

Growth of *Salmonella* spp. and *Vibrio cholerae* in Reconditioned Wastewater[†]

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ABSTRACT

Many food-processing plants are looking to increase the use of reconditioned water beyond the currently approved uses for initial cleaning (vegetables) and scalding water (meat and poultry). The preliminary survey showed that the reconditioned water from a local meat plant could support bacterial growth. The growth potential of *Salmonella* spp. and *Vibrio cholerae* (starting level of 3 to 4 log CFU/ml) in the reconditioned wastewater from this plant (with and without added thiosulfate) was studied at temperatures from 5 to 42°C. Bioassays for the assimilable organic carbon and coliform growth response suggest that this reconditioned water contained sufficient nutrients to support bacterial growth. Both pathogens grew in the unchlorinated reconditioned and chlorinated reconditioned water containing 10 mg of sodium thiosulfate per ml to neutralize the residual chlorine. Cell counts declined rapidly in chlorinated water without thiosulfate. The results of this study emphasized the importance of maintaining residual chlorine levels (0.2 mg/liter) in both reconditioned and potable waters to prevent pathogen growth.

Key words: *Salmonella*, *V. cholerae*, reconditioned water, bacterial growth

As communities increase in size, residential communities and neighboring industries compete for the use of existing water resources. In addition, environmental capacity to handle untreated wastewater is becoming limited (7). Increased requirements by regulatory agencies to reduce the biological oxygen demand and total solids of wastewater has encouraged expansion in wastewater reclamation. Wastewater reclamation was reported by Miller (13) as a means to decrease pollution and to supplement available resources. Reconditioned water is currently being used for agricultural and urban landscape irrigation and for selected industrial purposes (13, 17). The food industry is looking to increase reconditioned wastewater usage beyond that already approved.

The potential survival and/or growth of waterborne bacterial pathogens in the reconditioned wastewater used by the food industry is a public health concern. Black and Finch (3) reported survey results on the occurrence of waterborne bacterial pathogens. *Salmonella* spp. survived and were recovered from surface waters (3, 9), wastewater (9, 16), sea water (14) and bottled water (21). *Vibrio* spp. were found in drinking water (3, 8), surface water (5, 8, 9, 15), and ship ballast, bilge and sewage (10). As awareness increases that these pathogens may survive and grow in waters, Rice et al. (18) developed a bioassay procedure to assess the potential of drinking water to support coliform bacterial growth.

The objective of this study was to assess the ability of reconditioned wastewater to support the growth of *Salmonella* spp. and *Vibrio cholerae* and to correlate the water-quality bioassay levels of coliform growth response (CGR) and assimilable organic carbon (AOC) to the growth potential of the reconditioned wastewater.

METHODS AND MATERIALS

Microorganisms

Growth determination. A three-strain mixture of either *Salmonella* spp. or *Vibrio cholerae* was used for the growth studies. *Salmonella enteritidis* was obtained from the Microbial Food Safety Research Unit culture collection. *Salmonella typhimurium* 798 and *Salmonella choleraesuis* 38 were obtained from the National Animal Disease Center, Ames, Iowa. The stock cultures of each strain were maintained in brain heart infusion broth (BHI) Difco Laboratories, Inc., Detroit, MI) and stored at 4°C. *Vibrio cholerae* strains N16961-O1 Inaba-El Tor-toxigenic, O139 (non-O1)-1837, and O1-569B were obtained from the FDA, Washington, D.C. The stock cultures of each strain were maintained in BHI broth and stored at room temperature.

Overnight cultures of the three individual strains of the *Salmonella* spp. or *Vibrio* spp. were cultured by transferring 0.1 ml of each culture to 50 ml of BHI broth contained in a 250-ml Erlenmeyer flask and placed on a rotary shaker at 150 rpm (Model 3520, Lab-Line Instrumentation, Inc., Melrose Park, IL) at 37°C. The overnight cultures were centrifuged at 3300 × g for 15 min to concentrate the cells and the supernatant fluid was decanted and discarded. The cells were washed once to remove any nutrients in sterile deionized water and then resuspended in sterile deionized water. The three cultures of *Salmonella* spp. or *Vibrio* spp. were

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combined and diluted in sterilized deionized water to yield a concentration of 10^5 to 10^6 CFU/ml. A final dilution to yield 3 to 4 log CFU/ml was made in the reconditioned test water sample.

Microorganisms for bioassay determinations. *Enterobacter cloacae* was used for the coliform growth response (CGR) bioassay. *Pseudomonas fluorescens* P-17 and *Spirillum* NOX were used to determine the assimilable organic carbon (AOC). The inoculum was prepared as described in *Standard Methods for the Examination of Water and Wastewater* (1).

Experimental design

Indigenous microflora background study. During the summer of 1993, once a week for 5 weeks potable (as the control) and chlorinated reconditioned waters were obtained in the morning from a local pork-processing plant equipped with a wastewater treatment facility (12). The plant's potable water served as the control. Residual chlorine was neutralized in half of the potable and chlorinated reconditioned wastewater by the addition of 0.01% (wt/vol) sodium thiosulfate (Sigma Chemical Co., St. Louis, MO). The neutralized and nonneutralized water samples were surface plated using a Spiral Plater (Spiral Systems, Inc., Cincinnati, OH) on standard plate count (SPC), Hektoen enteric (HEKT), and MacConkey (MAC) agars (Difco). All plates were examined after 24 h of incubation at 37°C for the SPC, HEKT, and MAC plates and after 48 h for MAC plates only. The water samples were divided and transferred into 50-ml tubes before being incubated without shaking at 5, 19, and 37°C for up to 6 weeks. Samples were surface plated weekly on the three agars to determine background indigenous microflora.

Growth potential study I. Potable and reconditioned (unchlorinated and chlorinated) waters were obtained on the morning the study began. Portions of the potable and chlorinated reconditioned waters were neutralized with 0.01% (wt/vol) sodium thiosulfate. An aliquot of the unchlorinated reconditioned water was sterilized using a 0.2- μ m pore size Nalgene® filter (Nalge Co., Rochester, NY). Each of 5 different water samples were inoculated with the same three-strain mixed culture of *Salmonella* spp. or *Vibrio* spp. to achieve a starting level of 3 to 4 log CFU/ml. The inoculated water samples were immediately assayed, and then incubated at 5, 12, 19, 28, 37, and 42°C for *Salmonella* spp. and 5, 12, 19, 28, and 37°C for *Vibrio* spp. The water samples were surface plated for *Salmonella* spp. on double-modified lysine iron agar (DMLIA, Oxoid, England) (2) and for *Vibrio* spp. on TCBS cholera medium (TCBS, Oxoid). The plates were incubated at 37°C for 18 to 24 h before enumeration.

Growth potential study II. Chlorinated and unchlorinated reconditioned waters were collected on the day the study started. Samples of the waters were shipped on ice to the Environmental Protection Agency Cincinnati, OH laboratory for bioassay studies (CGR and AOC). A liter of the unchlorinated reconditioned water was filter sterilized using a 0.2- μ m pore size filter (Nalgene®) and inoculated with either the mixed culture of *Salmonella* spp. or *Vibrio* spp. to achieve a starting level of 3 to 4 log CFU/ml. After mixing, 12 ml of the inoculated unchlorinated reconditioned water was distributed into a duplicate set of sterile "L"-shaped test tubes placed in the temperature-gradient incubator (Model TN-3F, Advantec, Toyo Roshi Inter., Co., Dublin, CA) with the gradient set between 3.5 and 55.3°C. Growth was monitored by plating on tryptic soy agar (TSA, Difco) and incubating the plates at 37°C for 18 to 24 h before counting. The actual gradient temperature range was verified at the end of each study using the thermocouple sensor fitted in the gradient incubator. The growth study for each microbe was repeated two times.

Viability of the culture during the growth studies was deter-

mined by the LIVE/DEAD *BacLight*™ Viability Kit (Molecular Probes, Inc. Eugene, OR). The cells were prepared according to the recommended procedure of concentrating by centrifugation $2000 \times g$ for 15 min, resuspending in filtered (0.2- μ m pore size filter) sdw, and staining with the mixture of *BacLight*™ nucleic acid stains. The stained cells were viewed under an Olympus BH2 Epifluoresce microscope (Olympus, Tokyo, Japan) fitted with a blue dichroic filter for a wavelength of 490 nm.

Bioassay studies. For the CGR and AOC bioassay studies the test water samples were heated to 70°C for 30 min and inoculated with the test organisms (CGR, *E. cloacae*; AOC, *P. fluorescens* P17 and *Spirillum* NOX) as described in reference (1). The CGR level was determined by log transformation of the ratio between the level of bacteria present at the end of the 5-day incubation period (N_5) versus the initial level (N_0): $CGR = \log(N_5/N_0)$.

Values for AOC measurements were based upon the empirical growth yields of *P. fluorescens* P17 and *Spirillum* NOX and reported as micrograms of acetate carbon equivalents per liter for strain P17 and as micrograms of oxalate carbon equivalents for strain NOX. AOC values were calculated from the published yield factor of 6.613 log CFU/ μ g acetate carbon equivalents for P17 and 6.462 log CFU/ μ g oxalate carbon equivalents for NOX (1).

RESULTS

Indigenous microflora background study. No growth was observed for the weekly chlorinated reconditioned and potable water samples (neutralized or nonneutralized) when plated on SPC, HEKT, and MAC agars (data not shown). Each week the 4 water samples were split and incubated at 5, 19, and 37°C. No growth was observed when any of the water samples were plated on the HEKT or MAC agars after 6 wks incubation. A mixed microflora was observed on SPC agar after incubation for 6 weeks. No attempt was made to identify the observed background microflora.

Growth potential study I. Neither *Salmonella* spp. nor *Vibrio* spp. were recovered from the inoculated potable or chlorinated reconditioned waters after incubation at 5 to 37°C for up to 15 days, indicating that the plant has no problems maintaining water safety under current chlorination conditions. The results of the recoverable *Salmonella* spp. and *Vibrio* spp. from the inoculated neutralized potable water (PT), neutralized chlorinated reconditioned water (RT), and filtered unchlorinated reconditioned (FUR) water are presented in Table 1. The PT water did not support growth, as defined as an increase of 1 log CFU/ml, for the inoculated *Salmonella* spp. or *Vibrio* spp. after incubation for up to 22 and 15 days, respectively. *Salmonella* survived in low numbers from PT samples incubated at 5, 12, 19, and 28°C, where as *Vibrio* did not survive in any of the inoculated PT water samples. The RT water supported growth of *Salmonella* when incubated at 28 and 37°C and the FUR at 19, 28, and 37°C. The *Vibrio* spp. grew in the RT and FUR samples after 2 days of incubation at 12, 19, 28, and 37°C and cell counts decreased by 7 days.

Growth potential study II. The profiles of the growth potential for the three-serotype cocktail of *Salmonella* spp. in the filtered unchlorinated reconditioned (FUR) water between 3.5 and $51 \pm 1^\circ\text{C}$ were collected and are presented in abridged form in Figure 1. At 3.5 to $12 \pm 1^\circ\text{C}$ *Salmonella*

TABLE 1. Growth potential of *Salmonella* spp. and *Vibrio cholerae* from various water types using selective media^a

Incubation temp (°C)	Water type ^b	<i>Salmonella</i> (log CFU/ml)					Water type	<i>V. cholerae</i> (log CFU/ml)			
		Incubation time (days)						Incubation time (days)			
		0	2	7	15	22		0	2	7	15
5	PT	1.32	1.92	1.32	— ^c	—	PT	—	—	—	—
	RT	2.02	1.92	1.72	1.86	1.49	RT	3.58	—	—	—
	FUR	2.02	1.97	2.13	1.62	1.32	FUR	3.91	4.08	1.49	—
12	PT	—	1.79	1.49	—	—	PT	—	—	—	—
	RT	—	2.19	1.79	1.49	1.62	RT	—	6.88	3.87	—
	FUR	—	2.19	2.32	1.97	—	FUR	—	5.38	3.76	—
19	PT	—	2.1	—	—	—	PT	—	—	—	—
	RT	—	2.02	—	1.32	—	RT	—	5.64	4.08	—
	FUR	—	2.3	3.46	5.46	1.97	FUR	—	5.65	4.53	—
28	PT	—	1.72	1.02	—	—	PT	—	—	—	—
	RT	—	3.11	4.48	2.73	3.33	RT	—	6.11	—	—
	FUR	—	3.21	4.54	2.98	—	FUR	—	4.52	4.74	—
37	PT	—	—	—	—	—	PT	—	—	—	—
	RT	—	5.12	4.97	6.04	—	RT	—	4.93	2.66	—
	FUR	—	5.13	5.05	6.22	—	FUR	—	5.08	2.1	2.06

^a *Salmonella* spp. recovered on double modified lysine iron agar (DMLIA); *Vibrio* spp. recovered on thiosulfate citrate bile sucrose agar (TCBS).

^b Water types: potable water neutralized with 0.01% (wt/vol) sodium thiosulfate (PT); unchlorinated reused, neutralized with 0.01% (wt/vol) sodium thiosulfate (RT); and filtered unchlorinated reused (FUR).

^c Below 1.32 log CFU/ml, lower limit of detection.

spp. remained viable and were recovered, but no growth occurred. Growth occurred in the FUR water between 12.4 and 40 ± 1°C. The cell population reached a maximum between 17 to 37 ± 1°C, with a 3-log-unit increase at temperatures between 26.4 and 30 ± 1°C. In the FUR water the *Salmonella* counts dropped rapidly from 40 to 51 ± 1°C.

Growth potential data of the mixed strains of *V. cholerae* in the FUR water over the temperature range of 4.7 to 51.1 ± 1°C were collected and are presented in abridged form in Figure 2. The FUR water did not support growth of the *Vibrio* spp. in the temperature ranges of 4.7 to 9.7 ± 1°C and 39 to 51 ± 1°C, but cells survived and were recoverable. Growth of the vibrios occurred from 11 to 37 ± 1°C, with a

2-log-unit increase (maximum growth) occurring between 15.3 and 25 ± 1°C. In the temperature range between 20.4 and 37 ± 1°C maximum growth occurred within 2 days and then decreased rapidly.

The cultures of *Salmonella* spp. and *Vibrio* spp. in which no viable cells were detected (minimum level of detection was <21 CFU/ml) were examined by the LIVE/DEAD BacLight[®] viability test kit. No viable cells were observed, indicating that nonculturable cells were also nonviable.

Bioassays. The results of the bioassays for assimilable organic carbon (AOC) and coliform growth response (CGR) on the unchlorinated reconditioned water samples (UR) used

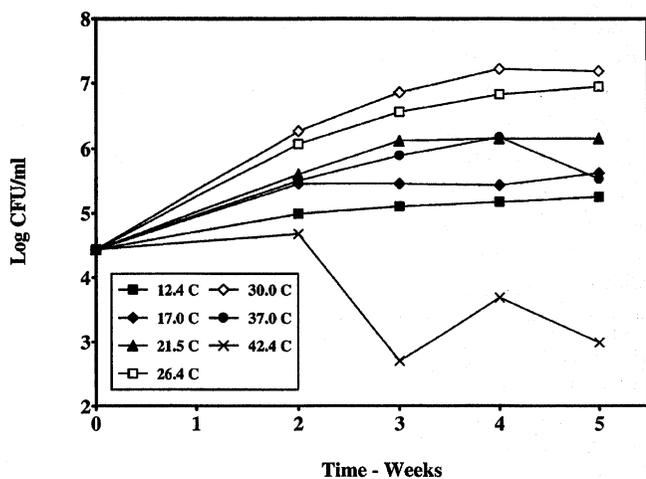


FIGURE 1. Growth potential of a mixed *Salmonella* culture in filtered unchlorinated reconditioned water at 3.5 to 51.1°C.

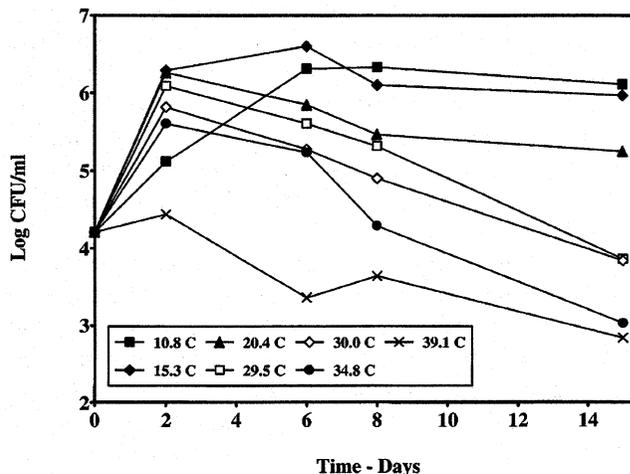


FIGURE 2. Growth potential of *Vibrio cholerae* strains in filtered unchlorinated reconditioned water at 4.7 to 51.1°C.

TABLE 2. Coliform growth response (CGR) and assimilable organic carbon (AOC) analysis of potable (P), chlorinated reconditioned (R), and unchlorinated reconditioned (UR) water

Water sample no.	Water type	Assimilable organic carbon ^a (µg of carbon equivalents per liter)			Coliform growth response ^b log(N _t /N ₀)
		P-17 ^c	NOX ^c	Total	
1	P	100	365	465	1.59
	R	222	966	1188	1.96
2	P	6	7	13	0.36
	UR	10243	1207	11270	3.58
3	UR	804	862	1666	3.25
	R	34	1586	1620	4.14
4	UR	8	458	466	2.07
	R	12	1586	1598	ND ^d
5	UR	10	362	372	2.09
	R	110	1207	1317	2.17

^a AOC >100 µg of C equivalents per liter suggest water contains nutrients capable of supporting growth.

^b CGR ≥1.0 suggest water contains nutrients capable of supporting growth. CGR organism: *E. cloacae*.

^c *P. fluorescens* P-17; *Spirillum* NOX.

^d ND, not determined.

for the growth studies (Fig. 1 and 2) are presented in Table 2. Water samples 2 and 5 were used for the growth trials (Fig. 1) in the gradient incubator using the *Salmonella* inoculum. The AOC levels were 11,270 and 372 µg of C equivalents per liter respectively and CGR levels were 3.58 and 2.09 log N_t/N₀, respectively. Water samples 3 and 4 were used for the *Vibrio* study in Fig. 2. The AOC and CGR levels for the first growth trial with *Vibrio* spp. were 1,666 µg of C equivalents per liter and 3.25 log N_t/N₀, respectively, and for the second trial 466 µg of C equivalents per liter and 2.07 log N_t/N₀. The unchlorinated reconditioned water AOC and CGR levels indicated the presence of sufficient nutrients to support bacterial growth. One potable water sample also had AOC and CGR levels high enough to support bacterial growth.

DISCUSSION

Several research groups have shown that drinking water, bottled water, and wastewater can support the growth of such microbes as *Bacillus* spp. and *Pseudomonas* spp. (4, 21) and *Salmonella* spp. (14, 20). The mixed flora, which we observed in the reconditioned water, were not identified other than to confirm that coliforms were not identified by growth on either MAC or HEKT agars. The reconditioned water was filtered sterilized before use for the growth studies to avoid any possible interference from the indigenous microflora.

A growth study comparing filtered unchlorinated and neutralized chlorinated reconditioned water showed that the nutrient levels were sufficient for limited *Salmonella* and *Vibrio* growth, but filtered unchlorinated reconditioned water supported growth more consistently. In this and the indigenous background growth studies, selective media (DMLIA, TCBS, MAC, and HEKT), were used to enumerate and recover the pathogens. McKay (11) reported that bacteria in water with extremely limited nutrients were not

recovered on the selective media under laboratory conditions. Roszak et al. (19) reported that better recovery of *Salmonella* spp. from water was obtained using a nonselective medium. In addition, Huck (6) reported that chlorination can affect the nutrient content of the water. Chlorine oxidizes refractory larger molecular weight compounds to smaller molecular weight compounds, which are more readily assimilated. The chlorinated compounds probably do not serve as a nutrient source (6). We decided that filtering the unchlorinated water enabled us to use a nonselective recovery media (TSA) and to control the water environment by not adding any nutrients.

Using the temperature-gradient incubator and enumerating on TSA, we were able to study the growth potential of *Salmonella* spp. and *V. cholerae* in the unchlorinated reconditioned water over a wide temperature range (4 to 50°C) and to compare the AOC and CGR levels to the observed growth potential of the water. The bioassays showed that sufficient nutrients were available in the unchlorinated reconditioned water to support growth. The filtered reconditioned water used in the first growth potential study for *Salmonella* achieved a higher cell population which correlated with the higher AOC and CGR levels. In the first experiment where the AOC and CGR levels were high, *Vibrio cholerae* survived better in the water at the lower temperature range and over the entire temperature range for the second. While *Vibrio cholerae* is not typically considered to grow at the lower temperatures, Wong et al. (22) reported the isolation of other vibrios which survived better at the low temperatures. Further studies are needed to determine the stress of limited nutrients in reconditioned water on the recoverability of these pathogens.

In conclusion, chlorine levels (0.2 mg/liter) must be maintained to prevent pathogen growth in any reconditioned water used in a food-processing plant.

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