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THE EFFECTS OF SIX ENVIRONMENTAL VARIABLES ON *PASTEURELLA MULTOCIDA* POPULATIONS IN WATER

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ABSTRACT: The effects of protein, pH, temperature, sodium chloride (NaCl), clays, sucrose, and their interactions on the survival and growth of *Pasteurella multocida* were evaluated. Pasteurellae populations declined rapidly in waters maintained at 2 C, compared to 18 C. Increasing water soluble proteins by 175 μg/ml, and NaCl by 0.5%, greatly enhanced survival of *P. multocida*, whereas variations in pH, clays, and sucrose had relatively minor effects. *Pasteurella multocida* survived for over 1 yr in some samples of water. This is the longest known survival of these bacteria in water.

Key words: Avian cholera, environmental microbiology, fractional factorial design, *Pasteurella multocida*, water microbiology, experimental study.

INTRODUCTION

Avian cholera has been recognized as a distinct disease since 1782 and has been identified as a serious problem among wildfowl in North America since 1944 (Rosen, 1971). In California, mortality typically ranges from 15,000 to 30,000 wildfowl each winter. Avian cholera has been reported among wildfowl on an irregular basis at six sites in Humboldt and Del Norte Counties (California, USA) since 1945 (Hazlewood et al., 1978; Oddo et al., 1978; Titche, 1979). Of these, the Centerville Gun Club has been the most commonly affected site.

The Centerville Gun Club (40°30′N, 124°10′W) is composed of low lying pasturelands adjacent to the Eel River Delta and is used for grazing cattle and sheep between January and October each year. Two temporary ponds, approximately 5.5 and 3.9 ha, respectively, lie within 700 m of the Pacific Ocean, and are used for waterfowl hunting from October to January. Their water depth does not exceed 1.2 m. Starting in September each year, the ponds are filled by water from an on-site well; this is supplemented by rainfall runoff. Recent studies at this site suggested that *Pasteurella multocida*, the bacterium causing avian cholera, could not survive well in soil or water, even during an avian cholera epornitic (Backstrand and Botzler, 1986).

Identifying the environmental factors influencing the survival and growth of *P. multocida* may provide insights for reducing the survival and transmission of these bacteria before and during avian cholera epornitics. Past studies suggest that *P. multocida* growth and survival are affected by protein (Rosen and Bischoff, 1950; Titche, 1979), pH (Dimov, 1964; Rosen, 1971), temperature (Rosen, 1971; Smith, 1974; Wilson and Miles, 1975), clays (Olson and Bond, 1969; Alexander, 1977), and sucrose (Carter and Bain, 1960). Due to the proximity of these ponds to the ocean, and their exposure to irrigation runoff water, we speculated that NaCl also may have an impact on any *P. multocida* populations in the water. However, there have been no studies evaluating the simultaneous effects and interactions of these environmental variables on *P. multocida* populations. The purpose of this study was to evaluate the effects of six environmental factors on the survival and growth of an avian strain of *P. multocida* in water, and to identify any interactions among the variables. The six variables were water soluble proteins, temperature, pH, NaCl, clays, and sucrose.

MATERIALS AND METHODS

A two-level fractional factorial design (Box et al., 1978) was used to evaluate the effects of

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TABLE 1. Adjustments of protein, pH, temperature, NaCl, clays and carbohydrates in a 2^{6-1} fractional factorial design experiment with pond water taken November 1984 from the Centerville Gun Club, Humboldt County, California.

Protein	
+	175 μg/ml protein[a] added
−	No protein added
pH	
+	Adjusted to pH of 6.3 (±0.05)
−	Adjusted to pH of 7.3 (±0.05)
Temperature	
+	Water maintained at 2 C
−	Water maintained at 18 C
NaCl	
+	5 g/liter NaCl added
−	No NaCl added
Clays	
+	0.374 g/liter added
−	No clays added
Sucrose	
+	2 g/liter sucrose added
−	No sucrose added

[a] Bacto-Beef Extract, Difco Laboratories, Detroit, Michigan 48232, USA.

protein, pH, temperature, NaCl, clays, and sucrose, as well as interactions between these variables, on the survival and growth of *P. multocida* in water (Tables 1, 2). Fractional factorial designs usually are employed in an iterative fashion during the exploratory stages of investigations. Each iteration is not meant to be a definitive study, but rather leads to more refined designs for future studies. We used this statistical model because there have been no in-depth studies of these factors and their interactions on the survival and growth of *P. multocida*. This was an exploratory study designed to provide a basis for further studies on the effects of environmental variables on *P. multocida* populations. For each environmental variable, two disparate values were chosen which were believed to reflect variation often encountered by *P. multocida* under natural conditions.

All protein concentrations were determined with a Spectronic 20 spectrophotometer (Baush and Lomb, Rochester, New York 14692, USA), at 500 nm, using the Folin-Lowry photometric assay (Clark and Switzer, 1977). Concentrations of NaCl were measured using a Buchler-Cotlove chloridometer, automatic titrator (Buchler Instruments Inc., Fort Lee, New Jersey 07024, USA). Clays were measured using adaptations of Black (1965), as reported by Bredy (1987).

TABLE 2. Variable combinations used in assessing the effects of environmental factors on the survival of *Pasteurella multocida* in water taken November 1984 from the Centerville Gun Club, Humboldt County, California.

	Variable					
Jar	Protein	pH	Temperature	NaCl	Clays	Sucrose
1	−[a]	−	−	−	−	−
2	+	−	−	−	−	+
3	−	+	−	−	−	+
4	+	+	−	−	−	−
5	−	−	+	−	−	+
6	+	−	+	−	−	−
7	−	+	+	−	−	−
8	+	+	+	−	−	+
9	−	−	−	+	−	+
10	+	−	−	+	−	−
11	−	+	−	+	−	−
12	+	+	−	+	−	+
13	−	−	+	+	−	−
14	+	−	+	+	−	+
15	−	+	+	+	−	+
16	+	+	+	+	−	−
17	−	−	−	−	+	+
18	+	−	−	−	+	−
19	−	+	−	−	+	−
20	+	+	−	−	+	+
21	−	−	+	−	+	−
22	+	−	+	−	+	+
23	−	+	+	−	+	+
24	+	+	+	−	+	−
25	−	−	−	+	+	−
26	+	−	−	+	+	+
27	−	+	−	+	+	+
28	+	+	−	+	+	−
29	−	−	+	+	+	+
30	+	−	+	+	+	−
31	−	+	+	+	+	−
32	+	+	+	+	+	+

[a] See Table 1 for definition of + and − values of each variable.

Total carbohydrate concentration was measured on a Spectronic 20 spectrophotometer, at 500 nm, using the Anthrone photometric assay of color (Plummer, 1978). All pH determinations were made with a Corning model 10 pH meter (Corning Scientific, Corning, New York 14830, USA), after all other materials required by the experimental design were added.

Surface water was collected from the center of the west, 5.5-ha pond on 26 November 1984. Analysis of the water for baseline values of these variables inadvertantly was not done. On 21 December 1985 (for protein and carbohydrates) and 16 January 1986 (for NaCl), the following

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values were recorded from a sample of this water that had been stored continuously at 2 C: protein—75 μg/ml; total carbohydrates—less than 5 μg/ml; NaCl—0.38 g/liter. No clays were present.

The combinations of variables were adjusted for jars 1 to 32 in 3.8-liter glass jars, using 2 liters of water, as outlined in Tables 1 and 2. Beef extract (Difco Laboratories, Detroit, Michigan 48232, USA) was added to jars requiring protein such that the water received an additional 175 μg/ml of protein. Limited amounts of 1 M HCl and 1 M KOH were used to change the pH in those samples requiring adjustment. Five g/liter NaCl was added to jars of water requiring salt. Clays were prepared (Black, 1965; Bredy, 1987) with soil collected in the summer of 1984 from the dried bottom of the west, 5.5-ha hunter pond and added to the appropriate water samples. Two g/liter sucrose was added to jars of water requiring carbohydrates. All jars were autoclaved for 45 min at 121 to 123 C. Those water samples held at 2 C were stored in a walk-in refrigerator. The samples held at room temperature were stored in a cabinet. All samples were kept in the dark. No further measurements of variables were made after sterilization.

A lyophylized strain of *P. multocida* (P3916, serotype 1) was obtained from the U.S.D.A. National Animal Disease Center (Ames, Iowa 50010, USA). This strain originally was isolated from the heart blood of a coot (*Fulica americana*) dying from avian cholera on the Centerville Gun Club in January 1979.

On 2 December 1984, 0.65 ml of a 24-hr culture of *P. multocida* in Brain Heart Infusion (BHI) broth was added to each of jars 1 to 16, resulting in a final concentration of 3.19×10^5 bacteria/ml of water. On 17 December, 0.65 ml of a 24-hr *P. multocida* culture in BHI broth was added to each of jars 17 to 32, resulting in a final dilution of 1.83×10^5 bacteria/ml of water. Although the numbers of pasteurellae added to the first set of jars was almost twice that added to the second set, we did not view this as a substantial logarithmic difference. All statistical analyses were based on logarithmic conversions of population estimates. No additional jars or controls were evaluated beyond those listed in Table 2.

Pasteurella multocida populations of each jar were determined daily for the first week, on alternate days during the second week, and once each week thereafter. Water was drawn from the top 5 cm of the water column and serial dilutions were cultured in duplicate on DAS medium (Das, 1958; Rosen, 1972). Water was not mixed before sampling.

Prior to onset of contamination, representative isolates from the inoculated water samples were visually compared to the original culture of *P. multocida* and tested by Gram stain, oxidase test and catalase test to support the visual observations that they were *P. multocida*. After contaminants first appeared, diagnosis of *P. multocida* for any colony that was questionable was based on the Gram stain; oxidase; catalase; motility at 37 C; fermentation of glucose, lactose, mannitol and rhamnose; production of H_2S, urease and indole; and growth on MacConkey Agar and Simmon's Citrate, as outlined in MacFaddin (1980).

The data were analyzed using the 1982 BMDP 2V computer package (BMDP Statistical Softwares, University of California, Los Angeles, California 90024, USA) on a Control Data Corporation 170/720 Cyber computer. The effects were calculated according to procedures developed by Box et al. (1978). An effect of a factor was defined as the change in response from the − to the + version of that factor. For example, if the log of the mean bacterial population with no protein added was 2.00, and with added protein the log was 3.50, the effect of protein would be +1.50.

An interaction between two variables occurred when the combination of variables resulted in significantly higher populations of *P. multocida* than would be expected from summing the log effects of the two variables individually. A half-fraction design such as used in this study results in a confounding of the main effects with higher order interactions; the other half of the fractional design would have to be conducted to resolve these confounding effects. We present two-factor interactions, but do not report third order or higher interactions, based on the evidence of Box et al. (1978) that these higher level interactions usually can be ignored. Although third-order interactions sometimes are statistically significant, it often is very difficult to assign a biological meaning to them.

The log effects of each variable and their interactions were determined for numbers of *P. multocida* over 14 days. Significant effects are reported for days prior to day 14, even though there is a risk of some dependence between daily measures of *P. multocida* in any given jar. Effects were not calculated after the fourteenth day due to the onset of contamination. It is not possible to get accurate bacterial counts on contaminated plates; the unwanted organisms could outgrow and overgrow the pasteurellae. However, the use of DAS medium greatly facilitated the estimation of *P. multocida* numbers in comparison to BHI and other general purpose media, probably owing to suppression of some con-

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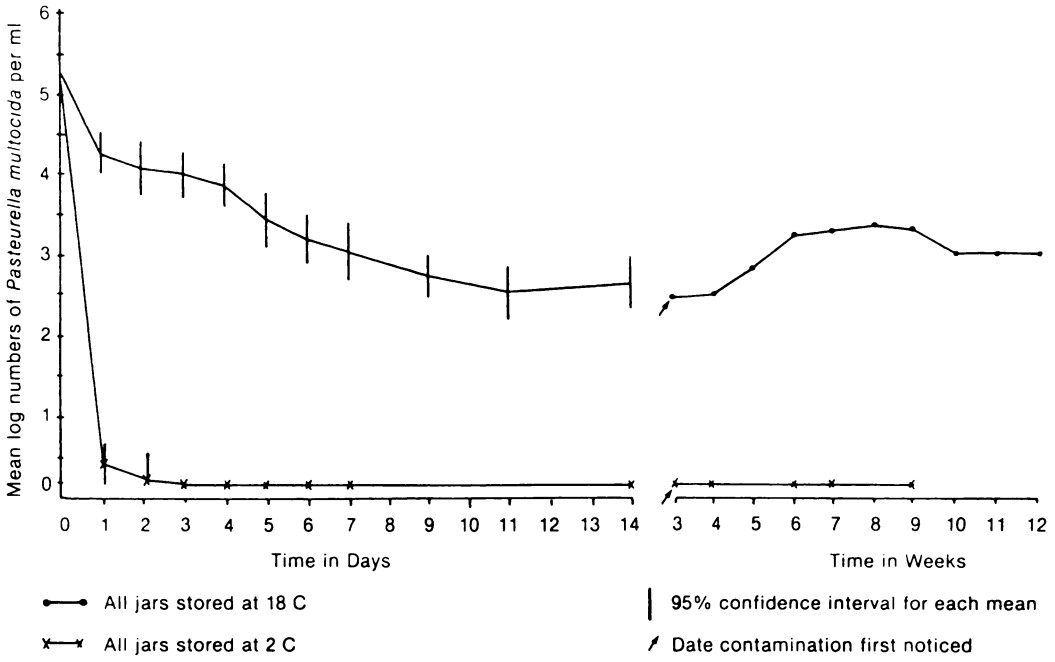


FIGURE 1. Survival of *Pasteurella multocida* at 2 C and 18 C in water taken from the Centerville Gun Club, Humboldt County, California, and inoculated on 2 or 17 December 1984.

taminants by the DAS medium (Das, 1958; Rosen, 1972). Population counts of *P. multocida* based on these contaminated plates are treated as minimum estimates of their true populations. The pH and the concentrations of proteins, salts and carbohydrates in the jars were not measured at the end of the study.

RESULTS

Main effects

Temperature was the single major factor affecting *P. multocida* numbers (Fig. 1). There were significantly ($P < 0.0001$) higher levels of *P. multocida* in the waters held at 18 C compared to bacterial levels in waters held at 2 C for days 1 to 14. Assuming that the population estimates of the pasteurellae in samples with evident contamination are minimum estimates, it appears that the influence of temperature was evident for at least 12 wk (Fig. 1).

The addition of 0.5% NaCl increased survival of *P. multocida* (Fig. 2). There were significantly ($P < 0.005$) higher populations of *P. multocida* in jars with added NaCl from days 1 to 14 compared to bacterial levels in jars without NaCl added. After contamination was evident, the minimum estimates of *P. multocida* appeared to decrease from weeks 3 through 12 in jars with salt, while increasing in jars without salt (Fig. 2).

Protein also had an impact on *P. multocida* survival. The *P. multocida* populations in jars to which 175 μg/ml of protein was added were significantly ($P < 0.05$) lower on day 1 and higher on day 9 compared to jars without added protein. There also were detectable differences ($P < 0.10$) on days 6, 7, and 14. After contaminants appeared, the influence of protein gradually increased through the twelfth week (Fig. 3).

Jars with water adjusted to a pH of 6.3 had significantly ($P < 0.05$) higher populations of *P. multocida* on days 1 and 3 only. Clays and sucrose had no significant ($P > 0.05$) effects over days 1 to 14.

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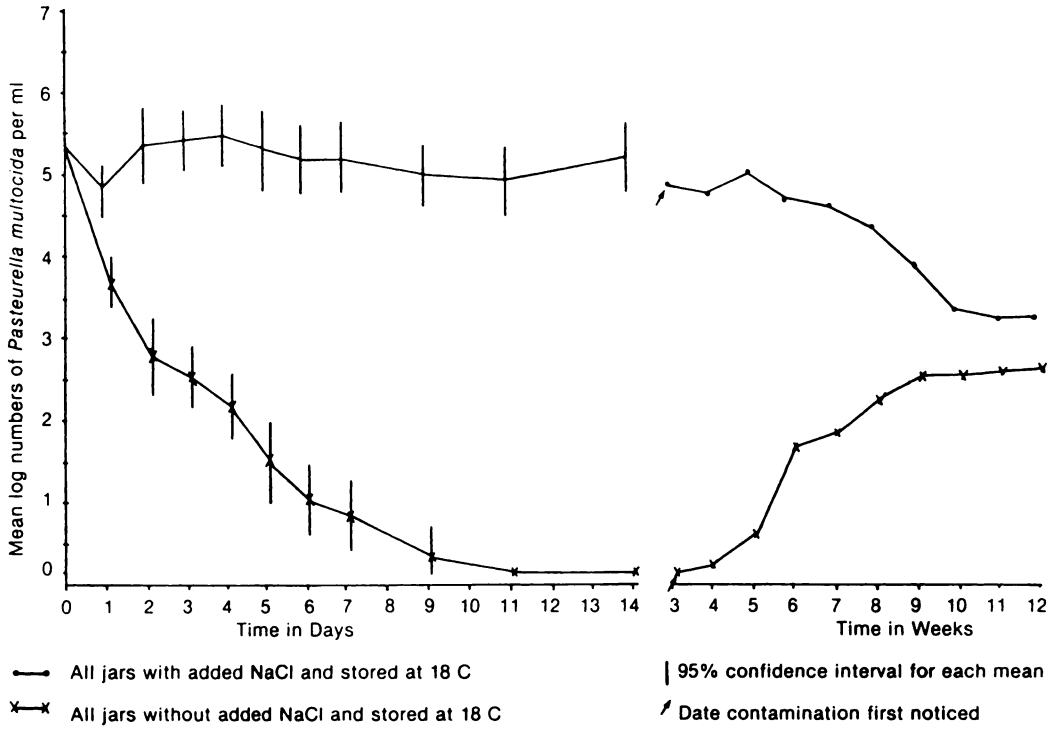


FIGURE 2. Survival of *Pasteurella multocida* with and without added 0.5% NaCl in water taken from the Centerville Gun Club, Humboldt County, California, inoculated on 2 or 17 December 1984, and incubated at 18 C.

Two-factor interactions

The interaction of temperature and NaCl had the greatest impact on numbers of *P. multocida*. Jars of water stored at 18 C, to which 0.5% NaCl was added, had significantly ($P < 0.005$) greater bacterial numbers on days 1 to 14, compared to jars at 2 C with no NaCl added. The interaction of an 18 C temperature and 175 μg/ml added protein had a significant ($P < 0.005$) impact on pasteurellae populations on day 9; there also were noticeable effects ($P < 0.10$) on days 6, 7, and 14. An 18 C temperature and 6.3 pH interaction resulted in higher *P. multocida* populations in water on day 1 ($P = 0.054$) and day 3 ($P < 0.10$).

The interaction of added protein and added NaCl to water resulted in significantly ($P < 0.05$) higher populations of *P. multocida* on days 3 and 4. There also were detectable effects ($P < 0.10$) on days 9 and 14. Increases in both protein and clays resulted in a detectable ($P < 0.10$) increase of *P. multocida* populations on day 3.

A 6.3 pH and 2 mg/ml added sucrose interaction resulted in significantly ($P < 0.05$) higher populations of *P. multocida* on day 7 in water. There also were detectable differences ($P < 0.10$) from days 3 to 6, and on day 9. A 6.3 pH and added NaCl interaction resulted in significantly ($P < 0.05$) higher levels of *P. multocida* on day 1. There was no significant ($P > 0.05$) effect of any other two-factor interaction on *P. multocida* populations from days 1 to 14.

DISCUSSION

Numerous authors (Olson and Bond, 1969; Titche, 1979; Price and Brand, 1984;

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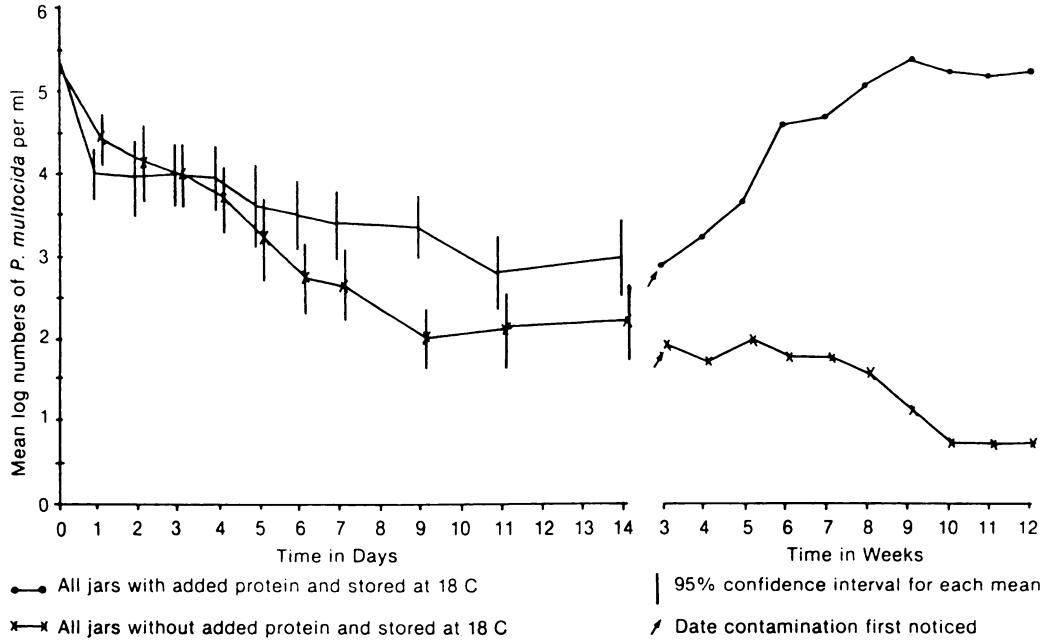


FIGURE 3. Survival of *Pasteurella multocida* with and without added protein in water taken from the Centerville Gun Club, Humboldt County, California, inoculated on 2 or 17 December 1984, and incubated at 18 C.

Backstrand and Botzler, 1986) have reported that *P. multocida* does not survive well in water. However, in this study, temperatures of 18 C, addition of 0.5% NaCl, and addition of 175 μg/ml protein were conditions very favorable for *P. multocida* survival. Survival of >1 yr occurred with the *P. multocida* in jars 11 and 26 (Table 2). The factors common to both jars were an NaCl supplement, storage at 18 C, and the presence of contaminating organisms. This is the longest known survival of *P. multocida* in water. Other reports of survival of *P. multocida* in pond and marsh water include 3 days (Price and Brand, 1984), 13 days (Backstrand and Botzler, 1986) and 30 days (Titche, 1979).

Compared to 2 C, a temperature of 18 C strongly favored ($P < 0.0001$) survival and growth of *P. multocida*. Therefore, in all interactions involving temperature, temperature was probably the major contributing factor. The poor survival of the bacteria at 2 C is surprising, since Dimov (1964) found the best survival of *P. multocida* occurred at 3 C in soils. However, others have reported the temperature range for growth of *P. multocida* as varying from 20 to 45 C (Rosen, 1971), 25 to 42 C (Smith, 1974), and 12 to 43 C (Wilson and Miles, 1975). These latter reports tend to support our finding that 2 C does not support *P. multocida* populations.

The clay did not appear to affect the numbers of *P. multocida*. However, the water jars were not mixed prior to sampling. Many bacteria adhere to clay particles. It is possible that the water samples with clay had more *P. multocida* than indicated in these results if the pasteurellae were attached to clay particles on the bottom of the jars.

With the poor survival observed for *Pasteurella multocida* at low temperatures, it is odd that the majority of avian cholera mortality in waterfowl is reported during the winter. However, the dense concentrations of waterfowl on the wintering

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grounds may allow for a transmission of the pasteurellae that is efficient enough to offset their poor survival in water at low temperatures. Further, other variables not considered in this study may play an important role. For example, Windingstad et al. (1984) found that specific conductance, calcium hardness and magnesium hardness were higher in water samples taken from avian cholera problem areas in Nebraska (USA), compared to nonproblem areas. They also reported high sulfate levels in the water of some avian cholera problem sites. Later, Windingstad et al. (1988) speculated that low concentrations of calcium and magnesium in water may contribute to reducing avian cholera mortality.

Although not formally evaluated by this study, other microorganisms also may have significant impacts on *P. multocida* growth. Observed numbers of *P. multocida* generally were higher after microbial contamination became evident. For example, after 2 wk with no detectable pasteurellae in water samples lacking NaCl, *P. multocida* again became detectable with the onset of contaminants (Fig. 2). Contaminants included molds tentatively identified as *Rhizoctonia* sp. and *Paecilomyces* sp., and bacteria tentatively identified as *Acinetobacter* sp. and *Aerococcus* sp. (Bredy, 1987).

The decreasing effect of NaCl on *P. multocida* populations over time (Fig. 2) may have been due to an increase of salts produced as metabolic by-products by contaminants in the waters with no added salt. No attempt was made to analyze these latter samples for the presence of salts. The role of other microorganisms on the survival and growth of *P. multocida* in aquatic systems deserves future study.

In the sense that our jars served as small microhabitats for *P. multocida*, we speculate that environmental factors enhancing *P. multocida* survival would not need to be present everywhere in an environmental system to support an avian cholera epornitic. Smaller pockets of habitat with appropriate ambient conditions could enhance *P. multocida* survival and growth.

Based on our findings, keeping water used by waterfowl as cold as possible and reducing addition of protein material and salt could help reduce the levels of *P. multocida* in water.

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