DAME: Destruction of Aqueous Microfibers Enzymatically

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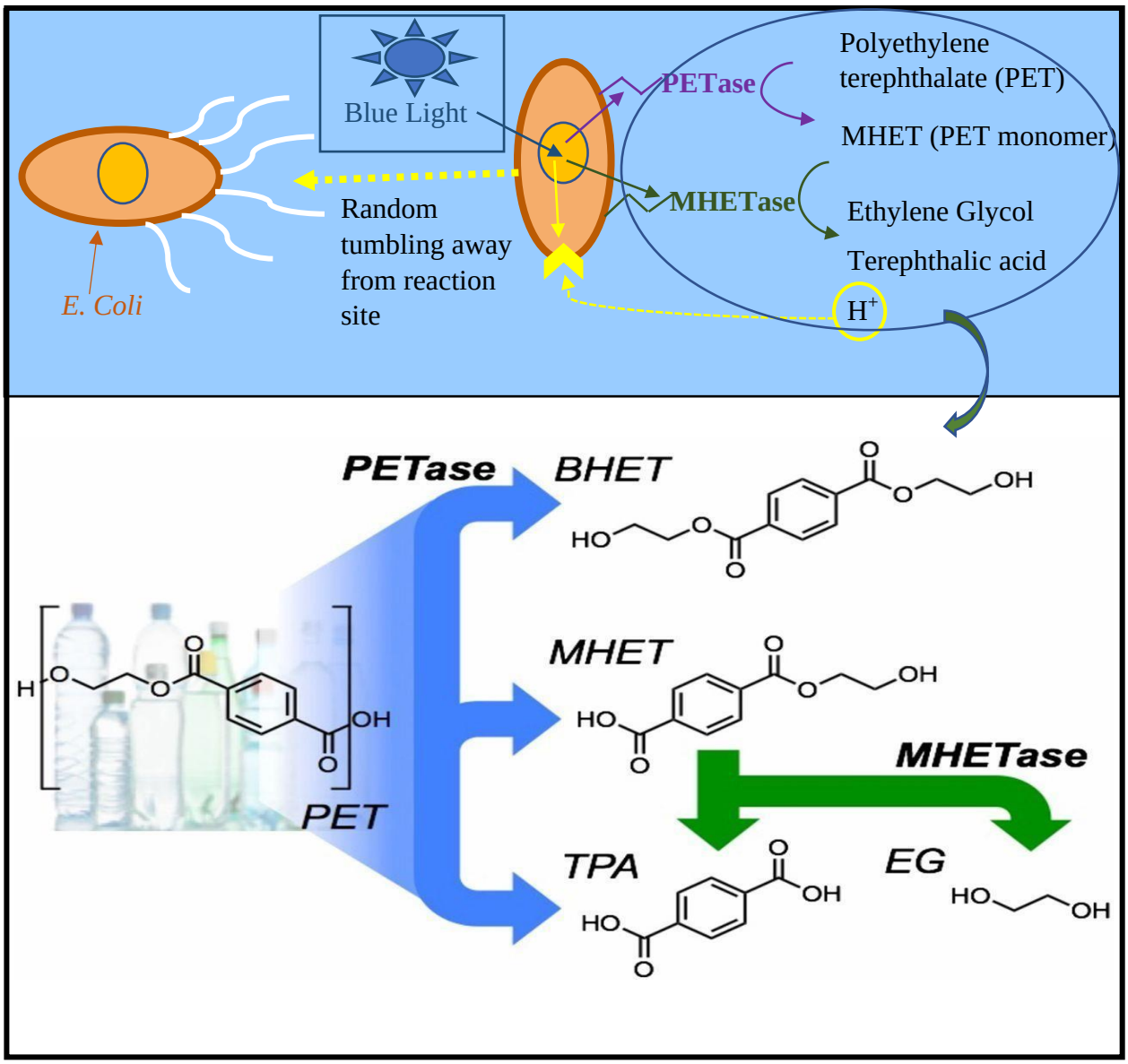
Abstract Figure



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Purpose

Microplastics are plastic particles that are less than a millimeter in length, of which the most common type are microfibers. Microfibers are pieces of synthetic textile that are usually created from the agitation of synthetic clothing in washing machines[1]. They can serve as vectors for chemical toxins and microbial pathogens, leading to the potential for biomagnification of these contaminants in organisms that consume them[2]. As well, they can be on the micrometer scale, in terms of size[1].

Sadly, microfiber pollution is an under-represented issue in public environmental affairs. One explanation could be that "more severe" issues, such as global warming, have taken center-stage in public debate. However, there is serious need for rallying around this subject.

Microfibers are accumulating across marine habitats worldwide. These pollutants have been detected on shorelines spanning nearly 18 sites in 6 continents[2]. A possible explanation for this could be due to atmospheric causes of microplastic transport. An example of such atmospheric transport is the shocking presence of microplastics in relatively remote corners of the world[37].

Importantly, the average garment is enhancing this pollution problem by contributing more than 1900 microfibers per wash[2]. The total number of microfibers in water sources will only expand as the human population surges and our dependence on synthetic textiles grows.

Initially, these aqueous microfibers travel to waste-water treatment plants (WWTPs). WWTPs are intended either for water purification aimed at public use or for dumping into marine environments. Yet, most WWTPs do not filter for microfibers due to the lack of cost-effective technology to do so. Moreover, one rare study that attempted to filter microfibers in WWTPs required nearly 3 levels of expensive filtration before reaching an efficient purification[1].

Of note, human health is directly being impacted by our negligence towards this issue. Interesting studies have found high levels of deleterious microfibers in commercial fish[3] and even in deep sea organisms[4], both of which may be causing ecological damage. Furthermore, humans are currently consuming and inhaling several thousands of microplastic particles

annually which poses serious health risks[38]. Microplastics can translocate into human tissues, producing immune responses, and release toxic heavy metals and pesticides into the body[39]. One obvious solution is for humans to curb their use of synthetic polymers but given that the textile industry is a multibillion-dollar trade, this is unlikely to happen.

Chemically, the most common types of synthetic textile polymers are polyamides and polyesters[5,6]. Within polyesters and polyamides, the most common compounds are polyethylene terephthalate (PET) and nylon (Figure 1). There are no conclusive studies comparing differences in degradation time for polyesters and polyamides enzymatically. Keeping the former in mind and with the hope of tackling one issue rather than bungling multiple, I have decided to tackle polyester-based microfiber pollution. I plan to use a synthetic biology approach to enzymatically destroy polyethylene terephthalate (PET) ultimately to benign natural small molecules.

To be more specific, I propose using synthetically engineered *Escherichia coli*, expressing enzymes that can degrade PET, upon blue-light induction, in a new type of water chamber located right before final effluent is released from a WWTP. The specific details of this system will be elucidated further but a general overview is provided (Figure 2).

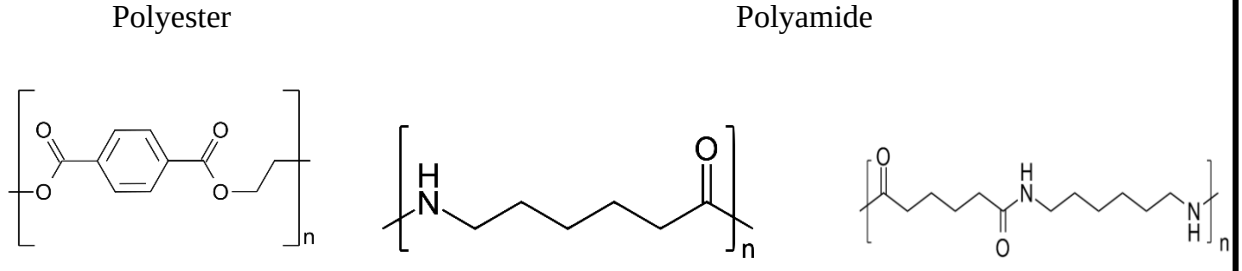

Figure 1. Comparing the structures of polyamides vs polyesters. From left to right: PET, Nylon 6 and Nylon 6,6.

Competing Technologies

Only one competing technology exists that has been used at the WWTP level and is published in scientific literature. The AnMBR pilot system uses a 1900L anaerobic bioreactor with a ceramic disc membrane unit capable of microfiltration[1]. The positives of this system

include the fact that it is capable of eliminating 99.1% of microfibers and is the first known system published in the scientific literature[1]. Some issues with this system include the requirement for 3 levels of filtration to receive an efficient microfiber reduction, the usage of a relatively small 1900L tank for filtration and the requirement for a microfiltration membrane disc which can cost upwards of 700 US dollars[1].

Two other alternatives involve tackling the same issue (microfiber pollution) but require

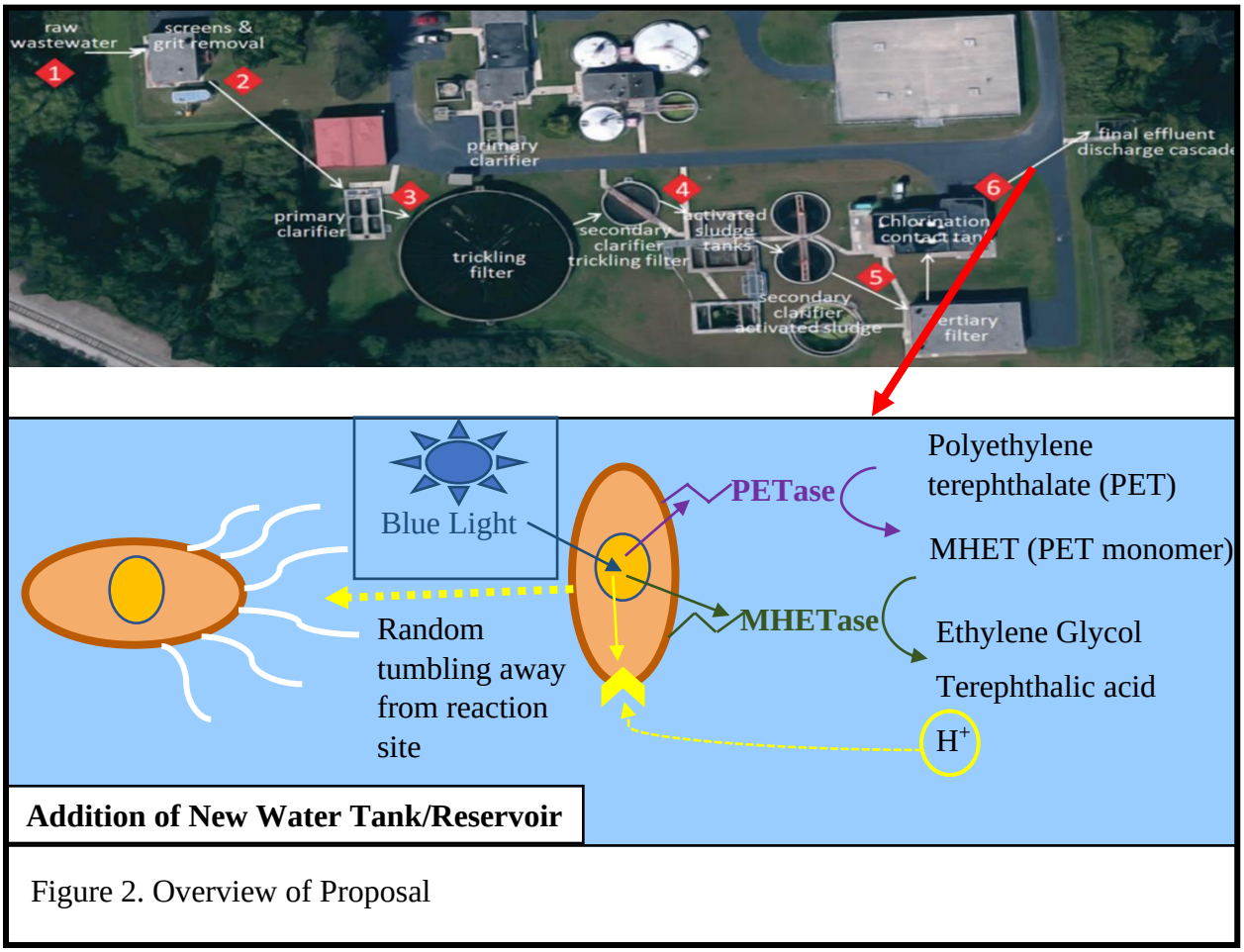

Figure 2. Overview of Proposal

consumers to take initiative to prevent microfiber pollution in their own washing machines.

The first solution is the Guppyfriend which is a washing machine-friendly bag that allows synthetic textile clothing to be inserted inside of it. The product claims to prevent the creation of microfibers and capture those that are released. The major benefit is that it gives environmentally-conscious consumers the choice to minimize their personal impact on the environment. Some issues though include the fact that it claims to capture 86% of potential

microfibers without unbiased, independent scientific review and that the marketing/awareness of this product is low.

The final competing technology of note is the Cora Ball, which is more like Guppyfriend than AnMBR. Consumers can buy this product, which goes into their washing machine, that claims to pick up most microfibers. It has the same benefits and issues as the Guppyfriend with a noted difference being the percentage of microfibers it can prevent being released. An independent study found that only 26% of microfibers were caught by this technology[7].

Background and Design

Polyethylene terephthalate (PET) can be broken into monomers by many processes including hydrolysis[8]. However, a novel enzymatic route was discovered only a few years ago. In 2016, researchers in Japan found a bacterial species, known as *Ideonella sakaiensis* 201-F6, that lived on thin PET film. It utilized two novel enzymes, PETase/MHETase, and used the enzymatic reaction products (Terephthalic Acid/Ethylene Glycol) as an energy source[9,10] (Figure 3). Specifically, the hypothesized metabolization of the enzymatic reaction products is as follows: the cell uptakes the Terephthalic Acid through the TPA transporter enzyme and produces NADPH with the final metabolite being a tricarboxylic acid[9]. The byproducts of this metabolic pathway are CO_2 and H_2O. PETase/MHETase require no known co-factors to function. UniProt IDs for PETase and MHETase are A0A0K8P6T7 and A0A0K8P8E7, respectively.

Of note, *I. sakaiensis*, the organism used in previous PET-degradation studies, has not been studied or utilized extensively. Rather, the enzymes of interest (PETase/MHETase) have been investigated. PETase specifically has been structurally deciphered using crystallography and was codon optimized for high expression using a plasmid in *E. coli*[10,11]. Specific details, such as active site flexibility, have also been characterized[12]. Finally, production of this enzyme extracellularly has been achieved[13].

At an overview, I propose creating a new 2000L microfiber-destroying water chamber in WWTPs right before final effluent is released. This chamber would contain water that is nearly pH-neutral, relatively nutrient depleted and around room temperature. I will use synthetically engineered bacteria to destroy aqueous microfibers in this specialized tank. Considering the rapid

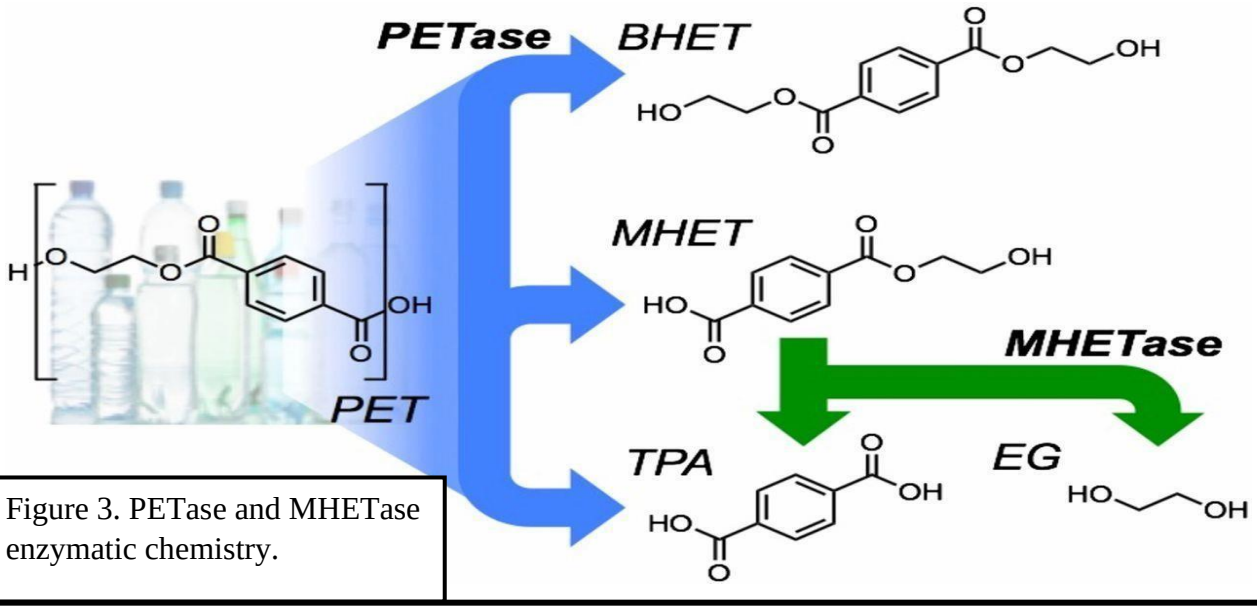

Figure 3. PETase and MHETase enzymatic chemistry.

division rate and scientists strong understanding of them, *E. coli* are the best option for a chassis. The engineered *E. coli* must survive in this aqueous environment which should not be an issue as bacteria can live in a variety of aqueous settings including well, lake, river and waste water[14-18].

The input to my system will be blue-light which will interact with a blue-light inducible promoter[19] in my engineered cells. This will induce an increase in the transcription of enzymatic genes that will ultimately destroy PET into its simple monomers of ethylene glycol and terephthalic acid. The sudden increase in acidity in the microenvironment by terephthalic acid will cause a negative chemotactic response from the cells[40]. Hence, the cells will randomly tumble away from the finished reaction site. This reaction will be allowed to take place over multiple hours whereby the starved *E. coli* cells (many of which could be dead) as well as the byproducts will be flushed with the effluent.

The engineered cells will be flushed with effluent to avoid the expensive cost of buying and maintaining microfilters. Since the engineered cells are being flushed, this system does require the addition of engineered cells upon each batch of the water chamber. This may necessitate the creation of a small building to maintain bacterial cultures. However, other collaborative solutions are possible. This could be an opportunity for collaboration between

government and industrial biotechnology companies whereby the companies maintain bacterial cultures whilst the government focuses on water purification.

In relation to the small molecule by products, terephthalic acid has very low toxicity with an LD_{50} over 1g/kg in an oral mouse model[20]. However, ethylene glycol is toxic with an oral LD_{Lo} of 786 mg/kg[21]. Yet, this should not pose a large risk for our system. Even though microfibers are ubiquitous, in terms of the solute/solution ratio, they would be quite dilute in a 2000L water chamber as proposed.

In addition, there are several other genetic components involved in my system. Besides, the blue-light inducible promoter, I would attach a membrane-anchored[22] PETase[23], a membrane-anchored[22] MHETase[24] and a proton-based negative chemotaxis protein[25]. As a point of clarification, the blue-light inducible promoter will control expression of all the parts.

Initially, I would like to create a plasmid containing all compoenents mentioned in the above paragraph. To do so, I would take the individual parts and use a method such as Golden Gate cloning[26] to combine them into one plasmid. To verify inclusion of all parts, we could take the "final product" of these modular cloning events and use High-Performance Liquid Chromotography-Mass Spectrometry (HP/LC-MS) techniques to quantify if the proteins of interest are found in an in-vitro medium[27].

Instead of inserting the plasmid into the engineered cells, I would like to insert this novel genetic material into the bacteria's chromosome through "recombineering" via Lambda Red. Lambda Red is an efficient method to insert novel genetic material onto chromosomal targets[28,29]. A similar HP/LC-MS technique as mentioned above can be used to verify that the recombination event has happened successfully.

Testing

Since this design has multiple complex components, I will focus on quantification of the metabolic products in this system, Ethylene Glycol and Terephthalic Acid. Initially, I would take a small beaker (500mL) and fill it with distilled water. Next, I would weigh a small piece of pure PET film and shred it using a micro-shedder. I would add a pre-determined quantity of bacteria with the shredded film into the water. Finally, I would shine strong blue light over the beaker to induce transcription of the necessary enzymatic genes.

After 4-5 hours, I would measure the amount of Ethylene Glycol/Terephthalic Acid that is produced as a ratio of PET (Ethylene Glycol or Terephthalic Acid ÷ initial PET). Small molecule quantification can be achieved via LC-MS[30]. This testing protocol will improve the overall system by allowing the fine-tuning of bacterial concentration needed in solution to effectively produce the by-products in a timely manner. Similarly, we can use these quantitative results to investigate the necessity of improving the enzymatic activity of the membrane-bound enzymes, PETase and MHETase.

Furthermore, testing may reveal greater potential for the system. Common polyester derivatives such as Polybutylene Terephthalate or Polytrimethylene Terephthalate could be added to see if they produce Terephthalic Acid upon contact with these polyester-degrading enzymes. This is a plausible idea due to the similar chemistry of the substrate and the relative lack of study on these enzymes. Even more radical, what if these enzymes can degrade polyamides? No matter how unlikely, no scientific literature to date has disproven this enzymatic function.

Expected Results

When the designed system is working perfectly, the blue light would be captured by microbes in the water. This would induce transcription of genes of interest whereby the PETase and MHETase go with an anchor protein to the membrane and the chemotaxis protein goes there as well. The system would react with nearby microfibers and degrade them to harmless by products. The increase in acidity due to the byproducts would trigger the proton-based negative chemotaxis protein, moving the bacterium away from the reaction site. Just like that, we have taken polluting synthetic polymers and turned them into natural small molecule products.

Potential Problems

There are many potential issues with this system. One could be that the blue light is not strong enough to induce transcription in all microbes present in the water chamber. A potential solution to this could be constitutive expression of genes of interest but this destroys the “ON” switch ability of this system. Another issue could be incomplete chemical activity. The enzymes may not be chemically active enough to hydrolyze substrates or incomplete reactions could occur (i.e. production of MHET). A remedy to this could be site-directed mutagenesis, which has been

achieved previously[11,31]. Finally, making sure that the membrane-bound enzymes are facing outwards to the extracellular matrix and not inwards to the cytoplasm could pose a problem. This could be fixed by using other membrane tags such as PelB[41].

Evolution of the engineered cells could be beneficial if they can survive without glucose and instead use the natural by-products as food. In fact, we could drive this process by selecting for cells that are able to grow on PET film, analogous to the natural selection conducted upon the original *I. sakaiensis* strain, before we introduce them to our water chamber. However, evolution could also cause these organisms to stop responding to blue-light and stop producing enzymes if they are nitrogen-depleted or in arrestation via RNA polymerase, sigma S (RpoS). RpoS arrestation of these bacteria is a valid concern that may cause inefficient ability to produce protein products of interest and prolong the bacteria's life[32,33]. Deletion of the RpoS gene could be a solution to non-functioning cells caused via arrestation of these cells[34].

Some shortcomings of this system include the fact that it requires a separate building/entity to maintain the cell culture since microbes are lost in each water chamber batch. Similarly, a new water chamber needs to be built that also utilizes strong blue light. This may become a serious monetary issue. As a final point, people may be scared by the idea that dead and/or alive bacterial cells will leave with the final effluent. The benefits of this release will be discussed further in the subsequent paragraph. Some solutions to this problem could be utilizing microfilters[1] or more specifically carbon nanotube filters[35,36]. Yet, both options are quite expensive.

This design, as it stands, does not pose major dangers to the environment or to public safety. If anything, there are potential benefits for releasing these bacteria into freshwater or into public water lines as a means of cleaning up any PET microfibers that are found. Once again, these microfibers are deleterious to our environment and to multiple species, including us. Nevertheless, evolution is complex and genetic changes in the synthetically introduced parts could cause novel chemistry and by products to form, which may be deleterious.

Other than money and labor, the risks of this proposal are minimal. The microfiber pollution problem has been caused by humanity's insatiable desire for synthetic polymers without much thought to its potential effects. Now, we could be at the cusp of reducing the biomagnification of pollutants in our environment and improving human health.

Conclusion

There have been very few novel systems like the Destruction of Aqueous Microfibers Enzymatically (DAME) system I have proposed. To be precise, there is only one competitor, the microfiber filtering AnMBR. This competitor was used temporarily for a study and does not seem to be operational at the current moment. My system would use a reactor with fewer mechanical parts and pose less of a biohazard. As well, the AnMBR system used ceramic disc microfilters to keep their microorganisms from being released due to potential hazards[1]. The usage of approximately 700 dollar microfilters is unnecessary given the fewer hazards my system presents.

The human population will rise. Our demand for synthetic polymers, like PET, in textiles will follow suit. I do not want to sit by idly in a world that accepts the havoc that these microfibers produce. Biomagnification of PET-bound toxins in marine ecosystems and in our daily lives is a serious concern. We understand the issues it causes in marine ecosystems, but the scariest part is that we haven't thoroughly/fully characterized its effects on human health. One can assume the effects would be deleterious but the absolute ignorance we have for this issue will need to be remedied. Research on the human health effects of PET and the toxins it binds must be conducted. While researching these health impacts, we must work to destroy/reduce microfiber presence in our world. We should all be remembered for leaving our world a better place than we found it. This is what drives me to the creation of new ideas such as DAME. To conclude, this system is imperative for improving our ecosystem and consequently, our health.

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