

Water Resources Center Annual Technical Report FY 2005

Introduction

The Minnesota WRR program is a component of the University of Minnesota's Water Resources Center (WRC). The WRC is a collaborative enterprise involving several colleges across the University, including the newly created College of Food, Agriculture and Natural Resource Sciences (CFANS; formerly the separate Colleges of Natural Resources and Agriculture, Food, and Environmental Sciences), the Minnesota Extension Service (MES), and the University of Minnesota Graduate School. The WRC reported to the Dean of CNR this past year. In addition to its research and outreach programs, the WRC is also home to the Water Resources Sciences graduate major. The WRC has two co-directors, Professor Deborah Swackhamer and Professor James Anderson, who share the activities and responsibilities of administering its programs.

The WRC funds 3-4 research projects each year, and the summaries of the current projects are found in the rest of this report.

Research Program

Photochemistry of Antibiotics and Estrogens in Surface Waters: Persistence and Potency

Basic Information

Title:	Photochemistry of Antibiotics and Estrogens in Surface Waters: Persistence and Potency
Project Number:	2003MN32G
Start Date:	9/1/2003
End Date:	2/28/2006
Funding Source:	104G
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Research Category:	Not Applicable
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Descriptors:	
Principal Investigators:	Kristopher McNeill, Deborah L. Swackhamer

Publication

1. J.J. Werner, A.L. Boreen, B. Edlund, K.H. Wammer, E. Matzen, K. McNeill, W.A. Arnold, Photochemical transformation of antibiotics in Minnesota waters, *CURA Reporter* 2005, 35(2), 1-5.
2. D. E. Latch, Environmental photochemistry: Studies on the degradation of pharmaceutical pollutants and the microheterogeneous distribution of singlet oxygen. Ph.D., University of Minnesota, Minneapolis, MN, 2005, 256 pp.
3. A.L. Boreen, Enhanced photolysis in natural waters: naturally occurring sensitizers and substrates and application to the fate of aquatic pollutants. Ph.D., University of Minnesota, Minneapolis, MN, 2006, 263 pp.
4. B. L. Edlund, W. A. Arnold, K. McNeill, Aquatic Photochemistry of Nitrofurantoin Antibiotics, *Environ. Sci. Technol.* in revision.
5. Werner, J.J.; Arnold, W.A.; McNeill, K., 2006. Water Hardness as a Photochemical Parameter: Tetracycline Photolysis as a Function of Calcium Concentration, Magnesium Concentration, and pH, *Environ. Sci. Technol.*, accepted.
6. Wammer, K.H.; LaPara, T.M.; McNeill, K.; Arnold, W.A.; Swackhamer, D.L., 2006. Changes in Antibacterial Activity of Triclosan and Sulfa Drugs due to Photochemical Transformations, *Environ. Toxicol. Chem.*, in press.
7. A.L. Boreen, W. A. Arnold, K. McNeill, Triplet-sensitized photodegradation of sulfa drugs

- containing six-membered heterocyclic groups: Identification of an SO₂ extrusion photoproduct, *Environ. Sci. Technol.* 2005, 39, 3630-3638.
8. J.J. Werner, K. McNeill, W.A. Arnold, Environmental photodegradation of mefenamic acid. *Chemosphere* 2005, 58, 1339-1346.
 9. D.E. Latch, J.L. Packer, B.L. Stender, J. VanOverbeke, W.A. Arnold, K. McNeill, Aqueous photochemistry of triclosan: Formation of 2,4-dichlorophenol, 2,8-dichlorodibenzo-p-dioxin and oligomerization products, *Environ. Toxicol. Chem.* 2005, 24 (3), 517-525.
 10. A.L. Boreen, W.A. Arnold, K. McNeill, Photochemical fate of sulfa drugs in the aquatic environment: Sulfa drugs containing five-membered heterocyclic groups, *Environ. Sci. Technol.*, 2004, 38, 3933-3940.
 11. J.J. Werner, Photolysis of pharmaceuticals in water and soil matrices: agricultural antibiotics and mefenamic acid. Ph.D. University of Minnesota, Minneapolis, MN, 2006, forthcoming.
 12. W.A. Arnold and H. Dolliver. Pharmaceuticals in Groundwater: Fate, Transport, and Effects. Minnesota Groundwater Spring 2006 Conference, St. Paul, MN, April 12, 2006.
 13. B.L. Edhlund, W.A. Arnold, K. McNeill, Aquatic Photochemistry of Nitrofurantoin Antibiotics. Celebrating Women Chemists Luncheon, University of Minnesota, Minneapolis, MN, February, 2006.
 14. K. McNeill, Photochemical approaches to environmental pharmaceutical pollutants, American Chemical Society (ACS) National Meeting Symposium: Strategies and Molecular Mechanisms of Contaminant Degradation Chemistry, Washington, D.C., August 28-Sept. 1, 2005.
 15. K. McNeill, Photo-generated Reactive Species and the Degradation of Pharmaceutical Pollutants, Stanford University, Civil and Environmental Engineering Student Seminar Series, May 20, 2005.
 16. K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, Cornell University, Department of Chemistry, March 7, 2005.
 17. K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, University of Rochester, Department of Chemistry, March 4, 2005.
 18. K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, University of California, Berkeley, Department of Chemistry, February 25, 2005.
 19. K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, Northwestern University, Department of Chemistry, February 18, 2005.
 20. K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, 3M, November 12, 2004.
 21. A.L. Boreen, W.A. Arnold, K. McNeill, Photochemical fate of sulfa drugs in the aquatic environment. Physics and Chemistry Colloquium, Concordia College, Moorhead, MN, November, 2004.
 22. Werner, J.J.; Arnold, W.A., McNeill, K. The environmental photochemical kinetics of tetracycline as a function of pH and water hardness. To be presented in Environmental Chemistry Awards, Environmental Chemistry Division, American Chemical Society National Meeting, San Francisco, CA, September, 2006.
 23. Werner, J.J.; Arnold, W.A.; McNeill, K., 2005. Water Hardness as a Photochemical Parameter: The Case of Tetracycline. Paper presented in Emerging Contaminants, Pacifichem 2005, Honolulu, HI, December 15-20, 2005.
 24. Werner, J.J.; Arnold, W.A.; McNeill, K., 2006. Environmental photochemistry of the antibiotic compound tetracycline: dependence on acid-base and metal binding speciation. Paper presented at the Minnesota Water 2005 and Annual Water Resources Joint Conference, Brooklyn Center, MN, October 25-26, 2006.
 25. B.L. Edhlund, W.A. Arnold, K. McNeill. Aquatic Photochemistry of Nitrofurantoin Antibiotics. Poster

- Presentation. ENVR, 230th ACS National Meeting, Washington, DC, August 2005.
26. B.L. Edhlund, W.A. Arnold, K. McNeill. Aquatic Photochemistry of Nitrofurans Antibiotics. Poster Presentation. Minnesota Water 2005 and Annual Water Resources Joint Conference, Brooklyn Center, MN, October 2005.
 27. B.L. Edhlund, W.A. Arnold, K. McNeill. Aquatic Photochemistry of Nitrofurans Antibiotics. Poster Presentation. Minnesota ACS Meeting, St. Paul, MN, November 2005.
 28. B.L. Edhlund, W.A. Arnold, K. McNeill. Aquatic Photochemistry of Nitrofurans Antibiotics. Poster Presentation. Environmental Sciences: Water Gordon Research Conference, Plymouth, NH, June 25-June 30, 2006.
 29. J.J. Werner, K. McNeill, W.A. Arnold, Speciation-dependent photochemistry of tetracycline antibiotics: acid-base speciation and metal-binding effects. Oral Presentation. Midwest Environmental Chemistry Workshop, Madison, WI, October 16-17, 2004.

Photochemistry of Antibiotics and Estrogens in Surface Waters: Persistence and Potency

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Start date: 9/01/2003

End date: 2/28/06

Executive Summary

Antibiotics and estrogens are two classes of wastewater contaminants that have been detected in US surface waters. The potentially adverse effects of these pollutants on water quality are unknown, but will be determined, in part, by their persistence and the biological activity of both the parent compound as well as the degradates. Photolysis is one possible loss process, and the direct and indirect photolysis of five sulfa drug antibiotics, four nitrofurantoin antibiotics, four fluoroquinolones, and tetracycline has been investigated. The structure of the R-substituent on the sulfa drugs controls the reactivity; those containing six-membered substituents degrade through both direct photolysis and reaction with triplet dissolved organic matter. Both processes result in SO₂ extrusion. The photochemical kinetic rate constants for the loss of tetracycline under natural sunlight are a function of its various environmentally-relevant aqueous chemical species, including acid-base equilibria and metal-binding. Direct photolysis has been found to be the major photochemical degradation pathway for the nitrofurantoin antibiotics, with the formation of a photostationary state between the *syn* and *anti* isomers occurring in the first several minutes of light exposure. All antibacterial compounds tested, three sulfa drugs and triclosan (an antimicrobial agent), photodegraded to products with no observable antibacterial activity.

Introduction

Reports of pharmaceuticals and personal care products (PPCPs) in natural waters have recently appeared with increasing frequency.¹⁻⁵ Two important subclasses of these emerging contaminants are particularly worrisome due to their potential to adversely affect surface waters: antibiotics and environmental estrogens. Estrogenic compounds have a demonstrated ability to interfere with the development of aquatic organisms,^{5,6} while there is concern that the presence of antibiotics in natural waters will lead to an increase of antibiotic resistant bacteria.^{7,8} These compounds are released into surface waters as a result of human use, through discharge of

treated and untreated wastewater. An additional, major source of antibiotics comes from their wide use in the production of food animals and in fish farming.¹⁻⁵

The magnitude of the effects and potential threat to water quality due to antibiotics and hormones is, in part, determined by the compounds' persistence in aquatic systems. The principle goal of this proposed study is to understand one aspect of their persistence—their degradation by photochemical processes. Based upon our work⁹⁻¹⁵ and that of others,^{11, 16-22} we believe that photodegradation may be a major loss process for these compounds in sunlit waters. Thus, it is important to understand the photochemical processes that degrade these chemicals in surface waters, to identify intermediates and products that are formed, and to assess the biological activity of these products.

Methods

Direct and natural water photolysis experiments

Photolysis experiments were performed outdoors under natural sunlight or indoors under medium pressure Hg-vapor lamps or a Suntest CPS+ solar simulator equipped with a Xe-arc lamp and a UV Special Glass filter to mimic the solar spectrum. Sample solutions were contained in quartz test tubes (OD = 13 mm, ID = 11 mm, V = 10 mL). For kinetic analyses approximately 0.5 mL samples were withdrawn from the quartz tubes at predetermined intervals and analyzed on an 1100 Series Hewlett Packard HPLC equipped with UV-absorbance detection and a computer driven data acquisition system. In experiments designed to probe for pH effects, various buffer solutions were employed to set the pH values. Solar quantum yields were calculated by comparing the rate constant for the disappearance of the PPCPs under either natural sunlight or the Suntest CPS+ solar simulator with the rate constant for the disappearance of a *p*-nitroanisole actinometer. For toxicity experiments, test tubes were sacrificed at pre-selected time intervals and saved for HPLC analysis of remaining antibiotic concentration and subsequent antibacterial activity testing. The wavelength dependence of the direct photolysis of the nitrofurantoin antibiotics was probed using a series of cut-off filter tubes (absorbing $\lambda < 320$ nm, 280 nm, and 220 nm). Quartz test tubes containing the photolysis solutions were placed inside the filter tubes during photolysis.

Natural water photolysis experiments were performed in 0.2 μm filtered Lake Josephine (LJW) water or Lake Superior (LSW) water. To determine which pathways were responsible for the photodegradation, various quenchers were added to or removed from the water samples (sodium azide or DABCO for $^1\text{O}_2$, isopropanol for radicals, oxygen and isoprene for triplet DOM) and the substrate was also photolyzed in DI water in a separate tube.

Speciation dependent behavior of tetracycline

Association constants which determine the speciation of calcium- and magnesium-tetracycline complex formation were measured by pH titrations (pH 3 to 11) performed at various constant metal concentrations and the collection of UV-vis spectral data. The first order rate constant for the loss of tetracycline under simulated sunlight (Suntest CPS+ photosimulator, Atlas) was observed at various pH, calcium, and magnesium concentrations. Kinetic experiments were performed as detailed above. The concentration-dependent initial rate of photochemical degradation was monitored for various initial tetracycline concentrations and extrapolated to infinite dilution to determine the first-order rate constant for the loss of tetracycline in the absence of self-sensitization.

Singlet oxygen (1O_2)

Singlet oxygen reaction kinetics were measured in one of three ways, directly by laser flash photolysis (LFP), or indirectly by either steady-state photolysis (SSP) or thermal generation of 1O_2 . In both LFP and SSP experiments the substrate (typically at micromolar concentrations) and 100 μ M perinaphthenone, a well-defined singlet oxygen sensitizer, were dissolved in aqueous buffer solutions. In the LFP experiments, a pulse of laser light excites the sensitizer, which then produces singlet oxygen after the excited-state sensitizer is quenched by dissolved molecular oxygen. A sensitive Ge-photodiode detector then monitors the phosphorescence emission from singlet oxygen. The rate of disappearance of the singlet oxygen phosphorescence signal is a measure of a substrate's activity toward singlet oxygen. The resulting total quenching rate constant (k_{tot}) is the sum of the chemical reaction and physical quenching rate constants.

In SSP experiments, the samples were photolyzed continuously and small aliquots were removed for analysis by HPLC. In this case, the disappearance of the PPCP was monitored (as decreases in peak area), rather than the singlet oxygen signal. This allows for determination of the chemical reaction rate constant (k_{rxn}) for the PPCP with singlet oxygen.

To avoid any competing photochemical reaction occurring in SSP, thermal generation of 1O_2 was used. In these experiments, 1O_2 was generated through the reaction of hydrogen peroxide (H_2O_2) and molybdate (MoO_4^{2-}).²³⁻²⁵ H_2O_2 (1 M) was added to a buffered solution containing MoO_4^{2-} (1 mM), a reference compound of known k_{rxn} (FFA; 100 μ M), and substrate (100 μ M). Aliquots of the reaction solutions were added to an aqueous solution of sodium azide (507 mM) at a series of time points to quench the reaction. Samples were then analyzed for both reference compound and substrate degradation via HPLC.

Product identification

Since large volumes of photolysate were required for product identification, photolyses were executed using a higher intensity light source (450 W medium pressure Hg-vapor lamp) which was completely immersed in the photolysis solution (100 μ M substrate, 300 mL). After photolysis, the solution was concentrated to a total volume of 2 mL and the desired photoproduct was isolated using preparative HPLC. Sufficient amounts of product for analysis were obtained by combining the collected fraction from multiple injections of the photolysate on the preparative HPLC column.

Following isolation, the product was identified using an array of analyses including mass spectrometry, infrared spectroscopy, and nuclear magnetic resonance (NMR). Mass spectral data were obtained for both the raw photolysate and the isolated products using a Bruker BioTOF ESI-TOF mass spectrometer. High resolution mass spectra were obtained using an internal standard of poly(ethylene glycol). Infrared absorbance spectra were acquired using a MIDAC Corporation M-Series FT-IR by placing a solution of the isolated photoproduct in methanol- d_4 between two NaCl plates. The 1H -NMR and ^{13}C -NMR spectra of isolated photoproducts were obtained on a Varian Inova 300 MHz spectrometer. A quantitative 1H -NMR spectrum of the same sample was acquired using an internal standard.

Biological activity

The ability of the antibacterial compounds and their photolysis products to inhibit bacterial growth was tested using *E. coli* DH5 α . The bacteria were maintained on agar plates and grown up overnight on Iso-Sensitest broth (ISB) (Oxoid, Inc.) prior to testing. One mL of antibacterial compound or photolysis product and 100 μ L of *E. coli* were added to test tubes

containing nine mL of ISB prepared in a pH 7 phosphate buffer (9.7 g KH_2PO_4 and 19.4 g Na_2PO_4 per liter deionized water). The solutions were incubated in the dark at 37 °C while being shaken. Bacterial growth was assessed after 8 hours by measuring optical density at 600 nm (OD_{600}).

The antibacterial compounds and their photolysis products were also tested for their ability to inhibit bacterial respiration. The respiration assay used was based on the ability of the bacteria to reduce iodinitrotetrazolium chloride. *E. coli* (400 μL) was added to 40 mL of ISB and incubated at 37 °C. Once the OD_{600} of this solution had reached 0.4 (in the exponential phase of the growth curve), 1 mL aliquots were centrifuged at 19,000g for five minutes. The supernatant was decanted, and 0.5 mL of antibiotic or photolyzed antibiotic was added. The bacterial pellet was resuspended, and the tubes were then incubated in the dark at 37 °C while being shaken. After one hour of incubation (approximately one generation time), 0.5 mL of a 5 mM solution of the tetrazolium salt was added and the tubes were incubated for an additional hour. The tubes were then centrifuged, the supernatant decanted, and 1 mL of an organic solution (1:1 dimethylformamide: ethanol) was added to the bacterial pellet to extract the formazan. The pellet was resuspended, and the tubes were incubated in the dark at room temperature for one hour. After centrifuging, the absorbance of the supernatant was measured at 464 nm to quantify the amount of formazan formed.

Results to date

Photodegradation of the Sulfa drugs

The photolysis rates of the sulfa drugs containing six-membered heterocyclic substituents (sulfachloropyridazine, sulfadiazine, sulfamerazine, and sulfamethazine) in Lake Josephine ($\text{DOC} = 5.9 \text{ mg/L}$) water were enhanced by a factor of 1.4-2.6 relative to the photodegradation rates in DI H_2O . The enhancement in the natural water has been attributed to reaction of the sulfa drugs with excited triplet dissolved organic matter (^3DOM). Verification that the reaction is sensitized by ^3DOM was provided by the characteristic enhancement of the degradation upon eliminating oxygen from the system and suppression of the degradation upon addition of isoprene, quenching of triplet-excited state perinaphthenone during LFP experiments, and the lack of reaction between the sulfa drugs and $^1\text{O}_2$ as measured using thermal generation methods. The natural water photodegradation of sulfadimethoxine matched the degradation in DI H_2O , and the degradation was thus attributed solely to direct photolysis. The direct photolysis of sulfadimethoxine is pH dependent, and is explained by differing reactivity of the protonation states. The remaining sulfa drugs' direct photolysis and triplet-sensitized degradations are not pH dependent over the pH range 6-9.

The primary product of both direct photolysis and triplet-sensitized degradation was identified as an SO_2 extrusion product (Figure 1). The yield of this product from sulfamethazine was found to be 64%.

Tetracycline

The pseudo-first-order rate constant for the photochemical loss of tetracycline was observed, under environmentally-relevant conditions, to be dependent on pH and both calcium and magnesium concentration. For each of the four acidic protons in tetracycline, deprotonation leads to both increased solar action spectrum and increased rate constant for photochemical degradation. The binding of tetracycline species to either calcium or magnesium leads to a further increase in the action spectrum for solar absorption. In the laboratory, the high

tetracycline concentrations (1 to 10 μM) led to significant self-sensitization, especially at higher pH values. For example, at a pH of 7.5, the observed pseudo-first-order rate constant appeared to double when increasing the initial tetracycline concentration from 1 to 15 μM , with a linear dependence on initial tetracycline within the concentration range. As an example of the rapid kinetics, the half-life of tetracycline extrapolated to infinite dilution at pH 7.5 was 9.9 minutes, where the experimental light intensity was approximately the same as that of a clear summer day, noon, 45° latitude.

Photochemical behavior of the nitrofurans antibiotics

The photodegradation of the nitrofurans antibiotics (furaltadone, furazolidone, and nitrofurantoin; Table 1) was found to be dominated by direct photolysis with the formation of a photostationary state between the *syn* and *anti* isomers occurring during the first minutes of photolysis. The direct photolysis rate constant and quantum yield were calculated for each of the nitrofurans. Environmentally relevant half-lives were determined for mid-season surface waters at 45°N latitude and range between 0.08 – 0.44 hours for mid-summer. Reaction rate constants with $^1\text{O}_2$ and $\cdot\text{OH}$ were also measured. Half-lives for these processes were calculated using environmentally relevant concentrations; 66 – 488 hours and 74 – 82 hours for $^1\text{O}_2$ (10^{-12} M) and OH (10^{-15} M), respectively. When compared with direct photolysis half-lives, it is clear that indirect photochemical processes will not compete with direct photodegradation.

The photodegradation pathway is summarized in Figure 3. The major photodegradation product of the nitrofurans was identified as nitrofuraldehyde, which is also photolabile. Direct photolysis of nitrofuraldehyde was found to produce NO which is easily oxidized to form nitrous acid. Studies determining the pH dependence of the photolysis of the nitrofurans concluded that the degradation is acid catalyzed, indicating that the acid produced from photolysis of the nitrofurans further catalyzes their degradation, leading to autocatalytic behavior. Natural waters were found to buffer the initial acid formation.

Biological Activity

Comparing the growth of *E. coli* DH5 α in the presence of unphotolyzed sulfathiazole (Figure 2, open circles) versus in the presence of partially photolyzed sulfathiazole (Figure 2, closed triangles) revealed little difference in the inhibition of bacterial growth as a function of sulfathiazole concentration. Any photolysis products generated at a given point along the curve and present in the samples in the photolyzed series in addition to the sulfathiazole would be responsible for deviations from the unphotolyzed sulfathiazole series. The concentration at which sulfathiazole has reached half of its maximum effective concentration (EC_{50} values) for these two curves were statistically similar. This suggests that the products of the photolysis do not retain any significant ability to inhibit bacterial growth; that is, the antibacterial activity of the photolyzed solution only comes from the unreacted sulfathiazole. Similar results were observed for sulfamethoxazole, sulfachloropyridazine, and triclosan.

Summary of findings

The photodegradation mechanism for the sulfa drugs containing six-membered substituents involves both direct photolysis and reaction with triplet dissolved organic matter generating an SO_2 extrusion photoproduct. Comparison of these results with those obtained for the sulfa drugs containing five-membered substituents reveals that minor structural changes can

give rise to disparate environmental loss mechanisms. The photochemical kinetic constants for the loss of tetracycline under natural sunlight are a function of its various environmentally-relevant aqueous chemical species, including acid-base and metal-bound forms. Direct photolysis has been found to be the major photochemical degradation pathway for the nitrofurantoin antibiotics, with the formation of a photostationary state between the *syn* and *anti* isomers occurring in the first several minutes of light exposure. All antibacterial compounds tested, three sulfa drugs and triclosan, photodegraded to products with no observable antibacterial activity.

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List of publications & presentations resulting from this project

Peer Reviewed Publications

B. L. Edhlund, W. A. Arnold, K. McNeill, Aquatic Photochemistry of Nitrofurantoin Antibiotics, *Environ. Sci. Technol.* in revision.

Werner, J.J.; Arnold, W.A.; McNeill, K., 2006. Water Hardness as a Photochemical Parameter: Tetracycline Photolysis as a Function of Calcium Concentration, Magnesium Concentration, and pH, *Environ. Sci. Technol.*, accepted.

Wammer, K.H.; LaPara, T.M.; McNeill, K.; Arnold, W.A.; Swackhamer, D.L., 2006. Changes in Antibacterial Activity of Triclosan and Sulfa Drugs due to Photochemical Transformations, *Environ. Toxicol. Chem.*, in press.

A.L. Boreen, W. A. Arnold, K. McNeill, Triplet-sensitized photodegradation of sulfa drugs containing six-membered heterocyclic groups: Identification of an SO₂ extrusion photoproduct, *Environ. Sci. Technol.* **2005**, 39, 3630-3638.

J.J. Werner, K. McNeill, W.A. Arnold, Environmental photodegradation of mefenamic acid. *Chemosphere* **2005**, 58, 1339-1346.

D.E. Latch, J.L. Packer, B.L. Stender, J. VanOverbeke, W.A. Arnold, K. McNeill, Aqueous photochemistry of triclosan: Formation of 2,4-dichlorophenol, 2,8-dichlorodibenzo-p-dioxin and oligomerization products, *Environ. Toxicol. Chem.* **2005**, 24 (3), 517-525.

A.L. Boreen, W.A. Arnold, K. McNeill, Photochemical fate of sulfa drugs in the aquatic environment: Sulfa drugs containing five-membered heterocyclic groups, *Environ. Sci. Technol.*, **2004**, 38, 3933-3940.

Popular Articles

J.J. Werner, A.L. Boreen, B. Edhlund, K.H. Wammer, E. Matzen, K. McNeill, W.A. Arnold, Photochemical transformation of antibiotics in Minnesota waters, *CURA Reporter* **2005**, 35(2), 1-5.

Dissertations

D. E. Latch, Environmental photochemistry: Studies on the degradation of pharmaceutical pollutants and the microheterogeneous distribution of singlet oxygen. Ph.D., University of Minnesota, Minneapolis, MN, **2005**, 256 pp.

A.L. Boreen, Enhanced photolysis in natural waters: naturally occurring sensitizers and substrates and application to the fate of aquatic pollutants. Ph.D., University of Minnesota, Minneapolis, MN, **2006**, 263 pp.

J.J. Werner, Photolysis of pharmaceuticals in water and soil matrices: agricultural antibiotics and mefenamic acid. Ph.D. University of Minnesota, Minneapolis, MN, **2006**, forthcoming.

Invited Presentations

W.A. Arnold and H. Dolliver. Pharmaceuticals in Groundwater: Fate, Transport, and Effects. Minnesota Groundwater Spring 2006 Conference, St. Paul, MN, April 12, 2006.

B.L. Edlund, W.A. Arnold, K. McNeill, Aquatic Photochemistry of Nitrofurantoin Antibiotics. **Celebrating Women Chemists Luncheon**, University of Minnesota, Minneapolis, MN, February, 2006.

K. McNeill, Photochemical approaches to environmental pharmaceutical pollutants, **American Chemical Society (ACS) National Meeting Symposium: *Strategies and Molecular Mechanisms of Contaminant Degradation Chemistry***, Washington, D.C., August 28-Sept. 1, 2005.

K. McNeill, Photo-generated Reactive Species and the Degradation of Pharmaceutical Pollutants, **Stanford University**, Civil and Environmental Engineering Student Seminar Series, May 20, 2005.

K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, **Cornell University**, Department of Chemistry, March 7, 2005.

K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, **University of Rochester**, Department of Chemistry, March 4, 2005.

K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, **University of California, Berkeley**, Department of Chemistry, February 25, 2005.

K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, **Northwestern University**, Department of Chemistry, February 18, 2005.

K. McNeill, Mechanistic Environmental Chemistry: Photo-generated Reactive Species and Pollutant Degradation, **3M**, November 12, 2004.

A.L. Boreen, W.A. Arnold, K. McNeill, Photochemical fate of sulfa drugs in the aquatic environment. **Physics and Chemistry Colloquium**, Concordia College, Moorhead, MN, November, 2004.

A.L. Boreen, W.A. Arnold, K. McNeill, Photochemical fate of sulfa drugs in the aquatic environment. **Celebrating Women Chemists Luncheon**, University of Minnesota, Minneapolis, MN, September, 2004.

K. McNeill. Photochemical fate of pharmaceutical pollutants. **Gordon Research Conference, Environmental Sciences: Water**, June 27 - July 2, 2004.

A.L. Boreen, W.A. Arnold, K. McNeill. Photochemical fate of pharmaceuticals in the environment: Sulfa drugs. *Oral presentation*. 9th Biennial **MN Water Conference**, Minneapolis, MN, March 23, 2004.

Conference Presentations

Werner, J.J.; Arnold, W.A., McNeill, K. The environmental photochemical kinetics of tetracycline as a function of pH and water hardness. To be presented in "Environmental Chemistry Awards," Environmental Chemistry Division, American Chemical Society National Meeting, San Francisco, CA, September, 2006.

Werner, J.J.; Arnold, W.A.; McNeill, K., 2005. Water Hardness as a Photochemical Parameter: The Case of Tetracycline. Paper presented in Emerging Contaminants, Pacificchem 2005, Honolulu, HI, December 15-20, 2005.

Werner, J.J.; Arnold, W.A.; McNeill, K., 2006. Environmental photochemistry of the antibiotic compound tetracycline: dependence on acid-base and metal binding speciation. Paper presented at the Minnesota Water 2005 and Annual Water Resources Joint Conference, Brooklyn Center, MN, October 25-26, 2006.

B.L. Edhlund, W.A. Arnold, K. McNeill. Aquatic Photochemistry of Nitrofurantoin Antibiotics. *Poster Presentation*. ENVR, **230th ACS National Meeting**, Washington, DC, August 2005.

B.L. Edhlund, W.A. Arnold, K. McNeill. Aquatic Photochemistry of Nitrofurantoin Antibiotics. *Poster Presentation*. **Minnesota Water 2005 and Annual Water Resources Joint Conference**, Brooklyn Center, MN, October 2005.

B.L. Edhlund, W.A. Arnold, K. McNeill. Aquatic Photochemistry of Nitrofurantoin Antibiotics. *Poster Presentation*. **Minnesota ACS Meeting**, St. Paul, MN, November 2005.

B.L. Edhlund, W.A. Arnold, K. McNeill. Aquatic Photochemistry of Nitrofurantoin Antibiotics. *Poster Presentation*. **Environmental Sciences: Water Gordon Research Conference**, Plymouth, NH, June 25-June 30, 2006.

J.J. Werner, K. McNeill, W.A. Arnold, Speciation-dependent photochemistry of tetracycline antibiotics: acid-base speciation and metal-binding effects. *Oral Presentation*. **Midwest Environmental Chemistry Workshop**, Madison, WI, October 16-17, 2004.

K. McNeill, W.A. Arnold. Contribution of photochemistry to the fate of pharmaceuticals and personal care products in surface waters. *Oral Presentation*. ENVR, Presented at the special symposium on Environmental aspects of pharmaceuticals and personal care products at the 228th **ACS National Meeting**, Philadelphia, PA, August 2004.

A.L. Boreen, W.A. Arnold, K. McNeill. Photochemical fate of sulfa drugs in the aquatic environment. *Oral Presentation*. ENVR, Presented at the special symposium on Environmental aspects of pharmaceuticals and personal care products at the 228th **ACS National Meeting**, Philadelphia, PA, August 2004.

K.H. Wammer, K. McNeill, T.M. LaPara, W.A. Arnold, D.L. Swackhamer. Changes in potency of antibacterials in the environment due to photochemical transformations. *Poster Presentation*. ENVR, Presented at the special symposium on Environmental aspects of pharmaceuticals and personal care products at the 228th **ACS National Meeting**, Philadelphia, PA, August 2004.

J.J. Werner, K. McNeill, W.A. Arnold, Kinetics of the environmental photodegradation of mefenamic acid. *Poster Presentation*. ENVR, Presented at the special symposium on Environmental aspects of pharmaceuticals and personal care products at the 228th **ACS National Meeting**, Philadelphia, PA, August 2004

W.A. Arnold, J.J. Werner, K. McNeill, Kinetics of the environmental photodegradation of mefenamic acid. *Poster presentation*. **Environmental Sciences: Water Gordon Research Conference**, Plymouth, NH, June 27-July 2, 2004.

K.H. Wammer, D.L. Swackhamer, W.A. Arnold, K. McNeill, Photochemical transformations of antibacterial compounds. *Poster presentation*. **Environmental Sciences: Water Gordon Research Conference**, Plymouth, NH, June 27-July 2, 2004.

J.J. Werner, K. McNeill, W.A. Arnold, Photochemical fate of pharmaceuticals in the environment. *Poster presentation*. 9th Biennial **MN Water Conference**, Minneapolis, MN, March 23, 2004.

Statement of related grants submitted or funded as a result of this project

Dr. McNeill, Dr. Arnold, and Dr. Swackhamer continue to actively seek funding to continue this avenue of research. Dr. Arnold has received funding from the Center for Urban and Regional Affairs (University of Minnesota) to investigate the photodegradation of selected antibiotics in Minnesota waters in 2003-2004. Dr. Arnold is also a co-investigator on a United States Department of Agriculture grant (2003-2006) to investigate the loss of veterinary antibiotics in soil systems. Drs. Arnold, McNeill and Swackhamer also recently received funding from the National Science Foundation (2006-2008) to study the photolysis of triclosan and polybrominated diphenyl ethers in both the laboratory and in the field.

Description of student training provided by project:

Name: Anne L. Boreen

Program: Department of Chemistry, University of Minnesota

Degree earned: Ph.D. (2006)

Name: Betsy L. Edhlund

Program: Department of Chemistry, University of Minnesota

Degree being sought: Ph.D.

Name: Douglas E. Latch
Program: Department of Chemistry, University of Minnesota
Degree earned: Ph.D. (2005)

Name: Jeffrey J. Werner
Program: Water Resources Science, University of Minnesota
Degree earned: M.S. (2004)
Degree earned: Ph.D. (2006)

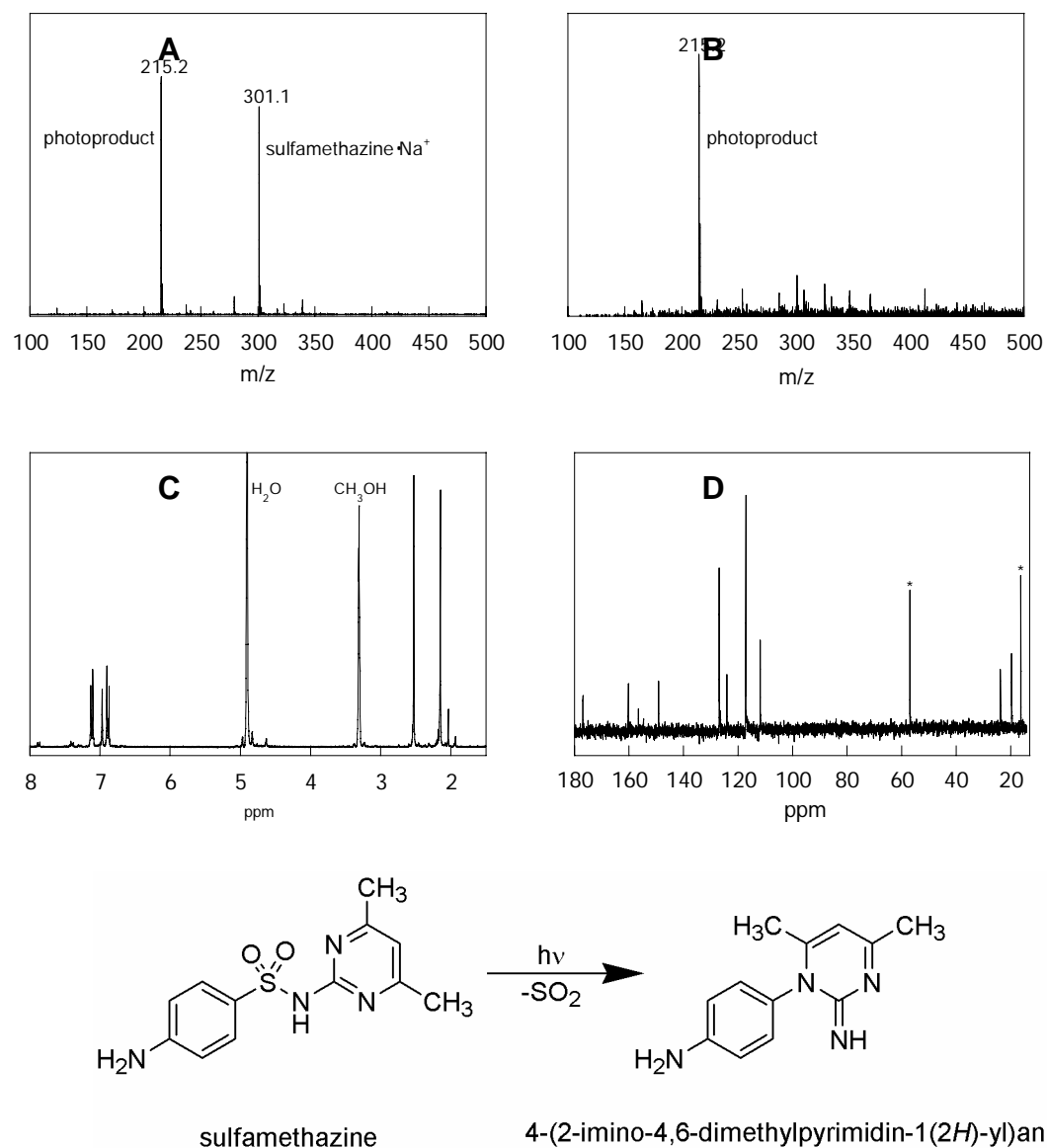


Figure 1. Characterization data for the primary photoproduct of sulfamethazine. (A) ESI-TOF mass spectrum of the raw photolysate of sulfamethazine showing the parent ion (m/z 301.1, MNa⁺) and the photoproduct (m/z 215.2). (B) ESI-TOF mass spectrum of the isolated photoproduct (m/z 215.2). ¹H-NMR (C) and ¹³C-NMR (D; * denotes ethanol peaks) of the isolated photoproduct.

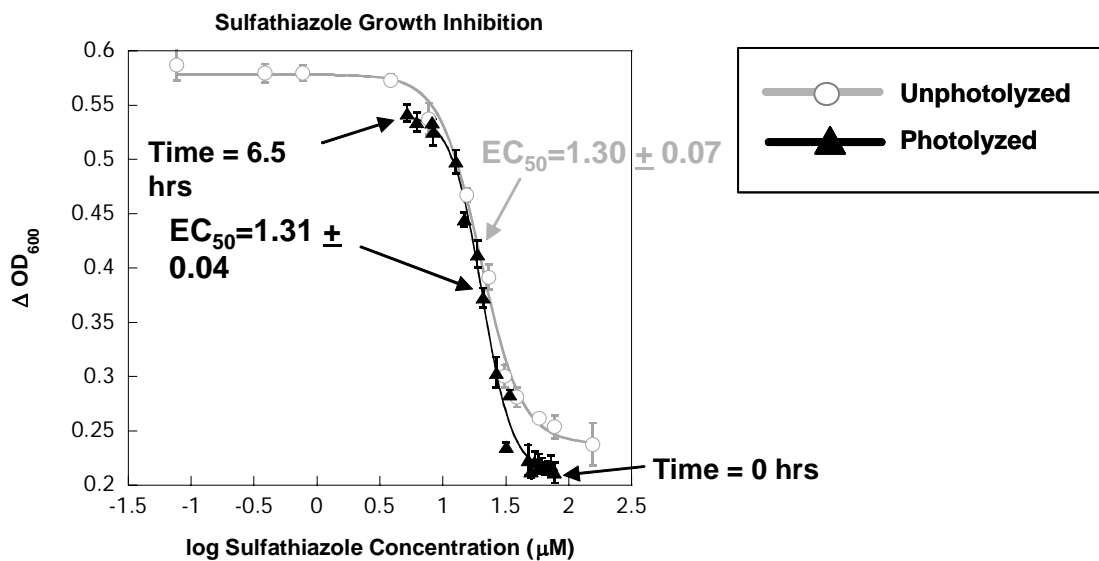


Figure 2. Change in optical density at 600 nm after 8 hours for *E. coli* DH5 α in the presence of sulfathiazole (open circles) and sulfathiazole plus photolysis products (closed triangles). Remaining sulfathiazole concentration is plotted (log of concentration (μM)). Initial and final sulfathiazole concentrations during photolysis (77 μM at 0 hours and 5.2 μM at 6.5 hours) are labeled.

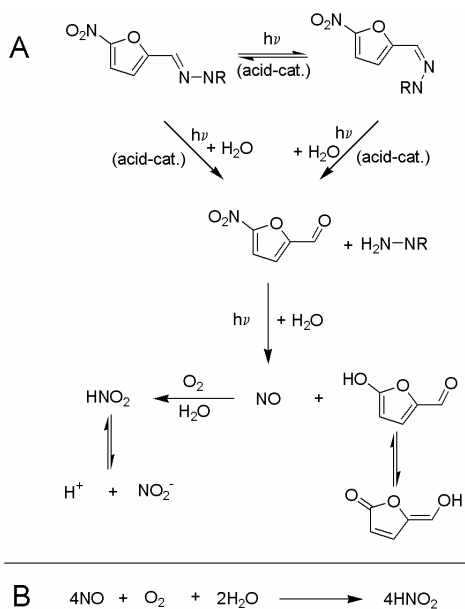
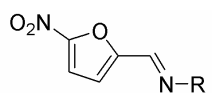


Figure 3. (A) Photodegradation scheme of the nitrofurantoin antibiotics. Parent and isomer conformations were arbitrarily set to anti as the parent and syn as the isomer. For clarity, the reaction of NO to HNO₂ is not balanced. (B) Balanced reaction for NO oxidation to HNO₂.



R				
Name	Furaltadone	Furazolidone	Nitrofurantoin	Nitrofurazone

Table 1. The general structure of the nitrofuran antibiotics, with the varying substituents (R) shown within the table.

Phyto-enhanced remediation: A wetland treatment system for surface water protection

Basic Information

Title:	Phyto-enhanced remediation: A wetland treatment system for surface water protection
Project Number:	2004MN48B
Start Date:	3/1/2004
End Date:	2/28/2006
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Focus Category:	Wetlands, Non Point Pollution, Treatment
Descriptors:	None
Principal Investigators:	William Alan Arnold, Timothy Michael LaPara

Publication

1. DeJournett, T.D.; LaPara, T.M.; Arnold, W.A., 2006. Fate of 14C-cis-dichloroethylene in wetland microcosms containing cattail and giant bur-reed. Poster presented at the Minnesota Water 2005 and Annual Water Resources Joint Conference, Brooklyn Center, MN, October 25-26, 2006.
2. DeJournett, T.D., Arnold, W.A., LaPara, T.M. 2005. Methanotrophic bacterial populations in constructed wetland sediments. Paper presented in In-situ Methods and Investigations in Environmental Science Geochemistry Division, American Chemical Society National Meeting, Washington, DC, August 28-September 1, 2005.
3. Characterization of methanotrophic bacterial populations in the rhizosphere of emergent wetland plants. Poster N-096 American Society for Microbiology 104th Annual Meeting, New Orleans, LA, May 23-27 2004.
4. Stimulation of methanotrophic bacteria in a wetland treatment system. Environmental Engineering Seminar, University of Missouri-Rolla November 7, 2003.
5. Phyto-Enhanced Remediation: A constructed wetland for removal of chlorinated ethylenes from groundwater Poster Presentation, Frontiers in Assessment Methods for the Environment (FAME), Minneapolis, MN August 10-13 2003.
6. Phyto-Enhanced Remediation: A constructed wetland for removal of chlorinated ethylenes from groundwater Poster Presentation, CSWEA/AWMA Conference on the Environment, Bloomington,

MN, November 20, 2003.

7. DeJournett, T.D., Fritsch, J.M., McNeill, K., and Arnold, W.A., 2005. Preparation of ^{14}C -cis-1,2-dichloroethylene from ^{14}C -trichloroethylene using a cobalt porphyrin catalyst. *Journal of Labelled Compounds and Radiopharmaceuticals*, 48(5): p. 353-357.
8. DeJournett, T.D.; Arnold, W.A.; LaPara, T.M., 2006. The Effect of Vegetation on Methanotrophic Bacterial Populations in a Constructed Wetland, *Applied Soil Ecology*, in review.
9. DeJournett, T.D., 2006. Removal of cis-1,2-dichloroethylene from groundwater using a restored wetland. Department of Civil Engineering, University of Minnesota, Minneapolis, MN, 140 pp.

Phyto-enhanced remediation: A wetland treatment system for surface water protection

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Start date: 3/01/2004

End date: 2/28/2006

Executive summary

Halogenated solvents, such as dichloroethylene (DCE), present a challenging remediation problem due to their prevalence and persistence in the environment. In groundwater contamination scenarios where the source pools cannot be located/removed, there is great demand for a long-term cost effective alternative to treat the contaminant plume. Wetland treatment is an attractive alternative because of its passive nature and low operation/maintenance costs. A wetland treatment system was implemented as a remedial action to protect Lake Minnetonka from a DCE plume emanating from a former manufacturing facility in Mound, Minnesota. This work was initiated to address a lack of data regarding the role of wetland vegetation in the removal of DCE by the constructed wetland. Work conducted to date suggests that wetland vegetation did not affect the size or structure of methanotrophic bacterial communities in the field, and cometabolic oxidation of DCE by methanotrophs was not a significant fate mechanism in laboratory microcosm studies. In the case of cattails, transport from the subsurface to the atmosphere via plant tissues is the primary fate mechanism for DCE in laboratory microcosms. Uptake was faster than predicted, and this phenomenon may be attributed to volatilization/gas-phase diffusion of DCE through gas-filled voids (aerenchyma tissue) in cattail plants. Previously published models are based on terrestrial plants, such as hybrid poplar trees, which lack aerenchyma tissue. Cattails also prevented the accumulation of vinyl chloride, an anaerobic biodegradation product of DCE. Because DCE removal by cattails is strongly influenced by transpiration rate, it may be possible to adapt wetland management practices to enhance DCE removal or to moderate DCE efflux to the atmosphere if necessary.

Introduction and Objectives

Halogenated solvents, such as chlorinated methanes, ethanes, and ethylenes, are among the most prevalent pollutants at contaminated sites on the National Priorities List as well as sites owned by the Department of Defense and Department of Energy. Contamination is also often observed at dry-cleaning and degreasing operations. Halogenated solvents pose an extremely difficult remediation problem. These compounds generally have low aqueous solubility and collect at impermeable layers forming pools of non-aqueous phase liquid (1).

While several remediation techniques are currently available for the removal or degradation of chlorinated compounds at contaminated sites, these techniques are subject to significant technical and economic limitations. Phytoremediation is a burgeoning technology that utilizes living plants to help remove contaminants from the environment. Phyto- and phyto-enhanced

remediation are potentially low cost and aesthetically pleasing remediation alternatives. One example of phytoremediation is a wetland treatment system, in which wetland plants facilitate the removal of contaminants from water as it flows through the wetland. Wetland treatment systems are becoming widely used to treat municipal wastewater (2-5) as well as numerous other waste streams including landfill leachate and acid mine drainage (6, 7). Wetlands offer a unique remediation environment, as shown by wastewater treatment applications that take advantage of the ability of the diverse microbial population supported by the wetland environment to degrade a variety of contaminants. The root zone, or rhizosphere, of wetland plants may play an important role in supporting essential, waste-degrading microbes.

Wetland treatment systems also have great potential for removing chlorinated solvents from groundwater (8). Wetlands have been shown to support microbes, such as methanotrophic bacteria, capable of degrading chlorinated solvents (8-11). Wetland plants may also have the capability to take up and transpire/mineralize chlorinated solvents (12, 13), although this has yet to be specifically demonstrated for most wetland plants. Root systems of wetland plants may also enhance the bacterial mineralization of chlorinated solvents in the rhizosphere through the excretion of root exudates and oxygen (14, 15).

The objective of this research is to determine the specific roles of the soil and plants and the impact of plant-microbial interactions in the removal of chlorinated ethylenes in a constructed wetland. Additionally, this study will elucidate the effect of wetland vegetation on the growth of methanotrophic bacteria in wetland sediment.

Methodology

Field Mesocosms Three field mesocosms (one unvegetated, two planted with a mixture of cattails, giant bur-reed, bottlebrush sedge, and bulrush) were observed from April-October (the growing season for Minnesota). Porewater samples were collected from the mesocosms via stainless steel microwells embedded in the sediment at 13-cm intervals. Samples were drawn from the microwells via a glass gas-tight syringe and Teflon-lined tubing. Porewater samples were analyzed for chlorinated ethenes and methane via gas chromatography. Dissolved oxygen, sulfate, and sulfide were measured using a handheld colorimetric test kit (CHEMetrics Company Vacu-Vials™).

The effect of the root systems of wetland plants on methanotrophic biomass levels was evaluated via sampling of mesocosm sediment. Soil cores were taken from each mesocosm cell in November 2002, May 2003, and July 2003. Soil cores were split in half along the longitudinal axis, and 2-gram composite soil samples were taken at 13-cm intervals along the length of the core. These samples were stored on ice for transport to the laboratory and immediately frozen at -20°C upon their arrival. DNA was extracted from soil samples using a FastDNA spin kit for soil (Qbiogene) and methanotrophic biomass was quantified via competitive polymerase chain reaction (cPCR) focusing on 16S rRNA genes for Type I and Type II methanotrophs. Competitor DNA was prepared using 16S rRNA material from *M. methanica* (Type I) and *M. trichosporium* (Type II). Additionally, methanotrophic community structure was evaluated using nested PCR and denaturing gradient gel electrophoresis.

Laboratory Microcosms. Laboratory microcosm studies were conducted to evaluate the fate of DCE and the effect of wetland plants on methanotrophic bacterial populations in a controlled system. Experimental treatments applied include: wetland plants growing in hydroponic solution and wetland plants growing in sediment from the site. Controls consisting of a glass rod in place of the wetland plant stem were included to account for any leakage through the plug. Experiments were conducted in triplicate using microcosms consisting of a root compartment and shoot compartment separated by a wax/clay composite seal. Either ¼-strength Hoagland's solution (hydroponic experiments) or synthetic groundwater with methane (plants with soil) were fed to the root compartment via flexible carboys under constant hydrostatic pressure. The air in the shoot compartment was exchanged continuously using a vacuum system. Exhaust air from the shoot compartments was passed through an activated carbon trap and two sequential potassium hydroxide traps. Replicate microcosms were spiked with a mixture of unlabeled and ^{14}C -*cis*-DCE. A new method for converting ^{14}C -TCE to ^{14}C -*cis*-DCE using Ti(III) citrate and a cobalt-porphyrin catalyst was developed as an economical alternative to purchasing commercially-synthesized ^{14}C -*cis*-DCE.

Aqueous samples (1-mL) were collected from the root compartments and analyzed for methane as well as *cis*-DCE, vinyl chloride, and ethylene were monitored using headspace analysis on a GC equipped with a flame ionization detector. A separate 0.5-mL aqueous sample was collected from the root compartment and added to a sealed 10-mL vial containing 3 mL of hexane and 1 mL of 1 M KOH solution. The vials were equilibrated overnight and the two phases were sampled separately and analyzed for ^{14}C via liquid scintillation counting (LSC) in order to determine the relative amounts of ^{14}VOC and $^{14}\text{CO}_2$ present in the root compartment solution. The activated carbon traps were extracted in hexane, and this extract was analyzed by LSC to determine the amount of ^{14}VOC transported through plant tissues. KOH traps were sampled and analyzed via LSC to determine the amount of $^{14}\text{CO}_2$ transported through the plant tissues. Transpiration was tracked by weighing the flexible carboys.

At the end of each experiment, the microcosms were dismantled, and soil was sampled for PCR and ^{14}C analysis. The plant roots were gently rinsed with DI water, and blotted dry. The plants were then divided into root, submerged shoot, and emergent shoot sections. Each section was weighed, flash-frozen in liquid nitrogen, and stored in Teflon-capped glass jars at $-20\text{ }^\circ\text{C}$. DNA from triplicate soil samples was extracted as previously described and subjected to the aforementioned cPCR and nested PCR analyses. Additionally, DNA was extracted from the frozen/pulverized root tissue and subjected to the PCR analyses.

Data Analysis. Mass transport coefficients for transport of DCE through cattail tissues were determined using a finite difference model adapted from Burken and Schnoor (6):

$$U = M_{DCE} \times T \times C_{bulk, avg} \quad (1)$$

Where U is the ^{14}C -VOC captured on the activated carbon over the sampling interval (dpm/hr), M_{DCE} is a dimensionless mass transport coefficient, T is the volume of water transpired over the sampling interval (mL/hr), $C_{bulk, avg}$ is the average aqueous ^{14}C -VOC concentration over the sampling interval (dpm/mL).

Mass transport coefficients for DCE transport through bur-reed and dead plant tissues were determined using a modified form of Fick's Law:

$$UR = M'_{DCE} \times C_{bulk,avg} \quad (2)$$

Where UR is the incremental rate of ^{14}C -VOC capture on activated carbon, (dpm/hr), M'_{DCE} is the mass transport coefficient (L/hr), and $C_{bulk,avg}$ has units of dpm/L. In each case, the mass transport coefficient was determined by fitting the model to microcosm data using the least squares fitting algorithm in Microsoft Excel.

Principal Findings and Significance

Field Mesocosms. Chlorinated ethylenes were not detected at any depth in any of the mesocosms. Data provided by Barr Engineering Company indicated that both DCE and vinyl chloride were present in substantial amounts in the deeper aquifer (15-20 ft). Additionally, the vertical groundwater gradient in the vicinity of the mesocosms was neutral, indicating minimal influence of groundwater discharge on subsurface conditions in the mesocosms. Large amounts of methane were detected in all three mesocosms, with methane concentration increasing with depth in each mesocosm. While methane concentration profiles were similar for the vegetated and unvegetated mesocosms during the Spring and Fall, vegetated mesocosms exhibited depressed methane concentrations in the upper 30 cm during the height of the growing season.

While large quantities of both Type I and Type II methanotrophic bacteria were detected in all three mesocosms, no trends in population size were observed with respect to time of year, depth, or presence/absence of vegetation. The qualitative analysis of methanotroph population structure revealed the following seasonal population shifts for Type I methanotrophs: appearance of *Methylocaldum* sp. in Fall, appearance of *Methylobacter* sp. in Spring, and appearance of *Methylomonas* sp. in Summer. No trends in Type I methanotrophic population structure were observed with respect to depth or presence/absence of vegetation. No trends in Type II methanotrophic population structure was observed with respect to time of year, depth, or presence/absence of vegetation.

Laboratory Microcosms-DCE Fate. DCE depletion followed pseudo-first-order kinetics, and occurred more rapidly in the vegetated microcosms than in the controls (Figure 1). While microcosms with living plants generally had higher rate coefficients compared to dead plant controls, these differences were statistically significant ($P = 0.001$) for bur reed but not for cattail ($P = 0.08$). Hydroponic cattail microcosms had a significantly lower rate coefficient than the dead cattail microcosms ($P = 0.002$). Microcosms with live bur reed plants had rate coefficients that were significantly greater than their cattail counterparts ($P = 0.007$ and $P = 0.014$ for hydroponic and plant/sediment conditions, respectively). Rate coefficients for dead bur reed and dead cattail were similar ($P = 0.145$). Under hydroponic conditions, more ^{14}C was removed from aqueous solution in bur reed microcosms (8% remaining) than in cattail microcosms (25% remaining). In the presence of sediment, the extent of ^{14}C removal was similar for cattail and bur reed microcosms (2%-5% remaining). While substantial ^{14}C removal also occurred in the case of dead cattail and dead bur reed microcosms (11%-16% remaining), removal was less than for live microcosms.

The transport of ^{14}C -VOCs to the shoot compartment was the dominant fate mechanism in microcosms with live cattails, accounting for 50% and 80% of the final activity balance in hydroponic cattail and cattail/sediment microcosms, respectively. Transport of ^{14}C -VOCs to the shoot compartment was less important for microcosms containing bur reed, accounting for 22%-

30% of the final activity balance. These results were similar to those obtained for dead cattail and dead bur reed microcosms, which transported 29%-32% of the added label to the shoot compartment as ^{14}C -VOC. Sequestration in plant tissues was a minor sink for ^{14}C , accounting for less than 1% of the final balance in all cases. Likewise, $^{14}\text{CO}_2$ was not detected in either the root compartment, or in the CO_2 traps for any of the treatments.

In cattail/sediment and hydroponic cattail microcosms (Figure 2), the initial ^{14}C removal from the root compartment was rapid (80% and 50% removal over the first 100 hours, respectively). Discharge of ^{14}C into the shoot compartment occurred more slowly. Thus, total ^{14}C recovery was initially low (approximately 40%), but improved as ^{14}C was discharged into the shoot compartment over time.

In the bur reed/sediment and hydroponic bur reed microcosms, 80% to 90% of the ^{14}C was removed from the root compartment over the first 100 hours. Discharge of ^{14}C to the shoot compartment was slower than cattails. Total ^{14}C recovery declined over the course of the study. In dead cattail and dead bur reed microcosms, 50% of the ^{14}C in the root compartment was removed over the first 130 hours. ^{14}C discharge to the shoot compartment was slow for all dead plants. Total ^{14}C recovery declined over the course of the study period.

Transport of ^{14}C -VOCs through cattail tissues was strongly correlated to the product of aqueous ^{14}C -VOC concentration and incremental transpiration (see eqn. 1). Transport of ^{14}C -VOCs through live and dead bur reed tissues, however, was correlated to aqueous ^{14}C -VOC concentration (eqn. 2). Radiolabeled VOC transport was also correlated to aqueous ^{14}C -VOC concentration in one of the three dead cattail replicates. Relevant mass transport coefficients calculated using equations 1 and 2 for the various treatments are summarized in Table 1.

Laboratory Microcosms-Methanotrophic Populations. The largest populations of Type I methanotrophs (1×10^6 to 5×10^6 gene copies/gram wet sediment) were observed in cattail/sediment microcosms. The type I methanotrophic population sizes in all three cattail/sediment replicates were an order of magnitude greater ($P = 0.01$) than in the sediment controls. Type I methanotrophic populations in the sediment controls declined over the course of the experiment. Type I methanotrophic population sizes declined in two of the bur reed/sediment replicates ($\sim 10^5$ gene copies/gram wet sediment) and remained unchanged in the third replicate ($\sim 10^6$ gene copies/gram wet sediment). Type I methanotrophic population sizes remained unchanged in the dead cattail microcosms. Bur reed/sediment microcosm 3 ($P = 0.01$) and all three dead cattail microcosms ($P < 0.02$) had Type I methanotrophic populations that were significantly greater than the sediment control.

The Type II methanotrophic populations observed in the microcosms (5,000 – 25,000 gene copies/gram wet soil) were significantly smaller than the Type I populations. As observed with Type I methanotrophs, the greatest stimulatory effect occurred in cattail/sediment microcosm 2 (25,000 gene copies/gram wet sediment). Cattail/sediment microcosm 1 also exhibited an increase in Type II methanotrophic population size (10,000 gene copies/gram wet sediment), whereas the population size declined in cattail/sediment microcosm 3 (2,500 gene copies/gram wet sediment). Type II methanotrophic population sizes also increased in two of the three sediment controls (10,000 – 15,000 gene copies per gram wet sediment), and in one of the three

bur reed/sediment replicates (~7,500 gene copies/gram wet sediment). Type II methanotrophic population sizes remained stable in two of the live bur reed replicates, as well as all three of the dead cattail replicates.

Significance. Transport through plant tissues is the most important fate mechanism for DCE in the case of live cattails, accounting for 50% of the added activity in the case of hydroponic cattail microcosms and 88% in the case of cattail/sediment microcosms. This transport mechanism is strongly correlated to the product of aqueous DCE concentration and transpiration rate.

While the form of the mass transport coefficient for living cattails resembles the transpiration stream concentration factor (TSCF; θ), the values (2.51 to 5.94) are much greater than the predicted TSCF value of 0.79. Cattails therefore possess a mechanism capable of transporting DCE much more rapidly than terrestrial plants, where vascular uptake is the dominant transport mechanism (*e.g.*, hybrid poplar trees). A likely transport mechanism in cattails is convective air flow from young leaves down into root aerenchyma, and out through older leaves (humidity-induced convection). Such convective flow is driven by the humidity gradient between the interior and exterior of the leaf. Transport would thus be correlated to transpiration rate because transpiration is also driven by the same humidity gradient. Methanotrophic population data also indicated enhanced oxygen transport to the rhizosphere by living cattail plants.

Transport of ^{14}C -VOCs through plant tissues was not the dominant fate mechanism in bur-reed, accounting for only 22% and 30% in the hydroponic and bur-reed/sediment microcosms, respectively. ^{14}C -VOC transport through bur-reed tissues occurred in similar fashion in both living and dead specimens and was strongly correlated to aqueous ^{14}C -VOC concentration. This phenomenon is consistent with gas-phase diffusion, indicating the absence of a convective gas transport mechanism in bur-reed.

Summary of findings

While wetland plants do not appear to significantly affect the size or structure of the methanotrophic bacterial populations in a constructed wetland, they can play a significant role in removal of DCE from groundwater via uptake/volatilization/convective transport through tissues. The results of this work demonstrate the significance of convective transport mechanisms in gas exchange between subsurface wetland sediments and the atmosphere. In addition to the obvious consequences for transport of volatile pollutants like DCE, convective transport is also important in oxygen transport to plant roots and associated aerobic microflora. The results of this work regarding the fate of DCE in giant bur-reed illustrate the need for further study of the remedial potential of this species.

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List of Publications

Articles in Referred Scientific Journals

DeJournett, T.D., Fritsch, J.M., McNeill, K., and Arnold, W.A., 2005. Preparation of ¹⁴C-*cis*-1,2-dichloroethylene from ¹⁴C-trichloroethylene using a cobalt porphyrin catalyst.

Journal of Labelled Compounds and Radiopharmaceuticals, 48(5): p. 353-357.

DeJournett, T.D.; Arnold, W.A.; LaPara, T.M., 2006. The Effect of Vegetation on Methanotrophic Bacterial Populations in a Constructed Wetland, *Applied Soil Ecology*, in review.

Dissertations

DeJournett, T.D., 2006. Removal of *cis*-1,2-dichloroethylene from groundwater using a restored wetland. Department of Civil Engineering, University of Minnesota, Minneapolis, MN, 140 pp.

Description of student training provided by project

Name: Todd D. DeJournett

Program: Department of Civil Engineering, University of Minnesota

Degree earned: Ph.D.

Seminar/Poster Presentations

DeJournett, T.D.; LaPara, T.M.; Arnold, W.A., 2006. Fate of ^{14}C -*cis*-dichloroethylene in wetland microcosms containing cattail and giant bur-reed. Poster presented at the Minnesota Water 2005 and Annual Water Resources Joint Conference, Brooklyn Center, MN, October 25-26, 2006.

DeJournett, T.D., Arnold, W.A., LaPara, T.M. 2005. Methanotrophic bacterial populations in constructed wetland sediments. Paper presented in "In-situ Methods and Investigations in Environmental Science" Geochemistry Division, American Chemical Society National Meeting, Washington, DC, August 28-September 1, 2005.

Characterization of methanotrophic bacterial populations in the rhizosphere of emergent wetland plants. Poster N-096 American Society for Microbiology 104th Annual Meeting, New Orleans, LA, May 23-27 2004.

Stimulation of methanotrophic bacteria in a wetland treatment system. Environmental Engineering Seminar, University of Missouri-Rolla November 7, 2003.

Phyto-Enhanced Remediation: A constructed wetland for removal of chlorinated ethylenes from groundwater Poster Presentation, Frontiers in Assessment Methods for the Environment (FAME), Minneapolis, MN August 10-13 2003.

Phyto-Enhanced Remediation: A constructed wetland for removal of chlorinated ethylenes from groundwater Poster Presentation, CSWEA/AWMA Conference on the Environment, Bloomington, MN, November 20, 2003.

Statement of related grants submitted or funded as a result of this project

None.

Table 1. Mass transport coefficients determine in the cattail and bur reed microcosm experiments.

Treatment	M_{DCE}^a
Hydroponic Cattail	4.02 ±1.32
Cattail + Sediment	4.30±1.63
	M'_{DCE}^b
Dead Cattail	0.22 ^c
Hydroponic Bur Reed	0.51±0.34
Bur Reed + Sediment	2.20±1.77
Dead Bur Reed	0.68±0.21
Hydroponic Control	na
Sediment Control	na

^a Units are mg volatilized/(mg/L in bulk solution × L water transpired).

^b Units are mg volatilized/(hr × mg/mL in bulk solution).

^c Only one of three replicates resulted in a correlation between volatilization rate and bulk concentration.

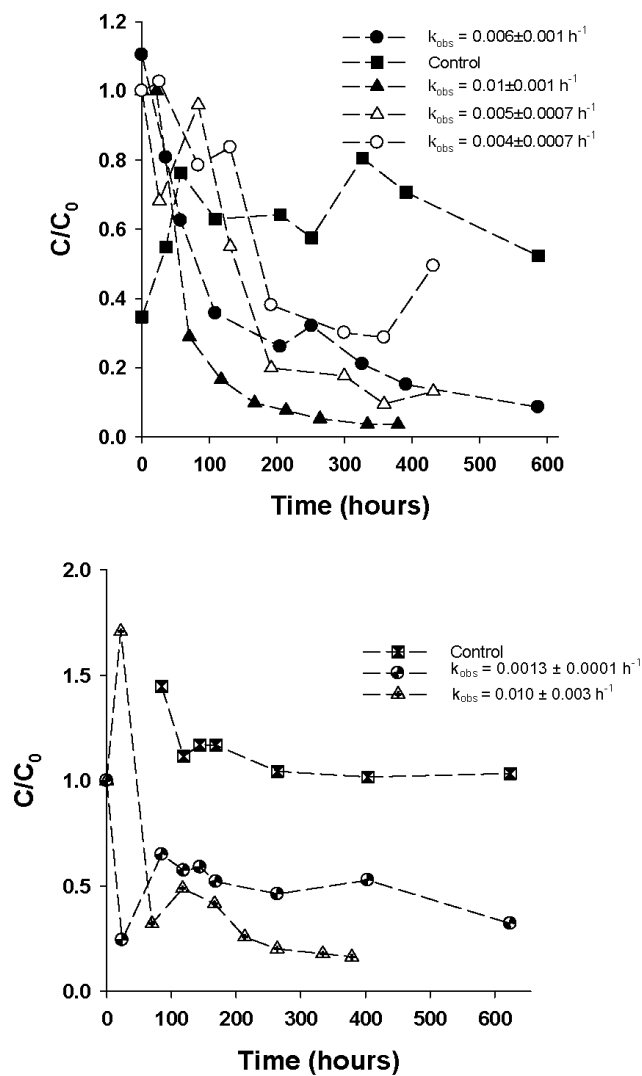


Figure 1: Depletion of DCE in the root compartments of wetland microcosms with sediment (top) and under hydroponic conditions (bottom). Data are average values of triplicate microcosms. Treatments shown are the sediment control (■), bur-reed/sediment (▲), cattail/sediment (●), dead bur-reed (△), dead cattail (○), hydroponic control (semi-filled squares), hydroponic cattail (semi-filled circles), and hydroponic bur reed (crossed triangles). microcosms Dashed lines are for visualization only.

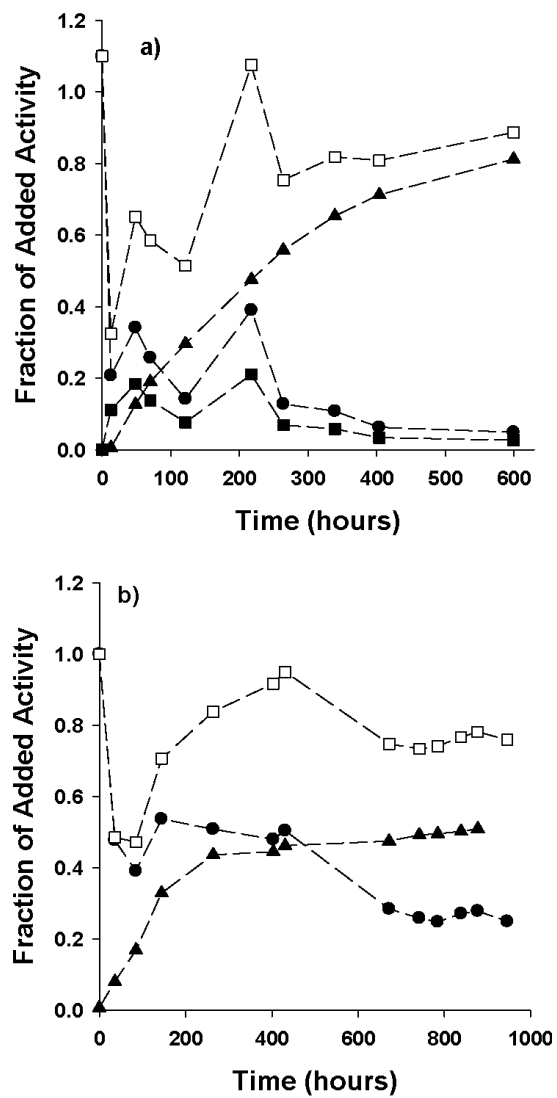


Figure 2: Radioactivity balance for cattail/sediment (a) and hydroponic cattail (b) microcosms. Data shown are aqueous ^{14}C -VOC (●), estimated ^{14}C -VOC sorbed to sediment (■), ^{14}C -VOC captured on activated carbon trap (▲), and total ^{14}C recovery (□). In each case, the majority of added ^{14}C was expelled into the shoot compartment by the plant.

ASSESSING THE ECOTOXICOLOGY OF 4-NONYLPHENOL, A UBIQUITOUS ENVIRONMENTAL ESTROGEN, IN TWO ORGANISMAL BIOASSAYS

Basic Information

Title:	ASSESSING THE ECOTOXICOLOGY OF 4-NONYLPHENOL, A UBIQUITOUS ENVIRONMENTAL ESTROGEN, IN TWO ORGANISMAL BIOASSAYS
Project Number:	2004MN82B
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Descriptors:	None
Principal Investigators:	Heiko L Schoenfuss, Matthew Julius, Larry B Barber

Publication

1. Bistodeau, T.J., L.B. Barber, S.E. Bartell, R.A. Cedic, K.J. Grove, J. Klaustermeier, J.C. Woodard, K.E. Lee and H.L. Schoenfuss. In Press. Larval exposure to environmentally relevant mixtures of alkylphenoethoxylates reduces reproductive competence in male fathead minnows. *Aquatic Toxicology*.
2. October 2004. Schoenfuss. Simple Questions - Complex Answers: Investigating the Effects of Alkylphenols on Aquatic Organisms. Invited Seminar, University of Nebraska, Omaha.
3. April 2005. Schoenfuss, H.L., T. Bistodeau, S. Bartell, J. Woodard, L. Barber, K. Lee, L. Zintek, A. Alwan, J. Lazorchack (2005) Concentration Dependent Effects of 4-Nonylphenol on Male Fathead Minnows in a Competitive Reproductive Assay. Oral Presentation at the Annual Meeting of the Midwest Division of the Society for Environmental Toxicology and Chemistry, Madison, WI, April 5, 2005.
4. April 2005. Bistodeau, T., R. Cediell, K. Groove, J. Klaustermeier, H. L. Schoenfuss (2005) Reproductive Consequences of Environmentally Relevant Exposures of Fathead Minnow Larvae to a Mixture of Alkylphenol Ethoxylates. Poster Presentation at the Annual Meeting of the Midwest

Division of the Society for Environmental Toxicology and Chemistry, Madison, WI, April 5, 2005.

5. May 2005. Schoenfuss, H.L. (2005) Biological Effects of Biologically Active Compounds: Experimental Considerations in the Study of Endocrine Disrupting Chemicals. Symposium Presentation at the Annual Meeting of the American Oil Chemists Society, Salt Lake City, UT, May 3, 2005.
6. October 2005. Schoenfuss. Concentration dependent effects of 4-nonylphenol on male fathead minnows in a competitive reproductive assay. MN Water 2005 Oral Presentation.
7. March 2006. Schoenfuss. Reproductive Consequences of Alkylphenol Exposure on Male Fathead Minnows. Midwest meeting of the Society for Environmental Toxicology & Chemistry, St. Cloud, MN March 20-22, 2006.

Assessing the Ecotoxicology of 4-Nonylphenol, An Ubiquitous Environmental Estrogen, in Two Organismal Bioassays

Principal Investigators

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Research Assistants

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Start date: 3/21/2004

End date: 2/28/2006

Executive summary

Alkylphenols (APs) were recently discovered in many surface water samples and even in some drinking waters in Europe and North America. Wastewater effluent was identified as a major source of contamination and found to revert much of the metabolic products of these biologically active compounds back to their most potent form, 4-nonylphenol. Alkylphenols are used in large quantities (thousands of tons annually in North America) as surfactants in industrial and domestic settings and are known to bind to the estrogen receptor of mammalian cells.

Environmental estrogens such as APs are known to disrupt normal endocrine hormones that are central to maturation and reproduction in fishes, and the ubiquitous presence of these biologically active compounds in surface waters should be of environmental and human health concern. In this study we propose to examine the effects of 4-nonylphenol on two organismal bioassays, representing the base and apex of the aquatic food chain. At the base of the food chain, diatoms, a group of photosynthetically active organisms, serve as a preferred food source for larval fish. Near the top, fathead minnows represent an important link in the food chain as a consumer of diatoms and as a food source for game fish. Previously we have demonstrated that both of these organisms are sensitive to aquatic pollution. Diatoms will reduce their lipid content, which makes them a lower quality food source for developing fish larvae, while fathead minnows exposed to pollutants will be less likely to reproduce. Our results indicate that the quality of diatoms as food source will diminish rapidly if they are exposed to 4-nonylphenol at concentrations frequently measured in rivers below treated sewage effluent outfalls. Male fathead minnows exposed to similar, environmentally relevant concentrations of 4-nonylphenol also appear to be less likely to reproduce than unexposed males. Based on the results of this study, we conclude that 4-nonylphenol represents a substantial pollution source in many riverine systems in Minnesota and the US and might partially account for the decrease in reproductive ability in exposed fishes.

Introduction

This study set out to determine whether 4-nonylphenol, the most biologically potent alkylphenol (APs), has significant effects on reproduction and health of two model organisms. Alkylphenols, a well recognized class of “environmental estrogens”, contribute significantly to the estrogenicity

of wastewater effluents (Field 1996; Barber 2000; Farre 2002), however, the effects of these compounds on exposed organisms is largely unknown. The few studies that have exposed aquatic vertebrates to APs in the laboratory have focused on sub-organismal endpoints such as gene expression of zona radiata protein (Arukwe 2001; Ackermann 2002), MCF-7 breast tumor cell proliferation (E-screen) (Gutendorf 2001; Folmar 2002), rainbow trout hepatocyte cultures (Madigou 2001), insulin-like growth factor (Le Gac 2001), and yeast based estrogen receptor assays (Gutendorf 2001; Madigou 2001; Folmar 2002). By far the most commonly used endpoints relate to the synthesis of the egg-yolk protein vitellogenin (VTG) (Foran 2000; Arukwe 2001; Hemmer 2001; Nichols 2001; Ackermann 2002; Folmar 2002; Villeneuve 2002) and the induction of the hepatic VTG mRNA (Hemmer 2001). The aforementioned endpoints have merits in determining acute exposure of oviparous vertebrates to compounds (“environmental estrogens”) binding to the estrogen receptors; however, their value in assessing the reproductive consequences for exposed organisms is limited. Several studies have, therefore, employed biomarkers more closely related to the reproductive competence of exposed organisms. As environmental estrogens, including APs, affect the hypothalamic-pituitary-gonadal steroidal axis, it seems intuitive to analyze hormones of these endocrine pathways. Estradiol (E2) concentrations increased in male and female fathead minnows exposed to nonylphenol in studies by Giesy and colleagues (2000) but did not exhibit a similar response in fathead minnow studies by Nichols and colleagues (2001) or in carp exposed by Villeneuve and colleagues (2002). The latter two studies also analyzed testosterone concentrations in the exposed organisms and did not report any significant differences from the respective control treatments (Nichols 2001; Villeneuve 2002). Harris and colleagues (2001) exposed female rainbow trout to nonylphenol and reported a decrease follicle stimulating and luteinizing hormones, which are both central to the fecundity of female fishes. Gametogenesis, the production of eggs and/or sperm, was measured directly in fathead minnows (Nichols 2001) and trout (Le Gac 2001). Several studies have also assessed sex ratios and the induction of intersex, the presence of female reproductive tissues in the testis of exposed male organisms (Jobling 1997; Gray 1999; Ackermann 2002). However, none of these endpoints allows for a direct assessment of the reproductive fitness of AP exposed animals.

The present study investigated two objectives to establish the effects of 4-nonylphenol through the development of two organismal bioassays. These objectives are:

(1) Determining the impacts of 4-nonylphenol exposure on the physiological development of a ubiquitous diatom species. Diatoms appear to be a particularly satisfactory food source for many aquatic animals, including fingerling fish (Volkman 1989, Ahlgren 1990). In addition, they are well known as highly sensitive indicators of environmental change (see Stoermer 2000). A laboratory experiment is proposed, examining the effects of 4-nonylphenol concentrations on the gross morphology and physiology of the diatom species *Cyclotella meneghiniana*. Ultimately, this experiment will lay the foundation for determining the impacts of pharmaceutical contamination on the development and food quality of species in the primary production community. If effects are confirmed, this study will also serve as the impetus for developing protocols for rapidly and inexpensively triaging other alkyphenol effects using certain diatom species as model organisms.

(2) Determine the effects of 4-nonylphenol exposure on reproductive success in laboratory fishes. The fathead minnow was chosen for this study as it is a tier one screening organism for endocrine- disrupting compounds (Ankley 1998), is easily maintained in the laboratory, and

reproduces year round in the laboratory. Fathead minnows are particularly well suited for a competitive assay as they establish a dominance hierarchy and compete for nest sites in which females then deposit eggs (Unger 1983, 1988; Sargent 1988, 1989). Thus, reproductive success of male fathead minnows is linked to its ability to acquire and defend a nest site until larvae hatch. In this study we introduced direct competition between males by limiting the number of available nest sites. By exposing male fathead minnows to APs and allowing these males to compete directly with control males we assessed the effects of this exposure on their reproductive competence.

Methods

Two bioassays will be utilized to meet the objectives of this study. To examine the effects of 4-nonyphenol on the gross morphology and physiological development of the diatom species *Cyclotella meneghiniana* we compared cell density, lipid composition, fatty acid concentrations, and the electron microscope ultra-structure in control and 4-nonyphenol exposed cultures. This diatom was selected because it commonly occurs in most freshwater environments and has been the subject of other toxicological studies. This provides a framework for structuring this project's experimental design. An adequate literature base also exists for evaluating results of this experiment. The species grows rapidly and is easy to maintain in culture. The use of a phytoplankton species as a test organism complements the fish portion of the study by considering effects at the apex and base of the food chain.

In a second bioassay we attempted to determine whether 4-nonyphenol exposure has adverse effects on the reproductive success of male fathead minnows. The fathead minnow was chosen as model species for this assay, as it is a widely used model organism for toxicological studies and has been named a tier one screening organism for endocrine disrupters by the US EPA (Ankley 1998). Furthermore, detailed protocols for handling fathead minnows for experimental testing exist (Denny 1987) and were incorporated in our previous studies of endocrine disrupters (Schoenfuss 2001; Schoenfuss 2003). Finally, we have developed DNA microsatellite primers to allow for paternity determination of offspring in the reproductive assay.

The fathead minnow is an attractive model for studies of the effects of aquatic contaminants because of the reproductive strategy of this species. Fathead minnows are nest breeders, with the male establishing the nest site and defending it until larvae hatch (Unger 1983; Unger 1988; Sargent 1989). The aggressive behavior of the male during nest holding is directly controlled by the endocrine system through testosterone release and is therefore vulnerable to endocrine disruption. Furthermore, reproductive success relies on the ability of the male to defend the nest site until larvae hatch. Any weakening of the male due to contaminant exposure may prevent him from defending the nest site and will result in reproductive failure (Sargent 1988). Fathead minnows in the reproductive assay were exposed to low and reasonable concentrations of 4-nonyphenol, which have been identified as a ubiquitous contaminant in European and North American surface waters. Dosage of 4-nonyphenol in this study was adjusted to bracket values found by the USGS in an ongoing survey of alkyphenol contamination in Minnesota.

Determine the impacts of 4-nonyphenol exposure on the physiological development of the ubiquitous diatom species Cyclotella meneghiniana.

Cyclotella meneghiniana were isolated into culture during the spring of 2002 from Mississippi

River collections obtained at a sampling location on SCSU's campus. An individual specimen was extracted from the algal sample to initiate the cultures, ensuring the cultures are unialgal and homozygous. The stock culture were maintained in WC media (Guillard 1975) at 20°C on a 16:8 light/ dark cycle at 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, achieving an approximate density of 100,000 cells/ml. For experimental purposes, stock cultures were divided among six 2-L acid washed flasks. Two flasks were used as a control and two were exposed to one of two concentrations of 4-nonyphenol. Exposure to 4-nonyphenol concentrations were initiated at 2pm each afternoon, corresponding to the 8th hour of the light period. The experiment ran for 10 days and culture flasks were sampled on day 0, 1, 3, 5, 7, and 10. This 10-day experiment was repeated once. Sub samples were enumerated to evaluate changes in cell density over time. Lipid concentrations were quantified for the sub samples using an Iatroscan Mark IV (Sicko-Goad 1993). Additionally, fatty acid concentrations were quantified for sub samples using a gas chromatograph (Sicko-Goad 1988).

Determine the effects of 4-nonyphenol exposure on reproductive success in the fathead minnow (*Pimephales promelas*).

Juvenile fathead minnows (3 months old) were purchased from Kurt's Fisheries, PA in the spring of the first year of this study. Fish were held in flow-through 500L stock tanks under constant conditions (16:8 light:dark, 25-27°C, fed *ad libitum*, 2L water/fish density) and reared to maturity (approx. 5-6 months old) in our fish holding facility. Water was supplied through an in-house well, avoiding premature exposure of fathead minnows to alkyphenols in surface waters. Upon maturation fish were separated by sex, and density was lowered to avoid changes in hormone concentrations due to male/male and male/female interactions. Fish were then randomly placed into groups of 8 males and exposed (in duplicate) to one of four concentrations of 4-nonylphenol (0.19; 0.25; 0.84; 3.2 $\mu\text{g}/\text{L}$ NP) or a well water control for 28 days.

After exposure, male fathead minnows were marked with fin clips to allow for identification of individual fish throughout the reproductive assay. Each exposed fish was matched with a control fish and placed into the competitive spawning scenarios for seven days. Fish were observed twice daily during this period to determine which male was defending the nest site. Eggs were counted once daily. After seven days, just prior to hatching of the first larvae, the experiment was terminated. All male fish were processed for secondary sexual characters (tubercle distribution and prominence of the dorsal pad), relative size of testis (GSI) and liver (HSI), plasma vitellogenin concentrations, and gonadal histology.

Results

Diatom Exposures. Exposure of the diatom culture to a mixture of alkyphenols that represents the APs component of a major municipal wastewater treatment plant results in an increase in chlorophyll A production (a sign of environmental stress in this organism) and a reduction in lipid content (Fig. 1). The combined effects of those two density changes is a dramatic loss in food quality of exposed diatoms to larval and fingerling fish that preferentially feed on this usually high quality food source. At the highest AP mixture concentration, cell death result in a decrease of chlorophyll A density in the tissue culture.

Fathead Minnows. Fathead minnows were exposed to a graded series of 4-nonylphenol concentrations in two exposure experiment (Fig. 2). Concentrations between the experiments

overlapped with exposures ranging from 0.19-3.2 µg/L in the first experiment (Fig.2 left) and 0.1-15 µg/L in the second experiment (Fig. 2 right).

Statistically significant ($p < 0.05$) vitellogenin induction above background levels present in most fish was only found in the highest 4-nonylphenol treatment (Fig. 3 right). Secondary sexual characters varied between treatment and were significantly depressed only in the highest 4-nonylphenol treatment in the first experiment (fig. 4 left). However, this finding was not present in the second exposure experiment despite 4-nonylphenol concentrations almost 5 times higher (Fig. 4 right). The gonadosomatic index (Fig. 5) followed the indications of secondary sexual characters and was only depressed in the highest exposure in the first experiment (Fig. 5 left), but was not depressed in any treatment in the second experiment (Fig. 5 right). When 4-nonylphenol exposed male fathead minnows were allowed to compete directly with control males for spawning opportunities, a parallel result emerged in both exposure experiments. In the lower concentration treatments, 4-nonylphenol exposed fish out-competed control males with a reversal of this result in the higher treatments.

Summary of findings

It appears that a mixture of APs, including 4-nonylphenol at concentrations well below the US EPA criterion for chronic exposure (6.1µg/L) results in diminished lipid content in exposed diatoms. As a result larval and fingerling fish, which feed preferentially on this food source, will be faced with a lower quality food source. Nonylphenol also exhibits adverse effects on exposed male fathead minnows. These effects are more subtle, however at the highest concentration used in both exposure experiments (3.2µg/L and 15 µg/L, respectively), fish exhibited consistent effects, including diminished secondary sexual characters, lower GSI, and reduced nest holding ability. The combination of these factors is indicative of reduced reproductive competency in exposed male fathead minnows. Further experiments are under way to ensure the repeatability of our results.

List of publications & presentations resulting from this project

Bistodeau, T.J., L.B. Barber, S.E. Bartell, R.A. Cedic, K.J. Grove, J. Klaustermeier, J.C.

Woodard, K.E. Lee and H.L. Schoenfuss. In Press. Larval exposure to environmentally relevant mixtures of alkylphenoethoxylates reduces reproductive competence in male fathead minnows. *Aquatic Toxicology*.

October 2004. Schoenfuss. Simple Questions - Complex Answers: Investigating the Effects of Alkylphenols on Aquatic Organisms. Invited Seminar, University of Nebraska, Omaha.

- April 2005. Schoenfuss, H.L., T. Bistodeau, S. Bartell, J. Woodard, L. Barber, K. Lee, L. Zintek, A. Alwan, J. Lazorchack (2005) *Concentration Dependent Effects of 4-Nonylphenol on Male Fathead Minnows in a Competitive Reproductive Assay*. Oral Presentation at the Annual Meeting of the Midwest Division of the Society for Environmental Toxicology and Chemistry, Madison, WI, April 5, 2005.
- April 2005. Bistodeau, T., R. Cediell, K. Groove, J. Klaustermeier, H. L. Schoenfuss (2005) *Reproductive Consequences of Environmentally Relevant Exposures of Fathead Minnow Larvae to a Mixture of Alkylphenol Ethoxylates*. Poster Presentation at the Annual Meeting of the Midwest Division of the Society for Environmental Toxicology and Chemistry, Madison, WI, April 5, 2005.
- May 2005. Schoenfuss, H.L. (2005) *Biological Effects of Biologically Active Compounds: Experimental Considerations in the Study of Endocrine Disrupting Chemicals*. Symposium Presentation at the Annual Meeting of the American Oil Chemists Society, Salt Lake City, UT, May 3, 2005.
- October 2005. Schoenfuss. Concentration dependent effects of 4-nonylphenol on male fathead minnows in a competitive reproductive assay. MN Water 2005 Oral Presentation.
- March 2006. Schoenfuss. Reproductive Consequences of Alkylphenol Exposure on Male Fathead Minnows. Midwest meeting of the Society for Environmental Toxicology & Chemistry, St. Cloud, MN March 20-22, 2006.

Statement of related grants submitted or funded as a result of this project

Drs. Schoenfuss, Barber, Julius, and Norris have been awarded a US EPA STAR grant to further the investigation into the effects of alkylphenols on aquatic vertebrates and to develop rapid assessment tools for their detection. Drs. Schoenfuss, Barber and Lee received a grant from the MN Pollution Control Agency to continue our work on the effects of 4-nonylphenol on aquatic vertebrates in Minnesota. Drs. Schoenfuss, Julius and Barber received a grant from the National Water Resources Institute to assess the trophic cascade effects of mixtures of estrogenic compounds.

Description of student training provided by project:

Name: Travis Bistodeau

Program: Department of Biological Sciences, St. Cloud State University, St. Cloud, MN
Degree being sought: Masters of Science (received 4/2005).

Name: Jason Koch

Program: Department of Biological Sciences, St. Cloud State University, St. Cloud, MN
Degree being sought: Masters of Science (expected 4/2007).

Name: Kent Groove

Program: Department of Biological Sciences, St. Cloud State University, St. Cloud, MN
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Name: Maria Legatt

Program: Department of Biological Sciences, St. Cloud State University, St. Cloud, MN
Degree being sought: Masters of Science (received 12/2004).

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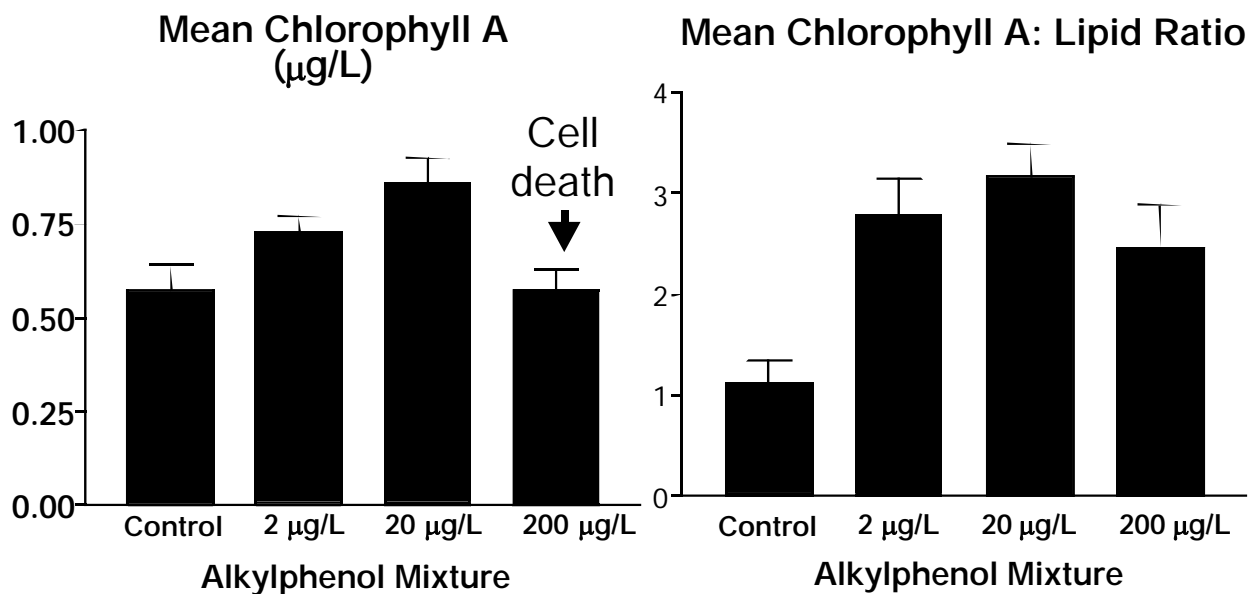


Figure 1. Effects of a mixture of alkylphenols on the production of chlorophyll A and lipids in diatoms (combined total alkylphenol concentration is provided).

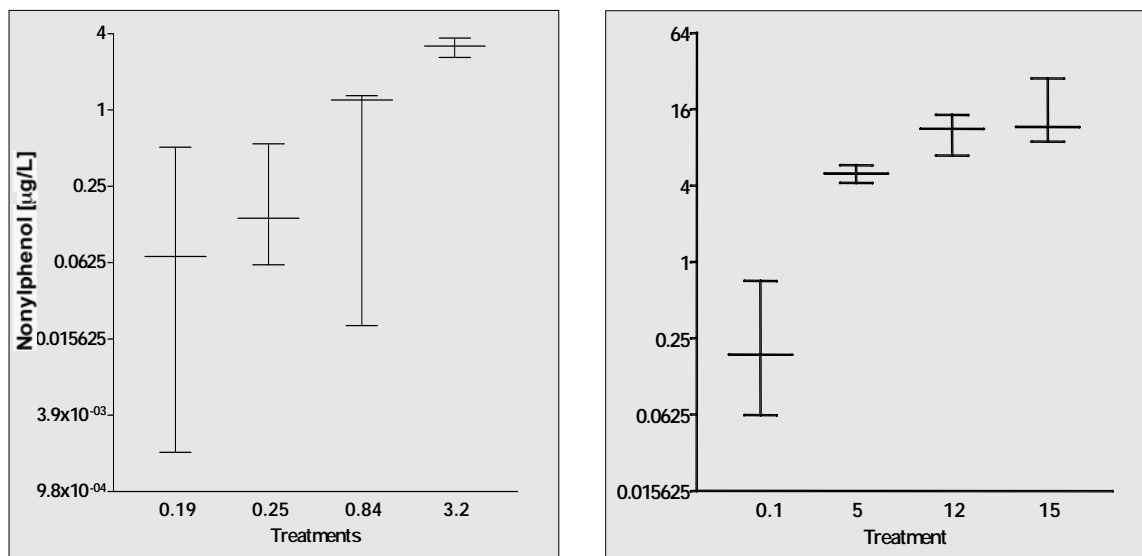


Figure 2. Measured 4-nonylphenol concentrations in two four week exposure experiments.

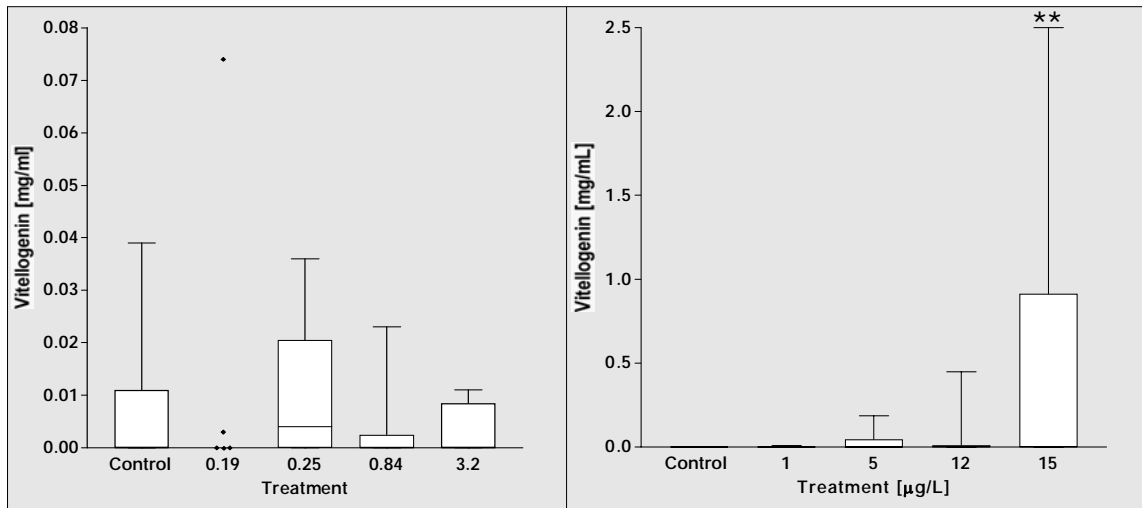


Figure 3. Vitellogenin concentrations in fishes exposed to 4-nonylphenol for four weeks in two exposure experiments (in $\mu\text{g/L}$). Line indicates median score, box 25 and 75 percentile, bars indicate data range. * indicates significance at $p < 0.05$.

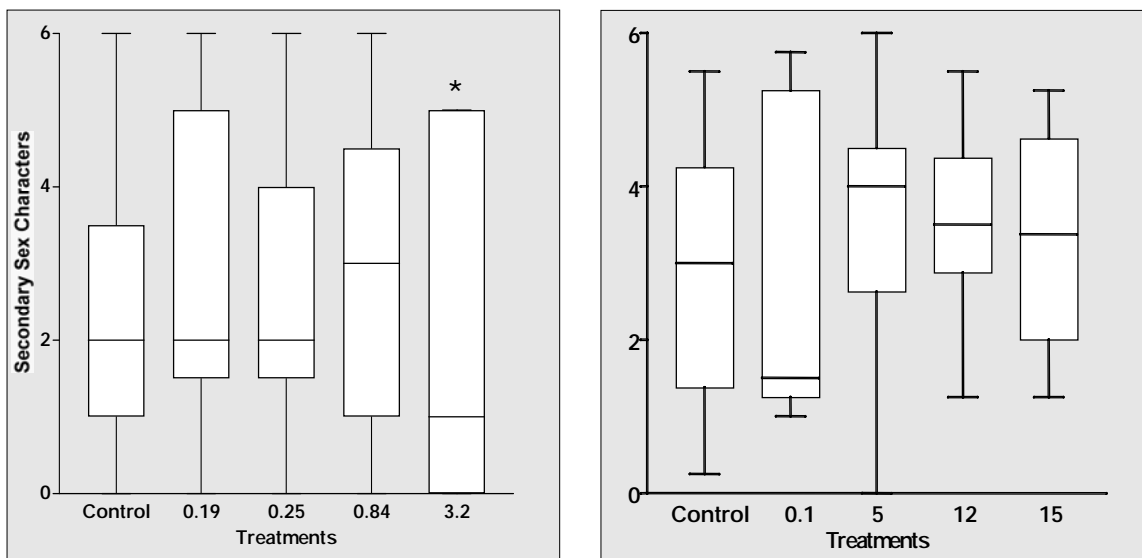


Figure 4. Secondary sexual characters (combined 0-3 score of tubercle and dorsal pad prominence) for male fathead minnows exposed for four weeks to 4-nonylphenol. Line indicates median score, box 25 and 75 percentile, bars indicate data range. * indicates significance at $p < 0.05$.

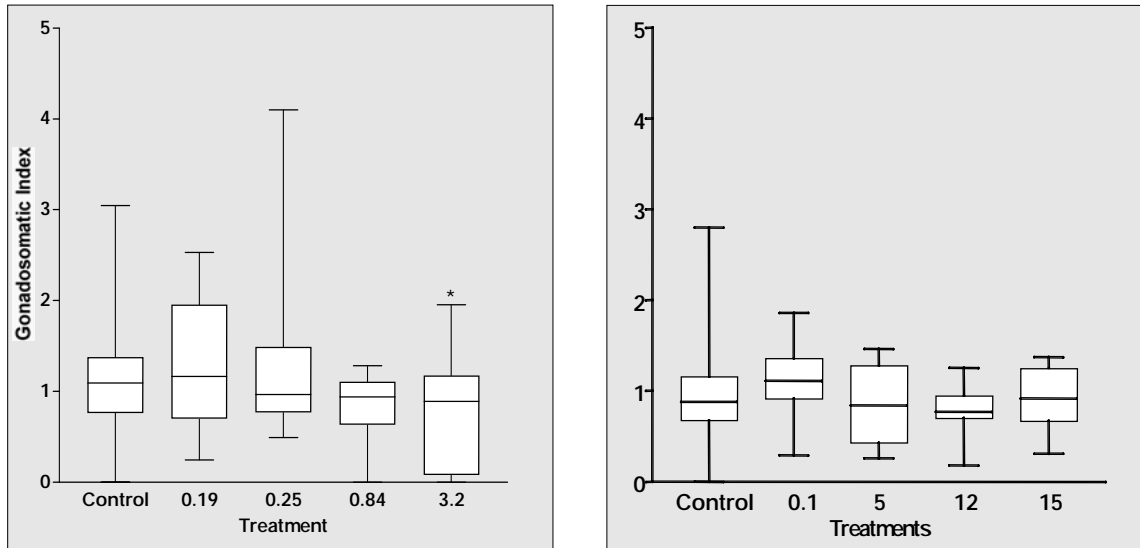


Figure 5. Gonadosomatic Index for male fathead minnows exposed for four weeks to 4-nonylphenol. Line indicates median score, box 25 and 75 percentile, bars indicate data range. * indicates significance at $p < 0.05$.

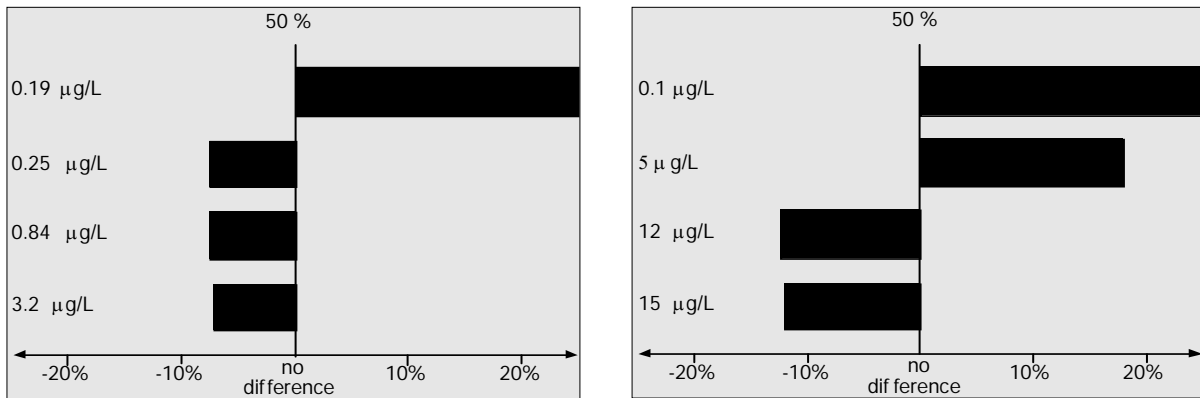


Figure 6. Results of a competitive spawning assay pairing control and 4-nonylphenol exposed male fathead minnows in small aquaria with two female fish and one nest site. Bar indicates the divergence in nest holding success from the expected 50:50 ratio between treated and control males.

Wireless Technologies Applied to Environmental Variables and Nutrient Loadings

Basic Information

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Wireless Technologies Applied to Environmental Variables and Nutrient Loadings

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Executive summary

The design and deployment of a real-time wireless sensor network for monitoring stream water quality parameters has been investigated. Multiple networking systems have been studied for efficiency and applicability in the field. These include the Crossbow Technologies Xmesh Mote Sensor Network and the Campbell Scientific PakBus Network. The Campbell Scientific system was selected considering its reliability, advanced software applications, and general ease of use. A laboratory grade nitrate biosensor was adapted for *in situ* field measurements to be used in conjunction with the concurrently developed sensor network. The measurement longevity is only restricted by the limited nitrate biosensor lifetime. A protocol has been developed for maintaining and sustaining the biological component of the sensors here at the University of Minnesota, thus reducing biosensor cost and startup time. The wireless technology will be deployed in the summer of 2006 to establish a real-time sensor network along Minnehaha Creek, Minnesota. With adjustable sampling protocols and sampling intervals of five minutes, the network will monitor turbidity, temperature, water level, and nitrate concentration. The network will provide unparalleled spatial and temporal resolution of the monitored parameters. The acquired data will then be compared to isolated USGS grab samples which are made typically on a biweekly time interval. The network data will provide a basis for quantifying temporal and spatial heterogeneities of nitrate, temperature, turbidity, and discharge in the Minnehaha Creek. Water quality will be observable as a dynamic response to land use gradients and hydrological transients rather than as an equilibrium described by average properties. This approach will enable process-based scaling and forecasting of water quality in streams from the in-stream processes to the watershed level. The wireless network developed in this project is focused on an urban stream, but can be expanded to include other watersheds with different land uses in the future. Generated data and scaling relationships will transform urban planning practices and management of water quality in streams draining urban land.

Introduction

Increasing urbanization across the world has progressively degraded the water quality of streams draining urban land (Booth et al., 2005; Welch et al., 2005a). Streams and rivers draining urbanizing watersheds are ecologically degraded and generally experience a consistent suite of effects named the “urban stream syndrome” (Welch et al., 2005b). General symptoms of the urban stream syndrome include more frequent large flow events with a shorter duration of the storm hydrograph, reduction in channel complexity, elevated concentrations of contaminants and pollutants, reduced retention of natural organic matter (NOM), elevated turbidity, and increased

biomass of filamentous algae (Hatt et al., 2004; Meyer et al., 2005). The mechanisms driving the urban stream syndrome are complex and interactive. The complexity of urban land use with associated heterogeneity in physical, chemical, and biological variables presents a research challenge of quantifying the mechanisms by which urban-derived stressors control water quality in streams. To obtain a complete diagnosis of the symptoms of even a single compound within a watershed requires adaptive and frequent event-driven sampling protocols over the range of scales. Wireless sensors and wireless networks are emerging technologies that are designed for concurrent measurements of parameters that experience spatial and temporal variability (Crossbow Technologies; Delin et al., 2004). These technologies are extremely well-suited for quantifying mechanisms between urban-derived stressors and stream water quality.

The deployment of a field-based wireless network for the concurrent measurement of environmental conditions, variables, and contaminants or surrogates for contaminants with adaptive sampling protocols is critical for understanding the input and fate of biological and chemical species throughout a watershed. Many inputs are non-steady state in nature. For example, pesticides enter streams after storm events, particularly at certain times of the year, the outflow of estrogenic compounds from wastewater treatment plants varies with time (*e.g.*, Schoenfuss et al., 2002), and the loading of NOM to a watershed will vary significantly with season and hydrologic events. In conjunction with non-steady state input flows of various species of interest, processes themselves often are either inherently non-linear in nature (the microbial transformation of compounds, for example) or are linear, but dependant on non-linear drivers. An example of the latter is the photo-transformation of pesticides in the environment. Direct photolysis rates are linear with respect to compound concentration and light intensity (Mill, 1999), but light intensity is inherently non-linear over time and space. Indirect photolysis in agricultural and urban streams is even more complicated. This process is a second-order reaction with a dependence on the concentration of hydroxyl radical. The hydroxyl radical concentration, in turn, is dependant on nitrate concentration, alkalinity, light intensity, and light penetration depth (Brezonik and Fulkerson-Brekken, 1998). The non-steady state nature of meteorological and hydrological processes, coupled with biogeochemical processes which are either non-linear processes or linear processes that are dependent on non-linear drivers, result in a system that is fundamentally variable with time and space and inherently far from equilibrium. Such a system requires frequent, tightly-spaced measurements to capture the dynamics of various pollutants entering, flowing through, and reacting or accumulating within the system boundaries.

Methods

Crossbow Mote Work

Software

The first step in this research was the acclimatization of the network operating environment. This included a familiarization with the TinyOS operating system developed at UC Berkeley and the C programming language (Kernighan and Ritchie, 1988). Both frameworks are the primary protocols for the Crossbow sensor network and function in a Linux based system. A 2-Day Wireless Network Training Seminar was attended at Crossbow Technologies in San Jose, CA to learn more about the system structure and how to design and deploy a useful sensor network.

Other available resources were used, such as customer support and user forums in learning the mote network system.

Hardware

The initial networking platform tested was developed by Crossbow Technologies. Prior to attending the workshop, a MICA2 Basic Kit was purchased for testing. A minimal Crossbow sensor network consists of two major parts (Fig. 1 & 2). The first is a remotely located mote/sensor node used for sampling, processing and radio transmission. The second is a server/gateway node used for radio receiving, processing and storage of experimental network data in a database format. An initial test-bed network was built in order to research the applicability of the network. The mote/sensor node was built using a MICA2 mote and a MDA300 generic sensor board.

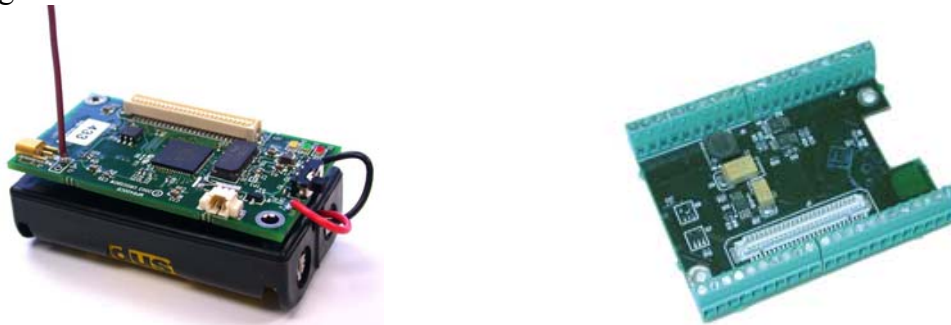


Figure 1: MICA2 mote (left) used for processing and communication; MDA300 sensor board (right) used for generic sensor sampling. This is known as the mote/sensor node. (Not to scale)

Several standard voltages were attached to the sensor board inputs in order to test the operation and applicability of the network as it applies to the transfer of measurement data. Sampling was done at near real-time, first through a gateway board directly connected to a PC. Data was logged in a generic database format on the PC. This was done to become familiar with the operation of the system. Preparations for outdoor deployment were also designed for this system. The transmission range of the motes was tested and they were then outfitted with higher quality antennae and ground plates to increase the transmission range. Designs were also made for power supply systems and housing enclosures. A remote server/gateway node was then built using a Stargate Gateway developed for the Crossbow by Intel, Inc. (Fig. 2). It uses a compact flash memory card for database storage and a PCMCIA wireless card for wireless communication.



Figure 2: Stargate Gateway used to receive network data packets for database storage. This is known as the server/gateway node.

A radio link for transmission of data was setup between the mote/sensor node and the server/gateway node (Fig. 3). A wireless link was setup between a PC and the sever/gateway. Transmission tests were performed to test the acquisition of sensor data at the mote/sensor node as well as radio transfer to the server/gateway node and wireless transfer to the PC.

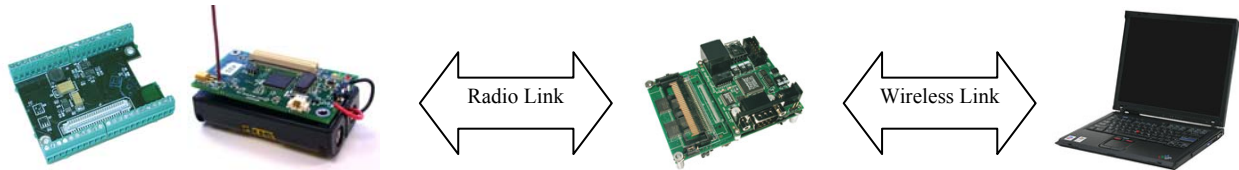


Figure 3: Crossbow test-bed setup used for transfer of data from field to PC. From left to right: sensor/mote, server/gateway and PC.

The Stargate runs a PostgreSQL database on a Linux operating system. It has low power consumption and can be outfitted with a wireless air card, allowing it to be placed in the field and contacted via a cellular network for network database access. Attempts were made to customize the sampling code executed by the mote/sensor node. This would allow the sensor measurements to be customized. Also, the Crossbow developed MoteView software platform was tested for managing the database of measurements stored on the Stargate server.

Campbell Scientific Work

Research into the Campbell PakBus Network was done in order to determine if it was a viable option for the creation of a wireless sensor network. Planning was done for a sensor network consisting of one sampling SubStation and one sampling BaseStation. The SubStation is run by a CR206 datalogger (Fig. 4) which samples four sensors (turbidity, depth, temperature and nitrate concentration).



Figure 4: Campbell Scientific CR206 (left) and CR1000 (right).

This station is in radio contact with the BaseStation, which is run by a CR1000 datalogger and samples the same four types of sensors (Fig. 4). The BaseStation will be in cellular contact with the University of Minnesota, Saint Anthony Falls Laboratory. The setup allows both stations to be accessed from the laboratory at any time. Both stations are solar powered and are entirely self-sufficient.

Locations for data collection in the Minnehaha Creek are currently being considered. The desired site will have the following attributes:

- At or near a USGS grab sample station.

- The network will spatially span a potential nitrate loading point caused by natural organic matter, storm water discharge or fertilizer run-off.
- The stations will be isolated from hard wired power to test its solar capabilities.
- The stations will be isolated from internet technology to test its data transfer capabilities.

Unisense Nitrate Biosensors

Many options are available for measuring nitrate at high levels, using ion reactive membranes. However, in order to measure nitrate at the typically low levels associated with river monitoring, alternative methods need to be employed. In collaboration with Unisense, Denmark, we have adopted a sensor designed for measuring nitrate concentrations in the laboratory and modified the sensor for field application (Fig. 5). The biosensor has a 90% response time of 90 sec and a minimum detection limit of 2 $\mu\text{g/L}$ as N. Detecting nitrate concentrations at low concentrations is a unique feature of this sensor. The NO_3^- ion enters the sensor by molecular diffusion through an ion-permeable membrane. Active denitrifying bacteria in the sensor reduce NO_3^- to N_2O , which is detected by an N_2O transducer. The current through the electrode is converted into a voltage by means of a picoammeter. The voltage is proportional to the concentration of nitrate present in the water sample. The nitrate-sensitive biochamber is replaceable and has a lifetime of about 2 months.

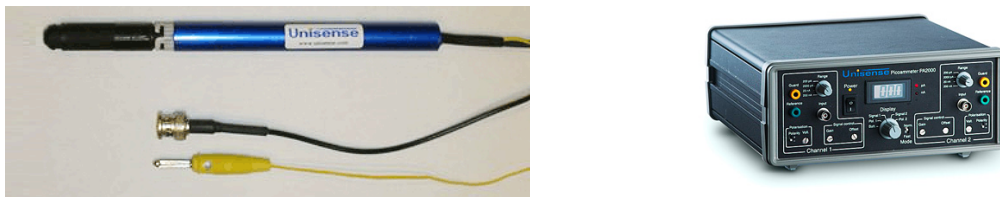


Figure 5: Nitrate biosensor (left) and measurement picoammeter (right).

Results to date

Crossbow Mote Work

A test-bed system was constructed and radio communication was established between the sensor/mote and server/gateway. Code was written to allow wireless communication with the server/gateway and a wireless connection to the server/gateway was made with a PC. However, connections and data transfers were very unreliable. Consequently, one of the major downfalls of the Crossbow system was found to be the complexity of its architecture and the lack of research friendly software. While connections were made, there were problems transferring data into the database from the sensor/mote as well as accessing the database for data retrieval from the PC. While some attempts to customize the sampling code were successful, the complexity of the programming framework was an important limitation of the setup. The system was unstable and difficult to work with. For example, due to lack of a hardware clock on the motes, the time stamping of measurements would need to be done computationally using a timer. This would lead to temporal sample inaccuracies throughout the network and also would make synchronization of the networking system virtually impossible. Temporal accuracy of a

measurement system is of utmost importance and the Crossbow system does not address these issues. Continuation with the system would have required a vast amount of background work in the area of computer programming and a fair amount of custom program code. Due to project constraints this was not a viable option. The decision was then made to begin researching the possibility of using another system to form the network.

Campbell Scientific Work

All of the appropriate sensor equipment needed to form a field sensor network has been ordered and received. Power tests have been run to ensure that the solar powered charging system will be able to maintain the network. A collective sensor housing unit has been designed using the four sensors (Fig. 6). The unit will be attached to a fence post in the Minnehaha creek and the sensor height can be easily adjusted using a hose clamp. The sensors will be attached to the station via a conduit to an onshore tripod which supports an enclosure, solar panel and a radio and/or cellular antenna. Contained within the enclosures are the datalogger, radio/cell phone, picoammeter and batteries.

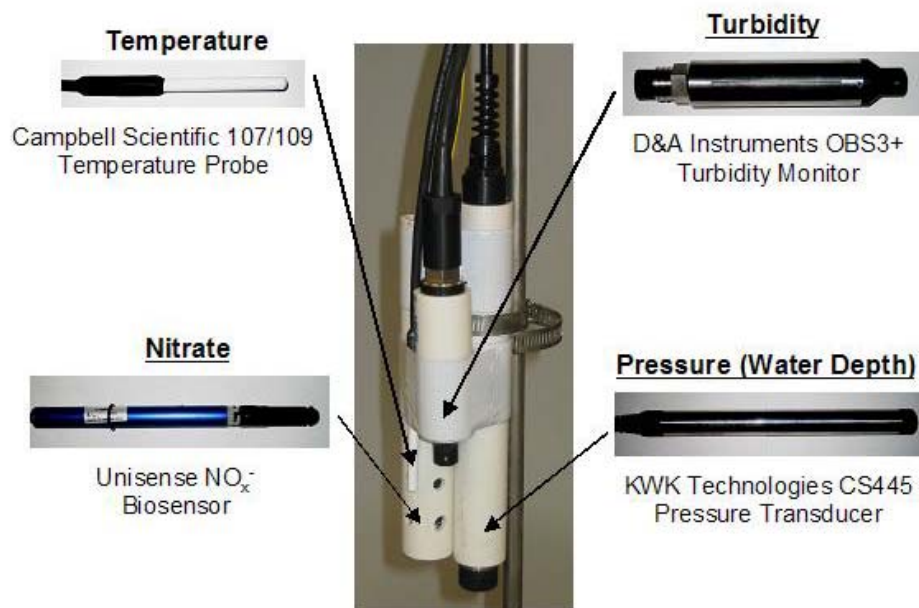


Figure 6: Sensor housing unit used with each station (UofMN design).

Programs have been written for both dataloggers that take one sample per minute for five minutes, and then record the average in a database. These programs can be easily edited to alter the temporal resolution of the network. They are also easily edited to perform any number of statistical or computational operations on the data itself. Programming algorithms have been developed to control power supply to the sensors, powering them only when necessary for a measurement. An optimization of power usage is a very important requirement for wireless networks which are powered by solar radiation. Radio communication has been established between the base and sub stations for two way data transfer. Programs and commands can be sent to either station and data can be downloaded from the stations. The variables running in the program can also be monitored in real-time. Once deployed, the stations need periodic visits for

routine physical maintenance or the occasional troubleshooting. The stations are currently being bench tested in the Saint Anthony Falls Laboratory and deployment is scheduled for the middle of June.

Unisense Nitrate Biosensors

A culture of the denitrifying bacteria used in the biosensors was acquired from Unisense. The bacteria were successfully inoculated, cultured and placed in cold storage. As a result, sterile chambers may now be purchased from Unisense at a considerably lower cost and the inoculation of the biochambers can be performed at the University of Minnesota. This will also eliminate the biosensor inactivation time that is commonly associated with the shipment of pre-inoculated chambers. The sampling code for the dataloggers also includes a procedure to measure the voltage put out by the biosensor picoammeter. The code samples and processes the voltage into a nitrate concentration based on a user entered linear calibration unique to each biosensor. Then the nitrate concentration is stored in the database along with the other sensor measurements.

Summary of findings

- Crossbow Technologies has developed a very compact and power efficient XMesh Mote Network system that offers many possibilities for scientific data acquisition. The system does not provide adequate communication software support needed for practical applicability in aquatic environments. Major deficiencies with the technology include:
 - time stamping of measurements at the time of acquisition;
 - sampling program versatility allowing the researcher to easily customize the sampling technique; and
 - inefficient database management.

For these reasons, the Crossbow XMesh Mote Network was not chosen for developing a stream monitoring wireless sensor network.

- The Campbell Scientific PakBus Network was found to be much better suited for the task of creating a sensor network. User-friendly communication software enables quick and reliable integration of a variety of sensors in the network. A two station network has been developed and is ready for deployment in the Minnehaha Creek. The network will monitor turbidity, depth, temperature, and nitrate concentration at two different locations with adjustable sampling intervals (from minutes to hours) along the Minnehaha Creek. The network can later be easily expanded to more stations to increase spatial resolution.
- A design protocol for the integration of the Unisense laboratory biological nitrate sensor into the sensor network has been completed, thereby allowing the sensor to be placed in an *in situ* measurement environment.

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Jazdzewski, J.D., M. Hondzo and W.A. Arnold, (2006) *Stream Water Quality Monitoring using Wireless Embedded Sensor Networks*. Future poster presentation at the Minnesota Water 2006 and Annual Water Resources Joint Conference, October 24-25, 2006, Minneapolis, MN.

Hondzo, M.; Arnold, W.A.; Novak, P.J.; Hozalski, R.M., 2006. Wireless technologies and embedded networked sensing: application to integrated urban water quality management. Paper presented at the ASCE World Environmental and Water Resources Congress, Omaha, NE, May 21-26, 2006.

Statement of related grants submitted or funded as a result of this project

This project facilitated funding by the National Science foundation (NSF) for two grants at the University of Minnesota.

Award: NSF-IGERT (Graduate education grant)

Principal Investigators: C. Neuhauser (PI), R. Hozalski, M. Hondzo, S. Sugita, C. Paola, W. Arnold N. Jindal, S. Shekhar

Title: IGERT: Non-equilibrium dynamics across space and time: a common approach for engineers, earth scientists, and ecologists

Amount: \$2,819,194

Period of Support: 08/01/05-07/31/10.

Award: NSF

Principal Investigators: M. Hondzo (PI), W. Arnold, R. Hozalski, N. Jindal, and P. Novak

Title: Wireless technologies and embedded networked sensing: Application to integrated urban water quality management

Amount: \$250,440

Period of Support: 08/01/06-07/31/08.

Description of student training provided by project:

Name: Jeremiah D. Jazdzewski

Program: Department of Civil Engineering, University of Minnesota

Degree being sought: Masters of Science

Estrogens and Estrogenic Activity in Swine Manure

Basic Information

Title:	Estrogens and Estrogenic Activity in Swine Manure
Project Number:	2005MN102B
Start Date:	3/1/2005
End Date:	2/28/2006
Funding Source:	104B
Congressional District:	4
Research Category:	Water Quality
Focus Category:	Toxic Substances, Agriculture, None
Descriptors:	None
Principal Investigators:	Kuldip Kumar, Satish C. Gupta, Ashok K Singh

Publication

1. Kumar, K., Satish Gupta, Ashok Singh, Shveta Gupta, Yogesh Chander, Lakhwinder Hundal, Albert Cox, and Thomas Granato. 2006. Occurance of Estrogenic Compounds in Manures and Biosolids. Invited Paper in Special Symposium on Emerging Contaminants and Land-applied Biosolids and Manures: State of the Science and regulatory implications. ASA-CSSA-SSSA International Annual Meetings. November 12-16, 2006.

Estrogens and Estrogenic Activity in Swine Manure

Principal Investigators

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Start date: 3/1/2005

End date: 2/28/2006

Executive Summary

Naturally occurring estrogens in animal waste can cause negative environmental impact through disruption of endocrine system in wild life, domesticated animal, and humans. Very little information is available on the type and the extent of estrogenic activities in swine manure. This is partially due to lack of analytical ability for estrogen analysis in manures. The goal of this study was to develop procedures for analyzing different estrogens and estrogenic activities in different types of swine wastes. The wastes included samples of urine and feces from pregnant female pig, non-pregnant pig of similar age, and a boar. The ELISA and HPLC-UV based methods were developed to quantify estrone (E1), 17 β estradiol (E2), and estriol (E3) and their conjugates. HPLC analysis showed many organic compounds in manure which had properties similar to that of conjugates of the parent estrogen compounds. Therefore, we concentrated on analyzing only the parent compounds E1, E2, and E3 in this study. In general, ELISA gave higher concentrations of these compounds compared to HPLC-UV analysis. The total concentration of estrogens was more in feces than in urine and followed the trend: pregnant female > non-pregnant female > boar. The concentration of various estrogens in swine waste was variable with concentrations as low as traces to 84 μ g/L of E1 in manure from nursery pigs and 1398 μ g/L of E2 in pits from finishing pigs. The concentrations of E2 in manure increased by as much as 50-100% on treatment with glucuronidase and sulfatases enzymes indicating that significant concentrations of conjugates were present in manure. Although these conjugates do not have much estrogenic activity, they can convert to free estrogens in manure lagoons thus leading to higher estrogenic activities.

Introduction and Objectives:

Estrogen hormones are emerging contaminant that are attracting public attention because at low concentrations these pollutants can adversely affect the reproductive biology of vertebrates such as fish, turtle, frogs; wild animals; and humans. One source of these contaminants in water bodies is through runoff from manure-applied fields. Currently, there is limited understanding of the toxicological significance (in particular low level long-term exposure) of these contaminants not only on human health but also on aquatic life. Several researchers have shown that estrogen concentrations as low as 10 – 100 ng/L in waterways can adversely disrupt the normal functioning of endocrine system of many species thus affecting their reproductive biology (Irwin et al., 2001; Hanselman et al., 2004). Swine farming is one of the major food-animal industries in the nation. In 2003, pig growers in the US had an inventory of 59.6 million pigs and hogs. Minnesota is #3 in swine production.

With wide spread scare about bird flu and consistent demand for while meat, a major increase in swine production facilities is expected over next several years. This means increased problems associated with disposal of swine waste and the risk of soil and water contamination.

Although estrogens excreted by the animals have been recognized as a potential pollutant, research data is lacking to make scientific assessment of the scope of estrogen contamination. An important reason for this lack of scientific data is an absence of analytical methods for the screening and conformation of estrogens, especially the conjugated estrogens, in swine waste. On an average, a pig produces about 3.9 kg of manure (a slurry of urine and feces)/day (Hamilton et al., 2003) which mean about 2.3×10^8 kg of manure is generated each day in the US by the pig industry. Furthermore, studies have shown that potent estrogens such as estrone (E1), estradiol (E2), and estriol (E3) are excreted into the environment in the urine and feces of all farm animals (Hanselman et al., 2003).

Estrogens are excreted in two forms: free estrogens such as E1 and E2 that are mostly present in feces, and conjugated estrogens such as E1S, E2S, E1G, and E2G that are mostly present in urine. Thus, stored manure generally contains both forms of estrogens. Unlike for free estrogens, the fate of conjugated estrogens in waste is not fully understood. Waste microorganisms, via their glucuronidase enzyme, have been shown to hydrolyze estrogen-glucuronide conjugate into free estrogens (Dray et al., 1972). It has also been shown that free-estrogens, and not their conjugated forms, are bioactive at very low concentrations (1 to 100 ng/L) (Matthiessen and Sumpter, 1998). Also, the potency of E2 is several folds greater than E1 and E3.

The goal of this study was to develop new methods and/or validate existing methods for detection and confirmation of estrogens, and then use the validated methods to evaluate the levels and fate of estrogens in swine manure. Specifically, the objectives were:

1. Develop ELISA methods for screening and HPLC-UV method for confirmation of free and conjugated estrogens in swine manure.
2. Develop methods for hydrolysis of conjugated estrogens using enzymes.
3. Develop methods to quantify estrogenic potency of manure samples using estrogen-receptor positive cells.

Materials and Methods:

Objective 1: Following ELISA kits were obtained from Japan Envirochemicals, Ltd, Japan to analyze estrogens in urine and manure of swine:

Total Estrogen ELISA: This kit measured total (estrone E1, estradiol E2 and estriol E3) estrogens in aqueous samples. The analysis is based on a competitive reaction where enzyme labeled standard competes with free estrogens in the sample for binding to a specific monoclonal antibody immobilized to the surface of the microtiter plate. The amount of labeled estrogen bound to the plate is determined by addition of a non-colored substrate, which is converted into a colored product. The color intensity is measured at 450 nm and is inversely proportional to the amount of estrogen in the sample. The assay is calibrated using a standard solution of estrogens supplied with the kit. The assay is highly sensitive, simple and rapid to perform. The standard curve working range is 0.1-3.0 $\mu\text{g/L}$. Before ELISA test, a simple solid phase extraction was performed on urine, feces or manure samples as described in the kit. Three kits used in estrogen analysis were: (i) total estrogens (E1+E2+E3), (ii) E1

kit, and (iii) E2 kit. The amount of E3 was then estimated by subtracting the concentration of E1 and E2 from total estrogens concentrations. The kit exhibited excellent linearity and recovery for all three estrogens in free or conjugated forms.

Individual estrogen ELISA: The positive samples were analyzed by using the estrogen specific kits for further identification. These kits did not distinguish between free and conjugated estrogens. When added to manure samples, different estrogens exhibited variable recoveries (25 to 50%), possibly due to their binding to the manure's solid particles.

HPLC-UV: Since the ELISA method did not differentiate between free and conjugated estrogens, this was achieved through the use of HPLC-UV technique. We are able to detect both free and conjugated estrogens simultaneously. Since the HPLC-UV is less sensitive than the ELISA methods, 5 to 10 ml of each sample was pooled and extracted with solid-phase extraction prior to estrogen analysis.

Objective 2: It is well established that the free, and not the conjugated estrogens are biologically active. Furthermore, the soil enzymes can convert conjugated estrogens into free estrogens, thus increasing the samples estrogenicity. Therefore, to determine the total estrogenic potential of a sample, it is important to convert the conjugated estrogens into free estrogens prior to analysis. To achieve the second objective, we spiked the manure samples with glucuronide and sulfate (5 ng each) conjugates of each estrogen and then incubated the samples in a mixture containing glucuronidase and sulfatase enzymes at 37 °C for 24 h. As shown in Figure 1, the conjugated estrogens were effectively converted into free estrogens thus facilitating the quantification of total estrogenic potential of four types of swine manures samples.

Briefly, the procedure for hydrolysis of conjugated estrogens into free-estrogens in swine manure involved drying the extract containing conjugated estrogens at 55 °C under nitrogen, dissolving the dried extract in 5 mL 0.2 M sodium-acetate buffer, and then incubating it with 100 µl β-glucuronidase and sulfatase enzymes (H2 type, activity 100,000 E/mL; sulfatase activity 5000 E/mL, respectively) at 37 °C for 24 h. The hydrolyzed extract is then extracted with a combination of C18 and NH₂ columns and analyzed either with ELISA or HPLC-UV. The specificity of the method is determined by incubating the samples with the β-glucuronidase inhibitor *d*-saccharic-1-4 lactone (100 mmol/L) prior to adding glucuronidase. The procedure described above yields the following fractions from each sample: (1) Fraction containing free estrogens (E1, E2, E3, 16-OH-E1, and 6-OH-E1). (2) Fraction containing conjugated estrogens (E1G, E2G, E1S, E2S, and E3G). (3) Hydrolyzed fraction containing free estrogens.

Objective 3 (in progress): We could not calibrate this method in time to perform this assay on manure samples in this project. However, we are in the process of growing an estrogen-positive cell lines for the purpose of determining the samples estrogenic potency. We expect to complete the developmental work for this project over next two months.

Results:

A comparison of ELISA and HPLC-UV results shows that solvent extraction of feces, urine, and manure slurries was sufficient to quantify the free estrogens in manure samples (Tables 1 and 2). Many organic compounds present in the manure eluted early during HPLC

determination and thus interfered with the separation of conjugated estrogens peaks. Thus, further testing of the methods was done only for free estrogens in manure and urine samples. We are continuing with efforts to develop clean up procedures for separating these conjugate estrogens peaks for swine manure samples. However, for this report only the data for free estrogens E1, E2, and E3 are reported.

In general ELISA and HPLC-UV techniques gave similar trends with ELISA giving higher concentrations of estrogens in fresh feces, urine, and manure samples. The reason for these differences may be that ELISA is based on immunological and colorimetric reaction test and conjugated estrogens and/or degradation products may be reacting with antibodies thus giving higher concentrations. Both ELISA and HPLC-UV techniques showed that there was a large variation in the concentrations of a given estrogen between the pigs urine and feces obtained from different facilities (Tables 1). For example, the concentrations of E2 in feces ranged from 1498 to 2022 $\mu\text{g/L}$ as compared to 180 to 490 $\mu\text{g/L}$ in urine samples using the ELISA test (Table 1). In general, higher concentrations of free estrogens were present in feces than urine and followed the trend: pregnant pigs > non-pregnant pigs > boar (Table 1).

The estrogen concentrations in actual manure samples also varied widely, with only trace concentrations of E2 in manure samples from nursery pigs to as high as 1398 $\mu\text{g/L}$ in manure samples obtained from pits of a feeder-finish facility (Table 2). In general, solid manure samples obtained from hoop structures contained lower concentrations of estrogens compared to the manure obtained from lagoons or pits. This may be due to three reasons (i) better composting of solid manure leading to rapid decay of these compounds, (ii) estrogens from urine percolated below the bedding materials into the soil, and (iii) low recoveries of estrogens from solid manures (mixture of bedding and feces) as compared to liquid manures from pits and lagoons.

We also tested the hypothesis that conjugated estrogens (no significant estrogenic activity) may be present in manures. This was done through the addition of β -glucuronidase and sulfatase enzymes. The results showed that concentration of estrogen E2 increased from as much as 40-100% in the four manures when treated with β -glucuronidase and sulfatase (Figure 2). This shows that in addition to free estrogens, concentrations of conjugated estrogens may be significant in manures and needs further quantification. During manure storage in pits or lagoons, there is a potential that some of the conjugated estrogens may be converted back to free estrogens and thus increasing the estrogenic activity of manure.

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2. Publications:

Kumar, K., Satish Gupta, Ashok Singh, Shveta Gupta, Yogesh Chander, Lakhwinder Hundal, Albert Cox, and Thomas Granato. 2006. Occurance of Estrogenic Compounds in Manures and Biosolids. Invited Paper in Special Symposium on Emerging Contaminants and Land-applied Biosolids and Manures: State of the Science and regulatory implications. ASA-CSSA-SSSA International Annual Meetings. November 12-16, 2006.

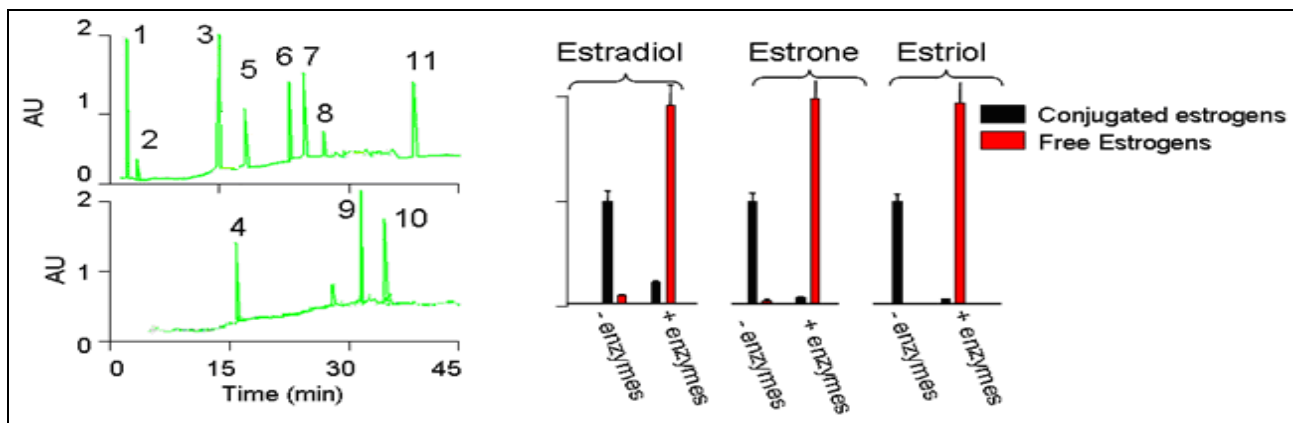


Figure-1: Hydrolysis of conjugated estrogens using a mixture consisting of glucuronidase and sulfatases. Top chromatograph shows conjugated estrogens and bottom chromatograph shows the enzyme hydrolyzed free estrogens. Almost 100% of the conjugated estrogens were hydrolyzed by the present method. 1 E3G, 2 E3S, 3 E2G, 4 E3, 5 E1G, 6 E2S, 7 E1S, 8 unknown, 9 E2, 10 E1, 11: unknown.

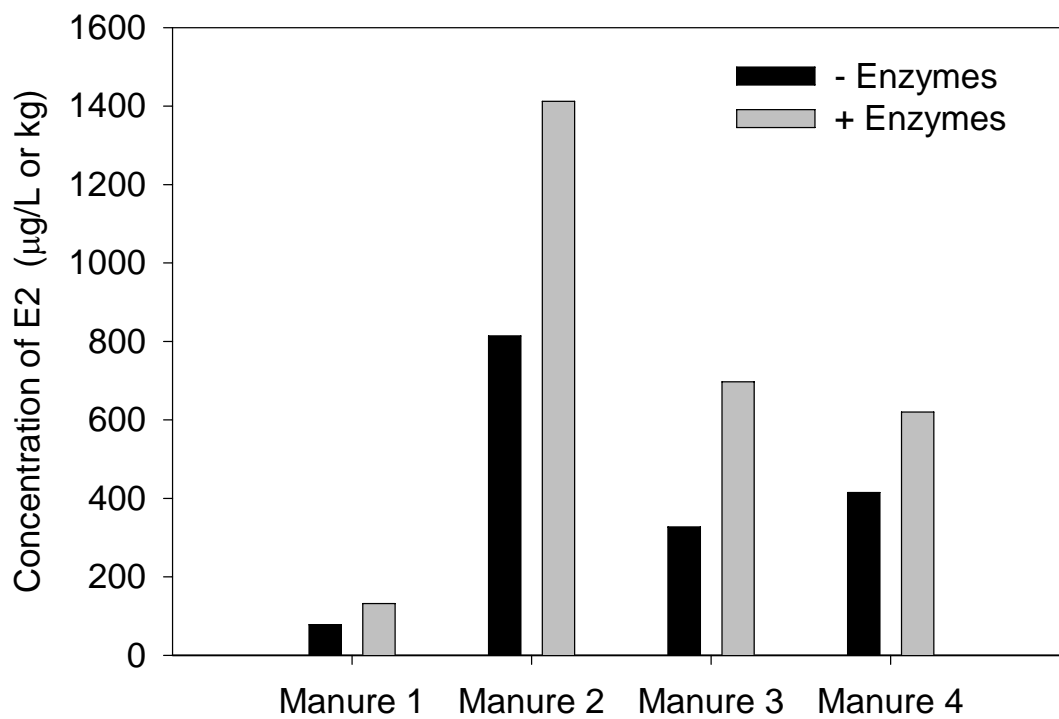


Figure 2: Hydrolysis of conjugated estrogens present in four swine manure samples using a mixture consisting of glucuronidase and sulfatase.

Table 1: Concentration of E1, E2, and E3 in feces and urine obtained from pregnant female pig, non-pregnant female pig and a boar using ELISA and HPLC-UV analysis.

	Feces ($\mu\text{g}/\text{kg}$ dry weight)						Urine ($\mu\text{g}/\text{L}$)					
	Pregnant Pig		Non-Pregnant		Boar		Pregnant Pig		Non-Pregnant		Boar	
	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC
E1	2409	2350	2221	2418	1127	1188	780	598	430	348	424	455
E2	2022	1790	1871	1316	1498	830	490	792	378	180	180	66
E3	3478	3380	1078	998	974	524	1084	898	904	504	90	26
Total	7909	7520	5170	4732	3599	2542	2354	2288	1712	1032	694	547

Table 2: Concentration of various forms of estrogens in swine manures obtained from different facilities.

Estrogen	Nursery pigs (n=2)		Finishing hoops ($\mu\text{g}/\text{kg}$) (n=3)		Feeder-Finish pits ($\mu\text{g}/\text{L}$) (n=3)		Finishing lagoons ($\mu\text{g}/\text{L}$) (n=3)	
	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC
E1	84 \pm 37	ND	149 \pm 56	90 \pm 45	648 \pm 319	455 \pm 203	1098 \pm 312	-
E2	Traces	ND	85 \pm 42	42 \pm 32	1398 \pm 418	915 \pm 157	327 \pm 156	-
E3	Traces	ND	203 \pm 72	104 \pm 56	58 \pm 26	43 \pm 18	208 \pm 92	-

ND – Not detected; - Not analyzed.

Use of *Arthrobacter aurescens* for Remediation of Groundwater Contaminated with Triazine Herbicides

Basic Information

Title:	Use of <i>Arthrobacter aurescens</i> for Remediation of Groundwater Contaminated with Triazine Herbicides
Project Number:	2005MN103B
Start Date:	3/1/2005
End Date:	2/28/2006
Funding Source:	104B
Congressional District:	4
Research Category:	Water Quality
Focus Category:	Water Quality, Groundwater, Treatment
Descriptors:	None
Principal Investigators:	Mike Sadowsky, Lawrence Phillip Wackett, Marc G. Von Keitz

Publication

Use of *Arthrobacter aureescens* for Remediation of Groundwater Contaminated with Triazine Herbicides

Principal Investigators:

Michael J. Sadowsky, Department of Soil, Water, and Climate, University of Minnesota
Lawrence P. Wackett, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota
Marc von Keitz, Biological Process Technology Institute, University of Minnesota

Start date: 3/1/2005

End date: 2/28/2006

Executive Summary:

The specific objectives of the proposed research are to evaluate the usefulness of the newly developed, stable and highly-active biocatalyst formulations for the bioremediation of atrazine-contaminated aquifer materials (water and sediments). The biocatalysts used in these studies were TrzN (triazine hydrolase) from *A. aureescens* strain TC1 and AtzB (hydroxyatrazine aminohydrolase) from *Pseudomonas* ADP. These two enzymes catalyze the first two steps in the atrazine biodegradation pathway, the dechlorination and subsequent hydrolysis of atrazine to non-toxic byproducts. In our studies we proposed to examine the stability, longevity, and activity of biocatalytic formulations in pilot scale simulated aquifers and wells. To achieve this goal, we firstly developed methods to overproduce the required enzymes, the used large scale fermentations to produce large quantities for cells for enzyme extraction. We are now developing methods to link these enzymes to solid support beads (EMPHAZE[®]) to maintain activity. This project is being done in conjunction with funding from the University of Minnesota Biocatalysis initiative, which provided funds to support the development of shelf-stable, highly active biocatalytic particles.

Methodology:

Expression and purification of TrzN. The TrzN was purified essentially as described by Shapir et al. (2006). Briefly, plasmid pAG, containing the chaperones groEL and groES, was transformed into *E. coli* BRL21(DE3) containing plasmid pET28b+::trzN. Strain BRL21(DE3) (pET28b+::trzN) was grown in LB medium containing kanamycin (50 µg/ml) and chloramphenicol (30 µg/ml) at 15°C, with shaking at 150 rpm. When cultures reached an optical density of 0.5 at 600 nm, the chaperones were induced by the addition of 0.0015% L-arabinose, and 1.5 µM IPTG (isopropyl-β-D-thiogalactopyranoside) was added after an additional 90 min of incubation at 15°C. Induced cells were grown for an additional 16 h under the same conditions, cultures were centrifuged at 10,000 x g for 10 min at 4°C, washed three times with 0.85% NaCl, and cell pellets were resuspended in 30 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 10% glycerol. Cells were broken by passage, three times, through a chilled French pressure cell operated at 140 Mpa, and cell-free extracts were obtained by centrifugation at 18,000 x g for 90 min at 4°C. Lysates were applied to a 5-ml HisTrap chelating column (Amersham Pharmacia Biotech, Piscataway, NJ), complexed with

Ni²⁺, according to the manufacturer's instructions. The column was washed with 15 ml 0.1 M sodium phosphate buffer, pH 7.0, followed by two washes with the same buffer supplemented with 0.1 M and 0.25 M imidazole, respectively. All buffers contained 10% glycerol. Enzyme was eluted from the column with 15 ml of 0.5 M imidazole in 0.1 M sodium phosphate buffer, pH 7.0, and the purified enzyme was concentrated using a Centricon-30 filtration unit (Amicon, Beverly, MA). Imidazole was removed from the enzyme preparation by dialysis, twice, at 4°C for 4 hr against 4 L of 0.1 M sodium phosphate buffer, pH 7.0 containing 10% glycerol. Enzyme purity and subunit molecular weight were estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay. Enzyme activity was measured by monitoring the disappearance of the substrate ametryn at 264 nm by using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). Reactions (1 ml) were carried out at 37°C in 0.1 M sodium phosphate buffer, pH 8.0, containing 132 µM ametryn. Reactions were initiated by the addition of enzyme, and the molar absorbance at 264 nm for ametryn under these conditions was determined to be 5 mM⁻¹cm⁻¹. Enzyme activity was also followed using a colorimetric assay that we previously developed (Shapir et al. 2005).

Expression and purification of AtzB: The enzyme AtzB was essentially produced as described by Boundy-Milles et al. (1977). The *atzB* gene was cloned into plasmid pACYC184, under control of the *atzA* promoter. *E. coli*(pATZB) was grown in LB medium containing tetracycline (15 mg/ml) and ZnSO₄ incubated at 37°C. Overnight cultures were centrifuged at 12,000 x *g* for 10 min at 48C, and pellets were washed twice with 25 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 6.9) and resuspended in the same buffer on ice. Cold cell suspensions were broken by three consecutive freeze-thaw cycles followed by sonication with a Biosonik sonicator (Bronwill Scientific, Rochester, N.Y.). Sonication was carried out three times at 80% probe intensity with intermittent cooling on ice. The broken cell suspensions were centrifuged at 17,000 3 *g* for 90 min at 48C to obtain crude cell extracts. Authentic samples of atrazine, hydroxyatrazine, and *N*-isopropylammelide were used to prepare 100-mg/ml stock solutions in 25 mM MOPS (pH 6.9). The crude extracts were diluted in 25 mM MOPS (pH 6.9) to obtain a final protein concentration of 50 mg/ml and amended with either atrazine, hydroxyatrazine, or *N*-isopropylammelide (100 mg/ml). Reaction mixtures were incubated at room temperature. We are currently optimizing large scale production and purification protocols for AtzB. Results of this work will be soon submitted to Applied and Environmental Microbiology.

Large scale fermentations: To produce sufficient quantities of the enzymes for immobilization and subsequent longevity studies, we firstly needed to produce large amounts of cells overproducing the enzymes. To do this, we used the fermentation facilities of the University of Minnesota Biotechnology Resource Center (BRC). A total of five fermentation runs were performed, totaling 750 liters of culture medium. This resulted in the production of 4,985 g of *E. coli* cells overproducing TrzN, and 10,340 og of cells overproducing AtzB. For fermentation of TrzN, *E. coli* BRL21(DE3) containing plasmid pET28b+::trzN and containing the chaperones groEL and groES was grown 300L DCI reactor at 15°C in TB medium containing kanamycin (50 µg/ml),

chloramphenicol (30 µg/ml), and ZnSO₄ (140 mg/l). When absorbance at 600nm reached 1.34, chaperone expression was induced by addition of L-arabinose to 0.015 g/L final concentration. After one doubling time (absorbance at 600nm = 2.56), trzN production was induced by the addition of IPTG to a final concentration of 2.0 µM. The culture was allowed to grow for 36 hours past the transition into stationary phase of growth for protein expression. 96 hr post-inoculation (final ABS₆₀₀ =13), the culture was cooled and harvested by continuous centrifugation in the Sharples AS-16 centrifuge. The cell paste was frozen in liquid nitrogen and stored at -80°C.

Enzyme Activity of TrzN: Crude extract was tested for activity, and found to degrade 5.8 umoles atrazine/min/mg cells. This activity is quite excellent, as much smaller batch reactions (up to 2 L) produced a maximum degradation of 2.5 umoles atrazine/min/mg cells.

Project Significance:

TrzN catalyses the first reaction step in the degradation of as many as 400 different s-triazine compounds (Strong et al. 2002), chemicals that migrate into the environment as they are used in pesticide/herbicide/antimicrobial applications. This protein is being used in a prototype biocatalyst to reduce atrazine concentration below the maximum level allowed by law in drinking water of 3ppb. The Biocatalyst is formed by immobilizing enzyme onto solid beads (EMPHAZE™ beads manufactured by 3M, 140µm diameter) via amino-functional ligands that form stable amide bonds with proteins. The resulting biocatalyst is packed into a plug-flow reactor configuration suitable for scale-up to purification of millions gallons drinking water per day.

Conservative analysis of research to date shows that reducing atrazine concentrations 93% (to 1ppb) in 1 million gallons per day would require a reactor containing 340 ft³ of media. Our current research focuses on improving this catalyst to reduce the required reactor size. The goal is to reduce capital and operating costs to confer affordability to governments in agricultural districts, where the need for this product is greatest.

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A Rapid Bioassessment Approach for Integrating Biological Data into TMDL Development for Organic Enrichment of Streams in Urbanizing Watersheds

Basic Information

Title:	A Rapid Bioassessment Approach for Integrating Biological Data into TMDL Development for Organic Enrichment of Streams in Urbanizing Watersheds
Project Number:	2005MN118B
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End Date:	2/28/2006
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Congressional District:	5 (=Minneapolis)
Research Category:	Water Quality
Focus Category:	Water Quality, Nutrients, Models
Descriptors:	None
Principal Investigators:	Leonard Charles Ferrington Jr.

Publication

A Rapid Bioassessment Approach for Integrating Biological Data into TMDL Development for Organic Enrichment of Streams in Urbanizing Watersheds

Principal investigators

Leonard C. Ferrington Jr., Professor, Department of Entomology & WRS

Research Assistants

Adam W. Sealock, WRS Program

Brenda Asmus, WRS Program

Start date: 03/01/2005

End date: 02/28/2006

Introduction

The Federal Pollution Control Act Amendments of 1972 (PL 92-500), as supplemented by the Clean Water Act of 1977 and the Water Quality Act of 1987 (in conjunction with more recent amendments) serve as the foundation for protecting the quality of our surface waters. Present-day implementation of Section 303d of the Clean Water Act focuses on ambient water quality standards, and requires states (1) to identify surface waters that are not meeting ambient water quality standards appropriate for their designated use categories and (2) to define the pollutants and their sources that are responsible for non-attainment of the ambient water quality standards. Section 303d further requires states to establish Total Maximum Daily Loads (TMDL) for pollutants impairing the surface waters and to develop strategies for reducing both point and non-point sources of the pollutants in order for the non-attaining waterbodies to meet ambient water quality standards.

Biological data are typically integrated into the above process as “front-end” input, being used (1) to assist in development of designated use categories and (2) in monitoring efforts to ensure that ambient water quality standards are met. However, prediction of biological responses that are likely to result from implementation of TMDL plans is not a fundamental element of the TMDL process. In a recent overview of the TMDL approach to water quality management requested by the US Congress, the National Research Council (2000, the full is text available on line at <http://nap.edu/openbook/0309075793/html/6.html>) made several recommendations for integration of biological data into the TMDL process. Among the recommendations, the report states “*EPA should promote the development of models that can more effectively link environmental stressors (and control actions) to biological responses*” and “*Monitoring and data collection programs need to be coordinated with anticipated water quality and TMDL modeling requirements*”. This proposal is to employ a newly tested rapid bioassessment technique developed for assessing organic enrichment in urban areas of Minneapolis/Saint Paul (Minnehaha Creek) in a second watershed (Vermillion River catchment) that is undergoing rapid urban development.

Incorporation of biological monitoring and numeric biological criteria by states into water quality standards has been a slow process. As of 1999, only two states had incorporated explicit monitoring and biocriteria into their statutes defining water quality standards, and fifteen others were at various stages of developing and codifying biological parameters into their

standards (Karr and Chu, 1999). At the urging of the National Academy of Sciences (2001) several additional states have more recently picked up momentum in this regard, but most cite shortages of federal, state and local sources of funding along with staffing deficiencies as impeding their progress. The long turn-around time for comprehensive biological surveys is also cited as contributing to the problem. Development of Rapid Bioassessment Protocols (e.g., Plafkin *et al.* 1989, Barbour *et al.* 1999) have represented attempts to reduce both costs and turn-around times for benthological surveys.

Previously, I was involved with the U. S. EPA Region VII in Kansas City to develop an alternative protocol using Chironomidae (Ferrington, 1987) as the basis for a Rapid Bioassessment Protocol (RBP). Our intent was to develop a protocol that could be employed in highly disturbed urban streams and rivers where the more standard procedures and metrics, at that time being developed by Plafkin *et al.* (1989), showed little promise for application due to the lack of biodiversity of Plecoptera, Ephemeroptera and Trichoptera. Chironomidae (Diptera) often predominate in highly disturbed urban streams (Ferrington 1990, Ferrington *et al.* 1991), increasing in relative abundance and richness as the degree of pollutant stress increases. Barbour *et al.* (1999) subsequently revised some of the protocols to more effectively utilize Chironomidae in a few of the metrics, but the potential for their cost-efficient use remains largely underdeveloped. In this project I employed a methodology for cost-effective analysis of Chironomidae for a rapidly urbanizing area, the Vermillion River watershed, with the objective of providing data to be integrated into setting goals for TMDL's related to organic enrichment in the watershed.

Methods

Description of Methodology for Cost-effective Analysis of Chironomidae in the Vermillion River Watershed- In this project collections of surface floating pupal exuviae (SFPE) were used to generate information about chironomid communities at 10 sites longitudinally within the Vermillion River watershed. Since this monitoring technique is little known among and poorly understood by water quality managers in the United States, I provide a detailed description of the technique in the following paragraphs.

Although not widely used in ecological investigations in the United States, collecting SFPE is not a new approach for gathering information about Chironomidae communities. It was first suggested by Thienemann (1910), but only occasionally used in taxonomic and biogeographic studies (Thienemann 1954, Brundin 1966) or ecological studies (Humphries 1938) until more recently. During the last 35 years, however, there has been increasing use of pupal exuviae collections in chironomid studies. Reiss (1968) and Lehmann (1971) used collections SFPE to supplement their larval collections when investigating Chironomidae community composition. In Western Europe and England collections of SFPE have been used extensively for surface water quality monitoring (McGill *et al.* 1979, Ruse 1995a, b; Ruse & Wilson 1984, Wilson 1977, 1980, 1987, 1989; Wilson & Bright 1973, Wilson & McGill 1977, Wilson & Wilson 1983). In North America the methodology has been successfully used in studies of phenology (Coffman 1973, Boerger 1981, Wartinbee & Coffman 1976), diel emergence patterns (Coffman 1974), ecology and community composition (Blackwood *et al.* 1995, Chou *et al.* 1999, Ferrington 1998, 2000,

Ferrington *et al.* 1995, Kavanaugh 1988), microbial decomposition (Kavanaugh 1988), assessment of effects of point sources of enrichment (Coler 1984, Ferrington & Crisp 1989), non-point pesticide effects (Wright & Ferrington 1996), and effects of agricultural practices (Barton *et al.* 1995). In England and the United States SFPE collections have been used to study water and sediment quality (Ruse & Wilson 1984, Ruse *et al.* 2000, Ferrington 1993b), and used in Australia for measuring phenology (Hardwick *et al.* 1995) and effects of stream acidification on Chironomidae (Cranston *et al.* 1997). The following paragraphs briefly describe aspects of the methodology common to most of the above applications.

Chironomid larvae live in soft sediments or on rocks and interstitial materials in stream beds, where they generally attain densities of 1000 or more larvae per square meter in healthy stream systems (Coffman & Ferrington 1995), and often more than 30,000 larvae per square meter in organically enriched streams (Ferrington 1990). Upon completion of the larval life they attach themselves with silken secretions to the surrounding substrates and pupation occurs. When the developing adult matures the pupa frees itself from the silken chamber and swims to the surface of the water where the adult emerges from within the pupal skin (or exuvia). The exuvia fills with air and by virtue of an outer waxy layer of the cuticle (which has non-wettable properties) it remains floating on the water surface until bacteria begin to decompose the wax layer. Floating exuviae are concentrated by stream currents into eddy areas or into regions such as slack water areas downstream of rocks or points where riparian vegetation or fallen trees contact the water surface. By collecting exuviae from these "natural" collection points, one can rapidly evaluate the emergence of Chironomidae from a broad spectrum of microhabitats in the stream. Emergence frequencies are then calculated for all species in the SFPE sample.

Field collection of SFPE is accomplished by dipping an enameled pan into the water downstream of areas where pupal exuviae accumulate. Water, detritus and floating pupal exuviae flow in as one edge of the pan is dipped beneath the surface of the water. After the pan has filled with water, the contents are passed through a U.S. Standard Testing Sieve with aperture of 125 microns. Detritus and exuviae are retained by the sieve. The entire procedure of dipping and sieving is repeated until a large amount of detritus and exuviae is accumulated in the sieve. Contents of the sieve are then transferred to a sample jar and field preservative of 80% ethanol added, along with a sample label. Exuviae are sorted from detritus in the laboratory under 12X magnification to insure all specimens are found and removed. It has been my experience that 10 minutes of collecting provides sufficient sample size for impact assessments in streams moderately to severely impacted by organic enrichment in eastern Kansas, with samples often containing several hundred to a thousand or more exuviae. The SFPE protocol is accepted as a Standard Operating Procedure (SOP) and a Rapid Bioassessment Protocol for water quality investigations by Region VII of the U.S. Environmental Protection Agency (Ferrington 1987).

Recently published identification guides for identifying pupal exuviae by Coffman and Ferrington (1984, 1995), Wiederholm (1986), Wilson and McGill (1982) and Langton (1991) have facilitated more extensive use of the method and easier adoption by monitoring agencies.

Sampling Design: This project was designed as a one-year study and initiated in March 2005. Ten sample sites were selected after consultation with MCES and Dakota County scientists. Eight sites were selected to bracket areas known to have contrasting water quality conditions

upstream and downstream of WWTP effluent outfalls. The remaining sites were located at successively greater distances downstream of the Empire WWTP effluent outfall, since this stretch of river is expected to experience the greatest improvement in water quality after the planned upgrade is completed. Samples were collected at monthly intervals during ice-free period, as weather and discharge conditions permitted. This design generate 90 samples. Based upon my past research in urban streams (Ferrington and Crisp 1989, Ferrington 1990) and research in progress in Minnehaha Creek (currently funded by WRS) it was anticipated that 60-65 genera, representing 115-130 species with a variety of tolerances to Phosphorus concentrations, sedimentation, and differing dissolved Oxygen concentrations, would occur in the watershed.

Results to date

Sixty-eight genera and approximately 118 species of Chironomidae were collected across the ten sample sites. Seasonality of emergence was distinct, with a progression from Orthoclaadiinae dominating emergence in early spring, Tanytarsini in late spring and Tanypodinae/Chironomini species being most common in mid to late summer. Prodiamesinae and Diamesinae were only encountered at sites furthest downstream of each WWTP, and were most numerous early and late in the ice-free period. By contrast, Tanypodinae/Chironomini were only abundant at sites located closest to each WWTP.

One site in the upper portion of the Vermillion River was located approximately on kilometer downstream of a tributary that receives outflow from Rice Lake near Lakeville, MN. Chironomidae emergence at this site was strongly influenced by the tributary and emergence in late spring and early summer was dominated by species that are more common in lentic habitats. This site dried during late summer but was quickly re-colonized by both lentic and lotic species that began emerging within 5 weeks of resumption of stream flow. The resilience of chironomids contrasted strongly with other aquatic insects such as mayflies and caddisflies, which took considerably longer to recolonize and grow to maturity.

Preliminary modeling using PCA and CONOCO has been used to search for clusters of species that most closely reflect declining water quality associated with effluents of the WWTPs. Comparisons with similarly designed studies in urban streams of the Greater Kansas City area show that water quality of the Vermillion river is much higher and there is less modification of the Chironomidae community composition downstream of WWTP effluent outfalls.

Ongoing work

We are continuing to refine our identification of species collected during this study and expect that more than 70 genera and 130 species will ultimately be discovered in the Vermillion River. Future modeling attempts will be done to quantify the extent of distance downstream of effluent outfalls that are impacted. More effort will be directed to resolving the temporal patterns of resilience of chironomids after loss of surface flow.

Summary of findings

(1) The longitudinal and temporal patterns of Chironomidae emergence have been documented for 118 species occurring in 68 genera.

- (2) Relative abundances of taxa have been determined and site-specific patterns relative to WWTP effluent outfalls have been quantified.
- (3) Preliminary modeling shows that effluents are only having minimal, but distinctly detectable, influence on chironomid community composition.
- (4) Chironomid community composition is most strongly modified at sites closest to WWTP effluent outfalls.
- (5) Outflow from Rice Lake that forms a tributary to the upper portion of the Vermillion River has a substantial modifying effect, shifting community structure strongly to species characteristic of lentic habitats.
- (6) Resilience to loss of surface flow, as measured by recolonization and time to emergence, is very high. Chironomidae that rapidly recolonize and grow to maturity after resumption of surface flow consist of a mixture of both lentic and lotic species.

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List of publications & presentations resulting from this project

None to date.

Description of student training provided by project:

Name: Adam W. Sealock

Program: WRS

Degree being sought: Masters of Science

Name: Brenda Asmus

Program: WRS

Degree being sought: Master of Science

Name: R. Will Bouchard

Program: Entomology

Degree being sought: Ph.D.

Name: Moriya Rufer

Program: Entomology

Degree being sought: Master of Science

Water Quality Monitoring Strategy Based on Agroecoregion Boundaries in the Minnesota River Basin

Basic Information

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Publication

Water Quality Monitoring Strategy Based on Agroecoregion Boundaries in the Minnesota River Basin

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Introduction

To better direct the funding of water quality monitoring and watershed remedial programs a better understanding of the relative importance of the factors associated with an elevated risk of water quality impairment in agricultural watersheds is needed. These factors include topographic slope steepness, the density and size of animal feedlots, stream channel gradient, watershed storage, stream channel density, and precipitation intensity. A land classification system developed in Minnesota by Hatch et al. (2001) incorporates many of these factors into areas termed agroecoregions. These agroecoregions are delineated based on data related to soil internal drainage, surficial geology, climatic patterns, topographic slope steepness, and land use. They represent relatively homogeneous areas and each have distinctly differing potentials for producing non-point source pollution from agricultural landscapes. Birr and Mulla (2002) found that this Minnesota agroecoregion framework was effective at characterizing regional lake water phosphorus concentration trends. The objective of this study is to combine the agroecoregion land classification system with information about animal feedlot density to assess whether agroecoregions provide a valid means to direct future monitoring efforts and remedial practices on a minor watershed scale.

Study Area

Four agricultural watersheds were monitored in south-central Minnesota (Table 1). They are located within the Steeper Till agroecoregion. Topography within the Steeper Till agroecoregion is generally steep in slope and prone to surface runoff (Table 2). In the Steeper Till agroecoregion, the soils are mixed between poorly and more moderately drained soils and surface water ponding is limited to poorly drained soils. The soil associations found within the Steeper Till agroecoregion watersheds are predominantly Webster, Clarion, and Nicollet.

Table 1. Site descriptions of the four watersheds monitored in south-central Minnesota (MN)

Site Name	Area (ha)	Mean Slope	Ag Land (%)	Outlet Description
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		(%)		
NEofNwUlm	2971	4.7	94	County Ditch #38
NWofTrvse	1839	2.7	96	Rogers Creek & County Ditch #78
Hueskmp1	1160	2.9	>90	Huelskamp Creek & County Ditch #85
Hueskmp2	1131	3.2	>90	Huelskamp Creek & County Ditch #80

The predominant animal type within each of the four watersheds monitored is swine, ranging between 67 and 81% of the total animal units (AUs) permitted. Dairy animals are the second most prominent animal type within four of the four watersheds monitored, ranging from 9 to 23% of the total AUs permitted. The AU density of permitted animals observed ranges from 0.74 to 5.87 AU ha⁻¹ across all four monitored watersheds (Table 2).

Table 2. Livestock population within the five watersheds monitored in south-central MN

Site Name	Permitted Animal Units (AUs)	AU Density (AU/ha)	Swine (AUs)	Beef (AUs)	Dairy (AUs)	Chicken (AUs)
NEofNwUlm	6683	4.13	5058	103	1522	0
NWofTrvse	8825	5.87	6687	423	755	960
Hueskmp1	2122	1.83	1420	327	375	0
Hueskmp2	834	0.74	674	17	143	0

Methods

Water Quality Data

Each of the four watersheds were equipped with monitoring equipment at the watershed outlet throughout the 2004 and 2005 summers. ISCO 6700 autosamplers were installed along with ISCO 730 bubbler modules and ISCO 750 area velocity probes to monitor the stage of the stream at the sampling location. At two of the sites, Campbell Scientific Inc. DB1 nitrogen gas liquid level sensors were used to monitor the stream stage. The autosamplers were programmed to initiate sampling during rainfall events when a significant increase in stream stage occurred (>3cm). Samples were collected at 15-30 minute intervals throughout the rising limb of the hydrograph and 1-2 hours intervals throughout the falling limb. Base flow grab samples were also taken at approximately two week intervals. The monitoring was conducted during ice-free periods extending from mid-May through the end of October.

The samples were typically collected within 24 hours of each storm event and refrigerated at 4°C until analysis. Samples were analyzed for total suspended solids (TSS), dissolved phosphorus (DP), total phosphorus (TP), and nitrate nitrogen (NO₃-N) using standard methods.

A rating curve was built for each site in accordance with U.S. Geological Survey (USGS) procedures (1984). Stage data were collected at 5 minute intervals with

Campbell Scientific Inc. DB1 nitrogen gas liquid level sensors and ISCO 730 bubbler modules. Velocity readings were taken with ISCO 750 area-velocity probes and wading measurements were taken with a standard AA current meter. All of these data were utilized in the construction of rating curves for the various sites.

The loads for each watershed were determined by integrating the product of each analyte (TSS, DP, TP and NO₃-N) concentration and the five minute flow volume. Concentrations were assigned to each five minute interval by using the concentration from one sample to the next. Annual loads were calculated for each year by summing the total calculated load discharge from each watershed between May 21st and October 31st.

To compare the analyte loads discharged from each watershed the annual loads are normalized by the watershed area and cm of runoff per watershed hectare. To do this the following equation was used:

$$N_y = T_y / (T_r / W_a)$$

N_y = Normalized yield (kg/ha/cm runoff)
 T_y = Total analyte yield (kg)
 T_r = Total runoff (cm)
 W_a = Watershed area (ha)

Results

Pollutant Loads

The mass of each analyte load measured annually and the discharge of water from each watershed throughout the monitoring period are shown in Table 3.

Table 3. The average analyte load and volume of water discharged from each watershed between May 21st and October 31st of each monitoring year

Site Name	Discharge (m ³ /s)	TSS (kg)	Total P (kg)	Dissolved P (kg)	Nitrate N (kg)
2004-2005 Average					
NEofNwUlm	0.146	116,575	733	432	54,618
NWofTrvse	0.142	60,465	863	521	105,267
Hueskmp1	0.085	158,379	1188	565	65,077
Hueskmp2	0.138	252,059	602	212	35,238

The rainfall measured at the mouth of each watershed during the 2004 monitoring period ranged from 50.44 cm (19.86 in) to 63.60 cm (25.04 in). During the 2005 monitoring season all four sites experienced a smaller total amount of rainfall ranging from 40.64 cm (16.00 in) to 61.62 cm (24.26 in). Averaging these rainfall totals over both years of the study, the range between the highest and lowest recorded rainfall equals 15.29 cm (6.02 in).

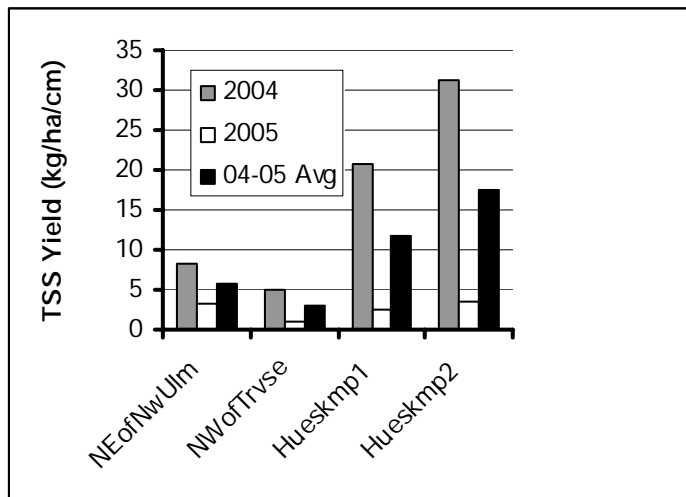
Total Suspended Solids (TSS) Yields

The normalized TSS yields measured from each watershed were higher in 2004 than in 2005, at least partly due to the higher amounts of rainfall experienced in the earlier year (Fig. 2). At 'Huelskmp1', and 'Huelskmp2' the differences between the two years appear large, while the remaining two sites, 'NEofNwUlm' and 'NWofTrvse', do not look quite as variable. Looking at the average normalized TSS over both years of monitoring both 'Huelskmp1' and 'Huelskmp2' exported the most sediment. The mean

topographic slopes observed within each watershed do not correlate closely with the TSS yields measured from each watershed. The steepest watershed, 'NEofNwUlm' exported the second lowest average TSS yield.

The average discharges measured annually (Table 3), as well as over both years, also do not closely relate to the TSS yields observed. The lowest TSS yields were observed at the 'NEofNwUlm' and 'NWofTrvse' sites, which had the highest average discharges. The lowest average discharge observed each year occurred at the 'Huelskmp1' site. However this site yielded the second largest TSS yield in 2004 and the third largest TSS yield in 2005.

Figure 2. Normalized TSS yield measured at the mouth of each watershed throughout the 2004 and 2005 monitoring periods.



Discharge per unit area is more closely correlated with TSS yields than average discharge. The largest discharge per unit area ($12 \times 10^{-4} \text{ m}^3/\text{s}/\text{ha}$) occurs in the 'Hueskmp2' watershed, which has the highest TSS yield. The lowest discharge per unit area ($0.5 \times 10^{-4} \text{ m}^3/\text{s}/\text{ha}$) occurs in the 'NEofNwUlm' watershed, which has the second lowest TSS yield. Discharges per unit area in the other two watersheds are roughly $0.75 \times 10^{-4} \text{ m}^3/\text{s}/\text{ha}$, but the TSS yield for 'NWofTrvse' is much smaller than the yield for 'Hueskmp1'.

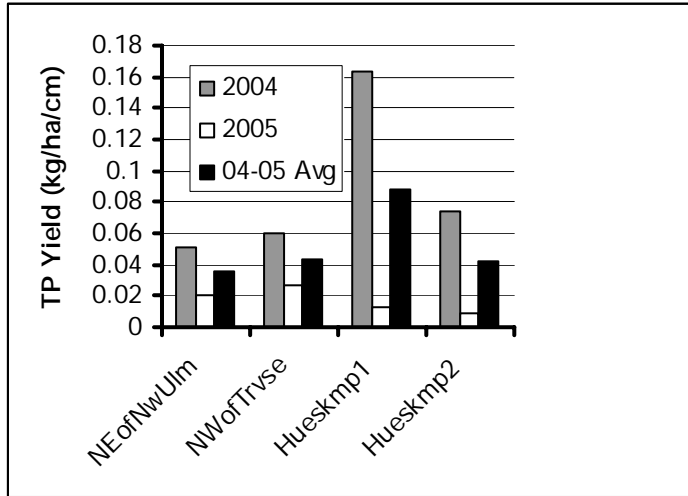
The influence of animal agriculture on the TSS load measured from the monitored watersheds may offer the best explanation for differences in observed TSS loads across watersheds. Repeated applications of manure to cropland soils build up the organic content of the soils. In addition, manure improves soil structure and soil aggregates become more stable and can better resist erosive processes (Gilley and Risse, 2000). The higher density of animal units observed in the 'NEofNwUlm' and 'NWofTrvse' watersheds and the lower TSS loads observed at these two sites compared to the 'Hueskmp1' and Hueskmp2' sites suggests that the applications of manure occurring within the former watersheds may be partially responsible for reductions in TSS loads.

Phosphorus Yields

The Total Phosphorus (TP) yields measured at each site were reasonably consistent across both years at the 'NEofNwUlm' and 'NWofTrvse' sites. However the

'Huelskmp1' and 'Huelskmp2' TP yields varied significantly over the two years that monitoring data was collected. The trend in total rainfall observed at each site closely matches the trends in TP yields across years.

Figure 3. Normalized Total Phosphorus (TP) yield measured at the mouth of each watershed throughout the 2004 and 2005 monitoring periods.



The average TP yields observed in each watershed are again apparently unrelated to differences in average topographic slope. For example, the steepest slopes occur in the 'NEofNwUlm' watershed, and this watershed has the lowest average TP yield. The 'Hueskmp1' watershed has the highest average TP yields and the second flattest watershed slopes.

As with TSS yields, there appears to be an inverse relationship between animal unit (AU) densities observed within each watershed and the normalized TP yields measured. For example, the highest animal densities occur in the 'NWofTrvse' and 'NEofNwUlm' watersheds, which also have the lowest average TP yields. The second lowest animal densities occur in the 'Hueskmp1' watershed, which also has the highest TP yields. Since TP yield is dominated by particulate phosphorus which is transported on eroded sediment, the similarities in relationships between animal density and either TSS or TP yields are reasonable.

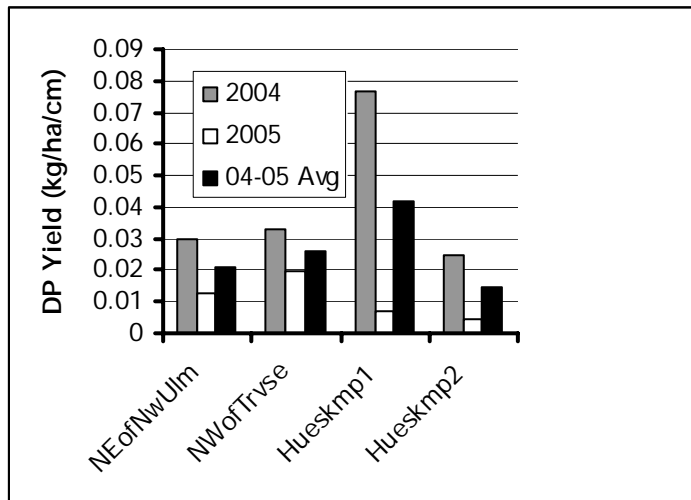
In view of the relationships between TSS and TP, it is instructive to compare the ratio of TP to TSS observed at each site relative to the animal density within the watershed (Table 4). The larger this ratio is the more TP is being measured relative to the amount of TSS discharged from each watershed. The average ratio of TP:TSS (0.012) observed at the sites with higher animal densities appears larger than the average ratio (0.004) at the sites with lower animal densities. Repeated manure applications may have built up the phosphorus content of the soil within these watersheds with higher animal densities, resulting in higher concentrations of P in runoff and eroded sediment relative to the sediment transported to the stream in watersheds with low animal densities.

Table 4. Total P to TSS normalized annual load ratios measured from each watershed over both years of monitoring.

Site Name	TP: TSS (kg/ha/cm runoff)			AU Density (AU/ha)
	2004	2005	04-05 Average	
NEofNwUlm	0.006	0.006	0.006	4.13
NWofTrvse	0.012	0.024	0.018	5.87
Hueskmp1	0.008	0.005	0.006	1.83
Hueskmp2	0.002	0.003	0.0025	0.74

Dissolved phosphorus (DP) yields across the four watersheds were not closely related to differences in animal density. For example, the 'Hueskmp1' and 'Hueskmp2' watersheds had the highest and lowest average DP yields but the lowest animal densities. In contrast, however, the ratios of DP:TP were closely related to animal density. The ratios of DP:TP were larger in the 'NEofNwUlm' (0.59) and 'NWofTrvse' (0.6) watersheds (having higher animal densities) than the corresponding ratios in the 'Hueskmp1' (0.47) and 'Hueskmp2' (0.35) watersheds. This difference indicates that the TP yields measured at the mouth of the 'Hueskmp' sites are made up of less DP than at the other two sites. The remaining P measured is assumed to be primarily particulate phosphorus (PP) bound to soil particles transported in stream flow. Again, it appears that watersheds in which large amounts of animal manure are applied to the land help reduce erosion and particulate phosphorus, while increasing the dissolved phosphorus relative to watersheds with lower animal densities.

Figure 4. Normalized Dissolved Phosphorus (DP) yield measured at the mouth of each watershed throughout the 2004 and 2005 monitoring periods.



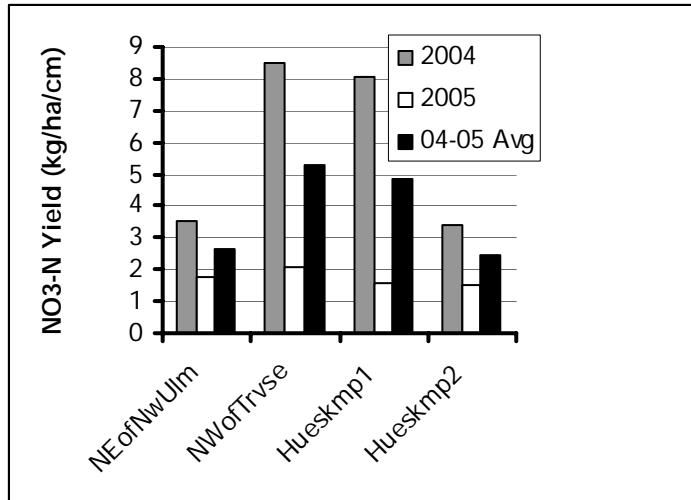
Nitrate-Nitrogen (NO₃-N) Yields

The normalized nitrate-nitrogen (NO₃-N) yields measured at each site located within the Steeper Till agroecoregion on average appear quite consistent across the four sites monitored (Figure 5). The average NO₃-N yield measured at these sites was 2.70 kg/ha/cm runoff.

The predominance of animal agriculture within the monitored watersheds correlates to some extent with the NO₃-N loads that were measured. The three sites

demonstrating the highest concentrations of animal units ('NEofNwUlm', 'NWofTrvse', and 'Huelskmp1') also reflect the largest average loads of NO₃-N measured from each watershed. The 'Huelskmp2' average NO₃-N load is less than those observed in the watersheds with much larger populations of livestock. The extremely close proximity of this watershed to 'Huelskmp1', may offer some explanation for this. A portion of the manure produced within the 'Huelskmp1' watershed is very likely applied within the 'Huelskmp2' watershed.

Figure 5. Normalized nitrate-nitrogen (NO₃-N) yield measured at the mouth of each watershed throughout the 2004 and 2005 monitoring periods.



Of perhaps greater importance for NO₃-N yields than animal density, however, is watershed slope. The steepest watersheds 'NEofNwUlm' and 'Hueskmp2' had the lowest nitrate yields, while the flattest watersheds 'NWofTrvse' and 'Hueskmp1' had the highest nitrate yields. Flatter watersheds tend to have more poorly drained soils and a greater density of subsurface tile drains than steeper watersheds. Nitrate is transported to surface waters through these subsurface tiles, so it is reasonable for flatter watersheds with a greater density of subsurface tile drains to transport more nitrate-N than steeper watersheds.

Conclusions

This study focused on differences in water quality across four watersheds located within the Steeper Till agroecoregion. Watersheds differed primarily in slope steepness and animal density. In general, total suspended solid (TSS) yields were lower in watersheds with higher densities of animal livestock, presumably because of the beneficial impacts of land applied manure on soil structure and infiltration. Discharge per unit area also explained some of the differences in TSS yields across watersheds, with higher discharges being associated with higher TSS yields. Total phosphorus (TP) yields were also inversely related to animal density, with lower TP yields in watersheds with higher animal densities. This is probably due to the reduction in erosion and particulate phosphorus that occurs in areas receiving land applied manure. However, the ratio of TP:TSS was greater in watersheds with higher animal densities, indicating that sediment eroded from watersheds with higher animal densities is enriched in phosphorus relative to

sediment from watersheds with lower animal densities. There were no effects of animal density on dissolved phosphorus (DP) yields. However, the ratio of DP:TP was greater in watersheds with higher animal densities than in watersheds with lower animal densities, indicating that there is a greater propensity for loss of DP than TP per unit of TP lost in watersheds with higher animal densities. Nitrate-N yields varied across watersheds in response to slope steepness, with flatter watersheds having higher nitrate yields. This is probably due to greater densities of subsurface tile drainage in flatter watersheds with more poorly drained soils. Nitrate-N yields were also somewhat related to differences in animal density, with higher yields in watersheds with a higher density of livestock.

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Hatch, L.K., A.P. Mallawatantri, D. Wheeler, A. Gleason, D.J. Mulla, J.A. Perry, K.W. Easter, P. Brezonik, R. Smith, and L. Gerlach. 2001. Land Management at the major watershed – agroecoregion intersection. *J. Soil Water Conservation*. 56:44-51.

Birr, A.S. and D.J. Mulla. 2002. Relationship between lake and groundwater quality patterns and Minnesota agroecoregions. *Hydrological Sci. Tech.* 18(1-4):31-41.

List of publications and presentations resulting from this project

None yet, results just summarized for first time a few months ago.

Description of student training provided by project:

Birr, A. 2005. Paired watershed studies for nutrient reductions in the Minnesota River Basin. Ph.D. Dissertation. Water Resources Science. College of Agricultural, Food and Environmental Sciences. Univ. Minnesota. St. Paul, MN. 169 pp.

Stuwe, L. 2006. Agricultural nitrogen and phosphorus mass-balances in south-central Minnesota. M.S. Thesis. Water Resources Science. College of Agricultural, Food and Environmental Sciences. Univ. Minnesota. St. Paul, MN. 169pp.

Assessing the ecotoxicology of alkylphenol mixtures across the aquatic food chain

Basic Information

Title:	Assessing the ecotoxicology of alkylphenol mixtures across the aquatic food chain
Project Number:	2005MN147G
Start Date:	9/1/2005
End Date:	8/31/2007
Funding Source:	104G
Congressional District:	6
Research Category:	Not Applicable
Focus Category:	Water Quality, Toxic Substances, Ecology
Descriptors:	None
Principal Investigators:	Heiko L Schoenfuss, Larry B Barber, Matthew Julius

Publication

Assessing the Ecotoxicology of Alkylphenol Mixtures Across the Aquatic Food Chain

Principal investigators

Heiko L. Schoenfuss PhD., Department of Biological Sciences, St. Cloud State University; Matthew L. Julius PhD., Department of Biological Sciences, St. Cloud State University; and Larry B. Barber PhD., Water Resources Division, US Geological Survey, Boulder, Colorado.

Research Assistants

KJ Grove, JK Koch, C Gamble, N. Jahns, R. Cediell, Department of Biological Sciences, St. Cloud State University

Start date: 9/1/2005

End date: 8/31/2007

Executive summary

Alkylphenol polyethoxylates represent a surfactant class of chemicals that has been detected in most anthropogenically altered surface waters in Europe and North America. The documented estrogenic potential of these compounds linked with their ubiquitous presence in the aquatic environment warrant a close examination of the effects of these compounds on the aquatic food chain. In this study, we explore the effects of alkylphenols as singularly compounds and in mixtures representing their common state in treated wastewater effluents on several trophic tiers of the aquatic food chain. Preliminary findings to date indicate the degrading effects of alkylphenols on the primary producer community, especially on diatoms which represent the preferred food source of many larval and fingerling fishes. In addition, we have found that diatoms are more sensitive to alkylphenolic compounds than they are to the known potent estrogen 17 β -estradiol suggesting that the effects of alkylphenolic compounds might disrupt differing pathways at subsequent tiers of the trophic cascade. Ongoing exposure experiments of daphnia magna and fathead minnows will further elucidate the effects of alkylphenolic compounds on several tiers of the trophic cascade. Finally, we have established protocols to link these tiers through feeding trials in which exposed diatoms will be pelleted and fed to daphnia and fathead minnows to assess the effects of alkylphenol exposure across the aquatic trophic cascade.

Introduction & research objectives

Alkylphenols, a group of compounds used in large quantities as industrial and household surfactants, are ubiquitously present in treated wastewater effluents which serve as their portal into the aquatic ecosystem. Alkylphenols are known to bind to the estrogen receptor of mammalian cells and disrupt the homeostasis of the internal milieu of the organism. Environmental estrogens such as alkylphenols are known to disrupt normal endocrine hormones that are central to maturation and reproduction in fishes, and the ubiquitous presence of these biologically active compounds in surface waters should be of environmental and human health concern. To date, alkylphenol studies have focused on 4-nonylphenol, the metabolic product of both aerobic and anaerobic microbial degradation of higher-chained alkylphenols and the US EPA has recently proposed effluent emissions criteria for this compound. However, mixtures of

nonylphenol and higher chained alkylphenols are found routinely in effluents and their combined action is entirely unknown. In this study, we propose to examine the effects of alkylphenol mixtures on three tiers of the aquatic food chain: the primary producer community (diatoms), a primary consumer (daphnia), and a vertebrate near the top of the food chain (the fathead minnow). We furthermore will link all three tiers through feeding trials to examine the effects of alkylphenol mixtures on the aquatic food chain. The present study proposes three objectives to determine the relationship between alkylphenol contamination of surface waters and adverse organismal effects. These are (1) determine the effects of alkylphenol exposure on the reproductive success of three tiers of the aquatic food chain; (2) determine the impacts of alkylphenol mixtures across the food chain; and (3) to test the three assays at a field site known to discharge alkylphenols.

To date we have completed the first round of fish and diatom exposures with alkylphenol mixtures. We have been able to demonstrate that the combined effects of alkylphenols exceeds that of individual alkylphenols in the fathead minnow and have also established that diatoms serve as sensitive indicators of biological disruption caused by the presence of alkylphenol mixtures. Furthermore, we have established protocols for the daphnia magna exposure and the linkage of these three tiers of the food chain through pelleting large quantities of alkylphenol exposed diatoms for larval fathead minnow feeding. Finally, we are currently exposing fathead minnows to undiluted sewage effluent and larval fathead minnows to a graded series of diluted effluents to determine whether the effects of alkylphenol mixtures can account for adverse responses in treated wastewater effluent exposed larval and adult fathead minnows. In summary, we are well underway to complete all proposed components of the National Institute of Water Resources funded study.

Methodology & preliminary findings.

We have completed several rounds of diatom exposures (*M. varians*) to graded concentrations of 4-nonylphenol (NP) singularly and to mixtures of alkylphenolic compounds (Table 1) including NP, nonylphenol-1-ethoxylate (NP1EO), nonylphenol-2-ethoxylate (NP2EO), nonylphenol-1-carboxylate (NP1EC), and nonylphenol-2-carboxylate (NP2EC). In addition, we exposed diatoms to 17 β estradiol, a compound with known endocrine disrupting activity that served as a reference exposure for this study.

Table 1: Concentrations used in *M. varians* exposures for 4-nonylphenol (4-NP), 17- β estradiol (E2) and the alkylphenol mixture (AP).

Treatment	Low Exposure Concentration (g/L)	Medium Exposure Concentration (g/L)	High Exposure Concentration (g/L)
17 β estradiol	3	30	300
4-nonylphenol	2	20	200
Alkylphenol mixture*	74.5	373	746

* sum of several alkylphenolic compounds (see Table 2).

Table 2: Alkylphenol compounds detected in the Metropolitan treated wastewater effluent (St. Paul, MN) and their environmental concentrations, used for determining experimental dose values.

Compound	Concentration (g/L)
NP	2.11
NP1EO	3.536
NP2EO	6.987
NP1EC	25.201
NP2EC	33.618
SUM	71.5

For the diatom exposures, monocultures of *Melosira varians* were grown in sterile WC media, then exposed to pre-determined test chemical concentrations and incubated in diurnal growth chambers with a 12:12 light:dark cycle for a period of ten days. Procedures were as follows:

100 ml of homogenized culture aliquots were added to 900 ml of sterile media and allowed a period ≥ 24 hours to acclimate. Due to its affinity for binding to glass, sterile polystyrene cell tissue rollers were used in the case of the 4-nonylphenol exposures, and 2 liter glass Florence flasks were used for the estradiol trials. Treatments consisted of control, low, medium and high exposure concentrations. Once treated, samples were taken on day one for cell count and chlorophyll-a analysis. Exposed cultures were then allowed to grow for a period of ten days, after which samples were once again obtained for chlorophyll-a and cell count. The chlorophyll-a content of the cells was measured using a fluorometer and averages for each treatment group were determined in order to quantitatively assess diatom health. Elevated chlorophyll A tissue concentration indicate a stress-effect caused by the exposure. Data are presented for chlorophyll A concentrations of three exposure experiments: Exposure to a graded series of E2 (Figure 1), a graded series of NP (Figure 2), and a graded series of the alkylphenol mixture (Figure 3).

When chlorophyll A was analyzed for each experiment, the results indicated a very similar pattern with no statistical significance amongst the treatments. Low concentrations of E2 and 4-NP individually and 4-NP included in the mixture, elicited a dose response indicating enhanced performance that peaked at a medium dose and then decreased as the chemical concentration increased (Fig.'s 1-3). Furthermore, diatom response to the synthetic hormone 17 β estradiol indicated that it was not negatively affected on the same scale as vertebrate species. Current EPA protocol calls for E2 monitoring in the ng/L range, which does not appear to have a significant effect on diatom physiology. We determined that the dose responses of *M. varians* to the alkylphenol (AP) mixture and 4-NP alone were similar to each other rather than 4-NP being similar to E2.

In addition to the diatom exposures, we have also completed the first round of fathead minnow exposures to several alkylphenolic compounds (NP, NP1EO, NP2EO, NP1EC, NP2EC) singularly and in mixture. Male fathead minnows were exposed for 28 days and then allowed to compete directly with control males for reproductive opportunities. Upon completion of the seven day competitive spawning trial, all male fish were sacrificed and sampled for plasma vitellogenin, relative size of gonads and livers, and analyzed for histopathology. We are currently analyzing these data and will present them in the final technical report.

2. PUBLICATIONS RESULTANT FROM NIWR/WRC FUNDING

- Schoenfuss, H. L., J. T. Levitt, G. Van Der Kraak, and P. W. Sorensen. 2002. Ten week exposure to treated sewage effluent discharge has small, variable effects on reproductive behavior and sperm production in goldfish. *Environmental Toxicology & Chemistry* 21 (10): 2185-2190.
- Schoenfuss, H.L., Martinovic, D., and Sorensen, P.W. 2001. Effects of exposure to low levels of water-borne 17 β -estradiol on nest holding ability and sperm quality in fathead minnows. *Water Resources Update* 120: 49-55.
- Schoenfuss, H.L. 2003. The need for novel approaches in assessing the biological impact of biologically active compounds. *In: Proceedings of the 3rd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals In Water*, R. Masters (ed.), Minneapolis, MN, March 19-21, 2003, pp. 103-121.
- Martinovic, D., P.W. Sorensen and H.L. Schoenfuss. 2003. Low levels of water-borne estrogen suppress androgen levels and the ability to male fathead minnow to reproduce in the competitive spawning scenario. *In: Proceedings of the 3rd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals In Water*, R. Masters (ed.), Minneapolis, MN, March 19-21, 2003, pp. 125-133.
- Levitt, J. T., H. L. Schoenfuss, I. R. Adelman. 2001. Possible Effects of Endocrine Disrupting Compounds on Walleye, *Stizostedion vitreum*, near the Metro Sewage Treatment Plant, Saint Paul, MN. *Proceedings of the Second International Conference on Pharmaceutics and Endocrine Disrupting Chemicals in Water*, D. Guth (ed.), Minneapolis, MN, October 9-11, 2001, pp. 191-202.

3. STUDENTS SUPPORTED BY THE PROJECT

Kent Grove (MS expected Fall 2007)
Jason Koch (MS expected Spring 2007)
Nathan Jahns (MS expected Spring 2008)
Roberto Cediell (MS expected Summer 2007)
Carolyn Gamble (MS expected Fall 2007)
Angela Allen (undergraduate project Summer 2006)
Tim Loes (undergraduate project Summer 2006)
Bradley Sivanich (undergraduate project Summer 2007)

5. SEMINARS & PRESENTATIONS

Schoenfuss, HL* and TJ Bistodeau. 2006 Midwest SETAC Meeting, St. Cloud, MN March 20-22, 2006.

Gable, C*, A. Gikineh and ML Julius. 2006 Midwest SETAC Meeting, St. Cloud, MN March 20-22, 2006.

Allen, AK*, T Loes and HL Schoenfuss. 2006 Midwest SETAC Meeting, St. Cloud, MN March 20-22, 2006.

Grove, KJ*, RA Cediell and HL Schoenfuss. 2006 Midwest SETAC Meeting, St. Cloud, MN March 20-22, 2006.

Koch, JK*, M Minger and HL Schoenfuss. 2006 Midwest SETAC Meeting, St. Cloud, MN March 20-22, 2006.

Information Transfer Program

Student Support

Student Support					
Category	Section 104 Base Grant	Section 104 NCGP Award	NIWR-USGS Internship	Supplemental Awards	Total
Undergraduate	2	2	0	0	4
Masters	7	5	2	0	14
Ph.D.	4	2	0	0	6
Post-Doc.	1	0	0	0	1
Total	14	9	2	0	25

Notable Awards and Achievements

Arnold, William. 2005 Excellence in Review Award from Environmental Science and Technology.

Werner, Jeffrey. 2006 Paper Award from the American Chemical Society, Division of environmental Chemistry. Mr. Werner is a PhD student in the Water Resources Science program. Mr Werner is supported with Drs. William Arnold and Kris McNeill's grants.

Newman, Raymond. 2005. College of Natural Resources' Richard C Newman Art of Teaching award.

Werner, Jeffrey. 2006 Graduate Student Award in Environmental Chemistry from the American Chemical Society. Mr. Werner is a PhD student in the Water Resources Science program. Mr Werner is supported with Drs. William Arnold and Kris McNeill's grants.

Serieyssol, Claire, 2005 Dayton Wilkie Grant, University of Minnesota. Ms Serieyssol is a PhD student in the Water Resources Science program. She is supported with Dr. Ferrington's grant.

Publications from Prior Projects

- 2002MN2B ("Effects of riparian forest harvest on instream habitat and fish and invertebrate communities") - Articles in Refereed Scientific Journals - Laudon, Matthew C.; Bruce Vondracek; Julie K. H. Zimmerman, December 2005, Prey Selection by Trout in a Spring-Fed Stream: Terrestrial Versus Aquatic Invertebrates, Journal of Freshwater Ecology, Volume 20, Number 4, 723-734
- 2000MNBN-04 ("Assessing the Effects of endocrine disrupters from St. Paul Sewage treatment plant on sperm viability and testicular development in fish") - Other Publications - Schoenfuss, H.L. 2003. The need for novel approaches in assessing the biological impact of biologically active compounds. In: Proceedings of the 3rd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals In Water, R. Masters (ed.), Minneapolis, MN, March 19-21, 2003, pp. 103-121.
- 2000MNBN-04 ("Assessing the Effects of endocrine disrupters from St. Paul Sewage treatment plant on sperm viability and testicular development in fish") - Articles in Refereed Scientific Journals - Schoenfuss, H. L., J. T. Levitt, G. Van Der Kraak, and P. W. Sorensen. 2002. Ten week exposure to

treated sewage effluent discharge has small, variable effects on reproductive behavior and sperm production in goldfish. *Environmental Toxicology & Chemistry* 21 (10): 2185-2190.

4. 2000MNBN-04 ("Assessing the Effects of endocrine disrupters from St. Paul Sewage treatment plant on sperm viability and testicular development in fish") - Water Resources Research Institute Reports - Schoenfuss, H.L., Martinovic, D., and Sorensen, P.W. 2001. Effects of exposure to low levels of water-borne 17 β -estradiol on nest holding ability and sperm quality in fathead minnows. *Water Resources Update* 120: 49-55.
5. 2000MNBN-04 ("Assessing the Effects of endocrine disrupters from St. Paul Sewage treatment plant on sperm viability and testicular development in fish") - Conference Proceedings - Martinovic, D., P.W. Sorensen and H.L. Schoenfuss. 2003. Low levels of water-borne estrogen suppress androgen levels and the ability to male fathead minnow to reproduce in the competitive spawning scenario. In: *Proceedings of the 3rd International Conference on Pharmaceuticals and Endocrine Disrupting Chemicals In Water*, R. Masters (ed.), Minneapolis, MN, March 19-21, 2003, pp. 125-133.
6. 2000MNBN-04 ("Assessing the Effects of endocrine disrupters from St. Paul Sewage treatment plant on sperm viability and testicular development in fish") - Conference Proceedings - Levitt, J. T., H. L. Schoenfuss, I. R. Adelman. 2001. Possible Effects of Endocrine Disrupting Compounds on Walleye, *Stizostedion vitreum*, near the Metro Sewage Treatment Plant, Saint Paul, MN. *Proceedings of the Second International Conference on Pharmaceutics and Endocrine Disrupting Chemicals in Water*, D. Guth (ed.), Minneapolis, MN, October 9-11, 2001, pp. 191-202.
7. 2004MN82B ("Assessing the Exotoxicology of 4-Nonyphenol, A Ubiquitous Environmental Estrogen, in Two Organismal Bioassays") - Articles in Refereed Scientific Journals - Bistodeau, T.J., L.B. Barber, S.E. Bartell, R.A. Cedie, K.J. Grove, J. Klaustermeier, J.C. Woodard, K.E. Lee and H.L. Schoenfuss. In Press. Larval exposure to environmentally relevant mixtures of alkylphenolethoxylates reduces reproductive competence in male fathead minnows. *Aquatic Toxicology*.
8. 2004MN82B ("Assessing the Exotoxicology of 4-Nonyphenol, A Ubiquitous Environmental Estrogen, in Two Organismal Bioassays") - Other Publications - October 2004. Schoenfuss. Simple Questions - Complex Answers: Investigating the Effects of Alkylphenols on Aquatic Organisms. Invited Seminar, University of Nebraska, Omaha.
9. 2004MN82B ("Assessing the Exotoxicology of 4-Nonyphenol, A Ubiquitous Environmental Estrogen, in Two Organismal Bioassays") - Conference Proceedings - April 2005. Schoenfuss, H.L., T. Bistodeau, S. Bartell, J. Woodard, L. Barber, K. Lee, L. Zintek, A. Alwan, J. Lazorchack (2005) Concentration Dependent Effects of 4-Nonylphenol on Male Fathead Minnows in a Competitive Reproductive Assay. Oral Presentation at the Annual Meeting of the Midwest Division of the Society for Environmental Toxicology and Chemistry, Madison, WI, April 5, 2005.
10. 2004MN82B ("Assessing the Exotoxicology of 4-Nonyphenol, A Ubiquitous Environmental Estrogen, in Two Organismal Bioassays") - Conference Proceedings - April 2005. Bistodeau, T., R. Cediel, K. Groove, J. Klaustermeier, H. L. Schoenfuss (2005) Reproductive Consequences of Environmentally Relevant Exposures of Fathead Minnow Larvae to a Mixture of Alkylphenol Ethoxylates. Poster Presentation at the Annual Meeting of the Midwest Division of the Society for Environmental Toxicology and Chemistry, Madison, WI, April 5, 2005.
11. 2004MN82B ("Assessing the Exotoxicology of 4-Nonyphenol, A Ubiquitous Environmental Estrogen, in Two Organismal Bioassays") - Conference Proceedings - May 2005. Schoenfuss, H.L. (2005) Biological Effects of Biologically Active Compounds: Experimental Considerations in the Study of Endocrine Disrupting Chemicals. Symposium Presentation at the Annual Meeting of the American Oil Chemists Society, Salt Lake City, UT, May 3, 2005.
12. 2004MN82B ("Assessing the Exotoxicology of 4-Nonyphenol, A Ubiquitous Environmental

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