**Date of Final Report:** October 6, 2000

**Date of Work Program Approval:**
**Project Completion Date:** October 6, 2000
**1997 Subd. 14 (c)**
**1998 Ch. 401 Sec 3.**

**LCMR Work Program 1997**

I. **PROJECT TITLE:** Water quality indicators of endocrine disrupting chemicals.

**Project Manager:** Dr. Judy Helgen
**Affiliation:** Minnesota Pollution Control Agency, Water Quality Division
**Mailing address:** 520 Lafayette Road, St. Paul, Minnesota 55155
**Telephone Number:** 612-296-7240 **E-Mail:** Judy.Helgen@PCA.State.MN.US
**FAX:** 612-297-8683
**Web Page address:**

**Total Biennial Project Budget:** $250,000

**$ LCMR:** $250,000
**-LCMR Amount Spent: $250,000**
**=LCMR Balance: $0**

A. **Legal Citation: ML 1998, Chap. 401, Sec.3. S.F. 3353**
Project Number: J3
WATER QUALITY INDICATORS OF ENDOCRINE DISRUPTING CHEMICALS
This appropriation is from the trust fund to the pollution control agency to monitor and research the effects of endocrine disrupting chemicals in surface waters on fish and wildlife through analysis of biological effects.

The availability of the appropriation in Laws 1997, chapter 216, section 15, subdivision 14, paragraph (c), to monitor and research the effects of endocrine disrupting chemicals in surface waters is extended to June 30, 2000.

II. **PROJECT SUMMARY AND RESULTS:** The overall results of the project will be used to determine the presence of endocrine disrupting chemical (EDC) activity in surface waters in Minnesota in relation to point and non point sources of contaminants, and to evaluate the appropriate use of biological indicators of EDC activity for surface water quality assessment. The data from the assays of serum yolk protein (VTG) and sex steroid hormones in male fish and VTG in frogs will be used to indicate locations and extent of estrogenic EDC activity in rivers and ponds within at least two river basins. Assays of native ranging fish will indicate overall river water condition. Frog assay data will indicate condition of ponds or wetlands.

Morphological indicators of endocrine disruption will be sought by histological examination of the gonads of fish and frogs and other tissues. An outcome of the project will be a refinement of protocols for the analysis of EDC activity in surface waters using biological response indicators for water quality monitoring programs.

Cooperative work with other agencies is being sought so that chemical analysis on the water from the assay sites can be done. There is no funding in this project for water chemistry, as there had been in the original proposal before it was reduced. Region V EPA has plans to provide water sampling in conjunction with the sampling of three wastewater treatment plants in September, 1997.

This project is not an extension of the investigation of deformed frogs carried out in 1996 with LCMR funding. Frogs are included in this project for serum analysis to look for endocrine disrupting activity. This may not relate at all to the kinds of abnormalities seen in frogs in Minnesota in 1996.

## III. PROGRESS SUMMARY:

**Final Results Summary, October, 2000**

**Result 1.** Fish serum EDC activity in streams final report.

The results for the analysis of several biological indicators of endocrine disrupting chemicals, or hormonally active agents (HAA) are given in the final report "Use of Biological Characteristics of Common Carp (*Cyprinus carpio*) to Indicate Exposure to Hormonally Active Agents in Selected Minnesota Streams, 1999" received by MPCA from the U.S. Geological Survey and attached to this final workplan report.

Biological characteristics were analyzed in serum of 201 female and 221 male carp from 22 locations in 15 streams in central and south central Minnesota. Serum measures included plasma vitellogenin (VTG) and the plasma sex steroid hormones, 17B-estradiol and 11-ketotestosterone. The specific criteria for indication of HAA activity are given in Table 14 of the USGS report.

Generally, if VTG was unusually low in females, or present in males above a designated background level, it was taken to indicate HAA activity. In female fish, if estradiol was too low, or testosterone was too high, or if testosterone was higher than estradiol, then HAA activity was indicated. In male fish, if VTG was present above a given background level, if estradiol was high, if testosterone was low, or if there was more estradiol than testosterone, then HAA activity was indicated.

Referring to USGS Table 14, VTG was documented in male carp at 7/21 stream locations in Minnesota. These sites were in the Cedar River downstream of Austin, in the

discharge channel below the Metropolitan Waste Water Treatment Plant in St. Paul, in the Shell Rock River near Albert Lea, in the South Fork of the Crow River upstream of Hutchinson, in the Rock River upstream of Luverne, in the Sauk River upstream of Sauk Center, and in the Watonwan River upstream of Madelia.

Separating the plasma indicators from the morphological indicators, and adding the total number of positive indicators for male and female carp combined shows the greatest number of fish serum indicators of HAA activity in the Des Moines River downstream of Windom, and in the Rock River upstream of Luverne. These indicators include VTG and imbalances in sex steroid hormones. Refer to the USGS report for background and more detail on the results from the 22 stream locations.

**Result 2.** EDC activity in frog serum final report.

The final report by Dr. Brent Palmer of University of Kentucky, "Analysis of Adult Male Frogs (*Rana pipiens*) From Wetlands in Minnesota for Evidence of Endocrine Disruption," is attached to this final workplan report. Dr. Palmer analyzed the plasma of adult male frogs for the presence and amount of the yolk protein vitellogenin (VTG). None of the 59 adult male frogs from 10 wetlands in Minnesota showed evidence of induction of VTG. Since Dr. Palmer has established that male frogs will produce VTG in the presence of estrogenic substances, this result suggests that the frogs that were analyzed were not exposed to levels of estrogenic chemicals sufficient to induce VTG synthesis in male frogs. Sex steroid hormones were not measured in the adult male frogs.

**Result 3.** Morphological indicators of EDC activity in fish and frogs.

*Morphological indicators in fish, final report*
The final report by USGS, "Use of Biological Characteristics of Common Carp (*Cyprinus carpio*) to Indicate Exposure to Hormonally Active Agents in Selected Minnesota Streams, 1999" is attached to this final workplan report. Morphological indicators of gonad development and gonad histopathology were analyzed. The criteria to indicate evidence of estrogenic effects are summarized in Table 14 of the USGS report. Evidence of reduced weight of ovaries or testes in relation to body weight (low gonadal somatic index) was seen in female fish from 10/21 stream sites, and in male fish from 7/21 stream sites.

Other morphological indicators on a cellular level were included as an "early warning of sublethal effects" of hormonally active agents. These indicators were an increase in the percentages of atretic eggs in females and an increase in the amount of ceroid/lipofuscin staining in the gonads of female and male fish. Atretic eggs are eggs that are degenerating and undergoing resorption and not able to become mature oocytes. Ceroid/lipofuscin deposits are areas of yellow brown pigment found, for example, in cerosis of the liver from accumulation of waxlike metabolic waste products that are

normally broken down. In this study, ceroid/lipofuscin deposits were analyzed in both ovaries and testes. Elevated percentages of degenerating, or atretic, oocytes were observed in female fish from 8/21 stream sites. Increased deposits of ceroid/lipofuscin pigment were recorded in female fish from 8/21 stream sites, and in male fish from 8/21 stream sites.

*Morphological indicators in frogs*
*Frog testes final report*

The results of morphological examination of the testes from 59 adult male frogs from ten wetlands in different areas in Minnesota, *Rana pipiens*, are given in the final report by Dr. Palmer, "Analysis of Adult Male Frogs (*Rana pipiens*) From Wetlands in Minnesota for Evidence of Endocrine Disruption," which is attached to this final workplan report. No abnormalities were seen in the testes, including malformations in tubules, presence of ovarian follicles in the testes, lesions or tumors. Detailed analysis of the dimensions of the tubules in the testis showed some differences in tubule size among the wetland sites, especially in one wetland.

The stage of sperm development was analyzed in all the frogs. There were significant differences in the stage of development. The adult males in one wetland with significant malformed juvenile frogs had very advanced sperm development. In contrast, in another wetland that had significant malformed juvenile frogs, the sperm development was the least advanced of all the sites. Analysis will be done to relate the findings to other data on the frog populations as it becomes available. All the frogs were collected around the time of reproduction, that is in May and June, so they would be expected to be sexually mature. None of the frogs analyzed by Dr. Palmer were malformed. MPCA rarely has found malformations in adult frogs, and when they have been found they are minor in nature. The assumption is that the young metamorphosed frogs which display the malformations are not surviving through the winter and do not develop to sexual maturity.

*Frog larynges final report*
The final report by Dr. Blinda McClelland, "Male *Rana pipiens* Laryngeal Morphology from nine Sites in Minnesota; Summer, 1999," is attached to this final workplan report. The larynx was investigated because its development is directed both by thyroid hormone and by androgen hormones, so irregularities in morphology or development might be indicative of disruption in these hormone signals. The larynges of 59 male *Rana pipiens* from 9 sites in Minnesota were examined for malformations and no abnormalities were observed. Structures of the larynx were examined for size differences, but none were found other than the expected changes with body mass of the adult male frogs. Dr. McClelland concludes there is no evidence of contamination or endocrine disruption reflected in the development of the larynges of the male frogs.

**Progress Summary, November 1, 1999**

MPCA has established a partnership with the U.S. Geological Survey (USGS) to provide coordination of the planning and field work for the fish component of this project with MNDNR. USGS staff will coordinate the fish work and partnership with MNDNR. MPCA staff will coordinate the frog work. Two interagency planning meetings have been held with staff from USGS, MPCA, U. Minnesota, Metropolitan Council, and other entities. Plans are in process for forming the contracts for the analytical work, site selection and the field work for summer, 1999 for the fish assessments. The planning for the fish work is being coordinated by Kathy Lee of USGS with MPCA and MDNR. MPCA is coordinating the frog component of this project and is planning with Dr. Brent Palmer at U. Kentucky for frog VTG analysis. Plans are being made for the frog larynx analysis with Dr. McClelland of U. Texas. Plans are being made for the methods and sites for the spring, summer and fall field collections of adult frogs. The workplan was revised in March of 1999 to reflect the new partnership with USGS and the extended end date of the project to June of 2000.

Between May 1, 1999 and September 1, 1999, USGS finalized its contracts for laboratory work for fish serum and tissue analysis, they worked with MPCA to select locations for sampling fish. USGS and DNR staff collected fish from 22 sites from 14 different streams located in central and southern MN. They collected blood serum and tissue samples from male and female carp from each site and shipped all samples to the laboratories doing the analysis of serum vitellogenin, estrogen and testosterone and histopathology of liver, gonads and spleen. Preliminary hydrologic analysis of the streams was completed. A summary of the USGS progress is attached.

MPCA collected adult male leopard frogs, *Rana pipiens*, for analysis for evidence of endocrine disruption in blood and larynx from 12 sites and shipped them to U. Kentucky where blood serum samples and gonads were removed and the larynx tissue dissected and shipped to U. Texas Austin for analysis. Carcasses were frozen and returned to MPCA for further analysis. Adult frogs were collected using drift fences and funnel traps initially, but it was more efficient to capture frogs by hand netting. A minimum of six adult male frogs were collected from each site and shipped in containers with sterile sphagnum moss moistened with water from the sites. Three females were shipped from one site in addition to the males. Collecting equipment was rinsed carefully and frogs were handled with gloved hands. A summary spreadsheet of the frogs collected is attached.

At University of Kentucky immediately upon receiving frogs, they were checked for health and condition, and cross-checked with the shipping list. For experimental frogs, blood samples were collected. The plasma from the blood is stored at -70$^0$C. Following sedation, the gondads, reproductive tracts and a portion of liver were removed and preserved. The larynx was dissected to include surrounding tissue and frozen at -70$^0$C and shipped frozen to Dr. McClelland at U. Texas Austin. Frogs for positive controls

were exposed to an estrogenic chemical in their water to induce the plasma vitellogenin to standardize the assays. Blood was withdrawn and frozen at two week intervals.

## IV. OUTLINE OF PROJECT RESULTS:

Result 1. Fish serum EDC activity in streams.
EDC activity in rivers and streams will be indicated by the presence of elevated vitellogenin (VTG), an egg yolk protein, and abnormal sex steroid levels in ambient male fish from approximately 35 total sites within at least two major river basins in relation to specific sources of pollution. Sites will be distributed among reference sites with minimal disturbance and potentially impacted sites where fish would likely be exposed to EDC's.

Reference sites will be selected based on EPA criteria of minimal disturbance, having undisturbed riparian areas and minimal influence from point or nonpoint-source pollution. The timing of field collections will be tied to the times of greatest potential exposure to EDC's, such as low-flow conditions below discharges or after storm events from nonpoint source areas. For any comparisons among sites, fish of the same species, age, gender and stage in reproductive cycle will be assayed to minimize natural variations in fish VTG and sex steroid concentrations.Vitellogenin and sex steroid concentrations in fish will be compared between potentially impacted and reference streams.

The science of assay techniques for EDC activity in fish and frogs is developing now in research laboratories. By the start date of the field component of this project, there may be a more efficient method available for assaying vitellogenin using its messenger RNA (mRNA) from liver tissue. The best and most efficient methods available for measuring vitellogenin will be used.

Result 1 Budget: Fish serum collections, fish cages, fish serum assays collections, equipment, supplies and travel: U.S. Geological Survey (USGS)- $25,000 ($17,000 for Kathy Lee and one student, $4,500 for travel, and $3,500 for equipment and supplies); Minnesota Department of Natural Resources (DNR) - 20,000 (8,000 personnel, $9600 equipment and supplies, $2400 travel); U Florida - $25,000 (fish serum assays).

Result 1 Budget Total: $70,000 Balance: $0
Completion date: Field Work – November, 1999; Lab Analyses – April, 1999

Result 2. EDC activity in frog serum.
Endocrine disruptor activity in standing waters will be indicated by assays of serum from male Leopard Frogs, *Rana pipiens*, analyzed for vitellogenin and sex steroid

hormone levels from 6 - 12 locations in two major river basins. Elevated vitellogenin will be related to frogs of similar age and reproductive stage collected from reference sites. Reference sites will be selected on the basis of minimal human disturbance and having a low (<1%) or nonexistant frequency of abnormal frogs at the site. Collections will be made of adult males as frogs leave overwintering sites (deeper ponds, streams, rivers) and migrate to the breeding ponds and during summer and early fall after maximum exposure time to the landscape.

Result 2 Budget: Frog serum collections, frog serum assays, supplies, travel, equipment: MPCA - $15,000 temp coordinator; $5697 seasonal intern 1999, $5,000 supplies, shipping, $2,500 administrative, $2,303 travel); U. Kentucky- $13,500 (frog serum assays).

Result 2 Budget: $44,000 Balance: $0

Completion date: Field work Nov. 1, 1999; Analysis April 1, 1999

Result 3. Morphological indicators of EDC activity in fish and frogs.
Histopathological examination of fish and frogs will show if there are morphological alterations in the sex organs in male fish or frogs. This could indicate EDC activity during previous development. The VTG is generally thought of as a short term response that reflects conditions (presence and concentrations of estrogens or chemicals in the water) at or near the time of sampling. Morphological changes can indicate past exposure during development. Evidence of abnormal gonadal tissues in fish (presence of ovotestis and reduced spermatid development in males) will be sought. Testis and ovary condition of fish and frogs from reference sites will be compared with those from reference sites. In frogs, analysis will be made of male seminiferous epithelia, retention of cortical or follicular structures, stage of sperm development and presence of pyknotic nuclei or Sertoli cells. Other tissues may be used as indicators of EDC activity. Histological examination of the larynx of male frogs will be done to reveal possible disruption of thyroid or androgen hormones by analysis of larynx size, shape, and stage of cellular maturation. The development of the larynx in males is driven by androgen hormone, but without the early presence of normal thyroid hormone, the larynx of the male frog will not develop.

Progress from Dr. Palmer U Kentucky:
Result 3 Budget. Histology of gonads of fish and frogs; histology of other tissues, (USGS-BRD: fish histopathology: $30,000; Frog histopathology contract $22,500);

Result 3 Budget: $52,500 Balance: $0
Completion Date: Fish and frog field work done November, 1999, fish and frog Laboratory Analyses done April 2000.

Result 4. Analysis and reporting.
Analysis and reporting of the data and protocols will be compiled into a final, evaluative report. Coordination, communication and planning will be ongoing among USGS, MPCA and MNDNR. The final report will relate the findings to issues of the feasibility of using the serum assays in surface water quality monitoring, the relationship to selected sources of pollution of the indicators of estrogenic and other endocrine disruption found in the various rivers, streams and wetlands in Minnesota, and discuss the findings in the perspective of other work nationally.

Analysis and final report USGS and PCA $83,000
Result 4 Budget: $68,000 USGS, $15,000 PCA Balance: $0

V. **DISSEMINATION:** The results of this project will be written into scientific publications and will be presented at scientific conferences and other meetings and conferences. Results will be presented to staff and managers within the MPCA for future planning of monitoring strategies for surface waters.

**VI. CONTEXT:**

A. **Significance:** Endocrine disruption is a new environmental concern and a new issue for water quality monitoring agencies whose water quality standards have been previously developed to protect the aquatic life in surface waters. Because this issue area is so new and so complex, most states, if not all, have no monitoring for endocrine disruptive chemicals (EDC's) in their water quality programs. Yet there are demonstrated effects on some aquatic life including fish. Additional surveys for evidence of biological effects endocrine disruptive chemicals in surface water and a discussion about the feasibility of monitoring for endocrine disrupting chemicals (EDC's) is needed. Exposure to EDC's in surface waters has resulted in infertility, poorly developed sex organs, intersexed animals and endogenous hormonal imbalances in all classes of egg-laying animals, including fish, amphibians, reptiles and birds (Colburn et al, 1993). In wildlife, endocrine disrupting chemicals have been demonstrated to cause male animals to become feminized and female animals to become masculinized or hyperfeminized. In Florida, reduced testosterone and alterations of sex organs in alligators and intersex in fish were attributed to EDC's in surface waters (Guillette et al, 1995). In Minnesota, work done by Minnesota Department of Natural Resources (MDNR) and U.S. Environmental Protection Agency (U.S. EPA) has found elevated yolk protein and depressed testosterone in male carp in the Mississippi River near the metropolitan area (Folmar, L.C. et al, 1996).

Estrogenic effects are not the only area where endocrine disrupting chemicals might alter aquatic organisms. Thyroid hormone (TH) has a chemical structure similar to DDT, PCB's and dioxin. TH is essential for normal metamorphosis in frogs and has long been known to be a key hormone for the differentiation of bone

in animals. It is possible that some EDC's which mimic thyroid hormone could alter development in aquatic species.

A broad spectrum of chemicals has been demonstrated to disrupt hormones: organochlorine pesticides, such as DDT, methoxychlor, endosulfan, some PCB's, dioxins, surfactants like alkylphenols from detergents, nonylphenols and phthalates from plastics, petrochemicals, and residuals from pharmaceuticals. Chemical monitoring for wide the range of EDC's may not be feasible because of cost. In this project, the feasibility of using biological assays for EDC activity is proposed as a first step in identifying the extent of estrogenic activity in surface waters.

State-wide monitoring using biological assays of blood serum sex steroid and yolk protein levels in male fish and frogs will most effectively locate waters with endocrine disruptive chemicals which have estrogenic effects. A cooperative effort by the USGS MPCA, MDNR, U.S. EPA, University of Florida, U. Kentucky, U. Texas Austin and University of Minnesota will provide the biological effects data. The results of the project will be used to evaluate whether it is necessary and feasible to begin monitoring for EDC's in Minnesota surface waters.

This project is not a continuation of the 1996 investigation of deformed frogs in Minnesota funded by LCMR on an interim basis. Instead, this project is exploring whether frog serum VTG, sex hormones and the morphology of frog sex organs and larynx can be used as indicators of endocrine disrupting activity in standing water. The very limited population surveys funded in this project are included as part of the criteria for selecting the reference sites for the frog serum assay work. These limited surveys do not constitute an investigation of frog deformities.

The workplan timeline for the 1996 investigation of deformed frogs ended in June, 1997 even though the appropriation goes to Dec. 1997. Additional funding for the investigation of deformed frogs will come from non-LCMR sources.

**B. Time:** The necessary followup of the proposed project is the 'tier II' phase which would require a proposal to LCMR for 2000-2004. This second phase would address the question of the feasibility of water chemistry monitoring for EDC's as part of a water quality monitoring program. This would involve water chemistry testing in the reference areas and the sites where serum assays of fish and frogs indicated EDC activity was present.

**C. Budget Context:**

| July 1995-June 1997 | July 1997-June 2000 | after June 2000 |
|---|---|---|
| **Prior expenditures on this project** | **Project period** | **Future expenditures on this project** |

| | | | |
|---|---|---|---|
| **LCMR** | | $250,000 | |
| **Other State** | | | |
| **Non State** | | | |
| **Match** | | | |
| **In kind** | | | |
| **Total** | | $100,633 | |
| | $0 | $250,000 | $0 |

**1997 LCMR BUDGET:**

| | |
|---|---|
| Personnel: | $136,697 DNR $8000 temp; USGS $93,000: 1 full time and 1 student employee); PCA $35,697 ($30,000 .5 fte, temp coordinator; $5,697 seasonal field staff ). |
| Equipment: | $14,000 ($9,600 DNR; USGS $3,500;$5000 PCA for supplies, shipping, vehicle rental, equipment, and rental vehicles, micro-centrifuge for blood serum centrifugation) |
| Acquisition: | |
| Development: | |
| Fish Serum assays: | $20,000 (technical contract U Florida) |
| Frog Serum Assays: | $13,500 (technical contract U. Kentucky) |
| Fish Histopathology: | $30,000 |
| Frog Histopathology: | $22,500 (technical contract U. Kentucky) |
| Travel: | $8,803 (DNR $2,400; PCA $2303) |

**VII. COOPERATION:** Dr. Leroy Folmar (5% time, no cost to project), Dr. John Fournie, U.S. EPA Florida; Dr. Nancy Denslow, University of Florida; Jack Enblom (5% time, no cost) MN DNR, Judy Helgen (5% no cost) MPCA. Dr. Brent Palmer, U.Kentucky, Dr. Blinda McClelland, University of Texas at Austin, Kathy Lee United States Geological Survey.

**VIII. LOCATION:** This project will take place in at least two major river basins in Minnesota, in other words it is a statewide project.

**IX. REPORTING REQUIREMENTS:** Work program progress reports and associated products will be submitted November 1, 1999, June 30, 2000.

**X. RESEARCH PROJECTS:** See attached addendum for this project.