Characterizing the diversity of the eukaryotic microbiome in marine crustacean zooplankton.

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology
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Abstract

Understanding zooplankton productivity is critical for modeling marine food web function, of which one poorly known factor is the influence of zooplankton symbionts. Zooplankton protist symbiont diversity is underestimated due to the limited surveys and techniques previously used. Using 18S V4 metabarcoding, I characterized the eukaryotic microbiomes associated with crustacean zooplankton from the northern Strait of Georgia, BC. Apostome ciliates were most abundant in all hosts except for cyclopoid copepods, which were dominated by Syndiniales. Most symbiont lineages were more abundant in one or two hosts, suggesting some degree of host preference. Microbiome data also provided information on diet, confirming increased diatom consumption during spring in calanoid copepods and consumption of crustaceans by Cyphocaris and Corycaeus. These data also suggest that zooplankton feed on siphonophores, a previously unrecognized interaction with the Cnidaria. My work contributes to resolving the interactions between zooplankton and alveolate symbionts, and the host-specificity of potential parasites.

Keywords

Protists, zooplankton, symbiosis, parasitism, commensalism, diet, lower marine food web, Strait of Georgia, metabarcoding, microbiome.
Summary for Lay Audience

Marine zooplankton are small animals and microorganisms that live in the ocean and eat other organisms. Animal zooplankton include well known groups such as copepods, krill, jellyfish, the larval stages of fish, crabs, lobsters, and shrimp, and many more. Zooplankton are crucial components of the marine food web because many of them feed on photosynthetic microorganisms called phytoplankton (including cyanobacteria, diatoms, and dinoflagellates) at the base of the food web. Zooplankton are themselves consumed, which transfers energy up the food web to support the growth of fish, whales, and birds at the highest trophic levels. It is important to understand how the growth of zooplankton is influenced by their environment, including their interactions with other organisms, due to their importance in food web stability. One interaction that is often overlooked or less understood is that between zooplankton and their microbial symbionts. Symbionts of zooplankton may live attached to their surface or inside their body and can have a range of effects on their hosts, from beneficial to harmful, the latter referred to as parasitism. Symbiotic microorganisms have been observed in zooplankton since at least the early 1900’s, although the true diversity of these symbionts is not known for most zooplankton, let alone their effects on the host. Microscopy is inadequate to detect and identify these symbionts, whereas DNA sequences can be used instead to determine their presence in zooplankton. This thesis is one of the first studies to characterize the microbial symbionts associated with various species of marine zooplankton using DNA sequencing methods. Ciliate and dinoflagellate lineages were the most abundant symbionts associated with crustacean zooplankton, such as copepods. Most symbionts were found associated with almost every zooplankton host analyzed, but some degree of preference for particular hosts was observed. Sequence data also provided insight into potential components of zooplankton diet including herbivory of diatoms in spring, and carnivory of crustaceans and cnidarians – which has not been known before. The high diversity of symbionts associated with zooplankton, many of which were previously undescribed, indicates potentially important and unrecognized interactions in the marine food web.
Co-Authorship Statement

The research described in this thesis is the work of Rose-Lynne Savage. The study itself was conceived by Dr. Vera Tai and was conducted in collaboration with the Hakai Coastal Initiative’s Marine Food Webs Working Group. As part of the collaboration, Drs. Brian Hunt, Colleen Kellogg, Jacqueline Maud, and Caterina Giner contributed to sample collections and zooplankton identifications. Rose-Lynne Savage collected samples and conducted all laboratory and data analyses for this thesis. Manuscripts will be developed by Rose-Lynne Savage and Dr. Vera Tai.
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<th>Full Form</th>
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<tbody>
<tr>
<td>18S V4</td>
<td>Hypervariable region of the 18S ribosomal small subunit gene</td>
</tr>
<tr>
<td>ANCOM</td>
<td>Analysis of Composition of Microbiomes</td>
</tr>
<tr>
<td>ASV</td>
<td>Amplified sequence variant</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved organic matter</td>
</tr>
<tr>
<td>PCoA</td>
<td>Principal coordinate analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>POM</td>
<td>Particulate organic matter</td>
</tr>
<tr>
<td>PR2</td>
<td>Protist Ribosomal Reference Database</td>
</tr>
<tr>
<td>QIIME2</td>
<td>Quantitative Insights in Microbial Ecology version 2</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SoG</td>
<td>Strait of Georgia</td>
</tr>
<tr>
<td>SSU</td>
<td>Small subunit</td>
</tr>
<tr>
<td>UNM</td>
<td>Universal non-metazoan</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Ecological significance of marine zooplankton

The Earth’s surface is mostly covered by its oceans, leading to the nickname “blue planet”. Marine ecosystems provide critically important ecological services such as atmospheric gas regulation (e.g., through carbon sequestration), nutrient cycling (e.g., nitrogen cycling), and food production (Costanza & Limburg, 1997; Falkowski, 2012). Marine ecosystems support diverse communities of organisms, many of economic and cultural importance to humans, including fish, seals, and cetaceans. The organic matter used as a source of energy supporting the growth and productivity of all heterotrophic organisms in marine ecosystems is mainly generated by phytoplankton, which are microbial photosynthetic primary producers. Most larger consumers are not able to access primary production energy directly and must feed on smaller organisms, such as small fish and zooplankton (Calbet & Landry, 2004; Mackas & Tsuda, 1999). Zooplankton include unicellular and metazoan heterotrophic eukaryotes that live in the water column, some of which are primary consumers, directly grazing on phytoplankton, and others are secondary consumers, preying on the primary consumers. Zooplankton communities are a crucial component linking the lower food web to higher trophic levels, contributing to the complexity of the marine food web. Understanding zooplankton dynamics and productivity is critical for understanding ecosystem function and ensuring production of higher trophic levels.

1.1.1 Zooplankton – consumers at the top of the lower marine food web

The lower marine food web consists of the zooplankton, phytoplankton, bacteria, and viruses living in the marine environment. At the top of the lower marine food web are metazoan zooplankton and below them are the eukaryotic and prokaryotic microorganisms and viruses. Metazoan zooplankton include cnidarians and larval stages of fish, molluscs, and cephalopods, but major groups of crustacean zooplankton – copepods, euphausiids (krill), amphipods, and decapods – dominate the zooplankton community and are especially important prey for carnivorous crustaceans (Dalpadado et
al., 2008) and higher consumers such as fish, birds, and whales (Mackas & Tsuda, 1999; Turner, 2004). Crustacean zooplankton have a diet ranging from phytoplankton (Calbet & Landry, 2004; Turner, 2004) to mesozooplankton, namely unicellular eukaryotes such as flagellates and ciliates, and small metazoans, such as nauplii, young copepodite stages, and larvae (Fig. 1.1) (Azam et al., 1983; Calbet & Saiz, 2005). Microorganisms (Bacteria, Archaea, Fungi, and unicellular eukaryotes, also referred to as protists) are ubiquitous across environments and play important roles in many global biogeochemical cycles (Falkowski, 2012). In marine ecosystems, energy from phytoplankton is transferred through bacteria, protists, and viruses (the microbial loop) or transferred to higher trophic levels by zooplankton consumption (Fig. 1.1) (Azam et al., 1983). Sinking zooplankton detritus and waste (i.e. particulate organic matter or POM) also contribute to the biological pump. The biological pump is the process in which organic matter is exported and sequestered in the deep ocean (Turner, 2015). The population dynamics and productivity of zooplankton are central to their role in the transfer of energy between primary producers and higher consumers, to the microbial loop, and to the deep sea via the biological pump.

Figure 1.1: Lower marine food web. Solid grey lines indicate the transfer of carbon through consumption, predation and herbivory. Dotted lines indicate sources of
particulate organic matter (POM; i.e. detritus, waste, sloppy eating, etc.), which decompose chemically or biologically (mainly by bacteria) into dissolved organic matter (DOM).

1.2 Zooplankton population dynamics and productivity

Population dynamics are concerned with the overall gain and loss of individuals within a population over time. For zooplankton, population growth includes reproduction and recruitment, whereas overall mortality accounts for population loss (Ohman, 2012). Zooplankton productivity is the conversion of energy from primary producers into zooplankton biomass, or the rate of change of total zooplankton biomass in the environment and is a component of population growth. Zooplankton population dynamics, life histories, and productivity are sensitive to environmental changes such as physical factors (e.g., rough winds and water turbulence), temperature, light, predation, and food availability (Peterson, 2001; Edwards & Richardson, 2004; Batchelder et al., 2013; Sodré & Bozelli, 2019). Particularly in temperate environments, zooplankton productivity is strongly tied to seasonal changes such as the spring phytoplankton bloom and warming water temperature.

1.2.1 Seasonal influences on productivity

Many marine ecosystems annually experience seasonal changes in environment which may support or impede the fitness and productivity of zooplankton. Changing environmental conditions will affect the diversity and productivity of zooplankton and progression through their developmental stages. Higher temperatures and increased food availability (e.g., a phytoplankton spring bloom) are good predictors of increased zooplankton productivity in some marine ecosystems. For example, higher temperatures are correlated with increased developmental and growth rates of copepods (Hirst & Bunker, 2003; Peterson, 2001), decapods (Lindley, 1998), cladocerans, and rotifers (Gillooly et al., 2002). In addition, in regions experiencing an annual spring phytoplankton bloom, warming temperature may act as a proxy or indicator for optimal food availability, triggering zooplankton spawning and growth (Mackas et al., 2012). Food abundance also influences reproductive and developmental growth in important groups such as copepods (Calbet et al., 2002; Peterson, 2001) and euphausiids (Pinchuk
& Hopcroft, 2006). During transitions between periods of cold temperature and low productivity (winter) to warm and highly productive conditions (spring/summer), some ecosystems experience a shift in the predominant zooplankton taxa to those whose productivity thrives under the conditions of each season. These shifts are correlated with temperature cues and the occurrence of a spring bloom (Ivory et al., 2019; Mahara et al., 2019; Tommasi et al., 2013). Zooplankton in the western Pacific showed a change in copepod communities as waters warmed (Chiba et al., 2006). During spring and early summer, cool water species such as *Neocalanus* spp., *Eucalanus bungii*, and *Metridia* spp., predominated the community. A shift in dominance from these cool water species to warm water species occurred throughout summer and late into autumn. This also demonstrated a shift from larger species, which primarily feed on diatoms in spring, to smaller omnivores in autumn (Chiba et al., 2006).

Physical changes that occur seasonally in the water column may also influence zooplankton production and growth. In the tropical Mexican Pacific Ocean, periods of mixed, upwelling waters in early spring and summer have caused increased zooplankton abundance relative to when water is stratified in late summer and winter (Ambriz-Arrreola et al., 2018). Shifts in predominating taxa of zooplankton in response to annual environmental cues demonstrate the influence of environmental changes on zooplankton productivity and succession.

Different life histories among zooplankton taxa may also influence their seasonal changes in productivity. Life cycles vary in generation time, spawning timing, and dormancy periods. Zooplankton can experience one or more generations a year (Dole-Olivier et al., 2000; Miller et al., 1984), and breeding periods can occur at different times depending on the taxa and the geographic region (Dvoretskii, 2007; Ross et al., 1982; Shebanova et al., 2011). Major reproductive periods can be linked to the occurrence of a spring bloom (Daase et al., 2013; Takahashi & Ide, 2011) or occur consistently throughout seasons (Batchelder, 1985). Periods of dormancy or migration to deep waters to survive unfavorable winter conditions also influence the abundance and diversity of zooplankton in the water column. Large herbivorous copepods such as *Calanus marshallae*, *Neocalanus plumchrus*, and *Eucalanus bungii*, are known in winter to diapause at late copepodite life stages, to be present in lower abundances and to be inactive (Harrison et al., 1983; Shoden et al., 2005). Other species of copepods, such as *Metridia pacifica*, are
present consistently throughout the year and may have multiple generations (Batchelder, 1985; Padmavati et al., 2004). *M. pacifica* also overwinters in deep waters but actively continues to feed instead of entering diapause (Padmavati et al., 2004; Tommasi et al., 2013). Different seasons (e.g. spring versus winter) may support contrasting life histories throughout the year, affecting the taxa that dominate in different seasons.

1.2.2 Diet and food availability

Seasonal changes in food availability and zooplankton feeding strategies also affect their productivity. Species, primarily phytoplankton, may exhibit peaks during different times of the year. Some zooplankton have been seen to shift their feeding strategies, from herbivory to omnivory, in response to changes in phytoplankton abundance (Nakagawa et al., 2000; Kraft et al., 2013; Cleary et al., 2017; Saiz & Calbet, 2011). Zooplankton feeding can also be influenced by prey shape and size. The quantity and quality of food is important for growth, reproduction, and survival. Copepod diets can be diverse, consisting of phytoplankton, heterotrophic microorganisms, as well as other copepods and their composition can influence important physiological processes such as reproductive development (Calbet et al., 2002; Castellani et al., 2005; Kiørboe & Nielsen, 1994; Kleppel, 1993). Diet has been studied especially in the copepod genus *Calanus*, using fatty acids and stable isotopes (Søreide et al., 2008; Wold et al., 2011), gut content analysis (Pasternak & Schnack-Schiel, 2001), and DNA metabarcoding (Cleary et al., 2017; Yeh et al., 2020), indicating a diet dominated by phytoplankton (mostly diatoms) during months of high primary productivity. Similarly, during periods of a spring bloom euphausiids are mainly herbivorous, with a diet dominated by diatoms, but can shift to carnivory of copepods and other protists in summer and autumn months when food conditions change (Gibbons et al., 1991; Nakagawa et al., 2000; Kaartvedt et al., 2002). Despite the large contribution of diatoms to the diet of zooplankton, diatoms produce potentially harmful compounds. For copepods, diatom-dominated diets caused deleterious effects on egg survival and copepod development (Miralto et al., 1999; Carotenuto et al., 2002), and may dominate diet when primary production is high (Saiz & Calbet, 2011). Furthermore, peak growth rates of Antarctic euphausiids have occurred during a diatom bloom while they are feeding on a mixed diet of diatoms and protozoans (Schmidt et al., 2006). Shifting diet composition and feeding strategies in response to
food availability are clearly important for zooplankton production, affecting their growth and survival.

1.2.3 Zooplankton mortality

Opposite to growth, mortality is also a major component of zooplankton population dynamics and is estimated from death rates caused by all sources, *i.e.* predatory and non-predatory (Ohman, 2012). The effects of non-predatory mortality, such as senescence, starvation (Ohman, 1995), toxicity (Dhanker et al., 2015; Kâ et al., 2014), environmental stress (Eiane & Daase, 2002; Tang et al., 2014), and parasitism (Kimmerer & McKinnon, 1990), are challenging to evaluate in zooplankton communities (Daase et al., 2014). However, non-predatory mortality is becoming increasingly recognized as an important negative factor to zooplankton productivity. Non-predatory mortality has been estimated to account for up to a quarter of the total mortality in global copepod populations (Hirst & Kiørboe, 2002). It has been measured to account for up to 54% of the mortality of the copepod *Calanus helgolandicus* in the English Channel (Maud et al., 2018). Lethal parasites have killed mass numbers of hosts (Gómez-Gutiérrez, 2003) and reduced host density down to 50% (Johnson et al., 2009). Furthermore, high density infestations of commensal epibionts have also been associated with increasing mortality rates of their host (Allen et al., 1993). Parasitism, however, has been difficult to measure in zooplankton populations because of the unknown prevalence within communities and the variable effects that parasitic lineages can have on their hosts. Shifts in parasitic load or increased prevalence of seemingly harmless symbionts could cause increased mortality rates of important zooplankton populations that may initially appear unaffected. Many microbial symbionts (as parasites, mutualists, and commensals) have been described, but in general the diversity of symbionts associated with zooplankton and their influences on zooplankton fitness, mortality, and productivity are poorly known. Symbiosis in zooplankton populations requires further exploration in view of these clear examples of their implications to zooplankton productivity.

1.2.4 Symbiosis

There is a long record describing the diversity of symbionts associated with zooplankton populations, most of which focuses on parasitic symbionts (Ho & Perkins, 1985; Coats,
1999; Théodoridès, 1989), although their contribution to the regulation and mortality of marine zooplankton is often overlooked. Symbiotic relationships can be categorized by their effect on each of the participating organisms. The main types of symbiotic relationships include mutualism, commensalism, and parasitism. In mutualism, both the host and the symbiont benefit from the association. In commensalism, the symbiont benefits from the interaction while the host is unaffected. In parasitism, the symbiont may cause physical damage to the host or derive its nutrition or habitat from its host causing harm. Parasitism is an especially significant symbiotic association as almost all organisms have parasites which negatively influence their fitness. Food web models tend to underrepresent or exclude the effect of parasitic relationships that influence population dynamics and the transfer of energy between trophic levels (Amundsen et al., 2009; Lafferty et al., 2008). This is particularly problematic for zooplankton communities as the diversity and effects of their parasites are often unrecognized.

Model host-parasite systems have been studied extensively in freshwater zooplankton as epidemics commonly occur and are responsible for depleting host abundances (Cáceres et al., 2014). There are also numerous examples of parasitic infections influencing the productivity of marine zooplankton, reducing host growth and reproduction, or causing death (Coats, 1999; Kimmerer & McKinnon, 1990; Lynn et al., 2014). But despite our knowledge of several parasitic marine protists and the widespread occurrence of parasitism in marine zooplankton populations, the effect of parasitism on the productivity of marine zooplankton is not equally understood. Some symbionts do not obviously affect host productivity and are considered commensals. However, high infestation causing increased zooplankton mortality has been documented. Infestations may inhibit host mobility, increasing host risk to predation (Weissman et al., 1993), increasing filtering feeding (Allen et al., 1993), and reducing fertility (Stirnadel & Ebert, 1997). Mutualistic associations between zooplankton and microbes are less well known than commensalism and parasitism, although these may still influence zooplankton host productivity (Moss et al., 2001; Gaevskii et al., 2004; Shoemaker & Moisander, 2015). Symbiotic relationships have been proven to influence zooplankton productivity and it is crucial to expand our understanding of their roles in population dynamics.
1.3 Protist symbionts of crustacean zooplankton

A wide diversity of organisms, including bacteria, protists, fungi, and metazoans, associate with crustacean zooplankton (Bojko & Ovcharenko, 2019; Ho & Perkins, 1985). Most known zooplankton symbionts, however, are protists. Some protist symbionts live inside the body of their hosts, taking up space in the digestive tract or growing into host tissues – these are called endosymbionts. Ectosymbionts (or epibionts) live attached to or form external structures on the host surfaces, for example the exoskeletons of crustacean zooplankton (Ho & Perkins, 1985). Some epibionts are seemingly harmless filter feeders that use the host as a substrate, while others feed on host tissue or cause damage and growth impediments (Grimes & Bradbury, 1992; Shields, 1994).

The effect that protist symbionts have on their hosts may vary among lineages and between host taxa. Diverse protist lineages have been reported in association with zooplankton hosts, many of them belonging to the Alveolata – a group that includes major lineages such as ciliates, dinoflagellates, and apicomplexans (Ho & Perkins, 1985; Shields, 1994). Less studied are the non-alveolate symbionts, which include diatoms (Bacillariophyta) (Gómez et al., 2018), rhizarians (Rhizaria) (Skovgaard & Daugbjerg, 2008), fungi, oomycetes (Stramenopiles) (Wolinska et al., 2009), and euglenids (Euglenida) (Willey et al., 1990).

1.3.1 Alveolate symbionts

1.3.1.1 Dinoflagellates

Many dinoflagellate symbionts are considered parasitic as they are known to have mild to severe consequences on zooplankton fitness. Dinoflagellates of the genus Blastodinium infect digestive tracts and establish in the stomachs of copepods. Infections can impede reproductive development and cause host castration (Shields, 1994). Blastodinium infections also cause host lethargy, starvation, reduced respiration rates, and failure to moult to adult stages (Fields et al., 2015) but are generally less lethal to hosts than other groups of parasites (Coats, 1999). The Syndiniales are an exclusively parasitic group of dinoflagellates, several genera of which infect crustaceans (Shields, 1994). Species of the
The genus *Syndinium* cause high mortality in populations of the copepod *Paracalanus indicus* (Kimmerer & McKinnon, 1990). Copepods infected with *Syndinium* sp. appear opaque as the plasmodium (a multinucleate life stage of the parasite) invades the tissues and organs of the host. This eventually leads to the death of the host by rupturing of the exoskeleton. (Shields, 1994).

### 1.3.1.2 Ciliates

Many ciliate symbionts of zooplankton are epibionts. Some lineages of apostome ciliates (Apostomatida) are virulent towards diverse zooplankton groups, with distinct life stages for transmission, dormancy, and feeding. The apostome *Vampyrophrya pelagica* rests dormant on the surface of copepods as a cyst, called a phoront, until the host is damaged or molts, allowing the ciliate to enter the host and feed on its tissue. Once fed, the ciliate will begin to divide, eventually leave the carcass, and encyst on a new surface (Grimes & Bradbury, 1992). Other apostome ciliates can be even more harmful to their hosts. *Pseudocollinia* spp. are parasitoids that have caused large population declines in their krill hosts (Gómez-Gutiérrez et al., 2006; Lynn et al., 2014). These parasites have been reported in krill species, and because of their high lethality, they likely contribute to the regulation of krill populations (Cleary et al., 2019). The apostome *Fusiforma themisticola* has been reported once, infecting the amphipod *Themisto libellula*, but is suggested to be lethal to the host based on the high abundance of infected carcasses and its close evolutionary relationship to the *Pseudocollinia* genus (Chantangsi et al., 2013). However, no other evidence of lethality has yet been observed.

Other ciliates, such as suctorians and peritrichs, are common epibionts of crustacean zooplankton, many of which are considered commensals (Fernandez-Leborans & TatoPorto, 2000a, 2000b). During feeding, suctorians are attached to their host as epibionts and undergo budding for dispersal of motile life stages, called tomites, to find the next host (Kobayashi et al., 2011), some showing high host specificity (Sherman & Schaner, 1965; Turner et al., 1979). Although no immediate harm is experienced by the hosts, high infestation rates of commensal epibionts are suspected to influence host survival by reducing host mobility or sinking, thereby increasing their risk to predation (Weissman et al., 1993). Some ciliates show evidence of co-evolution with their
respective hosts. *Ephelota* spp. are commensal suctorian ciliates of various zooplankton, including krill (Nicol, 1984), copepods (Purushothaman et al., 2019), cnidarians (Tazioli & Di Camillo, 2013), and decapods (Sawyer et al., 1976). The lifecycle of *Ephelota plana* was investigated in relation to its krill host *Euphausia pacifica* by Endo et al. (2017), who found that ciliate infestation and size increased with moult stage, suggesting that these ciliates have a lifecycle adapted to the moulting of the krill host.

1.3.1.3 Other alveolates

Ellobiopsidae is a group of crustacean ectoparasites belonging to the alveolates. These ectoparasites appear morphologically similar to a fungus and will puncture the host exoskeleton to feed on host tissues or for dispersal (Silberman et al., 2004; Théodoridès, 1989). Several species of the genus *Ellobiopsis* have been described infecting copepods and are considered parasitic castrators (Shields, 1994), with some populations of copepods experiencing infestations in the population up to 12% (Bielecka & Boehnke, 2014). These parasitic species can reduce the development of gonads in female copepods, cause the feminization of males, and impede host moulting (Albaina & Irigoien, 2006). *Thalassomyces* is another genus belonging to the Ellobiopsidae; it parasitizes various crustacean taxa including euphausiids, amphipods, and decapods (Théodoridès, 1989). *Thalassomyces* infection begins inside host tissues, targeting tissues near host nerves, and eventually penetrating through the exoskeleton to form an external reproductive structure (Shields, 1994). Depending on the species of *Thalassomyces*, infection can cause castration of the host or potentially influence host vision, which could interfere with host behavior such as vertical diel migration or predation (Shields, 1994).

1.3.2 Non-alveolate symbionts

Various lineages of non-alveolate taxa have been described from zooplankton hosts, some of which have adverse effects on their hosts. *Paradinium pouchetti*, a rhizarian originally described as a parasitic dinoflagellate, has been reported infecting multiple genera of copepods (Skovgaard & Daugbjerg, 2008). This species produces a plasmodium in copepod tissues and in late stage infection forms an external sporangium for dispersal to new hosts. Other symbionts include parasitic and epibiotic euglenids of copepods and other crustaceans (Willey et al., 1990, Skovgaard, 2014). High euglenid epibiont
infestation causes increased zooplankton susceptibility to fish predation under experimental conditions (Willey et al., 1990). Moss et al. (2001) reported amoebae and other protists living on the surface in distinct regions on a host ctenophore body. They suggested that one species of amoeba was mutualistic with the ctenophore, grazing on the bacteria present on the host surface, whereas another amoeba was potentially parasitic – feeding on host cilia. A high diversity of microsporidian parasites is associated with amphipods and decapods; many infections have been seen to cause muscle tissue damage and host feminization, to influence host behavior, or result in host death (Bojko & Ovcharenko, 2019). Finally, various yeasts and fungi also colonize and harm some zooplankton species. Parasitic species of the yeast Metschnikowia have been described infecting various crustacean zooplankton including cladocerans (Auld et al., 2012), copepods (Seki & Fulton, 1969), krill (Cleary et al., 2019), and decapods (Chen et al., 2003), although it is not yet clear whether these relationships are always parasitic. In freshwater systems, Daphnia populations commonly experience infections with Metschnikowia biscuspidata (Ebert et al., 2000), which has become a model system for studying host-parasite dynamics (Cáceres et al., 2014).

1.3.3 Crustacean zooplankton as intermediate hosts for parasites

Some symbionts, mainly parasites, may not use crustaceans as a primary host but as an intermediate host before they go on to colonize another organism. Metazoan parasites such as worms and cnidarians are known to use various zooplankton as intermediate hosts before being transmitted to some fish, their primary hosts, which they parasitize and harm (Bartholomew et al., 1997; Gregori et al., 2012; Marcogliese, 2002; Sichrowsky et al., 2013). For microbial symbionts, some fungal and protist lineages require an intermediate host to complete part of their life cycle while others may use crustaceans as a vessel to access their primary host (i.e. through predation). For example, the yeast Metschnikowia biscuspidata was reported to cause the death of farmed chinook salmon after being fed a brine shrimp that were harboring the yeast (Moore & Strom, 2003). The rhizarian Marteilia refrigens is a parasite of oysters, causing declines in economically important populations, and is confirmed to have an intermediate life stage which proliferates in copepods; however it was not clear that copepods were negatively affected (Audemard et al., 2002; Carrasco et al., 2008). As M. refrigens was present in gonad tissues (Arzul et
al., 2014) as well as in eggs and nauplii from infected individuals (Boyer et al., 2013),
vertical transmission to offspring in copepods was suspected, but it is not known how the
infection is passed from copepod to oyster (Carrasco et al., 2008).

Although many symbiotic taxa are known, most studies are limited to observations of a
particular symbiont associated with a particular host species. Despite the potential
influence that these protist symbionts have on host reproduction and fitness, it is
generally not known to what degree these interactions or infections are prevalent in
marine environments, and the degree to which they influence host dynamics. The
prevalence of these symbiotic interactions is dependent on several factors, including
specificity of the symbiont in associating with a host, encounter rates with their host, and
mode of transmission, among others.

1.4 Zooplankton host-symbiont interactions

1.4.1 Host specificity

The specificity of symbionts, especially parasites, colonizing zooplankton hosts remains
largely unexplored in marine environments. Host and symbiont genetics and physiology
as well as environmental conditions can influence colonization or infection of symbionts
within hosts. In freshwater systems, parasites of *Daphnia* spp. have shown both narrow
and broad ranges of host specificity potentially due to host immunity and physical
characteristics of lake environments (Stirnadel & Ebert, 1997; Duffy et al., 2010). Due to
the relatively low number of studies concerned with marine parasites of zooplankton, and
the fact that most such studies are focused on specific symbionts of specific host taxa, the
true specificity or global distribution of many parasites is generally unknown. Some
lineages have relatively well-known host ranges. Species of *Blastodinium* exhibit host
specificity, infecting multiple groups of copepod species (summarized by Skovgaard et
al., 2012) and have high infection occurrences in specific copepod species (Skovgaard &
Saiz, 2006).

Zooplankton may also be intermediate hosts for symbionts, which expands the specificity
of organisms that a symbiont interacts with. For example, *Hematodinium* spp. are
dinoflagellate parasites of crustaceans that have caused major losses of lobster and crab
populations but may also use amphipods as an intermediate host (Stentiford & Shields, 2005). Limited studies on zooplankton symbionts have been done to discover the true range of host specificity. Increasing the diversity of study regions and host taxa investigated in zooplankton symbiont studies will lead to a better understanding of the true prevalence and specificity of these parasites in marine ecosystems.

1.4.2 Seasonal influences on host-symbiont association

Host-symbiont interactions can be influenced by various factors such as host abundance, temperature, or the life history of the zooplankton host and the symbiont. These factors also interact, for example, as temperature influences the developmental rates of both zooplankton hosts and their symbiont. Seasonal patterns of parasitic infections have been reported, where increased infections and epibiont attachment of dinoflagellates and ciliates have been correlated with increased host abundance (Ianora et al., 1987; Skovgaard & Saiz, 2006) and water temperature (Bojko & Ovcharenko, 2019; Ohtsuka et al., 2004). Changing water temperature and zooplankton host abundance are closely linked to seasonal changes, as previously discussed. Temperature may also affect the free-living life stages of symbionts. For example, warmer temperatures may promote the transition into infective free-living life stages, potentially accounting for higher abundances of symbionts in zooplankton during warmer months. Furthermore, spore dispersal can be triggered by temperature changes as seen in the rhizarian parasite *Paradinium* sp. (Shields, 1994) and parasitic dinoflagellates (Coats, 1999). Dinoflagellate infections have been reported at lower frequency during colder months when host abundance is low (Kimmerer & McKinnon, 1990; Skovgaard & Saiz, 2006). Seasonal changes in the free-living microbial communities that are also known to associate with zooplankton hosts may influence infection dynamics and potential environmental drivers of microbiome composition.

1.4.3 Symbiont transmission between zooplankton

We currently do not fully understand many of the lifecycles of protist zooplankton symbionts, as they elude cultivation efforts in laboratories. For many of the symbionts that have been studied, our understanding of their lifecycles and transmission is based on their first descriptions (Shields, 1994). Transmission is a critical component of a
symbiont’s life cycle. Without adequate transmission, symbionts would be unable to find a new host, which would reduce their prevalence within the host population. Symbionts can encounter or become established with a host through either horizontal or vertical transmission. Horizontal transmission occurs as symbionts are passed from one individual to the next by dispersal through the environment and contact of the symbiont with a suitable host. Vertical transmission that occurs from parent to offspring (Bright & Bulgheresi, 2010).

Horizontal transmission of ciliates has been observed in both apostome and suctorian ciliates with infective life stages that move through the water column to attach or settle on a new host (Grimes & Bradbury, 1992; Stankovic et al., 2002). Some dinoflagellate symbionts are also transmitted horizontally. Parasitic spore stages, potentially released from the host carcass or excreted in fecal pellets, are found in the water column where they are ingested by the host or attached to their exoskeleton (Coats, 1999; Shields, 1994). Other interactions, such as mating, can facilitate horizontal transmission. Epibiont diatoms of *Corycaeus* spp. were found at higher densities on the regions of contact when these copepods mate, suggesting that transmission between individuals occurred during copulation (Russell & Norris, 1970).

Evidence for vertical transmission is scarcer in marine than freshwater ecosystems. Various lineages of microsporidian parasites in amphipod hosts can be transmitted either horizontally or vertically. Vertically transmitted microsporidians may cause feminization of their hosts and distort the sex ratio in the host population (Haine et al., 2007; Bojko & Ovcharenko, 2019). *Nosema* sp. is a parasite of the brackish water amphipod *Gammarus dubeni* that is passed from parent to eggs and offspring. The parasite causes the feminization of males and impedes egg production and growth in females (Terry et al., 1998).

Together, the interactions of zooplankton with the microbial community of their environment result in diverse associations, either by contact with their external surfaces or through consumption and infiltration of internal tissues. These associations can be necessary for the development and survival of zooplankton, or the microorganisms may be digested as nutrition. As we know relatively little about zooplankton symbionts and
their mutualistic, commensal, and parasitic roles are not often clearly defined, each are associated with their zooplankton host and are part of the zooplankton microbiome.

1.5 Microbiome of zooplankton

The community of microorganisms residing in specific regions or habitats are often complex and diverse, with unique functions and interactions, and is referred to as a microbiome (Berg et al., 2020). Microbiomes contribute to the ecology of open environments, such as soils, freshwater, seawater, and sediments (Berendsen et al., 2012; Herlemann et al., 2011). They also include the microbial communities associated with other organisms, such as animals, which provide habitats to colonizing or symbiotic microorganisms (McFall-Ngai et al., 2013). Much of the focus of zooplankton microbiome research has been directed to the diversity and metabolic contribution of bacterial communities that colonize hosts (Olszewski et al., 2020; Tang et al., 2011; Moisander et al., 2015; Corte et al., 2018; Datta et al., 2018). The protist microbiome of zooplankton is less well explored. Previously, much of the detection of protist symbionts has relied on the use of microscopy to observe and identify symbionts in zooplankton (Shields, 1994; Skovgaard & Saiz, 2006). This method has limited our current knowledge to the few symbionts that are easily identifiable or to those at a late stage of their life cycle or at high-density infestations. Recognizing the lack of knowledge of protist symbiont diversity associated with zooplankton, metabarcoding (DNA sequencing of barcode genes for taxonomic identification) has been used to detect symbionts, and importantly parasites, within hosts, and to identify free living life stages in the marine environment (Cleary et al., 2019; Vargas et al., 2015).

Most metabarcoding investigations of zooplankton are done primarily to investigate diet, but have unintentionally revealed the diversity of symbionts, providing insight into their protist microbiome and global prevalence (Cleary et al., 2018; Yeh et al., 2020). Therefore, molecular data can provide insights into both feeding and symbiotic trophic links within the zooplankton.

With the ability to process a greater number of samples without dependence on visual identification, molecular methods provide a clearer understanding of the range of hosts of a symbiont and a more complete survey of symbiont diversity. Since symbiotic
relationships can have a significant influence on host fitness but the diversity of symbionts are poorly known, this thesis will aim to explore the protist microbiome of marine crustacean zooplankton using molecular methods and to gain insight into the symbiotic relationships that potentially contribute to zooplankton population dynamics.

1.6 Study region

The protist microbiome of predominant taxa of crustacean zooplankton from the northern Strait of Georgia, British Columbia was investigated. The Strait of Georgia (SoG) is a coastal semi-enclosed basin located between the west coast of British Columbia and Vancouver Island, Canada (Harrison et al., 1983). The SoG is a critical region involved in the breeding of salmon and herring, commercially important populations, which rely on zooplankton for food (Hay & McCarter, 1997).

The SoG experiences strong temporal and spatial fluctuations in productivity, typical of a temperate coastal region (Jackson et al., 2015). During spring there is a marked increase in primary production, allowing higher trophic levels to flourish. This period of high productivity, called the spring bloom, occurs when the amount of sunlight reaching surface waters increases and winter mixing has supplied these waters with high amounts of nutrients (Allen & Wolfe, 2013).

Seasonal shifts are seen in the communities of phytoplankton and zooplankton that dominate the SoG throughout the year (Harrison et al., 1983; Peña et al., 2016). Common species of phytoplankton in the SoG include the diatomsSkeletonema costatum, Thalassiosira spp., Coscinodiscus spp. and Chaetoceros spp., and the dinoflagellates Gymnodinium spp., Peridinium spp., and Dinophysis spp (Harrison et al., 1983). Predominant crustacean zooplankton species include the copepodsEucalanus bungii, Calanus spp., Metridia spp., Paraeuchaeta spp., Pseudocalanus spp., Oithona spp., the euphausiidsEuphausia pacifica and Thysanoessa spp., the amphipodsThemisto pacifica, Primno abyssalis, Cyphocaris challengeri, and the ostracodsDiscoconchoecia spp. and Alacía spp (Mackas et al., 2013). Other common non-crustacean groups such as chaetognaths, polychaetes, siphonophores, ctenophores, and pteropods are also prevalent in the SoG (Mackas et al., 2013). Strong seasonal shifts of productivity and of these zooplankton communities can vary regionally within the strait. In the northern SoG,
primary production in the summer is lower than in the southern region due to limiting nutrients – lower nitrate concentration from low winds and water mixing (Peña et al., 2016). The timing of the spring bloom varies from year to year due to environmental variables such as light, temperature, and water mixing (Allen & Wolfe, 2013; Mahara et al., 2019), but on average the SoG spring bloom forms in mid-April (Peña et al., 2016). Following the increase in phytoplankton productivity, there is an increase in zooplankton biomass. Zooplankton communities in the northern SoG change seasonally – in winter, spring, and summer months, and this seasonal succession of zooplankton is a result of the distinct life histories of zooplankton species responding to seasonal and environmental changes (Mahara et al., 2019). These trends in host abundance and life histories (e.g. reproduction, feeding, diapause) may influence the diversity of symbionts and, as a result, host productivity seasonally. This thesis will explore the diversity of symbionts associated with crustacean zooplankton communities of the northern SoG ecosystem.

1.7 Study objectives

The overall purpose of this research is to survey the diversity of zooplankton symbionts associated with crustacean zooplankton in the northern SoG using high throughput DNA metabarcoding techniques. The thesis has the following objectives:

1) To characterize the diversity and specificity of symbiotic protists associated with predominant crustacean zooplankton hosts.

2) To identify seasonal changes in protist symbiont composition associated with zooplankton.

Using these same data, a third objective is:

3) To describe the diets of the dominant crustacean zooplankton.
Chapter 2

2 Methods

2.1 Field sampling

2.1.1 Field station

The Hakai Research Institute has established multiple permanent marine stations on the coast of British Columbia, Canada throughout the Strait of Georgia (SoG) and nearby regions. The samples for this thesis were collected at station QU39 (50.0307, -125.0992), located in the northern SoG, near Quadra Island (Fig. 2.1). Station QU39 is located 130 m from the east shore of Quadra Island and the local depth is approximately 265 m.

Figure 2.1: Map of the Strait of Georgia, BC. Hakai Institute marine station QU39 (50.0307, -125.0992) is located in the northern region of the strait.
2.1.2 Field sampling

Depth-integrated plankton tows were used to collect zooplankton at QU39 from August 28th to 30th 2018 and July 11th to 18th 2019. Plankton nets with 250 µm or 350 µm mesh were used in 2018 and 2019, respectively, and towed vertically from depth to the surface. Following collection, the contents of the net were gently rinsed with 0.22 µm filtered sea water, size fractioned using 1000 µm and 250 µm sieves, and fixed in 95 % ethanol. Samples from 2018 were collected in late morning or early afternoon. In 2019, one tow of zooplankton was collected each day in the afternoon, and samples were not size fractioned prior to fixation.

From Hakai Institute’s ongoing oceanography monitoring program at QU39, plankton tows that had been collected and preserved using the same methods were also analyzed. Specifically, zooplankton samples collected on February 2nd, April 21st, June 13th, August 28th, October 23rd, and December 4th, 2017 were used to examine seasonal patterns in microbiome composition.

2.2 Subsampling of the dominant crustacean zooplankton

Crustacean zooplankton species selected for this investigation were the numerically predominant species based on initial observations of the plankton tows from the 2018 samples and on previous research describing the taxa known to be abundant in this region (Mackas et al., 2013). The sub-sampled zooplankton taxa were amphipods *Cyphocaris challengeri* and *Themisto pacifica*, copepods *Calanus pacificus*, *Corycaeus* sp., *Eucalanus bungii*, *Metridia pacifica*, and *Oithona similis*, krill *Euphausia pacifica*, and ostracods.

Zooplankton were filtered out of the 95 % ethanol and resuspended in sterile artificial seawater in a Bogorov tray for sorting under a dissecting microscope. Identification of zooplankton to genus or species was based on reference taxonomy keys for copepods (Gardner & Szabo, 1982). All but two zooplankton taxa were identified to species. The copepod *Corycaeus* sp. was not identified to species because its small size made it difficult to discern species-specific features, but identification to the genus level was clear.
based on their distinct body shape and pigmented eye. *Oithona* copepods are also small, approximately 2 mm, but because of the typically high abundance of *O. similis* in this environment, it was assumed that the species *O. similis* was collected. Lastly, ostracods were not identified to genus or species, so these collections potentially comprise more than one species of ostracods.

Individuals were picked with sterile forceps, rinsed in 0.22 µm-filtered seawater or sterile artificial seawater, and transferred to 95% ethanol in screw capped tubes until the time of DNA extraction. Replicate groups of 10 or 20 individuals were collected. The number of individuals per group depended on numerical abundance in the samples as well as the size of the zooplankton. Almost all zooplankton, except *O. similis*, were large (> 4 mm) or with lower abundance and were collected in groups of 10. As *O. similis* is so small, 20 individuals were collected to ensure enough biomass would be sampled to amplify symbiont DNA. *Corycaeus* spp. were also small but due to the lower abundance of this taxon only 10 individuals were pooled per replicate. Three replicate groups were collected for each species (Table 2.1).

From the 2017 samples, it was not possible to collect three replicates for each taxon. Taxa of zooplankton were collected if abundance was high enough to sample an adequate number of individuals (10 or 20 individuals) and replicates (three replicate groups) (Table 2.1).
Table 2.1: Number of individuals pooled per replicate group of adult host taxa. Year and month indicate the time the sample was collected from QU39 and the number of replicate groups from each time point.

<table>
<thead>
<tr>
<th>Individuals pooled</th>
<th>Host (total replicates)</th>
<th>2019</th>
<th>2018</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FEB</td>
<td>AUG</td>
<td>FEB</td>
</tr>
<tr>
<td>10</td>
<td><em>Metridia pacifica</em> (21)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td><em>Calanus pacificus</em> (24)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td><em>Eucalanus bungii</em> (22)</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td><em>Corycaeus</em> sp. (19)</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td><em>Oithona</em> sp. (21)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td><em>Euphausia pacifica</em> (12)</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td><em>Cyphocaris challenger</em> (6)</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td><em>Themisto pacifica</em> (10)</td>
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</tr>
<tr>
<td>10</td>
<td><em>Ostracoda</em> (6)</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

2.3 Molecular methods

2.3.1 DNA extraction

Bead beating was used to homogenize all zooplankton tissues prior to DNA extraction using the GeneJET Genomic DNA Purification Kit (Thermo Fisher). To homogenize larger zooplankton, namely, *Calanus pacificus*, *Cyphocaris challenger*, *Eucalanus bungii*, *Euphausia pacifica*, *Metridia pacifica*, ostracods, and *Themisto pacifica*, 0.3 g of 1 mm and 0.2 g of 0.5 mm zirconium silica beads (BioSpec Products) were added to the groups of zooplankton plus 180 µL digestion buffer from the extraction kit. For smaller bodied copepods, *Oithona* sp. and *Corycaeus* sp., 0.5 g of 0.5 mm beads were used. Zooplankton were homogenized using a Bullet Blender (Next Advance) for 10 min at the instrument’s speed setting of 10. Using the homogenate, DNA extraction followed the GeneJET purification protocol and DNA samples were stored at -20 °C.

2.3.2 PCR of the V4 region of the 18S ribosomal RNA gene for metabarcoding

The V4 region of the 18S ribosomal RNA gene (18S V4) was targeted using oligonucleotide sequences that are designed to be biased against metazoans, but otherwise amplify all other eukaryotes (Table 2.2) (Bower et al., 2004; del Campo et al.,
These oligonucleotides will hereafter be referred to as universal non-metazoan, or UNM. The purpose of the UNM oligonucleotides is to reduce amplification of host DNA. The length of the region targeted by these oligonucleotides is approximately 600 bp.

A two-step PCR protocol was developed to amplify the 18S V4 region targeted by the UNM oligonucleotides while also adding adaptor and sample-specific index sequences necessary for multiplex sequencing with an Illumina MiSeq instrument (Fig. 2.2) (Gohl et al., 2016). The first PCR step is target specific and amplifies the 18S V4 region while also adding on part of an Illumina sequencing adaptor (Table 2.2, Fig. 2.2 A).

![Two-step PCR protocol](https://example.com/figure2.png)

**Figure 2.2:** Two-step PCR protocol for generation of 18S V4 dual indexed sequencing amplicons. A) First PCR is marker-specific. Primers include part of the Illumina-compatible adaptor sequence and the universal non-metazoan oligonucleotide targeting the 18S V4 region (Adaptor+UNM_F and R). B) Second PCR amplifies the product of the first amplification, and in the process adds sample specific indices and remaining adaptors to each end of the amplicon. C) The resulting PCR product is compatible with Illumina MiSeq sequencing.

For the first PCR step, the reaction mix comprised 1 µL of template DNA, 12.5 µL of Phusion Hot Start Flex 2X Master Mix (New England BioLabs, Ipswich, MA), 0.4 µM of forward and reverse Adaptor+UNM primers (Table 2.2), and 0.1 µg/µL BSA (New England BioLabs, Ipswich, MA), in a final volume of 12 µL. Initial denaturation at 95 °C for 5 min was followed by 20 cycles of; 95°C denaturation for 10 sec, 51.5 °C annealing for 30 sec, and 72 °C elongation for 1 min. A final elongation at 72 °C for 5 min followed
to conclude the first PCR. The product from this target-specific PCR was directly used as the template for the barcoding PCR.

The second PCR, referred to as the index PCR, added unique 8 bp sequences (an index) to distinguish each sample and the remainder of the Illumina adaptors to the V4 amplicons (Table 2.2, Fig 2.2 B), producing dual indexed amplicons (index sequences on each end of the amplicon) compatible with an Illumina MiSeq instrument (Fig. 2.2 C). The second PCR was conducted in 30 µL reactions and included: 2 µL of the first step PCR product, 15 µL Phusion Hot Start Flex 2X Master Mix (New England BioLabs, Ipswich, MA), 0.2 µM Adaptor+Index_F and Adaptor+Index_R (Table 2.2), 0.1 µg/µL BSA (New England BioLabs, Ipswich, MA). Initial denaturation at 95°C for 5 minutes was followed by 10 cycles of; 95 °C denaturation for 30 seconds, elongation at 55 °C for 30 seconds, and elongation at 72 °C for 1 minute, and then a final elongation at 72 °C for 5 minutes ended the second PCR. For each sample, two replicate two-step PCRs were conducted.

Agarose gel electrophoresis was used to confirm that the size of the final product was consistent with the predicted size of approximately 770 bp, and also to quantify the PCR product. The intensity of the band was compared to the intensity of bands from a 100 bp DNA ladder for which the DNA concentration is known (FroggaBio). For each replicate, approximately 10 ng of each dual indexed amplicon product were pooled together. This pooled library of DNA was cleaned using a PureLink™ PCR Purification Kit (Thermo Scientific) prior to sequencing.
Table 2.2: Universal non-metazoan (UNM) primers specific for 18S V4 rRNA barcode gene amplification with MiSeq sequencing adaptors. Underlined sequence indicates universal non-metazoan oligonucleotides. Bolded sequence indicates complementary sequences for priming the second step PCR.

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequence</th>
<th>Melting temperature of UNM oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adaptor+UNM_F</td>
<td>TCGTCGGCAGCGTCAGATGTGTATAAAGAGACAGGTGCCAGCAGCCGCG</td>
<td>70.4 °C</td>
</tr>
<tr>
<td>Adaptor+UNM_R</td>
<td>GTCTCGTGGGCTCGGAATGTGTATAAGACACATTAAATTCAGCCTTGCG</td>
<td>61.0 °C</td>
</tr>
<tr>
<td>Adaptor+Index_F</td>
<td>AATGATACGGCGACCACCAGATCTACACNNNNNNNNTCTCGTGCCACGCAGT</td>
<td>46.2 °C</td>
</tr>
<tr>
<td>Adaptor+Index_R</td>
<td>CAGCAGAAGCPGCATACGAGATNNNNNNNNTCTCGTGCCACGCAGT</td>
<td>50.1 °C</td>
</tr>
</tbody>
</table>

2.4 Sequencing

The DNA library was sequenced using an Illumina MiSeq instrument (and V2 chemistry) at the London Regional Genomics Center. Instead of the standard protocol of the 250 bp sequencing in each direction, sequencing was run for 300 cycles in the forward direction and 200 cycles in the reverse direction. Only forward reads were analyzed for this thesis because the amplicons are too long to merge the forward and reverse reads successfully, and the V4 region is more variable and thus more informative at this end of the amplicon.

2.5 Bioinformatics and statistical analyses

2.5.1 Sequence processing and taxonomic assignment

Sequences were trimmed to 285 bp and filtered for quality control, and taxonomic classifications were assigned using tools made available in QIIME2 (version 2019.4) (Bolyen et al., 2019). Using DADA2, sequences were trimmed to remove primer sequences and bases with a quality score lower than 25, chimeric sequences were removed, and sequencing errors were corrected, resulting in a list of unique amplicon sequence variants (ASVs). ASVs represented by a low abundance of sequence reads (<10) were removed. ASVs were taxonomically classified using a Scikit-learn Naive Bayes classifier trained on the Protist Ribosomal Reference database (PR²) for the V4 region of the 18S rRNA gene (Guillou et al., 2013).
### 2.5.2 Molecular confirmation of zooplankton identification

To confirm identification of the zooplankton based on morphology, all ASVs classified as “Crustacea” were analyzed. For each zooplankton, the crustacean ASV with the highest relative abundance was assumed to be from the zooplankton itself, *i.e.* the host (and not from their diet, or contamination). Other crustacean ASVs that were at least 97% similar to the most abundant crustacean ASV were also considered as likely host sequences. Genetic similarity was calculated in R version 3.6.2 (R Core Team, 2019), using the package ape (Paradis & Schliep, 2019). These ASVs were aligned to crustacean sequences from the PR2 database using MAFFT (Katoh et al., 2002), the alignment was trimmed using trimAl (Capella-Gutiérrez et al., 2009), and the best maximum likelihood phylogenetic tree was constructed using RAxML with statistical support assessed by 500 bootstrap replicates (Stamatakis, 2014). The tree was rooted with the species *Munida quadrispina*, commonly known as a squat lobster, acting as an outgroup.

### 2.5.3 Protistan microbiome analyses

To examine the eukaryotic microbiome of the zooplankton, all metazoan and plant ASVs were excluded. Statistical and phylogenetic analyses and graphs were done using several packages implemented in R version 3.6.2 (R Core Team, 2019), including phyloseq (McMurdie & Holmes, 2014), vegan (Dixon, 2003), and ggplot2 (Wickham, 2016).

The alpha diversity of the complete eukaryotic microbiome (*i.e.* of all protist and fungal lineages) for each zooplankton host was determined by calculating the Shannon diversity index in phyloseq (McMurdie & Holmes, 2014). To visualize the relative abundance of eukaryotic lineages associated with each host, ASV counts were transformed into relative abundances and ASVs with < 1% relative abundance in a sample were removed from the data before generating barplots with ggplot2 (Wickham, 2016).

Due to dominance of alveolate ASVs, the data were split into two categories: alveolate-only ASVs and non-alveolate ASVs. In phyloseq, alveolate and non-alveolate data were rarified to even sampling depth and the Bray-Curtis dissimilarity index was calculated as a measure of community composition differences among hosts (McMurdie & Holmes, 2014). Principal Coordinates Analysis (PCoA) was used to visualize similarities in the community structure of eukaryotic microbes.
To identify alveolate ASVs that were significantly different in abundance among hosts, Analysis of Composition of Microbiomes (ANCOM) was performed as implemented in QIIME2 version 2019.4 (Mandal et al., 2015; Bolyen et al., 2019). ASVs identified as differentially abundant but with poor taxonomic classification (e.g. not to family or genus) were placed into a reference phylogenetic tree using Phylogenetic Placement (pplacer) to identify closely related species or groups (Matsen et al., 2010). The data were split by sampling month for each zooplankton to investigate seasonal changes in alveolate ASVs. Alveolate-only data were rarefied, and Bray-Curtis dissimilarity indices calculated to assess beta diversity among hosts using PCoA as implemented in phyloseq (McMurdie & Holmes, 2014). To visualize changes in the diversity of the alveolate microbiome, relative abundances of major lineages were plotted by sampling month.

Lastly, the data were split by sampling month for each zooplankton to investigate seasonal changes in diet, including all protist and fungal ASVs, and more specifically diatoms (Bacillariophyta) ASVs. Barplots of diatom ASVs with a relative abundance ≥ 1% were examined to assess changes in diatom diet throughout the sampling periods.

2.5.4 Hydrozoan diversity

The diversity of hydrozoans ASVs associated with zooplankton was determined by phylogenetic analysis with 18S V4 sequences from known Hydrozoa and other Cnidarians. ASVs classified as Hydrozoa with a relative abundance ≥ 1% in each sample were aligned to representative hydrozoan and cnidarian sequences using MAFFT (Katoh et al., 2002), the alignment trimmed using trimAl (Capella-Gutiérrez et al., 2009), and a maximum likelihood phylogenetic tree was constructed using RAxML with statistical support assessed by 500 bootstrap replicates (Stamatakis, 2014).
Chapter 3

3 Results

3.1 Sequencing output

After error correction and chimera removal, a total of 5,699,343 sequences of the V4 region of the 18S rRNA gene (18S V4) region were produced from 140 samples of crustacean zooplankton using non-metazoan biased PCR primers. These sequences comprised 1,698 unique amplified sequence variants (ASVs). Approximately half (54.82\%) of the ASVs were classified as metazoan, of which 82.14\% were from the host zooplankton taxa (see section 3.2), and the other half, 45.14\%, were protist and fungi sequences (Table 3.1). When host ASVs were removed, the zooplankton microbiome consisted mostly of protists and hydrozoans (Fig. 3.1). Rarefaction curves of protist and fungi ASVs of 133 samples plateaued, or almost plateaued, indicating that most of the eukaryotic microbial diversity was captured by this sequencing effort (Appendix A, Fig. A.1 to A.9).

Table 3.1. Percentage of 18S V4 sequences for major taxonomic groups. The sample mean is the mean per zooplankton sample ± the standard deviation (SD). Also shown are the Metazoa split into host and Hydrozoa sequences (the percentage of non-host metazoan sequences is not shown).

<table>
<thead>
<tr>
<th>Sequence count</th>
<th>% Metazoa</th>
<th>% Host(^a)</th>
<th>% Hydrozoa</th>
<th>% Plant</th>
<th>% Protist &amp; fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>5,699,343</td>
<td>54.82</td>
<td>45.03</td>
<td>9.48</td>
<td>0.04</td>
</tr>
<tr>
<td>Sample mean</td>
<td>40,709</td>
<td>55.06</td>
<td>46.45</td>
<td>8.12</td>
<td>0.08</td>
</tr>
<tr>
<td>± SD</td>
<td>± 33,090</td>
<td>± 27.90</td>
<td>± 26.00</td>
<td>± 16.60</td>
<td>± 0.21</td>
</tr>
</tbody>
</table>

\(^a\) Sequences identified as the host genera, either: *Calanus, Conchoecia* (ostracods), *Corycaeus, Cyphocaris, Eucalanus, Euphausia, Metridia, Oithona*, or *Themisto* (*Parathemisto*) (see section 3.2).
Host identity confirmation

For each host, sequences assigned to Crustacea were examined to confirm the morphological identification of the zooplankton. The crustacean ASV with the highest relative abundance (no less than 50%) was assumed to belong to the host. Other ASVs with ≥97% similarity to the representative host ASV were also considered as representing the host species. A phylogenetic tree of these crustacean host sequences supports the morphological identification of the zooplankton hosts to genus level (Fig. 3.2), with the exception of Cyphocaris and ostracods (Discoconchoecia), for which 18S rRNA sequences of Cyphocaris and these particular ostracod taxa are unavailable.
For each zooplankton, host sequences accounted for most of the crustacean sequences in the data. For *C. pacificus, E. bungii, M. pacifica, E. pacifica*, and *T. pacifica*, host ASVs were cumulatively > 90 % of the crustacean sequences (Fig. 3.2). However, *Corycaeus* sp. and *C. challenger* host sequences accounted for only 86.8 % and 86.3 % of crustacean sequences, respectively, from these hosts (Fig. 3.2). In *Corycaeus* samples 9.4 %, 1.2 %, and 1.0 % of crustacean sequences came from other small copepod genera *Oithona, Paracalanus,* and *Pseudocalanus,* respectively. Amphipod *C. challenger* samples had relatively high proportions of *Themisto* (6.0 %) and *Oithona* (1.5 %) sequences from the crustacean ASVs. These sequences are likely a component of their prey as these zooplankton are known carnivores.

Hosts *Oithona* sp. and ostracods had lower proportions of the predominant host ASVs, with two dominating ASVs per host (Fig. 3.2). *Oithona* samples were identified as *O. similis,* one of the predominant *Oithona* species found in the SoG, and 31.0 % of the crustacean ASVs from the *Oithona* samples are likely to be that species (Fig. 3.2). The more predominant *Oithona* ASV (62.2 %) had > 99 % similarity to *O. atlantica,* the other species of *Oithona* present in the SoG, so the samples collected likely comprise both *Oithona* species.

During zooplankton sorting, ostracods were not identified to genus or species, only as the class Ostracoda, so a diversity of ostracod ASVs was expected. All ostracod ASVs were assigned to the same genus, *Conchoecia,* a close relative of the genus *Discoconchoecia,* which is known to occur in the SoG. Since the *Discoconchoecia* sequences are not in the PR² database, it is possible that these sequences assigned to *Conchoecia* in fact belong to *Discoconchoecia.* Two ASVs dominated ostracod sample data, accounting for 55.5 % and 21.1 % of ostracod crustacean sequences (Fig. 3.2). These likely comprise two species of ostracods.
3.2 Maximum likelihood phylogenetic tree of 18S V4 sequences of the zooplankton hosts. The squat lobster, *Munida quadrispina*, was used as the outgroup. Ostracods placed with the genus *Conchoecia*, although may represent the genus *Discoconchoecia*, which occurs in the Strait of Georgia. Only bootstrap values $\geq 75\%$ are shown. Bolded names indicate the amplified sequence variants (ASVs) of each zooplankton host in this study and the percentage of crustacean ASVs that are $> 97\%$ similar. Non-bolded names indicate similar reference sequences from The Protist Ribosomal Reference Database with their Genbank accession number.

### 3.3 Eukaryotic microbiome

For the microbial eukaryotes (i.e. protists and fungi), alpha diversity was similar among hosts, with cyclopoid copepods and *C. challengeri* having the highest variation among samples (Fig. 3.3). For all hosts, most of the sequences belonged to alveolates, and among them, mainly dinoflagellates and ciliates (Fig. 3.4). There were higher proportions of dinoflagellates in cyclopoid copepods, also slightly higher in amphipods, whereas other copepods, krill, and ostracods were dominated mostly by ciliates. Also, there was a relatively high abundance of Ellobiopsidae associated with amphipods, indicating a
preference for amphipod hosts (Fig. 3.4). Besides alveolates, ochrophytes (mostly diatoms) consistently comprised the largest relative abundance of sequences from non-alveolate lineages, most likely indicative of diatoms consumed as part of their diet. There was a higher relative abundance of cercozoan sequences associated with both cyclopoid copepods, the majority of which were identified as *Paradinium pouchetti*, a known parasite of copepods (Skovgaard & Daugbjerg, 2008).

Figure 3.3: Shannon diversity indices of protist and fungal microbiomes associated with crustacean zooplankton. The black dots indicate the calculated Shannon index for each sample. The midline indicates the median and the upper and lower half of the box represents the upper and lower quartile, respectively. Whisker lines indicate the minimum and maximum values. Colour indicates each zooplankton taxa.
Figure 3.4: Relative abundance of protist and fungal amplified sequence variants (ASVs) associated with crustacean zooplankton Amplified sequence variants with less than 1% relative abundance were not included.

PCoA analysis was used to assess community similarity among zooplankton hosts. PCoA plots of all eukaryotic microbes (all protists and fungal lineages) (Appendix A Fig. A.10) and for only alveolates (Fig. 3.5) showed similar results, suggesting that alveolate diversity is the main driving factor in community composition differences among hosts. In the alveolate-only PCoA, 42.6% of the variability in community composition was explained by the first three principal components (Fig. 3.5). There is variability in community composition among samples from the same host, but *E. bungii* was clearly different from the other zooplankton suggesting a species-specific microbiome for *E. bungii* (Fig. 3.5A). Overall, four general clusters of samples were observed indicating similarity in their microbiomes: calanoid copepods split into two groups (*C. pacificus* and *M. pacifica* in one group, and *E. bungii* in another), cyclopoid copepods (*Oithona* sp. and *Corycaeus* sp.), and the *Themisto-Euphausia* group (*T. pacifica* and *E. pacifica*) (Fig. 3.5B).
Not all *Corycaeus* sp. samples cluster with *Oithona*, and cluster on their own. Ostracods cluster on their own, with some variability. *C. challengeri* samples show high variability, with samples not forming a cluster.

When analyzing non-alveolates microbial eukaryotes, the samples no longer clustered by host (Fig. 3.6), again indicating that the alveolates are driving the similarities in symbiont communities within a host and between some hosts. There are likely fewer non-alveolates with distinct host preferences.

**Figure 3.5:** Biplots of principal coordinates analysis of alveolate communities of crustacean host taxa. (A) First and second principal components and (B) first and third principal components.
3.4 Differentially abundant alveolates and host specificity

Focusing on the alveolates, most of the alveolate ASVs were apostome ciliates and Syndiniales dinoflagellates, both of which include known symbiont (commensal and parasitic) lineages (Fig. 3.7). The relative abundances of the alveolate ASVs and an ANCOM analysis indicating which ASVs were significantly differentially abundant among the host taxa (Fig. 3.8) reflect the clusters observed in the PCoA analysis. In predicting the relationships of the potential symbionts with their host, ASVs of apostome ciliates were labeled as commensals or parasites if this is known for organisms with the same classification. Unclassified apostomes were labeled as symbionts since they were not related to lineages of known parasites or commensals, and both interactions are equally likely for these undescribed apostomes (Fig. 3.8 A). All Syndiniales ASVs were labeled as parasitic as all Syndiniales characterized to date are parasitic (Guillou et al., 2008), but these predicted interactions were not confirmed. Some replicates showed high variability in the presence and relative abundance of alveolate ASVs (Appendix A Fig. A.11).
Most alveolate lineages were present in multiple hosts, but some ASVs had a significantly higher relative abundance in certain hosts, demonstrating some host specificity or preference. For the two clusters of calanoid copepods recognized in the PCoA analysis, *M. pacifica* and *C. pacificus* communities were dominated by an apostome ciliate of the genus *Chromidina*, whereas *E. bungii* was mostly dominated by other unclassified apostome ciliates. Of the differentially abundant ASVs, five of the six *Chromidina* ASVs were significantly more abundant in *M. pacifica* and *C. pacificus*, suggesting some preference for these hosts. These ASVs were approximately 94% to 95% similar to a *Chromidina* sp. infecting squid and cuttlefish from the Mediterranean Sea but were not phylogenetically placed with this sequence, and likelihood ratios for placement of these ASVs with other known *Chromidina* were very low (0.05 to 0.07). It is likely that these *Chromidina* ASVs (all with >97% similarity to each other) represent undiscovered species of *Chromidina*.

In *Oithona* sp. and *Corycaeus* sp., *Chromidina* and apostome ciliates, although present, were much less predominant than in the three calanoid copepods. These copepods had high abundances of Syndiniales groups associated with them (Fig. 3.7). Group II Syndiniales ASVs were consistently more abundant in *Oithona* sp. and to a lesser extent *Corycaeus* sp. (Fig 3.8 B). Ciliates of the genus *Trochilia* (Order Dysteriida) were exclusively found in *Corycaeus* sp. (Fig 3.8 A). These differences between *Oithona* and *Corycaeus* communities may account for some *Corycaeus* samples clustering closer to *E. bungii* than the cyclopoid cluster in the PCoA (Fig. 3.5). The differentially abundant ASVs from Syndiniales group IV were of the known genera *Hematodinium* and *Syndinium*. *Hematodinium* had a higher relative abundance in *Oithona* sp., but also in *E. bungii*, and *C. challengeri*. *Syndinium* has a higher relative abundance in *Oithona* sp. and *M. pacifica*.

Although dominated by apostome ciliates, *Chromidina* ciliate ASVs were less abundant in the other non-copepod groups of zooplankton (amphipods, euphausiids and ostracods). Amphipod *T. pacifica* alveolate communities were dominated by two apostome ciliate ASVs, one classified as the parasite *Fusiforma themistocola* and the other unclassified.
Apostome ciliate 15 was found in higher relative abundances in *T. pacifica*, and apostome ciliate 16 was high in both *T. pacifica* and *E. pacifica* likely driving the similarity of their alveolate communities as observed in the PCoA analysis. Unclassified apostome ciliates 15 and 16 are phylogenetically most closely related to *Gymnodinioides* and *Hyalophysa* (likelihood ratios 1, and 0.75, respectively). *Gymnodinioides* and *Hyalophysa* are known to feed on the fluid from hosts shed exoskeleton (exuviotrophic) and are considered harmless to the host zooplankton (Landers et al., 1996; Ohtsuka et al., 2011).

Furthermore, both *E. pacifica* and *T. pacifica* had significantly lower relative abundances of other apostome ciliates and Syndiniales than observed in other hosts. In contrast, *Pseudocollinia oregonensis* ASVs were significantly more abundant in *E. pacifica* than in other hosts, including *T. pacifica*.

Ostracods are particularly distinct in the ANCOM analysis of differentially abundant ASVs. Several *Pseudocollinia* sp. and other Pseudocolliniidae ciliate ASVs were of significantly higher relative abundance within ostracods (Fig. 3.8 A). Unclassified Pseudocolliniidae that associated exclusively with ostracod hosts were placed phylogenetically near this family, although all placement likelihood ratios were low (< 0.66) except for Pseudocolliniidae 1 (0.97), which were placed basal to the *Pseudocollinia* spp. and *Fusiforma* sp. These ASVs likely represent currently unknown parasites from the family Pseudocolliniidae, exclusive to ostracod hosts.

Ostracods and *E. pacifica* shared a high relative abundance of suctorian ciliates, specifically ASVs classified as *Ephelota plana* that were persistent across other hosts but at low relative abundances (Fig. 3.7, 3.8 A). *C. challengeri* alveolates were more variable among replicates (Fig. 3.5), but high relative abundances of ASVs classified as the apostomes *Fusiforma* and *Chromidina*, Syndiniales group II, and *Hematodinium* were observed (Fig. 3.8).
Figure 3.7: Relative abundance of alveolate amplified sequence variants (ASVs), associated with crustacean zooplankton. ASVs with less than 1% relative abundance were not included.
Figure 3.8: Bubble plot of mean relative abundance of (A) ciliate and (B) dinoflagellate amplified sequence variants (ASVs) found to be differentially abundant among zooplankton host taxa. Size of solid bubble corresponds to the mean relative abundance of alveolate ASV from a host taxon. Upper standard deviation indicated by the outer bubble outline. ASVs are labeled with the taxon name of the lowest taxonomic rank for which the ASV was classified. Classification was confirmed using phylogenetic placement (using pplacer). Predicted biological relationship of the ASV with their host is based on phylogenetic relatedness to known alveolates and indicated to the right of the plot.
3.5 Seasonal changes in alveolate microbiomes

Seasonal changes in alveolates were examined in copepods *C. pacificus*, *Corycaeus* sp., *E. bungii*, *M. pacifica*, and *Oithona* sp. Due to the limited number of samples collected because of low prevalence of certain zooplankton taxa, insufficient seasonal data were collected for *E. pacifica*, *C. challenger*, ostracods, and *T. pacifica* (Table 2.1). PCoA plots were used to assess similarities in communities based on sampling month. Seasonal changes were evident, but the dynamics differed depending on the host.

*C. pacificus* alveolate communities were more similar among spring, fall, and winter, than summer (Fig. 3.9A). At a higher taxonomic rank, high relative abundances of apostome ciliates were observed across each sampling period, and the proportion of Syndiniales increased in spring (April 2017) (Fig. 3.10A). This is inconsistent with the PCoA plot, which shows summer alveolate communities are different from fall, winter, and spring. However, the distinct communities observed in summer months are likely due to a potential seasonal shift in apostome ciliate symbionts for *C. pacificus*. Trends in the relative abundances of individual alveolate ASVs show that in fall (October 2017), spring (April 2017), and winter (February and December 2017), *C. pacificus* was dominated by an unclassified apostome ASV, whereas in summer (June and August 2017, August 2018, July 2019) alveolates were dominated by a *Chromidina* ASV. Most of the Group II Syndiniales ASVs which were in high proportions in April 2017 belonged to the genus *Amoebophyra* (Fig. 3.11), which is a genus known to infect bloom-forming dinoflagellates (Guillou et al., 2008) not zooplankton.

For *M. pacifica* alveolate-associated communities, spring samples (April 2017) were more similar to those of summer months from August 2017 and different from fall (October 2017), winter (February and December 2017), and other summer samples (August 2018 and July 2019) (Fig. 3.9B). Like *C. pacificus*, *M. pacifica* was generally dominated by apostome ciliates throughout the sampling periods but was consistently dominated by *Chromidina* apostomes ASVs, one predominant in April and August 2017 and another dominating the rest of the sampling dates (June and October 2017, August 2018, July 2019) (Fig. 3.12), which was the same *Chromidina* ASV seen in *C. pacificus* summer samples (Fig. 3.11). During spring (April 2017) there was an increase in
Syndinium (Syndiniales group IV) in *M. pacifica*, a group that has yet to be described infecting *Metridia* spp.

*E. bungii* summer alveolate communities clustered together, along with October and December 2017 samples, but seasonal patterns were not consistent likely due to high variability among replicates in a sampling period (Fig. 3.9C). Apostome ciliates also dominated *E. bungii* in each sampling period with a high relative abundance of a *Chromidina* ASV in February and April 2017 (the same ASV as in *M. pacifica* in April and August 2017), which was replaced by an unclassified apostome ASV in the other sampled months (Fig. 3.13). February and April 2017 were also distinct with a higher relative abundance of Syndiniales ASVs (Fig. 3.10C). Specifically, *Hematodinium* (Syndiniales group IV) ASVs increased in February and April 2017, as well as *Amoebophyra* and other Syndiniales group II in April 2017 only (Fig. 3.13).

Interestingly, each calanoid copepod was associated with a different dominating Syndiniales ASV in April 2017, despite being collected on the same date and experiencing the same environment in the water column. *C. pacificus* experienced highest abundance of an *Amoebophyra* ASV, *M. pacifica* had the highest abundance of *Syndinium* ASV, and *E. bungii* experienced a high abundance of *Hematodinium* and *Amoebophyra*. This suggests a species-specific interaction between Syndiniales and calanoid copepod hosts.

For the cyclopoid copepod *Corycaeus* sp., samples were not collected in April and February 2017. Two distinct *Corycaeus* sp. clusters were observed in the PCoA analysis; June and October 2017, and December 2017 and July 2019 (Fig. 3.9 D). For summer months, the composition was not consistent in June 2017, August 2017 and 2018, and July 2019 samples, indicating significant interannual variability (Fig. 3.10 D). *Corycaeus* sp. were dominated by unclassified apostome ciliates in June and October 2017 and were otherwise dominated mainly by Syndiniales (group I and II) and by the ciliate *Trochilia* sp. (Order, Dysteriida) (Fig 3.10 D). In June and October 2017, the microbiome was dominated by the same apostome ASV prevalent in *C. pacificus* (Fig. 3.14). *Oithona* sp. samples did not cluster by season or sampling months (Fig. 3.9 E). All *Oithona* sp. samples were dominated by Syndiniales Group II and IV, with lower relative
abundances of Syndiniales Group I in August and October 2017 (Fig. 3.10E). Like Corycaeus sp., Oithona sp. did not have consistent alveolate microbiomes in the sampled summer months across years. Syndiniales group I was most abundant in August through October 2017, but the same high relative abundance was not seen in August 2018 and July 2019 samples. A Syndiniales group II ASV belonging to Amoebophyra (same ASV observed dominating in other hosts) was relatively high in all sampling periods except October 2017. Other Syndiniales group II ASVs were abundant mostly in the August 2018 and July 2019 samples. The two crustacean parasites belonging to Syndiniales group IV were found in Oithona sp. The Hematodinium ASV was of low abundance with a small increase in February 2017 whereas the Syndinium ASV was abundant throughout 2017, peaking in August, but did not reach the same prevalence in August 2018 and July 2019 (Fig. 3.15).

Figure 3.9: Biplots of principal coordinates analyses of alveolate communities associated with the crustacean zooplankton taxa Calanus pacificus (A), Metridia pacifica (B), Eucalanus bungii (C), Corycaeus sp. (D), and Oithona sp. (E). The season and sampling date at which the zooplankton were collected are indicated by colour and shape, respectively.
Figure 3.10: Relative abundances of alveolate amplified sequence variants (ASVs) associated with crustacean zooplankton *Calanus pacificus* (A), *Metridia pacifica* (B), *Eucalanus bungii* (C), *Corycaeus sp.* (D), and *Oithona sp.* (E) grouped by sampling date. Dates without bars do not have associated samples. ASVs with less than 1% relative abundance were not included. Dotted line separates 2017 sampling dates from summer months in 2018 and 2019.
Figure 3.11. Mean relative abundance of alveolate amplified sequence variants (ASVs) associated with *Calanus pacificus*, chronologically through sampling periods. Lines join unique symbols for individual ASVs of a taxon. A gap in the lines separates the end of 2017 timeseries data and 2018 and 2019 data.
Figure 3.12. Mean relative abundance of alveolate amplified sequence variants (ASVs) associated with *Metridia pacifica*, chronologically through sampling periods. Lines join unique symbols for individual ASVs of a taxon. A gap in the lines separates the end of 2017 timeseries data and 2018 and 2019 data.
Figure 3.13. Mean relative abundance of alveolate amplified sequence variants (ASVs) associated with *Eucalanus bungii*, chronologically through sampling periods. Lines join unique symbols for individual ASVs of a taxon. A gap in the lines separates the end of 2017 timeseries data and 2018 and 2019 data.
Figure 3.14. Mean relative abundance of alveolate amplified sequence variants (ASVs) associated with Corycaeus sp., chronologically through sampling periods. Lines join unique symbols for individual ASVs of a taxon. A gap in the lines separates the end of 2017 timeseries data and 2018 and 2019 data.
Figure 3.15. Mean relative abundance of alveolate amplified sequence variants (ASVs) associated with *Oithona* sp., chronologically through sampling periods. Lines join unique symbols for individual ASVs of a taxon. A gap in the lines separates the end of 2017 timeseries data and 2018 and 2019 data.

3.6 Seasonal changes in potential prey of non-alveolates

Non-alveolate ASVs largely consisted of diatoms (Bacillariophyta, within the Ochrophyta) (Fig. 3.4), which are known to be consumed by zooplankton, so these data were analyzed with a focus on zooplankton diets. From PCoA plots of community composition from each sampling month, general clustering patterns were similar to those as observed for only alveolate ASVs, except for *C. pacificus* and *E. bungii*, where spring samples were more distinct from other seasons (Fig. 3.16). For *C. pacificus*, summer months clustered independently of fall and winter (Fig. 3.16 A) and *E. bungii* summer, fall, and winter samples clustered apart from February 2017 (Fig. 3.16 C). *M. pacifica*, *Corycaeus* sp., and *Oithona* sp. did not cluster by season similarly to the alveolate results (Fig. 3.16B, D, E). Spring was particularly distinct for non-alveolates, but for *Oithona* sp.
and Corycaeus sp. there were no spring samples collected, so any potential differences in non-alveolate composition was not determined.

The distinct spring samples (April 2017) observed for C. pacificus and E. bungii were due to a large proportion of ochrophytes, however C. pacificus also had high relative abundances of ochrophytes during August 2018 (Fig. 3.17A, C). The majority of ochrophytes were diatoms (Bacillariophyta made up 99.4% of Ochrophyta ASVs). Most diatoms are considered as potential prey items of zooplankton, although some are known to be zooplankton symbionts. M. pacifica exhibited a lower relative abundance of diatoms in spring, compared to C. pacificus and E. bungii, but experienced another high relative abundance of diatoms in August 2017 (Fig. 3.17 B). Corycaeus sp. had a high relative abundance of diatoms in August 2018 but much lower in August 2017 (and all 2017 samples) and not in July 2019 (Fig. 3.17 D). Oithona sp. showed a similar pattern to Corycaeus sp., with the highest abundance of diatoms in August 2018 (Fig. 3.17 E).

For the zooplankton (C. pacificus and E. bungii) with high abundance of spring diatoms in April 2017, diatom diversity was relatively consistent among zooplankton consisting mostly of *Thalassiosira*, but relatively few *Detonula*, *Skeletonema*, and *Chaetoceros*. *Chaetoceros* dominated all other samples in 2017 except in December. Ochrophytes associated with C. pacificus in December consisted only of non-diatom Chrysophyte ASVs (data not shown) and E. bungii was dominated by *Navicula* and *Skeletonema* (Fig. 3.18 A, C). *M. pacifica* had a contrasting trend with high *Thalassiosira* in April and August, but high *Chaetoceros* in June and October 2017. *M. pacifica* had high *Minidiscus* and *Licmophora* in December 2017. In August 2018 all C. pacificus, M. pacifica, and E. bungii, are dominated by *Pseudo-nitzschia*. In July 2019 there was more variability among hosts; C. pacificus dominated by *Minidiscus* and *Synedra*, M. pacifica and *Synedra* exclusively, and E. bungii with *Chaetoceros*, *Minidiscus*, *Skeletonema*, and *Synedra* (Fig. 3.18 A, B, C).

Corycaeus sp. 2017 samples were dominated by *Chaetoceros*, *Skeletonema*, and *Pseudohimantidium*. *Pseudohimantidium* is a genus of epibiont diatoms known to associated with *Corycaeus* spp. copepods (Gómez et al., 2018), and so it is unlikely consumed as diet. High abundances were only observed in October and December 2017
samples indicating a preference for these hosts during colder months. (Fig. 3.18 D). In August 2018 Corycaeus spp. were also relatively more abundant with Pseudo-nitzschia and Chaetoceros, and July 2019 samples were dominated by Minidiscus and Arcocellulus.

From February, June, August, and October 2017, Oithona was dominated by Chaetoceros diatoms, with higher relative abundance of Thalassiosira in December 2017. Again, August 2018, was mostly composed of Pseudo-nitzschia. July 2019 samples were diverse, mainly Chaetoceros, Arcocellulus, Synedra, and Minidiscus (Fig. 3.18 E).

Figure 3.16: Biplots of principal coordinates analyses of protist and fungal communities associated with the crustacean zooplankton taxa Calanus pacificus (A), Metridia pacifica (B), Eucalanus bungii (C), Corycaeus sp. (D), and Oithona sp. (E). The season and sampling date at which the zooplankton were collected are indicated by colour and shape, respectively.
Figure 3.17: Relative abundances of protist and fungal amplified sequence variants (ASVs) associated with crustacean zooplankton *Calanus pacificus* (A), *Metridia pacifica* (B), *Eucalanus bungii* (C), *Corycaeus sp.* (D), and *Oithona sp.* (E) grouped by sampling date. Dates without bars do not have associated samples. ASVs with less than 1% relative abundance were not included. Dotted line separates 2017 sampling dates from summer months in 2018 and 2019.
Figure 3.18. Relative abundances of diatom amplified sequence variants (ASVs) associated with crustacean zooplankton *Calanus pacificus* (A), *Metridia pacifica* (B), *Eucalanus bungii* (C), *Corycaeus sp.* (D), and *Oithona sp.* (E) grouped by sampling date. Dates without bars do not have associated samples, except for *C. pacificus* where no diatom ASVs were present in December 2017. ASVs with less than 1% relative abundance were not included. Dotted line separates 2017 sampling dates from summer months in 2018 and 2019.

3.7 Abundance and diversity of unassigned hydrozoans

An unexpectedly large proportion of sequences belonging to the Hydrozoa
(approximately 9.5% of total sequences) were consistently observed across most samples (Table 3.1), and were found at higher proportions in amphipods, krill, and the copepod *E. bungii* (Fig. 3.1). Most hydrozoan sequences (30 of 36 Hydrozoa ASVs) could not be further classified past the rank Hydrozoa, indicating either that they are distinct from previously described hydrozoans or that the sequenced region cannot resolve the diversity of hydrozoans associated with zooplankton. Less than 1% of the sequences were classified to previously sequenced hydrozoans: *Aglaura hemistoma* (subclass Trachylina), *Cordagalma cordiforme* (order Siphonophora), and *Stegopoma plicatile* (order Leptotheccata). Using BLASTN and the GenBank database, unclassified ASVs had high percentage identity (> 99%) and coverage to multiple species of hydrozoans indicating that the 18S V4 region alone is likely unsuitable for hydrozoan identification. However, a phylogenetic tree constructed for the most abundant hydrozoan ASVs (present at ≥ 1% relative abundance in all samples) shows that the hydrozoans associated with zooplankton are siphonophores (Fig. 3.19).

**Figure 3.19: Maximum likelihood phylogenetic tree of hydrozoan 18S V4 amplified sequence variants (ASVs).** Hydrozoa ASVs from this study are in bold. The phylogenetic tree includes 18S V4 sequences from known species of Hydrozoa from the orders Siphonophora, Leptotheccata, Anthothecata (suborder Aplanulata and Filifera), and Limnomedusae (subclass Trachylina). Tip labels are coloured based
on their classified order or suborder. Outgroup sequences are from other medusozoans (Cnidaria). Bootstrap values ≥ 75% shown.

Chapter 4

4 Discussion

This is one of the first studies that explicitly characterizes the eukaryotic microbiome of crustacean zooplankton using a metabarcoding approach, and addresses symbiont diversity. The eukaryotic microbiome of nine predominant crustacean zooplankton taxa from the Strait of Georgia, BC comprised a diversity of protist symbionts, largely belonging to the ciliate and dinoflagellate lineages of the alveolates. Alveolates are one of the better studied lineages of crustacean symbionts (Ho & Perkins, 1985; Shields, 1994), although there is still much to discover regarding their life cycles, infectivity, influence on productivity, and prevalence as symbionts in zooplankton communities globally. This study also provided insight into the diets of these zooplankton, comprising diatoms, but and potentially siphonophores and other crustacean zooplankton. These roles, prey or symbiont, were inferred based on phylogenetic relatedness to lineages known to be symbionts, then otherwise considered to be prey. Based on sequencing data alone it is not possible to determine whether the organisms are truly symbiotic with the host or if there is any influence on the productivity or fitness of the host zooplankton. Furthermore, because some symbionts are transmitted to their host through ingestion and establish in their gut, the roles of prey or symbiont are intertwined, making it hard to separate them based on these roles. Nevertheless, the diversity and abundance of sequences closely related to known symbionts are high, suggesting important lineages to investigate further.

4.1 Symbiont microbiome

Previous studies of zooplankton protist symbionts have mostly investigated specific species of symbionts from specific hosts in various regions around the world (Ianora et al., 1990; Kimmerer & McKinnon, 1990; Nicol, 1983), but by relying on morphological identification and microscopy, could not reveal the full extent of the diversity of symbionts harboured by zooplankton (Skovgaard & Saiz, 2006). Previous metabarcoding studies have focused on single host species, specific symbiotic lineages, or on diet,
instead of the complete eukaryotic microbiome (Guo et al., 2012; Cleary & Durbin, 2016; Yeh et al., 2020). I also used metabarcoding, but to reveal a more complete picture of the diversity of symbionts associated with zooplankton hosts. Alveolate lineages of ciliates and dinoflagellates accounted for the majority of symbionts associated with the zooplankton of this study, consistent with the high diversity of alveolates known to associate with crustacean zooplankton (Ho & Perkin, 1985; Shields, 1994; Fernandez-Leborans & Tato-Porto, 2000; Skovgaard, 2015).

By surveying the diversity of symbionts associated with several taxa of zooplankton from the same environment, I was able to show that many of the protist symbionts are likely generalists, as they were observed in association with multiple hosts. However, some symbiont ASVs had a significantly higher relative abundance in particular hosts indicating a degree of host preference, or symbiont lineage-specificity given the conserved nature of the 18S V4 region.

4.1.1 Symbiont host specificity

This study shows that most symbiont ASVs were not exclusively found associated with a single zooplankton species but did show a higher prevalence or preference to one or two hosts. This suggests that the symbionts may be interacting with multiple hosts in the pelagic community, but for many known symbionts, these relationships have not yet been described. For example, the ciliates *Fusiforma* and *Pseudocollinia*, which have been described as parasites of *Themisto* and krill species, respectively (Chantangsi et al., 2013; Lynn et al., 2014), and had the highest relative abundances with these zooplankton in this study, were also observed associated with other non-preferred hosts but at a lower relative abundance (Fig. 3.8). Another example of host preference was observed for the suctorian ciliate *Ephelota*. ASVs classified as *Ephelota* were found at high relative abundances associated with *E. pacifica* and ostracods, both of which have been previously described harbouring *Ephelota* ciliates (Endo et al., 2017; Chatterjee et al., 2019), but these ASVs were also sequenced from all other hosts. These interactions demonstrate the broad range of zooplankton hosts of protist symbionts, suggesting a generalist lifestyle. However, host preferences may change regionally, emphasizing the importance of extensive global surveying of zooplankton. Sequence presence or absence
alone cannot determine if the ciliates affect other non-preferred hosts, but my results bring up the possibility of broader interactions among zooplankton and alveolate symbionts than previously known.

As the effects of many zooplankton symbionts on their hosts are poorly characterized, it is not clear what impact the broad specificity of symbiotic protists has on the host populations. Metabarcoding data alone provide no information on potential effects other than inferring similarities to known symbionts. But at the very least, this study suggests potential host preferences and interactions with non-preferred hosts. Interactions with non-preferred hosts in the environment may interfere with transmission of symbionts to the preferred host. Non-preferred hosts feeding on the free-living life stages of symbionts would remove free-living stages from the environment, reducing efficiency of the transmission to a new host (Thieltges et al., 2008).

4.1.1.1 Diversity and ecology of apostome ciliates

Apostome ciliates are well-known symbionts of zooplankton, and primarily of crustaceans (Lindley, 1978; Grimes & Bradbury, 1992; Gómez-Gutiérrez, 2003), but also associate with other animals such as ctenophores and chaetognaths (Skovgaard, 2014). In the present study, apostome ciliates were generally prevalent across all taxa except *Oithona* sp. and *Corycaeus* sp. which both had lower diversity of apostome ciliate ASVs than other hosts.

In crustacean zooplankton, apostome ciliates are known to range from commensal to parasitic, and related sequences were found in all zooplankton hosts examined here. Some of these ASVs were similar to those of known commensals, but for many a symbiotic relationship could not be predicted since the classification was to the order Apostomatida. Many of the apostome ciliate ASVs that were not classified to known species (Fig 3.8, Apostome 2, 9, 15, and 16) were similar to the sequences of *Gymnodinioides* and *Hyalophysa*, which are genera known as commensals of crustaceans and generalists (Landers et al., 1996; Bradbury, 2005; Bradbury, 1994). Copepods, including species of calanoids, from various regions in the Pacific and
Atlantic Oceans, have previously been observed to harbour a high diversity of taxa closely related to *Gymnodinioides* and *Hyalophysa* genera based on 18S molecular data (Guo et al., 2012).

Host selection for lineages of apostome ciliates is not well-understood. However, in the Seto Island Sea, Japan, the apostome *Vampyrophrya pelagica* has been reported at high abundances across copepod taxa, although *Oithona similis* (and other *Oithona* species) were rarely infested (Ohtsuka et al., 2004), which is consistent with low relative abundance of apostomes in *Oithona* species from the SoG reported in this study. In Japan, the contrasting infestation of apostomes on copepod taxa was not related to copepod taxonomy, size, or feeding behaviour, and is still unexplained. Ohtsuka et al. (2004) suggested that chemical cues released by *Oithona* species could possibly cause apostomes to avoid these copepods. Moulting frequency was hypothesized as the cause of fluctuations of phoront abundance on copepod hosts, as increased moulting would shed apostomes from copepod surfaces. In Japan, larger copepods (e.g. *Calanus* sp.) known to overwinter did not moult frequently, whereas small copepods with shorter life spans would more frequently shed their exoskeleton along with their apostome phoronts (Ohtsuka et al., 2004). This may account for the lower prevalence of apostomes in *Oithona* and *Corycaeus* spp. observed in this study.

The most abundant apostome in this study, *Chromidina*, is likely to use zooplankton as a secondary host. *Chromidina* ASVs dominated the apostome (and alveolate) communities of calanoid copepods *C. pacificus* and *M. pacifica*. *Chromidina* spp. are known parasites of cephalopods (ex. octopus, cuttlefish, squid), infecting the renal cavity of the host and causing tissue damage (Souidenne et al., 2016). The complete lifecycle of this apostome has yet to be documented, but the life stages associated with monotomy (budding) and palintomy (division) have been well described within the cephalopod host (Bradybury, 1994). After the ciliate leaves the cephalopod in an infecting life stage (tomite), it then encysts (as a phoront) to colonize an intermediate host and by an unknown route will complete its lifecycle by infecting a cephalopod again. The intermediate host for *Chromidina* spp. has not been confirmed (Souidenne et al., 2016), but due to the prevalence of apostome ciliates associated with crustaceans, they are likely a candidate (Gestal et al., 2019; Bradbury, 1994). Cephalopods have a diverse diet, some species
feeding on detritus (Hoving & Robison, 2012), fish, crustaceans, and other invertebrates (Ohkouchi et al., 2013; Olmos-Pérez et al., 2017), and various species of squid and octopuses have been observed to consume zooplankton such as copepods, euphausiids, amphipods, and decapods (Chen et al., 1996; Olmos-Pérez et al., 2017; Villanueva et al., 2017). The overwhelming abundance of Chromidina associated with the crustacean zooplankton in this study, supports the hypothesis that crustaceans – especially calanoid copepods – may act as intermediate hosts. Further investigation (e.g., microscopy, parasite transmission experiments, and further sequencing with more specific genetic markers) of the true association of these ciliates with crustacean hosts should confirm this component of the Chromidina life cycle and determine if zooplankton hosts are affected by this symbiotic association.

The apostomes associated with the zooplankton in this study also include known parasitic lineages, some of which are lethal in host populations. The Pseudocolliniidae are a parasitic group known to be extremely lethal in krill and potentially lethal in amphipods (Gómez-Gutiérrez et al., 2006; Chantangsi et al., 2013). Ciliate ASVs of the family Pseudocolliniidae, including known parasitic genera Pseudocollinia and Fusiforma (Chantangsi et al., 2013; Lynn et al., 2014), were found in all hosts but at significantly higher proportions in E. pacifica and T. pacifica, respectively (Fig. 3.8). The presumed parasite F. themistocola has only recently been described infecting the arctic amphipod Themisto libellula (Chantangsi et al., 2013), a close relative to T. pacifica investigated in this study. It is possible that the ASVs assigned to this parasite in this study may represent a different species of Fusiforma that infect Themisto spp. in the eastern Pacific. Pseudocollinia spp. have been reported more extensively from euphausiids in the Pacific, Atlantic, and Southern (Antarctic) Oceans (Lynn et al., 2014; Cleary et al., 2019), including infections of P. oregonensis causing mass deaths in E. pacifica populations off the coast of Oregon, USA (Gómez-Gutiérrez et al., 2006), which is in the same general North Pacific region as the SoG. In this study, host preferences for ASVs classified as F. themistocola and P. oregonensis were consistent, suggesting that these two genera of parasites specifically infect Themisto and Euphausia.

This study revealed that parasites of the family Pseudocolliniidae also interact with other zooplankton taxa, particularly ostracods, which appear to host a diversity of
Pseudocolliniidae distinct from the known euphausiid and amphipod infecting species. As they are not similar to those of known Pseudocolliniidae, these unique ASVs are likely to represent newly uncovered species. Further investigation should characterize these new lineages of Pseudocolliniidae specific to ostracods and determine if these lineages are similarly lethal or detrimental to ostracod productivity to those infecting amphipods and euphausiids. Ostracods are not as abundant as copepods and euphausiids in the SoG, but they are consistently present throughout the year and considered a major group in this region (Mackas et al., 2013). Furthermore, ostracods are an important component of the food web, having been observed to contribute to the diet of some salmon and herring species in the SoG (Osgood et al., 2016). It is reasonable to infer that these ciliates, which are so closely related to known parasites and parasitoids, may be detrimental to ostracod fitness with important effects in the SoG ecosystem.

Due to the extensive distribution of apostomes in crustacean zooplankton, Guo et al. (2012) hypothesized that they must be of importance in global marine ecosystems, but their roles are currently unknown. However, metabarcoding studies may overestimate the abundance of these organisms. Some protists, such as ciliates, are known to have high copy number of 18S rDNA, which may result in an overestimated proportion in the environment (Prescott, 1994). In this study, however, there was a significant difference in apostome composition between calanoid and cyclopoid copepods, so that at the very least the relative differences among hosts are an accurate representation of the host microbial communities. Although their precise abundances could not be determined, this study also supports the prevalent symbiotic association of apostome ciliates with crustacean zooplankton, suggesting their potentially significant negative impacts on productivity and their role as secondary hosts. This relationship should be further explored across both symbiont and host taxa to determine potentially unknown important ecological roles.

4.1.1.2 Diversity and ecology of Syndiniales

Apostomes were followed by syndinians in order of predominance in the zooplankton investigated in this study. Syndiniales are a diverse and geographically widespread parasitic group of marine dinoflagellates, included in the Marine alveolates (MALV)
Syndiniales not only associate with crustaceans, but also protists, such as other dinoflagellates, radiolarians, and cercozoans, as well as other metazoans (e.g. fish eggs) (Stentiford & Shields, 2005; Skovgaard, 2014; Clarke et al., 2019).

Syndinium and Hematodinium (Syndiniales group IV) are known to infect amphipods, decapods, and copepods, causing serious damage to or even death of the host (Stentiford & Shields, 2005; Shields, 1994). Hematodinium ASVs were present in cyclopoid copepods, albeit at a low relative abundance. This may have been due to the detection at an early stage of infection, which would underestimate their significance on host productivity. In contrast, Syndinium in Oithona sp. (and M. pacifica in April 2017) had a high relative abundance. This genus is known to be extremely parasitic to copepods, causing them to rupture and die (Shields, 1994), and has been responsible for up to a third of copepod host mortality (Kimmerer & McKinnon, 1990). Syndinium has not been described infecting Metridia spp, although it has been described infecting calanoid genera such as Paracalanus, Calanus, and Eucalanus as well as other species of Oithona and Corycaeus (Ho & Perkins, 1985; Skovgaard et al., 2005). High abundance of Syndinium in Oithona sp. in this study indicates a potentially significant source of mortality in Oithona populations in the SoG.

A high abundance of Syndinium spp. in copepod hosts was unexpected in view of their high lethality. In contrast, Pseudocollinia, another highly lethal parasite, was observed at relatively lower proportions in the krill E. pacifica, suggesting that potential hosts infected with large numbers of this parasite were killed and therefore not sampled. The time required for this parasitoid to establish infection and kill the host is relatively short, 33 to 73 hours, supporting the hypothesis that infected krill could have been killed quickly and sunk out of the water column, precluding their collection (Gómez-Gutiérrez et al., 2012). Syndinium infections have been reported to kill the host within an hour of infection with spores (Kimmerer & McKinnon, 1990), although these observations were based on microscopy. High abundances observed by metabarcoding could indicate an early stage infection that may not be identifiable by microscopy or that the time required to kill the host is longer than previously thought. This would support a higher prevalence of the parasite in a host than would otherwise be measured by microscopy alone.
Comparing the diversity of protist symbionts associated with live and dead crustacean zooplankton has yet to be investigated and may identify symbionts associated with dead zooplankton, thereby demonstrating their ability to cause zooplankton death. Parasite prevalence in dead zooplankton can be investigated using staining methods that determine if zooplankton individuals are dead or alive at the time of collection (Elliott and Tang, 2009; Maud et al., 2018). When live zooplankton and their carcasses can be captured (e.g. through a zooplankton net tow or sediment traps), live/dead staining methods in combination with metabarcoding could document differences in the eukaryotic microbiome before and after host death and investigate the prevalence of parasitism in non-predatory zooplankton mortality.

Syndiniales from groups I and II are not usually associated with crustaceans, and their prevalence in Oithona or Corycaeus spp. may be due to direct or indirect consumption. Hosts feeding on free-living dinoflagellates, radiolarians, or cercozoans, which are infected by these group I and II Syndiniales, could explain the occurrence of these Syndiniales in these zooplankton. However, there is not a corresponding high proportion of free-living dinoflagellates, cercozoans, or radiolarians in the Oithona sp. or Corycaeus sp. samples, which are typically infected by group I and II Syndiniales. Cercozoans associated with Oithona and Corycaeus were classified as Paradinium pouchetti, a known parasite of copepods (Skovgaard, 2014), and could potentially host these syndinian parasites. Protist parasites can infect other protist parasites, as is known for the apostome ciliate Photorophrya which parasitizes other apostomes (Ohtsuka et al., 2015), and this may be occurring with SoG Syndiniales. Alternatively, group I and II Syndiniales may in fact have infected free-living dinoflagellates that persisted beyond the time that the dinoflagellate host was digested. If these Syndiniales can survive digestion and evade the immune system of the host, they may be able to extend their survival inside the copepod host or even use the host for dispersal through excreted fecal pellets. This hypothesis has not been documented for Syndiniales and crustacean hosts, and further investigation into host digestion and immune system would be required to resolve the interaction.
It may be that the Syndiniales are symbionts that have yet to be described with these zooplankton hosts. The occurrence of free-living Syndiniales was significantly correlated with the abundance of copepods in inlets near southern Vancouver Island, BC and the activity of copepods was correlated with that of group II Syndiniales, but not with group I Syndiniales, suggesting parasitic or prey interactions with the former (TorresBeltrán et al., 2018). There are clearly intriguing correlations between Syndiniales and zooplankton in coastal marine environments, but their actual interactions need further investigation to determine whether they function as parasites, prey, or associates of other hosts.

4.1.2 Seasonal changes in copepod alveolate communities

In the northern SoG, a temperate location, the environment is strongly seasonal, with cyclic trends in primary productivity, phytoplankton and, zooplankton community composition, and nutrient and light availability (Harrison et al, 1983; Peña et al., 2016), and for this reason the diversity of symbionts associated with crustacean zooplankton was expected to undergo seasonal shifts, as has been observed previously. Seasonal trends in symbiont abundance or diversity associated with zooplankton hosts have not been studied extensively and observed trends may only be representative of the specific ecosystems in which they are recorded. General seasonal patterns in alveolate symbiont abundance have been observed in zooplankton populations. Seasonal patterns of dinoflagellate parasite infections were more noticeable when copepod host density was high, indicating that host density and life history (reproduction, growth, periods of peak biomass) influences the prevalence of some parasites in copepod populations (Ianora et al., 1990; Skovgaard & Saiz, 2006). In the northern SoG, the zooplankton species identified in this study generally increase in abundance following the spring bloom and in summer months (Mahara et al., 2019). For calanoid copepods, *C. pacificus*, *E. bungii*, and *M. pacifica*, apostome ciliate relative abundance was consistently high throughout the year and did not seem to rise in summer months when population density is highest. Apostome ciliates were relatively abundant regardless of relatively high or low host abundance. The calanoids (apart from *M. pacifica*) did exhibit lower relative abundance of apostome symbionts in early spring 2017, potentially due to growth and molting triggered by the spring bloom, which causes the loss of apostomes from copepod surfaces. Other apostome ciliates have been seasonally associated with copepods due to seasonal changes
in temperature influencing ciliate life cycle (cold period slowing development to infective stages) and host life history (hosts moulting less frequently in colder temperature and accumulating apostomes) (Ohtsuka et al., 2004).

Few specific symbiont ASVs had seasonal trends in their relative abundances. *Chromidina* ASVs associated with *C. pacificus* were more abundant during summer months and were replaced by unclassified apostome ciliates during colder months. For the other calanoids, dominating apostomes did not shift between warmer and colder months and *Chromidina* spp. associated with *M. pacifica* were at a high abundance consistently throughout the sampling periods. During colder months, *C. pacificus* is in diapause, unlike *M. pacifica*, which remains active throughout the year (Tommasi et al., 2013; Johnson & Checkley Jr., 2004). Perhaps because *C. pacificus* is not shedding its exoskeleton as frequently as *M. pacifica*, which is reproductively active and moults throughout the year (Padmavati et al., 2004), *Chromidina* accumulation is higher.

Copepods may be a secondary host of *Chromidina* spp., which are primarily known as parasites of cephalopods. Perhaps, cephalopods prefer copepods infested with *Chromidina* sp., in which case the copepods would be at a higher risk of predation by cephalopods, which would reduce the abundance of *Chromidina* measured in the population. The mode of transmission and recognition between the intermediate copepod host, cephalopod predator, and *Chromidina* ciliate requires further investigation to determine if predation accounts for the dynamics of *Chromidina* ASVs in copepods populations.

Seasonal trends were also seen in Syndiniales symbionts of zooplankton. A marked increase in the relative abundance of Syndiniales was observed in calanoid copepods during spring, and in February 2017 for *E. bungii*. During spring, *C. pacificus* and *E. bungii* had higher proportions of group II Syndiniales than *M. pacifica*. Syndiniales have been observed to have a higher relative abundance during the spring bloom, potentially due to increased availability of their hosts *e.g.*, dinoflagellates (Kellogg et al. 2019). The increase of group II Syndiniales associated with *C. pacificus* and *E. bungii* in spring could be the result of directly ingesting infective spores, which could be more abundant in the environment. Alternatively, group II Syndiniales could be infecting the blooming dinoflagellates that are consumed by these copepods, as discussed previously.
In contrast to the seasonal variation in the associated Syndiniales with calanoid copepods, the high relative abundance is seasonally consistent with *Oithona* sp. and *Corycaeus* sp. There is currently no known preference of Syndiniales parasites to infect cyclopoid copepods, as they are known to infect various groups of copepods, including calanoids and cyclopoids, as well as other crustaceans such as amphipods (observed hosts summarized in Shields, 1994). Perhaps the small size of these copepods allows them to feed on a higher proportion of Syndiniales spores directly, as the spores are less than 10 µm in length (Coats & Park, 2002), whereas adult calanoids are potentially too large to preferentially feed on small Syndiniales spores. However, this relationship remains unknown.

*E. bungii* had a higher proportion of the *Hematodinium* parasite (Syndiniales group IV) in February and April 2017, which was rare or absent in the other copepod hosts during the same sampling dates, indicating a potentially host-specific association with *E. bungii*. There is potentially a seasonal prevalence in hosts which are susceptible during this time of high productivity due to increased feeding as prey biomass is high, as previously discussed. *Syndinium* (Syndiniales group IV), a known parasite of copepods (Shields, 1994, Skovgaard et al., 2005), is prevalent mostly in *Oithona* sp. The presence of *Syndinium* in *Oithona* sp. increased throughout the warmer months and decreased in colder months in 2017. This observation is consistent with other studies where *Syndinium* infections of copepods were more prominent in warmer months when host abundance is higher (Ianora et al., 1990).

Parasitism causes mass mortality or significantly inhibits reproduction in zooplankton communities resulting in a loss of biomass for their predators, which feed on them throughout the year. Seasonal fluctuations in parasitism are important to understand as climate and the environment experienced by these zooplankton and symbionts changes, potentially opening new niches for infective stages of parasite life cycles or increasing host stress, rendering them more susceptible to infection. This study attempted to evaluate seasonal changes in symbiont, including parasite, diversity, but data varied between samples and years. Incomplete sampling of each zooplankton group further limited the diversity of zooplankton included in the seasonal analysis. Due to limited sampling time, this study included equidistant samples (each one month apart) from only
six months in 2017. Further studies of the seasonal composition of zooplankton symbionts should include a consistent sampling plan, with samples collected more frequently or from consecutive months to observe trends which may be occurring in a shorter period. Data should also be collected over more than one year, as there is clear interannual variability among summer samples.

### 4.2 Diet

Although the primary focus of this study was to characterize the symbionts associated with crustacean zooplankton, the metabarcoding analysis also provided insight into the potential components of zooplankton diet. Feeding behaviour and diet composition are extensively studied in marine zooplankton due to the importance of zooplankton in marine food webs. The studies aim to resolve trophic links between zooplankton and the flow of carbon through the food web. Copepods are extremely abundant globally as they constitute crucial links in the flow of carbon transfer through their feeding (Kleppel, 1993). Recently, DNA metabarcoding methods have been used to elucidate the feeding of certain copepod species and changes in their preferred prey both regionally and seasonally (Cleary et al., 2017; Yeh et al., 2020). These studies were focused on the diet of Arctic species *Calanus finmarchicus* and *Calanus glacialis*. Metabarcoding studies have been done on copepods, including tropical *Pseudocalanus* spp. (Cleary et al., 2016) and *Calanus* spp. in Norway (Ray et al., 2016). This is one of the first studies to use metabarcoding data to assess the eukaryotic diet of copepod species, with various feeding behaviors: *Calanus pacificus, Metridia pacifica, Eucalanus bungii, Oithona* spp., and *Corycaeus* sp.

#### 4.2.1 Diatoms

The most abundant ASVs that are likely to have a role in zooplankton diet are the diatoms. A large increase in the relative abundance of diatom ASVs was observed in April 2017 for calanoid copepods, correlating with the spring bloom. Metabarcoding studies of *Calanus finmarchicus* have shown that the diatoms *Thalassiosira* and *Chaetoceros* were abundant in their diets (Yeh et al. 2020). These were also two of the most abundant diatoms present in the copepods of the present study. The diet of *Calanus*
glacialis also had high abundances of diatoms, particularly during the spring bloom in April. In April Thalassiosira spp. were especially abundant (Cleary et al., 2017), consistent with their high abundance in the calanoids C. pacificus, M. pacifica, and E. bungii. However, not all patterns in diatom diets were consistent. Overall, the results of this study support the importance of diatoms, particularly Thalassiosira and Chaetoceros, in the diet of marine copepods, especially during the spring bloom when primary production is highest.

The composition of diatoms in the SoG changes in a seasonal cycle. The spring bloom is dominated by Thalassiosira spp., later followed by Skeletonema spp., Chaetoceros spp., Leptocylindrus spp. and Pseudo-nitzchia spp. In the fall, diatom abundance decreases, and resting spores are formed (Harrison et al., 1983). These seasonal trends in composition are consistent with the diatom composition associated with the copepod hosts, indicating that at the level of diversity captured by the 18S V4 there was not a strong diet preference, particularly in calanoid copepods. However, the diatom diet was dominated by Chaetoceros in summer 2017, by Pseudo-nitzchia in 2018, and by Navicula and others in 2019. Interannual variability in the timing of the spring bloom in the SoG is common (Allen & Wolfe, 2013), and shifts in the timing of the spring bloom may also affect the timing of the diatom succession later in the summer, which could account for the differences observed here among the three summers sampled. To investigate this issue more specifically, I will examine the phytoplankton composition and 18S V4 metabarcoding data collected from water samples at QU39 from 2017 to 2019 from a long-term monitoring program.

4.2.2 Crustaceans

Although most of the metazoan data were host sequences, the remaining sequences are likely to have originated from prey. The prevalence of cannibalism cannot be determined (as prey DNA cannot be distinguished from the host’s). The consumption of conspecifics is known for several species of zooplankton, including C. challengeri, T. pacifica, C. pacificus, and M. pacifica (Haro-Garay, 2003; Landry, 1981; Halsband-Lenk, 2005). This study does provide evidence for the consumption of Oithona, Paracalanus, and Pseudocalanus copepods by Corycaeus and Oithona, and for the consumption of
Themisto by Cyphocaris. Each taxon is known to be carnivorous, and these results are consistent with previous observations on their diet. Corycaeus spp. are known predators of nauplii and small bodied copepods (Landry et al., 1985) and C. challengeri has been observed feeding on Themisto pacifica as well as small copepods (Haro-Garay, 2003). It is possible that sample contamination was the source of non-host crustacean ASVs for hosts Corycaeus and Cyphocaris. This is unlikely, however, because individuals were carefully groomed of other zooplankton tangled by their appendages during collection (small copepods such as Oithona spp. can get caught in the legs and arms of the amphipods during collection) and these zooplankton were not detected in the other zooplankton microbiomes.

Oithona sp. samples also had a relatively high abundance of non-host crustacean ASVs. Approximately 4.9% of crustaceans ASVs from Oithona sp. samples were identified as Paracalanus copepods. The diet of Oithona spp. is still relatively unknown, although species have been observed feeding on motile prey, including dinoflagellates, ciliates, and copepod nauplii (Lampitt & Gamble, 1982; Saiz et al., 2014). However, individuals keyed to Paracalanus may have been misidentified as Oithona during sorting. Like Oithona, Paracalanus is also very small, with its adult total body length ranging from approximately 0.8-1.0 mm and approximately 0.5-1.0 mm in total length for Oithona (Gardner & Szabo, 1982). Because of the small size of both copepods, potential misidentification of Paracalanus as Oithona may have occurred. The relative abundance of Paracalanus ASVs is low and if Paracalanus was misidentified as Oithona during collection it likely represents a small number of individuals. Molecular data have previously demonstrated carnivorous feeding in Calanus copepods (Yeh et al., 2020; Cleary et al., 2017) as well as in other calanoids (Cleary et al., 2016), and my results provide evidence for carnivorous components of crustacean zooplankton diet in the northern SoG, which were detected using metabarcoding.

4.2.3 Hydrozoans

The high relative abundance of unclassified hydrozoan diversity was unexpected, although some hydrozoans are known to be epibions of crustaceans (Fernandez-Leborans & Gabilondo, 2005). The significance of hydrozoans in zooplankton diets is
poorly known, but recent data on the gut contents of eel larvae show that they consume siphonophores as an important component of their diet (Ayala et al., 2018). There are no observational data indicating that crustacean zooplankton directly consume adult hydrozoans or any cnidarians, and instead it has been suggested that zooplankton may feed on cnidarian detritus or waste (through marine snow) or even their larvae (Yi et al., 2017). Another possibility is that cnidarian tentacles may have attached to the zooplankton during sample collection in the plankton net and contaminated the zooplankton microbiome. However, multiple studies analyzing the gut or stomach contents of zooplankton have frequently shown the presence of cnidarians (Cleary et al., 2017; Yeh et al., 2020), which adds confidence to the notion that the hydrozoans found in this study are components of their diet and not contamination. Siphonophores were also observed at an unexpectedly high proportion in the gut of Antarctic krill (Cleary et al., 2018), supporting the hypothesis that siphonophores are a significant component of zooplankton diets. The consumption of cnidarians, perhaps through marine snow, has not been previously documented in zooplankton, and would represent an unrecognized path fueling zooplankton productivity and energy transfer in marine ecosystems, with significant consequences for carbon and nutrient cycling and the biological carbon pump.

### 4.3 Conclusions

This thesis assessed the eukaryotic microbiome of multiple species of crustacean zooplankton from the northern Strait of Georgia by metabarcoding of the V4 region of the 18S rRNA gene. Symbiotic relationships between known commensals and parasites of copepods, euphausiids, ostracods, and amphipods were inferred, and unknown symbiont species or unclassified symbiotic relationships were exposed. Many of these symbiotic relationships have been reported for different regions and for different host taxa but have not, until now, been described for the northern SoG. These results uncovered a diversity of zooplankton symbionts that were not previously discovered and described. The study reveals the prevalence of symbiotic relationships in all zooplankton populations investigated. Lineages of apostome ciliates and Syndiniales were the most abundant symbionts elicited in this study. As these lineages include known commensals and parasites found consistently and at high proportions within the sampled hosts, they may make an important contribution to the productivity and mortality of their
zooplankton hosts. Also, many more taxa are known to be parasites of other animals, such as cephalopods, indicating that ecological impacts of zooplankton symbionts may be complex and directly influence higher trophic levels. However, their ecological effects could be inferred only, and remain unproven.

Investigating the interactions between symbionts and hosts should help characterize the influence of symbiotic relationships on zooplankton regulation and productivity. Symbionts are an understudied factor influencing zooplankton productivity with potential to significantly influence zooplankton population dynamics. It is critical to gain a better understanding of the diversity of zooplankton symbionts across many hosts and geographic regions in order to assess the true prevalence of symbionts in marine environments across a broader range of potential hosts. Also, experimental infection or infestation of zooplankton with symbionts would provide a means of elucidating their impact gaining a better understanding of their life cycles and transmission between hosts. Continued efforts in this field will not only resolve important symbiotic connections in the lower marine food web but will improve our understanding of a critical component of zooplankton population dynamics.
References


https://doi.org/10.1016/j.pocean.2004.08.001


**Hematodinium** species and **Hematodinium**-like infections in marine crustaceans. *Diseases of Aquatic Organisms*, 66, 47–70. https://doi.org/10.3354/dao066047


Appendices

Appendix A: Chapter 3 supplementary material

Figure A1. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from *Calanus pacificus* samples.

Figure A2. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from *Metridia pacifica* samples.
Figure A3. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from *Eucalanus bungii* samples.

Figure A4. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from *Oithona* spp. samples.
Figure A5. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from Corycaeus sp. samples.

Figure A6. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from Euphausia pacifica samples.
Figure A7. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from *Themisto pacifica* samples.

Figure A8. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from *Cyphocaris challengeri* samples.
Figure A9. Rarefaction curves for protist and fungal amplified sequence variants (ASVs) sequenced from ostracods samples.
Figure A10. Biplot of principal coordinates analysis of protist and fungal communities between crustacean host taxa. First and second principal components are plotted.
Figure A11. Relative abundances of alveolate amplified sequence variants (ASVs) associated with crustacean zooplankton. Each bar represents the mean relative abundance of ASVs for the taxon sequenced from a host by sampling date. Error bars represent the upper limit of the standard deviation. Grey and white colours separate the samples of each zooplankton host taxa. ASVs with less than 1% relative abundance were not included.
Appendix B: A problematic amplification in amphipods

PCR amplification of amphipods *Themisto pacifica* and *Cyphocaris challenger* DNA samples consistently produced a second product that was larger, approximately 1000 bp, than the expected product size of 770 bp. To determine if the unexpected product was a larger, true 18S V4 amplicon or a product of nonspecific amplification. Both products were cloned and sent for Sanger Sequence at Robarts Research Institute. The unexpected 1000 bp product sequences were 98.84% and 96.95% similar to that of *Parathemisto pacifica* (KC428925) and the low expected band (770 bp) was 99.82% similar to that of the ciliate *Fusiforma themistocola* (KF516511). Based on these results, the high band being amplified from both amphipod DNA samples (*T. pacifica* and *C. challenger*) is suspected to be the amphipod 18S V4 region. Instead of size selecting for the expected band size of 770 bp, to exclude amphipod DNA, these products were included in the library prepared for sequencing. This would ensure that no abnormal protist DNA could be potentially excluded from sequencing and include host DNA that would be used for host taxa confirmation.
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