Part A – Applicant

A.1 Applicant

Name student (initials, first name, last name, student number): A. Alejandro Baars. 5325978

Affiliation (university/institute + department): Utrecht University / Master programme Bioinformatics & BioComplexity

Name first examiner: prof. dr. Han Wosten

Affiliation (university/institute + department): Utrecht University / Dep Biologie - Afd Biodynamics and Biocomplexity

Name second examiner: dr. Robin Ohm

Affiliation (university/institute + department): Utrecht University / Dep Biologie - Afd Microbiologie

Part B – Scientific proposal

Plastic degradation by the fungus Zalerion maritimum: Gene discovery and characterization.

Abstract

Plastic waste poses a significant threat to human health and the biosphere. While various ways exist to re-use or recycle plastic waste, they are hard to use on a sufficient scale to deal with the waste. In particular, microplastics and nanoplastics can negatively influence the health of humans, animals, and other organisms. Of the various plastic types produced, polyethylene is the most prominent in terms of production and waste generation. There are some microorganisms that have started to show the capability to (partially) degrade plastics waste, but they are often slow on larger particles, but some show promising results on micro- and nanoplastics. *Zalerion maritimum* degrades polyethylene under laboratory conditions, with evidence suggesting that it uses polyethylene as a carbon source. However, the mechanisms underlying this degradation of polyethylene are not known. This research proposal aims to identify these mechanisms by identifying the genes involved in degradation of polyethylene and by characterizing their encoding proteins. We will also perform a toxicity test of *Z. maritimum* for various additives present in polyethylene products. With this research, we will contribute to biological solutions to the growing threat of plastics.

Layman's Summary

Plastic waste poses a significant threat to human health and our environment. Over the past seventy years, plastic production has skyrocketed and has outpaced our ability to properly deal with the waste produced. There are various ways to re-use or recycle plastics, such as making fuel or plastic bags, or even using them to make concrete or roads. However, these methods are not used sufficiently to take care of the waste currently produced, let alone the waste that has already seeped into the environment. Plastic waste can be a choking, strangling, and restraining hazard for aquatic life. Initiatives like The Ocean Cleanup do their best to remove floating plastics from the ocean. While their work is admirable and important, the floating plastics are not the biggest problem when it comes to plastic pollution, but rather the smaller particles called micro- and nanoplastics. These small plastic particles consist of particles made at the micro- and nanometer size (used in

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cosmetics, drugs, etc.) and particles that have broken off from larger pieces. Due to their small size, they can easily enter our bodies through food, water, skin, and air. Their large surface area compared to their volume makes that they can easily release various additive chemicals that are present in the plastics upon contact, which are often harmful to our health. A big portion of plastics produced and discarded is polyethylene (PE), which is used in daily life in bags, bottles, food packaging, cable insulation, and many other applications. In fact, almost a third of all plastic produced and discarded is PE. To make an impact on the growing amount of plastic waste, this plastic is important to tackle.

Plastics are popular due to their versatility, low production cost, and resilience against biological and chemical breakdown. That being said, there are some bacteria and fungi that have the capability to break down plastics, or at least partially. These bacteria and fungi are often nature's 'clean-up crew' when it comes to new chemicals that are introduced into the environment. Due to the large number of different plastics and additives and the immense production of plastics in general, the bacteria and fungi that clean up cannot keep up. Their plastic degrading capabilities are in their infancy. However, with proper research, we may be able to help nature along and harness these capabilities to clean up our mess, in particular with microplastics, which are hard to filter from the water.

In this research proposal, we aim to identify the mechanisms by which *Zalerion maritimum*, a fungus found on the coast of Portugal, breaks down PE pellets. In addition, we will perform a toxicity test of *Z. maritimum* for various additives present in PE. This research is important to pave the road to a biological solution for a man-made problem.

Keywords
Zalerion maritimum, microplastics, biodegradation.

1. The plastic threat

1.1 The plastic soup

Plastics are materials that consist of long-chain polymers. They are commonly made from oil, and thus rich in hydrogen and carbon. Plastic polymers are typically formed through addition polymerization of carbon double bonds (olifins), creating carbon chains (polyolifins). About 60% of plastic production is formed by this reaction. Another common method is a condensation reaction between a carboxylic acid and an alcohol/amine group, creating a polyester or polyamine plastic respectively [1].

Plastics are extensively used in today's society due to their light weight, flexibility, strong plasticity, insulation capabilities (both electrical and thermal), resistance to corrosion and biological degradation, and low production cost. Since its introduction to our society in the 1950s, the use of plastics has grown exponentially. While worldwide production in 1950 amounted 2 million tonnes, in 2021 over 390 million tonnes were produced, and the production of plastics is expected to double within the next 20 years. It is estimated that between 8.3 and 9.2 billion tonnes has been produced between 1950 and 2017, of which more than half has since been discarded as waste. When discarded, a part of the plastic waste is recycled or incinerated. However, most plastic ends up in landfills and (often subsequently) in the environment, primarily in the groundwater and in the oceans. These plastic accumulations are often called by the nickname 'plastic soup' [2] [3] [4] [5].

1.2 Microplastics & nanoplastics

The increasing demand and production of plastics and the simultaneous failure to properly discard them are a threat to the biosphere and human health [6] [7]. Larger plastic particles can be a choking hazard for various animals, create malformed growth due to entanglement, or create a false sense of satiation due to ingestion and deprive animals of nutrients [6] [8] [9]. However, the larger pieces of plastics are not the only problem. Microplastics (MPs, <5 mm) and nanoplastics (NPs, <1 μm) can infiltrate organisms through their digestive tract, airways, or other pores and accumulate within their bodies [10] [11]. These MPs and NPs can be originally manufactured as small particles, which are commonly used in, for example, cosmetics, cleaning agents, or drug delivery. These particles are referred to as primary MPs/NPs [6]. Most MPs/NPs originally come

from larger plastic particles, which have eroded over time due to mechanical stress, photodegradation, and various other processes. These are called secondary MPs/NPs [6].

The full effect of these MPs and NPs on human health and ecosystems is still unknown, but initial reports are worrying. Research shows that MPs negatively impact reproductive capabilities, growth, detoxification, and immune capabilities in marine phytoplanktons, zooplanktons, invertebrates, and plants [10] [11] [12] [13]. Furthermore, MPs and NPs accumulate in larger fauna through trophic transfer from prey to predator. Studies have shown that organic pollutants and toxic trace elements, such as mercury and cadmium, can be absorbed or attached to MPs/NPs, meaning that MPs/NPs can potentially serve as a vector for harmful substances and pathogens [12] [14] [15]. Furthermore, the chemical additives that are present in plastics to modify their characteristics include several toxic substances, like phthalates for plasticity, polybrominated diphenyl ethers as flame retardants, and cadmium and lead compounds as heat stabilizers or as dyes. These chemicals are easily spread through MPs and NPs due to the increased surface area compared to larger plastic particles [16]. While there is a lack of *in vivo* toxicity data for humans, there is evidence that indicates that MPs/NPs are detrimental for human health. Studies in mouse models indicate that ingested MPs/NPs cause reduction of the gut mucus [17], gut barrier dysfunction, changes in gut microbiota composition [18], inflammation of intestines and liver [17] [19], lipid accumulation, and changes in lipid metabolism in the liver [17]. Several reports indicate that MPs/NPs have been detected in human lungs, gut, liver, kidney, muscles, and blood [20] [21] [22]. To combat the current widespread contamination of MPs and NPS and limit the future spread, solutions must be found and applied to 1) efficiently deal with the volume of plastic waste produced, 2) remove existing plastic waste from the environment, and 3) remove plastics from human bodies.

1.3 Current methods for dealing with plastic waste

To limit the amount of waste discarded into the environment, the European Commission has laid out an action plan for a ‘circular economy,’ a principle with the intention that all plastic packaging must be recyclable by 2030 [23]. As single-use packaging is the largest contributor to plastic waste, accounting for 44% of all plastic used in the EU plus Norway, Switzerland, and the UK in 2021 [2], tackling these plastics will greatly affect plastic waste production. Plastics can be recycled to a degree: By shredding, cleaning, and separating the plastics from non-plastic attachments (paper, dirt, metal, etc.), they can re-enter the market as recycled plastic granules. From these granules, grocery bags, blinds, shutters, and various other home items can be made [24]. Alternatively, some plastics can be turned into fuel by pyrolysis [25] or liquefaction [26], or they can be used in tar production [27] and road construction [28], and concrete production [29] [30]. Some of these methods are incredibly efficient at reducing plastic waste; road construction and concrete production in particular [30]. Pyrolysis and liquefaction-created fuel can substitute a part of traditional fuels and lessen the strain on oil reserves, but the processes are both very energy-intensive. Liquefaction also uses a lot of water [30]. Recycling plastics is also very energy-intensive and has the added detriment that the resulting recycled plastics are often significantly less durable. Furthermore, variations in plastic additives, with over 10 000 chemicals used in the production of plastics identified [31], and plastic film coatings make the recycling process harder [32]. Other than that, it is generally a cost-effective way of putting discarded plastics to use. The main problem with these methods is that they are not used sufficiently to deal with the yearly increase in discarded plastic [3]. Furthermore, while these methods are a good alternative for larger plastic waste, they are not suitable for MPs and NPs [33].

Not every type of plastic contributes to the problem in equal measure. Plastics are typically characterized by the monomer(s) used to create the polymer. It should be noted, though, that plastics comprised of the same monomer can have vastly different characteristics, depending on polymer length, methods used in the creation, and various additives. Of all plastics in existence, there are seven types that are the most prominent in plastic production; polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), polyethylene terephthalate (PET), polyurethane (PUR), and polyester, polyamide, and acrylic (PP&A) fibers make up over 90% of worldwide plastic production (figure 1). The largest group, PE, comprises 28.5% of the total plastic production in 2015 [4]. Furthermore, 32.1% of all plastic waste generated in 2015 belongs to PE plastics (figure 1) [4]. PE is also the most dominant group of plastics found in MP and NP research, albeit closely followed by PP [34]. This is due to its prominence in packaging material, which generally has a short usage lifespan [35]. PE is also the greatest potential vector for chemical pollutants [14]. Therefore, solutions for the reduction of PE plastic waste, both larger particles and MPs/NPs, will greatly impact global plastic waste.

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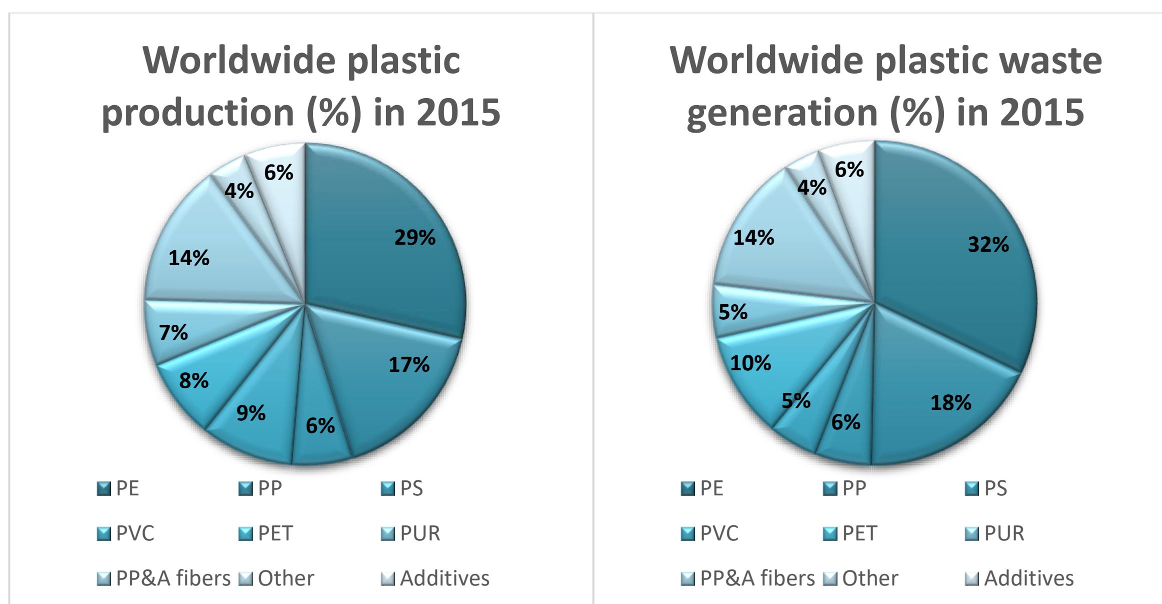

Figure 1: The worldwide production of plastics in 2015 sorted per type (left) and worldwide plastic waste generation in 2015 per plastic type (right). The biggest group of plastics produced and waste generating is polyethylene (PE). In total, PE, polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), polyethylene terephthalate (PET), polyurethane (PUR), and polyester, polyamide, and acrylic (PP&A) fibers make up over 90% of worldwide plastic production and waste generation.

2. A biological solution

2.1 Life always finds a way

In the biosphere, microorganisms play an important role in many environmental processes. They have evolved over the ages to break down and mineralize many different compounds, including xenobiotics in recent history. Their processes prevent bioaccumulation, as they consume and recycle these substances into smaller, reusable compounds instead. Many of these microbes have shown the capability to evolve their metabolic capacity to tackle new substances through various genome modifications. This metabolic flexibility functions as a 'self-cleaning' function for the biosphere. The advancements in synthetic compounds and huge increase in their production in the last seventy years exceed the ecosystems capacity to adapt to these new xenobiotics, resulting in their accumulation in the environment. Recently, though, some microbes have been shown to have developed the capability to degrade plastics, either fully or partially. However, the speed of the degradation processes is not sufficiently fast to keep up with the influx of plastic waste into the environment. Still, microorganisms show potential in dealing with plastic waste. This goes for microplastics in particular, as there are no existing methods to recycle microplastics described within the current literature [1].

2.2 Biological degradation of plastics

Various microbes isolated from plastic-rich environments (such as landfills, littered beaches, floating plastics in the ocean, etc.) have shown biodegradation capabilities for plastics. The capacity differs between species, as not all of them are able to break down polymers fully into minerals, or can only work on smaller molecules. The process of biodegradation can be laid out in four steps: (Bio)deterioration, (bio)fragmentation, assimilation, and mineralization [1].

The first step, biodeterioration, consists of the first, superficial degradation and mechanical and chemical alterations made to the polymer. Microbes can attach themselves to the polymer's surface through biofilm formation (bacteria) [36] or with their hyphae (fungi) [37]. The attached microbes exercise physical and chemical stress onto the polymer; biofilm formation often increases the surface contact between the microbe and the polymer [36], while attached fungi grow on the polymer and create localized swellings [1]. This process is often complemented by abiotic factors, which consist of mechanical stress, UV light, or environmental chemicals. These can change the physiological and chemical composition of the surface by, for example, introducing hydrophilic groups that can more easily facilitate microbe attachment. During the initial breakdown of the polymer and microbial growth, microbes typically feed on smaller molecules present within the surface of the polymer, such as residual mono- and oligomers, but also plasticizers and other additives [1] [38].

The second step, biofragmentation, entails the enzymatic cleavage of the polymers into smaller molecules by extracellular enzymes and microbe-produced highly-reactive molecules called free radicals [39]. This process has two principal reactions: The reduction in polymer molecular weight and the oxidation of the lower-weight molecules [40]. These resulting molecules are further degraded by hydrolytic cleavage, facilitated by enzymes, of the glycosidic, ester, and peptide bonds within the plastics. After this step, the remaining molecules can be oligomers or polymers that can be readily assimilated into the cell, such as ethylene glycol and terephthalic acid, or they may need further degradation before assimilation [41]. This step can also be complemented by chemicals in the environment, either produced by the microbe or through other means. These can range from inorganic compounds, such as ammonia and hydrogen sulphide, to organic acids, such as citric acid, fumaric acid, etc. [42].

Once the polymer has been sufficiently degraded outside of the cell, it can be assimilated into the cell. Due to the hydro-carbon-rich nature of polymers, it has been theorized that assimilation occurred through similar means. Research on the assimilation of polymers and degradation products of polymers is limited, research has shown that both active and passive transport are utilized [1]. Finally, once assimilated, the degradation products are further subjected to enzymatic reactions that complete the degradation process into oxidized metabolites, such as carbon dioxide, nitrogen, methane, and water. Alternatively, the intermediate products may be channeled into different chemical pathways, such as the Krebs cycle or lipid formation [43].

2.3 Plastic-degrading microbes

Plastic-degrading microbes are spread across both prokaryotic and eukaryotic kingdoms in the tree of life; bacteria, fungi, and even some algae with plastic-degrading capabilities have been identified [1]. The bacterial genera *Pseudomonas*, *Escherichia*, *Bacillus*, *Streptomyces*, *Thermoactinomyces*, and *Actinomadura* spp., and the species *Rhodococcus ruber* and *Nocardiopsis* sp. have been shown to possess plastic-degrading activity on PET, PE, PP, and p-Nitrophenyl esters [44] [45] [46] [47]. They have a wide variety of hydrolytic enzymes and other bioactive metabolites that they use in growing on and breaking down plastic polymers [48]. While algae are well-studied for their natural bioremediation capabilities and application in diverse industrial processes [49], their capability to break down plastic polymers is not. Nevertheless, there are a few studies that have researched various algae. Many algae species are able to ingest plastics, but there is no evidence supporting that they can all degrade it [50]. However, the species *Scenedesmus dimorphus*, *Anabaena spiroides*, and *Navicula pupula* have been shown to break down small amounts of PE [51]. For fungi, most studies regard *Aspergillus* as the most prominent fungal genus in plastic biodegradation. Most notably, *A. clavatus*, *A. fumigatus*, and *A. niger* have been shown to degrade PE, PU, and PP respectively [52] [53] [54]. Other species with plastic-degrading capabilities include *Fusarium solani*, *Alternaria solani*, *Spicaria* spp., *Geomyces pannorum*, *Phoma* sp., *Penicillum* spp., [47].

Most of this research has been done on large plastic particles rather than MPs and NPs. This methodology may be a reason why the measured degradation rates are low. For example, one isolate of *Nocardiopsis* sp. was able to reduce the PE weight by 22% after two months [46]. The algae *Scenedesmus dimorphus*, *Anabaena spiroides*, and *Navicula pupula* have been shown to degrade low-density PE 3.74%, 8.18%, and 4.44% respectively after 30 days of incubation [51]. *A. clavatus* degrades <25% of low-density PE films after 90 days of incubation [52]. In comparison, the fungal species *Zalerion maritimum* (also referred to as *Z. maritima*) degrades >95% of the dry weight of artificial PE MPs *in vitro* [55]. There is also evidence that suggests that *Z. maritimum* can use PE as a nutrient source [55]. Despite this efficient breakdown, little is known about the fungus itself. It was discovered in 2006 in the Mira river salt marsh and occurs naturally in the coastal waters of Portugal [55] [56]. Earlier this year, the second version of the genome was published on NCBI [57], which consists of 2208 scaffolds. Given its efficient breakdown of PE, this fungus has a lot of potential as a bioremediation tool. However, it has only been tested with pure PE pellets without additives. Therefore, we propose to investigate the underlying mechanisms that are involved in the breakdown of PE in *Z. maritimum*. To this end, we will investigate which genes are involved in degradation and characterize the coding proteins. Furthermore, we will perform a toxicity test on *Z. maritimum* for various additives present in PE products.

3. Workplan

To answer the research questions, we will investigate the sequenced *Z. maritimum* strain ATCC 34329. The standard growth conditions will be in a minimal growth medium with sea salts at 25°C as described by Paço *et. al.* [55], and pH 8 as seawater's pH is typically between 8.08 and 8.33 [58]. In our first work package, we will make an improved genome assembly by performing a hybrid assembly of second generation (Illumina) and third generation (Oxford Nanopore) sequencing reads. This genome will be used in gene prediction and expression analysis to determine significant expression in the presence of PE in our second package. We will test *Z. maritimum*'s resilience against plastic additives in our third work package by subjecting it to PE products used in daily life, as existing research on this fungus only used pure PE pellets [55]. In our fourth work package we will determine which proteins are involved in PE degradation by performing knock-outs on genes previously found by the expression analysis in our second package. In our fifth and final work package, we bring these proteins to expression in *E. coli*, isolate them, and determine their enzyme kinetics.

3.1. WP-1: Genome assembly improvements

Currently, there is a published reference genome [57] which was constructed using only short sequencing reads, which consists of 2,208 scaffolds and has a total length of 58.43 Mb. Consequently, the genome sequence has to be improved. To this end, we will use hybrid assembly [59]. This is a method where both short sequencing reads (100-1000 bp) and long sequencing reads (>10,000 bp) are used. While the shorter sequencing reads are more accurate, longer reads can help bridge gaps and reduce structural errors on repeating sequences [59]. While short-read sequences are already available on NCBI, we will obtain long sequencing reads using the Oxford Nanopore Technologies (ONT) MinION platform and perform a *de novo* genome hybrid genome assembly using both short and long reads.

3.1.1. WP-1.1: DNA isolation and sequencing.

Z. maritimum mycelium will be obtained by growing the fungus under standard conditions. DNA isolation will be performed in accordance to the protocol by Möller *et. al.* [60], which has been used to isolate DNA in the related species *Z. pseudomaritima* [61]. Quantification of the DNA concentration, and DNA quality check, enrichment, adapter ligation, sequencing, basecalling, and adapter trimming, as well as read quality control will be done as described by He *et. al.* [62]. If there are many short fragments in the sequenced reads, they will be filtered out by selecting only fragments >100 bp.

3.1.2. WP-1.2: *De novo* genome assembly.

Both the long reads and the short reads will be used to do a *de novo* hybrid assembly using SPAdes [63]. We will perform additional consensus runs on the assembled contigs and reads using Racon [64] to improve the assembly. While we do not expect or try to obtain a chromosome-level assembly, we are aiming to get an assembly with <100 scaffolds. The current reference assembly has a size of 58.43 Mb. The hybrid assembly will likely be larger due to a proper assembly of repeat sequences. We estimate the final assembly to be between 58 Mb and 62 Mb.

3.2. WP-2: Gene prediction and analysis

RNA will be isolated, sequenced using Illumina, followed by filtering of the mRNA reads. We will subsequently map the reads to the genome for gene prediction. To maximize genes discovered, RNA will be isolated from *Z. maritimum* mycelium grown at different pH and temperature conditions, with different carbon sources, and with and without PE pellets. With our predicted genes and RNA sequencing data, we can also determine which genes show significantly different expression in the absence and presence of PE pellets. Finally, we will do a functional gene analysis to see which types of genes are differentially expressed and search for orthologs in *A. clavatus* that is known to have limited PE degrading capabilities [52].

3.2.1. WP-2.1: RNA isolation and gene prediction

Z. maritimum mycelium will be obtained by growing the fungus under standard conditions and variations on it in single samples per condition. The fungus will be grown at pH 5, 8, and 10 using the standard medium. Also, *Z. maritimum* will be grown in variations on the standard media in which glucose and malt extract (standard conditions) have been replaced by lactose, corn starch, and cellulose. Also, the fungus will be grown at 5°C,

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25°C, and 37°C. From these incubations, samples will be taken after 7 days of incubation. Finally, *Z. maritimum* will be cultured under standard conditions with and without PE pellets as described by Paço *et. al.* [55] in biological triplicates for expression analysis. Samples of these incubations will be taken after 0, 7, 14, 21, and 28 days [55]. RNA isolation, RNA quality control, library preparation, sequencing, and read quality control will be performed according to the methods described by Antonieto and Silva [65].

3.2.2. WP-2.2: Gene annotation and expression analysis.

Reads from all previously specified conditions will be pooled and used to predict genes in our assembled genome using the FunGAP pipeline [66]. With the resulting gene annotation, we will perform functional annotation of the predicted genes using InterProScan [67]. By aligning the reads to the genome with BWA-MEM [68], we can generate gene counts using featureCounts [69]. Using EdgeR [70], we will determine which genes show significantly different expression in conditions with and without PE pellets, according to the EdgeR handbook [71]. Finally, we will search for orthologs in *A. clavatus* of all significantly differentially expressed genes found using OrthoMCL [72].

3.3. WP-3: Knock-out experiments

Based on the results obtained from the differential expression analysis, functional annotation, and ortholog results, genes will be selected for knock-out experiments based on their characteristics, such as whether or not they are extracellular, are predicted to be an enzyme, and whether their original function has to do with breaking down natural polymers. The genes we deem most likely to be involved in PE degradation based on these factors will be knocked out using CRISPR-Cas9, after which the knock-out strains will be subjected to media with PE pellets and monitored for PE degradation.

3.4.1. Knock-out using CRISPR-Cas

The knock-outs in *Z. maritimum* will be performed using the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) system designed for filamentous fungi by Nødvig *et. al* [73]. Single-guide RNA (sgRNA) sequences will be designed and verified *in silico* before *in vitro* application using BE-Designer and BE-Analyzer respectively [74]. The PE degradation will be measured in all knock-outs and the unmodified strain in biological triplicates as described by Paço *et. al.* [55].

3.4. WP-4: Determining protein characteristics

When impactful proteins have been identified by the expression analysis and knock-out experiments, they will be brought to expression in *E. coli*, after which they will be purified from the medium and their enzyme kinetics determined.

3.4.1. Enzyme kinetics

The proteins of interest will be brought to expression in *E. coli*. They will then be purified, verified by protein sequencing, and the enzyme kinetics at different temperatures and pH will be determined according to the methods described by Linde *et. al.* [75].

3.5. WP-5: Toxicity testing for plastic additives

A toxicity test will be performed with various PE sources used in daily life and analyze the ability of *Z. maritimum* to grow in their presence, and its ability to break down PE microplastics with additives. It should be noted that this method doesn't test specific additives, but rather the end-product plastics. This is mainly because investigating the over 10,000 different additives [31] individually is a task that doesn't fit the scope of this research project.

3.5.1. Incubation with daily-life sourced PE

To determine *Z. maritimum*'s ability to break down PE with various additives, we will perform the same growth experiments as described earlier with the methods by Paço *et. al.* [55], with the difference being that, instead of pure PE pellets, PE from packaging foils, plastic bags, agricultural mulch, cable insulation, and bottles will be used. These incubations will be performed in triplicates for 28 days in total, with sample collection at days 7, 14, 21 and 28.

3.6. Project duration and planning

The expected duration of this project is about two years. A more detailed time estimation can be found in the Gantt diagram (Table 1) below.

Table 1: Gantt diagram of expected duration of the discussed subsections of the methods. Each rectangle represents one month of time for a total of 24 months.

WP-1.1 DNA Isolation & sequencing																								
WP-1.2 *De novo* genome assembly																								
WP-2.1 RNA isolation & gene prediction																								
WP-2.2 Gene annotation & expression analysis																								
WP-3 Knock-out using CRISPR-Cas																								
WP-4 Enzyme kinetics																								
WP-5 Toxicity testing for plastic additives																								

4. Risk assessment

4.1. WP-1: Genome assembly improvements

DNA isolation (WP1) could result in low yield, low quality, or high fragmentation. As the proposed method has been used in a closely related species, we estimate the latter problem to be more likely than the others. The solution is to try and adapt the method, or try different methods. It is recommended that adjustments or new methods are based on enzymatic/chemical lysis, such as the methods by Lech *et. al.* [76], as they typically result in higher molecular weight DNA isolated. It is also possible that we fail to achieve an assembly with <100 scaffolds with the suggested methods. A possible alternative is to polish the existing assembly with the long reads using Racon.

4.2. WP-2: Gene prediction and analysis

It is possible that the fungus will not grow under some of the proposed conditions. If this is the case, we will adjust the conditions to be closer to the standard growth conditions. Secondly, RNA isolation may also result in a low yield, low quality, or high fragmentation. Like with DNA isolation, adjustments or different methods can be tried to alleviate the problems encountered. In terms of the bioinformatics part, not much can go wrong. The gene prediction will not be 100% accurate; some genes will be incorrectly predicted, and some existing genes may be missed. However, given the wide range of conditions for our RNA pool, the vast majority should be included, and the number of false positives and negatives is negligible.

4.3. WP-3: Knock-out experiments

Workplan 3 involves several risks, which stem from the inherent problem that research in *Z. maritimum* is quite limited so far. If we find that the initial attempts are unsuccessful, the problem may lie in the sgRNA sequence, the promotors used, or an inherent intolerance to any of the proteins involved. While the last one can only be solved by trying alternative methods, sgRNA sequences can be redesigned and optimized, and also be transformed as a pre-assembled complex [77]. Transformation in this non-model fungus may also prove difficult as well. There are various methods of transformation and other adjustments that can be made to the proposed methodology [78].

4.4. WP-4: Determining protein characteristics

There are also several risks involved in workplan 4. First, it's possible that some of our proteins of interest are toxic to *E. coli*. This can be resolved relatively simply by choosing a different host such as *Saccharomyces cerevisiae*. Secondly, the proteins of interest may require post-translational modifications that *E. coli* cannot provide. In this case, it will be necessary to isolate the protein from the native species or from another fungus such as *S. cerevisiae* [79].

4.5. WP-5: Toxicity testing for plastic additives

We do not expect failure of these experiments. If we find mixed results in the triplicates, we will repeat the growth experiment with that plastic on a larger scale (10 samples) for a more definitive answer as to how efficient *Z. maritimum* degrades daily-life PE products compared to pure PE pellets.

5. Impact on science and society

In light of the MP's and NP's crisis as explained in section 1, any research regarding potential (partial) solutions to the problem is of importance. In the short term, it will not do much to immediately alleviate the problem. It will add to our current insights into biological plastic degradation. It does open up possibilities for enzymatic cleaning of water, but more research is required as to what kind of intermediate and end products are produced by the genes we will find. In the long term, it may open up possibilities to clean water of PE MP/NP contamination in bioreactors using the fungus itself or the enzymes it produces; possibly in tandem with other plastic-degrading enzymes or organisms. It depends on the enzyme characteristics, the fungus' resilience against PE additives, and further research into this fungus and other plastic-degrading organisms as to what the best course of action will be. Either way, it is important to have sufficient information to make the right choices as soon as possible, and this research will assist in that goal.

6. Ethical considerations

This research has no direct negative impact on humans, animals, or our environment. That being said, researching plastic-degrading enzymes opens up possibilities further down the line for genetically modified organisms (GMOs) that can break down plastics at a faster rate. Optimizing the process of breaking down plastics is very important if we want to limit the effects of plastic pollution, and developing these GMOs for laboratories and/or reactors is a logical and important next step. One ethical consideration would be the question if we should release these inevitable GMOs into the environment to clean it up directly, rather than in reactors or a similar setup. Releasing GMOs into the environment is not something that should be done lightly, as they can have unforeseen effects on the ecosystem. However, setting up sufficient reactors or facilities to clean up the entire ocean may be impossible, in terms of both the material and finance required. With the growing plastic production, and thus the growing amount of plastic waste, we may not have a choice but to release cleaning GMOs into the ocean. While this prospect is still far away, it should be taken into consideration, as current GMO laws in many areas would not permit it.

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