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Application of Ozone Disinfection To Remove *Enterococcus seriolicida*, *Pasteurella piscicida*, and *Vibrio anguillarum* from Seawater

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Survival of bacterial fish pathogens, including *Enterococcus seriolicida*, *Vibrio anguillarum*, and *Pasteurella piscicida*, in ozonated seawater was determined in a batch system. Bacterial counts of all fish pathogens decreased at more than 0.040 to 0.060 mg of total residual oxidants (TROs) per liter, whereas no decrease in viable counts was observed at less than 0.018 to 0.028 mg of TROs per liter. The 99% inactivation point was achieved at concentrations of 0.111 mg/liter for *E. seriolicida*, 0.063 mg/liter for *P. piscicida*, and 0.064 mg/liter for *V. anguillarum* within 1 min. Moreover, the mean 99 and 99.9% killing concentration-contact time ($C \cdot t$) products were 0.123 and 0.186 mg · min/liter for *E. seriolicida*, 0.056 and 0.084 mg · min/liter for *P. piscicida*, and 0.081 and 0.123 mg · min/liter for *V. anguillarum*, respectively. However, the mean 99 and 99.9% $C \cdot t$ products for the mixed population in coastal seawater were 0.200 and 0.621 mg · min/liter. These results strongly suggest that ozone treatment at more than 1.0 mg of TROs per liter for several minutes is able to disinfect seawater for mariculture efficiently.

Successful use of ozone as a disinfectant for freshwater aquaculture systems has been recognized (2, 9). Recently, ozonation of seawater for marine fish farms and aquaria has also received considerable attention (4, 12, 14). However, ozone reacts with bromide (Br^-) and chloride (Cl^-) ions to form oxidants in seawater, so it has been suggested that the bactericidal activity of ozonated seawater may differ from that of ozonated freshwater (3, 20). Therefore, the objective of the present study was to determine the relationship between total residual oxidant (TRO) doses and the contact times needed to inactivate three bacterial fish pathogens that often occur in marine fish farms (8, 10).

The bacterial fish pathogens used in this study included *Enterococcus seriolicida* YT-3 (= ATCC 49156), *Vibrio anguillarum* ATCC 19264, and *Pasteurella piscicida* K-III, which had been isolated from a cultured specimen of yellowtail (*Seriola quinqueradiata*) with pseudotuberculosis. These bacteria were grown in brain heart infusion broth (Difco, Detroit, Mich.) supplemented with 0.5% NaCl for 24 h at 25°C, harvested by centrifugation at 5,000 × *g*, washed three times, and suspended in the sterile seawater at a concentration of 10^9 CFU/ml.

Ozone was generated from oxygen gas by an OZSD-1000 ozonator (Ebara Jitsugyo, Tokyo, Japan) and dispensed into 1,000 ml of seawater in an air-purging glass bottle. The seawater, collected from an unpolluted area of Shimoda, Shizuoka Prefecture, was filtered through a 0.22-μm-pore-size membrane filter (GS; Millipore Corp., Bedford, Mass.). The chemical profile of the seawater used was as follows: salinity, 34.4‰; pH 7.95; NH_3-N, 0.00 ppm; NO_2-N, 0.00 ppm; NO_3-N, 0.08 ppm; COD, 0.5 ppm; Br^-, 81.5 ppm. TROs in seawater were measured spectrophotometrically by a modification (15) of Shechter's method (11). Ozonated seawater was mixed with an equal volume of 2% potassium iodide in seawater and adjusted to pH 7.0 in a dark bottle. The potassium iodide was oxidized by TROs in the ozonated seawater within 10 min at 25°C to liberate triiodide ion (I_3^-), which was then measured with a UV-260 spectrophotometer (Shimadzu, Kyoto, Japan) at 352 nm by using a cuvette with an either 10- or 50-mm light path. One milliliter of a standard iodine solution (containing 6.4 g of potassium iodide and 1.2692 g of iodine in 1,000 ml of distilled water) was calculated as 240 μg of TROs. TRO contents of 0.005 to 2.0 mg/liter in seawater could be determined by this procedure.

At a final concentration of 10^6 CFU/ml, each bacterial pathogen was inoculated into 50 ml of the seawater containing 0.018 to 0.992 mg of TROs per liter in a dark glass bottle and mixed thoroughly by a magnetic stirrer at 25°C. Samples were taken at various times after inoculation, and excess residual oxidants of the sample were immediately quenched by mixing with brain heart infusion broth (1). The sample was serially diluted, plated on brain heart infusion agar in triplicate, and then incubated at 25°C for 72 h. Percent survival rates of each bacterial pathogen were calculated from the number of colonies grown. The same experiment was undertaken twice for each bacterial pathogen.

The viable counts of all fish pathogens decreased at more than 0.040 to 0.060 mg of TROs per liter, and killing activity accelerated with increasing TRO concentrations (Fig. 1 to 3). Complete bacterial inactivation was observed within 4 min at more than 0.096 mg of TROs per liter. In contrast to this, no decrease in bacterial counts was observed at concentrations of less than 0.018 to 0.028 mg of TROs per liter, suggesting that there are threshold values for the fish pathogens. A close relationship was observed between the TRO concentration and the contact time necessary for bacterial inactivation. To ascertain this relationship, the Chick-Watson equation (6, 21) was employed, as follows: $K = C^n \cdot t$, where K is a constant, C is the concentration (milligrams per liter) of TRO, t is the contact time (minutes) for a fixed percentage of bacterial inactivation, and n is the coefficient of dilution. Data analyses were conducted with the SYSTAT statistical program (SYSTAT Inc., Evanston, Ill.) operating on a

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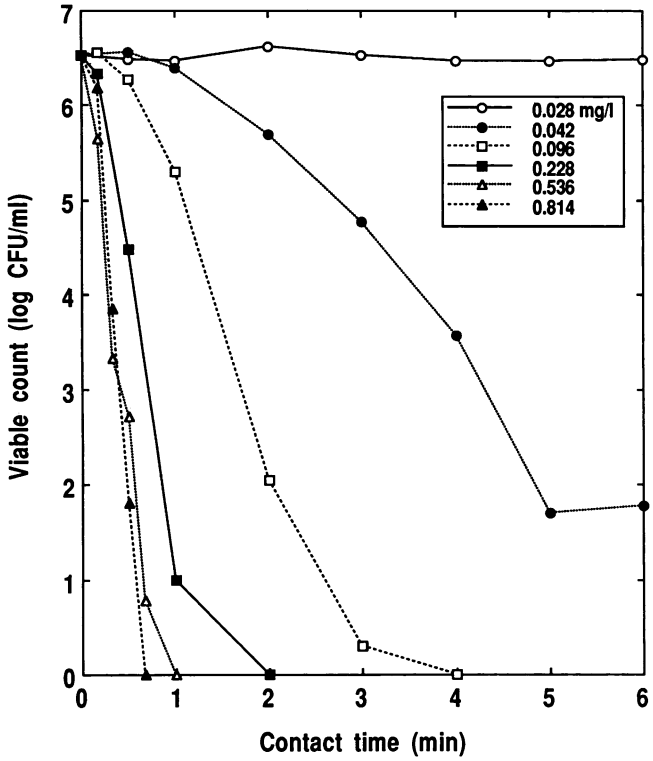

FIG. 1. Effect of TRO concentration on inactivation of *E. seriolicida* in seawater.

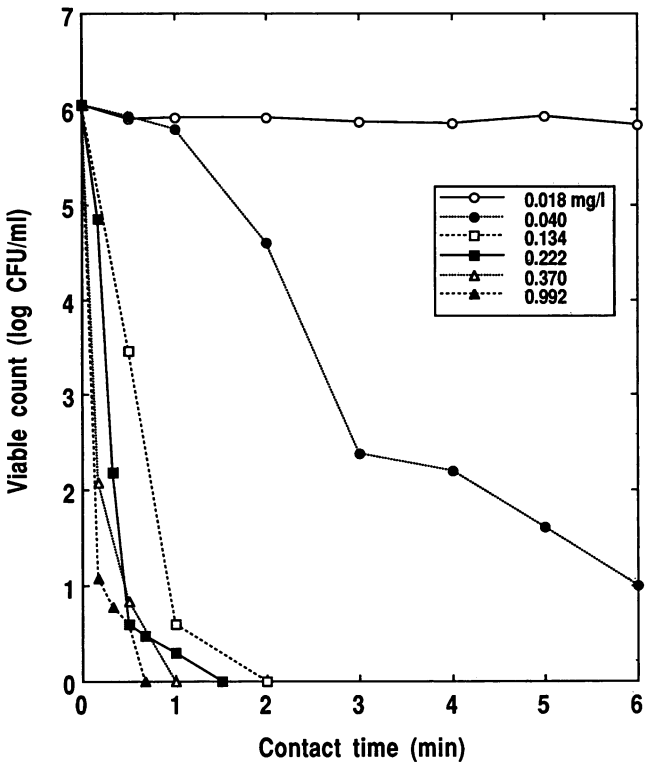

FIG. 2. Effect of TRO concentration on inactivation of *P. piscicida* in seawater.

Macintosh IIsi personal computer (Apple Computer, Cupertino, Calif.). The general linear-model procedure was used to fit a regression equation to predict inactivation time and to perform the analysis of variance. The resultant n values were 0.87 to 0.88 for *E. seriolicida*, 1.13 to 1.14 for *P. piscicida*, and 0.85 to 0.87 for *V. anguillarum* at levels of 99 to 99.9999% inactivation (Table 1). The n value (of greater than 1) of *P. piscicida*, unlike those of the other two pathogens, indicates that the TRO concentration is more important than the contact time for killing of this organism, although similar phenomena were observed with other disinfectants (21). Additionally, the mean 99 and 99.9% killing concentration-contact time ($C \cdot t$) products were 0.123 and 0.186 mg · min/liter for *E. seriolicida*, 0.056 and 0.084 mg · min/liter for *P. piscicida*, and 0.081 and 0.123 mg · min/liter for *V. anguillarum*. These results, along with the n values, show that *P. piscicida* is more sensitive to TROs than are the other two bacterial species, although the detailed mechanism responsible for the difference among fish pathogens remains to be elucidated. Furthermore, Katzenelson et al. (7) and Wedemyer and Nelson (18) revealed that the 99% killing $C \cdot t$ products for ozone were 0.02 mg · min/liter for *Escherichia coli*, 0.002 mg · min/liter for the enteric redmouth bacterium, and 0.006 mg · min/liter for *Aeromonas salmonicida*. These results suggest that the killing activity of TROs against bacterial pathogens is somewhat weak compared with that of ozone.

From the Chick-Watson equation it was determined that bacterial pathogens at a density of 10^6 CFU/ml could be diminished to 10^0 CFU/ml for a period of 1 min at concentrations of 0.393 mg of TROs per liter for *E. seriolicida*, 0.165 mg of TROs per liter for *P. piscicida*, and 0.229 mg of TROs per liter for *V. anguillarum* (Table 1). These results may show that ozone treatment at more than 0.5 to 1.0 mg of TROs per liter for 1 to several min is able to effectively eliminate fish-pathogenic bacteria from seawater because the bacterial density of 10^6 CFU/ml seems to be the upper level of heterotrophic bacteria in fish farming waters (13, 17).

In addition, to ascertain the effectiveness of ozone in the disinfection of natural seawater, aliquots (20 to 50 ml) of a water sample collected from a coastal region of Tokyo Bay were mixed with ozonated seawater to prepare TRO concentrations of 0 to 1.933 mg/liter and kept at 25°C for 3 min. After being quenched, the surviving bacteria in the seawater were grown at 25°C for 10 days on 1/20PYBG agar (16), which contains (per liter of 50% seawater) 0.5 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 0.25 g of Phytone peptone (BBL), 0.1 g of Lab-lemco powder (Oxoid, Hampshire, United Kingdom), 0.1 g of Bacto yeast extract (Difco), 0.1 g of glucose, and 15 g of agar, and adjusted to pH 7.5. As a result, the heterotrophic bacteria in the coastal seawater, with a population density of 2.5×10^5 CFU/ml, were completely inactivated within 3 min at more than 0.773 mg of TROs per liter, although disinfection was not complete at 0.357 mg of TROs per liter (Fig. 4). The mean 99 and 99.9% killing $C \cdot t$ products for the natural bacterial population (0.200 and 0.621 mg/liter, respectively) were much higher than those of the above-described pathogens (0.056 to 0.123 and 0.084 to 0.186 mg · min/liter, respectively). The difference in TRO resistance between the pure culture of specific pathogenic bacteria (Fig. 1 to 3) and the mixed bacterial population (Fig. 4) may be attributed to the likelihood that the microbial population in the coastal seawater comprises many species of bacteria with different degrees of TRO resistance. In other words, a disinfection

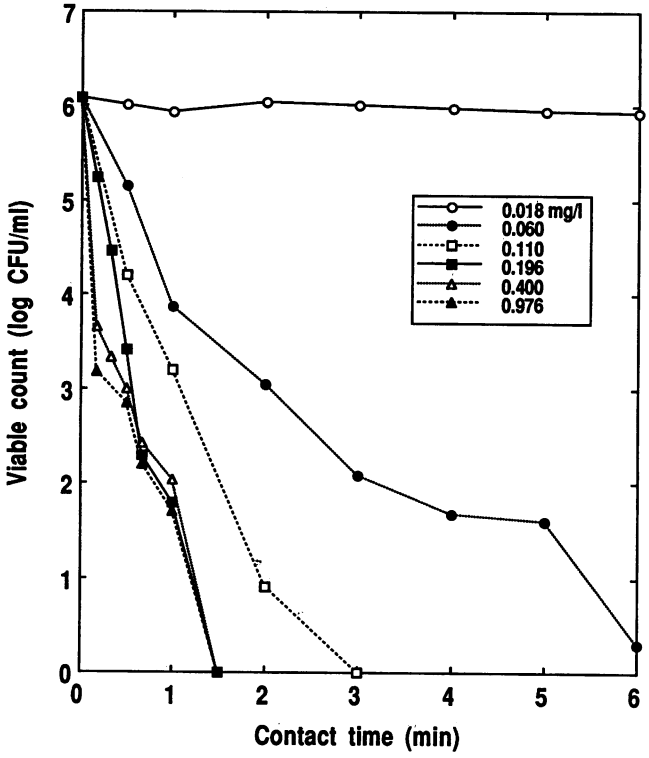

FIG. 3. Effect of TRO concentration on inactivation of *V. anguillarum* in seawater.

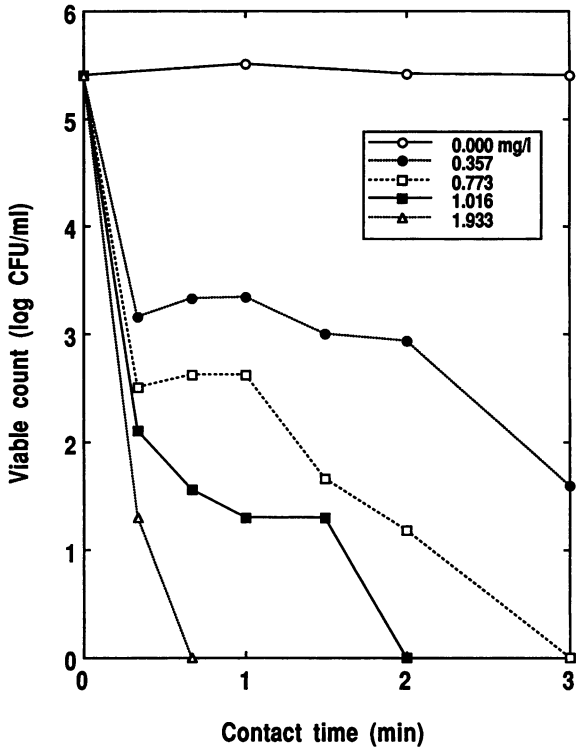

FIG. 4. Effect of TRO concentration on inactivation of heterotrophic bacteria in coastal seawater.

experiment with pure cultures is not the same as working with a mixed, heterotrophic population. Nevertheless, these results strongly suggest that ozone treatment at more than 1.0 mg of TROs per liter for several minutes is able to efficiently disinfect coastal seawater within which fish pathogens exist.

On the other hand, the toxicity of ozone has been recognized by many workers. Wedemeyer et al. (19) reported that the 96-h 50% lethal concentration was 9.3 mg/liter for rainbow trout (*Oncorhynchus mykiss*), and DeManche et al. (5) noted that trace concentrations of ozone were lethal to larval oysters (*Crassostrea gigas*). Although little information is available on levels of TROs that are lethal to fish and shellfish, it can be presumed that 1.0 mg of TROs per liter may be toxic to fish and shellfish. Therefore, TROs in ozonated seawater must be removed through activated charcoal before the seawater is transferred into fish aquaria and culture ponds (12).

TABLE 1. Chick-Watson parameters[a] for microbial inactivation by TROs

Bacterium	Inactivation (%)	*n*	*K*	*P* value[b]	Estimated TRO concn (mg/liter) at each % inactivation within 1 min
E. seriolicida	99	0.874	0.146	<0.005	0.111
	99.9	0.883	0.217	<0.005	0.177
	99.99	0.882	0.291	<0.005	0.246
	99.999	0.879	0.366	<0.005	0.319
	99.9999	0.877	0.441	<0.005	0.393
P. piscicida	99	1.14	0.0423	<0.01	0.063
	99.9	1.14	0.0637	<0.01	0.089
	99.99	1.14	0.0846	<0.01	0.115
	99.999	1.14	0.107	<0.01	0.140
	99.9999	1.13	0.129	<0.01	0.165
V. anguillarum	99	0.870	0.0921	<0.05	0.064
	99.9	0.853	0.144	<0.05	0.103
	99.99	0.850	0.189	<0.05	0.141
	99.999	0.850	0.233	<0.05	0.180
	99.9999	0.852	0.284	<0.05	0.229

[a] $K = C^n \cdot t$: C, TRO concentration (milligrams per liter); t, contact time (minutes); n, coefficient of dilution.
[b] Based on F-statistical analysis.

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