Report for 2004MN82B: Assessing the Exotoxicology of 4-Nonyphenol, A Ubiquitous Environmental Estrogen, in Two Organismal Bioassays

- Other Publications:
  - 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.
  - 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.
  - 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.

Report Follows

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

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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 know to bind to the estrogen receptor of mammalian cells. Environmental estrogens such as APs 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. 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 preliminary 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. We are currently attempting to repeat these results in a second series of exposures and link the two assays by feeding exposed diatoms to fish larvae. If our preliminary results are confirmed in these experiments, then 4-nonylphenol might represent a substantial pollution source in many riverine systems in Minnesota and the US and might partially account for the decrease in some fish populations.

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-nonyphenol through the development of two organismal bioassays. These objectives are:

(1) Determining the impacts of 4-nonyphenol 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-nonyphenol 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-nonyphenol 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 μE·m-2·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).

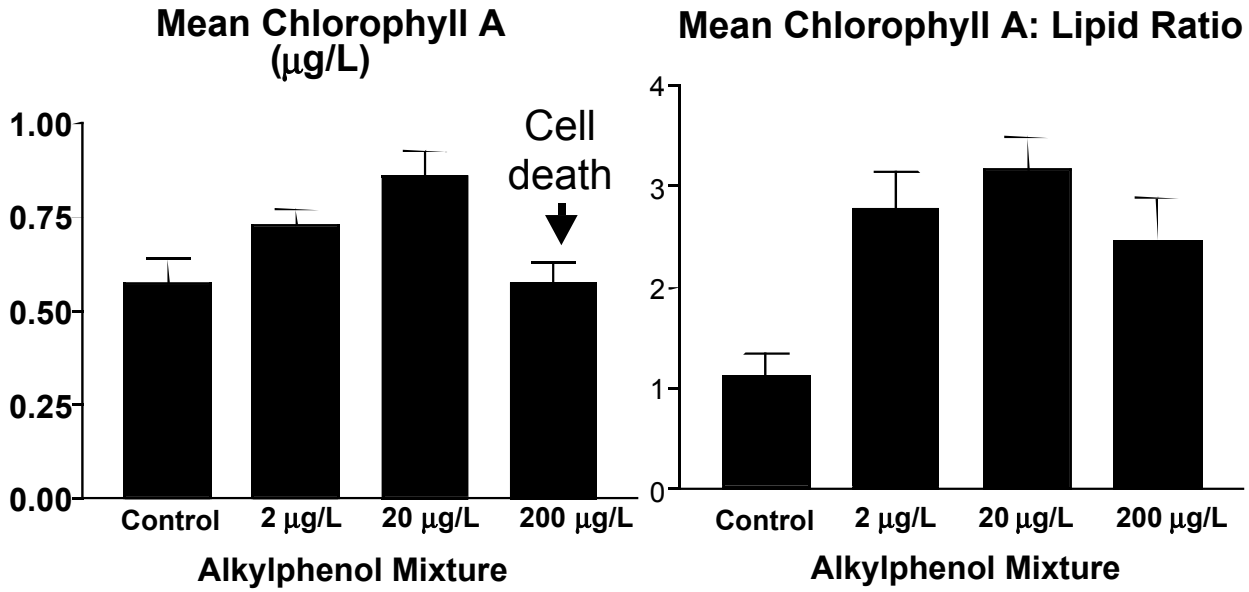
Juvenile fathead minnows (3 months old) were purchased from Kurt's Fisheries, PA in the spring of the first year of this study. Fish were held in flow-through 500L stock tanks under constant conditions (16:8 light:dark, 25-27ºC, fed *ad libitum*, 2L water/fish density) and reared to maturity (approx. 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 μg/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 to date

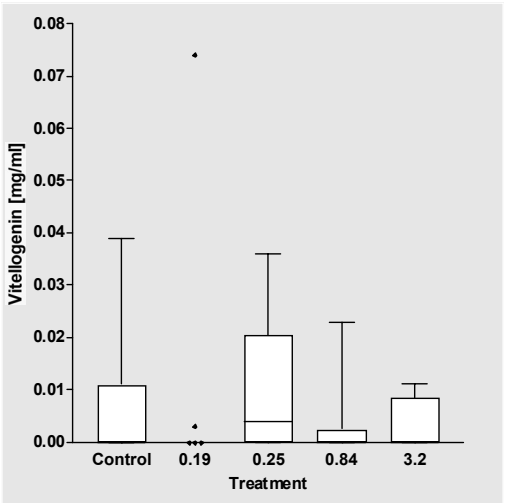
We were able to complete a full round of exposures of diatoms to a mixture of APs and to expose the fathead minnow to a series of 4-nonylphenol concentrations. Experimental protocols for both experiments were strengthened and subsequent exposure experiments are underway.

Diatom Exposures



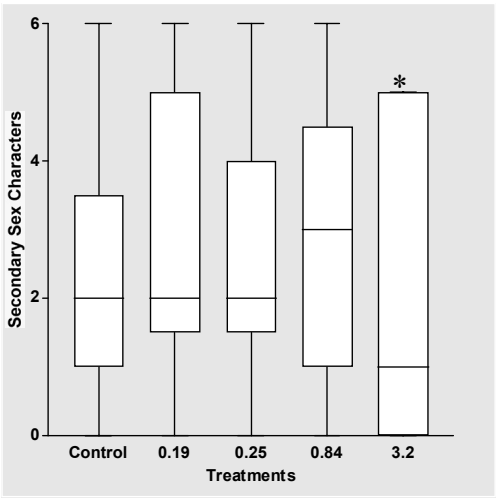
Exposure of the diatom culture to a mixture of alkylphenols that represents the APs component of a major municipal wastewater treatment plant results in an increase in chlorophyll A production (a sign of environmental stress in this organism) and a reduction in lipid content. The combined effects of 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 Minnow

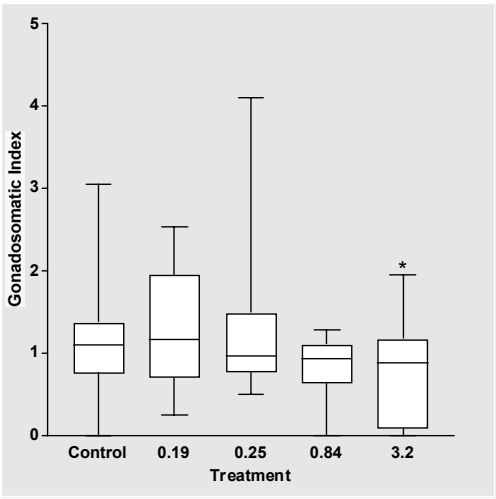


Vitellogenin [mg/ml]

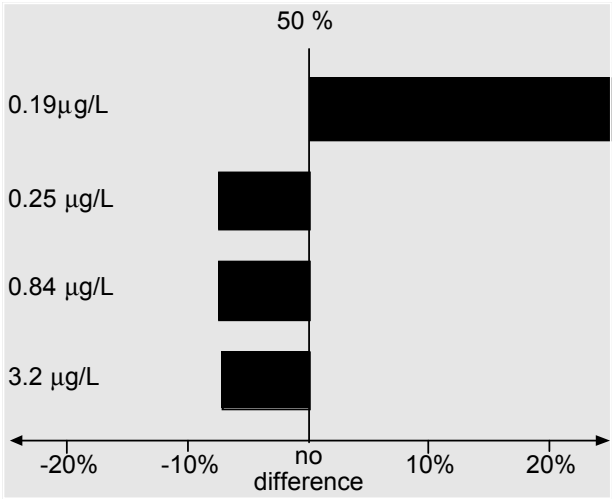
Secondary Sexual Characters



Gonadosomatic Index



Nest Holding Ability



Male fathead minnows exposed to 4-nonylphenol did not exhibit vitellogenin induction after a 28 day exposure. However, at the highest exposure concentration (3.2 µg/L), fish exhibited reduced secondary sexual characters, reduced testis size, and reduced nest holding ability.

Ongoing work

We are currently completing a second round of exposures and are extending our investigation into the effects of 4-nonylphenol on larval fathead minnows during sexual differentiation.

Summary of findings

To date it appears that a mixture of APs, including 4-nonylphenol at concentrations well below the US EPA proposed criterion for chronic exposure (5.9µg/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 this experiment (3.2µg/L), 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.

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

Drs. Schoenfuss, Barber, Julius, and Norris have submitted a US EPA STAR grant application 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.

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

Name: Jason Koch
Program: Department of Biological Sciences, St. Cloud State University, St. Cloud, MN
Degree being sought: Masters of Science

Name: Kent Groove
Program: Department of Biological Sciences, St. Cloud State University, St. Cloud, MN
Degree being sought: B.S., and Masters of Science