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Atlantic salmon water pathogens inactivation by UV irradiation

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Abstract

Open sea cage Atlantic salmon (*Salmo salar* L.) farming is currently facing major challenges related to sea lice infestations, sea lice treatment, fish mortality, infectious diseases and fish escapees. Semi-closed containment systems may have the potential to resolve these challenges, by separating the rearing unit from the external environment. UV disinfection is suggested as a possible water treatment method, to ensure optimal rearing environment and safe water supply within the system. The necessity and effect of such a treatment on pathogen inactivation needs to be evaluated before being implemented in semi-closed containment systems. There is also a need for more information on specific UV dose requirements to inactivate pathogens infecting farmed salmon during the seawater rearing phase.

This study investigates the required reduction equivalent UV doses for complete (99.9%) inactivation of specific Atlantic salmon pathogens, the impact of UV treatment on seawater microbial communities' equilibrium and the assessment of UV induced DNA damages in a salmon parasite. Bench-scale trials were conducted using two Collimated Beam Apparatus: a low- and medium-pressure UV mercury lamp. The targeted pathogens were *Moritella viscosa,* the infectious salmon anaemia virus (ISAV) and the ectoparasite *Lepeophtheirus salmonis.* DNA damages in *L. salmonis* were assessed using Comet assay analysis. Seawater samples from a salmon farm and a reference station were exposed to UV radiation and the effects on seawater microbial communities' equilibrium were evaluated.

For *M. viscosa* all UV doses employed in this experiment resulted in an inactivation greater than log 3. The lowest doses were 3 mJ/cm^2 using low-pressure UV and 2.3 mJ/cm^2 using medium-pressure UV. Contrary to this, none of the UV doses resulted in an inactivation of log 3 or higher in ISAV. The highest inactivation was log 2.59 and was achieved by 22.5 mJ/cm^2 with medium-pressure UV. Using low-pressure UV, the highest log inactivation achieved was 2.40 exposed to 2 mJ/cm^2. As for *L. salmonis*, none of the UV doses resulted in 99.9% mortality rate. The highest mortality was 47.1% and was achieved by 199.3 mJ/cm^2 using a medium-pressure UV apparatus. For low-pressure UV, the highest achieved mortality rate was 24.5% when exposed to 126 mJ/cm^2. The medium-pressure UV lamp induced a medium to high level of DNA damage in *L. salmonis* cells at doses of 5, 10, 20 and 40 mJ/cm^2. The seawater samples exposed to 25 mJ/cm^2 medium-pressure UV presented only residual colony forming bacteria, illustrating a significant disturbance on the seawater microbial communities' equilibrium.

In conclusion, UV disinfection can be used to prevent disease caused by *M. viscosa*. ISAV needs somewhat higher UV doses, but within feasible ranges. Control of *L. salmonis* is not possible with the tested technology as the UV dose required are too high.

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Abbreviations

ASK	Atlantic salmon kidney
CBA	Collimated Beam Apparatus
CFU	Colony forming units
CMS	Cardiomyopathy syndrome
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
HSMI	Heart and skeletal muscle inflammation
IPN	Infectious pancreatic necrosis
ISA	Infectious salmon anaemia
ISAV	Infectious salmon anaemia virus
LD_{50}	50% Lethal dose
LP	Low-pressure
MP	Medium-pressure
NaCl	Sodium chloride
PCR	Polymerase chain reaction
PD	Pancreas disease
RNA	Ribonucleic acid
S-CCS	Semi-closed containment system
TCBS	Thiosulfate citrate bile salts sucrose
TCID50 assay	Tissue culture infectious dose
UV	Ultraviolet
UVT	Ultraviolet transmission

1 Introduction

1.1 Atlantic salmon production

The breakthrough of Norwegian aquaculture came in the early 1970s. The pioneers Ove and Sivert Grøntvedt built the first successful salmon farm on the island of Hitra (Hovland et al., 2014). This was the first time Atlantic salmon (*Salmo salar* L.) was cultured to marketable size in sea cages (FAO, 2006). Currently, Norway is the world leading producer of farmed Atlantic salmon, with more than 50% of total production (Iversen et al., 2020). In 50 years, the aquaculture industry has grown from nearly nothing to one of the most important industries in Norway. Currently, seafood is the fourth largest export industry in the country (SSB, 2021). In 2019, fish from aquaculture in Norway amounted to 1.45 million tons at a first-hand value of NOK 71.7 billion, shown Figure 1 (SSB, 2020). Norwegian aquaculture is predominantly a monoculture (Hovland et al., 2014). Farmed Atlantic salmon made up 93.9% of the first-hand value in 2019. Including other farmed salmonids, they made up 99.6% of the first-hand value in 2019 (SSB, 2020).

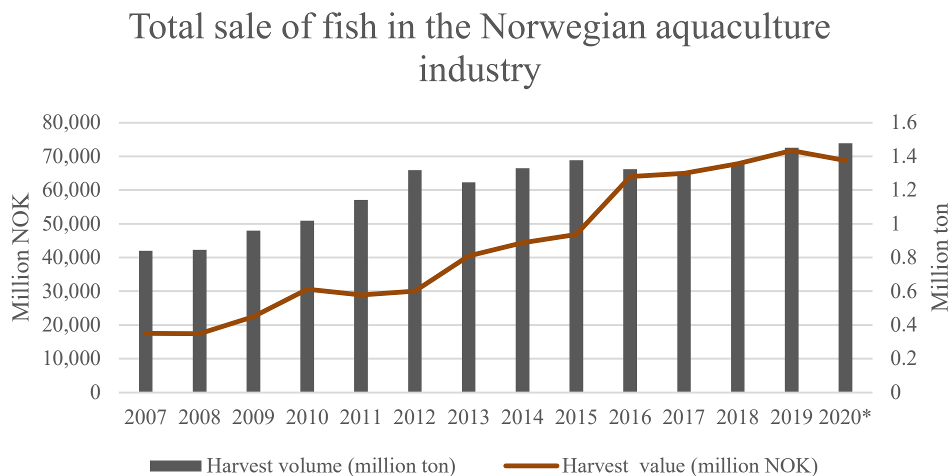

Figure 1. Total first-hand value (in 1000 NOK) of slaughtered fish in the Norwegian aquaculture industry in the period 2007 to 2020. * Preliminary figures. Retrieved from Directorate of Fisheries, 2021.

This spectacular course of development in Norwegian aquaculture has been made possible by several factors. The favourable hydrographic conditions laid the foundation of the emergence of aquaculture industry. The ocean temperatures and salinities ocean are stable and suited for Atlantic salmon. The available sites are sheltered and with good water exchange. Although farmed Atlantic salmon is genetically more adapted for aquaculture, they have the same origins as the wild salmon, which mature relatively late. Late maturation is advantageous as the salmon can reach marketable size and undesired effects of sexual maturation can be avoided (FAO, 2006).

Commercial production of Atlantic salmon (Figure 2) is a result of systematic breeding to ensure the best and most appealing product possible. The best quantitative traits of the broodstock are embodied in the next generation to make sure they are well adapted for intensive aquaculture. These include high growth rate, high utilization of feed, resistance against infectious diseases, flawless exterior, right colour, and texture of the flesh. The carefully selected broodstock are transferred to freshwater cages or tanks in land-based facilities where the eggs are stripped and fertilized with milt. The eggs are incubated until hatching and the fry are nourished by the yolk sac before being fed with pellets. The fry is kept in land-based freshwater tanks until they have undergone the seawater preparatory transformation, known as smoltification. The smolts are then transferred to sea cages (Hovland et al., 2014).

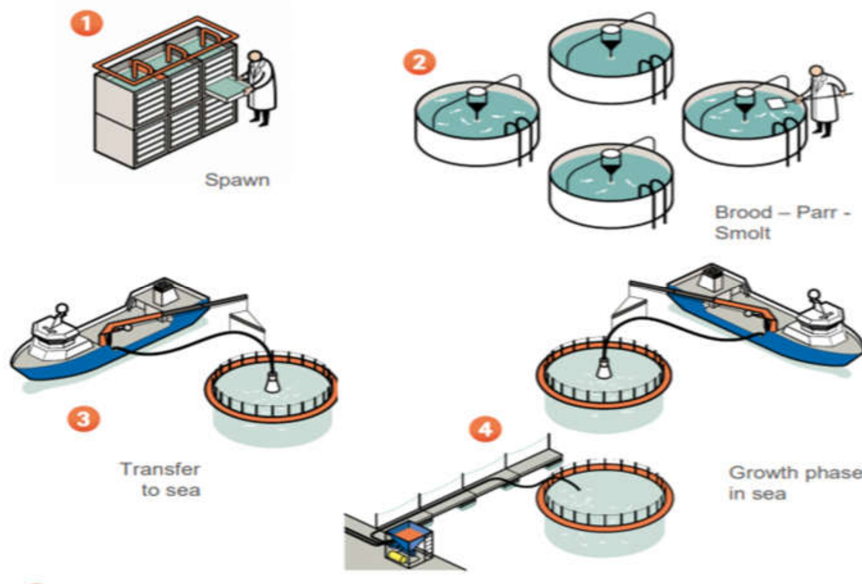

Figure 2. The different stages (1-4) of the Atlantic salmon production cycle. Retrieved from Marine Harvest, 2018.

The salmon are considered post-smolts after they have entered and acclimated to seawater. This process is a sensitive phase of the life cycle of farmed salmon (Stradmeyer, 1994). Smoltification is a complex and energy demanding transformation, making the smolt more responsive to stressors (Jarungsriapisit et al., 2016). The physiological changes following smoltification can supress components of the immune system, making the smolt more susceptible to diseases. In addition, the transfer and adaption to open sea cages itself is an especially stressful event for farmed salmon (Roberts & Pearson, 2005). Farmed Atlantic salmon spend the rest of their life cycle in sea cages, before they reach market size after around 15-18 months (Hovland et al., 2014).

1.2 Loss in aquaculture

Around 15% of farmed salmon are lost during production at sea (Norwegian Veterinary Institute, 2020). A substantial part of these losses occurs shortly after the farmed fish have been transferred to seawater (Bleie & Skrudland, 2014). Loss of farmed fish affects both the economic and environmental sustainability, as well as the reputation of the salmon industry. The causes of the mortality are also affecting the welfare of farmed fish (Diserud et al., 2019). Some of the losses are caused by production diseases and other disorders (Bruno et al., 2013). Other losses are linked to escaped farmed salmon, raising concern about negative impact on wild stocks (Diserud et al., 2019). Although substantial control measures may prevent disease outbreaks and lead to an overall improvement in the health of farmed fish, infectious diseases account for considerable parts of these losses (Norwegian Veterinary Institute, 2020).

Infectious diseases can be caused by different fish pathogenic bacteria, viruses, parasites or fungal diseases. In open aquatic environment, exposure to pathogenic microorganisms is impossible to avoid. The high fish stocking density in intensive aquaculture, in the case of Atlantic salmon reaching 200 000 fish per cage, constitutes a large reservoir for pathogenic organisms, making them highly prone to epidemics (Bruno et al., 2013).

Bacterial infections in farmed salmon are moderate and generally under control. However, bacterial diseases are detected periodically. The most common bacterial diseases are mycobacteriosis, yersiniosis, furunculosis, pasteurellosis, and winter ulcer. It is challenging to estimate the exact occurrence of winter ulcer, although it has been reported by the farmers as a major cause of reduced welfare of the farmed fish (Norwegian Veterinary Institute, 2020).

Viral infections account for large parts of losses in salmon farming. The most common viral diseases are cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation (HSMI), pancreas disease (PD), infectious salmon anaemia (ISA) and infectious pancreatic necrosis (IPN) (Madhun et al., 2020). Number of ISA cases are dramatically increasing in Norway. The number of ISA cases in 2020, were the highest recorded in the last 30 years. The outbreaks were detected all along the Norwegian coast (Norwegian Veterinary Institute, 2020).

The salmon louse, *Lepeoptherius salmonis,* are among the dominating causes of loss of farmed fish. However, mechanical treatment strategies are also leading to major losses (Norwegian Veterinary Institute, 2020).

1.2.1 Winter ulcer

Winter ulcer is a bacterial disease leading to increased mortality during the sea-phase and reduced fillet quality at slaughter (Norwegian Veterinary Institute, 2020). It is a considerable welfare problem for farmed salmon in Norwegian aquaculture. The fish can survive for long periods of time while exhibiting substantial skin lesions, shown in Figure 3 (Løvoll et al., 2009). The disease is usually a problem during autumn and winter, although it may occur all year around (Lunder, 1990). Winter ulcer most commonly affect smolts right after sea transfer but can affect farmed salmon at all stages of the seawater phase, often after management procedures including fish handling and stress (Norwegian Veterinary Institute, 2020). When seawater is added to freshwater for smolt acclimation, the disease can also infect fish at land-based hatcheries (Greger & Goodrich, 1999). The disease occurs in salmon farms along the entire Norwegian coast. However, it is difficult to estimate the exact occurrence as it is a non-notifiable disease (Norwegian Veterinary Institute, 2020).

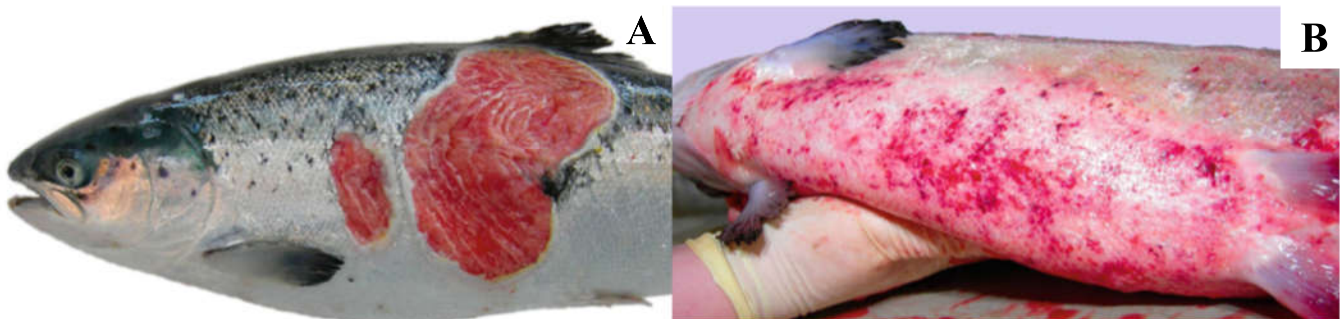

Figure 3. Winter ulcers in Atlantic salmon caused by *M. viscosa* (A). Ventral hemorrhage in Atlantic salmon from systemic *M. viscosa* infection (B). Retrieved from Bruno et al. 2013.

There are different types of ulcer syndromes correlated with farmed salmon in cold seawater. 'Typical' winter ulcer is the most common type, whereas the main causative agent is the bacteria *Moritella viscosa* (Norwegian Veterinary Institute, 2020). *M. viscosa* is a motile gram-negative and psychrophilic bacterium. It is also flagellated and has a curved rod shape (Bruno et al., 2013). *M. viscosa* can be cultivated in media containing Sodium chloride (NaCl) and form viscous colonies at 4°C to 21°C (Benediktsdóttir & Heidarsdóttir, 2007). *M. viscosa* extracellular products are cytotoxic to the cells of the fish and can be lethal to Atlantic salmon (Karlsen et al., 2017). The bacteria transmit horizontally in water (Norwegian Veterinary Institute, 2020).

The severity of the lesions can vary from superficial wounds to substantial wounds which can extend into muscle (Lunder et al., 1995). *M. viscosa* is commonly causing systemic infections, which may result in bleedings or circulatory failure in the internal organs (Norwegian Veterinary Institute, 2020). The bacteria can also cause eye infections (Tunsjø et al., 2007). Winter ulcer is generally easily diagnosed in the field based on the characteristic lesions (Løvoll et al., 2009). Other bacteria, such as *Tenebaculum* spp. and *Aliivibrio wodanis*, are also often found in diagnostic investigations, making the bacteriological picture complex (Norwegian Veterinary Institute, 2020).

Most of all farmed salmon are vaccinated against *M. viscosa* infection. However, the vaccine does not provide full protection. Antibiotic treatment has variable effect and is rarely used. Ensuring good skin health, avoid handling at low water temperatures and removal of fish showing visible lesion are preventive measurements. The less common type is 'Atypical' winter ulcer, also called 'tenecibaculosis'. It is a serious condition that can be characterised by deep lesion of the jaw and high mortality (Norwegian Veterinary Institute, 2020).

1.2.2 Infectious salmon anaemia

ISA is caused by the aquatic orthomyxovirus, infectious salmon anaemia virus (ISAV) (Mjaaland et al., 1997; Norwegian Veterinary Institute, 2020). The disease severely affects farmed Atlantic salmon after sea transfer or in freshwater hatcheries where seawater has been added (Nylund et al., 1994). ISA result in major fish and economic loss (Falk et al., 1997). It was first seen in farmed Atlantic salmon in Norway in 1984 (Thorud & Djupvik, 1988). After major disease outbreaks in the end of the 1980s, several measurements against the disease were implemented in Norwegian aquaculture. Measures like separating year classes, health certification, disinfecting effluent water from slaughtering facilities, fallowing of sites were used against ISA (and other diseases). This reduced the impact of the disease, and outbreaks declined (Bruno et al., 2013). Despite a decrease in number of outbreaks, there has still been occurrence of the disease every year since. In 2020 it was registered 23 ISA outbreaks in Norway, which is the highest recorded number in the last 30 years (Norwegian Veterinary Institute, 2021).

ISA is a notifiable disease in Norway. Occurrence or suspicion of ISA must immediately be reported to The Norwegian Food Safety Authority. Detection of ISAV shall also be reported to the World Organisation for Animal Health (Norwegian Veterinary Institute, 2020).

ISA virus is a 100-130 nm pleomorphic enveloped virus with 10-12 nm projections on the surface. The genome consists of single-stranded RNA with eight segments of negative polarity, whereas the total length of the segments is approximately 14.3 kb. Atlantic salmon kidney (ASK) cells are favored cell line for primary isolation. The optimal temperature for replication in susceptible fish cell lines is 10-15°C (Rimstad et al., 2011). ISA virus primarily attacks blood vessels and heart, through the mucous membranes on the skin and gills of the fish (Norwegian Veterinary Institute, 2020).

ISAV causes severe anaemia, circulatory problems and bleedings in internal organs and skin. Other symptoms of the disease can be pale gills, oedema, accumulation of fluid in the abdomen, swollen spleen or kidney, dark liver, or accumulation of blood in the intestine, shown in Figure 4 (Thorud & Djupvik, 1988; Evensen et al., 1991; Nylund et al., 1993). The disease spreads mainly through horizontal transmission, although vertical transmission most likely also occurs (Nylund et al., 1994; Norwegian Veterinary Institute, n. d.)

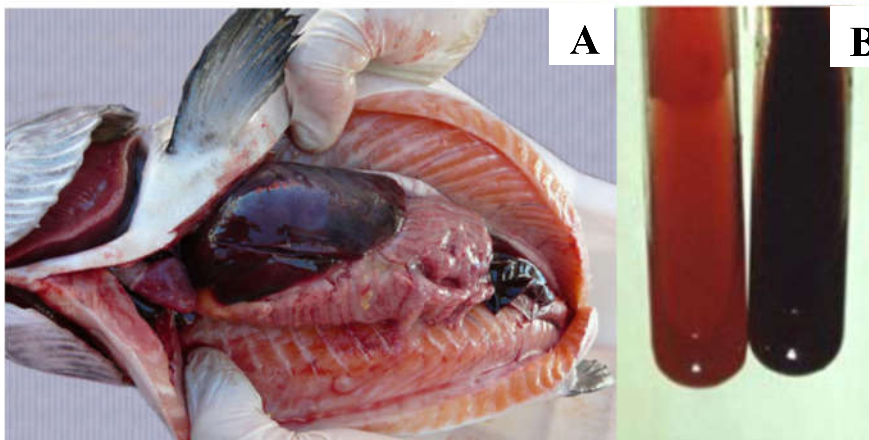

Figure 4. Characteristic dark liver in Atlantic salmon with ISA (A). Retrieved from Bruno et al., 2013. Anaemic blood from Atlantic salmon with and without ISA (B). Photo: Trygve Poppe.

The symptoms of ISAV infected fish can often be vague or inconsistent (Thorud, 1990). This can make it difficult to detect the disease under certain circumstances. In order to identify the disease, detection of histopathological changes characteristic of ISA and immunohistochemistry is necessary. In addition, virus must be detected with real-time PCR or cell culture to confirm an ISA diagnosis. As ISA is a notifiable disease in Norway, outbreaks are treated adopting strict measures. The Norwegian Food Safety Authority establishes a control area and a surveillance area with specific regulations. Infected fish would also normally be removed and slaughtered immediately to prevent transmission to other facilities. There is also developed a vaccine against ISA which are used in some cases, but it does not provide full protection (Norwegian Veterinary Institute, n. d).

1.2.3 *Lepeoptheirus salmonis*

The salmon louse, *L. salmonis,* is an obligate ectoparasite copepod on wild and farmed salmonids (Aarseth & Schram, 2002). It is currently one of the largest challenges in Norwegian aquaculture, causing major economic loss in salmon farming (Norwegian Veterinary Institute, 2020; Bruno et al., 2013). It is naturally occurring in the marine environment in the northern hemisphere with highest incidence during autumn (Norwegian Veterinary Institute, 2020). *L. salmonis* life cycle consists of ten stages from hatching to adult stage. These are two free-swimming naupliar stages, one free-swimming infective copepodid stage, four attached chalimus stages, two preadult stages, and an adult stage (Johnson & Albright, 1991). The three first stages are pelagic, the four next stages are fixed to the host, while the three last stages are mobile on the host (Aarseth & Schram, 2002).

L. salmonis attaches to external surface of the fish. They feed on mucus, epidermal, dermal or subdermal cutaneous tissue of the host. The feeding behaviour result in skin ulceration, petechiae (skin spots) and following hyperpigmentation. These lesions, showed in Figure 5, may break the osmotic barrier of the fish which may be fatal or result in sites for secondary infections (Bruno et al., 2013). Without treatment, the number of *L. salmonis* in a facility may increase significantly and result in severe fish skin lesions and mortality (Bruno et al., 2013). Each adult female copepod can produce thousands of eggs, number increasing with increasing water temperature. At the planktonic stages, the larvae may disperse several kilometres and infect farmed fish in sea cages (Norwegian Veterinary Institute, 2019). Intensive surveillance and prophylactic treatments are therefore necessary to reduce infestations (Bruno et al., 2013).

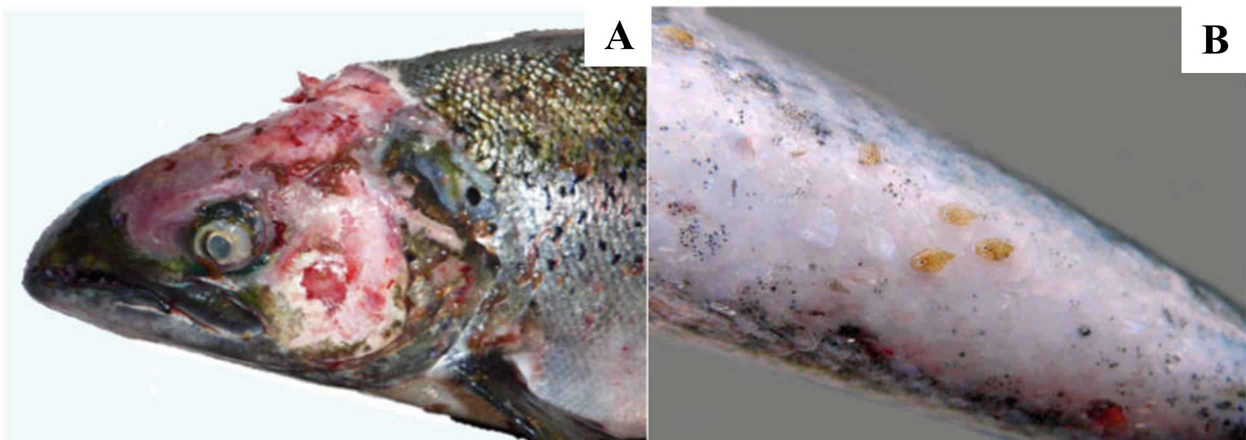

Figure 5. Severe skin lesions caused by *L. salmonis* on Atlantic salmon (A*). L. salmonis* chamilus larvae on ventral part of peduncle of Atlantic salmon (B). Retrieved from Bruno et al. 2013.

Norwegian legislation defines the maximum burden of permitted *L. salmonis* in a facility. All salmon farms are required by law to monitor and report number of *L. salmonis* every week to Norwegian Food Safety Authority (Forskrift om lakselusbekjempelse, 2016). Both pharmaceutical and non-pharmaceutical methods are used as treatments against *L. salmonis*. As levels of resistance against pharmaceuticals are increasing, non-pharmaceutical treatments are now the main control methodology. These are mainly thermal, mechanical and freshwater treatments (Norwegian Veterinary Institute, 2019). Cleaner fish are used as a preventative measure and as a form of biological control in addition to other treatments (Bruno et al., 2013). High frequency of treatments has resulted in a major rise in production costs in open cage salmon farming. Treatments also lead to considerable consequences regarding welfare of the fish, as there is a high risk of injury and mortality (Norwegian Veterinary Institute, 2020) and anti-sea lice pharmaceuticals may have detrimental effects on non-target species when released in the marine environment (Urbina et al., 2019).

1.3 Closed containment aquaculture system

Even though prophylaxis and vaccines are advancing, challenges related to sea lice infestations, sea lice treatment, mortality, infectious diseases and escapees, are limiting factors for further expansion and sustainability of the industry (Rud et al., 2017). Future expansion might therefore depend on development of alternatives to open sea cages or reduce time spent in open sea cages (Norwegian Veterinary Institute, 2020).

Floating semi-closed containment system (S-CCS) are one of the new production technologies for salmon sea farming. S-CCS are still at a development stage, although several companies have built and put to test different prototypes. The capacity and design among these are very different. A general principle of the system is shown in Figure 6. In these systems the floating rearing units are separated from the external environment by a physical barrier (Rosten et al., 2011). The barrier can be a semi-permeable or watertight structure (Van de Vis et al., 2020).

Intake water is actively transported through the system. It is pumped from a deeper part of the water column to prevent contamination from surface water. This also provides more stable temperature conditions and allows control of oxygen within the rearing unit (Van de Vis et al., 2020). The impact on the natural environment is also reduced with S-CCS. The system design reduces the risk of escaped farmed fish, as well as making waste product removal possible (Norwegian Veterinary Institute, 2020). S-CCS are mainly intended for production of

post-smolt up to 1 kg. By then the farmed salmon will have better ability to cope with environmental challenges, diseases, and transfer to open sea cages will be improved. This way S-CCS are not replacing open sea cages, rather supplementing them by reducing time spent in open sea cages (Calabrese, 2017).

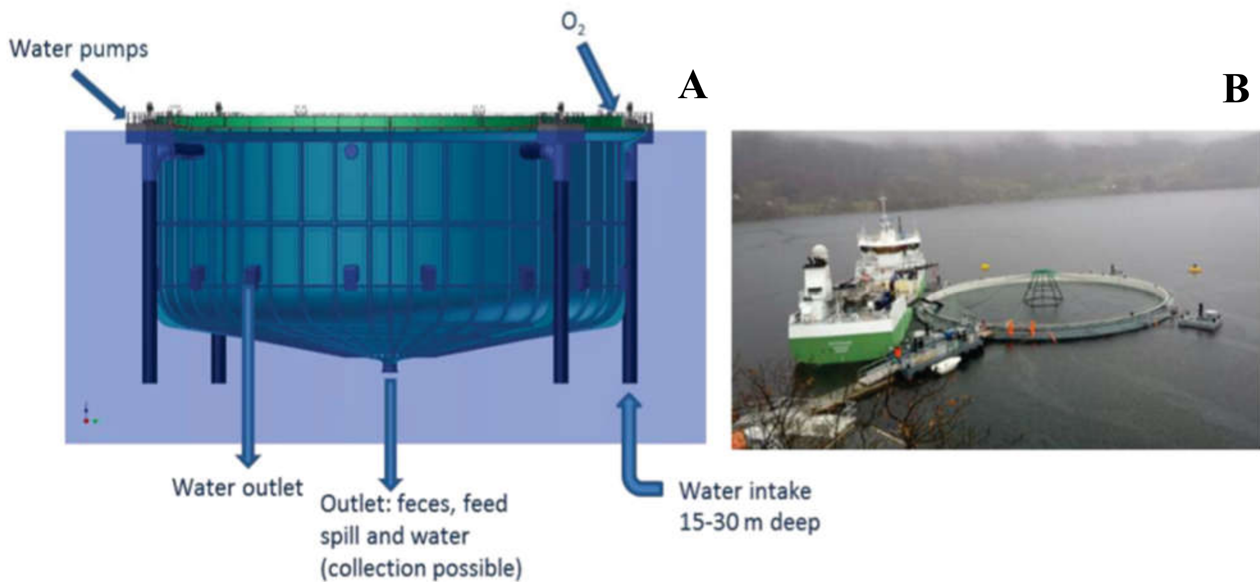

Figure 6. Mowi and AquaFarm Equipment sea-based closed-containment model (A) and prototype semi-closed system Neptune at Molnes (B). Retrieved from: AquaFarm equipment AS/Mowi.

S-CCS are operated as flow-through systems. The intake water is coarse filtrated, but not further treated. The water velocity is mainly responsible for self-cleaning of the tanks (Calabrese, 2017). The quality of the water in S-CCS are highly dependent on water volume, temperature, biomass, current speed and feeding (Norwegian Veterinary Institute, 2020). Water with optimal temperature is pumped from desirable depth between 15 to 30 m. Surface water, where sea lice prevalence is high, is avoided (Nilsen et al., 2017). However, pumping high volumes of water can potentially cause upwelling of sediment from the seabed, which might contain pathogenic species, like *M. viscosa* (Colwell & Morita, 1964; Urakawa et al., 1998). In addition, prevalence of pathogens in the sea column will naturally occur (Rud et al., 2017). On that account, infectious agent might find their way into the system. Nilsen et al. (2017) found that sea lice have minimal effect on farmed salmon in floating enclosures. Nevertheless, according to Handeland et al. (2015) sea lice still find their way into the system (as cited in Haaland, 2017). Water treatment methods used in other aquaculture systems could potentially be adapted, in order to ensure safe water supply and optimal rearing environment in S-CCS (CtrlAQUA, 2015).

1.4 Ultraviolet disinfection in aquaculture

Ultraviolet (UV) irradiation in the UV-C spectral is currently used for inlet water, recirculating water, and wastewater in land-based aquaculture systems (Liltved et al, 2006). UV-C irradiation is also suggested as a preventative measure in disinfection of sea lice eggs, as a treatment method of wastewater from delousing or other operations in the aquaculture industry (Barrett et al., 2019). UV-C irradiation is effective against most fish pathogenic bacteria and some viruses (Liltved & Landfald, 2000). In addition to the efficiency, it is a method that does not leave residual by-products behind (Liltved et al, 2006).

The most influential effect of UV-C irradiation is causing damage in the DNA or RNA of the microorganism in the form of photo-induced dimerisation of opposite pyrimidine (formation of pyrimidine dimers) in the nucleic acid strand. This results in blockage of replication of the nucleic acid or formation of mutant daughter cell (Liltved et al., 2006). These structural and functional changes will ultimately destroy or inactivate the cells of circulating germs. There are currently two different UV technologies for water and wastewater disinfection: low-pressure (LP) UV and medium-pressure (MP) UV (Mamane, 2008).

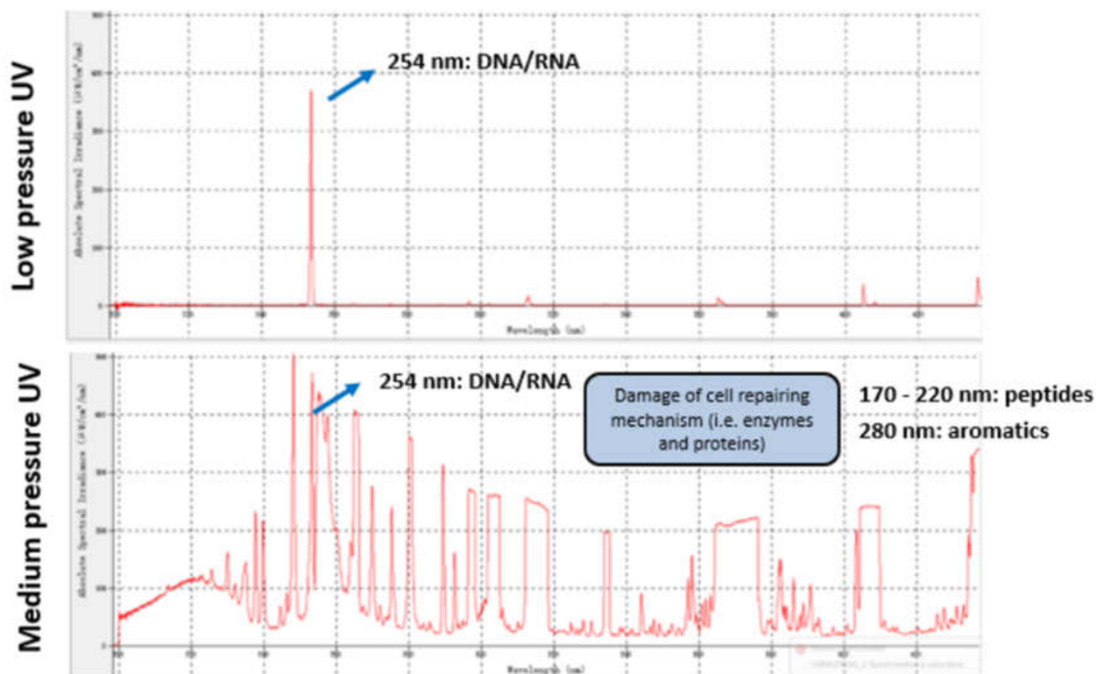

Figure 7. Wavelength spectrum of the two UV technologies. The low-pressure UV lamp (upper figure) emits one single wavelength (254 nm). The medium-pressure UV lamp (lower figure) emits a broad band of wavelength in the germicidal area (220- 300 nm). Retrieved from: https://atlantium.com/

1.4.1 Low-pressure UV

In order to cause DNA-damage, the UV has to be at wavelengths at which the nucleotides can absorb (Mamane, 2008). The nucleotide bases absorb UV wavelengths with a peak absorption close to 260 nm (Gates, 1929). LP UV mercury vapour lamps emit most of the energy output within a single wavelength at 254 nm, shown in Figure 7, making it a monochromatic lamp. This technology is used because of its high microbicidal efficiency, without causing photochemical changes in the water constituents (Mamane, 2008).

1.4.2 Medium-pressure UV

UV with wavelengths lower than 200 nm are not able to penetrate water, while wavelengths higher than 300 nm will not be absorbed in the DNA. Although UV with wavelength at 254 nm is causing DNA damage, other wavelengths can damage enzymes and other molecules. MP UV mercury lamps emit multiple wavelengths. It has a broad polychromatic spectrum ranging from 220 to 300 nm, shown in Figure 7. MP UV lamps require less space and fewer number of lamps, due to a remarkable high energy output. A broad spectrum of UV is also favourable, as it also causes damage in enzymes and other mechanisms responsible for DNA-repair, making the damages irreversible (Mamane, 2008).

1.4.3 UV-treatment in semi-closed containment systems

UV-irradiation has been proposed as a possible water treatment technology to implement in S-CCS. This could potentially result in increased biosecurity, reduced mortality, better fish health, better fish welfare and better control of the water (CtrlAQUA, 2015; Van de Vis et al., 2020). Due to UV irradiation adverse effect on fish, water needs to be treated before entering the rearing unit (Barrett et al., 2020). Although, placement of the water treatment unit is still a not determined. Mounting the treatment system directly on the floating tank is one alternative considered. Water treatment solutions for systems operating on large intake water flow, like S-CCS, are limited and require substantial investments (CtrlAQUA, 2015).

1.4.4 Assessment of DNA damage

Assessment of DNA damage in irradiated organisms can be used to evaluate the effect of UV radiation. Different types of DNA damage have been identified, such as single strand break, double strand break, cyclobutene pyrimidine dimers, 6-4 photoproducts and their Dewar valence isomers (Kumari et al., 2008). DNA damage can result in misincorporation of bases in replication or alkylating agents modifying bases. Hydrolytic damage in the DNA can lead to deamination of bases, depurination, and depyrimidination. Interaction with ionizing

radiations, certain genotoxic chemicals or UV radiation-induced free radicals or reactive oxygen species can result in oxidative DNA damage. Further, oxidative DNA damage can result in formation of different DNA lesions, such as cyclobutene pyrimidine dimers, pyrimidine 6-4 pyrimidone photoproducts and their Dewar isomers (Rastogi et al., 2010)

There are several strategies that are commonly used for the detection and quantification of DNA damage. Polymerase chain reaction (PCR), terminal deoxyribonucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling assay, halo assay, fluorescence in situ hybridization, high-performance liquid chromatography electrospray tandem mass spectrometry, flow cytometry, immunological assay including immunofluorescent and chemiluminescence thymine dimer detection, enzyme-linked immunosorbent assay, immunohistochemical assay, gas chromatography-mass spectrometry, radio immunoassay, annexin V labelling, electrochemical methods and comet assay are all used for assessing DNA damage in different organisms (Kumari et al., 2008).

Comet assay, or single-cell gel electrophoresis, is a method used for estimation of DNA damage at individual cell level and for damage distribution in a population of cells. The method is mainly used to identify single-strand break, double-strand break, oxidative DNA damage and single-strand break related to deficient excision repair sites. These types of damages may be a result of electromagnetic frequency radiation, ultrasound, UV radiation etc. (Kumari et al., 2008). Comet assay is widely applied in genotoxicity testing, human biomonitoring, epidemiology and fundamental research in DNA damage and repair. Advantages of the method include sensitivity, versatile in use, low cost, simplicity, and rapidness. It is mainly a method to measure the extent of DNA damage present in cells, although it is also possible to determine what form it is taking. Detection of pyrimidine dimers, oxidized bases and alkylation damage is, for example, possible through introduction of lesion-specific endonucleases (Collins, 2004).

1.5 Previous studies on UV-treatment on pathogens in aquaculture

Numerous studies have shown UV radiation ability to impair reproduction and survival of marine copepods (Klugh, 1929; Karanas et al., 1979, 1981; Dey et al., 1988; Kouwenberg et al., 1999). Barrett et al. (2019) found a cumulative UV-C dose at 90 mJ/cm^2 induced 95% mortality in *L. salmonis* eggs. Aarseth & Schram (2002) exposed *L. salmonis* to UV-B radiation and found 50% lethal dose (LD_{50}) was 84 kJ/m^2 (8400 mJ/cm^2). The copepod also

demonstrated ability to photorepair (Aarseth & Schram, 2002). Øye & Rimstad (2001) reported the inactivation dose for ISAV to be 7.9 J/m^2 using LP UV. Liltved et al. (2006) achieved a 99.9% inactivation of ISAV with a UV dose of 7.5 mJ/cm^2. Inactivation doses for *M. viscosa* is unknown. There is a need for additional information on the effect of the different water treatment technology, as well as the specific UV dose required to inactivate pathogens infecting farmed salmon in the seawater phase.

1.6 Background

There are major limiting factors for the expansion of the aquaculture industry, especially related to fish losses in open sea ages caused by exposure to pathogenic organisms (Norwegian Veterinary Institute, 2020). Floating semi-closed containment system at sea is one of the emerging production technologies for salmon farming. It allows to control and reduce the interactions between farmed fish and the external environment. The system can be used for post-smolt production before they are transferred to open sea cages. As follows, post-smolts are able to handle transfer better and total time spent in open sea cages is reduced (Rosten et al., 2011). Assuming that it is possible to efficiently prevent pathogenic organisms from entering the system, S-CCS in sea have the ability to gain valuable control over the rearing environment. Eliminating infestations of sea lice and pathogenic microorganisms, could increase biosecurity, provide a better control of the water quality, improve health and welfare of the farmed fish, as well as reduce mortality (CtrlAQUA, 2015).

UV-treatment of intake water has been proposed as a potential treatment method used in S-CCS. However, there is a need to evaluate the necessity and the effect of such a treatment (CtrlAQUA, 2015). Precise dose requirements of UV irradiation to obtain pathogen inactivation is crucial to establish a firm basis for the design of disinfection systems. In addition to optimisation of operation and control of existing systems, specific UV doses required for inactivation can be used to target pathogenic organisms infecting farmed salmon (Liltved et al., 2006).

CtrlAQUA is a centre for research-based innovation (SFI) doing research on closed-containment aquaculture systems, which develop biological and technological innovations making closed systems a reliable and economically viable technology. This study is a part of the INTAKE 2020 which is one of the projects of CtrlAQUA. The project contributes to development of UV technology used for water treatment by investigating the minimum required doses to inactivate Atlantic salmon post-smolt pathogens (CtrlAQUA, 2020).

Winter ulcer, ISA and sea lice are among the greatest challenges resulting in major loss in the seawater phase of farmed fish. Winter ulcer is reported as one of the most significant fish welfare problem, as well as downgrading the quality of the fillet at slaughter, leading to major economic loss. Neither antibiotic prescriptions, vaccines nor measures have been able to eliminate winter ulcer (Norwegian Veterinary Institute, 2020; Løvoll et al., 2009). ISA was a major problem in aquaculture in the late 1980s. Several preventative measures contributed to a substantial decrease in outbreaks, although occurrence of this disease has been reported every year since then (Bruno et al., 2013). In 2020, the number of ISA outbreaks were the highest in the last 30 years, which shows that ISA might be a recurring problem. Sea lice infestations, and following delousing operations, remain the current largest problem in aquaculture, causing major loss, adverse effects of fish welfare and the sustainability of the industry as a whole (Norwegian Veterinary Institute, 2020).

1.7 Thesis objective

The overall objectives for this thesis are to determine the minimum required UV doses for the complete (99.9%) inactivation of relevant Atlantic salmon pathogens, the impact of UV treatment on seawater microbial communities' equilibrium and to assess DNA damages in *L. salmonis* cells caused by UV treatments. To achieve this, bench-scale trials were conducted using two CBAs: a LP and MP UV mercury lamp. Pathogens were quantified before and after UV exposure. Plating and counting colony forming units (CFU) were used for bacterial analysis. Tissue culture infectious dose (TCID50 assay) were used for virus. Count of reactive individuals were used to estimate the survival of ectoparasites. The targeted pathogens were the bacterium *M. viscosa,* the infectious salmon anaemia virus (ISA) and the ectoparasite *L. salmonis.* Seawater samples taken from different depths at two marine sites, one with Atlantic salmon sea cages and one without sea cages were exposed to UV radiation to determine the impact of UV treatment on the seawater microbial communities' equilibrium. In order to evaluate the effect of UV radiation, DNA-damages in *L. salmonis* were assessed using Comet assay analysis.

2 Materials and methods

2.1 Experimental details

The experiments were performed at the laboratory facilities of the Norwegian College of Fishery Science at University of Tromsø and of Nofima Tromsø. *M. viscosa* and ISAV were isolated from material collected in previous work by Nofima and cultured in the Nofima laboratory facility. *L. salmonis* were obtained from Tromsø Aquaculture Research Station. Seawater samples were collected at two different sites in Kvalsundet, Tromsø. Comet assay analysis were performed at the Norwegian College of Fishery Science.

2.2 Experimental set-up

Pathogens and seawater samples were prepared for CBA trial by diluting them in artificial seawater, making up the test solution. The absorbance for all test solutions were measured and separately exposed to a series of UV doses. After UV irradiation, the pathogens were quantified. In *L. salmonis,* cellular DNA-damages potentially induced by UV treatments were assessed by Comet assay analysis.

2.3 General

The minimum required UV dose for achieving a complete (99.9%) inactivation of Atlantic salmon pathogens and the impact of UV treatment on seawater microbial communities' equilibrium were determined. For this purpose, two different bench-scale UV technologies were used: a CBA with a LP and a MP UV set up. The CBA enables measurement of the UV-dose response of the target microorganism.

2.4 Preparations before UV exposure

2.4.1 *Moritella viscosa*

M. viscosa, with serial number LFI 5006/2, was isolated from Atlantic salmon by Nofima from work carried out in 2014. After being preserved in glycerol at -80°C, the pathogen stock was defrosted and inoculated onto plates of blood agar 3% NaCl, presented in **Appendix A**. The plates were incubated for five days at 12°C. Three colonies were diluted in 12 ml liquid marine broth, presented in **Appendix A**, in 50 ml flask (Shake flask, Bellco, USA) and shaken for 24 hours in an orbital shaker (KS501 digital, IKA, Germany). A volume of 5 ml of the bacterial suspension was transferred to Eppendorf tubes and centrifuged (Centrifuge 5810 R, Eppendorf, Germany) at 3500 g for 10 minutes. The excess medium was removed and resuspended in 10 ml 30 ppt artificial seawater (Sea Salt from Aquaforest, Marine aquarium

products manufacturer, Poland). The solution was centrifuged again at 3500 g for 10 minutes. The artificial seawater was removed, and the pellet of bacteria was diluted in 240 ml 30 ppt artificial seawater, making up a concentration of $1.54*10^{-3}$ CFU/ml.

2.4.2 Infectious salmon anemia virus

ISAV was isolated from Atlantic salmon liver collected by Nofima from work carried out in 2009. The virus had been frozen in serum at -80°C before it was defrosted around an hour before the experiment. A volume of 2.5 ml of the virus solution was diluted in 247.5 ml in 30 ppt sterile Artificial Seawater, making up a virus titer of around $2.81*10^{6}$ TCID50/ml.

2.4.3 *Lepeophtheirus salmonis*

Living copepodites of *L. salmonis* were obtained from the Nofima Aquaculture Research Station in Tromsø. Egg strings from adult *L. salmonis* were detached from the body and incubated for 14 days at 9°C. Six 2L-bottles each containing approximately 1000 copepodites were used. They were stored overnight at 12°C before being used.

2.4.4 Seawater samples

Seawater samples were collected at two sites at Kvalsundet (Figure 8) located between Ringvassøya and Kvaløya (Tromsø, Norway). At this site there is a commercial aquaculture location with 7 active sea cages with Atlantic salmon (1400 tons) run by SalMar Farming AS. Samples were collected close by the sea cages (69°53'53" N 18°43'10" E) and around 12 km away from the sea cages (69°54'33" N 18°41'59 E). At both sites (aquaculture and reference station), samples were collected at 2- and 40-meters depth, using a water sampler (Ruttner Water Sampler 1L, KC Denmark AS Research equipment, Denmark). The samples were kept in sterile bottles and stored overnight at 4°C. The undiluted seawater samples were exposed to UV radiation (see § 2.6).

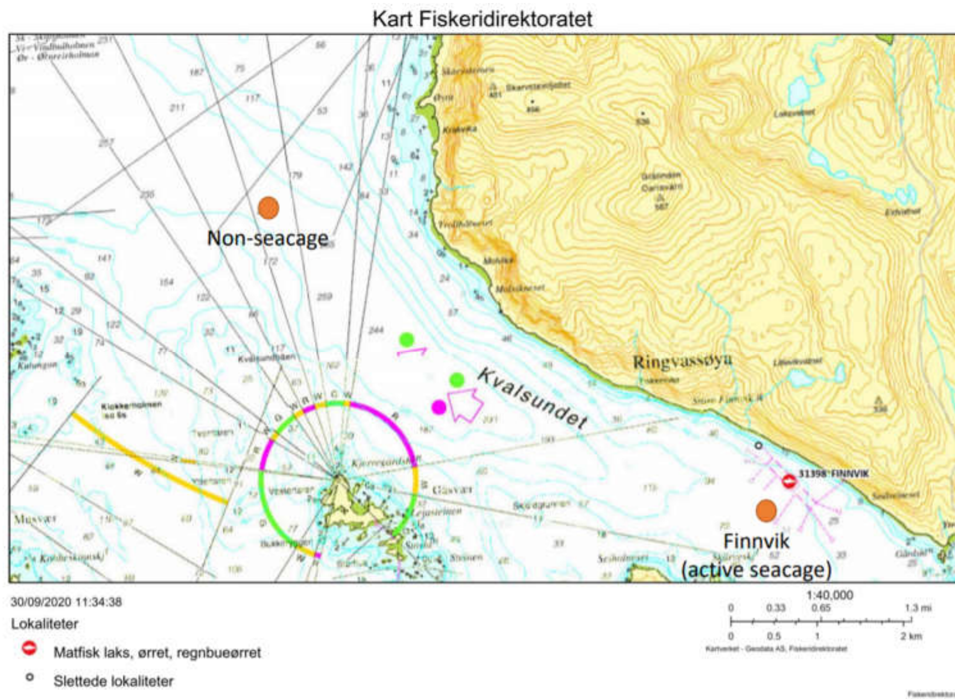

Figure 8. Map of the two different sites (aquaculture and reference station) where the seawater samples were collected (October 2020) in Kvalsundet, Tromsø. Source: Directorate of Fisheries, n.d.

2.5 Absorbance

For LP UV, the absorbance of the water sample at 254 nm was used to calculate the ultraviolet transmission (UVT) value. For MP UV, the absorbance of the water sample at every wavelength between 220 to 300 nm were used to calculate the UVT-value. Absorbance of the water sample was measured using a spectrophotometer (UV-1800, Shimadzu Corporation, Shimadzu USA manufacturing Inc., USA/Japan) in a 1 cm quartz cuvette.

2.6 Collimated Beam Apparatus

CBA is an experimental bench-scale setup, (Figure 9) developed to irradiate microorganisms in a water sample (Lichi, 2011). In this experiment, one CBA was equipped with a low-pressure monochromatic mercury lamp, while the other with a medium-pressure polychromatic mercury lamp. Both CBAs were provided by Atlantium (UV sterilizer manufacturer; Israel). Before use, the CBAs were calibrated, and the UV intensity values determined. The UV intensity values were necessary to calculate UV doses for the different pathogens. As the UV intensity was fixed, the UV doses were determined according to sample's exposure time.

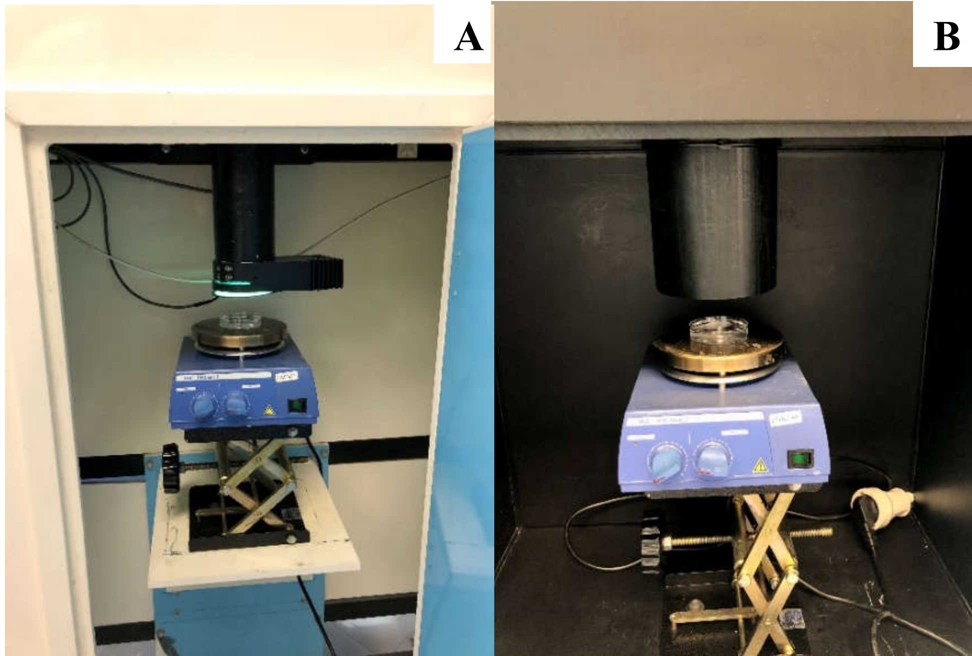

Figure 9. The two different collimated beam apparatus equipped with a medium-pressure UV lamp (A) and low-pressure UV lamp (B) exposing a water sample to UV radiation on a magnetic stirrer. Photo: Kari Elisabeth Justad.

2.6.1 Calculation of UV-doses

In order to calculate UV-doses to be irradiated for each pathogen, experimental specific conditions were taken into account for each CBA test to obtain the respective exposure times. The doses of the LP UV were calculated according to Equation 1. As the UV-doses were determined by the time of exposure, Equation 1 was modified to isolate exposure time, expressed in Equation 2. The time of exposure to obtain different doses of MP UV was calculated according to Equation 3.

Equation 1:

$$D = E_i P_f (1 - R) \frac{L\,(1 - 10^{-ad})}{(d - L)\, ad \ln{(10)}} t$$

Equation 2:

$$t = \frac{D}{E_i P_f (1 - R) \frac{L\,(1 - 10^{-ad})}{(d - L)\, ad \ln{(10)}}}$$

Equation 3:

$$t = \frac{D}{\sum_{i\,=\,220}^{300} E_i P_f (1 - R) \frac{L\,(1 - 10^{-ad})}{(d - L)\, ad \ln{(10)}}}$$

D = UV-dose (mJ/cm^2)

E_i = Average UV irradiance (mW/cm^2)

P_f = Petrifactor (unitless)

R = Reflectance at the air-water interface at 254 nm (unitless)

L = Length from centerline of lamp to suspension surface (cm)

d = Depth of the suspension (cm)

a = UV absorption coefficient of the suspension at 254 nm (cm^{-1})

t = exposure time

2.6.2 UV exposure

A volume of 20 ml of the test solution was pipetted in a 55 x 14.2 mm Petri dish with a magnetic stirrer, homogenizing the sample during exposure. The CBA emitted UV irradiation through a shutter onto the Petri dish containing the test solution, which was placed on a horizontal surface at a predetermined distance. The door was shut during the exposure time, to keep the radiation within the CBA. After the exposure time, the shutter closed by the operator, and the samples taken out. After UV-exposure the microorganisms were quantified.

2.7 Pathogen quantification after UV exposure

2.7.1 *Moritella viscosa*

Each sample, with 20ml of pathogen solution, was exposed to different UV doses at both LP and MP UV. After UV-exposure, all Petri dishes were kept on ice and the samples 10-fold diluted. A volume of 100 µl of the different dilutions was dispersed onto marine agar 3% NaCl plates (see **Appendix A**) using a pipette. A sterile spreader was gently moved back and forth, dispersing the sample throughout the plate. The plates were then left undisturbed on the bench for approximately 15 minutes before being incubated for 7 days at 12°C. The CFUs were registered, using countable ranges of 30 to 300.

Figure 10 Colonies of *M. viscosa* growing on marine agar 3% NaCl. Photo: Kari Elisabeth Justad.

2.7.2 Infectious salmon anemia virus

After UV exposure, a sample volume of 25µl was transferred and mixed with serum to 6 replicate wells in a 96 microwell plate, using a pipette. There were made 10-fold dilutions, whereas 25µl of each one of the first 6 replicate wells were transferred and mixed with the serum to the next 6 replicate wells and throughout 6 rows, using a multichannel pipette. The remaining 2 rows did not contain virus and were used as controls. After removing the medium of a microwell plate containing ASK-cells, the content of each well containing serum and sample solutions were transferred to corresponding wells in the plate with ASK-cells. These plates were incubated at 15°C for 11 days in a Refrigerated Incubator (KBP 6087, Termaks, Bergen). The cytopathic effect was then examined in each well.

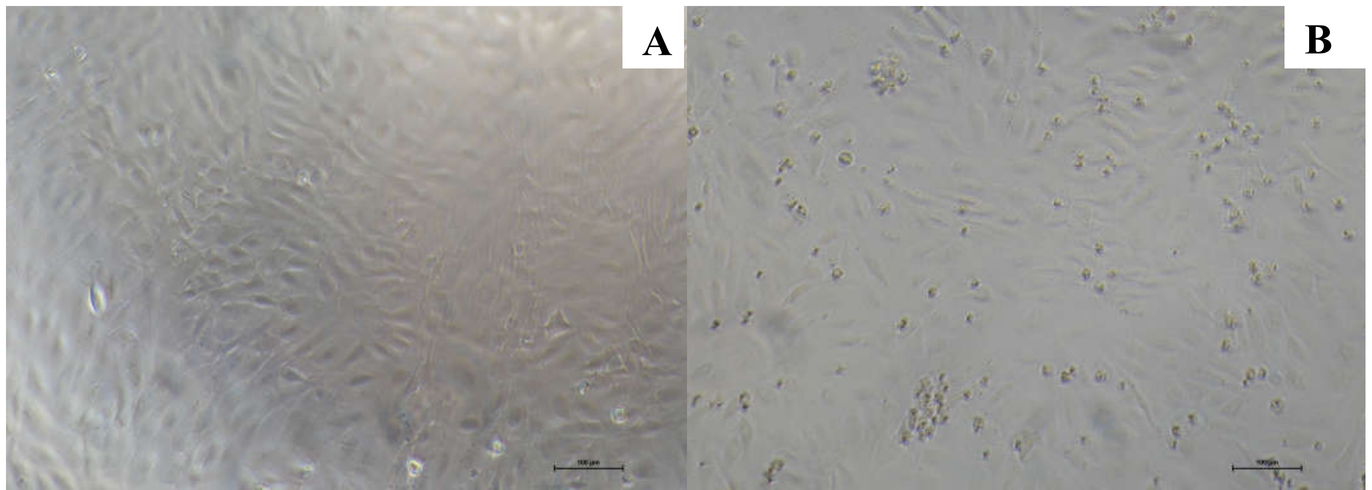

Figure 11. Microscopy ISA virus infected Intact Atlantic salmon kidney cells (A). Atlantic salmon kidney cells at 11 days postinfection (B). The scale bars represent 100 µm. Photo: Elin Sandaker.

2.7.3 *Lepeophtheirus salmonis*

Mortality

Alive and dead individual copepodites were counted before UV exposure and 24 hours after UV exposure and the corresponding mortality rate calculated. Individuals who were not moving after around 10 seconds and a gentle stimulus, were considered as dead.

Petri dishes containing 20 ml of test solution were exposed to UV radiation, and the content transferred to a beaker glass until approximately 50 individuals were irradiated. The test solution was filtered through a cell strainer by use of a pipette. The copepodites were briefly rinsed with distilled water and transferred to a Petri dish, by flushing them out of the cell strainer using distilled water. The excess distilled water was removed to increase the copepodite concentration in the sample. Around 0.8 ml of copepodites and distilled water were transferred to cryotubes. Approximately 50 control and UV-exposed copepodites collected at the end of the experiment were stored in 1ml mixture of 4:1 ratio of RPMI-1640™ cell culture medium and dimethyl sulfoxide (DMSO) prior slow freezing at -1°C/min in a freezer using the Corning® Coolcell™ Freezer container for approximately 80 minutes. Samples were then stored directly in the freezer at -80°C prior to analyses.

Figure 12. *L. salmonis* copepodite (incubated for 14 days at 9°C) obtained from Havbruksstasjonen Tromsø used in this study. The scale bars represent 100 µm. Photo: Kari Elisabeth Justad.

Comet assay analysis

The preserved samples were removed from the freezer, thawed, and kept on ice. The copepodites were gently crushed with a pestle and further vortexed to release its cell contents. This mixture was quickly spun down before pipetting and discarding the excess supernatant, leaving mainly the crushed copepodites. Approximately 250 µl mixture of Ca^{2+} and Mg^{2+} free Phosphate buffered saline (PBS) containing 20mM EDTA was added and mixed with the crushed copepodites before centrifuging (CT15RE, VWR, Hitachi Koki Co., Ltd, Japan) at 250 g for 10 minutes at 4°C, this procedure was then repeated in a two-step washing procedure to remove excess cell culture medium and DMSO. The cells were then re-suspended in 100µl Ca^{2+} and Mg^{2+} free PBS before being mixed with low-melting (LM) agarose.

The LM agarose (0.5% solution) was warmed up using a microwave, cooled, and maintained in a molten state at 37°C in a water bath until use. A 1:3 ratio mixture of cell suspension and LM agarose respectively was prepared and 50 µl this mixture was quickly pipetted on to a slide pre-coated with 1% standard agarose and covered with slips. All these procedures were done maintaining a temperature of approximately 37°C before refrigeration (4°C) in the dark for 30-40 minutes.

The cover slips were carefully removed from the slides, placed on a slide holding rack and gently dipped in a cold lysis solution kept on ice for 45 minutes. Samples were then tranfered into an alkaline unwidning solution (200 mM NaOH, 1mM EDTA, pH>13) kept at room temperature for 20 minutes prior to alkaline electrophoresis.

The electrophoresis unit (Figure 13) was kept refrigerated by cooling elements placed underneath the electrophoresis chamber (CometAssay Electrophoresis System II, Bio-Techne, USA) during use to maintain a cold temperature. A volume of 850 ml of freshly prepared and cooled alkaline electrophoresis solution (200 mM NAOH, 1mM EDTA pH>13) was poured into the cooled electrophoresis tank. The slides were carefully inserted onto the slide tray and the power supply was set to 21 V and 0.4 A and run for 30 minutes.

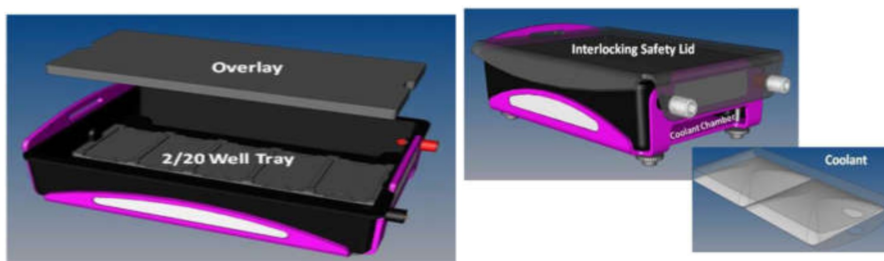

Figure 13 Electrophoresis system showing the electrophoresis tank, well tray, interlocking safety lid, coolant chamber and coolant. Retrieved from RnDsystems.

The slides were then washed twice in distilled water for 5 minutes each followed by 70% ethanol for 5 minutes. The slides were dried at 37°C for 15 minutes. A volume of 100 µl of DAPI was pipetted onto the slides and spread evenly. After 5 minutes the slides were washed briefly in distilled water. The slides were then let to completely dry for 1 hour at 37°C. All above operations were performed under dark conditions to prevent DNA photo-reactivation.

The slides were viewed by epifluorescence microscopy using a Leitz Aristoplan (Leica Microsystems GmbH, Germany) equipped with a Filter Cube A for UV (excitation filters BP 340-380 nm) and digital camera (Flexcam C1). Pictures (10x objective) were processed using Leica Application Suite v.4.2 (Leica Microsystems AG, Switzerland) and stored as tiff. files for further analysis. A COMET analysis software (Trevigen®) was employed to automatically locate and score comets and to characterize and quatify the degree of DNA damage (percentage of DNA in tail relative to cell nucleus and tail length) in control and treated groups. An example of intact and damage cells revealed by the comet assay and using this image processing method and is reported in Figure 14.

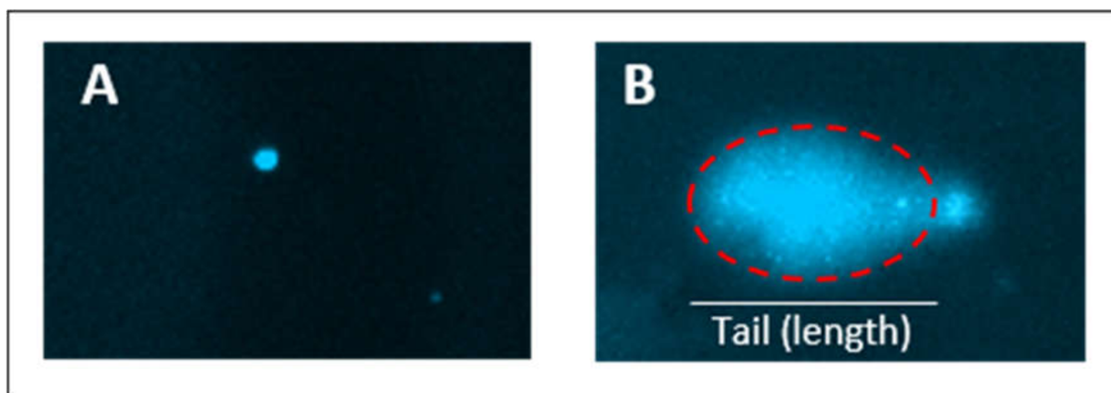

Figure 14. Representative pictures of cells of *L. salmonis* showing (A) intact and (B) damaged nuclei following alkaline CometAssay and DAPI staining. Photo: Stefano Peruzzi.

Alkaline COMET assay's experimental conditions were verified by use of commercial available reference cells (CometAssay® Control Cells, Trevigen Inc., Gaithersburg, MD20877, USA) showing no DNA damage (negative control) as well as different levels of nuclear damage (low, medium and high) following etoposide treatment. The results are reported as percentage DNA in tail (mean ± Standard Error, SE) and presented in **Appendix B.**

2.7.4 Seawater microbial communities

After UV-exposure, the sea water samples were 10-fold diluted three times. Two drops with 20 µl of each dilution, including undiluted sample, were seeded on marine agar and thiosulfate citrate bile salts sucrose (TCBS) agar, showed in Figure 15. The plates were incubated for 8 days at room temperature. The dilution which contained formation of bacteria colonies were recorded.

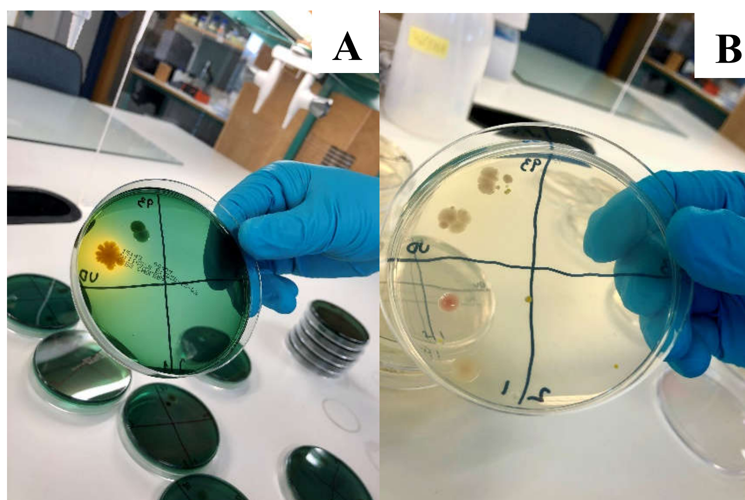

Figure 15. Bacteria colonies from different dilutions of the seawater samples collected (October 2020) from Kvalsundet on Thiosulfate citrate bile salts sucrose agar (A) and marine agar (B). Photo: Kari Elisabeth Justad.

2.8 Statistical analysis

All statistical analyses were done in SPSS. For *M. viscosa*, statistical analysis was performed on data that resulted in log 3 inactivation or higher. As the UV did not achieve a log 3 inactivation of ISAV, statistical analysis was performed on data resulted in log 2.5 or higher. For *L. salmonis,* percentage mortality of all UV-doses was included in the statistical analysis. For the Comet assay data, statistical analysis was done on results where DNA damage were found. All data were checked for homogeneity of variances and normality assumptions using Levene's test and Shapiro-Wilks test respectively.

Analysis for differences between UV-technologies for *M. viscosa* and ISAV was performed with independent t-test, while analysis of difference between *M. viscosa* and ISAV within the same UV technology was performed by chi-square (χ^2) test.

One-way ANOVA was used to test the difference among UV doses to achieve the same mortality for the copepodites. As this data was expressed in percentage, it was arcsine-transformed before analysis.

Kruskal-Wallis test was used to test for difference between UV doses of percentage DNA in comet tail and comet tail length. Result of percentage DNA in comet tail was transformed using arcsine before analysis to improve normality. Pairwise Comparisons were then used to identify specific UV doses with significant differences.

Overall confidence interval used in this study was 95% (p=0.05).

3 Results

3.1 *Moritella viscosa*

All UV doses of 3 to 59 mJ/cm^2 with LP and 2.3 to 45.7 mJ/cm^2 with MP achieved a log inactivation of *M. viscosa* greater than 3 (99.9%) (Figure 16). There were no significant differences between the two UV technologies. As the lowest possible dose were limited to 1 s of exposure with MP UV, it was not possible to register the exponential phase of the inactivation of *M. viscosa*. However, the lowest exposure time of 13 seconds under LP UV (Table 1) and 1 second under MP UV (Table 2) were sufficient to achieve 3 log inactivation. Accordingly, the minimum required UV-dose for a log 3 inactivation in this work was 3 mJ/cm^2 and 2.3 mJ/cm^2 under LP UV and MP UV, respectively.

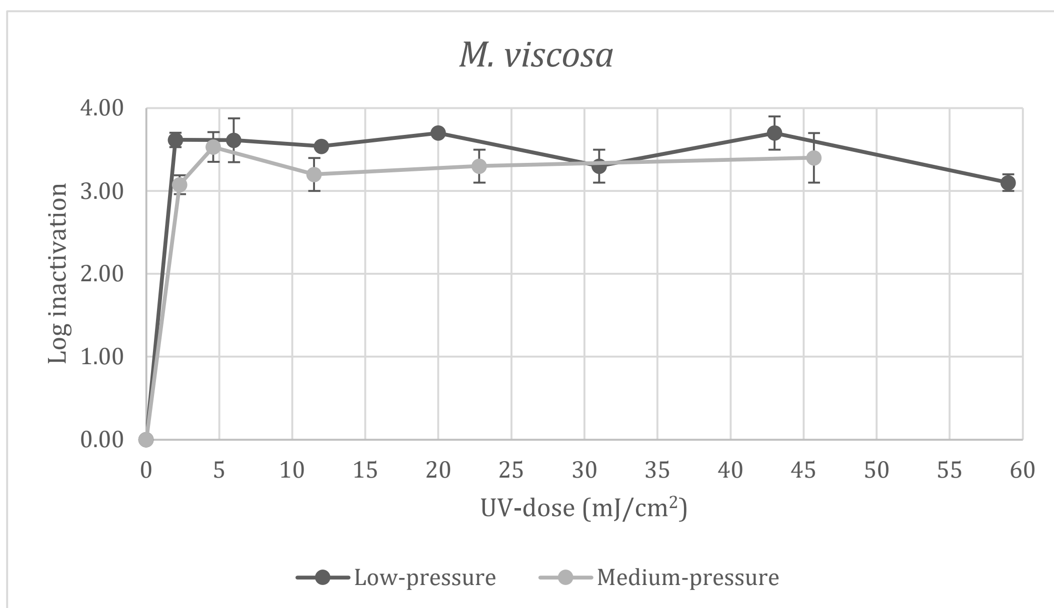

Figure 16. Mean log values ± standard deviations at different UV-doses of *M. viscosa* exposed to low-pressure and medium-pressure UV. Results from UV doses 0 to 15 mJ/cm^2 were obtained from this study. Results from UV doses 15 to 59 mJ/cm^2 are retrieved from previous work on the same project (Guerreiro, 2020).

Table 1. Mean and standard deviation of CFU/ml and mean log values of *M. viscosa* for the different doses and exposure time of low-pressure UV

Low-pressure UV					
UV dose (mJ/cm^2)	Exposure time (s)	CFU/ml		Log. Inactivation	
		Mean	STD	Mean	STD
0	0	3.16E+06	7.40E+05		
2	13	9.68E+02	7.84E+02	3.62	0.09
6	34	8.10E+02	4.74E+02	3.61	0.26
12	64	7.65E+02	1.50E+01	3.54	0.00
20	107	3.40E+02		3.70	0.00
31	172	8.65E+02	5.18E+02	3.30	0.20
43	236	3.40E+02	1.86E+02	3.70	0.20
59	322	1.33E+03	1.59E+02	3.10	0.10

Table 2. Mean and standard deviation of CFU/ml and mean log values of *M. viscosa* for the different doses and exposure time of medium-pressure UV

Medium-pressure UV					
UV dose (mJ/cm^2)	Exposure time (s)	CFU/ml		Log. Inactivation	
		Mean	STD	Mean	STD
0	0	3.05E+06	5.68E+05		
2.3	1	9.32E+02	5.48E+02	3.08	0.11
4.6	2	6.25E+02	1.22E+02	3.53	0.18
11.5	5	7.34E+02	1.84 E+02	3.20	0.20
22.8	10	5.52 E+02	2.44 E+02	3.30	0.20
45.7	20	5.48 E+02	3.43 E+02	3.40	0.30

3.2 ISAV

None of the UV doses of 2 to 8 mJ/cm^2 with LP and 2.2 to 33.7 mJ/cm^2 with MP achieved a log 3 (99.9%) inactivation of ISAV (Figure 17). There were no significant differences between the two UV technologies. The highest log inactivation was 2.59 (Table 4) and was achieved by a UV dose of 22.5 mJ/cm^2 under MP UV. The highest log inactivation by LP UV was 2.40, (Table 3) achieved by 2 mJ/cm^2.

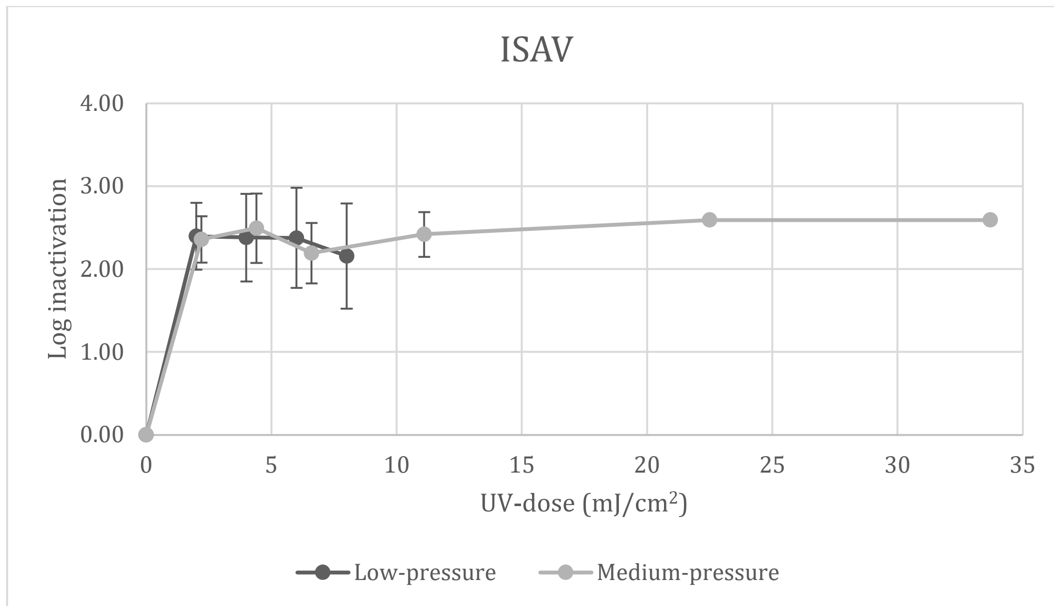

Figure 17. Mean log values ± standard deviations at different UV-doses of ISAV exposed to low-pressure and medium-pressure UV.

Table 3. Mean and standard deviations of TCID50/ml and mean log values of ISAV for the different doses and exposure time of low-pressure UV.

Low-pressure UV					
UV dose (mJ/cm^2)	**Exposure time (s)**	**TCID50/ml**		**Log. Inactivation**	
		Mean	**STD**	**Mean**	**STD**
0	0	4.41E+00	4.66E-01		
2	11	2.01E+00	3.94E-01	2.40	0.40
4	21	2.07E+00	4.17E-01	2.38	0.53
6	32	2.03E+00	4.71E-01	2.38	0.6
8	43	2.25E+00	4.60E-01	2.16	0.64

Table 4. Mean and standard deviations of TCID50/ml and mean log values of ISAV for the different doses and exposure time of medium-pressure UV.

Medium-pressure UV					
UV dose (mJ/cm^2)	**Exposure time (s)**	**TCID50/ml**		**Log. Inactivation**	
		Mean	**STD**	**Mean**	**STD**
0	0	4.22E+00	2.59E-01		
2.2	1	1.87E+00	2.36E-01	2.36	0.11
4.4	2	1.73E+00	4.68E-01	2.49	0.18
6.6	3	2.03E+00	4.71E-01	2.19	0.20
11.1	5	1.80E+00	3.00E-01	2.42	0.20
22.5	10	1.70E+00	0	2.59	0.00
33.7	15	1.70E+00	0	2.59	0.00

3.3 *Lepeophtheirus salmonis*

L. salmonis was exposed to doses ranging from 16 mJ/cm^2 to 157 mJ/cm^2 with LP UV and 21 mJ/cm^2 to 199.3 mJ/cm^2 with MP UV. None of the UV-doses allowed to achieve a mortality of 99.9% (Figure 18). There were no significant differences in terms of mortality among the different UV doses. For LP UV, the highest achieved mortality rate was 24.5% in samples exposed to 126 mJ/cm^2 (Table 5). The highest mortality rate was 47.1% and was achieved by 199.3 mJ/cm^2 under MP UV shown in Table 6.

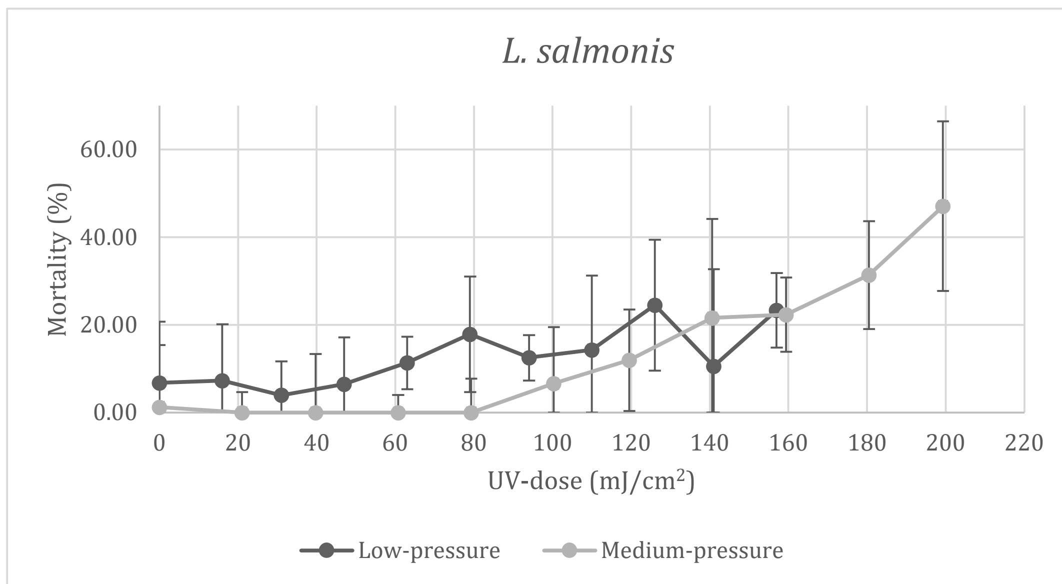

Figure 18. Mean percentage mortality ± standard deviations of *L.salmonis* 24 hours after exposure to different doses of low- and medium-pressure UV.

Table 5. Mean and standard deviations of percentage mortality of *L. salmonis* for the different doses and exposure time of low-pressure UV.

Low-pressure UV			
UV dose (mJ/cm^2)	Exposure time (s)	Mortality (%)	
		Mean	STD
0.0	0	6.74	14.02
16.0	84	7.27	12.90
31.0	168	3.98	7.73
47.0	252	6.45	10.74
63.0	336	11.34	5.98
79	420	17.87	13.16
94	503	12.50	5.19
110	587	14.30	16.93
126	671	24.52	14.93
141	755	10.56	22.11
157	839	23.33	8.48

Table 6. Mean and standard deviations of percentage mortality of *L. salmonis* for the different doses and exposure time of medium-pressure UV.

Medium-pressure UV			
UV dose (mJ/cm^2)	Exposure time (s)	Mortality (%)	
		Mean	STD
0	0	1.23	14.18
21	9	0.00	4.67
39.7	17	0.00	13.33
60.7	26	0.00	4.00
79.3	34	0.00	7.74
100.3	43	6.59	12.91
119.5	51	11.94	11.57
140.6	60	21.61	22.58
159.4	68	22.32	8.48
180.5	77	31.35	12.29
199.3	85	47.08	19.32

3.3.1 Comet assay analysis

A total of 25 randomly selected cells were analysed per UV irradiated copepodite sample. Among these cells, the number of intact cells and the level of DNA damage were registered. There were intact cells in most of the irradiated copepodite samples. Number of intact cells exposed to the respective doses of low-pressure UV is reported in Table 7. The number of intact cells exposed to the respective doses of MP UV is shown in Table 8.

Table 7. Number of intact cells, percentage of DNA found in comets' tail and the comet tail length out of a total of 25 cells exposed to different doses of low-pressure UV.

Low-pressure UV			
UV dose (mJ/cm^2)	Number of intact cells	DNA in tail (%)	Tail length (µm)
0	25	0	0
2	25	0	0
4	25	0	0
5	25	0	0
10	25	0	0
20	25	0	0
40	25	0	0

Table 8. Number of intact cells, percentage of DNA found in comets' tail and the comet tail length out of a total of 25 cells exposed to different doses of medium-pressure UV.

Medium-pressure UV			
UV dose (mJ/cm^2)	**Number of intact cells**	**DNA in tail (%)**	**Tail length (µm)**
0	25	0	0
2	25	0	0
4	25	0	0
5	16	0	0
8	25	0	0
10	0	0	0
16	25	0	0
20	0	0	0
40	0	0	0

There was no DNA damage for copepodite cells exposed to UV doses from 0 to 40 mJ/cm² low-pressure UV. However, there were DNA damages for copepodite cells exposed to certain doses of MP UV. MP UV doses causing DNA damage were 5, 10, 20 and 40 mJ/cm². Number of cells' DNA comets, percentage DNA in comet tail and the comet tail length reported in Table 9.

Table 9. Number of comets, percentage of DNA found in comets' tail and tail length out of total 25 cells exposed to different doses of medium-pressure UV.

Medium-pressure UV			
UV dose (mJ/cm^2)	**Number of comets**	**DNA in tail (%)**	**Tail length (µm)**
0	0	n.a.	n.a.
5	9	99.60	96.00
10	25	94.25	70.06
20	25	98.64	96.56
40	25	98.11	107.30

The level of DNA damage induced on *L. salmonis* copepodites following the UV treatments is reported as comet tail length (Figure 19) and percentage of DNA in the tail (Figure 20). Both measurements yielded significant differences (p<0.001) among UV doses. Overall, the samples exposed to 10 mJ/cm² showed a level of DNA damage lower (p<0.001) than all other treatments, both in terms of tail length (Figure 19) and percentage DNA in the tail (Figure 20).

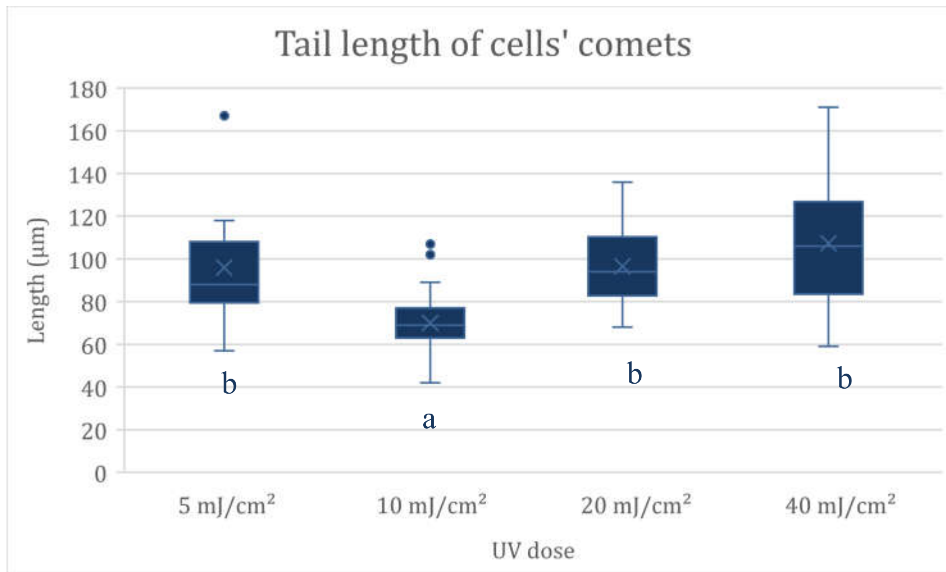

Figure 19. Boxplots showing the comets' tail length (µm) of cells irradiated with different doses (mJ/cm²) of medium-pressure UV. The boxplots report the upper and lower quartiles, the confidence interval around the median, the median and standard error. Outliers are shown as dots. Letters indicate significant differences between groups.

Compared to the reference cells (see **Appendix B**), these results show a medium to high level of DNA damage in copepodite cells treated at 10 mJ/cm² (ca. 70% as tail length) in contrast to a high level of DNA damage (>88.87%) in all other treatments.

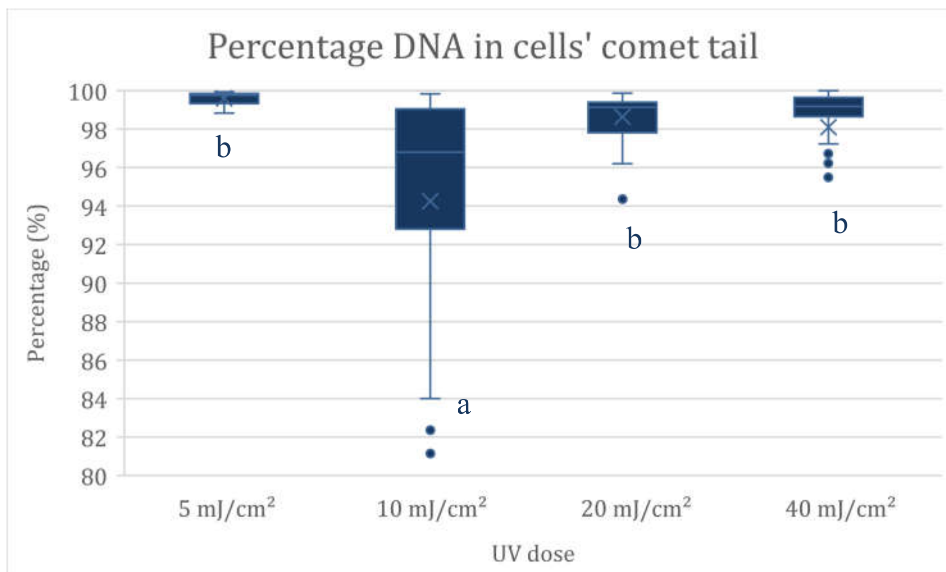

Figure 20. Boxplots showing the percentage DNA in comets tails of cells in irradiated with different doses (mJ/cm²) of medium-pressure UV. The boxplots report the upper and lower quartiles, the confidence interval around the median, the median and standard error. Outliers are shown as dots. Letters indicate significant difference between groups.

3.4 Seawater microbial communities

Water parameters recorded at different depths at “Aquaculture location” close by sea cages and “Reference location” around 12 km away from the sea cages in Kvalsundet are shown in Table 10. Sea water samples were collected at these sites and exposed to UV treatment. After UV radiation the samples were 10-fold diluted and seeded onto marine agar and TCBS agar plates.

Table 10. Water parameters recorded at the two locations where seawater samples were collected.

	Aquaculture location **69°53'53'' N 18°43'10'' E**		**Reference location** **69°54'33'' N 18°41'59 E**	
	2 m	**40 m**	**2 m**	**40 m**
Temperature (°C)	9.00	9.30	9.00	9.30
Salinity (ppt)	30.90	31.50	31.40	31.50
pH	10.50	10.50	10.50	10.50
O2 (%)	87.10	95.50	94.80	95.70

The recorded bacterial growth on marine agar from the samples exposed to MP UV dose of 25 mJ/cm^2 are presented in Figure 21. Results from samples with no UV exposure are presented in Figure 22.

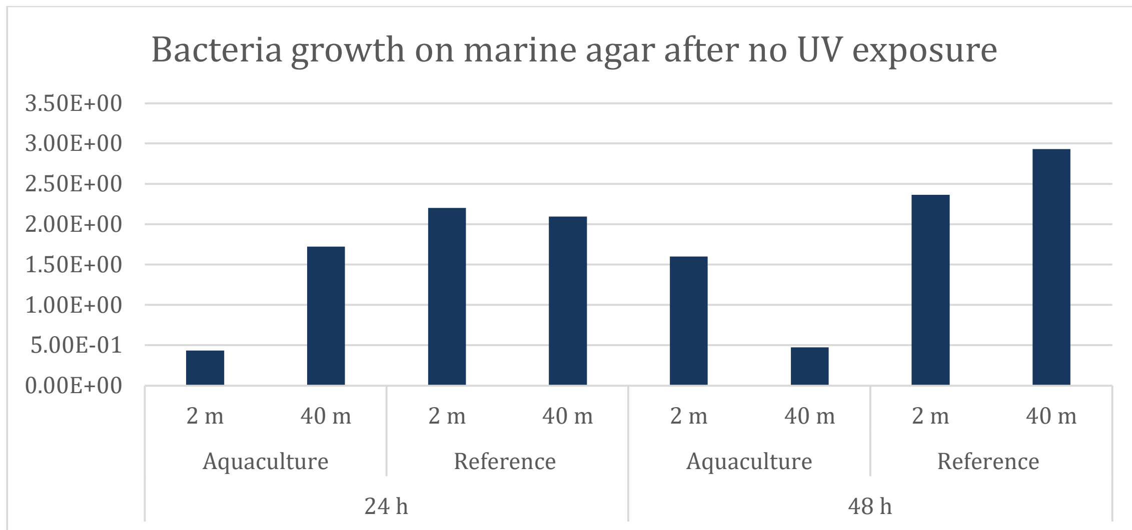

Figure 21. Bacterial growth from seawater samples collected at different depths (m) at Aquaculture location and Reference location on marine agar with no UV exposure. Bacterial growth was recorded according to the logarithmic dilution (y-axis).

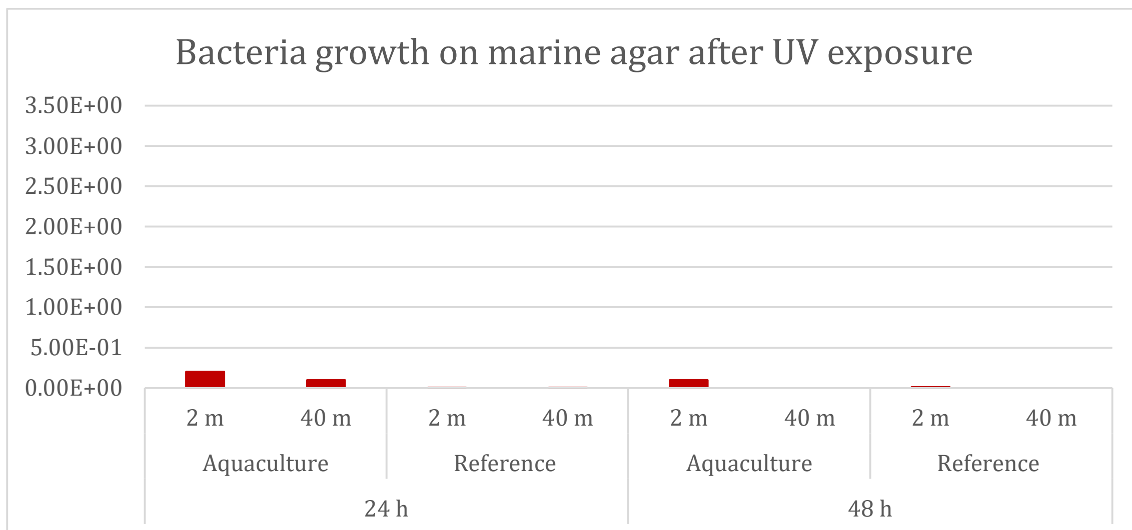

Figure 22. Bacteria growth from seawater samples collected at different depths (m) at Aquaculture location and Reference location on marine agar after MP UV exposure of 25 mJ/cm^2. Bacteria growth was recorded according to the logarithmic dilution (y-axis).

The bacterial growth on TCBS from the samples exposed to MP UV dose of 25 mJ/cm^2 are presented in Figure 23. Results from samples with no UV exposure are presented in Figure 24.

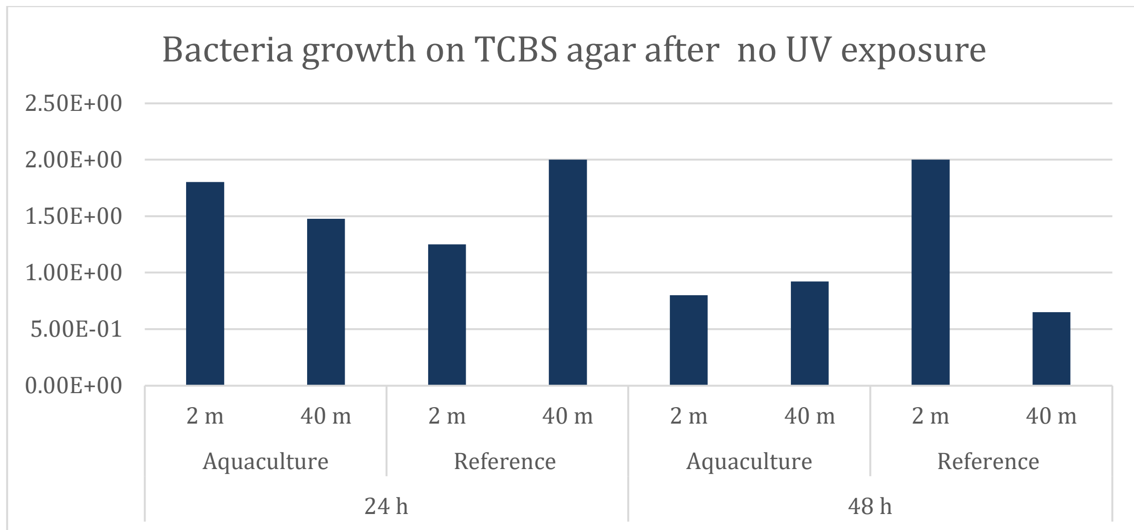

Figure 23. Bacterial growth from seawater samples collected at different depths (m) at Aquaculture location and Reference location on TCBS agar with no UV exposure. Bacterial growth was recorded according to the logarithmic dilution (y-axis).

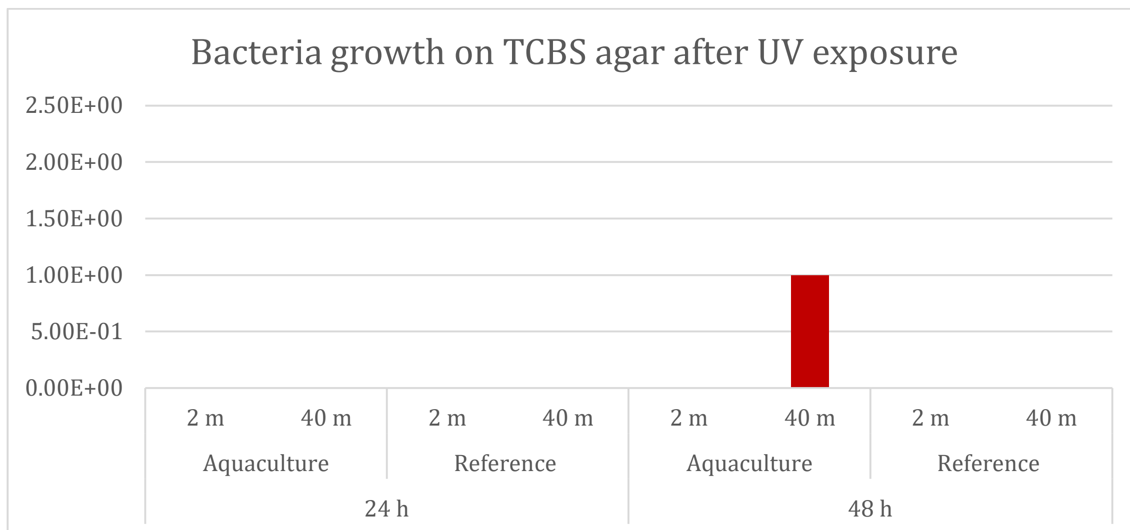

Figure 24. Bacterial growth from seawater samples collected at different depths (m) at Aquaculture location and Reference location on TCBS agar after MP UV exposure of 25 mJ/cm^2. Bacterial growth was recorded according to the logarithmic dilution (y-axis).

4 Discussion

4.1 Implementing S-CCS in today's production regime

Development of S-CCS is still at an early stage. Prototypes with wide range of solutions, are still being tested. In the future, S-CCS might be a possible supplement to production of Atlantic salmon in traditional open sea cages. Implementing these systems to current production regimes could prevent disease outbreaks and loss of fish, improve fish health and welfare, as well as reducing the environmental impact of aquaculture. This would reduce the production costs, increase the effectiveness of the Atlantic salmon production cycle, improve the social acceptance of Atlantic salmon operations and overall contribute to a more sustainable aquaculture. Development of other new production technologies, like offshore facilities, might also increase the need for more robust post-smolt at sea transfer. Accordingly, the advantages of different production strategies may be utilized in different ways, resulting in an overall optimization of the production cycle.

Regardless of the overall benefits of S-CCS, this technology may not necessarily apply to all Atlantic salmon production sites. Along the Norwegian coast, there are major variations among localities. Some sites have significant defies related to sea lice and diseases, while other sites have low occurrence of these challenges. Using S-CCS as a supplement to traditional open sea cage culture, might therefore not necessarily be beneficial nor cost-effective for every single salmon production site in Norway. Considerations about use of alternatives to open sea cages needs to be taken into account at each site (Haaland, 2017).

Implementation of S-CCS in current production is dependent on increasing efficiency and production volume, in order to secure a cost-effective production and be competitive to commercially open sea cages. It is difficult to estimate the cost of producing S-CCS, but this is expected to require higher investment and running cost, when compared to open sea cages. On the other hand, less mortality and operations involving handling of the fish while the fish are not fed, will result in production volume gains. The strategy might also contribute to a more efficient production at sea, as transfer to open sea cages becomes possible throughout the year and its ability to produce closer to maximal allowed biomass (Iversen et al., 2013). Moreover, if S-CCS can eliminate challenges related to lice and diseases, certain production costs would consequently decrease and production volumes increase.

S-CCS are highly dependent on optimal conditions within the rearing unit. To achieve this, it is essential to ensure a safe water supply to the system. Treatment of the intake water could reduce the risk of introducing pathogenic agents into the systems (Haaland, 2017). Water treatment for disinfection purposes may be viewed as necessary biosecurity measure by many farmers. The natural microbial community within the intake water may be introduced to a different environment inside the rearing unit, whereas the organic load, nutrient profile, environmental conditions are shifted may induce disturbance of the natural microbial balance and growth of potential pathogenic opportunistic species. In addition, the potential of causing upwelling of sediment harbouring pathogens, may introduce pathogens into the system with the intake water (CtrlAQUA, 2015).

4.2 Treatment technologies

Before implementing S-CCS to current production regimes, it is relevant to evaluate water treatment's necessity and effect. Water treatment technologies used in other aquaculture systems might potentially be adapted for use in S-CCS. To remove the largest parasites in the intake water, filtration processes could become effective with pores size less than 200 µm. However, in order to remove the smaller parasites, e.g. the amoeba *Paramoeba perurans*, pore sizes smaller than 20 µm would be necessary. Even with these small pore sizes, the amoeba could find a way through the filter, due to its flexible characteristics. Filtration processes are therefore not effective enough by themselves, although a combination with another treatment method would increase the efficiency (CtrlAQUA, 2015). Ozone and advanced oxidation process are actual water treatment methods already used in other aquaculture systems. Potential formation of toxic by-products are major limiting factors for these treatment methods. S-CCS would also need to handle large flows of intake water.

4.3 How results can be applied to S-CCS

UV treatment is an effective method for large water flows which do not leave any toxic residual behind, a potential alternative to adjust and implement in S-CCS (Liltved et al, 2006). In the present work, seawater samples were collected from different sites in order to evaluate the necessity and effect of UV treatment. Seawater treated with 25 mJ/cm^2 MP UV presented only residual colony forming bacteria, indicating a strong disturbance on the seawater microbial communities' equilibrium. The information generated in this study contributes to new knowledge on *M. viscosa* and the copepodid *L. salmonis* and adds to current knowledge base on UV radiation as a treatment strategy. Specifically, completion of bench scale trials on

UV treatment resulted in required doses to inactivate Atlantic salmon pathogens, impact on seawater and assessment of DNA damage in *L. salmonis* cells caused by UV radiation. This information can be applied to evaluate the necessity and effect of the low-pressure and medium-pressure UV lamp. It can also be used to determine the dose to be applied to the intake water, as well as target inactivation of specific pathogens before entering the closed containment system.

4.4 Inactivation doses for pathogens

The different pathogens tested in this study showed a degree of variation in both required UV dose, and resulting degree of inactivation. *M. viscosa*, ISAV and *L. salmonis* are different in size, taxa and genome characteristics. Smaller organisms could be more susceptible to UV since there will be less tissue for attenuation of UV. Regardless of size of the organism, there are also significant variations in biologically evolved UV resistance between different species (Barrett et al., 2019). The nature of the genome of the organisms has also been associated to UV susceptibility. For instance, viruses with double-stranded genome exhibit higher resistance to UV compared to ones with single-stranded genome (Liltved et al., 2006).

4.4.1 *Moritella viscosa*

In this study, all tested UV doses, resulted in a log 3 inactivation or higher of *M. viscosa*, whereas a dose of 2.3 mJ/cm^2 were the lowest tested UV dose in this study. The minimum required UV dose for *M. viscosa* could therefore potentially be lower. There were no previous studies done to determine the minimum required UV dose for log 3 inactivation, and there is a need for more information on this issue. Although, potential use of UV as treatment of intake water in S-CCS would probably apply a higher UV dose than 2.3 mJ/cm^2 in order to inactivate more resistant pathogens.

4.4.2 Infectious salmon anaemia virus

None of the UV doses in this study resulted in a log 3 inactivation or higher of ISAV. Thus, the minimum required UV dose for log 3 inactivation could not be determined for ISAV. Higher UV doses are necessary in order to determine the minimum UV dose for 3 log inactivation of ISAV. However, previous studies reported the inactivation dose to be considerably lower. Øye & Rimstad (2001) reported the inactivation to be 7.9 J/m^2 using LP UV. Liltved et al. (2006) found the inactivation dose to be of 7.5 mJ/cm^2 using LP UV.

The required inactivation doses for ISAV obtained in this study demonstrated some deviations from results stemming from previous studies (Liltved et al., 2006). These deviations may be influenced by variations in experimental factors, including storage temperature of the pathogens, salinity and water quality of the pathogen suspension, adherence to particles, arrangement of the UV lamp, quantification methods and deviations in intensity measurements (Liltved et al., 1995). Effectivity of UV lamps being used also depends on several factors. This includes the age and condition of the lamp, the cleanliness of lamp surface, the lamp intensity, the distance between UV source and the target organism, the type of target organism, the particles' content in the water and the duration of UV exposure (Lekang, 2020).

4.4.3 *Lepeophtheirus salmonis*

The minimum required UV dose to achieve 99.9% mortality were not determined for *L. salmonis.* The highest UV dose tested in this study were 199.3 mJ/cm^2 with MP UV which induced 47.1% mortality rate. The highest achieved mortality rate using LP UV was 23.3% with an irradiation dose of 157 mJ/cm^2. Higher UV doses are necessary in order to determine the minimum UV dose for 99.9% mortality of *L. salmonis.*

The methodology employed for the quantification of the mortality rate could however be a source of error. When counting mortality before and after UV exposure, individuals that showed no indication of movement over a period of 10 seconds, were considered as dead. It is possible that additional time would be necessary to accurately determine the mortality. The general movement of the copepodites were also low before UV exposure, leaving the possibility that mortality was overestimated before UV. On the contrary, the movement of the copepodites were slightly higher after UV exposure, making it easier to estimate the mortality. This could have led to results indicating a lower difference in mortality before and after UV exposure and corresponding underestimation of the mortality rate.

Aarseth & Schram (2002) found that a dose of 8400 mJ/cm^2 induced 50% mortality rate in *L. salmonis* using UV-B radiation. As *L. salmonis* normally are present in surface water exposed to UV-B radiation from the sun, it is likely that they could be more resistant to these wavelengths (Heuch et al., 1995). Barrett et al. (2019) reported a dose of 90 mJ/cm^2 induced 95% mortality in *L. salmonis* eggs using a low-pressure UV-C lamp. However, it can be hypothesised that *L. salmonis* eggs are more susceptible to UV radiation than copepodites, given that a smaller sized organism will have reduced attenuation through their tissues. On

that account, although the dose applied in S-CCS is not high enough to cause mortality directly into the infective copepodid stage, it could potentially inhibit egg strings to successfully molt into copepodites, thus resulting in an overall reduction in salmon louse production (Barrett et al., 2019).

DNA damages in the copepodite cells, percentage DNA in tail and tail length were expected to increase proportionally with higher UV doses, while number of intact cells were expected to decrease. However, results in this study showed some level of inconsistency. While UV doses of 5, 10, 20 and 40 mJ/cm² induced high DNA damage, UV doses of 8 and 16 mJ/cm² induced no damage. These particular observations are, however, originating from different batches of copepodites. This may point at an unknown experimental error, either biological or technological one.

The copepodite samples where DNA damages were found, were all induced by MP UV. This may demonstrate the copepodites tissues ability to photorepair at this UV intensity. The broad spectrum of wavelengths emitted by MP UV might affect the cellular mechanisms for DNA repair, unlike low-pressure UV which emits most of the output within a single wavelength. In theory, low-pressure UV irradiated samples could induce temporary DNA damages, which could be later repaired by photoreactivation.

4.5 Further work

In addition to UV treatment, other treatment strategies should be assessed for closed-containment aquaculture. The economic aspect, in form of investment and running costs, of implementation of a water treatment unit should be estimated and assessed up against the potential benefits achieved. Moreover, there is a need for more information and to continue the work on determination of the minimum required UV dose to effectively inactivate other relevant Atlantic salmon pathogens to increase the knowledge base for use of UV radiation as a treatment strategy in semi-closed containment systems.

Although all doses *M. viscosa* were exposed to resulted in a log 3 inactivation or higher, lower UV doses should be investigated to determine the actual minimum required dose. Higher UV doses need to be studied in the case of ISAV and *L. salmonis* in order to determine the minimum required dose for a log 3 inactivation and 99.9% mortality in these two pathogens. There is also a need to further investigate the impact on UV treatment on the

seawater microbial communities' equilibrium. Furthermore, estimation of bacterial activity and cultivable bacteria quantification should be investigated for the seawater samples

5 Conclusion

In this study, the lowest dose required to achieve a log 3 inactivation of *M. viscosa* with medium-pressure UV was 2.3 mJ/cm^2. As *M. viscosa* require a low UV dose to achieve complete inactivation, sites where it is a *M. viscosa* is a problem may use UV radiation to reduce winter ulcer outbreaks in S-CCS.

As for ISAV, none of the doses ISAV resulted in a log 3 inactivation with the highest inactivation being log 2.59 and achieved at 22.5 mJ/cm^2 using medium-pressure UV. ISAV needs a higher UV dose than *M. viscosa*, although still within reasonable range of UV doses. Future studies may repeat the experiments to estimate the dose required to 3 log.

Regarding *L. salmonis,* none of the UV-doses employed in this experiment resulted in 99.9% mortality rate with the highest mortality being 47.1% achieved at 199.3 mJ/cm^2 by use of medium-pressure UV. Control of *L. salmonis* using UV radiation is not feasible with the tested technologies as the required UV dose is too high. There were induced medium to high levels of DNA damages for cells exposed 5, 10, 20 and 40 mJ/cm^2 after treatment with a medium-pressure UV.

Seawater samples exposed to 25 mJ/cm^2 emitted by a medium-pressure UV presented only residual colony forming bacteria. General application of 25 mJ/cm^2 of UV seems to a reduce CFU, whether this is beneficial for Atlantic salmon rearing environment still needs to be tested.

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Appendix

A) Medium preparations

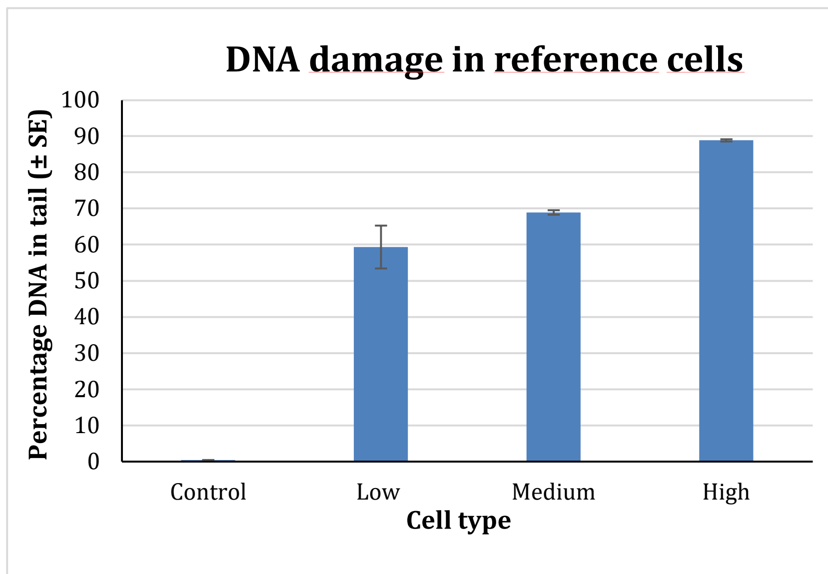
a) Blood agar 3% NaCl plates: 17.5 g of sodium chloride (1.06404.1000, Emsure, USA) and 28.0 g of blood agar (CM0271, Oxoid, England) were diluted in 700 ml of distilled water in an Erlenmeyer flask. The solution was stirred and autoclaved. 35 ml of full blood were added after the solution had cooled down. The solution was plated in sterile 92*16 mm Petri dishes and kept at 21°C for 24 hours. The Petri dishes were stored at 6°C for further use.

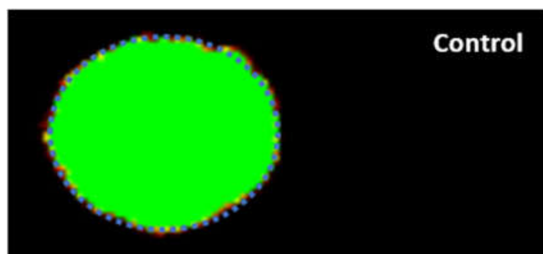
b) Liquid marine broth 3% NaCl: 26.2 g marine broth (279110, Difco, USA) and 7.0 g sodium chloride (1.06404.1000, Emsure, USA) were diluted in 700 ml distilled water in an Erlenmeyer flask. The solution was heated to boiling point, while stirred, before it was cooled down. The solution was then filtrated in a Büchner flask with funnel and 90 mm glass microfiber filter (Cat. No. 1822090, Whatman, USA) and autoclaved. The solution was stored at 12°C for further use.

c) Marine agar 3% NaCl plates: 26.2 g marine broth (279110, Difco, USA) and 7.0 g sodium chloride (1.06404.1000, Emsure, USA) were diluted in 700 ml distilled water in an Erlenmeyer flask. The solution was heated to boiling point, while stirred, before it was cooled down. The solution was then filtrated in a Büchner flask with funnel and 90 mm glass microfiber filter (Cat. No. 1822090, Whatman, USA). 10.5 g bacto agar (214010, Difco, USA) was added, before the solution was autoclaved. The solution had cooled down to approximately 40°C, it was plated in sterile 92*16 mm Petri dishes and kept at 21°C for 24 hours. The Petri dishes were stored at 6°C for further use.

B) CometAssay reference cells

	%DNA in Tail Mean	Standard Error (SE)
Control	0.39	0.06
Low	59.38	5.93
Medium	68.9	0.61
High	88.87	0.34


DNA damage in reference cells
Percentage DNA in tail (± SE)
100
90
80
70
60
50
40
30
20
10
0
Control
Low
Medium
High
Cell type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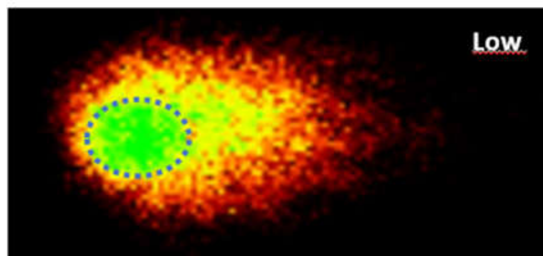
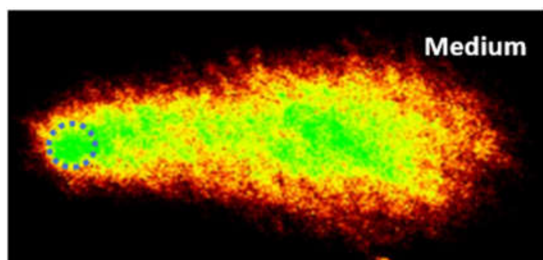
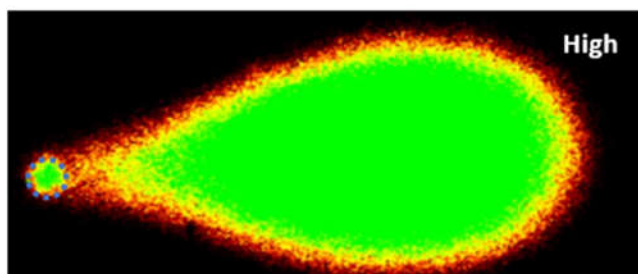

Low


Medium


High