Report as of FY2007 for 2005MN147G: "Assessing the Ecotoxicology of Alkylphenol Mixtures Across the Aquatic Food Chain"

Publications

- 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. 2006. Larval exposure to environmentally relevant mixtures of alkylphenolethoxylates reduces reproductive competence in male fathead minnows. Aquatic Toxicology 79: 268-277.
  - Julius, M.L., Stepanek, J., Tedrow, O., Gamble, C. and H.L. Schoenfuss. 2007.Estrogen-receptor independent effects of two ubiquitous environmental estrogens on Melosira varians Agardh, a common component of the aquatic primary producer community. Aquatic Toxicology 85: 19-27.
  - Schoenfuss, H.L., S.E. Bartell, T.B. Bistodeau, R.A. Cediel, K.J. Grove, L. Zintek, K.E. Lee, and L.B. Barber. In Press. Impairment of the reproductive potential of male fathead minnows by environmentally relevant exposures to 4-nonylphenol. Aquatic Toxicology.

Report Follows

ASSESSING THE ECOTOXICOLOGY OF ALKYLPHENOL MIXTURES ACROSS THE AQUATIC FOOD CHAIN

FINAL REPORT, OCTOBER 2007

PROJECT NUMBER: 2005MN147G

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Abstract

This study was designed to elucidate the effects of alkylphenols singularly and in mixtures on different levels of the aquatic food chain. Among estrogenic endocrine disrupting compounds, alkylphenolic surfactants stand out due to their ubiquitous presence in anthropogenically altered surface waters and their occurrence in complex mixtures. Although the parent compounds (nonylphenol and octylphenol) of most alkylphenol polyethoxylates are orders of magnitude less estrogenic than 17ß-estradiol, they are also found in concentrations orders of magnitude greater then the natural estrogen in many treated wastewater effluents and receiving streams and rivers. In addition, the longer-chained alkylphenol polyethoxylates are altering the bioavailability of nonylphenol and octylphenol, thus potentially facilitating the uptake of the more potent parent compounds by aquatic organisms exposed to these alkylphenol mixtures. Furthermore, the chemical nature of surfactant raises the specter that organisms at different levels of the trophic cascade may experience differential effects that may be estrogen receptor independent (diatoms) or estrogen receptor mediated (daphnia and fathead minnow). As a consequence, we proposed to test the effects of an alkylphenol

polyethoxylate mixture, realistic in composition and concentration, on tiers of an abbreviated aquatic food chain: the primary producer community (diatoms); a primary consumer (*Daphnia magna*); and a secondary consumer (fathead minnow, *Pimephales promelas*). Our findings from the single organism mixture exposure experiments indicate a 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 potent estrogen 17b-estradiol suggesting that the effects of alkylphenolic compounds might disrupt receptor independent pathways at subsequent tiers of the trophic cascade (Julius et al. In 2007). As a consequence of the diatom exposure, the nutritional value of diatoms for *D. magna* and larval fathead minnows was greatly diminished. In contrast, we did not find adverse effects of alkylphenol exposure on daphnia at any environmentally relevant concentration. However, we did find a significant reduction in the reproductive ability of male fathead minnows exposed to environmentally relevant concentrations of 4-nonylphenol, the parent compound and most estrogenic alkylphenols (Schoenfuss et al. In Press). Furthermore, we established that alkylphenol mixtures have a more potent effect on larval fathead minnows than the parent compound nonylphenol alone (Bistodeau et al. 2006). Together, these results suggest that alkylphenols contribute significantly to the observed estrogenicity of many municipal wastewater effluents, adversely affect the primary producer community and may have profound reproductive effects on adult fishes.

Introduction & research objectives

Endocrine disrupting compounds have been detected in many anthropogenically altered surface waters in North America (Kolpin et al. 2002, EST 36: 1202-1211), and Europe (Desbrow 1998, EST 32: 1549-1558). Several classes of endocrine disrupting compounds are usually recognized, including natural/synthetic hormones (estrone, estradiol, ethynylestradiol), personal care products (i.e., the antimicrobial soap ingredient Triclosan) and alkylphenolic surfactants. The latter have been found almost ubiquitously in anthropogenically altered surface waters in part because they use is inherently water related. Alkylphenols are a group of compounds used in large quantities as industrial and household surfactants and have been found to be estrogenic (Hemmer et al. 2001, ETC 20:336-343). 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 hormone 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 magna*), and a vertebrate near the top of the food chain (the fathead minnow).

Methodology & Results.

We 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 17β 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 (mg/L)	Medium Exposure Concentration (mg/L)	High Exposure Concentration (mg/L)
17β 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 (mg/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 $\geq$ 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. In three exposure experiments using graded series of 17ß-estradiol (experiment 1: 2µg/L, 20µg/L, 200µg/L: experiment 2: 4µg/L, 40µg/L, 400µg/L; experiment 3: 8µg/L, 80µg/L, 800µg/L) treatments at or above 80µg/L consistently found statistically significant (one way ANOVA, $p<0.05$) increases in chlorophyll A: lipid ratio indicating a reduced nutritional value of diatoms for higher levels of the trophic cascade. Clearly these 17ß-estradiol concentrations are well beyond environmentally relevant concentrations and indicate that 17ß-estradiol does not adversely affect exposed diatoms. In contrast, three exposure experiments using series of alkylphenols (experiment 1: 2µg/L, 20µg/L, 200µg/L: experiment 2: 4µg/L, 40µg/L, 400µg/L; experiment 3: 8µg/L, 80µg/L, 800µg/L) found statistically significant (one-way ANOVA, $p<0.05$) increases in the Chlorophyll A ; lipid ratio at and above 40µg/L (Figure 1 a-d from Julius et al. 2007). This concentration of total alkylphenols has been exceeded in many treated wastewater effluents and indicates that the primary producer community is likely adversely affected by environmental concentrations of alkylphenols.

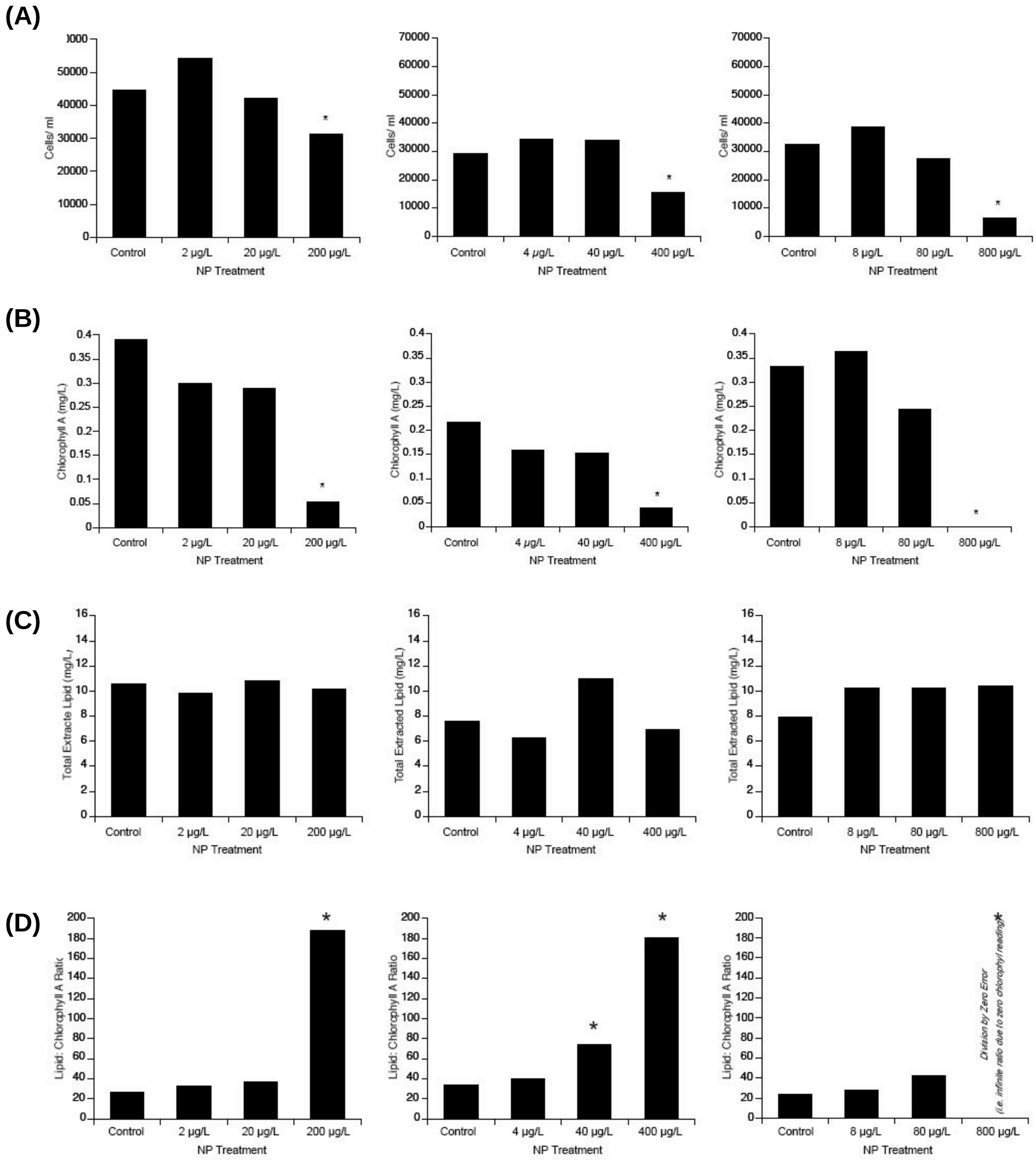


Figure 1. Alkylphenol exposures pf diatoms result in significant differences in cell counts (A); chlorophyll A concentrations (mg/L) (B); total lipid content (mg/L) (C); and lipid : chlorophyll ratios (D).

Daphnia magna were the model organism for our mid-food chain tier exposures. Cultures of daphnia were obtained from US EPA laboratories and maintained following standard US EPA procedures (US EPA 2002). Exposure experiment utilized neonates less than 24 hours. Neonates were carefully transferred individually into 50mL beakers (10 beakers - 10 daphnia per treatment) and exposed using a static renewal assay with 50% of each medium being exchanged daily for 21 days. Number of offspring was recorded for each beaker starting on day 6 (7 day life cycle from neonate to maturity) and continued through day 21. All offspring at day 21 were observed under a light microscope to determine presence of morphological abnormalities. A series of alkylphenol exposures up to 500µg NP/L medium were performed and did not result in significant effects on daphnia survival or developmental defects.

Fathead minnows, *Pimephales promelas*, were exposed in this study at two stages of development: as larvae and as mature adult males. Larval fathead minnows were exposed for 67 days post-hatch to either nonylphenol singularly at an environmentally relevant concentration, or to a mixture of alkylphenols representing in composition and concentration a major municipal wastewater effluent (Table 3, Bistodeau et al. 2006).

Table 3. Fish exposure experiments and nominal dosing concentrations for the alkylphenol (APE) mixture and nonylphenol (NP) only exposures and total NP concentrations utilized in each exposure.

Experiment Name	*Total NPE/OPE Concentration (μg/L)	Total NP (μg/L)
200% APE	148	4.2
100% APE	73.9	2.1
50% APE	38.1	1.1
NP	5	5.0

* APE mixture consists of NP, NP1EO, NP2EO, NP1EC, OP, OP1EO, and OP2EO in a ratio of 2.8:5.1:9.3:33.7:44.9:0.2:0.7:3.1 respectively.

Mortality was observed throughout the exposure and beyond the exposure where fish were reared in clear well water to maturity. Once mature, male fathead minnows from the exposures were paired with control males and allowed to compete for reproductive opportunities (Figure 2, Bistodeau et al. 2006).

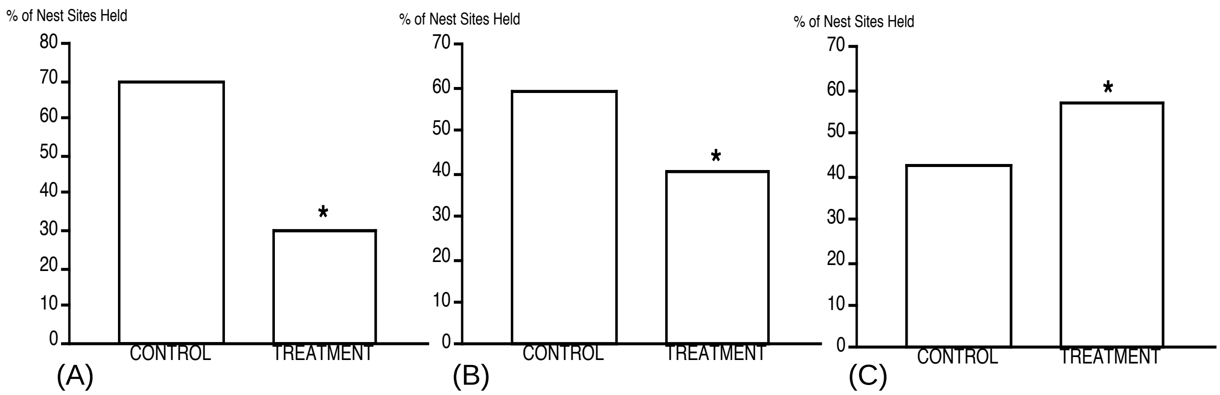


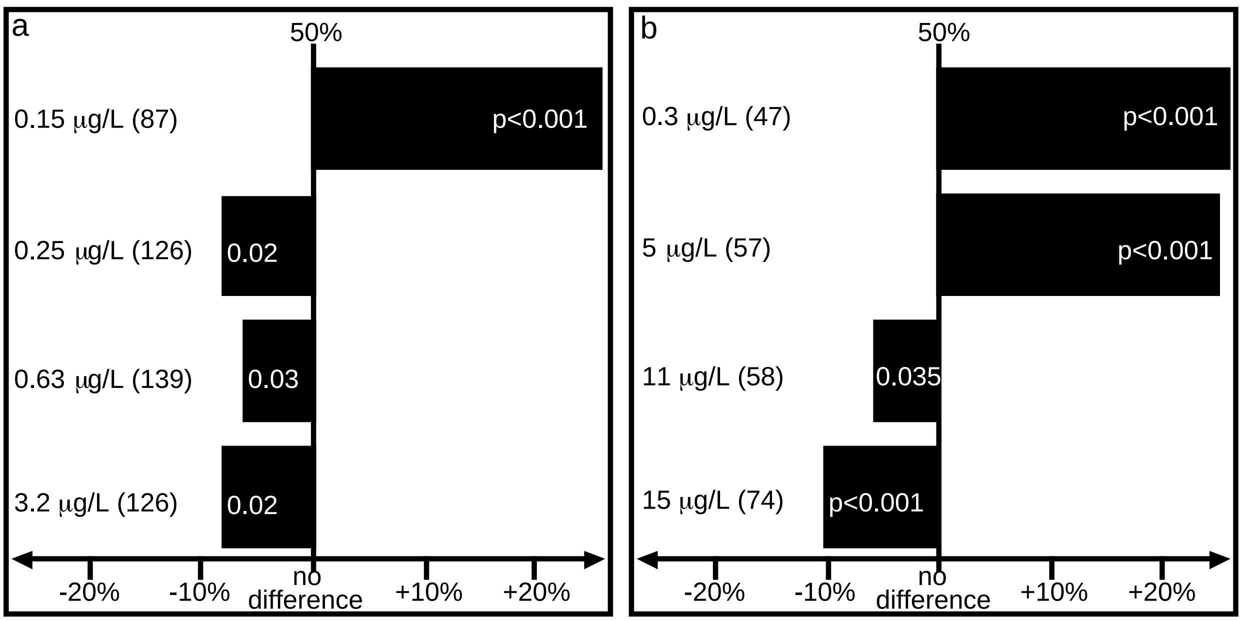
Figure 2. Competitive spawning results for (A) the 100% alkylphenol (APE) exposure experiment, (B) the 50% NPE/OPE exposure experiment, and (C) the 4-nonylphenol (NP) only exposure experiment. Bars indicate the percentage of nest sites held by either the control fish or the treated fish in each assay. (*p<0.005).

Our results indicate that reproductive competence is impaired in male fathead minnows that were exposed to the mixture for 67 days post-hatch at realistic concentrations (Fisher's Exact test; p<0.05). In addition, secondary sexual characters and the gonadosomatic index are significantly reduced when compared to control males (Student t-test, p<0.05). Even at a mixture concentration representing 50% the mixture concentration measured in the treated effluent, reproductive competence was significantly reduced. In contrast, nonylphenol alone had an excitatory effect on nest holding ability (Fisher's Exact test, p<0.05) that is likely the result of a priming effect of the low-concentration estrogenic compound. Detail results of the fathead minnow exposures are published in Bistodeau et al. (2006).

Our exposures of adult male fathead minnows further elucidated the effects of alkylphenols on exposed fishes and their reproductive potential. We exposed mature male fathead minnows for 28 days to graded concentrations of nonylphenol and followed the exposure first with the same competitive spawning scenario employed in the larval exposure experiment and then assessed vitellogenin induction, secondary sexual characters, and histopathology (Schoenfuss et al. In Press).

Exposure resulted in no discernable effects on the fishes gonadosomatic or hepatosomatic index. Secondary sexual characters were also not affected and vitellogenin induction was inconsistent. However, the competitive spawning assay revealed a marked decline in the exposed males ability to hold nests sites when nonylphenol concentrations exceeded 5 µg Np/L (Figure 3, Schoenfuss et al. In Press).

Figure 3. Nest holding ability of nonylphenol exposed male fathead minnows in direct



competition with control males in (a) experiment 1 and (b) experiment 2. [Numbers in parentheses indicates total observations of nest holding; p values are presented for each treatment, Fisher's exact test].

PUBLICATIONS RESULTANT FROM NIWR FUNDING

Bistodeau, T.J., L.B. Barber, S.E. Bartell, R.A. Cediel, K.J. Grove, J. Klaustermeier, J.C. Woodard, K.E. Lee and H.L. Schoenfuss. 2006. Larval exposure to environmentally relevant mixtures of alkylphenolethoxylates reduces reproductive competence in male fathead minnows. Aquatic Toxicology 79: 268-277.

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SEMINARS & PRESENTATIONS

(* indicates student presentation)

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

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

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

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

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

Schoenfuss, HL, Bistodeau, TJ 2006 Minnesota Water, Brooklyn Park, MN, October - oral presentation.

Schoenfuss, HL, Bistodeau, TJ, Society for Environmental Toxicology and Chemistry, Montreal, Canada, November 2006 - oral presentation.

Julius, ML, Society for Environmental Toxicology and Chemistry, Montreal, Canada, November 2006 - poster presentation.

Schoenfuss, HL. American Water Resources Association, Vail, Colorado, June 27, 2007 - Invited symposium presentation.

STUDENTS SUPPORTED BY THE PROJECT

Kent Grove (MS expected Fall 2007)

Jason Koch (MS expected Spring 2007)

Nathan Jahns (MS expected Spring 2008)

Roberto Cediel (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)

Josh Stepanek (undergraduate project academic year 2006-07)