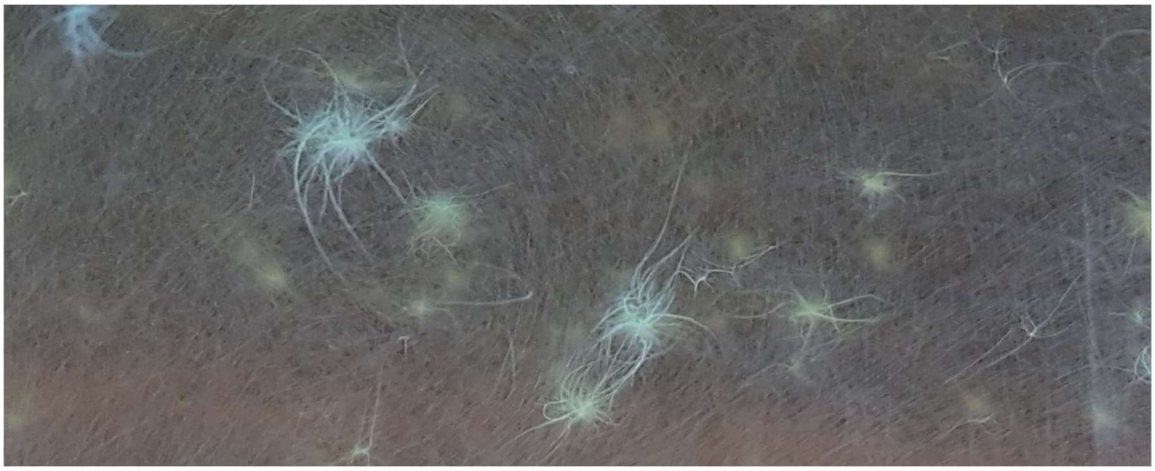
Effects of microcystin on the host-parasite interaction between brown trout (*Salmo trutta*) and the early life stages of the freshwater pearl mussel (*Margaritifera margaritifera*)



Master thesis in environmental toxicology

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Highlights:

- Effects of microcystin (MC) on the early life stages of the endangered freshwater pearl mussel (*Margaritifera margaritifera*) is unknown.
- *M. margaritifera* release their parasitic glochidia larvae during autumn, when blooms of toxin-producing cyanobacteria are most prevalent.
- Glochidia exposed to MC at 20 µg/L did not get a decrease in mortality.
- Ability of glochidia to attach to gill of brown trout (*Salmo trutta*) decreased when exposed to 100 µg/L MC for 2 hours prior infection.
- Negative effects on glochidial encystment also occurred during ten-day exposure of 40-70 µg/L MC on hosts. Haematocrit samples did not differ between host groups.
- More knowledge on the interaction of MC on *M. margaritifera* is crucial for further conservation.

Frontpage photo:
Unidentified colony of cyanobacteria in Kvernavatnet, Austevoll. By Katrine Åmdal Sundt

Abstract

Blooms of harmful cyanobacteria are increasing in magnitude and occurrences in freshwater habitats, with no signs of stopping. At the same time, species of freshwater mussels are experiencing a decline in populations worldwide, as is the case for the endangered freshwater pearl mussel *Margaritifera margaritifera*. Both species development is largely due to increasing eutrophication of their habitats. The bloom events often occur in the autumn months, around the same time the freshwater pearl mussel release their glochidia larvae to infect the gill of host fish. This thesis investigates the effects of the cyanotoxin microcystin (MC), a common type of toxin produced by cyanobacteria, and its effects on the early life stages of *M. margaritifera*. Three experiments were conducted, where mortality and infection ability of glochidia after exposure to MC were tested, as well as the attachment rate of glochidia after host exposure. Twenty-four-hour exposure to 20 µg/L MC showed no effect on glochidia mortality, but a two-hour exposure to 100 µg/L MC decreased infection success of the glochidia. For host-parasite interaction, hosts were exposed 45-55 days after glochidial infection. Host with ten-day exposure to 40-70 µg/L MC had fewer infections on the gills than the control group. The haematocrit did not differ between the host groups. These results indicate that the MC cyanotoxin might interfere with the survival of *M. margaritifera* during their early life stages.

M. margaritifera, a species adapted to oligotrophic conditions, might experience several negative effects of eutrophication of their habitats. Further knowledge on MCs and their effects on the freshwater pearl mussel is needed to properly conserve the species.

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Contents

1. Introduction

Cyanobacteria, previously known as blue-green algae, are the oldest photosynthesising organisms known (Paerl & Otten, 2013). They were largely responsible for the increase in atmospheric oxygen over 2 billion years ago, and they were the beginning of eukaryotic photosynthesising cells, being the origin of the chloroplast organelle (Davankov, 2020; Huisman et al., 2005). Without their alteration of atmospheric O_2 and immersion with other cells, the world's atmosphere and species would not be the same that it is today (Davankov, 2020). Cyanobacteria have a high ability to exploit available nutrients, and they are highly adaptable to changes in the habitat or climate, which is why these bacteria can thrive in most types of environments (Paerl & Paul, 2012; Whitton & Potts, 2012).

However, there is a downside to their success as these robust species can be harmful when growing uncontrollably. Harmful algal blooms (HABs) are high-density blooms consisting of algae or other plant-like organisms and are a natural phenomenon known to occur throughout history (Hallegraeff, 1993; Pavagadhi & Balasubramanian, 2013). During the last decades, incidents, intensity, and distribution of HABs have increased, causing an increasing concern worldwide (Anderson et al., 2002; Hallegraeff, 1993; Paerl & Otten, 2013). These blooms are considered harmful for several reasons. The high density gives poor conditions for other organisms due to unclear water and oxygen depletion when a bloom collapses (Paerl & Otten, 2013). A major concern with cyanobacterial HABs is the ability for some species to produce cyanotoxins as secondary metabolites (Paerl & Otten, 2013). Cyanobacteria can produce cyanotoxins like neurotoxins, hepatotoxins, cytotoxins, and irritating dermal toxins (Carmichael, 2001). These toxins are known to affect several organs, the nervous system, and can in severe cases lead to death (Carmichael, 2001; Jochimsen et al., 1998).

1.1. HABs - An increasing phenomenon

The reason why HABs are increasing is mostly due to human activities in the surrounding areas, such as alteration of habitat (dams and sluices), agriculture, and pollution (Paerl & Otten, 2013; Smith & Schindler, 2009; Xu et al., 2010). These activities can lead to eutrophication of waters, which is an increasing problem around the world (Yang et al., 2008). The eutrophication of habitats contributes to the growth and presence of toxin-producing cyanobacteria (Rantala et al., 2006; Smith & Schindler, 2009). An increase of

nutrients like nitrogen and phosphorous favour growth of cyanobacteria and high amount of P correlates to increased toxin production in some species (Kotak et al., 2000; Xu et al., 2010). Weather can further increase favourable nutrient supply and growth conditions. Rain events are a significant factor in lake eutrophication since they can cause nutrient runoff from surface water and nearby sources (Paerl & Otten, 2013). A mix of rainy periods followed by periods of warm temperature and drought creates conditions especially favourable for blooms (Paerl & Otten, 2013; Reichwaldt & Ghadouani, 2012; Weber et al., 2020). The effects of climate change are thus a concern regarding HABs. Climate change alters weather patterns, causing more extreme weather events both in terms of rain and drought (Paerl et al., 2016; Paerl & Otten, 2013). The general temperature increases, and high temperature is one of the most critical factors for occurrences of HABs (Paerl et al., 2016; Wicks & Thiel, 1990; Xu et al., 2010). Mild winters also allow cyanobacteria to overwinter and run photosynthesis all-year-round, giving them a head start to bloom in the spring and summer (Weber et al., 2020; Xu et al., 2010). Although HABs can occur during various seasons, they are most common and prominent in the late summer months and early fall (Lehman et al., 2008; Naveen et al., 2010).

To avoid HABs, strategies such as re-establishing of habitats, vegetation buffer zones, reduction of fertilisers and use of low P fertilisers in vulnerable areas are often used (Jeppesen et al., 2007; Weber et al., 2020). Nevertheless, lakes can have a lot of phosphorous in the sediment and this can take decades to normalise (Ho & Michalak, 2017). Thus once a HAB occurs, it can remain for several months independent of nutrient supply (Ho & Michalak, 2017; Paerl & Otten, 2013). Also, when climate change is such a significant contributor, the incidents are expected to increase regardless of nutrient-limiting strategies (Paerl et al., 2016; Reichwaldt & Ghadouani, 2012). Because of this, an increase of incidents and magnitude of HABs are expected. It is therefore important to gain more knowledge on HABs and how to reduce the problem and map the possible risks it poses for ecosystems.

1.2. The microcystins (MCs)

Among the cyanotoxins, the most frequently occurring are the microcystins (MCs) (Catherine et al., 2017). There are over 100 different variants of these hepatotoxins discovered, where microcystin leucine-arginine (MC-LR) is the most common and toxic (Catherine et al., 2017; Wu et al., 2019). These cyclic peptides can inhibit serine/threonine-specific protein

phosphatases, which play an essential role in cell signalling and physiological processes (Le Manach et al., 2019; Pereira et al., 2013). The interference with protein phosphatases will disturb cellular processes and can lead to the destruction of hepatocytes (Butler et al., 2009). Although MCs mainly targets the liver, it can be found in several other tissues after exposure (Cazenave et al., 2005; Malbrouck & Kestemont, 2006). There are several strategies to remove MCs and other cyanotoxins from the water, but ozone is considered to be the most effective (Rositano et al., 2001; Svrcek & Smith, 2004).

Fish and other species in the aquatic environments are usually the ones most frequently and directly exposed to potential toxins from HABs and have exhibited a higher tolerance for MCs compared to most terrestrial animals (Malbrouck & Kestemont, 2006). The tolerance within fish species has also been shown to vary, and species in oligotrophic habitats are generally more vulnerable than species in eutrophic habitats (Malbrouck & Kestemont, 2006; Råbergh et al., 1991; Snyder et al., 2002). HABs, and thus MCs, are more common in eutrophic environments, and organisms in eutrophic habitats have most likely developed a higher tolerance for the toxin compared to species accustomed to oligotrophic habitats (Malbrouck & Kestemont, 2006; Snyder et al., 2002). Due to species being adapted to their natural habitat, eutrophication has a large impact on the species composition of oligotrophic habitats (Sand-Jensen et al., 2018).

1.3. The fate and life of the freshwater pearl mussel, *Margaritifera margaritifera*

An example of a species adapted to oligotrophic conditions is the freshwater pearl mussel, *Margaritifera margaritifera*. This species, and many other freshwater mussel species worldwide, are threatened and on the brink of extinction (Atkinson et al., 2013; Strayer et al., 1999; Strayer et al., 2004). Their decline can have severe effects on their ecosystems, as healthy populations provide clear water and balance the nutrients available (Atkinson et al., 2013; Vaughn & Hoellein, 2018).

M. margaritifera are long-lived, and often reach an age of over a 100 years, but its populations in Europe is overaged, and many have not had successful reproduction for the last 30 to 50 years (Geist, 2010; Ziuganov et al., 2000). The early life stages are glochidia larvae, which are parasites on the gills of a host fish. The phases as free-living glochidia and

juveniles are probably the most vulnerable phases in their life cycle. (Fig. 1) (Bauer, 1988; Taskinen et al., 2011).

The female mussel releases parasitic glochidia larvae in late June to September and they attach to the gills of either salmon (*Salmo salar*) or brown trout (*Salmo trutta*) (Hastie & Young, 2001, 2003; Salonen et al., 2017). A formation of a protecting cyst of epithelial cells occurs on the gill, and the encysted glochidia remain there for about 9-11 months whilst it undergoes metamorphosis (Bauer, 1988; Hastie & Young, 2001; Jacobson et al., 1997; Karna & Millemann, 1978). Both free and attached glochidia, as well as the newly released juveniles, are susceptible to cyanotoxins as their release happens in summer/fall when HABs are most likely to occur.

Eutrophication is already known to provide poor conditions for juvenile mussels, as it leads to more mud, algae, and little oxygen in the sediments (Bauer, 1988; Geist, 2010; Strayer et al., 2004). Anthropogenic activities are likely more harmful than direct threats such as pearl fishing and predation (Geist, 2010). Industry and agriculture activities are thus limited along rivers containing threatened mussel populations (Larsen, 2018; Strayer et al., 2004).

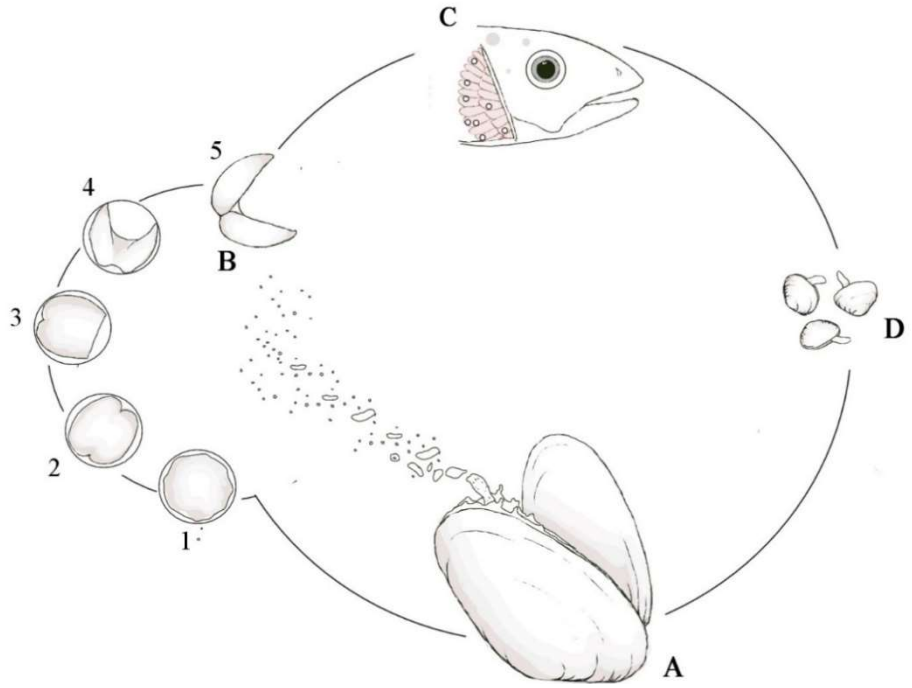

Figure 1: The life cycle of the freshwater pearl mussel, Margaritifera margaritifera. *A: Adult mussels are filter feeders and usually mate with dioecious reproduction (Bauer, 1987). B: glochidia larvae (60-80 µm) is released by a female adult (Ziuganov et al., 1994). C: Glochidia larvae attach to the gill of a suitable host fish and complete metamorphosis on the gill (Bauer, 1988). D: after ~11 months the glochidia excyst from the gill as a juvenile mussel (360-500 µm) (Bauer, 1994; Hastie & Young, 2001). Some glochidia might be released prematurely (1-4), but only stage 5 can infect host (Pichler-Scheder et al., 2011). Illustrated in Krita 4.2.8.*

1.4. Known effects of microcystins on the species studied

There have been documentations on the effect of MC on brown trout (*Salmo trutta*). They report damage to organs such as the liver, gills and kidney, and deaths due to this (Bury et al., 2003; Rodger et al., 1994). MC exposure has also lowered the haematocrit value and suppressed the immune responses in several species of fish (Lin et al., 2017; Rymuszka & Adaszek, 2012; Rymuszka et al., 2007; Zhang et al., 2007).

Species of freshwater mussels have showed increased inflammatory reactions, oxidative stress and accumulation in hepatopancreas when exposed to MCs (Gélinas et al., 2014; Kim et al., 2017; Wang et al., 2018; Yang et al., 2012). However, there is no documentation on the effects of MC on *M. margaritifera.* MC can have adverse effects on animals ingesting the toxin, and it has the potential to interfere with interactions between species in an ecosystem (Pavagadhi & Balasubramanian, 2013). There is a need for research considering the toxicity of MC in relation to biological interactions.

1.5. The focus of this study

This thesis investigates possible risks when HABs expands to oligotrophic systems, and if it is yet another factor amplifying the adverse effects of eutrophication on the freshwater pearl mussel. All experiments were performed at Kvernsmolt, Austevoll, a facility dedicated for conservation of *M. margaritifera* by breeding juvenile mussels. Some years before, the survival of the juvenile mussels severely declined, as a result of cyanobacterial HABs in Kvernavatnet, the lake supplying the facility with water. Having both the mussel facility and NIVA's algae lab available gave us a rare opportunity to investigate the effects of MCs on the early life stages of *M. margaritifera.* Three experiments were conducted, investigating the lethal and sublethal effects on glochidia larvae and the host/parasite interaction during exposure to MC extract and MC-producing *Microcystis aeruginosa.* The host used for parasite infection is brown trout (*Salmo trutta)*. The null hypothesis is that MC does not interfere with the mortality of glochidia larvae, infection rate and survival on host gills.

2. Materials and methods

To evaluate the effects of MC exposure on the infective and parasitic stages of *M. margaritifera*, three experiments (E) were conducted:

- **E1:** Investigation of lethal effects after up to 24 h exposure to 20 µg/L MC extract on glochidia larvae (2.2).
- **E2:** Investigation of sublethal effects on glochidia larvae exposed to 100 µg/L MC extract for 2 h. A comparison of infection success on the gills of brown trout (*Salmo trutta*) (2.3).
- **E3:** Investigation of the host-parasite interaction between brown trout and glochidia during host exposure to MC-producing cyanobacteria (*Microcystis aeruginosa*) (2.4)

All experiments were performed at Kvernsmolt, a facility at Austevoll leased by the University of Bergen. Water used was supplied from Kvernavatnet, a lake struggling with HABs. The water was thus treated twice with ozone to eliminate MCs present.

2.1. Materials

2.1.1. Test species

Glochidia larvae (*M. margaritifera*):

The Kvernsmolt facility kept adult mussels collected from Norwegian rivers to collect glochihidia and breed juveniles. E1 and E2 were performed in the fall of 2018, and the glochidia larvae used, for both experiments, were from the *M. margaritifera* population from Femanger, Bjørnefjorden. E3 was conducted in 2019, and collected glochidia larvae came from Skoelva, Telemark.

The release of glochidia happened late July – mid-September. When the adult mussels released glochidia, they were collected, and the stage was determined (Stereomicroscope, 40x magnification). Once glochidia were stage 5, as described in the introduction (Fig. 1), they were used in the experiments within 10 h after collection. Free glochidia larvae can survive for up to 10 days at temperatures <5 C°, but the length of survival decrease to 2-3 days when temperatures are 16°C or higher (Jansen et al., 2001). Collection occurred once a day, so the collected glochidia could have been released up to 24 h earlier. They were stored at <4°C

after collection.
In the individual experiments, the glochidia used were most likely spat from the same female.

Brown trout (*Salmo trutta*):
0+ brown trout from the population in Sirdalsvannet, produced by Norsk Ørret at Heggland, Tysnes was used in E2 and E3. The batches differed between the experiments, as E2 was performed in 2018 and E3 in 2019.
Due to the presence of the parasite *Ichthyobodo necator*, the brown trout for E3 were treated with formalin (~1/ 4000 in twenty minutes) two days before beginning the experiment and ten days before being infected with glochidia.

2.1.2. Source of microcystin (MC)

Cyanobacteria (*Microcystis aeruginosa*): For E3, two strains of *M. aeruginosa* were used: one with (NIVA-CYA 160/1) and one without (NIVA-CYA 144) the ability to produce MC. Both strains *M. aeruginosa* were supplied from NIVA's algal culture collection (https://niva-cca.no).
Both *M. aeruginosa* isolates were cultured in plastic bags with 20 L cultures (19.5 L water, 0.5 L algae culture and 20 mL cell medium (Standard solution 100 g Varicon Aqua Cell-hi F2F in 1 L water)). All equipment used were sterilised with 70% ethanol in advance. The temperature was stable at ~20 C°, and there was constant bubbling in the cultures. At first, the water used was ozonated once. Due to a lack of growth in the cultures and contamination of other algae from nearby cultures and water, the water was additionally boiled and cooled down before use, without successfully ending the problem.

MC extract: Concentrated MC solutions derived from freeze-dried *Planktotrix prolifica* (NIVA-CYA 98 from NIVA's culture collection) was used in E1, E2 and E3. Since all three experiments were performed on separate occasions, there were three different extracts with various concentrations: 1640 µg/L, 1500 µg/L and 7200 µg/L.
The solutions mainly consisted of MC-LR.

2.2. E1: Lethal effects of MC on *M. margaritifera* glochidia

To test for the eventual lethal effects on glochidia when exposed to MCs, groups of glochidia were exposed to MC-extract in three separate time periods. The glochidia in E1 were exposed to 20 µg/L MC for up to 24 h. This concentration was prepared before exposure by diluting

the 1640 µg/L MC extract (246.95 mL water, 3.05 mL 1640 µg/L MC. Total 250 mL). One 21 well plate contained 20 µg/L MC solution (Exposed) and one 21 well plate contained water (Control), and each well was filled with 10 glochidia and 5 mL of either MC solution or water. Each well plate was divided into three groups with different exposure length: 2, 16 and 24 h (Table 1).

Table 1: ***Control and exposed group in the experiment of lethal effects of MC on glochidia.*** *Both control and exposed had three different groups with different lengths of exposure (2, 16 and 24 h). All groups had seven wells, containing ten glochidia each.*

	Control (water)	Exposed (20 µg/L)
2 h	7 wells x 10 glochidia. N = 7	7 wells x 10 glochidia. N = 7
16 h	7 wells x 10 glochidia. N = 7	7 wells x 10 glochidia. N = 7
24 h	7 wells x 10 glochidia. N = 7	7 wells x 10 glochidia. N = 7

When testing for mortality, 20 µL of citric acid was pipetted into the well while carefully observing the response of the glochidia. Active glochidia reacted by closing their valves when exposed to outer stressors such as salt and acid (Fig. 2) (Gene et al., 2019; Zale & Neves, 1982). All responding glochidia were counted as living, whilst the ones not responding were presumed dead.

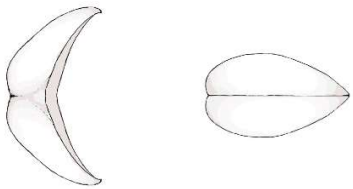

Figure 2: Stage five glochidia (M. margaritifera) in open (left) and closed (right) form. *By adding an acid or other outer stressors, living glochidia respond by closing, whilst non-active/dead glochidia are unresponsive (Gene et al., 2019; Zale & Neves, 1982). Illustrated in Krita 4.2.8.*

2.3. E2: Sublethal effects of MC on *M. margaritifera* glochidia

To test if MC interferes with the glochidial ability to infect their host, glochidia were exposed to 100µg/L MC-extract for 2 h before infection of brown trout. The glochidia was added directly in the MC dilution (931.5 mL water, 1.5 mL glochidia and 67 mL 1500 µg/L MC. Total 1 L). The glochidia in the control group were MC-free for an equal amount of time

(998.5 mL water, 1,5 mL glochidia. Total 1 L). Exposed and control group were each added to one 60 L aquarium, each containing 30 0+ brown trout (60 in total) (Table 2).

Table 2: ***Control and exposed group in the experiment of sublethal effects after 2 h MC exposure on glochidia.*** *The exposed group used glochidia exposed to MC (100 µg/L) before infection, whereas control had non-exposed glochidia. Both groups contained 30 brown trout, whereas 20 were collected for infection observation.*

Control	Exposed
1 L (glochidia: 500 per host) 30 trout (20 checked).	1 L (100µg/L MC, glochidia: 500 per host). 30 trout (20 checked).

The two bottles containing glochidial solutions were made from a dense solution of glochidia. To determine the glochidial density, 250 µl of homogenised glochidial solution was spread out in four of the pits in a counting chamber (total: 1 mL). Counting was done by using a Wild M3 stereomicroscope (x40 magnification), and the mean number from the four pits gave a likely estimate of glochidia density. The required amount was approximately 500 glochidia per fish, and 1.5 mL of the dense solution contained ~15 000 glochidia. Once added in the bottles, they stood still for 2 hours before being used for infections.

The solution containing exposed glochidia left a concentration of ~1.66 µg/L MC (1 L 100 µg/L MC added in 60 L aquarium) in the aquarium. The brown trout in this experiment was therefore exposed to a very low concentration of MC. The non-exposed glochidia were added to the control aquarium. Two days after the infection, 20 trout in each group were measured, and the gills were removed. Pressed between two glass plates, the number of glochidia on gills were counted using a Wild M3 stereomicroscope (40x magnification).

2.4. E3: Ten-day MC exposure of host during glochidial infection:

To address the crossed effect of glochidial infection and long-term exposure to MC on brown trout, four groups were used to cover the different effects (Table 3). The method of MC exposure was altered during the course of the experiment. At first, all the hosts in the groups were exposed to *M. aeruginosa,* but two different strains of the species were used. Control and infected groups had the non-toxic strain, whereas toxic and combined groups had the MC-producing strain. The brown trout were to be exposed one week before glochidial infection and for the following 40 days. Due to lack of growth and MC production in both

Table 3: ***Groups in the experiment of host-parasite interaction during host MC exposure.*** *N is the number of brown trout individuals in each group. The experiment used both non-toxic and MC-producing strains of M. aeruginosa.*

	Non-toxic *M. aeruginosa*	Toxic *M. aeruginosa*
Not infected	**Control:** Brown trout exposed to non-toxic *M. aeruginosa*. N = 38	**Toxic:** Brown trout exposed to toxin-producing *M. aeruginosa*. N = 38
Infected	**Infected:** Brown trout exposed to non-toxic *M. aeruginosa*, infected with glochidia (200 per host). N = 38	**Combined:** Brown trout exposed to toxin-producing *M. aeruginosa*, infected with glochidia (200 per host). N = 38

M. aeruginosa cultures, in addition to contamination from other algae, the algae exposure was terminated 23 days after infection. The method of exposure to MC was therefore changed and MC-extract was used instead of *M. aeruginosa* cells. Exposure by MC extract was used 44 – 55 days after infection, ten days in total. The bottle of MC extract had a volume of 1.7 L and a microcystin concentration of 7200 µg/L. This was enough to reach 51 µg/L by adding 425 mL in the two exposed groups two times (4x425 mL): at the start of the exposure and on day five. Infected and control group got 425 mL of water instead of the MC – solution.

2.4.1. Preparations before exposure

Two water systems were prepared for the four exposure groups; one for control and infected groups (non-toxic *M. aeruginosa*) and one for toxic and combined groups (MC producing *M. aeruginosa*). Each system had two 85 L aquaria, and two 182 L trays: one at the top and one at the bottom (Fig. 3). Filled up, each water system contained ~396 L water. Both trays had plant lights (Aquael Leddy Tube RetroFit 18W Plant) above the trays to promote algae growth. The systems were disinfected with chlorine dioxide treatment (1 tablet DryOx Deep clean by Dryden Aqua), and thoroughly cleaned and flushed afterwards to avoid traces of chlorine. Water used in the systems was filtered down to 1 µm and ozonated twice before being aerated and matured for four days. The fork length of the brown trouts was measured before being placed in the water systems on 05.08.19. Nineteen L of the *M. aeruginosa* cultures were added in each system the following day.

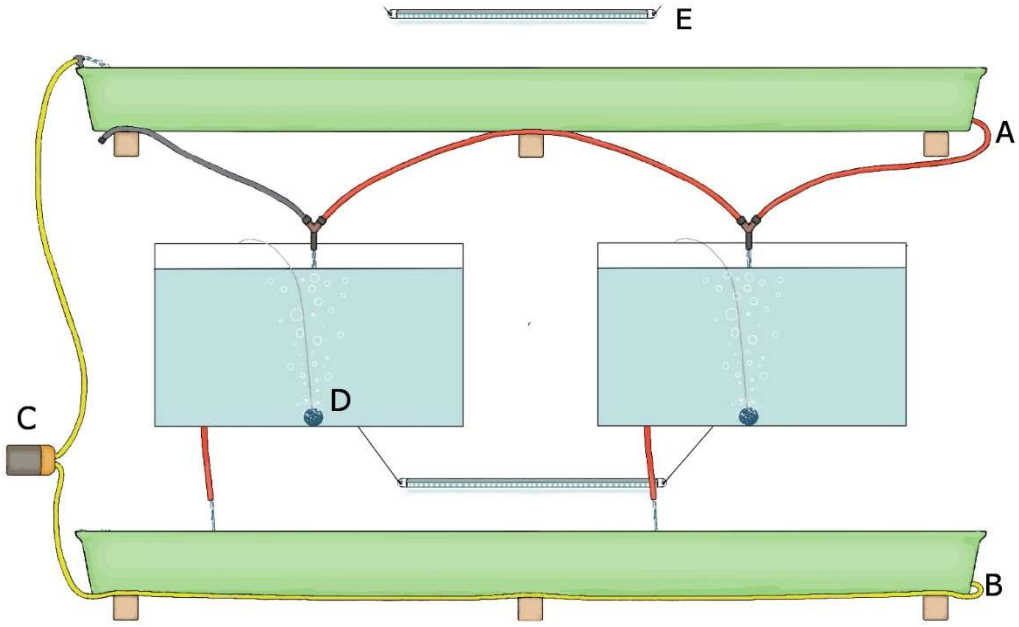

Figure 3: Illustration of one of the water systems providing cyanobacteria-containing water to two groups. *A: Red hoses transported water down to the aquaria and then further down the bottom tray. B. Water got transported up to the top tray through yellow hoses by the help of a pump (C). D: Aquarium air stones provided circulation and oxygen. These are also present in the bottom tray to avoid anoxic conditions. E: Plant lights (Aquael Leddy Tube RetroFit 18W Plant) above trays to promote cyanobacterial growth. The system contains ~396L water. Illustrated in Krita 4.2.8.*

2.4.2. Glochidial infection

Infected and combined group were infected with *M. margaritifera* glochidia on 13.08.19. The density of glochidia was estimated to be 1600 glochidia/mL. Two bottles were prepared to give the desired infection of 200 glochidia per trout (4.75 mL glochidia, 99.525 dL water. Total 1L).

Only the host was to be exposed in this experiment, so the glochidial infection had to be performed in the absence of MC to avoid confounding effects on the exposed host fish. The groups were removed temporarily from the water systems in separate 60 L aquaria with bubbling and no water input during infection. Infected and combined group got 1 L with infectious glochidia, whereas toxic and control got 1 L of water. After infection, water input was turned on, and the groups remained in the aquaria for two days (15.08.19) before being moved back to the water systems.

2.4.3. Feeding and water quality

Feeding was performed with caution, as too much feed could accumulate and pollute the systems. Each group got ~4 g of pellets (Skretting Nutra Sprint, 0.5 mm), which was mixed with water and pipetted slowly into the aquaria to assure that the feed did not float on the surface and fell to the bottom tray. Ammonium, nitrite, nitrate and phosphate levels were

monitored by using a Winlab® Data Line Photometer. The measurements were performed according to the attached test instructions manual (Table 4). The measurement range of ammonia was 0.05-3 mg/L, and to avoid exceeding this level, the water and cyanobacteria in the trays were changed weekly. When adding the cyanobacteria, 19 L of each culture was added, but the amount could be adjusted according to algal density in the water systems and cultures. Before and after the water change, 3 dL from each water system was filtered using GF/F filtration (Glass microfibre filter, 55 mm, pore size: 0.7µm). The green colouration on the filter gave an estimate of the density of cyanobacteria in the water, and the amount added was adjusted accordingly. On suspicion of lower growth in one of the *M. aeruginosa* cultures, the cultures would also get filtered.

Ammonia should not exceed levels higher than 0.1 mg/L, and nitrite should not exceed levels 0.3 mg/L, as higher levels could be harmful to the host (Noble et al., 2018; Wedemeyer, 1996). The levels of ammonia were calculated from the ammonium concentration by taking temperature and pH levels into account (Wetzel, 1983).
In the water systems, samples were taken from each system. Once the MC-exposure changed, samples were taken from all four groups, and only ammonium and nitrite were measured, as these are nutritious compounds and there were no cyanobacteria present. Samples for MC analyses were taken in glass tubes, due to MCs ability to react to plastic (Metcalf et al., 2000). Three mL samples of water from each system (each aquarium after changed exposure) got frozen at -20 C° for later MC analysis. Photometer samples and MC samples were taken three times a week. Once a week, pH levels were measured by using a calibrated pH meter (Sension+ PH31 Advanced GLP laboratory pH) at 18-19 C°.

The lack of growth and MC-production in the algal cultures made it necessary to use MC extract instead. When starting exposure with MC extract, the groups were moved to four separate 60 L aquaria, where toxic and combined group were exposed to MC extract starting 27.09.19, 44 days post-infection. In 60 L aquariums, it was expected high levels of ammonia over time. The aquaria got two bubbling stones each, and the trout was starved during this exposure time to avoid a rapid increase in ammonia. Once the levels of ammonium were 3 mg/L (02.10.19), ~50 L of the water was replaced, and the last dose of MC was added. The end of exposure and collecting of samples started on the 07.10.19

Table 4: ***Procedure and products used for measurements of ammonium, nitrite, nitrate and phosphate on Winlab® Data Line Photometer****. The procedures described are shortened versions of Winlab® Data Line Photometer Test Instructions (not available online), page number applies to this manual. Ammonium and phosphate were measured at a wavelength of 700 nm, nitrite and nitrate at 520 nm. All reactants for the treated cuvette are listed in order and amount for each procedure. Reactions that require mixing and reaction time are specified.*

Compound/test kit	Procedure
Ammonium, page 15 Product number: 37440 Measuring range: 0.05 – 3 mg/L NH_4^+	Blank cuvette: 5 mL. Treated cuvette: 5 mL. Reagent (R)1 (Sodium hydroxide): 0.6 mL. Mixed. R2 (Dichloroisocyanuric Acid Sodium Salt): One blue spoon, mixed. 5 min reaction time. R3 (Nitroprusside sodium): Three drops, mixed. 7 min reaction time. Method selected on photometer: "Ammonium +" The cuvettes were placed in the cuvette slot and covered with a cap. The blank was calibrated, and then the treated sample was measured, same for all procedures. For ammonium, the result appeared in mg/L NH_4^+.
Nitrite, page 99 Product number: 37450 Measuring range: 0.01 – 2 mg/L NO_2^-	Blank cuvette: 5 mL. Treated cuvette (large): 20 mL. Reagent (Tartric acid): two blue spoons. Dissolved. 3 min reaction time. ~5 mL poured over in small cuvette. Method: "Nitrite +" on photometer, results appeared in mg/L NO_2^-
Nitrate, page 95 Product number: 37449 Measuring range: 0.01 – 30 mg/L NO_3^-	Blank cuvette: 5 mL. Treated cuvette: 5mL Buffer A (tartaric acid): One green spoon. Dissolved *Buffer B (not specified): Three drops. Mixed.* *R1* (sulfanilic acid; zinc powder): One green spoon. Shaken for 30 sec. *R2:* (boric acid; N-2-Aminoethyl-1-naphthylamine dihydro chloride): One green spoon. Mixed for 30 sec, 5 min reaction time. *Method:* "Nitrate low +" on photometer. Results in mg/L NO_3^-
Phosphate page 125 Product number: 37451 Measuring range: 0.01 – 5 mg/L PO_4	Blank cuvette: 5 mL. Treated cuvette (large): 20 mL. R1 (sulfuric acid; ammonium molybdate tetrahydrate): 12 drops, mixed. R2 (not specified): 2 drops, mixed. 5 min reaction time Method: "Phosphate low +" on photometer. Results in mg/L PO_4.

2.4.4. Collecting samples from experimental fishes

The collecting of samples started on the 07.10.19 and lasted until the 10.10.19. Before starting, the following equipment was needed:

>760 x 1,5 mL Eppendorf tubes
>152 x heparinised haematocrit tubes (75 x 1.1-1.2 mm)
Formaldehyde solution (VWR Q-Path Chemicals Formalin 10%, buffered, pink)
RNAlater stabilization solution (ThermoFicher AM2021)
Aqui-S vet. anaesthetics, haematocrit centrifuge, weight, dissecting kits

4 mL of a standard solution of Aqui-S vet. (standard solution 1:10: 4,5 ml Aqua-S vet. and 45 mL of water. Tot: 49,5 mL) was added into 1 L of water. One trout from each group was selected in sequence to control for time effects. It was anesthetised until there were signs of unconsciousness: the trout started breathing at an uneven pace and rolled over with the abdomen upwards without any movement (~1 min.). It was then measured and weighed, and then euthanised by a spinal transition (neck cut) before taking blood samples. This method was used to avoid unnecessary mechanical damage to gills.

Due to the small size of the trout, the blood could not be collected easily by a needle. The tail was instead cut off right before the tail fin, and immediately a haematocrit tube was placed by the dorsal aorta for blood collection. A small drop of blood was placed on a microscope slide to create a blood smear. The drop was smeared carefully back by a cover glass before being pulled forward, spreading the blood drop evenly across the slide. The slide was laid to dry. About $1/6^{th}$ of the liver, not much bigger than a match head was collected in RNAlater to check for markers of oxidative stress. Half of the head kidney was collected in RNAlater for analysis of inflammatory markers. For histological examination, samples of gill, liver and head kidney were collected and preserved in formaldehyde. A small piece from the 2^{nd}-gill arch, half of the head kidney and the remaining parts of the liver were put in an Eddendorf tube. The rest of the brown trout was put in a plastic bag along with the individual number, and then frozen.

2.4.5. Microcystin analyses

The water samples from the toxic and combined group were tested for microcystin content by using a Microcystin-ADDA ELISA kit (Abraxis, product No. 520011). Three samples from the non-exposed groups were also tested to reaffirm that there were no high levels of

microcystin present. The ELISA is effective between 0 and 5 µg/L MC. Therefore, the samples from the toxic and combined group were diluted 20 times (100 µl sample, 1900 µl distilled water. Total 2 ml). Three dilutions were made for each sample. The anticipated concentration used in the experiment was 51 µg/L MC, so the dilutions had an anticipated value of a maximum of 2.55 µg/L MC. Six standard solutions containing small amounts of MC (0, 0.15, 0.40, 1.0, 2.0, 5.0 ppb, 1 mL), one control (0.75 ± 0,185 ppb, 1 mL) and a sample diluent (laboratory reagent blank, 25 mL) were provided in the kit. 50 µL of these standards and the diluted samples were pipetted into a 96 well plate according to the plate scheme (Fig. 4). Method proceeded as explained in the user guide: https://abraxis.eurofins-technologies.com/home/products/rapid-test-kits/algal-toxins/algal-toxin-elisa-plate-kits/microcystinsnodularins-adda-epa-etv-epa-method-546-elisa-96-test/.

Concentration of MC was calculated from the absorbance of the wells, measured in a Multiscan FC microplate reader.

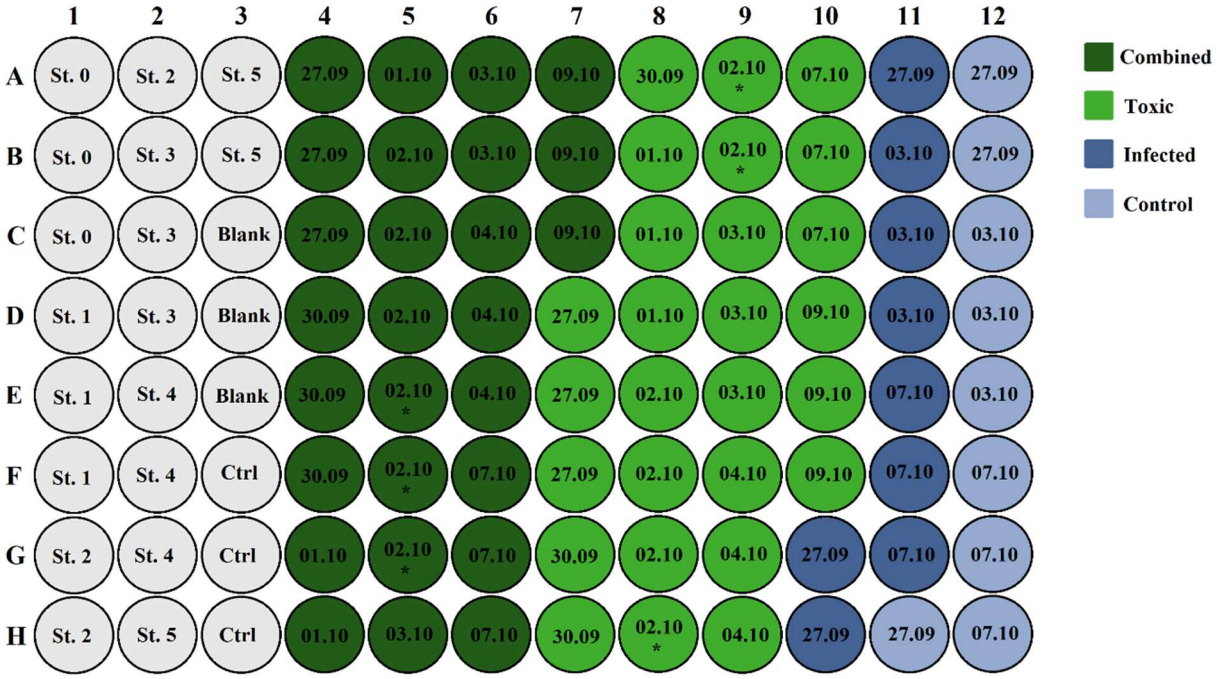

Figure 4: Plate scheme for the Microcystin ELISA test*. Standards, blank and control were added in the grey area, water samples were added from A4 to H12. Combined and toxic group (MC-LR exposed) had 9 samples each." *" Marks after exchange of water and MC-LR. Three samples from infected and combined group (no microcystin) were tested to prove there were no microcystin present.*

2.4.6. Haematocrit

Two haematocrit tubes were put in the haematocrit centrifuge at a time, and they were prepared as soon as the samples were ready. The tubes were placed opposite of one another in the centrifuge chamber|, so that the weight in the chamber was equally dispersed. The samples were centrifuged at 7000 rpm for 10 minutes, causing the blood within the tube to separate into the red blood cells and plasma. The ratio of red blood cells and plasma gives the haematocrit value.

2.4.7. Blood smears

In order to observe cells in the blood smears, a selection of dried smears were fixed and dyed with a Colorrapid-kit (LucernaChem). The smears were dipped in methanol fixation, red dye and blue dye for 5x1 seconds in each solution. After this, the slides were carefully rinsed in water and dried. At 200x magnification in a stereo microscope, the cells types were at best ability identified.

2.4.8. Samples from gill, liver and head kidney

Due to complications, mainly the COVID-19 shutdown, the samples for histology, oxidative stress and inflammatory markers could not be analysed. These results could therefore not be presented in this thesis.

2.4.9. Count of infections

After sampling, the gill arches on the left side were damaged. The gill arches on the right were intact and were examined to estimate glochidia infections on the gills of infected and combined group. The gill arches were collected from the thawed specimen, and the infections were counted in the same manner as the preparatory experiment for sublethal effects (2.4).

2.5. Statistical analysis:

For statistical analyses, the significance level of which H0 is rejected is set at $p < 0.05$. All statistical analyses were performed in R (R Studio version 1.2.5033).

Similar analyses were used for glochidial attachment in E2 and E3. The size of hosts was tested separately for significance using a two-sample t-test. A glm-model of infections excluding size as a predictor variable was also made. A combined glm-model was created for testing the effects of both predictor variables. The models belonged to the poisson family due to the response variable (infections) being count data. Due to overdispersion of data, quasipoisson and a following "F" test was used.
Model for experiment E2:

```
>fit1.glm <- glm(Infections~Treatment*Size, family=quasipoisson, data
 =sublethal.df)
>anova(fit1.glm, test="F")
```

For nitrite levels in the groups of E3, a one-Way ANOVA model was used.

```
>fit.lm <- lm(Nitrite~Treatment, data=nitrite.df)
>anova(fit.lm)
```

A glm model was also used for haematocrit analyses in E3, with binomial distribution. Due to the value being with decimals between 0-1, quasibinomial was used as error term.

```
>fit1.glm <- glm(haematocrit~Treatment, family=quasibinomial, data=
 hematocrit.df)
>anova(fit1.glm, test="F")
```

3. Results:

3.1. E1: Lethal effects after MC exposure of glochidia

Mortality of glochidia did not seem to be affected by the MC exposure of 20 µg/L (Table 5). Each group had seven wells containing ten glochidia each, and the total number for each exposure time was 70 glochidia. The highest mortality in the exposed group at 2 h was thus below 3%, and the three casualties occurred in separate wells. There was no mortality in either group at 24 h exposure.

Table 5: ***Lethal effects after 2-24 h MC exposure on glochidia.*** *Exposed group were exposed to 20 µg/L MC. Mortality was tested after 2-, 16- and 24-h exposure. Each exposure at each exposure time had seventy glochidia dispersed in 7 wells.*

	2 h	16 h	24 h
Control	1	1	0
Exposed	3	1	0

3.2. E2: Infection rate of glochidia after MC exposure

Although hosts were randomly selected from a same-age population, there was a slight difference in the length of the hosts between the groups (two-sample t-test: $t = -2.25$, $df = 36$, $p = 0.03$)(Fig. 5). The infection count differed significantly between the groups (GLM: $p = 0.006$), where the exposed group had fewer infections compared to the control.

The model that includes both size and infections showed differences in the infection trend between the two exposure groups (Fig. 6). The control group showed a typical increase of infections with increasing size of the host, but this effect was lessened when exposed to MC. Treatment significantly influenced infection trend (GLM: $p = 0.005$), but size also had an effect (GLM: $p = 0.007$). The results displayed a lower number of glochidia on the MC exposed group.

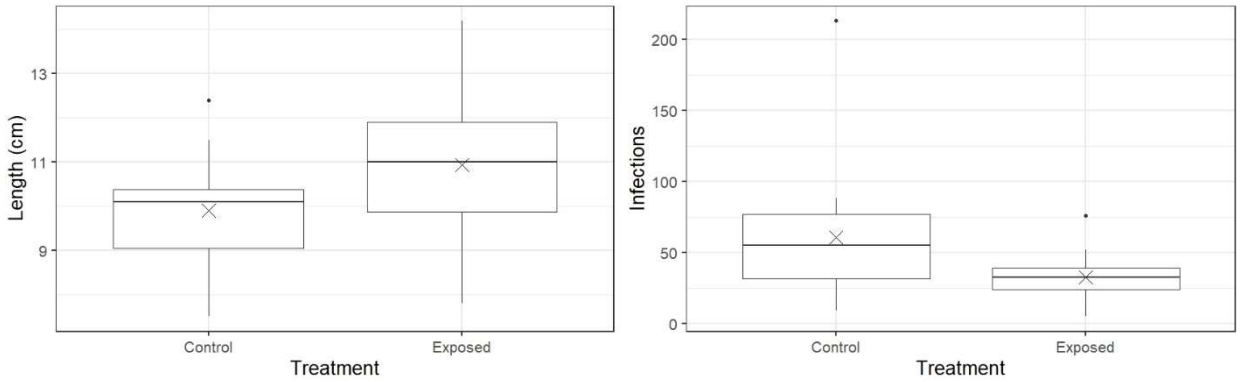

Figure 5: Host length (left) and infection number (right) after 2 h MC-LR exposure on glochidia in E2. *There is a significant difference in both host size (p=0.03) and infection number (p=0.005) between the two exposure groups. Infections refer to amount of glochidia attached to host gills. Mean of host length were 9.9 cm for control and 10.9 cm for exposed. Mean of infection number were 60.5 for control and 32.6 for exposed group.*

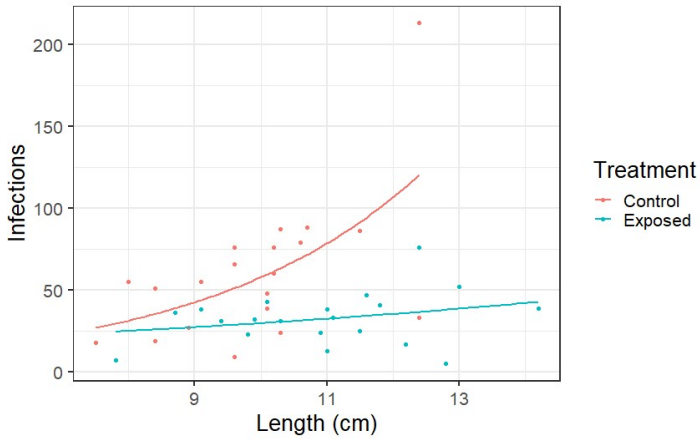

Figure 6: Infection rate in relation to host length after 2 h MC-LR exposure on glochidia in E2. *Infections refer to amount of glochidia attached to host gills. Both predictors had a significant effect on infection trend: treatment (p = 0.005) and length (p = 0.007).*

3.3. E3: Host-parasite effects: attachment success after host exposure

3.3.1. Background results: water quality

Ammonium, nitrite, nitrate and phosphate were all kept at a stable level until the cyanobacteria present in the waters was removed due to contamination of *Scenedesmus* and *Pseudoanabaena* sp 04.09.19 (Fig. 7). With no cyanobacteria in the water, the levels of ammonium, nitrite, nitrate and phosphate increased, and the levels of nitrogen increased the most. The highest level of nitrogen measured between before algal depletion was 0.82 mg/L, after it peaked at >30 mg/L. The mean before algal depletion was 0.44 mg/L for both of the systems, and the overall mean was 6.22 mg/L. Phosphate remained below 0.6 mg/L, reaching levels above 0.3 mg/L only after the loss of the algae. Mean of the two groups before algal depletion was 0.07 mg/L, and the overall mean was 0.17 mg/L.

Nitrite levels remained relatively stable with algal exposure and were not regulated by a large degree after water change. Without algae the levels peaked at 0.48 mg/L. The means of all the groups were between 0.172-0.188 mg/L, and the groups did not significantly differ from each

other (one-Way ANOVA, p=0.9). Out of the total ammonia nitrogen (TAN) in the water, the level of harmful undissociated ammonia, NH_3, varied depending on pH and temperature. Levels of NH_3 in TAN is around 1:3000 at pH 6 and 1:300 at pH 7 (Wetzel, 1983). pH levels and temperature were stable at about 6.6 and 16 C° in all groups throughout the experimental period. The highest levels of ammonium were at ~3 mg/L, and thus the highest levels of ammonia were likely below 0.003 mg/L.

Nitrite and ammonium levels were measured during exposure to MC extract (Fig. 8). Ammonium increased over time, and levels were reduced with each water change (02 Oct), whereas nitrogen remained relatively stable.

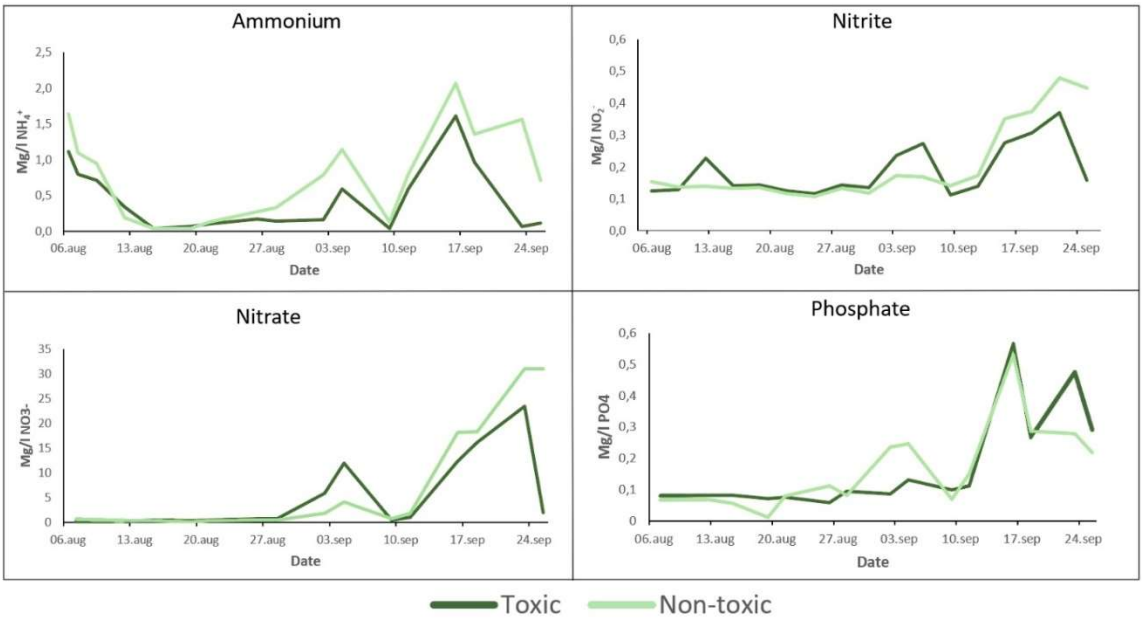

Figure 7: Levels of ammonium, nitrite, nitrate and phosphate in mg/L during cyanobacteria exposure in E3. *The non-toxic water supplied control and infected group, and toxic water supplied toxic and combined group. Ammonium and nitrite were measured from 06.08 – 25.09, nitrate and phosphate from 07.08 – 25.09.*

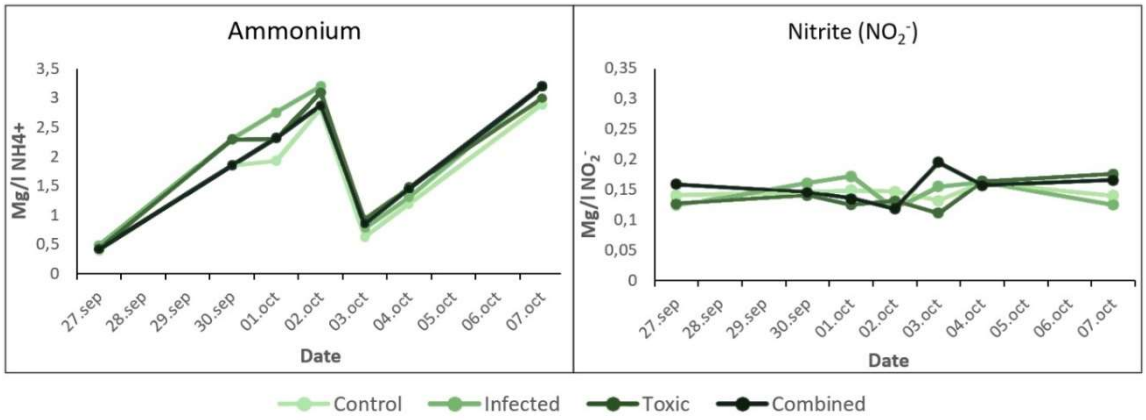

Figure 8: Levels of ammonium and nitrite in mg/L during MC-LR exposure in E3. *All four groups were in individual aquariums, where toxic and combined group are exposed to MC-LR. Water in all aquariums were changed 02.10.19.*

Samples from the period of cyanobacterial exposure showed that there were no high levels of MC (<5 µg/L) before being exposed to MC extract.

During the ten days of MC exposure, levels were above the desired level of 50 µg/L MC for several days (Fig. 9). The increase on 02.10 is from the change of water and new supply of MC. After adding of MC, the levels gradually decreased. From the measured values, the toxic group had an average of 50 µg/L MC and the combined group had 43 µg/L over ten days. For about 8 of those ten days, levels were above 40 µg/L. These results show that the two groups were successfully exposed to MC during their ten-day exposure. All analyses were done in three parallels, but not all parallels were successfully measured. Due to this, some dates lack error bars.

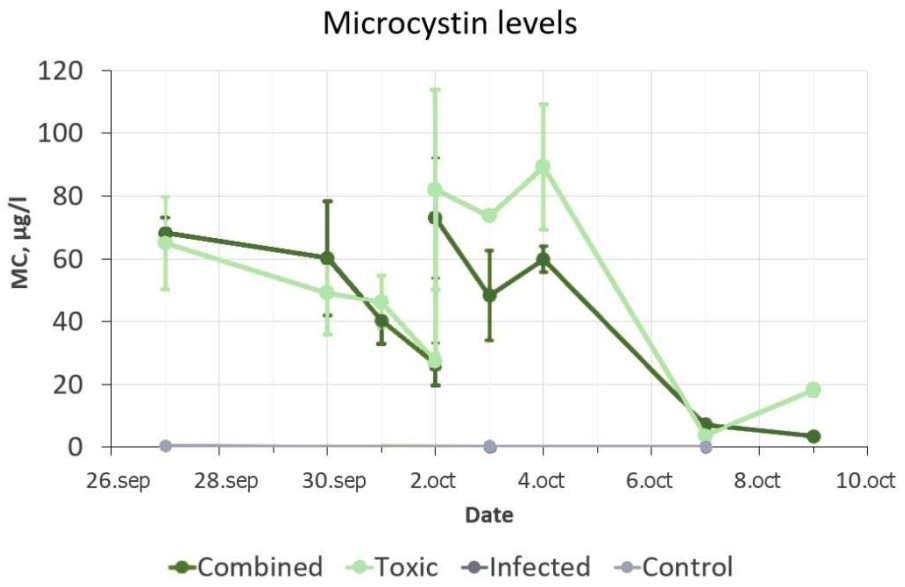

Figure 9: Concentration of MC-LR in the groups in E3 during ten-day exposure. *Control and infected group were not exposed to MC, and their samples did not contain MC. Toxic and combined group were both exposed to MC levels above 70 µg/L, and their concentrations were generally higher than 40. All samples have 3 parallels, providing error bars (1.96: standard error). Not all parallels were successfully measured (error bars missing).*

3.3.3. Haematocrit and blood smear from host exposure

The haematocrit values did not differ a lot between the exposure groups (GLM: P=0.8) (Fig. 10). The majority of all the groups had a haematocrit value of ~0.3, which is a regular and healthy value (Wells & Weber, 1991). Data from some individuals got lost due to broken samples or unsuccessful centrifugation, and this occurred in all groups. All the groups had n >30 (control: n=36, infected: n=31, toxic =34, combined = 33).

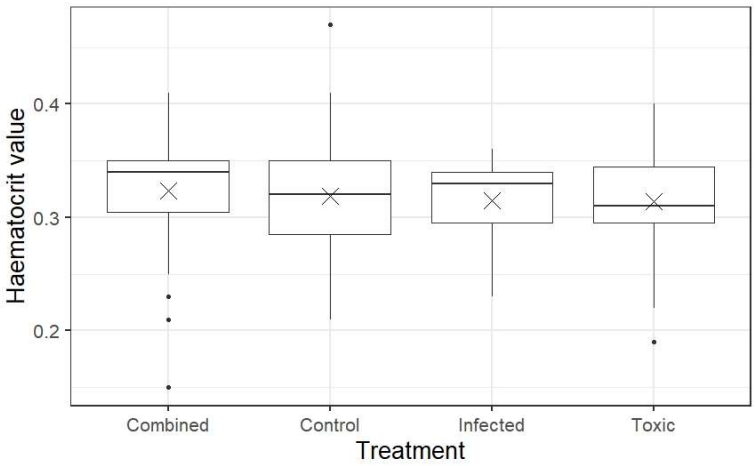

Figure 10: Haematocrit values in E3 after 55 days infection and 10 days MC-LR exposure. *All groups remain within the normal range of haematocrit values. The value of the groups did not differ between them (ANOVA: p=0.8). Mean of groups: Control: 0.32, Infected: 0.31, Toxic: 0.31, Combined: 0.33.*

Some of the blood smear samples were dyed and photographed, but the counting of the cells was not performed (Fig. 11). This was due to the uncertainty of unevenly smeared samples, damaged cells and the difficulty in separating erythrocyte from other cells. By visual observation, there were no apparent differences between the groups.

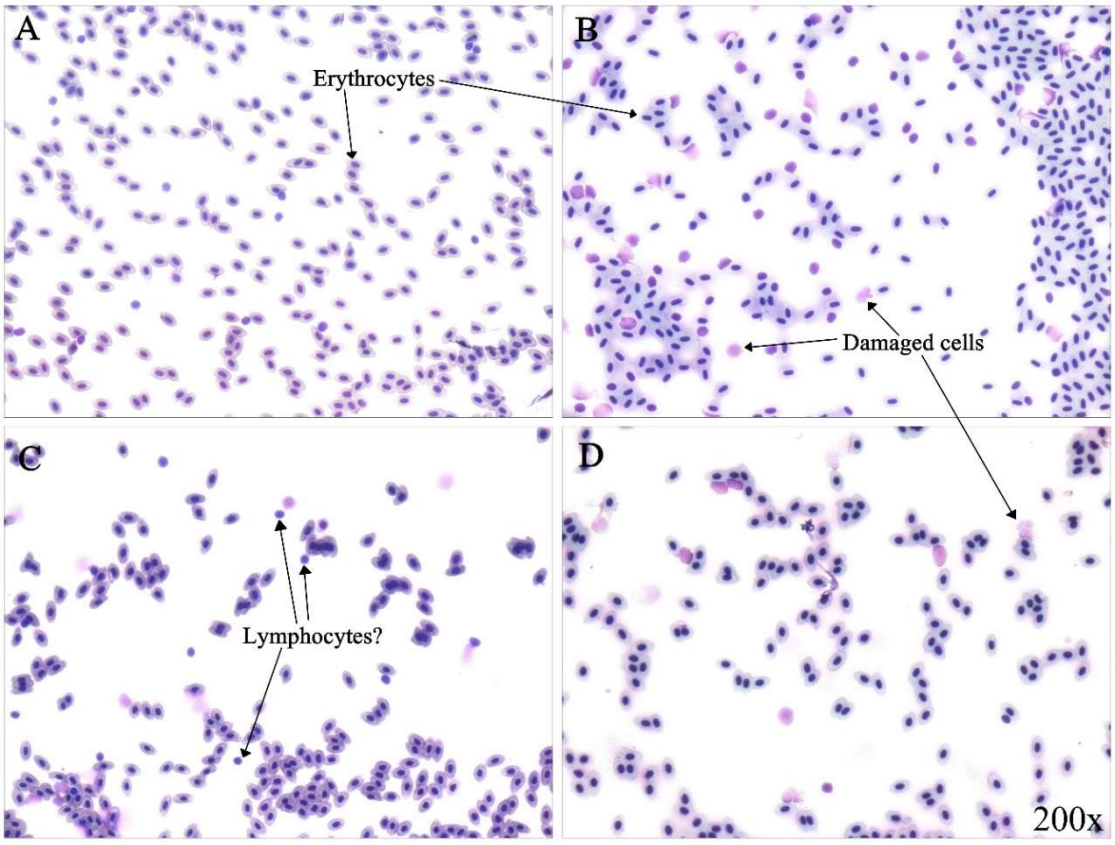

Figure 11: Example from blood smear slides from each group in E3. *A: Individual from control group, B: individual from infected group, C: individual from toxic group and D: individual from combined group. Erythrocytes and damaged cells are marked, lymphocytes were hard to distinguish from damaged cells.*

3.3.4. Glochidia infections after host exposure

There was no considerable differences in fork length of hosts between the groups (two-sample t-test: t-value = -0.22, df = 30 P= 0.8), while infections varied significantly between the treatment groups (GLM: P= 0.001) (Fig. 12). The combined group displayed fewer infections compared to the infected group.

The model including both size and infection also showed a difference in infection trend between the treatment groups (Fig. 13). The infected group, 55 days after infections, had a trend where larger fish had fewer gill infections. The combined group did not have a similar trend to the same extent. In this model, treatment significantly influenced infections (GLM, p = 0.001), but not length (GLM, p = 0.3). The data is based on the glochidial counts from the gill arches on one side, and several of the individuals had no infections to be counted. Empty cysts were observed in combined group.

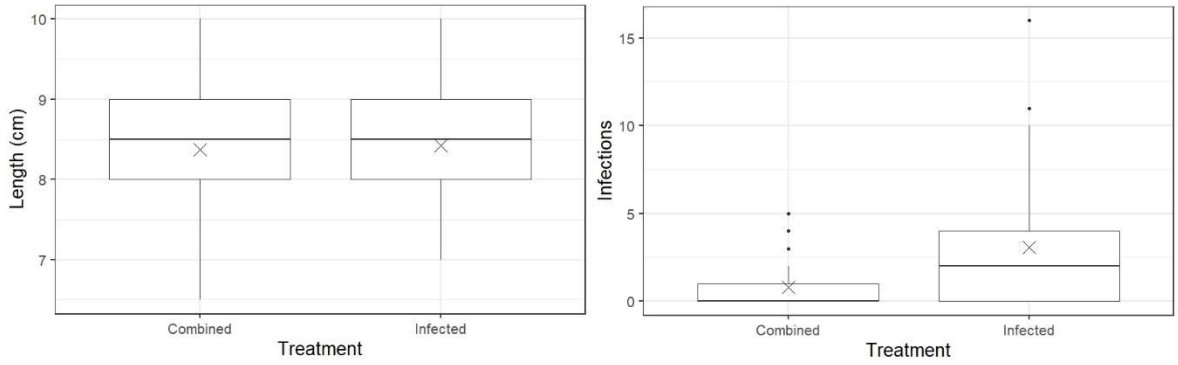

Figure 12: Length and infections of glochidia in E3 after ten-day MC exposure on host, and 55 days after infection. *The length of host fish does not differ (t-test, p=0.8), but infections did vary between treatments (ANOVA, p=0.001). Infections show the number of infections on the gill arches on the right side of the trout and not the total infection number on each host. Mean host length was 8.42 cm in control and 8.37 cm in combined group. Mean infection number was 3.1 for control and 0.8 for combined group.*

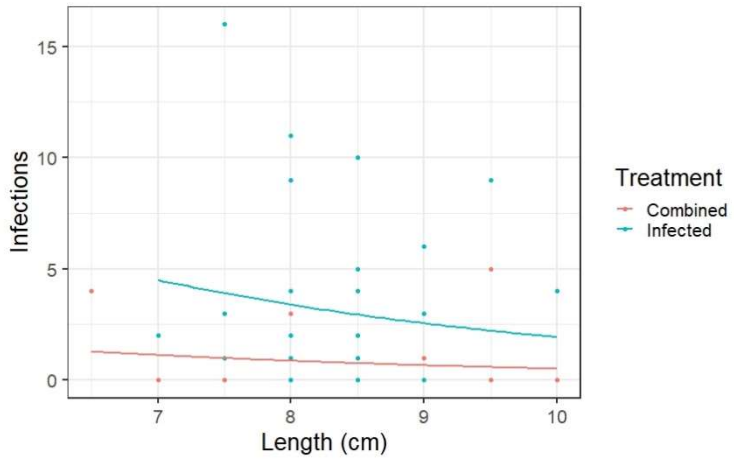

Figure 13: Infections rate of glochidia in relation to length after ten-day MC exposure on host, and 55 days after infection*. Infections show amount of infections on the gills on one side of the trout. Of the predictors, treatment had a significant effect (ANOVA, p=0.001) and length did not (ANOVA, p=0.3).*

4. Discussion

While no effects on lethality occurred on glochidia exposed to 20 µg/L MC for 24 h, there was negative effects on attachment ability of glochidia exposed to 100 µg/L for 2 h. A longer exposure of ten days on their host at ~40-70 µg/L MC also negatively affected the number of remaining glochidia on the gill. High MC-concentration thus seemed to decrease infection success after both glochidia and host exposure but did not affect the mortality rate at <20 µg/L.

The three experiments are discussed as separate and individual events due to the differences in MC concentration, populations and exposure time used. Due to limited access of MC, E1 and E2 could not be performed with a gradient of MC concentration. The MC extracts for these experiments were initially used for another ongoing experiment, and there were thus limitations of MC extract available. As the availability of MC was limited, we first tried to expose the hosts of E3 to MC by growing two different strains of *M. aeruginosa*, one with MC-production and one without. Had E3 had an exposure from *M. aeruginosa*, the concentration would fluctuate and be unpredictable, and could not be expected to match concentrations from previous experiments. However, the *M. aeruginosa* cultures did not grow properly and did not produce MC as expected. Hence, MC extract from NIVA was used, with a concentration high enough to provide the desired concentration above 50 µg/L.

4.1. E1: Mortality after glochidial exposure to MC

M. margaritifera is a mussel species bound to oligotrophic conditions and has been previously shown to be sensitive to eutrophication (Bauer, 1988; Geist & Kuehn, 2008). It was therefore expected that the glochidia of *M. margaritifera* would have a low tolerance to MC, a cyanotoxin following HABs and eutrophic conditions. The 24-h exposure groups showed no lethality at 20 µg/L MC, and no wells had more than one non-responding glochidia. A similar study performed by Gene et al. (2019) found that the glochidia larvae of the freshwater mussel *Lampsilis siliquoidea* did not see a significant decrease in mortality up to 72 h exposure of up to 100 µg/L MC-LR. They performed their study with *Microcystis aeruginosa* cells, testing a gradient where the lysed cells had MC-LR concentrations between 2.5 - 100 µg/L. A statistically insignificant decrease in viability was observed >25 µg/L after 48 h.

M. margaritifera did also tolerate MC-concentrations of at least 20 µg/L, but there is still a knowledge gap concerning this species and higher concentrations of MC.

The method used in E1 with response to outer stressors is a standard method for testing the survival of glochidia (Gene et al., 2019; Zale & Neves, 1982). Glochidia larvae are small organisms with a low metabolic rate that likely perform respiration, osmoregulation and nutrition activities over microvilli covering the larvae (Nezlin et al., 1994). The microvilli would thus likely be the route the MC in water would affect primarily. If the glochidia in E2 stopped responding to outer stressors, the glochidia was presumed dead. If this occurred following MC exposure, other possible reasons for lack of response could be defects in their sensory tufts or decreased ability to use their closing muscle (Nezlin et al., 1994). Still, the survival of glochidia is dependent on the ability to respond and attach to host gill (Jacobson et al., 1997). The mortality is nonetheless unaffected by a 24 h exposure to 20 µg/L MC. The non-responding glochidia in this experiment were not correlated to MC exposure, as no high numbers were observed and the longest exposure of 24 h had only responding glochidia.

4.2. E2: Sublethal effects (and viability) after glochidial exposure to MC

Exposing *M. margaritifera* to 100 µg/L MC showed a strong significant decrease in infections on the gills of the unexposed brown trout. Host fish were picked randomly. However, there was a slight but significant difference in the fork length between the groups. The length being larger in the exposed group was unfortunate, and was noticed too late to be corrected, but it does not discredit the findings on infections. According to previous studies, larger and older fish, naïve to glochidial infection, are expected to have a higher infection number (Hastie & Young, 2001; Marwaha et al., 2019). This trend is visible in the control group of E2 (Fig. 6). The exposed group, having generally larger hosts, does not show this trend to the same extent, and had fewer overall infections on the gills (Fig. 5). Although the groups were not equal from the start, the difference between them does not explain the difference in infection numbers. Thus, MC is considered to be a limiting effect on attachment success.

Glochidia are believed to release substances to trigger the host immune defence and accelerate encystment on the host gill, and a cyst formation is usually complete after ~12 h (Nezlin et al., 1994). Immune response in hosts is crucial for successful attachment because without encystment glochidia would fail to remain attached to the gill (Clements et al., 2018; Dodd et al., 2006; Marwaha et al., 2017; Nezlin et al., 1994). The brown trout of this experiment were

not exposed to MC, and they are expected to have a normal immune response in each group. It may be speculated that MC reduces the glochidial ability to get a proper immune response from their host. As the ability to attach to host gill is crucial for survival, the results of E2 would mean an MC exposure of 100 µg/L for 2 h decreases glochidia viability. It must also be noted that E1 was not performed at 100 µg/L, so effects in E2 could also be due to lethality or effects on sensory organ or closing muscle. If E1 was performed with a similar concentration, we could better determine whether the effect is due to lack of grasping the gill, or if it effects the glochidia's ability to form a cyst on the gill.

4.3. E3: Glochidial attachment after long-term host exposure to MC

The brown trout's in E3 were treated with formaldehyde a week and a half before being infected with glochidia. Before being separated into groups, the presence of the ectoparasite *Ichthyobodo necator* was observed, and a formaldehyde treatment was thus necessary. The presence of the *Ichthyobodo necator* parasite and formaldehyde could have adverse effects on the hosts. However, both parasite infection and formaldehyde treatment happened before separating the hosts in individual groups, and these factors should thus not differ between the groups. They also had a recovery period of 10 days before being infected with glochidia, hence it is unlikely it affected glochidial attachment.

Why the growth of the *M. aeruginosa* cultures was unsuccessful remains a mystery. During a pilot study, before the E3 experiment started, the cultures of *M. aeruginosa* did grow, and the toxic strain had an MC concentration of ~70 µg/L. During E3, there was no MC in the cultures. The *M. aeruginosa* cultures must have stopped growing at some point before the experiment started, and thus became more prone to contamination, or the growth could have been limited due to contamination. The contamination of algae, consisting of *Scenedesmus* and *Pseudoanabaena* sp appeared in both of the cultures. *Scenedesmus* had a similar green colour, and the contamination was not noticed until the culture turned slightly brown. *Scenedesmus* was cultivated only a few meters from the *M. aeruginosa* bags, and cells of *Scenedesmus* have found a way into the cultures despite sterilising all equipment. Transfer through air or pipette filter are among the theories as to why this happened. *Pseudoanabaena* sp has likely arrived in the cultures through incomplete water filtering, as the water first used in the cultures was only treated once. Of the two, *Scenedesmus* was the dominant species in the cultures. *Scenedesmus* is a rather harmless green algae, but the *Pseudoanabaena* cyanobacterias have reportedly produced MC (Oudra et al., 2002). However, in the samples

there were no high levels of MC reported (<5 µg/L).
Trying once again with new *M. aeruginosa* cultures, the temperature was stable at ~20 C° to maximise growth, and the water used was boiled in advance. There was no growth in the cultures for two weeks, and then it turned green. A sample of the culture in a microscope showed that the cells in the cultures were, once again, *Scenedesmus*. Around the same time, the synchronised cultures at NIVA also had poor production of MC. It may be speculated that the poor growth and MC production had to do with the biorhythm of the *M. aeruginosa*.

The mussels spat their glochidia larvae once a year in a limited time period, so the experiment could not be replicated the same year with *M. aeruginosa* cells once the cultures were unsuccessful. The use of MC extract was thus the only way to complete the experiment. The limitations of MC made it necessary to use a smaller volume of water, and the groups were moved to 60 L aquariums. Due to the low water volume, the fish had to be starved to avoid high amounts of waste products.

If we could have used *M. aeruginosa* cells whilst feeding, that would have been preferable, considering the main uptake of MC is through the gastrointestinal tract (Tencalla, Dietrich, & Schlatter, 1994). The MC analyses showed high amounts of MC present during the ten-day exposure, but uptake from immersion without feeding would be through drinking and absorption over gills and body surface (Cazenave et al., 2005). If the samples from host organs were analysed, the impact of MC exposure on the host might be less of a mystery. However, there is a significantly lower infection rate for the hosts exposed to MC, and the only significant difference accounted for was the MC exposure.

4.3.1. Water quality

The results of ammonium, nitrite, nitrate and phosphorous show a similar development among the groups (Fig. 7 & 8), with a clear indication that algae and cyanobacteria had an impact on the stability of presence of the compounds. The measurements were done to have a rough estimate of the presence of nutritious and harmful compounds. However, some inaccuracy must be expected as measuring reagents and buffer manually, with spoons and drops, will give some inequality between the reactions of each sample.

Ammonia levels were determined by considering ammonium, pH and temperature, giving a low value of <0.003 mg/L. This is far below the maximum value of 0.1 mg/L, but since the ammonium levels increased after water exchange, the levels were kept under the maximum

range of the Winlab photometer at 3 mg/L. Nitrite can be harmful at low levels and can become toxic for salmonids above 0.3 mg/L (Wedemeyer, 1996). The levels measured were higher than 0.3 mg/L the days before starting MC exposure, peaking at 0.48 mg/L (Fig. 7). The levels of nitrite did not seem especially affected by water exchange, and the results showed there were no significant difference in nitrite levels between the exposure groups.

Maximum measurement of nitrate was >30 mg/L, with a mean of 0.44mg/L before algal depletion and an overall mean of 6.22 mg/L. Nitrate increased when algae disappeared, but is acceptable in amount up to 100 µg/L (Noble et al., 2018). For phosphate, the maximum level was 0.56 mg/L with a mean of 0.07 mg/L before the algal depletion and an overall mean of 0.17 mg/L. Phosphate are known to promote growth of potentially harmful organisms such as cyanobacteria in eutrophic conditions (Correll, 1998). Growth of cyanobacteria being the goal, eutrophic levels of ~0.1 mg/L phosphorous was achieved (Prepas & Charette, 2003).

4.3.2. Microcystin analysis and exposure

The highest MC concentrations measured were between 60-95 µg/L, even though the maximum levels expected were ~50 µg/L. Some uncertainties are expected with the ELISA-kit used. As explained by Metcalf et al. (2000), small differences can occur if the equipment used consists of plastic, since MC can be absorbed by the plastic. Contact with plastic pipettes occurred only briefly during pipetting, and this effect is expected to be minimal. The kit is usually used to monitor levels in drinking water, and thus operates with a maximum range of 5 µg/L. The samples tested from the toxic groups thus had to be diluted 20 times, and this is likely the main source of variation in the measurements.

Variable measurements did occur, as the levels of MC show an increase three days after water change (Fig. 9) and the extract provided by NIVA seemed to have a higher amount of MC than expected. The concentration for the MC extract from NIVA was measured with the same technique, and from the results, the concentration may have been a little higher than 7200 µg/L. The higher concentration of the extract improved the exposure of the experiment, considering the levels of MC decreased over time, probably due to the bubble circulation in the aquariums. The increase in concentrations in toxic and combined group three days after adding MC is also likely to be a result of measurement errors, but the measurements are within the confidence limits from the first day after adding MC (02.10). The measurements do either way prove there was a significant exposure of >50 µg/L, varying over time and clearly

increasing after change of water and MC. As the concentration of MC decreased over time and the analysis has low precision, the assumed concentration of the ten-day exposure was between 40-70 µg/L.

4.3.3. Missing results

The samples of gill, liver and kidney were ready for analysis in October 2019. Due to unforeseen circumstances, the analysis was delayed, with hopes of being analysed in March 2020. Unfortunately, after the COVID-19 lockdown, it was decided not to include these samples in the thesis.

Brown trout are considered to be more sensitive to MCs compared to several other species of fish, especially those bound to eutrophic habitats (Bury et al., 2003; Malbrouck & Kestemont, 2006; Råbergh et al., 1991; Snyder et al., 2002). There have been reports of brown trout mortality following HABs, and Rodger et al. (1994) investigated liver, kidney and gills of deceased brown trout following a HAB in Loch Leven, Scotland. Liver histology revealed necrosis and loss of cell structures in the hepatocytes. Oedema and inflammatory regions were also observed, and they linked their findings to typical MC-exposure. The kidney had enlarged capillaries, and the gills had signs of irritation. However, the length of exposure was probably no longer than three days as decomposing of algae scum occurred 2-3 days before. MCs was reported in both water and algae scum of Loch Leven, but the exact concentrations the brown trout had been exposed to is unknown. It is also uncertain if other compounds are the cause of effects on gill and kidney.

Brown trout have been shown to be affected by the toxin when exposed by injections, forced feeding and immersion. The most commonly reported symptom is alterations to the liver. Direct effects like loss of cell structure, necrosis of hepatocytes and inflammation, and indirect effects like increased liver enzymes in the blood have been reported (Bury et al., 2003; Tencalla et al., 1994). In contrast to Rogder et al. (1994), injection studies have reported signs of kidney impairment on crucian carp (*Carassius auratus*) at 50 µg/L MC-LR (Zhang et al., 2007). A study by Kotak et al. (1996) found that rainbow trout, when injected with 1000 µg/kg MC-LR, had severe kidney damage in the form of necrosis and swelling in tubules, whereas the liver was less affected. The different target organs could be due to the route of exposure or differences in the species. In naturally occurring scenarios, the main route of exposure is through the gastrointestinal tract, and the liver becomes the main target

(Tencalla et al., 1994). In carp (*Cyprinus Carpio*), gill histology showed a loss of structure, mucus and necrosis of epithelial cells (Carbis et al., 1996; Jiang et al., 2011). Jiang et al. (2011) used a low MC exposure of 10 µg/L for 14 days, and changes in gill cells as well as an increased distance of gill lamellae. This effect could have been similar in our study, as it was performed with a higher concentration for ten days.
In this experiment, glochidial infections would also possibly show alterations in the gill tissue. Previous histology studies describe the capsule around the glochidia, and the glochidia have been observed to "grasp" the lamella capillary, reducing the lamellae tissue and its function for respiration (Karna & Millemann, 1978).

Immersion is usually the route of exposure with the least adverse effects, but the concentration should affect the hosts nonetheless. A study with 63-day exposure to 41-68 µg/L MC-LR showed reduced growth in brown trout (Bury et al., 1995). Another immersion study of 50 µg/L MC-RR on three species of fish (*Corydoras paleatus*, *Jenynsia multidentata* and *Odontesthes bonariensis*) for 24 h showed accumulation in liver, gills and muscles of all the species (Cazenave et al., 2005). It is thus fair to assume that the exposure of 40-70 µg/L MC in E3 affects the brown trout, and MC have been taken up and been distributed to liver and gills. It is also fair to assume, due to the exposure to >50 µg/L MC, there would be altered structures on both gill tissue and hepatocytes. Documentations on structure effects on kidneys is rather variable, so no assumptions are made on this matter. The missing data would have provided results that could be compared with previous experiments in order to verify the toxic effect of MC on the fish in this experiment.

4.3.4. Haematocrit and blood smears

Haematocrit values did not differ between the groups and did not show unusual values. Thus, either infection with glochidia or exposure to MC seem to have affected the brown trout. Regular values of haematocrit are ~0.3 (Wells & Weber, 1991), and the groups all had mean values between 0.31-0.33.
Heavy infections of glochidia have previously increased haematocrit values due to respiratory stress (Marwaha et al., 2019), but infection with glochidia in this experiment were unlikely high enough to cause respiratory stress. MC is also known to affect the haematocrit value. Decreased values have been documented in crucian carp (*Carassius auratus*) after injection of 50 µg/kg body weight MC-LR, and in the African mud catfish (*Clarias gariepinus*) after a 14-

day exposure to 200 µg/L (Isibor, 2017; Zhang et al., 2007). These studies used a higher exposure to MC, as immersion is a less direct way of exposure compared to injection. In this experiment, haematocrit values were not affected, which could be due to a lower exposure or the fact that there were different test species. Brown trout is, however, known to be less tolerant to MC than other species of fish, such as carp (Bury et al., 2003; Malbrouck & Kestemont, 2006). Another aspect to consider is that Zhang et al. (2007) found normal haematocrit values seven days after ending the exposure. The MC levels in E3 were relatively low at the time of sample collection, but they did not have several days without a significant concentration. Some recovery could have occurred, but the concentrations of MC were ~50 µg/L only 2-3 days before collecting samples, thus complete recovery is unlikely. There was some loss of samples due to unsuccessful centrifugation and broken tubes, and difficulties with collecting blood resulted in tubes with small volumes in them. The difficulties of collecting should not have a large impact on the results. The concentration of 40-70 µg/L MC could not be proved to affect the haematocrit value of brown trout.

The blood smears were dyed and photographed, but the smearing technique itself requires practice and precision. The samples were not performed by a practised hand and counting of cells from blood smears is a time-consuming method that is rarely used. Other methods are usually used when determining differences is red and white blood cells, e.g. by using a hemocytometer (Zhang et al., 2007). There was a limitation of host blood, and due to the tail cut, several samples had traces of mucus and dermal cells in them. There were also some damaged cells in the smears, and sometimes these could resemble a typical lymphocyte (Fig. 11). Erythrocytes were the only type of cells that were easy to identify, and the cells were unevenly dispersed across the slide. Some areas had many cells that could appear to be leukocytes, some had none, and some areas had a lot of scattered cells. The blood smears were for these reasons not counted, and no conclusive results were presented.

4.3.6. Glochidia attachment when only host was exposed

An effect of MC was expected, but rather than a decrease in infections in E3, a likely scenario would be more infections on the gills of the brown trout. This scenario appeared likely as MC is known to decrease immune responses in fish (Lin et al., 2017; Rymuszka et al., 2007). After 55 days, the humoral response of the host fish would be enabled, resulting in sloughing of glochidia (Bauer & Vogel, 1987; Meyers et al., 1980). With a suppressed immune system,

more infections could remain on the gill. However, this is not what happened. The decreased number of infections could have been due to other factors, such as MC present in the host gill or blood. Once encapsulated, glochidia are considered relatively protected from external environmental factors, as it does not get affected by substances such as copper, iron and aluminium (Jacobson et al., 1997; Taskinen et al., 2011). This protection is somewhat discussed, as some authors have reported a loss of infections after exposure to other compounds, such as chloride (Beggel & Geist, 2015). Why some harmful compounds affect glochidia, and some do not, could be due to a change in factors inside the gill. The capsule is covering the outer parts of the glochidia, whereas inside the gill lamellae, the glochidia are in contact with the lamellae capillary (Karna & Millemann, 1978). When exposed to MC, the toxin has been found in both blood and gill tissue of fish (Cazenave et al., 2005), and this could be a route of which glochidia gets directly exposed.

There could also be indirect factors involved, such as changes within the host following MC exposure. The host specificity of free glochidia of *M. margaritifera* is mainly due to their dependence on the specific host immune response (Clements et al., 2018; Marwaha et al., 2017). The dependence on the immune response would have been a considered response if the experiment went according to plan, and host were exposed to MC before and after infection. Moreover, the dependence of immune responses might be of some importance later in the infection period as well.

Intended exposure was between one week before infection and the following 40 days. The actual exposure was 44 – 55 days after the infection. By this time, the glochidia would already be encapsuled, and some glochidia would have been shed off due to the humoral immune response (Bauer & Vogel, 1987; Nezlin et al., 1994). There were relatively few infections on the gill of both infected and combined groups. The glochidial infection might have been low, and there would have already been some loss of attached glochidia due to the immune response. The results were from only one of the host gills, as the other side was used for histology samples. Still, there were significantly fewer infections on the gills of the exposed hosts. Empty cysts were observed in combined group, which indicate recent shedding of glochidia. It is uncertain if this effect is due to direct toxic effects, as the glochidia could have been exposed directly to MC inside the gill, or if it is a secondary effect due to toxic effects on the brown trout.

4.4. Study design of E1, E2 and E3

Using a gradient of concentrations of, e.g. 20, 50 and 100 µg/L MC, in E2 and E3 would provide better knowledge at what levels of MC adverse effects occur, if they occur at all in E1. Monitoring the glochidia of E1 for up to 48 h could also better describe their survival under bloom conditions.

E2 and E3 were performed with only one aquarium each, and the data from each group thus arrive from the same aquarium and bottle of glochidia. Having more aquaria would provide more precise estimates and improve the randomisation and the independence of the data, but this is not always practically feasible. For both issues concerning using a gradient and having several replicates, the availability of MC extract would have to be sufficient. The workload is also a limiting factor, and due to this, the sample group of 30 in E2 was reduced to 20. The two water systems used from the beginning of E3 required a lot of space and maintenance. To accomplish the use of more replicates, one would need more people and more space than what was available at the time.

However, with only one aquarium per treatment, there is an uncertainty regarding pseudoreplication and eventual effects in aquaria or glochidia bottles. In E2 the host were in the aquaria for 48 h, and eventual differences would be in the glochidia bottle. In E3 it could be differences in both glochidia bottle and aquarium effects. At best ability, any differences between groups were avoided. The glochidial soup was shaken to even out differences, and the density and amount of glochidia added was carefully measured, with no reasons for large differences to occur. In E3, feeding, water quality and treatments were performed identically in the groups. The only difference accounted for was the MC exposure, and the results of both E2 and E3 strongly indicate a negative effect of MC exposure.

4.5. Impact and future perspectives

Cyanobacteria have gas vacuoles and tend to migrate towards the surface water (Reynolds et al., 1987). During HAB season in lakes, MC levels in surface water have been recorded up to 29 000 µg/L (Nasri et al., 2004). Although this represents an extreme incident, the levels of MC in the surface water is known to reach high levels during blooms. The water running down from the lakes to the river or streams, is surface water, indicating a potential of high concentrations of MC in river habitats. MC from lakes and rivers have even travelled to marine ecosystems, as marine mussels have contained MC levels harmful for the surrounding

habitat (Miller et al., 2010; Peacock et al., 2018). It is thus not unlikely that harmful MC levels can occur near *M. margaritifera* populations. All concentrations used therefore represent realistic levels that can interact with the freshwater pearl mussels during their life stages.

The results strongly indicate the infection rate and success of the early life stages of *M. margaritifera* decrease in the presence of high levels of MC, and HABs in lakes connected to rivers could therefore affect the survival of a struggling species at their most vulnerable life stages. Eutrophication of the habitats of *M. margaritifera* is already known to be harmful, and these results further emphasize the harm of eutrophication of waters connected to oligotrophic rivers.

MC can affect the free-living glochidia itself in higher concentrations, but after infection and cyst formation, external factors should not affect the glochidia as easily. Whether or not the lower infections are caused due to alterations in the immune response of the brown trout or direct toxic effects of MC itself is still unknown. Similar experiments should be performed to say more about the effect and mechanism of toxicity, and it would be preferable using several concentrations of MC. Nonetheless, these experiments highlight a problem not previously studied on *M. margaritifera*, as both exposure of glochidia larvae and host showed adverse effects for the parasite at exposure 40-100 µg/L.

There are other aspects of the lifecycle of *M. margaritifera* to investigate concerning MC exposure. It would be interesting to see the outcomes of longer exposures during host-stage and development of juveniles feeding on toxic cyanobacteria. Gene et al. (2019) found that juveniles of *Lampsilis siliquoidea* had an LC50 value of 2.1 µg/L MC-LR during a 28-day exposure. There are no juvenile studies performed on *M. margaritifera*, and the other life stages of the mussel. Adult mussels of *M. margaritifera* can filter up to 50 L water a day and are thus easily exposed if there are cyanobacteria in the water (Larsen, 1997). Studies examining the effects of this, and potential effects on the glochidia released by exposed mussel would also be a new aspect to examine concerning MC exposure. The effects of HABs following eutrophication on oligotrophic species such as the freshwater pearl mussel is not thoroughly studied, and more knowledge is needed on this problem.

5. Conclusion

Considering that the experimental groups were treated identically and the results strongly point towards a negative effect on infections in both E2 and E3, the overall indication is that MC can negatively affect the early life stages of *M. margaritifera*. Adverse effects on attachment ability occurred when glochidia larvae were exposed to 100 µg/L, and no increased mortality occurred when glochidia were exposed to 20 µg/L. When hosts with established infections on their gills had a ten-day exposure to 40-70 µg/L MC, there was also a negative effect of MC exposure on the attachment of glochidia. Haematocrit samples did not differ between host groups.

The conclusion is therefore that higher concentrations between 40-100 µg/L MC negatively affect infection and the attachment of glochidia, and thus rejects the null hypothesis. With a rather low concentration of 20 µg/L MC, the survival of glochidia in E1 was not affected, and the null hypothesis is thus not rejected. The HABs that derives from further eutrophication of freshwater lakes and rivers can pose a threat to *M. margaritifera* populations, and this is something to consider and further investigate for the future preservation of the species.

If repeated with more resources, i.e. using more replicates and a concentration gradient to conclude at which concentrations *M. margaritifera* are negatively affected, the data would have greatly improved.

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