Can environmental toxins increase parasite fitness? Ecotoxicological studies on the effects of microcystin on the host-parasite dynamics of *Schistocephalus solidus*

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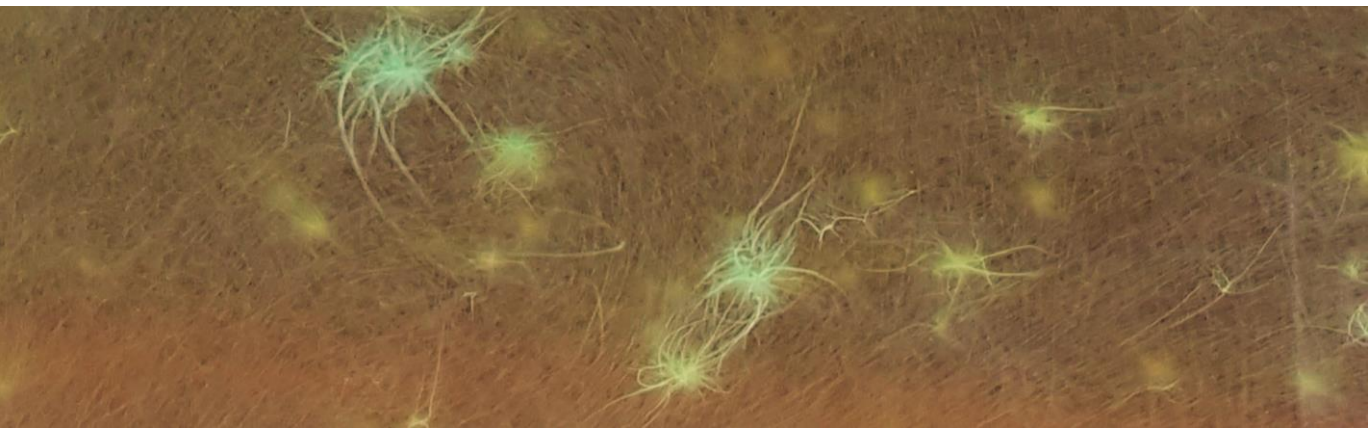

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Abstract

Background: Eutrophication of aquatic biomes and exacerbated climate change effects are expected to result in a global increase in harmful cyanobacterial blooms. Cyanotoxins are detrimental to animal health, but how they affect the dynamics within ecosystems is still mostly unknown. With a host-parasite system acting as a microcosm, I wanted to explore the changes in host-parasite dynamics with a cyanotoxin present.

Methods: In a set of laboratory studies with the copepod-*Scistocephalus solidus* system, I looked at the host-parasite dynamics in the presence of the hepatotoxin microcystin. In four different groups (control, toxin-only, infection-only, toxin-infection combined) of individually isolated copepods, I examined if mortality increased in the first intermediate host, or if the toxin would increase mortality or curb the growth in the parasite.

Results: While the presence of toxin alone increased copepod mortality significantly, microcystin did not exhibit any toxin-parasite interaction leading to increased mortality. However, the host's ability to hinder parasite growth was affected. Since tapeworms accumulate environmental toxins, I expected a lower growth rate of the parasites in the toxin group, but procercoids from toxin-parasite groups were found to have a significantly larger surface area (P.007) than procercoids from the infection-only group.

Conclusions: The increased growth of parasites in the presence of microcystin, suggests a change in the host-parasite dynamic. While host mortality was not significantly affected by the parasite infection. Increased procercoid growth points to a rise in pathogen virulence or weakened immunity in the host, which could be detrimental in less robust host individuals.

Frontpage picture: *The beginning of a cyanobacterial bloom, up close. Photo by Katrine Åmdal Sundt, 2018.*

Background

The work in this thesis was carried out on behalf of the university project Cultivation Project of River Mussels for Reintroduction (Kultiveringsprosjekt av elvemusling for gjenutsetting). The project is administered under the Department of Biology, University of Bergen, and funded by the Norwegian Directorate for Nature Management (Direktoratet for naturforvalting) and Lerøy Vest AS.

The goal of this thesis was to examine the dynamics between host and parasite, and how their systems relate to their environment and disrupting factors within it. While not directly connected to the freshwater pearl mussels (*Margaritifera margaritifera*), microcystins seem to be harmful to all organisms in aquatic biomes. Gaining a more in-depth knowledge of how cyanotoxins affect other organisms inhabiting the same habitat is required for a more holistic approach to ecology and ecotoxicology.

The thesis is equal parts ecotoxicology, parasitology and ecology, and might thus not fit any one of these just so. However, I feel the interdisciplinarity of research is another crucial step in understanding the "bigger picture" of ecological principles and furthering knowledge in this field, so this was also an exercise in juggling the specifics of several fields of research.

Acknowledgements

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Contents

1 Introduction

Human interference is affecting nature at an alarming pace and on a staggering scale, extensive enough to be known as the sixth mass extinction of the Anthropocene (GSDRC, 2009; IPBES, 2019). These are some of the contributing factors: fragmentation and loss of habitat, the increased spread of invasive species, anthropogenic global warming, nutrient depletion or eutrophication, soil and water acidification, and a wide variation of pollution agents—from light and noise to soil, water, and air. Having a solid understanding of ecological principles will help untangle the interconnected threads that make up a natural balance nearing its tipping point.

One field that has arisen in an effort to monitor the state of ecosystems in the Anthropocene is environmental parasitology, which lies within the intersection of ecology, toxicology, and classic parasitology. It examines the interactions between pollutants and parasites through an ecological lens. The rapid shift in the impacts on anthropogenic nature makes this field more relevant than ever, as the overall sensitivity of parasites to toxicants and other environmental disturbances makes them excellent indicators for the general state of an ecosystem (Sures et al., 2017). Host-parasite systems can also work as microcosms for ecological dynamics on a grander scale. This thesis examines one such host-parasite system as the basis of a toxicological study of the hepatoxic cyanotoxin.

Cyanobacteria are free-living, photosynthetic microbes that produce a wide range of toxins, which can be detrimental to human, animal, and environmental health (Pandhal et al., 2018). There is increasing evidence that the change in global climate directly influences the rate of harmful algal and cyanobacterial blooms, commonly referred to as harmful algal blooms (HABs) (Gobler, 2020; Visser et al., 2016; Weber et al., 2020). HABs can lead to the toxification of waterways and anoxic benthic conditions in rivers and lakes (Havens, 2008). The global cyanobacteria occurrence is also proportional with increased eutrophication and the exacerbation of climate change effects (Rastogi et al., 2015).

Among the most common of cyanotoxins are the hepatotoxic microcystins, of which microcystin-LR (MC-LR) is the most harmful. MC-LR was thus the toxicant of choice in the environmental parasitological study using a well-established host-parasite system—the first intermediate stage of the fish tapeworm *Schistocephalus solidus* life cycle. Such studies examine any toxin-infection interactions, as well as the effect on the parasite itself.

The goal of this thesis was to examine the effects of the cyanotoxin microcystin on the host-parasite dynamic of the cyclopoid copepod *Macrocyclops albidus* and the cestode *Schistocephalus solidus*. By monitoring the mortality of both host and parasite, as well as measuring the area of grown parasites, I attempted to test for two possible outcomes:

1. Whether the combination toxin and an active parasite infection would increase mortality in the first intermediate host (*Macrocyclops albidus).*
2. Whether the toxin will increase mortality or curb the growth rate in the parasite.

1.1 Biological Background and Research Organisms

1.1.1 Host-Parasite System

Schistocephalus solidus, a freshwater tapeworm, has been used for decades in studies of aquatic host-parasite systems, particularly that of its second intermediate host, the morphologically diverse three-spined stickleback (*Gasterosteus aculeatus)* (Barber, 2006; Barber and Scharsack, 2010; Scharsack et al., 2007). As *S. solidus*' first intermediate host, the copepod *Macrocyclops albidus* is also included in this system (Hafer-Hahmann, 2019; Wedekind, 1997). Both intermediate hosts are subject to host manipulation by the infective stage of the parasite. Infected copepods become more active than uninfected ones, and as manoeuvrability decreases, predation pressure from sticklebacks increases as the procercoids mature within their host (Hammerschmidt et al., 2009; Wedekind and Milinski, 1996). The manipulation in the next intermediate host is even more dramatic, including phenotypic changes in predator-response behaviours, as well as a significant loss in swimming ability (Grécias et al., 2017), turning the sticklebacks into easy prey for the final host—a piscivorous bird.

In the first intermediate stage of infection, *S. solidus'* genetic background and *M. albidus'* phenotypic characteristics determine the rate of infection. Two discrete steps have been identified as crucial for a successful continuation of the parasite's life cycle (Veen and Kurtz, 2002):

1. The establishment phase, in which the hunting ability, appetite, and immunity will affect the rate of successful infection.
2. The growth phase, which is influenced by features in both host and parasite, such as morphology and condition of the host, or the intrinsic mortality of the parasite.

According to Veen and Kurtz, the establishment phase is the most crucial, as the copepod is unlikely to be able to eliminate the parasite once it has penetrated the gut wall and entered the

haemocoel. A successful establishment hinges on the parasite's ability to overcome (a) behavioural resistance in the host, (b) the mechanical and immunological barrier of the gut wall, and (c) the innate immunity of the body cavity.

Males and copepodites continuously show a higher infection rate but also exhibit higher overall mortality (Veen and Kurtz, 2002; Wedekind, 1997). This sex-linked infection bias does not seem to primarily be a result of hormones, as is observed in many species of mammal (Skorping and Jensen, 2004). When one accounts for size and age, what bias there is—whether male or female—is explained by resource allocation due to sexual selection with hormonal effects playing a lesser role (Sheridan et al., 2000). When controlling for size and age, there is nonetheless a significant sex bias in the immune response to helminth infections in this particular species of copepod (Wedekind and Jakobsen, 1998).

The choice is thus one between high infection rates and high mortality, or lower infection rates but more copepods surviving until termination of the experiment. Given that copepodite stages are hard to identify and difficult to sex, adult females were the "safe" choice in a setting which explores chronic pathology.

1.1.1.1 Schistocephalus solidus

Helminths are a polyphyletic group of parasitic worms defined by certain morphological and life history traits. Those that are not free-living tend towards endoparasitism, and all share the general body plan of a worm. Thus, "helminth" is used interchangeably with "parasitic worm". The group is polyphyletic and consists of members from several phyla: Annelida (segmented worms), Platyhelminthes (flatworms), Nematoda (roundworms), and Acanthocephala (thorny-headed worms). The total number of helminth species has been estimated to be between 75,000 and 300,000 (Dobson et al., 2008). Cestodes (class Cestoda), or tapeworms, belong to the phylum of Plathyhelmintes, and all of the c. 8,000 species are endoparasites in their adult stage (Gibson et al., 2014). Highly complex life cycles are typical among tapeworms, and many species require more than one intermediate host before a definite vertebrate host can complete the cycle.

The diphyllobothrid cestode (fish tapeworm) *Schistocephalus solidus* has a life cycle that includes an arthropod and a fish as intermediate hosts, and birds or mammals as definite hosts (***Fig. 1***). It has been found to mature in over 40 types of piscivorous waterfowl, but also

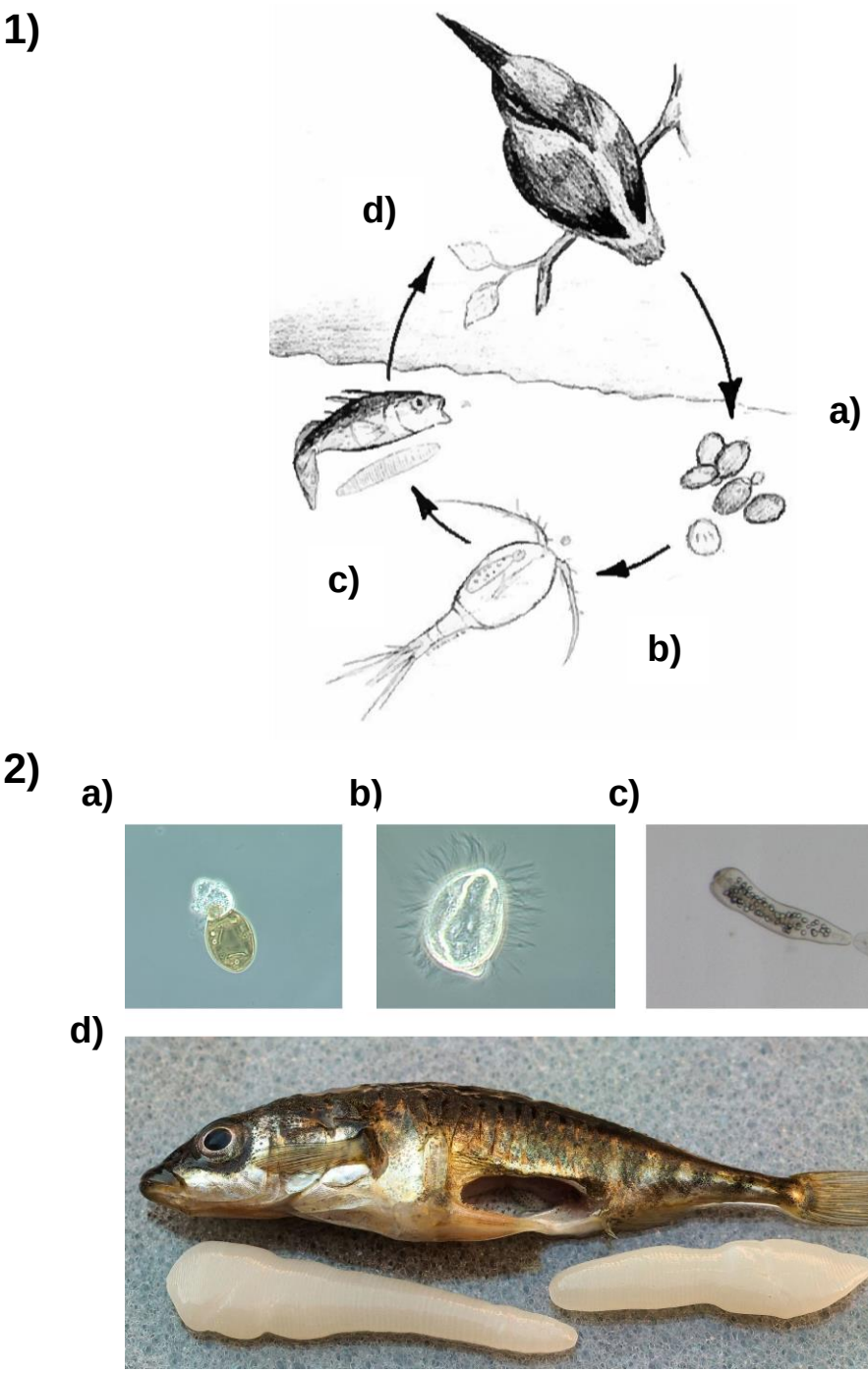

Figure 1. Life cycle and stages of *Schistocephalus solidus*. Starting at **a)** *S. solidus* eggs are released into the environment in bird droppings. Triggered by sunlight the eggs develop into **b)** free-living and motile coracidia ingested by a copepod intermediate host. In the copepod gut, the coracidium sheds its outer coat, bores into the copepod haemocoel, and matures into **c)** a procercoid. If the infected copepod is ingested by a three-spined stickleback (*Gasterosteus aculeatus*) the procercoid develops into the final larval stage, **d)** a plerocercoid. If the fish is eaten by the final host, a bird, adults parasites breed in the avian intestine and release new eggs into the environment along with the excrement.

Adaptation of schematic of life cycle (**1**) drawn by Claus Wedekind, 2005. Photos of individual stages (**2**) **a)** – **b)** Per Johan Jakobsen, **c)** Kristin Lian Aa, 2019 **d)** Jarle Tryti Noreide 2016. Images used with permission or distributed under a CC BY-SA 3.0 licence

pigeons, rodents, and aquatic mammals, such as otters (Clarke, 1953; Hoberg et al., 1997; McCaig and Hopkins, 1963). Development of plerocercoids in the final host is temperature-dependent, with somatic growth having a peak efficiency near 23 °C and secondary maturation with an optimum of near 40 °C (Sinha and Hopkins, 1967).

S. solidus' natural range is limited by that of its most common second intermediate host, the three-spined stickleback (*Gasterosteus aculeatus*) (Poulin et al., 2011). Three-spined sticklebacks thrive in the Northern hemisphere, in circumarctic and temperate freshwater lakes or coastal waters. In Greenland, Eurasia, North America, North-Africa, and in Eastern Asia north of Japan (Page et al., 1991, 663p.). However, *S. solidus* is not able to cope with the increased salinity of marine ecosystems, and only inhabits the body of freshwater and anadromous populations of stickleback (Barber, 2006).

S. solidus does not exhibit stenoxenous preference before the plerocercoid stage and can infect a wide range of freshwater adult and sub-adult cyclopoid copepods, including *Macrocyclops albidus* (Hahn et al., 2019; Wedekind, 1997) (***Fig. 2***). However, while the parasite can infect other fish for its second intermediate host, it is highly adapted to its local variation of three-spined stickleback (Henrich et al., 2013).

Paraphrasing Aristotle's *On the Generation of Animals*, it is said that "[…] *the business of most animals is to reproduce"*—which parasitic worms excel at to an extraordinary degree. Helminths divide most of their bodily resources into matters of sex, and as simultaneous hermaphrodites, *S. solidus* employs a mixed-mating system (Christen and Milinski, 2003). Parasite loads greater than one (1) individual unlocks the option of mutual cross-fertilization (outcrossing), while single individuals will resort to self-fertilization (autogamy).

Figure 2. Lateral view of adult female *Macrocyclops albidus*. Microscopy by Kristin Lian Aa, 2019.

During outcrossing, *S. solidus* can also breed with siblings (incrossing) if no other mates are available. Incrossing results in inbreeding depression over time and is less advantageous than non-incestuous cross-fertilization. However, any type of cross-fertilization is more beneficial than autogamy due to the lack of genetic variation (Schjørring, 2004; Schjørring and Jäger, 2007).

With phylum Cestodas' unique life histories come unique terminology. The cycle starts with *Schistocephalus* eggs hatching into *coracidia*. A coracidium represents a *hexacanth* larva enclosed by embryonic envelopes, which are parts of the embryo itself (Conn and Swiderski, 2008). In most cestodes, this structure is non-motile, and the hexacanth larva is enclosed by two embryonic envelopes. However, in some families, *Schistocephalus* among these, there is only the inner envelope covered in cilia.
The coracidia are free-swimming, and the embryo metabolizes granules of phospholipids and polysaccharides anaerobically to propel themselves through the water in random motions. At temperatures of 5-8 °C, coracidia from *S. solidus* are alive and motile for up to 120 hours (Dubinina 1966 cited by Smyth and McManus, 1989). Because the hatching of the coracidia is triggered by light of wavelengths mimicking visible sunlight in shallow waters (495-570 nm), I chose white LED aquarium lamps to trigger this response.

Hexacanth larva are named such because of a ring of six (three pairs) hooks associated with contractile cell bodies (myocytons) (Basyoni and Rizk, 2016). These articulated hooks work in conjunction with secretions from a penetration gland to invade the abdominal cavity of their first intermediate host by boring through the midgut wall (He et al., 2017). Once inside the copepod abdominal cavity, the hexacanth matures into a *procercoid*, which is regarded as the first true larval stage in many cestodes (***Fig. 3***).

Procercoids are characterized by a *cercomer* (caudal appendage) and a protective tegument (Jakobsen et al., 2012; Pospekhova and Regel, 2013). The latter allows the procercoids to pass unscathed through the acidic digestive enzymes of their second intermediate host, and only shed this when exposed to fish bile (Marwaha et al., 2013).

S. solidus procercoids are functionally mature when the cercomer appears, usually 9-11 days post-infection (Benesh and Hafer, 2012). The larva will continue to grow exponentially until day 30 in room temperature, at which point it reaches its asymptotic size (Hammerschmidt and Kurtz, 2009, p. 15).

Because of this, I chose to terminate the experiments between day 11 and day 30. I also chose to study the *S. solidus* system because it has an extensive catalogue of prior research and it is one of the few tapeworms that can easily be made to breed *in vitro* (Weinreich et al., 2014).

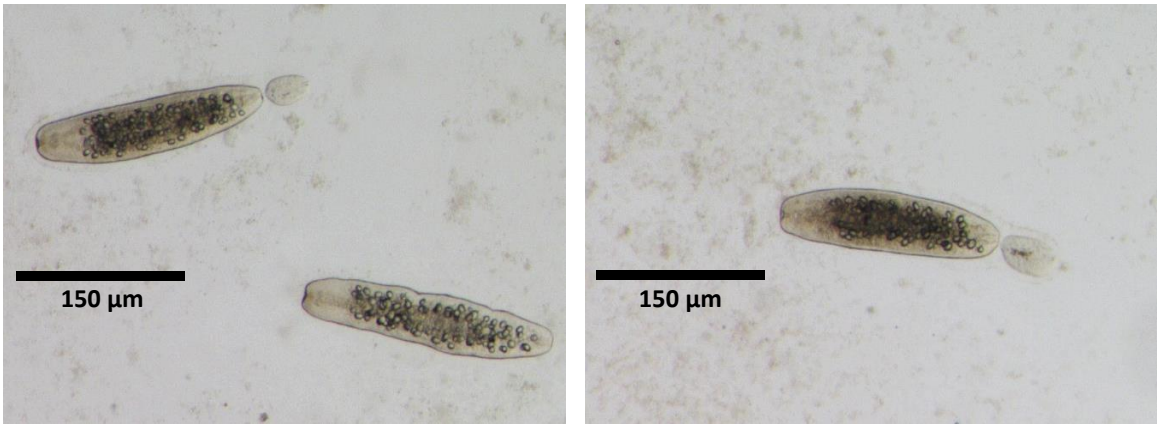

Figure 3. Mature *Schistocephalus solidus* procercoids with intact and lost cercomers. Twin and solo infections dissected from haemocoel of adult female *Macrocyclips albidus*. Microscopy by Kristin Lian Aa, 2019.

1.1.1.2 Parasite Ecology and Environmental Parasitology

The tremendous influence parasites have on their ecosystems causes both bottom-up and top-down cascades, shaping the life history of their hosts (Cable et al., 2017). This life history theory addresses the "life schedule" for an organism, from birth to death, as well as how natural selection has shaped the organism's fecundity, longevity, survival, age of sexual maturity, and reproductive strategy (Kochin et al., 2010). Environmental parasitology examines the interaction between parasites and their environment. Parasites can be both accumulation indicators and "pollution sinks" in that endoparasites, in particular, are very sensitive to the effects of xenobiotics (Sures et al., 2017). Incidentally, intestinal parasites acting as pollution sinks seem to provide the host with a barrier of protection towards environmental stress. Helminths can accumulate heavy metals from the host's internal environment and end up with concentrations several times higher than that of their host's tissues (Hassan et al., 2018; Teimoori et al., 2014).

Studies examining how temperature influences fitness trade-offs in the *S. solidus* system, suggest that increasing environmental temperatures promote the parasite rather than the host (Franke et al., 2017). Heatwaves associated with global warming have also been shown to immunocompromise the intermediate host in this system (Dittmar et al., 2014). How, then will the changes in toxic algal blooms affect these interactions?

Like environmental parasitology, the field of ecological immunology has seen a rapid development during the past 20 years. As an interdisciplinary field of research, it views an individual's immune response as being subject to optimization by the ecological situation in which an animal lives (Schulenburg et al., 2009). Rather than looking at the precise molecular mechanisms of immunity, it aims to understand immunological variance in the light of evolution and ecology (Schmid-Hempel, 2009).

While the effects of parasitism on the individual can be detrimental, a hallmark of a healthy ecosystem is a rich parasite community (Hudson et al., 2006). Parasites, both macro and micro, are prone to rapid evolution. The speed at which parasites evolve and adapt to their surroundings might lead to the beginning or end of epidemics (Duffy et al., 2009), or a co-evolutionary arm's race with their host—a principle dubbed "the Red Queen hypothesis", first proposed by Leigh Van Valen in 1973 (Holmgren et al., 2017). This evolutionary hypothesis is named after a character in Lewis Carroll's 1871 novel, *Through the Looking-Glass*, in which the protagonist, Alice, races with the Red Queen. No matter how fast Alice runs, she

doesn't move forward, but neither does the Red Queen. When Alice asks why, the Queen explains that " […] it takes all the running you can do, to keep in the same place" (Carroll, 1871).

Fish tapeworms have a broad host range (euryxenous) to species from several genera of cyclopoid copepod at the coracidium-procercoid stage (Scholz et al., 2019). Even species that were previously thought of as having a narrow host range (stenoxenous), such as *Bothriocephalus claviceps*, have since been shown to infect a wide range of species (Scholz, 1997). The fitness of helminths depends on the probability of reaching its ultimate host. This thesis sought to examine how toxins would affect this relationship, and whether the host, parasite or both would have a decrease in respective fitness.

1.1.2 Cyanobacteria

Cyanobacteria is an ancient phylum of photosynthetic prokaryotes that emerged somewhere between 3.6 ± 0.2 billion years ago (Garcia-Pichel et al., 2019). Although most reports and literature on cyanobacteria deal with temperate freshwater, they are found in a variety of environments, ranging from temperate streams and rivers, Arctic and Antarctic polar deserts (Friedmann, 1980) (Wierzchos et al., 2006), icy alkaline lakes, and hot springs (Glaring et al., 2015; Krienitz et al., 2003; Mohamed, 2008). Several species exhibit extremophile tendencies but do not, as a group, thrive in acidic waters with pH values lower than 5 (Rampelotto, 2013). Precursors to cyanobacteria likely caused the onset of one of the most notable evolutionary events—the Great Oxidation Event (GOE) of 2.46 to 2.43 billion years ago (Garcia-Pichel et al., 2019; Gumsley et al., 2017). Here, oxygen from primary photosynthetic producers reformed the anoxic, CO_2-dominated atmosphere and paved the way for oxygen-dependent life. This simultaneously caused the Earth's first mass extinction, in which primary production by anaerobic bacteria collapsed with the rise in atmospheric oxygen (Hodgskiss et al., 2019). The Boring Billion (1800 to 800 Ma) following the GOE saw cyanobacteria as the dominant lifeform before eukaryotic cells started showing up in the fossil record (Brasier and Lindsay, 1998; Mukherjee et al., 2018).

Cyanobacteria also played a starring role in Lynn Margulis' 1967 endosymbiosis theory, later dubbed "symbiogenesis", in which she propositioned that eukaryotic life had its origin in prokaryotic mutualism, born from either predation or parasitism (Sagan, 1967). Symbiogenesis resulted in eukaryotic metabolism and the precursors to multicellular life, the main component of which is the integration of proteobacteria and cyanobacteria, preceding

the eukaryotic organelles, which we now know as mitochondria and chloroplasts, respectively (Esposti, 2014; Falcón et al., 2010). Some genera of cyanobacteria form dense colonies in many aquatic environments (***Fig. 4***). When these colonies of picoplanktonic or filamentous bacteria grow large enough to constitute a bloom, they can be detrimental to ecosystems and human health (Huisman et al., 2018).

The chief harm in such blooms are toxins released into the environment, outcompeting phytoplankton, blocking sunlight for aquatic plants, or by creating dense, smothering mats of dead bacteria that sink and cause hypoxic or anoxic benthic conditions (Bartoli et al., 2018). This group of cyanobacteria are known as "bloom-forming harmful cyanobacteria" (CyanoHABs) (Paerl, 2014).

Figure 4. Masked lapwing (*Vanellus miles)* wading in a *Microcystis aeruginosa* outbreak on the southwest side of Lake Albert, Australia. *M. aeruginosa* is one of several genera of microcystin-producing cyanobacteria that can cause harmful algal blooms (HABs). Photograph by Bidgee, 2018 distributed under a CC BY-SA 3.0 licence.

1.1.3 Cyanotoxins and Harmful Algal Blooms (HABs)

As Charles Darwin put it in *The Origin of Species*, "[…] from so simple a beginning, endless forms most beautiful and most wonderful," The evolutionary playground that is multicellular life might, in turn, have pushed cyanobacteria as a group into a more diverse bouquet of extant morphotypes, leaving us with thousands of species of extremely well-adapted microbes (Schirrmeister et al., 2015). As of 2013, there were 2,698 species of cyanobacteria described, with the group containing an estimated 6,280 species in total (Nabout et al., 2013). This diversification has led to a spectacular array of noxious strategies to combat competition.

While some cyanobacteria are relatively harmless, even beneficial as a foundation for fertilizer and biofuels (Singh et al., 2016), many genera are known for producing bioactive compounds that will poison the well of their aquatic environment. These compounds are known as "cyanotoxins" and will burst forth as the cell wall breaks down in the dead bacterium.

Cyanotoxins provide a stunning array of harmful consequences for the organisms that happen to ingest them. They can enter the food net and bioaccumulate from low trophic organisms, leading to second-hand human ingestion (Ferrão-Filho and Kozlowsky-Suzuki, 2011). Secondary poisoning by toxins from algae or cyanobacteria in mussels is often called "shellfish poisoning" (Ballot et al., 2010; Gibble et al., 2016). These pathologies can range from acute to chronic and harm several different organs and organ systems (Dittmann et al., 2013).

Hepatotoxins include microcystins, nodularins, and cylindrospermopsins. Neurotoxins include anatoxin-a and saxitoxins, dermatotoxins and cytotoxins the lyngbatoxin-a and aplysiatoxins, besides a great many lipopolysaccharides found in or on the bacterial cell that function as endotoxins (Huisman et al., 2005, p. 11-12).

1.1.4 Nutrients and Hydrology

Commercial fertilizers come with an NPK ratio listed on the back of the container which refers to nitrogen (N), phosphorous (P) and potassium (K), given as stochiometric values of crucial nutrients in relation to one other. In most cases (i.e. Liebig's law of the minimum), such as environments supporting photosynthetic life, one of these elements will be a limiting factor (Taylor and Terry, 1984). That is to say: the element of which there is a shortage will first stop further growth of a specific organism, regardless of how much of the two others are

added. Nitrogen and phosphorous are often required in more substantial quantities due to various cellular processes (chiefly in the formation of ATP, amino acids, and nucleic acids) requiring a larger relative amount. Thus, excessive N or P lead to eutrophic conditions with an increased amount of chlorophyll-a—an indicator of phytoplankton activity (Lürling et al., 2017).

These conditions are exacerbated when nutrients accumulate in the sediments of shallow bodies of water, such as lakes and reservoirs. Sedimentation of phosphorous and nitrogen provides a store of nutrients, which becomes available occasionally throughout the season. Phosphorous is released due to oxygen depletion, often as a consequence of microbial hypoxia or anoxia (Hupfer and Lewandowski, 2008). Storms, flooding, or other disturbances might release nutrients trapped in sediment, as will the seasonal spring and fall turnovers (Jeke and Zvomuya, 2018; Paerl, 2017; Reynolds and Davies, 2001). These nutrients get into waterways through several pathways, the most common of which are industrial and agricultural runoff (Pelley, 2016), wastewater (Vasconcelos and Pereira, 2001), and land clearing and deforestation (Bastida et al., 2015).

Other sources only found a weak correlation between these factors and cyanobacteria abundance, stating that eutrophication should not be regarded as a regulating factor (Okogwu and Ugwumba, 2009). Instead, temperature pH and dissolved oxygen were shown to be more important in the increase of cyanobacteria proliferation (Sinden and Sinang, 2016). Rising global temperatures also become more relevant as the mean optimum growth temperatures for both cyanobacteria and green algae is between 20 °C and 30 °C (Konopka and Brock, 1978; Lürling et al., 2013). However, in boreal lakes, the threshold for total phosphorous (TP) seems to be the limiting factor, with the most substantial decrease in cyanobacterial biomass occurring at P concentrations below 50 $\mu g\ l^{-1}$ (Vuorio et al., 2019). It is also suggested that the N/P relationship is more important than an abundance of P itself (Mellios et al., 2020), with low nitrogen-to-phosphorus ratios being predictors of blooms and increased cyanobacterial activity.

This all points to a complex interplay of factors that might vary significantly both geographically and climatically. pH is also a direct impact of blooms, with proliferation limited by pH levels that are too high and hardly affected by projected decreases (Hinners et al., 2015). Increased photosynthetic activity leads to enhanced absorption of CO_2 and an increase in water pH (Paerl and Paul, 2012). The productivity of phytoplankton is inversely

proportional to water pH, which leads to a window of cyanobacterial domination before they, too, die off as water pH rises and resources are depleted (Sinden and Sinang, 2016).

The Antarctic has seen a mean temperature increase by 0.5 °C per decade since the 1960s (Turner et al., 2005), while the Arctic is heating up three times as fast as the global mean (Bintanja, 2018), leading to permafrost warming and melting at a global scale (Biskaborn et al., 2019). With these changes comes an increase in local rainfall and atmospheric temperatures, which in turn cause an increase in extreme weather, such as heavy rainfall, increased storm severity, and floods (Kleinteich et al., 2012; Marsooli et al., 2019; Paerl and Paul, 2012). Increase in concentrations of atmospheric CO_2 and elevated UV fluxes will also affect the proliferation of cyanobacteria (Bláha et al., 2009).

These conditions will vary with genera and local conditions, and synergies between them can affect the dynamics of blooms (Rastogi et al., 2015). There has been an increase in HABs since the 1980s (Hinners et al., 2015), and with rising temperatures, an increase in storms and floods following climate change. This combination of warmer waters, water eutrophication, and disturbances will most likely lead to a rise in conditions favoured by cyanobacteria (Gobler, 2020; Kleinteich et al., 2012).

Cyanobacteria's general adaptivity and ability to prevail in complex stressor regiments have made predicting their bloom hydrology a challenge. However, machine learning tools have shown great promise after being used to predict with more than 95% accuracy the severity of harmful blooms and categorize the risk levels in lakes based on water depth and levels of nitrogen and chlorophyll *a* (Mellios et al., 2020).

1.2 Chemical Background and Toxicology of Microcystins

Microcystins (or cyanoginosins) are cyclic heptapeptides. A cyclic peptide is any compound in which a polypeptide chain of the molecular structure contains a circular sequence. Out of all cyanotoxins, microcystins are the most commonly recorded, with 63% of all toxin reports globally (Svirčev et al., 2019). Cyclic peptides occur naturally in animals, plants, and bacteria, and show anti-microbial properties and remarkable biological stability (Craik, 2006). These compounds can withstand the hydrolytic activity of digestion, which eliminates an essential first line of defense against potentially harmful biological compounds surviving the route through the human digestive tract, allowing the cyclic peptides to reach the liver.

Microcystins are monocyclic, meaning that there is only the one circular sequence. There are 111 congeners (structural variants) that have been described as of 2020 (Ballot et al., 2020; Welker and von Döhren, 2006), and with biochemical transformation variants, there are likely over 300 MCs (Bouaïcha et al., 2019). The microcystin ring sequence is formed by six amino acids (AA), four of which are non-protein AAs and two of which are protein AAs. A side chain is formed by a non-protein AA called ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (***Fig. 5***) The ADDA sidechain is present in all congeners and is used to quantify the presence of microcystins, regardless of the structural variant (Ballot et al., 2020).

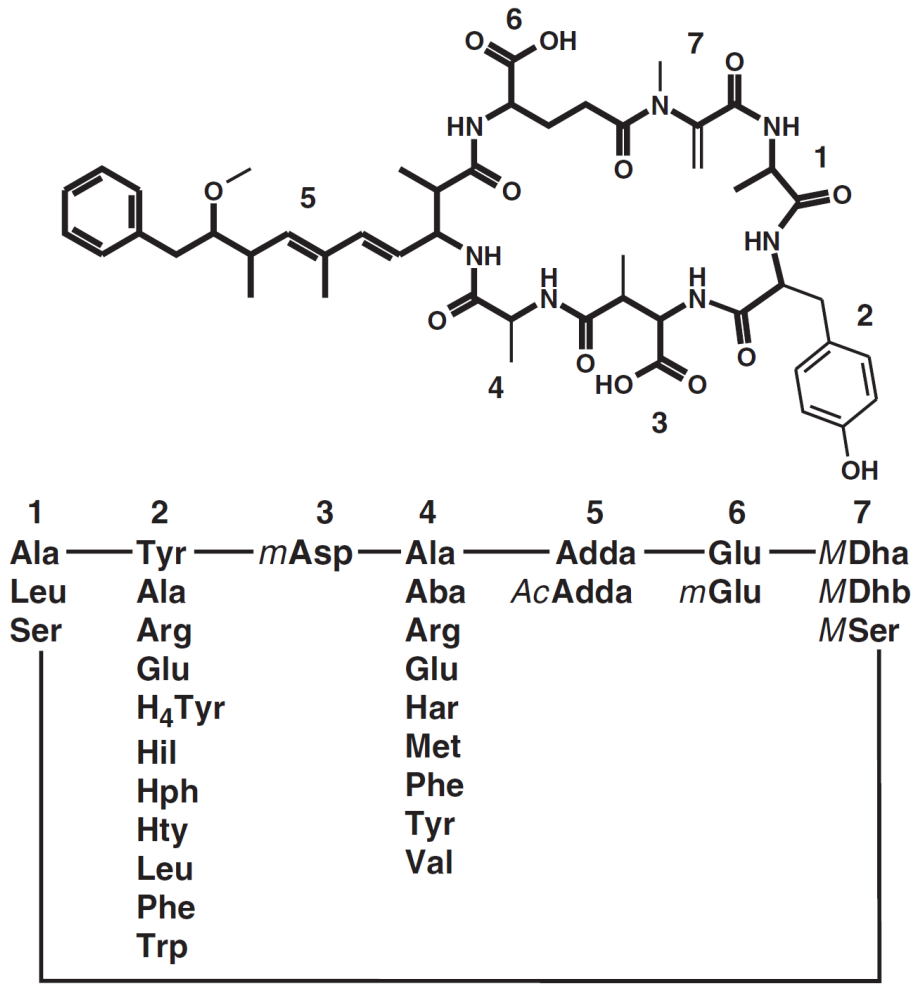

Figure 5. Molecular structure of microcystin-LA together with a schematic of the general structure of possible microcystin peptides. Microcystin-LA differs from microcystin-LR with the substitution of alanine in place of the arginine. ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) represents a non-protein amino acid side chain present in all microcystin congeners. Molecular structure from Botes et al., 1984 adapted with added schematic by Welker and von Döhren, 2006.

Microcystin-Leucine Arginine (MC-LR) is both one of the most common and the most toxic of the microcystins. For this, most toxicological studies on microcystins involve MC-LR, and which is used to describe the worst-case scenarios of HABs. Structurally, positions 1, 3, and 7 contain D-Leu, D-Asp, and Dha. At positions 2 and 4, L-Leu and L-Arg are located, which leaves position 5 and 6 for Adda and D-Glu, respectively (Bouaïcha et al., 2019).

MC-LR has both genotoxic and cytotoxic effects (Ding et al., 1999; Herrera et al., 2018; Nong et al., 2007). It induces reactive oxygen species (ROS) and lipid peroxidation formation, leading to DNA strand breaks and cytoskeletal disruption and apoptosis in vertebrate hepatocytes (Jiang et al., 2013; Nong et al., 2007).

In general, microcystins can bind to the catalytic subunit of protein phosphatase 2A (PP2A), which inhibits the action of this enzyme (Liang et al., 2011), though the exact mechanisms of binding and its effects are not fully understood. Still, through this pathway, MC-LR can alter the microtubule post-translational modifications, which disrupt the cytoskeleton and contributes to its cytotoxicity. In vertebrates, the consequent on inhibition of PP1A and PP2A causes massive hepatic haemorrhage (Dawson, 1998). MC-LR also leads to an upregulation in the expression of CYP2E1 mRNA, which might be a potential source responsible for ROS formation (Nong et al., 2007).

It is not unheard of for toxin levels in HABs to surpass extreme levels of 40,000 µg/L (Mathys and Surholt, 2004). While this is detrimental to the greater aquatic invertebrate community, crustaceans generally seem to exhibit a high tolerance toward the effects of microcystin (Bownik, 2013; Ger et al., 2016; Samdal et al., 2020) by either avoiding grazing on cyanobacteria or having a natural tolerance to the toxin.

In toxicity studies, 0.14 mg/L of MC-LR was required before chronic effects manifested in two species of copepod (Ger et al., 2009). However, the effects seem to be somewhat species-dependent (Lawton et al., 1995; Zhang et al., 2019) and the experiment set out to mimic levels realistic to the environment in question. For this reason, I chose 350 µg/L as a level of MC-LR that would produce sub-chronic effects, even if the true levels turned out to be somewhat lower.

1.3 Experimental Design

Milinski, 1997, proposed a list of "seven deadly sins" associated with behavioural study design. The study design, as outlined in the following ***Materials and Methods***, strives to avoid these pitfalls and create a solid foundation for empiricism. Chief in any scientific experiment is the avoidance of bias, defined as the "[unfair] inclination or prejudice for or against a group", or in the case of scientific testing, the inclination to confirm, rather than falsify one's hypothesis.

Of course, true objectivity is nigh impossible as long as human beings are the ones to carry out the experiments and analyze the data. Perhaps sometimes the best one can manage is to be conscious of one's own biases, identify them, and try to mitigate them through a structurally sound experiment.

The list of "sins" in its entirety paraphrased from the 1997 publication is as follows:

1. Unjustified conclusions drawn from correlational data
2. Data are not independent (pseudoreplication)
3. Treatments confounded by time and sequence effects
4. No efforts made to avoid observer bias
5. Potential artefacts arise when animals not accustomed to experimental procedures
6. Unsuitable controls
7. An attempt to "prove" the null hypothesis with small samples

In these experiments, randomization of the placement of copepods, as well as times of feeding and water change, was implemented to avoid edge effects and unconscious bias. In order to mitigate the number of artefacts, copepods were allowed to breed for several generation cycles before being utilized. The culture water was inoculated using dead vegetation from the same location as the copepods.

Pseudoreplication is the act of using inference statistics on an (artificially) inflated number of samples or replicates, or in terms of statistical analyses (ANOVA), when the error term does not fit the proposed hypothesis (Hurlbert, 1984). To avoid non-independent data, each copepod was isolated from the rest and treated exactly like its cohort in each group.

Small samples are always a problem when using statistical inference, so there is still a balance between the ideal sample size and resources available. To increase the chance for adequate

data in infection groups, the group sizes are uneven to mitigate the potential loss of data points if the infection rate was 50% or lower.

A truism dubbed Harvard's law is likely to make an appearance in any behavioural design. This "law" states that: "Under carefully controlled experimental circumstances, an animal will behave as it damned well pleases.", and while tongue-in-cheek, spontaneous behaviour *is* nonetheless a genuine factor that can affect the results in most animal studies (Maye et al., 2007). One can do little to weed out spontaneous behaviour in a tapeworm or minute crustacean, but one can endeavour to adhere to empirical rigidity.

2 Materials and Methods

2.1 Field Probe and First Experiment (E1)

Animals for E1 were wild-caught and kept in laboratory conditions until use.
Lake Skogseidvatnet (60°13′ N, 5°53′ E) in Fusa, Norway, has previously been a reliable source of sticklebacks (*Gasterosteus aculeatus*) utilized in research due to their consistently high parasite loads (Fossås, 2013, Kalbe et al., 2016). A field probe of the area on June 28, 2018, showed expected conditions, and plerocercoids of the cestode *Schistocephalus solidus* removed from hosts on July 3, 2018, were successfully made to breed. Matured eggs harvested from the pilot on June 6, 2018, successfully hatched into coracidia when triggered by radiation from 495-570 nm white LED aquarium lamps overnight. An excursion on June 26, 2019, yielded more infected sticklebacks, the plerocercoids of which were utilized in the first experiment conducted during July of 2019.

Sticklebacks were caught in the field using an electrofishing device and kept in aquariums in the lab until euthanasia and dissection. The fish were euthanized with an overdose of eugenol (>1500 µL/L) solved in 70% ethanol (Strykowski and Schech, 2015, Davis et al., 2015) as per the regulation concerning the use of animals for scientific purpose § 16 on humane euthanasia of laboratory animals. Before dissection, the sticklebacks were also washed in 75% ethanol to reduce bacterial contamination.

2.1.1 Host and Parasite Cultures

S. solidus was cultured *in vitro* based on methodology originating with Smyth, 1946-1954, and modified by Wedekind, 1997. Plerocercoids harvested from stickleback abdomens were immediately eased into medium-moist Visking tubing in pairs. The tubing was further filled with culture medium consisting of Minimum Essential Medium with Earle's salts, L-glutamine, with 25 mm HEPES-buffer and 6 g of D-glucose. Antibiotics (40 mg/ml gentamicin) was added to this mixture to prevent bacterial growth.

Tubes were then tied and suspended in sterilized 250 ml bottles filled with the same medium solution. The bottles were kept in a water bath for three days at a constant 40 °C to mimic the conditions of avian intestines. Eggs are denser than water and gather at the bottom of the tubing, clearly visible as black, grainy patches. Post breeding, worms were extracted, and the eggs harvested. To remove fouling, the eggs were rinsed with tap water. After maturing for 21 days at 18 °C, they were put into storage at 4 °C to prolong longevity, according to Scharsack

et al., 2007. Eggs were stored in light-proof plastic containers filled with fresh tap water until use. Eggs from at least five clutches were mixed to randomize the results of the copepod infection.

Macrocyclops albidus were caught in 15 cm x 60 cm simple plankton nets (70 µm mesh) in the central pond of Nygårdsparken city park (60° 22'54.9 "N 5° 19'49.8 "E) on July 21, 2018. After two weeks, the culture was filtered through stages of sieves of decreasing mesh size in order to separate the wild-caught F1 adults (500-100 µm) from F2 nauplii (70-20 µm) and other planktonic organisms. F2 nauplii where then put into separate tanks to encourage culture homogeneity and to make sure individuals used in the experiment were similarly preconditioned.

Copepods were kept in culture, and later in wells according to Veen and Kurtz, 2002. Aquaria sat in room temperature (18 °C) and contained ciliate inoculated freshwater in which the copepods could develop naturally throughout the experiment. Adult females without egg sacs were selected before the experiment and maintained in 2 mL wells in 13 x 24 ELISA plates. Laboratory light was kept on between 9 AM and 7 PM with an otherwise dark room to mimic photoperiod under fluorescent light conditions. The placement of the ELISA plates was rotated randomly every day to avoid any light-related edge-effects in the wells. Copepods that expired prior to termination of the experiment were fixated in osmotic 4% formalin to be dissected later according to the protocol of Haney and Hall, 1973.

Each well containing a single copepod fed one live *Artemia* rinsed in tap water every two days throughout the experiment. Water was changed every third day, and mortality logged twice per day, morning and evening. A copepod was considered deceased when the animal did not respond at all to disturbances in the water. A total of four groups of copepods were used in a 2 x 2 matrix: control, infection-only, toxin-only, and combined toxin and infection. The experiment contained a total of 312 copepods divided into a 1:3 ratio between non-infection and infection groups. As such: control (39), single toxin (39), single infection (117), combined toxin and infection (117).

In order to avoid systematic differences between groups, copepods were infected one by one. Each copepod in infection groups was fed two motile coracidia on day 10. The parasites were then allowed to develop for 18 days, at which the experiment was terminated, and copepods euthanized and dissected.

2.2 The Second Experiment (E2)

Heavy rainfall during the summer of 2019 led to severe flooding of Lake Skogseidvatnet, which prevented sticklebacks from being caught for E2 in October 2019. Because of this, the University of Münster donated eggs from two families of *S. solidus.* These were bred and matured according to the methodology outline above. Both lineages hail from Hörsteller Aa (52° 17'31.2 "N 7°36'47.0"E), a brook in Hörstel, Germany.

Descendants of the E1 copepods were also utilized in E2, and the regiment of feeding, toxin concentration, and water change remained the same. The total number of copepods and the number in each group was also identical to that of E2. The previous F2 culture was filtered and separated with sieves like in E1 once more before the start of E2. Environmental culture conditions were otherwise similar, except for E2 copepods being mass-fed live *Artemia* nauplii once a week one month prior to E2. The number of parasites was reduced to one coracidium per well on day nine, and copepods were euthanized and processed like in E1 but on days 13 and 14 post-infection.

2.3 Extraction of Microcystin and Toxin Sampling

Microcystins were extracted from cultures of *Microcystis aeruginosa* (NIVA-CYA 160/1), which is sustained by Z8 medium. Cells from the culture were harvested through centrifuging, and then freeze-dried, after which the dried mass was stored frozen. Before each experiment, the dry mass was resuspended in 1L ultrapure water and set on a magnetic stirrer for 2 hours. After stirring, particles were settled by centrifugation at 3,000 rcf, and the supernatant finally filtered over GF/F filters. The main toxin variant in this solution is Microcystin-LR.

The concentration of total microcystin is measured through an indirect competitive-ELISA immune assay with an Abraxis Microcystins-ADDA ELISA kit (Product No. 520011). Samples were thinned and measured on microplates (MultiScan FC from Thermo Scientific) at a wavelength of 450 nm. The kit includes standards for calibrations of the response curve. As the kit has a measuring range of 0-5 µg/L, it was necessary to dilute the concentrated stock microcystin solution up to 3-4000 X to obtain reasonable concentration estimates. The stock solution was then used to obtain desired concentrations (350 µg/L) by diluting in tap water for each water exchange during the experiments.
Small water samples were taken at intervals from two wells in each experiment, to track the microcystin concentrations. These 1-2 mL samples were taken in small glass vials, and frozen

immediately. After the termination of each experiment, these samples were analyzed using the same ADDA Elisa kit as above. Samples were diluted 150X in ultra-pure water to get within the measuring range of the kit.

2.4 Image Processing and Statistical Analysis

After euthanasia, copepods from infection groups were dissected on moist microscopy slides with microdissection tools (Item no. RS-6061, RS-6063, RS-6067) under a Leica MZ95 high-performance stereomicroscope. Any slides containing procercoids were transferred to a Leica M-125 encoded stereo microscope and photographed at 10x magnification. Photographs were processed in ImageJ, which was used to trace the outline of each procercoid and calculate surface area.

For all statistical analyses, the open source programming language R was used with the following packages installed: tidyverse (ggplot2), survminer, survival, ggpubr, magrittr, svglite. The mean area of both groups was compared using a t-test and a one-way ANOVA. Survival analysis was used to plot mortality curves and predict the day of death of all four copepod groups.

See ***Appendix 1*** for R codes for all analyses, and ***Appendix 2*** for copepod mortality in E1 and E2, and parasite abundance and procercoid area data in E1.

3 Results

3.1 Experiment 1 (E1)

Procercoids from combination group were found to have a significantly larger surface area (*P*=.007) than procercoids from the infection group in both t-test and one-way ANOVA. The mean of the surface area of the toxin-infection combination group was 0.041 mm^2, while the infection-only group had a mean of 0.036 mm^2 (***Fig. 6***).

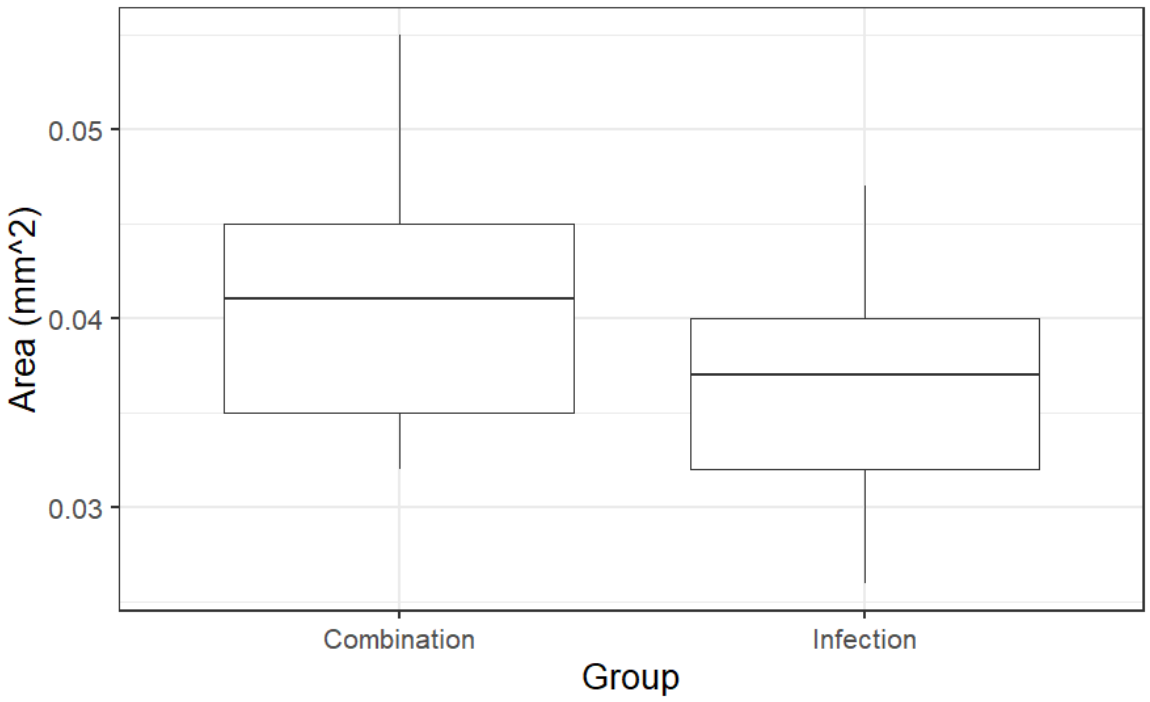

Figure 6. Boxplot of the surface area (mm^2) in E1 combination and infection group. Whiskers represent the spread of the size in the procercoids.

In the survival analysis, there was also a significant difference in mortality between the toxin-infection combination group and the control (P<.001) and parasite (*P*=.002), but not *between* the two toxin groups (***Fig. 7***). Mortality in the infection-only group was significantly higher (*P*<.001) than that of the control group.

The scale parameter 8.22, being larger than 1, indicates that the slope of the hazard increases with age in this study, i.e. instantaneous risk of death increases. The survival prediction analysis determined the day of death of the control group to be on average 90. For the infection, toxin and combination group these were 34, 31, and 30 respectively.

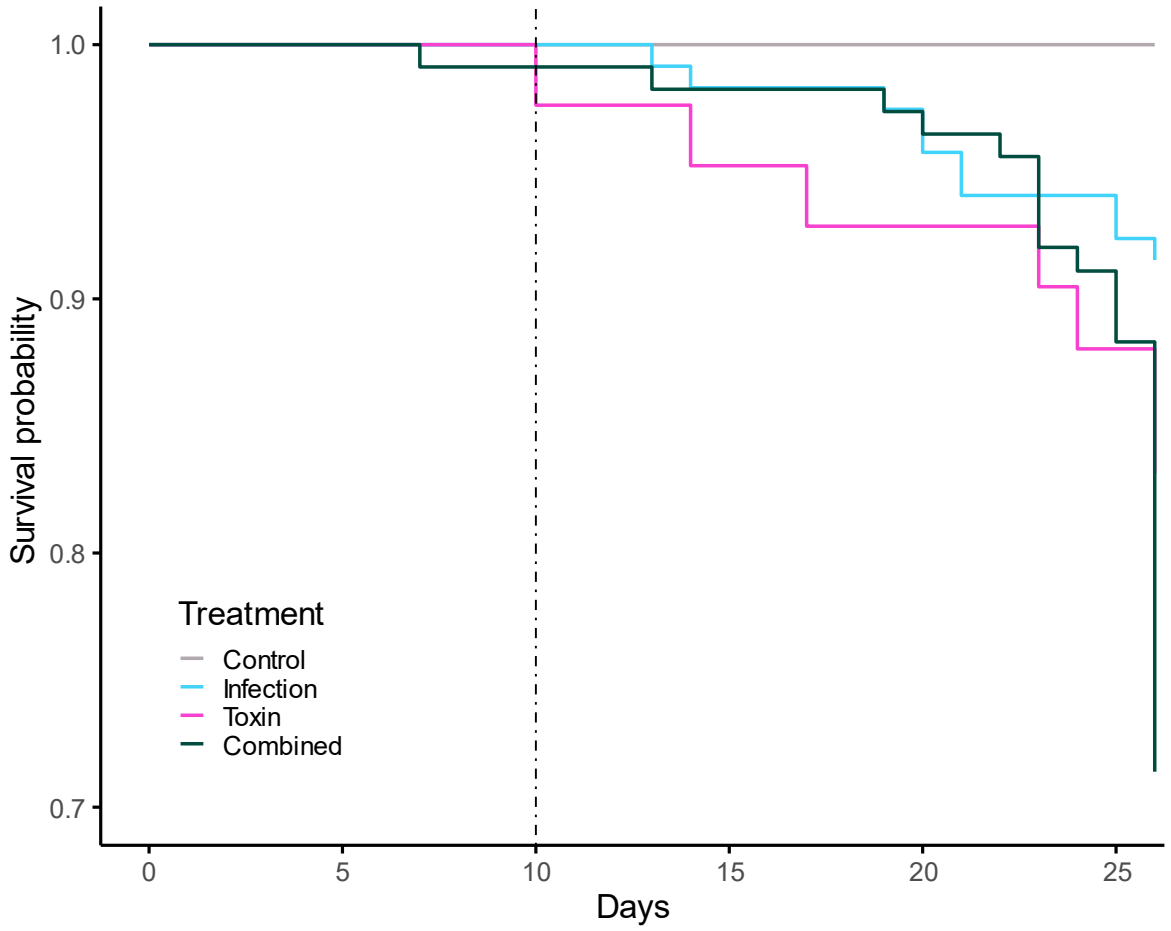

Figure 7. Survival analysis mortality curve for the four copepod groups in Experiment 1 (E1). The intercept (dashed line) has been added to show the curves in relation to the day of infection (10).

During dissection, 63 out of a possible 234 gave a total infection rate of 27% based on available data. Infections in copepods that were deceased prior to the termination of the experiment were left ambiguous. Separated into groups, the infection-only group had a slightly lower rate (25%) than that of the combination group at 29%. The two groups had an equal number (7) of observed twin infections, although it is impossible to say how many of the lost data points represented infection-related mortality, to say nothing of the twin or single nature of these.

Water levels of microcystins in toxin groups were measured over three days at the end of the experiment. Measurements hovered between 250-500 µg/L for values that weren't too great to be measured accurately by the test kit (***Fig. 8***).

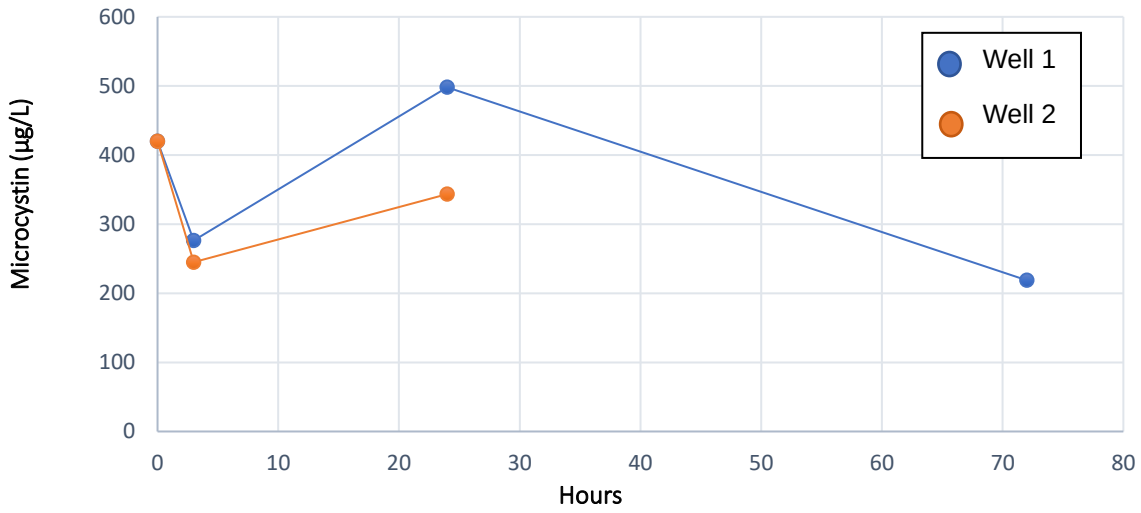

Figure 8. Representation of microcystin levels in toxin groups in Experiment 1. Measurements based on separate sample wells containing a single copepod.

3.2 Experiment 2 (E2)

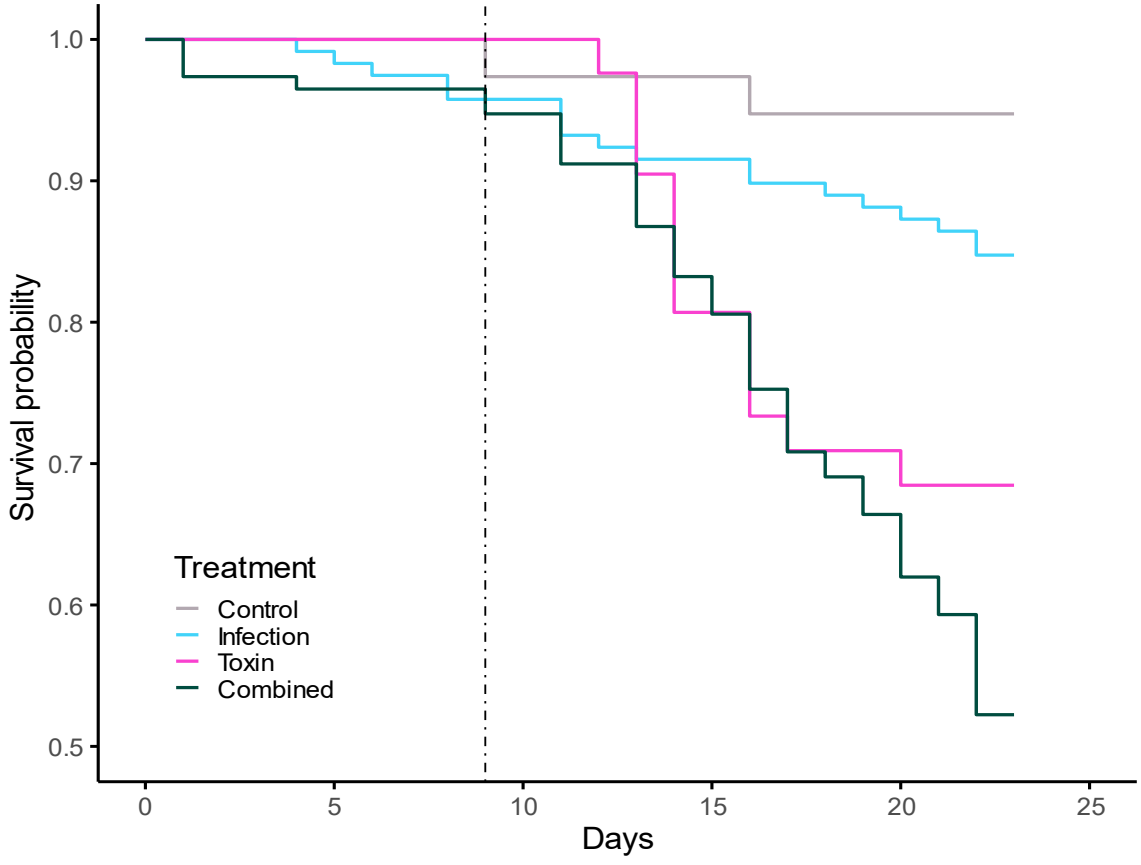

Figure 9. Survival analysis mortality curve for the four copepod groups in Experiment 2 (E2). The intercept (dashed line) has been added to show the curves in relation to the day of infection (9).

Despite promising hatching rates (>50%) and highly motile coracidia, only one procercoid (from the combination group) could be identified upon the termination of the second experiment (E2). Unlike in E1, copepods from parasite groups were also dissected throughout instead of being preserved in formalin for later analysis.

At no point was the physical presence of *S. solidus* procercoids detected. While there was a significant difference in mortality between control and the combination group (P=.001), and combination and infection (P<.001). There was no significant difference in mortality between the two toxin groups (***Fig. 9***) or between infection and control.

The survival prediction analysis determined the day of death in the control group to be day 86 on average. For the infection, toxin and combination groups, the days of death were 51, 34, and 28, respectively. The toxin is thus seen to be a more significant cause for mortality than parasitism alone, and no interaction effects between toxin and parasite could be detected. Unlike in E1, water levels of microcystins in toxin groups were measured every day from start to finish. Measurements hovered between 200-325 µg/L for most of the period, except for the three days of the last feeding-water change-cycle, upon which it dipped dramatically below 15 µg/L (***Fig. 10***).

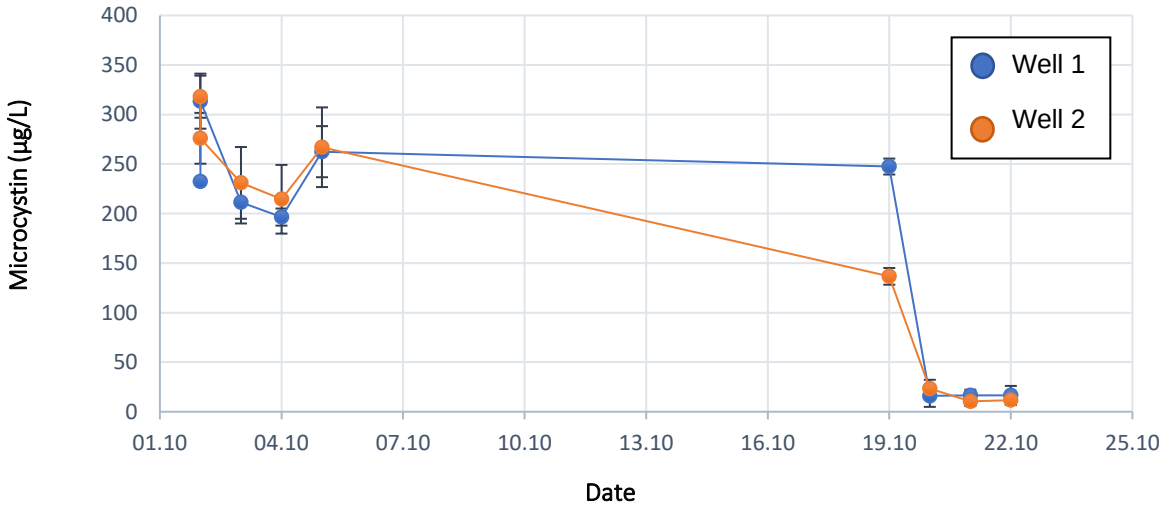

Figure 10. Representation of microcystin levels in toxin groups of Experiment 2. Measurements based on separate sample wells containing a single copepod. Error bars represent standard error.

4 Discussion

While no significant difference could be detected in the survival analyses between parasite and the combination group, the parasite seemed to hurt the survival of the host when compared to the control. This was expected, as, like most parasites with complex life cycles, diphyllobothrids lower the fitness of their intermediate hosts (Benesh and Hafer, 2012; Lievens et al., 2018).

Interestingly, the parasites in the combination group had a significantly larger surface area than those in the infection-only group. This difference might be an artefact of small sample size or inconsistencies in harvesting data. However, if not an artefact of small sample size, this effect could be due to differences in resource allocation with the toxin present. Unfortunately, only one set of area data became available, since the second experiment (E2) failed to provide enough mature procercoids for study.

There are several problems with small sample sizes and statistical inference methods, the chief of which being the low statistical power of the sample, but also low reproductivity, false positives, and an inflated rate of discovery and effect size estimation (Button et al., 2013; Colquhoun, 2014; Forstmeier et al., 2017). However, the total number of copepods (312) was deemed adequate after studies with similar a methodology (Hafer and Milinski, 2015) used numbers not much higher (744) divided on a whole team of researchers.

4.1 Concentrations of Microcystin

Measuring the exact concentration of microcystins in the toxin groups of E1 and E2 was difficult. Easier with E2, in which the sample number was increased, but still not without limitations. In spite of the dilution of the samples, several measurements were actually above the measuring range and had to be disregarded.

There are a wide variety of methods available to detect the presence of microcystins and other cyanotoxins. Among these are bioassays, antibody or enzyme-based assays, or liquid chromatography and other chemical analyses (Lawton et al., 1995; Mathys and Surholt, 2004; Shamsollahi et al., 2015). For rough estimates in natural environments, real-time PCR assays of chlorophyll-a have also been used to indicate toxin levels in harmful blooms (Mathys and Surholt, 2004). The standard method for analysis of microcystins is high-performance liquid chromatography (HPLC). However, HPLC is costly, time-consuming, and requires specialized equipment. For this reason, the method used to measure microcystin

concentrations was the Abraxis Microcystins-ADDA ELISA, which is routinely used to estimate microcystin levels also in Norwegian freshwater monitoring. This kit has some limitations as it is designed mainly to determine water safety. The method does not differentiate between microcystin congeners or exclude *nodularin*s (cyclic pentapeptides). It is even suggested in the kit protocol that positive samples should be confirmed with more conventional methods.

During E2, a biofilm started to form on the inside of the stock solution bottle with the microcystin concentrate. The bottle had been frozen until use, and the nature of the biofilm was not qualified, but it likely contained sulfur bacteria as the solution started to smell unmistakably of hydrogen sulfide gas (H_2S). The odour was faint but pervasive, and the solution was centrifuged and resuspended to clear it for impurities. Microcystin-levels during this time remained steady until the solution was filtered through a 0.45 μm polyethersulfone (PES) membrane filter. This last step completely cleared the concentrate for biofilm, but the levels of toxin also dropped dramatically (***Fig. 10***). This surprising turn of events, fortunately, happened during the last three days of the experiment.

Aerosolized microcystin particles measure between 1-30 μm and microcystin solved in water is not known to form micelles or aggregates (Cheng et al., 2007). In theory, the toxin concentration should have remained similar before and after filtering. The reason for the drop might have been because the toxin molecule was covalently bound to cysteine residues of proteins (Zilliges et al., 2011). The toxin could also have been trapped in porous particles large enough to be caught in the PES filter. Overall, the toxin levels were well above what is considered high in a natural system.

There were several faults with the first experiment (E1) that I attempted to avoid in E2. The main issue with E1 is the likely loss of crucial data points. Copepods that expired before the termination of the experiments, where fixated in osmotic 4% formalin to be dissected later, when equipment and training became available.

Limnologists have implemented the protocol of utilizing 4% sucrose-formalin to fixate and preserve morphological detail in zooplankton samples since the 1970s (Haney and Hall, 1973). While the exoskeleton was preserved perfectly, its content had run together into a fuzzy, brown substance, and no signs of parasites were apparent. It is unknown whether this was because the parasite had left the dying host, failed to establish, the host died before the growth phase, or because the procercoid too had been degraded. The standard for soft tissue

preservation among histologists is 10% neutral buffered formalin (NBF) (Matsuda et al., 2011), and perhaps this method would have made plankton samples more viable for dissection as well.

To detect the presence of parasites in degraded samples, one could, in theory, use quantitative PCR (qPCR) to measure differential RNA expression in some genes from fresh or snap-frozen and pooled samples (Pawluk et al., 2018). RNA extraction of snap-frozen samples could also possibly have worked on single copepods, even if tiny tissue samples can be problematic (Grinstein et al., 2018). Looking at protein or enzyme activity, or other markers for oxidative stress, such as the presence of reactive oxygen species (ROS) or ROS tissue damage, would be very difficult, if not impossible with the small sample size that the fixated copepods provided (Berg et al., 2014).

The pooling of individuals might have been possible, but the cost of these procedures would have run over budget. Because of these difficulties, it was decided it would be prudent to run an improved version of the experiment once more. However, while E2 shaped up to be a more streamlined experiment overall, procercoids failed to develop in all but one copepod in an infection group.

4.2 Lack of Procercoids in E2

Due to severe flooding and poor weather conditions, *S. solidus* breeding stock could not be obtained in time from our usual source of Lake Skogseidvatnet (60°13′ N, 5°53′ E) in Fusa, Norway. As a backup, eggs from two families of laboratory-bred parasites were used instead. Both lineages hail from Hörsteller Aa (52°17'31.2"N 7°36'47.0"E), a brook in Hörstel, Germany. Previous laboratory studies using this lineage had an infection rate of 50% for C4 and C5 copepods, and an infection rate of up to 80% for C3 and earlier stages (Scharsack, personal communication, May 2020). The observed hatching rate of these eggs in E2 was c. 50%, if not more, and the coracidia were motile, indicating successful hatching and 48 hours viability at 18 °C (Smyth and McManus, p. 199, 1989).

The method for infection was also the same as for E1, and copepods were observed to grab and ingest the coracidium put into their well immediately. It remains a mystery why all but one copepod in the infection groups failed to show any signs of an active infection. As stated, the mortality in the infection-only group was significantly higher than that of the control group, so the parasite likely had some negative effect on the host's fitness. However, chemical

or molecular immune parameters were not measured during this experiment. Various methods outlined above were discussed with Jörn Peter Scharsack and Joachim Kurtz of the University of Münster, but time and financial limitations made these impractical.

Naturally, parasite prevalence varies greatly with season and habitat, as well as between and within host populations. For example, in two *Eucyclops serrulatus* populations from Northern Germany, the parasite prevalence ranged from 0-3% (Becker, 2004). In Scottish populations of a possible *S. solidus* host, the copepod *Cyclops strenuus*, the prevalence of its fish tapeworm (*Diphyllobothrium* spp.) showed a seasonal variation in prevalence of 0.5-35% between August and October (Pasternak et al., 1995). Strains of *S. solidus* varies in how virulent they are in the second intermediate host (Ritter et al., 2017), but no studies showing how establishment differs between populations of copepod species.

Kalbe et al., 2016 found that the infection phenotype of the parasite-stickleback-system of *S. solidus* was dependent on both variability within host and parasite populations, but not the interaction between them. The Norwegian parasites (also from Lake Skogseidsvatnet) showed higher infectivity than their German counterparts, and Norwegian sticklebacks had a higher parasite resistance overall. The three critical stages of infection outcome as proposed by van der Veen and Kurtz, 2002, identifies an establishment phase (ingestion), gut penetration, and growth phase. The latter two are both likely candidates for why the parasite failed to manifest. During the gut penetration phase, larger host individuals are protected mechanically from infection based on tissue thickness.

The peritrophic matrix (PM) is a chitinous membrane secreted by the cells in the arthropod midgut, which protects the gut wall from abrasions and mechanical damage. Parasites that have a growth stage in the haemocoel, need to penetrate this layer (Miller and Lehane, 1993). The PM is thicker in species of free-living copepods, and proportionally thicker in larger individuals (Yoshikoshi and Kô, 1988). Thus, this might provide an additional barrier against tissue invasion from parasites. PM degradation is associated with reduction of growth and survival (Minoo Sajjadian and Hosseininaveh, 2015). Copepods in E2 might have been well-nourished and large enough for *S. solidus* to properly penetrate the gut wall, but still be left with debilitating damage to the gut tissue after the phase two was attempted. This would account for the increase in mortality in the infection-only group versus control. Copepods are unable to eliminate parasites once they have penetrated into the haemocoel, possibly due to immune suppression or biochemical camouflage (Loker, 1994).

4.3 E1 and E2 Culture Differences

While the E1 and E2 copepods came from the same culture, they were treated somewhat differently prior to the experiments. The E1 copepods lived mainly on ciliates and other protozoans present in the inoculated water. The culture after E1 was maintained similarly but also got a supplement of live *Artemia* nauplii once a week in the months prior to E2. This would result in a more thorough inoculation by facilitating bacterial growth from *Artemia* that were not preyed upon. It would also provide copepods with a nutritional boost that would result in higher female fecundity, which would end in least three new egg sacs after being isolated from the rest of the population in E2.

Both eggs and the copepods had stores of reddish lipid droplets, most likely esterified astaxanthin, a carotenoid pigment (Gilchrist et al., 1960). Astaxanthin (both free and esterified) has been shown to protect against oxidative stress from UV radiation and xenobiotics (Caramujo et al., 2012; Moeller et al., 2005). More recent research has suggested that carotenoid-accumulation in body tissues also has an immunorestorative effect in crustaceans (Weaver et al., 2018). It is likely that this might have affected the general receptiveness to all infections, including macroparasites.

Astaxanthin accumulation might also have been a result of parasite manipulation of the host, as pigment changes serve to make intermediate hosts more conspicuous to the final or nest intermediate host. *S. solidus* host manipulation is well-known in both intermediates (Hafer and Benesh, 2015; Scharsack et al., 2007). However, to date, there have been no published studies that also show pigment manipulation, such as how Bakker et al., 1997 described parasite-induced changes in colour in another crustacean prey of the stickleback.

Both virulence and the chances of establishing an infection increases with the number of coracidia administered intermediate hosts of *S. solidus* (Heins et al., 2019; Wedekind, 1997). Because of the possibility of a higher infection rate and a more dramatic parasite-toxin-interaction, two coracidia was given to each copepod in the infection groups of E1. However, while twin infections were counted in E1, procercoids from confirmed double infections were excluded from area measurements since intraspecies competition leads to proportionally smaller parasites and single-worm infections seem to be the norm in this host-parasite system (Wedekind, 1997). Administering exactly two coracidia is also manually a more difficult task than isolating one. For E2, it was thus decided to risk a slightly lower infection rate to remove a variable and gain a larger number of viable data points for the area measurements.

In insects, evidence of adaptive or specific mechanisms is suspected though not confirmed, though poorly understood and might also be a "loitering" innate response (Cooper and Eleftherianos, 2017). In crustaceans, adaptive immune responses have yet to be detected (Vazquez et al., 2009) and host-parasite interactions in copepods seem largely based on innate immunity (Kurtz, 2007). Kurtz and Franz, 2003, were able to show that while copepods infected by *S. solidus* cannot reject the parasite once established, their innate immunity does limit the growth of the procercoid. According to Scharsack et al., 2007, both host and parasite suffer from reduced to no reproduction during infection.

However, most E1 and E2 copepods were seen to produce new egg sacs post-infection. This was especially apparent in the E2 cohort, in which some females sustained as many as two new clutches post-infection. No differences were observed in any of the groups, although the exact number of clutches wasn't logged for all individuals due to the rapid rate at which they developed and started the cycle anew.

It is possible that parasites in E2 could have failed to develop due to troubles penetrating the gut wall, a more robust host immune system due to astaxanthin accumulation, acute microcystin toxicity, seasonal variation, host compatibility, or a combination thereof. It is unlikely to be changed by resource depletion in the host after the parasite had established itself, especially since E2 copepods were very well-fed and had continued fecundity throughout the experiment.

As suggested by Franz and Kurtz, 2002, there is no effect of the *S. solidus* infection on the muscles or storage lipids of its copepod host. Rather than direct host-manipulation, the physical condition of the host would be a more likely cause of altered host behaviour (Hafer and Benesh, 2015). Something that was also touched upon by Michaud et al., 2006, who found that multiple infections far exceeds the energetic requirement of a single procercoid.

4.4 Parasite-Microcystin Synergies

In E1, procercoids from the combination group were shown to have a significantly larger surface area than parasites from the infection-only group. Given this is not an artefact of small sample sizes, this might be indicative of an infection-toxin synergy. If the toxin affects the host, it will also have an effect on the parasite inhabiting it—the question is, how?

It is in the parasite's best interest for the host to survive for long enough for the parasite to reach the next stage in its life cycle. Virulence is typically defined as the severity of

pathogenicity, or the organism's ability to inflict damage (Pirofski and Casadevall, 2012). There are several evolutionary factors influencing virulence. A common trade-off is between fecundity and longevity in both parasite and host (Frank, 1996). It was believed for some time that a parasite's virulence was ever decreasing during a species' evolutionary timeline, and that severe infections were the mark of novel pathogens. This is because it was thought that infections leading to the disability or death of the host would also be harmful and unwanted to any parasite inhabiting it. Pathogens would thus evolve towards avirulence, or mutualism (Alizon et al., 2009).

This avirulence hypothesis was later replaced by a new model of explanation in the 1980s. Anderson and May (1982) and Ewald (1983) challenged the previous model by pointing out that a pathogen that is too restrained, will eventually lose out to eventual competition, such as a more aggressive strain. What was dubbed the "trade-off hypothesis" represents somewhat of a paradigm shift in the world of parasitology and epidemiology. The trade-off hypothesis would later be modified to include other life history traits and mechanisms (Alizon et al., 2009). We now operate with models of optimal virulence—the knife edge of virulence and avirulence on which a parasite balances in order to maximize its own reproductive success (Bull and Lauring, 2014).

The prevalence of virulence is also expected to relate to host stamina. Once established, the copepod cannot get rid of the parasite, but they can still regulate parasite growth (Benesh and Hafer, 2012; Scharsack et al., 2007). So, when potent toxins like microcystin is present in the environment, this might lower the capacity of an already weakened copepod to regulate this system. The well-fed and robust E2 copepods might thus have been better at fighting off infection early on, or prevent growth, even with the toxin present.

As previously mentioned, helminths act as pollution sinks for several types of xenobiotics, including heavy metals and persistent organic pollutants (POPs) (Hassan et al., 2018; Le et al., 2014; Teimoori et al., 2014). Palikova et al., 2013 were able to show that the diphyllobothrid *Khawia sinsensis* can also accumulate microcystins in a similar manner to that of other pollutants. The effects of the toxin on the parasite, or how it affects host-parasite dynamics is still unknown, and it is unfortunate that these findings are, as of yet, singular due to lack of research in this area.

Because procercoid and plerocercoid growth in intermediate hosts may affect the transmission rate and adult size, larval development is conditionally optimized (Michaud et al., 2006). In

general, procercoid growth is density-dependent, with maximum natural growth occurring in infection-onlys (Dupont and Gabrion, 1987). The development of procercoids appears to be independent of the life-stage of the copepod host, but nauplii and copepodites are more readily infected (Oliver and Boyd 1969 cited by Smyth and McManus, 1989). However, while procercoids commonly develop to a larger total size in adult females, they are proportionally larger compared to the size of adult male hosts (Wedekind et al., 2000).

In vitro studies mimicking the internal environment of copepods found that procercoids could grow longer than their largest intermediate hosts (1.5 mm) when unencumbered by mechanical and immunological factors (Jakobsen et al., 2012). Environmental contaminants such as polychlorinated biphenyls (PCBs) produce immunosuppressive effects which facilitate infections by the trematode *Anguillicola crassus* in eels (*Anguilla* spp.). In the roach (*Rutilus rutilus*), endocrine disruption due to chemical activity is exasperated by fish tapeworms, such as *Ligula intestinalis* (Sures, 2006). In invertebrates, this interaction was also demonstrated with how molluscs' response to pathogens changes with the presence of environmental contaminants (Morley, 2010).

Global and local changes in climatic conditions affect both bioavailability and toxic effects of pollutants (Schiedek et al., 2007), as well as host susceptibility and parasite development and transmission (Harvell et al., 2002). This change in environmental stressors could affect both host-parasite dynamics as well as the biology of parasite populations (Morley et al., 2006). Toxicant-parasite interactions on lower trophic levels might lead to bottom-up effects in which higher-trophic organisms, or the entire ecosystem, would be affected.

5 Conclusions

After two studies, neither hypothesis on the effects of microcystin on the copepod-*Schistocephalus*-system could be fully supported by the data. Microcystin did however prove to increase mortality significantly in both toxin-only and toxin-infection combination groups. This might point to a toxin-parasite interaction in which the host's immune system is weakened by the toxin and accidentally turns the infection more virulent. Based on how increasing environmental temperatures promote *S. solidus* fitness and growth (Franke et al., 2017) one can also surmise that an increase in environmental toxins could also change the host-parasite dynamics.

The parasites in the combined toxin-infection group also grew larger than the procercoids of the infection-only group. The immune response of the host most likely hindered the growth of

the parasites in the infection only groups. As the parasites themselves seem to be equally infectious and vital in both groups, this effect could be attributed to a weakening of the host's immune system, leading to increased parasite growth.

Toxin-infection interactions did not show any significant effect on the mortality of the host or the parasite. This was unexpected, as microcystin has shown to have detrimental effects on all trophic levels in aquatic ecosystems. Likely, *Macrocyclops albidus* is able to cope well with high concentrations of microcystin, and further research might look at more extreme concentrations to uncover more dramatic synergies.

Given more resources I would also have liked to study the immunological parameters of both host and parasite using biomolecular methods. This would give me additional insight into the host-parasite relationship and its interaction with environmental factors.

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Appendix 1

See files attached.

Appendix 2

See file attached.