Research Note

Effect of Alfalfa Seed Washing on the Organic Carbon Concentration in Chlorinated and Ozonated Water†

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ABSTRACT

The bioassays assimilable organic carbon (AOC) and coliform growth response are better indexes than biological oxygen demand to determine water quality and water’s ability to support the growth of bacteria. Ozonated (5 mg/liter) and chlorinated tap water were used to wash alfalfa seeds for 30 min. After washing in the ozonated tap water, the AOC concentration increased 25-fold, whereas the dissolved ozone decreased to undetectable levels. The AOC levels for the chlorinated water after washing the seeds also increased. These increases are due to ozone’s strong oxidizing ability to break down refractory, large-molecular-weight compounds, forming smaller ones, which are readily used as nutrient sources for microorganisms. This same phenomenon was observed when using ozone in the treatment of drinking water. The AOC value increased from 1,176 to 1,758 μgC-eq/liter after the reconditioned wastewater was ozonated. When the ozonated wastewater was inoculated with Salmonella serotypes, the cells survived and increased generation times were observed. The increased nutrients would now become more readily available to any pathogenic microorganisms located on alfalfa seed surface as seen with the increase in the inoculated levels of Salmonella in the ozonated wastewater. If the washing process using ozonated water is not followed by the recommended hypochlorite treatment or continually purged with ozone, pathogen growth is still possible.

Ozone, a strong oxidizing agent, has many commercial uses and reacts chemically with inorganic and organic materials (12). In water treatment plants, ozone is used for potable water purification and disinfection. Some treatment plants use ozone as a preoxidant to aid in the filtration process for the removal of turbidity or suspended solids (12). During this process, organic and inorganic materials are oxidized, and lower-molecular-weight compounds or biodegradable dissolved organic compounds are formed (1). The organic by-products formed can be organic acids, aldehydes, and ketoacids (31). There is a concern that these resulting smaller chain organic compounds can potentially become the carbon source for bacteria, causing regrowth of bacteria in the water distribution system (31).

Since potable (drinking) water can contain these small chain organic compounds, which enable regrowth of bacteria, more sensitive water quality test methods have been recommended (14–17). Rice et al. (27) determined that the coliform growth response (CGR) bioassay procedure is a more reliable monitor of water quality than biological oxygen demand (14–17). Rice et al. (27) determined that the coliform growth response (CGR) bioassay procedure is a more reliable monitor of water quality than the heterotrophic noncoliform bacteria method to determine regrowth. They also showed that the CGR bioassay test is a viable method for determining the biological stability of drinking water from different geographical areas (28). The CGR bio-

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† Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.
O157:H7 on inoculated alfalfa seeds using ozone water resulted in a 0.40- to 1.75-log reduction (30). In the Guidance to Industry, the FDA recommends disinfection for the seeds using a 20,000-mg/liter active chlorine treatment (6).

When alfalfa seeds are hydrated, they release organic materials, some of which could react with the ozone. There is limited information about the water quality after hydrating the alfalfa seeds in tap, chlorinated, or ozonated water. This study determined the CGR and AOC levels before and after soaking the alfalfa seeds in tap water chlorinated to a free chlorine level of 1.005 mg/liter or ozonated tap water (5 mg/liter) and the effect on percentage of germination. The growth potential of Salmonella spp. in ozonated and nonozonated reconditioned wastewater (nutrient-limiting conditions) was also compared.

**MATERIALS AND METHODS**

**AOC and CGR studies.** The bioassay for CGR uses E. cloacae. For the AOC bioassay, Spirillum NOX and P. fluorescens P-17 are used. The inoculum and test samples were prepared according to the method described by Rice et al. (27). Test water samples were heated to 70°C for 30 min. For CGR, water samples were inoculated with E. cloacae. CGR was determined by log transformation of the ratio between the levels of bacteria present after a 5-day incubation period (Nt) versus initial level (No). For the AOC assay, water samples were inoculated with P. fluorescens P-17 and Spirillum NOX. The AOC values were calculated from the published yield factor of 6.613 log CFU/µg acetate carbon equivalents (µg acetate carbon/liter) for P-17 and 6.462 log CFU/µg oxalate carbon equivalents for NOX (27).

**Gradient growth temperature study.** Unchlorinated reconditioned wastewater and ozonated wastewater were both obtained from a local food processing plant with an in-house water treatment plant. The water samples were collected just before chlorination (unchlorinated) and after ozonation (ozonated). After arrival in the laboratory, the water samples were filter sterilized and kept refrigerated until used in the growth studies. Separate water samples, taken at the same time, were sent on ice by overnight carrier to the U.S. Environmental Protection Agency laboratory in Cincinnati, Ohio, for the CGR and AOC bioassays. The biological oxygen demand (BOD) for the reconditioned wastewater was conducted according to the standard method (2) and was obtained from company record.

A cocktail of Salmonella Enteritidis, Typhimurium, and Choleraesuis 38 was prepared according to the procedure reported by Rajkowski et al. (24). For use in the growth study, individual strains were cultured overnight, concentrated, washed once in sterile deionized water, and combined to a concentration of 10⁸ to 10⁹ CFU/ml.

A liter of the filtered, unchlorinated, reconditioned (FUR) wastewater or the filtered, ozonated, reconditioned (FOR) wastewater was mixed with the inoculating cocktail to achieve a starting level of 10⁸ to 10⁹ CFU/ml. Twelve milliliters of the inoculated FUR or FOR wastewater was distributed into a duplicate set of L-shaped test tubes placed in a temperature-gradient incubator (model TN-3F, Advantec, Toyo Roshi International Co., Dublin, Calif.) set between 3.5 and 39.4°C. Growth was monitored by spiral plating the samples on tryptic soy agar (TSA) and incubating at 37°C for 18 to 24 h before counting. The actual gradient temperature range was verified using the thermocouple sensor fitted in the gradient incubator. The differences in growth and/or survival response for the Salmonella spp. in the FUR and FOR waters were calculated using the general linear model procedure of the SAS/STAT software system (29). The study for each water sample was repeated.

**Preparation of chlorinated tap water.** Municipal tap water, containing a chlorine residual of approximately 1 mg/liter (pH 8.9, 22 to 24°C), was superchlorinated by the addition of reagent-grade sodium hypochlorite to produce a chlorine residual of 1,000 mg/liter. Chlorine levels were determined by the N-N-diethyl-p-phenylenediamine colorimetric method (2). The biocidal action of chlorine was quenched by the addition of sodium thiosulfate.

**Preparation of ozonated tap water.** A flow-through ozone contactor was used to produce ozonated tap water. The initial tap water conditions were the same as for the chlorination studies. The water flow-through rate for the contactor was 435 ml/min, with an applied ozone dose of 8.2 mg/liter. Effluent samples were collected from the contactor and used for the ozone exposure experiments. Ozone levels were determined by the indigo trisulfonated method (2). Dissolved ozone levels were quenched by the addition of sodium thiosulfate.

**Treatment of alfalfa seeds with chlorinated or ozonated tap water.** Alfalfa seeds used for sprouting were purchased from International Specialty Supply (Cookeville, Tenn.). Twenty-five grams of alfalfa seeds was placed in 1 liter of the test waters at room temperature (22 to 24°C) and was continuously stirred using a magnetic stirrer. The seed water samples consisted of the alfalfa seeds washed in Cincinnati tap water (pH 8.9), in chlorinated tap water to approximately 1,000 mg/liter, or in ozonated tap water to approximately 5 mg/liter. The seeds were removed after an exposure time of 30 min. Sodium thiosulfate was added to each water sample to stop the action of chlorine or ozone. Control water samples were treated in the same way as the test samples but without seeds. All water samples were analyzed for AOC and AOC. The AOC assays were conducted in duplicate, and the CGR assays were conducted in quadruplicate for chlorine and ozone exposed seed water.

**Microbial analysis of residual water after treatment.** Heterotrophic plate counts of the tap water and tap water plus seeds were performed by spread plates on R2A agar, which were incubated 7 days at 25°C then counted. The results are the means of duplicate procedures (2).

**Effect of treatment on germination of alfalfa seeds.** After each treatment, 100 alfalfa seeds were placed on moist filter paper in petri dishes to determine the percentage of germination after being left at room temperature for 48 h.

**RESULTS AND DISCUSSION**

**Gradient growth temperature study.** The recovery of Salmonella from the nonozonated and ozonated reconditioned wastewater was performed using TSA. In a previous study, Rajkowski and Dudley (21) showed that recovery was greater using this nonselective agar. In this study, as in the previous study with Salmonella (20, 24), growth is defined as an increase of more than 1 log CFU/ml. The data were obtained using a gradient heat block shaker with two sample ports for each temperature. The results presented in Table 1 are the growth and/or survival data for both treatment waters over the temperature range of 4.5 to 39.3 ± 1°C. Survival of Salmonella occurred over the test temperature range in both test waters (FUR and FOR), whereas growth under these limiting nutrient con-
TABLE 1. Fate of Salmonella spp. cocktail inoculated into the nonozonated and ozonated reconditioned wastewater held at the temperature range of 4.3 to 39.3 °C

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Nonozonated (log CFU/ml)</th>
<th>Ozonated (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 days</td>
<td>7 days</td>
</tr>
<tr>
<td>4.3</td>
<td>4.73</td>
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</tr>
<tr>
<td>8.4</td>
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</tr>
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<td>9.8</td>
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</tr>
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</tr>
<tr>
<td>39.3</td>
<td></td>
<td>6.39</td>
</tr>
</tbody>
</table>

a CGR = 2.06 and AOC = 372 μgC-eq/liter.

b CGR = 3.6 and AOC = 1,758 μgC-eq/liter.

c Results are for one trial.

TABLE 2. Generation time of Salmonella spp. grown in nonozonated and ozonated reconditioned wastewater

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Nonozonated</th>
<th>Ozonated</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.1</td>
<td>80.5</td>
<td>51.4</td>
</tr>
<tr>
<td>23.4</td>
<td>63.2</td>
<td>38.5</td>
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<td>31.0</td>
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<td>34.3</td>
</tr>
<tr>
<td>32.8</td>
<td>39.4</td>
<td>35.9</td>
</tr>
</tbody>
</table>

a Significantly different in generation time at P < 0.05.

Water quality after seed washing. Alfalfa seeds were washed in tap, chlorinated, and ozonated water and removed for germination testing. The percentage of germination of the alfalfa seeds was not affected by the chlorine or ozone-treated water, which agrees with previous reports (19, 20, 30).

The microbial load of the water samples was tested before and after hydration of the alfalfa seeds. The results of the heterotrophic plate counts were negative (<1 CFU/ml) before treatment. The counts after treatment for the tap, chlorinated, and ozonated water samples were 10, 130, and less than 1 CFU/ml, respectively.

The bioassays results for CGR and AOC are presented in Table 3. After a 30-min exposure time in the tap water (control), the alfalfa seeds impart a large amount of nutrients into the water as seen by the CGR level increasing fourfold (from no growth to 4.07 and 3.85 log N_0/N_0) and the total AOC increasing from 140 to 13,430 μgC-eq/liter. Similar increases for the CGR and AOC levels were also observed for the chlorinated tap water after washing the alfalfa seeds (Table 3). This increase in nutrient content (organic material) can support microbial growth. The high levels of chloride had little effect on the biostability of the water when the seeds were present. When the alfalfa seeds were washed in the ozonated water, the CGR and AOC levels increased approximately fivefold and 25-fold, respectively (Table 3). The alfalfa seeds released a large amount of nutrients (organic carbon) as seen by the increased CGR and AOC levels into ozonated water, and the high level of ozone (5 mg/liter) had little effect on the biostability of the water when the seeds were present. Restaino et al. (26) showed that ozonated water was effective as long as there was no organic carbon present. With the release of nutrients by the seeds, the washing in ozone water is ineffective in reducing the microbial population.

Using waste alfalfa seed irrigation water, Howard and Hutcheson (7) reported that a Salmonella enterica strain grew using the released seed nutrients. When ozone was added to the irrigation and hydration water, the ozone oxidized these released nutrients, increasing the AOC and CGR levels (Table 3). When ozone reacts chemically with the released seed nutrients, the ozone level in the water
decreases, leaving little or no concentration to act as a disinfectant. This ozone depletion could explain the reduction and nonelimination of microbial counts as reported by Naito and Shiga.

| TABLE 3. Coliform growth response (CGR) and assimilable organic carbon (AOC) |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Tap water                  | NG\(^a\)                   | 20                          | 120                         | 140                         |
| Tap water and alfalfa seeds| 4.07                       | 8,290                       | 5,140                       | 13,430                      |
| Chlorinated tap water      | 1.35                       | 12                          | 113                         | 125                         |
| Chlorinated tap water and alfalfa seeds | 4.20              | 8,540                       | 3,760                       | 12,100                      |
| Tap water                  | NG\(^a\)                   | 27                          | 59                          | 86                          |
| Tap water and alfalfa seeds| 3.85                       | 7,560                       | 1,379                       | 8,939                       |
| Ozonated tap water         | 0.22                       | 78                          | 384                         | 462                         |
| Ozonated tap water and alfalfa seeds | 4.15          | 9,024                       | 2,896                       | 11,920                      |

\(^a\) NG, no growth.

REFERENCES


