Anthropogenic modifications and their impacts on shellfish physiology

by

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B.Sc., University of Victoria, 1994

A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

Supervisory Committee

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Abstract

Humans have been modifying marine habitats for centuries to enhance productivity and facilitate the collection of natural food sources such as fish and shellfish. Anthropogenic alterations and impacts on marine habitats include coastal development, aquaculture, fishing, agriculture, transportation and waste disposal, which have led to a decrease in habitat complexity and a loss of biological diversity. The maintenance, regulation and protection of healthy aquatic habitats and the ecosystem services they provide is a global concern. In this study transcriptional analysis was utilized to investigate physiological responses of shellfish to two different types of anthropogenic marine impacts; clam garden habitat modifications and microplastic pollution.

Clam gardens are examples of ancient anthropogenic modifications built by the Northwest Indigenous Coastal peoples of America to enhance clam habitat productivity, providing secure and reliable food sources. Physiological differences of *Leukoma staminea* (Littleneck clams) transplanted in unmaintained clam garden beaches for 16 weeks compared to clams in unmodified reference beaches were investigated using metrics of gene expression, growth and survival. This study found no statistically significant differences in growth and survival but did find statistical differences in expressed biological pathways in clams between clam gardens and reference beaches. Most biological pathways in both groups were associated with environmental stress, suggesting both habitats contained their own unique multiple stressors. There were also no statistically significant differences in sediment carbonate, organic content, or grain size distributions between the sediment from clam garden beaches compared to reference beaches. An interesting finding in this study was a significant negative correlation between sediment carbonate content and survival. The presence of several highly upregulated

viral transcripts from the Dicistroviridae family had significant correlations with geographical proximity and survival, further confirming that other factors (such as geographical location and sediment characteristics) had a greater influence on Littleneck clam survival and immune status if a beach had been modified or not

Microplastics are emerging anthropogenic pollutants found in marine habitats worldwide, including key aquaculture and fisheries species such as bivalves. To examine the impacts of environmentally relevant concentrations of microplastics on the highly commercial Pacific oyster (*Crassostrea gigas*), 102 adult oysters were exposed to microplastics (5 microplastic fibers per litre) in microalgal diets for 30 days and impacts were assessed using gene expression, condition index, microplastic load and lysosomal membrane stability. Results were compared to control (n= 102) oysters receiving microalgal feed and held in the same experimental conditions but with no microplastic exposure, and background counts of microplastic load in seawater and microalgal production were also assessed. There were no statistically significant differences observed in survival, condition index or lysosomal membrane stability between control and exposed oysters. However, there were statistically significant differences in microplastic load and gene expression between the exposed and control oysters. There was an upregulation in biological pathways associated with immunity and stress and a downregulation in pathways associated with reproduction in the exposed oysters, highlighting the potential long-term negative consequences of environmental microplastics on long-term population stability, especially if microplastic concentrations continue to increase.

This study found that previous beach modifications (clam gardens) did not positively affect clam growth, survival or physiology, and that regional environmental stressors played a greater role in survival. Environmentally relevant microplastic exposures over the 30-day study

period was found to elicit an immune response and have negative implications for reproductive success.

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Acknowledgements

I would like to extend my gratitude for the opportunity to live and learn in the unceded traditional territories of the sḵwx̱wú7mesh (Squamish), seĺiĺwitulh (Tsleil Waututh), Stó:lō (Sto:lo), and xʷməθikʷəy̓əm (Musqueam) Nations during my graduate studies. My clam garden research was conducted in Kanish Bay on Quadra Island, British Columbia (B.C.), which lies in the unceded territories of the Northern Coast Salish and the Southern Kwakwaka'wakw First Nations.

I would also like to express my deepest gratitude to my supervisors, and committee members; Dr. Helen Gurney-Smith for her unwavering commitment, guidance, and intellectual, professional, and personal support, and Dr. Ben Koop, Dr. Sarah Dudas, and Dr. Chris Pearce for their insight and continuous support, and encouragement.

Thank you to Kayla Balmer, Morgan Black, Brenna Collicutt, Garth Covernton, Kieran Cox, Maggie Dietterle, Colleen Haddad, Karia Kaukinen, Laurie Keddy, Jong Leong, Kayla Long, Matt Miller, Tobi Ming, Dr. Eric Rondeau, Caitlin Smith, and Dr. Ben Sutherland for all your assistance along the way, I couldn't have done this without your help and support.

Thank you to Dr. Stewart Johnson, Dr. Kristi Miller, and Kathryn Temple at the Pacific Biological Station, DFO, Nanaimo, B.C., for allowing me to use their lab facilities and equipment.

Thank you to the Tula Foundation for use of their facilities on Quadra Island, B.C., for supporting the clam garden research financially and for the knowledge and assistance provided by Clam Garden Network colleagues.

Thank you to the Department of Fisheries of Oceans for supporting the microplastics project.

Finally, thank you to my friends, family, and my children for their continued love and support.

Dedication

I would like to dedicate this to Wilhelmina and Rintje Raap; my mom and dad, for always encouraging me to study and learn, for their love and support, and always believing in my abilities.

Chapter 1: Introduction

1.1. Anthropogenic modifications

It is increasingly difficult, if not impossible, to find an ecosystem in the world that has not been somehow altered or influenced by human activity. Intertidal and marine habitats and ecosystems have been modified and utilized extensively for human use, for example fishing, aquaculture, coastal development, land reclamation and accessibility, industry practices, recreation, transportation and waste disposal (Airoldi 2007). For centuries coastal shorelines have been altered to increase productivity and facilitate the harvesting of natural resources. Ancient fishing weirs and clam gardens are examples of shoreline constructions built to trap fish and increase clam habitat respectively, providing productive and predictable food sources for coastal peoples (Caldwell et al. 2012, Deur et al. 2015). Fish traps are found worldwide and consist of stone and wood structures placed in semi-circular and linear arrangements, creating obstructions along estuaries, stream mouths, or tidal shorelines which trapped fish close to shore at low tides and facilitated easy capture (Moss 2013). Clam gardens of the Pacific Northwest Coast were constructed by removing rocks from clam bed areas and using them to build walls at the lowest exposed tide line; this created a flatter, larger clam bed area as the up shore beach side of the wall filled with silt and sediment (Groesbeck et al. 2014).

The increases in coastal human populations during the last century has increased pressure on coastal ecosystems through habitat manipulations, demand for natural resources, and pollution. These activities have often resulted in a decrease in structural habitat complexity, which has a detrimental impact on species diversity and composition (Smokorowski and Pratt 2007). Coastal wetlands comprise some of the most valuable ecosystems and are threatened by

coastal developments and land reclamation, which result in losses of important nursery grounds and refugia for aquatic species (Sheaves et al. 2014). Destructive fishing practices such as trawling, dredging, and overexploitation have caused significant loss to biogenic habitats created by plants and animals, such as coral and oyster reefs, and eelgrass meadows; this causes a decline in aquatic populations (such as oysters), water quality, and an increase in disease outbreaks (Rothschild et al. 1994).

The exponential growth in the human population has also resulted in an increase in anthropogenic pollution and debris in aquatic and terrestrial habitats worldwide from industrial, domestic and agricultural activities (Browne et al. 2015). The pollutants of major marine ecosystem concern include pesticides and fertilizers from agriculture practices, domestic and municipal wastes and sewage, oils, heavy metals, organic compounds (e.g. organochlorines, organophosphates, polycyclic aromatic hydrocarbons (PAHs) and organometals), sediments (erosion), eutrophication and algal blooms, biological pollution (pathogens and invasive species) and plastics (microplastics) (Shahidul Islam and Tanaka 2004). Marine pollutants cause a range of environmental and habitat alterations, including changes in water chemistry, nutrient ratios, dissolved oxygen, phytoplankton biomass and large scale changes in species diversity of benthic and fish communities (Shahidul Islam and Tanaka 2004). Many pollutants interact with physiological processes of marine organisms (e.g. growth and reproduction) potentially leading to serious declines in animal populations and reproductive function (Hamza-Chaffai 2014).

1.2. Shellfish aquaculture and habitat modifications

Global bivalve aquaculture production (predominantly oysters, mussels, clams, and scallops) has steadily increased since the 1990's, altering and impacting surrounding environments in both

positive and negative ways (Gallardi 2014). Both wild and cultured bivalves provide important ecosystem functions integral to healthy habitats, by improving water quality and mixing and flushing sediments (Norkko and Shumway 2011). As suspension feeders, bivalves filter and remove particles (phytoplankton, organic and inorganic matter) from the water column and discharge biodeposits in the sediment, helping buffer the shallow waters of estuaries and coastal waters against excessive phytoplankton, and harmful algae blooms (the latter a response to excessive nitrogen from anthropogenic loading) (NRCC 2010). Therefore shellfish can improve water quality by countering the negative effects of eutrophication, allowing deeper light penetration and thus growth of submerged aquatic vegetation important in nursery habitats (Norkko and Shumway 2011). Bioturbation, bioirrigation and the sediment modification by burrowing bivalves are integral processes for healthy soft-sediment ecosystems (Norkko and Shumway 2011).

Habitat alterations and disturbances resulting from aquaculture activities are dependent on culture factors (type, scale, intensity techniques used) as well as physical geographic and oceanographic conditions (Gallardi 2014). In general, aquaculture activities in high energy, well-flushed areas have environmental impacts on the surrounding benthic community than low energy levels, due to the dispersal of organic biodeposits (Gallardi 2014). Bivalve aquaculture can modify estuarian and coastal environments by altering planktonic and benthic food webs, ultimately altering food availability and resources to other species. The increased density of bivalves can lead to depletion of phytoplankton for other suspension feeders and when combined with alterations in physio-chemical characteristics of the sediment beneath oyster cultures, can cause an increase in smaller opportunistic deposit feeders such as scavengers, carnivores, and hydrogen sulphide-tolerant species (Gallardi 2014). The accumulation of biodeposits (feces and

pseudofeces) under culture operations can reduce sediment grain size, increase organic content and elevate nitrogen levels, thereby altering nitrogen cycling (Dumbauld et al. 2009).

Organic enrichment of marine sediments provides food for benthic organisms and increases benthic diversity, abundance and biomass (Norkko and Shumway 2011). An increase in sediment microbial activity also increases the sediment oxygen demand (Holmer et al. 2005). Prolonged periods of high oxygen demand can result in enhanced anaerobic activity and an increase in sulfate reducing bacteria and sulfides causing adverse effects on aerobic bacteria, plants, and fauna (Holmer et al. 2005). However, these effects are highly dependent on water flow and hydrodynamic setting (Norkko and Shumway 2011). Beaches with less tidal flushing are more sensitive to the effects of high organic sediment content than locations with greater tidal currents and wave action (Norkko and Shumway 2011). The oxidation of organic waste at the sediment surface is greater with higher temperatures in the summer months (Gosling 2008). Coastal sediments usually contain < 5% (of sediment dry weight) organics and > 10 % represents high organic content (Holmer et al. 2005).

Aquaculture activities can also physically disrupt coastal habitats through the use of husbandry equipment, which can alter water flow, sediment composition and sedimentation rate, encourage biofouling, and reduce eelgrass (Forrest et al. 2009). Worn and degrading aquatic plastic infrastructures (e.g. cages, floats, netting, and ropes) can also potentially contaminate aquatic habitats by contributing anthropogenic plastic and microplastic debris (Dumbauld et al. 2009). In clam aquaculture, alterations to the physical environment occur due to the removal of intertidal rocks, wood debris, and competing species (non-target species of clams, mussels, barnacles and predators) (Gallardi 2014). The presence of clam anti-predator netting may have negative consequences by trapping fish, increasing abundances of deposit-feeding polychaetes

and increasing sedimentation rates, resulting in sediments with enhanced organic content and lower dissolved oxygen (Dumbauld et al. 2009).The transfer and movement of bivalve stocks can also modify natural habitats with the intentional introduction and proliferation of non-native species to be cultured in aquaculture and the unintentional introduction and proliferation of non-native invasive species and pests (such as tunicates, macroalgae, and gastropods) and disease (Gallardi 2014).Pulse disturbances, caused by activities like harvesting, are damaging to benthic habitats (Dumbauld et al. 2009). In some aquaculture operations, bivalves are cultured on suspended racks or ropes that can be harvested with limited interfering with the environment, which is preferable to harvesting methods which physically disrupt the intertidal or seabed (Dumbauld et al. 2009). Harvesting methods like digging and raking are used with burrowing bivalves and bottom cultures can disturb and cause significant decreases in total macrobenthos, benthic polychaetes and other bivalves (Mosbahi et al. 2016). In fact, the hand collection of burrowing bivalves like clams results in the least amount of habitat disturbance compared to manual and hydraulic raking (Munari et al. 2006, Mosbahi et al. 2016). Other bivalve industries such as shellfisheries, also can alter coastal habitats where mechanized harvesting methods like dredging can be very damaging to benthic habitats (Mercaldo-Allen and Goldberg 2011). Here the dredge physically disrupts the intertidal and ocean floor by dragging collection nets, which stirs up sediment, ploughs over and uprooting rocks, sponges, and seagrass, and can dislodge, bury, or kill non-target species such as worms, snails, crabs, and fish (Mercaldo-Allen and Goldberg 2011).

The ability of shellfish aquaculture developments and shellfisheries to disturb and alter estuarian ecosystems, the importance of these ecosystems as nursery grounds for juvenile fish and other aquatic species and the reliance of these industries on productive habitats for bivalve

cultivation highlights the importance of continued research into impact assessments, improved management practices, and mitigation measures, to ensure healthy and productive coastal marine ecosystems.

1.3. Impact Assessment

Healthy marine habitats are vital for food security and for the other ecosystem services that marine ecosystems provide, such as recreational harvesting of seafood, harvesting of seaweed and other products for food and medicine, recreation/sports (boating, diving, surfing, kayaking), important breeding and nursery habitats, research and education, climate regulation, air purification, shoreline stabilization, and cultural heritage benefits (Barbier 2017). Surveying and assessing anthropogenic impacts on marine environments for the preservation of healthy, productive and ecologically sustainable marine habitats are ever-increasingly important tasks as the human population continues to expand. Shellfish are not only important in terms of food security (aquaculture, fisheries, recreational harvesting), but also as ecosystem engineers providing habitat and improving water quality. The sedentary nature of bivalves like mussels and oysters, their wide geographical distribution, and their ability to accumulate pollutants make them important indicator species of intertidal habitats and have therefore been used for assessing coastal water quality in international biomonitoring programs (e.g. Mussel Watch (international), RNO (France)) (Hamza-Chaffai 2014). It is important to monitor the potential impacts of environmental changes, such as climate change and pollution. New next generation technologies like RNA sequencing allows for the assessment of physiological responses to different biological and environmental challenges, with greater power to capture a wider scope of physiological changes.

1.4. RNA sequencing technology

Transcription is the process where a segment of DNA is copied into a corresponding messenger RNA (mRNA). These mRNAs are processed to become transcripts, which then serve as templates for protein synthesis, subsequently performing necessary cell functions. The transcriptome is the complete set of transcripts in a cell and their quantity (Wang et al. 2009). Studying transcriptomic responses to environmental change contributes to understanding the genetic basis for adaptation to climate change, temperature, and other environmental and biological stressors (Smith et al. 2013). Microarray technology is a hybridization-based method which involves specially designed pre-sequenced transcripts spotted onto glass slides, to which labeled samples either hybridize or not. RNA sequencing, also known as RNA-Seq, is a more recently developed high-throughput DNA sequencing method for quantifying and mapping entire transcriptomes (Wang et al. 2009). RNA-Seq (compared to DNA microarrays) has very low background signals, has a larger dynamic range for measuring very low and highly expressed genes (i.e. does not reach saturation), and has also been shown to be highly accurate with a high level of reproducibility for technical and biological replicates (Wang et al. 2009). Following sequencing, all generated reads are either mapped against an existing reference genome or a transcriptome can be assembled *de novo* without any prior genomic information, making this technique an attractive technology for studying non-model organisms. A genetic signature is a pattern of specific detectable nucleic acids that identify a tissue's state of function under a certain surrounding, or environmental phenomenon. The genes in the signature, and the associated biological processes, contribute to assessing the physiological condition of that tissue of that individual under the exposure conditions. Genomic signatures have been reported using

microarray technology in Pacific oysters (*Crassostrea gigas*) distinguishing oysters which died and survived an unexplained summer mortality event, often called the Pacific oyster mortality syndrome (POMS) (Chaney and Gracey 2011), and in sockeye salmon (*Oncorhynchus nerka*) under unusually high levels of pre-spawning mortality in the Fraser River salmon (Miller et al. 2011). RNA-Seq has been used to study transcriptional changes induced by temperature stress in Rainbowfish (*Melanotaenia duboulayi*) (Smith et al. 2013), and in Pacific oysters (*C. gigas*) relating to osmotic/salinity stress (Zhao et al. 2012, Meng et al. 2013) and *Vibrio* infections (de Lorgeril et al. 2011).

1.5. Thesis goals

This thesis examined two different aspects of human impacts on marine ecosystems. One chapter (clam gardens) examined the impacts of habitat modification for productivity enhancement and the other (microplastics) examined the impacts of anthropogenic debris on a socio-economically globally important shellfish species.

Clam gardens are examples of ancient anthropogenic habitat modifications, built and managed by Indigenous peoples to increase food production and security. Culturally and ecologically important, these 'gardens' also typically have a high concentration of small shell fragments (shell hash), which has been shown to increase bivalve recruitment and increase pH and saturation state by buffering sediments (Green et al. 2012). Chapter 2 of this thesis will focus on physiological impacts of habitat modification (clam gardens) on the culturally and recreationally important Littleneck clam species (*Leukoma staminea*).

Microplastics are small fragments of plastic between 100 nm and 5 mm in size and are emerging pollutants of concern in the marine environment. They are found in oceans worldwide and are present in most organisms, but their impacts on biological activities remains largely

unknown. Chapter 3 of this thesis will focus on the physiological impacts of emerging pollutants (microplastics) on the commercially important Pacific oyster species (*Crassostrea gigas*).

Chapter 2: Impacts of clam gardens on Littleneck clam *(Leukoma staminea)* physiology

2.1.1. Introduction

The sea plays a central role in Northern Coast Salish life as a major source of fish, sea mammals, shellfish, and marine plants (Caldwell et al. 2012, Lepofsky et al. 2017). The importance of shellfish as a staple food source is evidenced by white shell middens (discarded shells close to clam processing sites), that are characteristic of the coast line and hallmarks of coastal settlements in British Columbia (Lepofsky et al. 2017). Clam gardens are examples of ancient mariculture beach modifications and constructions and were used by the Indigenous peoples of the Northwest coast of North America to enhance shellfish production, cultivating a sustainable and predictable nutrient rich food source, which enhanced food security and community survival in nearby villages (Deur et al. 2015, Neudorf et al. 2017). Construction of these clam gardens is estimated to have began ~ 1000 – 1700 years (Neudorf et al. 2017), long before European contact, and were documented as still being tended up until the 1930's (Deur et al. 2015). Clam gardens are found in the low intertidal from Alaska, throughout British Columbia (B.C.) and into Washington State (Groesbeck et al. 2014). Northern Quadra Island (QI) B.C., has among the highest density of clam gardens on the Pacific Northwest coast (Neudorf et al. 2017), with 45 in Kanish Bay and 49 in Waiatt Bay on the northeast side of the island (Groesbeck et al. 2014). To the Kwakwaka'wakw Indigenous peoples whose territory spans Northwest Vancouver Island and across the Queen Charlotte Strait to the mainland of B.C., clam gardens are known as *loxiwey* meaning "to roll" (Deur et al. 2015). Clam gardens are

still important to Indigenous peoples (Deur et al. 2015). They provide an important insight into cultural food practices, traditional technologies, economies, values, and ancestral practices (Deur et al. 2015). Ancient mariculture research may also offer insights into future management strategies for food security for coastal communities (Groesbeck et al. 2014).

Clam gardens (Figure 1) are developed by increasing or creating an intertidal area by moving beach boulders to develop a wall; this allows for the deposition of sediment behind the wall, creating a wide intertidal shellfish habitat area with a reduced slope, facilitating more habitat for shellfish to grow and be cultivated (Neudorf et al. 2017).

Figure 1: Clam garden wall at low tide. (Photo credit: Monique Raap)

This flat zone creates a "garden" bed that is submerged at high tide and exposed only at very low tide, reducing desiccation risk and maximizing submersion time for feeding and therefore growth (Deur et al. 2015). In addition, the reduced beach slope allows a thin layer of

water to remain on the accumulated sediments, facilitating clams to remain in shallow, accessible portions of the tidal column (Deur et al. 2015). The removal of large rocks facilitated clam harvesting. Clam gardens were created at ideal tidal heights for shellfish such as Littleneck clams (*Leukoma staminea*) and expanded their habitat (Groesbeck et al. 2014). The other side of the clam garden walls creates rocky reef habitat for many other harvestable marine invertebrates including octopus, sea cucumber, whelks, chiton, red turban snails, Dungeness and red rock crabs (Caldwell et al. 2012, Deur et al. 2015, Lepofsky et al. 2017).

Clam beds were tended by selective harvesting with yew wood sticks, the removal of large shells and debris, and the mechanical aeration of the sediment (Deur et al. 2015). Smaller clams were not collected by harvesters in order to minimize localized over-harvesting. Anaerobic sediments can be found in an untended clam gardens, where the sediment is dark with a hydrogen sulfide smell, resulting in clams with an unpleasant taste (Deur et al. 2015).

Leukoma staminea (native Littleneck clams) are one of the 4 major species of bivalves in clam gardens harvested by the Kwakwaka'wakw, along with *Tresus nuttallii* (horse clams, or Pacific gaper), *Saxidomus giganteus* (butter clams) and *Clinocardium nuttallii* (cockles) (Deur et al. 2015). The sediments of the Kanish and Waiatt Bay clam gardens contains these native bivalve communities, as well as *Macoma spp*. (macoma clams), *Tresus capax* (also called horse clams, or fat gaper) and non-native *Venerupis philippanarum* (also known as Manila clams or Japanese Littlenecks) (Groesbeck et al. 2014).

2.1.2. Clam garden research

Clam garden research is an increasing area of both anthropological and scientific interest, with a developing body of litreature regarding the traditional management of marine and

terrestrial ecosystems among coastal Indigenous peoples (Lepofsky et al. 2017). Clam gardens along with stone fish traps, wooden fish weirs, size selective fishing practices, seaweed picking, root gardens and the reduction of predatory sea otters to increase shellfish abundance are all examples of marine technological and management strategies developed by Indigenous communities along the Northwest coast of North America, to cope with unexpected natural shifts or disasters that would affect resource availability (Groesbeck et al. 2014, Jackley et al. 2016). A growing body of evidence suggests that the development and refinement of diverse conservation and management strategies over millennia was designed to enhance food production and increase food security (Jackley et al. 2016). Maintaining healthy habitats is essential for sustaining global food production to meet rising demands from an increasing human population. Gaining insight from past practices using traditional knowledge may provide practical strategies for the management of current and future food resources.

Research conducted on the central coast of B.C. (Kwakshua Channel between Calvert and Hecate Islands) observed that the walled beaches found along the mouths and/or edges of small inlets had shallower slopes compared to the unmodified beaches (Jackley et al. 2016). The clam gardens of Quadra Island have rock walls constructed in the mid-intertidal zone, between 0.5-1.8m above chart datum (LLWLT: lowest low water large tide) creating shallow intertidal terraces at tidal heights that are optimal for shellfish growth and survival (Groesbeck et al. 2014).

Clam garden studies have found significantly higher densities of Littleneck clams and increased survival of clam recruits in walled compared to non-walled beaches in Kanish and Waiatt Bay on Quadra Island (Groesbeck et al. 2014, Jackley et al. 2016). On the central coast of B.C., butter clam (*S. giganteus*) density was on average 2.44 times greater in clam gardens than non-walled beaches (Jackley et al. 2016). This increase was at the top end of the beach in

particular, between tidal heights of 1.0 to 1.5 m, where clam habitat had been extended in clam gardens through the creation of a flatter beach (Jackley et al. 2016). In Kanish and Waiatt Bay on Quadra Island, clam garden beaches had 4 times more *S. giganteus* and 2 times more *L. staminea* than non-walled beaches (Groesbeck et al. 2014). On Quadra Island, transplanted *L. staminea* grew 1.7 times faster in walled relative to non-walled beaches (Groesbeck et al. 2014). On the central coast of B.C. clam community composition was found to change depending on whether beaches were clam or non-clam garden beaches, with both containing *S. giganteus, Macoma nusata* (bentnose macoma), *L. staminea*, and *C. nuttallii* with *M. balthica* (Baltic macoma) and non-native *V. philippinarium* only on non-walled beaches (Jackley et al. 2016).

Clam garden beaches on Quadra Island were observed to contain more shell hash (crushed shells) and gravel in their sediment compared to non-walled beaches (Groesbeck et al. 2014). A study on the central coast of B.C. examined 32 kilometres of intertidal and foreshore coastline (10 clam garden beaches and 16 unmodified non-walled beaches) and observed that the sediment from clam gardens was composed mainly of shell hash and gravel, whereas the sediment from the non-walled, unaltered beaches was primarily composed of silt, sand, and mud (Jackley et al. 2016).

Ocean acidification from increasing ocean carbon dioxide (pCO_2) concentrations results in a decrease in seawater pH and an alteration of carbonate chemistry through the increase in hydrogen and bicarbonate ions (Evans et al. 2014). The increase in ocean acidity reduces calcium carbonate ($CaCO_3$) saturation states (Ω) to corrosive levels (i.e., $\Omega < 1$) making the calcification process for the formation of shells more difficult for marine molluscs and other calcifiers (Green et al. 2012, Evans et al. 2014, Waldbusser and Salisbury 2014). Corrosivity in coastal waters surrounding Quadra Island, B.C., was found to be highest in the winter months from December

to February, lowest in the spring and summer months, and increased again in the fall (Smith et al. unpublished). Sediment carbonate saturation state is a significant chemical cue for the settlement of juvenile infaunal bivalves (Green et al. 2012). In the field bivalve recruitment was increased by a factor of 3 over a 30-day period by raising the pH (~0.3) and saturation state of surface sediments by buffering sediments with crushed shells ($CaCO_3$) (Green et al. 2012). However, average daily growth of early post-settlement Manila clams (*Venerupis philippinarum)* measured over two years was negatively correlated with carbonates, organics, and nitrogen in the sediment (Munroe 2016). These researchers also found clam growth also tends to increase with increasing gravel in the sediment (Munroe 2016). Buffering sediment with shell hash has not yet been implemented as an adaptation measure and could result in intertidal fauna smothering. Recent research measuring the effect of shell hash on porewater pH in Burrard Inlet B.C. as an effective mitigation measure for acidic sediment conditions was inconclusive (Doyle 2016).

Many factors affect clam feeding, reproduction, growth and survival. Temperature, salinity, turbidity, exposure to wave action and currents, sediment composition and water residency all play a role in clam distribution, and abundance (Gosling 2008). Clams occupy the broadest range of habitats of the four bivalve groups (Gosling 2008). Their habitats range from open coast to sheltered, saline and estuarine environments. They can settle from upper intertidal to subtidal regions in either or combinations of mud, sand or gravel (Gosling 2008). As burrowing bivalves, they prefer soft substrates with the highest numbers found in sand, moderate amounts found in sand and mud, and the fewest numbers found in mud. Littleneck clams habitats consists of estuarine locations in the intertidal to shallow intertidal zone, in mud and gravel, and their optimal temperature for somatic growth is 15° C, with a range of 12° – 18° C (Bernard 1983).

2.1.3. Chapter objectives

Clam gardens, ancient intertidal beach modifications built and used by the Indigenous people of the Northwest Pacific coast, increased food production, and food security (Deur et al. 2015, Neudorf et al. 2017). The construction of a rock wall at the low tide line created an expanded intertidal habitat for clams, once filled with silt and sediment, increasing the horizontal area at the tidal height of 1-2 m where clams are mostly found (Groesbeck et al. 2014, Deur et al. 2015). Clam gardens are often associated with large shell middens created by the historical processing of clams on site (Deur et al. 2015). It has been thought that beaches with increased levels of shell hash (crushed shells) in the sediment have increased clam settlement and productivity (Green et al. 2012, Groesbeck et al. 2014).

The objectives of this chapter were: 1) to determine whether clam gardens have a significant positive effect on Littleneck clam (*Leukoma staminea*) physiology; 2) to determine whether sediment carbonate and organic content influence clam growth and survival, and; 3) to determine if there are significant differences in Littleneck clam physiology at different geographic locations. To assess whether clam gardens or walled beaches provide a more suitable or more productive environment for clams than non-walled beaches, Littleneck clams were transplanted onto 3 unmanaged clam garden beaches and 3 non-walled beaches in Kanish Bay and in the adjoining Small Inlet, Quadra Island, B.C. After 16 weeks *in situ* clam growth and survivorship data was recorded, and surviving clams were sampled for genomic response analysis. Physiological responses of Littleneck clams were examined in clam garden beaches compared to non-clam garden beaches, in relation to beach carbonate and organic sediment content and overall clam health and survival. To survey whether clam garden beach sediment is

distinctly different than non-walled reference beach sediment, and to determine if sediment (and in particular shell hash) has any influence on shellfish productivity, sediment samples were collected and grain sizes, percent carbonates and organics determined and analyzed in conjunction with physiological data.

Null hypotheses

H_0 = Clam gardens do not have a significant positive effect on Littleneck clam physiology, growth or survival measured after 16 weeks *in situ* from beginning of May to the end of August.

H_0 = Sediment carbonate and organic content do not have any effect on clam growth and survival measured after 16 weeks *in situ* from beginning of May to the end of August.

H_0 = There are no significant differences in clam physiology, growth or survival at different geographic locations measured after 16 weeks *in situ* from beginning of May to the end of August.

2.2 Methods

Study Region

The intertidal coastlines of Kanish Bay and adjoining Small inlet, Quadra Island, B.C., Canada, were chosen for the study regions because of their abundance of clam gardens (Figure 2). Three clam garden (walled) and 3 non-walled (reference) sites were selected based on exposure, location, physical characteristics, and were also being concurrently studied facilitating field work. The following beach descriptions are anecdotal. Beach A (clam garden) was a southwest facing bay exposed to wave action from within and outside of Kanish Bay. The surface sediment appeared predominantly sandy with whole and crushed clam shells. Beach B

(reference beach) was a northeast facing bay exposed to wave action within Kanish Bay. The surface sediment was covered with rocks, barnacles, and is comprised of coarse sand. Beach C (clam garden) was within Small Inlet, south facing, sheltered and not exposed to wave action from within Kanish Bay. The surface sediment was observed to be predominantly fine sand with whole and crushed shells. Beach D (reference) was across from beach C within Small Inlet, north facing, and sheltered from wave action. The surface sediment appeared to contain high amounts of very fine sand and plenty of whole clam shells and was noticeably absent of crushed shells. Beach E (clam garden) was north facing, on the south side of Kanish Bay, and exposed to wave action within the bay. The surface sediment contained predominantly rocks covered in barnacles, coarse sand, silt, and some shells and fine shell hash. Beach F (reference) was directly adjacent to beach E and the surface sediment contained small rocks covered in barnacles, sand, and silt with a nearby shell midden. Specific locations of clam garden beaches are not provided, to respect the wishes of coastal communities. These clam gardens have likely not been tended in accordance with Indigenous practices since the early-to-mid 1900s (Deur et al. 2015).

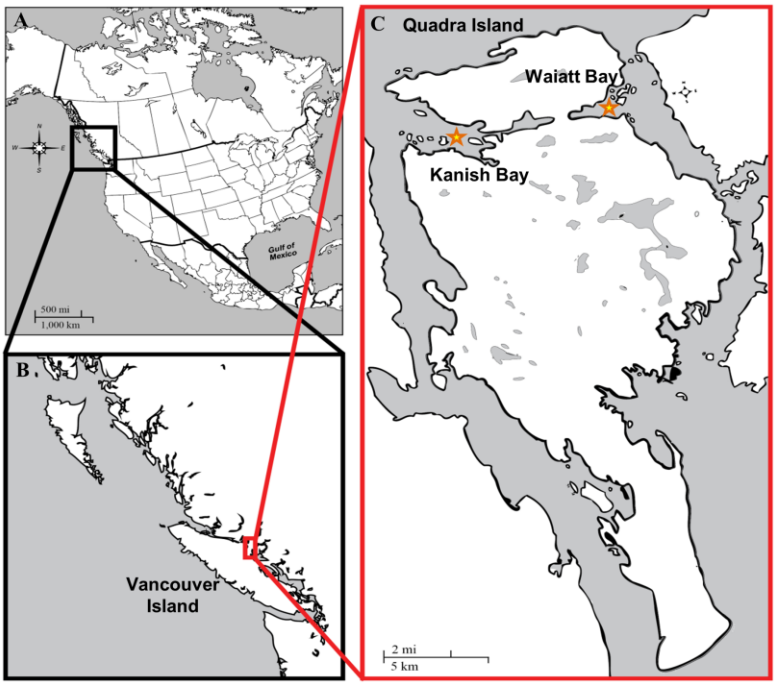


Figure 2: Broad locations of clam gardens area on Quadra Island, B.C., Canada and study site in Kanish Bay. Image taken from Groesbeck et al., 2014.

Clam transplantation experiment

Approximately 400 Littleneck clams were hand collected from a beach in Kanish Bay, individually weighed, measured (height), and randomly divided into 18 groups of 20 clams. The clams ranged in height (as defined by hinge line to the shell margin) from 11 – 21 mm with a mean height of 14 ± 2 mm. They ranged in weight from 0.6 – 3.6 g with a mean weight of 1.5 ± 0.6 g. Vexar™ high density polyethylene plastic mesh cubes (n=18), 20 x 20 x 4 cm in size, were fastened together with plastic cable ties, to contain each group/plot of 20 clams. Clams were deployed May 10-12th, 2016 depending on location. At each beach the tidal height was measured, and the 1.5-1.8 m intertidal zone was marked with flags. Each of the 3 Vexar™ cubes of 20 clams were buried in the top 20 cm of the sediment, 5 m apart, parallel to the shore in the middle of the 1.5 - 1.8 m mid intertidal zone at each beach. Each Vexar™ cube is defined as one plot. A length of rebar was hammered into the sediment and marked with flagging tape and a 'please do not disturb', and identification tag. Transplanted clams were left *in situ* for 16 weeks and collected August 30-31st, 2016. Clams and sediment were expediently transported in coolers and bags to the Hakai Institute laboratory facilities in Heriot Bay on Quadra Island. Clam survivorship, weight, height, length (the widest part across the shell at 90 degrees to the height), and National Aquatic Animal Health Program observational data were recorded. The gill and digestive gland tissues were sampled from all survivors for gene expression analysis.

RNA sampling, extractions and visual health observations

For gene expression analysis a small (~ 2 x 2 mm) section of gill and digestive gland tissue was excised from each surviving clam immediately after the clam valves were opened, using sterile techniques. Gills were chosen because they are in constant contact with their environment and therefore often used in environmental stress studies as more immediate environmental response indicators (Milan et al. 2011). Digestive glands were chosen as they are accumulatory organs and are used in toxicological and immunology studies to examine long-term effects of environmental change and exposure (Milan et al. 2011). Briefly the sampling area was disinfected with fresh (made daily) 0.5% solution of sodium hypochlorite, and sampling scissors, forceps and scalpels disinfected by 2 min. immersion in 0.5% sodium hypochlorite solution, dipped in water, and dipped briefly in 100% methanol before passing through a flame. Clams were opened with a sterile scalpel, and scissors and forceps used to remove a small cube of tissue (approximately 2 mm^3), from the gill first, and then the digestive gland, and stored in RNAlater as per protocol (Ambion, Carlsbad, CA).

RNA from tissue sections of 25 - 30mg were individually extracted from each tissue using RNeasy kits (Cat No./ID: 74106, Qiagen, Maryland). Tissues were homogenized in 2 mL tubes of Lysing Matrix D (SKU 116913500, MP Biomedicals, Solon, OHIO) in a Tissuelyser II (Cat. No. 85300,Qiagen, Maryland) at 25 Hz for 2 min. To eliminate DNA from contaminating the samples, a DNase treatment was applied using Turbo DNA-free Kits (SKU# AM1907, Ambion, Carlsbad, CA) and followed the product routine treatment protocol. The RNA concentration was quantified on a Nano-drop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) before and after DNase digestion.

At the time of clam sampling, a number of gross visual observations were made on animal condition, following the protocols developed for the Canadian National Animal Aquatic Health Program. This included (but was not limited to) observations of animal state (body condition, response), digestive gland and gill colouration, any nodules indicating disease, any parasites present and any internal shell deposits.

Library preparation for RNA sequencing

Fourteen of the 18 plots had ≥ 5 survivors (only one plot out of 3 on beach E (clam garden) and beach F (reference) had ≥ 5 survivors). Therefore, for RNA sequencing, pools were created of 5 randomly selected (where possible) individuals from each of these plots. This is a total of 14 pools per tissue and 28 RNA pools for library synthesis and sequencing. RNA quantities were normalized, and pools of RNA were created for each tissue of the 5 selected individuals from each plot. Prior to sending samples for library generation the RNA quality and quantity of each pool was determined using the Agilent RNA 6000 Nano chip (No.5067-1511, Mississauga, ON) on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

All RNA library synthesis and next-generation sequencing was conducted at Genome Québec Innovation Centre (Montreal, Québec, Canada). Briefly, mRNASeq stranded paired-end (2 x 100 bp) library synthesis methods was as follows: total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and its integrity was assessed on a 2100 Bioanalyzer (Agilent Technologies). Libraries were generated from 250 ng of total RNA where : mRNA enrichment was performed using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs) and cDNA synthesis was achieved with the NEBNext RNA First Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis

Modules (New England BioLabs). The remaining steps of library preparation were performed using and the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Adapters and PCR primers were purchased from New England BioLabs. Libraries were quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). Average fragment size was determined using a LaB.C.hip GX (PerkinElmer) instrument. Libraries were run on 4 lanes of an Illumina HiSeq4000 PE 100 platform (Illumina, San Diego, CA, USA) and the mean reads per library was 108 ± 18 million.

Transcriptome assembly and analysis

For the clam transcriptome *de novo* assembly, Trinity (v2.5.1, --min_kmer_cov 2) was used to assemble the 28 libraries. Before running the assembly, Trimmomatic (v0.36, ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) was used to clean the read data to obtain a more accurate assembly. With the resulting Trinity assembly (1,695,678 sequences) theTransDecoder pipeline (v5.0.1) was used to predict likely coding sequences (1,277,478), which uses homology (pfam-a, UniProt) and open reading frame (ORF) information. As multiple transcripts can represent a single gene, the best representative transcript was chosen for each gene predicted by TransDecoder which had an ORF type of 'complete'. These predicted complete ORFs required protein homology to be considered a representative. In cases where there were multiple transcripts with complete ORFs for a gene, the transcript with the largest ORF was chosen. There was a total of 54,337 transcripts, each representing a single gene.

To filter out transcripts that were too small and could introduce error to the analysis, only

transcripts that were ≥ 100 bp (54,126 transcripts) were selected. Finally, the pfam-a and Uniprot annotations were scanned to remove repetitive element related sequences using a keyword search for terms such as 'transposon', 'long terminal repeat', 'transposase', 'long interspersed element', etc. The final set contains 52,000 transcripts. With the finalized 52,000 set of transcripts, CLC Workbench's RNA-Seq Analysis tool (v9.5.4, minimum length fraction = 0.90, minimum similarity fraction = 0.95, maximum number of hit reads = 10) was used to map the raw reads back. The reads were mapped in pairs to determined Fragments Per Kilobase Million (FPKM) expression values. Without a genome, the number of transcripts can be large and can include tens or hundreds of contigs accounting for fragmented transcripts, repeats, transposons, alleles, and alternate transcripts (Conesa et al. 2016).

RNA sequencing data analysis

The edgeR (Robinson et al. 2010) package (Version 2.6.9) was used to analyze the RNA sequencing data and to detect significantly differentially expressed genes (DEGs) between clam garden and reference beach libraries. After genes with very low counts were filtered out, and data was normalized for library size multi-dimensional scaling (MDS) plots were run as a first analysis step to examine samples for outliers and other relationships. MDS is a type of unsupervised clustering function in edgeR that plots the RNA samples in which distances correspond to leading log-fold-changes between each pair of RNA samples. Generalized linear models were used to identify DEGs between the clam garden and reference beaches libraries. Heatmaps were run in edgeR. Up- and downregulated DEGs ($p \leq 0.015$, fold change (FC) $\geq \pm 2$) were analyzed separately using DAVID (v6.8) (Huang et al. 2007), for enrichment analysis of biological, and functional pathways. A probability value of ≤ 0.05 was used as a cut-off for significantly enriched functional clusters.

Sediment characteristics

To determine grain size distribution, the sediment samples were dried at 100^{o}C for 24 hours. They were then weighed for total dry weight and then transferred to the top sieve of sieve set with the following sizes: 4.75 mm, 2 mm, 1 mm, 500 µm, 250 µm, 125 µm, and a bottom collection pan. The sieve stack was placed in a shaker and shaken for 15 min. Each size fraction was then weighed and recorded.

Sediment carbonate and organic content determination

To determine sediment carbonate and organic content, the sediment samples were first dried in previously baked crucibles (450^{o}C for 8 hours) at 100^{o}C for 48 hours, and the sediment and crucible dry weight recorded, and sediment dry weights subsequently calculated. Sediment organic content was then determined by further drying in a muffle furnace at 435^{o}C for 8 hours. After cooling the samples in the desiccator for 1 hour, the weight of the samples was then recorded. To determine the sediment carbonate content these same previously weighed crucibles were then placed in a muffle furnace at 950^{o}C for 2 hours and sample weights were recorded after a cooling period of 2 hours in a desiccator.

Sediment data analysis

Sediment data was analyzed using the statistical program R (R Core Team. 2016). Nested ANOVAs (where replicates were nested under beach location) were run using the linear mixed effects function in the nlme package run in R to identify significant ($p \leq 0.05$) differences between clam garden and reference beaches in clam survival average growth, and individual

sediment characteristics. Linear mixed effects models were run with type of beach (clam garden or reference) as the fixed effect, and plots nested within beach as the random effect. A model, summary, and ANOVAs were run for each of the response variables: percent survival, growth, and sediment characteristics (carbonate, organic, rocks, small rocks, very coarse sand, coarse sand, sand, fine sand, very fine sand, and silt). Nested ANOVAs were run using linear mixed effects model to identify sediment characteristics that had a significant correlation with survival and/or growth. Models were run with survival and average growth as the response variables, with plots nested within beach as the random effects, and each sediment characteristic as the predictor

Plots were generated with growth and survival as the response (y axis), and each sediment characteristic on the x axis. A plot of percent survival with percent carbonates suggested a linear relationship, and the model with survival as the response variable and carbonate sediment content as the predictor variable was the only model that had a significant *p* value (0.014), For these reasons the following linear mixed effects models with beach as the random effect, survival as the response variable and with combinations of carbonates, and presence of wall as the predictor variables were fit using the lmer function in the R package lme4 (Bates et al. 2015):

```
model0 <- lmer(surv ~ 1 + (1|beach), data = clam, REML = FALSE)
model1 <- lmer(surv ~ carb + (1|beach), data = clam, REML = FALSE)
model2 <- lmer(surv ~ wall + (1|beach), data = clam, REML = FALSE)
model3 <- lmer(surv ~ wall + carb + (1|beach), data = clam, REML = FALSE)
model4 <- lmer(surv ~ wall + carb + wall:carb + (1|beach), data = clam, REML = FALSE)
```

These five models were compared using AICc (Akaike Information Criteria corrected) values due to the small sample sizes. The AICc values were: model0 = 176.1, model1 = 172.7, model2 = 179.4, model3 = 176.3, and model4 = 180.1. Model1: lmer(survival ~ % carbonates + beach as the random component) had the lowest AICc value and was therefore the best fit model. This model fit the linear model assumptions of homogeneity, independence, and normality of variance.

2.3 Results

Clam growth and survival

Leukoma staminea (Littleneck clams) adults between 1.1 – 2.1 cm in height (distance from umbo to valve edge), and 0.6 – 3.6 g in weight (n = 360) were collected in Kanish Bay, divided randomly into 18 groups (n = 20) and transplanted into 18 plots on 6 beaches (3 plots per beach) in the middle of the 1.5 – 1.8 m intertidal height zone. Three of these beaches were clam garden walled beaches and three beaches were reference non-walled beaches. Following 16 weeks *in situ*, clams were collected and surviving clams (n = 222) ranged from 1.2 – 3.0 cm in height, and 0.6 – 12.4 g in weight. The mean initial clam heights were 15.6 ± 1.8 mm (Figure 3). The mean final clam garden and reference beach clam heights were 20.3 ± 3.0 mm, and 21.5 ± 4.5 mm (Figure 4).

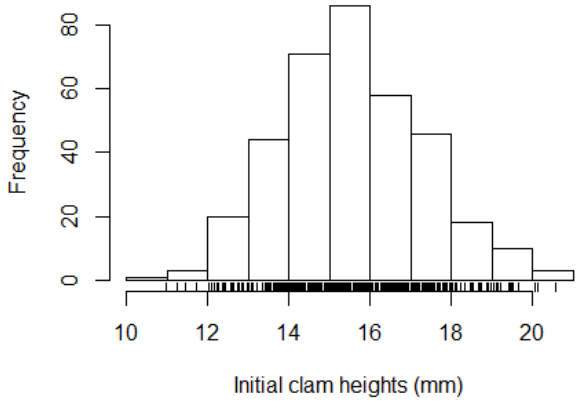


Figure 3: Histogram of initial clam heights

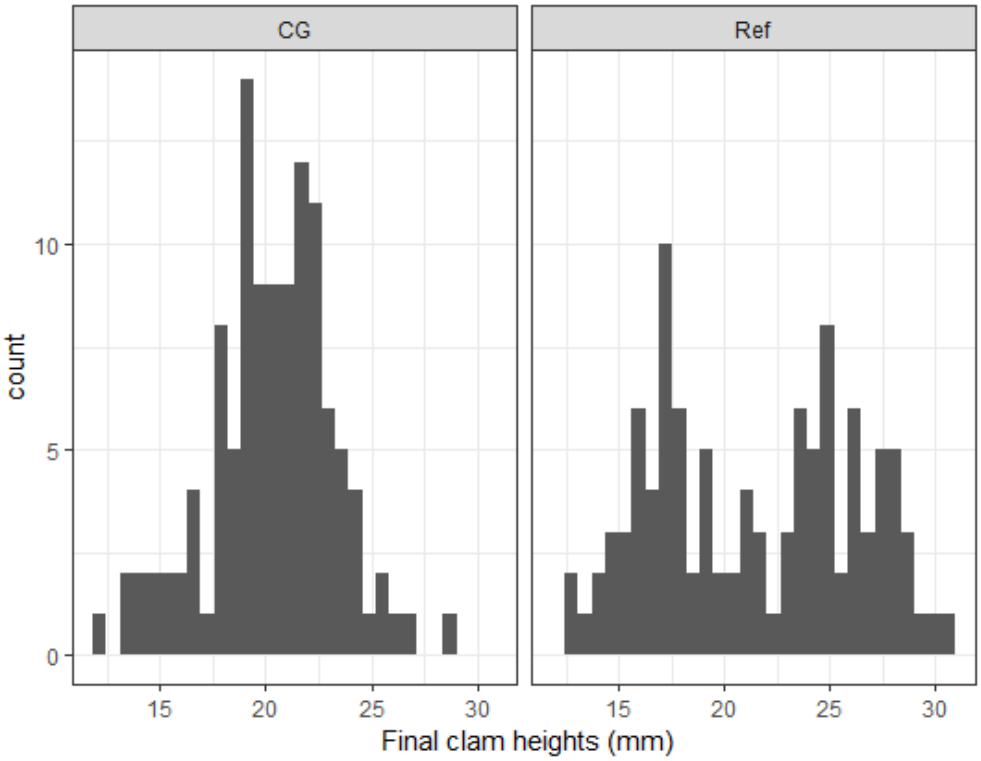


Figure 4: Histogram of final clam heights in clam gardens (CG), and reference (Ref) beaches

Nested ANOVAs of linear mixed effects models with type of beach (clam garden or reference beach) as the fixed effect, and plots nested as a random effect of beach were run to test for significant differences in initial and final clam heights in clam garden beaches compared to

reference beaches. We found there were no significant differences in initial clam heights between clam garden and reference beach clams (p = 0.17). There were also no significant differences in final clam heights between clam garden and reference beach clams (p = 0.17).

The mean percentage of survival of Littleneck clams on clam garden beaches was 63 ± 28 %, compared to 58 ± 37 % on reference beaches. The mean percent average growth of Littleneck clams on clam garden beaches was 155 ± 94 %, compared to 155 ± 122 % on reference beaches. Nested ANOVAs of linear mixed effects models with type of beach (clam garden or reference beach) as the fixed effect, and plots nested as a random effect of beach were run to test for significant differences in survival and growth in clam garden beaches compared to reference beaches. There were no significant differences in percent survival (p=0.86) or average growth (p=0.67) between walled and non-walled beaches (Figure 5).

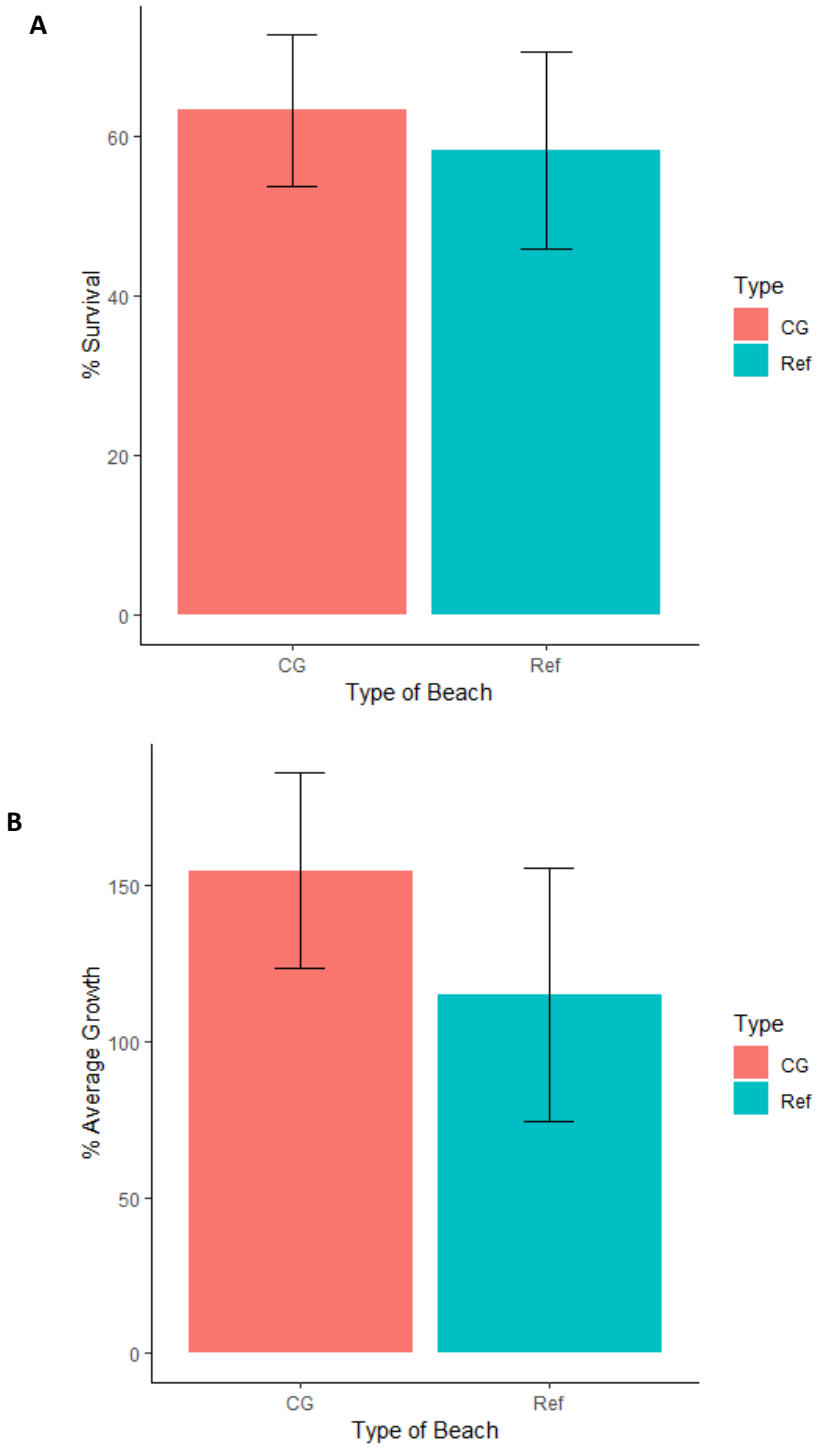


Figure 5: Percentage survival and growth increase of Littleneck clams after 16 weeks of field transplantation in clam garden and non-walled reference beaches. A; survival, B; growth. Columns represent means with standard error bars

Sediment results

Grain sizes

Sediment from each plot on each beach was analyzed for eight grain sizes, and are characterized in mean percentages as follows: rocks > 4.75 mm from clam gardens was 16 ± 7 % and from reference beaches was 15 ± 6 %; small rocks 2 – 4.75 mm from clam gardens was 12 ± 5 % and from reference beaches was 13 ± 3 %; very coarse sand 1- 2 mm was 12 ± 3 % from clam gardens and 13 ± 4 % from reference beaches; coarse sand was 14 ± 3 % in clam gardens and 13 ± 4 % in reference beaches; sand 250 – 500 µm was 20 ± 6 % from clam gardens and 18 ± 4 % from reference beaches; fine sand 125 – 250 µm was 19 ± 6 % from clam gardens and 17 ± 3 % from reference beaches; very fine sand 63 – 125 µm was 6 ± 2 % from clam gardens and 8 ± 4 % from reference beaches, and; silt < 63 µm there was 2 ± 1 % in clam gardens and 3 ± 1 % in reference beaches. Linear mixed effects models were used to examine any significant differences in the individual grain sizes between clam garden and reference beaches. There were no significant differences between clam garden sediment and reference beach sediment with any of the grain sizes (Figure 6).

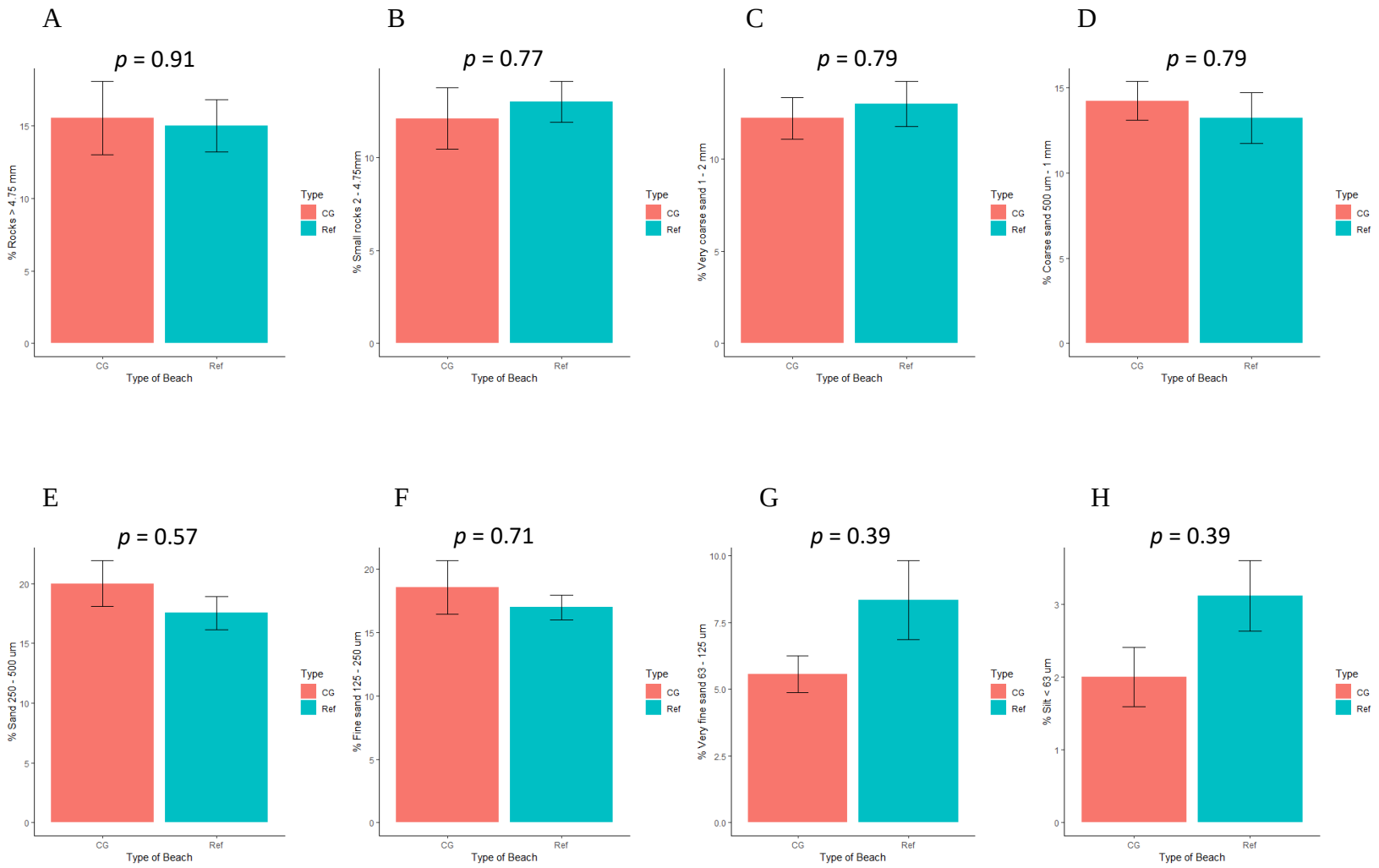


Figure 6: Percentages of each grain size of A rocks, B small rocks, C very coarse sand, D coarse sand, E sand, F, fine sand, G very fine sand and H silt in clam garden and reference beaches. Columns represent means and standard error bars, probability values are from linear mixed effects model statistical comparisons.

Carbonates and organics

Sediment samples from each plot on each beach were analyzed for carbonate and organic content (Figure 7). The mean percentage of carbonates in sediment samples from clam garden beaches was 7.5 ± 1.9 % and 7.2 ± 5.2 % in sediment from reference beaches. The mean percentage of organics in clam garden beaches was 1.3 ± 0.6 % and 1.1 ± 0.5 % in reference beaches. Linear mixed effects models were used to identify significant differences between carbonates and organics in clam garden and reference beaches, ANOVAs of linear mixed effects models with type of beach as the fixed effect, plots nested within beach as the random effects and percent carbonates and organics each as the response variables. We found there were no significant differences between the percent of carbonates ($p = 0.92$), or organics ($p = 0.72$) in clam garden beaches compared to reference beaches (Figure 7).

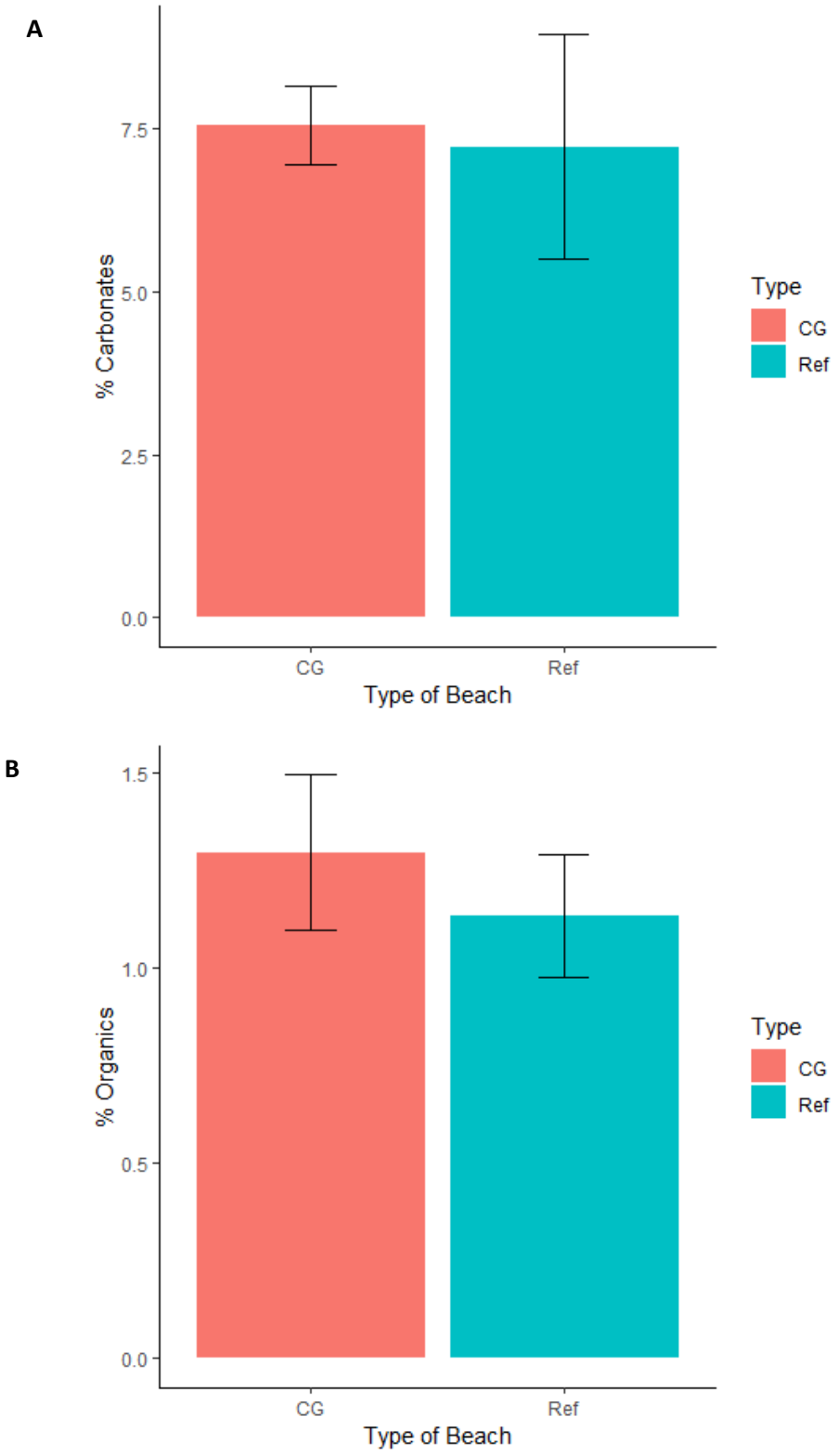


Figure 7: Mean sediment carbonate (A) and organic (B) percent content between clam garden and reference beaches. Bars represent means with standard errors.

Effects of carbonates and organics on survival and growth

To explore whether percentage sediment carbonates and organics impacted clam survival and growth, plots (Figures 8 and 9) and accompanying linear mixed effects models were employed.

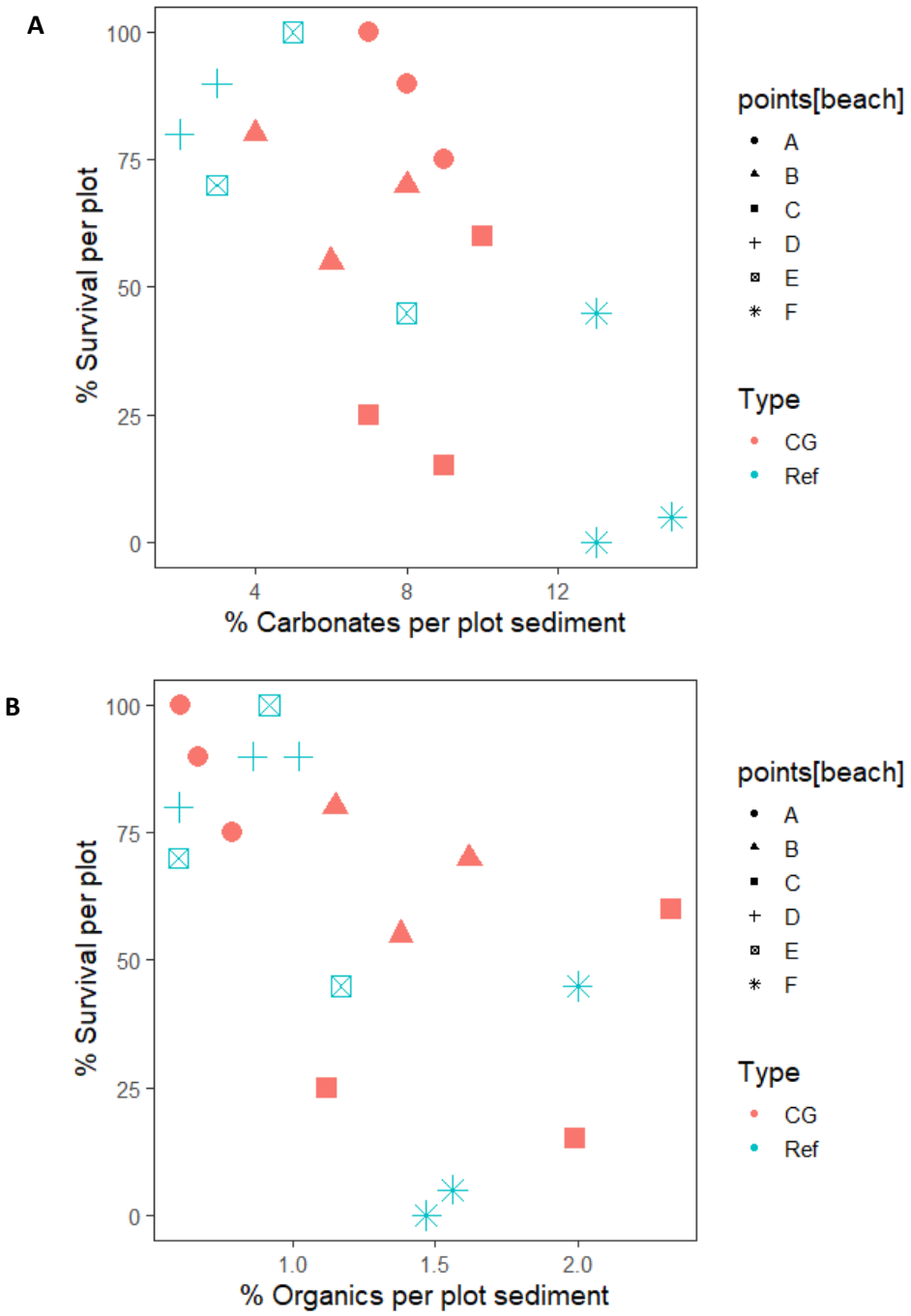


Figure 8: Littleneck clam mean survival on clam garden and reference beaches with sediment carbonate (A) and organics (B). Beach pairs are indicated by corresponding shapes, and beach type by colour.

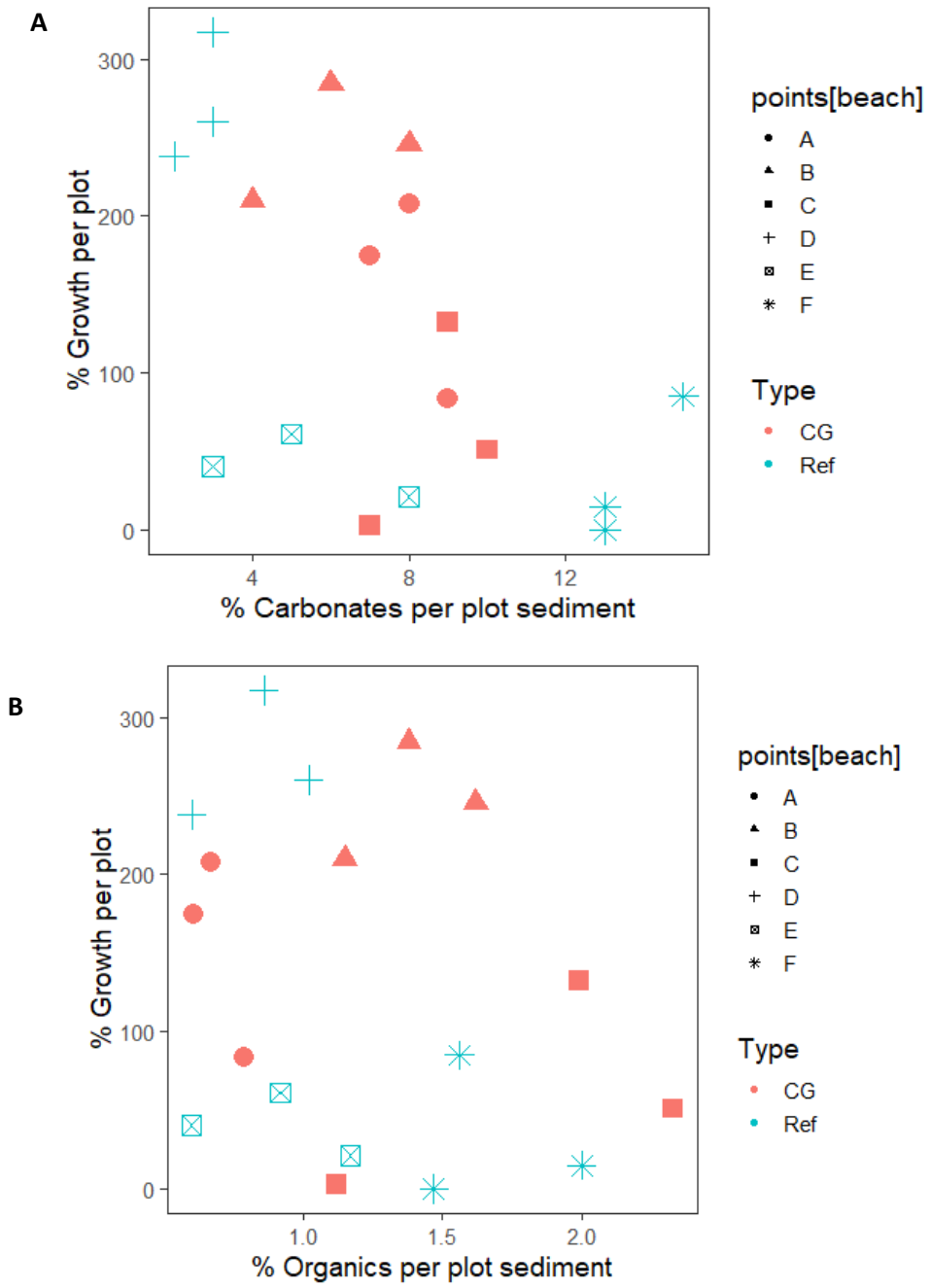


Figure 9: Littleneck clam mean growth on clam garden and reference beaches with sediment carbonate (A) and organics (B). Beach pairs are indicated by corresponding shapes, and beach type by colour.

As carbonates are an important component necessary for shell formation and the only significant sediment type or grain size found with survival, linear mixed effects models with combinations of carbonates and type of beach as the fixed effects, with survival as the response variable and plots nested within beach as the random effect were run. Percent organics ($p = 0.53$) or other grain sizes were not included in the model as none of them were significant with survival or growth. The model with survival as the response variable with carbonates as the predictor and with beach as the random effect had the lowest AICc value, indicating the best model fit. This model fit the linear model assumptions of homogeneity, independence, and normality of variance. The correlation coefficient for percentage carbonates was -0.89, and the *p* value was 0.0002. The linear plot and confidence interval for carbonates was negative indicating carbonates in the sediment had a negative effect on survival. A negative correlation of survival with carbonates was not expected and indicates the need for further research into optimal clam habitat sediment composition and OA mitigation measures.

The equations for each beach with this model were:

Pair 1:

Clam garden beach A: % survival = 118 - 5.7 * % carbonates

Reference beach B: % survival = 102.5 – 5.7 * % carbonates

Pair 2:

Clam garden beach C: % survival = 102.8 – 5.7 * % carbonates

Reference beach D: % survival = 102.6 – 5.7 * % carbonates

Pair 3:

Clam garden beach E: % survival = 93.2 – 5.7 * % carbonates

Reference beach F: % survival = 99 – 5.7 * % carbonates

Survival, growth, sediment data, and visual observations were used to describe the dominant type of sediment in each beach. By comparing individual beach results to overall averages (Table 1), and photos of sediment surfaces (Figure 10), some general observations were made about sediment type and productivity. Table 2 shows above and below average descriptions of productivity (growth and survival) and sediment qualities, surface cover, and shell hash type for each beach. Beaches A, C, and D which had higher than average survival and growth contained average to below average amounts of carbonates. Their surface sediments were covered in clam shells and the dominant form of shells in the sediment were whole clam shells and shell pieces. Beaches E and F which had below average growth and survival, had above average amounts of carbonates, organics, and silt. The surfaces of the sediments were covered in rocks covered in barnacles, and the sediment contained finely crushed shell hash. Beaches E and F had high percentages of organics (1.1 – 2.3 %) along with beach C (1.2 – 1.6 %), but beaches E and F had below average growth and survival compared to above average growth and survival at beach C. Beach B had above average survival, below average amounts of carbonates, was covered in rocks covered in barnacles, and the shells in the sediment were in small pieces. In summary, the least productive sediments in terms of survival and growth were high in carbonates, organics, silt, and finely crushed shells. The most productive beaches had sediment that contained average to below average amounts of carbonates and silt, were covered in clam shells, and contained whole clam shells and shell pieces, but was absent of finely crushed hash.

Table 1: Range and mean percentages of survival, growth, and sediment carbonate, organic, and grain sizes in all beaches.

Metric	Range (%)	Mean (%)
Survival	0 - 100	61 ± 32
Growth	0 - 317	135 ± 107
Carbonates	2 - 15	7.3 ± 3.8
Organics	0.6 - 2.3	1.2 ± 0.5
Rocks	3 - 25	15.3 ± 6.4
Small rocks	7 - 21	12.5 ± 4.1
Very coarse sand	6 - 19	12.5 ± 3.4
Coarse sand	9 - 21	13.7 ± 3.9
Sand	13 - 29	18.8 ± 4.9
Fine sand	11 -27	17.8 ± 4.8
Very fine sand	3 - 14	6.9 ± 3.6
Silt	0 - 5	2.4 ± 1.4

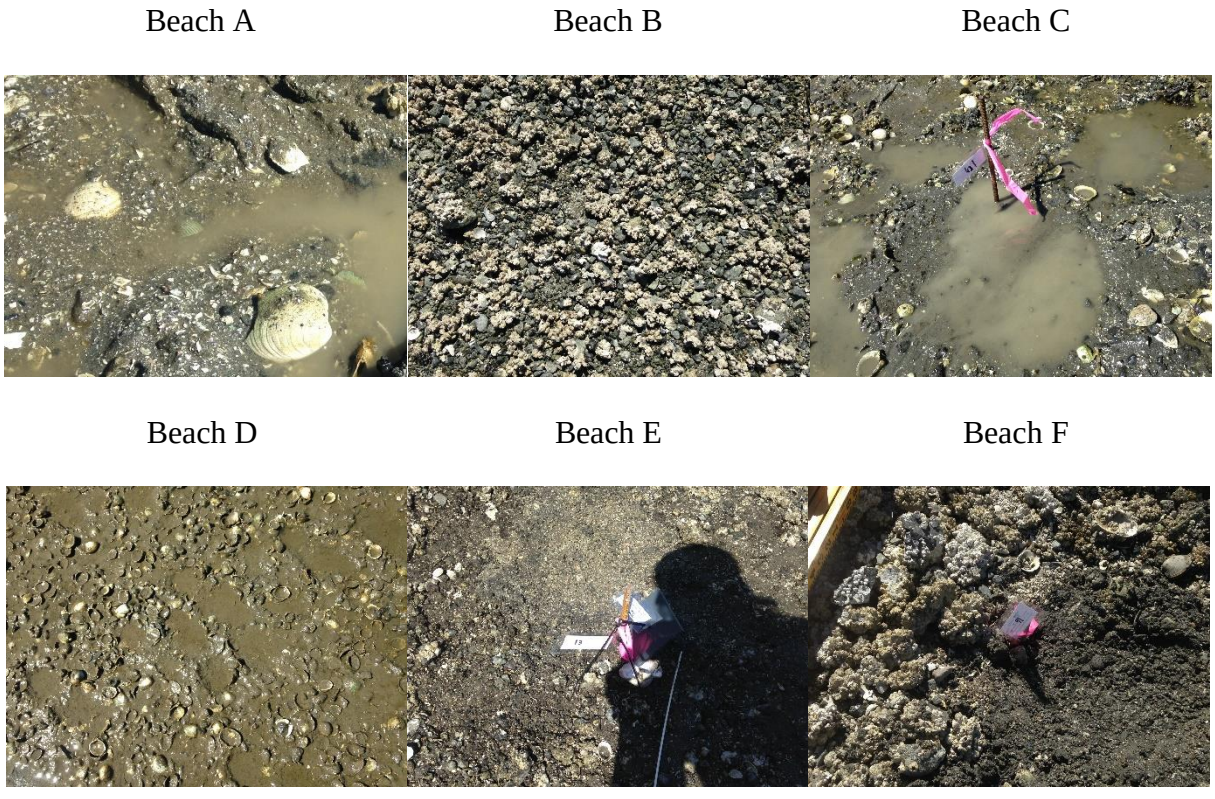


Figure 10: Photos of surface sediments of each beach (A – F)

Table 2: General beach descriptions from growth, survival, sediment analysis and visual observations. Beaches highlighted in green were the most productive with above average growth and survival.

Beach	Type	Above average [1]	Below average [1]	Surface appearance	Observed dominant form of shells in sediment
A	CG	growth, survival, coarse sand, sand, and fine sand	rocks , very fine sand, silt	rocks, clam shells	shell pieces
B	Ref	survival, very coarse sand, coarse sand, sand	carbonates, growth, sand, very fine sand	rocks covered in barnacles	shell pieces
C	CG	growth, survival, organics, rocks, fine sand	small rocks, very coarse sand	seaweed, clam shells	clam shells
D	Ref	growth, survival, very fine sand	carbonates, very coarse sand, coarse sand, sand	small clam shells	clam shells
E	CG	carbonates, organics, rocks, small rocks, very coarse sand, silt	growth, survival, sand, fine sand	rocks covered in barnacles	fine hash
F	Ref	carbonates, organics, small rocks, very fine sand, silt	growth, survival, rocks, coarse sand	rocks covered in barnacles	fine hash
		1: ≥ 1.5 % above or below average, except with organics above is 1 - 2 % organics, and below is < 1 % organics			

Gene Expression

A *de novo* transcriptome of the 28 libraries was assembled, resulting in 54,337 single gene transcripts based on protein homology and largest ORF. Small transcripts, < 100 bp, and sequences with keywords such as transposons, and long-terminal repeats, were filtered out for a final set of 52,000 transcripts of which 42,708 of these with Uniprot IDs.

Sequencing depth and coverage is dictated by the average numbers of reads that align to known reference bases and an increase in the number of reads (in the millions for RNA-Seq experiments) equals greater coverage of the transcriptome and greater confidence in the results (Sims et al. 2014). It is estimated that > 200 million paired-end reads are required to detect the full range of transcripts in human samples (Sims et al. 2014). A total of 3 billion paired-end reads were generated in this study equaling 305 billion nucleotides. A single lane of Illumina HiSeq4000 paired-end reads (2 x 100 bp) produced a mean of 762 ± 215 million reads. The total

reads/library ranged from 84 million to 159 million reads with a mean of 108 ± 19 million reads per library. There was a total of 14 gill and 14 digestive gland libraries sequenced (7 clam garden and 7 reference for each tissue type). Each library was constructed from a pool of five randomly chosen survivors from each plot. Due to financial constraints, it was not possible to sequence individual clams and therefore gene expression results are more from a population perspective than showing potential individual phenotypic plastic responses. There was a total of 1.50 billion gill reads, with a mean of 108 ± 22 million reads per library, and a total of 1.54 billion total reads from the digestive gland libraries, with a mean of 110 ± 17 million reads per library. The sequences ranged in length from 102 bp – 29, 916 bp. Base calling accuracy measured by the phred quality score was 38.9 ± 0.3, which indicates a base call accuracy of > 99.9%. After the data for each tissue was normalized to correct for differences in library sizes, multi-dimensional scaling (MDS) plots in edgeR were generated to determine the greatest source of variation between libraries and to identify outliers. MDS is a type of unsupervised clustering function in edgeR that plots the RNA samples in which distances correspond to leading log-fold-changes between each pair of RNA samples. MDS plots are visualizations of a principle component analysis (PCA). An MDS plot of all libraries combined (Figure 11) shows the gill libraries separate from the digestive gland libraries. There are two possible outliers (one from each tissue) from reference beach D but were not removed from the data analysis as these were from a plot and beach with high survival and there was no reason to conclude that the observed data response was not genuine.

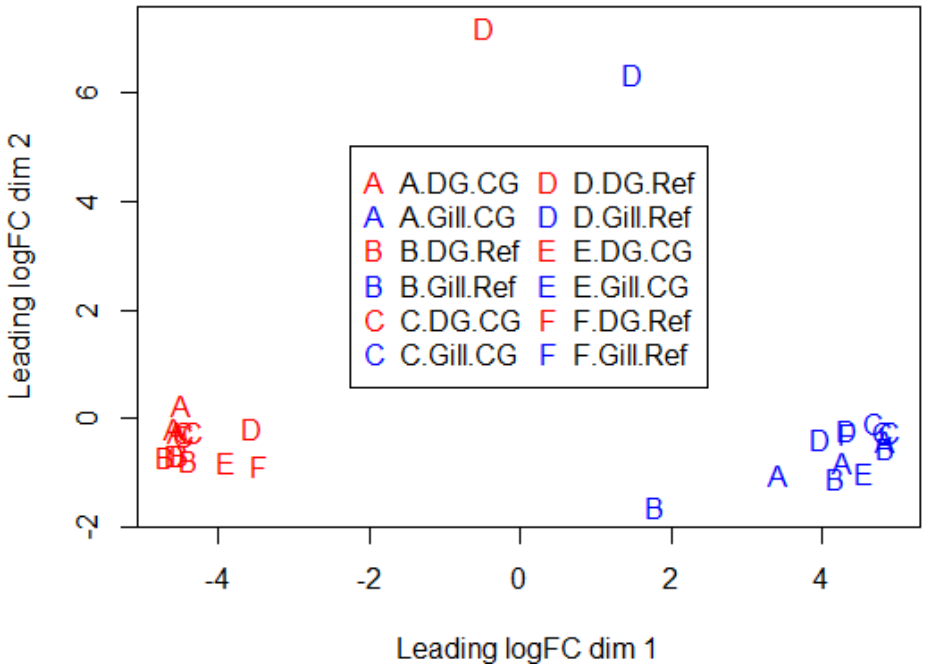


Figure 11: Multidimensional scaling of Littleneck clam gill and digestive gland tissue combined libraries. log FC; gene expression fold change. Beaches A, C, E are clam gardens and B, D and F are reference beaches.

A comparison of all the Uniprot IDs expressed in each tissue shows 11,673 Uniprot IDs (79 %) were shared between the two tissues, and 2,182 IDs (15 %) were uniquely expressed in only the gill libraries, and with 916 unique Uniprot IDs (6%) in the digestive gland libraries (Figure 12).

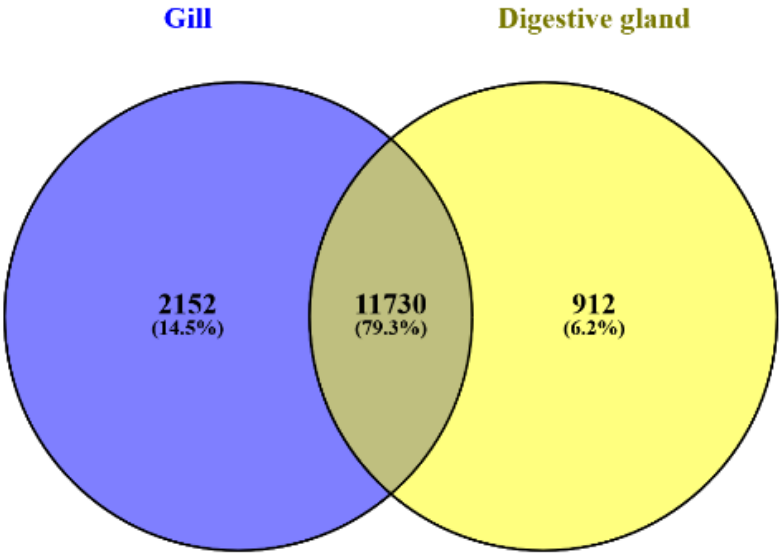


Figure 12: Comparison of unique and shared Uniprot IDs expressed in Littleneck clam gill and digestive gland libraries.

An MDS plot of the gill libraries (Figure 13) shows no evidence of clustering by beach location or beach type.

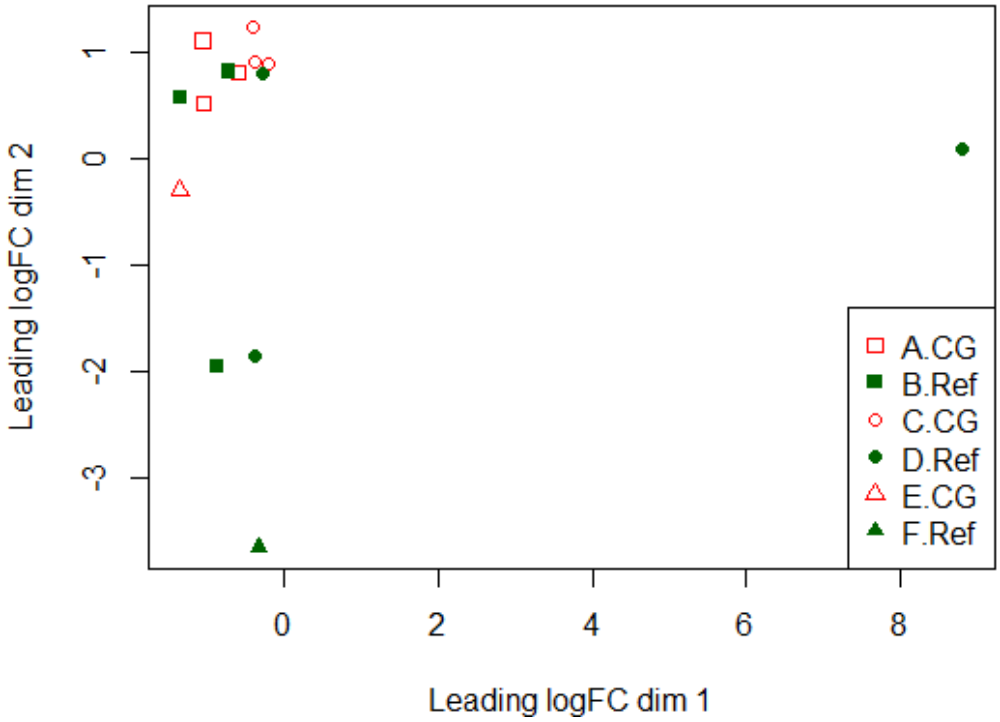


Figure 13: Multidimensional scaling of Littleneck clam gill gene expression libraries. log FC; gene expression fold change, A, C, E are clam gardens and B, D and F are reference beaches.

A digestive gland MDS plot (Figure 14), although shows no observable beach clustering, does show some potential clustering of clam garden and reference beaches.

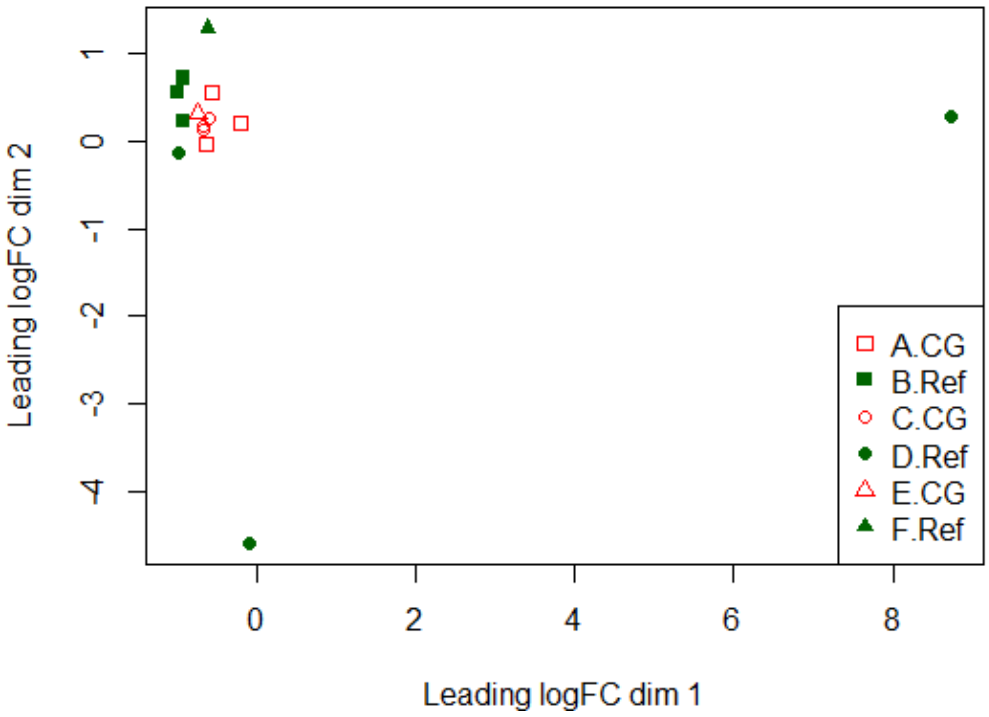


Figure 14: Multidimensional scaling of Littleneck clam digestive gland libraries in clam garden and reference beaches.log FC; gene expression fold change, A, C, E are clam gardens and B, D and F are reference beaches.

To examine physiological differences between Littleneck clams on clam garden beaches and reference beaches RNA sequencing data for each tissue (gill and digestive gland) were separately analyzed for significant differentially expressed genes. The sequencing depth of the treatment groups was well balanced with a total of 745 million reads in the gill clam garden group, and 761 million reads in the reference group. In the digestive gland there were 794 million reads in the clam garden group and 746 million reads in the reference group. The ability to find transcripts and detect differential expression is very much determined by the sequencing depth (Tarazona et al. 2011). For differential expression analysis balanced sequencing depth between conditions is advisable as greater sequencing depth identifies more transcripts and the

expression values are higher which leads to more accurate estimations of gene expression (Tarazona et al. 2011).

To analyze sequencing data for differentially expressed genes in each tissue between the two types of beaches ANOVAs of generalized linear models (GLMs) were run using the edgeR package in the R software environment. A heat map (Figure 15) of the most significant 75 DEGs ($p < 0.001$) in gill with genes (rows) and libraries (columns) showed evidence of significant differential expression between the two groups.

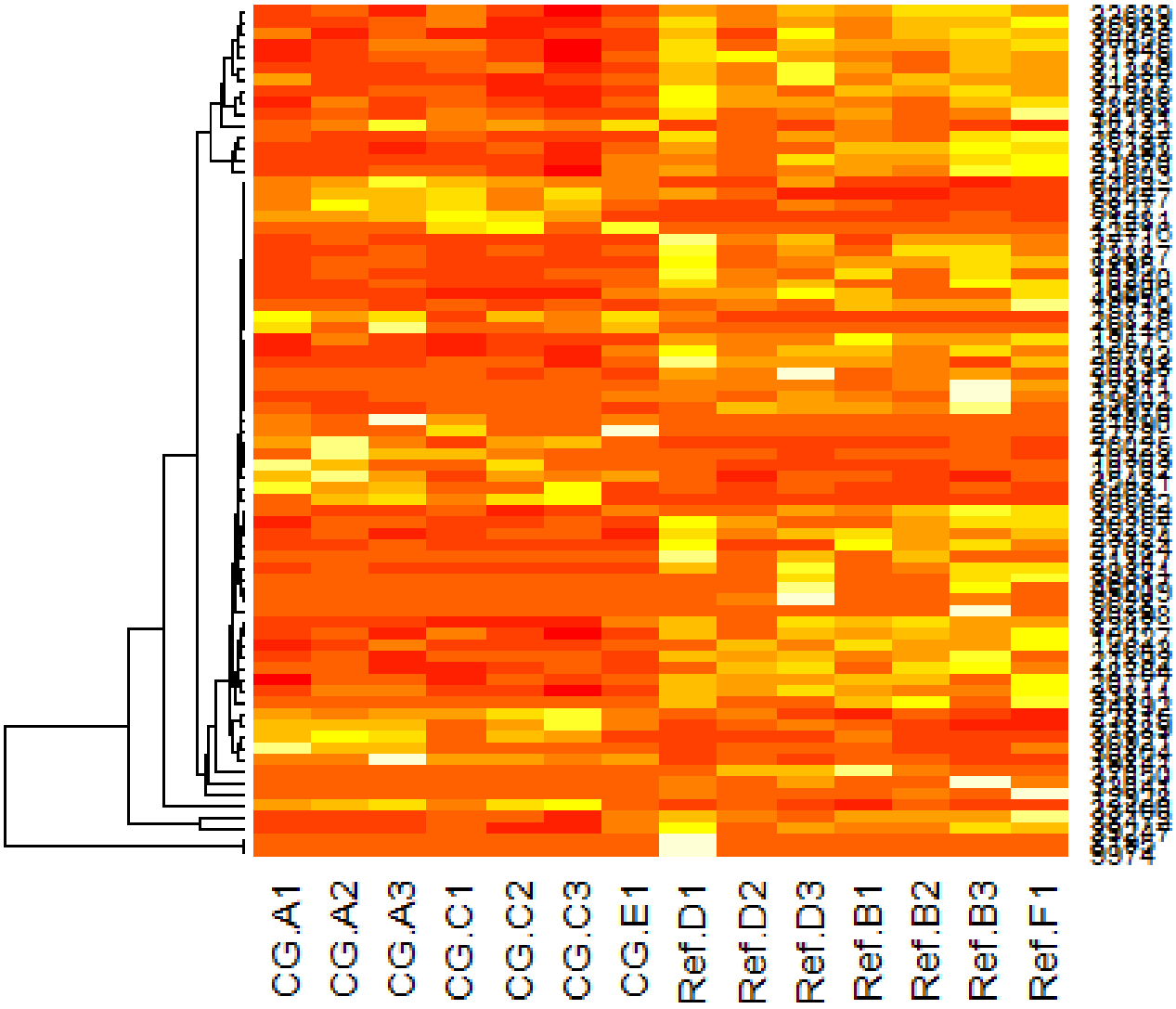


Figure 15: Heatmap of the top 75 Littleneck clam gill differentially expressed genes in clam garden and reference non-walled beaches. Yellow/white bands are upregulated genes, and orange/red bands are downregulated genes.

A heatmap of the 75 most significant DEGs ($p \leq 0.001$) in the digestive gland (Figure 16) also showed evidence of significant differential expression between the clam garden and reference beach libraries with a noticeable upregulation in the digestive glands of the reference beach libraries.

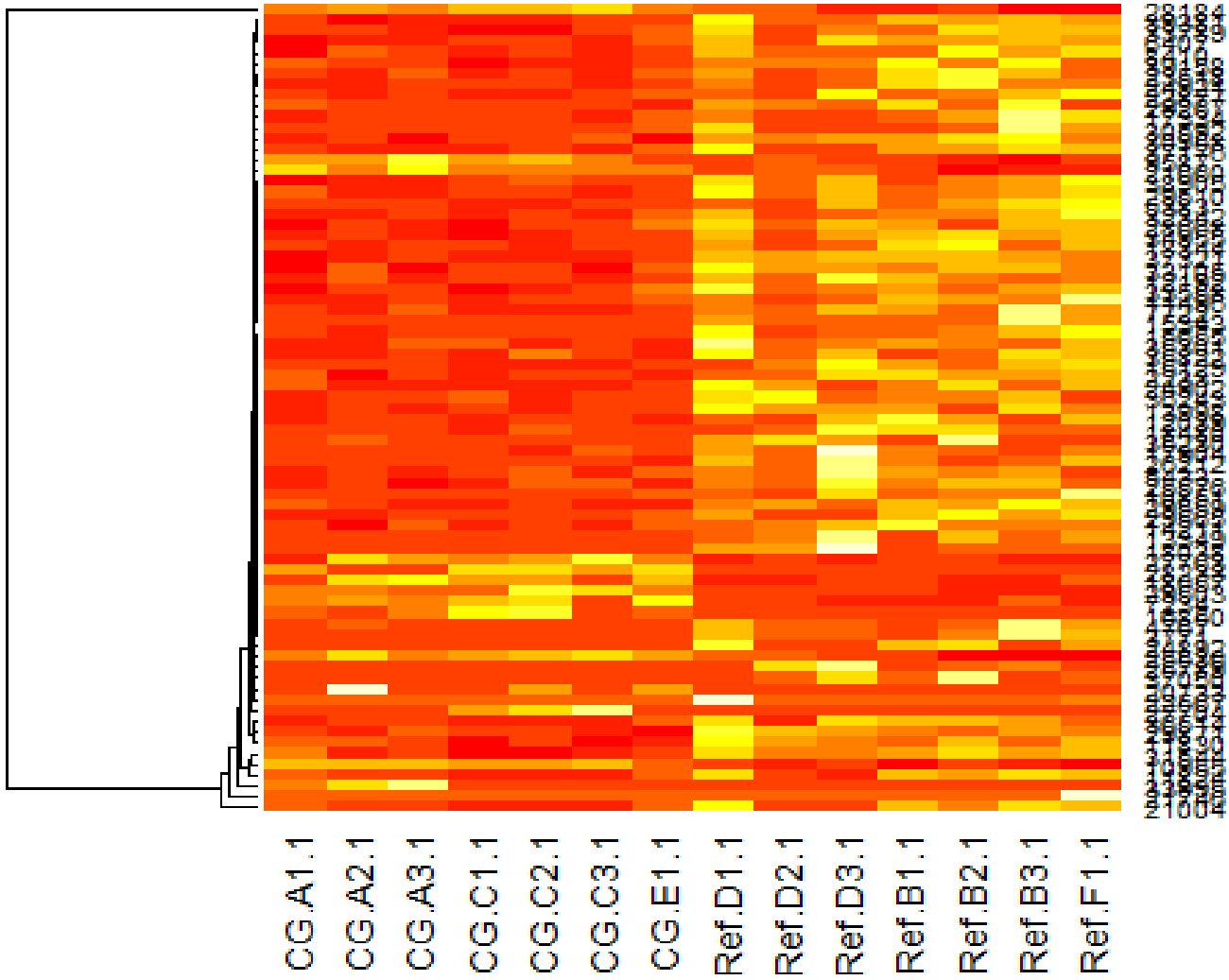


Figure 16: Heatmap of the top 75 Littleneck clam digestive gland differentially expressed genes in clam garden and reference non-walled beaches. Yellow/white bands are upregulated genes, and orange/red bands are downregulated genes.

Further analysis of the differentially expressed genes in the gill using a cut off probability value of 0.015 for each individual transcript and a FC $\geq \pm 2$ revealed 661 DEGs, of which 103 were upregulated in the clam gardens, and 558 were downregulated in clam gardens. In the

digestive gland libraries using the same cut off criteria revealed that there were 643 significantly DEGs, of which 110 were upregulated, and 507 were downregulated in the clam garden libraries. To analyze significant DEGs for biological interpretations, up (positively expressed) and down (negatively expressed) regulated genes for each tissue were analyzed separately using the DAVID enrichment analysis tool (Huang et al. 2007). In the gill libraries the significant ($p \leq 0.05$) biological process protein ubiquitination, and molecular functions ligase activity and ubiquitin-protein transferase activity were upregulated in the clam garden libraries compared to the reference beach libraries (Table 3). Protein ubiquitination, also known as the "kiss of death", tags proteins with ubiquitin for degradation in the proteasome and also plays a major role in apoptosis. It also regulates a number of other biological functions including DNA repair, and transcriptional activation and silencing, therefore affecting virtually all aspects of cell biology including division, growth, communication, signaling, movement, and cell death (Johnson 2002, Pickart and Eddins 2004, Sun and Chen 2004). Protein ubiquitination is essential for many physiological processes by selective degradation of target proteins, and its upregulation in the gills from clam garden clams indicates cellular physiological differences between the clam garden and reference beach clams; however, the specific implications of this requires more investigation.

Table 3: Biological process and molecular functions upregulated in Littleneck clam gill tissues from clam gardens.

Category	**Term**	**count**	***p value***
Biological process	GO:0016567~protein ubiquitination	4	0.004
Molecular function	GO:0016874~ligase activity	4	0.002
	GO:0004842~ubiquitin-protein transferase activity	4	0.004

The significant downregulated biological processes in the gill clam garden libraries were DNA repair, and establishment of planar polarity. Planar polarity establishes correct patterning and polarization of epithelial and mesenchymal cells and is essential for morphogenesis and function of all organs and organisms (Carvajal-Gonzalez and Mlodzik 2014). The downregulated molecular function in this case was transmembrane receptor protein tyrosine phosphatase activity and the clustered ontology terms were post-translational modification, protein turnover, and chaperones (Table 4). Post-translational modifications are protein modifications following protein synthesis and chaperones assist in protein folding.

Table 4: Biological process, cellular component, molecular function, and pathways downregulated in Littleneck clam gills from clam gardens

Category	**Term**	**count**	***p value***
Biological process	GO:0006281~DNA repair	8	< 0.001
	GO:0001736~establishment of planar polarity	3	0.004
Molecular function	GO:0005001~transmembrane receptor protein tyrosine phosphatase activity	3	0.004
COG_ONTOLOGY	Posttranslational modification, protein turnover, chaperones	5	0.012

A closer examination of the most significantly differentially expressed genes between clam garden and reference gill libraries and their biological processes shows several genes involved in redox, adhesion, apoptosis, immune response, viral entry, and signaling upregulated in the reference libraries. In the clam garden libraries genes involved in signaling, adhesion, and innate immunity for upregulated. These biological processes indicate that the reference beach clams were possibly virally challenged. An upregulation of genes involved in adhesion, signaling and innate immunity in the gills indicate the clam garden clams were also biotically or abiotically stressed (Table A12).

In the digestive gland the only upregulated enriched molecular function ($p \leq 0.05$, count ≥ 3) in the clam garden libraries was catalytic activity (4 transcripts) and 3 of these upregulated transcripts are involved in the biosynthesis of antibiotics KEGG pathway (Table 5).

Table 5: Biological processes upregulated in Littleneck clam digestive glands from clam gardens.

Category	**Term**	**count**	***p value***
Molecular function	GO:0003824~catalytic activity	4	0.017
KEGG_PATHWAY	mmu01130:Biosynthesis of antibiotics	3	0.011

The downregulated biological processes and molecular functions in the digestive gland clam garden libraries were receptor-mediated endocytosis, sprouting angiogenesis, calcium ion binding, Rab GTPase binding, and scavenger receptor activity (Table 6). Angiogenesis is the growth of blood vessels and would be upregulated in times of growth, and wound healing. The downregulation of heatshock protein 90 by 95-fold in the clam garden clams, which corresponds to an upregulation in the reference beach clams, indicates that the angiogenesis process upregulated in the reference beaches could be a result of cellular damage.

Table 6: Gene ontology terms downregulated in Littleneck clam digestive glands in clam gardens

Category	**Term**	**count**	***p value***
Biological process	GO:0006898~receptor-mediated endocytosis	7	0.001
	GO:0002040~sprouting angiogenesis	3	0.011
Cellular component	GO:0016020~membrane	26	0.002
	GO:0005615~extracellular space	17	0.009
	GO:0005887~integral component of plasma membrane	17	0.014
	GO:0000139~Golgi membrane	10	0.011
	GO:0043202~lysosomal lumen	5	0.002
	GO:0005604~basement membrane	4	0.013
Molecular function	GO:0005509~calcium ion binding	14	< 0.001
	GO:0017137~Rab GTPase binding	5	0.009
	GO:0005044~scavenger receptor activity	4	0.003

Analysis of DEGs between beaches with high and low survival revealed several significantly highly upregulated (> 400 FC) viral transcripts associated with factors, such as geographic location and survival (Table 7). Beaches A, C, and D were geographically nearby to each other and had higher survival. Beaches E, and F were side by side, on the far side of the bay from the other beaches and had low survival. The extensive sequencing depth of these libraries (84 – 159 million reads per library) allows for the potential of this data to be mined for complete viral transcripts. The number of base pairs sequenced from each of these viral transcripts ranged from 2733 – 7371 bp. Of the 15 significantly differentially expressed viral transcripts identified, only one was expressed in the gill and the others were all in the digestive gland. There were seven uniprot identified viral transcripts identified from six different viruses. These unique Uniprot IDs coded for structural (capsid) and replicase viral polypeptides.

The majority of the highly upregulated viral transcripts found were positive sense single stranded RNA viruses from the family Dicistroviridae (Drosophilia C virus, Cricket paralysis virus, and acute bee paralysis virus). Many of these viruses have been characterized after crashes of lab insect colonies. They have a broad host range, primarily infect the gut, have a poly A tail, and Internal Ribosome Entry Site (IRES) which allows them to enter host ribosomes to be translated in proteins. Most Dicistroviridae result in subtle disease (reduced longevity & fecundity), and some result in relatively rapid paralysis. There is currently limited research on host population and ecology impacts. In the sequencing data there were also differentially expressed single transcripts from feline calicivirus, cherry raspberry leaf, and nora virus.

The results of the gross observational data (following the National Animal Aquatic Health Program protocols) showed that the majority of the survivors on beaches A,B,C,D (which had $\geq$ 68 % survival) were healthy looking with medium or average tissue fatness, normal mantle recession, absent of conchiolin (internal shell) deposits, lesions, and had normal digestive gland colouration. Fourteen percent of these survivors had pale digestive glands. The survivors from beaches E and F, which had $\leq$ 35 % survival, appeared weak, emaciated and/or watery, with 71 % of them having slightly to extremely pale digestive glands.

Table 7: Viral genes significantly upregulated in Littleneck clam gill (Gill), and digestive gland (DG) tissues

Tissue	Beaches	% Survival	Uniprot [BP]	Gene ID	Stored sequence from	bp
Gill	C/D, A	81 ± 13	O36966 [1,2]	replicase polyprotein (RNA directed RNA polymerase)	Drosophila C virus	5070
DG	C/D, A	81 ± 13	Q9IJX4 [1,2]	nonstructural polyprotein (CrPVgp1)	Cricket paralysis virus	5619
DG	C/D, A	81 ± 13	O36966 [1,2]	replicase polyprotein (RNA directed RNA polymerase)	Drosophila C virus	4980
DG	C/D, A	81 ± 13	P13418	structural capsid polyprotein (CrPVgp2)	Cricket paralysis virus	7371
DG	A/C	78 ± 16	P27406	capsid protein	Feline calicivirus	1479
DG	E/F/B	41 ± 33	P13418	structural capsid polyprotein (CrPVgp2)	Cricket paralysis virus	2733
DG	E/F	25 ± 23	Q9DSN9 [1,2]	replicase polyprotein (RNA directed RNA polymerase)	Acute bee paralysis virus	3648
DG	E/F	25 ± 23	Q9DSN8	capsid protein (ABPVgp2)	Acute bee paralysis virus	2685
DG	D	87 ± 6	Q27YG9	replication polyprotein	Nora virus	6453
DG	E	17 ± 25	Q6EWG9 [1,2,3]	RNA1 polyprotein	Cherry rasp leaf virus	5910

BP: Biological Processes, 1: GO:0006351~transcription, DNA-templated, 2: GO:0039694~viral RNA genome replication, 3: GO:0018144 ~ RNA-protein covalent cross-linking

Throughout the sequencing data there were multiple transcripts with the same gene name and Uniprot ID but different lengths and sometimes expression patterns. For example, in the digestive gland two transcripts of prosaposin of different lengths (375 bp and 330 bp) were down regulated in the clam garden libraries. Aligning the two prosaposin sequences using the National Centre for Biotechnology Information (NCBI) database revealed that the two nucleotide sequences overlapped 59 %, and when each translated into protein sequences they overlapped 79 %. Multiple viral transcripts of different lengths with the same Uniprot ID overlapped only 2 – 6 % when the nucleotide sequences were aligned and overlapped 16 – 46 % when each sequence was first translated into protein sequences and then aligned and compared. These transcripts possibly represent homologs (genes with shared ancestry), spliced isoforms (alterations in splicing RNA), or variations among members of a gene family (similar genes).

2.4. Discussion

This study compared the physiology of Littleneck clams by means of growth, survival, and gene expression, following transplantation onto unmanaged clam garden and non-clam garden, reference beaches in Kanish Bay, Quadra Island, B.C., for 16 weeks from the beginning of May to the end of August in 2016. The sediment from each plot of clams was analyzed for grain size, carbonates, and organic content, and comparisons were made between clam garden and reference beaches in terms of growth, survival and sediment characteristics.

The null hypothesis of sediment carbonate and organic content do not have any effect on clam growth and survival was also unsupported. In our data there was a significant negative linear relationship between percentage carbonates in the sediment and clam survival. The beaches that had the highest percentage carbonates in their sediment had the poorest growth and survival, and the beaches with the lowest sediment carbonate contact had the highest growth and survival. This provides evidence that high concentrations of shell hash do not always benefit clam survival and growth, and is supported by recent research which observed that acidic sediment conditions were not influenced by shell hash content, suggesting that augmenting coastal sediment with shell hash is potentially not a suitable mitigation measure for ocean acidification (Doyle 2016). However, the most corrosive time of year in terms of aragonite saturation is during the winter months, so future studies investigating beneficial affects of carbonate should investigate influences of sediment parameters on full reproductive cycles of clams. Sediment organic content was ≤ 2.3 % in all beaches and although the least productive beaches had the highest organic sediment content, percent organics did not have a significant relationship between percentage growth or survival, nor relationship between organic content and beach type (clam garden or reference). The organic enrichment (> 10 % of sediment dry

weight) of sediments (eutrophication) can have adverse effects such as sediment oxygen depletion and benthic community alterations. These negative impacts are greater in areas with poor water and current flow and less in areas with greater wave action and tidal flushing. Our beaches were within the normal range of most coastal areas (< 5 %) and are not considered organically enriched.

Optimal clam habitat can be influenced by many variables. Temperature is an important environmental variable affecting metabolic performance. A comparison between clam garden and reference beaches found that clam garden sediment experienced less temperature variability, and was cooler in the summer months, and warmer in the fall than reference beaches (Salter 2018). Other important variables which affect clam density were water residency, sediment carbonate and silt content, and aragonite saturation (Salter 2018). In terms of clam biomass, this study found that temperature and carbonate content were the most important factors followed by silt, water residency, and aragonite saturation state. In our study the most productive beaches in terms of growth and survival in general had low amounts of carbonates, silt, and organics, were covered in whole and broken clam shells, and the sediment contained whole shells or shell pieces. The least productive beaches had high carbonate content, silt, organics, were covered in rocks covered in barnacles, and the sediment contained finely crushed shell hash. There were no observable correlations with survival, growth and beach exposure. Our results are therefore contrary to the previously mentioned study that found higher clam growth in beach plots with the addition of crushed shell hash (Salter 2018). Buffering sediments with crushed shell hash has also been shown to increase porewater calcium-carbonate saturation state and increase bivalve recruitment 3-fold in a 30-day study, by raising the pH and saturation state of surface sediments (Green et al. 2012). However, results are still inconclusive as a two-year study on *Venerupis*

philippinarum clams found that average daily growth of early post-settlement clams was negatively correlated with sediment carbonates and organics (Munroe 2016).

The use of shell hash for buffering sediments and enhancing clam habitats can have negative ecological implications. A study on habitat complexity and olfactory cues augmented soft-sediment roughness around experimental clam (*Mercenaria mercenaria*) plots and found whelk predation on clams surrounded by shells pieces was significantly higher than on clams surrounded by natural sediments (Ferner et al. 2009). This observation was attributed to whelk hunting behaviour, where bottom roughness increased mixing of prey chemicals or disrupted prey ability to detect and respond to predator odors (Ferner et al. 2009). Also, the addition of fine shell hash could have negative effects as it may blanket and smother the sediment preventing respiration of the benthic flora and fauna (Shahidul Islam and Tanaka 2004), thereby altering benthic community diversity and abundance. The scale of beneficial additional carbonates for corrosive environments is likely to be on the microscale and only during times of corrosivity.

The null hypothesis no physiological differences between Littleneck clams on clam garden beaches compared to reference beaches was unsupported, as heatmaps of differentially expressed genes from RNA sequencing data showed evidence of significant differential gene expression in both gill and digestive glands tissues. However, there was no observable significant differences between clam garden and reference beaches in terms of growth, and survival, which may be a function of the relatively short duration of this experiment. Therefore, the null hypothesis of no differences between clam garden and reference beach growth and survival was found to be supported in this study. This finding is in contrast to an earlier study

where clam garden beaches had higher survival and growth than non-clam garden beaches (Groesbeck et al. 2014).

In the clam garden libraries there was significant ($p \leq 0.001$, FC $\geq \pm 2$) upregulation of several biological pathways responsive to environmental stressors, such as protein ubiquitination in the gill tissues and in the digestive glands cell an upregulation of genes involved in catalytic activity and synthesis of antibiotics. Protein ubiquitination, the "kiss of death", marks proteins for destruction, and is an important component of the immune response, upregulated in response to viral infections (de Lorgeril et al. 2011). Oysters exposed to *Vibrio* infection, oyster mass mortality syndrome, and anthropogenic impacts all had an upregulation of genes involved in immune response, innate immunity, apoptosis, and protein degradation (de Lorgeril et al. 2011) (Chaney and Gracey 2011, Gavery and Roberts 2012) . The knowledge that the clam garden clams contained highly expressed viral transcripts combined with the upregulation in immune, apoptosis, and protein ubiquitination pathways further supports that these clams were physiologically stressed at the cellular level and were responding immunologically to their environment.

Many of the biological pathways upregulated in the reference beach libraries also play roles in responses to environmental stressors. There was an upregulation of biological pathways associated with DNA repair, establishment of planar polarity, post-translational modification, protein turnover, and molecular chaperones in the gills, and an upregulation of receptor-mediated endocytosis, scavenger receptor activity, and sprouting angiogenesis in the digestive glands. DNA repair elements are part of the immune system and are upregulated in response to viral infections (de Lorgeril et al. 2011). Outbreaks of harmful algal blooms (HABs) are predicted to increase in future climates, due to changes in ocean temperature, pH and nutrients. Okadaic acid

the active constituent in Diarrhetic Shellfish Poisoning (DSP), and is a biotoxin produced during HABs. It also has genotoxic properties causing DNA oxidative damage, and double stranded DNA breaks, potentially upregulating DNA repair mechanisms involved in the maintenance of genome integrity (Suarez Ulloa 2017). Saxitoxin, the principal phytotoxin contributing to paralytic shellfish poison (PSP), is largely produced by the marine microalgae of the genus *Alexandrium*, which causes massive deaths in fish and other marine species whereas marine bivalves can accumulate this toxin. The transcriptomic response of Chilean mussels, *Mytilus chilensis* hemocytes exposed to saxitoxin involves an upregulation of genes involved in mussel immunity (Toll-like receptors, tumour necrosis factor receptors, scavenger-like receptors), apoptosis (apoptosis inducible factor), signaling, and protein processing (Detree et al. 2016). Physiologically PSP may induce a decline in reproduction and growth rates in marine bivalves and cause a reduction in filtration, feed and respiration. (Detree et al. 2016). The exposure of animals to xenobiotics (chemically synthesized unnatural compounds, *i.e*. pharmaceuticals, halocarbons, polychlorinated biphenyls) causes oxidative damage and either induces or inhibits antioxidant enzyme activity, and immunological defenses depending on the animal tissues and species (Milan et al. 2011). Transcriptomic responses from xenobiotic exposure involve genes with roles in antioxidant defense, drug metabolism and detoxification (glutathione S-transferase, cytochrome P450, sulfotransferase) (Milan et al. 2011). Some contaminants may also cause immunosuppression increasing an organisms susceptibility to disease (Milan et al. 2011). Planar polarity is the process by which a cell develops a specific orientation with the plane of the epithelium, critical for multiple developmental processes and defects are capable of causing developmental impairment and disease (Vladar et al. 2009, Hazelwood and Hancock 2013). Receptor mediated endocytosis is part of the immune system responsible for the uptake,

sequestering, and presentation of foreign antigens into the cell and exploited by pathogens to modulate the immune system (Mak and Saunders 2006). Scavenger receptors are cell surface receptors that bind and internalize ligands, or modified lipoproteins, and play critical rolls in innate immunity, apoptotic cell clearance and tissue homeostasis (Greaves and Gordon 2005). Sprouting angiogenesis is fundamental in many biological processes including development, reproduction, and wound repair (Guerra et al. 2018). The upregulation of several biological pathways involved in immunity, DNA and wound repair, apoptosis, redox, and stress, again combined with the evidence of viral presence in most beaches is evidence that the clams from the reference beaches were most likely experiencing immunological and potentially other biotic (virus, and HABs) and abiotic (xenobiotic) stressors.

Although there was evidence of significantly differentially regulated biological processes between the clams from the two types of beaches, showing cellular physiological differences, most of the enriched pathways upregulated in both groups of clams were processes associated with responses to environmental stress. Transcriptomic responses to environmental stresses are complicated and difficult to interpret with single or few biological pathways (Li et al. 2013). Marine bivalves inhabit variable and unstable conditions, and as filter feeders in the intertidal zone bivalves potentially experience multiple biotic and abiotic stressors including pathogens, periodic hypoxia, hyposaline, temperature fluctuations caused by natural and sometimes extreme tidal cycles and anthropogenic influences such as pollution and climate change (Li et al. 2013). These stressors trigger a series of response mechanisms to restore cellular homeostasis which include molecular chaperones in protein folding, xenobiotic detoxification, antioxidant system, apoptosis, and immune, and osmotic stress response (Zhang et al. 2016). All the clam libraries in this study showed a significant upregulation of different viral entry and response genes. The

upregulation of these processes in both the clam garden and reference beach clams, high amounts of viral gene expression, and the upregulation of different genes with similar biological processes likely represents individual and population differences in cellular physiological responses. Perhaps the presence of the wall, which has been shown to lower temperatures and cause higher water residency in clam garden beaches, influences the genetic responses of clams compared to those on reference beaches (Salter 2018). More investigation is needed at this point to attempt to differentiate the stressors in the beaches and whether the differences in transcriptional responses are a result of beach type.

Genetically identical individuals exposed to the same environment and conditions can have different phenotypes (Lin et al. 2016). This variability, considered a response to microenvironmental changes, is termed microenvironmental plasticity and is due to individualized gene expression profiles (Lin et al. 2016). A study examining 730 individual *Drosphilia menlanogaster* of 16 fixed genotypes found that this plasticity in gene expression was dependent on genotype and sex (Lin et al. 2016). In our study we sequenced pools of five randomly selected surviving individuals from each plot and with four of the six beaches we sequenced three libraries (plots) of five survivors per beach; however, with beaches E (clam garden), and F (reference beach) there was only one plot from each beach that had five survivors to sequence. Pooling of samples was necessary due to financial considerations and therefore these gene expression results are more on a population level and cannot account for one individual's responses that may have showed significant differences in gene expression levels compared to its plot companions. Therefore, by sequencing pools of individuals from the same plot this study provided with an average gene expression profile for each plot. The additional sequencing of more biological replicates dramatically increases the ability to detect genes with

small fold-changes (Schurch et al. 2016) and so for this reason this study examined only differentially expressed genes with ≥ 2-fold-changes for population level responses. In our study, the collection of the clams from one population at one beach was conducted to reduce background genotypic variation that may exist between Littleneck clam populations from different locations due to local adaptation and selection. However, the movement of clams from one originating beach to other locations may have also affected their responses to a new environment; other sediment samples and clam tissues from clams from the originating beach that were not transplanted could have been sequenced and analyzed to examine if this was a significant influence or not. A further study for an extended time period encompassing a full reproductive cycle is also recommended.

Whole transcriptome analysis can reveal underlying cellular and organismal responses to physiological and pathological stresses (Milan et al. 2011). Reference based transcriptome assembly identifies genes that match a previously sequenced reference sequence. *De novo* assembly allows for the assembly of transcriptomes from non-model organisms where no reference sequence exists. Comparing differentially expressed genes from RNA-Seq data assembled *de novo* or reference based genomes reported seven times more differentially expressed genes with *de novo* assembly (Gavery and Roberts 2012). *De novo* assembly results in the discovery of novel transcripts and multiple sequences with the same gene identity (Gavery and Roberts 2012). Sequence alignment of multiple clam and viral transcripts with the same Uniprot ID were significantly differentially expressed in the digestive glands of clam garden clams, showed their nucleotide sequences overlapped 2 - 59 %; when translated into protein sequences the sequences had alignments of 16 - 79 %. These transcripts possibly represent homologs, spliced isoforms, or variations among members of a gene family. The sequence read

ends are trimmed before transcriptome assembly because they may contain adaptor sequences and low-quality reads and results in a transcriptome that more accurately represents the original sequence. As all the genes in the background are used for enrichment analysis and *de novo* assembly provides a more accurate representation of the background genes, *de novo* -based enrichment analysis is possibly more biologically relevant (Gavery and Roberts 2012).

The third null hypothesis was there are no significant differences in clam cellular physiology at different geographic locations was again rejected. Another interesting discovery in our RNA sequencing data was evidence of biotic stressors that could impact clam gene expression and physiology. A survey of transcripts of significantly differentially expressed in clams from high and low survival groups revealed several highly upregulated ($\sim \geq 400$ FC) viral transcripts. These genes were involved in viral genome replication, transcription, and capsid formation. Most of these transcripts were from positive sense single stranded RNA viruses from the family Dicistroviridae in the order Picornavirales. Eight such transcripts were found in the digestive gland tissues and one transcript in the gill tissues. Geographically adjacent beaches contained the same viral transcripts, and beaches farther from all the other beaches that were more exposed (in terms of waves and weather) had the least number of viral transcripts. The clams from beaches that had high growth and survival had transcripts from the genus Cripavirus (Cricket paralysis virus and Drosophila C virus), and a Picornaviridae-like virus (Nora virus). Clams from beaches that had poor growth and survival contained transcripts from the genus Aparavirus (Acute bee paralysis virus), and Cripavirus (Cricket paralysis virus). The Dicistroviridae family formerly known as “cricket paralysis-like viruses” are widely distributed in nature with the broadest host range of all invertebrate small RNA viruses (Bonning 2009). Their genome is 8.5 to 10.2 kb in size and contains two open reading frames (ORF) of

approximately 5,500 and 2,600 nucleotides. The first ORF encodes for non-structural proteins (helicase, protease and RNA-dependent RNA polymerase) and the second ORF encodes the structural proteins (capsid proteins) (Bonning 2009). As the Dicistroviridae viral sequences isolated in our study ranged in size from 1479 – 5619 nucleotides we can deduce that they represent viral transcripts, mRNA to be translated into viral replicase and capsid proteins to support viral replication, instead of viral genomes. The viral genome is infectious and can function as a genome and as mRNA to be translated into proteins to support viral replication immediately upon infection. When sufficient positive-stranded RNA progeny and virion proteins have accumulated, virions are assembled and released upon cell lysis (Bonning 2009). Dicistroviruses, in the vinegar fly (*Drosophila* melanogaster), primarily infect the gut and enter cells via clathrin-mediated endocytosis (Bonning 2009). As evident in our data, some dicistroviruses can be non-pathogenic or cause subtle disease (reduced longevity and fecundity) while others result in rapid paralysis (Bonning 2009). Nora virus, which was detected in clams in a beach with high growth and survival, is a persistent non-pathogenic virus of *Drosophila melanogaster* (Lopez et al. 2018). Littleneck clams from beaches that had high growth and survival did not exhibit any observable signs of pathophysiology, however 71 % of the clams from beaches with poor growth and survival were weak, emaciated or watery, and had slightly to extremely pale coloured digestive glands which could also indicate parasitic infection. Parasites such as paramyxean, and percinosis are responsible for disease in marine mollusks and infection is linked to digestive tropism interfering with food adsorption and causing pale-yellowish digestive glands with thin watery flesh in mussels and oysters (Carella et al. 2011).

A survey of the background genes (lists of all transcripts sequenced) from the gill and digestive gland for viruses, bacteria and parasites revealed an abundance of sequences.

Background gene lists were searched with the following keywords; ‘virus’, ‘bacterium’, ‘trypanosoma’, and ‘vibrio’. In the gill background gene list there were in fact, 86 ‘virus’ transcripts (50 Uniprot IDs), from 29 different viruses as well as 21 *Mycobacterium* transcripts (14 Uniprot gene IDs), from 10 different *Mycobacterium*, 15 *Vibrio* transcripts (7 Uniprot IDs) from 6 different *Vibrio* species, and 9 *Trypanosoma* (parasite) transcripts from three different species. In the background digestive gland gene list there were 99 ‘virus’ transcripts (53 Uniprot IDs), from 31 different viruses. There were also 15 *Vibrio* transcripts, 5 *Trypanosoma* transcripts, and 38 *Mycobacterium* transcripts in the digestive gland background gene list.

RNA sequencing technology has revolutionized virus detection and allows for the screening of a broad range of symptomatic and asymptomatic virus species in a non-discriminatory manner (Nagano et al. 2015). In our study, the *de novo* transcriptome assembly was able to detect novel transcripts and allowed for the detection of highly upregulated viral transcripts associated with high and low survival. Deep sequencing of the libraries which enables the sequencing of genes with low expression allowed for the detection of an abundance of transcripts from different viruses, bacteria, and parasites in the background gene lists. The significant upregulation of genes involved in immune, defense and viral response, interferon, and apoptosis in the digestive glands of most clams, and of genes involved in viral entry in the gills indicates that most of clams were allocating energy to defend against a biotic stress. In future studies RNA sequencing with high numbers of viral transcripts, combined with tissue histology and gross observational data of disease presence, would further elucidate the viruses and their pathogenicity and the presence of other potential biotic stressors (parasites or bacteria).

In conclusion we did not find any significant differences in sediment between clam garden and reference beaches. The only sediment characteristic that had a significant correlation

with growth or survival was sediment carbonate content. We found that percentage of carbonates in the sediment was negatively correlated with survival. In our study the most productive sediments in terms of clam growth and survival contained low amounts of carbonates, organics, silt, and fine shell hash. The most productive beaches were covered in whole or pieces of clam shells. The less productive beaches were covered in barnacles and contained finely ground shell hash. We also did not find significant differences in growth, or survival between clams transplanted on clam garden and reference beaches after 16 study weeks from the beginning of May to the end of August 2016. Although we found significantly differentially expressed genes after 16 weeks between clam garden and reference beach clam gill and digestive gland tissues, many genes were involved in similar biological processes. With the presence of highly expressed viral transcripts in the clams from all the beaches it cannot be determined in this study if the significant differences in gene expression between clam garden and reference beach clams were because of beach type or, which is more likely, a combination of multiple environmental influences such as geographic location, transplantation stress, sediment type and viruses. Finally, our data shows the ability of RNA sequencing to detect, and potentially obtain, complete viral RNA transcripts of viruses associated with geographic location and survival and may also provide a genomic resource for the future classifying of bivalve viruses.

Chapter 3: Impacts of microplastic fibers on Pacific oyster *(Crassostrea gigas)* physiology

3.1.1. Introduction

Human activities are responsible for increasing levels of debris pollution in the marine environment (Desforges et al. 2014), where increases in human population densities have been positively related to microplastic abundance (Browne et al. 2011). Since the mid-1950's annual global plastic production has increased steadily to 322 million tonnes in 2015 (PlasticsEurope 2016), of which 10% is estimated to ultimately end up in the oceans (Cole et al. 2011). These production values may underestimate plastic pollution, as they do not include polyethylene-terephthalate, polyamides, polypropylene, and polyacrylic fibers. Large plastic debris greater than 5mm are known as 'macroplastics' and smaller plastics between 100 nm and 5mm are classified as 'microplastics'. Nanoplastics are defined as plastics < 100 nm (Cole and Galloway 2015). Manufacturer micro- and nanoplastics are considered a primary source of contamination and include those used in industry (nurdles), facial products and cosmetics (microbeads), powdered coatings and paints (Cole and Galloway 2015). Secondary micro- and nanoplastics are generated from the breakdown of larger macroplastics.

Microplastics are considered emerging pollutants of concern that have been found in sediment and fresh and marine waters around the world (Browne et al. 2011). Industrial coastal areas, shorelines, and mid-ocean gyres are hot spots for microplastic accumulation (Cole et al. 2011, Wright et al. 2013) and there is a significant positive relationship between microplastic abundance and human population-density (Browne et al. 2011). In coastal waters microplastic

concentrations reaching 100,000 particles per m^{-3} have been reported, adjacent to a polyethylene production plant in a Swedish harbour area (Wright et al. 2013). Microplastics have also been found in salt products destined for human consumption; 21 sea and well salts from Spain contained 50-280 microplastics per kg of salt, with polyethylene-terephthalate (PET) as the most frequent polymer, followed by polypropylene (PP) and polyethylene (PE) (Iniguez et al. 2017). In China, sea salt was found to contain significantly more microplastics than lake salt and rock/well salt, with fragments and fibers being the predominant types of particles identified in sea salt (Yang et al. 2015). Microplastics have also been reported in honey, beer and in wild and commercial seafood (bivalves, fish, and shrimp) (Van Cauwenberghe and Janssen 2014, EFSA 2016).

Microplastics can enter the ocean from a variety of region-specific land and sea-based sources. Significant contributors of sea-based plastic pollution arise from the accidental loss of plastic pellets being transported at sea, from fishing gear lost by accident or from abandonment and deliberate disposal in regions of intensive fishing (GESAMP 2016). The distribution and abundance of microplastics in subsurface sea waters in the Salish Sea in the Northeast Pacific had concentrations ranging from 8 – 9,200 particles m^{-3}, with a mean of 3210 ± 628 microplastic particles m^{-3} (Desforges et al. 2014). Particle size distributions were in the 513 ± 494 µm size range, with 75% of the particles in the collective data set were fibers or filaments. Aquaculture uses a variety of plastics (ropes, netting, floats, and tanks), which may also contribute to microplastic marine contamination (Hinojosa and Thiel 2009, Desforges et al. 2014, Mathalon and Hill 2014, Davidson and Dudas 2016). The east coast of Vancouver Island is a key production area for shellfish. Surface seawater samples from Cowichan Bay, Nanaimo, Big Qualicum and Deep Bay contained an average of 660 ± 520 microplastics particles m^{-3}, the

majority (93%) of which were identified as fibrous plastics (Collicutt 2016). A study of British Columbian coastal water and sediments sampled from Baynes Sound, the Discovery Islands, Okeover Inlet, and Clayquot Sound found much lower microplastic loads, with mean microplastic concentrations of 2.31± 2.48 particles per L in water and 1.37 ± 1.56 particles per L in sediment samples, of which 97% and 99% respectively were fibers (Covernton et al. 2019). In the intertidal ecosystem surrounding Halifax harbor in Nova Scotia, significantly more microplastics were enumerated in farmed mussels compared to wild counterparts (Mathalon and Hill 2014). In a contrasting study in Baynes Sound in B.C., there was no significant difference between concentrations of microplastics in Manila clam tissues between aquaculture and non-aquaculture sites (Davidson and Dudas 2016).

Fibers are among the most common types of microplastics found in the natural environment (Browne et al. 2011, Gago et al. 2018). In a global sediment survey (Browne et al. 2011) of marine sewage disposal and reference sites, sewage effluent from treatment plants, and washing machine effluent across 6 continents, the proportions of synthetic fibers found in sewage and sediments resembled those used in textiles, suggesting that domestic washing machines could be an important source of the synthetic fibers found in marine environments. Abundances of fibers from the 18 sites ranged from 2-31 fibers per 250mL of sediment, of which polyester, acrylic, polypropylene, polyethylene, and polyamide fibers were the most prevalent (Browne et al. 2011). A recent review of all the scientific litreature on synthetic microfibers in the marine environment found a range of 0 to 450 microfibers m^{-3} in seawater (Gago et al. 2018), with polypropylene found to be the most common polymer type in seawater and sediments, followed by polyethylene in seawater and polyester in water and sediments respectively. Wastewater treatment plants can be effective in removing microplastics from

effluent; however even a small amount of released microplastic per litre can result in significant amounts of microplastics into the environment, due to the large volume of influent requiring processing (Browne et al. 2011, Murphy et al. 2016). Further research suggests that wearing clothing sheds textile fibers, which are then passively dispersed as atmospheric dust before entering terrestrial and aquatic

Microplastics are hydrophobic and can adsorb and concentrate persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and the insecticide dichlorodiphenyltrichloroethane (DDT) (Teuten et al. 2009, GESAMP 2016). Plastic debris also contain organic contaminants from the manufacturing process and adsorption from the surrounding seawater (Teuten et al. 2009). Styrofoam debris, collected from coastal waters and sea-floating buoys, and its additive flame retardant chemical hexabromocyclododecane (HB.C.D) was detected extensively along the Korean coastline (Jang et al. 2017).

3.1.2. Impacts of microplastics

Microplastics prevalence, persistence, ingestion potential (Van Cauwenberghe et al. 2013, Davidson and Dudas 2016), adsorbed persistent organic pollutants (POPs) (Teuten et al. 2009), biofilms of pathogenic organisms (Oberbeckmann et al. 2015, Kirstein et al. 2016) and capacity to be transported between trophic levels (Farrell and Nelson 2013) suggest that microplastics could have multiple negative impacts on the health of coastal ecosystems. The estimated decomposition time of plastics in the marine environment is hundreds to thousands of years, during which time larger plastics are being continuously worn down and fragmented into

smaller microplastics (Barnes et al. 2009). Microplastics are bioavailable to organisms throughout the food web due to their small size and ingestion potential (Cole et al. 2011). Microplastic contamination has been documented in over 100 marine species, ranging from plankton, to worms, crabs, shellfish, fish, marine mammals and birds (GESAMP, 2016). Microfibers have also even been found inside deep-sea organisms (depth of 334-1783m, and 954-1062m) in the equatorial mid-Atlantic and SW Indian Ocean (Taylor et al. 2016). There are several ways in which microplastics interact with marine organisms; adherence, direct or indirect ingestion, and absorption. Microalgae exposed to 1μm PVC spheres exhibited reduced growth and photosynthesis (Zhang et al. 2017). Zooplankton (*Temora longicornis*) have been shown to ingest, egest, and adhere to very small (~ 0.4 μm to 30.6 μm) polystyrene beads, resulting in decreased feeding, decreased fecundity, and the potential for possible reduced mobility, all of which results in limiting abilities to detect prey, feed, reproduce and evade predators (Cole et al. 2013, Cole et al. 2015). Filter feeding sessile and intertidal bivalves accumulate microplastic amounts that correspond positively with the pollution characteristics of their habitat (Thushari et al. 2017). Wild Manila clams from Baynes Sound, B.C., were found to contain 0.9 +/- 0.9 microplastic particles per g of clam tissue, which was not significantly different to that found in cultured Manila clams (*Venerupis philippinarum*) from the same area, which contained 1.7 +/- 1.2 particles per g tissue, *p* value = 0.289 (Davidson and Dudas 2016).

Harmful impacts on animal health have been observed in several invertebrates when exposed to microplastics, including zooplankton (Cole et al. 2013), lugworms (Besseling et al. 2013), mussels (von Moos et al. 2012) and oysters (Sussarellu et al. 2016). Negative impacts include a reduction in feeding in the Lugworm *Arenicola marina* and zooplankton *T. longicornis* (Besseling et al. 2013, Cole et al. 2013), weight loss in *A. marina* (Besseling et al. 2013),

inflammatory response, lysosomal membrane destabilization in *Mytilus edulis* (von Moos et al. 2012), and reproductive impairment in *Crassostrea gigas* (Sussarellu et al. 2016). Lugworms ingesting PVC particles made worms >30% more susceptible to oxidative stress (Browne et al. 2013). Polystyrene beads increased mortality and reduced posterior segment regeneration rate in the commercially important polychaete *Perinereis aibuhitensis* (Leung and Chan 2017). Microbeads have been shown to alter metamorphosis of juvenile development of sea urchin larvae and juvenile tunicates (*Ciona robusta)* (Messinetti et al. 2017). However, the cultivated green-lipped mussel *Perna perna* showed no significant physiological effects of environmentally realistic microplastics concentrations of PVC particles (0.125g per litre) over a 90 day period, as assessed by ingestion rate, assimilation efficiency, growth rate, cellular biomarkers (lysosomal integrity, lipid peroxidation, and DNA damage) and condition index (Santana et al. 2018).In vertebrates (for example the Zebrafish, *Danio rerio*) polystyrene microplastics induced inflammation, lipid accumulation in the liver and significantly increased superoxide dismutase and catalase activity, thereby disturbing lipid and energy metabolism (Lu et al. 2016). In European seabass, *Dicentrarchus labrax*, microplastics caused neurotoxicity, lipid peroxidation in brain and muscle, and altered energy-related enzyme activity (Barboza et al. 2018).

Due to their small size and potential to accumulate, microplastics are capable of tissue translocation, and trophic transfer. Translocation of microplastics from the gut to the circulatory system has been seen in mussels exposed to 3 and 9.6 μm sized polystyrene microspheres (Browne et al. 2008). Trophic level transfer and the translocation of microplastics has also been shown in the haemolymph and tissues of crabs fed microplastic-contaminated mussels that were previously exposed to 0.5 μm polystyrene microspheres (Farrell and Nelson 2013). To further investigate trophic transference, crabs were fed microplastic contaminated mussels for 10 days,

and then allowed to depurate for 10 days by eating uncontaminated puffer fish. After depuration the crabs did not contain any microplastics in their gut or tissues, suggesting there may be a reduced risk of microplastics on higher trophic levels (Santana et al. 2017).

Adhered and absorbed chemicals on microplastics can also cause issues for marine organisms. The transfer of chemical pollutants and products (nonylphenol, phenanthrene, antimicrobial Triclosan, flame retardant PBDE-479) and plastic additives from microplastics in the lugworm gut to its surrounding tissues reduced the phagocytic activity of coelomocytes (used to clear pathogenic bacteria from coelomic fluid) and caused mortalities (Browne et al. 2013). Virgin polystyrene (PS) and high density polyethylene (HDPE) particles have also caused adverse effects in sea urchin embryos during fertilization and development from the leaching of co-contaminant chemicals (additives, unreacted residual monomers, and by-products such as Bisphenol A (BPA), styrene, benzene, phthalates, citrates, solvents) (Martinez-Gomez et al. 2017). Microplastic physiological impact studies on larval zebrafish assessed using quantitative polymerase chain reaction (qPCR) gene expression levels examined effects of micro PVC particles with co-contaminants of phenanthrene (PAH) and 17 alpha ethinylestradiol (a synthetic oestrogenic substance); a positive linear relationship was found between co-contaminant concentration and expression of gene transcripts *cyp1a* (induced by PAH, involved in xenobiotic metabolism) and *vtg* (biomarker used in exposure to environmental estrogens) (Sleight et al. 2017). Xenobiotic metabolism can lead to detoxification or activation to reactive intermediates. Oyster larval gene expression was altered in response to extremely high (> 1000 polystyrene microspheres per L) microplastic exposures in microarray studies, where exposed larvae exhibited molecular signatures of energy allocation shifts from reproduction to structural growth, as well as endocrine disruption (Sussarellu et al. 2016). Mussels exposed to naïve PE and PS and

pyrene-treated particles showed transcriptional changes of several genes involved in DNA repair, detoxification, lysosomal metabolism, immunological functions and oxidative processes (Avio et al. 2015).

Surfaces, including microplastics, in the marine environment quickly absorb organic nutrients and subsequently attract microbial colonisers (Oberbeckmann et al. 2015). Over time, microplastics are subject to biofouling, collecting biofilms of bacteria and microorganisms. Microplastic biofilms have several potential ecological implications: firstly, biofilms can help 'disguise' microplastics as food (Vroom et al. 2017); secondly microplastics can serve as vectors for the dispersal of pathogenic organisms (Kirstein et al. 2016, Oberbeckmann et al. 2017); thirdly, can potentially alter natural bacterial abundances (Zettler et al. 2013) and; lastly can affect the physical properties of microplastics, reducing buoyancy and potentially leading to accelerated sedimentation and increasing accessibility to benthic organisms (Oberbeckmann et al. 2015).. Microplastic microbial communities, called the 'plastisphere', have been characterized with next-generation sequencing, revealing a diverse community of heterotrophs, autotrophs, predators, and symbionts, as well as several hydrocarbon-degrading bacteria, and several *Vibrio* species (Zettler et al. 2013). This study also observed that the plastisphere was distinctly different from the communities in the background water, and less diverse in bacterial flora. Pathogenic bacteria including *Vibrio* spp. (Kirstein et al. 2016), *Aeromonas salmonicida* (Virsek et al. 2017) as well as parasitic and saprophytic fungi have also been identified living on microplastic particles in the marine environment (Kettner et al. 2017). Cyanobacteria, frequently the cause of harmful algae blooms, are another important component of the plastisphere (Yokota et al. 2017). Harmful algae blooms are expected to increase in the future due to anthropogenic climate change and increased nutrient loads from terrestrial outputs and so will microplastics if

the human population increases, so the interactions of increased pollution with changing ocean conditions could further compound negative effects on the cellular physiological impacts on marine organisms.

The negative impacts of microplastics on primary producers, microalgae, and zooplankton, plus linkages between primary producers and higher trophic levels, highlight the potential risk for food web alterations due to microplastic contamination. In many species microplastic interactions led to reduced growth, decreased reproduction, and viability, which over time could lead to population declines. Trophic transfer and ingestion of contaminated microplastics could lead to bioaccumulation of POPs that could impact the health and resilience of higher trophic levels. The reduced feeding of zooplankton exposed to microplastics could over time led to reductions in ingested carbon biomass, which could have ocean acidification and ecological implications (Cole et al. 2015). Despite this research to date, there is still a lack of studies on microplastic impacts on organisms exposed to realistic levels of contamination found in nature. The majority of published microplastic litreature on exposure studies use commercially available microbeads or particles and concentrations 2-7 orders of magnitude higher than those found realistically in the receiving environment (Lenz et al. 2016). The level of risk to human health from the consumption of contaminated sea products and seafood is still uncertain and requires further research.

3.1.3. Chapter objectives

Microplastics are emerging pollutants found in terrestrial and aquatic habitats worldwide, and are ubiquitous and persistent in marine environments (Cole et al. 2011). Whilst negative physiological impacts have been observed in invertebrate laboratory exposure studies with

concentrations of microplastics 2-7 orders of magnitude larger than found in natural habitats (Lenz et al. 2016), there is a knowledge gap on the impacts of environmentally relevant microplastic concentrations on organism physiology.

The objectives of this chapter were to determine whether environmental concentrations and types of microplastics have an impact on Pacific oyster cellular physiology on the gene expression level, or by applying metrics of growth, survival, and membrane stress assessments. To assess the potential impacts of microplastics on health and physiology, Pacific oysters (*Crassostrea gigas*) were exposed to environmentally relevant (empirical local data) concentrations of microplastics in their algal feed (Desforges et al. 2014). Adult Pacific oysters were randomly assigned to groups for exposure or non-exposure to microplastic fibers and tissue samples were taken over a 28-day period; these tissues were examined for cellular physiological impacts and stress responses by analyzing gene expression patterns, condition indices, and histological assessments (Neutral Red Retention assay (Lowe et al. 1995) following protocols developed for oysters (Méthé et al. 2017)). Comparison of gene expression tissue responses (gill and digestive gland) between groups was used to assess if microplastic exposure may affect shellfish immune function, reproduction and growth and therefore subsequently affect their productivity and survival.

Null hypotheses

H_0 = Environmentally relevant concentrations and types of microplastics found in local waters do not have an impact within 30 days on Pacific oyster cellular physiology

H_0 = Environmentally relevant concentrations and types of microplastics found in local waters do not have a cellular physiological impact within 30 days ↕on Pacific oysters when applying metrics of growth, survival and membrane stress assessments.

3.2 Methods

Microplastic stressor experiment

The experimental procedures in this study were performed in strict accordance with the guidelines and recommendations of the Canadian Council of Animal Care (CCAC). The protocols were approved by the Department of Fisheries and Oceans (DFO) Pacific Region Animal Care Committee (Animal Use Protocol Number: 16-012). For the exposure experiment, 250 adult Pacific oysters (*Crassostrea gigas*) were collected from a shellfish aquaculture operation (Mac's Oysters Ltd.) in Fanny Bay. B.C., and brought to the laboratory facilities at PBS, Nanaimo, B.C. The area of the wet lab used to house the oysters for the experiment was sectioned off from the rest of the lab with cotton curtains from ceiling to floor, and wall to wall, to help reduce any background microplastic contamination. The oysters were acclimatized in a flow-through seawater system for 5 weeks, with seawater that was initially filtered to 20µm filtered, followed by 1µm filtration. During this acclimation time the oysters were fed 4 g *Chaetoceros muelleri* **Cm** (CCMP 1316) algal paste and 3 g *Isochrysis aff. galbana* 'Tahitian isolate' **Tiso** (CCMP 1324) paste, once a week for the 40-day acclimation period. During feeding the flow-through water was turned off for 2 hours. Prior to experimental start, the oysters were weighed (mean 21.4 ± 5.4 g) and measured (mean length 6.0 ± 0.6 cm) and 204 animals were randomly divided among 12 glass tanks (20L volume, length x width x height: 40 cm x 20 cm x 26 cm). Each of these 20 L tanks then contained 16 L of filtered seawater and 17 oysters. A randomized block design was employed, where 12 experimental tanks containing oysters and 6

empty experimental tanks used as controls were equally divided into three block units. The experimental units were placed in shallow rectangular tanks (1.2 m x 0.9 m x 0.3 m) filled with circulating seawater which acted as a temperature bath to stabilize experimental tank temperatures within the block unit (filtered flow-through seawater at ambient seawater temperatures of 10-11 ^{o} C) to help maintain the individual tank water temperatures, and covered with 100% cotton canvas to protect the glass aquaria tanks from external microplastic contamination (Figures 17 and 18). Each cotton covered fiberglass tank or 'tent' served as a blocking factor to account and control for background sources of variation or 'noise'. Each tent or blocking factor contained 6 randomly placed tanks, comprising of 2 containing oysters exposed to microplastics in microalgae feed, 2 containing oysters with microalgae feed only, and 2 tanks containing no oysters (blanks) which did not receive microalgae and were controls for external microplastic contamination. Each tent therefore constituted one block, and a total of 3 blocks were used in this experiment. The position of each type of tank (blank, control, or exposed) was randomized in each tent using random number generator tables. The oysters were acclimatized in their tanks for one week in a static system with 1μm filtered seawater (following 20 μm initial filtration) that was replaced daily by gentle siphoning, so as not to disturb the oysters. Each tank also contained an air stone and a HOBO® UTBI-001 TidbiT v2 water temperature data logger which recorded the temperature at 5 min intervals . Seawater filters were replaced weekly.

Figure 17: 'Tented' fiberglass tanks, each containing six individual experimental tank units.

Figure 18: Experimental tank units per tented block in randomized locations. Six 20 L tank units (2 blank, 2 control, and 2 exposed) in a larger seawater bath.

Water conditions (temperature, dissolved oxygen) and tank cleanliness were monitored daily, and ammonia levels were assessed on day 7 and 9 (day 1 being time = 0), one hour after morning feeding and just before the morning feed. Oyster faeces were siphoned out daily, and any mortalities were recorded and removed. Feeding rations were adjusted for any removal of oysters for sampling or due to mortality.

After acclimatization for 40 days the control (non-exposed) tanks (n = 6) containing oysters were fed cultured microalgae, at a 37.5: 25: 37.5 ratio of algal cell numbers of *Chaetoceros muelleri* **Cm** (CCMP 1316), *Tetraselmis suecica* (CCMP 904), *Isochrysis aff. galbana* 'Tahitian isolate' (CCMP 1324). The microplastic-exposed tanks (n = 6) were fed

microplastic fibers at a concentration of 5 microplastic fibers per litre with the same microalgae diet fed to control oysters, with the assumption that each oyster filtered 1 litre per hour for 24 hours (Ren et al. 2000). Amounts of microalgae delivered to each tank depended on the number of oysters present, as oyster numbers decreased during the study due to sampling. The oysters were all fed two times a day Monday – Friday in the morning and late afternoon. Faeces and water were siphoned out everyday (leaving 6 cm of water in tank to keep oysters submerged at all times) at 4 pm 7 days / week. On feeding days (Monday – Friday) the tanks were siphoned out immediately preceding the second feeding (around 4 pm).

Preparation of microplastic fibers

Oysters were fed four types of microplastic fibers: polypropylene/polyethylene mixed fibers from degrading turquoise rope, polyamide (nylon) fibers from green fish netting, acrylic fibers from yarn, and polyester fibers from polar fleece clothing fabric. The fibers were collected by agitating each material individually in a domestic washing machine for 2 hours and then collecting the fibers by filtering the washing machine water through a 63μm sieve. The material was then dried in a domestic dryer and the fibers collected from an uncontaminated lint collector.

Each collection of fibers was resuspended in 1μm filtered seawater. The concentration of each fiber solution was determined by counting the number of fibers in twenty aliquots of 10μL on a dissecting microscope, and then averaging to provide a concentration estimate (57 ± 25 fibers / mL). All microplastic fibers found in oysters were between 100 – 5000 μm. Aliquots of equal numbers of fibers were then made for each feeding day and tank. The total amount of fibers needed per day for each tank was calculated by multiplying the number of oysters in the tank by an estimate of the number of litres filtered by each oyster per hour by 24 hours at 10-13 ^{o}

C (Ren et al. 2000). On feeding days each microplastic aliquot was divided between the morning and afternoon feeding.

Sampling

Oysters were sampled at time zero, 3 hours after first microplastic feeding, 14 days, and at 30 days at which point the experiment was concluded. One oyster from each of the 12 tanks was weighed, measured (umbo to posterior edge), and sampled at each time point for gene expression analysis, and Neutral Red Retention assay, an indicator of stress response (Méthé et al. 2017). One oyster from each tank was also sampled at time zero, 14 days, and 30 days to analyze the amount of microplastics per animal, otherwise described as microplastic load (stored at – 20 ^{o} C). Two oysters per tank were sampled at time points zero, 15 days, and 30 days for condition indices (Rainer 1992), and stored at – 20 ^{o} C. For gene expression, 2 – 3 mm^3 gill and digestive gland tissue sections were excised and preserved according to methodologies below.

To determine if there were additional sources of microplastic introduction into the experimental units the seawater from the individual tanks, and the microalgal feed was sampled for microplastic load analysis. 500mL of microalgal feed of the 3 microalgae species mixed together was sampled each day of feeding (5 days a week) for microplastic analysis using previously unused Mason™ jars, rinsed with 1 µm filtered water, filled to the brim with microalgal diet and subsequently sealed with airtight lids prior to storing at 4 ^{o} C. 500mL seawater samples from each tank (n = 18) were taken at each sampling time point, as well as every second day of the experiment for microplastic concentration determination. Care was taken to prevent other sources of microplastic contamination in these samples (*i.e.* blue cotton coveralls were worn at all times).

RNA sampling, extractions and visual health observations

To analyze samples for gene expression, a section of gill and digestive gland tissue was excised from each oyster immediately after the oyster valves were opened. Gills were chosen because they are in constant contact with their environment and therefore often used in environmental stress studies as more immediate environmental response indicators. Digestive glands were chosen as they are accumulatory organs and are used in toxicological and immunology studies to examine long-term effects (Milan et al. 2011).

Briefly, the sampling area was disinfected with fresh (made daily) 0.5% solution of sodium hypochlorite, and sampling scissors, forceps and scalpels disinfected by 2 min. immersion in 0.5% sodium hypochlorite solution, dipped in water, and dipped briefly in 100% methanol before passing through a flame. Oysters were opened with a sterile scalpel, and scissors and forceps used to remove a small cube of tissue (approximately 2 - 3 mm^3), from the gill first, and then the digestive gland, and stored in RNAlater as per protocol (Ambion, Carlsbad, CA). At the time of oyster sampling, a number of gross visual observations were also made on animal condition, following the protocols developed for the Canadian National Animal Aquatic Health Program. This included (but was not limited to) observations of animal state (body condition, response), digestive gland and gill colouration, any nodules indicating disease, any parasites present and any internal shell deposits.

RNA from 25-30mg tissue sections was individually extracted using RNeasy RNA from tissue sections of 25-30mg was individually extracted from each tissue using RNeasy kits (Cat No./ID: 74106, Qiagen, Maryland). Tissues were homogenized in 2 mL tubes of Lysing Matrix D (SKU 116913500, MP Biomedicals, Solon, OHIO) in a Tissuelyser II (Cat. No. 85300,Qiagen,

Maryland) at 25 Hz for 2 min. To eliminate DNA from contaminating the samples, a DNase treatment was applied using Turbo DNA-free Kits (SKU# AM1907, Ambion, Carlsbad, CA) and clean-up followed the product routine treatment protocol. The RNA concentration was quantified on a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) before and after DNase digestion.

Library preparation for RNA sequencing

For subsequent RNA sequencing analysis, 4 oyster individuals were randomly selected from both the control and exposed treatment groups at the time = 0, time = 3 hours, and time = 14 days sampling time points for library synthesis and sequencing. This resulted in 24 libraries per tissue (4 oysters x 2 treatments x 3 time points), and 48 libraries in total. Prior to library generation RNA quality and quantity of each oyster RNA sample was determined using the RNA 6000 Nano chip (No.5067-1511, Mississauga, ON) on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

Library synthesis and sequencing

All RNA library synthesis and next-generation sequencing was conducted at Genome Québec Innovation Centre (Montreal, Québec, Canada). mRNASeq stranded paired-end (2 x 125 bp) library synthesis methods were as follows: total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and its integrity was assessed on a 2100 Bioanalyzer (Agilent Technologies). Libraries were generated from 250 ng of total RNA as following: mRNA enrichment was performed using the NEBNext Poly(A) Magnetic Isolation Module (New England BioLabs). cDNA synthesis was achieved with the NEBNext RNA First

Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs). The remaining steps of library preparation were performed using and the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs) with adapters and PCR primers from New England BioLabs. Libraries were quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems). Average size fragment was determined using a LaB.C.hip GX (PerkinElmer) instrument. Libraries were then subsequently sequenced on an Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA). Eight libraries were sequenced per lane resulting in a mean of 63 ± 26 million reads per library.

Microplastic load determinations and condition indices

Oyster tissue preparation

Frozen oysters for condition indices and microplastic enumeration were thawed from -20°C before processing. Oyster samples were processed in a laminar flow hood, to prevent contamination from airborne particles settling on/in samples or in the workspace. Before processing, a single 500 ml Mason™ jar was labelled with a corresponding number to the oyster sample to be processed, and a piece of steel mesh was placed over the opening of the sample jar and secured using an aluminum ring. Each jar was weighed individually, and weights were recorded for later use. These weighed jars were rinsed three times with filtered deionized water and covered with the steel mesh and ring to ensure no plastics remained in the jar before adding the oyster sample.

Each oyster was removed from its individual sample bag and its length, width, and shell depth recorded (cm). Oysters were then rinsed with filtered water to remove any possible

microplastics on the exterior of the shell. The upper and lower shell valve were separated using a scalpel that was rinsed three times with filtered deionized water. Once the shell valves were partially open, the oyster tissue was rinsed three times with water to remove any undigested materials. Once rinsed, the upper shell mantle tissue was quickly separated from the shell by gently scraping with the rinsed scalpel to remove all tissues, before repeating this to remove the tissue from the other valve; the whole wet weight oyster tissue mass was then deposited into its pre-labeled and weighed Mason jar before closing the jar with the steel mesh and aluminum ring. For condition index measurement samples, the oysters were removed from their shells and placed in their individual jars, the jars were reweighed to determine the total wet weights of oyster tissue. During each sample period, three controls were also created that mimicked laboratory procedures, but without the oyster, to provide background microplastic contamination data.

The jars containing the oysters were then placed in the drying oven at 60°C where they remained until a constant dry weight was achieved (~0.001 g), which was approximately 6 days for the oysters used in this study. Oyster condition indices (CI) were then calculated using the formula (Rainer & Mann, 1992):

$$CI = (\text{Dry tissue weight (g)} / \text{Dry shell weight (g)}) \times 100$$

After the oyster samples were dried to a constant weight, a subsequent digestion was applied for microplastic enumeration. Briefly, digestion proceeded by first gently unscrewing the top of the Mason™ jar and then quickly pouring ~25 mL of 10% potassium hydroxide (KOH) solution into the jar and then replacing the lid loosely on top of the sample jar. A piece of aluminum foil was then rinsed three times on each side with filtered deionized water and then

used to replace the steel mesh and aluminum ring and was secured with a natural rubber band and set aside. This was completed for all individual samples and controls. The samples were then placed back in the drying oven at 60°C for 24 hours. After 24 hours, the samples were removed from the drying oven in preparation for filtration.

Prior to sample filtration all funnels and funnel bases were rinsed three times with filtered deionized water and covered with a piece of rinsed aluminum foil. 8µm polycarbonate (PCTE) membrane filters (Sterlitech) were rinsed and placed onto the funnel base with the rinse funnel placed on top and secured in place with a clamp, and samples were filtered individually. Sample filtrations were followed with a deionized rinse of the funnel filter to remove any tissue or liquid that may have adhered to the side. The filter paper was then quickly and carefully removed and placed into a rinsed and labeled Petri-slide using rinsed forceps. Once this was completed for all samples and controls, the filter papers were examined under the microscope for microplastic abundance, size, colour, and type (fiber or fragment).

Microplastic Enumeration

An Olympus CX42 compound microscope was used for microplastic identification. To prevent contamination of samples with airborne microplastic fibers, the microscope housing was securely wrapped with transparent plastic sheeting. To minimize contamination all work was conducted in a laminar flow hood, water and solutions used were filtered through a 1.6 µm Whatman GF/C filter paper, and cotton coveralls and headscarves were worn. To account for background contamination, procedural controls, or blanks, were run with every batch of samples processed. The number of fibers were averaged, rounded to the nearest whole number, and subtracted from the number of clear fibers in each sample processed in that batch. Sample filter

papers were analyzed under 100x magnification by photography using a cellSens (Den Haag, Netherlands) digital camera, and recorded in a database. When a microplastic was encountered, identification followed protocols as described in the Marine & Environmental Research Institute "A Guide to Microplastic Identification" (Barrows 2017). Briefly, characteristics such as shape, colour, tensile strength and fraying were used to identify microplastics. Colored fragments are generally always microplastics but were always probed to confirm a correct identification.

Microplastic descriptions

The microplastic fibers the oysters were exposed to were highly identifiable because of their shape and markings; uniform in width and absent of striations, and colours; red, turquoise and shades of green (Figure 19).

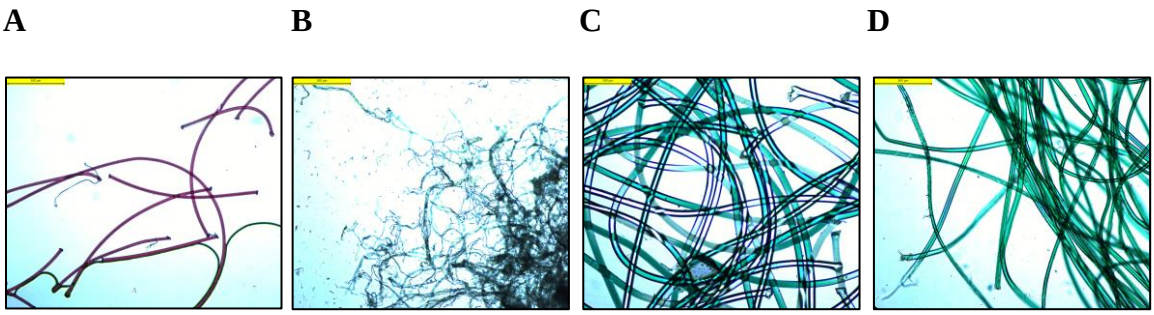


Figure 19: Images of microplastics exposed to oysters, captured afterwards to show shape and texture and are not representative of the actual length of fibers exposed to the oysters, A; red polyester polar fleece fibers, B; turquoise polyethylene, and polypropylene rope fibers, C; light green nylon fibers, D; dark green acrylic fibers. Yellow size bar in top left corner represents 500 µm.

The coloured fibers found in the oysters ranged in size from 100 – 5000 µm, were red and turquoise, uniform in width, and absent of striations (Figure 20). All the fibers in the background controls representing background contamination were blue and clear fibers with

striations (Figures 21). Blue and cotton fibers were chemically identified using a Cary 660 FTIR Spectrometer (Agilent) and identified as cellulose and rayon fibers (Covernton et al. 2019).

A B

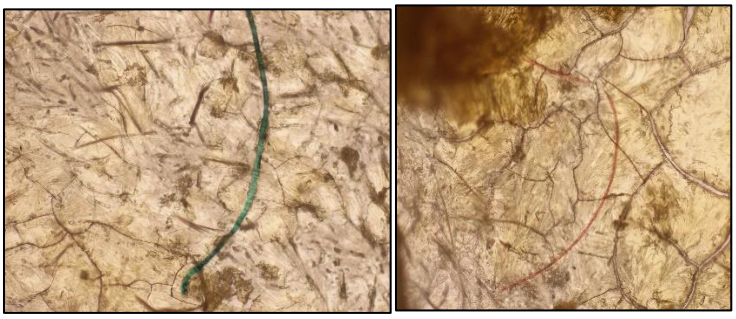


Figure 20: Coloured fibers found in oysters, A: turquoise – green fibers, B: red fibers

A B C

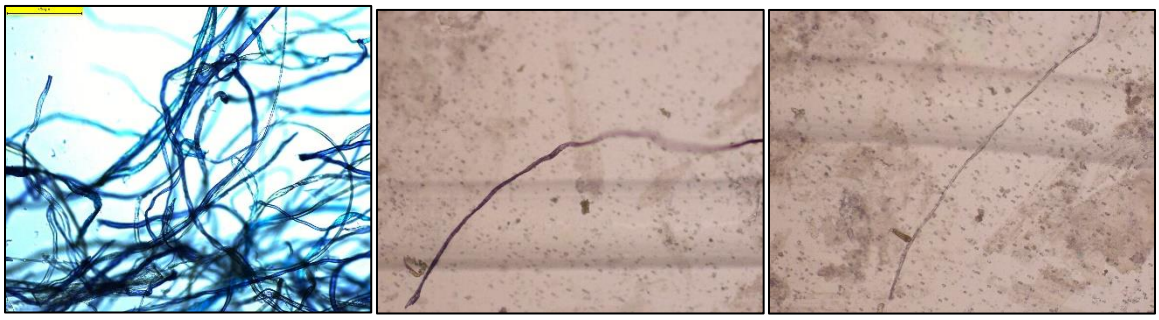


Figure 21: Images of background fibers, A: blue cotton overall fibers, B: blue cotton fiber found in oyster, C: clear fiber found in oyster.

Neutral Red Retention assay

The Neutral Red Retention (NRR) assay is an *in vivo* cytochemical method based on the retention of neutral red within lysosomes. Lysosomes are very sensitive to minimal concentrations of toxic chemicals that penetrate in the cells (Martinez-Gomez et al. 2015).

Lysosomes in healthy cells take up and retain larger quantities of neutral red longer than those from damaged cells (Martinez-Gomez et al. 2015). Lysosomal membrane stability (LMS) is a general stress biomarker of chemical pollution used to monitor environmental chemical quality of coastal waters with most countries using mussels as a target species (Martinez-Gomez et al. 2015). Membrane stability is visualized under a microscope with the uptake of neutral red into the cells and then the leakage back into the cytosol seen in damaged cells (Lowe et al. 1992). The lysosomal membrane destabilization index (LDI) represents the count of hemocytes (out of 100) having destabilized lysosomes evident with leakage of neutral red into the cytosol or enlarged lysosomes. A modified oyster protocol has been developed (Méthé et al. 2017), which was employed in this study.

Neutral-red dye stock solution was made fresh on each sampling day by dissolving 2.28 mg of neutral red powder (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, N4638, Sigma-Aldrich, Oakville, Ontario, Canada) in 1 ml of dimethyl sulfoxide (DMSO, D8418, Sigma-Aldrich). The experimental working neutral red solution applied to the samples was made by diluting 8.5µl of stock solution with 500µl of 0.22 µm filtered mantle fluid. Using a sterile pipette tip, hemolymph was sampled from the pericardial cavity of each oyster and placed in a corresponding labeled Eppendorf tube. Then 40µl of hemolymph fluid was transferred onto a Poly-L-Lysine treated microscope slide. Each slide was placed in a humidity chamber (a black plastic box where slides sit above ~ 0.6 cm of water at room temperature) for 15 minutes to adhere the cells to the slide surface. Excess hemolymph was poured off the slide to remove any fluid not containing haemolymph, and 20µl of neutral red working solution was added to attached hemocytes on the slide surface. Slides were then placed in humidity chamber again for 15 minutes for the cells to adsorb the dye. Cover slips were placed on each slide on removal

from the humidity chamber, and slides were observed under a Nikon microscope every 15- 20 minutes for 1 hour. Digital photos were taken each time point.

Data Analysis

Microplastic load and condition index data analysis

Plots were generated and condition index and microplastic load data was analyzed using the statistical program R (R Core Team. 2016). Nested ANOVAs of linear mixed effects models from the lme4 package, were employed to identify significant ($p \leq 0.05$) differences in condition indices, and microplastic load over time with microplastic exposure (treatment), with block as the random effect. Each tent is considered a true replicate as there were no significant differences between blocks in terms of microplastic load and condition indices.

Gene expression

The initial bioinformatics (trimming, transcriptome assembly, annotating, and calculation of read counts) was performed at the Biomedical Research Centre at the University of Victoria. The *Crassostrea gigas* genome assembly (NCBI GCA_000297895.1) composed of 557,735,934 bp in 7,659 scaffolds was RepeatMasked (v4.0.6, default parameters, RepeatMasker.lib) to reduce the number of simple repetitive elements (155,878 simple repeats representing 6,987,111 bp or 1.25%, 23,514 low complexity regions representing 1,122,256 bp or 0.20%). As sequencing read ends are low quality and have introduced adaptor sequences, these were removed or trimmed to generate a transcriptome more representative of the original sequence. Transcriptome assembly with a reference genome, as is the case here, will only contain transcripts from the reference genome. Forty-eight RNA-Seq libraries, representing gill and the

digestive system under various conditions, were compared to the results of the RepeatMasked oyster genome to produce a gene model. STAR 2.5.1b (runMode --alignReads --outSAMstrandField intronMotif --outFilterIntronMotifs RemoveNoncanonical --outSAMtype BAM Unsorted SortedByCoordinate) was used to produce coordinate-sorted alignments of the RNA-Seq libraries to the masked genome. Cufflinks (v2.21, -u --total-hits-norm) was run on the resulting sorted alignments to produce individual GTF files. GTF files were merged using Cuffmerge. TransDecoder 5.0.1's. Cufflinks_gtf_genome_to_cdna_fasta.pl was used to extract transcripts (208,905) from the merged GTF file. TransDecoder 5.0.1's cufflinks_gtf_to_alignment_gff3.pl was used to extract a gff3 annotation file.

Open reading frames (ORFs) (3,401,696) were predicted on the 208,905-transcript set using TransDecoder.LongOrfs 5.0.1 (-m 30). The longest peptide ORFs were retained from this prediction and compared for protein homology (blastp -max_target_seqs 1 -outfmt 6 -evalue 1e-5) against UniProt. ORFs were also compared to PfamA (hmmscan). The results of these homology scans were incorporated into TransDecoder.Predict to produce a transcript set of 196,737 sequences that contained protein homology and ORF evidence. Gene loci from this transcript set were chosen based off homology evidence (27,945). For each loci, the best transcript was chosen according to ORF size. Transcript annotations were filtered for unwanted transposable element keywords (transposon, long terminal, repeat, gag, bpol, long interspersed element, etc.). Distinctly non-overlapping loci were retained, resulting in a final 21,084 set of transcript coding domain sequences (CDSes).

Differential gene expression data analysis

The edgeR (Robinson et al. 2010) package (Version 2.6.9) was used to analyze the RNA sequencing data and to detect significantly differentially expressed genes (DEGs) between control and exposed oysters. After genes with very low counts were filtered out and data was normalized for library size, multi-dimensional scaling (MDS) plots were run as a first analysis step to examine samples for outliers and other relationships. MDS is a type of unsupervised clustering function in edgeR that plots the RNA samples in which distances correspond to leading log-fold-changes between each pair of RNA samples.

To find significant DEGs a generalized linear model approach was used as it allows for an infinite variety of contrasts to be tested between the groups. An ANOVA-like test in R was performed to find DEGs between groups without specifying before hand which groups might be different, this is analogous to a one-way ANOVA test (edgeR user manual reference insert here). To find DEGS between the exposed and control oysters at the 3 time points the groups that were compared were:

1) Ex.vsC.0h = GroupEx.0h - GroupControl.0h

2) Ex.vsC.3h = (GroupEx.3h - GroupEx.0h) - (GroupControl.3h - GroupControl.0h)

3) Ex.vsC.14d = (GroupEx.14d - GroupEx.0h) - (GroupControl.14d - GroupControl.0h)

Genes were selected as differentially expressed due to microplastic exposure when 1) the ANOVA probability (*p*) value was less than or equal to 0.015 and 2) at time = 0 there was less than or equal to 1.4-fold change difference between the control and exposed groups and 3) at time = 3 hours, and / or at time = 14 days there was a greater than or equal to ± 2-fold change in gene expression between the control and exposed libraries. A *p* value cut-off of 0.015 was chosen instead of using the corrected *p* value (also called False Discovery Rate, FDR) of 0.05-0.01, as there were only 4 biological replicates sequenced per treatment and time point.

The Uniprot accession numbers of the resulting gene lists for up- and downregulated genes in the exposed oysters at 3 hours and 14 days were analyzed for enriched biological themes and GO terms using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (Huang et al. 2008, Huang da et al. 2009) against the background gene list of each respective tissue. Biological processes and GO terms (default settings) were considered significantly enriched when the *p* value was less than or equal to 0.05.

3.3 Results

Condition index

During the microplastic exposure experiment, oyster productivity was assessed with condition index (CI) measurements. Six oysters (one from each tank) were randomly selected from each treatment group at 0, 14, and 30 days for condition index analysis. CI values at time zero averaged 6.4 ± 1.6 in the control group and 6.1 ± 1.9 in the exposed group. After 14 days the control group had an average CI of 5.8 ± 1.8 and the exposed group had an average of 6.4 ± 1.7. After 30 days the CI of the control group averaged 5.9 ± 1.9 and 5.6 ± 2 in the exposed group. An ANOVA of a linear mixed effects model to test for significant changes in CI over time with treatment produced probability values of 0.98 for the effect of treatment, $p = 0.64$ for the effect of time, and $p = 0.59$ for the effect of treatment and time, indicating that the presence of microplastics in feed did not significantly alter body condition during the 30-day course of the study (Figure 22).

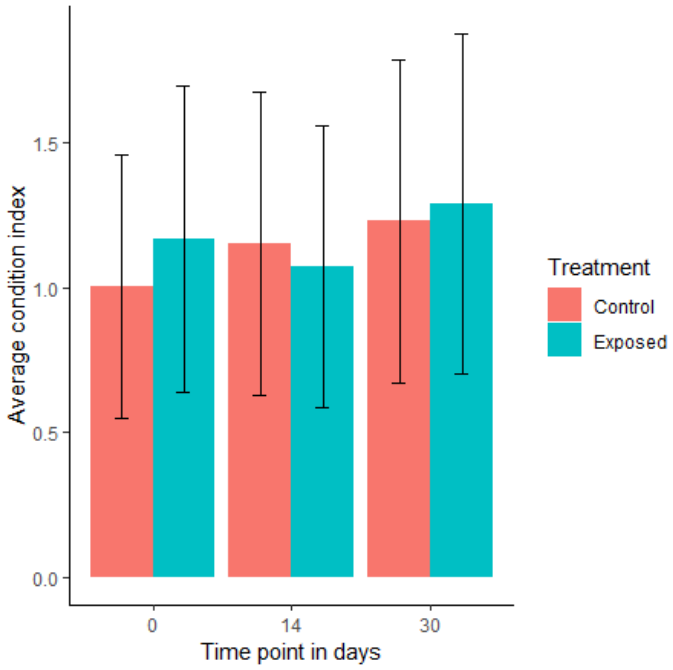


Figure 22: Mean Pacific oyster condition indices over 30 days of microplastic exposure compared to controls. Error bars represent standard errors.

Microplastic concentrations in oysters, water, and algal feed

Background controls for microplastic load analysis

Throughout the exposure experiment microplastic load was evaluated in oysters, water samples, and algae feed samples (fed naïve microplastics consisting of red, green and turquoise fibers). Most (93–100%) of the fibers found in the background controls were clear, with the only other colour of fiber being blue (striated and easily distinguishable from turquoise samples), which can be attributed to the blue cotton overalls worn during the microplastic experiment and the processing of samples. In a companion experiment, Fourier-transform infrared spectroscopy (FTIR) analysis found that many of the clear fibers were cellulose (Covernton et al. 2019). Background controls run alongside sample processing were averaged and subtracted from the number of clear fibers in each sample.

Microplastic load in oyster tissue

The total number of microplastic particles found in the control oysters at time 0 hours ranged from 0 to 7 fibers per oyster (n=6) and the number of microplastic particles in the exposed oysters at time = 0 hours ranged from 0 to 3 fibers per oyster (n = 6) following their acclimation period prior to experimental start. The total number of microplastics found in the control oysters at time 14 days ranged from 0 - 1 with one outlier that had 17 fibers (which was omitted from calculation of average fibers per oyster). This outlier was removed as it was greater than 1.5 times above the third quartile of the data set containing the numbers of microplastic fibers found in all the control oysters. The oysters analyzed for microplastic load were not the same animals sampled for RNA sequencing, so the removal of this outlier did not affect the gene expression results. The total number of microplastics in the exposed oysters at 14 days ranged from 0-9 fibers per oyster (n = 6) . An ANOVA of a linear mixed effects model with block as the random effect indicated that treatment had a significant effect on number of coloured microplastics found per oyster (p = 0.02) (Figure 23). Note that it was not possible to calculate microplastic load per gram of dried oyster tissue, as dry weights of samples analysed for microplastic load were not recorded due to a miscommunication. Differences in the numbers of microplastic fibers per oyster could be due to the differences in the microplastic microenvironment and functioning of each oyster, such as some oysters having a high filtration rate than others, or some being closer to air stones with a suggestion of disrupted feeding.

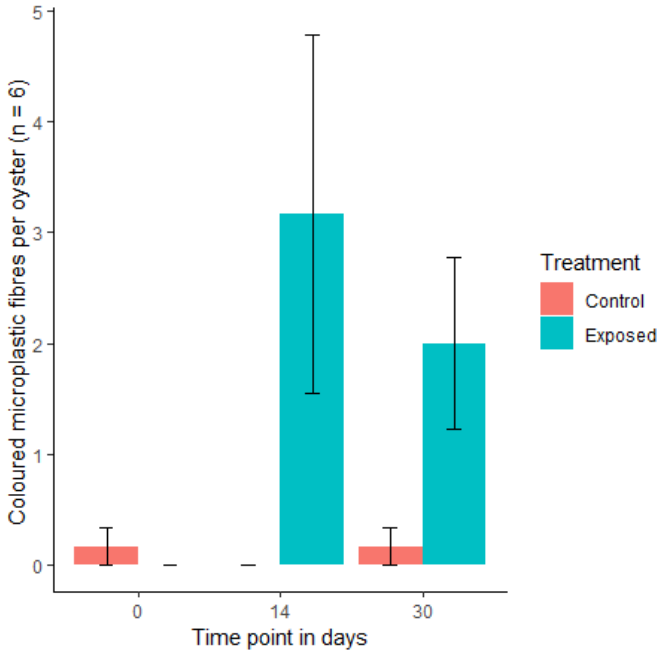


Figure 23: Mean microplastic load per individual Pacific oyster exposed to microplastics or unexposed controls.

Microplastics in tank seawater

To determine the amounts of microplastic particles in the tank seawater, 500 mL of seawater from each of the 18 tanks was sampled every 2–3 days throughout the exposure experiment. Water samples from each tank from each of the four sampling time points (time zero, 3 hours, 14 days, and 30 days) were analysed for microplastic content (n=18). After accounting for background errors, an ANOVA of a linear mixed effects model testing for significance in number of coloured microplastics per 500 mL of sampled tank water with time and treatment, and tent as a random effect, resulted in a significant difference in microplastics over time with treatment (p = 0.02) (Figure 24). There are decreased amounts of microplastics in water samples at day 14 and day 30 compared to time 0, as introduced microplastic load was a

function of the number of oysters present in the tank, which decreased due to sampling over time.

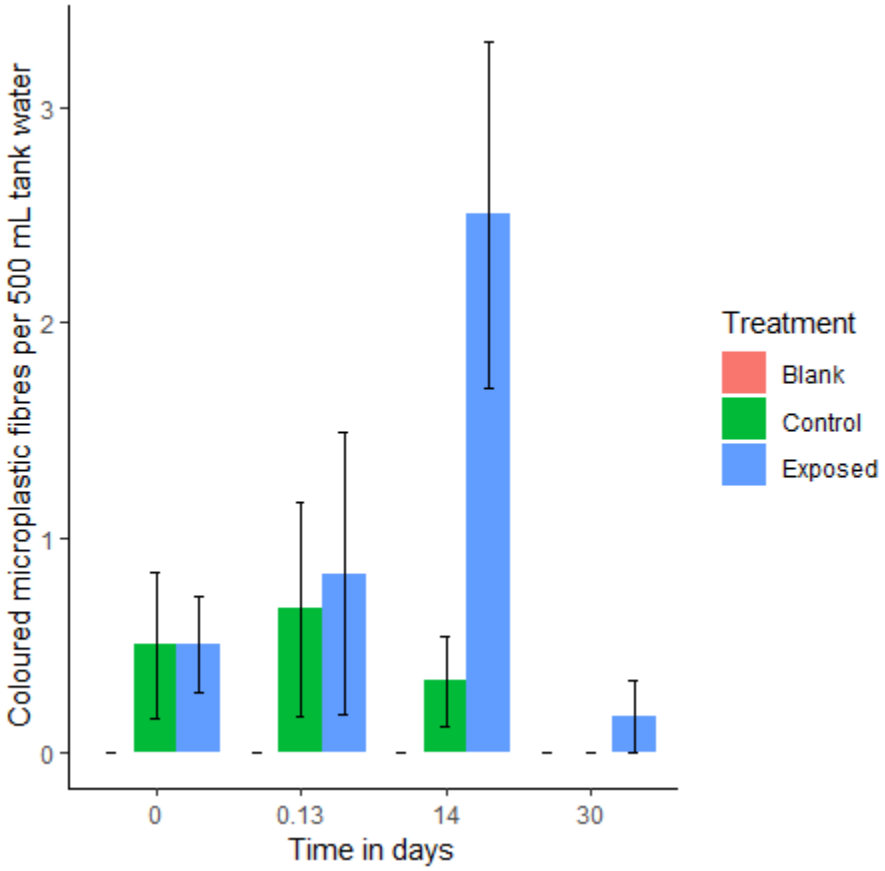


Figure 24: Mean seawater microplastic load in experimental tank units. Error bars indicated standard errors.

Microplastics in algal feed

During the exposure experiment oysters were fed a 37.5: 25: 37.5 daily ratio of *Chaetoceros muelleri* **Cm** (CCMP 1316), *Tetraselmis suecica* (CCMP 904), and *Tahitian Isochrysis* spp. **Tiso** (CCMP 1324) Monday through Friday. The volume of algae feed fed to each tank was related to oyster numbers per tank, which changed due to sampling over time. As

the algal production process is a potential source of microplastic contamination, one Mason™ jar (500 mL) of algae feed mix of the three algae species combined was sampled each day for microplastic content. The average number of microplastic fibers in the algae samples (n=21) was 0.007 ± 0.006 per mL of algal feed, 90% of which were clear, 3% red, 1% turquoise, and 6% blue, and these values show algal feed contributed negligible background microplastics to this study. Therefore, these amounts of microplastics were not subtracted from microplastics in oyster tissue.

Gene Expression

Sequencing depth and coverage is dictated by the average numbers of reads that align to known reference bases and an increase in the number of reads (in the millions for RNA-Seq experiments) equals greater coverage of the transcriptome and greater confidence in the results (Sims et al. 2014). It is estimated that > 200 million paired-end reads are required to detect the full range of transcripts in human samples (Sims et al. 2014). A total of 3 billion paired-end reads were generated in this study equaling 380 billion nucleotides. A single lane of Illumina HiSeq2000 paired-end reads (2 x 125 bp) produced a mean of 507 ± 90 million reads. The total reads/library ranged from 34 million to 175 million reads with a mean of 63 ± 26 million reads/library. There was a total of 1.6 billion gill reads, with a mean of 66 ± 23 million reads / library, and a total of 1.5 billion total reads from the digestive glands with a mean of 61 ± 30 million reads / library. The number of reads were well balanced between treatment groups in the digestive gland study with 735 million total reads in the control group, and 729 million reads in the exposed group. There was total of 918 million reads sequenced in the control group of the

gill libraries, and 665 million reads in the exposed group. An analysis of total and average reads per tissue, treatment and time point is in the appendix Table A14.

The 48 RNA-Seq libraries were aligned to RepeatMasked (to reduce the number of simple repetitive elements) *Crassostrea gigas* genome assembly (NCBI GCA_000297895.1) to produce a gene model. After alignments were sorted, open reading frames (ORFs) were predicted, and the longest peptide ORF was compared for protein homology against UniProt protein data base, resulting in a transcript set of 196,737. Gene loci were then chosen based on protein homology alone (resulting in a transcript set of 27, 945). The best transcript was chosen from each loci according to ORF size and then filtered for unwanted transposable elements. Distinct non-overlapping loci were retained for a final set of 21,084 coding domain sequences (CDSs) (putative-transcripts). The sequences ranged in length from 90 bp – 23,124 bp. Base calling accuracy measured by the phred quality score was 34.5 ± 1, which indicates a base call accuracy of > 99.9%.

To explore impacts of microplastic exposure, significantly differentially-expressed genes (DEGs) between the exposed and control groups at three time points and in two different tissues (gills and digestive glands) were identified using the edgeR package (Robinson et al. 2010), in the software program environment R (RCoreTeam 2015). Of the 21,084 CDSs, 18,315 of them mapped to a Uniprot identifier and were retained for DEG analysis. After low count reads were filtered out there remained 15,562 gill and 15,175 digestive gland putative transcripts, which will be called genes from this point for this thesis. Of these background genes there were 92 % Uniprot IDs shared between the two tissues, and 4 % uniquely expressed in each tissue (Figure 25).

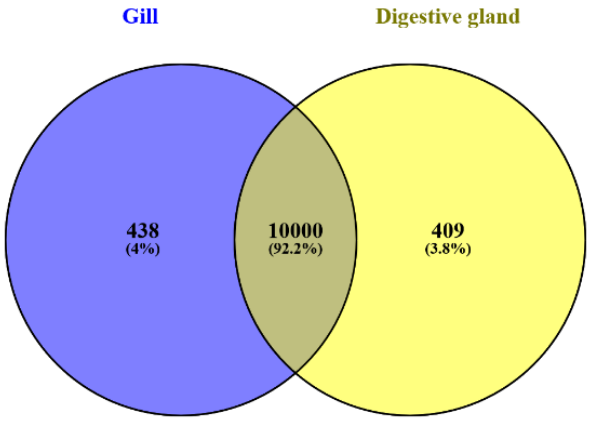


Figure 25: Venn diagram of a comparison of unique Uniprot IDs expressed in gill, unique digestive gland tissues and shared genes (overlapping 92.2%).

To analyze sequencing data for differentially expressed genes in each tissue between the two treatment groups, and the three-time points ANOVAs of generalized linear models (GLMs) were run using the edgeR package in the R software environment. Differentially expressed genes were identified with an ANOVA run between 3 groups; exposed vs control at time= 0 (Ex.vsC.Oh), exposed vs control at time = 3 hours (Ex.vsC.3h), and exposed vs control at time = 14 days (Ex.vsC.14d). Each group was calculated as follows:

Equation 1) Ex.vsC.0h = GroupEx.0h - GroupControl.0h

Equation 2) Ex.vsC.3h = (GroupEx.3h - GroupEx.0h) - (GroupControl.3h - GroupControl.0h)

Equation 3) Ex.vsC.14d = (GroupEx.14d - GroupEx.0h) - (GroupControl.14d - GroupControl.0h)

To find DEGs at the 0-time point between the exposed and control groups, the control group gene expression was subtracted from the exposed group gene expression (Equation 1). To find genes that were in response to microplastics in the exposed group at the 3- hour time point

firstly the 0 time point of the control group was subtracted from the 3-hour time point control group, and the 0 time point from the exposed group was subtracted from the exposed group at 3 hours and then the control oysters were subtracted from the exposed oysters (Equation 2), and then the same for the 14-day time point (Equation 3).

Heatmaps of the top 150 genes ($p \leq 0.007$) in each tissue identified by the ANOVAs to be differentially expressed were generated to examine any large-scale evidence of differential expression. In the gills there is evidence of upregulation, especially in two of the exposed libraries at 14 days (Figure 26).

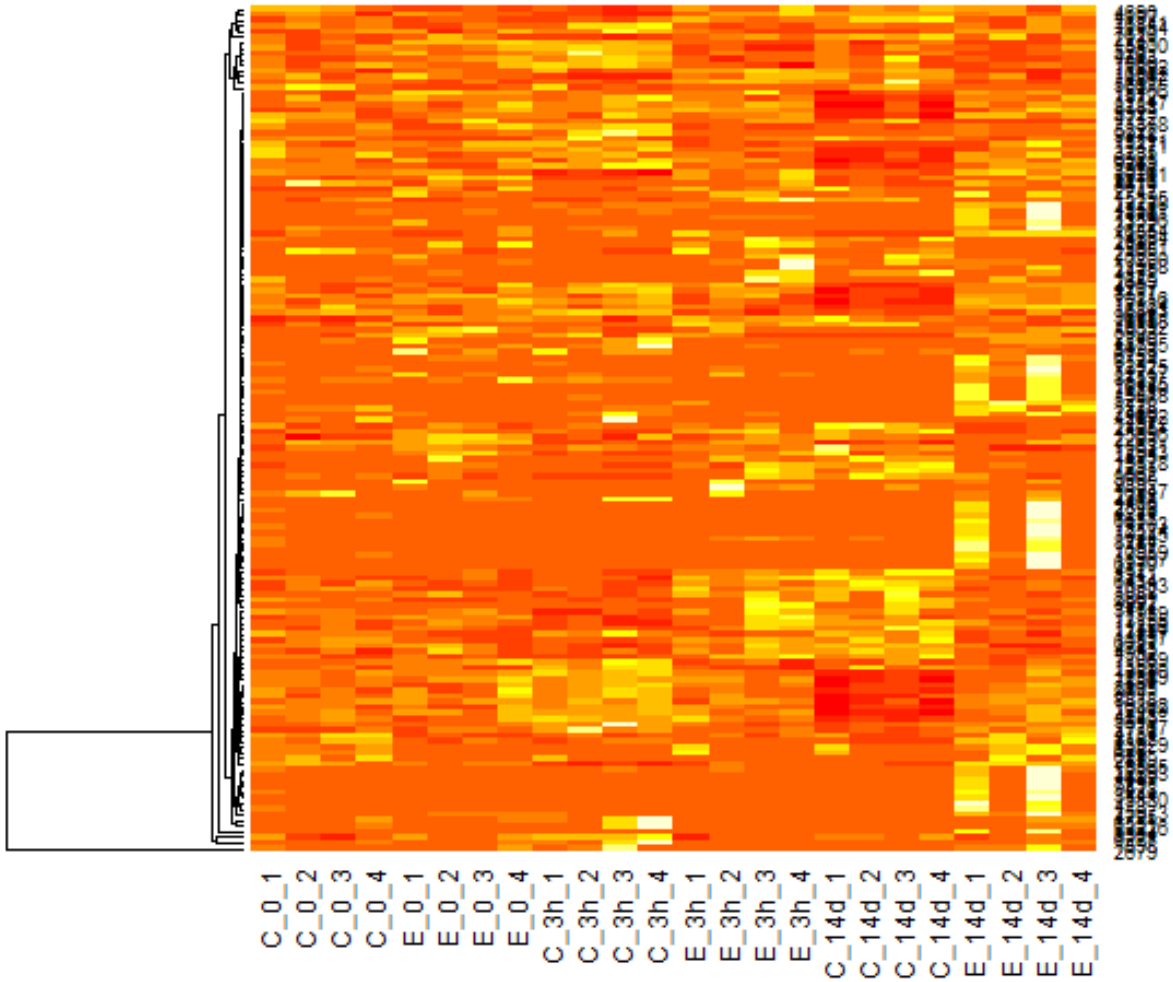


Figure 26: Heatmap of top 150 differentially expressed genes between control (C) and exposed (E) Pacific oyster gill tissues at time points 0 (0), 3 hours (3h), and 14 days (14d). Yellow/white bands are upregulated, and orange/red bands are downregulated genes.

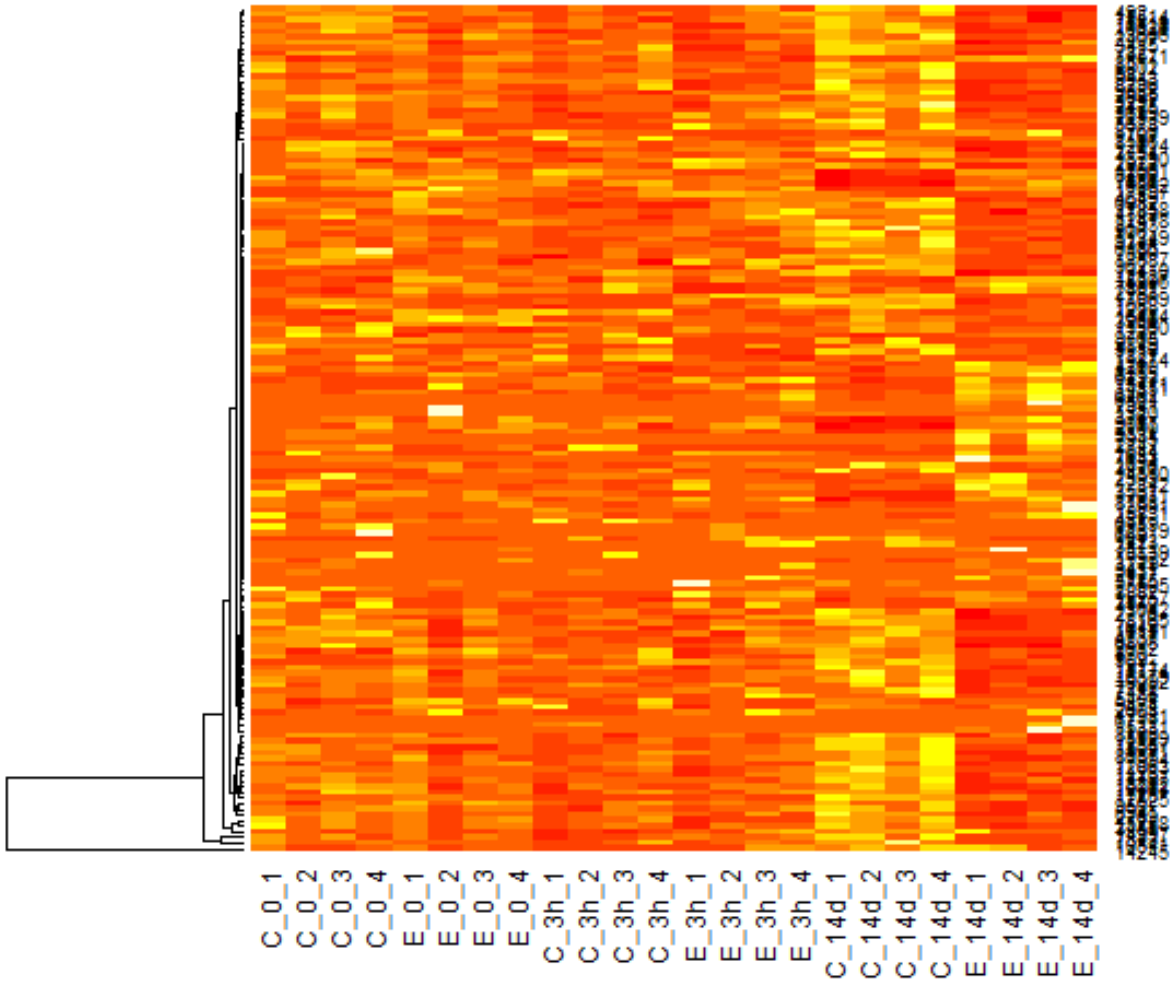


Figure 27: Heatmap of top 150 differentially expressed genes between control (C), and exposed (E) Pacific oyster digestive gland tissues at time points 0 (0), 3 hours (3h), and 14 days (14d). Yellow/white bands are upregulated, and orange/red bands are downregulated genes.

A heatmap of the top 150 DEGs ($p \leq 0.005$) in the digestive gland libraries (Figure 27) shows evidence of upregulation in the control group at 14 days (downregulation in the exposed group at 14 days).

In the gills there were 82 DEGs ($p \leq 0.015$ for each gene and fold change (FC) $\geq \pm 2$) between the time = 0 exposed and controls groups (Equation 1). There were 476 DEGs between

the time = 3 hour exposed and control groups (Equation 2), and 373 DEGs between the exposed and control groups at time = 14 days (Equation 3). In the digestive gland there were 185 DEGs at time = 0 between the exposed and control groups (Equation 1), 265 DEGs at the time = 3 hour (Equation 2), and 741 DEGs at time = 14 days (Equation 3). The numbers of up and down regulated genes in the exposed oysters in relation to the control oysters is shown in Table 8. These results show that the number of DEGs between the control and exposed oyster digestive glands increased over time, indicating that transcriptional, thus physiological, differences were increasing between the treatment groups with time of microplastic exposure. This pattern may have continued in the 30-day samples, which were unfortunately not processed due to resource limitations. In contrast, gill differential responses reached a peak after 3 hours and had decreased by day 14 of sampling.

Table 8: Total and up and down differentially expressed genes in exposed oysters compared to control Pacific oysters in gill and digestive gland tissues and time points 0, 3 hours, and 14 days.

Tissue	**Time**	**DEGs ($p \leq 0.015$, FC $\geq \pm 2$)**		
		Total	**Up**	**Down**
Gill	0 h	82	28	54
	3 h	476	245	231
	14 days	373	303	70
Digestive Gland	0 h	185	76	109
	3 h	265	127	138
	14 days	741	261	480

To select genes that were more indicative of a response to the microplastics only genes that were not significantly differentially expressed (FC ≤ 1.4) between the control and exposed groups at time zero but were significantly differentially expressed (FC ≥ 2, p value ≤ 0.015)

between the two groups at 3 hours and after 14 days of being exposed to microplastics, were selected. With these requirements in the oyster gills there were 68 DEGs at the 3-hour time point and 87 DEGs at the 14-day time point identified. In the digestive glands there were 64 DEGs at the 3-hour time point and 128 DEGs after 14 days of microplastic exposure, again showing that the number of DEGs between the control and exposed oysters increased with time, indicating that the microplastics are potentially impacting transcription.

Enrichment analysis on the upregulated DEGs (68 genes) in the gills from the exposed oysters from the 14-day time point identified the innate immune response as the most significantly enriched biological process ($p = 0.05$, Table 9). The 3 genes identified in this cluster were complement receptor type 1 (CR1), complement C1q B chain (C1QB), and cyclic GMP-AMP-synthase (MB21D1). Other immune-related genes upregulated in the exposed group at 14 days include macrophage mannose receptor 1 (Mrc1, FC 38.5, $p = 0.002$), beta-1,3-glucan binding protein (BGBP, FC 22, $p = 0.003$), and cadherin-99C (Cad99C, FC 15.6, $p = 0.002$). These results indicate that the microplastics are activating the immune system of the exposed oysters.

Table 9: Biological processes upregulated in the gills of exposed oysters at 14 days.

Category	**Term**	**count**	***p value***
Biological Process	GO:0045087~innate immune response	3	0.049
UP_KEYWORDS	Signal	9	0.005
UP_KEYWORDS	Innate immunity	3	0.015
UP_KEYWORDS	Complement pathway	2	0.022
UP_KEYWORDS	Immunity	3	0.050

The next most significantly enriched keyword in the exposed oyster gills after 14 days contained genes involved in signaling ($p = 0.005$). The 9 genes in this cluster were ganglioside GM2 activator (GM2A), low density lipoprotein receptor related protein 4 (LRP4), tyrosine-

protein kinase (TEK) also known as angiopoietin-1 receptor, complement C1q B chain (C1QB), complement C1q-like 4 (C1QL4), complement receptor type 1 (CR1), mammalian ependymin-related 1 (EPDR1), lactase-phlorizin hydrolase (LCT), and mitochondrial import receptor subunit TOM7 homolog (TOMM7). Many of these upregulated genes are involved in signaling are also involved in cell adhesion (LCT, GM2A, C1QL4, EPDR1). Other significantly upregulated genes in the exposed oysters after 14 days that are involved in cell adhesion were neuropilin-2 (Nrp2, FC 26.6, $p = 0.007$), cell adhesion molecule 2 (cadm2, FC 26.4, $p = 0.011$), mesenchyme-specific cell surface glycoprotein (msp130, FC 58.4, $p = 0$ .01), and adhesion G protein-coupled receptor L3 (ADGRL3, FC 6.4, $p = 0.008$).

Cell adhesion molecules link cells and can be involved in signaling, cell migration, tissue development and are essential components of invertebrate immune function (Johansson 1999). Upregulation of cell adhesion molecules has been found in response to pathogens in white shrimp (Lin et al. 2010), and in Pacific oysters from a high anthropogenic impact site compared to a low anthropogenic impact site (Gavery and Roberts 2012). The upregulation of cell adhesion molecules suggests the oysters exposed to the microplastics in their feed were physiologically more immunologically or environmentally stressed than those fed algae alone.

Other genes significantly upregulated in the exposed oysters gills after 14 days were involved in cytokine activity; complement C1q tumor necrosis factor-related protein 4, (C1qtnf4, FC 23.2, $p = 0.01$), defense and oxidative stress; eosinophil peroxidase (Epx, FC 4.2, $p = 0.006$), degradation; cholinesterase (B.C.HE, FC 104.2, $p = 0.015$), arylsulfatase B (Arsb, FC 8.9, $p = 0.005$), and cathepsin L1 (CTSL, FC 43.4, $p = 0.012$), and stress; heat shock protein 70 kDa protein 12A (HSPA12A, FC 5.1, $p = 0.011$), cyclin-dependent kinase 11B (Cdk11b, FC 2.1, $p = 0.014$), cysteine proteinase inhibitor 8 (OC-VIII, FC 32.6, $p = 0.014$), and a structural component

matrilin-3 (MATN3), was upregulated 50-fold (p = 0.014) (Appendix, Table A15). The upregulation of genes involved in cytokine activity, stress, and defense again suggest the microplastics are physiologically having a negative impact on the gills of the exposed oysters, even after short periods of exposure.

There were no significantly enriched functional clusters identified by DAVID from the lists of DEGs upregulated (35 genes) at 3 hours or downregulated (33 genes) at 3 hours, and 14 days (19 genes) in the exposed oysters gills. However, many genes that were upregulated in the exposed oysters at 14 days were also upregulated (but not to the same degree) after 3 hours. These were genes involved in cell adhesion; cadm2 (FC 2.03, p-value = .011), EPDR1 (FC 2.49, p = 0 .007), degradation; CTSL (FC 2.31, p = 0 .012), immunity; Mrc1 (FC 6.68, p = 0 .002), BGBP (FC 2.03, p = 0 .003), signaling and wound repair; TEK (FC 2.56, p = 0 .001), and structural constituents; MATN3 (FC 2.11, p = 0 .014). There were also genes that were still upregulated at 14 days, but the peak of upregulation occurred after only 3 hours exposure. These were genes involved in signaling; LRP4 (FC 90.8, p = 0 .007), Arsb (FC 11.01, p = 0 .005), and stress; heat shock 70 kDa protein 12 B (HSPA12B, FC 28.3, p = 0 .008). Other significantly upregulated genes in the exposed oysters after 3 hours were involved in signaling (possibly apoptosis); caprin-2 (CAPRIN2, FC 21.2, p = 0 .007), stress response to starvation, hypoxia, and bacteria; caveolin-1 (CAV1, FC 5.2, p = 0 .012), and wound healing; FGFR1 oncogene partner 2 (FGFR1OP2, FC 4.4, p = 0.001) (Appendix, Table A16).

In the digestive glands, the significantly enriched biological features upregulated (from 38 genes) in the exposed oysters after 14 days were cell adhesion and signaling (Table 10). The genes associated with these features were tenascin-R (TNR, FC 11.4, p = 0.013), protocadherin-9 (PCDH9, FC 7, p = 0.001), and protocadherin-11 Y-linked (PCDH11Y, FC 3.9, p = 0.005).

Another gene upregulated involved in cell adhesion and signaling was protocadherin-11 X-linked (PCDH11X, FC 18.9, $p = 0.0002$).

Table 10: Sequence feature, and keyword upregulated in the digestive gland of exposed oysters at 14 days.

Category	**Term**	**count**	***p value***
UP_SEQ_FEATURE	signal peptide	4	0.036
UP_KEYWORDS	Cell adhesion	3	0.005

Other highly upregulated genes in the digestive gland of exposed oysters after 14 days were involved in binding; collagen alpha-1 (II) chain (Col2a1) part of the T-cell co-signaling pathway was upregulated 96-fold ($p = 0.002$) in the exposed oysters as was myosin-IIIb (Myo3b, FC 77, $p = 0.002$), bacterial dynamin-like protein (Npun, FC 18.4, p-value = .002), and L-rhamnose-binding protein (ELEL-1, FC 6.2, $p = 0$ .005). Plasminogen (PLG) involved in degradation and inflammation was upregulated 18-fold ($p = 0.007$). Glutathione S-transferase A (GSTA) involved in stress and detoxification was upregulated 24-fold ($p = 0.007$). Aquaporin-8 (AQP8), a water channel involved in transport, was upregulated 12-fold ($p = 0.01$), and fibrillin-2 (Fbn2) a structural constituent was up 16-fold ($p = 0.007$) in the exposed oysters after 14 days (Appendix, Table A17).

Functional enrichment analysis in DAVID of the 89 downregulated DEGs did not identify any clusters with significant (≥3 genes with $p \leq 0.05$) biological process GO terms . However, further analysis of the individual genes downregulated in the exposed oysters at 14 days (or upregulated in the control oysters at 14 days) identified several highly downregulated genes with GO terms involved in reproduction (Table 11). The most highly downregulated gene

(FC -41) is involved in muscle organ development, followed by genes involved in hematopoietic progenitor cell differentiation (involved in the production of hemocytes), regulation of neuron apoptotic process (negative regulation of cell death), spermatogenesis (the development of sperm cells), luteinization (process of discharging eggs in reproduction), cell morphogenesis (cell differentiation), and liver, bone, heart, kidney and central nervous system development. This upregulation in the control samples or downregulation in the exposed oysters at 14 days was evident in the heatmap (Figures 26 and 27), and the further discovery that many of these genes are involved in reproduction indicates that the microplastics could physiologically (at the gene transcription level) be slowing or preventing maturation for reproduction in the exposed oysters.

Table 11: Biological processes of individual genes downregulated in the control oysters in the digestive glands at 14 days.

Biological Process	**uniprot**	**FC**	***p value***
GO:0007517~muscle organ development	Q6DIB5	-41	0.008
GO:0002244~hematopoietic progenitor cell differentiation	B9EKR1	-17	0.005
GO:0043523~regulation of neuron apoptotic process	A4IF63	-16	0.008
GO:0001889~liver development	Q8BXB6	-4	0.007
GO:0007283~spermatogenesis	Q9JHE4	-4	0.007
GO:0000902~cell morphogenesis	P48441	-4	0.004
GO:0006351~transcription, DNA-templated	Q86WZ6	-4	0.009
GO:0060348~bone development	Q6PFY8	-4	0.002
GO:0008202~steroid metabolic process,	P24453	-4	0.003
GO:0003382~epithelial cell morphogenesis	Q54I71	-3	0.005
GO:0007283~spermatogenesis	P07872	-3	0.001
GO:0003007~heart morphogenesis	P17336	-3	0.009
GO:0001822~kidney development	D4ACX8	-3	0.005
GO:0001553~luteinization	Q8CHN6	-3	0.002
GO:0007417~central nervous system development	P50430	-2	0.004
GO:0050679~positive regulation of epithelial cell proliferation	P11047	-2	0.014
GO:0001649~osteoblast differentiation	P00432	-2	0.002
GO:0002053~positive regulation of mesenchymal cell proliferation	O75197	-2	0.013
GO:0000910~cytokinesis	Q6GP52	-2	< 0.001
GO:0030097~hemopoiesis	Q9JLT4	-2	0.006
GO:0030148~sphingolipid biosynthetic process	Q6Y1H2	-2	0.011

In the digestive glands after 3 hours of microplastic fiber exposure there were no significantly enriched functional clusters identified using DAVID but there was an upregulation in genes with the keywords glycoprotein and signal (Table 12. Further investigation of the upregulated DEGs identified genes involved in stress; HSPA12B (FC 42.7, $p = 0.006$), signaling; LRP4 (FC 47, $p = 0.013$), parathyroid hormone 2 receptor (PTH2R, FC 11, $p = 0.01$), and corticotropin-releasing factor receptor 2 (Crhr2, FC 7.3, $p = 0.004$), cell adhesion; ganglioside GM2 activator (GM2A, FC 10.4, $p = 0.004$), binding; ELEL-1 (FC 35.6, $p = 0.004$), degradation; carboxypeptidase B (CPB1, FC 5.2, $p = 0.006$), inflammation; PLG (FC 22.8, $p = 0.007$), C3

and PZP-like alpha-2-macroglobulin domain-containing protein 8 (CPAMD8, FC 4.6, p = 0.0009), oxidizing; cytochrome P450 (Cyp2b15, FC 6.6, p = 0.008), and wound healing; FGFR1OP2 (FC 4.1, p = 0.00004) were upregulated in the exposed oysters (Appendix, Table). The upregulation of genes involved in stress, cell adhesion, degradation, inflammation, and wound healing suggest the microplastics are causing an immune or environmental stress physiological response.

Table 12: Terms upregulated in digestive glands of exposed oysters after 3 hours of microplastic exposure.

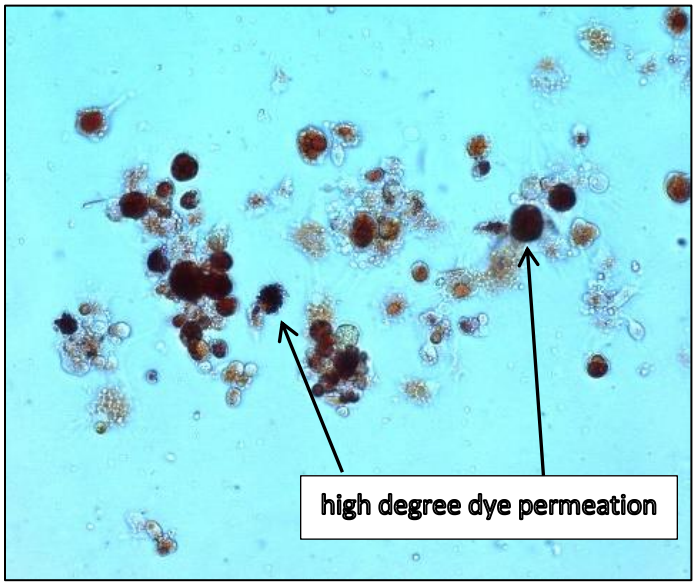
Category	**Term**	**count**	***p value***
UP_KEYWORDS	Glycoprotein	7	0.011
UP_KEYWORDS	Signal	6	0.034
UP_SEQ_FEATURE	glycosylation site: N-linked(GlcNAc…)	7	0.008
UP_SEQ_FEATURE	signal peptide	6	0.016

Two genes that were highly upregulated (FC 28 – 91) in both gill and digestive gland tissues after 3 hours, but not after 14 days, of microplastic exposure were LRP4 (signaling), and HSPA12B (stress). Genes that were upregulated in the digestive glands at both 3 hours and 14 days were ELEL-1, PCDH11X (both involved in binding), and PLG (inflammation). The consistent upregulation of genes and biological processes involved in immunity, cell adhesion, inflammation, stress, wound healing in both gill and digestive gland tissues is evidence that microplastics concentrations equivalent to 5 microplastics per litre (environmentally relevant concentrations (Desforges et al. 2014)) are initiating an immune response and are causing physiological cellular stress in oysters that could impact health and reproductive success.

Neutral Red Retention Assay

The Neutral Red Retention assay at the 0 and 3-hour time points did not show any effects of microplastic exposure on cell membrane stability (Figure 28). This assay requires hemocyte samples to be immediately processed in a time dependent manner and due to time constraints, it was difficult to process more than 12 samples in one day. Also, as the quantifying and scoring process is subjective and labour intensive this was found to be an unsuitable assay for quantifying stress in this type of experiment with the number of samples taken.

A



B

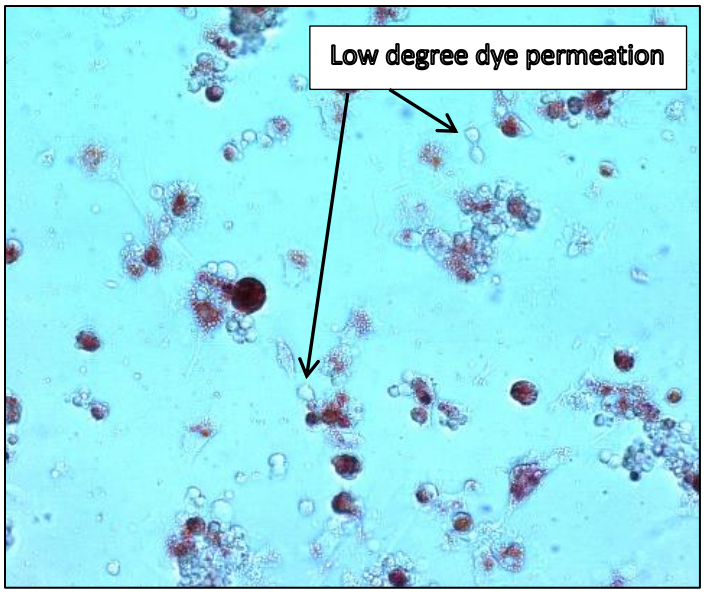


Figure 28: Hemocytes from exposed (A), and control (B) Pacific oysters after 3 hours of microplastic exposure, stained with neutral red dye, and incubated for 30 minutes (60X magnification).

3.4. Discussion

In this study, impacts of microplastic contamination in our local marine waters were assessed by comparing the cellular physiology of Pacific oysters exposed to environmentally relevant concentrations and types of microplastics over a 30-day period. Physiology was compared using condition index, survival, Neutral Red Retention assay (NRR), and gene expression analysis. Exposed oysters were fed an equivalent of 5 microplastic fibers (nylon, polypropylene, polyethylene, acrylic and polyester) per litre. This concentration, and the types of fibers of microplastics, were chosen to mimic the microplastics found in local marine waters on the east side of Vancouver Island based on scientific litreature and in companion studies which confirmed microplastic type by FTIR analyses (Desforges et al. 2014, Collicutt 2016, Covernton et al. 2019).

Our null hypothesis stating environmentally relevant concentrations and types of microplastics do not have a physiological impact on Pacific oysters when applying metrics of growth, survival and membrane stress assessments was supported. There were no statistical differences in condition index, survival, or lysosomal membrane stability observed between the two treatments. The lack of differences between condition indices and survival may be related to the short duration of the experimental study. A longer exposure time would be required to identify possible impacts in terms of condition indices and survival, and what the impacts may then be on population productivity from a growth and survival perspective. The NRR assay was found to be extremely difficult to implement in this study, due to the time taken to process each sample and the numbers of samples involved in this study. Analysis is also subjective as it involves visual determinations of membrane dye permeability, therefore requiring much skill in processing samples; our initial 3-hour findings showed no significant differences between treatments.

Our data rejects the null hypothesis stating environmentally relevant concentrations and types of microplastics do not have an impact on Pacific oyster gene expression physiology. Our data shows significantly differentially expressed genes between the exposed and control oysters in the digestive gland and gill tissues over time, indicating diverging physiologies at the transcriptomic level due to microplastic exposure. Heatmaps of these significantly differentially expressed genes gave evidence of significant physiological changes between the exposed and control group at 14 days, in both the gill and digestive glands tissues. Gill and digestive gland tissues are both suitable target tissues for pollution monitoring. Gills were chosen because they are in constant contact with their environment and therefore often used in environmental stress studies as more immediate environmental response indicators. Digestive glands were chosen as they are accumulatory organs and are used in toxicological and immunology studies to examine long-term effects (Gosling 2008, Moreira et al. 2015).

Functional enrichment analysis programs, which group the up- or downregulated genes (with a Uniprot ID, or other unique identifier) at each time point, for each tissue, into biological pathways, provided further insight into oyster health and function in response to microplastic exposure. Enriched biological processes and genes (involved in immunity, defense, degradation, stress, signaling, wound repair, structural constituents, oxidizing, and cell adhesion) had significantly increased levels of expression in the gills and digestive glands of animals exposed to microplastics compared to oysters not exposed to microplastics after only 3 hours and at 14 days of exposure.

Analysis of repeated exposure (as was the case in this study), and a recovery period, of *Mytilus galloprovincialis* exposed to polyethylene microbeads resulted in the production of stress and immune-related proteins and as a consequence a diminution of energy allocated to growth

(Detree and Gallardo-Escarate 2018). During the recovery period, there was an activation of apoptotic processes and an upregulation of immune receptors and stress related proteins (glutathione peroxidase, *hsp*70) (Detree and Gallardo-Escarate 2018). These results suggest that a recovery period after an exposure event is not sufficient to counteract the physiological stress induced by the first exposure (Detree and Gallardo-Escarate 2018), and therefore although our study exposure time was relatively short (at 30 days), there may be implications for long-term oyster health. The results of this study also point to the long-term consequences of microplastic and anthropogenic pollution on immunological and reproductive health of coastal organisms, and the possible negative population impacts on bivalve and other marine species.

The innate immune response is one of the major defense pathways and non-specific mechanisms responding to foreign bodies (xenobiotics) commonly seen in individuals exposed to air pollution (Lee et al. 2015), and have been considered appropriate biomarkers for pollution monitoring in bivalves (Skouras et al. 2003). Upregulation of genes involved in immunity suggest the microplastic fibers are activating the immune system. Cell adhesion is also an essential component of invertebrate immunology (Johansson 1999). Cell adhesion molecules are ligands (*e.g.* collagen, laminins, fibronectin) that bind to specific receptors (*e.g.* integrins, selectins, cadherins) and play a primary role in maintaining cell tissue structure, cell signaling, immunity, tissue repair and wound healing (Farahani et al. 2014). An upregulation of genes involved in adhesion and the innate immune response was also seen in Pacific oysters gills in response to salinity stress (Zhao et al. 2012), as well as in Pacific oyster hemocytes responding to *Vibrio* infections (de Lorgeril et al. 2011). A study on Pacific oysters comparing transcriptomes of oysters from low and high anthropogenic impacted harbors in Puget Sound in Washington (USA) also identified an enrichment of genes in the gills involved in cell adhesion in the oysters

from the highly impacted, more polluted harbor (Gavery and Roberts 2012). Upregulation of other defense pathways; degradation, stress, and wound repair genes also suggest the microplastics are causing cellular damage and stress, and indicates that microplastics are negatively impacting immune function, and as a consequence may have decreased levels of energy available for other processes such as growth, as seen in a recent 18 day transcriptomic study with mussels exposed to single and repeated exposures of 4.6 E +5 polyethylene microbeads per L (Detree and Gallardo-Escarate 2018).

Another very significant finding in the digestive glands of the exposed oysters was a down regulation of genes involved with biological processes associated with reproduction and growth, providing evidence that the microplastics were having a negative impact on oyster growth and reproduction. In this study the oysters were sampled in April and May, during their seasonal reproductive cycle as gametogenesis in Pacific oysters proceeds over winter months, accelerates in spring and spawnings occur July - September (Gosling 2008). Therefore, the presence of microplastics downregulating reproductive genes may have an impact on oyster reproductive capacity and long-term population stability. This corroborates with negative effects on reproductive health (decreased oocyte number, diameter, and sperm velocity, larval yield and development) seen in Pacific oyster larvae exposed to virgin micro-polystyrene 2 and 6 μm in diameter at microplastic concentrations of 0.023 mg per L (Sussarellu et al. 2016). Our study therefore supports this finding and suggests potential long-term negative implications on the health, reproduction and survival of oysters, which would have an effect on natural and cultured populations of oysters, especially if microplastic contamination in our local waters increases.

A gene expression study identified a signature associated with unexplained mass mortality events occurring during the summer months in adult Pacific oysters, finding an upregulation of

genes associated with cell death, lysosomal, proteolysis, and cellular assembly and organization (Chaney and Gracey 2011). Four genes upregulated in the mortality signature were shared among genes upregulated in the Pacific oysters exposed to microplastics in our study. These genes were cathepsin L (upregulated 43-fold in the exposed gills), and ras-related protein (upregulated 3-fold in the gills), and in the digestive glands of the exposed oysters there was myosin (upregulated 76-fold in exposed oysters), and Sushi von-Willebrand factor type A (upregulated 2-fold in exposed oysters). The activation of these genes in our samples may mean long-term negative or potentially fatal effects due to microplastic exposure.

These results suggest that even at very low environmentally relevant concentrations and with short exposure times, microplastics can cause changes in the transcription of genes with potential long-term impacts. Our study did not observe any changes at the cellular/morphological level or impacts on condition or visual examinations of health; this may be a function of the short duration of the experiment (30 days). At the gene expression level, the presence of microplastics did upregulate several biological pathways associated with oyster cellular defense strategies in the gill and digestive gland tissues. Microplastics also resulted in a shift in energy allocations in the digestive glands of exposed oysters from gametogenesis and reproduction to biological pathways involved in stress, detoxification, and inflammation. A reduction in oyster reproduction would have serious implications in terms of oyster productivity and survival, negatively affecting one of the largest aquaculture industries worldwide. These findings highlight the potential risk for aquatic organism health with long-term microplastic particle exposure. Future studies should examine longer-term exposure effects on growth, survival and body condition as well as any accumulation over time, which may be of concern to human consumers of shellfish products. In addition, longer-term studies are required to examine the impacts of microplastics on the full

reproductive cycle of marine bivalves, with implications for population productivity and the shellfish aquaculture industry. This study employed naïve microplastics, whereas in the marine environment biofilms and adhered, adsorbed or leaching xenobiotic pollutants may increase the health implications of microplastic exposure for both bivalves and consumers of bivalves. Future studies should examine the health implications of a range of concurrent xenobiotic pollutants on marine organisms, to provide an overall context of microplastic impact exposure. Microplastics represent one of the range of plastics with implications for marine ecosystems, but future studies should also address the possibility of tissue translocation of smaller microplastics and nanoplastics on organismal health and function. The significantly up and down regulated genes in this study due to microplastic exposure, are potential biomarkers for future use in microplastic, xenobiotic, and pollution biomonitoring.

Chapter 4: Conclusions

Anthropogenic alterations of marine habitats and ecosystems are threatening the biological diversity of species throughout the planet. Although modifications to enhance habitats and productivity of natural species have existed for centuries, the negative effects of human activities on habitats and ecosystems have become a global concern. Transcriptomics, also known as gene expression analysis, is a powerful approach for exploring organismal cellular physiological responses to environmental changes. This technique was used in this thesis to investigate the impacts of two different types of anthropogenic modifications of marine habitats on shellfish physiology.

In chapter 2 physiological differences between Littleneck clams in clam garden beaches compared to clams from reference beaches was investigated. Clam gardens are examples of ancient anthropogenic modifications built to enhance clam habitat and productivity, to help maintain a secure and reliable food source for the northwest coastal people of North America. There were no significant differences found between clam garden and reference beaches in terms of growth and survival of transplanted clams. There were also no significant differences in sediment carbonate, organic content, or grain size distributions between the sediment from clam garden beaches compared to reference beaches. An interesting finding in this study was a significant negative correlation between sediment carbonate content and survival; the beaches with the lowest carbonate content had the highest survival, and the beaches with the highest carbonate content had the lowest survival.

RNA sequencing of tissue samples from the gill and digestive glands of surviving individuals gave evidence of significant transcriptional differences between the two groups. Although there were differentially expressed biological pathways in clams from clam gardens

and reference beaches, most pathways in both groups were associated with environmental stress suggesting both habitats contained their own unique multiple environmental influences, and further investigations is needed to identify the potentially unique combinations of stressors associated with each type of habitat.

RNA sequencing of the Littleneck clams also revealed several highly upregulated, and potentially complete, viral transcripts from different viruses from the Dicistroviridae Family (Picornaviral Order) of single stranded RNA viruses, that had significant correlations with survival and geographical proximity. Clams from nearby beaches with high survival contained viral transcripts from both Cricket paralysis, and Nora virus, and other beaches adjacent to each other with low survival contained Acute bee paralysis viral transcripts, and Cricket paralysis virus. However, it should be noted that the clam garden beaches used in this thesis were not actively managed and therefore these study findings may not truly represent conditions found in a clam garden in active use.

In chapter 3 the impacts of environmentally relevant concentrations and types of microplastics on Pacific oyster physiology was investigated. Microplastics are emerging anthropogenic pollutants contaminating aquatic and terrestrial habitats worldwide. Pacific oysters were exposed to environmentally relevant concentrations of microplastics (5 microplastic fibers per litre). Condition index, microplastic load, lysosomal membrane stability, and gene expression analysis were metrics utilized to assess impacts of microplastic contamination. There were no significant differences observed in condition index or lysosomal membrane stability, which may be related to study length. There were however significant differences in microplastic load and gene expression between the exposed and control oysters. In the exposed oysters there was an upregulation in biological pathways associated with immunity and stress, and a

downregulation in pathways associated with reproduction, corroborating with previous microplastic research and highlighting the potential long-term negative consequences in terms of species survival and diversity, especially if marine microplastic concentrations continue to escalate.

In conclusion, this thesis provides insights into ancient anthropogenic mariculture intertidal modifications for habitat enhancement to increase clam productivity and provides evidence of negative impacts of microplastic contamination on shellfish physiology. It also demonstrates the power of RNA sequencing in biomonitoring for its increased sensitivity over metrics such as growth, survival, and lysosomal stability in assessing cellular physiological impacts, particularly in short-term experimentation. It also highlights benefits of using such techniques to detect potential particular biotic stressors and implications for long-term population stability and be utilized in non-model organisms without any prior genomic or transcriptional knowledge.

Bibliography

Airoldi, L., Beck, M.W. (2007). "Loss status and trends for coastal marine habitats of europe." Oceanography and Marine Biology: An Annual Review **45**: 345-405.

Avio, C. G., S. Gorbi, M. Milan, M. Benedetti, D. Fattorini, G. d'Errico, M. Pauletto, L. Bargelloni and F. Regoli (2015). "Pollutants bioavailability and toxicological risk from microplastics to marine mussels." Environ Pollut **198**: 211-222.

Barbier, E. B. (2017). "Marine ecosystem services." Curr Biol **27**(11): R507-R510.

Barboza, L. G. A., L. R. Vieira, V. Branco, N. Figueiredo, F. Carvalho, C. Carvalho and L. Guilhermino (2018). "Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the european seabass, dicentrarchus labrax (linnaeus, 1758)." Aquat Toxicol **195**: 49-57.

Barnes, D. K., F. Galgani, R. C. Thompson and M. Barlaz (2009). "Accumulation and fragmentation of plastic debris in global environments." Philos Trans R Soc Lond B Biol Sci **364**(1526): 1985-1998.

Barrows, A. P. W., Neumann, C.A., Pieper, C., Berger, M.L., Shaw, S.D. (2017). A guide to microplastics identification: A comprehensive methods guide for microplastics identification and quantification in the laboratory. M. a. E. Research. Blue Hill, ME.

Bates, D., M. Mächler, B. Bolker and S. Walker (2015). "Fitting linear mixed-effects models usinglme4." Journal of Statistical Software **67**(1).

Bernard, F. R. (1983). Physiology and the mariculture of some northeastern pacific bivalve molluscs. Canadian Special Publication of Fisheries and Aquatic Sciences.

Besseling, E., A. Wegner, E. M. Foekema, M. J. van den Heuvel-Greve and A. A. Koelmans (2013). "Effects of microplastic on fitness and pcb bioaccumulation by the lugworm *arenicola marina* (l.)." Environ Sci Technol **47**(1): 593-600.

Bonning, B. C. (2009). "The dicistroviridae: An emerging family of invertebrate viruses." Virologica Sinica **24**(5): 415-427.

Browne, M. A., P. Crump, S. J. Niven, E. Teuten, A. Tonkin, T. Galloway and R. Thompson (2011). "Accumulation of microplastic on shorelines woldwide: Sources and sinks." Environ Sci Technol **45**(21): 9175-9179.

Browne, M. A., A. Dissanayake, T. S. Galloway, D. M. Lowe and R. C. Thompson (2008). "Ingested microscopic plastic translocates to the circulatory system of the mussel, *mytilus edulis* (l)." Environ Sci Technol **42**(13): 5026-5031.

Browne, M. A., S. J. Niven, T. S. Galloway, S. J. Rowland and R. C. Thompson (2013). "Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity." Curr Biol **23**(23): 2388-2392.

Browne, M. A., A. J. Underwood, M. G. Chapman, R. Williams, R. C. Thompson and J. A. van Franeker (2015). "Linking effects of anthropogenic debris to ecological impacts." Proc Biol Sci **282**(1807): 20142929.

Caldwell, M. E., D. Lepofsky, G. Combes, M. Washington, J. R. Welch and J. R. Harper (2012). "A bird's eye view of northern coast salish intertidal resource management features, southern british columbia, canada." The Journal of Island and Coastal Archaeology **7**(2): 219-233.

Carella, F., S. Aceto, P. Maiolino and G. De Vico (2011). "What is your diagnosis? Pale yellowish digestive gland and watery tissues in mediterranean mussels." Vet Clin Pathol **40**(2): 273-274.

Carvajal-Gonzalez, J. M. and M. Mlodzik (2014). "Mechanisms of planar cell polarity establishment in *drosophila*." F1000Prime Rep **6**: 98.

Chaney, M. L. and A. Y. Gracey (2011). "Mass mortality in pacific oysters is associated with a specific gene expression signature." Mol Ecol **20**(14): 2942-2954.

Cole, M. and T. S. Galloway (2015). "Ingestion of nanoplastics and microplastics by pacific oyster larvae." Environ Sci Technol **49**(24): 14625-14632.

Cole, M., P. Lindeque, E. Fileman, C. Halsband and T. S. Galloway (2015). "The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod *calanus helgolandicus*." Environ Sci Technol **49**(2): 1130-1137.

Cole, M., P. Lindeque, E. Fileman, C. Halsband, R. Goodhead, J. Moger and T. S. Galloway (2013). "Microplastic ingestion by zooplankton." Environ Sci Technol **47**(12): 6646-6655.

Cole, M., P. Lindeque, C. Halsband and T. S. Galloway (2011). "Microplastics as contaminants in the marine environment: A review." Mar Pollut Bull **62**(12): 2588-2597.

Collicutt, B. (2016). The anthropogenic influence of shellfish aquaculture and microplastics on juvenile pacific salmon on the east coast of vancouver island. MSc, University of Victoria.

Conesa, A., P. Madrigal, S. Tarazona, D. Gomez-Cabrero, A. Cervera, A. McPherson, M. W. Szczesniak, D. J. Gaffney, L. L. Elo, X. Zhang and A. Mortazavi (2016). "A survey of best practices for rna-seq data analysis." Genome Biol **17**: 13.

Covernton, G. C., B. Collicut, H. J. Gurney-Smith, C. M. Pearce, J. F. Dower, P. S. Ross and S. E. Dudas (2019). "Microplastics in bivalves and their habitat in relation to shellfish aquaculture proximity in coastal British Columbia, Canada." Aquaculture Environment Interactions. *In press.*

Davidson, K. and S. E. Dudas (2016). "Microplastic ingestion by wild and cultured manila clams (*venerupis philippinarum*) from baynes sound, british columbia." Arch Environ Contam Toxicol.

de Lorgeril, J., R. Zenagui, R. D. Rosa, D. Piquemal and E. Bachere (2011). "Whole transcriptome profiling of successful immune response to *vibrio* infections in the oyster *crassostrea gigas* by digital gene expression analysis." PLoS One **6**(8): e23142.

Desforges, J. P., M. Galbraith, N. Dangerfield and P. S. Ross (2014). "Widespread distribution of microplastics in subsurface seawater in the ne pacific ocean." Mar Pollut Bull **79**(1-2): 94-99.

Detree, C. and C. Gallardo-Escarate (2018). "Single and repetitive microplastics exposures induce immune system modulation and homeostasis alteration in the edible mussel *mytilus galloprovincialis*." Fish Shellfish Immunol **83**: 52-60.

Detree, C., G. Nunez-Acuna, S. Roberts and C. Gallardo-Escarate (2016). "Uncovering the complex transcriptome response of mytilus chilensis against saxitoxin: Implications of harmful algal blooms on mussel populations." PLoS One **11**(10): e0165231.

Deur, D., A. Dick, K. Recalma-Clutesi and N. J. Turner (2015). "Kwakwaka'wakw "clam gardens"." Human Ecology **43**(2): 201-212.

Doyle, B. (2016). Investigating a mitigation strategy for acidic sediment conditions in support of first nation bivalve restoration initiatives: The effect of shell hash on porewater ph and carbonate saturation states in burrard inlet, british columbia, canada MSc, Royal Roads University, Victoria, B.C.

Dumbauld, B. R., J. L. Ruesink and S. S. Rumrill (2009). "The ecological role of bivalve shellfish aquaculture in the estuarine environment: A review with application to oyster and clam culture in west coast (USA) estuaries." Aquaculture **290**(3-4): 196-223.

EFSA (2016). "Presence of microplastics and nanoplastics in food, with particular focus on seafood." EFSA Journal **14**(6): 30.

Evans, W., J. T. Mathis and J. N. Cross (2014). "Calcium carbonate corrosivity in an alaskan inland sea." Biogeosciences **11**(2): 365-379.

Farahani, E., H. K. Patra, J. R. Jangamreddy, I. Rashedi, M. Kawalec, R. K. Rao Pariti, P. Batakis and E. Wiechec (2014). "Cell adhesion molecules and their relation to (cancer) cell stemness." Carcinogenesis **35**(4): 747-759.

Farrell, P. and K. Nelson (2013). "Trophic level transfer of microplastic: *Mytilus edulis* (l.) to *carcinus maenas* (l.)." Environ Pollut **177**: 1-3.

Ferner, M. C., D. L. Smee and M. J. Weissburg (2009). "Habitat complexity alters lethal and non-lethal olfactory interactions between predators and prey." Marine Ecology Progress Series **374**: 13-22.

Forrest, B. M., N. B. Keeley, G. A. Hopkins, S. C. Webb and D. M. Clement (2009). "Bivalve aquaculture in estuaries: Review and synthesis of oyster cultivation effects." Aquaculture **298**(1-2): 1-15.

Gago, J., O. Carretero, A. V. Filgueiras and L. Viñas (2018). "Synthetic microfibers in the marine environment: A review on their occurrence in seawater and sediments." Marine Pollution Bulletin **127**: 365-376.

Gallardi, D. (2014). "Effects of bivalve aquaculture on the environment and their possible mitigation: A review." Fisheries and Aquaculture Journal **05**(03).

Gavery, M. R. and S. B. Roberts (2012). "Characterizing short read sequencing for gene discovery and rna-seq analysis in *crassostrea gigas*." Comp Biochem Physiol Part D Genomics Proteomics **7**(2): 94-99.

GESAMP (2016). Sources, fate and effects of microplastics in the marine environment: Part 2 of a global assessment (kershaw, p.J., and rochman, c.M., eds). .
.
(IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection). Rep. Stud. GESAMP No.93, 220p.

.
Gosling, E. (2008). Bivalve molluscs: Biology, ecology and culture, Wiley.

Greaves, D. R. and S. Gordon (2005). "Thematic review series: The immune system and atherogenesis. Recent insights into the biology of macrophage scavenger receptors." J Lipid Res **46**(1): 11-20.

Green, M. A., G. G. Waldbusser, L. Hubazc, E. Cathcart and J. Hall (2012). "Carbonate mineral saturation state as the recruitment cue for settling bivalves in marine muds." Estuaries and Coasts **36**(1): 18-27.

Groesbeck, A. S., K. Rowell, D. Lepofsky and A. K. Salomon (2014). "Ancient clam gardens increased shellfish production: Adaptive strategies from the past can inform food security today." PLoS One **9**(3): e91235.

Guerra, A., J. Belinha and R. N. Jorge (2018). "Modelling skin wound healing angiogenesis: A review." J Theor Biol **459**: 1-17.

Hamza-Chaffai, A. (2014). "Usefulness of bioindicators and biomarkers in pollution biomonitoring." International Journal of Biotechnology for Wellness Industries **3**(1): 19-26.

Hazelwood, L. D. and J. M. Hancock (2013). "Functional modelling of planar cell polarity: An approach for identifying molecular function." BMC Dev Biol **13**: 20.

Hinojosa, I. A. and M. Thiel (2009). "Floating marine debris in fjords, gulfs and channels of southern chile." Mar Pollut Bull **58**(3): 341-350.

Holmer, M., D. Wildish and B. Hargrave (2005). Organic enrichment from marine finfish aquaculture and effects on sediment biogeochemical processes. Environmental effects of marine finfish aquaculture**:** 181-206.

Huang da, W., B. T. Sherman and R. A. Lempicki (2009). "Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists." Nucleic Acids Res **37**(1): 1-13.

Huang, D. W., B. T. Sherman and R. A. Lempicki (2008). "Systematic and integrative analysis of large gene lists using david bioinformatics resources." Nature Protocols **4**: 44.

Huang, D. W., B. T. Sherman, Q. Tan, J. Kir, D. Liu, D. Bryant, Y. Guo, R. Stephens, M. W. Baseler, H. C. Lane and R. A. Lempicki (2007). "David bioinformatics resources: Expanded annotation database and novel algorithms to better extract biology from large gene lists." Nucleic Acids Res **35**(Web Server issue): W169-175.

Iniguez, M. E., J. A. Conesa and A. Fullana (2017). "Microplastics in spanish table salt." Sci Rep **7**(1): 8620.

Jackley, J., L. Gardner, A. F. Djunaedi and A. K. Salomon (2016). "Ancient clam gardens, traditional management portfolios, and the resilience of coupled human-ocean systems." Ecology and Society **21**(4).

Jang, M., W. J. Shim, G. M. Han, M. Rani, Y. K. Song and S. H. Hong (2017). "Widespread detection of a brominated flame retardant, hexabromocyclododecane, in expanded polystyrene marine debris and microplastics from south korea and the asia-pacific coastal region." Environ Pollut **231**(Pt 1): 785-794.

Johansson, M. W. (1999). "Cell adhesion molecules in invertebrate immunity." Dev Comp Immunol **23**(4-5): 303-315.

Johnson, E. S. (2002). "Ubiquitin branches out." Nat Cell Biol **4**(12): E295-298.

Kettner, M. T., K. Rojas-Jimenez, S. Oberbeckmann, M. Labrenz and H. P. Grossart (2017). "Microplastics alter composition of fungal communities in aquatic ecosystems." Environ Microbiol **19**(11): 4447-4459.

Kirstein, I. V., S. Kirmizi, A. Wichels, A. Garin-Fernandez, R. Erler, M. Loder and G. Gerdts (2016). "Dangerous hitchhikers? Evidence for potentially pathogenic vibrio spp. On microplastic particles." Mar Environ Res **120**: 1-8.

Lee, A., P. Kinney, S. Chillrud and D. Jack (2015). "A systematic review of innate immunomodulatory effects of household air pollution secondary to the burning of biomass fuels." Ann Glob Health **81**(3): 368-374.

Lenz, R., K. Enders and T. G. Nielsen (2016). "Microplastic exposure studies should be environmentally realistic." Proc Natl Acad Sci U S A **113**(29): E4121-4122.

Lepofsky, D., N. F. Smith, N. Cardinal, J. Harper, M. Morris, Gitla, R. Bouchard, D. I. D. Kennedy, A. K. Salomon, M. Puckett and K. Rowell (2017). "Ancient shellfish mariculture on the northwest coast of north america." American Antiquity **80**(02): 236-259.

Leung, J. and K. Y. K. Chan (2017). "Microplastics reduced posterior segment regeneration rate of the polychaete *perinereis aibuhitensis*." Mar Pollut Bull.

Li, Q., X. Zhao, K. Kong and H. Yu (2013). "Transcriptomic response to stress in marine bivalves." Invertebrate Survival Journal **10**: 84-93.

Lin, Y., Z. X. Chen, B. Oliver and S. T. Harbison (2016). "Microenvironmental gene expression plasticity among individual drosophila melanogaster." G3 (Bethesda) **6**(12): 4197-4210.

Lin, Y. C., C. M. Tayag, C. L. Huang, W. C. Tsui and J. C. Chen (2010). "White shrimp litopenaeus vannamei that had received the hot-water extract of spirulina platensis showed earlier recovery in immunity and upregulation of gene expressions after ph stress." Fish Shellfish Immunol **29**(6): 1092-1098.

Lopez, W., A. M. Page, D. J. Carlson, B. L. Ericson, M. F. Cserhati, C. Guda and K. A. Carlson (2018). "Analysis of immune-related genes during nora virus infection of *drosophila melanogaster* using next generation sequencing." AIMS Microbiol **4**(1): 123-139.

Lowe, D. M., M. N. Moore and B. M. Evans (1992). "Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab limanda limanda." Marine Ecology Progress Series **91**: 135-140.

Lowe, D. M., C. Soverchia and M. N. Moore (1995). "Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene." Aquatic Toxicology **33**: 105-112.

Lu, Y., Y. Zhang, Y. Deng, W. Jiang, Y. Zhao, J. Geng, L. Ding and H. Ren (2016). "Uptake and accumulation of polystyrene microplastics in zebrafish (*danio rerio*) and toxic effects in liver." Environ Sci Technol **50**(7): 4054-4060.

Mak, T. W. and M. E. Saunders (2006). List of abbreviations. The immune response. Burlington, Academic Press**:** VII.

Martinez-Gomez, C., J. Bignell and D. Low (2015). Lysosomal membrane stability in mussels. Techniques in Marine Environmental Sciences. ICES. **56:** 41 pp.

Martinez-Gomez, C., V. M. Leon, S. Calles, M. Gomariz-Olcina and A. D. Vethaak (2017). "The adverse effects of virgin microplastics on the fertilization and larval development of sea urchins." Mar Environ Res **130**: 69-76.

Mathalon, A. and P. Hill (2014). "Microplastic fibers in the intertidal ecosystem surrounding halifax harbor, nova scotia." Mar Pollut Bull **81**(1): 69-79.

Meng, J., Q. Zhu, L. Zhang, C. Li, L. Li, Z. She, B. Huang and G. Zhang (2013). "Genome and transcriptome analyses provide insight into the euryhaline adaptation mechanism of *crassostrea gigas*." PLoS One **8**(3): e58563.

Mercaldo-Allen, R. and R. Goldberg (2011). Review-ecological-effects-of-dredging-to-harvest-molluscs. Massachusetts, NOAA.

Messinetti, S., S. Mercurio, M. Parolini, M. Sugni and R. Pennati (2017). "Effects of polystyrene microplastics on early stages of two marine invertebrates with different feeding strategies." Environ Pollut.

Méthé, D., L. A. Comeau, H. Stryhn, J. F. Burka, T. Landry and J. Davidson (2017). "Haemolymph fluid osmolality influences the neutral-red retention assay in the eastern oystercrassostrea virginica." Journal of Molluscan Studies.

Milan, M., A. Coppe, R. Reinhardt, L. Cancela, R. B. Leite, C. C. Saavedra, G. Chelazzi, S. Bortoluzzi and L. Bargelloni (2011). "Transcriptome sequencing and microarray development for the manila clam, *ruditapes philippinarum:* Genomic tools for environmental monitoring." BMC Genomics **12**: 234.

Miller, K. M., S. Li, K. H. Kaukinen, N. Ginther, E. Hammill, M. R. C. Janelle, D. A. Patterson, T. Sierocinski, L. Donnison, P. Pavlidis, S. G. Hinch, K. A. Hruska, S. J. Cooke, K. K. English and A. P. Farrell (2011). "Genomic signatures predict migration and spawning failure in wild canadian salmon." Science **331**(6014): 214-217.

Moreira, R., P. Pereiro, C. Canchaya, D. Posada, A. Figueras and B. Novoa (2015). "Rna-seq in *mytilus galloprovincialis:* Comparative transcriptomics and expression profiles among different tissues." BMC Genomics **16**: 728.

Mosbahi, N., J. P. Pezy, J. C. Dauvin and L. Neifar (2016). "Immediate effect of clam harvesting on intertidal benthic communities in the mudflat zones of kneiss islands (central mediterranean sea)." Journal of Aquaculture Research & Development **7**(11).

Moss, M. L. (2013). Fishing traps and weirs on the northwest coast of north america: New approaches and insights. In *oxford handbook of wetland archaeology.* Oxford, Oxford University Press.

Munari, C., E. Balasso, R. Rossi and M. Mistri (2006). "A comparison of the effect of different types of clam rakes on non-target, subtidal benthic fauna." Italian Journal of Zoology **73**(1): 75-82.

Munroe, D. (2016). "Habitat effects on early post-settlement growth of intertidal clams,venerupis philippinarum(a. Adams & reeve, 1850)." Journal of Molluscan Studies **82**(4): 507-514.

Murphy, F., C. Ewins, F. Carbonnier and B. Quinn (2016). "Wastewater treatment works (wwtw) as a source of microplastics in the aquatic environment." Environ Sci Technol **50**(11): 5800-5808.

Nagano, A. J., M. N. Honjo, M. Mihara, M. Sato and H. Kudoh (2015). "Detection of plant viruses in natural environments by using rna-seq." Methods Mol Biol **1236**: 89-98.

Neudorf, C. M., N. Smith, D. Lepofsky, G. Toniello and O. B. Lian (2017). "Between a rock and a soft place: Using optical ages to date ancient clam gardens on the pacific northwest." PLoS One **12**(2): e0171775.

Norkko, J. and S. E. Shumway (2011). Bivalves as bioturbators and bioirrigators. Shellfish aquaculture and the environment. S. E. Shumway, John Wiley & Sons, Inc.

NRCC (2010). 7 ecosystem services of bivalves: Implications for restoration. Washington, DC, The National Academies Press.

Oberbeckmann, S., B. Kreikemeyer and M. Labrenz (2017). "Environmental factors support the formation of specific bacterial assemblages on microplastics." Front Microbiol **8**: 2709.

Oberbeckmann, S., M. G. J. Löder and M. Labrenz (2015). "Marine microplastic-associated biofilms – a review." Environmental Chemistry **12**(5).

Pickart, C. M. and M. J. Eddins (2004). "Ubiquitin: Structures, functions, mechanisms." Biochim Biophys Acta **1695**(1-3): 55-72.

PlasticsEurope (2016). Plastic-the facts 2016: An analysis of european plastics production, demand and waste data. Brussels, Belgium.

Rainer, J. S., Mann, R. (1992). "A comparison of methods for calculating condition index in eastern oysters, *crassostrea gigas* (gmelin, 1791)." Journal of Shellfish Research **11**: 55-58.

RCoreTeam (2015). R: A language and environment for statistical computing. R. F. f. S. Computing. Vienna, Austria.

Ren, J. S., A. H. Ross and D. R. Schiel (2000). "Functional descriptions of feeding and energetics of the pacific oyster *crassostrea gigas* in new zealand." Marine Ecology Progress Series **208**: 119-130.

Robinson, M. D., D. J. McCarthy and G. K. Smyth (2010). "Edger: A bioconductor package for differential expression analysis of digital gene expression data." Bioinformatics **26**: 139-140.

Rothschild, B. J., J. S. Ault, P. Goulletquer and M. Heral (1994). "Decline of the chesapeak bay oyster population: A century of habitat destruction and overfishing." Marine Ecology Progress Series **111**: 29-39.

Salter, N. (2018). Ancient clam gardens magnify bivalve production by moderating ambient termperature and enhancing sediment carbonate. MSc, Simon Fraser University.

Santana, M. F. M., F. T. Moreira, C. D. S. Pereira, D. M. S. Abessa and A. Turra (2018). "Continuous exposure to microplastics does not cause physiological effects in the cultivated mussel *perna perna*." Arch Environ Contam Toxicol **74**(4): 594-604.

Santana, M. F. M., F. T. Moreira and A. Turra (2017). "Trophic transference of microplastics under a low exposure scenario: Insights on the likelihood of particle cascading along marine food-webs." Marine Pollution Bulletin **121**(1): 154-159.

Schurch, N. J., P. Schofield, M. Gierlinski, C. Cole, A. Sherstnev, V. Singh, N. Wrobel, K. Gharbi, G. G. Simpson, T. Owen-Hughes, M. Blaxter and G. J. Barton (2016). "How many biological replicates are needed in an rna-seq experiment and which differential expression tool should you use?" RNA **22**(6): 839-851.

Shahidul Islam, M. and M. Tanaka (2004). "Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: A review and synthesis." Mar Pollut Bull **48**(7-8): 624-649.

Sheaves, M., R. Baker, I. Nagelkerken and R. M. Connolly (2014). "True value of estuarine and coastal nurseries for fish: Incorporating complexity and dynamics." Estuaries and Coasts **38**(2): 401-414.

Sims, D., I. Sudbery, N. E. Ilott, A. Heger and C. P. Ponting (2014). "Sequencing depth and coverage: Key considerations in genomic analyses." Nat Rev Genet **15**(2): 121-132.

Skouras, A., K. Broeg, H. Dizer, H. von Westernhagen, P.-D. Hansen and D. Steinhagen (2003). "The use of innate immune responses as biomarkers in a programme of integrated biological effects monitoring on flounder (platichthys flesus) from the southern north sea." Helgoland Marine Research **57**(3-4): 190-198.

Sleight, V. A., A. Bakir, R. C. Thompson and T. B. Henry (2017). "Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish." Mar Pollut Bull **116**(1-2): 291-297.

Smith, C., H. J. Gurney-Smith, K. Mohns, M. Raap and W. Evans (unpublished). Immune response of *Mytilus edulis* (l.) hemocytes to changing coastal ocean conditions on Quadra Island, B.C.

Smith, S., L. Bernatchez and L. B. Beheregaray (2013). "Rna-seq analysis reveals extensive transcriptional plasticity to temperature stress in a freshwater fish species." BMC Genomics **14**: 375.

Smokorowski, K. E. and T. C. Pratt (2007). "Effect of a change in physical structure and cover on fish and fish habitat in freshwater ecosystems – a review and meta-analysis." Environmental Reviews **15**(NA): 15-41.

Suarez Ulloa, M. V. (2017). Transcriptomic and epigenetic responses to environmental stress in marine bivalves with a focus on harmful algal blooms.

Sun, L. and Z. J. Chen (2004). "The novel functions of ubiquitination in signaling." Curr Opin Cell Biol **16**(2): 119-126.

Sussarellu, R., M. Suquet, Y. Thomas, C. Lambert, C. Fabioux, M. E. Pernet, N. Le Goic, V. Quillien, C. Mingant, Y. Epelboin, C. Corporeau, J. Guyomarch, J. Robbens, I. Paul-Pont, P. Soudant and A. Huvet (2016). "Oyster reproduction is affected by exposure to polystyrene microplastics." Proc Natl Acad Sci U S A **113**(9): 2430-2435.

Tarazona, S., F. Garcia-Alcalde, J. Dopazo, A. Ferrer and A. Conesa (2011). "Differential expression in rna-seq: A matter of depth." Genome Res **21**(12): 2213-2223.

Taylor, M. L., C. Gwinnett, L. F. Robinson and L. C. Woodall (2016). "Plastic microfiber ingestion by deep-sea organisms." Sci Rep **6**: 33997.

Teuten, E. L., J. M. Saquing, D. R. Knappe, M. A. Barlaz, S. Jonsson, A. Bjorn, S. J. Rowland, R. C. Thompson, T. S. Galloway, R. Yamashita, D. Ochi, Y. Watanuki, C. Moore, P. H. Viet, T. S. Tana, M. Prudente, R. Boonyatumanond, M. P. Zakaria, K. Akkhavong, Y. Ogata, H. Hirai, S. Iwasa, K. Mizukawa, Y. Hagino, A. Imamura, M. Saha and H. Takada (2009). "Transport and release of chemicals from plastics to the environment and to wildlife." Philos Trans R Soc Lond B Biol Sci **364**(1526): 2027-2045.

Thushari, G. G. N., J. D. M. Senevirathna, A. Yakupitiyage and S. Chavanich (2017). "Effects of microplastics on sessile invertebrates in the eastern coast of thailand: An approach to coastal zone conservation." Mar Pollut Bull **124**(1): 349-355.

Van Cauwenberghe, L., M. Claessens and C. R. Janssen (2013). "Selective uptake of microplastics by a marine bivalve *(mytilus edulis*)." Commun Agric Appl Biol Sci **78**(1): 25-27.

Van Cauwenberghe, L. and C. R. Janssen (2014). "Microplastics in bivalves cultured for human consumption." Environ Pollut **193**: 65-70.

Virsek, M. K., M. N. Lovsin, S. Koren, A. Krzan and M. Peterlin (2017). "Microplastics as a vector for the transport of the bacterial fish pathogen species *aeromonas salmonicida*." Mar Pollut Bull **125**(1-2): 301-309.

Vladar, E. K., D. Antic and J. D. Axelrod (2009). "Planar cell polarity signaling: The developing cell's compass." Cold Spring Harb Perspect Biol **1**(3): a002964.

von Moos, N., P. Burkhardt-Holm and A. Köhler (2012). "Uptake and effects of microplastics on cells and tissue of the blue mussel *mytilus edulis* l. After an experimental exposure." Environmental Science & Technology **46**(20): 11327-11335.

Vroom, R. J. E., A. A. Koelmans, E. Besseling and C. Halsband (2017). "Aging of microplastics promotes their ingestion by marine zooplankton." Environ Pollut **231**(Pt 1): 987-996.

Waldbusser, G. G. and J. E. Salisbury (2014). "Ocean acidification in the coastal zone from an organism's perspective: Multiple system parameters, frequency domains, and habitats." Ann Rev Mar Sci **6**: 221-247.

Wang, Z., M. Gerstein and M. Snyder (2009). "Rna-seq: A revolutionary tool for transcriptomics." Nat Rev Genet **10**(1): 57-63.

Wright, S. L., R. C. Thompson and T. S. Galloway (2013). "The physical impacts of microplastics on marine organisms: A review." Environ Pollut **178**: 483-492.

Yang, D., H. Shi, L. Li, J. Li, K. Jabeen and P. Kolandhasamy (2015). "Microplastic pollution in table salts from china." Environ Sci Technol **49**(22): 13622-13627.

Yokota, K., H. Waterfield, C. Hastings, E. Davidson, E. Kwietniewski and B. Wells (2017). "Finding the missing piece of the aquatic plastic pollution puzzle: Interaction between primary producers and microplastics." Limnology and Oceanography Letters **2**(4): 91-104.

Zettler, E. R., T. J. Mincer and L. A. Amaral-Zettler (2013). "Life in the "plastisphere": Microbial communities on plastic marine debris." Environ Sci Technol **47**(13): 7137-7146.

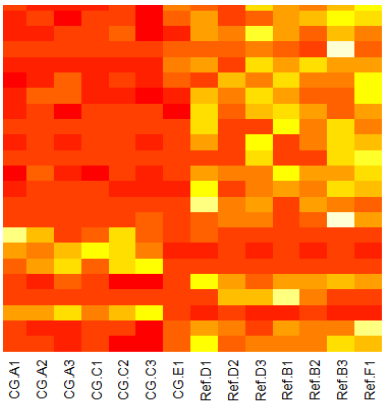
Zhang, C., X. Chen, J. Wang and L. Tan (2017). "Toxic effects of microplastic on marine microalgae skeletonema costatum: Interactions between microplastic and algae." Environ Pollut **220**(Pt B): 1282-1288.

Zhang, G., L. Li, J. Meng, H. Qi, T. Qu, F. Xu and L. Zhang (2016). "Molecular basis for adaptation of oysters to stressful marine intertidal environments." Annu Rev Anim Biosci **4**: 357-381.

Zhao, X., H. Yu, L. Kong and Q. Li (2012). "Transcriptomic responses to salinity stress in the pacific oyster *crassostrea gigas*." PLoS One **7**(9): e46244.

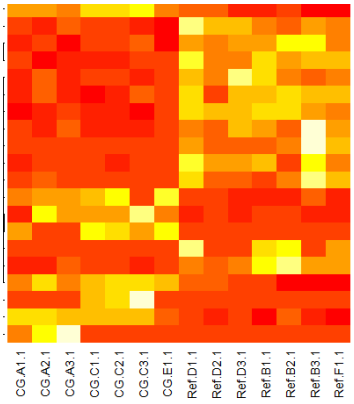
Appendix

Table A12: Gene Expression of the 23 most DEGs between clam garden and reference gill libraries



FC	ID	Biological processes
-1.8	L-threonine 3-dehydrogenase	L-threonine catabolic process to glycine, redox
-1.5	Epithelial cell-transforming sequence 2 oncogene-like	Signaling, apoptosis, development, angiogenesis, adhesion
-1.4	Uncharacterized protein C7orf72	
-9.0	Claudin-1	Adhesion, viral entry into host cell
-2.2	Nck-associated protein	Apoptosis, CNS development, viral process, signaling pathway involved in phagocytosis
-2.3	Helicase with zinc finger domain	DNA transcription, regulation of transcription from RNA polymerase II promoter
-1.8	Junction-mediating and -regulatory protein	DNA repair, regulation of signal transduction by p53 (tumor suppressor) class mediator
-2.9	Aminopeptidase N	Angiogenesis, viral entry into host cell
-23.9	Monocarboxylate transporter 2	Lactate and pyruvate transmembrane transport
-5.1	Platelet endothelial aggregation receptor 1	Apoptosis, signaling
-120.8	Solute carrier family 28 member 3	Nucleoside transport across plasma membrane
-2.3	Putative ATP-dependent RNA helicase	Male meiosis
-6.6	Protein crumbs	Morphogenesis of a polarized epithelium, cell-cell junction assembly
-21.7	Angiotensin-converting enzyme	Kidney development, neutrophil mediated immunity
-17.5	Baculoviral IAP repeat-containing protein	Regulates apoptosis, immune response, redox response, protein ubiquination, organismal development…
14.2	Fibrillin-1	Skeletal system development, signaling, cell adhesion mediated by integrin
9.0	C-type lectin lectoxin-Thr1	Innate immunity, mannose-binding, agglutinates cells, true venom family (snakes)
52.7	Beta-1,3-galactosyltransferase 5	Protein glycosylation
-1.6	Tyrosyl-DNA phosphodiesterase 1	DNA repair
-342.4	E3 ubiquitin-protein ligase	Sprouting angiogenesis, protein ubiquitination,
5.8	Protein Star	Regulation of epidermal growth factor receptor signaling pathway
-1.5	Nephrocystin-4	Structural organization, adhesion, signaling
-1.5	DNA-dependent protein kinase catalytic subunit	DNA repair

Table A13: Gene Expression of the 23 most DEGs between clam garden and reference digestive gland libraries



FC	ID	Biological process
2	Soma ferritin	Iron ion storage and transport, redox
-2	Zinc transporter ZIP14	Iron ion transport, cellular zinc ion homeostasis
-2	Low affinity immunoglobulin epsilon Fc receptor	Immune response
-2	Platelet endothelial aggregation receptor 1	Apoptosis, signaling
-2	C-1-tetrahydrofolate synthase	Redox, coenzyme metabolism, neutrophil homeostasis
-2	Guanylate-binding protein 1	Defense response to bacterium and virus
-2	Zinc finger CCCH-type antiviral protein 1	Response to virus, interferon production
-3	Prosaposin	Lysosomal sphingolipid degradation,catalytic activity
-4	Prosaposin	Lysosomal sphingolipid degradation,catalytic activity
-3	BTB and MATH domain-containing protein 38	
-24	Angiotensin-converting enzyme (Fragment)	Kidney development, neutrophil mediated immunity
5	Macrophage-expressed gene 1 protein	Innate immunity
13	Platelet endothelial aggregation receptor 1	Apoptosis, signaling
21	Tropinone reductase-like 2	Redox
-224	Heat shock 70 kDa protein 12A	Heat shock response
-3	Persulfide dioxygenase ETHE1	Redox, iron binding, glutathione metabolism
2	Retinol dehydrogenase 7	Retinol and redox activity
369	Replicase polyprotein, Acute bee paralysis virus	Viral RNA genome replication
2	Adenosylhomocysteinase	Cysteine and methionine metabolism
615	Replicase polyprotein, Cricket paralysis virus	Viral RNA genome replication

Table A14: Total and average millions of reads per tissue (gill and digestive gland), treatment group (control and exposed), and time point (0, 3 hours, and 14 days).

Tissue	Treatment	n	Time	Total reads (millions)	AV million reads/library
Gill	Control	4	0	236	59 ± 11
		4	3 h	384	96 ± 11
		4	14 d	297	74 ± 31
Gill	Exposed	4	0	208	52 ± 4
		4	3 h	238	60 ± 20
		4	14 d	219	55 ± 5
Digestive Gland	Control	4	0	217	54 ± 9
		4	3 h	242	61 ± 31
		4	14 d	276	69 ± 29
Digestive Gland	Exposed	4	0	209	52 ± 5
		4	3 h	321	80 ± 63
		4	14 d	199	50 ± 5

Table A15: Genes upregulated in microplastic-exposed gill at 14 days.

Biological function	UniProt	Gene identification	Abbrev.	Fold change	*p*-value
Immunity	P02746	Complement C1q suB.C.omponent subunit B	C1QB	33.1	0.011
Immunity	P17927	Complement receptor type 1	CR1	4.8	0.013
Immunity	Q8N884	Cyclic GMP-AMP synthase	MB21D1	4.4	0.013
Immunity	Q61830	Macrophage mannose receptor 1	Mrc1	38.5	0.002
Immunity	Q8N0N3	Beta-1,3-glucan-binding protein	BGBP	22.1	0.003
Immunity	Q9VAF5	Cadherin-99C	Cad99C	15.6	0.002
Signaling	O75096	Low-density lipoprotein receptor-related protein 4	LRP4	2.9	0.007
Signaling	Q9Pou1	Mitochondrial import receptor subunit TOM7 homolog	TOMM7	2.6	0.002
Signaling	P31030	Serine--pyruvate aminotransferase	AGXT	2.2	0.009
Signaling, Wound repair	Q02763	Angiopoietin-1 receptor / Tyrosine-protein kinase	TEK	3.2	0.001
Signaling, cell adhesion	P17900	Ganglioside GM2 activator	GM2	65.7	0.003
Signaling, cell adhesion	Q86Z23	Complement C1q-like protein 4	C1QL4	41.2	0.014
Signaling, cell adhesion	O97827	Adhesion G protein-coupled receptor L3	ADGRL3	6.4	0.008

Signaling, cell adhesion	Q9N0C7	Mammalian ependymin-related protein 1	EPDR1	41.6	0.007
Cell adhesion	P08472	Mesenchyme-specific cell surface glycoprotein	msp130	58.4	0.010
Cell adhesion	O35276	Neuropilin-2	Nrp2	26.6	0.007
Cell adhesion	Q6DJ83	Cell adhesion molecule 2	cadm2	26.4	0.011
Cell adhesion	P09848	Lactase-phlorizin hydrolase	LCT	26.1	0.011
Cytokine activity	Q8R066	Complement C1q tumor necrosis factor-related protein 4	C1qtnf4	23.2	0.013
Degradation Hydrolyzes	P21927	Cholinesterase	B.C.HE	104.2	0.015
Degradation, Lysosomal	Q9GL24	Cathepsin L1	CTSL	43.4	0.012
Degradation, Lysosomal	P50429	Arylsulfatase B	Arsb	8.9	0.005
Defense, Oxidative stress	P49290	Eosinophil peroxidase	Epx	4.2	0.006
Stress	O43301	Heat shock 70 kDa protein 12A	HSPA12A	5.1	0.011
Stress, Response to starvation, hypoxia, bacteria	P46892	Cyclin-dependent kinase 11B	Cdk11b	2.1	0.015
Stress, Plant defense	Q10J94	Cysteine proteinase inhibitor 8 / Orzacystatin -VIII	OC-VIII	32.6	0.014
Structural constituent	O42401	Matrilin-3	MATN3	50.5	0.014

Table A16: Genes upregulated in microplastic-exposed gill at 3 hours.

Biological function	**UniProt**	**Gene identification**	**Abbrev.**	**Fold change**	***p*-value**
Immunity	Q61830	Macrophage mannose receptor 1	Mrc1	6.68	0.002
Immunity	Q8N0N3	Beta-1,3-glucan-binding protein	BGBP	2.03	0.003
Signaling	O75096	Low-density lipoprotein receptor-related protein 4	LRP4	90.83	0.007
Signaling, maybe apoptosis	Q61MN6	Caprin-2	CAPRIN2	21.18	0.007
Signaling, Wound repair	Q02763	Angiopoietin-1 receptor / Tyrosine-protein kinase	TEK	2.56	0.001
Cell adhesion	Q6DJ83	Cell adhesion molecule 2	cadm2	2.03	0.011
Cell adhesion	Q9N0C7	Mammalian ependymin-related protein 1	EPDR1	2.49	0.007

Degradation / Lysosomal	P50429	Arylsulfatase B	Arsb	11.01	0.005
Degradation / Protein	Q9GL24	Cathepsin L1	CTSL	2.31	0.012
Stress	Q96MM6	Heat shock 70 kDa protein 12B	HSPA12B	28.33	0.008
Stress, Response to starvation, hypoxia, bacteria	Q00PJ9	Caveolin-1	CAV1	5.18	0.012
Wound healing	Q5ZKJ4	FGFR1 oncogene partner 2	FGFR1OP 2	4.44	0.001
Structural constituent	Q61554	Fibrillin-1	Fbn1	2.13	0.012
Structural constituent	O42401	Matrilin-3	MATN3	2.11	0.014

Table A17: Genes upregulated in microplastic-exposed digestive gland at 14 days.

Biological function	**UniProt**	**Gene identification**	**Abbrev.**	**Fold change**	***p*-value**
Binding, Part of T-cell co-signaling pathway	P05539	Collagen alpha-1(II) chain	Col2a1	96.1	0.0018
Binding actin, ATP, Protein kinase	Q1EG27	Myosin-IIIb	Myo3b	76.7	0.0023
Binding, hemagglutinin	C0HK24	L-rhamnose-binding lectin	ELEL-1	6.2	0.0045
Binding, hydrolase	B2IZD3	Bacterial dynamin-like protein	Npun	18.4	0.0017
Cell adhesion	Q71M42	Protocadherin-11 X-linked	PCDH11X	18.6	0.0002
Cell adhesion	Q92752	Tenascin-R	TNR	11.4	0.0125
Cell adhesion	Q9HC56	Protocadherin-9	PCDH9	7	0.0011
Cell adhesion	Q9BZA8	Protocadherin-11 Y-linked	PCDH11Y	3.9	0.0045
Degradation, Inflammation	P12545	Plasminogen	PLG	17.6	0.0066
Stress oxidative, Defense	P33248	Thymosin beta-12	TMSB12	2.9	0.0001
Stress, Detoxification	P30568	Glutathione S-transferase A	GSTA	23.6	0.0070
Stress, Plant defense	P48809	Heterogeneous nuclear ribonucleoprotein 27C	Hrb27C	3.6	0.0018
Structural constituent	Q61555	Fibrillin-2	Fbn2	16.3	0.0074
Transport / Water channel	QF14F8	Aquaporin-8	AQP8	12.2	0.0102

Table A18: Genes upregulated in microplastic-exposed digestive gland at 3 hours.

Biological function	**UniProt**	**Gene identification**	**Abbrev.**	**Fold change**	***p*-value**
Binding lectin, hemagglutinin	C0HK24	L-rhamnose-binding lectin	ELEL-1	35.6	0.00449
Cell adhesion	Q71M42	Protocadherin-11 X-linked	PCDH11X	11.5	0.00019
Cell adhesion	P17900	Ganglioside GM2 activator	GM2A	10.4	0.00372
Cell adhesion	P11047	Laminin subunit gamma-1	LAMC1	3.9	0.01363
Cell adhesion	Q9HC56	Protocadherin-9	PCDH9	2.7	0.00110
Degradation	P04069	Carboxypeptidase B	CPB1	5.2	0.00628
Degradation, Inflammation	P12545	Plasminogen	PLG	22.8	0.00661
Immunity	Q81ZJ3	C3 and PZP-like alpha-2-macroglobulin domain-containing protein 8	CPAMD8	4.6	0.0009
Oxidizer	Q64583	Cytochrome P450 2B15	Cyp2b15	6.6	0.00771
Signaling	P49190	Parathyroid hormone 2 receptor	PTH2R	11.1	0.01142
Signaling	O42603	Corticotropin-releasing factor receptor 2	Crhr2	7.3	0.00442
Signaling	O75096	Low-density lipoprotein receptor-related protein 4	LRP4	47	0.01379
Stress	Q96MM2	Heat shock 70 kDa protein 12B	HSPA12B	42.7	0.00596
Stress, Detoxification	Q9ZRW8	Glutathione S-transferase U19	GSTU19	3	0.00465
Stress, Oxidative	G9JJU2	Glutathione peroxidase	GPx	2.2	0.00748
Wound healing	Q5ZKJ4	FGFR1 oncogene partner 2	FGFR1OP2	4.1	0.00004