# Plastic Degrading Potential of Arctic Deep-Sea Hydrothermal Microbiomes

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# Abstract

Plastic pollution is a significant environmental and global problem that has been on the rise for several decades. The use of plastic has increased dramatically, with a global production of over 350 million tons each year, and only a small percentage of plastic is recycled or incinerated, causing an accumulation in the environment. Plastic pollution into the environment has widespread impacts on nature and human health. Previously, enzymes from microorganisms originating from the inaccessible deep-sea hydrothermal vents along the Arctic Mid-Ocean Ridges (AMOR), have shown promise for the degradation of industrial relevant polymers. Currently, there is strong focus on plastic waste reduction, however, the potential use of microorganisms for plastic degradation is still in its infancy.

The aim of this thesis was to investigate the potential of using targeted *in situ* incubations for microbial plastic degradation in hot sediments from the Aegirs kilde hydrothermal vent field. The *in situ* incubations were amended with synthetic polymers of low- and high-density polyethylene (LDPE, HDPE), polyethylene terephthalate (PET) and polyurethane (PU), when offered as the sole carbon source. After recovery, the *in situ* incubations were used as inoculum for lab-grown enrichment cultures to identify potential microorganisms with plastic-degrading capabilities. The abundance and growth of microorganisms in the cultures was investigated by using a combination of counting chamber and quantitative PCR. In addition, activity assays was conducted by using agar plates with substrate to get an indication of the exoenzymatic degradation of the cultures.

The results indicated successful growth on most of the plastic substrates. Interestingly, cultures added high density polyethylene (HDPE) as the sole carbon source showed stable growth after every transfer to new culture media, with the bacterial abundance stable after the first transfer. The growth curves show exponential growth after only 15 hours of incubation. Overall, the results obtained show promise to elucidate the potential for plastic degraders at deep-sea hydrothermal vent fields at AMOR. However, to reveal the true plastic degradation of the HDPE culture, additional experiments are required such as using isotopic labelled plastic substrates.


Keywords: deep-sea hydrothermal vents, in situ incubations, plastic biodegradation, high density polyethylene (HDPE)

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# 1. Introduction

The production of plastics has increased dramatically from 1.5 million tonnes in the 1950s to over 350 million tonnes in 2019, causing a significant environmental problem as it accumulates as a pollutant (Danso et al., 2019; Xanthos & Walker, 2017). Plastics are man-made, organic polymers from non-renewable petrochemicals like oil, gas, and coal. It is made up of hundreds to thousands organic subunits linked together by covalent bonds. This makes the plastics very durable and adaptable. It also has a light weight, a low production cost, and is resistant to environmental influence and microbial activity (Atanasova et al., 2021). Therefore, it has a wide range of applications, examples include containers and packaging materials, textile, pharmaceuticals, cosmetics, detergents and in transportation and construction. The rise of growing technology and increasing demand for cost-effective, long-lasting and adaptable products has opened up for plastics to be an important substitute for traditional natural materials like wood, leather, stone and glass (Vaksmaa et al., 2021). This makes plastics an unavoidable part of our daily life.

## 1.1 Plastic Pollution: The Environmental Crisis of our Time

Plastic pollution is an accelerating global environmental problem. Over the years, the irresponsible and unethical disposal of plastics has resulted in a build-up of plastic waste in the environment, leading to unforeseen and significant issues for both nature and human health that are difficult to reverse (Chia et al., 2020).

Plastic debris enters the marine environment through rivers, drainage, and sewage systems and with the wind, and can be found both at the shorelines, the ocean surface, and in the deep-sea (Thushari & Senevirathna, 2020). According to the UN, some 11 million tonnes of plastic waste flow annually into oceans, which may triple by 2040 (Bailey et al., 2020). Over 800 marine and coastal species are affected by this pollution through ingestion, entanglement, and other dangers (Harding, 2016). Exposure to plastics can also harm human health, potentially affecting fertility, hormonal, metabolic, and neurological activity, and open burning of plastics contributes to air pollution (Bailey et al., 2020; Flaws et al., 2020).

The research done on the effects of microplastics on humans is still limited. However, existing research suggests that these particles may pose a variety of risks, such as inflammation,

oxidative stress, and damage to organs and tissues. Humans are exposed to plastic particles through diet, oral inhalation, and dermal contact (Prata et al., 2019). Microscopic plastic fragments have also been found in drinking water around the world (Amereh et al., 2020). Lately, plastic particle pollution has been discovered and quantified in human blood too (Leslie et al., 2022).

A study done on fish exposed to microplastics showed that ingesting plastic debris may have altered their endocrine system function, which can be critical for development of reproductive systems and potentially populations (Rochman et al., 2014). A Norwegian group of research institutes has found methods for quantification of microplastic in fillets and organs of farmed and wild salmonids, however, biological effects were not evaluated within that study (Gomiero et al., 2020). To fully understand the potential risk of microplastic to human health, more research is needed, especially because of the expected increase in synthetic polymers (Prata et al., 2019).

## 1.2 Global Commitments

Various countries have implemented the United Nations sustainable development goals (SDGs[1]) to achieve sustainability. They are based on the collective decision by all developed and developing countries to take action against the most critical multi-dimensional socio-economic environmental global threats to tackle global challenges such as poverty, inequality, injustice, and climate change (Kumar et al., 2021). One of the SDGs targets plastic pollution directly, SDG 14: *life below water*. The goal is to prevent or reduce marine (micro)plastic debris pollution from land-based activities in the ocean by 2025[2]. Indirectly, plastic pollution can be incorporated into all the other SDGs, especially in food security and hunger, since microplastics reach the marine ecosystems threaten marine health.

According to a press release by the United Nations Environment Program (UNEP), nations have endorsed a historic resolution to end plastic pollution and forge an international legally binding assessment by 2024. The resolution addresses the full lifecycle of plastic, including its production, design, and disposal (UNEP, 2022).

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[1] https://sdgs.un.org/goals
[2] https://sdgs.un.org/goals/goal14

## 1.3 Impact of (Micro)Plastics on Ecosystems

There are several damaging impacts of plastic pollution on ecosystems. Manufactured or processed solid material discharged in marine or coastal environments is defined as marine debris. Besides metal, glass, paper, wood, and clothing, the most abundant material found is plastic, and it is of particular concern due to its persistence and fragmentation into microplastics (<5 mm) and nanoplastics (<0.1 μm) (Gall & Thompson, 2015; Oliveira et al., 2021). According to the surface-to-volume-ratio principle, the smaller an item is the bigger the surface (Ahmed et al., 2021). This allows for contaminants to get transported with the microplastic. Contaminants constitute for example the substances the plastic consists of, such as monomers, oligomers, bisphenol-A, phthalate, or brominated flame retardants. Other toxic substances in the ocean can also be adsorbed to the surface of the plastic (Gall & Thompson, 2015). Once organisms ingest the (micro)plastic, the toxic substances can persist within their tissues (Verma et al., 2016).

The deleterious effects of plastic pollution on wildlife are not only significant, but also visually apparent, as they get entangled and can restrict movement, and can cause starvation or drowning. This type of pollution poses a direct threat to a variety of animals, such as fish, seabirds, marine mammals, and benthic invertebrates. These animals are prone to mistaking plastic debris for food, which can lead to a many health issues. Ingesting plastic can cause a reduction in feeding stimuli, gastrointestinal blockage, decreased levels of enzymes and hormones, and ultimately, reproductive defects. It is worth noting that the plastic particles persist in the digestive systems of these animals, exacerbating the severity of their deleterious effects (Webb et al., 2013).

## 1.4 Plastic Degradation in the Environment (Biodegradation)

Degradation of plastic polymers is done by different factors and mechanics, such as thermal degradation, photo-induced degradation, mechanical degradation, hydrolytic degradation, ultrasonic degradation, or chemical degradation (Nkwachukwu et al., 2013). The first step of plastic degradation in the environment is caused by mechanical disruption by natural forces, such as wind and waves, or by UV light exposure. The chemical, physical and mechanical properties of plastic change and result in microplastic (Danso et al., 2019). Another type of polymer degradation is by biological activity, known as biodegradation. Biodegradation is defined as any physical or chemical change in a material caused by biological activity, including

bacteria, fungi, and actinomycetes. The biodegradation of polymers by microorganisms is a prolonged process (Alshehrei, 2017). Microorganisms can attach to the surface of the (micro)plastic, and then grow using the polymer as a carbon source. Secreted exoenzymes degrade the large polymers into smaller subunits that can pass through the semi-permeable outer bacterial membranes into the microbial cell. Once in the cells, either the oligomers or the degradation products of these are funneled through the classical degradation pathways to yield energy and/or serve as building blocks for catabolism or metabolism (Lucas et al., 2008). Microbes can also develop new enzymes and pathways to break down anthropogenic compounds through the natural selection of mutants, and novel metabolic pathways can be discovered (Amobonye et al., 2021).

During aerobic biodegradation of plastics, carbon dioxide, and water is produced as by-products in the breakdown of organic chemicals into smaller organic compounds, by using oxygen as an electron acceptor. While in anaerobic biodegradation of plastics, the electron acceptor can, for example, be sulphate, iron, or nitrate, where carbon dioxide and water as well as other metabolic products are produced (Alshehrei, 2017).

Recent research has also opened the opportunity for potential microbial consortia in the biodegradation of plastic. Skariyachan et al. (2022) define microbial consortia as the combinations of various microorganisms that can degrade polymeric materials with more significant biodegradation potential than individual isolates (Skariyachan et al., 2022). These consortia can either be natural or artificial. The engineering of microbial consortia allows for selection of strains with desired characteristics. Cooperative microbial activity on plastic polymers enables the distribution of the metabolic products to be utilized by different bacteria strains, and some functions can also be impossible for only one strain to perform (Brenner et al., 2008; Han et al., 2020). Additionally, in an engineered consortium, the populations are more robust to changes in the environment than individual isolates because of the metabolic diversity (Brenner et al., 2008).

## 1.5 Plastic Polymers as a Carbon Source

The research into biodegradation of polymers is increasing (Ren et al., 2019). As a result, bacteria associated with the degradation of plastic polymers have been found in various locations, including dumpsites, recycling sites, landfills, cold marine environments, and even in the gut of insects (Amobonye et al., 2021). However, the synthetic nature, hydrophobicity,

and inaccessibility features of plastic make it a difficult target for animal, fungal, and microbial-derived enzymatic activities (Sanluis-Verdes et al., 2022).

### 1.5.1 Polyethylene (PE)

Among the most used plastics is polyethylene (PE), as it is used for shopping bags, containers, and bottles. Low density polyethylene (LDPE) and high density polyethylene (HDPE) differ in density, the degree of branching, and the availability of functional groups on the surface (Restrepo-Flórez et al., 2014). PE has a high molecular weight, a hydrophobic nature, and lacks functional groups that microbial enzymatic systems can recognize. Therefore it is considered non-biodegradable (Kundungal et al., 2019). Waste degradation of PE in landfills can have a negative effect on soil quality, and disturb soil fauna (Ali et al., 2016). The incineration of PE produces vapors containing toxic compounds and emission of greenhouse gases (Kundungal et al., 2019).

The research on PE biodegradation by microorganisms has therefore increased, resulting in several isolates capable of interacting with PE (Restrepo-Flórez et al., 2014). Additionally, enzymes from the waxworm *Galleria Melonella* gut microbiome, the invertebrate itself, or both, have shown to degrade PE (Bombelli et al., 2017; Sanluis-Verdes et al., 2022). However, despite it being the plastic type with the highest global annual production, there are currently no biochemically characterized enzyme capable of degrading this polymer (Buchholz et al., 2022; Restrepo-Flórez et al., 2014).

### 1.5.2 Polyethylene Terephthalate (PET)

Polyethylene terephthalate (PET) is also among the most used plastics and shares many of the properties as PE, and is mostly produced as bottles, packaging materials and as synthetic fibers in the textile industry (Webb et al., 2013). PET can be recycled into new materials by thermally degrading it. However, the reprocessed materials cannot be degraded thermally a second time and are therefore often incinerated, generating greenhouse gasses. Another solution for PET waste is "chemical recycling", a process where PET is decomposed into monomers that can be repolymerized into new plastic. However, chemical recycling is more expensive than using monomers from fossil fuels (Hiraga et al., 2019).

Biodegradation of PET is needed to prevent the accumulation of plastic in nature. One of the best-studied models for enzymes capable of degrading PET is from the isolated *Ideonella*

*sakaiensis* 201-F6, by Yoshida et al. (2016), which is described to use PET as its energy and carbon source and convert it into its monomers. The biodegradation is started by the excreted PET hydrolases (PETase) from the attached bacteria, breaking down its ester bonds. The products are terephthalic acid (TPA) and ethylene glycol (EG), mono(2-hydroxyethyl) terephthalate (MHET), and bis-(2-hydroxyethyl) terephthalate (BHET). MHET can be further hydrolyzed into TPA and EG by MHET hydrolase (MHETase) (Qi et al., 2021). The PET film was almost completely degraded after six weeks at 30°C (Yoshida et al., 2016).

### 1.5.3 Polyurethanes (PU)

Polyurethanes account for 6-7% of the plastics generated and have a wide range of applications (Biffinger et al., 2015). PU is catalytically synthesized from different isocyanates and a balanced mixture of glycols (Liu et al., 2021). Depending on the additives, polyurethanes range from flexible to rigid and from liquid to solid (Peng et al., 2014). It is used in foams, fibers, adhesives, coatings, etc.

Studies have shown that proteins and organisms have been able to degrade polyurethanes (Biffinger et al., 2015). The microbial degradation of PU can be validated using Impranil®DLN (Impranil) (Bayer Materials Science, PA), where the culture goes from a milky liquid to a clear one or as clearing zones in agar. There are two types of polyurethanes, polyester, and polyether, where polyester polyurethane is more susceptible to microbial degradation (Osman et al., 2018). A research article from Hung et al. (2016) showed that extracellular enzymes from Pseudomonas species degraded Impranil (Hung et al., 2016). However, because of the complexity of the polymer, efficient biodegradation of a promising rate has not been reported (Liu et al., 2021).

### 1.5.4 Biodegradable Polymers

Polycaprolactone (PCL) is recognized as a fully biodegradable synthetic polymer, where its ester bonds are degraded by hydrolysis (Reshmy et al., 2021). Bacteria, fungi, and algae can, in a bioactive environment, degrade biodegradable polymers through enzymatic action (Gross & Kalra, 2002). PCL is commonly used to produce specialized polyurethanes (PU) (Jha & Kumar, 2019). The polymer can be used in packaging but also in tissue engineering, and other biomedical applications, because of its long degradation time, when compared to natural biopolymers like starch and collagen (Jha & Kumar, 2019; Lu Wang et al., 2013).

## 1.6 Hydrothermal Vent Systems

Hydrothermal vent systems are formed when cold seawater seeps into the rocks heated up from lava along the mid-ocean ridges. The water is then heated and rises back up to the surface as extremely hot fluid. This fluid is enriched with minerals extracted from the rocks below (Dick, 2019). Hydrothermal deposition is due to the rapid temperature decrease when the hydrothermal fluid enters the cold oceanic water. The minerals precipitate and build up to form the unique chimney structures. The chemical composition of the vent fluid varies depending on the underlying rocks and affects the structure and composition of the vent community (Amend et al., 2011; Desbruyères et al., 2001).

The discovery of deep-sea hydrothermal vents with unique ecosystems represents one of the most fundamental scientific breakthroughs of the 20th century. They were first discovered in the late 1970s in the Galapagos rift (Corliss et al., 1979) and harbor more life than what was expected. Before this time, it was unimaginable that the deep-sea could sustain organisms living solely on chemical energy from the Earth's interior. What makes these systems so extreme is that they are found along the spreading ridges in the deep-sea, where the hydrostatic pressure is high. The surrounding water is cold (2°C), compared to the emerging fluids from the hydrothermal vents which can reach up to 400 °C (Wirth, 2017). Vent fields have been discovered along spreading ridges from the southern Bransfield Strait close to Antarctica to the northern Central Arctic Ocean (Klinkhammer et al., 2001; Ramirez-Llodra et al., 2022) (Figure 1). The deep-sea is known for being a resource-scarce environment, where nutrients mainly come from the sinking of organic matter from surface waters. This leads to low levels of productivity, but supports a diverse range of benthic communities which are mainly composed of deposit and filter-feeding organisms (Georgieva et al., 2021). However, compared to non-chemosynthetic deep-sea benthos, the hydrothermal vents are characterized by communities with a high abundance of a few organism species (Ramirez-Llodra et al., 2007). Meanwhile, hydrothermal vents possess one of the highest microbial diversity worldwide (Synnes, 2007).
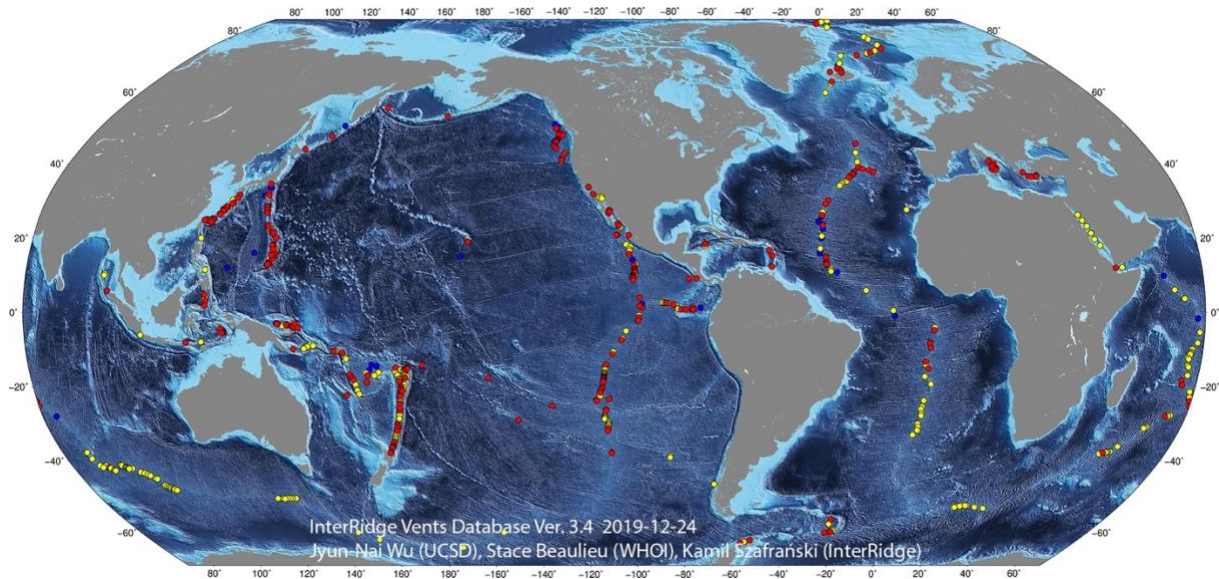
**Figure 1:** Global map of the discovered hydrothermal systems. Systems that are confirmed are in red, inferred in yellow and inactive ones in blue. Image is retrieved from the InterRidge Vents database Version 3.4, CC BY-NC-SA 4.0 (Beaulieu & Szafranski, 2020).

### 1.6.1 Microbial Diversity of Deep-Sea Hydrothermal Vents and Their Habitats

Microorganisms in hydrothermal vents live in different habitats defined by physical, chemical, and biological characteristics. The structures of microbial communities identified in hydrothermal fluids are limited by the amount of energy available (Storesund & Øvreås, 2013). Temperature is a significant factor influencing microbial diversity, with anaerobic thermophiles (optimum growth at >50°C) and hyperthermophile (optimum growth at >80°C) microorganisms found in the hot, anoxic fluids streaming through the chimneys (Arbab et al., 2022). Plumes and subsurface seafloor areas also harbor diverse microbial communities, along with animal symbionts. Hydrothermal vent ecosystems rely on chemosynthesis, with bacteria using chemicals from the vents to produce energy for themselves and their hosts. Despite high temperatures and toxic chemicals, these bacteria provide carbon to their hosts and can switch between different ways of producing carbon depending on their environment (Dick, 2019).

### 1.6.2 Arctic Mid-Ocean Ridge (AMOR)

As defined by Pedersen et al. (2010), the Arctic Mid-Ocean Ridges (AMOR) is a 4000 km long ultra-slow spreading ridge, extending from the Icelandic shelf to the Laptev Sea. The ridge can

be divided into six segments: the Kolbensey Ridge, Mohns Ridge, Knipovich Ridge, Molloy Ridge, Lena Trough and the Gakkel Ridge (Rolf B. Pedersen, Thorseth, et al., 2010). From the southern regions of the Kolbensey Ridge, the depth is approximately 1000 m, impacted by the Iceland hotspot. North towards the Knipovich Ridge, the depth increases to approximately 2500-3000 m, while at the Gakkel Ridge, the depth can reach up to approximately 5300 m (Cruz et al., 2016). In the Mohns ridge segment, the first active vent fields discovered were the Jan Mayen vent fields at the southern end and Loki´s Castle at the northern end, in 2005 and 2008, respectively (R. B Pedersen et al., 2005; Rolf B. Pedersen, Rapp, et al., 2010). Since then, more active vent fields in Mohns Ridge have been discovered and analyzed, like Fåvne discovered by the Norwegian Petroleum Directorate (Stenløkk et al., 2019). The Aegirs kilde vent sites was discovered on an expedition with R/V G.O Sars by UiB in 2015 (R. B. Pedersen & Bjerkgård, 2016) (Figure 2). The depth of Aegirs kilde is about 2300 m. The vent field covers about 5000 m$^2$ and consist of five to six clusters of relatively small chimneys, also with diffuse venting from the chimneys and the seabed (Rolf B Pedersen et al., 2021).
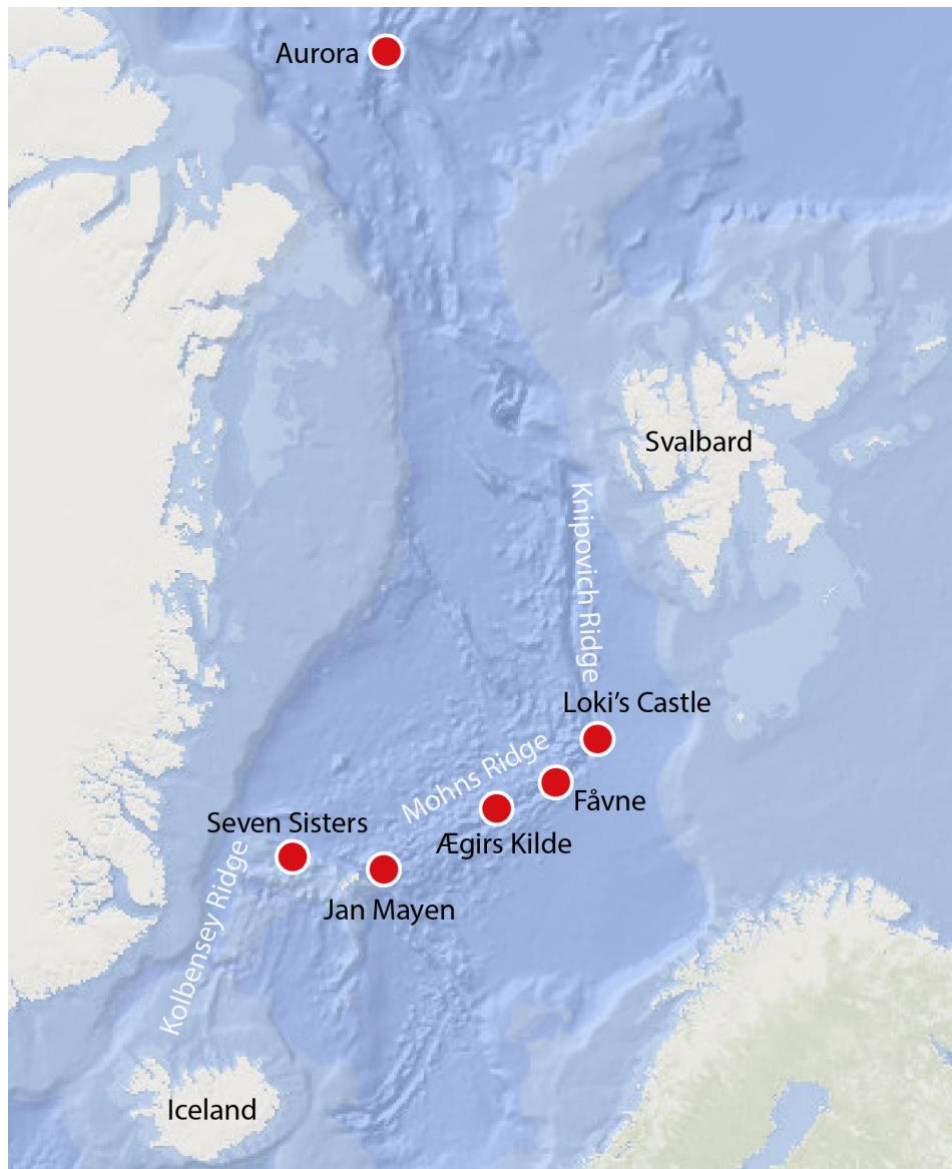
**Figure 2:** Map of Mohns Ridge at the Arctic Mid-Ocean Ridge (AMOR), with the active vent fields Jan Mayen, Aegirs Kilde, Fåvne and Loki´s Castle.

## 1.7 Bioprospecting of deep-sea hydrothermal vents

Microorganisms found in extremes of temperature, pressure, salinity, and pH are called extremophiles (Van den Burg, 2003). Survival in extreme environments requires adaptions of all biomolecules within the cells, and the extremophiles produce different enzymes capable of catalyzing specific biochemical reactions under these harsh conditions (Synnes, 2007). The exceptional characteristics of these organisms have led to the development of novel applications of enzymes in industrial processes (Van den Burg, 2003). The collection and screening of biological material for commercial purposes is described as bioprospecting

(Synnes, 2007). Over the past 20 years, there has been a significant increase in the number of studies on deep-sea extremophiles. However, only a minor fraction of these enzymes are applied in industry (Jin et al., 2019).

## 1.8 Aim

The bacterial and archaeal communities in this study were collected on a research cruise with the University in Bergen to Mohns Ridge in July 2022. Studies of deep-sea vent microbes aim to identify novel enzymes for industrial bioprospecting. Microbial communities can overcome various environmental challenges by modifying their metabolic capacity through genome alterations. This adaption can enable them to integrate new substrates into their metabolic pathways (Amobonye et al., 2021). As plastic pollution into the environment is among the most problematic environment-related issues presently, there is a significant interest in finding a solution to it (Citterich et al., 2023). With much attention drawn to microplastic as an emerging threat to wildlife and humans, this microbiology study is directed at the potential of enzymes from Arctic deep-sea hydrothermal microbiomes degrading microplastics (Fackelmann et al., 2023).

The aim of this thesis is to investigate the potential of *in situ* enriched bacterial and archaeal communities from sediment slurries collected at the Arctic Mid-Ocean Ridge hydrothermal vent fields to degrade the synthetic polymers low- and high-density polyethylene (LDPE, HDPE), polyethylene terephthalate (PET) and polyurethane (PU) when offered as the sole carbon source.

The study will be achieved by setting up enrichment cultures of potential plastic-degrading microorganisms with subsequent plastic-degrading capabilities using lab-grown cultures. Then to measure the abundance of microorganisms in the cultures, a qPCR will be done on the starting material and after every culture transfer. An activity assay will be conducted using agar plates with the substrate to indicate the exoenzymatic degradation of the cultures. The growth of the culture was also researched by counting the number of cells and by qPCR.

# 2. Materials and Methods

## 2.1 Site Description and *in situ* Incubation

The Aegirs Kilde vent field is located at the Arctic Mid-Ocean Ridges (AMOR), between Svalbard and Jan Mayen (R. B. Pedersen & Bjerkgård, 2016). In July 2022, as part of a research cruise arranged by the Center for Deep-Sea Research (CDeepSea, UiB), a remotely operated vehicle (ROV) deployed incubators near an active vent site at Aegirs Kilde (Figures 3 and 4). The five titanium incubators had four chambers each (Stokke et al., 2020). Each incubator contained hydrothermal sediment mixed with a different type of plastic; polyethylene terephthalate (PET), low-density polyethylene (LDPE), polyurethane (PU) pellets and foam, and a control with three chambers (containing no substrate). The temperature varies in the sediments close to the vent sites, creating a temperature gradient for each chamber in the incubators. While incubated, incubators 3 and 4 were equipped with an internal temperature probe that measured the temperature at 2-hour intervals. After six days of incubation, the incubators were collected and sampled for microbial composition.
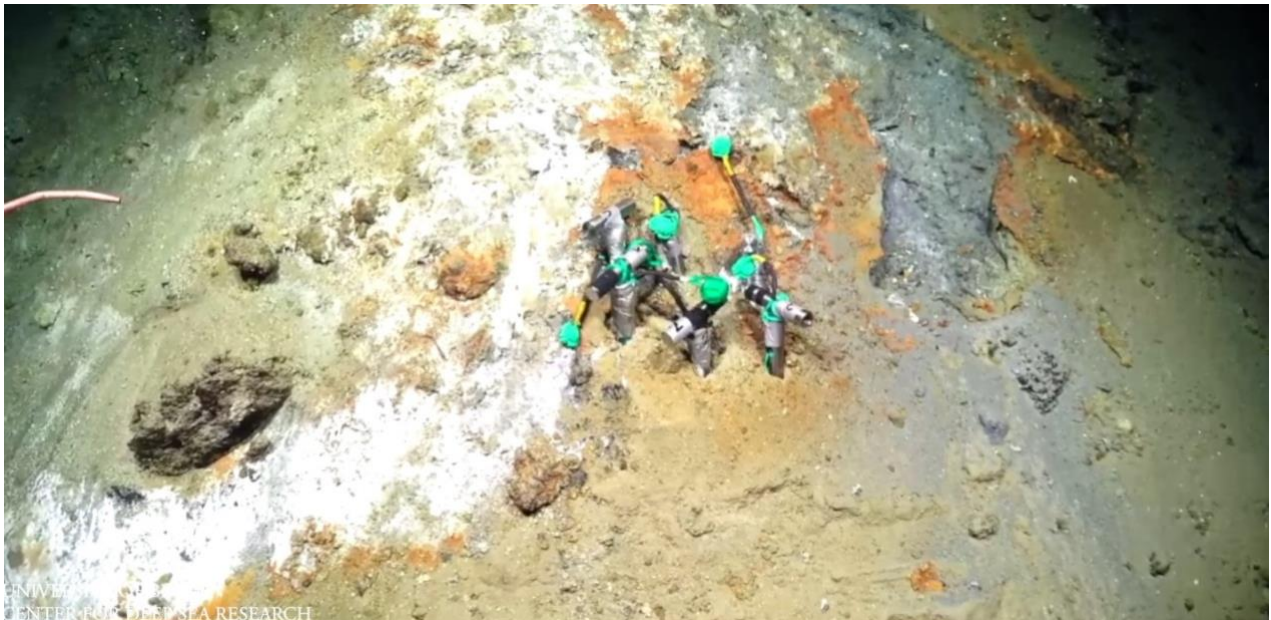


**Figure 3:** Image of the surface of sediments where the *in situ* incubators were deployed at Aegirs kilde hydrothermal vent field.
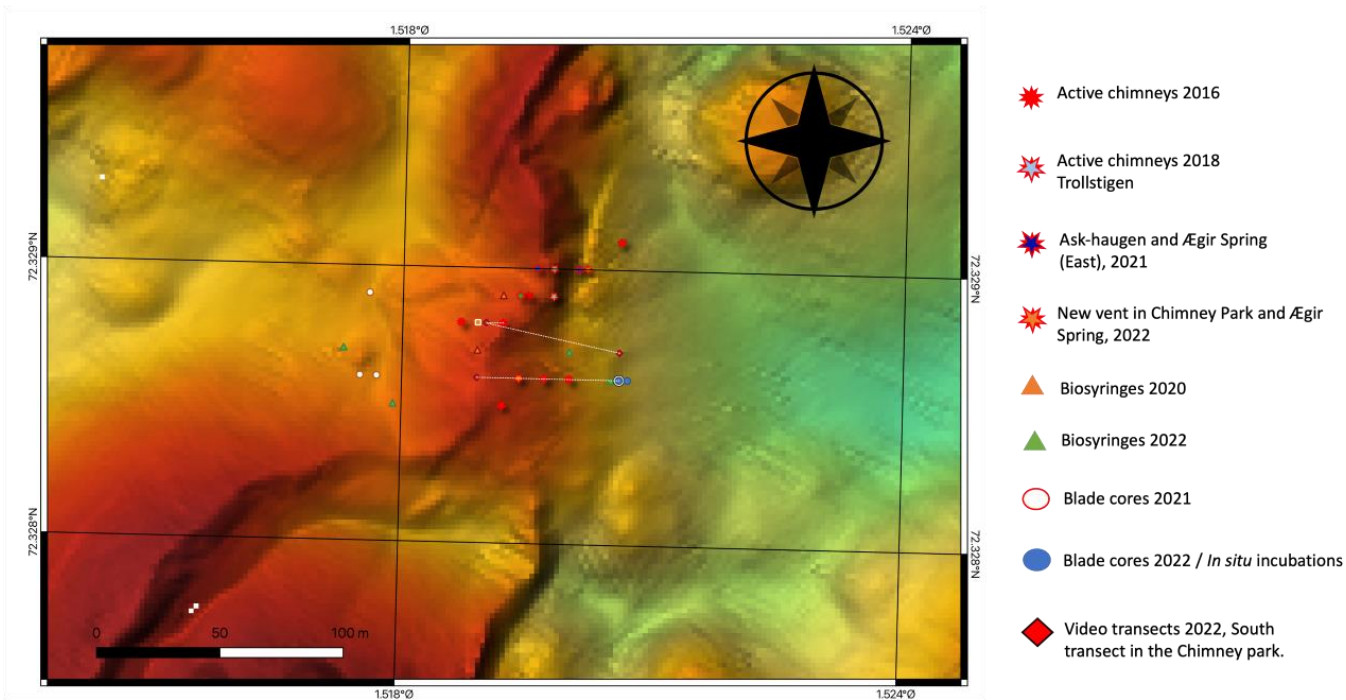
**Figure 4:** Bathymetry map of Aegirs kilde with active chimneys and sampling marked according to the information on the right side. *In situ* incubations were placed where the blue circle is located near active venting chimneys. Bathymetry data and image in courtesy of Thibaut Barreyre (CdeepSea) and Runar Stokke, respectively.

The *in situ* incubations with the substrates were collected after the six days incubation period. From each chamber, subsamples were taken (Figure 5). Subsamples were taken and fixed for FISH (fluorescence in situ hybridization) and SEM (Scanning Electron Microscopy) to visualize and identify the possible microorganism in the samples. Another subsample from each chamber was directly frozen to preserve the microbial communities. The last subsample was combined with sediment and medium to make slurries for long-term storage. The slurries were used as inoculum for the different enrichments and plates for cultivation in the current study.

**Figure 5:** Image of one of the titanium incubators and subsampling after retrieval.

## 2.2 Cultivation of Microbes Supplemented with Plastic

The microbes were cultivated using specialized media and specific growth conditions. All samples were cultivated in aerobic or anaerobic liquid medium, and on aerobic PBS agar plates. The incubation temperature was 30, 55, and 65°C.

### 2.2.1 Cultivation in Liquid Medium

The cultivation of microorganisms was done in anaerobic and aerobic medium. The cultures were incubated at 30, 55, and 65°C. The substrates used were PU (Impranil DLN Suspension, Covestro), PET (300 Mikron, half-crystalline, copolymer, powder, Goodfellow), LDPE (300 Mikron, powder, Goodfellow) and HDPE (Thickness 0.01 mm, film, Goodfellow). The cultivation was done in 100 ml serum bottles, with 50 ml medium.

To cultivate anaerobic microorganisms, anaerobic liquid medium was made according to the procedure described by Leigh et al. (1981) (Leigh et al., 1981), with the following changes. The amount per liter basal medium of the following components was changed to; 1ml resazurin, 10 g NaCl, and 0.1 g yeast extract. $Na_2S \cdot 9H_2O$ and $NaHCO_3$ were not added, however, 5.4 g of $KHCO_3$ was added. The amount of $Na_2HPO_4 \cdot 2H_2O$ in the buffer medium was changed to 8.9 g/L. From the enrichment medium described in the procedure, 0.1 g of yeast extract and 10 ml vitamin solution was added. The HDPE, LDPE, and PET substrates were added to anerobic medium in a COY Lab Vinyl Anaerobic Chamber. The samples and equipment were placed in the airlock, and the air was flushed four times with nitrogen gas and one time with a mix of nitrogen gas and hydrogen gas with a vacuum phase in between. When the oxygen level in the chamber reached 0 ppm and the hydrogen between 1-3%, the chamber was anoxic. A few flakes of the HDPE film substrate were added to each serum bottle. Enough amount of LDPE and PET powder substrates were added to their corresponding serum bottles just to cover the surface of the medium. For PU, a syringe was used, adding 100μl to each serum bottle. Active cultures were transferred to fresh medium by adding 1 ml into a fresh medium.

For the cultivation of aerobic microorganisms, the culture media was based on the M9 mineral medium from the *Molecular biological methods for Bacillus* book by Harwood (Harwood & Cutting, 1990). Since no glucose was added, the amount of sterile water added was 887 ml instead of 867 ml.

Activity in the cultures was observed using a microscope or through direct observation. After activity was observed, 1 ml of the culture was transferred to fresh medium with substrate. For each culture transfer, a subsample was collected for downstream qPCR experiments and stored in a freezer at -20°C.

## 2.3 Activity Assay

An activity assay was conducted by using agar plates with substrate to get an indication of the exoenzymatic degradation of the samples. Samples of 500 µl were centrifuged for 10 min at 14 000 rpm to divide the cell material and the enzymes released by the cells. A few drops of the sample were placed on the agar plates and incubated at the respective temperatures (30, 55 and 65°C.)

Plates containing the polymer substrates polycaprolactone (PCL) and bis(2-hydroxyethyl) terephthalate (BHET) were made to assess the bacterial activity, according to the procedure made by Pérez-Carcía et al. (Pérez-García et al., 2021). Plates containing polyurethane (PU) Impranil®DLN (Impranil) (Covestro Leverkusen, Germany) were made according to the procedure made by Molitor et al. (Molitor et al., 2020).

## 2.4 Growth Curve – Cell Counting

Growth curves were made to measure the growth of the cultures and calculate the generation time. The growth of cells in active aerobic culture HDPE (30°C) was measured by counting in a microscope over a set period. A particular slide for direct microscopic count (DMC) called the Petroff-Hausser counting chamber was used, according to the counting manual associated with the counting chamber at BIO (Thoma, Assistent®). The counting chamber is a glass plate with an engraved grid and a profile giving the preparation a given thickness. The size and depth of each route is known, and therefore the volume can be calculated. The Thoma chamber has a depth of 0.02 mm, and a surface of $0.04 mm^2$. This gives a volume of $5*10^{-8} mL$.

To get familiarized with the growth of the sample, a test count was done over three days, where there were two different dilutions, one where 0.5 ml of active sample was added to 50 ml of fresh medium, and one where 1 ml was added. Then, another count was done to look for improvements, such as more frequent counting. For the final counting of cells, the cells were counted two times on the first day, three times on the second and third days, and once on the fourth day. The number of cells against the time (hr) were plotted into an Excel sheet, and a line graph was made.

The generation time was measured using the numbers from the counting with this exponential growth model:

$$y = Ae^{Bx} \text{ (Formula 1)}$$

Where *y* is the population size at time *x*. *A* is the initial population size. *B* is the exponential growth rate. This model can be used as the population size at any time *x* and can be predicted if the population size at time *x* represents the number of cells in the culture with a constant growth rate. The calculation of generation time (*G*) is the time it takes for the cells in the culture to double, which they do during the exponential phase. The generation time is calculated using the exponential growth rate (*B*) and the natural logarithm (*ln*) with the following equation (Widdel, 2010):

$$G = \frac{\ln(2)}{B} \textbf{ (Formula 2)}$$

For each time the cells were counted, a subsampling to do a qPCR was done.

## 2.5 Growth Curve – qPCR

For every transfer of active culture to fresh medium, subsampling was done to extract the DNA and to do a qPCR. Subsamples were also taken from each time the cells were counted. Quantitative PCR (qPCR) is used to quantify gene copy numbers in a sample. Like in a normal polymerase chain reaction (PCR), the target gene is amplified with a heat-stable Taq polymerase, primers and nucleotides through cycles of denaturation, annealing and elongation (Joshi & Deshpande, 2010). This method enables fast and effective quantification of target sequences within a diverse community (Smith & Osborn, 2008). To quantify the number of PCR amplicons, two fluorescents detection methods can be used. One is with a SYBR Green dye that binds to double-stranded DNA and emits fluorescence that can be detected after each cycle (Wittwer et al., 2018). On the other hand, the dye is not specific and will bind to other double-stranded DNA as well, which can lead to false positives (Tajadini et al., 2014). The other method is with fluorescently labeled probes. A TaqMan probe is nucleotides labeled with a fluorescent reporter dye and a quencher that hybridizes to the target sequence and when the polymerase reaches it, the reporter dye is released from the quencher molecule, and emits fluorescence (Tajadini et al., 2014). With both methods, the quantity of DNA amplicons is measured by the amount of fluorescence after each cycle. One limitation when quantifying the cell numbers of an unknown species in a diverse community sample is the variations of gene copy numbers of the 16s rRNA (Smith & Osborn, 2008).

A qPCR was done in this case instead of Optical Density (OD) measurements due to the samples containing plastic substrates, which could influence the results.

### 2.5.1 DNA Extraction

DNA from 14 of the subsamples from transfer of active cultures, and ten samples from the cell counting analysis were extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). The samples were the aerobic HDPE 6-1 (30 ℃), the anaerobic LDPE 6-1 (50℃), and the anaerobic LDPE 6-1 AN (65℃) from five culture transfers (four for the anaerobic LDPE 6-1 (65℃) culture). The culture number refers to titanium incubator (first digit) and the chamber (1-4) (last digit). The volume of each sample was 1.5 ml. The DNA was extracted according to the manufacturer's protocol with the following changes. These samples were not soil samples; therefore, the samples were centrifuged at 16162 x g for 15 minutes to create a bacterial pellet, which was mixed with the Sodium Phosphate Buffer in the protocol. The elution volume of the extracted DNA was 50 µl.

DNA concentration was measured by an Invitrogen Qubit™ 4 fluorometer device (Invitrogen) according to the manufacturer's protocol to check the quantity before continuing with qPCR.

### 2.5.2 Preparation of DNA Standards

Prior to the qPCR, standards were made for the machine (thermocyclerCFX96™ Real-Time System (BIO-RAD)) to have concentrations to compare with. The sample used for the standards was a HDPE culture with high turbidity and biofilm. The quality of the DNA extraction of the standard was verified in a gel electrophoresis. Then, a PCR of the wanted sequence was done. The PCR product was then cleaned and concentrated, and a 1:10 dilution series was made.

#### 2.5.2.1 Gel Electrophoresis

First, an agarose gel (1% TAE) was made to check the DNA quality. GelRed® Nucleic Acid Gel Stain 10,000X (Biotium) was used according to manufacturer's protocol to stain the DNA in the gel. For approximate quantification of the sample in the gel, 5 µl GeneRuler 1 kb DNA Ladder (Thermo Scientific™) was used. The dye used to monitor the migration of DNA in the gel, was the 6X DNA loading dye (Thermo Scientific™), 1 µl was mixed with 5 µl of the

sample. The gel was run for 45 minutes at 200V. The bands in the gel were then visualized under UV light with the ChemiDoc™ XRS+ Gel Imaging System (BIO-RAD).

### 2.5.2.2 PCR of the Standard

A PCR was performed on the sample used as standard. The PCR was done according to the HotStarTaq Master Kit (QIAGEN) protocol. Bacterial 16S rRNA was amplified with the primers Bac 338 Forward 5´-3´(ACTCCACGGGAGGCAGCAG) 10 µM and Prun518 Reverse 5´-3´(ATTACCGCGGCTGCTGG) 10 µM (De Mulder et al., 2019). For the positive control, a colony from *Escherichia coli* was used. The cycling program was set according to manufacturer's protocol with an initial heat activation for 15 min at 95℃, denaturation for 1 min at 94℃, annealing temperature for 1 min at 56℃ and an elongation time of 30 seconds at 72℃.

To ensure that the amplicon was the right size and that there was no contamination, gel electrophoresis of the samples was done, see Section 2.5.2.1.

### 2.5.2.3 Clean and Concentrate PCR Product

The PCR product was then purified according to the DNA clean & concentrator -5 (Zymo research) protocol to a volume of 18µl. The DNA concentration was measured on Qubit™ 4 fluorometer device (Invitrogen) according to their protocol.

### 2.5.2.4 Dilution Series of the Eluted DNA

A 10-fold dilution series of the clean and concentrated PCR product was made to give the qPCR machine (thermocyclerCFX96™ Real-Time System (BIO-RAD)) a range of gene copy numbers to compare the number of genes in the samples. The standard had to have a higher and lower number of gene copies compared to the samples. The standard (18µl) was diluted with 27µl RNase-free $H_2O$ to make the dilution series.

### 2.5.3  Trial qPCR Run

A trial qPCR run was made to determine what sample concentration best suit the standards. This is so the qPCR machine can compare the samples and so that the samples have good qPCR efficiencies.

The samples were mixed well and then diluted into three concentrations: 10 ng/µl, 1 ng/µl and 0.1 ng/µl. A master mix was made with the components needed in qPCR, and a 96-well plate layout with the different concentrations was set up. The qPCR was run, and the optimal DNA concentration for the samples was determined.

### 2.5.4   qPCR of Samples

After the trial qPCR run with different DNA concentrations, an optimal concentration was determined according to the melt curve of the qPCR analysis (Figure 12, Appendix A). All the samples were then diluted to this concentration. The SssoFast$^{TM}$ EvaGreen$^{®}$ Supermix-kit (BIO-RAD) was used for the qPCR reaction according to the manufacturer's protocol. The thermocyclerCFX96$^{TM}$ Real-Time System (BIO-RAD) was used according to the manufacturers protocol, with an enzyme activation temperature of 95°C for 2 minutes, and a denaturation temperature of 95°C and annealing temperature of 56°C for 30 seconds each in 40 cycles.

The number of copies of a template present in the sample was determined using a calculator provided by Andrew Staroscik at URI Genomics and Sequencing Center (Staroscik, 2004). The formula used is:

$$number\ of\ copies = \frac{(amount * 6.022 * 1023)}{(length * 1x109 * 650)}$$

The average weight of a single base pair (bp) is assumed to be 650 Daltons, and one mole of a bp weighs 650 grams. From this, the template's estimates molecular weight was calculated, multiplying the length in bp with 650. The inverse of the molecular weight is the number of moles of template present in one gram of material. To calculate the number of molecules of the template per gram of material, the inverse of the molecular weight can be multiplied with Avogadro's number (6.022*10²³ molecules/mole):

$$\frac{Mol}{g} * \frac{molecules}{mol} = \frac{molecules}{g}$$

To convert this number into the number of copies of the template in the sample, it is converted to nanograms (multiplied with $1 \cdot 10^9$) and multiplied by the amount of templates in the sample (in nanograms).

## 2.6 Nanopore Sequencing

In the project description, long-read nanopore sequencing was added as a wild card if time allowed. However, due to time constraints, this was not feasible within the project period. However, Nanopore sequencing was performed by my supervisors Katharina Sass and Runar Stokke and the microbial composition was implemented in the discussion.

# 3. Results

## 3.1 Temperature Profile of *in situ* incubators and On-Board Work

Each titanium incubators used in this study consisted of 4 chambers with a volume of 16 ml (2.5 cm radius and 5.5 cm in length) with a pore size of 1 mm (Stokke et al., 2020).

Incubators 3 and 4 contained a temperature probe to measure the temperature in the sediments where incubation occurred (Figure 6). The temperature measurements were done in a 2-hour interval throughout the whole incubation time (146 hours). Each chamber within the incubator has an approximate length of 5.5 cm, resulting in the total depth of the incubator to 22 cm into the sediments.

For incubator 3 (INC3), the chambers with depths of 16.5 and 11 cm shows the highest temperature of around 90℃. The chamber with the deepest placement in the sediments (22 cm), had a temperature of about 80-85℃. The chamber closest to the surface (5.5 cm deep) had the lowest temperature of approximately 70℃.

Regarding the temperature measurements of incubator 4 (INC4), all the chambers exceed a temperature of 95℃. The chamber placed deepest in the sediments had the highest temperature of 105-106℃, while the second deepest (16.5 cm) had a temperature of 102-104℃. The temperature measured for the two shallowest chambers (5.5 and 11 cm) had temperatures from 94-100℃.
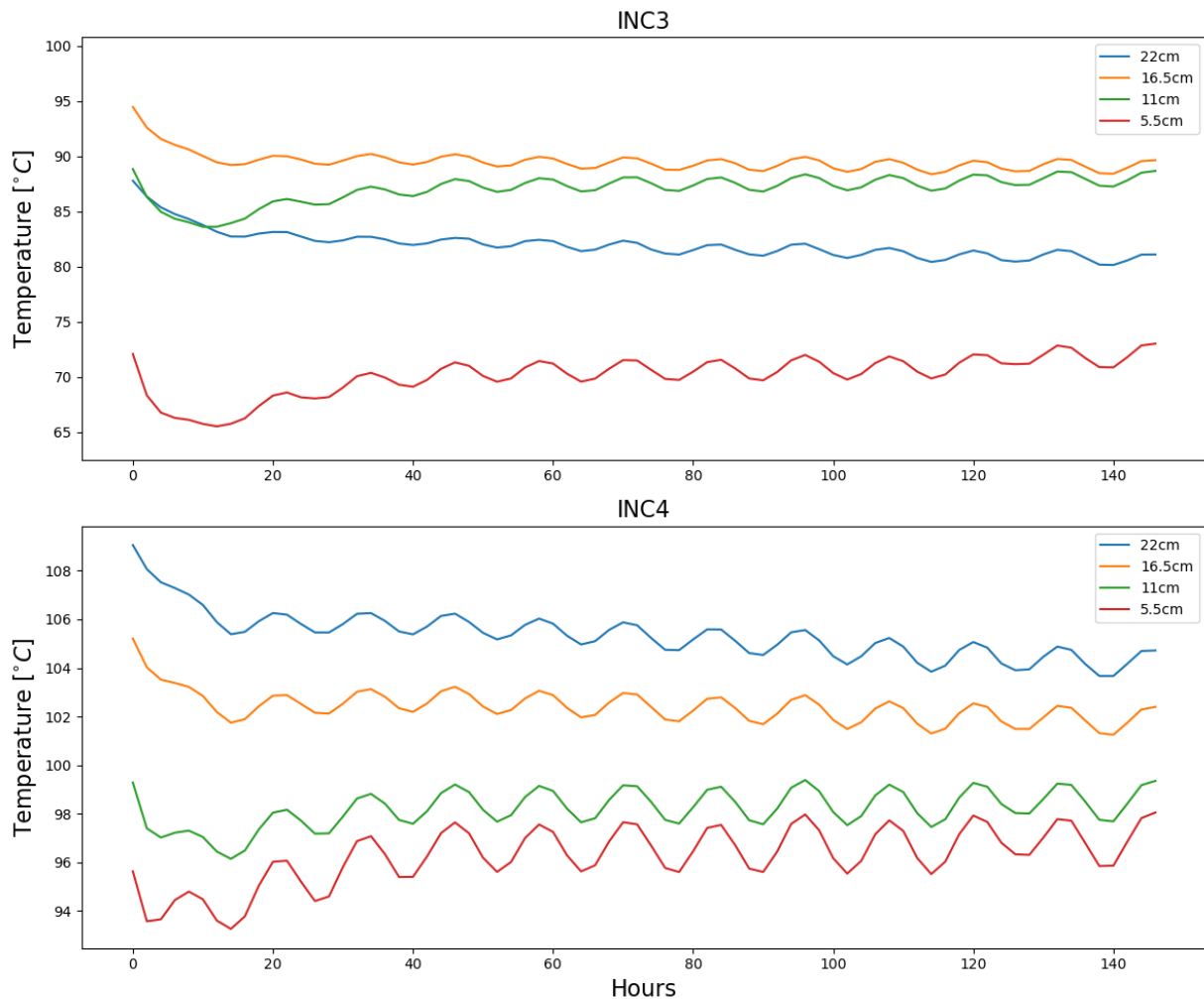
**Figure 6:** Temperature profiles of incubators 3 and 4 incubated in hot sediments at Aegirs kilde. The measured temperature is represented on the y-axis, while the x-axis shows the time interval (0h – 146h). Each incubator consists of four chambers, which are distinguished by different colors to indicate their respective depth (ranging from 22 cm to 5.5 cm).

Following the incubation period, the incubators were retrieved, and subsamples were collected from each chamber of each incubator for FISH, SEM, and slurries. These slurries were utilized for the cultivation and enrichment of microorganisms. Additionally, the research cruise involved several interdisciplinary sampling and experimental procedures, in which I contributed to sample handling and logging of ROV video material.

## 3.2 Cultivation of Microbes Supplemented with Plastic

Culturing microbes in media enriched with LDPE, HDPE, PET and PU polymers was done at three different temperatures: 30, 55, and 65℃. The cultures were separated into aerobic and anaerobic conditions. The cultures are separated according to which chamber they were taken from in the incubator. The first number is related to the incubator, and the second number is to the chamber, where chamber one is closest to the surface. Once activity was seen in the microscope or by direct observation, the culture was transferred to fresh medium (Table 1).

The cultures with the highest observed growth activity were the aerobic HDPE cultures incubated at 30℃ (Table 1). These cultures were successfully transferred seven times and, thus, described further in detail in this thesis. The anaerobic LDPE 6-1 cultures incubated at 55℃ and 65℃ were also transferred 6 and 4 times, respectively. Some of the cultures with PU as substrate at different incubation temperatures also showed growth, and were transferred a couple of times, except for the aerobic PU 4-2 and PU 4-4 cultures incubated at 30℃, which were transferred six and five times, respectively.

**Table 1:** Overview of the cultivated cultures. Their substrates were HDPE, LDPE, PET, and PU. The culture number refers to titanium incubator (first digit) and the chamber (1-4) (last digit). The number of transfers varies between 1-7. Each culture had an incubation temperature of either 30, 55, or 65℃. The level of activity was divided into very active with high visible growth (+++), less active (++), and low growth (+). Cultures without visible growth were described as not active (-).

| | AEROBIC | | | | ANAEROBIC | | | |
|---|---|---|---|---|---|---|---|---|
| **CULTURE** | Transfers | 30℃ | 55℃ Activity* | 65℃ | Transfers | 30℃ | 55℃ Activity* | 65℃ |
| | 7 | +++ | | | 3 | + | | |
| **HDPE 6-1** | 1 | | - | | 1 | | - | |
| | 1 | | | - | 1 | | | - |
| | 7 | +++ | | | 2 | + | | |
| **HDPE 6-2** | 1 | | - | | 4 | | ++ | |
| | 1 | | | - | 1 | | | - |
| | 7 | +++ | | | 1 | - | | |
| **HDPE 6-3** | 1 | | - | | 1 | | - | |
| | 1 | | | - | 1 | | | - |
| **HDPE 6-4** | 7 | +++ | | | 3 | + | | |

| Sample | N | | | | N | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | - | | 1 | | - | |
| | 3 | | | + | 1 | | | ++ |
| **LDPE 6-1** | 3 | ++ | | | 2 | - | | |
| | 1 | | +++ | | 6 | | +++ | |
| | 3 | | | + | 4 | | | +++ |
| **LDPE 6-2** | 4 | - | | | 2 | - | | |
| | 1 | | - | | 1 | | - | |
| | 1 | | | + | 3 | | | +++ |
| **LDPE 6-3** | 2 | + | | | 1 | - | | |
| | 1 | | + | | 1 | | +++ | |
| | 1 | | | - | 1 | | | - |
| **LDPE 6-4** | 2 | - | | | 5 | +++ | | |
| | 1 | | - | | 1 | | - | |
| | 1 | | | + | 2 | | | - |
| **PET 2-1** | 2 | - | | | 1 | - | | |
| | 2 | | + | | 2 | | ++ | |
| | 1 | | | - | 1 | | | - |
| **PET 2-2** | 3 | + | | | 1 | - | | |
| | 1 | | - | | 1 | | - | |
| | 1 | | | - | 1 | | | - |
| **PET 2-3** | 3 | ++ | | | 1 | - | | |
| | 1 | | - | | 1 | | - | |
| | 1 | | | - | 1 | | | - |
| **PET 2-4** | 1 | + | | | 1 | - | | |
| | 1 | | - | | 1 | | - | |
| | 1 | | | - | 1 | | | - |
| **PU 3-1** | 2 | - | | | 1 | - | | |
| | 1 | | - | | 2 | | - | |
| | 3 | | | + | 1 | | | - |
| **PU 3-2** | 2 | + | | | 2 | - | | |
| | 1 | | - | | 2 | | ++ | |
| | 1 | | | +++ | 2 | | | + |
| **PU 3-3** | 2 | +++ | | | 1 | - | | |
| | 1 | | - | | 2 | | ++ | |
| | 1 | | | +++ | 2 | | | +++ |
| **PU 3-4** | 2 | + | | | 1 | - | | |
| | 1 | | - | | 1 | | - | |
| | 1 | | | +++ | 2 | | | + |
| **PU 4-1** | 2 | +++ | | | 1 | - | | |
| | 1 | | - | | 2 | | - | |
| | 1 | | | +++ | 2 | | | ++ |

| | Count | | | | Count | | | |
|---|---|---|---|---|---|---|---|---|
| **PU 4-2** | 6 | +++ | | | 1 | - | | |
| | 1 | | - | | 1 | | - | |
| | 1 | | | +++ | 2 | | | + |
| **PU 4-3** | 2 | ++ | | | 1 | - | | |
| | 1 | | - | | 1 | | - | |
| | 1 | | | +++ | 2 | | | + |
| **PU 4-4** | 5 | + | | | 1 | - | | |
| | 2 | | - | | 2 | | ++ | |
| | 1 | | | - | 2 | | | + |

*Activity is based on observed visible growth.

## 3.3 Activity Assay

Activity assay with the different plastic substrates on agar plates was made to check for activity by exoenzymes secreted by the bacteria in the cultures. Clearing zones (haloes) around the sample in the agar is an indicator of activity on the substrate.

After six days of incubation, clear haloes were observed (Figure 7, 1A-C) on plates incubated at 30℃ in aerobic conditions. The PU 3-2, PU 4-2, and PU 4-3 cultures created haloes on PCL agar plates. The PU 3-2 culture also created haloes on the BHET agar plate. The HDPE 6-1, HDPE 6-2, and HDPE 6-4 cultures showed haloes on the agar plates with PCL as substrate but not on the BHET agar plate. After 18 days of incubation, the clearing zones on the agar plates had increased in size (Figure 7, 2A-C).
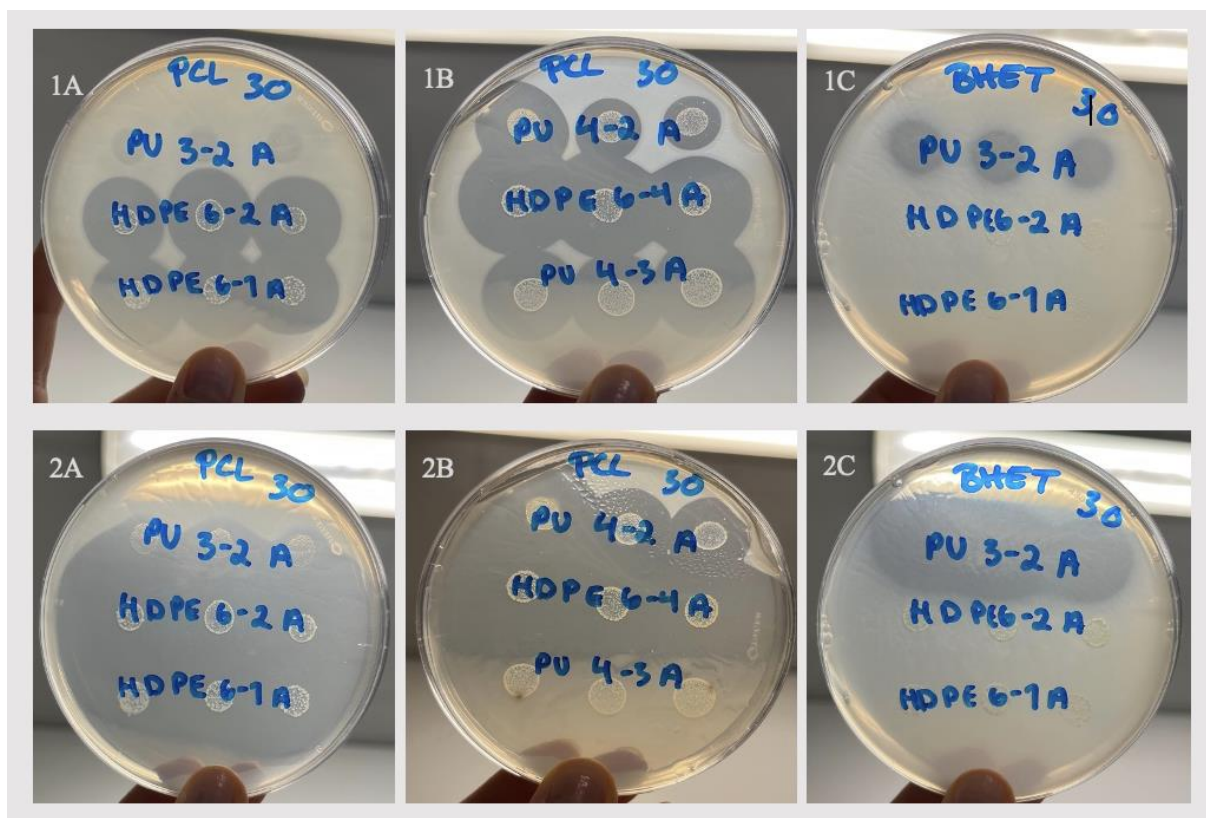
**Figure 7:** Plate assay for detection of exoenzymatic activity on PCL and BHET substrate, incubated at 30°C in aerobic conditions. Enzyme activity is detected by the presence of a halo around the sample. Agar plates 1A-C is after 6 days of incubation with their cultures and enrichments. 1A) Agar plate enriched with PCL with haloes from PU 3-2, HDPE 6-2, and HDPE 6-1 cultures. 1B) Clearing zones from PU 4-2, HDPE 6-4 and PU 4-3 cultures on agar plates enriched with PCL. 1C) Agar plate enriched with BHET, with clearing zones from PU 3-2 culture. Agar plates 2A-C is after 18 days of incubation of the same plates.

## 3.4 Growth Curve – Cell Counting

A growth curve was made to measure the growth of cells in the aerobic HDPE 6-1 culture incubated at 30°C. By plotting the number of cells at different time points a growth curve was made. The count was done twice.

The growth curve can be divided into three parts: a lag phase, an exponential phase and a stationary phase. The growth in the HDPE culture has a slow increase in cell number from right after incubation in fresh medium with HDPE as substrate (Figure 8). After 30 hours of incubation, the cells grow exponentially, increasing until 50 hr after inoculation. The number

of cells per ml at the peak of the growth curve is $1.9 \cdot 10^{13}$. The culture then begins to slowly decrease after 50 hr of incubation.
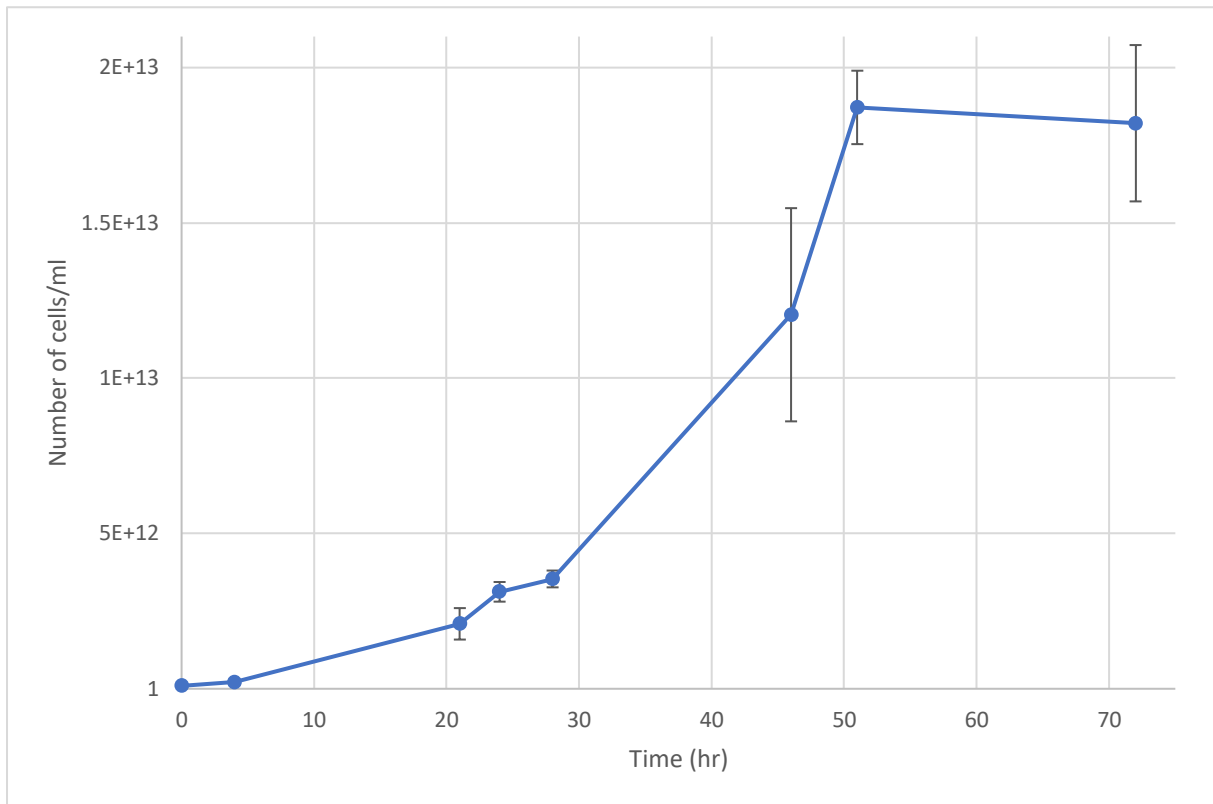


**Figure 8:** Graphical representation of the bacterial growth in aerobic medium with HDPE substrate incubated at 30°C. The number of cells/ml is on the y-axis, and the time after incubation is on the x-axis.

The growth rate was retrieved from the exponential part of the growth curve with the exponential growth model ($y = 6E+11e^{0,067x}$) (Formula 1). To calculate the generation time of the culture in this count, the natural logarithm (ln 2) is divided by the growth rate (Formula 2):

$$G = \frac{\ln 2}{0.067}$$
$$G \approx 10.3$$

For the second count of cells, the culture has a similar growth curve as the first count (Figure 9). From about 40 hr until 45 hr after incubation, there is exponential growth, but after that the growth stops. The peak number of cells per ml, is $2.4 \cdot 10^{11}$.
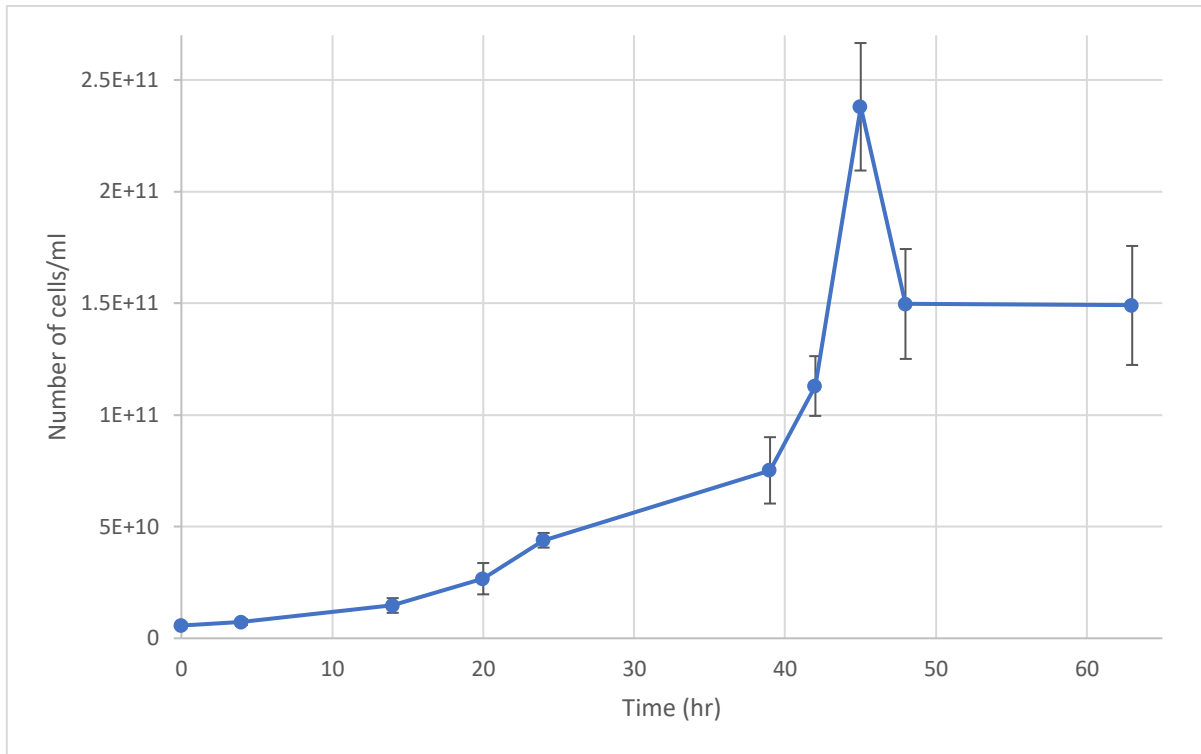
**Figure 9:** Growth curve of cells in aerobic medium with HDPE as the sole carbon source using a counting chamber. The number of cells/ml is on the y-axis, and the time after incubation is on the x-axis.

The growth rate of the second count was retrieved from the formula of the trendline of the exponential growth in the curve ($y = 7E{+}09e^{0,0708x}$). The generation time was calculated by dividing the natural logarithm (ln 2) by the growth rate.

$$G = \frac{\ln 2}{0.0708}$$
$$G \approx 9.8$$

## 3.5 Growth Curve – qPCR

To compare the number of cells from cultivation of cultures and from counting, a qPCR was done.

### 3.5.1 DNA Extraction

DNA was extracted from the subsampling for each transfer of culture. Also, the DNA from each counting in the second growth curve was extracted (Table 2). Some of the culture transfer

samples had too low DNA concentrations for the Invitrogen Qubit fluorometer to detect (4, 5, 7, 8, 10 and 11). While samples 0, 3, 6, 9 and 12 had 51.0, 56.0, 48.6, 55.0, and 36.9 ng/μl, respectively. Samples 0, 3, 6, 9 and 12 are the aerobic HDPE (30℃) culture from the transfers, while 1, 4, 7, 10, and 19 were from the anaerobic LDPE (65℃). The remaining concentrations from the DNA extraction are from the anaerobic LDPE (55℃) culture transferred. The standard (S) used in dilutions for reference for the qPCR had 55.0 ng/μl.

The two first samples from the DNA extractions of the growth curve samples, had too low DNA concentrations for the Qubit measurements. In the rest of the samples (2-9), the DNA concentrations increased, except for sample 6 and 7 which showed a slightly lower concentration (55.0 and 52.0 ng/μl, respectively).

**Table 2:** Measured Qubit values of culture transfer samples, and growth curve samples after DNA extraction.

| Culture transfer samples | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | S |
| 51.0 | 0.0510 | 0.115 | 56.0 | Too low | Too low | 48.6 | Too low | Too low | 55.0 | Too low | Too low | 36.9 | Too low | 55.0 |
| Growth curve samples | | | | | | | | | |
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Too low | Too low | 0.202 | 5.62 | 12.6 | 60.0 | 55.0 | 52.0 | 60.0 | 60.0 |

### 3.5.2   Preparation of DNA Standards

The DNA extraction was checked via gel electrophoresis to ensure high quality and quantity; see Section 2.5.2.1 (Figure 13, Appendix A). A PCR was done on the 16S rRNA gene of the standard. As mentioned in Section 2.5.2, the standard used was a culture growing on HDPE, with high turbidity and biofilm. The PCR of the standard sample was done to verify the size and no contamination, where the expected amplicon size was 180 base pairs see Section 2.5.2.2 for the designed primers, Bac338 and Prun518, used in amplification. The amplicon was checked in gel electrophoresis (Figure 14, Appendix A), where the amplicon appears to have a size of less than 250 base pairs, compared to the DNA Ladder. There are no other visible bands in the gel, and therefore no contamination. The PCR product was then cleaned and concentrated.

The final concentration of the purified amplicon was measured to 50 ng/µl. The amount of DNA was about 3.3 ng. With the length of 180 base pairs and the amount of 3.3 ng, the template copy number was calculated to $1.7*10^{10}$, for the $10^{-1}$ dilution, $1.7*10^{9}$ for the $10^{-2}$ dilution etc.

### 3.5.3   qPCR

A qPCR was done to monitor the abundance of microorganisms in the samples. The changes in the microbial community were tracked by quantifying the amount of DNA from all bacteria containing the 16S rRNA gene. To measure the number of cells in the samples in the qPCR, the SYBR®Green dye (BIO-RAD) was used. The resulting $R^2$ value was 0.995, and the PCR efficiency was 61.5%.

The resulting bar chart of the qPCR of each transfer of the aerobic HDPE 6-1 (30°C) culture shows a decrease in the number of cells after transfer 1 (Figure 10). The number of cells in the first transfer was almost $3.0 \cdot 10^8$. From the other transfers, the culture has approximately $1.5 \cdot 10^8$ cells/ml or less (transfer 2-5).
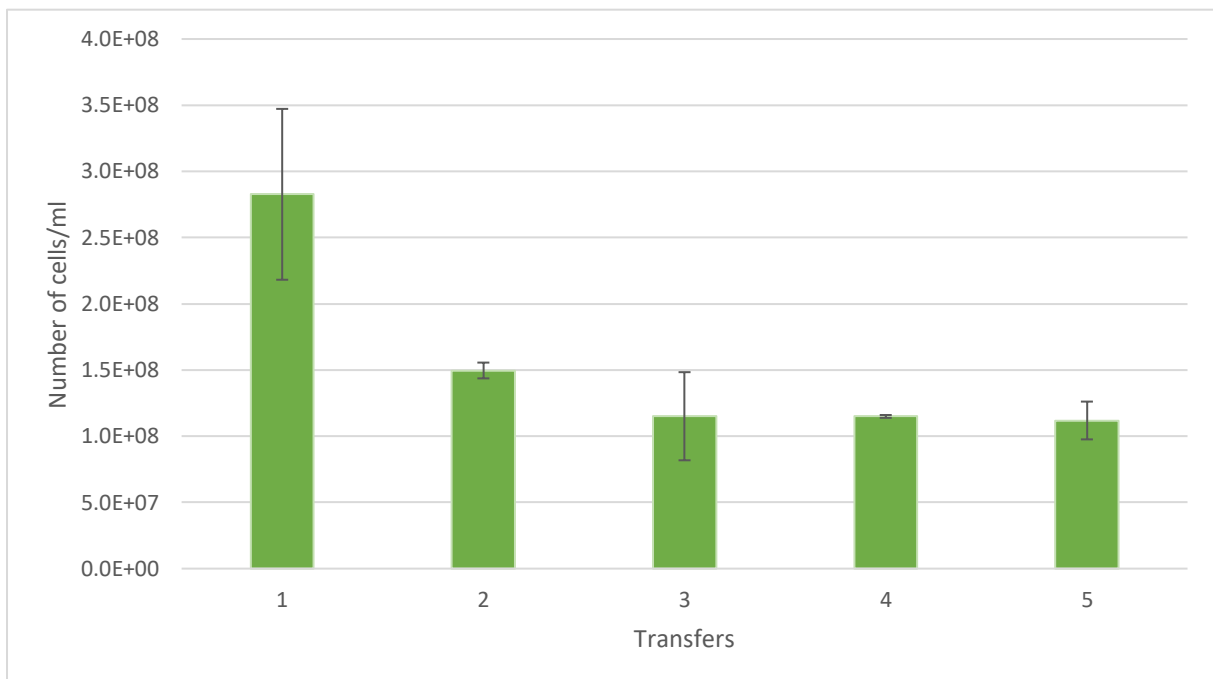


**Figure 10:** Bar chart of the aerobic HDPE 6-1 culture incubated at 30°C after the number of transfers (x-axis), with the number of cells per ml on the y-axis.

The growth curve measured by a qPCR has the same trends as the ones where the counting chamber was used (Figure 11). There is an increase in the number of cells/ml after 15 hours, then exponentially up until about 40 hours. Then there is a decrease before the number of cells reaches a new peak of about $4.5 \cdot 10^8$ cells/ml. After this peak, the number of cells slowly declines. The generation time (G) of the growth curve made by qPCR was calculated to be 5.8. The generation time was calculated using the growth rate from the exponential phase in the curve and by dividing it by the natural logarithm (Formula 2). The resulting $R^2$ value of this qPCR was 0.995, and the PCR efficiency was 60.2%.
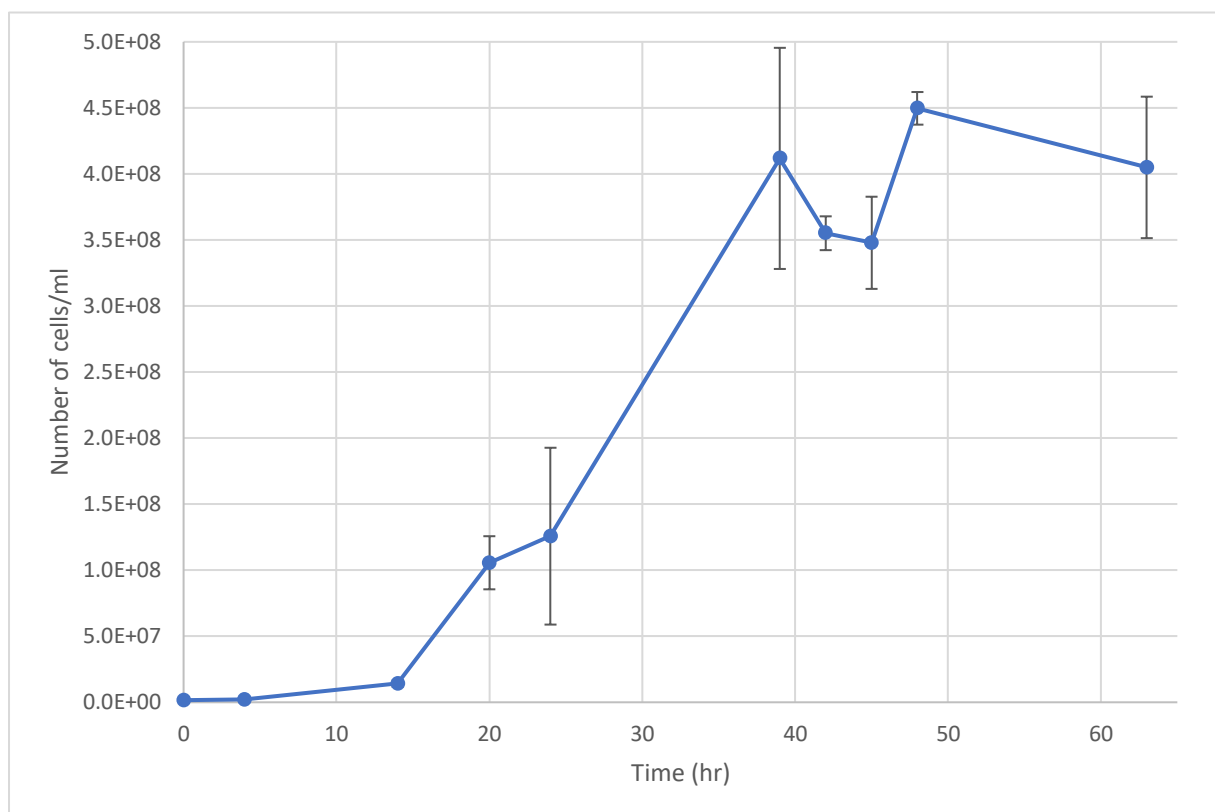


**Figure 11:** Growth curve of cells in aerobic medium with HDPE as the sole carbon source measured with qPCR. The time is on the x-axis, and the number of cells/ml on the y-axis.

# 4. Discussion

In this thesis, the samples were collected as part of an ongoing project at the Deep-Sea Research Centre (RCN-funded DeepSeaQuence, 315427). The samples were collected on an annual research cruise in the Nordic Sea. A remote-operated vehicle (ROV) was used to both incubate the titanium chambers as well as collect them from the sediments at Aegirs kilde as previously shown (Stokke et al., 2020). The aim of this thesis was to investigate the potential of bacterial and archaeal communities from sediment slurries collected at the Arctic Mid-Ocean Ridges hydrothermal vent fields to degrade the synthetic polymers low- and high-density polyethylene (LDPE, HDPE), polyethylene terephthalate (PET) and polyurethane (PU), when offered as the sole carbon source. To research this potential, several experiments took place. First, cultivation of the cultures was done to observe which cultures were able to grow on the substrates. Due to the sampling being close to hydrothermal vents, the incubation temperatures of the samples were done at 30, 55, and 65°C. To break down polymers into simpler forms, microbes can secrete enzymes, known as exoenzymes, making it more available. Therefore a plate assay on agar with BHET, PCL and PU was made to check if the cultures had any exoenzymes working on those substrates. After cultivating cultures with the different substrates, the aerobic HDPE 6-1 culture incubated at 30°C was observed as the most active. Therefore, this culture was used in a qPCR and for the growth curve.

## 4.1 Temperature Gradients in Sediments Close to Hydrothermal Vents

Internal temperature probes in incubators 3 and 4 measured the temperature in the sediments incubated close to hydrothermal vents throughout the incubation time (Figure 6). The temperature of incubator 3 ranged from approximately 65-95°C, while the temperatures in the sediments of incubator 4 ranged from approximately 94-106°C. The four incubators were incubated within the same square meter, see Section 2.1, and therefore shows the steep temperature gradients that could occur in the sediments close to hydrothermal vents. For incubator 3, the temperature for each depth shows that the chamber with the shallowest position in the sediments has the lowest temperature. The chambers placed deeper into the sediments have a higher temperature, although the deepest chamber has a slightly lower temperature than the chamber in the middle of the incubator. The depth difference in the sediments also shows that deeper down, the temperature is higher for incubator 4. The incubation temperature of the cultures cultivated from the slurries from these incubators was 30, 55, and 65°C. The incubation

temperatures are much lower than the temperature of the sediments used to incubate the titanium chambers.

## 4.2 Cultivating Extreme Microbes from Hydrothermal Vents

Microorganisms found close to hydrothermal vents are characterized as extreme because of the high temperatures, pressure, and chemical gradients. Due to these dynamic characteristics, the microbes have evolved to adapt uniquely (Minic & Thongbam, 2011). These adaptions allow them to carry out metabolic processes and survive. Hydrothermal vent systems are a quite new habitat in research since its first discovery in 1977, it is expected to find new and interesting enzymes from microbes there (Corliss et al., 1979). For example, *Thermus aquaticus*, an extremophilic bacteria, found in hydrothermal vents and hot springs have brought the polymerase chain reaction (PCR) to life (Taq DNA polymerase) (Brock, 1997). Exploring new enzymes is important to gain insights into the adaptions of the bacteria living here and develop innovative solutions for various industrial and environmental challenges (Synnes, 2007).

The cultivation of cultures enriched with HDPE, LDPE, PET and PU polymers showed activity in many of the cultures (Table 1). Many of the cultures were active initially, but the activity stopped after a few transfers. The reason for this could be that the microbes had brought nutrient sources with them from the incubation in the sediments. After some time, these nutrients would have been used up, and the culture no longer active. The most active ones were the aerobic HDPE cultures incubated at 30℃, the anaerobic LDPE 6-1 cultures incubated at 55℃ and 65℃, and some aerobic PU cultures.

HDPE is a high-density polyethylene and is designed to resist natural degradation processes, with its crystalline structures (Byrne et al., 2022). Until now, no known enzyme can degrade crystalline polyethylene on a biochemical level (Buchholz et al., 2022). Therefore, this aerobic HDPE 6-1 (30℃) culture was chosen for further experiments due to its unexpected high activity after every transfer. The HDPE polymer was added to the medium without physical or chemical pretreatment. Other experiments could have been done on the aerobic HDPE 6-1 (30℃) culture, which could potentially give more information on how the culture grows with HDPE as its sole carbon source. Analyzing and measuring the waste products in the medium as well as gases in the headspace of the sealed incubated bottles, could show differences between samples and a control.

The qPCR analysis of the aerobic HDPE 6-1 (30°C) culture revealed that the initial transfer likely brought in nutrients from the starting material and increased microbial diversity, while subsequent transfers resulted in a more stable culture that grew on the given substrate (Figure 10).

LDPE is the low-density version of polyethylene and has branched polymers, made to be softer and more flexible (Byrne et al., 2022). Due to the ongoing activity of the anaerobic LDPE 6-1 (55°C and 65°C) culture after each transfer, the DNA was extracted. From the measurements of the DNA concentration on the Qubit fluorometer, the concentrations were too low (Table 2). Growth of this culture was observed in the microscope; however, compared to the growth of the aerobic HDPE 6-1 (30°C) as observed during the growth curve experiment, the cell density was not near the same.

For the cultivation of bacteria in medium enriched with PU, the activity was measured by how clear the medium had turned from the "milky" look it had right after incubation. Biffinger et al. (2015) have noted that while clearing studies using Impranil can indicate the degradation of plastic by microorganisms or enzymes, such studies need to be complemented with quantitative molecular spectroscopic methods to confirm the extent of the degradation (Biffinger et al., 2015).

## 4.3 Activity Assays to Evaluate for Biodegradation of Plastic

Bacterial activity on plastic substrates checked by indicator agar plates showed clearing zones indicating exoenzyme secretion (see Section 3.3). Clear haloes were observed after six days of incubation (Figure 7, 1A-C), with increased size after 18 days (Figure 7, 2A-C). Also, after just 24 hr, clearing zones started to appear. The haloes are created when bacteria have secreted enzymes that can degrade the plastic substrate.

The two plates with the most activity was on those enriched with PCL. Given that PCL is a biodegradable plastic, despite its petrochemical origin (see Section 1.5.4), it is designed to be broken down into molecules that could be easier metabolized by microbial activity (Narancic et al., 2018). Researchers have isolated bacterial strains from deep-sea sediments capable of degrading PCL, but not other biodegradable polymers. These findings suggest that these strains could be used in the identification of new biodegradable polymers. This study by Sekiguchi et al. (2010) is particularly significant, given that the deep-sea can be a final site of plastic waste (Sekiguchi et al., 2010).

On the plate enriched with BHET, the aerobic PU 3-2 (30°C) culture showed activity. The haloes on the agar plate created by the culture doubled in size after 12 days (Figure 7, 1C and 2C). BHET is one of two products made by degrading PET (Qi et al., 2021). Therefore, the aerobic PU 3-2 (30°C) medium culture was transferred to liquid medium supplemented with PET, to analyze for growth. This culture was transferred twice, but then stopped growing. Hence, further experiments could be done. This would likely involve analyzing the culture's growth media and waste products and conducting genetic analyses to determine which enzymes and metabolic pathways the cells in the culture use to break down BHET.

## 4.4 Growth Curve Analysis to Measure Cell Growth on HDPE

To get a measure on how the aerobic HDPE 6-1 (30°C) culture was growing, a growth curve was made by counting the cells during a time interval. As this culture contains plastic pieces of different sizes, the cells had to be counted in a microscope. Using this method, the morphological structure of the cells can be observed. Another advantage of counting using the microscope is that it is easy and requires minimum equipment.

### 4.4.1    Analysis of Growth Curves

When following the growth curve of a model organism like *Escherichia coli* in a culture liquid medium, each phases represents a distinct period of growth associated with typical physiological changes in the cell culture (Pletnev et al., 2015).
During the initial phase of the growth curve, the cells are in a new environment with potentially different nutritional conditions. They need to adapt to grow and thrive in the new environment metabolically (Navarro Llorens et al., 2010). This phase is called the lag phase because of the absence of growth. From the first growth curve, the lag phase is visible from the beginning up until 30 hr after transferring to fresh medium (Figure 8). The second growth curve has a slightly longer lag phase (Figure 9). On the other hand, the log phase in the growth curve from the qPCR of the culture (Figure 11) appears to be about 15 hours long. When the cells have adapted to the new conditions in the environment, the exponential (log) phase starts, and the cells divide by binary fission and reach their maximum growth rate. From this phase, the generation time is calculated (Liyun Wang et al., 2015). The rate depends on how nutrient-rich the mediums are, with nutrient-rich medium giving a higher growth rate, and in a nutrient-limited medium, the growth rate is lower (Navarro Llorens et al., 2010). When comparing the growth curves from

cell counting with the one from the qPCR, they peak after approximately 40-50 hours. However, the amount of cells/ml varies, with $1.9 \cdot 10^{13}$ and $2.4 \cdot 10^{11}$ for counting cells, while just $4.5 \cdot 10^8$ cells/ml from the qPCR analysis. When nutrients get limited or depleted, the culture reaches the stationary phase, where the division of cells is at the same rate as cells dying. Other factors also inhibit growth, such as the accumulation of toxic products from catabolism. Then lastly, there is a death phase, where the number of dead cells exceeds that of live cells (Liyun Wang et al., 2015). By comparing the growth curves derived from cell counting and qPCR, it was observed that both techniques depict similar trends across the various phases of microbial growth. This correspondence between the growth curves suggests that the growth pattern in this experiment accurately represents the growth of the culture in these conditions.

### 4.4.2   Analysis of Generation Time

To calculate the generation time, an exponential growth model was used (Widdel, 2010). This model can be used as the population size at any time x can be predicted if the population size at time x represents the number of cells in the culture with a constant growth rate. The generation time (G) calculation is the time it takes for the cells in the culture to double, which they do during the exponential phase. The generation time is inversely proportional to the exponential growth rate, where a high growth rate gives a short generation time, while a low growth rate gives a long generation time.  The calculated generation time to the growth curves where the cells were counted was 10.3 hr and 9.8 hr, respectively. This means that the bacteria in the aerobic HDPE (30°C) culture doubles every 10 hr approximately. On the other hand, the calculated generation time for the growth curve made from the qPCR results was 5.8 hr. A study done by Pramila et al. (2012) on biodegradation showed that in minimal medium with powdered LDPE as the sole carbon source, bacteria isolated from soil in landfills had generation times of 28, 79, 80, and 167 minutes for the different bacterial species (Pramila et al., 2012). They also verified the growth by measuring carbon dioxide. But as mentioned, presently, there are no known biochemically characterized enzyme capable of degrading polyethylene (low density and high density)(Buchholz et al., 2022).

### 4.4.3   Challenges with Microscopic Counting

Counting in the microscope using a counting chamber can be imprecise. The same culture has different growth rates and generation times (approximately 10 hours for the microscopic

counting of cells, 5.8 hours for the qPCR results). The cells move; therefore the same cell could have been counted more than once. Also, there is no way to differentiate between dead and alive cells. Another factor influencing the number of cells, was that this culture generated biofilms. The biofilms had a high cell density, making it impossible to count. The biofilms make the culture very unevenly distributed with cells and may be the reason for the decrease in cell number after the peak of the second growth curve.

Other methods for measuring the growth curve and determining the generation time of a culture can be used to get more accurate results. One is the Coulter Counter, where the medium with the bacteria cells passes through a pore in a membrane, which changes the electrical impedance of the fluid (Hurley, 1970). The passing of each cell causes these changes, therefore the number of cells can be determined by analyzing the frequency and amplitude created by the membrane (Zhe et al., 2007). Another method used for counting cells, is through flow cytometry. This method is very accurate when it comes to counting cells. The cells pass through a narrow channel where a laser beam hits them. The resulting light scatter from the beam hitting the cells is measured by detectors. The signal detected can be analyzed, and the data can be used to count the cells (Adan et al., 2017). Optical density (OD) measurements are also a method for counting cells based on light measurements. This is the most used method for counting cells, as it is fast, simple, inexpensive, and easily automated (Beal et al., 2020). The reason for not using this method for the growth curve was that the plastic polymers in the culture could interfere with the OD measurements.

## 4.5 DNA Extraction and Quantification of 16S rRNA Genes using qPCR

### 4.5.1 DNA Extraction

The DNA extractions of the anaerobic 6-1 LDPE (55 and 65°C) cultures showed insufficient DNA in many of the samples for the Qubit fluorometer to detect (Table 2). While for the aerobic 6-1 HDPE (30°C) culture, the concentrations were sufficient to run these samples in a qPCR. The DNA extractions made of the samples from the growth curve, had an increasing concentration (Table 2). The extractions from the two first counts were too low to measure, but then the concentration of the DNA extractions increased. This increase is already an indicator of what the growth curve would look like.

### 4.5.2 Quantification of 16S rRNA using qPCR

The target gene for the qPCR was the 16S rRNA gene, which is a structurally conserved molecule existing in all prokaryotes (Vargas-Albores et al., 2017). The gene consists of conserved regions and hypervariable regions. The V3 region, with position 341-534 in the 16S rRNA molecule is one of the most hypervariable regions (Bodilis et al., 2012). In this study, the amplified sequence had position 338-518 in the 16S rRNA, thereby a part of the V3 region. Instead of amplifying the whole 16s rRNA gene, the shorter hypervariable regions of the gene are used. The V3 region is used because it is relatively short, it has many differences in the sequence and the primer pairs used when amplifying this sequence are less likely to produce biased results compared to other primer pairs (Ziesemer et al., 2015). Targeting the hypervariable regions of the 16s rRNA gene, enables quantification of specific phylum and species (Smith & Osborn, 2008). As mentioned in Section 2.5, quantifying the cell number with qPCR is limited because the number of copies of the 16s rRNA gene varies between species. Analysis of the data from the sequencing of the aerobic HDPE (30°C) culture done by the supervisors in this thesis, found 9 copies of the 16s rRNA gene in most abundant bacteria in the sample. Sequencing can therefore be an important part when doing qPCR. The number of cells present is significantly reduced when divided by 9. The PCR efficiency of the qPCR from the culture transfers and the growth curve from Section 3.5.3 was 61.5% and 60.2%, respectively. The PCR efficiency is defined as the fraction of target molecules copied in one cycle and should be at least 90%. Factors affecting the PCR efficiency can be the characteristics of primers and target sequence, other substances from previous steps inhibiting the PCR as well as the concentrations and protocol (Svec et al., 2015). The $R^2$ value is a statistical measure of how well the qPCR data fit the regression line. The $R^2$ values in the qPCR amplification were 0.995 for both and are within the desired range from 0.980-1.00. (Bivins et al., 2021).

## 4.6 Future Perspectives

### 4.6.1 Techniques to measure plastic degradation

Other techniques that can be used to assess plastic degradation by microbial action can be divided into mass loss, $CO_2$ evolution, chemical analysis of concentrations, and surface analysis (Chamas et al., 2020).

Another way to determine the biodegradation of plastic polymers by microorganism is by using stable isotope labeling (SIP). This method can more accurately assess plastic degradation by

microorganisms by tracking the formation of reaction products and growth using a stable isotope label ($^{13}$C). The plastic is labeled, and if the microorganism has incorporated it into its growth, then the isotope ratio can be measured (Wegener et al., 2016). In a study by Goudriaan et al. (2023) on PE mineralization by the well-studied bacterium *Rhodococcus ruber*, they concluded that carbon from the plastic was used in cell growth (Goudriaan et al., 2023). However, the microbial mineralization rates were low.

### 4.6.2   Possible Improvements

The plastic substrates used in this study were not pre-treated. Chemical, physical and thermal pretreatment have been shown to enhance the microbial degradation of plastics by disrupting the large-scale molecular structures of the plastic polymers (Yasin et al., 2022). Pretreatment of the plastic polymers in this study could have transformed the polymer into a more easily accessible one.

Also, the manufacturer of the plastic polymers used in this study have no information on the production. Possible trace compounds on the plastic substrates during the production can be used as a carbon source by the microbes. On the other hand, the culture showed degradation (clearing zones) on the PCL assay activity plates (Figure 7). This shows the potential to find plastic degrading-enzymes in these cultures.

Another factor that may have influenced the cultures is the incubation temperature. The temperature of the sediments the microbes were collected from had a much higher temperature than expected. The incubation temperature used in cultivation of the cultures were much lower and may have had an impact on the general growth of all cultures.

### 4.6 Conclusion

Plastic pollution is a global issue with increasing production and limited recycling, leading to environmental accumulation and negative impacts on nature and human health. Deep-sea hydrothermal vent microorganisms have shown promise for degrading industrial polymers.

The aim of this thesis was to investigate the potential of *in situ* enriched bacterial and archaeal communities from sediment slurries collected at the Arctic Mid-Ocean Ridge hydrothermal vent fields to degrade the synthetic polymers low- and high-density polyethylene (LDPE,

HDPE), polyethylene terephthalate (PET) and polyurethane (PU) when offered as the sole carbon source.

The study was achieved by setting up enrichment cultures of potential plastic-degrading microorganisms. Microorganism abundance was measured by qPCR. Activity assay was set up for indication of exoenzymatic activity. Growth curves by cell counting, and qPCR, was done to measure growth and calculate generation time.

Growth on most plastic substrates was observed. However, HDPE cultures were the most active and stable ones after transferring. Furthermore, growth curve analysis showed that the cells in the HDPE culture had exponential growth after 15 hours of incubation. These findings suggest a potential for plastic degradation by microorganisms in deep-sea hydrothermal vent fields at AMOR. However, further experiments are needed to fully understand the plastic degradation capabilities of the HDPE culture, including the use of isotopically labeled plastic substrates.

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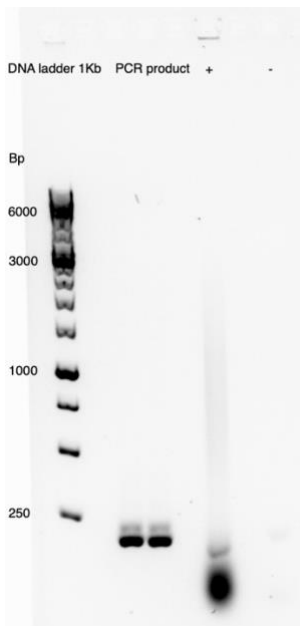
# Appendix A
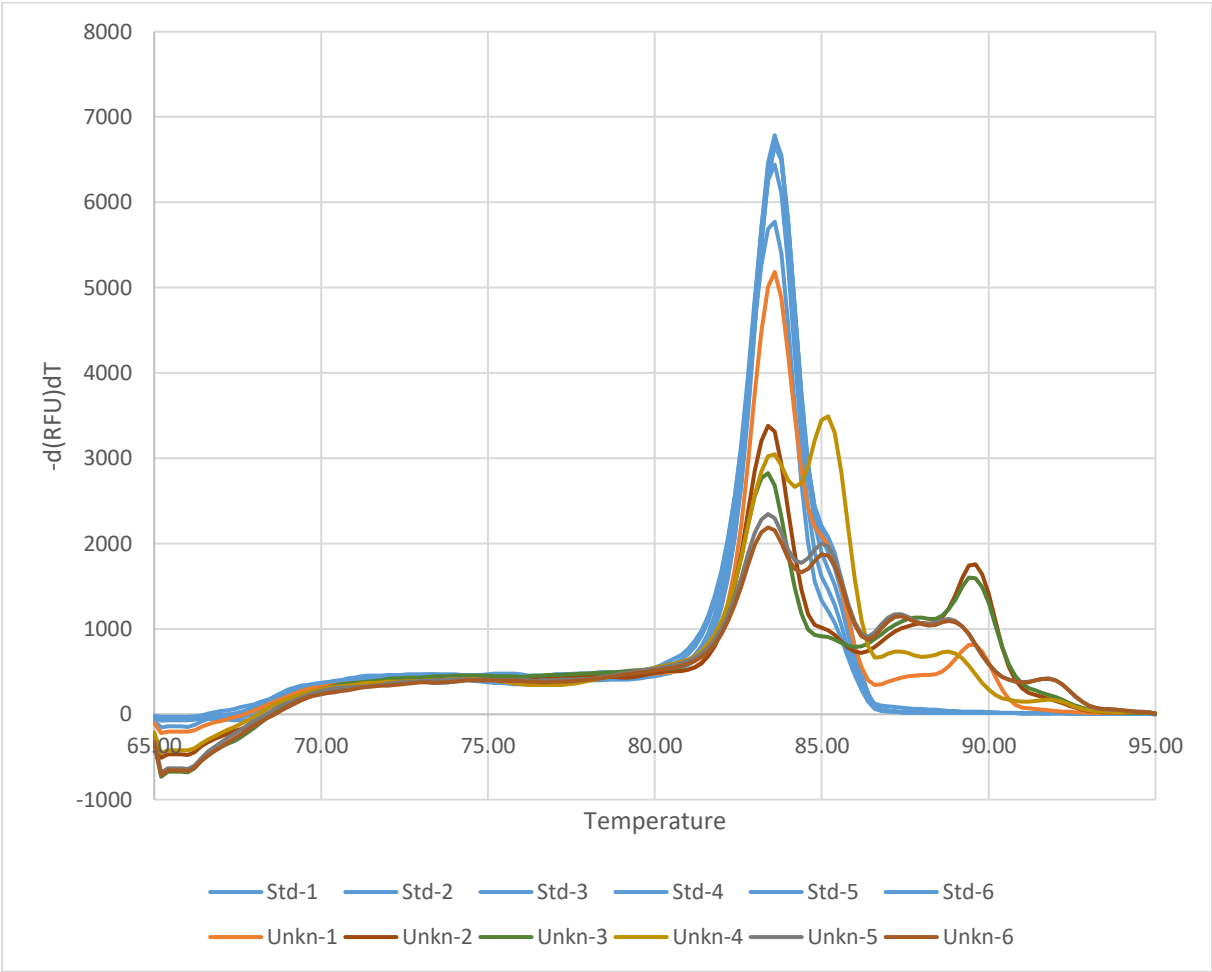


**Figure 12:** Melt curve derivative results from test qPCR of the cultures. Unknown sample 1 is closest to the standards and was therefore chosen as the optimal DNA concentration for further qPCR processes.
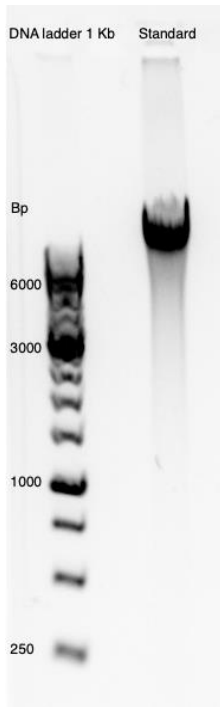
**Figure 13:** Gel electrophoresis of the DNA extraction of the Standard (S) to check the quality and quantity.



**Figure 14:** Gel electrophoresis to check for the correct size of the PCR product. The PCR product is 180 base pairs, which seems to be the correct size according to the Ladder. The positive control is from an *E. coli* colony. No other bands were visible, indicating that there was no contamination.