Mutagenicity of Poultry Chiller Water Treated with either Chlorine Dioxide or Chlorine

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U.S. Department of Agriculture regulation of poultry processing requires the eviscerated warm carcasses to be quickly chilled to ensure microbiological safety and wholesomeness of the products. This is achieved by immersing carcasses in icy water for about 1 h. In addition, chlorine is widely used to control the microbial population in poultry chiller water (PCW), and until 1996 it was the only approved disinfectant. Recently, chlorine dixoide has also been approved the U.S. Food and Drug Administration and the U.S. Department of Agriculture as a disinfectant in PCW. Its potential mutagenicity was investigated using the Ames mutagenesis assay with *Salmonella typhimurium* strain TA 100 without S-9 mix, the protocol formerly demonstrated to be senstive to chlorine-treated PCW. Chlorine dioxide was found to impart negligible mutagenicity to PCW when tested at 4 times the level required for disinfection.

Keywords: *Poultry chiller water; chlorine dioxide; chlorine; hypochlorite; Ames mutagenicity; mutagens; microbiology; bacteria*

INTRODUCTION

The U.S. poultry industry produces annually about 22 billion pounds of chicken and 5 billion pounds of turkey in ready-to-cook forms [U.S. Department of Agriculture (USDA), 1994]. Automated production processes are of multimillion unit capacity, where birds are slaughtered, defeathered, eviscerated, rinsed, chilled, and packed for the market on a continuous production line. Chilling carcasses rapidly is critical for minimizing microbial growth and preserving carcass quality. USDA regulation requires that carcasses must be chilled to 40 °F, or less, within 4−8 h, depending on their weight, from the time of slaughter (USDA, 1987). The chilling is accomplished by immersing rinsed carcasses in icy water in one or more long tanks, or chillers, for approximately 1 h. Many processors also use chlorine to control the microbial population in poultry chiller water (PCW). On the basis of our unofficial survey, 60% (or more) of U.S. poultry products have been exposed to chlorine during chilling. Until 1996, chlorine and its hydration products, hypochlorous acid and hypochlorite, had been the only disinfectants permitted by both the U.S. Food and Drug Administration and the U.S. Department of Agriculture for use in PCW (USDA, 1993). In March 1995, the FDA approved chlorine dioxide to be used as an antimicrobial agent in poultry processing at a residual level not to exceed 3 mg/L (*Federal Register*, 1995). Chlorine dioxide was tested in PCW by several poultry processors under the supervision of the FSIS and received final approval in 1996 (FSIS, 1996).

Because of its high organic matter content (Tsai et al., 1992), treating PCW with chlorine results in the formation of trihalomethanes, primarily chloroform (Robinson et al., 1981; Tsai, unpublished data), plus other mutagenic compounds that have not yet been identified (Masri, 1986; Schade et al., 1990).

Poultry meats account for about 36% of the meat consumption in the American diet, equivalent to a per capita value of 44 kg (96 lb) (USDA, 1994). The wholesomeness of poultry products may thus have a profound effect on public safety and health. While work is underway to establish the impact of chlorine treatment, other disinfection methods are also being investigated. Such development will not only provide an alternative, should the mutagens prove to be a safety concern, but may also offer a more efficient and safe method for disinfecting PCW.

Chlorine dioxide could be one such substitute for chlorine. It is superior to chlorine in at least two respects. First, chlorine dioxide is a more potent bactericide than chlorine in PCW. It was reported to be more efficient than chlorine in reducing bacteria in the PCW and from turkey skin surfaces (Baron et al., 1973) and in the control of *Salmonella* contamination on turkey carcasses (Villarreal et al., 1990). Chlorine dioxide was shown to be 7 times more effective than chlorine in controlling the aerobic bacteria in broiler-processing PCW (Lillard, 1979). The *Salmonella* incidence of broilers was reduced to zero by treating PCW at 5 mg/L chlorine dioxide (Lillard, 1980) or at 1.39 mg/L (Thiessen et al., 1984).

Second, chlorine dioxide, like chlorine, is an oxidant, but its redox potential in aqueous solution, 1.15 V (ClO_2 + e = ClO_2^-), is less than that of hypochlorous acid, 1.49 V ($HClO$ + H^+ + 2e = Cl^- + H_2O) (White, 1986). Chlorine dioxide is, therefore, likely to be less reactive and produce fewer byproducts. For example, the chlorine atom in chlorine dioxide incorporated much more slowly with unsaturated lipids than chlorine itself in aqueous solution (Ghanbari et al., 1982). Also, chlorine dioxide is relatively inert compared to chlorine in reacting with individual amino acids (Tan et al., 1987), although both chlorine and chlorine dioxide react so quickly with peptides and proteins that the reaction rates were impossible to differentiate. Chlorine dioxide is also known to form no trihalomethanes in drinking water as does chlorine (Symons et al., 1981). Trace amounts of chloroform were found in chlorine dioxide water containing chicken carcasses (Robinson et al.,

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1981), but it was attributed to chlorine contamination of the chlorine dioxide solution.

One of the primary concerns associated with using chlorine dioxide is the production of chlorite and chlorate during disinfection (Masschelein, 1979). Chlorite and chlorate are additives regulated in drinking water by the U.S. Environmental Protection agency (U.S. Environmental Protection Agency, 1993). In the Disinfectants/Disinfection Byproducts Rules to be implemented in 1998, the maximum contaminant level (MCL) for chlorite in drinking water will be set at 1.0 mg/L. The accumulation of chlorite and chlorate residue in chlorine dioxide-treated PCW was thoroughly investigated (Tsai et al., 1995). Under normal operating conditions, when the chlorine dioxide demand of PCW is 0.30 mM (20 mg/L), it is possible to achieve effective disinfection with minimum accumulation of chlorite and chlorate and low health risk from the standpoint of chlorite and chlorate contamination.

Although chlorine dioxide is known to form no trihalomethane when used in drinking water, this is not necessarily so for PCW. Whereas the principal mutagen precursor in drinking water is humic acid (Meier, 1983), the organic component of PCW is primarily lipids and <0.1% proteins (Tsai et al., 1992). The finding of mutagenicity in chlorine-treated PCW and the lack of similar studies on chlorine dioxide treatment prompted us to carry out the following investigation. This knowledge was critical for the approval of chlorine dioxide in any food-processing water that is in direct or indirect contact with foods.

MATERIALS AND METHODS

Poultry Chilling Water. Water samples were obtained from the prechiller of a local commercial broiler processor as described previously (Tsai et al., 1987). The PCW was collected in Teflon bottles from the side trough after the chiller had operated for 5 h or more. They were transported in packed ice to the authors' laboratory within 1 h, screened with a No. 35 sieve (500 μm opening, U.S. Standard Testing Sieve, W. S. Tyler, Inc., Mentor, OH), and stored at 2–4 °C. Samples were discarded after 7 days.

Chlorine Dioxide Synthesis and Determination. Chlorine dioxide solution, free from hypochlorite, was prepared by slowly adding 23 mL of hydrochloric acid (3 M) to 80 mL of 7.5% sodium chlorite solution (Masschelein, 1979) in a 150 mL three-neck flask. The chlorine dioxide generated was stripped by N_2 and passed through three glass traps (40 mL in volume) connected in series into 125 mL of organic-free water in a glass cylinder immersed in crushed ice. Traps 1 and 3 were empty. Trap 2 contained 20 mL of 10% sodium chlorite and 1% sodium hydroxide. Concentration of chlorine dioxide in the receiving glass reached 1.0–1.3% in 60 min. This stock solution was stable for several months when kept in a sealed amber bottle under refrigeration.

Chlorine dioxide concentration was determined at room temperature using an amperometric method (Aieta et al., 1984). Titrant, phenylarsine oxide solution (0.00564 N, Shape Products Co., Oakland, CA), was added automatically with a Metrohm Titroprocessor (Brinkmann Instrument Co., Westbury, NY) at 0.05 mL increments. At pH 7, each mL of phenylarsine oxide used is equivalent to 0.028 mM or 1.9 mg/L chlorine dioxide. The optimum range of response for the Metrohm Titroprocessor is between 0 and 0.056 mM (3.8 mg/L). Titration accuracy is ±0.0014 mM (0.095 mg/L) chlorine dioxide, or 5% at 0.028 mM. Chlorine dioxide stock solution was titrated at pH 7 and then at pH 2 before it was used. The ratio of titrant at pH 2 to that at pH 7 must be 4 ± 0.1 to ensure that the chlorine dioxide solution is free from chlorine contamination (Aieta et al., 1984). Since chlorine is carefully

excluded, measurement at pH 7 is presumably all chlorine dioxide. Blank titration indicates the absence of any oxidants in solution.

Chlorine Stock Solution and Determination. Chlorine (sodium hypochlorite) stock solutions were prepared from chlorine gas (high-purity grade, Union Carbide Corp., Linde division, Danbury, CT) and solutions of sodium hydroxide (Baker analyzed reagent grade, J. T. Baker Inc., Phillipsburg, NJ) in the manner described previously (Schade et al., 1990). Total residual chlorine was measured according to a modified amperometeric titration procedure (APHA-AWWA-WPCF, 1989) using the Metrohm Titroprocessor equipped with a polarized platinum wire electrode. Duplicate samples were diluted with 18 MΩ water (prepared with a Milli-Q system) to <2 mg/L chlorine titrated with 0.00564 N phenylarsine oxide. The titrant was added with continuous mixing in constant increments (e.g., 0.05 mL) by the Titroprocessor until the endpoint was reached, which was indicated by an abrupt change of current. Each milliliter of phenylarsine oxide used was equivalent to 1 mg/L chlorine. The optimum range of chlorine concentration for titration is from 0 to 2 mg/L.

Chlorine Dioxide and Chlorine Treatment of PCW. To test the mutagenic effect of chlorine dioxide, PCW was treated with 100 mg/L chlorine dioxide. For comparison at equal bactericidal potency, PCW was treated with 400 mg/L chlorine. One liter of the screened PCW sample was treated with disinfectant in a 1 L glass bottle with a Teflon-lined cap (Wheaton, NJ) under constant stirring for 4 h at 21 ± 1 °C. The chlorine-treated samples were adjusted to the pH of the original PCW, pH 7.5 ± 0.5, with 6 N HCl. No such adjustment was needed for the chlorine dioxide-treated samples. At the end of the treatment period, the chlorine dioxide-treated samples were sparged with N_2 for 15 min and immediately extracted with dichloromethane (DCM).

Extraction. Treated PCW was extracted using the procedure described by Schade et al. (1990). Briefly, the PCW was adjusted to pH 11.0–11.2 and extracted twice with 250 mL of DCM. The two DCM extracts were combined and concentrated to about 8–10 mL using a Kuderna-Danish apparatus. The extract was then evaporated to dryness under a N_2 stream, redissolved in 10 mL of DCM, and stored in the dark at 4 °C. This concentrated base extract from 1 L of PCW was expressed as 0.1 L of PCW/mL of DCM (0.1 L/mL).

The aqueous phase of the extracted PCW was then adjusted to pH 1.8–2.0 with 6 N HCl, extracted, and treated as above. The resulting 10 mL of DCM solution contained the acidic materials, equivalent to 0.1 L of PCW/mL of DCM (0.1 L/mL).

Mutagenicity Assay. DCM extracts of treated and untreated PCW were tested for mutagenicity according to the Ames *Salmonella* assay using the standard top agar overlay method (Maron and Ames, 1983). *Salmonella typhimurium* strain TA 100 without S-9 mix was chosen for these assays on the basis of its relative sensitivity to mutagens found in chlorine-treated chiller water (Masri, 1986). A dilution series of the DCM solution was prepared and then dried under a N_2 stream. The resulting solids were resuspended in 100% ethanol and added to top agar at a volume of 0.1 mL per plate. Vigorous agitation immediately before plating was found necessary to obtain homogeneous suspensions. Some PCW samples formed globules in the agar, making colony counting difficult and partially obscuring the lawn. Where necessary, colonies were counted manually and restreaks were made to ensure against false positives. The highest concentration of DCM extracts that could be assayed with confidence for all samples was 0.5 L of PCW/mL of ethanol. Mutagenicity is expressed as revertant colonies per plate. Dose response data were collected in the linear range (<1200 colonies per plate). Positive control plates were added to confirm tester strain response and contained methyl methanesulfonate (MMS) at 0.4 μL per plate. Single numbers represent an average of duplicate plate counts.

Bactericidal Potency Evaluation. Chlorine dioxide- and chlorine-treated PCWs were evaluated for total aerobic bacteria within 3–5 min of treatment with Petrifilm aerobic count plates (Medical-Surgical Division/3M, St. Paul, MN) incubated for 48 h at 31–33 °C. Dilutions of each sample were prepared
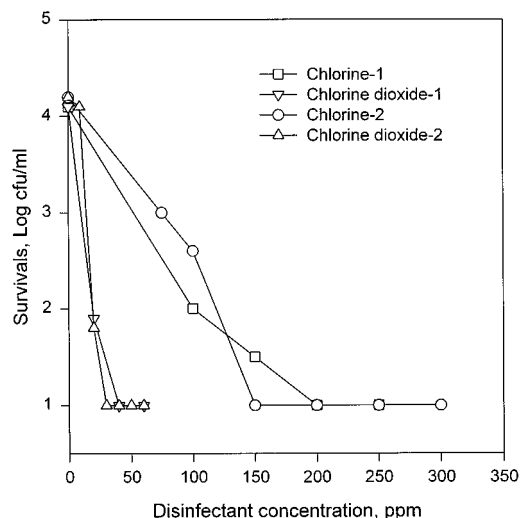
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Figure 1. Disinfecting efficacy of chlorine and chlorine dioxide in PCW. Total aerobic plate counts (i.e. survivals) were enumerated within 3–5 min of treatment.

Table 1. Proximate Analysis (Percent) of PCW[a]

sample	total solids	fat	ash	total N
PCW 177	0.105	0.056	0.045	0.0034
PCW 178	0.118	0.043	0.048	0.0016

[a] Screened with No. 35 sieve (500 μm opening; W. S. Tyler, Inc.).

with sterile 0.1% peptone solution. The counts reported represent the average of duplicate plates of each dilution.

Proximate Analysis. Methods of the Association of Official Analytical Chemists (1980) were used to determine total solids (Section 33.041), total nitrogen (Section 2.057), and ash (Section 14.006). Lipid content was determined from the total dry solids (Section 7.056).

RESULTS AND DISCUSSION

Although chlorine is commonly used to disinfect PCW, the amount used by each processor varies and has not been reliably determined due to the lack of a precise and uniform measuring protocol. Where chlorine is added automatically to PCW of high chlorine demand, it is conceivable that some chillers may use up to 200 mg/L. To determine mutagenesis in a worst case scenario, a concentration of twice what may be used in the field, 400 mg/L, was selected.

The chlorine dioxide concentration for this study was selected on the basis of its bactericidal potency relative to chlorine. Lillard compared the bactericidal efficiency of chlorine to chlorine dioxide in a commercial poultry chiller and found that a 7-fold difference, i.e. using 34 mg/L chlorine or 5 mg/L chlorine dioxide, resulted in approximately the same bacterial kill (Lillard, 1979). Microbial assays conducted in our laboratory showed a 3-log reduction of the natural flora in PCW with 30–40 mg/L chlorine dioxide or 150–200 mg/L chlorine (Figure 1). Chlorine dioxide thus proved to be 4-fold more potent than chlorine. The difference between our results and Lillard's could be attributed to a variability in testing conditions or the chlorine and chlorine dioxide demands of the individual PCW (Tsai et al., 1992, 1995). To achieve a comparable level of bactericidal potency in the PCW used for this study, the level of chlorine dioxide chosen reflects a 4-fold difference, i.e. 100 mg/L chlorine dioxide vs 400 mg/L chlorine.

Mutagenicity studies were carried out on PCWs sampled on three different dates over a period of 3 months. The proximate analyses of screened PCWs 177 and 178 are given in Table 1. The compositions of the two samples were quite similar. PCW 180 was not analyzed; however, the proximate composition of the unscreened PCWs from the same commercial operation, analyzed over a period of 5 years, indicated that the total solids of the PCW (with mean equal to 0.35%; RSD, 40%) did not vary significantly (Tsai, 1992).

The mutagenicity of pH 11 and pH 2 extracts of disinfectant-treated PCW is summarized in Tables 2 and 3, respectively. Extracts of PCW 177 were assayed for mutagenicity once and provided the effective range of concentrations. Extracts of PCW 178 were divided into two equal portions and assayed in two separate experiments, 7 days apart. Extracts of PCW 180 were also assayed in duplicate, 4 days apart. Untreated PCW, extracted at pH 11 or pH 2, showed no mutagenic response at all PCW volumes tested. Chlorine dioxide treatment showed a slight repeatable increase in revertants, but never exceeded 2-fold background, even at the highest plate concentration. A 2-fold increase is the minimum level suggested by Ames to qualify as mutagenic (Ames, 1975). The chlorine-treated samples were highly mutagenic. Doses of 50 and 25 mL/plate were toxic and had to be diluted and tested at lower levels.

Table 2. Mutagenicity of pH 11 Extracts of PCW Treated with Chlorine Dioxide or Chlorine

treatment	PCW, mL/plate[b]	revertants/plate[a] PCW 177	PCW 178A	PCW 178B	PCW 180A	PCW 180B	mean	RSD, %
untreated	50	214	174	190	180	178		
	25	183	185	168	180	178		
	13	196	198	141	187	173		
100 ppm of ClO$_2$	50	76	282	265	309	308		
	25	192	240	214	230	228		
	13	217	219	200	216	224		
	6.3	200	209	175	182	210		
	3.1	185						
400 ppm of Cl$_2$	13		607	489	705	630		
	6.3	748	363	317	383	377		
	3.1	423	245	222	321	284		
	1.6	269	223	213	212	216		
background[c]		176	181	160	175	169	172	5
MMS[d]		1070	>1200[e]	>1200	>1200	>1200		

[a] Average of duplicate plates. [b] Each plate was inoculated with 0.1 mL of PCW extract in ethanol. [c] Plated with 0.1 mL of ethanol. [d] Plated with 0.4 μL of methyl methanesulfonate. [e] Numbers >1200 were not quantified.

Table 3. Mutagenicity of pH 2 Extracts of PCW Treated with Chlorine Dioxide or Chlorine[a]

		revertants/plate[a]						
treatment	PCW, mL/plate[b]	PCW 177	PCW 178A	PCW 178B	PCW 180A	PCW 180B	mean	RSD, %
untreated	50	107	164	149	166	128		
	25	151	198	173	164	169		
	13	146	191	147	187	179		
100 ppm of ClO_2	50	238	327	270	297	263		
	25	242	284	197	251	227		
	13	224	221	202	228	186		
	6.3	180	229	137	205	163		
400 ppm of Cl_2	25					>1200		
	13		449	296	772	802		
	8.3	649	345	279	586	492		
	6.3	527	291	230	469	377		
	4.2	439	278	192	359	295		
background[c]		176	181	160	175	169	172	5
MMS[d]		1070	>1200[e]	>1200	>1200	>1200		

[a] See footnotes to Table 2.

Table 4. Mutagenicity of Chlorine Dioxide- and Chlorine-Treated PCW

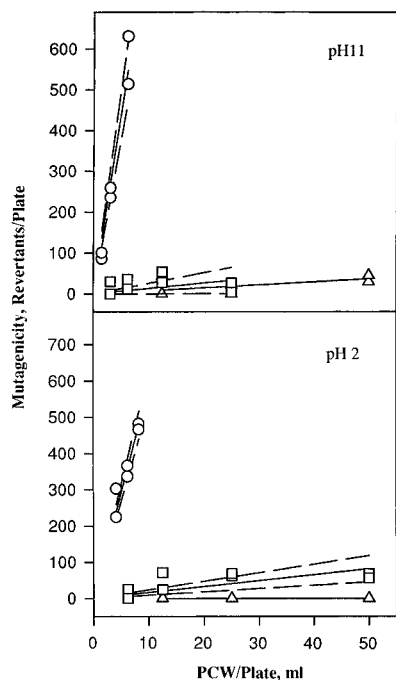|	revertants/L							revertants/L			
	untreated			100 ppm of ClO_2				400 ppm of Cl_2			
sample	pH 11	pH 2	total	pH 11	pH 2	total	total[a]	pH 11	pH 2	total	total[a]
PCW 177	740	0	740	1320	1630	2950	30	87520	57430	144950	362
PCW 178	350	130	480	2160	2650	4810	48	29110	16050	45160	113
PCW 180	270	130	400	2710	2300	5010	50	38410	44190	82600	207
mean (3)			540			4260	43				227
RSD, %			33			27	26				55

[a] Revertants/mg of chlorine dioxide or chlorine.

Figure 2. Mutagenicity of untreated PCW, PCW 177 (△), PCW treated with 100 mg/L chlorine dioxide (□), and PCW treated with 400 mg/L chlorine (○). Plotted values represent net increase over background (172 revertants/plate). Solid lines represent the linear regression fits and broken lines the 95% confidence intervals.

Figure 3. Mutagenicity of untreated PCW, PCW178 (△), PCW treated with 100 mg/L chlorine dioxide (□), and PCW treated with 400 mg/L chlorine (○). Plotted values represent net increase over background (172 revertants/plate). Solid lines represent the linear regression fits and broken lines the 95% confidence intervals.

The mutagenicity and linear regression fit of treated and untreated samples are plotted in Figures 2–4, after adjustment for the background. Figure 2 shows that the low, repeatable mutagenic response of chlorine dioxide treatment was not significantly different (95% confide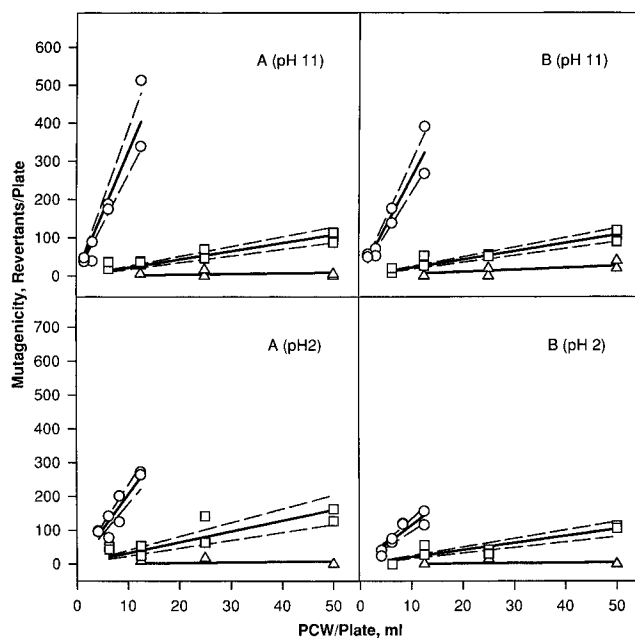nce) from the untreated samples. Figures 3 and 4 do show a significant difference between chlorine dioxide-treated and untreated PCWs, although neither mutagenic value exceeds 2 times background. The mutagenic effect of chlorine treatment is verified in all results except the pH 2 extracts of PCW 178, for which the samples were apparently too dilute. There, the mutagenicity was linear but <2-fold background.
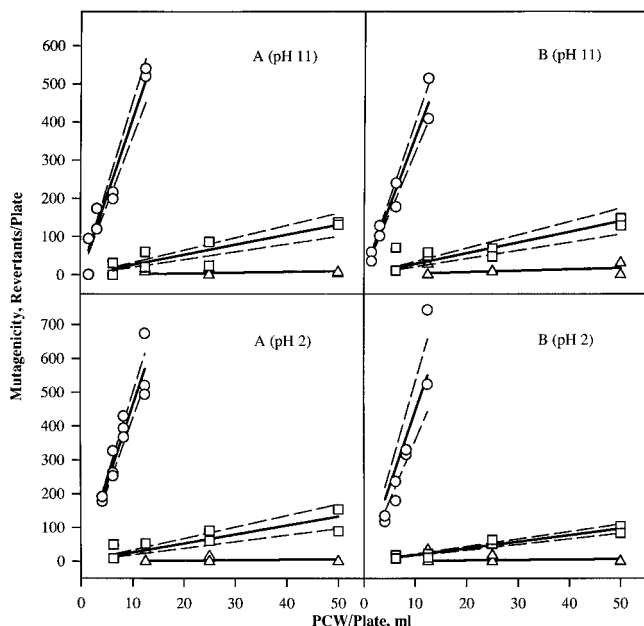
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Figure 4. Mutagenicity of untreated PCW, PCW 180 ($\triangle$), PCW treated with 100 mg/L chlorine dioxide ($\square$), and PCW treated with 400 mg/L chlorine ($\bigcirc$). Plotted values represent net increase over background (172 revertants/plate). Solid lines represent the linear regression fits and broken lines the 95% confidence intervals.

The mutagenicities, calculated from linear regression analysis and expressed as revertants per liter of PCW, are given in Table 4. Extraction of PCW at pH 11 recovered basic and neutral organic components, and that at pH 2 recovered acidic compounds (U.S. EPA, 1979). Extraction at both pH values in series ensured a complete recovery of total mutagenicity in PCW. At a comparable level of bactericidal potency, the mutagenicity of the extract of the chlorine-treated PCW was 21-fold that of chlorine dioxide-treated PCW. Assuming the effects of chlorine dioxide and chlorine on PCW to be linear, the mutagenic effect of chlorine dioxide would be 43 revertants/mg. The effect of chlorine would be 227 revertants/mg, about 5-fold more mutagenic than chlorine dioxide.

At a 95% confidence interval, the total mutagenicity of the untreated samples was estimated to be 540 ± 356 revertants/L. Therefore, it was possible to use 21 mg/L chlorine dioxide without a significant increase in mutagenicity over the control untreated samples. In a separate study, we have demonstrated that it is feasible to control the chlorine dioxide demand of PCW at 20 mg/L under commercial operating conditions and to effectively disinfect PCW with low health risk from the standpoint of chlorite and chlorate contamination (Tsai et al., 1995). The work presented here demonstrates further that using chlorine dioxide at about the 20 mg/L level will be safe from the standpoint of mutagen formation.

Although chlorine treatment at 400 mg/L resulted in mutagenic PCW, it cannot be assumed that chlorine use at lower levels will have the same effect. Neither Masri (1986) nor Schade et al. (1990) presented a linear dose effect in their papers on chlorine treatment of PCW and, therefore, their conclusions can be treated only qualitatively. Work is needed on the isolation and identification of mutagen(s) from chlorine-treated PCW for assessing the risk of chlorine treatment at the commonly used levels, i.e. 20–50 mg/L.

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