American Fisheries Society Symposium 61:000–000, 2008

Reductions in Bacterial Microorganisms by Filtration and Ozonation of the Surface Water Supply at the U.S. Fish and Wildlife Service Northeast Fishery Center

PATRICIA BARBASH
U.S. Fish and Wildlife Service, Northeast Fishery Center, Fish Health Center
Post Office Box 155, Lamar, Pennsylvania 16848, USA

JOHN FLETCHER AND ANTHONY CARTA
U.S. Fish and Wildlife Service, Northeast Fishery Center
Post Office Box 155, Lamar, Pennsylvania 16848, USA

STEVEN SUMMERFELT
Conservation Fund Freshwater Institute
Post Office Box 1889, Shepardstown, West Virginia 25443, USA

DUNCAN CREASER
U.S. Fish and Wildlife Service, Region 5 Contracting and General Services
300 Westgate Center Drive, Hadley, Massachusetts 01035, USA

ABSTRACT.—A water filtration and ozonation system was recently installed to treat creek water used to culture species of concern at the U.S. Fish and Wildlife Service's Northeast Fishery Center, Lamar National Fish Hatchery (NFH). Past experience with fish culture indicates that the following bacterial pathogens are endemic to the creek water supply: *Aeromonas salmonicida*, *Yersinia ruckeri*, *Flavobacterium columnaris*, and *Flavobacterium psychrophilia*. Water samples were collected from sites located before and after filtration and ozonation and examined for culturable bacteria. Variable operation of the filtration/ozonation system was used to examine (1) the effect of microscreen filtration (i.e., using drum filters containing 37-µm sieve panels) on ozone inactivation of bacterial microorganisms, (2) the effect of dissolved ozone contact times on inactivation of bacterial microorganisms, and (3) the effect of water quality fluctuations on the dissolved ozone demand measured during the course of these tests. Inactivation exceeded 98% for all bacteria when ozone *C*t* values were about 1.0 and reached 100% at 21.3, regardless of water quality parameters or implementation of microscreen filtration. These results indicate that the use of ozonation to treat surface water supplies used for fish culture facilities will effectively inactivate the majority of bacteria entering the system and will likely serve to prevent introduction of bacteria that can be pathogenic to fish.~~and Erie, which facilitated recovery of the burbot populations there. Although sea lampreys have been controlled in Lake Ontario, alewives are probably still too abundant to permit burbot recovery.~~

* Corresponding author: patricia_barbash@fws.gov

Introduction

Surface water use in flow-through aquaculture systems poses a risk of contamination from microorganisms that are known fish pathogens. Pathogen exposure of fish maintained under intensive culture conditions often leads to infection, clinical disease, and production losses. Methods used by fish culturists to control a disease outbreak often include chemotherapies, which are limited because of Food and Drug Administration licensing restrictions, costly, and are sometimes ineffective. The only effective technique for eliminating a pathogen from an aquaculture facility is to depopulate and disinfect the entire facility. Even then, potential for introduction of the same pathogens via the surface water source is equal to before, unless the water is decontaminated prior to its use. When valuable fish are raised at a facility that must rely upon surface water sources, prevention of pathogen introduction through water source treatment is necessary (Timmons et al. 2001).

An example of a fish culture facility having known fish pathogens in its surface water supply is the Intensive Culture (IC) Building at the U.S. Fish and Wildlife Service's Northeast Fishery Center in Lamar, Pennsylvania (NEFC). Until recently, the facility utilized untreated surface water from Fishing Creek for both its flow-through and recirculation systems. Contamination from fish pathogenic organisms endemic in this water supply has resulted in periodic losses of several species of fish cultured in the IC building. Infections caused by the following pathogens have been documented at the NEFC: parasites—*Saprolegnia*, *Trichodina*, *Epistylis*, *Trichophora*, *Chilodonella*, *Ichthyopthirius*, *Ichthyobodo,* *Scyphidia*, *Myxobolus cerebralis*, *Gyrodactilus*, *Dactylogyrus*; viruses—infectious pancreatic necrosis virus (IPNv), an unknown (possibly viral) agent causing cytopathic effect on cell lines from tissues of Atlantic sturgeon juveniles showing clinical signs of disease; bacteria—*Aeromonas salmonicida* (bacterial furunculosis), *Yersinia ruckeri* (enteric redmouth disease), *Flavobacterium columnare* (columnaris disease), and *Flavobacterium psychrophilum* (coldwater disease). Losses of production and research specimens due to these disease outbreaks necessitated disinfection of the water supply.

To eliminate fish pathogens, a water filtration and ozonation system was installed to treat the surface water entering the IC building. The system is described extensively by Summerfelt et al (2007, this volume). Previous literature on the elimination of bacterial pathogens through ozonation indicates that ozone concentration and contact time (C^*t) requirements ranged widely but were approximately 0.05–0.6 mg/L · min for *Aeromonas salmonicida*, 2.8 mg/L · min for *Flavobacterium*, and 0.005–1.0 mg/L · min for *Yersinia ruckeri* (Summerfelt et al. 2007). The system consists of two 60-μ sieve panel drum filter (microscreen) for filtration of the surface water and an ozonation system as described by Summerfelt et al. (2007). The objective of our study was to assess the ability of the treatment system to effectively inactivate bacterial organisms present in the surface water entering the culture facility. We investigated how the performance of the treatment system responded to variations in water flow, turbidity, temperature, ozone concentrations and contact times, and presence or absence of pathogenic bacteria.

Methods

Sample Sites

We collected water samples for our analysis from two locations within the ozone treatment system at the NEFC. A "preozonation" collection site was used to sample raw surface water after microscreen filtration with the 60-μm sieve mesh drum filter unit. This location was sampled with the microscreen filtration "online" as well as with it "offline" to determine the difference in ozone inactivation of bacteria with or without microscreen filtration. A "postozonation" sample site was located at a valve directly exiting the ozone contact tank. The valves were open and wa-

ter was allowed to run for several minutes prior to collection of samples.

Water Quality Sample Methods

Total flow was monitored using an electromagnetic flowmeter (Aquaflux 020K, Krohne, Inc., Peabody, Massachussetts). Water quality samples were tested immediately after collection for dissolved ozone concentration using a Palintest dissolved ozone test (Palintest, Ltd., Tyne & Wear, England). Dissolved ozone data were used to calculate a residual concentration in mg/L (C) for a given time in minutes (t) to produce the value C^*t as described by Summerfelt et al. (2007). Water samples were stored in bottles on ice and typically tested within 24–48 h for turbidity level. Turbidity was measured in nephelometric turbidity units (NTU) with a Hach Chemical Company Ratio/XR turbidimeter using APHA (1985) method 214 A.

Bacterial Enumeration from Water

Methods described by Ford (1994) were employed to quantify the number of heterotrophic bacteria in water samples and to select for the fish pathogens *A. salmonicida, Y. ruckeri*, *F. columnare*, and *F. psychrophilum*. Using 300–500 mL of water collected from each sample site, five replicate samples were obtained in sterile collection containers. Samples were kept cold until plate inoculation was completed, usually within 2 h of collection. Preozonation water samples were diluted in sterile distilled water at ratios of 1:10, 1:100, and 1:1000 (v/v) depending upon the turbidity of the sample. Postozonation water samples were not diluted. A total of 100 mL of each sample replicate (in diluted or undiluted form) was then filtered through a sterile membrane filter (0.45-µ pore) mounted on a filter apparatus that was surface disinfected with 70% isopropyl alcohol. After filtration, the filter was removed from the apparatus using sterile forceps, and placed face down on a Petri plate containing selective media. The filters were aseptically removed after 5–10 min of contact with the media.

Bacterial Isolation and Identification

Coomassie Brilliant Blue agar (CBB) was used for isolation and quantification of heterotrophic bacteria, as well as for presumptive isolation of *A. salmonicida*, as described by Cipriano and Bertolini (1988). Bacterial cell counts on CBB plates and colony isolations were conducted after approximately 48 h of incubation at 20°C. All CBB plates were rechecked for signs of delayed *A. salmonicida* growth after 72 h incubation. Tryptone–yeast-extract-gelatin (TYG) agar was utilized for isolation of yellow pigmenting bacterial species (Bullock et al. 1986). Colony counts of yellow pigmenting bacteria were conducted after 72–96 h of incubation at 15°C. All bacterial counts were reported as colony forming units of bacteria per milliliter (cfu/mL) of water sampled.

Bacterial identifications were performed on two of the sample dates to determine the dominant species of bacteria entering the ozone treatment system and to identify those surviving the treatment. Identifications were conducted on representative colonies from CBB plates. Single colonies were transferred to tryptic soy agar (TSA) plates for identification using the identification scheme of MacFadden (1985) and the API 20E system (BioMeriex). Gram-positive bacteria were incubated for several additional days and observed microscopically for development of spores. Most gram positive rod bacterial isolates produced spores and were given the general identification of *Bacillus* species.

Yellow pigmenting bacteria were enumerated, and then single colonies were transferred to TYG plates. Isolates were tested for growth on TSA and those that were able to grow within 24 h were identified as non- *F. columnaris* and *F. psychrophilum* yellow pigmenters (Thoesen 1994).

Results

Water flow was controlled so that variations approximated 700, 1,500, and 2,300 L/min. The ozone C^*t was also maintained at levels between 1.1 and 7.0, except for one instance

when the system was tested to determine its maximum output, which reached a level of 21.3 C^*t (Table 1). Throughout the test period, natural fluctuations in water quality were observed, which provided for a wide variety of parameters under which disinfection by filtration/ozonation could be tested. Water temperatures fluctuated from a low of 4.8–17°C (Table 1). Water turbidity (NTU) was recorded as low as 1.9 to the high turbidity level of 49.3 NTU measured during a rain storm event (Table 1).

Pathogenic bacterial organisms were not found in any of the water samples collected during this study. Heterotrophic bacteria ranged from 19.2 to 867.6 cfu/mL water and were inactivated by ozonation at rates exceeding 99.3%. Yellow pigmenting bacteria enumerated in the preozonated water samples ranged from 5.6 to 340.8 cfu/mL water, and inactivation rates were equal to or greater than 98.5% postozonation. Disinfection by ozonation produced 2–4 $\log_{(10)}$ reductions in surface water bacteria per milliliter of water (Table 1; Figure 1). Inactivation rates did not vary with flow (Figure 2), turbidity (Figure 3), water temperature (Figure 4), or ozone C^*t (Figure 5). Inactivation of bacteria exceeded 99% even when microscreen filtration was taken offline (Table 1). Complete (100%) water decontamination of both heterotrophic and yellow pigmenting bacteria occurred when the maximum ozone C^*t value of 21.3 was recorded during the study (Table 1; Figure 5).

There was a significant correlation between ozone C^*t and heterotrophic bacterial inactivation ((Pearson's correlation coefficient); $r = 0.64$, $P = 0.0462$), but no correlation was evident with turbidity level ($r = –0.30$, $P = 0.394$) and flow ($r = –0.25$, $P = 0.478$). Percent reduction of yellow pigmenting bacteria was also correlated with ozone C^*t ($r = 0.4862$, $P = 0.1542$). There was no significant effect of the drum filter on bacterial inactivation (heterotrophic: $t = 0.64$, $P = 0.54$; yellow pigmenters: $t = 0.03$, $P = 0.97$).

Pseudomonas fluorescens and other nonfermenting bacteria comprised the highest percent prevalence in water samples that were collected at the preozonation site with reduced prevalence postozonation, while *Bacillus* species increased in percent prevalence postozonation (Table 2). All bacteria species identified in water samples are considered common water/soil inhabitants, including *Aeromonas hydrophila*, *Bacillus* species, and nonfermenting bacteria such as *Psedumonas fluorescens* (Bergey et al. 1994).

Discussion

A significant correlation between bacterial inactivation with ozone C^*t was detected. Inactivation exceeded 98% for all bacteria when ozone C^*t values were about 1.0 and reached 100% at 21.3 (Figure 5). The total number of heterotrophic bacteria remaining in water samples collected from the post ozonation site averaged 2.2 cfu/mL (range 0.004–18.4), regardless of bacterial load in the prefiltered samples (Figure 1). Although a slight improvement in percent bacterial inactivation can be made with increasing ozone C^*t values, the treatment system passed less than one cfu/mL of live bacteria in most cases. At an ozone C^*t level of 5.9, disinfection was 99.9–100% complete, indicating that ozone concentrations probably do not need to reach 21.3 to achieve complete disinfection (Table 1).

Turbidity levels even as high as those reached during a rain storm event that increased the water turbidity to 49.3 NTU did not influence bacterial inactivation by ozonation (Figure 3). Varying flow (Figure 2) and temperature (Figure 4) had insignificant effects on the ability of ozonation to inactivate water bacteria as well. Fish culture facilities that utilize surface water are often prone to seasonal fluctuations of temperature and flow, as well as temporary fluctuations of temperature, flow, and turbidity caused by rain storm events. The data presented here indicates that a water treatment system utilizing ozonation as described in these proceedings (Summerfelt et al. 2007) will successfully inactivate the majority of bacteria entering the system throughout the seasonal and temporary fluctuations of water flow,

TABLE 1. Inactivation by ozonation of heterotrophic and yellow pigmenting bacteria under varying water quality conditions.

Sample ID	Water quality parameters				Heterotrophic bacteria effects of ozonation			Yellow pigmenting bacteria effects of ozonation		
	Water temp °C	Flow (L/min)	Turbidity (ntu)	Ozone (C^*t)	cfu/mL[a] before ozone	cfu/mL[a] after ozone	% bacteria inactivated	cfu/mL[a] before ozone	cfu/mL[a] after ozone	% bacteria inactivated
26Mam	4.8	788	1.9	5.9	160.0	0.14	99.9	302.2	0.06	100.0
26Mpm	5.0	2,237	3.1	2.7	150.6	1.07	99.3	93.8	0.30	99.7
27Mam	4.9	737	42.3	1.1	80.4	1.06	98.7	10.2	0.07	99.4
27Mpm[b]	5.1	737	49.3	1.2	867.6	1.60	9.8	340.8	0.13	100.0
16Aam	12.0	850	3.3	2.1	63.2	0.35	99.4	25.0	0.05	99.8
16Aam	14.0	1,465	3.2	1.5	58.8	0.60	99.0	20.6	0.08	99.6
16Apm	17.0	2,213	4.2	1.0	240.0	0.70	99.7	30.2	0.22	99.3
17Aam	13.5	768	2.8	21.3	181.4	0.00	100.0	42.8	0.00	100.0
17Aam[b]	13.8	757	2.5	2.6	133.8	0.20	99.9	22.2	0.06	99.7
17Apm[b]	17.0	1,469	7.5	7.0	19.2	0.09	99.5	5.6	0.04	99.3

[a] Cfu/mL indicates colony forming units of bacteria per milliliter of sample.

[b] Indicates microscreen filtration was offline for sample.

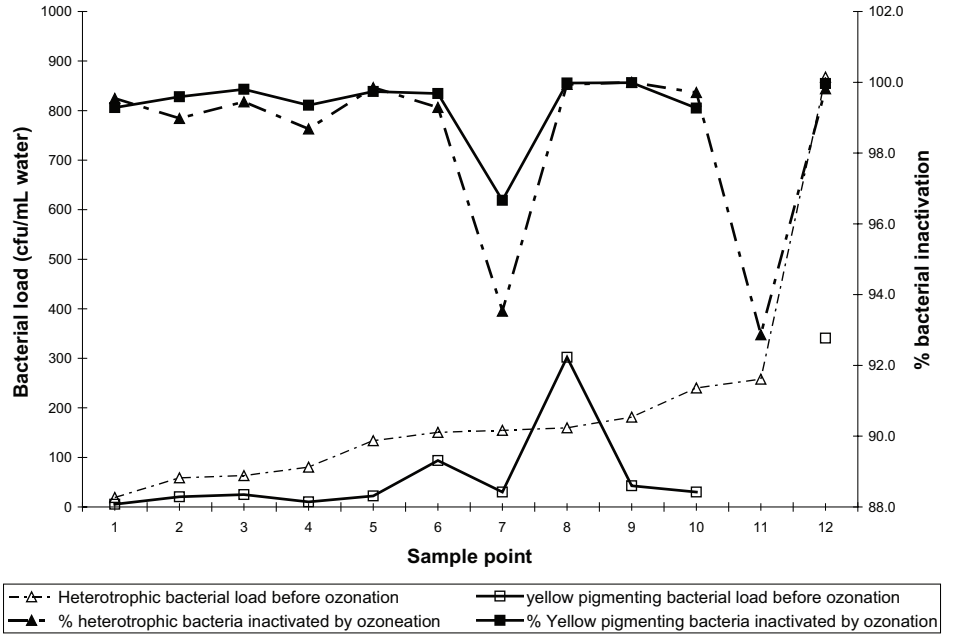

FIGURE 1. Bacterial loads of heterotrophic and yellow pigmenting bacteria at the preozonated sample site and percent of bacteria inactivated by ozonation.

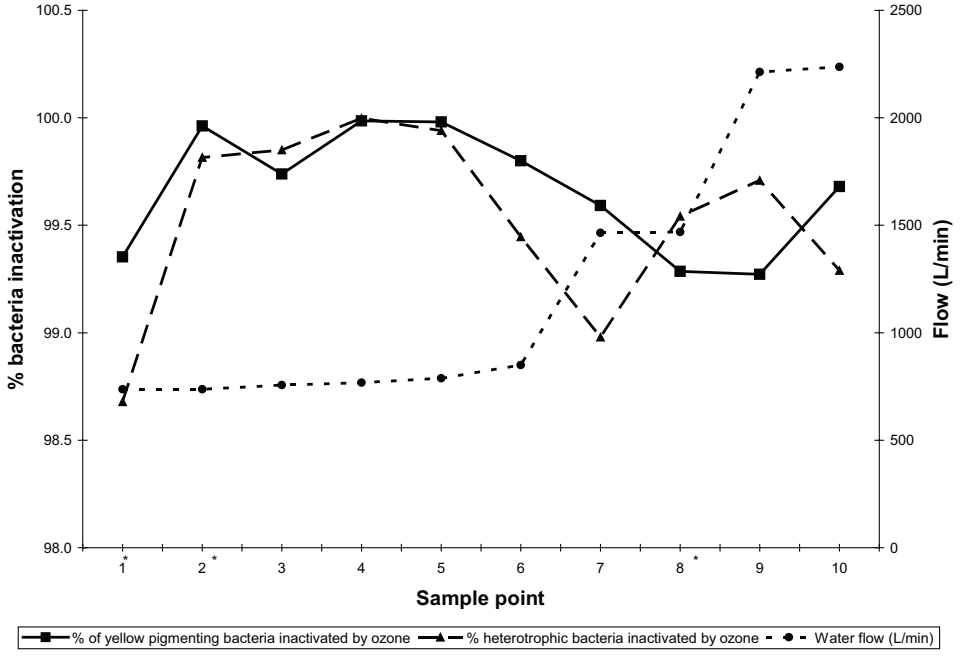

FIGURE 2. Percent bacterial inactivation of yellow pigmenting and heterotrophic bacteria in water after ozonation in relation to increasing water flow. Asterisk indicates sample point when microscreen filters were offline.

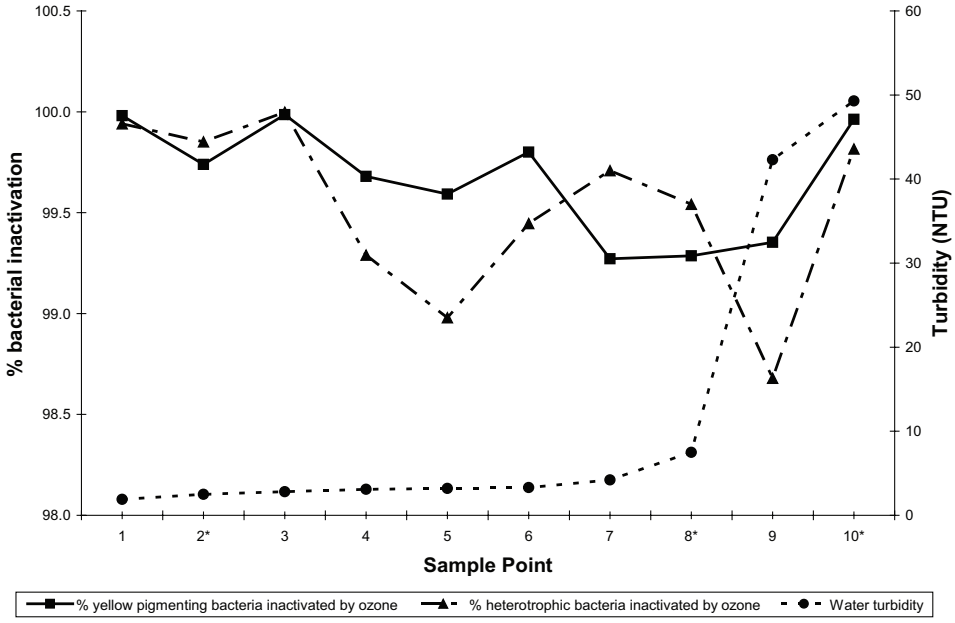

FIGURE 3. Percent bacterial inactivation of yellow pigmenting and heterotrophic bacteria in water after ozonation in relation to increasing water turbidity. Asterisk indicates sample point when microscreen filters were offline.

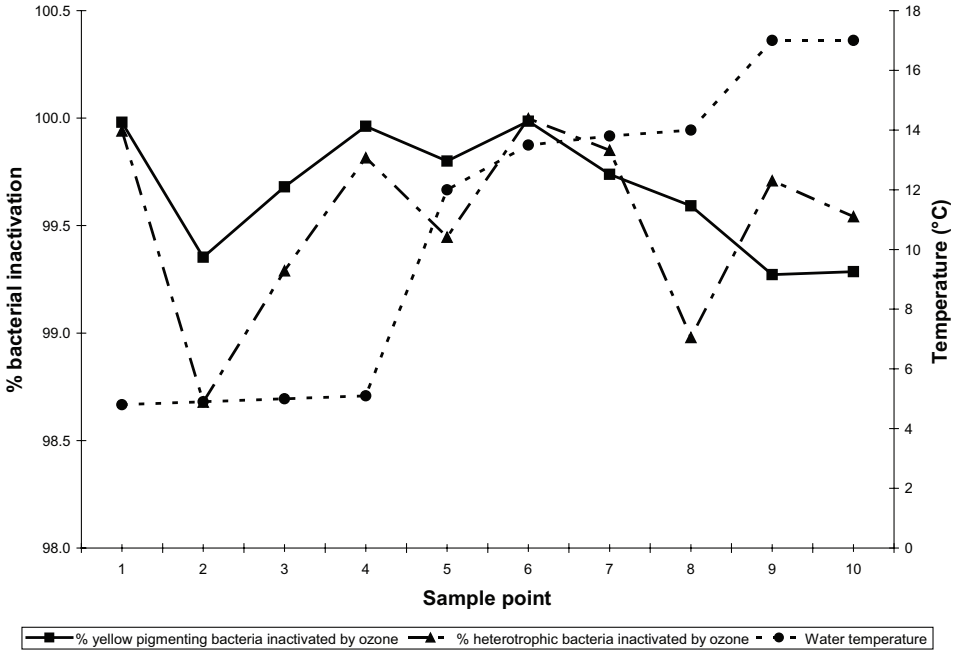

FIGURE 4. Percent bacterial inactivation of yellow pigmenting and heterotrophic bacteria in water after ozonation in relation to increasing water temperature.

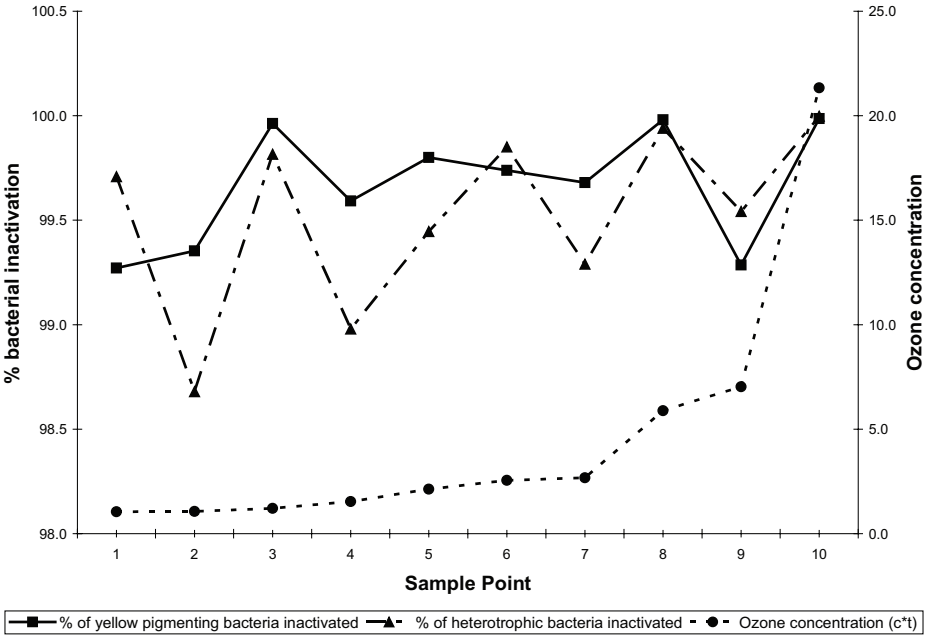

FIGURE 5. Percent bacterial inactivation of yellow pigmenting and heterotrophic bacteria in water after ozonation as measured by increasing C^*t. Asterisk indicates sample point when microscreen filters were offline.

temperature, and turbidity that are common to surface water supplies. Although drum filtration did not affect bacterial inactivation during our study, there is an inherent need to remove large particles of organic debris in order for the ozone system to function efficiently (Timmons et al. 2001). Large amounts of organic material will affect a treatment system's ability to maintain ozone residuals at levels required to inactivate microorganisms present in the water during a given contact time. Additionally, bacteria and other types of microorganisms embedded in large pieces of debris may be protect-

TABLE 2. Comparison of percent sample composition of each major bacterial group between preozonated and postozonated sample locations.

Sample point	Percent composition nonfermenting bacteria	Percent composition *Bacillus* sp.	Percent composition *Aermonas hydrophila*
	Preozonated sample site		
1	65.3	12.2	0.0
2	no data	no data	0.0
3	68.1	4.5	0.0
4	57.2	0.0	8.6
	Postozonated sample site		
1	17.7	58.8	0.0
2	9.5	35.0	0.0
3	29.4	11.8	29.4
4	25.3	47.4	5.9

ed from oxidation by the ozone, presenting a contamination hazard. Nevertheless, this study demonstrated that, even during storm events that resulted in high turbidity levels of the surface water, the NEFC ozone treatment system was able to maintain ozone residuals at levels required for efficient bacterial inactivation, even without microscreen filtration.

We did not detect any fish pathogenic bacterial species from our water samples, which made it impossible to test the effectiveness of the system on bacteria that are known to cause diseases in fishes. The bacterial species endemic to the creek water supply, as indicated in the introduction, are gram-negative, nonspore-forming rod bacteria. Previous authors have evaluated average ozone concentrations needed to achieve more than 99% disinfection of these bacterial species (Timmons etal, 2001), and all are at similar concentrations to those achieved by the system described herein. It is, therefore, reasonable to assume that the successful disinfection of the common water/soil bacteria isolated from the creek water would approximate the effective disinfection of pathogenic species as well. Our data suggest that the spore-forming bacteria *Bacillus* species was the predominant survivor of ozonation. However, the reduction in numbers of this species after ozonation did not exceed 0.1 cfu/mL postozonation (Table 2). These results provide evidence that spores of these bacteria may be able to withstand the ozonation to some extent, just as they are able to resist environmental adversities such as extreme heat and desiccation. On the other hand, it was not determined if the isolation of Bacillus at both the pre- and postozonation sample site may involve both live bacterial cells as well as vegetative spores, so survival of the spore form of *Bacillus* after ozonation is not conclusive. Further speciation of these isolates could have allowed for better speculation on this issue. There are very few published instances where spore-forming bacteria have been associated with infectious disease in fish (Cann and Taylor 1982, 1984; Oladosu et al. 1994), and infections of fish caused by these bacteria have never been diagnosed at the NEFC. Therefore, it is unlikely that the spore-formers we observed would pose a significant threat to fish in the reduced concentrations that we observed after the ozone treatment.

Fish culture facilities that utilize water supplies that are influenced by streams, rivers, surfacing springs, and lakes are at risk for contamination by microorganisms that are pathogenic to fish (Wedemeyer 2002). Unfortunately, treatment systems built to disinfect surface water may not always work under the extremes of water quality parameters such as flow, turbidity, and temperature. This study has established that, under specific design and operation parameters, ozonation will successfully disinfect a surface water source under a variety of water quality conditions, making ozonation an important tool for aquaculture. As concern grows about the introduction and spread of not only pathogenic organisms, but also aquatic invasive species that are detrimental to aquaculture systems, additional studies should focus on the ability of similar ozone systems to decontaminate water supplies of other microorganisms, such as zebra mussel veligers and aquatic plants.

Acknowledgments

We would like to acknowledge John Sweka, U.S. Fish and Wildlife Service, Northeast Fishery Center for providing the statistical analysis. John Coll, Mike Millard, and Jerre Mohler provided review of the manuscript.

References

Bergey, D. H., J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. 1994. Bergey's manual of determinative bacteriology, 9th edition. Williams & Wilkins, Antioch, California.

Bullock, G. L., T. C. Hsu, and E. B. Shotts, Jr. 1986. Columnaris disease of fishes. U.S. Fish and Wildlife Service, Fish Disease Leaflet 72, Washington, D.C.

Cann, D. C., and L. Y. Taylor. 1982. An outbreak of botulism in rainbow trout, *Salmo gairdneri* Richardson, farmed in Britain. Journal of Fish Diseases 5(5):393–399.

Cann, D. C., and L. Y. Taylor. 1984. An evaluation of residual contamination by *Clostridium botulinum* in a trout farm following an outbreak of botulism in the fish stock. Journal of Fish Diseases 7(5):391–396.

Cipriano, R. C., and J. B. Bertolini. 1988. Selection for virulence in the fish pathogen *Aeromonas salmonicida*, using Coomassie brilliant blue agar. Journal of Wildlife Diseases 24:672–678.

Ford, L. A. 1994. Detection of *Aeromonas salmonicida* from water using filtration method. Aquaculture 122(1):1–7.

MacFadden, J. F. 1985. Media for isolation-cultivation-identification-maintenance of medical bacteria, volume I. Williams and Wilkins, Baltimore, Maryland.

Oladosu, G. A., O. A. Ayinla, and M. O. Ajiboye. 1994. Isolation and pathogenicity of a *Bacillus* species associated with a septicaemic condition in some tropical freshwater fish species. Journal of Applied Ichthyology 10(1):69–72.

Thoesen, J. C. 1994. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 4th edition, version 1. American Fisheries Society, Fish Health Section, Bethesda, Maryland.

Timmons, M. B., J. M. Ebeling, F. W. Wheaton, S. T. Summerfelt, and B. J. Vince. 2001. Recirculating aquaculture systems. Cayuga Aqua Ventures, Ithaca, New York.

Wedemeyer, G. A., editor. 2002. Fish hatchery management, 2nd edition. American Fisheries Society, Bethesda, Maryland.