

## Research Note

**Survival and Growth Potential of *Aeromonas hydrophila* in Reconditioned Pork-Processing-Plant Water<sup>†</sup>**

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(MS# 95-254: Received 6 October 1995/Accepted 26 January 1995)

**ABSTRACT**

The growth and survival of *Aeromonas hydrophila* K144 was studied in reconditioned pork-processing-plant water. Neutralization of residual chlorine by thiosulfate permitted growth and long-term survival of the bacterium at temperatures ranging from 5 to 28°C; growth was also observed at 37 and 42°C but survival times were shorter. The coliform growth response, a bioassay system to measure the amount of nutrients available for microbial growth, for the reconditioned water was  $2.91 \pm 0.61$ , which agreed with our observation that this water contained sufficient nutrients to support about 3 log units of growth of *A. hydrophila*. Our results indicate that residual chlorine levels are necessary to prevent the growth of any *A. hydrophila* which might contaminate reconditioned water.

Key words: Pathogen survival, pathogen growth, reused water, *Aeromonas hydrophila*, pork processing

Members of the *Aeromonas hydrophila* group occur widely in the aquatic environment (4, 5, 6, 9). Their numbers vary widely and are a function of both trophic state (14, 15) and temperature (3, 11, 12, 20). Research has determined that these bacteria can survive long periods (7) and often grow in water; this survival and growth is a function of various factors such as nutrient types and levels (16–19). The roles of *A. hydrophila* and *A. sobria* as foodborne pathogens were reviewed by Buchanan and Palumbo (2).

Water conservation and water use in food-processing plants is of increasing concern, because the increased initial cost of water and sewage fees are based on the volume and biological oxygen demand of the waste water released from

the plant. To reduce usage and costs, various food-processing plants are reconditioning waste water and then using this reconditioned water for different food-processing operations. We investigated the safety of using reconditioned water in certain pork slaughter operations (8). Specifically, the meat plant operates a water-treatment plant on their premises and purifies the water by pH adjustment, dissolved air flotation, denitrification, nitrification, clarification, sand filtration, and chlorination. This reconditioned water is used primarily for carcass scalding and dehairing. We determined that the bacteriological quality (pathogen level and total aerobic plate count) of carcasses was similar whether they were washed with potable or reconditioned water (8).

In view of the widespread occurrence of *A. hydrophila* in the aquatic environment, its ability to grow and survive in many water types, its putative role as a human pathogen, and its known role as a food-spoilage bacterium, we investigated the growth and survival potential of *Aeromonas hydrophila* in reconditioned water from this pork packing plant and determined the influence of temperature and residual chlorine levels. Previous work (8) showed that *A. hydrophila* generally was not present in reconditioned water from the plant or was present at very low levels; our study would simulate the fate of this bacterium if it were to contaminate reconditioned water.

**MATERIALS AND METHODS***Organism*

*Aeromonas hydrophila* K144 from the Microbial Food Safety Research Unit's culture collection was used as a representative strain. All experimental samples were inoculated from a starter flask of brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI) incubated overnight (ca. 18 h) at 28°C and agitated at 100 rpm. Before inoculation into the different waters, the cultures were centrifuged for 10 min at setting 5 in an IEC clinical centrifuge (International Equipment Co., Needham, MA), washed once with sterile distilled water, and diluted to the desired concentration ( $10^5$  CFU/ml as determined by plate counting on

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<sup>†</sup> Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TSA, see below) with sterile distilled water; this formed the inoculum.

#### Water samples

Potable (P) municipal drinking water from the plant's supply, and reconditioned water samples, before (NC) and after (R) chlorination, were obtained from the water-treatment facility at Hatfield Quality Meats (Hatfield, PA). Samples (at least 1 liter) were collected in sterile polypropylene containers and stored at 5°C until used (usually within 24 h of collection).

#### Experimental procedure

Portions of each water type (700 ml) were transferred to sterile glass containers. To selected samples of either potable or chlorinated reconditioned water, 0.7 ml of a 10% (wt/vol) solution of sodium thiosulfate was added to 700 ml of water to yield a final concentration of 0.01% (100 mg/l) (1); this amount will neutralize ca. 15 mg/l of residual chlorine. Some nonchlorinated water samples were filter sterilized (0.22- $\mu$ m-pore-size filter Nalge NYL 153) to remove the background microflora. Aliquots (49.5 ml) were transferred to sterile polypropylene tubes (Falcon) and 0.5 ml of the inoculum was added to yield a starting count of ca.  $10^3$  CFU/ml. Samples were then incubated at 5, 12, 19, 28, 37, or 42°C. At intervals appropriate to the experiment, aliquots of each water sample were removed, diluted as needed in 0.1% peptone water, and surface plated (Spiral plater, Model D, Spiral Biotech, Bethesda, MD) on tryptic soy agar (TSA) (Difco) and starch ampicillin (SA) agar (10), and incubated for 24 h at 28°C. Colonies on TSA for total aerobic plate count were counted with a laser counting system (Spiral Biotech) and amylase-positive colonies on starch ampicillin agar plates (*Aeromonas hydrophila*) were determined by the addition of Lugol's iodine as described by Palumbo et al. (10).

#### Water analysis

The nutrient content of the various water samples used in these studies was determined by the coliform growth response (CGR) as described and performed by Rice et al. (13). In this procedure, the water to be analyzed is inoculated with a strain of *Enterobacter cloacae* and incubated at 20°C, and viable counts are determined at the beginning and end of the 5-day incubation. CGR is determined by the formula  $\log(N_5/N_0)$ , where  $N_5$  is the number of CFU per ml at day 5 and  $N_0$  is the number of CFU per ml at day 0. According to Rice et al. (13), a CGR  $\geq 1$  indicates that the water can support microbial growth.

## RESULTS AND DISCUSSION

Although several bacteria might have been selected for a study of their growth and survival potential in water, *A. hydrophila* was chosen because of its longstanding association with many types of water and its ready isolation from various water supplies. Though the reconditioned water studied previously was shown to contain few or no *A. hydrophila* (8), the potential exists for recontamination to occur. The putative role of *A. hydrophila* as a human pathogen (2) and its known role as a spoilage bacterium further supported our choice of *A. hydrophila* in this study of growth and survival potential in reconditioned water.

Previous studies indicated the necessity of adding sodium thiosulfate to the reconditioned water to neutralize any residual chlorine and permit determination of its bacte-

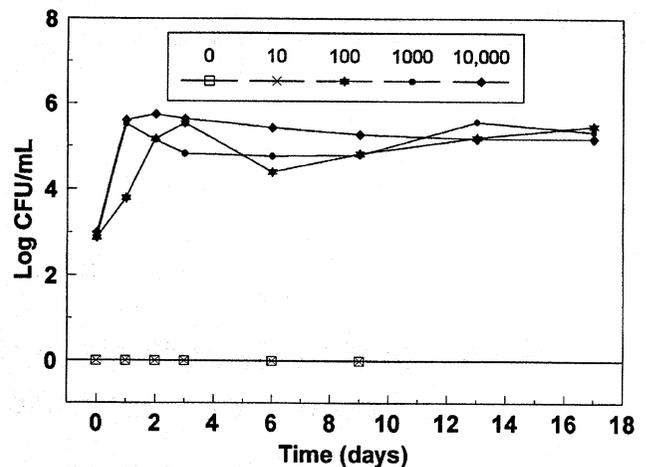


FIGURE 1. Effect of sodium thiosulfate concentration on the growth and survival of *A. hydrophila* K144 in reconditioned water held at 19°C; counts on SA agar. Levels of sodium thiosulfate, mg/l: 0, 10, 100, 1,000, 10,000.

rial content (8). The possibility thus existed that residual chlorine in the reconditioned water might interfere with the survival of *A. hydrophila*. This possibility was investigated in a dose-response study (Fig. 1). Different levels of sodium thiosulfate were added to reconditioned water and the growth and/or survival of the bacterium determined over time. These data indicate that 100 mg/l, the amount recommended in *Standard Methods for the Examination of Water and Wastewater* (1), is adequate and this amount was utilized in the rest of this study whenever thiosulfate was added; amounts in excess of this level did not permit greater recovery and growth of the bacterium.

Representative data are presented in Figures 2 and 3. Each study was repeated twice and some three times and similar responses were observed. However, because sampling times differed and the reconditioned water samples (both chlorinated and nonchlorinated) were different with respect to organic content, it was not possible to analyze the data statistically.

After determining the optimum level of thiosulfate for growth and recovery of *A. hydrophila*, the three water types (P, R, NC) in all combinations (with and without thiosulfate; with and without *A. hydrophila*; nonchlorinated with and without filtration through a 0.22- $\mu$ m-pore-size filter to remove the background flora, both living and dead) were screened for the response of the bacterium. Data for the three water types held at 12°C are shown in Figure 2. These data indicate that the following, when inoculated with *A. hydrophila*, supported growth and long-term survival of the bacterium: nonchlorinated unfiltered (NCA) and filtered (NCF) water, and reconditioned water plus thiosulfate (RTA). There also appeared to be a resident flora of presumptive *Aeromonas* (amylase positive on the selective SA medium) in the nonchlorinated water and this natural *Aeromonas* flora increased during incubation at temperatures of 37°C and below. Based on these observations, only RTA, NC, NCA, and NCF were further tested.

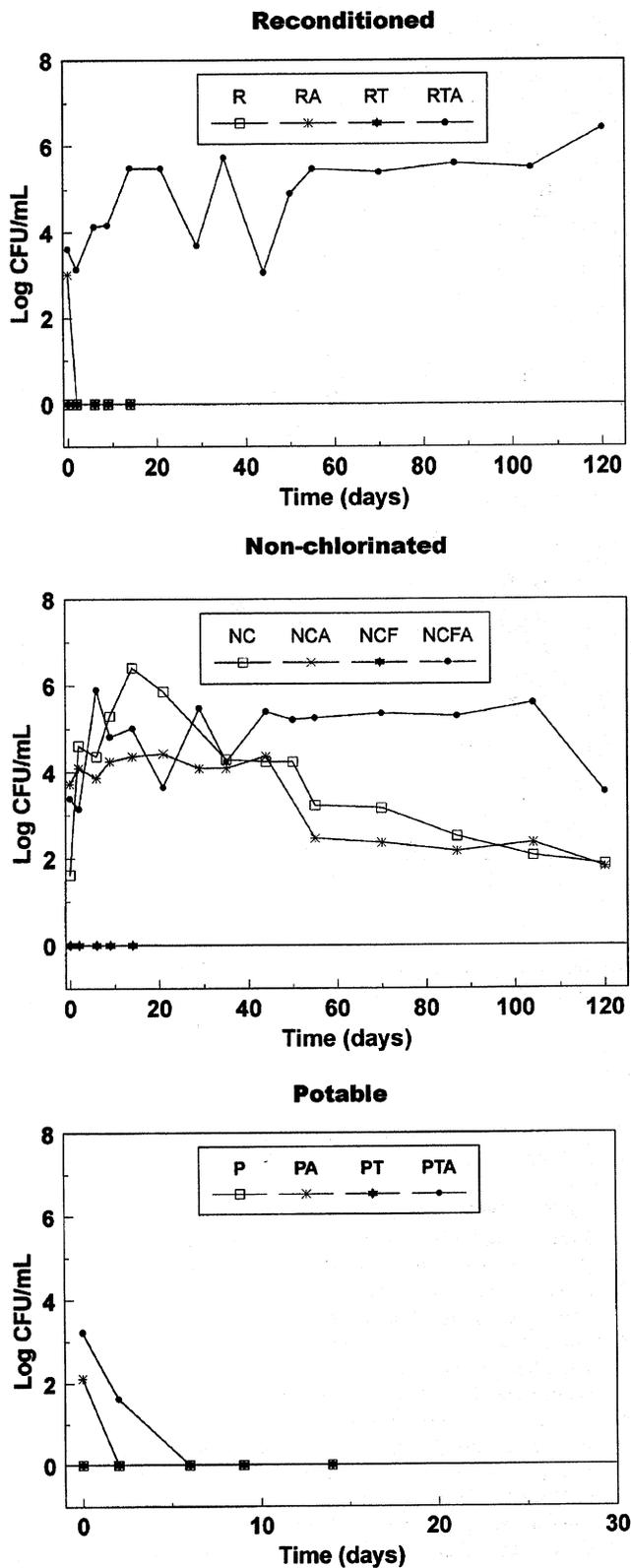


FIGURE 2. Growth and survival of *A. hydrophila* K144 in various waters from the pork-processing plant water-treatment facility; waters held at 12°C; counts on SA agar. P, potable; R, reconditioned; NC, nonchlorinated; F, filtered through 0.22- $\mu$ m-pore-size filter; A, inoculated with *A. hydrophila* K144; T, 100 mg/l sodium thiosulfate added.

The influence of multiple factors (temperature, presence and/or absence of background flora, water type) on the growth and survival of *A. hydrophila* is shown in Figure 3. As was shown in Figure 2, only RTA, NC, NCA, and NCFA permitted consistent growth and survival; potable water was included as a negative control and as shown in Figure 2, *A. hydrophila* rapidly declined in number. While because of sampling times it was not possible to determine which temperature supported the fastest growth, survival tended to be longer at temperatures of 19°C and below. Temperatures higher than 28°C (37 and 42°C) supported growth but shorter survival (data not shown). Since higher temperatures would not likely be encountered except during the lethal temperatures of sanitation, only temperatures between 5 and 28°C were evaluated. The detection of the bacterium in the samples without added *A. hydrophila* was not unanticipated. In our previous study (8), very low levels of *A. hydrophila* were detected in reconditioned water. There are conflicting reports on the resistance of *A. hydrophila* to chlorine, and some investigators have detected the bacterium in chlorinated drinking water. The level of *A. hydrophila* in NC was always lower than in the NCA samples, indicating that the natural flora aeromonads did not interfere with the detection of the bacterium.

It can be seen in both Figures 2 and 3 that filtering the nonchlorinated reconditioned water to remove the background bacteria contributed to the long-term survival of the bacterium. This suggests that the background flora of the water was competing with *A. hydrophila* for the nutrients in the reconditioned water.

Using the coliform growth response (CGR) technique to measure the amount of nutrients potentially available for microbial growth, various reconditioned (R) and nonchlorinated (NC) water samples, those used in experiments presented (Fig. 1, 2, and 3) as well as in other studies not shown, were analyzed. For 17 samples, the CGR was  $2.90 \pm 0.61$ . According to Rice et al. (13), this CGR indicates that the water is capable of supporting microbial growth. The CGR determined for the 17 samples, 2.90, supports our observation of an approximately 3 log-unit increase in numbers of *A. hydrophila* seen in these studies. This agreement of the CGR value with the observed amount of growth of *A. hydrophila* K144 suggests that *A. hydrophila* K144 has nutritional requirements similar to *E. cloacae*. The potable water used as the negative control had a CGR of  $<0.50$  and therefore should not support growth (see Fig. 2).

In conclusion, the data presented here indicate that the reconditioned water from this meat-processing plant can support the growth of *A. hydrophila* K144 if the residual chlorine is neutralized or if the reconditioned water is used without chlorination. This emphasizes the importance of residual chlorine levels in this water. Survival is increased at low temperatures and survival times of  $>120$  days have been observed (data not presented). Reconditioned water can be a viable alternative for certain operations in the processing plant, but residual chlorine levels must be maintained to prevent regrowth of *A. hydrophila* as well as other pathogens which might recontaminate this water.

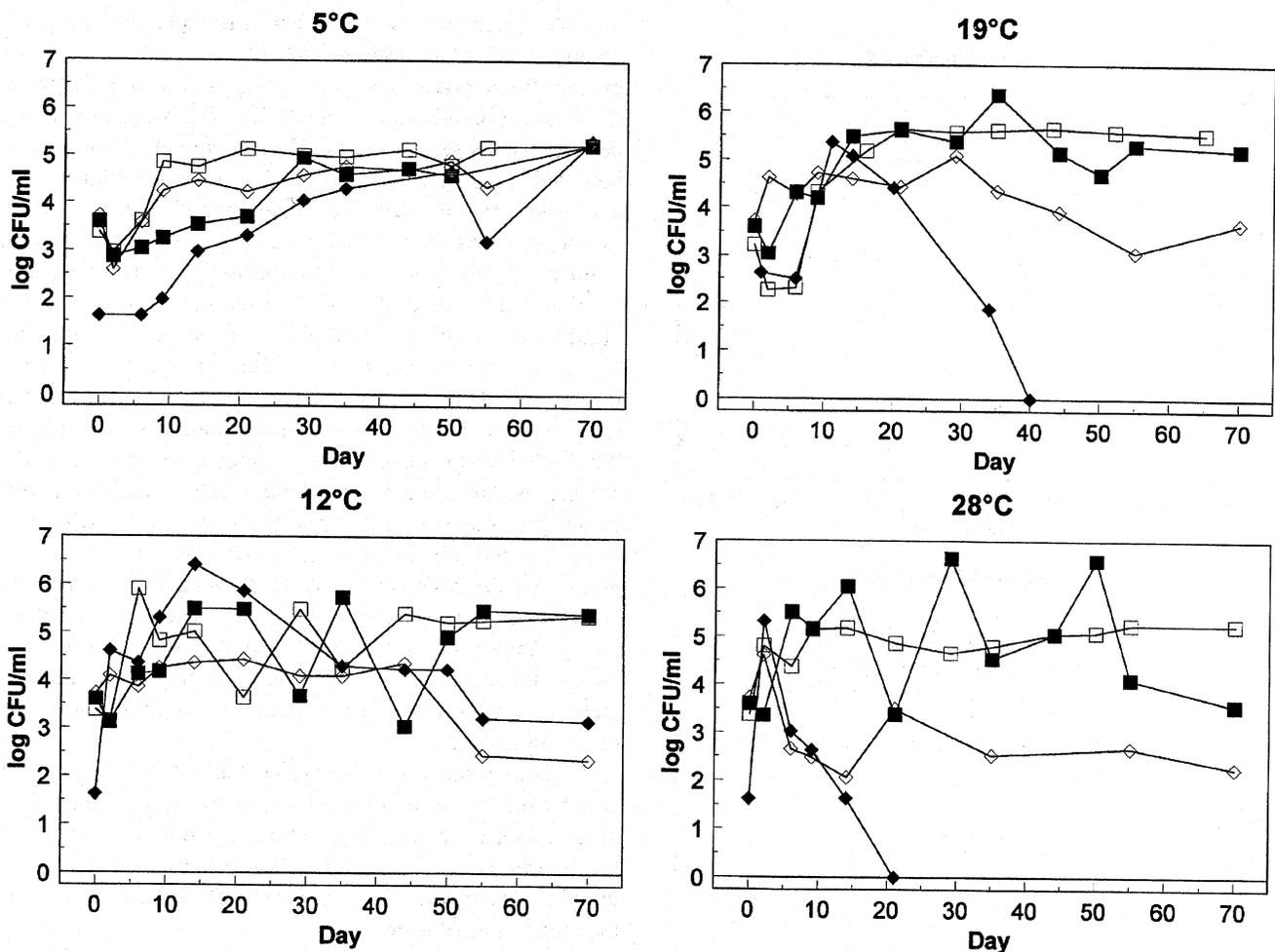


FIGURE 3. Influence of temperature and water type on the growth and survival of *A. hydrophila* K144; counts on SA agar. A, inoculated with *A. hydrophila* K144; T, 100 mg/l sodium thiosulfate added; R, reconditioned; NC, nonchlorinated; F, filtered (sample filtered through a 0.22  $\mu\text{m}$ -pore-size filter). RTA, ■; NC, ◆; NCA, ◇; NCF, □.

Residual chlorine will also suppress the natural flora *A. hydrophila*.

#### ACKNOWLEDGMENTS

We thank Eugene Rice of the EPA, Cincinnati, Ohio, for performing the coliform growth-response assays and Allan Pickard for technical assistance.

#### REFERENCES

1. A. E. Greenberg, R. R. Trussell, and L. S. Clesceri (ed.). 1986. Standard methods for the examination of water and wastewater, p. 975. American Public Health Association, Washington, D.C.
2. Buchanan, R. L., and S. A. Palumbo. 1985. *Aeromonas hydrophila* and *Aeromonas sobria* as potential food poisoning species: a review. *J. Food Safety* 7:15-29.
3. Burke, V., J. Robinson, M. Gracey, D. Peterson, and K. Partridge. 1984. Isolation of *Aeromonas hydrophila* from a metropolitan water supply: seasonal correlation with clinical isolates. *Appl. Environ. Microbiol.* 48:361-366.
4. Hazen, T. C., C. R. Fliermans, R. P. Hirsch, and G. W. Esch. 1978. Prevalence and distribution of *Aeromonas hydrophila* in the United States. *Appl. Environ. Microbiol.* 36:731-738.
5. Knochel, S. 1990. Growth characteristics of motile *Aeromonas* spp. isolated from different environments. *Int. J. Food Microbiol.* 10:235-244.
6. LeChevallier, R., J. Seidler, and T. M. Evans. 1980. Enumeration and characterization of standard plate count bacteria in chlorinated and raw water supplies. *Appl. Environ. Microbiol.* 40:922-930.
7. Lowcock, D., and C. Edwards. 1994. Survival of genetically-marked *Aeromonas hydrophila* in water. *Lett. Appl. Microbiol.* 19:121-123.
8. Miller, A. J., F. J. Schultz, A. Oser, J. L. Hallman, and S. A. Palumbo. 1994. Bacteriological safety of swine carcasses treated with reconditioned water. *J. Food Sci.* 59:739-741, 746.
9. Nakano, H., T. Kameyama, K. Venkateswaran, H. Kawakami, and H. Hashimoto. 1990. Distribution and characterization of hemolytic, and enteropathogenic motile *Aeromonas* in aquatic environments. *Microbiol. Immunol.* 34:447-458.
10. Palumbo, S. A., F. Maxino, A. C. Williams, R. L. Buchanan, and D. W. Thayer. Starch-ampicillin agar for the quantitative detection of *Aeromonas hydrophila*. *Appl. Environ. Microbiol.* 50:1027-1030.
11. Pathak, S. P., J. W. Bhattacharjee, N. Kalra, and S. Chandra. 1988. Seasonal distribution of *Aeromonas hydrophila* in river water and isolation from fish. *J. Appl. Bacteriol.* 65:347-352.
12. Rhodes, M. W., and H. Kator. 1994. Seasonal occurrence of mesophilic *Aeromonas* as a function of biotype and water quality in temperate freshwater lakes. *Water Res.* 28:2241-2251.
13. Rice, E. W., P. V. Scarpino, G. S. Logsdon, D. J. Reasoner, P. J. Mason, and J. C. Blannon. 1990. Bioassay procedure for predicting

- coliform bacterial growth in drinking water. Environ. Technol. 11:821-828.
14. Rippey, S. R., and V. J. Cabelli. 1985. Growth characteristics of *Aeromonas hydrophila* in limnetic waters of varying trophic state. Arch. Hydrobiol. 104:311-319.
  15. Rippey, S. R., and V. J. Cabelli. 1989. Use of the thermotolerant *Aeromonas* group for the trophic state classification of freshwaters. Water Res. 23:1107-1114.
  16. Rippey, S. R., M. A. Troy, and V. J. Cabelli. 1994. Growth kinetics of *Aeromonas hydrophila* in freshwaters supplemented with various organic and inorganic nutrients. World J. Microbiol. Technol. 10:159-164.
  17. van der Kooij, D. 1991. Nutritional requirements of Aeromonads and their multiplication in drinking water. Experimentia 47: 444-446.
  18. van der Kooij, D., and W. A. M. Hijnen. 1988. Nutritional versatility and growth kinetics of an *Aeromonas hydrophila* strain isolated from drinking water. Appl. Environ. Microbiol. 54:2842-2851.
  19. van der Kooij, D., A. Visser, and W. A. M. Hijnen. 1980. Growth of *Aeromonas hydrophila* at low concentrations of substrates added to tap water. Appl. Environ. Microbiol. 39:1198-1204.
  20. Williams, L. A., and P. A. LaRock. 1985. Temporal occurrence of *Vibrio* and *Aeromonas hydrophila* in estuarine sediments. Appl. Environ. Microbiol. 50:1490-1495.