Effects of Total Residual Oxidant on Oxidative Stress in Juvenile Olive Flounder *Paralichthys Olivaceus*

Han Seok Ryu, Jung Ho Han, Jin Ah Song & Cheol Young Choi

Taylor & Francis
Taylor & Francis Group

Check for updates

Effects of Total Residual Oxidant on Oxidative Stress in Juvenile Olive Flounder *Paralichthys Olivaceus*

Han Seok Ryu*[a], Jung Ho Han*[b], Jin Ah Song[a], and Cheol Young Choi[a]

[a]Division of Marine BioScience, National Korea Maritime & Ocean University, Busan 49112, Korea; [b]Chemical and Biological Engineering, Jeju National University, Jeju 63243, Korea

ABSTRACT

We investigated oxidative stress in olive flounder *Paralichthys olivaceus* induced by exposure to total residual oxidant (TRO) at three different concentrations (20, 40, and 60 µg/L) over 14 days to determine concentrations of TRO appropriate for aquaculture. We analyzed survival rate, antioxidant enzyme (superoxide dismutase [SOD] and catalase [CAT]) mRNA abundance, and heat shock protein 70 (HSP 70) expression (mRNA and protein) in the gills and liver, in addition to levels of hydrogen peroxide in plasma. Furthermore, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used to measure apoptosis in the gills. All flounder exposed to 40 and 60 µg/L TRO died by day 14 and day 7, respectively. Antioxidant enzyme mRNA and HSP 70 expression (mRNA and protein) in the gills and liver significantly increased with increasing TRO concentration but decreased on day 14 in the 20 µg/L TRO group. In the gills, TUNEL-positive cells increased, and histological modifications were observed at 40 and 60 µg/L TRO; however, these differences were not observed in the 20 µg/L TRO group. These results indicate that 20 µg/L TRO does not significantly affect olive flounder survival after 14 days or the indicators of oxidative stress between 7 and 14 days. This target concentration of TRO (TRO 20) may be suitable for water treatment in recirculating aquaculture systems containing olive flounder.

Introduction

Ozone (O₃), an allotrope of oxygen with strong oxidizing capacity, has the ability to decompose organic matter and is used for water sterilization in various applications, including aquaculture. The injection of ozone into water treatment systems to induce oxidation and remove microorganisms has been referred to as the 'ozone oxidation method' (Oh, Kim, and Cho 1999; Park et al. 2013). The ozone oxidation method is often applied in recirculating aquaculture systems that require purification and reuse of the process water (Schroeder et al. 2011). This method has been reported to be effective for the elimination of pathogenic microorganisms and viruses, and for the removal of organic matter resulting from metabolic activities of aquatic organisms (Sharrer and Summerfelt 2007; Wold et al. 2014). The removal of organic matter by ozone is not only attributable to ozone itself, but also to the effects of hypobromous acid (HOBr) and hypobromite ion (BrO⁻), which are powerful oxidants formed by a reaction between ozone and Br-. In this process, the oxidants that are created by ozone that function to destroy

microorganisms are called total residual oxidants (TRO) (Buchan, Martin-Robichaud, and Benfey 2005).

Studies on the effects of ozone-induced TRO on seawater sterilization and fish toxicity have been undertaken. Recently, studies on the optimal TRO concentration for direct application in aquaculture have become increasingly important (Jung et al. 2018). Even when small amounts of TRO exist in water, sterilization occurs following the destruction of bacterial cell membranes through its powerful oxidative capacity. In addition, TRO and components of fish excreta, such as organic matter and colloids, cohere to bromoform (CHBr₃), which can be easily removed by skimmers and other filter equipment (Haag and Hoigné 1984; Liltved et al. 2006). However, when organic matter that can react with TRO no longer exists in the water, TRO is difficult to remove from the water and is known to be toxic to fish and other aquatic organisms (Hofmann 2000). Direct exposure to TRO induces damage in red blood cells and tissues such as gills and may increase mortality rates in severe cases (Fukunaga et al. 1992a; Kim, Oh, and Jung 1999; Yoshimizu 1995). While ozone is rapidly converted into

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/bose.

secondary oxidants such as TRO in the seawater (Perrins et al. 2006; Tango and Gagnon 2003), TRO is relatively stable and can, therefore, be used as an indicator of potential toxicity when ozone is used for water sterilization (Jones et al. 2006).

TRO induces oxidation-reduction reactions in the body and forms reactive oxygen species (ROS) that cause oxidative stress in cells and tissues (Fukunaga et al. 1992b). Representative ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^\cdot OH$), and singlet oxygen ($^1O_2$) (Roch 1999), which impart negative effects on cell viability when present in excess by inducing cellular damage, aging, and apoptosis (Nordberg and Arnér 2001; Slaninova et al. 2009). Because of these toxic oxidative effects, organisms have antioxidant defense mechanisms that convert ROS into nontoxic substances through the actions of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (McFarland et al. 1999). Antioxidant enzymes are mainly expressed in the liver and kidney of fish, and SOD primarily converts O_2^- into O_2 and H_2O_2 (Basha and Rani 2003). Since H_2O_2 is also an ROS, it must be secondarily converted to nontoxic H_2O and O_2 by CAT (Hansen et al. 2006; Kashiwagi et al. 1997).

Cellular damage caused by TRO promotes the synthesis of heat shock protein (HSP), a stress-related protein (Kregel 2002). HSP plays an important role in external contaminant and abiotic stress responses, in addition to its role in high temperature stress, and helps to prevent cellular damage and maintain homeostasis (Yamashita, Yabu, and Ojima 2010). Despite the fact that TRO is an important byproduct in the ozone sterilization method, few studies have investigated the effects of TRO on fish antioxidant systems and stress responses.

This study was conducted to investigate the effects of TRO at different concentrations on the stress response of olive flounder *Paralichthys olivaceus*. Park et al. (2013) reported that TRO at 20 and 40 μg/L was effective for reducing organic matter and bacteria in a recirculating seawater system used for the culture of black seabream *Acanthopagrus schlegelii*. Based on these concentrations, olive flounders were exposed to available concentrations of TRO (20, 40, and 60 μg/L) that could be applied for the purpose of water sterilization in aquaculture. In this study, we analyzed the expression of SOD and CAT mRNA in the gill and liver tissues of olive flounder maintained in seawater treated with ozone in order to investigate the induction of oxidative stress over time at different TRO concentrations. In addition, the concentration of H_2O_2 in plasma, and HSP 70 mRNA expression and activity in the gill and liver tissues were analyzed. The degree of apoptosis in the gill tissue was also analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The results of this study provide an understanding of safe concentrations of TRO that can be applied in aquaculture.

Materials and methods

Experimental fish

Juvenile olive flounders (length 10.5 ± 0.4 cm; mass 9.2 ± 0.5 g) were purchased from a fish farm in a Pohang, Korea, and were allowed to acclimate for two weeks in twelve 150 L recirculation filter tanks in the laboratory (Figure 1). Experiments were performed in three 150 L recirculation filter tanks (skimming filtration type) for each TRO concentration, with 25 fish in each tank. The fish were exposed to a simulated natural photoperiod (27 W white flight light bulb as 12 h light:12 h dark photoperiod;
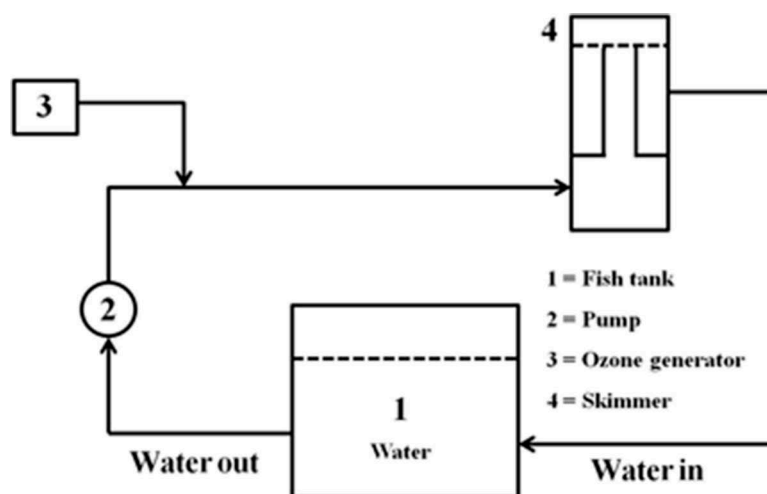

Figure 1. Ozone recirculation system used in current study. Flow rate = 15 ± 0.5 L/min.

was 20 ± 0.5 °C. The fish were fed 0.6 ± 0.05 g of commercial feed twice daily (09:00 h and 17:00 h) and feed was withheld for 48 h prior to sampling.

TRO treatment and sampling

To investigate the effects of TRO on oxidative stress in olive flounder, fish were maintained in water enriched with differing amounts of ozone to achieve TRO concentrations of 0, 20, 40, and 60 µg/L. Ozone was generated from four microplasma ozone generators (MP1-1003; Ozonaid Co. Ltd., Jeju, Korea) intermittently to maintain TRO concentrations, and TRO concentration was measured using the N,N-diethyl-p-phenylenediamine procedure and a spectrophotometer (DR3900; HACH, Loveland, CO, USA) five times daily at 06:00, 10:00, 14:00, 18:00, and 22:00. All fish were anesthetized using 20 µg/L of 2-phenoxyethanol (Sigma, St. Louis, MO, USA) and blood was collected from the caudal vein using a 1 mL syringe coated with heparin. Fish were decapitated prior to tissue collection. Plasma samples were separated by centrifugation (1000 × g for 15 min at 4 °C) and stored at −80 °C until analysis. Tissues (gill and liver) were sampled from five different fish from each tank on days 0, 1, 3, 7, and 14. All tissues were collected and immediately stored at −80 °C until total RNA was extracted.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from each tissue sample using TRI Reagent® (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription of 2 µg of total RNA was performed in a 20 µL reaction mixture using an oligo-(dT)$_{15}$ anchor and M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. The resulting cDNA was diluted and stored at 4 °C for use in real-time quantitative polymerase chain reaction (qPCR) analysis.

Real-time qPCR

The qPCR analysis was conducted to determine the relative expression of SOD, CAT, and HSP 70 mRNA using the cDNA synthesized from total RNA extracted from the gill and liver of olive flounder. The qPCR primers were designed using known olive flounder sequences (Table 1). We conducted the qPCR amplification using a Bio-Rad iCycler iQ multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and iQ SYBR green supermix (Bio-Rad) following the

Table 1. Primers used for qPCR amplification.

Genes (Accession no.)	Primer	DNA sequences
SOD (**EF681883**)	Forward	5′-CGT TGG AGA CCT GGG GAA TGT G-3′
	Reverse	5′-ATC GTC AGC CTT CTC GTG GAT C-3′
CAT (**GQ229479**)	Forward	5′-CCA AAC TAC TAT CCC AAC AGC-3′
	Reverse	5′-CCA CAT CTG GAG ACA CCT T-3′
HSP 70 (**AF053059**)	Forward	5′-GCA AAC AGG TTG AGC AG-3′
	Reverse	5′-ATC GTG TCC CTC TTC AGC-3′
β-actin (**HQ386788**)	Forward	5′- GGA CCT GTA TGC CAA CAC TG-3′
	Reverse	5′- TGA TCT CCT TCT GCA TCC TG −3′

manufacturer's instructions. The β-actin gene served as an internal control and was also amplified in each sample. All data were expressed relative to the corresponding β-actin threshold cycle (Ct) number. The Ct levels were defined as the PCR cycle in which the fluorescence signal crossed a threshold during the exponential phase of the amplification curve. Sample transcript abundance was expressed relative to internal control (β-actin) abundance using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{internal\ control}$. After the PCRs were completed, the qPCR data from three replicate samples were analyzed using the Bio-Rad software to estimate transcript copy numbers in each sample.

HSP 70 enzyme-linked immunosorbent assay (ELISA)

HSP 70 activities in gills and liver were analyzed using a commercial ELISA kit (MBS007829; Mybiosource Inc., San Diego, CA, USA). Five hundred micrograms of tissue was homogenized with 500 µL phosphate buffered saline and homogenates were centrifuged (1,500 × g for 15 min at 20 °C) according to the manufacturer's instructions. The absorbance at 450 nm was measured using a spectrophotometer.

Plasma H_2O_2 analysis

Plasma was separated from heparinized blood samples by centrifugation (1,500 × g for 15 min at 4 °C). Plasma samples were assayed immediately and stored at −80 °C. Plasma H_2O_2 levels were measured using a Peroxide Detect™ Kit (Sigma).

TUNEL assay

To evaluate the apoptotic response in fish gill cells exposed to TRO, we performed the TUNEL assay using a commercial *in situ* cell death detection kit (catalog number 11 684 795 910; Roche, Basel, Switzerland). Olive flounder gill tissue was washed, fixed with 4% buffered paraformaldehyde, and permeabilized with freshly prepared 0.1% Triton X-100 and 0.1% sodium

citrate solution. For the paraffin-embedded tissue sections, the slides were dewaxed and fixed according to standard protocols, and then treated as described above. The gill tissue was then incubated with the TUNEL reaction mixture for 1 h at 37 °C in a humidified chamber. The slides were washed three times with phosphate-buffered saline, and the incorporated biotin-dUTP was detected under an optical microscope (Eclipse Ci; Nikon, Tokyo, Japan).

Statistical analysis

All data were analyzed using the SPSS statistical package (version 19.0; SPSS Inc., USA). A one-way ANOVA followed by Tukey's post hoc test was used to compare differences in the data ($P < .05$). The values are expressed as the mean ± standard error (SE).

Results

TRO concentration

The TRO concentrations measured during the experimental period are listed in Table 2. Concentrations were measured at 06:00, 10:00, 14:00, 18:00, and 22:00 each day for the entire duration of the experiment in the three replicate tanks for each treatment.

Survival rate

Survival rate decreased with time and TRO concentration in all groups during the 14 d exposure period (Figure 2). Survival rates in the 40 and 60 μg/L TRO groups were decreased relative to the survival rates in the 0 and 20 μg/L TRO groups. Survival in the 40 μg/L TRO group decreased rapidly on day 7 and all flounder had died by day 14. Likewise, survival in the 60 μg/L TRO group decreased rapidly on day 3 and all flounder had died by day 7.
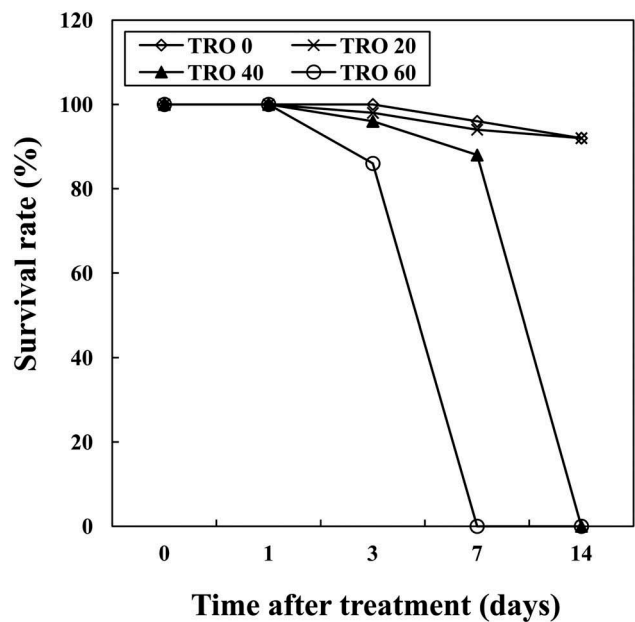

Figure 2. Survival rate of olive flounders in control and experimental groups exposed to three different total residual oxidant (TRO) concentrations (20, 40, and 60 μg/L) for 14 days.

Changes in SOD and CAT mRNA expression

SOD and CAT mRNA expression in the gills and liver significantly increased over time in all groups exposed to TRO (Figure 3). In the gill tissue, SOD and CAT mRNA expression in the 20, 40, and 60 μg/L TRO groups significantly increased in a dose-dependent manner by day 3. However, gill SOD and CAT transcript abundances in the 20 μg/L TRO group decreased to control levels on days 7 and 14, respectively. In the liver, SOD and CAT mRNA expression also increased in a dose-dependent manner by days 1 and 3, respectively. No difference in CAT mRNA expression in the liver was observed between days 7 and 14 in the 20 μg/L TRO group.

Table 2. TRO concentrations during the experimental period.

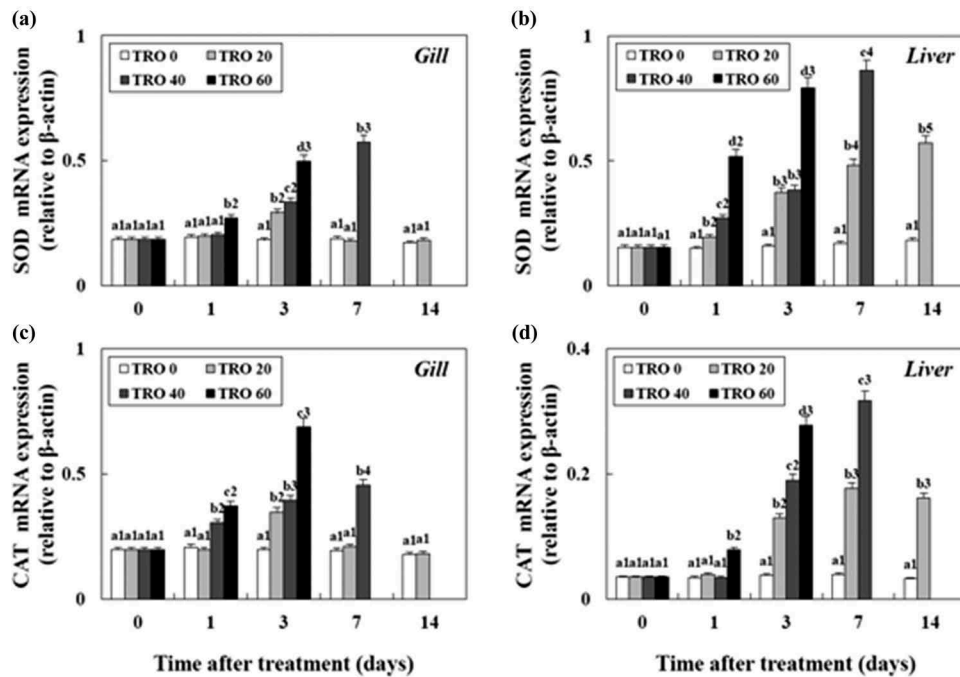| Time (hour)	Concentration (μg/L)	1 day	3 day	7 day	14 day
06:00	TRO 20	21.9 ± 0.82	22.0 ± 1.00	21.7 ± 1.53	22.0 ± 1.00
	TRO 40	42.0 ± 1.00	41.3 ± 1.15	42.3 ± 0.58	42.0 ± 1.00
	TRO 60	62.3 ± 0.58	62.3 ± 0.58	62.3 ± 0.58	62.3 ± 1.15
10:00	TRO 20	21.7 ± 0.58	21.7 ± 0.58	20.3 ± 1.53	21.0 ± 2.00
	TRO 40	40.7 ± 1.53	41.0 ± 1.00	41.7 ± 0.58	41.3 ± 2.08
	TRO 60	61.7 ± 0.58	61.3 ± 1.15	61.3 ± 1.53	60.3 ± 1.15
14:00	TRO 20	21.3 ± 0.58	21.3 ± 1.15	20 ± 2.65	19.7 ± 1.53
	TRO 40	42.0 ± 1.00	40.3 ± 0.58	41.0 ± 1.00	41.0 ± 1.73
	TRO 60	58.7 ± 1.00	60.7 ± 2.31	60.3 ± 2.52	61.0 ± 2.65
18:00	TRO 20	21.7 ± 1.15	21.3 ± 2.31	21.3 ± 0.58	21.3 ± 1.15
	TRO 40	40.3 ± 0.58	42.0 ± 1.73	41.3 ± 1.15	41.0 ± 1.00
	TRO 60	60.3 ± 0.58	61.3 ± 1.53	62.0 ± 1.00	62.7 ± 0.58
22:00	TRO 20	21.0 ± 1.73	20.7 ± 2.52	20.0 ± 1.73	20.0 ± 0.58
	TRO 40	40.0 ± 2.65	39.7 ± 2.89	39.7 ± 2.08	41.0 ± 1.00
	TRO 60	60.0 ± 1.00	61.3 ± 2.08	61.0 ± 2.00	62.7 ± 0.58

Data presented as mean ± SD.

Figure 3. Expression of superoxide dismutase (SOD) mRNA in the gills (a), SOD mRNA in the liver (b), catalase (CAT) mRNA in the gills (c), and CAT mRNA in the liver (d) of olive flounder in control and experimental groups exposed to total residual oxidant (TRO) at different concentrations (20, 40, and 60 μg/L) for 14 days. Different numbers indicate significant differences among the different exposure periods at the same TRO concentration ($P < .05$). The lowercase letters indicate significant differences among the different TRO concentrations for the same exposure periods ($P < .05$). All the values are means ± SE ($n = 5$).

Changes in HSP 70 mRNA and protein expression

HSP 70 mRNA expression and activity in the gills and liver significantly increased over time in all groups exposed to TRO (Figure 4). Significant dose-dependent increases were also observed in HSP 70 mRNA expression and protein levels in both the gills and liver between the control and TRO treatment groups; however, mRNA expression and protein levels on day 14 were lower than those on day 7 in both tissues. In addition, HSP 70 activity in the gills of the control and the 20 μg/L groups did not differ on day 14.

Changes in plasma H₂O₂

Plasma H_2O_2 levels significantly increased over time in all groups exposed to TRO (Figure 5). Significant dose-dependent increases in plasma H_2O_2 were also observed between the controls and each treatment group by day 3. On day 14, no significant difference in plasma H_2O_2 was observed between the control and the 20 μg/L TRO group.

TUNEL assay

The TUNEL assay was used for the detection of apoptotic cells (Figure 6). We observed an increase in the number of labeled cells between the control group and the experimental groups (40 μg/L TRO on day 7 and 60 μg/L TRO on day 3). However, there was no visible difference in the number of apoptotic cells between the control group and the 20 μg/L TRO group on days 3, 7, and 14. Also, hypertrophy and deformation of gill tissues were observed after exposure to 40 μg/L TRO for 7 d and 60 μg/L TRO for 3 d.

Discussion

In this study, we examined changes in the stress response and antioxidant capacity of olive flounder after exposure to TRO, an indicator of toxicity used in ozone-mediated water sterilization in aquaculture. Three concentrations of TRO (20, 40, and 60 μg/L) were used. Olive flounder survival in the 0 and 20 μg/L TRO groups was 92% on day 14; however, in the 40 and 60 μg/L TRO groups, all individuals died by day 14 and day 7, respectively. Similarly, Kim, Oh, and Jung (1999) investigated the survival rate of olive flounder exposed to seawater treated with different concentrations of TRO (0–47 μg/L) for 96 h, reporting a gradual decrease in survival with increases in TRO and 0% survival at 47 μg/L TRO. Based on this and findings of previous studies, it appears that concentrations of TRO up to 20 μg/L can be safely
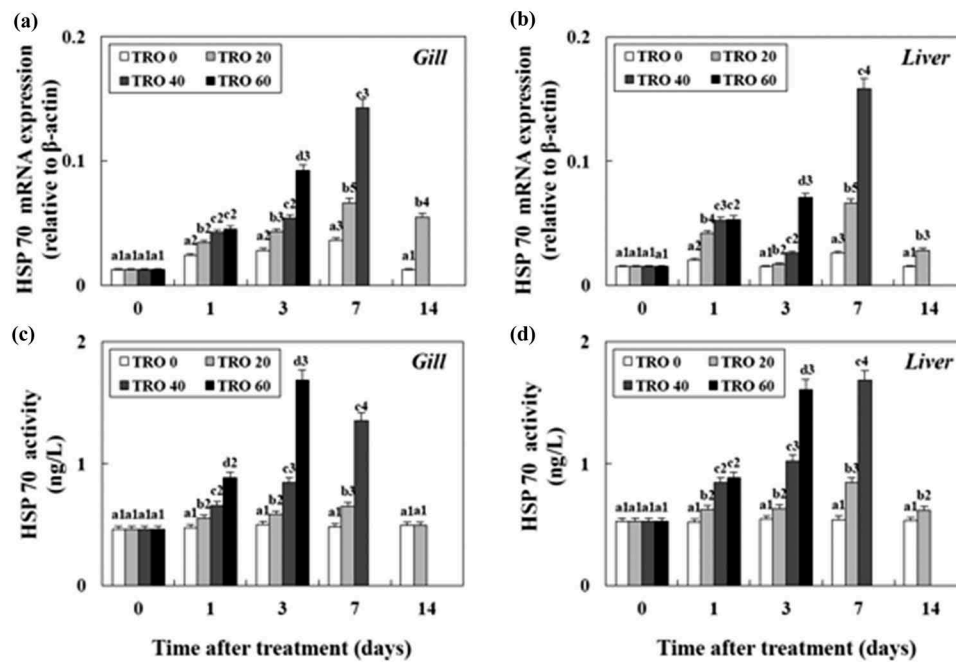
Figure 4. Expression of heat shock protein 70 (HSP 70) mRNA in the gills (a), HSP 70 mRNA in the liver (b), HSP 70 protein in the gills (c), and HSP 70 protein in the liver (d) of olive flounder in control and experimental groups exposed to total residual oxidant (TRO) at different concentrations (20, 40, and 60 µg/L) for 14 days. Different numbers indicate significant differences among the different exposure periods at the same TRO concentration ($P < .05$). The lowercase letters indicate significant differences among the different TRO concentrations for the same exposure periods ($P < .05$). All the values are means ± SE ($n = 5$).

Figure 5. Plasma H_2O_2 in olive flounder in control and experimental groups exposed to total residual oxidant (TRO) at different concentrations (20, 40, and 60 µg/L) for 14 days. Different numbers indicate significant differences among the different exposure periods at the same TRO concentration ($P < .05$). The lowercase letters indicate significant differences among the different TRO concentrations for the same exposure periods ($P < .05$). All the values are means ± SE ($n = 5$).
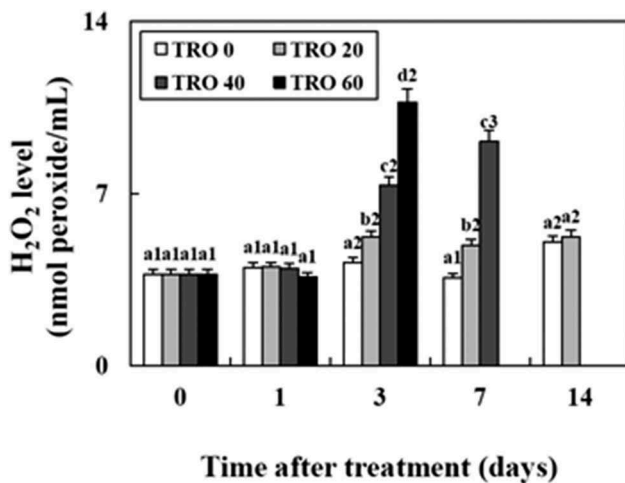
used for olive flounder culture without causing significant increases in fish mortality.

In this experiment, expression of mRNA for the antioxidant enzymes SOD and CAT in the gill and liver tissues of olive flounder significantly increased in relation to TRO concentration and exposure time. Interestingly, SOD and CAT mRNA expression in the gills decreased to control levels on days 7 and 14, respectively, in the 20 µg/L TRO group. A previous study reported that short-term exposure to 5.2 µg/L of ozone-treated freshwater in rainbow trout *Oncorhynchus mykiss* culture significantly increased the activities of SOD and CAT in the gills and liver, but that enzyme activities were decreased to control levels after 48 h (Ritola et al. 2002). It has also been reported that TRO in freshwater caused by ozone damages the gill tissue of rainbow trout (Wedemeyer, Nelson, and Yasutake 1979). Similar to previous studies, TRO caused damage to the gill and liver tissues and induced oxidative stress in olive flounder in this study, as indicated by the increased expression of mRNA for the antioxidant defense enzymes, SOD and CAT. The return of SOD and CAT transcript abundance in the gill and liver to control levels after 3 days suggests an adaptation to TRO exposure in olive flounder over time.

In this study, HSP 70 mRNA expression and activity in the gills and liver of olive flounder were significantly increased by TRO in relation to exposure time and concentration. However, in the 20 µg/L TRO group, HSP 70 mRNA expression and activity in the gills and
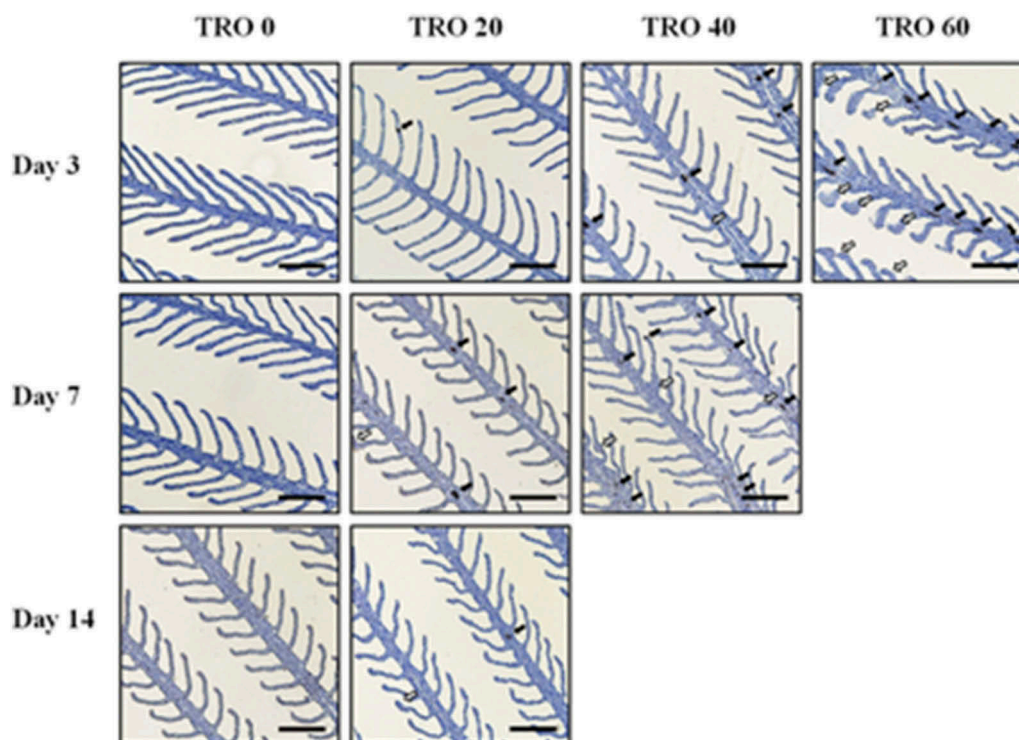
Figure 6. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay using gill sections from olive flounder in control and experimental groups exposed to total residual oxidant (TRO; 20, 40, and 60 μg/L) for 3, 7, and 14 days. Dark-colored cells indicated by black arrows are apoptotic. White arrows indicate gill deformation. Scale bars = 100 μm.

liver on day 14 were lower than those on day 7, and HSP 70 activity in the gill was not different from that in the controls. Similarly, Reiser et al. (2011) exposed turbot *Psetta maxima* to 0.15 mg/L ozone-treated seawater and reported significantly higher HSP 70 mRNA expression on days 1 and 7 that recovered to control levels by day 21. The transient increase in HSP 70 in the 20 μg/L TRO group suggests adaptation to TRO over time in olive flounder, similar to that observed for SOD and CAT.

Plasma H_2O_2 concentration in olive flounder significantly increased with increasing TRO concentration and exposure time within TRO exposure groups in the current study. Fukunaga et al. (1999) exposed rainbow trout to 20 mg/L ozone-treated freshwater for a short period and observed a significant increase in red blood cell H_2O_2 levels. These authors also reported that ozone exposure resulted in membrane damage that induced the production of H_2O_2. Therefore, it is suggested that exposure of olive flounder to TRO generated by ozone injection in seawater causes oxidative stress and cellular damage through the generation of ROS, including H_2O_2. Meanwhile, H_2O_2 concentrations on day 14 were significantly higher in the 20 μg/L TRO groups relative to the control group at other sampling times, but it is assumed that dissolved ozone temporarily increases the H_2O_2 in water (Glaze 1986).

Increases in apoptotic (TUNEL-positive) cells and hypertrophy and deformation of gill tissues were observed after exposure to 40 μg/L TRO for 7 days and 60 μg/L TRO for 3 days; these concentration and exposure time combinations also coincided with the highest rates of mortality. In a previous study, Paller and Heidinger (1980) reported that the gill tissue of bluegill *Lepomis macrochirus* was damaged by exposure to ozone-treated freshwater at 90 μg/L for 6 h. Jung et al. (2018) exposed olive flounder to 25 μg/L TRO-treated seawater for 26 days, reporting hypertrophy and necrosis in the epithelial cells of gills. In general, it is known that cellular damage caused by external stressors can induce apoptosis (Häcker 2000). Therefore, in this study, it is suggested that apoptosis was induced by the damage inflicted to gill tissues as a result of direct exposure to TRO.

Conclusion

In summary, the results of this study suggest that 20 μg/L TRO does not significantly affect the survival of olive flounder over 14 days. At higher concentrations (40 and 60 μg/L), TRO not only causes oxidative stress, but also induces deformation and apoptosis of gill tissue, which together negatively affect survival. A concentration of 20 μg/L TRO may be suitable for water treatment in

recirculating aquaculture systems containing olive flounder. Further studies on the concentrations of TRO that cause physiological stress in other important aquaculture fish species, and long-term exposure to diverse concentrations of TRO, are needed.

References

Basha, S. P., and U. A. Rani. 2003. "Cadmium-induced Antioxidant Defense Mechanism in Freshwater Teleost *Oreochromis Mossambicus* (tilapia)." *Ecotoxicology and Environmental Safety* 56 (2):218–21. doi:10.1016/S0147-6513(03)00028-9.

Buchan, K. A., D. J. Martin-Robichaud, and T. J. Benfey. 2005. "Measurement of Dissolved Ozone in Sea Water: A Comparison of Methods." *Aquacultural Engineering* 33 (3):225–31. doi:10.1016/j.aquaeng.2005.02.002.

Fukunaga, K., N. Nakazono, T. Suzuki, and K. Takama. 1999. "Mechanism of Oxidative Damage to Fish Red Blood Cells by Ozone." *IUBMB Life* 48 (6):631–34. doi:10.1080/713803566.

Fukunaga, K., T. Suzuki, M. Arita, S. Suzuki, A. Hara, K. Yamauchi, and K. Takama. 1992a. "Acute Toxicity of Ozone against Morphology of Gill and Erythrocytes of Japanese Charr *Salvelinus Leucomaenis*." *Comparative Biochemistry and Physiology Part C Comparative Pharmacology and Toxicology* 101 (2):331–36. doi:10.1016/0742-8413(92)90283-D.

Fukunaga, K., T. Suzuki, A. Hara, and K. Takama. 1992b. "Effect of Ozone on the Activities of Reactive Oxygen Scavenging Enzymes in RBC of Ozone Exposed Japanese Charr *Salvelinus Leucomaenis*." *Free Radical Research Communications* 17 (5):327–33. doi:10.3109/10715769209079525.

Glaze, W. H. 1986. "Reaction Products of Ozone: A Review." *Environmental Health Perspectives* 69: 151–57. doi: 10.1289/ehp.8669151

Haag, W. R., and J. Hoigné. 1984. "Kinetics and Products of the Reactions of Ozone with Various Forms of Chlorine and Bromine in Water." *Ozone: Science & Engineering* 6 (2):103–14. doi:10.1080/01919518408551009.

Häcker, G. 2000. "The Morphology of Apoptosis." *Cell and Tissue Research* 301 (1):5–17. doi:10.1007/s004410000193.

Hansen, B. H., S. Rømma, Ø. A. Garmo, P. A. Olsvik, and R. A. Anderson. 2006. "Antioxidative Stress Proteins and Their Gene Expression in Brown Trout *Salmo Trutta* from Three Rivers with Different Heavy Metal Levels."

Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 143 (3):263–74. doi:10.1016/j.cbpc.2006.02.010.

Hofmann, R. 2000. "Using ammonia to inhibit bromate formation during ozonation." phD diss., University of Toronto.

Jones, A. C., R. W. Gensemer, W. A. Stubblefield, E. Van Genderen, G. M. Dethloff, and W. J. Cooper. 2006. "Toxicity of Ozonated Seawater to Marine Organisms." *Environmental Toxicology and Chemistry* 25 (10):2683–91. doi:10.1897/05-535R.1.

Jung, S. M., W. G. Park, J. H. Park, J. W. Kim, and P. K. Kim. 2018. "growth Performance and Physiological Changes of Olive Flounder *Paralichthys Olivaceus* by Concentration of Ozone Produced Oxidants in semi-RAS. [In Korean]." *Korean Journal of Fisheries and Aquatic Science* 51 (6):688–96.

Kashiwagi, A., K. Kashiwagi, M. Takase, H. Hanada, and M. Nakamura. 1997. "Comparison of Catalase in Diploid and Haploid *Ranarugosa* Using Heat and Chemical Inactivation Techniques." *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 118 (3):499–503. doi:10.1016/S0305-0491(97)00216-2.

Kim, H. Y., M. J. Oh, and S. J. Jung. 1999. "acute Toxicity of Ozone on Survival and Physiological Conditions of Olive Flounder, *Paralichthys olivaceus*.[In Korean]." *Journal of Fish Pathology* 12 (1):32–41.

Kregel, K. C. 2002. "Heat Shock Proteins: Modifying Factors in Physiological Stress Responses and Acquired Thermotolerance." *Journal of Applied Physiology* 92 (5):2177–86. doi:10.1152/japplphysiol.01267.2001.

Liltved, H., C. Vogelsang, I. Modahl, and B. H. Dannevig. 2006. "High Resistance of Fish Pathogenic Viruses to UV Irradiation and Ozonated Seawater." *Aquacultural Engineering* 34 (2):72–82. doi:10.1016/j.aquaeng.2005.05.002.

McFarland, V. A., L. S. Inouye, C. H. Lutz, A. S. Jarvis, J. U. Clarke, and D. D. McCant. 1999. "Biomarkers of Oxidative Stress and Genotoxicity in Livers of Field-collected Brown Bullhead, *Ameiurus Nebulosus*." *Archives of Environmental Contamination and Toxicology* 37 (2):236–41. doi:10.1007/s002449900510.

Nordberg, J, and E. S. Arnér. 2001. "Reactive Oxygen Species, Antioxidants, and the Mammalian Thioredoxin System." *Free Radical Biology and Medicine* 31 (11):1287–312. doi:10.1016/S0891-5849(01)00724-9.

Oh, M. J., H. Y. Kim, and H. S. Cho. 1999. "Disinfection of Culture Water Supply by Ozonization I. Susceptibility of Some Fish-Pathogenic Bacteria Isolated from Cultured Marine Fish. [In Korean]]." *Journal of Fish Pathology* 12 (1):42–48.

Paller, M. H., and R. C. Heidinger. 1980. "Mechanisms of Delayed Ozone Toxicity to Bluegill *Lepomis Machrochirus* Rafinesque." *Environmental Pollution Series A, Ecological and Biological* 22 (3):229–39. doi:10.1016/0143-1471(80)90017-3.

Park, J., P. K. Kim, T. Lim, and H. V. Daniels. 2013. "Ozonaton in Seawater Recirculating Systems for Black Seabream *Acanthopagrus Schlegelii* (bleeker): Effects on Solids, Bacteria, Water Clarity, and Color." *Aquacultural Engineering* 55: 1–8. doi: 10.1016/j.aquaeng.2013.01.002

Perrins, J. C., W. J. Cooper, J. H. Van Leeuwen, and R. P. Herwig. 2006. "Ozonation of Seawater from

Different Locations: Formation and Decay of Total Residual Oxidant-implications for Ballast Water Treatment." *Marine Pollution Bulletin* 52 (9):1023–33. doi:10.1016/j.marpolbul.2006.01.007.

Reiser, S., S. Wuertz, J. P. Schroeder, W. Kloas, and R. Hanel. 2011. "Risks of Seawater Ozonation in Recirculation aquaculture-Effects of Oxidative Stress on Animal Welfare of Juvenile Turbot *Psetta Maxima*." *Aquatic Toxicology* 105 (3–4):508–17. doi:10.1016/j.aquatox.2011.08.004.

Ritola, O., D. R. Livingstone, L. D. Peters, and P. Lindstrom-Seppa. 2002. "Antioxidant Processes are Affected in Juvenile Rainbow Trout *Oncorhynchus Mykiss* Exposed to Ozone and Oxygen-supersaturated Water." *Aquaculture* 210: 1–19. doi: 10.1016/S0044-8486(01)00823-7

Roch, P. 1999. "Defense Mechanisms and Disease Prevention in Farmed Marine Invertebrate." *Aquaculture* 172 (1--2):125–45. doi:10.1016/S0044-8486(98)00439-6.

Schroeder, J. P., P. L. Croot, B. Von Dewitz, U Waller, and R Hanel. 2011. "Potential and Limitations of Ozone for the Removal of Ammonia, Nitrite, and Yellow Substances in Marine Recirculating Aquaculture Systems." *Aquacultural Engineering* 45: 35–41. doi: 10.1016/j.aquaeng.2011.06.001

Sharrer, M. J., and S. T. Summerfelt. 2007. "Ozonation Followed by Ultraviolet Irradiation Provides Effective Bacteria Inactivation in a Freshwater Recirculating System." *Aquacultural Engineering* 37 (2):180–91. doi:10.1016/j.aquaeng.2007.05.001.

Slaninova, A., M. Smutna, H. Modra, and Z. Svobodova. 2009. "Reviews; Oxidative Stress in Fish Induced by Pesticides." *Neuroendocrinology Letters* 30 (1):2–12.

Tango, M. S., and G. A. Gagnon. 2003. "Impact of Ozonation on Water Quality in Marine Recirculation." *Aquacultural Engineering* 29 (3–4):125–37. doi:10.1016/S0144-8609(03)00061-X.

Wedemeyer, G. A., N. C. Nelson, and W. T. Yasutake. 1979. "Physiological and Biochemical Aspects of Ozone Toxicity to Rainbow Trout *Salmo Gairdneri*." *Journal of the Fisheries Research Board of Canada* 36 (6):605–14. doi:10.1139/f79-088.

Wold, P. A., A. B. Holan, G. Qie, K. Attramadal, I. Bakke, O. Vadstein, and T. Leiknes. 2014. "Effects of Membrane Filtration on Bacterial Number and Microbial Diversity in Marine Recirculating Aquaculture System (RAS) for Atlantic Cod *Gadus Morhua* L. Production." *Aquaculture* 422: 69–77. doi: 10.1016/j.aquaculture.2013.11.019

Yamashita, M., T. Yabu, and N. Ojima. 2010. "Stress Protein HSP 70 in Fish." *Aqua-BioScience Monographs* 3 (4):111–41. doi:10.5047/absm.2010.00304.0111.

Yoshimizu, M. 1995. *Diseases in Asian Aquaculture II: Disinfectant Effect of Oxidant Produced by Ozonization of Sea Water on Fish Pathogenic Viruses, Bacteria, and Ciliata.* Manila: Asian Fisheries Society.