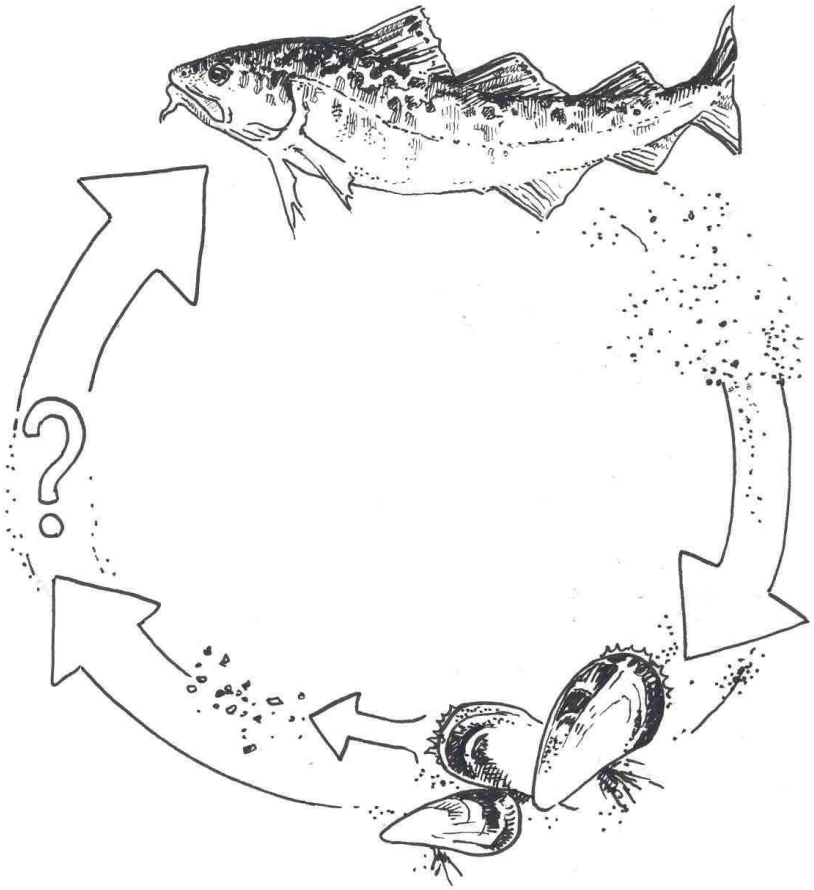
Observations on the survival of *Francisella noatunensis* in water and in blue mussels (*Mytilus edulis*)



Thesis for the degree

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Abstract:

Francisellosis was discovered in farmed Atlantic cod (*Gadus morhua*) in the western parts of Norway in 2004. The bacterium *Francisella noatunensis* was identified as the causative agent. Today, francisellosis is known as one of the most severe diseases affecting farmed cod, and it has resulted in great economical losses for the industry. The knowledge on mechanisms involved in the spreading of the pathogen is scarce; however transmission has been shown by experimental cohabitation. Vertical transmission may also be possible, as *F. noatunensis* have been detected in cod eggs and in farmed juveniles. The bacteria have been detected in a number of wild fish species, in blue mussels (*Mytilus edulis*) and edible crab (*Cancer pagurus*). In the present study, four experiments were conducted in order to increase the knowledge concerning survival of *F. noatunensis* in freshwater and seawater at different temperatures, and the potential role blue mussels' play in spreading of the bacterium.

The results indicate that both temperature and salinity have an impact on the culturability of *F. noatunensis*. Whether the bacteria are dead or have entered a viable but non culturable state, could not be determined, hence further research is needed to verify this state in *F. noatunensis* and its significance. *Francisella noatunensis* was rapidly filtered by the blue mussel and transported to the digestive diverticulae. The bacteria passed through the entire digestive system, and experiments showed that they were alive and infective in faeces shed by blue mussels. The mussels are thus clearly not capable of killing all *F. noatunensis* which pass through the digestive system. A cohabitation experiment with cod and blue mussels' previously exposed to *F. noatunensis* did not lead to infection in cod; hence the role as a reservoir seems unlikely. Further, no evidence suggesting that the bacteria are capable of persisting and multiplying in the mussel tissues was found. Bacterial clearance from the mussels was relatively fast, however faeces particles with live and infective bacteria may be passed on to the next trophic level.

Experience is the name everyone gives to their mistakes.

Oscar Wilde, *Lady Windermere's Fan, 1892, Act III*

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I. Introduction:

Atlantic cod (*Gadus morhua*) is one of the most exploited cold-water fish species on the northern hemisphere. It is distributed on both sides of the Atlantic Ocean in several stocks, were each stock has its own distinct life history and migration pattern. The North-East Arctic cod is the largest stock and migrates from feeding areas in the Barents Sea to the spawning grounds at Lofoten and along the Norwegian coast (Svåsand et al. 2004). Processing and turnover of both North-East Arctic- and coastal cod have influenced the settlement and social infrastructure in Norway through thousands of years (Borthen et al. 2005). As early as in the ninth century Norsemen had already established plants for processing dried cod in Norway and were trading the surplus in Northern Europe. The fish stocks in the North Atlantic were in 1946 at a peak level, as a result of six years with limited fishing activity during world war II (Kurlansky 1998). This resulted in increased landings, and since then, the annual catch from most of the wild stocks has been declining due to decreasing stocks (Svåsand et al. 2004). In 1989 the Norwegian government decided to restrict the fishery and after two years, measurements showed that the cod stock was on a rice again (Kurlansky 1998). Despite this, the coastal cod has been on Norway's red list of endangered species since 2006 and in 2008 it was measured to a historically low level (Berg 2009, Svåsand et al. 2009).

Cultivation of Cod

Stock enhancement programs, with the hatching of cod eggs and release of yolk sack larvae were started as early as in the 1880's at the Institute of Marine Research in Flødevigen, Arendal, Norway (Svåsand et al. 2004, Borthen et al. 2005). This was done as an attempt to increase the Atlantic cod stock (Svåsand et al. 2004, Øiestad 2005, Svåsand et al. 2007). Although the benefits of the release were never documented, this practice continued for nearly 90 years (Svåsand et al. 2004). Extensive production experiments were started in the mid 1970's (Svåsand et al. 2007) and a few years later successful mass production in large enclosures were made possible (Øiestad et al. 1985).

The cod farming industry have continued to grow during the recent years, and the total production in Norway increased more than ten-fold from 2003 to 2006, giving a total production of approximately 10 000 tons (Svåsand et al. 2007). Further growth during the coming years due to increased market needs and the diminishing supply from fisheries is expected (Brown et al. 2003, Rosenlund & Skretting 2006).

Some biologists believe that gadoid culture have the potential to reach the same production levels as salmon farming within the next 15 – 20 years, and a worldwide production of 150 – 200 000 tons by 2010 has been predicted (Brown et al. 2003, Rosenlund & Skretting 2006). Commercial farming facilities of cod are established in Norway, the United Kingdom, on the east coast of USA and Canada, in addition to some smaller farming facilities on Iceland (Rosenlund & Skretting 2006). In Norway an estimated 13 500 tons of farmed cod were slaughtered in 2008, this represent an increase of approximately 25% from 2007 (Lassen 2009).

Whether this growth continues and predictions come true depends largely on the ability to prevent and treat diseases. Fish cultivated in large densities in small net pens are likely to experience an increased rate of infection compared to wild populations. The high density of fish kept in relatively small areas compared to the situation in the wild, will give pathogenic microorganisms great advantages like easy access to new hosts. In addition, the amount of stress which the fish is experiencing due to large densities and handling, might make the host even more susceptible to opportunistic pathogens (Bergh 2002).

Diseases affecting cod

In farming of salmonids, transfer from freshwater to seawater represents a barrier to a wide range of parasites and other infectious agents. Gadoids who live their entire life in seawater lack this barrier and may therefore be more vulnerable to pathogenic parasites, bacteria and viruses (Kjesbu et al. 2006). Parasitic infections in skin and gills caused by *Ichtyobodo* spp., *Trichodina* spp. and *Gyrodactylus* spp. cause problems in the cultivation of cod (Karlsbakk et al. 2009). Some viruses have also caused diseases in farming of gadoids such as infectious pancreatic necrosis virus (IPNV), nodavirus, and viral haemorrhagic septicaemia virus (VHSV) (Bricknell et al. 2006). Nevertheless, bacterial diseases (like vibriosis, francisellosis etc.) are considered to be one of the largest problems in Norwegian cod farming industry today.

Bacterial diseases affecting cod

For a long time, vibriosis has been one of the most serious diseases in cod farming (Samuelsen et al. 2006, Hellberg et al. 2009). It is caused by *Vibrio anguillarum,* and according to Bricknell et al. (2006) *V. anguillarum* serotype 02β is emerging as the major pathogenic serotype. Vibriosis is manifested as an acute haemorrhagic septicaemia and the main clinical signs are erythema of the head region and fin erosion (Larsen & Pedersen 2002,

Samuelsen et al. 2006). High initial mortalities without the characteristic symptoms can also be observed. The fish eventually becomes anorectic and dark pigmented with ulcers of varying sizes (Larsen & Pedersen 2002, Samuelsen et al. 2006).

Another serious bacterial infection in cod farming is atypical furunculosis which is caused by an atypical strain of *Aeromonas salmonicida* (Eggset & Gudmundsdottir 2002). The bacterium has been found in both wild and cultured Atlantic cod (Wiklund & Dalsgaard 1998, Magnadottir et al. 2002). The atypical strain may cause skin ulceration, with haemorrhages on the snout/mouth and the base of the fins, in addition to granulomas in most of the internal organs (Wiklund & Dalsgaard 1998, Eggset & Gudmundsdottir 2002, Magnadottir et al. 2002). From 2007 to 2008 an increased amount of outbreaks of atypical furunculosis was registered in Norwegian cod farming (Hellberg et al. 2009).

In 2004, a new systemic granulomatous disease affecting larger cod was detected in western Norway. The causative agent was shown to be an intracellular bacterium related to *Francisella philomiragia* (Nylund et al. 2006, Olsen et al. 2006). The disease is associated with mortalities and economical losses due to reduced quality or discarding of the fish, and francisellosis is at present defined as the most severe disease in Norwegian cod farming (Hellberg et al. 2009).

Francisella species and *Francisella*-like fish diseases

Francisella tularensis is a zoonotic bacterial disease, and probably the best known species in the genus *Francisella* which until recently comprised only *F. tularensis* and *F. philomiragia* (Tärnvik & Berglund 2003, Sjöstedt 2005) .

Francisella tularensis is known to be a serious human pathogen more commonly associated with rodents, and is one of the most infectious bacteria known (Dennis et al. 2001). Shape ranges from coccoid to short rod, it is a strictly aerobic, intracellular, Gram negative bacterium (reviewed by Tärnvik & Berglund 2003). As reviewed by Ellis et al. (2002) tularaemia is found in various terrestrial and aquatic animals like ground squirrels, rabbits, hares, voles, muskrats, water rats and other rodents, and thought to be maintained in the environment by these animals. Further on a range of ticks, biting flies and mosquitoes have been implicated as vectors and the ability the bacteria have to persist in water may be associated with amoebae. It has been shown that *F. tularensis* is capable of survival and growth inside *Acanthamoeba castellanii*, which is commonly found in natural aquatic systems (Abd et al. 2003).

Francisella philomiragia was first isolated from sick muskrats (*Ondatra zibethica*) and water samples in 1969 and believed to belong to genus *Yersinia* (Jensen et al. 1969). In 1989 *Yersinia philomiragia* was transferred to genus *Francisella* as *Franciella philomiragia* due to its considerable genetic relatedness to the species (Hollis et al. 1989). *Francisella philomiragia* is a small, non motile, strictly aerobic, intracellular, Gram – negative coccobacilli (Hollis et al. 1989, Wenger et al. 1989). The bacterium is less pathogenic than *F. tularensis* and has been isolated from water, muskrats (*O. zibethica*), and humans (near-drowning victims) (Hollis et al. 1989).

Rickettsia-like organisms (RLO) was first observed in diseased puffers (*Tetrodon fahaka*) in the Nile River in Egypt as early as in 1939. Later RLO's and PLO's (Piscirikettsia-like organisms) have been detected in a number of fish species around the world (reviewed by Mauel & Miller 2002). The PLO group have been shown to include both the *Pisckirickettsiaceae* and the *Francisellaceae* families, which is relatively closely related (Mikalsen 2008). *Piscirikettsia salmonis* is a Gram-negative obligate intracellular bacterium. It causes serious disease among salmonids and other fish in the marine environment with clinical signs like dark pigmenting, lethargia and macroscopic changes such as skin lesions, swollen spleen and discoloured kidney (reviewed by Fryer & Hedrick 2003).

Similar organisms have in later years been reported from both marine and fresh-water species worldwide (Fryer & Mauel 1997, Mauel & Miller 2002, Fryer & Hedrick 2003). In 2003 a novel intracellular bacterium was characterized in Hawaiian tilapia (*Oreochromis mossambicus* and *Sarotherodon melanotheron*). The most prominent clinical signs were pale fish which swam erratically and had internal macroscopic changes like enlarged spleens with multiple white granulomas. The bacterium had many characteristics in common with *P. salmonis*, though it was different in size, host, active temperature, genetics, pathology and antigenic variance. It was proposed that the bacterium should be considered a *Piscirickettsia*-like bacterium (Mauel et al. 2005).

Occurrences of other PLO's have also been reported in tilapia from the continental United States, and Tasmanian farmed Atlantic salmon (Corbeil et al. 2005, Mauel et al. 2005). These cases of PLO's have retrospectively been confirmed as infections with *Francisella* spp. (Hsieh et al. 2006, Birkbeck et al. 2007, Mauel et al. 2007*)*.

Kamaishi et al. (2005) reported the first verified *Francisella* infections and case of francisellosis in farmed three-line grunt (*Parapristipoma trilineatum*) in Japan. Affected fish showed signs of granulomas in kidney and spleen. On the basis of the phylogenetic analysis, the closest relative organism was *Francisella philomiragia*. *Francisella* like organisms have

also been detected in hybrid striped bass (*Morone chrysops* x *M. Saxatilis*) and ornamental cichlids (Ostland et al. 2006, Hsieh et al. 2007). Ottem et al (2009) proposed the name *F. noatunensis* subsp. *orientalis* for *Francisella* sp. from *P. trilineatum* and most *Francisella* isolates from tilapias worldwide have been confirmed to belong to that subspecies (Ottem et al. 2009).

Francisellosis – a relatively new problem in Norwegian aquaculture

The causative agent to the bacterial disease subsequently known as francisellosis which were detected in western Norway in 2004, was determined to be most closely related to *Francisella philomiragia* (Nylund et al. 2006). The novel bacterium was proposed as both a new species, *F. piscicida* (Ottem et al. 2007b) and a new subspecies, *F. philomiragia* subsp. *noatunensis* (Mikalsen et al. 2007). The two research groups responsible for the names agrees that they dealt with the same species of bacterium and an elevation of the senior heterotypic synonym *Francisella philomiragia* subsp. *noatunensis* was proposed by Ottem et al. (2009) and Mikalsen & Colquhoun (unpublished results). The name *Francisella noatunensis* will therefore be used in this thesis.

Affected fish showed signs of reduced swimming performance, loss of appetite and dark pigmentation (Nylund et al. 2006, Olsen et al. 2006). Few other external signs were found, except for some individuals who had granulomas in the skin, around gills and in the oral cavity (Nylund et al. 2006). Internal signs ranged from slightly swollen spleen and kidney to white granulomas covering and infiltrating the spleen, kidney and heart (Nylund et al. 2006, Olsen et al. 2006). Histological examination revealed an extensive chronic granulomatous inflammation in these organs toghether with the lamina propria of the intestine (Nylund et al. 2006, Olsen et al. 2006). Focal granulomatous inflammation were visible in the epicardium and spongious myocardium of the heart, white muscle and in filaments and lamellae of the gills. Granulomas were also detected in the external eye muscle and chroid rete of the eye (Olsen et al. 2006).

The bacterium was characterized as a facultative intracellular Gram negative bacterium, with a shape ranging from coccid to short rod, with a size range of 0.5 µm-1.5 µm. It is aerobic, with a growth temperature of 10 – 25˚C, with an optimum at ca 20˚C. The bacterium is oxidase negative and weakly catalase positive. It does not produce H_2S on triple sugar iron agar (TSI), does not hydrolyze gelatine and addition of cystein to the growth medium enhances growth (Olsen et al. 2006, Ottem et al. 2007a).

F. noatunensis is found to be present in phagocytes in the spleen and kidney of infected fish, but it is also found in endothelial cells lining the heart chambers and in leucocytes attached to the blood vessel walls in the liver, pseudobranch and gills. This may indicate that the target cells are phagocytes and other cells with phagocytic activity (Nylund et al. 2006). As the disease progress, the granulomas consist mainly of host cells (phagocytes, fibroblasts and lymphocytes) organized in concentric cellular layers, with little or no bacteria present in the centre (Nylund et al. 2006, Olsen et al. 2006). In the last stage of the disease there is a prominent necrosis in the core, and the dead cells in the centre are replaced by transparent liquid. At this stage no bacteria can be detected by microscopy in the core vacuole (Nylund et al. 2006).

Horizontal transfer of *F. noatunensis* has been shown in laboratory experiments (Nylund et al. 2006, Nordstrøm 2008, Mikalsen et al. 2009) and it has during the later years shown a great potential to cause severe problems in cod farms (Hellberg et al. 2009). From 2004 to 2006 a screening of both farmed and wild Atlantic cod off the coast of Norway was done to determine the prevalence of *F. noatunensis*. Results showed that farmed cod from most counties in Norway were positive for *F. noatunensis* when tested with real-time RT-PCR. The examination of wild cod showed that of 422 sampled cod were 6.6% positive for *F. noatunensis* (Ottem et al. 2008). As stated earlier is the bacteria readily transmitted horizontally over short distances. However, Ottem et al (2008) postulate that the presence of the bacteria in wild cod is probably not a result of farming activities alone. The bacterium may be shed into water by faecal matter but the distribution route and potential vectors are not yet fully understood (Mikalsen et al. 2009).

Parker et al. (1951) have stated that the *F. tularensis* bacterium is capable of surviving one year in the aquatic environment. It is still not certain whether this also applies to *F. noatunensis* or not, although the bacterium have been observed to survive on the same agar plate for one year (Nylund & Ottem 2006a). The fact that there is little or no knowledge on the survival of *F. noatunensis* in the marine environment is of great concern when new farming facilities are to be established; and a better understanding of the risk of transmitting *F. noatunensis* from one farming facility to another is necessary.

There is no published data on the accumulation or survival of *F. noatunensis* in bivalve molluscs or other filtrating invertebrates, and their potential role as trophic transmission. Their potential as a reservoir in the dispersion of francisellosis is therefore still unknown. Blue mussels (*Mytilus edulis*) are widespread in the marine environment and commonly present on farming facilities in Norway. The bivalve and its ability to filtrate and

clear water of particles (clearance rate) have been widely studied. An ongoing review which has compared the clearance rate (CR) in mytilid species from 61 studies state that the mean CR is 2.6 L $g^{-1}h^{-1}$, this shows that the mussel is capable of filtrating large quantities of water (Cranford et al. in prep). The ability the mussel has to retain particles from water depends on the size of the particle. In general will bivalves completely retain particles above 4 µm, the efficiency in retaining particles below 2 µm decrease to between 35 – 70% and to approximately 20% of particles of 1 µm (Birkbeck & McHenery 1982, Riisgård 1988). However, studies have shown that mussels are capable of retaining virus for shorter or longer periods of time when exposed through cohabitation (Mortensen et al. 1992, Skår & Mortensen 2007).

The infectious salmon anaemia virus (ISAV) was readily removed (after 4 days) while the infectious pancreatic necrosis virus (IPNV) persisted for at least 50 days, though this may be due to differences in the virus' ability to survive (Mortensen et al. 1992, Skår & Mortensen 2007). Marine bivalve molluscs have also been reported to serve as potential reservoirs of certain finfish pathogens (Meyers 1984). A study on the clearing of the Gram negative intracellular bacterium *Renibacterium salmoninarum* from seawater by blue mussel was done by Paclibare et al. (1994). Findings in this experiment point toward the fact that the bivalve is capable of inactivating the bacterium in the digestive glands, hence it is unlikely to serve as a long term reservoir. After 21 days in clean water only two cells of the *R. salmoninarum* bacterium were detected (Paclibare et al. 1994)(Starliper & Morrison 2000).

No studies have been conducted to determine the blue mussels role in the spreading of *F. noatunensis*, however it has been isolated from blue mussels (*Mytilus edulis*) from the environment of infected cod farms (Ottem et al. 2008). It is therefore highly relevant to determine the role of the blue mussel in the survival and spreading of *F. noatunensis*.

Aims of the study:

The aim of the present study was to contribute to knowledge on the spreading and transmission routes of *F. noatunensis* in the marine environment, with a special focus on the role of blue mussels. This study consists of four experiments investigating the survival of *F. noatunensis* in seawater and freshwater at different temperatures, the blue mussels' ability to kill *F. noatunensis* in the digestive system, and a cohabitant challenge to see whether cod was infected after cohabitation with blue mussels previously exposed to *F. noatunensis*.

II. Material and methods:

Experimental animals and *Francisella noatunensis* culture

Blue mussels

The blue mussels used in the experiments originated from a wild population at Svindal, Lindås, North of Bergen, Norway, with no fish farms in the vicinity of the collection site in April 2008. Mussels with a shell length of approximately 5 cm were selected and kept in a storage tank with running filtered seawater at approximately 9ºC at the Institute of Marine Research. As a control for unexposed blue mussels in the cohabitant experiment (exp.4), 5 mussels from the batch used were sampled and analysed with real-time RT-PCR (see page 27-30) at the start of the experiment. An additional 10 negative control samples were taken at day 88 and 5 at the end of the experiment, all negative controls were taken from the storage tank.

Atlantic cod

Cod used in experiment 2 & 3 originated from Parisvatnet, near Bergen, Norway. They had been dip vaccinated with Norvax- Compact 6 when they were approximately 5 - 10 g. At the start of the experiment the fish had a mean weight of 170 g. The cod were kept in 250 L tanks, with a water flow of 10 L/min, a temperature of 14˚C ± 0.1, salinity of 34.5‰ and oxygen saturation of 7.5 – 8.5 mg/L.

Ten cod, acting as a negative control group for experiments 2 and 3, were anesthetised to death by benzocaine prior to the experiment and kidney samples were analysed with real-time RT-PCR for the presence of *F. noatunensis*. In addition an untreated negative control group were kept at the same conditions as the injected groups.

The cod used in the cohabitation experiment (exp. 4) also originated from Parisvatnet. They were unvaccinated and had been given a prophylactic treatment with oxolinic acid for 3 months prior to the experiment. These cod were kept in 80 L tanks, with a water flow of 80 L/min, salinity of 34.5‰ and a temperature of 9˚C ± 0.1 for one month before the temperature was raised to 14˚C ± 0.1˚C for two months. Ten cod from the stock were anesthetised to death prior to the experiment and kidney samples were collected. In addition a negative control group were kept at the same conditions and handled as the cohabitation groups and samples were collected at termination of the experiment. All negative control groups were analysed with real-time RT-PCR for the presence of *F. noatunensis* (p. 27-30).

Anaesthesia used in this experiment was benzocaine (200g benzocaine in 1L ethanol), 2.5-3.0 ml in 10 litres of water in order to sedate cod, and 6.0 ml in 10 litres of water to anesthetise the cod to death.

Francisella noatunensis

The *Francisella noatunensis* strain (GM2212) used throughout this experiment originated from a disease outbreak in 2004, where it was isolated from the head kidney of Atlantic cod (Nylund et al. 2006).

***Francisella noatunensis* antiserum**

The anti-sera used for the detection of *F. noatunensis* in this thesis, were made from the *F. noatunensis* strain GM2212. The bacterium was grown on cystein heart agar plates (CHAB, see appendix 2), transferred to phosphate buffer and injected in rabbit (done by a laboratory in Belgium). The anti-sera had a titer of 1: 600 000, and have not been absorbed. It agglutinates *F. noatunensis*, and to some degree the *F. philomiragia* strain.

Experiment 1: Observation on the survival of *Francisella noatunensis* freshwater and seawater at different temperatures

An overview

Cells were harvested from agar plates and subjected to different environmental conditions in axenic cultures. At fixed times, broth was added and subsamples collected. 16S rRNA concentrations in subsamples were estimated by real-time RT-PCR. The tubes containing broth were incubated for 3 weeks and 16S rRNA concentrations were again determined. An increase in 16S rRNA concentration was regarded as an increase in cell number, and hence a proof of cell survival.

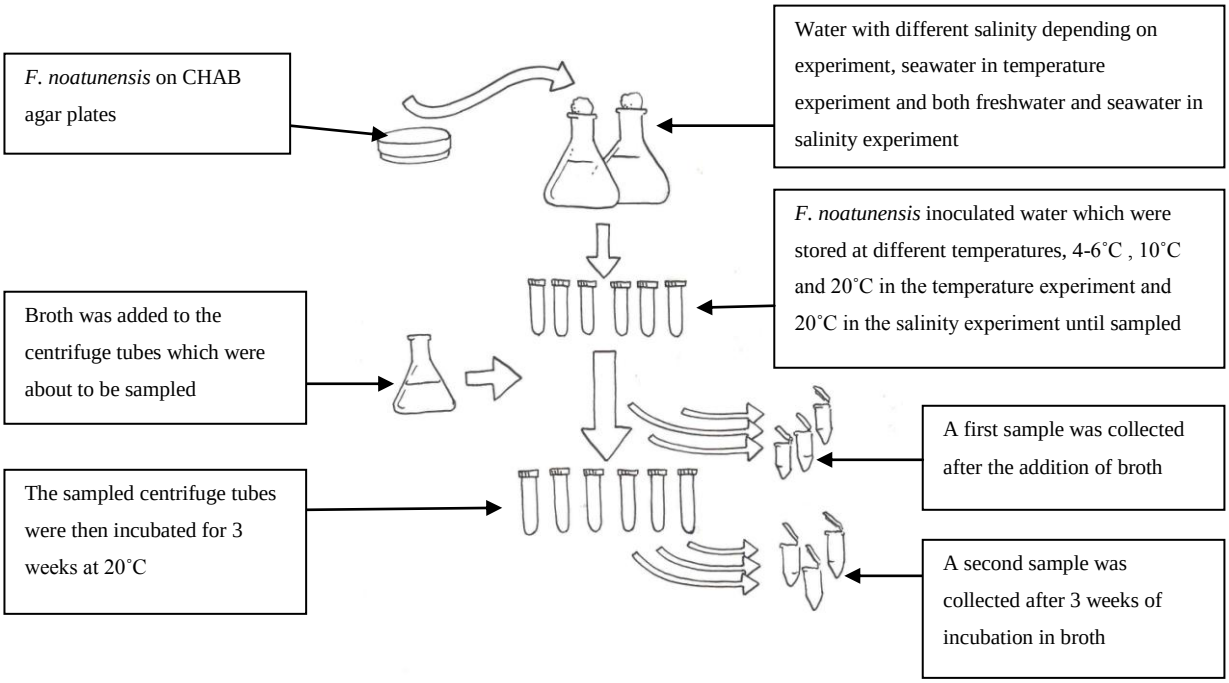


Fig. 1: Schematic overview of temperature and salinity experiment.

Prior to the *in vitro* experiment a pre-culture was made in order to test if the bacterium was able to survive and grow in a 1:4 water/broth ratio. The bacteria were cultured in 2.5 ml seawater mixed with 7.5 ml broth at 20˚C for three weeks. The cultivation of *F. noatunensis* in B1817 (see appendix 3) showed the most rapid growth compared to Bacto™ Eugon broth (see appendix 3), and based on these results the B1817 growth medium was used throughout this study.

Experimental design: temperature experiment

The survival of *F. noatunensis* at different temperatures was tested. Three different temperature intervals were used: 4-6°C, 10°C and 20°C. Water with an approximate salinity of 33‰ was collected, autoclaved and filter sterilized through 0.2 µm syringe filters prior to the addition of *F. noatunensis*. The bacteria were scraped of CHAB agar plates, and were not washed prior to the transfer to seawater. The *F. noatunensis* concentration was subsequently determined by real-time RT-PCR to a Ct value of 15.6 which corresponds to 1 x 10^8 bacteria pr ml (see p.34). A total of 135 sterile centrifuge tubes (50 ml), 45 for each temperature, were filled with 10 ml of inoculated water at day zero and stored at the respective temperatures in a stagnant system. Seawater from the batch used in the experiment was added to three 50 ml centrifuge tubes as a negative control. These were not inoculated with *F. noatunensis* and were sampled at time zero, at eight weeks and at the end of the experiment.

Experimental design: freshwater and seawater experiment

The survival of *F. noatunensis* in seawater and freshwater was tested. Water was collected with an approximate salinity of <0.5‰ (tap water) and 33‰, autoclaved and filter sterilized through 0.2 µm syringe filters. The collected freshwater and seawater were inoculated with *F. noatunensis* from CHAB agar plates in two Erlenmeyer flasks as described above in temperature experiment. The concentration was subsequently determined by real-time RT-PCR to a Ct value of 17.8 in seawater and 16.6 in freshwater which corresponds to 3 x 10^7 and 5 x 10^7 bacteria pr ml respectively (see p.34). A total of 90 centrifuge tubes, 45 for each of the two salinities were filled with 10 ml of the inoculated water at day zero and stored at 20˚C in a stagnant system. As a negative control, both fresh- and seawater used in the experiment was added in six 50 ml centrifuge tubes. These were not inoculated with *F. noatunensis* and were sampled at time zero, at eight weeks and at the end of the experiment in the same matter as the tubes containing *F. noatunensis* as described below.

Sampling

At sampling (day zero, 1 week, 2 weeks, 4 weeks, 8 weeks, 12 weeks and 24 weeks) were the centrifuge tubes, three for each of the two salinities, and three for each of the three temperatures, filled with 30 ml of B1817 growth medium (see appendix 2). A sample of 1 ml was collected from the 15 tubes immediately after the adding of broth and the samples were stored at -80˚C before analysed. All sampled centrifuge tubes were incubated at 20˚C at 150 rpm in a shaking incubator (Unitron, Infors AG) for three weeks before a second sample was collected and stored at -80˚C. RNA from all samples were extracted and analyzed by real-time RT-PCR according to protocols and normalised against the exogenous control *Halobacterium salinarum* (see p.28).

Experiment 2: Cod inoculated with tissue homogenate from *Francisella noatunensis* exposed blue mussels

An overview

Experiment 2 was conducted to examine the ability of blue mussels' to kill *F. noatunensis* in the digestive gland. Blue mussels were left in a tank containing seawater contaminated with *F. noatunensis*. The mussels were transferred to a flow through system, in order to let them process the filtrated bacteria. A tissue homogenate was made from the digestive gland and intraperitoneally injected in cod. These fishes were kept for nine weeks, until they were anesthetised to death and samples were collected.

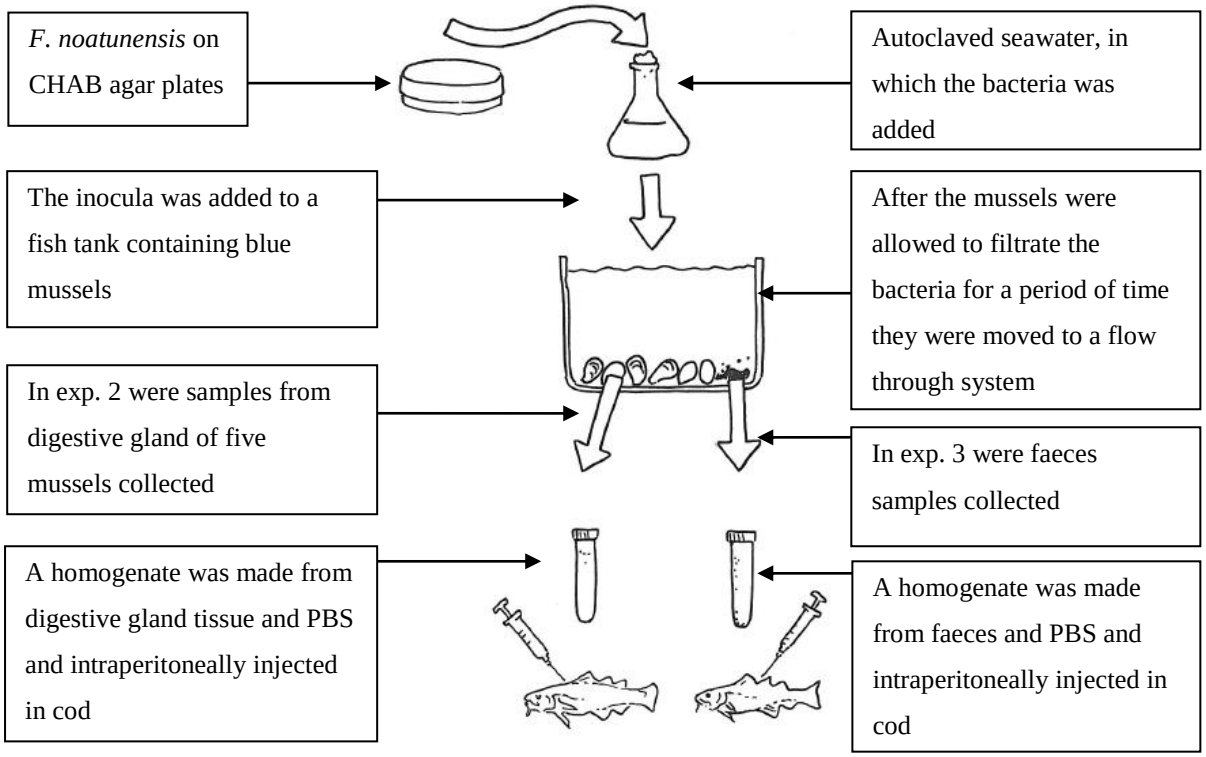


Fig. 2: Schematic overview of experiment 2 & 3: Injection of cod with homogenate from contaminated blue mussels.

Experimental design:

An aquarium was placed in a temperature controlled room, giving a water temperature of 8˚C. The tank was filled with 30 L aerated seawater and 60 blue mussels were added. *Francisella noatunensis* were grown on CHAB agar plates and mixed with 800 ml autoclaved seawater before it was added to the tank, the bacteria were not washed prior to adding. The concentration of *F. noatunensis* in the aquarium was determined by real-time RT-PCR to a Ct value of 13.6 corresponding to approximately 3 x 10^8 bacteria pr ml (p. 34).

Samples from the digestive gland were collected at day two and four in five mussels, and analysed by real-time RT-PCR as a control for the uptake of *F. noatunensis*. After six days exposure, the mussels were removed and transferred to a flow through system where they were kept for five days. The aquarium containing *F. noatunensis* contaminated water was not emptied, and 52 days past inoculation of the blue mussels a 100 µl water sample were plated out on CHAB agar, to test if the bacteria were still alive.

Digestive gland tissue from five mussels were diluted 1/10 in PBS and homogenised before it was transferred to a 14 ml centrifuge tube, and centrifuged (54 x g, 2 min, 20˚C and 149 x g, 1 min, 20˚C) to remove particulate material. The supernatants were transferred to a new tube and further diluted 1/5. A sample was collected for later estimation of bacterial numbers. The tissue homogenate was tested with real-time RT-PCR and had a Ct value of 29.7 corresponding to approx. 2 x 10^4 bacteria pr ml and hence 4000 bact. x $fish^{-1}$(see p. 34). Samples from the digestive gland of contaminated mussels used to prepare the homogenate were collected analysed with real-time RT-PCR. The mussel digestive gland homogenate in PBS was injected intraperitoneally (0.2 ml) in each of 10 benzocaine sedated fish. After nine weeks at 14˚C the fish were anesthetised to death with benzocaine and samples were collected and stored at -80˚C for later RNA extraction and real-time RT-PCR.

Experiment 3: Cod inoculated with faeces from *F. noatunensis* exposed blue mussels

An overview

The third experiment was conducted in order to determine whether the bacteria still alive and infective when shed with faeces of blue mussels. Faecal pellets homogenate in PBS from blue mussels exposed to *F. noatunensis* were injected intraperitoneally in cod (se fig. 2). The fish were kept for nine weeks, until they were anesthetised to death by benzocaine and samples were collected.

Experimental design:

A fish tank was placed in a temperature controlled room, giving a water temperature of 8˚C. The tank was filled with 30 L aerated seawater and 60 blue mussels, which constituted the negative control group, were added. After three days the mussels were moved to a flow through system where they were left for five days before the tank was flushed and thoroughly washed in order to remove all faeces particles. A new fish tank was prepared in the temperature controlled room as described above. *F. noatunensis* were grown on CHAB agar plates and mixed with 800 ml autoclaved seawater before it was added to the tank, the bacteria were not washed. The bacteria concentration in the tank was subsequently determined by real-time RT-PCR to a Ct value of 18.1 corresponding to 2×10^7 bacteria pr ml (se p. 34).

After three days the mussels were moved to a flow through system and kept for five days before the tank was flushed and thoroughly washed in order to remove all faeces particles. The following day were faeces from contaminated mussels (502 mg) and faeces from the negative control mussels (511 mg) collected and transferred to two 14 ml centrifuge tubes containing 4.5 ml PBS. These samples may have contained both faeces and pseudofaeces and these were not distinguished. The tubes were vortexed and left on the laboratory bench for 2 minutes in order to let the faeces particles sediment. Then 2 ml each supernatant was transferred to a tube containing 8 ml PBS.

A sample from each of the two faeces homogenates were collected and kept at -80˚C for later analysis with real-time RT-PCR for the presence of *F. noatunensis*. The faeces homogenate from contaminated mussels had a Ct value of 27.1 which correspond to approximately 8×10^4 bacteria pr ml, hence 16000 bact. x $fish^{-1}$ (see p. 34). The faeces homogenate from unexposed blue mussels had a Ct value of 32.8 when tested for *F.*

noatunensis which correspond to a bacterial concentration of c. 2 x 10^3 bacteria pr ml, hence 400 bact. x $fish^{-1}$ (see p. 34). Faeces samples were collected at day 1, day 5, day 12 and day 19 after transfer to flow through system.

Ten cod in each group were sedated using benzocaine and intraperitoneally injected with 0.2 ml of the faeces homogenate. After nine weeks at 14˚C the fish were anesthetised to death with benzocaine and kidney samples were collected and later analysed for *F. noatunensis* with real-time RT-PCR.

Experiment 4: Cohabitation of cod with blue mussels contaminated with *Francisella noatunensis*

An overview

The fourth experiment was designed to determine whether if cod became infected by cohabitation with mussels previously exposed to *F. noatunensis*. Mussels in two tanks were allowed to filtrate water containing the bacteria before cod was added. The fish were kept for 13 weeks, until they were killed by anesthetisation and samples were collected.

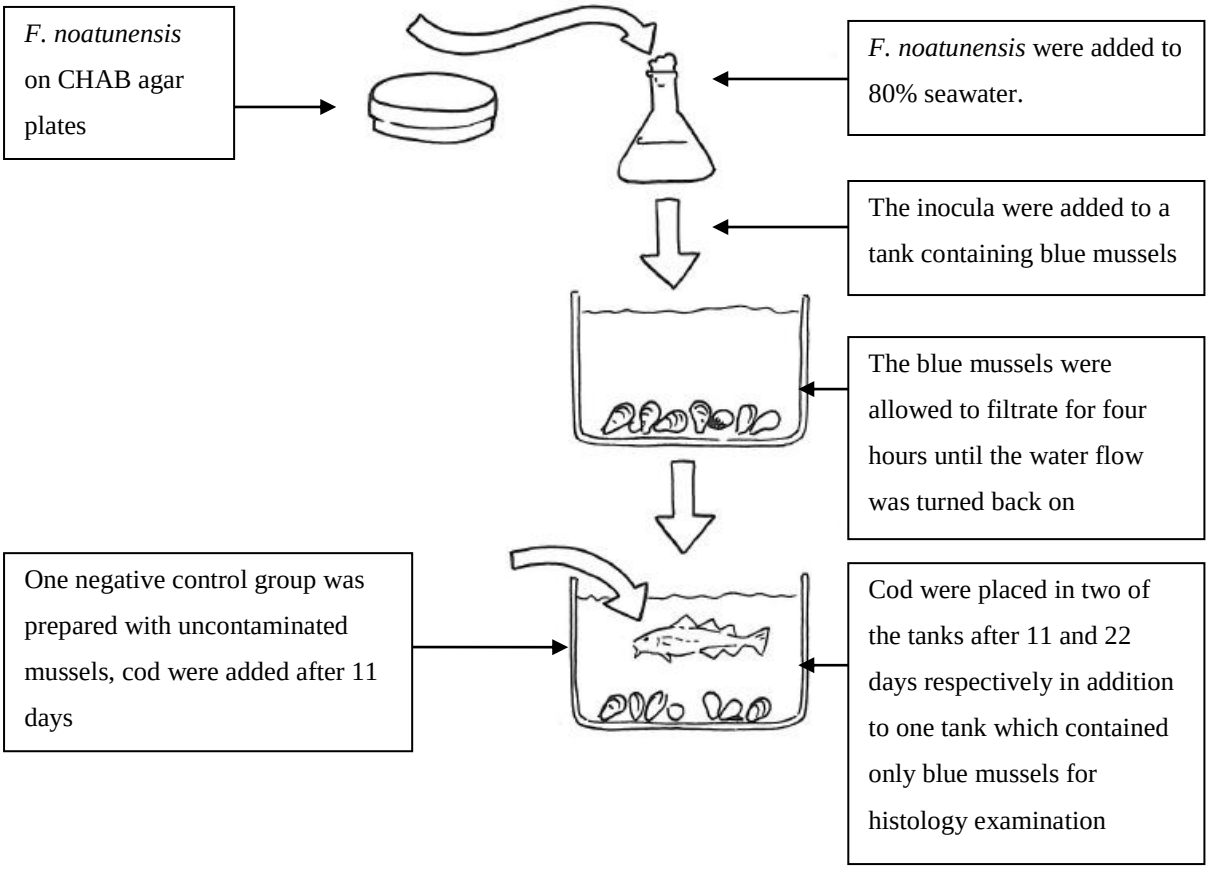


Fig. 3: Schematic overview over experiment 4: cohabitation of cod with blue mussels contaminated with *F. noatunensis*.

Experimental design:

In four tanks receiving continuous water were 60 blue mussels added. A *F. noatunensis* suspension was prepared form CHAB agar plates day 12 post inoculation in a total of 150 ml water. The suspension was distributed to six tubes, washed (centrifuged 10 minutes at 4303 x g), resuspended in 80% seawater and further diluted 1:10. From this solution a tenfold dilution series was made. The 10^{-2} dilution was counted in a counting chamber (Improved Neubauer) and 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated out on CHAB agar in triplicates. The agar plates were examined after approximately two weeks in order to determine the concentration of bacteria in the inoculum. Colony forming units (CFU) from inoculum used in the cohabitation experiment were counted and are presented in
table 1.

Table 1: CFU counts from dilution 10^{-4}, 10^{-5} and 10^{-6} from the inocula in the cohabitation experiment.

Dilution	1 x 10^{-4}	1 x 10^{-5}	1 x 10^{-6}
	>500	434	78
CFU	>500	463	85
	>500	454	83
Mean value CFU	>500	450	82

These counts correspond to a concentration of 8.2 x 10^{8} in the inocula, and a final concentration of 1.4 x 10^{7} in the blue mussel tanks. The bacterial suspension were transferred to three 500 ml bottles and stored on ice until inoculation. The 500 ml of suspension was added in each of the three tanks, containing 30 L of water. The suspension was added over a period of one hour in order to avoid cessation of filtration by the blue mussels, the filtration activity was monitored closely during this period. The blue mussels were allowed to filtrate for four hours until the water flow was slowly turned back on. In the first tank nothing was added and acted as a negative control group. Ten cod was added after 11 days. In the second tank mussels received *F. noatunensis* suspension and this group was sampled for histology and real-time RT-PCR analyses at day 1, 3, 7, 11, 22, 46, 69 and 113 with 5 mussels at each sampling according to protocols. In the last two tanks (3 & 4) bacterial suspension was added as described above. The tanks were thoroughly flushed and washed to remove faeces and pseudofaeces from the blue mussels before ten cod were added after 11 days (day 11 group) in tank three and 22 days (day 22 group) in tank four. The fish and blue mussels were kept together at 9˚C for four weeks, before the blue mussels were removed and the temperature raised to 14 ˚C. The group intended for histology were kept on 9˚C throughout the entire

experiment in order to avoid spawning. The fish were kept in the tanks for a total of 13 weeks, before they were sampled and analysed with real-time RT-PCR.

Methods:

Cultivation of *Francisella noatunensis*

The bacteria were grown on cysteine heart agar (Difco™) with 5% chocolatized sheep blood (CHAB) and incubated at 20°C (see appendix 2).

Sampling for real-time RT-PCR and histological assay

Blue mussel

The tip of a sharp knife was carefully inserted between the shells at the ventral lip and run dorsally between the shells until the posterior adductor muscle was cut. A cross section of approximate 5 mm was removed from the blue mussel using a scalpel and placed in a tissue cassette for histology. The cassette was placed in a jar filled with Davidson's fixative (see appendix 2) for 48 hours. Samples were processed by an automatic tissue processor (Reichert Jung Histokinette 2000), and embedded in paraffin (see p. 36). For real-time RT-PCR analysis an additional sample of tissue was cut from the blue mussel's digestive gland and put in a 1.5 ml centrifuge tube on dry ice until it was stored at -80˚C.

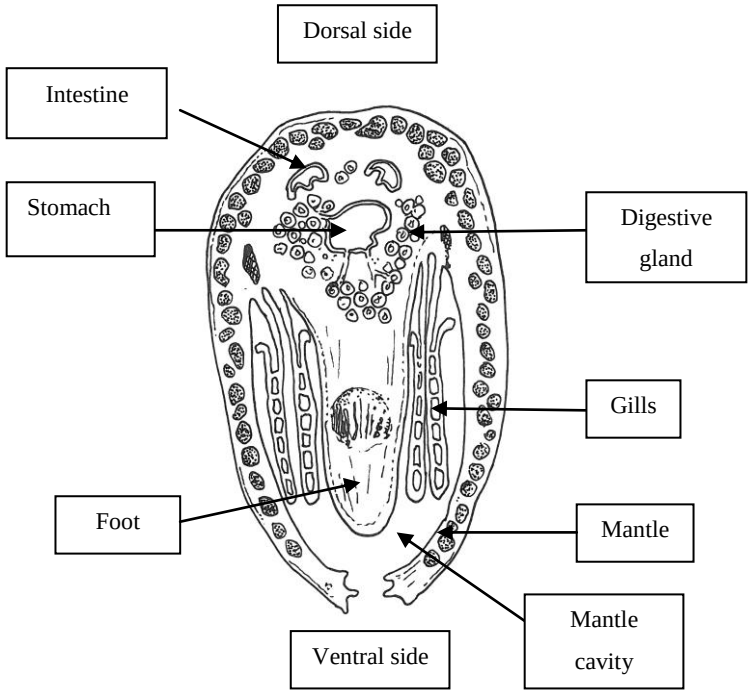


Fig. 4: Schematic overview over a section through a blue mussel.

Atlantic cod

All fish were anesthetised to death by benzocaine, weight and length were registered. The abdominal cavity was carefully cut open with a scalpel inserted by the pectoral fin and run back in a ventral and postal direction to the anal fin. Macroscopic signs of disease were registered, and a section of the spleen, heart (atrium and ventricle) and kidney was cut and transferred to a 1.5 ml centrifuge tube and left on dry ice until it was stored at -80˚C until analysed. Only the kidney samples were analysed for *F. noatunensis* by real-time RT-PCR.

From cod in the cohabitation experiment (exp. 4), additional samples of approximately 5 mm were cut from visible granulomas in liver, in addition to sections from spleen, heart and kidney. These were put in a tissue cassette and fixed in 4% phosphate-buffered formaldehyde (see appendix 2) for 48 hours.

Extraction of total RNA

Total RNA was extracted from water samples, fish tissue and mussels using the RNeasy Mini Kit (Qiagen®) according to the manufacturer's recommendations for tissue samples. The extreme halophile bacterium *Halobacterium salinarum* (type strain DSM 3754/ATCC 33171) was selected as an exogenous control for the real-time RT-PCR assays. The bacteria was cultivated at 37˚C in broth recommended by DSMZ, to an optical density OD_{600nm} of 2.0, which was estimated by counting chamber to approximately 5.5 x 10^{11} bacteria per ml. The bacteria were aliqoted at this concentration and stored at -80˚C. Of this stock were 2 µl added to all samples prior to RNA extraction. RNA quantity from tissue samples were controlled using Nano Drop ND-1000 spectrophotometer (Thermo Scientific). RNA quantities from water samples were not measured. RNA in all tissue samples (cod and blue mussels) were diluted to an approximate concentration of 45 ng/µl prior to real-time RT-PCR screening.

RNA quality from a selection of 12 samples, 6 from cod tissue and 6 from blue mussel were analysed using RNA 6000 Nano Assay Kit with the Agilent Bioanalyzer 2100 (see appendix 1).

Extraction of Total RNA from Animal Tissues with Qiagen RNeasy Mini Kit

Tissue pieces of approximately 60 mg were cut by eye measure in order to ensure good quality of the RNA. The procedure was carried out as fast as possible to avoid thawing of the tissue sample before it was added to the lysis buffer. The entire tissue piece was transferred directly from storage at -80˚C into a 2 ml Lysing Matrix D tube (MP Biomedicals) containing 700 µl RTL (lysis buffer) and 7 µl β-Mercaptoetanol. Subsequently 2 µl of *Halobacterium salinarum* stock solution was added. Samples were homogenized by a Fast prep™ FP120 (Bio 101 Thermo electron corporation) for 20 seconds. The lysate was pipetted out and 350 µl were transferred to a 1.5 ml tube containing 350 µl ethanol, the suspension was mixed immediately by pipetting. The sample was pipetted to an RNeasy spin column placed in a 2 ml collection tube before it was centrifuged for 15 seconds at ≥8 000 g. The flow through was discarded. The RNeasy spin column was filled with 350 µl of Buffer RW1 and centrifuged for 15 seconds at ≥ 8 000 g. Flow through was discarded, before this step was repeated once. Then 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 seconds at ≥ 8 000 g. The flow through was discarded and the step repeated and centrifuged for 2 minutes at ≥8 000 g. The RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute. The RNeasy spin column was then transferred to a new 1.5 ml collection tube. The spin colum membrane was filled with 50 µl RNase-free water and centrifuged for 1 minute at ≥8 000 g. The last step was repeated once, 1 µl of the extracted RNA were tested with nano-drop (Thermo Scientific) before it was stored at -80˚C.

Extraction of Total RNA from water samples with Qiagen RNeasy Mini Kit

Water samples were thawed on ice and 100 µl were transferred to a 1.5 ml centrifuge tube containing 350 µl RLT Buffer and 3,5 µl β-Mercaptoetanol before 2 µl of *H. salinarum* was added. Then 350 µl of 70% ethanol was added and mixed immediately with the lysate. The RNeasy spin column was filled with 450 µl of the sample before it was centrifuged for 15 seconds at ≥8 000 g. The flow through was discarded. The last step was repeated with the remaining volume of the 1.5 ml centrifuge tube. The flow through was discarded. Buffer RW1 was added and the rest of the procedure was as described above.

Real – time RT-PCR

For real-time RT-PCR an assay (Fc50) specific for the 16S rRNA from *F. noatunensis* were used (Ottem et al. 2008). The elongation factor from cod (EF1AA) was used as an internal control (Olsvik et al. 2006) and *H. salinarum* (sal) were used as an exogenous control (Andersen et al. in prep.). In all runs negative template controls (NTC) and negative controls from the RNA extraction was included. One positive control for *F. noatunensis* was also included in all runs to ensure that the reaction mix was working.

Verso^{TM} 1-step QRT-PCR ROX Kit (Thermo Scientific) was used for the real time RT-PCR assays. The reaction mixture was as follow; 6.25 µl 2X 1-step QPCR Rox Mix (Verso), 0.125 µl Enzyme mix, 0.625 µl RT-enhancer, primers and probes depending on assay (see p. 32) and 2 µl of total RNA (90 ng for tissue samples) as template. The total volume was adjusted to 12.5 µl by adding DEPC H_2O.

ABI 7500 sequence detection system (Applied Biosystems), were used to perform the analysis. The reaction was one cycle of 15 minutes at 50ºC (reverse transcriptase step), 15 minutes at 95ºC (polymerase activation step), 45 cycles at 95°C for 15 seconds (DNA-dissociation) followed by 1 minute at 60°C (annealing and elongation). Threshold values were set at 0.003 for the Fc50, 0.008 for EF1AA and 0.001 for *H. salinarum*. All samples were run in duplicates and a standard deviation of maximum 0.6 was set as a limit for samples used in relative quantification, samples exceeding this value are marked with a * in appendix. Primer and probe sequence for the 3 assays are shown in table 2.

Table 2: Primer and probe sequence for the 3 real-time RT-PCR assays used in this thesis

Target	Assay	bp	Sequence	Position	Acc. #	Source
Elongation factor alfa	EF1AA - F - primer	93	5'- CGGTATCCTCAAGCCCAACA – 3'	100-119	CO541952	Olsvik et al (2006)
	EF1AA - R - primer		5' – GTCAGAGACTCGTGGTGCATCT – 3'			
	EF1AA - Probe		G-FAM-TCACCTTCGCCCCC-MGB			Nordstrøm (2008), developed by Olsvik et al (2006)
Francisella noatunensis	Fc50 - F - primer	101	5'– AACGACTGTTAATACCGCATAATATCTG – 3'	123-151	DQ309246	Ottem et al 2008
	Fc50 - R - primer		5' – CCTTACCCTACCAACTAGCTAATCCA – 3'	224-198		
	Fc50 - Probe		FAM – 5' – GTGGCCTTTGTGCTGC – 3' - MGB	161-177		
Halobacterium salinarum	Sal - F - primer	59	5' – GGGAAATCTGTCCGCTTAACG – 3'	541-562	AB219965	Andersen (unpubl.)
	Sal - R - primer		5' – CCGGTCCCAAGCTGAACA – 3'	582-600		
	Sal - Probe		VIC – 5' – AGGCGTCCAGCGGA – 3' - MGB	566-579		

Optimization of primer and probe concentrations

Primer and probe concentration were optimized for the three different assays. The RNA template used was extracted from uninfected cod; cod infected with *F. noatunensis* and a 100 µl water sample spiked with 2 µl *H. salinarum*. Forward and reverse primers were tested in 9 different concentrations rangin from 300/300 to 900/900 with 3 triplicates (see appendix 1) After the optimal primer concentration for the three different assays was determined, the probe was tested in 7 different concentrations ranging from 75–225 nM (see appendix 1). The same RNA template was used and all concentrations were analysed in triplicates.

The optimal primer and probe concentration for the different assays are shown in table 3. These were selected based on the observation of the concentration which gave the lowest Ct value and the highest ΔRn (fluorescence value).

Table 3: Optimal forward and reverse primer and probe concentration for the 3 assays used for real-time RT-PCR

Assay	Forward primer	Reverse primer	Probe
EF1AA	600nM	900nM	125nM
Fc50	600nM	900nM	175nM
SAL	600nM	900nM	175nM

Efficiency test

The efficiency of *F. noatunensis*, *H. salinarum* and elongation factor for cod assays were determined. The efficiencies of the three assays were tested by a tenfold dilution series of RNA extracted from a water sample containing *F. noatunensis* and *H. salinarum* in addition to RNA extracted from kidney tissue from cod. The RNA template was diluted using 45 µl yeast t-RNA (20 ng/µl) and 5 µl template RNA, as yeast t-RNA have been shown to stabilize the kinetics during the dilution series (Ståhlberg et al. 2004). All samples were analyzed in triplicates using real-time RT-PCR. The standard curves created by the ABI 7500 sequence detecting system (Applied Biosystems) were used (see appendix 1). The standard curve is made from the mean value of the triplicates plotted against the serial logarithmic dilutions. The amplification efficiency was calculated using the formula: $(10^{-1/\text{-slope}})$-1.

The standard curve of the *F. noatunensis* assay had a slope of -3.3865 an intercept of 13.8941 and a R^2 of 0.9919. The efficiency was :$(10^{-1/-3.3865}) - 1 = 0.9737$

The standard curve for the *H. salinarum* assay had a slope of -3.3553 an intercept of 15.9541 and R^2 of 0.9987. The efficiency was: $(10^{-1/-3.3553}) - 1 = 0.9863$.

The standard curve for the elongation factor EF1AA had a slope of -3.352923, an intercept of 10.582047 and R^2 of 0.999604. The efficiency was: $(10^{-1/-3.3529}) - 1 = 0.9872$. The three standard curves are shown in appendix 1.

Sensitivity test for the *F. noatunensis* assay was taken from (Ottem et al. 2008) and set to be Ct value 37.5.

Relative quantification of *Francisella noatunensis* in water samples

Relative quantification of *F. noatunensis* RNA from water samples were done using the Microsoft- Excel® based computer software Q-Gene, the principles are reviewed by (Muller et al. 2002, Simon 2003). The mean Ct values of duplicates from real-time RT-PCR runs with the *F. noatunensis* assay were normalised against a reference gene, in this case Ct values from the *H. salinarum* assay. This Microsoft- Excel® based computer software calculates a mean normalised expression on the basis of the efficiency of the assays.

$$\text{MNE} = \frac{(E_{\text{reference}})^{\text{Ct reference, mean}}}{(E_{\text{target}})^{\text{Ct target, mean}}}$$

Samples were run in duplicates and a limit was set at a standard deviation of maximum 0.6 between these duplicates. The mean Ct value of the two duplicates were calculated and plotted in Q-gene (procedure 1). The mean normalized expression values from samples collected at time 0, immediately after the adding of broth, were compared with the corresponding mean normalised expression values after three weeks incubation.

Determination of concentration of bacteria in inocula

A dilution series was made in order to determine the amount of *F. noatunensis* in the different inocula relative to the Ct value from the real-time RT-PCR. The bacteria, grown on CHAB agar plates 12 days in advance, were washed off two petri dishes with 3 ml of 80% autoclaved seawater. This 3 ml suspension was further diluted in a tenfold dilution series in 9 tubes, and the 10^{-2} dilution were counted three times in a counting chamber (Improved Neubauer). The 10^{-6}, 10^{-7}, 10^{-8} tubes were plated out on CHAB and colony forming units (CFU) were counted after approximately two weeks. The entire dilution series was stored at -80˚C and analysed with real-time RT-PCR in duplicates.

The 10^{-2} dilution was counted 3 times in a counting chamber, and gave 194, 197 and 155 bacteria which give a mean value of 182 bacteria. This corresponds with 1.8×10^{8} bacteria per ml in the 10^{-2} dilution and c. 1.8×10^{10} pr ml in the undiluted sample.

The colony forming units counts are presented in table 4.

Table 4: Colony forming units counts from dilution 10^{-6}, 10^{-7} and 10^{-8} of inocula

Dilution	1×10^{-6}	1×10^{-7}	1×10^{-8}
	>300	148	34
CFU	>300	160	13
	>300	147	20
Mean value CFU	>300	152	23

This gives a CFU of approximately 2×10^{10} bacteria per ml in the undiluted sample, which corresponds well with the results from the counting chamber. The bacterial concentration in the inocula used in the experiments was calculated based on the growth function (exponential regression) in Microsoft Excel® based on numbers given in table 5. The Ct values were set as the known x values, the bacteria pr ml number as the known y values and Ct values from inocula with unknown bacteria concentration was plotted in as the unknown x value.

Table 5: Dilution series based on counted numbers/CFU compared to Ct values, run in duplicates, from real-time RT-PCR.

Dilution	Ct value	Mean Ct value	Standard deviation	Bacteria per ml
10^{-9}				$2x10^{1}$
	Undetermined	42,32	-	
	42,3195			
10^{-8}	36,5833	36,18	0,57	$2x10^{2}$
	35,7818			
10^{-7}	31,767	31,91	0,21	$2x10^{3}$
	32,0584			
10^{-6}	28,1661	28,05	0,17	$2x10^{4}$
	27,9272			
10^{-5}	25,5549	25,68	0,18	$2x10^{5}$
	25,8063			
10^{-4}	21,4837	21,85	0,51	$2x10^{6}$
	22,2091			
10^{-3}	18,5249	18,35	0,25	$2x10^{7}$
	18,176			
10^{-2}	12,3742	12,51	0,20	$2x10^{8}$
	12,6527			
10^{-1}	10,1002	9,84	0,37	$2x10^{9}$
	9,57728			
Undiluted	10,3105	10,4	0,12	$2x10^{10}$
	10,4856			

Table 6: Ct values from different inocula used in the experiments and corresponding concentrations of bacteria. These values were calculated on the basis of a dilution series (table 5) made and the growth function (exponential regression) in Microsoft excel®.

Inoculum	Mean Ct value	Calculated concentration (bacteria pr ml)
Saltwater inocula (salinity exp)	17.8	3×10^{7}
Freshwater inocula (salinity exp.)	16.6	5×10^{7}
Inocula (temperature exp)	15.6	1×10^{8}
Tissue homogenate from digestive glands of contaminated blue mussels	29.7	2×10^{4}
Faeces homogenate from contaminated blue mussels	27.1	8×10^{4}

Histology

Dehydration / paraffin infiltration

The formaldehyde fixed tissue samples were transferred to tissue cassettes and placed in the histokinette. The tissue was then automatically transferred through 12 different solutions; time and solution are shown in table 7.

Table 7: Dehydrating and paraffin infiltrating baths.

Solution	Time
4% phosphate buffered formaldehyde	1hour
50% ethanol	1hour
70% ethanol	1hour
80% ethanol	1hour
96% ethanol	1hour
96% ethanol	1hour
100% ethanol	1hour
100% ethanol	1hour
Xylen	2hours
Xylen	2hours
Paraffin	2hours
Paraffin	2hours +

Paraffin embedding

Samples were transferred from the histokinette to the paraffin embedding machine. A metal mold was filled with liquid paraffin and the tissue was placed in the mould. It was then transferred to ice in order let the tissue stick to the bottom of the mould, before the tissue cassette were placed over and filled with paraffin. The tissue cassette was then placed in a freezer for 5-10 minutes before it was removed from the metal mould.

Sectioning

Paraffin around the edges of the tissue cassette was cut off before the cassette was inserted in the microtome. The block was adjusted in order to get a clean cut, and sections of approximately 3 µm were cut from the tissue. The section was carefully transferred to a microscope slide, and put in a water bath to ensure that the section was sufficiently extended. The section was then transferred to a microscope slide and left on a heating block for a short period of time.

Immunohistochemistry

Sections were placed in a heating chamber for 30 min at 60˚C, before they were hydrated in 7 different solutions according to table 8 in a fume hood.

Table 8: Deparaffinising and rehydrating baths for sections used for immunohistochemistry.

Bath	Time
Xylen	10 min
100% ethanol	5 min
100% ethanol	5 min
96% ethanol	5 min
70% ethanol	5 min
50% ethanol	5 min
Running water	5 min

The sections were left to dry overnight at room temperature in a vent. They were marked with a pap-pen (Dako A/S) to ensure complete staining. All incubations were performed in a humidity chamber in fume hood at room temperature (20˚C). In order to prevent non-specific antibody binding, sections were blocked by using Tris-hydroxymethyl-amino methan buffer (TRIS) with 5% bovine serum albumin (BSA). The primary polyclonal rabbit antisera: anti-*Francisella*, were diluted 1:2000 in TRIS-buffer with 2.5% (BSA). Avidine-biotin-alkaline phosphatase complex reaction kit (biotinylated secondary antisera and ABC-AP complex) (Vectastain® universal ABC-AP Kit AK 5200, Vector lab) and Fuchin substrate-chromagen (substrate) (KO624, Dako A/S) were used to visualize positive staining and prepared according to the manufacturer's recommendation with a slight alteration. Shandon's haematoxylin (Thermo Fisher Scientific Inc.) was used for counterstaining and cover glass was glued on by Aquatex (BDH VWR Chemicals). Sections were stored in the dark. One positive tissue control from cod infected with *F. noatunensis* was included for each staining, and unchallenged mussels and cod from the negative control group were used as negative control. A Leica DMBE microscope equipped with a Micro publisher 5.0 RTV (Q-Imaging) was used to examine and photograph the sections. Incubation time and solution are shown in table 9.

Table 9: Incubation of sections in the different solutions during immunostaining

Solution	Time	Temperature
TRIS with 5% BSA	20 min	
Primary antisera	30 min	
TRIS buffer	5 min	
Secondary antisera	30 min	
TRIS buffer	5 min	Room temperature in humidity chamber
ABC – complex (prep. 30min prior to use)	30 min	
TRIS buffer	5 min	
Substrate	5 min	
Running water	5 min	
Haematoxylin	1.5 min	
Running water	4 min	

III. Results

Experiment 1: Observation on the survival of *Francisella noatunensis* in freshwater and seawater at different temperatures

Temperature experiment

At the 4 weeks sampling, there were distinct differences in turbidity between the low (>10) temperature groups compared to the high temperature group. The tubes incubated at 20˚C showed no sign of increased turbidity (based on visual observation) after incubation with B1817 broth for 3 weeks in a shaking incubator. Samples after 8 weeks were not analysed as the bacteria showed no signs of growth at 8 weeks.

The negative water control which was sampled at time zero in the experiment gave a Ct value of 35.46; however the sample was negative after it had been incubated at 20˚C for 3 weeks with B1817 broth. The negative control sample after 8 weeks were negative. All RNA extraction controls and NTC were negative except the RNA extraction control for samples collected after 2 weeks at 20˚C (second samples). The Ct value was 41.4 in one of the two duplicates. RNA extraction control for the first sample for the three temperatures collected after 8 weeks were by mistake not analysed. Results are shown in figure 2, Ct values are listed in appendix 3.

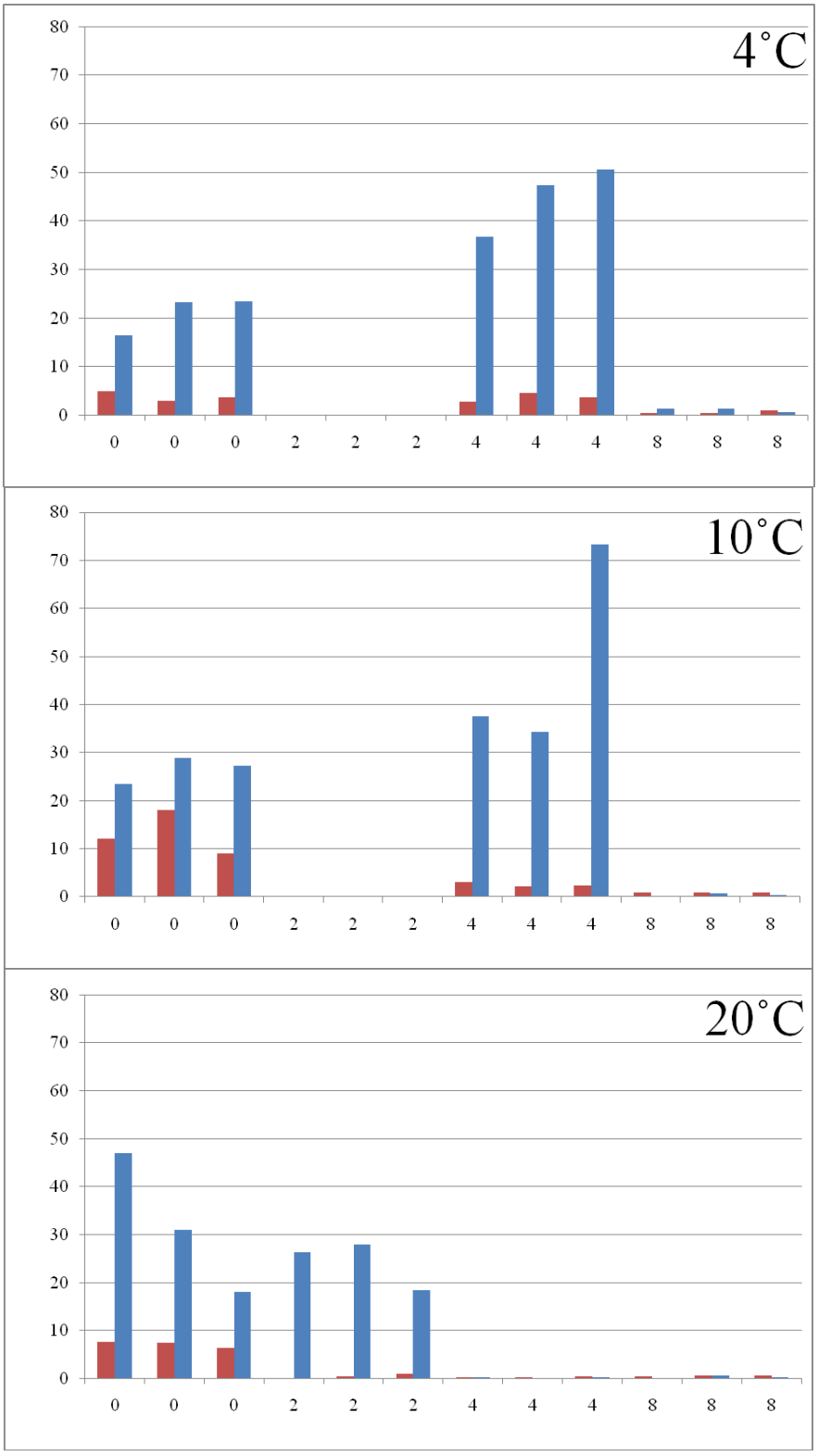


Fig. 5: Cultivability of *F. noatunensis* at 4˚C, 10˚C and 20˚C in seawater, samples were analysed in triplicates for each temperature, at time 0, 2 (only 20˚C) ,4 and 8 weeks. Red bars represent samples collected immediately after adding of broth (sample 1). Blue bars represent samples collected after the bacteria were allowed to grow for 3 weeks at 20˚C in the added broth (sample 2). The y-axis represents mean normalised expression which is a value calculated on the basis of Ct values and the efficiency of the real-time RT-PCR assay. *Francisella noatunensis* and the exogenous control *H. salinarum* are compared in the Microsoft- Excel® based computer software Q-Gene, which calculates the mean normalized expression. These values from sample one and two were compared.

Freshwater and seawater experiment

After four weeks there were no signs of increased turbidity in seawater between the first and the second sample, based on visual observations. The tubes containing fresh water were not culturable at one week. Samples at 2 weeks in freshwater and four weeks in seawater were not analysed as the bacteria were not culturable at these points. The negative water control sample collected at time zero was negative in both seawater and freshwater, however the second sample had a Ct value of 39.2 in freshwater. The negative water control samples (both freshwater and seawater) collected at eight weeks were negative. One RNA extraction control was positive and this is representative for the samples collected immediately after the adding of broth at one and two weeks in freshwater, and two and four weeks in seawater. The RNA extraction control had a Ct value of 41.4. All NTC included in the real-time RT-PCR runs were negative.

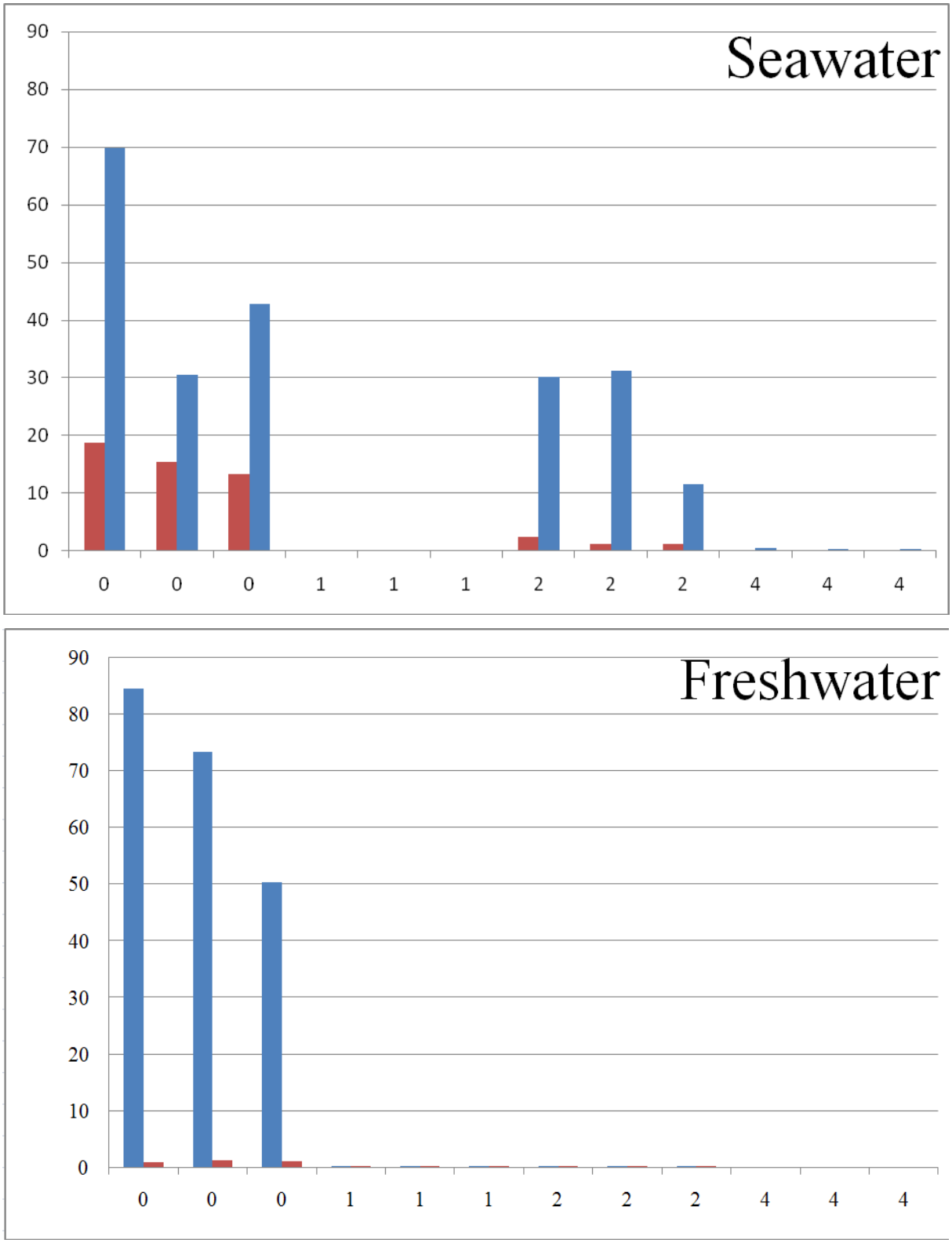


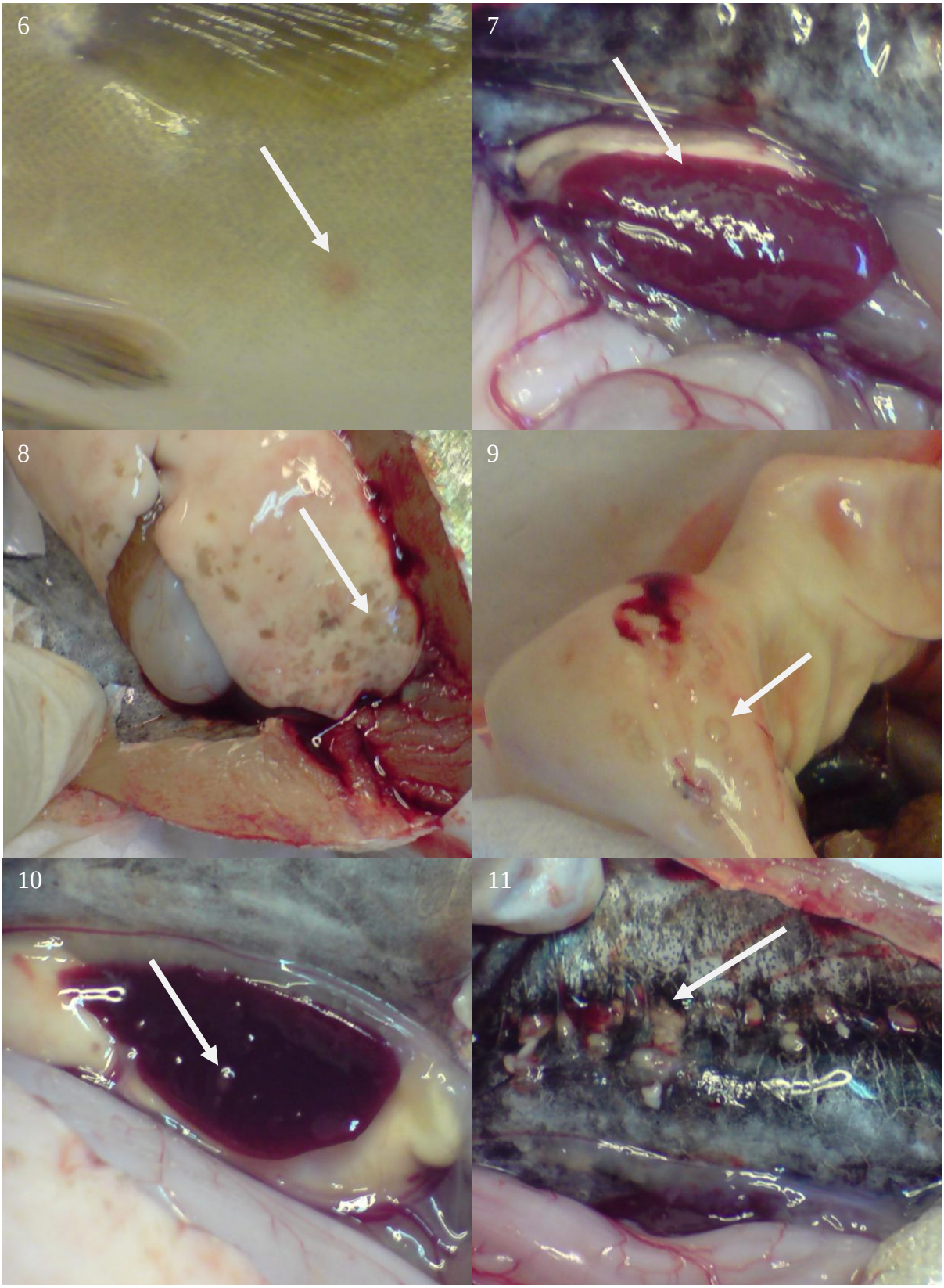
Fig. 4: Cultivability of *F. noatunensis* at 20˚C in seawater and freshwater. Samples was analyzed in triplicates for each of the two salinities, at time 0, 2 and 4 weeks in seawater and 0, 1 and 2 weeks in freshwater . Red bars represent samples collected immediately after adding of broth (sample 1). Blue bars represent samples collected after the bacteria were allowed to grow for 3 weeks at 20˚C in the added broth (sample 2). The y-axis represents mean normalized expression which is a value calculated on the basis of Ct values obtained from real-time RT-PCR. *F. noatunensis* and the exogenous control *H. salinarum* are compared in the Microsoft- Excel® based computer software Q-Gene, which calculates a value (mean normalized expression) based on the Ct values and their efficiency. Mean normalized expression values from sample one and two were compared.

Experiment 2: Cod inoculated with tissue homogenate from *Francisella noatunensis* exposed blue mussels

The ten cod from the stock which were analysed and tested for *F. noatunensis* prior to the experiment were all negative, weight and length ranged from 116 g to 492 g (mean 265 g) and from 23.0 cm to 35.0 cm (mean 28.0 cm). Weight and length for the cod inoculated with tissue homogenate was at the end of this experiment from 243 to 499 g (mean 358g) and from 29.9 to 35.5 cm (mean 31.8 cm). Seven cod showed clear signs consistent with francisellosis when killed nine weeks after IP injection with homogenate from mussels previously exposed to *F. noatunensis*. Macroscopic signs observed were granulomas in liver, spleen and the inside of the abdominal wall in addition to one possible granuloma in the skin (figs. 6-11) All seven cod were positive for *F. noatunensis* when analysed by real-time RT-PCR (Ct value range 26.2-38.8). The three fishes in the experimental group with no macroscopic signs of disease were all *F. noatunensis* negative when tested with real-time RT-PCR. The biological seawater sample from the aquarium which was plated out 52 days past inoculation of the blue mussels, showed growth of bacterial colonies, and this was confirmed to be *F. noatunensis* by real-time RT-PCR. The digestive gland samples collected from mussels used in tissue homogenate was analysed with real-time RT-PCR gave Ct values ranging from 29.6 to 37.5 when tested with the *F. noatunensis* assay.

Table 10: Ct values, in duplicates of gills and digestive gland from blue mussels sampled at day 2 and 4 after exposure with *F. noatunensis* in a closed system. (ME = mussel)

gills	Ct value	Ct value	digestive gland	Ct value	Ct value
Day 2			**Day 2**		
Me 1	15.8	16.5	Me 1	18.4	18.3
Me 2	16.4	17.4	Me 2	23.0	23.2
Me 3	15.9	15.9	Me 3	20.5	22.6
Me 4	17.1	17.4	Me 4	20.1	21.0
Me 5	18.0	17.1	Me 5	19.7	21.1
Day 4			**Day 4**		
Me 1	17.4	16.2	Me 1	23.6	24.1
Me 2	19.2	19.7	Me 2	21.3	21.8
Me3	19.4	19.7	Me3	25.3	25.3
Me 4	19.0	18.7	Me 4	21.3	20.5
Me 5	19.0	19.2	Me 5	21.1	20.8



Figs. 6 – 11: Macroscopic signs of disease in cod injected with tissue homogenate from *F. noatunensis* contaminated blue mussels. *F. noatunensis* infected cod show signs like granulomas in skin (fig. 6), swollen spleen (fig. 7), granulomas in liver (fig. 8 &9) granulomas in spleen (fig. 10) and the abdominal wall (fig. 11).

Experiment 3: Cod inoculated with faeces from *Francisella noatunensis* exposed blue mussels

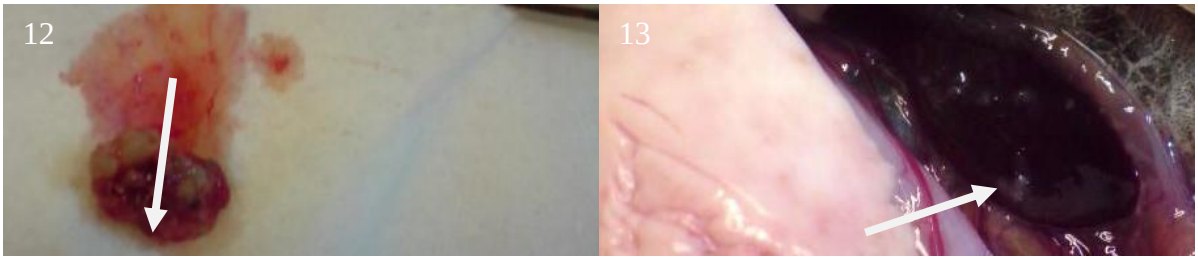
The ten cod from the stock which were analysed and tested for *F. noatunensis* prior to the experiment were all negative, weight and length ranged from 116 g to 492 g (mean 265 g) and from 23.0 cm to 35.0 cm (mean 28.0 cm). The negative control group had weight and length ranging from 317 g to 675 g (mean 530 g) and 30.2 cm to 39.0 cm (mean 36.2 cm). These were negative when tested for *F. noatunensis* with real-time RT-PCR.

The control group injected with faeces from unexposed blue mussels had weight and length ranging from 295 g to 550 g (mean 445 g), ranging from 31.3 cm to 37.0 cm (mean 34.1 cm). These were also negative for *F. noatunensis* when tested with real-time RT-PCR, however single granulomas were seen in kidney and spleen in one individual from the negative control group and one individual from the group injected with faeces from unexposed mussels (fig. 12 & 13). One fish in the untreated negative control group was killed due to eye damage, and one cod in the faeces control group probably died due to injuries caused by the injection.

The mean weight and length of this group at the end of the experiment were not measured, neither were the macroscopic signs of disease. All cod in this group were analysed with the *F. noatunensis* real-time RT-PCR assay and all were positive with Ct values ranging from 25.1 to 33.9. Ct values from faeces samples collected at day 1, 5, 12 and 19 are presented in table 5. Samples were analysed with the *F. noatunensis* assay (Fc50) in real-time RT-PCR.

Table 11: Ct values, in duplicates, from faeces samples analysed with real-time RT-PCR (*F. noatunensis* assay) collected at day 1, 5, 12 and 19 after the mussels were transferred to a flow through system.

Faeces samples	Ct value	Ct value
Day 1	18.1	17.4
Day 5	28.9	30.2
Day 12	36.0	36.1
Day 19	33.9	33.9



Figs. 12 – 13: Fig. 12 show granulomas in kidney from one fish in the negative control group in experiment 3. Fig 13. Show granulomas in spleen of one fish injected with faeces from uncontaminated blue mussels, both fishes were negative for *F. noatunensis* when tested with real-time RT-PCR.

Experiment 4: Cohabitation of cod and blue mussels contaminated with *F. noatunensis*

The fish from the stock used in this experiment which were sampled prior to the experiment had weight and length ranging from 114 g to 184 g (mean 142 g) and 22.2 cm to 26.0 cm (mean 23.9 cm) respectively. All cod were negative when tested with real-time RT-PCR for *F. noatunensis*.

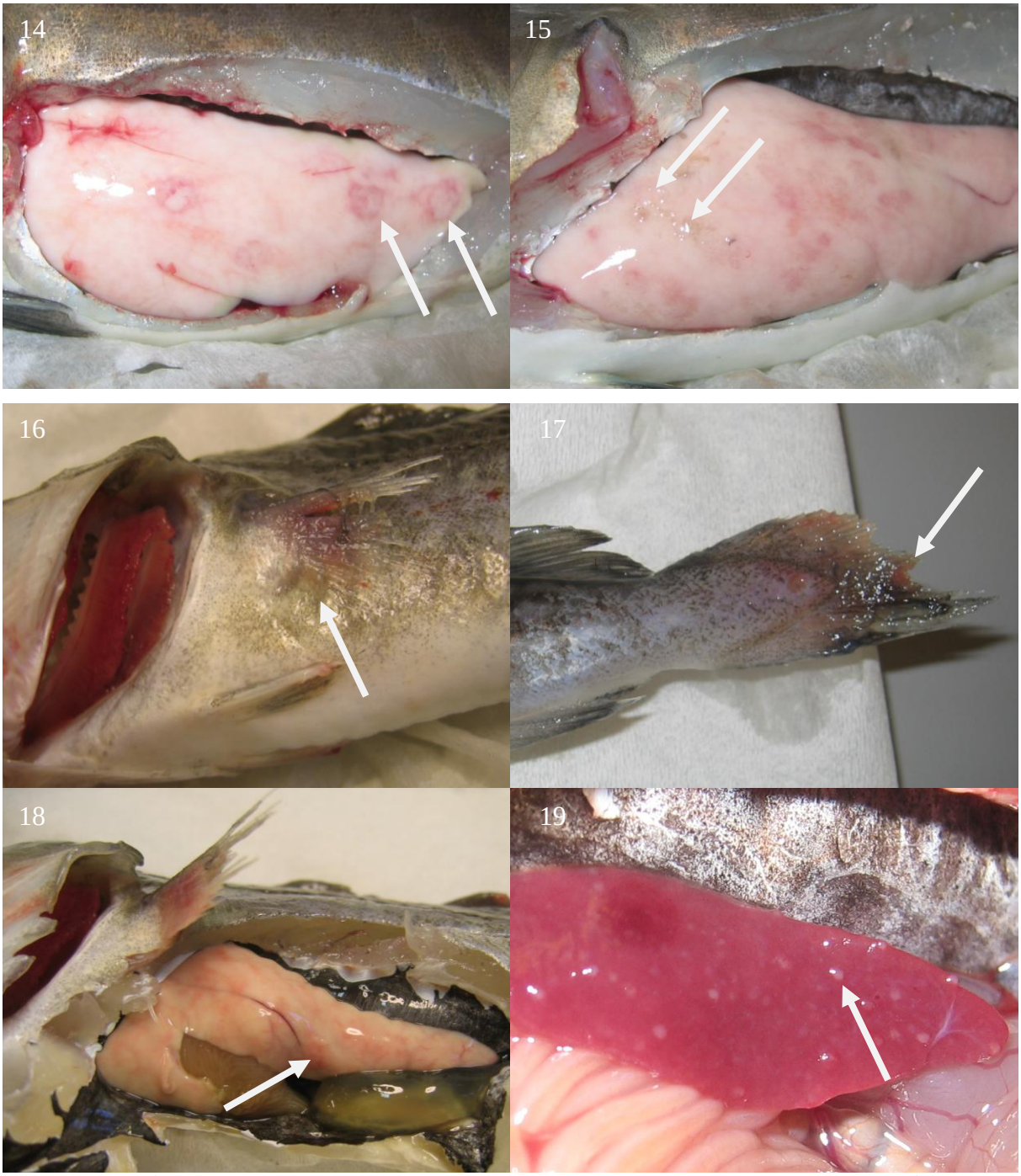
The group where cod were added 11 days after the mussels were exposed to *F. noatunensis* had weight and length ranging from 69 g to 137 g (mean 101 g) and 20.3 cm to 28.5 cm (mean 22.9 cm) respectively. One fish died during the three months duration of the experiment and samples were not taken from this individual.

Four of the nine remaining fishes had granulomas in the liver (fig. 15), however they were all negative for *F. noatunensis* when tested with real-time RT-PCR. One of the duplicates of one fish came out positive with a Ct value of 39.0 when tested for *F. noatunensis*, when the run was repeated it was negative.

In the group where cod were added 22 days after the blue mussels were exposed to *F. noatunensis*, four of ten fishes died during the experiment. Samples for real-time RT-PCR analysis were collected; however samples for histology were not taken. For the remaining six cod weight and length ranged from 72 g to 173 g (mean 125 g) and 19.9 cm to 26.5 cm (mean 23.5 cm) respectively. The deceased fish showed sign of disease as haemorrhages on snout/mouth and fins, ascites, bleedings in liver in addition to granulomas in spleen and liver (figs. 14-19). Samples were analysed with real-time RT-PCR for *F. noatunensis* and were all negative.

In the group where cod was added to a tank containing unexposed blue mussels, four of the ten cod died prior to the termination of the experiment. Three of these were sampled for real-time RT-PCR analysis, samples for histology were not taken. The deceased fish showed signs of haemorrhages on snout/mouth and fins (figs 16 & 17), no granulomas were observed either in spleen or liver. The remaining six fishes had weight and length ranging from 69 g to 133 g (mean 97 g) and 19.7 cm to 25.6 cm (mean 22.5 cm).

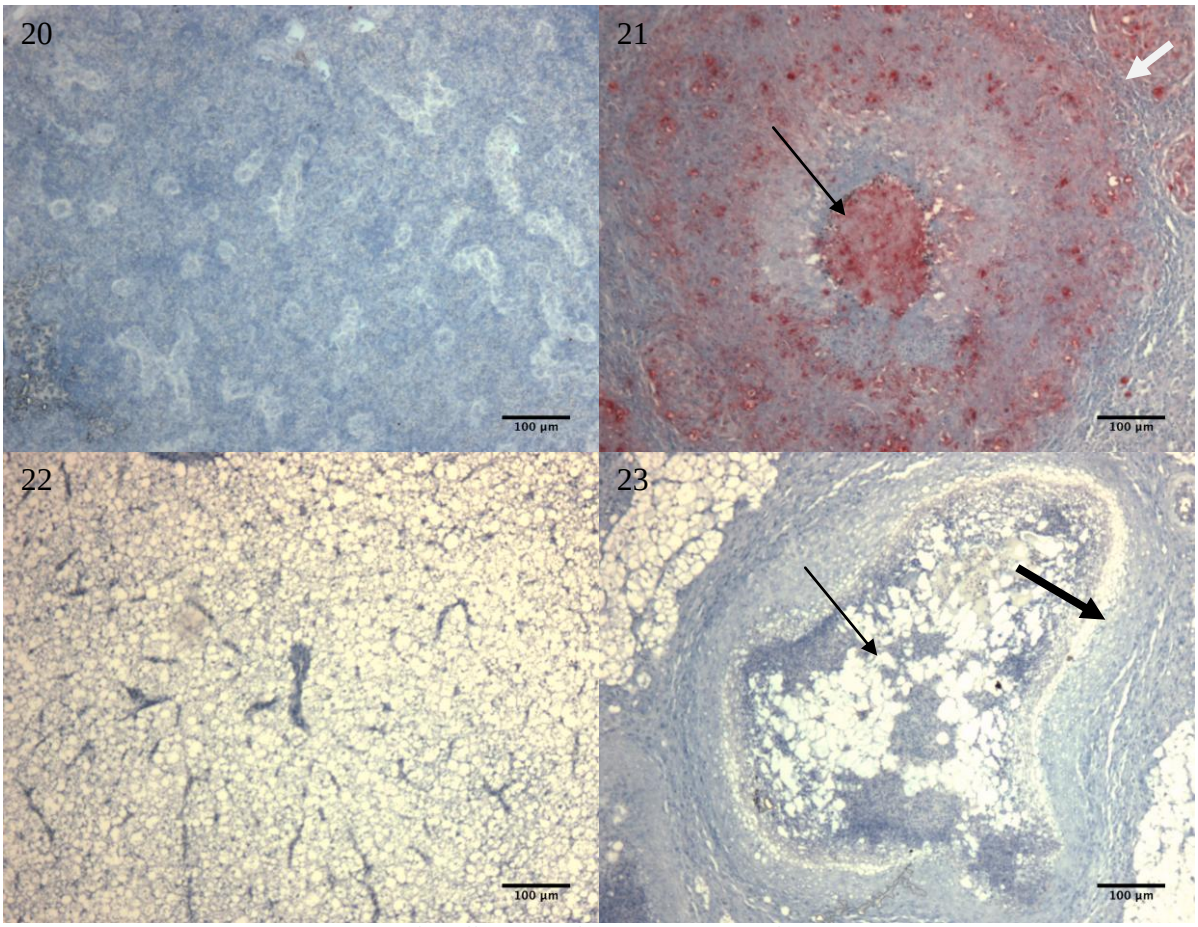
From one of the recently deceased cod a kidney smear was inoculated on 5% blood agar (Oxoid nutrient, Oxoid LtD), after 14 days no bacterial growth was observed. The nine cod which were analysed for *F. noatunensis* with real-time RT-PCR were all negative.



Figs. 14 – 19: Cod from cohabitation experiment with blue mussels contaminated with *F. noatunensis*. Macroscopic signs of disease in the fish in the day 11 group consisted of bleedings (fig. 14) and apparent granluomas (fig. 15) in the liver. In the day 22 group, several individuals showed signs of wounds and haemorrhages on fins (figs. 16 & 17) in addition to bleedings in liver (fig 18). One individual also had small granulomas in spleen (fig. 19)

Immunohistochemistry of cod

All histological samples from cod in experiment 4 were negative for *F. noatunensis* (figs. 20, 22 and 23) when analysed by immunohistochemistry. The standard positive control, which was tissue from *F. noatunensis* infected cod, was positive and showed red coloration (fig. 21).



Figs. 20 – 23. Immunohistochemical staining of paraffin sections from spleen and liver of Atlantic cod. Avidine-biotin-alkaline phosphatase method, primary polyclonal rabbit antisera: anti-*Francisella*, and Shandon haematoxylin counterstained. Positive immunohistochemical staining is visualized by red colour (fig. 21). Fig. 20 show spleen from unchallenged cod. Fig. 21 show spleen from cod suffering from francisellosis. Black arrow show the centre of the granuloma, with aggregates of bacteria. Around the granuloma (white arrow) the formation of connective tissue can be seen. Fig. 22 show normal liver from unchallenged cod. Fig 23 show granuloma in liver from cod in the cohabitation experiment. Narrow arrow show the centre of the granuloma with the presence of leucocytes and necrotic tissue, no bacteria was observed. Bold arrow show the edge of the granuloma with the formation of connective tissue. No coloration of the granulomas in cod in the cohabitation experiment were observed (fig. 23) which indicated that *F. noatunensis* was not present.

Immunohistochemistry with corresponding real-time RT-PCR of blue mussel

No positive immune staining was observed in the digestive diverticulae of unexposed control mussels. However a staining pattern that could be interpreted as positive was observed at different sites. A diffuse, light red staining of intestinal epithelia was observed in most specimens (fig. 24 narrow black arrow). In addition focal aggregates of haemocytes and/or brown cells which contained a red-brownish granulation were observed. In four specimens, small, red, positively stained particles were observed inside haemocytes. Samples from the digestive gland of blue mussels (N=20) in the negative control group were analysed by real-time RT-PCR for *F. noatunensis* in duplicates. In six mussels (ME 1, 2, 7, 12, 16 & 19, see table 7) both duplicates were positive, in six mussels (ME 5, 6, 8, 10, 11 & 17) one of the duplicates were positive. The remaining eight mussels were negative. Mussels 1 – 5, 6 – 10 and 16 – 20 had one positive NTC (Ct value 39.4) and RNA extraction control was negative. Mussels 11-15 had negative NTC and negative RNA extraction control. There was no consistency between the red coloration of negative control mussels in immunohistochemistry and negative control mussels which came out positive when tested with real-time RT-PCR. A test of a polyclonal anti-serum for nodavirus were performed on the unexposed blue mussels, and no red staining was observed in the epithelia of the gut.

Table 12: Ct values, in duplicates, from unexposed control mussels in the cohabitation experiment. A total of 20 mussels were tested for the presence of *F. noatunensis*, prior, during and after the experiment (ME = mussel).

Mussel	Ct value	Ct value	Mussel	Ct value	Ct value
Me 1	37.2	37.0	Me 11	39.4	Undetermined
Me 2	37.2	37.6	Me 12	27.1	27.8
Me 3	Undetermined	Undetermined	Me 13	Undetermined	Undetermined
Me 4	Undetermined	Undetermined	Me 14	Undetermined	Undetermined
Me 5	Undetermined	39.1	Me 15	Undetermined	Undetermined
Me 6	Undetermined	38.6	Me 16	35.0	35.1
Me 7	34.3	34.2	Me 17	Undetermined	39.1
Me 8	Undetermined	31.1	Me 18	Undetermined	Undetermined
Me 9	Undetermined	Undetermined	Me 19	39.1	39.6
Me 10	38.0	Undetermined	Me 20	Undetermined	Undetermined

Samples from challenged mussels also revealed the diffuse staining of stomach and intestine wall epithelia and the brownish, focal granulation observed in unexposed control specimens. In addition a clear red immune staining, different from the control mussels, was observed in digestive cells in the digestive diverticulae. One day after exposure to *F. noatunensis*, positive

immune staining was observed as weakly red points or areas in the digestive cells in the digestive diverticulae of three out five mussels sampled (Fig. 25 bold black arrow & fig. 29). This staining was not observed in other tissues. At day three, the positive staining was observed in all five specimens. The number of positive points, as well as the intensity of the staining, was variable. At day seven, a moderate but variable staining was observed in the digestive diverticulae. In one specimen a strong positive staining was observed in the lumen of a primary digestive duct (fig. 26 white arrow). At day 11, four out of five specimens revealed a positive but variable staining as described above. Two of these also showed a few positive particles in the intestinal lumina (fig. 27 narrow black arrows). At days 46 and 70, no immunohistochemical staining different from the control specimens was observed, however in one specimen sampled at day 46, a few positive particles were observed in haemocytes. The mussels were also tested for *F. noatunensis* with real-time RT- PCR in duplicates. At day one they had a mean Ct value from 33.1 to 38.9 at day three mean Ct value ranged from 35.7 to 38.1. The mussels sampled at day seven had a Ct value from 36.4 to 38.7, in addition to one negative individual. At day 11 one mussel were negative, while the remaining four had Ct values from 35.8 to 39.8. At day 22 three mussels was negative for *F. noatunensis* while one had a Ct value of 33.2, while the last one had one negative duplicate and one with a Ct value of 39.1. At day 70 all the five mussels tested for F. noatunensis with real-time RT-PCR were negative, however at day 113 (termnation of the experiment) one of the mussels had a mean Ct value of 34.8 while the remaining four where negative. This run had one positive NTC of 39.4.

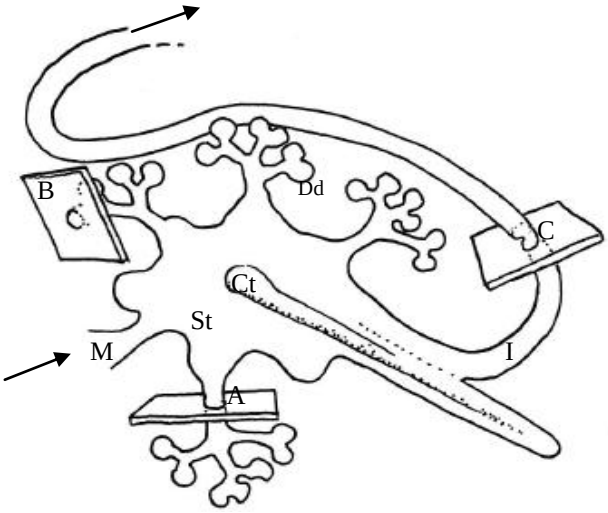
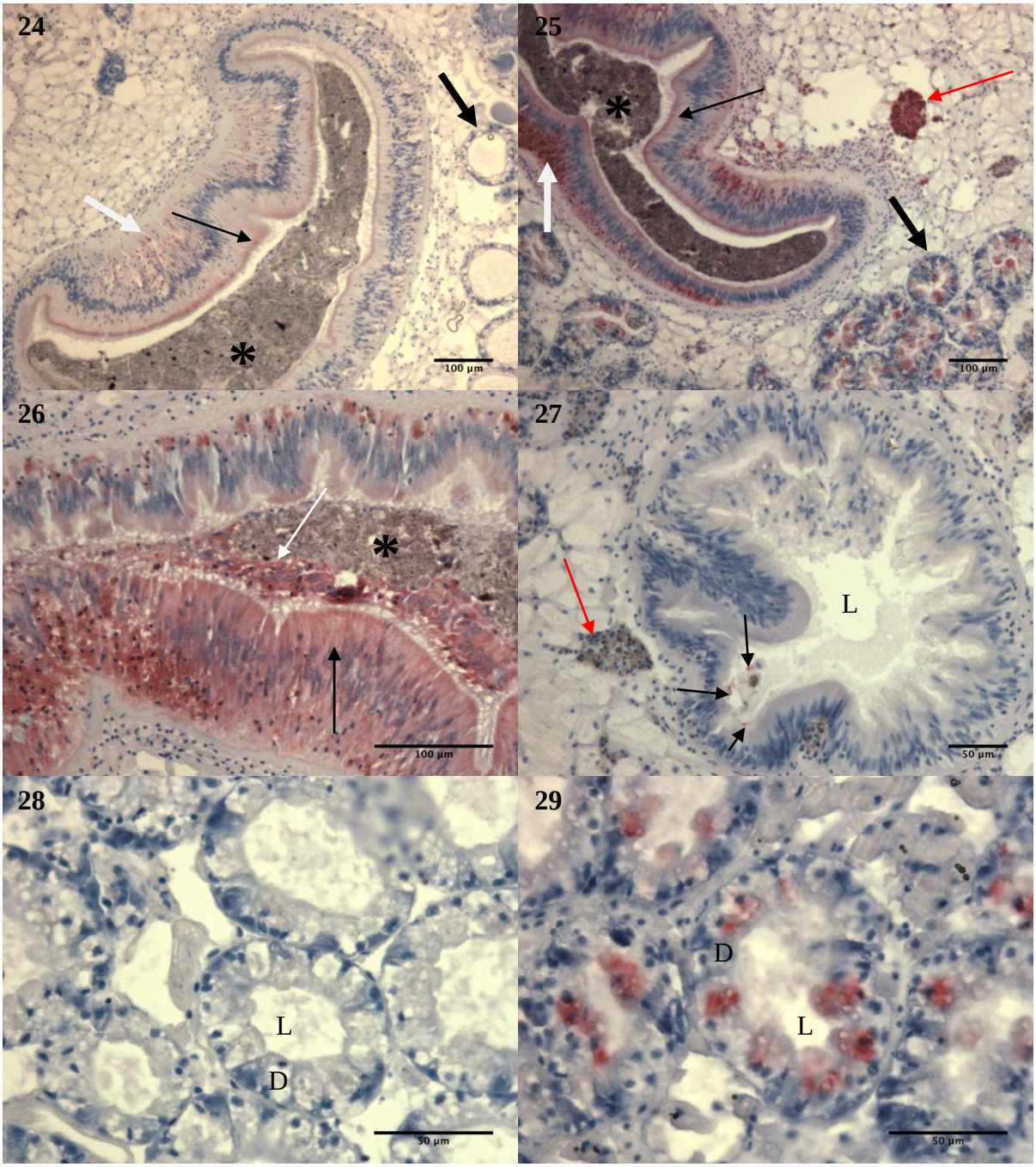


Fig. 23a: Schematic overview of the digestive system in blue mussels. Sections through the different parts (shown in figs. 24-29) are shown by A, B and C, where A represents the opening of the ducts, B the digestive diverticulae and C the intestine. Particles enter the mouth (M) before it is transported from the oesophagus to the stomach (St) where enzymes will be released from the crystalline style (Ct). Food material will be directed toward the opening of the ducts (A) leading to the digestive diverticulae (Dd). The remaining material will be passed into the intestine (I) and excreted.



Figs. 24 – 29 Immunohistochemical staining of paraffin sections from blue mussels. Avidine-biotin-alkaline phosphatase method, primary polyclonal rabbit antisera: anti-*Francisella*, and Shandon haematoxylin counterstained. Positive immunhistochemical staining is visualized by red colour. * represents contents of the intestine. L represent lumen and, D the digestive epithelia of the diverticulae. Fig. 24 shows an untreated control mussel, red coloration are shown in the epithelial cells lining the intestine (narrow black arrow), however red coloration were not seen in the digestive ducts or diverticulae in any of the control mussels (bold black arrow). Some red coloration was also seen in individual cells (presumably haemocytes) (white bold arrow). Fig. 25 shows the intestine and digestive diverticulae 7 days after inoculation with *F. noatunensis*. The narrow black arrow shows coloration in the intestinal epithelia. The white arrow shows a stronger coloration inside the epithelium. Coloration is also seen in the digestive diverticulae (bold black arrow). An aggregate of unknown contents show coloration (red arrow), the same kind of aggregate can be seen in fig. 27 (red arrow), with a weaker coloration. Fig. 26 show the gut of a blue mussel inoculated with *F. noatunensis*, red coloration are seen in the epithelial cells lining the stomach (narrow black arrow), coloration are also seen in the contents of the intestine (narrow white arrow). Fig 27, show coloration of what might be bacteria in the lumen of a primary digestive duct (narrow black arrows). Fig. 28 shows digestive diverticulae from a negative control mussel. Fig 29 show the diverticulae from of a blue mussel inoculated with *F. noatunensis*. The red coloration is interpreted as the presence of bacteria in the digestive epithelia 3 days after inoculation.

IV. Discussion

What is the source of *Francisella noatunensis*?

There is little information available on the presence and survival of *F. noatunensis* in the marine environment, and the only known source is infected cod. However other *Francisella* species like *F. philomiragia* and *F. tularensis* have been isolated from water and soil (Larson et al. 1955, Jensen et al. 1969, Hollis et al. 1989, Forsman et al. 1995, Barns et al. 2005, Petersen et al. 2009). The *F. tularensis* bacterium has also been associated with crayfish (*Procambarus clarkia*) fishing, and it is capable of surviving more than a year in water or mud (Parker et al. 1951, Anda et al. 2001). In addition were 21 *F. philomiragia* and 3 *F. tularensis* species detected in samples collected from and around a brackish water pond on Martha's Vineyard,USA (Berrada & Telford). One of the partial 16S rRNA *F. philomiragia* sequence (EU503153) submitted to the Genebank database is identical to *F. noatunensis* bacterium (Karlsbakk 2009). *Francisella noatunensis* has also been detected in a number of fish species, blue mussels and crab (Ottem et al. 2008). Such observations may indicate that *Francisella* species are widespread in the environment and capable of surviving in the environment for prolonged periods of time.

Survival of *F. noatunensis* in water

The *in vitro* studies revealed differences in culturability of *F. noatunensis* relative to both temperature and salinity. The bacteria were not culturable after one week in freshwater, hence it seems to be less tolerant to freshwater than to seawater.

Another intracellular pathogen, *Piscirikettcia salmonis*, which causes disease in salmonids cultured in seawater in Chile, also showed rapid inactivation of the bacteria in freshwater (Lannan & Fryer 1994). Despite the fact that freshwater seem to kill these bacteria relatively fast compared to seawater have *Piscirikettcia salmonis* also been detected in coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) in freshwater (Bravo 1994). A *Francisella* sp. closely related to the *F. noatunensis*, were detected as the etiological agent in an outbreak of a granulomatous disease in Atlantic salmon (*Salmo salar*) in Chile (Birkbeck et al. 2007).

The lack of growth may be due to the bacterium entering a "Viable but Nonculturable State" (VBNC) as reviewed by Oliver (2005). VBNC is defined as the lack of growth on routine bacteriological media on which the bacteria normally grow. The reviewed results indicate that the bacteria are alive and capable of renewed metabolic activity. The VBNC

response is assumed to be a result of some form of natural stress like starvation, incubation outside the temperature range of growth, oxygen concentrations or exposure to white light (Oliver 2005). It has been claimed that *F. tularensis* is able to enter such a state after starvation in cold water (Forsman et al. 2000). However the study failed to show resuscitation of the bacteria and these were not virulent when injected in mice. It cannot be excluded that *F. noatunensis* is able to enter a VBNC state, a problem that could be examined through injection in cod. Hence further research is needed to verify this state in this bacterium, including cohabitation trials to examine the possible epizootiological significance of the VBNC state.

To our knowledge only one experiment has been performed to determine the survival of *F. noatunensis* in water, however these results are not yet published (Duodu & Colquhoun unpublished results). Based on the lack of information a basic *in vitro* experiment was conducted under axenic conditions. Axenic incubation, in a closed system with no supply of additional oxygen or nutrients, is not optimal. These conditions do not mimic the situation in natural water very well, since they do not supply additional oxygen or give access to nutrients normally present in water. Still, closed and sterile environments represent readily repeatable and controllable entities, where the impact of various environmental factors on bacterial survival can be examined. However it must be noted that *F. noatunensis* is likely to show a very different survival in natural water, where factors like competition with other bacteria or predation (bacterivory, filtering) may potentially reduce the survival. On the other hand the occurrence of nutrients or possible temporary host might increase the survival of *F. noatunensis*. Live *F. noatunensis* were detected in a water sample plated out from an aquarium with heavy microbial growth 52 days past inoculation at 8˚C. In the axcenic incubation the bacteria were unculturable between 4 and 8 weeks. This observation suggests that *F. noatunensis* will be alive and culturable for a longer period of time in natural seawater with the supply of oxygen and nutrients.

The real-time RT-PCR assay used for *F. noatunensis* targets the 16S rRNA, this method is a fairly good way to measure differences in RNA, however as with all other methods there are sources of error. Some differences in the amount of RNA both in the samples collected immediately after the adding of broth and in samples collected after three weeks incubation in broth were observed. The same inocula were added to all the tubes in the salinity experiment and freshwater & seawater experiment, respectively, and the amount of bacteria should therefore be similar. Marine *Vibrio* spp. only retain between 10-26% of their original rRNA content after starvation for 15 days (Kramer & Singleton 1992). As the

bacteria in our experiment were added to an axenic environment they were subject to starvation, which may cause depletion in RNA in samples collected immediately after adding of broth. However since these tendencies are not studied in *Francisella* no assumption wheter this applies to this species can be made. Further, results published by Kerkhof & Kemp (1999) show that 16S rRNA levels are not linearly related to growth rate in most of the nine strains of proteobacteria analysed in the experiment. This also implies that the 16S rRNA amount in bacteria varies according to which state of growth the bacterium is in at the time of sampling. It is therefore difficult to relate the rRNA amount to bacterial numbers, and we can only state that the bacteria are alive/culturable or not.

In the observation on the survival of *F. noatunensis* experiment RNA extraction control had a Ct value of 41.4 in one of the two duplicates, when tested for *F. noatunensis*. All the unknown samples in this experiments had Ct values lower than 25, and due to the large difference between the RNA extraction control and the unknown samples, it is considered legitimate to ignore the positive RNA extraction control (Bustin & Nolan 2004). From our experiments it may be concluded that *F. noatunensis* reaches an uncultivable state within 4 – 8 weeks at low temperatures (≤10), which is representative for the winter-spring situation in Norway. Further research is needed to examine whether unculturable bacteria are able to infect cod.

Transmission of *F. noatunensis*

Knowledge of transmission mechanisms of *F. noatunensis* is scarce, however horizontal transmission by cohabitation in tanks at high temperatures have been shown in laboratory experiments (Nylund et al. 2006, Nordstrøm 2008, Mikalsen et al. 2009). Field observations support this findings, as the prevalence within infected stock often are high (Colquhoun et al. 2008). The bacteria has been detected in skin and mucus of infected individuals (Nylund & Ottem 2006b). Granulomas have also been detected in the intestine, which may indicate faecal shedding of *F. noatunensis* by infected fish (Mikalsen et al. 2009). Whether vertical transmission occurs is not yet determined, however *F. noatunensis* have been detected in cod eggs, and in farmed juveniles (Karlsbakk et al. 2008).

Francisella noatunensis have been detected in wild cod in the southern parts of Noway, in addition to other fish species like saithe (*Pollachius virens*), pollock (*Pollachius pollachius*), poor cod (*Trisopterus minutes*), mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*), megrim (*Lepidorhombus whiffiagonis*), angler-fish (*Lophius piscatorius*), flounder (*Platichthys flesus*) and farmed Atlantic salmon (*Salmo salar*) (Ottem et

al. 2008). Archive samples from wild cod in the south-east part of the North sea dated back to the 1980's (Van Banning 1987) have subsequently been diagnosed as francisellosis (Colquhoun et al. 2008). Further on have the cause of an outbreak of a systemic granulomatous disease in cod off the west coast of Sweden during the summer of 2004 (Alfjorden et al. 2006) also retrospectively been diagnosed as *F. noatunensis* (Colquhoun et al. 2008).

These reports indicate that the bacterium is present in wild cod and other fish species off the Norwegian and Swedish coast. However the role these fish species play in the spreading of *F. noatunensis* is still unknown. The presence of *F. noatunensis* in wild cod is mainly found in the southern parts of Norway, and the presence of the bacterium in cod farms in the northern parts of Norway may be a result of transportation of infected cod to these areas (Ottem et al. 2008). Further on it is likely that the water temperatures may have an impact on the outbreak of francisellosis, as most outbreaks occur during the warmer parts of the year. The southern parts of Norway experience temperatures close to the optimal temperature for *in vitro* growth for *F. noatunensis*, during the summer, which also may have an impact on the immunocompetence in cod (Ottem et al. 2008).

The fate of *F. noatunensis* in mussels

How *F. noatunensis* is released from infected fish is still unknown, though two hypotheses have been presented earlier. The bacteria have been detected in the skin and mucus of infected fish, in addition to granulomas in the intestine (Nylund & Ottem 2006b, Mikalsen et al. 2009). This implies that the bacteria either is shed directly into water or by faecal shedding Zooplankton and bivalve mollusks like blue mussels are very common in the marine environment, and such organisms feed on small particles in water, which also include bacteria e.g. (Rivkin et al. 1999)

Bacteria are ingested and assimilated as food in mussels, though in varying degree (Zobell & Feltham 1937, McHenery & Birkbeck 1985). The uptake of particles is indiscriminate in blue mussels however they show selectivity once the particles have been taken up (McHenery & Birkbeck 1985). The digestion of bacteria is dependent on each species abilities to resist the enzymes present in the digestive system of the bivalve, and lyzosyme resistant bacteria are rejected without degradation (Prieur et al. 1990). As a result blue mussels may function as a biological filter or a reservoir for different bacteria. They have been shown to be capable inactivate fish pathogens like the ISA virus, and the intracellular bacterium *Renibacterium salmoninarum* (Paclibare et al. 1994, Skår & Mortensen 2007).

However, marine bivalve mollusc have also been shown to function as reservoirs of certain viral finfish pathogens (Meyers 1984).

Ottem et al. (2008) detected *F. noatunensis* in both blue mussels and edible crab (*Cancer pagurus*) sampled in the vicinity of a cod farm with francisellosis. For the blue mussel to act as a reservoir for *F. noatunensis* to cod it must either:

1: Accumulate *F. noatunensis*, and release live and infective bacteria, which infect cod through water.

2: Accumulate live and infective *F. noatunensis*, and act as prey for cod, leading to the ingestion of live bacteria.

3: Accumulate bacteria; act as prey for small fish and, which in turn is eaten by cod, and *F. noatunensis* leads to infection.

In our experiments we wanted to expand the knowledge around blue mussels and their potential to act as biological filter or reservoir for the *F. noatunensis* bacterium. Real-time RT-PCR results from blue mussels' gills and digestive glands sampled at 2 and 4 days after exposure to *F. noatunensis* in water, show that the bacterium is present in large amounts in both organs. These bacteria were proven to be live and infective as the fish injected with tissue homogenate from the digestive gland showed *Francisella noatunensis* infections consistent with francisellosis. Immunohistochemistry of the digestive system of the mussels also show an occurrence of the bacterium in the digestive diverticulae. Hence the blue mussels exposed to *F. noatunensis* had taken up the bacteria from water. Evidence has also been presented that the bacteria are present in the lumen of the gut and in the lumen of the digestive tract which indicate that the bacteria pass through the entire digestive system.

Samples from faeces particles were also collected and analyses with real-time RT-PCR of these shows a clear presence of *F. noatunensis*. This agrees with results from the experiments where fish showed signs of infection with *F. noatunensis* after being injected with tissue and faeces homogenate from previously exposed blue mussels. Therefore it may be concluded that the blue mussels are not capable of killing all the filtrated *F. noatunensis* bacteria. However, the mussels are not likely to serve as a reservoir as immunohistochemistry results indicate that the bivalves rid themselves with the *F. noatunensis* bacterium relatively fast. Further, we found no evidence suggesting that the bacterium is capable of persisting or multiplying in the digestive tissue of the mussels, despite the fact that they were seen in haemocytes in some of the mussels sampled.

In both the immunohistochemistry and in the real-time RT-PCR some of the samples from unexposed blue mussels were positive when tested for *F. noatunensis*. In the real-time RT-PCR this may be a result of contamination from other positive samples on the same reaction plate since one NTC came out positive. However mussel 12, which had both negative RNA extraction control and NTC, had a mean Ct value from the two duplicates of 27.4, this is a relatively high Ct value, and rather unlikely to be a result of contamination. And as suggested by (Bustin & Nolan 2004) should a Ct value of a unknown sample that differs more than 5 from the NTC be regarded as positive and not a result of contamination. The sample from mussel 12 should therefore be sequenced in order to determine whether it is a bacterium, and if so which bacterium is causing the positive real-time RT-PCR results.

In the tissue homogenate from unexposed mussels, a Ct value of 32.8 were obtained when tested for *F. noatunensis* with real-time RT-PCR, in this case both the RNA extraction control and NTC's were negative. A Ct value of 32.8 corresponds to approximately 2 x 10^3 bacteria pr ml (during growth), hence c. 400 bact. x $fish^{-1}$. Despite this, all fish tested in this group were negative for *F. noatunensis*. It is therefore reason to believe that the blue mussels either contain partly degraded *F. noatunensis* or a structure which gives cross-reaction and false positive samples in the real-time RT-PCR runs.

In the examination of the immunohistochemistry sections, a red staining was observed in the epithelia of the gut of unexposed blue mussels, this staining pattern were also observed in the exposed mussels. The anti-serum used in this thesis is polyclonal and not absorbed, and the structure to which the anti-serum binds is therefore unknown. It is possible that the anti-serum binds to a structure present in the epithelia of the gut of blue mussels, which is not necessarily *F. noatunensis* or bacteria from the *Francisella* species. Further, a polyclonal anti-serum specific for nodavirus were tested on the unexposed mussels, and there were not observed a similar staining pattern of the epithelia (these results are not presented in this thesis). Further experiment is needed to examine the unspecific coloration and positive real-time RT-PCR results.

At day 11 the immunohistochemistry and real-time RT-PCR showed that the blue mussels contain small amounts of *F. noatunensis*, hence the lack of infection may be a result of the dose of the bacteria the fish were exposed to. A very small proportion of a group of cod juveniles, which where bath challenged with relatively high doses ($\leq 10^6$ bacteria pr ml) of *F. noatunensis* became infected after 11 weeks (Omdal et al. 2009). Differences between cod juveniles and cod used in our experiments may be expected due to differences in size. The dose of *F. noatunensis* the blue mussels were exposed to were approximately 10^7 bacteria pr

ml, hence the dose which cod were subjected to 11 and 22 days later are probably substantially lower as the blue mussels seem to rid themselves with the bacterium. Another hypothesis for the lack of infection in cohabitated cod is the fact that *F. noatunensis* may be encapsulated by faeces particles when shed into water by the blue mussel. This may require the bacteria to be ingested by cod in order to cause infection. Further research is needed on this point, in order to determine the role of blue mussels and other invertebrates in the spreading of francisellosis.

In the cohabitation experiment a total 8 cod died prior to the end of the experiment, and the hours from death occurred until they were sampled may therefore wary greatly. As a result of this the quality of RNA may be of variable and potentially poor quality, however the Ct value of the elongation factor were compared and no major differences from deceased fish were observed. These cod were not sampled for histological purposes as the degradation of tissue starts quickly after death and it may be difficult to discriminate between post mortem degradation and pathological signs of disease.

The cause of the disease were not verified, however it may have been caused by an atypical strain of *Aeromonas salmonicida*, which causes atypical furunculosis in cod. Macroscopic signs consisted in haemorrhages on snout/mouth, skin ulceration, pale gills and granulomas in spleen and liver and histological examination revealed no signs of bacteria in the centre of the granulomas, this agrees with results presented by Magnadottir et al. (2002). Atypical furunculosis has the later years become a known differential diagnosis to francisellosis, due to the resemblance in macroscopic signs.

Conclusion

The survival of *F. noatunensis* under axenic conditions is related to both salinity and temperature, with the longest survival in seawater at low temperature. Based on samples from crude seawater containing *F. noatunensis*, it might be expected that the bacteria is capable to survive for a longer period of time in natural seawater with available oxygen and nutrients compared to axenic conditions.

The mechanism of how the bacteria are shed in water is unknown, though the bacteria have been detected in skin and epithelia of infected cod. Horizontal transmission has also been detected between cohabitated cod in laboratory experiments. When the bacteria have been shed from infected cod it may be taken up by animals like marine bivalves. These species are very common in the marine environment and filtrate large volumes of water. The blue mussels were found to be incapable of killing all *F. noatunensis* ingested, and bacteria were shed live and infective into water with faeces particles. The mussels rid themselves with the bacteria, and no evidence of persistence or multiplication by the *F. noatunensis* were shown in this study. However, small faeces particles may be taken up by zooplankton or other filtering invertebrates present in water, and this may lead to *F. noatunensis* being transmitted through trophic levels. Hence, further studies are needed to determine the potential role these small invertebrates may play in the transmission of *F. noatunensis* in the marine environment.

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VI. Appendix:

Appendix 1: Bioanalyzer & real-time RT-PCR

Agilent 2100 bioanalyzer procedure and results

RNA 6000 Nano Assay Kit

The RNA samples used was diluted to a concentration of approximately 200ng/µl. The RNA samples we wanted to test and the RNA ladder was denaturised for 2min at 70˚C. And all reagents and samples were allowed to equilibrate to room temperature for 30min before use. The electrodes were decontaminated according to manufacturer's manual before the gel was prepared. Then 550µl of RNA 6000 Nano gel matrix was placed into the top receptacle of a spin filter. The spin filter was centrifuged for 10min at 1500g ± 20%. The filter was discarded and 65µl of the filtered gel were transferred to 0.5ml RNase-free microfuge tubes and stored at 4˚C. The Gel-Dye Mix was prepared as follow: All reagents were allowed to equilibrate to room temperature for 30min before use. The RNA 6000 Nano dye concentrate was vortexed for 10s and spun down. Then 1µl of dye was added to a 65µl aliquot filtered gel. The tube was vortexed thoroughly and spun for 10min at 13000g at room temperature. The gel-dye mix was pipetted in a volume of 9µl to the bottom of the well marked G. The timer was set to 30s, and the plunger at 1ml before the Priming station was closed. The plunger was pressed until it was held by the syringe clip. After 30 seconds the plunger was released and after additional 5 seconds the plunger was slowly pulled back to the 1ml position. The priming station was opened and 9.0µl of the gel-dye mix were added in each of the wells marked G. Then 5µl of the RNA 6000 Nano Marker was pipetted into the well marked with the ladder symbol and each of the 12 sample wells. The well with the ladder symbol was filled with 1µl of the ladder. After denaturizing the samples 1µl was loaded into each of the 12 sample wells. The chip was vortexed for 1min at the IKA vortexer set-point (2400 rpm). The chip was inserted in the Agilent 2100 Bioanalyzer and the run was started.

2100 expert_EukaryoteTotal RNA Nano_DE34903001_2009-01-19_13-01-07.xad Page 1 of 5

Assay Class: EukaryoteTotal RNA Nano
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Created: 1/19/2009 1:01:07 PM
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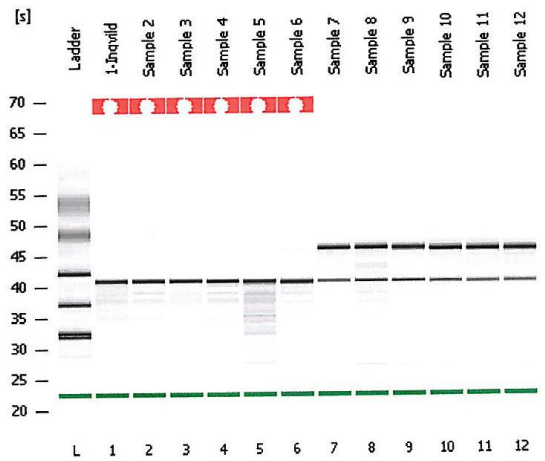


Instrument Information:

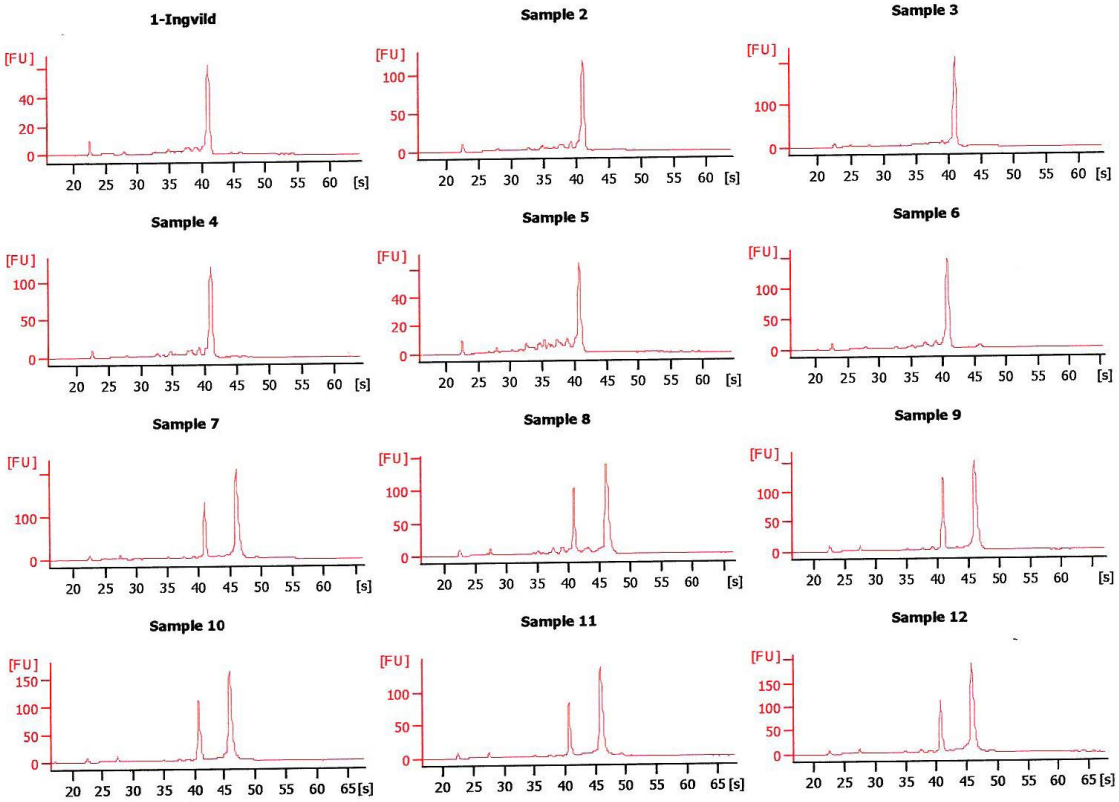
Instrument Name:	DE34903001	Firmware:	C.01.069
Serial#:	DE34903001	Type:	G2938C

Assay Information:

Assay Origin Path:	C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Eukaryote Total RNA Nano Series II.xsy
Title:	Eukaryote Total RNA Nano Series II
Version:	2.5
Assay Comments:	Copyright © 2003-2006 Agilent Technologies

Chip Information:

Chip Lot:	
Reagent Kit Lot:	
Chip Comments:	



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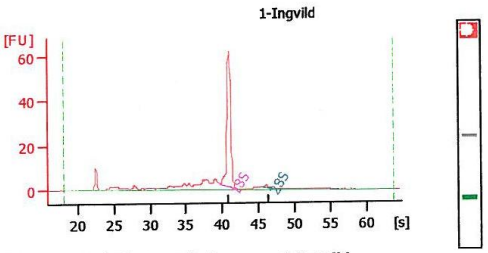
Modified: 1/19/2009 1:25:08 PM

Electropherogram Summary

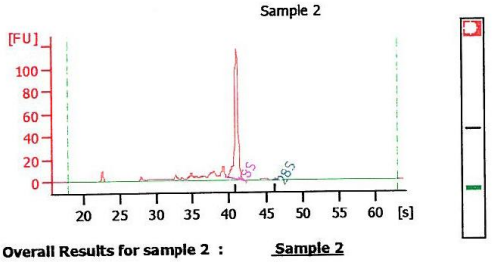


Overall Results for sample 1 : 1-Ingvild

RNA Area:	191.7
RNA Concentration:	278 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	N/A (A.01.01)

Fragment table for sample 1 : 1-Ingvild

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.63	42.04	85.5	44.6
28S	45.60	46.85	1.7	0.9

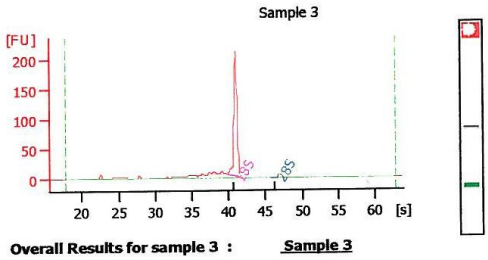


Overall Results for sample 2 : Sample 2

RNA Area:	289.1
RNA Concentration:	419 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	N/A (A.01.01)

Fragment table for sample 2 : Sample 2

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.70	42.07	164.0	56.7
28S	45.87	46.65	0.4	0.1

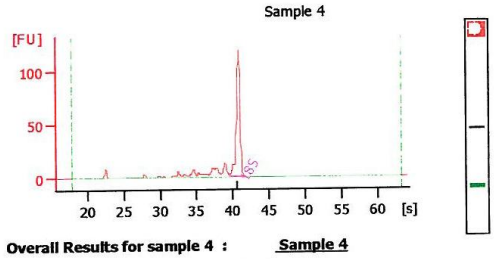


Overall Results for sample 3 : Sample 3

RNA Area:	504.8
RNA Concentration:	732 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	N/A (A.01.01)

Fragment table for sample 3 : Sample 3

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.58	41.99	275.9	54.7
28S	45.82	46.69	1.0	0.2



Overall Results for sample 4 : Sample 4

RNA Area:	297.0
RNA Concentration:	431 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	N/A (A.01.01)

Fragment table for sample 4 : Sample 4

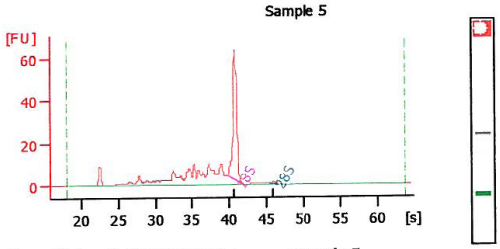
Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.63	42.11	163.8	55.1

Assay Class: EukaryoteTotal RNA Nano
Data Path: C:\... EukaryoteTotal RNA Nano DE34903001 2009-01-19 13-01-07.xad

Created: 1/19/2009 1:01:07 PM
Modified: 1/19/2009 1:25:08 PM

Electropherogram Summary Continued ...

Sample 5



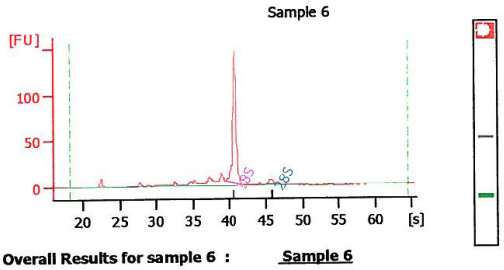
Overall Results for sample 5 : Sample 5

RNA Area:	266.4
RNA Concentration:	386 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	N/A (A.01.01)

Fragment table for sample 5 : Sample 5

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.68	41.81	89.0	33.4
28S	45.51	46.57	0.9	0.3

Sample 6



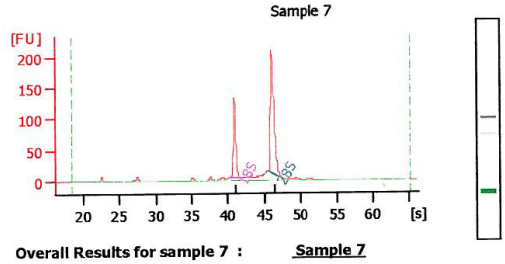
Overall Results for sample 6 : Sample 6

RNA Area:	358.4
RNA Concentration:	520 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	N/A (A.01.01)

Fragment table for sample 6 : Sample 6

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.39	41.91	227.1	63.4
28S	44.95	46.73	8.4	2.3

Sample 7



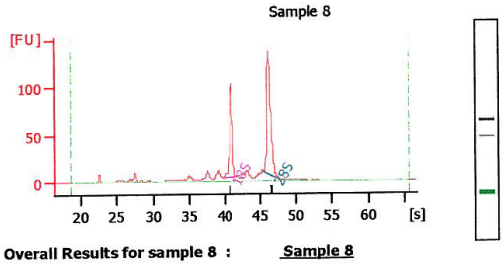
Overall Results for sample 7 : Sample 7

RNA Area:	616.7
RNA Concentration:	894 ng/µl
rRNA Ratio [28s / 18s]:	2.0
RNA Integrity Number (RIN):	9.7 (A.01.01)

Fragment table for sample 7 : Sample 7

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.23	41.97	123.9	20.1
28S	45.47	47.46	249.3	40.4

Sample 8



Overall Results for sample 8 : Sample 8

RNA Area:	565.0
RNA Concentration:	820 ng/µl
rRNA Ratio [28s / 18s]:	1.7
RNA Integrity Number (RIN):	8.9 (A.01.01)

Fragment table for sample 8 : Sample 8

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.71	41.62	107.9	19.1
28S	45.52	47.76	184.3	32.6

Assay Class: EukaryoteTotal RNA Nano
Data Path: C:\... EukaryoteTotal RNA Nano DE34903001 2009-01-19 13-01-07.xad

Created: 1/19/2009 1:01:07 PM
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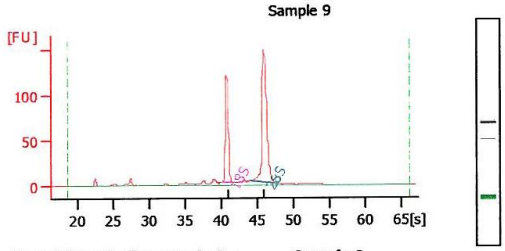
Electropherogram Summary Continued ...

Sample 9



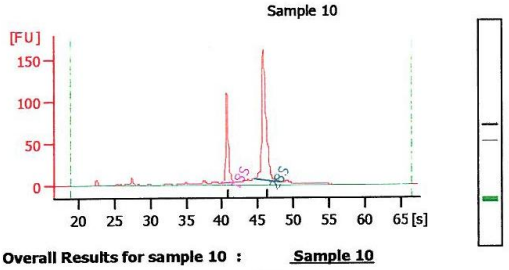
Overall Results for sample 9 : Sample 9

RNA Area:	491.7
RNA Concentration:	713 ng/µl
rRNA Ratio [28s / 18s]:	2.0
RNA Integrity Number (RIN):	9.9 (A.01.01)

Fragment table for sample 9 : Sample 9

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.20	41.55	117.4	23.9
28S	44.18	47.93	230.8	46.9

Sample 10



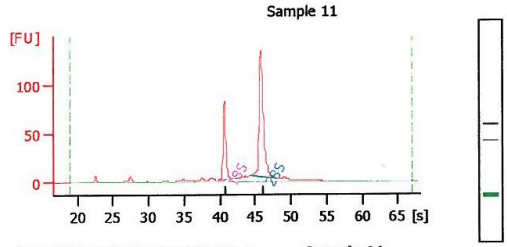
Overall Results for sample 10 : Sample 10

RNA Area:	549.9
RNA Concentration:	798 ng/µl
rRNA Ratio [28s / 18s]:	2.2
RNA Integrity Number (RIN):	9.9 (A.01.01)

Fragment table for sample 10 : Sample 10

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.73	42.00	114.8	20.9
28S	44.52	48.10	247.9	45.1

Sample 11



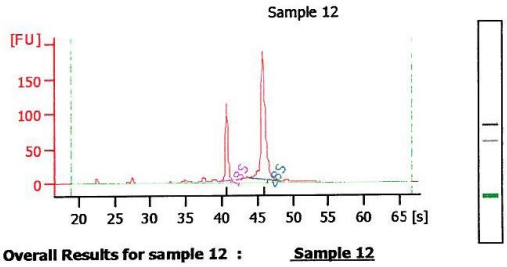
Overall Results for sample 11 : Sample 11

RNA Area:	480.6
RNA Concentration:	697 ng/µl
rRNA Ratio [28s / 18s]:	2.4
RNA Integrity Number (RIN):	9.7 (A.01.01)

Fragment table for sample 11 : Sample 11

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.65	41.94	86.2	17.9
28S	44.22	47.96	205.2	42.7

Sample 12



Overall Results for sample 12 : Sample 12

RNA Area:	659.1
RNA Concentration:	956 ng/µl
rRNA Ratio [28s / 18s]:	2.5
RNA Integrity Number (RIN):	9.7 (A.01.01)

Fragment table for sample 12 : Sample 12

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.67	41.85	119.8	18.2
28S	43.88	48.20	303.3	46.0

Real-time RT-PCR

Real-time RT-PCR is a technique which targets RNA, hence a refinement of the original RT-PCR technique developed by Higuchi et al (Kubista et al. 2006). The PCR reaction performed on DNA needs two oligonucleotide primers, dNTPs which are the four nucleotide triphosphates, a heat stable polymerase and magnesium ions in the buffer. The reaction is performed by temperature cycling where initial high temperature is applied to separate the DNA stands, before the temperature is lowered in order to let the primers anneal, before the temperature is increased to around 72˚C to allow the polymerase to extend the primers by incorporating the dNTPs (Kubista et al. 2006). As Real-time RT-PCR targets RNA an initial step of reverse transcription is required in order to convert RNA to cDNA. This can be done in a two-step or one-step procedure. In the two-step reaction RNA is first reverse transcribed before an aliquot of the reverse-transcription reaction is added to the real-time PCR. In a 1-step procedure the reverse-transcription takes place in the same tube as the real-time PCR, and requires a cDNA synthesis step of 15min at 50˚C. There are different methods for detecting the PCR products, like SYBR® Green which is a fluorescent dye that binds to double-stranded DNA or TaqMan® probes which are fluorescently labeled sequence-specific probes. With the use of SYBR® Green nonspecific PCR products and primer-dimers will also contribute to the fluorescent signal. The curve goes into an exponentially phase as the signal accumulates, before it levels off and saturates. A threshold for the fluorescence signal level is set and the difference is quantified by the comparing of the number of amplification cycles required to reach this threshold, also called the Ct value (Kubista et al. 2006).

Optimization of primer and probe concentration

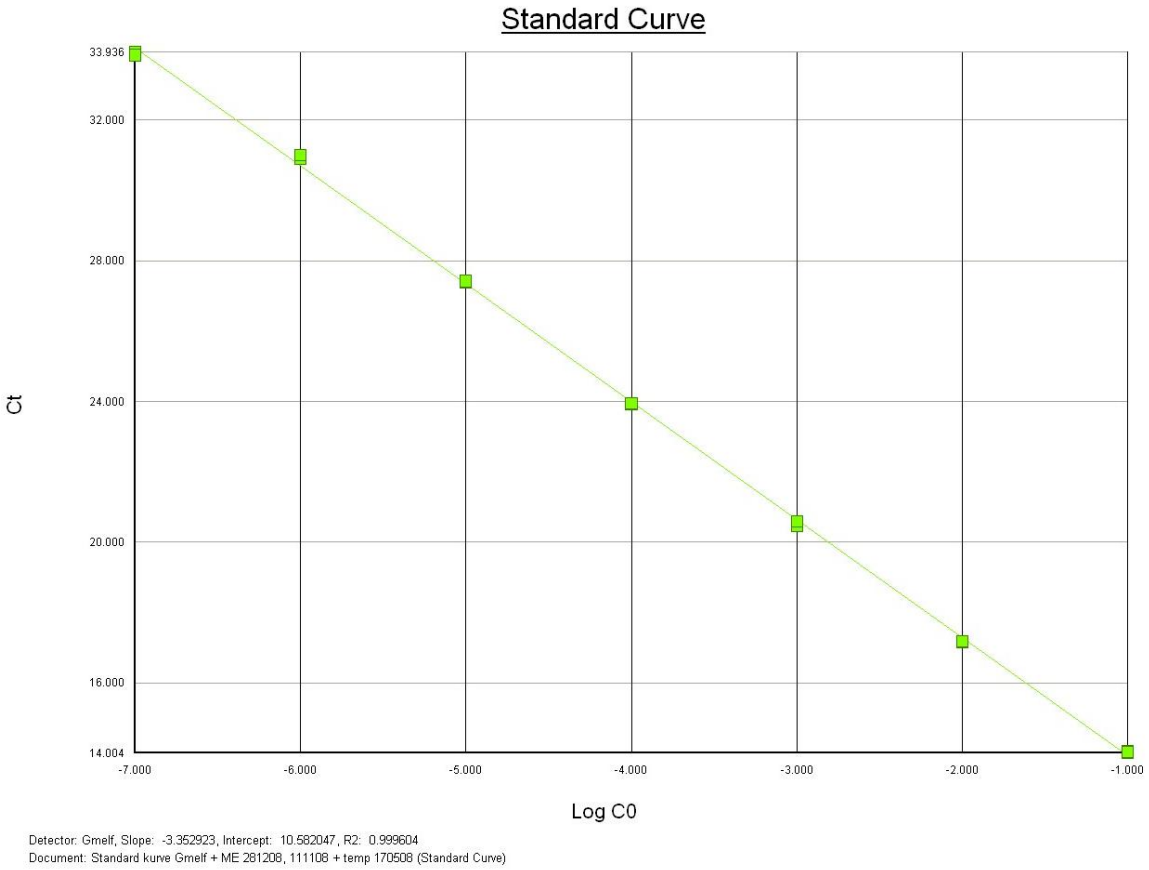
Table 13: Primer optimization setup for the 3 different assays

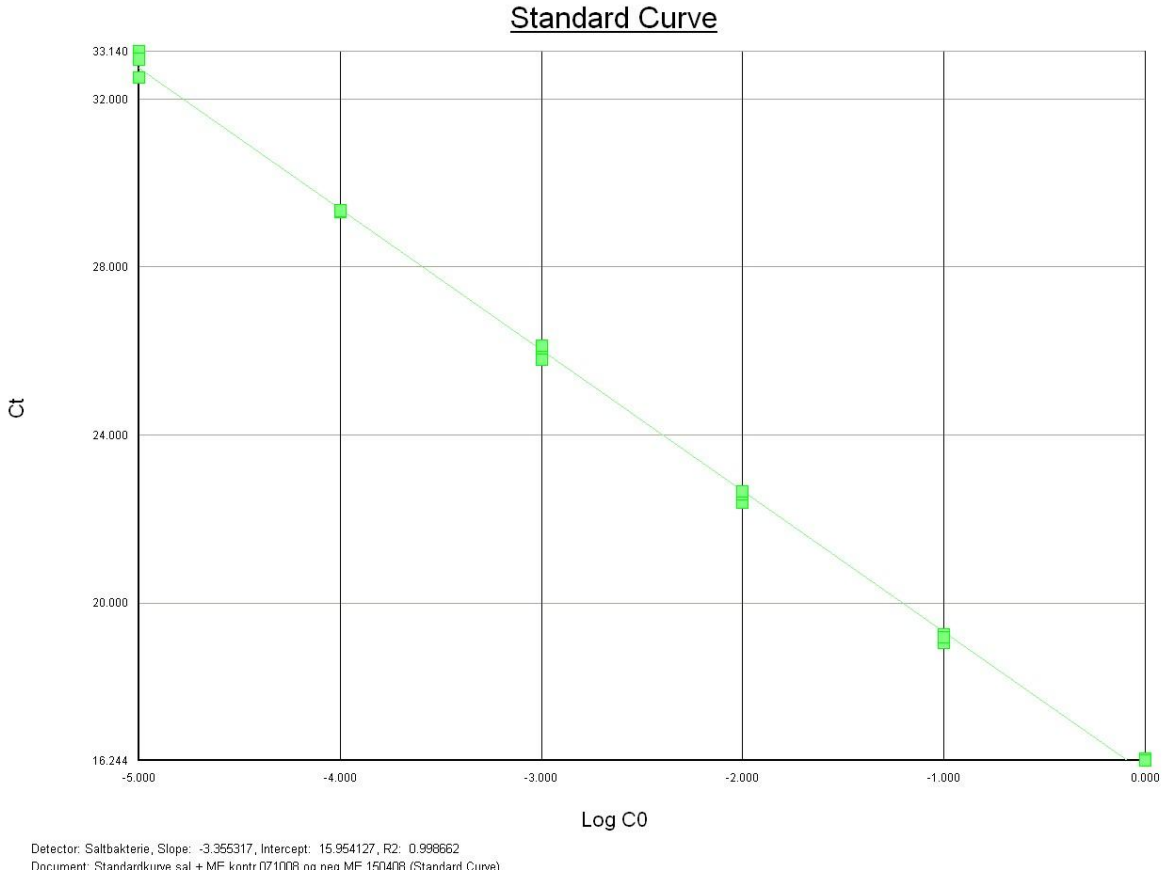
Primer concentr.	NTC	300/300	300/600	300/900	600/300	600/600	600/900	900/300	900/600	900/900
Master Mix	7	7	7	7	7	7	7	7	7	7
F primer	0.375	0.375	0.375	0.375	0.75	0.75	0.75	1.125	1.125	1.125
R primer	0.375	0.375	0.75	1.125	0.375	0.75	1.125	0.375	0.75	1.125
Probe	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Template	0	2	2	2	2	2	2	2	2	2
Water	4.5	2.5	2.125	1.75	2.125	1.75	1.375	1.75	1.375	1
Total volume	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5

Table 14: Probe optimalisation setup for the 3 different assays, a and b represent optimized forward and reverse primer concentration.

Probe conc. [nM]	NTC	75	100	125	150	175	200	225
Master mix [µl]	7	7	7	7	7	7	7	7
F primer	a	a	a	a	a	a	a	a
R primer	b	b	b	b	b	b	b	b
Probe	0.125	0.093	0.125	0.156	0.188	0.219	0.250	0.281
Template	0	2	2	2	2	2	2	2
Water	3.875	1.156	1.125	1.093	1.063	1.031	1	0.969
Total Volume	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5

Standard curves





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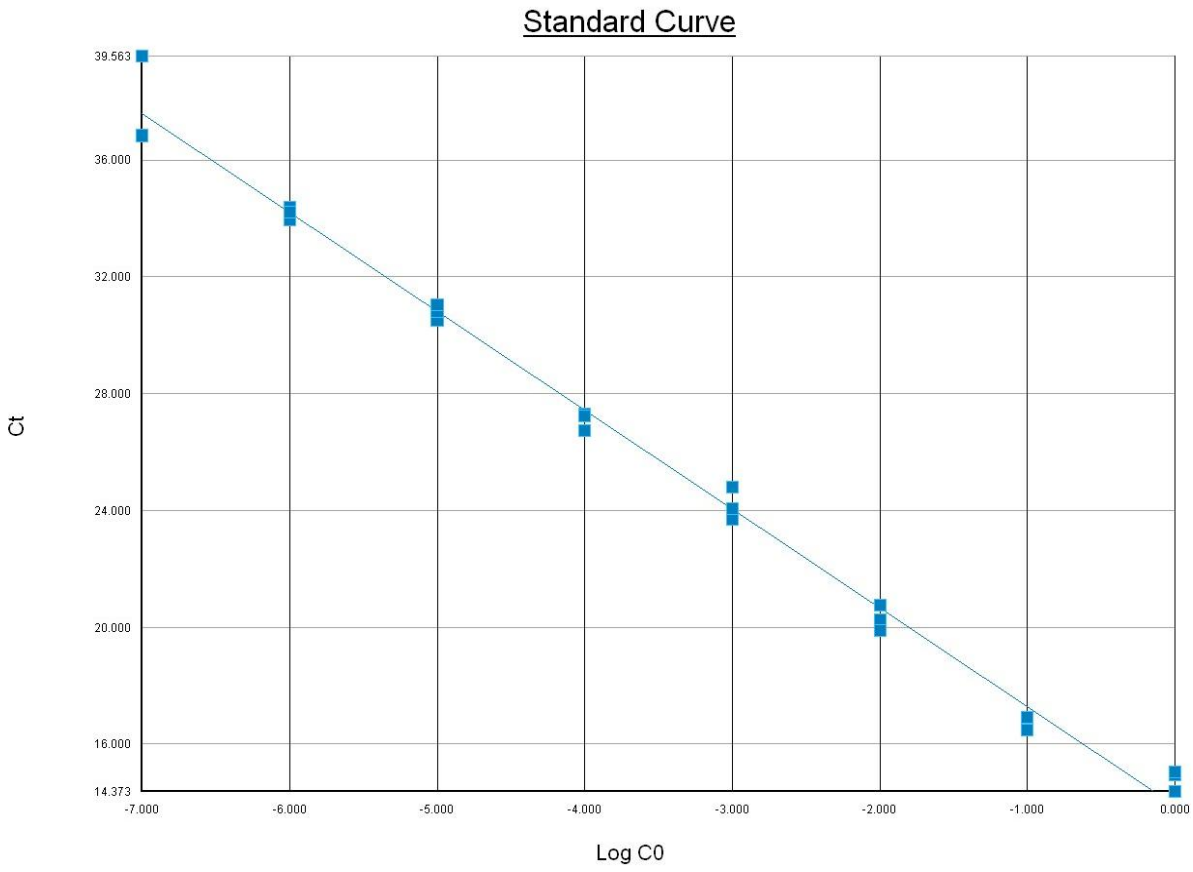
Document: Standardkurve sal + MF kontr 071008 og neg MF 150408 (Standard Curve)



Detector: Fc50, Slope: -3.386518, Intercept: 13.894060, R2: 0.991913
Document: Standard kurve (Standard Curve)

Appendix 2: Recipes

Culture medium

Bacto™ Eugon Broth (Becton, Dickinson and Company) was made as described in the manufacturer's manual. Bacto™ Eugon Broth was autoclaved and then mixed with filter sterilized $FeCl_3 \cdot 6H_2O$ (Merck™) to a final concentration of 2mM according to (Kamaishi et al. 2005)

The B1817 growth medium consists of 450ml Marine Broth (Difco), 50ml of fetal calf serum (Gibco/BRL™), 30ml of Yeastolate utrafiltrate (Gibco/BRL™), 20ml L-cystein·HCl (Merck) sol 6,3g/l dH_20 and 20ml D-glucose (Merck) sol 200g/l dH_2O as described by (Ottem et al. 2007b). All the constituent parts were filter sterilised through 0.2µm syringe filter. Finally ampicillin and fungizon was added to a final concentration of 50 – 100ng/µl.

Cysteine heart agar with chocolatized 5% sheep blood (CHAB)

Cystein heart agar (Difco) (10.2g) was solved in 100ml dH_2O and boiled to solve the agar. Cystein with a concentration of 10% and fungizon and ampicillin with a final concentration of 50 – 100ng pr ml were filter sterilized through 0.2µm syringe filter. The cystein heart agar was cooled down to about 60 ˚C before the sheep blood was added. The medium was additionally cooled before cystein, fungizon and ampicillin were added. The agar was transferred to petri dishes and left to cool for 20 - 40min before they were stored in a refrigerator with a shelf life of 2-4 weeks

Davidson's fixative solution

Davidson's fixative solution consist of 200 ml 37% formaldehyde, 100ml glycerol, 300ml 95% ethanol, 300 ml filtered seawater and 100ml acetic acid, The acitic acid was added slowly shortly before use (Shaw & Battle 1957).

4% phosphate-buffered formaldehyde

4% phosphate-buffered formaldehyde concist of 100ml of 37% formaldehyde, 8.15g $Na_2HPO_4 \times 2H_2O$, 4.00g $Na_2H_2PO_4 \times H_2O$ and 400ml tap water. Solve the phosphate salts in lukewarm water before the formaldehyde solutions is added. Control the pH which should be 7.2.

Appendix 3: Ct values and normalised expression

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std sal	Mean normalized expression
Day zero	13.298			15.0352			
4˚C P1	13.0284	13.16	0.19	15.6597	15.35	0.44	4.88
	13.6445			15.2234			
4˚C P2	14.1107	13.88	0.33	15.385	15.30	0.11	2.89
	13.2375			15.3875			
4˚C P3	14.1671	13.70	0.66*	15.5389	15.46	0.11	3.66
	35.4608			14.9909			
4˚C kontr	34.4528	34.96	0.71*	15.357	15.17	0.26	-
	11.9093						
10˚C P1	11.7898	11.85	0.08	15.3685	15.37	-	12.1
	11.306			15.4001			
10˚C P2	11.1464	11.23	0.11	15.3089	15.35	0.06	18.1
	12.0201			15.4353			
10˚C P3	12.6274	12.32	0.43	15.3912	15.41	0.03	9.01
	12.1903			15.0973			
20˚C P1	12.6917	12.44	0.35	15.4685	15.28	0.26	7.61
	12.9493			15.4635			
20˚C P2	12.5997	12.77	0.25	15.7122	15.59	0.18	7.50
	12.1535			15.1043			
20˚C P3	13.1802	12.67	0.73	15.4388	15.27	0.24	6.45
Day zero + 3 weeks incubation							
	12.0229			16.0531			
4˚C P1	12.1305	12.08	0.08	16.0614	16.06	0.01	16.5
	11.4766			16.0554			
4˚C P2	11.4987	11.49	0.02	15.8766	15.97	0.13	23.2
	10.8342			15.2716			
4˚C P3	11.0334	10.93	0.14	15.5852	15.43	0.22	23.5
4˚C kontr	Undetermined			15.7782			
	Undetermined	-	-	16.2513	16.01	0.33	-
	11.0904			15.459			
10˚C P1	10.9057	11.00	0.13	15.514	15.49	0.04	23.4
	10.9812			15.3732			
10˚C P2	10.8879	10.93	0.07	16.0872	15.73	0.50	28.9
	10.8052			15.5183			
10˚C P3	11.0584	10.93	0.18	15.7764	15.65	0.18	27.3

Day zero + 3 weeks incubation	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std sal	Mean normalized expression
	10.6821			16.0236			47.0
20˚C P1	10.4324	10.56	0.18	16.12	16.07	0.07	
	11.1696			15.9836			
20˚C P2	10.9007	11.04	0.19	15.8871	15.94	0.07	31.0
	12.4356			16.3717			
20˚C P3	12.3441	12.39	0.06	16.61	16.49	0.17	18.0

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std Sal	Mean normalized expression
2 weeks							
	18.0006			14.646			
20˚C P1	17.7274	17.86	0.19	14.9093	14.78	0.19	0.135
	16.0913			14.6076			
20˚C P2	16.2279	16.16	0.10	14.6013	14.60	0.00	0.380
	14.7672			14.4127			
20˚C P3	14.323	14.55	0.31	14.4732	14.44	0.04	1.02
2 weeks + 3weeks incubation							
	10.4817			15.1012			
20˚C P1	10.4561	10.47	0.02	15.1766	15.14	0.05	26.3
	10.7261			15.5252			
20˚C P2	10.6882	10.71	0.03	15.3908	15.46	0.10	27.9
	10.5117			14.9794			
20˚C P3	11.0145	10.76	0.36	14.8418	14.91	0.10	18.5

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expression
4 weeks	16.5921			17.4405			
4˚C P1	15.72	16.16	0.62*	17.591	17.52	0.11	2.82
	14.8603			16.9662			
4˚C P2	15.1175	14.99	0.18	17.1475	17.06	0.13	4.55
	14.719			17.0507			
4˚C P3	16.0787	15.40	0.96*	17.2811	17.17	0.16	3.71
	16.012			17.5881			
10˚C P1	15.9679	15.99	0.03	17.2872	17.44	0.21	2.99
	16.4129			17.1111			
10˚C P2	15.8538	16.13	0.40	17.067	17.09	0.03	2.14
	16.2022			17.0374			
10˚C P3	15.644	15.92	0.39	16.8736	16.96	0.12	2.26
	18.9579			17.2085			
20˚C P1	18.7839	18.87	0.12	17.1627	17.19	0.03	0.356
	19.5125			17.197			
20˚C P2	19.5242	19.52	0.01	17.1389	17.17	0.04	0.226
	18.3007			17.0631			
20˚C P3	18.8234	18.56	0.37	16.9701	17.02	0.07	0.391
4 weeks + 3 weeks incubation							
	11.296			16.3758			
4˚C P1	11.1188	11.21	0.13	16.3436	16.36	0.02	36.8
	11.0944			16.6223			
4˚C P2	11.1344	11.11	0.03	16.6376	16.63	0.01	47.4
	11.1974			17.2289			
4˚C P3	11.8478	11.52	0.46	17.0347	17.13	0.14	50.5
	11.1868			16.8851			
10˚C P1	12.002	11.59	0.46	16.657	16.77	0.16	37.6
	12.4627			17.2416			
10˚C P2	11.8641	12.16	0.58	17.1536	17.20	0.06	34.3
	10.7045			16.9867			
10˚C P3	10.5258	10.62	0.42	16.5728	16.78	0.29	73.3
	19.6041			17.1861			
20˚C P1	19.1552	19.38	0.13	17.0546	17.12	0.09	0.240
	19.743			16.6857			
20˚C P2	19.4777	19.61	0.32	16.8091	16.75	0.09	0.173
	19.5825			17.0653			
20˚C P3	19.9352	19.76	0.19	17.1966	17.13	0.09	0.197

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std Sal	Mean normalized expression
8 weeks							
	17.8129			16.3393			
4˚C P1	17.8083	17.81	0.00	16.1521	16.25	0.13	0.384
	17.0252			15.7099			
4˚C P2	16.5442	16.78	0.34	15.5248	15.62	0.13	0.502
	17.4186			17.0716			
4˚C P3	17.1607	17.29	0.18	16.7981	16.93	0.19	0.871
	17.7593			17.3348			
10˚C P1	17.4698	17.61	0.20	17.3417	17.34	0.00	0.929
	17.6665			17.2501			
10˚C P2	17.5503	17.61	0.08	17.3722	17.31	0.09	0.910
	17.9288			17.6967			
10˚C P3	17.7056	17.82	0.16	17.3682	17.53	0.23	0.917
	18.8909			17.8473			
20˚C P1	19.0328	18.96	0.10	17.6517	17.75	0.14	0.491
	18.6571			17.6901			
20˚C P2	18.6322	18.64	0.02	17.4917	17.59	0.14	0.574
	17.6852			17.0411			
20˚C P3	17.8571	17.77	0.12	17.1145	17.08	0.05	0.697
8 weeks + 3 weeks incubation							
	14.7481			15.0023			
4˚C P1	14.6383	14.69	0.08	15.1002	15.05	0.07	1.40
	17.2142			17.4426			
4˚C P2	17.1776	17.20	0.03	17.3554	17.40	0.06	1.28
	17.6844			16.5206			
4˚C P3	17.46	17.57	0.16	16.6662	16.59	0.1	0.570
	18.0483			15.1964			
10˚C P1	18.0081	18.03	0.03	15.4305	15.31	0.17	0.173
	18.0818			17.6645			
10˚C P2	18.1303	18.11	0.03	17.0944	17.38	0.40	0.679
	18.356			16.3874			
10˚C P3	18.0055	18.18	0.25	15.5257	15.96	0.61	0.245
	19.2733			16.323			
20˚C P1	19.4018	19.34	0.09	16.3986	15.21	0.05	0.0664
	17.2626			16.6148			
20˚C P2	16.9429	17.1	0.23	16.1153	16.37	0.35	0.675
	18.3589			16.4468			
20˚C P3	18.296	18.33	0.04	16.4106	16.43	0.03	0.305

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std Sal	Mean normalized expression
Day zero							
FW P1	18.2989			18.036			
	18.5511	18.43	0.18	18.0516	18.04	0.01	0.860
FW P2	18.1714			18.1305			
	17.5921	17.88	0.41	18.0948	18.11	0.03	1.31
FW P3	17.7007			17.7461			
	17.8129	17.76	0.08	17.5949	17.67	0.11	1.05
SW P1	11.8354			16.0558			
	12.0052	11.92	0.12	16.0955	16.08	0.03	18.7
SW P2	11.9518			15.8861			
	12.1605	12.06	0.15	15.9704	15.93	0.06	15.4
SW P3	11.8036			15.3728			
	12.0839	11.94	0.20	15.8192	15.60	0.32	13.3
Day zero + 3 weeks incubation							
FW P1	9.18024			15.5393			
	9.1149	9.15	0.05	15.5202	15.53	0.01	84.4
FWP2	9.57862			15.792			
	9.68536	9.63	0.08	15.8158	15.80	0.02	73.3
FW P3	11.4183			17.0959			
	11.7661	11.59	0.25	17.2867	17.19	0.13	50.2
SW P1	9.5793			16.0138			
	9.85672	9.72	0.20	15.6192	15.82	0.28	69.9
SW P2	11.918			16.9131			
	12.1676	12.04	0.18	16.9067	16.91	0.00	30.5
SW P3	10.4735			15.8481			
	10.3157	10.39	0.11	15.6983	15.77	0.11	42.9

Sample	Ct value Fc50	Std Fc50	Mean value Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expression
2 weeks							
FW P1	19.5886			16.1214			
	19.5518	19.57	0.03	15.7502	15.94	0.26	0.0937
FW P2	20.6557			16.2696			
	20.8553	20.76	0.14	16.1859	16.23	0.06	0.0509
FW P3	20.5771			16.2851			
	20.6986	20.64	0.09	16.9983	16.64	0.50	0.0732
SW P1	15.9542			16.8291			
	15.7439	15.85	0.15	17.189	17.01	0.25	2.45
SW P2	16.2958			16.8324			
	17.2145	16.76	0.65*	16.7275	16.78	0.07	1.13
SW P3	15.8969			16.2549			
	16.234	16.07	0.24	16.1124	16.18	0.10	1.19
2 weeks + 3 weeks inc.							
FW P1	22.2764			17.4189			0.0348
	22.4556	22.37	0.13	17.128	17.27	0.21	
FWP2	22.147			16.8012			
	22.949	22.55	0.57	17.0342	16.92	0.16	0.0242
FW P3	21.9436			17.1033			
	22.6484	22.30	0.50	16.6707	16.89	0.31	0.0281
SW P1	12.4848			17.2227			
	12.4513	12.47	0.02	17.4085	17.32	0.13	30.2
SW P2	12.5438			17.5247			
	12.9433	12.74	0.28	17.7458	17.64	0.16	31.3
SW P3	14.182			17.9755			11.5
	14.9185	14.55	0.52	17.9806	17.98	0.00	

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expression
4 weeks							
SW P1	19.1339			15.1961			
	19.197	19.17	0.04	15.0775	15.14	0.08	0.0710
SW P2	19.2175			15.8928			
	19.3128	19.27	0.07	15.5138	15.70	0.27	0.0975
SW P3	18.873			15.7572			
	19.1127	18.99	0.17	15.5462	15.65	0.15	0.0114
4 weeks + 3 weeks incubation							
							0.439
SW P1	18.8708			17.0393			
	18.1602	18.52	0.5	17.2574	17.15	0.15	
SW P2	21.7273			17.1403			
	22.0126	19.14	0.33	17.8098	17.48	0.47	0.361
SW P3	18.4705			16.8486			
	18.6303	18.55	0.11	16.5706	16.71	0.2	0.318

Sample	Ct value Fc50	Std Fc50	Mean value Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expexpression
1 week							
FW P1	21.2457			16.2487			
	21.2352	21.24	0.01	15.6986	15.97	0.39	0.0307
FW P2	19.8885			15.4472			
	20.0076	19.95	0.08	15.2638	15.36	0.13	0.0486
FW P3	20.1991			15.6515			
	20.6681	20.43	0.33	15.3426	15.50	0.22	0.0386

Sample	Ct value Fc50	Std Fc50	Mean value Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expexpression
1 week + 3 weeks incubation							
FW P1	22.3944			17.6592			
	22.3774	22.39	0.01	17.6304	17.64	0.02	0.042
FW P2	22.9716			17.5839			
	22.2943	22.63	0.48	17.5803	17.58	0.00	0.0361
FW P3	23.1244			17.3392			
	22.8539	22.99	0.19	17.2554	17.3	0.06	0.0233

Table 15: Samples from the cod stock, prior to experiment 2 & 3, tested for *F. noatunensis*

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	Undeterermined	-	19.40	0.22	15.41	0.11
GM 2	Undeterermined	-	22.25*	0.88*	14.27	0.58
GM 3	Undeterermined	-	18.20	0.20	28.06	-
GM 4	Undeterermined	-	19.79	0.37	15.39	0.12
GM 5	Undeterermined	-	21.14	0.00	16.61	0.26
GM 6	Undeterermined	-	21.48	0.27	15.88*	0.78*
GM 7	Undeterermined	-	19.47	0.26	16.37	0.27
GM 8	Undeterermined	-	18.62	0.05	15.86	0.23
GM 9	Undeterermined	-	20.24	0.18	15.35	0.15
GM 10	Undeterermined	-	18.28	0.09	15.52	0.17

Table 16: Samples from cod inoculated with tissue homogenate tested for *F. noatunensis*

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	34.04	0.06	18.51	0.11	15.33	0.18
GM 2	33.52	0.24	18.57	0.67*	15.15	0.26
GM 3	28.01	0.88	19.09	0.58	15.56	0.06
GM 4	-	-	17.54	0.23	14.72	0.16
GM 5	34.08	1.21	18.47	0.18	15.58	0.14
GM 6	38.76	1.93	18.55	0.50	14.91	0.22
GM 7	26.15	0.97	16.93	0.09	16.13	0.10
GM 8	31.26	1.03	18.09	0.11	15.81	0.10
GM 9	-	-	19.68	0.47	15.36	0.00
GM 10	-	-	19.77	0.42	15.42	0.54

Table 17 Samples from cod inoculated with tissue homogenate, tested for *F. noatunensis*

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	27.8991	0.07	20.44	0.15	15.17	0.00
GM 2	29.66835	0.02	19.47	0.10	15.33	0.05
GM 3	34.96155	0.40	22.07	0.30	15.17	0.39
GM 4	29.96215	0.63	21.84	0.31	15.23	0.57
GM 5	25.08215	0.58	21.30	0.09	15.90	0.19
GM 6	32.0882	0.38	20.53	0.09	16.10	0.00
GM 7	30.9205	0.22	21.16	0.12	15.20	0.04
GM 8	28.33605	0.46	21.61	0.35	15.16	0.04
GM 9	33.879	0.73	21.09	0.19	15.63	0.02

Table 18: Samples from cod injected with faeces from unexposed blue mussels, tested for *F. noatunensis*

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	Undeterermined	-	19.39	0.07	14.61	0.20
GM 2	Undeterermined	-	19.52	0.01	14.37	0.04
GM 3	Undeterermined	-	18.72	0.06	14.63	0.02
GM 4	Undeterermined	-	20.10	0.11	14.37	0.08
GM 5	Undeterermined	-	19.97	0.04	14.40	0.25
GM 6	Undeterermined	-	19.13	0.07	14.12	0.13
GM 7	Undeterermined	-	19.48	0.12	14.29	0.09
GM 8	Undeterermined	-	19.26	0.02	13.87	0.06
GM 9	Undeterermined	-	19.07	0.26	14.09	0.10

Table 19: negative control group for experiment 2 & 3, tested for *F. noatunensis*

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	Undeterermined	-	Undeterermined	-	15.16	0.16
GM 2	Undeterermined	-	Undeterermined	-	15.27	0.05
GM 3	Undeterermined	-	Undeterermined	-	15.48	0.18
GM 4	Undeterermined	-	Undeterermined	-	14.59	0.13
GM 5	Undeterermined	-	Undeterermined	-	14.70	0.09
GM 6	Undeterermined	-	Undeterermined	-	14.78	0.10
GM 7	Undeterermined	-	Undeterermined	-	15.03	0.24
GM 8	Undeterermined	-	Undeterermined	-	14.90	0.06
GM 9	Undeterermined	-	Undeterermined	-	14.92	0.15

Table 20: Cod from stock prior to the experiment, tested for *F. noatunensis*

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	Undeterermined	-	22.07	0.47	15.30	0.10
GM 2	Undeterermined	-	24.78	0.52	15.83	0.40
GM 3	Undeterermined	-	22.30	0.12	15.03	0.03
GM 4	Undeterermined	-	23.41	0.24	15.60	0.28
GM 5	Undeterermined	-	22.18	0.72*	15.05	0.00
GM 6	Undeterermined	-	23.81	0.35	14.20	0.01
GM 7	Undeterermined	-	22.19	0.16	14.68	0.14
GM 8	Undeterermined	-	23.38	0.19	15.74	0.23
GM 9	Undeterermined	-	22.49	0.05	15.57	0.01
GM 10	Undeterermined	-	22.60	0.02	15.17	0.02

Table 21: Negative control group, cohabitated with unexposed blue mussels, tested for F. noatunensis.

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	Undeterermined	-	22.15	0.08	14.88	0.34
GM 2	Undeterermined	-	21.30	0.03	15.07	0.01
GM 3	Undeterermined	-	22.12	0.15	14.77	0.24
GM 4	Undeterermined	-	22.38	0.17	15.05	0.02
GM 5	Undeterermined	-	22.00	0.59*	14.97	0.20
GM 6	Undeterermined	-	20.65	0.03	16.75	0.11
GM 7	Undeterermined	-	20.30	0.08	14.58	0.41
GM 8	Undeterermined	-	22.28	0.41	15.51	0.37
GM 9	Undeterermined	-	22.50	0.00	14.59	0.33

Table 22: Cod cohabited with mussels exposed to *F. noatunensis* 22 days earlier, tested for *F. noatunensis*

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	Undeterermined	-	21.63	0.20	15.67	0.07
GM 2	Undeterermined	-	20.12	0.48	16.97	0.14
GM 3	Undeterermined	-	20.79	0.03	17.59	0.09
GM 4	Undeterermined	-	21.19	0.27	15.23	0.14
GM 5	Undeterermined	-	23.28	0.48	14.48	0.21
GM 6	Undeterermined	-	23.97	0.05	14.36	0.22
GM 7	Undeterermined	-	23.09	0.11	14.83	0.00
GM 8	Undeterermined	-	22.75	0.29	15.16	0.14
zGM 9	Undeterermined	-	20.82	0.15	15.21	0.15
GM 10	Undeterermined	-	22.61	0.05	15.07	0.06

Table 23: Cod cohabitated with mussels exposed to F. noatunensis 11 days earlier, tested for F. noatunensis

Sample	Mean value Fc50	Std Fc50	Mean value Sal	Std Sal	Mean value GMelf	Std Gmelf
GM 1	Undeterermined	-	23.15	0.28	15.11	0.05
GM 2	Undeterermined	-	24.09	0.13	14.88	0.02
GM 3	Undeterermined	-	23.48	0.13	15.15	0.12
GM 4	Undeterermined	-	23.33	0.17	16.39	0.36
GM 5	Undeterermined	-	22.58	0.66*	15.29	0.02
GM 6	Undeterermined	-	23.32	0.08	14.92	0.14
GM 7	Undeterermined	-	23.17	0.80*	15.82	0.22
GM 8	Undeterermined	-	22.71	0.29	15.12	0.05
GM 9	Undeterermined	-	22.96	0.36	15.93	0.09

Table 24: Blue mussels exposed to *F. noatunensis*, tested for F. noatunensis

Sample	Ct value Fc50	Std Fc50	Mean value Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expression
Day 1							
211008							
ME 1	33.2458			19.7628			
	33.0251	33.14	0.16	19.8262	19.79	0.04	1.29×10^{-4}
ME 2	35.8026			21.0093			
	35.8091	35.81	0.00	20.7706	20.89	0.17	4.48×10^{-5}
ME 3	36.1563			20.4579			
	35.5399	35.85	0.44	20.2384	20.35	0.16	3.01×10^{-5}
ME 4	Undetermined			20.3333			
	38.8952	-	-	20.5258	20.43	0.14	-
ME 5	34.8557			21.8273			
	35.0759	34.97	0.16	22.1003	21.96	0.19	1.65×10^{-4}
Day 3							
231008							
ME 1	Undetermined			20.7061			
	38.098	-	-	21.0232	20.86	0.22	-
ME 2	35.3894			19.9981			
	36.0125	35.70	0.44	19.8872	19.94	0.08	2.52×10^{-5}
ME 3	36.126			22.2172			
	36.8517	36.49	0.51	21.7417	21.98	0.34	5.97×10^{-5}
ME 4	36.2502			21.4759			
	36.4887	36.37	0.17	21.4011	21.44	0.05	4.47×10^{-5}
ME 5	37.6486			20.518			
	37.3135	37.48	0.24	20.5386	20.53	0.01	1.13×10^{-5}
Day 7							
271008							
ME 1	37.862			20.2672			
	39.4294	38.65	1.11*	20.525	20.40	0.18	4.65×10^{-6}
ME 2	38.254			20.293			
	38.89	38.57	0.45	20.0625	20.18	0.16	4.22×10^{-6}
ME 3	Undetermined			18.8826			
	Undetermined	-	-	18.6784	18.78	0.14	-
ME 4	37.7231			20.5549			
	38.4235	38.07	0.50	20.5654	20.56	0.01	7.96×10^{-6}
ME 5	36.6588			21.1442			
	36.0908	36.37	0.40	21.1468	21.15	0.00	3.66×10^{-5}

Sample	Ct value Fc50	Std Fc50	Mean value Fc50	Ct value Sal	Mean value Sal	Std Sal	Mean normalized expression
Day 11							
311008	Undetermined			21.4541			
ME 1	Undetermined	-	-	21.4948	21.47	0.03	-
	37.4161			21.88			
ME 2	37.142	37.28	0.19	21.7215	21.80	0.11	3.08×10^{-5}
	Undetermined			21.5319			
ME 3	39.6233	-	-	21.0075	21.27	0.37	-
	40.243			21.5773			
ME 4	39.4395	39.84	0.57	21.4507	21.51	0.09	4.43×10^{-6}
	35.5635			21.4482			
ME 5	36.0988	35.83	0.38	21.2718	21.36	0.12	6.11×10^{-5}
Day 22							
111108	Undetermined			23.618			
ME 1	Undetermined	-	-	23.9475	23.78	0.23	-
	39.1093			22.2359			
ME 2	Undetermined	-	-	21.8037	22.02	0.31	-
	33.463			21.3087			
ME 3	33.0255	33.24	0.31	21.5144	21.41	0.15	-
	Undetermined			22.3368			
ME 4	Undetermined	-	-	22.1881	22.26	0.11	-
	Undetermined			20.4018			
ME 5	Undetermined	-	-	20.1422	20.27	0.18	-
Day 40							
291108				21.4201			
ME 1				21.6934	21.56	0.19	
				21.3811			
ME 2				21.1161	21.25	0.19	
				20.7584			
ME 3				20.8668	20.81	0.08	
				20.1536			
ME 4				20.441	20.30	0.20	
				20.5376			
ME 5				20.5426	20.54	0.00	

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std Sal	Mean normalized expression
Day 70	Undetermined						
281208	Undetermined			19.9338			
ME 1		-	-	19.9837	19.96	0.04	
ME 2	-	-	-	-	-	-	-
	Undetermined			20.6516			
ME 3	Undetermined	-	-	21.1539	20.90	0.36	
	Undetermined			19.8686			
ME 4	Undetermined	-	-	19.8646	19.87	0.00	
	Undetermined						
ME 5	Undetermined			21.0862			
		-	-	21.0242	21.06	0.04	
Day 113							
100209	Undetermined			20.4473			
ME 1	Undetermined	-	-	20.7382	20.59	0.21	
	Undetermined			20.1823			
ME 2	Undetermined	-	-	19.9929	20.09	0.13	
	Undetermined			20.1971			
ME 3	Undetermined	-	-	20.1531	20.18	0.03	
	Undetermined			20.3664			
ME 4	Undetermined	-	-	20.5695	20.47	0.14	
	34.8611			21.2957			
ME 5	34.7959	34.83	0.05	20.984	21.14	0.22	
Me positive							
150408	38.5624			20.9818			
ME 1	36.3704	37.47	1.55	20.7204	20.85	0.18	
	31.076			20.0263			
ME 2	30.6704	30.87	0.29	19.8874	19.96	0.10	
	31.115			18.4991			
ME 3	31.4137	31.26	0.21	18.4047	18.45	0.07	
	33.2661			19.3484			
ME 4	33.6634	33.46	0.28	18.8484	19.10	0.35	
	29.4453			20.1371			
ME 5	29.8096	29.63	0.26	19.5668	19.85	0.40	

Table 25: Blue mussels sampled in exp. 2, tested for *F. noatunensis*

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std Sal	Mean normalized expression
ME 060408 digestive gland	18.351			21.6192			
Me 1	18.2779	18.31	0.05	21.9341	21.78	0.22	
	23.0338			20.2201			
Me 2	23.1555	23.09	0.09	20.0364	20.13	0.13	
Me 3	20.5437			19.1175			
	22.5951	21.57	1.45*	18.7928	18.96	0.23	
	20.0702			20.7612			
Me 4	21.0289	20.55	0.68*	20.7096	20.74	0.04	
	19.7347			19.9623			
Me 5	21.1354	20.44	0.99*	19.4593	19.71	0.36	
ME 080408 digestive gland	23.6441			19.3425			
Me 1	24.0954	23.87	0.32	18.8773	19.11	0.33	
	21.2906			17.4586			
Me 2	21.7699	21.53	0.34	17.1214	17.29	0.24	
Me 3	25.3433			20.1756			
	25.3382	25.34	0.00	19.3724	19.77	0.57	
	21.3125			19.5695			
Me 4	20.466	20.89	0.60	19.5126	19.54	0.04	
	21.0705			19.278			
Me 5	20.7572	20.91	0.22	20.1948	19.74	0.65	
ME 060408 gills	15.7894			19.4044			
Me 1	16.5271	16.16	0.52	18.3346	18.87	0.76*	
	16.4026			21.0962			
Me 2	17.3917	16.90	0.70*	20.4006	20.75	0.49	
	15.9088			18.5319			
Me 3	15.9471	15.93	0.03	19.5165	19.02	0.70*	
	17.0925			20.6659			
Me 4	17.3729	17.23	0.20	20.1715	20.42	0.35	
	18.0203			19.339			
Me 5	17.1376	17.58	0.62*	19.3704	19.35	0.02	

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std Sal	Mean normalized expression
ME 080408							
gills	17.363			18.64			
Me 1	16.1542	16.76	0.85*	18.3738	18.51	0.19	
	19.1743			18.512			
Me 2	19.6654	19.42	0.35	18.3092	18.41	0.14	
	19.4029			19.1706			
Me 3	19.6781	19.54	0.19	19.135	19.15	0.03	
	19.001			19.113			
Me 4	18.6614	18.83	0.24	19.0574	19.09	0.04	
	19.0472			18.9495			
Me 5	19.1956	19.12	0.10	18.9599	18.95	0.01	

Table 26: Unexposed mussels tested for *F. noatunensis*

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Mean value Sal	Std Sal	Mean normalized expression
Exp. 2	39.2186			17.6779			
ME 1	38.9767	39.10	0.17	17.6095	17.64	0.05	
	Undetermined			19.3319			
ME 2	Undetermined	-	-	19.1583	19.25	0.12	
	Undetermined			18.367			
ME 3	Undetermined	-	-	18.5854	18.48	0.15	
	29.3828			19.1407			
ME 4	30.6497	30.02	0.90*	19.1786	19.16	0.03	
Exp 3							
digestive gland	Undetermined			18.722			
ME 1	Undetermined	-	-	18.4051	18.56	0.22	
	Undetermined			20.1188			
ME 2	Undetermined	-	-	19.6863	19.90	0.31	
	Undetermined			19.9399			
ME 3	Undetermined	-	-	20.0269	19.98	0.06	
	Undetermined			20.1703			
ME 4	Undetermined	-	-	19.2892	19.73	0.62*	
	Undetermined			19.4634			
ME5	Undetermined	-	-	18.9531	19.21	0.36	
Exp 3							
gills	39.4192			18.1157			
ME 1	Undetermined	-	-	17.8324	17.97	0.20	
	Undetermined			18.7558			
ME 2	39.5792	-	-	18.1335	18.44	0.44	
	Undetermined			18.204			
ME 3	Undetermined	-	-	18.103	18.15	0.07	
	Undetermined			19.4622			
ME 4	Undetermined	-	-	19.2218	19.34	0.17	
	Undetermined			19.0609			
ME 5	Undetermined	-	-	18.7433	18.90	0.22	

Table 27: Unexposed mussels tested for *F. noatunensis*

Sample	Ct value Fc50	Std Fc50	Mean value Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expression
Day 1 exp 4							
digestive gland	39.4303	-	-	21.2542			
ME 1	Undetermined			21.3294	21.29	0.05	
	27.0595			Undetermined			
ME 2	27.8092	27.43	0.53	Undetermined	21.88	0.11	
	Undetermined			21.7993			
ME 3	Undetermined	-	-	21.9545	21.34	0.39	
	Undetermined			21.059			
ME 4	Undetermined	-	-	21.6156	21.32	0.20	
	Undetermined			21.1771			
ME 5	Undetermined	-	-	21.4546	21.88	0.11	
Day 88							
digestive gland	Undetermined			20.0417			
ME 1	38.5708	-	-	20.0569	20.05	0.01	
	34.3163			20.7978			
ME 2	34.2481	34.28	0.05	20.4904	20.64	0.22	
	Undetermined			19.4563			
ME 3	31.1333	-	-	19.5165	19.49	0.04	
	Undetermined			21.8234			
ME 4	Undetermined	-	-	21.3266	21.58	0.35	
	37.959			20.5762			
ME 5	Undetermined	-	-	20.193	20.38	0.27	
	35.0324			19.8354			
ME 6	35.0522	35.04	0.01	19.4684	19.65	0.26	
	Undetermined			21.3271			
ME 7	39.0514	-	-	21.0586	21.19	0.19	
	Undetermined			20.4708			
ME 8	Undetermined	-	-	20.0172	20.24	0.32	
	39.0741			19.1751			
ME 9	39.5959	39.34	0.37	19.1706	19.17	0.00	
	Undetermined			20.7961			
ME 10	Undetermined	-	-	20.4451	20.62	0.25	

Sample	Ct value Fc50	Std Fc50	Mean value Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expression
Termination of exp.							
digestive gland	37.2207			19.6699			
ME 1	37.0087	37.11	0.15	19.9243	19.80	0.18	
	37.2241			19.965			
ME 2	37.5938	37.41	0.26	19.8133	19.89	0.11	
	Undetermined			20.1683			
ME 3	Undetermined	-	-	19.706	19.94	0.33	
	Undetermined			19.3131			
ME 4	Undetermined	-	-	19.336	19.32	0.02	
	Undetermined			20.7145			
ME 5	39.1131	-	-	20.7529	20.73	0.03	

Table 28: Samples collected in exp. 2 & 3 and tested for *F. noatunensis*.

Sample	Ct value Fc50	Mean value Fc50	Std Fc50	Ct value Sal	Std Sal	Mean value Sal	Mean normalized expression
ME faeces	18.0521			17.685			
sample 110408	17.4104	17.73	0.45	17.5715	17.63	0.08	
Me faeces	28.8551			17.0314			
sample 150408	30.1595	29.51	0.92	17.0118	17.02	0.01	
Me faeces	35.3453			19.6233			
Sample 220408	36.1388	35.74	0.56	20.0323	19.83	0.29	
Me faeces	33.8486			18.1486			
Sample 230408	34.0433	33.95	0.14	18.2157	18.18	0.05	
Me faeces	33.8651			18.3613			
Sample 290408	33.9405	33.90	0.05	18.8266	18.59	0.33	
Control faeces (injected in cod) Me 160608	32.9749			16.6558			
	30.6748	31.82	1.63	17.1011	16.88	0.31	
Water sample aquarium exp 2	13.7883			15.4111			
	13.4284	13.61	0.25	15.4164	15.41	0.00	
Water sample aquarium exp 3	18.2003			16.4124			
	18.0517	18.13	0.11	16.5185	16.47	0.08	