# Lower Red River and Lake Winnipeg South Basin Pathogen – Parasite Survey Report, Fall 2006

**Brian Souter, Andy Dwilow, Sandra Aldous, Jason Allen, Ken Rowes, Bill Franzin and Doug Watkinson**

**Freshwater Institute, Fisheries and Oceans Canada, Winnipeg, MB**

## Summary


- 10 species of fish were targeted for collection, 60 fish per species were to be collected.

- 9 of the 10 targeted species were successfully sampled. Only 7 channel catfish were captured. Total of 547 fish were screened for bacterial and viral pathogens of concern.

- 60 lake whitefish were tested for the presence of *Myxobolus cerebralis* (causative agent of whirling disease).

- There was no clinical evidence of disease suggestive of an infection with any bacterial and viral pathogens of concern in any of the fish examined. Lymphocystis and dermal sarcoma, both neoplasias having viral etiologies were observed in a small number of walleye and sauger.

- Virus was not isolated from any of the samples received, nor were any bacterial pathogens of concern (e.g. Aeromonas salmonicida, causative agent of furunculosis, and Yersinia ruckeri, causative agent of enteric redmouth disease).

- Myxobolus cerebralis was not detected in the lake whitefish sample.

- The Winnipeg Fish Health Laboratory used the indirect fluorescent antibody technique to stain kidney smears to screen for the prescence of *Renibacterium salmoninarum* (causative agent for bacterial kidney disease). The pathogen was not detected by this method in either the samples processed in the Winnipeg Fish Health Laboratory, nor the stained slide preparations received from the Bozeman Fish Health Center. The inter-laboratory exchange of material was undertaken to compare the results obtained in the two laboratories because distinctly different methods of detection for this pathogen were used in the respective laboratories.

# Devils Lake/Lake Winnipeg Pathogen/Parasite Survey Report Fall 2006

## Relevant Decision Points Arising from Monitoring Workshop held in Winnipeg, August 23 – 24, 2006

- Kidneys and spleens will be sampled from all fish. In addition, swimbladder tissue will be collected from Centrarchids.
- Tissue preparations for virus detection: either the filtration or antibiotic method is suitable. Differences in viral detection methods of DFO and USFWS labs are minor; therefore, methods are considered equivalent.
- USFWS will use the CHSE, EPC, CCO and BF-2 cell lines; Canada will use CHSE, EPC and CCO (no centrachid samples will be collected in Canada, therefore, the BF-2 cell line is not required).
- The detection of an unknown pathogen in samples obtained during this survey will be reported to the team with the isolate(s) of same being retained for possible further investigation.
- DELTS will be recorded by field fishing crews. They will use a readily available reporting form from EPA or Ohio EPA. Peters and Franzin will agree on a form to use.
- Any abnormality observed during fish scans will be sampled for bacterial or histopathology at the discretion of the investigators.
- Lumsden and Souter will exchange possible positive R. salmoninarum detections (kidney tissue) for PCR confirmations by USFWS staff at the Bozeman lab (Hudson); samples of positive USFWS ELISA will be sent to DFO Winnipeg for fluorescent antibody staining.
- Sixty lake whitefish and sixty lake ciscoes from Lake Winnipeg will be assayed by DFO for M. cerebralis; PCR confirmation, if necessary, will be done by DFO, Nanaimo, BC or by USFWS, Bozeman.

## Bacterial, viral and parasitological work performed by Fisheries and Oceans Canada at the Winnipeg Fish Health Laboratory, Winnipeg, Manitoba

### Sample Collection

Ten species from the lower Red River and south basin of Lake Winnipeg were targeted for collection. The species, number collected and dates of collection are shown in Table 1. Maps showing sampling locations are shown in Appendix 2.

Table 1. Targeted species, (numbers collected) and dates of collections

| **Species** | | | | | | **Totals** |
|---|---|---|---|---|---|---|
| Channel catfish | Oct 10 (6) | Oct 19 (1) | | | | 7 |
| Emerald shiner | Oct 4 (60)* | Oct 16 (60) | | | | 120 |
| Fathead minnow | Oct 20 (60)* | Oct 30 (60) | | | | 120 |
| Yellow perch | Oct 4 (120)** | | | | | 120 |
| White bass | Oct 4 (120)** | | | | | 120 |
| Walleye | Oct 16 (2) | Oct 17 (18) | Oct 18 (8) | Oct 19 (20) | Oct 23 (12) | 60 |
| Northern pike | Oct 16 (4) | Oct 17 (17) | Oct 18 (10) | Oct 19 (12) | Oct 23 (17) | 60 |
| Brook stickleback | Oct 20 (60)* | Oct 23 (60) | | | | 120 |
| Sauger | Oct 16 (14) | Oct 17 (26) | Oct 19 (17) | Oct 26 (3) | | 60 |
| Goldeye | Oct 10 (20) | Oct 16 (40) | | | | 60 |

* Due to small size of fish, entire sample was fixed for histopathology.
** Due to small size of fish, half of the sample (i.e. 60 fish) was fixed for histopathology; the remaining 60 were processed for bacteriology and virology.

Fish were captured by a number of methods including seines, gill nets and electro-shocking. Small fish were placed in bags of water, the bags sealed, placed on crushed ice in coolers and transported to the Winnipeg Fish Health Laboratory. Larger fish were placed on ice in ice chests and transported to the lab. The majority of fish were processed for virology, bacteriology and histopathology within 10 hours from the time of capture. In one instance, fathead minnows captured on Friday Oct 20th were held in a live tank until Monday Oct 23rd at which time they underwent bacterial and viral testing. Three other small species including yellow perch, white bass and brook sticklebacks were kept alive and necropsied the morning following their collection.

**Virology**

Sixty fish samples were collected for nine of the ten targeted species. However, declining water temperatures and increasingly inclement weather made it difficult to capture channel catfish. It was decided that no further attempt would be made to collect the required sample. Only 7 channel catfish were collected.

Approximately equal amounts of kidney and spleen tissues were collected from all species, and, in the majority of cases, the tissues were pooled in five fish pools. In instances were odd numbers of fish were delivered to the lab, pools of tissues consisting of less than five fish were prepared.

The methodology used for virus detection was that described in the Fish Health Protection Regulations: Manual of Compliance (Department of Fisheries and Oceans, 1984, revised). Briefly, this involved the preparation of 2% tissue homogenates prepared in pH adjusted Hanks balanced salt solution (HBSS). Homogenates were centrifuged for 15 mins. at 2500 x g at $4^0$C. Approximately 5 ml of supernatant was removed and filter sterilized using sterile, low protein binding disposable filters (0.45 um pore dia.). Cell lines used for virus detection included the chinook salmon embryo (CHSE-214), epithelioma papulosum cyprini (EPC) and channel catfish ovary (CCO). Twenty-four hours before inoculation. duplicate preformed monolayers were prepared in 24 well multi-well plates. For all species except channel catfish, multi-well plates were drained of their growth medium and then inoculated with 0.1 ml of filtrate, allowed to adsorb for I hr. at $15^0$C and then overlayed with 1 ml of Eagles minimum essential medium (MEM) plus HEPES buffer. The inoculated plates were incubated at $15^0$C for 21 days and observed at least twice per week for the presence of CPE. The procedure for filtrates derived from channel catfish tissues was the same as that for filtrates derived from the other nine species with the exception that channel catfish derived filtrates were inoculated onto duplicate monolayers of CCO cells in addition to CHSE and EPC cells. The temperature for virus adsorption and incubation involving the CCO cell line was $22^0$C. If CPE was not observed during the 21 day incubation period the results were considered negative and the plates were discarded.

**Bacteriology**

**Bacterial Isolation and Identification**

Brain heart infusion agar (BHIA) was used to isolate bacteria from fish kidney tissue. Briefly, this involved inserting a flame sterilize inoculating loop into the posterior region of the kidney and streaking this material onto individually labelled plates of BHIA. The inoculated BHIA plates were incubated at $20^0$C and observed at 24, 48 and 72 hours for the presence of bacterial growth. Representative isolated colonies were selected and restreaked onto fresh plates of BHIA. The restreaked plates were incubated at $20^0$C to obtain pure cultures. When pure cultures were obtained, Gram stains were prepared; Glucose Motility Deep (GMD) media was stab inoculated to determine motility and the ability of the isolates to oxidatively or fermentatively utilize glucose; and API-20E strips were inoculated following the manufacturer's instructions. Inoculated GMD tubes and the API-20E strips were incubated at $20^0$C and observed at 24 and 48 hrs. Motility and glucose utilization were read at 48 hrs. At this time the required reagents were added to the API-20E strips. Results from the API-20E strips were read and the biochemical profiles determined. The biochemical profiles were checked against known profiles for Yersinia ruckeri (enteric redmouth bacterium) and Aeromonas salmonicida (causative agent of furunculosis) and then entered into the APILAB Plus bacterial identification program and the bacterial identification results recorded.

**Indirect Fluorescent Antibody Staining (IFAT) for Renibacterium salmoninarum (R. s.) causative agent of Bacterial Kidney Disease**

At the time that BHIA plates were being streaked for bacterial isolation, multiwell slides were prepared for eventual IFAT staining. This involved inserting a cotton swab or flame sterilized loop into the posterior region of the kidney and then smearing this material onto duplicate wells of a labelled multiwell slide (ten or twelve wells/slide). The slides were air dried and stored in slide boxes until they could be stained. Prior to staining the slides were fixed in acetone for ten minutes. The preparation of reagents and the staining procedure used is described in Appendix 1. The reagents, serum and staining protocol was provided by the Fish Health Laboratory, Department of Microbiology, University of Guelph, Guelph, Ontario, Canada. When each batch of slides was stained, an R. s. positive control slide was stained to ensure integrity of the staining procedure. Stained smears were examined at 1000X magnification using a Zeiss Axioskop 40 transmitted/UV microscope. Kidney tissue and/or kidney/spleen homogenates from pooled viral test samples were retained at -80$^0$C pending IFAT screening results. At the August workshop it was decided that the Winnipeg Fish Health Lab and the Bozeman Fish Health Center would exchange any kidney material that gave R. s. positive or suspect positive results by the methods used in the respective labs.

**Parasitology (Myxobolus cerebralis)**

Screening for the presence of spores of the myxosporean parasite Myxobolus cerebralis involved acquiring sixty lake whitefish from a commercial fisher. Lake ciscoes were not obtained. The fish were held at -20$^0$C until they could be processed. The method used for screening for the presence of spores of M. cerebralis was the crainial digest method using pepsin-HCL described in Section XI, Procedures for the Detection of Certain Parasites in the FHPR Manual of Compliance with the following exception. Following de-fleshing of the heads, cranial tissue and gill elements were pooled in five fish pools to reduce the dilution factor associated with processing a larger volume of tissue as a single pool. Pelleted material from each of the twelve pools was used to prepare individual slides. A minimum of 100 fields per slide were examined for the presence of M. cerebralis spores at a magnification of 400X.

**Results**

**Virology**

A total of 114 pools of kidney/spleen tissues were prepared from the ten targeted species. Cytopathic effect (CPE), indicative of a viral infection in any of the species tested, was not observed in any of the three cell lines used. Cell destruction was observed in both inoculated and negative control wells of CCO cells inoculated with filtrates derived from channel catfish tissues. Cell destruction was not observed in the corresponding CHSE and EPC wells. Supernatant from the affected inoculated wells and the negative control wells was harvested separately, filter sterilized (0.45 um pore dia), diluted $10^{-1}$ and $10^{-3}$ and 0.1 ml of each dilution inoculated onto fresh duplicate preformed monolayers of CCO cells. The multi-well plates were incubated at 22$^0$C and observed for a further seven days. Cell destruction attributable to viral CPE or cytotoxicity was not observed during this time. Cell destruction observed in the CCO cells in the initial

multi-well plates was attributed to deterioration of the monolayers due to the rapid growth and subsequent death of the cells which produced an alkaline growth environment.

**Bacteriology**

A total of 111 bacterial isolates were cultured from the 10 targeted species (Table 2) of which 100 pure cultures were obtained for identification purposes. No attempt was made to screen out any of the isolates on the basis of phenotypic characteristics such as colony morphology, colour, texture, etc. If this had been the case, the number of cultures subjected to biochemical testing would have been significantly less.

Table 2. Number of bacterial isolates cultured from each of the targeted species of fish

| Species | Number of isolates cultured from each species |
|---|---|
| Walleye | 1 |
| Sauger | 14 |
| Yellow perch | 10 |
| Fathead minnow | 6 |
| Emerald shiner | 13 |
| Goldeye | 33 |
| Northern pike | 8 |
| Channel catfish | 1 |
| White bass | 10 |
| Brook stickleback | 15 |
| Total | 111 |

The results of isolate identification and the species of fish from which they were isolated are shown in Table 3. The majority of the isolates were Gram negative rods, many of which were non-motile. Only 14 of 100 isolates were identified with any degree of confidence using the API-20E system. Aeromonas hydrophila was the most common identifiable bacterium; it was present in four species of fish. Pseudomonas. aeruginosa was the next most common isolate; it was isolated from three species followed by Hafnia alvei, Pseudomonas. fluorescence, Flavobacterium sp. and Plesiomonas shigelloides which were each isolated from one species of fish. The API-20E system is routinely used to identify two of the fish pathogens being screened for in this survey (i.e. Y. ruckeri and A. salmonicida, neither of which were detected). However, the product is designed to identify enteric bacteria which are routinely incubated at 35 – 37$^0$C. The lack of definitive identifications obtained for the majority of the bacterial cultures isolated in this survey, may be a reflection of a less than optimum incubation temperature for the majority of isolates to properly metabolize the various substrates used in the API-20E system, or the number and variety of substrates used in the API-20E system is inadequate to properly identify non-enteric organisms. None of the isolates produced biochemical profiles consistent with those for known bacterial fish pathogens of concern.

Table 3. Bacterial identification results and the fish species from which the isolates were cultured.

| Bacterial Identifications and Species of Fish | | | | | |
|---|---|---|---|---|---|
| H. alvei | A. hydrophila | Ps. aeruginosa | Ps. fluorescence | Flavobacterium sp. | Plesiomonas shigelloides |
| YP* | YP<br>FHM (2)**<br>WB (3)<br>CC | YP<br>FHM<br>WB | ES | S | WB |

*Abbreviations: YP = yellow perch, FHM = fathead minnow, ES = emerald shiner, S = sauger, WB = white bass, CC = channel catfish

** Number of isolates identifed

## Screening for R. salmoninarum (BKD) by IFAT Staining

Duplicate kidney smears from 547 fish were examined following IFAT staining. All IFAT stained R. s. positive control slides stained at the same time as the kidney smears provided positive results demonstrating that the IFAT reagents performed properly. A minimum of 25 fields was examined on each duplicate kidney smear. There was no evidence of fluorescing bacteria of typical size and shape in any of the 1094 smears examined that would suggest the presence of R. s. in any of the 547 fish examined.

## Comparison of IFAT, ELIZA and PCR Screening Methods for R. salmoninarum

Because the two participating laboratories used distinctly different methods for R. s. detection, the laboratories were asked to exchange samples of kidney material that had been screened by the methods used in the respective labs (i.e. ELIZA followed by PCR confirmation at the Bozeman Lab, and by IFAT at the WFHL). This exchange took place to compare the R. s. test results obtained in the respective laboratories. Although there was no clinical evidence infection with R. s. in any of the fish tested in the WFHL, nor was there any evidence of the pathogen in any of the IFAT stained kidney smears, a sub-sample of 60 tissues (i.e. 10 kidney tissue samples, and 50 kidney/spleen tissue samples consisting of kidney tissue from which smears were prepared) was sent to Bozeman. The WFHL received 48 kidney smears prepared from Devils Lake fish. Each laboratory provided material collected from seven species of fish, four of which were the same (walleye, northern pike, white bass and yellow perch). Methanol fixed kidney smears were received from the Bozeman laboratory. In addition to the four common species, the Bozeman sample also included kidney

smears prepared from black crappie, white sucker, and fathead minnow. Table 4 shows the ELIZA/PCR results obtained in the Bozeman laboratory, and the IFAT results obtained in the WFHL for the 48 Bozeman samples.

Table 4.

**Devils Lake/Lake Winnipeg Pathogen/Parasite Survey - Comparison of Results for Samples Exchanged for R. salmoninarum Screening**

| | Devils Lake | Bozeman Fish Health Center Results | | | | | WFHL | |
|---|---|---|---|---|---|---|---|---|
| Species | Sample # | Mean ELIZA OD | ELIZA C/O | | ELIZA P/N | PCR confirm'n P/N | IFAT Results P/N | |
| NOP | 18 | 0.096 | 0.088 | P | | ND | N | |
| NOP | 24 | 0.104 | 0.088 | | P* | N* | | N' |
| NOP | 26 | 0.108 | 0.088 | | P | N | | N |
| NOP | 27 | 0.106 | 0.088 | | P | N | | N |
| NOP | 30 | 0.108 | 0.088 | P | | ND | N | |
| NOP | 32 | 0.102 | 0.088 | P | | ND | N | |
| NOP | 35 | 0.106 | 0.088 | P | | ND | N | |
| NOP | 36 | 0.121 | 0.088 | P | | ND | N | |
| NOP | 37 | 0.103 | 0.088 | P | | ND | N | |
| NOP | 44 | 0.106 | 0.088 | P | | ND | N | |
| NOP | 47 | 0.096 | 0.088 | P | | ND | N | |
| NOP | 48 | 0.111 | 0.088 | P | | ND | N | |
| NOP | 55 | 0.09 | 0.088 | P | | ND | N | |
| NOP | 56 | 0.15 | 0.088 | P | | ND | N | |
| NOP | 58 | 0.119 | 0.088 | P | | ND | N | |
| BLC | 4 | 0.077 | 0.088 | N | | ND | N | |
| BLC | 5 | 0.079 | 0.088 | N | | ND | N | |
| BLC | 6 | 0.078 | 0.088 | N | | ND | N | |
| WHS | 4 | 0.082 | 0.079 | P | | ND | N | |
| WHS | 5 | 0.081 | 0.079 | N | | ND | N | |
| WHS | 8 | 0.082 | 0.079 | P | | ND | N | |
| WHS | 12 | 0.082 | 0.079 | P | | ND | N | |
| WHS | 18 | 0.085 | 0.079 | P | | ND | N | |
| WHS | 20 | 0.088 | 0.079 | P | | ND | N | |
| WHS | 22 | 0.088 | 0.079 | P | | ND | N | |
| WHS | 25 | 0.091 | 0.079 | P | | ND | N | |
| FHM | 1 | 0.115 | 0.089 | | P | N | | N |
| FHM | 2 | 0.113 | 0.089 | | P | N | | N |
| FHM | 3 | 0.129 | 0.089 | | P | N | | N |
| WHB | 2 | 0.097 | 0.089 | | P | N | | N |
| WHB | 8 | 0.09 | 0.089 | P | | ND | N | |
| WHB | 9 | 0.084 | 0.089 | N | | ND | N | |
| WHB | 47 | 0.085 | 0.089 | N | | ND | N | |
| WHB | 51 | 0.198 | 0.089 | | P | N | | N |
| WHB | 59 | 0.104 | 0.089 | | P | N | | N |
| YEP | 2 | 0.098 | 0.089 | | P | N | | N |
| YEP | 4 | 0.091 | 0.089 | | P | N | | N |
| YEP | 9 | 0.095 | 0.089 | | P | N | | N |

| | | | | | |
|---|---|---|---|---|---|
| WAE | 16 | 0.08 | 0.088 N | ND | N |
| WAE | 18 | 0.078 | 0.088 N | ND | N |
| WAE | 19 | 0.08 | 0.088 N | ND | N |
| WAE | 20 | 0.079 | 0.088 N | ND | N |
| WAE | 22 | 0.079 | 0.088 N | ND | N |
| WAE | 24 | 0.081 | 0.088 N | ND | N |
| WAE | 36 | 0.081 | 0.088 N | ND | N |
| WAE | 40 | 0.079 | 0.088 N | ND | N |
| WAE | 42 | 0.08 | 0.088 N | ND | N |

**P*** : results shown in bold letters represent results for all three test methods
P/N: positive/negative
ND: not done
C/O: cut off or neg/pos threshold

The Bozeman laboratory obtained positive ELIZA results for 32 of 48 samples sent to the WFHL All 48 corresponding kidney smears were negative following IFAT staining at the WFHL. Twelve the 32 positive ELIZA samples had C/O values above a designated minimum threshold level of detection. These samples were subjected to confirmation testing using the highly sensitive PCR method. All 12 samples were negative by PCR.

**Screening for Myxobolus cerebralis**

To our knowledge there are no reports in the literature of M. cerebralis infecting lake whitefish. However, M. cerebralis does infect members of the Salmonidae family (i.e. various species of trout and salmon). Because this species is a member of the Salmonidae family, and it could be readily acquired from Lake Winnipeg, it was decided it would be prudent to test lake whitefish for the parasite. Using the pepsin-HCl digest method, all twelve pools of lake whitefish crainial/gill element tissue were negative for spores of M. cerebralis.

**Discussion:**

All fish sampled in this initial survey appeared healthy and there was no clinical evidence of either a bacterial or viral infection with pathogens of concern. The Gram negative organisms positively identified in this survey are ubiquitous water-borne bacteria that can be readily isolated from the gastro-intestinal tract and surfaces of fish. They are considered to be opportunistic pathogens capable of producing septicemic disease when fish are subjected to less than optimal environmental conditions such as elevated water temperature, low oxygen concentration and poor water quality, which stress the fish leaving them vulnerable to infection.

Clinical evidence of bacterial kidney disease was absent in all fish examined in the fall 2006 survey regardless of their source. Bullock et al. (1980) demonstrated that a minimum of $10^6$ R.s. cells $ml^{-1}$ are required to detect the presence of the pathogen in kidney preparations stained using either the direct or

indirect fluorescent antibody technique (FAT). Based on this minimum level of infection, if the pathogen was present in any of the species captured in the lower Red River and South Basin of Lake Winnipeg, it may have been present at levels undetectable by FAT staining. The methods used in the respective laboratories for R. s. detection were different in terms of what they are designed to detect. The ELIZA method detects a soluble extracellular protein secreted by R. s. cells; whereas, FAT detects intact cells (living or dead). Reasons discussed by Meyers et al. (1993) for the lack of agreement between ELIZA and FAT results in studies involving wild and cultured salmonid stocks included: the uneven or focal distribution of R. s. cells in kidney tissue resulting in too few cells present in the small amount of kidney tissue used to prepare a smear; and the number of bacterial cells present in fish recovering from an R. s. infection could be reduced, but the immune complexes produced in these fish would still contain soluble antigen available for detection by ELIZA.

The results obtained in the Bozeman laboratory for R. s. detection using the ELIZA method are similar to results reported in an earlier study (Hudson and Peters, 2005). In that study, the authors stated that the soluble antigen levels detected by ELIZA were very low when compared to ELIZA detection threshold levels established using standardized R. s. negative reference material derived from fall chinook salmon. Kidney tissue from the 32 ELIZA - positive fish used to prepare smears for IFAT screening at the WFHL would also be considered weakly positive by the ELIZA method. Therefore, a negative IFAT result is not surprising given the fact that the more sensitive PCR method failed to confirm the ELIZA results on all 12 of the selected ELIZA - positive samples. Hudson and Peters (2005) also stated that for the purposes of the National Wild Fish Health Survey, R. s. test results are considered inconclusive when ELIZA-positive results are not corroborated by PCR. To date, R. salmoninarum has not been conclusively demonstrated to be present in North Dakota (Peters 2002, Hudson and Peters 2005); therefore, it is reasonable to assume that the ELIZA-positive results obtained in the fall 2006 survey of Devils Lake fish represent false-positive findings.

*Note: This report does not include ELIZA/PCR results for the 60 tissue samples sent to the Bozeman laboratory. Should PCR confirm any presumptive ELIZA- positive result(s), the IFAT results obtained in the WFHL for the corresponding sample(s) would be interpreted as being false negative(s).*

**References**

Bullock, G. L., Griffin, B. R., Stuckey, H. M. (1980). Detection of *Corynebacterium salmoninus* by direct fluorescent antibody test. Can. J. Fish. Aquatic Sci. 37: 719 – 721.

Department of Fisheries and Oceans. 1984 Fish Health Protection Regulations: manual of compliance. Fish. Mar. Serv. Misc. Spec. Publ. 31 (Revised): 43 p.

Meyers, T. R., Short, S., Farrington, C., Lipson, L., Geiger, H.J., Gates, R. (1993). Comparison of the enzyme-linked immunosorbent assay (ELIZA) and the fluorescent antibody test (FAT) for measuring the prevalences and levels of *Renibaterium salmoninarum* in wild and hatchery stocks of salmonid fishes in Alaska, USA. Dis. aquatic Org. 16: 181 – 189.

Peters, K. (2002). Survey of specific fish pathogens in free-ranging fish from Devils Lake and the Sheyeen and Red rivers in North Dakota. U. S. Fish and Wildlife Service, Bozeman Fish Health Center Technical Report.

Hudson, C., and Peters, K. (20050. Survey of specific fish pathogens in free-ranging fish from Devils Lake, North Dakota. U. S. Fish and Wildlife Service, Bozeman Fish Health Center Technical Report 05-02.

## Appendix 1

## IFAT for detecting Renibacterium salmoninarum

### Part 1: Reagents and Serum

#### A. Rabbit anti – Renibacterium salmoninarum serum

- Rabbit anti – Renibacterium salmoninarum serum, was prepared by the Fish Health Laboratory (J. Daly and P. Senson). Serum dilutions were used in IFAT tests to determine the last easily-read dilution; the working concentration used is the dilution *before* that end point (i.e. 10 – fold more concentrated).
- The serum is stored frozen in 100 uL aliquots in bijoux bottles
- Add 4.9 mL PBS to rehydrate (1 in 50), mix gently.
- Filter through a 0.4r um filter unit
- Three bijoux bottles (approx 15 mL) will stain approx. 20 IFAT multi-well slides.

#### B. Phosphate buffer saline

- **PBS recipe:**

| | |
|---|---|
| - NaCl | 8.0 gm |
| - KCl | 0.2 gm |
| - $Na_2HPO_4.12H_2O$ | 2.84 gm |
| - $KH_2PO_4$ | 0.2 gm |
| - distilled water to 1L | |

#### C. Goat anti-rabbit Ig FITC/Rhodamine counterstain

**C.1 Goat anti- rabbit FITC:** Available commercially from Jackson Laboratories, supplied by Biocan Ltd., Biocan catalogue # 111-015-003. *Vials containing 50 uL of goat anti-rabbit FITC were provided by Steve Lord, Fish Health Laboratory, Department of Microbiology, University of Guelph.*

- Add 4.9 mL of PBS to a bijoux bottle containing 50 uL of goat anti-rabbit FITC. Mix well
- Three bijoux bottles (approx. 15 mL) will cover approximately 20 multi-well IFAT slides.

**D. Carbonate – Bicarbonate buffer**

This is composed of two stable solutions that can be made in advance. However, they should only be mixed together immediately before use as Solution C is not stable (it will absorb $CO_2$ and changes the pH).

Solution A:

$Na_2CO_3$ .....26.5 gm or
$Na_2CO_3.H_2O$..... 31.0 gm

Solution B:

$NaHCO_3$ .....21.0 gm

Solution C: PREPARE FRESH DAILY
Mix 1 part Solution A with 4 parts Solution B

| Total Vol of Soln C req'd | mL of Solution A | mL of Solution B |
|---|---|---|
| 250 | 50 | 200 |
| 500 | 100 | 400 |
| 750 | 150 | 600 |
| 1000 | 200 | 800 |
| 1250 | 250 | 1000 |
| 2000 | 400 | 1600 |
| 2500 | 500 | 2000 |
| | | |

**E. FA Mounting fluid**

*We used Zeiss immersion oil.*

**Part 2: Slide Preparation**

**A. Slides supplies**

1. **Multi-well slides:** 26 mm X 75 mm multiwell slides with 2 rows of 6 wells (7 mm dia) and frosted end. Order as: **Printed microscope slides #101007, Carlson Scientific Inc.,** 514 South Third St.

St., Peotone, Illinois, 60468

2. **Coverslips: (No.1) 22 mm x 50 mm**

**B. Tissue samples:**

- Run a flame sterilized inoculating loop or a sterile cotton swab along the entire length of the kidney. Prepare two smears per fish (i.e. in two wells, one above the other, on the multi-well slide).

**Control Slides:** A positive control slide prepared from known R. s. infected tissue is stained at the same time as each set of test slides, to ensure the staining worked and to provide a reference standard when examining the slides.

**Part 3: Staining procedure**

1. **Fix the slides by immersing in acetone for 5 to 8 minutes.**

- Place slides back-to-back in the slots of glass staining jars.
- Working in a fumehood, add fresh acetone to cover the slides in each jar.
- After fixing, remove the slides and place them on an aluminium tray of paper towelling. Allow the slides to air dry.
- Fix positive control slide separately from test slides.
- Discard used acetone in a container marked USED ACETONE.

2. Using a Pasteur pipette, cover the smears on each slide with a drop of Rabbit anti-R. s. serum.

3. Allow the slides to sit on the bench-top for 30 mins.

4. Rinse the antiserum from the slides with PBS, pH 7.2.

5. Soak slides in glass staining jars containing PBS for 10 mins.

6. Remove the slides an allow to completely air dry.

7. Cover each circle on the slides with the FITC conjugated goat anti-rabbit serum.

8. Stain the slides on the bench-top covered from light for 30 minutes.

9. Rinse the slide with carbonate-bicarbonate buffer.

10. Soak the slides for 10 minutes in carbonate-bicarbonate buffer in the glass jars covered from the light.

11. Rinse the slides thoroughly with distilled water. Rinse well to remove buffer mixture as residual buffer crystals will fluoresce.

12. Allow the slides to air dry in the dark. DO NOT BLOT DRY.

**Part 4: Slide examination**

1. Place a drop of Zeiss immersion oil in the center of each smear on the multi-well slide

2. Place a long coverslip (No. 1) 22 x 50 mm over the wells, and press out any air bubbles using the eraser end of a pencil. Place a drop of immersion oil on the coverslip above each smear. Wipe off any excess mounting fluid from the edges of the slides.

3. Using a UV light microscope and a 1000x mag., examine the positive control slide that was stained with the test slides that will be examined to ensure that the staining procedure worked. Small rod-shaped bacteria in singles and pairs that fluoresce when examined under a microscope (1000 x mag) equipped for epifluorescence.

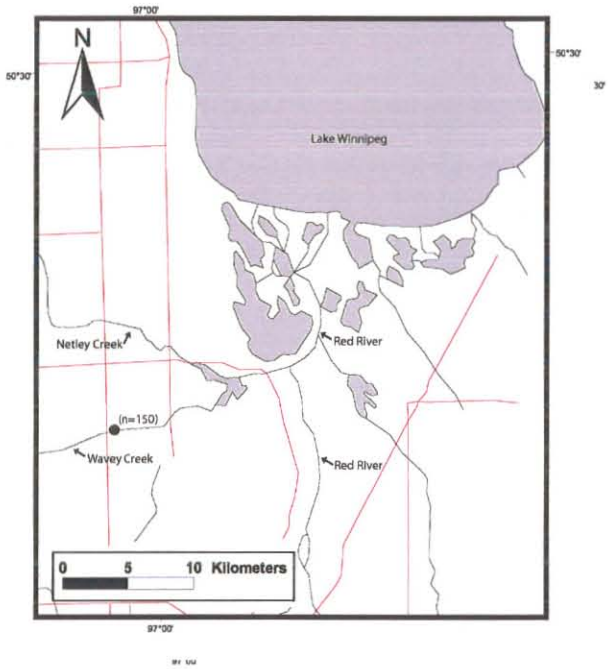
4. Read each well across its diameter. Read along the top row of wells and then back along the bottom row of wells. When the width of the well is measured and divided by the field width of the microscope, two passes of the well diameter provide the minimum of 50 fields examined per well (i.e. 100 fields examined per fish).

5. Record the results in the tally sheet. For positive wells indicate the number of positive fields observed, and, if possible, the number of bacteria per field. Indicate the number of fish positive out of 60 fish examined. Initial each set of results recorded.

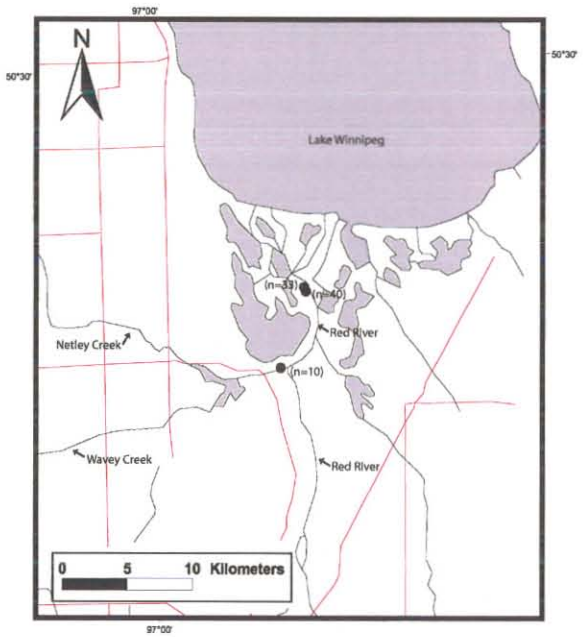
## Appendix 2

## Maps of Lower Red River/South Basin Lake Winnipeg Showing Collection Sites for Targeted Fish Species

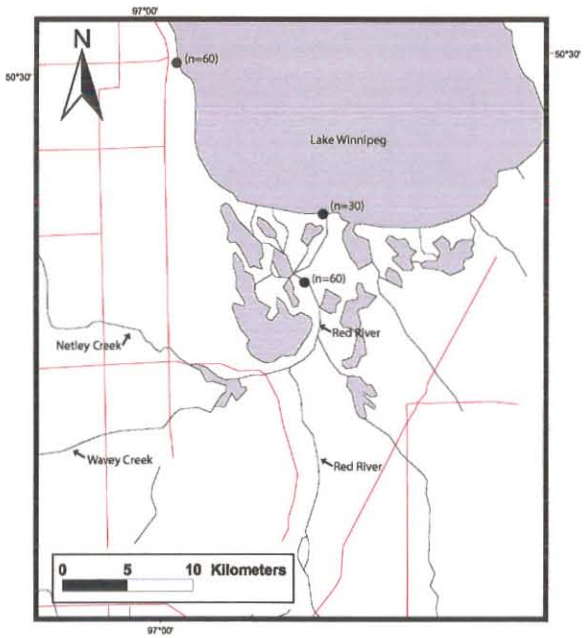
Fathead Minnow



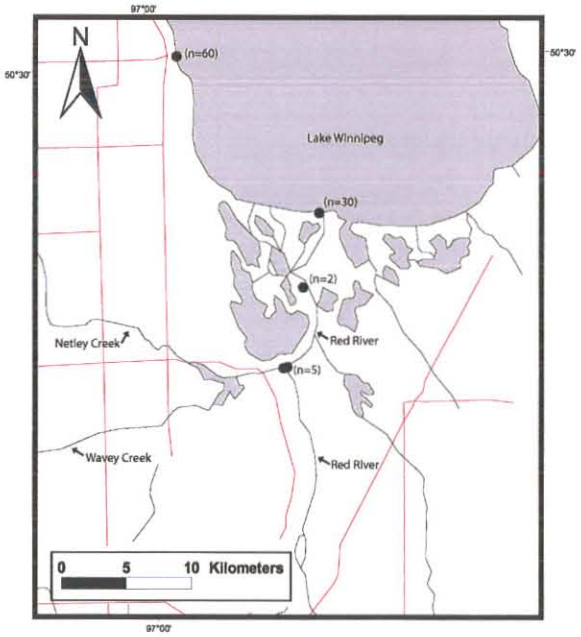

Goldeye



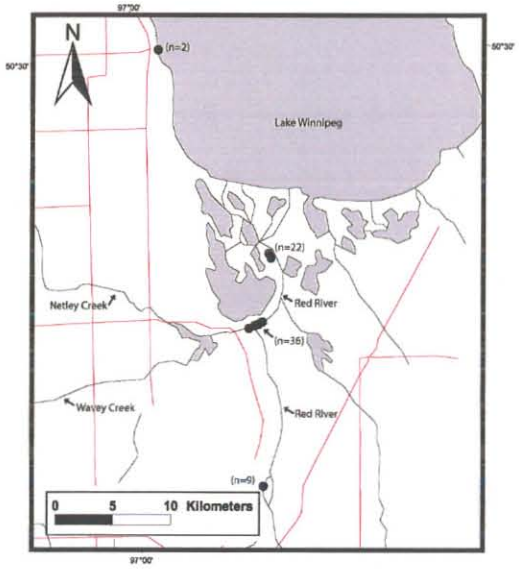

Emerald Shiner



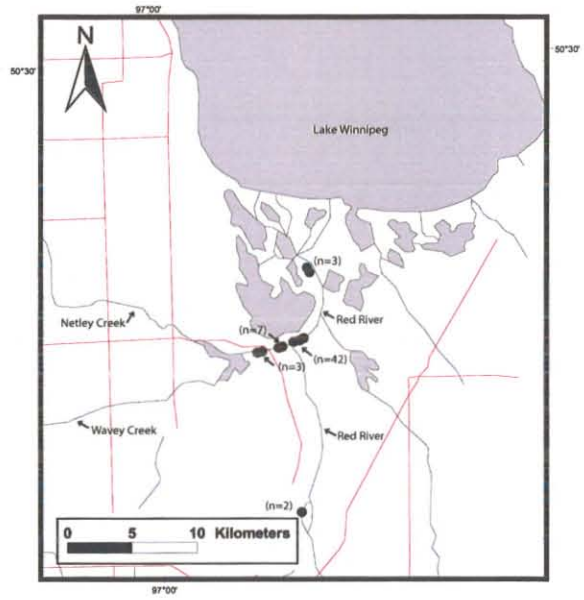

White Bass



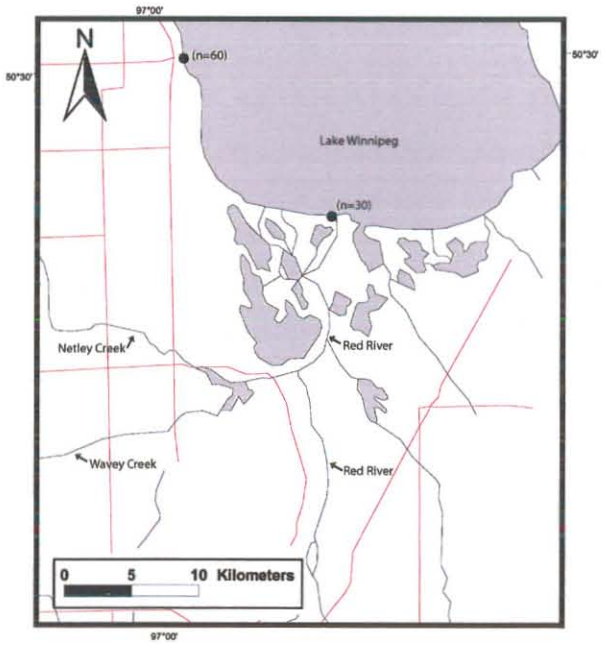
Sauger



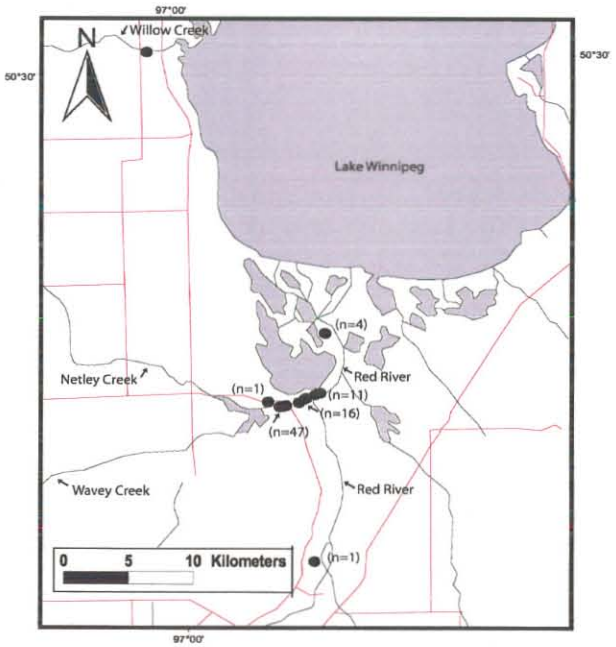

Walleye



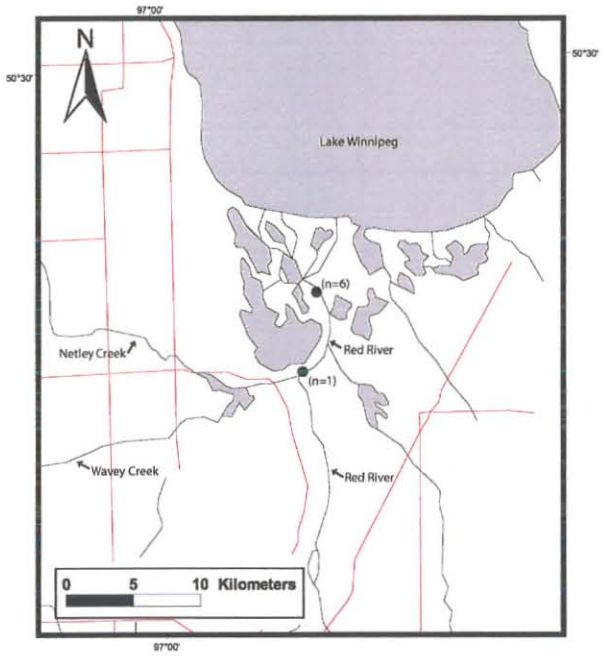

Yellow Perch



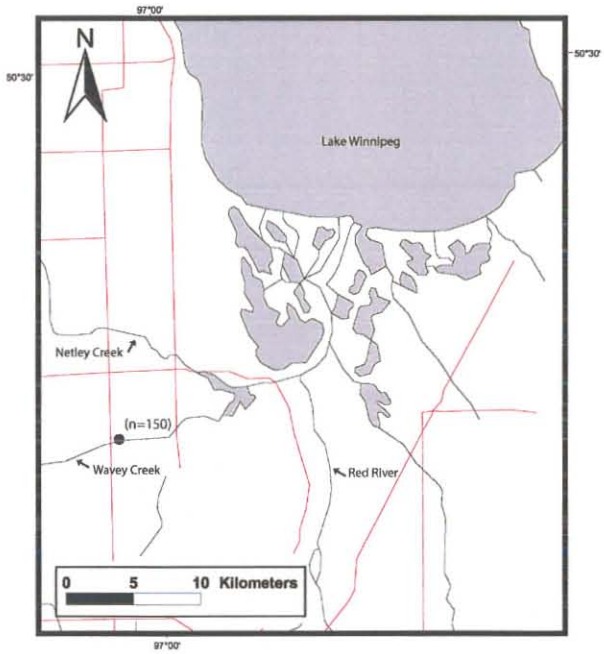

Northern Pike



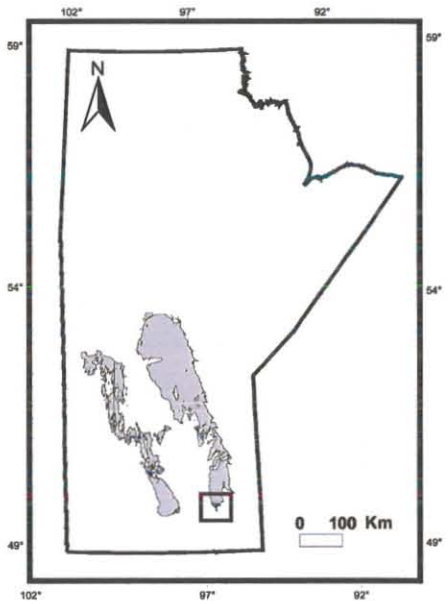
Channel Catfish




Brook Stickleback




Map of Manitoba



Lake Whitefish