A Risk Assessment of Coastal Nanoplastic Particles:

Enzymatic Pre-Treatment and Analytical Approaches at the Nanoscale

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Abstract

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Anthropogenic impacts on marine ecosystems have developed into a dominating and formative force within coastal regions. One of the strongest influences within these coastal anthropogenic zones is the deposition and life cycle of marine plastic waste. Macroplastic, mesoplastic, and microplastic waste fields have undergone significant growth in both study and remediation yet given the various mechanisms of degradation within the marine environment, all plastic waste categories without intervention will degrade onto the nanoscale, a class of waste termed nanoplastics.[2] The field of nanoplastics remains in its infancy,[1] it lacks both standardized definitions and operating procedures for sampling and analysis, however inferences on activity and environmental relationships can be derived from current and former literature on engineered nanomaterials. This work provides several approaches for addressing a risk assessment

for coastal micro and nanoplastic particles within the coastal zone scenario, with specific scoping towards the developmental endpoint. This risk assessment incorporates ranges of i) plastic concentrations, ii) plastic exposures through incidental marine ingestion and ingestion of seafood, iii) and constituent compound contents to better assess plastic risk from a holistic view. There is insufficient toxicological data to conduct a thorough quantitative risk assessment. Using available environmental monitoring data, we were able to apply the mixtures-based risk assessment developed within this work and we believe that coastal micro and nanoplastic mixtures pose low to no level of risk to the public regarding the developmental endpoint at present environmental concentrations. Although this study has determined low to no risk at present, we emphasize that this assessment and the data availability relating to hazard and exposure characterizations is underdeveloped and believed to be increasing in magnitude leading to a relevant yet likely inaccurate model. This study also addressed some of the analytical deficiencies identified through conducting a review of the literature relating to sample treatment and analysis for environmental nanoplastic particles from various media. This review revealed complex analytical considerations applied within the pretreatment schema we put forth for seafood and marine media. A methodological study and approach is applied here in where polyethylene (PE, 200-1000 nm), polyethylene terephthalate (PET, 60-500 nm), polymethyl methacrylate (PMMA, 150 nm), and carboxylated polystyrene (PS, 120 nm) self-synthesized and commercially procured nanoparticles underwent an enzymatic digestion to progress the state of viable pretreatment methods for marine micro and nanoplastic particle samples. Post-digestion particle size when assessed via spectroscopic methods yielded statistically significant increases of 23% (PS), 51.7% (PMMA), 43.9% (PE), 380.2%(PET) diameter across all polymers, while

assessment through microscopic techniques yielded a statistically significant increase and decrease of 65.7% and -72.4% for PS and PET respectively.

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Dedication

I would like to dedicate this work to my mother and father who have supported me tirelessly.

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1.1 Plastic Production and the Marine Environment

Coastal zones have held significance for human beings since the dawn of civilization. These zones provide various logistical advantages such as resource access, transportation, visibility, and recreational or cultural significance as an interface between the land and the ocean. Projections estimate that populations within the contiguous and hydrologically connected zone of land along the coast and below 10 m of elevation (LECZ) may hold 949 million people by 2030[31]. The United Nations set forth in 2017 that 40% of the world's population lives within 100 km of the coastline. Within this interactive zone, anthropogenic influence has become ubiquitous and has played a large role in marine ecosystem influence[49]. Synthetic polymers, also known as plastics, have incorporated themselves into our everyday lives. Currently, plastic production has become integral to global industry, however our ability to mitigate and manage the global plastic volume is underdeveloped[16]. From its beginnings in 1950, plastics have grown from an estimated 2 million metric tons (Mt) of resins and fibers produced annually onto an estimated 380 million Mt produced in 2015[11]. It is estimated that from the beginnings of plastics to present day the world has produced over 9 billion Mt of primary plastics, and the most common plastic species globally are polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyurethane (PUR), and polystyrene (PS). PE can come in various densities as can other plastic species, high-density polyethylene (HDPE) and low-density polyethylene (LDPE) are the main subsections of this class[11]. Conventional plastics pose a challenge due to their inability to effectively biodegrade, resulting in long residence times and accumulation in the environment. It is estimated PE (36%), PP (21%), PVC (12%), PET (<10%), PUR(<10%), and PS (<10%) comprise 92% of all plastics ever made[11]. In 2015, it

was estimated that Europe alone consumed 49 Mt of plastic products throughout various large market industries. The most significant industry consumers were the packaging (39.9%), building and construction (19.7%), automotive (8.9%), electrical and electronic (5.8%), and agricultural industries (3.3%), with the remaining 22.4% being attributed to various smaller consumers denoted as "Others" [12]. Based on present growth rates, global plastic production is anticipated to increase by 100% within the next 20 years. Alongside plastic production itself the utilization and societal role that plastics have adopted pose further challenges. It is estimated that of all plastics produced all time, 30% are currently in use, 60% have been discarded into landfills or environmental compartments, and the final 10% is unaccounted for [11].

Of concern within this body of work is plastic waste that has been or is more likely to be redistributed into the environment, commonly termed mismanaged plastic waste (MPW). It is estimated that 60-99 million Mt of MPW was produced in 2015 globally, however MPW figures have undergone significant growth in recent years, and it is projected that under a business-as-usual scenario MPW production could reach 155-265 Mt annually by 2060 [13]. The annual amount of MPW generated by populations living within 50 km of the coast was estimated at 31.9 million metric tons per year, giving insight into MPW more likely to be redistributed into marine environments [18]. In 2010, annual entrance of land-based plastic waste into marine systems was estimated to be between 4.8-12.7 million Mt [14]. Of the total mass of plastic waste produced in 2016, approximately 19-23 million Mt or 11% of the annual load entered aquatic ecosystems with this value anticipated to reach 53 million Mt by 2030 [15]. Comparing Lebreton et al. and Borrelle et al. there is a range of 49%-479% estimated growth of MPW entering the marine compartment between 2010 and 2016, a level of variability that prevents

effective assessment of and response to marine plastic waste. While fluctuating usage, production, and management influence this variability, it should also be noted that plastic releases entering the ocean via terrestrial, atmospheric, fluvial, and marine sources are dynamic and require further investigation[16, 22]. At present, the current understanding is that land-based sources remain the dominant route for plastic waste entering the oceans globally with the following contributing classes being marine based sources, fluvial sources, and atmospheric sources[16, 17, 22].

1.2 Definitions of Marine Plastic Waste

Various terminology exists for marine plastic and plastic wastes, however the definitions for marine plastic waste classes to be used within this work are as follows: macroplastics (large plastic objects, 1 m – 2.5 cm in size), mesoplastics (large plastic particles, 2.5 cm – 5 mm in size), microplastics (small plastic particles, 5 mm-1 μm in size), and nanoplastics (small plastic particles, ≤1000 nm in size) [21, 29, 30]. The class we are concerned with in this body of work is the transportation, formation, and activity of marine nanoplastic materials. The field of marine plastic study began to develop during the early 1970's alongside other budding environmental movements, however microplastics and nanoplastics as respective fields are still developing. In a meta study by Alprol et al conducted in 2021 (Figure 1) it was found that less than 50 publications containing the keywords, "microplastic(s), nanoplastic(s)" were in circulation in 2010, and by 2021 these keywords yielded 3,072 and 292 articles, respectively. At present there are still no standardized definitions for microplastics or nanoplastics. The working definition of microplastics used within this study is generally accepted, however the

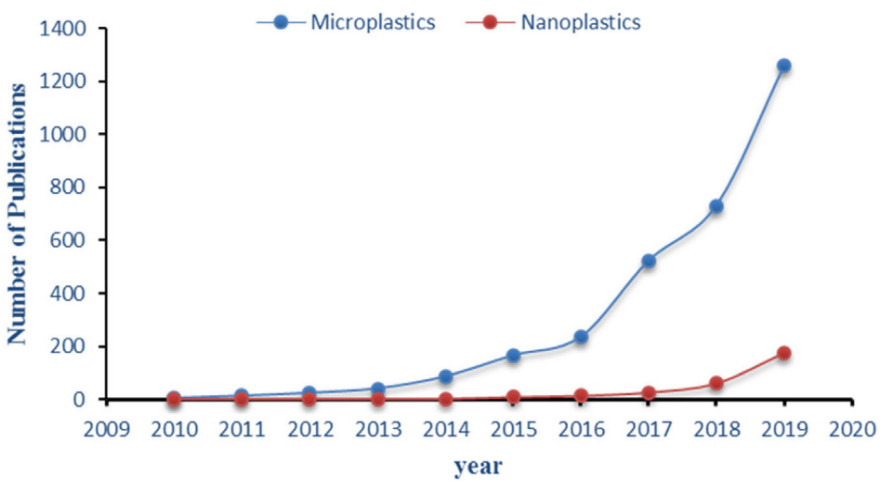


Figure 1. Literature on MPs and NPs in the environment web of science (a tool from Clarivate Analytics, 2020) since 2009, analyzed by years.[1]

definition of nanoplastic is still under debate [16, 20]. Some have recommended that this class solely include plastic particles below 100 nm in size, others below 1,000 nm, and others that colloidal behavior be the defining characteristic [19, 20, 61]. It is likely that these definitions were influenced by the US National Nanotechnology Initiative nanomaterial definition which is a material having at least one dimension between 1 and 100 nm and exhibiting properties not found at larger sizes of the same material [21]. While often applicable due to similarities in size and analytical instrumentation, engineered nanomaterials and environmental nanoplastic particles often contrast in physical condition, composition, and media considerations [50].

1.3 Degradation of Marine Plastic Waste

Using estimations of the global marine plastic fraction and known accumulation zones, guided assessments of marine plastic have yielded conflicting and concerning

observations [30, 51, 52]. At present, assessments of the global loading of plastic waste into marine systems have found that 95.3%-99% of anticipated plastics produced since 1960 are unaccounted for [14, 23]. There are likely many factors that play into such high discrepancies between the quantities of plastics produced and those that are being observed. One explanation could be that the magnitude of plastic waste entering the ocean has been overestimated, however it is highly unlikely that these figures would exhibit a 95.3%-99% level of inaccuracy. A more convincing argument, and of primary concern within this work, is a lack of persistence on the ocean's surface by small-scale marine plastics [24]. Many plastics are widely considered non-biodegradable and boast substantial half-lives, however through various passive and active marine mechanisms, it is clear that the primary fate of marine plastic without intervention is degradation into the nanoscale. Marine plastic degradation is a time-intensive process (on the order of decades to centuries), and can take place via biotic, abiotic, or abiotic-biotic pathways. It is assumed that abiotic processes predate biotic degradation during early stages of exposure at the macro and mesoplastic scale [32]. Once waste has undergone degradation onto the microscale it is more readily consumed and incorporated into organisms and biotic media accelerating and promoting biotic degradation in later stages of marine residency [32]. Most degradation occurs first at the polymers surface due to environmental accessibility, thus making microplastics and nanoplastics more susceptible to degradation pathways as they possess greater surface area:volume ratios [32].

1.3.1 Mechanical Degradation of Marine Plastic Waste

Mechanical pathways provide both a method of degradation as well as sedimentation for marine plastic waste. The majority of studies that verify this theory

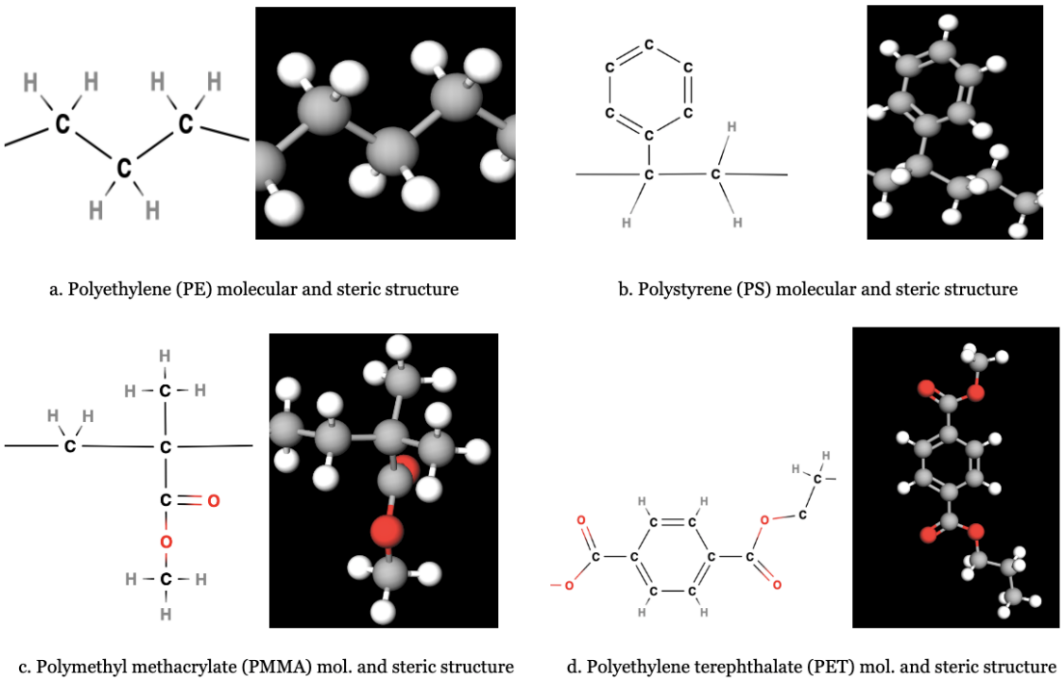
are simulated coastal weathering experiments that successfully degraded mesoplastics, microplastics, and nanoplastics into smaller plastic pieces. In a study by Song et al. 2017 PE, PP, and expanded PS were exposed to varying ultraviolet (UV) light over 12 month periods followed by 2 months of exposure to sand abrasion in a tumbling simulation of coastal environments. Minimal fragmentation occurred during PE (8.7 ± 2.5 particles/pellet) and PP models (10.7 ± 0.7 particles/pellet) that were unexposed to UV, however post UV exposure increases in degradation were observed among both species with greater effect being observed for PP (20 ± 8.3 particles/pellet and 6084 ± 1061, respectively). Expanded PS (EPS) also known as styrofoam fragments underwent significant degradation (4220 ± 33 particles/pellet) without UV exposure, however post-UV exposure the rate of degradation increased substantially (12,152 ± 3276 particles/pellet). Post-UV exposure and mechanical abrasion EPS saw extremes of 76.5% volume loss being unaccounted for lending towards the theory these particles reached the sub-micrometer scale [25]. In a similar study by Chubarenko et al. 2020, various films of common plastic polymers (non-buoyant PS, LDPE, buoyant PS, buoyant PP) were exposed to a simulated sea swash zone with varying sediment types such as sand, granules, small pebbles, and large pebbles. All plastics displayed some form of particle generation as a result of degradation over the 24 h abrasion period. PS, LDPE, EPS, and PP demonstrated 27%, 13%, 12%, <1% mean mass fragmentation post exposure [26]. Gerritse et al., 2020 generated a laboratory seawater microcosm for incubation of plastic waste composed of PE, PS, PP, PUR, cellulose acetate (CA), latex, polyester (PES), and polylactic acid (PLA) materials for 378-427 days. During this study, weight loss of plastic items from PE, PS, and PP classes was ≅ 1% per year, 3-5% for latex, PET, and PU, 15% for CA, and 7-27% for PES and PLA biodegradable plastic bags

[27]. Also observed within this study, microbial biofilms dominated by Cyanobacteria, Proteobacteria, Planctomycetes and Bacteriodetes grew on the plastic surfaces causing some PE pieces to sink [27]. Depending on age and composition of bacterial communities as well as residence time, it is likely that this phenomena could occur for various plastic species with densities lower than seawater. Mechanical fragmentation and deposition (e.g. sand settling, incorporation into beach media) are likely centralized to coastal zones and beach environments where the possibility of collision with more dense media and more intense wave-breaking occurs [29].

1.3.2 Photo-oxidative Degradation of Marine Plastic Waste

Photo-oxidative degradation is the process by which marine plastic waste at all scales is modified and weakened via UV (solar) radiation. This process takes place via free radical reactions and chain scission. As this form of degradation occurs at the molecular level, the efficiency and the events required in order for progression relies upon chemical structure of polymer species as well as constituent compound content. The composition of the nanoparticles in this work are PE, PMMA, PET, and Carboxylate modified-PS. The figure below provides the chemical composition of these species as well as their classification; it should be noted that polymers are generated through the covalent bonding of monomers into a polymer chain, these polymer chains then undergo further addition to generate plastic materials. The diagrams displayed in *Figure 2* only provide the "backbone" or monomer sections that combine to form a polymer [32]. The first stages in these degradative processes, initiation, requires the absorption of UV radiation by abnormalities or unsaturated chromophoric groups within the polymer, this excitation breaks covalent bonds resulting in free radical

formation [28, 32, 33]. These processes are autocatalytic as the next stage of the reaction, propagation, occurs when the newly formed polymer radical combines with oxygen,



a. Polyethylene (PE) molecular and steric structure

b. Polystyrene (PS) molecular and steric structure

c. Polymethyl methacrylate (PMMA) mol. and steric structure

d. Polyethylene terephthalate (PET) mol. and steric structure

Figure 2. Chemical structure and diagrams for polymer species PE, PS, PMMA, and PET (starting left, clockwise) (a tool from MolView, 2023).

generating a peroxy radical. This newly formed peroxy radical is then capable of reacting with the next substrate resulting in further radical generation [28, 32, 33]. The final stage, termination, occurs when radicals form inert compounds, the stages of this reaction are able to proceed as long as oxygen is readily available. The polyolefin PE is resistant to photo-oxidative degradation due to its inert nature, lack of UV-visible chromophores and C-C bonding in the backbone which is not readily able to undergo hydrolysis [32, 33]. PET, a polyester, is readily able to undergo hydrolytic cleavage and photo-oxidative degradation [33]. PS is readily able to degrade via photo-oxidative degradation, due to chain scission and cross-linking the resulting radicals later form olefins and ketones as

by-products[32]. PMMA, the most widely used polyacrylic, is considered to be one of the more stable polymer species [36]. In the modeling work by Chamas et al, extrapolated half lives for PET, HDPE (thick), HDPE (thin), LDPE, and PP considering solely thermal and photo-oxidative degradation in the marine environment were 2.3, 26, 530, 5, and 87 years respectively [33].

1.3.3 The Plastisphere and Biodegradation of Marine Plastic Waste

Biotic interactions with polymer species in the marine environment likely play a large role in the mass loss of marine plastic waste, and can serve as significant pathways for both degradation and trapping [4, 37, 38, 39]. Biodegradation relating to marine plastic waste refers to the incorporation, mineralization, and acceleration of plastic degradation by marine microbial life [4, 53, 54, 55, 59]. Many studies to date have investigated the interaction, ingestion, mineralization, and behavior of low diversity or monoculture microbial communities during and post exposure to microplastic and nanoplastic media [57]. Although valuable in developing our baseline knowledge of ecotoxicological interactions, studies such as these provide little insight into environmentally relevant community organization and interaction.

Within the marine environment, the plastisphere influences surrounding communities to adapt and incorporate plastics into their function, for microbial life this is attachment and subsequent biofilm production [59, 3]. Zettler et al. observed differing microbial community diversity with plastic pieces towed from sections of the Northern Atlantic exhibiting unique and shared Operational Taxonomic Units (OTUs) reads. Samples included PP MPW (n = 24,267), PE MPW (n=26,726), and open seawater (n=29,193) (OS) sequences which revealed 799, 413, and 1,789 unique OTUs,

respectively, whilst only sharing 16 (PP:OS), 117 (PE:OS), and 53 (PP: PE: OS) OTUs [3]. Pitting of plastic surfaces was noted in scanning electron microscopy (SEM) images (5-10μm scale resolution) of sampled marine plastic waste during said study that conformed to cell shape of local residents suggesting plastisphere members are hydrolyzing plastic waste in the environment [3]. Bacteria and fungi are the microorganisms of interest for the biodegradation of plastics.

As discussed in Sections 1.3-1.3.2, the efficiency of various degradation pathways is influenced by the physicochemical properties of the polymer species (e.g. first order structure characteristics). Biodegradation of marine plastic waste is unique from other previously mentioned exposure pathways as it is highly variable and heavily influenced by temperature, location, season, and exposures. Microbial community structure and characteristics, like physicochemical properties of the polymers, heavily influences the ability of microorganisms to degrade plastic species. Auta et al., observed significant plastic degradation of PP during exposure to *Bacillus* and *Rhodococcus* strains isolated from mangrove sediments in Malaysia [62]. Plastic degradation via bacterial strains was determined through gravimetric methods, 4.0% (*Bacillus)* and 6.4% (*Rhodococcus*) mass loss of PP microplastic was observed after a 40 day incubation period [62]. Another study conducted by Paço et al. observed significant degradation of PE microplastic pellets by the marine fungus *Zalerion maritimum* [63]. A positive correlation was recorded between increases in biomass weight percentage of *Z. maritimum* and increases in PE particle mass-loss throughout exposure trials (n = 4). One particularly interesting result saw fungi exposed for 14 days to PE particles experiencing a biomass variation of 82.0% ± 2.1% with a corresponding PE mass loss observation of 43% ±2.9% [63]. As formerly mentioned in *Section 1.3.1,* community composition and density of this plastisphere can

result in biofouling [60], or movement of smaller scale particles down the water column via surface residency [27]. Although on a larger scale, models assume that micro and nanoplastic particles manage to sediment or enter into brownian motion in the subsurface, this behavior is much more dynamic than our current understanding, requiring further information on particle wettability, functional grouping, and interparticle behavior [61].

1.4 Health Effects of Nanoplastics

Microparticles and nanoparticles are a current section of science and development that present promise in terms of energetic, commercial, and medical application[65]. This potential, although vast, should not overshadow the capacity for harm and the use of appropriate caution. Microplastics and nanoplastics present significant data gaps as to their imposed health effects, specifically the health and ecological effects of environmentally relevant micro and nanoplastic exposures. Literature reviews with key terms such as “microplastic”, “nanoplastic”, “developmental toxicity”, and “reproductive toxicity” at present yield conflicting and limited results. Toxicological and pharmacological assessment data at present for microplastic and nanoplastic is limited in its morphological and chemical profile, as well as information relating to constituent and sorbed compounds [44]. As toxicologists, oftentimes we are limited by the accessibility and cost of appropriate toxicological assessments, and as such we adopt structural or class-based approaches in order to apply informed risk decision making principles. A class-based approach will be followed in this work.

In recent years both public and scientific interest has surged regarding human health effects associated with micro and nanoplastics. We are aware that these materials

are nearly ubiquitous and through laboratory studies have observed the penetration of PS nanoparticles through the cellular membrane via endocytosis and passive pathways demonstrating translocation into human tissues for size ranges of 50 nm and 500 nm[68]. Nanoplastic penetration has been observed in mammalian tissue, microbiota, and plant materials [73, 74, 75]. As to relevant human exposures, development in study design and instrumentation has led to the successful detection of micro and nanoplastic particles in human stool, blood, and placental samples [69, 70, 71]. The current opinion is that polymers likely pose low chemical risk to human health due to their inert nature, however our understanding of interactions at the nanoscale as well as constituent compound content requires further investigation [21]. Laboratory studies to date include limited polymer and morphological variation, and are often conducted with virgin nanoparticles that hold little relevance to "real-world" micro and nanoplastic exposures. Models to date are often dominated by the use of virgin polystyrene micro and nanospheres, which hold little relevance in regard to environmental exposures both in the monoculture of polymer, the lack of constituent compounds, and the uniform morphology and size range of said micro and nanospheres [66, 68, 79]. While these studies provide a good foundation within the field, the physicochemical interactions and delivery processes are likely unique from particle to particle. Currently, the body of literature regarding the magnitude of nanoplastic and microplastic particle exposure that happens during pregnancy and early-stage development is lacking. The work by Sripada et al., 2022 notes that intake ratios per unit body weight are significantly higher during early-stage development [76]. Pregnant women and children likely represent sensitive populations within the field of nanoplastic and nanoparticle research as these populations will

experience these exposures during periods of significant neurological, behavioral, immune, metabolic, and cardiovascular system development [76].

Recent study has found concern for developmental and reproductive toxicity of these plastic particles to be valid. In a study by Huang et al. 2015, 20 to 500 nm carboxylate-modified PS nanoplastic particles were intravenously injected into pregnant mice to assess nanoplastic capability to pass the placental barrier [73]. At 4 h-post exposure, nanoparticle content of the fetal brain, lungs, and liver were too low for high performance liquid chromatography (HPLC) quantification, but were effectively detected in these locations via fluorescent microscopy (FM) [73]. Cytotoxic influence of these particles upon trophoblast cells observed significant increase in Caspase 3 activation, representing induction of apoptotic pathways, at size ranges of 20 - 500 nm and exposure concentrations of 50 - 500 $\mu g/mL$ [73]. In a study conducted by Fournier et al. 2020, they observed the fetal development and tissue of rats on GD20, post 24-hr acute respiratory exposure to 20 nm rhodamine doped polystyrene nanoplastic particles[66]. Fluorescent optical imaging of doped particles post exposure found significant nano PS deposition in the maternal lung, heart, spleen, and significantly elevated levels of polystyrene in gestational day (GD) 20 fetuses, fetal abdomens, and isolated livers compared to controls[66]. This would suggest that acute respiratory exposures, as well as a systemic circulation, of PS nanoplastics lead to maternal and fetal tissue particle deposition[66]. Litter characteristic assessment within this study also observed significant reductions in fetal and placental weight (7% and 8% respectively) within exposed rats as well as significant increases in resorption sites [66].

1.5 Role of Nanomaterials Concerning Antibiotic Microbial Resistance

Current estimates postulate that over 50,000 lives are claimed annually within both Europe and the United States due to antibiotic microbial resistance (AMR) with expectations of nearly 10 million annual AMR related deaths by 2050 globally [40]. To date there has been emphasis on the capacity of marine environments to serve as a reservoir for AMR as well as an interface for human-AMR interaction [41, 42]. Despite widespread production, the activity of chemicals associated with plastic pollution is still under investigation [43, 44, 45], and further understanding of nanoplastic prevalence and composition may hold a key to expanding this knowledge. Investigations of positive AMR relationships with metallic nanoparticles [5,6,7] and exhaust nanoparticles [8] have set the stage for investigation into plastic-based nanoparticle interactions. *E. coli* containing RP4 plasmid (donor) and supplemented with 50 mg/L kanamycin were cultured alongside *E. coli* K12 MG1655 (recipient) supplemented with 25 mg/L chloramphenicol and underwent a series (0.1, 1, 10, 100 mg/L) of nanoplastic exposures ranging in size (10 nm, 50 nm, 500 nm) for 24 h [9]. It was observed at the 10 nm scale that particles positively influenced gene transfer efficiency at 0.1, 1, and 10 mg/L with significant reductions observed at 100 mg/L. Across all concentrations higher efficiency was observed by 50 nm particle exposures with the 100 mg/L groups experiencing ~400% increases in efficiency relative to control groups [9]. The 500 nm groups saw no statistically-significant increases in gene transfer efficiency across all exposure concentrations [9]. Observation of antibiotic resistance gene (ARG) propagation within biological phosphorus removal systems found significant increases in ARG propagation upon micro and nanoplastic introductions [10]. Further studies have observed similar occurrences in nitrifying sludge emphasizing wastewater treatment systems and infrastructures as areas of concern relating to AMR propagation [77]. Environmental

mixtures including but not limited to nanoplastics, antibiotics, disinfectants, and metals are most likely of main concern in application [46, 47], however co-occurrent events may not be necessary to drive these outcomes. In a former study conducted by Li et al., there was observed co-occurrence in 5,436 bacterial genomes analyzed for metal resistant genes (MRGs) and ARGs with presence detected in 47% and 48% of all 5,436 bacterial genomes respectively [48]. A total of 0.8% of these genomes exhibited high abundance for said genes, with higher prevalence seen among bacteria associated with anthropogenic impacts and environments [48].

1.6 Methodology for Environmental Nanoplastic Study

The field of environmental nanoplastic study at present lacks a standardized definition, operating procedure, and analytical methodology. To date there are approximately 33 scientific studies that have successfully detected environmental nanoplastics, often times these studies are reliant on mass-based methodology such as Pyrolysis-Gas Chromatography-Mass Spectrometry (PyGCMS), Thermal Desorption-Proton Transfer Reaction- Mass Spectrometry (TD-PTR-MS), and Time-Of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) [82, 90, 92, 93, 94]. In order to better understand the role morphology, size distribution, and constituent compound content plays on the toxicological loading of these materials further development of viable non-degradative pre-treatment and concentration methodology is needed. Various studies have attempted enzymatic pre-treatment with enzyme profiles developed for specific targets such as marine filter samples and seafood tissue [95, 96]. Loder et al. conducted digestions on microscale articles (500um) alongside inclusion of strong oxidizing agents leaving said efficacy unknown at the nanoscale [96]. The digestion

set forth within Chang et al. saw effective application of lipase and corolase 7090 within a pretreatment scheme on the nanoplastics of concern. There were slight structural changes observed among the PET nanoparticles within the study, however it is unlikely that this enzyme cocktail is viable for both marine and fish tissue studies [95]. Within this work we set forth an investigation to determine the efficacy of an enzymatic digestion pretreatment schema on PS, PET, PE, and PMMA nanoplastic particles (<1000 nm).

1.7 Hypothesis

A. If constituent compounds are assessed along nanoplastic particles within a coastal risk assessment model, then significant levels of risk will be observed at environmentally relevant concentrations when considering the developmental endpoint.
B. If a selective enzymatic pretreatment is applied to PE, PET, PMMA, PS nanoplastic particle standards, then there will be no observed degradation among these species demonstrating viability for environmental application.

1.8 Specific Aims

Coastal Risk Assessment Investigation

Specific Aim 1A: Develop environmentally relevant profiles of nanoplastic and constituent compound content exposure.

Specific Aim 2A: Generate models to assess overall hazard given varying environmental concentration, content, and bioaccumulation factors.

Specific Aim 3A: Assess the level of influence constituent compounds have over hazard capacity.

Enzymatic Digestion Pretreatment Investigation

Specific Aim 1B: Effectively assess nanoplastic particle size and morphology for nanoplastic standards.

Specific Aim 2B: Effectively digest and recover nanoplastic particles of concern.

Specific Aim 3B: Assess non-degradative capacity of pre-treatment schema for nanoplastic standards in question.

2. Materials and Methods

2.1 Developmental Risk Assessment Methods, Coastal Zone

Toxicological advancement and development of mixtures-based human health assessments are limited in that toxicology studies often focus on single chemical exposures and are limited in their capacity to mimic real environmental exposures [72]. Due to the cost and time constraints of effectively assessing the toxic capacity of compounds, we stand to gain insight through the use of risk assessment frameworks through applying class-based and structure-activity relationship approaches in order to inform our decision-making process both at the public and private level. Both the Centers for Disease Control and Prevention's Agency for Toxic Substances and Disease Registry (ATSDR), as well as the U.S. Environmental Protection Agency (EPA), have set forth guidance for approaching mixtures-based health approaches [72,73].

As discussed in Section 1.4, micro and nanoplastic particles have exhibited toxic potential and transport within in-vivo models for the mammalian fetal compartment

and have been detected in human placental, blood, and stool samples [69,70,85]. Given our concern with the marine environment and coastal communities, the exposure routes that we will be assessing will be the ingestion of contaminated seafood and the accidental ingestion of marine waters during recreational activity. To properly scope this risk assessment, we will be centrally focused on both pregnant and nursing women in order to address their likelihood as a sensitive population.

Various assumptions are applied within this mathematical model, hence the necessity of incorporating various ranges for exposure scenarios. Given the various routes of passive degradation covered within the preliminary sections of the thesis, we are assuming that microplastics will be accompanied by the production or presence of nanoplastics. As such, exposures are assessed as a total concentration of plastic content in place of particle counts, thus ignoring morphology and size on toxic influence. Particles at the nanoscale have been proven to translocate into biological tissue and pass natural filters such as the mammalian fetal and blood brain barrier, lending to the assumption of total body distribution [68, 73, 74, 75, 79]. The plastic profiles that were selected for this study were derived from estimated total polymer production to date provided by Geyer et al. 2017[11], the mean profile of plastics detected in human stool provided by Schwabl et al. 2019[70], and finally the mean profile of plastics detected in human blood provided by Leslie et al. 2022[85]. These scenarios titled Scenario A, Scenario B, and Scenario C respectively were intentionally selected as representatives for plastics in the environment, plastics being excreted, and plastics that are in circulation in the human body and their content is displayed in *Figure 4* above. The European Chemical Agency has actively identified 1,550 plastic additives known to leach into the environment (ECA, 2019), these additives generally being non-covalently bound constituent compounds

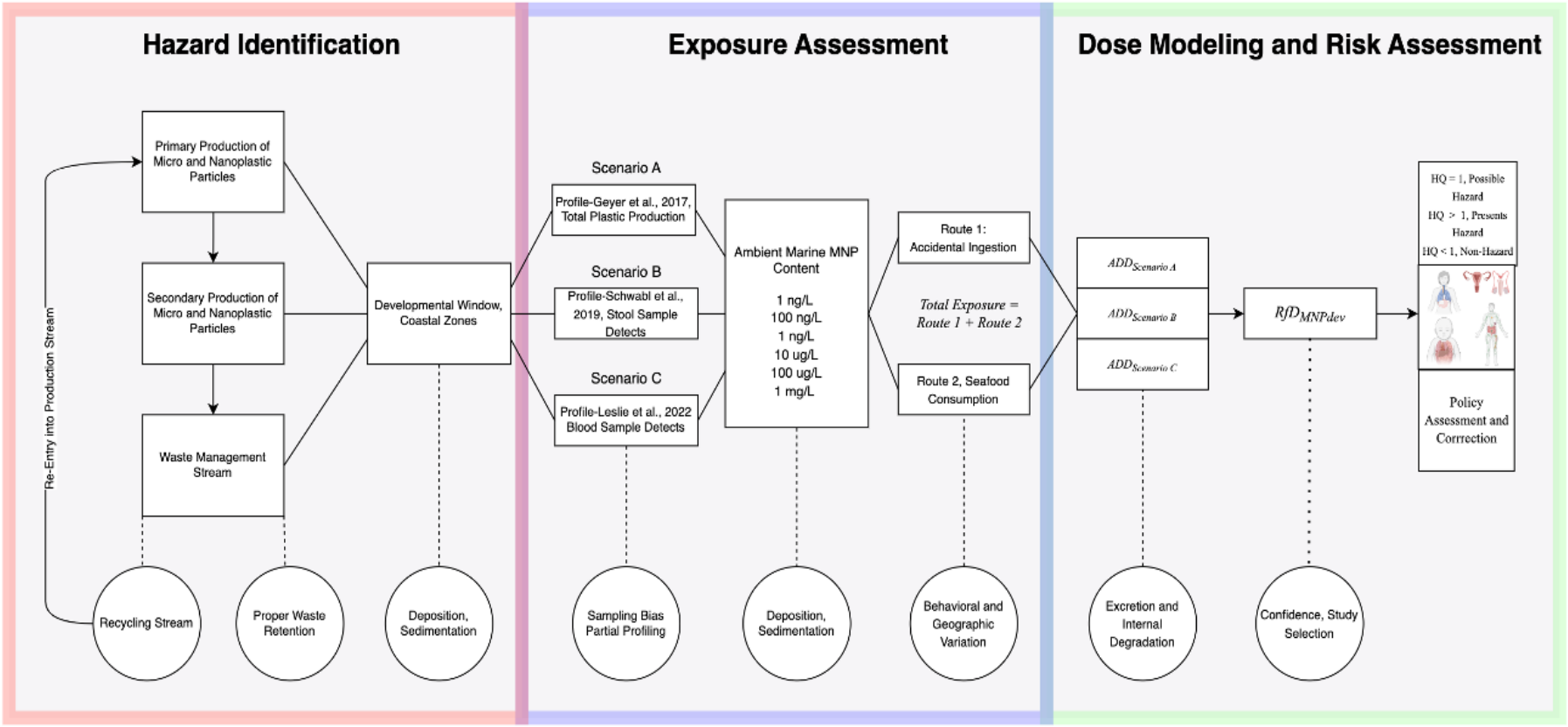


Figure 3, Coastal Zone Developmental Risk Assessment Conceptual Model and Scenario Mapping (Hazard Identification, Exposure Assessment, Dose Modeling)

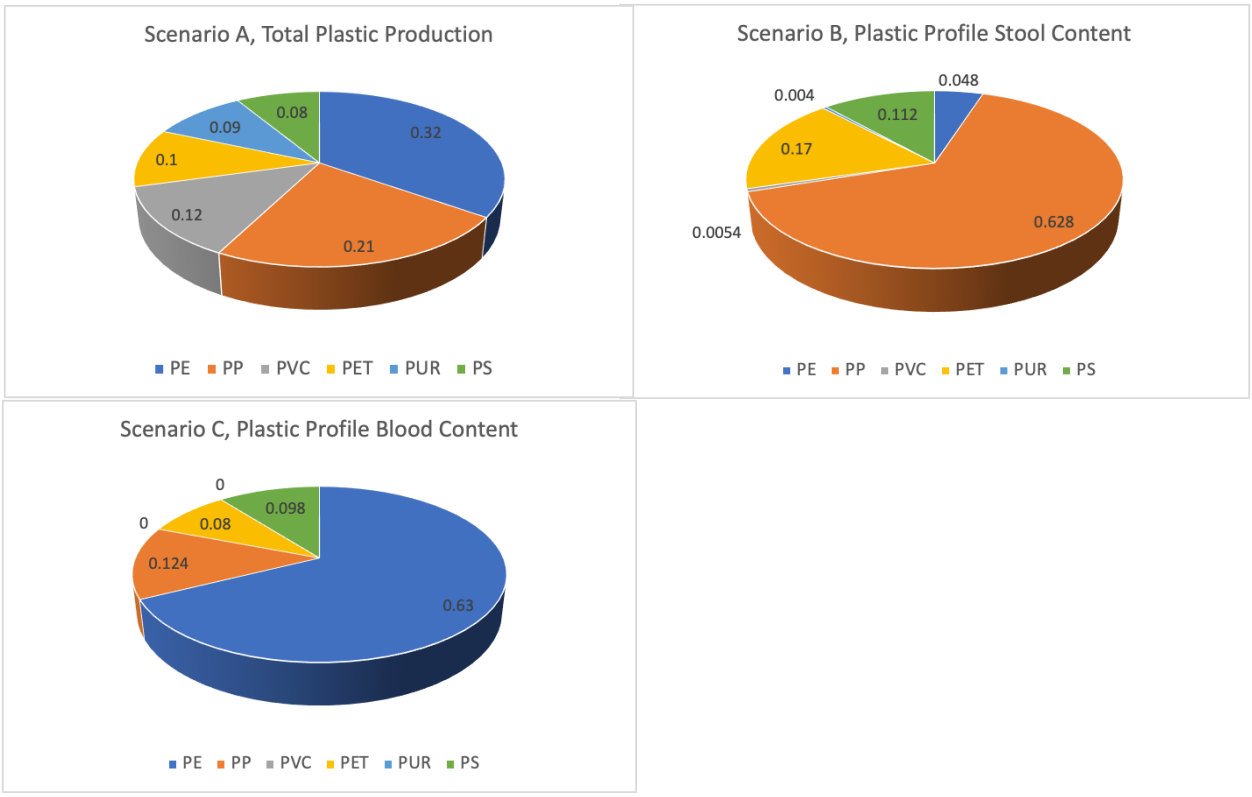


Figure 4, Plastic Profiles for Scenario A, B, and C (Gewert et al., Schwabl et al., and Leslie et al.)

which have potential to induce toxic effects in their own right [84]. Profiles for toxic constituent compounds were derived from the values gathered in Hahladakis et al., 2018 [84] and set forth in the EFSA Panel on Contaminants in the Food Chain (CONTAM), 2016 [80]. CONTAM sets forth that micro and nanoplastics on average are composed of 4% constituent compound content, whilst the table of values taken from Hahladakis et al. provides varying ranges of compound content dependent on species. Given this study's focus specifically on the developmental endpoint, we selected the classes of Plasticizers, Flame Retardants, Stabilizers, and Inorganic Pigments. These were selected due to their respective contents of various phthalates (Phth), polybrominated diphenyl ethers (PBDE), Bis-phenols (BPs), and heavy metals (HM) all of which have noted activity to

the developmental endpoint at relatively low concentrations (RfD<0.05 mg/kg/day)[84]. A summary of the values used for this risk assessment as well as their sourcing and role can be seen in *Table 1* below.

The equations which describe the risk assessment include Adjusted Daily Ingestion (ADI) formulas for our accidental ingestion exposure route

Parameter	Value	Unit	Reference	Context
Constants	-	-	-	-
MNP Concentrations	$1.00E^{-6}$-1.00	mg/L	(Materić et al., 2022)	Exposure
Adult accidental Ingestion (ai)	9.00	mL	(DeFlorio-Barker et al., 2018)	Exposure
BAFsea	()	-	-	Exposure
BAFhuman	()	-	-	Exposure
Exposure Duration	70	years	(ATSDR, 2005)	Exposure
Exposure Frequency	365	days	(ATSDR, 2005)	Exposure
Scenario, A	8.00-32.00	%	(Plastics Europe, 2017)	Exposure
Scenario, B	0.4-62.80	%	(Schwabl et al., 2019)	Exposure
Scenario, C	8.00-63.00	%	(Leslie et al., 2022)	Exposure
Constituent Content	4	% w/w	(EFSA (CONTAM), 2016)	Hazard Ident.
Constituent Content*	0.01-70	% w/w	(Hahladakis et al., 2018)**	Hazard Ident.
Bis-Phenol A RfD	0.016	mg/kg/day	(Willhite et al., 2008)	Dose Modeling
PBDE (47-88) RfD	0.0001	mg/kg/day	(EPA, 2017)	Dose Modeling
Phthalate Mixture RfD**	0.037	mg/kg/day	(Kortenkamp & Koch, 2020)	Dose Modeling
Inorganic Pigments RfD	0.0001	mg/kg/day	(ATSDR, 2005)	Dose Modeling
MNP Reference Dose	0.15	mg/kg/day	(Park et al., 2020)	Dose Modeling
Daily Seafood Ingestion (dsi)	878	mg/kg/day	(FAO, UN, 2020)	Dose Modeling
Weight, Expecting Mother	73	kg	(ATSDR, 2005)	Dose Modeling

*The profile was determined from values not only derived from Hahladakis et al., 2018, by also confirmed within Andrade et al., 2022, **Mean derived from RfD of DBP, DIBP, BBP, DEHP, DINP, (Kortenkamp & Koch, 2020)

Table 1, Coastal Zone Developmental Risk Assessment Table of Values, generated by Thomas Dugan 2023

(Route 1) and contaminated seafood ingestion route (Route 2) seen as *Equation A1* and *Equation A2* below.

$$ADI_{NPai} = \frac{C_{sw} * IR * ET * ED}{BW * AT}$$

Equation A1. Accidental Daily Ingestion Eq.

$$ADI_{NPdsi} = \frac{C_{fish} * BAF_{human} * DSI * ET * ED}{BW * AT}$$

Equation A2. Daily Ingestion Attributable to Seafood Eq.

Given the wide variety of reported values for marine micro and nanoplastic (MNP) concentration the concentration of MNPs in seawater (Csw) was assumed to be between 1 ng/L to 1 mg/L on a logarithmic-scale (1 ng/L, 10ng/ L, 100 ng/L, 1 µg/L, 10 µg/L, 100 µg/mL, 1 mg/L). The exposure duration (ED) and exposure time (ET) were 70 years and 365 days respectively, and our averaging time (AT) the 365 days multiplied by the 70 year duration. The values used for accidental ingestion rate and average body weight for an expecting mother were 0.009 L and 73 kg respectively [72,81]. In order to gauge the impact of bioavailability, or the amount of nanoplastics bioavailable and able to exert their toxic capacity and achieve residency, we will be examining the role of a bioavailability factor (BAF) on both human (BAFhuman) and fish (BAFfish) adjusted daily dosage. The concentration of MNPs within fish tissue was determined using *Equation 3*, in which the concentration of MNPs within fish tissue is dependent upon the concentration in their surrounding waters (Csw), the level of contamination in fish feed stock (Fs, generalized to a value of 1), and finally the BAFfish factor. To assess the total level of exposure within the coastal zone, the ADD's derived from the accidental ingestion scenario and the seafood ingestion scenario were summed, this can be seen in *Equation A4*.

$$C_{fish} = C_{sw} * BAF_{fish} * Fs$$

Equation A3. MNP Seafood Accumulation Eq.

$$ADI_{NPcz} = ADD_{NPai} + ADD_{NPdsi}$$

Equation A4. Adjusted Daily Dose for Coastal Exposure (Total).

The general equation for generating a hazard quotient can be seen below in *Equation A5*. The equation to be used for generating a hazard quotient for a mixtures-based exposure can be seen in *Equation A6*. The equation to be used specified to our profiles and constituent compounds is listed below as *Equation A7*.

$$HQ = \frac{ADI}{RfD}$$

Equation A5. Hazard Quotient Basic Eq.

$$\sum_{i=o}^{n} \frac{ADI_1}{RfD_1} + \frac{ADI_2}{RfD_2} + \frac{ADI_3}{RfD_3} + {}^{n}$$

Equation A6. Hazard Quotient Mixtures Based Eq.

$$\sum_{i=o}^{n} \frac{PE(ADI_{NPcz})}{RfD_1} + \frac{PP(ADI_{NPcz})}{RfD_2} + \frac{PVC(ADI_{NPcz})}{RfD_3} + \frac{PET(ADI_{NPcz})}{RfD_4} + \frac{PUR(ADI_{NPcz})}{RfD_5} + \frac{PS(ADI_{NPcz})}{RfD_6} + \frac{Phth(ADI_{NPcz})}{RfD_7} + \frac{PBDE(ADI_{NPcz})}{RfD_8} + \frac{BPA(ADI_{NPcz})}{RfD_9} + \frac{IOP(ADI_{NPcz})}{RfD_{10}}$$

Equation A7. Hazard Quotient Applied Mixtures Based Eq.

Reference dose (RfD) selection for Phths, BPs, PDBEs, and HMs were derived from the literature on oral dose toxicity with emphasis on the developmental endpoint [72,87,88,89]. Kortenkamp & Koch suggest revised reference doses of 6.7 µg/kg/d (DBP), 100 µg/kg/d (DIBP), 10 µg/kg/d (BBP), 10 µg/kg/d (DEHP), 59 µg/kg/d (DINP) for the phthalates assessed, given that the profiling described in Hahladakis et al. does not provide phthalate class specifics we assume the arithmetic mean of these values to be representative. A reference dose of 0.016 mg/kg/d was accepted as representative for BPA content. Given that HMs such as lead are not believed to have an acceptable daily intake, the RfD for cadmium (0.0001 mg/kg/d) was chosen as representative for inorganic pigment constituent compounds. The reference dose for all MNPs within this

study was derived from Park et al. 2020 which recommended after observation of 90-day chronic oral dosing of PE microplastics during gestation and early stage development that a no observed adverse effect level (NOAEL) for PE MNPs be set at 15 mg/kg/d [83]. By applying the NOAEL RfD extrapolation method and applying an interspecies factor of 10 as well as an uncertainty factor of 10, we applied a 0.15 mg/kg/d RfD for MNPs.

2.2 *Enzymatic Digestion Materials*

In order to address the need for analytical characterization within the field of marine nanomaterials, we set forth a pre-treatment method utilizing selective enzymatic digestion and set out to determine its viability as a non-degradative wash for the nanoplastic polymers PS, PMMA, PE, and PET. The plastic standards that were used consisted of particles at the micron and sub-micron scale, including three commercially procured standards as well as one self-synthesized particle solution. FluoSpheres Carboxylate-Modified Microspheres, 0.1 m, yellow-green fluorescent particles, 10-kDa MWCO Dialysis tubing, were purchased from ThermoFisher Scientific Inc. (Waltham, MA). Polymethyl-methacrylate (~100 nm) uncolored nanospheres were purchased from CD Bioparticles Inc. (Shirley, NY). Powderized uncolored PE nanospheres (9900 um - 200 nm) were purchased from Cospheric LLC (Santa Barbara, CA). PET stock plastic was taken from a recycled type-1 plastic bottle, nanospheres were self-synthesized using a modified version of the methods set forth by Rodriguez-Hernandez et al. 2019 [64]. Trifluoroacetic acid, 30% Hydrogen Peroxide, Sulfuric Acid, Lipase from Aspergillus Oryzae Sigma Aldrich Inc. (St. Louis, MO). Tween 80 was purchased from Research Products International Inc. (Mount Prospect, IL).

Polymer	Procurement	Provision	Media	Conc.	Size Dist. Thero (nm)
PS(COOH)	Commercially Procured	ThermoFischer Scientific Inc.	Liquid suspension	1 mg/mL	110nm
PMMA	Commercially Procured	CD Bioparticles Inc.	Liquid suspension	10 mg/mL	110nm
PE	Commercially Procured	Cospheric LLC.	Powdered Particles	1 gram	0.2nm-1μm*
PET	Self-Synthesized	TFA-based Synthesis	Liquid suspension	1 mg/mL	150nm

**Theoretical size distributions not met in initial assessments.*

Table 2, Standard Polymer Procurement, Method, Media, Concentration, and Theoretical Size Distribution Prior to Manipulation.

2.3 Dynamic Light Scattering Methods

Both within the method validation as well as sample analysis the dynamic light scattering (DLS) methods detailed below were used in order to determine particle polydispersity, hydrodynamic diameter, and zeta potential. The Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK) within the University of Washington, Clean Energy Institute, Research and Training Testbeds was used for all DLS trials.

Prior to all measurements, standards were diluted to final concentrations of approximately 100 μg/mL and then underwent probe ultrasonication (30W, 2 min). Aliquots of 1 mL (100 μg/mL) were run throughout DLS trials for both hydrodynamic diameter as well as zeta potential, all trials were performed within polystyrene-based cuvettes and polystyrene-based electrode cuvettes.

2.4 Scanning Electron Microscopy Methods

Both within the method validation as well as sample analysis the scanning electron methods (SEM) as well as the wafer preparation (WP) procedures detailed below were used for drop-casting as well as assessing particle morphology and size. The Apreo Variable-Pressure Scanning Electron Microscope (ThermoFisher Scientific, Waltham, US) within the University of Washington, Molecular Analysis Facility was used for all SEM imaging. Prior to imaging, both appropriate substrate selection and substrate preparation must take place; the methodology set forth by (Chou et al., 2022)[81] was adopted for sample preparation and drop-casting. Due to their mechanical stability, uniform surface, and lack of intrinsic signal silicon wafers were selected as the substrate of choice for this study. Silicon wafers were first split into approximately 1cm x 1cm squares. In order to obtain a super-hydrophilic surface prior to freeze drop-casting, wafers underwent a 10 minute acid piranha solution bathe (1:3 30% hydrogen peroxide, sulfuric acid), followed by a rinsing step (DI water), and then 85°C drying in an oven (ref. *Figure #* below). A freeze drop-casting method was selected in order to reduce and negate the effect of capillary flow upon particle distribution during evaporation, also known as the "coffee-ring effect"[81]. Super-hydrophilic wafers are placed onto a piece of dry ice until the surface is completely frozen. Once frozen, a 1 µL suspension was dripped onto the center of the surface via micropipette. In cases where solution concentration was found to differ significantly from theoretical concentrations, spin-coating methodology was employed. Cases where spin-coating was employed disqualified samples from particle concentration measurement but were viable for particle size and morphology assessment. The beam parameters for SEM imaging included a standard use case, with a field of 2keV, and a beam energy of 13 Pa. Particles

were generally imaged with a dwell time of 3 µs and a pixel density of 1536 x 1024 pixels. Upon centering the field of particles attached to the wafers surface, a series of five randomized images at working distance (WD) 4-6.3 mm and a magnification of 12,000x - 35,000x were taken in order to generate a randomized sample of particle size and morphology.

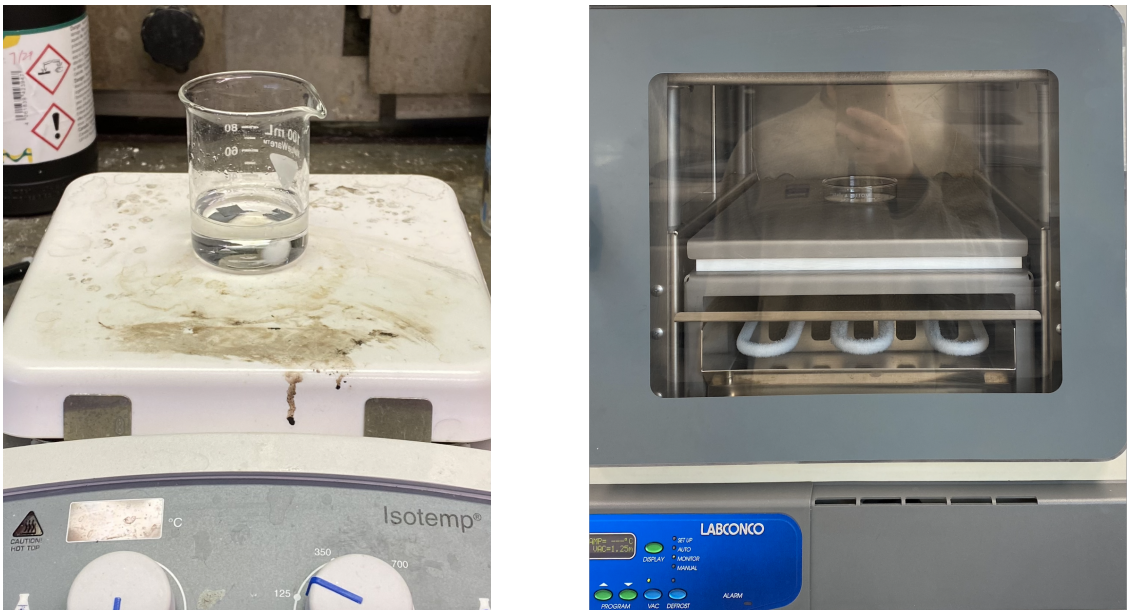


Figure 5, Acid Piranha Bath of Diced Silicon Wafer Fragments (left), subsequent Freeze-drying of Drop-Casted Wafers (right)

Images were subsequently analyzed by ImageJ software (LOCI, Madison, WI, USA) and morphology was assigned manually. Due to the electrostatic nature of the polymers used within the study, occasional particle charging was observed (specifically among the PE microspheres). This saturation behavior was selected as a defining characteristic when comparing suspected nanoparticles to background material during post-enzymatic digestion assessments.

2.5 Enzymatic Digestion of Nanoplastic Standards

The need for a pre-treatment methodology that is capable of purifying marine and fish tissue samples of organic background media while maintaining the structural integrity of nanoplastics has been summarized in *Section 1.6*. Alongside our risk

Figure 6, Pre-Treatment Schema for Enzymatic Digestion of Nanoplastic Standards

assessment, this body of work also sought to assess the viability of the enzymatic digestion and pre-treatment protocol set forth herein and its effect on particle integrity. The pretreatment scheme set forth and used within this work is shown in the flow diagram of Figure 6 above.

Given this study's scope of marine MNP assessment, as well as the risk assessment models consideration of both marine water MNP content as well as seafood MNP content, the pretreatment scheme is intended for both marine samples as well as fish tissue samples. Enzymes within this work were specifically selected with sample content in mind and were influenced by the digestion methods set forth in Loder et al. 2017 and the seafood and oyster extraction methods set forth within Chang et al. 2022. During the enzymatic digestion process, 100 μg/10 mL dilutions of stock MNP solutions underwent addition of enzymatic mixtures and a subsequent 100 nm aluminum oxide filtration. Stock Tris HCl buffer was used in instances where optimal enzymatic digestion occurred at pH 9. Stock sodium acetate buffer was employed in instances where optimal enzymatic digestion occurred at ph 5. All digestions occurred between 40-50°C and underwent incubation in a mixing unit at 80 RPM. Upon completion of the final digestion step, the addition of 10 mL of 10% SDS solution was applied in order to clean remaining protein residues. Ultra Dialysis occurred over a 24 hr period with 0.3 μm filtered, deionized water as the purifying solution. A review of the enzymes employed during this process, optimal temperature, optimal pH, enzymatic targets, and addition volumes can be found in Table 3 below.

Enzyme	*Temp. (℃)*	*pH*	*Incubation (hr)*	*Enzymatic Target*	*Volume (µL)*
Chitinase	*40*	*5*	*24*	*Aminopolysacchride Content*	*10*
Protease	*50*	*9*	*24*	*Hydrolysis of peptide bonds*	*10*
Lipase	*40*	*9*	*24*	*Lipid contents*	*10*
Cellulase	*40*	*5*	*24*	*Marine plant cell wall content*	*10*

Table 3, Pre-Treatment Enzymes, Procurement, Optimal Conditions, Targets, and Volume.

3. Results and Discussion

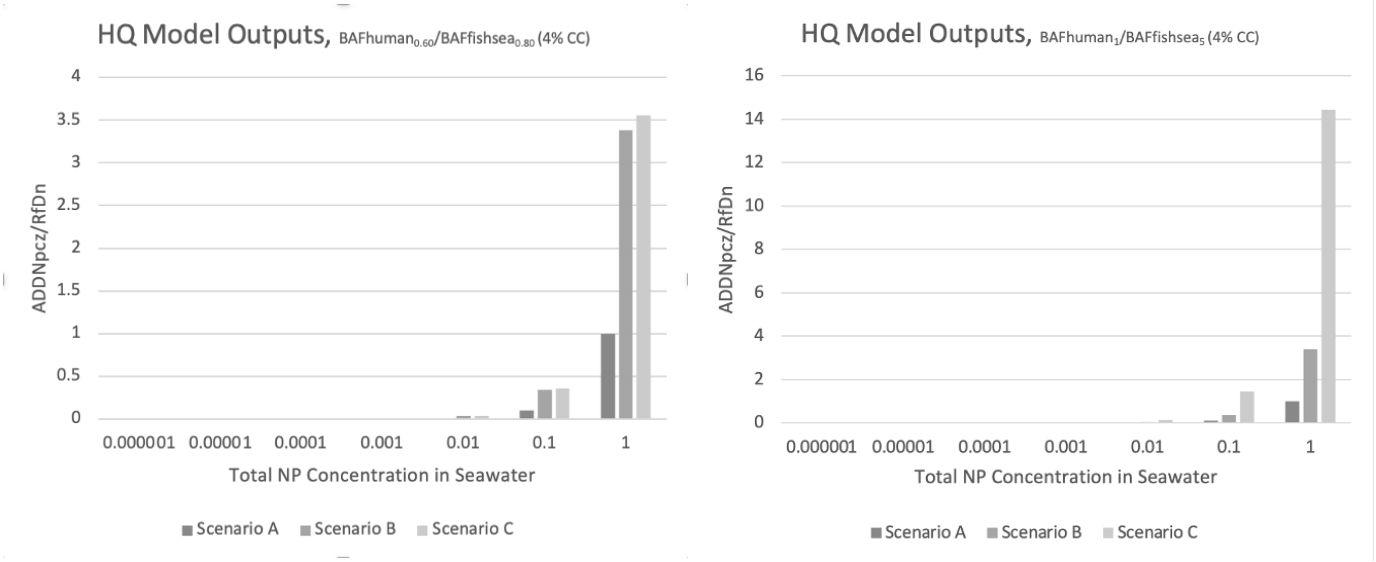


Figure 7, HQ Model Outputs for 4% Constituent Compound Profile for All Scenarios

Scenario	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*
Conc.	*1 ng/L*	*10 ng/L*	*100 ng/L*	*ug/L*	*10 ug/L*	*100 ug/L*	*1 mg/L*
Conc. (mg/L)	*1E-06*	*1E-05*	*1E-04*	*1E-03*	*1E-02*	*1E-01*	*1*
Scenario A, (Plastics Europe)	***3.38E-06***	***3.38E-05***	***3.38E-04***	***3.38E-03***	***3.38E-02***	***0.34***	***3.38***
Scenario B, (Schwabl, Stool)	***3.55E-06***	***3.55E-05***	***3.55E-04***	***3.55E-03***	***3.55E-02***	***0.36***	***3.55***
Scenario C, (Leslie Blood)	***3.42E-06***	***3.42E-05***	***3.42E-04***	***3.42E-03***	***3.42E-02***	***0.34***	***3.42***

Table 4, HQ Model Outputs for 4% Constituent Compound Profile, BAFhuman0.6 and BAFfishsea0.8

Scenario	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*
Conc.	*1 ng/L*	*10 ng/L*	*100 ng/L*	*ug/L*	*10 ug/L*	*100 ug/L*	*1 mg/L*
Conc. (mg/L)	*1E-06*	*1E-05*	*1E-04*	*1E-03*	*1E-02*	*1E-01*	*1*
Scenario A, (Plastics Europe)	***1.37E-05***	***1.37E-04***	***1.37E-03***	***1.37E-02***	***1.37E-01***	***1.37***	***13.7***
Scenario B, (Schwabl, Stool)	***1.44E-05***	***1.44E-04***	***1.44E-03***	***1.44E-02***	***1.44E-01***	***1.44***	***14.4***
Scenario C, (Leslie Blood)	***1.36E-05***	***1.36E-04***	***1.36E-03***	***1.36E-02***	***1.36E-01***	***1.36***	***13.6***

Table 5, HQ Model Outputs for 4% Constituent Compound Profile, BAFhuman1 and BAFfishsea5

3.1 Model Outputs, Risk Assessment

The model outputs for the *4% constituent compound profile* for the upper (1, 5) and lower limits (0.6, 0.8) of the bioaccumulation factors are presented above in Figure 6 and Tables 4-5 above. Environmental concentrations of micro and nanoplastics are expected to be highly variant dependent upon geographical location, and generally it is believed that they are in the ng/L-µg/L range [92, 93]. When observing model outputs, the HQ for MNP and constituent compounds in total are orders of magnitude below considerable risk levels at environmentally relevant concentrations. As these models, specifically upper limit runs (1, 5 BAF), approached concentrations of 10 µg/L we see HQ outputs that are one order of magnitude from a hazardous indication. The model indicated that MNP and constituent compound content for the given profile did in fact prove hazardous at concentrations of 1 mg/L for lower limit BAFs (0.6, 0.8) as well as concentrations of 100 µg/L and 1 mg/L for upper limit BAFs (1, 5). Average contributions to the HQ from the polymer MNPs as inert particles comprised 1.09%, with the remaining 98.91% being attributed to constituent compound content. Across both BAF scenarios, the polymer profile with the highest associated HQ at the 1 mg/L

concentration was derived from the Schwabl et al. 2019 stool profile.

The model outputs for the *Profile Specific constituent compound* exposure scenario for the upper (1, 5) and lower limits (0.6, 0.8) of the bioaccumulation factors are presented below in Figure 7 and Tables 6-7. When observing model outputs, the HQ for MNP and constituent compounds in total is orders of magnitude below considerable risk levels at environmentally relevant concentrations. As these models, specifically

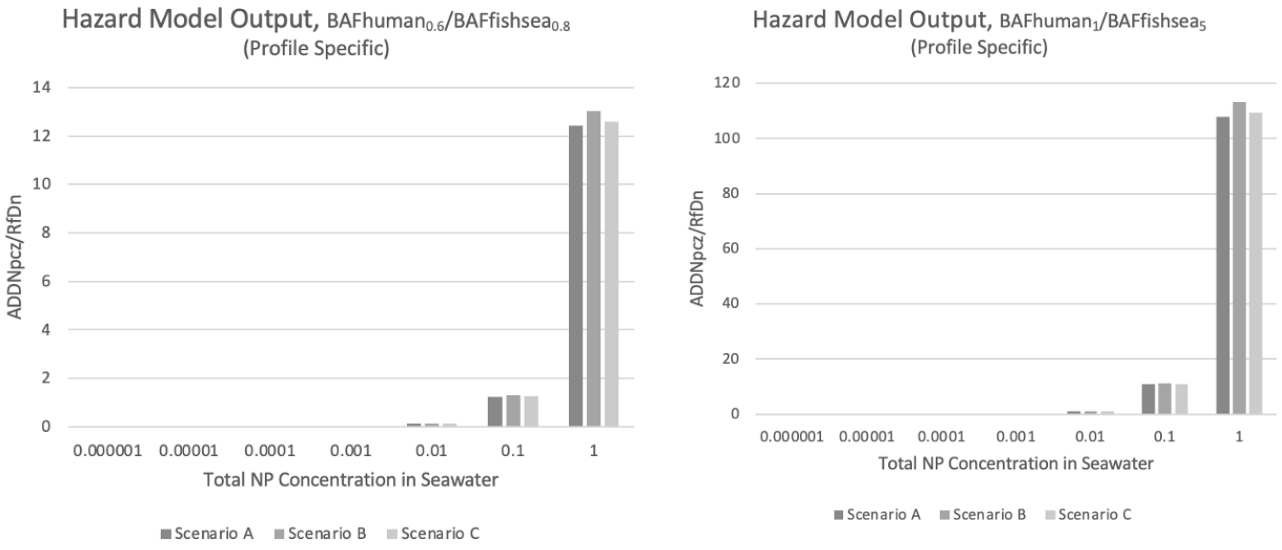


Figure 8, HQ Model Outputs for Profile Specific Constituent Compound Profile for All Scenarios

Scenario	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*
Conc.	*1 ng/L*	*10 ng/L*	*100 ng/L*	*ug/L*	*10 ug/L*	*100 ug/L*	*1 mg/L*
Conc. (mg/L)	*1E-06*	*1E-05*	*1E-04*	*1E-03*	*1E-02*	*1E-01*	*1*
Scenario A, (Plastics Europe)	***1.24E-05***	***1.24E-04***	***1.24E-03***	***1.24E-02***	***1.24E-01***	***1.24***	***12.4***
Scenario B, (Schwabl, Stool)	***1.30E-05***	***1.30E-04***	***1.30E-03***	***1.30E-02***	***1.30E-01***	***1.30***	***13.0***
Scenario C, (Leslie Blood)	***1.25E-05***	***1.25E-04***	***1.25E-03***	***1.25E-02***	***1.25E-01***	***1.25***	***12.5***

Table 6, HQ Model Outputs for Profile Specific Constituent Compound Profile, BAFhuman0.6 and BAFfishsea0.8

Scenario	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*	*HQ*
Conc.	*1 ng/L*	*10 ng/L*	*100 ng/L*	*ug/L*	*10 ug/L*	*100 ug/L*	*1 mg/L*
Conc. (mg/L)	*1E-06*	*1E-05*	*1E-04*	*1E-03*	*1E-02*	*1E-01*	*1*
Scenario A, (Plastics Europe)	***1.07E-04***	***1.07E-03***	***1.07E-02***	***1.07E-01***	***1.07***	***10.7***	***107***
Scenario B, (Schwabl, Stool)	***1.13E-04***	***1.13E-03***	***1.13E-02***	***1.13E-01***	***1.13***	***11.3***	***113***
Scenario C, (Leslie Blood)	***1.09E-04***	***1.09E-03***	***1.09E-02***	***1.09E-01***	***1.09***	***10.9***	***109***

Table 7, HQ Model Outputs forProfile Specific Constituent Compound Profile, BAFhuman1 and BAFfishsea5

upper limit runs (1, 5 BAF), approaching concentrations of 1 µg/L-10 µg/L, we see HQ outputs that are one order of magnitude from a hazardous indication. The model indicated that MNP and constituent compound content for the given profile did in fact prove hazardous at concentrations of 100 µg/L - 1 mg/L for lower limit BAFs (0.6, 0.8) as well as concentrations of 10 µg/L, 100 µg/L, and 1 mg/L for upper limit BAFs (1, 5). Average contributions to the HQ from the polymer MNPs as inert particles comprised 0.18%, with the remaining 99.82% being attributed to constituent compound content. Across both BAF scenarios, the polymer profile with the highest associated HQ at the 1 mg/L concentration was derived from the Schwabl et al. stool profile. It should be noted that within the upper limit scenario with BAFs (1, 5) in simulations where the concentrations within marine waters was 1 mg/L, the model indicated HQs exceeding 107 suggesting extreme hazard in these scenarios. The model showed general agreement across scenarios despite varying constituent compound profile, BAFs, and concentrations.

3.2 Dynamic Light Scattering Particle Characterization

The use of dynamic light scattering (DLS) was employed in order to assess particle hydrodynamic diameter (HD) within solution both prior and post enzymatic digestion treatments. Preliminary DLS trials indicated respective HDs of 124.6, 134.2, 66.7, and 478.3 nm for PS, PMMA, PET, and PE nanoparticles respectively. The standard deviations for polymer standards were 27.32, 29.57, 52.98, and 112.9 nm respectively. These measurements showed general agreement with both advertised sizing of polymer standards. Following the treatment of the nanoparticles detailed in *Figure 6,* DLS trials

Pre-ED Polymer	Avg. d (nm)	SD. d (nm)	Post-ED	Avg. d (nm)	SD. d (nm)	diff.d	diff.sd
PS	*124.6 nm*	*27.32*		*153.6 nm*	*13.01nm*	*29 nm* (≈23%)*	*-14.31 nm (≈-47%)*
PMMA	*134.2 nm*	*29.57*		*203.6 nm*	*48.11 nm*	*69.4 nm* (≈51.7%)*	*+18.54 nm (≈62.6%)*
PET	*66.7 nm*	*52.98*		*253.6 nm*	*86.6 nm*	*186.9 nm* (≈380.2%)*	*+33.62 nm (≈63.4%)*
PE	*478.3nm*	*112.9*		*688.1 nm*	*78.48 nm*	*209.8 nm* (≈43.9%)*	*-34.42 nm (≈69.5%)*

*Table 8, DLS Particle Characterization and Diff. p = 0.05, *statistically significant difference.*

indicated HDs of 153.6, 203.6, 253.6, and 688.1 nm for PS, PMMA, PET, and PE nanoparticles respectively. The standard deviations for polymer standards were 13.01, 48.11, 86.6, and 78.48 nm respectively. When comparing the HDs for these trials with a p-value of 0.5, all differences in HDs between fellow polymer assessments were found to

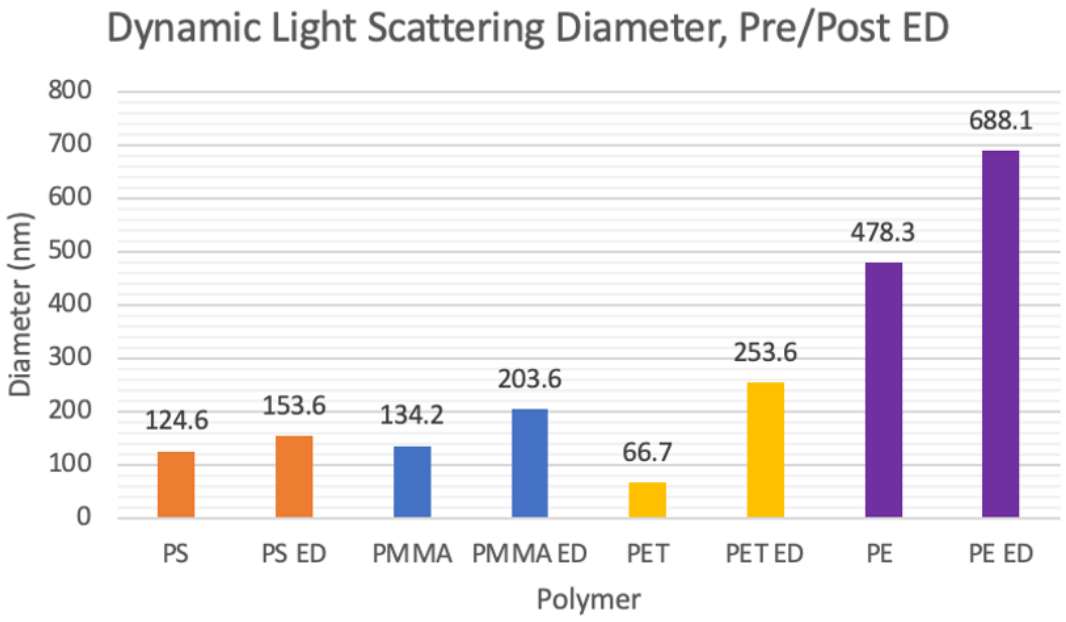


*Figure 9, DLS Particle Characterization and Diff. p = 0.05, *statistically significant difference.*

be statistically significant with all polymer classes showing a trend of increasing HDs post enzymatic digestion. The difference in HDs between pre and post enzymatic digestion nanoplastic particles were relative to preliminary measurements (diff.d <100%) for all nanoplastic standards with the exception of PET (380.2% diff). Differences in standard deviations across profiles were relative to preliminary measurements across all be statistically significant with all polymer classes showing a trend of increasing HDs post enzymatic digestion. When comparing the HDs for these trials with a p-value of 0.5, all differences in HDs between fellow polymer assessments were found to be statistically significant, with all polymer classes showing a trend of increasing HDs post enzymatic digestion. Differences in standard deviations across profiles were relative to preliminary measurements across all nanoplastics (diff.sd <100%). These data are presented within *Table 8* and *Figure 9* above.

3.3 Scanning Electron Microscopy Particle Characterization

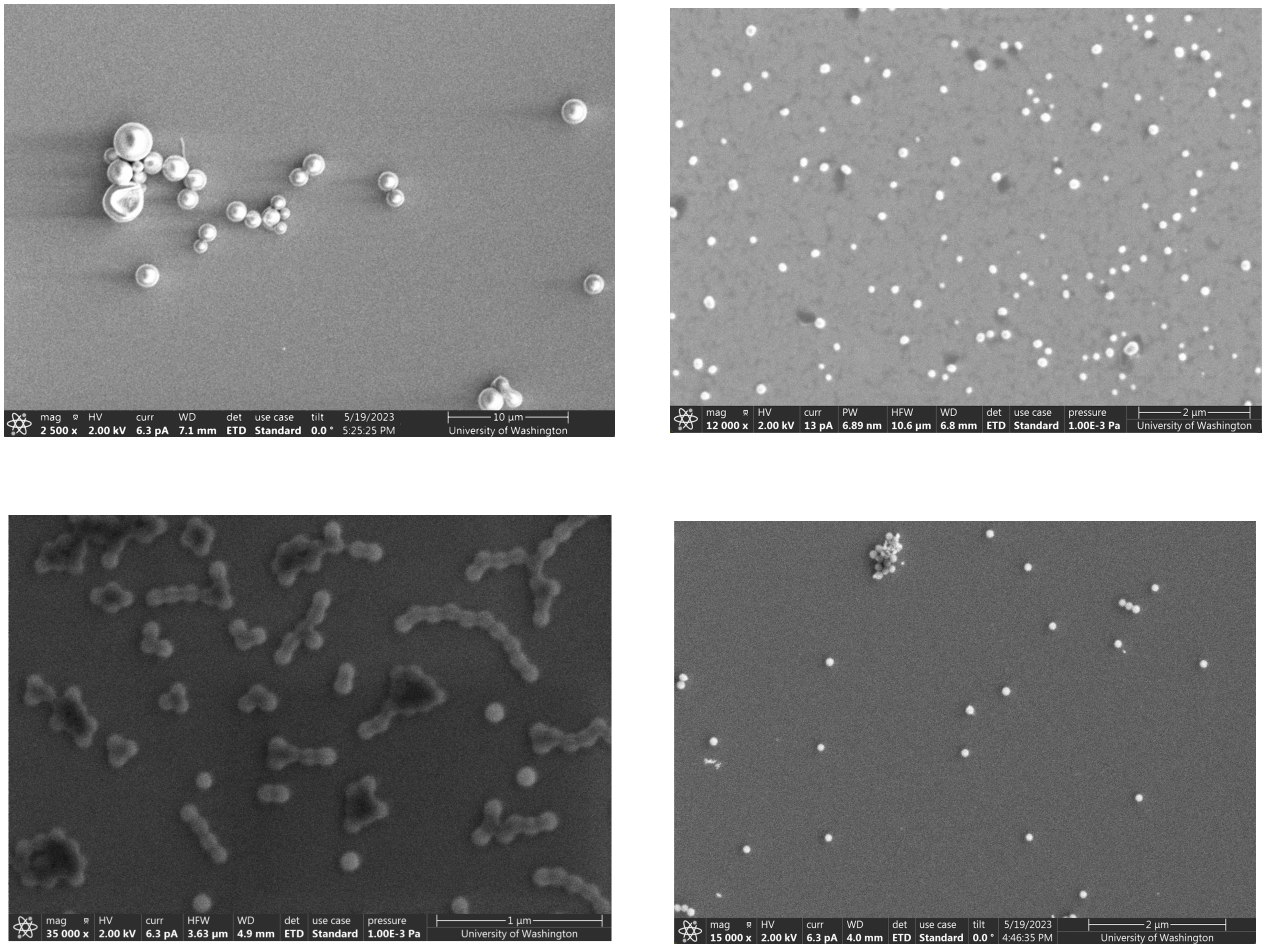


Figure 10, Scanning Electron Microscope Images for Determination of Morphology and Size of PE (top right), PET (top left), PMMA (lower left), and PS (lower right) standards.

SEM analysis was employed not only to confirm DLS trials but to assess the change in the morphology and surface of nanoplastic particles having undergone enzymatic pretreatment. As seen in *Figure 10* above, particles even at concentrations of $1E^{8}$-$2.7E^{9}$ demonstrated aggregative behavior within images of PE, PMMA, and PS. Generally across all images, particles were found to be uniform in spherical morphology

for PE, PMMA, and PS. PMMA and PS particles were confirmed to be uniform in spherical morphology during preliminary assessments.

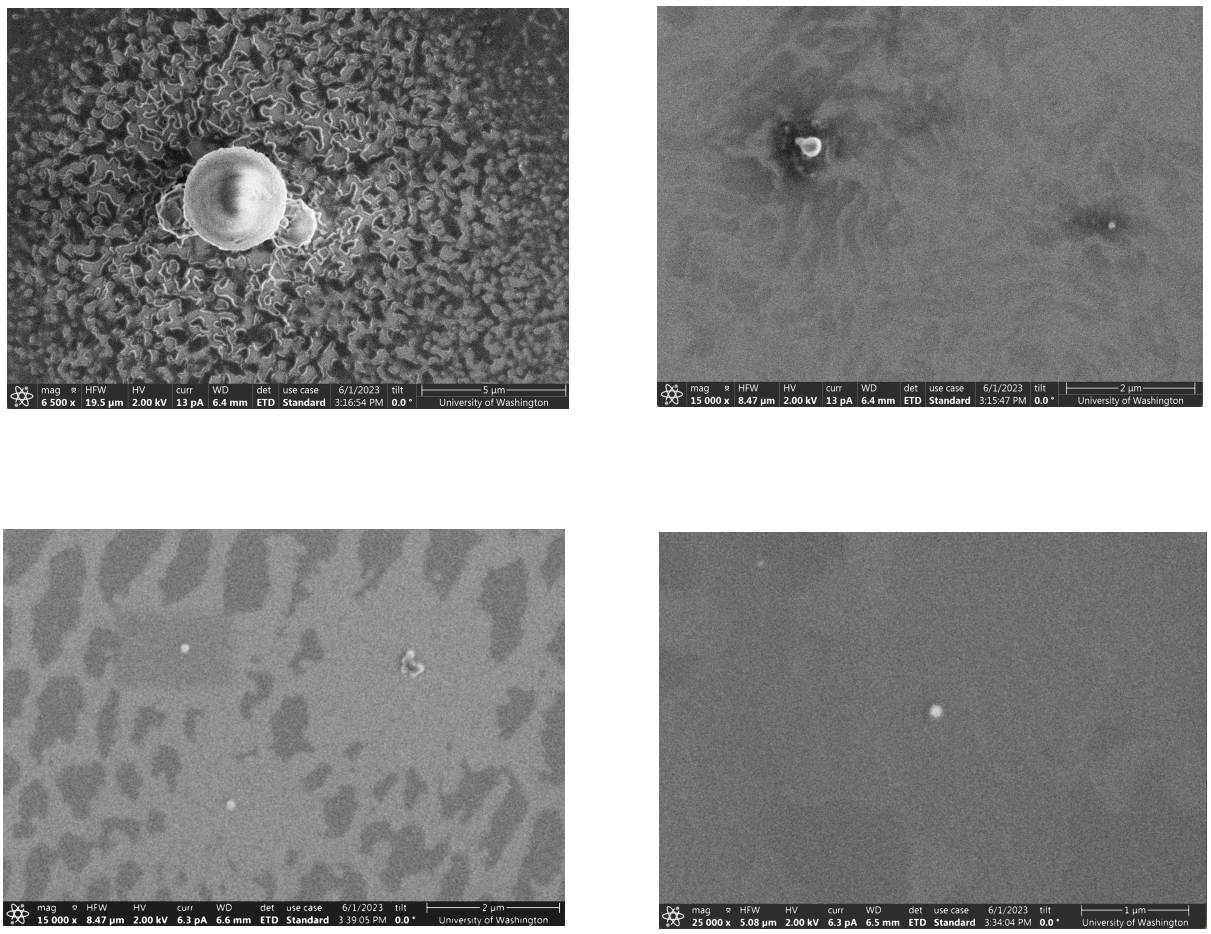


Figure 11, Scanning Electron Microscope Images for Determination of Morphology and Size of PE (top right), PET (top left), PMMA (lower left), and PS (lower right) post-digestion.

PE and PET were found to be irregular in morphology across preliminary assessments as can be seen in *Figure 10*. PET samples presented elliptical non-regular particles, and PE samples were found to be relatively spherical but slight indentation and irregularity dependent was noted across particle surfaces. Post enzymatic digestion morphology

assessment, as seen in *Figure 11*, shows relative agreement with preliminary assessment among all particle solutions. Of note across all post enzymatic digestion images was the presence of background media as can be seen forming on the surface of PE and PET particles, as well as changing the coloring of substrate surface in PS and PMMA images.

Polymer	*Pre-ED*	*Avg. d (nm)*	*SD. d (nm)*	*Post-ED*	*Avg. d (nm)*	*SD. d (nm)*	*diff.d*	*diff.sd*
PET		*116.7 nm*	*47.7 nm*		*84.5 nm**	*43.4 nm*	*32.2 nm (≅72.4%)*	*-4.3 nm (≅9.9%)*
PMMA		*105.3 nm*	*7.7 nm*		*104.6 nm*	*16.3 nm*	*0.7 nm (≅0.007%)*	*-8.6 nm (≅211%)*
PS		*100.3 nm*	*5.6 nm*		*166.2 nm**	*85.85 nm*	*65.9 nm (≅65.7%)*	*-80.25 nm (≅1533%)*
PE		*1356 nm*	*2850 nm*		*1764 nm*	*1143.9 nm*	*-408 nm (≅30%)*	*-1706.1 nm (≅40%)*

*Table 9, SEM Particle Characterization and Diff. p = 0.05, *statistically significant difference.*

ImageJ analysis of particle counts and sizing found preliminary diameters of 116.7, 105.3, 100.3, and 1356 nm for PET, PMMA, PS, and PE respectively. The standard deviations for polymer standards were 47.7, 7.7, 5.6, and 2850 nm respectively. ImageJ analysis of particle counts and sizing found post enzymatic digestion diameters of 84.5, 104.6, 166.2, and 1764 nm for PET, PMMA, PS, and PE respectively. The standard deviations for polymer standards were 43.4, 16.3, 85.85, and 1142.9 nm respectively. With a p-value of 0.5, SEM assessment found no significant change among PE and PMMA nanoparticles however there was observed significant change among PET and PS nanoparticles. These data can be observed in *Table 9* above and *Figure 12* below.

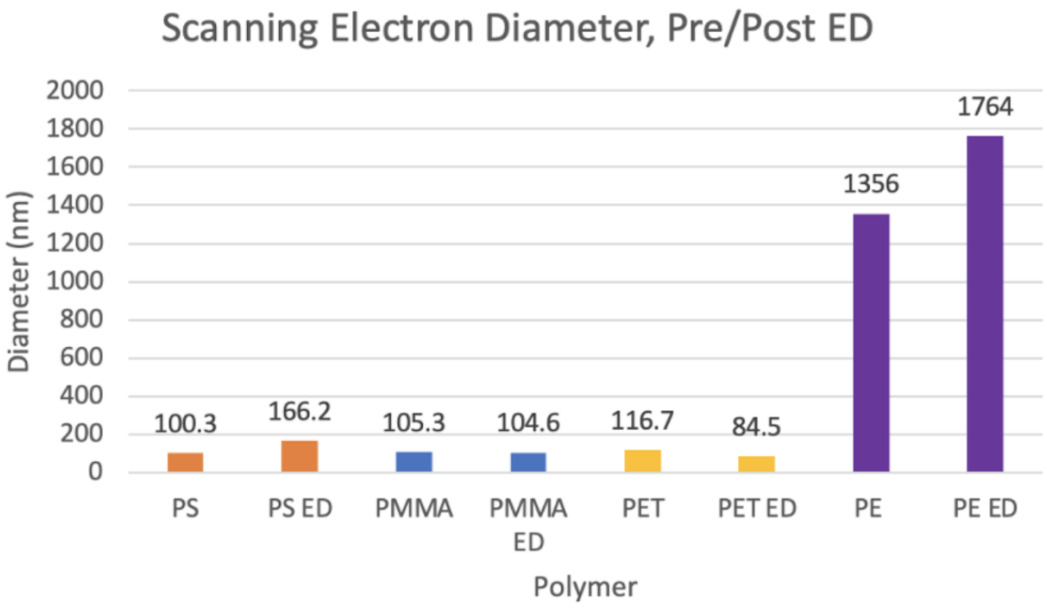


*Figure 12, SEM Particle Characterization and Diff. p = 0.05, *statistically significant difference.*

3.4 Conclusion

In conclusion, this study successfully developed a developmental risk assessment model for coastal zones focused on micro and nanoplastic particles with relevant constituent compound profiles. Model HQ outputs, given the current state of the literature, for what have been deemed environmentally relevant concentrations show an agreement between current understanding of micro and nanoplastic and the associated risk within the model. The model includes various assumptions which in turn could lead to conservative or liberal outcomes, and at present there is no section of the model that accounts for particle size distribution, morphology, and polymer specific reference doses due to limited data availability. Our model suggests there is no associated hazard with nanoplastic particles at environmentally relevant ranges (ng/L-µg/L), however as the model approached concentrations of 1 mg/L all model scenarios and profiles showed

agreement in classifying said exposure scenario as hazardous emphasizing the need to revisit plastic usage and policy.

This study also successfully conducted enzymatic digestions on a series of nanoplastic and microplastic particle standards in order to determine method viability as a non-degradative pretreatment step for nanoplastic marine media samples. Although particles were successfully recovered from the proposed procedure and effectively characterized by both DLS and SEM, there were statistically significant differences between pre and post digestion particle reads for all polymers in DLS trials and 50% of all polymers for SEM trials. The increase in particle diameter and variation through SEM imaging was largely attributed to improper removal of organic material, protein residue, and surfactant leading to surface buildup on nanoplastic particles. We still believe this method, when optimized and appropriately applied, shows promise in application at the nanoscale.

The results of these works indicate the need for further exposure assessment as well as improved particle analysis protocol and application for further development of environmental and *in vivo* nanoplastic particles.

3.5 Future Recommendations

At present, there is no classification of micro and nanoplastics as hazardous waste and to date risk assessments of these materials often lack data or consideration of constituent compound content. Constituent compounds, although selected for the developmental endpoint within this work, have the potential to impose carcinogenic, teratogenic, and mutagenic outcomes within biological systems. Considering Aim 1A, this study sought to develop environmentally relevant profiles of nanoplastic and

constituent compound content exposure and through the application of various studies providing industry production, human exposure, and chemical additive overview data was able to successfully do so. Relating to Aim 2A, the exposure scenarios set forth within Scenario A, B, and C provide a range of polymer exposures, and as a result of profile specific content, a range of constituent compound loadings. Variations in these profiles proved applicable in total HQ output for these models, however the contribution of nanoplastics was found to be less significant (<3% of HQ) when compared to that of the constituent compound content. Across all profiles and exposure scenarios the model indicated that at present concentrations there is no indication of significant hazard associated with these materials, however at what are considered extreme concentrations within the marine and aquatic space (>100 μg/L - 1 mg/L) the model indicated not only a significant level of hazard, but HQs exceeding 107 in liberal profile and BAF scenarios. Relating to Aim 3A, we believe that this model has effectively provided an understanding of the role constituent compounds play when considering the total hazard associated with nanoplastic materials. At present, given the attribution of over 98% of HQs being driven by constituent compound content, we highlight constituent compounds to be the primary concern when approaching nanoplastic risk. It is not to be understated how important particle dynamics and distributions will be in refining and improving the applicability of said models, as well as further discovery and assessment of nanoplastic concentrations within marine media and seafood tissue.

To date effective, non-degradative, pretreatment methodology is lacking within the marine micro and nanoplastic space. Relating to Aim 1B, this study was able to successfully assess various nanoplastic standards via DLS and SEM methodology with the express intent of determining the degradative capacity of the enzymatic

pretreatment schema set forth herein. Considering Aim 2B and 3B, the study was able to recover and observe an increase in the diameter of particles across both methods. Given the expectation of no-observed-change to degradative as stated within our hypothesis, the growth in observed particle sizing was an unexpected result. Upon further review of SEM images, it would appear that protein residue and surfactant buildup were likely candidates for mechanisms behind this increase in particle diameter. Further review and optimization of these methods is needed to establish them as a viable non-degradative process for marine and seafood tissue nanoplastic samples. Based on the findings within this work, we would recommend further investigation into appropriate exposure assessment of nanoplastic materials in environmental compartments with a specific focus on the marine compartment. Ideally, further research should focus on loading of nanoplastics in the human body such as the case profiles used in this work (Schwabl et al., Leslie et al.). We also recommend further investigation into the application of the enzymatic methods put forth herein so that optimization, appropriate recovery, and applicability alongside environmental samples can be assessed. Mass-based and spectroscopic analytical methods should also be applied in further iterations to assess any molecular changes during the pre-treatment process.

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