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Determining Toxicity Trends in the Ozonation of Synthetic Dye Wastewaters Using the Nematode *Caenorhabditis elegans*

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Abstract. The nematode *Caenorhabditis elegans* was used in 72-h toxicity tests to evaluate the influence of ozonation on the toxicity of three synthetic azo dye wastewaters (two reactive dyes and one acid-based dye). The two reactive dye wastewaters contained high concentrations of NaCl (89–112 g/L) in addition to potentially toxic dye components. To determine the contribution of NaCl to toxicity, simulated dye wastewater samples with and without NaCl were tested. Samples were collected at various times during ozonation (t = 0, 8, 32, 64 min); nematodes were exposed to the samples for 72 h. The influence of ozonation on toxicity varied between dye wastewater types. For the acid-based dye wastewater, toxicity increased as duration of ozonation increased. For the reactive dyes without NaCl, toxicity did not appear to be influenced by ozonation. For the reactive dyes with NaCl, mortality was 100% with or without ozonation. Range-finding experiments with NaCl in water and NaCl in dye wastewaters suggested an additive toxic interaction between NaCl and the dyes in wastewater to the nematodes. The duration of ozonation for acid-based dyes and the relatively high NaCl concentrations for the reactive dyes appear to influence effluent toxicity in the ozonated dye wastewaters.

The presence of potentially toxic compounds in wastewaters from textile dyeing industries (dyehouses) has led to environmental research to identify methods that can effectively treat these wastewaters. Dyehouse effluents generally contain chemicals with intense colors and the release of these effluents to receiving streams may be objectionable for aesthetic reasons. Research in the area of dye wastewater treatment includes identifying the toxic constituents of dyes and associated processing waters as well as assessing potential toxicity associated with the use of ozone treatment to reduce color (Carrière *et al.* 1993; Keqiang *et al.* 1994; Fouche 1995; Law 1995).

Wastewater color is one of the major problems facing industries involved in dyeing processes. Wastewater from dyehouses often carries high concentrations of excess dye that fails to adhere to substrate fibers. Ozonation of colored wastewaters from these industries is an effective treatment option for effluent decolorization. Ozone has been used to treat processing waters from several types of industries, including textiles and pulp and paper processors (Carrière *et al.* 1993). The decolorization of dyes in wastewaters results from direct and indirect reactions of ozone with the dye molecules. As ozone decomposes in water, hydroxyl and other free radicals are formed that may break double bonds, such as N = N and C = C. These double bonds link long conjugated chains of organic dye molecules that enable dye color to be seen by the human eye (Law 1995). Ideally, ozonation shifts the dominant wavelength of dye absorption to shorter values outside the visible range, resulting in decolorization. Wastewater treatment plants using biological processes do not effectively remove color, resulting in discharges that decrease the aesthetic value of receiving waters and may adversely impact aquatic biota (Law 1995). The potential release of toxic chemical constituents of the dyes and the dyeing process exists. Excess salinity used in the dyeing process to increase fixation of reactive dyes to fibers, as well as heavy metal components of some dye wastewaters, such as copper and zinc, may adversely impact the aquatic biota of the receiving waters (Wells *et al.* 1994; Law 1995). In previous work, the *Caenorhabditis elegans* aquatic toxicity test has been used to evaluate toxicity levels for the breakdown products of plant glucosinolates (Donkin *et al.* 1995) and to determine the source of toxicity for industrial effluents (one source being a textile dye operation) entering a waste water treatment plant (Hitchcock *et al.* 1997). In comparison, this study presents the ability of the nematode test to assess toxicity of synthetic dye wastewater byproducts after ozonation.

The objectives of this study were to determine if the synthetic dye wastewaters and/or ozonated dye wastewater byproducts are toxic to the nematode *C. elegans;* determine the toxic components in the synthetic dye wastewaters; and determine lethality trends over time during the ozone treatment of the synthetic dye wastewaters.

Materials and Methods

Nematode Culturing Methods

Caenorhabditis elegans cultures were maintained using published techniques (Williams and Dusenbery 1990; Donkin and Williams

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1995). The dauerlarva dormant life stage is exhibited by many free-living nematodes when subjected to environmental stresses such as starvation and overcrowding (Cassada and Russell 1975). One hundred to 200 dauers from a stock population of *C. elegans* were transferred to agar plates containing a food source consisting of an *Escherichia coli* (OP50 strain) bacterial lawn, which allows worms to mature (Brenner 1974). In 2 to 3 days, eggs from the mature adults were isolated using a 10% Clorox® solution followed by a rinse with K-medium (Donkin and Williams 1995). These eggs were allowed to hatch on agar plates with food source, resulting in synchronized adult worm populations to be used in toxicity testing. Agar plates containing approximately 250 adult worms (3 days old) were rinsed with K-medium to free the worms from the agar. The worms were then transferred by a sterile pipet into a 15-ml centrifuge tube and allowed to settle by gravity. The supernatant was removed with a sterile pipet, the worm pellet was suspended again with 10 ml of K-medium, and the worms were allowed to settle again. Once the worms settled, the supernatant was removed and 3 ml of K-medium was added to the centrifuge tube. The worms were suspended again before being transferred by pipet into a small glass petri dish and used to perform toxicity tests.

Toxicity Testing Methods

For each sample, the toxicity test consisted of 10 ($\pm$2) adult nematodes in four wells (Costar 3512 well dishes, Corning, Kennebunk, ME). Twenty-four- and 72-h toxicity tests were performed. For 24-h tests, 1-ml aliquots of sample were pipetted directly into each of four wells. For 72-h tests, a nematode food source was required. Upon test initiation, 15 ml of a given sample was added to a centrifuge tube containing a pelleted *E. coli* food source. This pellet was prepared prior to testing by centrifugation from 45 ml of saturated L-broth (a 3:1 broth to sample ratio) (Donkin and Williams 1995). The food source pellet was suspended into the sample with a sterile pipet. One-milliliter aliquots of the sample solution plus food source were pipetted into each of four wells. For each sample, eight to 12 worms each were transferred via pipet from the glass petri dish to the eight wells for both the 24- and 72-h toxicity tests. The actual number of worms placed into each well was recorded. Well dishes were covered and placed in a 20°C incubator. Counts of surviving worms were taken after 24 h ($\pm$1 h) or every 24 h ($\pm$1 h) for 72 h, depending on the test duration. Death was determined by visual observation and verified by gently probing the worms with a platinum wire to determine survival.

Toxicity Testing of Synthetic Dye Wastewaters

Toxicity tests were run for nonozonated dye wastewater samples to determine LC50 (lethal concentration of compound to 50% of test population) values. Samples tested in this experiment included two reactive azo dye wastewater samples (dye wastewater #4 and dye wastewater #9) and one acid-based azo dye wastewater sample (acid red). The ingredients (Table 1) were prepared in batches that resulted in a 30% dye dilution with tap water to represent simulated dye wastewater; this dilution percentage is based on the fact that industrial dye fixation averages are approximately 70%. The LC50s after a 72-h exposure duration were determined for acid red dye wastewater, dye wastewaters #4 and #9 with and without NaCl, and tap water equivalents containing 89 g/L NaCl and 112 g/L NaCl, respectively, for comparison to dye wastewaters #4 and #9. Salt, particularly NaCl, is commonly used in the dyeing process to increase the fixation of reactive dyes to their substrates (Apsland 1992; Keqiang *et al.* 1994), and the concentrations used in this study reflect those typically found in these dye wastewaters (Table 1). All pH levels for each sample were adjusted based on those necessary for optimal decolorization by ozonation (dye wastewaters #4 and #9 to pH 11; acid red dye wastewater to pH 4). Percentage-based volumetric dye wastewater dilutions in tap water were prepared based on initial range-finding tests for each type of sample. The surfactant Foam Buster®, used to inhibit foaming during ozonation, was also tested for toxicity using concentrations of 3–6 ml (at 1-ml intervals) per liter of tap water. All tests were run three times.

Table 1. Ingredients for simulated dye wastewaters (Hoechst-Celanese 1994)

Wastewater	Ingredient	Concentration (g/L)
#4	Remazol brill blue BB	0.32
	Intracron golden yellow	0.3
	Remazol red RB	0.08
	Alkodye 8526	0.5
	Assistant HOH	1.0
	Karadye	3.0
	Soda ash	4.5
	NaCl (optional)	89.0
#9	Remazol black B	0.75
	Remazol golden orange 3G-150	0.44
	Remazol red RB	0.11
	Alkodye 8526	0.5
	Assistant HOH	1.0
	Karadye	3.0
	Soda ash	4.5
	NaCl (optional)	112.0
Acid red	Acid red 337	0.3
	Triton X-100 (surfactant)	0.5
	Acetic acid	1.0

Ozonation Process

Simulated wastewaters were ozonated in an acrylic contact column with a volume capacity of 6.2 L. Ozone-bubble sparging was facilitated across the entire column cross-sectional area by a diffuser located at the bottom of the contact column (Figure 1). Input and output ozone concentrations were monitored by ultraviolet absorption type photometric monitors (Model HC; PCI Ozone and Control Systems, Inc., West Caldwell, NJ). Residual dissolved ozone in the wastewater was measured by a polarographic, membrane-type sensor with an electronic readout (Model 5160; Exidyne Instrumentation Technologies, Exton, PA) calibrated by KI titrations of wastewater drawn downstream of the sensor. Ozone was sparged into the column at a mean rate of 7.81 mg/L $\cdot$ min (steady state) (Law *et al.* 1996).

Ozonation of Tap and Deionized Water

Preliminary 24- and 72-h toxicity tests were run with nonozonated and ozonated tap water and deionized water to resolve any discrepancies in using either water source as a solvent in simulated dye wastewater mixtures. Preliminary tests were conducted to estimate nematode mortality due to the presence of ozone, independent of the presence of dye wastewaters. Preliminary tests were also conducted to resolve any issue of change in sample toxicity over time between withdrawing a sample from the ozone column and subsequent nematode exposure because of the potential of ozone to volatilize from solution. The following samples were used in preliminary toxicity tests for ozone treatment: (1) deionized water (pH = 7.9); (2) deionized water with adjustment to a pH of 11 using 10% NaOH; (3) tap water (pH = 7.9); and (4) tap water with a adjustment to a pH of 11 using 10% NaOH. This procedure was replicated twice for each sample type.

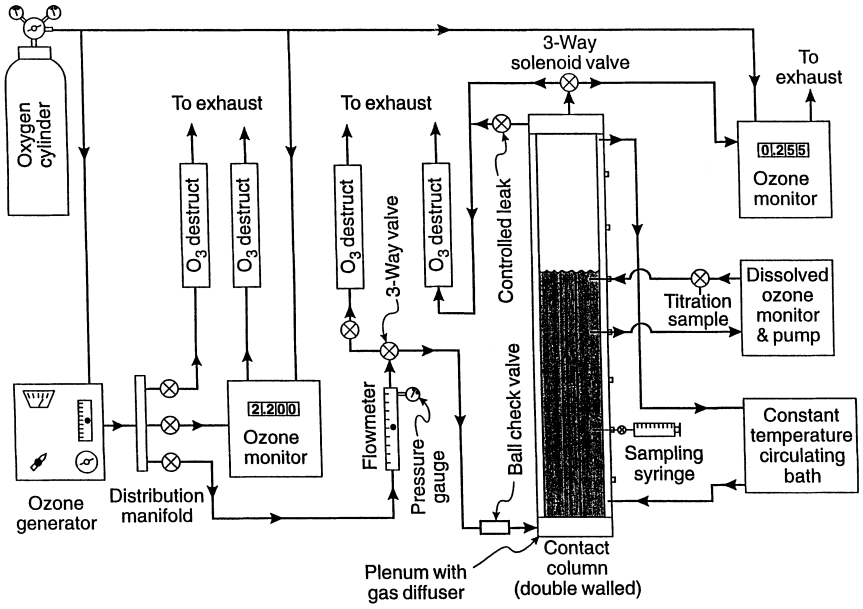

Fig. 1. Schematic diagram of the ozonation apparatus (Law *et al.* 1996)

The ozonation contact column containing 6.2 L of non-dye sample was brought to a steady state level of dissolved ozone content. The column remained at this steady state while samples were extracted for subsequent exposure to nematodes. A 50-ml ozonated sample with known dissolved ozone content was collected with a polyethylene syringe at time zero; this sample was subsequently divided into four open containers. Each of these four containers was allowed to sit in open air for a given time period (0 min, 32 min, 64 min, and 24 h) to allow for potential ozone volatilization. After each duration, toxicity tests were initiated for each sample.

Ozonation of Dye Wastewaters

The three synthetic dye wastewater types were ozonated and subsequently tested for toxicity using methods described above. The two reactive azo dyes (#4 and #9) were ozonated with and without NaCl salt concentrations, consisting of 89 g/L and 112 g/L, respectively. Also, pH was adjusted prior to ozonation for each sample type (dye wastewaters #4 and #9 to pH 11; acid red dye wastewater to pH 4). Foam Buster®, a dimethylpolysiloxane surfactant used to reduce bubbling during ozonation, was added to the acid red dye wastewater sample before ozonation (3–6 ml/L of dye wastewater). The testing of the five synthetic dye wastewaters was replicated three times for each sample. A 50-ml sample was collected from the ozonation column and tested for toxicity. Sample collection and subsequent 24- and 72-h toxicity testing was initiated after 8, 32, and 64 min of ozonation. Note that in testing tap and deionized water samples, treatment was considered to be sample retention time in open air for potential ozone volatilization; in testing ozonated dye wastewater samples, treatment was defined as the duration of sample treatment in the ozone contact column. Due to potential ozone volatilization from solution, a standard of 2 min was established as the mean time between dye wastewater sample extraction from the ozone column and nematode exposure to ensure consistency in dissolved ozone content in the sample.

Preparation of Ozonation Control Samples

Controls for column contamination, influence of additives to toxicity, nematode health, and pH adjustment were created for each test run. A 50-ml, time zero, nonozonated dye formula sample was taken from its original storage container after pH adjustment and before addition of Foam Buster®. This sample was used as a control for potential Foam Buster® effects and column contamination. Other controls consisted of regular tap water and adjusted tap water with a pH of 4 (adjusted with 10% HCl) or pH of 11 (adjusted with 10% NaOH), depending on the appropriate pH of the dye formula to be tested. Toxicity tests were considered invalid if nematode mortality counts in either tap water controls or pH adjusted tap water controls were greater than 10% (Williams and Dusenbery 1990; Donkin and Williams 1995).

Statistical Analyses

The level of significance for all statistical analyses conducted was 0.05. Mortality counts from nonozonated dye wastewater samples were tested for normal distribution using a Chi-square (goodness of fit) test for normality. Percentage-based concentration data were log-transformed (base ten) and LC50 values were determined by probit analysis using ToxStat© software (WEST, Inc.) (Gulley 1994).

Two-way analysis of variance (ANOVA) for repeated measures was used to determine significant differences in mean mortality data between exposure duration (24, 48, and 72 h) in each dye wastewater type. The ANOVA also tested for significant differences between treatment means taking into account the interaction between treatment time and length of exposure duration. Duncan's multiple range test was also used to determine significant differences in mortality data between treatment duration, including time zero nonozonated samples, of each dye wastewater type.

Results

Nonozonated Dye Wastewaters

Foam Buster® at concentrations as great as 6 mg/L demonstrated no significant mortality to *C. elegans* at 72 h of exposure. The LC50s for the five synthetic dye wastewater types are summarized in Table 2.

Table 2. Nematode LC50s based on 72-h mortality data for diluted synthetic dye wastewaters with and without NaCl and for tap water–NaCl solution equivalents (values are given as percent dilution)

Dye Wastewater	With NaCl	Without NaCl	Tap Water–NaCl
#4	19.2	62.0	27.8
#9	14.5	55.6	22.8
Acid red	NA	82.6	NA

NA = tests not conducted for LC50s

Ozonation of Tap and Deionized Water

Initial toxicity testing of ozonated tap and deionized water with and without pH adjustments showed no significant mortality for 24, 48, or 72 h of exposure ($\mu < 10\%$) due to either the ozonation process, the type of dilution water, or the pH adjustment to pH 11, except for deionized water at pH 11 at 72 h of exposure ($\mu = 25.8\%$). Therefore, tap water was used in dye wastewater recipes and controls for pH adjustment were established for each run of the experiment.

Ozonation of Synthetic Dye Wastewaters

Nematode mortality results of 72 h toxicity testing in 24-h increments as well as mortality data for each treatment time of ozonation (t = 0, 8, 32, and 64 min) are given in Table 3 for dye wastewater #4 without NaCl, dye wastewater #9 without NaCl, and acid red dye wastewater, respectively. For both dye wastewaters #4 and #9 with 89 g/L NaCl and 112 g/L NaCl, respectively, nematode mortality was 100% in all ozonated and untreated samples at all exposure durations.

For dye wastewaters #4 and #9 without NaCl, the impact on relative toxicities before, during, and after ozonation was evaluated. There was no significant difference in mortality data between ozone treated (t = 8, 32, and 64 min) and untreated dye wastewater #4 (t = 0) based on ANOVA and Duncan's multiple comparison test. There was a significant increase in nematode mortality from 24 to 48 h of exposure to dye wastewater #4, but no significant increase occurred from 48 to 72 h. Nematode mortality percentages increased as exposure duration increased, from 5–10% at 24 h to 72–77% at 48 h of exposure to dye wastewater #4 independent of ozonation treatment time.

Following the ozonation of dye wastewater #9, there was a significant difference in nematode mortality after 24 h of exposure based on Duncan's test for multiple comparisons; mortality decreased as the time of ozonation increased. The trend of decreased mortality with increased ozonation may occur at 48 and 72 h of exposure, but the differences were not significant. A significant increase in nematode mortality was demonstrated from 24 to 72 h of exposure to dye wastewater #9 for all treated and untreated samples.

Following ozonation of acid red dye wastewater, there was a significant increase in nematode mortality for 24, 48, and 72 h of exposure duration as the time of ozonation increased as shown by the repeated measures ANOVA and Duncan's test for multiple comparisons. At 24 and 48 h of exposure, there was no significant difference ($p > 0.05$) in nematode mortality between nonozonated acid red wastewater and wastewater that has been ozonated for 8 min. There were significant differences in mortality between 8, 32, and 64 min of ozonation of acid red dye wastewater; as the time of ozonation increased, mortality increased significantly ($p < 0.05$) after 24 and 48 h of exposure. There was no significant difference in nematode mortality after 72 h exposure in any of the samples.

Table 3. Results of Duncan's multiple range test for nematode mortality data after increasing durations of ozone treatment of synthetic dye wastewaters (values given are percent mortality means with standard errors in parentheses)

Sample Type	Duration of Ozone Treatment (min)	Exposure Duration		
		24 h	48 h	72 h
#4	t = 0	10 (±6.2)	74 (±13)	89.2 (±8.8)
	t = 8	6.6 (±2.7)	72.3 (±18.5)	75 (±20.4)
	t = 32	5.8 (±1.8)	73.3 (±17.7)	79.2 (±17)
	t = 64	7.1 (±3.2)	76.6 (±19.1)	76.6 (±20.6)
#9	t = 0	19.5 (±7)[a]	58.9 (±17.1)[a]	95.7 (±2.6)[a]
	t = 8	9.4 (±3.9)[b]	38.5 (±16)[a]	84.6 (±8.7)[a]
	t = 32	3.6 (±1.7)[b]	50.4 (±20.6)[a]	91.4 (±5.3)[a]
	t = 64	0.9 (±0.7)[c]	38.7 (±15.1)[a]	77.3 (±11.3)[a]
Acid red	t = 0	8.3 (±3.9)[a]	30.6 (±2.1)[a]	62.1 (±11.7)[a]
	t = 8	3.9 (±2.3)[a]	24.2 (±7.1)[a]	66.9 (±16.9)[a]
	t = 32	35.7 (±8.9)[b]	65.9 (±14)[b]	89.4 (±4.8)[b]
	t = 64	93.2 (±3)[c]	100 (±0)[c]	100 (±0)[c]

[a,b,c] Means in the same column with different letters are significantly different ($p < 0.05$) with respect to same sample type

Discussion

Ozone in the absence of dye wastewaters did not reduce the survival of the nematode *C. elegans* in 24- and 72-h tests, except in deionized water at pH 11. Donkin and Williams (1995) demonstrated that a low concentration of ions, such as that found in deionized water, may stress the nematode. This stress, along with the added stress of an alkaline environment, may have contributed to sample lethality.

For dye wastewaters #4 and #9 with NaCl (89 g/L and 112 g/L, respectively), mortality of *C. elegans* was 100% in all ozonated and untreated samples within 24 h. These dye wastewater samples contained NaCl concentrations greater than those that can be tolerated by most freshwater aquatic organisms. *C. elegans* can survive maximum salinities of 15.5 g/L NaCl and 11.5 g/L KCl in K-medium, and 20.9 g/L NaCl and 18.8 g/L KCl in moderately hard reconstituted water (per US EPA recipe [1993]) for 96 h (Khanna *et al.* 1997). The LC50s for NaCl to *C. elegans* range from 15 g/L in K-medium to 25 g/L in moderately hard reconstituted water in 48-h exposures (Cressman and Williams 1997). Due to the high concentrations of NaCl in these dye wastewaters, the impact of ozone treatment and its subsequent byproducts in these samples could not be evaluated.

Dye wastewater containing acid red was the only sample that became more toxic with increased ozonation duration. One major difference among dye types was the adjustment of pH in the samples; dye wastewaters #4 and #9 were ozonated at pH 11, while acid red dye wastewater was ozonated at pH 4. The ozonation of dyes can form organic acids (Fouche 1995) that

may lower the pH. *C. elegans* can tolerate a pH range of 3.2–11.8 for 96 h in K-medium and 3.4–11.7 for 96 h in moderately hard reconstituted water without significant mortality from concurrent tests with controls (Khanna *et al.* 1997). Acidic byproducts of dye wastewater ozonation could lower the initial pH of 4 to a level lethal to the nematode. For alkaline dye formulas, ozonation can lower pH by as much as 1.24 pH units (Keqiang 1994). In this study, ozonation decreased the initial pH of all wastewaters. After 64 min of ozonation, the pH of the salt-free reactive dyes shifted from 11 to 10.2, and the acid red dye wastewater shifted from pH 4.0 to pH 3.7. For ozonation of reactive dyes, the final pH (10.2) was within the maximum pH tolerated by *C. elegans* (11.7–11.8), while for the acid-based dye, the final pH (3.7) was near the nematode's minimum tolerable pH (3.2–3.4). Due to its complex nature, tap water constituents along with a lowered pH may have contributed to nematode mortality following ozonation.

Based on the results of this study, chemical byproducts from ozonation contributed to increased toxicity of certain dye wastewaters. For example, ozonation of metallized dyes may liberate toxic metal byproducts into solution. Results of previous toxicological studies have shown that ozonation of some reactive dyes increased toxicity to the duckweed species *Lemna minor* (Fouche 1995). In other studies with duckweed, the chelation and ozonation of dye wastewater was evaluated. Results demonstrated that ozonation did liberate metals (Cu, Cr, and Ni) into solution upon degradation of the dyes, thus increasing the toxicity to duckweed (Hill *et al.* 1996). In this study with *C. elegans*, the only metallized dye used was dye wastewater #4 (containing copper) and it exhibited no significant increased nematode mortality upon increased ozonation duration at 24, 48, or 72 h of exposure. The Remazol Blue BB dye component of dye wastewater #4 contains a copper compound (45–50%) and free copper (3.6%) (Hoechst-Celanese, Inc. 1994). Williams and Dusenbery (1990) and Freeman *et al.* (1998) have reported LC50s for *C. elegans* exposure to copper. In K-medium, the 24-h, 48-h, and 72-h LC50 for copper was 63, 1, and 0.49 mg/L, respectively. There was no significant difference in mortality at each exposure duration between nonozonated and ozonated dye wastewater #4 samples. Evidently, the concentration of copper compound of dye wastewater #4 was not high enough to show significant mortality in the nematode before ozonation, nor was enough copper liberated after ozonation to result in a concentration high enough for significant nematode mortality.

The 72-h LC50 values for nonozonated dye wastewater samples (Table 2) corresponded to the 72-h mortality results for the time zero non-ozonated samples tested during the ozonation experiments (Table 3). Nematode mortality for dye wastewater #4 at time zero of ozonation was 89.2%, while 100% dilution corresponded to over 80% predicted mortality of the nematode. Nematode mortality for dye wastewater #9 at time zero of ozonation was 95.7%, while 100% dilution resulted in 100% predicted nematode mortality. The *C. elegans* mortality for acid red dye wastewater at time zero of ozonation was 62.1%, while 100% dilution would give approximately 80% predicted nematode mortality.

LC50s for nonozonated samples show that NaCl was not the only factor contributing to toxicity of dye wastewaters. The LC50 for dye wastewater #4 with NaCl was 19.2% dilution, while the LC50 of its tap water equivalent was significantly higher at 27.8% dilution. For dye wastewater #9, the LC50 with NaCl was 14.5% dilution, while its tap water equivalent was 22.8% dilution. An increased ozonation duration for the acid-based dye wastewater resulted in increased nematode mortality, possibly due to lowered pH levels. High NaCl concentrations in the reactive dye wastewaters appeared to be the major factor influencing nematode mortality in the ozone treatment of these dye wastewaters.

Conclusions

Ozone treatment demonstrates tremendous applicability in the control and prevention of aesthetic pollution, particularly in the decolorization of textile dye wastewaters. Overall, optimal dye wastewater treatment results should exhibit the greatest degree of color removal with the least toxic end product. By analyzing trends in toxicity before, during, and after dye wastewater ozonation, the usefulness of this pollution prevention technique can be better evaluated for future use in industry.

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