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Acclimation Potential to a Future Ocean Acidification Scenario in the Key Copepod Species *Calanus finmarchicus*

- with emphasis on the genetic contribution
to the acclimation response

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Marine Coastal Development
Submission date: May 2014
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ACKNOWLEDGEMENTS

This master's thesis has been written at the department of biology at the Norwegian University of Science and Technology (NTNU) as part of the Master in Science program in Marine Coastal Development. The experiment was conducted at NTNU Centre of Fisheries and Aquaculture (SeaLab) from October 2012 to April 2013, and analyses were continued in laboratories at NTNU Gløshaugen until March 2014.

I would like to thank all the people who have been helping me in conducting the experiment and writing this thesis, I am very grateful for all the help I have received throughout the process.

First of all, I would like to thank my scientific supervisor Dr. Sindre Andre Pedersen for introducing me to the field of ocean acidification, and for all his help with conducting the experiment, as well as great guidance throughout the writing process. I would also like to thank Randi Røsbak for all her help in the laboratories at NTNU Gløshaugen, and for teaching me several laboratory techniques.
Thanks to my co-supervisor Henrik Jensen for helping me with the microsatellite part of the experiment, and especially with interpretation of the results.
Also, I am very grateful for all the help I have received from Dag Altin, BioTrix, at SeaLab, especially with everything related to the acute toxicity test. I also want to thank my fellow student and friend Solveig Nilsen, who took the time to proofread part of my thesis and give me valuable feedback.

I want to give a special thanks to my family and friends for always believing in me, and for being very supportive and encouraging.

Last, but not least, a very special thanks to my boyfriend Vegard for being an invaluable support throughout all five years at NTNU, you are the best!

Trondheim, May 2014

Marit Aase

ABSTRACT

Ocean acidification, as a result of the increased concentration of carbon dioxide in the ocean, poses a threat towards marine ecosystems through lowered pH and altered seawater chemistry. The marine copepod *Calanus finmarchicus* is an ecologically and socioeconomically important species. In this study *C. finmarchicus* collected in Trondheimsfjorden was exposed to a CO_2 concentration relevant for year 2300 (~2080 ppm) for two consecutive generations in the laboratory. The ability of *C. finmarchicus* to acclimate towards a pCO_2 scenario relevant for year 2300, as well as the genetic contribution to this ability, was investigated in the study. Individuals exposed to elevated CO_2 concentrations for two generations had significantly higher LC50 values than individuals kept under ambient CO_2 concentrations, which indicates that *C. finmarchicus* has the ability to acclimate to a pCO_2 scenario of 2080 ppm. Microsatellite analyses on the animals revealed a tendency of lower genetic diversity, measured as allelic richness and expected heterozygosity, in the populations exposed to high CO_2 concentrations compared to the control populations, although the difference was not significant. The microsatellite analysis thus indicated that some of the observed increase in tolerance among the CO_2 exposed animals may be due to genetic adaptation, but this needs to be further studied. The present study indicated that *C. finmarchicus* may be tolerant towards the direct effects of a pCO_2 scenario relevant for year 2300.

SAMMENDRAG

Havforsuring, som et resultat av den økte konsentrasjonen av karbondioksid i havet, utgjør en trussel for marine økosystemer gjennom å senke pHen og endre sjøvann kjemien. Den marine copepoden *Calanus finmarchicus* er en nøkkelart i mange havområder, og fungerer som en forbindelse mellom primærprodusenter og høyere trofiske nivåer. Den er også en viktig matkilde for flere kommersielle fiskearter og kan derfor anses for å være både en økologisk og sosioøkonomisk viktig art. I dette studiet ble *C. finmarchicus* individer samlet i Trondheimsfjorden eksponert for en CO_2 konsentrasjon relevant for år 2300 (~2080 ppm) i to etterfølgende generasjoner i laboratoriet. Evnen til å akklimatisere og genetisk adaptere til et pCO_2 scenario relevant for år 2300 ble undersøkt i studiet. Individer eksponert for forhøyede CO_2 konsentrasjoner i to generasjoner hadde signifikant høyere LC50 verdier enn individene kultivert under normale CO_2 konsentrasjoner. Dette indikerer at *C. finmarchicus* har evnen til å akklimatisere til et pCO_2 scenario relevant for år 2300. Mikrosatellittanalysene avslørte en tendens til lavere genetisk diversitet, målt som forventet heterozygositet og allelrikdom, i populasjonene som var eksponert for forhøyede CO_2 konsentrasjoner i forhold til kontroll populasjonene, men forskjellen var ikke signifikant. Mikrosatellittanalysen indikerer derfor at *C. finmarchicus* kan ha potensialet til å genetisk tilpasses til forhøyede CO_2 konsentrasjoner, men dette må undersøkes nærmere. Denne studien indikerer at *C. finmarchicus* kan ha evnen til å tolerere de direkte effektene av et pCO_2 scenario relevant for år 2300.

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Abbreviation

µg C L^{-1}	**m**icro**g**ram **C**arbon per **L**itre
µl	**m**icro**l**iter
µM	**m**icro**M**olar
A_N	**N**umber of **A**lleles
ANOVA	**A**nalisis **O**f **V**ariance
A_R	**A**llelic **R**ichness
Ar	**A**ragonite
CI-CVI	**C**opepodite stage **I-VI**
Ca	**C**alcite
CI	**C**onfidence **I**nterval
CO_2	**C**arbon **d**ioxide
CO_2^{3-}	**C**arbonate
DNA	**d**eoxyribonucleic acid
F1	**F**irst filial generation
F2	**S**econd filial generation
H_2CO_3	**C**arbonic acid
HCO_3^-	**B**icarbonate
H_{exp}	**E**xpected **H**eterozygosity
H_{obs}	**O**bserved **H**eterozygosity
HWE	**H**ardy-**W**einberg **E**quilibrium
LC50	**L**ethal **c**oncentration **50**
LSD	Fisher's **L**east **s**ignificant **d**ifference

ng	**n**ano**g**ram
OA	**O**cean **A**cidification
P	**P**arental generation
***p*CO$_2$**	**p**artial pressure of **c**arbon **d**ioxide
PCR	**p**olymerase **c**hain **r**eaction
pH	**p**ower of **H**ydrogen
pH$_{Tot}$	**T**otal **p**ower of **H**ydrogen
ppm	**p**arts **p**er **m**illion
SD	**S**tandard **D**eviation
SE	**S**tandard **E**rror
T_A	**T**otal **A**lkalinity

1 INTRODUCTION

1.1 Ocean acidification

Over the last decades there has been an increased focus on the emission of anthropogenic carbon dioxide (CO_2) leading to climate change, and its consequences on the environment. The greenhouse gas CO_2 contributes to absorption of energy reflected from the earth's surface and thus plays a role in trapping heat in the atmosphere. The increased concentration of CO_2 in the atmosphere is therefore contributing to increased temperatures on earth (Bolin et al., 1986). Even though the effects of global warming have received more attention so far, the effects of the increased concentration of CO_2 will also apply to the ocean, which is continuously interacting with the atmosphere. This interaction implies that an increased concentration of CO_2 in the atmosphere, as a result of higher consumption of fossil fuels and agriculture activities, also leads to an increased concentration of CO_2 in the ocean. The ocean represents an important sink for anthropogenic CO_2 as it has absorbed about one third of the total human-caused CO_2 emissions to the atmosphere since the industrial revolution (Sabine et al., 2004).

Even though ocean uptake of CO_2 slows down the process of global warming by removing the gas from the atmosphere it is not without consequences. When CO_2 is absorbed in the water it reacts in a series of chemical reactions resulting in an increase in the concentration of hydrogen ions, which further leads to a reduction of the pH of seawater. This process is referred to as ocean acidification (OA). An addition of CO_2 will also affect the seawater carbon chemistry. The uptake of CO_2 in the oceans the past 200 years has led to a reduction of the pH by approximately 0.1 units, from 8.21 to 8.10 (Doney et al., 2009). This corresponds to a 30 % increase in the concentration of hydrogen ions (H^+). It has been predicted that the emissions of CO_2 will lead to a larger reduction in pH over the next few centuries than what have been experienced the last 300 million years (Caldeira and Wickett, 2003). The concentration of CO_2 in the atmosphere in the period before the industrial revolution was about 280 parts per million (ppm). This concentration has increased rapidly since then, to a value of about 390 ppm today, and it is predicted that the

concentration will continue to increase. Some models are predicting that there is a possibility of an increase to over 1900 ppm by year 2300 (Caldeira and Wickett, 2003), and a "worst case" scenario of 8000 ppm has even been proposed (Caldeira and Wickett, 2005).

Not only is the level of CO_2 today higher than it has been for at least the last 800 000 years (Lüthi et al., 2008), during the last century the level has also been increasing at a rate up to a hundred times faster than any change observed in geological records during the past 650 000 years (Siegenthaler et al., 2005). This gives reason for concern about whether the changes are too rapid for the marine inhabitants to adapt to the new CO_2 conditions. More knowledge on how the marine biota will respond to future ocean acidification scenarios is needed.

1.1.1 CO_2 in seawater – the chemistry of OA

The atmosphere is continuously interacting with the ocean, exchanging soluble gasses like carbon dioxide. The exchange will continue until equilibrium is reached. This implies that an increase in the atmospheric CO_2 concentration also will lead to an increased concentration of the gas in the ocean. This exchange is described by the following equation:

$$CO_2(atm) \leftrightarrow CO_2(aq) \qquad (1.1)$$

When CO_2 dissolves in seawater it reacts through a series of chemical reactions resulting in a higher concentration of bicarbonate ions [HCO_3^-] and hydrogen ions [H^+], and a lower concentration of carbonate ions [CO_3^{2-}] (Doney et al., 2009). The first step is the production of carbonic acid, H_2CO_3:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \qquad (1.2)$$

Carbonic acid quickly dissolves into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and hydrogen ions (H^+), as shown in the following reactions:

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \qquad (1.3)$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \quad (1.4)$$

The increased concentration of hydrogen ions is lowering the pH of the seawater, thus making the ocean more acidic. Another effect of increasing [H^+] is a reduction in the concentration of carbonate ions, which affects calcification rates through lowering the calcium carbonate saturation states (Doney et al., 2009) .

1.2 Consequences of ocean acidification on the marine biota

The acidification of the ocean may have negative consequences for its inhabiting organisms. Species living in the ocean are adapted to their environment and when it changes it can affect the species' ability to function, especially if the changes are rapid so there is limited time to adapt. The adaptive potential of marine organisms to increasing CO_2 concentrations is not well known, but it is an important future research area (Doney et al., 2009). Due to the effect of ocean acidification on calcification, through lowering saturation states, several studies have been conducted on calcifying organisms (Havenhand et al., 2008, Ries et al., 2009). In addition to affecting the concentration of carbonate, CO_2 can also affect the marine biota by disturbing the acid/base regulation, affect growth, and also influence respiration, energy turnover and mode of metabolism (Pörtner et al., 2004). The sensitivity of an animal to increased concentrations of CO_2 is expected to be related to the species organization level, mode of life and energy requirements (Pörtner et al., 2004). For example, pelagic species are adapted to constant low concentrations of CO_2 and are thus expected to be more sensitive to changes than e.g. species living in the intertidal zone who are more used to variations (Pörtner et al., 2004).

Most of the studies done on the effects of ocean acidification on marine organisms so far have been short-term experiments (Doney et al., 2009). However, experiments of different durations have revealed pronounced differences even within the same species. For instance, a study done by Form and Riebesell (2012) on the cold-water coral *Lophelia pertusa* showed that calcification rates were reduced when exposed to increased concentrations of CO_2 for 8 days, but not when exposed to the same concentration for 178 days (Form and Riebesell, 2012). Another long-term

experiment on the effect of CO_2 on the sea urchin *Strongylocentrotus droebachiensis* revealed that while exposure to 1200 ppm CO_2 for 4 months had effects on fecundity and larval survival it had no effect when the exposure lasted for 16 months (Dupont et al., 2013). This emphasizes the importance of acclimation and of conducting long-term experiments. To get a better understanding of potential effects of chronic exposure to elevated CO_2 concentrations, it is important to conduct long-term experiments. The sensitivity towards CO_2 can vary between different life stages and young stages are generally more sensitive (Gibson et al., 2011, Pedersen et al., 2014). Therefore, it is necessary to conduct experiments covering the entire life cycle of species. Additionally, it is important to conduct multigenerational studies to investigate the adaptive potential to elevated CO_2 concentrations.

Elevated CO_2 can affect the acid/base regulation of organisms by diffusing into the body and making the internal environment more acidic (Pörtner et al., 2004). Species have different ways of regulating their internal pH, e.g. water breathers in most part rely on ion exchange mechanisms (Pörtner et al., 2004). Even though species may be able to compensate for the lowered pH this will require more energy spent on acid/base regulation and leave less energy available for other processes, like e.g. growth and reproduction.

An increasing amount of studies of ocean acidification are becoming available, but still there is a need for experiments investigating the long-term and multi-generational effects of elevated CO_2 concentrations.

1.3 *Calanus finmarchicus* (Gunnerus, 1770)

An important zooplankton species in Norwegian waters is *Calanus finmarchicus*. This copepod plays a key role in the marine ecosystem as it serves as a link between lower and higher trophic levels, i.e. between primary producers and consumers. The species is dominating in the northern North Sea and the North Atlantic waters, and the largest amount of *C. finmarchicus* can be found in the Norwegian Sea (Planque and Batten, 2000). It is a key stone species that serves as a primary food source for several economically important fish species like e.g. Norwegian spring-spawning herring (Kaartvedt, 2000, Dalpadado et al., 2000), mackerel and blue whiting

(Prokopchuk and Sentyabov, 2006) and cod larvae (Beaugrand et al., 2003) as well as other species like the North Atlantic right whale (Greene and Pershing, 2004). The ecological importance of *C. finmarchicus* makes it particularly important to investigate how it may be affected by future ocean acidification scenarios. Potential negative effects may also have large socioeconomic consequences due to the importance of *C. finmarchicus* as feed for several commercial fish species.

1.3.1 Life cycle

The life cycle of *Calanus finmarchicus* consists of six naupliar stages (NI-NVI) followed by five copepodite stages (CI-CV), before it enters the final copepodite stage, CVI, which is the adult stage consisting of sexually matured males and females (Figure 1.1). Each transition between development stages are followed by moulting (Carlotti et al., 1993). When stage CV is reached a proportion of the individuals will descend to deeper waters by vertical migration and enter a dormant state called diapause (Fiksen, 2000). This is an effective way of avoiding predation and unfavorable conditions. Before descending to deep waters, the copepodites accumulate storage materials in the form of wax esters kept in the fat sac (Fiksen, 2000). These fat reserves are used as an energy source during their dormant state and for maturation in the early spring (Fiksen, 2000).

The reproduction cycle of *C. finmarchicus* is closely related to the phytoplankton spring bloom (Diel and Tande, 1992, Plourde and Runge, 1993). The animals ascend from overwintering prior to the spring bloom, which means that young copepodite stages appear in time to take advantage of the increased phytoplankton biomass available (Kaartvedt, 2000).

The numbers of generations in one year vary with location. They generally have one generation in the northern and western part of the Norwegian Sea, while two generations may be found in the south and east (Broms and Melle, 2007). Fewer generations in the north and west can be explained by delayed phytoplankton development and lower temperatures in those regions (Broms and Melle, 2007).

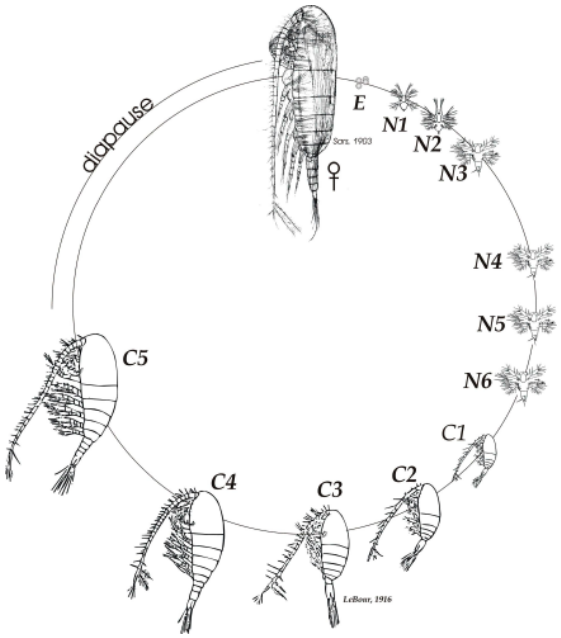

Figure 1.1 The life cycle of *C. finmarchicus*. Adult individuals produce eggs that develop into six consecutive naupili stages (NI-NVI). Then follows five copepodite stages (CI-CV). Some of the CV individuals enter diapause before finally becoming an adult male or female (CVI). Credit: Baumgartner, M. at Woods Hole Oceanographic Institution (Baumgartner, 2009).

1.3.2 *Calanus finmarchicus* and ocean acidification

So far only a few studies on the effects of ocean acidification on *Calanus finmarchicus* have been performed. One of them was done by Mayor et al. (2007) who showed that while growth of female individuals were not affected by a five days exposure to 8000 ppm CO_2, hatching success of *C. finmarchicus* eggs to nauplii was significantly impaired (Mayor et al., 2007). The exposure of CO_2 reduced the proportion of hatching eggs by 50 % (Mayor et al., 2007).

An experiment done by Pedersen et al. (2013) revealed that survival was not affected by an exposure to 3300 ppm for 28 days, but was significantly reduced at 7300 and 9700 ppm. The study also suggested that CO_2 retarded the rate of ontogenetic development. Pedersen et al. (2014) investigated whether the early stages were more vulnerable to elevated CO_2 concentrations by exposing eggs and nauplii to elevated concentrations for one week. With eggs as the starting point they observed no effect on hatching success when exposed to 8800 ppm, but there was a

significant reduction in nauplii survival as well as decreased ontogenetic development rate. With third-stage nauplii (NIII) as a starting point, there was no effect on survival and ontogenetic development at $pCO_2 \geq 7700$ ppm (Pedersen et al., 2014). The study indicates that early stages (NI and NII) are the most vulnerable and emphasizes the importance of doing studies that include all life stages (Pedersen et al., 2014). Collectively, these studies suggest that *C. finmarchicus* may be relatively robust against direct effects of the elevated CO_2 concentrations predicted for the near future (Pedersen et al., 2013, Pedersen et al., 2014).

Since reduced seawater pH is an effect of ocean acidification it is also relevant to investigate the consequences of lowered pH on *C. finmarchicus*. This was done by Marshall et al. (1935) who found that individuals exposed to a pH of 6.7 for 48 hours appeared to be unaffected by those conditions (Marshall et al., 1935). A study on the effect of pH on other zooplankton species, e.g. on *Calanus pacificus*, revealed that the sensitivity towards lowered pH was species specific and that the lethal concentration-50 (LC50) value increased with increasing exposure time (Yamada and Ikeda, 1999). This means that a smaller decrease in pH was needed to create 50 % mortality when exposure time was longer (Yamada and Ikeda, 1999).

There have not been many studies on *C. finmarchicus* and ocean acidification yet, but studies on related species can also give indications of how this species may respond to elevated CO_2 concentrations. In a study on the copepod *Acartia tsuensis* exposure to a concentration of 2380 ppm did not significantly affect survival, body size or development speed (Kurihara and Ishimatsu, 2008). Further, no significant effect on egg production or hatching rates was found. When *Acartia steueri* was exposed to 10000 ppm CO_2, the egg production rate decreased significantly (Kurihara et al., 2004). A study on the combined effect of CO_2 and temperature on *C. hyperboreus* and *C. glacialis* showed that a concentration of 3000 ppm had no effect on the performance of the species, but synergetic effects of elevated temperature and CO_2 were found in *C. hyperboreus* females as they lost significantly more carbon and nitrogen (Hildebrandt et al., 2014).

From the studies mentioned above it seems like *C. finmarchicus* generally require CO_2 concentrations well above those predicted within the next few centuries to induce direct effects (Pedersen et al., 2013). However, most of the studies have been

short-term exposures, and even though no immediate response is observed this does not necessarily imply that the animals will be unaffected by exposure over a longer period.

The most accurate understanding of the effects of ocean acidification will probably be obtained by exposing animals to CO_2 concentrations relevant for the near future for several generations, as this will be the most realistic simulation of a future scenario.

1.4 Phenotypic plasticity and the ability for genetic adaptation to OA

One important question when investigating the ecological effects of a changing environment is whether species will be able to adapt fast enough to cope with their new living conditions (Visser, 2008). Almost every change in the environment of a species, e.g. ocean acidification, is a potential source of new or intensified directional selection on fitness related traits (Gienapp et al., 2008). Populations exposed to new selection pressure can basically respond in three ways (Gienapp et al., 2008). The first possibility is to avoid the new selection pressure by dispersing to other suitable habitats (Gienapp et al., 2008). The second option is to adjust to the new conditions with means of a strictly phenotypic response based on phenotypic plasticity (Gienapp et al., 2008). Third, they can adapt to the changed environment by genetic adaption, i.e. evolution (Gienapp et al., 2008). A critical aspect of a species' ability to cope with a changing environment is therefore the ability to acclimate and genetically adapt. Organisms may be able to adjust their physiology to cope with new conditions either through acclimation or genetic adaptation, and it is important to be able to discriminate between the two strategies since they have different implications on genetics, ecology and conservation (Calosi et al., 2013).

Phenotypic plasticity is a term used for all environmentally induced phenotypic variation and is the basis for the ability of a species to acclimate (Stearns, 1989). The potential for an evolutionary response, i.e. genetic adaptation, to environmental changes is depending on the population's genetic diversity. Adaptation has taken place when the average fitness of the shifted phenotypic distribution is higher than the fitness of the original distribution in the new environment (Visser, 2008).

In the short term, the response to ocean acidification is determined by the acclimation capacity (Sultan, 2007). The existing phenotype repertoires will be crucial for the species persistence (Sultan, 2007). In the long term however, it is important to have the ability to pass alleles at the genes underlying the adaptations on to successive generations (Sultan, 2007).

Whether acclimation or adaptation is the favored strategy is determined by their respective costs (Calosi et al., 2013). Acclimation can require energy, which otherwise would have been used for other vital processes, like growth or reproduction (Calosi et al., 2013). Maintaining the sensory and regulatory machinery needed for acclimation can be a considerable cost (DeWitt et al., 1998). Therefore, it is unlikely that plastic responses provide long-term solutions for challenges faced by populations exposed to ongoing directional environmental changes (Gienapp et al., 2008). If the energy costs of phenotypic plasticity become high, genetic adaptation can be a better strategy and especially when the changes are ongoing (Calosi et al., 2013). However, genetic adaptation can also have costs as it can result in less genetic diversity or cause pleiotropic effects, and thus decrease the performance of other traits (Calosi et al., 2013).

So far, there have not been done any studies on the adaptive potential of *C. finmarchicus* to ocean acidification. However, a few studies on other species exist. Lohbeck et al. (2012) investigated the adaptive evolution of *Emiliania huxleyi*, an ecologically important phytoplankton species, and found that individuals kept under elevated levels of CO_2 for 500 generations had a higher growth rate under elevated CO_2 conditions than individuals kept under ambient CO_2 conditions (Lohbeck et al., 2012). Calcification rates were also higher in the exposed group and indicates that *E. huxleyi* has the ability to adapt to elevated levels of CO_2 (Lohbeck et al., 2012).

The study done by Form and Riebesell (2012) indicated that *Lophelia pertusa* also was able to acclimate to an increased CO_2 concentration (Form and Riebesell, 2012). Calosi et al. (2013) conducted an *in situ* study on polychaetes and their ability to acclimate or genetically adapt to high CO_2 concentrations. They found that both strategies might be important in making species able to cope with new CO_2 conditions. One of their test species, *P. dumerilii*, was able to genetically and

physiologically adapt while *A. mediterranea* responded by acclimation only (Calosi et al., 2013).

Selection is expected to result in reduced genetic variation in a population, since specific alleles will experience an increase in fitness and thus also increase in frequency, while other alleles will have a reduced fitness in the new environment and therefore decrease in frequency. Together, this will lead to a reduction in the genetic variation of the population. Therefore, it is possible to observe whether selection has taken place in a population by looking at the changes in genetic variation over time. This can be done by using neutral markers that are assumed to reflect the genome-wide genetic variation of a population. Since other evolutionary forces, like genetic drift, also can result in a reduction of the genetic variation in a population, it is important to be able to exclude the presence of such forces (Halliburton, 2004). To avoid the effect of random genetic drift it is important to have as high population sizes as possible. In addition, it is important to try to keep a constant population size between treatment populations so they at least are exposed to the same amount of genetic drift. Population bottleneck events will also entail an increased rate of genetic drift, and should therefore be avoided if the reduction of genetic variance should be used as an indication of selection. Thus, the most important thing when using changes in genetic variation as an indicator of selection is to keep all factors as equal as possible between treatments, except for the factor under study (here, CO_2 concentration).

1.5 Microsatellites as markers of genetic diversity

One of the most widely used genetic markers is microsatellites, which are non-coding DNA sequences containing variable numbers of short tandem units of 1-6 nucleotides length (Selkoe and Toonen, 2006). The advantage of using microsatellite loci when looking at changes in genetic variation is that they are known to have a high genetic diversity and a high mutation rate (Ellegren, 2004). Microsatellites are good genetic markers to estimate the level of genetic variation within and between populations of the same species in relatively few generations. The DNA sequences surrounding a microsatellite locus are called the flanking region, and are usually conserved across individuals of the same species (Selkoe and Toonen, 2006). This

makes it possible to identify a specific locus by its flanking region (Selkoe and Toonen, 2006). When amplifying microsatellite loci in polymerase chain reactions (PCR) the primers are designed to bind to the flanking region on each side of the tandem repeat unit, and the desired microsatellite region will thus be amplified (Selkoe and Toonen, 2006). Because different microsatellite alleles differ in the number of repeat units they contain, alleles differ in length and can be separated using electrophoresis. Polymerase chain reactions (PCR) make it possible to do studies based on minute tissue samples, and the fact that microsatellites are species-specific eliminates the problem of cross-contamination by non-target species (Selkoe and Toonen, 2006). When doing studies of selection over a few generations it is important to have loci that have multiple alleles in the studied population, and that have a flanking region with a low mutation rate that will bind PCR primers in the majority of the individuals in the population (Selkoe and Toonen, 2006).

Microsatellites are the most applied marker of genetic diversity (Hoshino et al., 2012) and estimates of the genetic diversity in a population can be obtained by estimating the expected heterozygosity and allelic richness at microsatellite loci (Kalinowski, 2005). In the present study, microsatellite analyses were used to investigate whether there were any signs of selection of CO_2-tolerant genotypes present in the populations exposed to elevated levels of CO_2. The microsatellites studied are neutral markers, but the genetic variation at the microsatellites is assumed to reflect the genome-wide genetic variation in the populations, and will therefore also reflect any reduced variation as a consequence of selection of CO_2-tolerant genotypes.

1.6 Aims of the present study

The purpose of this study was to determine whether *Calanus finmarchicus* populations have the ability to acclimate to a pCO_2 scenario relevant for year 2300 (2080 ppm), and to investigate whether there is a genetic component involved. This was done by exposing wild caught copepods (P generation) to an elevated pCO_2 level over two consecutive generations (F1 and F2). Changes in CO_2 tolerance and genetic variance in the populations were assayed among the CV stage individuals in the wild caught generation (P) and the last generation (F2). The potential to acclimate was investigated using a lethal concentration-50 (LC50) test to examine whether exposure to increased CO_2 levels lead to more CO_2 tolerant phenotypes. In addition, the possible genetic contribution to the phenotypic plasticity was examined by doing microsatellite analyses to determine the genetic variation in the different test populations. The changes in genetic variation were then used as information about the species' ability to adapt genetically to increased CO_2 levels.

Two different hypotheses were tested during the present experiment:

- Hypothesis 1:

C. finmarchicus populations are able to acclimate to elevated levels of CO_2.

- Hypothesis 2:

Selection of CO_2 tolerant genotypes contributes to the acclimation response of CO_2 exposed *C. finmarchicus* populations.

2 MATERIALS AND METHODS

2.1 Experimental set up

The experiment was conducted in a climate room at NTNU Centre of Fisheries and Aquaculture (SeaLab) in Trondheim from October 2012 to April 2013. The experiment was designed to investigate the adaptive potential of *Calanus finmarchicus* to elevated CO_2 concentrations by exposing two consecutive generations to either ambient CO_2 (390 ppm) or high CO_2 concentrations (2080 ppm). Field collected *C. finmarchicus* individuals (P generation) for the experiment were collected from Trondheimsfjorden at the beginning of the experiment. Subsamples of the population were collected for a test of acute CO_2 exposure tolerance and for microsatellite analyses before the exposure of CO_2 began on the two successive generations (F1 and F2). Two replicates for each exposure regime were included, making a total of four treatment containers. Subsamples for the acute toxicity test and microsatellite analyses were also collected from the F2 generation. After the exposure experiment was ended, microsatellite analyses were continued in laboratories at NTNU Gløshaugen.

2.2 Equilibration of experiment water

The seawater used in the experiment was pumped from Trondheimsfjorden at approximately 70 meters depth. To get the desired CO_2 concentrations in the experimental water, a custom-made gas mixer (HTK Hamburg®GmbH) was used. The gas mixer was adjusted to get the appropriate CO_2 concentrations by regulating the amount of air (compressed air distributed at SeaLab) and CO_2 (100%, from a pressure flask). The two gas mixtures (390 and 2080 ppm) were led to two different equilibrating column units, where seawater was calibrated with the gas mixtures. The calibration columns were made of two cylinders, one smaller cylinder inside a bigger one. Here, the water was led into an inlet at the top of the equilibration units and the gas mixture was bubbled into the bottom of the inner cylinder through a wooden air stone (Aqua Media GmbH, Germany). A submersible aquatic pump was situated at the top of the inner cylinder, causing downwards water movements in the inner

cylinder. At the same time, bubbles with the gas mixture were ascending, creating a counter-current system in the inner cylinder, which allowed effective equilibration of the seawater. A water outlet at the bottom of the columns led to the corresponding experimental tanks consisting of four 90 liters polystyrene containers. Each column led to two tanks (the replicates). The water from the equilibration column units entered the tanks at both the bottom and the top. A flow chart of the equilibration of the experimental seawater is shown in Figure 2.1.

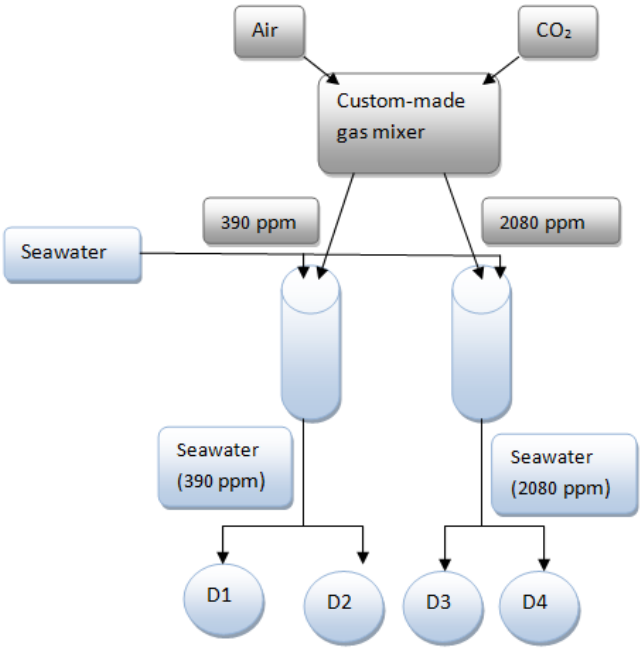

Figure 2.1 A flow chart illustrating the equilibration of experimental water. Compressed air and 100 % CO_2 entered the custom-made gas mixer, which produced gas mixtures with two CO_2 concentrations: 390 and 2080 ppm. The two different gas mixtures exiting the gas mixer entered one equilibration column unit each, which consisted of two cylinders; one smaller inside a bigger one. The equilibration units were also supplied with seawater, which was pumped downwards in the inner cylinder. At the same time, the gas mixture was bubbled into the bottom of the inner cylinder through a wooden air stone. The counter-current system created inside the equilibration column by ascending gas and descending seawater allowed for effective equilibration of the seawater. Then, the calibrated seawater entered the experimental tanks, which contained a secondary equilibration system. Seawater containing 390 ppm CO_2 was led to tank D1 and D2, while seawater from the equilibration column unit receiving a gas mixture of 2080 ppm CO_2 was led to tank D3 and D4.

To counteract the effect of air-water exchange of CO_2 in the tanks, a secondary equilibration system was also installed directly in the tanks. A column covered with a nylon mesh was placed in the middle of the tanks. The gas mixture was bubbled through a wooden air stone at the bottom of this column, and water was pumped downwards with a pump placed at the top of the equilibration system. Wastewater exited through an outlet at the top of the tank at a rate of 90 liters day^{-1} so that a volume corresponding to the total tank volume was exchanged every 24 hours. A heating system was also incorporated in the tanks to maintain a constant temperature of 11 °C during the experiment. The heating element was placed inside the equilibration column and the heat sensor was placed just outside of the column so it was approximately in the middle of the water column. The algae feed, provided by a peristaltic dosing pump, entered at the water surface of the tanks.

2.3 Water parameters

A set of regular measurements was taken throughout the experiment to make sure that the experimental tanks had the appropriate water conditions. It is not convenient to measure pCO_2 directly. Instead it can be determined by measuring two carbon parameters along with information about the temperature, salinity, pressure and the solubility constant for CO_2 in seawater, as well as the equilibrium constants for each of the acid-base pairs that are assumed to exist in the solution, and the total concentration of all the non-CO_2 acid-base pairs (Dickson et al., 2007). The two carbon parameters measured in this experiment were total alkalinity, A_T, and the spectrophotometric pH. The actual CO_2 concentrations in the experimental tanks were thus determined by doing regular measurements of total alkalinity, salinity, temperature and pH, and then using these data in the software calculating program CO2SYS-v2.1.xls (Pelletier et al., 2007). In addition, regular measurements of algae concentrations were taken to make sure the animals had the appropriate amount of feed.

2.3.1 Temperature

In the beginning of the experiment there was no regulation of the temperature in the tanks other than the room temperature, which was set to 10 °C. However, due to quite large fluctuations, a temperature controller system was installed in the tanks in January to provide a more stable environment for the animals. The target temperature was set to 11°C and was monitored by a temperature sensor placed in the water column. Whenever the temperature dropped below the set point, a heating element would warm the water until the set point was reached. The temperature in the tanks was also measured daily with a glass thermometer (VWR® Precision Thermometer, accuracy ±0.3 °C) to verify that the heating system was working properly. The temperature was increased with one degree (from 10 to 11 °C) to speed up the development of the animals.

2.3.2 Spectrophotometric pH

The pH in the tanks was measured spectrophotometrically every day throughout the experiment. The spectrophotometric pH measurements were based on a method described by Dickson et al. (2007). This method uses the total hydrogen ion concentration pH scale, and is based on the use of indicator dye m-cresol purple (2 mM) and its pH-dependent coloration. The second dissociation of the indicator dye is as following,

$$HI^{-}(aq) = H^{+}(aq) + I^{2-}(aq) \qquad (2.1)$$

Where I is the indicator, HI^- is the acidic form of m-cresol and I^{2-} is the basic form. The acidic and basic forms of the indicator have different absorbance maxima, which makes it possible to estimate the ratio of the concentration of the basic to the acidic form, $[I^{2-}] / [HI^-]$, based on the absorbance spectra. The ratio can further be used to find the total hydrogen ion concentration, $[H^+]$, and thereby also the pH (Dickson et al., 2007).

Seawater samples from each tank were collected in gas-tight glass bottles (50 ml). The samples were collected inside a filtering cup with small pore size (64 µm) to

prevent introduction of animals in the sample. The sample bottles were rinsed twice before the samples were collected and then transferred to a water bath set at 25 °C for equilibration. After equilibration, the seawater samples were transferred to an optical glass cuvette until a positive meniscus was obtained using a pipette. The cuvette was then sealed with a Teflon cap to remove excess water and air bubbles. The first spectrophotometric reading was done only on seawater samples to obtain the background absorption. Then, a new reading was performed on the seawater samples together with the indicator dye. Indicator dye (50 µl) and seawater was mixed in the optical glass cuvette prior to the analysis. Both the background reading and the reading with indicator dye were performed at the same three wavelengths (434, 578 and 730 nm) by using the RNA-DNA sub-menu of the Cary WinUV software. The wavelengths 434 and 578 nm correspond to the absorption maxima of the acidic (HI^-) and the basic (I^{2-}) forms of the indicator, respectively, while 730 nm corresponds to a non-absorbing wavelength for m-cresol and is included to make correction of any baseline shifts possible (Dickson et al., 2007). The measurements were done using Cary 50 Bio UV-visible Spectrophotometer (Varian Inc., Mulgrave, Australia), a Cary Single cell peltier accessory (Varian, Inc., Mulgrave, Australia) and a Micro-Jet MC450 multi-use micro-pump (Aquarium Systems, Sarrebourg, France). The measurements done on only the seawater (blank measurements) were used to correct for background absorption by subtracting them from the measurements done on the seawater samples containing indicator dye (m-cresol measurements). The results from the absorption measurements were inserted into a custom-made excel sheet that automatically calculated the pH according to the following formula:

$$pH = 8.0056 + \log\left(\frac{\left({}^{A1}/_{A2} - 0.00691\right)}{\left(2.2220 - \left({}^{A1}/_{A2} \times 0.1331\right)\right)}\right) \qquad (2.2)$$

where

$$\frac{A1}{A2} = \frac{(\mathrm{A578cresol} - \mathrm{A578blank} - (\mathrm{A730cresol} - \mathrm{A730blank}))}{(\mathrm{A434cresol} - \mathrm{A434blank} - (\mathrm{A730cresol} - \mathrm{A730blank}))} \qquad (2.3)$$

2.3.3 Total alkalinity

Total alkalinity (A_T) was measured daily in one of the four tanks. The samples for the measurements were collected in the same way as described for pH measurements, and transferred to a water bath (25 °C) for equilibration. The method employed was based on an open-cell titration method described by Xiaowan et al. (2009) (Xiaowan et al., 2009). The measurements were done using an automatic potentiometric titrator (TitraLab TIM860 Titration Manager, Radiometer Analytical SAS; Villeurbanne, France) with a PHC2001-8 pH electrode. The titrant used was a solution of 0.02 M HCl with 0.68 M NaCl maintained at 25 °C.

Air bubbles in the tubing system of the titrator had to be removed before analysis to avoid inaccurate measurements. A sample with a volume close to 25 ml was transferred from the seawater sample to the titration cell together with a magnet. The exact volume of the sample (measured on a weighing scale) was inserted into the program, and titrant was added in steady doses (except a larger first addition) until the titration curve was determined. The alkalinity was automatically determined by the apparatus based on the volume of titrant necessary to reach the equivalence point obtained during the titration. The total alkalinity concentration, M_{At} (eq/l), was calculated as follows:

$$M_{At} = c_{HCl} \times \frac{V_{HCl}}{m_{sw}} \qquad (2.4)$$

where

c_{HCl} = molality of the HCl solution

V_{HCl} = volum of HCl consumed (ml)

m_{sw} = mass (g) of seawater sample

To ensure that the method was working properly, a measurement of a reference material (certified seawater, Scripps Institution of Oceanography, La Jolla, USA) with known A_T was done before measuring the daily seawater samples.

2.3.4 Salinity

Salinity in the tanks was determined daily by using a refractometer (H_2Ocean ATC salinity). The refractometer was calibrated with standard seawater with a known salinity (33.3) before the salinity of the seawater in the tanks was determined. Standard seawater (approximately 0.5 ml) was placed on the sample plate with a plastic pipette and the cover was gently closed. After a few seconds of equilibration, the salinity was read by looking into the ocular while it was pointed towards a light source. If the salinity reading was incorrect it was adjusted to the correct value (33.3) by using a screwdriver. After the calibration, the seawater salinity was determined by placing a few drops of the seawater sample onto the sample plate and reading off the salinity by pointing the refractometer towards the same light source used during the calibration.

2.3.5 Feed concentration

The feed composition in the experiment was chosen based on previous successful culturing of *C. finmarchicus* by Hansen et al. (2007) using a mixture of three unicellular algae species; *Isochrysis galbana*, *Dunaliella tertiolecta* and *Rhodomonas baltica* (Hansen et al., 2007). Even though *C. finmarchicus* probably do not actually feed on *I. galbana* due to its small size, it was included since this mixture has proven to be successful. Campbell et al. (2001) found that the critical concentration for normal development and growth in *C. finmarchicus* is 100 µg C L^{-1} (Campbell et al., 2001). A combined target value of 250 µg C L^{-1} was chosen as this would mean that the feed concentration was sufficiently high to allow the animals to grow and develop, and at the same time it was not too high, since an excess of feed potentially could mask energetic effects of elevated CO_2. The feed was supplied continuously to the tanks from a daily-prepared stock solution by the use of a peristaltic dosing pump.

To make sure that the organisms had the appropriate level of feed, measurements were performed on a regular basis (usually every other day) using a coulter counter (Multisizer™3 Coulter Counter® Beckman Coulter Inc., USA). Samples for the feed concentration analysis were collected using a long tube with a mesh at the end to prevent any individuals from entering the sample. This sampling procedure was

adopted to get a sample from the entire water column. The water collected in the tube was transferred to a cell counter cup (25 ml), which was placed on a stirring table (Labotron 960742, Infors HT, Bottmingen) until it was analyzed to prevent sedimentation of the feed particles. The coulter counter determined the feed concentration (cells mL^{-1}) by taking three consecutive measurements from the same water sample. A curve showing the integrated number of particles against particle diameter was created. This curve was used to integrate the appropriate size range (5.333-9.651 µm), and the obtained concentration was then transformed into carbon equivalents (µg C L^{-1}).

2.4 The three experimental generations

Individuals of *C. finmarchicus* collected from Trondheimsfjorden were divided randomly among four experimental tanks, which were labeled D1, D2, D3 and D4. Throughout the entire P generation all tanks had ambient CO_2 concentrations of 390 ppm. Exposure with elevated CO_2 concentrations was started on the F1 generation and continued throughout the F2 generation. Two of the tanks, D1 and D2, were maintained at an ambient CO_2 level (390 ppm) and thus served as control tanks, while D3 and D4 had an elevated concentration of 2080 ppm CO_2 and were thus called CO_2 tanks. The same set up was used for the F2 generation. A summary of the treatment terms used later in this paper, along with the most important characteristics, is presented in Table 2.1.

Table 2.1 Experimental treatments and their characteristics. The involved tanks (D1, D2, D3 or D4), generation (P, F1 or F2) and CO_2 concentration (390 or 2080 ppm) for each treatment are listed.

Treatment	Tanks	Generation	pCO_2 (ppm)
P	D1-D4	P	390
F1-390 1	D1	F1	390
F1-390 2	D2	F1	390
F1-2080 1	D3	F1	2080
F1-2080 2	D4	F1	2080
F2-390 1 (Control 1)	D1	F2	390
F2-390 2 (Control 2)	D2	F2	390
F2-2080 1 (CO_2 1)	D3	F2	2080
F2-2080 2 (CO_2 2)	D4	F2	2080

A total of nine treatments are described. The tanks D1 and D2 are considered as replicates of the 390 ppm treatment, and D3 and D4 are considered as replicates of the 2080 ppm treatment for F1 and F2. The replicates F2-390 1 and F2-390 2 are collectively called F2-390 (control treatments). The replicates F2-2080 1 and F2-2080 2 are collectively called F2-2080 (CO_2 treatments). No tests were conducted on the F1 generation.

2.4.1 Starting a new generation - Collection of nauplia

When starting a new generation, the nauplia had to be separated from the adult individuals to prevent predation as *C. finmarchicus* may display a relatively high degree of cannibalism (Basedow and Tande, 2006). This was done using custom-made nauplia traps attached to each tank. The trap consisted of a small aquarium that was placed on the side of each tank. A PVC-tube was leading from the tank to the aquarium and served as a siphon. At the end of the tube a plankton mesh prevented adult stages from entering. A lamp was installed just inside of the mesh to attract phototactic nauplia. The aquarium had an outlet that also was covered with a plankton mesh. This had an even smaller mesh size to prevent nauplia from exiting the aquarium. A syringe was used to start the siphon. The water level in the aquarium was adjusted by regulating the height of the tube outlet. The traps were used for a few days until it seemed like a good amount of nauplia had been collected.

Before the nauplia collected in the traps were transferred back to the tanks, the adult individuals in the tanks had to be removed. This was done by transferring the water inside the tanks to buckets (2 x 50 L for each tank) using a siphon. The end of the tube that was submerged in the tank water had a filter at the end that prevented any animals from being transferred to the bucket. The water was thus removed while the adult animals remained in the tanks. The last few liters of water in the tanks, including the adult animals, were emptied out through the drain at the bottom of the tank. The tanks were then properly cleaned and the filter cloth covering the equilibration column in the tank was changed. The water collected in the buckets was then transferred back to the tanks, and the nauplia collected in the traps were transferred to their corresponding tank.

2.5 LC50 test

An acute CO_2 toxicity test was conducted on individuals from the P and F2 generation. The purpose of the test was to determine the lethal concentration 50 (LC50), which is a measure of the concentration of a substance in a medium that will kill half of the exposed population. The value LC50 is useful because it allows for the comparison of the toxicity of different chemicals, and in this case it was used to investigate potential differences in the tolerance against elevated CO_2 among the populations from tanks with a pre-history of exposure to ambient vs. elevated pCO_2. A high LC50 means that a higher concentration is required to kill 50 % of the exposed animals, and is thus a sign of higher tolerance towards the substance. Seven different elevated concentrations of CO_2 and a control with ambient concentration were used in this part of the study. For each concentration, three replicates were included, while the control of the P generation had six. Each replicate consisted of a 0.5 L gas-tight bottle of seawater that contained between five to eight CV individuals. The exposure lasted for 96 hours and mortality was recorded after 6, 12, 24, 48, 72 and 96 hours. The set up was static, i.e. the test solution remained unchanged throughout the experiment. No food was provided throughout the experiment.

2.5.1 Equilibration of test water

The target CO_2 concentrations in the test were as follows: control (390 ppm = 0.00039 %), 2.25 %, 2.89 %, 3.7 %, 4.75 %, 6.08 %, 7.8 % and 10 %. Before the test started, seawater with the different CO_2 concentrations was prepared by bubbling filtrated seawater with a mixture of air and CO_2. The seawater was first filtrated through a 0.22 µm filter, and then transferred to eight different bottles (2 L) containing enough water for all the replicates. A gas mixer was then used to get the target CO_2 concentrations in the different bottles. The gas mixer had two inlets, one for air and one for 100 % CO_2. Inside the gas mixer the gases went through capillaries of different lengths, which ended in eight different outlets, one for each treatment. The gas was bubbled through a wooden air stone at the end of the tube. The relationship between the length of the air capillary and the CO_2 capillary determined the

concentration of CO_2 in the gas leading through the outlets. The mixing of gas and water continued for two hours to make sure that the water was properly equilibrated. The equilibrated seawater was then carefully transferred to smaller bottles (0.5 L), three for each CO_2 treatment and six for the control treatment of the P generation, using a siphon tube. An illustration of the equilibration of the LC50 test water is shown in Figure 2.2.

After the water was transferred to its respective bottle, five to eight CV individuals were transferred to each replicate in a random order in respect to the CO_2 concentration. It has been shown that different development stages in copepods can display different tolerances towards elevated CO_2 concentrations, and it was therefore deemed important to use the same copepodite stage in all the LC50 tests (Cripps et al., 2014). Before collecting the animals, the water in the exposure tank was stirred carefully, but thoroughly. A glass bowl was used to collect a subsample of the tank population. Then, the individuals were transferred to the test bottles using a plastic pipette with a wide opening. When transferring individuals from the glass bowl to the bottle, care was taken to make sure that they were chosen as randomly as possible, by stirring in the bowl as well as collecting from random locations within the bowl when taking out the animals. Due to some loss of water during transfer of the animals, an extra bottle of bubbled water was used to refill the bottles completely to make sure they were filled to the top. Finally, the bottles were sealed with a gas tight lid.

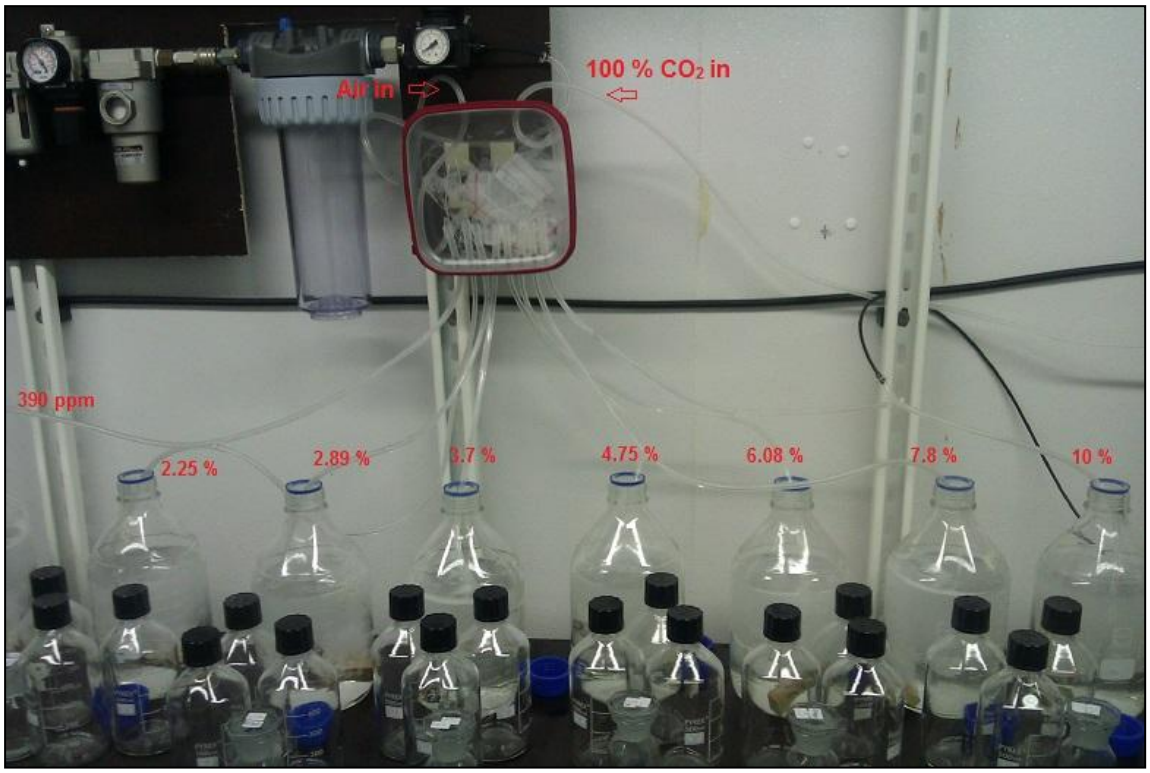

Figure 2.2 The equilibration of LC50 test water. Air and 100 % CO_2 are entering through tubes at the top of the gas mixer and going through capillaries of different lengths inside the mixer, resulting in different concentrations, before exiting through eight outlets. Each outlet lead to a 2 L bottle filled with filtered seawater, and was bubbled out through a wooden air stone. For each concentration, the water was transferred to three smaller bottles (0.5 L) after equilibration. Then, these bottles were added *C. finmarchicus* individuals.

2.5.2 Calculating the actual CO_2 concentrations

To determine the actual CO_2 concentrations in the test, a set of seawater measurements were taken from each bottle at the end of the LC50 test. Temperature, salinity, total alkalinity and pH in each bottle were determined, and were then used to calculate the actual pCO_2 by using the software calculating program CO2SYS-v2.1.xls (Pelletier et al., 2007). Temperature and pH were measured immediately after the exposure was terminated, while salinity and total alkalinity were measured shortly after.

The pH in each bottle was determined potentiometrically with a pH-meter (pH 1000 T, pH-enomenal), which was two-point calibrated using IUPAC precision pH buffer 4.005 and 7.000 (Radiometer Analytical). The electrode (pH-enomenal® 111) was

left in seawater for about 20 minutes so it would have time to equilibrate to the higher ion strength of seawater after the calibration. Then, the pH was determined by placing the electrode directly into the bottles and letting the pH stabilize before the pH value was read. The pH-meter was also showing the temperature, which was recorded for each bottle. Alkalinity and salinity were determined as described in section 2.2.

2.5.3 Oxygen measurements

The oxygen contents of the exposure bottles were measured after the test period the first time the LC50 test was conducted to make sure that mortality was not caused by oxygen deficiency. The measurements were conducted with an YSI Dissolved Oxygen Meter (Model 58). According to OECD's guideline for testing of chemicals, the oxygen concentration should not be less than 60 % of the air saturation value (Oecd, 1994).

2.5.4 LC50 calculations and statistics

The number of dead copepods in the parallel bottles of each concentration of exposure media was not pooled prior to calculations or corrected for any mortality in the controls, as all control mortalities were lower than the threshold value of 10 %. The number of dead copepods was calculated as percentage of the initial number. The calculations of the LC50 values, along with their 95 % confidence intervals, were done using a non-linear model based on a sigmoidal dose-response model with variable slope (four-parameter logistic equation) using GraphPad Prism version 6.0 (GraphPad Software, San Diego California USA):

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC50 - X) - Hillslope}} \quad (2.5)$$

Here, *Bottom* is the Y value at the bottom plateau, *Top* is the Y value at the top plateau, *LogEC50* is the X value when the response is halfway between Bottom and

Top. The *HillSlope* describes the steepness of the curve and has no units. If this value is positive, the curve increases as X increases. If it is negative, the curve decreases as X increases. A standard sigmoid dose-response curve has a HillSlope of 1.0. When HillSlope is less than 1.0, the curve is shallower. When HillSlope is greater than 1.0, the curve is steeper. A variable slope rather than a standard slope was chosen in this experiment since the curve was based on many data points.

When performing the calculations, constraints on the dose-response curve was placed both on top and bottom, forcing the effect to be calculated within the interval 0-100 % effects (mortality).

To determine statistical differences between LC50 values the 95 % confidence intervals calculated in GraphPad Prism were used. Treatments with non-overlapping intervals could be considered significantly different with 95 % certainty (Knezevic, 2008). When confidence intervals overlapped, a test based on the square root of the sum of standard errors was applied to determine whether the LC50 values were significantly different (Knezevic, 2008). The values were considered significantly different when:

$$(x_1 - x_2) > 1.96\sqrt{SE_1^2 + SE_2^2} \qquad (2.6)$$

Here, x_1 and x_2 are the LC50 values and SE_1 and SE_2 are their corresponding standard errors (Knezevic, 2008).

Graphs were created in GraphPad Prism and in Sigmaplot.

2.6 Microsatellite analyses

After the end of the CO_2 exposure experiment, microsatellite analyses of the DNA from individuals collected from the P and F2 generation was carried out in the molecular genetics laboratories at NTNU Gløshaugen. In addition, some individuals from the continuous lab culture maintained at NTNU SeaLab were sampled for analysis.

2.6.1 Sampling and preservation of DNA

Individuals (CV stage) from the P generation and the F2 generation were sampled for microsatellite analyses. A total of 46 individuals were preserved from the P generation and 24 individuals were used from each of the four tanks in the F2 generation (F2-390 1 and 2, F2-2080 1 and 2), making a total of 96 individuals from F2. A total of 23 individuals were collected from the lab culture at SeaLab. These individuals will be referred to as culture animals. The analysis of the culture animals was conducted to get an insight of how the effects of genetic drift may reduce the genetic variation in laboratory cultures of this species. The culture animals have been cultured at SeaLab for approximately 40 generations. As a relatively high mortality was observed among the test animals, a comparison of the genetic diversity in the test population versus the culture animals could provide information on how large effect the reduction in population size may have had on the genetic diversity.

The individuals were chosen at random by mixing the water in the tanks gently, both vertically and horizontally, before samples were collected in a glass bowl. From the bowl they were transferred individually to a well in a 96-well plate and stored in ethanol (200 µl, 96 %) at room temperature until the analysis.

2.6.2 DNA extraction

DNA extraction was done by modifying a method employed for extraction of DNA from house sparrow blood. The entire individual was used in the extraction. The animals were carefully dried to remove ethanol before being transferred to a new 96-

well plate by using a toothpick. A mixture was prepared from 6050 µl Lairds buffer and 440 µl Proteinase K (20 mg ml^{-1}) for each plate (96 DNA samples). A volume of 60 µl of this mixture was added to each well. The plate was then centrifuged and incubated on 55 °C and 210 rpm shaking for 20 hours. After incubation, the plate was centrifuged again, and the rest of the extraction was done by an automated extraction method carried out on a Beckman Coulter NXp pipetting robot (Beckman Coulter, USA) by using a Relia Prep Large Volume HT gDNA Isolation System (Promega, USA). To investigate the DNA concentrations in the extracted samples some of the concentrations were read with a plate reader by using a Fluostar Omega scanner and software, while some of the other samples were checked on NanoDrop ® ND-1000 spectrophotometer.

2.6.3 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify chosen microsatellite loci from the extracted DNA. Two different PCR methods were employed: Multiplex PCR using 12 microsatellite loci for the P generation, and singleplex PCR followed by multiplexing of the PCR products prior to fragment analysis using six microsatellite loci on the F2 generation and the culture animals. The change in PCR method was done due to problems in the F2 generation with amplification using the first method. These amplification problems are discussed in part 4.6.2.1 of this paper. The method was thus adjusted and six of the loci causing problems were removed from the analysis. Therefore, only six of the twelve loci initially amplified in the P generation were used in further analyses.

2.6.3.1 Multiplex PCR in the P generation

Multiplexing using a fluorescently labeled universal primer, in combination with modified locus-specific primers with 5` universal primer sequence tails, was employed as the PCR procedure for the P generation. This is a so-called three primer approach, which is more cost-effective and less time consuming than directly labeling locus-specific primers (Blacket et al., 2012). The three primer technique uses universal primers that can be combined with multiple fluorophores, which makes it

possible to co-amplify multiple loci (Blacket et al., 2012). During PCR the tailed locus-specific primer is depleted and the fluorescently labeled universal primer is subsequently incorporated (Blacket et al., 2012). Multiplexing is possible by using different fluorescent dyes and having products with different size ranges (Blacket et al., 2012). The two universal primer sequences along with the respective fluorophore, basepair length (bp), the GC content (%) and the melting temperature, T_m (°C), are shown in Table 2.2.

Microsatellite loci were chosen by searching through the literature. Based on loci described by Parent et al. (2012) and Provan et al. (2007) a total of 14 microsatellite loci were tested (Parent et al., 2012, Provan et al., 2007). The parent population was used when testing the loci as this group was expected to display the highest genetic variation. Amplification success and presence of variation at loci were investigated in the test. Two of the 14 loci were not variable and where thus excluded from further analysis. As a result, two multiplex panels were designed based on 12 of the 14 loci. For the P generation the individual genotypes were determined using the following 12 microsatellite loci: FK868270, FG632811, FK670364, FK867682 (Parent et al., 2012), EL696609, EL585922, EH666870, EL773359, EL773519, EH666474, EL586088 and EL585872 (Provan et al., 2007). In parts of this paper the loci will be abbreviated using the two first letters and the last three numbers of the locus names. The microsatellites were labeled with one of the two following fluorescent dyes: NED (yellow) and FAM (blue). This made it possible to separate the different loci based on both color and size by electrophoresis. The panels were used to score the genotypes of the parent generation. Each panel had a total of six loci, as shown in Table 2.3. Wherever two loci in a panel had overlapping size ranges, the loci were labeled with different dyes.

Table 2.2 Universal primers used in PCR and their tail sequence, fluorophore, length (bp), GC content and melting temperature (Primer Tm, °C).

Labeled universal primer	Tail sequence (5'-3')	Fluorophore	Length (bp)	GC content (%)	Primer Tm (°C)
Primer A	GCCTCCCTCGCGCCA	FAM	15	80	63
Primer B	GCCTTGCCAGCCCGC	NED	15	80	57

Table 2.3 Overview of microsatellite loci in the two respective panels, as well as both forward (F) and reverse (R) primer sequences, and labeled universal primer used in the P generation. Loci marked with an asterisk (*) were used in genotyping of individuals from both generations and on the culture animals. The highlighted part of the forward sequences is the sequence of the universal primers.

Panel	Microsatellite loci	Primer sequence (‘5-‘3)	Labeled universal primer
1	FK868270	F-**GCCTCCCTCGCGCCA**GAAAGAGGAGGCTAAAAAGC R-CATACTGAGGAGTGGAGGAA	FAM- Primer A
	FK867682*	F-**GCCTTGCCAGCCCGC**AGACTGGAGAAGATCATTGC R-TGCTCTGTTTTCACTACACG	NED- Primer B
	EL696609*	F-**GCCTTGCCAGCCCGC**GATCTGCGCTAGGCTAGTGAT R-GTCTGAACATCCACGCAAGA	NED- Primer B
	EL585922	F-**GCCTCCCTCGCGCCA**AGCAGTAGAGGAAGCAAAGGT R-TCTTGCTGCCTCAGTTTCTG	FAM- Primer A
	EH666474*	F-**GCCTTGCCAGCCCGC**ACCAAAAGATGCTGCCAAAG R-GAAGCTATCGGCTGACTTGG	NED- Primer B
	EL585872	2 F-**GCCTCCCTCGCGCCA**TTTAAGCTGGGATGGGACAG R-GTCGTAGGCGGTGAAACACT	FAM- Primer A
2	FG632811*	F-**GCCTTGCCAGCCCGC**ATGAAAGTGCCTCTGATAGC R-CTGTTGAATGATCCTGGTCT	NED- Primer B
	FK670364	F-**GCCTCCCTCGCGCCA**ACTATGGTGTTGCTGAAGGT R-CAGCCAGTCTAGGATTGTTC	FAM Primer A
	EH666870	F-**GCCTCCCTCGCGCCA**CAACATGTCAGGGTCCAACA R-TGGGGGTGGTTGACTAAGAT	FAM- Primer A
	EL773359*	F-**GCCTCCCTCGCGCCA**CATAGCTAGTCATACATGGCAT R-GGAAAAACAAAGATTTATTAACAAA	FAM- Primer A
	EL773519*	F-**GCCTCCCTCGCGCCA**ACTAGAGCAGACCCCAAGCA R-CTCTGTGGGGCTCACTAAGG	FAM- Primer A
	EL58608	F-**GCCTTGCCAGCCCGC**CGAAGAGGAATTAGGAGTCTCG R-GGGCGCCTATAATTTCTCAC	NED- Primer B

2.6.3.2 PCR for the F2 and culture animals

As mentioned, only six of the 12 loci were used when genotyping the F2 generation and the culture animals. The six loci used were: FG632811, FK867682, EL696609, EL773359, EL773519 and EH666474. Instead of multiplex PCR, which was used when genotyping the P generation, singleplex PCR followed by multiplexing prior to the fragment analysis was conducted for F2 and the culture animals. Prior to fragment analysis the PCR products from each locus were combined into the same

sample as all loci either had different size ranges or were labeled with different colors. There were thus six PCR reactions for each individual and the products from these reactions were combined after the PCR procedure by mixing either 2 or 4 µl of each product before using this mixture in the electrophoresis and fragment analysis. Whether 2 or 4 µl were used depended on the amplification strength of each locus: 4 µl was used of loci with weaker amplification strength and 2 µl were used for loci with higher signals.

2.6.3.3 PCR profile

The PCR amplification was executed in Applied Biosystems GeneAmp PCR system 9700 PCR machines (Applied Biosystems, USA).

Included in each PCR reaction mixture (10 µl) was 5 µl 2x QIAGEN Multiplex PCR Master Mix (QIAGEN Inc, USA), 0.2 µl tail A (2 µM), 0.2 µl tail B (2 µM), 0.2 µl of each reverse primer (2 µM), 0.05 µl (0.5 µM) of each forward primer, approximately 20-40 ng of DNA and a varying amount of RNase free water making the total volume equal to 10 µl.

The same touchdown profile was used in all the PCR reactions: First a denaturing step at 94 °C for 15 minutes followed by 15 cycles at 94 °C for 30 seconds, an annealing step initially at 68 °C for 90 seconds (successively reduced by 1°C each cycle for 15 cycles); and an elongation step at 72 °C for 60 seconds. Following this there were 30 cycles with 94 °C for 30 seconds, 53 °C for 90 seconds and 72 °C for 60 seconds. Finally, the PCR machine was run for 30 minutes at 60 °C, and the PCR products were then kept at 4 °C.
The PCR profile was chosen based on a test of three different profiles with a varying number of cycles.

For each sample, 1 µl of the PCR product was mixed with 0.5 µl of a size ladder (GeneScan LIZ 600, Applied Biosystems, USA) and 10 µl Hi-Di formamide solution (Applied Biosystems, USA). Separation of the PCR products was then performed using an automated 16 capillary electrophoretic analysis system: ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, USA). Genotypes of all individuals on the

microsatellite loci were scored using the software package GENEMAPPER 4.0 (Applied Biosystems, USA).

2.6.4 Microsatellite statistical analysis

In all tests the significance level was set to $p \leq 0.05$.

All loci were tested for Hardy-Weinberg equilibrium in each treatment using the software Cervus version 3.0.3 (Marshall, 1998). Although the test implied that some loci deviated from Hardy-Weinberg equilibrium in some of the treatments, it was assumed that this was due to a low sample size, and expected heterozygosity was thus still used to provide an estimate of the actual heterozygosity in the treatments. An estimation of null alleles, as well as calculations of observed and expected heterozygosity, was conducted in the same program. Some loci had a deficiency of heterozygote individuals and an excess of homozygotes. This could be explained by the Wahlund effect (Wahlund, 1928), inbreeding, selection favoring homozygote genotypes, scoring errors or the presence of null alleles (Degen et al., 1999). The most likely explanations in this case are probably scoring errors or the presence of null alleles, but could also be a result of sampling effects (Degen et al., 1999). Selection of homozygote genotypes is not likely since the genotyped microsatellite loci are assumed to be selectively neutral. That the deficiency of heterozygotes is due to the Wahlund effect is also an unlikely explanation in this case, as there was no possibility of migration between the different populations (treatments). Furthermore, no population substructure is expected to be present either in Trondheimsfjorden or within the different treatments. Inbreeding is also excluded as a possible explanation as the population sizes are assumed to be large enough to avoid deviations from panmixia (random mating). Loci with positive F(Null) values (indicating the presence of null alleles) were not omitted due to a low number of loci to begin with, the possibility of sampling effect being the cause of the positive values and because no locus had high null allele frequencies in all treatments.

Allelic richness and expected heterozygosity were used as measures of the genetic diversity within each treatment.

Allelic richness was determined using the software HP-Rare (Kalinowski, 2005). Allelic richness is a measure of genetic diversity, which in cases with equal sample sizes can be calculated simply as the average number of alleles over loci (Kalinowski, 2005). In this study however the sample sizes varied, and since it is expected that a large sample will have more alleles than a small sample some compensation for differences in sample sizes had to be made (Kalinowski, 2005). The HP-Rare software uses a rarefraction technique which compensates for unequal sample sizes, and this method was therefore applied in the present study (Kalinowski, 2005).

The level of genetic differentiation between treatments was estimated using the population genetics software Genepop (Raymond and Rousset, 1995). Pairwise F_{ST} values were then calculated for all possible pairs of populations.

Differences in expected heterozygosity were investigated in the statistical package SPSS (IMB® SPSS® Statistics version 21) using one-way analysis of variance (ANOVA) followed by *post hoc* LDS to determine significant differences. No significant difference from homogeneity of variances was detected between the samples.

The same procedure was conducted to investigate significant differences in allelic richness between treatments. No significant difference in homogeneity of variances was detected for allelic richness either. All graphs were created in Sigmaplot.

3 RESULTS

3.1 Water parameters

As shown in Table 3.1, the actual CO_2 concentrations in the tanks differed slightly from the target values. For the two 390 ppm treatments, the actual values were somewhat higher than the target value, with 472.93 ppm and 466.93 ppm, respectively. For the elevated CO_2 treatments, on the other hand, the actual concentrations were slightly lower than the target concentration, with a mean of 1747.90 and 1814.33, respectively.

Table 3.1 The mean pH ($pH_{Tot,25°C}$), temperature (Temp., °C), total alkalinity and salinity in the experimental tanks throughout the exposure period, along with the standard deviation, based on the measurements done during the exposure period, i.e. on the F1 and F2 generation. Calculated pCO_2 (ppm), $pH_{Tot,in\ situ}$ and Ω aragonite (Ar) and calcite (Ca) (mean±SD) are also shown.

Treat-ment	$pH_{Tot,25°C}$	Temp. (°C)	Total alkalinity	Salinity	pCO_2(ppm)	$pH_{Tot,in\ situ}$	Ω Ar	Ω Ca
F2-3901	7.75±0.02	10.3±1.1	2242±30.29	33.3 ±0.33	472.93±33.28	7.97±0.02	1.64±0.09	2.59±0.14
F2-3902	7.76 ±0.02	10.4±0.9	2242±30.29	33.3±0.33	466.93±30.93	7.97±0.02	1.68±0.09	2.65±0.14
F2-20801	7.26±0.06	10.4±0.9	2242±30.29	33.3 ±0.33	1747.90±263.75	7.45±0.06	0.51±0.08	0.81±0.12
F2-20802	7.25±0.06	10.2±1.0	2242±30.29	33.3 ±0.33	1814.33±269.81	7.43±0.06	0.49±0.08	0.78±0.12

3.2 The LC50 test

3.2.1 Actual concentrations in exposure bottles

The actual CO_2 concentrations in the glass bottles after the LC50 exposure period are shown in Table 3.2. The calculations revealed that the actual CO_2 concentrations were generally higher than the target values. The difference between the nominal and actual value was quite large in some cases, so the actual CO_2 concentrations were used when calculating the LC50 values. However, for simplicity they will still be referred to as their nominal value.

Table 3.2 Actual CO_2 concentrations (%) in the exposure bottles used in the LC50 test. The actual concentrations are calculated from measurements of pH, temperature, alkalinity and salinity. The mean of the replicates (N=3, except control treatment of the P generation where N=6) along with standard deviation is shown for each treatment and nominal concentration.

Nominal conc. (%)	**Actual concentrations in the toxicity test**				
	Treatment				
	P	**F2-390 1**	**F2-390 2**	**F2-2080 1**	**F2-2080 2**
0.00039	0.00085 ±0.00002	0.00075 ±0.00007	0.00069 ±0.00001	0.00069 ±0.00002	0.00068 ±0.00002
2.25	2.30±0.23	3.53±0.08	3.42±0.02	3.39±0.03	3.67±0.01
2.89	3.61±0.11	4.38±0.11	4.32±0.01	4.25±0.02	4.55±0.04
3.70	4.6±0.03	5.41±0.04	5.36±0.05	5.26±0.06	5.67±0.02
4.75	5.74±0.06	6.46±0.03	6.43±0.10	6.32±0.05	6.78±0.05
6.08	7.33±0.25	8.20±0.03	8.26±0.11	7.84±0.10	8.64±0.04
7.80	7.62±0.33	9.03±0.29	8.78±0.10	8.55±0.05	9.24±0.05
10	10.55±0.17	12.26±0.07	11.92±0.13	11.38±0.02	12.26±0.03

3.2.2 LC50 values

The mortalities in the controls were all lower than the 10 % threshold, as shown in Table A.6 in Appendix A. All bottles also had an oxygen content that exceeded 88 %, and were thus well above the threshold of 60 % set by OECD (Oecd, 1994), as can be seen in Appendix A, Table A.7.

The calculated LC50 values of each treatment at 96 hours of exposure are presented in Figure 3.1, along with the corresponding 95 % confidence intervals. The F2-2080 treatments (LC50= 3.896 and 4.273) displayed significantly higher LC50 values compared to the F2-390 treatments (LC50= 3.502 and 3.251) with a 95 % certainty, as can be seen by their non-overlapping confidence intervals. Although the confidence intervals of the two control treatments do not overlap, they are both significantly lower than the CO_2 treatments as well as the P treatment.

The highest LC50 values were observed in the P generation (LC50=4.320) and in the F2-2080 treatments, which were not significantly different from each other.

The result output from the sigmoidal dose-response model used to calculate the LC50 values and corresponding 95 % confidence intervals in GraphPad for the P generation and all F2 treatments at all monitoring points is shown in Table A.8-A.12 in Appendix A. The mortality data that these calculations are based on is shown in Table A.1-A5 in Appendix A.

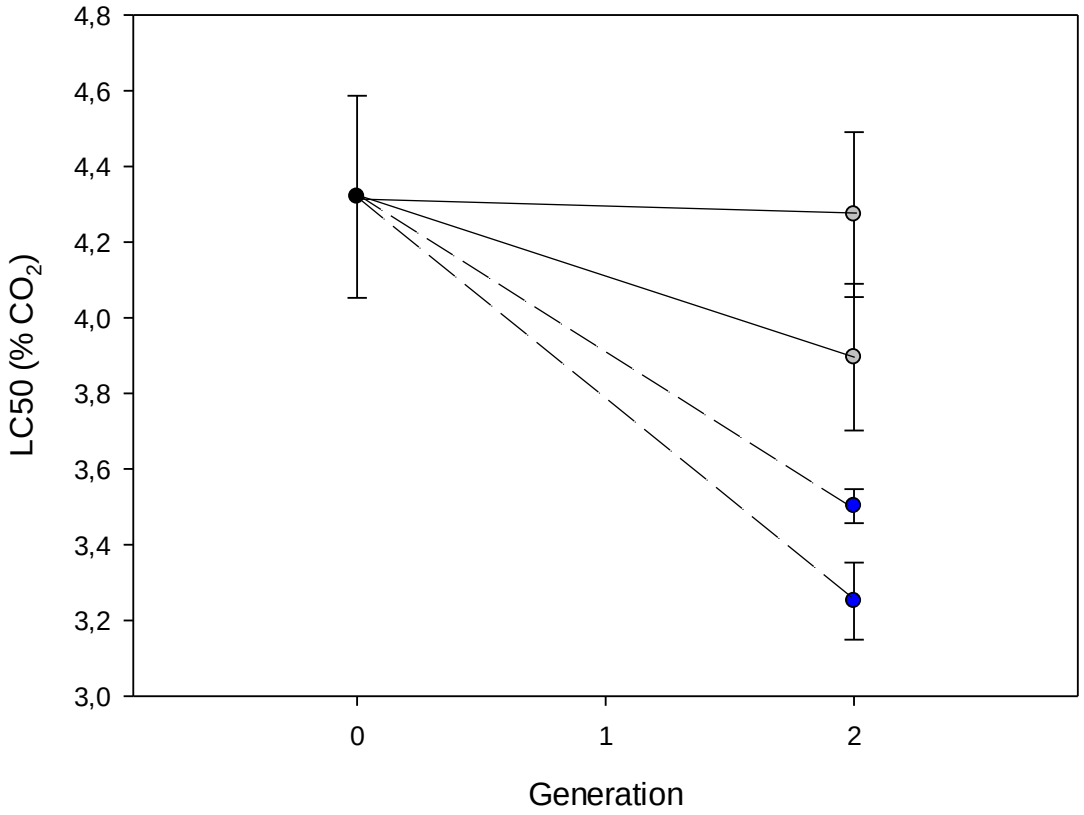

Figure 3.1 The LC50 values and 95 % confidence intervals of each treatment at 96 hours of exposure are plotted against generation. Treatments are indicated using different colors: Black: P generation, blue: F2-390 and grey: F2-2080. Solid (between P and F2-2080) and dashed (between P and F2-390) lines are used to illustrate that the F2 generation originated from the P generation.

The LC50 values of the parent generation and the two F2 treatments for each monitoring point are shown in Figure 3.2. The P generation displayed the highest LC50 value for all the monitored time points, except after six and 12 hours, where F2-2080 had the highest values. For the rest of the monitored time points, F2-2080 had somewhat lower values than P, but well above F2-390. F2-2080 displayed a sharp decrease in LC50 after six hours. Then, the decrease slowed down after 24 hours and stabilized for the rest of the exposure period. The control treatments had the lowest LC50 values for all the monitored time points. The control displayed a large drop in LC50 between 6 and 12 hours, then, the decrease slowed down and

stabilized. The P generation seemed to have an almost linear decrease in LC50 throughout the exposure period.

Percent mortality as a function of CO_2 concentration (%) for all treatments after 96 hours of exposure is shown in Figure 3.3. The F2 control treatments displayed a higher mortality compared to the P and F2-2080 treatments at any given CO_2 concentration.

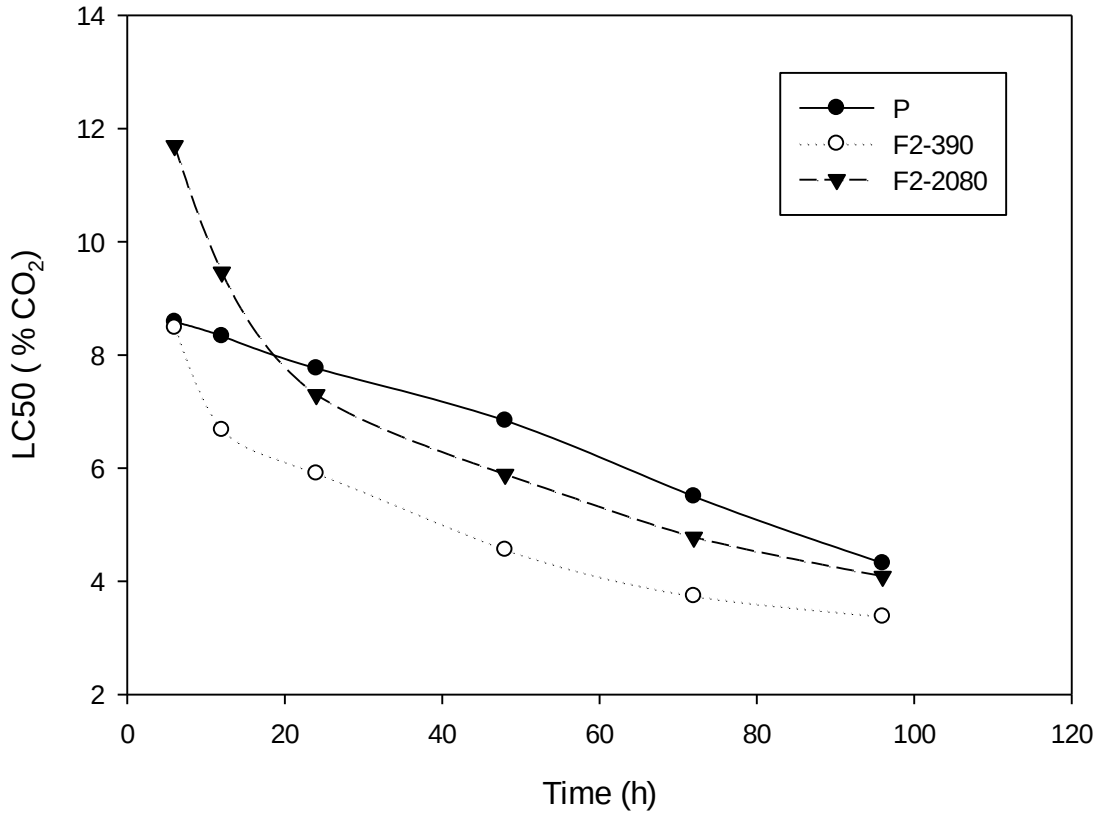

Figure 3.2 LC50 values (% CO_2) in the different treatments at the different time points for monitoring (6, 12, 24, 48, 72, and 96 h). Mean LC50 values are shown for the F2 treatments (N=2).

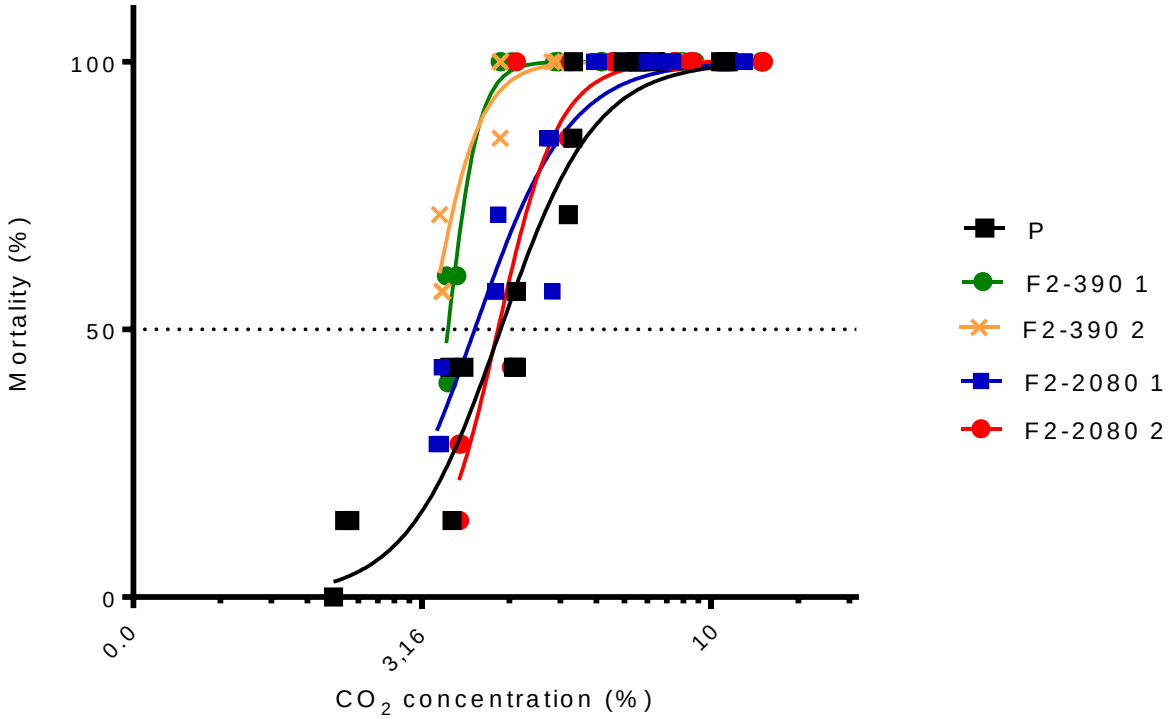

Figure 3.3 Percent mortality as a function of CO_2 concentration (%) for each treatment at 96 hours of exposure. The different treatments are indicated using different symbols and colors: P generation (black squares and lines), F2-390 1 (green circles and lines), F2-390 2 (orange crosses and lines), F2-2080 (blue squares and lines), F2-2080 2 (red circles and lines). The dashed horizontal line is drawn at the point of 50 % mortality.

Percent mortality as a function of CO_2 concentration is shown for each monitored time point for all treatments in Figure 3.4. The parent population (Figure 3.4 A) has a low increase in mortality at the beginning of the exposure, as seen by the short distance between the earliest monitoring lines. That is, the CO_2 concentration required to kill a certain amount of the population remains pretty similar for the first monitoring points. Then, the increase in mortality increased rapidly as exposure time becomes longer. This means that at the beginning of the exposure the concentration that is required to kill a certain amount of the test population remains almost constant. Then, as exposure time increases the concentration of CO_2 that is required to kill the same amount of the population decreases rapidly as exposure time increases.

For all F2 populations (Figur 3.4 B-E) the concentration of CO_2 that is required to kill a certain amount of the population seems to have a more even decrease as time increases for the entire exposure period since there appear to be a more constant distance between all monitoring lines.

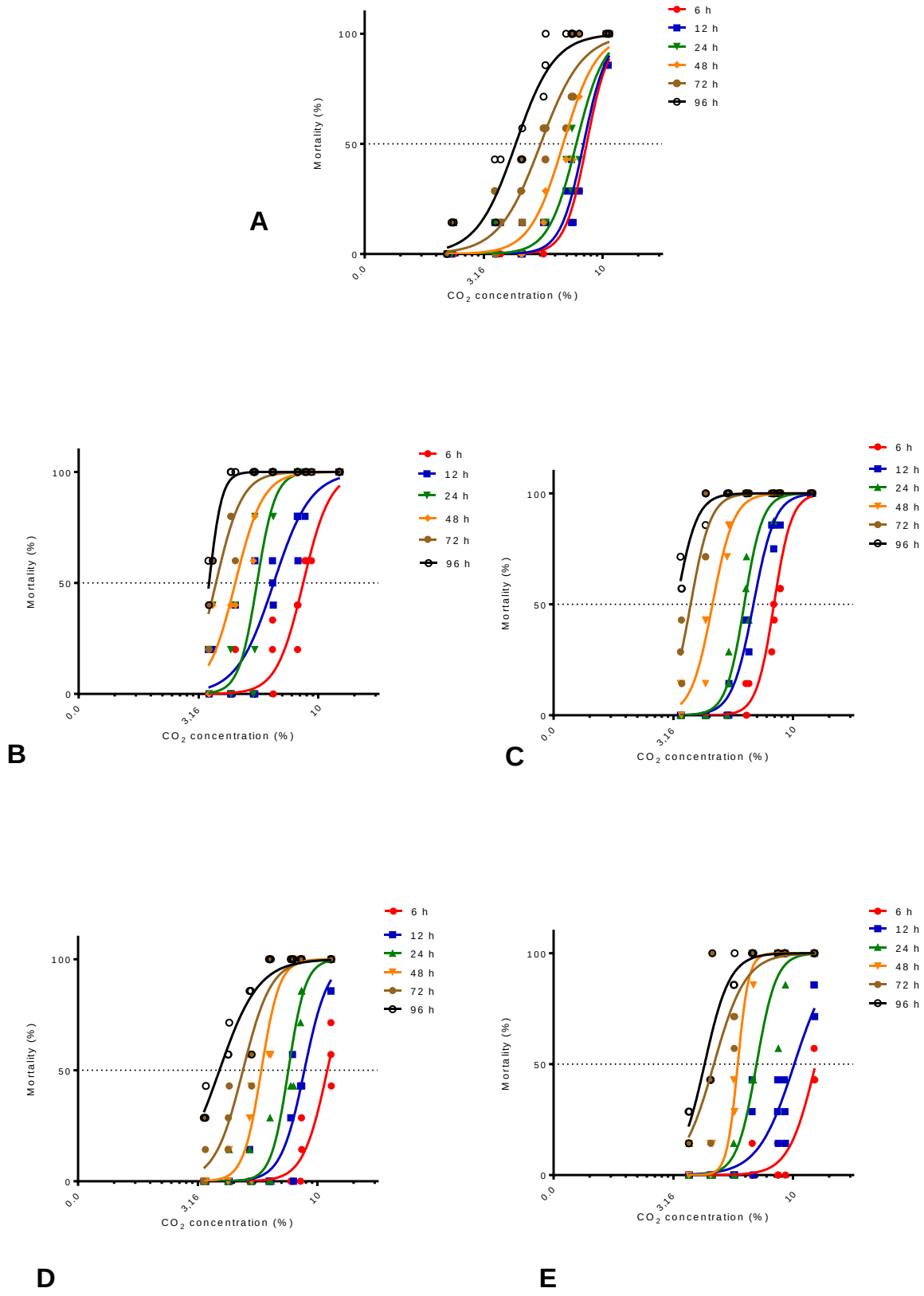

Figure 3.4 The concentration-mortality curve at all monitoring points for the five treatments. A) P generation, B) F2-390 1, C) F2-390 2, D) F2-2080 1 and E) F2-2080 2.

3.3 The microsatellite analysis

3.3.1 Genotyped loci

A total number of 12 microsatellite loci were investigated in the P generation, but the results of six of those were not included since they were not successfully genotyped in the F2 generation. Of the six loci included the size range of the products from each locus is shown together with the total number of alleles in Table 3.4. The number of alleles per locus ranged from eight to 16.

Table 3.3 Size range (bp) of the products and total number of alleles at each locus genotyped in the P generation and the F2 generation in the experiment.

Locus	Size (bp)	Number of alleles
FG811	247-274	12
FK682	171-207	16
EL609	128-140	8
EL359	128-156	8
EL519	186-207	10
EH474	144-165	9

The number of successfully scored individuals, observed and expected heterozygosity, Hardy-Weinberg estimates, null alleles and allelic richness at each locus in the six treatments are shown in Table B.1 in Appendix B. Mean expected heterozygosities, allelic richness and private allelic richness for each treatment is shown in Table B.2 in Appendix B.

3.3.2 Expected heterozygosity and allelic richness

Mean expected heterozygosity is plotted against generation in Figure 3.5. Although no statistical significant differences were found between the expected heterozygosity of the control (H_{exp}=0.6592 and 0.5640) and CO_2 treatments (H_{exp}=0.4825 and 0.5267) there was a tendency of lower expected heterozygosity for the elevated CO_2 treatments (p=0.095). Neither the control nor the CO_2 treatments were significantly different from the parent generation (H_{exp}=0.6053). However, there was a tendency of

lower expected heterozygosity in the CO_2 treatment compared to the P generation (p=0.065). The only significant difference in H_{exp} was observed for the culture treatment (H_{exp}=0.2095), which displayed significantly reduced expected heterozygosity compared to all the other treatments ($p \leq 0.001$). The culture animals are estimated to have gone through approximately 40 generations in the continuous laboratory culture.

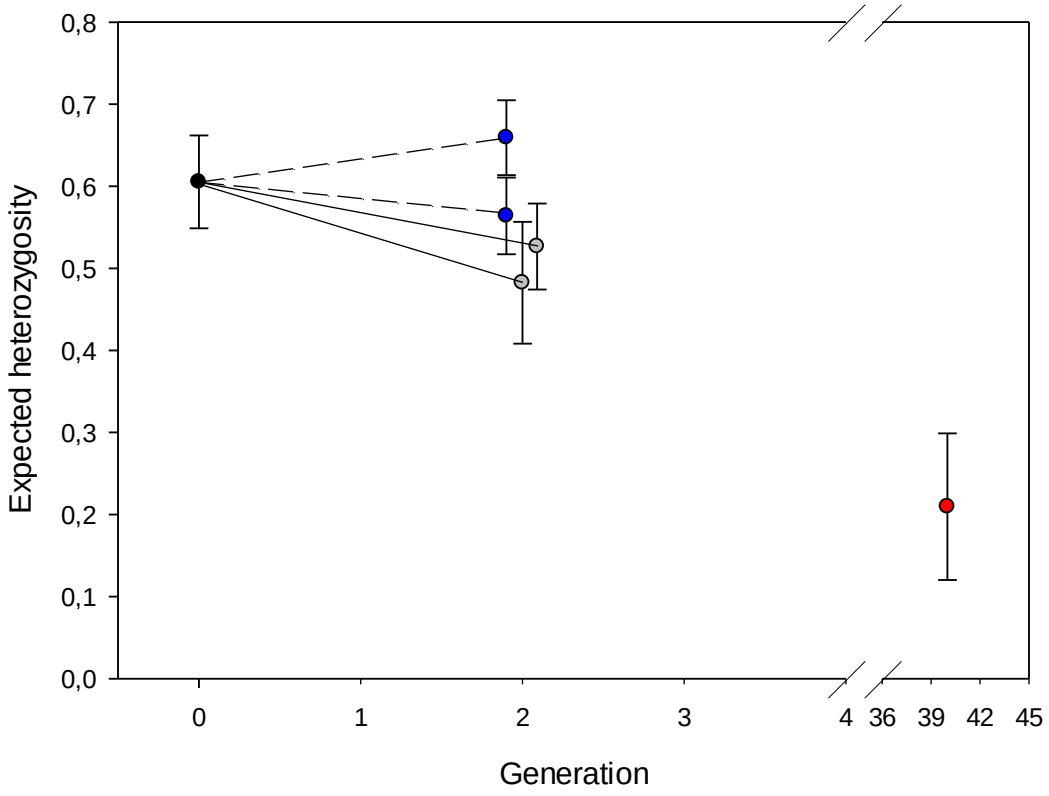

Figure 3.5 Mean expected heterozygosity, including standard error bars, as a function of generation. The different treatments are distinguished using different symbols: Black:P, blue:F2-390, grey:F2-2080, red:Culture. The F2 generation samples are plotted with a small offset along the x-axis even though they all belong to generation two. Solid (between P and F2-2080) and dashed (between P and F2-390) lines are used to illustrate that the F2 generation samples all originated from the P generation.

Mean allelic richness is plotted against generation in Figure 3.6. A pattern similar to the one observed for expected heterozygosity was observed. Again, no significant difference was found between the parent generation (A_R=3.0618) and the F2 control treatment (*p*=0.341). However, the CO_2 treatment had significantly lower allelic richness than the parent generation (*p*=0.038). The culture animals displayed a significantly reduced allelic richness (A_R=1.5712) compared to all the other populations (*p≤0.003)*. As for H_{exp}, the CO_2 treatments (A_R=2.3765 and 2.5360) appear to have somewhat lower allelic richness than the controls (A_R=2.9973 and 2.5864), but the difference is not significant (*p*=0.151).

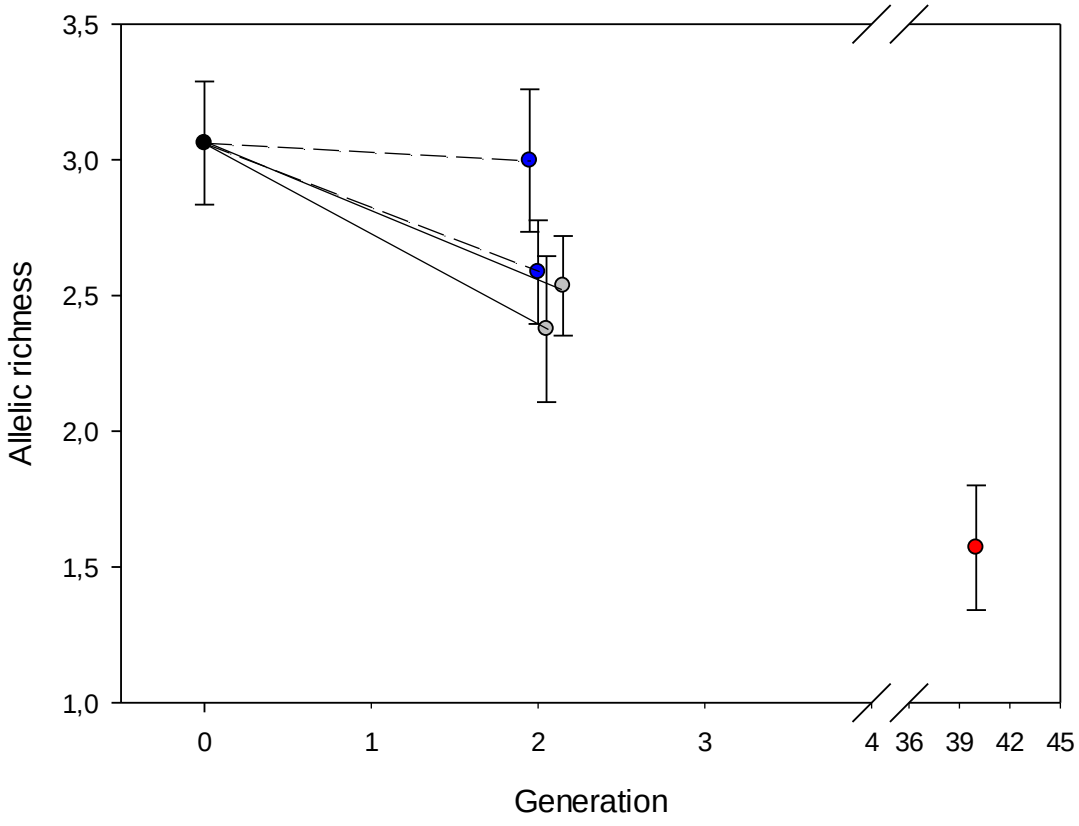

Figure 3.6 Mean allelic richness, including standard error bars, versus generation. The different treatments are distinguished using different symbols: Black:P, blue:F2-390, grey:F2-2080, red:Culture. The F2 generation samples are plotted with a small offset along the x-axis even though they all belong to generation two. Solid (between P and F2-2080) and dashed (between P and F2-390) lines are used to illustrate that the F2 generation samples all originated from the P generation.

3.3.3 Genetic differentiation

Pairwise F_{ST} values between all possible pairs of populations are shown in Table 3.4. A low F_{ST} value means that populations have a low degree of genetic differentiation, and vice versa. The control treatments are less genetically differentiated from the parent generation compared to how differentiated the CO_2 treatments are from the parent generation. Control 1 is less differentiated from control 2 than from the CO_2 treatments. Control 2, on the other hand, is more differentiated from control 1 than from the CO_2 treatments. The CO_2 populations are less differentiated from each other than from any other population. The highest degree of differentiation is found between the culture and all the F2 populations.

Table 3.4 Level of genetic differentiation (F_{ST}) between all pairs of populations calculated in Genepop.

	P	**Culture**	**Control 1**	**Control 2**	**$CO_2$1**
Culture	0.2795				
Control 1	0.1661	0.3307			
Control 2	0.1997	0.3904	0.0721		
$CO_2$1	0.2997	0.5255	0.0995	0.044	
$CO_2$2	0.2544	0.4325	0.0923	0.024	0.0272

4 DISCUSSION

4.1 Water parameters

The water parameters controlled during the exposure period revealed that there were some deviations between target and observed values. The difference between target and actual CO_2 concentrations observed in the tanks can be explained by the fact that there has been a certain degree of gas exchange with the surroundings, as well as the fact that there have been some fluctuations in the parameters affecting the CO_2 concentration, e.g. temperature and alkalinity. As a consequence the concentrations in the control tanks were somewhat higher than the target value (mean 472±33 and 466±30), while the concentrations in the CO_2 treatment tanks were lower than the target value (mean 1747±263 and 1814±269), and this study will thus reflect a CO_2 scenario relevant for a time period somewhat prior to year 2300.

The pH remained fairly constant between the replicates of both the control (both 7.97) and the CO_2 treatments (7.45 and 7.43), and the temperature did not seem to differ notably between any of the treatments, mean ranging from 10.2-10.4. The measured temperatures do however have quite large standard deviations ($0.9 \leq SD \leq 1.1$), which can be explained by the fact that there was no heating system in the tanks at the beginning of the experiment, and the temperature was thus about 1°C lower at the beginning than after the heating system was installed. The variations in temperature should however be equal for all tanks and should thus not affect any potential differences between the control and the CO_2 treatments.

4.2 The acclimation potential of *C. finmarchicus* towards elevated CO_2

Based on the results from the acute toxicity test it is possible to say something about the ability of *C. finmarchicus* to acclimate to an elevated concentration of 2080 ppm CO_2. When comparing the LC50 values of treatment F2-390 and F2-2080, the significantly higher value of the F2-2080 individuals shows that they require a higher concentration of CO_2 to reach 50 % mortality in the sample population, and this further indicates that they have attained a higher tolerance towards elevated CO_2 from being exposed to an elevated concentration of 2080 ppm CO_2 over two generations (Figure 3.1). The F2-2080 treatment populations did however, not display a higher tolerance than the P generation population. This could possibly be explained by the fact that individuals cultured in the laboratory tend to have a smaller size and fat content compared to wild animals, as a result of a higher development rate due to increased temperatures (Campbell et al., 2001). This can reduce the ability of cultivated animals to cope with elevated CO_2 levels compared to wild caught animals. This is supported by the fact that the control populations in the F2 generation have lower CO_2 tolerance than the P generation, as they otherwise would have been expected to have equal tolerances. Therefore it may be misleading to compare the LC50 value of the P generation with the F2 generation, since more than just the CO_2 factor differs between those generations. A better understanding of the tolerance towards CO_2 can probably be attained from comparing the control and CO_2 treatments within the F2 generation, which both were assumed to be exposed to the same conditions except for the difference in CO_2 concentrations.

The largest advantage of previous exposure to elevated CO_2 concentrations is attained when exposure time is short, as can be seen by the notably higher LC50 value of F2-2080 at 6 hours exposure compared to the other two treatments, which both have approximately the same value at 6 hours (Figure 3.2). After six hours the advantage declines, as the LC50 value rapidly decrease and stabilize at a value somewhat lower than the P generation for the rest of the exposure time. Still, the advantage is present throughout the entire exposure period as the F2-2080 has higher LC50 values than the F2-390 treatment at all times. The advantage of previous exposure to elevated CO_2 concentrations remains almost constant from 12-96 hours of exposure, but there is possible to observe a small decrease in the

advantage at 72 and 96 hours of exposure, as the difference in LC50 values between the control and elevated CO_2 populations becomes somewhat smaller. In addition, it seems like the largest advantage is attained when CO_2 concentrations are relatively low, as shown in Figure 3.3, since the mortality of the control treatment increase rapidly even at the lowest CO_2 concentrations, while the increase in mortality for the CO_2 treatment is somewhat slower at the beginning. When the concentration reaches a certain point it seems like the apparent advantage of F2-2080 is negligible as mortality rapidly increases and levels off at 100 %. This could probably be explained by the fact that there is a large gap between the highest CO_2 concentrations in the acute toxicity test, ranging from 2.25-10%, and the CO_2 concentration the F2-2080 treatments were exposed to prior to the test (~2080 ppm). Even though exposure to 2080 ppm CO_2 for two generations seems to give *C. finmarchicus* a higher tolerance towards elevated CO_2 concentrations, it might not be surprising that the tolerance towards e.g. 10 % CO_2 is not significantly affected.

The reaction patterns in mortality as exposure time increases at the different concentrations differ the most between generations rather than between treatments (Figure 3.4). An increasing exposure time does not have much effect on the mortality in the parent generation in the beginning of the exposure. In other words, there were not much happening in the exposure bottles between six and 12 hours of exposure. Then, as exposure time increases the effect on mortality is larger, i.e. the concentration required to kill a certain amount remains almost constant in the beginning and then, a notably lower concentration is required to kill the same amount as the exposure time becomes increasingly longer. This means that a higher number of individuals in the bottles died in between monitoring points when the exposure time had lasted for a while, compared to how many individuals that died between monitoring points in the beginning of the test. The F2 generation, on the other hand, displays a more constant reaction pattern throughout the entire exposure period. The effect on the mortality as a function of exposure time is more evenly distributed, and it may even seem that the effect on mortality is largest at first. This indicates that wild animals may be able to cope relatively better with increased CO_2 concentrations at short exposure times than the cultured animals. The seemingly higher initial CO_2 tolerance among the wild animals compared to the animals reared in the laboratory might again be explained by the observation that wild *C. finmarchicus* often have a

considerably larger size and fat content than cultured individuals due to the effects of increased temperature on development rate and body size (Campbell et al., 2001). Increased temperature increase the development rate and leads to smaller body size (Campbell et al., 2001, Carlotti et al., 1993). There does not seem to be a difference in the reaction pattern between the control and elevated CO_2 treatments of the F2 generation regarding the exposure duration. However, it is possible to observe a tendency of a general higher mortality in the control treatments, which also are evident by the observed significantly lower LC50 values of the control treatments (Figure 3.1 and 3.2). This is e.g. easy to see on the 6 hours exposure line, where the control treatments have points ranging from 0 to 100/ almost 100 %, while the mortality of the CO_2 treatments only ranges from 0 to almost / just above 50 %.

The results of the LC50 test thus indicated that *C. finmarchicus* from Trondheimsfjorden may have the ability to adapt to elevated concentrations of CO_2 relevant for the year 2300. This study is therefore consistent with previous studies showing that exposure over a longer period leads to smaller effects of the elevated CO_2 concentrations on the organisms, as they will have the time to acclimate (Calosi et al., 2013, Lohbeck et al., 2012). In addition, the present study indicates that *C. finmarchicus* may be robust towards the direct effects of a future ocean acidification scenario, which also is consistent with previous studies (Pedersen et al., 2013, Pedersen et al., 2014).

4.3 Genetic basis for the acclimation response to high CO_2

The tendency of reduced expected heterozygosity and allelic richness observed in the treatments exposed to elevated CO_2 concentrations in the F2 generation compared to the control treatments indicate that the F2-2080 populations may have a lower level of genetic variation than the F2-390 populations, although the difference is not significant. This possible reduction in genetic variation could be the result of directional selection towards high CO_2 tolerant phenotypes (adaptive evolution) (Halliburton, 2004). The increased concentration of CO_2 can entail a selection pressure on the exposed individuals, and thus reduce the genetic variation in the population by causing selection against certain phenotypes (Halliburton, 2004). To which degree selection will reduce the genetic variation is dependent on the strength of the selection (Halliburton, 2004). Strong directional selection against some phenotypes under elevated CO_2 conditions, e.g. by affecting the survival, will rapidly reduce the genetic variation (Halliburton, 2004). Large selective advantages of some phenotypes will cause them to rapidly increase in frequency, and as a consequence the variation will also rapidly be reduced (Halliburton, 2004). However, if there is only a weak selective advantage the increase of those phenotypes, and simultaneously decrease of others, will be slower (Halliburton, 2004). Therefore, the probability of being able to detect selection over only a few generations increases if selection is strong (De Kovel, 2006). The previous studies done on *C. finmarchicus* exposed the species to higher CO_2 concentrations compared to the concentration used in the present study (Mayor et al., 2007, Pedersen et al., 2013, Pedersen et al., 2014). Still, they revealed that *C. finmarchicus* was relatively unaffected by these exposure levels (Mayor et al., 2007, Pedersen et al., 2013, Pedersen et al., 2014). This indicates that the selection pressure caused by increased CO_2 concentrations on *C. finmarchicus* populations probably is weak, and might therefore explain why only tendencies and no significant reductions in genetic variation were observed in the present study. If the exposure had lasted for more than two generations it might have been possible to observe larger differences in the genetic diversity between the control and exposed populations, since it takes more time to create differences when selection is weak (De Kovel, 2006).

Lower genetic variation could also be caused by genetic drift, which probably have occurred to some degree in all treatments as an effect of high mortality and therefore reduced population sizes in the laboratory. However, since all factors were the same between the treatments apart from the difference in CO_2 concentration the most plausible explanation for the observed tendency of reduced genetic diversity among the high CO_2 populations is that they have experienced a directional selection of CO_2 resistant phenotypes. The difference in expected heterozygosity and allelic richness between the P generation and the control populations is small and not significant, which indicates that there has not been a high level of genetic drift, since it would be expected that the control treatments would have lower genetic diversity than the parent generation if genetic drift was a significant force. However, it is worth noticing that one of the control populations had both a lower expected heterozygosity and allelic richness than the other control population. The most likely explanation of this observation is that there has been a higher degree of genetic drift in the control tank with the lowest genetic diversity, which probably is due to a higher mortality rate causing a lower population size in that tank. Genetic drift is inversely related to population size, which means that a lower population size in one treatment tank will result in a higher degree of genetic drift (Halliburton, 2004). It was observed that there seemed to be some differences in population size between the tanks, but the differences were never quantified. Genetic drift and directional selection both work by reducing the genetic variation in a population (Halliburton, 2004), and the presence of genetic drift could therefore make it more difficult to detect a selection effect. The presence of genetic drift is therefore a possible explanation of the non-significant difference between control and CO_2 treatments. However, as mentioned the difference in genetic diversity between the parent generation and the control populations are not large, so genetic drift was probably only present to a small degree, but evidently it seemed to be more significant in the control with the lowest genetic variation. It would be desirable to keep the population size in each tank equal since then it could be assumed that all populations experienced the same amount of genetic drift. In that case, the difference in genetic variation between treatments could be explained by selection with a higher degree of certainty compared to when there is a varying population size. However, in this experiment the amount of genetic drift was probably varying between tanks depending on their population size and this is making it more difficult to say something for certain about whether selection of CO_2

tolerant genotypes was the reason for the reduced genetic diversity observed in the elevated CO_2 populations.

The fact that there is a significant reduction in the allelic richness of the populations exposed to elevated CO_2 compared to the parent generation, at the same time as there is no significant reduction in the allelic richness of the control populations compared to the parent generation, is also indicating the presence of selection in the CO_2 populations.

If the observed microsatellites were known to be in linkage disequilibrium with the genes affecting the ability of *C. finmarchicus* to cope with elevated CO_2 concentrations, the reduced genetic variation in exposed populations at the genotyped loci would be safer signatures of selection (De Kovel, 2006, Li et al., 2010). This is not the case in the present study. However, if the selection of CO_2 is causing genome-wide selection signatures the observed changes will reflect selection.

The significantly lower genetic diversity found in the culture population compared to all other populations can be explained by ongoing genetic drift for approximately 40 generations, as a consequence of low population size compared to wild populations, and possibly also selection towards the laboratory environment. The results give information about how cultivation of *C. finmarchicus* reduces the genetic diversity of the population. The significantly lower genetic diversity of the cultured animals compared to the wild animals emphasizes the importance of genetic drift in a relatively small laboratory culture.

If the ability to adapt to increased CO_2 concentrations has a genetic component it would be expected that the control treatments were less differentiated than the CO_2 treatments from the P generation, at the same time as each of the replicates would be less differentiated from each other than from the other treatment. The observed pattern in the results almost fit those expectations with the exception of control replicate 2, which is less differentiated from both CO_2 treatments than from the other control replicate (Table 3.4). All the pairwise F_{ST} values of the culture and the F2 populations are high, indicating that the culture is highly differentiated from all other treatments as expected considering this population has been in the laboratory for many generations. The parent generation is more differentiated from all F2

treatments than the F2 treatments are from each other. The fact that one of the control treatments is less differentiated from both CO_2 treatments than from the other control replicate could possibly be a consequence of random genetic drift. This is supported by the fact that the same control population displays lower expected heterozygosity and allelic richness compared to the other control population. Lower differentiation between CO_2 1 and CO_2 2 than between control 1 and control 2 supports the theory that selection has taken place. Genetic drift is a random process, which means that it will lead to different alleles increasing and decreasing in frequency in different populations, i.e. there is no direction on the allele frequency changes (Andrews, 2010). Directional selection on the other hand will give rise to the same alleles increasing and decreasing in frequency as some are related to higher and lower fitness (Andrews, 2010). The observation that the control populations are more differentiated from each other compared to how differentiated the CO_2 populations are from each other indicates that genetic drift is the dominating force in the controls, while selection is the dominating force in the CO_2 populations. As genetic drift seems to be a more significant force in one of the control population it is possible that the random reduction in genetic diversity has caused the population to be less differentiated from the elevated CO_2 population than from the other control population simply due to random events.

The present study indicates that genetic adaptation could explain part of the higher CO_2 tolerance observed among the *C. finmarchicus* populations maintained at high CO_2 for two generations. The ability to acclimate indicates that *C. finmarchicus* has the phenotypic plasticity required to cope with increased pCO_2 and the microsatellite analysis suggests that some of the phenotypic variation might have a genetic component. The fact that there seems to be a genetic component involved in the adaptation towards elevated CO_2 concentrations means that CO_2 tolerant phenotypes will pass alleles contributing to this tolerance on to successive generations, which can contribute to maintaining the fitness of *C. finmarchicus* populations under future pCO_2 scenarios.

4.4 Main findings in relation to previous studies

There is generally a lack of knowledge about the adaptive potential of marine organisms to ocean acidification (Doney et al., 2009). Several studies have been done on the effects of climate change, but there is a lack of evidence of evolutionary adaptation towards climate change stressors (Gienapp et al., 2008). This is because many studies done on the adaptive potential of species only investigated phenotypic acclimation without studying the genetic contribution. In addition, some studies designed to investigate the genetic contribution to the acclimation ability have been based solely on phenotypic data, and the interferences about the genetic contribution in those studies are therefore not always credible (Gienapp et al., 2008).

To the author's knowledge this study is the first examination of the adaptive potential of *C. finmarchicus* to ocean acidification. However, a few studies have been done on other species. Lohbeck et al. (2012) investigated the adaptive evolution of the phytoplankton species *Emiliania huxleyi* and found that individuals kept under elevated levels of CO_2 for 500 generations had a higher growth rate under elevated CO_2 conditions compared to individuals kept under ambient CO_2 conditions. In addition, the exposed group had higher calcification rates. This indicated that *E. huxleyi* has the ability to adapt to elevated levels of CO_2 (Lohbeck et al., 2012), which also seems to be the case with *C. finmarchicus*. The study did not look at whether the response had a genetic component.

The study mentioned earlier done on *Lophelia pertusa* also showed that this coral was able to acclimate to an increased CO_2 concentration (Form and Riebesell, 2012). While a short-term exposure of only one week resulted in decreased calcification, a long-term exposure of 6 months revealed slightly increased calcification rates (Form and Riebesell, 2012). This study did not investigate whether there was a genetic component to the acclimation potential either.

However, a study investigating both the phenotypic plasticity and the genetic adaptation potential was recently done by Calosi et al. (2013). They conducted an *in situ* study on polychaetes and examined their ability to acclimate or genetically adapt to high CO_2 concentrations. The study revealed that both strategies may be important in making polychaete species able to cope with new CO_2 conditions. One of their test species, *P. dumerilii*, was able to genetically and physiologically adapt, while *A.*

mediterranea responded by phenotypic acclimation (Calosi et al., 2013). The study thus indicated that while an evolutionary response may be important for some species, other cope with increased CO_2 concentrations simply by the means of a plastic response.

A study done on purple sea urchins (*Strongylocentrotus purpuratus*) cultured under different CO_2 concentrations demonstrated that ocean acidification generated patterns of genome-wide selection (Pespeni et al., 2013). Larval development and morphology showed little response to the elevated CO_2 exposure, but there were found substantial allelic change, and especially in genes related to growth and biomineralization (Pespeni et al., 2013). The study therefore emphasizes the fact that even though there seems to be no effects of elevated CO_2 concentrations there might be substantial shifts in the underlying processes working to maintain the processes of the species, like e.g. development. Rapid evolution in changing environments is likely to depend to a higher degree on the existing genetic variation rather than new mutations (Pespeni et al., 2013). This makes it more likely that an evolutionary response will occur when a population is large and exists in a variable environment (Pespeni et al., 2013). The *C. finmarchicus* population in Trondheimsfjorden is large, but is not experiencing the same variation in pH as the purple sea urchin. Still, they will experience some fluctuations in pH e.g. during diapauses as they encounter different environments at different depths and might therefore contain the genetic variation necessary to cope with elevated CO_2 concentrations.

The findings in the present study indicating that selection of CO_2 tolerant genotypes may explain part of the acclimation potential of *C. finmarchicus* are consistent with the fact that species with short life spans and fast generation times generally are likely to have a greater capacity for evolutionary change (Gibson et al., 2011).

Studies done on the effects of ocean acidification on marine organisms have revealed diverse results, even within closely related species. This means that it is difficult to draw general conclusions on the response of a taxon based on studies of only one or a few of its species. However, *C. finmarchicus* does seem to be among the more robust species when it comes to the direct effects of elevated pCO_2.

4.5 Ecological implications of the results and future research

Since *C. finmarchicus* is a key stone species it is important to do thorough investigations of how it might be affected by future pCO_2 scenarios. The results of the present study indicate that *C. finmarchicus* may be resilient when it comes to a future pCO_2 scenario of approximately 2080 ppm, which is relevant for year 2300. This is consistent with other studies done on *C. finmarchicus,* and other related species, which also indicate that these copepods have the ability to cope with elevated CO_2 concentrations predicted for the near future (Pedersen et al., 2013, Kurihara and Ishimatsu, 2008). The results are good news also for other species since *C. finmarchicus* is a key species serving as an important food source for many marine organisms. The importance of the species to many commercial fish species makes the apparent tolerance towards CO_2 important in a socioeconomic aspect, as well as an ecological.

Caution should however be taken when making conclusions about the effects of ocean acidification on natural populations based on laboratory studies (Collins et al., 2014). It has to be kept in mind that laboratory experiments, even though useful, represent simplified models of natural systems as they in the present study e.g. remove the effects of predation and fluctuations in temperature, food availability and food type. The release of CO_2 from combustion of fossil fuels and altered land-use also has other implications on the environment since the increased atmospheric CO_2 concentrations e.g. lead to higher temperatures both on land and in the ocean. The combined effects of climate change stressors may affect *C. finmarchicus* differently than the stressor of increased CO_2 alone. Another example is that genetic constraints are less likely to inhibit evolution in the laboratory where only a few traits are under selection (Collins et al., 2014). The effects of elevated CO_2 concentrations could thus be masked in a laboratory environment, which could result in an underestimation of the consequences of a future pCO_2 scenario.

The fact that the changes in ocean acidity are happening at a fast rate is giving additional reason for concern about the ability of species to cope with the changing environment (Gibson et al., 2011). It is generally a lack of knowledge about whether adaptive genetic change can occur at the rate needed to avoid extinctions (Gibson et

al., 2011). In the present study the animals were exposed to an immediate increase in CO_2 concentration. The rate of change was thus much higher in the experiment than it will be in nature, and the fact that *C. finmarchicus* was able to adapt to the immediate change indicates that they may be able to deal with the rate of change projected to occur in nature.

In the present study it was revealed that *C. finmarchicus* seems to have the ability to genetically adapt, but further investigations should be done preferentially using protein-coding loci known to affect the ability of the copepod to cope with CO_2. However, this might be difficult to realize as it would require many generations. In addition to revealing whether directional selection has taken place, studies on protein-coding loci can give information on which enzymes and proteins that are being selected, and thereby give insight on which physiological mechanisms the animals use to cope with elevated CO_2 concentrations. To get a broader picture of the adaptive potential of *C. finmarchicus* to the projected environment conditions in year 2300, it would be interesting to investigate the combined effects of CO_2 and increased temperatures or other climate change related stressors. Even though it seems like *C. finmarchicus* is able to acclimate this might have costs that prevent the acclimation strategy from being sustainable in the long term. Therefore, the costs related to acclimation should also be further studied.

4.6 Evaluation of the methods

4.6.1 Acute toxicity test

The OECD guideline was used as guidance when setting up the LC50 test, but due to some complications some of the guidelines had to be adjusted. Due to a low number of stage CV individuals left in the tank at the start of the test on the F2 generation only five individuals were used in the LC50 test of the animals from tank D1 (F2-390 replicate 1), while seven individuals were used for the other populations. In addition, due to shortage of exposure bottles only three replicates were used as controls in the F2 generation, while six were used in the P generation. As fewer individuals were used in the LC50 test of F2-390 replicate 1 this test had a lower sample number, and could thus be somewhat less representative of the population than the other LC50 tests. The fact that few CV individuals were left in the tank at the time of sampling for the LC50 test may also have biased the result towards individuals with the slowest growth rate, the majority of the population had already reached the adult stage. Whether a slow growth rate might affect the LC50 value is uncertain. However, the LC50 values of F2-390 replicate 1 and 2 seemed to be similar for all monitoring points so the effect may be assumed to be negligible.

4.6.2 Microsatellite analyses

There were some issues with the microsatellite analysis that should be addressed. First of all the number of DNA samples from each treatment were low with N ranging from 16 to 45. As the actual population size in all treatments were considerably higher than this there will be an uncertainty related to whether the sampled individuals reflect the actual gene pool of the populations in the different treatments. The smaller the sample size is related to the population size, the higher the uncertainty. Therefore, this is most relevant for the parent generation, since a much lower percent of the actual Trondheimsfjorden population were samples compared to the percent sampled of the F2 generations. The low sample sizes probably explain why some of the loci deviated from Hardy-Weinberg equilibrium. Another issue that has to be kept in mind is that the number of replicates was very low. The control (390

ppm) treatment and the elevated CO_2 (2080 ppm) treatment had only two replicate tanks each. Even though it would have been desirable to have both a higher sample size and more replicates, the present study may still give valuable information regarding the ability of wild caught *C. finmarchicus* populations in Trondheimsfjorden to adapt to elevated CO_2 concentrations in the ocean, but the results have to be interpreted with care.

The microsatellite loci used in the present study are assumed to be selectively neutral and should thus not be related to the ability of the organism to cope with elevated CO_2 concentrations. A change in the genetic diversity at the loci does therefore not necessarily indicate that the organism has experience genetic adaptation towards CO_2. However, since the only difference between control and CO_2 treatment should be the CO_2 concentrations in the tanks it is reasonable to assume that a difference in genetic diversity between treatments is caused by adaptation towards elevated CO_2. The microsatellite analysis was therefore used as an indication of possible genetic adaptation, but cannot be used to say anything with certainty. A more confident result could probably be attained by using DNA loci known to affect the CO_2 tolerance of *C. finmarchicus* in some way. A critical assumption of the microsatellite analysis is thus that any difference in genetic diversity between treatments reflects genetic adaptation towards increased CO_2 concentrations. Still, useful information can be obtained as long as this underlying assumption is kept in mind.

4.6.2.1 Genotyping problems

In some cases, some of the microsatellite loci did not amplify. This could possibly be explained either by degraded DNA, null alleles or some issues with the GeneMapper program (some of the samples displayed an error message during genotyping). In cases were no loci amplified for an individual the cause was probably degraded DNA or GeneMapper issues, while cases where some loci amplified and others did not for the same individuals the reason is probably null alleles or some interaction between loci in the panels causing amplification problems. In the F2 generation there were many problems with genotyping using 12 loci divided into the two multiplex panels

that were used for the parent generation. There were occasional problems with amplification in the parent generation as well, but it was still possible to obtain good genotype profiles for most of the individuals. Several explanations of the issues arising in genotyping of the F2 generation are possible. One of them was that DNA concentrations either were too low or too high for the F2 samples, but this explanation was rejected as concentration readings using the Nanodrop showed that the DNA concentrations were comparable to that of the P generation. In addition a test where the amount of DNA used in each reaction was adjusted to a constant level did not improve the results of the genotyping. Another possibility is that the purity of the DNA samples was insufficient, which maybe could be explained by a high fat content of *C. finmarchicus*. Therefore, another DNA extraction method using phenol and chloroform was employed on some of the F2 individuals. However, using DNA extracted with this method did not improve the genotyping results. As none of the alterations to the method improved the results the six loci that most often caused problems were omitted and singleplex PCR followed by multiplexing the individual microsatellites prior to the fragment analysis were adopted with a successful outcome. The difficulties with obtaining good genotype profiles thus seem to be caused by issues with the panels, e.g. due to primer-primer interactions. However, why the panels were causing more problems in the F2 generation than for the parent generation are not clear. To get a better result from the multiplex PCR procedure more time should perhaps have been invested in optimizing the set up. In the present study equal amounts of all primers were used in the reaction mixture, but ideally those should have been adjusted according to their amplification strength (Henegariu et al., 1997). Four different PCR profiles were tested regarding to the number of cycles, but it would have been even better to also test a different set of annealing and extension temperatures as well (Henegariu et al., 1997).

5 CONCLUSIONS

- Hypothesis 1: *Calanus finmarchicus* populations are able to acclimate to elevated levels of CO_2 relevant for year 2300.

The results of the acute toxicity test indicate that the *C. finmarchicus* population in Trondheimsfjorden has the potential to acclimate to elevated concentrations of CO_2, as individuals exposed to a CO_2 concentration of 2300 ppm in the course of two generations have a significantly higher tolerance towards elevated CO_2 levels than individuals exposed to ambient concentrations. Hypothesis 1 cannot be rejected.

- Hypothesis 2: Selection of CO_2 tolerant genotypes contributes to the acclimation response of CO_2 exposed *C. finmarchicus* populations.

The tendency of lower genetic diversity in the treatments exposed to elevated pCO_2 indicates that selection of CO_2 tolerant genotypes may explain part of the observed plasticity, but this should be further studied. Hypothesis 2 cannot be rejected.

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APPENDIX A – Acute toxicity data

Table A.1 Recorded mortalities in the exposure bottles for the P generation. Each bottle had seven individuals.

Time	Control (390 ppm)						2.25 %			2.89 %			3.7 %			4.75 %			6.08 %			7.8 %			10 %		
(h)	1	2	3	4	5	6	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
6	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	2	1	1	2	2	1	6	6	7
12	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0	1	1	1	2	1	1	2	3	2	7	6	7
24	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0	1	1	1	3	2	2	3	3	4	7	7	7
48	0	1	0	0	0	0	0	1	1	1	2	0	0	2	0	1	2	2	3	3	3	5	5	7	7	7	7
72	1	1	0	0	0	0	0	1	1	1	2	0	2	3	1	4	4	3	4	5	5	7	7	7	7	7	7
96	2	1	0	0	0	0	0	1	1	3	3	1	3	4	3	5	7	6	7	7	7	7	7	7	7	7	7

Table A.2 Recorded mortalities in the exposure bottles for F2-390 replicate 1 (Tank D1). Each bottle had five individuals, except 2.25 % replicate 3 and 4.75 % replicate 1 which each had six individuals.

Time (h)	Control (390 ppm)			2.25 %			2.89 %			3.7 %			4.75 %			6.08 %			7.8 %			10 %		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
6	0	1	0	0	1	0	0	1	0	0	0	0	2	0	1	2	2	1	3	3	3	5	5	5
12	0	1	0	0	1	1	0	2	0	0	0	3	3	2	3	4	3	4	5	4	5	5	5	5
24	0	1	0	0	2	1	1	2	0	1	0	4	6	4	5	5	5	5	5	5	5	5	5	5
48	0	1	0	0	2	1	2	2	2	4	3	5	6	5	5	5	5	5	5	5	5	5	5	5
72	0	1	0	1	3	2	4	3	4	5	5	5	6	5	5	5	5	5	5	5	5	5	5	5
96	0	1	0	2	3	3	5	5	5	5	5	5	6	5	5	5	5	5	5	5	5	5	5	5

Table A.3 Recorded mortalities in the LC50 test exposure bottles for F2-390 replicate 2 (Tank D2). Each bottle had seven individuals, except 6.08 % replicate 1 which had eight individuals.

Time (h)	Control (390 ppm)			2.25 %			2.89 %			3.7 %			4.75 %			6.08 %			7.8 %			10 %		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
6	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	4	3	2	4	7	4	7	7	7
12	0	0	0	0	0	0	0	0	0	0	1	0	2	3	3	6	6	6	6	7	7	7	7	7
24	0	1	0	0	0	0	0	0	0	2	1	0	3	4	5	7	7	7	7	7	7	7	7	7
48	0	1	0	0	1	1	1	3	3	6	6	5	7	7	7	8	7	7	7	7	7	7	7	7
72	0	1	0	3	1	2	5	7	5	7	7	7	7	7	7	8	7	7	7	7	7	7	7	7
96	0	1	0	4	4	5	7	7	6	7	7	7	7	7	7	8	7	7	7	7	7	7	7	7

Table A.4 Recorded mortalities in LC50 exposure bottles for F2-2080 replicate 1 (Tank D3). All bottles had seven individuals.

Time (h)	Control (390 ppm)			2.25 %			2.89 %			3.7 %			4.75 %			6.08 %			7.8 %			10 %		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	3	5	4
12	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	4	2	3	3	3	7	6	6
24	0	0	0	0	0	0	1	0	0	0	1	0	0	2	0	3	7	3	6	5	6	7	7	7
48	0	0	0	0	0	0	1	0	0	3	2	0	4	7	4	7	7	7	7	7	7	7	7	7
72	0	0	0	0	1	2	3	1	2	4	4	3	7	7	7	7	7	7	7	7	7	7	7	7
96	0	0	0	2	2	3	5	4	4	6	6	4	7	7	7	7	7	7	7	7	7	7	7	7

Table A.5 Recorded mortalities in LC50 exposure bottles for F2-2080 replicate 2 (Tank D4). All bottles had seven individuals.

Time (h)	Control (390 ppm)			2.25 %			2.89 %			3.7 %			4.75 %			6.08 %			7.8 %			10 %		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	3	4	3
12	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	1	2	3	1	2	3	5	6	6
24	0	0	0	1	0	0	0	0	0	0	0	1	3	3	3	4	7	7	6	7	7	7	7	7
48	0	0	0	1	0	1	1	0	1	2	2	3	6	7	7	7	7	7	7	7	7	7	7	7
72	0	0	0	1	1	1	7	1	3	4	5	5	7	7	7	7	7	7	7	7	7	7	7	7
96	0	0	0	2	1	2	7	3	3	6	7	6	7	7	7	7	7	7	7	7	7	7	7	7

Table A.6 The percentage mortality in the controls for each treatment at all monitoring points.

	Mortality in controls (%)		
Time (h)	P	F2-390	F2-2080
6	2.38	2.78	0
12	2.38	2.78	0
24	2.38	5.56	0
48	2.38	5.56	0
72	4.76	5.56	0
96	7.14	5.56	0

Table A.7 Average oxygen content in exposure bottles in the P generation.

Treatment	0.00039	2.25	2.89	3.7	4.75	6.08	7.8	10
O_2-content (%)	92.55	89.13	92.03	93.83	93.6	93.93	94.47	88.9

The output from the calculations of LC50 values and corresponding 95 % confidence intervals in GraphPad for all treatments at all monitoring times are shown in Table A.1-A.5. The EC50 (effective concentration-50) is the LC50 value. Standard error (SE), 95 % confidence intervals and HillSlope are also among the stated values in the output.

Table A.8 The output from the calculations of the LC50 values and the 95 % confidence intervals of the parent generation at all monitoring points.

	Nonlin fit	A	B	C	D	E	F
		6 timer	12 timer	24 timer	48 timer	72 timer	96 timer
		Y	Y	Y	Y	Y	Y
3	Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
4	Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
5	LogEC50	0.9338	0.9210	0.8901	0.8350	0.7403	0.6354
6	HillSlope	9.378	8.864	7.384	6.146	4.921	5.292
7	EC50	8.587	8.337	7.765	6.839	5.499	4.320
8	Std. Error						
9	LogEC50	0.009387	0.01207	0.01001	0.01616	0.01592	0.01320
10	HillSlope	1.350	1.845	1.454	1.467	0.8055	0.7923
11	95% Confidence Intervals						
12	LogEC50	0.9142 to 0.9535	0.8957 to 0.9463	0.8692 to 0.9111	0.8012 to 0.8688	0.7069 to 0.7736	0.6078 to 0.6631
13	HillSlope	6.551 to 12.20	5.002 to 12.73	4.341 to 10.43	3.075 to 9.217	3.235 to 6.607	3.634 to 6.950
14	EC50	8.207 to 8.984	7.865 to 8.836	7.399 to 8.148	6.327 to 7.393	5.093 to 5.937	4.053 to 4.603
15	Goodness of Fit						
16	Degrees of Freedom	19	19	19	19	19	19
17	R square	0.9303	0.8809	0.9105	0.8272	0.8920	0.9309
18	Absolute Sum of Squares	1298	2315	1982	4486	2914	1829
19	Sy.x	8.265	11.04	10.21	15.37	12.38	9.812
20	Constraints						
21	Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
22	Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
23	Number of points						
24	Analyzed	21	21	21	21	21	21

Table A.9 The output from the calculations of the LC50 values and the 95 % confidence intervals of the F2-390 1 treatment at all monitoring points.

Nonlin fit	A	B	C	D	E	F
	6 timer	12 timer	24 timer	48 timer	72 timer	96 timer
	Y	Y	Y	Y	Y	Y
Best-fit values						
Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
LogEC50	0.9370	0.8139	0.7444	0.6536	0.5731	0.5443
HillSlope	7.725	5.615	11.41	7.453	7.931	16.08
EC50	8.649	6.515	5.551	4.504	3.742	3.502
Std. Error						
LogEC50	0.009803	0.01853	0.01504	0.009944	0.008469	0.002668
HillSlope	1.596	1.192	4.395	1.149	1.281	3.134
95% Confidence Intervals						
LogEC50	0.9165 to 0.9575	0.7751 to 0.8527	0.7129 to 0.7758	0.6328 to 0.6744	0.5554 to 0.5908	0.5387 to 0.5499
HillSlope	4.384 to 11.07	3.120 to 8.109	2.209 to 20.60	5.048 to 9.858	5.250 to 10.61	9.523 to 22.64
EC50	8.250 to 9.068	5.959 to 7.124	5.163 to 5.968	4.293 to 4.725	3.592 to 3.898	3.457 to 3.547
Goodness of Fit						
Degrees of Freedom	19	19	19	19	19	19
R square	0.9140	0.8338	0.7898	0.9201	0.8940	0.9500
Absolute Sum of Squares	2161	4973	7119	1744	1143	293.6
Sy.x	10.67	16.18	19.36	9.581	7.757	3.931
Constraints						
Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
Number of points						
Analyzed	21	21	21	21	21	21

Table A.10 The output from the calculations of the LC50 values and the 95 % confidence intervals of treatment F2-390 2 at all monitoring points.

Nonlin fit	A	B	C	D	E	F
	6 timer	12 timer	24 timer	48 timer	72 timer	96 timer
	Y	Y	Y	Y	Y	Y
Best-fit values						
Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
LogEC50	0.9202	0.8346	0.7963	0.6635	0.5724	0.5120
HillSlope	11.79	9.677	11.29	9.660	10.31	10.13
EC50	8.321	6.833	6.256	4.608	3.736	3.251
Std. Error						
LogEC50	0.007283	0.005986	0.006076	0.006284	0.006887	0.006604
HillSlope	3.771	0.9784	1.746	1.161	1.401	1.978
95% Confidence Intervals						
LogEC50	0.9049 to 0.9354	0.8221 to 0.8471	0.7835 to 0.8090	0.6503 to 0.6766	0.5579 to 0.5868	0.4981 to 0.5258
HillSlope	3.902 to 19.69	7.629 to 11.73	7.634 to 14.94	7.230 to 12.09	7.380 to 13.24	5.985 to 14.27
EC50	8.034 to 8.618	6.638 to 7.033	6.075 to 6.441	4.470 to 4.749	3.614 to 3.862	3.149 to 3.356
Goodness of Fit						
Degrees of Freedom	19	19	19	19	19	19
R square	0.9232	0.9793	0.9732	0.9671	0.9291	0.9170
Absolute Sum of Squares	2330	794.9	1102	872.2	980.6	325.1
Sy.x	11.08	6.468	7.615	6.775	7.184	4.136
Constraints						
Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
Number of points						
Analyzed	21	21	21	21	21	21

Table A.11 The output from the calculations of the LC50 values and the 95 % confidence intervals of treatment F2-2080 1 at all monitoring points.

Nonlin fit	A	B	C	D	E	F
	6 timer	12 timer	24 timer	48 timer	72 timer	96 timer
	Y	Y	Y	Y	Y	Y
Best-fit values						
Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
LogEC50	1.041	0.9451	0.8781	0.7654	0.6886	0.5906
HillSlope	8.509	8.835	12.06	11.44	7.360	5.280
EC50	10.98	8.813	7.553	5.827	4.882	3.896
Std. Error						
LogEC50	0.007852	0.01020	0.009896	0.009111	0.01070	0.01063
HillSlope	1.326	1.867	3.000	2.342	1.170	0.6836
95% Confidence Intervals						
LogEC50	1.024 to 1.057	0.9238 to 0.9665	0.8574 to 0.8988	0.7463 to 0.7845	0.6662 to 0.7110	0.5684 to 0.6129
HillSlope	5.735 to 11.28	4.927 to 12.74	5.783 to 18.34	6.535 to 16.34	4.911 to 9.808	3.849 to 6.711
EC50	10.58 to 11.41	8.391 to 9.257	7.201 to 7.921	5.576 to 6.088	4.637 to 5.140	3.702 to 4.101
Goodness of Fit						
Degrees of Freedom	19	19	19	19	19	19
R square	0.8951	0.9047	0.9104	0.9395	0.9250	0.9137
Absolute Sum of Squares	947.7	2152	3163	2470	2034	1133
Sy.x	7.063	10.64	12.90	11.40	10.35	7.720
Constraints						
Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
Number of points						
Analyzed	21	21	21	21	21	21

Table A.12 The output from the calculations of the LC50 values and the 95 % confidence intervals of treatment F2-2080 replicate 2 at all monitoring points.

Nonlin fit	A	B	C	D	E	F
	6 timer	12 timer	24 timer	48 timer	72 timer	96 timer
	Y	Y	Y	Y	Y	Y
Best-fit values						
Bottom	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0	= 100.0
LogEC50	1.094	1.005	0.8475	0.7692	0.6695	0.6307
HillSlope	7.637	5.684	10.53	18.93	6.496	8.234
EC50	12.42	10.11	7.039	5.877	4.672	4.273
Std. Error						
LogEC50	0.007837	0.01577	0.008750	0.004978	0.01732	0.01087
HillSlope	1.123	1.164	1.852	4.311	1.541	1.599
95% Confidence Intervals						
LogEC50	1.078 to 1.111	0.9719 to 1.038	0.8292 to 0.8658	0.7587 to 0.7796	0.6332 to 0.7057	0.6080 to 0.6535
HillSlope	5.286 to 9.987	3.247 to 8.121	6.652 to 14.40	9.906 to 27.95	3.271 to 9.721	4.888 to 11.58
EC50	11.96 to 12.90	9.374 to 10.91	6.748 to 7.342	5.738 to 6.020	4.298 to 5.079	4.055 to 4.503
Goodness of Fit						
Degrees of Freedom	19	19	19	19	19	19
R square	0.8950	0.8209	0.9510	0.9679	0.8153	0.8765
Absolute Sum of Squares	614.8	3031	1907	1147	4472	2210
Sy.x	5.688	12.63	10.02	7.771	15.34	10.79
Constraints						
Bottom	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0	Bottom = 0.0
Top	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0	Top = 100.0
Number of points						
Analyzed	21	21	21	21	21	21

APPENDIX B – Microsatellite analysis

Table B.1 Number of successfully scored genotypes, N_T, observed heterozygosity, H_{obs}, expected heterozygosity, H_{exp}, Hardy-Weinberg significance, null alleles, allelic richness and private allelic richness, A_R.

Treatment	Locus	N_T	Hobs	Hexp	HW	Null alleles	Allelic richness	Private A_R
P	FG811	42	0.762	0.682	NS	-0.0746	3.1340	1.0026
	FK682	43	0.279	0.423	NS	+0.1914	2.3506	0.66300
	EL609	45	0.622	0.789	NS	+0.1109	3.7271	0.53040
	EL359	43	0.953	0.782	NS	-0.1111	3.6628	2.19300
	EL519	43	1.000	0.658	***(p =3.5x10^{-6})	-0.2341	2.8831	0.60440
	EH474	45	0.489	0.568	NS	+0.0620	2.6132	0.19920
∑		46		0.6501			3.06	0.87
F2-390 1	FG811	17	0.353	0.672	NS	+0.3253	2.9477	1.37610
	FK682	18	0.389	0.817	NS	+0.3409	3.9298	1.24970
	EL609	16	0.500	0.653	*(p=0.0063)	+0.1755	3.1257	0.42900
	EL359	18	0.333	0.508	NS	+0.1940	1.9760	0.00000
	EL519	16	0.389	0.567	NS	+0.1899	2.7447	1.10630
	EH474	24	0.438	0.738	NS	+0.2473	3.2598	0.28430
∑		24		0.6592			3.00	0.74
F2-390 2	FG811	21	0.571	0.470	NS	-0.1169	2.0517	0.18170
	FK682	23	0.391	0.604	NS	+0.1891	2.8106	0.64430
	EL609	23	0.478	0.633	NS	+0.1597	2.9232	0.25890
	EL359	24	0.375	0.566	NS	+0.1855	2.3012	0.00930
	EL519	24	0.458	0.397	NS	-0.1164	2.2011	0.50260
	EH474	24	0.458	0.714	NS	+0.2042	3.2303	0.18060
∑		24		0.5641			2.59	0.30
F2-2080 1	FG811	16	0.125	0.748	*(p=0.0063)	+0.7042	3.4795	0.81340
	FK682	19	0.789	0.590	NS	-0.2056	2.7483	0.77900
	EL609	19	0.211	0.240	ND	-0.1227	1.6707	0.03230
	EL359	19	0.632	0.512	NS	-0.1176	1.9792	0.00000
	EL519	19	0.263	0.330	ND	+0.0804	1.9820	0.32530
	EH474	19	0.474	0.475	NS	+0.0164	2.3994	0.11700
∑		24		0.4827			2.38	0.34
F2-2080 2	FG811	22	0.591	0.587	NS	-0.0325	2.6352	1.27250

	FK682	24	0.708	0.573	NS	-0.1770	2.9082	0.81840
	EL609	24	0.625	0.605	NS	-0.0112	2.9039	0.31910
	EL359	24	0.417	0.550	NS	+0.1332	2.2070	0.00570
	EL519	24	0.292	0.267	ND	-0.0712	1.7865	0.14690
	EH474	24	0.542	0.578	NS	+0.0324	2.7752	0.22730
∑				0.5266			2.54	0.47
Culture	FG811	19	0.000	0.000	ND	ND	1.0000	0.00000
	FK682	19	0.105	0.102	ND	-0.017	1.2945	0.00000
	EL609	19	0.316	0.289	ND	-0.778	1.8644	0.03600
	EL359	19	0.737	0.613	NS	-0.1147	2.5402	0.35890
	EL519	19	0.158	0.149	ND	-0.0331	1.4120	0.12350
	EH474	19	0.105	0.104	ND	-0.0175	1.3158	0.14860
∑		23		0.2096			1.57	0.11

Table B.2 Mean expected heterozygosity, allelic richness and private allelic richness across all loci in each treatment along with the standard error of the means.

Treatment	Hexp	Allelic richness	Private allelic richness
P	0.6501±0.057	3.06±0.227	0.87±0.286
F2-390 1	0.6592±0.046	3.00±0.262	0.74±0.235
F2-390 2	0.5641±0.047	2.59±0.191	0.30±0.096
F2-2080 1	0.4827±0.074	2.38±0.269	0.34±0.150
F2-2080 2	0.5266±0.052	2.54±0.184	0.47±0.197
Culture	0.2096±0.089	1.57±0.230	0.11±0.056