MICROBIOLOGICAL CONSIDERATIONS OF OZONE TREATMENT OF REUSE WATER IN FISH HATCHERIES

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PATRICIA JEANNE COLBERG

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TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

MICROBIOLOGICAL CONSIDERATIONS OF OZONE TREATMENT OF REUSE WATER IN FISH HATCHERIES

ABSTRACT

by

Patricia Jeanne Colberg

The effectiveness of ozone as an alternative to current ultraviolet disinfection of makeup water and its potential for treatment of recycled water in commercial reuse hatcheries was considered in this study. Comparative survival rates in water were established for four bacterial fish pathogens (Aeromonas salmonicida, Aeromonas liquefaciens, Pseudomonas fluorescens, and the causative agent of Hagerman Redmouth Disease) and spores of Bacillus polymyxa during batch and continuous flow ozonation in the laboratory. A mixed bacterial-protozoan population isolated from soil was also subjected to ozonation, with protozoan survival monitored by hemacytometer.

A specific microbial ozone demand was exerted in batch ozonation, while greater than 99% mortality of the fish pathogens was observed within 60 seconds contact during continuous flow exposure at all concentrations of ozone applied. Spores of B. polymyxa were resistant at a residual concentration of 1.0 mg/l. The oxidation of the combined bacterial-protozoan biomass closely approximated oxidation

rates established for the pure culture studies with no significant difference in relative survival rates between bacteria and protozoa. Increased ozone concentrations caused increased mortality rates, while elevated carbon levels did not appear to exert a preferential ozone demand when added to suspensions of test organisms. Results of the survival studies refute the "all-or-none" phenomenon reportedly associated with ozone treatment.

Oxidation of carbon, ammonia, and nitrite by ozone was rapid at low ozone concentrations with carbon and ammonia oxidation rates exhibiting pH-dependence. The oxidation capacity of ozone in water was greatest at elevated pH even though the measurable concentration of ozone in solution was low. It is believed that the pH effect is due to free radical formation subsequent to ozone decomposition in aqueous systems.

INTRODUCTION

The reduced availability of quality water for commercial aquaculture operations makes reuse hatchery design both economically and environmentally desirable. Yet recycling water in commercial fish culture is plagued by some of the problems characteristic of single-pass operations, in addition to some difficulties unique to the recirculation of rearing water.

Since chemically treated intake and recycle water is unsuitable for fish culture, recycle hatcheries subject incoming water to sand filtration and ultraviolet treatment, while recycled water is passed to biological nitrification beds. Ultraviolet radiation is often incapable of adequately penetrating a stream of inflowing water, while system maintenance and replacement costs are additional disadvantages. Biological treatment of reuse water is subject to fluctuations in efficiency.

The purpose of this study was to assess the effectiveness of ozone as an alternative technology to current hatchery treatment by examining some questions of microbiological importance.

REVIEW OF LITERATURE

The reduced availability of quality water for commercial aquaculture operations makes single-pass hatchery design obsolete. Water consumption by salmonid hatcheries alone exceeds 250 million cfs per day in the United States (Liao and Mayo, 1972). The need for recycling and reuse of water is an obvious one (Liao and Mayo, 1974).

Dworshak National Fish Hatchery in Idaho is the world's largest steelhead trout hatchery and served as a model in this research. For both economic and environmental reasons, this facility was designed as a reuse system, requiring approximately 90% recirculation of rearing water. In addition to traditional problems encountered in fish culture, there are difficulties unique to the reuse system.

In reuse hatcheries there is accumulation of both fish wastes and potentially toxic metabolites. Of particular concern is the production of ammonia, due to microbial degradation of waste products and unused food, and accumulation of nitrite, due to partial oxidation of ammonia. Even at sublethal concentrations, ammonia is capable of inhibiting fish growth, as well as producing an increased susceptibility to other unfavorable conditions of the culture system (Spotte, 1970). Nitrite levels in excess of

0.2 mg/l are related to anoxia and heavy mortalities in hatchery operations (Liao and Mayo, 1972).

Another problem characteristic of a recycle unit is depletion of dissolved oxygen due to microbial uptake during decomposition of accumulated organic matter in the system (Liao and Mayo, 1972). The result can be an insufficient supply of oxygen for fish respiration.

In addition to the disease potential of incoming water, disease problems may be intensified with the recirculation of untreated water. Recycled water may provide a suitable environment for culture of fish pathogens and serve as a reservoir for reinfection upon circulation.

Each of these problems has traditionally been addressed by modifying some part of the system. In an effort to avoid ammonia accumulation, recycled water is passed to biological nitrification beds where ammonia is oxidized to nitrate by the resident population of nitrifying bacteria. The problem of dissolved oxygen depletion is usually resolved by simple mechanical aeration. The omnipresent disease potential has been alleviated by sand filtration and ultraviolet sterilization of makeup water, with the application of chemotherapeutic agents to affected rearing ponds in specific disease outbreaks. However, recycled water in the Dworshak system is not subjected to any disinfection regime before returning to the raceways.

Biological oxidation of ammonia is subject to fluctuations in efficiency (Kawai et al., 1965; Johnson and Sieburth, 1974). Ultraviolet radiation is often incapable of adequately penetrating a stream of inflowing water (Sanders et al., 1972; Honn and Chavin, 1976). Recently, Wedemeyer and Nelson (1977) outlined the three phases of reuse hatchery practice in which disease control is essential: (1) disinfection of makeup water to permit use of sources currently unsuitable because of enzootic fish disease; (2) disinfection of recycled water; (3) treatment of discharge water from fish-holding facilities, preferably with a nonpersistent agent, to prevent release of exotic fish pathogens into receiving waters.

Modification in hatchery design or operation has resulted in a piecemeal approach to a problem which may well require a more comprehensive solution. One especially promising technique, which may possess such diverse application, involves the use of ozone for treatment of makeup and recycled water in a commercial reuse system.

Ozone is an allotropic form of oxygen with a half-life in water of approximately 15 min. With twice the oxidation potential of chlorine as hypochlorite ion, its oxidative capacity is exceeded only by fluorine, fluorine oxide, and monomolecular oxygen. Ozone is ten times more soluble in water than oxygen, but due to a low partial pressure, it is difficult to obtain greater than a few

milligrams per liter in water under normal conditions of temperature and pressure (Rosen, 1972).

At present, most commercial ozone generators operate on the corona discharge principle. Oxygen or compressed air is passed between electrodes, across which is maintained a high voltage differential. Commercial production of ozone by this technique is inherently inefficient. Only 10% of the energy supplied for the reaction is used to make ozone, with the remainder lost as light, sound, and heat (Rosen, 1973). The only process which may compete with corona discharge in the near future is chemonuclear generation, that is, nuclear power supplying the energy required for conversion of oxygen to ozone. Research at Brookhaven National Laboratories (Steinburg and Beller, 1970) indicates the feasibility of producing hundreds of tons of ozone per day by this process. Low level ozone production may also be obtained by electrolysis of perchloric acid or exposure of oxygen to ultraviolet radiation.

There is relatively little known concerning the chemistry of ozone in aqueous systems. Since ozone is an extremely unstable molecule in water, a number of workers (Weiss, 1935; Alder and Hill, 1950; Kilpatrick et al., 1956; Hewes and Davison, 1971; Hoigne and Bader, 1976) studying the reaction kinetics and dissociation of ozone in water have noted the formation of free radical species, particularly hydroxyl ions (OH^-). Peleg (1976) and Hoigne and

Bader (1976) have proposed that free radicals may, in fact, be responsible for the oxidation properties normally associated with the ozone molecule itself. These species are generally considered to be the same biologically active products of irradiation (McLean et al., 1973; Hoigne and Bader, 1976). Other reactive species such as superoxide (O_2^-), hydroperoxyl (HO_2^-), hydrotrioxyl (HO_3^-), and oxide (O^-) are also known to occur (McGrath and Norrish, 1958; Norrish and Wayne, 1965; DeMore, 1973; Gorbenko-Germanov and Kozlova, 1973, 1974), with reactions between decomposition products resulting in formation of other potentially oxidizing species (Thomas, 1965) or in neutralization (Czapski and Biehki, 1963). In alkaline solutions, the formation of O^- radicals becomes increasingly important (Hochanandal, 1962) and may react with oxygen to form the ozonide ion (O_3^-) (Adams, 1965). Since the half-life of ozone and such intermediate ions is extremely short, the only by-product of ozone treatment is molecular oxygen (Rosen, 1973).

The potential role of OH^- radicals in waste treatment systems was studied by Bishop and his co-workers (1968), indicating a strong similarity to normal ozone application. Hewes and Davison (1972) have shown that the rate of ozone oxidation of organic compounds in wastewaters is pH dependent. Reicherter and Sontheimer (1973) agreed that pH affects the rate of ozone purification of wastes and maintained that the radical mechanism is probably responsible

for oxidation in aqueous systems. Recently, Hoigne and Bader (1975) have shown experimentally that as the pH increased, the kinetics of ozonation of organic matter changed. In a study of the oxidation of ammonia by ozone, Singer and Zilli (1975) have concluded the reaction is first-order with respect to substrate concentration and is catalyzed by OH^- over the pH range of 7-9.

The use of ozone for disinfection of municipal drinking water antedates chlorination. As early as 1873, Fox reported destruction by ozone of bacteria and fungi. Almost 20 years passed before Ohlmuller (1892) demonstrated that ozone killed typhoid and cholera bacteria and inactivated anthrax spores. Van Ermengen (1895) continued studies of the bactericidal properties of ozone and inactivated numerous sporeformers, while Calmette and Roux (1899) reported sterilization of raw river water following exposure to ozone. The first commercial application of ozone in water treatment was operational in Nice, France, by 1906.

By 1930, approximately 200 municipal plants in Europe were employing ozone for treatment of water supplies. In 1937, de Lipkowski reported the virtual disappearance of typhoid fever and other waterborne infections in those cities where the water supply was treated with ozone. Lebout (1950) concluded that of 92 reported cases of typhoid and paratyphoid fevers occurring in Nice in

1948, none were attributable to transmission by ozonated drinking water.

The commercial use of ozone in the United States has been supplemental to chlorination, employed primarily for the elimination of odor, color, and taste in but a few public water supplies. Consequently, microbiological data are exiguous. Ferkinoff (1935) reported complete elimination of Escherichia coli from ozonated river water. Following installation of ozone generating equipment in the treatment facilities of Whiting, Indiana, Bartuska (1941) recorded an average reduction of 95-97% in the coliform index, in addition to alleviation of taste and odor problems in the industrially polluted water supply. Consoer (1941) reported similar results in Hobart, Indiana, and Denver, Pennsylvania. In 1949, Philadelphia, Pennsylvania, installed the world's largest ozone plant for removal of tastes, odor, and manganese from Schuykill River water (Bean, 1959). The plant recorded 95-99.9% reduction in coliform counts and an estimated 85-99.8% drop in total bacterial load.

Rosen (1973) has summarized the potential advantages of ozone in tertiary wastewater treatment, which include reduction of the Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), increased dissolved oxygen content, oxidation of secondary sludge organics, disruption of filamentous bacterial growth and colloidal structure,

disinfection of combined stormwater and overflows, and odor control. More detailed evaluations of ozone treatment of drinking water (Hann, 1956), wastewaters (Rosen, 1973), and chemical impurities persistent in some waters (O'Donovan, 1965), have been considered by other investigators. A comprehensive paper discussing some of the technical and economic aspects of ozone installation has been published by Majumdar and Sproul (1974).

Reliable data evaluating the effects of ozone on specific groups of microorganisms are scattered. One of the earliest reports considered the comparative effects of chlorine and ozone on cysts of Entamoeba histolytica, the causative agent of waterborne amoebiasis (Kessel et al., 1944). Results of the ozone application indicated a high cysticidal efficacy and prompted Newton and Jones (1949) to pursue a similar study to determine the effect of ozone on cysts of E. histolytica under various conditions. It was concluded that ozone in aqueous solution is also highly cysticidal for the organism with 96-99% destruction after 1 min, but that cysticidal action does not appear to be influenced by pH, temperature, or organic nitrogen levels. Ingram and Haines (1949) reported susceptibility of fungi and bacteria was decreased by at least one order of magnitude if the organisms were exposed to ozone in nutrient media. Giese and Christensen (1954) attempted to microscopically examine the effects of ozone on protozoa, bacteria, rotifers, and sea urchin larvae. They also

investigated changes in yeast respiration and adaptive enzyme formation following ozone exposure. Their observations led them to suggest that the mode of action of this agent differed from chlorine in that damage was irreversible once ozone had penetrated the cell membrane. Utilizing a laboratory procedure approximating continuous flow conditions, Dickerman and his colleagues (1954) subjected both pure cultures and mixed populations of bacteria to ozonation. Residual concentrations of 2 mg/l ozone for 5 min was recommended for satisfactory disinfection of raw water. Following ozonation of suspensions of E. coli, Fetner and Ingols (1956) observed what they termed the "all-or-none effect." This phenomenon subscribes to the belief that no bactericidal effects are apparent below a certain critical concentration of disinfectant; above this concentration there are no detectable survivors. In other words, mortality is inevitable for the entire population once the threshold concentration of ozone is reached. Contrarily, chlorination produces mortality in geometric progression as a function of time or concentration. McNair Scott and Lesher (1963) also measured the survival of E. coli during ozone treatment while attempting to elucidate its mode of action at the cellular level. In this case, however, survival data did not exhibit the kinetics predicted by the Fetner-Ingols model. Burleson et al. (1975) noted rapid inactivation of several bacteria and viruses of public

health significance, but likewise never observed the all-or-none phenomenon. The all-or-none effect was observed in another study (Broadwater et al., 1973) testing ozone sensitivity of E. coli, Bacillus cereus, and Bacillus megaterium. With a uniform contact time of 5 min, the lethal threshold concentration for vegetative cells was less than 0.2 mg/l, while spores required as much as 2.29 mg/l ozone for kill.

Since many viruses resist chlorination, numerous reports concerning virus inactivation by ozone are available (Kessel et al., 1943; Yakovieva and Le'Nitskii, 1967; Carazzone and Vanini, 1969; Perlman, 1969; Pavoni et al., 1972; Burleson et al., 1975; Munger et al., 1977). Majumdar et al. (1973) established 1.0 mg/l as the threshold concentration for inactivation of poliovirus type 1. Continuous flow studies were incomplete, but the authors reported the data followed trends similar to rate equations for the batch runs. In one of the most recent investigations (Snyder and Chang, 1975), a continuous flow apparatus was designed to evaluate the relative resistance of eight human enteric viruses to ozonation in the laboratory. It was found that the time required to inactivate a virus is a function of the nature of the water in which the virus is suspended.

These data generated from organism-oriented studies have prompted a number of investigators to consider the mode of action of ozone in biological systems. The earliest of

such speculations (Ingram and Haines, 1949) maintained that ozone destroys the dehydrogenase enzymes in microorganisms with consequential interference with cellular respiration. Giese and Christensen (1954) observed structural alteration of the membrane surface, with subsequent oxidation of cell organelles. Other researchers (Bringeman, 1954; Fetner and Ingols, 1956) have classified ozone as a general protoplasmic oxidant. McNair Scott and Lesher (1963) have postulated that primary attack is on the cell wall or cell membrane by probable reaction with the double bonds of lipids, resulting in increased permeability, leakage of cell contents, or complete lysis. Early work by Christensen and Giese (1954) and Giese et al. (1952) examined the comparative effects of ozone and ultraviolet radiation on absorption spectra of nucleic acids, proteins, and amino acids. In related work, Mudd et al. (1969) were unable to determine whether the protein or the lipid portion of the biomembrane was damaged first. McFarlane and McNulty (1966) examined permeability changes in eucaryote systems following ozone exposure. Unlike McNair Scott and Lesher (1963), they observed increased permeability resulting from an unspecified effect on the membrane structure. In another eucaryote system (Pace et al., 1969), human fetal lung epithelium was exposed to ozone in an effort to assess the possibility of tolerance development during <u>in vitro</u> culture of the cell line. Low concentrations of ozone retarded cell

proliferation and interfered with mitosis. The effect of ozone was dependent upon concentration and length of exposure with *in vitro* tolerance developed due to suspected stimulation of the pentose-phosphate pathway (Stokinger and Scheel, 1962). In a 1974 report (Hamelin and Chung, 1974), it was suggested that ozone directly exerts both lethal and mutagenic effects on cells via a primary effect on the permeability of the cell membrane. In a later paper, the same authors (Hamelin and Chung, 1975) reported isolation of ozone-induced maltose and methionine mutants of *E. coli* K-12, which possessed similar characteristics of mutants obtained after ultraviolet irradiation.

Ozone related studies have been confined almost exclusively to treatment of municipal or industrial wastes. A brief paper by Benoit and Matlin (1966) described use of ozone for control of *Saprolegnia* growth on eggs of rainbow trout, which resulted in high uninfected yields upon hatching. Another aquaculture application was more recently advocated by Honn and Chavin (1976). Water was ozonated in a closed marine aquarium housing four nurse sharks (*Ginglymostoma cirratum*). Ammonia and nitrite levels were reduced significantly following ozonation. Nitrate levels increased slightly but were well within acceptable limits, while bacterial counts were much lower after ozonation as compared with aquarium and algal bed-bacterial filter

effluents. The use of ozone as a supplemental system was highly recommended for marine systems.

Seaworld of Ohio has maintained a recycled, ozone-treated aquarium system since 1974, established primarily for BOD reduction. It has been reported (Murphy, 1975) that as of 1975 the system still contained its original water. The water quality was described as "exceedingly high" with all animals in excellent health.

In another model experiment, the Cubic Corporation of San Diego (Ciambrone, 1975) has designed a packaged sewage treatment system which utilizes ozone for oxidation and disinfection of domestic sewage for use in controlled mariculture. In addition to reducing by half the growing time for marketable oysters (Crassostrea virginica), reduction of BOD, COD, settleable solids, suspended solids, and coliforms has been impressive.

To date, only two reports have been published concerning use of ozone in reuse hatcheries. In a preliminary report by Rosenlund (1975), hatchery influent was subjected to ozone, though power oscillations and other technical difficulties prevented completion of experiments as planned. It was noted, however, that fish exposed to freshly ozonated water experienced irritation and, in some groups, mortality.

Wedemeyer and Nelson (1977) have determined inactivation curves for two bacterial fish pathogens during both batch ozonation and chlorination. HRM, the causative agent

of Hagerman Redmouth Disease and tentatively designated Versinia ruckeri, was more sensitive to batch application of ozone in all water types tested than Aeromonas salmonicida, the etiologic agent of furunculosis. Yet survival of HRM exceeded that of A. salmonicida when suspended in untreated lake water. A longer contact time was required for inactivation of both organisms when suspended and ozonated in hard and soft lake water.

MATERIALS AND METHODS

Ozone Production

Ozone was produced from oxygen in the laboratory by a reconstructed, water-cooled Welsbach model Cl-D corona discharge generator (Welsbach Corp., Philadelphia, PA). A glass contact column (Fig. 1) (Pyrex No. 29/42) was modified for survival and oxidation studies allowing for syringe withdrawal of samples without interruption of the ozone flow. Ozone entered the column of water through a sintered glass (Corning grade) dispersion tube suspended near the base of the contact chamber. Tygon tubing (Norton Plastics and Synthetic Div., Akron, OH) was threaded into the column and sealed in place to prevent escape of ozone into the laboratory. A 10 ml syringe was used to initiate sample flow with 1 ml or 2 ml samples withdrawn by sterile 1 ml or 5 ml glass syringes, respectively. Ozone concentrations in water were determined by the spectrophotometric method of Schechter (1973).

Preparation and Enumeration of Cultures for Survival Studies

The four bacterial fish pathogens tested, Aeromonas liquefaciens, Pseudomonas fluorescens, HRM (recently designated Versinia ruckeri; also known as Hagerman Redmouth Disease, ERM, or Enteric Redmouth Disease), and Aeromonas

Fig. 1.--Continuous flow apparatus

salmonicida, were originally isolated from confirmed disease cases and maintained under lyophilization. The test organisms were propagated in shake flasks of Trypticase Soy Broth (TSB) at 25 C for 18 hr. Aliquots of the culture were centrifuged at 3020 x g for 10 min, washed once in sterile 0.02 M phosphate-buffered saline (PSB), and resuspended in sterile PBS.

Spore suspensions of Bacillus polymyxa were prepared following propagation on bottle-slants of Trypticase Soy Agar (TSA) for 5-7 days at 30 C. Cells were harvested, suspended in sterile PBS, heat-shocked in a boiling water bath at 80 C for 15 min, and sonicated for 30-60 sec with a Biosonik III unit (Bronwell Scientific, Rochester, NY). The resulting suspension was centrifuged and washed three times in sterile PBS.

A mixed population of bacteria and biflagellated protozoans was isolated from soil and grown at room temperature on plates of 0.1% tryptone and 1.5% agar, with a 9 ml surface layer of 0.1% tryptone broth. The mixed culture was harvested after 48 hr, centrifuged, washed once, and resuspended in sterile PBS.

Survival of bacterial fish pathogens and B. polymyxa spores during exposure to ozone was monitored by surface spread plating in triplicate on TSA, while bacteria in the mixed soil culture were plated in triplicate on 0.5% tryptone and 1.5% agar. Serial dilutions were made in 9 ml

volumes of 0.1% tryptone broth. Fish pathogen and soil culture plates were incubated at 25 C for 24-48 hr. Bacillus polymyxa spores were incubated at 30 C for 18 hr. Protozoan survival was followed by direct counts in a hemacytometer under a phase contrast microscope.

Batch and Continuous Flow Ozonation

Batch ozonation was accomplished by gas flow into a 180 ml volume of sterile water or buffer for a 15 min period followed by addition of the test organisms and sample withdrawal at 1 min intervals. Continuous flow ozonzation differed in that the flow of ozone was not suspended after addition of the organisms or carbon and nitrogen addenda. Syringe withdrawal of 1 ml volume samples was achieved without interruption of the gas flow. The action of ozone was stopped by immediate addition of the sample to a 9 ml dilution blank containing 0.1% tryptone broth. All experiments were conducted at room temperature, with oxygen flow controls, in pH 7.0 distilled water or in distilled water buffered at the desired pH.

Glucose additions to organism survival systems were made at BOD levels of 5, 10, 25, 40, 50, and 100 mg/l. Test conditions at pH 6.0 and pH 7.2 were maintained by 0.02 M phosphate buffer, while 0.0125 M borate buffer allowed for oxidations at pH 8.2 and pH 9.3.

Carbon, Ammonia, and Nitrite Oxidations

A 5 ml glass syringe was used to withdraw 3 ml volume samples at 1 min intervals, which in turn were dispensed in triplicate as 1 ml volumes into a precombusted glass ampule containing 5 ml water, 0.2 g potassium persulfate ($K_2S_2O_8$), and 0.25 ml of a 6% phosphoric acid solution. The ampules were sealed and autoclaved for 4 hr at 17 psi. Carbon as glucose was measured by infrared spectroscopy using the Model 0524B Total Carbon System (Oceanography International Corp., College Station, TX).

A 5 ml glass syringe was employed to withdraw 4 ml volume samples at 1 min intervals, which were dispensed in triplicate as 2 ml volumes for duplicate nitrate or nitrite determinations. The samples were added directly to the respective reagents in order to facilitate inactivation of ozone. Oxidation of ammonia as ammonium chloride (NH_4Cl) was allowed as nitrate formation by the Chromotropic Acid Method (APHA, 1976). Oxidation of nitrite as sodium nitrite ($NaNO_2$) was monitored as nitrite disappearance by the Sulfanilic Acid Method (APHA, 1976).

Plotted values of experiments represent mean values of a minimum of three runs in the survival studies and two determinations in the case of carbon, ammonia, and nitrite oxidations.

RESULTS AND DISCUSSION

Results of batch ozonation studies in pH 7.0 distilled water are plotted in Fig. 2. A specific microbial ozone demand was exerted by Aeromonas liquefaciens, Pseudomonas fluorescens, and HRM (Versinia ruckeri), while Aeromonas salmonicida appeared the most sensitive to ozone exposure at 1.0 mg/l ozone. These results are contrary to data reported in studies by Wedemeyer and Nelson (1977) in that survival of A. salmonicida during ozone exposure was reportedly greater than that of HRM. Though the concentration of ozone in this study remained constant, these data may be used to explain the frequency of the so-called "all-or-none effect," a reportedly characteristic feature of ozone treatment (Fetner and Ingols, 1956; Perlman, 1969). The methodology by which all-or-none data have usually been gathered is by some type of batch exposure system (Broadwater et al., 1973; Majumdar et al., 1973). The subsequent interpretation of such data has resulted in support of the all-or-none concept. In batch studies of poliovirus inactivation, Majumdar et al. (1973) reported their findings as threshold concentrations, yet both methodology and results were similar to those for batch ozonation of bacteria reported in this research.

Figures 3, 4, 5, and 6 summarize results of continuous flow ozonation. The rates of die-off in pH 7.0 adjusted distilled water for A. liquefaciens, P. fluorescens, and HRM (V. ruckeri) increased as the concentration of ozone in solution increased. Likewise, survival of A. salmonicida (Fig. 6) seemed dependent on ozone concentration yet, as in batch application, exhibited greater sensitivity to ozone. The more rapid die-off of A. salmonicida is clearly evident in Fig. 7, which compares the relative survival of the four bacterial fish pathogens during continuous flow at 0.1 mg/l ozone. The other organisms appear comparably sensitive to ozone treatment. Survival curves for continuous flow studies agree well with reports of other investigators (McNair Scott and Lesher, 1963; Burleson et al., 1975), who also failed to observe the all-or-none effect in inactivation studies of a variety of microorganisms. There are no reports in the literature to explain the consistently observed sensitivity of A. salmonicida to ozonation.

In addition to survival monitored in distilled water, carbon as glucose was introduced at concentrations equivalent to BOD levels of 5, 10, 25, 40, 50, and 100 mg/l. No differences in bacterial survival were detectable at elevated carbon levels under the same experimental conditions of continuous flow ozonation. The effect of dissolved organic matter on survival of organisms exposed to

Fig. 2.--Survival of Aeromonas liquefaciens, Pseudomonas fluorescens, HRM (Versinia ruckeri), and Aeromonas salmonicida during batch ozonation at 1.0 mg/l ozone in pH 7.0 distilled water

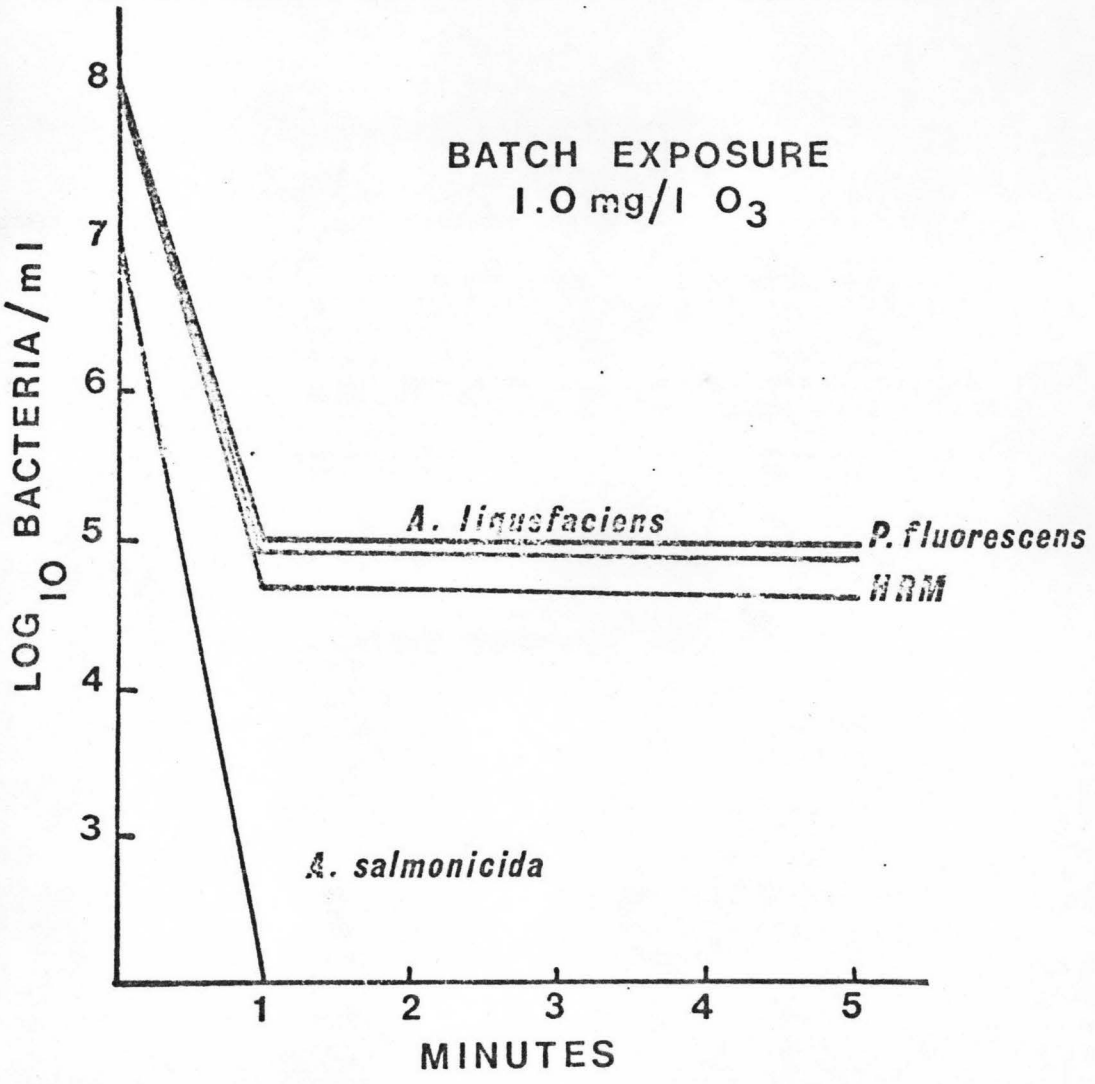


Fig. 3.--Survival of Aeromonas liquefaciens during continuous flow ozonation at varying ozone concentrations in pH 7.0 distilled water

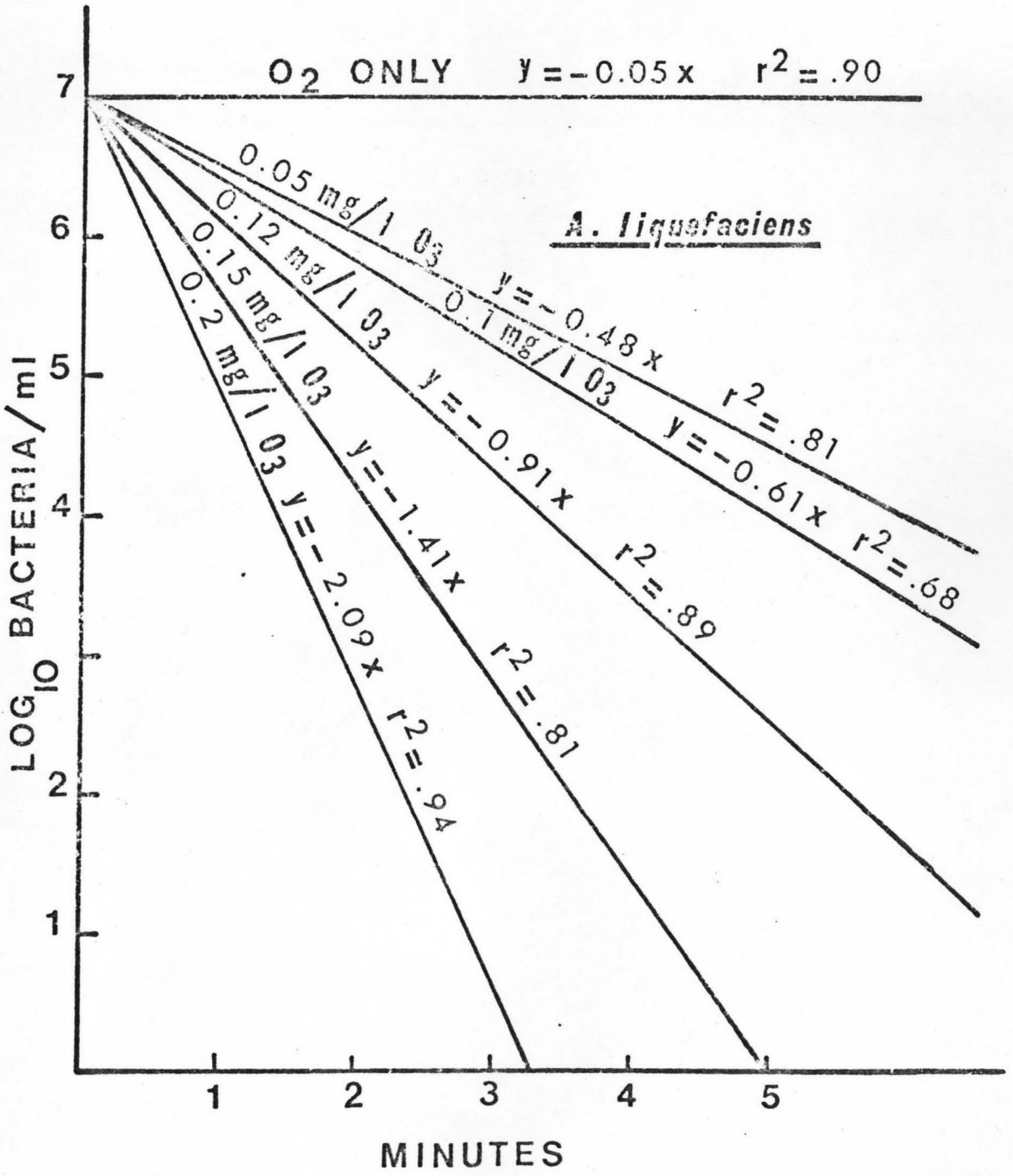


Fig. 4.--Survival of Pseudomonas fluorescens during continuous flow ozonation at varying ozone concentrations in pH 7.0 distilled water

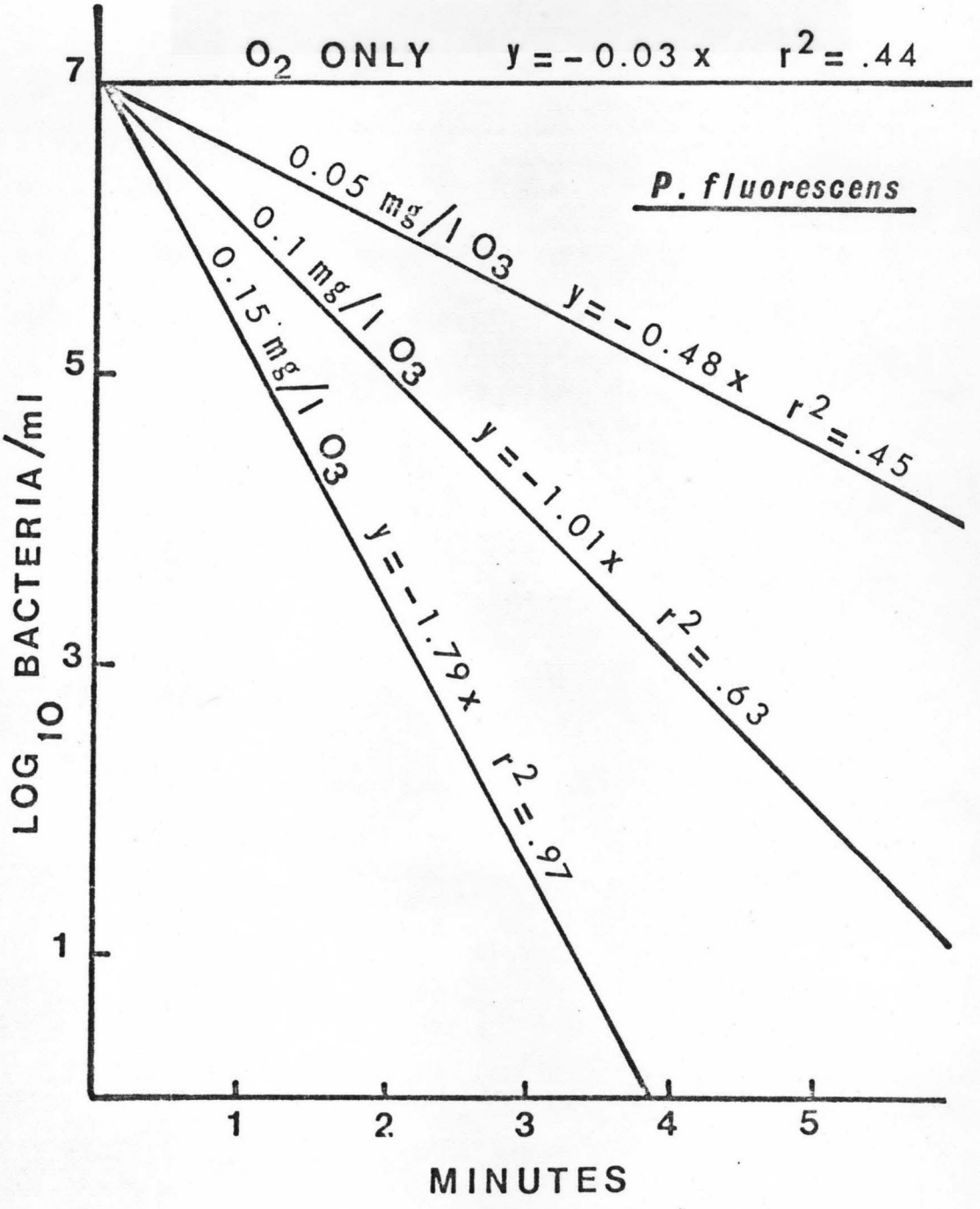


Fig. 5.--Survival of HRM (Versinia ruckeri) during continuous flow ozonation at varying ozone concentrations in pH 7.0 distilled water

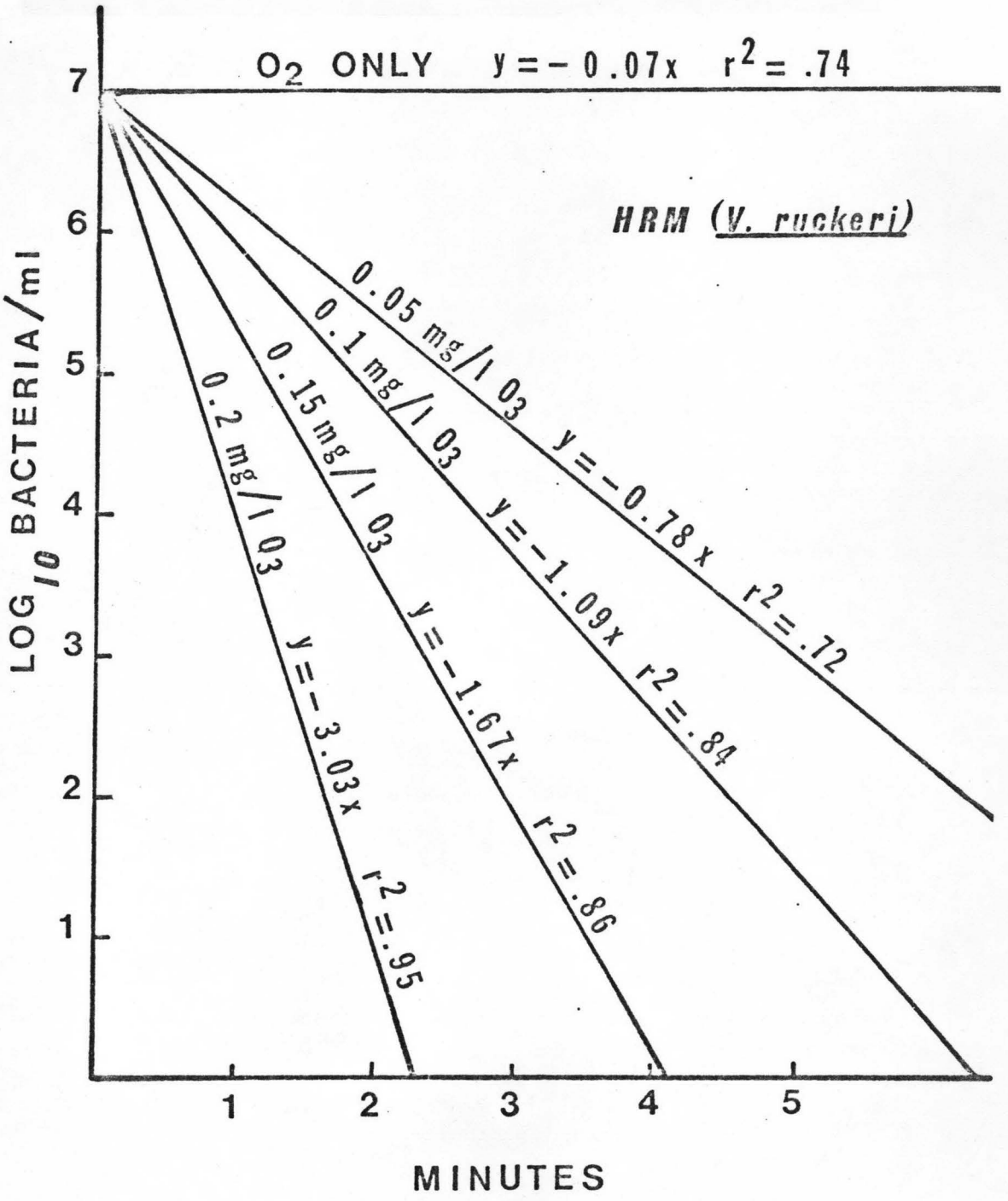


Fig. 6.--Survival of Aeromonas salmonicida during continuous flow ozonation at varying ozone concentrations in pH 7.0 distilled water

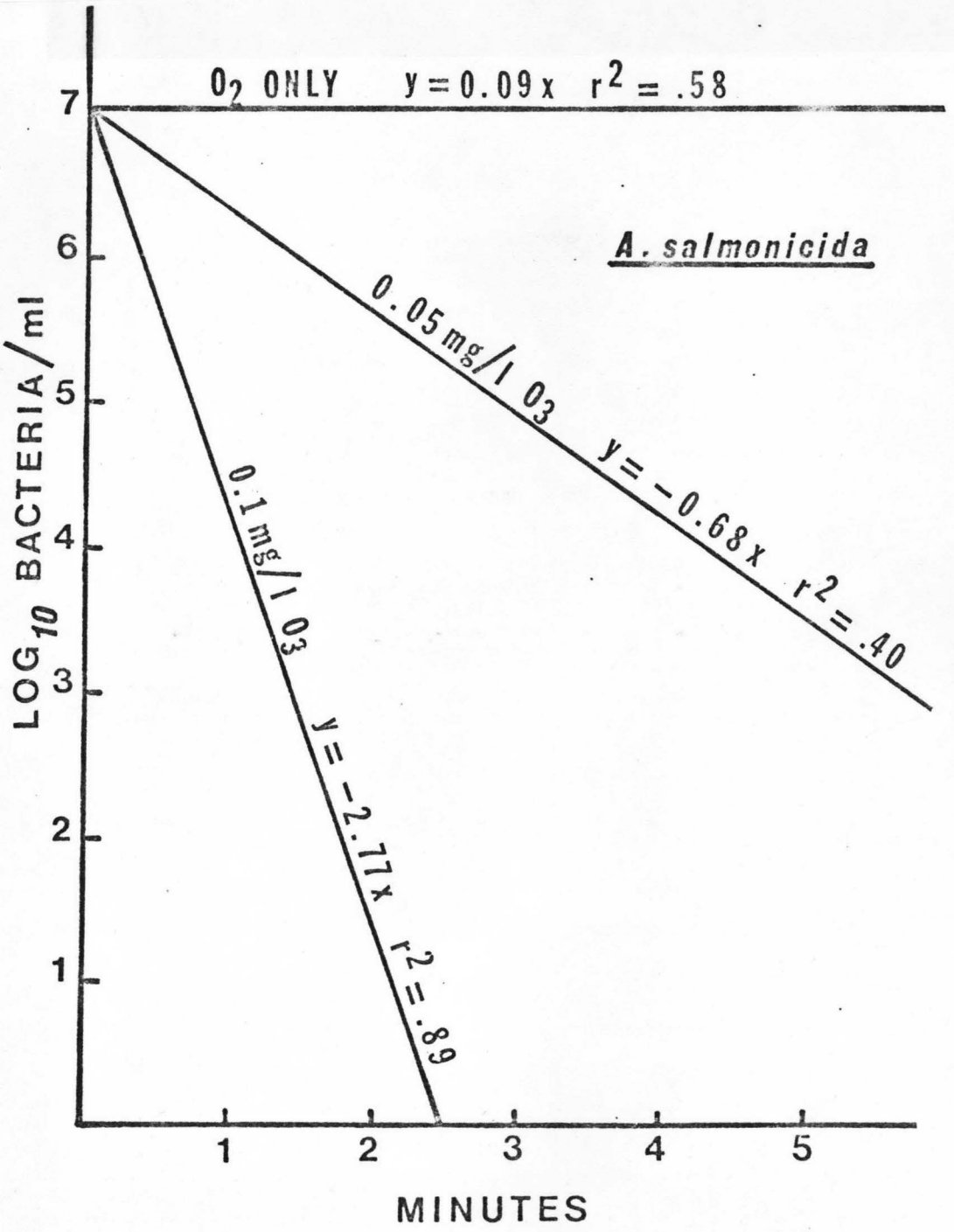
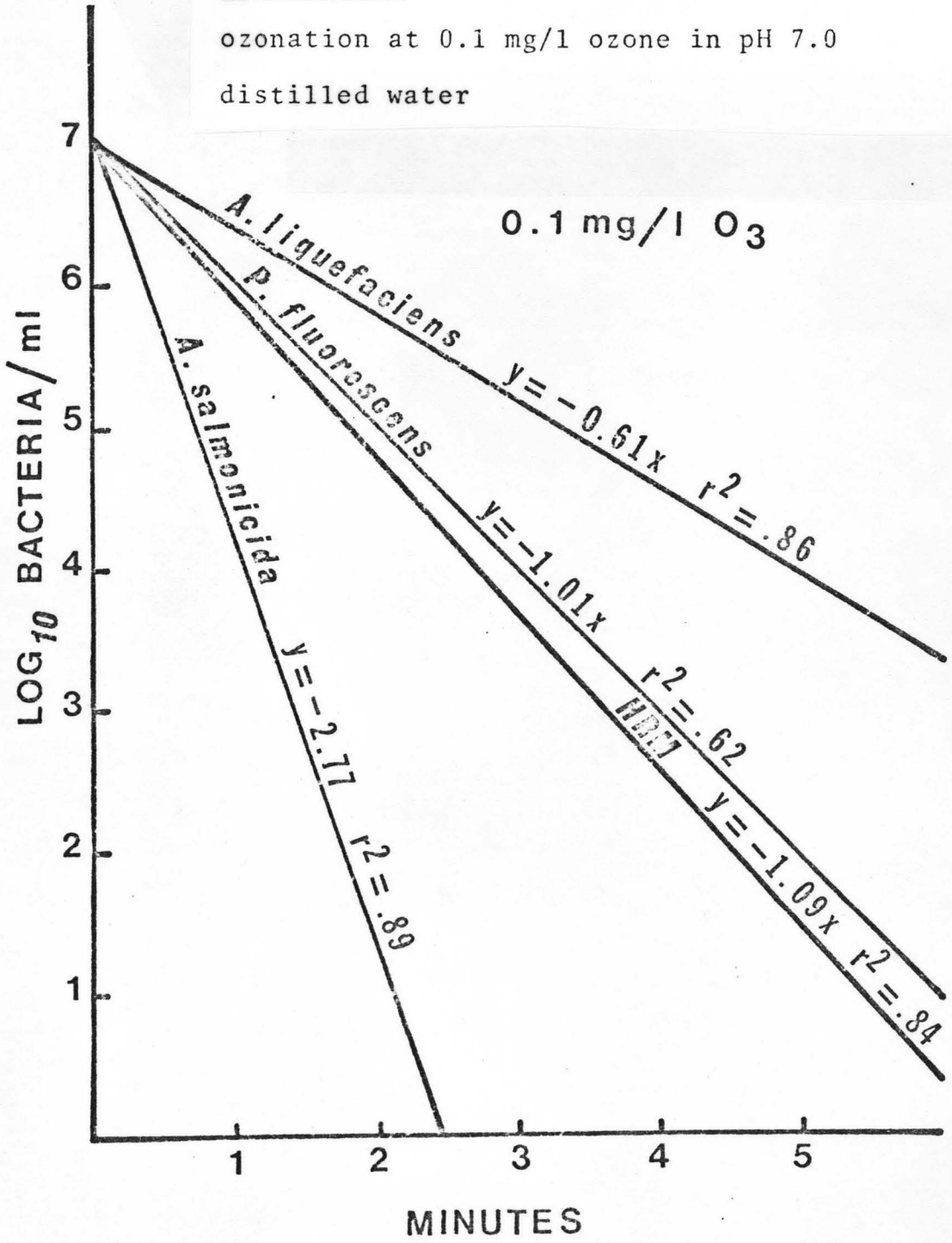


Fig. 7.--Relative survival rates of Aeromonas liquefaciens, Pseudomonas fluorescens, HRM (Versinia ruckeri), and Aeromonas salmonicida during continuous flow ozonation at 0.1 mg/l ozone in pH 7.0 distilled water

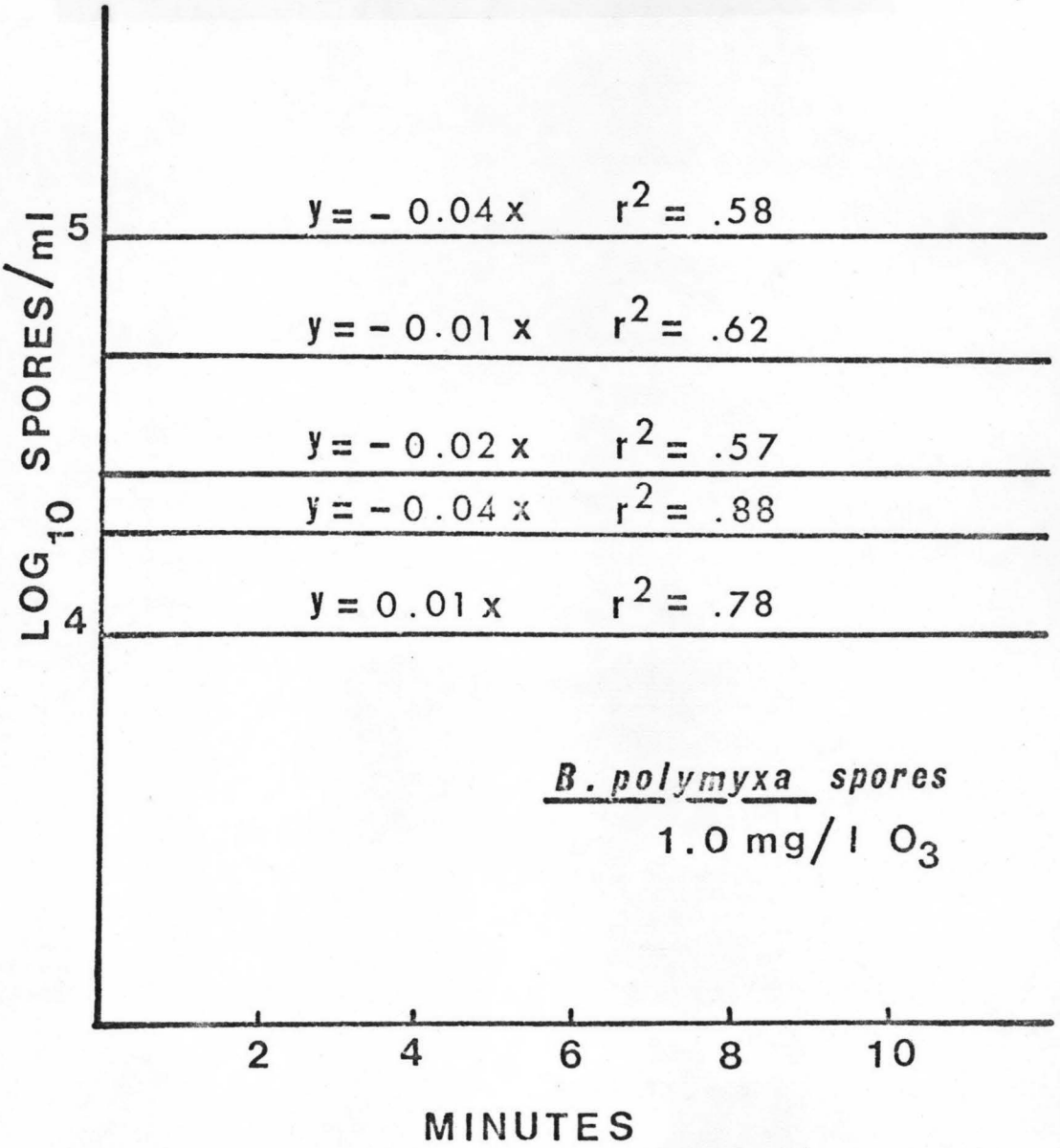


ozone-treated water has received some attention (Ingram and Haines, 1949; Dickerman et al., 1954; Broadwater et al., 1973). Results of such studies confirm that organic matter in solution may compete with microorganisms by exerting an ozone demand, resulting in less efficient disinfection of water. These reports, however, considered much higher carbon levels than were added to test systems in this study.

These data suggest that greater than 99% mortality of dense suspensions of pure cultures of the four fish pathogens tested can be achieved within 60 sec of contact in water at low ozone concentrations. Increased ozone concentrations yielded increased rates of mortality, while elevated levels of soluble carbon did not appear to exert a preferential ozone demand when added to suspensions of test organisms.

Spore suspensions of Bacillus polymyxa were subjected to ozone as a model of the most resistant organisms. The maximum concentration of ozone that was consistently generated with the available equipment was approximately 1 mg/l ozone. At that level, spore viability was unaffected by continuous flow exposure to ozone as monitored for 10 min (Fig. 8). Survival data for ozone inactivation of bacterial spores is restricted to a paper by Broadwater et al. (1973). Their results subscribe to the all-or-none phenomenon with threshold concentrations established between 2.03 and 2.29 mg/l ozone for spores of Bacillus cereus and Bacillus

Fig. 8.--Survival of Bacillus polymyxa spores during continuous flow ozonation at 1.0 mg/l ozone in pH 7.0 distilled water



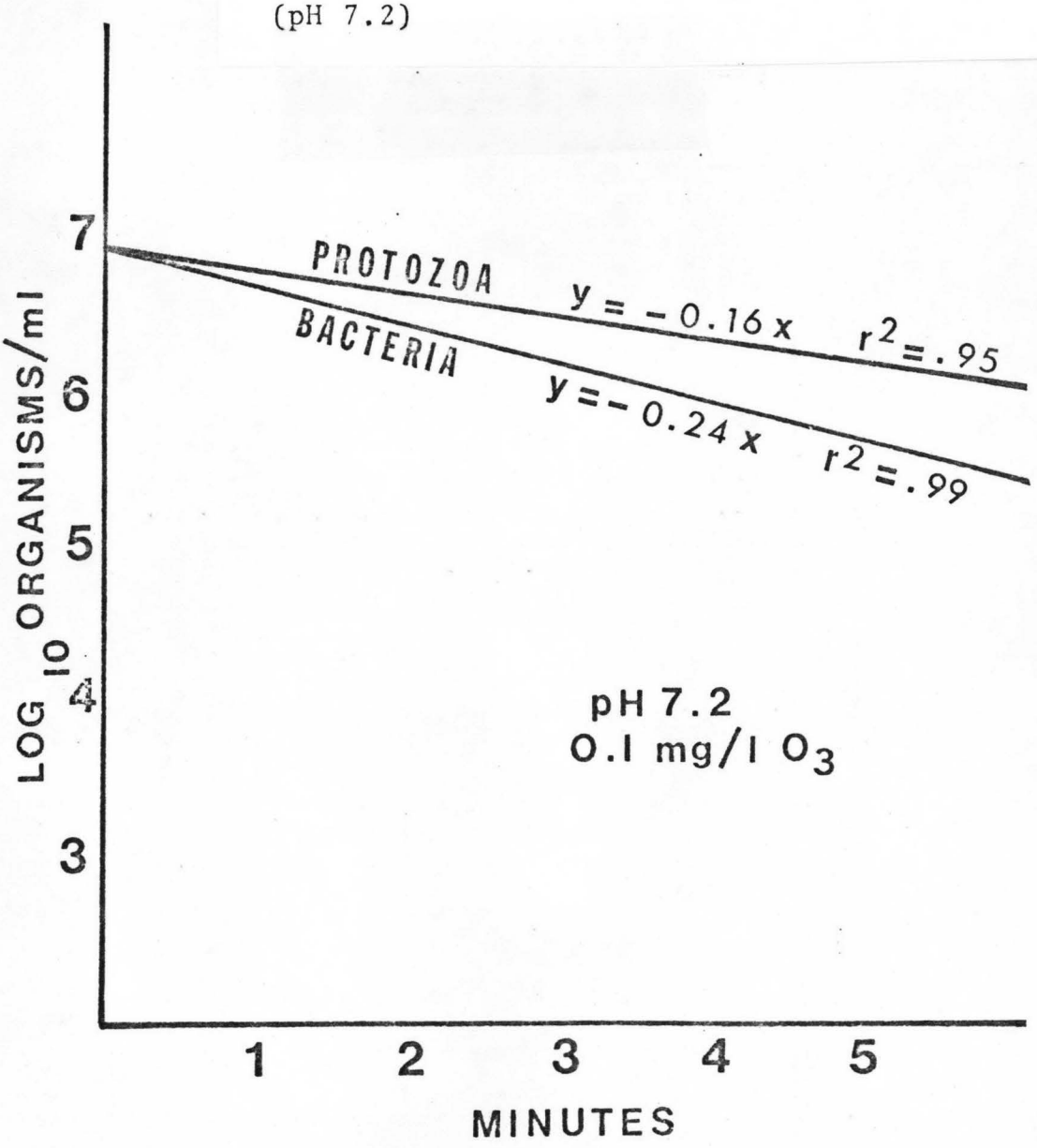
megaterium during batch ozonation. To date, no continuous flow data for bacterial spores are available.

Since many protozoans pathogenic to fish are Sporozoans (Amlacher, 1970) and difficult or impossible to culture by ordinary means, a mixed population of bacteria and biflagellated protozoans was isolated from soil. Survival rates during ozonation of the mixed culture can be seen in Fig. 9. The oxidation of the combined biomass at pH 7.2 closely approximates oxidation rates established for the pure culture studies (Figs. 3, 4, 5). Analysis of variance indicates no significant difference in the relative survival rates between bacteria and protozoa at 0.1 mg/l ozone. Research on ozone inactivation of protozoans is limited to some early microscopic observations by Giese and Christensen (1954) and evaluations of the cysticidal potential of ozone on cysts of the waterborne agent of amoebiasis (Kessel et al., 1944; Newton and Jones, 1949).

The concentration of ozone in aqueous systems appears to be pH-dependent as summarized in Fig. 10. Buffer solutions at four pH levels were ozonated for 15 min with subsequent measurement of the final ozone concentrations. As the pH increased, the concentration of ozone decreased.

This variance in oxidation potential with increased pH was examined more closely in a series of measurements of carbon oxidation. Carbon as glucose was subjected to

Fig. 9.--Comparative survival rates of mixed bacterial-protozoan population during continuous flow ozonation at 0.1 mg/l ozone in sterile phosphate buffer (pH 7.2)



BUFFER SYSTEM	pH	mg/l O_3
0.0125 M BORATE	9.3	0.15
0.0125 M BORATE	8.2	0.25
0.02 M PHOSPHATE	7.2	0.38
0.02 M PHOSPHATE	6.0	0.44

Fig. 10.--Effect of pH on concentrations of ozone in solution after continuous flow for 15 min

ozonation at 1.0 mg/l ozone with oxidation rates measured in buffers of pH 6.0, 7.2, 8.2, and 9.3, as monitored by infrared carbon analysis (Fig. 11). The oxidation activity of ozone was greater at elevated pH, even though the measurable ozone concentration was actually lower (Fig. 10).

Since the influence of pH seemed substantial for carbon oxidation, one of the fish pathogens, HRM, was tested in buffer solutions at the same pH levels. No significant differences in survival at 0.1 mg/l ozone were evident (Fig. 12) as compared to survival in pH 7.0 distilled water (Fig. 5). Likewise, as depicted in Fig. 13, no significant differences in survival of the mixed bacterial-protozoan population were observed as the pH increased. Though pH had no apparent effect on rate of kill in either organism test system, carbon oxidation clearly exhibited pH-dependence. Consequently, the biocidal activity of ozone appears due to a mechanism more specific than random oxidation.

Since oxidation of ammonia in recycled water is essential for maintenance of aquatic animals (Spotte, 1970; Johnson and Sieburth, 1974), ammonia as ammonium chloride (NH_4Cl) was exposed to 1.0 mg/l ozone with oxidation followed as nitrate formation. Oxidation of ammonia also appeared to be pH-dependent (Fig. 14). There was only negligible nitrate formation at pH 7.2.

Fig. 11.--Oxidation rates of carbon (as glucose) at pH 6.0, 7.2, 8.2, and 9.3 during continuous flow ozonation at 1.0 mg/l ozone

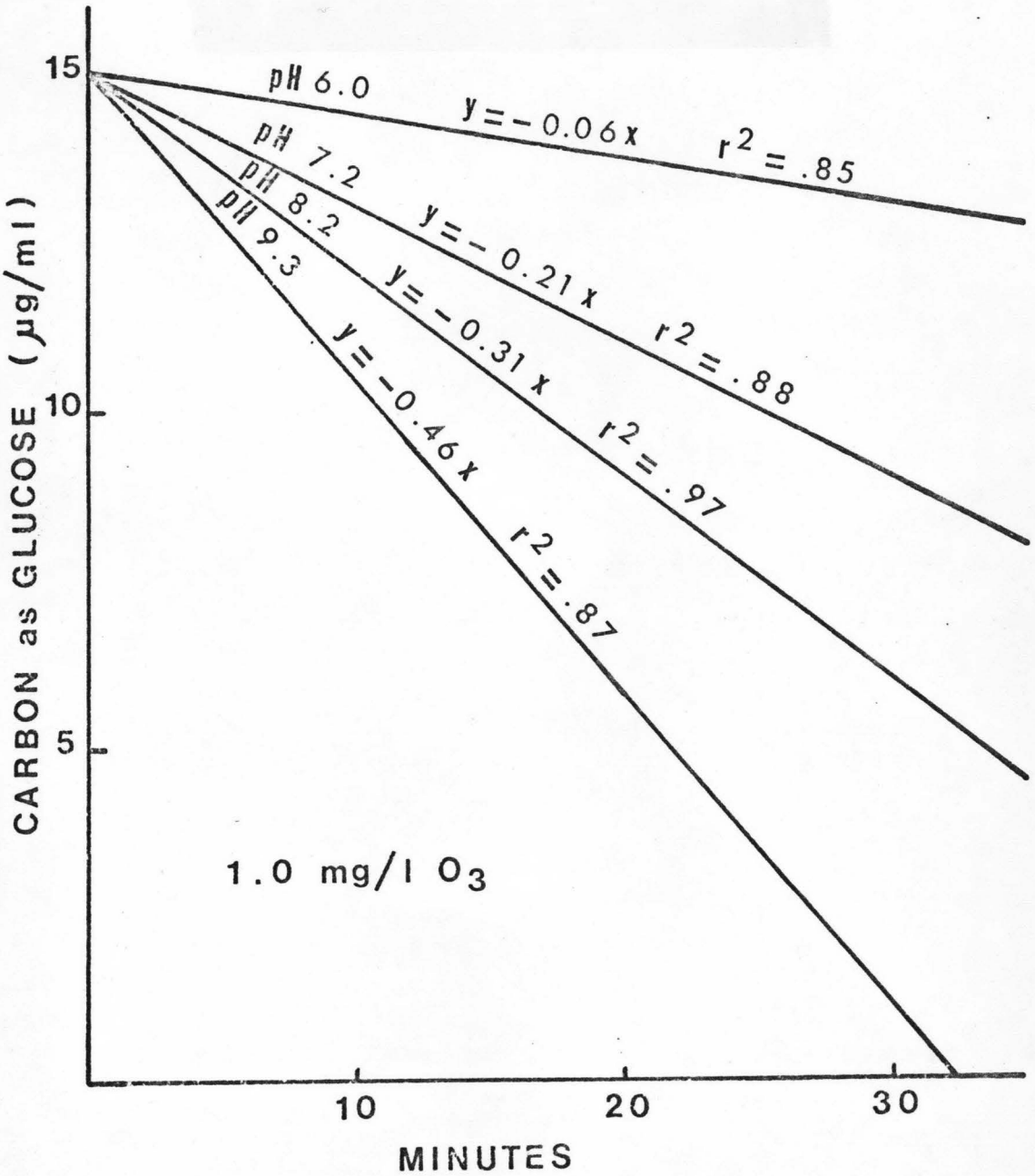


Fig. 12,--Influence of pH on survival of HRM (Versinia ruckeri) during continuous flow ozonation in buffer solutions at 0.1 mg/l ozone

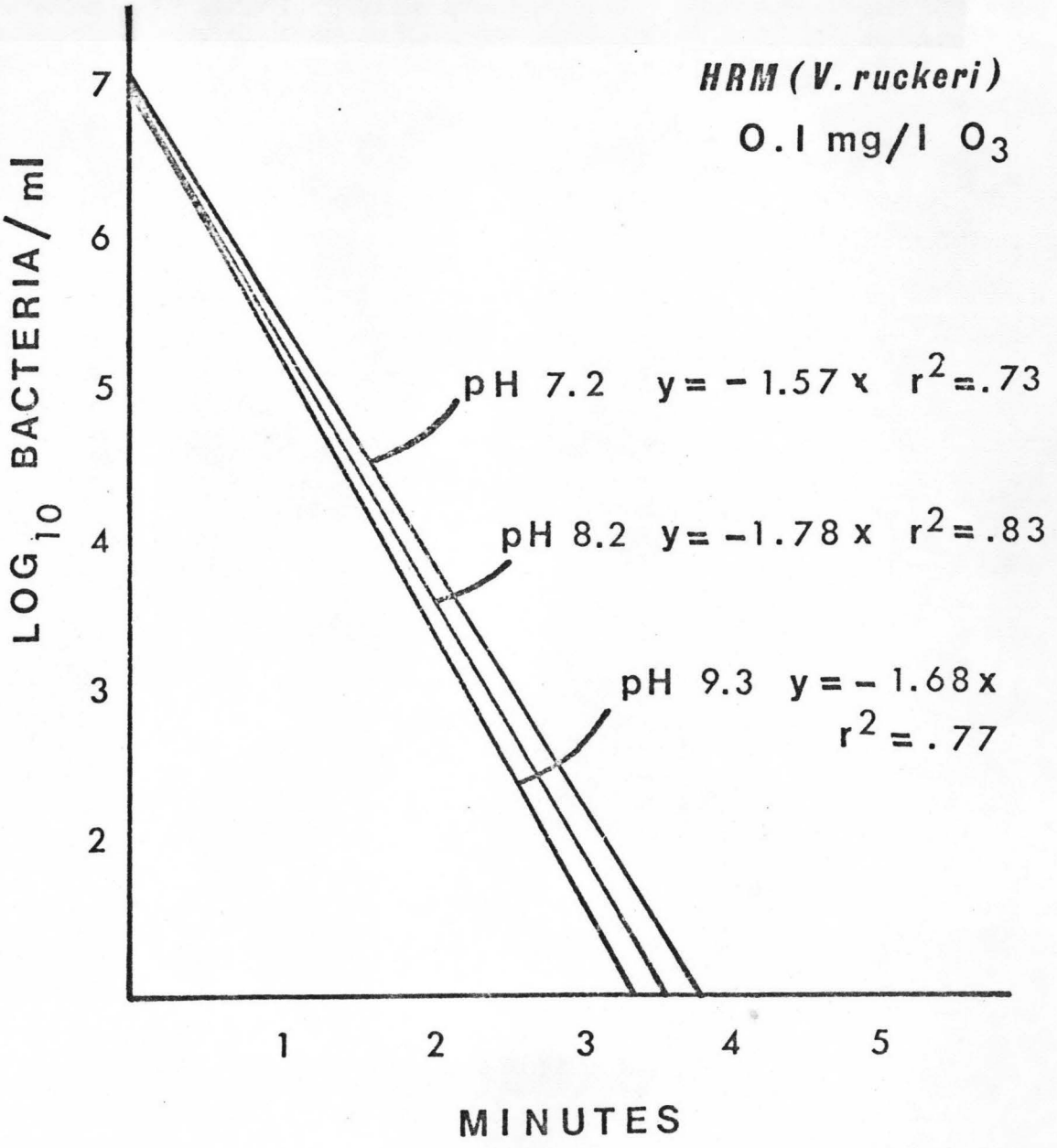
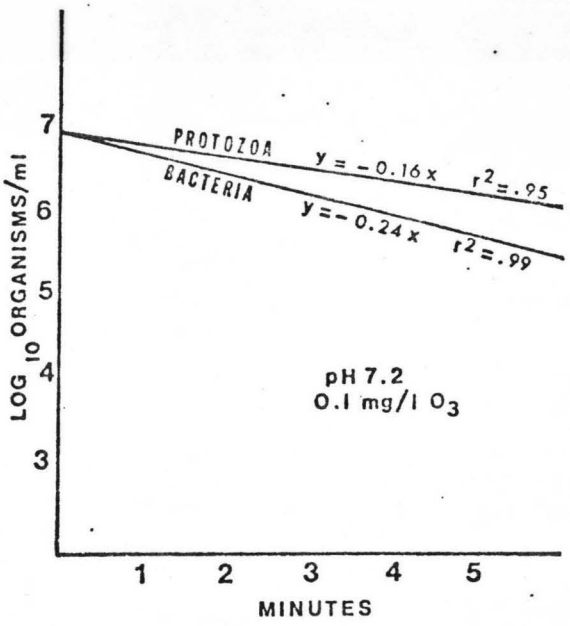
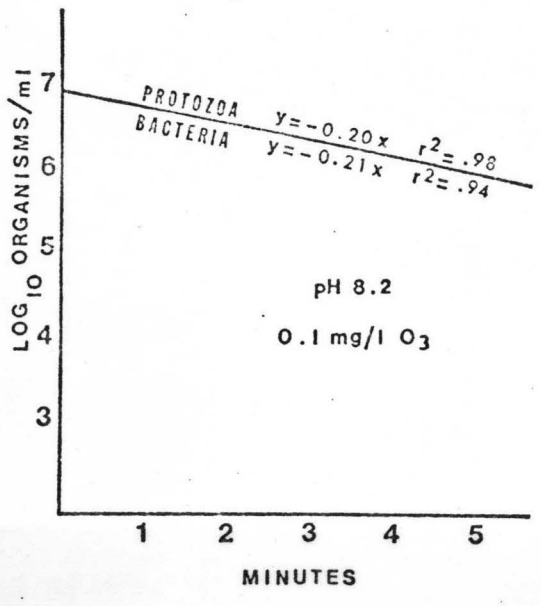


Fig. 13.--Survival rates of mixed bacterial-protozoan population during continuous flow ozonation in buffer solutions at pH 7.2, 8.2, and 9.3 at 0.1 mg/l ozone





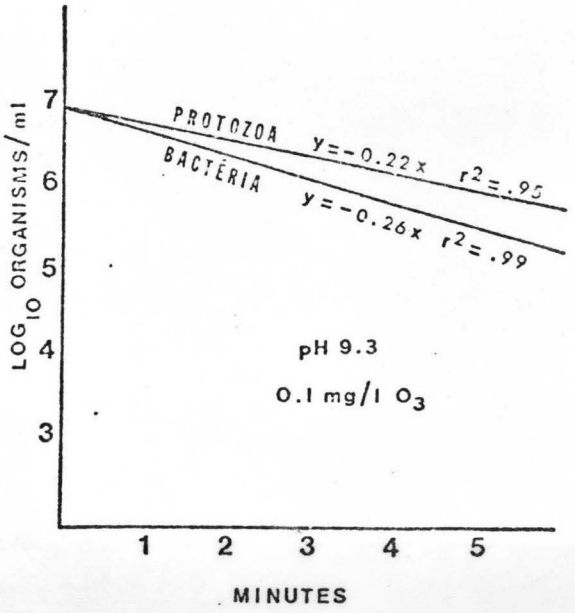
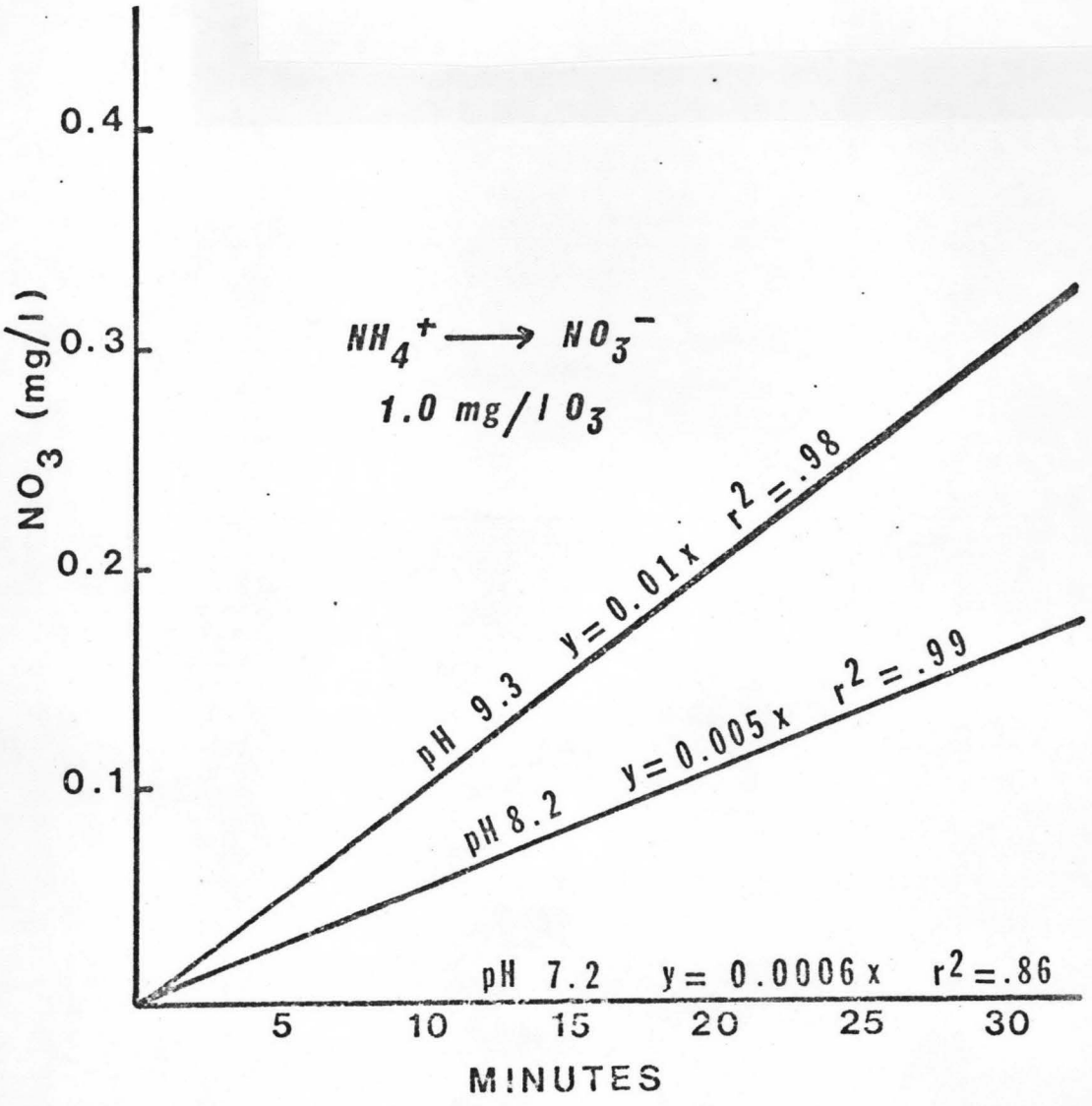


Fig. 14.--Influence of pH on oxidation of ammonia as NH_4Cl during continuous flow ozonation in buffer solutions at 1.0 mg/l ozone



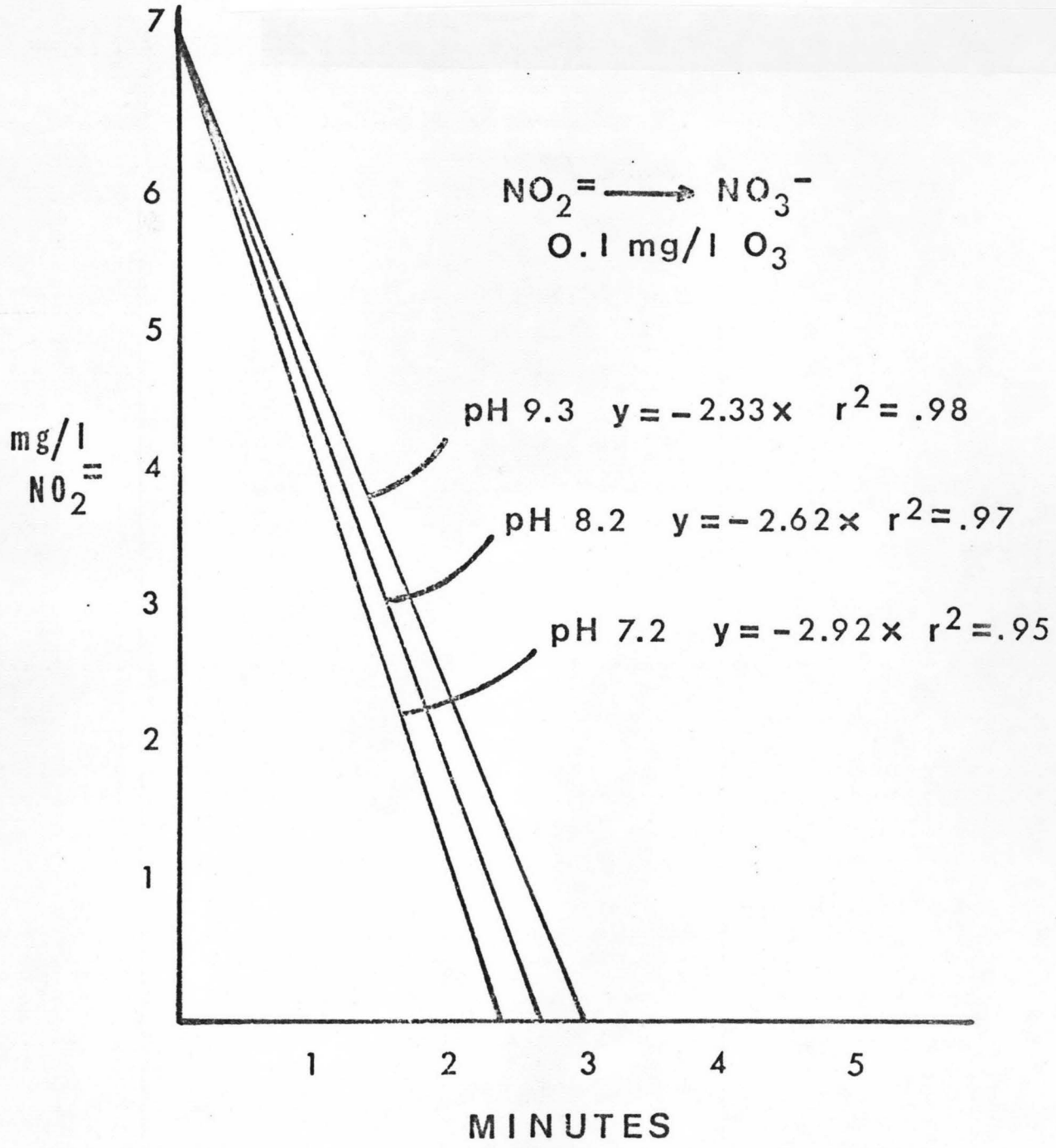
An intermediate state of ammonia removal by biological nitrification is nitrite. Nitrite oxidation, monitored as nitrite disappearance, was rapid even at 0.1 mg/l ozone (Fig. 15), with the pH effect statistically insignificant in this case.

Formation of free radical species due to ozone decomposition in aqueous systems (Weiss, 1935; Alder and Hill, 1950; Kilpatrick et al., 1956; Thomas, 1965; Hewes and Davison, 1971; DeMore, 1973; Gorbenko-Germanov and Kozlova, 1973, 1974; Hoigne and Bader, 1975, 1976; Peleg, 1976) would account for the decreased ozone concentration in water at elevated pH (Fig. 10). Carbon oxidation data reveals the oxidation potential of such free radicals in water (Fig. 11).

No pH effect was evident on survival of either HRM or the mixed bacterial-protozoan population during ozonation. This suggests that the mode of action of ozone on biological matter is not necessarily influenced by free radical formation, but that oxidation readily occurs without regard for the pH of the system nor the available oxidizing species.

Data for ammonia oxidation by ozone agree with recent work by Singer and Zilli (1975). The lack of ammonia oxidation at pH 7.2 could easily be alleviated in an aquaculture system by lime clarification (Singer and Zilli, 1975).

Fig. 15.--Oxidation of nitrite as $NaNO_2$ during continuous flow ozonation in buffer solutions at 0.1 mg/l ozone



Recent evidence suggests that nitrite levels in excess of 0.2 mg/l can be related to anoxia and heavy mortalities in hatchery operations (Liao and Mayo, 1972). One of the persistent problems with the biological nitrification system at the Dworshak hatchery has been incomplete ammonia oxidation, with subsequent nitrite accumulation. It is apparent from results of this study that this problem could be efficiently resolved by ozonation.

On the basis of this research, it is easy to visualize an on-line ozone treatment system effectively alleviating the disease potential associated with large-scale fish culture, while efficiently oxidizing potentially toxic metabolites such as nitrite and ammonia. However, successful installation and operation of such a facility is dependent on the economics of such an investment (Majumdar and Sproul, 1974) as well as provision of a mechanism for ozone removal prior to recirculation. There is concern that even low ozone "residuals," though short-lived, may cause gill adhesions and mortality in fish exposed to freshly ozonated water (Rosenlund, 1975). A short-term holding tank, step-ladder return flow to the raceways, or passage through an activated charcoal filter are potential methods of ozone stripping. If these problems are overcome, the potential of ozone treatment of water in reuse hatcheries is both warranted and promising.

SUMMARY

Results of laboratory survival studies of four bacterial fish pathogens originally isolated from disease cases and a mixed bacterial-protozoan population isolated from soil suggested ozone to be an extremely efficient biocidal agent in water at low concentrations. The all-or-none effect commonly reported a characteristic feature of ozone treatment was never observed. Bacterial spores exposed to ozone were resistant at a residual concentration of 1.0 mg/l ozone. Increased ozone concentrations yielded increased rates of mortality, while elevated carbon levels did not appear to exert a preferential ozone demand in organism test systems.

Oxidation of carbon, ammonia, and nitrite by ozone was rapid at low ozone concentrations, with carbon and ammonia oxidation rates dependent on pH. The oxidation potential of ozone in water was greatest at elevated pH even though the measurable ozone concentration was low. It is believed the pH effect is due to free radical formation subsequent to ozone decomposition in water.

On the basis of these findings, it was concluded that ozone has the potential for both effective reduction of the disease potential of influent and recycled water,

and for efficient elimination of ammonia and nitrite accumulation in reuse aquaculture systems.

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