11/16/05
Study Update

The Pittsburgh Fish Consumption Study has completed river fish collection for 2005. All fish have been dissected as detailed in the Implementation Plan (below). Store-bought fish are now also being prepared for analysis. Analysis of filets for metal content and estrogen binding capacity will begin shortly.

Implementation Plan

Development of a Community Based Participatory Environmental Research Project: Focus Groups to Investigate Fish Consumption Patterns. A Screening Assessment for Metals and Estrogenicity in White Bass and Channel Catfish Caught in the Three Rivers Area of Pittsburgh, Pennsylvania

Conrad Daniel Volz, DrPH, MPH[1,2,3], Devra Davis, PhD, MPH[4,5], Myron Arnowitt[6], Marcia Barr, BSCE [7], Sean Brady[8], Patricia Eagon, PhD[9], Linda Pearce, PhD[1], Jim Peterson, PhD[1], Ravi Sharma, PhD[10], Nancy Sussman, PhD[1]

1. Graduate School of Public Health, Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, Pennsylvania
2. Consortium for Risk Evaluation with Stakeholder Participation (CRESP), Piscataway, New Jersey
3. Graduate School of Public Health, Scientific Director, Center for Healthy Environments and Communities, University of Pittsburgh, Pittsburgh, Pennsylvania
4. Graduate School of Public Health, Department of Epidemiology, University of Pittsburgh, Pittsburgh, Pennsylvania
5. University of Pittsburgh Cancer Institute, Director-Center for Environmental Oncology, Pittsburgh, Pennsylvania
6. Clean Water Action (CWA), Pittsburgh, Pennsylvania
7. University of Pittsburgh Cancer Institute, Environmental Assessment, Monitoring and Control Division Director, Center for Environmental Oncology, Pittsburgh, Pennsylvania
8. Venture Outdoors, Pittsburgh, Pennsylvania
9. Department of Gastroenterology, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania
10. Graduate School of Public Health, Department of Behavioral and Community Health Services, University of Pittsburgh, Pittsburgh, Pennsylvania

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I. Study Purpose

Subsistence fishing provides nutrition for some low income residents of the Allegheny County area. African Americans disproportionately rely on subsistence fishing. Additionally, sports fishing groups increasingly angle in the waters of the Allegheny, Monongahela and Ohio Rivers, known as the Three Rivers Area (TRA), for predatory species including largemouth and smallmouth bass. Information is not generally available regarding the demographics of these fishermen in the TRA; the species and sizes of fish caught and preferred by each group for consumption; the methods used to clean and prepare as well as cook wild fish for eating; and the types and amounts of fish consumed in any given time period of each season. Initial information from both representatives of sport-fishing and meat-fishing groups indicates that the White Bass and the Channel Catfish are important fish for table usage in Western Pennsylvania and Allegheny County, descriptive information obtained from the Pennsylvania Fish and Boat Commission (PFBC) on each fish is presented in Appendix A.

There is abundant evidence that legacy wastes from the Iron and Steel Industry (ISI) have and continue to contaminate fish habitat through runoff and leeching from RCRA, Superfund and Brownfield sites; transport from contaminated surface soils, subsurface media and groundwater transport; and residual toxins deposited in river bottom sludge and sediment. Ongoing industrial operations, overflow from municipal wastewater plants and urban runoff add contaminants including polycyclic aromatic hydrocarbons (PAH), household chemicals and products, estrogenic compounds and heavy metals to each rivers ecosystem.

Upon entering the rivers, water contaminants enter a complex food web and can move between trophic levels. These contaminants can also be deposited in river sediment. Plants and animals that are low on the food chain take up contaminants as well as nutrients via bio- concentration and bio- accumulation. Bio-magnification of contaminants can occur as you move up the food chain to predatory fish through processes such as the concentration of many organic pollutants in lipids and the binding of heavy metals to proteins. Sediments on river bottoms act as a sink and source of contaminant dispersion during high water periods. Additionally, sediments containing contaminants deposit in slow water pools behind dams, built for navigation.

There exists the potential for human exposure to a variety of contaminants from ingestion of fish caught in the TRA. Detailed studies of the Mahoning River (MR), used for ISI and municipal waste disposal in analogous fashion, to the TRA of Pittsburgh, have shown bottom sediment containing extremely high levels of heavy metals (mercury, lead, zinc, copper, cadmium, silver and iron), grease, oil, organic compounds, PCBs and PAH and pesticides. According to the Pennsylvania Department of Environmental Protection (DEP) Watershed Management tool

eMapPA major river segments of both the Three Rivers and major feeder streams are considered impaired under their 305(b) layer. This means that impaired sections of the river and streams are not attaining at least one of the following uses;

- Aquatic Life use attainment - The integrity reflected in any component of the biological community. (i.e. fish or fish food organisms)
- Human Health use attainment - The risk posed to people by the consumption of aquatic organisms (ex. fish, shellfish, frogs, turtles, crayfish, etc.) or the ingestion of drinking water
- Recreational use attainment - The risk associated with human recreation activities in or on a water body. (i.e. exposure to bacteria and other disease causing organisms through water contact recreation like swimming or water skiing)

Particularly disturbing is information from the Pennsylvania Fish and Boat Commission that spots for catching large predatory fish are located at the mouth of streams feeding the Allegheny River, many of which are continuously or intermittently impaired.

It has been reported that the estrogenic activity of effluent-dominated streams is increasing. These estrogenic compounds are theorized to come from pharmaceuticals - especially hormone replacement drugs, cosmetics and creams, animal feedlot estrogenic compounds, building materials and household products and natural products. Since rivers and streams have been channelized causing substantial runoff from urban areas and suburban developments, without sufficient time from swamp, eddy or pooling sedimentation or soil and sub-surface cleansing of water, these estrogenic compounds quickly find their way into large stream bodies and the municipal water supply. There is no treatment for these chemicals at system intakes presently. Fish can serve as the "canary in the mine" for human exposure.

Screening for estrogenic effects in male fish in Germany, Norway and the United States has shown highly elevated levels of blood plasma vitellogenin concentrations in male fish. Vitellogenin is a protein used for egg yolk production and would not be expected to be found in high concentrations in male fish. The British Royal Society released a report in June, 2000 which states that all sewage effluents in the UK have enough estrogenic activity to feminize fish, further they have discovered that some fish in ALL rivers of the United Kingdom are intersex. The Royal Society study found that in rivers with high levels of effluent that all male fish were intersex to varying degrees. Similar findings have been described in the Upper and Lower Potomac River by the United States Geological Survey (USGS). Fish in the Upper Potomac were being studied in order to determine if there was a link to higher than normal human cancer rates in the area.

Store bought fish consumption is also an important component of the diet of anglers and non-anglers in the Pittsburgh area. There exists no data on their fish purchasing

and consumption patterns or methods of cooking. Data are also missing on the level of contaminants in store bought fish in the Pittsburgh area, of particular interest are those fish labeled organic in stores. Both White Bass (or equivalent trophic level) and Channel Catfish are commercially available.

II. Study Objectives

1. Identify fishing groups to partner with for the study and explore the TRA for fishing holes to identify groups of meat-fishers and low income fishers. Conduct two (2) focus group meetings with identified groups in order to better understand qualitatively – group demographics, attitudes to river water quality, the locations of fishing, types of fish taken, sizes preferred, number of fish meals eaten per week, the serving size eaten at each meal, and the methods used to clean and cook each type of fish. Build community environmental capacity within these groups to include an education program regarding contaminants in fish so that groups can actively participate in the initial screening and possible downstream research studies and understand the risks associated with wild fish consumption.
2. In conjunction with Objective #1, obtain nautical maps of the TRA for the purpose of establishing the boundaries of the study. Maps will be digitized so that the locations of fishing spots and all catches may be geo-referenced. Additionally, the locations of sewer outflows, water intakes, active and inactive industries, and brownfield, CERCLA and RCRA sites will be shown on finished maps.
3. Catch 40 White Bass and 40 Channel Catfish by rod and reel (the way that fishers catch their fish so that more accurate risk assessments of the fish can be made). The study will attempt to achieve a balanced design so that 10 fish of each species come from the Allegheny, Monongahela and Ohio Rivers. Additionally, 10 fish of each species will be caught in the upper reaches of the Allegheny and Monongahela Rivers and 10 fish of each species will be bought commercially.
4. Prepare all fish for analysis including division of fish into two fillets- one for metals analysis and one for estrogenic activity. Each organism's kidneys, gonads, heart and liver will be archived for latter studies.
5. Screen all fish for metal contamination. Fish will be analyzed by ICP-AES for a suite of metals including: mercury, cadmium, arsenic, manganese, lead, chromium, copper, zinc, selenium and cobalt. Report measures of central tendency and the variability of each contaminant in each fish species in the TRA and Upper River areas. Evaluate the metal concentrations between all sampling sites to the extent possible given the small sample sizes.
6. Screen all fish for estrogenic activity using two assays (See methods below). Evaluate the efficacy of testing the fish extracts in 3 breast cancer cell lines.
7. Prepare a report detailing the results of the focus group work, analyze fish consumption patterns qualitatively. Report the results of and analyze the metals and estrogenicity fish screens. Include all maps generated during the study. Develop a detailed list of recommendations for further study of TRA fish consumption patterns and fish at each tropic level including their habitat and comment on the policy implications of findings.
8. Preliminarily assess health risk from eating locally and commercially caught White Bass and Channel Catfish.

III. Study Methods and Detailed Protocols

The methods of the study can be divided into four major parts;

A. Focus Groups

At least two focus groups will be convened to obtain in-depth qualitative information on fish consumption patterns, size and species preferences, favorite fishing areas, and methods of cleaning and cooking. One focus group will be made-up of sports fishing group members and another from subsistence style fishers. These focus groups will be informal and participants will be encouraged to talk about fishing experiences, perceived needs, and observations and perceptions of water and fish quality. Each session will last approximately 90 minutes and the group will not contain more than 10 members to encourage interaction between members. One objective of the focus groups is to gain information to develop a Community Based Participatory (CBP) approach to to help define the research objectives for further research on the TRA.

Subsistence type and sports fishermen will be reached by river bank and power boat surveys on the Three Rivers. Launch areas will be targeted. It is anticipated that sports fisherman can be reached through regular meetings of their groups while the subsistence group will be primarily contacted while fishing.

Additional Information for the Behavioral and Community Health Study Section

Fisherman Interviews

Charles Christen and the PI have developed an initial list of questions (included in the Institutional Review Board {IRB} approved submittal attached as Appendix B). Both Mr. Christen, a doctoral student in Behavioral and Community Health Services {BCHS} and Dr. Volz will interview fisherman on the Three Rivers (TR) using this initial list of questions as a starting point for a more in depth anthropological study of the culture of fishing and attitudes and beliefs regarding fishing as well as the water quality on the TR. This information will be used to develop a qualitative manuscript on fishing attitudes and beliefs and river quality feelings. This information will also help inform the development of additional questions for the focus groups described above. During these interviews it is expected that recruitment for these fisherman-based focus groups will be accomplished.

Interviews of the General Public

Interviews of the general public will be performed at Wholey's Fish Market, Whole Foods, as well as at a Giant Eagle in a primarily African –American neighborhood. We anticipate the usage of graduate students doing independent study as well as undergraduate researchers helping in this process. The general IRB questionnaire

will be amended for this group and additional questions will be added according to qualitative techniques established by Kidder and Fine.

B. Fish Catch

Fish will be caught from shore and from boats by fisherman enlisted by the PI and his assistants. All fishermen will follow the regulations of the Pennsylvania Fish and Boat Commission. GSPH researchers will be assisted in fishing spot locations, and fish identification by Venture Outdoor Director, Sean Brady. The weight, sex, standard, fin and total length, head, snout and post-orbital length, body depth and girth of all fish species caught will be noted. The GPS coordinates of all White Bass and Channel Catfish will be recorded. Each fish will have a unique specimen number and will include the initials of the river the fish was caught in, the date caught, the fish type, and the tissue type (See Below). The heart, gonads, liver and kidneys of each fish will be archived for analysis in subsequent studies (See Labeling Below). Dissections will be performed in the field where possible. Other species of interest, caught by researchers, which can be legally taken will be sampled and archived for analysis in subsequent studies. Samples in excess of those that will be analyzed as part of this study are being taken so that the sampling distribution of each type of fish falls within plus or minus one (1) standard deviation unit of the mean where possible.

C. Protocols, Three Rivers White Bass and Channel Catfish Study- Fish Retention

Fishermen volunteering to fish for this study were told to keep fish alive as long as possible after catch in live wells if caught on a boat. On return to land, fish are killed by a cut through the gills. Fish are then stored on ice and given to the PI or representative within 24 hours.

Fish caught on land and donated to the study by fisherman will be/were put directly on ice and taken to a storage facility, where they were immediately dissected or frozen for dissection at some future time.

D. Sample Preparation

Dissection of specimens was/will be done as soon as possible but within 5 days after freezing or immediately if specimen is not frozen. Gross dissection for fillets, heart, liver, kidney and gonads as well as skin and stomach contents will be done outdoors on board a boat or at the PI's storage facility. Gloves will be changed between each sample, and aluminum foil (used to wrap frozen fish) will be discarded between each sample. Dissection tools will be washed as a group between samples. All surfaces and instruments will be treated with isopropyl alcohol after each specimen dissection.

E. Numbering System for Fish Caught & Catalogued for the Pittsburgh Fish Consumption Study

General Numbering

See Figure 3

a. Code Sequence

Study Number (001-100) – Date Caught (month-day) – Fish Type Code – River Code

b. Study Number – Fish will be numbered consecutively starting with the first fish processed and ending with the last fish processed. Beginning number is 001.
c. Date Caught – Fish will be caught in the fall of 2005 through the spring of 2006. The date caught consists of first the month caught and than the date of that month caught. So 1029 means that the fish was caught on October 29th of 2005.
d. Fish Type Code
    a. Channel Catfish
    b. Hybrid Stripped Bass
    c. White Bass
e. River Code-Where Caught.
    a. Monongahela River
    b. Allegheny River
    c. Ohio River
    d. Store Bought (Specify)
        a.) Whooleys
        b.) Whole Foods
        c.) Giant Eagle
    e. Mahoning River
    f. Other (Specify in database)
        a.) Point

See Figure 4 for Further Examples. 004-1004-2-2 - This code represents the fourth fish processed in the study, caught on the 4th of October, 2005. The type of fish in the sample is white bass, which was caught on the Allegheny River.

This code will be put on the 1 or 2 gallon outside packaging containing all the split, replicate, archived and retained samples from that specific fish.

Analysis Stream Numbering For Filets Undergoing Immediate Analysis

Each fish will be divided into two filets. Channel catfish will be skinned before filleting and white and hybrid striped bass will be de-scaled before filleting. Filets will be put in 1 gallon containers and labeled according to instructions contained in item I of this procedure. Labeling will continue in metals and Estrogenicity activity laboratories according to Figure 1 presented below.

Example; *089-0123-1-4a-E* – This denotes the 89th fish processed, caught on the 23rd of January, 2005. The fish was a Channel Catfish store-bought at Whole Foods

market. This section of the filet is slated for estrogen analysis as the primary sample to be analyzed.

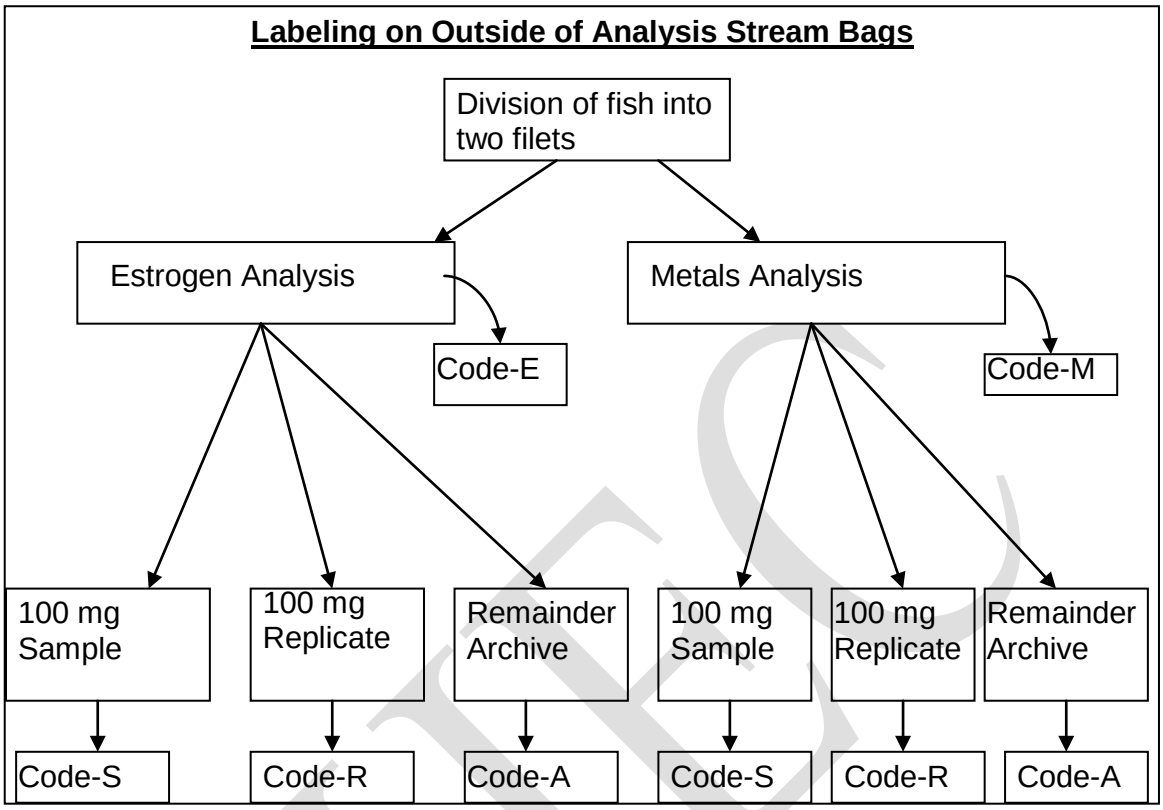

Figure 1. Major Analysis Stream Bags

Tissues of Individual Fish, Retained for Possible Analysis, Codes

Individual fish will be dissected according to the project plan and labeled as shown in Figure 2. See Figure 4 for numbering examples that include organs.

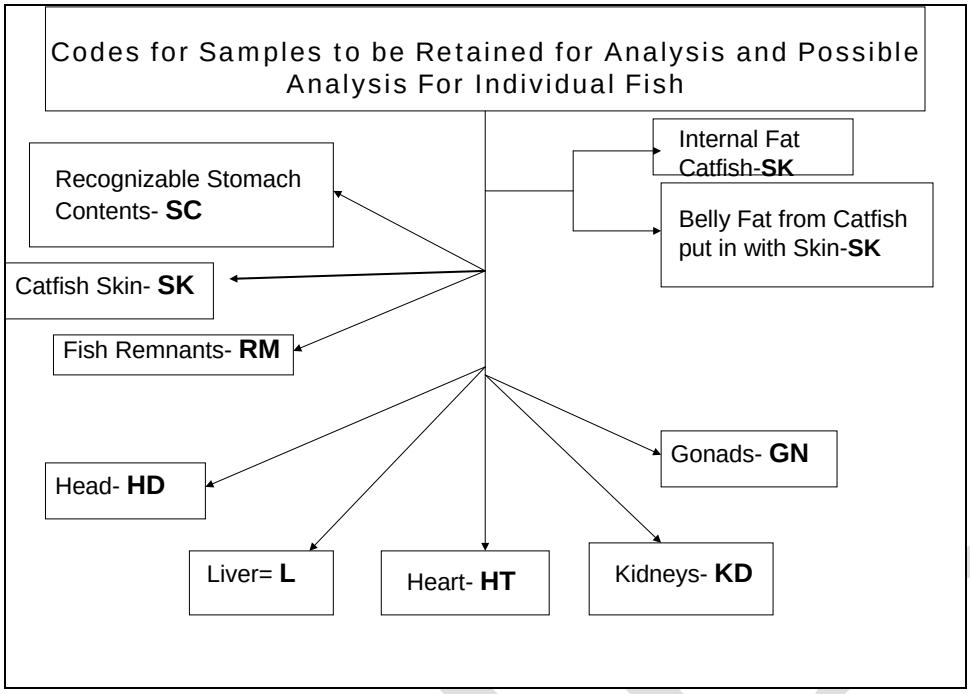

Figure 2. Tissues of Individual Fish, Retained for Possible Analysis, Codes

Numbering System for Individual Fish

1. **First number represents the order in which the fish were initially processed (001 to 100 or the highest number of fish dissections).**
2. **The second four digit number represents the date the fish was caught. For instance 1015 represents that the fish was caught on the 15th day of October. The Study PI will strive to catch all fish in the year 2005, if fish catch needs to be extended into 2006 the same system will be used. Since no fish are expected to be caught after April 2006, the numbering system representing date can be used and the year is understood.**
3. **The third number represents the type of fish caught:**

1) Channel Catfish.

2) Hybrid Stripped Bass.

3) White Bass.

6S) Shad (A baitfish at a lower trophic level).

4. **The fourth number represents the river on which the fish were caught:**

1.) Monongahela
2.) Allegheny
3.) Ohio
4.) Store-bought
5.) Mahoning
6. Other Specify
a.) PT= Point

Figure 3. Capsule-Numbering System for Individual Fish

Numbering Examples

A. Individual Fish

1. 004 -1019 -1- 1 This represents a fish that was dissected fourth, caught on 10/19/05 and it is a Channel Catfish caught on the Monongahela River.

2. 087-1109-3-6PT This represents a fish dissected 87th caught on 11/09/05 and it is a White Bass caught at the Point.

B. Fish Samples and Organs

1. 093-1115-1-2HT This code represents the Heart of a Channel Catfish caught on the Allegheny River on 11/15/05. It was the 95th fish dissected.

2. 063-1031-3-3GN This code represents the Gonads of a White Bass caught on the Ohio River on 10/31/05. It was the 63rd fish dissected.

Figure 4. Capsule Examples of Numbering System

Sample Packaging

The above samples from each fish will be put in quart bags and labeled according to the outlined scheme. All samples from the same fish will than be put into one gallon plastic bag, which has the omnibus sample number for that fish written on the outside of the bag

Sample Storage

At the PI's storage facility, located at 166 Shephard Road, Gibsonia PA 15044 samples that have been processed will be placed in a standard household freezer. These samples will be transferred to Dr. Pat Eagon's laboratory and kept at minus 60 degrees Centigrade until metal or Estrogenicity testing.

Chain of Custody

All samples will have a separate Chain of Custody form attached to them linking them uniquely to the original specimen.

Specimen Handling at UPMC & EOH

a.) **Specimen tracking -** A specimen tracking system has been set up by Chuck Christen, who will manage the electronic data base with the help of Tiffany Green during all analysis flows as well as tracking split samples, which may go to an EPA laboratory. The database was prepared using SPSS Version 12.01.

b.) **Specimen selection -** Specimens to be prepared each day will be selected by Eagon, Peterson and Volz. All steps will be recorded in a laboratory notebook, and later entered into the computer data base. Christen and Green may assist in this process as needed.

c.) **Specimen handling-** Specimens will be defrosted and 100 gram tissues measured. Samples for replicate and archival streams will measured and labeled for retention at that time.

Species Identification

Important aspects of biological sampling - There are three features of biological collection are crucial to QA/QC: (1) Correct identification of species. (2) Adequate description of collection location physically. (3) Adequate description of habitat location.

a.) **Correct identification of species -** All personnel involved in collection of any biological samples will be under the direction of someone who is expert in the identification of the species, and has been involved in collections previously: See Appendix A for identification of White Bass and Channel Catfish.
b.) **Voucher specimens-** A voucher specimen from each species will be collected whole, frozen entirely.
c.) **Photographic voucher-** A digital photograph will be taken of each species type for purposes of future verification if necessary.
d.) **Collection location physically-** The locations of all collections will be recorded in the field and in the laboratory notebooks. All physical locations will be noted using GPS in digital format.

Protocol for Estrogenicity Assay & Downstream Work

Fish parts would be extracted by organic solvent extraction as determined, and samples would be tested in two assays to determine estrogenicity. A screening assay, the competitive cER-α assay, would be used to screen 100 samples. It is less expensive, and generally predicts interactions with estrogen receptors. Depending on the results of this assay, we predict that we would screen about 20% of the same samples using the cER-ß assay, which is more expensive but can detect other types of estrogenic substances. After analysis of these data, we would evaluate the efficacy of testing the fish extracts in the breast cancer cell lines. We predict that 20% of the samples would be tested in our standard assay (3 different cell lines, as noted below).

1. **Competitive <u>in vitro</u> cytosolic estrogen receptor (cER) assay**: This assay determines the ability of agents to interact with the estrogen receptor and generally predicts estrogenicity. Details of our specific assay have been published (1 and references therein). Briefly, aliquots of cytosol prepared from livers of ovariectomized female rats (a source of ER-α) or cytosol from ventral prostate of male rats (a source of ER-ß) are incubated with 5nM [^{3}H]-E_2 in the absence (control) and presence of test substances. Estrogens E_2 and diethylstilbestrol (DES), the potent and nonaromatizable androgen

dihydrotestosterone (DHT), and the phytoestrogens genistein and/or coumestrol will be used in concentrations of 5nM-50mM, which represent a range of 1X-10,000X the concentration of labeled E_2. The individual **FISH** extracts will be tested from dilutions of 1/20th to full-strength, or more dilute as needed. The mixtures are incubated at 4°C overnight, and bound ligand is separated from free by use of spin columns made of P6 resin (BioRad Inc.) (2) or by dextran-coated charcoal treatment (3). The ER binding assay can be used to estimate an "E_2 equivalence" of the crude extracts by comparing the competition of the extract with a dose-response curve generated using either E_2 or DES as a standard competitor. All competition is expressed relative to the results for control binding (100%) in which there is no competitor present.

In similar assays, we will determine if the extracts contain any components which might interact with the progesterone and androgen receptors. Our previous studies have determined that extracts of wild yam and squaw vine compete for binding to the progesterone receptor. The assays used for these studies have been published by our group (4-7). To date, we have not found any extract that interacts with the androgen receptor.

2. **Breast cancer cell lines (Aim #1):** We use a number of breast cancer cell lines that vary in their expression of ERs (MCF-7, HTB-129 (also known as T47D), BT-20) and normal breast cells (HMEC line). The MCF-7 cell line expresses primarily ER-α, HTB-129 express primarily ER-ß, and BT20 cells are ER-negative.

   Initial end points measured will be changes in cell number and viability. The proliferation assay is performed in phenol red free RPMI medium supplemented with 10% charcoal/dextran-stripped fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells will be seeded into 96 well plates at a concentration of 5000 cells/well. Twenty-four hours post plating, the cells will be treated with either **FISH** extract, estradiol ($1x10^{-9}$) *or both*. The plates are allowed to incubate for 72 hours, at which time the proliferation index relative to untreated cell controls and to the E_2-treated controls (10nM) will be determined using a commercial assay kit, MTS Proliferation Assay (Promega). Other treatment controls could include the *phytoestrogens genistein and coumestrol* (each at several doses, 1-100nM). An increase in proliferation index will be taken as an estrogenic response. In contrast, a reduction in the E_2-stimulted proliferation due to the presence of the extract will be taken as evidence that the extract may contain antiestrogenic substances. Use of the ER-negative BT-20 cells allows us to detect any cell toxicity that is not related to hormonally active components of the extracts. To confirm the findings that cell proliferation is mediated by ERs, another set of cells treated with extract will be simultaneously treated with the antiestrogen tamoxifen or ICI 182,780. **FISH** extracts will be tested over a

broad range of concentrations, to be determined by preliminary experiments.

If funding is increased over the course of this project the following detailed work can be done-also if there are findings that indicate that this work should be done the PI will immediately notify UPCI-CEO.

Once growth or inhibition due to treatment with a given **FISH** extract has been established, cells will be grown in either T-75 or T-150 flasks with an optimum dose of extract to obtain sufficient cells for RNA and/or protein extraction, to be used for other assays as noted below. Specifically, we will quantitate mRNA for ER-α and ER-ß to determine if the treatment alters the expression of these receptors. We will also quantitate mRNA for the estrogen-responsive markers progesterone receptor (PgR), c-Myc, cyclin D1, and pS2 by either Northern blots or real-time PCR, our methods for which are published (4). Apoptotic gene expression will also be measured. The balance between expression of the anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax is considered a good indicator of apoptotic activity of tumor cells. Bcl-2 and Bax expression seem also to individually play a prognostic role in breast cancer (8) and the ratio of Bcl-2 and Bax are useful indicator of the apoptotic rate in breast cancer tissue (8-11). Bcl-2, an anti-apoptotic factor, can prevent death if overexpressed. In contrast, Bax, a strong pro-apoptotic factor, can induce apoptosis if overexpressed (11). The susceptibility of tumor cells to apoptosis induced by anti-tumor drugs appears to depend on the balance between pro-apoptotic and anti-apoptotic signals. Therefore, the ratio of Bcl-2 and Bax will be determined in these cells and in the animal model described in Specific Aim #3. The Bcl-2 and Bax ratio will be measured by western blot analysis using monoclonal anti-Bcl-2 (catalog #sc-7382) and anti-Bax (catalog #sc-7480) bodies purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA) and as described (12).

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Protocols for Metals Analysis

One fillet of each fish will be used for metals analysis. Amounts over those necessary to perform the analysis will be achieved for latter use. Tissues will be digested by a nitric acid/hydrogen peroxide method – typically 2 mL 12 M ('metal-free') HNO_3 + 1 mL 30% (w/w) H_2O_2 added to ~1 g tissue, dissolved in 2% HNO_3 after the instrument-controlled microwave-based digestion cycle. Microwave-based approaches enable us to routinely prevent background contaminants from entering the samples.

Samples will be analyzed using Inductively Coupled Plasma- Atomic Emission Spectroscopy (ICP-AES) for a suite of metals including As, Cd, Cr, Mn, Pb, Se, Co, Cu, Fe, Ni, Zn. Mercury (Hg) will be measured by a Cold Vapor Technique.

III. Project Responsibilities

Responsibilities of C. D. Volz, DrPH, MPH – Project Principal Investigator

1. Create the descriptive study design and make alterations to the initial design with input from project participants and approval of Dr. Davis, UPCI, CEO.
2. Develop project plans and timetables.
3. Oversee all aspects of the study for compliance with the project plan and anticipated timetable. Report all potential problems to Dr. Davis, UPCI, CEO.
4. Obtain IRB approval for the study.
5. Recruit focus group subjects.
6. Hold focus groups and write observations, summarize findings for final report and manuscripts.
7. Insure that Co-Principal Investigators for metals and estrogenicity analysis report results on time and budget.
8. Review all metals and estrogenicity data for completeness and trends.
9. Insure data transfer to the project biostatistician.
10. Insure that fishing spots and catch are geo-coded.
11. Supervise Chuck Christen, Project GSR.
12. Assemble, write and edit final report and project manuscript.
13. Present report to UPCI-CEO and funders.

Responsibilities of Patricia Eagon, PhD – Co-Principal Investigator Estrogen Activity Analysis

1. Preliminarily assess the estrogen receptor binding strength of fillet tissue, liver tissue and gonadal tissue. Provide PI QA/QC procedures.
2. In discussions with the PI and Dr. Davis determine the sequence and types of tissues to be analyzed for alpha and beta estrogen receptor binding and cell line proliferation.
3. Perform analysis as agreed upon.
4. Inform PI of any significant findings as they are discovered. Change analysis course dependant on findings and discussions with the PI and Dr. Davis.
5. Enter all data into a laboratory book and electronic data base that is compatible with the system used by the PI and biostatician.
6. Write final report of results of tissue analysis and submit to the PI.

Responsibilities of Jim Peterson, PhD – Co-Principal Investigator Metals Analysis

1. Develop detailed processes and QA/QC procedures for metals analysis to include homogenization and analytical techniques.
2. Perform analysis of metals as proposed on all fillet samples and on other samples in discussion with the PI.
3. Inform PI of all significant findings as they occur. Change analysis course dependant on findings and discussions with the PI and Dr. Davis.

4. Enter all data into a laboratory book and electronic data base that is compatible with the system used by the PI and biostatician.
5. Write final report of results of tissue analysis and submit to the PI.

Responsibilities of Nancy Sussman, PhD – Project Statistician

1. Assist in the development of data tracking methods.
2. Provide descriptive statistics for both estrogen activity and metals analysis.
3. Mine data to look for associations between estrogen activity/cell proliferation and metals or groupings of metals.
4. Assist PI with provision of histograms and figures for the final report.

Responsibilities of Ravi Sharma, PhD – Map Generation for Sampling Locations and Sources

1. Geo-code positions of fish take, areas fished routinely by area fisherman, PA Fish and Boat Commission Launches and sources of contamination.
2. Generate maps, with accompanying methodology, for final report.

Responsibilities of Venture-Outdoors – Contact Sean Brady

1. Add to the project knowledge base regarding fish actually caught and eaten by fisherman in the Three Rivers Area (TRA).
2. Identify locations where these fish can be caught most easily.
3. Identify locations where both meat and recreational fisherman fish.
4. Assist in the identification of boats that can be used to provide an adequate platform for mass fish catch and processing.
5. Provide manpower to catch fish in the field and provide fish to project scientists. Assist in enlisting project volunteers to catch fish in the field and project scientists with fish cataloguing. Provide tackle and bait to catch white bass as well as channel catfish and other fish that might be necessary to round out the project.
6. Provide a few fish (3-5 of each species if caught and of size not to disturb breeding potential in area) from your Wednesday fishing at the point. Bring dead whole fish to Dr. Volz
7. Assist in determination of focus group identification of fisherman from the TRA. Give the PI names and phone numbers of potential group members.
8. Write a narrative regarding the methods used to catch target fish in the TRA including bait usage and provide logs of fish caught during the groups Wednesday fishing for statistical analysis by Pitt researchers. Include in these narratives anecdotal information on fishing over the years in the TRA by interviews with known long time fisherman.
9. Assist in the analysis of data and as data comes in suggest other fishing areas dependant on project needs.
10. Assist in the development of the final report and review same for accuracy.

Responsibilities of Clean Water Action – Contact Myron Arnowitt

1. Add to the project knowledge base regarding areas in the Three Rivers of critical concern due to either past or current industrial or municipal contamination sources. Especially important is the identification of sites that Clean-Water Action has some environmental data concerning, including the mining operations in the Ohio River. Provision of data on contaminant monitoring that can be linked to contaminant loads in fish.
2. Provide manpower (1 at least) to catch fish in the field and provide fish to project scientists (at least 1 day for one CWA employee-we want everyone to be comfortable with methods). Assist in enlisting project volunteers to catch fish in the field and project scientists with fish cataloguing.
3. Identify through your sources locations where both meat and recreational fisherman fish.
4. Assist in determination of focus group identification of fisherman from the TRA. Give the PI names and phone numbers of potential group members. Assist in the determination of fish- buying focus group members; give names and addresses to the project PI.
5. Write a short narrative regarding the areas on the river of greatest concern to CWA, include why they are of concern and provide data as per item (1).
6. Assist in the analysis of data and as data comes in -suggest other fishing areas dependant on project needs.
7. Assist in the development of the final report and review same for accuracy.

IV. Project Timetable

The project process is being tracked on Microsoft Project. Charts can be sent if the receiver has this software.

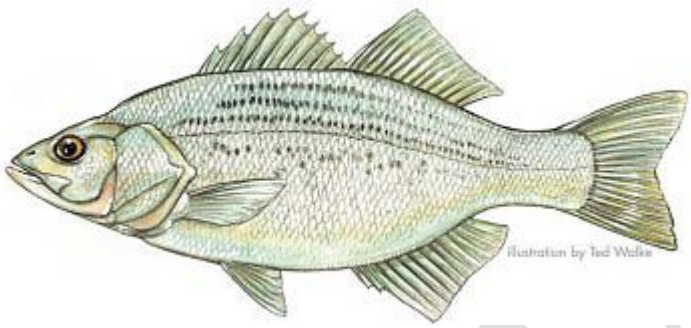
ID	Task Name	Duration	Start	Finish	Predecessors
1	Develop Fish Numbering and Identification System	6 days	Thu 9/15/05	Thu 9/22/05	
2	Assemble Fishing Groups	15 days	Mon 9/26/05	Fri 10/14/05	
3	Fish Spots on the Three Rivers per Proposal	45 days	Sat 10/15/05	Thu 12/15/05	2
4	Meet with Sportsman Groups	90 days	Wed 10/5/05	Mon 2/6/06	
5	Produce Initial List of Interview Questions	4 days	Mon 8/29/05	Thu 9/1/05	
6	Prepare and Submit IRB	2 days	Mon 10/3/05	Tue 10/4/05	
7	IRB Approval	30 days	Wed 10/5/05	Mon 11/14/05	6
8	Fish Dissections	50 days	Sat 10/15/05	Thu 12/22/05	
9	Hiring of Graduate and Undergraduate Help	30 days	Tue 10/4/05	Fri 11/11/05	
10	Initial Ethnograph Interviews of Fisherman	60 days	Mon 11/14/05	Fri 2/3/06	9
11	Focus Group Formation	90 days	Tue 10/4/05	Fri 2/3/06	
12	Focus Group Observations	16 days	Mon 2/6/06	Mon 2/27/06	11
13	Analysis of Samples for Metals	75 days	Thu 12/15/05	Wed 3/29/06	
14	Analysis of Samples for Estrogen Activity	75 days	Wed 12/21/05	Tue 4/4/06	
15	Descriptive Data Analysis	35 days	Wed 4/5/06	Tue 5/23/06	14
16	Focus Group Analysis	40 days	Mon 3/6/06	Fri 4/28/06	
17	Final Report	28 days	Wed 5/24/06	Fri 6/30/06	15

Appendix A

Descriptive Information on the White Bass and the Channel Catfish

White Bass *Morone chrysops*

Species overview: The white bass is a freshwater fish, with its largest populations in the Great Lakes and the Mississippi River system. In Pennsylvania the white bass is native to the western counties, especially Lake Erie and the Ohio River watershed. Its species name "chrysops" refers to the fish's golden eye.



Identification: The white bass is a medium-sized fish, silvery, with an arched look to its back. The maximum size is about 18 to 20 inches, with a two- or three-pounder a trophy. The more usual size is one-half to about two pounds. White bass have a deep body, compressed laterally. The back is blue-gray or steel-gray. The base color of the sides is silvery-white to silvery pale-green, with a yellow tinge on the lower edge. The body is marked with four to seven gray-brown or black horizontal stripes, not as distinct as the stripes of the striped bass. The two dorsal fins are separated by a notch, and the anal fin has three spines and 12 to 13 soft rays. The eye is yellow and the dorsal and caudal fins are clear to gray. White bass have teeth in a patch on the base of the tongue, unlike the white perch, which has a thin band of teeth around the front edge of its tongue. The white bass's mouth is basslike. The lower jaw projects beyond the upper jaw.

Habitat: White bass inhabit large lakes and small to large rivers. They prefer water that is relatively clear, and they rarely maintain a population in lakes less than 300 acres. Prime white bass habitat includes extensive water acreage deeper than 10 feet, and gravelly shoals or rock- rubble reefs on which the fish can spawn. In recent years, white bass fishing has been exceptional at the Allegheny Reservoir, Warren County.

Life history: White bass are school fish, spawning, feeding and traveling in compact groups. In late April to early June, schools of white bass migrate to spawn over rocky or gravelly shoals, either going to that habitat in a lake or traveling upstream in a river to reach it. The bass appear to return to the same spawning site each spring. Spawning takes place near the surface in six or seven feet of water, at 58 to 64 degrees. The females release 25,000 to one million minute eggs into the current, accompanied by several spawning males. The eggs are adhesive, drifting to the bottom and sticking to the stones. They hatch in two or three days. Successful hatching depends on favorable conditions of current or wave action, and temperature. White bass produce abundant year-classes intermittently. Spawning success and year-class survival usually depend on a variety of environmental conditions.

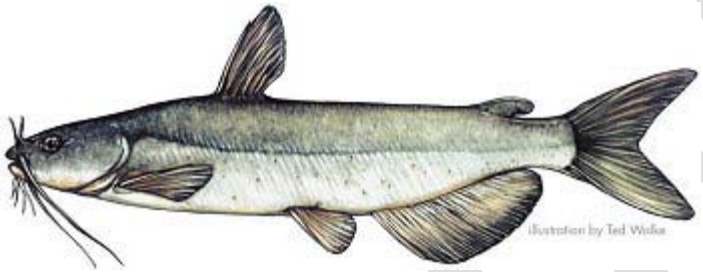
Young white bass quickly show their schooling tendencies, drifting in large groups and eating zooplankton. As they grow they switch to larger prey, like aquatic insects, crustaceans and their primary food, fish, especially consuming schooling forage fish like gizzard shad. White bass show

several daily peaks in feeding activity, which vary seasonally. They retire to deeper water by day and swim toward shallower water at nightfall. Aggressive feeders, white bass may make a great commotion on the surface when they attack a school of forage fish or during spawning activities, a tip-off to anglers of this fish's presence

Channel Catfish *Ictalurus punctatus*

Species overview: Next to the flathead catfish, the channel catfish is the largest catfish in Pennsylvania. Weights of up to 15 pounds are not unusual at lengths of about 30 inches. The state record is over 35 pounds. Channel "cats" are avidly sought sport fish and are raised commercially for the table. They are found statewide, introduced where they did not occur naturally. The native range of channel catfish is believed to be the Great Lakes and St. Lawrence River watershed, the Missouri River system, the Mississippi River watershed, Gulf of Mexico

watershed and parts of Mexico. They were not native to the Atlantic Coast north of Florida. The channel catfish's species name "punctatus" means "spotted," referring to the small, dark spots on its sides. The channel catfish is the only catfish that has these dots.



Identification: The channel cat has a deeply forked tail, with tail lobes that are sharply pointed. In bigger fish, the fork is less noticeable or disappears. Channel cats have 24 to 30 rays on the anal fin, a small, fleshy adipose fin that is separated from the tail, and typical catfish spines on its dorsal and pectoral fins. The barbels are black and long. The back is blue-gray to slate-gray or bluish olive. The sides tend to be silvery-gray, and the belly is whitish. Except for some large adults, especially the males, channel catfish have small, irregular spots on the sides and back. None of the other catfishes has these spots. Males become darker, almost blue-black, during spawning time.

Habitat: The channel catfish is an adaptable fish, usually found in clear, warm lakes and moderately large to large rivers, over clean sand, gravel or rock-rubble bottoms. It is generally not found in the muddied, weed-choked waters that some other catfish species frequent. Channel cats, especially young fish, may be found in fast-flowing water. Usually, channel catfish prefer deep pools and runs in rivers that have alternating pool and riffle habitats. It is also found in reservoirs, lakes and farm ponds, and even in some of the larger trout streams.

Life history: Channel catfish spawn in May to early June, when the water temperature ranges from 75 to 85 degrees, with 80 degrees the optimum. The male prepares the nest, which is usually a depression or hole in an undercut bank, or an excavated burrow under logs or rocks. Sometimes channel cats spawn in sunken, hollow logs or abandoned muskrat holes. In clear ponds, spawning channel cats must have semi-darkened shelters, either natural or provided. From reservoirs, channel catfish sometimes move upstream to spawn in tributary rivers. A female channel cat may lay 2,000 to 70,000 eggs per year, depending on her size. After spawning, the males protect the adhesive egg mass and aerate and clean the eggs by fanning their fins. The males also guard the hatched fish for a time. Young channel cats are insect-eaters, feeding on mayfly nymphs, caddis larvae and midge larvae. As they grow, they switch to fish, crayfish and mollusks, but still feed on aquatic insects, and occasionally eat plant matter. Yearling and subadult channel cats are more tolerant of fast water than larger adults. They move out of slow water into the quicker current or swim short distances into

tributary streams to feed. Channel cats feed mostly at night, but may forage on the bottom, where it's dim during the day. Channel catfish, especially young fish, have been known to feed on the surface. Like other catfish, at night they depend on their barbels and their sense of taste to find food. Even so, channel cats are believed to be more of a sight-feeder than other catfishes, because of their clear-water habitat.

The above taken from the Pennsylvania Fish and Boat Commission Website.

Appendix B

Approved Institutional Review Board Number & Important Sections

Approval Number <<0510031 x Volz app 110705.pdf>>

Dear Dr. Volz:

An electronic version of your IRB approval letter is attached. You may now go forward with your research project. If you have any questions, please don't hesitate to contact the IRB office.

Kathy Yobbi
Institutional Review Board
3500 5th Avenue
Suite 105
Pittsburgh, PA 15213

Script

(a) (Changed Script)-----The University of Pittsburgh, Graduate School of Public Health, Department of Environmental and Occupational Health is asking people who fish in the Three Rivers Area to participate in focus groups so that we can get some idea of the type(s) and amount(s) of fish caught and eaten in the TRA basin. Fishers under 21 years of age will not be able to participate in the study. We would like to know more about your use of fish from the Three Rivers Area so that we can better understand the risks and benefits of eating these fish. Any information that you give us will be kept confidential and you will be informed if you wish of the results of both our focus group study as well as contaminant levels in TRA fish. You have the right to withdraw from this study at any time and no identifying information will be kept by Dr. Volz without your expressed written permission. If you agree to enter the study we ask you to participate in three 1 hour sessions . At these sessions we will discuss your fishing methods, fish taken home and eaten as well as cooking methods. If you attend all three sessions we will give you a new rod and reel after the last session. We would appreciate your help, would you join the study? Please provide us with your name and

phone number so that we can contact you regarding the time and place of the first focus group meeting.

Initial List of Focus Group Questions

This is a descriptive, qualitative study. Initial focus group questions will be;

1. Where do you fish most often in the Three Rivers area? Do you move your fishing spot often? Do you have a number of favorite spots? Do you collect different fish at different places?
2. How often do you fish? Do you fish year round or fish during specific seasons?
3. What is your favorite fish for eating? Can you list the best fish for eating from top to bottom? What fish will you not eat and why?
4. What size of fish do you prefer to catch for eating? Does this size vary for each type of fish that you like to eat? Is there a certain size range that you use to judge the fish you might keep?
5. How do you clean your fish for eating? Do you clean the fish at the river or at home ?
6. Do you do any other trimming or cleaning before you cook your fish?
7. What are your favorite ways to prepare your favorite fish? Could you give us your favorite recepies? What kind of sauces or oils do you use to prepare your fish?
8. How often do you eat fish from the rivers? What portion size do you eat per meal (show photos of different portion sizes for reference)? Do you share your catch with anyone else or your meal with others? How often do you eat store-bought fish?
9. Are you concerned about contaminants in the water and sediment in the river contaminating the fish? Are you aware of fish advisories put out by the Pennsylvania Fish and Boat Commission or the Environmental Protection Agency? Have you read about the proper way to prepare fish in your fishing liscense booklet? Would you eat more fish if you thought the river was cleaner? Would you spend more time fishing if you knew the river water was getting cleaner?
10. Do you do anything to remove contamination from the fish you catch? What do you do specifically?
11. What do you know about the specific kind of contaminants in the Three Rivers Area? Would you like more information on contaminants in the fish that you like to eat?