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The efficacy of ozonated seawater for surface disinfection of haddock (*Melanogrammus aeglefinus*) eggs against piscine nodavirus

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Abstract

Piscine nodavirus, also known as viral nervous necrosis (VNN), is a lethal, vertically transmitted virus that causes severe mortality in fish. It affects primarily marine species, including cultured haddock (*Melanogrammus aeglefinus*). Ozone has been used successfully to disinfect Atlantic halibut (*Hippoglossus hippoglossus*) eggs against nodavirus. Fertilized eggs from different species of fish can tolerate varying levels of dissolved ozone, so specific exposure levels need to be determined for individual species. The objectives of this study were to investigate the tolerance of newly fertilized haddock eggs to dissolved ozone and to determine if this exposure is sufficient to disinfect against piscine nodavirus. Eggs were exposed to an ozone concentration of 3.0(±0.3) mg/l of total residual oxidants (TRO) of Cl_2 for CT units (TRO × duration of exposure in min, mg/l/min) of 0, 5, 10, 15, 20, 25, 30, 40 and 50. A decrease in survival was observed when the exposure exceeded 30 CT units. Following this, other fertilized haddock eggs were submerged in nodavirus suspensions with densities of $10^{2.5}$ and $10^{3.5}$/0.1 ml $TCID_{50}$ units for 30 min, followed by exposure to ozonated seawater at a concentration of 3.0 mg/l for CT units of 0, 10, 20 and 50. Viable VNN was detected by cell culture using striped snakehead (SSN-1) cell lines. The positive controls (exposed to 0 CT units) all tested positive for nodavirus, while all but one of 24 egg samples exposed to ozonated seawater tested negative. This indicates that ozone can be successfully used to disinfect haddock eggs against nodavirus at a concentration of 3(±0.3) mg/l TRO as Cl_2 for 3.3–6.7 min.

Keywords: Haddock; Nodavirus; VNN; Ozone; Egg disinfection

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1. Introduction

Haddock (*Melanogrammus aeglefinus*) are a cold water, fast growing, high value fish (Waiwood, 1994), making them suitable for aquaculture in Atlantic Canada (Frantsi et al., 2002). They are serial spawners that will spawn under culture conditions, allowing for the collection of fertilized, pelagic eggs from spawning tanks. Eggs must be surface disinfected prior to incubation to minimize transmission of surface contaminating bacteria and viruses.

Nodavirus is the causative agent of viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER), or fish encephalopathy. Nodavirus infections are a worldwide problem affecting over 30 species of marine finfish including Atlantic halibut (*Hippoglossus hippoglossus*) (Grotmol et al., 1995), sevenband grouper (*Epinephelus septemfasciatus*) (Tanaka et al., 1998), Japanese flounder (*Paralichthys olivaceus*) (Nguyen et al., 1994), red drum (*Sciaenops ocellatus*) (Oh et al., 2002), winter flounder (*Pseudopleuronectes americanus*) (Barker et al., 2001), Atlantic cod (*Gadus morhua*) (Johnson et al., 2002) and haddock (Harmon et al., 2003). Clinical signs of the disease include anorexia, lethargy, pale-gray colouration, hyperinflation of the swim bladder and erratic swimming. Infected fish have been observed swimming in a corkscrew-like pattern, sinking to the bottom and then floating up to the surface (Chi et al., 1997). The causative virons have been detected in eggs, larvae and broodstock of striped jack (*Pseudocaranx dentex*), indicating that spawners can be a source of infection (Arimoto et al., 1992). Nodavirus is durable and tolerant to various environmental conditions (Frerichs et al., 2000). However, ozone has been shown to inactivate nodavirus, although specific VNN titers were not reported (Arimoto et al., 1996; Grotmol and Totland, 2000). Fertilized eggs from different species of fish can tolerate varying levels of dissolved ozone, so specific exposure levels need to be determined for each species (Grotmol et al., 2003).

Ozone oxidizes non-selectively (Benoit and Matlin, 1966) and disinfects by destroying bonds found in organic compounds (Evans, 1972). Scott and Lesher (1963) concluded that the primary attack of ozone on *Escherichia coli* was on the cell membrane of the bacteria, most likely at the lipid double bonds, causing cell lysis. Virus particle membranes are destroyed in a similar fashion, along with the destruction of their RNA, thereby disabling replication (Roy et al., 1981).

The objectives of this study were to investigate the tolerance of newly fertilized haddock eggs to dissolved ozone and to determine if this exposure is sufficient to disinfect against piscine nodavirus.

2. Materials and methods

2.1. Eggs

Eggs were collected from a broodstock tank containing 37 adult haddock (19 males and 18 females) at the St. Andrews Biological Station (Fisheries and Oceans, Canada). Haddock spawn naturally under culture conditions, enabling collection of their buoyant fertilized eggs using a surface skimming egg collector. The collector was emptied and the developmental stage of the eggs checked every day to ensure only newly fertilized eggs were collected and used for this experiment.

2.2. Ozonation system

A bench-scale ozonation system was developed using a 200 mg/h adjustable corona discharge ozone generator (Red Sea Fish Pharm, TX, USA). The gaseous ozone was pumped into a 500 ml gas-washing bottle (AM Glassware Ltd., Aberdeen, Scotland), modified by the addition of spigots at the top and bottom, and dispersed into the water column via an air stone (porosity of 40–100 μm). Seawater was pumped into the washing bottle through the bottom spigot using a peristaltic pump, where it came in contact with the ozone, allowing some of the ozone to dissolve into the water column. The ozonated seawater was then pumped from the cylinder into a 500 ml glass beaker at a rate of 37 l/h. Water was continuously pumped through the system, constantly replenishing the ozonated water in the beaker and maintaining a constant temperature of 6 °C (±1 °C). A magnetic stir bar in the treatment beaker ensured an even mixing of water and ozone. Eggs were disinfected in this solution and ozone concentrations were measured before, during and after treatments. The oxidative power of the ozonated seawater was determined using the

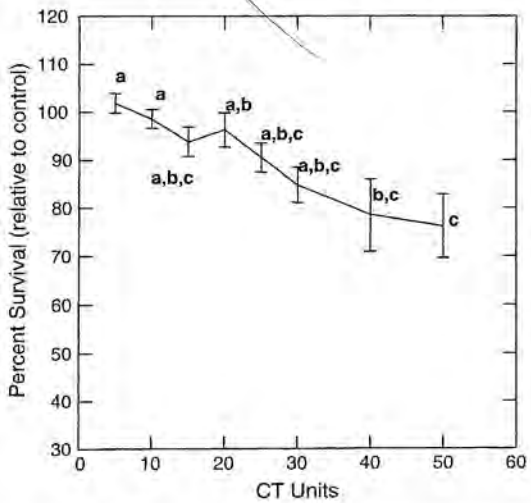


Fig. 1. Haddock egg survival after different ozone exposures. Three trials were conducted with three different batches of eggs, requiring separate controls (0 CT units) for each trial. Percent survival is relative to the control for the corresponding trial. Some treatments at 5 and 10 CT units had better survival than their corresponding control. Bars represent standard errors, with values with the same letter being not significantly different from each other using the Student–Newman–Keuls test.

there were several dead eggs in all beakers, but this is normal as unfertilized eggs settle out at this point (S. Warrington, DFO St. Andrews Biological Station, personal communication). Hatched larvae from all treatments appeared to have developed normally and exhibited normal swimming patterns. Egg survival was affected by exposure to ozone ($P < 0.003$; ANOVA), decreasing as time of exposure to ozone increased (Fig. 1). There was no statistically significant difference in survival between controls and ozone doses up to 30 CT units. Survival rates after exposures to 25 and 30 CT units were not significantly different from the lower survival rates at 40 and 50 CT unit exposures. It was, therefore, decided that the optimum dosage was 20 CT units. Using an ozone concentration of 3.0 mg/l equates to a 6.7 min maximum exposure time.

3.2. Egg exposure to nodavirus and ozone

All positive controls (i.e., eggs exposed to nodavirus followed by non-ozonated seawater) tested positive for nodavirus, indicating that the diagnostic test was sensitive enough to detect viable nodavirus at the concentrations used for this experiment. All but one of the other treatments tested negative for nodavirus, with the one exception being a virus concentration of $10^{2.5}$/0.1 ml $TCID_{50}$ for an exposure of 20 CT units. Therefore, an exposure of 10 CT units is sufficient to inactivate nodavirus.

4. Discussion

Ozone can successfully disinfect fertilized haddock eggs against nodavirus. It is recommended that fertilized haddock eggs be disinfected with an ozone dose of 3.0 mg/l TRO for 3.3–6.7 min (10–20 CT units). This exposure inactivates nodavirus but does not lower the hatching survival of eggs. No visible differences were observed between the hatched larvae that were exposed to ozone and controls. The larvae seemed to have developed normally and normal swimming patterns were observed. It appears that if eggs are not exposed to an ozone concentration that impedes hatching, there are no detrimental effects of the ozonation process. However, long-term effects of ozone on fertilized fish eggs are currently unknown.

Eggs that were exposed to high ozone levels appeared to be alive during the incubation stage but did not hatch when the untreated/lower CT treated eggs did. They remained alive but unhatched for 3–4 more days, and then died. These embryos appeared to have developed normally inside the eggs as their heartbeats were easily observed prior to death, but they were not able to hatch. Other researchers observed a similar inability of some ozone-treated eggs to hatch (Hall et al., 1981; Arimoto et al., 1996; Mimura et al., 1999; Grotmol and Totland, 2000). Grotmol and Totland (2000) suggested that this could be due to a reduced secretion of hatching enzyme or a reduction of its effectiveness because the eggshell protein polymer was altered by ozone. This exemplifies the need to determine maximum doses of dissolved ozone that eggs from different species of fish can tolerate.

It is important to know that ozone gas can be harmful to humans and certain safety precautions need to be followed. Proper ventilation is mandatory and the use of an air filter or fume hood is strongly

N,N-diethyl-*p*-phenylenediamine (DPD) colorimetric method (American Public Health Association, 1992), using a spectrophotometer (Swan Analytical, Chematest 20, Switzerland) reporting units of total residual oxidants (TRO) in mg/l as Cl_2 (Buchan et al., 2005).

2.3. Ozone tolerance of haddock eggs

One millilitre lots of eggs (~400 eggs) at the 4–16-cell stage were suspended in 3(±0.3) mg/l TRO as Cl_2 ozonated water using dip nets for 0.0, 1.7, 5.0, 6.7, 8.3, 10.0, 13.3 and 16.7 min resulting in ozone doses of 0, 5, 10, 15, 20, 25, 30, 40 and 50 CT units (TRO × duration of exposure in minutes). Each treatment was conducted in triplicate within a trial, with a total of three trials. Replicates within a trial came from the same batch of eggs, but each trial used a different batch of eggs. After ozone treatment, eggs were transferred to 250 ml clear glass beakers filled with 150 ml of 6 °C autoclaved seawater. The eggs were incubated at 6 °C in a climate-regulated room, under constant light, generally at 40 lux except when dead eggs or live larvae were being removed at which time the light intensity was increased to 1500 lux. Eggs that turned opaque or had sunk to the bottom of the beakers were determined to be dead and were counted and removed every 2–3 days. Water was exchanged with new 6 °C autoclaved seawater once mid-way through the approximately 2 week incubation period. Hatched larvae were counted and removed from the beakers using pipettes. Percent survival was determined, and compared to the control (no exposure to ozone) of that particular batch of eggs to determine if a difference in survival existed.

2.4. Egg exposure to nodavirus and ozone

Nodavirus preparations were obtained by passaging brain and eye tissue that was cultured from infected haddock, on striped snakehead (SSN) cell line. This was followed by polymerase chain reaction (PCR) techniques to verify the growth as VNN (Merritt, 2003). One hundred milliliters of haddock eggs (~40 000 eggs) were then submerged in a virus concentration of either $10^{2.5}$/0.1 ml or $10^{3.5}$/0.1 ml $TCID_{50}$ (tissue culture infectious dose for 50%) units for 30 min to provide infected eggs for ozone treatments. Eggs exposed to either viral load were then submerged into ozonated seawater. Ozone treatments were administered to 1 ml lots of eggs that were placed in histology tissue cassettes (3.3 cm × 2.8 cm × 0.7 cm) and then suspended in ozonated seawater for 0, 10, 20 or 50 CT units. Each treatment was repeated in duplicate. Negative controls, with no exposure to nodavirus, were administered as well. The eggs were diluted with 1–2 ml of Hanks Balanced salt solution (HBSS, pH 7.6) in a whirlpak bag, homogenized manually (with a roller) and filtered aseptically through a 0.45 μm pore diameter membrane filter. 0.1 ml of each filtrate was added in duplicate to 24 well plates (Linbro-ICN) containing striped snakehead (SSN-1) cells using the simultaneously applied cells and test sample method as described in the Fish Health Protection Regulations Manual of Compliance (Department of Fisheries and Oceans, 1984). The SSN-1 cell line used for the assays was maintained at 25 °C in 75 cm^2 cell culture flasks (Corning-Costar) in Leibovitz L-15 medium containing Hanks salts, glutamine, 5% fetal bovine serum (FBS) and antibiotic–antimycotic mixture (1000 units/ml penicillin, 1 mg/ml streptomycin and 2.5 μg/ml amphotericin B; GIBCO-BRL). Viral cultures were incubated at 20 °C and examined for cytopathogenic effects (CPE) caused by viral agents for a minimum of 28 days. If CPE were detected in any of the samples, the agent causing CPE was confirmed as VNN using RT-PCR analysis (Merritt, 2003). Two tests were conducted for each sample of eggs.

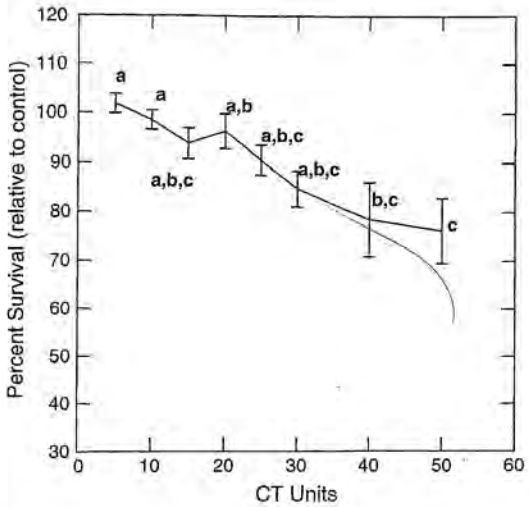
2.5. Statistical analysis

The proportion of surviving eggs was analysed using a mixed model ANOVA, where trial (three levels) was a random factor and ozone exposure (nine levels) was a fixed factor. The assumption of homogeneity of variance was tested using Cochran's test. This assumption was not violated. Multiple comparisons were done using Student–Newman–Keuls (SNK).

3. Results

3.1. Haddock egg tolerance of ozone

The haddock eggs appeared to develop normally after the ozone treatments. Two days after treatments,



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It is important to know that ozone gas can be harmful to humans and certain safety precautions need to be followed. Proper ventilation is mandatory and the use of an air filter or fume hood is strongly

recommended. An Ecosensors EcoZone EZ-1X (Santa Fe, NM, USA) ozone monitor was used to monitor ozone levels in the air surrounding the ozone system and an Extract-All Air Impurities Removal System (Milwaukee, WI, USA) with an activated carbon filter was used to filter any ozone present.

Due to the threat of nodavirus in marine fish hatcheries, a disinfection protocol for large-scale ozone disinfection of marine fish eggs should be developed. As our findings and others studying other marine species demonstrate, the use of ozonated seawater to disinfect eggs can be beneficial (Ben-Atia et al., 2001), especially against nodavirus (Arimoto et al., 1996; Grotmol and Totland, 2000). The system described, here, is too small for large-scale disinfection of egg batches. The higher organic load of large egg biomasses will significantly shorten the half-life of ozone (which is normally about 15 min; Lawson, 1995) so sufficient water volume and ozone flow is necessary to maintain appropriate disinfection doses. We suggest that eggs should be disinfected in the egg incubator by adding ozone directly with an air stone while the seawater flow is temporarily turned off. After the desired treatment interval the water can be turned on, flushing out any residual ozone. At a concentration of 3.0 mg/l TRO, the safe but effective treatment time is 3.3–6.7 min, thereby, allowing sufficient time to conduct this procedure and allow effective disinfection.

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