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Nanoplastic Neurotoxicity in the Marine Mussel *Mytilus galloprovincialis*

Master's in Marine and Coastal Systems
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Declaration of Work (Declaração de autoria de trabalho)

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

I declare to be the author of this work, which is original and unpublished. Authors and works consulted are duly cited in the text and are included in the list of references.

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Muito obrigada a todos!

Abstract

Plastic is a ubiquitous, long-lasting, problematic form of litter. It has been found everywhere on Earth, from the bottom of the ocean's deepest trench to the top of the world's tallest mountain. As the COVID-19 pandemic rages on, so does the global dependence on the usage of single use plastics (SUPs), such as disposable face masks and takeout food containers. Once released into the environment, plastic fragments break down into microplastics (1-5 mm) and nanoplastics (1-100 nm) due to fragmentation and degradation processes. Compared to microplastics, nanoplastics have a much larger surface area to volume ratio, which makes them more reactive since they can adsorb different chemicals and travel across biological barriers.

The present research was conducted to unravel potential toxicity responses caused by nanoplastics. This suggests that the seeping of plastics through cell membranes can affect all organisms, including those in higher trophic levels that consume contaminated prey. Toxins caused by nanoplastics may travel up the food chain and pose a risk to human health. This study confirmed neurotoxic effects in the gills of marine mussels *Mytilus galloprovincialis* after an exposure of 10 μgL^{-1} of Polystyrene (PS) nanoplastics after 21 days of contamination followed by 14 days of depuration using the biomarker Acetylcholinesterase (AChE).

Keywords: Nanoplastics, ecotoxicology, neurotoxicity, *Mytilus galloprovincialis*.

Resumo

Plástico é um componente do lixo omnipresente, duradoura e problemática. Encontra-se em todos os lugares da Terra, desde a maior profundidade do Oceano até o topo da montanha mais alta do mundo bem como nas calotes polares. Com o avanço da pandemia de COVID-19, observou-se um considerável aumento da dependência de plásticos de uso único (SUPs), como máscaras descartáveis e recipientes de embalagem de alimentos. Ao entrarem no ecossistema marinho, os plásticos estão sujeitos a processos de degradação e fragmentação alcançando tamanhos menores como os microplasticos (MPs; 1 – 5 mm) e nanoplasticos (NPs; 1 – 100 nm). Os NPs, em comparação com os MPs, têm uma área superfícial maior, e à medida que o tamanho dos NPs diminui, esta relação aumenta. No ecossistema marinho, esta característica dá aos NPs uma maior reatividade, possibilitando a entrada através de barreiras biológicas como também a absorção de outros compostos químicos presentes no meio ambiente.

Este estudo teve por objetivo avaliar as potenciais respostas de toxicidade causada por NPs, uma vez que a facilidade destas partículas atravessarem barreiras biológicas poderá afetar todos os organismos, incluindo níveis tróficos mais elevados por consumo de presas contaminadas por NPs. Além disso, os efeitos tóxicos causados por NPs podem também, devido a biomagnificação na cadeia alimentar, representar um risco para a saúde humana. Este estudo confirmou a existência de efeitos neurotóxicos nas brânquias de mexilhões marinhos *Mytlius galloprovincialis* após uma exposição de 10 µg/L de nanoplasticos de poliestireno (nPS) durante 21 dias, seguido de 14 dias de depuração, utilizando como biomarcador a acetilcolinesterase (AChE).

Palavras-chave: Nanoplastics, *Mytilus galloprovincialis,* Depuração, AChE

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List of Abbreviations

AChE — Acetylcholinesterase
ALAD — Delta-aminolaevulinic acid dehydratase
BA — Bioaccumulation
BBB — Blood-Brain Barrier
CAT — Catalase
CDC — The Center for Disease Control
ChE — Cholinesterase
CI — Condition Index
CT — Control
CYP — Cytochrome P450s
DG — Digestive Gland
DLS — Dynamic Light Scattering
DO — Deionized Water
EC — Emerging Contaminant
ECDC — European Center for Disease Prevention and Control
EEA — European Environmental Agency
G — Gonads
GHG — Greenhouse Gas
GPx — Glutathione Peroxidases
HDPE — High-Density Polyethylene
LDPE — Low-Density Polyethylene
LPO — Lipid Peroxidation
MP — Microplastic
MT — Metallothionein
NGO — Non-Governmental Organization
NP — Nanoplastic
OP — Organic Pollutant
R — Remainder
PAH — Polycyclic Aromatic Hydrocarbons
PCB — Polychlorinated Biphenyls
PCP — Personal Care Product
PET — Polyethylene Terephthalate
PIA — Plastics Industry Association
PS — Polystyrene $(C_8H_8)_n$
PP — Polypropylene
PPT — Plastic Particle Toxicity
POPs — Persistent Organic Pollutants
PVC — Polyvinyl Chloride
ROS — Reactive Oxygen Species
SOD — Superoxide Dismutase
SPI —Society of the Plastics Industry
SW —Natural Seawater
SUP —Single Use Plastic
TP —Total Proteins
WHO —World Health Organization
WWF —World Wildlife Fund
WWTP —Wastewater Treatment Plants
UNEP —United Nations Environment Program

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Chapter 1. Introduction

1.1 The Current Scenario of Plastic Production Worldwide

Plastic and plastic polymers have become ubiquitous in the context of our daily lives (Thompson *et al.,* 2009; Barnes *et al.,* 2009), with applications in furniture, construction, packaging, food preservation, textiles, household items, electronics, and the medical field (Pinto Da Costa *et al.,* 2020; Thompson *et al.,* 2009). Plastics are lightweight, durable, strong, inexpensive materials, that are easy to produce (Thompson *et al.,* 2009) and can last hundreds of thousands of years in the natural environment (Hopewell *et al.,* 2009). The manufacturing process to create plastics combines ingredients such as carbon, silicon, hydrogen, oxygen, and chloride (Shah *et al.,* 2008) with various polymers and additives through polymerization and polycondensation reactions to form polymer chains (Stapleton, 2019). The polymers and additives used in the production process vary from product to product, and can include ingredients such as phthalates, metals, and brominated flame retardants (Ologbonjaye *et al.,* 2019; Teuten *et al*. 2009). The Society of the Plastics Industry, (SPI), has classified a waste management guideline for plastics based on the polymers used to produce them.

1 - Polyethylene Terephthalate (PET);
2 - High-Density Polyethylene (HDPE);
3 - Polyvinyl Chloride (PVC);
4 - Low-Density Polyethylene (LDPE);
5 - Polypropylene (PP);
6 - Polystyrene (PS); and
7 - Other as not identified above, including Polycarbonate and Polylactide (nylon)

Plastic products with the resin ID number 1-6 collectively account for nearly 90% of the total plastic produced worldwide (Andrady and Neal, 2009; Li *et al.,* 2016). The most common properties of plastic are strength, resiliency, pliability, malleability, and longevity (Thompson *et al.,* 2009). Other plastics may have desirable properties such as high electrical and thermal insulation, corrosion resistance (Thompson *et al.,* 2009.), or as a sealant to barricade against moisture (Andrady, 2011). Because of their valuable benefits, plastic production has drastically increased since the 1950s (PlasticsEurope, 2019) (Figure 1). Between 2002 and 2018, plastic production increased 79.5% from 200 to 359 metric tons per year, and over half of the plastic produced between 1950 and 2016 occurred since 2000 (Shanmugam, 2020; Wit *et al.,* 2019). Due to the chemical stability of plastics, their manufacturing process cannot be reversed, leaving them to accumulate in the environment unless they get recycled or incinerated (Stapleton, 2019).

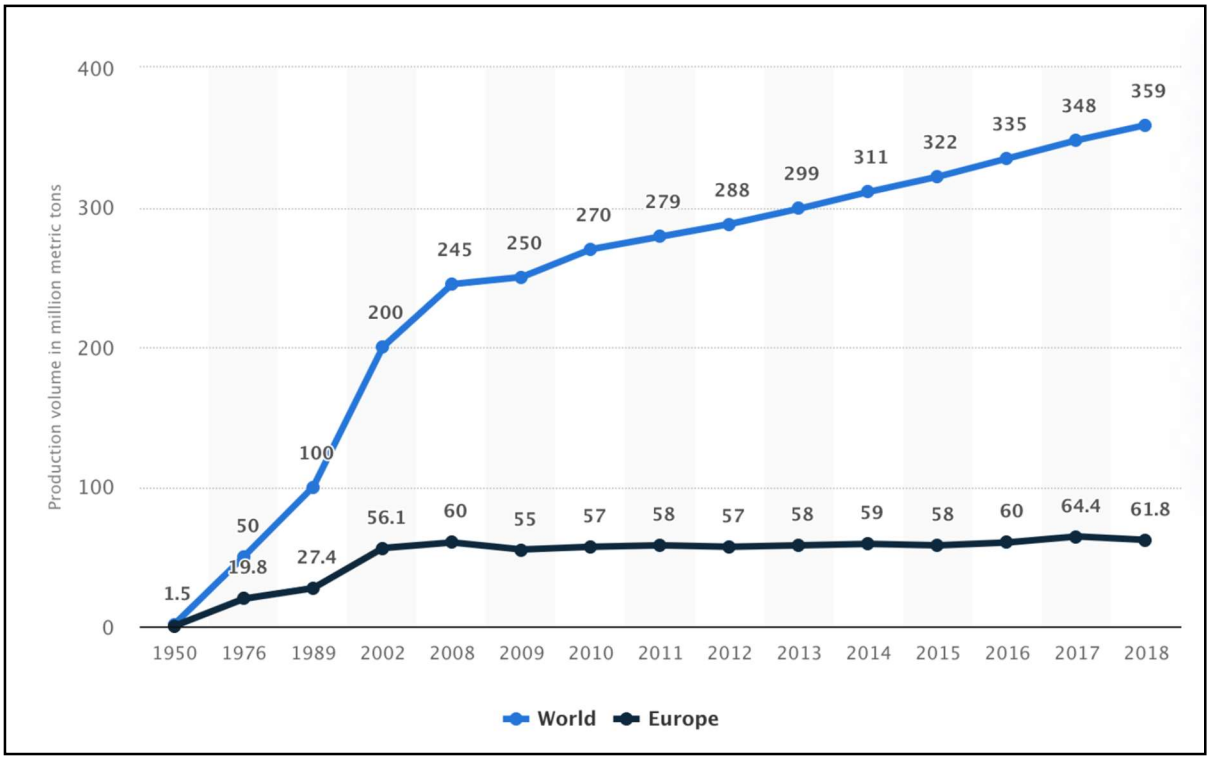

FIGURE 1. GLOBAL PLASTIC PRODUCTION (PLASTICSEUROPE, 2019)

In 2020, over 380 million tons of plastic were manufactured globally. Of this amount, 9% was recycled, 12% was incinerated, and 79% accumulated in either landfills or the natural environment (Geyer, *et al.,* 2017). By 2030, the amount of globally manufactured plastics may double, and by 2050 it is estimated that there will be nearly 12 billion tons of plastic in landfills and natural environments (Geyer *et al.,* 2017; Silva *et al.,* 2021). Existing as an ever-growing "throwaway culture," most of all plastic ever produced is already waste (Wit *et al.,* 2019). Plastic materials made with the intent of only being used once and disposed of within one year of manufacture are known as single use plastics (SUPs) (Hopewell *et al.,* 2009). The most common SUP products are consumer goods such as shopping bags, cutlery, and packaging, which account for 40% of all produced plastic (Ericksen *et al.,* 2014; Chen *et al.,* 2021). While properly disposed SUPs are routed to landfills or incineration facilities, over 89% of SUPs accumulate in soils, rivers, and oceans (UNEP, 2018), resulting in a loss of valuable resources (Boucher *et al.,* 2019; Law *et al.,* 2010; Briassoulis *et al.,* 2015; Wang *et al.,* 2018a, 2018b). In a report commissioned by the WWF (World Wildlife Fund), the cost of plastic to society, the economy, and the environment is 10 times higher than the market price of virgin plastic (Dalberg, 2021). Specifically, the cradle-to-grave cost of plastic produced in 2019 is estimated to be US$3.7 trillion, with US$32 Billion devoted to the collecting, sorting, disposing, and recycling of plastic at waste management facilities. US$15 Billion is spent by governments, NGOs (non-government organizations), and citizens for organized clean-up events, and US$7 Billion is revenue loss from tourism, fishing, and aquaculture due to marine plastic pollution (Dalberg, 2021). Additional costs of plastic come from the exposure of marginalized communities located near incineration plants or oil and gas refineries (Dalberg, 2021).

1.2 The Pandemic's Effect on Plastic Consumption

Despite a growing awareness regarding the plastic pollution problem, the outbreak of SARS-CoV-2 in 2019 has led to an unprecedented rise in the demand for SUPs such as personal protective equipment (PPE) for health and safety reasons (Silva *et al.,* 2020; Syam, 2020; Nzediegwu and Chang, 2020). To reduce the potential airborne human-to-human transmission pathways of COVID-19, many countries have employed the use of single-use facemasks to combat the spread of COVID-19 (CDC, 2020; ECDC, 2020; WHO, 2020). N95 masks recommended by the CDC are made of plastic nanofiber strands with threads ranging from < 1-10 μm in diameter (Aragaw, 2020; Prata *et al.,* 2018; Xu & Ren, 2021). These strands are composed of polypropylene, polyethylene terephthalate, polyurethane, polyacrylonitrile, polystyrene, polycarbonate, polyethylene, and polyester plastics (Fadare & Okoffo, 2020; Silva *et al.,* 2020).

Globally, the production of masks, face shields, gloves, gowns, containers for hand sanitizer, and other disposable PPE equipment used in the medical field has increased by 17% since May 2020 (Silva *et al.,* 2020). China, a major producer of facemasks, increased its facemask exports by 450% from January 2020 to February 2020 (da Costa, 2021). Collectively, the 27 EU Member States imported an additional 17,000 tons of face masks than their business as usual (BAU) values (Figure 2), which were calculated using data from the 14 months preceding the pandemic's arrival in Europe (EEA, 2021).

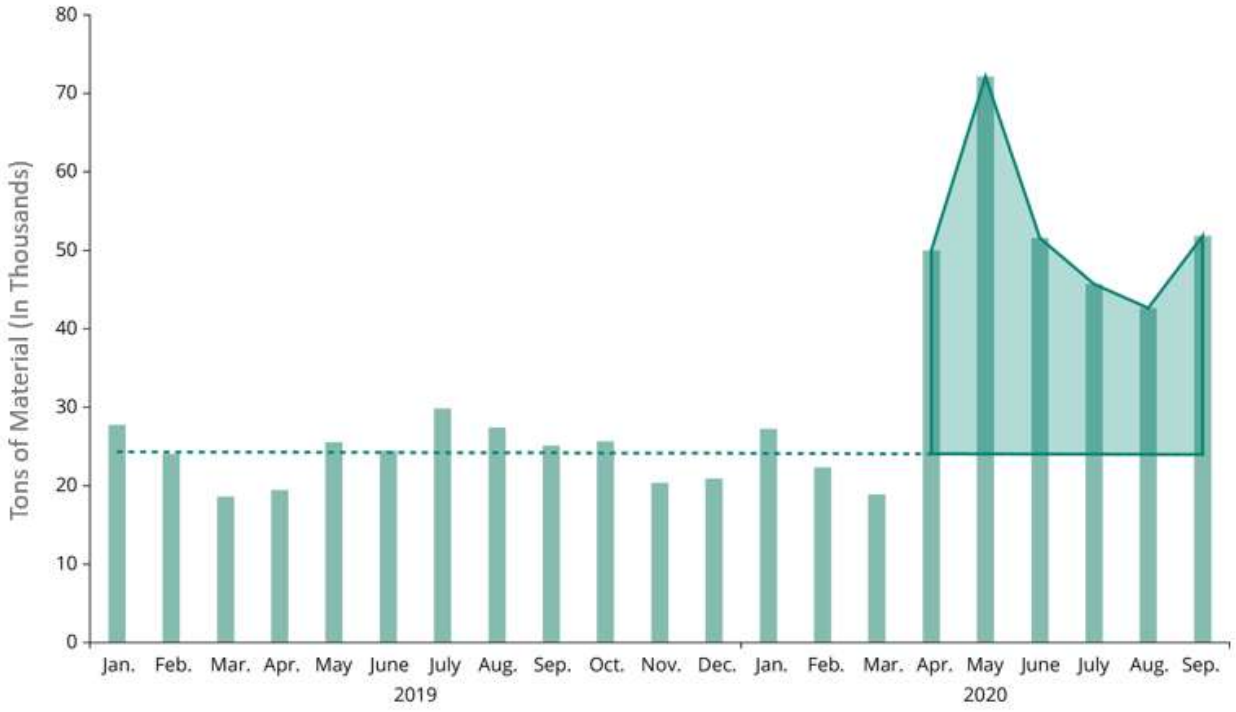

FIGURE 2. FACE MASK IMPORTS (IN THOUSAND-TONS) TO THE EU (EEA, 2021)

As a consequence of implemented safety recommendations, stringent directives, and an increase of PPE production, an estimated 3.4 billion single-use face masks and face shields are discarded daily, resulting in 1.6 million tons of plastic waste per day (Benson *et al.,* 2021). Current studies estimate that within one year, 0.15-0.39 million tons of mismanaged plastic waste from COVID will enter global oceans (Chowdhury *et al.,* 2021).

Figure 3 shows a map that highlights the number of masks, by country, that are disposed of daily, leading with China, India, the United States, Brazil, and Indonesia (Benson, 2021). From this, it is estimated that 1.56 billion masks have already entered the marine environment (Bondaroff & Cooke, 2020). Once in marine waters, disposable masks will break down faster than bulk plastics (such as plastic bags) due to the thin strands of plastic that are used to construct them (Bondaroff & Cooke, 2020; Xu & Ren, 2021).

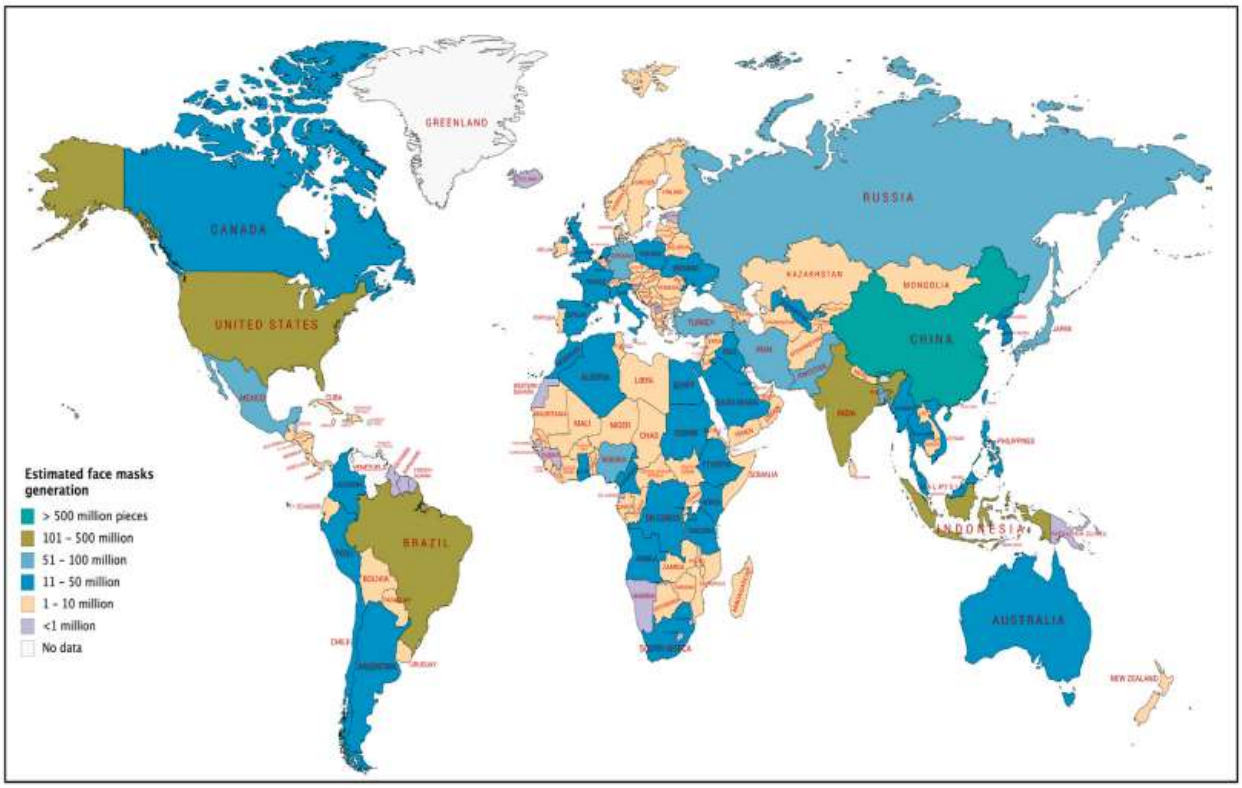

FIGURE 3. QUANTITY OF DAILY DISCARDED SINGLE-USE MASKS BY COUNTRY (BENSON ET AL., 2021)

Since the beginning of the COVID-19 global pandemic, there has been a dramatic increase in waste worldwide (Chen *et al.,* 2021). The Plastics Industry Association (PIA) claimed that reusable grocery bags could be a vector for COVID-19, leading to a delay, suspension, or cancellation of local plastic pollution reduction policies (Pinto da Costa *et al.,* 2021), which have led to a widespread resurgence of single-use polyethylene plastic packaging for foods and consumer products (Pinto da Costa *et al.,* 2021). After discovering that the highly contagious virus responsible for COVID-19 could survive plastic surfaces for several days if not properly disposed of (Bronson *et al.,* 2021), all PPE relating to COVID was classified as medical waste. Before being

sent to a landfill, all medical waste must first be incinerated (Silva *et al.,* 2020). However, this unforeseen increase in medical and domestic waste has overloaded some municipality's ability to manage it adequately (Bondaroff & Cooke, 2020), which has led to the bulk mismanagement in waste collection and treatment services. This has led to an increase of litter as well as blockages in sewage systems around the world (Bronson *et al.,* 2021). In one day, the city of Wuhan (China) produced 200 tons of medical waste, or quadruple the amount of waste their local facilities could treat (Silva *et al.,* 2020). For this specific scenario, the city of Wuhan was able to import mobile waste management facilities, however that is not always the case as many countries struggle to meet the increase in medical waste (Chen *et al.,* 2021; Silva *et al.,* 2020).

1.3 Routes and Fate of Plastics into the Ocean

Oceans are the ultimate sink for plastic waste, as over 12 million tons of plastic enters the sea every year (Pico *et al.,* 2019). The major source of land-based litter is from solid waste disposal management facilities, and their accidental discharge (Peng *et al.,* 2020). Plastics may enter the marine environment via the discharge of effluents from point sources such as wastewater treatment plants, or in runoff from streams and storm drains carrying products such as cleaning agents, textile fibers, and personal care products directly into the waterways (Barnes *et al.,* 2009; Carr *et al.,* 2016; Alprol *et al.,* 2021). With aid from wind and rain, rogue litter will be transported to the ocean where it will degrade and fragment into smaller pieces of marine litter (Figure 4) (Barnes *et al.,* 2009). Due to the current, tides, and wind, not a single beach on Earth is safe from plastic pollution, regardless of proximity to human activity (Horton & Barnes, 2020).

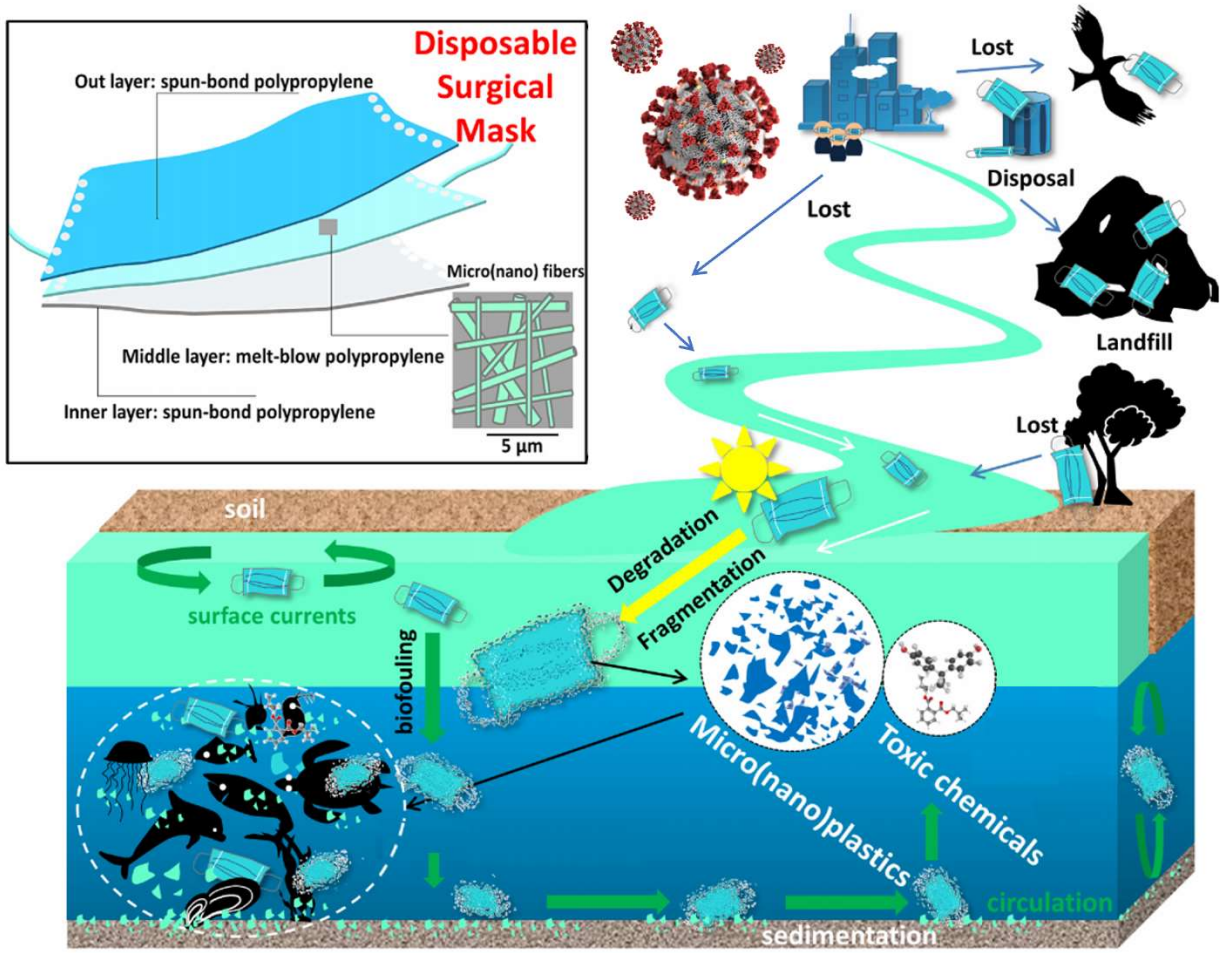

FIGURE 4. ROUTES OF SINGLE-USE SURGICAL MASKS INTO THE OCEAN (ADAPTED FROM XU & REN, 2021)

Even with modern filtration techniques, many global wastewater management systems are not adequately equipped to remove MPs from the effluents (Ou & Zeng, 2018). Approximately 83% of all the plastic debris in the world's oceans originate from a mere 20 countries, most of which are categorized as developing nations with poor waste management systems and the absence of stringent regulations (Tibbetts, 2015; Mitrano & Wohlleben, 2020). Collectively, these 20 countries (including China, the Philippines, and the US) are responsible for releasing 275 million metric tons of plastic waste per year into the ocean (Tibbetts, 2015).

Advances in wastewater treatment plants (WWTPs) technologies have revealed successful removal rates of microplastics from sewage wastes, (Carr *et al.,* 2016), however, WWTPs are still the main source of microplastics entering the marine environment through effluent and sludge (Liu *et al.,* 2021), as MPs were found in 90% of treated water worldwide (Mason *et al.,* 2018). To remove MPs, WWTPs use a variety of filtration techniques including primary settling treatments with flocculation, bioreactor systems, advanced oxidation, and membrane filtration methods, with filter-based treatment methods having the highest removal rate (Liu *et al.,* 2021). These mechanical, chemical, and biological treatment processes remove approximately 99% of microplastics entering a WWTP (Ziajahromi *et al.,* 2016). Although these results seem promising,

only countries that can afford these techniques have implemented them, and there are still 5.00×10^5–1.39×10^{10} microplastic particles being released into the marine environment every day (Liu *et al.,* 2021). Although successful methods have been developed to remove NPs in lab settings, there are not yet any methods to eliminate NPs on a larger scale at WWTPs (Murry & Örmeci, 2020).

Marine-based debris enters the ocean both accidently and intentionally while at sea and makes up 20% of marine pollution (Barnes *et al.,* 2009). The debris may originate from offshore petroleum platforms, as well as from fishing, shipping, military, research, and recreational vessels (Sheavly & Register, 2007). Accidental debris dumping can include events such as losses in transport or accidents, while intentional dumping occurs when trash originating from a vessel is thrown overboard, or when other equipment such as buoys, pots, traps, and nets fail to be retrieved by fishing vessels via either loss or abandonment (Barnes *et al.,* 2009; Sheavly & Register, 2007). The most common marine litter is abandoned fishing gear and consumer items such as plastic bottles, plastic bottle lids, straws, grocery bags, food wrappers, and cigarettes (Figure 5) (Barnes *et al.,* 2009; NOAA, 2021; Williams, 2021).

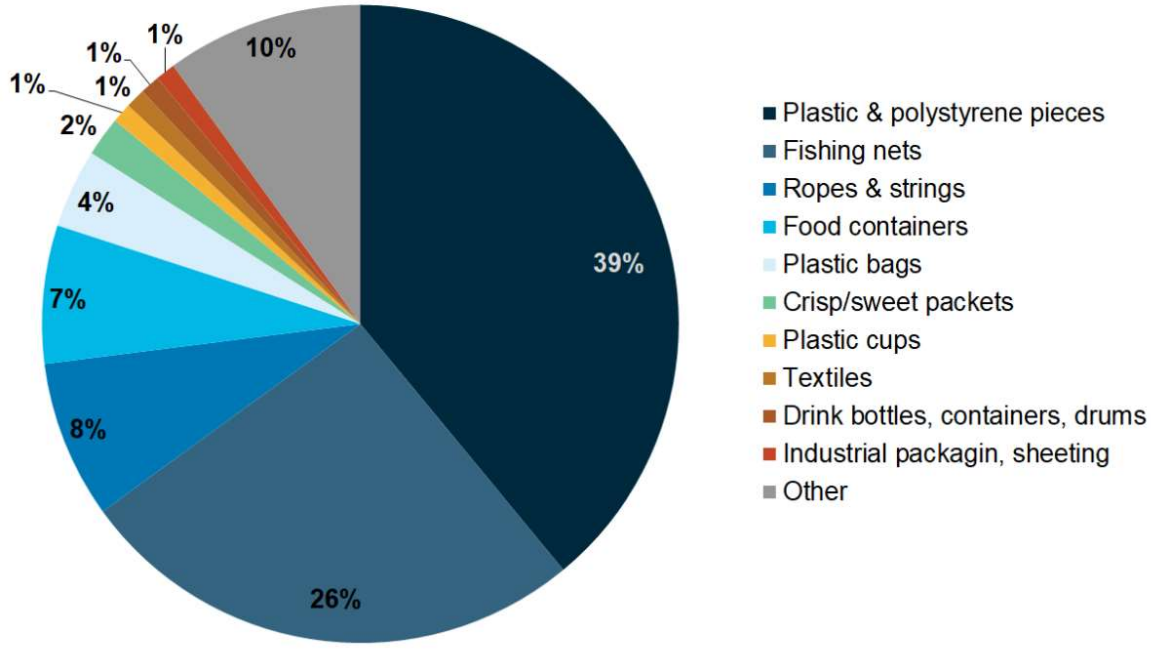

FIGURE 5. BREAKDOWN OF COMMON MARINE LITER (FLEET *ET AL.*, 2019)

Once plastics enter the ocean, they can be found on shorelines, the sea surface, in benthic ecosystems, and throughout the entire water column (Thushari & Senevirathna, 2020). On average, 50-80% of shoreline litter is plastic (Barnes *et al.,* 2009). Because 60% of produced plastics are less dense than water, ocean currents can transport floating plastics thousands of kilometers over many years (Lebreton *et al.,* 2018; Thompson, *et al.,* 2011) and accumulate in gyres based on the shape and composition of individual pieces (Lusher, 2015) due to ocean currents caused by the Coriolis effect (Chen *et al.,* 2017). After reaching the ocean, polymers may begin to break down into smaller pieces through fragmentation and degradation, as well as bond-breaking reactions

induced by chemical, physical, and biological events (Figure 6) (Andrady, 2011; Mattson *et al.,* 2018). Degradation includes the following processes: biodegradation, photodegradation via UV-B radiation, thermo-oxidative degradation, hydrolysis, thermal degradation, and mechanical degradation, (Barnes *et al.,* 2009; Gonçalves & Bebianno, 2021; Thushari & Senevirathna, 2020), introducing critical concerns associated with micro/nanosized plastics (MPs and NPs, respectively).

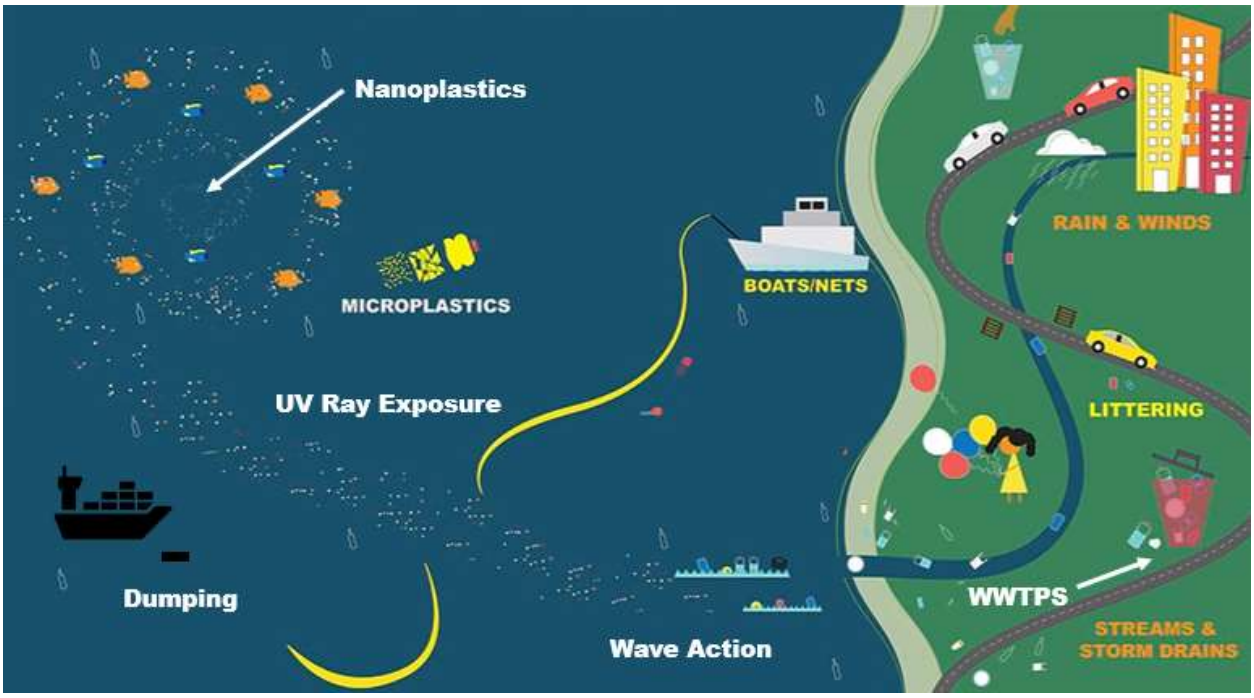

FIGURE 6. ROUTES OF PLASTIC INTO THE OCEAN (ADAPTED FROM NOAA, 2021)

Sizes of plastic found in the ocean have been categorized as macro (>20 mm), meso (5-20 mm), and micro (1-5 mm), whereas nanosized plastics fall within the range of other types of nanoparticles (1-100 nm) (Gigault *et al.,* 2018; Gonçalves & Bebianno, 2021). It is estimated that 98% of MP and NP contamination in the marine environment originates from land (Rochman, 2018; Mofijur *et al.,* 2021), and that around 80% of these plastics in the oceans originated from tires, city dust, cosmetic industry, manufacturing, fishing industry, packaging, and plastic bottles (Figure 6) (Mattsson *et al.,* 2018).

MPs and NPs may be categorized as primary or secondary classes. Primary plastics are those that enter the marine environment as they were manufactured: as microscopic virgin resin pellets applied in cosmetics, facial cleansers, industrial abrasives and air blasting, waterborne paints, electronics, coatings, as well as the micro/nanosized plastics applied in medicine as vectors for drugs (Alprol *et al.,* 2021). Primary plastics also include nurdles, which are manufactured plastic pellets ranging 1-5mm diameter that are shipped globally to be melted and molded into common plastic products such as bottles, bags, sunglasses, and cutlery (Tunnell *et al.,* 2020).

There is an extensive report of the distribution and abundance of MPs in coastal compartments, including seawater column, sediment, and beaches (Li *et al.,* 2019). Nurdles have been reported

washing up on beaches all over the world (Fernandino *et al.,* 2015). An estimated 230,000 tons of nurdles enter the oceans annually (Sherrington, 2016), with 167,000 tons from Europe alone (Hann *et al.,* 2018). Nurdles can enter the environment during all stages of the plastic supply chain, from manufacturing to transportation, during loading/storage, and at the fabrication facilities (Karlsson *et al.,* 2018). MPs have been found in every ocean (Barnes *et al.,* 2009), remote polar areas (Machado *et al.,* 2019), on top of the highest mountain, in oceanic sediment (Zheng *et al.,* 2019), and at the bottom of the seafloor (Van Cauwenberghe *et al.,* 2013; Barnes *et al.,* 2009). MPs have been discovered in organisms at the ocean surface (Barnes *et al.,* 2009) as well as at depths of 10,890 m (Jamieson *et al.,* 2019).

Classified as contaminants of emerging concern (CECs) (Mateos-Cardenas *et al.,* 2021), MPs have been discovered in zooplankton, phytoplankton, earthworms, soils, plants, and aquatic macrophytes (Chang *et al.,* 2019; Mateos-Cardenas *et al.,* 2021). This finding reveals that MPs can be transported by soil, air, and water (Alimi *et al.,* 2018), and that all living organisms at the bottom of food webs are subjected to harms from microsized plastics, further generating an environmental concern for trophic transfer, with potential effects on human health (Mateos-Cardenas *et al.,* 2021).

The threats posed by MPs to marine ecosystems and respective biota are well documented in current literature. The main consequences of MP contamination on aquatic organisms are the exposure, ingestion, and accumulation of MPs in tissues, ranging from effects on behavior (Mattsson *et al.,* 2015), metabolism (Lu *et al.,* 2016), and reproduction (Sussarellu *et al.,* 2016).

Ingestion of MPs may potentially expose organisms to toxic chemicals (Vethaak and Leslie, 2016) as reactions may result from the presence of additives, adsorbed chemicals (Barboza *et al.,* 2019), leaching pollutants, and dissociating external contaminants that bind to the MPs (Campanale *et al.*, 2020).

Exposure to MPs has neurotoxic effects on both marine vertebrates (Barboza *et al.,* 2020) and invertebrates (Tlili *et al.,* 2020; Ribeiro *et al.,* 2017, Oliveira *et al.,* 2018). In fish species *Dicentrachus labrax, Trachurus trachurus,* & *Scomber colias*, an increase of brain acetylcholinesterase activity was detected after exposure to MPs (Barboza *et al.,* 2020). Additionally, Tlili *et al*. (2020) proved that ingesting MPs inhibited AChE activity in the gills of wedge clams (*Donax trunculus*) after the first 4 hours of exposure using realistic concentrations of PE & PP MPs [0.06 g/Kg of sand; 0.05-5 µm] in the lab. Exposure to PS MPs in bivalves including *Scrobicularia plana* [1mgL^{-1};20µm] and *Corbicula fluminea* [0.13 mg L^{-1}; 1-5 µm] also all resulted in an inhibition of AChE activity in the gills (Ribeiro *et al.,* 2017; Oliveira *et al.,* 2018).

1.4 The Emerging Threat of Nanoplastics

Nanoplastics are small pieces of plastic that are either manufactured for a specific process and lost in the environment, or they are formed in the environment due to the degradation of MPs and other larger plastic pieces. Primary NPs found in the marine environment can be the result of drug delivery, paint production, adhesives, coatings, biomedical products, electronics, medical diagnostics, optoelectronics, magnetics (Koelmans *et al.,* 2015), toothpaste, cosmetics, and other

PCPs (personal care products) (Luo *et al.,* 2015). However, the majority of NPs in the marine environment are secondary plastics, derived from the fragmentation and degradation of macro and micro plastics (Andrady, 2011; Cole *et al.,* 2011; Mattsson *et al.,* 2017). Degradation of plastics in the marine environment occurs through hydrolysis, mechanical/physical and thermo oxidative degradation, photodegradation, and/or biodegradation (Andrady, 2011). The decrease in size dimension and increase in surface area makes NPs more reactive in the aquatic environment than the respective bulk plastic material due to changes in physical and chemical properties, such as strength and conductivity. The chemical composition of NPs has a polarization that influences the hydrophobicity and hydrophilicity of the particles, which may lead to the adsorption of chemical contaminants to their surface (Liu *et al.,* 2016; Song *et al.,* 2019), in addition to the polymers that were used to produce them (Costa *et al.,* 2016; Gaylarde *et al.,* 2020; Li *et al.,* 2020). This can cause NPs to physically or chemically absorb compounds present in their environment such as persistent organic pollutants (POPs), natural organic macromolecules, microbes, metals (Galloway *et al.,* 2017; Ashton *et al.,* 2010; Velzeboer *et al.,* 2014; Wang *et al.,* 2016), and biomolecules in living organisms such as proteins and lipids (Galloway *et al.,* 2017; Walker & Chan, 2012).

With nanosized dimensions, NPs have the physical features that enable it to aggregate and travel across biological barriers such as organs and tissues, resulting in a higher ingestion and biological reactivity (Rist & Hartmann, 2017; El-Hadri *et al.,* 2020; Costa *et al.,* 2016; Gaylarde *et al.,* 2020; Li *et al.,* 2020), with an ultimate impact on the behavior and metabolism of biota (Worm *et al.,* 2017; Mattson *et al.,* 2018). The combination of these features may enhance NPs' bioavailability and transference over the marine food chain, with potential and unknown biological impacts across trophic levels (Mattson *et al.,* 2015; Venâncio *et al.,* 2019).

Size cutouts of NPs are arbitrary, as they can exist as spheres (PCPs), fragments (bulk fragmentation), fibers (polyester clothing), films, and foams (Hartmann *et al.,* 2019). NPs, like MPs, can enter the environment as primary (engineered) or secondary plastics (Hartmann *et al.,* 2019). Because NPs rely on wave-like light rays to be measured, they are much harder to quantify, and thus regulate, than MPs (Gigault *et al.,* 2018; Mitrano *et al.,* 2021). In lab settings, NPs can be counted and characterized using dynamic light scattering (DLS), Raman spectroscopy, transmission electron microscopy, hyperspectral microscopy, and mass or size-based particle counter methods (Wagner & Reemtsma, 2019). Common modifications to easily characterize NPs are the addition of a metallic core, or a surface modification such as radioactive or fluorescent labeling (Sander *et al.,* 2019; Mitrano *et al.,* 2019). Although there is currently no way to accurately measure the quantity of NPs in the environment, it is estimated that the present concentration of NPs is over 1,000 times higher than current concentrations of microplastics (Besseling *et al.,* 2019), or < 1 mg L^{-1} in natural seawater (Lenz *et al.,* 2016). Biological and ecological implications surrounding NPs are more complicated than that of microplastics due to their smaller size and ability to travel biological barriers (Rist & Hartmann, 2017).

In marine biota, NP toxicity is associated with a decrease in growth rates, energy, stress, inflammation, and malformations (Kögel *et al.,* 2020). In algae, NPs have affected cell density and growth rate (Sjollema et al., 2016), as well as larvae development, embryo malformation, and oxidative stress-induced damage of lipid membranes in crustacea (Lee et al., 2013).

Figure 7 demonstrates the functional changes that may occur when particles of different size classes interact with biological organisms (green) or to chemical transport of particles in different environments (purple). Compared to larger particles, NPs can enter an organism through inhalation, ingestion, through biological barriers, and biological uptake into cells (Mitrano *et al.,* 2021). Impacts caused by PS NP translocation and deposition in invertebrates are yet to be fully studied, although effects relating to nanomaterial transport and systemic toxicity are expected (Stapleton *et al.,* 2012).

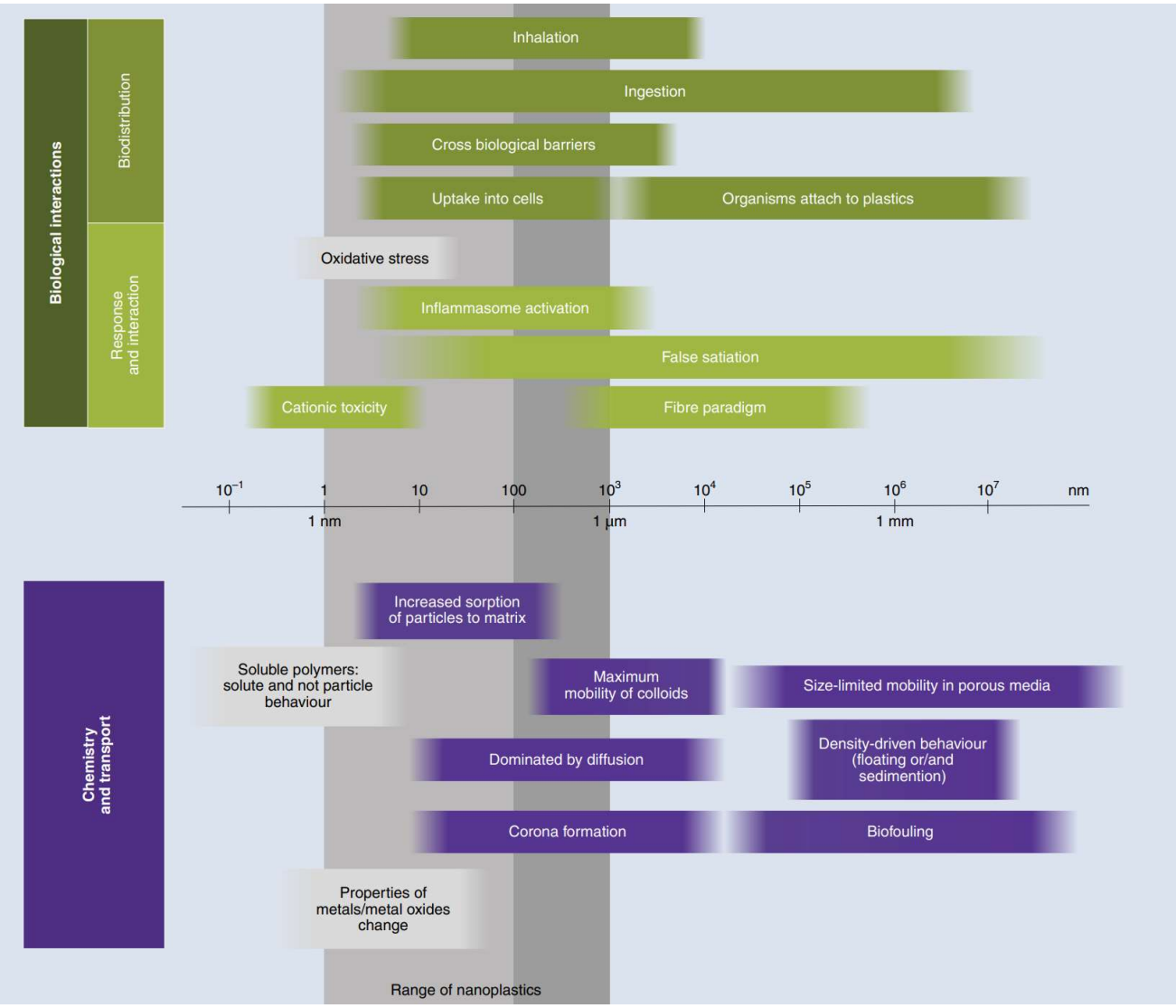

FIGURE 7. INTERACTIONS BETWEEN DIFFERENT SIZE PARTICLES BETWEEN BIOLOGICAL ORGANISMS (GREEN) AND CHEMICAL TRANSPORT OF PARTICLES (PURPLE) (MITRANO *ET AL.*, 2021)

1.4.1 The Impacts of NPs on Marine Biota

The toxicity due to the presence of NP is related to the polymer type, size, concentration, exposure time, as well as other specific environmental factors such as the presence of other contaminants, the availability of food, the species, and the developmental stage of the species (Kögel *et al.,* 2020).

Chronic exposure to NPs in bivalves may lead to reactive oxygen species (ROS) generation, which may cause oxidative stress and oxidative damage to the liver and intestines (Deng *et al.,* 2017; Joeng *et al.,* 2016; Kyun-Woo *et al.,* 2013).

The first reported case of a vertebrate ingesting a piece of plastic from the ocean was in the early 1970s, with the discovery of plastic particles in the larvae, juvenile, and adult pelagic and demersal fish. In 1990, Hoss and Settle (1990) conducted an experiment to study the interaction between microplastics and marine biota in which 5 out of 6 species of fish ingested plastic pellets ranging in size from 100–500 μm. The initial focus of this study was only to assess potential physical blockages in the fish. However, currently it is known that persistent, bioaccumulative, and toxic hydrophobic pollutants in the ocean are capable of adsorbing onto plastic debris, affecting the health of marine biota and the entire food web (Cole *et al.,* 2011; Thompson *et al.,* 2009).

Due to limited plastic removal strategies, ingestion and biomagnification of plastics present a large concern for marine biota as they may consume it at any level throughout the ocean (Figure 8) (Barnes *et al.,* 2009; Ologbonjaye *et al.,* 2019). On the surface and in the photic ocean layer, plastics are distributed similarly to phytoplankton, and can be mistakenly consumed (Andrady, 2017). On the seafloor, aggregated plastics have been found in the sediment, where they can also be mistaken for food (Cole *et al.,* 2011).

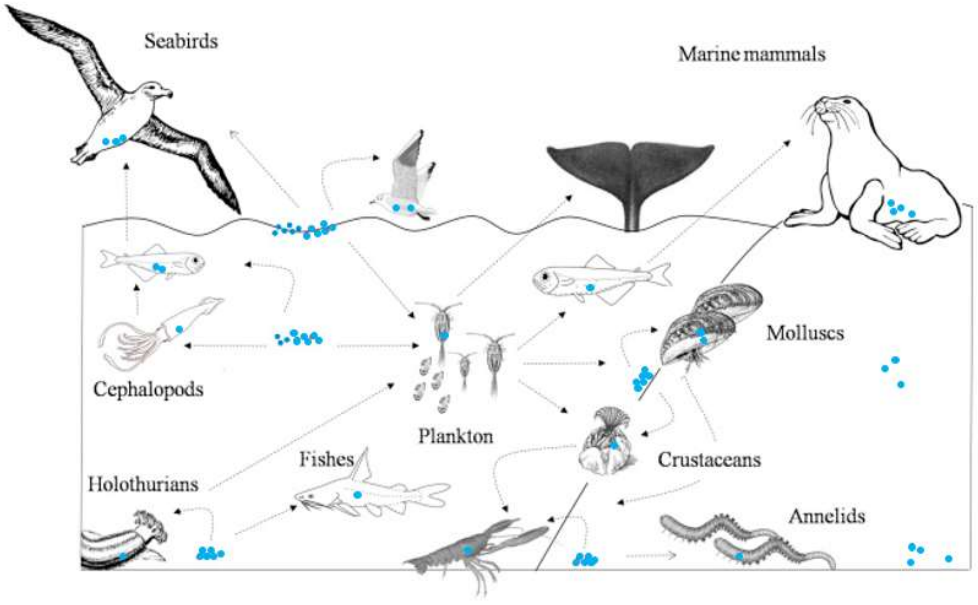

FIGURE 8. POTENTIAL INGESTION AND BIOMAGNIFICATION RISKS (ADAPTED FROM SUL & COSTA, 2014)

Inside an organism, NPs can cross the blood brain barrier (BBB) and enter different organs and tissues due to their small size (Costa *et al.,* 2016; Von Moos *et al.,* 2012). This crossing has caused observable behavioral disorders in fish, (Mattson *et al.,* 2017) and can lead to further morphological disorders (Kik *et al.,* 2020). After exposure to 30 nm PS NPs, the blue mussel,

Mytilus edulis, reduced its filtering activity and increased its production of pseudofeces (Wegner *et al.,* 2012).

Exposure to certain chemicals can have sublethal effects that alter an organism's behavioral, morphological, or reproductive systems (Costa *et al.,* 2016). Plastic Particle Toxicity (PPT) can occur in marine invertebrates, and depends on a particle's size, concentration, exposure time, and polymer type (Kögel *et al.,* 2020). Common symptoms of PPT are increased stress and reduced growth, energy, movement, hormonal regulation, and development in organisms (Kögel *et al.,* 2020). Exposure to such contaminants can induce oxidative stress, which can lead to oxidative damage (Li *et al.,* 2020), cellular damage, and an increased chance of developing neuronal disorders (Prüst *et al.,* 2020). Concerns about long-term toxicity effects surrounding PPT arise when chemicals from the NPs leach into the tissues and cells of organisms (Costa *et al.,* 2016).

Although not much is known about invertebrates, plastic particles with diameters less than 10 μm can penetrate organisms through their skin, respiratory system, and digestive track (Kik *et al.,* 2020; Tallec *et al.,* 2018) and accumulate in alveoli (Hoet *et al.,* 2004), which can result in consequences to the reproductive ability, fitness, and success of certain species (Gallo & Tosti, 2019).

1.5 Bivalves as Bioindicators

Filtering organisms are highly susceptible to pollution (Staichak *et al.,* 2021). Specifically, marine invertebrates play an important role in ecosystem biodiversity and can be applied in the assessment of marine pollution caused by emerging contaminants (EC) due to their ability to filter large quantities of water (Deidda, 2021). Sessile bivalves are used to assess environmental quality, particularly due to their filter feeding habits (Fabra *et al.,* 2021). Certain species of mussels are capable of filtering between 25 and 50 L of water through their gills per day and can be used to monitor their surrounding environments (Bottollier-Depois, 2019; EPA, 2014). Because bivalves can accumulate plastics from the environment, they have been used to draw attention to the health effects from human consumption of filtering organisms (Tomazelli *et al.,* 2003). A study by Wang *et al.,* (2020) found accumulated plastic particles in the digestive track, rather than the gills, indicating translocation of particles within the organism, with potential to travel up the food web (Staichak *et al.,* 2021). This is a growing concern as most seafood (mollusks, oysters, mussels, scallops, and clams) consumed by humans are filtering organisms (Ding *et al.,* 2020; Tomazelli *et al.,* 2003), highlighting shellfish consumption as an important route of MP and NP exposure to humans (Cho *et al.,* 2019).

Common characteristics of successful bioindicators are short life cycles, easily measurable responses, dose-dependent behavior to pollutant exposure, sensitivity to pollution, and a link to adverse health effects (Serafim, 2019). Bivalves are used to monitor contaminant exposure through cellular, biomechanical, or molecular alterations, which can be measured in tissues, cells, or fluids (Hulka, 1990; Nikinmaa, 2014).

Bioindicators may also provide a delineation of events between exposure and disease, establish a measurable dose response, identify mechanisms where exposure and disease are related, reduce misclassification of exposures or risk factors and disease, and enhance individual and group risk assessments (Mayeux, 2004). Although proficient at detecting and responding to single pollutants,

bioindicators are not sensitive enough in areas with mixed pollutants, as mixtures of different chemicals can have different effects (Mayeux, 2004). Bioindicators are used for both monitoring disease progression and risk prediction (Mayeux, 2004). In this study, bioindicators will be used to monitor both disease progression during exposure of NPs, and risk prediction, during the depuration phase.

Mussels have been used in a variety of studies to monitor MPs in soft tissues, feces and pseudofeces (Staichak *et al.,* 2021). Their simple anatomy allows for specific studies to isolate the digestive glands, gills, gonads, or blood. Additionally, their nervous system, which is composed of neuronal and glial cells, exhibits similarities with vertebrate nervous systems in biochemical and functional ways (Diedda, 2021). Factors such as neuroinflammation, oxidative stress, and altered levels of neurotransmitters are all neurotoxic effects that occur in both humans and marine invertebrates such as mussels (Diedda, 2021). As a result of consuming MPs, mussels may also experience physiological responses, histological changes, energy alteration, genotoxicity and transcriptional responses, and neurotoxic effects as well as effects on the immune systems (Li *et al.,* 2019).

As suspension feeders, bivalves are capable of ingesting nano and micro size particles (Canesi, 2012). This characteristic enables bivalves to be one of the species most impacted by plastic pollution (Ward *et al.,* 2019). Concern has recently been drawn to the internalization, retention time, and effects of MPs and NPs in aquatic biota (Sendra *et al.,* 2021). A study by Browne *et al.,* (2008) showed that *Mytilus edulis* ingested and accumulated MPs in the first 12 hours of the experiment, and Álvarez-Ruiz & Campo (2021) found most of the ingestion occurred within the first 48 hours of exposure to pollutants.

Mussels have recently been observed regulating their filtration rates based on stress intensity (Li *et al.,* 2021). This behavior has been observed during uptake, translocation, and elimination processes of MPs including ingestion, adherence, and fusion (Kolandhasamy *et al.,* 2018; Li *et al.,* 2019b). Li *et al.,* (2021) suggests mussels have selective uptake techniques regarding MPs due to the difference in MP levels in mussel tissues and the marine environment. Upon ingestion, MPs enter the mussels through the siphon and either assimilate over the gill epithelium or are transported to the digestive system via microvilli activity and endocytosis (Von Moos *et al.,* 2012). Other organs, including the gonad, mantle, adductor, visceral, and foot, are controlled by the adherence of MPs (Kolandhasamy *et al.,* 2018). Selective elimination of MPs occurs through excretion and difference in retention time of various MPs in mussels (Li *et al.,* 2021). In some cases, mussels have produced pseudofeces using a specialized method of expulsion to reject plastic before entering the digestive tract (Lusher, 2015), but this can lead to starvation over time due to the extra energy required to coat the plastics in mucus (Wegner *et al.,* 2012).

1.5.1 The Use of Mussels *Mytilus galloprovincialis* as Sentinel Species

Mussels have a wide geographical distribution, proving they are very resilient in different environments (Bottollier-Depois, 2019). *Mytilus galloprovincialis* (Figure 9) is a filter-feeding bivalve native to the Atlantic shores of the Iberian Peninsula (Veiga *et al.,* 2020), found along rocky coasts, sheltered harbors, and estuaries, such as in intertidal zones, to depths of 40 m (Bonham *et al.,* 2021). Their solid shells are variable in color and shape, but tend to be dark blue,

purple, or brown (Anacleto *et al.,* 2016). Although adults can reach up to 15 cm long, most grow to between 5 and 8 cm (Anacleto *et al.,* 2016).

FIGURE 9. M. GALLOPROVINCIALIS

The average lifespan for *M. galloprovincialis* is 8 years, allowing for stable populations that permit repeated sampling (Serafim, 2019). Additionally, *M. galloprovincialis* are easily collected and maintained, allowing for easy transport and handling (Serafim, 2019).

Due to its ability to reproduce quickly, tolerate a wide range of environmental conditions, and outcompete native mussels, *M. galloprovincialis* is considered an invasive species (Bonham *et al.,* 2021). Although it is cultivated in 19 different countries, including Australia, Albania, Bulgaria, Canada, China, Croatia, Egypt, France, Greece, Italy, Morocco, Portugal, the Russian Federation, Spain, South Africa, Turkey, the United States, Ukraine, the former Yugoslavia, and Scotland, it can also be found along the pacific coast of North America, Japan, Hong Kong, South Africa, Chile, and Australia which allows for comparison between different locations (Bonham *et al.,* 2021).

M. galloprovincialis is used as a sentinel species because of its high levels of consumption and commercial value, contributing to public health importance (Serafim, 2019). Additionally, they can easily bioaccumulate dissolved substances in their gills (Gomes *et al.,* 2012). Gills are the main tissue involved in filtration and are most frequently in direct contact with contaminants in the water. Mussels are also susceptible to oxidative stress induced by the accumulation of contaminants in their gills, digestive glands, and gonads (Ribeiro *et al.,* 2017). Although contaminants accumulate in the gills, they are stored in the digestive gland, which makes it possible to study the concentration of contaminants both during periods of contamination and recovery. Common ecotoxicological effects in bivalves range from physical damages such as weight loss, reduced feeding activity, and growth inhibitions, to metabolic disorders, oxidative stress, genotoxicity, lysosomal perturbations, and inhibition of AChE activity (Canesi *et al.,* 2012; Fabra *et al.,* 2021; Foley *et al.,* 2018).

1.6 Acetylcholinesterase (AChE) Activity and Neurotoxicity

Neurotoxic responses are measured via enzymatic activity of acetylcholinesterase (AChE) in the gills. AChE is a serine protease, whose main function is to inactivate acetylcholine (ACh) after its nerve signal transfer by cleaving ACh molecules into acetate and choline, through hydrolysis. Cholinesterase (ChE) enzymes inside the nervous tissue, muscles, and red cells in the CNS, catalyze the hydrolysis of ACh (Dingová & Hrabovská, 2015). During normal transmission, nerve impulses cause the release of ACh from the presynaptic membrane to postsynaptic membranes. As ACh passes between the pre and post synapsis, it binds to the cholinergic receptors on the postsynaptic membrane and the ACh relays a message by stimulating a transmission of impulse (Figure 10). Once the message has been conveyed, the ACh leaves the receptor and returns to the presynaptic membrane.

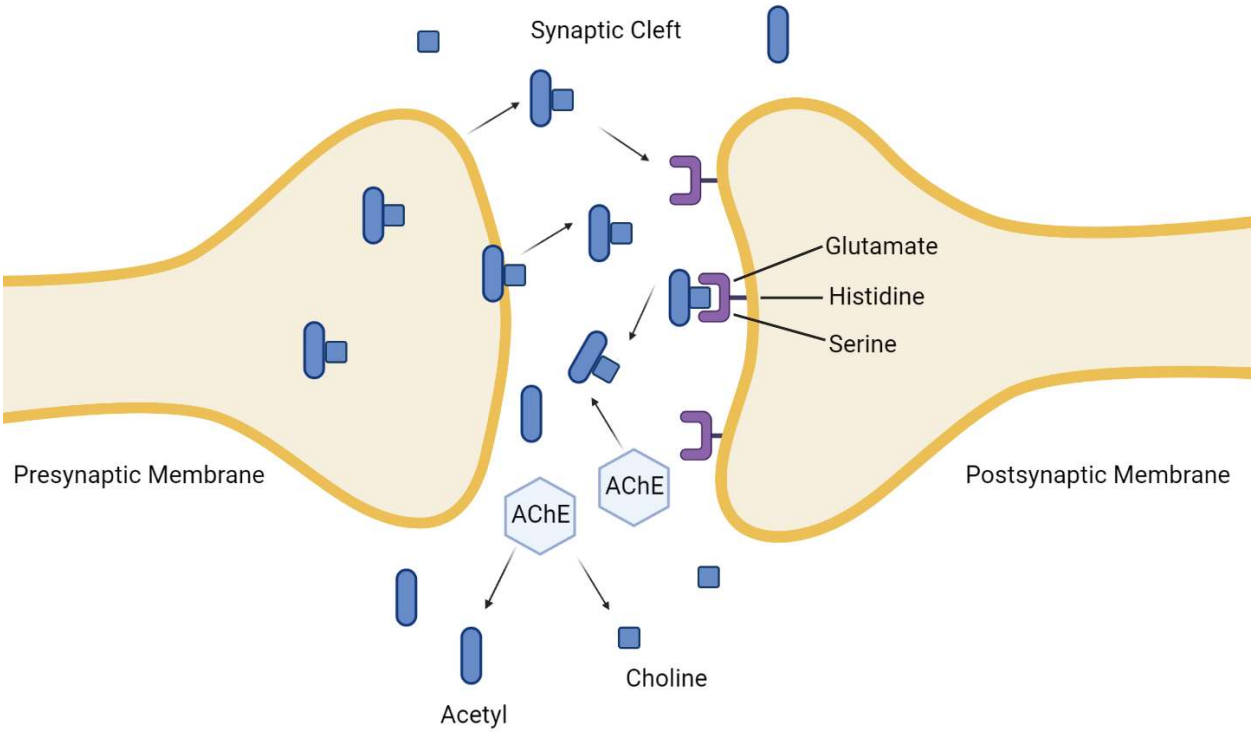

FIGURE 10. ACHE CLEAVING ACH INTO ACETYL AND CHOLINE (ADAPTED FROM STANCIU *ET AL.*, 2019)

Termination of the response occurs through the cleaving of ACh by the action of AChE. The binding site where the cleaving action takes place is at the catalytic triad, a molecule composed of 3 amino acids; Glutamate, Histidine, and Serine (Colović *et al.,* 2014). While the serine binds with the acetate, the choline molecule diffuses away. Water, which is present in the environment, donates a proton to serine, causing a new bond to form. This reaction causes the bond between carbon and acetate to break, and acetate forms a new bond with the oxygen from the water molecule. From here, the acetate diffuses away from the active site, restoring the enzyme to its initial reactive state (Figure 11).

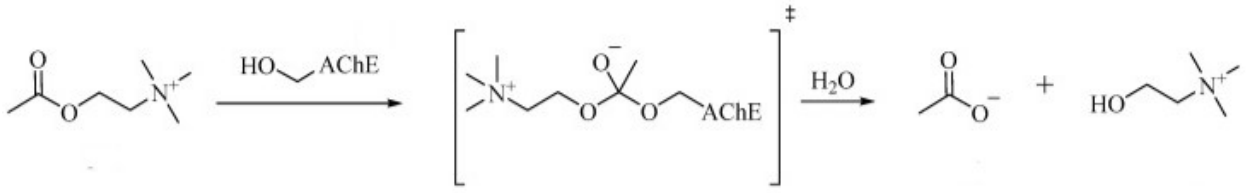

FIGURE 11. HYDROLYSIS OF ACH DUE TO ACHE (CAVALCANTE *ET AL.*, 2020)

In the presence of an AChE inhibitor, the acetylcholine is unable to be cleared, leading to cholinergic excess (Figure 12).

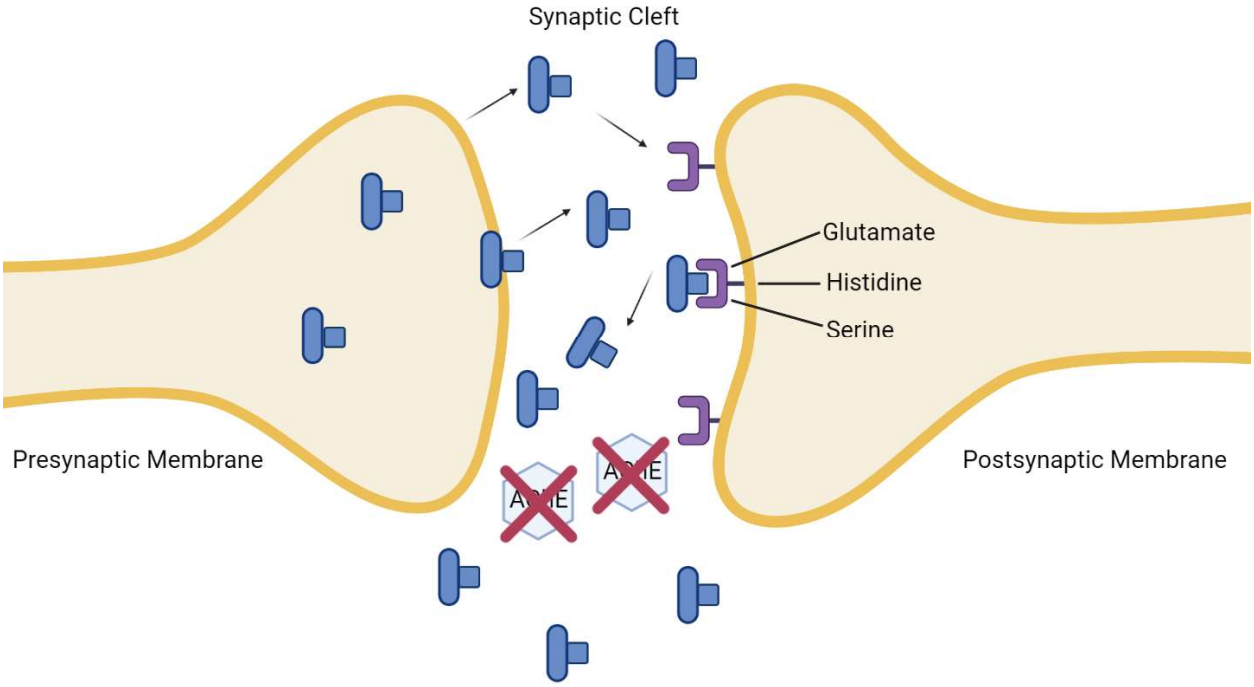

FIGURE 12. TRANSMISSION MECHANISM OF ACHE WITH INHIBITION (ADAPTED FROM STANCIU *ET AL.*, 2019)

AChE has commonly been used as a biomarker to study organophosphate pesticides in the tissues of aquatic invertebrates (Bocquené & Galgani, 1990; Cavalcante *et al.,* 2020; Campillo *et al.,* 2013; Perić *et al.,* 2017). It has been found that plastic particles inhibit AChE activity, thus altering neurotransmitter levels, and changing behavior in certain aquatic invertebrates (Yong *et al.,* 2020). Biomarkers are defined as "cellular, biochemical, or molecular alterations that are in biological media such as tissues, cells, or fluids that can be measured as indicators of biological, pathogenic, or pharmacological responses to a therapeutic intervention" that can act as early warning signals of environmental damage (Lionetto *et al.,* 2013). These early warnings, caused by contamination, may impact entire populations and effect reproduction (Canesi, 2012; Lionetto *et al.,* 2013).

1.7 Hypothesis and Objectives

A wide array of research has been conducted regarding the biochemical effects triggered by PS microplastics in marine biota. Data gathered has proven the relevance of marine mussels as a good sentinel species in the assessment of MPs, particularly showing the potential of these contaminants of emerging concern (CECs) to trigger neurotoxic responses. Because the impacts caused by NPs have not yet been uncovered, the focus of this study is to assess the neurotoxic effects of PS NPs in the marine mussel *M. galloprovincialis* after 21 days of exposure. Moreover, the present study also aimed to investigate the potential recovery of mussels after a 14-day depuration phase. To accomplish this goal, the inhibition of the AChE enzyme was monitored to determine the neurotoxicity caused by the long-term exposure of marine mussels to NPs (10 μg L^{-1}: 55 nm]. Current MP research supports the present hypothesis that mussels *M. galloprovincialis* subjected to a 21-day exposure to nanosized plastics may result in neurotoxic effects. In addition, this work was supposed to quantify the amount of NP ingestion during the experiment but due to the pandemic of Covid-19 this part of the work was not accomplished.

Chapter 2. Materials and Methods

2.1 NP Characterization

10 mL of fluorescent Flouresbrite Plain YG 0.05 Micron Microspheres (9003-53-6) were purchased from Polysciences Inc (CAT# 17149-10). To measure the hydrodynamic diameter of NPs in ultrapure water (7732-18-5) and filtered seawater (S=36.5 ± 0.75), at 25 ºC, a particle sizer (ZetaSizer Nano ZS90, Malvern Inc.) was used, assessing the NP diameter in suspensions over a period between 2 and 12 h. Aggregate kinetics were evaluated by using DLS, with a 50 second time gap between the beginning of aggregation and data collection. These commercially available NPs (Lot#A780141) arrived in an aqueous suspension (2.6%) with the following properties (Table 1) and kept under 4 °C.

TABLE 1. FLOURESBRITE NP PROPERTIES

Flouresbrite Plain YG 0.05 Micron Microsphere Properties	
Concentration	3.64 x 10^{14} particles mL^{-1}
Diameter	55 nm
Density	1.05 g cm^{-1}
Excitation Maximum	441 nm
Emission Maximum	486 nm

2.2 Experimental Design

M. galloprovincialis (n = 320) were collected from the Ria Formosa Lagoon (Faro, Portugal; 37°00'30.6"N 7°59'39.6"W) (Figure 13), during the low tide, and immediately transported alive to the laboratory, where individuals were acclimated over 7 days in water with relatively consistent physical and chemical conditions.

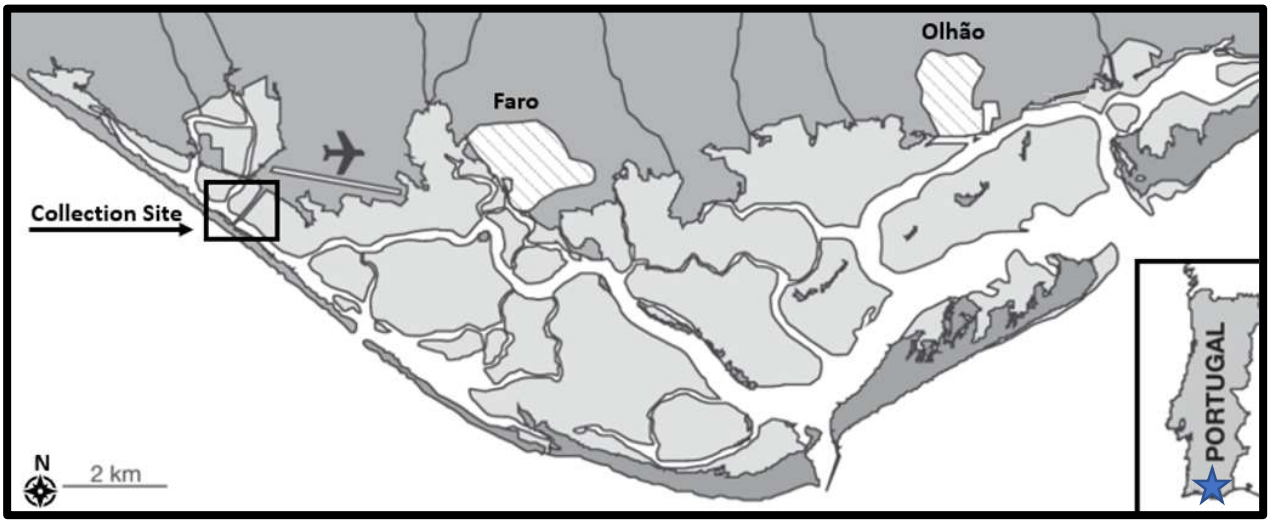

FIGURE 13. MAP OF COLLECTION SITE (ADAPTED)

Upon mussel collection, temperature (20 °C), pH (7.9), and salinity (45) values were registered at the natural site with the aid of a multiparametric probe (Odean Range Probe) and refractometer (Figure 14).

FIGURE 14. ODEAN RANGE PROBE (LEFT) AND HANDHELD REFRACTOMETER (RIGHT)

Before being placed into aquariums, all mussels were lightly scraped off using a metal shucker to eliminate any interference on NPs ingested by barnacles. Mussels were evenly distributed into 8 25 L decontaminated glass aquariums (Figure 15), filled with 20 L of natural seawater (2 mussels L^{-1}), set under constant aeration using glass pipettes with O_2 values of 8.9 mg L^{-1} ± 0.3, water temperature at 20.4 °C ± 1.2°C, a pH of 8.1 ± 0.2, and a salinity of 44.3 ± 2.9. Artificial light was provided by ceiling lights with a photoperiod of 12 h light and 12 h darkness. Every 2-3 days, all 8 aquariums were completely drained and then refilled using filtered seawater piped directly from the Ria Formosa. Water drained from the CT aquariums was disposed of on site, and water drained from the NPs aquariums was collected for future studies. The SW piped directly from the Ria Formosa Lagoon provided enough nutrients for mussel maintenance during the experiment.

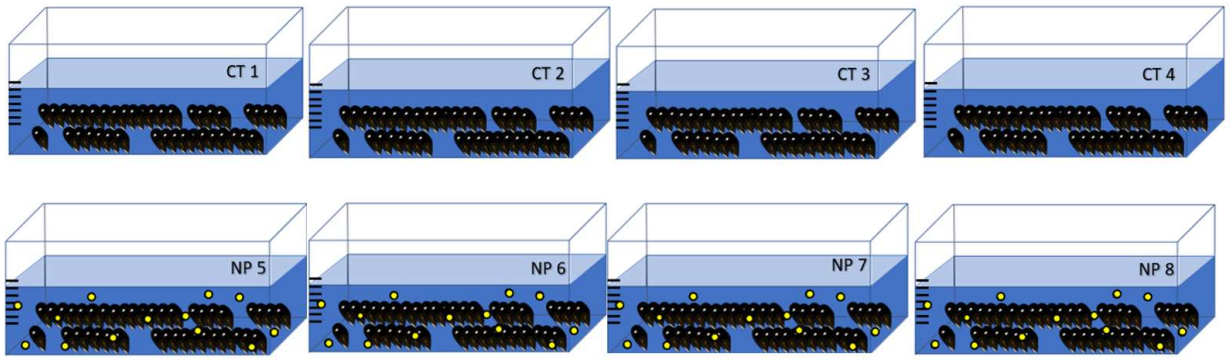

FIGURE 15. INITIAL AQUARIUM SETUP

Two treatment groups were established, in quadruplicate: the control and the NP systems contaminated with an aqueous concentration of [10 µg L^{-1}; 55 nm] NPs. The exposure part of the experiment was carried out over 21 days, followed by 14 days of depuration, when no NPs were added. The entire experiment lasted for 35 days. Natural seawater from the Ria Formosa was renewed every 48 hours and NPs were pipetted into aquariums 4-8 by a micropipette from the NPs stock solution. Mussels were collected from the systems at the following days: day 0 (beginning of the experiment), 7, 14, 21 (exposure period), and 28, and 35 (depuration period).

Throughout the whole bioassay, a constant ratio of 2 mussels L^{-1} of seawater was maintained in the aquarium, such that the nominal concentration of NPs was achieved. At a stock concentration

of $3.64*10^{14}$ particles per 1 µL stock solution, calculations were performed to determine the volume of NPs needed to add to each tank (Equation 1).

EQUATION 1. CALCULATIONS FOR PS NPS

$$\frac{PS\ Particles}{(L)} = \frac{6W * 10^{12}}{\rho * \pi * \omega^3} * 1000$$

Where:

$$W = \left(\frac{PS\ (g)}{latex\ (ml)}\right)$$

$$\rho = Density\ of\ PS\ \left(\frac{g}{mL}\right)$$

$$\varphi = Diameter\ of\ latex\ particles\ (\mu m)$$

To attain this, every time mussels were removed from the aquariums, the ratio of mussels to SW and SW to NPs was preserved by decreasing the volume of SW to create the contamination schedule (Table 2). To avoid cross contamination of the exposure systems to plastics other than the NPs supplied from Polysciences Inc, the use of gloves and other plastic items were restricted. NP-contaminated water from aquariums was retained for proper disposal.

TABLE 2. EXPERIMENTAL SCHEDULE (PER AQUARIUM) FOR CT AND NP AQUARIUMS

	Acclimation Period	Exposure			Depuration		
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
CT Mussels Remaining	40	35	30	25	20	15	10
NPs Mussels Remaining	40	35	30	25	20	15	10
Volume of SW to add (L)*	20	20	17.5	15	12.5	10	N/A
Volume of NPs to add (µl/L)**	N/A	6	5.25	4.5	N/A	N/A	N/A

*To maintain the ratio of 2 mussels L^{-1}

** To maintain the concentration of 10ug PS NPs L^{-1}

2.2.1 Mussel Dissection

Dissections were performed in a sterilized lab setting on days 0, 7, 14, 21, 28, and 35. Dissections took place in glass petri dishes using a 24-gauge metal scalpel and metal tweezers (Figure 16). All equipment was sterilized using 70% alcohol between the dissection of each mussel.

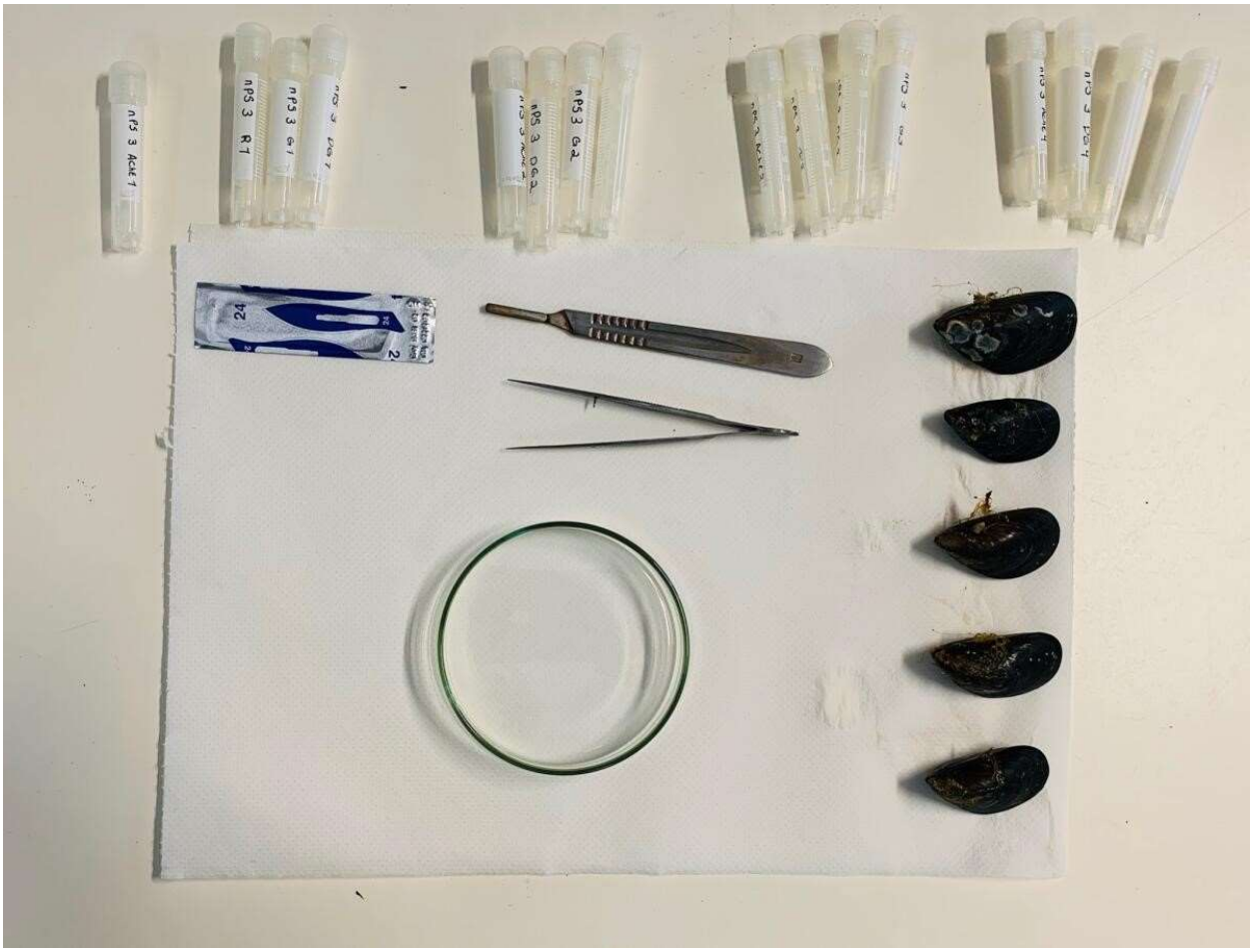

FIGURE 16. DISSECTION DAY LAB SETUP

Five mussels were collected from each aquarium (n = 20, per treatment) and the gills (Figure 17) dissected from each mussel were collected and put into individual microtubes, which were immediately submerged in liquid nitrogen and stored in a freezer at -80 °C, until the AChE analysis. One dead mussel was found during dissection at day 0 in a CT aquarium. This mussel was replaced with an extra mussel from the same aquarium.

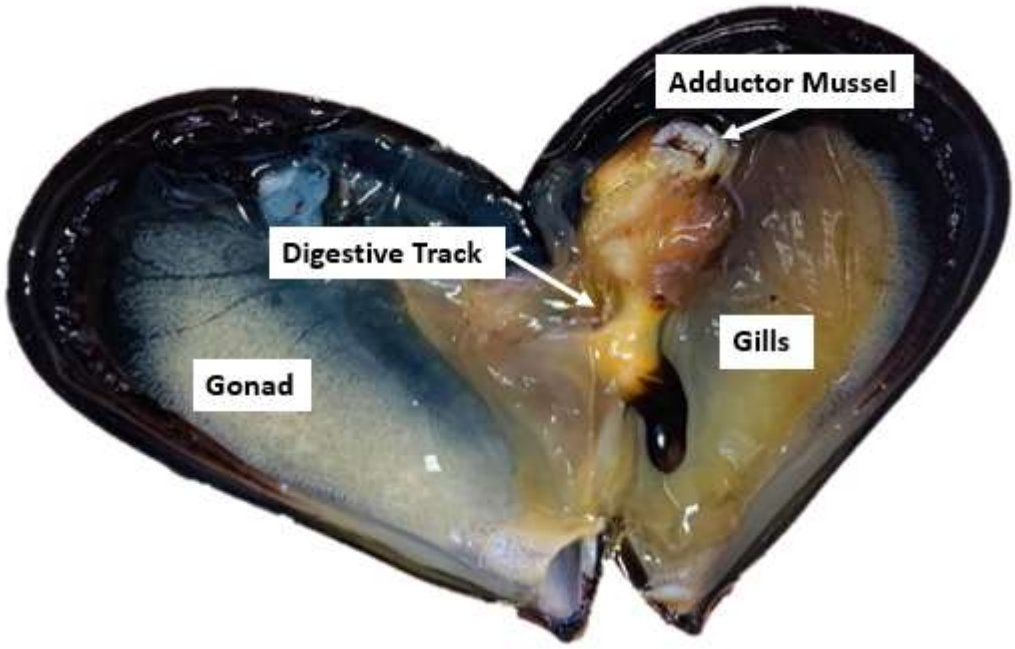

FIGURE 17. M. GALLOPROVINCIALIS READY FOR DISSECTION

2.3 Tissue Preparation and Homogenization

To prevent temperature from affecting enzyme activity, the experiment was carried out in low-temperature conditions and samples were kept on ice. After slowly thawing, gill samples from days 0, 7, 14, 21, 28, and 35 were individually homogenized with 100 mM Tris-HCl buffer containing Tris-(hydroxymethyl)-aminomethane ($NH_2C(CH_2OH)_3$) and Milli-Q water (pH 8.0; 0.1% Triton). After 7 minutes of homogenization (VWR Star-Beater), samples were centrifuged at 12000 *g* for 30 minutes, at 4˚C. Cytosolic supernatants were evenly distributed into two microtubes, whereby aliquots (150 µL) were separated for total protein (TP) and AChE experiments.

2.4 Total Protein Determination

The concentration of total solubilized proteins was quantified in gills from *M. galloprovincialis* according to the Bradford Assay (Bradford *et al.,* 1976), through a colorimetric method that uses bovine serum albumin (BSA) as a standard (1 mg mL^{-1}). The Bradford Method, adapted for the microplate reader, relies upon the addition of the acidic dye Coomassie Brilliant Blue G-250 to the protein solution, yielding a visible color change according to the protein content, within the absorbance range of 470-595 nm. Once the dye binds to a protein, it will be converted into a stable blue solution, at an absorbance of 595 nm (Reisner *et al.,* 1975).

Briefly, a standard BSA solution of 1.0 mg BSA mL^{-1} was prepared, followed by serial dilutions for the preparation of standard concentrations for the calibration curve, from 0.005 to 1.0 mg BSA mL^{-1}. One part of Dye reagent concentrate (BioRad; catalog number 500-0006) was added to four

parts of distilled water. Further, aliquots of BSA standards and tissues samples were inserted in 96-well microplates, followed by the addition of Bradford Dye reagent. After a 20-min incubation, the absorbance at 595 nm was read using a microplate reader (Infinite ® 200, Pro-Tecan), and was directly proportional to the amount of bound protein. Protein concentrations were expressed as mg g^{-1} wet weight tissue. Using a graphing program, a linear equation was created from the data to form a calibration curve.

After a standard curve was created to determine the sample concentration, TP was calculated using the following formulas (Equation 2) (Equation 3).

EQUATION 2. TP CONCENTRATION CALCULATION

$$TP = C\ x\ \frac{V_B}{W_T}$$

Where:

$TP = Total\ Protein\ \left(\frac{mg}{g}\right)$
$C = Concentration\ (\frac{mg}{g})$
$V_B = Buffer\ Volume\ (ml)$
$W_T = Weight\ of\ Individual\ Gill\ Tissue\ (g)$

EQUATION 3. CALCULATING TP

$$|TP| = \frac{|Abs - b|}{a} * Df$$

Where:
$TP = Total\ Protein$
$Abs = Absorbance$
$a = slope\ coefficient\ obtained\ from\ Bradfor\ Method$
$b = y - intercept\ coefficient\ obtained\ from\ Bradford\ Method$
$Df = Dilution\ factor$

2.5 Neurotoxicity (AChE)

For both treatments, AChE activity in the gills of *M. galloprovincialis* was used to assess the neurotoxicity according to Ellman's colorimetric method (Ellman *et al.,* 1961) in triplicate. This method relies on measuring the rate of production of thiocholine as acetylthiocholine becomes hydrolyzed by AChE. Thiocholine from the sample reacts upon the addition of the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce 5-mercapto-2-nitrobenzoato (Colovic *et al.,* 2014). To test AChE activity, samples were first slowly thawed over ice and then pipetted into a 96-well microplate with 200 µl of DTNB and 50 µl of AChE buffer (Tris-HCl; 100 mM; pH 8). After incubating for 5 minutes, 50 µl of the catalyst solution, ATC (Acetylcholine & Milli-Q water), was added to each well. After an additional 10 minutes of incubation at room temperature, absorbance

was then measured at 412 nm for 5 minutes, in intervals of 30 seconds, at room temperature (Ribeiro *et al.,* 2017). The results were expressed as nmol min^{-1} mg^{-1} of protein.

2.6 Condition Index

The condition index (CI) was used to estimate the physiologic status of the mussels with respect to treatment, time of exposure, and depuration (0, 7, 14, 21, 28, and 35 days) (n=15 per treatment). The CI (%) was calculated using the ratio of the drained weight of soft tissues (g) and the total weight of each mussel (soft tissue and shell) (g), as described by Gomes *et al*. (2013).

2.7 Statistical Analysis

Statistical analyses were carried out using the software GraphPad Prism 9. Data was checked for assumptions of normality and homogeneity of variance by using the Shapiro-Wilk and Levene's tests, respectively. Once assumptions were met, experimental data was subjected to one-way analysis of variance (ANOVA), followed by the post-hoc multicomparison Tukey test. Results were significant when $p < 0.05$.

Chapter 3. Results

3.1 NP Characterization

Characteristics of 55 nm PS were investigated using the hydrodynamic diameter and ζ-potential with measurements every 50 seconds in ultrapure and filtered seawater, for a period between 2 and 12 h. The average size of NPs used was approximately 25 nm, less than the size reported by Polysciences. Results show that in ultrapure water the size of PS NPs was consistent, suggesting the NPs did not aggregate in these conditions, and the NPs were stable. This confirmed the ζ-potential to be approximately -68.8 ± 0.66 mV, which aligns with what was expected from the hydrodynamic diameter of the NPs. Upon dispersal in filtered seawater, the size of the NPs increased (852 ± 103 nm), confirming the occurrence of aggregation because of the ionic strength in filtered seawater. Under these conditions the ζ-potential was very low (-0.068 ± 0.23 mV), confirming the occurrence of aggregation.

3.2 Condition Index

No significant alterations were registered in the CI over the 21 days of the bioassay (Table 3), either in the CT ranging from 18.40 to 26.74 ($p > 0.05$) or from mussels exposed to NPs, ranging from 26.49 to 43.58 ($p > 0.05$), respectively. However, there was a significant difference between the CI from organisms collected under exposure to NPs and the depuration time, specifically NP3 vs NP21 and NP3 vs NP28 ($p < 0.05$). The high standard deviation can be attributed to various calibration errors on the lab scale during the second half of the experiment.

TABLE 3. CONDITION INDEX (MEAN ± S.D.) OF *M. GALLOPROVINCIALIS* MASS EXPOSED TO NPS DURING CONTAMINATION (RED), DEPURATION (GREEN), AND THE LAST DAY OF CONTAMINATION (RED WITH GREEN HASHES). N = 20.

Time (day)	CT (g)	NPs (g)
0	20.39 ± 2.75	27.99 ± 2.92
7	19.66 ± 2.70	26.93 ± 4.48
14	18.40 ± 3.01	26.49 ± 6.04
21	26.74 ± 5.41	43.58 ± 24.62
28	25.70 ± 4.72	42.85 ± 22.97
35	23.15 ± 4.21	41.17 ± 23.68

3.3 Acetylcholinesterase Activity

Findings from the present study showed no alterations in AChE activity assessed in control groups, except for the last day of sampling (T35), which indicated its decline, creating significant differences compared to CT0 (Aa; 35.35 nmol ACTC min^{-1} mg $prot^{-1}$) and CT7 (Aa; 34.64 nmol ACTC min^{-1} mg $prot^{-1}$) (Figure 18). Regarding NP exposure, mussels experienced a significant 4-

fold decrease between T7 (Aa) and T21 (Bb), by dropping from 27.81 (nmol ACTC min^{-1} mg $prot^{-1}$) to 6.93 (nmol ACTC min^{-1} mg $prot^{-1}$).

No statistical differences occurred in the contaminated mussels between T14 (Bb; 6.93 nmol ACTC min^{-1} mg $prot^{-1}$), T21 (Bb; 5.63 nmol ACTC min^{-1} mg $prot^{-1}$), T28 (Bb; 3.41 nmol ACTC min^{-1} mg $prot^{-1}$), and T35 (Bb; 2.48 nmol ACTC min^{-1} mg $prot^{-1}$). However, there were statistical differences between the CT mussels between T14 (Aab; 29.70 nmol ACTC min^{-1} mg $prot^{-1}$), T21 (Aab; 24.95 nmol ACTC min^{-1} mg $prot^{-1}$), T28 (Aab; 20.83 nmol ACTC min^{-1} mg $prot^{-1}$), and T35 (Ab; 15.28 nmol ACTC min^{-1} mg $prot^{-1}$).

On the 14th day of the bioassay, mussels' gills exposed to NPs showed a significant and steep decrease in AChE activity regarding the respective time-control group ($p < 0.05$), which is different from the enzymatic profile registered in mussels exposed to NPs at the 7th day ($p < 0.05$). On the following contamination day (T21) and depuration days (T28, T35), AChE activity kept decreasing in mussels (5.63 nmol ACTC min^{-1} mg $prot^{-1}$, 3.41 nmol ACTC min^{-1} mg $prot^{-1}$, 2.48 nmol ACTC min^{-1} mg $prot^{-1}$) previously exposed to NPs, maintaining the significant difference from CT0 and CT7, although no significant differences were detected between NP-treated mussels ($p > 0.05$). Results from the mediated toxicity demonstrated that PS NPs, at a concentration 10 µg L^{-1}, significantly yielded neurotoxic responses in gills from *M. galloprovincialis*.

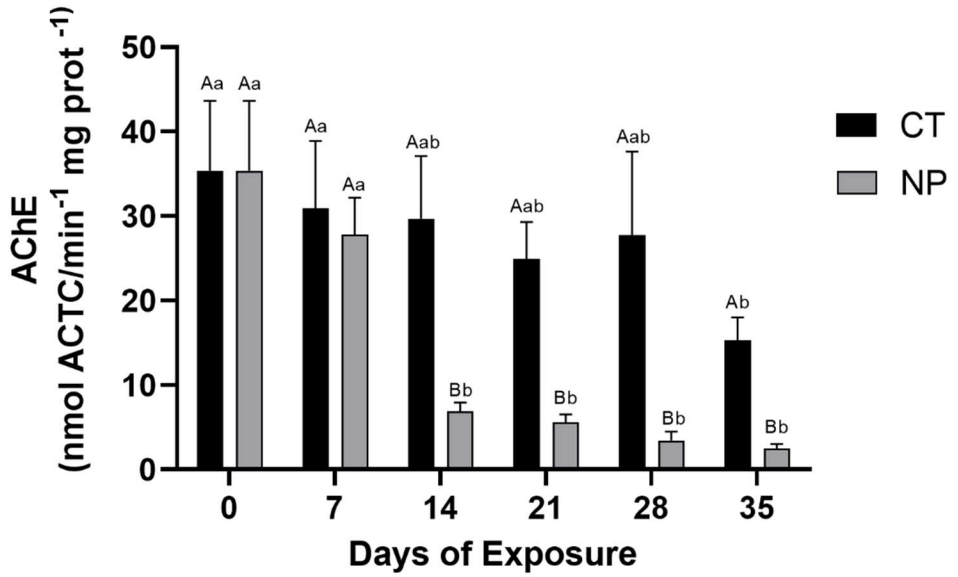

FIGURE 18. BIOCHEMICAL BIOMARKERS - ACHE ACTIVITY (MEAN ± SD) IN GILLS OF MUSSELS *M. GALLOPROVINCIALIS*, UNEXPOSED AND EXPOSED TO NPS (10 µG L^{-1}). CAPITAL AND LOWER LETTERS INDICATE SIGNIFICANT DIFFERENCES BETWEEN TREATMENTS AT EACH EXPOSURE TIME ($P < 0.05$).

Chapter 4. Discussion

As the COVID-19 pandemic surges on, many people are still reliant on SUPs such as masks and takeout containers in their everyday lives, further contributing to the global problem of plastic waste. The entrance of primary plastics to the ocean and the fragmentation and degradation of plastic debris in the seawater column have led to the ubiquitous occurrence and abundance of plastic particles in the marine environment (Andrady, 2011; Mattson *et al.,* 2018; Hernandez *et al.,* 2017) as MPs (1-5 mm) and NPs (1-100 nm) (Gigault *et al.,* 2018; Gonçalves & Bebianno, 2021). Buoyant plastics are carried by the current to gyres, and more dense plastics sink to the bottom of the seafloor, where they can either settle into the sediment, or continue to travel by underwater currents (Chen *et al.,* 2017).

In mammals, long term exposure to phthalates, which make plastic flexible, are also endocrine disrupting chemicals that affect neurodevelopment, which can lead to behavioral outcomes such as lower IQ, hyperactivity, problems paying attention, and poor social communication (Ejaredar *et al.,* 2015). Phthalates have strong estrogenic activity and can travel through biological barriers and enter breast milk (Ejaredar *et al.,* 2015). When aquatic organisms are exposed to NPs, this may induce oxidative stress, resulting in cellular damage and an increased probability of developing neuronal disorders and behavioral changes via the inhibition of AChE activity and altered neurotransmission levels (Prüst *et al.,* 2020).

There is a biological change in cells exposed to plastics that alter their metabolism (Legler *et al.,* 2021). It has been well documented that the exposure to MPs in the marine environment causes irritation and injuries to the digestive system which may lead to malnutrition and starvation in marine biota (Marques-Santos, 2018). Moreover, MPs have also led to behavioral changes, oxidative stress (Tlili *et al.,* 2020), oxidative damage, histopathological injuries, as well as changes in reproduction and genotoxicity, in a wide array of marine organisms, such as bacteria (Sun *et al.,* 2018), algae (Bhattacharya et al. 2010; Gonçalves & Bebianno 2021), rotifers (Manfra *et al.,* 2017), zooplankton (Lee *et al.,* 2013), mollusks (Wegner *et al.,* 2012; Brandts *et al.,* 2018; Yong *et al.,* 2020; Li *et al.,* 2020; Gonçalves & Bebianno, 2021; Ribeiro *et al.,* 2017), and fish (Almeida *et al.,* 2019; Lee *et al.,* 2019). After 14 days of exposure to 20 μm PS MPs [1mg L^{-1}] and 7 days of depuration, *Scrobicularia plana* exhibited effects such as antioxidant capacity, genotoxicity, neurotoxicity, and oxidative damage (Ribeiro *et al.,* 2017).

By ingesting plastics, bivalves and other marine organisms are exposed to chemicals which may lead to multigenerational consequences (Lee *et al.,* 2013). Neurotoxic effects in marine biota are heavily linked with the increase of AChE activity and changes in their behavior (Bhattacharys *et al.,* 2010; Li *et al.,* 2020). While the effects of microplastics on mammalian cells are well-studied, far less information is known about the potential biological and ecological consequences from the exposure of marine biota to NPs present in the marine environment (Canesi *et al.,* 2012; Della Torre *et al.,* 2014). As NPs accumulate in the marine environment, it is of the utmost importance to assess their effects on living organisms due to their ability to adsorb pollutants from within the environment (Lee *et al.,* 2019) as they can travel across cell membranes and tissues in invertebrates, putting all the organisms in higher trophic levels at risk of exposure to plastics, and

the chemicals they contain. There are not many studies relating AChE to NPs, emphasizing the importance of this study. In other marine species, alterations in AChE levels result in oxidative stress (Bhattacharys *et al.,* 2010; Li *et al.,* 2020), behavioral changes (Wegner *et al.,*2012), physiological changes (Brandts *et al.,*2018), reproductive changes (Lee *et al.,*2019), and even mortality (Lee *et al.,* 2013; Manfra *et al.,* 2017). This array of effects from ingesting plastics may cause many disruptions throughout the food web.

In marine algae (*Scenedesmus spp.),* after 24 h of exposure, PS NPs (20 nm; 4 mg mL^{-1}) were absorbed into the cellulose, which inhibited photosynthesis and caused oxidative stress (Bhattacharya et al. 2010). A significant mortality rate was registered for rotifers *Brachionus koreanus* exposed over 24 h to 50 nm PS NP, at a concentration of 10 µg mL^{-1} (Manfra *et al.,* 2017). In zooplankton, copepods (*Tigriopus japonicus*) exposed to 50 nm PS (1.25, 12.5, and 25 µg mL^{-1}) for 96 hours experienced a significant increase in mortality at 12.5 µg mL^{-1}, causing the subsequent generation to experience a decrease in survival at just 1.25 µg mL^{-1} (Lee *et al.,* 2013). Three species of mollusks including *Mytilus edulis* (Wegner *et al.,* 2012), *M. galloprovincialis* (Brandts *et al.,* 2018), and *Dreissena polymorpha,* (Li *et al.,* 2020), all experienced effects from PS NPs. When exposed to 30 nm PS particles [0.1, 0.2, and 0.3 g L^{-1}], *M. edulis* displayed a change in ingestion by reduced feeding and producing pseudofeces (Wegner *et al.,* 2012). In *M. galloprovincialis*, results showed a decrease in cholinesterase activity, inhibited neurotransmission, and an increase in oxidative stress, LPO, genotoxicity, and altered gene expression when exposed to 106 ± 10 nm PS at [0.5-50 mg L^{-1}] for 96 hours (Brandts *et al.,* 2018). After 96 h of exposure to 80 nm PS NP at [0.1, 1, 5 mg L^{-1}] in freshwater *C. fluminea*, several effects were noted including the accumulation of NP in several organs such as the mantle, liver, stomach, and gill, as well as an increase in oxidative stress and oxidative damage in all concentrations tested (Li *et al.,* 2020). Li *et al.,* (2020) demonstrated NP-induced damage to the liver and gut by studying the uptake and excretion of NPs (Li *et al.,* 2020). Furthermore, *C. fluminea* also experienced oxidative stress in the visceral mass, gill, and stomach, as well as an imbalance in the antioxidant system, leading to liver damage, neurotoxicity, and intestinal inflammation (Li *et al.,* 2020). The fish *Dicentrarchus labrax (DLB-1)* exhibited a decrease in cell viability after a 24 h in vitro exposure to 100 nm PS at [1 mg L^{-1}) (Alemida *et al.,* 2019). Lee et al (2019) noted the freshwater *Danio rerio* experienced higher mortality and deformation rates and a decrease in hatching rate when exposed to 50 nm PS [10 mg mL^{-1}] for 24 hours. It was also noted that NPs penetrated the chorion and developing embryo, and accumulated throughout the whole body, but was especially prevalent in lipid-rich regions (Lee *et al.,* 2019). Additionally, the exposure altered ROS and pro-inflammatory responses, and caused mitochondrial damage at subcellular levels (Lee *et al.,* 2019). ROS production, cell death, and pro-inflammatory responses were all enhanced due to the size and concentration of NPs, with smaller particles causing the most damage (Lee *et al.,* 2019).

After confirmed exposure, both NPs and MPs have been proven to climb up the food web via trophic transfer (Chae *et al.,* 2018; Farrell & Nelson, 2013; Mattsson *et al.,* 2015; Mattsson *et al.,* 2017). Firstly, Farrell & Nelson (2013) confirmed the trophic transfer of MPs from a bivalve (*Mytilus edulis)* to a crustacean (*Carcinus maenas).* After an exposure of 1 h to 500 nm PS MPs

to *M. edulis*, C. *maenas* was given 45 minutes to eat the mussels before being euthanized (Farrell & Nelson, 2013). Results from their study showed trophic transfer of PS MPs from a bivalve to a crustacean, and translocation to the hemolymph and tissues of *C. maenas* (Farrell & Nelson, 2013).

To study changes in behavior and metabolism of fish using trophic transfer of NPs, Mattsson *et al.,* 2015 contaminated algae (*Scenedesmus sp.)* with 24 nm and 27 nm PS particles [9.34×10^{12} particles L^{-1}], which were fed to zooplankton (*Daphnia magna*), that were then fed to top-consumer fish (*Carassius carassius)* [1×10^{13} particles L^{-1}]. As a result of the NP trophic transfer of NPs, *C. carassius* experienced significant effects on feeding and shoaling behavior as well as metabolism (Mattsson *et al.,* 2015). This time, the authors discovered NP particles in the brains of *C. carassius*, demonstrating another consequence of the uptake of NP through the food web (Mattsson *et al.,* 2017). The authors also noted smaller NPs at higher concentrations were more harmful, in that after 24 hours at [0.025 g L^{-1}], all *D. magna* were alive, however, at [0.075 g L^{-1}] all *D. magna* had died within 13 hours (Mattsson *et al.,* 2017). In 2018, Chae *et al.,* again, confirmed trophic transfer between marine organisms, this time with 4 species. Algae (*Chlamydomonas reinhardtii),* water flea (*D. magna)*, fish (*Oryzias sinensis)* and consumer-fish (*Zacco temminckii)* were tested using 57.29 nm, 57.45 nm, and 60.39 nm PS NPs [50 mg L^{-1}] (Chae *et al.,* 2018). Along with confirming trophic transfer, the authors were able to witness induced histopathological changes in the livers of exposed *Z. temminckiis*, as well as NPs that had entered the embryo and penetrated the yolk sacs of hatched juveniles (Chae *et al.,* 2018). Combined, these studies confirm the threat of NPs in the marine environment. Not only can NPs affect each level of the food web, but they can also enter at any level, and cause magnifying effects throughout the entire ecosystem. As filter feeders, *M. galloprovincialis* ingests organisms that also ingest and are affected by NPs, and themselves undergo chronic neurotoxic effects from NP pollution. Close to the bottom of the food web, other marine organisms are expected to consume NPs. Due to a maintained inhibition of AChE, it is expected that some NPs will bioaccumulate within *M. galloprovincialis*, causing NPs to travel up the food web, affecting every species as it travels, and becoming more concentrated in top predators.

Neurotoxicity is a noncancer endpoint used for risk assessments by regulatory agencies (Tilson *et al.,* 1995). The quantity of neurotoxicants that pose a risk for human health and ecosystems are increasing, as are the number of evaluations aimed at predicting and preventing harm to human populations (Legradi *et al.,* 2018). Neurotoxic pollutants such as inorganic metals, organometals, pharmaceuticals, and pesticides (Roberts *et al.,* 2015) may change an organism's ability to survive, reproduce, or adapt to shifting environmental conditions, which may reduce an individual's fitness and lead to population declines, therefore impacting entire ecosystems (Legradi *et al.,* 2018). Tilson *et al.,* (1995) estimated that 30% of all commercially used chemicals may be neurotoxic. Early identification of neurotoxicity enables early intervention and allows for improved outcomes and using biomarkers to study neurotoxicity allows for continual monitoring (Roberts *et al.,* 2015). Invertebrates and other marine organisms are commonly used in ecological studies regarding the nervous system to study pollutants that may be harmful to human health (Deidda *et al.,* 2021).

Mussels were exposed to 10 μg L^{-1} of PS NPs for 21 days before a depuration period of 14 days. Results showed that after a 21-day exposure, there are significantly lower levels of AChE activity in gills after the 21st day in the NP treatment, in comparison to respective controls, confirming a neurotoxic effect of PS NPs. Surprisingly, CT mussels had results reflecting inhibited AChE, though at much lower doses/amounts on day 35. This profile may be interpreted similarly to that of Fernandes *et al*. (2020), which presents a steep decrease in AChE between day 3 and day 7. In that study, the authors attribute their results to the absence of microalgae for the first few days of the experiment. However, Matozzo *et al*. (2019), also reported a sharp linear decrease of AChE in the CT group over 14 days when a constant supply of microalgae was available. This result could be attributed to preexisting NPs in the water used for the experiment. Conclusions based on this data validate the decrease in AChE activity of the CT treatment through the 35-day experiment. Inhibiting AChE may lead to an excess of ACh and a deficiency in choline. This imbalance may cause confusion in the transmission from nerves to muscles, leading to paralysis or death. If this were to occur in *M. galloprovincialis*, they would stop filtering as much water, leaving murkier water and making it difficult for primary producers to get energy from the sun, therefore affecting the entire ecosystem.

In summary, results obtained from this study confirm previous research suggesting that ingestion of PS NPs [10 μg L^{-1}] cause tissue-specific and time-dependent effects in *M. galloprovincialis* (Brandts *et al.,* 2018; Capolupo *et al.,* 2021; Gonçalves and Bebianno, 2021) such as AChE inhibition after 21 days of exposure and effects continue during a 14-day depuration.

Chapter 5. Conclusion

NPs found in the marine environment are becoming a more serious threat. Their small size and increased reactivity allow them to translocate through and internally disrupt organisms on a cellular level. NPs are of an increased concern because plastics containing new combinations of pollutants can be more harmful than individual pollutants. As more data becomes available on this EC, more research is being done to study the impact of NPs on human health through trophic transfer.

The results from the present thesis indicated neurotoxic effects after 21 days of exposure and a maintained effect after 14 days of depuration. This indicates that mussels were still having effects from the presence of NPs after 2 weeks after exposure, leading to the conclusion that PS NPs cause chronic neurotoxic effects in *M. galloprovincialis*.

Overall, health effects associated with NPs are a cause for concern and need to be studied further. Even though plastics improve our quality of life, it is essential to shift towards more sustainable alternatives.

5.1 Future Perspectives

The results from this study call for further research to acquire a more comprehensive understanding of the effects of NPs on marine life. Some suggestions are to:

- Study the ingestion of the NPs and calculate what percentage of the contaminant stays within the organism and where it accumulates.
- Increase the depuration stage from 14 days to 21 days to see if the mussels can recover from the same amount of exposure after a longer depuration period.
- Study the toxicity of NPs combined with other pollutants. There is growing concern about the risks of multiple contaminants such as gold or other endocrine disruptors due to their increased reactivity.

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