Temperature-mediated Biodegradation of Plastics in Marine Environments

By

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A Thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of

Master of Engineering

Faculty of Engineering and Applied Science

Memorial University of Newfoundland

May 2025

St. John’s, Newfoundland and Labrador, Canada

ABSTRACT

The global ocean accumulates massive plastic waste, raising concerns over environmental impacts. Plastic biodegradation is a promising solution; however, the efficiency of this process is highly temperature-dependent. Despite its importance, the comprehensive understanding of how temperature affects microbial dynamics in plastic degradation across diverse marine climates, particularly in colder regions, remains limited.

This study begins with a literature review on temperature-mediated biodegradation of plastics in marine environments. Evidence suggests that elevated temperatures generally promote biofilm growth and enzymatic activity. Cold-tolerant bacteria produce extracellular polymeric substances (EPS) to stabilize biofilms at lower temperatures. At moderate temperatures, Proteobacteria dominate the initial degradation phase, while Actinobacteria, Firmicutes, and Cyanobacteria contribute to various stages of degradation. Psychrophilic and thermophilic bacteria facilitate degradation in extreme climates. Enzymes such as cutinases, lipases, and depolymerases facilitate partial degradation of hydrolyzable plastics, while non-hydrolyzable plastics remain recalcitrant, relying on enzyme-generated reactive oxygen species (ROS) for gradual breakdown.

Additionally, controlled laboratory experiments were conducted to evaluate the biodegradation of petroleum-based low-density polyethylene (LDPE), bio-based polylactic acid (PLA), and polyhydroxyalkanoates (PHAs) at various temperatures (4, 15, and 22 °C) using a cold-tolerant *Alcanivorax* strain isolated from North Atlantic Ocean. Compared to LDPE and PLA, results showed that PHA films supported substantial

bacterial growth, displayed considerable morphological damage, and released more microplastics (MPs) and dissolved organic carbon (DOC) across all temperatures. Notably, degradation by-products of PHA at 22°C exhibited the highest toxicity to *Vibrio fischeri*, highlighting temperature's role in biodegradation rates and associated ecological risks. These findings from both the literature review and experiment studies underscore the critical influence of temperature on plastic biodegradation and provide fundamental knowledge for mitigating plastic pollution in diverse marine climates.

CO-AUTHORSHIP STATEMENT

This thesis includes collaborative work, and I, Yuanmei Zhang, am the first author for both included chapters. The contributions for each co-authored section of the thesis are outlined below, addressing design and identification of the research topic, practical aspects of the research, data analysis, and manuscript preparation.

Chapter 2, based on an article prepared for submission titled *A Critical Review on Temperature-Mediated Marine Plastic Biodegradation*, was co-authored with Dr. Yiqi Cao, Dr. Bing Chen, and Dr. Baiyu Zhang.

1) Design and Identification of the Research Topic: I led the conceptualization and framework development for the review in collaboration with Dr. Yiqi Cao.
2) Practical Aspects of the Research: I conducted the literature search, compiled relevant studies, and structured the review.
3) Data Analysis: I synthesized and critically analyzed the findings from the literature to develop key insights and conclusions.
4) Manuscript Preparation: I drafted and edited the manuscript, with additional editing and review provided by Dr. Yiqi Cao. Dr. Bing Chen and Dr. Baiyu Zhang contributed to supervision, conceptual guidance, and further editing.

Chapter 3, based on the published article *Marine Biodegradation of Plastic Films by Alcanivorax Under Various Ambient Temperatures: Bacterial Enrichment, Morphology Alteration, and Release of Degradation Products* (Zhang Y, Cao Y, Chen B, Dong G, Zhao Y, Zhang B, 2024, *Science of the Total Environment*), was co-authored with Dr. Yiqi Cao, Dr. Bing Chen, Dr. Guihua Dong, Dr. Yuanyuan Zhao, and Dr. Baiyu Zhang.

1) Design and Identification of the Research Topic: I contributed to the conceptualization of the study in collaboration with Dr. Yiqi Cao, Dr. Bing Chen, and Dr. Baiyu Zhang.
2) Practical Aspects of the Research: I conducted the experiments, including bacterial enrichment, morphological studies, and degradation product characterization, with guidance from Dr. Yiqi Cao and Dr. Baiyu Zhang.
3) Data Analysis: I performed all primary data analysis, including interpreting experimental results and identifying key patterns and trends.
4) Manuscript Preparation: I drafted and edited the manuscript. Dr. Yiqi Cao provided input on manuscript review and editing. Dr. Guihua Dong and Dr. Yuanyuan Zhao reviewed and provided additional edits. Dr. Bing Chen and Dr. Baiyu Zhang supervised the work and contributed conceptual guidance and final manuscript editing.

All co-authors have reviewed and approved their contributions and consent to the inclusion of these works in this thesis.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my supervisors, Dr. Baiyu Zhang and Dr. Bing Chen, for their invaluable guidance, support, and mentorship throughout my research journey. Their expertise and encouragement have been instrumental in shaping my academic growth and achievements.

I gratefully acknowledge the financial support provided by the Natural Sciences and Engineering Research Council of Canada (NSERC), Environment and Climate Change Canada (ECCC), the Canada Foundation for Innovation (CFI), and the Canada Research Chair (CRC) program. Their generous funding made this research possible.

I extend my heartfelt appreciation to my co-authors, Dr. Yiqi Cao, Dr. Guihua Dong, and Yuanyuan Zhao, for their collaboration and contributions to my published paper. I am also deeply thankful to my colleagues at the Northern Region Persistent Organic Pollution Control (NRPOP) Laboratory, Lidan Tao, Dr. Min Yang, Dr. Liu Bo, Dr. Qiao Kang, Hemeihui Zhao, and Jiayi Chen, for their constant support, insights, and encouragement.

Lastly, I am profoundly grateful for my family's unwavering love and support. To my husband, Haoming Zeng, for his understanding and encouragement, my parents for their constant belief in me, and my one-year-old son, Mason, whose laughter and joy have been a source of endless motivation and happiness.

Thank you all for being part of this journey and for your indispensable support.

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LIST OF SYMBOLS AND ABBREVIATIONS

MPs	Microplastics
NPs	Nanoplastics
DOC	Dissolved organic carbon
EPS	Extracellular polymeric substances
ROS	Reactive oxygen species
PE	Polyethylene
LDPE	Low-density polyethylene
PLA	Polylactic acid
PHAs	Polyhydroxyalkanoates
PET	Polyethylene terephthalate
PP	Polypropylene
PS	Polystyrene
PHB	Polyhydroxybutyrate
PHBH	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PHBV	Polyhydroxybutyrate-co-hydroxyvalerate
PBAT	Poly(butylene adipate-co-terephthalate)
PBS	Polybutylene succinate
PBSA	Poly(butylene succinate-co-butylene adipate)
PCL	Polycaprolactone
P(3HB)	Poly(3-hydroxybutyrate)
PA4	Polyamide 4

PBSC	Poly(butylene succinate-co-carbonate)
PBAF	Poly(butylene adipate-co-furanoate)
PBSe	Polybutylene sebacate
PBSeT	Polybutylene sebacate co-butylene terephthalate
PVA	Polyvinyl alcohol
PES	Poly(ethylene succinate)
PEA	Poly(ethylene adipate)
ATR	Attenuated total reflection
FTIR	Fourier-transform infrared spectroscopy
SEM	Scanning electron microscope
XPD	X-ray diffraction
ICP-OES	Inductively coupled plasma optical emission spectroscopy
GC/MS	Gas chromatography/mass spectrometry
PCR	Polymerase chain reaction
GPC	Gel permeation chromatography
HPLC-MS	High-performance liquid chromatography-mass spectrometry
FAME	Fatty acid methyl esters
Csp	Cold-shock proteins
MHET	Mono(2-hydroxyethyl) terephthalate
BHET	Bis(2-hydroxyethyl) terephthalate
TPA	Terephthalic acid
EG	Ethylene glycol

PhaZ	PHA depolymerases
scl-PHAs	Short-chain-length PHAs
mcl-PHAs	Medium-chain-length PHAs
C-C	Carbon-carbon
AlkB	Alkane monooxygenase
MSM	Mineral salts medium
OD	Optical density
BCA	Pierce bicinchoninic acid
SDS	Sodium dodecyl sulfate
TOC	Total organic carbon
POPs	Persistent organic pollutants
TCA	Tricarboxylic acid
ANOVA	Analysis of variance

CHAPTER 1 INTRODUCTION

1.1 Background

Plastics are integral to modern life, with applications in packaging, construction, healthcare, and electronics due to their affordability, durability, and versatility. However, their resistance to degradation and prolonged lifespans have resulted in a global environmental crisis. Since their widespread adoption in the mid-20th century, plastic production has surged, reaching 413.8 million metric tons in 2023 (Statista, 2024). Of this total, an estimated 320.2 million metric tons become waste, much of which is poorly managed and infiltrates natural ecosystems (OECD, 2024).

In marine environments, plastics accumulate through direct sources such as improper waste disposal, abandoned fishing gear, and industrial discharges, and indirect sources like terrestrial runoff and fragmentation of larger debris (Fig. 1.1) (Amaral-Zettler et al., 2020; Andrady, 2022). Once in the oceans, plastics follow a complex lifecycle: they float, sink, or remain suspended based on density, undergoing processes such as colonization by microbes, biofouling, and degradation. Over 99% of plastic debris resides below the surface, either in the water column or on the ocean floor, significantly impacting marine ecosystems (Amaral-Zettler et al., 2020). Over time, plastics break down into smaller particles via mechanical wear, ultraviolet radiation, and oxidative processes, forming microplastics (MPs, <5 mm) and nanoplastics (NPs, <100 nm) (Thompson et al., 2004). MPs are particularly concerning due to their pervasive presence in sediments, water columns, and marine organisms, including commercially harvested species consumed by humans (Prata et al., 2020). The ingestion of MPs by marine organisms impairs feeding efficiency, growth, and overall health. Additionally, plastics can alter habitats, smother

coral reefs, and disrupt ecological functions. Beyond their physical harm to marine life, MPs act as vectors for persistent organic pollutants (POPs) and heavy metals, compounding their ecological toxicity and further highlighting the urgent need for mitigation (Moyal et al., 2023).

Plastics can be degraded through physical, chemical, and biological processes, with biodegradation offering a promising solution (Amobonye et al., 2021). As illustrated in Fig. 1.2, microorganisms initiate biodegradation process by secreting plastic-degrading enzymes, such as PETase, esterases, and cutinases. These enzymes adsorb onto the plastic surface and catalyze its hydrolysis, breaking it into short degradation intermediates. The intermediates are then assimilated into the microbial metabolic pathway, entering the core metabolisms like tricarboxylic acid (TCA) cycle. This process converts the intermediates into carbon dioxide, water, and other metabolic byproducts, effectively recycling the plastic into usable carbon sources for microbial growth and energy production. This enzymatic mechanism highlights the potential of biodegradation as an eco-friendly solution to plastic waste (Mohanan et al., 2020).

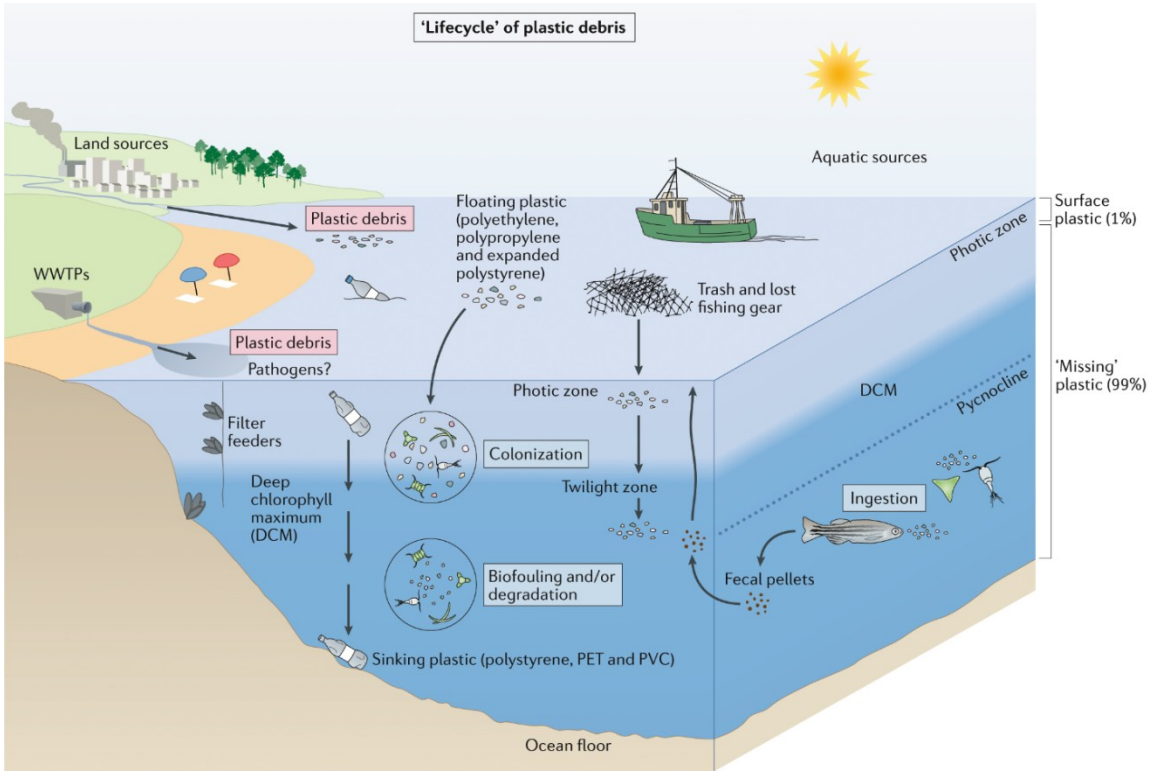


Figure 1.1 The lifecycle of plastic litter. Adapted from Amaral-Zettler et al. (2020).

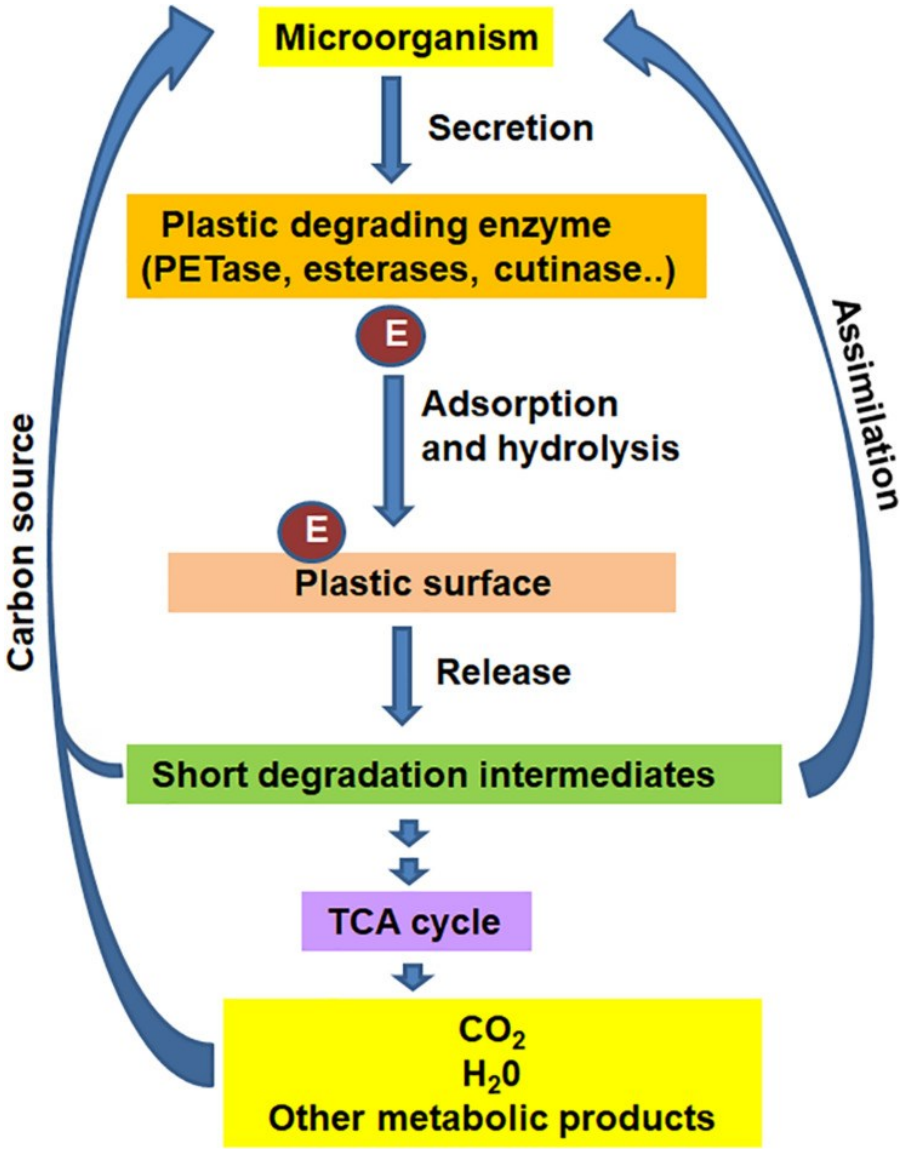


Figure 1.2 The general mechanism for biological degradation of plastics. Adapted from Mohanan et al. (2020).

Despite its potential, the biodegradation rate of conventional plastics like polyethylene (PE) and polyethylene terephthalate (PET) is slow under natural conditions. To address this challenge, biodegradable plastics such as polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) were developed. These materials are engineered to be degraded under specific conditions, such as industrial composting environments with controlled temperature, humidity, and microbial activity (Song et al., 2009). However, their degradation remains insufficient in marine ecosystems, requiring specific environmental triggers and extended periods (Samalens et al., 2022).

Marine plastic biodegradation is driven by diverse microbial communities influenced by environmental conditions, such as temperature, salinity, and nutrient availability. Microorganisms like *Pseudomonas*, *Alcanivorax*, and *Bacillus* produce specialized enzymes, including cutinases, hydrolases, and lipases, which catalyze polymer breakdown and initiate the depolymerization process (Danso et al., 2019; Du et al., 2022). Temperature plays a pivotal role in marine plastic biodegradation, influencing both microbial activity and the physicochemical properties of plastics. Warmer temperatures enhance microbial metabolic rates and enzyme activity, whereas colder temperatures, such as in polar regions, slow these processes. Temperature also influences the chemical properties of plastics, such as crystallinity and hydrophobicity for microbial attack (L. Liu et al., 2022). Understanding the role of temperature in plastic biodegradation is thus crucial for understanding the fate of plastics in the oceans under the changing climate and addressing marine plastic pollution.

1.2 Statements of Problems

(1) Lack of a comprehensive literature review regarding temperature effects on marine plastic biodegradation

Plastic pollution in marine environments has reached alarming levels, with an estimated 8 to 11 million tons of plastic entering the oceans each year, constituting up to 79% of global waste (OECD, 2024). Despite its widespread use, only 9% of plastic is recycled, leading to severe environmental consequences (Geyer et al., 2017). Temperature plays a critical role in shaping microbial dynamics, biofilm formation, and enzymatic activity, influencing plastic breakdown (Moyal et al., 2023). Plastics are dispersed across diverse marine environments, from tropical seas to Arctic regions (Peeken et al., 2018). However, the effects of temperature on plastic biodegradation remain inadequately explored. In addition, most studies focus on single-temperature conditions, neglecting the broader range of temperatures that plastics experience in natural settings. This oversight hinders our understanding of how temperature fluctuations affect degradation rates, especially under the stress of climate change. Extreme temperatures may either enhance or inhibit microbial activity and enzymatic processes (X.-F. Wei et al., 2024). A comprehensive literature review addressing the dynamic interplay between temperature, microbial communities, and plastic biodegradation across diverse marine environments is thus crucial for informing effective mitigation strategies.

(2) Lack of marine plastic biodegradation analysis in the northern regions

Marine plastic pollution is a growing global concern. MPs have been found and accumulate in northern regions due to slower degradation rates caused by reduced microbial activity and enzymatic efficiency in cold climates (Bergmann et al., 2022). Additionally, climate change is expected to exacerbate the persistence of plastics in these areas as melting sea ice releases more MPs into the environment (Ford et al., 2022). Plastics in cold marine ecosystems persist for extended periods, worsening their ecological impact, particularly in fragile ecosystems (Urbanek et al., 2018). While research on plastic biodegradation in marine environments has been increasing, the effects of low temperatures on microbial degradation are still underexplored (Ross et al., 2021). Most existing studies have focused on warmer climates or optimal conditions for microbial degradation (Matjašič et al., 2021). Thus, there is a need for further research on marine plastic biodegradation in cold climates such as northern regions to assess the full scope of temperature-induced effects on microbial activity and biodegradation rates. It is essential to develop effective strategies to mitigate plastic pollution in these vulnerable areas.

1.3 Research Objectives

To address the research gaps identified in Section 1.2, this thesis investigates temperature-mediated marine plastic biodegradation. It entails two main tasks: (1) reviewing current research on temperature-induced biofilm formation, microbial succession, and enzymatic depolymerization, and (2) experimentally examining the biodegradation of plastic films by *Alcanivorax* under various ambient temperatures in the

north. The study focuses on understanding how bacterial activity, particularly enzyme production and biofilm formation, drives the breakdown of microplastics in marine environments. By exploring these mechanisms, the research aims to provide insights into the role of microorganisms in mitigating plastic pollution.

For the literature review, studies on temperature-induced biofilm formation, microbial succession, and enzyme efficacy in the depolymerization of plastics were compiled to evaluate existing knowledge and gaps. Emphasis was placed on microbial activity across temperature ranges (e.g., polar to tropical), enzyme function under fluctuating temperatures, and environmental factors influencing microbial degradation of common plastics, such as PET and PE. This analysis highlights the critical role of bacteria in initiating and accelerating plastic degradation through enzymatic hydrolysis and oxidative modifications. Specifically, it explores how temperature variations influence microbial metabolism, enzyme stability, and biofilm dynamics, which are key factors in determining the rate and extent of plastic biodegradation. This analysis aimed to clarify how temperature affects microbial behavior and the degradation rates of plastics.

The experimental research utilized *Alcanivorax*, a cold-tolerant marine bacterium with demonstrated plastic-degrading abilities, to study temperature effects on plastic biodegradation. The selection of Alcanivorax as the model organism is based on its well-documented hydrocarbon-degrading capabilities, its prevalence in marine environments, and its ability to thrive in cold temperatures, making it an ideal candidate for studying biodegradation in diverse climatic conditions. Experimental parameters included bacterial enrichment on plastic films, morphological alterations, and the release of degradation

products across temperature gradients. Factors such as ambient temperature variation and microbial succession over time were monitored to assess microbial responses and biofilm formation. By focusing on *Alcanivorax*, the study provides a detailed understanding of how specific bacterial strains contribute to plastic degradation and how their activity is influenced by temperature. The experimental findings provided insights into temperature-dependent microbial efficiency and plastic degradation rates.

The findings of the thesis work are expected to advance our understanding of temperature-mediated plastic biodegradation and inform strategies to mitigate plastic pollution in marine environments. By elucidating the relationship between bacterial activity and microplastic degradation, this research contributes to the development of targeted bioremediation strategies, particularly in cold regions where plastic accumulation is a growing concern. Additionally, the study underscores the importance of selecting appropriate microbial strains, such as *Alcanivorax*, for future biodegradation research and applications.

1.4 Thesis Structure

This thesis is organized into four chapters. Chapter 1 introduces the study by highlighting the critical role of temperature in marine plastic biodegradation. It defines the research problem, outlines the objectives, and discusses the significance of the study. Chapter 2 offers a comprehensive literature review on temperature-mediated plastic biodegradation in marine environments. This chapter examines microbial mechanisms of plastic

degradation, temperature effects on various plastic types (PET, PLA, PHAs, PE, polypropylene (PP), and polystyrene (PS), enzymatic degradation processes, and microbial succession under both ambient and extreme temperature conditions. Chapter 3 presents the experimental investigation of plastic film biodegradation by *Alcanivorax* species under different ambient temperatures. It explores bacterial enrichment, morphological changes in plastic films, and the release of degradation products throughout the process. Finally, Chapter 4 concludes the thesis by summarizing the key findings, offering recommendations for future research, and discussing potential practical applications in marine waste management.

CHAPTER 2 CRITICAL REVIEW OF TEMPERATURE EFFECTS ON MARINE PLASTIC BIODEGRADATION[1]

[1] This chapter is based on an article currently under review in *Eco-Environment & Health* (Special Issue: *Persistent Toxic Substances & Health*), *A Critical Review on Temperature-Mediated Marine Plastic Biodegradation* by Yuanmei Zhang, Yiqi Cao, Bing Chen, and Baiyu Zhang. Contributions: Yuanmei Zhang – conceptualization, methodology, manuscript drafting and editing; Yiqi Cao – conceptualization, manuscript editing, review; Bing Chen – conceptualization, supervision; Baiyu Zhang – conceptualization, supervision, review, and editing.

2.1 Background

Plastics are indispensable in our daily lives due to their durability and low cost. However, only about 9% of plastics are recycled, while an overwhelming 79% end up in landfills or the environment, and between 8 million and 11 million tons of plastic waste enter the ocean every year (Geyer et al., 2017; OECD, 2024). The biodegradation of plastic waste functions as a natural defense mechanism, mitigating the accumulation of synthetic pollutants in marine ecosystems. Biodegradable plastics have been promoted as a potential solution to the plastic pollution crisis. The global bioplastics production capacity is projected to increase significantly, from around 2.18 million tonnes in 2023 to approximately 7.43 million tonnes by 2028 (European Bioplastics, 2023).

The plastic degradation process in marine environments is affected by intrinsic properties (e.g., composition, material, structure, and existence form of plastics) as well as external environmental conditions (e.g., temperature, pH, humidity, microorganisms, kinetic factors, hydrolysis, catalysts, enzymes) (L. Liu et al., 2022). Among these, temperature is critical in shaping microbial dynamics, biofilm formation, plastic properties, and enzyme activity (Moyal et al., 2023). Plastics are distributed across diverse marine environments, from tropical waters to more extreme conditions. For instance, MPs have been detected in Arctic ice and the deep sea (Peeken et al., 2018), highlighting the presence of plastic waste across a broad temperature range from -2°C in polar regions to over 30°C in tropical and temperate waters (Van Rossum, 2021; Zhi Xiang et al., 2023).

Moreover, climate change amplifies the impacts of marine plastic pollution through mechanisms such as ice melting, extreme weather events, and ocean acidification. For instance, the melting of Arctic sea ice is projected to release trillions of microplastic particles into marine systems within the next decade (Ford et al., 2022; Haque & Fan, 2023). Rising ocean temperatures driven by climate change further complicate degradation processes by accelerating plastic fragmentation (X.-F. Wei et al., 2024). Additionally, extreme events like storms and cyclones, which are becoming more frequent due to climate change, disrupt microbial communities and influence biofilm formation on plastics (Sunil et al., 2024). Typically, higher temperatures promote faster biodegradation as long as the temperature stays within the optimal range for microbial and enzymatic activity (Sudhakar et al., 2008). However, extremely high and low temperatures may hinder these processes by either denaturing enzymes or reducing microbial activity. Thus, understanding the temperature dynamics across different marine regions is crucial for assessing the biodegradation potential of plastics in a warming world.

Research is increasingly focused on understanding the degradation mechanisms of both conventional and biodegradable plastics in marine environments, using in-situ and laboratory-based biodegradation experiments. However, many investigations of marine plastic biodegradation remain limited to a single temperature range (Giacomucci et al., 2020; Khandare et al., 2021). It lacks a broad comparison across the diverse thermal environments. As a result, critical gaps remain in our understanding of how different temperatures affect plastic degradation rates and microbial succession, particularly

concerning seasonal variations and extreme conditions. Besides, studies overlook the long-term impacts of temperature fluctuations, given the prolonged persistence of plastics in marine environments. Addressing these gaps is essential for developing effective strategies to mitigate marine plastic pollution.

Using a systematic literature review methodology, we identified relevant publications by applying the search terms “ocean OR marine OR seawater” AND “biodegradation OR microbial degradation” AND “plastics OR microplastics” across all fields from the last 10 years (Fig. 2.1). To refine the focus, we further filtered articles by adding keywords of “temperature OR temperature effects”. The decreasing number of such publications highlights a significant research gap in this area (Fig. 2.1). To address this gap, this review evaluates current research on the effects of temperature on marine plastic biodegradation, with representative studies summarized in Table 2.1. It highlights key aspects such as biofilm development, microbial succession, and enzyme-based depolymerization under various temperature conditions (Fig. 2.2). Furthermore, the review examines the environmental and ecological implications of temperature-mediated plastic degradation and proposes strategies for mitigating the growing crisis of plastic pollution in marine environments.

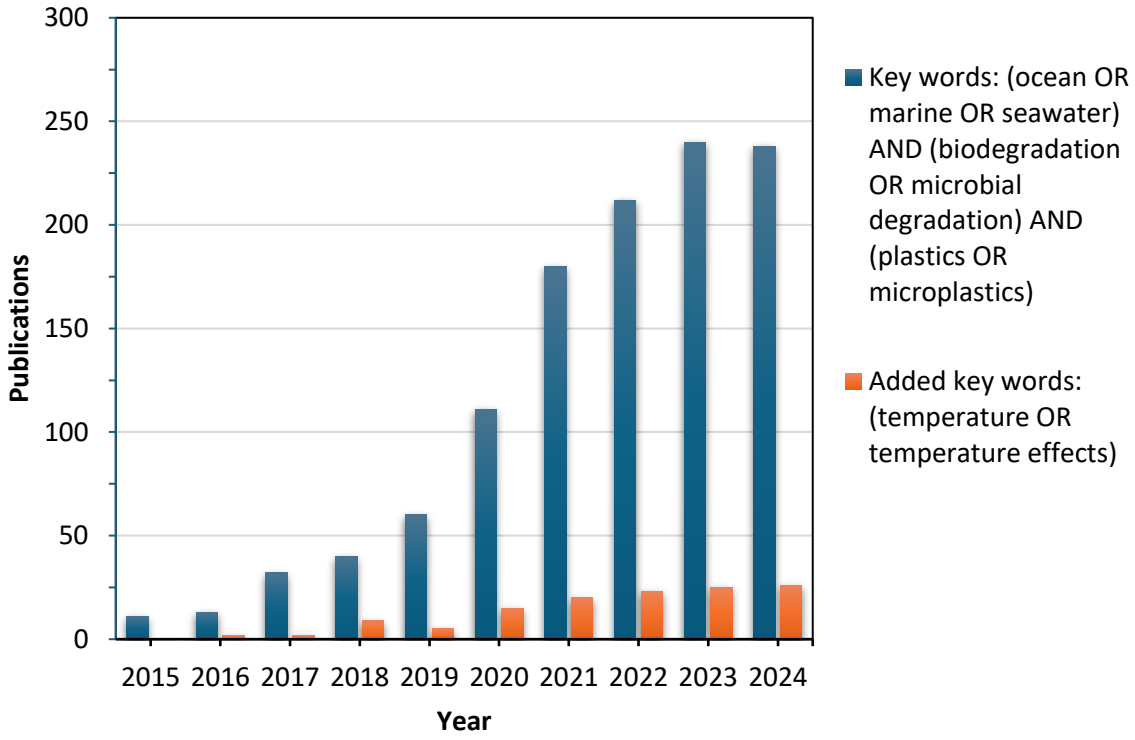


Figure 2.1 Publication trends derived from literature search.

Table 2.1 Summary of studies on the temperature effects on biodegradation efficiency of various plastics in marine environments.

Plastic types	Form	Bacteria	Temperature	Duration	Location	Conditions	Result	Assessment	Reference
PHB	Powder, Film	*Shewanella*	4°C, 15°C, 25°C, 30°C, 37°C, 50°C, 60°C	10 days	Yaizu, Suruga Bay, Japan	Lab	Clear zone on P(3HB) medium was largest at 15°C, strain grew well at 30 to 37°C.	Clear zone	Sung et al., 2016
PHBH, PLA, PBAT, PBS, PBSA, PCL	Film	*Glaciecola*, *Aestuariibacter halophilus*, *Pseudoalteromonas*	11°C, 14°C, 20°C	1 month	Takasago harbor, Japan	Lab	PHBH and PCL films showed degradation, with biofilm formation being crucial for degradation efficiency.	Observation, 16S rRNA	Morohoshi et al., 2018
PHBH, PP, Cellulose	Sheet, Flake	*Clostridiales*, *Gemmatales*, *Phycisphaerales*, *Chlamydiales*	25°C (Room temperature)	148-195 days	Georgia, United States	Lab	Anaerobic sludge produced more methane with PHBH compared to cellulose. PHBH had variable CO_2 production under aerobic conditions.	Gas evolution (CO_2, Methane)	Wang et al., 2018
P(3HB)	Film	*Alcanivorax dieselolei* (MC1)	4°C, 20°C, 25°C, 30°C, 37°C, 50°C	5 days	Microbiota of cheeses, Japan	Lab	MC1 formed clear zones at 30 to 37°C, inactive at 4°C. Nutrient-rich conditions suppressed P(3HB) degradation.	Clear zones	Tachibana et al., 2019

PHBH	Film	*Bacillus sp.*, *Alteromonas*, *Psychrobacter*	4°C	1 month	Deep-sea sediment, Japan	Lab	PHBH degraded by bacteria from deep-sea environments at low temperatures and high pressure.	Microbial composition	Kato et al., 2019
P3HB	Film	-	10°C, 20°C, 27°C	4 weeks	Osaka South Port and Osaka Bay	Lab and field	PHAs biodegraded about 25%, higher temperature increased degradation.	BOD test (lab); Weight loss and molecular weight (field)	Nakayama et al., 2019
PET	Film	*Vibrio* sp. (bacteria), *Aspergillus* sp. (fungi)	25°C, 35°C, 45°C	6 weeks	Bay of Bengal	Lab	Plastic bottle waste sample degraded 35% by bacterial strains and 22% by fungal strains. Best rate of degradation at 35°C.	Weight loss, FTIR, SEM, XRD	Sarkhel et al., 2020
P(3HB)	Film	*Nocardioides* sp. OK12	4°C, 15°C, 25°C, 30°C, 37°C, 40°C, 50°C	7 days	Okinoshima beach, Japan	Lab	Optimum degradation at 30°C. Biofilm formation enhanced degradation.	Clear zone, weight loss, FTIR, genetic analysis	Suzuki et al., 2021
PHAs	Film	*Bacillus* sp. JY14	20°C, 30°C, 37°C, 42°C	5 days	Marine soil, Korea	Lab	Highest PHB degradation at 30°C (40% in liquid culture).	Weight loss, clear zone	Cho et al., 2021
PA4	Powder	*Pseudoalteromonas sp. Y-5*	15 °C	4 days	Marine environment	Lab	Maximum PA4-degrading activity observed after 4 days of incubation. The purified enzyme successfully hydrolyzed PA4 into gamma-aminobutyric acid oligomers.	Enzyme activity assays, mass spectrometry	Saito et al., 2023

PET	Bottle	marine microbial community (Pseudoalteromonas 21%, Alteromonas 11%)	25 °C	150 days	Shuangyue Bay in Huizhou	Field	Light, high-pressure heat, and humid environments significantly affect degradation; high-salt environments have less effect. Ordinary cleaning processes are insufficient to remove inorganic substances.	SEM, Elemental analysis, tensile test, viscosity	Wu et al., 2023
PE, LDPE	Film (from bottles, gloves)	*Pseudomonas aeruginosa, Halomonas venusta*	30 °C ± 2 °C	3 months		Lab	Light promotes the leaching of harmful additives; microbial activity, salinity, pH, and aeration significantly influence biodegradation. PE is more susceptible to degradation than LDPE.	SEM, FTIR, ICP-OES, GC/MS	Dimassi et al., 2024
PLA, PBS, PBAT, PBSC, PBAF	Film (0.15 and 0.5 mm thick)	Marine microbial community	min: 11.6 °C (winter), max: 22.8 °C (summer)	12 months	Pohang-si, South Korea	Field	Weight loss rate of polyesters directly affected by water temperature; average rate in summer (23°C) is 1.9 times higher than in winter (12°C). No surface modification was observed at 4 and 15°C, holes started to appear on the surface, and these became larger and deeper with time at 25°C.	SEM, molecular weight loss	Shin et al., 2024
PBS/PBAT	Rope	-	4 °C, 15 °C, 25 °C, 40 °C, 60 °C	18 months	Brest estuary	Lab	No significant loss in molecular weight at low temperatures; higher temperatures led to faster loss of properties.	Tensile test, Molecular weight loss, SEM	Le Gué et al., 2023

PHB, PBSe, PBSeT, LDPE	Film	-	20 °C (lab), 12 to 30 °C (field)	331 days	Seawater taken at Seccheto, Isola d'Elba, Italy	Lab and field	Biodegradation half-life varies significantly by climate, habitat, and material; LDPE showed no biodegradation.	CO_2 release	Lott et al., 2021
PE, PLA, tire particles	Particles	Marine microbial community	5 °C and 25 °C	60 days	Pingdingshan, China	Lab	Temperature influences catalase and neutral phosphatase activities; tire particles increase microbial diversity and alter community structure.	PCR, SEM, FTIR, enzyme activity	Guo et al., 2024
PCL, PBS, PBAT	Film	*Vibrio* species dominant	Lab: room temp, Field: 3°C (winter), 27°C (summer)	1 year	Odo 1-ri, Heung-hae-eup, Buk-gu, Pohang City, Gyeongsangbuk-do, South Korea	Lab and field	PCL degraded at the rate of 89 μm/month in a coastal marine environment; ranking of decomposition rates was PCL > PBS > PBAT. After 12 months, tensile strength of PCL decreased to 15 MPa and elongation to almost 0%.	Molecular weight loss, tensile test, SEM	Lee et al., 2024
PE, PET	Film	*Pseudomonas, Bacillus, Vibrio*	26 °C (room temperature)	4 weeks	Huiquan Bay Qingdao, China	Field	Molecular weight loss and surface erosion observed in both PET and PE samples.	16S rRNA, SEM, FTIR, GPC), XRD, HPLC-MS	Gao & Sun, 2021
PCL	Film	*Pseudomonas pachastrellae*	4, 20, 25, 30, 37, and 40 °C	1 week	Okinoshima coastal water, Chiba, Japan	Lab	Strain TKCM 64, closely related to *Pseudomonas pachastrellae*, degraded PCL film at a rate of 1.39 ± 0.09 mg $cm^{-2}{\cdot}day^{-1}$; hydrolytic activity induced by PCL and its hydrolysate 6-hydroxyhexanoic acid.	PCR, GC, FAME	Suzuki et al., 2018

PLA, PVA/starch blends, LDPE	Bag strip	22.86 ± 4.28 °C	6 months	Hong Kong	Field	All marine plastic samples show notable biofouling growth and fragmentation. PLA and PVA/Starch blends show larger mass losses by 23 to 100 % than the LDPE.	Weight loss, SEM, FTIR	Cheung & Not, 2024

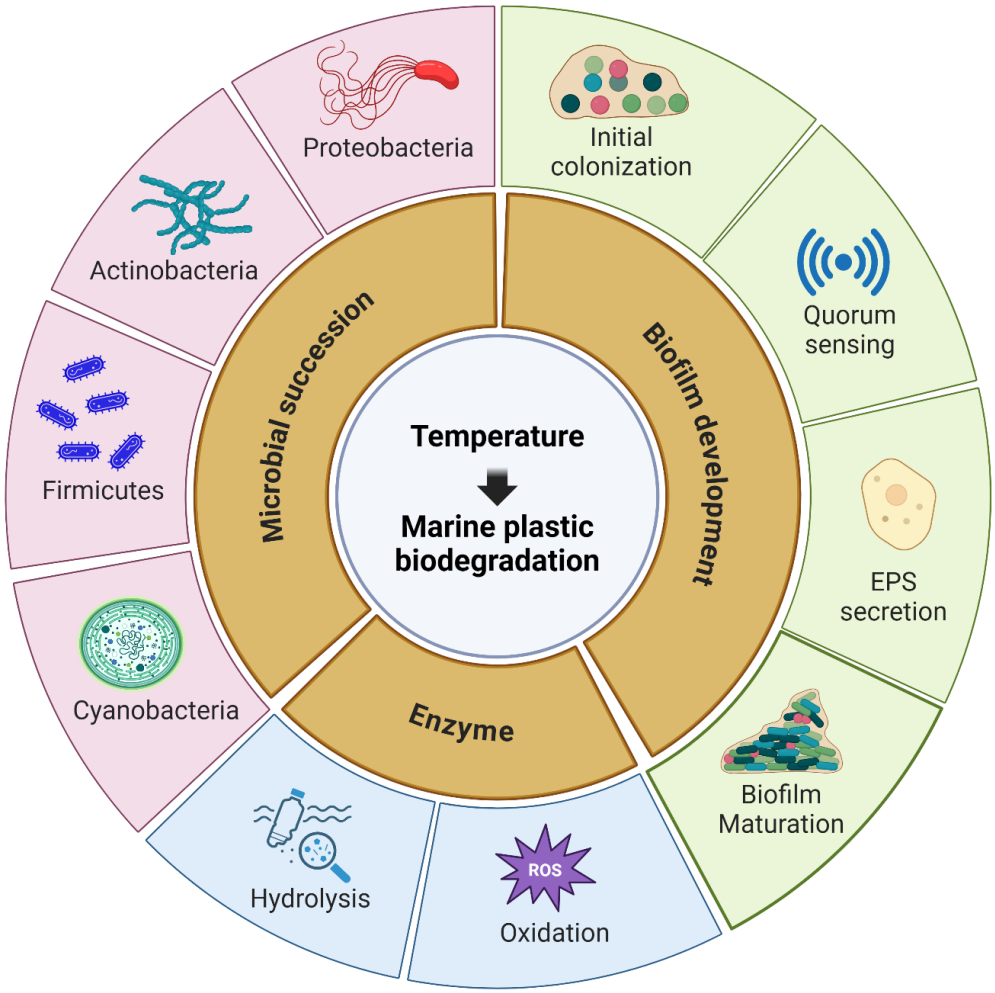


Figure 2.2 Summary of research focus on temperature-mediated plastic marine biodegradation.

2.2 Temperature Effects on Biofilm Development

Plastic pollution in marine environments was first recorded in the 1970s, particularly in the North Atlantic Ocean, where plastic particle concentrations reached up to 3,500 pieces per km^2 (Law & Thompson, 2014). Plastics often undergo physical and chemical weathering before and after entering the ocean. These processes generate MPs (<5 mm) or NPs (1 nm to 1 μm), which have garnered significant attention due to their direct impacts on marine ecosystems and interactions with other contaminants (P. Liu et al., 2020). Microorganisms colonize these MPs' surfaces, forming biofilms named plastisphere (Amaral-Zettler et al., 2020). These biofilms enhance survival by aiding stable microbial consortia, horizontal gene transfer, nutrient accumulation, and protection from toxic substances (Sooriyakumar et al., 2022). Within the plastisphere, pioneer microorganisms colonize the plastic surface, initiating primary biodegradation by fragmenting the plastic into smaller pieces through the breakdown of polymer chains. Secondary colonizers then produce extracellular polymeric substances (EPS) to form more irreversible attachments of biofilm. These fragments are eventually mineralized by various enzymes secreted within the biofilms into CO_2 (Du et al., 2022). The duration of each step varies; initial colonization occurs within minutes, while secondary biofilm development may take months, and final mineralization takes even longer (Du et al., 2022).

Higher temperatures generally promote the early stages of biofilm formation by accelerating the growth of pioneer colonizers, such as members of the Gammaproteobacteria. These pioneer organisms stabilize the biofilm by releasing organic

substrates, which support the development of more complex microbial communities (Dang & Lovell, 2016). Despite this, lower temperatures can enhance biofilm formation by accumulating cyclic-di-GMP, a second messenger that regulates biofilm development, motility, and EPS production (Römling et al., 2013). As illustrated in Fig. 2.3, increased cyclic-di-GMP levels at low temperatures enhance bacterial communication and biofilm formation, creating a more stable microbial community on plastic surfaces (Lin et al., 2024). For instance, cold-tolerant bacteria like *Erythrobacter* have been shown to colonize PS and PE in waters of 17°C (Oberbeckmann et al., 2018). *Vibrio cholerae* can form more complex biofilms at lower temperatures (15 and 25°C) than 37°C (Townsley & Yildiz, 2015). *Pseudoalteromonas* have been observed to produce more EPS at lower temperatures, and *Pseudomonas aeruginosa* forms denser biofilms under reduced temperature conditions (Bisht et al., 2021). Additionally, cyclic-di-GMP plays a crucial role in flagellar regulation, downregulating motility and contributing to biofilm stability. Besides, temperature fluctuations can also influence the activity of extracellular attachment structures like flagella (Moyal et al., 2023; Su et al., 2022).

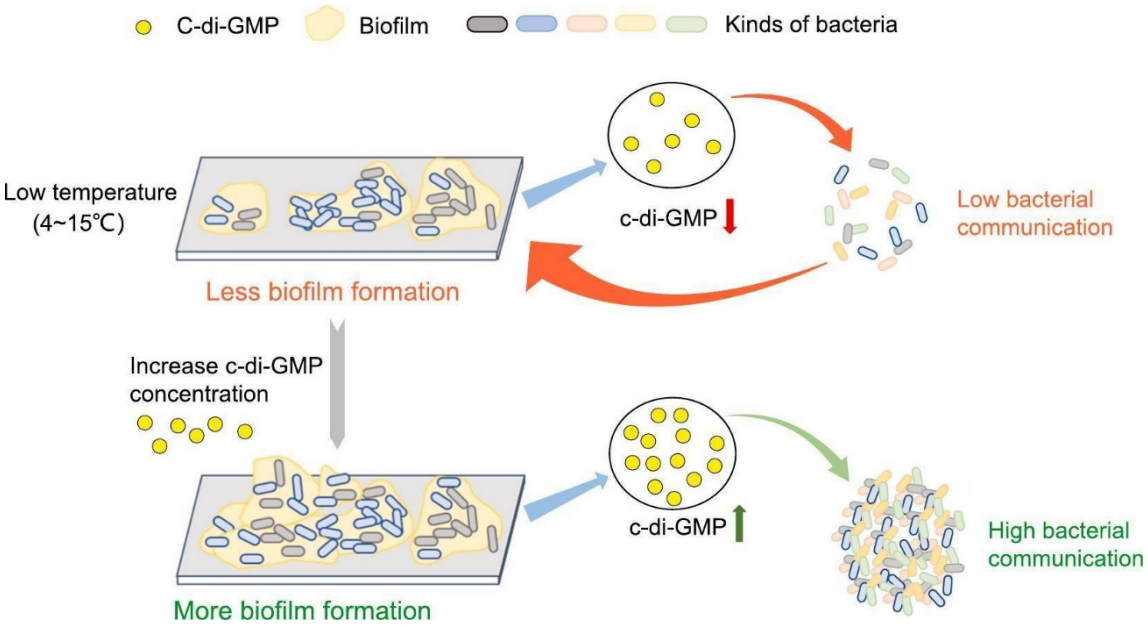


Figure 2.3 The mechanism of temperature on biofilm development at low temperature. Adapted from Lin et al. (2024).

However, current research on biofilm formation and its role in plastic degradation faces several limitations. Most studies are conducted over short timeframes, which may not capture the long-term dynamics of biofilm development and plastic degradation. For example, while initial colonization and biofilm stabilization can occur within days or weeks, the complete mineralization of plastics often takes months or even years, depending on environmental conditions. Long-term studies are needed to better understand how biofilms evolve over time and how their composition and activity influence plastic degradation rates.

Another challenge is isolating the effects of temperature from other environmental factors, such as salinity, pH, and nutrient availability. In natural marine environments, these factors often interact in complex ways, making it difficult to determine the specific contribution of temperature to biofilm formation and plastic degradation. Controlled laboratory experiments, while useful, may not fully replicate the dynamic conditions of real-world marine ecosystems. Future research should aim to bridge this gap by combining controlled experiments with field studies to provide a more comprehensive understanding of biofilm dynamics under varying environmental conditions.

Besides, the diversity and structure of mature biofilms depend on the microorganisms' ability to adapt to temperature changes. Microorganisms with broader temperature tolerance are typically more dominant in colonizing plastics in marine environments. Various studies have explored the influence of environmental factors on plastic biofilm development, such as seasonality, temperature, and light. In-situ experiments have provided valuable insights into the ecological dynamics of bacterial communities (Misic

& Covazzi Harriague, 2019; Pinnell & Turner, 2020). For instance, Pinnell & Turner (2020) reported the critical impacts of seasonal variation on microbial diversity. However, investigating sole temperature effects on microbial dynamics remains challenging as field trials often have complex conditions such as different salinities and pH.

In summary, temperature plays an essential role in the formation and development of biofilms on marine plastic surfaces. While higher temperatures generally accelerate the early stages of biofilm formation, lower temperatures may enhance biofilm stability through mechanisms such as increased EPS production via cyclic-di-GMP regulation. However, the full extent of temperature's influence on biofilm dynamics, particularly concerning long-term plastic degradation, remains insufficiently explored, especially given the complexity of microbial communities and the variety of plastic substrates. Further research is needed to unravel how these factors interact over time to influence the degradation process and to better understand how temperature fluctuations affect biofilm resilience and plastic breakdown in diverse marine environments. Addressing these gaps will require a combination of long-term studies, advanced analytical techniques, and interdisciplinary approaches to account for the multifaceted nature of biofilm-mediated plastic degradation.

2.3 Temperature-induced Microbial Succession

Temperature is a key driver of microbial succession in the degradation of plastics in marine environments, profoundly influencing the composition and activity of microbial

communities. Phylum Proteobacteria dominate initial colonization at ambient temperatures (Sooriyakumar et al., 2022). Besides, phyla Actinobacteria, Firmicutes, and Cyanobacteria further contribute to plastics biodegradation. In extreme environments, temperatures shape microbial communities differently: psychrophilic bacteria dominate in colder waters, while thermophiles take over in warmer regions, such as deep-sea black and white smokers (Atanasova et al., 2021). The following sections delve deeper into the temperature effects on microbial succession, summarized at the phylum level, as illustrated in Fig. 2.4, which highlights the temperature ranges for marine plastic-degrading bacteria across different phyla.

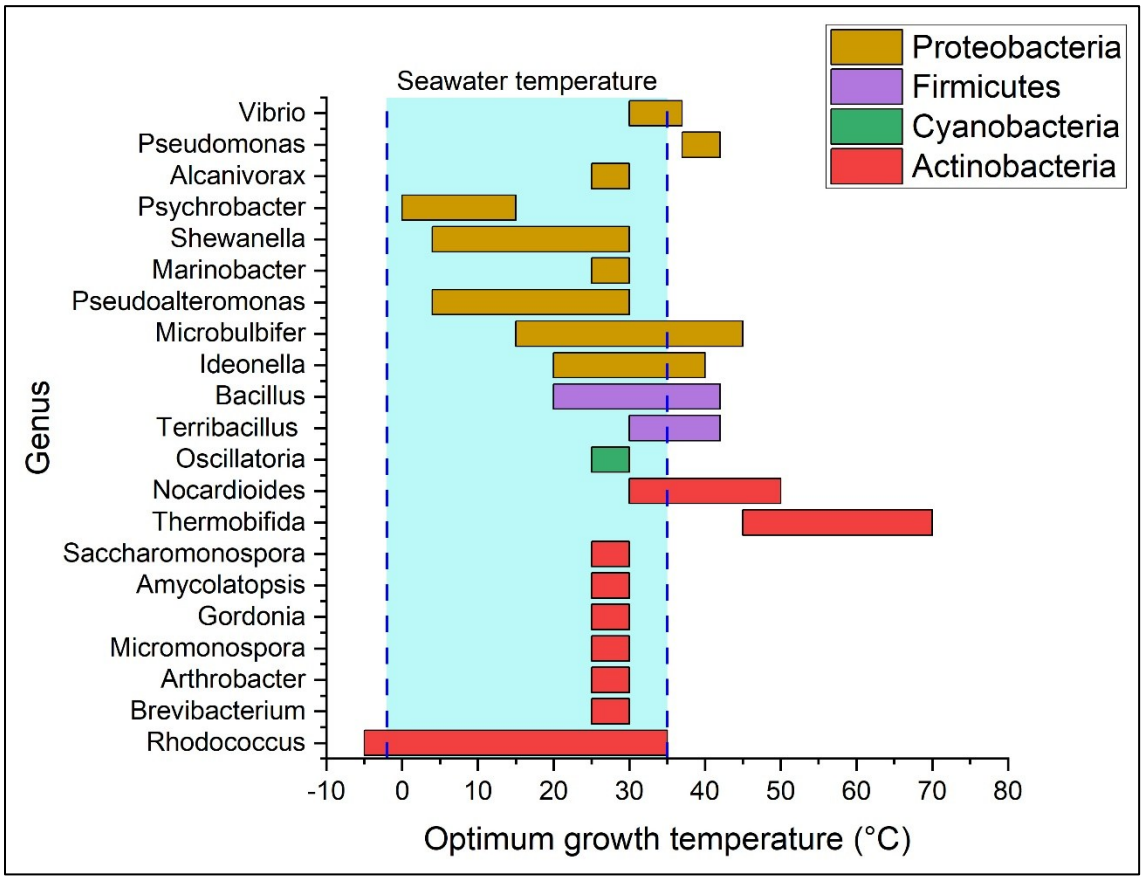


Figure 2.4 Temperature ranges for marine plastic-degrading bacteria across different phyla.

2.3.1 Proteobacteria

As one of the largest bacterial phyla, Proteobacteria encompasses a variety of species with notable plastic-degrading capabilities in marine ecosystems due to their metabolic diversity and adaptability to various environmental conditions. A prominent example from the Betaproteobacteria class is *Ideonella sakaiensis,* primarily recognized for its ability to degrade PET at 30°C (Denaro et al., 2020).

Among the Gammaproteobacteria, several marine-adapted species stand out for their plastic-degrading abilities. For instance, *Alcanivorax* species, a vital member of the hydrocarbonoclastic clade, have been shown to contribute significantly to the degradation of PE (Delacuvellerie et al., 2019), polyhydroxybutyrate (PHB) (Cao, Zhang, et al., 2022) and PS (Lv et al., 2024) under moderate temperatures. Notably, it was reported that higher ambient temperatures can enhance the release of fragments and degradation by-products during the breakdown of PE, PLA, and PHAs by *Alcanivorax* (Y. Zhang et al., 2024). Studies on oil-degrading bacteria have also isolated *Alcanivorax* species from cold marine environments (Cai et al., 2014). Its cold adaptation aids plastic degradation; for example, PP demonstrated more efficient breakdown at 10°C than at 20°C in mesopelagic environments (Koike et al., 2023).

Another member of the Gammaproteobacteria, *Pseudomonas*, can survive at temperatures from 4°C to 42°C (LaBauve & Wargo, 2012). It has been shown to degrade various plastics in marine environments, such as polycaprolactone (PCL) in coastal waters (Suzuki et al., 2018; Wilkes & Aristilde, 2017). Its metabolic versatility, particularly in

degrading PET, has been observed under deep-sea conditions with high pressure and low temperatures (R. Liu et al., 2024). Moreover, *Pseudomonas aeruginosa* displayed the highest cell abundance on PE and degradation activity at 23°C than 44°C (Mouafo Tamnou et al., 2021). Mixed cultures of *Pseudomonas aeruginosa* and *Brevibacterium sp.* degraded LDPE, with weight loss of 5.22% after 30 days at 25°C and decreased at higher temperatures, with 4.14% loss at 30°C and only 2.24% at 35°C (Dwicania et al., 2019). These findings indicate that *Pseudomonas* species may achieve optimal plastic degradation at moderate temperatures around 25°C, with their activity declining as temperatures increase.

The genus *Vibrio* demonstrates varied responses to temperature, with some species thriving between 30 and 37°C (Fig. 2.4), while others exhibit more complex or even inverse reactions (Sheikh et al., 2022). Notable, Studies reveal that *Vibrio* species form more substantial biofilm biomass at 25°C than at higher temperatures on LDPE, PP, and PS (Leighton et al., 2023). Besides, biofilm formation on plastics enhances plastics degradation and increases their dispersal across marine ecosystems. Given the pathogenic nature of many *Vibrio* species and their ability to travel on MPs, they pose ecological and public health risks (Zhai et al., 2023). As global ocean temperatures rise, *Vibrio* may present increased challenges, with warmer conditions potentially amplifying both plastic degradation and the spread of pathogenic strains (Sheikh et al., 2022).

Marinobacter species, belonging to the Alteromonadales order, demonstrate strong capabilities in degrading PE across a wide temperature range (4 to 30°C) in marine environments (Branchu et al., 2017). Additionally, Alteromonadales are identified as

primary degraders of PHAs in biofilms on plastic films submerged in seawater. Studies show that increasing seawater temperatures from 11°C to 20°C correlates with enhanced PHA degradation (Table 2.1) (Morohoshi et al., 2018).

In cold environments, psychrophilic bacteria like *Psychrobacter sp. NJ228*, isolated from Antarctic sea ice, plays a crucial role in marine plastic biodegradation by producing cold-adapted enzymes such as laccase. This enzyme shows optimal activity in temperatures between 10 and 20°C while still retaining functionality at 0°C (Fig. 2.4) (A. Zhang et al., 2022). *Psychrobacter* and other psychrophilic bacteria thrive in cold conditions by modifying the composition of their cell membranes. Specifically, they increase the proportion of unsaturated and short-chain fatty acids, which helps maintain membrane fluidity at low temperatures. This adaptation preserves cell integrity and allows efficient nutrient transport and enzyme activity (Nogi, 2011). Moreover, psychrophilic bacteria also produce cold-shock proteins (Csp) to stabilize RNA structures at low temperatures and ensure stable protein synthesis and enzyme production (Poli et al., 2017). Thus, maintaining membrane fluidity and the production of Csp is crucial for sustaining plastic biodegradation by psychrophilic bacteria in cold marine environments.

2.3.2 Actinobacteria

Actinobacteria, a prominent phylum recognized for their role in plastic biodegradation, demonstrate a wide range of temperature adaptability. These bacteria are also renowned for their ability to break down petroleum hydrocarbons alongside plastics (Rathore et al., 2021). In addition, marine-derived Actinobacteria not only biodegrade conventional

plastics like LDPE and PS but can also utilize these plastics as a carbon source to produce biodegradable PHA bioplastics (Oliveira et al., 2022). This adaptability, particularly under mesophilic conditions, underscores the ecological versatility of Actinobacteria in facilitating marine plastic biodegradation.

Within the family of *Nocardiaceae*, the genus *Rhodococcus* stands out due to its remarkable temperature adaptability. It can grow across a wide temperature range, from -5 to 50°C, optimally at 37°C (Fig. 2.4) (Xiang et al., 2022). Besides, the ability of *Rhodococcus* to degrade long-chain alkanes and other recalcitrant compounds further reinforces their significance in regulating marine biogeochemical cycles (Pátek et al., 2021). In cold environments, *Rhodococcus* species continue to play a vital role. *Rhodococcus sp. JG3*, isolated from the Antarctic Dry Valley permafrost, has been observed to grow at temperatures as low as -5°C. This cold tolerance is primarily attributed to gene expression adaptations that enhance energy production and redox homeostasis under cold stress (Pátek et al., 2021). Similarly, *Rhodococcus fascians* from Antarctica has been reported to produce bioemulsifiers that enhance the biodegradation of hydrocarbons like hexadecane and biphenyl between 4 and 35°C (Rathore et al., 2021).

Nocardiopsis, another critical genus from *Nocardiaceae*, has been identified for its efficiency in plastic degradation under mesophilic conditions. For instance, *Nocardiopsis* isolated from marine sediments, was found to excrete extracellular PHB depolymerase and grew efficiently on PHB and its copolymers as the sole carbon source at 30°C (Ghanem et al., 2005). Additionally, it demonstrated significant degradation of poly(3-hydroxybutyrate) (P(3HB)) films and formed biofilms on P(3HB) and PP surfaces, with

optimal growth occurring at 30°C, although it did not survive at temperatures above 50°C (Table 2.1) (Suzuki, Tachibana, Takizawa, et al., 2021).

2.3.3 Firmicutes

Within the phylum Firmicutes, numerous species, particularly those in the *Bacillaceae* family, are known for their plastic-degrading abilities at 25 to 37°C (Fig. 2.4). The genus *Bacillus* is widely studied. For instance, *Bacillus infantis* PD3 achieved a remarkable 98.71% degradation of PHB film within five days at 37°C in a mineral medium (Jeon et al., 2023). Similarly, *Bacillus sp.* JY14, a thermotolerant strain, can degrade PHB film from 20°C to 42°C, with the highest efficiency observed at 30°C (Table 2.1) (Cho et al., 2021). This adaptability underscores its potential for plastic degradation in environments with fluctuating temperatures. Alongside *Bacillus*, *Terribacillus* shows notable plastic degradation abilities, demonstrating activity on poly(butylene adipate-co-terephthalate) (PBAT) and PCL and degrading polybutylene succinate (PBS) at 42°C. The clear zone expansion was slower at 42°C than at 30°C and 37°C, suggesting greater efficiency at moderate temperatures (S. H. Kim et al., 2022).

2.3.4 Cyanobacteria

Cyanobacteria, a diverse group of photosynthetic bacteria, play an increasingly recognized role in marine plastic degradation. *Oscillatoria subbrevis* is particularly effective at degrading plastics from 25 to 30°C (Sarmah & Rout, 2018). Additionally, filamentous cyanobacteria such as *Leptolyngbya*, *Limnothrix*, *Phormidium*, *Prochlorothrix*, and *Rivularia* colonize plastic debris in regions like the Great Pacific

Garbage Patch and the North Atlantic (Rogers et al., 2020). Seasonal shifts affect biofilm composition and activity; for instance, it is reported that *Pseudophormidium* tends to dominate biofilms on PS particles in warmer months, while *Synechococcus* becomes more prevalent in cooler conditions, influencing carbon and nutrient cycling in marine environments (Du et al., 2022). With rising ocean temperatures, Cyanobacteria's role in plastic degradation and nutrient regulation is likely to expand, potentially impacting marine food webs and biodiversity.

Taking all this together, temperature is a crucial factor in microbial succession during marine plastic degradation, influencing which phyla dominate and how effectively they degrade plastics. Proteobacteria initiate the breakdown at moderate temperatures, while Actinobacteria, Firmicutes, and Cyanobacteria continue to serve in subsequent stages, adapting to varied temperatures and further facilitating degradation. In extreme conditions, psychrophilic and thermophilic bacteria drive degradation within cold and warm environments, respectively. Seasonal shifts also alter the biofilm composition of Cyanobacteria, with microbes like *Pseudophormidium* and *Synechococcus* affecting nutrient cycles. Rising global temperatures may accelerate some degradation rates but also slow degradation for bacteria with moderate-temperature optima, as exceeding these ranges can reduce their efficiency. Additionally, warmer conditions heighten ecological risks by facilitating the spread of pathogens colonized on MPs across marine ecosystems. Understanding these temperature-driven microbial dynamics is essential for predicting plastic degradation pathways and environmental impacts.

2.4 Enzyme-based Plastic Depolymerization under Different Temperatures

Enzymes catalyze the breakdown of complex polymer chains into smaller fragments to facilitate microbial degradation and assimilation. The degradation rate is generally influenced by temperature and varies significantly with the plastic types (Viel et al., 2023). Plastics are broadly classified into two categories: hydrolyzable and non-hydrolyzable plastics. Each one exhibits distinct degradation behaviors. Hydrolyzable plastics, including PET, PCL, PLA, and PHAs, are more prone to enzymatic degradation due to the presence of cleavable ester or amide bonds. In contrast, non-hydrolyzable plastics like PE, PP, and PS resist enzymatic attacks due to the absence of such bonds. Here, we selected representative plastics to summarize the key enzymes involved in their depolymerization (Table 2.2) and the resulting morphological changes under different temperatures (Fig. 2.5).

While hydrolyzable plastics are relatively well-studied, the degradation of non-hydrolyzable plastics remains poorly understood, particularly in marine environments. The recalcitrant nature of PE and PS, coupled with their widespread use, poses significant challenges for biodegradation. Current research on these plastics is limited by a lack of long-term studies and a limited understanding of the enzymatic mechanisms involved. Addressing these gaps is critical for developing effective strategies to mitigate plastic pollution.

Table 2.2 Key enzymes in marine plastic degradation and their temperature ranges.

Enzyme	**Plastic Type**	**Observed Degradation Temperature (°C)**	**Role in Degradation**
Cutinase	PET, PCL	30-70	Hydrolyzes ester bonds in PET, breaking it down into terephthalic acid and ethylene glycol.
PHA Depolymerase	PHAs	4-40	Depolymerizes PHAs by breaking ester bonds, producing hydroxyalkanoic acids.
Proteinase K	PLA	40-60	Hydrolyzes ester linkages in PLA, breaking it down into lactic acid monomers.
Lipase	PCL, PHAs, PLA	25-37	Breaks down PCL by hydrolyzing ester bonds, particularly at interfacial areas.
Laccase	PS, PE, PLA	30-50	Catalyzes the oxidation of the polymer backbone, facilitating microbial attack.
Alkane Hydroxylase	PP, PE	25-40	Initiates oxidation of hydrocarbons, aiding in the breakdown of PP and PE.
Monooxygenase	PE, PP, PS	30-35	Generates ROS, oxidizing C-C bonds for further breakdown
Ring-Hydroxylating Dioxygenase	PS	25-35	Cleaves the aromatic ring structure in PS, leading to partial degradation.

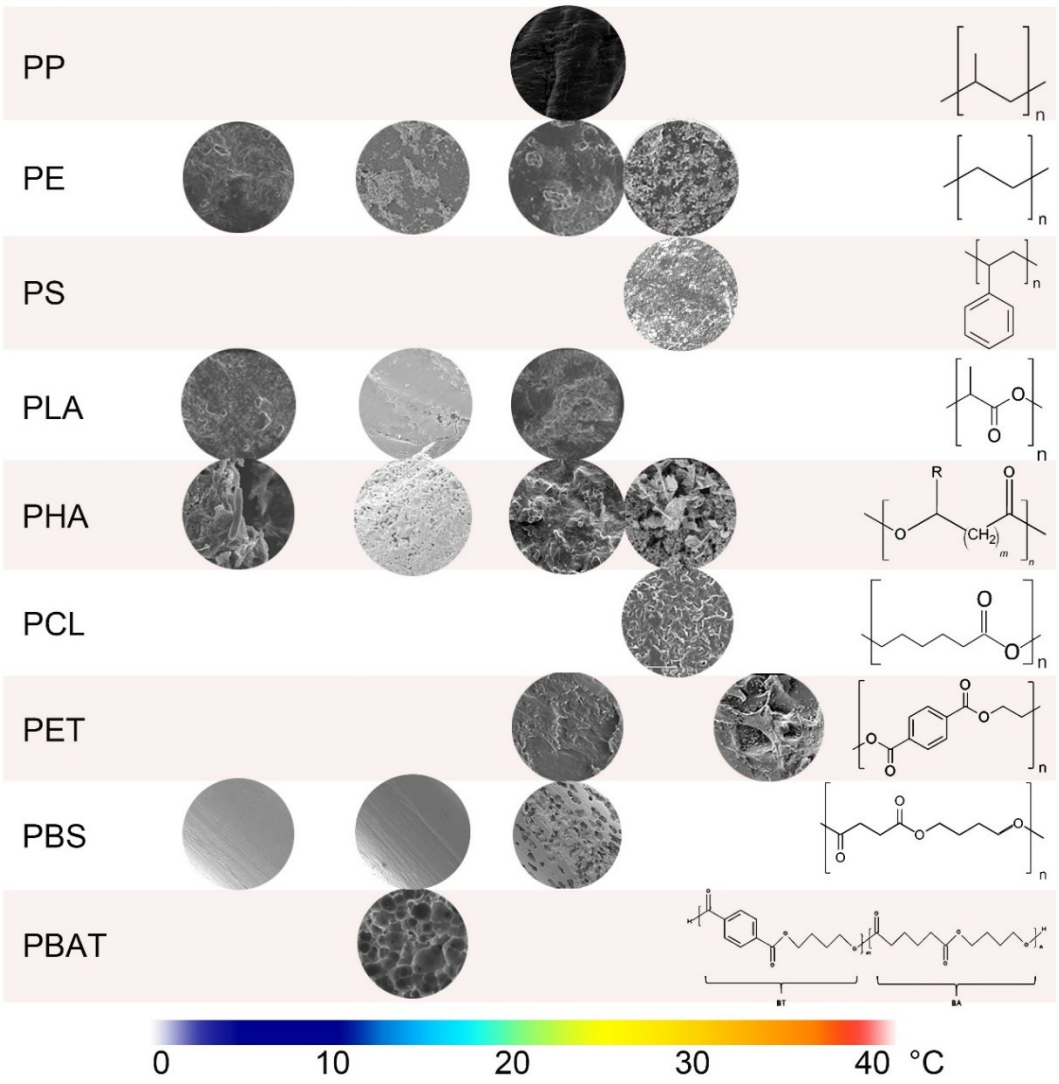


Figure 2.5 Morphological changes of various plastics under different temperature ranges.

(SEM images of PP: Wang et al., 2018; PE: Dimassi et al., 2024; Eich et al., 2015; Guo et al., 2024; PS: Syranidou et al., 2017; PLA: Guo et al., 2024; Zhang et al., 2024; PHA: Cho et al., 2021; Kato et al., 2019; Wang et al., 2018; Y. Zhang et al., 2024; PCL: Suzuki et al., 2018; PET: Gao & Sun, 2021; Sarkhel et al., 2020; PBS: Le Gué et al., 2023; PBAT: Shin et al., 2024)

2.4.1 Polyethylene terephthalate (PET)

PET is among the most widely produced thermoplastics, extensively used in packaging and textile production due to its durability and resistance to degradation. Crystallization initiates at 70°C, theoretically providing optimal conditions for degradation by balancing chain mobility and enhanced enzyme activity (Danso et al., 2019). However, achieving these conditions in marine environments is challenging, as the limited prevalence of plastic-degrading enzymes and pelagic conditions suggests a lower degradation potential than in terrestrial ecosystems (Danso et al., 2018). In colder regions like the Arctic, where temperatures near 0.5°C, PET degradation proceeds extremely slowly, requiring approximately 162 years for 50% depolymerization. Conversely, in tropical regions at around 35°C, PET achieves 50% degradation in 4.5 years (Stanica-Ezeanu & Matei, 2021).

PET depolymerization typically undergoes three stages: First, PET is broken down into two primary intermediates by PETase: mono(2-hydroxyethyl) terephthalate (MHET) and bis(2-hydroxyethyl) terephthalate (BHET). These intermediates are then further degraded into terephthalic acid (TPA) and ethylene glycol (EG) through the action of enzymes such as MHETase and cutinase, which cleave the ester bonds (Tournier et al., 2023). Finally, mineralization occurs, converting intermediates into non-toxic by-products, though this process may be inhibited in marine environments due to factors such as salinity and low temperatures (Tournier et al., 2023).

One notable enzyme in PET depolymerization is *Is*PETase, secreted by *Ideonella sakaiensis*. It exhibits a high degradation capacity at moderate temperatures (~30°C). It aligns with scanning electron microscope (SEM) observations, which show no signs of degradation on PET films at lower temperatures; however, at 36°C, the PET surface loses smoothness, with visible cracks observed (Fig. 2.5) (Sarkhel et al., 2020). Furthermore, MHETase exhibits optimal activity at moderate temperatures (30 to 35°C) when transforming the PET oligomer to TPA and EG (Tournier et al., 2023). Besides, carboxylic ester hydrolases from *Pseudomonas aestusnigri*, have been identified to hydrolyze PET films at around 30°C (Bollinger et al., 2020). However, *Is*PETase loses functionality at higher temperatures due to thermal denaturation (S. Yoshida et al., 2016). To overcome this limitation, Son et al. (2019) employed a rational protein engineering strategy to enhance its thermal stability, thus improving the complete biodegradation of PET under broader temperature ranges.

2.4.2 Polycaprolactone (PCL)

PCL is a biodegradable plastic with a low melting point (Tm = 58 to 63°C), a low glass transition temperature (Tg ≈ -65°C), and high crystallinity, leading to its widespread use in biomedical applications. Optimal degradation of PCL typically occurs between 30 and 60°C (Marten et al., 2003). Compared to other bioplastics such as PLA, PBS, and poly(butylene succinate-co-butylene adipate) (PBSA), PCL demonstrates faster enzymatic degradation rates in marine environments due to its lower Tm and Tg (Table 2.1) (Nakayama et al., 2019). PCL has also been used as a model substrate to replace PET due to its less complex structure. Suzuki et al. (2018) observed that the pristine PCL film

was very smooth and became much rougher at 30°C (Fig. 2.5). Several enzymes, including esterases, lipases, and carboxylesterases, are involved in PCL hydrolysis by catalyzing the breakdown of the ester bonds. For instance, lipases from mesophilic organisms such as *Pseudomonas aeruginosa* typically show optimal activity between 30 and 40°C, with peak catalytic efficiency at 37°C (Jaeger & Eggert, 2002). Additionally, new strategies, such as incorporating marine-derived proteins into PCL biocomposites, have shown higher degradation rates at 20°C over 56 days, as protein fillers serve as nutrients for microorganisms (K. Yoshida et al., 2024).

2.4.3 Polylactic acid (PLA)

PLA, one of the most widely used biodegradable plastics, is popular in packaging and medical devices. Its degradation is primarily facilitated by enzymes such as Proteinase K, lipases, esterases, and cutinases, which hydrolyze ester bonds (Shalem et al., 2024). At higher temperatures (40 to 60°C), enzyme activity increases, accelerating hydrolysis due to improved enzyme-substrate interactions and greater flexibility in PLA chains. While PLA degrades rapidly under composting conditions at 60°C, it remains recalcitrant in cooler marine environments. For example, Bagheri et al. (2017) observed no significant mass loss of PLA after 400 days in seawater at 25°C. Chamas et al. (2020) estimated that, although PLA degrades about 20 times faster than HDPE on land, it also has a similarly limited degradation in marine environments. Other studies also found no significant morphological changes on PLA surfaces between 4 and 25°C after two months (Fig. 2.5) (Guo et al., 2024; Y. Zhang et al., 2024). Royer et al. (2023) reported no signs of PLA

degradation after prolonged 428 days in the colder waters off California (13 to 23°C), indicating slower depolymerization in cold marine environments.

Notably, Cheung & Not (2024) observed a different outcome, with PLA samples disintegrating within one month at an average temperature of 23°C in Hong Kong's coastal waters, outperforming LDPE (Table 2.1). They hypothesized that the warmer and more stable seawater in Asian subtropical regions may create a more favorable environment for PLA hydrolysis compared to European temperate or Mediterranean climates.

Moreover, Proteinase K emerges as a critical enzyme for enhancing hydrolysis. It is particularly effective under optimal conditions around 37°C (Shalem et al., 2024), and its efficiency and thermal stability in PLA hydrolysis have been documented at temperatures near 50°C (Q. Huang et al., 2020; Tokiwa & Calabia, 2006). Embedding Proteinase K in PLA matrices has been proven to significantly accelerate degradation, achieving a 78% weight loss of PLLA films within 96 hours (D. Huang et al., 2020). He et al. (2024) also demonstrated substantial weight loss in PLA copolymers exposed to Proteinase K, underscoring the enzyme's potential to enhance PLA biodegradation in marine environments.

2.4.4 Polyhydroxyalkanoates (PHAs)

Bioplastic PHAs synthesized naturally by organisms are considered superior to other bioplastics due to their complete biodegradability in marine ecosystems (Zhou et al., 2023). PHAs exhibit significant biodegradability across a wide temperature range in

various marine environments, including coastal, shallow-water, and deep-sea regions (Suzuki, Tachibana, & Kasuya, 2021). The average degradation rate of PHAs in marine settings is estimated to be between 0.04 and 0.09 $mg \cdot day^{-1} \cdot cm^{-2}$, suggesting that an 800 μm thick PHA-based water bottle could take approximately 1.5 to 3.5 years to be fully biodegraded (Dilkes-Hoffman et al., 2019).

Early stages of PHAs degradation involve enzymatic breakdown into smaller oligomers, primarily mediated by enzymes such as PHA depolymerases (PhaZ), carboxylesterases, and lipases (Cao et al., 2024). The enzymatic systems differ between short-chain-length PHAs (scl-PHAs) and medium-chain-length PHAs (mcl-PHAs). Scl-PHA depolymerases operate optimally across a broad temperature range of 30 to 90°C, while mcl-PHA depolymerases are most effective between 35 and 70°C (Urbanek et al., 2020). P(3HB), the most common PHA produced by microorganisms, exhibits optimal degradation between 37 and 55°C (Suzuki, Tachibana, & Kasuya, 2021).

The enzyme efficiency of PHA degradation generally increases with higher temperatures as enzymes become more flexible and effectively cleave ester bonds in PHA polymers. Several studies found that abundant bacterial cells were observed and attached to plastic surfaces. The surface became porous, and pore size increased along with incubation time and temperatures (Fig. 2.5). For instance, Deroiné et al. (2014) found that polyhydroxybutyrate-co-hydroxyvalerate (PHBV) degraded significantly faster in seawater at 40°C compared to 4 or 25°C. In contrast, it is reported that PHA degradation slows considerably in colder marine environments (below 15°C) as reduced enzymatic

activity and lower microbial metabolism inhibit the breakdown process (Table 2.1) (Kato et al., 2019; Sekiguchi et al., 2011).

A distinctive feature of PHA is its release of by-products such as dissolved organic carbon (DOC) and MPs, as demonstrated in short-term microcosm studies. Temperature notably influences the extent of this release. Studies indicate that PHAs become increasingly porous (Fig. 2.5), resulting in a more significant release of MP fragments and DOC than PLA or PE at temperatures of 4, 15, and 22°C after 60 days; higher temperatures further accelerate this release (Y. Zhang et al., 2024). PHAs also release oxidized EPS-related fragments within a few days (Cao et al., 2024). These findings highlight temperature's critical role in accelerating PHA degradation rates and influencing the quantity and size of MP particles entering the marine environment.

2.4.5 Polyethylene (PE) and Polypropylene (PP)

PE and PP are among the most widely produced plastics globally, highly valued for their durability and chemical stability. This stability primarily results from their non-hydrolyzable carbon-carbon (C-C) backbone and the absence of functional groups, such as ester bonds, which make them inherently resistant to microbial degradation and hydrolysis (Gewert et al., 2015). For instance, the PP surface remained unchanged after incubation of 195 days at 24°C (Fig. 2.5). In marine environments, the biodegradation of PE and PP depends on the formation of reactive oxygen species (ROS) generated by enzymes such as catalase-peroxidase, catalase, superoxide dismutase, cytochromes P450, and alkane monooxygenase (AlkB) (Y. Zhang et al., 2022). These ROS facilitate initial

oxidative changes within the polymer structure, creating weak points in the C-C backbone by forming hydroxyl and carbonyl groups to enhance susceptibility to microbial degradation (Fig. 2.6) (Wright et al., 2020). These enzymes produce modified fragments that are smaller, more accessible, and more readily biodegradable by microbial consortia (Andrady et al., 2022). A representative marine species is *Alcanivorax borkumensis*: they can utilize ROS to oxidize the PE and PP's surface and improve access for subsequent enzymatic assimilation (Delacuvellerie et al., 2019; Koike et al., 2023). Typically, elevated temperatures can accelerate the effectiveness of these oxidative and microbial processes in marine environments (Shyam & Sarma, 2023). For example, cracks and pitting appeared on the PE surface at lower temperatures. More significant morphological changes, such as small holes and grooves, appeared under higher temperatures (30°C) (Fig. 2.5).

However, the overall temperature-mediated degradation rate of PE and PP in marine environments remains limited, as most studies are conducted over relatively short observation periods, which overlooks the long-term temperature effects. Further research is needed to establish a more comprehensive understanding of how temperature variations over extended periods impact the biodegradation pathways of these recalcitrant plastics in marine ecosystems. Additionally, the enzymatic mechanisms involved in the degradation of PE and PP are not fully understood, particularly in cold marine environments. Future studies should focus on identifying and characterizing novel enzymes capable of breaking down these plastics under a wider range of temperatures.

2.4.6 Polystyrene (PS)

PS is another commonly produced plastic that poses significant challenges for degradation due to its aromatic ring structure and C-C backbone. Similar to PE and PP, ROS initiates the enzymatic degradation of PS. However, this process requires substantial energy input and progresses slowly, even under favorable conditions. Enzymes such as cytochrome P450s, alkane hydroxylases, and monooxygenases contribute to the degradation of PS's C-C backbone (Hou & Majumder, 2021). Moreover, the aromatic ring in PS makes enzymatic depolymerization particularly challenging compared to PE and PP. Only limited ring-hydroxylating dioxygenases in certain marine bacteria are reported can cleave PS side chains and oxidize the aromatic ring, breaking it into smaller, more manageable compounds (Gallego et al., 2014; Hou & Majumder, 2021).

Based on current knowledge, predicting an optimal temperature for the enzymatic degradation of PS remains challenging due to the limited studies on this topic and the incomplete understanding of the biochemical mechanisms associated with specific enzymes. Only a few studies have shown the potential of PS marine biodegradation. Syranidou et al. (2017) utilized marine consortia to degrade PS films at 28°C and found the signs of biodegradation, such as fissures, cracks, and roughness on the PS film surface after 6 months (Fig. 2.5). In addition, indigenous marine bacteria isolated from mangrove PS pollutants show a 2.66-7.73% degradation rate at 28°C over one month (R. Liu et al., 2023). Cooler marine temperatures (15 to 25°C) may limit microbial activity and enzyme efficiency of PS biodegradation (Zhai et al., 2023; Y.-B. Kim et al., 2024).

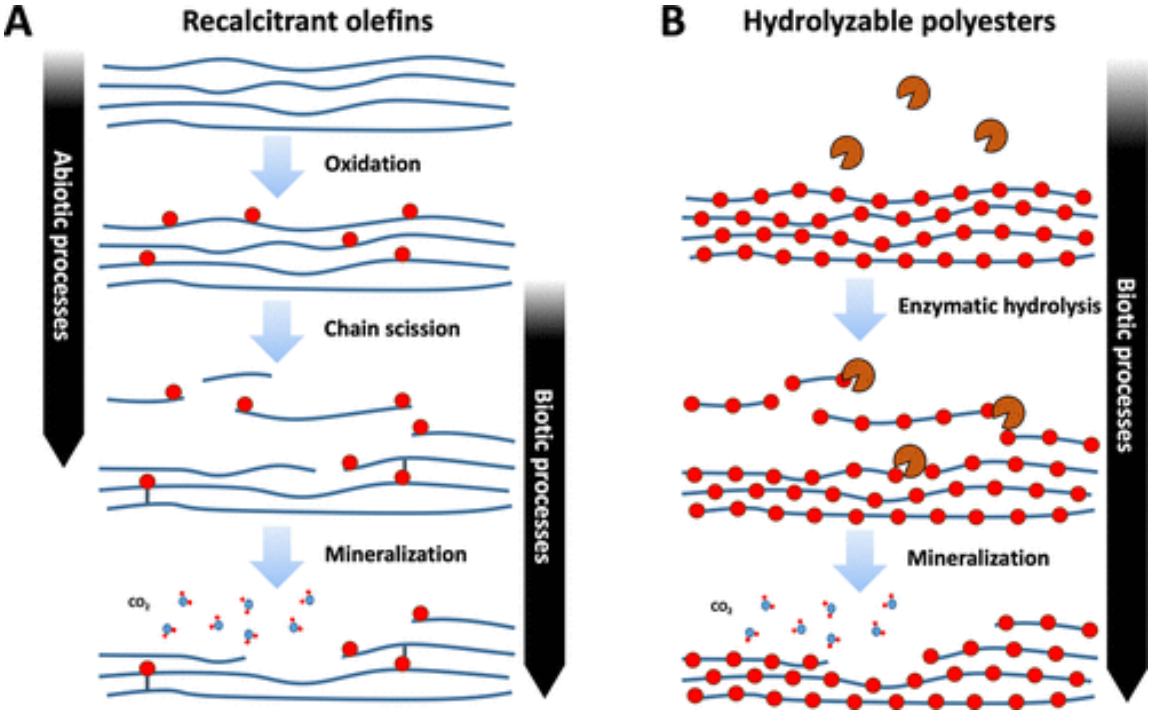


Figure 2.6 Steps required for recalcitrant (a) and hydrolyzable polymer degradation (b). Those steps that are most likely carried out by abiotic and biotic processes are highlighted. Blue lines represent polymeric chains and red circles represent oxygen groups. Hydrolytic enzymes are represented in brown. Adapted from Wright et al. (2020).

In brief, the degradation of hydrolyzable plastics primarily occurs through hydrolysis at moderate temperatures, facilitated by enzymes such as cutinases, lipases, and depolymerases (Fig. 2.6). This process results in notable morphological changes, including surface erosion and increased porosity, particularly in PHAs. In contrast, non-hydrolyzable plastics with a resilient C-C backbone rely on enzymes such as monooxygenases and peroxidases to generate ROS that initiate oxidative modifications, breaking the polymer into monomers for further enzymatic breakdown (Fig. 2.6). These polymers generally retain a smoother surface after degradation. Due to their slow biodegradation rates and limited biodegradability, the effects of temperature on this process are not readily apparent. Moreover, elevated temperatures typically accelerate degradation rates and increase the release of MPs and DOC from PHAs. Although lower temperatures may slow degradation, it still occurs, leaving persistent fragments in the marine environment. Current studies on the long-term effects of temperature on marine plastic biodegradation remain limited, which restricts a comprehensive understanding of how temperature variations may impact degradation rates.

2.5 Summary

This study underscores the critical role of temperature in marine plastic biodegradation, impacting biofilm formation, microbial succession, and degradation pathways. Findings reveal that higher temperatures generally stimulate early biofilm formation, enhancing microbial colonization. In specific cases, lower temperatures promote EPS production to stabilize biofilms for cold-tolerant bacteria. Hydrolyzable plastics, such as PHAs, degrade most effectively at moderate temperatures due to optimal enzymatic activity, resulting in significant morphological alteration. On the other hand, non-hydrolyzable plastics depend on oxidative modifications initiated by ROS to enhance their susceptibility to microbial degradation. Despite these insights, several research gaps persist. Current experiments are often limited to short observation periods, restricting understanding of long-term degradation dynamics and morphological changes. Field studies also struggle to isolate the sole effects of temperature on degradation, as real-world conditions involve multiple interacting environmental factors. Additionally, while many bacteria exhibit plastic-degrading potential, optimal activity is typically observed at temperatures between 25 and 30°C, with specific resilient strains required to thrive at lower temperatures. To address these gaps, future research should adopt a more holistic approach that considers the interplay between microbial communities, enzyme activity, and environmental conditions. Comprehensive studies that integrate these factors will provide a deeper understanding of the mechanisms driving plastic biodegradation and enable the development of more effective waste management strategies.

Expanding research on temperature effects and incorporating specialized microbial communities tailored to specific plastic types could significantly enhance waste management strategies, enabling more efficient and sustainable biodegradation processes in diverse marine ecosystems. For example, studies should explore how microbial consortia, interact with plastics under varying temperatures. This approach would better reflect the complexity of natural marine environments, where multiple microbial species coexist and collaborate in degradation processes.

The future of marine plastic biodegradation hinges on understanding and mitigating temperature-related impacts, especially as climate change intensifies ocean warming, seasonal variability, and extreme environmental events. Integrating microbial genetic databases and machine learning holds immense promise for optimizing plastic degradation across diverse thermal conditions. Microbial databases can identify key plastic-degrading enzymes and metabolic pathways, revealing adaptive functions that enable microorganisms to thrive in temperature extremes, such as cold polar waters or increasingly warm tropical seas. These insights can guide the design of microbial consortia tailored to degradation processes that remain efficient under varying thermal regimes.

Machine learning can complement these efforts by predicting how enzymes interact with their environments and optimizing microbial assemblages to enhance biodegradation under temperature fluctuations. By modeling enzyme stability and activity, machine learning can inform enzyme selection or engineering for superior performance in both cold and warm marine conditions. Tools like AlphaFold can further refine enzyme design,

enabling the development of thermally resilient enzymes suited for degrading specific plastic types in distinct marine environments. For instance, machine learning algorithms could be used to predict the optimal temperature ranges for specific enzymes and identify potential modifications to enhance their stability and activity under extreme conditions.

As climate change drives shifts in ocean temperatures and disrupts ecosystems, these advancements will be critical for developing resilient biodegradation strategies. Warmer oceans may enhance enzymatic activity and microbial growth in some regions, while polar and deep-sea environments demand innovations to overcome the constraints of low temperatures. By addressing these challenges, these temperature-focused solutions can contribute to more effective plastic pollution management in a rapidly changing climate, safeguarding marine ecosystems from the growing threat of plastic waste. Ultimately, a multidisciplinary approach that combines microbiology, enzymology, environmental science, and computational modeling will be essential for advancing our understanding of plastic biodegradation and developing sustainable solutions to mitigate plastic pollution in marine environments.

CHAPTER 3 EXPERIMENTAL ANALYSIS OF MARINE PLASTIC BIODEGRADATION UNDER VARIOUS TEMPERATURES[2]

[2] The chapter is based on a published article: Zhang Y, Cao Y, Chen B, Dong G, Zhao Y, Zhang B (2024) *Marine biodegradation of plastic films by Alcanivorax under various ambient temperatures: Bacterial enrichment, morphology alteration, and release of degradation products*. *Science of the Total Environment*, 170527. https://doi.org/10.1016/j.scitotenv.2024.170527. Contributions**:** Yuanmei Zhang – methodology, experimentation, data analysis, manuscript drafting and editing; Yiqi Cao – conceptualization, manuscript editing, review; Bing Chen – conceptualization, supervision; Guihua Dong – review and editing; Yuanyuan Zhao – review and editing; Baiyu Zhang – conceptualization, supervision, review and editing.

3.1 Background

Global plastic production reached about 390.7 million metric tons annually in 2021, with over half of all plastics ending as waste (Plastics Europe (PEMRG), 2022). An estimated 9.5 million metric tons of plastics, including macro (>5mm) and micro- (<5mm) plastics, enter the ocean annually (Lau et al., 2020). These conventional (or petroleum-based) plastics will take millions of years to decompose and have caused detrimental environmental issues. Replacing petroleum-based plastics with bioplastics (including biodegradable plastics) has been recognized as a promising solution (Nanda et al., 2022), and the global production capacity of bioplastics will be increased from around 2.46 million tons in 2022 to approximately 6.95 million tons in 2027 (European Bioplastics, 2023) . All these plastic wastes will experience diverse physical, chemical, and biological processes in marine environments. Therein, marine biodegradation is an indispensable approach to combat the challenges of plastic wastes (Wayman & Niemann, 2021).

Marine biodegradation is intricately intertwined with microorganisms, plastic properties, and surrounding environmental conditions. Marine microbes, especially plastic-degrading bacteria (e.g., *Alcanivorax, Pseudomonas, Vibrio*, etc.), have been reported to erode on the surface of plastics and utilize the plastic-associated carbons (Roager & Sonnenschein, 2019). These microorganisms will colonize plastics' surface and alter associated physical and chemical properties. Meanwhile, the process will result in the release of fragments like MPs and plastic-related chemicals like DOC (Ren et al., 2023). MPs could absorb other contaminants (e.g., polycyclic aromatic hydrocarbons, heavy metals, and antibiotics) for increased environmental toxicity or influence microbial behaviors for

modified environmental processes (Amelia et al., 2021). For instance, MPs could alter the microbial population and affect aquatic food web dynamics and biogeochemical processes in the aquatic environment (Yang et al., 2020); MPs would also aggravate other environmental stressors, such as water acidification and global warming (Bertucci & Bellas, 2021; Chang et al., 2022). Besides, it is estimated that up to 23,600 metric tons of DOC are leaching from plastics annually (Romera-Castillo et al., 2018), and approximately 60% of the released DOC is bioavailable for continuously promoting microbial activity in the ocean (Eronen-Rasimus et al., 2022). Moreover, environmental conditions will affect the biodegradation process significantly. The marine environment is typically considered oligotrophic, and research has demonstrated that the degradation rates of petroleum-based polymers are slower in the ocean than in landfills (Chamas et al., 2020). Since the ocean acts as the ultimate pool receiving massive and diverse plastic wastes, these concerns have raised extensive research on a systematic understanding of the marine biodegradation processes of various plastics.

Given that the global ocean has broad and fluctuating temperatures as the seawater is distributed from pole to equator with various depths and potential climate change impacts, the temperature is a critical environmental parameter influencing the marine biodegradation process (Viera et al., 2021). It will affect the physiological activity of microorganisms and the rate of enzymatic reactions in cells during microbial degradation (Sooriyakumar et al., 2022). Elevated temperatures can enhance microbial activity and promote the assimilation of plastic monomers, while lower temperatures may lead to longer persistence of MPs and pose greater risks to ecosystems (X. F. Wei et al., 2021;

Zadjelovic et al., 2022). However, most studies are conducted at optimal room temperature, while plastic marine degradation is constantly exposed to a broad range of natural temperatures, particularly to sub-optimal conditions like low temperatures (Matjašič et al., 2021; X. F. Wei et al., 2021). For instance, MPs have been detected in near-surface seawater across the European and North American Arctic, including the North Pole (Ross et al., 2021). It is necessary to answer whether plastics can be biodegraded in cold regions and to elucidate the dynamic bioprocess of the whole lifecycle of plastics' marine biodegradation under different environmental temperatures (Urbanek et al., 2018).

Taking all these factors together, in this study, we investigated marine biodegradation of various plastics under different ambient temperatures (i.e., 4, 15, and 22°C). We chose the commercially used petroleum-based LDPE, bio-based PLA, and PHAs plastic films as target plastic wastes. The marine psychrotolerant bacteria *Alcanivorax* species isolated from the North Atlantic Ocean was chosen as the model species to simulate the accelerated biodegradation process (Cao, Zhang, et al., 2022). *Alcanivorax*, a globally distributed hydrocarbon-degrading bacteria in the ocean, along with other hydrocarbon-degrading bacteria found within the plastisphere, is associated with the degradation of plastics (Denaro et al., 2020; W.-L. Li et al., 2019; Nauendorf et al., 2016; Roager & Sonnenschein, 2019; Zadjelovic et al., 2020). We systematically evaluated the marine biodegradation processes, including bacterial growth, alteration of plastic films, and the release of degradation products. We also highlighted the impacts of plastic type and

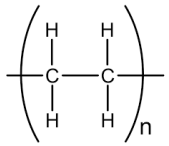
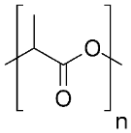
temperature on marine biodegradation and raised potential environmental concerns for marine resiliency to plastic wastes.

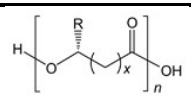
3.2 Materials and Methods

3.2.1 Materials

The petroleum-based LDPE plastic films were commercially available and collected from food container packaging. Bio-based plastic films PLA (ME33-FM-000150) and PHA (BV301025) were purchased from Sigma-Aldrich. All films were sterilized with 70% ethanol and cut into the size of 1 cm squares using a scalpel. The detailed properties of the used plastics are listed in Table 3.1.

Table 3.1 The properties of used plastics in the experiment.

Plastics	Chemical structure	Thickness	Density (g/cm^3)	Properties	Applications	Reference
LDPE		Approximately 36 µm	0.915-1.00 Average: 0.923	• Flexibility • Low crystallinity • Low permeability • Impact resistance • Cost-effective, economical, and widely recyclable	• Food-safe bagging, waterproof packaging, and other packaging materials • Extrusion coating • Wire and cable	("Goodfellow Polyhydroxybutyrate / Polyhydroxyvalerate (PHB 92/PHV 8)," 2023; "Low Density Polyethylene (LDPE)," 2022)
PLA		50 µm	1.00-2.47 Average: 1.28	• Bio-origin (made of renewable resources such as corn or sugar cane) • Can be solvent welded (such as with dichloromethane) • Low heat resistance • Comparatively low strength	• Food containers and medical devices • 3D printing feedstock • Medical implants • Compost bags, food packaging, disposable tableware, and loose-fill packaging • Disposable clothing, feminine hygiene products, and nappies	("Overview of Materials for Polylactic Acid (PLA) Biopolymer," 2023; "What is pla? (everything you need to know)," 2023)

PHA		25 µm	1.25	• Bio-origin (processing by microorganisms) • Piezoelectricity and excellent barrier properties • Poor mechanical properties • High production cost • Limited functionalities • Incompatibility with conventional thermal processing techniques	• Moulding containers such as shampoo bottles, disposable razors • Packaging film • Biomedical applications such as heart valves and other vascular applications	(“Goodfellow Polyhydroxybutyrate / Polyhydroxyvalerate (PHB 92/PHV 8),” 2023; Kovalcik et al., 2019; Z. Li et al., 2016)

3.2.2 Biodegradation experiment

The biodegradation of the plastic films was conducted in a volume of 10 mL glass tubes covered with tin foil caps. In each test tube, there was 3 mL of autoclave-sterilized marine mineral salts medium (MSM) along with a single piece of sterilized plastic film (Fig. 3.1). The MSM per liter consists of 20g NaCl, 5 g $(NH_4)_2SO_4$, 3.4 g KH_2PO_4, 4.4 g $K_2HPO_4 \cdot 3H_2O$, 1.02 g $MgSO_4 \cdot 7H_2O$, 0.00028 g $FeSO_4 \cdot 7H_2O$, and 0.5 mL trace element (0.516 g $ZnSO_4$, 0.24 g $CaCl_2$, 0.368 g $CuSO_4 \cdot 5H_2O$, 0.223 g $MnCl_2 \cdot 4H_2O$) (Cao, Kang, et al., 2022). Plastic-degrading bacteria *Alcanivorax* sp. N3-2A isolated from the Northern Atlantic Ocean was used for the marine biodegradation experiment (Cao, Zhang, et al., 2022). Briefly, the cells were enriched using Marine Broth 2216 (Difco #279110) supplemented with sodium pyruvate (1%) for 3 days. Cell culture was washed twice using MSM and adjusted to an optical density (OD) at 600 nm of 1.5. Then 100 µL of the washed *Alcanivorax* was added to each test tube. All test tubes were divided into three groups and incubated at 4, 15, and 22 °C. On days 7, 30, and 60, the plastic films and the culture medium were subjected to respective analyses.

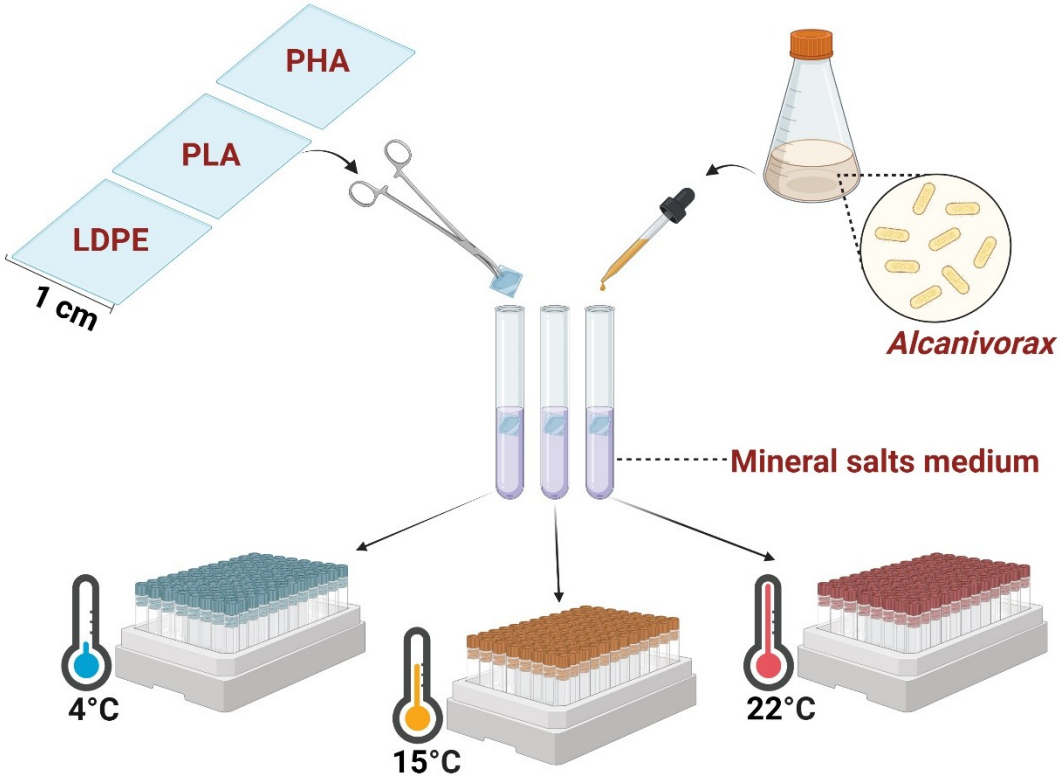
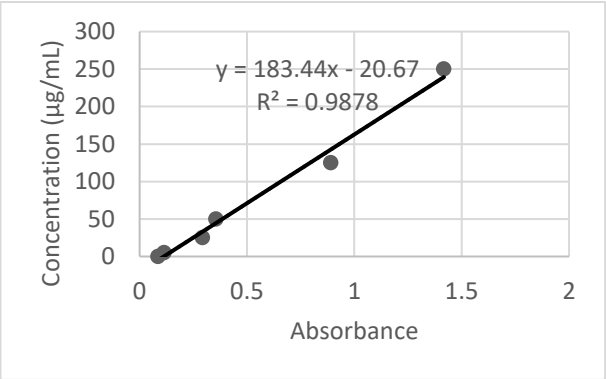


Figure 3.1 Diagram of plastics marine biodegradation experiment.

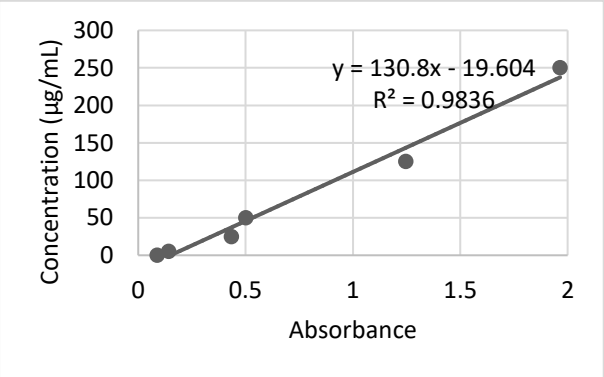
3.2.3 Characterization of bacterial growth

Bacterial abundance on plastic surfaces and in culture medium was quantified using Pierce bicinchoninic acid (BCA) total cellular protein assay (Zadjelovic et al., 2020). The entire test tube of the medium, including the plastic film, was added to a 15-ml Falcon tube, which was centrifuged at 6000 rpm for 10 min. The supernatant was removed without disturbing the cell pellet, and the cell pellet was washed with distilled water. Total cellular protein was extracted using 1 mL of 2% sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl buffer with 2% [wt/vol] SDS) followed by 60 °C incubation for 30 min (Overholt et al., 2016). Samples were sonicated for 2 min and then centrifuged at 6,000 rpm for another 10 min. Total cellular protein was measured at 562 nm absorbance following the BCA protein assay protocol (Thermal Scientific). The dilution scheme was the enhanced test tube protocol with an operating range of 5 to 250 μg/mL. The standard curves of BCA analysis on each day are shown in Fig. 3.2.

(a) Day 7



(b) Day 30



(c) Day 60

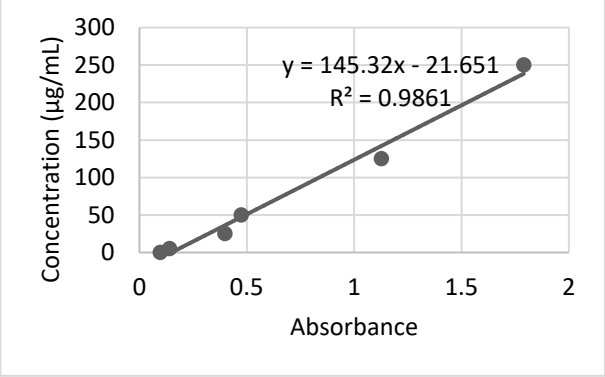


Figure 3.2 BCA standard curve used for total protein assay on (a) day 7, (b) day 30, (c) day 60.

3.2.4 Characterization of plastic films

The plastic films were collected from the test tubes on specific days (i.e., days 7, 30, and 60), then rinsed with distilled water before being transferred to the glass slides. After drying at room temperature, the slides were covered and stored for further assessment, including optical imaging, contact angle analysis, SEM, and Attenuated total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR) characterization.

The plastic films were first subjected to optical microscopy (Leica DM 2500M optical microscope) (Cao et al., 2021). The contact angle analysis was performed utilizing our previously developed static sessile drop method with a DSA-25S goniometer (KRÜSS Scientific) (Yu et al., 2022). Briefly, a 5 μL distilled water droplet was dispensed onto each plastic film. The angle formed between the liquid-solid interface is the contact angle. In addition, for SEM characterization, samples were sputter-coated with a thin film of gold to improve the electrically conducting properties for SEM imaging preparation. The surface of the films and the morphology of the fragments were examined by the FEI MLA 650FEG SEM in a high-vacuum mode operating at 5 kV (Yang et al., 2021). The ATR-FTIR characterization was carried out over the wavelength range of 900-4000 cm^{-1} (FTIR, INVENIO, Bruker) (Cao et al., 2021).

3.2.5 Characterization of water phase

3.2.5.1 Plastic particle size and count

After 60 days of biodegradation, the images with a remarkable abundance of plastic particles were analyzed using ImageJ v.1.53k software. We set the scale bar based on

SEM magnification and subsequently measured the diameters of particles with well-defined boundaries. The detailed scheme of particle size analysis on plastic films is shown in Fig. 3.3.

Moreover, we quantified the plastic particles released into the medium after 60 days using a hemocytometer integrated with ImageJ software. The detailed procedure is shown in Fig. 3.4. A hemocytometer is a counting-chamber device used for counting blood cells and has been used to count MPs in water samples (Elizalde-Velázquez et al., 2020). In brief, the hemocytometer comprises nine quadrants of 1 mm^2, with a height of 0.1 mm, which results in regions with a volume of 100 nL/each (Malafaia et al., 2022). After injecting 10 µL medium into the hemocytometer, we captured the images of the hemocytometer under the optical microscope at 10×40 magnification. Then the particles on the images were quantified using an automated counting function of ImageJ (X. F. Wei et al., 2021).

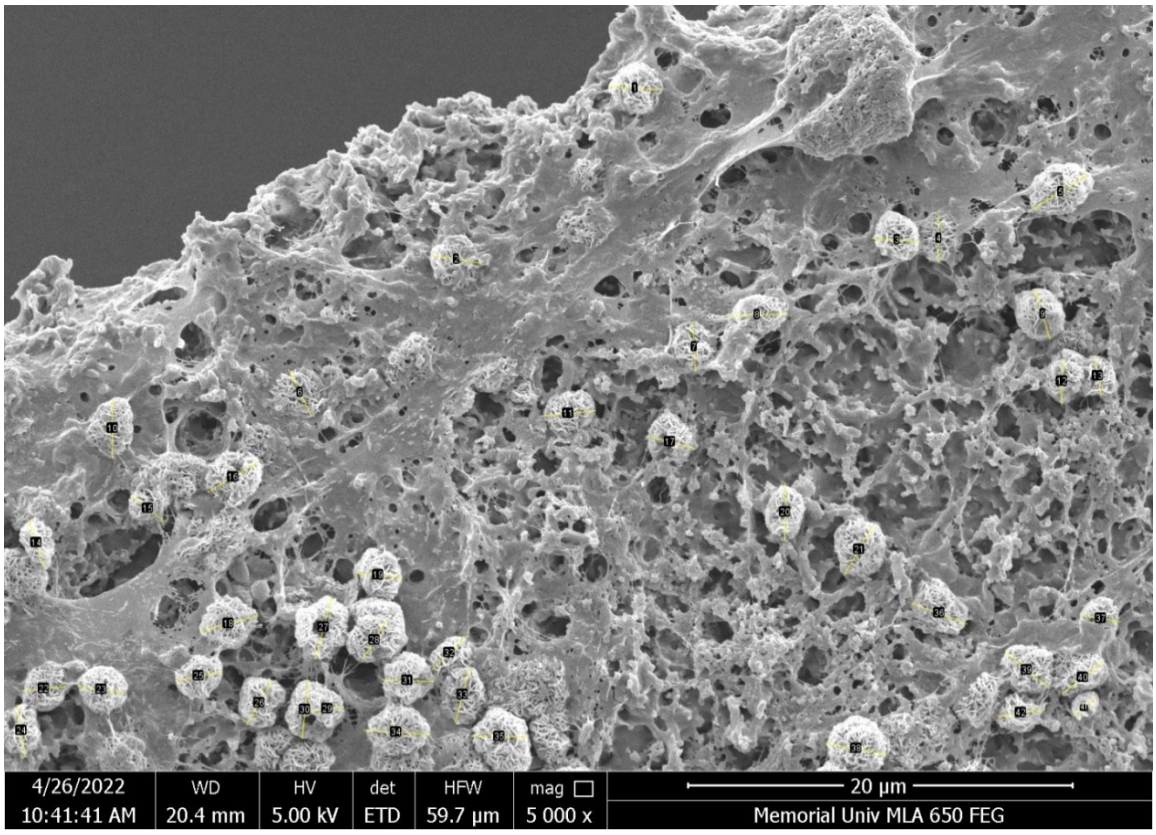


Figure 3.3 A diagram showing particle size measurements from SEM images using ImageJ. The example was taken using the PHA plastic film after marine biodegradation.

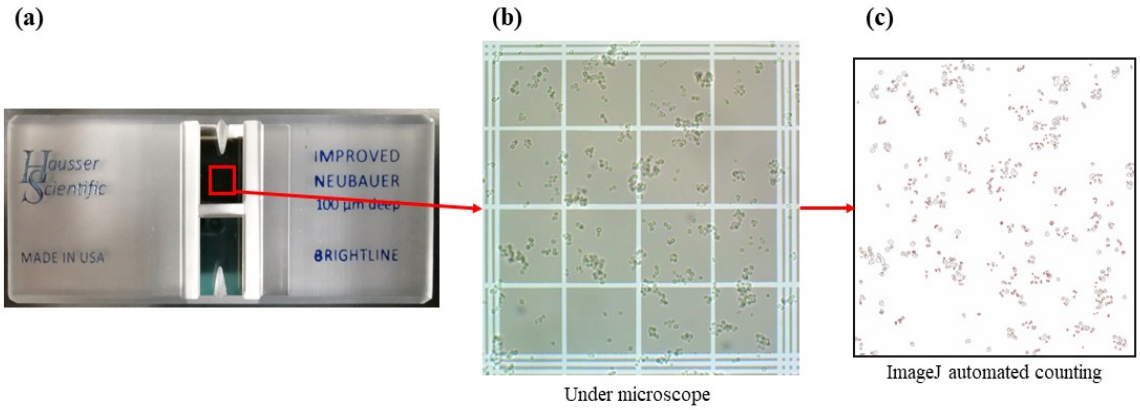


Figure 3.4 The scheme of particle counting procedures.

(a) The red square is the counting chamber of the hemocytometer after injecting 10 µL of the water phase. (b) counting chamber is observed under a microscope. (c) The particles on the microscopic images are quantified using the automated counting function by ImageJ.

3.2.5.2 DOC analysis and acute toxicity assessment

DOC is the indicator of available carbon sources in the water phase and was assessed by a total organic carbon (TOC) analyzer (Dong et al., 2022). Briefly, phosphoric acid was added to the medium samples to ensure the pH was less than 1. The TOC of the medium was measured by the burning oxidation-non-dispersive infrared absorption method using the Shimadzu Toc-L Total Organic Carbon Analyzer with an ASI-L autosampler. Milli-Q water was set as a control.

In addition, the acute toxicity of the water phase was measured by the Microtox® (Model 500) analyzer after 60 days of biodegradation. It measured the changes of light produced naturally in samples exposed to the luminous bacterium (*Vibrio fischeri*) (Dong et al., 2021). The luminometer and a standard log-linear model were used to determine a 50 percent light loss in the test bacteria expressed as the effective concentration (EC50) value (Dong et al., 2021).

3.2.6 Statistical analysis

All experiments were conducted in triplicate. The data were analyzed using one-way analysis of variance (ANOVA) and Tukey's significant difference test (p-value < 0.05) on OriginLab 2021 and were expressed as means ± standard deviation.

3.3 Results

3.3.1 Bacterial growth

Bacterial growth was evaluated within 60 days (i.e., 7, 30, and 60 days) when utilizing different plastics (i.e., LDPE, PLA, and PHA) as sole carbon sources under 4, 15, and 22°C, respectively. Since bacteria could attach to plastic surfaces and form insoluble clumps, their growth was evaluated by total protein assay. Notably, the bacteria showed different growth behaviors among plastic types. Within 60 days, bacterial growth on LDPE and PLA accounted for similarly low protein contents (less than 10 μg/mL) and was slightly affected by temperatures (Fig. 3.5a-b). From day 7 to day 30, the bacterial cell concentrations in LDPE and PLA remained low under all temperatures (Figs. 3.5a-b). After 60 days, we observed a slight increase in LDPE samples under 22°C, but the total protein level in PLA samples remained consistently low. By contrast, when grown on PHA, the total proteins were strongly influenced by temperatures and time duration (Fig. 3.5c). On day 7, the bacterial abundance in PHA increased along with temperature, with the highest level (24.55 μg/mL) achieved under 22°C (Fig. 3.5c). On day 30, PHA enriched the bacterial abundance under both 4 and 15°C but this was not apparent under 22°C (Fig. 3.5c). The decrease under 22°C implies that labile components of PHA could be exploited thoroughly within a short period. However, after 60 days, PHA had a relatively high total protein content of 22.60 μg/mL under 22°C and 27.54 μg/mL under 15°C. It reached the highest level of 43.09 μg/mL under 15°C (Fig. 3.5c). Overall, we observed continuous bacterial growth under low and medium temperatures (i.e., 4°C and 15°C) when grown on PHA. The stable bacterial growth under the low temperature

identified the cold adaptation of this bacterium for degrading plastics, particularly for PHA.

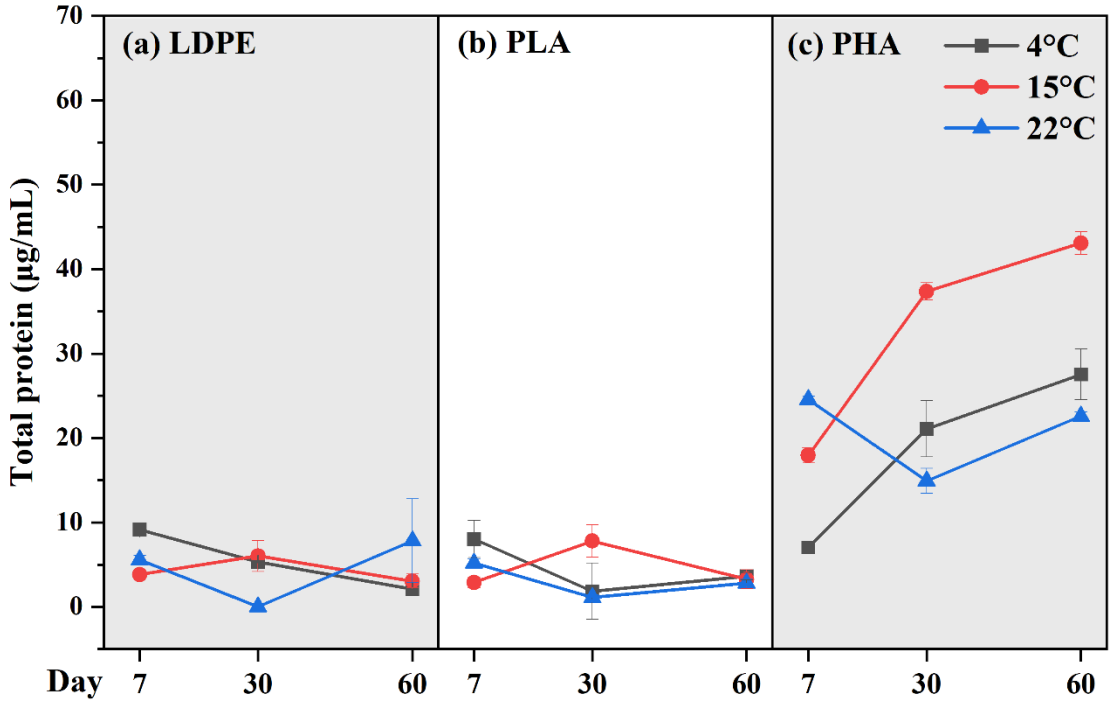


Figure 3.5 The bacterial growth of *Alcanivorax* in studied plastics (a) LDPE, (b) PLA, and (c) PHA measured by total protein assay on days 7, 30, and 60.

3.3.2 Plastic films characterization

3.3.2.1 Optical imaging

To assess the surface characteristics of the plastic films, we initially examined their morphology using an optical microscope. The representative images after 60 days of biodegradation are shown in Fig. 3.6. We found LDPE was floated on the water surface due to a lower density (0.91-0.93) g/cm^3 than the water, while PLA and PHA sank at the bottom with densities of 1.21-1.25 g/cm^3 and 1.18-1.26 g/cm^3, respectively (Figs. 3.6a-c and 3.7) (Naser et al., 2021; Sastri, 2022). After 60 days of biodegradation, turbidity was observed at the bottom of all samples, which may be caused by the formation of degradation products like plastic fragments or particles (Figs. 3.6a-c and 3.7). When comparing the results among different temperatures, more noticeable turbidity was observed under a higher temperature (22°C) than others (Fig. 3.7). After being collected from the medium and air-dried, both LDPE and PLA films retained their shape with limited noticeable signs of degradation (Fig. 3.6d-e). By contrast, the PHA plastic film became excessively soft and seriously deformed (Fig. 3.6f). More detailed images are provided, including observational photos of all plastics at different temperatures (i.e., 4°C, 15°C, and 22°C) over 60 days in test tubes (Fig. 3.7), as well as optical microscope images of LDPE (Fig. 3.8), PLA (Fig. 3.9), and PHA (Fig. 3.10).

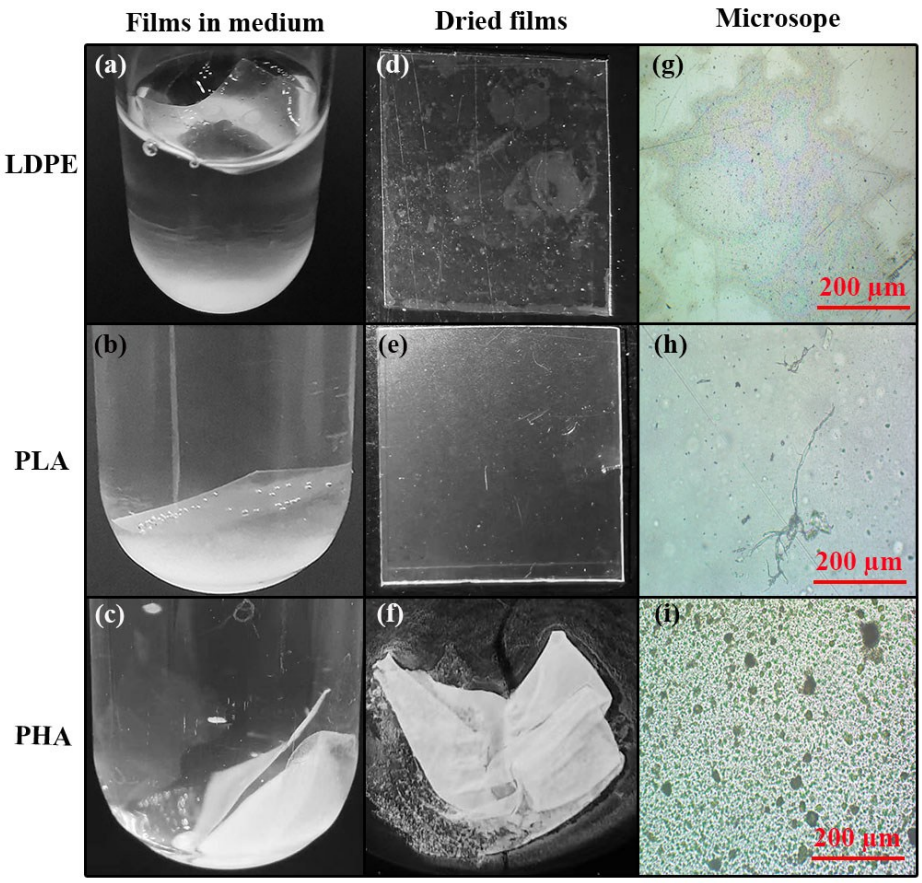


Figure 3.6 Plastic film samples in medium (a-c), after air drying (d-f), and under the microscope (g-i) after 60 days of biodegradation.

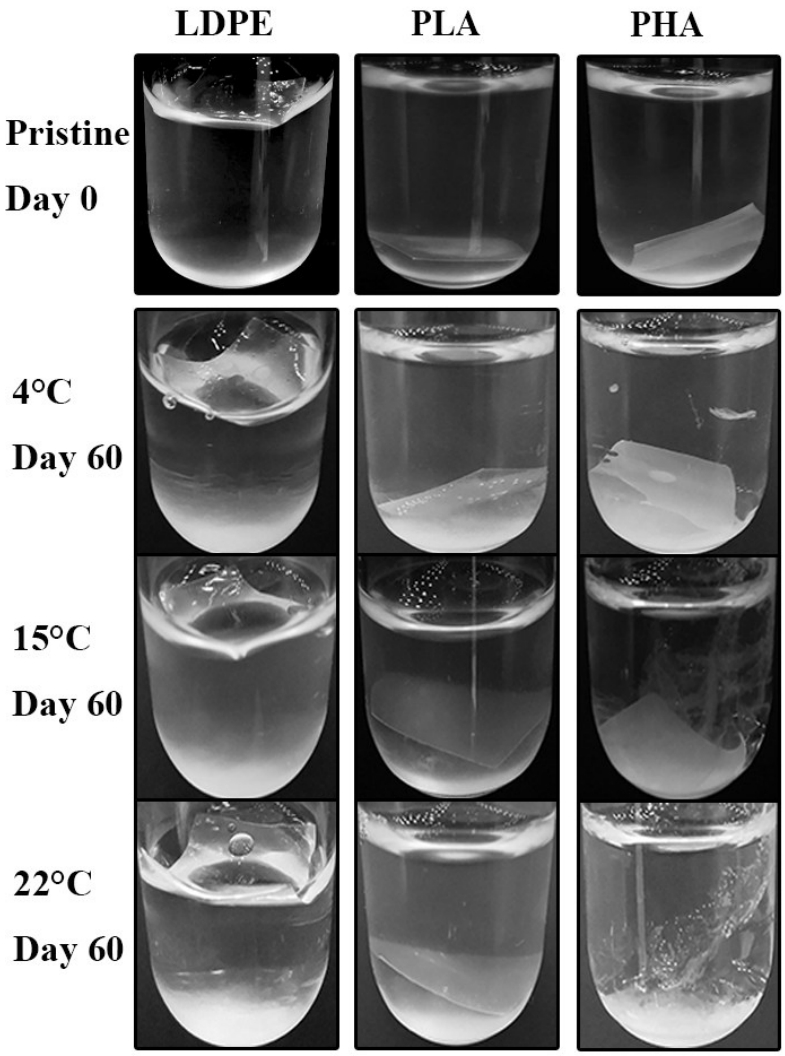


Figure 3.7 Observation photos of incubation tubes for LDPE (first column), PLA (second column), and PHA (third column) before (first row) and after 60 days of marine biodegradation under 4°C (second row), 15°C (third row), and 22°C (fourth row).

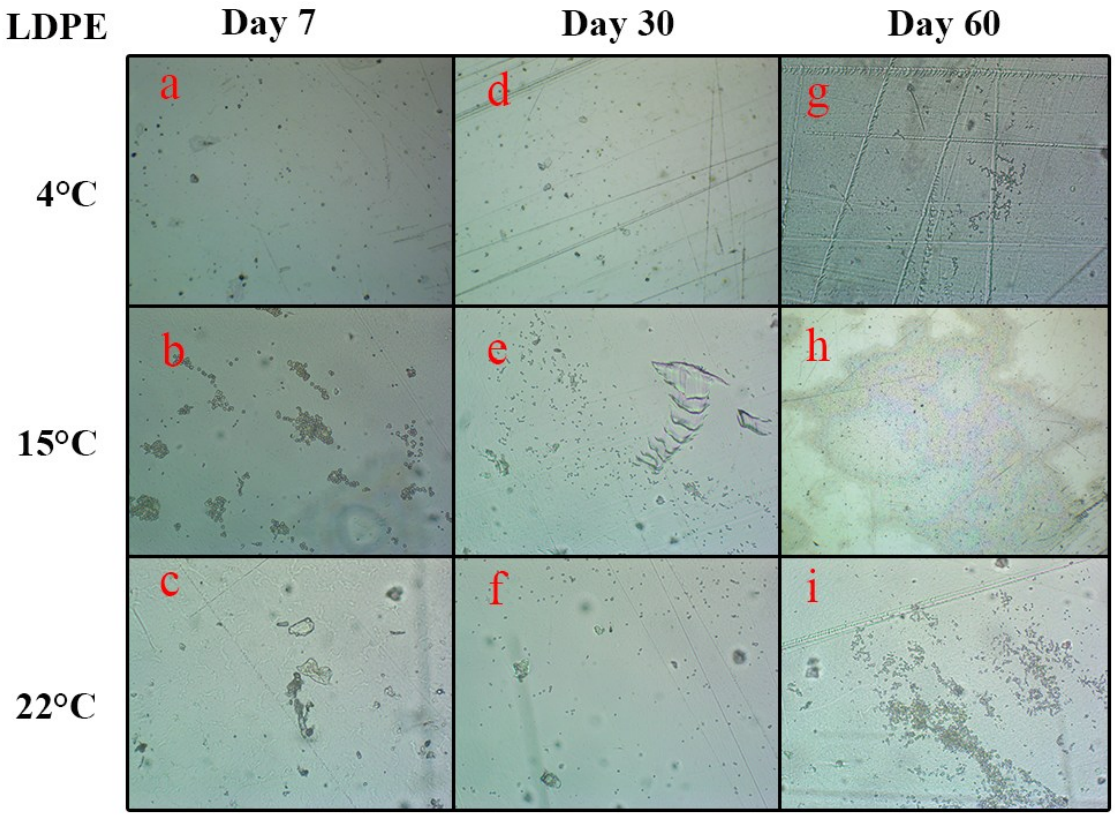


Figure 3.8 Optical microscope images (magnification 10 × 40) of LDPE after marine biodegradation under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a), day 30 (column d), and day 60 (column g).

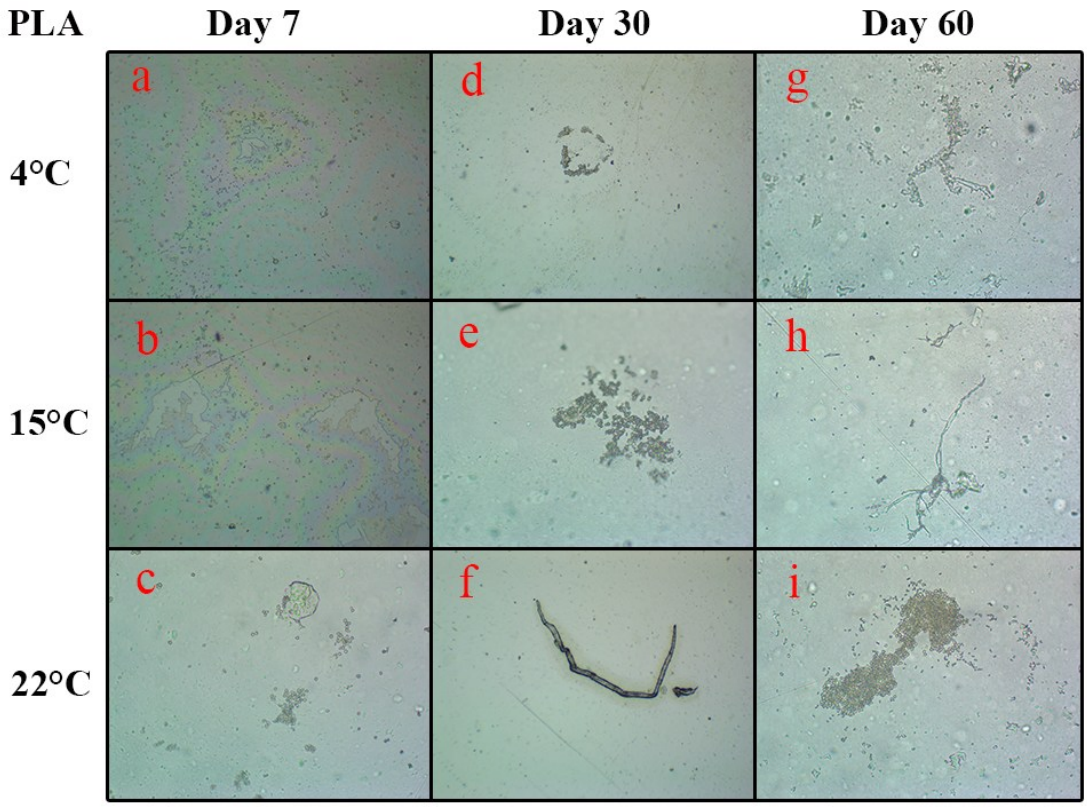


Figure 3.9 Optical microscope images (magnification 10 × 40) of PLA after marine biodegradation under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a), day 30 (column d), and day 60 (column g).

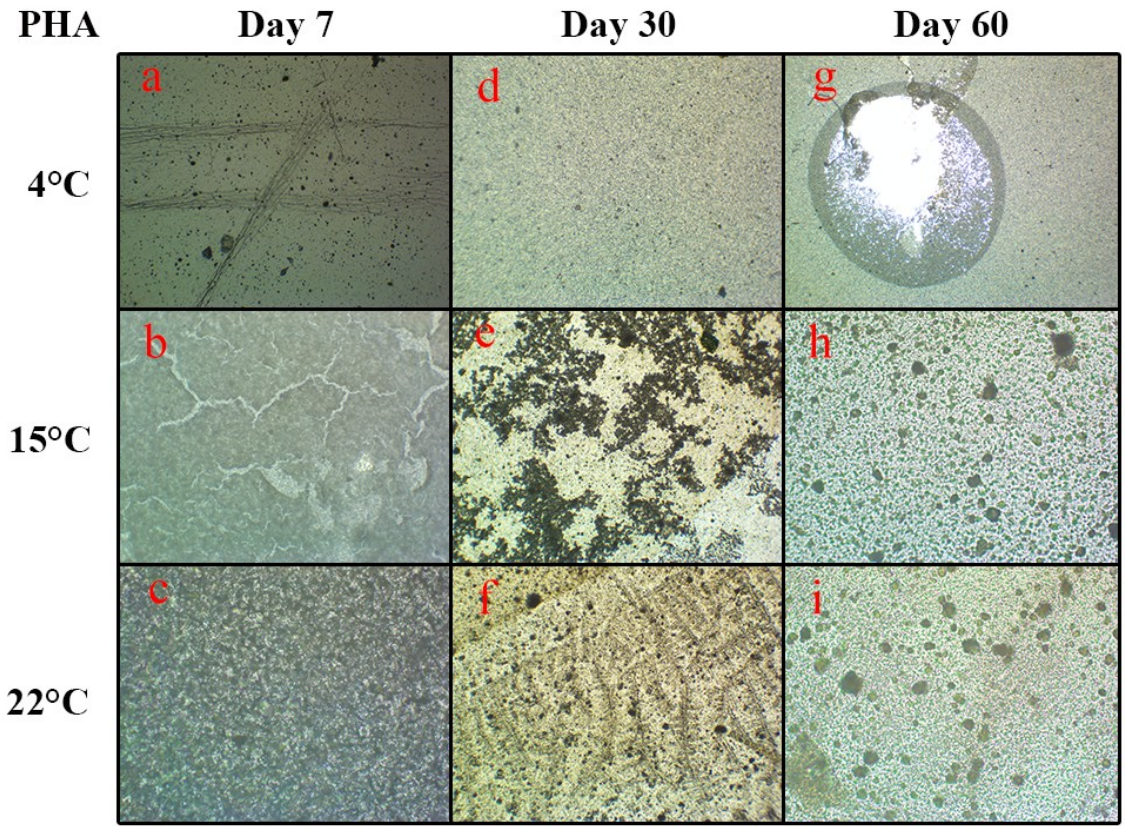


Figure 3.10 Optical microscope images (magnification 10 × 40) of PHA after marine biodegradation under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a), day 30 (column d), and day 60 (column g).

Optical microscopy analysis revealed that numerous particles and fibers formed on their surfaces during the degradation of LDPE and PLA. (Figs. 3.6g, 3.6h, 3.8, and 3.9). We also observed the presence of oil layers on the surface of LDPE (Fig. 3.6g), which may be due to the generation of intermediate alkanes, essential oils components, during LDPE biodegradation. (Khandare et al., 2022). Besides alkanes, other degradation by-products such as fatty acids, ester group of alcohol, eicosane, and hexaoxane were reported during the degradation of LDPE by *Pseudomonas* species (Kyaw et al., 2012). By contrast, we observed numerous holes on the surface of PHA (Figs. 3.6i and 3.10). The material also exhibited a softer, glue-like texture that posed challenges for further assessments, such as contact angle and SEM imaging. The results of optical observation and bacterial growth assay indicated that higher temperature induced accelerated bacterial activities for the fragmentation of plastic films when grown on PHA, while temperature influences on LDPE and PLA degradation were not significant.

3.3.2.2 Contact angle analysis

The water contact angle is an indicator of the hydrophilicity of plastic surfaces. A lower contact angle indicates a higher hydrophilicity and surface energy (Chamas et al., 2020; Han et al., 2020). In this study, all three pristine plastics have a mean contact angle of less than 90° (Figs. 3.11 and 3.12). Specifically, LDPE had the highest contact angle (89.77°), followed by PLA (78.53°) and PHA (72.40°), representing the higher hydrophobicity of LDPE than bioplastics.

Marine biodegradation of LDPE led to a limited contact angle alteration than the two bioplastics types. The contact angle of LDPE decreased evidently only after a long-term (60 days) biodegradation under 15°C, reaching mean values of 65.86° ($p = 0.015$) (Fig. 3.11a). The contact angles of PLA plastic decreased to 43.70°- 62.99° regardless of the degradation time and temperature (Fig. 3.11b). By contrast, the contact angles of PHA decreased significantly ($p < 0.001$) on day 7 under 15°C, and 22°C, resulting in 21.37° and 19.79°, respectively (Fig. 3.11c). Besides, the contact angle of PHA did not change significantly along with time under 4°C (Fig. 3.11c). Since the surface structure of PHA was severely damaged after biodegradation at 15 and 22 °C on days 30 and 60, the contact angles under these scenarios could not be measured. The contact angle analysis indicated that the biodegradation of these three plastics underwent different trends; particularly, PHA film experienced more significant biodegradation than the other two types of plastics.

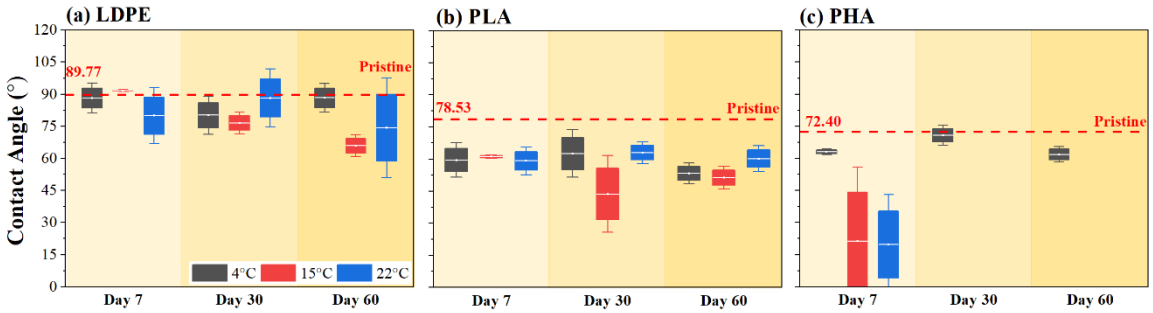


Figure 3.11 The measured water contact angle on (a) LDPE, (b) PLA, and (c) PHA surfaces within 60 days of marine biodegradation.

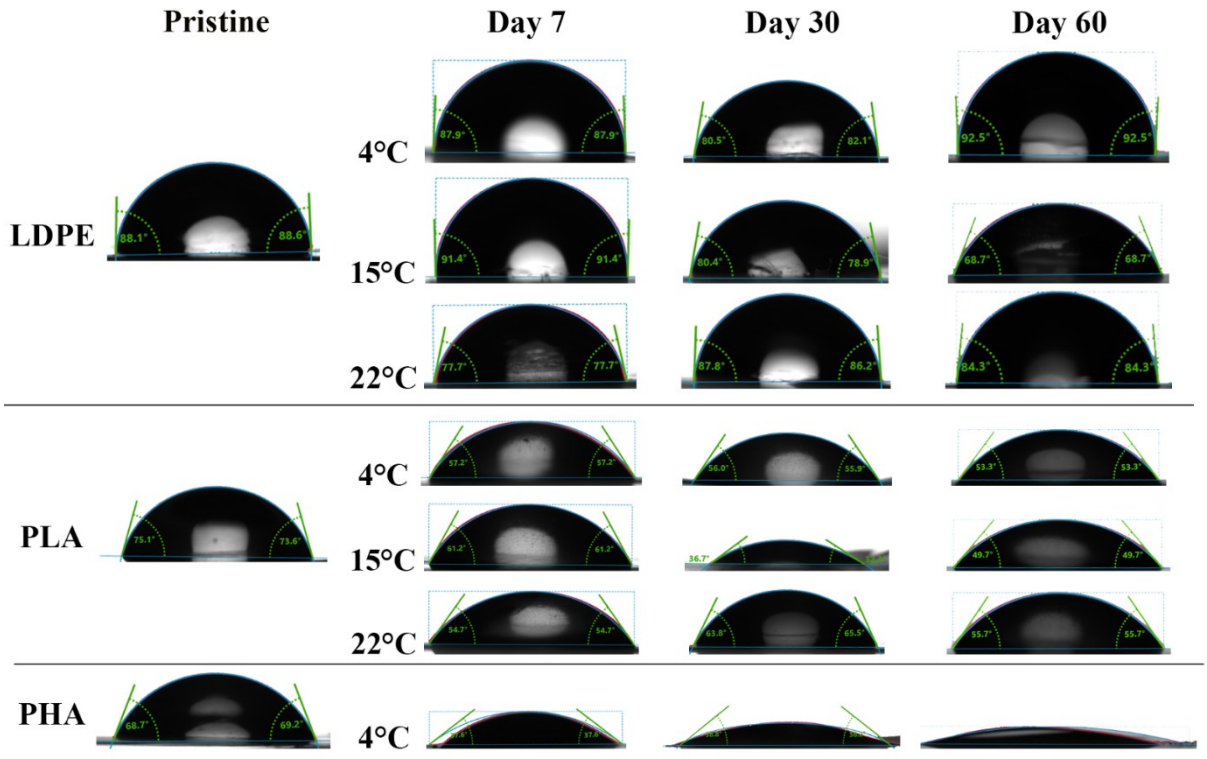


Figure 3.12 The droplets of water contacting the plastic surface during the contact angle assessment.

3.3.2.3 SEM analysis

We applied SEM characterization for a deeper view of the film's surface and edge changes. These representative images are shown in Fig. 3.13. Before biodegradation, all plastic surfaces were relatively smooth (Figs. 3.13a, 3.13e, and 3.13i), and the edges were sharp and clear (Figs. 3.13c, 3.13g, and 3.13k). The LDPE (Fig. 3.14-3.16), PLA (Fig. 3.17-3.19), and PHA (Fig. 3.20-3.22) imaged under different temperatures over time are included in this section.

For LDPE, compared with the pristine surface, the biodegradation did not induce significant alteration (Fig. 3.14b); however, we still observed fibers and particles on the surface (Figs. 3.14 and 3.16), and flaking started from the edge (Figs. 3.13d and 3.15). In particular, the edge of LDPE became rougher and created more hidden space for microbial adhesion after 60 days under all the temperatures (Figs. 3.13d and 3.15).

Similarly, for PLA, the temperature had a minor impact on the morphological changes (Figs. 3.13f and 3.17). Its surface was considerably smooth, with only a few dents and undulation over time (Fig. 3.17). Biodegradation induced the edge of PLA to become slightly rougher (Figs. 3.13g-h and 3.18). Particles and fibers (Fig. 3.19) were also observed on the surface under all temperatures.

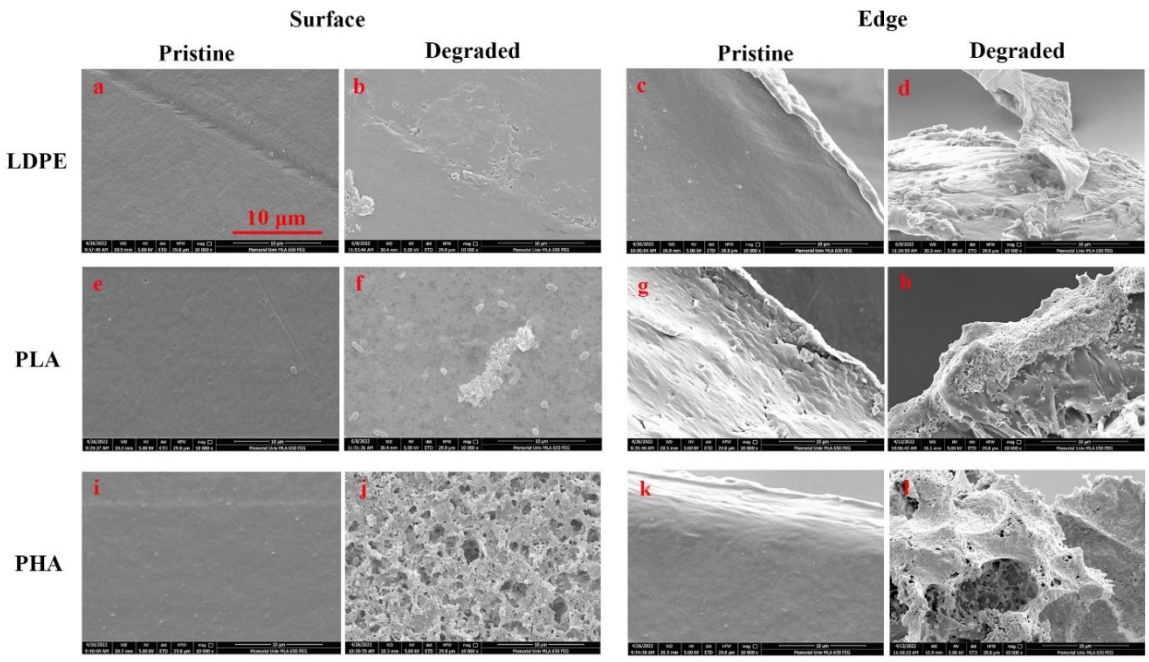


Figure 3.13 The representative SEM images of plastic films' surface and edge throughout 60 days of marine biodegradation.

(a) Pristine and (b) biodegraded LDPE surface, (c) pristine and (d) biodegraded LDPE edge; (e) pristine and (f) biodegraded PLA surface, (g) pristine and (h) biodegraded PLA edge; (i) pristine and (j) biodegraded PHA surface, (k) pristine and (l) biodegraded PHA edge.

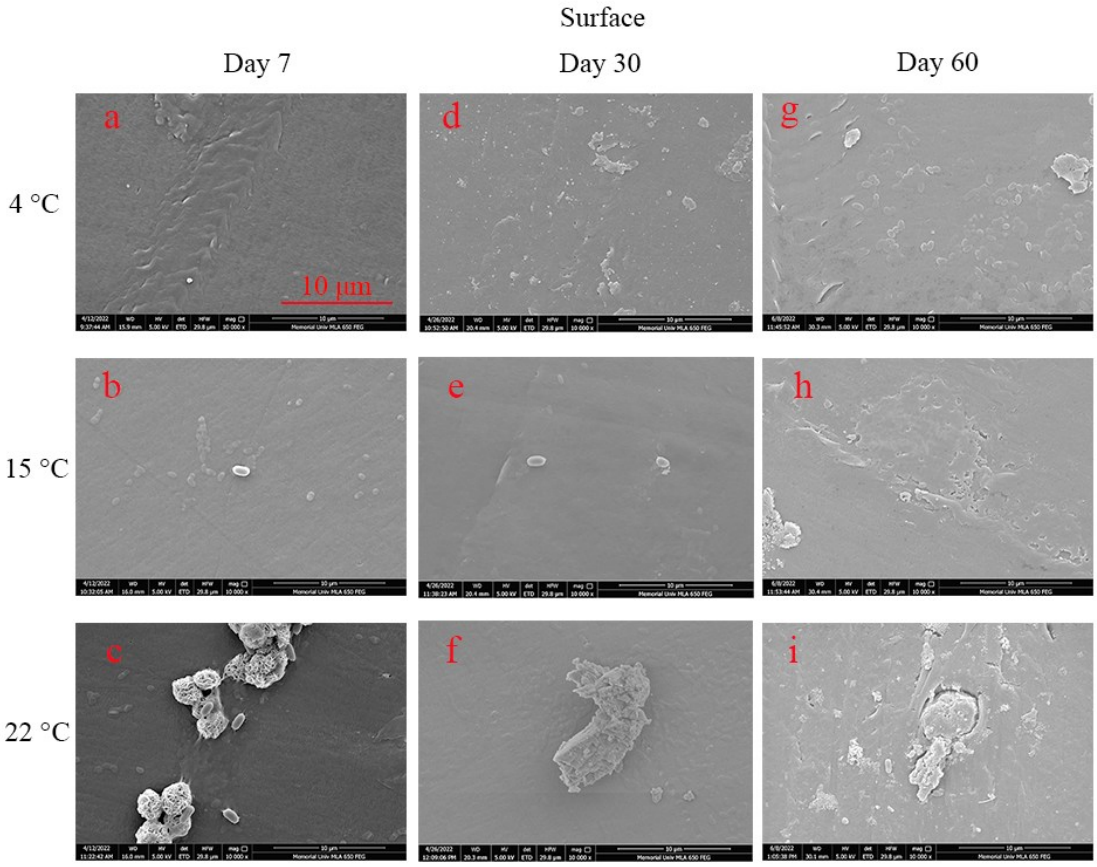


Figure 3.14 SEM images of LDPE surface under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a), day 30 (column d), and day 60 (column g).

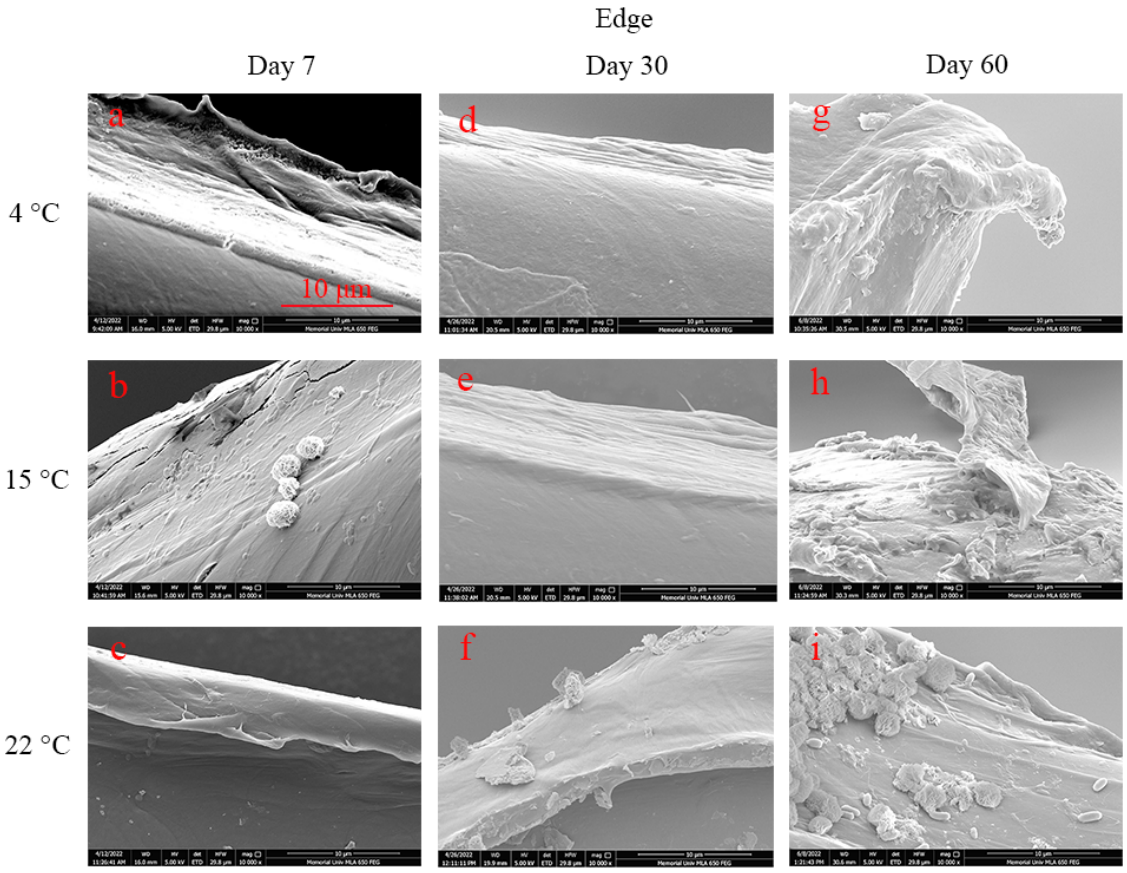


Figure 3.15 SEM images of LDPE edge under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a), day 30 (column d), and day 60 (column g).

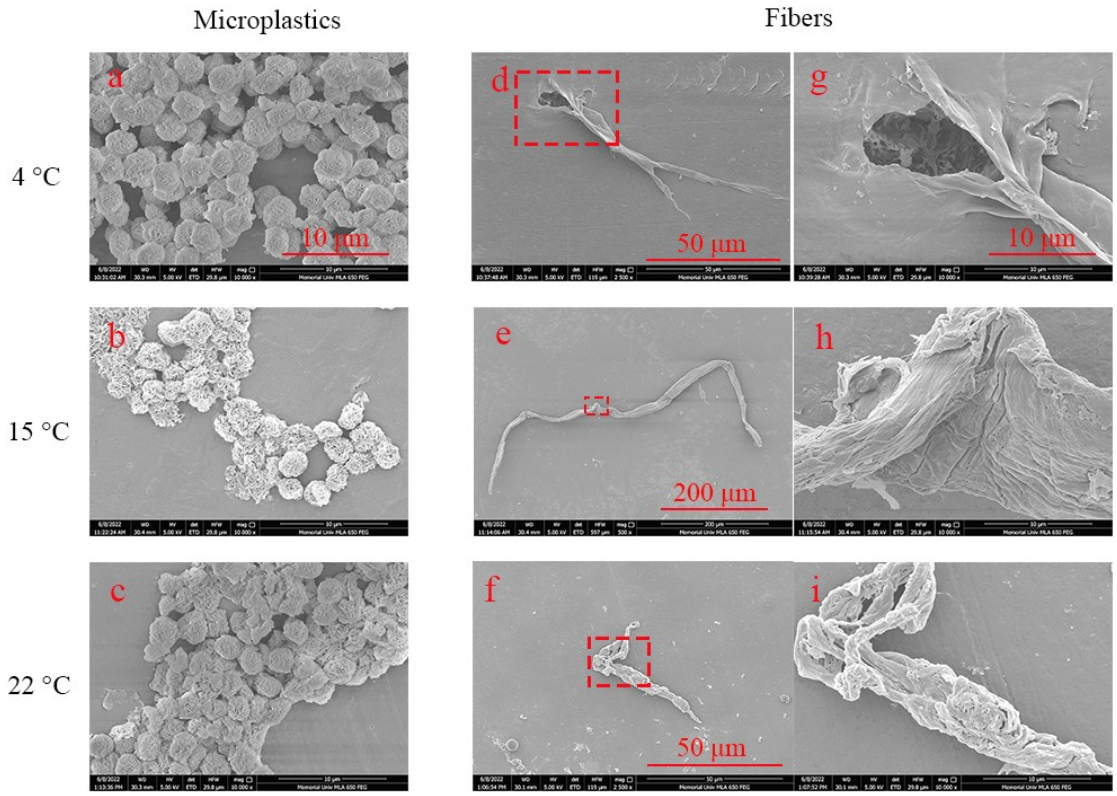


Figure 3.16 SEM images of degradation products from LDPE under 4°C (row a), 15°C (row b), and 22°C (row c).

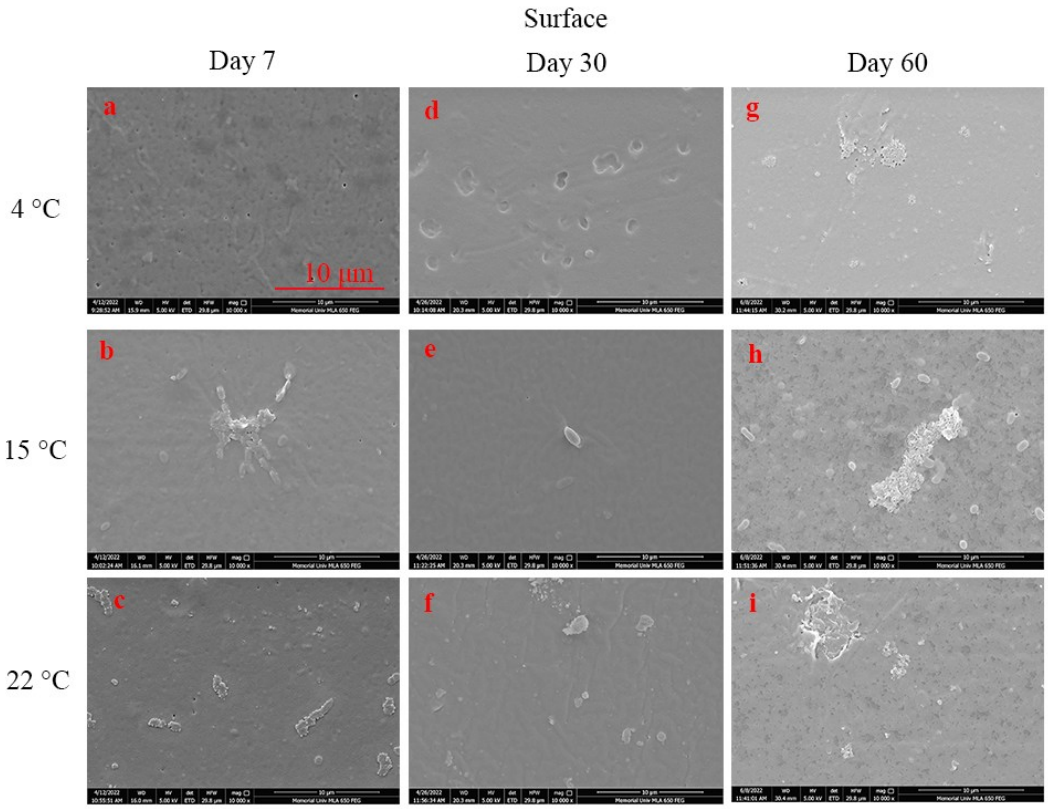


Figure 3.17 SEM images of PLA surface under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a), day 30 (column d), and day 60 (column g).

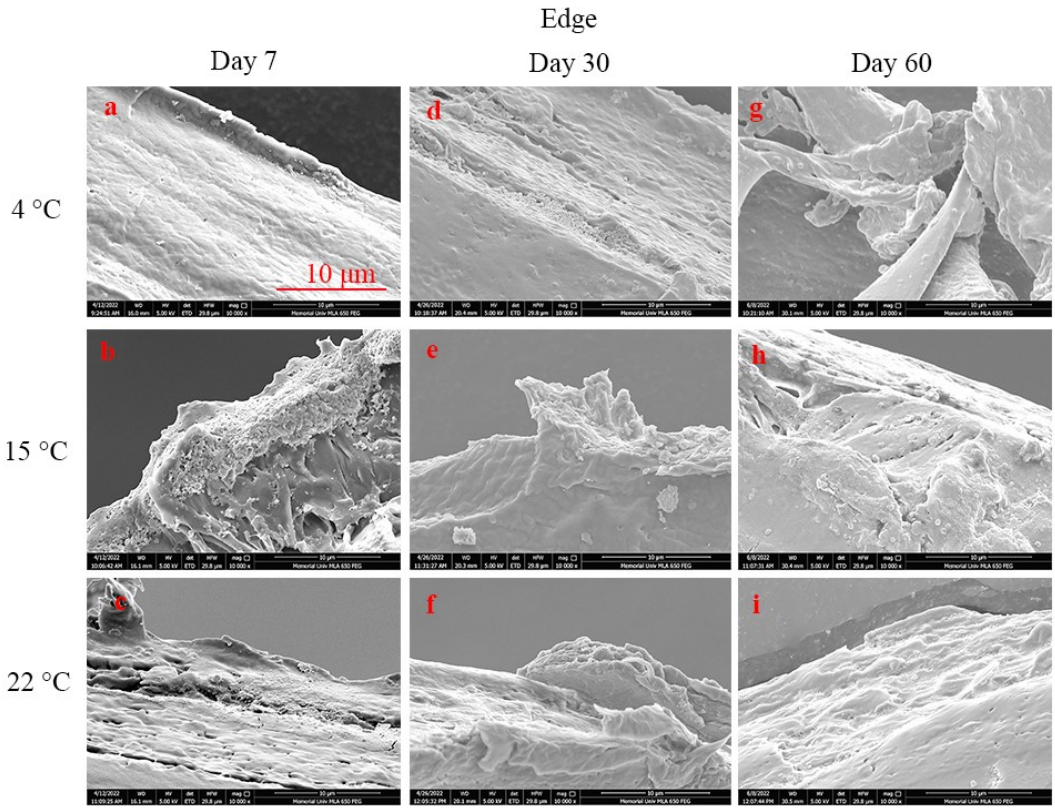


Figure 3.18 SEM images of PLA edge under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a), day 30 (column d), and day 60 (column g).

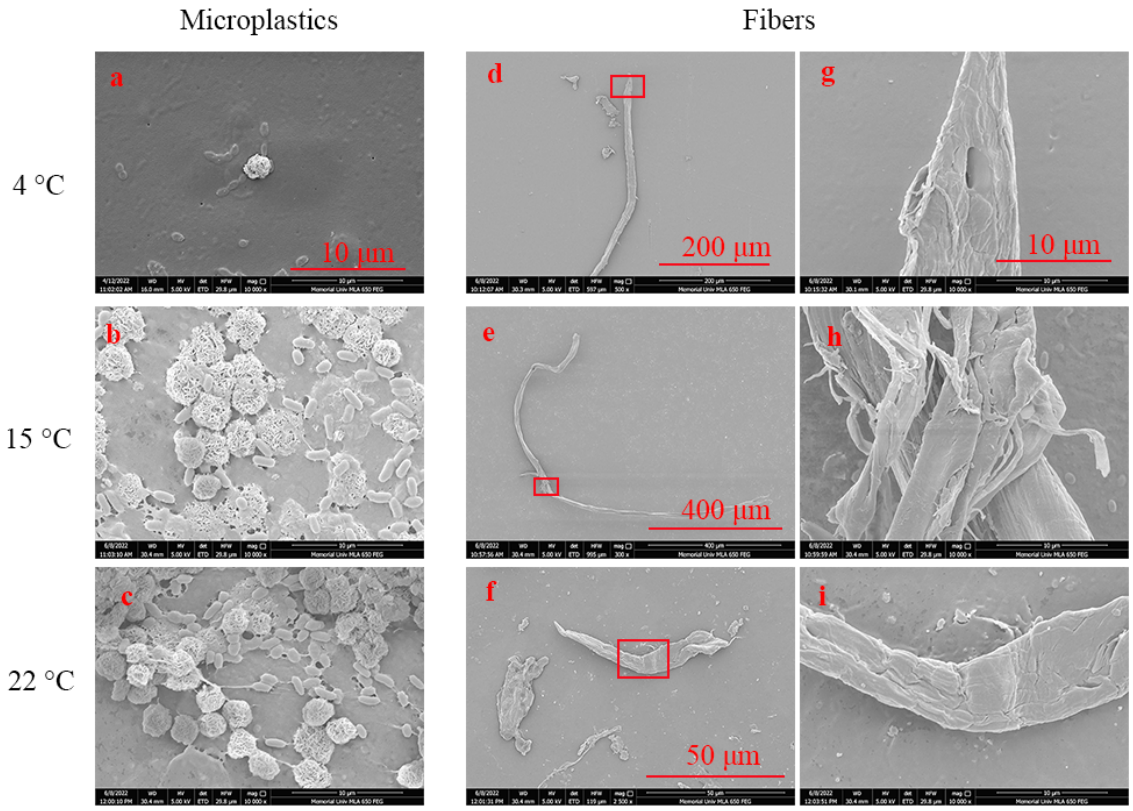


Figure 3.19 SEM images of degradation products from PLA under 4°C (row a), 15°C (row b), and 22°C (row c).

In contrast, there were apparent morphological changes during the biodegradation of PHA films. Within 7 days, creases and holes appeared throughout the films (Fig. 3.20). As incubation time increased, we observed a thoroughly porous surface (Figs. 3.13j and 3.20), an unstructured edge (Figs. 3.13l and 3.21), and remarkable fragmentation. MPs and bacteria were also found embedded in the holes (Figs. 3.20-3.22). The SEM characterization of PHA after long-term biodegradation was omitted as the films were completely damaged. Nevertheless, PHA's short-term morphology changes reflected that its biodegradation performance was dominant among the three plastic types.

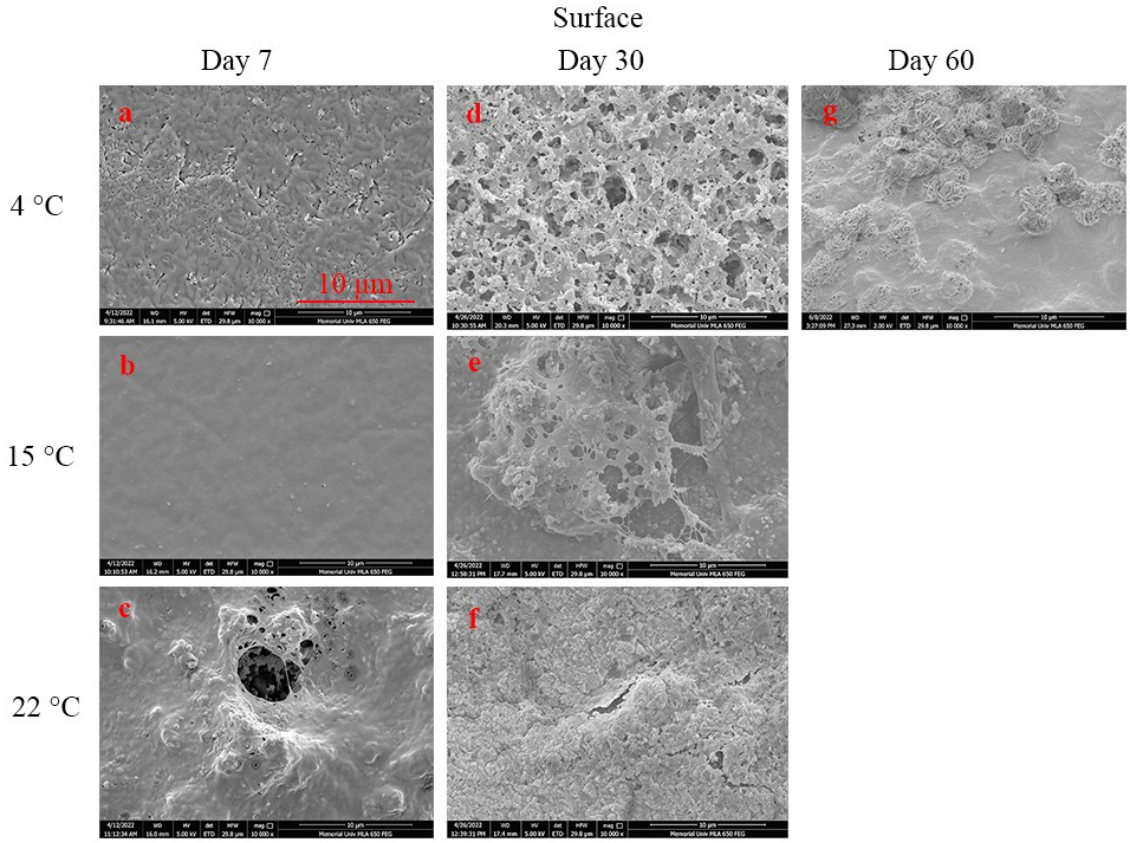


Figure 3.20 SEM images of PHA surface under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a), day 30 (column d), and day 60 (g). Due to the deformation of PHA, the SEM images of PHA after 30 days at 15 and 22°C could not be obtained.

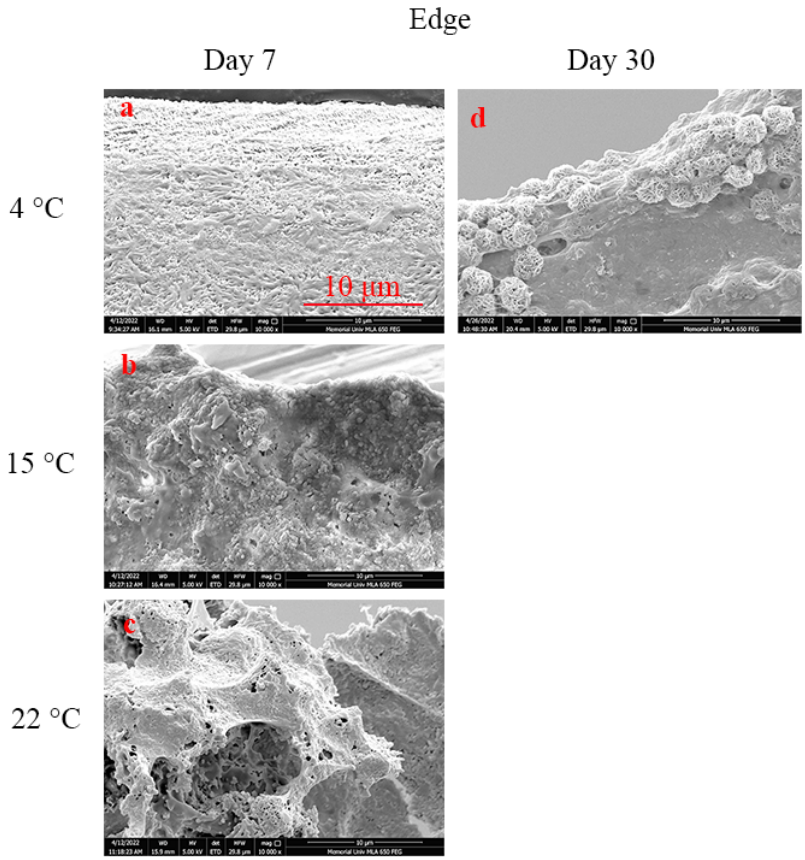


Figure 3.21 SEM images of PHA edge under 4°C (row a), 15°C (row b), and 22°C (row c) on day 7 (column a) and day 30 (d). Due to the deformation of PHA, the SEM images of PHA after 7 days at 15 and 22°C could not be obtained.

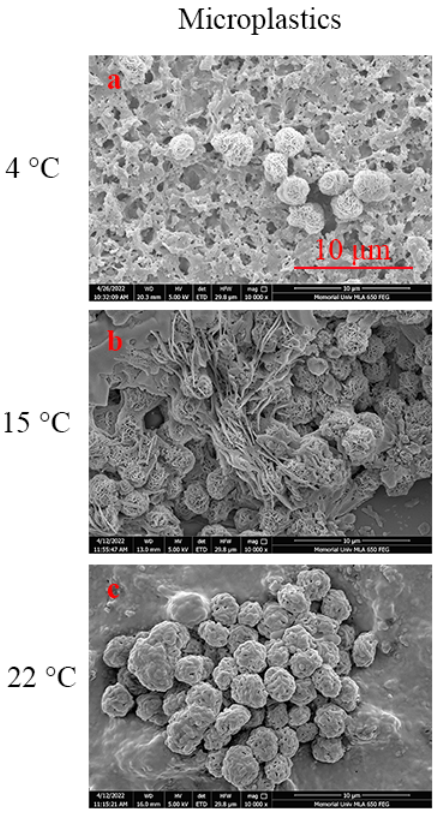


Figure 3.22 The representative SEM images of degradation products from PHA under 4°C (a), 15°C (b), and 22°C (c).

3.3.2.4 ATR-FTIR analysis

The effect of biodegradation on the alteration of functional groups was further assessed by ATR-FTIR. For pristine and incubated LDPE films, they have similar peaks at 2934 cm^{-1} and 1458 cm^{-1} representing CH stretch and CH_2 bends, respectively (Fig. 3.23a). After 60 days of biodegradation, we did not observe peaks corresponding to oxidation functional groups, such as carbonyl C=O (1720 to 1740 cm^{-1}) and hydroxyl O-H (3300-3400 cm^{-1}) groups. The pristine and biodegraded PLA films also have representative functional groups with similar intensity, including carbonyl C=O asymmetric stretching (1747 cm^{-1}), which appears asymmetric due to the unequal distribution of electron density in the carbonyl bond, resulting in a non-uniform vibrational mode. The C-O-C stretching region (1079-1077 cm^{-1}) and CH stretching region (3000-2879 cm^{-1}) were also observed (Fig. 3.23b). These results are in line with the contact angle and SEM analysis, indicating limited oxidation of LDPE and PLA plastic films after 60 days of marine biodegradation. Uniquely, comparing with pristine PHA, we observed decreased peaks after biodegradation in the regions of 2934 cm^{-1}, 1458 cm^{-1}, and 1381 cm^{-1} corresponding to CH, CH_2, and CH_3 groups, respectively (Fig. 3.23c). Besides, the peaks at 1724 cm^{-1}, 1280 cm^{-1}, 1185 cm^{-1} denoting carbonyl C=O, C-O, and C-O-C, respectively, were also decreased after biodegradation. The vibrational frequencies at 1280 and 1185 cm^{-1} correspond to the two distinct C-O bonds in the ester functional group. The peak at 1280 cm^{-1} represents the C-O bond stabilized by resonance with the adjacent carbonyl group, while the peak at 1185 cm^{-1} corresponds to the non-resonant C-O bond. New peaks of amide group I and amide group II appeared at 1635 cm^{-1} and 1538 cm^{-1}, respectively,

indicating the formation of proteins on PHA (Fig. 3.23c). These proteins may be associated with bacterial cells or EPS. A new peak area at 3100-3600 cm^{-1} representing the hydroxyl O-H groups further confirmed the strong oxidation of PHA during biodegradation (Dong et al., 2022).

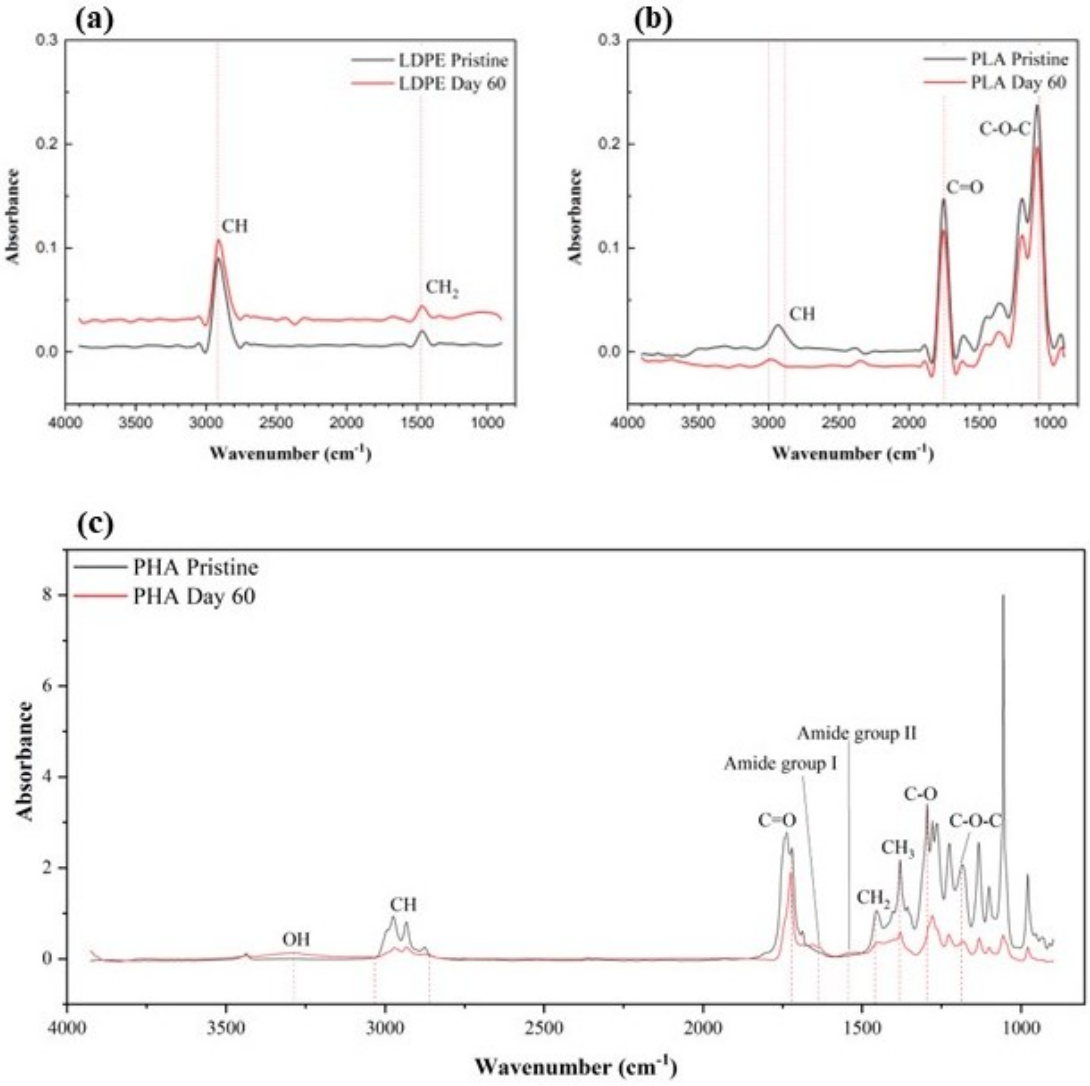


Figure 3.23 ATR-FTIR spectrum of (a) LDPE, (b) PLA, and (c) PHA before and after 60 days of degradation.

3.3.3 Release of degradation products

3.3.3.1 Release of plastic particles

Numerous particles released from plastic biodegradation appeared on both plastic films and in the water phase of all samples (Fig. 3.24). For LDPE, the particles occurred together with filaments (Fig. 3.24a). These particles were distributed in singular or cluster forms on the PLA surface (Fig. 3.24b and 20). On the contrary, we observed more significant amounts of particles and evenly distributed holes on PHA surface (Fig. 3.24c). Taking a closer look at these particles via SEM, they were spherical particles with a rough surface for all these types of plastics (Fig. 3.24g-i). When in a cluster form, the particles usually interacted with microbial colonization. For LDPE and PLA, abundant particles and microbial colonization were spotted on edge during biodegradation (Figs. 3.24d and 3.24e). Notably, for PHA, particles were harbored in its numerous holes (Figs. 3.24f and 3.24i). Maclean et al. (2021) also observed that these rounded aggregates were inside the surface cracks and tightly attached to strings of EPS matrix. With the existence of EPS, the bacterial cells may facilitate the metabolization and release of particles.

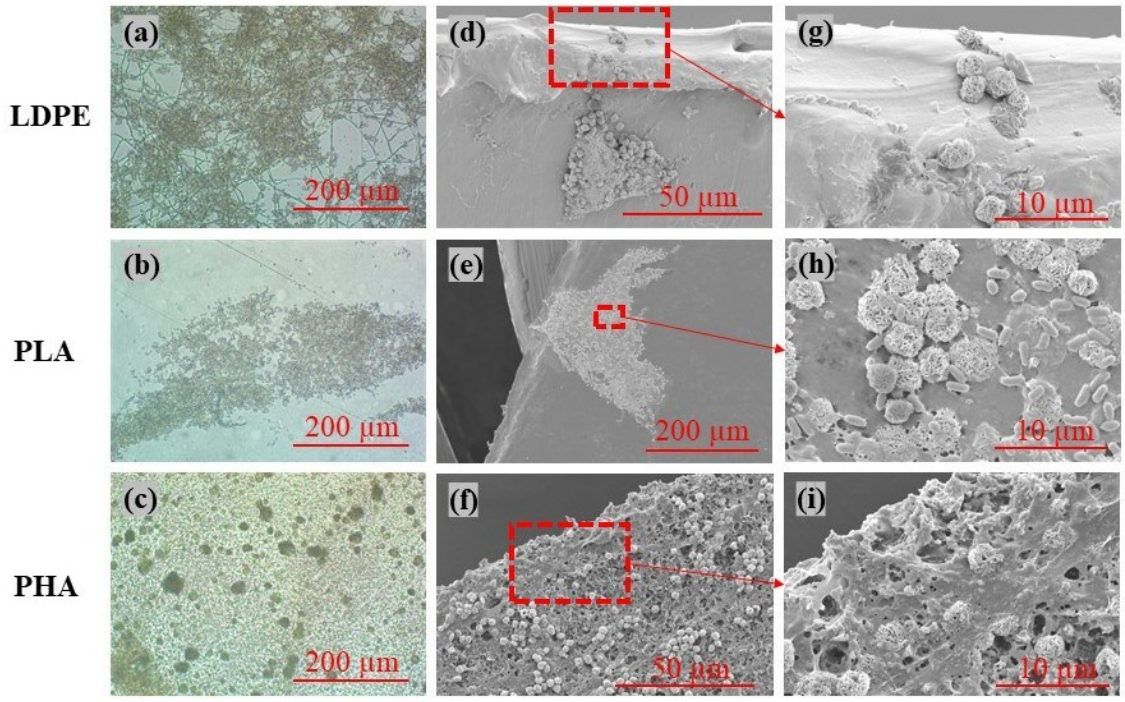


Figure 3.24 Optical microscopic (column a) and SEM images (column d-g) of representative released particles on LDPE (row a), PLA (row b), and PHA (row c) plastics after 60 days of marine biodegradation.

The size and quantities of particles were evaluated after 60 days of biodegradation (Fig. 3.25). Using SEM integrated with ImageJ software (Fig. 3.3), we measured the particle size of LDPE, PLA, and PHA ranged from 1.72 to 3.93 μm, 1.95 to 3.87 μm, and 1.49 to 5.19 μm, respectively (Fig. 3.25a). Under different temperatures, the average particle size remained within a tight range of 2.66 μm to 2.99 μm (mean size of 2.8 μm). Based on the size of these particles, they can be defined as ball-liked MPs. It also indicated minor impacts of temperature and plastic types on the size of released MPs during marine biodegradation.

Further, we calculated the numbers of emitted MPs using a hemocytometer integrated with ImageJ software (Fig. 3.4). We found millions of MPs released to the water phase after 60 days of biodegradation for all plastics, even under the lower temperature of 4°C (Fig. 3.25b). We also observed that the number of MPs increased as the temperature rose for all plastics. For LDPE, the quantities of released MPs increased from 5.55×10^6 under 4°C to 5.63×10^7 under 15°C, then reached 1.65×10^8 under 22°C. The numbers of MPs released from PLA under 4°C and 15°C were close to 2×10^7. As the temperature increased to 22°C, the quantities increased approximately 10 times. As expected, PHA released the highest number of MPs compared with others under all temperatures.

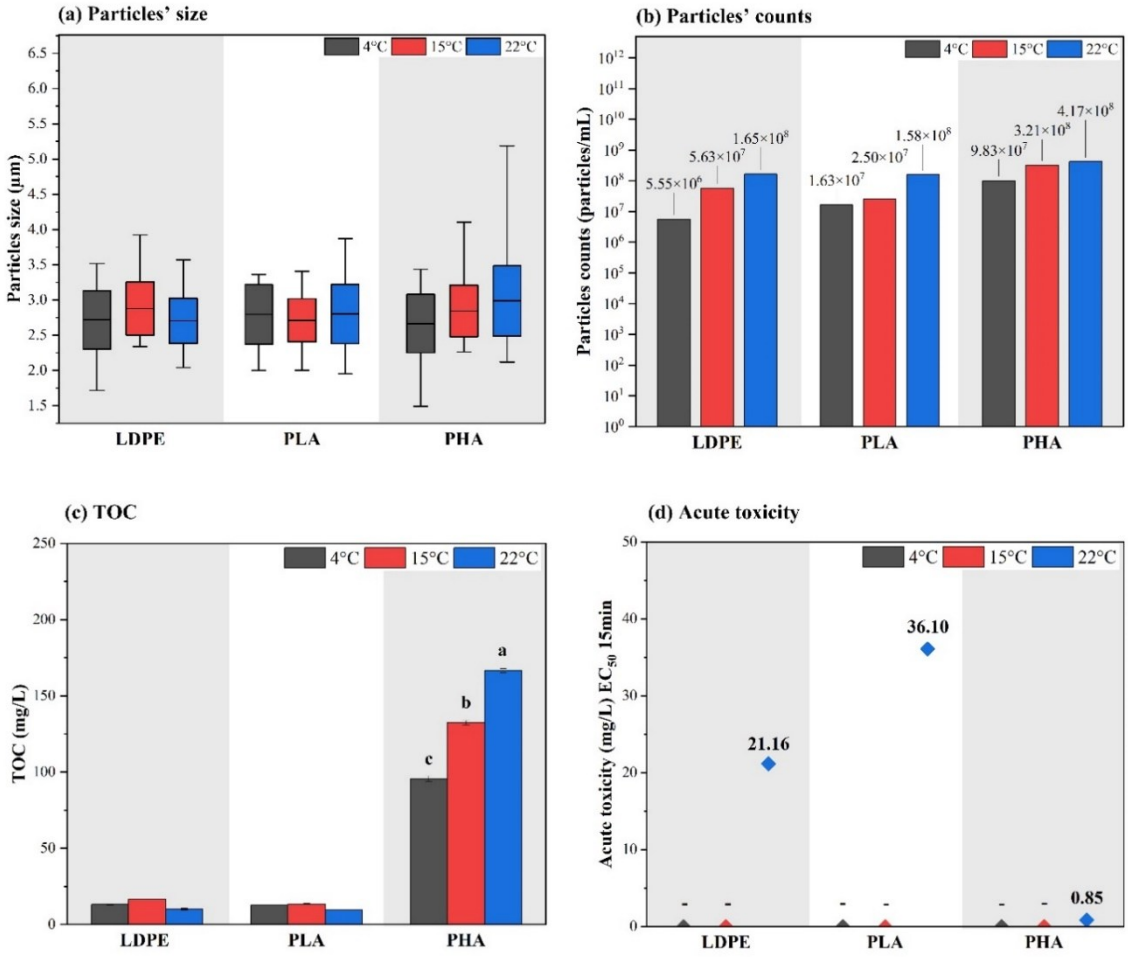


Figure 3.25 Degradation products released from LDPE, PLA, and PHA films after 60 days of marine biodegradation.

(a) Released particle size distribution measured on SEM images; (b) released particle quantities estimated by microscope integrated with ImageJ; (c) TOC of the water phase representing DOC. Lowercase letters indicate significant differences between temperatures ($p < 0.05$); (d) acute toxicity of the degradation medium to *Vibrio fisheri*. '-' means acute toxicity was not detected in samples.

3.3.3.2 Release of DOC and the associated acute toxicity

During marine biodegradation, DOC comprises the degradation products dissolved in the water phase (Zadjelovic et al., 2022). We assessed the DOC release of the three plastics after 60 days of biodegradation under different temperatures. We perceived that LDPE and PLA released 9.45-16.71 mg/L of DOC to the water phase under different temperatures (Fig. 3.25c), and the DOC concentration was steady without significant differences under the three temperatures. Distinctively, PHA released DOC of 95.67 mg/L under 4°C, 132.43 mg/L under 15°C, and 166.55 mg/L under 22°C. It showed that biodegradation of PHA induced the highest release of DOC than the other two types of plastics. Additionally, higher temperatures only enhanced DOC release when degrading PHA plastics but not for the LDPE and PLA.

The leaching of chemicals may result in potential toxicity (Lambert et al., 2017). We further investigated the acute toxicity to *Vibrio fischeri* of the water phase after 60 days of biodegradation using Microtox®. We detected the acute toxicity (EC_{50} value) of the water phases after biodegrading LDPE (21.16 mg/L), PLA (36.10 mg/mL), and PHA (0.85 mg/L) under 22°C (Fig. 3.25d). The value of 0.85 mg/L was extremely low, indicating marine biodegradation of PHA plastics under 22°C induced the highest acute toxicity. The heightened acute toxicity, potentially caused by the rapid and substantial release of MPs and DOC during PHA biodegradation, could inevitably lead to disruptions in marine ecosystems. Nevertheless, MPs made from PHA were reported with a relatively short persistence in marine environments due to the microbial depolymerases, for example, the lifespan of a 35 μm PHA bag ranges from 25 days to several months (Dilkes-Hoffman et

al., 2019). Moreover, this represents an accelerated biodegradation process, where the toxic effects may diminish as a result of natural dilution within marine ecosystems. Conversely, for LDPE and PLA, the acute toxicity observed at 22°C may primarily stem from the presence of released MPs, as DOC emissions remained minimal across all temperatures. However, it is important to consider the long-term toxicity effects caused by the bioaccumulation of LDPE and PLA, given their longer lifespans.

3.4 Discussion

3.4.1 How PHA, LDPE and PLA act differently during marine biodegradation?

Plastic biodegradation relies on microbial colonization on plastic surfaces and utilizing plastic monomers as carbon sources (Zeenat et al., 2021). In the present study, the enrichment of microbes on LDPE and PLA only occurred at the early stage (Figs. 3.5a-b). Results for LDPE biodegradation are in line with other studies, which showed *Oleiphilus* was enriched on LDPE only at the early stage (after 2 days) of colonization in the seawater and tended to decrease after a long term (Yakimov et al., 2022). Though PLA is a type of bio-origin plastic made of renewable resources such as corn or sugar cane (Table 3.1), its biodegradation in the marine environment is still limited. By contrast, PHA is well known to be degradable in marine environments; as further confirmed in this study, the bacteria could burgeon on PHA derived from microorganisms throughout 60 days of biodegradation.

The optical and SEM images presented minor morphological changes in LDPE and PLA plastics, while momentous alteration was observed on the PHA plastics as numerous

holes appeared. These porous structures can enhance the exposed surface area for enzymatic binding and attack (Meereboer et al., 2020). Indeed, we discovered bacteria and MPs in these porous structures and edges. These structures' further detachment and fragmentation could cause more MPs to release (X.-F. Wei, Capezza, et al., 2022). In this study, we observed millions of MPs released after 60 days of marine biodegradation for all the plastics. A similar result was reported by X.-F. Wei et al. (2022), who found that more than one million MPs were generated from 0.1 g of PCL film within three days of biodegradation. The released MPs from LDPE, PLA, and PHA had a size of 2.75 μm, 2.74 μm, and 2.84 μm, respectively (Fig. 3.25a). The MPs with a similar size (i.e., 2.8 μm) were released from PCL/PE 90/10 blend plastic in another study (X.-F. Wei, Hedenqvist, et al., 2022). These small and massive numbers of MPs could inevitably risk marine food webs and ecosystems. The ingestion of MPs has adverse physical effects on marine life, such as abrasions and blockage of digestive organs (Hong et al., 2018). Furthermore, MPs originating from petroleum-based plastics (i.e., PE) may contain chemical additives and POPs, resulting in adverse toxic effects on marine organisms. Conversely, MPs derived from bioplastics are generally acknowledged to exhibit reduced toxicity due to their natural origins. Nevertheless, it is important to note that certain additives and impurities present in bioplastics could potentially pose risks (Andrady, 2011).

Biodegradable plastics exhibit variable degradation rates depending on the environmental conditions. Even though PLA is promoted as bioplastic, its biodegradability is only presented under industrial composting and anaerobic digesting conditions. Other studies

also identified that the biodegradation of PLA does not differ significantly from petroleum-based plastics in aqueous environments (Narancic et al., 2018). PHA displays efficient marine biodegradation compared to other bioplastics, such as PLA, poly(ethylene succinate) (PES), and poly(ethylene adipate) (PEA), PBAT (Choe et al., 2021; Suzuki, Tachibana, & Kasuya, 2021). Within 60 days, we identified PHA plastics derived from microorganisms could be well marine biodegraded, with the fragmentation of plastic films and release of plenty of MPs and DOC; oppositely, marine biodegradation primarily led to the release of MPs for LDPE and PLA and limited impacts on the alteration of the plastic film itself and DOC release.

Acknowledging the limitation of relying on a single species, such as *Alcanivorax*, to represent the entire microbial community in environmental studies is crucial. For example, the degradation of PHAs involves a diverse range of bacteria utilizing extracellular depolymerases, contrasting with *Alcanivorax*'s reactive oxygen species mechanism. While some *Alcanivorax* strains may possess alpha/beta hydrolases for PHA degradation, the underlying molecular mechanisms behind the ability of these strains to degrade such polyesters remained unknown. Besides, research indicated that *Alcanivorax borkumensis* encodes the enzyme ABO2449, exhibiting strong hydrolytic activity on PLA (Zadjelovic et al., 2020). Furthermore, LDPE degradation, primarily driven by oxidation initiated by UV light and/or heat, involves impurities like catalyst residues. While *Alcanivorax* may produce extracellular reactive oxygen species during this process, the role of additives in the initial stages is substantial (Zadjelovic et al., 2022). This underscores the need for a multi-

species approach in environmental plastic degradation studies to capture the diverse enzymatic capabilities and degradation pathways among different microbes.

3.4.2 How does temperature affect marine plastic biodegradation?

So far, there have been multiple studies investigating plastic biodegradation in the air (Ding et al., 2020), soil (Pischedda et al., 2019), compost (Al Hosni et al., 2019), freshwater (X. F. Wei et al., 2021) and even marine sediment (K. Zhang et al., 2021). However, to our best knowledge, the effects of temperature on the biodegradation performance of various plastic types in the marine environment are still poorly understood. Given the global ocean has broad temperatures as the seawater is distributed from pole to equator with various depths (Chen et al., 2021; Peeken et al., 2018), as well as the potential climate change impacts, investigating the temperature influence can advance our understanding of marine biodegradation of plastics.

Some studies suggested that high temperatures encourage biodegradation by stimulating microbial metabolic activities. PLA can achieve high degradation rate under a higher temperature (i.e., >58°C) in soil or composting conditions (Chamas et al., 2020; Rudnik & Briassoulis, 2011). However, higher temperatures may enhance the release of degradation products such as DOC and MPs. Naturally occurring DOC is the primary carbon source for microbes at the base of marine food webs. Nevertheless, the new form of organic matter released from synthetic plastics could interfere with the activity and composition of marine microbial communities (Zhu et al., 2020). Additionally, MPs with a high surface area can trap additives and other pollutants and cause binary toxicity

(Bacha et al., 2023). In this study, we observed elevated levels of MPs and detected acute toxicity to *Vibrio fischeri* in all plastic samples when subjected to higher temperatures (i.e., 22°C) (Fig. 3.25d). The increased release of DOC along temperature was only observed for the biodegradation of PHA but not for LDPE and PLA. It further indicated that the high release of both DOC and MPs during the biodegradation of PHA might be responsible for the induced highest acute toxicity (Fig. 3.25). The acute toxicity caused by biodegrading LDPE and PLA under 22°C may be derived primarily from the released MPs as DOC was rarely emitted. These findings challenge the traditional view that bioplastics are a promising solution to plastic pollution.

Besides, plastic pollution is pervasive in cold climate areas such as the Arctic, even in areas without apparent human activity (Bergmann et al., 2022; Waller et al., 2017). Arctic sea ice is an essential sink for transporting MPs (Peeken et al., 2018). Climate change will further raise the risk as substantial quantities of legacy MPs may be released into the ocean as the sea ice melts (Chen et al., 2021; Obbard et al., 2014). Although previous studies investigated plastic biodegradation by cold-tolerant bacteria, most of the investigated temperature was set at room temperature. According to a review of over 145 studies by Matjašič et al. (2021), 43.4% of them were conducted at temperatures between 25 and 40°C. The present study deployed the *Alcanivorax* species as the model cold-tolerant bacteria to simulate the accelerated marine biodegradation process. It demonstrated that all plastics were fragmented during marine biodegradation under a low temperature (i.e., 4°C) with the release of millions of MPs per cm^2 of exposed surface area. It emphasized that biodegradation of plastics can occur even in low-temperature

regions. More future research is needed on unraveling degradation products during natural bioprocesses and the associated toxicity and risks to cold marine ecosystems.

3.5 Summary

This study systematically compared the biodegradation performance of petroleum- and bio-based plastics under three ambient temperatures by evaluating the bacterial enrichment, morphology alteration, and release of degradation products (i.e., MPs and DOC). The biodegradation rate of PHA is higher than LDPE and PLA, which has been evidenced by the rapid bacterial growth observed on PHA and the resulting more pronounced morphological damage on the plastic surface. Its release of MPs and DOC was more vulnerable to temperature influence. Conversely, PLA and LDPE films had a similarly limited biodegradation performance, and marine biodegradation primarily induced the release of MPs but not DOC. All plastic films could release more than millions of MPs per cm^2 of surface area after 60 days of biodegradation, even under a low temperature (4°C). These findings indicate that marine biodegradation of plastics may further threaten the ecologically fragile cold regions where plastics are also widely distributed. Furthermore, during marine biodegradation, the toxic effects observed under high temperatures are not limited to conventional plastics; they can also arise from bioplastics (i.e., PHA) within 60 days. The future production, marketability, and discharge of the so-called environmentally friendly bioplastics should comprehensively consider their environmental impacts and risks to marine ecosystems.

CHAPTER 4 CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

This thesis evaluates temperature effects on plastics biodegradation in marine environments by conducting a comprehensive literature review and an experimental analysis. Both emphasize the interplay between microbial activity, biofilm formation, enzymatic processes, and plastic biodegradation.

As shown in our critical review, at higher temperatures, microbial metabolic rates and enzymatic activities are generally enhanced to promote faster degradation of hydrolyzable plastics such as PCL and PHAs. Warm temperatures also accelerate biofilm formation and stimulate enzymatic degradation processes, where enzymes such as lipases, cutinases, and depolymerases exhibit optimal activity, enabling efficient breakdown of plastic polymers into smaller and relatively accessible monomers for biomineralization. Conversely, colder temperatures in marine environments typically slow down microbial processes and enzymatic activity. A notable and different finding is that cold-adapted microbes and specific bacterial strains, such as psychrophilic species, exhibit unique adaptations under these conditions. These bacteria enhance the stability of biofilms by producing EPS to aid in the gradual breakdown of plastics. This adaptation underscores the complex nature of plastics biodegradation across different temperatures. Further, non-hydrolyzable plastics such as PE and PS are more resistant to biodegradation due to their structural properties, particularly their robust carbon-carbon backbone. These plastics undergo oxidative modifications facilitated by ROS, generated by specific microbial enzymes.

In the experimental phase, the biodegradation of LDPE, PLA, and PHAs was assessed under varying temperature conditions (4°C, 15°C, and 22°C). The results demonstrated that PHAs supported the highest bacterial growth, exhibited significant morphological damage, and released the most MPs and DOC across all temperatures. Importantly, the degradation by-products of PHAs at 22°C exhibited the highest acute toxicity to *Vibrio fischeri*, highlighting the potential ecological risks associated with the release of plastic degradation products at elevated temperatures.

In sum, the evaluation of temperature-mediated plastic biodegradation has significant implications for addressing marine plastic pollution. The findings are crucial for supporting the development of region-specific strategies to tackle plastic pollution. For instance, in warmer regions, policymakers could prioritize the use of hydrolyzable plastics like PHAs, which degrade more efficiently at higher temperatures, while in colder regions, strategies should focus on reducing the accumulation of non-hydrolyzable plastics like PE and PS, which persist longer. Additionally, the findings highlight the need for improved waste management systems to prevent the release of microplastics and toxic by-products into marine ecosystems, particularly in areas experiencing temperature fluctuations due to climate change.

4.2 Contribution

The contribution of this work lies in advancing our understanding of plastic biodegradation, with particular focus on the role of temperature in microbial activity,

biofilm formation, and enzymatic processes. The critical review synthesizes existing research, providing a nuanced understanding of how temperature influences these factors. It highlights the roles of diverse microbial phyla, including Proteobacteria, Actinobacteria, Firmicutes, and Cyanobacteria, as well as psychrophilic and thermophilic bacteria, in adapting to temperature variations. This integrative perspective bridges knowledge gaps and establishes a foundational framework for studying biodegradation, particularly in marine environments.

The experimental analysis extends this understanding by utilizing a cold-tolerant *Alcanivorax* strain to investigate plastic biodegradation across temperature gradients (4, 15, and 22 °C). This approach offers valuable insights into the biodegradation performance of both petroleum-based and bio-based plastics. It provides empirical evidence of how temperature affects degradation efficiency and the release of by-products such as microplastics and dissolved organic carbon. Notably, PHA exhibit greater susceptibility to marine biodegradation, with a pronounced temperature dependence. In contrast, PLA and LDPE show similarly limited degradation rates across all tested temperatures, predominantly contributing to microplastic release over time. This underscores the persistence of plastic fragments in marine ecosystems. Interestingly, all three plastics release substantial quantities of microplastics, even at low temperatures (4°C).

From an environmental perspective, the findings emphasize the ecological risks associated with temperature-mediated plastic degradation, particularly the increased release of microplastics and dissolved organic carbon at elevated temperatures for PHA.

These risks are heightened under climate change scenarios, where temperature fluctuations can alter microbial dynamics and plastic degradation rates, leading to prolonged pollution and ecosystem damage. Policymakers can use these insights to create guidelines for the use, disposal, and labeling of biodegradable plastics. For instance, there is a need to differentiate between industrial composting conditions and marine environments, where plastics like PLA may persist. Effective policies can help mitigate ecological risks and promote the development of sustainable solutions for managing plastic waste.

In addition, the study encourages greater community and consumer awareness about plastic biodegradation, particularly in colder environments where plastics degrade more slowly. This calls for specialized waste management practices and a focus on addressing persistent marine plastic pollution in cold regions. Consumers of bioplastics are also urged to make informed decisions regarding the use and disposal of plastics. It is essential to understand that biodegradable plastics like PHA degrade effectively in marine environments, while PLA does not. Proper waste segregation and recycling are crucial to minimizing environmental harm and promoting sustainability.

4.3 Recommendations

The future of marine plastic biodegradation depends on understanding temperature-related impacts, especially as climate change intensifies ocean warming, seasonal variability, and extreme events. Integrating microbial genetic databases could greatly

enhance our ability to optimize plastic degradation across diverse thermal environments. These databases can help identify key plastic-degrading enzymes and metabolic pathways, providing insights into how microorganisms adapt to temperature extremes, such as the cold of polar waters or the warmth of tropical seas.

Additionally, advanced imaging techniques such as atomic force microscopy and confocal laser scanning microscopy can be employed to study biofilm formation and plastic surface changes at the micro- and nanoscale. Omics approaches, including metagenomics, transcriptomics, and proteomics, can provide deeper insights into the genetic and enzymatic mechanisms driving plastic biodegradation. These methods will help identify key microbial genes, enzymes, and metabolic pathways involved in the process.

Long-term field studies across various marine climates are crucial for understanding how temperature fluctuations impact microbial communities and plastic degradation pathways. Such research could reveal how microbial communities adapt to temperature shifts, including both seasonal variations and the effects of climate change on marine ecosystems. Additionally, long-term monitoring of recalcitrant plastics, which are more resistant to degradation, can shed light on how temperature influences their biodegradation over time.

Encouraging the production and use of biodegradable plastics, such as PHAs, in marine settings could mitigate the environmental impact of plastic waste. However, it is equally important to evaluate the ecological risks associated with their degradation by-products across different temperatures. By advancing these research directions, this study lays a

foundation for innovative approaches to managing marine plastic pollution and restoring ecological balance in diverse marine environments.

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