PRESENCE OF TETRACYCLINE ANTIBIOTICS IN SURFACE WATER
A STUDY OF THE PRESENCE / ABSENCE OF TETRACYCLINE IN THE RACCOON RIVER WATERSHED
DES MOINES WATER WORKS LABORATORY

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I. Introduction

Available information on the presence of antibiotics in surface water is very limited. Low concentrations of antibiotics in waste water and surface water have been detected in recent studies (Hartmann et al., 1998; Hirsch et al., 1999; Hartig et al., 1999; Meyer et al., 2000; Alder et al., 2000; Nipales et al., 2000; Frick et al., 2001). Despite this detection, little is known about the distribution of antibiotics in the environment, their mobility and their persistence in natural and engineered waters.

Antibiotics are considered emerging microcontaminants in water because of their potential adverse effects on ecosystems and human health. Large antibiotic usage may result in 90% of prescribed antibiotics excreted into environmental waters without undergoing metabolism (Levy, 1992). These antibiotics are released into waterways via wastewater effluent and agricultural runoff as result of incomplete metabolism, ineffective treatment removal and improper disposal. Two potential environmental and human health effects of antibiotics which have been recognized are an increase in the proliferation of antibiotic-resistant pathogens and the disruption of microbial ecology. It stands to reason that water industries may soon be presented with the issue of water quality with respect to antibiotics and should be encouraged to start resource planning now. Des Moines Water Works undertook this study of the distribution of antibiotics in the Raccoon River watershed as a first step in evaluating their presence in source waters.

II. Antibiotics in Livestock

Information on of animal antibiotic use is limited due to poor documentation by producers and the lack of public access to records. The Union of Concerned Scientists (UCS) (Mellon et al., 2001) issued a report on the estimates of antibiotic use in beef cattle, swine and poultry production in the U.S. Their findings focused on non-therapeutic uses of antibiotics in livestock for growth promotion and disease prevention. These antibiotics increase the animal's ability to absorb feed, reach market weight quicker, and counteract the effects of over-crowded living conditions and poor hygiene in confinements.

More than 40% of antibiotics produced in the U.S. are used as feed supplements according to Environmental Media Services (2000). The antibiotic dosage varies from 1 to 100 g Mg^{-1} of feed depending on the animal species and antibiotic administered. The concentrations of antibiotics also vary between animal species, so the types of antibiotic compounds that are found in surface water will strongly depend upon the types of livestock operations within the watershed. Quantities of tetracycline (chlortetracycline and oxytetracycline in greatest quantity), macrolide (tylosin in greatest quantity), sulfonamide (sulfamethazine), aminoglycoside (lincomycin and apramycin) and B-lactoam (penicillin) antibiotics are commonly added to the

feed of beef cattle, poultry and swine. Land application of animal waste also provides routes for agricultural antibiotics to enter aquatic environments which eventually reach drinking water supplies.

Beef cattle confinements outnumber swine and poultry operations in the Raccoon River watershed. Therefore, antibiotics fed to beef cattle will be of consideration. Table 1 below indicates the levels of tetracycline antibiotics present in cattle manure.

Table 1: ESTIMATES OF CONCENTRATIONS OF ANTIBIOTICS IN MANURE OF BEEF CATTLE* IN A CONFINED FEEDING OPERATION

Antibiotics	Class	Antibiotic Concentration (ug of antibiotic/kg of manure)
Chlorotetracycline Sulfamethazine	Tetracycline Sulfonamide	3973
Tylosin	Macrolide	1946
Bacitracin	Polypeptide	1703
Oxytetracycline	Tetracycline	1064
Chlortetracycline	Tetracycline	993
Erythromycin Thiocyanate	Macrolide	730

*Average weight: 700-1200 pounds

- The amount of manure produced is estimated based upon data found in the literature (Miner et al., 2000).
- The amount of antibiotic excreted is estimated based upon the antibiotic dosage reported by the UCS and in the Feed Compendium (2000), assuming up to 80% of the administered antibiotic is excreted unmetabolized.

Based on this data, and because beef cattle are the primary livestock in the Raccoon River watershed, and because the tetracycline antibiotic group is the most-utilized antibiotic for beef cattle, tetracycline was chosen for this study.

Researchers have also found that the drug metabolites of chlorotetracycline excreted by medicated livestock are decomposed by bacterial action in liquid manure and reconverted into active drugs (Warman and Thomas 1981) thus posing potential antibiotic contamination of surface water.

Public and water utilities are becoming increasingly concerned with the contribution of livestock-borne antibiotics in the Raccoon and Des Moines River watersheds due to the high numbers of confinement operations in these areas. Figure 1 below shows the locations of Concentrated Animal Feeding Operations (CAFOs) in Iowa. Figure 2 below shows locations of manure management sites in the Raccoon River Watershed.

Figure 1: Iowa Concentrated Feeding Operations

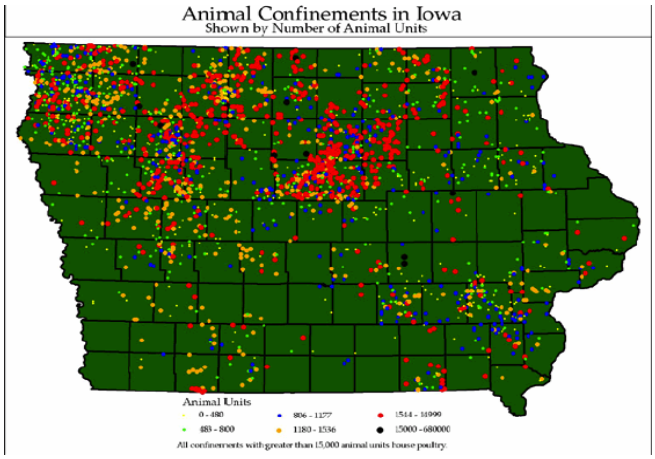
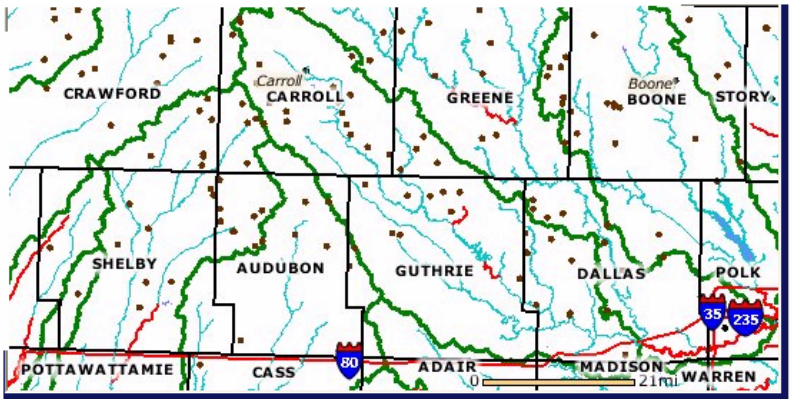


Figure 2: Manure Management Locations in the Raccoon River Watershed



III. DMWW Tetracycline Testing

A. Method Description

The analytical method used was the Ridacreen tetracyclin enzyme immunoassay (ELISA) for the quantitative analysis of tetracycline, adapted to the analysis of surface water by Dr. K. Kumar, Department of Soil, Water, & Climate, University of Minnesota, St. Paul, MN.

ELISA assays are commercially available for testing antibiotic residues in milk. A 1:1 dilution of surface water with buffer is the only modification to the method. The ELISA tetracycline kit was purchased from r-Biopharm, Marshall, MI.

The basis of the test is the antigen-antibody reaction. The process is shown graphically in Figure 3 below:

Figure 3: ELISA Test for Tetracycline

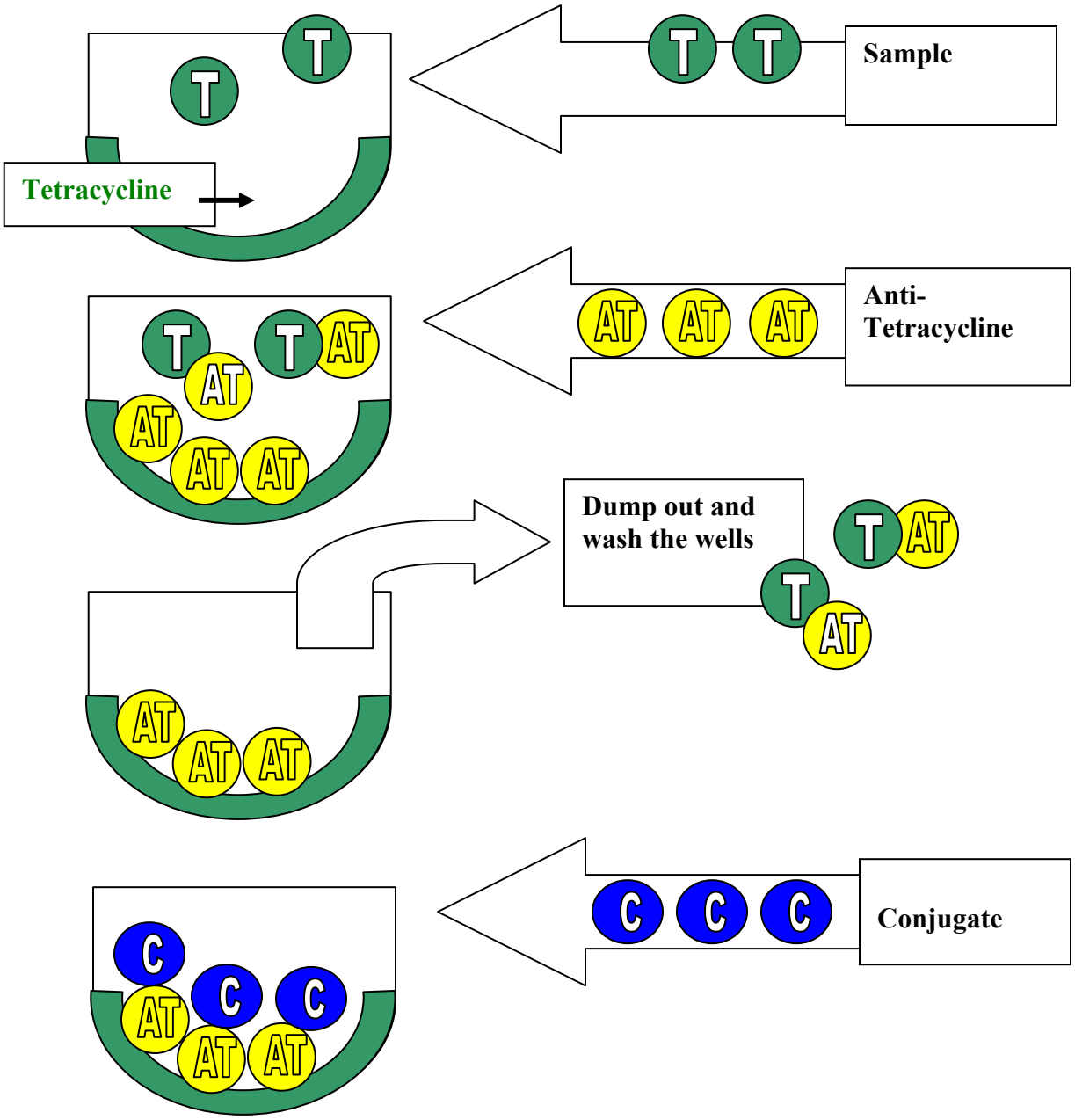
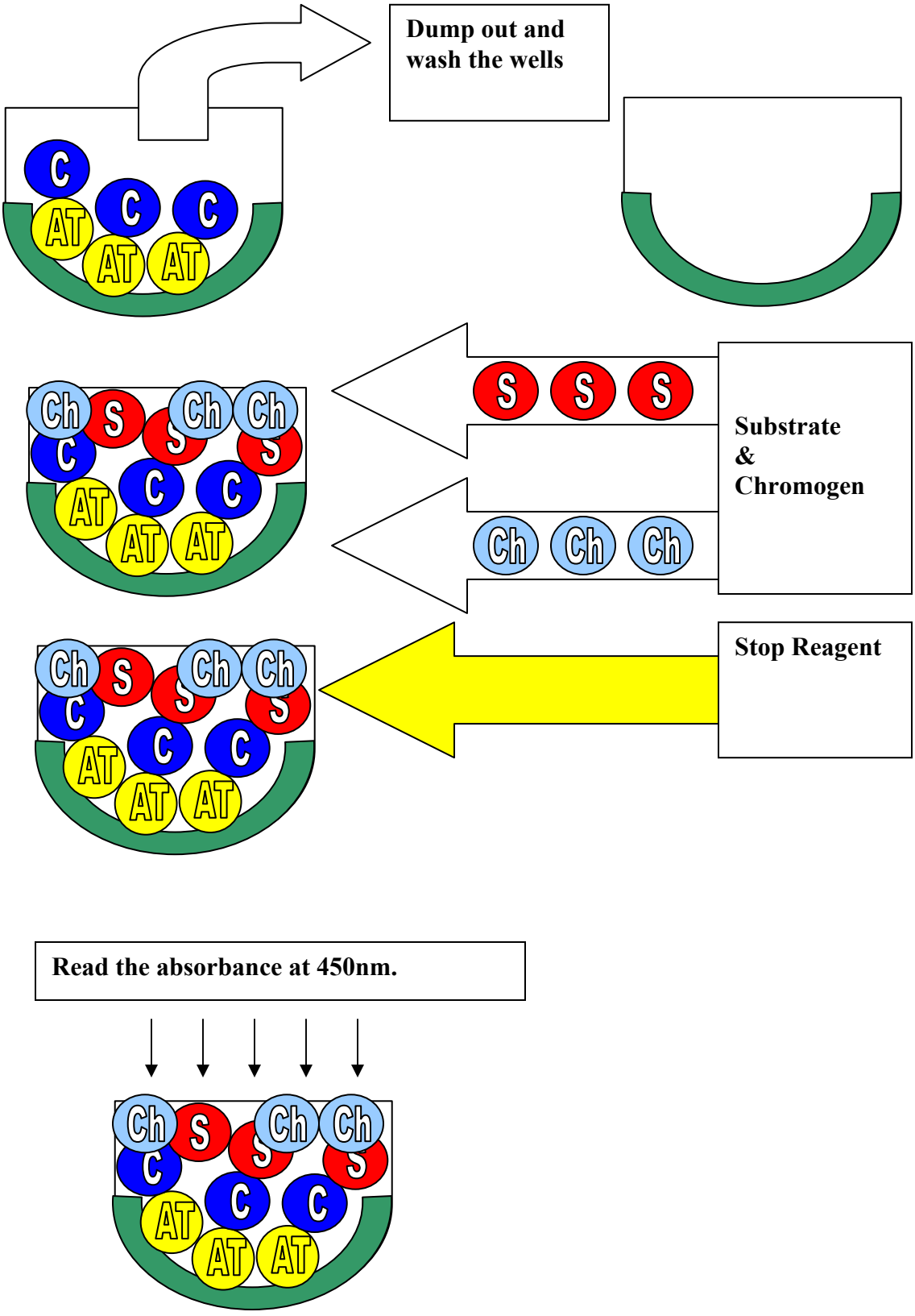


Figure 3, Continued



The microtiter wells are coated with tetracycline-protein-conjugate. Tetracycline standards or sample solutions and anti-tetracycline antibodies are added. Free tetracycline and immobilized tetracycline compete for the tetracycline antibody binding sites. Unbound antibody is then removed in a washing step. The enzyme-labeled secondary antibody, which is directed against the anti-tetracycline antibody, is then added. After removing unbound enzyme-labeled antibodies by a washing step, enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen into blue product. The addition of the stop reagent leads to a color change from blue to yellow. The measurement is made photometrically at 450nm. The absorption is inversely proportional to the tetracycline concentration in the samples. The test measures tetracycline, chlortetracycline, minocycline and rolitetracycline as one.

B. Analytical Steps

1. prepare standards: 450µl buffer + 50µl of each concentrated standard concentrations 0, 0.05, 0.15, 0.45, 1.35, 4.05 ppb in glass vials
2. prepare samples: (1:1 dilution with same buffer used to make standards)
3. insert sufficient number of wells into microwell holder for both samples and standards
4. Add 50µl each of prepared water samples and standards to microtiter wells of the ELSIA plate – duplicate!
5. Add **50µl** of anti-tetracycline antibody solution to each well. Mix gently by rocking the plate manually and incubate for **1 hour** at room temperature (20 – 25^{O}C / 68 – 77 ^{O}F).
6. Pour liquid out of wells and tap the microwell holder upside down vigorously (3X) against absorbent paper to ensure complete removal of liquid from wells. Fill all wells with 250 µl washing buffer and pour out the liquid. Repeat two more times.
7. Add **100 µl** of enzyme conjugate to each well. Mix gently by rocking the plate manually and incubate for **15 min** at room temperature.
8. Pour liquid out of the wells and tap the microwell holder upside down vigorously 3X in a row against absorbent paper to ensure complete removal of liquid from wells. Fill the wells with 250 µl washing buffer and pour out the liquid again. Repeat 2 more times.
9. Add **50µl** substrate and **50µl** chromogen to each well. Mix gently by rocking the pate manually and incubate for **15 minutes** at room temperature in the dark.
10. Add **100 µl** stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 60 minutes after addition of the stop solution.

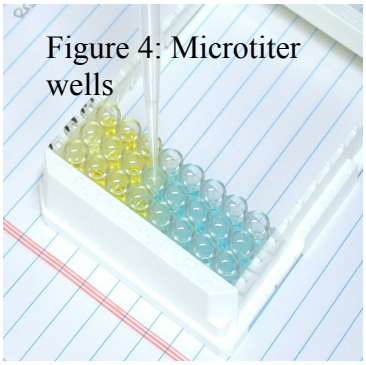


Figure 4: Microtiter wells

11. Samples are read utilizing Des Moines University research laboratories microtitler plate reader with the assistance of Dr. Bryan Larson and Michael Essmann. Absorbances obtained from the microtiter plate reader are then entered into the r-biopharm software for the analysis of tetracycline and concentrations, ppt, are derived.

Figure 5: Plate Reader

IV. Results

A. Trial Study

1. Sampling Plan

a) Collect samples following a rain event since tetracycline binds to soil
b) Sample a minimum of four sites in Sac County – heavy cattle confinement area
c) Sample two urban sites: Beaver and Walnut Creek
d) Sample DM waste water effluent
e) Analyze for tetracycline using ELISA test procedure
f) Also analyze samples for anions and coliforms to evaluate connection with tetracycline.

Trial Study Sample Locations

Site Code	Description
RR-1	LAKE CREEK @HIGHWAY 20 EAST OF ROCKWELL CITY
RR-2	BIG CEDAR @ HIGHWAY 20 EAST OF SAC CITY
RR-3	RACCOON RIVER @ HIGHWAY 20 AT SAC CITY
RR-4	RACCOON RIVER @ HIGHWAY 196 NORTH OF ULMER
RR-5	MIDDLE RACCOON RIVER AT CARROLL
RR-6	STREAM ON N50 BY RACCOON RIVER AT DICKENSON TIMBER PRESERVE BY LIDDERDALE
RR-7	BEAVER CREEK @ 141
WW	DES MOINES WASTE WATER TREATMENT PLANT

2. Results: March 29, 2005

SITE	CHLORIDE ppm	NITRATE NO3-N ppm	PHOSPHATE PO4-P ppm	TOTAL COLIFORMS TC/100mL	E.COLI EC/100mL	TETRACYCLINE ppt
RR-1	101	7.24	0.22	14,390	2780	89.75
RR-2	27.7	10.23	<0.1	517	17	65.69
RR-3	34.1	14.2	0.41	411	36	113.3
RR-4	31.5	11.48	0.28	579	33	<50.00
RR-5	18.6	9.42	<0.1	921	77	<50.00
RR-6	52	11.2	<0.1	613	5	77.12
RR-7	30.1	11.63	<0.1	1203	25	101.92
WW	NA	NA	NA	NA	NA	55.29

3. Discussion of Results

Tetracycline was detected in 5 of the 8 sites sampled. Whether or not the tetracycline was introduced into the surface water by humans or livestock cannot be defined. Personal communications with Dr. Kumar confirmed that the amount of tetracycline detected falls into expected concentration ranges for surface water influenced by animal waste or wastewater discharge. Tetracycline concentrations do not strongly correlate with anion or coliform results.

B. Lake Creek Study

1. Overview

Lake Creek just east of Rockwell City was sampled during the trial study. The Lake Creek sample contained 14,390 TC/100 mL total coliforms, 2,780 EC/100 mL *E.Coli*, and 89.75 ppt tetracycline when sampled on March 29th. The goal of the Lake Creek study was to determine whether or not Lake Creek is contaminated with Tetracycline and *E. coli* from Rockwell City's wastewater treatment plant or from agriculture confinement operations. Several samples were collected along Lake Creek from Rockwell City down to its confluence with the North Raccoon River.

Research of literature and personal communication with Dr. Kumar supported a hypothesis of tetracycline absorption onto soil particles. Dr. Kumar supplied an extraction procedure to remove the bound tetracycline from sediments in order to determine the total amount of tetracycline present in an aqueous sample. This enabled analysis of the Lake Creek samples for both dissolved and total tetracycline.

Lake Creek Sample Locations

SITE Code	Site Location	Physical Site Observations
NR	Rainbow bridge ½ mile down stream from confluence of Lake Creek and North RR	Woodlands, park
LC2	Approx. 1 mile downstream from 100 head feed lot	Pasture, riparian, woodlands
LC8	Rockwell City wastewater effluent	Effluent into Lake Creek, grass
LC7 ALT	Across from feedlot east of Rockwell City on Hwy 20	Feedlot access to creek, pasture
LC7	Feedlot east of Rockwell City on Hwy 20	Downstream from wastewater treatment plant & before feedlot, pasture
LC11	Lake creek at 250	Some buffer, tree lined pothole to water cattle, evidence of cattle path, row crop
LC15	Wastewater effluent @ Pomeroy	Lagoon treatment plant discharging twice per year. Started discharging week before.

2. Results: April 19, 2005

SITE #	CHLORIDE ppm	NO3-N ppm	PO4-P ppm	TOTAL COLIFORMS TC/100mL	E.COLI EC/100mL	Dissolved TETRA-CYCLINE ppt	Total TETRA-CYCLINE (PPT)
NR	27.61	13.23	<0.2	6,300	2,000	<50	3090
LC2	46.63	14.98	0.44	686,700	488,400	<50	3176
LC8	>200	8.32	1.38	816,400	727,000	314	1869
LC7 ALT	64.33	10.15	<0.2	1,986,300	1,413,600	<50	2195
LC7	68.29	10.14	0.15	9,208	1,565	<50	3338
LC11	35.45	10.17	<0.2	2,046	2,382	<50	3000
LC15	>200	0.25	2.32	2,046	262	<50	3500

3. Discussion

All samples were visually turbid. The nature of the suspended particles varied greatly. Some samples had fairly large suspended particles up to 3mm in length with the water otherwise appearing clear. Others contained fine particles that made the samples look homogeneously turbid. The Pomeroy wastewater treatment plant sample was very green. The color was determined to be due *Euglena*.

Somewhat unexpectedly, dissolved tetracycline was detected only in the Rockwell City wastewater treatment plant effluent. The samples were collected after a rain and in areas densely populated with cattle. Sample analysis revealed *E.Coli* counts in the range of 2,000 to 730,000/100 mL.

Substantially higher results for total tetracycline supports lends evidence to the hypothesis of tetracycline absorption onto soil particles. These results were consistent with those obtained by Dr. Kumar and John Vargo of the University of Iowa Hygienic Laboratories. It became apparent that further study on surface water samples would require the extraction procedure to free tetracycline absorbed onto soil particles.

4. Tetracycline Extraction Procedure

I. Divide the sample into two aliquots
   a) Filter one aliquot through the 0.45 micron filter
   b) Mix this aliquot1:1 with dilution buffer.

II. Add 200mg EDTA to the second aliquot - 20mL water sample, (EDTA will extract any bound tetracycline from soil particles and colloids thus enabling quantitative analysis of the amount of tetracycline bound to the soil particles and colloids)
   a) Mix on a shaker for 1 hour
   b) Filter through a 0.45 micron filter and dilute 1:1 with buffer.

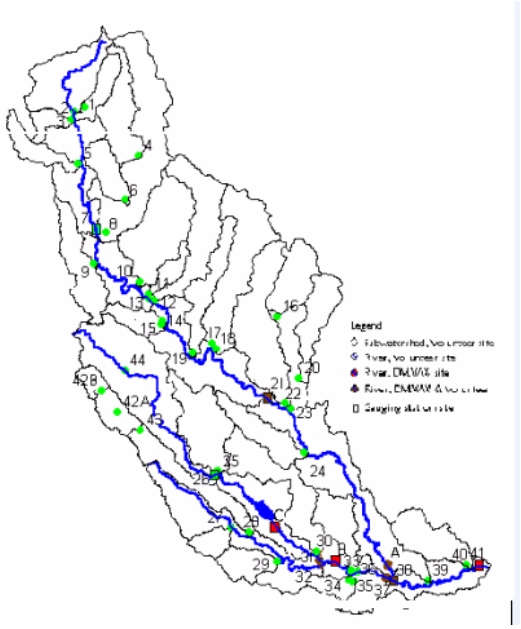
III. Estimate the solids in the water sample.
   a) The difference between the 2^{nd} and 1^{st} aliquot will provide an estimate of amount of tetracycline absorbed on sediments or colloids.

C. Agriculture Clean Water Alliance Samples

1. Overview

For the past six years, the Des Moines Water Works has partnered with Agriculture's Clean Water Alliance to study the Raccoon River Watershed. The purpose of this partnership was to provide a snapshot of where high nitrate levels originated in the watershed, with the idea remedial efforts could be focused in these areas. This tetracycline study utilized these same samples for the determination of the presence-absence of tetracycline throughout the Raccoon River watershed.

2. Sample Locations



Site	Description
C	Raccoon River at Panora
5	Outlet Creek
7	North Raccoon River 2
8	Cedar Creek
12	Lake Creek
14	Elk Run A
43	Brushy Creek

3. Results: April 28, 2005

SITE	CHLORIDE ppm	NITRATE NO3-N ppm	Ortho-PHOSPHATE ppm	TOTAL COLIFORMS TC/100mL	E.COLI Count/100mL	Dissolved TETRACYLCINE ppt	Total TETRACYCLINE ppt
C	25.30	7.52	<0.1	1411	38	NA	641
5	21.75	16.09	<0.1	>2419	1414	NA	342
7	27.31	15.25	<0.1	1300	77	NA	279
8	29.31	16.40	<0.1	1553	205	NA	356
12	38.16	15.21	<0.1	2419	308	NA	434
14	33.14	31.11	<0.1	>2419	1553	NA	439
43	15.96	15.34	<0.1	>2419	>2419	152	589

4. Discussion

Tetracycline was detected at all volunteer monitoring locations that were tested. Results were not that variable from one site to another, and there does not seem to any correlation with the other water quality parameters.

D. Process Water Samples

1. Overview

Source, process, and finished water samples were collected at both the Fleur Drive and Maffitt Treatment Plants. Source water at both plants includes both shallow groundwater under the influence of the Raccoon River and surface water—reservoir and quarry pit water at Maffitt and river water at Fleur.

2. Results 5-16-05

a) Fleur Drive

Sample	Dissolved Tetracycline (ppt)	Total Tetracycline (ppt)
Raccoon River	272	966
Des Moines River	562	1014
Infiltration gallery	<50.0	NA
Gallery Recharge Pond	155	713
Filter Effluent	<50.0	NA
Finished Water	<50.0	NA

b) Maffitt

Sample	Dissolved Tetracycline (ppt)	Total Tetracycline (ppt)
Reservoir	272	900
Quarry Pit	<50.0	745
Total Raw Water	<50.0	NA
Filter Effluent	<50.0	713
Finished Water	<50.0	NA

3. Discussion

It was discovered that extracting low-turbidity samples (such as groundwater, finished water, and filter effluent water) produced false positives, so only source surface water samples were extracted. It stands to reason that since suspended solids are absent in the finished water samples, that any tetracycline present would have to be in the soluble form.

The above results indicate that tetracycline is being removed by the treatment process. This is not a surprise since lime softening treatment, which is implemented at both plants, removes virtually all suspended solids from the water.

V. Conclusions

The investigator has concluded that tetracycline is indeed present in the waters of the Raccoon River watershed and in the surface water sources of the Des Moines Water Works. Sources of the tetracycline likely include both humans and livestock. Large variations throughout the watershed were not observed. Correlations with other water quality data, such as nitrate and coliform bacteria, were not strong.

Apparently tetracycline is completely removed at both of DMWW's treatment plants. This is due in large part to tetracycline's propensity to bind strongly with suspended particles. Lime softening, the method of treatment at both plants, precipitates and removes large quantities of suspended materials; this enables tetracycline to attach to insoluble material and then settle from the water as the water is processed through the treatment train. Nonetheless, DMWW will remain vigilant in assessing tetracycline, along with other emerging contaminants, in the source, process, and finished waters.