I)}$ (Fraction 30): Hydrolysis of MUF-butyrate increased from 0.74 mU·mL^{-1} at pH 5 to 2.45 mU·mL^{-1} at pH 7. At pH 8, the activity decreased again and ceased at pH 9. Similarly, hydrolysis of MUF-heptanoate increased from pH 5 (1.25 mU·mL^{-1}) to pH 7 (10.29 mU·mL^{-1}) and pH 8 (10.62 mU·mL^{-1}) and decreased at pH 9 (5.56 mU·mL^{-1}). No hydrolysis of MUF-oleate was present in E_I (Figure 3a).

$E_{(II)}$ (Fraction 37): Highest hydrolysis rates of MUF-butyrate appeared at pH 5 to pH 8 (7.54 to 8.1 mU·mL^{-1}). The activity decreased at pH 9, reaching 2.53 mU·mL^{-1}. Hydrolysis of MUF-heptanoate showed a broad pH maximum between pH 5 to pH 8 and a slight decrease of activity at pH 9 (4.55 mU·mL^{-1}). No hydrolysis of MUF-oleate was detected (Figure 3b).

$E_{(III)}$ (Fraction 41): Hydrolysis of MUF-butyrate increased from pH 5 (3.3 mU·mL^{-1}) to pH 6 (6.71 mU·mL^{-1}) and decreased continuously toward pH 9 where it reached 1.94 mU·mL^{-1}. The hydrolysis rate of MUF-heptanoate was lower than that of MUF-butyrate but less variable over the investigated pH-range. The activities ranged from 1.84 mU·mL^{-1} at pH 5 to 3.58 mU·mL^{-1} at pH 8. As in $E_{(I)}$ and $E_{(II)}$, no hydrolysis of MUF-oleate was detected (Figure 3c).

$E_{(IV)}$ (Fraction 45) showed the highest hydrolysis rate of all MUF-derivatives. Hydrolysis of MUF-butyrate was highest at pH 5 (38.62 mU·mL^{-1}) and decreased continuously toward pH 8 (2.52 mU·mL^{-1}). No hydrolysis was present at pH 9. Similarly, MUF-heptanoate showed highest degradation at pH 5 (58.85 mU·mL^{-1}) with a continuous decrease at higher pH. No activity was present at pH 9. Hydrolysis of MUF-oleate increased from pH 5 (13.32 mU·mL^{-1}) to pH 6 (24.69 mU·mL^{-1}) and decreased at higher pH towards 4.73 mU·mL^{-1} at pH 8. No activity remained at pH 9 (Figure 3d).

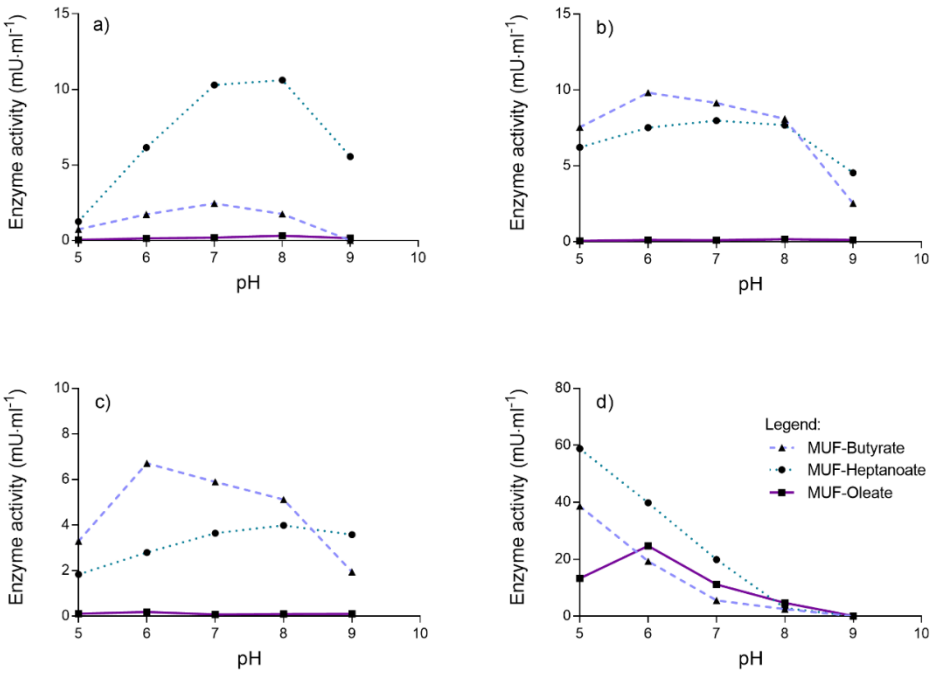

Figure 3: Esterase activities of a) $E_{(I)}$, b) $E_{(II)}$, c), $E_{(III)}$ and d) $E_{(IV)}$, measured with MUF-butyrate, MUF-heptanoate and MUF-oleate at pH 5 to pH 9.

3.3.3 Effect of SDS on enzyme activities

The presence of 0.1 % SDS reduced significantly the esterase activity in the gastric fluid and, thus, the hydrolysis rates of all tested substrates (Paired t-test: $p < 0.01$). The activities of the fractions were not equally affected by SDS.

While the activity in $E_{(I)}$ dropped by 34.7 % for MUF-butyrate and 55.1 % for MUF-heptanoate, the activity in $E_{(II)}$ dropped by over 90 % to residual activities of 3.4 % and 7.4 %, respectively (Figure 4a).

The hydrolysis rate of MUF-butyrate by $E_{(III)}$ dropped by 63.8 % while the hydrolysis of MUF-heptanoate completely ceased (0.4 % residual activity – Figure 4b). The hydrolysis rate of all three substrates by $E_{(IV)}$ extinguished almost completely (0.2 - 2.3 % residual activity - Figure 4c).

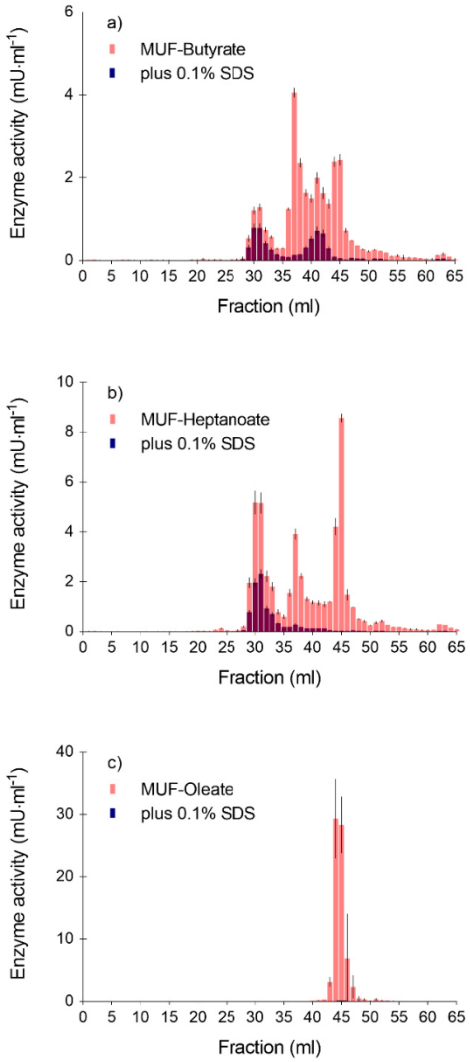

Figure 4: Esterase activity (in mU·mL^{-1}) of each fraction at pH 7 with and without addition of 0.1% sodium dodecyl sulfate (SDS) with a) MUF-butyrate, b) MUF-heptanoate and c) MUF-oleate (means ± SD, n = 3).

3.4 Hydrolysis of BPE-AMF-PLA by gastric fluid fractions

The fractions around $E_{(I)}$ to $E_{(IV)}$ with high carboxylesterase activity were pooled and will be denoted in the following as Pool $E_{(I)}$ to Pool $E_{(III/IV)}$ (Figure 5). $E_{(III)}$ and $E_{(IV)}$ were combined to one Pool $E_{(III/IV)}$, because the esterase peak in $E_{(III)}$ was missing in some samples. Proteins in Pool $E_{(I)}$ (Fractions 29-32), Pool $E_{(II)}$ (Fractions 36-38), and Pool $E_{(III/IV)}$ (Fractions 41-47) were concentrated and their potential to hydrolyze BPE-AMF-PLA was tested. As a negative control, fractions without esterase activity were pooled, concentrated, and tested (Fraction 60-65).

Pools $E_{(I)}$ to $E_{(III/IV)}$ were able to hydrolyze the BPE-AMF-PLA microparticles (Figure 5). Pool $E_{(I)}$ showed the highest hydrolysis rate (8.1 ± 4.4 nmol·min^{-1}), followed by Pool $E_{(III/IV)}$ (5.4 ± 1.3 nmol·min^{-1}). Pool $E_{(II)}$ showed the lowest hydrolysis rate (1.4 ± 1.3 nmol·min^{-1}). The negative control showed no hydrolysis rate.

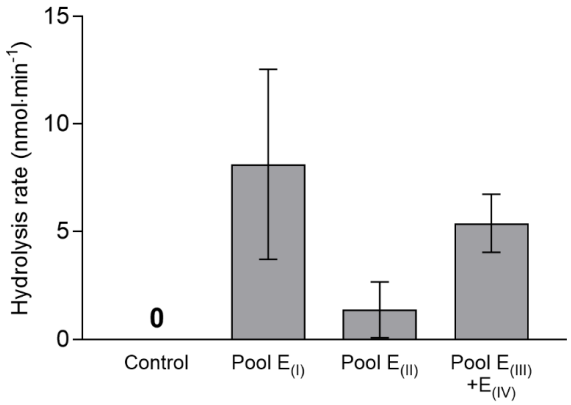

Figure 5: Hydrolytic degradation of BPE-AMF-PLA by the pooled fractions with esterase activity of the gastric fluid of *Cancer pagurus* measured by pH-Stat titration at 30 °C, pH 8.2 and 32 ppt salinity (means ± SD, n =3). A zero indicates no measured hydrolysis.

3.5 Electrophoretic separation of proteins

SDS-PAGE with subsequent Coomassie staining showed different protein bands in the four investigated fractions. Incubation with MUF-substrates showed clear activity bands in $E_{(I)}$ and $E_{(III)}$.

$E_{(I)}$ showed a faint activity band at around 45 kDa (Figure 6a, Lane 1). Another faint band, which appeared at the top of the gel, is considered an artefact due to the unrealistic high molecular mass (~250 kDa), which may result from protein agglutinations. A strong activity band appeared after incubation with MUF-heptanoate at around 45 kDa and a faint signal at 40 kDa (Figure 6a, Lane 2). No activity bands were visible after incubation with MUF-oleate (Figure 6a, Lane 3).

$E_{(III)}$ showed clear activity bands at 40 kDa for both MUF-butyrate and MUF-heptanoate (Figure 6 c, Lane 1-2). MUF-butyrate showed also a faint activity band 35 kDa. Similar to $E_{(I)}$, $E_{(III)}$ showed no activity bands after incubation with MUF-oleate (Figure 6c, Lane 3).

$E_{(II)}$ and $E_{(IV)}$ showed only faint activity bands after incubation with any of the MUF-substrates. The positions of these bands are indicated by white ticks (Figure 6 b, d).

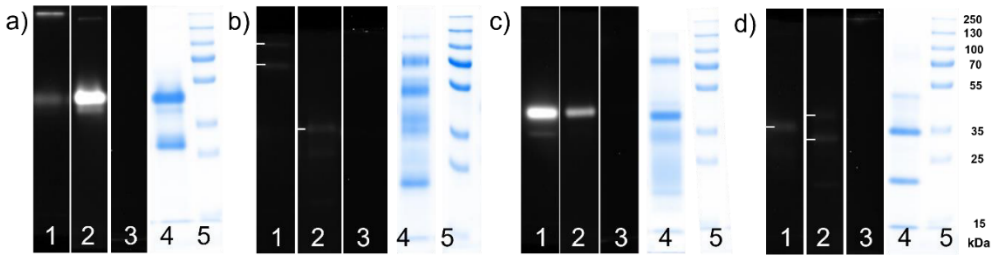

Figure 6: Snippets of polyacrylamide gels with proteins separated by SDS-PAGE of a) E_{I}, b) E_{II}, c) E_{III} and d) E_{IV}. The gels were incubated with MUF-butyrate (Lane 1), MUF-heptanoate (Lane 2), MUF-oleate (Lane 3) and Coomassie (Lane 4). Lane 5 shows the molecular weight marker.

In summary, several AMF-PLA-degrading carboxylesterases were identified in the gastric fluid of the Edible crab *Cancer pagurus*. They exhibit different characteristics such as pH optimum, inhibition by SDS and substrate specificity, and their molecular weights are in the range of 40 kDa (Table 3).

Table 3: Characteristics of the carboxylesterases present in the fractions $E_{(I)}$ to $E_{(IV)}$. n.d. = not defined.

Fractions	**Substrate**	**pH optimum**	**Inhibition by SDS (%)**	**Molecular weights (kDa)**
$E_{(I)}$	Butyrate (C4)	7	35	45
	Heptanoate (C7)	8	55	45, 40
	Oleate (C18)	n.d.	n.d.	n.d.
$E_{(II)}$	Butyrate (C4)	6	97	n.d.
	Heptanoate (C7)	7	93	n.d.
	Oleate (C18)	n.d.	n.d.	n.d.
$E_{(III)}$	Butyrate (C4)	6	64	40, 35
	Heptanoate (C7)	8	> 99	40
	Oleate (C18)	n.d.	n.d.	n.d.
$E_{(IV)}$	Butyrate (C4)	(<) 5	98	n.d.
	Heptanoate (7)	(<) 5	> 99	n.d.
	Oleate (C18)	6	> 99	n.d.

4 Discussion

Crustaceans play an economically important role as marine food resource and are ecologically crucial in a multitude of different habitats and food webs. The crustacean order of Decapoda shows various feeding habitats, covering a wide range of feeding strategies that implicate the uptake of microplastic particles, whether as predator, herbivore, omnivore or filter feeder (D'Costa, 2022). Microplastics were found in the gastrointestinal tracts and stomachs in various decapod species collected in natural environments (e.g. Stasolla et al., 2015; Piarulli et al., 2019; Li et al., 2021, Joyce et al., 2022).

Adaptation to different ecological niches and the associated food sources yielded a broad range of digestive hydrolytic enzymes (Vogt, 2021). Besides a large number of proteinases, the gastric fluids of decapods contain glucosidases, esterases, and lipases (Vogt, 2021), which were also confirmed for *C. pagurus* by ApiZym-screening. This variety of highly active digestive enzymes allows decapods to utilize natural polymers efficiently, including chitin and cellulose (Linton et al., 2014 and literature cited therein). Accordingly, it appears reasonable that gastric enzymes may also be able to hydrolyze biodegradable plastics. The Edible crab, *Cancer pagurus* served in our study as a model species. It allowed to withdraw gastric fluid and to simulate *in vitro* the enzymatic processes that appear within the stomach of the crab.

The *in-vitro* degradation experiments were conducted at 15 °C, which is the upper temperature range of *Cancer pagurus* in its preferred sublittoral habitat (Cuculescu et al., 1998; Bakke et al., 2019). Among the five plastics incubated with gastric fluid, BPE-AMF-PLA showed the highest hydrolysis rate. BPE-AMF-PLA is a biobased biodegradable plastic blend consisting of the polymer components PLA and PBAT. PLA is an aliphatic polyester that can be degraded by several hydrolytic enzymes, but predominantly proteases (Lim et al., 2005; Kawai, 2010; Miksch et al., 2021). PBAT is an aromatic aliphatic polyester, which can be degraded by esterases and lipases (Wallace et al., 2017; Kanwal et al., 2021). The enzyme screening of the gastric fluid of *C. pagurus* via ApiZym strips revealed high activities of both proteolytic and lipolytic enzymes. Accordingly, either of these enzyme classes in the gastric fluid can be responsible for the observed hydrolysis of BPE-AMF-PLA. However, previous studies have shown, that BPE-AMF-PLA was preferably degraded by lipase from the yeast *Candida antarctica* under the same *in-vitro* conditions as in the present study (Miksch et al., 2022). No hydrolysis was observed by protease from *Bacillus licheniformis* suggesting that the lipolytic enzymes rather than the proteolytic enzymes in the gastric fluid of *C. pagurus* are responsible for the degradation of the material. So we focused the further investigations on gastric enzymes with lipolytic activity.

After anion exchange chromatography, the separated fractions were screened for their esterase activity using MUF-derivatives of different length. Four fractions ($E_{(I)}$ to $E_{(IV)}$) with distinct hydrolytic potential to cleave either MUF-butyrate (C4), MUF-heptanoate (C7), or MUF-oleate (C18) were identified. Short-, medium- or long-chain fatty ester acids were hydrolyzed differently by $E_{(I)}$ to $E_{(IV)}$. Therefore, these results indicate the presence of several different esterases with lipolytic activity in the gastric fluid of *C. pagurus*. $E_{(I)}$ contains an esterase, which tends to cleave esters of medium-chain fatty acids, whereas

enzymes in $E_{(II)}$ and $E_{(III)}$ are hydrolyzing both C4- and C7-chain length fatty acids at similar rates. This is different to lipases from other crustaceans like penaeids, which rather hydrolyze longer chain substrates (del Monte et al., 2002; Forrellat Barrios et al., 2004; Rivera-Pérez et al., 2011). Esterases in $E_{(IV)}$ showed higher specificity towards the long-chain substrate at pH 7, but were also capable of hydrolyzing short- and medium chain substrates at high rates. Such wide substrate specificity is unusual for digestive lipases. However, Cherif et al. (2007) observed a similar specificity in a digestive lipase from the European shore crab *Carcinus maenas*. Accordingly, it can be suggested that lipases with a wide specificity are common in decapod crustaceans.

The fractions $E_{(I)}$ to $E_{(IV)}$ showed different pH optima. Although the pH in the gastric fluid is around 5.8 (Navarette del Toro et al. 2006), the lipolytic activity in $E_{(I)}$ peaked at neutral and slightly alkaline values of pH 7 and pH 8, respectively. A similar pH-optimum was observed for the endopeptidase trypsin in the gastric fluid of *C. pagurus*, which was almost inactive at pH below 6 but rose with increasing pH (Saborowski et al., 2004). Unlike trypsin, carboxylesterases in $E_{(II)}$ and $E_{(III)}$ were active over the range of pH 5 to pH 9, albeit with decreasing activity towards the acidic and the alkaline margins. Furthermore, different to trypsin of the gastric fluid, the carboxylesterase activity in $E_{(IV)}$ strongly decreased with increasing pH. The activity was highest at lower pH values of 5 and 6, which is in agreement with other studies of gastric lipase from crustaceans (O'Connor and Gilbert, 1968).

Fractions with high esterase and lipase activities were grouped in pools ($E_{(I)}$, $E_{(II)}$, $E_{(III)+(IV)}$) and tested again for BPE-AMF-PLA hydrolysis by pH-Stat titration. All pools, which previously exhibited lipolytic activity, were also able to hydrolyze BPE-AMF-PLA, whereas the negative control lacking lipolytic activity showed no hydrolysis of BPE-AMF-PLA. The selected pool for the negative control included fractions, which were the last to elute from the anion exchange column and showed a high protein peak (Figure 4a). These fractions possess high tryptic activity, which was shown in a study by Hehemann et al. (2008). Trypsin is a protease that has been shown to hydrolyze PLA (Lim et al. 2005). However, the trypsin in the gastric fluid of *C. pagurus* does not hydrolyze BPE-AMF-PLA. These results affirm the assumption, that the enzymes responsible for the degradation of the biodegradable plastic are lipases that hydrolyze the PBAT fraction of the compound, rather than proteases that may hydrolyze the PLA fraction. Furthermore, there seems to be a relation between MUF-heptanoate activity and BPE-AMF-PLA hydrolysis. Fractions with higher activity towards MUF-heptanoate also showed higher hydrolysis rates of BPE-AMF-PLA (Figure 4b, 7). This indicates that lipases from the gastric fluid, which are able to cleave esters from medium-chain fatty acids, are contributing most to the hydrolysis of PBAT-based plastics, at least at the given conditions of pH 8.2 and 32 ppt salinity.

Esterase activity was measured in all four fractions ($E_{(I)}$ to $E_{(IV)}$). However, the activity disappeared almost entirely in $E_{(II)}$ and $E_{(IV)}$ after separation by SDS-PAGE. The loss of activity was caused by the addition of 2% SDS during sample preparation, which was confirmed by the inhibition of esterase/lipase activity in single LC-fractions after addition of SDS (Figure 4). Surfactants such as SDS can have various consequences on the molecular structure and activity of enzymes (Miller et al., 2000; Antipova

et al., 2001). SDS at low concentrations forms complexes with enzymes. It inhibits the activity of lipase under simulated gastrointestinal conditions (Delorme et al., 2011; Li and McClements, 2011). However, fraction $E_{(I)}$ and $E_{(III)}$ showed less inhibition by SDS, resulting in bright activity bands on the gels.

The activity bands showed apparent molecular masses of 40 and 45 kDa. Lipase in other crustaceans, such as *Penaeus vannamei*, showed a similar molecular mass of 44.8 kDa (Rivera-Pérez et al., 2011). Lipases are a subclass of esterases with a unique mode of action. Unlike other enzymes, they catalyze reactions at solid-water interfaces by adsorption to the surface of water-insoluble substrates (Hasan et al., 2009), which might be advantageous in the hydrolysis of microplastics. Lipases were studied in several other crustacean species, such as *Homarus americanus* (Brockerhoff et al., 1970), *C. maenas* (Cherif et al., 2007) or *Penaeus vannamei* (Rivera-Pérez et al., 2011). Preliminary pH-Stat experiments showed that the gastric fluid of *H. americanus* was able to hydrolyze BPE-AMF-PLA to a similar extent as *C. pagurus* (L. Miksch, unpublished data). Therefore, screening of additional species is required to obtain an overview whether crustaceans and other invertebrates are generally capable of hydrolyzing biodegradable plastics after ingestion. However, not all species that are exposed to microplastic in the environment are large enough to provide enough gastric fluids for analysis. In those cases, molecular approaches and de novo peptide sequencing techniques would be a suitable step, comparing the sequences with known data from other crustacean species.

Hydrolysis rates of biodegradable plastics are lower than those of natural polymers, such as collagen (Miksch et al., 2022). Nevertheless, hydrolysis of biodegradable microplastics in the stomach of *C. pagurus* may have various consequences to the organism. The degradation of ingested bioplastic particles can facilitate the release of toxic additives (Koelmans et al., 2013; Gewert et al., 2021). Degradation products and intermediates may interact with digestive enzymes and inhibit their hydrolytic capacities, which, in turn, would hamper nutrient utilization from ingested food. The efficiency of the degradation process and, thus, the amount of degradation products also depends on the residence time of ingested particles within the digestive tract. Rapid egestion or regurgitation of microplastics, which was observed in some crustacean species (Saborowski et al., 2019; Korez et al., 2020), might prevent the microplastics from being hydrolyzed. Nonetheless, ingestion of biodegradable plastics might impact crustaceans differently than conventional microplastics. Whether this happens in a positive or negative way, is question to further research.

5 Conclusions

Biodegradable plastics are a promising alternative to conventional plastics due to their expected shorter residence time in the environment. However, the degradability of these plastics under marine conditions is under debate and there is no information about their fate upon ingestion by marine invertebrates. The gastric fluid of the Edible crab *C. pagurus* contains several enzymes with carboxylesterase activity, which are capable of hydrolyzing a plastic blend based on PLA/PBAT. Since carboxylesterases are common digestive enzymes, this study suggests that a multitude of marine invertebrates are able to degrade biodegradable plastics. However, the nature of the degradation products and their biochemical effects in the intestine of animals is unknown and demands further research.

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Data availability statement

Upon acceptance of this manuscript, datasets presented in this study will be uploaded to PANGEA online repository to be openly available.

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Supplementary Material

Table S1: Specification sheets of the used polymers.

Designation	Base polymer	Producer	Application	Biodegradability
BPE-C-PLA	PLA/PBS	ABM	Cutlery	No information
BPE-RP-PLA	PLA/PBS	ABM	Rigid packaging	Manufactured from compostable plastic (EN 13432)
BPE-AMF-PLA	PLA/PBAT	NaturePlast	Mulch films	No information
BPE-SP-PBS	PBS	NaturePlast	Soft packaging	Industrially compostable (NF EN 13432:2000)
BPE-T-PHBV	PHBV	NaturePlast	Toys	Industrially compostable (ASTM D6400)
PMMA	PMMA	KFG	Industry	Not biodegradable

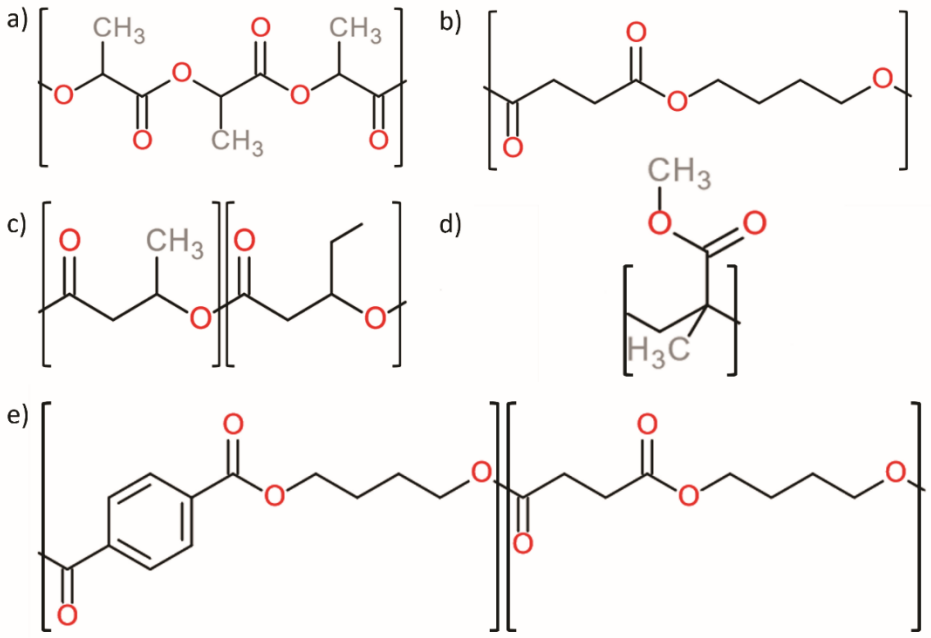

Figure S1: Structural formulas of the base polymers of the test materials used in this study. a) Polylactic acid (PLA), b) Polybutylene succinate (PBS), c) poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV), d) poly(methyl methacrylate) (PMMA), e) polybutylene adipate terephtalate (PBAT).

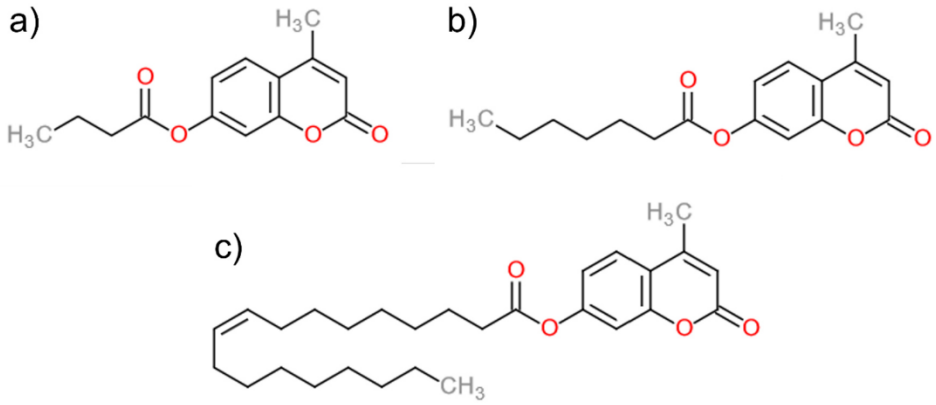

Figure S2: Structural formulas of the fluorogenic MUF-derivatives from fatty ester acids used for enzyme activity examination. a) MUF-butyrate (C4), b) MUF-heptanoate (C7), c) MUF-oleate (C18).

3.4 Publication IV (Manuscript 2)

Toxicity of bioplastics and bioplastic additives on *Brachionus plicatilis* (Rotifera) and *Artemia persimilis* (Crustacea, Brachiopoda)

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Toxicity of bioplastics and bioplastic additives on *Brachionus plicatilis* (Rotifera) and *Artemia persimilis* (Crustacea, Brachiopoda)

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Abstract

Bioplastics are promising alternatives to conventional petroleum-based plastics in pursuing the goal to reduce environmental plastic pollution. Nevertheless, first studies report the presence of bioplastics and bioplastic fragments in seawater and marine sediments, where they may accumulate and interact with biota. Therefore, we studied the acute toxic effects of seawater leachates from five novel bioplastics based on polylactic acid (PLA), polybutylene succinate (PBS), polybutylene adipate terephthalate (PBAT), and poly(hydroxybutyrate-co-hydroxyvalerate (PHBV), as well as five common chemical additives of bioplastics. Leachates were prepared with 5 $mg \cdot ml^{-1}$ of microparticles (< 200 µm) made from bioplastics or variable amounts of additives, which were incubated for 24 h in artificial seawater. Test organisms were incubated in the filtered seawater medium containing the leachates. Acute toxicity tests were done with rotifers according to ISO Standard 19820 and with nauplii of the Brine shrimp *Artemia persimilis*. The mortality of the test organisms was determined after 24 and 48 hours, respectively. Leachates of one of the five bioplastic materials, PHBV, caused high toxicity in both test species. Subsequent testing with the pure and unprocessed base material showed no adverse effects on rotifers and *Artemia* nauplii. Leachates of the additive benzophenone showed high toxicity to both test species, whereas leachates of glycerol triacetate proved to be toxic to rotifers but not to the Brine shrimp nauplii. Accordingly, bioplastic products can have adverse toxic effects on small marine organisms. However, the toxicity was primarily ascribed to the additives in the formulated compound rather than the polymeric material as such.

Keywords: bioplastics, leachates, additives, polymers, acute toxicity, marine zooplankton

1. Introduction

The progressing pollution of the environment with plastics leads to a rethinking of plastic consumption and substitution by new and more environment-friendly alternatives. A promising approach is the development and application of biodegradable plastics. The aim is to replace conventional non-biodegradable plastics by plastic that can be degraded to harmless compounds and, at the best, to CO_2 and water (Tokiwa et al., 2009). Biodegradable plastics can either be based on fossil resources or on renewable biomass resources. The latter are progressively introduced into modern biodegradable plastics, combining an increased environmental compatibility with a lower carbon footprint and resulting in products with maximized sustainability. Common novel polymers that are used for such plastics are for example polylactic acid (PLA), polybutylene succinate (PBS) or polyhydroxyalkanoates (PHAs). However, physical properties of biodegradable plastics are often inferior to conventional, non-degradable plastics. To match the requirements for the wide range of plastic applications, additives, fillers, and copolymers are applied to enhance their performance (Khan et al., 2017).

The matrix of a plastic material, either biodegradable or conventional, consists not only of one or more polymers, but also of various other chemicals (OECD, 2004). Additives are found in almost every plastic product. Their quantities and compositions vary depending on the base polymers, the production process, and the intended application of the final products (OECD, 2004). Additives in common plastic products include ultra-violet (UV)-stabilizers, anti-hydrolysis agents, antioxidants, plasticizers, impact modifiers, flame retardants, and many other (Hahladakis et al., 2018). Furthermore, plastics may contain non-intentionally-added chemicals that are formed as side or breakdown products during degradation or interaction with packaged foodstuff (Muncke, 2009). When discarded into the environment, additives, degradation- and reaction-products, as well as not polymerized residual monomers might leach out of the plastic (Sheftel, 2000; OECD, 2004). The release of leachates depends not only on the material composition, but also on the processing and weathering of the material (Sheftel, 2000; Koelmanns et al., 2013; Rochman et al., 2013).

Once in the environment, substances that leach out of discarded plastic may harm organisms (e.g. Kim et al., 2006; Lithner et al., 2009; Capolupo et al., 2020; Rhendell-Bhatti et al., 2021). In marine environments, leachates can induce biological effects already at low concentrations (Oehlmann et al., 2009). Biobased and biodegradable plastics particularly rely on the use of additives, to improve their limited physical properties (Beach et al., 2013; Khan et al., 2017). Zimmermann et al. (2020a) showed that the majority of biobased and biodegradable plastics

contain high numbers of chemicals and toxic compounds. Biobased and biodegradable plastics are advertised as safe alternative to conventional plastics. However, the impact of substances leaching into the environment is largely unknown. Several studies indicate a connection of toxicity with plastic degradation (Jahnke et al., 2017; Ouyang et al., 2021; Quade et al., 2022).

The growing demand of bio-based and biodegradable plastics boost their production, which is projected to increase from 2.4 million tons in 2021 to 7.6 million tons in 2026, bypassing the 2% share of the total global plastic production (European Bioplastics, 2021). As a consequence, higher amounts of bio-based and biodegradable plastics are likely to be discarded into the environment. Low consumer attention to correct disposal because of the "biodegradable" status of such products might contribute to further environmental pollution (Taufik et al., 2020). Accordingly, there is a need for rapid and accurate evaluation of the potential impacts of biodegradable plastics on biota.

In the present study, we examined the toxicity of leachates from five biobased biodegradable plastics and common additives on two test species. We employed rapid acute toxicity tests with the rotifer *Brachionus plicatilis* following the ISO Standard 19820 and with nauplii of the Brine shrimp *Artemia persimilis*. Bioplastics that caused elevated toxicity to either species were examined in more detail by determining LC_{50} values and compared with the pure base polymer.

2. Materials and Methods

2.1. Bioplastics

The plastic compounds used in this study (Table 1) were provided within the framework of the Horizon 2020 EU-project "Bio-Plastics Europe (BPE)". The compounds were produced and supplied by Arctic Biomaterials OY (ABM, Tampere, Finland), and Natureplast SAS (Ifs, France). Native granules of PHBV, which were used for the compound formulation of T-PHBV, were provided by Natureplast SAS.

Table 1: Specification of the bioplastic compounds and polymers used for this study.

Compound	Polymer type	Producer	Application
BPE-C-PLA	PLA/PBS	ABM	Cutlery
BPE-RP-PLA	PLA/PBS	ABM	Rigid packaging
BPE-AMF-PLA	PLA/PBAT	Natureplast	Agricultural mulch films
BPE-SP-PBS	PBS	Natureplast	Soft packaging
BPE-T-PHBV	PHBV	Natureplast	Toys
PHBV (base polymer)	PHBV	Natureplast	Plastic component

2.1.1. Preparation of microparticles

Compounds and polymers were ground to a fine powder by cryogenic milling (6775 Freezer/Mill, Spex SamplePrep) to increase the surface of the plastics and facilitate the leaching of associated chemicals. The cryogenic mill was loaded with one gram of material per grinding process. Before the start of the grinding program, the materials were cooled for 15 min to achieve a sufficient brittleness, making them easier to process. Grinding was done in four to eight cycles, with each cycle including 1 to 2 min of grinding and 1 to 2 min of cooling. Grinding speed was set to 15 impacts per second. After each grinding process, the material was sieved to obtain the size fraction smaller than 200 µm.

2.2. Additives

Five commonly used additives were selected to evaluate their toxicological effect on marine organisms (Table 2). The additives were provided by ArcticBiomaterials (ABM, Tampere, Finland) and are commonly used in formulations of bio-based and biodegradable plastics.

Table 2: Specifications of the additives used for toxicity testing.

Additive	Application	Producer	Concentration used
Benzophenone	UV absorber	Sigma	0.1 $mg \cdot mL^{-1}$
CT-L03	PLA melt strength enhancer	Polyvel Inc.	10 $mg \cdot mL^{-1}$
Elvaloy™ PTW	Impact modifier	Dow Chemical	100 $mg \cdot mL^{-1}$
Exolit® AP 422	Flame retardant	Clariant	1 $mg \cdot mL^{-1}$
Glycerol triacetate	Plasticizer	Sigma	4.3 $\mu l \cdot mL^{-1}$

2.3. Toxicity of bioplastic leachates and additives

2.3.1. Toxicity screening with the marine rotifer Brachionus plicatilis

Fifty mg of microplastic particles of each compound were filled into separate 15-mL glass test tube. Ten mL of artificial seawater were added to the test tube, resulting in a concentration of 5 mg microplastics per mL. Ten mL of medium without microplastics were filled in a glass test tube and used as control. The test tubes were closed with Teflon seals and placed in a rotator mixer (RM Multi-1, Starlab Group, Hamburg, Germany) with non-stop orbital rotation at a speed of 9 rpm for 24 hours at 20 ± 1°C. After 24 hours of incubation, the media were filtered

through 0.45 µm cellulose acetate syringe filters (Minisart, Sartorius, Göttingen, Germany) to obtain particle free fluids.

Toxicity tests were performed with the commercially available RotoxKit M testkit (Microbiotests, Gent, Belgium, order code TK22) according to ISO Standard 19820 as per the supplier´s instructions. Hatching of the cysts of the rotifer *B. plicatilis* was initiated in a hatching well by placing the cysts in 20 ppt artificial seawater and incubating them for 24 to 26 hours at 25 ± 1°C with continuous illumination of at least 3000 lux. Each test medium was transferred to one rinsing and six test wells of a PVC plate as provided by the kit. About 50 hatched rotifers were first transferred to each rinsing well filled with 0.7 mL of the different test media or control medium, to avoid a dilution of the test solution with hatching medium in the test wells. The rotifers were kept in the rinsing wells for approximately one hour to let them adapt to the salinity change from 20 to 35 ppt. Subsequently, five rotifers from each rinsing well were transferred to each of the six test wells filled with 0.3 mL test medium, resulting in 30 rotifers per treatment. Thereafter, the rotifers were incubated at 25 °C in darkness and mortality was inspected after 24 and 48 hours under a dissecting microscope. Rotifers were considered dead when they showed no movement after five seconds of observation.

2.3.2. Toxicity screening with nauplii of the brine shrimp Artemia persimilis

The preparation of the leachate medium for *Artemia* tests was analogue to that of *Brachionus plicatilis*, with minor changes of the standard seawater. Fifty mg of microplastic particles of each compound were filled in a 15-mL glass test tube and 10 mL of tap water were added. The test tubes were closed with a Teflon-sealed screw cap and placed in a rotator mixer (RM Multi-1, Starlab Group, Hamburg, Germany) with non-stop orbital rotation at a speed of 9 rpm for 24 hours at 20 ± 1 °C. After 24 hours of incubation, the media were filtered through 0.45 µm cellulose acetate syringe filters (Minisart, Sartorius, Göttingen, Germany) to obtain particle free medium. Then, *Artemia* salt containing dried microalgae as food (JBL, Neuhofen, Germany) was added to obtain a salinity of 23 ppt. Each test medium was transferred to one rinsing and three test wells of a 24-well PVC cell-culture plate (Orange Scientific, Braine-l'Alleud, Belgium, cat no. 4430300).

Cysts of *Artemia persimilis* (brand ´Black Label´) were purchased from REBIE-Onlineshop (Bielefeld, Germany). 0.8 g of brine shrimp cysts were added to 500 mL of artificial seawater in a glass beaker. The cysts were incubated at 25 °C for 48 hours at continuous aeration and illumination (minimum 3000 lux). Hatched brine shrimp nauplii were first transferred to the

rinsing wells filled with 1 mL of test medium to avoid a dilution of the test solution during transfer. After 1 hour, 10 *Artemia* nauplii from the rinsing well were placed in each of the three test wells filled with 1 mL test medium or control medium. This procedure was repeated for every treatment, resulting in a total of 30 brine shrimp larvae exposed to each test medium and the control. The larvae were incubated at 25 °C in darkness for 24 hours, after which they were observed under a dissecting microscope. The *Artemia*-nauplii were considered dead if they did not show any movement during ten seconds of observation.

2.3.3. Toxicity of the base polymer PHBV

Bioplastic compounds that showed increased toxicity to *B. plicatilis* or *A. persimilis* nauplii, were subsequently tested for the toxicity of their basic components. Suspensions of PHBV particles in medium were prepared analogously to the leachate toxicity tests with the plastic compounds (Section 2.3.1 and 2.3.2). Briefly, 50 mg of particles were suspended in 10 mL medium and agitated for 24 hours in a rotator mixer. The particles in the suspension were then eliminated by filtering through 0.45 µm cellulose acetate syringe filters. Subsequently, the medium was used for mortality tests with rotifers and *Artemia* nauplii in the same manner as described for the plastic leachate tests.

2.3.4. Toxicity of chemical additives

Different amounts of additives were used (Table 2), based on available LC_{50} data for marine organisms taken from the respective safety data sheets of the substances. The additives were filled in 15 mL glass test tubes and 10 mL of the respective medium either for rotifers or *Artemia* was added (see section 2.3.1 and 2.3.2). The control was sole medium without additive. The additives showed low solubility and, with the exception of the liquid glycerol triacetate, are present in solid form. Therefore, the test tubes were placed in a rotator mixer (RM Multi-1, Starlab Group, Hamburg, Germany) with non-stop orbital rotation at a speed of 9 rpm for 24 hours at 20 ± 1°C. After 24 hours of incubation, the media with benzophenone and Elvaloy PTW were filtered through 0.45 µm cellulose acetate syringe filters (Minisart, Sartorius, Göttingen, Germany) to eliminate particulate residues. The respective medium with the dissolved additive was then used for mortality tests with rotifers or *Artemia* nauplii. The mortality tests were conducted as described above (2.3.2 and 2.3.3), but with additive medium instead of leachate medium.

2.4.5. Definitive tests

Bioplastic leachates and additives that showed significantly higher toxicity than the controls were subjected to LC_{50} determination. A two-fold dilution series of the originally used concentration (100 %, 50 %, 25 %, 12.5 % and 6.25 %) was prepared, to determine the range of the lowest concentration inducing 100 % mortality and the highest concentration inducing 0 % mortality. Experiments with these concentrations were performed in triplicate for both rotifers and *Artemia* nauplii.

2.5. Statistics

Mortality data were statistically analyzed and visualized with the software GraphPad Prism Version 7.05 for Windows (La Jolla, CA, USA, www.graphpad.com). The relative mortalities were compared by a 1-factorial analysis of variance (ANOVA) with multiple comparisons (n = 3). Prior to the ANOVA, the data were tested for heteroscedasticity by Brown-Forsythe test. The significance level of all statistical analyses was $\alpha = 0.05$. To determine the range of the highest observed concentrations inducing 0 % mortality for LC_{50} calculation, concentrations that did not differ significantly from the control were considered as zero mortality. The LC_{50} values were calculated after logit and probit transforming of the data and subsequent linear regression, and by applying the Quest Graph™ LC_{50} Calculator from AAT Bioquest Inc. (https://www.aatbio.com/tools/lc50-calculator).

3. Results

3.1. Mortality of Brachionus plicatilis

3.1.1. Mortality by bioplastic leachates

The sensitivity of the rotifers to plastic leachates was observed after 24 and 48 hours of exposition. The mortality in the control group did not exceed 10 % at any given time. After 24 hours, all rotifers died, which were exposed to the leachates of T-PHBV. All other treatments showed very little (≤ 1.2%) or zero mortality after 24 hours (Figure 1a). After 48 hours, there was a slight increase in mortality of 1.1 to 6.7 % in all treatments except C-PLA and T-PHBV. The highest mortality besides T-PHBV after 48 hours was induced by AMF-PLA with 7.8 ±

7.7 %. However, the mortality after 48 hours was not significantly higher than the mortality in the control after 48 hours (Figure 1b).

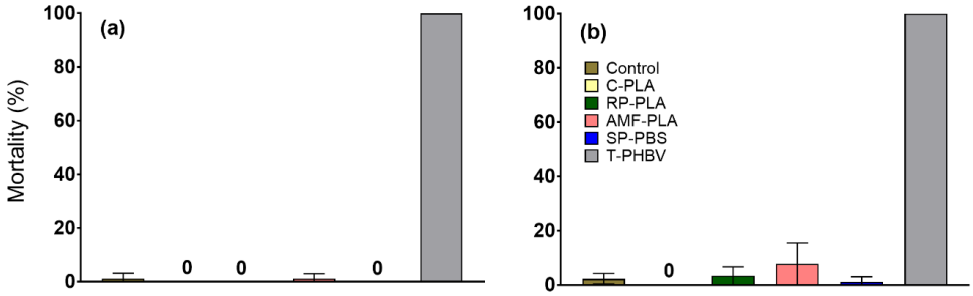

Figure 1: Relative mortality of rotifers, *Brachionus plicatilis*, after (a) 24 hours and (b) 48 hours exposure to plastic leachates and a control medium (means ± SD, n = 3). The number zero ´0´ indicates no mortality.

3.1.2. Mortality by additives

The commercial additives induced differential mortalities in rotifers exposed for up to 48 hours. The mortality of the control group did not exceed 10 % at any time. After 24 hours, rotifers exposed to benzophenone showed the highest mortality of 22.7 ± 24.2 %, followed by glycerol triacetate (5.6 ± 9.6 %). Rotifers exposed to Exolit showed only 1.1 ± 1.9 % mortality, while animals exposed to Polyvel and Elvaloy showed no mortality at all (Figure 2a). After 48 hours, the mortality increased in all treatments. The highest increase to 85.5 ± 10.1 % was observed in the rotifers exposed to glycerol triacetate. 73.8 % ± 23.2 % of the rotifers exposed to benzophenone died. In all other treatments, the mortality was less than 5 % after 48 hours (Figure 2b).

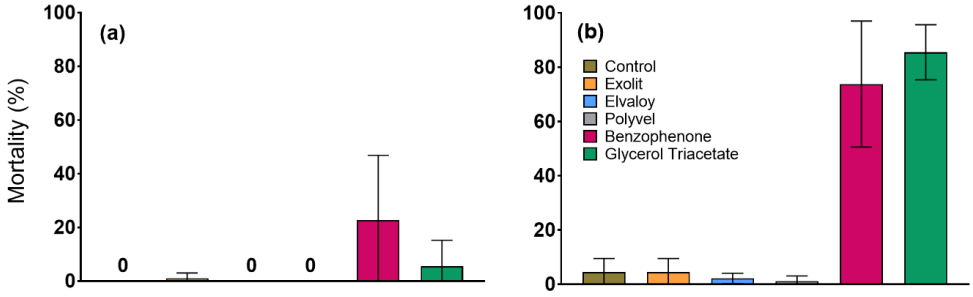

Figure 2: Relative mortality of rotifers, *Brachionus plicatilis*, after (a) 24 hours and (b) 48 hours exposure to additives and a control medium (means ± SD, n = 3). The number ´0´ indicates no mortality.

3.2. Mortality of Artemia persimilis nauplii

3.2.1 Mortality by plastic leachates

The mortality of *Artemia* nauplii in the control and in all plastic leachate treatments except T-PHBV stayed below 5%. 94.4 ± 1.9 % of the nauplii exposed to the leachate of T-PHBV died after 24 hours (Figure 3a).

3.2.2. Mortality by additives

For *Artemia* nauplii exposed to the different commercial additives, benzophenone was most toxic inducing 60.0 ± 0.0 % mortality. In the control and all other treatment groups, only around 5% of nauplii died after 24 hours, with no statistical differences between the treatments (Figure 3b).

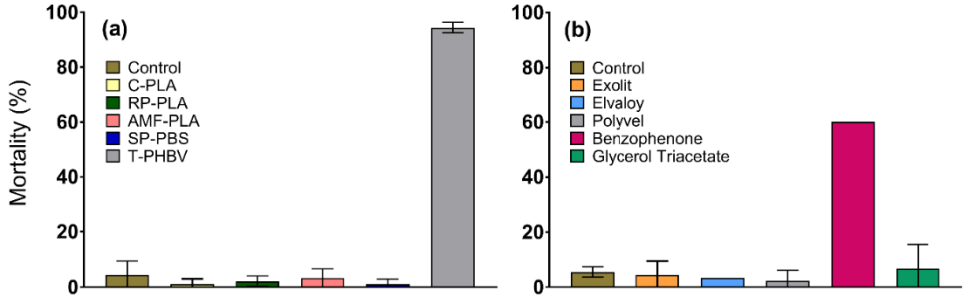

Figure 3: Relative Mortality of brine shrimp nauplii, *Artemia persimilis*, exposed to (a) plastic leachates and (b) additives for 24 hours (means ± SD, n = 3).

3.3. Toxicity of the base polymer PHBV

Brachionus plicatilis and *A. persimilis* nauplii that were incubated in leachates of PHBV microparticles did not show significantly higher mortalities than individuals in the control treatment.

3.4 Median lethal concentration of plastic leachates (LC_{50})

Brachionus plicatilis exposed to microparticles of T-PHBV showed mortality at the highest concentration of the definitive test. The lowest observed concentration inducing 100 % mortality was 5 $g \cdot L^{-1}$. Also for rotifers exposed to benzophenone, only the highest tested concentration of 150 $mg \cdot L^{-1}$ induced a mortality that was significantly higher than the control. For glycerol triacetate, the lowest observed concentration inducing 100 % mortality in rotifers was 6 $ml \cdot L^{-1}$ and the highest concentration inducing 0 % mortality was 1.5 $ml \cdot L^{-1}$. The data obtained for T-PHBV and benzophenone covered a range, which was too small to linearize them with logit and probit functions. Accordingly, LC_{50} values for *B. plicatilis* ranged between 0.16 $g \cdot L^{-1}$ for Benzophenone and 4.32 $g \cdot L^{-1}$ for T-PHBV (Table 3).

For *A. persimilis* nauplii exposed to microparticles of T-PHBV, the lowest observed concentration inducing 100 % mortality was 8 $g \cdot L^{-1}$ and the highest concentration causing no mortality was 2 $g \cdot L^{-1}$. For nauplii exposed to benzophenone, these concentrations ranged between 200 $mg \cdot L^{-1}$ and 50 $mg \cdot L^{-1}$. Accordingly, LC_{50} values varied for *A. persimilis* between 0.07 $ml \cdot L^{-1}$ for benzophenone and 3.4 $g \cdot L^{-1}$ for T-PHBV (Table 3).

Table 3: **LC_{50} values for *B. plicatilis* after 48 h**

Plastic/Additive	Logit (P) function	Probit (P) function	Quest Graph™
T-PHBV	-	-	4.32 $g \cdot L^{-1}$
Benzophenone	-	-	0.16 $g \cdot L^{-1}$
Glycerol triacetate	3.52 $ml \cdot L^{-1}$	3.49 $ml \cdot L^{-1}$	3.74 $ml \cdot L^{-1}$
LC_{50} values for *A. persimilis* after 24 h			
T-PHBV	3.39 $g \cdot L^{-1}$	3.32 $g \cdot L^{-1}$	3.40 $g \cdot L^{-1}$
Benzophenone	0.08 $g \cdot L^{-1}$	0.08 $g \cdot L^{-1}$	0.07 $g \cdot L^{-1}$

4 Discussion

Bioplastics are considered a more sustainable alternative to conventional plastics due to the substitution of non-degradable polymers by biodegradable polymers. In addition to the base polymers, bioplastic compounds usually contain several different additives to tailor the specific properties for the intended applications (Notta-Cuvier et al., 2014; O'Mahony et al., 2020; Milovanovic et al., 2022). This leads to a mixture of chemical substances in bioplastics that might be harmful or even toxic to biota. Bioplastic products can contain several thousands of chemicals (Zimmermann et al., 2020a), that can leak into the environment during degradation (Oluwasina et al., 2021). Therefore, it is important to acquire more information about the

toxicological impact of bioplastics on the environment before these plastics are produced routinely in huge amounts. To test for the toxicity of the components of bioplastic, we exposed the rotifer *Brachionus plicatilis* and nauplii of the brine shrimp *Artemia persimilis* to bioplastic leachates.

Zooplankton species are frequently used in toxicity standard tests to determine ecotoxicological impacts of chemicals and plastics (e.g. Beiras et al., 2018; Reddy and Osborne, 2020; Yoon et al., 2021). The use of *B. plicatilis* and *Artemia* nauplii in quantitative toxicological monitoring has several advantages. Handling is non-elaborate and time-saving due to their small size. Exposures can be done in tissue culture plates with small amounts of test materials. The dry cysts of rotifers and brine shrimps can be stored for months, but hatch on demand after few hours of incubation. Furthermore, early life stages of zooplankton organisms are generally more sensitive to pollutants than adult species. They show quick responses making them excellent indicator species of water quality (Gannon and Stemberger, 1978).

Both rotifers and brine shrimp nauplii in this study were negatively affected by bioplastic leachates from one out of five tested compounds. The investigated plastic materials are based on different raw materials, including PLA, PBS, PBAT and PHBV. The observed toxicity was caused by leachates of the compound T-PHBV, the only plastic that is based on the polymer PHBV. PHBV belongs to the family of polyhydroxyalkanoates (PHAs), and is an aliphatic copolymer from 3-hydroxybutanoic acid and 3-hydroypentanoic acid (Li et al., 2016). PHAs can be produced by bacterial fermentation. Such polymers can contain endotoxins, which need to be removed before further use of the PHA material (Sevastianov et al., 2003). Endotoxins can potentially harm zooplankton species (Gilbert, 1996; Barreiro et al., 2007). However, it is as yet not clear to which degree endotoxins can leach out of the plastics and affect organisms.

PHBV shows the highest biodegradability in seawater compared to all other polymers of our bioplastic selection (Rutkowska et al., 2008; Deroiné et al., 2015; Bagheri et al., 2017; Liu et al., 2022). This may lead to a higher concentration of degradation products in the incubation medium for *Artemia* nauplii and rotifers. However, toxicities of degradation products of PHAs are uncertain. Tanadchangsaeng and Pattanasupong (2022) reported no toxic effects on *Artemia franciscana* nauplii in similar acute toxicity tests with PHBV degradation products. Likewise, the leachate experiments with sole PHBV, which is the same but unprocessed polymer of the toxic plastic T-PHBV, showed no adverse effects. Therefore, a toxic effect originating from the polymer or degradation products thereof alone is unlikely.

The five bioplastics were designated for different applications, such as in cutlery, mulch films or toys. Accordingly, they differ in their compositions of additives. The toxicity of conventional microplastics for marine organisms was caused by functional additives (Beiras et al., 2021). This was also the case for bioplastics based on PLA or polyhydroxybutyrate (PHB), where leaching additives were responsible for the observed toxicity in several aquatic species (Zimmermann et al., 2020b; Quade et al., 2022; Uribe-Echeverría and Beiras, 2022). Due to confidentiality agreements, the manufacturers did not provide us with details about the employed additives. However, we received samples of five additives that are commonly used for compounding bioplastics. Of these five additives, benzophenone and glycerol triacetate showed toxic effects.

Benzophenone increased mortality in both rotifers and brine shrimp nauplii. Benzophenone is an organic compound. Derivatives of Benzophenone are widely used as UV filter in sunscreen agents (Liao and Kannan, 2014) and also in plastic products to block harmful UV irradiation and increase the weather resistance of polymers (Asimakopoulos et al., 2016). Benzophenones are considered endocrine disrupting chemicals, posing health risks to humans and animals (Suzuki et al., 2005; Ghazipura et al., 2017). They are known for acute and chronic toxicity on aquatic zooplankton, such as the mysid *Siriella armata* (Paredes et al., 2014), the water flea *Daphnia magna* (Sun et al., 2016; Song et al., 2021) and larvae of corals (He et al., 2019) or the sea urchin *Paracentrotus lividus* (Paredes et al., 2014). The leaching of benzophenones from the bioplastic compound may have induced the mortality in rotifers and brine shrimp. However, the presence of benzophenones in the leachates has to be verified by suitable analytical methods.

In contrast to benzophenone, glycerol triacetate (syn. triacetin) induced mortality only in the rotifer *B. plicatilis*. Glycerol triacetate is a common additive in food and cosmetics (Fiume, 2003), but is also used as plasticizer in plastic compounds (Zhu et al., 2013; Gama et al., 2019). Glycerol triacetate is considered safe for humans and wildlife (e.g. Fiume, 2003; Quinn Jr and Ziolkowski Jr, 2015) and is classified as non-hazardous by the European Chemicals Agency (ECHA, 2022). Standardized toxicity studies with other zooplankton species, such as *D. magna*, showed no toxicity in chronic exposition experiments over 21 days at concentrations of up to 100 mg·L^{-1} (ECHA, 2022). The LC_{50} (48 h) of glycerol triacetate for *D. magna* was 380 mg·L^{-1} (ECHA, 2022), which is approximately hundred times higher than the LC_{50} determined for *B. plicatilis* in our study. However, it is not clear why *B. plicatilis* is much more sensitive to this chemical than brine shrimp or water flea. More research on glycerol triacetate and its potential toxicity is needed to explore whether and why this reaction is specific to certain taxa or affects

a wider range of marine species or their development stages. Leaching of this additive from the T-PHBV compound might explain the mortality in rotifers, but not in *Artemia* nauplii. Nevertheless, the presence of glycerol triacetate must be verified by chemical analytical methods.

The LC_{50} values of benzophenone for *B. plicatilis* and *A. persimilis* nauplii determined in our study are much higher than any concentration that can be expected in the marine environment. However, because of its frequent usage, benzophenone can enter the marine environment through several gateways and can be found at environmentally relevant concentrations in the range of $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$ (Cuccaro et al., 2022 and the studies cited therein). The highest concentration of 1.395 $mg \cdot L^{-1}$ was measured in Saint John Island, Virgin Islands, USA (Downs et al., 2016) for benzophenone-3, a derivative of benzophenone. This is far below the LC_{50} for *A. persimilis* (80 $mg \cdot L^{-1}$) and *B. plicatilis* (160 $mg \cdot L^{-1}$). Similarly, the LC_{50} values of the compound T-PHBV were much higher than microplastic concentrations expected in the oceans (Isobe et al., 2017; Everaert et al., 2018; Wang et al., 2020), especially when considering that all bioplastics account for only about 1 % of all produced plastics (European Bioplastics, 2021). From this perspective, the impact of bioplastic leachates at environmentally relevant concentrations appears negligible.

However, organisms interact directly with microplastic particles in the environment, as reported by a multitude of studies (e.g. Wright et al., 2013; Kühn et al., 2015). Many marine zooplankton species are filter feeders that indiscriminately filter large volumes of water to obtain food particles. This mode of feeding makes them especially vulnerable to ingest suspended microplastic particles (Kaposi et al., 2014). Uptake and ingestion of microplastics results in a more frequent and extended exposure to potential substances leaching from plastic particles. Furthermore, microplastics might release more substances, when exposed to digestive fluids upon ingestion (Koelmans et al., 2013). Moreover, bioplastics may release more leachates than conventional plastics because of their higher degradability, because chemicals are increasingly leaching from weathered plastics (Koelmans et al., 2014). Therefore, an increased share of bioplastics on the market, according to future projections (European Bioplastics, 2021), might lead to higher amounts of bioplastic particles in the environment and an increased burden on species that are exposed to microplastics.

5. Conclusion

The exact additive compositions of the bioplastic compounds used in this study are unknown and require detailed chemical analyses in subsequent studies. Nevertheless, it is revealing that bioplastics compounds and additives used therein prove to be toxic for marine organisms. The use of toxic additives raises doubts about bioplastics having a better environmental compatibility than conventional plastics. To develop more sustainable bioplastics, ecotoxicological research and the bioplastic producing industry need to cooperate to identify and subsequently remove toxic components from these materials. A more transparent approach with plastic formulations and the included substances would help facilitate this process.

CRediT authorship contribution statement

Lukas Miksch: Conceptualization; Investigation; Data curation; Formal analysis; Validation; Writing - original draft; Writing - review and editing. **Ann-Christin Scheer:** Methodology, Data curation; Validation; Writing - review and editing. **Lars Gutow:** Conceptualization; Funding acquisition; Supervision; Formal analysis; Data curation; Writing - review and editing. **Reinhard Saborowski:** Conceptualization; Funding acquisition; Supervision; Data curation; Writing - review and editing.

Declaration of Competing Interests

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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4 Synoptic discussion

This thesis addresses the biodegradability of selected biobased and biodegradable plastics under marine conditions and their impact on marine invertebrates. Currently, the pollution by plastic waste burdens marine ecosystems and compromises the services they provide to humanity (Barbier, 2017; Sharma and Chatterjee, 2017; Beaumont et al., 2019). While the rising production and usage of biobased and biodegradable plastics might be a great opportunity to mitigate this problem, only little is known about their persistence in marine environments and their impact on marine biota. Recently, concerns about poor degradability and hardly discernible differences to conventional plastics have been raised (Nazareth et al., 2019; Wang et al., 2021a).

The studies presented in this thesis were focused on five biodegradable plastic compounds, which were designed to replace conventional plastics for different applications. Besides the published papers (I, II) and the completed manuscripts (III, IV), this synoptic discussion also draws on results from additional complementary experiments.

4.1 Degradation of plastics in the marine environment

To understand the biodegradation of plastics in the ocean, knowledge of the conditions that prevail in the sea and affect biodegradation of plastics are crucial. The oceans cover 70.9 % of the earth's surface and are the largest aqueous system, containing 97 % of the world's total water (Chahine, 1992). The conditions in marine environments differ from other environments and are strongly shaped by the specific characteristics of seawater. The temperature of seawater is usually low compared to other environments, varying with season and latitude. While the surface water temperatures in tropic environments can exceed 30°C, it can be as cold as -2 °C in polar waters (Baringer et al., 2020). The temperature also varies with the depth of the water body. The temperature of the deep water (below 2000 m) is generally between 0 and 4 °C (Russel, 1990). Furthermore, the average salinity of seawater is 35 ppt and the pH slightly alkaline with values of 7.5 to 8.4 (Munn, 2011).

These abiotic conditions play an important role in the degradation of plastics in the ocean. **Publication II** shows, that the temperature strongly affects the enzymatic degradation of biodegradable plastics. Microparticles from biodegradable compounds showed increased hydrolysis rates at temperatures above 25 °C, but almost no hydrolysis at temperatures below 20 °C. Although hydrolytic enzymes such as lipase or protease are capable of hydrolyzing the plastics, the low temperatures in seawater impair the enzymatic activity, resulting in a limited

degradation. Similar observations were made for plastic samples incubated for six months in seawater and estuarine mud. The results from the mass loss analyses and scanning electron micrographs support the assumption of slow degradation under marine conditions. Only one of the five plastics, the compound T-PHBV, showed signs of degradation. The mass of the T-PHBV samples decreased when incubated in seawater (1.6 % ± 0.16) and even more after incubation in estuarine mud (6.6 % ± 0.53) (**Appendix Table A1**). Before incubation, the surfaces of all test bars were uniformly smooth (**Figure 4.1 a1, b1, c1, d1, e1**). This did not change after incubation in seawater or estuarine mud except for T-PHBV. Scanning electron micrographs of T-PHBV samples showed clear signs of erosion after six months in seawater (**Figure 4.1 a2**). This was even more pronounced after six months in estuarine mud, where the test bars displayed large pits in the surface (**Figure 4.1 a3**). Hence, PHBV seems to be better degraded in mud than in seawater. A decisive factor for this difference in degradation might have been the anaerobic conditions in the mud (**Table A2**), since many PHAs are better degraded in anoxic environments (Abou-Zeid et al. 2001). All materials showed significantly reduced maximum tensile strengths after six months of incubation in seawater and mud suggesting compromised mechanical properties of the material. However, this was also observed for samples stored at room temperature for six months (**Appendix Figure A3**). Accordingly, this loss of stability seems not to be connected to the hydrolytic degradation of the material, but to an intrinsic ´aging´ process, which also seems to take place on air.

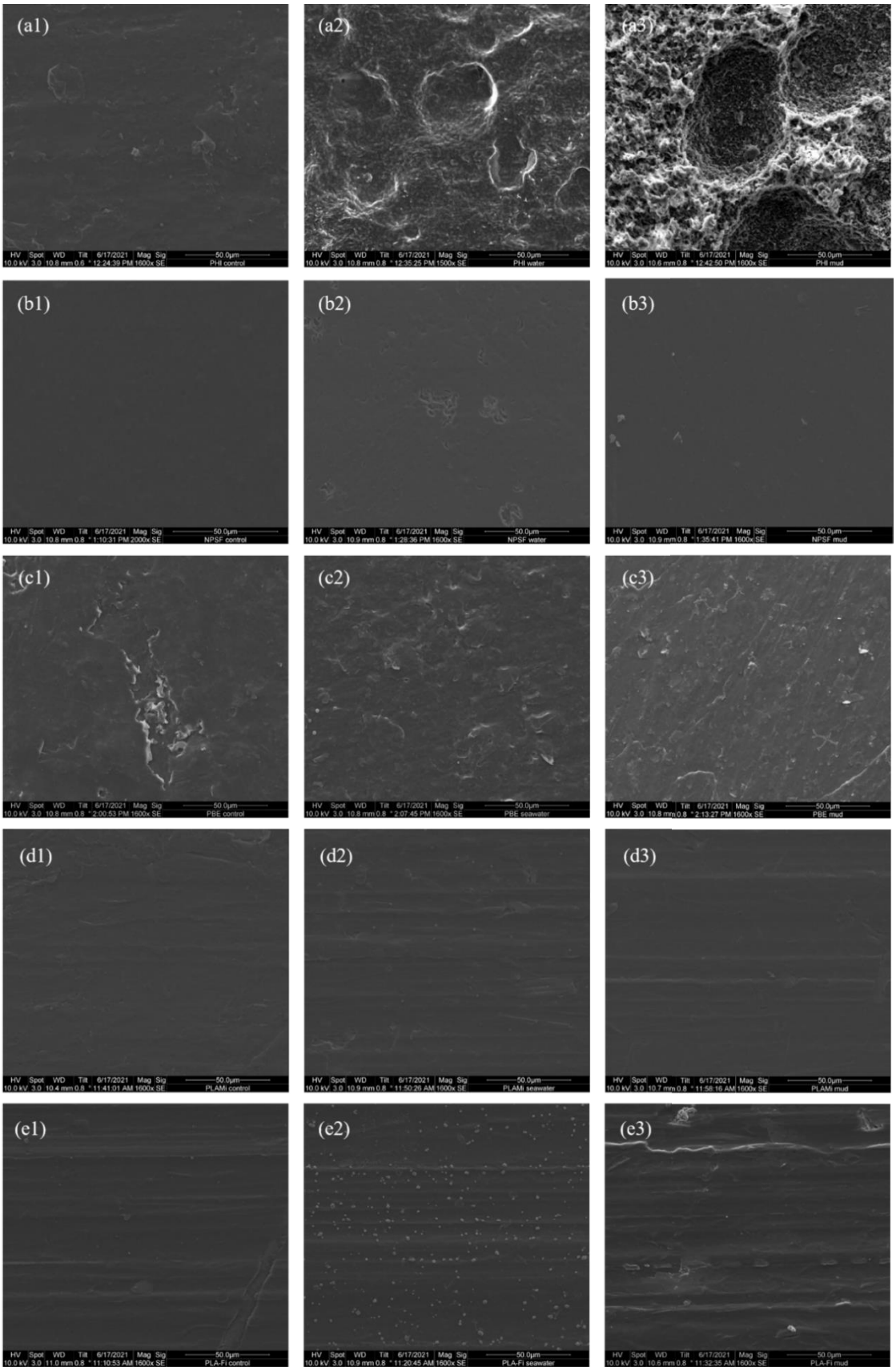

Figure 4.1: SEM photographs of the surfaces of (a) BPE-T-PHBV, (b) BPE-AMF-PLA, (c) BPE-SP-PBS, (d) BPE-RP-PLA, and (e) BPE-C-PLA test bars. The first column of micrographs shows the untreated plastic-bars (a1, b1, c1, d1, e1) and the second and third column shows the bars after six months exposure in seawater (a2, b2, c2, d2, e2) and estuarine mud (a3, b3, c3, d3, e3), respectively. All photographs were taken at the same magnification.

The degradation of PHBV-based materials in marine environments has been described previously. Volova et al. (2010) observed a total mass loss of 13 - 46 % for PHBV films after 160 days in seawater, while Rutkowska et al. (2008) reported a mass loss of 60 % after 42 days in seawater. This distinctly higher degradation can be attributed to the different conditions, but also the different thickness of the samples used. In both cited studies, the seawater temperatures ranged between 19 and 29 °C, which is higher than the constant 15 °C in my experiments. Furthermore, thin PHBV-films have a higher surface-to-volume ratio compared to the relatively thick (4 mm) plastic-bars, which provide a larger surface for enzymatic attack (Wright et al., 2013). According to this, degradation experiments with PHBV-bars of 2.5 mm thickness showed a mass loss of about 10 % after incubation in marine sediment for one year (Seggiani et al., 2018). This value is similar to my results of 6.6 % mass loss after six months in estuarine mud and underlines the relevance of the shape of the plastic material for the degradation rate.

The degradability of the base polymers used for the BPE test compounds differ between each other and in different environments (**Table 4.2**). For reasons of comparability only mass changes were considered in the following discussion. Other indicators of degradability are hardly comparable due to the variety of testing methods (Phua et al., 2012). PLA, PBS, and PBAT are best degraded in soil and compost while their degradation in seawater is negligible (e.g. Karamanlioglu and Robson, 2016; Muroi et al., 2016; Bagheri et al., 2017, Liu et al., 2022). The reasons for this difference are the favorable conditions in soil and compost for the prosperity of microorganisms that produce polymer-degrading enzymes and the enzymatic activities as such (Lenz, 1993; Lu et al., 2018; Wang et al., 2022). Composting conditions such as high temperatures (37 – 58 °C), good aeration, and neutral pH are more favorable than the conditions in the marine environment mentioned above. PHBV seems to be the only one of the tested polymers, which is degradable in almost all environments, although its degradation in seawater is slower than in other environments (e.g. Rutkowska et al., 2008; Volova et al., 2010; Boyandin et al., 2013). The degradability of PHBV in different habitats can be attributed to the availability of microbial depolymerases, which selectively degrade the polymer PHBV. Bacteria such as *Acidovorax* or *Bacillus* are widely distributed in aquatic and terrestrial ecosystems and are known to produce PHBV-degrading enzymes (Sudesh et al., 2000; Müller et al., 2001; Shah et al., 2007; Wang et al., 2012).

Table 4.1: Degradation of biodegradable base polymers by total mass loss in different environments.

Polymer	Environment	Duration	Total mass loss	Reference
PLA	Soil	28 days	none	Adhikari et al., 2016
	Soil	120 days	< 1%	da Silva et al., 2020
	Seawater	364 days	none	Wang et al., 2020a
	Seawater	12 months	none	Bagheri et al., 2017
	Freshwater	12 months	none	Bagheri et al., 2017
	Compost (37°C)	12 months	22%	Karamanlioglu & Robson, 2016
	Compost (50°C)	6 weeks	60%	Karamanlioglu & Robson, 2016
PBS	Soil	180 days	11%	Hoshino et al., 2001
	Soil	180 days	28%	Teramoto et al., 2004
	Seawater	364 days	< 3%	Wang et al., 2020a
	Seawater	440 days	4%	Liu et al., 2022
	Compost	90 days	14-72%	Zhao et al., 2005
	Compost (58°C)	24 weeks	30%	Puchalski et al., 2018
PBAT	Soil	168 days	22%	Muroi et al., 2016
	Soil	120 days	2%	Someya et al., 2007
	Seawater	12 months	none	Bagheri et al., 2017
	Seawater	56 weeks	< 5%	Wang et al., 2019
	Freshwater	12 months	none	Bagheri et al., 2017
	Compost (58°C)	60 days	8%	Ruggero et al., 2021
PHBV	Soil	365 days	35-61%	Boyandin et al., 2013
	Soil	200 days	0.03-0.64% /day	Mergaert et al., 1993
	Seawater	160 days	13-46%	Volova et al., 2010
	Seawater	180 days	36%	Deroiné et al., 2015
	Freshwater	254 days	100%	Eubeler et al., 2009
	Freshwater	358 days	77-100%	Mergaert et al., 1995
	Compost	50 days	80%	Luo & Netravali, 2003
	Compost	6 weeks	100%	Rutkowska et al., 2008

Besides the abiotic conditions, also biotic factors play a key role in the biodegradation of plastics. Since biodegradation is defined by the IUPAC (1997) as the "breakdown of a substance catalyzed by enzymes", the enzymes capable of hydrolyzing plastics need to be present in the respective environment. These enzymes are produced by bacteria, fungi, archaea, and other microorganisms. They cleave polymer chains into water-soluble oligo- and monomers (Amobonye et al., 2021). The degradation rate of synthetic polymers correlates positively with the number of microorganisms (Kasuya et al., 1998; Lu et al., 2018; Urbanek et al., 2018). The role of microorganisms and their extracellular enzymes were apparently also evident for the degradation of T-PHBV incubated in estuarine mud. In the natural untreated mud, biodegradation could be observed as significant mass loss after six months. In contrast, no

degradation occurred in the same mud that was previously autoclaved (**Figure 4.2**). Presumably, autoclaving eliminated the microbes in the mud and ceased enzymatic plastic degradation, despite the otherwise identical abiotic conditions (**Appendix Figure A1**). Extracts of untreated mud from the Weser estuary showed high activities of phosphatases, aminopeptidases, glycoside hydrolases, and esterases (**Appendix Figure A2**). This is in line with findings from several other studies, reporting the presence of aminopeptidases, esterases, glycoside hydrolases and metallo- and thiol proteases in different marine ecosystems (King, 1986; Mayer, 1989; Poremba, 1995). However, enzyme activities decreased with depth of the sediment from the Weser estuary (**Appendix Figure A2**). Microbial growth and, thus, extracellular enzymatic activity depends on the input of organic matter, occurring mostly by sedimentation onto the surface of the sediment. Consequently, the activity decreases with the depth of the sediment (Meyer-Reil, 1987; Fabiano and Danovaro 1998; Santos et al., 2006). A vertical decline of enzyme activity and microbial cell density was also observed in sediments from the deep North Atlantic (Poremba, 1995) and the Arctic Ocean (Sahm and Berninger, 1998).

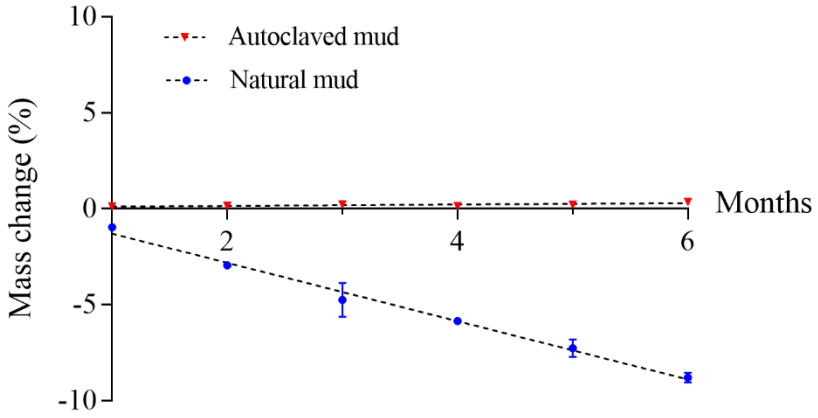

Figure 4.2: Mass change of plastic pieces of BPE-T-PHBV over the course of six months in autoclaved and untreated estuarine mud from the Weser estuary (means ± SD, n = 3).

Concluding these results, it can be said that although the tested polymers are considered biodegradable, the biodegradation of most of them seems to be limited under marine conditions. PLA, PBS and PBAT are enzymatically degradable by different hydrolytic enzymes, such as protease and lipase (see **Table 1.1**, section 1.3). These enzymes can be found in almost all organisms and they possess a rather broad substrate specificity. However, very low to zero biodegradation was observed for compounds based on these polymers. The reasons for this are the rate-limiting conditions in seawater and estuarine mud for enzymatic activity and a

presumed low abundance of microbes producing these polymer-degrading enzymes. The only material showing appreciable degradation under marine conditions was a compound based on PHBV. PHBV is specifically degraded by PHBV-depolymerases. Microorganisms producing such enzymes are present in a variety of terrestrial and aquatic environments, including seawater (Sudesh et al., 2000; Müller et al., 2001). Based on the low degradation rates of the compounds examined in this study, biodegradable plastics need to be further improved to achieve a better degradability in the marine environment. Accordingly, replacing conventional plastics with biodegradable plastics, which do not degrade fast enough, will inevitably lead to the accumulation of those materials in the marine environment.

4.2 Uptake and digestion of biodegradable plastics by aquatic invertebrates

Microplastics usually enter the digestive system of organisms deliberately or unintentionally while feeding (Devriese et al., 2015; Po et al., 2020). Non-selective feeding habits, passive ingestion with prey or confusion with food are mechanisms that apply to both the uptake of conventional and biodegradable plastics. However, reports on the abundance of biodegradable or biobased plastic in organisms are scarce. Steer et al. (2017) identified microplastic fibers composed of cellulose-based rayon in fish larvae. The low number of studies on biodegradable plastics in marine biota is probably due to the fact that biodegradable plastics account for only a small share of about 1% of global plastics (European Bioplastics, 2021) and are so far not very abundant in the environment. Nonetheless, the share of biodegradable plastic litter in the environment will increase and presumably accumulate in marine environments (see section 4.1.). The first step in assessing impacts of biodegradable microplastics on marine biota is the validation whether biodegradable microplastics are ingested by marine organisms.

Conventional microplastics are found in the intestines of a variety of aquatic invertebrates from different trophic levels (e.g. Murray and Cowie, 2011; Van Cauwenberge and Janssen, 2014; Desforges et al., 2015). Information about the microplastic uptake at the base of the food web is especially relevant, as it may result in the propagation of microplastics to higher trophic levels through trophic transfer (Costa et al., 2020a; Sarker et al., 2022; Uy and Johnson, 2022). Most zooplankton species are primary consumers, which, in turn, provide a food source for organisms at higher trophic levels (O'Brien, 1979; Lesutienė et al., 2007). Many zooplankton species are suspension feeders, obtaining their food from the surrounding water by filtering. This feeding mode makes them especially vulnerable to encounter and ingest microplastics (Kaposi et al., 2014). The feeding experiments with zooplankton conducted in my thesis have shown that

biodegradable microparticles are taken up in the same manner as commercial polystyrene (PS) microbeads. Rotifers, daphnids, and brine shrimp nauplii ingested microparticles of all five biodegradable BPE compounds (**Figure 4.3, Appendix Figure A4)**, independent of their chemical composition. The uptake of both conventional and biodegradable microplastics can be attributed to the similar feeding mode of all three species. The rotifer *Brachionus plicatilis* is a generalistic filter feeder. It generates currents with the cilia of the corona channeling food items into its mouth, poorly discriminating between size and quality of the particles (Rothhoupt, 1990). Daphnids are also filter feeders, that forage non-selectively on particles of various sizes (Rosenkranz et al., 2009; Rehse et al., 2016). The same applies to *Artemia* nauplii from the second larval stage (instar II) onward (Lavens and Sorgeloos, 1996; Makridis and Vadstein, 1999). Larval and adult *Artemia* naturally feed on detritus, algae, and bacteria (Van Stappen, 1996).

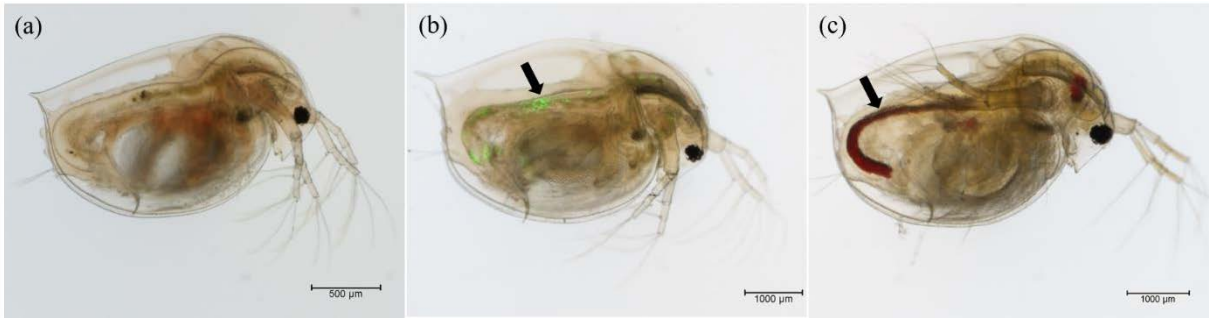

Figure 4.3: Lateral view of *Daphnia pulex* with (a) empty gut, (b) ingested green PS microbeads (9.9 µm diameter, G1000, Fluoro-Max™) and (c) ingested Nile Red-stained BPE-AMF-PLA particles (< 200 µm). Microparticles in the gut are marked with an arrow.

In contrast to the larvae, adult *Artemia* did not ingest microparticles derived from the biodegradable plastics, but only conventional microplastics. Although *Artemia* are considered non-selective filter feeders, there are indications for a preference for food of a specific size. Fernández (2001) reported for adult *Artemia* a notably higher ingestion rates for food particles between 6.8 and 27.5 µm diameter than for larger particles. The seawater medium, in which the feeding experiments with the brine shrimp were conducted, was prepared with *Artemia* salt that contained microalgae (< 10 µm). Accordingly, adult brine shrimp might have selectively been feeding on the microalgae instead of ingesting the larger plastic particles (< 200 µm).

The opossum shrimp *Mysis* spec. (Mysidacea) did not ingest biodegradable microplastics. Despite the larger body size of the shrimp compared to daphnids and *Artemia* nauplii, only the small PS microbeads of 9.9 µm diameter were ingested. Mysids can switch between two feeding

modes: filter feeding on detritus and phytoplankton or raptorial predation (Grossnickle, 1982). Depending on the food available, the feeding mode can be shifted, hereby also affecting the ingestion of microplastic particles (Lehtiniemi et al., 2018). In the presence of smaller particles around 10 µm, efficient filtering of these microplastic has been observed (Setälä et al., 2016), which is in line with my findings. In the predatorial mode, however, mysids do actively select prey (Viherluoto and Viitasalo, 2001). Bigger particles, such as microparticles from the biodegradable plastic compounds and food flakes, might have induced this mode. In this feeding mode, mysids were probably able to select fragments of the food flakes over particles from the biodegradable plastics.

Decapod crustaceans from higher trophic levels play important ecologically and economically roles. They inhabit a multitude of different ecosystems, including shallow and deep waters, as well as hard and soft bottoms (e.g. Martin and Haney, 2005; Pohle et al., 2011; Briones-Fourzán et al., 2020), where they are integral part of the respective food webs. Furthermore, they are important marine fishery targets and, thus, food resource for humans. Decapods exhibit herbivorous, omnivorous, or carnivorous feeding modes and possess efficient external and internal structures to capture and process food items (reviewed in D'Costa, 2022). Several studies report the presence of microplastics in the intestines and stomachs of cultured and wild-caught decapod crustaceans, such as the Norway lobster *Nephrops norvegicus*, the lesser swimming crab *Charybdis longicollis*, the Mediterranean green crab *Carcinus aestuarii* and many others (Stasolla et al. 2015; Welden and Cowie, 2016; Lusher et al., 2017; Piarulli et al. 2019, Li et al., 2021).

In my study, the common ditch shrimp, *Palaemon varians*, ingested both biodegradable microplastics and microbeads made of conventional plastics. However, microplastics were only ingested when offered together with food flakes. The microbeads and microplastics were present in the stomach of *P. varians* at high concentrations (**Figure 4.4**). Shrimps of the genus *Palaemon* use a wide spectrum of different food types but are usually detritivores (Aguzzi et al., 2005). As detritivores, they feed on decomposing organic matter, which is why micro-plastics are incidentally ingested during food uptake, rather than selectively foraged. The observation that microplastics are ingested when provided on food flakes but not without other food items supports this assumption. The uptake of inorganic particles while feeding has also been shown for the North Sea shrimp *Crangon crangon*, which ingest sand grains sticking to its common food (Schmidt et al., 2021). In contrast to my findings, Saborowski et al. (2022) showed the uptake of small microbeads by *P. varians* also in the absence of additional food.

However, this might be attributed to the different experimental setup. Saborowski et al. (2022) kept the microbeads in suspension whereas my experiments were not designed to keep the plastic particles suspended. A misperception of microplastics with food particles might be more likely when the microplastics float freely in the water column. Furthermore, the microplastic particles might be too small to be perceived and are ingested accidentally.

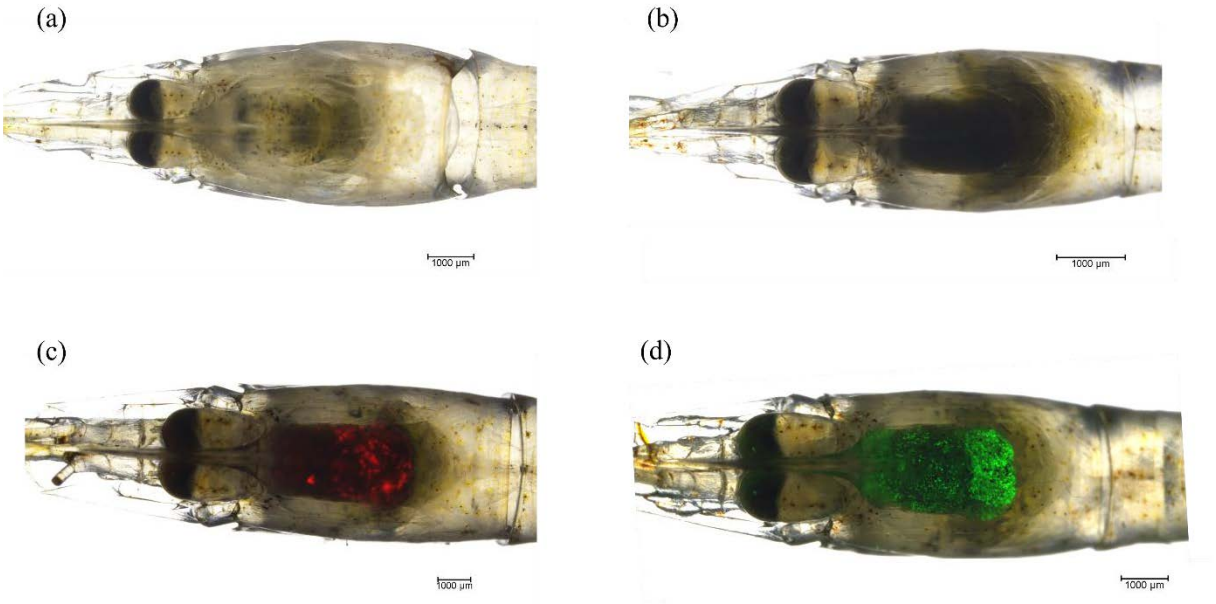

Figure 4.4: Dorsal view of the cephalothorax of *Palaemon varians* with (a) empty stomach, (b) stomach filled with food flakes, (c) stomach filled with Nile Red-stained BPE-AMF-PLA particles (< 200 µm) and (d) stomach filled with green fluorescent PS microbeads (9.9 µm diameter, G1000, Fluoro-Max™).

Additional feeding experiments with *Palaemon elegans* were carried out to test for differential uptake of conventional and biobased biodegradable microplastics. Food flakes were coated with Nile red-stained microparticles and fed to the shrimp. The shrimp were dissected 8 h after feeding and investigated for the presence of microparticles in the digestive tract. The abundance of microplastics in the stomach varied strongly between individuals. Animals fed with conventional LDPE microplastics showed lowest numbers, ranging from 0 to 228 ± 194 particles per stomach. Stomachs of shrimp fed with AMF-PLA particles contained between 281 ± 377 and 2299 ± 1069 particles, while those fed with T-PHBV showed the highest numbers of 2439 ± 1398 to 73,012 ± 23,298 particles. Although the size of all particles was roughly the same, microplastics from the biodegradable compounds appeared in higher numbers in the stomachs of the shrimp. This, however, is most likely attributed to the experimental design and the different densities of the materials. Microplastics tended to detach from the food flake because of maceration in the water and during feeding when the shrimp shredded the flake with

their maxillipeds to smaller pieces. Since LDPE has a lower density than water, it rises immediately to the surface after detachment from the flake, being inaccessible for the animals. This is not the case for the biodegradable compounds, since their density is higher than that of seawater. Nonetheless, the different amounts of biodegradable microplastics in shrimp fed with AMF-PLA compared to T-PHBV might indicate other means of food detection, e.g. chemoreception. Leaching chemicals from the different compounds might stimulate or prevent ingestion. A selective ingestion of microplastics with different chemical composition has been shown for organisms which rely on chemical senses for food uptake, such as the hard coral *Astrangia poculata* (Allen et al., 2017).

The current study indicates that both biodegradable and conventional microplastics are readily ingested by a variety of aquatic organisms. The chemical composition of particles seems to be irrelevant for small filtrating zooplankton organisms, as long as the particles are in a size range to be ingested. However, the disparity of microplastics from different compounds in the stomach of larger shrimp *Palaemon elegans* might indicate, that these animals are able to distinguish between different microplastic types, probably due to higher evolved sensory systems at their feeding appendices. Nonetheless, for the purpose of my study it can be stated that both *P. elegans* and *P. varians* did readily ingest various microparticles, including those derived from biodegradable materials. Other crustaceans as well, like the amphipod *Gammarus fossarum*, did not distinguish between conventional petroleum-based and biodegradable microplastics (Straub et al., 2017). PLA microplastic particles were also ingested by brine shrimp *Artemia franciscana* nauplii and the medusa *Aurelia* sp. (di Giannantonio et al., 2022). Both species are also known to ingest microparticles originating from conventional plastics (Peixoto et al., 2019; Costa et al., 2020b).

Microplastics have no nutritional value, except from a biofilm that might have developed on the surface of the particle upon exposure in the aquatic environment (Michels et al., 2018). Until recently, it was assumed that ingested microplastics are neither digested nor assimilated due to lacking catabolic enzymes and metabolic pathways for the utilization of synthetic polymers (Andrady, 2011). However, more recent studies revealed the capability of some organisms to enzymatically break down ingested plastics. Sun et al. (2022) found enzymes in the gut microbiome of *Zophobas morio* larvae (Insecta, Coleoptera), that are capable of degrading polystyrene (PS). Similarly, PS and polyethylene (PE) are degraded in the gut of larvae of the mealworm *Tenebrio molitor* (Yang et al., 2015a, b, 2018; Brandon et al., 2019). Lou et al. (2020) showed the degradation of PS and PE by larvae of the greater wax moth *Galleria*

mellonella and Song et al. (2020) suggested the partial biodegradation of PS by the land snail *Achatina fulica*. However, apart from these few studies with insect larvae and terrestrial gastropods, there is limited information about plastic biodegradation by freshwater and marine organisms that are particularly exposed to plastics (So et al., 2022). So far, studies focused mostly on conventional, petroleum-based plastics, that are considered non-degradable. However, along with the introduction of biodegradable plastics, these new materials might be much easier hydrolyzed by a wider range of enzymes after ingestion by metazoans (Tokiwa et al., 1990).

Basically, ingested microplastics are subject to the same digestive processes as natural food. In crustaceans, ingested food first enters the stomach where it is mechanically fragmented, macerated, and mixed with digestive enzymes (Vogt, 2021). The digestive enzymes are synthesized in the midgut gland and released into the stomach where they facilitate the initial degradation of the major food components but also structural materials like chitin and cellulose. Microbial enzymes seem to play a minor role in food digestion in the gut of crustaceans (Vogt, 2021).

My studies showed that enzymes from the gastric fluids of the marine crab, *Cancer pagurus,* and lobster, *Homarus americanus*, can hydrolyze certain types of biodegradable plastics *in-vitro* (**Figure 4.5**). The highest hydrolysis rates were observed for BPE-AMF-PLA, a plastic blend of PLA and PBAT. Detailed investigations of the gastric fluid of *C. pagurus* revealed high activities of carboxylesterases, which are suggested to be the major plastic-degrading enzyme (**Publication III/Manuscript 1**).

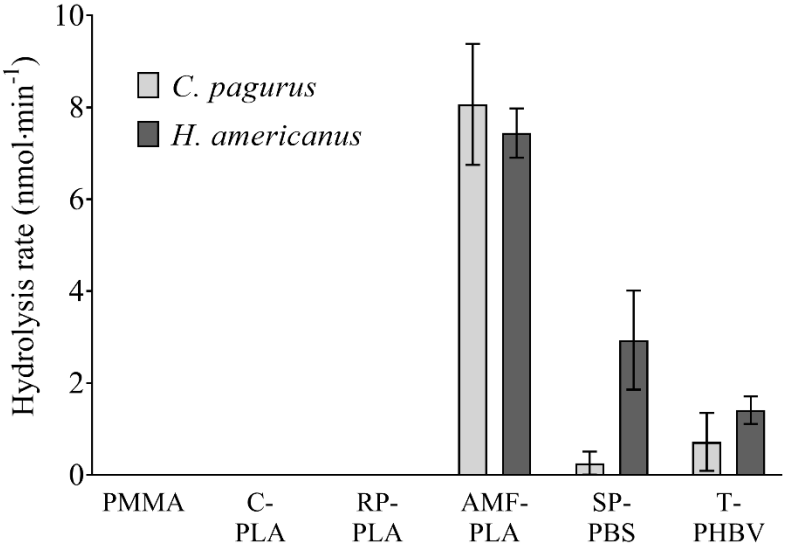

Figure 4.5: Hydrolytic degradation of biodegradable and non-degradable plastics by the gastric fluid of *Cancer pagurus* and *Homarus americanus* measured by pH-Stat titration at 15°C, pH 8.2 and 32 ppt salinity (means ± SD, n =3). Modified after Publication III.

Carboxylesterases comprise enzymes with different substrate specificities, such as quite unspecific esterases and more specific lipases (Chahiniana and Sarda, 2009). Lipases catalyze the hydrolysis of triglycerides to free fatty acids and glycerol (Joseph et al., 2008). Lipases are water-soluble enzymes that catalyze reactions with water-insoluble substrates after adsorption to their surface (Hasan et al., 2009). This mechanism might play an important role in the effective hydrolysis of microplastics. Several studies showed that lipases are able to hydrolyze synthetic biodegradable polymers, such as PBS or PBAT (Shi et al., 2019; Kanwal et al., 2022). Lipases are present in the digestive fluids of various crustaceans, including *Homarus americanus*, *Carcinus maenas* or *Penaeus vannamei* (Brockerhoff et al., 1970; Cherif et al., 2007; Rivera-Pérez et al., 2011). Accordingly, crustaceans with lipolytic activity in their gastric fluids might be able to hydrolyze ingested biodegradable microplastics based on PBS or PBAT.

In my experiments, *P. elegans* fed with biodegradable AMF-PLA particles showed a significant increase in carboxylesterase activities in midgut glands, when compared to the control shrimp fed with food flakes. This was evident at each time point (4 to 48 hours) and after 12 days of feeding when the short-chain carboxylester MUF-butyrate was used as substate (**Figure 4.6**). An increase in carboxylesterase activities was also observed with the medium-chain carboxylester MUF-heptanoate as substrate, although only 4 h after feeding (**Appendix, Figure A5**). No significant variation in carboxylesterase activity appeared with MUF-oleate as substrate (**Appendix, Figure A6**). Elevated activities might indicate an activation of the

digestive system of *P. elegans* and induction of carboxylesterase immediately after feeding with AMF-PLA microplastics.

Microplastic ingestion has been shown to alter digestive enzyme activities in various species, including fish, mollusks and crustaceans (Romano et al., 2018; Korez et al., 2019; Trestrail et al., 2021). Changes in enzyme activities can depend on the polymer type of the ingested microplastic (Trestrail et al., 2021), which was also evident in feeding experiments with *P. elegans*. Here, only the shrimp fed with AMF-PLA exhibited elevated carboxylesterase activities when compared to the control group. Shrimp fed with T-PHBV particles showed no significant increase. The induced esterase in *P. elegans* shows similar substrate specificity as esterases in the gastric fluid of *Cancer pagurus*. (**Publication III/Manuscript 1**).

The increased carboxylesterase activities may be explained by a molecular or sensory resemblance of the biodegradable plastic or chemicals leaching out of it with natural food (Luo et al., 2020). In invertebrates, this might stimulate the production and secretion of respective digestive enzymes (Mathers, 1973). However, it remains to be investigated whether this increase in carboxylesterase activity in *P. elegans* also results in an effective hydrolysis of the biodegradable plastics.

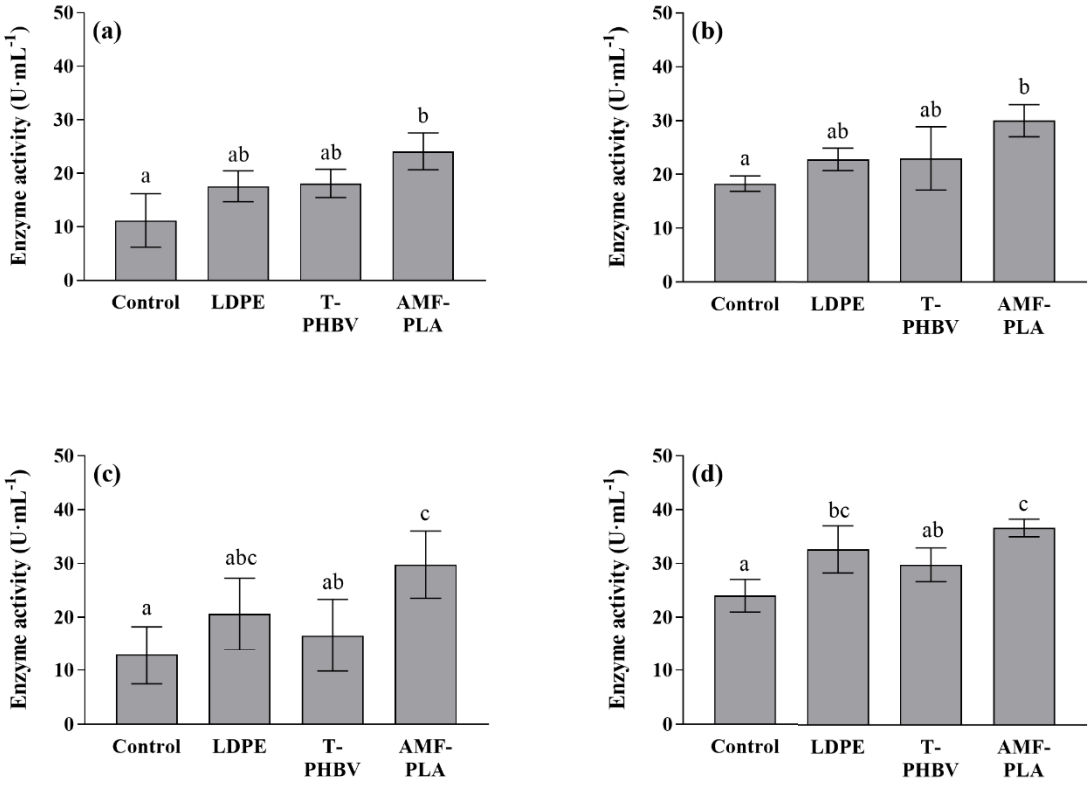

Figure 4.6: Carboxylesterase activities measured with MUF-butyrate in the midgut glands of *Palaemon elegans* fed with microparticles of the BPE-compounds (T-PHBV, AMF-PLA) and conventional plastic (LDPE) and a control after (a) 4 h, (b) 24 h and (c) 48 h of feeding and (d) repeated feeding for 12 days. Different letters indicate significant differences (means ± SD, n = 4).

Apart from carboxylesterases, a multitude of different highly active enzymes, including proteases, glucosidases and phosphoesterases are present in the gastric fluids of crustaceans (**Table 4.3**). Enzymes, that might potentially hydrolyze biodegradable plastics are also present in many other crustaceans. Besides lipases, proteolytic enzymes are also known to degrade plastics based on PLA and PBS (**Publication I**, Lim et al., 2005; Kawai, 2010). Furthermore, α-amylase is capable of degrading polymers based on thermoplastic starch (Vikman et al., 1999; Abbasi, 2012). The degradation of biodegradable plastic by digestive enzymes is not limited to *C. pagurus* and *H. americanus*. For example, ingested starch- and cellulose-based plastics were enzymatically degraded by the terrestrial isopod *Porcellio scaber* (Wood & Zimmer, 2014).

Table 4.3: Digestive enzymes in crustaceans (modified after Vogt, 2021).

Enzyme	Species	Reference
Aminopeptidase	*Procambarus clarkii*	De la Ruelle et al., 1992
Amylase	*Penaeus vannamei*	Van Wormhoudt et al., 2003
Aspartic protease	*Homarus gammarus*	Navarrete del Toro et al., 2006
	Homarus americanus	Rojo et al., 2010
Astacin	*Astacus astacus*	Bode et al., 1992
	Cancer pagurus	Navarrete del Toro et al., 2006
	Carcinus maenas	Roy et a., 1996
Carbohydrase	*Homarus gammarus*	Glass and Stark, 1995
Carboxypeptidase	*Astacus astacus*	Titani et al., 1984
Carboxylesterase	*Cancer pagurus*	Publication III (Manuscript 1)
Cellulase	*Cherax quadricarinatus*	Byrne et al., 1999
	Parasesarma erythodactyla	Bui and Lee, 2015
	Procambarus virginalis	Gutekunst et al., 2018
	Austrothelphusa transversa	Linton et al., 2006
Chitinase	*Penaeus monodon*	Proespraiwong et al., 2010
	Penaeus japonicus	Watanabe et al., 1998
Chitobiase	*Homarus americanus*	Brockerhoff et al., 1970
Chymotrypsin	*Homarus americanus*	Brockerhoff et al., 1970
	Crangon crangon	Saborowski et al., 2012
	Penaeus vannamei	van Wormhoudt et al., 1992
Cysteine protease	*Penaeus vannamei*	Le Boulay et al., 1996
	Metapenaeus ensis	Hu and Leung, 2007
Lipase	*Homarus americanus*	Brockerhoff et al., 1970
	Carcinus maenas	Cherif et al., 2007
	Penaeus vannamei	Rivera-Pérez et al. 2011
Phosphatase	*Homarus americanus*	Brockerhoff et al., 1970
	Homarus gammarus	Barker and Gibson, 1977
	Carcinus maenas	Compère et al., 1993
Trypsin	*Homarus americanus*	Brockerhoff et al., 1970
	Crangon crangon	Saborowski et al., 2012
	Paralithodes camtschaticus	Rudenskaya et al., 2014
	Astacus astacus	Titani et al., 1983

Biodegradable plastics can be hydrolyzed by digestive enzymes of crustaceans. However, it is yet unknown whether crustaceans are also able to utilize these materials metabolically. The metabolic utilization of biodegradable plastics involves three fundamental processes. First, the digestive fluids must be able to enzymatically break down the ingested plastics. This was already shown for e.g. *C. pagurus* in **Publication III/Manuscript 1** and was discussed previously. Second, the ingested plastics need to be exposed to the digestive enzymes long enough to liberate suitable amounts of metabolites with low molecular weight. Third, the

produced metabolites must be absorbed by the cells of the midgut gland and contribute to the intermediary metabolism.

Digestive enzymes are capable of hydrolyzing biodegradable plastics. However, the degree of hydrolysis depends on how long and if at all the plastic is exposed to the digestive enzymes. The hydrolysis rates of biodegradable plastics are lower than those of natural polymers. This was shown for the hydrolysis of collagen in comparison to the hydrolysis of biodegradable plastic compounds. The hydrolysis rate of collagen by protease was 6 to 60 times higher than that of biodegradable plastics by any of the tested enzymes (**Publication II**). Accordingly, the degradation of biodegradable plastic in the digestive system of crustaceans will be significantly slower than the degradation of natural dietary polymers. Hence, microparticles of biodegradable plastics would need to remain in the digestive tract of the crustaceans for a longer period of time than natural food, to achieve a similar degree of degradation. The retention time of food in the digestive tract of crustaceans varies among species and ranges from 1-2 hours in penaeid shrimps fed with shrimps, to 72 hours in the crab *Necora puber* fed with brown algae (McGaw and Curtis, 2013). In the European shore crab *Carcinus maenas* food is macerated by the gastric mill and mixed with digestive enzymes in the first 12 hours after ingestion. Subsequently, the food is sorted by size into a coarse fraction and a fine fraction. The coarse fraction is passed into the hindgut and is defecated. This happens within 12 to 48 hours after ingestion. The fine fraction, consisting of diminutive particles and liquids, passes the pyloric filter and enters the midgut gland (syn. hepatopancreas), where the nutrients are absorbed. The size of the particles passing the pyloric filter is between 5 and 30 nm in *C. maenas*. Remnants of hepatopancreatic digestion are returned to the midgut after 12 to 48 hours, passed to the hindgut and egested (Hopkin and Nott, 1980).

The size of particles that can pass the pyloric filter and enter the hepatopancreas differs between species. While particles in the size range of 100 nm and bigger are retained by pyloric filters in *Astacus astacus* (Vogt, 2002) and *C. maenas* (Hopkin and Nott, 1980), microplastics of this size range were found in the midgut gland of *Crangon crangon* and *Palaemon varians* (Korez et al., 2020; Saborowski et al., 2022). Microplastics that enter the hepatopancreas must either be ingested already in very small size, or remain long enough in the stomach to be further fragmented by enzymatic degradation and the action of the gastric mill. Some crustaceans have developed mechanisms to efficiently remove indigestible material from their stomachs. Korez et al. (2020) showed, that the North Sea shrimp *C. crangon* regurgitates microplastics of a certain size 6 to 12 hours after ingestion. Similarly, *P. varians* regurgitates plastic fibers, which

are accumulated in the stomach (Saborowski et al., 2019). However, there are indications, that microplastics are retained longer in the stomach, when they are ingested simultaneously with food (Saborowski et al., 2019). Considering the hydrolysis rates of biodegradable plastics, the retention time of food, and the mechanisms of stomach evacuation in crustaceans, it appears that biodegradable plastics might not provide as much assimilable metabolites as natural food would.

Depending on the composition of a biodegradable plastic, different products are formed during degradation. These products differ according to the base polymers and additives used in the matrix of the plastic. Besides the base polymer, a single plastic product can contain several thousands of chemicals (Zimmermann et al., 2020a), making it difficult to predict all degradation or intermediate products. The enzymatic degradation of a plastic involves the binding of the enzymes to the polymeric substrate, catalyzing the hydrolytic cleavage of the polymer chain (Tokiwa and Calabia, 2004). During cleavage, water-soluble intermediates such as oligomers, dimers, and monomers are formed (Göpferich, 1996).

In the case of PLA, these intermediates comprise lactic acid oligomers and monomers (Castro-Aguierre et al., 2017). Lactic acid is a metabolite that is also produced by anaerobic fermentation of glucose and other sugars, a metabolic pathway occurring in procaryotic and eucariotic cells (Reddy et al., 2008; Summermatter et al., 2012). Some studies indicate, that lactic acid displaces glucose in the energy metabolism of brain neurons in some mammals (Zilberter et al., 2010; Wyss et al., 2011). Lactic acid from hydrolyzed PLA microparticles might therefore also be available for the energy metabolism of crustaceans.

The degradation products of PHBV comprise 3-hydroxybutyric acid (HB) and 3-hydroxyvaleric acid (HV) (He et al., 2014). HB and HV are a so-called ketone-bodies that are natural metabolites of all organisms and are used as alternative energy source (Aneja et al., 2002; Krishnakumar et al., 2008). During prolonged nutrient deprivation or carbohydrate restriction, ketone bodies can be used for metabolic energy production by increasing the fatty acid availability (Cahill, 2006). In mammals, they are produced in the liver and transported to other tissues for oxidation (Robinson and Williamson, 1980).

Degradation of PBS yields 1,4-butanediol and succinic acid (SA) (Gamerith et al., 2017). Succinic acid is an integral component of several metabolic pathways of energy production. SA is produced in the mitochondria and is an essential intermediate of the citric acid cycle (Tretter et al., 2016).

Degradation products of biodegradable plastics can be utilized by microorganisms as energy source (Mergaert and Swings, 1996). There is a potential for crustaceans, and most likely other invertebrates, to utilize degradation products of biodegradable plastics as well. Wood and Zimmer (2014) reported the utilization of degradation products of starch- and glucose-based plastics by the terrestrial isopod *Porcellio scaber*. This seems not uncommon because the degradation products of these plastics, including glucose and cellobiose, are also found in the natural diet of crustaceans (Bergreen et al., 1961; Linton et al., 2006). It remains to be investigated whether the degradation products from other biodegradable plastics can be utilized by crustaceans and other organisms and how they might be incorporated in metabolic pathways.

Overall, digestive enzymes in crustaceans show a high potential to hydrolyze certain biodegradable plastics. Gastric fluids from *Cancer pagurus* and *Homarus americanus* hydrolyzed a biodegradable PLA/PBAT-blend *in-vitro*, probably due to the presence of digestive carboxylesterases. However, it is unclear if the retention time of ingested microplastics might suffice to degrade the biodegradable plastics, considering the low hydrolysis rates at seawater temperatures. Mechanisms to get rid of indigestible particles like regurgitation might further limit the hydrolysis of ingested microplastics (Saborowski et al., 2019; Korez et al., 2020). If degradation liberates intermediates that might be absorbed in the hepatopancreas, it is unknown whether the incorporation of degradation products causes health implications. Moreover, the nutritional value of a biodegradable plastic is very low when compared to natural food, as it primarily contains carbon and no nitrogen. This makes biodegradable plastics an insufficient source for nutrition.

4.3 Ecotoxicity of biodegradable plastics on marine invertebrates

Micro- and nanoplastics are known to interact with a variety of marine invertebrates. In crustaceans they were found at the gills, in the gastrointestinal tracts, and in the midgut gland and were associated with adverse effects on these organisms (reviewed in D'Costa, 2022). In contrast to conventional microplastics, there is limited information about the impacts of biodegradable plastic on marine invertebrates. Considering only the physical properties of biodegradable microplastics, similar effects can be expected as those caused by conventional microplastics. However, the chemical compositions of biodegradable plastics differ from those of conventional plastics, which could play an important role in the toxicity to organisms interacting with them. Especially in the case of ingested microplastics exposed to gastric fluids,

this could result in unforeseen effects caused by degradation products or toxic leachates (Koelmans et al., 2013; Endo et al., 2013).

Rockpool shrimp *Palaemon elegans* were fed with microparticles generated from biodegradable and conventional plastics. Microparticles from conventional plastics are known to provoke negative effects upon ingestion. As an acute toxic reaction, conventional plastics increased mortality of several decapod species, including *Emerita analoga*, *Litopenaeus vannamei* and *Charybdis japonica* (Horn et al., 2019; Hsieh et al., 2021; Wang et al., 2021c). However, in my study ingestion of microplastics did not induce elevated mortality in *P. elegans*, neither after a single short-term exposure nor after continuous exposure over 12 days. Moreover, the mortality did not differ between individuals fed with conventional microplastics and individuals that received biodegradable microplastics. Similarly, microplastic ingestion did not induce mortality in *Palaemon varians* and *Crangon crangon.* These species seem to be able to tolerate ingested particles because they live in habitats with high particle loads. They involuntarily take up microparticles with their food but the particles do not accumulate in the intestine because ingested microplastics were regurgitated and egested (Saborowski et al., 2019; Korez et al., 2020, Schmidt et al. 2021).

Besides conventional ecotoxicological end points with immediate implications, such as mortality (ECHA, 2011), there are also physiological end points that are less obvious but not less important. Initial responses of organisms to toxic effects or internal damage can occur on a cellular level. For example, the exposition of organisms to pollutants or pathogens can induce cellular oxidative stress (Akbulut et al., 2014; Duan et al., 2015). As a defense mechanism, cells rapidly release reactive oxygen species (ROS) in a process called respiratory burst (Lushchak, 2011). In crustaceans, ROS can be formed in the midgut gland (Liu et al., 2010). ROS can induce damage to DNA, enzymes, and cell constituents. To avoid or minimize damage of the own tissue, cells rely on an antioxidant defense system that removes ROS from the cells (Halliwell and Gutteridge, 1999). The process is initiated by antioxidants such as the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reductase (GR) (Matés and Sánchez-Jiménez, 1999; Sáez and Están-Capell, 2014). Elevated activities of these enzymes can indicate if an organism suffers from oxidative stress.

Microplastics can induce the intra- and extracellular generation of ROS (Zhu et al., 2020; Umamaheswari et al., 2021). An increase of antioxidant enzyme activities after ingestion of micro- and nanoplastics was reported for several crustacean species, including *Macrobrachium rosenbergii* (Jaikumar et al., 2021), *Procamparus clarkii* (Capanni et a., 2021), *Eriocheir*

sinensis (Yu et al., 2018) and *Litopenaeus vannamei* (Wang et al., 2021d). In contrast, microplastics seem to inhibit the antioxidant defense in *Charybdis japonica* (Wang et al., 2021c). Feeding conventional and biodegradable microplastics did not induce SOD activities in the midgut gland of *Palaemon elegans*, neither at different timepoints after single exposure, nor after repeated exposure over 12 days. However, 4 h after ingestion of microparticles, SOD activities were significantly higher in shrimp fed with AMF-PLA compared to shrimp fed with T-PHBV (**Figure 4.7a**).

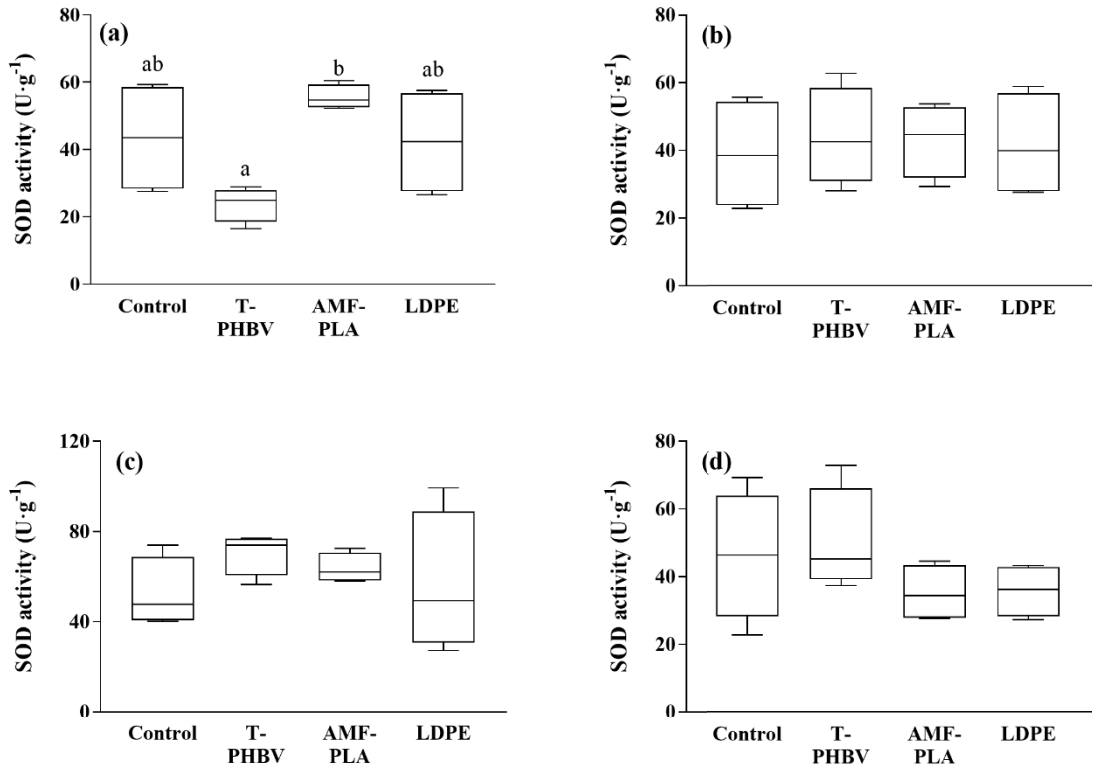

Figure 4.7: Superoxide dismutase (SOD) activities in the midgut gland of *Palaemon elegans* fed with microparticles of the BPE-compounds (T-PHBV, AMF-PLA) and conventional plastic (LDPE) and only food flakes as control after (a) 4 h, (b) 24 h and (c) 48 h of feeding and (d) after repeated feeding for 12 days. Different letters indicate significant differences (means ± SD, n = 4).

Palaemon elegans seems not to be affected by the ingestion of microparticles of conventional and biodegradable plastics whereas the congener *Palaemon varians* showed elevated SOD activities after ingestion of conventional microplastics (Saborowski et al., 2022). These different results could be due to the different size and shape of microplastics used in the experiments. Size and shape of microplastics do not only affect their availability to organisms, but can also affect the impact on an organism upon ingestion (Lee et al., 2013; Coppock et al., 2019; Schwarzer et al., 2022). For adult daggerblade grass shrimp *Palaemonetes pugio*, for

example, the size and the shape of ingested microplastics was decisive for their survival (Gray and Weinstein, 2017). While Saborowski et al. (2022) used spherical PS microbeads in the size range of 0.1 to 9.9 µm, I used irregular shaped microplastics of a bigger size range (< 200 mm). Smaller particles are more likely to pass the pyloric filter and enter the midgut gland of crustaceans, while particles larger than 100 nm are commonly retained by the pyloric filter system of crustaceans (Hopkin and Nott, 1980; Vogt, 2002; Korez et al., 2020). Similar to *P. elegans*, brown shrimp *Crangon crangon* did not show elevated activities of antioxidant enzymes after ingestion of PLA microplastics (Korez et al., 2022). The authors suggested that *C. crangon* may be adapted to the uptake of indigestible particles due to their feeding habit, since they occasionally ingest natural microparticles. A fine-meshed filter in the stomach prevents the passage of larger particles into the midgut gland and particles are regurgitated to void the stomach (Korez et al., 2022). *Palaemon elegans* are detritus feeders that might ingest natural particles as well during feeding. A similar ability as that of *C. crangon* could allow *P. elegans* to cope with microparticles of natural but also synthetic origin.

Apparently, SOD activities responded differently in shrimp exposed to T-PHBV or AMF-PLA. The SOD activity declined 4 h after ingestion of T-PHBV but increased after ingestion of AMF-PLA, resulting in significantly different SOD activities between both treatments. These different effects might be attributed to the different chemical compositions of the plastics. Plastic additives are known to induce oxidative stress in aquatic organisms, including crustaceans (Pérez-Albaladejo et al., 2020; Pires et al., 2022; Ru et al., 2022). Savva et al. (2022) identified several additives in single-use plastic items with biobased and biodegradable origin that induced oxidative stress in cytotoxic tests. Exposition to the plasticizer bisphenol A inhibited SOD and other antioxidant enzymes in the red swamp crayfish *Procambarus clarkii* (Zhang et al. 2020). In my experiments, *P. elegans* ingested roughly 30 times more T-PHBV particles than AMF-PLA particles, which could also lead to a higher exposure to leaching additives from the T-PHBV particles (Section 4.2). Antioxidant enzymes such as SOD can either increase at lower concentrations and short-term exposition to harmful substances, or can be inhibited at higher concentrations and long-term exposition (Frías-Espericueta et al. 2022). Thus, the different amount of ingested particles could explain the different SOD responses. The missing antioxidant response by LDPE could be due to even less microplastics being ingested by the shrimp, compared to microparticles of both biodegradable plastics (Section 4.2).

Exposition of zooplankton to conventional plastic leachates caused several implications. Lehtiniemi et al. (2021) showed elevated oxidative stress, impaired swimming activity, and increased mortality in the copepod *Limnocalanus macrurus* after exposure to leachates from plastics. Exposition of barnacle larvae, *Amphibalanus amphitrite*, to plastic leachates also resulted in increased mortality and reduced settlement of the cyprids (Li et al., 2016). The development of larvae of the sea urchin *Paracentrotus lividus* was negatively affected by exposure to leachates from polyvinyl chloride (PVC) (Oliviero et al., 2019; Rendell-Bhatti et al., 2021).

In my exposition experiments with the five different biodegradable plastics, leachates from compounds based on PLA, PBAT and PBS were not lethal to the rotifer *Brachionus plicatilis* and to nauplii of *Artemia persimilis*. However, leachates from a PHBV-based compound induced high mortality in both species (**Publication IV/Manuscript 2**).

The substances that can leach from plastics depend on the composition of the material. The largest part of a plastic is made up by the polymeric compounds, consisting of one or more base polymers. Accordingly, the toxicity of a plastic might be primarily determined by its polymeric composition. During the production of the polymers, some monomers might remain unpolymerized and leach out of the plastic into the environment, because they are only weakly bound to the polymeric matrix (Sheftel, 2000; OECD, 2004). However, in contrast to formulated plastics, leachates from virgin polymer from biodegradable and conventional plastics were not toxic to zooplankton (Zimmermann et al., 2020b; Beiras et al., 2021). Similarly, the unprocessed base polymer of BPE-T-PHBV was not toxic to *Brachionus plicatilis* and *Artemia* nauplii, but the formulated plastic induced high mortality in both species (**Publication IV/Manuscript 2**). So far, my studies indicate that toxicity of leachates less likely originates from the polymer type, but more likely from additional chemicals supplied to the plastic matrix.

Additives, that are not covalently bound in the plastic matrix, might leach out of the plastic and induce toxic effects in organisms (Bibi et al., 2012). Conventional plastics can include a variety of chemicals that are endocrine disrupting (e.g. bisphenole A), carcinogenic (e.g. benzene, 1,3-butadiene), mutagenic (e.g. phenol), and induce acute and chronic toxicity (e.g. benzene, toluene diisocyanate) (Paxéus, 2000; García Ibarra et al., 2018; Thaysen et al., 2018; Chen et al., 2019; Kong et al., 2020). Many studies have shown that the toxicity of plastics to zooplankton and other marine invertebrates is primarily based on associated toxic additives. Leaching additives from car tire rubbers induced acute and sub-lethal responses in the mussel

Mytilus galloprovincialis (Capolupo et al., 2020). Shore et al. (2022) showed reduced survival for larvae of the sea urchin *Strongylocentrotus purpuratus* exposed to four different additives. Beiras et al. (2021) reported the toxicity of plastic leachates to larvae of the copepod *Acartia clausi* and the sea urchin *Paracentrotus lividus*, but found that the leachates were harmless when using the respective virgin polymers without additives. Compared to conventional polymers, biobased and biodegradable polymers have inferior physical properties, especially with regard to impact and tensile strength, thermal stability, and permeability (Pawde et al., 2008; Sadasivuni et al., 2015; Sapuan et al., 2018). To improve the physical properties and the performance of the end-product, a variety of additives are supplied during production (Kahn et al., 2017; Zimmermann et al., 2020a).

The toxicity tests with additives, that are commonly used in formulations of biodegradable plastics, showed adverse effects on *Artemia* nauplii and rotifers of two out of five chemicals (**Publication IV/Manuscript 2**). The toxic additives were benzophenone, an UV absorber, and glycerol triacetate, a plasticizer. The exact compositions of the biodegradable plastics used for this study are unknown. Therefore, it cannot be excluded that the toxic additives might be present in the plastics, leach out, and cause the mortality.

When sea urchin larvae were exposed to biodegradable plastics in a mesocosm study, an initial toxicity during the first seven days of exposure was observed, indicating a rapid release of weakly bound additives from the plastics (Quade et al., 2022). Similarly, a growth inhibition of sea urchin larvae exposed to PHB leachates was attributed to the presence of high quantities of additives in the PHB compound (Uribe-Echeverría and Beiras, 2022). The additives tested in my study are not only used in biodegradable plastics, but also in conventional plastics. Specific additives aimed for biodegradable plastics are slowly being developed towards more natural and less toxic substances.

Additives as main driver of toxicity might explain inconsistent findings about the toxicity of plastics with the same base polymer, since the formulations of different products might differ in their additive composition. Furthermore, degradation of plastics promotes the leaching of additives (Gewert et al., 2021). This seem to apply to T-PHBV: the compound with the highest degradability also shows the highest toxicity. Accordingly, necessity to develop harmless additives for biodegradable plastics is even higher because they degrade faster than conventional plastics and, thus, release more additives.

Taken together, biodegradable plastics can have diverse effects on marine invertebrates. Ingestion of biodegradable plastics did not induce oxidative stress in *P. elegans*. This could be

attributed to the size and shape of the particles and the resilience of species to cope with ingested microplastics. Leaching chemicals from one of the compounds induced high mortalities in microzooplankton. The toxic effects do not derive from the sole polymers alone, but must be attributed to additives, which showed toxic effects on microzooplankton. This observation indicates, that the toxicity of a plastic compound is highly dependent on the additives which are used in the formulation.

4.4 Conclusive remarks

The objective of this thesis was to investigate the degradability of a set of biobased biodegradable plastics under marine conditions and their potential impacts on marine organisms. I used several approaches to study the *in-vitro* enzymatic degradation of biodegradable plastics in seawater. Long-tern exposition of bioplastic test bars was run under controlled laboratory conditions. Ingestion of plastic particles was tested with various crustacean species and intestinal degradation was tested with the gastric fluids of crab and lobster. The toxicity of plastic leachates was evaluated in exposition experiments.

Biodegradable plastics are intended to replace conventional plastics with the goal to counteract environmental pollution. The plastics can be degraded by a variety of enzymes, which was shown in **Publication I**. However, the degradation rates were low under marine conditions (**Publication II**). This is mainly due to the rate limiting effects of low temperatures on the activities of hydrolytic enzymes. With regard to *Research question I*, it can be concluded, that in most marine ecosystems only limited degradation of biodegradable plastics will occur. Accordingly, biodegradable plastic products and fragments thereof will accumulate in many marine ecosystems.

Microparticles resulting from the fragmentation of the biodegradable plastics are readily ingested by marine invertebrates in the same manner as conventional microplastics. After ingestion, the microplastics are exposed to a variety of digestive enzymes. They can be hydrolyzed by enzymes from the gastric fluid as shown for the compound AMF-PLA. Conventional microplastics, such as PMMA, and other biodegradable plastics were not hydrolyzed. Further analyses of the gastric fluid of the crab identified several carboxylesterases, which are responsible for the degradation of the PBAT fraction in the AMF-PLA compound (**Publication III/Manuscript 1**). Regarding *Research question II*, it can be stated that gastric enzymes of crustaceans such as *C. pagurus* and *H. americanus* are capable of hydrolyzing certain biodegradable plastics. Feeding experiments with *P. elegans* and microplastics from

conventional and biodegradable plastics complemented these finding. Significantly elevated carboxylesterase activities were observed in midgut glands of *P. elegans* when fed with AMF-PLA, indicating an induction of digestive enzymes. However, as degradation was indicated only for one of the five compounds, biodegradable plastics will be rarely assimilated and will not notably contribute to the energy metabolism of larger marine invertebrates.

In the environment, substances such as additives, degradation products, and non-polymerized monomers might leach from a plastic. By exposing the rotifer *Brachionus plicatilis* and *Artemia persimilis* nauplii to leachates of the five biodegradable plastics, leachates of T-PHBV induced acute mortality in both species. Further acute toxicity tests with the base polymer (PHBV) showed no toxic effect. However, several commercial additives for biodegradable plastics were tested, some of which caused mortality of the test species. Toxic effects from biodegradable plastics are most likely caused by toxic additives within the material (**Publication IV/Manuscript 2**). Feeding shrimp, *P. elegans*, with biodegradable microplastics induced no cellular response in terms of oxidative stress. The answer to *Research question III* is, that biodegradable plastics can be toxic to marine invertebrates. However, the toxicity might strongly depend on the additives that are used in the formulation of a plastic. Hence, environmental-friendly and non-toxic additives are required in future biodegradable plastics.

The findings of my thesis suggest that biodegradable plastics are not a panacea in mitigating marine plastic pollution. Their slow degradation in the oceans may lead to an accumulation of these materials in marine ecosystems in the same way as conventional plastics. With the increasing production of biodegradable plastics, marine species will inevitable be exposed to their microparticles. In their current state, it cannot be ruled out that interaction with biodegradable plastics might lead to harmful effects to organisms.

4.5 Perspectives

This work is an important contribution to a better understanding of the performance and impact of biodegradable plastics in the marine environment. However, during the process of this thesis, questions were answered but new knowledge gaps were pointed out and new questions arose. These questions were beyond the scope of this thesis, but emphasize the need for future research.

Screening polymers for their degradability with enzymes of marine origin

Global plastic production will further increase in the upcoming years (Statista, 2022), and concomitantly, the share of biobased and biodegradable plastics (Plastics Europe, 2021). My studies have shown that biodegradable plastics are slowly degraded in the marine environment. Therefore, development and testing of degradable polymer needs to be improved with the goal of rapidly designing new polymers or modifying existing ones. A helpful tool for the rapid screening of the biodegradability of a material is the pH-Stat titration assay (**Publication I**). Immediate screening of the enzymatic biodegradability of products can help to accelerate the development of new materials.

Identification of polymer-degrading digestive enzymes

Enzymes capable of hydrolyzing biodegradable plastics were discovered in the gastric fluid of the crab *C. pagurus* (**Publication III/Manuscript 1**). Beside a characterization of the substrate specificity, a more detailed identification of the polymer-degrading enzymes by proteomics and mass spectrometry would provide sequence information. A comprehensive analysis of the enzyme sequences with transcriptome data could provide functional information. In a next step, molecular screening of target enzymes in other taxa could reveal, whether polymer-degrading enzymes are also present in other species and whether these might be able to hydrolyze biodegradable plastics. This information, in turn, might support risk assessment for organisms, if exposed to biodegradable plastics in their environment.

Synergistic effect of additives

Biodegradable plastics had negative impacts on marine invertebrates as shown by acute toxicity tests. Most likely, leaching additives were responsible for mortality. However, to certainly identify and remove toxic additives from plastic formulations, a closer cooperation between the industry and science is desired. A more transparent disclosure of the chemicals used in plastic formulations would facilitate product-related research and thus, the development of environment-friendly plastics. Moreover, chemicals leaching out of the plastic may interact with each other and alter their properties (Liess et al., 2020). Therefore, combinations of additives used in plastic products must be evaluated for their toxicity.

Impact of plastic degradation products

Synergistic effects of leaching substances on organisms need to be considered to better evaluate the impact of biodegradable plastics on marine biota. Degradation products of biodegradable plastics might interact with other leaching chemicals. Up to now, little is known about the biochemical effects of intermediates of biodegradable plastics. Although some studies indicate no environmental risk by degradation of aliphatic-aromatic polyesters in the environment (Witt et al., 2001), others show slight to moderate acute toxic effects of degradation products on aquatic organisms (Kennedy Jr, 2002). For a more precise evaluation of the toxicity of degradation products, further tests are needed. Especially the potential interaction of degradation products with other substances leaching from the plastic matrix needs to be considered.

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6 Appendix

Supporting information to Chapter 4

Table A1: Weight change of the plastic-bars incubated for six months in seawater and estuarine mud (means ± SD, n = 4).

Plastic	Weight change in seawater (%)	Weight change in mud (%)
BPE-T-PHBV	-1.6 ± 0.2	-6.6 ± 0.5
BPE-AMF-PLA	+0.2 ± 0.2	-0.1 ± 0.1
BPE-SP-PBS	+0.1 ± 0.0	-0.0 ± 0.1
BPE-RP-PLA	+0.0 ± 0.0	-0.1 ± 0.0
BPE-C-PLA	+0.2 ± 0.1	-0.3 ± 0.0

Table A2: Abiotic conditions in estuarine mud and seawater used for the degradation experiment with the plastic bars of the different compounds (means ± SD).

Parameter	Seawater	Estuarine mud
Temperature (°C)	15.5 ± 0.5	13.5 ± 0.4
Redox potential (mV)	241.7 ± 48.9	-189.5 ± 28.2
pH	8.0 ± 0.1	7.3 ± 0.1
Salinity (ppt)	3.3 ± 0.2	-
Nitrate ($mg \cdot L^{-1}$)	3.9 ± 0.5	-
Nitrite ($mg \cdot L^{-1}$)	< 0.03	-
Ammonia ($mg \cdot L^{-1}$)	<0.02	-

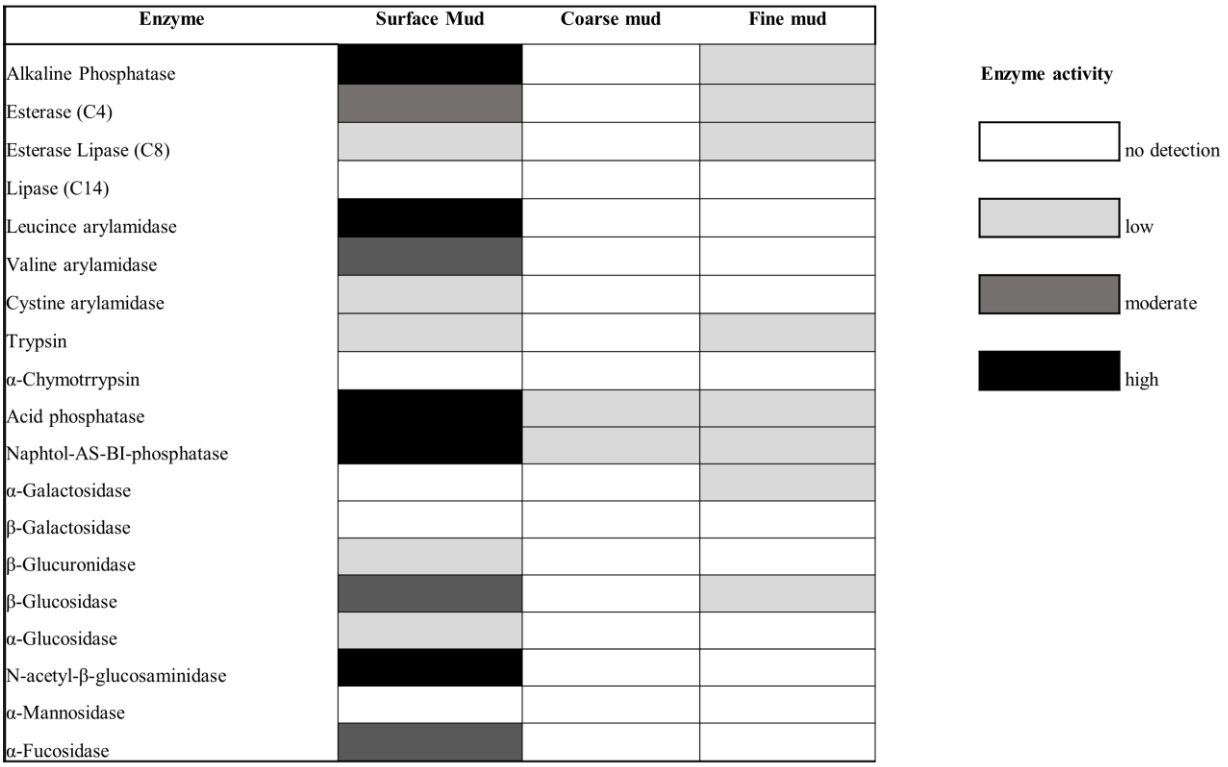

Figure A1: Semiquantitative analysis of activities of extracellular enzymes from estuarine mud samples. The shading in the boxes indicates the strength of activity. White: no detected enzyme activity (value 0), light grey: low enzyme activity (value 1), dark grey: moderate enzyme activity (value 2-3) and black: high enzyme activity (value 4-5).

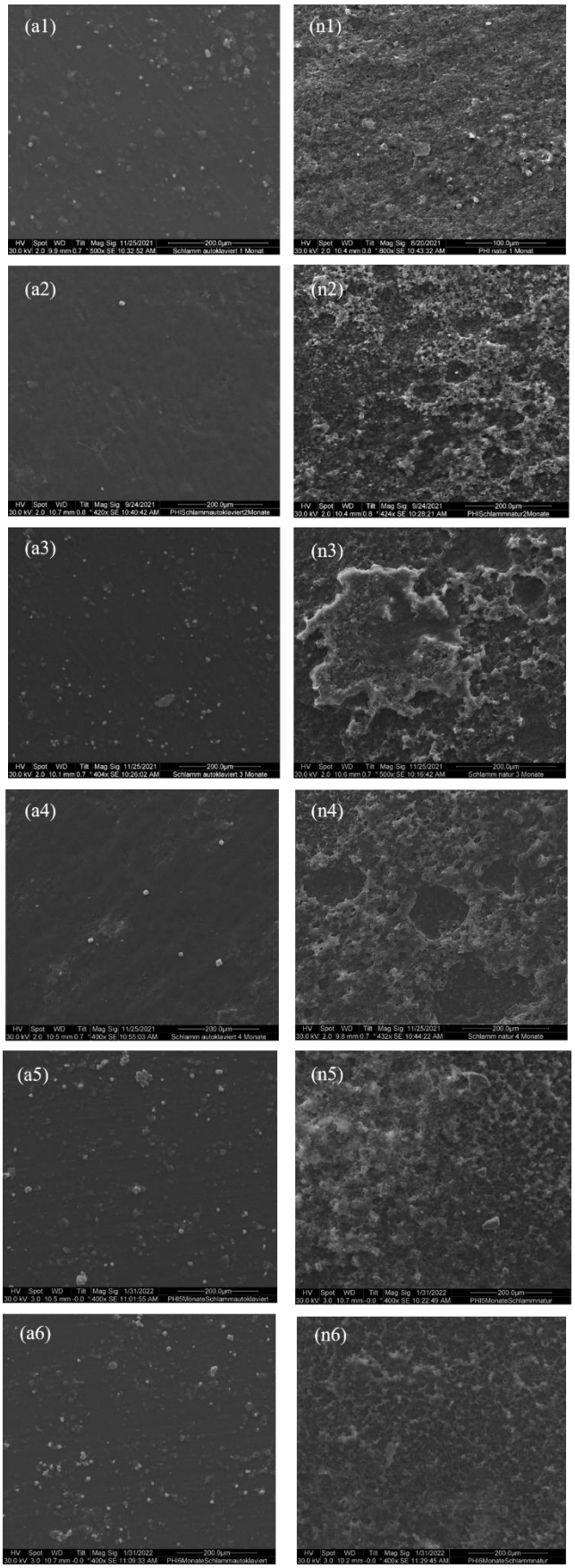

Figure A2: SEM pictures of the surfaces of BPE-T-PHBV pieces incubated in (a) autoclaved mud and (n) natural mud for up to six months. The numbers depict the duration of incubation in month.

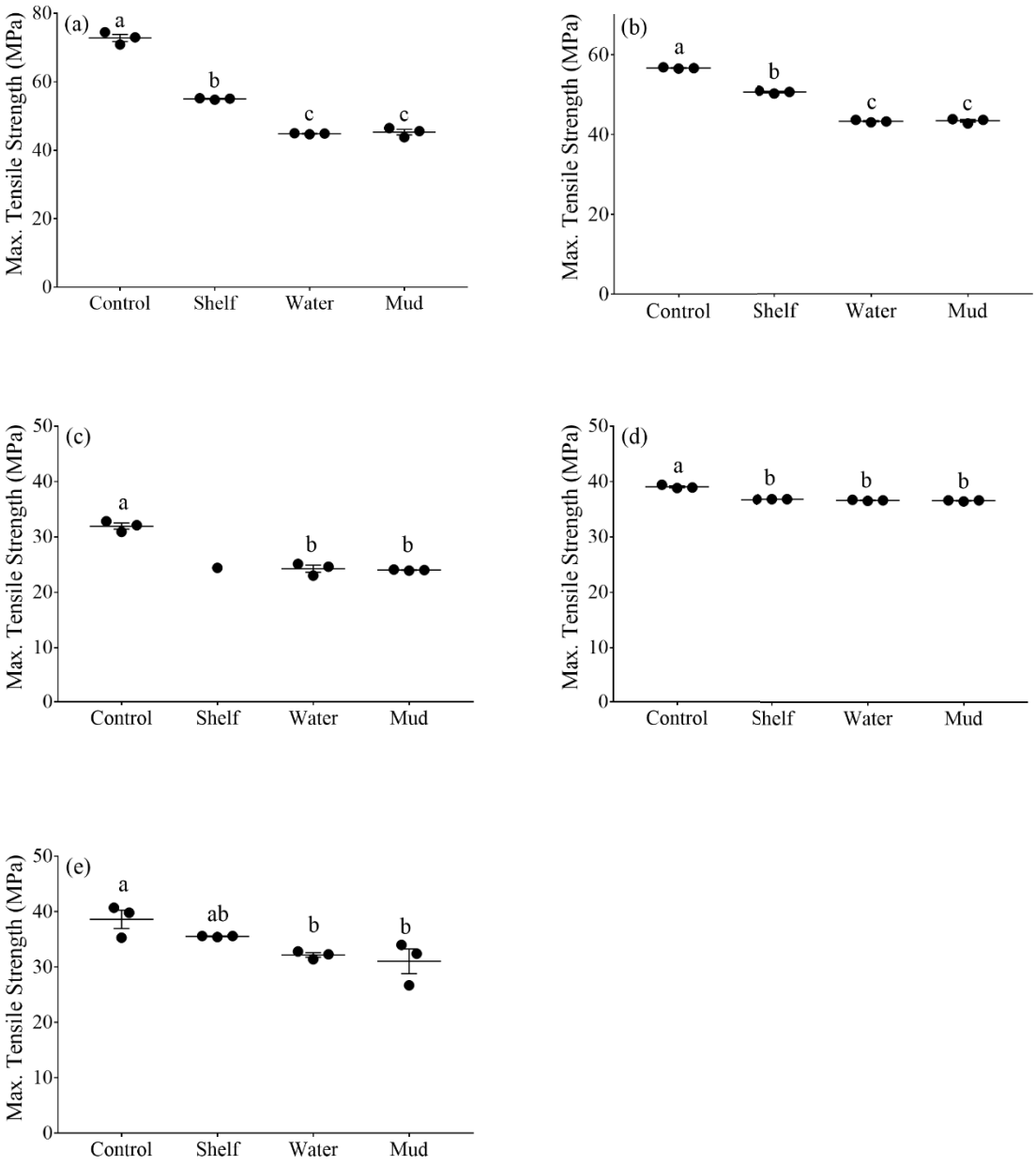

Figure A3: Maximum tensile strength of control BPE materials and BPE materials after 6 months at room temperature (shelf) and of exposure to seawater and mud. (a) BPE-C-PLA, (b) BPE-RP-PLA, (c) BPE-AMF-PLA, (d) BPE-SP-PBS and (e) BPE-T-PHBV. Each dot represents a single test bar (n=3, except AMF-PLA), the horizontal line is the average and the error bars represent the standard deviation. Different letters indicate significant differences.

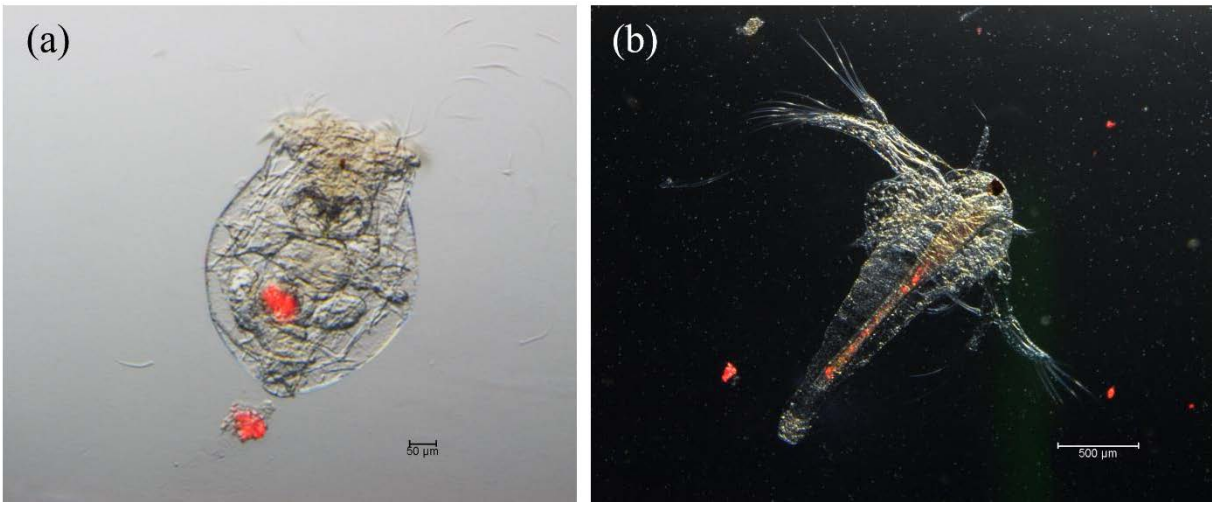

Figure A4: Nile Red stained microplastics from the BPE-compounds ingested by (a) *Brachionus plicatilis* and (b) *Artemia persimilis* nauplii. Pictures were taken by Ann-Christin Scheer.

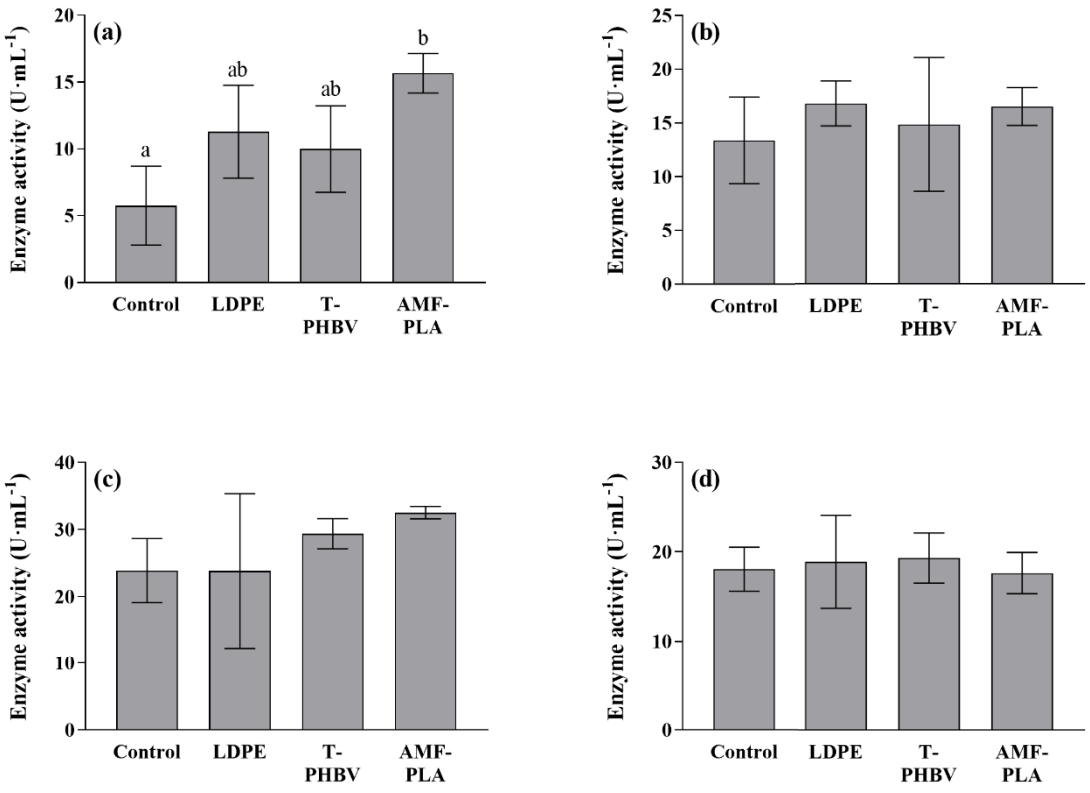

Figure A5: Carboxylesterase activities measured with MUF-heptanoate in the midgut glands of *Palaemon elegans* fed with microparticles of the BPE-compounds (T-PHBV, AMF-PLA) and conventional plastic (LDPE) and a control after (a) 4 h, (b) 24 h and (c) 48 h of feeding and (d) repeated feeding for 12 days. Different letters indicate significant differences (means ± SD, n = 4).

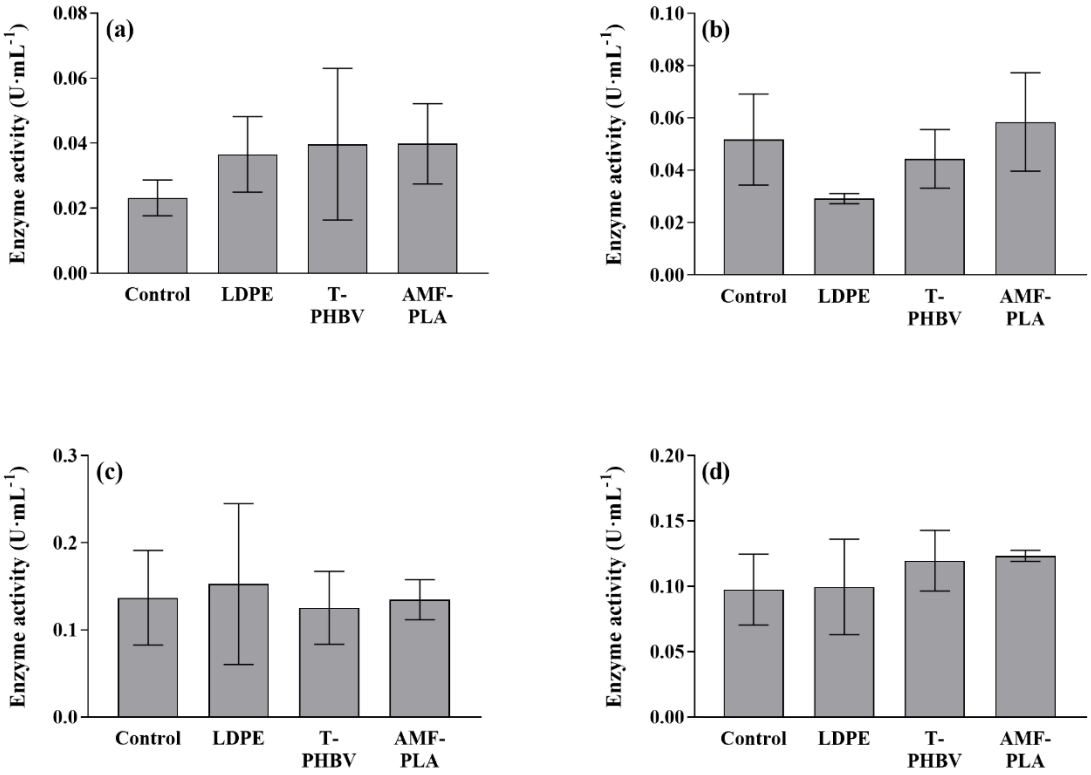

Figure A6: Carboxylesterase activities measured with MUF-oleate in the midgut glands of *Palaemon elegans* fed with microparticles of the BPE-compounds (T-PHBV, AMF-PLA) and conventional plastic (LDPE) and a control after (a) 4 h, (b) 24 h and (c) 48 h of feeding and (d) repeated feeding for 12 days (means ± SD, n = 4).

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