

Environmental Toxicology Laboratory
Toxicology Centre
University of Saskatchewan

STANDARD OPERATING PROCEDURE

UofS-ETL-EDNA-29

**Sub-chronic aqueous chemical exposure for assessing
gut microbiome perturbations in fishes**

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APPROVAL PAGE

Revisions to an existing SOP, addition of a SOP change form, or preparation of a new SOP must be reviewed, approved, and signed by the following:

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(QA Coordinator)

DEFINITIONS AND ACRONYMS

ATRF	Aquatic Toxicology Research Facility
CCAC	Canadian Council on Animal Care
PI Primary	Investigator
SOP	Standard Operating Procedure
QC	Quality Control
UCACS	University Committee on Animal Care and Supply
U of S	University of Saskatchewan
ETL	Environmental Toxicology Laboratory (University of Saskatchewan)
DQO	Data Quality Objective
DHSE	Department of Health Safety and Environment (University of Saskatchewan)
QA	Quality Assurance
SOP	Standard Operating Procedure
PPE	Personal protective equipment
LC-MS	Liquid chromatography tandem mass spectrometry

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1.0 PURPOSE

This SOP has been developed under University Committee on Animal Care and Supply guidelines to gain better insights into fish gut microbial function during an aqueous exposure to a toxicant.

2.0 SCOPE AND APPLICATION

This SOP applies to the ETL for gut microbial samples in order to better understand how toxicants may impact the gut microbiome of fishes.

3.0 SAFETY CONSIDERATIONS

Safety considerations are under accordance with the U of S Safety Resources procedures and the U of S ATRF Safety Manual.

When using chemicals proper PPE is required including lab coats, gloves, face masks and safety glasses in accordance to the safety protocols for the chemical of use.

When handling fish, proper protocols will be maintained including handling fish with care to reduce stress of the fish, and use of gloves, and lab coats to reduce the spread of potential diseases between fish and humans.

Working environments will be disinfected using 70% ethanol. All hazardous materials will be disposed of in appropriate containers. Disposal of hazardous materials, dead fish and biological material will be done in accordance with the U of S Safety Resources procedures.

According to the ATRF Management Committee, all effluent will be directed into the appropriate effluent sewers which will then run through decontamination media before it is disposed of into the municipal sewers.

4.0 EQUIPMENT, MATERIALS, AND REAGENTS

- One hundred adult mixed fathead minnows
- Twenty 20L-tanks
- Blood worms
- Chemical of interest (154.014 mg of fluoxetine)
- Eight Water heaters
- Five multiple output air pumps

- Twenty Aquarium filters
- Anesthetic MS-222
- pH and ammonia test kits
- Four 100L-carboy containers
- Dissecting scissors
- Forceps
- Razor blades
- KimWipes
- Buckets
- Nets
- Heparinized Capillary tubes
- Centrifuge tubes (micro, 2 or 1.5-mL?)
- 70% ethanol
- Air stones
- Breeding tiles
- Extension cords
- 4 water tables frames
- 8 thermometers
- PVC stands
- Rubber pipes to siphon water
- Liquid nitrogen

5.0 METHOD, PROCEDURES, AND REQUIREMENTS

Exposure system set up

- Four water tables will be needed to house 20, 20-liter tanks, 5 tanks per table (one table per exposure).
- Tanks will sit on PVC stands within water baths to allow the water to flow underneath the tanks.
- Two heaters will be placed in each water table – be sure to always have heaters completely submerged in water.
- Water will be added to tables so that tanks are submerged in approximately 3 inches of water.
- Add two thermometers to each water bath, one on each side to verify temperature.
 - o Temperature of water baths should be 25°C +/- 2°C as per OECD229.
- Water quality of tanks will be tested every day to observe pH, ammonia, nitrates and dissolved oxygen.
- Lighting will be linked to the ATRF timer of 16 light: 8 dark according to OECD 229

Carboy reservoir set up

- Four 100L labeled carboy reservoirs will hold each treatment concentration (control, low, medium, high $\mu\text{g/L}$).
- Carboys will be filled with 100L of ATRF dechlorinated water and the appropriate concentrations of chemical.
- Carboys will be filled after every renewal with consistent concentrations and adjust to 25°C.
- Dosing buckets, labeled, will be filled with treated water from the carboy holding containers to then fill tanks.

Tank set up

- Airline tubing with an air stone will be assigned for each tank.
- Airline tubing is connected to a 3 or 4-way splitter connected to air pump.
- One breeding tile (1/4" PVC pipe cut in half) positioned at the back of the tank.
- One aquarium filter with air stones will be placed on the side of each tank.

Cleaning

- Before exposures, clean tanks and water tables with vinegar to remove calcification.
 - o Vinegar in water should be 2-3%
- Let tanks and water tables soak in bleach overnight to disinfect.
 - o Bleach in water should be 5 to 10%
- All reusable items in-line and in direct contact with water for fish should be cleaned before and after each experiment following the steps below:
 1. Soaked and scrubbed in soap and water
 2. Rinsed 3 X with filtered water
 3. Soaked and scrubbed in diluted vinegar
 4. Rinsed 3 X with filtered water
 5. Soaked and scrubbed in diluted bleach
 6. Rinsed 3 X with filtered water
 7. Rinsed in diluted acetone bath
 8. Rinsed 10 X with filtered water

Effluent drainage

- Depending on the chemical, all waste-water should be disposed of down the rubber or carbon mulch before it is disposed of via the waste management facility.

Methods

This sub-chronic exposure will take place over 28 days. Overall, one hundred adult fish are required. There will be five replicates per treatment consisting of three treatments plus a control. Each replicate will consist of five fish per tank. Fish will be randomly assigned to each tank keeping in mind two males and three females per tank and fish will need to be acclimated at least 24 hours after transport before use.

- 1) Once tanks are set up and contain specified concentrations of chemical (control, low, medium, high), allow for water to adjust to 25°C, collect a sample for chemical analysis and record water quality (pH, dissolved oxygen ammonia and nitrogen).
- 2) Light/dark period should be adjusted 16:8 in accordance to the University Committee on Animal Care and Supply guidelines.
- 3) Randomly select five adult fish (2 males and 3 females), record weight and length and add them to the first tank. Do the same for the next 95 fish. Density of fish biomass per liter tank should be less than 0.5-1 g/L. Average adult fathead minnows weigh approximately 3-5g allowing the use of a 20L tank for five fish.
- 4) Do not feed for the first 12 hours to reduce ammonia concentrations since they will be too stressed to eat.
- 5) After the first 12 hours, feed fish twice a day with blood worms at 2-3.5% feed per gram of body weight.
- 6) For cleaning tanks, use rubber pipes with mesh at the bottom, as to not vacuum up fish, siphon out $\frac{3}{4}$ of tanks into biohazard bucket.
- 7) Dispose of contaminated water down carbon mulch waste-water pipes in the ATRF.
- 8) Using the carboy method, water changes should happen every other day unless ammonia levels are too high then water changes should occur every day.
- 9) Before every water change, a sample of carboy water will be taken to analyze exact concentrations using the LC-MS.
- 10) Record any anxiolytic, bottom or top dwelling or reduced feeding type behavior.
- 11) Exposure will be conducted 28 days.
- 12) The day before sacrificing the fish, fish should not be fed.
- 13) On the 28th day, all fish will be weighed, and length measured.
- 14) Using MS-222, fish will be anesthetized for blood extraction and body length and mass will be recorded.
- 15) Euthanize fish by decapitation (SOP E201).
- 16) Extract gut, spleen, liver, head kidney and gonads (SOP C301).

- 17) Add individual organs into labeled centrifuge tubes and immediately put them in liquid nitrogen to later be put into -80 °C freezer.
- 18) Labelling should include date, concentration fish was exposed to, and the organ that is in the tube.

6.0 POTENTIAL COMPLICATIONS AND TROUBLESHOOTING

Potential complications will be avoided by using proper techniques. However, complications such as death of fish due to transport will be circumvented by receiving more fish than are needed for exposures. Water quality will be tested every day, however, if ammonia or pH levels are not conducive, addition of air pumps, filtering of water and biofilms may be implemented.

7.0 SAFETY CONCERNING CHEMICAL OF USE (FLUOXETINE)

Fluoxetine is a selective serotonin reuptake inhibitor. The Chemical Abstracts Service (CAS) number is 56296-78-7. Hazard identification for this product include: Acute toxicity: Oral, Category 3; Eye irritation, Category 1; Aquatic toxicity (acute), Category 1 (Sigma Aldrich). Fluoxetine's solubility in water is 4 mg/mL and it is a basic, lipophilic compounds with an acid dissociation constant (pKa) value of 10.5, a log octanol-water partitioning coefficient (K_{ow}) of 1.22 and sorption coefficient ($\log K_{oc}$) of 4.65 (Adlard, Okafo, Meenanb, & Camilleri, 1995; Bi et al., 2018; Black & Armbrust, 2007). The LC_{50} for rainbow trout is 1.57 mg/L within a 96-hour exposure (Sigma Aldrich).

Methods developed using modifications from the EPA OCSP guideline 890.1350 (Ankley et al., 2001; U.S. Environmental Protection Agency, 2011)

8.0 REFERENCES

- Adlard, M., Okafo, G., Meenanb, E., & Camilleri, P. (1995). *Rapid Estimation of Octanol-Water Partition Coefficients using Deoxycholate Micelles in Capillary Electrophoresis*. *J. CHEM. SOC., CHEM. COMMUN* (Vol. 224). Retrieved from <https://pubs.rsc.org/en/content/articlepdf/1995/c3/c39950002241>
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algal species to triclosan, fluoxetine and their mixtures. *Scientific Reports*, 8(1), 15361. <https://doi.org/10.1038/s41598-018-33785-1>

Black, M., & Armbrust, K. (2007). *The Environmental Occurrence, Fate, and Ecotoxicity of Selective Serotonin Reuptake Inhibitors (SSRIs) in Aquatic Environments* / Research Project Database | Grantee Research Project | ORD | US EPA. Retrieved from https://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.highlight/abstract/1755/report/0

U.S. Environmental Protection Agency. (2011). *Standard Evaluation Procedure (SEP) Fish Short-Term Reproductive Assay OCSPP Guideline 890.1350*. Washington DC. Retrieved from https://www.epa.gov/sites/production/files/2015-07/documents/final_890.1350_fish_short_term_reproduction_assay_sep_10.6.11.pdf

APPENDIX A: TANK RECORD SHEET

Tank Record Sheet

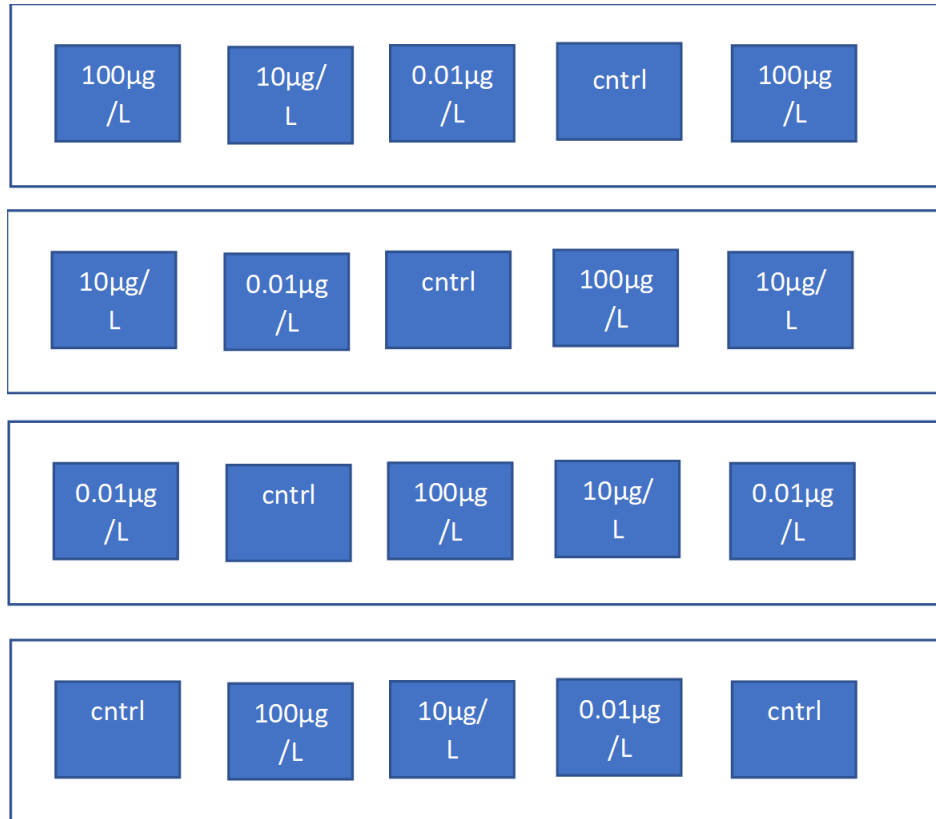
Project:		
Tank number		
Date:		Time:
Water Quality:	DO:	Ammonia:
	Water temp:	pH:
Description of behaviors/observations:		

APPENDIX B : CHECKLIST

Statue Number Item

- 100 adult mixed fathead minnows
- 20, 20L tanks
- Blood worms
- Chemical of interest (154.014 mg of fluoxetine)
- 8 Water heaters
- 20 Air pumps
- 20 Aquarium filters
- Anesthetic MS-222
- pH and ammonia test kits
- 4 100L carboy containers
- Dissecting scissors
- Forceps
- Razor blades
- KimWipes
- Buckets
- Nets
- Heparinized Capillary tubes
- Centrifuge tubes
- 70% ethanol
- Air stones
- Breeding tiles
- Extension cords
- 4 water tables frames
- 8 thermometers
- PVC stands
- Rubber pipes to siphon water
- Liquid nitrogen
- Sharps container
- Notebook
- Pencil
- Nitrile gloves

TANK LAYOUT



Depiction of water tables containing 5 20L tanks per table with specified concentrations.