Impact of temperature, food availability and life-history stages on the eDNA emission from *Pacifastacus leniusculus* and its obligate parasite *Aphanomyces astaci*

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Masteroppgave i økologi og evolusjon
ved Institutt for Biovitenskap

UNIVERSITETET I OSLO

2017

2017

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Abstract

Environmental DNA (eDNA) monitoring of common, invasive, and endangered species as well as pathogenic microorganisms is an increasing trend in biodiversity surveys, conservation work and surveillance programs. In Europe, *Pacifastacus leniusculus* (signal crayfish) is an alien North-American freshwater crayfish that carry and transmit the parasitic oomycete *Aphanomyces astaci*, which is causing crayfish plague and mass mortalities in indigenous *Astacus astacus* (noble crayfish) populations. In this master thesis, the relationship between *P. leniusculus* individual density and emission of *P. leniusculus* and *A. astaci* eDNA has been investigated including the potential effect of factors such as temperature, food availability and important life history stages as molting and death. Over the past years, several studies have proven that eDNA successfully can detect crayfish species and *Aphanomyces astaci* in freshwater. However, the use of eDNA to quantify crayfish species such as *P. leniusculus* needs to be developed further. In this thesis, aquarium experiments with *P. leniusculus* and *A. astaci*, filtration methods, DNA extraction and qPCR has been conducted. It was not found any significant relationship between *P. leniusculus* density and *P. leniusculus* and *A. astaci* eDNA concentration. For *P. leniusculus* it was observed a strong trend of increase in eDNA concentration when the temperature was 10 °C compared to 20 °C. For *A. astaci*, it was found a dramatic decrease in eDNA concentration at 20 °C. The presence of food lead to a significant decrease in *P. leniusculus* eDNA concentration, but does not affect *A. astaci* eDNA concentration. The effect of moulting and death was not tested statistically, but it was observed a strong increase in eDNA concentrations of both target organisms during moulting, especially in aquaria where the *P. leniusculus* exoskeleton remained present. Death apparently did not affect the abundance of *A. astaci* eDNA, while for *P. leniusculus* eDNA a marked increase was observed for the low-density experimental tank. For this reason, it is assumed that death is a factor that contributes to increase in *P. leniusculus* eDNA abundance. For the high-density tank, rapid fouling of the water prevented reliable results. It was also found by calculating a *P. leniusculus/A. astaci* eDNA ratio, that *P. leniusculus* eDNA nearly always was present in higher concentrations than *A. astaci* eDNA. This means that *P. leniusculus* emits much more of its own DNA, than from its parasite, *A. astaci.* The exception was during molting, when higher numbers of *A. astaci* eDNA than *P. leniusculus* eDNA was detected.

In conclusion, it was shown that estimating *P. leniusculus* numbers or population density from eDNA concentrations can be challenging, as no correlation between *P. leniusculus* eDNA emission and density/number of individuals was found. In addition, it was found that certain factors can affect the eDNA emission from *P. leniusculus* and *A. astaci*, which makes estimating abundance a demanding task. However, the results suggest that if the goal is detection, it is favorable target *P. leniusculus* eDNA above *A. astaci* eDNA in all seasons except moulting season. Moulting season is time of the year when detection of *A. astaci* is easiest, unless the water temperature is exceptionally high which would suppress sporulation and make detection of *A. astaci* difficult. It is important to note that *A. astaci* still is present in the carrier *P. leniusculus*, and it does not mean that they are eradicated from the area. eDNA from *A. astaci* was shown to be less affected by the presence of food/presence of microbiological activity, and could be favorable to detect when there is a lot of turbidity in the water. However, more research is needed on the intricate relationships between eDNA emission/concentration, life-history stages and environmental factors.

Sammendrag

Miljø-DNA (eDNA) overvåking av vanlige, invaderende og truede arter, samt patogene mikroorganismer er en økende trend i biodiversitetsundersøkelser, bevaringsarbeid og overvåkingsprogrammer. I Europa er *Pacifastacus leniusculus* (signalkreps) en invaderende nord-amerikansk ferskvannskreps som bærer og overfører den parasittiske oomyceten (eggsporesopp) *Aphanomyces astaci*, som forårsaker krepspest og massedød hos stedegne *Astacus astacus* (edelkreps) populasjoner. I denne masteroppgaven er forholdet mellom *P. leniusculus* tetthet og utslipp av *P. leniusculus* og *A. astaci* eDNA blitt undersøkt, inkludert den potensielle effekten av faktorer som temperatur, mattilgjengelighet og viktige livshistorie-stadier som skallskifte og død. I de siste årene har flere studier vist at eDNA vellykket kan oppdage kreps og *Aphanomyces astaci* i ferskvann. Imidlertid må bruk av eDNA for å kvantifisere krepsarter som *P. leniusculus* utvikles videre. I denne oppgaven har akvarieforsøk med *P. leniusculus* og *A. astaci*, filtreringsmetoder, DNA-ekstraksjon og qPCR blitt utført. Det ble ikke funnet noen signifikant sammenheng mellom *P. leniusculus* tetthet og *P. leniusculus* og *A. astaci* eDNA konsentrasjon. For *P. leniusculus* ble det observert en sterk trend at til at eDNA-konsentrasjon var høyere når temperaturen var 10 °C

sammenlignet med 20 °C. For *A. astaci* ble det funnet en dramatisk reduksjon i eDNA-konsentrasjon ved 20 °C. Tilstedeværelsen av mat førte til en signifikant reduksjon i *P. leniusculus* eDNA-konsentrasjon, men påvirket ikke *A. astaci* eDNA-konsentrasjon. Effekten av skallskifte og død ble ikke testet statistisk, men det ble observert en sterk økning i eDNA konsentrasjoner av begge målorganismer under skallskifte, spesielt i akvarier der *P. leniusculus* skallet var tilstede sammen med krepsen etter skallskifte. Død påvirket tilsynelatende ikke tilstedeværelsen av *A. astaci* eDNA, mens for *P. leniusculus* eDNA ble det observert en markert økning i tankene med lavest tetthet. Av denne grunn antas det at død er en faktor som bidrar til økning i *P. leniusculus* eDNA konsentrasjon. I tanken med høy tetthet av døde individer, ble det observert en høy grad av forurensning som forhindret pålitelige resultater. Ved en beregning av *P. leniusculus / A. astaci* eDNA ratio, ble det funnet at *P. leniusculus* eDNA nesten alltid var tilstede i høyere konsentrasjoner enn *A. astaci* eDNA. Dette betyr at *P. leniusculus* slipper ut mye mer av sitt eget DNA enn fra parasitten *A. astaci*. Unntaket var under skallskifte, da høyere antall *A. astaci* eDNA enn *P. leniusculus* eDNA ble påvist.

Det ble konkludert med at estimering av *P. leniusculus* antall eller populasjonstetthet fra eDNA-konsentrasjoner kan være utfordrende, da det ikke ble funnet noen korrelasjon mellom *P. leniusculus* eDNA-utslipp og tetthet/antall individer. I tillegg ble det funnet at flere faktorer kan påvirke eDNA-utslipp fra *P. leniusculus* og *A. astaci*, noe som gjør estimering av antall til en krevende oppgave. Resultatene tyder imidlertid på at hvis målet er påvisning, er det gunstig å undersøke *P. leniusculus* eDNA fremfor *A. astaci* eDNA i alle årstider unntatt rundt skallskifte. Tiden på året der skallskiftet skjer, er da påvisning av *A. astaci* er enklest, med mindre vanntemperaturen er unormalt høy, noe som ville undertrykke *A. astaci* sporuleringen og gjøre påvisning av *A. astaci* vanskelig. Det er viktig å merke seg at *A. astaci* fortsatt er tilstede i bæreren *P. leniusculus*, og det betyr ikke at de er utryddet fra området. eDNA fra *A. astaci* viste seg å være mindre påvirket av mikrobiologisk aktivitet, og det kan derfor være gunstig å gjennomføre målinger av *A. astaci* eDNA fremfor *P. leniusculus* eDNA i perioder med mye turbiditet i vannet. Det er imidlertid behov for mer forskning på de intrikate forholdene mellom eDNA-utslipp/konsentrasjon, livshistorie-stadier og miljøfaktorer.

Forord:

Takk til min hovedveileder Trude, for at jeg fikk tilliten til å skrive denne oppgaven og for all hjelp, kunnskapsdeling og støtte underveis. Takk til min veileder David, som har hjulpet meg med alle de store og små spørsmålene jeg har hatt. Takk til min veileder Tom, som har bidratt med uvurderlig hjelp til R og statistikk.

Takk til Johannes som har hjulpet meg med alt fra å håndtere aggressive kreps til å fikse vannlekkasjer og sette opp eksperimenter. Takk til Øystein Toverud ved Utmarksavdelingen Oslo og Akershus for hjelp til å gjennomføre krepsefisket i Øymarksjøen og Rødenessjøen. Takk til Elin Rolén som har gitt meg super hjelp på laben med blant annet isolering av DNA fra krepsevev. Og takk til alle andre hyggelige mennesker som har hjulpet meg underveis.

Og ikke minst, tusen takk til min kjære Øivind, som har støttet meg hele veien (nå er jeg ferdig!)

Charlotte Laurendz, oktober 2017

Abbreviations and glossary:

Experimental tanks – aquaria used in the experiments.

Population tank – communal housing tank.

Bp – base pair.

Carrier – an individual who harbours a disease-causing microorganism without ill-effects and who can transmit the microorganism to others. *

CPUE – catch per unit effort.

CTAB – cetyl trimethylammonium bromide (used in CTAB DNA extraction).

Cyst – protective coat surrounding resting cells, an encysted oomycete zoospore. *

eDNA – environmental DNA (DNA isolated from an environmental sample such as a water sample).

Host - an organism whose body provides nourishment and shelter for a parasite. *

Indigenous – describing a species that occurs naturally in a certain area, as distinct from one introduced by humans. *

Invasive species – a species that are non-native/alien to an ecosystem and whose presence causes or is likely to cause harm.

ITS – internal transcribed spacer (of the nuclear ribosomal DNA).

LOD – limit of detection.

LOQ – limit of quantification.

MGB – minor groove binder.

MQ (MilliQ) – ultrapure water.

Non-indigenous – non-native to an area. Species that have moved or been moved from their original ecosystem to a new ecosystem.

PCR – polymerase chain reaction. A technique used to replicate a fragment of DNA to produce many copies of a particular DNA sequence. *

qPCR – quantitative real-time PCR. Used for quantitative estimation of DNA amounts in a sample.

Parasite – organism which lives on or in another organism and draw nutrients on the expense of the host.

Pathogen – any disease-causing microorganism. *

Prevalence – the proportion of a population found to have a condition – e.g. being infected with a parasite

Primer - a short single-stranded DNA molecule that provides a starting point for DNA synthesis

Probe – a labelled oligonucleotide designed to identify complementary or homologues molecules to which it base-pairs.

Spore – a dormant reproductive cell formed by certain organisms. It is thick-walled and highly resistant to survive under unfavorable conditions so that when conditions revert to being suitable it gives rise to a new individual.

Virulence – 1. The relative ability of a microorganism to cause disease, degree of pathogenicity. 2. The capability of a microorganism to cause disease.

Vector – an agent that transmits the causative agent or disease-causing organism from the reservoir to the host.

Zoospore - a spore that possesses one or more flagella and is therefore motile. Released from a sporangium. *

*Oxford DICTIONARY OF Biology. 2008. Sixth edition.

Innhold

1 Introduction 1

1.1 Alien invasive species 1

1.2 Freshwater crayfish 1

1.3 Alien threats to European freshwater crayfish 2

1.4 *Aphanomyces astaci* – the crayfish plague pathogen 3

1.5 Introduction and spread of Aphanomyces astaci and its hosts in Europe 4

1.6 Crayfish plague outbreaks and signal crayfish introductions in Norway 6

1.7 Environmental DNA monitoring 8

1.8 Hypothesis and research questions 10

2 Materials and methods 11

2.1 *Pacifastacus leniusculus* origin and husbandry 11

2.1.1 *Pacifastacus leniusculus* origin 11

2.1.2 Husbandry and marking 12

2.2 Experimental designs and implementation 14

2.2.1 Experiment 1: Temperature, density, and food availability 14

2.2.2 Experiment 2: Moulting 16

2.2.3 Experiment 3: Death 16

2.2.4 Excluded experiment: Reproduction: 17

2.3 Sample preparation: 17

2.3.1 Water filtration 17

2.3.2 Tissue sampling and preparation 18

2.3.3 Research hygiene and biosecurity measures 18

2.4 Ethics statement - Animal welfare 19

2.5 Molecular analyses 19

2.5.1 DNA extraction from filter samples 19

2.5.2 DNA extraction from tissue 20

2.5.3 Quantitative real-time PCR 21

2.6 Statistical analyses. 24

2.7 Collaboration and shared work 25

3 Results 25

3.1 Overall result summary 25

3.2 ***Pacifastacus leniusculus*** **eDNA abundance** 27
3.2.1 **Effects of temperature, density, and food availability** 28
3.2.2 **Effect of moulting on *P. leniusculus* eDNA abundance** 29
3.2.3 **Effect of death on *P. leniusculus* eDNA abundance** 30
3.3 ***Aphanomyces astaci*** **eDNA abundance** 32
3.3.1 **Effects of temperature, food availability and temperature** 32
3.3.2 **Effect of moulting on *A. astaci* eDNA abundance** 34
3.3.3 **Effect of death on *A. astaci* eDNA abundance** 35
3.3.4 **eDNA ratios of *P. leniusculus* and *A. astaci*** 37
3.4 ***Pacifastacus leniusculus*** **measures and** ***Aphanomyces astaci*** **infection level** 37
3.5 **Summary of qPCR results from control samples** 38
4 **Discussion** 40
5 **Conclusion** 52
Referances 54
6 **Supplementary** 61

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1 Introduction

1.1 Alien invasive species

The invasion of alien species is a major threat to biodiversity worldwide (Clavero and Garcia-Berthou, 2005; Rahel and Olden, 2008). Alien species are organisms that are non-native to an ecosystem (COP6, 2002). Species adapt to their environment over a long evolutionary time. However, trade and travel facilitates introduction of alien species at rates that are much higher than their natural dispersal rate (Ricciardi, 2007). These rates do not allow for adaptation, neither for the alien species nor the native species. Invasive alien species may outperform native species, either through predation, superior fitness traits, or by carrying harmful pathogens, and thus have negative impacts on native species populations (Lowe et al., 2004). Historically, alien species have in several occasions carried pathogens that have locally eradicated native species populations or even lead to global extinction (Bakke and Harris, 1998; et al., 2004). In addition to biodiversity loss, extinction of a species can affect ecosystems negatively, and change food web structure (Zavaleta et al., 2001). In Norway, the invasion of alien species is increasing rapidly (Hendrichsen, 2014).

1.2 Freshwater crayfish

Freshwater crayfish are a part of the order Decapoda, which lies under the extensive phylum Arthropoda. Freshwater crayfish can belong to either superfamily Astacoidea or Parastacoidea, in which the latter comprises crayfish species on the southern hemisphere. Freshwater crayfish are keystone species in freshwater ecosystems; they transport energy from both decaying and living plant and animal material to higher trophic levels by functioning as food for birds, fish, and mammals. They also have other important ecological

roles as biodiversity indicators, water quality indicators and as ecological-engineers trough burrowing activity (Reynolds et al., 2013). The North-American freshwater signal crayfish (*Pacifastacus leniusculus,* Dana, 1852) and the European freshwater noble crayfish (*Astacus* astacus L. 1758) both belong to the Astacoidea superfamily (Crandall and De Grave, 2017).

1.3 Alien threats to European freshwater crayfish

Pacifastacus leniusculus carries one of the most serious threats to the native freshwater *Astacus* (Unestam and Weiss, 1970). *Pacifastacus leniusculus* is a chronic carrier and transmitter of the crayfish plague pathogen (*Aphanomyces astaci*, Schikora 1906), an oomycete (water mold) that is lethal to European crayfish including *A. astacus* (Holdich et al., 2009; Söderhäll and Cerenius, 1999). Crayfish species in North America, such as *P. leniusculus*, have undergone co-evolution with *A. astaci*, which act as a specialized and relatively harmless parasite on these species. Because of this, North American crayfish have evolved defense mechanisms against *A. astaci*, and this balanced host-parasite relationship is not present in European crayfish species (with exceptions discussed below). Crayfish plague infection has therefore caused mass mortalities and eradication of *A. astacus* populations all over Europe (Holdich et al., 2009), and the species has been and is still declining largely because of crayfish plague all over its natural range (Edsman et al., 2010). *Pacifastacus leniusculus* show higher reproductions rates, higher consumption rates, and has a stronger impact on submerged plants and on bottom conditions than *A. astacus* (Nyström and Strand, 1996). They are also more carnivorous and aggressive than *A. astacus* and their presence may lead to changes in the food web and negative effect on other macroinvertebrates (Moorhouse et al., 2014; Twardochleb et al., 2013). Further, *P. leniusculus* is also an active burrower and may have negative structural impacts on river banks (Guan, 1994).

Figure 1. *Pacifastacus leniusculus* (signal crayfish), left, and *Astacus astacus* (noble crayfish), right. Photo by David A. Strand.

1.4 *Aphanomyces astaci* – the crayfish plague pathogen

The crayfish plague pathogen *Aphanomyces astaci* is a sporulating, parasitic oomycete that infects crayfish tissue (Söderhäll and Cerenius, 1999; Unestam and Weiss, 1970). It reproduces asexually trough formation of mobile zoospores (fig. 2). The zoospores settle down on the host cuticle, where the zoospore encysts. After encystment, a germ tube penetrates the cuticle and hyphae branches throughout it. Depending on the host immune response, the infection is either encapsulated, or the hyphae continues to branch. In the latter case, the hyphae continue to grow through the host tissue and organs, eventually leading to death. At the end of the infection phase, the hyphae grow out from the crayfish cuticle and create sporangia. From the sporangia, a "spore ball" emerges, where primary spores develop flagella and becomes secondary zoospores. The zoospores eventually become released to the water. If the zoospores find a suitable host, the process repeats itself, and if not, new zoospores are developed from the cysts that failed to reach a new host (fig. 2). New zoospores can emerge up to three times from a cyst before they die (Söderhäll and Cerenius, 1999). Since the zoospores eventually dies, crayfish plague cannot persist in areas that lacks a resistant host. This is because the highly susceptible *A. astacus* dies from the infection, and the pathogen burns out relatively shortly after all the *A. astacus* are dead (Söderhäll and Cerenius, 1999). However, recent research has suggested that *A. astaci* may

live on alternative hosts such as freshwater crabs, which needs to be taken into consideration during conservation work (Svoboda et al., 2014).

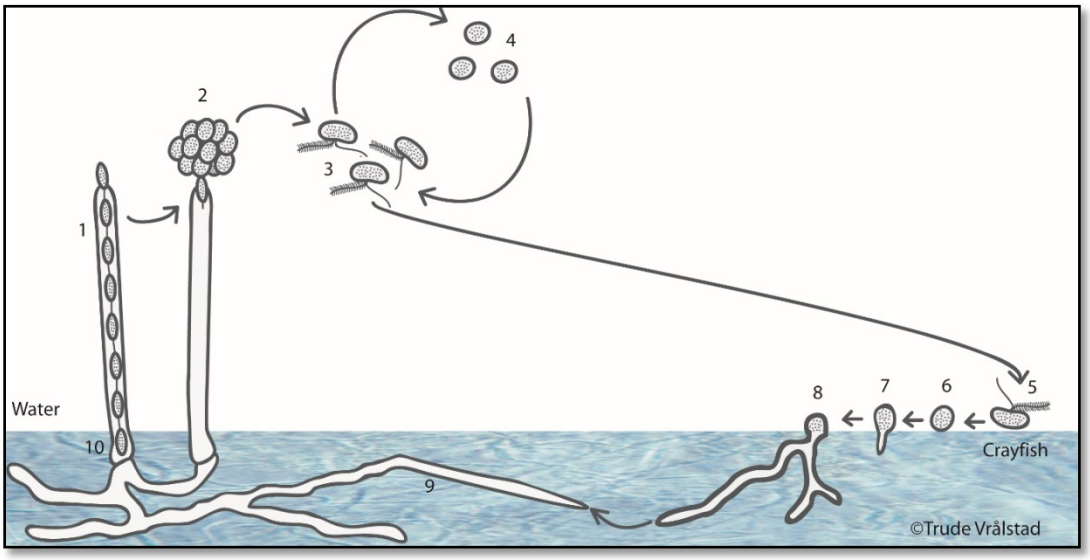

Figure 2. The *A. astaci* life cycle. Illustration from Vrålstad et al (2006). Originally modified after Bangyeekhun (2002).

1.5 Introduction and spread of Aphanomyces astaci and its hosts in Europe

Aphanomyces astaci has adapted to different host species of freshwater crayfish, and exists as strains of different genotypes. In total, four genotypes has been described from invasive North American crayfish species in Europe (Kozubikova et al., 2011; Söderhäll and Cerenius, 1999), while the genotype that is associated with the first introduction of crayfish plague to Europe has no known North American host (Söderhäll and Cerenius, 1999). Recently, a new microsatellite based method has enabled epidemiological tracing of genotypes from historical outbreaks of the crayfish plague pathogen (Grandjean et al., 2014; Vrålstad et al., 2014). The first outbreaks in Europe were caused by *A. astaci* genotype A starting in Italy in 1859 (Alderman, 1996; Söderhäll and Cerenius, 1999). The next outbreak followed in France in 1874. At that time, it was not known what caused mass mortalities of indigenous crayfish. After the outbreak in France, outbreaks followed in Central and Eastern Europe, facilitated via the large European rivers. *Aphanomyces astaci* came to Finland in 1893, and reached Sweden in 1907 as a result of import of diseases noble crayfish from Finland (Alderman, 1996; Edsman, 2004). The following years it spread through Sweden, but did not reach Norway until 1971 (Håstein and Unestam, 1972).

Before North American crayfish were known as *A. astaci* carriers, they were intentionally introduced to Europe for economic and culinary reasons. Further spread has also been shown to be facilitated by numerous vectors such as birds and mustelid crayfish predators; contaminated fishing gear and boats (Alderman et al., 1987), but the main source is human-assisted and often illegal spread of North American crayfish (Holdich et al., 2009). When it in 1972 became known that North American crayfish species were natural hosts and thus carriers of *A. astaci* (Unestam, 1972) it was in many cases too late. Today there are twice as many non-indigenous crayfish species in Europe than indigenous (Kouba et al., 2014). The *A. astaci* carrying North American crayfish *Pacifastacus leniusculus*, *Orconectes limosus* (Rafinesque, 1817) and *Procambrus clarkii* (Girard, 1852) were introduced before 1975. Later, North American crayfish species as *Orconectes immunis* (Hagen, 1870), *Orconectes juvenilis* (Hagen, 1870), *Orconectes virilis* (Hagen, 1870), *Procambrus sp.* (Ortmann, 1905) and *Procambrus acutus* (Girard, 1852) has been introduced. There are also other non-indigenous crayfish species, like the Australian species *Cherax destructor* (Clark, 1936) and *Cherax quadicarinatus* (Von Martens, 1968). It is the North American species *P. leniusculus*, *P. clarkii* and *O. limosus* that carry known *A. astaci* genotypes (genotype B, C, D and E, respectively)(Grandjean et al., 2014), but also the recently introduced North American species *Orconectes* cf. *virilis* is also confirmed carrier of *A. astaci* (Tilmans et al., 2014). *Pacifastacus leniusculus* has a known presence in 27 European countries, and is the only known non-indigenous crayfish species in Norway (Holdich et al., 2009). *Pacifastacus leniusculus* and other North American crayfish have evolved a fast immune response to the infection of crayfish plague zoospores (Söderhäll and Cerenius, 1999).

It has been found that *P. leniusculus* has a continuous production of high levels of phenol oxidase transcripts. The high transcription rate gives them the opportunity to rapidly encapsulate the infection in melanin. In this way, the *P. leniusculus* encapsulate hyphae within the exoskeleton and prevent them from penetrating deeper into the body cavity with soft muscle tissue and vulnerable organs (Söderhäll and Cerenius, 1999). Previously it has been suggested that the susceptible *A. astacus* lacks this high transcription rate, and their immune response fails to stop the infection of *A. astaci* and the crayfish dies (Cerenius et al., 2003). This view has been challenged by Gruber et al (2014). They found that *A. astacus* immune system produces even higher levels of phenol oxidase transcripts than *P.*

leniusculus, but it fails in making the product, melanisation. They suggested that when *A. astacus* invested more energy in immune defenses, their survival time shortened. Resource allocation to immune defense mechanisms could lead to exhaustion and death for *A. astacus*.

Recently, several studies have suggested that adaptation of *A. astaci* to a more biotroph relationship with *A. astacus* and other susceptible native crayfish is happening. Viljamaa-Dirks et al. (2011), Makkonen et al. (2012), Kusar et al. (2013), Viljamaa-Dirks et al. (2016), Maguire et al. (2016) and Martín-Torrijos et al. (2017), found native European populations of *A. astacus, Austrapotamobius torrentium* (Schrank 1803), *Astacus leptodactylus* (Eschscholtz 1823), and *Austropotamobius pallipes* (Lereboullet 1858) with persistent *A. astaci* infection, thus making them carriers. These crayfish carry *A. astaci* with genotype A, the genotype that first came to Europe. This could be a sign of co-evolution between native European crayfish species and *A. astaci* genotype A, in contrast to *A. astaci* genotype that B arrived later together with its host (*P. leniusculus*) and yields considerably higher mortality rates (Makkonen et al., 2012) (Viljamaa-Dirks et al., 2013).

1.6 Crayfish plague outbreaks and signal crayfish introductions in Norway

Revealing *P. leniusculus* and the associated *A. astaci* in a lake or river, often happens when they already are well established. Until 2006 it was believed that Norway did not have any populations of *P. leniusculus*, but in the 10 previous years, 6 illegally introduced *P. leniusculus* populations have been discovered (Johnsen and Vrålstad, 2017). An example is Lake Øymarksjøen. This is a large and complex lake, and it is challenging to cover the area sufficiently with traps. When *P. leniusculus* was discovered in Lake Øymarksjøen in 2008, the population showed signs that it had persisted in the area for several years (Vrålstad et al., 2011).

Vrålstad et al. (2014) found that the first outbreak in 1971-1974 in Veksa and the river Vrangselva near the Swedish border, was caused by the A/As genotype group of *A. astaci*, the same as the first genotype that entered Europe in the 1860s (Söderhäll and Cerenius, 1999). All the following outbreaks in Norway were caused by the *A. astaci* genotype group B/Ps1 (Vrålstad et al, 2014), which originated from the introduction of *P. leniusculus* to Sweden in the 1960s (Söderhäll and Cerenius, 1999). After the outbreaks in Veksa and Vrangselva, Norway's largest river, Glomma was hit in 1987. This outbreak eradicated the

local *A. astacus* populations in the watercourse downstream Kirkenær (Taugbøl et al., 1993). In 1989 there was an outbreak in the Norwegian/Swedish border Lake Store Le and the connected Norwegian Halden watercourse. An outbreak causing mass mortalities among *A. astacus* happened in the river Lysakerelva in Oslo in 1998. After the outbreak in Glomma in 1987, the *A. astacus* population was intentionally re-established (Taugbøl 2004). In 2003, another *A. astaci* outbreak again hit Glomma. The result was again eradication of the population. The same happened with re-established *A. astacus* populations in Lake Øymarksjøen in the Halden watercourse in 2005 (Vrålstad et al., 2009). It was not until 3 years later that *P. leniusculus* was discovered in Lake Øymarksjøen and could explain the outbreak in 2005 (Vrålstad et al., 2011).

In 2006 the first *P. leniusculus* population was discovered in Dammane (Telemark), Norway, and was a result of illegal introduction (Johnsen et al., 2007). Illegal introductions of *P. leniusculus* have since then been discovered in Lake Øymarksjøen (Østfold, 2008), in golf-ponds on the island Ostøya (Akershus, 2009), in Lake Skittenholvatnet and Lake Oppsalvatnet in Hemne (Sør-Trøndelag, 2011), in Lake Kvesjøen (Nord-Trøndelag, 2013) and in Lake Rødenessjøen (Østfold, 2014) (Johnsen and Vrålstad, 2017; Vrålstad et al., 2011).

After the crayfish plague outbreak in Lake Øymarksjøen in 2005, the water locks in Ørje were permanently locked by the Norwegian Food Safety Authority in order to prevent the spread of the disease and also to function as a migration barrier for the later discovered *P. leniusculus* population (Vrålstad et al., 2011). However, in 2014, the neighboring lake to Lake Øymarksjøen, Lake Rødenessjøen experienced *an A. astaci* outbreak. This outbreak was caused by human assisted, illegal introduction of *P. leniusculus,* leading to mass mortalities of the *A. astacus* population upstream the Ørje water lock (Johnsen and Vrålstad, 2017). There have also been recent outbreaks of crayfish plague in the River Buåa (2010), in Lake Mjær and River Hobølelva (2016) and Southern part of River Vrangselva (2016).

In Lake Stora Le, Lake Øymarksjøen and Lake Rødenessjøen, the presence of *P. leniusculus* could explain the *A. astaci* outbreaks leading to local extinction of the *A. astacus* populations. In the river Glomma, the infection source has not been found. In Vrangselva and Buåa, the outbreaks came from the Swedish side of the boarder (Vrålstad et al., 2017) Two populations of *P. leniusculus* were previously chemically exterminated, the one in Dammane (Telemark) and the one in Ostøya (Akershus). Chemical eradication is only

ecologically responsible in lakes that are relatively small and simple in structure, thus the *P. leniusculus* populations in the Halden watercourse appears unfortunately to be permanently established.

1.7 Environmental DNA monitoring

Early detection is very important when it comes to alien species. If the species is still low in abundance, it may be easier to carry out successful eradication measures. Environmental DNA monitoring is a relatively new, animal-friendly, time-saving, and cost-effective monitoring method that allows for early detection. Environmental DNA (eDNA) is DNA that is present in samples taken from the environment, such as water, soil and air (Thomsen and Willerslev, 2015). A common definition of eDNA is "genetic material obtained from environmental samples without any obvious signs of biological source material" (Thomsen and Willerslev, 2015). Environmental samples may contain DNA from many extinct or extant species that previously inhabited the area (Thomsen and Willerslev, 2015) but in environmental water samples, it has been shown that the eDNA content to a large extent give a snap-shot of the present living species, with only a few weeks time lag after a species disappear from the system until eDNA no longer can be detected (Dejean et al., 2011). Environmental DNA originates from DNA holding components that are shed from an organism into the environment. This could be skin cells, hair, saliva, eggs, mucus, feces etc. Following sampling, eDNA is extracted and analyzed by PCR and sequencing methods, or by quantitative real-time PCR (qPCR) (Bohmann et al., 2014). The former approach is commonly used for eDNA monitoring of biodiversity, and the latter is used for more targeted monitoring of specific species, including red list species, alien black list species and specific disease pathogens (Agersnap et al., 2017; Strand et al., 2014). Detection of alien species at low densities has positive implications for conservation purposes, because then necessary action at least in some cases can be taken before the population has grown out of control. With the new advances of eDNA monitoring of natural lakes, *P. leniusculus* can perhaps be discovered when the population still is in its establishment phase. Environmental DNA detection of *A. astaci* and *P. leniusculus* could also help determine when an area is fit for re-establishment of *A. astacus*. The risk of failure will then be smaller (Simberloff et al., 2013).

Over the past years, several studies have been done on eDNA detection of organisms in water bodies. Ficetola et al. (2008) suggested as early as in 2008 that eDNA methods could

be more efficient for discovering target species at low population densities than traditional methods. Most of the research has focused on fish and amphibians (Dejean et al., 2012; Doi et al., 2015a; Eichmiller et al., 2016; Ficetola et al., 2008; Goldberg et al., 2011; Gustavson et al., 2015; Jerde et al., 2011; Lacoursière-Roussel et al., 2016; Pilliod et al., 2013), but more recently, several studies on crayfish eDNA has emerged (Agersnap et al., 2017; Cai et al., 2017; Dougherty et al., 2016; Dunn et al., 2017; Ikeda et al., 2016; Larson et al., 2017; Tréguier et al., 2014). These studies have confirmed that it is possible to detect invasive and endangered freshwater crayfish species using eDNA. However, in most of the studies qualitative questions have been addressed, and a few focuses on quantitative relationships. For conservation purposes, monitoring of population development is important. This implies that quantitative methods need to be developed further and more data on DNA emission and degradation rates, seasonal impact, life cycle and other biotic and abiotic factors should be investigated further.

For *A. astaci*, quantitative eDNA detection of spores has proven to be successful. Strand et al. (2011) showed that as little as 1 spore/L could be detected from water samples using species specific TaqMan® minor groove binder qPCR (Vrålstad et al., 2009). Strand et al. (2012) also revealed details on *A. astaci* spore dynamics and optimized spore detection techniques. The eDNA monitoring concept of *A. astaci* was further developed and implemented for large freshwater lakes (Strand et al., 2014), a method that now is implemented in the crayfish plague disease surveillance work in Norway (Vrålstad et al., 2017).

The research projected TARGET (Targeted strategies for safeguarding the noble crayfish against alien and emerging threats), is a project granted by the Norwegian Research Council (NRC) and conducted by The Norwegian Veterinary Institute (NVI) in Oslo. The TARGET project also cooperates with other institutions and universities, both in Norway and abroad. The overall project goal is to "develop cost efficient and environmentally friendly monitoring tools and control strategies for better protection of the *A. astacus*". TARGET was established in April 2015 and is led by Senior Researcher Trude Vrålstad at NVI. The use of environmental DNA (eDNA) as a monitoring tool creates the basis for TARGETS work, which this thesis is a part of.

1.8 Hypothesis and research questions

The TARGET project aims to identify eDNA predictors for *P. leniusculus* population density and *A. astaci* prevalence. In this master thesis, eDNA predictor values will be determined more accurately based on aquaria experiments where the number of detectable eDNA copies/L from known numbers of *P. leniusculus* will be measured. ***The overall hypothesis is that eDNA emitted from P. leniusculus correlates with population density/number of individuals***. A further TARGET project aim is to develop eDNA predictors for relative crayfish plague prevalence in carrier crayfish populations deduced from eDNA ratios of the crayfish/pathogen. In this master thesis, detectable *A. astaci* eDNA copies in water will therefore be measured by means of eDNA methods and compared to *P. leniusculus* eDNA copies and crayfish tissue pathogen load (Vrålstad et al., 2009). It can be expected that several factors affect both the emission and detectability of eDNA from signal crayfish and its obligate parasite *A. astaci* into the ambient water, including varying environmental conditions and life history stages. Therefore, this thesis will specifically address the following research questions:

Is the measurable amount of *P. leniusculus* and *A. astaci* eDNA affected by

1. density of the *P. leniusculus* individuals?

2. water temperature?

3. food availability for the *P. leniusculus* individuals?

4. crucial *P. leniusculus* life history stages such as moulting, reproduction and death?

Three main aquaria experiments with *P. leniusculus* were conducted attempting to answer these questions using water filter samples where eDNA from *P. leniusculus* and *A. astaci* were measured by means of species specific qPCR.

2 Materials and methods

2.1 *Pacifastacus leniusculus* origin and husbandry

2.1.1 *Pacifastacus leniusculus* origin

The individuals of *P. leniusculus* that participated in this thesis were caught using traps in Lake Øymarksjøen and Lake Rødenessjøen (fig. 3), both known to harbor populations of *A. astaci* infected *P. leniusculus*. In total, 141 individual *P. leniusculus* from the two different lakes were caught.

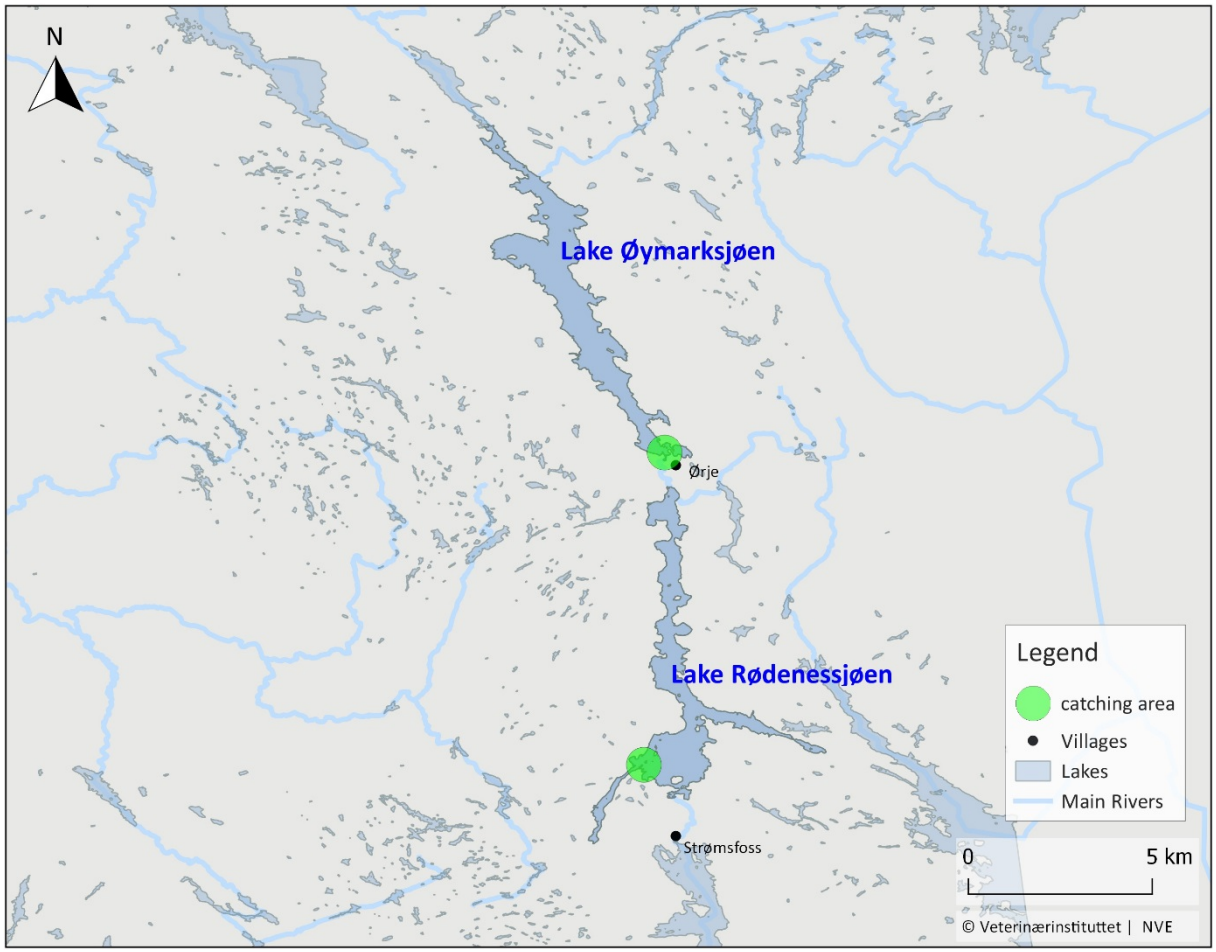

Figure 3. Lake Øymarksjøen and Lake Rødenessjøen are two large freshwater lakes where *P. leniusculus* and the associated parasite *A. astaci* are present. The lakes are situated in the municipalities Aremark and Marker, in Østfold County in the south-eastern part of Norway. Lake Rødenessjøen is connected to, and lies to the north of Lake Øymarksjøen. The locations are a part of the Halden watercourse, which consists of many lakes and rivers. Credit: Johannes Rusch.

Capture of *P. leniusculus* is illegal in Norway. The TARGET project has therefore applied for and got granted a legal permit from the Norwegian Food Safety Authority and the Norwegian Environmental Agency to capture *P. leniusculus* in the Halden watercourse, and

keep *P. leniusculus* in safe infection-controlled aquarium facilities at the Norwegian Veterinary Institute for research purposes (text S.1, text S.2), provided that the *P. leniusculus* were euthanized and not released after ended experiment. Individuals of *P. leniusculus* in this thesis originate from two sampling events. The first in Lake Rødenessjøen was conducted by the Agency for outlying fields, Akershus & Østfold in collaboration with TARGET in August and September 2015, were *P. leniusculus* were captured using traps baited with chicken during a survey to determine the distribution of *P. leniusculus* in the lake. The second was conducted by the TARGET project in October 2015, where 52 *P. leniusculus* were captured using baited crayfish traps in Lake Øymarksjøen. There were used 25 traps baited with chicken that were placed in 5 various sites approximately 5 meters from the shoreline. The traps were in the water from approximately 0700 pm until 0830 am. After capture, the *P. leniusculus* were kept safe in a Styrofoam box and transported to the research facilities at the NVI in Oslo.

2.1.2 Husbandry and marking

The *P. leniusculus* captured in Lake Rødenessjøen and Lake Øymarksjøen were kept together in a large communal housing tank. This was done to create a mixed population scenario for reducing of the effect population differences may have on treatment effects. The communal housing tank created the basis for the subsequent experiments.

The communal housing tank had oxygen supply and a water filtration system for ensuring good water quality. Temperature and oxygen measurements were conducted every day using OxyGuard Handy Polaris 2 D.O. meter (OxyGuard International, Farum, Denmark). In addition, pH, NO_{3-}, NO_{2-}, Cl_2, and water hardness were measured three times a week with JBL EasyTest 6-in-1 test strips (JBL, GmbH & Co., Neuhofen, Germany). Shelters were provided to reduce aggressive behavior. The shelters were made of plastic pipes.

Three different marking techniques were used to ensure that the *P. leniusculus* always could be identified with a number. The first method was to use a white marker and draw an assigned number onto the head part of the cephalothorax. The second method was to place a sticker with its number on the thorax part of the cephalothorax. The last marking method was done by punctuating the uropods with a needle, in a specific order (Guan, 1997)(fig. 4.a, b, c, d.).

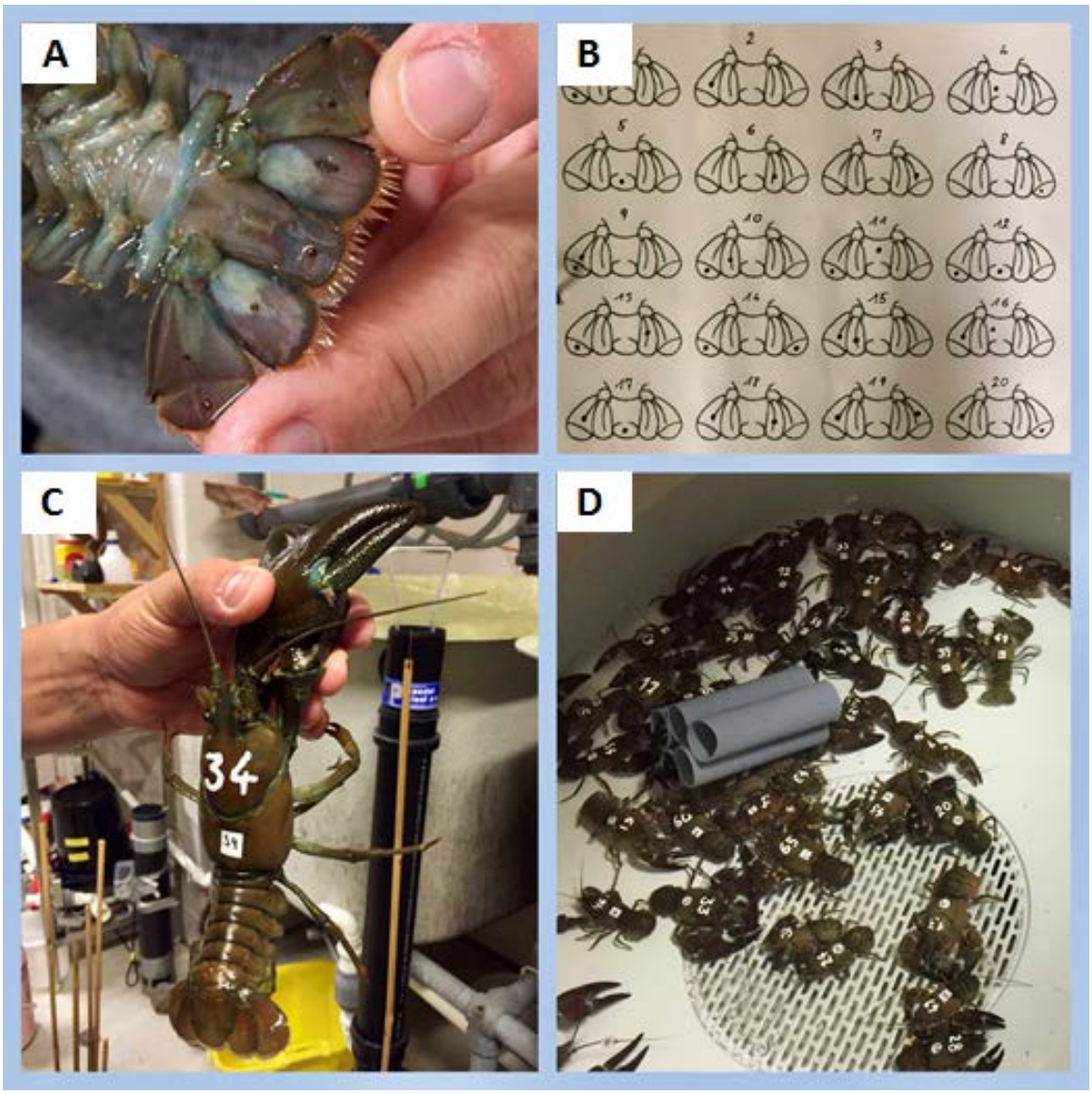

Figure 4. a) *Pacifastacus leniusculus* marked with needle. b) Tail marking schedule. c) *Pacifastacus leniusculus* with all three marking methods. d) Overview of communal housing tank with marked individuals.

2.2 Experimental designs and implementation

The experiments were conducted in the infection room of the common aquarium of NMBU (Norwegian University of life sciences) and NVI (The Norwegian Veterinary Institute) situated at Adamstuen in Oslo. The aquarium is approved/holds a permit for work with crayfish. This thesis consisted of three main experiments: 1) *Temperature, density, and food availability*; 2) *Moulting* and 3) *Death*. A forth experiment on *Reproduction* was attempted but not successfully carried through (see below). In addition, tissue samples were collected from *P. leniusculus* post mortem for analyzation of their infection level. Data on *P. leniusculus* origin, sex, length and assigned tanks can be found in table S.1.

2.2.1 Experiment 1: Temperature, density, and food availability

The first experiment was set up to investigate how density, temperature and food availability influenced the number of detectable eDNA copies/L. Four tanks were set up as respective experimental tanks for 2 fed, 2 not fed, 20 fed and 20 not fed *P. leniusculus* at the beginning of each week (fig. 5.). The three first weeks (replicates) represented summer temperature (20 °C), and the three last weeks (replicates) represented spring/autumn temperatures (10 °C) (fig. 6). Three days before starting the experiments, *P. leniusculus* in the communal housing tank were not given food to get the same basis. This series of experiments were carried out over six weeks, with three replicates for each temperature. One week equals one replicate (fig. 6).

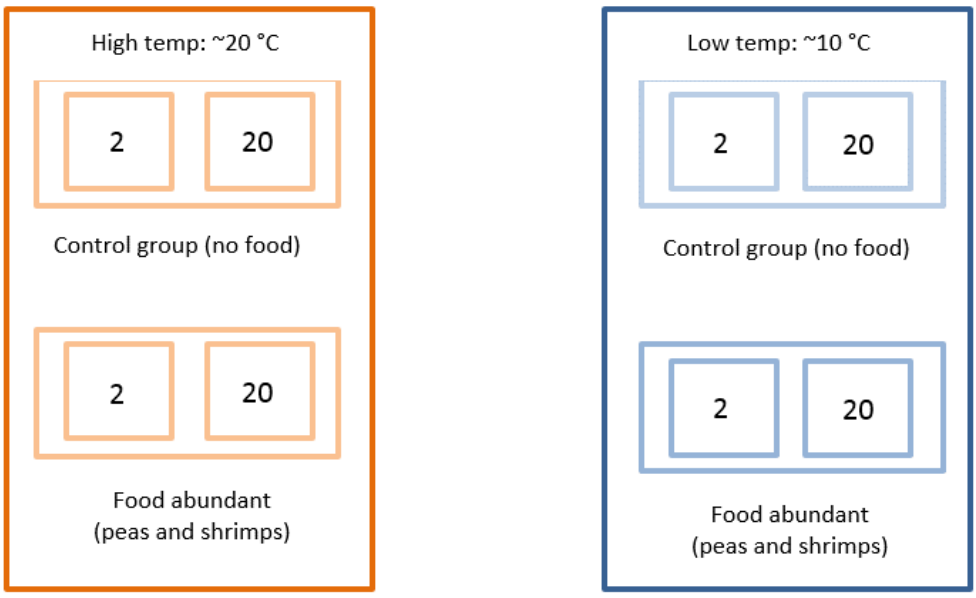

Figure 5. Experimental design Experiment 1: Temperature, density, and food availability. The number 2 and 20 refers to number of *P. leniusculus* in each tank.

Week 1 20 °C 9-16.11.2015 Replicate 1 20 fed 20 not fed 2 fed 2 not fed	Week 2 20 °C 23-30.11.2015 Replicate 2 20 fed 20 not fed 2 fed 2 not fed	Week 3 20 °C 9-16.12.2015 Replicate 3 20 fed 20 not fed 2 fed 2 not fed	Week 1 10 °C 6-13.1.2016 Replicate 1 20 fed 20 not fed 2 fed 2 not fed	Week 2 10 °C 18-25.1.2016 Replicate 2 20 fed 20 not fed 2 fed 2 not fed	Week 3 10 °C 1-8.2.2016 Replicate 3 20 fed 20 not fed 2 fed 2 not fed

Figure 6. Experiment 1 consisted of six replicates in total. Three replicates for each temperature, each replicate lasting one week. Each replicate consisted of four tanks containing 2 fed, 20 fed, 2 non-fed, and 20 non-fed *P. leniusculus*.

Table 1. Feeding regime given during Experiment 1: temperature, density, and food availability. Starting 3 days before each week (replicate), feeding of the *P. leniusculus* was terminated. At day 1 of the week (replicate) and every other day after that, food was given to the *P. leniusculus* that were in the feeding groups. At day 8 and after the end of the week (replicate), the *P. leniusculus* were transferred to the communal housing tank and the normal feeding regime was restored. Normal feeding regime includes regularly feeding with plant based nutrients as peas, and animal based nutrients as shrimps given in relation to the number of *P. leniusculus* (Rusch and Fureder, 2015).

Days												
Before replicate	***Day -3***	***Day -2***	***Day -1***	***Day 1***	***Day 2***	***Day 3***	***Day 4***	***Day 5***	***Day 6***	***Day 7***	***Day 8***	***After replicate***
Normal feeding in communal housing tank	No feeding in communal housing tank			2 *P. l* 2 peas ½ shrimp	2 *P. l* No food	2 *P. l* 2 peas ½ shrimp	2 *P. l* No food	2 *P. l* 2 peas ½ shrimp	2 *P. l* No food	2 *P. l* 2 peas ½ shrimp	2 *P. l* No food	Normal feeding in communal housing tank
				20 *P. l* 20 peas 2 shrimps	20 *P. l* No food	20 *P. l* 20 peas 2 shrimps	20 *P. l* No food	20 *P. l* 20 peas 2 shrimps	20 *P. l* No food	20 *P. l* 20 peas 2 shrimps	20 *P. l* No food	
				2 *P. l* No food	2 *P. l* No food	2 *P. l* No food	2 *P. l* No food	2 *P. l* No food	2 *P. l* No food	2 *P. l* No food	2 *P. l* No food	
				20 *P. l* No food	20 *P. l* No food	20 *P. l* No food	20 *P. l* No food	20 *P. l* No food	20 *P. l* No food	20 *P. l* No food	20 *P. l* No food	

Pacifastacus leniusculus were randomly chosen from the communal housing tank and assigned to their experimental tank. The *P. leniusculus* assigned number and sex were noted. The experimental tanks each contained 100 L tap water. At the end of every week, 3*1 L filter samples were taken from each tank (described below).

During the experiment, food was given every other day, starting on day 1 of each replicate. The 20 *P. leniusculus* were fed 20 frozen green peas and 2 shrimps. The two *P. leniusculus* were fed 2 frozen green peas and ½ shrimp.

Oxygen supply and shelters were provided in the experimental tanks. Water quality measurements were conducted in the same way as in the husbandry tank.

The mean temperatures during these experiments were 19.76 °C for the high temperature group, and 10.83 °C for the low temperature group.

2.2.2 Experiment 2: Moulting

To investigate the impact of moulting on the number of detectable eDNA copies/L in the water, moulting individuals of *P. leniusculus* were isolated.

By supervision of the communal housing tank, newly molted *P. leniusculus* were placed in separate tanks, together with their old exoskeleton whenever possible. To obtain the crayfish as fast as possible after moulting, the communal housing tank was observed a couple of times every day. Whenever a *P. leniusculus* showed signs of moulting, it was placed in its own separate experimental tank. If the *P. leniusculus* exoskeleton was discovered, it was placed in the experiment tank together with the *P. leniusculus.* When the exoskeleton was not present, the *P. leniusculus* possibly ingested it. The experimental tanks contained 100 L tap water. Newly molted *P. leniusculus* were kept in the experimental tank for one week. At the end of the week, 3*1 L water samples were filtered (described below). The *P. leniusculus* in this experiment were not fed. The mean water temperature during moulting experiments was 11.51 °C.

2.2.3 Experiment 3: Death

The experimental crayfish should at this state be euthanized, following the NFSA demand. To investigate the impact of death and consequently *P. leniusculus* decay on the eDNA content

in the water, an experiment was conducted in the end when all the other experiments were completed.

Three experimental tanks (100 L) were set up for the experiment. Individual *P. leniusculus* were euthanized by placing *P. leniusculus* placed in a Styrofoam box containing ice water to numb their nervous system. Using a scalpel, the brain was pierced from the back of its head to between the eyes, and then again across the back head (EFSA, 2005). The procedure was performed by a researcher with approved training in animal laboratory science. See text S.3 for the application text. Euthanized crayfish were placed in the experimental tanks, two tanks with 2 *P. leniusculus* each and one tank with 20 *P. leniusculus*. After one week 3*1 L filter samples were taken from each tank at the end of the week. Average temperature during this experiment was 12.75 °C.

2.2.4 Excluded experiment: Reproduction:

An experiment involving eDNA emission during *P. leniusculus* reproduction was attempted, but unfortunately not successfully carried through. *P. leniusculus* that showed signs of reproduction behavior were taken out of the communal housing tank and placed in separate tanks to measure the *P. leniusculus* eDNA emission from the reproducing couple. However, this interruption probably aborted the reproductive behavior, leading to uncertainty about whether *P. leniusculus* had reproduced. It did also not seem responsible to carry out the experiments due to signs of aggressive behavior from the *P. leniusculus* individuals involved. We thus failed to organize a controlled experiment that could answer if reproduction impacted on the amount of eDNA in the water, and further details of and data from this experiment are not included in the thesis.

2.3 Sample preparation:

2.3.1 Water filtration

The filtration method used was first described in Strand et al (2014). This filtration method was used to capture eDNA of both *A. astaci* and *P. leniusculus*. Components used during filtration were a peristaltic pump, tygon tubing with filter holder (Cole-Parmer, Illinois, USA), sterile 47mm Millipore glass fiber filters with 2 µl pore size (AP25, 47 mm diameter, Millipore, Billerica, Massachusetts, USA), and forceps.

At the end of each experiment, a volume of 1 L tank water was filtered through the glass fiber filter from each tank. This was repeated 3 times to generate 3 replicates. Prior to filtration, sterile filters were retrieved and placed in the filter holder using sterile forceps. Another forceps were used for retrieving filters after filtration and placing them separately in marked falcon tubes. Between tanks, clean tap water was pumped through the empty filter holder for 5 minutes. Then 1 L tank-specific water was pumped through before the filter was inserted. At the start of each experiment, control water samples (3*1 L) were taken from a clean bucket filled with water from the same source as used in the experiment. Post filtration, filters were frozen at -20 °C for storage.

The filter system was rinsed after each experiment. A 10% chlorine solution were pumped through the system for 10 minutes, then clean running water were pumped through for another 10 minutes, before 10% natrium thiosulfate were pumped through to remove chlorine residues. Then the tubing was emptied before being placed in the freezer at -22 °C.

2.3.2 Tissue sampling and preparation

After euthanization, tissue samples were taken of the uropods, soft cuticle of the abdomen, and the inner joint of the second walking leg. This procedure followed (Vrålstad et al 2011). Tissue samples were then stored at -20 ^{o}C prior to further analyses. Before laboratory procedures, samples were frozen down to -80 ^{o}C. Tissue samples were also taken from the uropods of frozen *P. leniusculus* who had died earlier during the experiments.

2.3.3 Research hygiene and biosecurity measures

To obtain controlled and uncontaminated experiments, many approaches were followed. Before each experiment/replicate, the experimental tanks, oxygen supply and when present immersion heaters, were disinfected using 10% Enduro Chlor solution and Virkon® S. Shelters were rinsed as carefully as possible. Control samples of 3*1 L were taken from the same tap water as the water in the experimental tanks for each experiment/replicate. Controls were also used in the laboratory procedures, as specified. The communal housing tank and the experimental tanks were placed in an isolated environment due to their risk of proliferating biological hazards, such as *A. astaci*. The draining system connected to the experimental tanks is connected to a basin where Virkon® S is added, followed by filtration, before the water is then transferred to the public sewage system. Forceps were sterilized with ethanol and flames during tissue sampling.

2.4 Ethics statement - Animal welfare

Unfortunately, some *P. leniusculus* died during the experiments, as conditions indoors never can be as satisfactory for a crayfish as its natural environment. To ensure satisfactory conditions, water quality was monitored, and shelters provided. It is also important to emphasize that crayfish can survive for long periods without food (Rusch and Füreder, 2015). *P. leniusculus* in this thesis had a maximum 11 days without food.

In the capture permit from the NFSA and NEA, euthanasia of the *P. leniusculus* after completion of research activity was demanded. Releasing the *P. leniusculus* back into their environment was not an option, as they are on the Norwegian black list of alien invasive species, and categorized among the "high risk" species (Gederaas et al., 2012).

Protocols for husbandry and euthanization followed the protocols approved by NFSA from other previous and on-going crayfish experiments of the TARGET project that involve an experimental load requiring own permission for use of experimental animals from the Food Safety Authority (former "Forsøksdyrutvalget").

2.5 Molecular analyses

2.5.1 DNA extraction from filter samples

To extract total genomic DNA from the filtered water samples, a large volume CTAB extraction procedure following (Strand et al 2014) was used. Before extraction, the samples were frozen at -80 ^{o}C prior to freeze drying. Then the filters were freeze dried for approximately 24 hours to remove excess water.

The procedure was conducted as follows: CTAB-buffer added 1% mercaptoethanol was heated at 65^{o}C for at least 10 minutes, and 4 ml CTAB buffer was added to each of the filter-tubes. The filter was then ripped apart inside the tube, using the pipette tip. The samples were thereafter frozen for 30 minutes at -80 C^{o}. The samples were then thawed at 65 C^{o} for 10-15 minutes. After that, 40µl Proteinase K was added directly to the filters and mixed carefully using the pipette tip. Then the samples were incubated for 60 minutes at 65 C^{o}. Following, 4 ml chloroform was added to the tubes, and then mixed gently with the pipette tip. Subsequently the tubes were centrifuged for 15 minutes at 4800 rpm. Then 3000 µl of the upper phase was transferred to two new tubes, marked A and B, with 1500 µl in each.

Then 500µl chloroform was added and the tubes were vortexed and centrifuged for 5 minutes at 12000 rpm. Then 1200 µl of the upper phase was transferred to new marked 1.5 ml Eppendorf® tubes. Thereafter, 800 µl ice cold isopropanol was added, and the tubes were inverted to start precipitation of DNA. The samples were placed in the fridge for 15 minutes at approximately 4 ^{o}C, and thereafter centrifuged for 15 minutes at 16000 g to pellet the DNA. The supernatant removed and 500 µl ice cold 70% ethanol was added to the tubes. The tubes were mixed carefully and centrifuged for 5 minutes at 16000 g, before using a pipette to gently remove the ethanol. The DNA pellets were dried for 15 min. at 45 ^{o}C in a vacuum drier, DNA mini Centrifugal Evaporator (Heto-Holten A/S, Allerød, Denmark). The last step was to add 100µl TE-buffer to the DNA pellet, vortex, centrifuge for 1 minute and let dissolve for 30 minutes. After extraction, the DNA samples were stored at 20 °C pending qPCR analysis.

Two different controls were created during the extraction procedure. One laboratory environmental control with 200 µl ddH2O in a 1.5µl Eppendorf® tube, stayed open during the extraction procedure. In addition, one extraction blank control (EBC) tube was included during each round of DNA isolation, following the same treatment as the rest of the samples.

2.5.2 DNA extraction from tissue

The tissue samples were transferred from their initial tubes to Precellys® tubes containing steel beads (Precellys MK28). Sample weight was noted. Then 450 µl ATL buffer was added to each tube. The Precellys®24 Homogenizer (Bertin Technologies, Montigny, France) was used to thoroughly homogenize the tissue samples using the following program, 6500 rpm for 1 minute for 3 sessions with a resting time of 2 minutes between the sessions (1:6500-3*60-120). Then the samples were centrifuged for 1 minute to remove any residues from inside of the lid. The samples were frozen for 10 minutes at -80 ^{o}C to break remaining cell walls. Then they were incubated at 56 ^{o}C until they were thawed. The samples were subsequently centrifuged for 1.5 minutes. Following that, 10 µl RNaseA (10 mg/ml) was added before centrifugation for 1 minute. Another 10 µl proteinase K (20 mg/ml) was added and mixed by vortexing. Subsequently the samples were incubated at 56 ^{o}C for 30 minutes, and then centrifuged for 5 minutes at 12000g. Finally, 200 µl of the resulting supernatant were added to new, marked tubes. The remaining part of the extraction process was

performed on a QIAcube DNA extractor (QIAGEN, GmbH, Hilden, Germany) using QIAcube "QIAamp® DNA Min Kit". After qPCR the DNA samples were stored at -20 °C.

One laboratory environmental control with 200 µl ddH2O in a 1.5µl Eppendorf tube, stayed open during the extraction procedure. One extraction blank control (EBC) followed the same treatment as the rest of the samples. In addition, each QIAcube run had a QIAcube blank control containing 200 µl of ddH_2O.

2.5.3 Quantitative real-time PCR

Two quantitative real-time polymerase chain reaction (qPCR) protocols were used for species specific detection and quantification of *P. leniusculus (*Agersnap et al., 2017*)* and *A. astaci* (Vrålstad et al., 2009).

2.5.3.1 Primers and probes

The *P. leniusculus* primers and probe developed by Agersnap et al (2017) detect a 65 basepair fragment of the mitochondrial cytochrome oxidase subunit 1 (mtDNA-CO1), which target the same sequence region as Treguiér et al (2014) used for eDNA detection of red swamp crayfish (*Procambrus clarkii*). The sequences for the forward primer Paclen_COI_F0336, reverse primer Paclen_COI_R0397, and probe Paclen_COI_P0357 are listed in table 2.

The qPCR assay used for detection of *A. astaci* is a TaqMan™ minor groove binder assay developed by (Vrålstad et al 2009) detecting a 57-base pair fragment of the internal transcribed spaced 1 (ITS1) region of nuclear ribosomal DNA (nrDNA). The sequences for the forward primer AphAstITS-39F (5 µm), reverse primer AphAstITS-97R (5 µm) and minor groove binder (MGB) probe AphAstITS-60T (5 µm) are listed in table 2.

Table 2. Overview of *P. leniusculus* and *A. astaci* primers and probes.

Species	Primer/probe name	Primer/probe sequence
Pacifastacus leniusculus	Paclen_COI_F0336	5'-AACTAGAGGAATAGTTGAAAG-3'
Pacifastacus leniusculus	Paclen_COI_R0397	5'-CGCTGCTAGAGGAGGATAA-3'
Pacifastacus leniusculus	Paclen_COI_P0357	Fam-AGGAGTGGGTACTGGATGAACT-BHQ-1
Aphanomyces astaci	AphAstITS-39F	5'-AAG GCT TGT GCT GGG ATG TT-3'
Aphanomyces astaci	AphAstITS-97R	5'-CTT CTT GCG AAA CCT TCT GCT A-3'
Aphanomyces astaci	AphAstITS-60T	5'-6-FAM-TTC GGG ACG ACC C-MGB-NFQ-3'

2.5.3.2 Standards

Standard dilution series with known DNA copy numbers of *A. astaci* and *P. leniusculus* are routinely made at the NVI, following the protocols described in Vrålstad et al (2009) and Agersnap et al (2017), respectively. These standards were available for this thesis, and were used to generate a standard curve for each target species. The standard was added to the qPCR plate in 10^1, 10^2, 10^3, and 10^4 dilutions to generate a standard curve (tab. S.2). The dilutions were made by adding 5 µl of the stock standard solution to 45 µl ddH2O. Then the standard was vortexed before 5 µl again was transferred to a new tube containing 45 µl ddH2O. The standard dilution series were then loaded on the qPCR plate with each concentration distributed in two wells each. The standard curve makes it possible to quantify target DNA and calculate the limit of quantification (LOQ). The LOQs have previously been shown to be 10 PCR forming units (PFU) for *P. leniusculus* and 50 PFU for *A. astaci,* for each PCR reaction. PFU refers to the amplifiable DNA copies in a PCR reaction (Vrålstad et al 2009). As described later, since the filter samples consists of one A and one B sample, the LOQ is multiplied by two. Thus, the used LOQ values are in this thesis 20 PFU for *P. leniusculus* and 100 PFU for *A. astaci*. Further, in this thesis, PFU will be referred to as detectable eDNA copies.

2.5.3.3 qPCR analyses

All DNA isolates from the experiments and tissue samples were diluted 10-fold in new Eppendorf® tubes prior to qPCR. In one qPCR run, 2 undiluted and 2 diluted samples were

run, in total 4 replicates per filter sample. The mastermix contained for each sample 12.5 µl TaqMan™ Environmental Master Mix 2.0 (Life Technologies (Thermo Fischer Scientific), Carlsbad, California, USA), 1.5 µl ddH2O, 2.5 µl forward primer, 2.5 µl reverse primer and 1 µl probe, yielding a concentration of 500 nm primer and 250 nm probe. To each well on the qPCR plate, 20 µl mastermix and 5 µl DNA isolate were added. The control samples from the filtration and extraction work were included in the respective qPCR runs. In addition, two wells containing only mastermix functioned as qPCR blank controls. The real-time quantitative PCR machine used was Stratagene 3005P. Pre-and post qPCR work were conducted in separate rooms according to the NVI standards.

The qPCR program used for *A. astaci* follow Vrålstad et al (2009) with modifications to the annealing/extension cycle (Strand et al. 2014), and consist of 2 minutes decontamination at 50 ^{o}C to allow optimal enzymatic activity of uracil-DNA glycosylase (UNG). Then 10 minutes at 95 ^{o}C to activate the DNA polymerase, deactivate the UNG and denature the template DNA. This is followed by 50 cycles of 95 ^{o}C for denaturation for 15 seconds and 62 ^{o}C for annealing and synthesis for 30 seconds.

The qPCR program used for *P. leniusculus* follow Agersnap et al (2017) and consists of 5 minutes decontamination at 50 ^{o}C, then 10 minutes denaturation at 95 ^{o}C. This is followed by 50 cycles of 95 ^{o}C denaturation for 30 seconds, and 56 ^{o}C annealing and synthesis phase for 1 minute.

The data from the qPCR reactions were analyzed in the MxPro software V.4.10. When analyzing the qPCR results, inhibition needs to be considered. Inhibition happens when other substances than target DNA interfere in the PCR reaction. This may lead to measurement errors. Measuring inhibition is done by calculating the difference in Ct-values between the undiluted and diluted samples (ΔCt) (Kozubíková et al 2011). If inhibition is absent the ΔCt equals 3.32. To account for errors in pipetting, amplification efficiency and other inaccuracies, a variance of 15 % were accepted (ΔCt range 2.82 to 3.82). In this case, the mean PFU value between the undiluted and 10-fold diluted (multiplied by 10) sample was used. If inhibition was present, ΔCt<2.82, the 10-fold diluted sample were used (multiplied by 10). If ΔCt>3.83, indicating that the 10-fold diluted sample was out of quantifiable range, only the undiluted sample were used.

The concentration of eDNA copies/L (PFU_L) were calculated according to (Agersnap et al., 2017) with some adjustments for replicates, using the following equation:

$PFU_L = ((PFU_A + PFU_B) * (V_e/V_r))/(V_W)$ where PFU_L = PCR forming units per volume (L) aquarium water, PFU_A = estimated PCR forming units for subsample A per reaction volume, PFU_B = estimated PCR forming units for subsample B per reaction volume V_e = total elution volume after extraction, V_r = volume of eluded extract used in the qPCR reaction, V_r = volume of filtered aquarium water.

2.6 Statistical analyses.

The values used are eDNA copy values from the A and B samples added together. The A and B samples together equals one filter. The eDNA copy values were then multiplied with 20 to get eDNA copies/L. For eDNA copies/L values with a Ct (cycle threshold) value of 41 or higher, the uncertainty is large, and it is common to set a cutoff at Ct 41 (Agersnap et al., 2017; Kozubikova et al., 2011), regarding these result as negatives. Here, these values were treated as 0 in the statistical analyses.

Further, to obtain measures on the crayfish individual level, these values where further divided by crayfish density (e.g. 2 or 20). It is the eDNA copy/L per crayfish values that are used in the statistical analyses. In the text these values are referred to as eDNA copies per individual.

The eDNA copies/L values from the qPCR reactions were converted using log (eDNA copies/L+1) to avoid log (0) = -∞ for the non-detect samples. The eDNA copies/L values range from 0 several millions, and because of this wide range, a logarithmic transformation was necessary before starting the statistical analyses.

For statistical computing, RStudio v.3.4.0 was used. For R-script see text S.2.

For analyses of *P. leniusculus* and *A. astaci* eDNA abundance in Experiment 1: temperature, density and food availability, linear mixed model effects (lme) were used to correct for pseudoreplication, because the 3 replicate filters from the same tank are not true replicates. A composite identifier representing tank within experiment (Tank: Experiment) is used as random effect to correct for pseudoreplication. For *P. leniusculus* and *A. astaci*, several

models were tested for the different treatments (details under results and in text S.3). After fitting the models with lme, they were refit from REML (restricted maximum likelihood) to ML (maximum likelihood) to perform likelihood ratio tests. AIC – Akaike tests were used to find the most adequate model.

The R package "lattice" was used to make boxplots for Experiment 1: temperature, density and food availability. Microsoft Excel 2016 diagrams were used when making a graphical overview of all the result. Here also the eDNA copy per individual values were log(x+1)-transformed. The same is valid for the experiments "Moulting" and "Death", as they did not contain enough samples to run statistical tests (tab. S.3).

2.7 Collaboration and shared work

Some of the work on this master thesis was done in collaboration with PhD-student Johannes Rusch, as this master thesis is a part of a larger project (TARGET). Examples of shared work that we collaborated on include husbandry and handling of *P. leniusculus*, the setup of aquarium experiments, daily measurements, eDNA and tissue sampling. The material generated in this master thesis will also be used by Johannes Rusch to generate further results by the use of droplet digital PCR (ddPCR), with the aim of a joint manuscript.

3 Results

Aquarium experiments with *Pacifastacus leniusculus* has been conducted to measure the resulting eDNA concentrations of *P. leniusculus* and its associated parasite *Aphanomyces astaci* in the water under different environmental conditions, including two different *P. leniusculus* densities, food availability versus no food available, two temperatures simulating summer and spring/autumn, and during the life history stages moulting and death.

3.1 Overall result summary

Figure 7 presents a graphical overview of all results. The overall results from the main experiment (Experiment 1) indicate that increased density of *P. leniusculus* with some exceptions does not significantly increase eDNA concentrations per individual of both target organisms. However, it is not either observed a ~10-fold increase in *P. leniusculus* and *A. astaci* eDNA concentrations when the density increased from two to twenty, which would have been expected if each crayfish individual produced eDNA copy numbers at a relative

equal rate. Feeding contributes to a significant reduction in detectable eDNA copies per individual from *P. leniusculus*, but does not affect *A. astaci* eDNA concentration. On the other hand, a temperature of 20 °C has a strong negative effect on *A. astaci* eDNA concentration. *Pacifastaculus leniusculus* eDNA concentration is also negatively affected by 20 °C, but the effect is weaker than for *A. astaci,* and non-significant. The highest eDNA concentrations from both target organisms are detected at 10°C, with a clear peak for *P. leniusculus* at high-density and no feeding. Moulting seemingly increases the amount of detectable eDNA copies per individual from both target organisms, although these data are not tested statistically due to small sample size. Death of the *P. leniusculus* had no apparent overall impact on eDNA concentrations of both target organism compared to the "normal state" results, but these results are strongly confounded by poor water quality in the high-density tank where extreme biofouling of the water was observed. In the low-density tank, dead crayfish clearly yield much higher eDNA copy numbers per L water than live crayfish (see below).

On average, a single *P. leniusculus* individual give rise to eDNA concentrations ranging from ~250-900.000 and ~1-6000 copies per L water for *P. leniusculus* and *A. astaci,* respectively, depending on tested condition (tab. 3, fig. 7). The number of detectable eDNA copies from all the experiments can be found in table S.4.

Table 3. Summary of the average detectable *P. leniusculus* eDNA *(P. l)* and *A. astaci* eDNA *(A. a)* copy numbers per liter water per *P. leniusculus* individual for the combinations of test conditions: density, food availability and temperature.

	Low density	Low density	High density	High density	
10°C	4580 (±3849)	5885 (±5152)	242 (±153)	883393 (±873732)	***P. l***
20°C	1283 (±1819)	18026 (±17090)	254 (±263)	2343 (±2239)	***P. l***
10°C	1354 (±2141)	698 (±838)	5869 (±8766)	6140 (±8883)	***A. a***
20°C	44 (±76)	5 (±6)	1 (±2)	4 (±7)	***A. a***
	Food	No food	Food	No food	

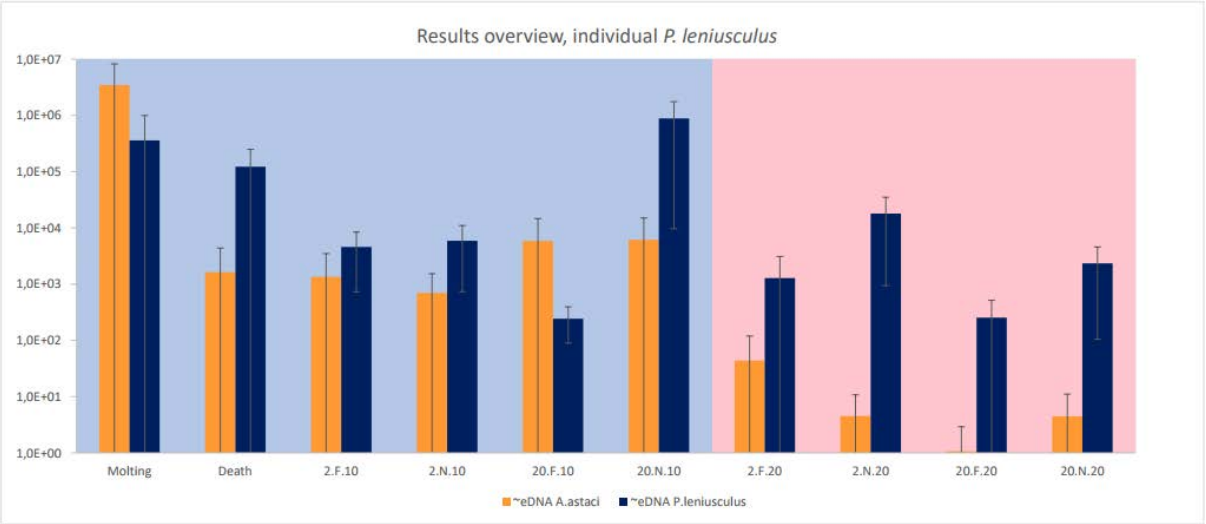

Figure 7. Graphical presentation of the overall results. All experiments and treatments are represented on the x-axis. The pink bars indicate a temperature of ~20 °C, and the blue bars indicate a temperature of ~10 °C. The values are shown in log10. The number of detectable eDNA copies per individual, both of *P. leniusculus* and *A. astaci*, increase during moulting. For *A. astaci*, the abundance is markedly lower at ~20 °C. The highest numbers of detectable eDNA copies per individual was observed in the 10 °C experiments.

2.F.10 Tank with 2 *P. leniusculus*, food and 10 °C water temperature

2.N.10 Tank with 2 *P. leniusculus*, no food and 10 °C water temperature

20.F.10 Tank with 2 *P. leniusculus*, with food and 10 °C water temperature

20.N.10 Tank with 2 *P. leniusculus*, no food and 10 °C water temperature

2.F.20 Tank with 2 *P. leniusculus*, food and 20 °C water temperature

2.N.20 Tank with 2 *P. leniusculus*, no food and 20 °C water temperature

20.F.20 Tank with 2 *P. leniusculus*, with food and 20 °C water temperature

20.N.20 Tank with 2 *P. leniusculus*, no food and 20 °C water temperature

3.2 *Pacifastacus leniusculus* eDNA abundance

In the main experiment, Experiment: 1, the effects of temperature, density and food availability on the amount of detectable *Pacifastacus leniusculus* eDNA copies per individual were tested. For *P. leniusculus* eDNA results, 93% of the filter samples yielded eDNA copy numbers above LOQ (20 eDNA copies/L) (fig. S.1). The data were tested with linear mixed model effects (LME), and some significant results were found (see below).

3.2.1 Effects of temperature, density, and food availability

Several models were tested to find the minimal adequate model that could represent the effects of temperature, density, and food availability on the amount of *P. leniusculus* eDNA. A model including only the additive effects of temperature and food was shown adequately represent the data, after excluding multiplicative (interactions) effects and density as explanatory variables with anova-tests (likelihood ratio tests)(text S.4). An outlier was identified, but not removed. The model proved to be robust and not affected by including the outlier. However, the p-value changed from significant (p=0.0430) to not-significant (p=0.0522) when the outlier was removed. It is important to note that both p-values lies close to 0.05, so that none of them are truly robust. The outlier was included because variation is normal in eDNA studies (see discussion). For plots on residuals and normal distribution see fig. S.3. The standard deviation (1.35^2) was larger than the residual variance (variance between tanks, 0.18^2), which shows the large contribution of between-filter variation (pseudoreplication) to the total unexplained variance (text. S.5).

The results show that increasing the temperature from 10 °C to 20 °C made the number of detectable *P. leniusculus* eDNA copies per individual decrease with a ~13-fold ($10^{-1.13}$) = 0.07 = 1/13) (p=0.0523) (fig. 8). Note that this effect had a p-value larger than 0.05 which means that it is estimated with high uncertainty. Removing the food source increased the number of detectable *P. leniusculus* eDNA copies per individual with a ~15-fold ($10^{1.19}$ = 15.53) (p=0.0430) (fig. 8). Density was not found to have any effect on the number of detectable eDNA copies per individual.

When considering only the average values (tab. 3), some trends can be found. When the number of *P. leniusculus* increased from low to high density (2 versus 20 individuals) in the non-fed group, the detectable number of eDNA copies per individual increased drastically with 877508 copies per individual from 5885 to 883393. Thus, there is seemingly an extra non-significant effect of increased density, yielding a 150-fold increase in the average of eDNA per *P. leniusculus* individual from the low-density group. The averages also underline the effect of food, with decreasing numbers when adding food in all groups. Details on the statistics can be found in text S.5.

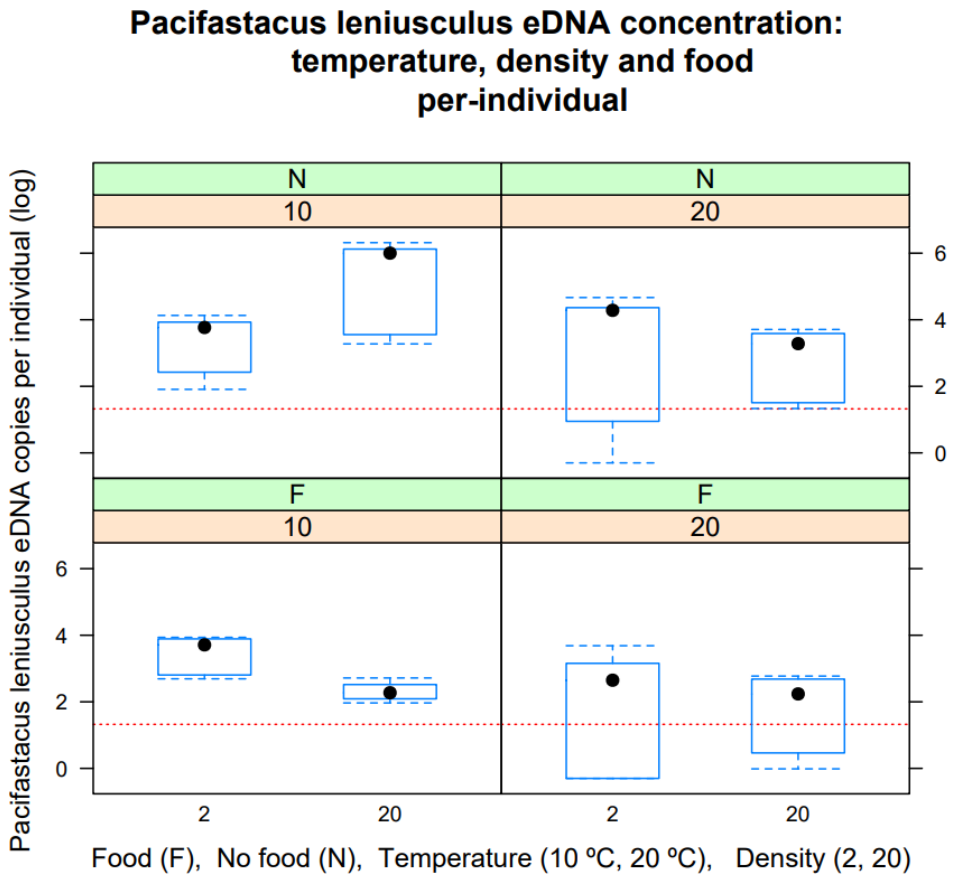

Figure 8. *Pacifastacus leniusculus* eDNA concentrations per individual water under different treatments. The stapled red line represents the LOQ (20). eDNA copies per individual increased visually with density only in the non-fed group at 10 °C. Feeding impact negatively on the number of eDNA copies per individual, in particular at high temperature and high density. High temperature decreases the number of eDNA copies per individual, but not significantly.

3.2.2 Effect of moulting on *P. leniusculus* eDNA abundance

A comparison of *P. leniusculus* eDNA copy numbers emitted from moulting versus non-moulting *P. leniusculus* was made by calculating the average number of detectable eDNA copies per individual for moulting and "normal state" *P. leniusculus*. To obtain the most comparable results, the average eDNA copy number per "normal state" individual was calculated from the experimental tanks with two non-fed *P. leniusculus* individuals kept at 10 °C (tab. 3). There were too few replicates in this experiment to perform any statistical tests. Each moulting event is based on one individual crayfish per tank. For the few moulting events observed and subsequently measured in terms of eDNA, there is a clear trend that

moulting increases the amount of detectable *P. leniusculus* eDNA copies per individual compared to non-moulting *P. leniusculus* (fig. 10). An average of 314464 eDNA copies per individual are detected during moulting (N = 5), compared to 5885 eDNA copies per individual in a "normal state" *P. leniusculus*. Most eDNA copies per individual are found when the *P. leniusculus* is kept in the tank together with its exoskeleton (N = 3), on average 535299 eDNA copies per individual compared to an average of 93630 eDNA copies per individual when the exoskeleton is absent (N = 2) (fig. 9).

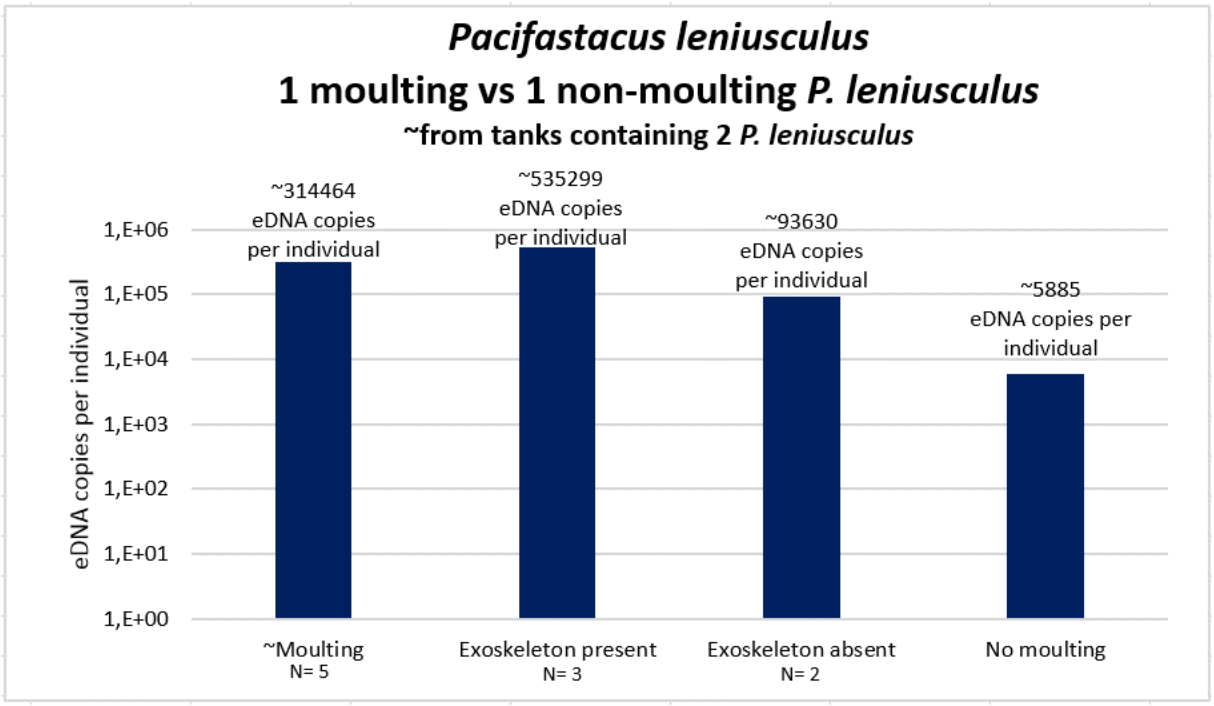

Figure 9. Moulting individuals of *Pacifastacus leniusculus* yield a higher amount of eDNA than non-moulting individuals. When the exoskeleton is kept in the tank together with the *P. leniusculus*, the highest number of eDNA copies per individual are detected. The number of eDNA copies per individual are averages from tanks containing 2 *P. leniusculus* from Experiment 1 and 2, and are not tested statistically due to the low replicate number.

3.2.3 Effect of death on *P. leniusculus* eDNA abundance

A comparison of the number of *P. leniusculus* eDNA copy numbers from dead versus live *P. leniusculus* was made by calculating the average number of eDNA copies per individual for one dead and one living *P. leniusculus.* To obtain the most comparable results, the average eDNA copy number for the living *P. leniusculus* were calculated from experimental tanks where two non-fed individuals were kept at 10 °C, and from experimental tanks where 20

non-fed individuals were kept at 10 °C (Experiment 1, tab. 3). The average number of eDNA copies per individual for dead *P. leniusculus* were calculated from the average values of the two experimental tanks where 2 dead *P. leniusculus* were kept, and another average from the one tank where 20 dead *P. leniusculus* were kept. The eDNA copy numbers were compared according to density, and calculated to consider each individual *P. leniusculus*. This experiment did not have enough replicates to be tested statistically.

In the two tanks containing 2 dead *P. leniusculus* individuals, the average *P. leniusculus* eDNA concentration was much higher than in the tank containing 2 living *P. leniusculus* (fig. 10). An average of 182912 eDNA copies per dead *P. leniusculus* was detected, in contrast to 5885 to eDNA copies per individual on average for one living *P. leniusculus* (tab. 3). The comparison between experimental tanks where 20 dead *P. leniusculus* and 20 living *P. leniusculus,* showed in contrast to the results from the previous comparison, that 20 living *P. leniusculus* visibly has a higher average number of detectable eDNA copies per individual than the dead (fig. 10). However, the extreme biofouling of the water in the tank with 20 dead crayfish probably impacted strongly on the observed low eDNA copy numbers.

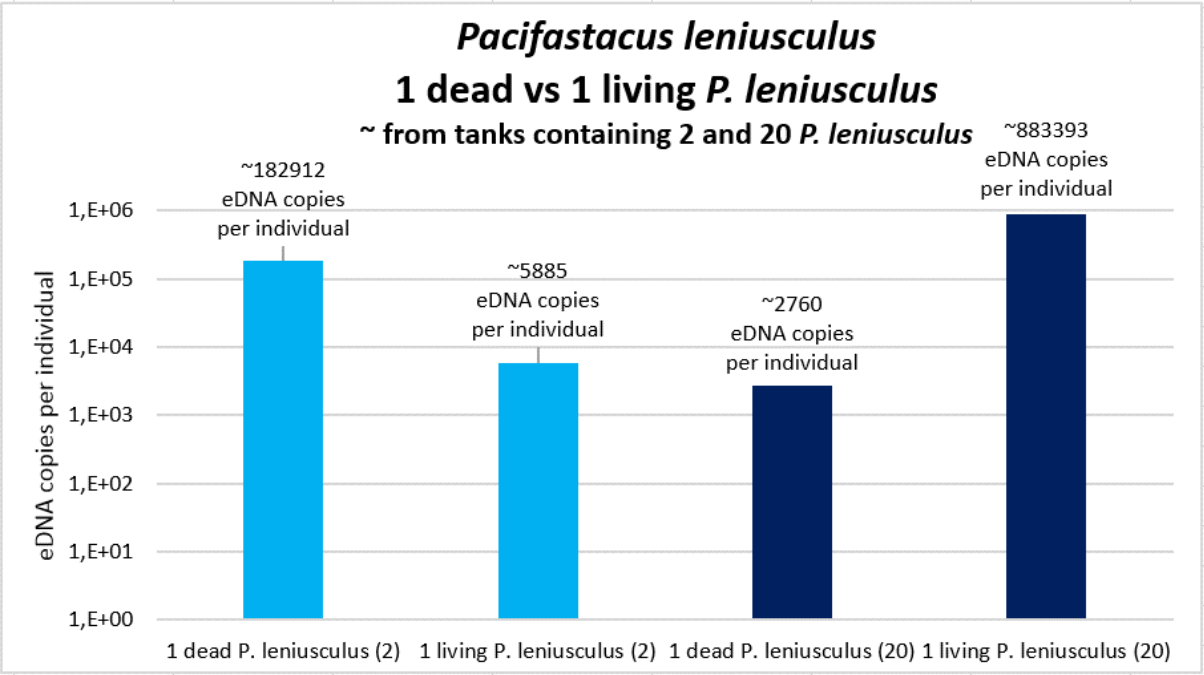

Figure 10. Mean *P. leniusculus* eDNA copy number per individual in water with dead compared to live crayfish. The number of eDNA copies per individual are averages from tanks containing 2 and 2 *P. leniusculus* respectively from Experiment 1 (2 P. leniusculus, 10 °C, no food) and 3, and are not tested statistically. The values are calculated to represent the mean emission of eDNA per-individual in tanks with low (2) and high (20) density. At low density, dead individuals give rice to a higher amount of eDNA compared to living individuals. At high density, the opposite is observed: a lower amount of eDNA per individual was detected in the water of 20 dead individuals compared to tanks were 20 living individuals were kept together.

3.3 *Aphanomyces astaci* eDNA abundance

In Experiment 1, the effects of temperature, density, and food availability on the number of *Aphanomyces astaci* eDNA copy number per individual were tested. For the *A. astaci* results, 68% of the filter samples yielded values above LOQ (100 eDNA copies/L) (fig. S.2).

3.3.1 Effects of temperature, food availability and temperature

After testing with lme and anova, it was found that temperature was the only factor that significantly affected the number of detectable *A. astaci* eDNA copies/L (text S.5). The standard deviation (0.67^2) was larger than the residual variance (0.59^2), indicating a major contribution from between-filter variation to the total unexplained variance (see Figure S.4 for plots on residuals and normal distribution).

The results show that increasing the temperature from 10 °C to 20 °C, made the number of detectable *A. astaci* eDNA copies per individual significantly decrease with a ~280-fold ($10^{-2.44}$ = 0.00356 = 1/280) (p=0) (fig. 11). Feeding and density did not have any significant effects on the number of detectable *A. astaci* eDNA copies per individual (fig. 11). Density showed a non-significant effect on *A. astaci* eDNA concentration, with an increase of 3.6~fold ($10^{0.56}$=3.64) (p=0.085). Note that this effect had a p-value larger than 0.05 which means that it is estimated with high uncertainty.

If looking at the average values in isolation, the conclusion from the statistics is underlined (tab. 3). At 20 °C, the lowest detected eDNA copy number per individual for all groups was below LOD (set to 1), and the highest 44. This contrasts with the range from 698-6140 eDNA copies per individual for the 10 °C groups (tab. 3). Even though not significant, it is rather clear from the average values that the number of detectable *A. astaci* eDNA copies per individual increases with a 6-fold when *P. leniusculus* density increases from 2 to 20 individuals per-tank for the fed and non-fed groups at 10 °C (tab. 3). Here, 1026 *A. astaci* eDNA copies per individual were produced at low *P. leniusculus* density, compared to 6006 eDNA copies per individual in the high-density groups. The lack of food-effect is also underlined by the average values (tab. 3).

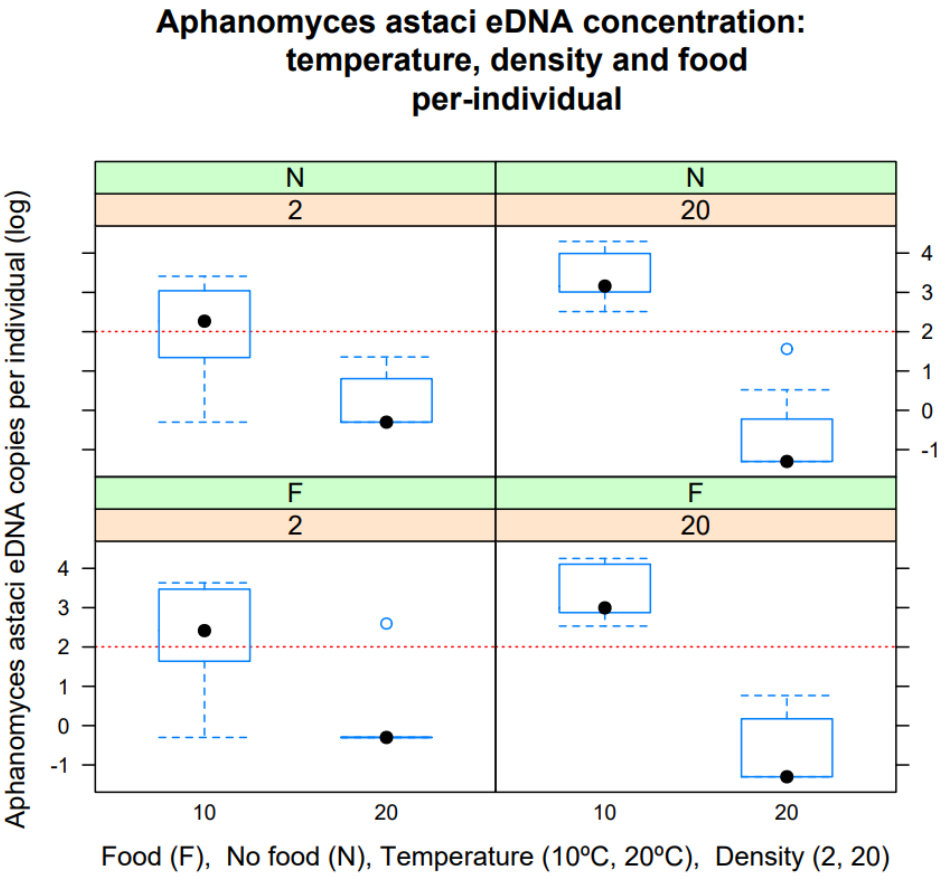

Figure 11. *Aphanomyces astaci* eDNA copies per individual. When the temperature is 20 °C, almost no *A. astaci* eDNA were detected. Feeding and density has no significant impact on the number of *A. astaci* eDNA copies per individual.

3.3.2 Effect of moulting on *A. astaci* eDNA abundance

The average *A. astaci* eDNA copy number per individual for moulting and non-moulting ("normal state") *P. leniusculus* were calculated to make a comparison between the groups. To obtain the most comparable results, the average eDNA copy number per non-moulting individual was calculated from Experiment 1 and experimental tanks with two fed/non-fed *P. leniusculus* at 10 °C (tab. 3). Each moulting event is based on one individual per tank. The number of replicates from this experiment is too few to perform any statistical tests.

The average values show a clear trend that moulting greatly increases the number of detectable *A. astaci* eDNA copies per individual compared to non-moulting *P. leniusculus* (fig. 12). On average, in a tank where moulting *P. leniusculus* are kept, 2915748 *A. astaci* eDNA copies per individual are detected, in contrast to 1026 in tanks with non-moulting *P. leniusculus*. Most detectable *A. astaci* eDNA copies per individual are found when the

moulting *P. leniusculus* is kept in the tank together with its exoskeleton, 5737170 versus 94327 respectively (fig. 12).

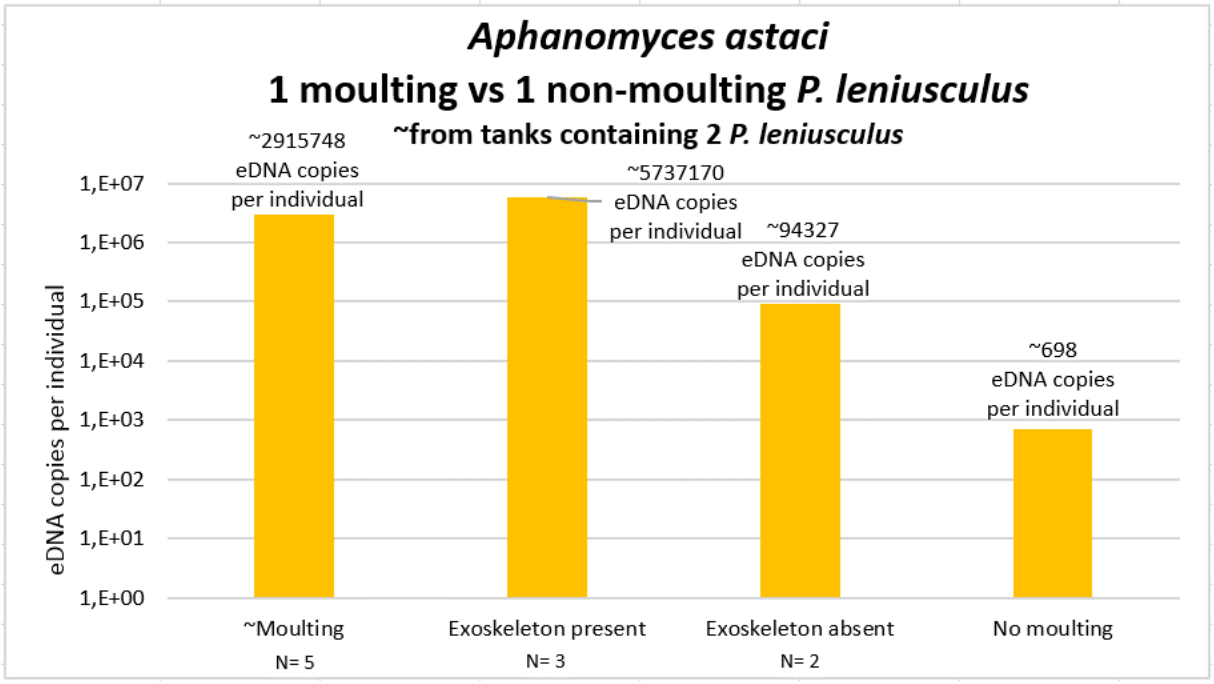

Figure 12. Moulting individuals of *Pacifastacus leniusculus* yields a higher amount of *Aphanomyces astaci* eDNA than non-moulting individuals. When the exoskeleton is kept in the tank together with the *P. leniusculus*, the highest number of eDNA copies per individual are detected. The number of eDNA copies per individual are averages from tanks containing 2 *P. leniusculus* from Experiment 1 (2 *P. leniusculus*, 10 °C, no food) and 3, and are not tested statistically.

3.3.3 Effect of death on *A. astaci* eDNA abundance

A comparison of the number of *A. astaci* eDNA copy numbers from dead versus live *P. leniusculus* was made by calculating the average number of eDNA copies per individual for one dead and one living *P. leniusculus.* To obtain the most comparable results, the average eDNA copy number for the living *P. leniusculus* were calculated from experimental tanks where two non-fed individuals were kept at 10 °C, and from experimental tanks where 20 non-fed individuals were kept at 10 °C (Experiment 1, tab. 3). The average number of detectable *A. astaci* eDNA copies per individual for dead *P. leniusculus* were calculated from the average of the two experimental tanks where two dead *P. leniusculus* were kept, and another average from the one tank where 20 dead *P. leniusculus* were kept. The eDNA copy

numbers were compared according to density, and calculated to consider each individual *P. leniusculus*. This experiment did not have enough replicates to be tested statistically.

In the two tanks were two dead *P. leniusculus* were kept, the average number of *A. astaci* eDNA copies per individual were 2476. This is more than double the amount of the average in tanks with 2 living *P. leniusculus*, which were 1026 eDNA copies per individual (fig. 14). In the tank where 20 dead individuals of *P. leniusculus* were kept together, almost no *A. astaci* eDNA was detected in contrast to tanks were 20 living individuals were kept together (fig. 13). In the tank with 20 dead individuals were kept, are the results strongly confounded by poor water quality. The average number of detectable *A. astaci* eDNA copies per individual in the tanks with living *P. leniusculus* was 6004.

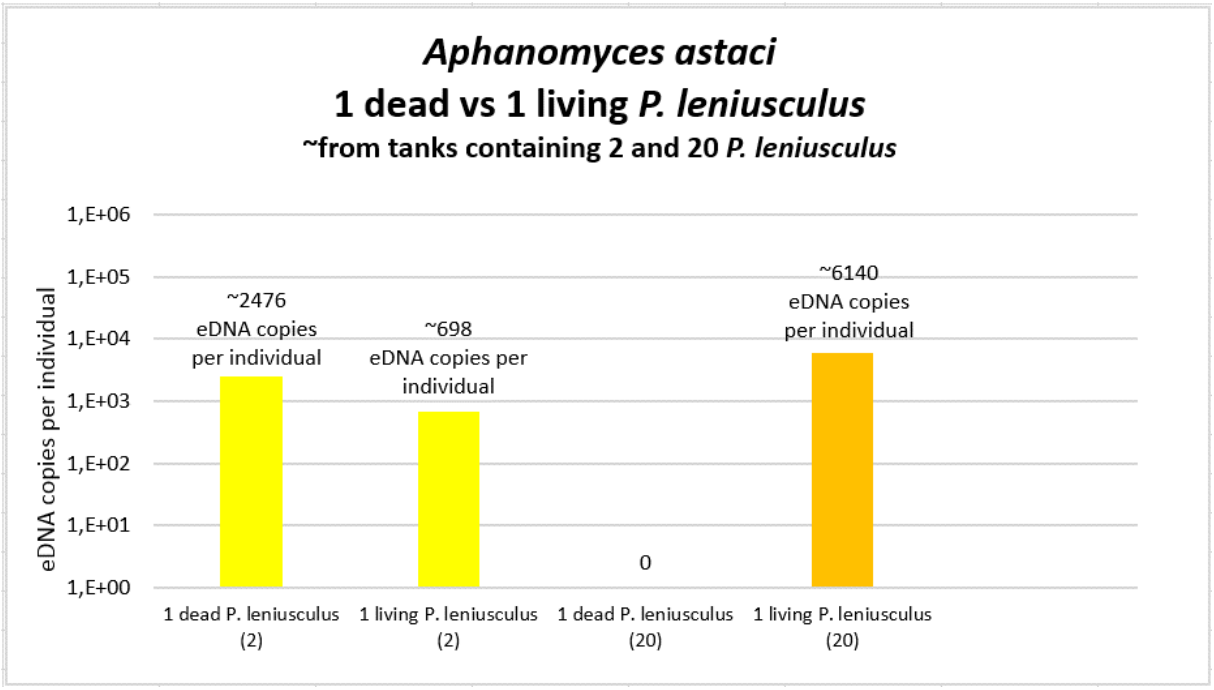

Figure 13. Mean *A. astaci* eDNA copy number per individual in water with dead compared to live crayfish. The number of eDNA copies per individual are averages from tanks containing 2 and 2 *P. leniusculus* respectively from Experiment 1 (2 P. leniusculus, 10 °C, no food) and 3, and are not tested statistically. The values are calculated to represent the mean emission of eDNA per-individual in tanks with low (2) and high (20) density. At low density, dead individuals give rice to a higher amount of eDNA compared to living individuals. At high density, the opposite is observed: a lower amount of eDNA per individual was detected in the water of 20 dead individuals compared to tanks were 20 living individuals were kept together.

3.3.4 eDNA ratios of *P. leniusculus* and *A. astaci*

With few exceptions, the eDNA values produced by individual *P. leniusculus* per L water are higher for *P. leniusculus* eDNA compared to *A. astaci* eDNA. This is most prominent for 20 °C where *A. astaci* largely disappeared from the water. However, also for the 10°C, the values of detectable *P. leniusculus* eDNA copies per individual are from 3 to nearly 3000 times higher than for *A. astaci* (tab. 4). The exceptions are the trials with high density and food (Experiment 1), where there is nearly 25 times more eDNA from *A. astaci* than from *P. leniusculus*, and for moulting (Experiment 2) where there is ~9 times more eDNA from *A. astaci* than from *P. leniusculus*.

Table 4. Mean eDNA ratios of *P. leniusculus*/*A. astaci* (mean eDNA values of *P. leniusculus* per crayfish individual divided with mean eDNA values of *A. astaci* per crayfish individual) from all experiments

Treatment/Temperature	**10°C**	**20°C**
Low density, Food	3.4	29.2
Low density, No food	8.4	3605.2
High density, Food	0.04	254
High density, No food	143.9	468.6
Low density, Moulting	0.11	
Low density, Death	73.9	
High density, Death	2760	

3.4 *Pacifastacus leniusculus* measures and *Aphanomyces astaci* infection level

The size of the *P. leniusculus* ranged from 72 mm to 150 mm with a mean of 104,9 mm. The 84 *P. leniusculus* involved had a sex ratio of 43:41 male: female. Of the total number of *P. leniusculus* that were a part of the study, 45 were tested for *A. astaci* infection. Of these were 10 negative and 35 positive, yielding an *A. astaci* prevalence of 78% in the experimental pool (tab. 5). Almost all the experimental tanks included some of the confirmed infected *P. leniusculus*. (tab. S.5). The agent levels in the examined tissue ranged from 12 to 33100 PFU (PCR forming units, equivalent to DNA copies) (tab. S.6)

Table 5. *Aphanomyces astaci* agent levels in analysed individuals of *Pacifastacus leniusculus* that participated in multiple experimental tanks. The agent level categories are based on number of observed PCR-forming units from *A. astaci* specific quantitative real-time PCR. The experiments included are: Experiment 1: temperature, density and feeding, and Experiment 3: Death. Experiment 2: Moulting, is not included.

Exp.	Tank	Treatment	# *P. leniusculus* in tank	#*P. leniusculus* analysed	# Positive *P. leniusculus* analysed	% Positive *P. leniusculus*	A_0	A_1	A_2	A_3	A_4	A_5
1	B5	20 °C, N.F	2	1/2	1/1	100%	0	0	0	1	0	0
1	B12	20 °C, F.	2	1/2	0	0	0	0	0	0	0	0
1	B13	20 °C, F.	20	11/20	9/11	82%	2	0	4	5	0	0
1	C13	20 °C, N.F.	20	13/20	11/13	85%	1	1	4	6	0	0
1	B5	20 °C, N.F.	20	11/20	9/11	82%	2	0	2	6	0	1
1	B12	20 °C, F.	20	11/20	9/11	82%	2	0	5	4	0	0
1	B13	20 °C, F.	2	2/2	2/2	100%	0	0	1	1	0	0
1	C13	20 °C, N.F.	2	2/2	1/2	50%	0	1	0	1	0	0
1	B5	20 °C, F.	2	2/2	1/2	50%	0	0	0	1	0	0
1	B12	20 °C, N.F.	20	10/20	8/10	80%	2	0	4	2	1	1
1	B13	20 °C, N.F.	2	1/2	1/1	100%	0	0	1	0	0	0
1	C13	20 °C, F.	20	12/20	10/12	83%	2	0	2	8	0	0
1	B5	10 °C, N.F.	20	12/20	10/12	83%	2	0	2	6	1	1
1	B12	10 °C, F.	20	13/20	10/13	77%	3	0	5	5	0	0
1	B13	10 °C, F.	2	1/2	1/1	100%	0	0	1	0	0	0
1	C13	10 °C, N.F	2	1/2	1/1	100%	0	0	0	1	0	0
1	B5	10 °C, F.	20	11/20	7/11	64%	4	0	4	3	0	0
1	B12	10 °C, N.F.	2	0/2	-	-	-	-	-	-	-	-
1	B13	10 °C, F.	2	2/2	1/2	50%	1	0	0	1	0	0
1	C13	10 °C, N.F.	20	6/20	6/6	100%	0	0	1	5	0	0
1	B5	10 °C, N.F.	2	2/2	1/2	50%	0	0	1	0	0	0
1	B12	10 °C, N.F	20	8/20	7/8	88%	1	0	3	4	0	0
1	B13	10 °C, F.	2	1/2	0	0	0	0	0	0	0	0
1	C13	10 °C, F.	20	8/20	6/8	75%	2	0	1	5	0	0
3	B13	10 °C	20	20/20	14/20	70%	6	0	4	10	0	0
3	B12	10 °C	2	2/2	1/2	50%	0	0	0	1	0	0
3	C13	10 °C	2	2/2	2/2	100%	0	0	1	1	0	0
			# *P. leniusculus*	# *P. leniusculus* analysed	# *P. leniusculus* positives	Total # prevalence	# analysed *P. leniusculus* agent levels					
		TOTAL	84	45	35	78%	9	1	20	12	1	2

Agent level 0 (A_0) = negative; A_1 = below level of detection ($PFU_{obs}<5$); $A_2 = 5 \leq PFU_{obs} \leq 50$; $A_3 = 50 \leq PFU_{obs} \leq 10^3$; $A_4 = 10^3 \leq PFU_{obs} \leq 10^4$; $A_5 = 10^4 \leq PFU_{obs} \leq 10^5$. Here, A_1 is also interpreted as a negative result (according to Vrålstad et al, 2009). N.F = No food, F = Food.

From the total of the 85 involved *P. leniusculus* individuals, 45 (57%) were analysed. Note, the individual *P. leniusculus* were involved in multiple time-replicates of experiment 1, as well as in experiment 2 and 3. Thus the situation per tank cannot be summed up as the total. Last column of the table sums up the actual numbers individuals used and analysed. In total 78 % of the analysed individuals were positive for *A. astaci* infection.

3.5 Summary of qPCR results from control samples

Four levels of controls were included in the experiment, as described in Materials and Methods. That includes filter controls where inlet water was filtered (filter controls), environmental control during DNA extraction, extraction blank control, and PCR blank control.

Of the total 62 *Pacifastacus leniusculus* control samples, 44 were negative, 3 were below LOQ, and 15 were unfortunately positive (fig. S.5). The contamination problem was seen for filter controls in experiment 1 and 2, but not for experiment 3. Minor contamination problems were also seen for an extraction blank control for the same experiments, here 1 sample was positive (<100 copies). All laboratory environmental controls and PCR blank controls remained negative (fig. S.5).

The same number of controls were taken for *Aphanomyces astaci,* and of the 62 control samples in total, 53 were negative, 7 were below LOQ and 2 were positive (fig. S.6). The positives were only seen in experiment 1. Traces of *A. astaci* DNA detected below LOQ were seen in one extraction blank control sample and one laboratory environmental control sample. All PCR blank controls remained negative (fig. S.6). For the *P. leniusculus* tissue samples, all controls remained negative (fig. S.7). More details on the controls are listed in Table S.7.

4 Discussion

In this thesis, a major aim was to investigate if eDNA emitted from *P. leniusculus* correlates with population density/number of individuals, and also examine the impact of various factors thought to influence *P. leniusculus* and *A. astaci* eDNA concentrations in the water. In general, the variance was very high and the results do not support that there are any constant numbers of *P. leniusculus* and *A. astaci* eDNA copies emitted per individual. Even though it was not found significant results on *P. leniusculus* density affecting the number of detectable *P. leniusculus* and *A. astaci* eDNA copies per individual, this is more likely due to the high variance. If eDNA from *P. leniusculus* was emitted at a rather constant rate per individual, it could be expected a 10-fold increase in eDNA copy number in tanks with 10 times more crayfish. This situation was never observed. On the contrary, increased density was observed sometimes to lower the average eDNA copy number per crayfish, in particular in tanks where food was available, or drastically increase the average copy number per crayfish such as in the 10 °C tanks without available food. Thus, the hypothesis that eDNA emitted from *P. leniusculus* correlates with population density/number of individuals was not supported. This is in concordance with Dunn et al. (2017), who suggested that eDNA from *P. leniusculus* is not released at a constant rate, because it was not found any relationship between biomass and eDNA concentration.

The rather huge variation in eDNA copies per individual and different environmental conditions in the experimental tanks further confounds a clear quantitative relationship between eDNA concentration and *P. leniusculus* population density. The temperature did not significantly affect *P. leniusculus* eDNA detectability, in contrast to *A. astaci* that had a strong drop in eDNA concentration at 20°C, which is more prominent than previously reported, and surprising taken into consideration that the described optimum temperature range of this genotype is from 16-20°C (Strand et al., 2012). On the other hand, the presence of food did not affect *A. astaci* eDNA concentrations, but lead to a significant decrease in the number of detectable *P. leniusculus* eDNA copies per individual that was most prominent at high temperatures. In the planning of the thesis, food was believed to increase the eDNA concentrations with increased activity level and feces production. However, the murky water resulting from the feeding regime most likely inflicted on the results and could have led to a

higher degradation of eDNA that was not protected in living cells – such as the *A. astaci* zoospores. The effect of moulting and death was not tested statistically, but it was during moulting observed a strong increase in eDNA concentrations of both target organisms, especially in aquaria where the *P. leniusculus* exoskeleton was present. An important result of this thesis is the observation that the *P. leniusculus*/*A. astaci* eDNA ratio is nearly always in favor of *P. leniusculus,* implying that the crayfish emit considerably more "self" eDNA than from its parasite *A. astaci*. The clear exception is during moulting, when there is on average ~9 times more eDNA from *A. astaci* than from *P. leniusculus*. Consequently, if *P. leniusculus* is present in a waterbody, it can be expected that eDNA monitoring of crayfish eDNA rather than parasite eDNA is better tool for monitoring and early detection of the alien species with exception for moulting periods, where *A. astaci* detection might be even more powerful.

It was not found significant evidence for that the measurable amount of *P. leniusculus* and *A. astaci* eDNA is affected by density of the *P. leniusculus* individuals, since increased *P. leniusculus* density was not shown to significantly increase neither *P. leniusculus* nor *A. astaci* eDNA concentrations per-individual. However, it was observed a strong trend where *P. leniusculus* eDNA concentration increased when the number of *P. leniusculus* increased from two to twenty in the non-fed groups at the lowest temperature. This could be due to density dependent factors, such as increased aggressive behaviour and stress (Hudina et al., 2015). In combination, the absence of food and the high density could probably contribute to increased fighting behaviour. Even though shelters were present for all crayfish, it was observed a prominent fighting behaviour during the trial replicate that yielded the highest *P. leniusculus* eDNA concentrations. Here, a larger portion of *P. leniusculus* limb losses and cannibalism were observed compared to any of the other trials, which most likely contributed to the elevated eDNA concentrations. Fighting also occurred in other tanks, but on a smaller scale. *Pacifastacus leniusculus* that died during the experiments were removed and replaced by a new individual from the communal housing tank. However, *P. leniusculus* that had lost limbs after fights were not removed, and neither their limbs. This could skew results to higher levels. Cai et al. (2017) removed crayfish that were visibly injured from fighting and replaced them as soon as detected to avoid excessive release of eDNA that could skew the results. This was not done in this thesis, as *P. leniusculus* are considered

aggressive (Hudina et al., 2015), and fighting including limb losses (that regenerates) (Durand, 1960) is a natural part of their behavior. By removing the injured *P. leniusculus* and limbs, the results would reflect natural conditions to a lesser degree. Dunn et al (2017) conducted a study where one of the experimental groups had their claws taped to prevent fighting. They did not find evidence for that fighting increased the *P. leniusculus* eDNA concentrations, however they did not either observe enough fighting events to conclude. This thesis suggests that fighting indeed can lead to elevated *P. leniusculus* eDNA concentrations, which likely results from increased levels of tissue- and haemolymph cells in the water after limb losses.

Some quantification studies have been conducted on freshwater invertebrates. Agersnap et al. (2017) observed a trend where more densely populated waters contained more eDNA than the less populated waters, but this must be studied further and with traditional abundance estimating methods as controls. This was also the conclusion from Cai et al. (2017), Svoboda et al. (2016) and Larson et al. (2017). It is logic that more densely populated waters contains more eDNA. However, there are a lot of factors that influence crayfish eDNA concentration (discussed below), and this study demonstrate that even under fairly controlled aquaria experiments it was not found a simple correlation between number of individuals and eDNA copy number.

Ikeda et al. (2016) used eDNA to successfully detect the endangered crayfish *Cambaroides japonicus* in streams. They compared the results with hand capturing methods and confirmed that the burrowing crayfish can be detected with 1 L water samples filtered through a 0,7 µm glass fiber filter, followed by DNA extraction and qPCR. The burrowing and hiding activity (Guan, 1994), of crayfish may affect their eDNA release to the water masses. Even though it is confirmed that burrowing species can be detected (Ikeda et al., 2016), it is important to consider the risk of false negatives, especially in large lakes. When crayfish stay in caves or under rocks, can DNA holding components be trapped in muddy sediments. Sediments tend to bind DNA-fragments and conserve them, thus making them unavailable to the water masses for sampling (Turner et al., 2015). Especially in the winter, when crayfish are more sedentary (Bubb et al., 2002), the eDNA concentration would be expected to be lower compared to other seasons. For *A. astaci*, it was shown that the eDNA could be detected all year round, but in smaller amounts during winter (Wittwer et al., 2017).

Compared to *P. leniusculus* eDNA, the *A. astaci* spores are alive and active swimmers, and are therefore not as prone to be trapped in sediments. In this thesis there was not any sediment present in the aquaria. The eDNA that were transmitted in various form from the crayfish would in our case most likely be suspended in the water column, and/or on the bottom of the aquaria, except from *A. astaci* spores. Here it would be exposed to swirling by small currents created by the oxygen stone and crayfish movements.

Cai et al. (2017) detected down to as small amounts of crayfish as one per paddy (artificial rice pond). They also detected crayfish inn all ponds containing crayfish, whereas bottle traps only had a detection rate of 68%. In their study, the ponds were quite small (range 7-500 m^2) compared to e.g. Lake Rødenessjøen at ~15 km^2 (Skulberg and Kotai, 1982) in size, and with possibly fewer ecological factors affecting eDNA persistence. Aquaria experiments cannot be compared with the rice paddy study, as eDNA in an aquarium would be more concentrated and less influenced by environmental factors. However, the more research on this, the more transferable will aquaria experiments be to field conditions.

Tréguier et al. (2014) addressed the advantages and technical limits in detecting invasive *Procambrus clarkii* in freshwater ponds. They found that eDNA methods perform better in detecting crayfish when the abundance is large, and trapping is difficult due to habitat, then when ponds are large, and abundance is low. Large abundance of crayfish would increase the eDNA concentration due to the number of crayfish releasing DNA, and the density factor slightly suggested in this thesis.

In this thesis, it was found that one *P. leniusculus* on average produces 5885 eDNA copies/L in 1 L water (low density, low temperature and no food), this equals a total of 588500 eDNA copies in 100 L (which was the volume of water in the aquaria) if we presume equal eDNA copy distribution. For simplicity, if we use the concentration 5000 copies/L at 100 L, and calculate the dilution effect in a larger system, this would correspond to 500 copies/L at 1 m3 water volume and 50 copies/L at 10 m3 water volume. With a detection limit of 5 eDNA copies per qPCR reaction, a totalt of 200 eDNA copies needs to be captured on the glass fiber filter and extracted during DNA extraction in order to get a positive detection using the described methodology. Thus, it is necessary to filter 4 lites of water in order to detect one crayfish individual per 10 m3 volume of water. This is only valid if there is an absence of inhibition in the qPCR, degredation of eDNA in the water and it is most likely that sediments,

organic matter and clay particles bind up a large portion of the emitted eDNA copies, as discussed above (Turner et al., 2015). For large ponds with low abundance, it would be more favourable to filter larger volumes of water (e.g. 5 L per sample; Strand et al 2014, Agersnap et al 2017).

The prevalence of *A. astaci* infected *P. leniusculus* vary from 0 to 100 % in Europe (James et al., 2017; Kozubikova et al., 2011; Vrålstad et al., 2011), and was in this thesis determined to be around 78% for the experimental crayfish inferred from uropod samples. Thus, experimental tanks with twenty *P. leniusculus* were more likely to house predominantly *A. astaci* infected *P. leniusculus* individuals than tanks with only two *P. leniusculus* where the presence of one non-infected individual would make a bit impact on the results. The detectable number of *A. astaci* eDNA copies/L is dependent on the number of spores in the water, which again is dependent on the *P. leniusculus* infection level. It could therefore be difficult to conclude whether or not density affects *A. astaci* eDNA concentrations.

A trend was observed that the measurable amount of *P. leniusculus* eDNA is affected by the water temperatures, and a temperature of 20 °C contributes to a decrease in the number of *P. leniusculus* eDNA copies/L. That was indeed the case also for *A. astaci*, where a temperature of 20 °C merely eliminated the presence of detectable *A. astaci* eDNA in the water. DNA degradation is known to increase with temperature (Dejean et al., 2011; Pilliod et al., 2014; Strickler et al., 2015; Lance et al., 2017) and the amount of eDNA present is a product of release rate from the target organism and degradation rate. The non-significant, but still rather marked decrease in *P. leniusculus* eDNA copies/L at 20 °C compared to 10°C in the fed groups, could be due to this. The observed water quality in the fed groups was markedly decreased (see fig S.8) and it was likely a rather high microbiological activity in the water that could lead to faster degradation of the *P. leniusculus* eDNA that is likely presented in terms of shed (and thus dead) cells. In contrast, this was likely not the case for the decline in *A. astaci* eDNA copy numbers. It is previously shown that *A. astaci* genotype B reduces is motile zoospore period when the temperature is above 18 °C (Diéguez-Uribeondo et al., 1995). This is in consistence with Strand et al. (2012) who found that there is a negative correlation between spore number and temperature, when the temperature increases from 17 °C to 23 °C. This agrees with the results from this thesis. However, a temperature of 20 °C was not expected to influence the *A. astaci* eDNA concentration this

much. It might be that the *P. leniusculus* immune defence works better at this temperature, and that the parasite spore production for this reason is inhibited. However, Jiravanichpaisal et al. (2004) found that *P. leniusculus* immune defence functions for the white spot syndrome virus are lowered at higher temperatures (22 °C), and better at lower temperatures. But, this is not comparable to the results in this thesis, because both the pathogen and temperatures are different. In Norway, genotype B is the only known *A. astaci* genotype that is present today (Vrålstad et al., 2014). In contrast, genotype D carried by the warm water adapted American crayfish species *Procambrus clarkii* have the ability to sporulate better than other genotypes when temperatures are above 20 °C (Diéguez-Uribeondo et al., 1995). It is surprising that 20 °C have such a marked negative impact on *A. astaci* sporulation taken into account that the described range for sporulation temperatures include 20 °C (Diéguez-Uribeondo et al., 1995) and that sporulation was still observed at 23 °C in the eDNA study of Strand et al. (2012). Perhaps the *A. astaci* genotype group B isolates present in Norway have lowered temperature optimum than those described in the literature. The results combined with previous research on the subject have implications for sampling in the field. If water temperature is about 20 °C and higher, less *A. astaci* eDNA can be expected to be detected. This does not mean that the *A. astaci/P. leniusculus* presence is decreasing, just that the sporulation activity is lower. In any case, from calculated eDNA ratios of *P. leniusculus/A. astaci*, monitoring of *P. leniusculus* eDNA in warm waters would provide the highest probability for detection success.

Pacifastacus leniusculus lower their activity level, and by that also their aggression level and metabolism when the temperature is below 7 °C (Bubb et al., 2002). The mean temperature during the low temperature replicates was 10 °C. This temperature is therefore not low enough to induce sedentary behaviour. However, Wittwer et al. (2017) found that their trap studies failed when the temperature was ~10 °C in a cold May. It is possible that some reduction in activity level is reached at 10 °C, and this could in field conditions lead to less detectability of eDNA from *P. leniusculus* (discussed later). It is clear from the results in this thesis that *A. astaci* eDNA is easier to detect when the temperature is 10 °C compared to 20 °C. Wittwer et al. (2017) also showed that *A. astaci* eDNA is detectable all year round, with a peak in October (that coincides with the mating season). They found that compared to trap studies of *P. leniusculus*, that during the winter months, *A. astaci* eDNA sampling would be

favourable over trapping. Trapping was according to this study most successful during the period of the year where the crayfish are at their most active behaviour (summer).

It was found significant evidence for that the measurable amount of *P. leniusculus* eDNA concentration is decreasing with the presence of food. *A*phanomyces *astaci* eDNA concentration is in contrast not affected by food availability for the *P. leniusculus* individuals. The results showed that feeding has a significant impact on the number of *P. leniusculus* eDNA copies/L as the number is decreasing when the *P. leniusculus* are fed compared to when they are not fed. This is most prominent when comparing the high-density groups for fed and non-fed *P. leniusculus* at low temperature. The presence of food is likely to increase microbiological activity trough increasing the excretion rate and degradation of the food itself. The presence of food has no significant impact on *A. astaci* eDNA concentration since the source of *A. astaci* DNA is the living zoospores and cysts that protect the DNA much better from microbiological degradation compared to eDNA originating from crayfish cell sheds. In addition, *Aphanomyces astaci* feeds directly off the hosts tissue, predominantly the tailfan and soft abdominal cuticle (Oidtmann et al., 2006; Vrålstad et al., 2011), and can therefore most likely draw nutrients and sporulate regardless of the hosts nutrition intake.

It was found that moulting increases the eDNA concentrations from both target organism dramatically. This coincides with other studies (Oidtmann et al., 2002; Svoboda et al., 2013), and implies that taking eDNA samples during the moulting season is favourable compared to other seasons. During the moulting incidents observed during this thesis, the *A. astaci* eDNA concentration was higher than *P. leniusculus* eDNA concentration. As discussed earlier, it was shown that the *A. astaci* eDNA concentration was very low in the 20 °C aquaria. Therefore, it is important to keep in mind the water temperature when planning to take samples in the moulting season. However, in Norway, the water temperatures are most likely rarely above 20 °C, especially at the bottom of the lakes where the crayfish lives, so this is not to be considered as an obstacle here.

Fish species has been used in eDNA studies to a larger degree than invertebrates eg. (Takahara et al., 2013; Thomsen et al., 2012)(see introduction). The advantage when

measuring eDNA from fish over crayfish eDNA comes from their large emissions of extracellular DNA from body mucus secretion (Livia et al., 2006). In this thesis, it was unfortunately not possible to create sufficient replicates for crucial life history stages, such as moulting, reproduction and death, that might affect the measurable amount of *A. astaci* and *P. leniusculus* eDNA. However, as mentioned, we did see very clear trends even though the limited number of replicates did not allow for statistical tests. Crayfish, like other invertebrates, has a solid exoskeleton that slows down the release of DNA from their bodies (Dougherty et al., 2016; Tréguier et al., 2014). Like observed in this thesis, moulting increases the amount of *P. leniusculus* and *A. astaci* eDNA concentrations. During moulting, the crayfish is vulnerable and soft bodied, and when not protected by the exoskeleton, the DNA release from body cells is probably increasing. Dunn et al. (2017) found that the presence of egg bearing females increases the crayfish eDNA concentration. Like moulting, reproductive behaviour and eggs increase the amount of slimy, non-exoskeleton covered tissue. This is likely to increase the eDNA concentrations.

Aphanomyces astaci spores are usually released from *A. astacus* in large amounts when they are diseased or dead (Makkonen et al 2013). For *P. leniusculus*, this mainly happens during moulting or death (Oidtmann et al., 2002). However, Strand et al (2012) showed that live *P. leniusculus* release a constant number of *A. astaci* spores in the absence of moulting and death, and observed a trend where there was an increase in eDNA concentration of *A. astaci* during moulting. Since *Aphanomyces astaci* infects the exoskeleton of *P. leniusculus* (Söderhäll and Cerenius, 1999; Unestam and Weiss, 1970), the parasitic *A. astaci* is likely to increase sporulation effort when the exoskeleton is shed during moulting, in order to re-infect its host (Svoboda et al., 2013). The number of *A. astaci* eDNA copies was at its highest in the tanks where both the *P. leniusculus* and its exoskeleton were found. Even though the results were not tested statistically, it is probable that the presence of exoskeleton increases spore-production, as it is where *A. astaci* is located.

The results of this thesis observed on average ~9 times more eDNA from *A. astaci* than from *P. leniusculus* during moulting, opposed to most other situations where *P. leniusculus* eDNA copies outperformed *A. astaci*. Consequently, for moulting periods, eDNA monitoring of *A. astaci* might be even more powerful tool for revealing *P. leniusculus* than *P. leniusculus* eDNA itself, provided that the population has a rather high prevalence, such as in this thesis.

From the overall results, death has no apparent impact on the number of eDNA copies/L for neither *P. leniusculus* nor *A. astaci*. However, looking more at the details there are certain trends and also most likely experimental conditions that negatively impacted on some of the results. The case of the tank containing 20 dead *P. leniusculus* where almost no *A. astaci* eDNA was detected, could be a result of excessive microbiological activity leading to rapid death/toxification and disappearance of the spores. When the *P. leniusculus* were euthanized, the stomach was in some cases damaged (as this lies in the head part of the animal where the scalpel went in). The stomach contents then contaminated the tanks, and this was especially prominent in the tank containing 20 *P. leniusculus*. It could be that the results from this tank should have been rejected, but due the low number of replicates and the fact that no statistical tests were conducted on these results, the results were included. When filtering this tank, the filter was clogged, and the filtration went slow. Regarding this, it is interesting that *P. leniusculus* eDNA is not affected in the same way, as their eDNA is more unprotected than the *A. astaci* spores. It would have been natural to think that *P. leniusculus* eDNA would have been degraded in at least the same degree as *A. astaci* eDNA. Several scenarios could explain this: 1. *P. leniusculus* eDNA was released at an enormous scale, and the amount that was left after degradation by the end of the week was only a fraction of the original. 2. The *A. astaci* eDNA concentration could have been affected by the fact that the *P. leniusculus* were placed in ice water before euthanization. Since *A. astaci* sits in the exoskeleton, ice water could have inhibited some of the spore production. However, since *A. astaci* was detected in the other tanks also containing *P. leniusculus* that were placed in ice water for sedation, the last explanation is not likely.

For *A. astacus* it was observed that the number of spores detected increased drastically 24 hours after death, with a peak 48-60 hour after death (Makkonen et al., 2013). However, this is not comparable to *P. leniusculus*, as their immune response hinders *A. astaci* to infect their entire system. In addition, the *P. leniusculus* in this thesis did not die of *A. astaci* infection, as the *A. astacus*, therefore it could be that infected *P. leniusculus* has a more moderate release of *A. astaci* eDNA after death compared to *A. astacus*. The filter samples in this experiment were taken after one week, and the sporulation peak (if this is present in *P. leniusculus*) could have already been reached. Despite this peak, the survival time for *A. astaci* spores and cysts are estimated to be at least 14 days (CEFAS, 2000; Cerenius and

Söderhall, 1985), so the detectability would not be severely affected after one week. However, a third explanation to the lack of detectable *A. astaci* eDNA in the tanks with many dead *P. leniusculus* could involve toxification or hypoxia (reduced oxygen content) due to the rapidly fouling of the water could have aborted or largely inhibited the *A. astaci* sporulation. This probably disturbed the measurements from this tank so much that it cannot be drawn any conclusions. In the study by Makkonen et al (2013) observing the eDNA content of *A. astaci* during disease and mortality of *A. astaci* infected *Astacus astacus*, the *A. astaci* eDNA increased rapidly until 48 hours post mortem, and declined then rapidly.

Environmental conditions affect the persistence of eDNA in aquatic environments (Barnes et al., 2014; Dejean et al., 2011; Lance et al., 2017) and other important factors than the ones included here needs to be taken under consideration. This include the presence of humic acids (Dougherty et al., 2016; Larson et al., 2017), water flow (Deiner and Altermatt, 2014; Jane et al., 2015), substrate, presence of other organisms, UV-degradation, and water quality parameters such as pH, for the results to be valid in a natural lake or river. Varying environmental factors could affect the results from presence and absence studies, and especially when conducting abundance studies. Cai et al. (2017) used paddy water to simulate field environment and to include the effect of possible qPCR interference from humic acids and other substances. This contrasts with the ambient water used in this thesis. The use of natural lake water demands more resources and planning, and it is important that the water originates from lakes where the target organism is not present.

Environmental DNA sampling in freshwater lakes for quantification purposes is recommended taking place from June to August. This is to take advantage in the lakes composition when temperature and circulation has stabilized after the spring turnover (Fossøy et al., 2017). This study was done on fish species, so it is not certain whether it is valid for crayfish, as their eDNA is sampled at the bottom of the lake, and not in the water masses.

DNA release and spore release are dependent on many factors. Reproduction behaviour is one factor that is important, that was not included in this thesis. Dunn et al. (2017) showed that by using egg-bearing female *P. leniusculus,* detection at low abundance was possible.

During the mating season, the aggression level in *P. leniusculus* is elevated, and more limb loss due to fighting is observed (Stebbing et al., 2003; Woodlock and Reynolds, 1988). It is reason to believe that this elevated aggression level combined with egg-bearing females would increase the eDNA concentration. There were plans of including a reproduction experiment in this thesis, but as mentioned earlier, this was concluded early because of high aggression level directed against the female *P. leniusculus* involved.

The methods used for molecular analyses are well implemented in monitoring of crayfish plague in Norway. Stochastic factors as amplification efficiency, manual pipetting amongst others can lead to variations in ΔCt. Cycle threshold values were treated to deal with inhibition, as explained under section 2.5.3.3. Values below LOQ are included in plots in the results section. Even if the values are more unreliable, a lot of information would be lost if they were omitted. For solving the issue of pseudo replication, linear mixed model effects (lme) were used in R to calculate statistics. If only the average values for the three filters were used, and no correction applied, a lot of information would be lost (residual variance). Log transformation, Log+1 were used in plots to convert PFU_L (eDNA copies/L) values to more statistically manageable values. The PFU_L values ranged from zero to several millions, but with the log+1 transformation the range was effectively downsized.

Agersnap et al., (2017) developed and tested qPCR assays for species specific detection and quantification of *A. astacus, P. leniusculus* and *Astacus leptodactylus*. For development of primers they used mitochondrial cytochrome oxidase 1(mtDNA-CO1). They successfully detected some or all of these crayfish species in Danish, Norwegian and Finnish freshwater samples originating from rivers and lakes with known populations of the mentioned crayfish. The *P. leniusculus* primers used in these experiments are because of this thoroughly tested. As for the *A. astaci* primers and probe, this has been used in several research projects, and has proven to be reliable and is thoroughly validated (Kozubikova et al., 2011; Strand et al., 2014; Tuffs and Oidtmann, 2011). The decision on making PFU_L values above Ct 41 negative is taken because detection at high Ct values (even above Ct 39) represents highly uncertain results in terms of specificity (could represent false positives) and is in any case detected at a level where the positive signal at the most represent below 5 copies (detection below LOD) (Agersnap et al., 2017; Kozubikova et al., 2011; Strand et al., 2014).

There is a large variation in the number of eDNA copies/L between tanks and filter samples from the same tank, and this corresponds with other studies on quantification of eDNA (Dunn et al., 2017; Klymus et al., 2015; Pilliod et al., 2014; Turner et al., 2014). For *P. leniusculus*, Dunn et al. (2017) suggests that the differences can originate from different excretion rates among *P. leniusculus* and dominance hierarchies over food. Further, as the target region is mitochondrial DNA, the number of mitochondria varies even between cell types. In the cases of injuries resulting from fighting, minor tissue pieces containing thousands, or millions of mitochondria could even be captured on individuals filters and thus lead to high differences between filter samples from the same tank. For *A. astaci*, it is normal that the eDNA copy numbers fluctuate between each sample, as sporulation starts with the ejection of a spore ball (aggregate of primary cysts) that give rice to individual zoospores (fig. 1). Thus, this aggregated distribution of sources to *A. astaci* eDNA will impact on the variance between filter samples from the same tank.

Filter samples were taken on an average water depth of approximately 10 cm. This was done under the assumption that sources of eDNA (commonly shed cells) floats freely in water (note that the presence of oxygen stones could help distribute the eDNA material). However, zoospores have targeted movements toward hosts (chemotaxis)(Unestam, 1969), and cysts have the ability to stick to surfaces and crayfish in the tank (Strand et al., 2012). This makes it more uncertain whether a representative amount of *A. astaci* eDNA is captured in relation to *P. leniusculus* eDNA.

The observed positive controls are most likely due to insufficient cleaning of the filtration equipment. There is a gasket in the middle of the two parts of the filter holder. This was not removed and cleaned separately during the rinsing process. This could be a contamination source. There was also used a presumably sterile bucket to retrieve the filter controls from, but this could have been contaminated. In the positive controls, the results for the associated filter samples from tanks were positive beyond the values of the filter controls. Therefore, the presence of false positives is not likely, but the results can have been skewed to a slightly higher level. It was chosen not to subtract the positive filter control values from the filter samples, as this not would seem to solve the problem correctly. In a report from NINA (Norwegian Institute for Nature Research), they recommend the use of disposable

filter holders (Fossøy et al., 2017). This could prevent positive controls, especially in the field where rinsing facilities are not as good as in the laboratory.

Droplet digital PCR (ddPCR) is a new PCR method that can detect even smaller amounts of DNA than qPCR, and is shown to be successful in several studies e.g (Doi et al., 2015a; Doi et al., 2015b; Fossøy et al., 2017). This implies that the limit of quantification can be lowered, and we will be able to quantify more of the results even when the density of the target DNA is low. In this thesis, qPCR was used, and it is reason to believe that ddPCR would have slightly increased the number of results above LOQ.

Like most studies, this thesis had benefited from more replicas and this could probably contribute to yield more significant results and make the variation lower.

5 Conclusion

When looking at the results from this thesis taken together, it is suggested that abundance estimation of *P. leniusculus* by eDNA measurements is a challenging task that depends on a lot of factors. It was not shown any correlation between *P. leniusculus* eDNA emission and number of individuals. The experiments were as mentioned only conducted in an artificial environment. It is expected that the lack of significant results is due to low number of replicates, that made the variation quite large. When measuring in field-conditions, even more factors affect eDNA emission from *P. leniusculus* and *A. astaci*, and the variation is thought to be even higher than seen in this thesis.

However, some interesting results were found. Even though temperature did not have significant effect on *P. leniusculus* eDNA concentration, more research on this area would be of interest, as the p-value were quite low (p=0.053). The fact that *A. astaci* eDNA was proven not to be affected by the presence of food, could propose that times when there is a lot of turbidity in the water, it could be favourable to use *A. astaci* eDNA for detection, because it is more resilient than *P. leniusculus* eDNA.

The *P. leniusculus/A. astaci* eDNA ratio, that was nearly always in favour *P. leniusculus*, shows that if the goal is detection of the invasive species, and not the measuring of an

outbreak of crayfish plague, *P. leniusculus* is the most reliable eDNA target. This is not valid in the moulting season, were *A. astaci* was proven to be in favour of *P. leniusculus*. When measuring in particularly vulnerable sites, or sites that are not screened for *P. leniusculus* and/or *A. astaci* earlier, it could be an extra security measure to analyse eDNA from both organisms. This is to ensure that there are no unknown influencing factors that could disguise the presence of the one of the organisms and contribute to a false negative.

The dramatic decline in *A. astaci* eDNA concentration at 20 °C suggest that it is important to consider temperature before taking measurements of *A. astaci* eDNA concentration in the field. These strong results call for more research on the *A. astaci* eDNA emission pattern, as they do not entirely coincide with previous studies.

Even though the experiment on dead crayfish got disturbed by poor water quality in the high-density tank, it was observed that dead *P. leniusculus* emitted more eDNA than the living ones. This could imply that in situations when there is e.g. a mass mortality event, more *P. leniusculus* eDNA than normal could be expected. For *A. astaci*, it is not good enough results to conclude. It also showed that death, which is a natural part of the life-cycle, is a factor that most likely contribute to the variation of *P. leniusculus* eDNA concentration in the field.

Monitoring of crayfish plague was commonly done using cage experiments, where healthy *A. astacus* were placed in cages in places where an outbreak was suspected. If the *A. astacus* died, it was further analysed to determine if crayfish plague infection was present (Vrålstad et al., 2009; Vrålstad et al., 2014; Vrålstad et al., 2017), which is problematic from an animal welfare perspective. Thus, the eDNA as a concept is more animal friendly.

Environmental DNA is a very promising monitoring method when it comes to detection, but it is important to be critical and consider all possible variables when it comes to quantification. This especially true for crayfish, which has proven difficult when it comes to linking biomass and eDNA emissions together. There is reason to believe that the use of eDNA for monitoring of endangered and invasive species is only getting started, and that we will see a lot of interesting results over the next years.

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6 Supplementary

Texts, figures and tables.

VETERINÆRINSTITUTTET OSLO
Postboks 750 Sentrum
0106 OSLO

v/ Trude Vrålstad

Deres ref:
Vår ref: 2014/220941
Dato: 01.10.2014
Org.nr: 985399077

Statens tilsyn for planter, fisk, dyr og næringsmidler

Mattilsynet

Dispensasjon fra soneforskrift i forbindelse med prøvetaking for kunnskapsutvikling under og etter krepsepestutbrudd i Rødenessjøen

Mattilsynet viser til deres brev av 1.10.2014 med søknad om dispensasjon fra soneforskrift i forbindelse med prøvetaking for kunnskapsutvikling under og etter krepsepestutbrudd i Rødenessjøen.

Søknaden er utarbeidet av Veterinærinstituttet v/Trude Vrålstad, forsker og seksjonsleder mykologi ved, på vegne av Veterinærinstituttet, NIVA, NINA og Utmarksavdelingen for Akershus og Østfold.

Representanter for organisasjonene i tillegg til Trude Vrålstad ved Veterinærinstituttet er:

- David Strand, forsker, Norsk institutt for vannforskning (NIVA)
- Øystein Toverud, utmarkssjef ved Utmarksavdelingen for Akershus og Østfold
- Stein Johnsen, forsker, Norsk institutt for naturforskning, NINA

Saken gjelder

- Forskningsaktiviteter ferskvannskreps

Mattilsynet, regionkontoret fatter med dette følgende vedtak:

Vedtak om dispensasjon fra forskrift

Veterinærinstituttet v/Trude Vrålstad, NINA v/ Stein Johnsen, NIVA v/David Strand og Utmarksavdelingen for Akershus og Østfold v/Øystein Toverud innvilges dispensasjon fra forskrift av 25.9.2014 om kontrollområde for å bekjempe krepsepest i Haldenvassdraget, Marker kommune i Østfold og Aurskog-Høland kommune i Akershus (kontrollforskrift for Haldenvassdraget) § 5 første ledd.

Dispensasjonen gis ved gjennomføring av følgende aktiviteter i Rødenessjøen:

- Innsamling av vannprøver.
- Innsamling av ferskvannskreps (edelkreps og signalkreps) som fanges enten ved hjelp av teiner eller ved dykking dersom det er for kaldt til å fange kreps med teiner.

Mattilsynet
Regionkontoret Oslo, Akershus og Østfold

Saksbehandler: Ingjerd Lassen
Tlf: 22 40 00 00 / 64944373
Moerveien 12, Ås
E-post: postmottak@mattilsynet.no
(Husk mottakers navn)

www.mattilsynet.no

Postadresse:
Felles postmottak, Postboks 383
2381 Brumunddal
Telefaks: 23 21 68 01

- Burforsøk med edelkreps for overvåkning av smittespredning.

Dispensasjonen innvilges med følgende vilkår:

Alle aktiviteter som skal gjennomføres skal skje på en slik måte at verken kreps, vann, båter, utstyr eller annet smitteførende materiale flyttes oppstrøms i sonen.

Ved fangst av edelkreps eller signalkreps skal følgende tiltak gjennomføres for å hindre smittespredning:

- Fanget kreps unntatt kreps til burforsøk jf. eget punkt og kreps som det skal tas prøver av skal ikke settes ut igjen. Disse krepsene skal håndteres på en av disse måtene: Krepsen kokes og leveres til destruksjon til Distriktskontoret for Indre Østfold og Follo.
- Kreps transporteres i tette, forseglede beholdere direkte til Veterinærinstituttets laboratorium og håndteres i samsvar med Veterinærinstituttets rutiner for avfallsbehandling av smitteførende materiale.

Fanget frisk edelkreps som skal benyttes i burforsøk skal kun flyttes til burforsøk nedstrøms i sonen.

Ved prøvetakning av levende kreps skal følgende tiltak gjøres for å hindre smittespredning:

- Kreps skal enten fikseres i etanol på stedet eller transporteres i forseglede beholdere direkte til Veterinærinstituttets laboratorium.
- Videre håndtering på laboratoriet skal skje i samsvar med Veterinærinstituttets rutiner for håndtering og avfallsbehandling av smitteførende materiale.

Ved uttak av vannprøver skal følgende tiltak gjøres for å hindre smittespredning:

- Vannprøver kan tas ved filtrering av vann på stedet eller ved direkte transport av vann i forseglede vannbeholdere til Veterinærinstituttets laboratorium.
- Vannprøver som tas ved filtrering skal pumpes opp og filtreres på stedet i tråd med sikre metoder utviklet i tidligere prosjekt. Filter med smittestoff skal overføres til 15 ml plastrør (falconrør) med tette lokk og fraktes i lukket fryseboks med kjøleelementer til laboratoriet.
- Videre håndtering på laboratoriet skal skje i samsvar med Veterinærinstituttets rutiner for håndtering og avfallsbehandling av smitteførende materiale.

Vedtaket er fattet med hjemmel i omsetnings- og sykdomsforskriften for akvatiske dyr § 47 Kontrollområde - ville akvatiske dyr jf. forskrift av 25.9.2014 om kontrollområde for å bekjempe krepsepest i Haldenvassdraget, Marker kommune i Østfold og Aurskog-Høland kommune i Akershus (kontrollforskrift for Haldenvassdraget) § 9.

Dispensasjonen gjelder fra 01.10.2014 til 31.12.2015

Dispensasjon fra omsetnings- og sykdomsforskriften for akvatiske dyr § 47 Kontrollområde - ville akvatiske dyr

Vi har observert:
Mattilsynet har den 25.9.2014 fastsatt forskrift om kontrollområde for å bekjempe krepsepest i Haldenvassdraget, Marker kommune i Østfold og Aurskog-Høland kommune i Akershus (kontrollforskrift for Haldenvassdraget). Forskriften er fastsatt i samsvar med forskrift av 17.6.2008 nr. 819 om omsetning av akvakulturdyr og produkter av akvakulturdyr, forebygging og bekjempelse av

smittsomme sykdommer hos akvatiske dyr (omsetnings- og sykdomsforskriften for akvatiske dyr) § 47. Forskriften er foreløpig ikke registret på Lovdata, men er kunngjort på Mattilsynets internettsider.

Dere søker om dispensasjon fra deler av forskriftenes § 5 om forbud og påbud for å hindre smittespredning for å kunne gjennomføre innsamling av prøver av vann og ferskvannskreps, samt overvåkning av smittespredningen ved utsetting av burforsøk med edelkreps.

Grunnlaget for søknaden er dels å teste metodikken som tidligere er utviklet i forbindelse med det NFR-finansierte forskningsprosjektet ”Avansert overvåking av introdusert krepsepest (Aphanomyces astaci) for bedre forvaltning av truet ferskvannskreps”. Samtidig ønsker dere å overvåke situasjonen med burforsøk, samt kartlegge med tanke på dødelighet av edelkreps og utbredelse av signalkreps.

I prosjektet ble det utviklet metodikk for direkte påvisning og kvantifisering av krepsepestsmitte i vann. I prosjektperioden var det ingen utbrudd av krepsepest i Norge og dere fikk i stor grad bare testet metodikken for innsjøer med smittet signalkreps. I forbindelse med pestutbrudd i Rødenessjøen mener dere at dere nå en unik mulighet til å sikre vannprøver som kan videreutvikle deres kompetanse på spredningsdynamikk og smittepress under og etter et pestutbrudd.

Det framgår av søknaden at de konkrete tiltak dere ønsker å utføre er:

1. Innsamling av vannprøver i regi av Veterinærinstituttet v/Trude Vrålstad, utført av NIVA v/David Strand.
2. Innsamling av ferskvannskreps i regi av NINA v/Stein Johnsen, utført av Utmarksavdelingen v/ Øystein Toverud. Ferskvannskreps (edelkreps og signalkreps) fanges enten ved hjelp av teiner eller ved dykking dersom det er for kaldt til å fange kreps med teiner.
3. Burforsøk med edelkreps i regi av og utført av Utmarksavdelingen v/Øystein Toverud. Her søkes det om en videreføring av pågående overvåkningsprogram i sonen, men med en økning på 4 ekstra bur i forhold til dagens 4 burforsøk.

Søknaden inneholder også en nærmere beskrivelse av hvordan disse aktivitetene skal gjennomføres og behandles for å sikre at smitte fra innsamlet materiale ikke spres ut av sonen til nye lokaliteter. Dette er beskrevet slik:

Prøver av kreps:

- Tillatelse for krepsing og fiske er søkt og godkjent av Fylkesmannen i Østfold v/fiskeforvalter Leif Roger Karlsen.
- Hele kreps fikseres i etanol på stedet. Dette vil både sikre forskningsmaterialet og samtidig deaktivere smitte.
- Levende kreps kan i spesielle tilfeller måtte fraktes fra smittesone til laboratorium for å sikre materiale for dyrking av agens. Transport vil i så tilfelle foregå i forseglede beholdere i bil og prøvene bringes direkte til Veterinærinstituttets laboratorium. Her vil kreps avlives og smitte deaktiveres etter Veterinærinstituttet gjeldende prosedyrer ved krepsepestdiagnostisk.

Vannprøver:

- Vann pumpes opp og filtreres på stedet i tråd med sikre metoder utviklet i tidligere prosjekt. Filter med smittestoff overføres til 15 ml plastrør (falconrør) med tette lokk og fraktes i lukket fryseboks med kjøleelementer til laboratorium, hvor prøvene fryses ned til -80 °C. Dette vil både sikre forskningsmaterialet og samtidig deaktivere smitte.

- Vannprøver kan i spesielle tilfeller måtte fraktes fra smittesone til laboratorium. Transport vil i så tilfelle foregå i forseglede vannbeholdere i bil og prøvene bringes direkte til Veterinærinstituttets laboratorium. Filtratvann vil enten autoklaveres eller behandles med klor (1 del klorin til 20 deler filtratvann i minimum 10 minutter) før utslipp i avløp fra laboratorium selv om det etter all sannsynlighet ikke lenger inneholder smitte.

Det er i søknaden også beskrevet aktuelle metoder for desinfeksjon av båt og annet utstyr (filtreringsutstyr, krepsetegner, klær, støvler etc).

Mattilsynet vurderer dette slik:
Av § 9 i kontrollforskrift for Haldenvassdraget framkommer det at Mattilsynet i særskilte tilfeller kan dispensere fra bestemmelsene i forskriften og sette vilkår for dispensasjonen.

Utvikling av metoder og vitenskapelig kompetanse i forhold til smittespredning og diagnostikk av krepsepest er viktig for ivaretakelse av den utrydningstruede edelkrepsbestanden i Norge. Gjennomføring av forskningsaktivitet vurderes derfor som et særskilt tilfelle som gir Mattilsynet anledning til å dispensere fra bestemmelsene i forskriften.

§ 5 i kontrollforskrift for Haldenvassdraget inneholder følgende bestemmelser:

Det er forbudt

1. å fange levende kreps eller å plukke død eller levende kreps, samt å oppbevare kreps i bur eller teiner i kontrollområdet,
2. å flytte levende og døde ferskvannskreps innen kontrollområdet,
3. å føre levende kreps og andre akvatiske organismer inn i kontrollområdet,
4. å føre levende og døde fisk og andre akvatiske organismer ut av kontrollområdet. Forbudet gjelder ikke andre døde akvatiske organismer enn ferskvannskreps, som skal gå direkte til konsum,
5. å føre ubehandlet avfall og ubearbeidede produkter av fisk og andre akvatiske organismer ut av kontrollområdet,
6. å føre ubehandlet vann ut av området eller oppstrøms innen kontrollområdet.

Det er påbudt

1. å sørge for at båter og vannsportutstyr, fangst- og fiskeredskaper, anleggsmaskiner, vannbeholdere og annet utstyr eller redskaper som har vært benyttet i området tørkes fullstendig og desinfiseres, før de benyttes utenfor området. Innenfor området skal tilsvarende tiltak gjennomføres dersom omtalte gjenstander tas ut av vannet før de flyttes oppstrøms,
2. å melde funn av døde eller syke kreps til Mattilsynet.

For å gjennomføre de aktivitetene som det søkes om, er det behov for dispensasjon fra bestemmelsene i § 5 første ledd. Vi vurderer at en dispensasjon kan gis fra disse bestemmelsene, forutsatt at det settes vilkår som sikrer at smittespredning unngås.

Bestemmelsene i § 5 annet ledd inneholder bestemmelser om desinfeksjon av båter, utstyr etc. og må ivaretas i aktivitetene som det søkes om og som det i søknaden også er beskrevet framgangsmåter for. Vår vurdering av er at det verken er behov for eller ønskelig å dispensere fra disse bestemmelsene. De desinfeksjonsmetodene som beskrives i søknaden anses som egnet.

Det er i søknaden beskrevet hvordan det skal sikres at smitte ikke spres ut av sonen til nye lokaliteter. Disse er etter vår vurdering i hovedsak tilstrekkelige, men de tar ikke i nødvendig grad hensyn til å unngå videre smittespredning innenfor kontrollsonen. Ved angivelse av vilkår for dispensasjonen har vi derfor tilføyd tiltak for å unngå dette.

Se vedlegg til tilsynsrapport.

Med hilsen

Jarle Bergsjø
seksjonssjef

Vedlegg:
Vedlegg til tilsynsrapport
Melding om rett til å klage over forvaltningsvedtak

VEDLEGG TIL TILSYNSRAPPORT

Hjemmel for tilsyn

Mattilsynet har i henhold til matloven § 23 hjemmel til å føre tilsyn og fatte vedtak for gjennomføring av bestemmelser gitt i medhold av loven.

Klagerett

Det er klagerett på enkeltvedtak. Fristen for å klage er tre uker etter at dere har mottatt informasjon om vedtaket, jf. forvaltningsloven §§ 28 og 29. Dere finner mer informasjon om klageretten i vedlegget Melding om rett til å klage over forvaltningsvedtak.

Virksomheten er vurdert etter følgende regelverk

- FOR 2008-06-17 nr 819: Forskrift 17. jun. 2008 nr. 819 om omsetning av akvakulturdyr og produkter av akvakulturdyr, forebygging og bekjempelse av smittsomme sykdommer hos akvatiske dyr (omsetnings- og sykdomsforskriften for akvatiske dyr)

Avsender	Melding om rett til å klage over forvaltningsvedtak (Forvaltningsloven § 27)	
Mattilsynet Regionkontoret Oslo, Akershus og Østfold Felles postmottak, Postboks 383 2381 Brumunddal		
Mottaker (navn og adresse)	**Dato**	**Klageinstans**
VETERINÆRINSTITUTTET OSLO Postboks 750 Sentrum 0106 OSLO	01.10.2014	Mattilsynet, Hovedkontoret

Denne meldingen gir viktige opplysninger hvis De ønsker å klage over vedtak De har fått underretning om.

Klagerett	De har rett til å klage over vedtaket.
Hvem kan De klage til	Klagen skal først sendes til avsenderen av denne meldingen. Dersom dette organet ikke endrer vedtaket som følge av klagen, vil den bli sendt videre til klageinstansen for avgjørelse.
Fristen til å klage	Klagefristen er 3 uker fra den dag dette brevet ble mottatt. Det er tilstrekkelig at klagen er postlagt innen fristen løper ut. Dersom De klager så sent at det kan være uklart for oss om De har klaget i rett tid, bes De oppgi dato når denne meldingen ble mottatt. Dersom klagen blir sendt for sent, er det adgang til å se bort fra den. Om De har særlig grunn til det, kan De likevel søke om å få forlenget klagefristen. De bør da i tilfelle nevne grunnen til forsinkelsen.
Rett til å kreve begrunnelse	Dersom De ikke allerede har fått begrunnelse for vedtaket, kan De sette fram krav om å få det. Slikt krav må settes fram i løpet av klagefristen. Klagefristen blir i så fall avbrutt, og ny frist begynner å løpe fra det tidspunkt De mottar begrunnelsen.
Klagens innhold	Klagen skal nevne det vedtak det klages over, og den eller de endringer som ønskes. De bør også nevne Deres begrunnelse for å klage og eventuelle andre opplysninger som kan ha betydning for vurderingen av klagen. Klagen må undertegnes.
Utsetting av vedtaket	Selv om De har klagerett, kan vedtaket vanligvis gjennomføres straks. De har imidlertid adgang til å søke om å få utsatt iverksettingen av vedtaket inntil klagefristen er ute eller klagen er avgjort
Rett til å se sakens dokumenter og til å kreve veiledning	Med visse begrensninger har De rett til å se dokumentene i saken, jf fvl §§ 18 og 19. De må i tilfelle vende Dem til det forvaltningsorgan som har sendt denne meldingen. Der kan De også få nærmere veiledning om adgangen til å klage, om fremgangsmåten ved klage og om reglene for saksbehandlingen ellers.
Kostnader ved klagesaken	De kan søke om å få dekket utgifter til nødvendig advokatbistand etter reglene om fritt rettsråd. Her gjelder imidlertid normalt visse inntekts- og formuesgrenser. Fylkesmannens kontor eller vedkommende advokat kan gi nærmere veiledning. Det er også særskilt adgang til å kreve dekning for vesentlige kostnader i forbindelse med klagesaken, for eksempel til advokatbistand. Dersom vedtaket er blitt endret til gunst for klageren, er det etter fvl § 36 også adgang til å søke dekning for vesentlige kostnader i forbindelse med saken. Klageinstansen vil om nødvendig orientere Dem om retten til å kreve slik dekning for sakskostnader.
Klage til Sivilombudsmannen	Det er også mulig å klage til Stortingets ombudsmann for forvaltningen (Sivilombudsmannen).

Norsk institutt for naturforskning
Postboks 5685 Sluppen
7485 Trondheim

Trondheim, 22.10.2015

Deres ref.:	Vår ref. (bes oppgitt ved svar):	Saksbehandler:
[Deres ref.]	2015/10115	Anne Kristin Jøranlid

Tillatelse til fangst av signalkreps i forbindelse med forskningsprosjekt

Vi viser til Deres søknad datert 25.09.2015 om tillatelse til fangst av signalkreps i forbindelse med forskningsprosjektet «Targeted strategies for safeguarding the noble crayfish against alien and emerging threats» (TARGET – NFR243907).

Miljødirektoratet kan treffe enkeltvedtak om høsting og annet uttak av ferskvannsorganismer til vitenskapelige formål, jamfør § 13 i lakse- og innlandsfiskeloven, samt § 18 bokstav f i naturmangfoldloven. Miljødirektoratet har ikke åpnet for fiske etter signalkreps, og det er dermed forbudt uten tillatelse.

Norges edelkrepsbestander har blitt svært viktige i europeisk sammenheng, selv om det har vært en betydelig nedgang i bestandene i Norge også. Det er sammensatte årsaker til den drastiske nedgangen i edelkrepsbestander i Europa og Norge, men signalkreps, som er bærer av krepsepest, ansees som den største trusselen mot edelkreps i dag. Signalkreps ble påvist for første gang i Norge i 2006. Edelkreps står på den norske rødlista i kategorien sterkt truet, og har status som sårbar på rødlista til IUCN (International Union for Conservation for nature). Edelkreps omfattes også av Bernkonvensjonens liste III og EU's habitatdirektiv.

Prosjektet søker om en generell tillatelse til å fange signalkreps i aktuelle lokaliteter i Norge. Fanget signalkreps skal benyttes i planlagte eksperimenter knyttet til eDNA og krepsepest. Prosjektet søker om tillatelse til fangst av signalkreps frem til prosjektet avsluttes i 2018.I tillegg til NINA er Veterinærinstituttet, NIVA og Utmarksavdelingen for Akershus og Østfold involvert i prosjektet.

Miljødirektoratet har ikke åpnet opp for ordinært fiske etter signalkreps, av bekymring for ytterligere spredning av signalkreps dersom det åpnes for fiske av arten. Miljødirektoratet ønsker å øke kunnskapen om den viktigste trusselen til norske edelkrepsbestander, og mener fangst av signalkreps til vitenskapelige formål, som TARGET, er viktig.

Vi mener at kunnskapsgrunnlaget for denne søknaden er tilstrekkelig, jf. § 8 i naturmangfoldloven. Miljødirektoratet har i behandlingen av søknaden også sett på naturmangfoldloven §§ 10-12, og

vurdert de som ikke relevante til denne søknaden.

Vedtak

Miljødirektoratet har vurdert søknaden ut ifra naturmangfoldloven §§ 8-12, og mener vi har et godt nok kunnskapsgrunnlag i denne søknaden.

Med bakgrunn i § 13 i Lov om laksefisk og innlandsfisk m.v, og § 18 bokstav f i Lov om forvaltning av naturens mangfold gis det med dette tillatelse til fangst av signalkrepstil vitenskapelige formål i forbindelse med forskningsprosjektet «Targeted strategies for safeguarding the noble crayfish against alien and emerging threats» (TARGET).

Tillatelsen forutsetter at fanget signalkreps blir avlivet etter endt forsøk i laboratoriet. Videre forutsettes det at veterinærfaglige kriterier er oppfylt, i henhold til Mattilsynets bestemmelser. Grunneiere, rettighetshavere og vedkommende politimyndighet skal om mulig varsles før fisket tar til. Det presiseres at tillatelsen kun gjelder for fangst til dette prosjektet, og tillatelsen er gyldig frem til 1.januar 2019.

Dette vedtaket kan påklages jf. forvaltningsloven § 29. Klagefristen er 3 uker etter at vedtaket er mottatt, og en eventuell klage sendes til Miljødirektoratet.

Hilsen
Miljødirektoratet

Dette dokumentet er elektronisk godkjent og har derfor ingen signatur

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Supplementary table 1. Overview over all tanks/aquaria and *P. leniusculus* sex, length and origin.

Overview of *P. leniusculus* involved in all the experiments, with lenght, sex and origin.				
Mixed = Øymarksjøen or Rødenessjøen (unknown origin).				
EX1 - 20 °C				
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B13 - # 20. Food.	96	M	101	Mixed pop.
	97	M	84	Mixed pop.
	27	M	119	Rødenessjøen.
	64	F	109	Rødenessjøen.
	23	F	119	Rødenessjøen.
	43	M	108	Rødenessjøen.
	25	F	114	Rødenessjøen.
	59	M	111	Rødenessjøen.
	41	M	115	Rødenessjøen.
	81	F	117	Rødenessjøen.
	54	M	109	Rødenessjøen.
	98	M	100	Mixed pop.
	99	F	94	Mixed pop.
	51	F	112	Rødenessjøen.
	37	F	116	Rødenessjøen.
	92	F	87	Øymarksjøen
	100	M	100	Mixed pop.
	101	F	84	Mixed pop.
	102	M	85	Mixed pop.
	103	F	Unknown	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B12 - # 2. Food.	45	F	121	Rødenessjøen
	3	M	117	Rødenessjøen
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
C13 - # 20. No food.	31	M	115	Rødenessjøen
	33	M	133	Rødenessjøen
	63	F	120	Rødenessjøen
	104	M	80	Mixed.pop
	2	M	115	Rødenessjøen
	6	M	125	Rødenessjøen
	11	M	137	Rødenessjøen
	22	M	122	Rødenessjøen
	44	F	128	Rødenessjøen
	38	F	100	Rødenessjøen
	16	F	130	Rødenessjøen
	86	F	105	Rødenessjøen
	91	F	100	Rødenessjøen
	62	F	115	Rødenessjøen
	105	M	95	Mixed.pop
	52	M	115	Rødenessjøen
	36	M	119	Rødenessjøen
	29	F	129	Rødenessjøen
	60	M	122	Rødenessjøen
	72	M	124	Rødenessjøen
Tank	***P. leniusculus* number**	Sex	**Lenght mm**	**Origin**
B5 - # 2. No food.	50	F	117	Rødenessjøen
	65	M	134	Rødenessjøen

EX2 20 °C				
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B12 - # 20. Food.	2	M	115	Rødenessjøen
	65	M	134	Rødenessjøen
	63	F	120	Rødenessjøen
	106	M	94	Mixed pop.
	107	F	85	Mixed pop.
	108	M	92	Mixed pop.
	91	F	100	Rødenessjøen
	29	F	129	Rødenessjøen
	54	M	109	Rødenessjøen
	47	F	125	Rødenessjøen
	77	F	113	Rødenessjøen
	56	F	111	Rødenessjøen
	76	M	112	Rødenessjøen
	51	F	112	Rødenessjøen
	68	F	110	Rødenessjøen
	8	M	117	Rødenessjøen
	25	F	114	Rødenessjøen
	102	M	85	Mixed pop.
	109	M	87	Mixed pop.
	110	M	80	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B13 - # 2. Food.	100	M	100	Mixed pop.
	12	F	131	Rødenessjøen
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B5 - # 20. No food.	111	F	72	Mixed pop.
	3	M	117	Rødenessjøen
	27	M	119	Rødenessjøen
	33	M	133	Rødenessjøen
	50	F	117	Rødenessjøen
	99	F	94	Mixed pop.
	97	M	84	Mixed pop.
	89	F	119	Rødenessjøen
	38	F	100	Rødenessjøen
	82	M	114	Rødenessjøen
	41	M	115	Rødenessjøen
	103	M	Unknown	Mixed pop.
	59	M	111	Rødenessjøen
	96	M	101	Mixed pop.
	112	M	89	Mixed pop.
	113	F	85	Mixed pop.
	114	M	85	Mixed pop.
	115	M	87	Mixed pop.
	60	M	122	Rødenessjøen
	64	F	109	Rødenessjøen
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
C13 - # 2. No food.	52	M	115	Rødenessjøen
	72	M	124	Rødenessjøen

EX3 20 °C				
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
C13 - # 20. Food.	60	M	122	Rødenessjøen
	82	M	114	Rødenessjøen
	51	F	112	Rødenessjøen
	104	M	80	Mixed pop.
	65	M	134	Rødenessjøen
	52	M	115	Rødenessjøen
	103	M	Unknown	Mixed pop.
	109	M	87	Mixed pop.
	25	F	114	Rødenessjøen
	29	F	129	Rødenessjøen
	23	F	119	Rødenessjøen
	100	M	100	Mixed pop.
	101	F	84	Mixed pop.
	36	M	119	Rødenessjøen
	93	F	87	Øymarksjøen
	106	M	94	Mixed pop.
	59	M	111	Rødenessjøen
	111	F	72	Mixed pop.
	41	M	115	Rødenessjøen
	102	M	85	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B5 - # 2. Food.	95	M	Unknown	Øymarksjøen
	108	M	92	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B12 - # 20. No food.	27	M	119	Rødenessjøen
	114	M	85	Mixed pop.
	89	F	119	Rødenessjøen
	68	F	110	Rødenessjøen
	115	M	87	Mixed pop.
	66	F	117	Rødenessjøen
	56	F	111	Rødenessjøen
	2	M	115	Rødenessjøen
	50	F	117	Rødenessjøen
	107	F	85	Mixed pop.
	38	F	100	Rødenessjøen
	77	F	113	Rødenessjøen
	6	M	125	Rødenessjøen
	91	F	100	Rødenessjøen
	63	F	120	Rødenessjøen
	94	F	85	Øymarksjøen
	96	M	101	Mixed pop.
	113	F	85	Mixed pop.
	99	F	94	Mixed pop.
	110	M	80	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B13 - # 2. No food.	3	M	117	Rødenessjøen
	12	F	131	Rødenessjøen

EX4 10 °C				
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B13 - # 2. Food.	12	F	131	Rødenessjøen
	43	M	108	Rødenessjøen
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B12 - # 20. Food.	52	M	115	Rødenessjøen
	89	F	119	Rødenessjøen
	51	F	112	Rødenessjøen
	100	M	100	Mixed pop.
	109	M	87	Mixed pop.
	76	M	112	Rødenessjøen
	102	M	85	Mixed pop.
	115	M	87	Mixed pop.
	107	F	85	Mixed pop.
	38	F	100	Rødenessjøen
	75	F	130	Rødenessjøen
	99	F	94	Mixed pop.
	56	F	111	Rødenessjøen
	63	F	120	Rødenessjøen
	117	F	86	Mixed pop.
	36	M (died, replaced by #110)	119	Rødenessjøen
	110	M	80	Mixed pop.
	60	M	122	Rødenessjøen
	2	M	115	Rødenessjøen
	118	F	80	Mixed pop.
	119	M	84	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
C13 - # 2. No food.	96	M	101	Mixed pop.
	116	M	105	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B5 - # 20. No food.	3	M	117	Rødenessjøen
	103	M	Unknown*	Mixed pop.
	94	F	85	Øymarksjøen
	91	F	100	Rødenessjøen
	82	M	114	Rødenessjøen
	101	F	84	Mixed pop.
	68	F	110	Rødenessjøen
	23	F	119	Rødenessjøen
	120	F	87	Mixed pop.
	98	M	100	Mixed pop.
	121	M	86	Mixed pop.
	87	F	107	Rødenessjøen
	50	F	117	Rødenessjøen
	97	M	84	Mixed pop.
	66	F	117	Rødenessjøen
	Unknown*		Unknown*	Unknown*
	41	M	115	Rødenessjøen
	6	M	125	Rødenessjøen
	112	M	89	Mixed pop.
	77	F	113	Rødenessjøen
	65	M	134	Rødenessjøen
	25	F	114	Rødenessjøen

EX5 10 °C				
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B13 - # 2. Food.	120	F	87	Mixed pop.
	56	F	111	Rødenessjøen
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B5 - # 20. Food.	126	F	100	Mixed pop.
	103	M	ukjent	Mixed pop.
	41	M	115	Rødenessjøen
	107	F	85	Mixed pop.
	99	F	94	Mixed pop.
	82	M	114	Rødenessjøen
	89	F	119	Rødenessjøen
	109	M	87	Mixed pop.
	77	F	113	Rødenessjøen
	93	F	87	Øymarksjøen
	94	F	85	Øymarksjøen
	98	M	100	Mixed pop.
	38	F	100	Rødenessjøen
	101	F	84	Mixed pop.
	25	F	114	Rødenessjøen
	75	F	130	Rødenessjøen
	51	F	112	Rødenessjøen
	91	F	100	Rødenessjøen
	127	F	103	Mixed pop.
	128	M	90	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B12 - # 2. No food.	50	F	117	Rødenessjøen
	6	M	125	Rødenessjøen
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
C13 - # 20 No food.	96	M	101	Mixed pop.
	123	F	80	Mixed pop.
	3	M	117	Rødenessjøen
	121	M	86	Mixed pop.
	76	M (died, replaced by # 68)	112	Rødenessjøen
	68	F	110	Rødenessjøen
	117	F	86	Mixed pop.
	112	M	89	Mixed pop.
	111	F	72	Mixed pop.
	116	M	105	Mixed pop.
	23	F	119	Rødenessjøen
	2	M	115	Rødenessjøen
	52	M	115	Rødenessjøen
	106	M	94	Mixed pop.
	43	M	108	Rødenessjøen
	110	M	80	Mixed pop.
	102	M	85	Mixed pop.
	125	F	75	Mixed pop.
	124	M	74	Mixed pop.
	63	F	120	Rødenessjøen
	115	M		

EX6 10 °C				
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B13 - # 2. Food.	75	F	130	Rødenessjøen
	43	M	108	Rødenessjøen
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B12 - # 20. No food.	51	F	112	Rødenessjøen
	50	F	117	Rødenessjøen
	107	F	85	Mixed pop.
	41	M	115	Rødenessjøen
	3	M	117	Rødenessjøen
	102	M	85	Mixed pop.
	99	F	94	Mixed pop.
	110	M	80	Mixed pop.
	106	M	94	Mixed pop.
	129	M		Mixed pop.
	94	F	85	Øymarksjøen
	130	M		Mixed pop.
	131	F		Mixed pop.
	60	M	122	Rødenessjøen
	82	M	114	Rødenessjøen
	120	F	87	Mixed pop.
	109	M	87	Mixed pop.
	101	F	84	Mixed pop.
	98	M	100	Mixed pop.
	128	M	90	Mixed pop.
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B5 - # 2. No food.	56	F	111	Rødenessjøen
	77	F	113	Rødenessjøen
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
C13 - # 20. Food.	68	F	110	Rødenessjøen
	89	F	119	Rødenessjøen
	63	F	120	Rødenessjøen
	103	M	ukjent	Mixed pop.
	127	F	103	Mixed pop.
	112	M	89	Mixed pop.
	38	F	100	Rødenessjøen
	113	F	85	Mixed pop.
	25	F	114	Rødenessjøen
	124	M	74	Mixed pop.
	126	F	100	Mixed pop.
	2	M	115	Rødenessjøen
	93	F	87	Øymarksjøen
	65	M	134	Rødenessjøen
	96	M	101	Mixed pop.
	123	F	80	Mixed pop.
	91	F	100	Rødenessjøen
	117	F	86	Mixed pop.
	115	M	87	Mixed pop.
	52	M	115	Rødenessjøen

Death				
Tank	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
C13 - # 2	65	M	134	Rødenessjøen
	77	F	113	Rødenessjøen
	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B12 - # 2	101	F	84	Mixed pop.
	52	M	115	Rødenessjøen
	***P. leniusculus* number**	**Sex**	**Lenght mm**	**Origin**
B13 - # 20	25	F	114	Rødenessjøen
	38	F	100	Rødenessjøen
	45	F	121	Rødenessjøen
	56	F	111	Rødenessjøen
	60	M	122	Rødenessjøen
	63	F	120	Rødenessjøen
	75	F	130	Rødenessjøen
	82	M	114	Rødenessjøen
	94	F	85	Øymarksjøen
	98	M	100	Mixed pop.
	103	M	unknown	Mixed pop.
	107	F	85	Mixed pop.
	112	M	89	Mixed pop.
	120	F	87	Mixed pop.
	124	M	74	Mixed pop.
	?1 (unknown)*			
	U1 (unknown)*			
	U2 (unknown)*			
	U3 (unknown)*			
	U4 (unknown)*			
**P. leniusculus* where marking is lost.				

Unknown = *P. leniusculus* where marking is lost.

Moulting is not included in this table.

Mixed = Øymarksjøen or Rødenessjøen (unknown origin).

Supplementary text 3. Søknadstekst som brukes for å søke om avlivning av kreps.

- Avlivingsmetoder som anses å minimere smerte og stress for tifotkreps er avkjøling i luft, avkjøling i isvann, nedsenkning i nellik olje bad eller elektriske metoder (EFSA 2005).
- Etter å ha tatt en blodprøve fra krepsen, enten fra ryggsiden eller fra et bein, vil kreps raskt fryses ned i luft (dypfryser) før videre prøvetaking av organer og muskler. I enkelte tilfeller vil det være behov for å ta ut ferske prøver til histologi. I de tilfellene vil krepsen avkjøles i en kort periode for bedøving, etterfulgt av to snitt i hoderegionen for å raskt ødelegge nervesystemet.»

Supplementary table 2. Overview over standard dilution values, used to generate a standard curve for each species.

Dilution	**PFU values *P. leniusculus***	**PFU values *A. astaci***
10^1	137101.00	890241.02
10^2	13710.10	89024.10
10^3	1371.01	8902.41
10^4	137.10	890.24

Supplementary text 4. R-script used for statistical calculations.

```
d <- read.table("Hovedtabell til R august 2017.txt", header=TRUE, sep="\t")
dim(d)
summary(d)
with(d, table(Experiment, Food))

# Focus on the experiments
Ex <- subset(d, Experiment %in% c("Ex1", "Ex2", "Ex3", "Ex4", "Ex5", "Ex6"))
dim(Ex) # 72 records

# Relevel factors for new subset
Ex$Experiment <- factor(Ex$Experiment)
Ex$Tank <- factor(Ex$Tank)
Ex$fNumber <- factor(Ex$Number)
Ex$Food <- factor(Ex$Food)
Ex$fTemp <- factor(Ex$Temp > 15, labels=c("10", "20"))

offset <- 1 # For log transformation (avoid log(0))
Ex$log.A.astaci.PFU <- log10(Ex$Tot.PFU.A.astaci + offset) #legger til offset
Ex$log.P.leniusculus.PFU <- log10(Ex$Tot.PFU.P.leniusculus + offset) #legger tl offset
Ex$log.A.astaci.Cray <- log10(Ex$Tot.PFU.A.astaci/Ex$Number + offset) #A. astaci eDNA per kreps
Ex$log.P.leniusculus.Cray <- log10(Ex$Tot.PFU.P.leniusculus/Ex$Number + offset) #P. leniusculus eDNA per
kreps

Ex <-Ex[, c("Experiment", "Tank", "Number", "fNumber", "Temp", "fTemp", "Food", "log.A.astaci.PFU",
"log.P.leniusculus.PFU", "log.A.astaci.Cray", "log.P.leniusculus.Cray" )]
with(Ex, table(fNumber, fTemp, Food))
with(Ex, table(Experiment, Tank)) # 3 filters from each Experiment : Tank = Unit
Ex$Unit <- with(Ex, factor(paste(Experiment, Tank, sep=".")))
summary(Ex)

library(lattice)
# Plot with LOQ: https://stackoverflow.com/questions/17601053/lattice-xyplot-panel-abline-how-to-add-
different-horizontal-lines-based-on-x-v
#Pacifastacus leniusculus
LOQ.P.leni <- 20
bw.loq.Pl <- function(...) {
  panel.abline(h=log10(LOQ.P.leni + offset), lty = "dotted", col = "red")
  panel.bwplot(...)
}
#all factors included
bwplot(log.P.leniusculus.Cray ~ fNumber | fTemp + Food, data=Ex, panel=bw.loq.Pl, main="Pacifastacus
leniusculus eDNA concentration:
      temperature, density and food
      per-individual",ylab="Pacifastacus leniusculus eDNA copies per individual (log)", xlab="Food (F),  No food
(N),  Temperature (10 ºC, 20 ºC),   Density (2, 20)" ) #Per crayfish

#statistical tests
#per crayfish
library(nlme)
summary(qm1 <- lme(log.P.leniusculus.Cray ~ (fTemp * fNumber * Food), random= ~1|Unit, data=Ex))
summary(qm2 <- lme(log.P.leniusculus.Cray ~ fTemp * Food, random= ~1|Unit, data=Ex))
anova(update(qm1, method="ML"), update(qm2, method="ML")) #0.2837

summary(qm3 <- lme(log.P.leniusculus.Cray ~ fTemp + Food, random= ~1|Unit, data=Ex))
anova(update(qm2, method="ML"), update(qm3, method="ML")) #p=0.6897
```

```
summary(qm4 <- lme(log.P.leniusculus.Cray ~ (fTemp * fNumber), random= ~1|Unit, data=Ex)) # p=0.95
summary(qm5 <- lme(log.P.leniusculus.Cray ~ (fTemp + fNumber), random= ~1|Unit, data=Ex))
anova(update(qm4, method="ML"), update(qm5, method="ML")) #p=0.6125
summary(qm6 <- lme(log.P.leniusculus.Cray ~ (fNumber * Food), random= ~1|Unit, data=Ex))
summary(qm7 <- lme(log.P.leniusculus.Cray ~ (fNumber + Food), random= ~1|Unit, data=Ex))
anova(update(qm6, method="ML"), update(qm7, method="ML")) #p=0.195
#no effects of interaction, choose the simplest model fTemp+fNumber+Food
summary(qm8 <- lme(log.P.leniusculus.Cray ~ (fTemp + fNumber + Food), random= ~1|Unit, data=Ex))
summary(qm9 <- lme(log.P.leniusculus.Cray ~ (fTemp + Food), random= ~1|Unit, data=Ex))
anova(update(qm8, method="ML"), update(qm9, method="ML")) #p=0.8807
#keep qm9
qm9.0 <- update(qm9, . ~ . , method="ML")
anova(qm9.0, update(qm9, . ~ . - fTemp, method="ML")) #p=0.0358
anova(qm9.0, update(qm9, . ~ . - Food, method="ML")) #p=0.0286
#still keeping qm9
plot(qm9)
qqnorm(residuals(qm9)) # 1 possible outlier?
k <- which.max(residuals(qm9))
Ex[k, ] # low density, high temp, no food - and high signal PFU
# Redo without high-residual obs.
summary(qm9.k <- lme(log.P.leniusculus.Cray ~ (fTemp + Food), random= ~1|Unit, data=Ex[-k, ]))
# Random effect / residual variance even higher, otherwise similar fixed effects
plot(qm9.k)
qqnorm(residuals(qm9.k)) # Nice!

#Aphanomyces astaci
LOQ.A.astaci <- 100
bw.loq.Aa <- function(...) {
  panel.abline(h=2.0043, lty = "dotted", col = "red")
  panel.bwplot(...)
}
#all factors
bwplot(log.A.astaci.Cray ~ fTemp | fNumber + Food, data=Ex, panel = bw.loq.Aa, main="Aphanomyces astaci
eDNA concentration:
     temperature, density and food
     per-individual", ylab="Aphanomyces astaci eDNA copies per individual (log)", xlab="Food (F),  No food (N),
Temperature (10ºC, 20ºC),  Density (2, 20)") #Per crayfish

#statistical tests
#per crayfish
library(nlme)
summary(rm1 <- lme(log.A.astaci.Cray ~ fTemp * fNumber * Food, random= ~1|Unit, data=Ex))
summary(rm2 <- lme(log.A.astaci.Cray ~ fTemp * Food, random= ~1|Unit, data=Ex))
anova(update(rm1, method="ML"), update(rm2, method="ML")) #0.0533
summary(rm3 <- lme(log.A.astaci.Cray ~ fTemp * fNumber, random= ~1|Unit, data=Ex))
anova(update(rm1, method="ML"), update(rm3, method="ML")) #0.99. remove food
summary(rm4 <- lme(log.A.astaci.Cray ~ fTemp + fNumber + Food, random= ~1|Unit, data=Ex))
summary(rm5 <- lme(log.A.astaci.Cray ~ fTemp + fNumber, random= ~1|Unit, data=Ex)) #keep this model
anova(update(rm4, method="ML"), update(rm5, method="ML")) #p=0.8917
rm5.0 <- update(rm5, . ~ . , method="ML")
anova(rm5.0, update(rm5, . ~ . - fTemp, method="ML")) #p= <.0001
anova(rm5.0, update(rm5, . ~ . - fNumber, method="ML")) #p=0.0625
#only temperature has significant effect
#Random effect variance is same order as residual variance
#Mixed effect correction for pseudoreplication is important
plot(rm5) #No indication of heteroscedasticity https://en.wikipedia.org/wiki/Heteroscedasticityr
qqnorm(residuals(rm5)) #Not bad...A.astaci residuals
```

Supplementary text 5. The statistical models used for a.) *P. leniusculus*, and b.) *A. astaci.*

a.

```
> summary(qm9 <- lme(log.P.leniusculus.Cray ~ (fTemp + Food), random=
~1|Unit, data=Ex))
Linear mixed-effects model fit by REML
 Data: Ex
       AIC      BIC    logLik
  87.35925 98.52978 -38.67962

Random effects:
 Formula: ~1 | Unit
        (Intercept)  Residual
StdDev:     1.35083 0.1805319

Fixed effects: log.P.leniusculus.Cray ~ (fTemp + Food)
                Value Std.Error DF   t-value p-value
(Intercept)  2.984516 0.4790101 48  6.230591  0.0000
fTemp20     -1.138074 0.5531132 21 -2.057579  0.0523
FoodN        1.191419 0.5531132 21  2.154024  0.0430
 Correlation:
        (Intr) fTmp20
fTemp20 -0.577
FoodN   -0.577  0.000

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-1.88259263 -0.49767167  0.05500089  0.39295835  3.49825774

Number of Observations: 72
Number of Groups: 24
```

b.

```
> summary(rm5 <- lme(log.A.astaci.Cray ~ fTemp + fNumber, random= ~1|Unit,
data=Ex)) #keep this model
Linear mixed-effects model fit by REML
 Data: Ex
       AIC      BIC    logLik
  177.3768 188.5474 -83.68842

Random effects:
 Formula: ~1 | Unit
        (Intercept)  Residual
StdDev:   0.6796505 0.5934047

Fixed effects: log.A.astaci.Cray ~ fTemp + fNumber
                 Value Std.Error DF   t-value p-value
(Intercept)  2.4474085 0.2690960 48  9.094928   0.000
fTemp20     -2.4482540 0.3107253 21 -7.879160   0.000
fNumber20    0.5616668 0.3107253 21  1.807599   0.085
 Correlation:
          (Intr) fTmp20
fTemp20   -0.577
fNumber20 -0.577  0.000

Standardized Within-Group Residuals:
          Min            Q1           Med            Q3           Max
-2.8228219015 -0.2384091894  0.0002886971  0.4371439177  3.2123169461

Number of Observations: 72
Number of Groups: 24
```

Supplementary table 3. Overview over statistical methods and methods used for making plots.

Pacifastacus leniusculus			
Experiment	Test	Figures	Program
Experiment 1: Temperature, density, food	NLME→ ML → anova	Boxplot	RStudio
Experiment 2: Moulting	None	Bar plot	Microsoft Excel 2016
Experiment 3: Death	None	Bar plot	Microsoft Excel 2016
Overview	None	Bar plot	Microsoft Excel 2016
Aphanomyces astaci			
Experiment 1: Temperature, density, food	NLME → ML → anova	Boxplot	RStudio
Experiment 2: Moulting	None	Bar plot	Microsoft Excel 2016
Experiment 3: Death	None	Bar plot	Microsoft Excel 2016

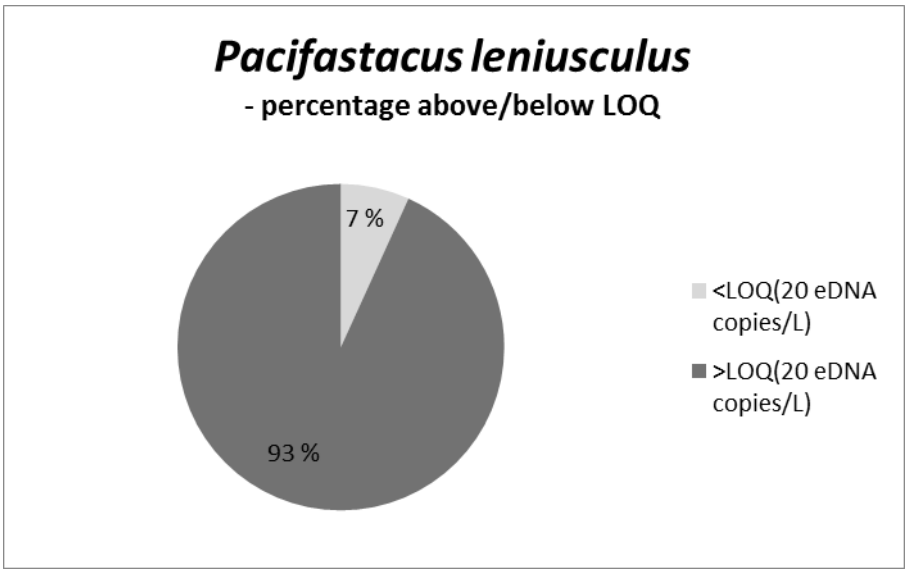
Supplementary table 4. qPCR results and data from all experiments.

Experiment	Tank	Number	Food	Temp	Filter	Av.Ct.A.astaci	Tot.PFU.A.astaci	Av.Ct.P.leniusculus	Tot.PFU.P.leniusculus	pH	Nitrate	Nitrite	Cl2	KH	Oxy.conc	Oxy.sat
Ex1	B5	2	N	18,70	CL9	41,000	0,000	40,210	16,726	6,80	0,00	0,00	NA	4,5	8,46	93,63
Ex1	B5	2	N	18,70	CL10	40,550	11,766	41,000	0,000	6,80	0,00	0,00	NA	4,5	8,46	93,63
Ex1	B5	2	N	18,70	CL11	41,000	0,000	41,000	0,000	6,80	0,00	0,00	NA	4,5	8,46	93,63
Ex1	B12	2	F	20,40	CL12	41,000	0,000	41,000	0,000	6,80	0,00	0,00	NA	6,0	7,85	89,88
Ex1	B12	2	F	20,40	CL13	41,000	0,000	41,000	0,000	6,80	0,00	0,00	NA	6,0	7,85	89,88
Ex1	B12	2	F	20,40	CL14	41,000	0,000	41,000	0,000	6,80	0,00	0,00	NA	6,0	7,85	89,88
Ex1	B13	20	F	21,25	CL15	41,000	0,000	40,070	18,358	7,27	18,33	1,58	NA	8,0	6,78	78,00
Ex1	B13	20	F	21,25	CL16	41,000	0,000	39,780	57,200	7,27	18,33	1,58	NA	8,0	6,78	78,00
Ex1	B13	20	F	21,25	CL17	41,000	0,000	38,450	56,600	7,27	18,33	1,58	NA	8,0	6,78	78,00
Ex1	C13	20	N	19,85	CL18	41,000	0,000	36,490	429,070	6,87	12,50	1,00	NA	4,0	8,00	89,75
Ex1	C13	20	N	19,85	CL19	41,000	0,000	36,125	576,400	6,87	12,50	1,00	NA	4,0	8,00	89,75
Ex1	C13	20	N	19,85	CL20	41,000	0,000	35,865	642,300	6,87	12,50	1,00	NA	4,0	8,00	89,75
Ex2	B5	20	N	18,94	CL21	41,000	0,000	28,600	77180,000	6,40	0,00	0,00	0,9	6,0	7,80	85,20
Ex2	B5	20	N	18,94	CL22	41,000	0,000	28,450	90460,000	6,40	0,00	0,00	0,9	6,0	7,80	85,20
Ex2	B5	20	N	18,94	CL23	36,620	725,600	28,265	102120,000	6,40	0,00	0,00	0,9	6,0	7,80	85,20
Ex2	B12	20	F	20,52	CL24	39,480	48,000	33,665	3375,200	6,40	0,00	0,00	0,8	6,0	6,46	73,60
Ex2	B12	20	F	20,52	CL25	40,220	28,800	33,185	3453,200	6,40	0,00	0,00	0,8	6,0	6,46	73,60
Ex2	B12	20	F	20,52	CL26	39,275	115,600	32,155	7106,000	6,40	0,00	0,00	0,8	6,0	6,46	73,60
Ex2	B13	2	F	20,36	CL27	37,970	787,000	31,710	9699,000	6,40	0,00	0,00	0,8	6,0	7,64	86,40
Ex2	B13	2	F	20,36	CL28	41,000	0,000	32,125	7626,000	6,40	0,00	0,00	0,8	6,0	7,64	86,40
Ex2	B13	2	F	20,36	CL29	41,000	0,000	33,590	2866,700	6,40	0,00	0,00	0,8	6,0	7,64	86,40
Ex2	C13	2	N	20,18	CL30	41,000	0,000	29,780	36032,000	6,40	0,00	0,00	0,8	4,5	8,78	98,80
Ex2	C13	2	N	20,18	CL31	40,615	44,528	29,690	38324,000	6,40	0,00	0,00	0,8	4,5	8,78	98,80
Ex2	C13	2	N	20,18	CL32	40,400	25,400	29,425	46160,000	6,40	0,00	0,00	0,8	4,5	8,78	98,80
Ex3	B5	2	F	19,72	CL33	41,000	0,000	35,755	776,000	6,40	13,75	0,75	0,8	6,0	7,87	87,50
Ex3	B5	2	F	19,72	CL34	41,000	0,000	34,990	1242,800	6,40	13,75	0,75	0,8	6,0	7,87	87,50
Ex3	B5	2	F	19,72	CL35	41,000	0,000	35,540	890,400	6,40	13,75	0,75	0,8	6,0	7,87	87,50
Ex3	B12	20	N	19,30	CL36	39,020	65,400	30,020	37801,000	6,40	23,75	1,88	0,8	6,0	7,37	80,50
Ex3	B12	20	N	19,30	CL37	41,000	11,000	30,080	38292,000	6,40	23,75	1,88	0,8	6,0	7,37	80,50
Ex3	B12	20	N	19,30	CL38	41,000	0,000	29,290	74160,000	6,40	23,75	1,88	0,8	6,0	7,37	80,50
Ex3	B13	2	N	18,15	CL39	41,000	0,000	29,405	68772,000	6,40	7,50	0,00	0,8	6,0	8,75	93,83
Ex3	B13	2	N	18,15	CL40	41,000	0,000	28,800	92940,000	6,40	7,50	0,00	0,8	6,0	8,75	93,83
Ex3	B13	2	N	18,15	CL41	41,000	0,000	31,230	42243,800	6,40	7,50	0,00	0,8	6,0	8,75	93,83
Ex3	C13	20	F	19,75	CL42	41,000	0,000	32,205	11866,000	6,60	17,50	1,25	0,8	6,0	7,54	79,00
Ex3	C13	20	F	19,75	CL43	41,000	0,000	32,205	9627,000	6,60	17,50	1,25	0,8	6,0	7,54	79,00
Ex3	C13	20	F	19,75	CL44	41,000	0,000	31,935	10079,000	6,60	17,50	1,25	0,8	6,0	7,54	79,00

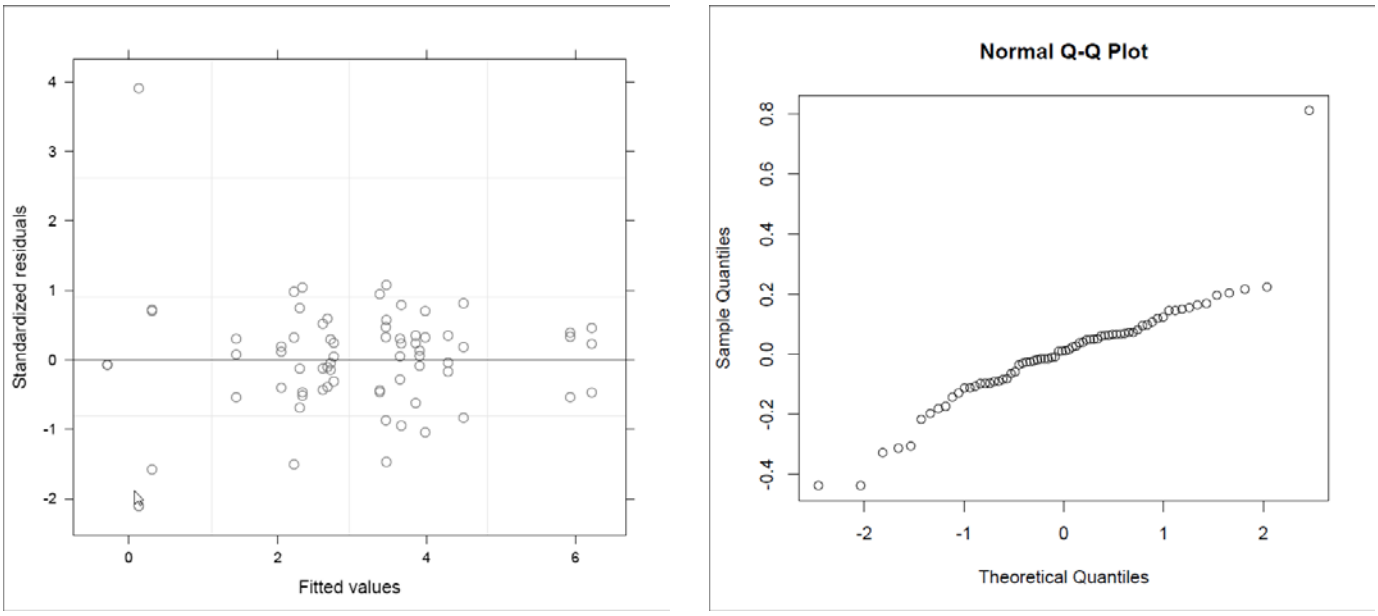
Experiment	Tank	Number	Food	Temp	Filter	Av.Ct.A.astaci	Tot.PFU.A.astaci	Av.Ct.P.leniusculus	Tot.PFU.P.leniusculus	pH	Nitrate	Nitrite	Cl2	KH	Oxy.conc	Oxy.sat
Ex4	B5	20	N	10,88	CL45	32,185	20468,000	20,425	26543000,000	6,60	2,50	0,13	NA	NA	9,41	86,38
Ex4	B5	20	N	10,88	CL46	31,985	23024,000	19,895	37102000,000	6,60	2,50	0,13	NA	NA	9,41	86,38
Ex4	B5	20	N	10,88	CL47	31,480	32188,000	19,740	41369000,000	6,60	2,50	0,13	NA	NA	9,41	86,38
Ex4	B12	20	F	11,00	CL48	31,645	255324,000	32,590	2860,000	6,60	0,00	0,00	NA	NA	8,49	80,13
Ex4	B12	20	F	11,00	CL49	27,905	347320,000	33,225	3744,000	6,60	0,00	0,00	NA	NA	8,49	80,13
Ex4	B12	20	F	11,00	CL50	27,865	356780,000	32,655	5678,000	6,60	0,00	0,00	NA	NA	8,49	80,13
Ex4	B13	2	F	11,66	CL51	33,410	8571,000	31,775	10286,000	6,50	6,25	0,50	NA	NA	10,15	94,38
Ex4	B13	2	F	11,66	CL52	34,040	5914,000	31,370	13412,000	6,50	6,25	0,50	NA	NA	10,15	94,38
Ex4	B13	2	F	11,66	CL53	33,495	8468,000	32,220	5844,000	6,50	6,25	0,50	NA	NA	10,15	94,38
Ex4	C13	2	N	10,95	CL54	36,885	1019,900	37,770	160,800	6,60	0,00	0,00	NA	NA	10,38	96,13
Ex4	C13	2	N	10,95	CL55	35,250	3630,400	37,040	386,600	6,60	0,00	0,00	NA	NA	10,38	96,13
Ex4	C13	2	N	10,95	CL56	34,210	5133,000	37,710	530,380	6,60	0,00	0,00	NA	NA	10,38	96,13
Ex5	B5	20	F	10,00	CL57	31,935	24578,000	33,700	2371,700	6,40	8,00	0,40	0,8	6,0	8,53	77,88
Ex5	B5	20	F	10,00	CL58	33,805	6794,000	33,880	1851,200	6,40	8,00	0,40	0,8	6,0	8,53	77,88
Ex5	B5	20	F	10,00	CL59	32,690	14582,000	33,495	2459,600	6,40	8,00	0,40	0,8	6,0	8,53	77,88
Ex5	B12	2	N	10,32	CL60	35,425	2202,800	30,885	16072,000	6,40	0,00	0,00	0,8	6,0	10,44	95,00
Ex5	B12	2	N	10,32	CL61	38,060	369,200	30,760	16950,000	6,40	0,00	0,00	0,8	6,0	10,44	95,00
Ex5	B12	2	N	10,32	CL62	39,285	153,600	31,375	10655,000	6,40	0,00	0,00	0,8	6,0	10,44	95,00
Ex5	B13	2	F	10,53	CL63	39,110	85,800	34,875	980,800	6,40	0,00	0,00	0,8	6,0	10,40	93,63
Ex5	B13	2	F	10,53	CL64	37,500	520,600	34,640	1278,200	6,40	0,00	0,00	0,8	6,0	10,40	93,63
Ex5	B13	2	F	10,53	CL65	41,000	0,000	34,630	1163,400	6,40	0,00	0,00	0,8	6,0	10,40	93,63
Ex5	C13	20	N	9,93	CL66	27,925	396460,000	28,770	71480,000	6,40	6,00	0,00	0,8	6,0	10,35	93,00
Ex5	C13	20	N	9,93	CL67	27,935	391940,000	28,870	66660,000	6,40	6,00	0,00	0,8	6,0	10,35	93,00
Ex5	C13	20	N	9,93	CL68	30,455	195166,000	31,400	37587,400	6,40	6,00	0,00	0,8	6,0	10,35	93,00
Ex6	B5	2	N	11,25	CL69	38,480	43,000	34,685	11699,600	6,40	0,00	0,00	0,8	6,0	10,35	96,63
Ex6	B5	2	N	11,25	CL70	41,000	0,000	31,425	22496,000	6,40	0,00	0,00	0,8	6,0	10,35	96,63
Ex6	B5	2	N	11,25	CL71	40,020	15,570	31,160	26982,000	6,40	0,00	0,00	0,8	6,0	10,35	96,63
Ex6	B12	20	N	11,00	CL72	30,095	28928,000	22,350	13199000,000	6,40	6,00	1,20	0,8	6,0	9,34	88,50
Ex6	B12	20	N	11,00	CL73	31,195	10492,000	22,020	20024000,000	6,40	6,00	1,20	0,8	6,0	9,34	88,50
Ex6	B12	20	N	11,00	CL74	31,925	6496,000	21,885	20598000,000	6,40	6,00	1,20	0,8	6,0	9,34	88,50
Ex6	B13	2	F	11,28	CL75	38,020	168,480	31,860	16652,000	6,40	0,00	0,00	0,8	6,0	10,30	96,50
Ex6	B13	2	F	11,28	CL76	35,450	639,000	31,920	15555,000	6,40	0,00	0,00	0,8	6,0	10,30	96,50
Ex6	B13	2	F	11,28	CL77	41,000	0,000	31,810	17264,000	6,40	0,00	0,00	0,8	6,0	10,30	96,50
Ex6	C13	20	F	11,25	CL78	30,525	16260,000	33,170	6604,000	6,40	6,00	0,10	0,8	6,0	9,50	89,13
Ex6	C13	20	F	11,25	CL79	30,235	19774,000	33,525	7654,000	6,40	6,00	0,10	0,8	6,0	9,50	89,13
Ex6	C13	20	F	11,25	CL80	30,655	14974,000	32,950	10406,000	6,40	6,00	0,10	0,8	6,0	9,50	89,13

Experiment	Tank	Number	Food	Temp	Filter	Av.Ct.A.astaci	Tot.PFU.A.astaci	Av.Ct.P.leniusculus	Tot.PFU.P.leniusculus	pH	Nitrate	Nitrite	Cl2	KH	Oxy.conc	Oxy.sat
Molting	B1	1	N	11,50	M1	29,010	137260,000	36,850	270,730	6,40	0,00	0,00	0,8	6,0	10,10	94,00
Molting	B1	1	N	11,50	M2	29,665	87800,000	36,625	362,940	6,40	0,00	0,00	0,8	6,0	10,10	94,00
Molting	B1	1	N	11,50	M3	30,275	57920,000	36,065	448,600	6,40	0,00	0,00	0,8	6,0	10,10	94,00
Molting	B2	1	N	NA	M4	41,000	0,000	27,515	210560,000	NA	NA	NA	NA	NA	NA	NA
Molting	B2	1	N	NA	M5	41,000	0,000	27,380	226060,000	NA	NA	NA	NA	NA	NA	NA
Molting	B2	1	N	NA	M6	41,000	0,000	28,210	124080,000	NA	NA	NA	NA	NA	NA	NA
Molting	B3	1	S	10,52	M7	27,530	373620,000	23,985	2599800,000	6,40	2,50	0,00	0,8	6,0	10,77	98,00
Molting	B3	1	S	10,52	M8	30,575	168314,000	27,070	990840,000	6,40	2,50	0,00	0,8	6,0	10,77	98,00
Molting	B3	1	S	10,52	M9	30,815	168592,000	27,160	882420,000	6,40	2,50	0,00	0,8	6,0	10,77	98,00
Molting	B4	1	S	11,22	M10	23,720	5872000,000	30,505	22444,000	6,40	0,00	0,00	0,8	6,0	10,34	95,60
Molting	B4	1	S	11,22	M11	23,620	6258000,000	30,540	20929,000	6,40	0,00	0,00	0,8	6,0	10,34	95,60
Molting	B4	1	S	11,22	M12	23,310	7688000,000	30,010	30954,000	6,40	0,00	0,00	0,8	6,0	10,34	95,60
Molting	B5	1	S	11,95	M13	23,025	9250000,000	28,465	87250,000	6,40	0,00	0,00	0,8	6,0	10,37	94,50
Molting	B5	1	S	11,95	M14	22,980	9566000,000	28,665	79560,000	6,40	0,00	0,00	0,8	6,0	10,37	94,50
Molting	B5	1	S	11,95	M15	22,600	12290000,000	28,210	103490,000	6,40	0,00	0,00	0,8	6,0	10,37	94,50
Graveyard	B13	20	NA	12,89	G1	41,000	0,000	31,105	53240,000	6,60	0,00	0,00	0,8	7,0	8,20	77,88
Graveyard	B13	20	NA	12,89	G2	41,000	0,000	30,240	68200,000	6,60	0,00	0,00	0,8	7,0	8,20	77,88
Graveyard	B13	20	NA	12,89	G3	41,000	0,000	30,595	44147,000	6,60	0,00	0,00	0,8	7,0	8,20	77,88
Graveyard	B12	2	NA	12,84	G4	34,390	5412,000	31,355	23362,000	6,40	0,00	0,00	0,8	6,0	10,03	95,50
Graveyard	B12	2	NA	12,84	G5	33,830	7458,000	31,410	25196,000	6,40	0,00	0,00	0,8	6,0	10,03	95,50
Graveyard	B12	2	NA	12,84	G6	31,670	15908,000	28,875	1476690,000	6,40	0,00	0,00	0,8	6,0	10,03	95,50
Graveyard	C13	2	NA	12,51	G7	41,000	0,000	28,040	242670,000	6,40	0,00	0,00	0,8	6,0	10,25	97,00
Graveyard	C13	2	NA	12,51	G8	37,870	495,800	28,555	226200,000	6,40	0,00	0,00	0,8	6,0	10,25	97,00
Graveyard	C13	2	NA	12,51	G9	40,095	130,400	28,470	200830,000	6,40	0,00	0,00	0,8	6,0	10,25	97,00

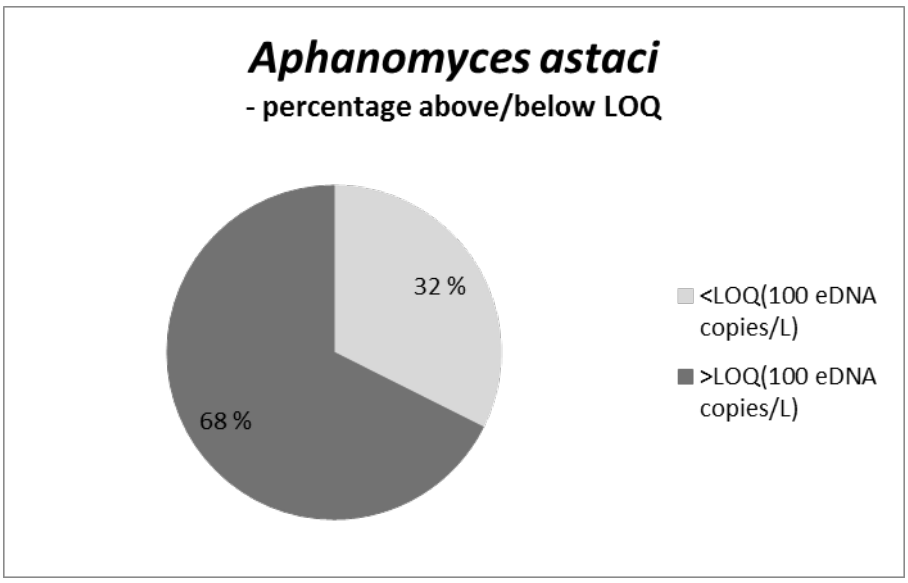
Ex1, Ex2, Ex3, Ex4, Ex5, Ex6 = Experiment 1: temperature, density and feeding regime.
Moulting = Experiment 2.
Death = Experiment 3.
PFU = detectable eDNA copies
Av.Ct.A.astaci = average CT value *Aphanomyces astaci* specific qPCR (A+B sample).
Av.Ct.P.leniusculus = average CT (value *Pacifastacus leniusculus* specific qPCR (A+B sample).
Tot.PFU.A.astaci = PFU/L *Aphanomyces astaci.*
Tot.PFU.P.leniusculus = PFU/L *Pacifastacus leniusculus*.
Temp = average temperature °C.



Supplementary figure 1. The percentage of the *Pacifastacus leniusculus* filter samples that yielded values above and below LOQ (limit of quantification).



Supplementary figure S.3. Residuals and normal distribution for *P. leniusculus* model in R. Valid for Experiment 1.



Supplementary figure 2. The percentage of the *Aphanomyces astaci* filter samples that yielded values above and below LOQ (limit of quantification).

Supplementary table 5. Overview over prevalence in experimental tanks in Experiment 1: temperature, density and food availability.

Ex1	Prevalence
#2 *P. leniusculus*, 20 °C, no food	½ tested. 1 positive.
#2 *P. leniusculus*, 20 °C, food	½ tested. 1 negative.
#20 *P. leniusculus*, 20 °C, no food	12/20 tested. 11/12 positive.
#20 *P. leniusculus*, 20 °C, food	11/20 tested. 9/11 positive.

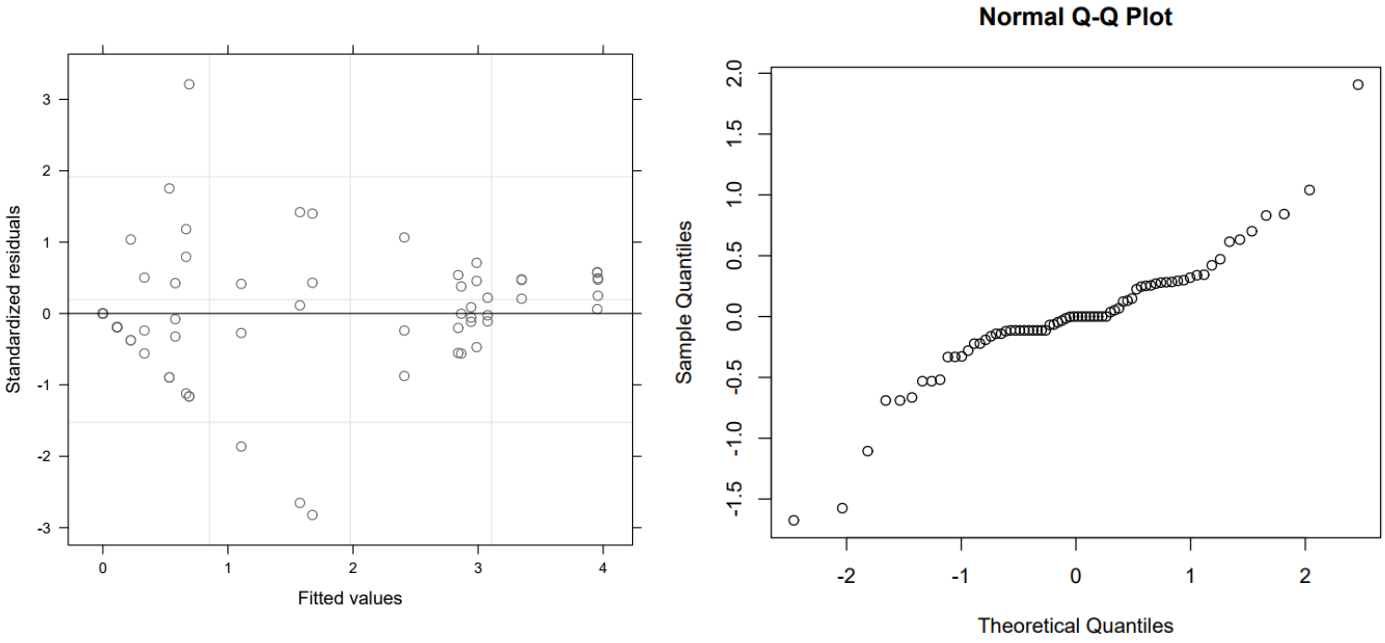
Ex2	Prevalence
#2 *P. leniusculus*, 20 °C, no food	2/2 tested. 2/2 positive.
#2 *P. leniusculus*, 20 °C, food	2/2 tested. 2/2 positive.
#20 *P. leniusculus*, 20 °C, no food	11/20 tested. 9/11 positive.
#20 *P. leniusculus*, 20 °C, food	11/20 tested. 9/11 positive.

Ex3	Prevalence
#2 *P. leniusculus*, 20 °C, no food	½ tested. 1 positive.
#2 *P. leniusculus*, 20 °C, food	2/2 tested. ½ positive.
#20 *P. leniusculus*, 20 °C, no food	10/20 tested. 8/10 positive.
#20 *P. leniusculus*, 20 °C, food	12/20 tested. 10/12 positive.

Ex4	Prevalence
#2 *P. leniusculus*, 20 °C, no food	½ tested. ½ positive.
#2 *P. leniusculus*, 20 °C, food	½ tested. ½ positive.
#20 *P. leniusculus*, 20 °C, no food	12/20 tested. 10/12 positive.
#20 *P. leniusculus*, 20 °C, food	13/20 tested. 10/13 positive.

Ex5	Prevalence
#2 *P. leniusculus*, 20 °C, no food	0/2 tested.
#2 *P. leniusculus*, 20 °C, food	2/2 tested. ½ positive.
#20 *P. leniusculus*, 20 °C, no food	6/20 tested. 6/6 positive.
#20 *P. leniusculus*, 20 °C, food	11/20 tested. 7/11 positive.

Ex6	Prevalence
#2 *P. leniusculus*, 20 °C, no food	2/2 tested. ½ positive.
#2 *P. leniusculus*, 20 °C, food	1/2 tested. 0/1 positive.
#20 *P. leniusculus*, 20 °C, no food	8/20 tested. 7/8 positive.
#20 *P. leniusculus*, 20 °C, food	8/20 tested. 6/8 positive.



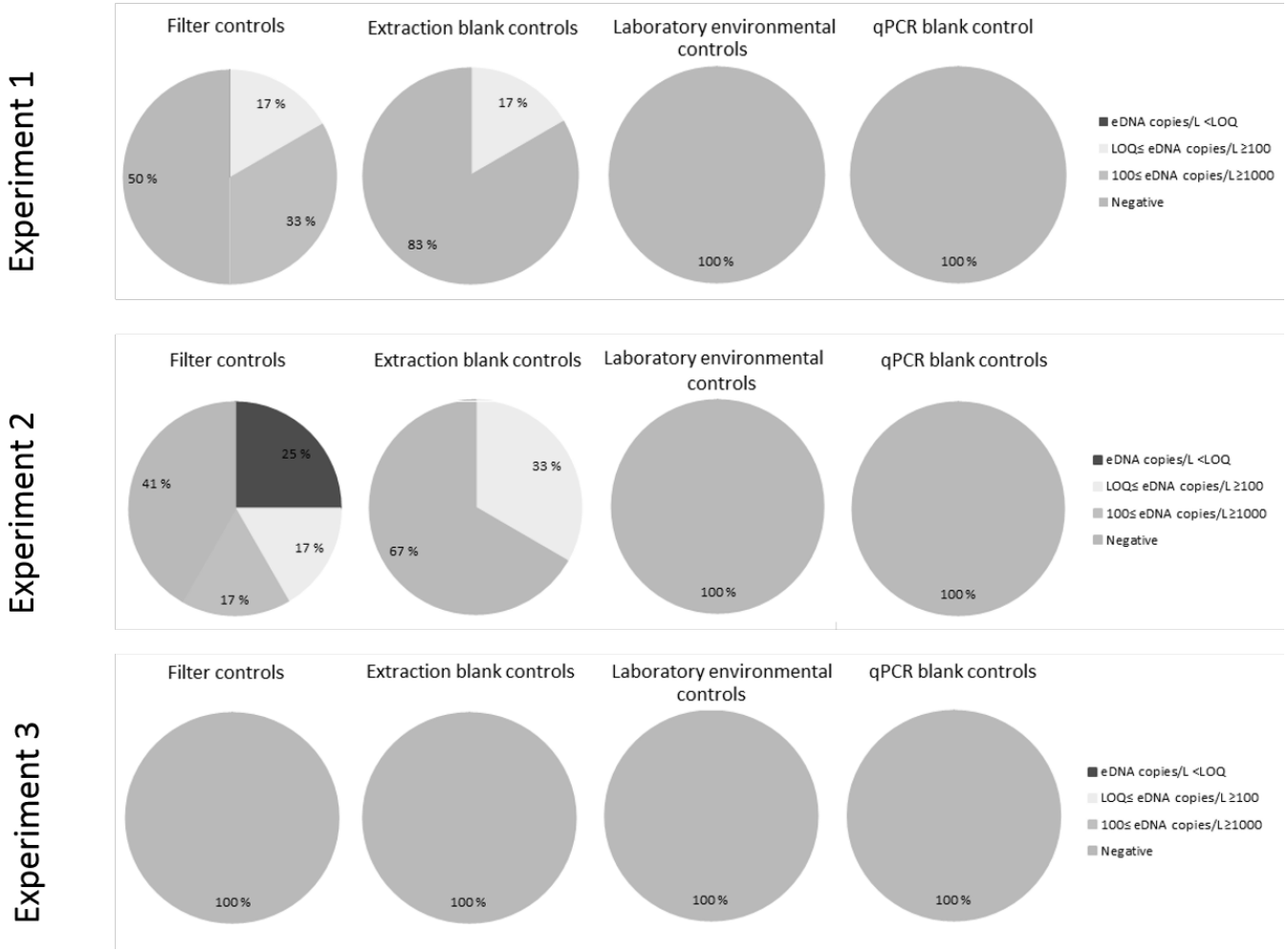
Supplementary figure 4. Residuals and normal distribution for Aphanomyces astaci model in Rstudio. Valid for Experiment 1.

Supplementary table 6. Results from *A. astaci* specific qPCR from uropod tissue samples.

Well Name	Ct	Ct*10	PFU	PFU*10
8Hx1 Krepsepest	36,79	0,00	28,20	0,00
12Hx1 Krepsepest	37,43	0,00	18,50	0,00
22Hx1 Krepsepest	34,12	39,16	166,00	5,88
27Hx1 Krepsepest	32,78	37,22	403,00	21,30
29Hx1 Krepsepest	34,50	38,56	129,00	8,78
31Hx1 Krepsepest	36,04	38,25	46,60	10,80
33HX1 Krepsepest	31,81	35,98	766,00	48,40
36Hx1 Krepsepest	37,28	41,50	20,50	1,25
37Hx1 Krepsepest	35,70	39,66	58,30	4,24
51Hx1 Krepsepest	36,26	0,00	40,10	0,00
59HX1 Krepsepest	33,66	38,27	225,00	10,60
62Hx1 Krepsepest	33,74	37,38	213,00	19,20
66Hx1 Krepsepest	30,46	34,90	1870,00	98,80
72Hx1 Krepsepest	40,02	41,28	3,33	1,45
76Hx1 Krepsepest	35,03	40,38	90,60	2,62
81Hx1 Krepsepest	37,29	40,24	20,30	2,89
86Hx1 Krepsepest	31,94	36,38	702,00	37,20
87Hx1 Krepsepest	26,12	30,27	33100,00	2130,00
95Hx1 Krepsepest	34,81	38,72	105,00	7,90
97Hx1 Krepsepest	37,34	0,00	19,60	0,00
99Hx1 Krepsepest	38,03	0,00	12,40	0,00
105Hx1 Krepsepest	36,23	40,24	41,10	2,87
108Hx1 Krepsepest	0,00	0,00	0,00	0,00
114Hx1 Krepsepest	27,83	32,37	10700,00	528,00
116Hx1 Krepsepest	34,65	38,66	117,00	8,21
118Hx1 Krepsepest	34,79	40,47	106,00	2,47
QCC1x1 Krepsepest	0,00	0,00	0,00	0,00
QCC2X1 Krepsepest	0,00	0,00	0,00	0,00
QCC3x1 Krepsepest	0,00	0,00	0,00	0,00
EBK1 Krepsepest	0,00	NA	0,00	NA
EBK2 Krepsepest	0,00	NA	0,00	NA
EMK1 Krepsepest	0,00	NA	0,00	NA
EMK2 Krepsepest	0,00	NA	0,00	NA
BLANK Krepsepest	0,00	NA	0,00	NA
BLANK Krepsepest	0,00	NA	0,00	NA
4^1 Krepsepest	21,23	NA	890000,00	NA
4^1 Krepsepest	21,13	NA	890000,00	NA
4^3 Krepsepest	24,65	NA	89000,00	NA
4^3 Krepsepest	24,56	NA	89000,00	NA
4^5 Krepsepest	28,19	NA	8900,00	NA
4^5 Krepsepest	27,93	NA	8900,00	NA
4^7 Krepsepest	31,73	NA	890,00	NA
4^7 Krepsepest	31,51	NA	890,00	NA

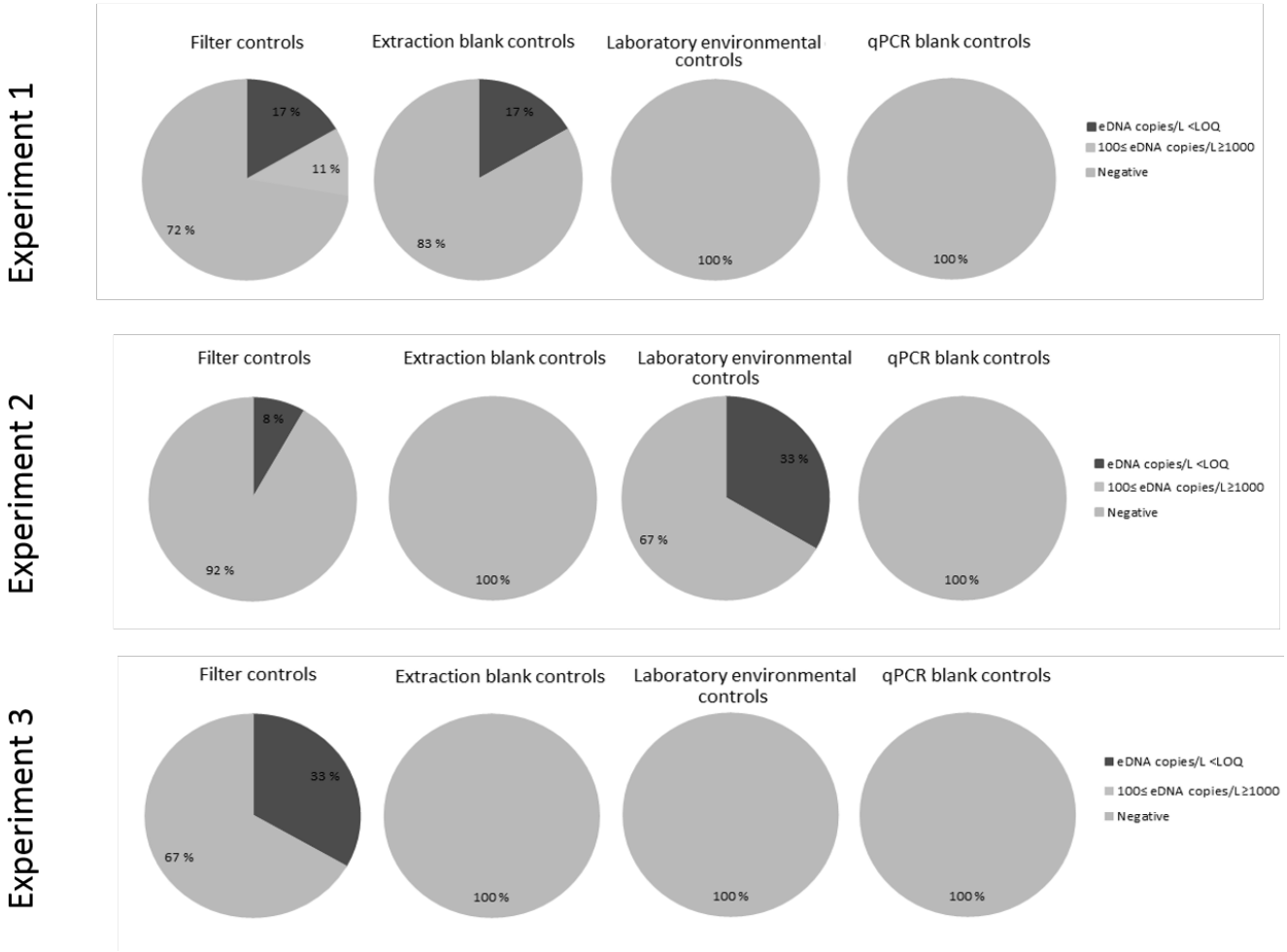
25Hx1 Krepsepest	34,16	38,47	108,90	5,82
38Hx1 Krepsepest	0,00	0,00	0,00	0,00
45Hx1 Krepsepest	0,00	0,00	0,00	0,00
52Hx1 Krepsepest	32,35	36,88	374,20	17,16
56Hx1 Krepsepest	0,00	0,00	0,00	0,00
60Hx1 Krepsepest	32,09	36,29	445,20	25,66
63Hx1 Krepsepest	36,76	0,00	18,51	0,00
65Hx1 Krepsepest	33,83	37,62	136,10	10,38
75Hx1 Krepsepest	0,00	0,00	0,00	0,00
77Hx1 Krepsepest	35,62	0,00	40,37	0,00
82Hx1 Krepsepest	34,50	40,21	86,58	1,78
94Hx1 Krepsepest	34,65	39,51	78,19	2,86
100Hx1 Krepsepest	33,68	38,92	151,00	4,26
101Hx1 Krepsepest	0,00	0,00	0,00	0,00
103Hx1 Krepsepest	0,00	0,00	0,00	0,00
107Hx1 Krepsepest	35,82	38,79	35,29	4,67
112Hx1 Krepsepest	33,60	40,09	159,20	1,92
120Hx1 Krepsepest	33,80	37,91	138,60	8,49
124Hx1 Krepsepest	33,04	39,29	232,80	3,32
?1Hx1 Krepsepest	33,66	39,51	153,20	2,87
U1Hx1 Krepsepest	0,00	0,00	0,00	0,00
U2Hx1 Krepsepest	36,59	39,38	20,86	3,13
U3Hx1 Krepsepest	35,83	0,00	35,00	0,00
U4Hx1 Krepsepest	34,87	39,24	67,17	3,44
QCC1 Krepsepest	0,00	0,00	0,00	0,00
QCC2 Krepsepest	0,00	0,00	0,00	0,00
EBK1 Krepsepest	0,00	NA	0,00	NA
EBK2 Krepsepest	0,00	NA	0,00	NA
EMK1 Krepsepest	0,00	NA	0,00	NA
EMK2 Krepsepest	0,00	NA	0,00	NA
BLANK Krepsepest	0,00	NA	0,00	NA
BLANK Krepsepest	0,00	NA	0,00	NA
4^1 Krepsepest	21,08	NA	890000,00	NA
4^1 Krepsepest	20,93	NA	890000,00	NA
4^3 Krepsepest	24,52	NA	89000,00	NA
4^3 Krepsepest	23,90	NA	89000,00	NA
4^5 Krepsepest	27,62	NA	8900,00	NA
4^5 Krepsepest	27,59	NA	8900,00	NA
4^7 Krepsepest	31,39	NA	890,00	NA
4^7 Krepsepest	30,92	NA	890,00	NA

Pacifastacus leniusculus



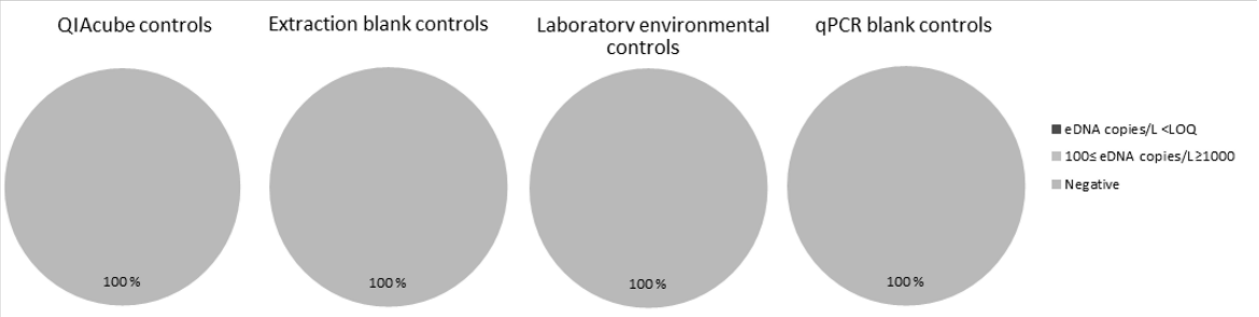
Supplementary figure 5. Overview over the *Pacifastacus leniusculus* control samples used in experiments 1-3.

Aphanomyces astaci



Supplementary figure 6. Overview over the *Aphanomyces astaci* control samples used in experiments 1-3.

Aphanomyces astaci



Supplementary figure 7. Overview over the *Aphanomyces astaci* control samples used in the tissue extraction procedure.

Supplementary table 7. Control values from all the experiments.

Experiment		Av.Ct.A.astaci	Tot.PFU.A.astaci	Av.Ct.P.leniusculus	Tot.PFU.P.leniusculus
Ex1	EX1C1	41,00	0,00	41,00	0,00
	EX1C2	41,00	0,00	41,00	0,00
	EX1C3	41,00	0,00	41,00	0,00
	EX1 EBK	41,00	0,00	41,00	0,00
	EX1 EMK	41,00	0,00	41,00	0,00
	EX1 BLANK	41,00	0,00	41,00	0,00
Ex2	EX2C1	39,20	58,40	39,51	22,20
	EX2C2	41,00	0,00	41,00	0,00
	EX2C3	39,93	35,20	41,00	0,00
	EX2 EBK	41,00	0,00	41,00	0,00
	EX2 EMK	41,00	0,00	41,00	0,00
	EX2 BLANK	41,00	0,00	41,00	0,00
Ex3	EX3C1	41,00	0,00	41,32	28,20
	EX3C2	40,43	12,00	40,34	26,40
	EX3C3	41,00	0,00	38,41	124,00
	EX3EBK	41,00	0,00	39,83	23,20
	EX3EMK	41,00	0,00	41,00	0,00
	EX3 BLANK	41,00	0,00	41,00	0,00
Ex4	EX4C1	41,00	0,00	41,00	0,00
	EX4C2	41,00	0,00	41,00	0,00
	EX4C3	41,00	0,00	41,00	0,00
	EX4 EBK	41,00	0,00	41,00	0,00
	EX4 EMK	41,00	0,00	41,00	0,00
	EX4 BLANK	41,00	0,00	41,00	0,00
Ex5	EX5C1	36,92	782,60	35,97	665,60
	EX5C2	41,00	0,00	35,47	658,40
	EX5C3	38,86	240,80	35,72	538,00
	EX5 EBK	40,41	34,80	82,00	0,00
	EX5 EMK	41,00	0,00	41,00	0,00
	EX5 BLANK	41,00	0,00	41,00	0,00
Ex6	EX6C1	41,00	0,00	39,62	178,80
	EX6C2	41,00	0,00	37,46	376,60
	EX6C3	41,00	0,00	41,00	0,00
	EX6 EBK	41,00	0,00	41,00	0,00
	EX6 EMK	41,00	0,00	41,00	0,00
	EX6 BLANK	41,00	0,00	41,00	0,00

Molting	MC1 17.2	41,00	0,00	37,24	188,40
	MC2 17.2	41,00	0,00	39,09	49,60
	MC3 17.2	41,00	0,00	37,59	211,60
	MC1 8.3	40,42	29,20	39,49	18,47
	MC2 8.3	41,00	0,00	39,82	14,56
	MC3 8.3	41,00	0,00	41,00	0,00
	MC1 9.3	41,00	0,00	41,00	0,00
	MC2 9.3	41,00	0,00	41,00	0,00
	MC3 9.3	41,00	0,00	39,62	16,42
	MC1 10.3	41,00	0,00	41,00	0,00
	MC2 10.3	41,00	0,00	37,60	69,40
	MC3 10.3	41,00	0,00	41,00	0,00
	EBK M1	41,00	0,00	41,00	0,00
	EMK M1	41,00	0,00	41,00	0,00
	BLANK M1	41,00	0,00	41,00	0,00
	EBK1 M2	41,00	0,00	38,74	70,93
	EMK1 M2	41,00	0,00	41,00	0,00
	EBK2 M2	41,00	0,00	41,00	0,00
	EMK2 M2	40,25	56,00	41,00	0,00
	BLANK M2	41,00	0,00	41,00	0,00
Graveyard	GC1	40,50	42,40	41,00	0,00
	GC2	41,00	0,00	41,00	0,00
	GC3	41,00	0,00	41,00	0,00
	EBK	41,00	0,00	41,00	0,00
	EMK	41,00	0,00	41,00	0,00
	BLANK	41,00	0,00	41,00	0,00

Ex1, Ex2, Ex3, Ex4, Ex5, Ex6 = Experiment 1: temperature, density and feeding regime.

Moulting = Experiment 2.

Death = Experiment 3.

PFU = detectable eDNA copies

Ct = cycle treshold

C1, C2, C3 = Filter controls

EBK = Extraction blank control

EMK = Laboratory environmental control

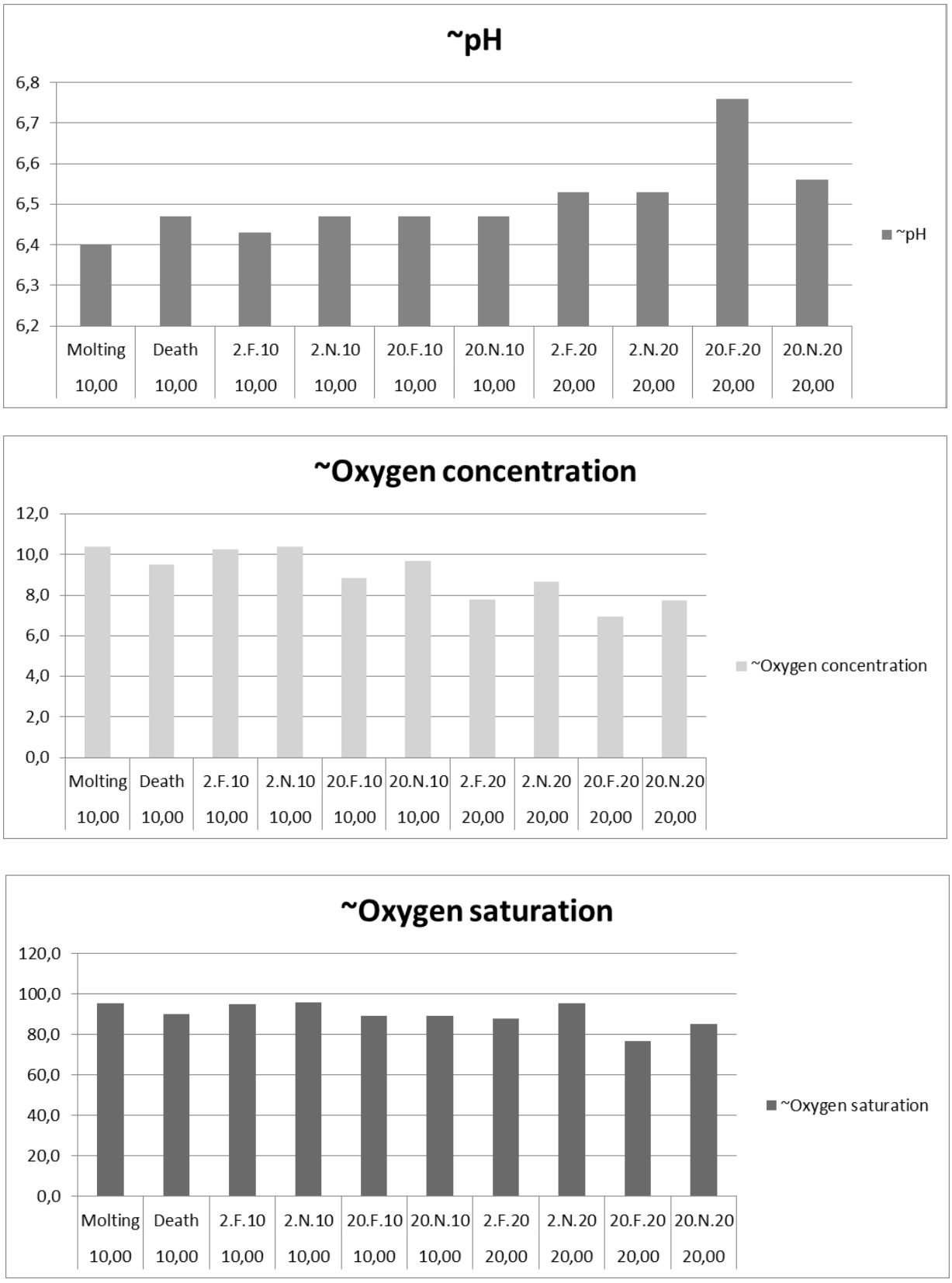
BLANK = qPCR blank control

Av.Ct.A.astaci = average CT value *Aphanomyces astaci* specific qPCR (A+B sample).

Av.Ct.P.leniusculus = average CT (value *Pacifastacus leniusculus* specific qPCR (A+B sample).

Tot.PFU.A.astaci = PFU/L *Aphanomyces astaci*.

Tot.PFU.P.leniusculus = PFU/L *Pacifastacus leniusculus.*



Supplementary figure 8. The water quality parametres a.) oxygen concentration., b.) oxygen saturation, and c.) pH.