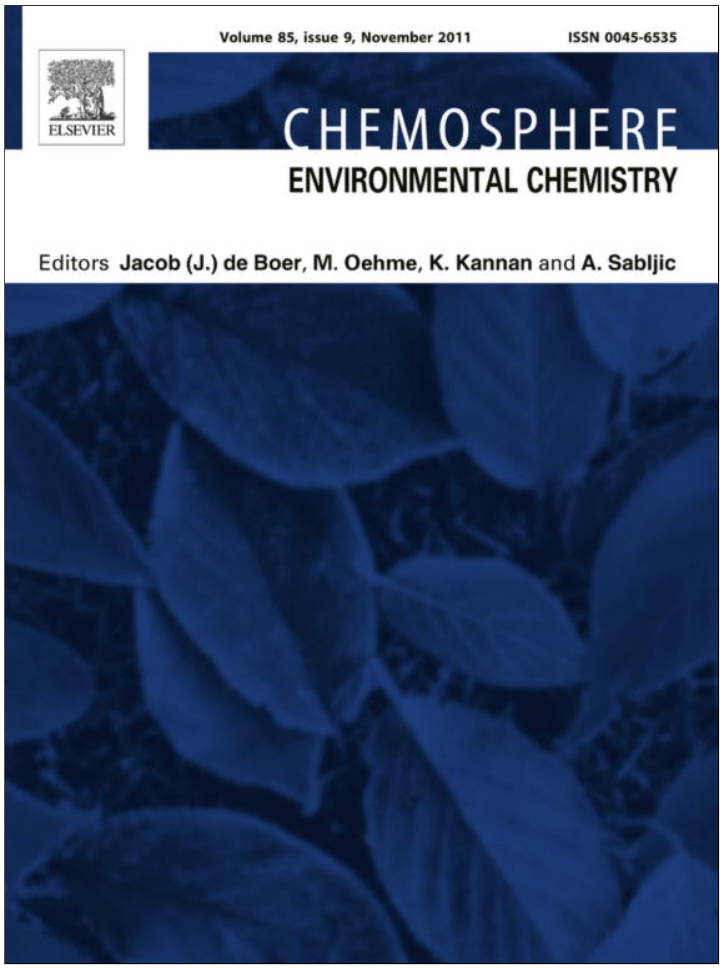

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Tetracycline photolysis in natural waters: Loss of antibacterial activity

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ABSTRACT

Previous work has shown that tetracycline undergoes direct photolysis in the presence of sunlight, with the decomposition rate highly dependent on conditions such as water hardness and pH. The purpose of this study was to examine the potential long-term significance of photoproducts formed when tetracycline undergoes photodegradation under a range of environmentally relevant conditions. Tetracycline was photolyzed in nine different natural and artificial water samples using simulated sunlight. The pH values of the samples ranged from 5 to 9. Total hardness values (combined Ca^{2+} and Mg^{2+} concentrations) varied from 30 to 450 ppm. Assays based on growth inhibition of two bacterial strains, *Escherichia coli* DH5α and *Vibrio fischeri*, were used to determine the antibacterial activity of tetracycline's photoproducts in these water samples. In all tested conditions, it was determined that the photoproducts retain no significant antibacterial activity; all observed growth inhibition was attributable to residual tetracycline. This suggests that tetracycline photoproducts formed under a wide range of pH and water hardness conditions will not contribute to the selection of antibiotic-resistant bacteria in environmental systems.

1. Introduction

The use of antibiotics in human and veterinary medicine is extensive and increasing (Sarmeh et al., 2006). Although reliable information about the quantities of antibiotics produced and used is not easily available, it is estimated that an excess of 50 million pounds are used annually in the United States alone (Levy, 1998; Verma et al., 2007a). Numerous studies have detected antibiotics in natural waters, and the main concern associated with the long-term presence of antibiotics in the environment is proliferation of antibiotic resistance among environmental bacteria. Resistant environmental bacteria are problematic because they may serve as a reservoir for antibiotic resistance genes that can potentially be transferred to pathogenic strains. Recent reviews have summarized the current state of knowledge in this area and point out the need for much more fundamental fate and effects data to accurately assess potential environmental risks of antibiotics in the aquatic environment (Kümmerer, 2009a,b). Tetracyclines (e.g. tetracycline, chlortetracycline, oxytetracycline) are used to treat infections in human and veterinary medicine and as growth promoters in agriculture, and account for approximately 29% of total antibiotic use, the largest class by volume (Khetan and Collins, 2007). The focus of this paper is on tetracycline (TTC, see Fig. 1), a member of the tetracycline class used primarily in human medicine.

Given the high usage rates and the fact that 80–90% of tetracycline is excreted unmetabolized (Hirsch et al., 1999), it is not surprising that tetracyclines have been detected in wastewater treatment plant (WWTP) effluents and receiving waters, in addition to waters impacted by agricultural runoff (e.g. Yang and Carlson, 2003; Miao et al., 2004; Karthikeyen and Meyer, 2006; Kim and Carlson, 2007). Photodegradation is expected to be a major fate of TTC and other members of the tetracyclines in many surface waters, with photolysis rates highly variable; Werner et al. (2006) found that pseudo-first-order rate constants for TTC photolysis can vary by up to an order of magnitude over the range of Mg^{2+} and Ca^{2+} concentrations relevant to natural waters. Several other studies have shown that the products of TTC photolysis can also be highly variable depending on factors such as pH and redox conditions (Davies et al., 1979; Hasan et al., 1985; Oka et al., 1989; Morrison et al., 1991; Addamo et al., 2005). In studies performed under conditions not necessarily relevant to surface waters, major products observed have included 5a,6-anhydrotetracycline and lumitetracycline (Hasan et al., 1985; Morrison et al., 1991). When photolyzing TTC under more environmentally relevant conditions, with borosilicate-filtered mercury vapor lamps and air-saturated solutions, Davies et al. (1979) and Addamo et al. (2005) both reported 4a,12a-anhydro-4-oxo-4-dedimethylaminotetracycline as a major degradation product, whereas Jiao et al. (2008) proposed products resulting from the loss of $-NH_2$ from the 2-carboxamide group and demethylation from the dimethylamine group at C-4,

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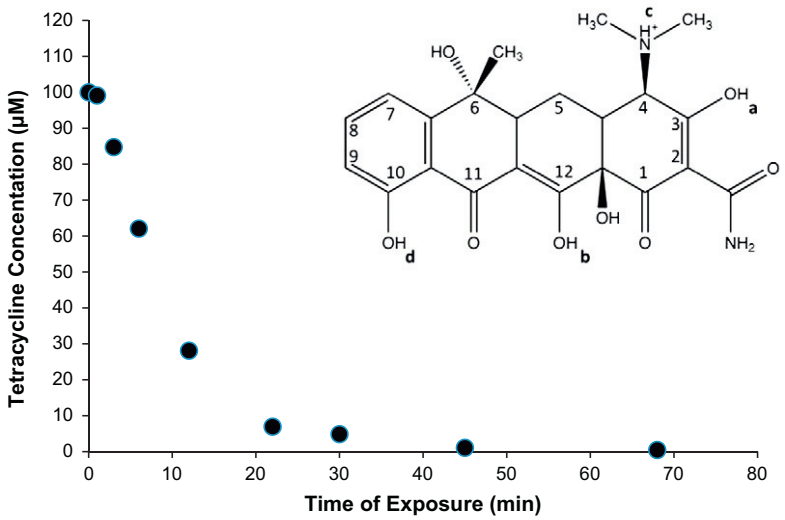

Fig. 1. A representative photolysis decay curve showing loss of tetracycline (μM) over time (min) in St. Paul tap water. The structure of tetracycline is also shown.

followed by loss of –OH at C-3 This variability potentially complicates assessment of the long-term risk of TTC in natural waters; if some photoproducts retain biological activity, specifically the ability to inhibit bacterial growth, it would be important to know under what conditions and at what rates they would be expected to form.

The goal of the present study was to determine whether any significant antibacterial activity is retained by products formed when TTC is photolyzed under a range of pH and water hardness parameters relevant to natural waters. Antibacterial activity was assessed by evaluating the overall ability of each photolyzed TTC solution to inhibit growth of susceptible laboratory bacterial strains. This strategy allowed a rapid assessment of whether photoproducts that retain the ability to inhibit bacterial growth, and are therefore of potential concern for contributing to long-term selection of resistant environmental bacteria, would be likely to form in natural systems.

2. Materials and methods

2.1. Chemicals

Tetracycline (TTC, 98% purity) was supplied by Sigma–Aldrich (St. Louis, MO), Iso-Sensitest bacterial growth medium by Thermo Fisher Scientific (Pittsburgh, PA), and Instant Ocean by Aquarium Systems Inc. (Mentor, OH). Other media components (tryptone, yeast extract, agar) were supplied by Becton, Dickinson & Company (Sparks, MD). Solvents (HPLC grade) and buffer components were supplied by VWR (Radnor, PA).

2.2. Natural and artificial water characterization

Water samples were collected from a rural lake (Eagle Lake, Monticello, MN), an urban lake (Lake Josephine, Roseville, MN), and a private suburban residential well (Woodbury, MN). Water samples were vacuum filtered through 0.2 μm pore size, 47 mm nylon filters and stored at 4 °C. In addition to these natural water samples, several artificial waters were created to broaden the range of pH and water hardness beyond that found in the three natural waters. These artificial waters were created by adding sodium hydroxide, hydrochloric acid, calcium chloride, and magnesium chloride to St. Paul city tap water; unmodified tap water was also used as an artificial water. Concentration of calcium and magnesium ions in appropriate dilutions of each natural and artificial water sample were determined using a Varian 55B Atomic Absorption Spectrophotometer equipped with a Ca/Mg cathode lamp. Absorption was measured at 422.7 nm for calcium ions and 285.2 nm for magnesium ions.

2.3. Tetracycline photolysis

Solutions of tetracycline (100 μM) were prepared in each natural and artificial water sample. Solutions were sonicated and then stored refrigerated in the dark. All solutions were used within 24 h of preparation; no TTC degradation was measured over this time scale. For each photolysis experiment, several 13 mm × 100 mm quartz test tubes containing the tetracycline solution were placed at a 45° angle to the light source. Photolysis experiments were conducted in an Atlas Suntest CPS + solar simulator equipped with a xenon lamp and an Atlas UV Suntest filter, to provide an emission spectrum accurately simulating that of natural sunlight with an irradiance setting of 765 W m^{-2}. Each test tube was removed from the instrument after a predetermined period of time and placed in the dark until all samples were ready for analysis.

Residual TTC concentration at each time point was determined by High Performance Liquid Chromatography (HPLC) analysis using an Agilent 1100 Series HPLC instrument equipped with a UV detector and a Supelco Discovery® RP Amide C16 column (10 cm × 4.6 mm, 5 μm particle size). The mobile phase consisted of an initial 90:10 mixture of pH 3 phosphate buffer (8.7 mM) : acetonitrile with a 12 min gradient to a 50:50 mixture of phosphate buffer:acetonitrile at a 1.0 mL min^{-1} flow rate. UV detector signals were read at 366 nm. TTC concentration was measured immediately prior to antibacterial activity tests, which were always initiated within 24 h of the photolysis experiments.

2.4. Antibacterial activity analysis

Antibacterial activity of TTC in each natural or artificial water was screened by measuring growth inhibition of the susceptible lab strain *Escherichia coli* DH5α by methods similar to those described previously (Wammer et al., 2006). *E. coli* were incubated at 37 °C overnight on Iso-Sensitest broth (ISB, made by adding 23.4 g of ISB powder per liter of pH 7 phosphate buffer (4.9 g KH_2PO_4 and 9.7 g Na_2PO_4 in deionized water) and sterilized by autoclaving (20 min; 121 °C; 97,000 Pa)). The appropriate volume

Table 1
pH and Magnesium and calcium ion concentrations of natural and synthetic water samples used in this study. Apparent pseudo-first-order rate constant (k_{app}) for photolysis of 100 μM TTC in each water sample.

Sample	pH	$[Mg^{2+}]$ (ppm)	$[Ca^{2+}]$ (ppm)	Total hardness (ppm)	Hardness classification	k_{app} ($\times 10^{-3}$ s^{-1})
Eagle Lake	8.4	27.5	17.8	45.3	Soft	1.97
Lake Josephine	7.8	24.5	8.3	32.8	Soft	2.23
Woodbury Well	7.3	30.4	63.0	93.4	Mod. hard	2.69
St. Paul tap	6.4	3.61	26.6	30.2	Soft	1.72
Hard adjusted tap	7.1	51.2	188	239	Hard	2.61
V. hard adjusted tap	7	106	343	449	Very hard	2.78
pH 5.2 adjusted tap	5.2	3.61	26.6	30.2	Soft	0.138
pH 6.0 adjusted tap	6.0	3.61	26.6	30.2	Soft	0.540
pH 9.0 adjusted tap	9.0	3.61	26.6	30.2	Soft	1.12

of unphotolyzed 100 μM tetracycline solution was added to test tubes containing 9.0 mL of sterile ISB and then the total volume was adjusted to 10.0 mL to obtain a range of tetracycline concentrations varying from approximately 0.01 to 10 μM. Aliquots of *E. coli* (100 μL) were then added to each tube. To assess the antibacterial activity of tetracycline photoproducts, 1.0 mL of photolysate (photoproduct mixture plus remaining parent tetracycline from each photolysis time point, as described in Section 2.3) was added to the test tubes in place of the unphotolyzed tetracycline. Test tubes were incubated in the dark for 6 h (37.0 °C, 190 rpm). Bacterial growth was assessed by measuring optical density at 600 nm and comparing to initial optical density of each solution (final OD_{600} − initial OD_{600}) using a Varian Cary 300 Bio UV–Visible Spectrophotometer. All bioassays were performed in triplicate.

An analogous bioassay was performed with *Vibrio fischeri*, ATCC# 7744, using a 300 μM TTC solution in St. Paul tap water because higher TTC concentrations were required to suppress growth of this organism. Bacteria were grown on photobacterium media (made by adding 15.0 g Instant Ocean, 2.5 g tryptone, 2.5 g yeast extract, and 1.5 mL of glycerol to 500 mL of DI water) at 26 °C overnight. Aliquots of *V. fischeri* (100 μL) were added to each test tube containing a mixture of ISB media and Instant Ocean. These assays were also performed in triplicate.

A phase contrast microscope (Axioskop 40, Carl Zeiss, Inc.) was used to verify that changes in sizes of individual bacteria during the course of the bioassays were not influencing optical density measurements. Images of representative individual bacteria were captured using a digital camera connected to the microscope at 1000× magnification.

3. Results and discussion

3.1. Water characteristics and photolysis

A variety of natural water characteristics may influence photodegradation of TTC. For example, indirect photochemical reactions could occur due to interactions with reactive oxygen species (ROS) formed in the presence of dissolved organic matter (DOM) and nitrates. Previous work, however, has demonstrated that ROS-mediated reactions are unlikely to be significant for photolysis of TTC in natural systems (Werner et al., 2006). Rather, direct photolysis is likely to dominate and be most heavily influenced by pH and water hardness, with the dominant TTC species present determined by the combination of these two factors. TTC has four acidic protons (a–d in Fig. 1) with p*K*a values (3.45, 8.00, 9.82, and 12.37) expected to shift significantly in the presence of calcium and magnesium (Regna et al., 1951; Rigler et al., 1965). Major species likely to occur in the range of pH and hardness values most commonly seen in natural waters include those with two or three acidic protons present (H_2L^-, H_3L^0), and those with one or two acidic protons plus one calcium or magnesium metal ion (MHL^0, MH_2L^+) (Werner et al., 2006). Therefore, if pH and calcium and magnesium ion concentrations are known, it should be possible to predict relative photolysis rates based on Werner et al.'s work.

Magnesium and calcium ion concentrations and pH for each of the three natural waters (Eagle Lake, Lake Josephine, Woodbury Well), St. Paul city tap water, and artificial waters created from tap water are shown in Table 1. Each sample is classified according to USGS total hardness guidelines as soft (0–60 ppm), moderately hard (61–120 ppm), hard (121–180 ppm), or very hard (over 180 ppm).

Fig. 1 shows loss of TTC when photolyzed in St. Paul tap water as a representative example. Photolysis of TTC in each water sample appeared to follow pseudo-first-order kinetics; however, Werner et al. (2006) showed that TTC photolysis includes some self-sensitization and is dependent on initial [TTC]. Therefore, the apparent rate constants included in Table 1 (k_{app}) should not be considered true first-order rate constants, especially because concentrations here needed to be high enough to test antibacterial activity and therefore are much higher than what would be expected in natural waters. Photolysis rates for 100 μM initial [TTC] varied over an order of magnitude in the nine water samples, with the fastest degradation in the presence of higher Mg^{2+} and Ca^{2+} ions and the slowest degradation at low pH. These relative rates matched very well with the predicted dominant TTC species in each water sample according to Werner et al. For example, relatively rapid degradation ($k_{app} = 2.78 \times 10^{-3}$ s^{-1}) was observed in the very hard adjusted tap water, where the dominant TTC species should be a complex with one metal ion and two acidic protons present (MH_2L^+). Much slower degradation ($k_{app} = 0.138 \times 10^{-3}$ s^{-1}) was observed in pH 5.2 adjusted tap water, where the dominant species is expected to have three acidic protons present and no metal ion (H_3L^0). The rate of pH 9.0 adjusted tap water ($k_{app} = 1.12 \times 10^{-3}$ s^{-1}), where the dominant species should have one metal ion bound and one acidic proton (MHL^0), falls, as predicted, between the two extremes. The application of the previously determined kinetics successfully predicts relative rates of TTC photolysis in water samples with a range of pH and water hardness values. Thus, the major question to be addressed in this study is whether the products produced in any of these waters retain measurable antibacterial activity.

3.2. Antibacterial activity: E. coli DH5α

The antibacterial activity assay used here measures bacterial growth, and the ability of compounds to inhibit that growth, as change in optical density over six hours. Growth in the presence of unphotolyzed TTC was compared to growth in the presence of a mixture of residual photolyzed TTC (as measured by HPLC) in addition to photoproducts generated at various time points. Phase contrast images of *E. coli* were taken when exposed to 2.0 or 0.5 μM TTC or photolyzed TTC at these same concentrations to ensure that differences in cell size did not contribute to changes in optical density in ways that would affect this comparison. At

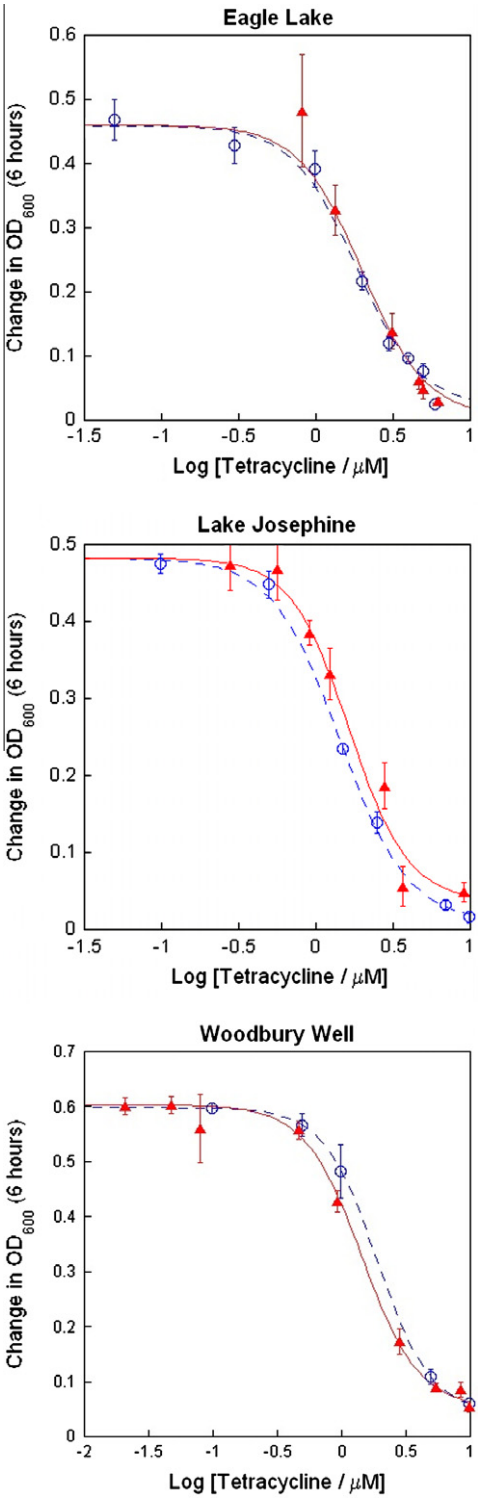

Fig. 2. Change in optical density at 600 nm after 6 h (ΔOD_{600}) for *E. coli* DH5α in the presence of a dilution series of unphotolyzed tetracycline (open circles, dashed line) and residual photolyzed tetracycline plus photolysis products (closed triangles, solid line) for three natural water samples. Error bars represent one standard error.

higher (2.0 μM) TTC, a slightly elongated cell phenotype was observed, but no differences in cell length were seen between photolyzed and unphotolyzed TTC (Fig. S1A).

Figs. 2, S2, and S3 show the results of the *E. coli* DH5α antibacterial activity assay. The data were fit using Igor Pro with a sigmoidal (Boltzmann) curve fit (Wammer et al., 2006):

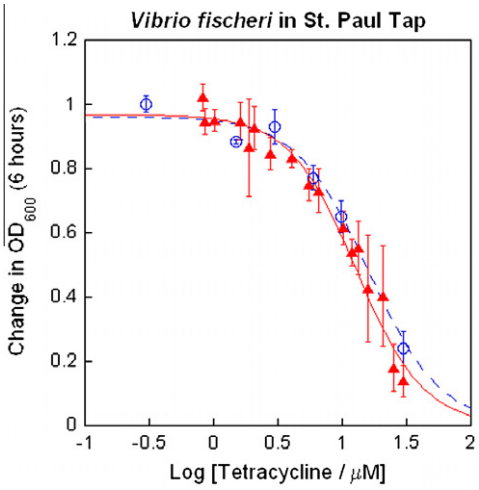

Fig. 3. Change in optical density at 600 nm after 6 h (ΔOD_{600}) for *V. fischeri* in the presence of tetracycline (open circles, dashed line) and tetracycline plus photolysis products (closed triangles, solid line) in St. Paul tap water. Error bars represent one standard error.

$$y = A_2 + \frac{(A_1 - A_2)}{1 + e^{\frac{(x - x_0)}{dx}}} \quad (1)$$

where x is the logarithm of tetracycline concentration, y is the change in OD_{600} over 6 h (ΔOD_{600}), A_1 is the value of y corresponding to no growth inhibition, A_2 is the value of y corresponding to maximum growth inhibition, dx is the slope at the midpoint of the curve, and x_0 is the midpoint. Values of A_1 were the same as those for positive control samples with no TTC or photoproducts present; values of A_2 were the same as those for negative controls with no change in OD_{600} over 6 h. In a few cases, A_1 and A_2 for the photolyzed data were held to the same value as the unphotolyzed data. This was the case for Eagle Lake (Fig. 2), pH 5.2 adjusted tap water and pH 6.0 adjusted tap water (Fig. S3), and *V. fischeri* in tap water (Fig. 3, A_2 only held).

The results for the three natural water samples are shown in Fig. 2. For the unphotolyzed tetracycline, the midpoint value x_0 corresponds to the concentration of tetracycline that is at half its maximum effective concentration (EC_{50}). This value cannot be correctly called an EC_{50} for the photolyzed tetracycline data if photoproducts with unknown concentrations potentially contribute activity, yet it is a useful value for comparison of antibacterial activity and is used as such here. These EC_{50} and midpoint values are listed in Table 2, along with 95% confidence intervals. The confidence intervals of the values for all six data series shown on Fig. 2 overlap. This shows that the activity of TTC itself is not significantly affected by any variations in water composition among these natural water samples. More significantly, the fact that the EC_{50} and midpoint values are statistically similar means that all growth inhibition in the photolyzed samples can be attributed to residual TTC. Additional activity attributable to any TTC photoproducts would result in a decrease of the midpoint value for the photolyzed TTC data. Therefore, no products with measurable antibacterial activity are formed during the course of the TTC photolysis in any of the natural water samples. If active products are formed, they are not formed at a high enough concentration to have a measurable effect.

Fig. S2 shows the results of the *E. coli* growth assay in soft, hard, and very hard water; EC_{50} and midpoint values are listed in Table 2. It is known that high Ca^{2+} and Mg^{2+} concentrations can decrease TTC activity in clinical settings (Dürckheimer, 1975). The EC_{50} values do seem to be slightly higher for harder water samples, but there is no clear trend when confidence interval overlaps are examined. This means it is unlikely that TTC activity will be

Table 2
EC_{50} values for unphotolyzed tetracycline solutions and midpoint values for photolyzed tetracycline solutions from *E. coli* antibacterial activity assays.

Sample	pH	Hardness (ppm)	Unphotolyzed EC_{50} (μM)	95% Confidence interval range (μM)	Photolyzed midpoint value (μM)	95% Confidence interval range (μM)
Eagle Lake	8.4	45.3	1.8	1.5–2.2	2.0	1.4–2.8
Lake Josephine	7.8	32.8	1.4	1.2–1.7	1.7	1.6–1.7
Woodbury Well	7.3	93.4	1.9	1.6–2.3	1.4	1.1–1.8
St. Paul tap	6.4	30.2	1.7	1.5–1.9	1.8	1.6–2.1
Hard adjusted tap	7.1	239	2.6	2.3–2.9	2.4	1.8–3.3
V. hard adjusted tap	7	449	1.9	1.6–2.2	2.5	2.1–2.8
pH 5.2 Adjusted tap	5.2	30.2	1.6	1.3–2.0	2.1	2.0–2.3
pH 6.0 Adjusted tap	6.0	30.2	1.9	1.4–2.6	2.0	1.8–2.2
pH 9.0 Adjusted tap	9.0	30.2	1.4	1.2–1.7	1.5	1.3–1.6

significantly affected even in very hard natural waters. In addition, as was seen for the natural water samples, the EC_{50} of each unphotolyzed sample is statistically similar to the midpoint value of the comparable photolyzed solution. Therefore, no active photoproducts are observed over a wide range of water hardness parameters.

Results of the *E. coli* growth assay in pH-adjusted tap water are shown in Fig. S3. All three EC_{50} values for the unphotolyzed data and all three midpoint values for the photolyzed data once again overlap the values for the natural waters; no products with measurable antibacterial activity are seen at any of the pH values tested.

3.3. Antibacterial activity: V. fischeri

Jiao et al. (2008) tested toxicity of TTC photoproducts using bioluminescence inhibition of *V. fischeri*. Enhanced inhibition was observed for samples that had been photolyzed for over 3 h, which suggested some acute toxicity attributable to those photoproducts. Here, we are specifically interested in the ability of photoproducts to retain antibacterial activity, and therefore potentially have long-term effects on antibiotic resistance, rather than acute toxicity. It has been shown that while the standard 30 min. *V. fischeri* bioluminescence assay (ISO/CD 11348) is useful for assessing acute toxicity, the assay is not long enough to cover the reproduction of *V. fischeri* and is thus unable to measure growth inhibition (Froehner et al., 2000). Jiao et al. cited the need for a more systematic study of photoproduct activity based on their results. Therefore, we performed the antibacterial activity assay using *V. fischeri*, to see if there were any organism-specific effects on growth.

Fig. 3 shows the results for the *V. fischeri* growth assay in St. Paul tap water. As was the case for *E. coli*, *V. fischeri* exposed to both unphotolyzed and photolyzed solutions after 4 h of incubation were similar in cell size (Fig. S1B). Growth of this wild strain is less consistent than for the *E. coli* DH5α, but once again no evidence was observed that suggests photoproducts are forming in high enough concentrations to significantly affect bacterial growth. The EC_{50} value (and 95% confidence interval range) for TTC is 16 μM (9–30 μM) while the midpoint of the TTC plus photoproducts curve is 13 μM (11–15 μM). Therefore, all observed activity is attributable to residual parent TTC concentration.

3.4. Environmental significance

Due to a wide range of pH and water hardness values, the nine water samples included here had variable relative concentrations of the major possible TTC species, which resulted in highly variable photolysis rates. Despite this, no active photoproducts were observed in any of the natural or artificial water samples at TTC concentrations much higher than those typically found in the environment. This makes it very unlikely that photoproducts able to inhibit bacterial growth will form in most natural waters and unlikely that photoproducts will therefore contribute to the development of antibiotic-resistant environmental bacteria. This does not rule out, however, other potential effects; Verma et al. (2007b) observed effects on protein production in river and wetland bacteria at TTC concentrations as low as 5 $\mu g\,L^{-1}$.

In the present study, no photoproducts were observed to form in significant concentrations during any of the photolysis experiments that retained the 360 nm chromophore of TTC. When absorbance of the other chromophore of TTC at 270 nm was monitored, one major photoproduct was commonly observed but it was photolabile and eliminated within three TTC half-lives. This means disruption of the four-ring structure has occurred for all major photoproducts, and all TTC derivatives with fewer than four rings are inactive (Dürckheimer, 1975. Therefore, this suggests that if photolysis is allowed to proceed long enough in oxygenated waters, the products will all be too small to retain biological activity. These observations are consistent with those of Oka et al. (1989), who photolyzed pH 7 solutions and isolated several products, all with three or less rings intact, after three hours of photolysis.

3.5. Conclusions

Photolysis of TTC was studied under a wide range of pH and water hardness conditions relevant to natural waters. In every case examined, no measurable antibacterial activity was observed that could be attributed to any photoproducts. This helps clarify the potential significance of TTC in surface waters. While TTC speciation is complicated and relative photolysis rates vary significantly by species, reasonable rate estimates are possible if pH and Ca^{2+} and Mg^{2+} ion concentrations are known. This study shows that such a prediction of parent TTC concentration is most likely adequate to assess potential for long-term effects. While the possibility that some transient photoproducts exhibit acute toxicity may need further examination, photoproducts are unlikely to affect bacterial growth and therefore unlikely to contribute to selection of antibiotic-resistant organisms.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.08.051.

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