Concentration and characterization of microplastics in Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) from Nova Scotia

by

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at

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Dalhousie University is located in Mi'kma'ki,
the ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

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Abstract

Microplastics (MPs) (<5 mm) have become an increasingly global concern due to their potential to impact both human health and the environment. This research explored the occurrence of microplastics in Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) from three fishing regions across Nova Scotia, Canada. This study also explored common and emerging methods for microplastic research in marine mussel and oyster species. Results found evidence of plastic in both bivalves. Average suspected microplastic (SMP) concentration was 4.25 ± 1.48 SMPs/g of wet weight (ww) tissue and 3.79 ± 1.27 SMPs/g of ww tissue in mussels and oysters, respectively. The average MP size classification was 2-10 μm and 10-20 μm for mussels and oysters, respectively. Results found that factors such as sampling location and species of bivalve influenced SMP concentrations. Plastic polymers were identified in bivalves including, polyethylene (PE), polyvinyl chloride (PVC), and polypropylene (PP). Potential sources include marine shellfish aquaculture equipment, packaging, and other land-based plastics. Results found trends in the research of MPs in bivalves and noted future considerations such as the use of temperature in processing and storage, as well as methods for analyzing small size MPs. The findings from this study highlight the need for standardized methods in MP research and further monitoring for MPs in shellfish farmed or caught for human consumption.

List of Abbreviations Used

ABS.....Acrylonitrile Butadiene Styrene
ANOVA.....Analysis of Variance
BPA.....Bisphenol
CFIA.....Canadian Food Inspection Agency
DFO..... Fisheries and Oceans Canada
ECCC .....Environment and Climate Change Canada
EPR .....Extended Producer Responsibility
FTIR.....Fourier-Transform Infrared
GPS.....Global Positioning System
HCl.....Hydrochloric Acid
HNO_3.....Nitric Acid
H_2O_2..... Hydrogen peroxide
KI .....Potassium Iodide
KOH .....Potassium hydroxide
LDPE.....Low Density Polyethylene
LMT.....Lithium Meta-Tungstate
PA.....Polyamide
PAH.....Polycyclic Aromatic Hydrocarbons
PAN.....Polyacrylonitrile
PC.....Polycarbonate
PCB..... Polychlorinated Biphenyl
PE.....Polyethylene
PET.....Polyethylene Terephthalate
PFAS.....Per- and Polyfluoroalkyl Substances
PLS.....Polysulfone
PP..... Polypropylene
PS.....Polystyrene
PVC .....Polyvinyl Chloride
PUR..... Polyurethane
Pyr-GC/MS.....Pyrolysis and Gas Chromatography and Mass Spectrometry
MT.....Metric Tonnes
MPA.....Marine Protected Area
MP.....Microplastic
MPs.....Microplastics
NaCl.....Sodium Chloride
QA/QC.....Quality Assurance and Quality Control
SEM.....Scanning Electron Microscopy
SEM-EDX.....Scanning Electron Microscopy Energy Dispersive X-ray Analysis
SMP..... Suspected Microplastic
SMPs.....Suspected Microplastics
TGA.....Thermogravimetric Analysis
UNEP.....United Nations Environment Programme
VI.....Visual Analysis
WW.....Wet Weight
$ZnCl_2$.....Zinc Chloride

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CHAPTER 1: Introduction

Marine plastics have increased exponentially within the last few decades due to our reliance on the convenience of plastic products (Ford et al., 2022). Synthetic monomers are the foundation of plastics and are ingrained within the current economy and found in everyday products such as cleaning supplies, construction materials, clothing, and packaging. It is estimated that 9.2 billion metric tonnes (MT) of primary fossil fuel-derived plastics were produced between 1950 and 2017, with the majority of this being produced post-2004 (Geyer, 2020). None of the commonly used plastics are biodegradable (Geyer et al., 2017). As a result, plastic pollution accumulates in landfills or the natural environment (Geyer et al., 2017). Canada estimates 29,000 tonnes of plastic waste makes their way into the environment, 9% is recycled, and 2.8 million MT end up in landfills (Government of Canada, 2021). Even in landfills, plastic litter can travel thousands of kilometres (km) from its initial disposal site through weather events and persist for decades due to its rigid structure (Cable et al., 2017; Geyer, 2020; Helm, 2020).

Plastic debris has been found in all major ocean basins, with an estimated 4 to 12 million MT of plastic waste generated on land entering the marine environment in 2010 alone (Geyer et al., 2017). Products such as plastic bags, bottles, fishing nets, and packaging can break down into smaller plastic particles through environmental processes such as weathering, erosion, and photodegradation (Chamas et al., 2020). Plastic particles between 5 mm and 1 μm are defined as microplastics (MPs) (Allen et al., 2022). Other size classes of plastic pollution include: macroplastics (>25 mm diameter), mesoplastics (5–25 mm), and nanoplastics (<1000 nm) (Napper et al., 2020). MPs have been

previously undetected in Earth's oceans due to their small size, but have been gaining awareness due to the increasing levels of plastic pollution globally (Windsor et al., 2019).

In marine aquatic ecosystems, MP particles are abundant and therefore bioavailable for organisms such as bivalves (Bom & Sá, 2021), crabs (Waite et al., 2018), fish (Wang et al., 2017), and seabirds (Sühring et al., 2022) to ingest. The literature documents the adverse effects MPs have on marine biota, such as reduced immune system functionality by reducing hemocyte count in bivalves (Le Guernic et al., 2020; Mkuye et al., 2022), and tissue damage and oxidative stress in fish (Bhuyan, 2022; Zitouni et al., 2021). This also poses a threat to human health due to the potential exposure to MPs from the consumption of seafood (Dehaut et al., 2016).

Shellfish species such as mussels and oysters are understood as key indicator species that are highly sensitive to environmental stressors, particularly from anthropogenic sources (Wootton et al., 2022). This makes them an ideal biomonitoring tool where pollutants are retained within their tissues, making these organisms potential vectors for pollutants to move through food webs and up trophic levels (Crooks et al., 2019). Research suggests that these bivalves are selective feeders who more readily ingest some MPs because they resemble their natural food sources in size, shape, and sometimes colour (Ward et al., 2019). In contrast, Ward et al. (2019) suggests that the selection of particles pre- and post- ingestion may lead to biased data and conclusions. These selective capabilities of bivalves may not in fact make them good bioindicator of MPs in the environment (Ward et al., 2019). However, they assert more research in the uptake, retention, and accumulation of MPs should be further studied to confirm or deny bivalves as a robust bioindicator of MP pollution. Regardless, due to their ubiquity and economic

significance, MPs in bivalves are studied to characterize potential human exposure to plastic pollution (Li et al., 2022).

Plastics and MPs are a growing concern in marine environments in Canada. Studies have examined marine plastics in western Canada, where Murphy (2018) found MPs in both sediment and water samples from the Strait of Georgia, off the Coast of British Columbia. They found MP within sediment cores in the estuarine environments from the protected area of Clayoquot Sound, and lacustrine sediments in Orchid Lake. Furthermore, researchers found MPs of various size classes in 16 sites within Lambert Channel and Baynes Sound, British Columbia which is a key growing area for the Pacific oyster (*Crassostrea gigas*) (Kazmiruk et al., 2018). They found that sediment around this key growing area was highly contaminated with MPs. In contrast, Covernton et al. (2019) found that MP concentrations did not differ between shellfish aquaculture and non-aquaculture sites for either bivalve species, sediment, or water samples. Within the province of Nova Scotia, only one study has examined suspected microfibers in bivalves. Mathalon and Hill (2014) found fibers on beaches on the Eastern Shore and compared both retail and wild Blue mussels. They found significantly higher abundances in farmed Blue mussels. The various methodologies and protocols for quantifying and characterizing MP pollution leads to difficulties comparing data across studies (Cowger et al., 2020). Future MP research should attempt to balance needed standardization within the field and the exploration of new protocols.

1.1 Project background

1.1.1 Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) in Nova Scotia

Blue mussels (*Mytilus edulis*) and eastern oysters (*Crassostrea virginica*) are native to various regions in Nova Scotia where climates accommodate their growth. Wild blue mussels are found in intertidal tidal zones across Nova Scotia from the southwestern shore to the Gulf of St. Lawrence region. They attach themselves with tough byssal threads to rocks, pilings, and buoys (Waite & Tanzer, 1981; Lee et al., 2006). Eastern oysters, also known as Atlantic or American oysters, grow along Nova Scotia's rugged coastline. Due to cold temperatures along the Nova Scotia coastline, reproductive oyster beds are very scarce (Gregoire, 2014). Established oyster beds are found in the Northern or Gulf region of Nova Scotia. Factors such as salinity, temperature, tidal shifts, and mineral and chemical composition play a role in the success of wild bivalve populations (Gregoire, 2014). Alterations or disturbances within the environment may put remaining beds at risk (Government of Canada, 2020)**.** In addition, shellfish aquaculture also represents a significant number of bivalves in Nova Scotia where, in 2021, 1275 tonnes of mussels and 492 tonnes of oysters were produced in the province and valued at $1.2 million and $4.7 million, respectively (DFO, 2021). Along the coasts of Nova Scotia, Blue mussels and Eastern oysters are both economically significant sources of food and integral to marine food webs.

1.1.2 Microplastics in marine aquatic ecosystems in Atlantic Canada

Due to their ubiquitous distribution and economic significance mussels and oysters have been used to investigate MP contamination in a variety of ecosystems globally (Bom & Sá, 2021). However, there is a knowledge gap in MP research within Atlantic Canada. Liboiron et al. (2020) reviewed findings of 57 published articles, reports, and grey literature and found a wide range of plastics in surface water and an increasing temporal trend in the abundance of plastics. In addition, a study by Smith et al. (2022) found in surface waters within Atlantic Canada an average density of 9669 plastic items/km^2 where 68% were MPs. Furthermore, Teddiman, (2021) found a range of 6-19 particles/m^2 in sediment on McNabs Island and Lake Bannok beaches. A previous study has looked at MP contamination in blue mussels in Nova Scotia (Mathalon & Hill, 2014). This study explored the concentration of microfibers in sediments of intertidal zones of one exposed beach and two protected beaches along the Eastern Shore of Nova Scotia. Blue mussels (*Mytilus edulis*) were analyzed for microfiberss. In this study, the 5 mussel subsamples contained an average of around 170 particles per 5 wild mussels and an average of about 375 particles per 5 retail mussels. They reported that the high number of microfibers in farmed mussels may be due to the use of plastic in mussel aquaculture practices and the use of polypropylene lines. However, within the study's methods, they did not use any procedures for characterizing polymer types and solely used visual analysis (Mathalon & Hill, 2014). Therefore, their results may not have accurately quantified or characterized plastics in mussels in Nova Scotia, and further research is needed.

1.1.3 Methods for quantifying and characterizing microplastics in bivalves

Methodologies used to quantify and characterize MPs in the marine environment include various processing stages to isolate particles from various environmental matrices and then analyzing them (Mariano et al., 2021; Huang et al., 2023). This can include a storage, digestion, density separation, and filtration stage aimed to separate suspected plastics from the matrix. Analysis methods such as light microscopy and spectroscopy are often used to gain information about size, morphology, and polymer type for MPs found in samples (Mariano et al., 2021; Huang et al., 2023). The various methodologies chosen for enumeration or analysis of MPs may lead to an over or under estimation of particles (Kotar et al., 2022). Due to this lack of standardization, it is difficult to compare studies (Cowger et al., 2020). In recent years, there have been trends towards standardization, however considerations for factors such as the use high temperature during processing have not been considered (Thiele et al., 2019). This lack of standardization in MP research may be due to factors such as the availability of resources, the use of highly technical methods, and shortcomings in the reporting of methods and comparable units (Adhikari et al., 2022). Therefore, future research and discussion is needed to create standardized protocols for MP isolation and analysis that is reliable and accessible. This thesis will be an adaptation of the study by Mathalon & Hill, (2014) and will aim to use alternative methods for accurately enumerating and characterizing MPs in both Blue mussels and Eastern oysters collected across Nova Scotia.

1.2 Thesis objectives

This thesis aims to assess the concentration and characterization of MPs found in Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) from Nova Scotia. Specifically, the objectives of this study are to:

1. Assess and compare the concentration of MPs in Blue mussels and Eastern oysters from Nova Scotia.
2. Characterize the polymer types of MPs found in Blue mussels and Eastern oysters from Nova Scotia.
3. Assess and compare the methods used to enumerate and analyze MPs in marine mussels and oysters and the challenges associated with methodologies of MP analysis.

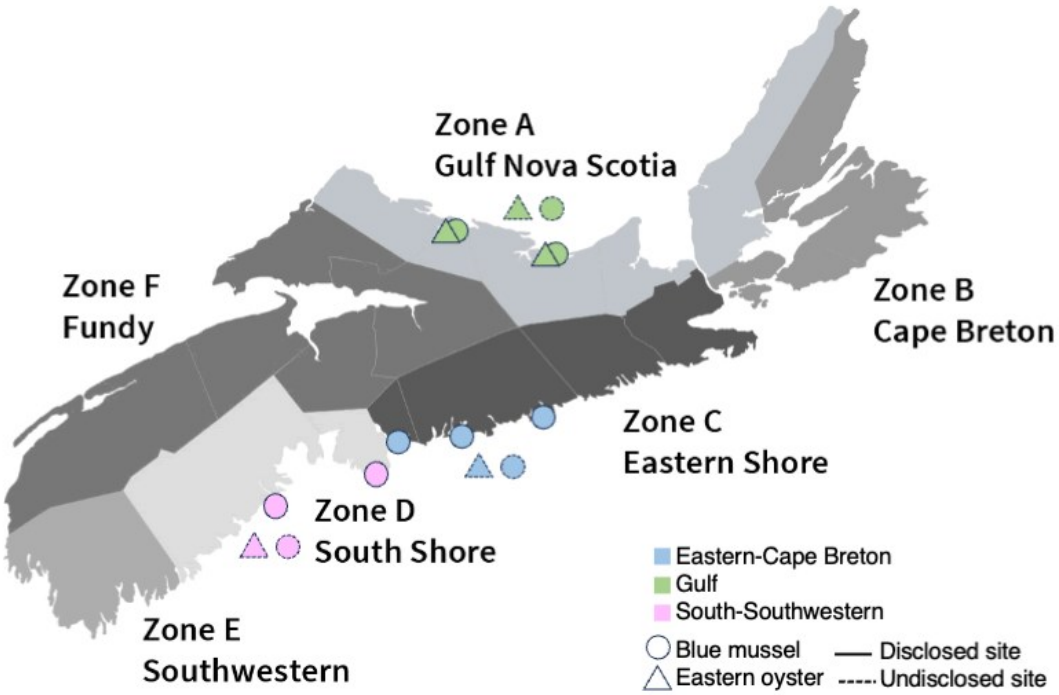


Figure 1.1 Map adapted from (Fish Harvesters, 2023) of sampling sites for Blue mussels and Eastern oysters from Nova Scotia. Note that oysters were sampled in colocation with mussels from both sites in the Gulf zone and not collected in other zones. Aquaculture locations denoted by a dashed line and undisclosed to protect the identity of businesses.

1.3 Methodology overview

For Objectives 1 and 2, Blue mussels and Eastern oysters were collected from sampling locations in Nova Scotia, selected based on local knowledge and in relation to Marine Protected Areas (MPAs) and shellfish closure areas implemented by Fisheries and Oceans Canada (DFO). One undisclosed site per species was collected within the Eastern-Cape Breton zone, the South-Southwestern zone, and the Gulf zone which are loosely defined based on the fishing zones of Nova Scotia (Fish Harvesters, 2023). These 5 sites were labelled undisclosed to protect businesses due to their sampling in proximity to shellfish aquaculture. The undisclosed sites as well as Site 2 (Halifax) were considered more 'anthropogenically influenced'. The remaining 7 of the 13 sampling sites were disclosed and approximate location is denoted in Figure 1.1. These sites were considered 'wild' sites where oyster or mussel beds may naturally occur. Blue mussels were collected from two sites in the South-southwestern zone and three sites on the Eastern zone. In the Gulf zone, two sites were selected to collect both oysters and mussels, as environmental conditions favoured their growth compared to the South-Southwestern and Eastern-Cape Breton zone. Samples were treated with an alkaline (10% KOH) followed by an oxidative (30% H_2O_2) digestion to remove the non-plastic material. A density separation step was carried out to separate plastics from the remaining matrix and filtered onto small pore-size filters to be analyzed for MPs. This research used methods of analyzing MPs of small sizes (<20 μm) such as Nile Red microscopy and micro-Raman spectroscopy. To complete objective three, a systematic literature review was conducted

to determine and compare the methods used to enumerate MPs in mussels and oysters in marine environments and the challenges associated with MP methodologies.

1.4 Thesis structure

This thesis comprises five chapters. Chapter 2 presents a literature review of the background and context relating to this thesis. Chapter 3 presents context, methodology, results, and discussion for the study of the concentration and characterization of MPs in Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) from Nova Scotia. Chapter 4 illustrates a systematic literature review of the current methods for enumerating and characterizing MPs in mussels and oysters and the advantages and limitations associated with current research in MPs. Chapter 5 provides the conclusions of this thesis and any recommendations for future plastics management in marine environments and MPs in shellfish research.

CHAPTER 2: Review of Literature

2.1 Evolution of plastic pollution

Plastics have been used for over a century. The first synthetic plastic was invented by Leo Baekeland in 1907 and used for products such as telephones, radios, and electrical insulation (Chalmin, 2019). Plastic use increased exponentially after World War II as the materials became cheaper and more accessible (Chalmin, 2019; Geyer et al., 2017). Plastics can be generally divided into two major categories: Thermos plastics and thermosetting plastics (Muzzy & Kays, 1984; Geyer et al., 2017). Thermoplastics can soften when heated and return to their original form, and include polyethylene (PE), polypropylene (PP), and polyvinyl chloride (PVC). Thermosetting plastics are plastics that once cooled or hardened cannot be retransformed into their original forms (Muzzy & Kays, 1984). Some of the most common plastic polymers include PE (high and low density), PP, PVC, polystyrene (PS), polyamides (PA), and polyethylene terephthalate (PET) (Bajt, 2021; Kole et al., 2017). Much of the plastics produced today are not biodegradable so when they are landfilled or littered, they accumulate within the natural environment rather than decompose (Geyer et al., 2017). The life cycle of plastics is often described from their production to past their disposal stage, where due to their properties they can persist in the natural environment as pollutants.

Global production of plastics has increased exponentially since the 1950s, as production levels reached an estimated 348 million MT in 2017 (Thompson et al., 2009; Plastics Europe, 2018). But this is predicted to double within 20 years (Lebreton & Andrady, 2019). It is estimated that plastic waste generation increased from one percent in 1960 to more than ten percent by 2005 (Jambeck et al., 2015). These estimates of

plastic pollution have increased significantly where Eriksen et al. (2023) found that based on their model, there were approximately 82–358 trillion plastic particles weighing 1.1–4.9 million tonnes afloat in global surface waters in 2019. In Canada, it is estimated that 29,000 tonnes of plastic waste makes their way into the environment, 9% is recycled, and 2.8 million MT end up in landfills (Government of Canada, 2021). After their initial disposal, plastic pollution can break down and can travel thousands of kilometres (km) (Horton & Dixon, 2018). Through weather events such as wind, storms, and atmospheric deposition, this pollution is transported to other compartments of the environment such as the air and soil. (Cable et al., 2017; Geyer, 2020; Helm, 2020). However, many of these plastics are deposited in the ocean, where an estimated 80% of marine pollution is from land-based sources (Ambrose et al., 2019). Along their journey, these plastics may undergo a variety of physical, chemical, and biological processes that cause them to degrade into smaller plastic particles (Chamas et al., 2020). Small plastic particles between 1 µm and 5mm in size are defined as MPs (Frias & Nash, 2019). Other size classifications of plastic pollution can include mesoplastics (5–20 mm), and nanoplastics (<1000 nm) (Napper et al., 2020).

2.2 Microplastic pollution transportation and fate in the environment

There are two main types of MP pollution. Primary MPs are defined as plastic particles that are produced in this size class by the industry as products such as microbeads. Primary MPs are used in cleaning products and personal care products (Praveena et al., 2018). Secondary MPs are generated from discarded plastics that fragment into smaller sizes through various physical, biological, and chemical processes.

The degradation of plastics into micro or nano plastics depends on various factors such as the type of the polymer, exposure to physical and mechanical weathering processes, and the rate of degradation (Chamas et al., 2020; Lin et al., 2022). Degradation in environments, such as marine ecosystems include processes such as mechanical (erosion, abrasion, wave action, and turbulence), thermal processes, photodegradation and chemical or biological processes. Furthermore, fragmentation processes that generate secondary MPs in water can include bio-fragmentation, assimilation, and biodeterioration (Emadian et al., 2017).

Plastic pollution of various sizes can be transported to benthic (Van Colen et al., 2021), Arctic (Bergmann et al., 2019), and remote island (Martins et al., 2020) ecosystems. The transportation of microplastic pollution is hydrological, where rivers, streams, and estuaries play a significant role in the translocation of pollution into marine environments (Windsor et al., 2019). The degree of weathering is thought to influence transportation mechanisms depending on other factors such as density, size, and shape of particles (Lin et al., 2022; Windsor et al., 2019). Transportation of MPs differs from macroplastics due to their size, where more energy is required to move larger plastics (Windsor et al., 2019). The distribution of MP pollution is poorly understood due to the wide array of meteorological, atmospheric, coastal and tidal processes that can influence the transportation, accumulation and dispersion of particles (Foekema et al., 2013; Windsor et al., 2019).

Within terrestrial systems, MPs in soils derive from point sources such as agricultural practices such as irrigation, rural and urban waste, wastewater treatment plant sludge, and atmospheric deposition (Lamichhane et al., 2022). Due to improper waste

management MPs in soils are prevalent and deteriorate soil characteristics such as porosity and texture (de Souza Machado et al., 2019; Guo et al., 2022). MPs in soils can also be remobilized and transported across environments to river systems, through flooding and storms where landfill plastics can be redistributed in the terrestrial environment (Guo et al., 2020; Windsor et al., 2019). MP contamination in surface soils can impact organisms such as worms (Huerta Lwanga et al., 2016), microorganisms (Bowley et al., 2021), and insects (Windsor et al., 2019).

MPs can be transported from terrestrial and marine environments to atmospheric systems through processes such as the combustion of waste plastics, wind erosion, urban dust, and heavy storms (Petersen & Hubbart, 2021). Precipitation such as snowfall and wet deposition are understood to be key drivers of MP deposition by atmospheric processes (Allen et al., 2022; Bergmann et al., 2019). MPs in snow have been recorded depositing particles in urban areas, the ocean, or Arctic regions (Allen et al., 2022; Windsor et al., 2019). MP pollution from atmospheric deposition has also been observed in other remote areas such as the Tibetan glaciers (Bergmann et al., 2019; Zhang et al., 2019). It is thought that winds in high latitudes may influence the deposition of MPs on glaciers (Windsor et al., 2019). From the current literature, atmospheric MPs are considered more as a temporary store and potential short-to-long-distance pathway for MP deposition.

The journey of MPs extends beyond atmospheric considerations to their interactions with aquatic environments such as freshwater systems. Freshwater environments are often considered a conduit for atmospheric and terrestrial MPs to marine ecosystems, facilitating long-range transport across land masses (Horton &

Dixon, 2018). Sources of MPs in freshwater systems include urban and rural litter, landfills, and wastewater treatment plants. Modern treatment plants can remove both large and small plastics from raw influent (95%-99%) but outflows are point sources of smaller size class particles that are directly released into freshwater systems (Murphy et al., 2016; Windsor et al., 2019). Treatment efficiencies for MP removal vary across wastewater treatment plants, and transportation of particles is typically through sludge where MPs have accumulated (Carr et al., 2016). Plastics in river systems may accumulate and pool in benthic sediments, acting as short-to-long-term storage for particles (Cable et al., 2017; Windsor et al., 2019). Despite the amount of research on the presence of MPs in freshwater systems, the net or total flux of plastics from terrestrial sources, through hydrological processes to marine systems remains poorly understood (Windsor et al., 2019).

After travelling long distances from their initial disposal, MPs often find their endpoints in marine systems, where particles are stored in benthic sediments and the water column (Jambeck et al., 2015; Windsor et al., 2019). MPs are also prominent in coastal zones due to their proximity to terrestrial inputs and tidal processes that provide favourable conditions for the accumulation of plastic debris (Windsor et al., 2019). A common pathway is the redeposition of plastic to coastal and beaches where litter from marine environments is transported through wave processes (Browne et al., 2011). In addition, a mechanism of MP transport from marine to atmospheric systems is through the sea surface microlayer where particles become aerolized by wind action (Wright & Kelly, 2017). In marine environments, MPs in benthic, surface, and the various zones of the water column are available for long-term storage (Windsor et al., 2019). The presence

of MPs has been evaluated in a wide array of environmental compartments, and despite this, their fate is uncertain due to their persistent properties and remobilization in the environment.

2.2.1 Chemical additives and microplastic-associated contaminants

MPs have infiltrated various aspects of the environment. However, their effects are poorly understood due to the wide diversity in their size, shape, polymer type, and concentration, which are factors that contribute to evaluating the risks to human health and environmental biota (Huang et al., 2021). MPs may be exposed to humans through pathways such as inhalation, absorption, or ingestion (Campanale et al., 2020). Plastics can contain additives can that improve resistance to degradation by temperature, radiation, mould, bacteria and mechanical, thermal and electrical resistance (Campanale et al., 2020; Hahladakis et al., 2018). Additives such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and bisphenol A (BPA) have been observed to accumulate on surfaces as well as inside MPs (Campanale et al., 2020; Huang et al., 2021). In addition, other additives such as plasticizers, antioxidants, UV stabilizers, and flame retardants have all been associated with MP toxicity (Campanale et al., 2020; Hahladakis et al., 2018). The toxicity of these chemicals can have effects at the cellular level, on organ systems, or the entire body (Campanale et al., 2020). However, studies examining the effects of plastic additives on humans through the ingestion of MPs are limited.

MP exposure to humans poses significant risks from plastic additives but also from associated contaminants such as heavy metals that are attracted to the surfaces of MPs

(Jan et al., 2015). As plastics degrade into smaller constituents the potential for ingestion and accumulation within the gut and tissues increases (Brennecke et al., 2016; Campanale et al., 2020). This is due to factors such as the increased surface area for potential contaminants to attach or leach out of MPs. Heavy metals such as cadmium, arsenic, chromium, lead, and copper are observed to be accumulating in marine biota and are linked to MPs (Brennecke et al., 2016). Heavy metals are naturally occurring in many environmental compartments. However, they can exist in elevated concentrations due to anthropogenic activities and intensive industrial projects. For instance, Cadmium (Cd) is a heavy metal often associated with PVC where human health effects include changes in metabolism, cellular apoptosis, and bone fractures in post-menopausal women (Campanale et al., 2020). In addition, studies have investigated the absorption of heavy metals in aged MPs. Brennecke et al. (2016) found the absorption of copper (Cu) and Zn leached from anti-fouling virgin PS beads and aged PVC fragments in seawater. Factors such as surface area, influence the creation of active sites on MP particles that may attract heavy metals (Campanale et al., 2020; Wang et al., 2018). However, their absorption onto MPs and potential transfer through food webs is poorly understood (Crooks et al., 2019; Huang et al., 2021).

In addition, MPs have also been associated with pathogens and harmful microorganisms within marine environments (Bowley et al., 2021; Naik et al., 2019). MP surfaces in aquatic environments are colonized by bacteria and biofilms through the attachment of nutrients, organic matter, and biomolecules to MP surfaces. Kirstein et al. (2016) found evidence that the potentially pathogenic *Vibrio parahaemolyticus* was observed on PE, PP, and PS particles from the North and Baltic Seas. The transportation

of MPs over long distances may indicate their potential as vectors for pathogenic transfer through ingestion. Within these areas, commercially significant species such as fish (Foekema et al., 2013) and bivalves (Van Cauwenberghe & Janssen, 2014) may ingest MPs with harmful contaminants and potentially become a vector for human exposure.

2.2.2 Potential impacts of microplastic pollution on human health

There is limited research on the effects of MPs found in humans. However, plastic particles have been found in numerous compartments of the human body. Pathways include ingestion and inhalation where exposure to MPs can occur, with endocytic and paracellular transfer across epithelial tissues (Wright & Kelly, 2017). MPs have found in various organ systems such as the nervous system, kidney system, digestive and excretory system, respiratory system, and skin (Campanale et al., 2020). Studies have also found PET, PE, and polymers of styrene in human blood samples (Lamichhane et al., 2022; Leslie et al., 2017). However, uptake is dependent on particle size, morphology, and surface chemistry (Yee et al., 2021). It is suggested that particles smaller than 150 μm can cross the gastrointestinal epithelium in mammals, and 0.3% of these are expected to be absorbed (Barboza et al., 2018; Campanale et al., 2020). The direct effects of MPs are still unclear, but mammalian models can be used to predict the toxicity and potential human effects (Lamichhane et al., 2022). For instance, Deng et al. (2017) exposed PS MPs (5 μm and 20 μm) to mice for about 28 days and found accumulation in the kidney, guts, and liver, leading to problems such as liver inflammation and lipid metabolism disorder. Additionally, studies in humans at the cellular level found that PS MPs can cause oxidative stress by reducing the expression of antioxidants, thereby leading to

apoptotic cytotoxicity in human vascular endothelial cells (Chen et al., 2023). The entry and effects of small-sized MPs should be further researched, as MPs under 20 μm can penetrate organs and those that are 10 μm can cross the blood-brain barrier and cell membranes as well as enter the placenta (Campanale et al., 2020; Tielman et al., 2022).

2.3 Microplastic pollution in the marine environment

Marine ecosystems are generally understood as plastic sinks due to the numerous pathways into oceans globally (Windsor et al., 2019). Land-based plastics can travel through freshwater systems into estuaries, harbours, and shorelines, which then transported into the ocean (Barboza et al., 2018; Cable et al., 2017). Hydrodynamic processes such as coastal currents and river outflows disperse MPs into marine environments (Windsor et al., 2019). Other sources of plastic also include materials from fishing activities, industrial spillage, or plastics from tourism (Walker et al., 2006). Industrial and commercial fishing plastics such as netting, sheeting, and ropes can degrade into MPs through physical, chemical, or biological processes (Government of Canada, 2020; Mascorda Cabre et al., 2021). Macro and MPs can be situated in subtidal sediments, surface water and the water column. Mechanical weathering within the open sea makes plastics more brittle and susceptible to breaking down, making them more prone to fragmentation over time (Jahnke et al., 2017). There are estimates of 51 trillion MP particles within the ocean surfaces globally (Agamuthu et al., 2019). MPs have been detected in marine ecosystems from all over the globe including but not limited to: the Northeast Atlantic Ocean (Lusher et al., 2014), the Irish Continental shelf (Martin et al., 2017), and the Bohai Sea in the western Pacific Ocean (Dai et al., 2018). Furthermore,

processes such as gyres, currents, and tides further degrade and disperse MPs within various zones of the marine environment (Windsor et al., 2019). Here, a wide array of organisms have been observed to ingest MPs such as zooplankton (Botterell et al., 2022), bivalves (Van Cauwenberghe & Janssen, 2014; Ward et al., 2019), and whales (Merrill et al., 2023; Moore et al., 2020).

Bivalves are especially studied due to their sensitivity to pollutants and their ubiquity across marine ecosystems (Cho et al., 2021 Forrest et al., 2019). Furthermore, they are economically significant organisms where between 2010-2015 the global production of marine bivalves reached more than 15 million tonnes per year (Wijsman et al., 2018). It is understood that ingestion is one of the main human exposure pathways to MPs (Smith et al., 2018). This has raised concerns about seafood consumption as a potential pathway of MPs to humans (Dehaut et al., 2016; Masiá et al., 2022).

2.3.1 Microplastics in marine mussels and oysters

Bivalves such as mussels and oysters have been observed to ingest MP particles from the water column and accumulate them within their tissues. Research in the concentration and abundance of MPs of mussels and oysters has been conducted in various countries including but not limited to Italy (Nalbone et al., 2021), South Korea (Cho et al., 2019), Australia (Klein et al., 2022), and South Africa (Sparks et al., 2021). A literature review looking at the concentration of MPs in bivalves found that many of the studies originated in Asia and Europe (Bom & Sá, 2021). Within this review, concentrations of MPs in mussels were found ranging from 0 (Schessl et al., 2019) to 20 (Kolandhasamy et al., 2018) MPs/gram wet weight of soft tissue (MPs/g ww), and the

mean values were between 0 and 3 MPs/g ww. In contrast, oysters had a smaller range of concentrations with a maximum of 7.2 MPs per gram of tissue (Li et al., 2018) and a mean of below 1 MPs/g ww tissue (Bom & Sá, 2021). Furthermore, a literature review by Wootton et al. (2022) found that globally, 94.4% of all oysters contained MPs, with an average of 1.41 ± 0.33 MPs/g ww. These discrepancies in concentrations or abundance of MPs in bivalves may be due to differences in physiology and feeding behaviours, sampling and laboratory methods, or analysis that may have influenced MP load (Li et al., 2019; Van Cauwenberghe & Janssen, 2014).

Within examining MP contamination in bivalves there have also been studies looking at the comparison of MP load between species. For example, in France, researchers identified that mussels *M. edulis* and oysters *C. gigas* have similar concentrations of MPs, with mean values of 0.23 ± 0.20 and 0.18 ± 0.16 MPs/g ww, respectively (Phuong et al., 2018). In addition, Exposito et al. (2022) found abundances of 18.6 ± 23.0 and 22.8 ± 14.4 particles per individual in *M. galloprovincialis* and *C. gigas,* respectively. MP load between mussels and oysters may be due to differences in physiology, sampling location, methods used, and habitat (Cho et al., 2019; Phuong et al., 2018). However, it is suggested that further research is needed to accurately characterize if there are species-level differences in the uptake and retention of MPs in bivalves.

Furthermore, the comparison of bivalves from different modes of life such as wild-caught or aquaculture raised has been studied to compare MP occurrence and characterization. It is estimated that between 2010 and 2015 89% of marine bivalve production was aquaculture-raised with 11% originating from wild fisheries (Wijsman et al., 2018). Aquaculture-raised bivalves are of particular interest due to their direct

exposure to humans (Covernton et al., 2019; Bringer et al., 2021). Studies have shown high concentrations of MPs in both aquacultures (Mathalon & Hill, 2014), and wild caught bivalves (Li et al., 2018). However, there is no consensus if there is a direct relationship between MP concentrations in bivalves and whether they were grown from aquaculture or not.

2.3.2 Characteristics of microplastics in mussels and oysters

Shape, size, colour, and polymer type are characteristics used to describe MPs within marine mussels and oyster species. Researchers look at these descriptors to identify and categorize various particles and gather information on the potential sources and transport of MPs. Within marine mussels and oysters, the main shapes of MPs found in bivalves are defined as fibers, pellets, fragments, and films (Bom & Sá, 2021). Current literature has found high concentrations of fibers in coastal waters around the world due to their size, low density, and wide distribution globally (Suaria et al., 2020). The literature review by Bom & Sá, (2021) identified fibers as the most common shape observed in bivalves followed by fibers. Also, it is important to note that the definitions for fibers and fragments may differ based on the methods employed as well as the description of the defined shapes. However, there have been efforts towards the standardization of definitions and how to deal with these discrepancies (Frias and Nash., 2019; Hartmann et al., 2019; Rochman et al., 2019). For instance, Hartmann et al., 2019 define fibers as plastics that are “significantly longer in one than wide in two dimensions (length-to-diameter ratio) and are commonly (and interchangeably) described as fibers or filaments, with both terms describing thread-like structures”. Fibers have also been defined in terms

of their visual characteristics such as “flexible, with equal thickness throughout and ends that are clean-cut, pointed, or fraying” (Rochman et al., 2019). Fragments have been described as “ having a rigid structure and sometimes irregular shape. They can be round, subround, angular, or subangular. They are not always equally thick throughout and can appear twisted or curled” (Rochman et al., 2015). For larger plastics morphology may be easier to distinguish where shapes may be more distinctive but for small sizes (<20 μm) MP characteristics such as shape may be less defined and much more difficult to identify (Lenz et al., 2015).

Various size classes of MPs have been found in marine mussels and oysters. Factors such as sampling location and the methodologies used can influence the observed occurrence of MP pollution bivalves. The sizes of MPs found within bivalves are also influenced by physiological processes and feeding behaviours that govern the selective ingestion of particles. In addition, the detection of small-size (<20 μm) MPs within the environment is dependent on the methods chosen (Li et al., 2019; Naidu, 2019). For instance, a literature review of MPs found in oysters globally found sizes ranged from >1 μm to 5 mm depending on the methods employed (Wootton et al., 2022). These lower limits can also be defined by filter sizes and the detection techniques used (Li et al., 2019; Bom & Sá, 202). Current literature has found that in mussels, plastic particles as small as 1 μm using Raman spectroscopy (Adhikari et al., 2022; Lenz et al., 2015; Xu et al., 2019). In addition, studies have also observed that MPs (~3.0 μm) have higher accumulation rates in mussel tissues (Kazour & Amara, 2020; Hermabessiere et al., 2019).

MP size has been measured by its longest dimension, but this does not consider three-dimensional shape. As particles are transported within mussels they are thought to rotate at different angles influencing their route throughout tissues, and retention time (Li et al., 2021). A laboratory study showed that oysters and mussels can ingest spheres and fibers larger than 1 mm, but these particles were released in pseudofeces and egested in the feces (Ward et al., 2019). There is potential for even smaller particles or nanoplastics to accumulate in the tissues of bivalves. However, the methods for reliably detecting these particles at smaller sizes have not been developed (Adhikari et al., 2022).

The colour of MPs found in bivalves has been reported in studies, however, there has been little consensus on whether bivalves actively select them. For instance, De Witte et al. (2014) suggested that since ingested orange synthetic fibers originate from PE dolly rope and fishing nets in harbours, orange foods could be a popular food choice for mussels. They also used colour to assess some of the sources of MPs where the MPs extracted from mussels were mainly dark in colour (black and blue), suggesting that the MPs were from similar sources (Sparks et al., 2021). There are various sizes and morphological differences in the types of MPs ingested by marine bivalves which may be due to factors such as the degree of weathering, point sources, and the polymer type (Phuong et al., 2018).

There are a wide variety of plastics produced, and therefore a wide array of MPs within the environment each with their suite of chemical and physical properties and behaviours. A review by Andrady, (2017) stated the global production of PE and PP (the most common in marine MPs) grew at the rate of 8.7% per year (1950–2012). This is consistent with baseline studies of MPs in shellfish where Hermabessiere et al. (2019)

found the presence of PE (36.8%) and PP (32.2%), respectively. PE is a low-density polymer (density: 0.962 gcm^3) and is available in the upper layer of the water column for mussels and oysters to ingest (Cho et al., 2019). In addition, Hidago-Ruz et al. (2012) also found similar results where PE, PP, and PS were the main polymers in marine environments, beach sediments and water columns. In contrast, in a literature review looking at MPs in mussels, Li et al. (2019) reported PE, PP, PS, PET, PVC, cellophane, and polyamide were some of the most reported polymers. Furthermore, a literature review found that within 93 bivalve studies, 63 of them identified PE as one of the main polymer types, followed by PP, PET, PE, cellophane, and PS (Li et al., 2019). This reinforces the notion that the variability in common polymer types might be attributed to regional differences (Bom & Sá, 2021).

From the identification of polymers in bivalves, researchers can make inferences about potential sources of MPs due to the plastics that may be likely present in specific marine environments (Browne et al., 2011). For instance, De Witte et al. (2014) identified orange fibers in farmed mussels suggesting that this may be because farmed mussels are grown in PP socks or lines (Sparks et al., 2021), although this could also be due to MP exposure before they arrive at the store (Mathalon & Hill, 2014). Identifying polymer types in bivalves can help inform some of the potential sources of MPs. However, it is almost impossible to tell the specific products or location from which plastic pollution was derived, due to the degree of weathering and hydrogeological processes that have transported/dispersed particles across the globe. Characteristics such as shape, size, colour, and polymer type can inform researchers about potential sources of MPs as well as play a role in the selective ingestion of bivalves.

2.3.3 Factors that influence uptake of microplastics in mussels and oysters

It is understood that MPs enter through the siphons with surrounding seawater and are then captured by the gills. On the gill surfaces, MPs are then incorporated by the gill epithelium or into the mouth and digestive system and absorbed by microvilli and endocytosis (von Moos et al., 2012). MP contamination has also been found in other organs such as the gonad, mantle, adductor, viscera and foot (Kolandhasamy et al., 2018; Li et al., 2021). Gills, palps, stomach and digestive glands are important organs involved in selecting and transferring MPs (Ward et al., 2019; Ward et al., 2019). On the gills and palps of mussels, MPs may be rejected as pseudofeces, directly assimilated by the gill epithelium, or transported into the mouth and digestive system. If MPs captured by the gill are discriminated against and rejected by mussels, they will be transported to specific sites on the mantle and expelled as pseudofeces. MPs can also be transported to the gut and incorporated into fecal material (Ward et al., 2019).

The size of MPs may be a factor in the uptake of MPs where for instance, researchers found 10 μm sized particles were the smallest size detected in mussels that were exposed to seawater containing three different-sized (10 μm, 30 μm, and 90 μm) MPs (Van Cauwenberghe et al., 2015). Furthermore, they found larger MPs were detected in the feces of field *M. edulis* (15–500 μm) compared with those in the soft tissue (20–90 μm). Ward et al. (2019) suggest that capture efficiency shows an increasing trend with increasing particle size above 1 μm to a maximum efficiency (near 100%) at the size of 2.5–3.5 μm. They suggest that although mussels have a high capture efficiency for MPs between 500 and 1000 μm, they often expel these particles as pseudofeces. In contrast,

Mladinich et al. (2022) found that on average, oysters rejected >45% of 500 μm fibers and >60% of 970 μm fibers, whereas mussels rejected >10% of 500 μm fibers and >25% of 970 μm fibers. In addition, they found that the polymer type did not influence the selective ingestion of similar-sized microfibers (nylon vs PES) or microspheres (PE vs PS) (Mladinich et al., 2022). They also found that oysters rejected a higher percentage of all particle types than mussels and significantly more in the cases of microfibers.

These differences between mussels and oysters may be due to anatomical variations. For instance, oysters have a more complex heterorhabdic gill structure, which performs bidirectional transport of particles and allows for particle selection on the gills (Li et al., 2021; Ward et al., 2019). This means that the oysters have two sites for particle selection whereas all potential particle selection in mussels happens on the labial palps. Li et al. (2021) suggest that it is difficult to assess the factors that lead to differential uptake of MPs of varying sizes and polymer types. Anatomical constraints also exist in the gill, labial palps, and mouth of mussels, which could reduce the ingestion of particles larger than 100 μm (Ward et al., 2019). For example, in some bivalve species, in labial palps, particles can be transported on the crests of the ridges both proximally and anteriorly and become trapped in the troughs between ridges. These can be then carried distally to the edge of the palp for rejection as pseudofeces (Ward et al., 2019; Garrido et al., 2012).

In addition to size, other factors influence the selectivity of particles including shape and degree of weathering. (Bråte et al., 2018; Qu et al., 2018). Laboratory studies have shown, for instance, that *Mytilus galloprovincialis* ingested significantly more weathered PE particles than virgin particles (Bråte et al., 2018). Furthermore, it is thought that colour may influence feeding strategies. However, there is no evidence that mussels

select them actively. If there is an active selection process, there are most likely other confounding factors that may make plastic particles found in the environment more susceptible to being ingested (Birnstiel et al., 2019; Li et al., 2021). Understanding feeding behaviours and factors that may influence MP ingestion mechanisms may be essential in characterizing the potential risks to the organism as well as the types of particles to which humans may be exposed.

2.3.4 Physiological and cellular effects of microplastics in mussels and oysters

Once MPs have been ingested by bivalves, they may accumulate within tissues and and may have physiological effects. These effects are poorly understood, however, there are several laboratory studies that observe the potential physiological, and molecular changes in various bivalves due to the ingestion and accumulation of MPs (Bom & Sá, 2021; Franzellitti et al., 2019; Ma et al., 2020). For instance, both physiological and cellular effects have been observed in oysters such as the initiation of oxidative stress (Kwon et al., 2021), disruption of feeding activity, metabolic, or energy balance (Gardon et al., 2018), and reductions in oocyte numbers and sperm velocities (Sussarellu et al., 2014, 2016). Furthermore, von Moos et al. (2012) found histological changes and a strong inflammatory response to the uptake of PE MPs in blue mussels (*Mytilus edulis*). The physiological effects of MPs ingested by mussels and oysters are also characterized by exposure to potential MP-associated contaminates. Zhu et al. (2020) found that the *in vivo* concentrations of Cd, Cr, Pb, and Cu enriched in oysters increased with the *in vivo* abundance of MPs, suggesting that the bioaccumulation of heavy metals can be magnified *in vivo* by MPs. The literature points towards the links between MP ingestion

and a suite of physiological, reproductive, and cellular effects in individuals that indicate a potential risk for humans if left able to bioaccumulate in tissues.

2.3.5 Ecosystem implications of microplastics in mussels and oysters

MP contamination in bivalves such as mussels and oysters may not only affect individuals but small populations and aquatic food webs. For example, Shang et al. (2021) studied the impact of SMPs on the energy budget of *Mytilus coruscus* and found suppression of cellular energy allocation (CEA) by MP exposure suggests that bioenergetics disturbances might lead to a decrease in growth and productivity of mussel populations in environments with heavy MPs loads. Additionally, laboratory studies have been performed to simulate trophic level transfer and characterize the potential transportation of MPs within food webs. This was investigated when Crooks et al. (2019) fed mussels 50 µl (~4.1×10^6) of 0.5 µm PS fluorescent MP spheres and then fed mussels to velvet swimming crabs (*Necora puberty*). MPs were present in all crab tissues sampled and remained present for the duration of the trial and both the testes and stomach showed a significant increase in the number of MPs present with the number of mussels consumed (Crooks et al., 2019). This may have implications for marine organisms across the food chain as well as humans through the consumption of seafood from higher trophic levels.

2.4 Plastic and microplastic pollution in Canada

Canada heavily relies on the plastics industry. The plastic production industry is valued at $35 billion, employing close to 100,000 people in nearly 2,000 businesses that make and recycle plastic products (Government of Canada, 2021). Every year Canadians produce an estimated 3 million MT of plastic waste from their homes and businesses. Almost half of that is packaging. The rest comes from sectors like construction, textiles, agriculture, automotive and electronics. Only nine percent of this plastic waste is recycled while the remaining amount is landfilled, sent as waste to energy facilities, or directly into the environment (Government of Canada, 2021). There are several sources of MPs in Canada's marine environment, including plastic litter, microbeads in personal care products, and synthetic textiles. To combat waste, Canada has implemented regulations to ban the use of microbeads in personal care products. In addition, the Canadian government has implemented several initiatives, including the Oceans Protection Plan, which includes measures to reduce plastic pollution and increase marine litter monitoring (Government of Canada, 2023). The Government of Canada released the 'Scientific Assessment of Plastic Pollution,' where they recommended that epidemiological studies in the general population should be performed to inform the human health impacts of MPs (Government of Canada, 2020). Their stance is that currently there is not enough literature to support MPs as a health concern but their presence in human tissues should be closely monitored (Government of Canada, 2020). While the long-term human health effects of MPs are unclear, more research should be completed to evaluate the risks of MPs and their associated contaminants to humans through exposure pathways such as the consumption of seafood.

2.4.1 Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) in Nova Scotia

Blue mussels (*Mytilus edulis*) and eastern oysters (*Crassostrea virginica*) are native to various regions in Nova Scotia where climates accommodate their growth. It takes blue mussels five to seven years to reach 7 cm, and they can grow up to 10 cm. Wild blue mussels are found in intertidal tidal zones across Nova Scotia from the southwestern shore to the Gulf of St. Lawrence region. They attach themselves with tough byssal threads to rocks, pilings, and buoys (Waite & Tanzer, 1981; Lee et al., 2006). Mussels have two siphons between their shells or valves. One siphon takes in water containing food and oxygen, and the second siphon is for the release of water and waste. (Inoue et al., 2021). Mussels usually spawn in spring, releasing eggs and sperm into the water. Some females may spawn as many as 12 million eggs.

Eastern oysters, also known as Atlantic or American oysters, grow along Nova Scotia's rugged coastline. These oysters are known for being indigenous to Malagash Harbour in Nova Scotia, where the native Mi'kmaq and European settlers originally gathered them in the early 1800s. The aboriginal Mi'kmaq made extensive historical use of oysters in addition to other shellfish and finfish in the region (Gregoire, 2014). Due to cold temperatures along the Nova Scotia coastline, reproductive oyster beds are very scarce (Gregoire, 2014). Established oyster beds are found in the Northern or Gulf region of Nova Scotia. Factors such as salinity, temperature, tidal shifts, and mineral and chemical composition play a role in the success of wild bivalve populations (Gregoire, 2014). These factors influence shell characteristics and even taste. Alterations or disturbances within the environment may put remaining beds at risk (Government of Canada, 2020).

In addition, shellfish aquaculture also represents a significant number of bivalves in Nova Scotia where, the annual average farm-gate value of mussel and culture in Canada was $44.7 million in the last five years (2011-2015) (Government of Canada, 2013). In 2021, 1275 tonnes of mussels and 492 tonnes of oysters were produced in the province and valued at $1.2 million and $4.7 million, respectively (DFO, 2021). Eastern Oysters are grown in the warm, shallow bays and estuaries of the southwestern Gulf of St. Lawrence along the coast of Prince Edward Island and New Brunswick, as well as in the coves of Cape Breton's Bras d'Or Lakes (Government of Canada, 2013). Oyster farming involves suspension of juveniles in secured floating devices in nutrient-rich subtidal water until they grow to market size (Government of Canada, 2013). In contrast, cultured mussels are not grown on the ocean bottom; rather, the seed or spat is transferred to grow to market size on mussel socks suspended from rafts or longlines near the surface of the water (Government of Canada, 2013). Mussels obtain all their nutritional requirements naturally from the marine environment and do not require additional feeding from farmers.

2.4.2 Marine bioregions regions in Nova Scotia

Nova Scotia is divided into two bioregions: the Scotian Shelf and the Gulf of St. Lawrence. These bioregions are based on geographic differences in ocean conditions and depth (Government of Canada, 2019). Two main current systems influence the Canadian Atlantic – the Labrador Current originating from the north, and the warm Gulf Stream from the south (Figure 2.1). For the past decade, ice volumes on the Gulf of St. Lawrence and the Scotian Shelf have generally been lower than normal reaching a record-low value

in the Gulf of St. Lawrence in 2021 (Government of Canada, 2022). In addition, bottom temperatures were considerably above normal across the zone, including record highs in the northern Gulf of St. Lawrence, and on the Scotian Shelf.

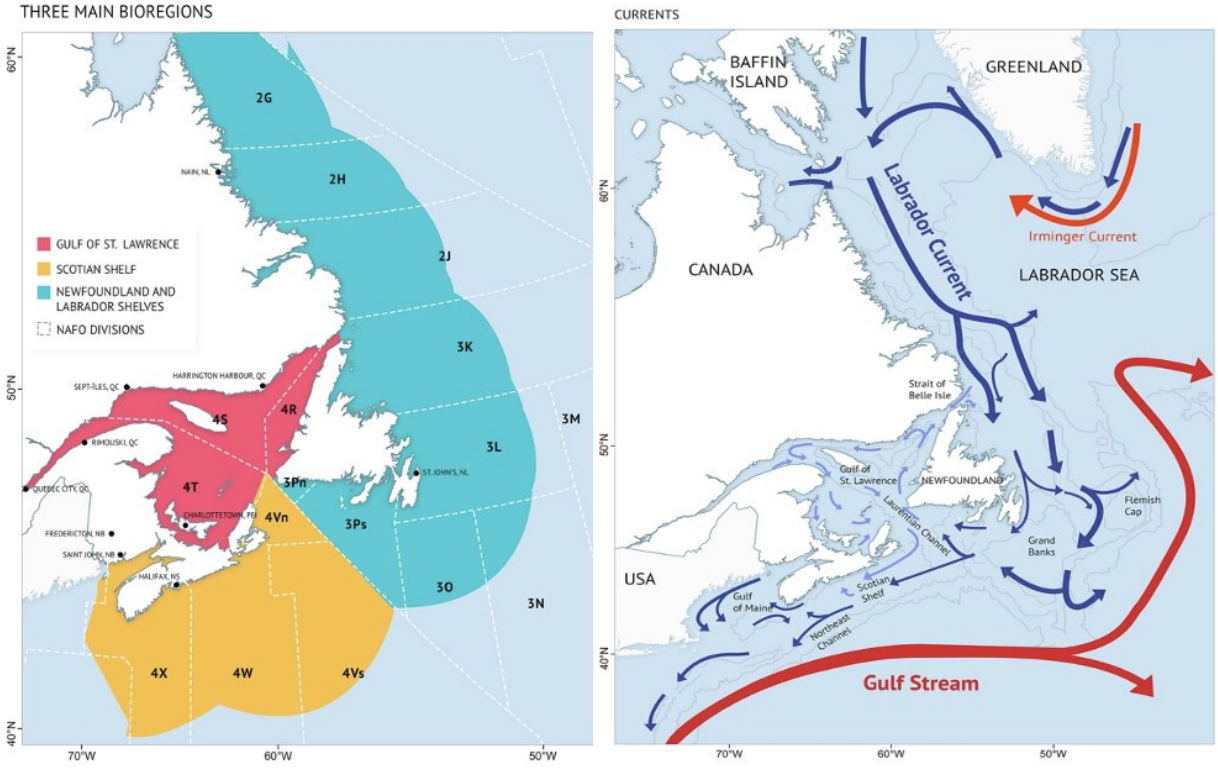


Figure 2.1 Map of Atlantic Bioregions (left), and map of Atlantic currents (right) (Government of Canada, 2019).

The Gulf region has lower levels of salinity in comparison to the Scotian Shelf due to the influence of the influx of freshwater from major rivers such as the St. Lawrence River, which drains a large portion of North America's Great Lakes system. The freshwater input from these rivers reduces the overall salinity in the Gulf (Government of Canada, 2019). In contrast, the Scotian Shelf, which refers to the waters off the coast of Nova Scotia, is more influenced by the Atlantic Ocean's saltwater. The

Gulf Stream, a warm ocean current, flows near the Scotian Shelf and contributes to higher salinity levels in that region (Government of Canada, 2019).

In addition, nutrient levels between the Gulf of St. Lawrence and the Scotian shelf differ. On average, the Gulf of St. Lawrence had a higher average deep nitrate inventory from 1999 – 2016 (Government of Canada, 2019). They found higher incidences of shellfish such as Atlantic rock crab (*Cancer irroratus*), Hyas crab (*Hyas coarctatus*), and softshell clams (*Mya arenaria*) over time due to warmer climates and more favourable growing conditions. These trends were also found in the Scotian Shelf where warming temperatures resulted in a decrease in pelagic species that require cooler temperatures and an increase in species that prefer warmer temperatures such as American Lobster.

Furthermore, in 2022, dissolved oxygen concentration generally declined in the deep waters of the Gulf of St. Lawrence and was at a record low in the Estuary (Government of Canada, 2022). In addition, levels of chlorophyll *a* have been above normal in most of the Gulf of St. Lawrence show a similar pattern to the 2018–2021 period (Government of Canada, 2022). Low zooplankton biomass was observed between 2015-2017 throughout most of the Atlantic zone and followed by small increases in subsequent years (Government of Canada, 2022). These factors may have had impacts on the occurrence of mussel and oyster beds found in the Gulf and Scotian Shelf region (Government of Canada, 2019).

2.4.3 Microplastic research in Atlantic Canada

Plastic and MP research are limited in this region. However, there are a handful of studies looking at baseline levels of contamination within these coastline provinces. A

study conducted in Newfoundland and Labrador synthesized the findings of 57 published articles, reports, and grey literature and found a wide range of plastics in surface water and found an increasing trend in the abundance of plastics over time (Liboiron et al., 2020). Furthermore, another study looked at MP contamination in Nova Scotia and Newfoundland Canada and found that surface water samples at all three sites contained plastic with an average abundance of 9669 items/km^2. They found that most plastics (68%) are sized as MPs (0.425-5 mm) (Smith et al., 2022). Further MP research in Atlantic Canada should be performed in various compartments to better characterize plastic pollution within this region.

Due to their importance in human diets, monitoring for toxins and other pollutants is significant for mitigating environmental and human health risks. It is estimated that the global human mean intake of MPs from shellfish consumption was 751 particles/per capita/year (Li, et al., 2022). Within Atlantic Canada, Mathalon and Hill, (2014) previously found an average of ~170 particles per 5 wild mussels and an average of ~375 particles per 5 retail mussels. However, polymer identification was not performed, and therefore the confirmation of plastics in bivalves was not established. Exploring MP characteristics such as morphology, size classes, and polymer type can provide information on the potential sources of pollution and potential risks to human health and the environment. Therefore, further research is needed in Atlantic Canada for quantifying and characterizing MP pollution in bivalves from various areas around Nova Scotia.

CHAPTER 3: Concentration and characterization of microplastics in Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) from Nova Scotia, Canada

3.1 Abstract

Plastics have become ubiquitous within society as production has increased over the last half-century. Due to their improper disposal, plastics have persisted and accumulated within coastal and marine aquatic ecosystems. Plastics of small size are abundant within marine ecosystems and are easily ingested by filter feeders such as mussels and oysters. Bivalves were collected from three zones across Nova Scotia, Canada. Mean concentrations of suspected microplastics (SMPs) were 4.25 ± 1.48 SMPs/g of wet weight tissue (48.59 ± 17.93 SMPs/individual) in Blue mussels and 3.79 ± 1.27 SMPs/g of wet weight tissue (53.54 ± 21.78 SMPs/individual) in Eastern oysters. Fragments or films were the most common morphology observed in both mussels and oysters across all sampling locations. SMPs were predominantly in the 2-10 μm size range in mussels and in the 10-20 μm size range in oysters. Polyethylene and polyvinyl chloride were the two dominant polymers observed based on micro-Raman results in both species. This study demonstrates that bivalves such as blue mussels and eastern oysters are suitable organisms for the assessment of MP marine pollution across locations. This research recommends future studies for exploring MPs in wild and farmed bivalves, as well as species differences in the accumulation and ingestion of MPs.

3.2 Introduction

Plastic and microplastic (MP) pollution in the ocean has become a global challenge as upwards estimates of 8 million metric tonnes (MT) of plastics enter the ocean annually. (Jambeck et al., 2015). It is estimated that 80% of marine plastic pollution is land-derived and can travel thousands of kilometers (km) from its initial disposal site (Horton & Dixon, 2018; Windsor et al., 2019). Various polymers such as polypropylene (PP), polyvinyl chloride (PVC), and polyethylene (PE) have properties that make them highly durable and persistent within the environment (Geyer, 2020; Geyer et al., 2017). Plastic debris can fragment through several degradative processes such as erosion, photodegradation, or biological mechanisms that produce smaller-sized particles known as microplastics (MPs) (Chamas et al., 2020; Lin et al., 2022). These are generally defined as plastic particles in the size range of 1 um to 5 mm (Thompson, 2015; Thompson et al., 2009). There are two categories of MPs. Primary MPs are industry derived and are produced as MPs. Secondary MPs are derived from the degradation of plastic debris through various mechanical, biological, and chemical mechanisms (Chamas et al., 2020; Lin et al., 2022). Most plastics are single-use and due to their improper disposal end up in aquatic ecosystems such as estuaries (Choong et al., 2021), Arctic ecosystems (Zhang et al., 2022), and coastal areas (Li et al., 2016). Within marine environments, MPs are ubiquitous in environmental matrices such as surface waters (Rakib et al., 2023; Smith et al., 2022) and sediment (Choong et al., 2021), as well as can be ingested by marine organisms such as fish (Karbalaei et al., 2019; Wang et al., 2017).

Filter feeders such as mussels and oysters are often studied for environmental pollutants due to their ubiquity in marine environments and economic significance (Bom

& Sá, 2021; Ding et al., 2022; Walker & MacAskill, 2014). Due to the small size of MPs, bivalves can ingest these particles and accidentally not reject them, therefore retaining them within their gut where translocation to other tissues may occur (Ward et al., 2019). Research suggests that these bivalves are selective feeders where some MPs are more readily ingested because they resemble their natural food sources in size, shape, and sometimes colour (Ward et al., 2019).

From the coasts of France (Phuong et al., 2018) and India (Saha et al., 2021), to benthic habitats in Argentina (Ríos et al., 2020) MPs in bivalves have been observed globally. Furthermore, toxicity and exposure studies have shown cellular and sub-cellular effects such as changes in gene expression and physiological responses in bivalves (Gardon et al., 2018; Kwon et al., 2021; Patra et al., 2022). MPs as vectors for pathogens (Kirstein et al., 2016; Bowley et al., 2021), heavy metals (Brennecke et al., 2016), and microorganisms (Bowley et al., 2021), have the potential to be transferred along marine food webs through bivalves (Crooks et al., 2019; Zhao et al., 2018). Bivalves therefore are also considered a potential vector for transferring contaminants to humans as they represent a significant food source globally (Dehaut et al., 2016; Masiá et al., 2022). MPs have been found in humans in tissue including the nervous system, respiratory system, and pancreas (Campanale et al., 2020). Therefore, the potential pathways of MP exposure to humans should be examined to characterize potential human health risks (Barboza et al., 2018; Walker et al., 2022; Wright & Kelly, 2017).

Within Atlantic Canada MP research in bivalves is limited, In previous studies, the average abundance of microfibers was observed in mussels across Nova Scotia (Mathalon & Hill, 2014). However, within the study's methods, they did not use any procedures for

characterizing polymer types and solely used visual analysis (Mathalon & Hill, 2014). Their results may not have accurately quantified or characterized plastics in mussels in Nova Scotia, and further research is needed. In addition, there have been no studies examining MP contamination in Eastern oysters within Nova Scotia. Therefore, research is needed to determine the MP load in these two species.

The aims of this study were: First, assess and compare the concentration of MPs in Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) from Nova Scotia. This includes a description of morphological properties such as size classification and shape. Second, characterize polymer types found in mussels and oysters from Nova Scotia. The implications of this study include a baseline study of MP concentrations in two bivalve species from Nova Scotia and the characterization of their polymers. Their importance as a food source highlights a potential pathway for exposure.

3.3 Methods

3.3.1 Study area and sampling locations

Nova Scotia is a province in Eastern Canada (Figure 3.1), which is well-known for its distinct fishing areas in the Atlantic Ocean, the Gulf of Maine, and the Bay of Fundy. Eastern Oysters are grown in the warm, shallow bays and estuaries of the southwestern Gulf of St. Lawrence along the coast of Prince Edward Island and New Brunswick, along the Atlantic coast of Nova Scotia from Whitehead to Argyle, and the coves of Cape Breton's Bras d'Or Lakes (Government of Canada, 2013). For this study 13 sampling locations were selected across Nova Scotia. Bivalve samples were selected based on shellfish closure information provided by the Department of Fisheries and

Oceans Canada (DFO), traditional knowledge, and previous studies on Nova Scotian bivalves. Before field collection, two scientific permits (Application for A Licence to Fish for Scientific, Experimental, Or Educational Purposes) were obtained from DFO, Gulf Region and DFO, Maritimes region to collect these organisms. Sampling sites were selected based on three Nova Scotia fishing zones: Eastern-Cape Breton, Gulf, and South-Southwestern zone (Fish Harvesters, 2023). A total of 13 sites were selected for the collection of bivalves. 10 locations were selected for the collection of Blue mussels and 5 for the collection of Eastern oysters. Two sites were selected where both mussels and oysters were collocated. Among sampling locations, oysters were only collected in the Gulf zone due to the suitable conditions for growth. The approximate location for 7 of the 13 sites is shown in Table 3.1. The disclosed locations represent where bivalves were found in the 'wild' which was defined loosely as sites where bivalve beds were thought to be naturally occurring. Three sampling locations for mussels and three sampling locations for oysters were marked as 'undisclosed sites' due to their proximity to shellfish aquaculture and considered 'more anthropogenically influenced'. Their approximate location and zone within Nova Scotia are shown in Table 3.1 and Figure 3.1. Exact locations were not disclosed to protect businesses. Site 2 (Halifax) was also considered 'more anthropogenically influenced' due to its proximity to trade, tourism, and residential activities.

Table 3.1 Approximate coordinates of sampling locations for Blue mussels and Eastern oysters in Nova Scotia. Some coordinates of sampling locations were undisclosed to protect businesses, approximate area was provided.

Organisms Collected	Sampling Location	Zone	Latitude (ºN)	Longitude (ºW)
Blue mussels	Halifax	Eastern-Cape Breton	44.629373°	-63.591135°
	Taylor Head		44.8068374°	-62.5571301°
	Martinique Beach		44.6893749°	-63.1405464
	Risser's Beach	South-Southwestern	44.2312641°	-64.238031°

	West Pennant		44.4695487°	-63.6535854°
	Undisclosed site	Eastern-Cape Breton	Approx. area – Eastern-Cape Breton zone	
	Undisclosed site	Gulf	Approx. Gulf zone	
	Undisclosed site	South-Southwestern	Approx. South-Southwestern zone	
Eastern oysters	Undisclosed site	Eastern-Cape Breton	Approx. Eastern-Cape Breton zone	
	Undisclosed site	Gulf	Approx. Gulf zone	
	Undisclosed site	South-Southwestern	Approx. Southern-Southwestern zone	
Blue mussels and Eastern oysters	Melmerby Beach		45.656361°	-62.507956°
	Tatamagouche		45.733508°	-63.285608 °

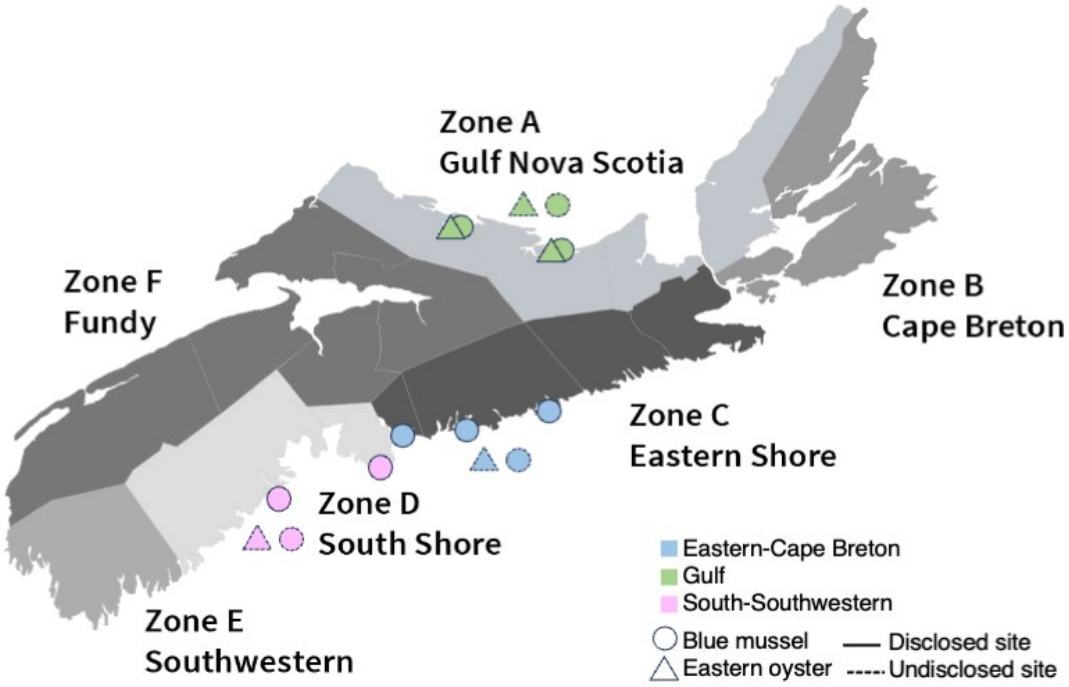


Figure 3.1 Map adapted from (Fish Harvesters, 2023) of sampling sites for Blue Mussels and Eastern oysters from Nova Scotia as described in Table 3.1. Note that oysters were sampled in colocation with mussels from both sites in the Gulf and not collected in other zones. Undisclosed sites denoted by a dashed line were plotted in the general zone to protect the identity of businesses.

3.3.2 Sample collection and storage

In the field, bivalves were collected by hand at low tide from May to August 2022 (Table 3.1) (Figure 3.1). Bivalves were rinsed with MilliQ® three times, placed in glass jars collectively, labelled corresponding to the site, and transported to the lab

immediately for initial processing. In the laboratory, six individuals were randomly selected from each site, and shell length, width, and depth were measured using a vernier calliper (Table 3.2). Individuals by site were then wrapped in tinfoil and numbered from 1-6. Bivalves were separated by species and site and were then collectively stored in glass jars submerged in a mixture of 100% ethanol and clove oil used to euthanize them. Glass jars were then labelled and refrigerated (4^{o}C) until further processing. Methods for the euthanasia of invertebrates were approved prior to collection by the Dalhousie University Committee on Laboratory Animals (Protocol No. 20-132).

Table 3.2 Mean ± standard deviation of shell length, shell width, shell depth, and soft tissues weight, and number of individual mussel and oyster samples from sites across Nova Scotia (M= mussels, O= oysters). Approximate zones include EC= Eastern-Cape Breton, G= Gulf, and SW= South-Southwestern.

Zone	Location	Code	Length (mm)	Width (mm)	Depth (mm)	Soft tissue weight (g/indi.)	# of indi.
EC	Undisclosed site	M1	63.00 ± 1.25	34.62 ± 1.83	27.80 ± 0.88	11.62 ± 1.13	6
	Halifax	M2	73.22 ± 4.42	31.22 ± 1.86	25.98 ± 2.04	11.31 ± 1.26	6
	Martinique Beach	M3	66.06 ± 3.89	37.48 ± 4.62	29.49 ± 4.13	11.91 ± 1.73	6
	Taylor Head	M4	71.95 ± 5.05	31.93 ± 2.93	26.15 ± 1.77	10.96 ± 1.10	6
G	Undisclosed site	M5	65.89 ± 2.44	34.29 ± 1.34	25.76 ± 1.79	13.82 ± 0.86	6
	Melmerby Beach	M6	66.12 ± 2.11	33.87 ± 2.36	28.53 ± 2.05	10.15 ± 0.40	6
	Tatamagouche	M7	62.79 ± 3.18	27.17 ± 0.70	18.31 ± 1.15	10.18 ± 1.03	6
SW	Undisclosed site	M8	66.77 ± 1.81	31.12 ± 1.75	25.13 ± 2.38	12.24 ± 1.79	6
	West Pennant	M9	63.05 ± 2.73	31.80 ± 3.44	26.75 ± 2.12	10.56 ± 1.13	6
	Risser's Beach	M10	68.02 ± 4.57	39.84± 1.34	36.67 ± 3.64	11.99 ± 1.30	6
EC	Undisclosed site	O1	68.32 ± 3.99	46.68 ± 1.74	23.52 ± 2.49	16.63 ± 0.67	6
G	Undisclosed site	O2	64.19 ± 2.22	55.34 ± 4.96	22.84 ± 2.70	13.59 ± 1.01	6
SW	Undisclosed site	O3	85.27 ± 2.92	46.43 ± 3.97	17.08 ± 2.69	13.17 ± 0.54	6
G	Melmerby Beach	O4	70.02 ± 4.67	45.38 ± 2.10	26.20 ± 4.20	14.22 ± 1.94	6
	Tatamagouche	O5	71.57 ± 4.09	42.01 ± 3.47	18.78 ± 2.65	11.55 ± 1.18	6

Table 3.3 Sample size, and mean ± standard deviation of shell length, width, depth, and wet weight tissue analyzed from sampled Blue mussels and Eastern oysters across Nova Scotia.

	Sample Size	Wet weight (g)	Shell length (mm)	Shell width (mm)	Shell depth (mm)
Blue mussels	n=60	11.47 ± 1.62	66.79 ± 4.80	33.33 ± 4.19	27.6 ± 4.92
Eastern oysters	n=30	13.83 ± 2.02	71.87 ± 9.19	47.17 ± 5.61	21.68 ± 4.48

3.3.3 Digestion

Mussel and oyster individuals were dissected and the whole soft tissue was weighed (g) and each transferred into a 250 mL test tube with 45 mL of filtered (1.2 µm) potassium hydroxide (KOH). Test tubes were placed on a heat block at 40ºC for 48 hours. 10 mL of hydrogen peroxide (H_2O_2) was pipetted into the test tube and taken off the heat until the reaction subsided. Samples were then placed on the heat block at 40ºC for an additional 24 hours. A maximum of 15 mL of ethanol was pipetted to the test tubes to prevent overflow. If the reaction was volatile and beginning to overflow, the contents were transferred to a 500 mL glass beaker until the reaction was subdued. The overflown test tubes were turned upside down into the beaker and covered with aluminum foil until all the contents were in the beaker. The contents for overflowed samples were only transferred back into a test tube and placed back on the heat block if the reaction subsided. Once tissues were digested based on the transparency of the remaining digestate, they were filtered using a vacuum filtration system on a glass microfibre filter with a pore size of 1.2 µm with an inner diameter of 22 mm (Whatman 1822-047 GF/C). To rationalize the methods used, Wang et al. (2021) reported in their study that using the combination of 30% H_2O_2 and 10% KOH at temperatures lower than 65˚C did not cause significant damage to analyzed polymers.

3.3.4 Density separation and filtration

Due to the incomplete digestion of tissue and additional organic material, non-plastic material was removed using a density separation protocol. Contents on the filter were washed with 100 mL of filtered $ZnCl_2$ (ρ = 2.91 g/cm³) and transferred into silicone

tubes to separate the MPs from the non-plastic material. The tubes were covered with aluminum foil and secured on a density separation instrument. The device was placed on an orbital shaker (New Brunswick orbital agitator) at 80rpm for 48 hours. The overlaying solution (~25%) was filtered through a vacuum filtration system onto a 1.2 μm glass microfiber filter (Whatman 1822-047 GF/C, diameter of 22 mm). Leftover $ZnCl_2$ was collected and re-filtered for further processing. Filters were stored in washed labelled aluminum tins until further observation.

3.3.5 Quality assurance and quality control measures

Quality assurance and control measures were taken throughout the lab to minimize background contamination. All surfaces before any sort of processing were rinsed three times with Milli-Q® water and covered with aluminum foil. Plastic equipment was reduced as much as possible. All solutions such as 100% ethanol, 10% KOH, $ZnCl_2$, and 30% H_2O_2 were filtered three times using a 1.2 μm pore filter and a vacuum pump before usage. For each filtration step, the inner walls were rinsed with MilliQ® three times, to reduce the loss of potential MPs. All instruments were covered with aluminum foil when not in use or transient usage to avoid background contamination. Cotton, linen, and non-plastic fabrics were always worn within the workspace. Procedural blanks were performed in parallel to sample processing to capture background contamination. 3 Blanks were conducted on average for every 7 bivalves processed. Blanks were subtracted from particle count accordingly based on the sample batch and date of the sample laboratory processing blanks. Blank correction data is available in the supplementary material (Appendix B). In addition, the ethanol storage

solution for samples of each was treated with 50mL of 30% H_2O_2 at room temperature for 24 hours and then filtered using a 1.2 μm pore filter for further analysis to capture potential MPs that may have been external to the bivalve, escaped the shell cavity or were egested as pseudofeces.

3.3.6 Nile Red and micro-Raman analysis

To quantify the number of SMPs, the filters were stained with a Nile Red solution (0.1 μg/L, in methanol as a solvent). Visual inspection of filters was carried out using a 40VA EPI-Fluorescence Trinocular Compound Biological Microscope (OMAX 40X-100X) at 10x objective and a total magnification of 100x. Filters were analyzed by imaging a vertical strip across from the inner diameter of one end of the filter to the other end representing about 12.9% of the total area of the filter (Allen et al., 2022; Erni-Cassola et al., 2017). Photos were taken with a digital camera attached to the fluorescent microscope and video/images were processed through Am Scope V4.11. From the Nile Red analysis, SMP sizes were assessed and distinguished as either fragment, film, or fibers (Figure 3.2). Images were processed and particles were analyzed for count and size in Image J (Version 2.90). Particles were distinguished as either fragments or fibers based on the value of circularity where a value of <0.33 indicated a fibrous shape. Particles smaller than 2μm were omitted to limit the detection of particles at a 100x magnification. The size classes were categorized into 6 groups: 2-10 μm, 10-20 μm, 20-30 μm, 30-40 μm, 40-50 μm, and >50 μm. The concentration of MPs was reported by the number of SMPs/g (wet weight tissue), or in abundance expressed as the number of SMPs per individual. Counts from the 12.9% of the filter were analyzed and scaled to represent the

whole inner diameter area of the filter and individual bivalves. It is recognized that using these methods, there may be particles >50 μm that may not have been captured by the analysis of the strip.

Polymer identification was done via spectroscopy using a micro-Raman confocal microscope (Renishaw inVia 830 nm) located at Memorial University in Saint John's Newfoundland and Labrador. One filter from each sample (~16% of total samples) that was not subjected to Nile Red treatment (n=15, 5 sites of oysters, 10 sites of mussels) was randomly selected and a straight line across the filter at a 20x objective with a total magnification of 200x was used to analyze particles (~4.8% of filter). The spectra were collected with a spectral range of 700-2000 nm^{-1} with an average exposure time of 10 seconds, between 0.001 and 100% laser power and 3 accumulations. The resultant spectra were matched against available spectral libraries SLOPP and SLOPP-E (Munno et al., 2020) and Open Space (Cowger et al., 2021). A >60% match rate was used across all sampled filters to characterize a snapshot of the potential polymer types found in mussels and oysters. Spectra were processed using Spectraglyph (v1.2.16.1). Random blanks (n=3) were also selected for μRaman analysis and a >60% match rate was also used. After filters were analyzed by the Raman, they were then analyzed using Nile Red microscopy.

3.3.7 Statistical analyses

All statistical analyses were completed using R Studio 4.3.0 (R Studio Team, 2020). A one-way analysis of variance (ANOVA) was used to examine the relationship between the concentration of SMPs in Blue mussels across sampling locations in Nova Scotia

(n=10). A one-way ANOVA was also used to examine the relationship between the concentration of SMPs in Eastern oysters across sampling locations in Nova Scotia (n=5). A two-way ANOVA was conducted to assess differences in SMP concentration among species and location within Melmerby Beach and Tatamagouche. It is hypothesized that there will be significant differences among sampling locations in mussels and oysters from Nova Scotia. It is also hypothesized that there will be significant differences among species and sampling locations within Melmerby Beach and Tatamagouche where bivalves were found co-located. For all tests, the observed SMP concentration data conformed to a normal distribution (Shapiro–Wilk, $p>0.05$) (CRAN package stats). and the variances were homogeneous (Levene's test, $p>0.05$) (CRAN package stats). For all tests, significant differences were determined by ANOVA Tukey-HSD post hoc investigation and plotted (Wickham et al., 2016). In addition, a two-way chi-square test of independence was used to examine the difference in proportions between species among the polymer classes. An alpha value of 0.05 was used for all tests.

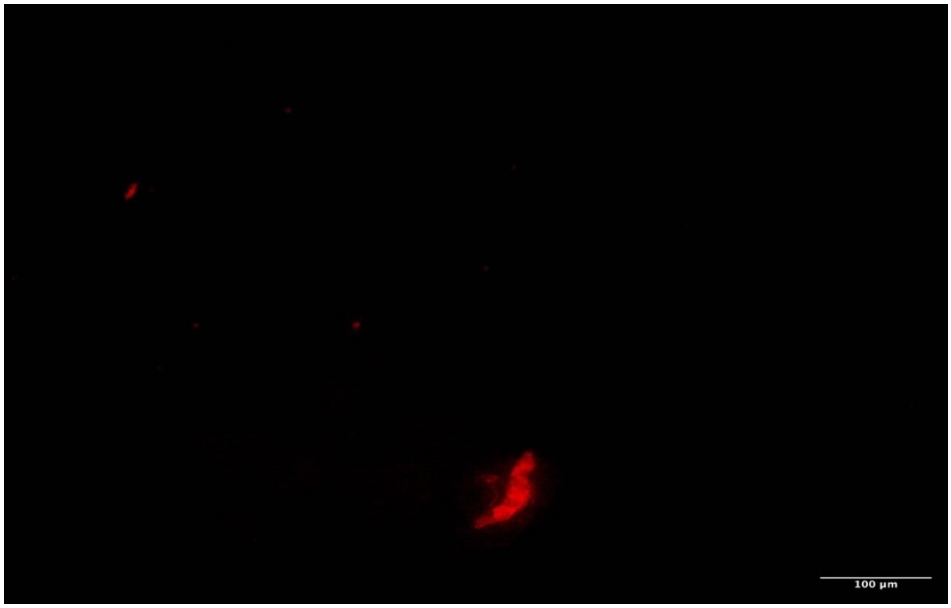
3.4 Results

3.4.1 Quality control and microplastic identification

The processing procedure was performed without any sample and analyzed using Nile Red Microscopy to evaluate any background contamination (Figure 3.4). Two blank tests were partially contaminated but corrected in corresponding samples that also displayed similar levels of apparent contamination due to Zinc chloride ($ZnCl_2$) spiked with microplastics. The outliers contained an estimated 597.55 and 2040.88 particles per filter. Omitting the two outliers, the blanks displayed an average of approximately 27.31

± 29.66 particles per filter (n=16). Within the blanks, on average 96.66% of identified particles were fragments or film and 3.08% were identified as fibers. The proportion of SMPs by size classes is as follows: 2-10 µm (61.22%), 10-20 µm (24.49%), 20-30 µm (8.16%), 30-40 µm (2.04%), and >50 µm (4.08%). Blank counts are shown in supplementary data Appendix B. The proportion of polymers that may have been found in the blanks was 50% PVC, 25% acrylonitrile butadiene styrene (ABS), and 25% PE. The ethanol storage solution for each site showed an average of approximately 4.13 ± 1.27 suspected particles per filter per 6 bivalves stored in ethanol.

a) Mussel



b) Oyster

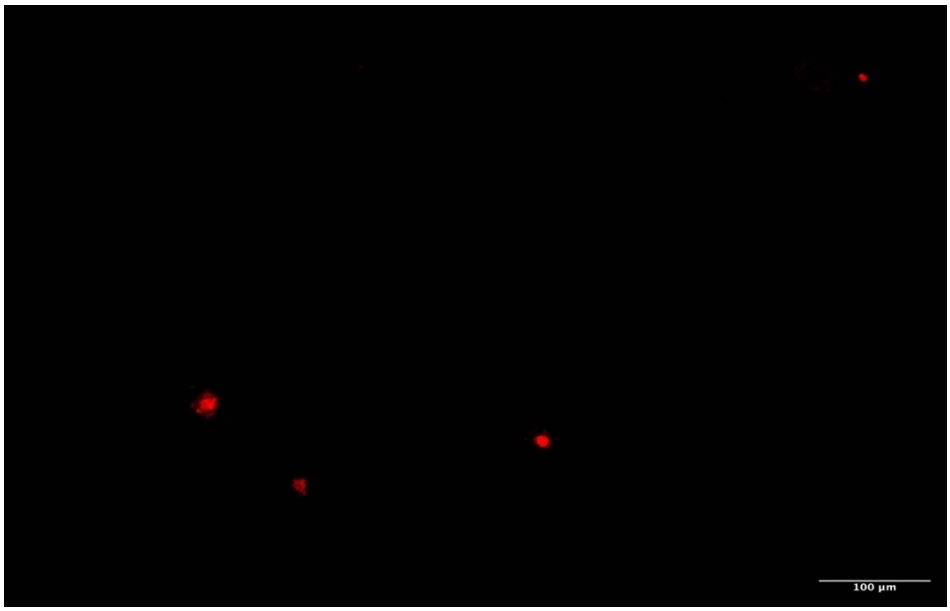


Figure 3.2 Nile red microscopy of fragments or films and fibers found in a) mussel and b) oyster samples.

3.4.2 Microplastics in Blue mussels from Nova Scotia

In Blue mussels, shell lengths ranged from 82.46 mm to 58.84 mm, shell widths ranged from mm to 46.17 mm to 26.19 mm and depths from 40.20 mm to 16.65 mm. Mean values are shown in Table 3.3. The frequency of SMPs was 98.33% in mussels across all sampling locations. The mean concentration of SMPs was 4.25 ± 1.48 SMPs/g of wet weight tissue (mean ± standard deviation) and 48.59 ± 17.93 SMPs per individual mussel (n=60) (Table 3.4). Using a one-way ANOVA and Tukey HSD tests, significant differences in SMP concentration were observed among mussels across sampling locations ($F = 84.81$, $df = 9$, $p = 1.39e\text{-}08$) in Nova Scotia (Figure 3.3). (Appendix B Table B6-7). SMP concentration at sites 1 (Undisclosed site in the Eastern-Cape Breton zone) and 2 (Halifax) were significantly higher than site 4 (Taylor Head) ($p<0.05$). Site 9 (West Pennant), and site 10 (Risser's Beach) showed significantly lower SMP concentrations in mussels than the undisclosed site in the South-southwestern zone (site

8) ($p<0.05$). In addition, across Nova Scotia, sites 9 and 10 had significantly lower SMP concentrations than sites 1, 2, 3 (Martinique Beach), 5 (Undisclosed in the Gulf zone), and 6 (Melmerby Beach) ($p<0.05$). SMP concentrations at site 9 were also significantly lower than at site 7 (Tatamagouche) ($p<0.05$). The most common size class of SMPs in Blue mussels was in the 2-10 µm (52.62%) range followed by 10-20 µm (27.86%), 20-30 µm (8.87%), 30-40 µm (5.06%), 40-50 µm (3.19%), and the >50 µm (2.40%) range (Figure 3.4). The proportion of suspected fragments or films to fibers was 92.55% and 7.45%, respectively Figure 3.4. From the micro-Raman analysis, the most identified polymer in mussels was PE (33.33%), followed by PVC (25.64%), ABS (15.38%), polyamide (PA) (12.82%), polysulfone (PLS) (5.13%), polypropylene (2.56%), polyacrylonitrile (PAN) (2.56%), and cellulose acetate (2.56%) as shown in Figure 3.6.

Table 3.4 Mean concentration or abundance (mean ± standard deviation) of SMPs in Blue mussels and Eastern oysters across Nova Scotia (M= mussels, O= oysters). Approximate zones include EC= Eastern-Cape Breton, G= Gulf, and SW= South-Southwestern.

Zone	**Location**	**Site #**	**Code**	**Concentration**			
				Mussel		**Oyster**	
				SMPs/g wet weight	**SMPs/ individual**	**SMPs/g wet weight**	**SMPs/ individual**
EC	Undisclosed site	1	M1	5.79 ± 0.55	67.25 ± 8.87		
	Halifax	2	M2	5.56 ± 0.79	62.08 ± 6.34		
	Martinique Beach	3	M3	4.41 ± 1.08	51.09 ± 7.23		
	Taylor Head	4	M4	3.27 ± 0.44	51.09 ± 4.14		
G	Undisclosed site	5	M5	5.22 ± 0.65	35.57 ± 6.99		
	Melmerby Beach	6	M6/O4	4.53 ± 0.63	45.91 ± 6.10	3.13 ± 0.38	43.97 ± 4.84
	Tatamagouche	7	M7/O5	4.14 ± 0.75	42.03 ± 7.89	2.00 ± 0.55	22.63 ± 4.71
SW	Undisclosed site	8	M8	4.98 ± 1.34	59.06 ± 11.51		
	West Pennant	9	M9	2.19 ± 1.40	21.99 ± 13.59		
	Risser's Beach	10	M10	2.44 ± 0.64	29.10 ± 8.31		
EC	Undisclosed site	11	O1			5.06 ± 0.61	84.07 ± 9.76
G	Undisclosed site	12	O2			4.93 ± 0.59	66.61 ± 6.50
SW	Undisclosed site	13	O3			3.85 ± 0.53	50.44 ± 5.54
	Mean mussels			4.25 ± 1.48	48.59 ± 17.93		
	Mean oysters					3.79 ± 1.27	53.54 ± 21.78

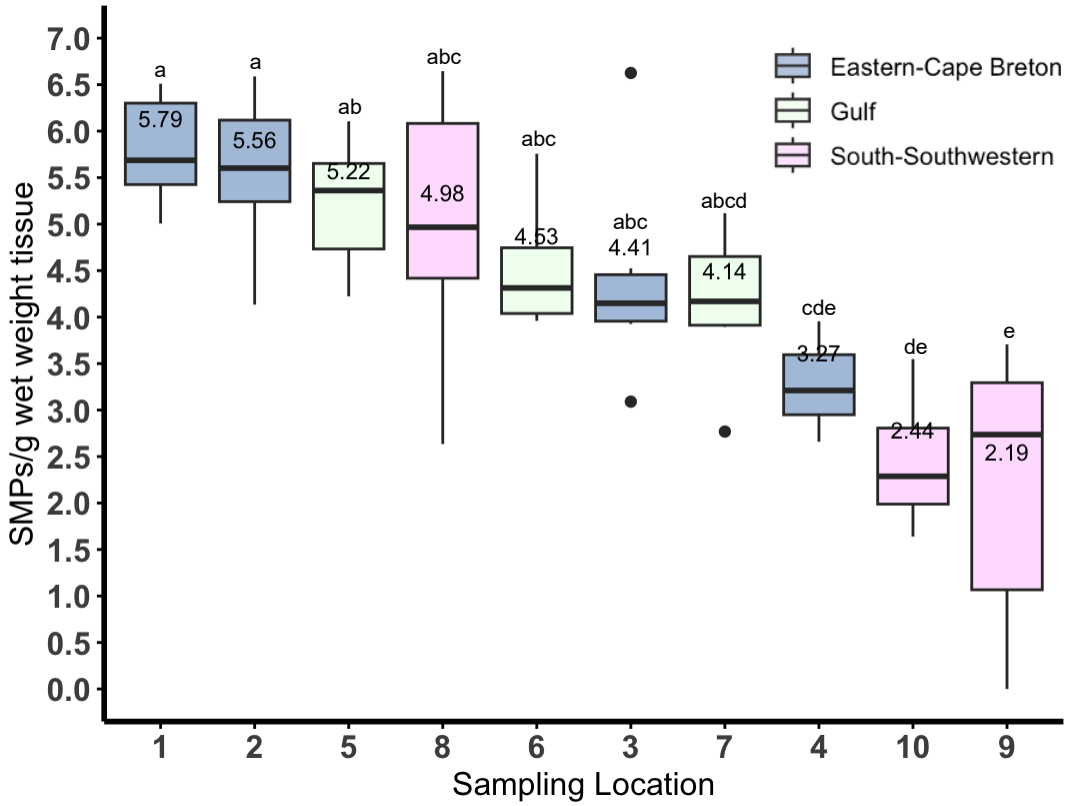


Figure 3.3 The concentration of SMPs/g of tissue in Blue mussels (n=10) from different sampling sites across Nova Scotia. Different letters indicate statistically significant differences between sites ($p<0.05$).

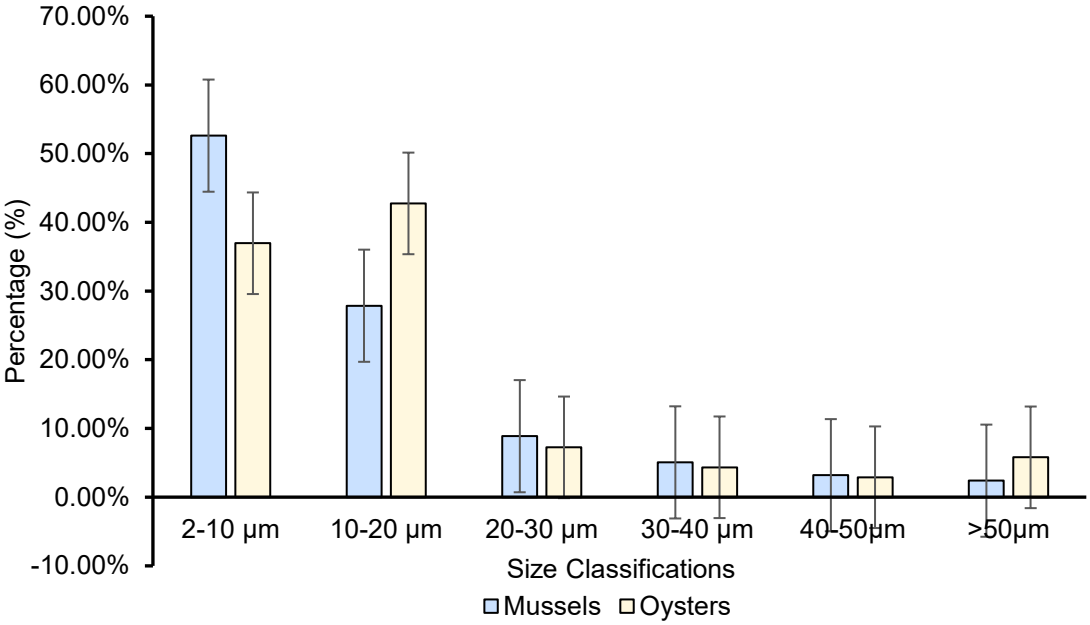


Figure 3.4 Mean size classifications of SMPs in mussels and oysters sampled from Nova Scotia. Error represented as standard error.

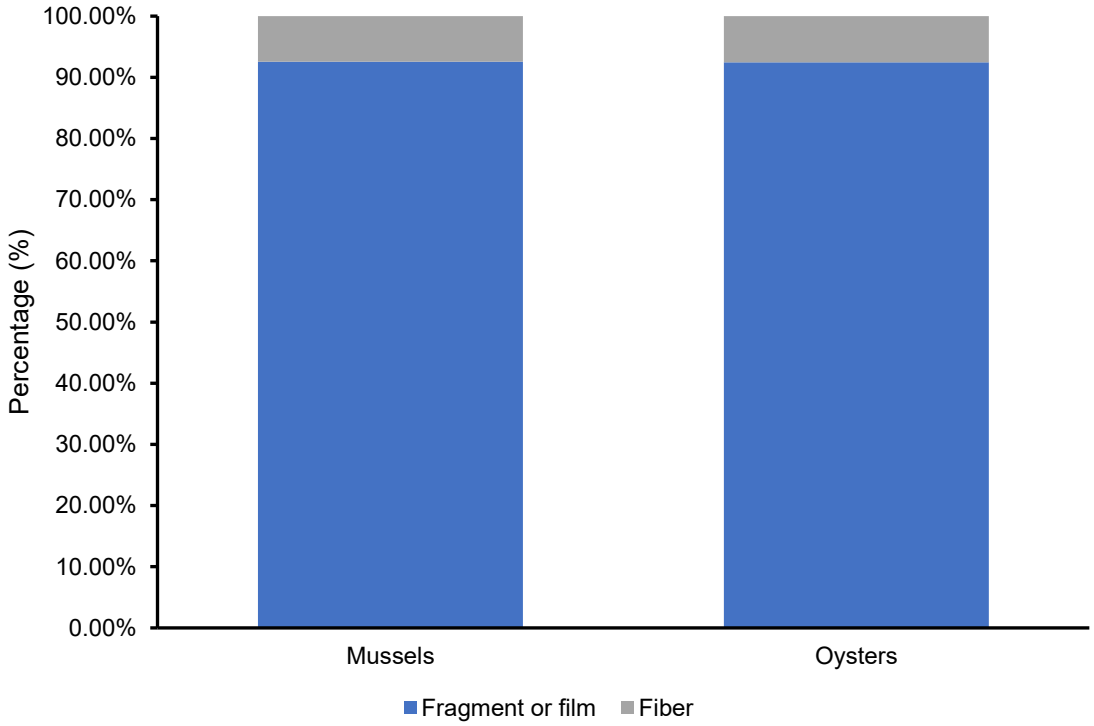


Figure 3.5 Proportion of the morphologies ('Fragment or film' or 'fibers') of SMPs in mussels and oysters sampled from Nova Scotia.

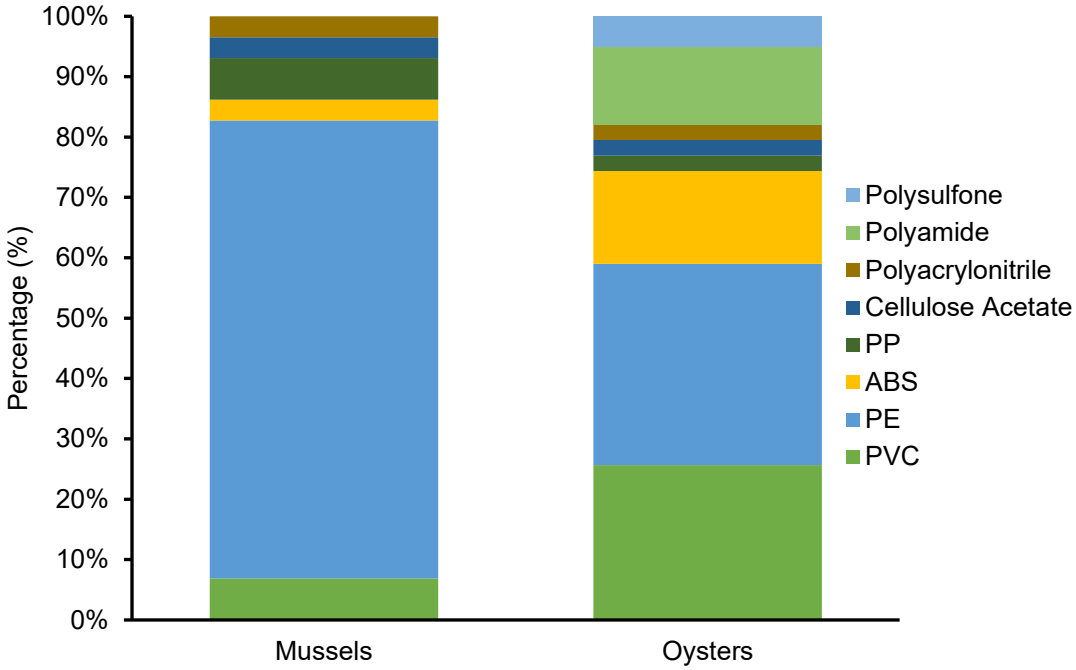


Figure 3.6 The proportion of polymers found in a) mussels and b) oysters from Nova Scotia. PVC = polyvinyl chloride, PE = polyethylene, ABS = acrylonitrile butadiene styrene PP = polypropylene.

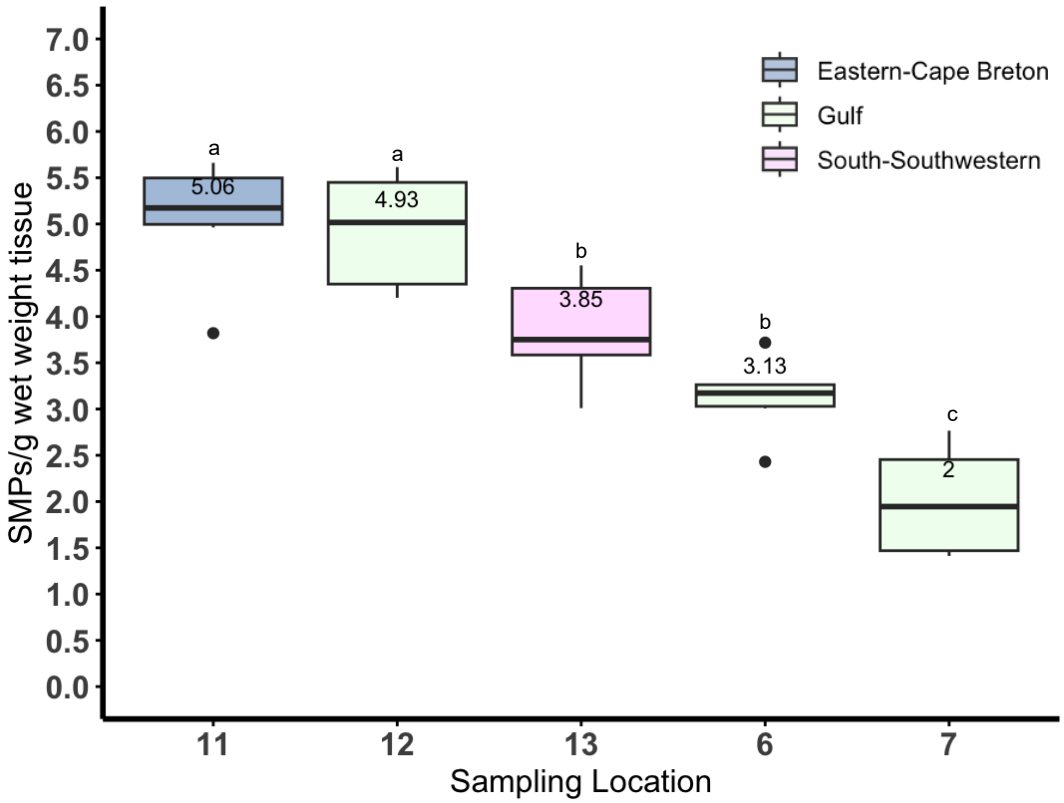


Figure 3.7 The concentration of SMPs/g of tissue in Eastern oysters (n=5) from different sampling sites across Nova Scotia. Different letters indicate statistically significant differences between sites ($p<0.05$).

3.4.3 Microplastics in Eastern oysters from Nova Scotia

In Eastern oysters, shell length ranged from 89.79 mm to 60.63 mm, shell width 63.82 mm from to 35.86 mm, and shell depth from 31.47 mm to 13.77 mm. Averages for these metrics are shown in Table 3.3. The frequency of SMPs was 100% in oysters across all sampling locations. SMPs were found in all oyster samples where the mean concentration of SMPs found in oysters was about 3.79 ± 1.27 SMP/g of wet weight tissue and about 53.54 ± 21.78 SMPs per individual (n=30) (Table 3.4). The mean approximate diameter of SMPs was 17.21 µm in oysters. Using a one-way ANOVA and a Tukey HSD test, significant differences in SMP concentration were observed among Eastern oysters across sampling locations ($F = 39.34$, $df = 4$, $p = 5.77e\text{-}09$) in Nova

Scotia (Figure 3.7) (Appendix B Table B8-9) The undisclosed site in the Gulf zone (Site 12) showed significantly higher SMP concentrations than sites 6 (Melmerby Beach) and site 7 (Tatamagouche) ($p<0.05$). SMP concentrations were significantly lower at site 7 than at site 6, 11 (Undisclosed in the Eastern-Cape Breton zone), 12 (Undisclosed in the Gulf zone), and 13 (Undisclosed in the South-Southwestern zone) ($p<0.05$). There were no significant differences between sites 11 and 12 ($p=0.99$) and 13 and 6 ($p=0.24$). The common size class for SMPs in Eastern oysters were in the 10-20 μm (42.57%) size class followed by 2-10 μm (36.96%), 20-30 μm (7.25%), 30-40 μm (4.35%), >50 μm (5.80%), and in the 40-50 μm (2.90%) range as shown in Figure 3.4. The proportion of suspected fragments or films to fibers in Eastern oysters was 92.43% and 7.57%, respectively (Figure 3.5). From the micro-Raman analysis, the most identified polymer that was found in Eastern oysters was PE (75.86%), followed by PVC (9.90%), PP (6.90%), ABS (3.45%), cellulose acetate (3.45%), and PAN (3.45%) as shown in Figure 3.6.

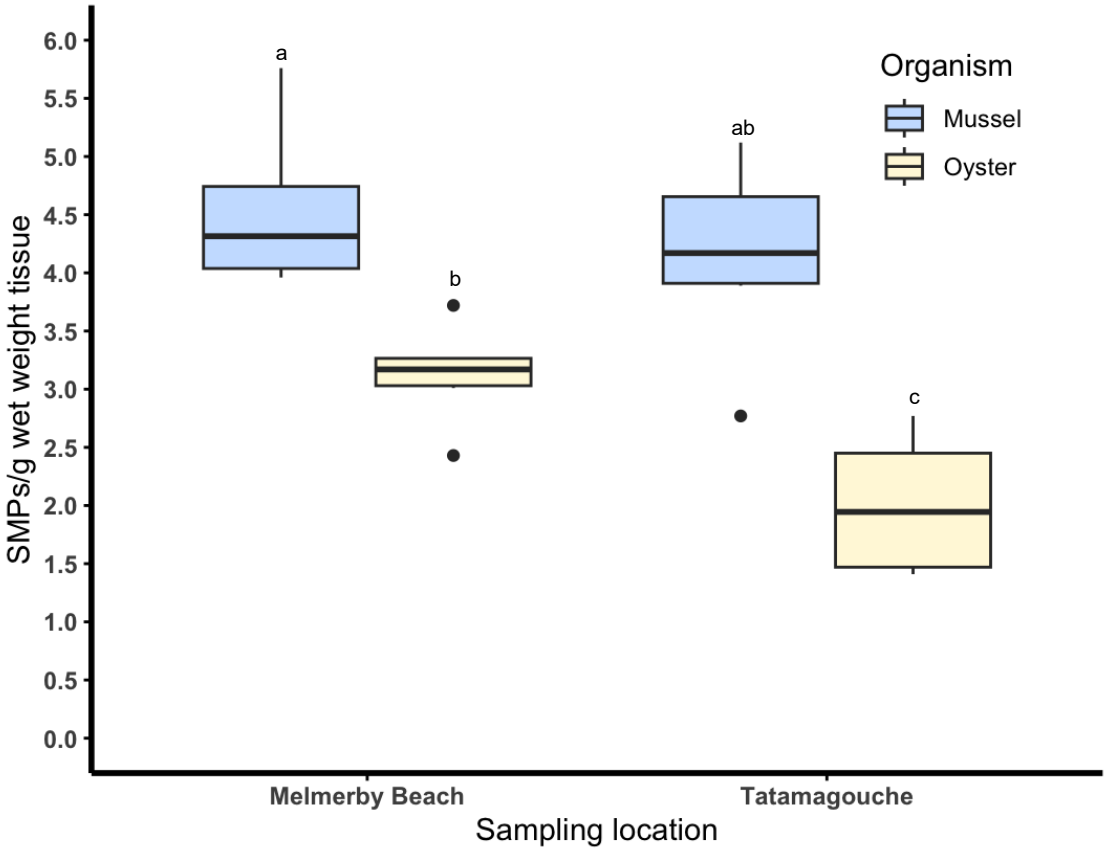


Figure 3.8 Concentration of SMPs in mussels and oysters from Melmerby Beach (Blue), and Tatamagouche (Yellow) from the Gulf zone of Nova Scotia. Different letters indicate statistically significant differences between sites ($p<0.05$).

3.4.4 Microplastics in mussels and oysters from Nova Scotia

The two locations in the Gulf zone where both mussels and oysters were co-located showed significant differences based on species and sampling location (two-way ANOVA). There were no interactive effects between the organism and sampling location observed within this dataset ($p=0.17$). There was a significant difference between species ($F = 8.19$, $df = 1$, $p=0.009$) and locations ($F = 44.96$, $df = 1$, $p = 1.59e-06$) (Appendix B. Table B10). Differences occurred in Melmerby Beach where SMP concentrations were significantly higher in Blue mussels than in Eastern oysters ($p<0.05$) (Figure 3.8). Differences also occurred in Tatamagouche where SMP concentrations were significantly

higher in Blue mussels than in Eastern oysters ($p<0.05$). SMP concentrations were significantly lower in Eastern oysters from Tatamagouche than in Blue mussels from Melmerby Beach ($p<0.05$). Furthermore, SMP concentrations were significantly higher in Eastern oysters from Melmerby Beach than in oysters from Tatamagouche ($p<0.05$). There were no significant differences observed in Blue mussels from Melmerby Beach and Tatamagouche ($p=0.73$). In addition, there were no significant differences in SMP observed between Eastern oysters from Melmerby Beach and Blue mussels from Tatamagouche ($p=0.058$).

Examining the proportions of polymers found in both bivalves, results from the two-way chi-square test showed there were no differences among species ($p=0.99$) (Figure 3.6) (Appendix B Table B12).

3.5 Discussion

3.5.1 Microplastics in Blue mussels and Eastern oysters from Nova Scotia

Results from this study were generally lower than findings from previous studies performed in Nova Scotia where Mathalon & Hill, (2014) observed microfibers in Blue mussels from Nova Scotia. They found the 5 mussel subsamples contained an average of 170 MP particles per five wild mussels and an average of 375 SMP particles per 5 retail mussels (Mathalon & Hill, 2014). However, within their methods, they did not use any procedures for characterizing polymer types in this study and solely used visual analysis (Mathalon & Hill, 2014). In other studies across Canada, concentrations of 212.80 to 77.12 MPs/g in oysters were found from coastal and aquaculture farms in British Columbia, Canada (Murphy, 2018). The lack of polymer identification in methodologies

may lead to the overestimation of MPs; as well, the use of harsh acids may additionally fragment or degrade plastics (Catarino et al., 2017; Courtene-Jones et al., 2017).

In contrast, Noel et al. (2022) and Covernton et al. (2022) found concentrations of 0.24 ± 0.04 and 0.04 ± 0.06 SMPs/g of tissue in mussels and oysters, respectively which are much lower. Other concentrations have been reported globally and are generally lower or similar to results from this study (Table 3.5, Table 3.6, and Table 3.7). The variations in methodology, particle sizes, and the reporting of MP load in bivalves make finding comparable studies challenging. For instance, research suggests that the counting of Nile Red images may be overestimated by 11-67% due to the presence of organics (Nel et al., 2021). However, the addition of H_2O_2, to further degrade organics may address some of these issues (Erni-Cassola et al., 2017).

Table 3.5 Summary of some research results on MPs in mussels.

Country	**Species**	**Digestion Method**	**Polymer Method**	**Concentration (MPs/g)**	**Abundance (MPs/ind.)**	**Ref.**
Canada, Nova Scotia	*M.edulis*	10% KOH + 30% H_2O_2	Raman	4.25 ± 1.48	48.59 ± 17.93	This study
Morroco, Tunisa	*M.galloprovincialis*	10% KOH	FTIR, SEM-EDX	1.27 ± 0.42		Abelouah et al. (2023)
India	*P.viridis*	10% KOH	Raman	3.28 ± 0.87		Dowarah et al. (2020)
China	*P.viridis*	10% KOH	FTIR	0.36 ± 0.81		Lin et al. (2022)
Canada, Nova Scotia	*M.edulis*	30% H_2O_2	N/A		~34 (wild) ~75 (farmed)	Mathalon & Hill, (2014)
Canada, British Columbia	*M.edulis*	Corolase 7090 (AB Enzymes)	FTIR	0.24 ± 0.04		Noel et al. (2022)
Thailand	*P.viridis*	10% KOH + 30% H_2O_2	FTIR	0.07 ± 0.19		Phaksopa et al. (2023)

Table 3.6. Summary of some research results on MPs in oysters.

Country	Species	Digestion Method	Polymer Method	Concentration (MPs/g)	Abundance (MPs/ind.)	Ref.
Canada, Nova Scotia	*C.virginica*	10% KOH + 30% H_2O_2	Raman	3.79 ± 1.27	53.54 ± 21.78	This study
Canada, British Columbia	*C.gigas*	10% KOH	FTIR	0.02 ± 0.03 to 0.04 ± 0.06		Covernton et al., 2019
Vietnam	C.gigas	10% KOH + 30% H_2O_2	FTIR	1.88 ± 1.58		Do et al. (2022)
Taiwan	*Crassostrea and Saccostrea*	30% H_2O_2	Raman	3.24 ±1.02		Liao et al. (2021)
Canada, British Columbia	*C.gigas*	68–70% HNO_3	FTIR	77.12 ± 126 (wild) 212.80 ± 153.80 (farmed)		Murphy, (2018)
Australia	*C.gigas S.glomerta*	10% KOH	FTIR	0.09 ± 0.01		(Wooton et al., 2022)

Table 3.7 Summary of some research results on MPs in both mussels and oysters where, M=mussel, O=oyster.

Country	Species	Digestion Method	Polymer Method	Concentration (MPs/g)	Abundance (MPs/ind.)	Ref.
Canada, Nova Scotia	*M.edulis C.virginica*	10% KOH + 30% H_2O_2	Raman	M: 4.25 ± 1.48 O: 3.79 ± 1.27	M: 48.59 ± 17.93 O: 53.54 ± 21.78	This study
China	*M.galloprovincialis, C. gigas*	KOH	Raman	M: 1.9 ± 2.1 O: 0.4 ± 0.4		Lerebours et al. (2022)
India	*Perna viridis, Crassostrea sp.*	30% H_2O_2	FTIR	M: 3.2 ± 1.8 O: 4 ± 2		Saha et al. (2021)
Germany	*M.edulis, C. gigas*	69% HNO_3	Raman	M: 0.36 ± 0.07 O: 0.47 ± 0.16		Van Cauwenberghe & Janssen (2014)
China	*M.edulis, C. gigas*	10% KOH	FTIR	M: 0.21 ± 0.21 O: 0.77 ± 0.81		Zhang et al. (2022)

Sampling location was observed to be a significant factor influencing MP concentrations within both Blue mussels and Eastern oysters. Mussel and oyster populations may be especially susceptible to MP pollution due to their proximity to

coastal areas, human activities, and land-based plastics (Cluzard et al., 2015; Klein et al., 2022; Mathalon & Hill, 2014). Nova Scotia has a unique shellfish aquaculture industry that spans all sides of the province, especially in high tourism areas such as Cape Breton and the Eastern Shore, areas known for having ports supporting fishing activities (Government of Canada, 2016). Within each zone, mean concentrations of SMPs found in both mussels and oysters were observed to be higher at undisclosed sites than disclosed sites within their respective zones regardless of whether they were significant or not. Undisclosed sites were categorized as 'more anthropogenically influenced' due to their collection in proximity to shellfish aquaculture. Site 2 (Halifax) was also considered 'more anthropogenically influenced' due to the high frequency of activities from residential areas, shipping, and tourism. This trend is evident, in mussels, where undisclosed sites 1 (Eastern-Cape Breton zone), 5 (Gulf zone), 8 (South-Southwestern zone), and site 2 (Halifax) were considered 'more anthropogenically influenced' and showed observed higher mean concentrations than sites 3 (Martinique Beach), 4 (Taylor Head), 6 (Melmerby Beach), 7 (Tatamagouche), 9 (West Pennant), and 10 (Risser's Beach) which were disclosed or considered 'wild' sites where mussel beds were thought to be found naturally occurring. Furthermore, in oysters, undisclosed sites 11 (Eastern-Cape Breton zone), 12 (Gulf zone), and 13 (South-Southwestern zone) had higher observed mean SMP concentrations than disclosed or 'wild' sites 6 and 7. This trend was particularly evident in oysters from the Gulf zone where the undisclosed site (site 12) showed significantly greater SMP concentrations than site 6 and site 7. Furthermore, in the South-Southwestern zone, mussels in the undisclosed site (site 8) showed significantly greater SMP concentrations than site 9 and site 10. Regardless of if these

sites were significantly different or not, potential trends in MP concentrations in bivalves from 'more anthropogenically influenced' and 'wild' sites should be further studied. This has implications for future studies examining MPs in aquaculture-raised and wild-caught bivalves which have been performed in other regions globally (Birnstiel et al., 2019; Phuong et al., 2018).

In addition, differences in concentrations across sampling sites may also be influenced by currents in Atlantic Canada. The Labrador and Scotian shelf currents may influence MP deposition from Cape Breton down towards the Southwestern shore (Government of Canada, 2019). The Gulf of St. Lawrence current may influence deposition across the Gulf zone. MPs may be deposited along the Northumberland Strait and up and around the Eastern-Cape Breton zone (Government of Canada, 2019). Deposition of MPs in mussels and oysters may be dependent on a variety of factors including hydrodynamic forces, wind currents, salinity, and polymer density, which may influence MP deposition and abundance in sediments and surface waters (Horton & Dixon, 2018; Thompson, 2015; Windsor et al., 2019).

Furthermore, the species of bivalve was also a factor that influenced concentrations and differences were observed where mussels and oysters were co-located. These differences were most apparent from the site of Melmerby Beach where tourism and recreation are common (Tourism Nova Scotia, 2023). This has also been found in other studies globally, where concentrations of MPs have been observed to be higher in mussels than in oysters from France (Lerebours et al., 2022), and China (Pan et al., 2022). In contrast, studies from Spain (Expósito et al., 2022), the Netherlands (Leslie et al., 2017), and India (Saha et al., 2021) found the opposite (Table 3.7). There are however

limited studies on the uptake of MPs from mussels and oysters from co-located sampling sites.

3.5.2 Characteristics of microplastics in mussels and oysters from Nova Scotia

The most common shape was fragments or films followed by fibers in both sampled mussels and oysters. In contrast, the most common shapes found on coastlines and surface waters are fibers (Carvalho Ferreira et al., 2023 Fagiano et al., 2023). Furthermore, studies from the United States (Klasios et al., 2021), Tunisia (Abidli et al., 2019), and China (Qu et al., 2018) found a higher proportion of fibers in comparison to other MP shapes. This may be due to common polymers found in waters along shorelines such as PE which can further degrade into fragments through photoaging (Sorasan et al., 2022). Fragments may settle faster in the water column than fibers and may be more available to mussels for ingestion (Mendrik et al., 2023). For instance, Mendrik et al. (2023) found that clean fibers of various polymers settled slower than fragments despite similar densities.

These findings about morphology are also consistent with results from other studies that have found fragments as the dominant MP shape from Korea (Cho et al., 2021), France (Phuong et al., 2018), and New Zealand (Webb et al., 2019). Furthermore, the differences in shape morphology among sites may be due to proximity to urban, fishing, and residential areas (Andrady, 2011; Thompson, 2015). For instance, Napper et al. (2022), found that PE fishing ropes shed more MP fragments than fibers from abrasion. This suggests that bivalves sampled in proximity to shellfish aquaculture may ingest more fragmented shaped MPs due to the shedding behaviours of PE fishing ropes.

The most common size class was in the 2-10 μm range and the 10-20 range for mussels and oysters, respectively. These results are consistent with findings from Murphy (2018) who found higher proportions of MPs in the <20 μm and 20-50 μm size class for oysters. In contrast, Joshy et al. (2022) found dominant size classes in bivalves in the >1mm range. The larger size classes observed in oysters may also be due to sampling location and the unique environments where bivalves are found (Ward et al., 2019). Within surface waters, observed size distribution at the sea surface generally shows, from large to small sizes, a gradual increase followed by a rapid decrease (Eo et al., 2018; Isobe et al., 2014). The decrease may be due to the hypothesis that the smallest fragments are selectively removed by sinking or biological uptake or because the mechanical energy required to produce such small fragments occurs more rarely (Aoki & Furue, 2021). This can also be influenced by proximity to cities where small MPs can bypass filters from washers, and therefore may potentially be available for bivalves to ingest (Carr et al., 2016; Kazour, Terki, et al., 2019; Leslie et al., 2017).

In addition, feeding strategies and preferences of mussels and oysters may influence MP size ingested and retained. Trends tend to show the abundance of MPs tended to decrease with increasing particle size (Liu et al., 2021). Smaller MPs at the < 20 μm range have been shown to transfer to different tissues and cause physiological effects on mussels (Browne et al., 2008). This is concerning, as most evidence, especially in mammalian and human observations, indicates that <130 μm could be the potential threshold for particle toxicology, with particles <10 μm potentially posing a greater risk (Browne et al., 2008; Papageorgiou et al., 2014; Volkheimer, 2001; Wright and Kelly, 2017). Ward et al. (2019) found that feeding behaviours in Blue mussels and Eastern

oysters rejected larger microspheres on average compared to smaller microspheres (Ward et al., 2019). For instance, Van Cauwenberghe et al. (2015) found larger MPs were detected in the feces of field *M. edulis* (15–500 μm) compared with those in the soft tissue (20–90 μm). Studies have also observed that smaller MPs (~3.0 μm) have higher accumulation rates in mussel tissues (Kazour & Amara, 2020; Hermabessiere et al., 2019).

Furthermore, differences in the size of MPs ingested in mussels and oysters may be attributed to physiological differences. Reasons for these observed differences may be due to the gill structure between mussels and oysters, where the heterorhabdic gill structure of oysters, which performs bidirectional transport and particle selection, differs from mussels, where their homorhabdic gill structure performs a predominately unidirectional transport (Ward et al., 2019). Oysters have two sites for particle selection whereas mussels only contain one (Ward et al., 2019). Two sites of selection may influence the accumulation of different-sized MPs in tissues. Other studies have suggested that the size of MPs influences the retention and elimination times of ingested MPs, as well as their potential to accumulate in tissues (Kinjo et al., 2019; Ward et al., 2019). Characteristics such as the size and shape of MPs ingested in bivalves can inform on the potential behaviours and sources of MPs, and their fate in the environment.

3.5.3 Polymer types and their potential effects on mussels and oysters from Nova Scotia

Polymer types assessed in this study were used to confirm the potential presence of plastics. Since a >60% hit rate was used this is a snapshot of potential polymers within bivalves found in Nova Scotia and further spectroscopic analysis with a higher threshold

is recommended. Major polymer groups detected in Blue mussels and Eastern oysters were both PE and PVC (Figure 3.6). This was confirmed by other studies, which observed the presence of PE and PVC in bivalves (Kor et al., 2023; Sparks et al., 2021; Wootton et al., 2022). The most widely produced polymers are estimated to be PE (38%), followed by PP (24%), and PVC (19%) in total global production (Andrady, 2011). Mid-density plastics such as PVC (ρ=1.38 g/cm^3) may be suspended within the water column for longer periods due to their similar density to seawater (Choy et al., 2019). Low-density plastics such as PE (ρ= 0.95–0.96g/cm^3) tend to float on surface waters but may be available for mussels and oysters at lower tides (Erini-Cassola et al., 2019; Expósito et al., 2022). In addition, sediment-dwelling bivalves may have more contact with sediment-bound MPs, which can include both low-density and medium-density plastics (Scott et al., 2019). Several studies showed that bivalves are capable of ingesting different types of polymers and the most common plastic inside their soft tissues differed from one study to another (Christoforou et al., 2020; Kazour et al., 2019).

In addition, proportions of PVC have been observed to be ingested in bivalves where potential sources may include film, pipe, containers, window frames, flooring, and shower curtains (Bom & Sá, 2021; Coyle et al., 2020). PVC is also used in finfish mariculture and pipe and valve fittings for offshore cages (Skirtun et al., 2022). Other polymers identified include ABS which is often used in outer casings for electronics and toys, while cellulose acetate is a synthetic biodegradable plastic present in products such as cigarette butts, which are often found on tourist beaches (Kühn et al., 2017). Cellulose acetate is naturally present in the marine environment but is also found in clothes, and various films but is understood to not persist due to its biodegradability (Kühn et al.,

2017; Yadav & Hakkarainen, 2021). Polyacrylonitrile (PAN), is an acrylic fibre used to make socks, hats, outdoor textiles, tents, and yacht sails due to its resistance to sun damage (Wright et al., 2020).

Polyamide (PA), or nylon, is a polymer related to carpet and staple fibers, textiles and industrial filaments including fishing nets, and lines (Fernández-González et al., 2021). These polymers may be derived from synthetic fibers released by wastewater treatment plants, or from fishing nets or other various aquaculture gear used in mussel aquaculture. In addition, PA were the second most common type of MPs found in the water column of the Gulf of Lions (Lefebvre et al., 2019) and large quantities of PA particles were discovered in shrimps (Hossain et al., 2020).

Lastly, polysulfone (PLS) was a polymer identified in blue mussels from Nova Scotia and is used in water treatment, fluid handling, medical applications, and the agricultural industry (Malankowska et al., 2021; Price, 2019). There is limited information on the release of PSU in the marine environment, but its application in wastewater treatment filtration membranes may be a potential source.

3.5.4 Environmental implications and limitations of measuring microplastics in mussels and oysters from Nova Scotia

Past research has confirmed the ingestion and accumulation of MPs within bivalve tissues which may have health implications for bivalves themselves and potentially humans. For instance, physiological and cellular effects have been observed in oysters such as the initiation of oxidative stress (Kwon et al., 2021), disruption of feeding activity, metabolic, or energy balance (Gardon et al., 2018), and reductions in oocyte

numbers and sperm velocities (Sussarellu et al., 2014; Sussarellu et al.,2016). This may have population-level implications where mussel beds located in MP-rich waters may have lower fitness due to cellular and physiological stress (Shang et al., 2021).

In addition, the transfer of MPs in bivalves across food webs is possible. Trophic level movement of MPs has been examined by Crooks et al. (2019), where they fed mussels 50 µL (~4.1×10^6) of 0.5 µm PS fluorescent MP spheres and then fed them to velvet swimming crabs (*Necora puberty*). MPs were present in all tissues sampled and remained present for the duration of the trial and both the testes and stomach showed a significant increase in the number of MPs present with the number of mussels consumed (Crooks et al., 2019). This poses potential human health risks, as species of mussels and crabs are economically significant seafood options for consumption (Dehaut et al., 2016). In contrast, Catarino et al. (2018) found that the potential for MP ingestion from shellfish consumption was lower in comparison to general air exposure from household dust (123-4620 particles/year/capita) than from food (13,731-68,415 particles/year/capita). However, they used concentrations of 0.031-0.086 particles per wet weight of tissue for their calculations which is much lower than the results present in this study.

Limitations of using mussels and oysters as biomonitoring tools are demonstrated by Ward et al. (2019) where they suggest that the selection of particles pre- and post-ingestion may lead to biased data and conclusions. These selective capabilities of bivalves may not in fact make them good bioindicators of MPs in the environment (Dimitrijevic et al., 2018; Ward et al., 2019). Furthermore, limitations of using bivalves to measure MP pollution may include the sizes analyzed. It is suggested that within bivalves, larger MPs are more likely to be rejected and are not retained in tissues for long

periods, while small MPs are internalized and retained longer (Ringwood, 2021). Therefore, the size distributions observed in this study may only provide a snapshot of the MP sizes existing within the marine environment.

3.5 Conclusion

This study has provided baseline concentrations and comparisons of MPs in Blue mussels and Eastern or American oysters from Nova Scotia. Factors such as sampling location may influence SMP concentration in Blue mussels and Eastern oysters. In addition, sampling location and type of bivalve may have influenced SMP concentrations in mussels and oysters from Melmerby Beach and Tatamagouche. Small-sized SMPs (<10 μm) were found in both mussels and oysters using visual methods such as Nile Red staining and microscopy, emphasizing the consideration of smaller-sized MPs for future studies. This study also confirms the presence of plastic polymers within mussels and oysters from Nova Scotia. This highlights the need for continued monitoring of MP pollution in shellfish and the broader marine environment. As the demand for seafood continues to rise, aquaculture has become an increasingly important source of food production. Future research on MP contamination found in aquaculture or wild-raised bivalves is recommended. The findings of this study provide a baseline for future research into the effects of MPs found in mussels and oysters from Nova Scotia and highlight the importance of implementing strategies to reduce MP pollution in the marine environment.

Chapter 4: Common and emerging methods for the analysis of microplastics in various marine mussel and oyster species

4.1 Abstract

The presence of microplastics (MPs) in marine environments poses a threat to aquatic ecosystems and human health. Mussel and oyster species have been used to measure MP pollution in the water column due to their abundance in the environment and economic importance. However, the lack of standardized methods in MP research has made it challenging to compare and interpret results across global studies. This review aims to provide an overview of common and emerging methods for MP analysis in mussels and oysters, using techniques and protocols from studies published between 2014 – March 2023. From the reviewed articles (n=97), alkaline reagents were the most common digestion method in both mussels (n=22), and oysters (n=13) and of the studies examined, 68% used a pore size of ≤5 μm for the processing of samples. The role of temperature is discussed in the context of both the storage of samples and the digestion step as a consideration for future protocols. Visual analysis approaches such as the use of microscopic identification and/or Nile red fluorescence are common methods to enumerate and investigate the morphology of suspected microplastics (SMPs). Common methods of polymeric identification include the use of Fourier transform infrared spectroscopy (FTIR) which was the most common method among both oysters and mussels (n=17) and (n=37), respectively. Future considerations and research for the effects of temperature within the digestion and storage phase is recommended.

4.2 Introduction

The production of plastics has become a growing concern due to their improper disposal, leading to their persistence in the environment (Jambeck et al., 2015). It is estimated that 380 million tons of plastic is produced each year, with up to 50% of that comprising single-use plastics (Geyer et al., 2017). From food packaging to electronics and cars, plastics are ubiquitous in nature and persistent in composition (Geyer, 2020; Walker et al., 2021). When improperly disposed of, plastics can pollute the environment and threaten the integrity of natural ecosystems (Windsor et al., 2019). These land-based plastics can travel thousands of kilometres from their disposal sites into aquatic environments such as freshwater systems, estuaries, and the marine environment (Andrady, 2011; Cole et al., 2011). Larger plastic debris will eventually break down into smaller plastic particles known as secondary microplastics (MPs). MPs are defined as plastic particles between 1 μm and 5 mm and are further categorized into primary and secondary. Primary MPs are plastic particles that are produced as MPs (Thompson, 2015). This can include glitter (Yurtsever, 2019), scrubbing agents in cleaning supplies (van Wezel et al., 2016), and exfoliants in personal care products (Praveena et al., 2018). Secondary MPs are produced by the degradation of larger plastics through processes such as mechanical weathering and erosion, biologically induced weathering, and photodegradation (Cole et al., 2011; Lin et al., 2022). As particles become smaller and break apart, weather events contribute to MP transportation in various remote environments such as Antarctic seawater (Zhang et al., 2022), remote islands (Ivar do Sul et al., 2013), and estuaries (Choong et al., 2021; Li et al., 2018).

In marine aquatic ecosystems, MP particles are available for organisms such as bivalves (Bom & Sá, 2021), crabs (Waite et al., 2018), and fish (Wang et al., 2017) to ingest. It is documented in the literature the adverse effects MPs have on marine biota, such as: tissue damage and oxidative stress in fish (Bhuyan, 2022; Zitouni et al., 2021) and inhibited energy metabolism in corals (Liao et al., 2021). MPs have been recently discovered at even smaller size classes in marine biota which may pose additional risks due to the increased surface area and abundance of smaller particles (Chubarenko, 2022) which could carry harmful and toxic substances (Bowley et al., 2021; Campanale, Massarelli, et al., 2020).

Bivalves such as mussels and oysters are often studied due to their ubiquity and sensitivity to environmental stressors (Wootton et al., 2022). Pollutants such as MPs and heavy metals have been shown to accumulate within tissues, making these organisms potential vectors for pollutants to move through food webs and up trophic levels (Crooks et al., 2019). Research suggests that these bivalves are selective feeders where some MPs are more readily ingested because they resemble their natural food sources in size, shape, and sometimes colour (Ward et al., 2019). Bivalves may accidentally not reject MPs and ingest them within their gut and soft tissues (Ward et al., 2019). In addition, both wild and aquaculture sourced bivalves have been assessed to compare MP abundance, potential sources of pollution, and characterize the potential risks of exposure from seafood consumption from these sources (De Witte et al., 2014; Mathalon & Hill, 2014). Research assessing baseline concentrations and the characterization of MPs to inform potential sources has increased significantly over the last decade due to the demand for seafood, and environmental monitoring of toxic pollutants (Bom & Sá, 2021).

Various methods for the extraction and analysis of MPs in marine biota have been produced to efficiently isolate suspected MPs from environmental samples. Procedures typically include a digestion step using various acids (Thushari et al., 2017), oxidative agents (Li et al., 2016), alkaline agents (Sparks et al., 2021), biological enzymes (Paradinas et al., 2021), or a mixture of these reagents (Gardon et al., 2021). Some methods use a density separation step to further isolate MPs with the use of a mid-high dense solution (Cho et al., 2019; Qu et al., 2018). Procedures include the use of various pore size filters for filtering samples such as > 50 μm large size (Baechler et al., 2020) to small size filters <5 μm pore sizes (Catarino et al., 2018; Naji et al., 2018), which determines the size classification of MPs captured (Ding et al., 2022). Various quality control measures are performed in standard procedures to reduce background contamination (Li et al., 2019). The analysis of MPs is typically performed using various visual analysis techniques ranging from stereoscopic, digital, fluorescent, or a combination of methods (Bom & Sá, 2021; Li et al., 2019). Suspected microplastics (SMPs) are then confirmed as plastics using spectroscopic methods such as Fourier transform infrared (FTIR) spectroscopy (De-la-Torre et al., 2022; Digka et al., 2018) or Raman spectroscopy (Van Cauwenberghe & Janssen, 2014). There are various methods within each step of the MP extraction and analysis process, which make results difficult to compare. The lack of standardization in MP research is due to factors such as the availability of resources, the use of highly technical methods, and shortcomings in the reporting of methods and comparable units (Adhikari et al., 2022).

This short systematic review will summarize the various approaches to isolating and analyzing MPs in mussels and oysters and the common trends in methodology. This

review will aim to discuss the advantages and limitations of common methods and produce a protocol informed by literature for the extraction and analysis of MPs in marine mussels and oysters. This review will also provide consideration for future protocols and research for the extraction and analysis of MPs in marine mussels and oysters.

4.3 Methods

A literature search was used to investigate common and emerging methods of extracting and characterizing MPs from marine mussel and oyster species. Studies that focused on determining baseline MP contamination in the natural environment were included. Methodology and laboratory studies were only included if their target organisms were marine oyster and mussel species, and environmental levels of MP contamination were analyzed. Studies looking at commercial or retail organisms were included if they were marine species.

The search engines Scopus, Novanet, and Google Scholar were used to identify studies by searching the title, abstract, and/or keywords using the query “microplastic” AND “marine” AND (“oyster” OR “mussel”), published between January 2014 and March 2023. A total of 1086 studies were found, and 429 duplicates were automatically removed by Covidence, (Covidence, 2023) a screening and data extraction tool for conducting systematic reviews bringing the total number of studies to be screened to 657. Additional criteria for screening articles were adapted from a review paper by Wootton et al., 2022 looking at MP contamination in oyster species globally. These criteria were based on methods looking at small particle sizes and the confirmation of plastic

polymers. Toxicity studies, freshwater studies, and review studies without an original component were all excluded. The screening criteria included the following requirements:

1. Species must be marine-derived or commercial species.
2. A sample size of 10 or at least soft tissues of 10 individuals of a targeted species to undergo extraction methods.
3. A chemical digestion step to dissolve organic matter (e.g., KOH, H_2O_2, HCl or other).
4. Use of small sieves or filters (<50 μm) to filter the material and a microscope for initial identification of MPs.
5. Use of quality assurance and quality controls as measures for reducing contamination such as the use of procedural or background blanks or the use of non-plastic equipment and clothing during processing and analysis.
6. Reported plastic load (MPs/gram wet weight, average value or range across all samples collected or in at least one of the targeted organisms, irrespective of if they contained MP or not).

A manual screening of the title, keywords, and abstract removed an additional 478 studies, bringing the potential studies to 179. Using the above criteria, the full text of the remaining studies was examined. Ninety-seven (n=97) studies were identified that are in scope for this literature review (Table 4.1- 4.3). This list is not all-inclusive, so some studies may have been unintentionally excluded from this query. Minor changes, such as the sample size of at least 10 being modified to at least 10 individuals to undergo sample extraction, were undertaken to capture studies with low sample sizes due to the pooling of

soft tissues for digestion. In addition, the parameter of a reported plastic load in a comparable unit was changed to capture studies that used methods such as Pyr-GC/MS, which reports plastic loads by weight of suspected MPs.

4.4 Results

4.4.1 Number of publications

MP studies in biota have been researched as early as the late 1970s. Research has expanded exponentially and particles have been found in all corners of the world, from remote islands (Martins et al., 2020) to marine bodies close to urban centers (Ríos et al., 2020). Baseline studies have been increasing at a high rate due to the interest in environmental concentrations of MPs to characterize the potential for human exposure. Studies with the criteria of adequate methods include 97 articles published from 2014 until March 2023 (Figure 4.1). Similar results were observed in a systematic literature review conducted by Bom & Sá (2021).

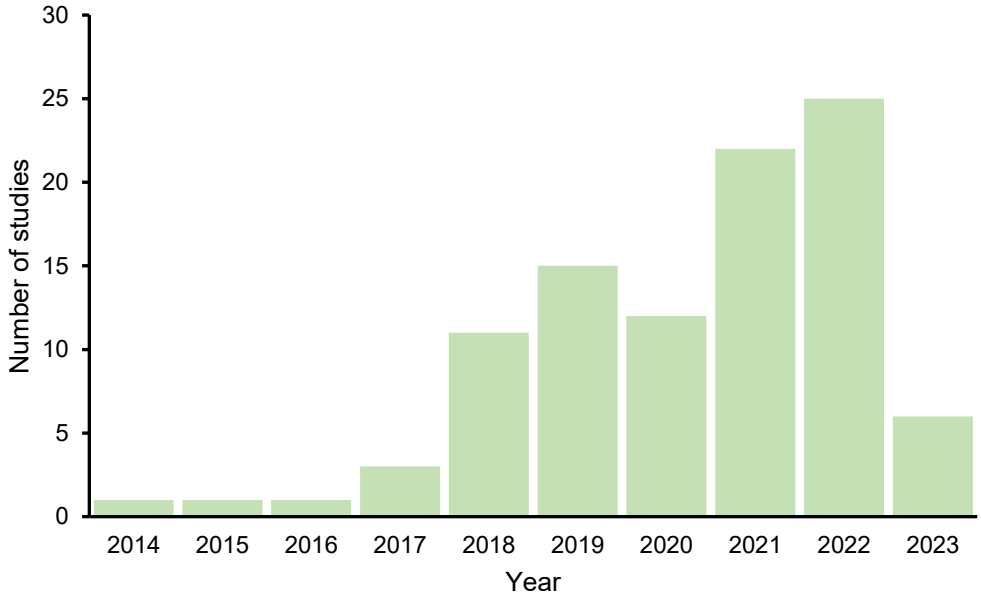


Figure 4.1 The number of articles using the methods and reporting criteria outlined in the methods section from January 2014 to March 2023, using a systematic literature review search.

4.4.2 Study areas and storage of samples

Global MP studies have increased significantly over the last decade, especially in examining baseline concentrations in marine biota. Sorting the number of studies by continent, research from Asia was the most abundant fitting the criterion for this review (43.30%) (n=42). This was followed by Europe (33.96%) (n=31), Oceania (7.22%) (n=7), North and South America (6.19%) (n=6), Africa (4.12%) (n=4), and Worldwide studies (1.03%) (n=1). In terms of research output from individual countries, China produced the most studies within the scope of this literature review with (n=21) studies. This was followed by India (n=6), and the United Kingdom, France, and Italy (n=5). Studies spanning multiple countries, Australia, and Thailand produced (n=4) studies each. Examining studies containing both mussel and oyster organisms, Asia produced the most studies (n=7), and China was the country that produced (n=4) the most studies that fit the

criterion for this literature review. For studies solely examining mussel species, Europe was the continent that produced the most studies within the scope of this literature review (n=25), with China producing the most from a single country (n=8), followed by the United Kingdom (n=5), followed by India and Italy with (n=4) studies each. For studies looking solely at oyster species, Asia was the continent that produced the most studies (n=20) with China producing the most studies for a single country (n=9).

Mode of life is also important when considering the occurrence of MPs in environmental biota. Studies examining both wild and commercially available seafood are important for understanding potential human exposure and ecosystem health. For the purposes of this study, Wild-Caught is defined as presently native species that are collected in a region locally without any interference, Aquaculture and/or Retail is defined as organisms collected directly or near aquaculture initiatives, as well as organisms collected from supermarkets or local fish markets.

Examining the scoped studies, 51.55% (n=50) have been identified as organisms that are solely Wild-Caught, followed by Aquaculture and/or Retail (35.05%) (n=34), and Wild-Caught + Aquaculture and/or Retail (13.40%) (n=13) (Figure 4.2). Separating the studies based on target organisms: examining mussels, oysters, or both, all categories showed a high representation of Wild-Caught studies with (n=31), (n=14), and (n=5), respectively.

The collection and storage of samples often employ a freezing or refrigeration step to euthanize or store the target organisms for various amounts of time before dissection and laboratory processing. Within the scope of this review, methodologies in the selected articles often froze samples at various temperatures (0^{o}C to -80^{o}C) until laboratory

processing (n=78), stored with ethanol or isopropanol to preserve the samples (n=3), refrigerated (n=1), or an unspecified storage method/temperature (n=15) (Figure 4.4).

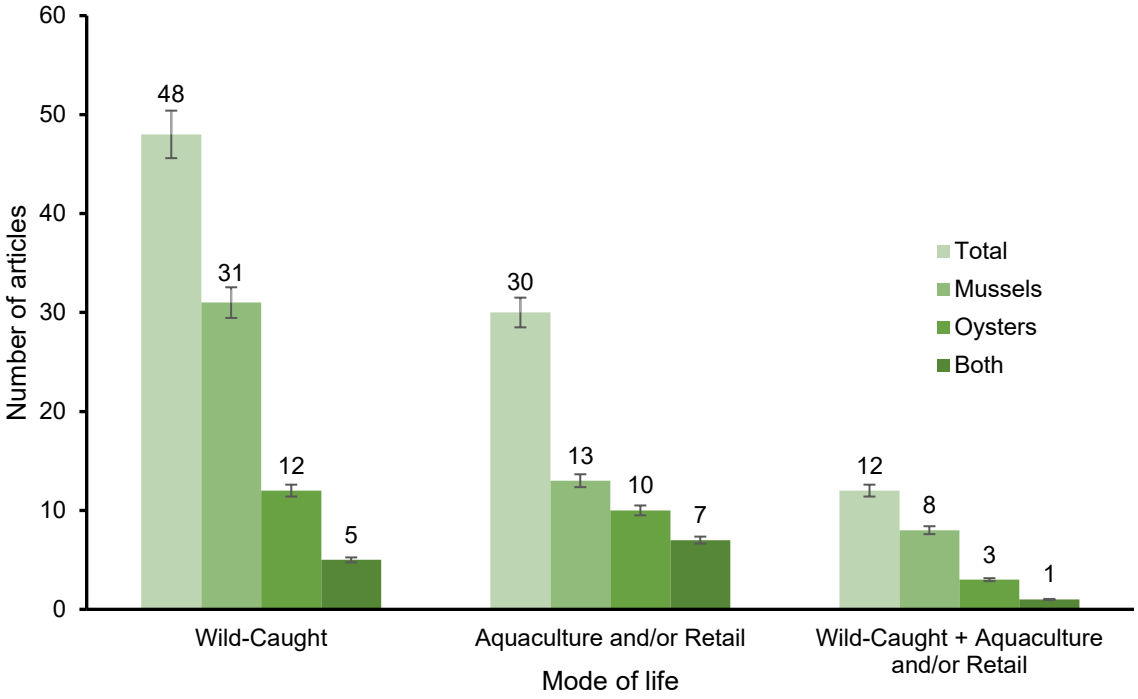


Figure 4.2. Number of studies looking at MP contamination in oysters and mussels globally from a set criterion and their modes of life at the time of collection. Error bars represent the percentage error range. Numbers above each bar represent the number of studies from each mode of life. The number above each column represents the number of studies from each country and source.

Mussel and oyster species	Year	Sampling area	Tissue type(s)	Digestion method	Density separation	Pore size (µm)	Analysis of microplastics	Number of microplastics reported	Unit	Reference
Mytilus galloprovincialis, Crassostrea gigas	2019	Tunisia	Whole soft tissue	10% KOH at 65°C for 24hrs, RT 24-48 h	NaCl	1	VI + FTIR	O: 1482.82 ± 19.20 M:~800 (not explicitly reported)	items/kg-1 WW	Abidli et al. (2019)
Mytilus edulis, Crassostrea gigas	2019	Korea	Whole soft tissue	10% KOH at 60°C overnight	LMT	20	VI + FTIR	O: 0.77 ± 0.74 M: 0.68 ± 0.64	n(items)/individual	Cho et al. (2019)
Mytilus edulis, Crassostrea gigas	2021	Korea	Whole soft tissue	10% KOH at 60°C + 35% H_2O_2 + Fe (II) at 75°C	LMT	20	VI + FTIR	O/M:0.33 +/- 0.23	n/g wet weight	Cho et al. (2021)
Mytilus galloprovincialis, Crassostrea gigas	2021	China	Whole soft tissue	10% KOH at 60°C 24 h	No	0.7	VI + FTIR	M: 0.8–2.1 O: 1.2–3.3	items/individual	Ding et al. (2021)
Mytilus galloprovincialis, Crassostrea gigas,	2022	Spain	Whole soft tissue	1) 2M KOH + 10% SDS at 40 °C 2) Protease, lipases, and celluloses + 33-35% H_2O_2 at 40 °C 3) Fe (II) + Chitinase at 40 °C	ZnCl2	10	VI + FTIR	M: 18.6 ± 23.0 O: 22.8 ± 14.4	MPs/individual	Exposito et al. (2022)
Mytilus edulis, Magallana gigas	2022	France	Whole soft tissue	10% KOH at 60ºC for 24 h	No	1.6	VI + Raman	M 1.9 ± 2.1 O 0.4 ± 0.4	MPs/g WW	Lerebours et al. (2022)
Crassostrea gigas, Mytilus edulis	2017	Netherlands	Whole soft tissue	Nitric acid (HNO_3) (microwave destruction) for 45min + NaOH with 30% $H2O_2$	No	0.7	VI + FTIR	M: 19 - 105 O: 30 - 87	MP/g DW	Leslie et al. (2017)
Perna viridis, Crassostrea hongkongensis	2022	China	Whole soft tissue	10% KOH at 65°C until digested	No	20	VI + FTIR	0.2-3.1 (all bivalves)	items/individual	Li et al. (2022)
Perna viridis, Crassostrea gigas	2022	China	Whole soft tissue	10% KOH at 60°C for 24 h	No	0.7	VI + Raman	M: 1.8 O: 1.55	items/individual	Pan et al. (2022)
Crassostrea gigas, Mytilus edulis	2018	France	Whole soft tissue	10% KOH at 60°C for 24 h	KI	12	VI + FTIR	M: 0.61 ± 0.56 O: 2.1 ± 1.7	MP/individual	Phuong et al. (2018)
Perna viridis, Crassostrea sp.	2021	India	Whole soft tissue	30% H_2O_2 at 65°C for 24 h, RT 24-48 h	No	5	VI + FTIR	M: 3.2 ± 1.8 O: 4 ± 2	MP/g WW	Saha et al. (2021)
Crassostrea gigas, Mytilus edulis	2014	Germany	Whole soft tissue	69% Nitric acid (HNO_3) for 2 h boiling (~80°C)	No	5	VI + Raman	M: 0.36 ± 0.07 O: 0.47 ± 0.16	particles/g WW	Van Cauwenberghe & Janssen (2014)
Crassostrea gigas, Mytilus edulis	2022	China	Whole soft tissue	10% KOH	No	8	VI + FTIR	M: 0.21 ± 0.21 O: 0.77 ± 0.81	MP/g-1 WW	Zhang et al. (2022)

Table 4.1 Adapted from (Dellisanti et al., 2022) summarizing the methods reported from studies published between January 2014 – March 2023. Proportion of the target organisms from studies analyzing both mussel and oyster species from selected publications from January 2014 – March 2023. Including the species of mussel and oyster, year of publication, sampling area, tissues or biomass targeted, digestion methods using various enzymes and chemicals, density extraction steps using zinc chloride (ZnCl2), potassium iodide (KI), sodium chloride (NaCl), sodium iodide (NaI), or lithium meta-tungstate (LMT), pore size reported in (µm), a suite of visual analysis techniques by visual analysis (VI) using various microscopes (stereo, light, dissecting, optical, inverted, compound, digital), through Nile red fluorescence and other fluorescent microscope techniques, and the polymer identification of MPs by

Fourier-transform infrared spectroscopy (FTIR), Raman spectroscopy, Scanning Election Microscope (SEM), or a combination of various methods, and the number of MPs reported and their units.

Oyster species	Year	Sampling area	Tissue type(s)	Digestion method	Density separation	Pore size (µm)	Analysis of microplastics	Number of microplastics reported	Unit	Reference
Crassostrea virginica	2022	USA	Whole soft tissue	0.1% tween + 30% H_2O_2 at 65ºC for 24 h	No	0.45	VI + Raman	5.6-7	MP/g-1 WW	Aung et al. (2022)
Crassostrea gigas	2020	Taiwan	Whole soft tissue	10% KOH at 50ºC for 24-48ch	No	20-25	VI + FTIR	107.9	MP/Kg-1 seafood tissue	Chen et al. (2020)
Crassostrea gigas	2020	Italy	Gills and hepatopancreas	30% H_2O_2 at RT-45ºC	No	0.2	VI + FTIR	Gills: 329,849 ± 1149 Hep: 238,931 ± 677	SMP g-1	Corami et al. (2020)
Crassostrea gigas	2022	Vietnam	Whole soft tissue	10% KOH + 30% H_2O_2 at 60ºC for 24 h	NaI	0.7	VI + FTIR	1.88 ± 1.58	particles/g (wet weight)	Do et al. (2022)
Crassostrea gigas	2022	China	Whole soft tissue	10% KOH + 30% H_2O_2 at 60ºC for 24 h	No	8	VI + FTIR	2.92 ± 0.10	items/individual	Du et al. (2022)
Pinctada margaritifera	2021	French Polynesia	Whole soft tissue	10% KOH 30% H_2O_2 at 50ºC for 2 h	No	1.2	VI + FTIR	2.1-125	MPs/gram DW	Gardon et al. (2021)
Unspecified oyster	2022	United Arab Emirates	Whole soft tissue	10% KOH 30% H_2O_2 at 50ºC for 24 h	No	11	VI + FTIR + Hot needle	101.2 ± 93.8	MP/Kg of sample	Hammadi et al. (2022)
Saccostrea glomerata	2019	Australia	Whole soft tissue	10% KOH at 60-65ºC for 24 h	NaI	1	VI + FTIR	0.25-0.83	MPs/gram WW	Jahan et al. (2019)
Spondylus spinosus	2019	Lebanon	Whole soft tissue	10% KOH at 60ºC for 24 h	No	1.6	VI + Raman	0.45 ± 0.3	MP/g-1WW	Kazour et al. (2019)
Saccostrea cuccullata	2023	Iran, Pakistan	Whole soft tissue	10% KOH at 40ºC for 72 h	NaI	8	VI + Raman	1.00 ± 0.0	n/individual	Kor et al. (2023)
Saccostrea cucullata	2022	Brunei	Whole soft tissue	10% KOH at 40ºC for 48 h	No	11	VI + FTIR	0.43-7.20	MP/g-1WW	Lee et al. (2022)
Saccostrea cucullata	2018	China	Whole soft tissue	10% KOH at 65ºC for 24 h, RT for 24 h	NaCl	20	VI + FTIR	1.5-7.2	items/g WW	Li et al. (2018)
Crassostrea and Saccostrea	2021	Taiwan	Whole soft tissue	30% H_2O_2 at 65ºC for 24 h	NaCl	5	VI + Raman	3.24 ± 1.02	MP/g-1 WW	Liao et al. (2021)
Crassostrea gigas	2020	USA	Whole soft tissue	30 H_2O_2 at 65ºC for 24-48 h	NaCl	5	VI + FTIR	0.02-0.14	MP/g-1 WW	Martinelli et al. (2020)
Pinctada radiata	2018	Iran	Whole soft tissue	30% H_2O_2	No	0.45	VI + FTIR + SEM-EDX + Hot needle	0.1	MPs/g-1 WW	Naji et al. (2018)
Magallana bilineata	2019	India	Whole soft tissue	10% KOH at 50ºC for 72 h	NaI	0.8	VI + FTIR + SEM-EDX	0.81 ± 0.45	MPs/g-1 WW	Patterson et al. (2019)
Crassostrea gigas	2020	Australia	Whole soft tissue	10% KOH at 60ºC for 24 h	No	2.7	No VI + Pyr-GC/MS	0.01	Mg/g-1	Ribeiro et al. (2020)
Crassostrea gigas, Crassostrea angulate, Crassostrea	2019	China	Whole soft tissue	10% KOH + 30% H_2O_2 at 60ºC for 48 h	No	1	VI + FTIR	0.62	items/g WW	Teng et al. (2019)

hongkongensis, and Crassostrea sikamea										
Saccostrea forskalii	2017	Thailand	Whole soft tissue	69% Nitric acid (HNO_3) at RT, then 100°C 2 h	No	5	VI + Raman	0.57-0.37	particles/g WW	Thushari et al. (2017)
Placuna placenta	2022	Indonesia	Whole soft tissue	30% H_2O_2 at 65°C for 24 h	NaCl	0.45	VI + FTIR	0.033	MP/g-1WW	Tielman et al. (2022)
Crassostrea Gasar	2021	Brazil	Hepatopancreas	10% KOH at 40°C for 48 h	NaCl	8	VI + SEM + EDS	9.6	mg-1 hepatopancreas	Vieria et al. (2021)
Crassostrea virginica	2022	USA	Whole soft tissue	10% KOH at 40°C for 24 h + 1.0M citric acid	No	1.2	VI + FTIR	2.43 ± 0.52	MP/g-1 WW	Walters et al. (2022)
Saccostrea cucullata	2021	China	Whole soft tissue	10% KOH + 30% H_2O_2 at 65°C for 8 h	No	2.7	VI + FTIR	1.84	n/g (wet weight)	Wang et al. (2021)
Crassostrea gigas	2019	China	Whole soft tissue	30% H_2O_2 + 65% HNO_3 for 48 h	NaCl	50	VI + FTIR + SEM/EDX	41 ± 15.5	Items/individual	Wang et al. (2019)
Crassostrea gigas Saccostrea glomerta	2022	Australia	Whole soft tissue	10% KOH at 60°C overnight	No	38	VI + FTIR	0.09 ± 0.01	MPs/g WW	Wootton et al. (2022)
Ostrea denselamellosa	2020	China	Whole soft tissue	10% KOH + 30% H_2O_2 at 60°C for 24 h	No	0.7	VI + FTIR	0.31 ± 0.10	particles/g (wet weight)	Wu et al. (2020)
Crassotrea gigas	2022	China	Whole soft tissue	10% KOH at 60°C for 24 h	No	2.7	VI + FTIR	0.92 ± 0.80	items/g WW	Zhang et al. (2022)
Not Specified (oyster)	2021	China	DG, Gills, Other soft tissues	10% KOH at 40°C for 48-72 h	No	5	VI + FTIR	7.05-0.59	MP/g-1 WW	Zhu et al. (2021)
Crassostrea hongkingensis	2019	China	Whole soft tissue	10% KOH at 40°C for 48-96 h	No	5	VI + Raman	3.2-8.6	MPs/individual	Zhu et al. (2019)

Table 4.2 Adapted from (Dellisanti et al., 2022) summarizing the methods reported from studies published between January 2014 – March 2023. Proportion of the target organisms in oyster species from selected publications from January 2014 – March 2023. Including the oyster species, year of publication, sampling area, tissues or biomass targeted, digestion methods using various enzymes and chemicals, density extraction steps using zinc chloride (ZnCl2), potassium iodide (KI), sodium chloride (NaCl), sodium iodide (NaI), or lithium meta-tungstate (LMT), pore size reported in (µm), a suite of visual analysis techniques by visual analysis (VI) using various microscopes (stereo, light, dissecting, optical, inverted, compound, digital), through Nile red fluorescence and other fluorescent microscope techniques, and the polymer identification of MPs by Fourier-transform infrared spectroscopy (FTIR), Raman spectroscopy, Scanning Election Microscope (SEM), or a combination of various methods, and the number of MPs reported and their units.

Mussel species	Year	Sampling area	Tissue type(s)	Digestion method	Density separation	Pore size (µm)	Analysis of microplastics	Number of microplastics reported	Unit	Reference
Mytilus galloprovincialis	2023	Morocco, Tunisia	Digestive gland and gills	10% KOH at 60°C for 24 h	No	0.8	VI + FTIR + SEM-EDX	1.27 ± 0.42	MP/g-1 WW	Abelouah et al. (2023)
Perna perna	2019	Brazil	Whole soft tissue	30% H_2O_2 at RT for 7days	NaCl	0.45	VI + FTIR	16.6 ± 6.6 to 31.2 ± 17.8	MP/g-1WW	Birnstiel et al. (2019)

Mytilus galloprovincialis	2023	Balkans	Whole soft tissue	10% KOH at RT	No	1.2	VI + FTIR	2.53 ± 1.1	items/individual	Bošković et al. (2023)
Mytilus spp. (Mytilus edulis, M. trossulus, M. galloprovincialis)	2018	Norway	Part soft tissue	10% KOH at 60ºC for 24 h	No	2.7	VI + FTIR	0.97 ± 2.61	MPs/gram WW	Bråte et al. (2018)
Mytilus spp. and Modiolus modiolus	2017	UK	Whole soft tissue	Protease at 60ºC overnight	No	0.8-1.6	VI + FTIR	Fibers (10.4 ± 3.42) Particles (0.9 ± 0.99) Films (1.3 ± 2.38)	MPs/individual	Catarino et al. (2017)
Mytilus spp. and Modiolus modiolus	2018	UK	Whole soft tissue	Corolase 7089 (Protease) 9.6 UHb/mL for Mytilus spp.and 19.3 UHb/m for M.modiolus at 60ºC overnight	No	0.8	VI + FTIR	Mytilus spp. 3.0 ± 0.9 M.modiolus 0.086 ± 0.031	MP/g-1WW	Catarino et al. (2018)
Perna viridis	2022	Thailand	Whole soft tissue	1% KOH + 30% H_2O_2 at 60ºC	NaCl	20	VI + FTIR	4.13-2.53	items/individual	Cherdsukjai et al. (2022)
Aulacomya atras	2022	Peru	Whole soft tissue	10% KOH at 60ºC for 24 h	No	20-25	VI + FTIR	0.56 ± 0.08	MP g-1	De-la-Torre et al. (2022)
Mytilus galloprovincialis	2018	Greece	Digestive gland and gills	30% H_2O_2 at 55-65ºC	No	1.2	VI + FTIR	1.83	Ind-1	Digka et al. (2018)
Mytilus galloprovincialis	2018	Greece	Digestive gland and gills	30% H_2O_2 at 55-65ºC	No	1.2	VI + FTIR	1.7–2	items/individual	Digka et al. (2018)
Perna viridis	2020	India	Whole soft tissue	10% KOH at 40ºC for 72 h, + 10% KOH for 24 h	No	11	VI + Raman	3.28 ± 0.87	microplastics/individual	Dowarah et al. (2020)
Mytilus galloprovincialis	2020	Turkey	Whole soft tissue	30% H_2O_2 at 65ºC for 72 h	No	1.2	VI + FTIR	0.06-2.47	Ind-1	Gedik and Eryasar (2020)
Mytilus galloprovincialis	2022	Turkey	Whole soft tissue	30% H_2O_2 at 65ºC for 3-5 days	No	1.2	VI + FTIR	0.11 to 4.58	MP/g-1 fresh weight	Gedik et al. (2022)
Mytilus galloprovincialis	2019	Italy	Whole soft tissue	Protease at 50 °C for 48 h, then 20 % KOH at 50 °C for 36 h,	NaI	0.2	VI + FTIR	0.24-1.33	MP/g-1WW	Gomiero et al. (2019)
Various	2020	Turkey	Whole soft tissue	30 % KOH + NaClO at RT for 10 days	KI	0.45	VI + Raman	0.6 +/- 0.1	MP/mussel-1	Gündoğdu et al. (2020)
Mytilus edulis	2019	France	Whole soft tissue	10% KOH at 60ºC for 24 h	No	1.6	VI + Raman	0.15 ± 0.06 - 0.25 ± 0.16	MP/g-1 WW	Hermabessiere et al. (2019)
M. bilineata, P. viridis,	2022	India	Digestive gland (DG) and gills (GI)	50ºC proteinase-K 2 h + sodium perchlorate at 60 °C for 20 min	No	1.2	VI + Raman	5.6 (DG) and 8.5 (GI)	items/g-1 tissue	Joshy et al. (2022)
Mytilus edulis	2020	France	Whole soft tissue	10 % KOH at 60 °C for 24 h	No	1.6	VI + Raman	0.61-1.67	G-1/WW	Kazour and Amara (2020)
Mytilus spp.	2022	Australia	Whole soft tissue	30% H_2O_2 at 45ºC for 48 h	$ZnCl_2$	1.2	VI + FTIR + Hot Needle	3.58 ± 8.18	particles/individual	Klein et al. (2022)
Perna viridis	2021	Hong Kong (China)	Whole soft tissue	10 % KOH + 14 % EDTA + 30 % H_2O_2 at 40 °C for 48 h	No	30	VI + Raman	0.21-1.83	MP/g-1 WW	Leung et al. (2021)
Mytilus edulis	2016	China	Whole soft tissue	30% H_2O_2 at 65ºC for 24 h RT for 24-48 h	NaCl	5	VI + FTIR + SEM-EDS	0.9 to 4.6	items/g	Li et al. (2016)
Mytilus edulis	2018	UK	Whole soft tissue	30% H_2O_2 at 65ºC for 24 h RT for 24-48 h	NaCl	5	VI + FTIR	0.7 to 2.9 (wild)	items/g WW	Li et al. (2018)

								0.9 to 1.4 (supermarket)		
Mytilus spp.	2019	China	Byssus wet tissue	30 % H_2O_2 at 65 °C for 24-48 h	No	5	VI + FTIR	3.69 to 9.16	Items/g	Li et al. (2019)
Perna viridis	2022	China	Whole soft tissue	10% KOH at 40ºC for 2-5 days	NaCl	0.45	VI + FTIR	0.36 ± 0.81	items/individual	Lin et al. (2022)
Mytilus galloprovincialis	2021	China	Whole soft tissue	10% KOH + 30% H_2O_2 at 60ºC for 48 h	No	8	VI + FTIR	0.19 to 1.76	items/g WW	Liu et al. (2021)
Mytilus edulis	2021	China	Whole soft tissue	10% KOH at 50ºC	NaI	0.45	VI + TGA-FTIR-GC/MS	0.58	MP/Kg-1 WW	Liu et al. (2021)
Magallana gigas	2021	Mexico	Soft tissue, Digestive system, gonad	30% KOH + 30% H_2O_2 at 40ºC for 72 h	No	2.7	VI + FTIR + Hot needle	0.06 ± 0.02	MPs/g (w.t)	Lozano-Hernandez et al. (2021)
Mytilus spp.	2021	Portugal	Whole soft tissue	10% KOH + 10% Tween 60 detergent at 60ºC for 24 h	No	1.6	VI + FTIR	0.54 to 3.0	/g-1 WW	Marques et al. (2021)
Mytilus galloprovincialis	2022	Spain	Whole soft tissue	H_2O_2 at 65ºC for 24 h then RT 24 h	No	0.45	VI + FTIR	0.55–3.20	items/g WW	Masiá et al. (2022)
Perna canaliculus	2022	New Zealand	Digestive tract + intestines	10% KOH at 20ºC for 24 h then 80ºC for 2 h	No	0.8	VI + FTIR	15	items/100g-1 WW	Mazlan et al. (2022)
Perna viridis	2019	India	Part of soft tissue	69 % HNO_3 at RT overnight then 80 °C for 2 h	No	5	VI + Raman	0.09 to 0.32	items/g WW	Naidu et al. (2019)
Mytilus galloprovincialis, Mytilus edulis	2021	Italy	Whole soft tissue	30% H_2O_2 at 65ºC for 36-48 h	NaCl	5	VI + FTIR	0.29 ± 0.38	items/g w.w	Nalbone et al. (2021)
Mytilus edulis, Mytilus californianus	2022	Canada	Whole soft tissue	Corolase 7090 (AB Enzymes) at 60ºC overnight	No	20	VI + FTIR	0.38 ± 0.04	particles/individual	Noël et al. (2022)
Mytilus edulis	2021	UK	Whole soft tissue	0.625% Trypsin at 38-42ºC for 30min	No	20-25	VI + FTIR	0 to 23.81	MP /gw.w	Paradinas et al. (2021)
Perna perna, Perna viridis	2021	India	Whole soft tissue	10% KOH + 30% H_2O_2 at 40ºC for 72 h	No	0.8	VI + FTIR + SEM-EDX	0.87 ± 0.55 to 10.02 ± 4.15	Ind-1	Patterson et al. (2021)
Mytilus galloprovincialis	2021	Portugal	Whole soft tissue	10% KOH at RT for 24 h	No	1	VI + FTIR	0.18 ± 0.31	MP/g-1 WW	Pequeno et al. (2021)
Mytilus chilensis	2020	Argentina	Whole soft tissue	30% H_2O_2 at 45ºC for 48 h, RT for 1 Hr and 55ºC for 15min	No	22	VI + Raman	8.6 ± 3.53	items/individual	Perez et al. (2020)
Perna viridis	2023	Thailand	Whole soft tissue	10% KOH + 30% H_2O_2 at 60ºC 24 h	NaCl	20	VI + FTIR	0.07 ± 0.19	MP/g-1 WW	Phaksopa et al. (2023)
Mytilus edulis	2018	France	Whole soft tissue	10 % KOH at 60 °C for 24 h	KI	0.23	VI + FTIR	0.23	G-1/WW	Phuong et al. (2018)
Mytilus edulis, Perna viridis	2018	China	Whole soft tissue	30% H_2O_2 at 65ºC for 24hrs RT for 24-48 h	NaCl	5	VI + FTIR	1.52-5.36	MP/g-1 WW	Qu et al. (2018)
Mytilus trossulus	2018	Finland	Whole soft tissue	Sodium Dodecyl sulphate + Detergent enzymes Lipase and protease and amylase at 37.5ºC for 48 h	No	20	VI + FTIR	3.5 ± 4.4	ML/g-1 WW	Railo et al. (2018)
Aulacomya atra	2020	Argentina	Whole soft tissue	30% H_2O_2 at 60ºC for 24-48 h	No	30	VI + SEM-EDS	0.3	MP/g-1 WW	Rios et al. (2020)
Mytella strigata, Mytella guyanensis	2022	Costa Rica	Whole soft tissue	10% KOH at 30ºC for 48 h	No	1.2	VI + FTIR	0.7 ± 0.7 to 2.8 ± 3.9	MPs/g-1	Rojas-Jimenez et al. (2022)

Mytilus edulis	2019	UK	Whole soft tissue	10% KOH at 70ºC for 48 h	No	50	VI + FTIR	1.43 to 7.64	Ind-1	Scott et al. (2019)
Choromytilus meridionalis, Mytilus meridionalis	2021	South Africa	Whole soft tissue	10% KOH at 60ºC for 24 h	No	20	VI + FTIR	0.04	MPs/g soft tissue	Sparks et al. (2021)
Perna viridis	2022	Thailand	Whole soft tissue	30% H_2O_2 + Fe (II) at 60ºC for 24 h	No	5.3	VI + FTIR	96 ± 19	particles/individual	Ta et al. (2022)
Mytilus galloprovincialis	2023	Italy	Whole GI (Gastic gland museels, and GI tract)	10% KOH at 50ºC for 24-48 h	No	25	VI + FTIR	0.08-0.76	MPs/g fresh weight	Trani et al. (2023)
Brachidontes rodriguezii	2021	Argentina	Whole soft tissue	10% KOH at 50ºC 48 h	No	0.70	VI + ATR + SEM-EDX	0.15 to 0.25	item/g-1 WW	Truchet et al. (2021)
Mytilus edulis	2015	Belgium and France, Dutch North Sea	Whole soft tissue	69% Nitric acid at boiling (~80ºC) for 2 h	No	5	VI + Raman	0.2 ± 0.3	microplastics/g-1	Van Cauwenberghe et al. (2015)
Mytilus edulis	2021	12 countries	Whole soft tissue	Sodium dodecyl sulphate (SDS), protease, lipase at 50ºC cellulase, H_2O_2, and chitinase at 37.5ºC	No	5	VI + FTIR + Raman	0.13 to 2.4	particles /gram wet weight (g ww)	Vinay Kumar et al. (2021)
Mytilus galloprovincialis	2021	Portugal	Whole soft tissue	10% KOH at 50ºC for 48 h	NaCl	5	VI + FTIR	2.6-0.6	MPs/individual	Vital et al. (2021)
Mytilus galloprovincialis	2022	Italy	Whole soft tissue	10% KOH at 45ºC overnight + 15% H_2O_2 to filters at 45ºC overnight	NaCl	8	VI + FTIR	3.13	microfibers/g-1 WW	Volgare et al. (2022)
Mytilus galloprovincialis	2020	Tunisia	Whole soft tissue	10% KOH at 60ºC for 48 h	NaI	0.8	VI + FTIR	2.6 ± 1.7 -12 ± 1.4	Items/mussel-1	Wakkaf et al. (2020)
Perna canaliculus	2019	New Zealand	Whole soft tissue	22.5M HNO_3 at RT overnight, then boiled (80ºC) for 2 h	No	1.2	VI + FTIR	0 to 1.5	particles/individual	Webb et al. (2019)
Gigantidas platifrons	2023	China	Whole soft tissue	65% HNO_3 at 80ºC for 4 h	No	0.7	VI + FTIR	0.13 ± 0.04	items/individual	Zhang et al. (2023)

Table 4.3 Adapted from (Dellisanti et al., 2022) summarizing the methods reported from studies published between January 2014 – March 2023. Proportion of the target organisms in mussel species from selected publications from January 2014 – March 2023. Including mussel species, year of publication, sampling area, tissues or biomass targeted, digestion methods using various enzymes and chemicals, density extraction steps using zinc chloride (ZnCl2), potassium iodide (KI), sodium chloride (NaCl), sodium iodide (NaI), or lithium meta-tungstate (LMT), pore size reported in (µm), a suite of visual analysis techniques by visual analysis (VI) using various microscopes (stereo, light, dissecting, optical, inverted, compound, digital), through Nile red fluorescence and other fluorescent microscope techniques, and the polymer identification of MPs by Fourier-transform infrared spectroscopy (FTIR), Raman spectroscopy, Scanning Election Microscope (SEM), or a combination of various methods, and the number of MPs reported and their units.

4.4.3 Isolation of microplastics

The types of tissues and organs selected for the isolation of MPs within this literature review include: the Whole soft tissue (n=84) followed by Part of soft tissue (n=2), Gastrointestinal tract/organs and/or Gills (n=6), and Other (n=5).

MPs are difficult to isolate and enumerate directly from the targeted biomass, so a digestion step is employed to extract the MPs from the tissue. The main methods of digestion found in this review were alkaline chemicals (44.33%) (n=43), followed by oxidative (22.68%) (n=22), mixed chemicals (20.62%) (n=20), and both enzymes and acids (6.59%) (n=6). The digestion step includes the use of acids (nitric acid (HNO_3)); alkaline solutions (potassium hydroxide (KOH)); oxidative chemicals (hydrogen peroxide (H_2O_2)), and enzymatic reactions (Trypsin, Protease, or Colorase); or a mix of reagents. The most common reagent was the use of potassium hydroxide KOH (n=60) followed by hydrogen peroxide H_2O_2 (n=39).

A digestion step alone is often insufficient for the removal of all non-plastic material, where sand particles, inorganic, and organic material may be left behind and clog small pore size filters. A density separation step is also common within MP extraction methods in marine biota where tissues are not fully digested. In the evaluated studies 68.04% did not use a density separation step (n=66), followed using sodium chloride (NaCl) (17.53%) (n=17), sodium iodide (NaI) (7.22%) (n=7), potassium iodide (KI) (3.09%) (n=3) and zinc chloride ($ZnCl_2$) and lithium meta-tungstate (LMT) (2.06%) (n=2).

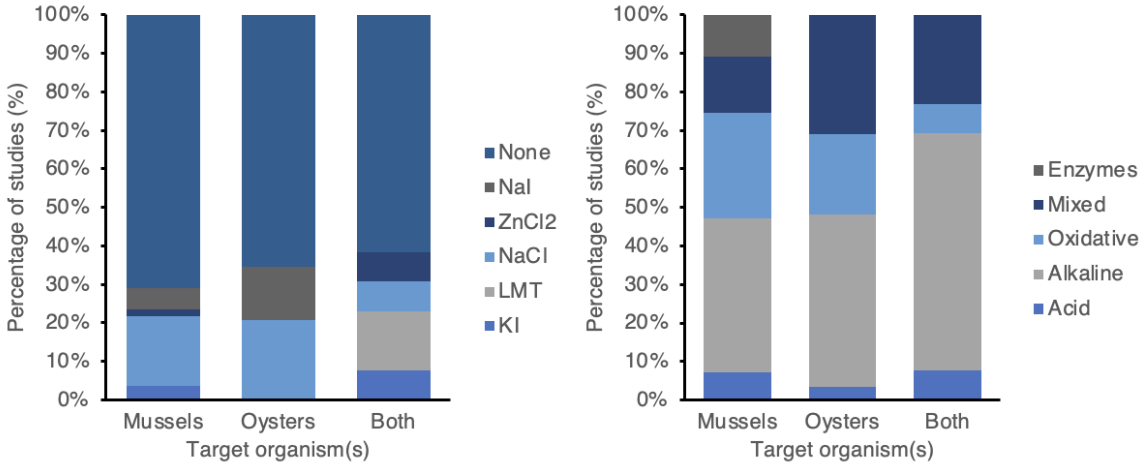


Figure 4.3 The proportion of publications between 2014 and March 2023 where chemical digestion methods were used to isolate MPs using enzymes, alkaline, oxidative, acid, or mixed methods, or a density separation step was used to isolate MPs from non-plastic material using lithium meta-tungstate (LMT) and potassium iodide (KI), sodium chloride (NaCl), sodium iodide (NaI), or zinc chloride ($ZnCl_2$) in mussels, oysters, or both target organisms.

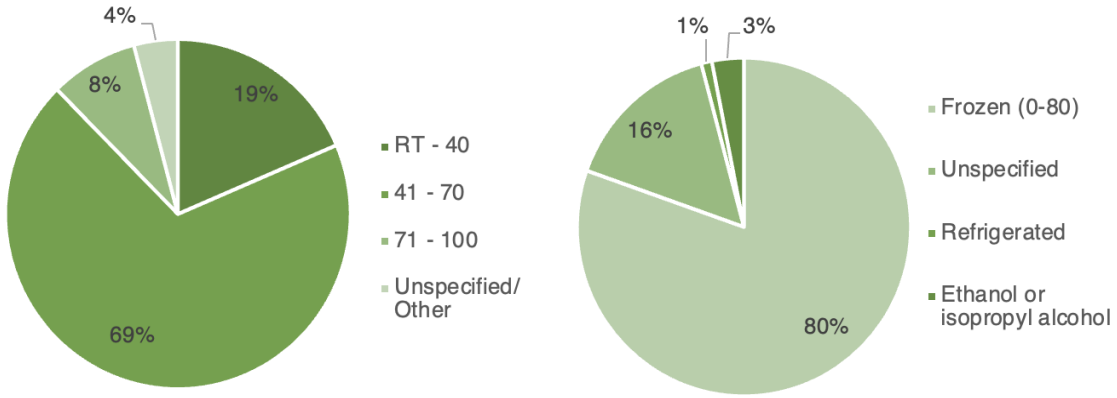


Figure 4.4 Storage methods or temperatures applied to bivalve samples before the lab processing phase (0ºC to -80 ºC) (left). Temperatures applied to samples during the digestion and density separation phase ranging from RT (room temperature) -40ºC, 40ºC-70ºC, 71ºC-100ºC, and unspecified.

The use of heating during both the digestion and density separation phase is common in MP extraction methods for increasing faster reaction rates. Most of the studies applied heat ranging from 41ºC-70ºC (n=67) (69.07%), followed by room temperature (RT) – 40ºC (n=18) (18.56%), 71ºC-100ºC (n=8) (8.25%), and (n=4) (4.12%) studies with

unspecified or other temperatures applied. Other methods used to apply temperature to assist with digestion include the use of microwave destruction as defined by the use of electromagnetic waves of certain frequencies to generate heat in a material (Leslie et al., 2017; Silva et al., 2014). There were no notable differences in the heating temperatures used between the target organisms.

Once the remaining non-plastic material was removed, the digestate was often filtered through a small pore size filters to capture the targeted MPs. Within the evaluated studies, 66 used a pore size of 0-5 μm, (n=66) followed by 10-20 μm (n=13), 5-10 μm (n=8), 20-30 μm (n=7), and 30-50 μm (n=3). There were no notable differences in the pore size used and the studies focusing on mussel, oyster or both tissues.

4.4.4 Visual analysis and characterization of polymers

Many of the evaluated studies used visual analysis or screening to sort suspected MPs, with the use of various microscopic techniques through microscopy (Stereo, light, dissecting, optical, inverted, compound, digital, or scanning electron microscopy (SEM) (n=88), through Nile red or other fluorescent techniques (n=7), or no visual or other methods (n=2) such as the use of a high-resolution scanner. There were no noticeable differences between the visual inspection methods between studies with differing target bivalves (mussels, oysters, or both).

Spectroscopic methods of determining polymer types are the most common technique, with FTIR spectroscopy being the most used (65.98%) (n=64), followed by Raman spectroscopy, (18.56%) (n=18), and FTIR + other (9.28%) (n=9), other techniques (5.15%) (n=5), and Raman + FTIR (1.11%) (n=1). Other methods of

confirmation include scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX), the use of a hot needle, and Pyr-GC/MS. It is noted that only SEM in conjunction with EDS/EDX was recognized as a method of polymer identification. While SEM can offer morphological information, this alone may not be sufficient for determining composition (Shi et al., 2022). Therefore, in conjunction with EDX, this tool provides quantitative information about the elemental composition of the analyzed sample (Shi et al., 2022).

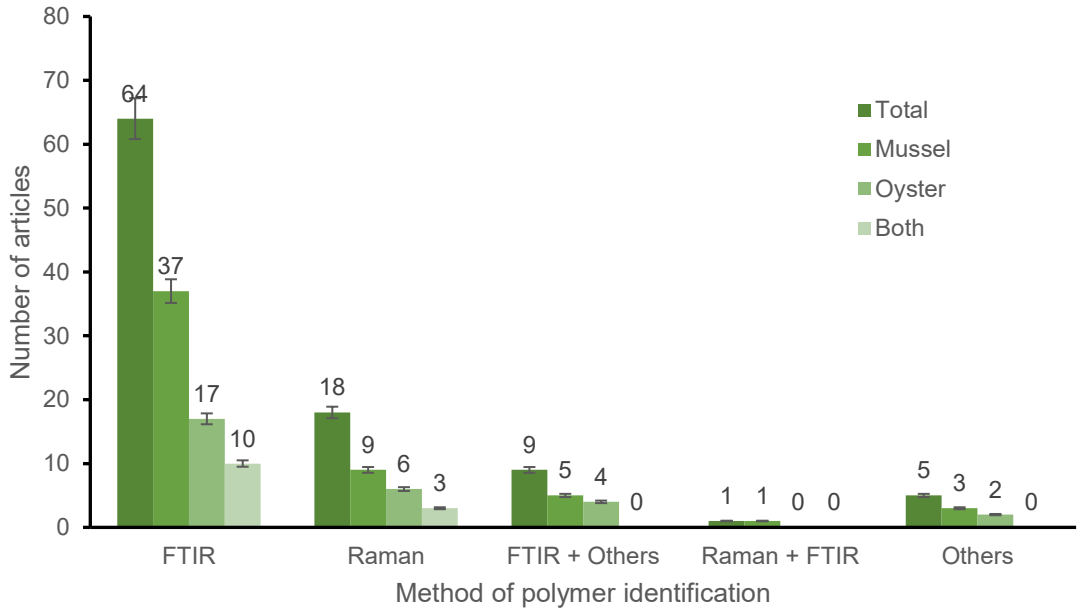


Figure 4.5 Distribution of the number of articles using various polymer identification methods such as: Fourier transformed infrared spectrometry (FTIR), Raman spectroscopy, scanning electron microscopy-energy dispersive X-ray analysis (SEM-EDX), hot needle test, and pyrolysis and gas chromatography and mass spectrometry (Pyr-GC/MS).

4.4.5 Quality assurance and quality control measures

Due to the small size of MPs, it is imperative that Quality assurance and quality control measures (QA/QC) measures are implemented to reduce the amount of

background and procedural contamination during sampling, and laboratory processing. Common steps in methodologies include: the use of non-plastic equipment such as glass, aluminum, and steel, the use of reverse osmosis, MilliQ® water, or alcohol to rinse equipment, the use of cotton or linen based clothing/lab coats during processing environments, as well as the use of 'blanks' or 'controls' in the field, processing, or analysis phase. In total 94.85% (n=92) of studies used 'blank' or 'control' measures to quantify the potential background contamination found in the field, within the ambient air during processing, or during the analysis (visual or polymer characterization) phase.

4.5 Discussion

Common methods of extracting and analyzing MPs from both marine oyster and mussel species from studies between January of 2014 and March of 2023 are summarized within Table 4.1 – 4.3. There are clear trends in methodology within MP research such as the use of alkali reagents (i.e., KOH) to digest tissues. However, there is still much to discuss in terms of the limitations of these methods as well as recommendations for producing more replicable, reliable and standardized studies (Cowger et al., 2020).

4.5.1 Sampling locations, target tissues, and storage methods

An examination of the distribution of studies for individual countries shows that China displayed the highest abundance for all three categories of target organisms. This is likely due to higher levels of plastic pollution observed in China and therefore a higher allocation of resources spent towards research (Li et al., 2019). In addition, population

size may be a factor when looking at the representation of studies by country (i.e., China has a population of 1.426 billion (as of 2022) or approximately 17.86% of the global population (Hackett, 2022).

Methods of sampling included collecting wild mussels from coastal, intertidal, and urban areas adjacent or in proximity to wastewater treatment plants or oyster and mussel farming zones (Keisling et al., 2020; Li et al., 2018; J. Li et al., 2016; Thushari et al., 2017). In contrast, aquaculture or commercial mussels were obtained through seafood markets, grocery stores, wholesalers, or directly from aquaculture sites (Cho et al., 2019; De-la-Torre et al., 2022; Sparks et al., 2021; Wu et al., 2020). Globally 51.55% (n=50) of the studies identified MP contamination in solely Wild-Caught organisms which may be due to the interest of the baseline abundance and characterization of MPs in the natural environment. This may also include locations in proximity to wastewater treatment plants (Leslie et al., 2017), urban centers (Ríos et al., 2020), or rural areas (Keisling et al., 2020). In addition, this could be due to difficulties involved when drawing comparisons about potential sources of MPs, as sources of contamination in retail or aquaculture sampling locations may be more uncertain due to the bivalve's mode of life. Studies examining modes of life within marine bivalves may be more comparable due to the use of similar methods, however, comparisons globally may be difficult due to differences in methodology and reporting (Vinay Kumar et al., 2021).

The use of various temperatures to store and digest environmental samples will be discussed in relation to isolating and preserving the MPs within various marine biota to minimize further degradation to accurately characterize MP size, shape, and abundance in natural environments (Thiele et al., 2019). The use of freezing and subsequently thawing

through often unreported methods for environmental samples may cause MPs to shed, fracture, split, or degrade, which may influence abundance, shape and size (Brostow et al., 2011; Chubarenko, 2022). A limitation of methods in the scope of this literature review are the potential data gaps in the accuracy of reporting where the freezing of marine biota before processing is a common method due to manay logistical and practical reasons such as the transportation of samples long distances (Hidago-Ruz et al., 2012). However, there is limited information on the nature of freezing (freezer, liquid nitrogen, or other) as well as the duration of freezing until processing which may influence plastic properties (Alimi et al., 2022; Niu et al., 2021). In addition, methods of processing organisms should be considered to preserve the MPs in their ingested state (Courtene-Jones et al., 2017). It is understood that decreasing temperature restricts molecule mobility and hence makes a polymer more brittle, which enhances the fragmentation of macro-plastic items and the generation of MPs (Brostow et al., 2011; Chubarenko, 2022). Furthermore, plastic materials at temperatures below their glass transition temperature or the brittle/ductile transition (Tg/Tbd) temperature are brittle (Brostow et al 2011). For instance, the approximate Tg/Tbd (applicable to the amorphous part of polymer, and crystalline part, respectively) for PET ranges from -40^{o}C to 75^{o}C, and for rigid PVC -10^{o}C to +1^{o}C in marine environments. For environmentally degraded plastics and MPs it is understood that the brittleness of plastics increases with aging (Brostow et al., 2011). Therefore, the effect of cold temperatures should be considered when developing a MP extraction methodology for accurately characterizing MP size and shape in marine bivalves.

The use of ethanol may be used to store and preserve bivalve samples before the processing phase. Ethanol functions through the extraction of water, denaturing of proteins and other biomolecules, preserving the structure and integrity of tissue (Kristoffersen & Salvanes, 1998; Kundu et al., 2017). Herrera et al. (2018) used 96% ethanol as a density separation treatment for vegetal-rich samples and observed recovery rates of 100% for PE, PP, PS, and PA pellets. Furthermore, Dawson et al. (2020) found that the addition of 100% EtOH did not induce physical or chemical degradation in polymers. Further research is needed on other preservatives to determine any potential effects on MPs as an alternative method for storage.

4.5.2 Target tissues, tissue digestion, and density separation

MPs can be extracted from a variety of tissues or organs to determine the accumulation of particles at specific sites (Ding et al., 2018; Sparks et al., 2021). The selection of target tissues may also be due to the MP size classifications analyzed where small sizes are able to pass through specific organs (Browne et al., 2008; Dellisanti et al., 2023). The whole soft tissues of bivalves may be more widely examined as they represent a portion of seafood (Dehaut et al., 2016). Furthermore, the dissection of specific tissues may introduce contamination through prolonged exposure to the air (Wesch et al., 2017).

There are various chemical digestion methodologies used to separate the targeted tissue from suspected MPs, including: alkaline or basic reagents (potassium hydroxide (KOH)), enzymes (protease, lipase, trypsin), oxidative chemicals (hydrogen peroxide (H_2O_2)), acids (nitric acid (HNO_3)), or a mixture of digestion methods (Dehaut et al., 2016; Mercogliano et al., 2021). Within the evaluated studies, the two main reagents used

were KOH and H_2O_2 for digesting bivalve tissues. In earlier methods, strong acids such as nitric acid at specific dilutions were recognized as efficient methods of degrading soft tissues. However, the use of strong acids has drawn criticism for potentially reducing the number of MPs recovered (Phuong et al., 2018; Mathalon & Hill, 2014). In addition, Gulizia et al. (2022) found HNO_3 to be the most destructive for PS MPs with alkali and oxidative reagents resulting in negligible changes in plastic properties. Enzymes can be used as a digestion method to break down various organic tissues. However, their application can be very time-consuming, and costly, and can contain multiple steps that may increase risks of contamination (von Friesen et al., 2019). Studies have looked at the use of Proteinase-K to digest tissues. However, the high costs associated with the digestion of 0.2g of tissue using Proteinase-K was found to be unsuitable and costly for digesting entire oyster tissues (Karlsson et al., 2017; Thiele et al., 2019). It has been observed that enzymes and the use of small size filters might be incompatible where digestion was only filterable with larger pore size filters such as 63 μm or 88 μm when using Trypsin (Courtene-Jones et al., 2017; Karami et al., 2017; Thiele et al., 2019).

The use of 10% KOH was the most common reagent used within the scope of this review. KOH as an effective but MP non-destructive method has been found to recover acrylic and rayon fibers, PP and PET in fibre-form and film of PVC and LDPE (Catarino et al., 2017; Karami et al., 2017; Thiele et al., 2019). This was confirmed by Liu et al. (2021), who found similar results where a 10% KOH digestion treatment was the most successful treatment showing acceptable recoveries (>97%) without degrading MPs. In addition to being widely effective, it has a lower hazard than other reagents, is easily filtered, and is relatively low-cost, which makes this a suitable and accessible reagent

(Thiele et al., 2019; Wang et al., 2017). This agrees with other studies that have stated that using 10% KOH is an efficient method of digestion without MP degradation (Covernton et al., 2019; Ding et al., 2022). However, acrylic may be particularly affected by alkaline reagents through being hydrolyzed which can cause polymer damage and changes in spectral output (Gupta et al., 2004; Thiele et al., 2019). Furthermore, Karami et al. (2017) found a reduced intensity at 1610 cm^{-1} in PET when exposed to KOH at 50 or 60 °C for 96 h. However, this could be due to the use of high temperatures. Overall, there are limited studies examining the effects of digestion on spectral intensities, although the abundance of studies using the 10% KOH seems to be acceptable for producing spectra for polymer identification. Therefore, 10% KOH is a suitable reagent for the digestion of bivalve samples without the further degradation of SMPs. However, due to the denaturing of proteins and absorption of water due to the use of ethanol as a preservative, 10% KOH alone may not be sufficient to digest whole tissues in conjunction with this storage method. If 10% KOH alone does not fully digest the tissue, small amounts of 30% H_2O_2 is recommended to be added to fully digest bivalve tissue (Do et al., 2022). This method of using both alkali and oxidative reagents has been tested and displayed high recovery rates and percentage of similarity for spectroscopy signatures (Munno et al., 2018).

In some studies**,** a single digestion step is insufficient for effectively isolating separated MPs from non-plastic material. Remaining digestate in environmental samples could include inorganic material such as sand or glass, remaining biomass, or other organic material that can clog small pore size filters, and cause obstructions during the analysis phase (Xu et al., 2020). The remaining sample is added to an often high-density

liquid solution to separate the MPs from the heavier material left in the sample, such as sodium chloride (NaCl) (ρ=2.16 g/cm^3), sodium iodide (NaI) (ρ=3.67 g/cm^3)., or zinc chloride ($ZnCl_2$) (ρ= 2.91g/cm^3). In comparison, common plastics such as polypropylene have densities around 0.87-0.92 g/cm³ and polystyrene with densities ranging from 1.04-1.08 g/cm³ (Grigorescu et al., 2019). The approach is that these low-density plastics would float to the surface usually in conduction with some form of agitation to further separate the material where MPs can be extracted and filtered for analysis (Mattsson et al., 2022; Mercogliano et al., 2021). However, the limitations of using a density separation step are due to the transfer of material, which can introduce contamination or even result in the loss of sample (Dellisanti et al., 2023).

In both the digestion and density separation steps, the use of high temperatures is commonly applied to speed up reaction time and facilitate the digestion of organic material. It is suggested that temperature plays an auxiliary role rather than a leading role in the process of plastic degradation, making particles more prone to breaking and cracking (Lin et al., 2022). Additional methods testing found that experiments using wet peroxide oxidation generated enough heat to result in the complete loss of some types of MP particles, and boiling tests confirmed that temperatures >70°C were responsible for MP loss (Munno et al., 2018). Furthermore, Dehaut et al. (2016) reported degradation in polycarbonate (PC), at heating temperatures of 60°C. Thiele et al. (2019) found that at differing temperatures KOH destroyed rayon at 60 °C but not at 40°C. Hence it is recommended to consider the effects of temperature by heating from a precautionary stance where the maximum temperature applied should be 40^o C for the recovery of various MP polymers from environmental samples (Thiele et al., 2019). In conjunction

with sample processing, various efficiency and recovery rate tests have commonly been performed to confirm the reliability of the chosen methods, and investigate any potential morphological effects to SMPs. (Catarino et al., 2017; Cho et al., 2019; Sparks et al., 2021; Wu et al., 2020). Spike recovery tests often involve the use of virgin MPs, which may be more durable than environmentally weathered MPs. The lack of morphological changes that may be observed in these tests using virgin plastics may not necessarily be indicative of the behaviours of the suspected plastics in environmental samples (Savino et al., 2022). In spiked tests, it is also observed that there are significantly lower recovery rates for small size MPs <500 μm. (Avio et al., 2015; Imhof et al., 2012; Thiele et al., 2019). It is suggested that this could be due to the handling process, therefore the flushing of equipment with >500 mL may be required for the reliable inclusion of small size MPs (Thiele et al., 2019).

While the use of digestion treatments is more standardized, the use of varying filters in the methodology of assessing MPs is far from being uniform and may influence MP abundance counts (Sparks et al., 2021; Kazour & Amara, 2020; Phuong et al., 2018). Within the scope of this literature review, 66 studies were identified using >5 μm pore size filters, which is recommended by ICES (J. Li et al., 2019; Vandermeersch et al., 2015). There were other studies using larger pore size filters such as 5 – 10 μm pore sizes (Expósito et al., 2022; Li et al., 2018), 10 – 30 μm pore sizes (Chen et al., 2020; Li et al., 2018) and <30 μm pore size filters (Scott et al., 2019). Filter pore size should be selected and related to the spectroscopic limits and visual analysis methods chosen for the assessment of MPs (Cai et al., 2020). The disconnect between the use of small pore size filters and the detection limits defined by methodology can result in an unoptimized

approach and a time-consuming process for the analysis of larger particles (Xu et al., 2020). Small size filters can be easily clogged without proper digestion methods and can introduce an increased risk of contamination due to longer processing times/steps. In addition, Phuong et al. (2018) found that 20 μm was the minimum particle size that could be detected using FTIR under manual inspection. For studies looking at mid-size particles or the use of FTIR where the size detection limit is larger, pore size should be considered.

4.5.3 Visual analysis

MPs within marine bivalves are typically categorized by visual microscopy using various microscopes such as: stereo, dissecting, digital, or compound microscopes using light to help identify particles (Huang et al., 2023; Kalaronis et al., 2022). MP particles are of various shapes but often transparent in colour that can be mistaken for biological material, or non-plastic material such as glass (Lenz et al., 2015). One of the advantages of visual sorting included allowing for a detailed qualitative assessment of the size colour and shape (Kotar et al., 2022; Phuong et al., 2018). However, these results often overestimated the number of MPs, as their composition could not always be verified through visual observation. As well, visual observation can be a quite time-consuming process (Mathalon & Hill, 2014; Phuong, et al., 2018). Visual screening using these techniques is also highly labour intensive and false identification rates are high (over 70%), especially for small and transparent particles (Shi et al., 2022; Shim et al., 2017). For visual identification, it is important to have specific criteria as part of the methodology (Hartmann et al., 2019; Rochman et al., 2019). These include looking at the homogenous thickness across the particles, various colours (red, blue, green, and black),

counting fibers, (Kazour et al., 2019; Kazour & Amara, 2020; Sparks et al., 2021). From the results of this literature search, 98.96% (n=96) of the studies reported some sort of visual microscopy for the preliminary identification of MPs. However, within this study, only 54.64% (n=53) reported criteria such as size and shape morphology. Overall, it is understood that visual microscopy is an effective and accessible skill for the quantification of particles (>50 μm) (Kotar et al., 2022). Although microscopy alone is less reliable for smaller particle sizes (>20 μm), additional methods should be considered such as staining with dyes, fluorescence, polarized light microscopy, or tactile examination (Kotar et al., 2022).

Five of the examined studies used Nile Red staining. This is a lipophilic dye that can be absorbed on the surfaces of MPs due to its hydrophobic nature (Nalbone, Panebianco, et al., 2021; Shim et al., 2016). Excitation wavelengths from 450-490 nm and emission wavelengths from 515-565 nm are noted as some of the most used wavelengths for MP detection (Shruti et al., 2022). Nile Red is dissolved in solvents such as ethanol, acetone, or methanol, which support the binding of Nile Red to plastics and influence spectral intensity (Shruti et al., 2022). Nile Red staining has proven efficient at distinguishing MPs from non-plastic materials such as amphipod carapaces, algae, seaweeds, wood, feathers, mollusk shells, chalk and sand particles using only blue light microscopy filters, or in combination with orange filters (Shim et al., 2016). Nile Red may be efficient for organic-rich samples where (Mai et al., 2018; Shim et al., 2016) found a recovery rate of 98% in MP detection. However, other studies have indicated chitin-based debris and natural fibers can still be stained and show fluorescence, leading to a potential misidentification of the particles present which should be further reported

(as % or rate of negative identifications) and reflected in the results (Stanton et al., 2019). Research also suggests that the counting of Nile Red images may overestimate by 11-67% due to the presence of organics (Nel et al., 2021). However, the addition of H_2O_2, as suggested by Erni-Cassola et al. (2017) may address issues where organic materials may fluoresce. Furthermore, there is potential for machine learning or algorithmic techniques to be applied during the processing phase of images to reduce human bias (Primpke et al., 2017; Primpke et al., 2018). Meyers et al. (2022) developed a semi-automated process training Plastic Detection Model (PDM) and a Polymer Identification Model (PIM) that predicted with high accuracy the plastic or natural origin of particles (95.8%), and the polymer types of the MPs (88.1%). Further research should be performed to determine the reliability and accuracy of trained models.

Other microscopic techniques for acquiring characteristics of MPs include the use of a scanning electron microscope (SEM), which is able to produce information about the morphological surface structure of MPs through the generation of high-resolution images (Mariano et al., 2021; Shi et al., 2022). Advantages of SEM include allowing for a greater depth than traditional visual microscopy, higher resolutions that can lead to details about surface textures for further classification of particles, as well as the potential to go down to the nanoplastics scale, where Shi et al. (2022) produced images down to a resolution of 1nm. Limitations of SEM include the expense of the equipment, as well as long sample preparation times (Mariano et al., 2021). As with Nile Red staining and fluorescent microscopy, it is recommended that future research is needed to create automated approaches to produce comprehensive datasets. From an examination of the various visual methods presented in studies examining MPs in marine oysters and mussels, it is

recommended that Nile Red fluorescence/fluorescent microscopy be used to capture and identify, large, hard to see, and transparent MPs. This is due to its suitability for identifying small size class particles, as well as its wider accessibility and lack of technical training requirements compared to other methods such as SEM.

4.5.4 Polymer identification

Methods for visually identifying but also confirming the presence of plastic particles include Raman spectrometry (Kazour, Jemaa, et al., 2019; Van Cauwenberghe & Janssen, 2014), Fourier-transformed infrared spectrometry (FTIR) (Pequeno et al., 2021; Zhang et al., 2023), pyrolysis-gas chromatography combined with mass spectroscopy (FTIR-GC/MS) (Liu et al., 2021), attenuated total reflectance (ATR) (Masiá et al., 2022), and SEM-EDS (Li et al., 2016) (Figure 4.5). The assessment of polymer categories through spectroscopic methods is useful due to their ability and efficiency in identifying polymer types through the acquisition of spectral data (Phuong et al., 2018b). Imaging is conducted at different wavelengths to capture varying sizes of MP pollution (Sparks et al., 2021; Phuong et al., 2018). FTIR in general is a more cost-effective option that requires less tedious sample preparation. FTIR is more suitable for larger-sized MPs and challenging for particles that are between or smaller than 10-20 μm (Cabernard et al., 2018). Furthermore, FTIR spectra can be affected by moisture and therefore may not be optimal for some aquatic environmental samples (Dellisanti et al., 2023; Mai et al., 2018). This was also confirmed by Käppler et al. (2016) who asserts FTIR imaging is currently the most convenient method for MP analysis due to short measurement times, and the investigation of large sample areas. However, given the size detection limit of

FTIR, the number of smaller-sized MPs currently in the environment may be underestimated using this method (Sparks et al., 2021). This is further suggested in previous studies, which have observed up to a 35% loss in the detection of small-size MPs (<20 μm) (Käppler et al., 2016; Vinay Kumar et al., 2021).

In comparison, Raman spectrometry measures the energy difference between the incident (laser) light and the scattered (detected) light. Filters should be smooth and unstructured to ease the detection of MPs, especially the smaller ones. This method has limitations which require a significantly longer duration for the same analysis (Käppler et al., 2016; Löder et al., 2015). The efficiency of the approach to analyze MPs smaller than 20 μm in general is significantly less in comparison to micro-Raman spectroscopy due to the diffraction limit of light (Käppler et al., 2016). The Raman approach theoretically can detect MPs in the 1 μm range and is resistant to water and moisture in environmental samples (Lenz et al., 2015). Other limitations include that organic colourants and pigments strongly fluoresce in visible light which can hinder spectral acquisition (Vinay Kumar et al., 2021).

In combination with reliable analysis algorithms, manual investigation on an individual particle basis could be made obsolete for counting, polymer type identification and size. Within spectroscopic approaches, the method of subsampling is often used due to the time-consuming effort of analyzing individual particles manually (El Khatib et al., 2023). Without costly particle identification add-ons such as Horiba's "ParticleFinder," the effort required to manually produce spectra for hundreds of identified particles can be tedious and time-consuming (Lenz et al., 2015; Pittroff et al., 2021). Sub-sampling at this phase is common where a number or a percentage of identified particles are analyzed

using spectroscopic methods (Brandt et al., 2021; El Khatib et al., 2023; Thaysen et al., 2020). Factors such as particles with different buoyancy may affect estimates and estimates from subsampling may not be indicative at all of what is truly in a sample (Dellisanti et al., 2023). Both FTIR and Raman spectra are matched with commercial or open-source libraries, where acquired spectra can be compared to known databases (Miller et al., 2022). It is recommended for the use of FTIR or Raman, that the spectral match rate is 75% as a standard threshold (Li et al., 2019).

Other methods include the hot needle test, or hot point test, which is used to identify suspected MPs under optical microscopy by observing their physical melt or deformation. This method is widely accessible due to its low cost and lack of the need for technical training (Beckingham et al., 2023; Devriese et al., 2015). Beckingham et al. (2023) found that in a single-blind trial of researchers applying different hot point conditions to a set of synthetic, semi-synthetic and natural fibres, synthetic and some natural fibres were accurately identified >70% of the time. They also found that cellulose acetate from cigarette filters was the most challenging to identify due to the variability in the response of individual fibres to heat and the difficult observation of small microfibers (Beckingham et al., 2023).

Another method is the use of a scanning electron microscope coupled with energy dispersive spectroscopy (SEM-EDS/EDX) to rule out non-plastics and screen for potential MPs, based on surface characteristics and elemental signatures (Wang et al., 2017). Chlorinated plastics such as PVC could be easily identified with SEM/EDS due to their unique elemental signatures including chlorine, as could mineral species that are falsely identified as plastics by optical microscopy (Wang et al., 2017). Research has also

utilized Pyr-GC/MS as a tool to determine chemical composition, as well as other environmental pollutants on the surfaces of MPs (Peters et al., 2018; Zhong et al., 2022). A limitation of this method is that it requires highly skilled operators as well as complex sample preparation. This technique is also not widely accessible and is destructive to the samples during analysis (Dellisanti et al., 2023).

In addition to a suite of individual methods for polymer identification, there are also studies that combine methods to measure both abundance and categorize polymer type. For instance, Liu et al., 2021 used thermal gravimetric analysis (TGA), FTIR and chromatography-mass spectrometry (TGA-FTIR-GC/MS) to determine both polymer type and quantity of MPs in the sample represented as mass. TGA is an analytical technique used to measure the weight loss of a sample as it is heated at a programmed rate in a controlled gaseous environment. Combined with GC/MS the products of pyrolysis can be analyzed using mass spectrometry (Liu et al., 2021).

4.5.5 Quality assurance and quality control measures

Due to the small size and abundance of MPs naturally occurring in anthropogenic environments, contamination assessments are completed to ensure that airborne contamination is captured in the data. Measures such as the use of non-leaching freezer bags and Petri dishes, multiple rinsing of lab equipment, drying materials in the oven or freeze drier, as well as the use of non-plastic equipment, can reduce potential contamination. In addition, the use of cotton clothes and lab coats reduced the occurrence of polyester MP fibres (Kazour et al., 2020). Other precautions taken include the use of ultra-pure, deionized, or MilliQ® water to wash equipment (Hermabessiere et al., 2019;

Phuong et al., 2018). Performing procedures in fume hoods with a switched-off aspiration system limited airborne particles in the ambient air (Hermabessiere et al., 2019). Atmospheric blanks or controlled air quality testing is also an efficient way to check for contamination within the ambient air at various stages during the procedure (Hermabessiere et al., 2019). Within the scope of this literature review, only five studies were identified that did not report any 'blank' or 'control' procedures. 'atmospheric', 'procedural', or 'airborne' blanks were loosely defined. Some studies reported using background or airborne blanks where an open petri dish or vial would be left open and later analyzed for suspected MPs and then subtracted from data (Dawson et al., 2023; Martin et al., 2018; Tsering et al., 2022). Other studies would perform 'procedural', 'airborne' or 'controls' where the laboratory procedure without a sample would be employed to capture any background contamination (Ding et al., 2022; Nalbone et al., 2021; Saha et al., 2021). This could be interpreted to assert that 'airborne' and 'procedural' blanks are equivalent when they are not. One of the limitations of this literature search is the lack of standardization in the reporting of methods. Additional QA/QC measures may have been taken but not explicitly stated. It is imperative that there is a more descriptive method section potentially within an expanded supplementary data section, where all methodological information can be accessed. Since MP research is an emerging field, the methodologies are just as important as the results and they should be transparent and made readily available to produce comparable data (Cowger et al., 2020).

4.5.6 Challenges in the reporting of microplastic data

In addition to the challenges associated with selecting a MP extraction and analysis procedure, there are also challenges in the reporting of MP data. There is high variability in the reporting of size range, making it difficult to draw comparisons between studies (Li et al., 2019). Larger sized MPs >100 μm have been more commonly observed in bivalves within the environment, due to the methods chosen which may not be suitable for the detection of small size MPs (Li et al., 2019; Naidu, 2019). This is also the case for MP shape classifications or morphology. Research reports various shapes such as fragments, fibers, spheres, pellets, and films (Digka et al., 2018; Dowarah et al., 2020; Phuong et al., 2018; Railo et al., 2018; Sparks et al., 2021). These are all common MPs shapes. However, current studies often do not define the parameters for classifying suspected MPs as such. There are also limitations in the comparison among studies when looking at the classification of MPs. It is also important to note the ratio of length to width often used to discern between fragments and fibers, but at small particle sizes this may be more difficult to differentiate. This lack of standardization in reporting is also exemplified in the reporting of MP loads. Units such as items/gram, items/individual, kg of dry weight, and MP detection as a percentage are used to report MP abundance, however they are not easily comparable (Bom & Sá, 2021; Cowger et al., 2020). It is recommended that SMPs/gram of wet weight of tissue (MPs/g WW) are used as standardized units for future studies to make better comparisons between global research (Bom & Sá, 2021; Li et al., 2019). It is also recommended that inclusion and exclusion criteria for size class, morphology, and other characteristics be explicitly defined. Standardization in the reporting of size classes, morphological classifications, and

reported units should be used to make research more replicable, comparable, and reliable (Cowger et al., 2020).

4.6 Future recommendations

This review has provided a short overview of current and emerging methods for extracting and analyzing MPs in both marine oyster and mussel species.

1. **Standardization and consideration of chemical reagents and the use of temperature:** the use of varying reagents may impact the recoverability of MPs. The use of temperature during processing can affect the detection and quantification of MPs. It is recommended that 10% KOH at a maximum of 40^{o}C heat be used to extract MPs from bivalve tissue. A <5 μm pore size is also recommended for the analysis of small size MPs. Considerations for temperature during storage is suggested and further research is needed to characterize the effects of low temperatures on MPs.
2. **Standardization of visual analysis and polymer identification methods.** It is recommended that Raman spectroscopy is used to analyze and characterize MPs due to their smaller detection limits. While there is a common trend in using FTIR as a spectroscopic technique, it is recommended that Raman spectroscopy be used for analyzing MPs in marine mussels and oysters to capture small particle sizes (>20 μm). Future research is recommended in comparing visual analysis methods using various microscopy and fluorescent microscopy techniques.
3. **Standardization and reporting of class sizes, shape classifications, and MP units:** Currently there is consensus on the specific size classes for MP research. In

consequence, this has made it difficult to compare abundance, morphology, and classification between studies. Future studies should report MP load in units such as particles/g, so data is more widely comparable (Bom & Sá, 2021).

4. **Further research in accessible methods:** Access to costly resources such as Nile red or spectroscopic methods may not be readily accessible, therefore the use of cost-effective dyes and fluorescent imaging should be explored and standardized to synthesize a to promote inclusive science (Sturm et al., 2023).

4.7 Conclusion

This review examines the common MP extraction and analysis methods in mussels and oysters from marine environments and provides new perspectives for accurately enumerating MPs. Common extraction, visual analysis, and polymeric identification approaches are still highly variable, which makes comparisons between studies difficult. It is imperative that future MP research should move towards standardization to become more comparable, transparent, and reproducible. Extraction methods for accurately enumerating MPs at smaller size classes are recommended, as well as using algorithmic techniques and machine learning during image processing, which may help remediate human error as well as any visual bias that is present within this field of research. Concerns over issues such as the use of temperature, filter pore size, and digestion reagents should be further researched to develop standard procedures. Further research in the effects of freezing and fixatives on MPs as a method of sample storage is recommended. Reagents such as KOH and/or H_2O_2 are commonly used to digest marine mussel and oyster tissues. Furthermore, it is suggested that in both the storage and

digestion phase, temperatures above 0° C and below 40° C be used to preserve MP morphology. To capture small size MPs, it is recommended that micro-Raman spectroscopy is used to characterize polymer types of suspected MPs. It is recommended that procedural blanks or controls be performed during the processing phase to capture background contamination. Overall, trends point to the need for a standardized approach to MP extraction and analysis, and the incorporation of new technologies to provide a more thorough understanding of the abundance, morphology, and characterization of MP pollution in marine bivalves.

CHAPTER 5: Conclusion

5.1 Summary of research

This study presents findings on the occurrence and characterization of MPs found in Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) across Nova Scotia. The three objectives of this study were to:

1. Assess and compare the concentration of MPs in Blue mussels and Eastern oysters from Nova Scotia.
2. Characterize the polymer types of MPs found in Blue mussels and Eastern oysters from Nova Scotia.
3. Assess and compare the common methods used to enumerate and analyze MPs in Blue mussels and Eastern oysters and the challenges associated with methodologies of MP analysis.

The first two objectives of this thesis were achieved through the collection of Blue mussels and Eastern oysters from 13 sampling sites across Nova Scotia. MP isolation and analyzing techniques, mixed alkaline and oxidative digestion, density separation, and small pore size filtration were used to isolate the MPs from the soft tissue of the collected bivalves. To analyze the MPs, Nile Red microscopy and micro-Raman spectroscopy were used to determine the abundance and concentration of SMPs and confirm specific polymer types. Concentration was reported SMPs/g of wet-weight tissue and abundance was reported as suspected MPs per individual. Following the study, the third objective was completed using a literature review search to explore the common and emerging methods of MP enumeration and analysis within marine mussels and oysters. Trends and

limitations of various methodologies for isolating and analyzing MPs from marine mussels and oysters were used and helped inform some of the methods chosen in the present study.

5.2 Research findings

This study confirms the presence of MPs within the soft tissue of Blue mussels (*Mytilus edulis*) and Eastern oysters (*Crassostrea virginica*) from Nova Scotia. Results have suggested that sampling location may influence SMP concentration in Blue mussels and Eastern oysters. In addition, sampling location and species of bivalve influenced SMP concentration in bivalves in Melmerby Beach and Tatamagouche. Overall abundance and concentration of SMPs were lower than in previous studies performed in Nova Scotia, which may be due to the differences in sampling location and the methodologies used. The results of this study indicate the need for further monitoring of MPs in various environmental media from Nova Scotia. These findings confirm the need for standardization in MP research, the reporting of comparable units, and transparency in methodologies. The results of this study have shown that bivalves such as mussels and oysters are suitable for measuring MP pollution in marine ecosystems across Nova Scotia.

This study has identified trends and presented considerations for future MP isolation and characterization protocols in marine mussel and oyster species. This includes future research or the consideration of alternative methods of storage for environmental samples in place of freezing to fully characterize MP pollution in marine biota. The use of KOH and of H_2O_2 has proven to be effective in digesting the soft tissue

of bivalve species. The use of temperatures above 0ºC to a maximum of 40ºC to progress digestion reactions should be considered to preserve MP size and shape and prevent further degradation through heating. Previous studies have shown that Nile red microscopy and micro-Raman spectroscopy are suitable methods for enumerating small size MPs in environmental biota. The inclusion of blank corrections and the nature of the blanks performed should be reported to avoid misrepresentation of the results. It is recommended that future discussions on the definition of terms such as 'procedural blanks' are defined to further promote standardization. Future studies should consider reporting MP load in concentrations such as MPs/g of wet weight tissue to make studies more comparable.

5.3 Study limitations

While this study provided a baseline assessment of MPs in Blue mussels and Eastern oysters from Nova Scotia, there were limitations that may inhibit a wide comparability of this study with other global assessments. Limitations include a low sample size due to the lengthy processing time, which was a limiting factor for producing a reasonable timeline. Other factors such as the laboratory spaces used for processing, and/or analysis may have introduced additional contamination. Limitations of this study might also include the proportion of mussels to oysters analyzed. For instance, the lack of oysters sampled was due to the environmental conditions and sparsity of oyster beds. Further research should be completed to further strengthen the comparison of MP concentrations found between these two bivalves. Within the methods, a potential limitation may be the use of Nile Red where staining may cause biological material such

as chitin to fluoresce, which may lead to the over or underestimation of SMPs (Stanton et al., 2019). In contrast, identification may be difficult for plastics with certain dyes such as black polyester, and Blue acrylic where weak fluorescence has been observed (Stanton et al., 2019). Limitations from this study also include the weak Raman signals obtained from the resulting particles, which may be due to steps in their processing, their small MP size, as well as the lack of expertise when handling the equipment. Due to the lengthy analysis process, a subset of the samples (~10%) were analyzed by micro-Raman spectroscopy and in addition, only a sub-section of the filter was analyzed for particles. Subsampling methods may not take into account factors such as the movement of particles with different buoyancy, which may create hotspots of potential MPs on filters and effect estimates, and therefore not be indicative of what is truly in a sample (Dellisanti et al., 2023). In addition, field and background blanks were not performed in this study which may lead to an overestimation of SMPs. Another limitation of this study was the lack of clear spectral results where a match rate of >60% was used. Generally, it is recommended that a match rate of >80% is used to accurately characterize particles. However, due to reasons such as the small sizes analyzed, as well as the methods used, weak signals were obtained from the micro-Raman and a lower match rate was used. In addition, the use of zinc chloride ($ZnCl_2$) and the density separation step could have influenced the spectral outputs. This is due to an observed thin film that may have been produced during the density separation step where the addition of water caused the precipitation of $ZnCl_2$ onto the resulting filters. Another limitation of this study was the presence of contamination found in the blanks, which may be a limiting factor in the reliability of this study. Within the Nile Red analysis, blanks were contaminated with

small size MPs. This contamination could be due to the lengthy laboratory process that presents an additional opportunity for contamination to reach samples, as well as small sizes. Even with cleaning and precautionary measures the presence of small-size MPs is inevitable in the ambient area due to the wide use of plastics in society, and their proliferation in outdoor and indoor environments is often undetected (Xie et al., 2022; Zhang et al., 2020). In comparison to the literature, some studies' blank corrections may be from solely airborne analysis and not fully procedural blanks. In addition, the long process and use of multiple steps for isolating the MPs from the soft tissue may have introduced contamination into the samples.

5.4 Recommendations and future research

1. **Future studies and biomonitoring opportunities**: Future studies should investigate MPs in bivalves from wild and aquaculture-raised organisms to further characterize potential risks from seafood consumption. In addition, further studies should examine the level of MP contamination in shellfish over a temporal scale from varying regions.
2. **Harmonization and standardization of methods:** Standardization in the storage digestion, analysis, and characterization of microplastics in marine mussels and oysters is recommended to improve comparability across studies. In addition, future research in cost-effective methods and fluorescent imaging should be explored to promote inclusive science (Sturm et al., 2023).
3. **Policy-based instruments for addressing plastic pollution:** The use of various policy instruments such as regulations be implemented federally to decrease the

production of virgin plastics and therefore decrease potential marine plastic pollution. This could be achieved by implementing recycled content regulations for plastics which will help government bodies regulate the amount and type of plastics on the market and potentially in the environment after their disposal (Vogt et al., 2021). The second is to reduce land-based plastics and reduce overall virgin plastics in the waste stream. This can manifest as diverting plastics from landfills, strengthening existing or weak recycling systems, as well as incentivizing the industry to redesign plastic products and packaging for circularity (Kahlert & Bening, 2022).

5.5 Conclusions

This study provides a baseline assessment of the MP contamination in mussels and oysters from Nova Scotia. Nile red analysis and micro-Raman spectroscopy were used to quantify and confirm MP polymers in these two bivalves. Results found that sampling location may have influenced SMP concentrations in Blue mussels and Eastern oysters. Results identified that sampling location and bivalve species were factors that influenced differences in the concentration of SMPs in Tatamagouche and Melmerby Beach. Results identified that there were no interactive effects between sampling location and the type of organisms in these locations. While the deviated from some of the current literature, the methods employed, and the sampling locations examined may have contributed to the observed differences Further studies using alternative methods with recovery rates and efficiency testing should be performed to assess the degree to which MP abundance/concentration may be over or underestimated. Nevertheless, MP particles

were found within both bivalve species across various locations in Nova Scotia. Future research in the analysis of MPs in seafood should be completed in Nova Scotia to determine the potential risk exposure of marine MPs to humans.

From the results of the review, there have been trends towards the standardization of methods in the quantification and analysis of MPs in marine mussels and oysters. Considerations and future research for the effects of temperature and fixatives such as ethanol on MPs in the storage and digestion phase are suggested. Methods such as Nile Red microscopy and plastic-confirming protocols such as FTIR or micro-Raman spectroscopy can be used for enumerating and characterizing MP pollution at varying sizes. Transparency in the reporting of methods and the reporting of MP concentrations in standardized units such as MPs/g of tissue should be considered to make data more comparable. Future research in cost-effective and efficient methods for MP research is recommended to improve more inclusivity and accessibility worldwide. Future research in the analysis of MPs in seafood should be completed in Nova Scotia to determine the potential risk exposure of marine MPs to humans through the aquatic environment or diets.

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Appendix A: Supplemental Maps and Images

Figure A1. Disclosed sampling locations for mussels in the South-Southwestern zone of Nova Scotia (Sites 9 and 10 (Right to Left)).

Figure A2. Disclosed sampling locations for mussels in the Eastern-Cape Breton zone of Nova Scotia (Sites 2, 3, and 4 (Left to Right)).

Figure A3. Disclosed sampling locations for mussels and oysters in the Gulf zone of Nova Scotia (Sites 6 and 7 (Right to Left)).

Appendix B: Microplastic Data and Analysis

Table B1. Plastic polymer legend.

Polymer abbreviation	Full name
PE	Polyethylene
PES	Polyester
PET	Polyethylene terephthalate
PP	Polypropylene
PS	Polysulfone
PVC	Polyvinyl chloride
Nylon/PA	Polyamide
ABS	Acrylonitrile butadiene
PUR	Polyurethane
PLS	Polysulfone

	M2-2	M2-2	M2-3	M2-4	M2-5	M2-6
total number of particles	7.00	10.00	9.00	7.50	10.00	8.50
fragment or film	7.00	10.00	9.00	7.50	10.00	8.50
fibre	0.00	0.00	0.00	0.00	0.00	0.00
Particle size (count)	M2-2	M2-2	M2-3	M2-4	M2-5	M2-6
2-10 µm	7.00	7.50	4.00	7.50	5.50	2.00
10-20 µm	0.00	1.50	4.00	0.00	3.50	6.50
20-30 µm	0.00	1.00	1.00	0.00	0.00	0.00
30-40 µm	0.00	0.00	0.00	0.00	1.00	0.00
40-50µm	0.00	0.00	0.00	0.00	0.00	0.00
>50µm	0.00	0.00	0.00	0.00	0.00	0.00
total	7.00	10.00	9.00	7.50	10.00	8.50
Count per filter	54.32	77.6	69.84	58.2	77.6	65.96
g/ sample	10.85	11.92	10.81	10.81	13.98	11.33
particles per g	5.01	6.51	6.46	5.38	5.55	5.82
	M2-1	M2-2	M2-3	M2-4	M2-5	M2-6
total number of particles	7.00	7.50	8.00	7.50	9.50	8.50
fragment or film	7.00	4.50	8.00	5.50	9.50	6.50
fibre	0.00	3.00	0.00	2.00	0.00	2.00
Particle size (count)	M2-1	M2-2	M2-3	M2-4	M2-5	M2-6
2-10 µm	0.00	3.50	5.00	4.50	4.50	5.50
10-20 µm	3.00	4.00	1.00	2.00	4.00	2.00
20-30 µm	0.00	0.00	1.00	0.00	1.00	0.00
30-40 µm	1.00	0.00	1.00	0.00	0.00	1.00
40-50µm	2.00	0.00	0.00	0.00	0.00	0.00
>50µm	1.00	0.00	0.00	1.00	0.00	0.00
total	7.00	7.50	8.00	7.50	9.50	8.50
Count per filter	54.32	58.2	62.08	58.2	73.72	65.96
g/ sample	13.14	10.93	10.56	9.39	11.19	12.65
particles per g	4.13	5.32	5.88	6.20	6.59	5.21
	M3-1	M3-2	M3-3	M3-4	M3-5	M3-6
total number of particles	5.50	8.00	7.50	6.50	6.50	5.50
fragment or film	4.50	8.00	7.50	6.50	4.50	5.50
fibre	1.00	0.00	0.00	0.00	2.00	0.00
Particle size (count)	M3-1	M3-2	M3-3	M3-4	M3-5	M3-6
2-10 µm	3.50	2.00	2.50	3.50	4.50	1.50
10-20 µm	0.00	3.00	4.00	3.00	1.00	3.00
20-30 µm	2.00	1.00	0.00	0.00	0.00	1.00
30-40 µm	0.00	0.00	0.00	0.00	1.00	0.00
40-50µm	0.00	1.00	0.00	0.00	0.00	0.00
>50µm	0.00	1.00	1.00	0.00	0.00	0.00
total	5.50	8.00	7.50	6.50	6.50	5.50
Count per filter	42.68	62.08	58.2	50.44	50.44	42.68
g/ sample	13.81	9.37	14.39	11.86	11.15	10.87
particles per g	3.09	6.63	4.04	4.25	4.52	3.93
	M4-1	M4-2	M4-3	M4-4	M4-5	M4-6
total number of particles	4.00	5.50	4.50	4.50	4.00	5.00
fragment or film	4.00	5.50	4.50	4.50	4.00	5.00
fibre	0.00	0.00	0.00	0.00	0.00	0.00
Particle size (count)	M4-1	M4-2	M4-3	M4-4	M4-5	M4-6
2-10 µm	2.50	2.00	1.00	4.50	4.00	1.00
10-20 µm	1.50	3.50	2.50	0.00	0.00	0.00
20-30 µm	0.00	0.00	0.00	0.00	0.00	1.00
30-40 µm	0.00	0.00	1.00	0.00	0.00	1.00
40-50µm	0.00	0.00	0.00	0.00	0.00	1.00
>50µm	0.00	0.00	0.00	0.00	0.00	1.00
total	4.00	5.50	4.50	4.50	4.00	5.00
Count per filter	31.04	42.68	34.92	34.92	31.04	38.8
g/ sample	10.65	12.68	11.43	9.51	11.67	9.81
particles per g	2.91	3.37	3.06	3.67	2.66	3.96

Figure B1. Sites M1-M4 Nile red blank-corrected particle counts per size range and concentration calculations.

	M2-2	M2-2	M2-3	M2-4	M2-5	M2-6
total number of particles	7.00	10.00	9.00	7.50	10.00	8.50
fragment or film	7.00	10.00	9.00	7.50	10.00	8.50
fibre	0.00	0.00	0.00	0.00	0.00	0.00
Particle size (count)	M2-2	M2-2	M2-3	M2-4	M2-5	M2-6
2-10 µm	7.00	7.50	4.00	7.50	5.50	2.00
10-20 µm	0.00	1.50	4.00	0.00	3.50	6.50
20-30 µm	0.00	1.00	1.00	0.00	0.00	0.00
30-40 µm	0.00	0.00	0.00	0.00	1.00	0.00
40-50µm	0.00	0.00	0.00	0.00	0.00	0.00
>50µm	0.00	0.00	0.00	0.00	0.00	0.00
total	7.00	10.00	9.00	7.50	10.00	8.50
Count per filter	54.32	77.6	69.84	58.2	77.6	65.96
g/ sample	10.85	11.92	10.81	10.81	13.98	11.33
particles per g	5.01	6.51	6.46	5.38	5.55	5.82
	M2-1	M2-2	M2-3	M2-4	M2-5	M2-6
total number of particles	7.00	7.50	8.00	7.50	9.50	8.50
fragment or film	7.00	4.50	8.00	5.50	9.50	6.50
fibre	0.00	3.00	0.00	2.00	0.00	2.00
Particle size (count)	M2-1	M2-2	M2-3	M2-4	M2-5	M2-6
2-10 µm	0.00	3.50	5.00	4.50	4.50	5.50
10-20 µm	3.00	4.00	1.00	2.00	4.00	2.00
20-30 µm	0.00	0.00	1.00	0.00	1.00	0.00
30-40 µm	1.00	0.00	1.00	0.00	0.00	1.00
40-50µm	2.00	0.00	0.00	0.00	0.00	0.00
>50µm	1.00	0.00	0.00	1.00	0.00	0.00
total	7.00	7.50	8.00	7.50	9.50	8.50
Count per filter	54.32	58.2	62.08	58.2	73.72	65.96
g/ sample	13.14	10.93	10.56	9.39	11.19	12.65
particles per g	4.13	5.32	5.88	6.20	6.59	5.21
	M3-1	M3-2	M3-3	M3-4	M3-5	M3-6
total number of particles	5.50	8.00	7.50	6.50	6.50	5.50
fragment or film	4.50	8.00	7.50	6.50	4.50	5.50
fibre	1.00	0.00	0.00	0.00	2.00	0.00
Particle size (count)	M3-1	M3-2	M3-3	M3-4	M3-5	M3-6
2-10 µm	3.50	2.00	2.50	3.50	4.50	1.50
10-20 µm	0.00	3.00	4.00	3.00	1.00	3.00
20-30 µm	2.00	1.00	0.00	0.00	0.00	1.00
30-40 µm	0.00	0.00	0.00	0.00	1.00	0.00
40-50µm	0.00	1.00	0.00	0.00	0.00	0.00
>50µm	0.00	1.00	1.00	0.00	0.00	0.00
total	5.50	8.00	7.50	6.50	6.50	5.50
Count per filter	42.68	62.08	58.2	50.44	50.44	42.68
g/ sample	13.81	9.37	14.39	11.86	11.15	10.87
particles per g	3.09	6.63	4.04	4.25	4.52	3.93
	M4-1	M4-2	M4-3	M4-4	M4-5	M4-6
total number of particles	4.00	5.50	4.50	4.50	4.00	5.00
fragment or film	4.00	5.50	4.50	4.50	4.00	5.00
fibre	0.00	0.00	0.00	0.00	0.00	0.00
Particle size (count)	M4-1	M4-2	M4-3	M4-4	M4-5	M4-6
2-10 µm	2.50	2.00	1.00	4.50	4.00	1.00
10-20 µm	1.50	3.50	2.50	0.00	0.00	0.00
20-30 µm	0.00	0.00	0.00	0.00	0.00	1.00
30-40 µm	0.00	0.00	1.00	0.00	0.00	1.00
40-50µm	0.00	0.00	0.00	0.00	0.00	1.00
>50µm	0.00	0.00	0.00	0.00	0.00	1.00
total	4.00	5.50	4.50	4.50	4.00	5.00
Count per filter	31.04	42.68	34.92	34.92	31.04	38.8
g/ sample	10.65	12.68	11.43	9.51	11.67	9.81
particles per g	2.91	3.37	3.06	3.67	2.66	3.96

Figure B2. Sites M5-M7 Nile red blank-corrected particle counts per size range and concentration calculations.

	M8-2	M8-2	M8-3	M8-4	M8-5	M8-6
total number of particles	9.33	9.00	6.67	7.33	8.33	5.00
fragment or film	9.33	9.00	6.67	5.33	6.33	3.00
fibre	0.00	0.00	0.00	2.00	2.00	2.00
Particle size (count)	M8-2	M8-2	M8-3	M8-4	M8-5	M8-6
2-10 µm	3.33	5.00	2.67	2.33	3.33	0.00
10-20 µm	2.00	1.00	0.00	0.00	3.00	1.00
20-30 µm	2.00	1.00	3.00	4.00	1.00	2.00
30-40 µm	2.00	2.00	0.00	1.00	1.00	1.00
40-50µm	0.00	0.00	0.00	0.00	0.00	1.00
>50µm	0.00	0.00	1.00	0.00	0.00	0.00
Total	9.33	9.00	6.67	7.33	8.33	5.00
Count per filter	72.43	69.84	51.73	56.91	64.67	38.80
g/ sample	11.54	10.51	11.72	10.34	14.60	14.73
particles per g	6.28	6.65	4.41	5.50	4.43	2.63
	M9-1	M9-2	M9-3	M9-4	M9-5	M9-6
total number of particles	4.00	1.00	3.00	4.33	0.00	4.67
fragment or film	4.00	1.00	3.00	3.33	0.00	3.67
fibre	0.00	0.00	0.00	1.00	0.00	1.00
Particle size (count)	M9-1	M9-2	M9-3	M9-4	M9-5	M9-6
2-10 µm	4.00	0.00	3.00	3.00	0.00	2.00
10-20 µm	0.00	0.00	0.00	0.00	0.00	1.67
20-30 µm	0.00	0.00	0.00	1.33	0.00	0.00
30-40 µm	0.00	0.00	0.00	0.00	0.00	0.00
40-50µm	0.00	1.00	0.00	0.00	0.00	1.00
>50µm	0.00	0.00	0.00	0.00	0.00	0.00
total	4.00	1.00	3.00	4.33	0.00	4.67
Count per filter	31.04	7.76	23.28	33.63	0.00	36.21
g/ sample	9.21	12.51	9.67	10.97	11.20	9.77
particles per g	3.37	0.62	2.41	3.07	0.00	3.71
	M10-1	M10-2	M10-3	M10-4	M10-5	M10-6
total number of particles	4.00	3.00	3.50	3.00	6.00	3.00
fragment or film	4.00	3.00	3.50	2.00	6.00	3.00
fibre	0.00	0.00	0.00	1.00	0.00	0.00
Particle size	M10-1	M10-2	M10-3	M10-4	M10-5	M10-6
2-10 µm	4.00	3.00	1.00	3.00	0.00	2.00
10-20 µm	0.00	0.00	2.50	0.00	0.00	0.00
20-30 µm	0.00	0.00	0.00	0.00	2.00	1.00
30-40 µm	0.00	0.00	0.00	0.00	2.00	0.00
40-50µm	0.00	0.00	0.00	0.00	2.00	0.00
>50µm	0.00	0.00	0.00	0.00	0.00	0.00
Total	4.00	3.00	3.50	3.00	6.00	3.00
Count per filter	31.04	23.28	27.16	23.28	46.56	23.28
g/ sample	10.59	12.02	11.17	14.20	13.12	10.86
particles per g	2.93	1.94	2.43	1.64	3.55	2.14

Figure B3. Sites M8-M10 Nile red blank-corrected particle counts per size range and concentration calculations.

	O1-1	O1-2	O1-3	O1-4	O1-5	O1-6
total number of particles	11.00	12.00	11.33	10.33	8.33	12.00
fragment or film	11.00	12.00	11.33	8.33	8.33	12.00
fibre	0.00	0.00	0.00	2.00	0.00	0.00
Particle size (count)	**O1-1**	**O1-2**	**O1-3**	**O1-4**	**O1-5**	**O1-6**
2-10 µm	5.00	9.00	3.33	3.33	3.33	5.00
10-20 µm	4.00	3.00	7.00	3.00	4.00	4.00
20-30 µm	1.00	0.00	1.00	2.00	1.00	1.00
30-40 µm	0.00	0.00	0.00	2.00	0.00	1.00
40-50µm	0.00	0.00	0.00	0.00	0.00	0.00
>50	1.00	0.00	0.00	0.00	0.00	1.00
Total	11.00	12.00	11.33	10.33	8.33	12.00
Count per filter	85.36	93.12	87.95	80.19	64.67	93.12
g/ sample	17.20	16.45	17.26	15.27	16.93	16.69
particles per g	4.96	5.66	5.10	5.25	3.82	5.58
	O2-1	**O2-2**	**O2-3**	**O2-4**	**O2-5**	**O2-6**
total number of particles	8.50	9.00	9.50	7.50	9.50	7.50
fragment or film	6.50	9.00	8.50	7.50	8.50	7.50
fibre	2.00	0.00	1.00	0.00	1.00	0.00
Particle size (count)	**O2-1**	**O2-2**	**O2-3**	**O2-4**	**O2-5**	**O2-6**
2-10 µm	2.50	1.00	1.50	4.50	1.50	3.50
10-20 µm	4.00	6.00	5.00	2.00	7.00	3.00
20-30 µm	0.00	1.00	1.00	0.00	0.00	0.00
30-40 µm	0.00	0.00	1.00	0.00	0.00	1.00
40-50µm	0.00	0.00	0.00	0.00	0.00	0.00
>50	2.00	1.00	1.00	1.00	1.00	0.00
total	8.50	9.00	9.50	7.50	9.50	7.50
Count per filter	65.96	69.84	73.72	58.20	73.72	58.20
g/ sample	15.45	12.84	13.13	13.85	13.52	12.67
particles per g	4.27	5.44	5.61	4.20	5.45	4.59
	O3-1	**O3-2**	**O3-3**	**O3-4**	**O3-5**	**O3-6**
total number of particles	6.00	6.33	7.33	7.33	6.67	5.33
fragment or film	6.00	5.67	7.33	6.33	6.67	5.33
fibre	0.00	0.67	0.00	1.00	0.00	0.00
Particle size (count)	**O3-1**	**O3-2**	**O3-3**	**O3-4**	**O3-5**	**O3-6**
2-10 µm	5.00	2.33	1.33	1.33	1.67	0.33
10-20 µm	1.00	4.00	5.00	4.00	4.00	2.00
20-30 µm	0.00	0.00	0.00	2.00	1.00	1.00
30-40 µm	0.00	0.00	1.00	0.00	0.00	0.00
40-50µm	0.00	0.00	0.00	0.00	0.00	1.00
>50	0.00	0.00	0.00	0.00	0.00	1.00
total	6.00	6.33	7.33	7.33	6.67	5.33
Count per filter	46.56	49.15	56.91	56.91	51.73	41.39
g/ sample	12.69	13.82	12.75	12.50	13.50	13.75
particles per g	3.67	3.56	4.46	4.55	3.83	3.01

Figure B4. Sites O1-O3 Nile red blank-corrected particle counts per size range and concentration calculations.

	O4-1	O4-2	O4-3	O4-4	O4-5	O4-6
total number of particles	5.00	5.50	6.50	5.00	5.50	6.50
fragment or film	5.00	4.50	6.50	5.00	5.50	6.50
fibre	0.00	1.00	0.00	0.00	0.00	0.00
Particle size (count)	**O4-1**	**O4-2**	**O4-3**	**O4-4**	**O4-5**	**O4-6**
2-10 µm	0.00	2.50	4.50	0.00	4.50	2.50
10-20 µm	1.00	0.00	2.00	5.00	1.00	3.00
20-30 µm	0.00	1.00	0.00	0.00	0.00	0.00
30-40 µm	0.00	0.00	0.00	0.00	0.00	0.00
40-50µm	2.00	1.00	0.00	0.00	0.00	1.00
>50	2.00	1.00	0.00	0.00	0.00	0.00
total	5.00	5.50	6.50	5.00	5.50	6.50
Count per filter	38.80	42.68	50.44	38.80	42.68	50.44
g/ sample	12.54	11.48	15.44	15.97	13.13	16.77
particles per g	3.09	3.72	3.27	2.43	3.25	3.01
	O5-1	**O5-2**	**O5-3**	**O5-4**	**O5-5**	**O5-6**
total number of particles	3.50	2.50	2.50	2.00	3.50	3.50
fragment or film	2.50	1.50	1.50	1.00	1.50	2.50
fibre	1.00	1.00	1.00	1.00	2.00	1.00
Particle size (count)	**O5-1**	**O5-2**	**O5-3**	**O5-4**	**O5-5**	**O5-6**
2-10 µm	0.00	2.50	2.50	2.00	0.00	0.00
10-20 µm	1.50	0.00	0.00	0.00	1.50	1.50
20-30 µm	0.00	0.00	0.00	0.00	0.00	2.00
30-40 µm	1.00	0.00	0.00	0.00	2.00	0.00
40-50µm	1.00	0.00	0.00	0.00	0.00	0.00
>50	0.00	0.00	0.00	0.00	0.00	0.00
total	3.50	2.50	2.50	2.00	3.50	3.50
Count per filter	27.16	19.40	19.40	15.52	27.16	27.16
g/ sample	9.82	13.48	12.45	10.99	10.89	11.64
particles per g	2.77	1.44	1.56	1.41	2.49	2.33

Figure B5. Sites O4-O5 Nile red blank-corrected particle counts per size range and concentration calculations.

	Blank 1	Blank 2	Blank 3	Blank 4	Blank 5	Blank 6	Blank 7	Blank 8	Blank 9	Blank 10	Blank 11	Blank 12	Blank 13	Blank 14	Blank 15	Blank 16	Blank 17	Blank 18
total number of particles	0.00	4.00	3.00	1.00	0.00	77.00	0.00	5.00	0.00	5.00	15.00	5.00	263.00	0.00	6.00	5.00	0.00	0.00
fragment or film	0.00	3.00	3.00	0.00	0.00	77.00	0.00	4.00	0.00	3.00	15.00	5.00	255.00	0.00	6.00	5.00	0.00	0.00
fibre	0.00	1.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	2.00	0.00	0.00	8.00	0.00	0.00	0.00	0.00	0.00
Particle size (count)	Blank 1	Blank 2	Blank 3	Blank 4	Blank 5	Blank 6	Blank 7	Blank 8	Blank 9	Blank 10	Blank 11	Blank 12	Blank 13	Blank 14	Blank 15	Blank 16	Blank 17	Blank 18
2-10 µm	0.00	2.00	1.00	1.00	0.00	68.00	0.00	4.00	0.00	2.00	12.00	4.00	217.00	0.00	3.00	1.00	0.00	0.00
10-20 µm	0.00	2.00	1.00	0.00	0.00	7.00	0.00	1.00	0.00	2.00	1.00	1.00	39.00	0.00	3.00	1.00	0.00	0.00
20-30 µm	0.00	0.00	0.00	0.00	0.00	2.00	0.00	0.00	0.00	1.00	1.00	0.00	6.00	0.00	0.00	2.00	0.00	0.00
30-40 µm	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00
40-50µm	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
>50 µm	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00
Total	0.00	4.00	3.00	1.00	0.00	77.00	0.00	5.00	0.00	5.00	15.00	5.00	263.00	0.00	6.00	5.00	0.00	0.00
Particles per filter	0.00	31.04	23.28	7.76	0.00	597.52	0.00	38.80	0.00	38.80	116.40	38.80	2040.88	0.00	46.56	38.80	0.00	0.00

Figure B6. Nile red blank particle counts per size range and concentration calculations.

Table B2: Mean concentration and abundance of microplastics in Blue mussels and Eastern oysters. Mean shell length, width and depth, wet weight of tissue analysed and ranges of shell length, width, and depth for mussels and oysters collected in Nova Scotia.

Measurement	Blue mussel	Eastern oyster
Concentration (MP/gram of tissue)	4.25 ± 1.48	3.79 ± 1.27
Abundance (MP/individual)	48.59 ± 17.93	53.54 ± 21.78
Mean shell length (cm)	66.79 ± 4.80	71.87 ± 9.19
Mean shell width (cm)	33.33 ± 4.19	47.17 ± 5.61
Mean shell depth (cm)	27.29 ± 4.92	21.50 ± 4.48
Wet weight of tissue analyzed (g)	11.47 ± 1.62	13.83 ± 2.02
Range of shell length (cm)	82.46-58.84	89.79-60.63
Range of shell width (cm)	41.53-26.83	63.82-35.86
Range of shell depth (cm)	46.17-26.19	31.47-13.77

Table B3. Mean concentration of microplastics, standard error (SE), and number of individuals analyzed in Blue mussels and Eastern oysters from various sampling locations across Nova Scotia. (M=mussel, O=oyster). Letters represent approximate zones (EC=Eastern-Cape Breton, G=Gulf, SW=South-Southwestern).

Zone	Location	Location #	Sample Code	Mean (SMPs/g)	SE	Mean particle Diameter (µm)	# of indi.
EC	Undisclosed Site	1	M1	5.79 ± 0.55	0.22	8.98 ± 2.49	6
	Halifax	2	M2	5.56 ± 0.79	0.32	14.10 ± 7.37	6
	Martinique Beach	3	M3	4.41 ± 1.08	0.44	14.42 ± 5.69	6
	Taylor Head	4	M4	3.27 ± 0.44	0.18	12.11 ± 8.83	6
G	Undisclosed Site	5	M5	5.22 ± 0.65	0.27	8.51 ± 1.72	6
	Melmerby Beach	6	M6	4.53 ± 0.63	0.26	11.86 ± 2.28	6
	Tatamagouche	7	M7	4.14 ± 0.75	0.30	16.10 ± 8.62	6
SW	Undisclosed Site	8	M8	4.98 ± 1.34	0.55	19.42 ± 5.04	6
	West Pennant	9	M9	2.19 ± 1.40	0.57	14.43 ± 14.79	6
	Risser's Beach	10	M1	2.44 ± 0.64	0.26	13.99 ± 12.63	6
EC	Undisclosed Site	11	O1	5.06 ± 0.61	0.25	14.02 ± 3.77	6
G	Undisclosed Site	12	O2	4.93 ± 0.59	0.24	18.05 ± 10.08	6
SW	Undisclosed Site	13	O3	3.85 ± 0.53	0.22	16.60 ± 8.69	6
G	Melmerby Beach	6	O4	3.13 ± 0.38	0.16	20.29 ± 15.3	6
	Tatamagouche	7	O5	2.00 ± 0.55	0.22	18.48 ± 14.77	6

$$\frac{(Area\ of\ captured\ field\ of\ view)}{(Area\ of\ Filter)} \times\ 100\% = Area\ scanned\ (\%)$$

$$\frac{(l\ \times w)}{(\pi r2)} \times 100\% = Area\ scanned\ (\%)$$

$$\frac{(14.62mm^2)}{(113mm^2)} \times 100\% = 12.9\%$$

Figure B7. Calculation of each filter scanned during Nile Red analysis.

$$\frac{(Area\ of\ captured\ field\ of\ view)}{(Area\ of\ Filter)} \times\ 100\% = Area\ scanned\ (\%)$$

$$\frac{(l\ \times w)}{(\pi r^2)} \times 100\% = Area\ scanned\ (\%)$$

$$\frac{(5.47mm^2)}{(113mm^2)} \times 100\% = 4.8\%$$

Figure B8. Calculation of each filter scanned during μ-Raman analysis.

Table B4. Number of microplastic particles identified within analyzed strip of Nile Red blanks collected, shapes identified and the most common shape, as well as the common size classification.

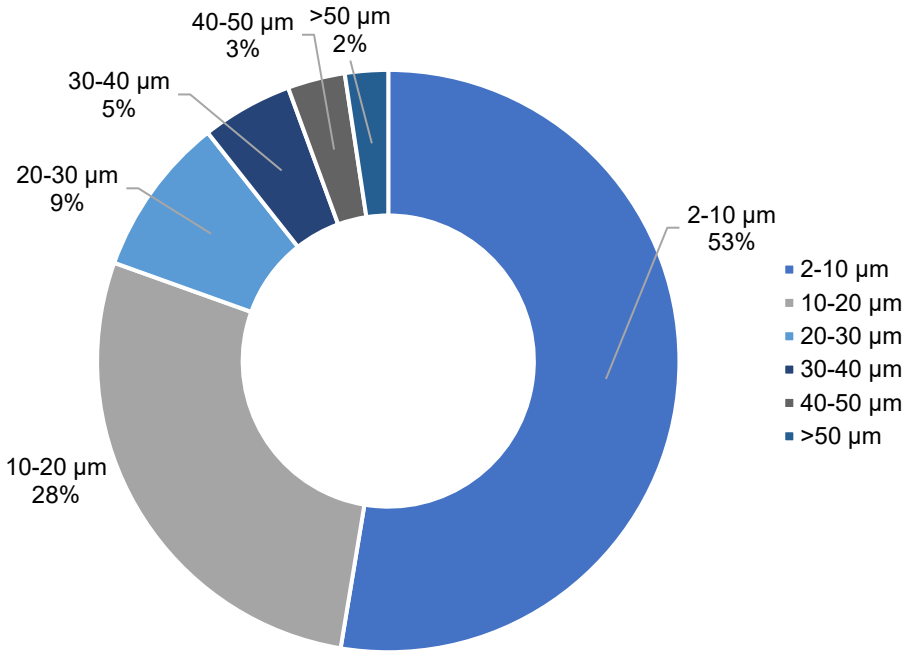
Blank	Number of particles identified within strip (~5%)	Shapes identified	Most common shape	Common size classification
Blank 1	0	N/A	N/A	N/A
Blank 2	4	Fragment or film, fibre	Fragment or film	10-20 μm
Blank 3	3	Fragment or film	Fragment or film	2.2-10 μm, 10-
Blank 4	1	Fragment or film	Fragment or film	20 μm, >50 μm
Blank 5	0	N/A	N/A	2-10 μm
Blank 6*	77	Fragment or film	Fragment or film	N/A
Blank 7	0	N/A	N/A	2-10 μm
Blank 8	5	Fragment or film, fibre	Fragment or film	N/A
Blank 9	0	N/A	N/A	2-10 μm
Blank 10	5	Fragment or film, fibre	Fragment or film	N/A
Blank 11	15	Fragment or film	Fragment or film	10-20 μm
Blank 12	5	Fragment or film	Fragment or film	2-10 μm
Blank 13*	263	Fragment or film, fibre	Fragment or film	5-10 μm
Blank 14	0	N/A	N/A	2-10 μm
Blank 15	6	Fragment or film	Fragment or film	N/A
Blank 16	5	Fragment or film	Fragment or film	2-10 μm
Blank 17	0	N/A	N/A	N/A
Blank 18	0	N/A	N/A	N/A

*Blanks that were contaminated with $ZnCl_2$ spiked with plastic

Table B5. Number of microplastic particles identified within analyzed strip of ethanol storage solution shapes identified/most common shape, as well as the common size classification.

Sample Code	Number of particles identified within strip (~5%)	Shapes identified	Most common shape	Common size classification
M1	0	NA	NA	NA
M2	0	NA	NA	NA
M3	2	Fragment or film, fibre	Fragment or film	30-40 µm
M4	0	NA	NA	NA
M5	0	NA	NA	NA
M6	0	NA	NA	NA
M7	2	Fragment or film	Fragment or film	20-30 µm
M8	0	NA	NA	NA
M9	2	Fragment or film	Fragment or film	>50 µm
M10	0	NA	NA	NA
O1	0	NA	NA	NA
O2	1	Fragment or film	Fragment or film	30-40 µm
O3	1	Fragment or film	Fragment or film	>50 µm
O4	0	NA	NA	NA
O5	0	NA	NA	NA
Blank 1	0	NA	NA	NA
Blank 2	0	NA	NA	NA
Blank 3	2	Fragment or film	Fragment or film	30-40 µm

a) Mussels



b) Oysters

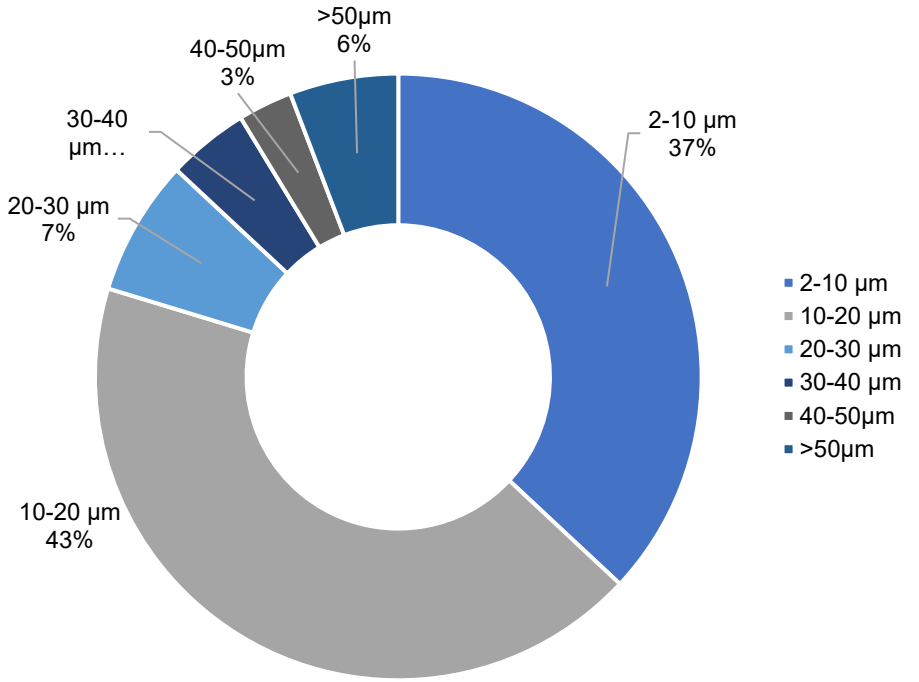
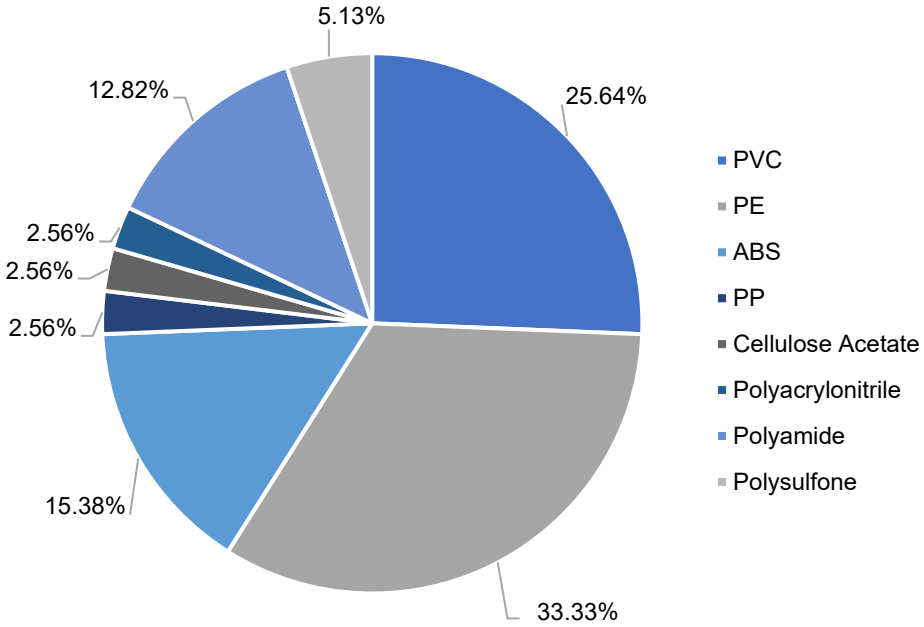


Figure B9. Relative particle size distribution within each group, a) mussels and b) oysters.

a) Mussels



b) Oysters

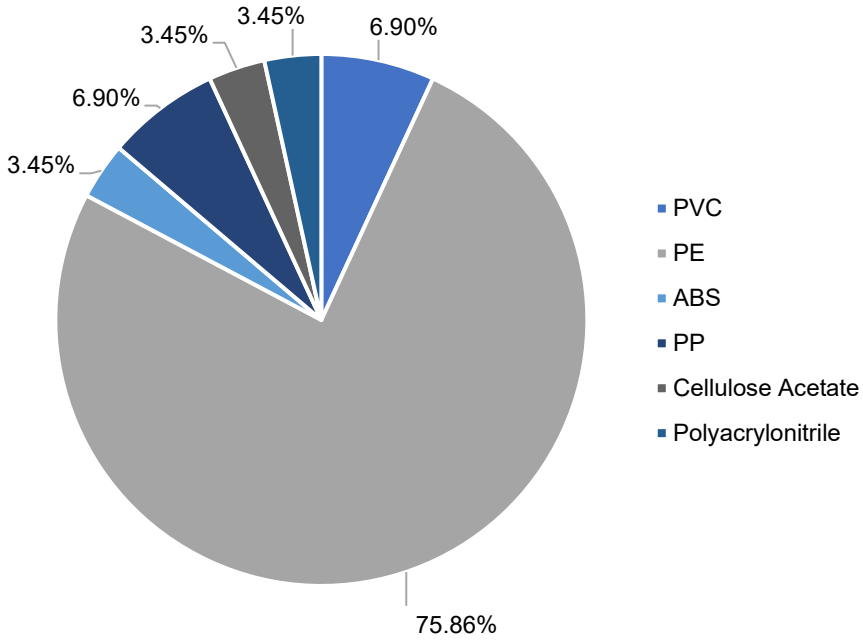


Figure B10. μ-Raman 60% hit rate results.

Table B6. Results of two-way ANOVA significant indicating differences in microplastic concentration in Blue mussels from various sampling and species from Nova Scotia. Bolded values indicated significant differences in microplastic concentration among factors.

Factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Location	9	84.81	9.42	9.99	**1.39e-08**
Residuals	50	47.13	0.94		

Table B7. Results of pairwise comparison analysis using a post-hoc Tukey HSD test indicating significant differences in SMP concentration in Blue mussels from various sampling locations across Nova Scotia. Bolded values indicated significant differences in microplastic concentration among pairs. Letters represent approximate zones (EC=Eastern-Cape Breton, G=Gulf, SW=South-Southwestern).

Comparison by site	Comparison by sampling location	Species	Interaction	*p-value*
2-1	Halifax - Undisclosed site (EC)	*M.edulis*	Sampling location	0.99
3-1	Martinique Beach - Undisclosed site (EC)	*M.edulis*	Sampling location	0.31
4-1	Taylor Head - Undisclosed site (EC)	*M.edulis*	Sampling location	**0.001**
5-1	Undisclosed site (G) - Undisclosed site (EC)	*M.edulis*	Sampling location	0.99
6-1	Melmerby Beach - Undisclosed site (EC)	*M.edulis*	Sampling location	0.44
7-1	Tatamagouche - Undisclosed site (EC)	*M.edulis*	Sampling location	0.12
8-1	Undisclosed site (SW) - Undisclosed site (EC)	*M.edulis*	Sampling location	0.91
9-1	West Pennant - Undisclosed site (EC)	*M.edulis*	Sampling location	**0.000002**
10-1	Risser's Beach - Undisclosed site (EC)	*M.edulis*	Sampling location	**0.000001**
3-2	Martinique Beach - Halifax	*M.edulis*	Sampling location	0.57
4-2	Taylor Head - Halifax	*M.edulis*	Sampling location	**0.005**
5-2	Undisclosed site (G) - Halifax	*M.edulis*	Sampling location	0.99
6-2	Melmerby Beach - Halifax	*M.edulis*	Sampling location	0.71
7-2	Tatamagouche - Halifax	*M.edulis*	Sampling location	0.28
8-2	Undisclosed site (SW) - Halifax	*M.edulis*	Sampling location	0.99
9-2	West Pennant - Halifax	*M.edulis*	Sampling location	**0.000009**
10-2	Risser's Beach - Halifax	*M.edulis*	Sampling location	**0.00004**
4-3	Taylor Head - Martinique Beach	*M.edulis*	Sampling location	0.58
5-3	Undisclosed site (G) - Martinique Beach	*M.edulis*	Sampling location	0.91
6-3	Melmerby Beach - Martinique Beach	*M.edulis*	Sampling location	0.99
7-3	Tatamagouche - Martinique Beach	*M.edulis*	Sampling location	0.99
8-3	Undisclosed site (SW) - Martinique Beach	*M.edulis*	Sampling location	0.99
9-3	West Pennant - Martinique Beach	*M.edulis*	Sampling location	**0.008**
10-3	Risser's Beach - Martinique Beach	*M.edulis*	Sampling location	**0.02**
5-4	Undisclosed site (G) - Taylor Head	*M.edulis*	Sampling location	**0.03**
6-4	Melmerby Beach - Taylor Head	*M.edulis*	Sampling location	0.44
7-4	Tatamagouche - Taylor Head	*M.edulis*	Sampling location	0.86
8-4	Undisclosed site (SW) - Taylor Head	*M.edulis*	Sampling location	0.09
9-4	West Pennant - Taylor Head	*M.edulis*	Sampling location	0.65
10-4	Risser's Beach - Taylor Head	*M.edulis*	Sampling location	0.89

6-5	Melmerby Beach - Undisclosed site (G)	*M.edulis*	Sampling location	0.96
7-5	Tatamagouche - Undisclosed site (G)	*M.edulis*	Sampling location	0.65
8-5	Undisclosed site (SW) - Undisclosed site (G)	*M.edulis*	Sampling location	0.99
9-5	West Pennant - Undisclosed site (G)	*M.edulis*	Sampling location	**0.00007**
10-5	Risser's Beach - Undisclosed site (G)	*M.edulis*	Sampling location	**0.0003**
7-6	Tatamagouche - Melmerby Beach	*M.edulis*	Sampling location	0.99
8-6	Undisclosed site (SW) - Melmerby Beach	*M.edulis*	Sampling location	0.99
9-6	West Pennant - Melmerby Beach	*M.edulis*	Sampling location	**0.004**
10-6	Risser's Beach - Melmerby Beach	*M.edulis*	Sampling location	**0.01**
8-7	Undisclosed site (SW) - Tatamagouche	*M.edulis*	Sampling location	**0.89**
9-7	West Pennant - Tatamagouche	*M.edulis*	Sampling location	**0.03**
10-7	Risser's Beach - Tatamagouche	*M.edulis*	Sampling location	0.09
9-8	West Pennant - Undisclosed site (SW)	*M.edulis*	Sampling location	**0.003**
10-8	Risser's Beach - Undisclosed site (SW)	*M.edulis*	Sampling location	**0.001**
10-9	Risser's Beach - West Pennant	*M.edulis*	Sampling location	0.99

Table B8. Results of one-way ANOVA significant indicating differences in microplastic concentration in Eastern oysters from various sampling and species from Nova Scotia. Bolded values indicated significant differences in microplastic concentration among factors.

Factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Location	4	39.34	9.84	28.35	**5.77e-09**
Residuals	25	8.67	0.35		

Table B9. Results of pairwise comparison analysis using a post-hoc Tukey HSD test indicating significant differences in SMP concentration in Eastern oysters from various sampling sites across Nova Scotia. Bolded values indicated significant differences in microplastic concentration among pairs. Letters represent approximate zones (EC=Eastern-Cape Breton, G=Gulf, SW=South-Southwestern).

Comparison by site	Comparison by sampling location	Species	Interaction	*p-value*
7-6	Tatamagouche – Melmerby Beach	*C.virginica*	Sampling location	**0.02**
11-6	Undisclosed site (EC) – Melmerby Beach	*C.virginica*	Sampling location	**0.00005**
12-6	Undisclosed site (G) – Melmerby Beach	*C.virginica*	Sampling location	**0.0001**
13-6	Undisclosed site (SW) – Melmerby Beach	*C.virginica*	Sampling location	0.24
11-7	Undisclosed site (EC) – Tatamagouche	*C.virginica*	Sampling location	**0.000001**
12-7	Undisclosed site (G) – Tatamagouche	*C.virginica*	Sampling location	**0.000001**
13-7	Undisclosed site (SW) – Tatamagouche	*C.virginica*	Sampling location	**0.0001**
12-11	Undisclosed site (G) – Undisclosed site (EC)	*C.virginica*	Sampling location	0.99
13-11	Undisclosed site (G) – Undisclosed site (EC)	*C.virginica*	Sampling location	**0.01**
13-12	Undisclosed site (SW) – Undisclosed site (G)	*C.virginica*	Sampling location	**0.02**

Table B10. Results of two-way ANOVA comparing SMP concentrations in Blue mussels and Eastern oysters found co-located from Melmerby Beach and Tatamagouche in the Gulf zone of Nova Scotia. Bolded values indicated significant differences in microplastic concentration among factors.

Factor/Interaction	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Organism	1	3.43	3.44	8.19	**0.009**
Location	1	18.67	18.87	44.96	**1.59e-06**
Organism: Location	1	0.83	0.83	1.98	0.17
Residuals	20	8.393	0.420		

Table B11. Results of pairwise comparison analysis using a post-hoc Tukey HSD test indicating significant differences in microplastic concentration in Blue mussels and Eastern oysters from the Gulf zone. Bolded values indicated significant differences in microplastic concentration among pairs.

Comparison by sample code	Comparison by location	Species	Interaction	*p-value*
M7-M6	Tatamagouche – Melmerby Beach	*M.edulis*	Sampling location	0.73
O4-M6	Melmerby Beach	*M.edulis, C.virginica*	Sampling location, Organism	**0.006**
O5-M6	Tatamagouche – Melmerby Beach	*M.edulis, C.virginica*	Sampling location, Organism	**0.000007**
O4-M7	Melmerby Beach – Tatamagouche	*M.edulis, C.virginica*	Sampling location, Organism	0.058
O5-M7	Tatamagouche	*M.edulis, C.virginica*	Sampling location, Organism	**0.00007**
O5-O4	Tatamagouche – Melmerby Beach	*C.virginica*	Sampling location	**0.032**

Table B12. Results of two-way chi squared test of independence to test differences in the proportions of polymer classes found among Blue mussels and Eastern oysters from Nova Scotia.

	X-squared	Df	*p-value*
Proportions of polymers among species of bivalve	0.55	7	0.99

Appendix C: Chapter 4 Literature Review – Included Studies

Table C1. Chronological list of studies included in Chapter 4: "Common and emerging methods for the analysis of microplastics in various marine mussel and oyster species"

Author(s)	Year	Bivalves(s) studied	Title
Van Cauwenberghe & Janssen	2014	Both	Microplastics in bivalves cultured for human consumption
Van Cauwenberghe et al.	2015	Mussel	Microplastics are taken up by mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) living in natural habitats
Li et al.	2016	Mussel	Microplastics in mussels along the coastal waters of China
Catarino et al.	2017	Mussel	Development and optimization of a standard method for extraction of microplastics in mussels by enzyme digestion of soft tissues
Thushari et al.	2017	Oyster	Effects of microplastics on sessile invertebrates in the eastern coast of Thailand: An approach to coastal zone conservation
Leslie et al.	2017	Both	Microplastics en route: Field measurements in the Dutch river delta and Amsterdam canals, wastewater treatment plants, North Sea sediments and biota
Railo et al.	2018	Mussel	Application of an enzyme digestion method reveals microlitter in *Mytilus trossulus* at a wastewater discharge area
Qu et al.	2018	Mussel	Assessing the relationship between the abundance and properties of microplastics in water and in mussels
Phuong et al.	2018	Both	Factors influencing the microplastic contamination of bivalves from the French Atlantic coast: Location, season and/or mode of life?
Catarino et al.	2018	Mussel	Low levels of microplastics (MP) in wild mussels indicate that MP ingestion by humans is minimal compared to exposure via household fibres fallout during a meal
Digka et al.	2018	Mussel	Microplastic Abundance and Polymer Types in a Mediterranean Environment
Naji et al.	2018	Oyster	Microplastics contamination in molluscs from the northern part of the Persian Gulf
Digka et al.	2018	Mussel	Microplastics in mussels and fish from the Northern Ionian Sea
Li et al.	2018	Mussel	Microplastics in mussels sampled from coastal waters and supermarkets in the United Kingdom
Li et al.	2018	Oyster	Microplastics in oysters *Saccostrea cucullata* along the Pearl River Estuary, China
Bråte et al.	2018	Mussel	Mytilus spp. as sentinels for monitoring microplastic pollution in Norwegian coastal waters: A qualitative and quantitative study
Phuong et al.	2018	Mussel	Quantification and characterization of microplastics in blue mussels (*Mytilus edulis*): protocol setup and preliminary data on the contamination of the French Atlantic coast
Cho et al.	2019	Both	Abundance and characteristics of microplastics in market bivalves from South Korea

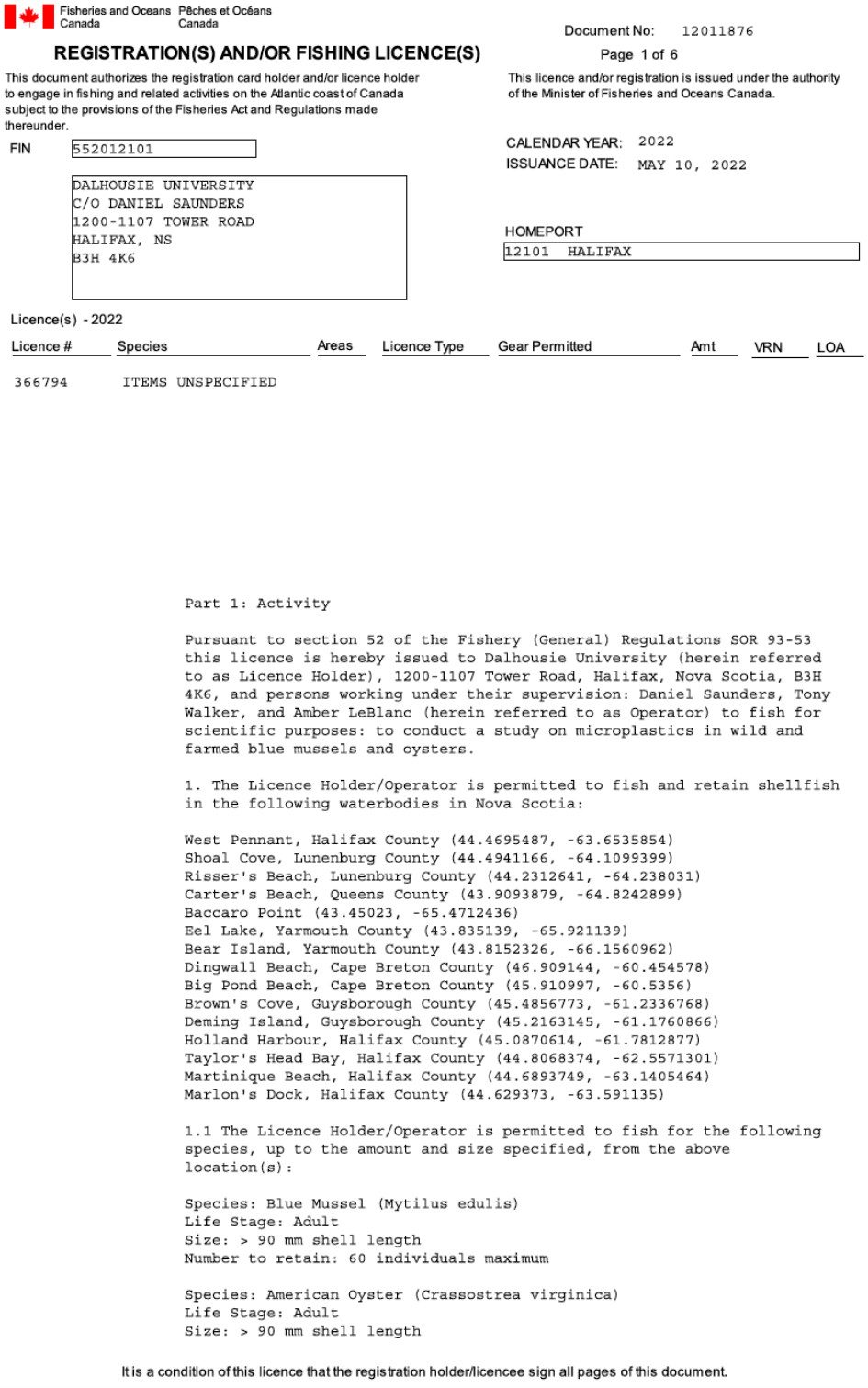
Birnstiel et al.	2019	Mussel	Depuration reduces microplastic content in wild and farmed mussels
Gomiero et al.	2019	Mussel	First occurrence and composition assessment of microplastics in native mussels collected from coastal and offshore areas of the northern and central Adriatic Sea
Jahan et al.	2019	Oyster	Interrelationship of microplastic pollution in sediments and oysters in a seaport environment of the eastern coast of Australia
Hermabessiere et al.	2019	Mussel	Microplastic contamination and pollutant levels in mussels and cockles collected along the channel coasts
Teng et al.	2019	Oyster	Microplastic in cultured oysters from different coastal areas of China
Zhu et al.	2019	Oyster	Microplastic pollution in the Maowei Sea, a typical mariculture bay of China
Abidli et al.	2019	Both	Microplastics in commercial molluscs from the lagoon of Bizerte (Northern Tunisia)
Webb et al.	2019	Mussel	Microplastics in the New Zealand green lipped mussel *Perna canaliculus*
Kazour et al.	2019	Oyster	Microplastics pollution along the Lebanese coast (Eastern Mediterranean Basin): Occurrence in surface water, sediments and biota samples
Scott et al.	2019	Mussel	Particle characteristics of microplastics contaminating the mussel Mytilus edulis and their surrounding environments
Naidu et al.	2019	Mussel	Preliminary study and first evidence of presence of microplastics and colorants in green mussel, *Perna viridis* (Linnaeus, 1758), from southeast coast of India
Patterson et al.	2019	Oyster	Profiling microplastics in the Indian edible oyster, *Magallana bilineata* collected from the Tuticorin coast, Gulf of Mannar, Southeastern India
Li et al.	2019	Mussel	Fusion of microplastics into the mussel byssus
Wang et al.	2019	Oyster	Typhoons increase the abundance of microplastics in the marine environment and cultured organisms: A case study in Sanggou Bay, China
Wu et al.	2020	Oyster	Accumulation of microplastics in typical commercial aquatic species: A case study at a productive aquaculture site in China
Rios et al.	2020	Mussel	Assessing urban microplastic pollution in a benthic habitat of Patagonia Argentina
Perez et al.	2020	Mussel	First report of microplastics presence in the mussel *Mytilus chilensis* from Ushuaia Bay (Beagle Channel, Tierra del Fuego, Argentina)
Martinelli et al.	2020	Oyster	Low incidence of microplastic contaminants in Pacific oysters (*Crassostrea gigas* Thunberg) from the Salish Sea, USA
Chen et al.	2020	Oyster	Microplastic contamination of three commonly consumed seafood species from Taiwan: A pilot study
Gedik and Eryasar	2020	Mussel	Microplastic pollution profile of Mediterranean mussels (*Mytilus galloprovincialis*) collected along the Turkish coasts

Wakkaf et al.	2020	Mussel	Microplastics in edible mussels from a southern Mediterranean lagoon: Preliminary results on seawater-mussel transfer and implications for environmental protection and seafood safety
Dowarah et al.	2020	Mussel	Quantification of microplastics using Nile Red in two bivalve species *Perna viridis* and *Meretrix meretrix* from three estuaries in Pondicherry, India and microplastic uptake by local communities through bivalve diet
Gündoğdu et al.	2020	Mussel	Stuffed with microplastics: Microplastic occurrence in traditional stuffed mussels sold in the Turkish market
Kazour and Amara	2020	Mussel	Is blue mussel caging an efficient method for monitoring environmental microplastics pollution?
Ribeiro et al.	2020	Oyster	Quantitative Analysis of Selected Plastics in High-Commercial-Value Australian Seafood by Pyrolysis Gas Chromatography Mass Spectrometry
Corami et al.	2020	Oyster	Evidence of small microplastics (<100 μm) ingestion by Pacific oysters (Crassostrea gigas): A novel method of extraction, purification, and analysis using Micro-FTIR
De-la-Torre et al.	2021	Mussel	ABUNDANCE AND CHARACTERISTICS OF MICROPLASTICS IN MARKET BIVALVE *Aulacomya Atra* (MYTILIDAE: BIVALVIA)
Sparks et al.	2021	Mussel	Abundance and characteristics of microplastics in retail mussels from Cape Town, South Africa
Vinay Kumar et al.	2021	Mussel	Analysis of microplastics of a broad size range in commercially important mussels by combining FTIR and Raman spectroscopy approaches
Paradinas et al.	2021	Mussel	A New Collection Tool-Kit to Sample Microplastics From the Marine Environment (Sediment, Seawater, and Biota) Using Citizen Science
Liao et al.	2021	Oyster	Assessment of microplastics in oysters in coastal areas of Taiwan
Leung et al.	2021	Mussel	Determination of microplastics in the edible green-lipped mussel *Perna viridis* using an automated mapping technique of Raman microspectroscopy
Vital et al.	2021	Mussel	Do microplastic contaminated seafood consumption pose a potential risk to human health?
Zhu et al.	2021	Oyster	Long-term trends of microplastics in seawater and farmed oysters in the Maowei Sea, China
Marques et al.	2021	Mussel	Major characteristics of microplastics in mussels from the Portuguese coast
Patterson et al.	2021	Mussel	Microplastic contamination in Indian edible mussels (*Perna perna* and *Perna viridis*) and their environs
Gardon et al.	2021	Oyster	Microplastics contamination in pearl-farming lagoons of French Polynesia
Truchet et al.	2021	Mussel	Microplastics in bivalves, water and sediments from a touristic sandy beach of Argentina
Ding et al.	2021	Both	Microplastics in four bivalve species and basis for using bivalves as bioindicators of microplastic pollution
Nalbone et al.	2021	Mussel	Microplastics in fresh and processed mussels sampled from fish shops and large retail chains in Italy

Pequeno et al.	2021	Mussel	Microplastics in Marine and Estuarine Species From the Coast of Portugal
Saha et al.	2021	Both	Microplastics in seafood as an emerging threat to marine environment: A case study in Goa, west coast of India
Cho et al.	2021	Both	Nationwide monitoring of microplastics in bivalves from the coastal environment of Korea
Vieria et al.	2021	Oyster	Occurrence of microplastics and heavy metals accumulation in native oysters *Crassostrea Gasar* in the Paranaguá estuarine system, Brazil
Liu et al.	2021	Mussel	Pollution Characteristics of Microplastics in Mollusks from the Coastal Area of Yantai, China
Wang et al.	2021	Oyster	Quantitative and qualitative determination of microplastics in oyster, seawater and sediment from the coastal areas in Zhuhai, China
Lozano-Hernandez et al.	2021	Mussel	Microplastic concentrations in cultured oysters in two seasons from two bays of Baja California, Mexico
Liu et al.	2021	Mussel	Separation and identification of microplastics in marine organisms by TGA-FTIR-GC/MS: A case study of mussels from coastal China
Zhang et al.	2022	Oyster	Abundance and characteristics of microplastics in shellfish from Jiaozhou Bay, China
Do et al.	2022	Oyster	Abundance of microplastics in cultured oysters (*Crassostrea gigas*) from Danang Bay of Vietnam
Volgare et al.	2022	Mussel	A versatile approach to evaluate the occurrence of microfibers in mussels *Mytilus galloprovincialis*
Zhang et al.	2022	Both	Distribution and Characteristics of Microplastics in Barnacles and Wild Bivalves on the Coast of the Yellow Sea, China
Ta et al.	2022	Mussel	Investigation of microplastic contamination in blood cockles and green mussels from selected aquaculture farms and markets in Thailand
Exposito et al.	2022	Both	Levels of microplastics and their characteristics in molluscs from North-West Mediterranean Sea: Human intake
Lee et al.	2022	Oyster	Microplastic accumulation in oysters along a Bornean coastline (Brunei, South China Sea): Insights into local sources and sinks
Joshy et al.	2022	Mussel	Microplastic contamination in commercially important bivalves from the southwest coast of India
Pan et al.	2022	Both	Microplastic contamination in seafood from Dongshan Bay in southeastern China and its health risk implication for human consumption
Wootton et al.	2022	Oyster	Microplastic in oysters: A review of global trends and comparison to southern Australia
Li et al.	2022	Both	Microplastics contamination in bivalves from the Daya Bay: Species variability and spatio-temporal distribution and human health risks
Noël et al.	2022	Mussel	Microplastics distribution in sediment and mussels along the British Columbia Coast, Canada
Lin et al.	2022	Mussel	Microplastics in biota and surface seawater from tropical aquaculture area in Hainan, China

Klein et al.	2022	Mussel	Microplastics in intertidal water of South Australia and the mussel *Mytilus spp.;* the contrasting effect of population on concentration
Masiá et al.	2022	Mussel	Microplastics in seafood: Relative input of Mytilus galloprovincialis and table salt in mussel dishes
Mazlan et al.	2022	Mussel	Microplastics in the New Zealand Environment
Cherdsukjai et al.	2022	Mussel	Preliminary Study and First Evidence of Presence of Microplastics in Green Mussel, *Perna viridis* from Phuket
Tielman et al.	2022	Oyster	Presence of Microplastics in Windowpane Oyster *Placuna placenta* and the waters from the Tambak Lorok Coastal Area in Central Java, Indonesia
Aung et al.	2022	Oyster	Prevalence of Microplastics in the Eastern Oyster Crassostrea virginica in the Chesapeake Bay: The Impact of Different Digestion Methods on Microplastic Properties
Walters et al.	2022	Oyster	Quantifying Spatial and Temporal Trends of Microplastic Pollution in Surface Water and in the Eastern Oyster Crassostrea virginica for a Dynamic Florida Estuary
Du et al.	2022	Oyster	Seasonal change of microplastics uptake in the Pacific oysters *Crassostrea gigas* cultured in the Yellow Sea and Bohai Sea, China
Gedik et al.	2022	Mussel	The microplastic pattern of wild-caught Mediterranean mussels from the Marmara Sea
Lerebours et al.	2022	Both	Spatio-temporal contamination of microplastics in shellfish farming regions: A case study
Hammadi et al.	2022	Oyster	Microplastic pollution in oyster bed ecosystems: An assessment of the northern shores of the United Arab Emirates
Rojas-Jimenez et al.	2022	Mussel	Presence of microplastics in six bivalve species (Mollusca, Bivalvia) commercially exploited at the Pacific coast of Costa Rica, Central America
Phaksopa et al.	2023	Mussel	Assessment of Microplastics in Green Mussel (*Perna viridis)* and Surrounding Environments around Sri Racha Bay, Thailand
Abelouah et al.	2023	mussel	Binational survey using Mytilus galloprovincialis as a bioindicator of microplastic pollution: Insights into chemical analysis and potential risk on humans
Kor et al.	2023	Oyster	Microplastic occurrence in finfish and shellfish from the mangroves of the northern Gulf of Oman
Zhang et al.	2023	Mussel	Microplastic sink that cannot be ignored in chemosynthetic organisms
Bošković et al.	2023	Mussel	Microplastics in mussels from the Boka Kotorska Bay (Adriatic Sea) and impact on human health
Trani et al.	2023	Mussel	Microplastics in water surface and in the gastrointestinal tract of target marine organisms in Salento coastal seas (Italy, Southern Puglia)

Appendix D: Additional Supplementary Material



Fisheries and Oceans Canada / Pêches et Océans Canada

Document No: 12011876

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REGISTRATION(S) AND/OR FISHING LICENCE(S)

This document authorizes the registration card holder and/or licence holder to engage in fishing and related activities on the Atlantic coast of Canada subject to the provisions of the Fisheries Act and Regulations made thereunder.

This licence and/or registration is issued under the authority of the Minister of Fisheries and Oceans Canada.

FIN 552012101

DALHOUSIE UNIVERSITY
C/O DANIEL SAUNDERS
1200-1107 TOWER ROAD
HALIFAX, NS
B3H 4K6

CALENDAR YEAR: 2022
ISSUANCE DATE: MAY 10, 2022

HOMEPORT
12101 HALIFAX

Licence(s) - 2022

Licence #	Species	Areas	Licence Type	Gear Permitted	Amt	VRN	LOA
366794	ITEMS UNSPECIFIED						

Part 1: Activity

Pursuant to section 52 of the Fishery (General) Regulations SOR 93-53 this licence is hereby issued to Dalhousie University (herein referred to as Licence Holder), 1200-1107 Tower Road, Halifax, Nova Scotia, B3H 4K6, and persons working under their supervision: Daniel Saunders, Tony Walker, and Amber LeBlanc (herein referred to as Operator) to fish for scientific purposes: to conduct a study on microplastics in wild and farmed blue mussels and oysters.

1. The Licence Holder/Operator is permitted to fish and retain shellfish in the following waterbodies in Nova Scotia:

West Pennant, Halifax County (44.4695487, -63.6535854)
Shoal Cove, Lunenburg County (44.4941166, -64.1099399)
Risser's Beach, Lunenburg County (44.2312641, -64.238031)
Carter's Beach, Queens County (43.9093879, -64.8242899)
Baccaro Point (43.45023, -65.4712436)
Eel Lake, Yarmouth County (43.835139, -65.921139)
Bear Island, Yarmouth County (43.8152326, -66.1560962)
Dingwall Beach, Cape Breton County (46.909144, -60.454578)
Big Pond Beach, Cape Breton County (45.910997, -60.5356)
Brown's Cove, Guysborough County (45.4856773, -61.2336768)
Deming Island, Guysborough County (45.2163145, -61.1760866)
Holland Harbour, Halifax County (45.0870614, -61.7812877)
Taylor's Head Bay, Halifax County (44.8068374, -62.5571301)
Martinique Beach, Halifax County (44.6893749, -63.1405464)
Marlon's Dock, Halifax County (44.629373, -63.591135)

1.1 The Licence Holder/Operator is permitted to fish for the following species, up to the amount and size specified, from the above location(s):

Species: Blue Mussel (Mytilus edulis)
Life Stage: Adult
Size: > 90 mm shell length
Number to retain: 60 individuals maximum

Species: American Oyster (Crassostrea virginica)
Life Stage: Adult
Size: > 90 mm shell length

It is a condition of this licence that the registration holder/licencee sign all pages of this document.

Figure D1. Fisheries and Oceans Canada (DFO) scientific license for the Maritimes region.

Pêches et Océans Canada | Fisheries and Oceans Canada

DEPARTMENT OF FISHERIES AND OCEANS
GULF REGION

LICENCE TO FISH FOR

Scientific Purposes

LICENCE No.: SG-RHQ-22-039

Pursuant to Part VII, Section 52, of the *Fishery (General) Regulations*, this licence is issued to **Daniel Saunders, Masters student** at **Dalhousie University**, 1200- 1107 Tower Rd, Halifax NS, B3H 4K6, (902) 292-9218. daniel.saunders@dal.ca

This licence is issued for the purpose of:

To conduct a study on microplastics in both wild and farmed blue mussels (Mytilus edulis) and American oysters (Crassostrea virginica) from Nova Scotia. This will be a spatial assessment from three regions (Gulf Region, Eastern Shore/Cape Breton, and the Southwestern Shore). This study will also look at potential sources of microplastic pollution. This will be conducted by collecting mussels and oysters at various points in Nova Scotia . The analysis will focus on the types of microplastics found within the samples and their potential sources. The bulk of the fieldwork/ sample collection will take place from May to August 2022.

THE FOLLOWING CONDITIONS APPLY TO THIS LICENCE:

The following persons are authorized to carry out activities under the authority of this licence:

Name	Organization	Cell / Phone
Daniel Saunders	Dalhousie University	902-292-9218
Amber LeBlanc	Dalhousie University	902-305-4275
Tony Walker	Dalhousie University	902-494-4478

AUTHORITY TO FISH

Fishing activities carried out under the authority of this licence must only be conducted under the direct supervision of the licence holder or authorized individuals as listed above. The licence holder is responsible to ensure that an authorized individual is present during any fishing activity authorized under this licence. Persons working under the authority of this licence must carry a copy of the licence while conducting fishing activities and while in possession of fish caught or fishing gear used for fishing under the authority of this licence. The operator of a vessel or persons authorized to carry out fishing activities must produce this licence upon request by a fishery officer or fishery guardian for inspection.

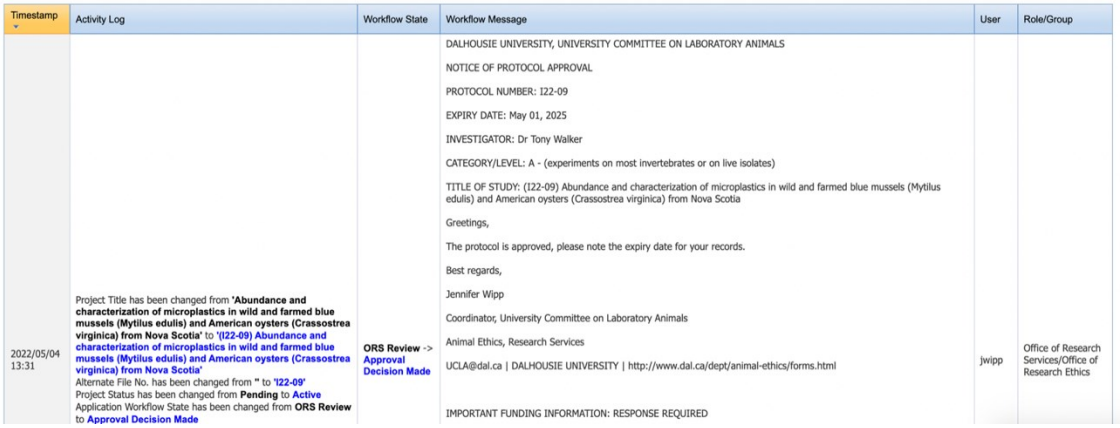
Prior to the commencement of a fishing trip, the licence holder/operator must check the Fisheries and Oceans Canada Orders Registry Website at http://www.inter.dfo-mpo.gc.ca/Gulf/Orders-Registry for any fishing prohibition notices applicable to this licence.

AREA OF ACTIVITIES

Fishing activities carried out under the authority of this licence must only be conducted within the following area:

Site area	North Latitude	West Longitude
Pugwash	45.845167 °	-63.664028
Horton Point	45.810317°	-63.431175 °
Tatamagouche	45.741383°	-63.266758 °
Powell's Point Provincial Park	45.6538°	-62.555503°
Quarry Island Rd	45.627308°	-62.492403°
Melmerby Beach	45.656361°	- 62.507956°

Figure D2. Fisheries and Oceans Canada (DFO) scientific license for the gulf region.



Timestamp	Activity Log	Workflow State	Workflow Message	User	Role/Group
2022/05/04 13:31	Project Title has been changed from **'Abundance and characterization of microplastics in wild and farmed blue mussels (Mytilus edulis) and American oysters (Crassostrea virginica) from Nova Scotia'** to **'(I22-09) Abundance and characterization of microplastics in wild and farmed blue mussels (Mytilus edulis) and American oysters (Crassostrea virginica) from Nova Scotia'** Alternate File No. has been changed from **''** to **'I22-09'** Project Status has been changed from **Pending** to **Active** Application Workflow State has been changed from **ORS Review** to **Approval Decision Made**	**ORS Review** -> **Approval Decision Made**	DALHOUSIE UNIVERSITY, UNIVERSITY COMMITTEE ON LABORATORY ANIMALS NOTICE OF PROTOCOL APPROVAL PROTOCOL NUMBER: I22-09 EXPIRY DATE: May 01, 2025 INVESTIGATOR: Dr Tony Walker CATEGORY/LEVEL: A - (experiments on most invertebrates or on live isolates) TITLE OF STUDY: (I22-09) Abundance and characterization of microplastics in wild and farmed blue mussels (Mytilus edulis) and American oysters (Crassostrea virginica) from Nova Scotia Greetings, The protocol is approved, please note the expiry date for your records. Best regards, Jennifer Wipp Coordinator, University Committee on Laboratory Animals Animal Ethics, Research Services UCLA@dal.ca | DALHOUSIE UNIVERSITY | http://www.dal.ca/dept/animal-ethics/forms.html IMPORTANT FUNDING INFORMATION: RESPONSE REQUIRED	jwipp	Office of Research Services/Office of Research Ethics

Figure D3. Dalhousie University Committee on Laboratory (UCLA) ethics approval.