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Master Thesis

When hungry meets harmful: investigating the toxic relationship between haptophytes and copepods

Predatory induced defenses in *Chrysochromulina leadbeateri*

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Marine Biology and Limnology

60 credits

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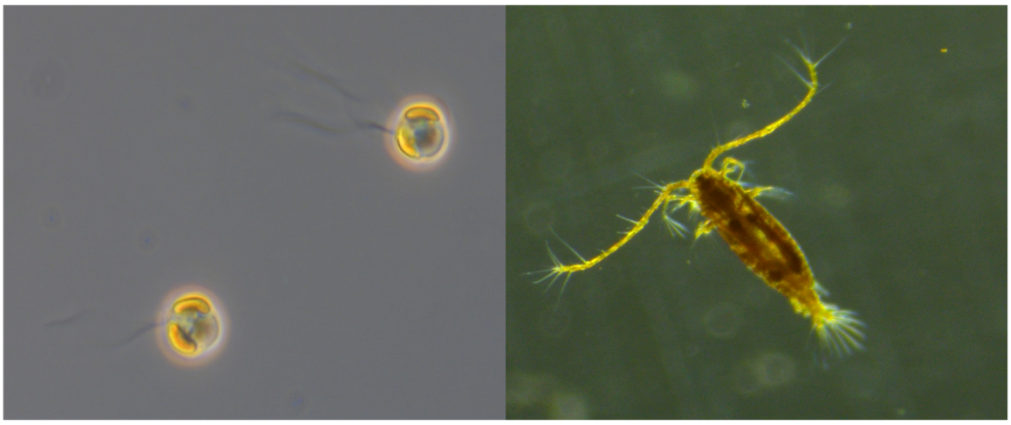

Abstract

Harmful algal blooms are a recurring phenomenon all around the world, causing devastating effects including massive fish kills. A bloom of the toxic haptophyte, *Chrysochromulina leadbeateri*, in Northern Norway during May-June 2019 was the largest bloom ever recorded in that area causing massive mortalities of farmed salmon. While past *C. leadbeateri* blooms are known to cause such damage, relatively little is known about the species, compared to other toxic haptophytes. Some studies have investigated the bloom dynamics and abiotic factors influencing its toxicity, but little is known about its biotic influences. Especially little is known about the predator-prey interactions with copepods. Past studies have found that when other species of algae are directly exposed to predation threats (e.g. herbivorous copepods), they will respond by inducing and/or increasing toxicity potential as a defense mechanism. Earlier studies have focused on toxic diatoms and dinoflagellates producing shellfish toxins, but the potential for fish-killing algae to be influenced by predation pressure remains to be explored.

In this thesis, I therefore examined the biotic interactions between the toxic *C. leadbeateri* and the grazing *Acartia* sp. copepods. I first conducted a series of 3-day tolerance experiments, exposing *Acartia* sp. to different *C. leadbeateri* concentrations to answer: What is the threshold *C. leadbeateri* concentration at which *Acartia* sp. can maintain survival and grazing? Then I conducted 3-day induction experiments, exposing *C. leadbeateri* to varying levels of grazing pressure, and *in vitro* tests using two fish cell lines to measure this potential grazing-induced toxicity to answer: Does the presence of copepod grazers affect toxicity in the ichthyotoxic haptophyte *C. leadbeateri*?

Through the initial tolerance experiments, I determined that ca. 5×10^4 cells ml^{-1} of *C. leadbeateri* was the threshold concentration at which enough *Acartia* sp. could survive and maintain feeding for the later toxicity induction experiments. *Acartia* sp. short-term tolerance to direct exposure to *C. leadbeateri* appeared to be concentration and time-dependent since the toxins need to be taken up by *Acartia* sp. to cause mortalities. Copepod grazing behavior observed during the induction experiment appeared unaffected when *C. leadbeateri* was the only food source, as evidenced by observed fecal pellet production. To accurately determine the effects of grazing on *C. leadbeateri*, future studies should observe grazing periodically with *Acartia* sp. exposed to different *C. leadbeateri* concentrations and non-toxic food alternatives.

I also examined whether the toxicity of *C. leadbeateri* was influenced by the presence of grazing copepods using *in vitro* tests on two fish (Rainbow trout and Atlantic salmon) cell lines to measure cell viability and light microscopy to look at morphological changes. I created different concentrations of algal extracts from crude *C. leadbeateri* material exposed to varying grazing levels. The different extracts were exposed to the gill cells in a microplate-based assay for 24 hours and at the endpoint, cell viability was measured using Alamar blue as an indicator dye. It seems that while increasing the algal extract concentration decreases cell viability, extract made from *C. leadbeateri* exposed to increasing grazing pressure does not increase the negative effects on cell viability. The light micrographs of the gill cells suggest the same conclusion, but with some inclination that grazing pressure does affect some toxicity potential

in *C. leadbeateri*. Unfortunately, due to the lack of more independent trials, there are no statistical analyses to back up these claims. More experiments would allow for proper analyses and a potentially more conclusive statement about the relationship between copepod grazing and the toxicity potential of *C. leadbeateri*. This thesis can be used as a starting point for future research on the dynamics between toxic haptophytes and their copepod grazers.

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1 Introduction

1.1 Harmful algal blooms in Northern Europe

Harmful algal blooms (HABs) are recurrent phenomena in northern Europe, particularly in the Kattegat-Skagerrak, North Sea, Norwegian Sea, Barents Sea, and along the Baltic Sea coasts (Karlson et al., 2021). These events have severe effects on tourism, recreation, human health, aquaculture, and fisheries (Berdalet et al., 2016). For event reporting and monitoring practices, HABs and their consequences are subdivided into two categories (Harmful Algae Event Database, HAEDAT, http://haedat.iode.org, 2021): 1) high algal cells density blooms covering large areas that cause fish and other marine fauna mortalities and/or damage to ecosystem function and 2) blooms even at low cell densities causing seafood contamination by phycotoxin accumulation, especially in shellfish. These two categories of HABs are functionally different from each other with the diversity of the HAB events paralleling that of the algal species responsible. The intensity and frequency of these blooms vary at the regional and local scale both with increasing or decreasing trends, and seemingly sporadic events, but with no general uniform trend (Hallegraeff et al., 2021). Specifically major fish-killing events in northern Europe are sporadic and oftentimes unpredictable, and the economic impacts affect the expansion of fish aquaculture activities along the Atlantic margin of Europe, including Norway (Bresnan et al., 2021).

These fish-killing blooms cause fish mortalities and morbidities, including acute gill irritation and damage to the fish gills (karyorrhexis and epithelial loosening), reduction of gas exchange efficiency and osmoregulatory failure (Andersen et al., 2015; MacKenzie et al., 2011; Tang & Au, 2004). Most of the fish-killing algal blooms in Scandinavia, including Norway, have been directly linked to harmful blooms of marine haptophytes, specifically members belonging to the genera *Prymnesium* and *Chrysochromulina*. The first recorded major HAB in Scandinavia linked to haptophytes occurred in May-June 1988, in the Kattegat-Skagerrak area and the eastward extension of the North Sea (Dahl, et al., 1989; Edvardsen & Paasche, 1998; Skjoldal & Dundas, 1991). It was caused by *Prymnesium polylepis* (formerly *Chrysochromulina polylepis*) and the

massive bloom, spanning approximately 75 km^2, caused major ecosystem disruptions, including mortalities observed in benthic communities and fish communities. This massive haptophyte HAB killed both wild and farmed fish populations (Gjøsæter et al., 2000).

The Norwegian fish farm industry, particularly with salmon, has periodically suffered coinciding with the massive fish-killing algal blooms over the past decades. From late July to early August 1989, a massive bloom of *Prymnesium parvum* caused 750 metric tons of caged Atlantic salmon (*Salmon salar*) and Rainbow trout (*Oncorhynchus mykiss*) mortalities from the nearby fish farms (Johnsen and Lein, 1989; Kaartvedt et al., 1991). From 1989 to 1995, there were *P. parvum* bloom annually in Ryfylke in western Norway, with mortalities of caged salmon reported in 1990, 1991, and 1995. There were no more *P. parvum* blooms and subsequent fish kills reported when the fish farmers moved out of the area in 1995, but when fish farming was reintroduced in 2005, *P. parvum* blooms reoccurred, killing 135 metric tons of caged salmon (Johnsen et al., 2010). The first recorded fish-killing HAB caused by *Chrysochromulina leadbeateri* was in late May 1991 in the Lofoten archipelago and Vestfjorden in Northern Norway (Rey, 1991). Losses from that HAB event then was 742 metric tons of farmed salmon mortalities and an estimated value of 3.5 million US dollars (USD). A smaller bloom of *C. leadbeateri* caused fish mortalities in the same area in 2008. Later in early May-June of 2019, the same species caused the largest fish kills caused by a HAB ever recorded in Norway and at least in economic terms, the largest in Northern Europe. Fish kills from the HAB were first reported in Astafjorden in Troms and Ofotfjorden in Nordland and further north to Vestfjorden and Troms (*Figure 1*). Fish death was relatively sudden with frequently observed gill damage. The losses were extensive with approximately 7-8 million farmed salmon mortalities (14,500 tons) (Samdal & Edvardsen, 2020).

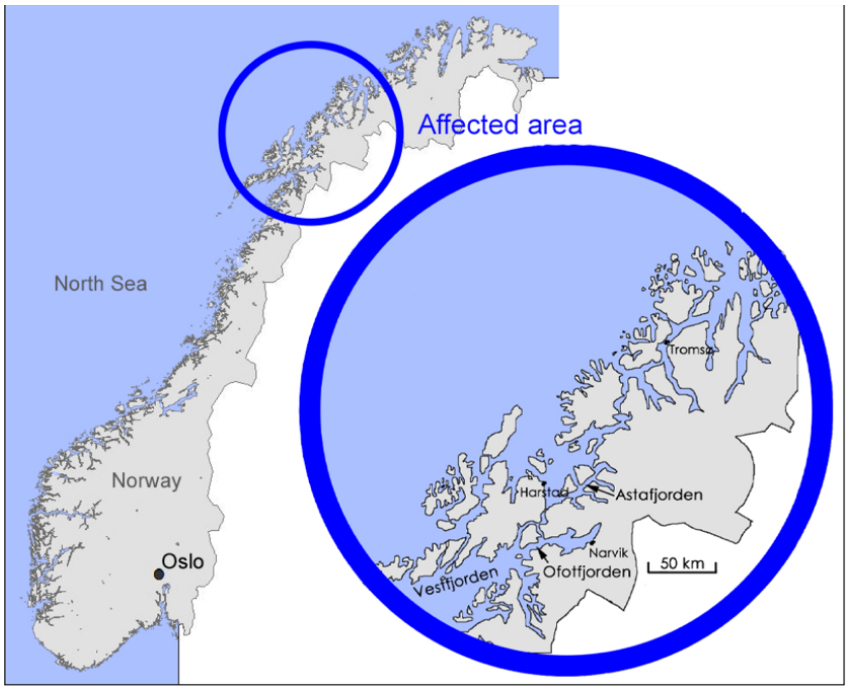

Figure 1. Map of Norway showing where the Chrysochromulina leadbeateri *bloom in May-June 2019 occurred. Figure was taken from (Samdal and Edvardsen, 2020).*

Direct and indirect gross economic losses from this bloom are estimated to be approximately 300 million USD (Marthinussen et al., 2020). In terms of contribution to economic growth in the Norwegian gross national product (GNP), the aquaculture and fisheries industries are some of the most successful and important with an average GNP increase of 19% since 1970, and so these massive-fish kills from HABs cause some considerable damage (Johansen et al., 2019). Given the magnitude of damage *C. leadbeateri* blooms have caused, not much is known about this species, specifically why this species bloomed again and how it became so toxic, compared to other ichthyotoxic haptophytes like *P. parvum* and *P. polylepis*.

Figure 2. The May-June 2019 Chrysochromulina leadbeateri *bloom causing a massive farmed fish kill. Photo showing crates of dead salmon being transported. Photo: Northern Lights Salmon.*

1.2 The background of *Chrysochromulina leadbeateri*

In Scandinavian waters, haptophyte blooms are common in fjords, estuaries, and coastal areas, such as Skagerrak. Some of these haptophytes are members of the order Prymnesiales and are known to produce high biomass blooms (>1 x 10^6 cells L^{-1}), during warm and sunny conditions in stratified water even when there is low inorganic nutrient content (nitrogen, phosphate) in the upper mixed layer (Lekve et al., 2006). The highest cell concentration of *Chrysochromulina leadbeateri* during the May-June 2019 bloom was 27.6 x 10^7 cells L^{-1}(John et al., 2022). It was also found that higher temperatures and dissolved inorganic nutrients (N,P) were positively correlated with *C. leadbeateri* cell abundance while salinity was negatively correlated with abundance. (John et al., 2022). This agrees with hypotheses from past studies of *Prymnesium* and *Chrysochromulina* species that high N:P nutrient ratios and low salinities favors haptophyte blooms. These studies concerning the key abiotic ecological factors favoring haptophyte blooms also hypothesized reduced vertical mixing and a high solar irradiance to be key factors (Edvardsen & Paasche, 1998; Lekve et al., 2006). There are also indications that

Chrysochromulina spp. not only thrive in high N:P ratios during low phosphate concentrations, but it could potentially promote toxicity if the condition becomes phosphate-limited (Dahl et al., 2005; Edvardsen & Imai, 2006). In the 1991 *C. leadbeateri* bloom, it was hypothesized that nutrient loading in the Ofotfjord area was enhanced and with the release of polyamines during the decay of dead fish could have enhanced the growth of the mixotrophic *C. leadbeateri* (Johnsen et al., 1999).

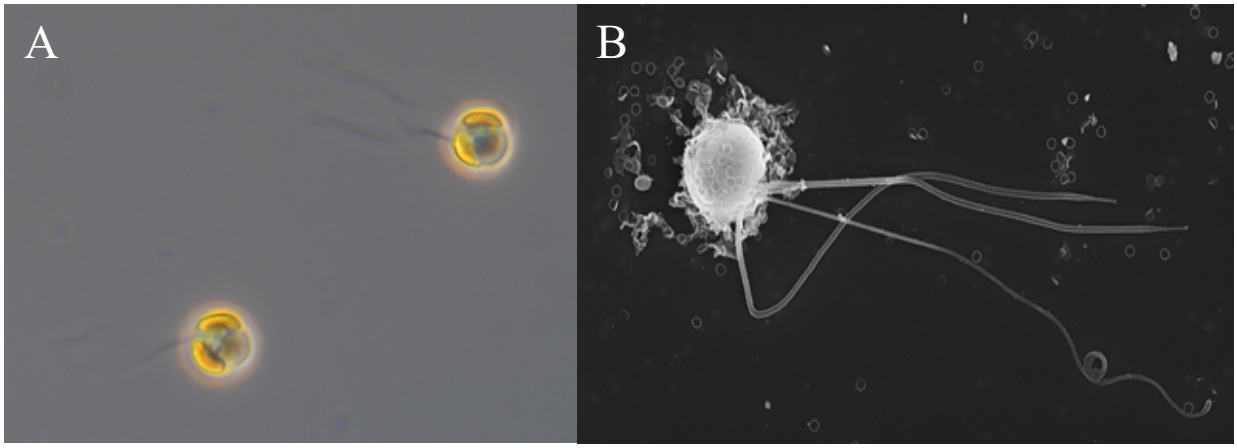

Figure 3. ***A)*** *Light micrograph of the two cells of the haptophyte* C. leadbeateri showing the chloroplasts and flagella. *Photo: Bente Edvardsen, University of Oslo.* ***B)*** *Scanning electron micrograph of* C. leadbeateri *showing the two flagella, the haptonema, and the scales covering the cell body. Photo: Wenche Eikrem and Antje Hofgaard, University of Oslo.*

Despite these implications, it is difficult to draw a clear connection between nutrients and *C. leadbeateri* blooms, at least in terms of determining bloom dynamics after seasonal bloom initiation. There are suggestions that environmental factors (e.g., salinity, inorganic nutrients, and temperature) are less important in driving later bloom dynamics than biotic interactions (Aalto et al., 2023; Lindh et al., 2015; Needham & Fuhrman, 2016). These papers were referring to microbial interactions, but interactions between prey and other predators of *C. leadbeateri* blooms should be considered too.

1.3 Harmful algae and grazer interactions

Phytoplankton, harmful algal species included, are grazed primarily by zooplankton which includes mesozooplankton and copepods are the most abundant (Turner & Tester, 1997;

Schminke, 2007). Haptophytes are part of the copepod diet and their grazing rates are in accordance with the abundance of haptophytes present (Meyer-Harms et al., 1999; Nejstgaard et al., 2003). When copepods grazed on known toxic haptophytes like *P. parvum* (both *P. parvum* f. *parvum* and f. *patelliferum*), there was no measured short-term mortality, but a strong reduction in secondary production of copepods in the event of a bloom (Sopanen et al., 2006). This reduction was based on the low number of pellets and egg production, signaling that the copepods exposed to these toxic haptophytes soon became inactive (Sopanen et al., 2006; Nejstgaard & Solberg, 1996). While there might not be a direct impact on the actual predation and prey, the fear of predation may have a bigger influence, especially in aquatic ecosystems (Preisser et al., 2005). This can be seen where copepods are the predators and phytoplankton the prey. As predators, copepods are able to detect and use chemical cues in their environment. These cues are bioactive compounds and involved in the main activities in a copepod's life including: survival, by avoiding predators, reproduction, by finding a mate, and foraging (Heuschele & Selander, 2014). The presence of these copepod predators and grazers also releases chemical cues into the surrounding waters and can serve as early warning signs for prey of predator presence (Selander et al., 2016).

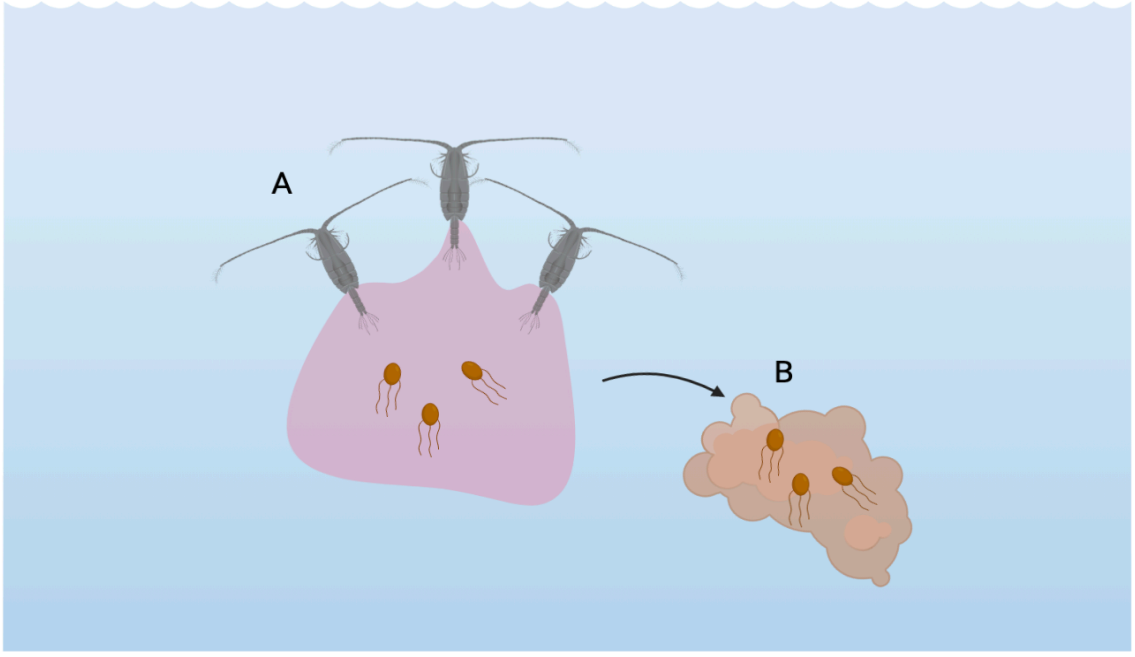

Figure 4. ***A)*** *The copepod emits chemical cues into the surround waters when grazing.* ***B)*** *The grazed algae detect these chemical cues and will release toxins to alleviate predation pressure. Figure created on Biorender.com.*

Phytoplankton can pick up on these cues and respond in a number of ways (Bjærke et al., 2015; Long et al., 2007; Selander et al., 2011). Some species reduce their swimming speed or colony size to decrease in size which would reduce encounter rates with grazers (Long et al., 2007; Selander et al., 2011). Some species increase bioluminescence or toxin production (Lindström et al., 2017; Lundholm et al., 2018; Selander et al. 2006). The effects of increased toxin production on grazers and herbivory however are less predictable because studies have shown both grazers avoiding toxic algae and feeding on the toxic algae, seemingly unaffected (Tammilehto et al., 2012; Turner & Tester, 1997).

1.4 Inducing toxicity among harmful algae through copepod grazing

Some species of harmful algae can thus detect the threat of predation and will respond by increasing toxin production. Increasing toxin production as a response to predation threat from copepods have been found in different microalgae groups. The dinoflagellate *Alexandrium minutum* was found to produce up 2.5 times more gonyautoxins, a type of paralytic shellfish toxin (PST) than controls when exposed to cues from copepods. Grazing copepods had a significantly higher effect in toxin production compared to starving copepods (Selander et al., 2006). Other studies showed that the diatom *Pseudo-nitzschia seriata* increased domoic acid (DA) toxin content up to 3300% when exposed to grazing *Calanus* copepods and copepodites, either through direct physical contact or separated by a membrane. An induction of DA in the previously non-toxic *Pseudo-nitzschia obtusa* was also observed (Harðardóttir et al., 2015; Tammilehto et al., 2015). It is suggested that the chemical cues excreted by grazing copepods responsible for triggering DA toxin production in *Pseudo-nitzschia* are specific polar lipids called copepodamides (Lundholm et al., 2018). Copepodamides are characterized by the scaffold and fatty acid attached to it (Grebner et al., 2019). Copepodamides have been previously measured by three species of calanoid copepods, *Centropages typicus*, *Calanus* sp., and *Pseudocalanus* sp. (Selander et al., 2015). An additional study found copepodamides in 10 common marine copepods, including *Acartia clausi*, present in the Northeast Atlantic surface waters (Grebner et al., 2019). While these toxin-inducing lipids and other chemical cues excreted from copepod

grazers were found in toxic algae affecting shellfish and human health, there were also chemical cues found in algae toxic to finfish, ichthyotoxic algae.

A study found that when the ichthyotoxic dinoflagellate, *Karenia mikimotoi*, was directly exposed to grazers, including the copepod *Pseudodiaptomus annandalei*, or waterborne chemical cues, its toxin production was induced. Specifically the production of hemolytic toxins and synthesis of eicosapentaenoic acid were induced (Dang et al., 2015). Not much more literature exists exploring the grazer-induced toxicity in ichthyotoxic algae. In general, more work can be done to study the toxicity inducing mechanisms behind ichthyotoxic algal species, specifically haptophytes. Even when it comes to some algal toxins (phycotoxins), most of them described are the ones that accumulate in shellfish and are toxic towards humans, like saxitoxin, one the toxins responsible for paralytic shellfish poisoning (PSP) (Rasmussen et al., 2016).

1.5 Ichthyotoxic haptophytes: toxicity and toxins

All species belonging to the genus *Prymnesium* are suspected to be toxic to gill-breathing organism (Edvardsen & Imai, 2006). One of the more well-studied toxic haptophyte species, P. parvum produce toxins with ichthyotoxic, cytotoxic, antibacterial, neurotoxic, and allelopathic activity (Shilo, 1981). Several toxic substances produced by P. parvum have been suggested to be responsible for fish mortality, including hemolysin 1, prymnesins 1 & 2 (Kozakai et al., 1982; Igarashi, et al., 1996). Recent studies have revealed the molecular structure of different prymnesins (type A, B, C). Another haptophyte species, *Phaeocystis pouchetii*, is shown to have caused cytotoxicity in cod larvae and the isolated polyunsaturated aldehyde was proposed to have caused the cell damage (Hansen et al., 2004). From the *Chrysochromulina* species, only *C. leadbeateri* have shown sustained toxicity. Several chemical compounds such as glycolipid and fatty acids have been isolated from *Prymnesium polylepis* extracts and shown hemolytic activity. However, it was found to have no differences in glycolipid and fatty acid compositions and toxic and non-toxic clones of *P. polylepis* (John et al., 2002). Still, compared to other known

ichthyotoxic haptophytes, the chemical characterization of *P. polylepis* toxins are lacking (Edvardsen & Imai, 2006).

This is even more of the case for *C. leadbeateri,* as little is known about the toxicity and physiology (but see Edvardsen 1993, Meldahl et al. 1994, Johnsen et al. 1999) of this species and toxins produced about this species are unknown. Even less is known about the biotic relationships, specifically copepod grazing, and the extent to which they influence *C. leadbeateri* blooms and toxicity. Much of the literature that exists of grazer interactions with toxic haptophytes are how the copepods feeding behavior and fecundity are affected (Sopanen et al., 2006, 2008; Witt et al., 2019). Other literature studying copepod grazing interactions focused on consumptive effects on HABs, but the mechanisms and relative significance behind these non-consumptive effects remain largely unknown among marine plankton (Stibor et al., 2004).

1.6 An *in vitro* approach to testing ichthyotoxicity

Scientists have routinely tested ichthyotoxic algal species directly on whole target organisms such as brine shrimp, larval and adult fish subjects (Edvardsen, 1993; Mooney et al., 2010). These *in vivo* approaches come with their own difficulties with special animal-rearing facilities and require ethical approvals, and it may be difficult to be reproducible. An alternative to testing on whole organisms is the development of primary cultures and animal cell lines which reduces the number of sacrificed animals and satisfies the ethical demands (Segner, 1998). Additionally, using the *in vitro* approach with cell lines allows for better control of experimental conditions by reducing variability due to unavoidable stress responses (Lee et al., 2009). This *in vitro* approach, however, can produce different results compared to working with whole organisms, at least when it comes to differing toxicokinetics between whole organisms and cells or tissues. Most studies, however, reported a high correlation between *in vivo* fish lethality data and *in vitro* cytotoxicity data from fish cell lines (Segner, 1998). In this thesis, the use of two epithelial gill cell lines were used to test the ichthyotoxicity of grazed microalgae of *C. leadbeateri*. The two cell lines RTgill-W1 and ASG-10 gill cells have been useful in past studies of gill diseases in Atlantic salmon and Rainbow trout (Dorantes-Aranda et al., 2011; Gjessing et al.,

2018; Solhaug et al., 2023). The two cell lines were not used for the purpose of a comparative study to determine which cell lines is best to measure ichthyotoxic effects but may indirectly do so anyway.

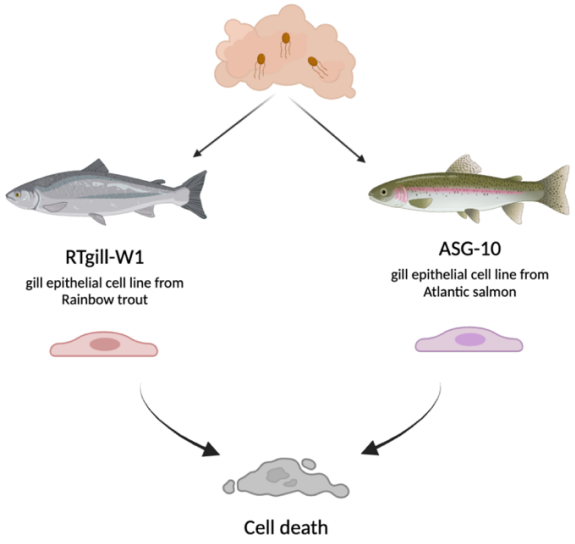

Figure 5. Graphical representation of the in vitro gill epithelial line bioassay used to test ichthyotoxic algae effects on fish. Figure created on Biorender.com and inspired by Solhaug et al. (2023).

1.7 Aims of the thesis

The presence of copepods can induce toxicity in known harmful algal species through the release of waterborne chemical cues and direct grazing. However, those interactions with copepods have been found in other microalgae groups such as diatoms and dinoflagellates. (Lundholm et al., 2018; Selander et al., 2006, 2015; Tammilehto et al., 2012, 2015). The potential for copepod grazing to induce toxicity in haptophytes has not been explored, at least not among *Chrysochromulina leadbeateri*. Not much is known about *C. leadbeateri* compared to better-studied toxic haptophytes, like *P. parvum* and *P. pouchetii*. Past studies have studied key abiotic ecological factors favoring *C. leadbeateri* blooms, concluding positive correlations with temperature, solar irradiance, and nitrate and phosphate concentrations, and negative correlations with salinity and vertical mixing of the water column (Edvardsen & Paasche, 1998.; John et al., 2022; Lekve et al., 2006). However, there are suggestions that these abiotic factors are less important in driving later bloom dynamics than biotic interactions (Aalto et al., 2023; Lindh et al., 2015; Needham & Fuhrman, 2016).

This thesis aims to study the biotic interaction between copepod grazers and ichthyotoxic haptophytes, and how copepod mortality and fish cell line toxicity can be influenced when the two interact. I used the copepod *Acartia* sp. to graze on *C. leadbeateri* to answer two essential questions (including sub-questions) about copepod grazing inducing toxicity in known ichthyotoxic haptophytes:

1. What is the threshold *Chrysochromulina leadbeateri* concentration that *Acartia* sp. can maintain survival and grazing?
   - Does the presence of the toxic *C. leadbeateri* influence the survival and activity of *Acartia* sp.?
   - What are the feeding responses (grazing and egestion) of *Acartia* sp. to *C. leadbeateri*?

2. Does the presence of copepod grazers affect toxicity of the ichthyotoxic haptophyte *Chrysochromulina leadbeateri*?
   - Can *C. leadbeateri* detect the direct predation threat of copepod grazing by responding with the triggering and/or increasing the toxicity?

1.8 Hypotheses

Based on these questions I formulated the following hypotheses about the expected outcomes of my study:

Hypothesis one: There will be an increase in short-term mortality of Acartia *sp. when directly exposed to increased concentrations of* Chrysochromulina leadbeateri *cells.*

Hypothesis two: The predation threat of direct Acartia *sp. grazing will cause increased toxicity potential in* Chrysochromulina leadbeateri*.*

2 Materials and methods

To answer the research questions posed, three different experiments were conducted, in a consecutive manner: 1) copepod survival exposed to a range of *Chrysochromulina leadbeateri* concentrations, 2) induction of *C. leadbeateri* toxicity production from copepod grazing, and 3) gill bioassay to test *C. leadbeateri* toxicity after exposure to grazing. The first determined which algal concentration the copepods survived at. Once it was determined at which algal concentration *Acartia sp.* had the highest survival rate, a second experiment compared the toxicity-producing potential of *C. leadbeateri* at varying levels of copepod grazing. The third experiment measured toxicity of the algal samples that experienced varying levels of grazing to establish if grazing has an inducing effect on *C. leadbeateri* toxicity production.

2.1 Culture maintenance

2.1.1 Culturing *Chrysochromulina leadbeateri*.

Chrysochromulina leadbeateri cultures were established of strain UIO-393, obtained from The Norwegian Culture Collection of Algae (NORCCA). This strain was isolated off Tromsøy in Troms, Norway (69.6469 ºN; 18.862667 ºE) on the 25th of May 2019 by Luka Supraha, University of Oslo. It was grown in the algal medium IMR 1/2 with salinity 30 PSU at temperature 14 °C and illumination (Osram l36w/830 warm white, Germany, under 70 W m^{-2} s^{-1} and a 12:12h L:D cycle. Using a transfer pipette, cells from the main culture were transferred into fresh IMR ½ medium (detailed recipe in Appendix A: Supplementary Methods) solution to create a separate culture. The copepods (*Acartia* sp.) were collected from a main culture stock established in 2019 and isolated from the Oslo fjord and kept at the University of Oslo. The temperature and salinities for the stock cultures were 17°C and 33 PSU.

2.1.2 Measuring algal cell concentration

Algal cell concentration was determined by cell counting under a light microscope using a Fuchs-Rosenthal counting chamber. Either all cells on the counting chamber were counted or at least 400 cells were counted as an estimate for cell concentration.

2.1.3 Culturing and handling *Acartia* sp.

Using a 500-ml glass beaker with a handle, water and copepods were collected from the main stock culture of *Acartia* sp. Next, an acrylic sieve (200 µm mesh size) was placed in a 50ml beaker. Then the water sample containing the copepods was poured through the sieve in the beaker. The filter in beaker was angled at a slant so the filtrate poured out into the 50 ml-beaker while the captured *Acartia* sp. were still within the filter, but not completely dry to prevent damaging the copepods. Finally, using a plastic pipette under a stereomicroscope, individual copepods were carefully picked and isolated into 24-well cell culture plates for later use. The copepods were always picked and isolated the same day as the experiments took place.

2.2 Copepod tolerance with varying *C. leadbeateri* cell concentrations

The aim of this experiment was to incubate *Acartia* sp. with varying *C. leadbeateri* concentrations to test the toxicity dynamics and survival over time and determine its Lethal concentration 50 (LC-50) toxicity (algal concentration causing 50% mortality). It was also to determine the optimal *C. leadbeateri* concentration to sustain *Acartia* sp. grazing, while also ensuring a sufficiently high concentration to detect any potential grazing-induced toxicity. Two methods of incubation: glass bottles and multi-well plates were used. Glass bottles were used to mimic the same chambers that would be used in the main induction experiment. Multi-well plates were later used as the incubation chamber for easier replication of the several trials that were conducted and to increase the number of replicates of each treatment.

Table 1. Full list of algal strains and concentrations that Acartia *sp. was exposed to in tolerance experiments.*

Algal strain	**Cell concentration (cells ml^{-1})**	**Experiment**	**Type of incubation**
Chrysochromulina leadbeateri (UIO-393)	200 000	3	Multi-well plate
	100 000	3	Multi-well plate
	50 000	3	Multi-well plate
	25 000	2, 3	Multi-well plate
	3 200	2	Multi-well plate
	2 000	1	Glass bottle
	1 600	2	Multi-well plate
	1 000	1	Glass bottle
	800	2	Multi-well plate
	500	1	Glass bottle
	400	2	Multi-well plate
	250	1	Glass bottle
	100	2	Multi-well plate
	0 (control)	1, 2, 3	Multi-well plate Glass bottle
Chrysochromulina throndsenii (UIO-135)	25 000 (control)	2	Multi-well plate
Haptolina ericina (NIVA/16-1)	200 000 (control)	3	Multi-well plate

2.2.1 Experiment 1, 2, and 3: Copepod tolerance to *Chrysochromulina leadbeateri*

Experiment 1

The glass bottle incubation to test copepod tolerance had five different treatments (concentrations of *C. leadbeateri*) with three replicates each. Each 100-ml glass reagent bottle (VWR® Borosilicate 3.3) represented one replicate for a total of 15 bottles and was labeled accordingly (e.g. "2000A"). The different treatments included: *C. leadbeateri* concentrations that followed a log 2 scale dilution series starting with 2,000 cells ml^{-1} and a control with only algal medium and no algae present. The treatments with different algal concentrations were established in the same method as described in the plate incubation experiments. Copepods were also picked and isolated as described above. What differed was the amount of algal culture and copepods prepared. After the different treatments of cell concentrations were made, each bottle was filled with the algal concentration that corresponded to its label to the "80 ml" mark. Ten adult copepods that were previously picked and isolated, were carefully transferred into each of the treatment wells using a plastic pipette. While pipetting up each copepod from the 24-well plate where they were being stored, no more than 0.5ml of seawater was also pipetted up and transferred into the bottle. Once all 10 of the copepods were added and had moved to the lower half of the bottle, more algal culture of the corresponding concentration was added. Algal culture was added until the liquid level reached to the rim and the bottle was completely full. Then, the bottle opening was covered with parafilm and sealed tightly with the bottle cap. It had to be made sure that the copepods did not move above the 80ml mark during the sealing process and no copepod adhered to the parafilm.

After successful sealing, the incubation bottles were placed in a climate room of ca 16°C for 72 hours periodically checked for any copepod mortalities after: 6, 24, 48, and 72 hours.

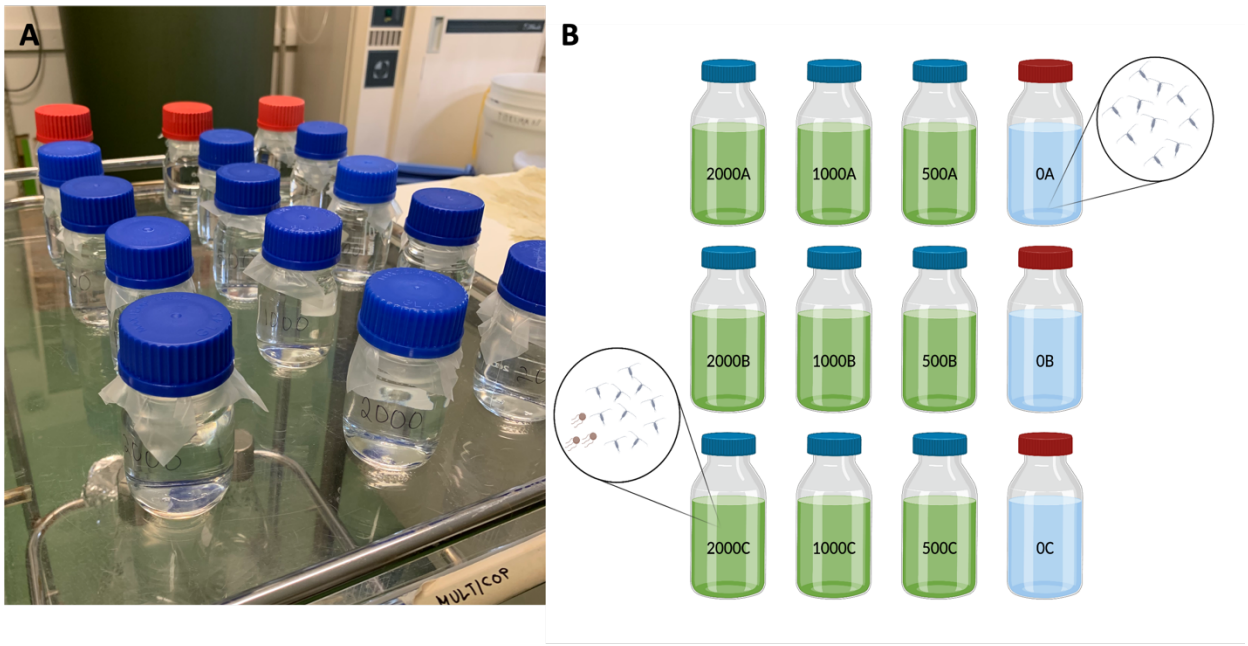

Figure 6. ***A)*** *A picture of the 1st tolerance experiment testing* Acartia *sp. tolerance to different* C. leadbeateri *concentrations in glass bottles.* ***B)*** *A schematic illustration of the experimental setup for the 1st tolerance experiment. Figure made in Biorender.com.*

The periodic checks during the glass bottle tolerance experiment proved difficult to determine whether the copepods showed activity or otherwise. That, combined with the different treatments the copepods were exposed to being such low concentrations, the results were omitted from the analysis. The results are in Appendix B: Supplementary Results.

Experiment 2 and 3

The second experiment had the aim to test copepod tolerance to eight different concentrations of *C. leadbeateri,* with five replicates of each concentration. The experiment was performed in a 6-well plate (3506 Costar, Modell, Company), where each well represented one replicate with 10 *Acartia* sp. individuals per well. In total 40 wells or seven 6-well plates was used. The *C. leadbeateri* concentrations loosely followed a log 2 scale dilution series starting with 25,000 cells ml^{-1}, a negative control of a non-toxic algal strain (*Chrysochromulina throndsenii*) with also 25,000 cells ml^{-1}, and another control with algal medium with no algae present. The treatments with different algal concentrations were established first in separate cell culture flasks from 300 ml of the highest concentration of 25,000 cells ml^{-1}. The other culture flasks for the different

treatments were labeled: 3200 cells ml^{-1}, 1600, 800, 400, 100, 0, and 25,000 (*C. throndsenii*). The culture flask labeled "0 cells ml^{-1}" was filled with 150 ml of IMR ½ algal medium and the flask labeled "25,000 (*C. throndsenii*)" was filled with 150 ml of 25,000 cells ml^{-1} *C. throndsenii*. The other culture flasks were diluted from the 25,000 cells ml^{-1} *C. leadbeateri* culture flask following the $C_1V_1=C_2V_2$ formula to determine how much the higher algal concentration and IMR ½ agar medium needed to make ca 150 ml of the intended concentration. For example, to dilute 25,000 cells ml^{-1} of *C. leadbeateri* to 3200 cells ml^{-1}, 20 ml of 25,000 cells ml^{-1} culture was added to ca 136ml IMR ½ medium to make ca 156 ml of 3200 cells ml^{-1} *C. leadbeateri*.
After the necessary dilutions were made, the 6-well plates were labeled and prepared for incubation. A randomized list of all the treatments and replicates (e.g., "100kA" for 100,000 cells of *C. leadbeateri*) was generated in RStudio and used to label each well accordingly. This limited the risk that one treatment would receive for example, more light exposure once incubated if all the replicate wells were on one plate. Then, using an automated pipette, 10ml of each treatment were transferred to the corresponding five labeled wells for that treatment. The pipette tip was changed when a new treatment was being transferred. After all the wells were filled with the different treatments, three adult copepods that were previously picked and isolated, were carefully transferred into each of the treatment wells using a plastic pipette. While pipetting up each copepod from the 24-well plate where they were being stored, no more than 0.5ml of seawater was also pipetted up and transferred into the treatment well. After successfully transferring the copepods, the incubation plates were placed in a climate room of ca 14°C under a lamp (Osram 49W/940 cool white, Germany, under ca. 54 W m^{-2} s^{-1}). More detailed light measurements are in Appendix B: Supplementary Results. The total incubation time was 72 hours, with periodic checks for copepod mortalities after 6 hours, 24, 48, and at 72 hours.

A second plate incubation experiment followed the same methods as in the first experiment, except with cell concentrations: 200,000 cells ml^{-1}, 100,000, 50,000, 25,000, and 0. The objective was to establish a potential higher *C. leadbeateri* concentration that could sustain *Acartia* sp. grazing and threshold concentration between *Acartia* sp. survival and death.

Additional trials of the plate incubation experiments testing several other *C. leadbeateri* concentrations were omitted from the analysis due to high copepod mortalities observed throughout all treatments. The results are in Appendix B: Supplementary Results.

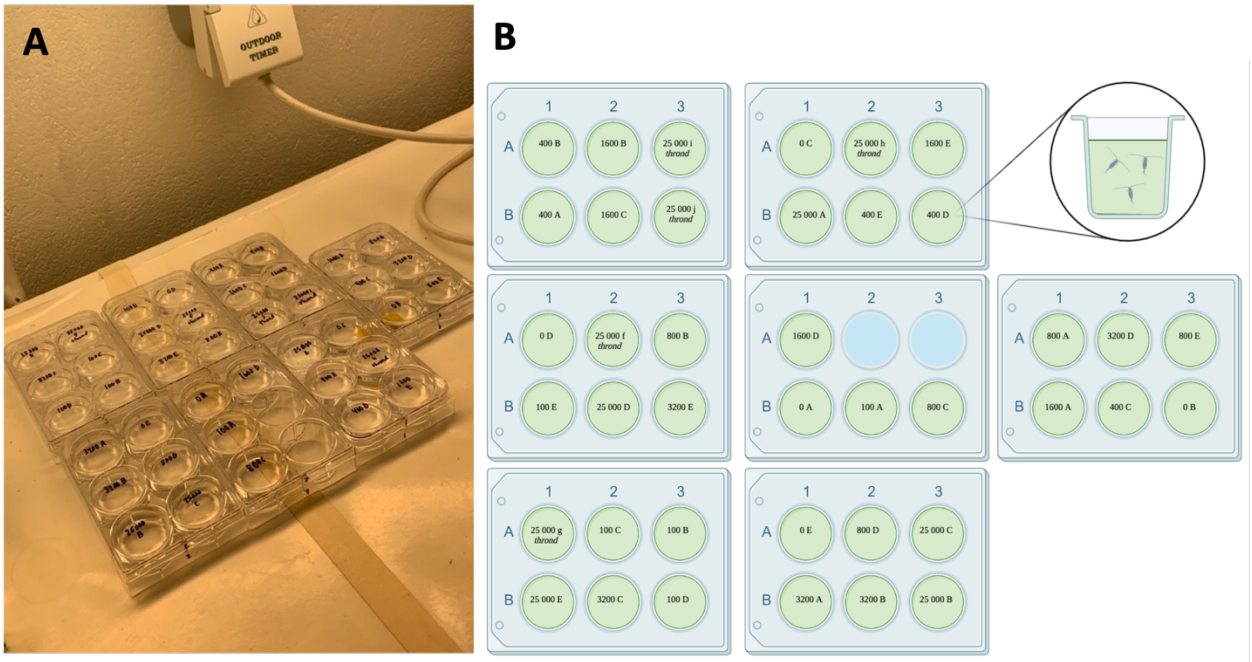

Figure 7. ***A)*** *A picture of the 2nd tolerance experiment testing* Acartia *sp. tolerance to different* C. leadbeateri *concentrations in multi-well plates.* ***B)*** *A schematic illustration of the experimental setup for the 2nd tolerance experiment. Figure made in Biorender.com.*

2.3 Inducing *C. leadbeateri* toxicity production with copepod grazing

The aim of this experiment was to expose *C. leadbeateri* to varying levels of copepod grazing to test if it induces or influences its toxicity potential. Two separate toxicity induction experiments are described below: 1) initial induction experiment with 250 000 cells ml^{-1} and 2) second induction experiment with 50 000 cells ml^{-1} *C. leadbeateri*. The initial induction experiment was noted to have >50% copepod mortalities at the of the incubation period and the high cell concentration was presumed to be the issue. So, that is what copepod tolerance experiments (earlier described) aimed to solve. It is still included in the methods as the algal samples were later used in toxicity tests. The second induction experiment incubated grazers with the *C. leadbeateri* concentration determined (in the tolerance experiments) to best sustain grazing.

2.3.1 Experiment 1 and 2: Toxicity induction through grazing

Experiment 1

The experiment was conducted in 100-ml glass bottles. Each treatment (control and grazing copepods) was replicated three times for a total of 12 glass bottles. To ensure an effective spreading of the inducing cues in the larger flasks, *C. leadbeateri* cells were exposed to direct grazing (Selander et al., 2006). Also assuming only a small fraction of *C. leadbeateri* would be eaten and the grazing frequency would not exceed the replication rate of *C. leadbeateri*, excessive feeding affecting toxicity could be excluded as a significant factor of lessening the predicted increase in toxicity. Grazing copepods were a varying number of 0, 3, 6, and 9 *Acartia* sp. individuals per bottle. The treatment control contained 0 individuals. Prior to the experiment, at least 54 adult *Acartia* sp. were picked out and isolated in 24-well plates (3527 Costar, Modell, Company) for later retrieval. The cell concentration of *C. leadbeateri* was determined using a Fuchs-Rosenthal counting chamber before addition to the culture flasks. All 12 bottles were each filled to the 100-ml mark and the correct number of copepods were gently pipetted from the wells into the designated treatment bottles. Then, the bottles were filled completely with the algal culture and carefully covered with parafilm before closing. It was made sure that there were no air bubbles before sealing. The bottles were placed on a plankton wheel in a climate room (rotating at 2 rpm, temperature 12°C, dim light). Incubation lasted for 72 hours and afterwards, the algal and copepod harvesting processes began.

Table 2. Number of grazing copepods from the initial toxicity induction experiment that were incubated with C. leadbeateri *cultures.*

Treatment (cells ml^{-1})	**# of *Acartia* sp. individuals**
C. leadbeateri 250 000	9
C. leadbeateri 250 000	6
C. leadbeateri 250 000	3
Algal medium (negative control)	0

Experiment 2

Once it was determined that *Acartia* sp. had highest survival rate at 50,000 cells ml^{-1}, the toxicity induction experiment followed. The experiment was conducted in 200-ml cell culture flasks (VMR, USA). Each treatment (control and grazing copepods) was replicated three times for a total of 15 cell culture flasks. To ensure an effective spreading of the inducing cues in the larger flasks, *C. leadbeateri* cells were exposed to direct grazing (Selander et al., 2006). Also assuming only a small fraction of *C. leadbeateri* would be eaten and the grazing frequency would not exceed the replication rate of *C. leadbeateri*, excessive feeding affecting toxicity could be excluded as a significant factor of lessening the predicted increase in toxicity. Grazing copepods were a varying number of: 0, 1, 5, 10, and 20 *Acartia* sp. individuals per bottle. The treatment control contained 0 and 1 individuals. Prior to the experiment, at least 108 adult *Acartia* sp. were picked out and isolated in 24-well plates (3527 Costar, Modell, Company) for later retrieval. The cell concentration of *C. leadbeateri* was determined using a Fuchs-Rosenthal counting chamber before addition to the culture flasks. All 15 flasks were each filled with the algal culture to the 150-ml mark. Then, the copepods were gently pipetted from the wells into the designated treatment flasks until each was had the correct number of copepods. Finally, all 15 flasks were filled with algal culture and sealed tight. All bottles were incubated in a 16°C climate room and under an average 59 µmol $m^{-2}s^{-1}$ illumination (12h:12h). More detailed recording of light measurements are in Appendix B: Supplementary Results. Incubation lasted for 72 hours and periodically checked for any copepod mortalities after: 6, 24, 48, and 72 hours. After the 72 hours, the algal and copepod harvesting processes began.

Table 3. Number of grazing copepods from the second toxicity induction experiment that were incubated with C. leadbeateri *cultures.*

Treatment (cells ml^{-1})	**# of *Acartia* sp. individuals**
C. leadbeateri 50 000	20
C. leadbeateri 50 000	10
C. leadbeateri 50 000	5
Algal medium (negative control)	1
Algal medium (negative control)	0

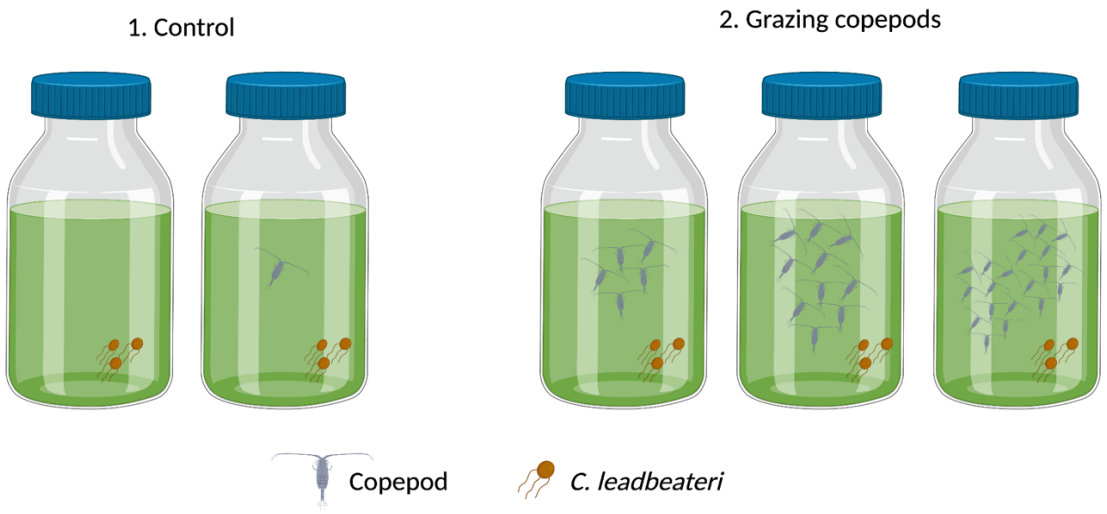

Figure 8. A schematic illustration of the different treatment types in the 2nd toxicity induction experiment with different number of copepods. The actual experiment was conducted in 200-mL culture flasks. Figure was made with Biorender.com.

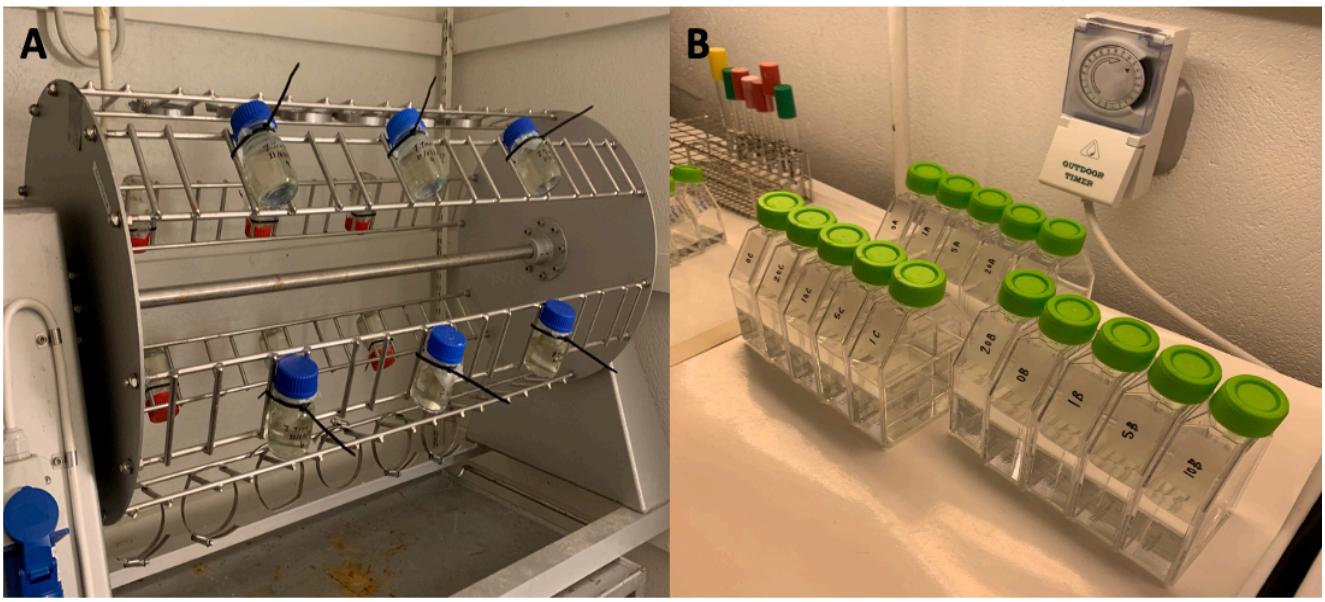

Figure 9. ***A)*** *First toxicity induction experiment with glass bottles on plankton wheel at 2 rpm.* ***B)*** *Second toxicity induction experiment with treatment flasks.*

2.3.2 Harvesting algal and copepod samples

After the conclusion of the toxicity induction experiment, *C. leadbeateri* exposed to grazing was harvested for later toxicity tests. First, a sieve (30-40 µm mesh size) was placed over a 250ml beaker. The contents in the flasks were then poured through the sieve to remove the copepods and fecal pellets from the algae. Each flask was rinsed down three times with filtered seawater to make sure the flask was emptied of its contents. The copepods and pellets were caught with the sieve while the algae samples flowed through the mesh and into the beaker. Using a spray bottle containing filtered seawater, the copepods and pellets were transferred from the sieve into a 25 ml glass vial. Then the samples in the vial were fixed with Lugol's solution.
Next, a subsample from the now isolated algal sample was collected. The subsample was used for later cell counting as an indicator of how much grazing took place during the induction experiment. Using an automated pipette, 10 ml of the algal sample was transferred from the beaker to a 25 ml glass vial then fixed with Lugol solution. In the end, there was 15 total glass vials from each replicate.

The remaining isolated algal sample was poured back into its original culture flask and tested for its pH using a pH meter. The whole process was repeated for each replicate, making sure to rinse each sieve with filtered seawater and beaker with distilled water through properly between each use.

The final step was to isolate the algae from the samples by filtration measure the amount by chlorophyll fluorescence. A filtration rack with a vacuum pump was used for algae filtration. The algal samples were poured into the filtration funnel and the algal cells were collected on a glass fiber Whatman GF-F filter (0.7 µm mesh size). Each replicate flask was filtered individually with its own filter and kept separate in 50 ml falcon tubes, totaling 15 falcon tubes. Each tube was kept on ice until filtering was completed and all the tubes containing the filters were frozen and kept in an 80°C freezer.

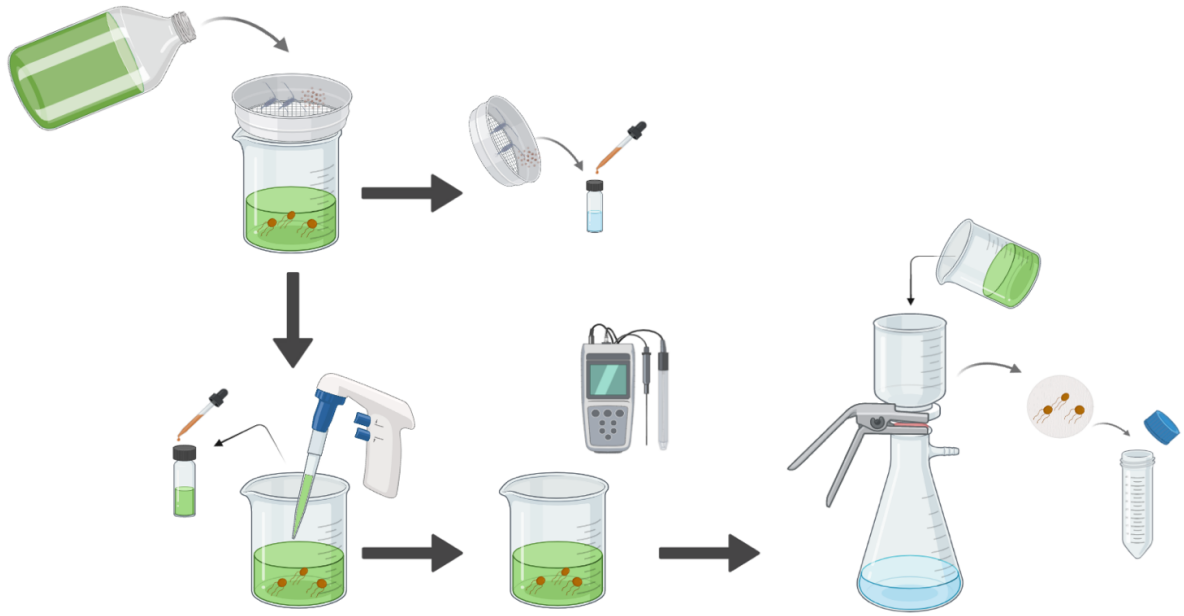

Figure 10. Schematic demonstration of how algal, copepod, and fecal pellet harvest was conducted after conclusion of the 2nd induction experiment. Figure was made with Biorender.com.

2.3.3 Determining grazing frequency

Counting algal cell concentration

The amount and general detection of copepod grazing during the 72-hour induction experiment was also investigated. In the previous step described 10 ml of subsample from each replicate was pipetted into a 25 ml glass vial and fixed with Lugol solution. Each of the 15 samples were manually counted under a light microscope (Zeiss Axiostar Plus Binocular) using a Fuchs-Rosenthal counting chamber. The cell count post-incubation of the induction experiment was used as an indicator of grazing frequency that took place during the 72 hours.

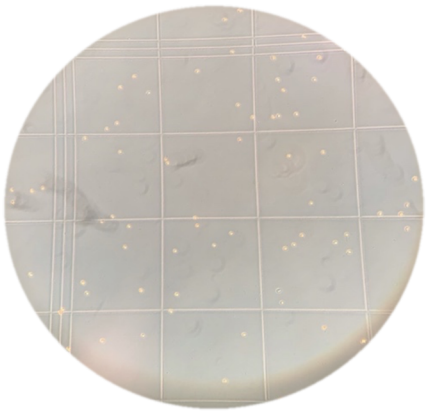

Figure 11. Counting C. leadbeateri *under a light microscope (100x magnification) with a Fuchs-Rosenthal counting chamber. A minimum of 400 cells were counted per sample.*

The grazing activity (filtration and ingestion rates) was calculated according to (Frost, 1972) for the 72-hour period from the treatment bottles with grazing copepods present. The growth constant for algal grown, k, was calculated from

$$C_2 = C_1 e^{k(t_2 - t_1)},$$

where C_1 and C_2 are *C. leadbeateri* cell concentrations (cells ml^{-1}) in the control flasks containing no copepod grazers at $t_1$and t_2, the times before and after the grazing period. Then for each treatment flask with copepod grazers present, the grazing coefficient, g, was calculated from

$$C_2^* = C_1^* e^{(k-g)(t_2 - t_1)},$$

where C_1^* and C_2^* are cell concentrations (cells ml^{-1}) at $t_1$and t_2, the times before and after grazing period.

The grazing values (g) and algal concentration counts after grazing are presented as *Table S9* and *Figure S9* in Appendix B: Supplementary Results.

Counting fecal pellets

To further the assess foraging and feeding activity of the copepods, fecal pellets from each replicate sample containing copepods were counted and measured using a stereomicroscope (Leica S9D, Boston Industries) and camera (Leica Flexacam C3). Fecal pellet volume was estimated by measuring the length of photographed pellets with the Fiji software and Leica

Application Suite X (Schindelin et al., 2012). At least three fecal pellets were recorded and measured. It was estimated how many fecal pellets each copepod individual ($\frac{\#\text{ of fecal pellets}}{\#\text{ of } Acartia \text{ sp.}}$) produced.

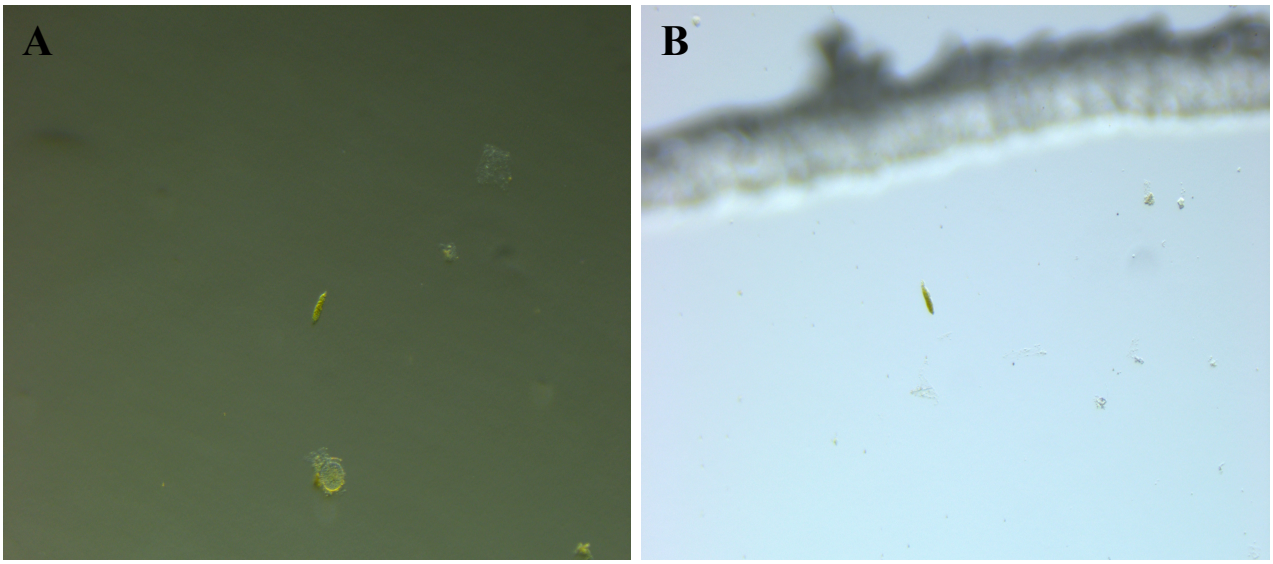

Figure 12. Fecal pellets examined under a stereomicroscope from the 20 grazing copepods treatments: ***A)*** *replicate 2 and* ***B)*** *replicate 3.*

2.4 *In vitro* bioassay using RT-gill W1 and ASG-10 gill cell lines

The *in vitro* bioassays aimed to test the toxicity of the crude algal extracts of *C. leadbeateri* that had been exposed to varying levels of grazing pressure. One bioassay was conducted using two different epithelial cell lines that originated from the gill filaments of 1) Rainbow trout *Oncorhynchus mykiss* (Bols et al., 1994) and 2) Atlantic salmon *Salmo salar* (Gjessing et al., 2018). The Rainbow trout cell line (RTgill-W1) was used in a trial experiment to test *C. leadbeateri* toxicity effects on a well-known cell line. It is noted that no copepod tolerance experiment was conducted prior to the RTgill-W1 testing to determine which *C. leadbeateri* concentration would "best" maintain sustained grazing from *Acartia* sp. Because of this, the concentrations from the *C. leadbeateri* harvested after the induction experiment were different for tests on the two cell lines. Despite this, both tests using the two cell lines are described as it gives valuable information to better answer the questions the thesis aims to answer. The bioassay with the two cell-lines each took place over the course of three days:

1. Preparing crude algal extracts and seeding
2. Making treatment dilutions and exposure
3. Cellular viability measurements using Alamar blue and imaging

2.4.1 Fish gill cell line: origin, maintenance, and culturing

The gill cell line RTgill-W1 was obtained from the American Type Culture Collection (CRL-2523, ATCC) and initiated from *O. mykiss* while the gill cell line ASG-10 was obtained from the Norwegian Veterinary Institute and initiated from *S. salar*. The growth and maintenance of these cell lines were in accordance with the procedure described in (Solhaug et al., 2023). Both cell lines were cultured at 19 °C in a non-ventilated cell culture flask with L15 medium which is L15 with 10% v/v Fecal Bovine Serum (FBS) and Penicillin-Streptomycin. The ASG-10 cell line was additionally supplied with 33 µM β-mercaptoethanol to provide the cells with anti-oxidants. The cultures were sub-cultured 1:2 every 10 days using tryPLE to detach the cells first from the sides of the flask. After preparing crude algal extracts, the bioassay with different cell lines followed the same procedure.

2.4.2 Preparing crude algal extracts

Centrifugation samples. For the RTgill-W1 cell bioassay, crude algal extracts were prepared from algal samples (250 000 cells ml^{-1}) that had been harvested through centrifugation after the first induction experiment. Extracts of the algal samples were prepared the day prior to the exposure experiment. For the gill bioassay, those samples were first thawed in room temperature before extraction. Next, 2 ml of 1:9 H_2O:MeOH was added to each tube with the defrosted samples. Then after sonication (20 min, 40 kHZ) and centrifugation (10 min, 4000 rpm, 7°C), the supernatant that was left was pipetted into a new glass container. All the replicates of the same treatment were combined into one container (e.g. three replicates for the '0 copepod' treatment were placed in one glass vial) and the samples were evaporated at 45°C under constant N_2 gas (Xcel/Vap^{TM} Evaporation/Concentration System).

Filtration samples. For the ASG-10 cell bioassay, crude algal extracts were prepared from the algal samples (50 000 cells ml^{-1}) that had been harvested onto glass fiber filters (Whatman GF/F) after the second induction experiment and kept at -80°C until later use. Before the gill bioassay was conducted, the samples were thawed at room temperature and 9 ml of 1:9 H_2O:MeOH was added to each tube, making sure each filter was completely submerged. The samples were sonicated and centrifuged as described in the RTgill-W1 bioassay. Once the algal material was extracted from the filters, the liquid sample was pipetted into spin column tubes and we centrifuged the samples. The spin column removed all glass fiber material from the extract, leaving the extract in the collection tube. The extract was transferred from the collection tube to a small glass vial and evaporated a final time at 45°C under constant N_2 gas. After all the methane had completely evaporated, the samples were kept in a -20°C freezer until dilutions were made the next day.

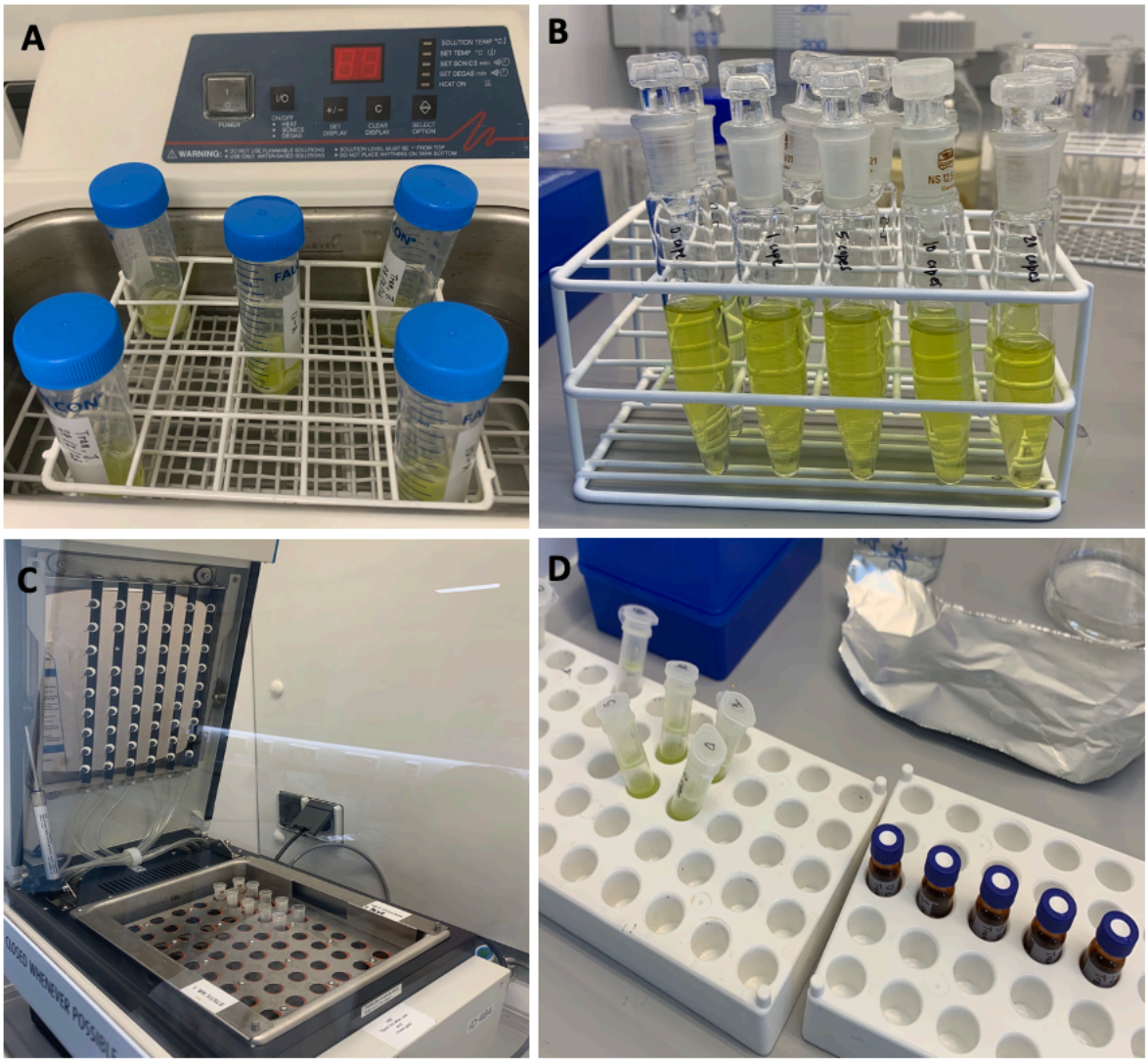

Figure 13. ***A)*** *Harvested algal samples after the induction experiment were sonicated as a step of the crude algal extract preparation.* ***B)*** *Algal samples after sonication, centrifugation, and removal of the glass fiber filter.* ***C)*** *First round of evaporation at 45 ℃ under constant N_2 gas.* ***D)*** *After the first evaporation round, the samples were placed in a spin column tube and centrifuged. The extract in the collection tube was transferred to small glass containers.*

2.4.3 Seeding gill cells

The seeding procedure took place one day before the exposure experiment and involved transferring the fish gill cells from the non-ventilated flask to a 96-multiwell plate (3598 Costar, Modell, Company). The L15 medium (with FBS and Penicillin-Streptomycin, (Lonza, Basel, Switzerland)) was removed from the culture flask with confluent RTgill-W1 or ASG-10 gill cells attached to the surface. Phosphate-Buffered Saline (PBS, 7.2 pH) was added to the flask and

washed the cells for 30 seconds. Once the PBS was removed, TrypLE was added to detach the cells from the flask's surface. After 5-10 minutes and gentle tapping of the flask, the cells would lose their flattened, irregular polygonal shape and turn into detached spherical cells. Complete L15 medium was added and the resulting cell suspension was transferred to a centrifuge tube. Then 4 mL of the cell suspension and 8 mL of complete L15 medium was added together to reseed the cells (1:2) to a new flask. The cells were counted with an imaging counter and diluted in complete L-15 medium to the desired cell concentration, 450 000 cells ml^{-1} in a final volume of 10 mL.

The plate was then prepared when 100 µL of PBS was pipetted into the outer wells of the plate. This was to prevent the undesired edge effect where because the contents in the outer wells would evaporate during incubation, they may behave differently than inner wells (e.g. metabolic activity) (Mansoury et al., 2021). Then, 100 µL of the diluted cell suspension was pipetted to the remaining 60 wells in a random order to prevent systematic bias during cell seeding. The final cell count was ca. 140 000 cells cm^{-2}. The plate was kept in the dark, in 19°C incubator overnight so that gill cells could attach to the surface of the wells. The next day, the seeded gill cells were checked under a light microscope to make sure the cells looked healthy and had reached full confluency before exposure.

2.4.4 Making dilutions and exposure for treatments

The day after the seeded cells had attached, dilutions of the extracts were made. The crude extract samples were taken out of the -20°C freezer and 1:9 H_2O:MeOH -in-water was added to each sample. Then the samples were vortexed, sonicated, and centrifuged. Once all the crude extract on the sides of the glass container were mixed into the methanol, the samples were evaporated at 45°C under constant N_2 gas to obtain the extracted biomass residue. After evaporations, dilutions were made of the extract in L-15/ex medium (OECD, 2021) to make the desired concentrations. Methanol was added to each sample making sure methanol content was equal in each sample and contained the same volume of methanol as the solvent control (1% methanol).

Table 4. List of amount of extract, L15/ex medium and methanol added to make each desired dilution for the in vitro bioassay using ASG-10 and RTgill-W1 cell lines.

ASG-10 gill cell bioassay					
Dilutions	**1%**	**0.5%**	**0.25%**	**0.1%**	**Solvent control (SC)**
Extract added (µL)	5	-	1.25	1	0
L15/ex added (µL)	495	-	495	990	495
Methanol added (µL)	0	-	3.75	9	5
RTgill-W1 cell bioassay					
Extract added (µL)	5	2.5	2.5	1	0
L15/ex added (µL)	495	495	990	990	495
Methanol added (µL)	0	2.5	7.5	9	5

Dilutions were made in Eppendorf tubes for each number of grazing copepods (treatment) with extract percentage (e.g. 0 copepods – 1%, 0 copes – 0.25%, 0 copes – 0.1%) with 20 Eppendorf tubes in total. Once the dilutions were made, the complete L15 medium was removed from all 60 wells of the incubated plate and replaced with 100 µL of the exposure solution (dilutions made). Each dilution was added into the designated well as shown in *Figure 9*. The plate was placed back into the dark in the 19°C incubator for 24 hours.

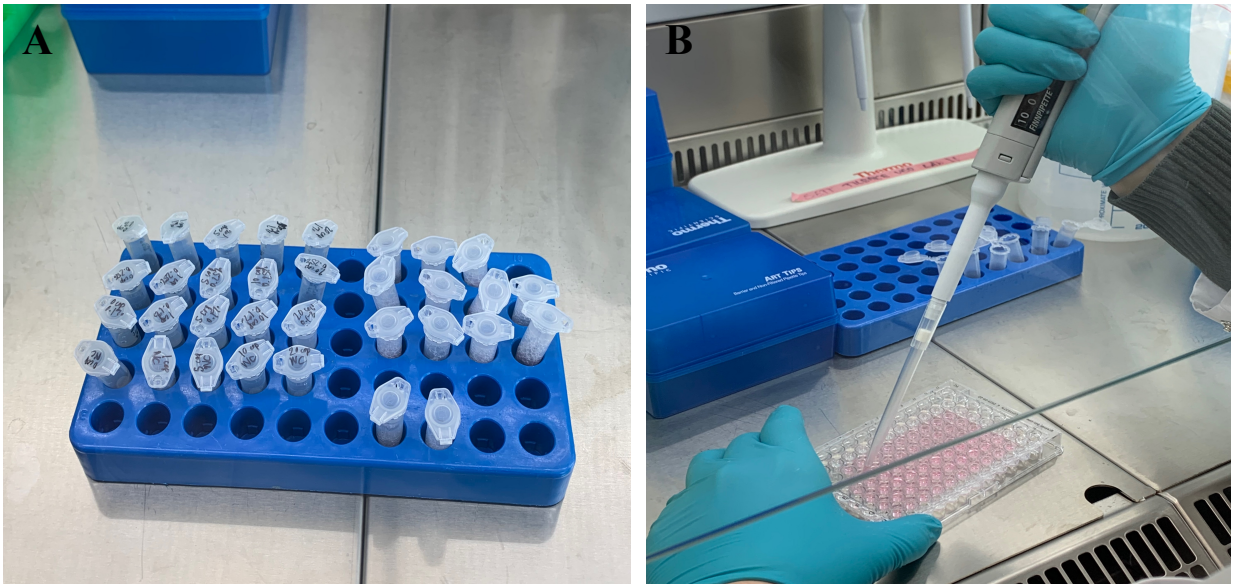

Figure 14. ***A)*** *Dilutions with different extract percentages made for each treatment.* ***B)*** *Pipetting out L15 medium to be later replaced with 100 µL of the exposure solution (dilutions made).*

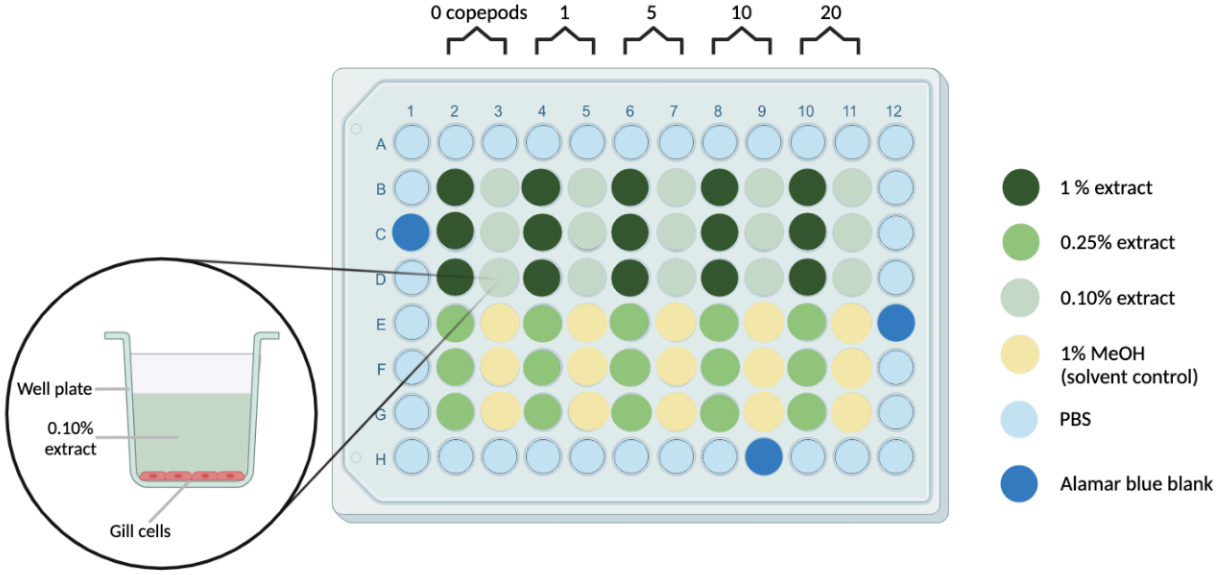

Figure 15. The plate setup used for the exposure experiment in the ASG-10 gill bioassay. The setup shows the arrangement of different extract percentages and treatments of grazing copepods. Also a close-up of what each well would look like inside with the layer of gill cells on the below and extract above is shown. Figure created with Biorender.com.

2.4.5 Cellular viability with Alamar blue and imaging

The final day of the three-day gill bioassay experiment involved measuring metabolic activity/cellular viability using Alamar Blue dye assay (Invitrogen, Thermo Fisher). The Alamar blue stock solution and L15/ex were prepped and warmed to room temperature. In a 1:2 ratio, Alamar Blue stock solution was diluted in L15/ex medium (e.g. 100 µL Alamar Blue with 200 µL L15/ex) and mixed well. Then, the plate was taken out of the incubator and 9 µL of the diluted Alamar Blue solution was pipetted into each well (that were exposed to the algal extract). Nine µL were also added into three exterior wells filled with PBS and served as a blank. Once completed, the plate was added back into the 19°C incubator and incubated there for two hours. After incubation, the plate was put in a SpectraMax i3x plate reader (Molecular Devices, San Jose, CA, USA) and its fluorescence was measured (excitation 555 nm with bandwidth 9nm/emission 585 nm with bandwidth 15 nm). The measured fluorescence is proportional to the number of viable cells because the dark blue oxidized form of Alamar Blue is reduced to a highly fluorescent form (resorufin) by mitochondrial or cytoplasmic enzymes (Rampersad, 2012). The viability results (*Figure 23*) were expressed as percentage of the readings compared to the solvent control (% of control).

Figure 16. The SpectraMax i3x plate reader used to measure gill cellular viability through fluorescence.

Once the plate was read, each treatment was examined under a microscope (Zeiss Observer A1). Phase contrast images of the gill cells were taken with the Axiocam 503 and analyzed using ZenLite software to assess morphological changes.

2.5 Statistical Analysis

2.5.1 Copepod survival as a function of *C. leadbeateri* concentrations

The "tolerance" of copepods was measured in terms of copepod survival. During the tolerance experiment, the activity of the copepods was visually checked at several time points (6, 24, 48, and 72 hours). Each copepod was marked "inactive" if the individual did not move for at least 10 seconds. At the conclusion of the experiment and 72-hour check, the copepods were given a more thorough and conclusive evaluation. The copepod that had not moved in at least 10 seconds were "poked" by a plastic pipette and if the individual did not physically respond by the disturbance, it was marked as a mortality. For the sake of evaluating copepod "survival", it was

assumed that any copepod that had been marked inactive during the visual checks, (6, 24, or 48 hours), but was marked as survived during the conclusive final check at 72 hours, was a human error. That copepod would be assumed to be active instead and marked as such for later data analyses.

The Cox proportional hazards model (Anderson & Gill, 1982) in R (library survival, Therneau and Grambsch (2000), R version (4.1.3) was used for copepod survival analysis. It was used to model the survival probability of copepods after exposure to different algal concentrations over time. A similar model also predicted the hazard ratio compared to the control for different algal concentrations, i.e. how much more likely the copepods were about to die compared to the 0 cells ml^{-1} control.

To analyze the effect of *C. leadbeateri* concentration on *Acartia* sp. survival, a reduced General Unified Threshold model for Survival (GUTS) (Jager et al., 2011; Jager and Ashauer, 2018) was also applied, assuming stochastic death (SD) as death mechanisms. The GUTS package of the BYOM framework under MATLAB (MATLAB 2021) was used and run by co-supervisor Jan David Heuschele. The model assumes that the toxic effect of *C. leadbeateri* is caused by the accumulation of damage in *Acartia* sp. once the toxin concentration exceeds a certain threshold level. Once this internal threshold is reached of how much *Acartia* sp. can tolerate, *C. leadbeateri* begins to interfere with the *Acartia* sp. biological processes, causing damage and eventually leading to mortality. The GUTS model fits a set of differential equations to the survival data, and estimates the parameters describing the uptake rate, threshold concentration, killing rate, and background mortality. The killing rate (slope of damage increase) parameter represents the relationship between *C. leadbeateri* concentration and the probability of *Acartia* sp. death, while the threshold concentration represents the *C. leadbeateri* concentration at which toxic effects on *Acartia* sp. begin to occur. The GUTS model predictions for survival and damage dynamics are plotted in *Figures S9* and *S10* in the Appendix B: Supplementary Results. The model estimated LC-50 values by calculating the slope and

threshold concentration parameters from the experimental data and predicted the concentration of *C. leadbeateri* at which 50% of the *Acartia* sp. population present would die.

2.5.2 Grazing rates during toxin induction experiment

Differences in grazing of varying number of *Acartia* sp. individuals present were tested with a 1-way ANOVA from simple linear regression models. The linear models tested the fecal pellet amount per individual and length (µm) as a function of the number of grazers. The analyses were performed with R version 4.1.3 (R Core Team 2022).

3 Results

The results are presented in the following order: 1) Second copepod tolerance experiment to *C. leadbeateri*, 2) Third copepod tolerance experiment to *C. leadbeateri*, 3) Second toxicity induction experiment of *C. leadbeateri* through grazing of *Acartia* sp., and 4) *in vitro* toxicity tests using two fish gill cell lines. As mentioned in the Materials and Methods section, the results from the first pilot tolerance experiment using glass bottle incubation and three trials using the multi-well plate incubation are presented in Appendix B: Supplementary Results. The results presented here thus begin with Experiment 2 testing *Acartia* sp. tolerance to cultures of *C. leadbeateri*.

The first and second part of the results addressed if the presence of toxic *C. leadbeateri* influenced the survival and activity of *Acartia* sp. Specifically, the experiments determined the optimal concentration of *C. leadbeateri* that enabled the copepods to survive and continue grazing.

The third part of the results presents the data from the second toxicity induction experiment that included the grazing frequency observed in each treatment of varying numbers of grazers present. Grazing was not observed in the first toxicity experiment which explains the absence of results from Experiment 1: toxicity induction through grazing.

3.1 Experiment 2: Copepod tolerance to *Chrysochromulina leadbeateri*

The aim of this experiment was to determine *Acartia* sp. tolerance as a function of C. *leadbeateri* concentration. The highest *C. leadbeateri* concentration at which *Acartia* sp. showed the highest survival rate was selected for the subsequent grazing-induced toxicity experiment.

The third tolerance experiment was carried out with eight experimental units (six different potentially toxic *C. leadbeateri* cell concentrations and two control treatments on *Acartia* sp.

The six concentrations were 25 000, 3200, 1600, 800, 400 and 100 cells ml^{-1}. The two control treatments were the non-toxic *Chrysochromulina throndsenii* (25 000 cells ml^{-1}) and IMR ½ medium with no algal cells. Five replicates of each algal concentration were used, with 10 copepods in each replicate (well). The tolerance to different algal concentrations and strains were measured by the number of *Acartia* sp. individuals that had survived at different time checkpoints (6, 24, 48, and 72 hours), which is summarized in *Figure 17*.

Points in *Figure 17A* represent the number of *Acartia* sp. individuals (of 3) that survived in each of the five replicates at each time checkpoint in response to different algal concentrations exposure. Points in *Figure 17B* represent the total number of *Acartia* sp. individuals from the five replicates in response to different algal concentrations exposure. The trendlines show the overall pattern of copepod survival for easier visualization.

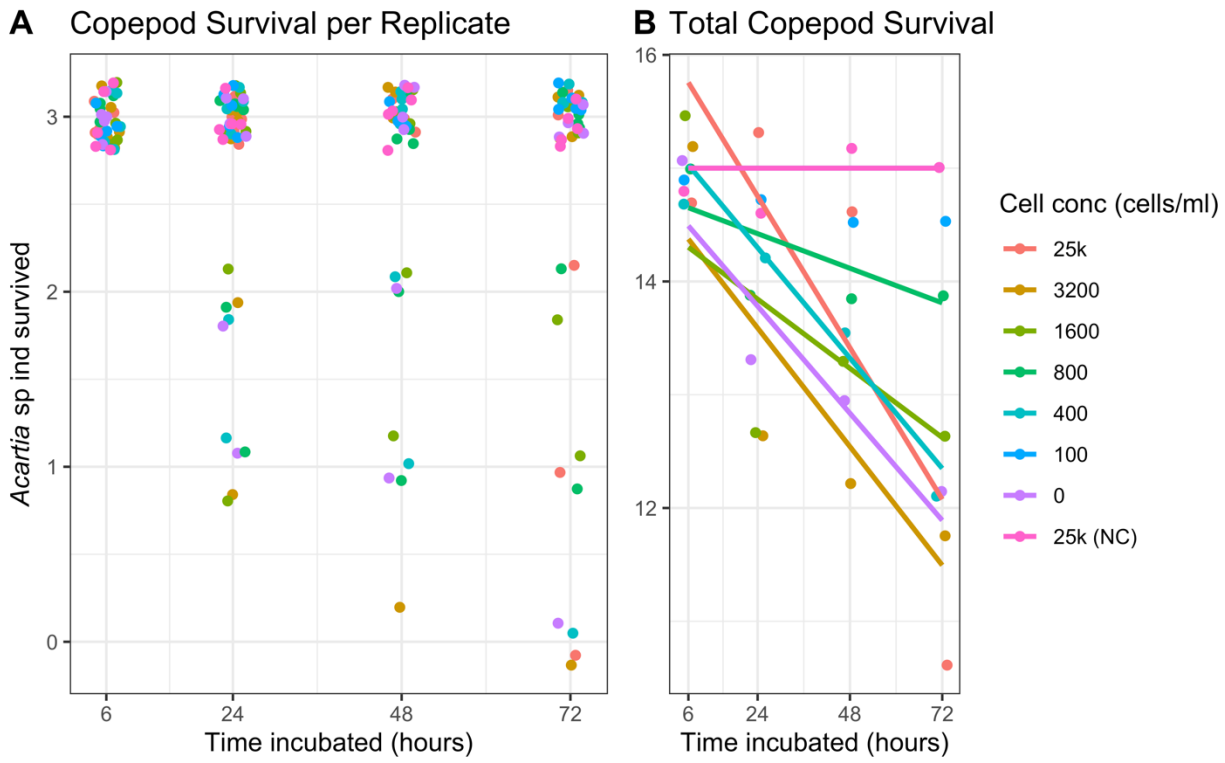

Figure 17. Number of Acartia *sp. individuals that survived at different time checkpoints during the second tolerance experiment.* ***A)*** *Copepods that survived at different algal concentrations during different time checkpoints. Each outcome of copepod survival from each replicate are plotted.* ***B)*** *The outcome of copepod survival from each replicate exposed to the same algal concentration are combined and plotted for a better visualization of the results. Trendlines showing the overall pattern of copepod survival are plotted for easier interpretation of the results.*

Figure 17 shows that all copepods survived after six hours incubation in any of the tried *C. leadbeateri* concentrations. Then there was a decrease in *Acartia* sp. survivors the longer the incubation, with all the concentrations except with 100 ml^{-1} of *C. leadbeateri* and at 25 000 cells ml^{-1} of the negative control, non-toxic *Chrysochromulina throndsenii*. This suggests that the toxic effect of *C. leadbeateri* on *Acartia* sp. is time dependent, as the toxins need to be absorbed by *Acartia* sp. and cause damage. The lowest survival appears to be at 25 000 cells ml^{-1} of the toxic *C. leadbeateri*. There are mortalities from the control containing no algae, but this may be from starvation or spontaneous mortality.

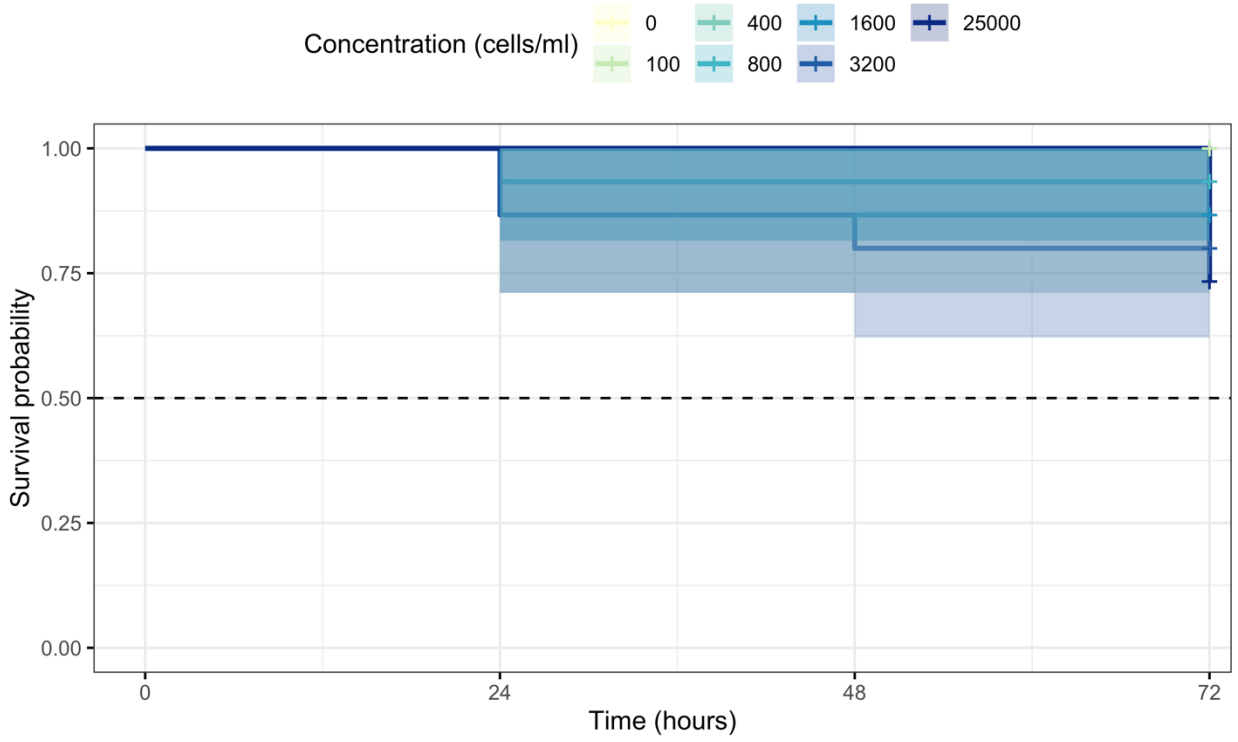

Figure 18. A survival plot showing the survival probability of Acartia *sp. when exposed to different* C. leadbeateri *concentrations (cells ml^{-1}) for 72 hours. This is from the 2nd copepod tolerance experiment.*

The survival probability across all tested *C. leadbeateri* concentrations was one for the first 24 hours of incubation, meaning all incubated copepods have survived (*Figure 18*). As time progresses, the survival probabilities for algal concentrations higher than 100 cells ml^{-1}, seem to decrease over time. At 24 hours onwards, survival scale lowers with higher *C. leadbeateri*

concentrations. This means the probabilities the copepods survive being incubated in those *C. leadbeateri* concentrations are time dependent since the toxins need to be taken up by *Acartia* sp. to cause mortalities.

When analyzing the data with a Cox proportional hazards model, the hazards ratio was very small per increase in cell concentration but was not significant (Wald test = 1.52 on 1 df, p = 0.2).

3.2 Experiment 3: Copepod tolerance to *Chrysochromulina leadbeateri*

The aim of this experiment, like the previous Experiment 2, was to determine *Acartia* sp. tolerance to various *C. leadbeateri* concentrations, but in a higher algal concentration range.

The third tolerance experiment was carried out with six experimental units (four different toxic *C. leadbeateri* cell concentrations and two control treatments on *Acartia* sp. The four different concentrations were 200 000, 100 000, 50 000, and 25 000 cells ml^{-1}. The two control treatments were of the non-toxic *Haptolina ericina* (200 000 cells ml^{-1}) and IMR ½ medium with no algal cells. Five replicates of each algal concentration were used, with three copepods in each replicate (well). This tolerance to different algal concentrations and strains were measured by the number of *Acartia* sp. individuals that had survived at different time checkpoints (6, 24, 48, and 72 hours), which is summarized in *Figure 19*.

Points in *Figure 19A* represent the *Acartia* sp. individuals that survived in each of the five replicates at each time checkpoint in response to different algal concentrations exposure. Points in (*Figure 19B*) represent the total number of *Acartia* sp. individuals from the five replicates in response to different algal concentrations exposure. The trendlines show the overall pattern of copepod survival for easier visualization. When each copepod survival from each replicate is plotted (*Figure 19A*, there appears to be mortalities in at least one replicate from every concentration but the non-toxic control. Mortalities also increased with longer incubation time.

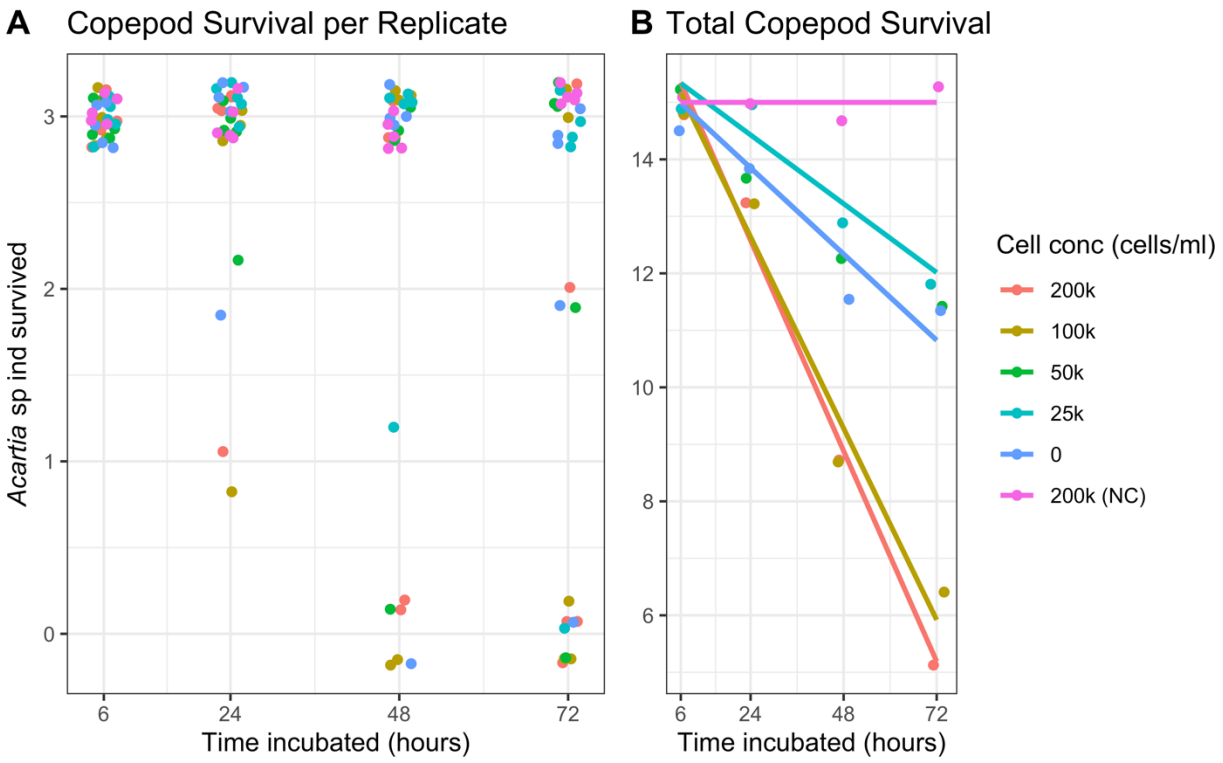

Figure 19. Number of Acartia *sp. individuals that survived at different time checkpoints during the third tolerance experiment.* ***A)*** *Copepods that survived at different algal concentrations during different time checkpoints. Each outcome of copepod survival from each replicate are plotted.* ***B)*** *The outcome of copepod survival from each replicate exposed to the same algal concentration are combined and plotted for a better visualization of the results. Trendlines showing the overall pattern of copepod survival are also plotted for easier interpretation of the results.*

Figure 19 shows that all copepods survived after six hours incubated in any of the tried *C. leadbeateri* concentrations. Then there was a decrease in *Acartia* sp. survivors the longer the incubation with all *Haptolina ericina* concentrations. This again suggests that the toxic effect of *C. leadbeateri* on *Acartia* sp. is time dependent, as it takes time for the toxins to be absorbed by *Acartia* sp. and cause damage. The lowest survival appears to be at 200 000 cells ml^{-1} of the toxic *C. leadbeateri*. There are mortalities from the control containing no algae, but this may be from starvation or spontaneous mortality.

When grouping the replicates of each algal concentrations and observing copepod survival (*Figure 19B*), it appears that there is the highest copepod survival when exposed to 200 000 cells ml^{-1} of the non-toxic *H. ericina* (negative control) with no mortalities witnessed during the

72-hour incubation. The lowest survival appears to be at the higher algal concentrations of *C. leadbeateri* at 100 000 and 200 000 cells ml^{-1}. Apart from the negative control, all treatments of *C. leadbeateri* concentrations show a downward trend in *Acartia* sp. survival. From the steps shown in *Figure 19A*, the general trend is that the higher the *C. leadbeateri* concentration is, the lower the *Acartia* sp. survival.

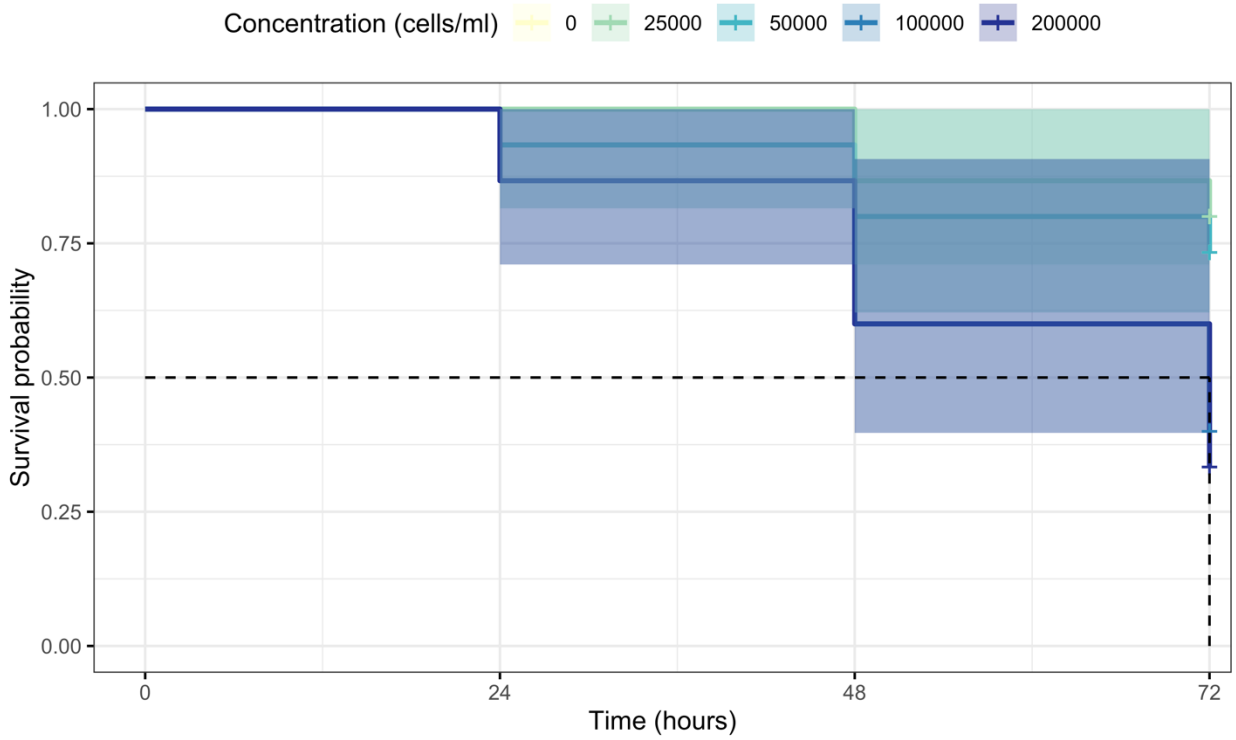

Figure 20. A survival plot showing the survival probability of Acartia *sp. when exposed to different* C. leadbeateri *concentrations (cells ml^{-1}) for 72 hours. This is from the 3rd copepod tolerance experiment.*

The survival probability across all tested *C. leadbeateri* concentrations was one for the first 6 hours of incubation, meaning all incubated copepods survived (*Figure 20*). As time progresses, the survival probabilities for concentrations, except 0 and 25 000 cells ml^{-1}, decrease over time. The lowest survival probabilities are from incubation past 24 hours with 200 000 cells ml^{-1} *C. leadbeateri*, with survival probability reaching below 0.5 after 48-72 hours incubation. This means the probabilities the copepods survive being incubated in those *C. leadbeateri* concentrations are time dependent since the toxins need to be taken up by *Acartia* sp. to cause mortalities.

When analyzing the data with a Cox proportional hazards model, the hazards rate was very small per unit increase in cell concentration (Wald test = 10.32 on 4 df, p = 0.04). To better interpret the results, cell concentration was therefore implemented as a factor to calculate the hazard ratios for specific concentrations. At 200 000 cells ml^{-1} of *C. leadbeateri*, *Acartia* sp. are 3.2 times more likely to die (exp(coef) = 3.1992, p = 0.05) than the control without *C. leadbeateri*.

The LC-50 values graph generated from the GUTS model provided a visual representation of the toxicity of *C. leadbeateri* to *Acartia* sp. over 15 days, but we focus on the first three days as that was the duration of the induction experiment (*Figure 21*). The GUTS model predicted the LC-50 value to be ca. 750 000 cells ml^{-1} of C. leadbeateri after one day, ca. 250 000 cells ml^{-1} the second day, and ca. 125 000 cell ml^{-1} the third day ($p<0.05$). From day one to two, the slope of the line is the steepest indicating the most rapid increase in toxicity, followed by day three and so on. The slope of the line decreases with increasing days, indicating a slower increase in toxicity.

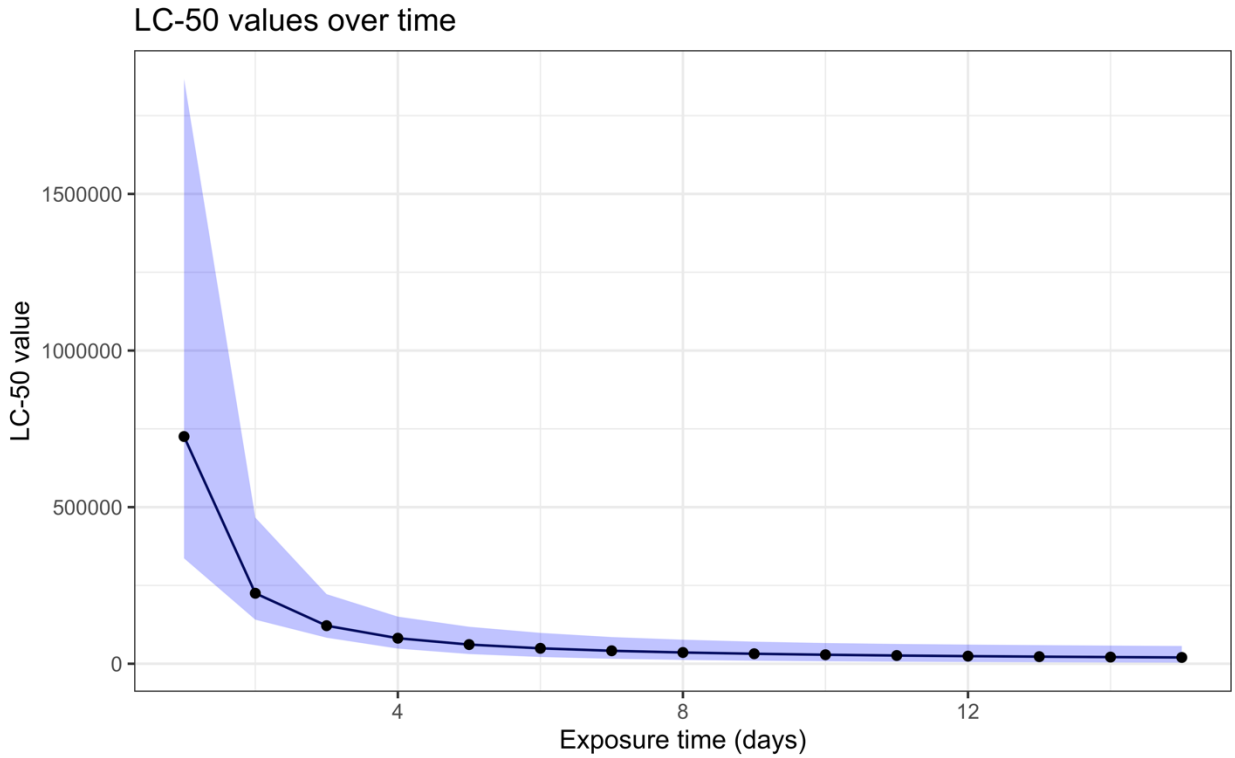

Figure 21. Predicted LC-50 values of C. leadbeateri *(cells ml^{-1}) over a 15-day exposure period using the estimated GUTS model parameters.*

The predicted LC-50 value was the highest at ca. 750 000 cells ml^{-1} after one day exposure, ca. 250 000 cells ml^{-1} after two days, and 125 000 cells ml^{-1} after three days exposure. In general, the LC-50 values are the highest at the beginning of the exposure period, indicating that the toxic effect of *C. leadbeateri* is time dependent since the toxins needs to be taken up and damage *Acartia* sp. needs to accumulate to cause mortality. However, at long exposure times, the LC-50 values decrease, indicating that *Acartia* sp. is not tolerate of high concentrations of *C. leadbeateri* over time.

The main objective of the copepod tolerance experiments was to find at which *C. leadbeateri* concentration *Acartia* sp. exposed to would have the highest survival rate. From both tolerance experiments, it was determined highest total *Acartia* sp. survival rate was at 100 cells ml^{-1} *C. leadbeateri*. However, that concentration was too low to obtain proper algal extracts from for the later *in vitro* toxicity tests. So, for the subsequent toxicity induction experiment, 50 000 cells ml^{-1} of *C. leadbeateri* was selected. The concentration was a high enough concentration to create extracts from while causing relatively "low" mortalities to the *Acartia* sp. exposed to it.

3.3 Experiment 2: Toxicity induction through grazing

3.3.1 Grazing rates

The production of fecal pellets by *Acartia* sp. can reflect its grazing and feeding activity during the experiment. The grazing values calculated according to Frost (1972), were positive, albeit not significantly different from zero (Appendix B: *Table S9*).

The fecal pellets produced per individual and average fecal pellet length as a function of the number of grazers are shown in *Figure 22.* The highest number of fecal pellets produced per individual is at the treatments with one copepod present, but at treatments with 5, 10, and 20 grazers, there seems to be an upward trend in pellets per individual. With fecal pellet length, except for the outlier at five copepods, fecal pellet lengths seem to decrease with increasing numbers of grazers present.

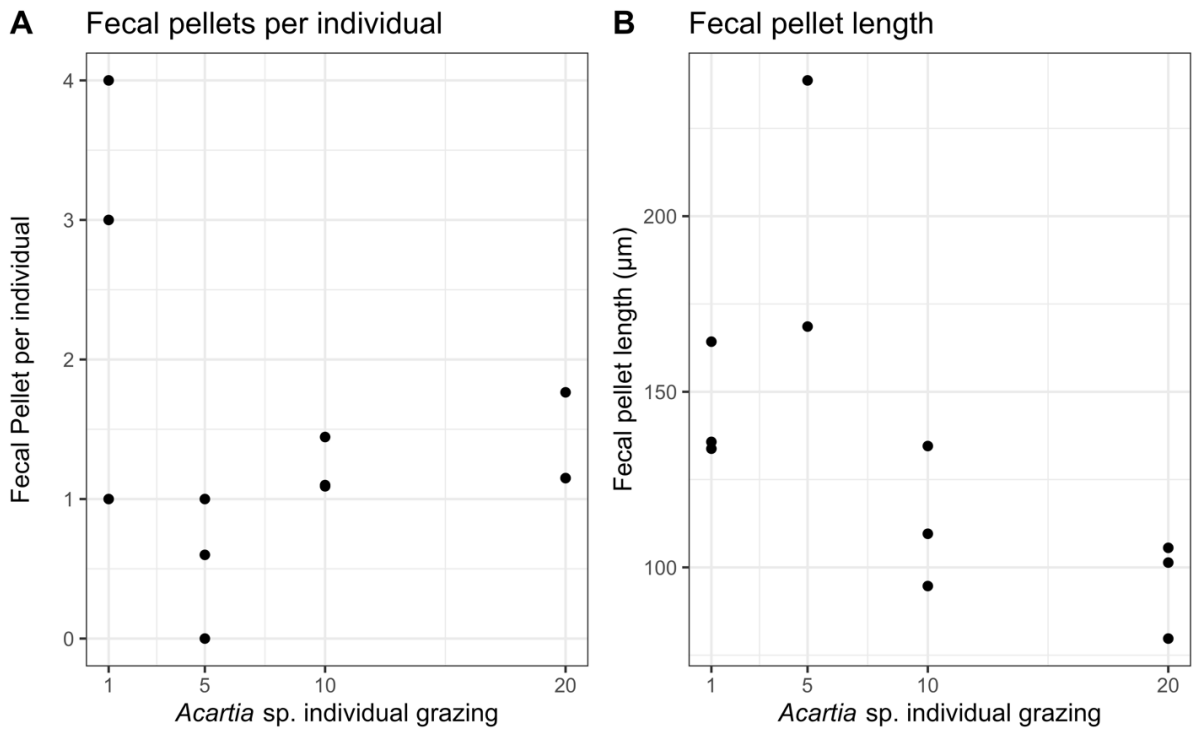

Figure 22. ***A)*** *Number of fecal pellets per individual* Acartia *sp. in each treatment of varying number of grazers present.* ***B)*** *Fecal pellet length (µm) from three pellets from each treatment of varying number of grazers present.*

This could be that with more grazers present, there is a possibility for copepods to encounter pellets while grazing and swimming around and break them apart into smaller pieces. So, one larger fecal pellet becomes multiple smaller pellets.

The functional response of *Acartia* sp. with *C. leadbeateri* was that fecal pellet length (µm) increased with increasing *Acartia* sp. grazers present (1-way ANOVA: $\mathrm{df}_{\mathrm{grazers,total}}$ = 3; $\mathrm{F}_{\mathrm{grazers}}$ = 8.7; p = 0.009). In contrast, it was not statistically significant that fecal pellets produced per individual increased with increasing grazers present (p=0.07).

3.4 *In vitro* toxicity tests using RT-W1 gill and ASG-10 gill cell lines

The experiment aims to test whether copepod grazing can induce toxicity in the presumed toxic haptophyte, *Chrysochromulina leadbeateri* through testing toxicity through two different fish epithelial gill cell lines.

3.4.1 Effect of grazed *C. leadbeateri* on Rainbow trout gill cellular viability

We observed grazed *C. leadbeateri*-induced toxicity (at 250 000 cells ml^{-1}) on RT-gill W1 (Rainbow trout) by measuring cellular viabilities (% of control) when exposed to different algal extract concentrations. The extracts were made from *C. leadbeateri* material exposed to varying numbers of grazing *Acartia* sp. individuals. When comparing the same algal extract concentration, there does not seem to be any clear decreases in average gill cellular viability with increases in *Acartia* sp. grazers present (*Fig. 23A*). However, it can be clearly observed that cellular viability reduces with increasing concentrations of algal extract. Average cellular viabilities at 1% algal extract were all ca. 10% for all varying number of grazing copepods while lower concentrations of algal extracts showed increases in cellular viabilities. Cells exposed to 0.5% algal extract, across all varying levels of grazing, showed ca. 20% cellular viability and at 0.1% extract, cellular viabilities reached 50% and increased. In general, and excluding the solvent control, cells exposed to the lowest algal extract at 0.1% increases in cellular viability. Exposure to the highest algal extract of 1% showed decreases in cellular viability.

3.4.2 Effect of grazed *C. leadbeateri* on Atlantic salmon gill cellular viability

We observed grazed *C. leadbeateri*-induced toxicity (at 50 000 cells ml^{-1}) on ASG-10 (Atlantic salmon) by measuring cellular viabilities (% of control) when exposed to different algal extract concentrations. The extracts were made from *C. leadbeateri* material exposed to varying numbers of grazing *Acartia* sp. individuals. When comparing the same algal extract concentration, there does not seem to be any clear decrease in gill cellular viability with increases in *Acartia* sp. grazers present (*Fig. 23B*). However, it can be clearly observed that

cellular viability goes down with increasing concentrations of algal extract. At the highest extract concentration of 1%, it appeared consistent across all treatments of grazing copepods that the cellular viabilities showed a sharp reduction compared to the gills exposed to the other extract concentrations. This indicated a negative effect towards gill cellular viability upon increasing *C. leadbeateri* exposure and confirmation of *C. leadbeateri* being ichthyotoxic. Unlike in the RTgill-W1 bioassay, it did not appear that the lowest algal extract concentration (0.1%), showed an increase in cellular viabilities compared to higher extract concentrations (1% and 0.25%). Between the treatment groups of varying grazer individuals, the highest average cellular viabilities observed alternated between 0.25% and 0.1% algal extracts. At 1, 10, and 20 grazing copepods, cellular viabilities increased (ca. 110%) at 0.1% algal extract compared to the other extracts in their group. However, at 0 and 5 copepods, the increased cellular viabilities were observed at 0.25% extract with ca. 100% and 110%, respectively.

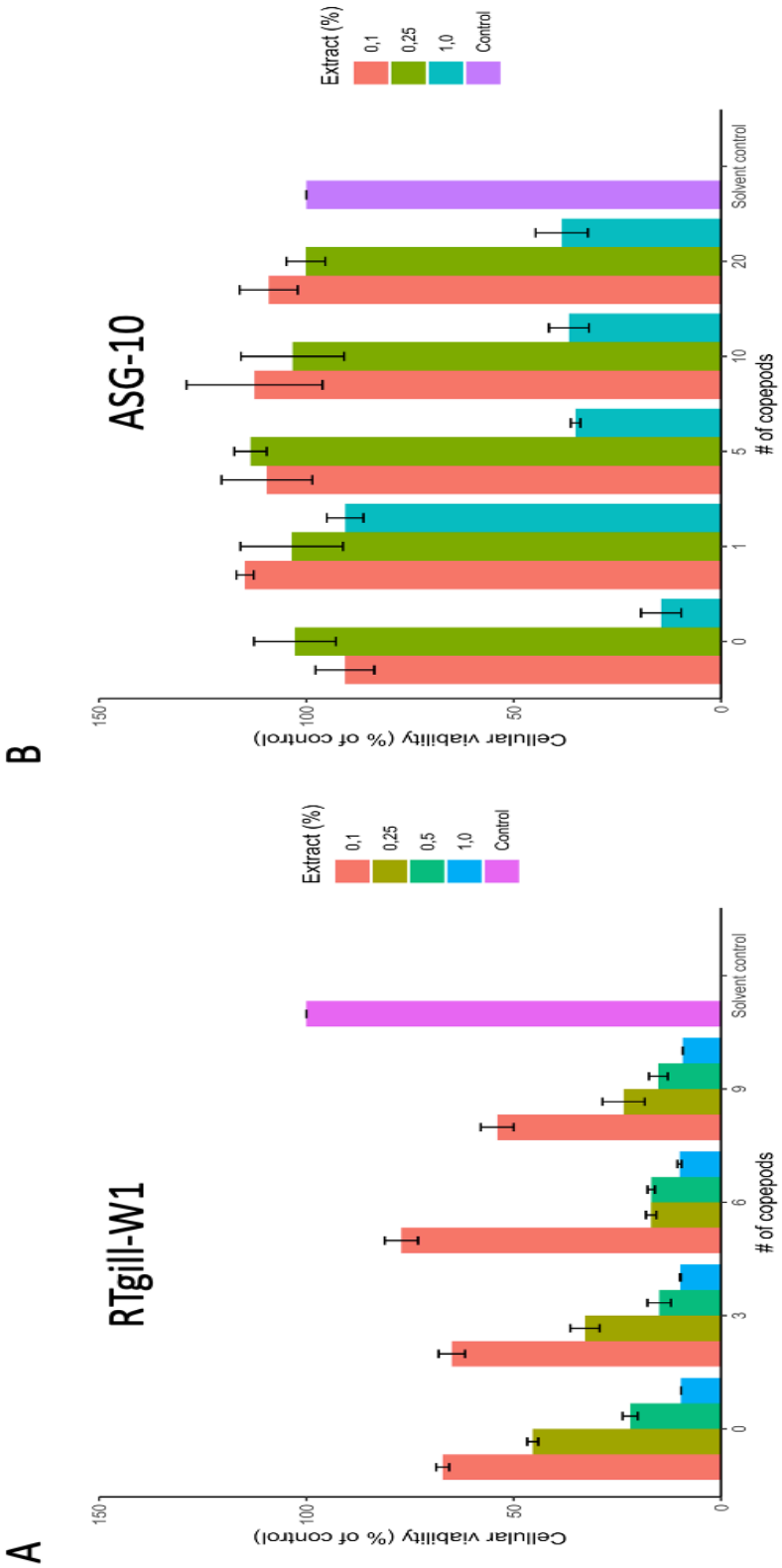

Figure 23. Viability of the gill cells after exposure to four concentrations of algal extract. The bars represent the mean and error bars the standard deviation of cell viability from triplicate wells containing 0.1%, 0.25%, 1%, and the solvent control. ***A)*** *RTgill-W1 bioassay with copepods grazing at 250 000 cells ml^{-1} C. leadbeateri and* ***B)*** *ASG-10 gill bioassay with copepods grazing at 50 000 cells ml^{-1}.*

3.4.3 Cytotoxicity of *C. leadbeateri* on Atlantic salmon epithelial gill cells

The cytotoxicity of *C. leadbeateri*, as a measure of cellular viability, was examined through investigating the morphology of Atlantic salmon epithelial gill cells after exposure. When comparing just between the algal extract concentrations groups, the higher algal extract concentrations are, the more damage it causes to the epithelial gill cells (*Figure 24*). The gill cells exposed to the lower algal extract concentrations like the solvent control (1% MeOH) and 0.1% extract, appeared plump and irregular-shaped polygonal. The cells also appeared more confluent with an even distribution. In contrast, at the higher algal extract concentrations of 0.25% and 1%, the cells appear shrunken or distorted, with irregular cell membranes and nuclei. The cells are not as confluent with the cells appearing to aggregate and clump together. In general, as the algal extract concentration that the epithelial gill cells are exposed to increases, there are corresponding observable damage to the gill cells, as evidenced by less confluent and shrunken cells, distorted cell membranes, less distinguishable nuclei, and aggregation or clustering of cells.

Interestingly, between the same algal extract concentrations exposed to different number of grazing copepods, some variations were observed especially in the higher algal extract concentrations. This can be seen when comparing the gill cells exposed to 0.25% extract of the algae with 0 versus 20 grazing copepods. The gill cells exposed to the more grazed upon algae observed more damage, at least with less defined cell membranes and less confluency which might suggest a stronger toxicity effect. Additionally, gill cells exposed to 1% extract of algae has observable damage with increasing number of grazing copepods. While there is still noticeable damage at 1% extract with one copepod, there are more defined cell membranes and less “shrinking” of cells compared to algae with higher numbers of grazers. However, it is noted that with zero copepods present, there was considerable decrease in viability (*Figure 23*) and that is seen also in *Figure 24* with the noticeable “shrinking” and less defined cell membranes.

Although the observable damages seen in epithelial gill cells are not as prominent as when comparing between increasing algal extract concentrations, there are some noticeable increases in gill cell damages between the same algal extract concentrations but different number of grazing copepods present. This could suggest grazing pressure plays some role in determine toxicity level in *C. leadbeateri* and subsequent damage to epithelial gill cells of Atlantic salmon.

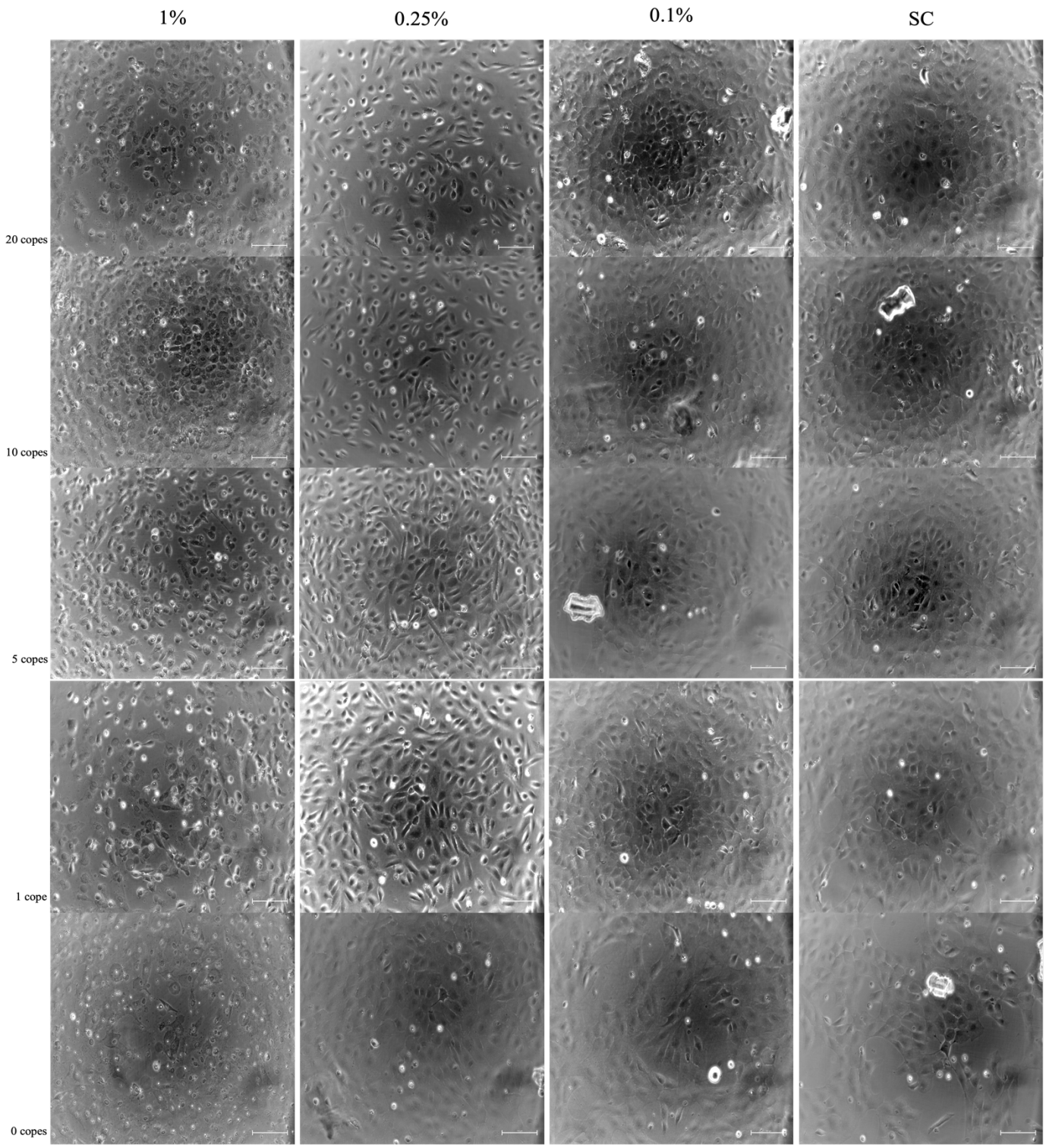

Figure 24. Microscope images from one replicate well of each treatment of ASG-10 gill cells after exposure to varying number of grazing copepods and algal extract concentrations of Chrysochromulina leadbeateri*. The original grazed algal concentration was 50 000 cells ml^{-1}. Magnification at 200x and bar = 100 µm.*

4 Discussion

This thesis aimed to gain more insight on the biology and toxicity potential of the toxic haptophyte, *Chrysochromulina leadbeateri*, by focusing on the predator-prey interactions with *Acartia* sp. My findings confirm that while *C. leadbeateri* is toxic to fish gills, grazing by *Acartia* sp. does not seem to induce nor influence toxicity in the haptophyte. My experiments show that grazing can enhance toxicity potential in *C. leadbeateri*, but there is a need for more data to conclusively state that increased grazing pressure influences *C. leadbeateri* toxicity production. Additionally, I found that the tolerance levels for *Acartia* sp. depend on the *C. leadbeateri* concentration and exposure time to *C. leadbeateri*. Grazing behavior of *Acartia* sp. also do not seem to be affected by *C. leadbeateri* in my experiments. However, there needs to be more data from trials exposing *Acartia* sp. to non-toxic food alternatives and different *C. leadbeateri* concentrations to make proper conclusions.

My study focuses on *C. leadbeateri,* which, compared to other toxic haptophytes like *P. parvum* and *P. polylepis*, is a relatively unknown species. While it has been established that *C. leadbeateri* is ichthyotoxic, my findings measuring toxicity are limited as the specific toxins of *C. leadbeateri* causing damage to fish gills are still unknown. Furthermore, although the actual induction experiment occurred under the favorable temperature and light conditions for algal growth, it did not occur in conditions (high N:P ratios, low salinities) that were found to promote haptophyte blooms (Edvardsen & Paasche, 1998; Lekve et al., 2006). This limits to how representative the results are for the toxicity of *C. leadbeateri* in nature if they were not in "optimal" toxicity conditions. Nonetheless, my results confirm that *C. leadbeateri* is toxic to fish gills and *Acartia* sp. They also suggest that grazing pressure does not influence *C. leadbeateri* toxicity capabilities.

I will first discuss the three parts of this thesis in the same consecutive order as previous sections: 1) Activity and survival of copepods from the tolerance experiments, 2) Grazing patterns from the toxicity induction experiment, and 3) The measured induced toxicity from the

in vitro toxicity tests using fish epithelial cell lines. This involves the questions they aimed to answer in the order they were answered, what the results indicate, the changes that could have been made to give more insight, and their place in the larger context of previous research. Then I will address the hypotheses originally made and how future studies can build on the results from this thesis to provide further insight into the research questions posed.

4.1 What is the threshold *Chrysochromulina leadbeateri* concentration that *Acartia* sp. can maintain survival and grazing?

The experiments' main focus has been determining the threshold *C. leadbeateri* concentration *Acartia* sp. could survive in to maintain grazing for use in the subsequent toxicity induction experiment. Through investigating this threshold, my experiments showed that *C. leadbeateri* influences the survival and activity of *Acartia* sp. depending on the concentration of *C. leadbeateri* cells and exposure time, which I discuss in further detail below.

Survival and activity of copepods

When incubated with the non-toxic algal controls (*Chrysochromulina throndsenii* and *Haptolina ericina*), even in high concentrations, *Acartia* sp. were active and survived throughout the whole 72-hour incubation period. There were copepod mortalities observed in the IMR ½ medium negative control, but that was most likely caused by starvation or spontaneous mortality. In contrast, copepods incubated with the toxic *Chrysochromulina leadbeateri* at high enough concentrations showed impaired movement with no movement after at least 10 seconds and marked mortalities with no signs of pipette avoidance after the final check at 72 hours. Also, my results showed that copepod mortalities increased with time incubation, because it takes time for the toxins to be taken up by *Acartia* sp. to cause damage and result in mortality. Although, it is important to note that mortalities and inactivity were present in all concentrations of *C. leadbeateri*. This is consistent with the findings from the Sopanen et al. (2006) study that found that any concentration of another toxic haptophyte, *Prymnesium parvum*, copepods were exposed to soon became inactive.

Previous studies on short-term toxicity of haptophytes on copepods have shown conflicting results. A study with a shorter incubation time of 48 hours with toxic *Prymnesium patelliferum*, showed no signs of acute mortality or inactivity of the exposed copepods (*Calanus finmarchicus* and *Acartia clausi*) (Nejstgaard et al., 1995). In accordance with this study, late copepodites and adult *A. clausi* showed no mortality even when kept in very dense cultures (10^6 cells ml^{-1}) of *P. patelliferum* for 48 hours (Nejstaard & Solberg, 1996). In contrast, another study found elevated mortalities in *Eurytemora affinis* when exposed to high concentrations of *P. patelliferum* compared to lower mortalities when exposed to lower concentrations and to non-toxic food alternatives (Koski, et al., 1999). It is important to note that the study found *P. patelliferum* harmful to copepods if offered as the sole food source, like I did in this thesis with *C. leadbeateri*. Other studies that tested copepod activity survival and fed copepods both toxic and non-toxic, high quality food sources simultaneously showed different results. When *E. affinis* was incubated in cell mixtures of both the toxic *P. parvum* and non-toxic *Rhodomonas salina*, no lethal effects were detected, and the majority of the copepods stayed active throughout the 72-hour experiment (Sopanen et al., 2008). This could be because copepods are more selective in their feeding, and when given a high-quality and non-toxic alternative, they would choose the non-toxic species. A study looking at short-term toxicity effects of *P. parvum* on different zooplankton communities found the responses to be species-specific and that copepods were impacted less short-term if given food alternatives (Witt et al., 2019).

The main objective of these tolerance experiments was to find the optimal *C. leadbeateri* concentration *Acartia* sp. could survive in and continually graze. It also gave some insight into how the toxic haptophyte, *C. leadbeateri*, could potentially affect the viability and grazing of *Acartia* sp. There are several native *Acartia* sp. species to Norway like *Acartia clausii*, *Acartia longiremis*, and *Acartia discaudata*, as well as non-native species like *Acartia tonsa* recorded in Norway (Husa et al., 2022; Moseid et al., 2021; Næss, 1996). With past *C. leadbeateri* blooms that have been recorded along the Northern Norwegian coastlines (Rey, 1991; Samdal & Edvardsen, 2020), it was useful to study these complex interactions between these predator and prey.

From my experiments, I conclude that *C. leadbeateri* influences the survival of *Acartia* sp., and this influence becomes greater with increasing cell concentration and time incubated. I rule out that the possibility that high concentration of algae per se caused potential adverse effects on copepod survival and feeding, since the all the copepods survived in the non-toxic high concentration treatment. Such effects are caused, for example, in high turbidities caused from silt in the water (Carrasco et al., 2013). This indicates further to the toxicity potential of *C. leadbeateri*. However, in reality, it may not be as big as an issue if copepods are more selective feeders and choose non-toxic food alternatives to *C. leadbeateri*, if provided the chance. Then the more selective copepod grazers would be less susceptible to *C. leadbeateri* toxicity effects, like with in the case with *P. parvum* (Witt et al., 2019). This leads to the next part in the discussion where actual copepod grazing was observed: the induction experiment.

Feeding behavior (grazing and egestion) of copepods

The main purpose of measuring fecal pellet production was to verify that *Acartia* sp. fed on *C. leadbeateri* during the induction experiment. The presence of fecal pellets after the toxicity induction experiment showed that *Acartia* sp. did graze and ingest *C. leadbeateri*. The positive grazing values calculated according to Frost (1972), meant grazing exceeded algal growth. At least, algal growth was not higher in the presence of copepods from possible enhanced nutrient regeneration as concluded by Sopanen et al. (2006).

Also, from my results and neglecting the one copepod grazers treatment, fecal pellets produced seem to increase per individual grazer with increasing grazers present (*Figure 22A*). However, this increase was not statistically significant. Fecal pellet length (µm) decreased with increased copepod presence. A possible explanation is that with more grazers present, there is a higher tendency of individuals to handle or graze on the pellets, breaking one larger pellet into multiple smaller pellets.

It is difficult to give more insight into the feeding responses of *Acartia* sp. to *C. leadbeateri* because there is insufficient information. Information on *Acartia* sp. grazing behavior when exposed non-toxic algal food alternatives would have been ideal. To gain more insight on the

influence of *C. leadbeateri* on copepod grazing, one needs to measure *Acartia* sp. grazing in non-toxic controls (Koski et al., 1999; Sopanen et al., 2006, 2008). Koski et al. (1999), found no significant differences in *Eurytemora affinis* ingestion rates between the toxic *P. patelliferum* and non-toxic controls. Sopanen et al. (2006, 2008), studies used *Rhodomonas salina* as non-toxic food source controls. Two copepod species (*Acartia bifilosa and E. affinis*) mostly avoided feeding on the toxic P*. parvum* which reflected in the low pellet production. Even the lowest concentration at 2000 cells ml^{-1} was enough to deter grazers (Sopanen et al., 2006). Other studies have shown similar avoidance in feeding or feeding in less amounts when copepods were exposed to *P. parvum* (Nejstgaard et al., 1995; Nejstgaard & Solberg, 1996). From my results, it seems like *Acartia* sp. fed as normal with *C. leadbeateri,* but with no non-toxic control, I cannot conclusively state that the toxic *C. leadbeateri* does not affect *Acartia* sp. feeding.

Further insight on the potential influence of *C. leadbeateri* on *Acartia* sp. could have been gained by exposing *Acartia* sp. to a range of algal concentrations and measuring fecal pellet production. This could have been done during the earlier copepod tolerance experiments. A study found that copepods exposed to higher *P. parvum* cell densities showed a significant decrease in grazing activities, as evidenced by lower fecal pellet production (Sopanen et al., 2008). In contrast, Koski et al. (1999), found no significant differences in *E. affinis* ingestion rates between *P. patelliferum* concentrations. Sopanen et al. (2006), found the feeding of *E. affinis* and *A. bifiolosa* too low on *P. parvum* to allow observations of any functional responses to increasing cell concentrations.

To conclude, grazing activity was observed in the toxicity induction experiment and was verified by fecal pellet production. Fecal pellet length (µm) was directly proportional to increasing amount of grazer presence. However, there was no significant relationship found between fecal pellets produced per individual and increased grazer presence. While it is possible that grazing activity, like survival and swimming activity, of *Acartia* sp. could be negatively affected by *C. leadbeateri*, there is, for now, too little information given to make those conclusions. Future

experiments measuring grazing and egestion activity when copepods are exposed to multiple *C. leadbeateri* concentrations, and a non-toxic algal food alternative would give us that necessary information.

4.2 Does the presence of copepod grazers influence toxicity in the ichthyotoxic *C. leadbeateri?*

Chrysochromulina leadbeateri has caused massive fish kills when they are in bloom (Rey, 1991; Samdal & Edvardsen, 2020). Additionally, studies have showed that at different abiotic factors like high N:P ratios, low salinities, reduced vertical mixing, and and high solar irradiance are favorable for haptophyte blooms (Edvardsen & Paasche, 1998; Lekve et al., 2006). This thesis, instead, focuses on the how biotic factors, like predator-prey relationships, can influence toxicity potential of *C. leadbeateri*. The main focus of the toxicity induction experiments and subsequent *in vitro* toxicity tests has been on the general question of whether or not the presence of copepod grazers influence toxicity in the ichthyotoxic *C. leadbeateri*. Through investigating these possible influences, my experiments showed that *C. leadbeateri* extracts does have some adverse effects on gill cell viability upon direct exposure, in both the RTgill-W1 and ASG-10 cell line. However, my results come with limitations which I discuss below, as well as comparisons between both cell lines results and morphological responses of ASG-10 gill cells to the extracts.

Predation pressure influences on *C. leadbeateri* toxicity potential

My experiments showed that *C. leadbeateri*, a known ichthyotoxic algae, reduces cellular viabilities in both RTgill-W1 (Rainbow trout) and ASG-10 (Atlantic salmon). The higher the *C. leadbeateri* extract percentages are exposed to the cell lines, the greater the reduction in cellular viabilities are. This is seen when comparing viability results from extract percentages across all treatments of varying number of grazers. However, a clearer inversely proportional relationship between algal extract percentages and cellular viability is more apparent in RTgill-W1 than in ASG-10. This is apparent from the highest extract percentage (1%) not always causing the strongest reduction in cellular viabilities in the treatment groups with varying

grazing pressure-induced toxicity (*Figure 23*). In general, the results might confirm that *C. leadbeateri* is indeed toxic. This is consistent with results from a study by Edvardsen (1993), using an *in vivo Artemia salina* (brine shrimp) bioassay to test for toxicity of *Chrysochromulina* species. The study found that water samples from the 1991 *C. leadbeateri* bloom were toxic to *Artemia* nauplii, although weakly toxic. In contrast, another study found *C. leadbeateri* to lose its toxicity over time (Meldahl et al., 1994) and another did not find *C. leadbeateri* to be toxic through the *Artemia* bioassay at all (Simonsen & Moestrup, 1997).

While my findings suggest *C. leadbeateri* exposure reduces cellular viabilities in both cell lines, it does not seem that increasing grazing pressure affect toxicity potential. I find this by comparing the cellular viabilities from the same algal extract percentages across different treatment groups (# of copepods present). From this comparison, it seems that grazing pressure from 1 to 20 *Acartia* sp. individuals, has no influence on the toxicity potential of *C. leadbeateri*. Additionally, I suggest that grazing by *Acartia* sp. does not also induce toxicity in *C. leadbeateri* either. In the control with no grazers present, there are still reductions in cellular viabilities observed in both cell lines. This indicates that *C. leadbeateri* has toxicity capabilities, reducing cellular viability and causing cell death, with or without the presence of grazing pressure from *Acartia* sp. It could be considered that grazing from other copepod species can promote toxin production and there is a potential prospect for future studies to utilize other grazing species.

These findings are in contrast with other studies who have also used *Acartia* sp. grazers. Selander et al., 2006 conducted a density-dependent experiment and found that the presence of female adult *Acartia tonsa* was directly proportional to increase in paralytic shellfish toxin (PST) production (Gonyautoxins 1, 2, and 4). The increase in PST production was stronger in the highest copepod density (16 copepod l^{-1}) compared to intermediate copepod densities (4 and 8 copepod l^{-1}). Harðardóttir et al. (2015) and Tammilehto et al. (2015) found that grazing *Calanus* copepods and copepodites not only increased the domoic acid production in the toxic *Pseudo-nitzchia seriata*, but induced toxicity in the previously non-toxic *P. obtusa*. Lundholm et al.

(2018) found increasing the number of grazing *Calanus* and *Acartia* copepods resulted in an increase in DA production from *P. seriata and P. obtusa*.

My conclusion comes with notable limitations. Though the cellular viabilities (*Figure 23)* are from the averages of triplicates, only one independent *in vitro* experiment for each cell line was conducted. Ideally 3-4 independent experiments would have been conducted with subsequent statistical analyses, like in Solhaug et al. (2023), to create a stronger basis for my findings. Also, the cellular viabilities were measured after 24 hours of exposure. If viabilities had been observed at more checkpoints like the Solhaug et al. (2023) study did for 24 and 48 hours, it could have given more insight on the toxicity effects of *C. leadbeateri* like if it was time dependent like the earlier-conducted *Acartia* sp. tolerance experiments results suggested.

4.3 Comparison between RTgill-W1 and ASG-10 cell lines

While I used two different fish epithelial cells to test for toxicity of *C. leadbeateri*, it was not for the purposes of comparative study between the well-known RTgill-W1 cell line (Bols et al., 1994) and the newly established ASG-10 cell line (Gjessing et al., 2018). The switch to testing on the ASG-10 cell line was for pragmatic reasons, but it is still worth discussing what the different results obtained from testing on the two cell lines are and why are they different. I compare the results of the two cell lines by discussing two main possible sources for inconsistent results between the cell lines: 1) the treatment of *C. leadbeateri* of which the extracts were made from and 2) extraction processes of *C. leadbeateri*.

Treatment differences

Toxicity effects of *C. leadbeateri* appeared greater when exposed to RTgill-W1 gill cells compared to ASG-10 cells. This was concluded by examining that the general cellular viabilities, across all extract percentages, were lower in the RTgill-W1 cells than the ASG-10 cells. This could be because the algal extracts were made from a much higher *C. leadbeateri* concentration for the RTgill-W1 bioassay at 2.5×10^5 cells ml^{-1} than the ASG-10 at 5×10^4 cells ml^{-1}. If this is as the *Acartia* sp. tolerance test results suggests, then *C. leadbeateri* toxicity

effects are concentration dependent. However, toxicity effects on the gill cells were still observed in both cell lines. When comparing these *C. leadbeateri* concentrations used in the induction experiment, to the past blooms cell concentrations of 2.5 x 10^5 and 5 x 10^5 cells ml^{-1}, these concentrations would have been greater than recorded abundances. In the 2019 *C. leadbeateri* bloom, the highest cell abundance was recorded to be 27.6 x 10^3 cells ml^{-1} (John et al., 2022). In comparison, in the 1991 event, *C. leadbeateri* dominated at total cell densities of >2 x 10^3 cells ml^{-1} (Johnsen et al., 1999).

To conclude, there is an overall greater reduction in cellular viabilities seen in RTgill-W1 compared to ASG-10. However, that might be because the algal extract exposed to RTgill-W1 was made from a higher *C. leadbeateri* concentration. Despite this, both cell lines were subjected to extracts made from high enough *C. leadbeateri* concentrations to create a toxic effect, if compared to recorded cell abundances from past blooms. This again, is evidenced by both RTgill-W1 and ASG-10 observing cellular viability reductions.

A recent study compared the two cell lines and their responses to certain triggers. In the Solhaug et al. (2023), study, the two salmonoid gill cell lines were compared in their tolerances to rotenone-induced toxicity. As part of the assessment of tolerance capabilities of both cell lines, the Alamar blue bioassay was also used to measure cellular metabolic activity and viability. The results showed that RTgill-W1 gill cells exhibited greater reductions in cellular viability when exposed to lower concentrations of rotenone. Also, toxicity effects of rotenone on RTgill-W1 were time dependent, unlike ASG-10. Cellular viabilities measured from the Alamar blue bioassay using two cell lines in this thesis was only measured once after 24-hours exposure to *C. leadbeateri* algal extracts, so I cannot also give conclusive evidence to a statistically significant degree on if *C. leadbeateri* toxicity effects on the lines were time-dependent.

Again comparing the cellular viability results between the two cell lines, there are inconsistencies between the extracts with different copepod numbers. While there seems to be

no clear viability decreases with increasing *Acartia* sp. grazing in both cell lines, accurate comparisons between the two are difficult. The grazing pressure *C. leadbeateri* was subjected to was different for RTgill-W1 and ASG-10. The greatest grazing pressure subjected to *C. leadbeateri* for the RTgill-W1 were nine adult *Acartia* sp. while it was 20 adult *Acartia* sp. for ASG-10. Furthermore, >50% of the grazing copepods were marked dead at the end of the first toxicity induction experiment. So, there is no guarantee that grazing potential was maximized for the grazed-*C. leadbeateri* exposed to RTgill-W1.

Summarizing my findings, I cannot conclusively state that the RTgill-W1 cell line is more sensitive to the toxic effects of *C. leadbeateri* compared to the ASG-10 cell line, because they were exposed to different treatments: two different *C. leadbeateri*-grazed concentrations and different grazing pressures. Also, because the cellular viabilities of the exposed gill cells were only checked one time, I cannot give insight into how the two cell lines might react differently to *C. leadbeateri* extract for longer exposure times.

Extraction procedures

Another possible reason why overall cellular viabilities in RTgill-W1 showed a greater reduction than ASG-10 could be the extraction procedures. The extraction processes for *C. leadbeateri* after direct grazing exposure differed for the bioassays on RTgill-W1 and ASG-10. As mentioned before, algal extracts made for RTgill-W1 cell line was harvested through centrifugation while extract for ASG-10 was harvested through filtration. Because of filtering, the algal material had to be separated from the glass fiber filter. To properly dissolve the algal biomass residue, additional MeOH was added. Then an extra round of centrifugation using the spin columns had to be done, in addition to the extraction procedure done for RTgill-W1. It is possible that the dissolution volume (MeOH) was too high and made the extract too “diluted”. Those extract dilutions were made with specific amounts of MeOH, L15/ex, and extracts. With the extra MeOH for the extracts exposed to ASG-10, it is possible that could have lessened the toxicity potency of the grazed *C. leadbeateri*. At least, it could be ruled out that the addition of 1% MeOH itself had an effect on gill cell viability (Dorantes-Aranda et al., 2011). Regardless, the

potential that the algal samples were diluted by the extra MeOH or that some algal material were still left on the discarded filters could not be ruled out.

In a novel study to assess ichthyotoxicity in harmful marine microalgae Dorantes-Aranda et al. (2011), cellular viabilities of the RTgill-W1 cell line were tested using similar procedures as this thesis. The gill cells were exposed to both live algae cultures and algal extracts. The algal extract used were fatty acid that were also dissolved in 1% MeOH. It was found that both extracts and live algae were harmful when exposed to RTgill-W1 cells and the cellular viability reductions were time-dependent. While the extraction process was similar to my methods, no mention on possible toxic potential limitations due to algal material preparation was discussed. Another study Mardones (2020), also used RTgill-W1 cell line to test the toxicity of multiple algal strains. The algal strains were prepared by sonication, like in my study, but filtration differed when a syringe with a nylon filter was used. It was found that some strains were toxic but there were no limitations on toxic potential due to sample preparation noted either. For future studies, it could be considered to use the syringe and nylon filtration method instead.

Despite the differences of RTgill-W1 and ASG-10, the *in vitro* toxicity tests performed on both cell lines were valuable alternatives to whole fish experiments. The *in vitro* approach saved time, money, laboratory facilities, and ethics approval procedures compared to testing on live animals (Dorantes-Aranda et al., 2011). Other methods of testing *C. leadbeateri* could have followed past studies like the *in vivo Artemia* bioassay (see Edvardsen, 1993; Meldahl et al., 1994; Simonsen & Moestrup, 1997), but testing on fish gill cell lines allowed for different insight on the ichthyotoxic capabilities of *C. leadbeateri*.

4.4 The morphological responses of epithelial gill cells to *C. leadbeateri*

Fish killed from *C. leadbeateri* blooms were frequently observed with gill damage (Edvardsen & Paasche, 1998; Samdal & Edvardsen, 2020). The purpose of taking micrographs was to have an idea of how the gill cells looked post-exposure. Alamar blue measured metabolic activity, but it was important to have a visual confirmation as well. The cytotoxicity of *C. leadbeateri* was

examined through micrographs of ASG-10 gill cells exposed to different extracts of different percentages and with different grazing pressure.

I reported that when comparing just between algal extract groups, the extract percentage and observable damage to the gill cells are directly proportional (*Figure 24*). Although damage is observed when exposed to all concentrations of algal extracts. Similar to my micrographs, scanning electron micrographs from Dorantes-Aranda et al. (2011) showed that RTgill-W1 cells observed damage at all algal extract concentrations. The gills had suffered membrane damage with big holes visible on the cell surface. Also like this study, there was minimal to no observed gill damage in the solvent control. Additionally, I find that grazing by *Acartia* sp. does not also induce toxicity in *C. leadbeateri* either. Like my results measuring cellular viabilities (*Figure 23*), the control with zero grazers at 1% algal extract show considerable damage. There was noticeable "shrinking" and less defined cell membranes. This again suggests that *C. leadbeateri* has toxicity capabilities, causing damage to gill cells, with or without the presence of grazing pressure from *Acartia* sp.

Based on my micrograph results, I cannot conclude that *C. leadbeateri* exposed to increasing grazing pressure causes more gill damage. There are however observable increases in cell damage when the gills are exposed to the same extract concentration, but different grazing pressure. For example, at 0.25% extract with 0 versus 20 grazing copepods (*Figure 24*). There was more observed damage to the gills at the 20 copepods treatment. Unfortunately, these observations are not supported with any statistical analysis and micrographs were taken from just one independent experiment using only the ASG-10 line. Nonetheless, it is still interesting to note.

To conclude, my micrograph results gave insight into how *C. leadbeateri* toxicity affects the morphology of ASG-10 epithelial gill cells. It seems that increasing algal extract concentrations increases cell damages including: less cell confluency, shrunken cells, distorted membranes, less distinguishable nuclei, and clustering of cells. Also, the observable damages from exposure to *C.*

leadbeateri to increasing grazing pressure is not as prominent, with some exceptions. Similar to the cellular viability results, *C. leadbeateri* seems capable of causing gill damage with or without direct grazing presence, as evidenced by the cells exposed to the solvent control. While these micrographs give us interesting insight into *C. leadbeateri* cytotoxic capabilities, there is no statistical analyses to back these claims so I cannot make any definitive assertions from these results.

4.5 Hypotheses revisited

At the beginning of this thesis, I formulated two hypotheses for what I expected the results of this thesis would show. Below, I readdress each hypothesis made and whether the results support my predictions.

Hypothesis one: There will be an increase in short-term mortality of Acartia sp. when directly exposed to increasing concentration of Chrysochromulina leadbeateri cells.

Hypothesis one is supported by the results of the 3-day copepod tolerance experiments and subsequent survival analysis. Examining the survival numbers in each tolerance experiment, we see copepod mortalities exposed to *C. leadbeateri*, but no mortalities when exposed to the non-toxic algal control. The Cox proportional hazards model and GUTS model showed that *C. leadbeateri* toxicity effects on *Acartia* sp. are time and concentration dependent. *Acartia* sp. were 3.2 times more likely to die after exposure to 200 000 cells ml^{-1} *C. leadbeateri* compared to 25 000 cells ml^{-1}. Predicted LC-50 values were the highest at the beginning of the exposure period, indicating the toxic effect of *C. leadbeateri* need to be taken up by *Acartia* sp. and damage to accumulate to cause mortality. LC-50 values decrease over longer exposure times, indicating that *Acartia* sp. is not tolerant of high concentrations of *C. leadbeateri* over time.

Hypothesis two: The predation threat of direct Acartia sp. grazing will cause increased toxicity potential in Chrysochromulina leadbeateri.

The results of the toxicity induction experiment do not support hypothesis two. They show that *C. leadbeateri* has toxicity capabilities towards fish epithelial gill cells without grazing pressure and do not seem to respond to increasing pressure with more toxicity.

4.6 Methods evaluation and future studies

This thesis aimed to give more insight on the predator-prey interactions between *Chrysochromulina leadbeateri* and copepods, specifically how these interactions might affect *Acartia* sp. survival and activity and, influence toxicity potential in *C. leadbeateri*. While I have shown that *Acartia* sp. tolerance to *C. leadbeateri* is concentration and time dependent, the toxicity effects might not be fully reflected in the observed mortalities due to the reasonable, but short duration of the experiments. Also, the toxicity effects on *Acartia* sp. activity, specifically grazing and egestion, was not properly explored. The lack of data observing *Acartia* sp. grazing activity when exposed to non-toxic food alternatives and different *C. leadbeateri* concentrations, did not allow for proper conclusions on the matter. Future studies could explore those interactions and possibly how *Acartia* sp. egg production is affected in the presence of *C. leadbeateri* for even further insight.

The *in vitro* toxicity tests on RTgill-W1 and ASG-10 cell lines have shown that while *C. leadbeateri* does indeed have toxic effects on fish gill cells, its toxic potential does not seem to be influenced nor induced by direct grazing pressure. The limited number of independent toxicity tests conducted prevent the use of statistical analyses to establish a strong basis for my conclusions regarding *C. leadbeateri* responses to grazing pressure. Also, the *C. leadbeateri* extraction process could be modified to prevent any possible "dilution" of toxicity when adding extra dissolution material. To build off the results from this thesis, future studies could conduct more independent experiments, check gill cellular viabilities at multiple checkpoints to see if *C. leadbeateri* toxicity is time-dependent and modify extraction protocol. In addition, if future studies tests *C. leadbeateri* under "optimal" toxicity conditions like reduced salinities and N:P ratios, that may give a more comprehensive look at *C. leadbeateri* toxicity potential. Nonetheless, this thesis used a promising alternative to testing on whole organisms when evaluating the effects on the ichthyotoxic *C. leadbeateri* as valuable resources were saved. There is much more to explore of the biotic factors influencing *C. leadbeateri* toxicity and in general, predator-prey interactions between haptophytes and copepods. This thesis has given more insight on the subject that while *C. leadbeateri* may be toxic to *Acartia* sp. and fish gill cells, it may not be as influenced by grazing pressure as some other known toxic haptophytes.

4.7 Conclusion

While most of the fish-killing algal blooms in Scandinavia, including Norway, have been directly linked to harmful algal blooms of haptophytes, there is still much more to explore about these toxic species. Especially with the species, *Chrysochromulina leadbeateri* whose bloom in May-June 2019 caused the largest fish kill from a HAB in Norway to date. I have focused on the biotic factors that influence the toxicity potential of *C. leadbeateri* by observing the predator-prey interactions between this species and *Acartia* sp. copepods.

I first found that when *Acartia* sp. is directly exposed to *C. leadbeateri* at higher concentrations and for longer times, its survival rates and activity levels are negatively affected. I theorize that this is because it takes time for the toxins to taken up by *Acartia* sp. to cause damage and result in mortality. Other *Acartia* sp. activities, like grazing and egestion, did not seem strongly affected by *C. leadbeateri*, at least at 5 x 10^4 cell ml^{-1} concentration, as evidenced by fecal pellet production. However, with the lack of grazing observations of *Acartia* sp. exposed to non-toxic food controls and different *C. leadbeateri* concentrations, it is difficult to make any accurate conclusions on how *C. leadbeateri* really affects *Acartia* sp. feeding behavior. I next measured the toxicity potential of *C. leadbeateri* after exposure to different grazing pressure by conducting *in vitro* toxicity tests using the RTgill-W1 and ASG-10 fish epithelial cell lines. Alamar blue was used as an indicator dye to measure gill cell viability after exposure to different algal extracts made from *C. leadbeateri* exposed to different grazing intensities. Micrographs were also taken to better understand cytotoxicity effects of *C. leadbeateri* by looking at morphological changes to the gill cells after exposure to the algal extracts. While there seems to be viability reductions in both cell lines after exposure to increasing extract concentrations, this claim is only based on only one independent experiment. The same goes for my finding that there seems to be no effect in cellular viability in the cell lines when they are exposed to *C. leadbeateri* subjected a range of grazing pressure. However, results from both the Alamar bioassay and micrographs indicate *C. leadbeateri* does have some toxic effects on fish gill cells.

Much remains to be explored about the complex predator-prey relationship between *C. leadbeateri* and copepods and really about *C. leadbeateri*, in general. The existing literature on toxic haptophytes has focused more on other toxic species like *P. parvum*, while the limited studies on *C. leadbeateri* have investigated more abiotic factors like nutrient limitations. In addition, literature on copepod-induced toxicity have only been described in diatoms and dinoflagellates. This thesis has hopefully laid part of the foundation for which future studies could build upon and give more insight into just how toxic this relationship between haptophytes and copepods could be.

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Appendix A: Supplementary Methods

Making IMR ½ algal medium

For the algae used in the tolerance and toxicity induction experiment, the different strains (*C. leadbeateri*, *C. throndsenii*, and *H. ericina*) were grown in medium. The medium used was IMR ½ (Eppley et al., 1967, modified by E. Passche, UIO) with PSU 30. The base for the algal medium is natural seawater which was collected from approximately 40 meters depth from the Oslo fjord near Drøbak. The seawater was filtered through GF/C glass fiber filters (Whatman Glass Microfiber Filters). Then, distilled H_2O and the rest of the stock solution (detailed below) was added to the filtered seawater. The salinity level was checked using the refractometer and autoclaved (HS 6610EC-1 Autoclave, Getinge, Sweden). The media were autoclaved with the program PO3 for wet autoclaving at 121°C for 20 minutes. The media were stored in a climate room at 14°C. The recipe for the IMR ½ algal medium is detailed below.

Table S1. Detailed recipe of material added to make the IMR ½ algal medium used to culture algae used in the experiments.

Material added	Amount added (mL)
Filtered Seawater	2700
Distilled H_2O	300
Nitrate (KNO_3)	1500
Phosphate (KH_2PO_4)	1500
Trace metal solution	1500
Vitamins	1500
Selenite solution	3000

The salinity level was checked using the refractometer and autoclaved (HS 6610EC-1 Autoclave, Getinge, Sweden). The media were autoclaved with the program PO3 for wet autoclaving at 121°C for 20 minutes. The media were stored in a climate room at 14°C.

Measuring light intensity

During the two experiments: 1) copepod survival exposed to varying levels of *Chrysochromulina leadbeateri* concentrations, 2) induction of *C. leadbeateri* toxicity production from copepod grazing, light intensity measurements were taken. This was done at the start and end of each experiment which both lasted 72-hours. The portable photometer (SpectraPen, Photon Systems Instruments) was placed adjacent to the where the plates or bottles were placed to measure the light.

Figure S1. Measuring the light intensity (in µmol $m^{-2}s^{-1}$) where the bottles were placed during the toxicity induction experiments, using the SpectraPen photometer.

Light intensity measurements were taken to ensure the copepods and algae were exposed to similar environmental conditions during the duration of the experiment. Maintaining similar environmental conditions throughout the experiment isolates better guarantees that any presumable toxicity changes in the algae would be from grazing only. The light measurement results can be seen in Appendix B: Supplementary Results.

Measuring pH

Like the light intensity measurements, pH was measured using a pH meter (VWR® pHenomenal pH 1100l) prior to and at the conclusion of the tolerance and toxicity induction experiments.

When algal cells grow and replicate, they respire and produce CO_2. This leads to a chemical reaction causing carbonic acid to produce, which lowers the pH of the surrounding water. By comparing the pH at the start and end of each experiment, it gives an indication of if the algal cells still grew and replicate during the 72-hours. The comparison can also give us an indication of whether the copepods were exposed to different environmental factors throughout the experiment.

Figure S2. Measuring the pH of each algal concentration bottle prior to adding grazing copepods during the toxicity induction experiment. Between each measurement, the sensor was rinsed with filtered seawater with a corresponding PSU.

Measurements were taken from all algal concentrations including 0 cells ml^{-1} and concentrations of the non-toxic algal controls. At the end of the experiments, all the replicates with the same algal concentration were combined and one measurement were taken per cell concentration. The pH measurement results can be seen in Appendix B: Supplementary Results.

Appendix B: Supplementary Results

Table S2. Trials of tolerance experiments with different C. leadbeateri *concentrations incubated in multi-well cell plates. There were three replicates of each treatment with three* Acartia *sp. individuals in each. The results from the replicates were combined and were not used in the data analysis because of its high mortalities.*

Algal concentration (cells ml^{-1})	*Acartia* sp. survived	Survival rate (%)
Trial #1		
200000	0	0
100000	0	0
50000	0	0
25000	1	11
12500	1	11
6250	0	0
Trial #2		
3000	2	22
1500	6	75
750	7	56
375	5	63
~188	7	78
~95	6	67
Trial #3		
2000	4	44
1500	6	67
1000	3	33
750	5	63
500	3	60
250	5	63
0	4	50

Table S3. Tolerance experiment with different C. leadbeateri *concentrations incubated in glass bottles. There were three replicates of each treatment with 10* Acartia *sp. individuals in each. The results from the replicates were combined and were not used in the data analysis because the checks for* Acartia *sp. survival were not thorough enough.*

Algal concentration (cells ml^{-1})	*Acartia* sp. survived	Survival rate (%)
Trial #1		
2000	15	50
1000	19	63
500	18	60
250	22	73
0	19	63

Table S4. Light intensity measurements taken in µmol $m^{-2}s^{-1}$ at the start and end of the tolerance and inducing toxicity experiments. Three light measurements were taken at each time and the bolded values are the corresponding averages.

	Start	End
Experiment 2: Tolerance		
	52.9	55.2
	54.1	53.3
	57.4	53.3
	54.8	**53.9**
Experiment 3: Tolerance		
	52.3	51.2
	54.5	51.4
	54.6	51.5
	53.8	**51.4**
Inducing Toxicity Experiment		
	58.7	57.9
	58.5	57.9
	58.4	58.0
	58.5	**57.9**

Table S5. pH measurements at the start and end of experiments 2 and 3 tolerance experiments. All replicates with the same algal concentration were combined to have one pH measurement per concentration at the end of the experiments.

Algal concentration (cells ml^{-1})	**Start pH**	**End pH**
Chrysochromulina leadbeateri		
200 000	8.32	8.20
100 000	8.24	8.06
50 000	8.18	7.96
25 000	7.90	7.96
	8.21	7.93
3 200	-	7.90
1 600	-	7.86
800	7.91	7.95
400	7.94	7.93
100	7.90	7.93
0	7.91	7.89
	8.17	7.91
Chrysochromulina throndsenii		
25 000	7.89	7.94
Haptolina ericina		
200 000	8.05	7.92

Table S6. pH measurements at the start and end of the 2nd toxicity induction experiment. Each replicate post-grazing was measured for pH and the average measurement is in the rightest column.

Grazing copepods		End pH		
	A	**B**	**C**	
0	8.62	8.66	8.58	8.62
1	8.64	8.60	8.62	8.62
5	8.57	8.58	8.61	8.59
10	8.55	8.58	8.48	8.54
20	8.55	8.49	8.51	8.52
		Start pH		8.43

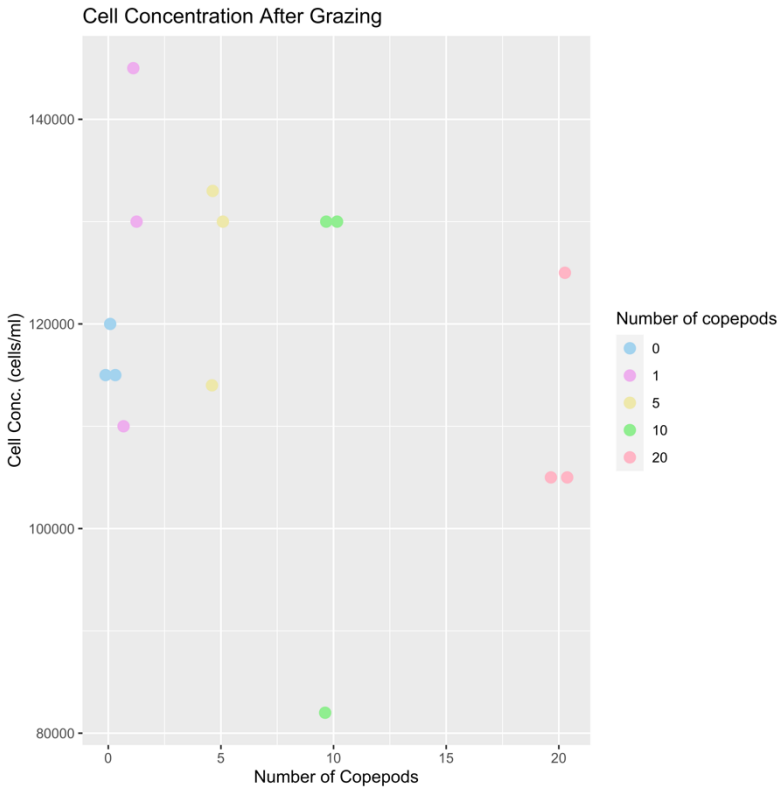

Figure S3. Cell concentration of C. leadbeateri *after 72 hours of exposure to varying number of grazing* Acartia *sp.*

Table S7. C. leadbeateri *cell concentrations after 72 hours exposure, during the 2nd toxicity experiment, to grazing and grazing values calculated according to Frost (1972). The pre-grazing concentration was 50 000 cells ml-1.*

Treatment (# of grazers – replicate name)	Post-grazing concentration (cells ml^{-1})	Grazing values
1-A	130 000	0.0088
1-B	145 000	0.0018
1-C	110 000	0.0041
5-A	114 000	0.0034
5-B	133 000	0.0012
5-C	130 000	0.0088
10-A	82 000	0.0047
10-B	130 000	0.0088
10-C	125 000	0.0017
20-A	105 000	0.0030
20-B	105 000	0.0030
20-C	112 000	0.0029

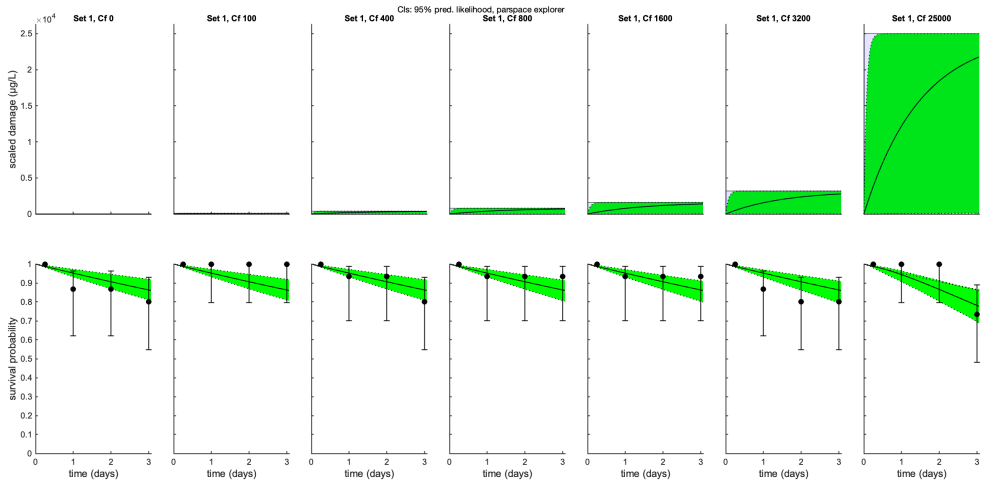

Figure S4. Modelled damage terms (upper row) and survival (lower row) for the different tested concentration of experiment 1. Mean and Se of the raw data are plotted represented as points and errorbars.

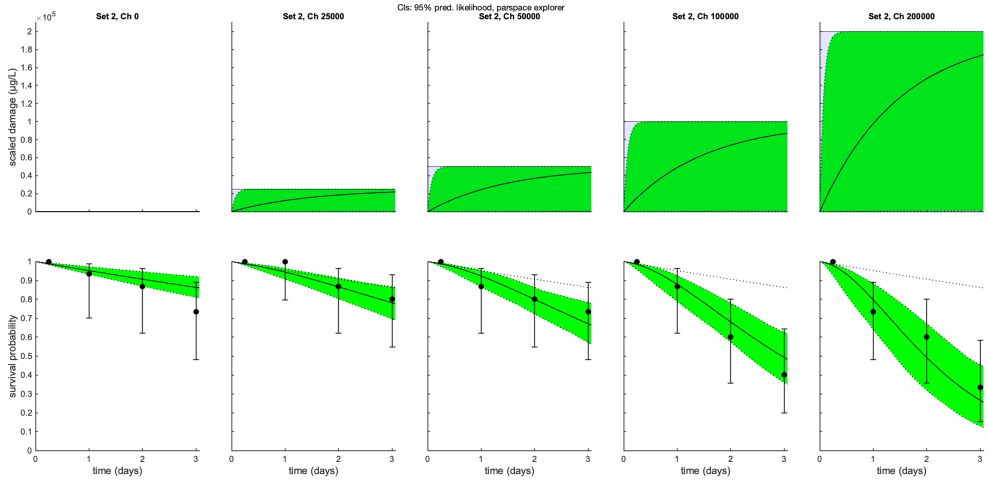

Figure S5. Modelled damage terms (upper row) and survival (lower row) for the different tested concentration of experiment 2. Mean and Se of the raw data are plotted represented as points and errorbars.

The estimates GUTS parameters values were a dominant rate constant (kd, uptake rate) 0.6700(0.001641 – 11.41) d-1; a threshold concentration (mw) of 5390 (0.02507 – 3.483e+04) cells ml^{-1} d-1, a background hazard rate (hb) of 0.04906 (0.02808 – 0.07000*) d-1, and a killing rate (bw) of 3.621e-06 (1.279e06 – 0.001316) d-1.

Appendix C: Scripts

Survival plots (Second Tolerance Experiment)

```
---
title: "Survival_plot"
author: "Jacquelynn Tran"
date: "3/23/2023"
output:
  word_document: default
  html_document: default
---

```{r setup, include=FALSE}
knitr::opts_chunk$set(echo = TRUE)
knitr::opts_knit$set(root.dir = "~/Documents")
```
```

Survival plot (with EDITED data)

```
#install.packages(c("survival", "survminer"))
#install.packages("tidyselect")

library(RColorBrewer)
library(survival)
library(survminer)
library(tidyverse)
library("cowplot")
library(gridExtra)

getwd()

## [1] "/Users/jacquelynntran/Documents"

survival_two <- readxl::read_excel("Tolerance_incubation_results.xlsx", sheet
="2nd_series_coef")

survival_two %>%
  group_by(species_name) %>%
  filter(survival == max(survival)) -> tempdf

tempdf %>%
  filter(survival == 1) %>%
  group_by(species_name) %>%
  filter(time_incubated == max(time_incubated)) -> tempdf1

tempdf %>%
  filter(survival == 2) %>%
```

```
  group_by(species_name) %>%
  filter(time_incubated == min(time_incubated)) -> tempdf2

tempdf1 %>%
  bind_rows(tempdf2) %>%
  rename(ID = species_name) -> survival_two_df


my_y_title <- expression(paste(italic("Acartia"), " sp ind survived"))


copepod_activity_2nd_total <- survival_two %>%
    filter(survival == 1) %>%
  mutate(treatment = factor(treatment, levels = c("200000-toxic", "100000-tox
ic", "50000-toxic", "25000-toxic", "0-toxic", "200000-nontoxic")),
         survival = as.factor(survival)) %>%
  group_by(treatment, survival, time_incubated) %>%
  dplyr::summarise(NrofIndividuals = n()) %>%
  ggplot(aes(x = time_incubated,
             y = NrofIndividuals,
             color = treatment)) +
  geom_smooth(method = "lm", se = FALSE) +
  geom_jitter(width = 2, height = 0.5)  +
  facet_grid(.~ survival) +
  theme_bw() +
  labs(x="Time incubated (hours)", y= NULL,
       colour = "Cell conc (cells/ml)",
       title = "Total Copepod Survival") +
       scale_color_discrete(labels=c('200k', '100k', '50k', '25k', '0', '200k
(NC)')) +
  scale_x_continuous(breaks=c(6,24,48,72)) +
  scale_y_continuous(breaks=c(seq(0, 16, by=2)))

## `summarise()` has grouped output by 'treatment', 'survival'. You can overr
ide
## using the `.groups` argument.

copepod_activity_2nd_reps <- survival_two %>%
  mutate(treatment = factor(treatment, levels = c("200000-toxic", "100000-tox
ic", "50000-toxic", "25000-toxic", "0-toxic", "200000-nontoxic")),
         survival = as.factor(survival)) %>%
  group_by(survival, well_number, time_incubated, treatment) %>%
  dplyr::summarise(NrofIndividuals = n()) %>%
  mutate(survival = ifelse(survival==2 & NrofIndividuals==3, 0, survival),
         NrofIndividuals = ifelse(survival==0, 0, NrofIndividuals),
         survival = ifelse(survival==0, 1, survival)) %>%
  filter(survival==1) %>%
  ggplot(aes(x = time_incubated,
             y = NrofIndividuals,
             color = treatment,
```

```
              #shape = toxicity,
              group = well_number
              )) +
  geom_jitter(width = 2, height = 0.2)  +
  labs(x="Time incubated (hours)", y= my_y_title,
       colour = "Cell conc (cells/ml)",
       title = "Copepod Survival per Replicate") +
      scale_color_discrete(labels=c('200k', '100k', '50k', '25k', '0', '200k
(NC')) +
  scale_x_continuous(breaks=c(6,24,48,72)) +
  scale_y_continuous(breaks=c(seq(0, 3, by=1))) +
      theme(legend.position="none")+
      theme_bw() +
      guides(color = FALSE) # Remove Legend

copepod_activity_2nd_total

copepod_activity_2nd_reps

library(cowplot)
plot_grid(copepod_activity_2nd_reps, copepod_activity_2nd_total, labels = c("
A","B" ), align="v")

ggsave("2nd_series_survival.png", dpi = 600)

## Saving 5 x 4 in image

res.surv.two <- coxph(Surv(time_incubated, survival) ~ concentration, data =
survival_two_df)

summary(res.surv.two)

## Call:
## coxph(formula = Surv(time_incubated, survival) ~ concentration,
##     data = survival_two_df)
##
##   n= 75, number of events= 30
##
##                     coef exp(coef)  se(coef)     z Pr(>|z|)
## concentration 6.974e-06 1.000e+00 2.337e-06 2.984  0.00284 **
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
##               exp(coef) exp(-coef) lower .95 upper .95
## concentration         1          1         1         1
##
## Concordance= 0.66  (se = 0.054 )
## Likelihood ratio test= 8.39  on 1 df,   p=0.004
## Wald test            = 8.9  on 1 df,   p=0.003
## Score (logrank) test = 9.59  on 1 df,   p=0.002
```

```
res.surv.two <- coxph(Surv(time_incubated, survival) ~ as.factor(concentratio
n), data = survival_two_df)
summary(res.surv.two)

## Call:
## coxph(formula = Surv(time_incubated, survival) ~ as.factor(concentration),
##     data = survival_two_df)
##
##   n= 75, number of events= 30
##
##                                     coef  exp(coef)   se(coef)      z Pr(>
|z|)
## as.factor(concentration)25000 -3.574e-01  6.995e-01  7.638e-01 -0.468   0.
6398
## as.factor(concentration)50000  4.151e-15  1.000e+00  7.071e-01  0.000   1.
0000
## as.factor(concentration)1e+05  1.038e+00  2.823e+00  6.017e-01  1.725   0.
0846
## as.factor(concentration)2e+05  1.163e+00  3.199e+00  5.927e-01  1.962   0.
0498
##
## as.factor(concentration)25000
## as.factor(concentration)50000
## as.factor(concentration)1e+05 .
## as.factor(concentration)2e+05 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
##                                exp(coef) exp(-coef) lower .95 upper .95
## as.factor(concentration)25000    0.6995     1.4297    0.1565     3.125
## as.factor(concentration)50000    1.0000     1.0000    0.2501     3.998
## as.factor(concentration)1e+05    2.8226     0.3543    0.8680     9.179
## as.factor(concentration)2e+05    3.1992     0.3126    1.0012    10.223
##
## Concordance= 0.671  (se = 0.051 )
## Likelihood ratio test= 11.03  on 4 df,   p=0.03
## Wald test            = 10.32  on 4 df,   p=0.04
## Score (logrank) test = 11.72  on 4 df,   p=0.02



fit <- survfit(Surv(time_incubated, survival) ~ concentration, data = cox_mod
el_2_df)

ggsurvplot(
  fit,
  data = cox_model_2_df,
  surv.median.line = "hv",
  # Add medians survival
  # Change legends: title & labels
  legend.title = "Concentration (cells/ml)",
```

```
  legend.labs = c("0", "25000", "50000", "100000", "200000"),
  # Add p-value and interval
  pval = F,
  conf.int = TRUE,
  # Add risk table
  risk.table = F,
  tables.height = 0.2,
  break.x.by = 24,
  xlab = "Time (hours)",
  ylab = c("Survival probability"),
  tables.theme = theme_cleantable(),
  palette = brewer.pal(n = 5, name = "YlGnBu") ,
  ggtheme = theme_bw(),  # Change ggplot2 theme
  ) -> survivalplot_2

survivalplot_2

#ggsave("Survival_plot_2nd_series.png", dpi = 600)
```

Survival plots (First Tolerance Experiment)

```
survival_one <- readxl::read_excel("Tolerance_incubation_results.xlsx", sheet
="1st_series_coef")

survival_one %>%
  group_by(species_name) %>%
  filter(survival == max(survival)) -> tempdf

tempdf %>%
  filter(survival == 1) %>%
  group_by(species_name) %>%
  filter(time_incubated == max(time_incubated)) -> tempdf1

tempdf %>%
  filter(survival == 2) %>%
  group_by(species_name) %>%
  filter(time_incubated == min(time_incubated)) -> tempdf2

tempdf1 %>%
  bind_rows(tempdf2) %>%
  rename(ID = species_name) -> survival_one_df

my_colour_title <- expression(paste(italic("C. leadbeateri"), " concentration
(cells/ml)"))
my_y_title <- expression(paste(italic("Acartia"), " sp ind survived"))


copepod_activity_1st_total <- survival_one %>%
```

```
    filter(survival == 1) %>%
  mutate(treatment = factor(treatment, levels = c("25000-toxic", "3200-toxic"
, "1600-toxic", "800-toxic", "400-toxic","100-toxic", "0-toxic", "25000-nonto
xic")),
         survival = as.factor(survival)) %>%
  group_by(treatment, survival, time_incubated) %>%
  dplyr::summarise(NrofIndividuals = n()) %>%
  ggplot(aes(x = time_incubated,
             y = NrofIndividuals,
             color = treatment)) +
  geom_smooth(method = "lm", se = FALSE) +
  geom_jitter(width = 2, height = 0.5)  +
  facet_grid(.~ survival) +
  theme_bw() +
  labs(x="Time incubated (hours)", y= NULL,
       colour = "Cell conc (cells/ml)",
       title = "Total Copepod Survival") +
       scale_color_discrete(labels=c('25k', '3200', '1600', '800', '400','100
','0', '25k (NC)')) +
  scale_x_continuous(breaks=c(6,24,48,72)) +
  scale_y_continuous(breaks=c(seq(0, 16, by=2)))

## `summarise()` has grouped output by 'treatment', 'survival'. You can overr
ide
## using the `.groups` argument.

copepod_activity_1st_reps <- survival_one %>%
  mutate(treatment = factor(treatment, levels = c('25000-toxic', '3200-toxic'
, '1600-toxic', '800-toxic', '400-toxic','100-toxic','0-toxic', '25000-nontox
ic')),
         survival = as.factor(survival)) %>%
  group_by(survival, well_number, time_incubated, treatment) %>%
  dplyr::summarise(NrofIndividuals = n()) %>%
    mutate(survival = ifelse(survival==2 & NrofIndividuals==3, 0, survival),
         NrofIndividuals = ifelse(survival==0, 0, NrofIndividuals),
         survival = ifelse(survival==0, 1, survival)) %>%
  ggplot(aes(x = time_incubated,
             y = NrofIndividuals,
             color = treatment,
             #shape = toxicity,
             group = well_number
             )) +

  geom_jitter(width = 2, height = 0.2)  +
  labs(x="Time incubated (hours)", y= my_y_title,
       colour = my_colour_title,
       title = "Copepod Survival per Replicate") +
      scale_color_discrete(labels=c('25k', '32k', '1600', '800', '400', '100'
, '0', '25k (NC)')) +
  scale_x_continuous(breaks=c(6,24,48,72)) +
```

```
  scale_y_continuous(breaks=c(seq(0, 3, by=1))) +
        theme(legend.position="none") +
        theme_bw() +
        guides(color = FALSE) # Remove Legend

## `summarise()` has grouped output by 'survival', 'well_number',
## 'time_incubated'. You can override using the `.groups` argument.

copepod_activity_1st_total

copepod_activity_1st_reps

plot_grid(copepod_activity_1st_reps, copepod_activity_1st_total, labels = c("
A","B" ), align="v")

ggsave("1st_series_survival.png", dpi = 600)

## Saving 5 x 4 in image

res.surv.one <- coxph(Surv(time_incubated, survival) ~ concentration, data =
survival_one_df)

summary(res.surv.one)

## Call:
## coxph(formula = Surv(time_incubated, survival) ~ concentration,
##     data = survival_one_df)
##
##   n= 105, number of events= 16
##
##                     coef exp(coef)  se(coef)     z Pr(>|z|)
## concentration 2.930e-05 1.000e+00 2.378e-05 1.232    0.218
##
##               exp(coef) exp(-coef) lower .95 upper .95
## concentration         1          1         1         1
##
## Concordance= 0.571  (se = 0.077 )
## Likelihood ratio test= 1.34  on 1 df,   p=0.2
## Wald test            = 1.52  on 1 df,   p=0.2
## Score (logrank) test = 1.58  on 1 df,   p=0.2
```

It looks the hazard ratio is sooooo small per increase in cell concentration that you do not even get a number here (exp(coef)), a potential workaround here is to use it as concentration as a factor.

```
res.surv.one <- coxph(Surv(time_incubated, survival) ~ as.factor(concentratio
n), data = survival_one_df)

## Warning in coxph.fit(X, Y, istrat, offset, init, control, weights = weight
s, :
## Loglik converged before variable 1 ; coefficient may be infinite.
```

```
summary(res.surv.one)

## Call:
## coxph(formula = Surv(time_incubated, survival) ~ as.factor(concentration),
##     data = survival_one_df)
##
##   n= 105, number of events= 16
##
##                                     coef  exp(coef)   se(coef)      z Pr(>
|z|)
## as.factor(concentration)100   -1.849e+01  9.314e-09  5.638e+03 -0.003    0
.997
## as.factor(concentration)400   -3.928e-02  9.615e-01  8.165e-01 -0.048    0
.962
## as.factor(concentration)800   -1.167e+00  3.113e-01  1.155e+00 -1.010    0
.312
## as.factor(concentration)1600  -4.206e-01  6.566e-01  9.129e-01 -0.461    0
.645
## as.factor(concentration)3200   1.979e-02  1.020e+00  8.165e-01  0.024    0
.981
## as.factor(concentration)25000  2.249e-01  1.252e+00  7.639e-01  0.294    0
.768
##
##                               exp(coef) exp(-coef) lower .95 upper .95
## as.factor(concentration)100   9.314e-09  1.074e+08   0.00000       Inf
## as.factor(concentration)400   9.615e-01  1.040e+00   0.19405     4.764
## as.factor(concentration)800   3.113e-01  3.212e+00   0.03238     2.993
## as.factor(concentration)1600  6.566e-01  1.523e+00   0.10972     3.930
## as.factor(concentration)3200  1.020e+00  9.804e-01   0.20586     5.054
## as.factor(concentration)25000 1.252e+00  7.986e-01   0.28018     5.596
##
## Concordance= 0.658  (se = 0.059 )
## Likelihood ratio test= 7.69  on 6 df,   p=0.3
## Wald test            = 1.84  on 6 df,   p=0.9
## Score (logrank) test = 5.31  on 6 df,   p=0.5



fit_1 <- survfit(Surv(time_incubated, survival) ~ concentration, data = cox_m
odel_df_1)

ggsurvplot(
  fit_1,
  data = cox_model_df_1,
  surv.median.line = "hv", # Add medians survival
  # Change legends: title & labels
  legend.title = "Concentration (cells/ml)",
  legend.labs = c("0", "100", "400", "800", "1600", "3200", "25000"),
  # Add p-value and interval
  pval = F,
  conf.int = TRUE,
```

```
  # Add risk table
  risk.table = F,
  tables.height = 0.2,
  break.x.by = 24,
  xlab = "Time (hours)",
  ylab = c("Survival probability"),
  tables.theme = theme_cleantable(),
  palette = brewer.pal(n = 7, name = "YlGnBu") ,
  #ggtheme = theme_dark() ,
  ggtheme = theme_bw(),  # Change ggplot2 theme
  ) -> survivalplot_1st

## Warning in .add_surv_median(p, fit, type = surv.median.line, fun = fun, :
Median
## survival not reached.

survivalplot_1st

survivalplot_1st$plot +
  geom_hline(yintercept = 0.5, linetype = 2)

ggsave("Survival_plot_1st_series.png", dpi = 600)

## Saving 5 x 4 in image

library(ggplot2)

# Create a data frame with the LC50 values and intervals
LC50_data <- data.frame(
  Days = 1:15, # Exposure time
  LC50 = c(7.255e+05, 2.250e+05, 1.215e+05, 8.150e+04, 6.124e+04, 4.925e+04,
           4.140e+04, 3.590e+04, 3.185e+04, 2.873e+04, 2.627e+04, 2.428e+04,
           2.263e+04, 2.125e+04, 2.007e+04), # LC50 values
  lower_CI = c(3.366e+05, 1.407e+05, 8.320e+04, 4.839e+04, 3.102e+04, 2.157e+
04,
               1.587e+04, 1.217e+04, 9631., 7813., 6466., 5439., 4639., 4004.
, 3492.), # Lower CI values
  upper_CI = c(1.866e+06, 4.667e+05, 2.222e+05, 1.504e+05, 1.180e+05, 9.851e+
04,
               8.517e+04, 7.676e+04, 7.045e+04, 6.611e+04, 6.322e+04, 6.116e+
04,
               5.942e+04, 5.794e+04, 5.666e+04) # Upper CI values
)

# Create a line graph with LC50 values and confidence intervals
ggplot(LC50_data, aes(x = Days, y = LC50)) +
  geom_line(size = 0.5, color = "black") +
  geom_ribbon(aes(ymin = lower_CI, ymax = upper_CI), alpha = 0.3, fill = "blu
e") +
  labs(x = "Exposure time (days)", y = "LC50 value") +
  ggtitle("LC50 values over time") +
```

```
  theme_bw() +
  geom_point(color = "black") +
  #scale_y_continuous(breaks=seq(0, 1500000, by=250000))
  scale_y_continuous(labels = scales::scientific_format(digits = 1))

## Warning: Using `size` aesthetic for lines was deprecated in ggplot2 3.4.0.
## ℹ Please use `linewidth` instead.

#ggsave("LC_50_GUTS.png", dpi = 600)
```

Acartia grazing frequency

```
---
title: "Acartia grazing frequency"
author: "Jacquelynn Tran"
date: "4/3/2023"
output:
  word_document: default
  html_document: default
---

```{r setup, include=FALSE}
knitr::opts_chunk$set(echo = TRUE)
knitr::opts_knit$set(root.dir = "~/Documents")
```
```

```
library(ggplot2)
library(readxl)
library(cowplot)

fp <- read_excel("Acartia_FP_measurements.xlsx", sheet ="fp_avg")
fp$treatment <- factor(fp$well, levels = c(1, 5, 10, 20))

my_title <- expression(paste(italic("Acartia"), " sp. individual grazing"))
my_y_title <- expression(paste(italic("Acartia"), " sp. individual grazing"))

my_legend_title <- expression(paste(italic("Acartia"), " sp individual grazin
g"))

ggplot(fp, aes(x=well, y=fp_length, )) +

geom_point(width = 2, height = 0.5)  +
  theme_bw() +
  labs(x=my_y_title, y= "Fecal pellet per individual",
       color = my_legend_title,
       title = "Fecal pellet amount per individual") +
          #guides(colour = guide_legend(title = my_title)) +
           #scale_color_discrete(labels=c('1', '5', '10', '20')) +
          scale_x_continuous(breaks=c(1,5,10,20)) -> ind_fp

ind_fp
```

```
ggplot(fp, aes(x=well, y=num_fp, )) +

geom_point(width = 2, height = 0.5)  +
  theme_bw() +
  labs(x=my_y_title, y= "Fecal pellet length (µm)",
       color = my_legend_title,
       title = "Fecal pellet length") +
          scale_x_continuous(breaks=c(1,5,10,20)) -> fecal_length
```

```
fecal_length

plot_grid(ind_fp, fecal_length, labels = c("A","B" ), align="v")

ggsave("Acartia_grazing_plots.png", dpi = 600)

## Saving 5 x 4 in image

model <- lm(fp_length ~ treatment, data = fp)
anova(model)

## Analysis of Variance Table
##
## Response: fp_length
##           Df  Sum Sq Mean Sq F value   Pr(>F)
## treatment  3 15791.8  5263.9  8.7005 0.009253 **
## Residuals  7  4235.1   605.0
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

summary(model)

##
## Call:
## lm(formula = fp_length ~ treatment, data = fp)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -35.048 -13.316  -3.367  14.842  35.048
##
## Coefficients:
##             Estimate Std. Error t value Pr(>|t|)
## (Intercept)   144.60      14.20  10.182  1.9e-05 ***
## treatment5     59.02      22.45   2.628   0.0340 *
## treatment10   -31.66      20.08  -1.577   0.1589
## treatment20   -49.05      20.08  -2.442   0.0446 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 24.6 on 7 degrees of freedom
##   (1 observation deleted due to missingness)
## Multiple R-squared:  0.7885, Adjusted R-squared:  0.6979
## F-statistic:   8.7 on 3 and 7 DF,  p-value: 0.009253
```

```
model <- lm(fp_ind ~ treatment, data = fp)
anova(model)

## Analysis of Variance Table
##
## Response: fp_ind
##           Df Sum Sq Mean Sq F value  Pr(>F)
## treatment  3 7.1582 2.38607  3.4666 0.07092 .
## Residuals  8 5.5065 0.68831
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

summary(model)

##
## Call:
## lm(formula = fp_ind ~ treatment, data = fp)
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.66667 -0.20490 -0.02256  0.35245  1.33333
##
## Coefficients:
##             Estimate Std. Error t value Pr(>|t|)
## (Intercept)   2.6667     0.4790   5.567  0.00053 ***
## treatment5   -2.1333     0.6774  -3.149  0.01361 *
## treatment10  -1.4549     0.6774  -2.148  0.06400 .
## treatment20  -1.3118     0.6774  -1.936  0.08883 .
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.8296 on 8 degrees of freedom
## Multiple R-squared:  0.5652, Adjusted R-squared:  0.4022
## F-statistic: 3.467 on 3 and 8 DF,  p-value: 0.07092

#hist(resid(model))

anova(model)

## Analysis of Variance Table
##
## Response: fp_ind
##           Df Sum Sq Mean Sq F value  Pr(>F)
## treatment  3 7.1582 2.38607  3.4666 0.07092 .
## Residuals  8 5.5065 0.68831
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

C. leadbeateri concentrations before and after grazing

```
library(ggplot2)


# create the scatter plot
ggplot(data = df, aes(x = number_of_copepods, y = after_grazing, group = numb
er_of_copepods, color = factor(number_of_copepods))) +
  geom_point(size = 3, position = position_jitter(height = 0.5)) +
  labs(x = "Number of Copepods", y = "Cell Conc. (cells/ml)", color = "Number
of copepods") +
  scale_color_manual(values = c("lightskyblue2", "plum2", "palegoldenrod", "p
alegreen2", "pink1")) +
  ggtitle("Cell Concentration After Grazing")

scale_x_continuous(breaks=c(0,1,5,10, 20))

## <ScaleContinuousPosition>
##  Range:
##  Limits:    0 --    1

  #theme_bw()
#ggsave("Cell conc after grazing.png", dpi = 600)
```

Gill bioassay plots

---
title: "Gill bioassay plots"
author: "Jacquelynn Tran"
date: "3/23/2023"
output:
 word_document: default
 html_document: default

```{r setup, include=FALSE}
knitr::opts_chunk$set(echo = TRUE)
```

```
rm(list=ls())

library(readr);library(dplyr);library(tidyverse);library(readxl)

##
## Attaching package: 'dplyr'

## The following objects are masked from 'package:stats':
##
##     filter, lag
```

```
## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union

## ── Attaching packages ─────────────────────────────────────── tidyverse 1.
3.2 ──
## ✓ ggplot2 3.4.0     ✓ purrr   1.0.1
## ✓ tibble  3.1.6     ✓ stringr 1.5.0
## ✓ tidyr   1.3.0     ✓ forcats 0.5.2
## ── Conflicts ────────────────────────────────────────── tidyverse_conflict
s() ──
## ✖ dplyr::filter() masks stats::filter()
## ✖ dplyr::lag()    masks stats::lag()

setwd("~/Documents")

getwd()

## [1] "/Users/jacquelynntran/Documents"
```

ASG-10 Gill Bioassay

```
df<- read_excel("2023_03_15_copepod_exp_UiO393_ASGgill.xlsx",sheet="graph")
df$copepods <- factor(df$copepods, levels = c('0','1','5','10','20','Solvent
control'))
df$percentage <- as.factor(df$percentage)


#ggplot(df, aes(x=copepods, y=new values, color=percentage)) +
 # geom_line() +
  #scale_y_continuous()

options(scipen=22500000)

ggplot(df, aes(x=copepods, y=mean, fill=percentage)) +
  geom_bar(stat='identity', position = position_dodge(preserve = "single")) +
  geom_errorbar(aes(ymin=mean-sd,
                    ymax=mean+sd),
                width=0.3, position = position_dodge(0.9, preserve = "single"
)) +
  scale_y_continuous(expand=c(0,0), breaks = c(0, 10, 20, 30, 40, 50, 60, 70,
80, 90, 100, 110, 120, 130, 140)) +
  coord_cartesian(ylim=c(0,140)) +
  theme_classic() +
  scale_x_discrete(limits=c(0,1, 5, 10, 20, "Solvent control")) +
  #theme_bw() +
  #theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank
()) +
  labs(x="# of copepods", y="Cellular viability (% of control)", fill = "Extr
act (%)")
```

```
ggsave("ASG_10_bioassay.png", dpi = 600)

## Saving 5 x 4 in image
```

RT-W1 Gill Bioassay

```
df1 <- read_excel("2022_11_03_copepod_exp_UiO393_RTgill.xlsx",sheet="graph")
df1$copepods <- factor(df1$copepods, levels = c('0','3','6','9','Solvent cont
rol'))
df1$percentage <- as.factor(df1$percentage)


#ggplot(df, aes(x=copepods, y=new values, color=percentage)) +
 # geom_line() +
  #scale_y_continuous()

options(scipen=22500000)

ggplot(df1, aes(x=copepods, y=viability, fill=percentage)) +
  geom_bar(stat='identity', position = position_dodge(preserve = "single")) +
  geom_errorbar(aes(ymin=viability-new_sd,
                    ymax=viability+new_sd),
                width=0.3, position = position_dodge(0.9, preserve = "single"
)) +
  scale_y_continuous(expand=c(0,0), breaks = c(0, 10, 20, 30, 40, 50, 60, 70,
80, 90, 100, 110, 120, 130, 140)) +
  coord_cartesian(ylim=c(0,140)) +
  theme_classic() +
  scale_x_discrete(limits=c(0,3, 6, 9, "Solvent control")) +
  #theme_bw() +
  #theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank
()) +
  labs(x="# of copepods", y="Cellular viability (% of control)", fill = "Extr
act (%)")

ggsave("RTgill_W1_bioassay.png", dpi = 600)

## Saving 5 x 4 in image
```