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TITLE: Real-Time Behavioral Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations

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This a report of activities performed under a collaborative agreement between the U.S. Army Center for Environmental Health Research (USACEHR) and the University of Maryland Aquatic Pathobiology Center (UM APC). These activities support the USACEHR and their US EPA EMPACT project entitled "Real Time Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations." The overall objective of the USACEHR - UM APC collaborative was to test a biomonitoring system with sentinel fish under laboratory conditions, with exposures to temperature fluctuation, hypoxia, and a harmful algal bloom toxin, brevetoxin. We also developed a $^{14}$C-labeled 2-deoxyglucose autoradiography method to examine changes in central nervous system activity, and conducted pathological examinations, in fish exposed to brevetoxin. In the temperature fluctuation experiment, each daily 5°C rise in temperature was associated with minor elevations in ventilatory rate and depressions in ventilatory depth. Fish exposed to hypoxia showed temporal elevations in VR with minor associated depressions in ventilatory depth, and elevations in cough rate. In a 19°C brevetoxin experiment (49µg/L), fish responded with a minor temporal elevation in ventilatory rate and a suppression of ventilatory depth. In a 25°C brevetoxin experiment 53µg/L, there was also a minor elevated spike in VR. However, there was also a major elevated spike in cough rate and percent movement. The brains of fish exposed to 49µg brevetoxin/L showed notably higher incorporation of 2-deoxyglucose compared with control and vehicle fish. Histopathological observations indicated no significant difference between control fish and brevetoxin exposed fish. Outreach for this project has been in the form of poster presentations at two well-recognized scientific meetings, and a website (http://aquaticpath.umd.edu/empact) developed and maintained by the UM Aquatic Pathology Center.

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## ABSTRACT

This is a report of activities performed under a collaborative agreement between the US Army Center for Environmental Health Research (USACEHR) and the University of Maryland Aquatic Pathobiology Center (UM APC). These activities support the USACEHR and their US EPA EMPACT project entitled "Real Time Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations." The overall objective of the USACEHR - UM APC collaborative was to test a biomonitoring system with sentinel fish under laboratory conditions, with exposures to temperature fluctuation, hypoxia, and a harmful algal bloom toxin, brevetoxin. We also developed a $^{14}C$-labeled 2-deoxyglucose autoradiography method to examine changes in central nervous system activity, and conducted pathological examinations, in fish exposed to brevetoxin. In the temperature fluctuation experiment, each daily 5°C rise in temperature was associated with minor elevations in ventilatory rate and depressions in ventilatory depth. Fish exposed to hypoxia showed temporal elevations in VR with minor associated depressions in ventilatory depth, and elevations in cough rate. In a 19°C brevetoxin experiment (49µg/L), fish responded with a minor temporal elevation of ventilatory rate and a suppression of ventilatory depth. In a 25°C brevetoxin experiment (53 µg/L), there was also a minor elevated spike in VR. However, there was also a major elevated spike in cough rate and percent movement. The brains of fish exposed to 45µg brevetoxin/L showed notably higher incorporation of 2-deoxyglucose compared with control and vehicle control fish. This indicated that there was a notable alteration in brain activity in brevetoxin exposed fish. Histopathological observations indicated no significant difference between control fish and brevetoxin exposed fish. Outreach for this project has been in the form of poster presentations at two well-recognized scientific meetings, and a website (http://aquaticpath.umd.edu/empact) developed and maintained by the UM Aquatic Pathobiology Center.

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## BACKGROUND

### *Project Personnel*

This is a report of activities performed under a collaborative agreement between the US Army Center for Environmental Health Research (USACEHR) and the University of Maryland Aquatic Pathobiology Center (UM APC). These activities support the USACEHR and their US EPA EMPACT project entitled "Real Time Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations." Project investigators include Andrew Kane (Principle Investigator, UM APC, laboratory biomonitoring component), Ellen Silbergeld (Co-Principle Investigator, UM Program in Human Health and the Environment, neurotoxicology component) and Renate Reimschuessel (Co-Principle Investigator, US FDA, pathology component, under separate contract with the Army). Mr. Geoffrey Gipson supported laboratory biomonitoring studies under the direction of Dr. Kane. Dr. Jennifer Sass and Ms. Jennifer Choich supported technical aspects of the neurtoxicology component under the direction of Dr. Silbergeld.

The collaborative agreement between USACEHR and the UM APC was generated when the UM APC was located at the UM School of Medicine in Baltimore. During the period of performance the UM APC was relocated to the UM College of Agriculture and Natural Resources, Department of Veterinary Medicine, at the College Park Campus. This report represents the final deliverable of the cooperative agreement as generated through Dr. Kane's appointment in the Department of Pathology, University of Maryland, Baltimore. However, all future correspondence with Dr. Kane should be directed to the new UM APC facility at College Park: Aquatic Pathobiology Center, Department of Veterinary Medicine, 8075 Greenmead Drive, College Park, MD 20742; 301-314-6808; akane@umaryland.edu.

### *EMPACT Project Background*

The USACEHR EMPACT project was designed to provide near real-time monitoring of potentially toxic waterway conditions using an automated biomonitoring system. The biomonitoring system consists of a series of eight flow-through plexiglas™ chambers that house sentinel fish. Fish respiratory response signals are non-invasively transmitted, amplified and uploaded into personal computers, where field signals from individual fish thriving in potentially

deleterious water conditions may be compared to the baseline signals from the same individual fish. The system is designed to generate data that health and environmental officials can use to provide timely advice on the safety of waters. This type of information has great potential to benefit commercial fisheries, recreation industries, and the general public by filling a critical need for objective, rapidly acquired, and readily understandable warning information to guide decisions regarding waterway closure and potentially hazardous conditions. This regional US EPA-supported EMPACT project represents a multi-organizational effort between US Environmental Protection Agency, US Army Center for Environmental Health Research, GEOCENTERS, Inc., University of Maryland Aquatic Pathobiology Center, Johns Hopkins University Applied Physics Laboratory, US Food and Drug Administration's Center for Veterinary Medicine and the US Army Medical Research Institute for Infectious Diseases.

***Project Goals***

The overall goal of this EMPACT project is to apply the Army's biomonitoring system to detect environmental perturbations, such as the presence of harmful algal blooms, particularly those of toxic *Pfiesteria*-like organisms. The objectives of this collaborative agreement are to test the biomonitoring system with sentinel fish under laboratory conditions with exposure to *Pfiesteria*-like dinoflagellates, and to develop a method to examine possible changes in central nervous system activity as well as pathological alterations in exposed fish.

However, since it has not been possible to acquire and dose fish with these dinoflagellates or culture supernatants during the period of performance of this collaborative agreement, an environmentally relevant surrogate biotoxin, brevetoxin (PbTx2), has been utilized as an exposure stressor. Brevetoxin is biologically formed by the dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve*), a common harmful algal bloom species on the US Atlantic coast.

The specific aims of this project focused on exposure effects of bluegill sunfish (Centrarchidae: *Lepomis macrochirus*) to a sublethal concentration of PbTx2. Efforts in these studies included the collection of behavioral (respiratory) data from exposed fish within the biomonitoring system, neurotoxicity data using PbTx2-exposed fish injected with radiolabeled 2-deoxyglucose, pathology data from exposed fish, and project outreach in the form of a website.

## METHODS

### *Animals*

Healthy, pond-reared bluegill were supplied by the Army to the Aquatic Pathobiology Center and laboratory acclimated for at least two weeks prior to any manipulations. Four weeks prior to exposure fish were acclimated to 24-h lights-on photoperiod. Fish were maintained in flow-through freshwater 200-liter aquaria and fed 38% protein fish chow (Zeigler Bros., Gardners, PA). For laboratory biomonitoring (respiratory) studies, light-acclimated fish were then acclimated to exposure chambers for 3 days prior to collecting baseline "control" data.

### *Preliminary Range-finding Study*

In order to determine an appropriate concentration of PbTx2 for the behavioral and neurotoxicology studies we conducted preliminary range-finding test. Fish were fasted for 48 hours prior to use in this study to reduce excretion of nitrogenous waste during the exposure. Groups of five fish were exposed in a replicated series (i.e., 10 fish exposed at each concentration) of PbTx2 concentrations (30, 40, 50 and 60 µg/L) in 3.5L media in 4.0L covered glass beakers. Twenty solvent control fish were also exposed. Vessels receiving PbTx2 also contained the vehicle Emulphur-620. The vehicle concentration remained consistent (0.0001%) in all PbTx2 exposure vessels. Fish were exposed for 1 hour to the respective treatments and then transferred to recovery beakers with only control water (no Emulphor, no PbTx). This design was chosen to evaluate responses to a "transient bloom," and to represent the exposure duration that was used in the biomonitoring study. Fish were considered "dead" when they no longer maintained their position in the water and did not respond to gentle prodding with a glass rod. Dead fish were removed upon observation. Twenty-four hour response data were evaluated using probit analysis.

### *Brevetoxin Source, Analytical Methods and Water Quality*

The brevetoxin toxin used in these studies was PbTx2 was purchased from Dr. Dan Baden, University of North Carolina at Wilmington. It was obtained as a dry, white, crystalline,

95% purified residue stored under $N_{2(g)}$, and was maintained at –20°C at the UM APC until it was put into solution. Superstock solutions were made by the addition of absolute ethanol. These superstock solutions were also stored at –20°C. Working stock solutions were generated by diluting the superstocks with exposure dilution media. This exposure dilution media consisted of non-chemically dechorinated tap water with 0.0001% of the surfactant Emulphor-620.

Actual exposure concentrations were determined using a radioimmunosorbant assay (RIA) by Dr. Mark Poli (US Army Medical Research Institute for Infectious Disease). This RIA is specific for brevetoxins sharing the PbTx2 type backbone and is fully described elsewhere (Poli and Heweston 1992; Poli et al. 1995). Standard curves were constructed by incubating antiserum (1:7.500 dilution in phosphate buffered saline containing 0.01% emulsifier) with increasing concentrations of PbTx2 in the presence of a constant concentration of [$^3$H]PbTx9 (0.1 nM) in a total volume of 1 mL. After incubation for at least 1 hour at 4°C, 0.5 mL of a 1:160 dilution of 10% dextran-coated charcoal in PBS was added, mixed, and incubated for an additional 15 minutes. The charcoal and serum-bound label was separated from free label by 15 minute centrifugation at
1,500 x g. Clear supernatant (1 mL) was transferred to scintillation vials, acidified with 50µL glacial acetic acid, and the bound radioactivity was counted on a scintillation counter. Results were quantified by comparison of unknowns to a standard curve and expressed as PbTx2 equivalents/mL.

Pond-reared bluegills were acclimated to a 24 h lights-on photoperiod for four weeks prior to exposure. Fish were maintained in flow-through 200 L aquaria and fed fish chow (Zeigler Bros., 38% protein). The water source for holding and testing was non-chemically dechlorinated Baltimore city municipal water (pH 6.8-7.0; hardness 78 mg/L as $CaCO_3$ equivalents). General holding conditions included dissolved oxygen >80% saturation and temperature 20°± 1°C.

***Laboratory Biomonitoring Studies***

The behavioral monitoring system was set up at the UM APC with the assistance of Tom Shedd and Mark Widder of USACEHR. A custom gravity-fed dilutor system was installed by Dr. Kane to deliver continuous pulse flow (35 mL/minute/chamber) to a single bank of eight

behavioral monitoring chambers (Figure 1). After laboratory and photoperiod acclimation, fish were acclimated to the behavioral exposure chambers for 3 days prior to collecting data. During chamber acclimation, chamber hardware and computer hardware and software observed for stable readings. Signal integrity from the exposure chambers was empirically verified electronically using an oscilloscope as well as visually using a remote video camera. Subsequent to chamber acclimation, "control" (baseline) behavioral data was recorded for each individual fish (n=6-8) for 4 days. Fish were monitored for up to 7 days and "exposure" behavioral data collected. Control and exposure behavioral data, as analyzed by the Army-supplied software, included ventilatory rate (VR), ventilatory depth (AD), cough rate (CR), and movement (%Mov) within the chambers.

Baseline and exposure data were obtained for 6-8 animals simultaneously in five separate trials. These trials consisted of a preliminary baseline exposure study to gain experience with the system; a temperature fluctuation study, a hypoxia exposure study; and two biotoxin exposure studies using PbTx2, one at 19°C and one at 25°C.

***Temperature Fluctuation Exposures.*** This study gathered response data from fish exposed to dilution water that fluctuated by 5°C daily, from 19° to 24° C. This was accomplished by exhausting the supply of warmed (24°C) dilution water from the 600-liter dilutor reservoir and then refilling it daily with 19°C water. Water in the reservoir was warmed using glass-encapsulated, self-regulating submersed heaters. This paradigm caused a slow rise (19-24° over approximately 22 hours) in the delivery water temperature as the reservoir warmed up, with a relatively rapid drop (24-19° in approximately 2 hours) in delivery water temperature as the vessels received reservoir water that was more recently replenished.

***Hypoxia Exposures.*** In order to test fish in the system for stress responses to water quality perturbations, we designed an experiment to examine the effects of depressed dissolved oxygen (i.e., hypoxia). Hypoxia was chosen as an initial stressor since it is a common cause of aquatic animal stress and fish kills along the U.S. Atlantic coast, and it is relatively easy to experimentally execute. The experimental design utilized 7 out of 8 chambers of the system for fish exposure and collection of biological data. The 8$^{th}$ chamber was used for monitoring

dissolved oxygen, pH and ammonia. Dissolved oxygen and pH were measured potentiometrically, and ammonia levels were measured using a nesslerization kit.

Initially we bubbled $N_2(g)$ in the splitter chamber, upstream of the exposure chambers, to reduce dissolved oxygen. However, $N_2(g)$ bubbling did not cause greater than a 25% reduction in the dissolved oxygen levels. In the final experiments, a sufficient decrease in dissolved oxygen was accomplished by temporarily stopping water flow into the individual exposure chambers and allowing the biological oxygen demand of the fish to naturally reduce the dissolved oxygen concentration. In the definitive hypoxia experiment, exposure baseline was established for 98 hours and then fish were repeatedly exposed to approximately 50% depressions in dissolved oxygen followed by recovery. These experimental hypoxic events were conducted at 98, 120, 165, 238, and 294 hours after the start of the experiment. Water quality and biological response data were recorded during the oxygen depression and recovery periods. When approximately 50% $O_2$ depression was achieved, water flow to the chambers was resumed and fish were allowed to recover. Use of multiple time points supported data collection relevant to direct biological response, recovery response, and the influence of prior hypoxic exposure to time-to-response or recovery.

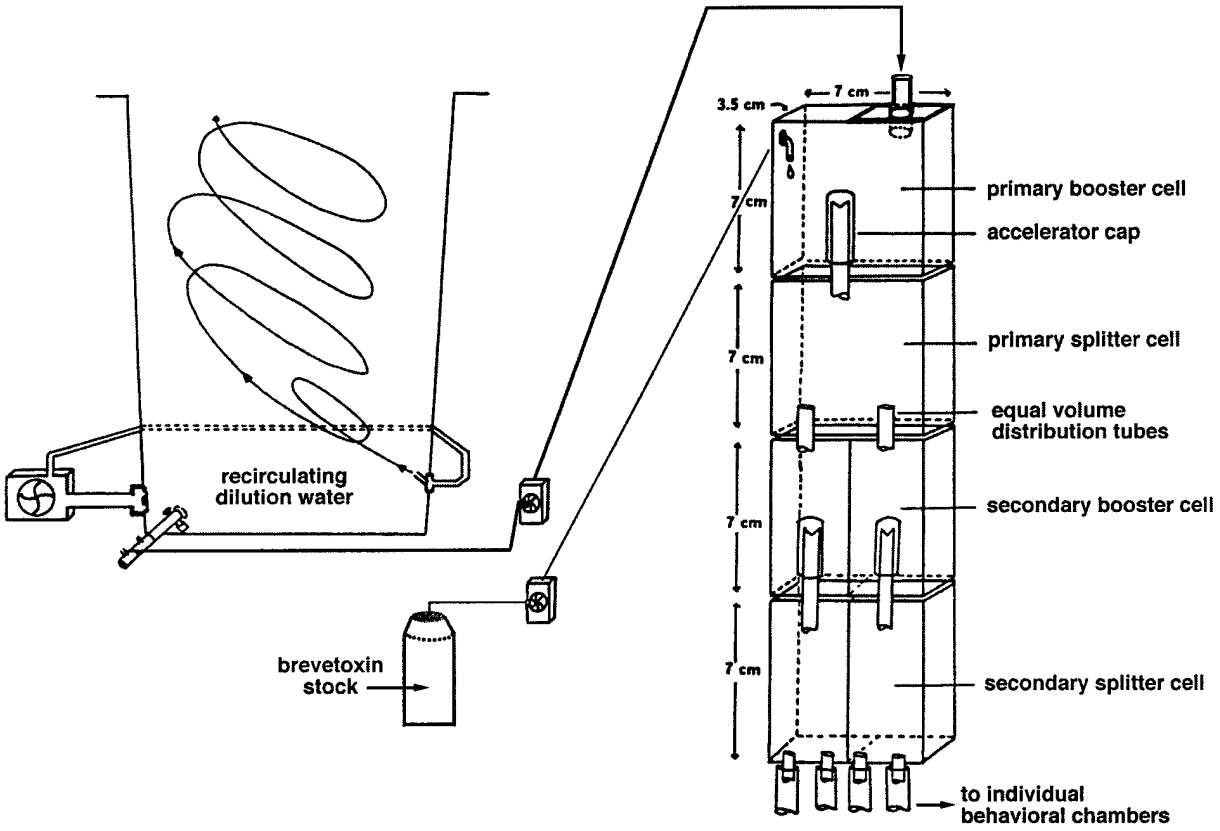


**Figure 1.** Schematic representation of dilutor cells used to split water and toxicant flow to individual exposure chambers. One set of two of splitter and accelerator cells (leading to 4 behavioral chambers) and one of two dilution water carboys are illustrated. Peristaltic pumps delivered dilution water and toxicant stock to the primary accelerator cell of each bank of splitter/accelerator cells. After Kane et al. 1988.

***Brevetoxin Exposures.*** After the fish were acclimated to the chambers and baseline data was collected, PbTx2 was added to the diluant flow by means of a peristaltic pump. Toxicant flow continued for one hour. Actual exposure concentrations were determined by Dr. Mark Poli (US Army Medical Research Institute for Infectious Disease) using a RIA, as described above. Diluent flow to the chambers continued for 23 additional hours after toxicant flow ceased.

*Neurotoxicity Studies*

For this aspect of the project we developed a novel system to better understand the mechanisms of environmental neurotoxins and pesticides that may present a hazard to fish. Specifically we evaluated PbTx2 according to specific regional changes in brain activity. Alterations in brain activity were detected using radiolabeled 2-deoxyglucose.

Fish were exposed to diluent water only, diluent water plus vehicle control (0.0001% Emulphor-620) or diluent water plus vehicle with 45 µg/L PbTx2. Exposures with 5 replicate fish were conducted in separate 4L beakers containing 2L of exposure media at 25°C. After one hour in the treatment beaker, each fish was injected intramuscularly below the dorsal fin with 2µCi of $^{14}$C-2-deoxyglucose (Amersham Pharmacia Biotech, Piscataway, NJ) and placed in a another beaker containing only freshwater for a thirty minute recovery period. Following the recovery period, fish were sacrificed by cervical dislocation, and whole brains removed. Brains were quick frozen on aluminum foil dipped in 2-methyl butane chilled over dry-ice, and were subsequently stored at –80°C.

Frozen whole fish brains were then horizontally cryosectioned at 12µm and thaw mounted directly onto microscope slides. Figure 2 indicates the plane of tissue sectioning. Slides were then coated with liquid emulsion (Ilford Nuclear Research, North Carolina) in a darkroom and placed flat into light-tight dessicator black boxes for 4 weeks at room temperature. Following development slides were removed from the black boxes in a darkroom and immersed into photographic developer (Kodak D-19) for 4 minutes, rinsed briefly in water, and then placed into photographic fixative (Kodak) for 2 minutes. Slides were then washed in water and analyzed by microscopy. Developed glass slides were viewed using light and dark field microscopy at 2x magnification. These autoradiograms were visualized with a video-based digital system by

Alpha Innotech Corporation (computer software AlphaImager 2000, version 4.03) and digital images were recorded.

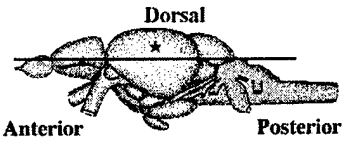


**Figure 2.** Cartoon showing anatomy of a teleost brain illustrated using a zebrafish model. The horizontal line indicates the point of horizontal sectioning through the experimental bluegill brains in the present study. Along the cut line, this fish brain shows, from left to right, an olfactory lobe (including an anterior olfactory bulb), a relatively large optic lobe (★), a cerebellum and a medulla (brainstem). After preparation the optic lobe is the dominant tissue remaining for observation.

***Pathology Studies***

Fish accessioned for pathology studies were taken at the time of death (or morbidity) from the range-finding exposure study at the UM APC. Specimens were necropsied (Kane 1996) and processed for routine histopathology (Profet et al. 1992). If exposure-related pathological alterations were noted from fish exposed in the range-finding study, additional exposures for pathology would be conducted. Glass slides were reviewed by Dr. Kane and forwarded to Dr. Reimschuessel at the FDA Center for Veterinary Medicine. Dr. Reimschuessel served as the primary pathologist for this project and was responsible for generating and compiling the pathology results of this report.

## RESULTS & DISCUSSION

### *Empirical Observations*

In order to verify the biological integrity of the response data from the experimental chambers, we observed the analog signal using an oscilloscope as well as visually using a remote video camera. Both the oscilloscope readings and the visual observations of mouth and opercular movement of fish in the exposure chambers were consistent with the ventilatory rate (VR) response signal and corresponding dataset. Coughs were also observed videographically and noted in corresponding cough response ventilatory data.

### *Preliminary Range-finding Study*

The dose response curve generated from this preliminary study indicated an estimated LC50 of 35 µg/L (95% CI: 22-42 µg/L). During the initial 3 hours of exposure (one hour of PbTx2, two hours in clean, recovery water) there were no gross signs of intoxication in PbTx2-exposed fish relative to control fish. However, after 8 hours some of the animals, particularly at the higher concentration showed signs of lethargy and morbidity. After 10 hours the majority of animals that were ultimately reported as dead or moribund at the end of the 24-h exposure were already dead or moribund.

The estimated LC50 derived from this preliminary study may be lower than an LC50 estimated from a more controlled, definitive experiment under flow-through conditions, or where loading of fish would be lower. Exposure vessel biomass in this experiment was approximately 7 grams/L (unionized ammonia reached as high as 200 µg/L; pH and DO remained within acceptable limits based on parallel exposures). Further, the animals used in this experiment had mild to marked parasite loading (cestodes[1] in and on the heart and liver; the animals were otherwise apparently normal and healthy under months of laboratory acclimation). Parasite loading may contribute to reduced resistance to toxic stress during the assay. However, similar data generated by USACEHR indicate that the estimated LC50 from our preliminary test was similar to a concentration previously derived for PbTx2 with bluegill.

[1] Most probable organism: certainly an encysted metazoan parasite. Differential diagnosis could include digenetic trematode.

***Laboratory Biomonitoring Studies***

Fish were exposed to either baseline conditions, fluctuating temperature, hypoxia or PbTx2 at 19° or 25°C treatments. Biomonitoring responses, including VR, CR, VD and %Mov for each of these treatment studies, are shown in Figures 3, 4, 5, 6, and 7 respectively).

***Hypoxia Study.*** We induced 5 dissolved oxygen (DO) depressions (i.e., hypoxic events) at 98, 117, 162, 235 and 288 hours after test initiation. This was accomplished by stopping water flow to the exposure chambers. Reduction in DO, therefore, was caused by the biological oxygen demand of the fish respiring in the chambers. DO concentrations in the exposure chambers were reduced by 39-60% while other measured parameters remained within acceptable limits (Table 1). Biological responses indicated a direct temporal relationship between hypoxia and increased VR (Figure 5a), as well as significant elevations in VR with minor associated depressions in AD and elevations in CR (Figure 5b).

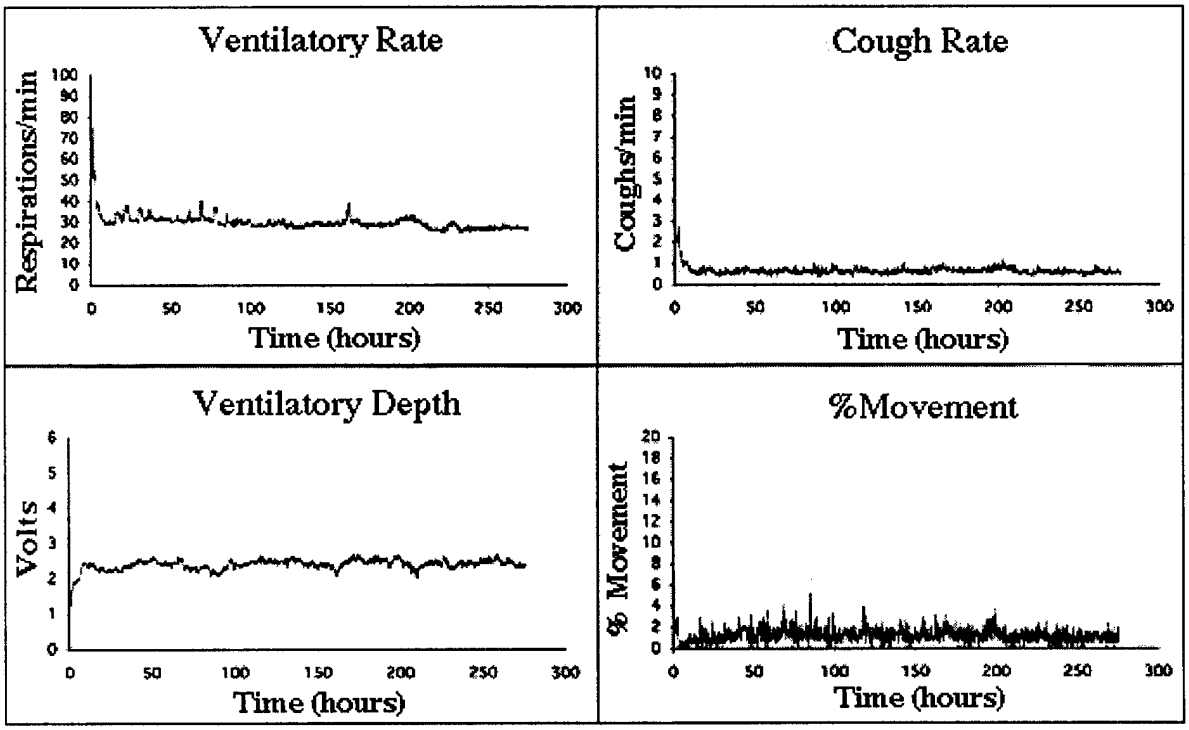

**Figure 3.** Fish responses to baseline exposure for over 250 hours at 19°C. Each graph shows response data averaged from 7 fish. There are only minor variations in the four response variables over time.

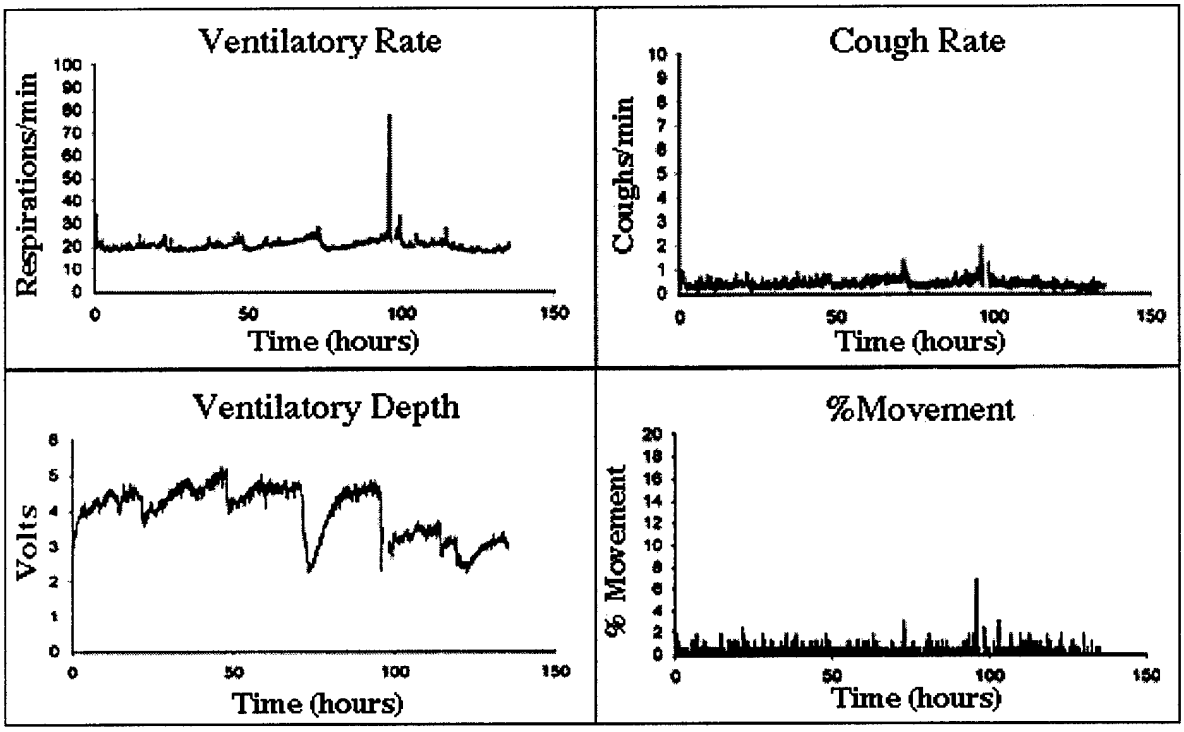


**Figure 4.** Fish responses to 5°C daily fluctuations in temperature. Fluctuations were caused by gently heating the source water in large delivery carboys over 24 hours, and then replenishing the source carboys with cooler (19°C) water at 24, 48, 72 and 96 hours. No other stressor was involved in this exposure. With each daily rise in temperature there was an associated minor elevation in ventilatory rate and depression in ventilatory depth. Cough rate and % movement did not appear notably effected. Note the spike in ventilatory rate and depth observed at 98 hours. This was caused by one of the investigators walking into the room and coming into visual contact with the exposure chambers. Each graph shows response data averaged from 7 fish.

| Exposure # | Total Ammonia (mg/L) | % Unionized Ammonia | Unionized [NH3](mg/L) | pH | [D.O.] (mg/L) | % D.O. drop |
|---|---|---|---|---|---|---|
| 1 @ flow shut off | 0.0 - 0.1 | 4.8% | 0.0048 | 8.1 | 7.0 | 39% |
| 1 @ flow resume | 0.1 - 0.2 | 2.0% | 0.0039 | 7.7 | 4.3 | |
| 2 @ flow shut off | 0.0 -0.1 | 5.1% | 0.0051 | 8.1 | 6.8 | 52% |
| 2 @ flow resume | 0.1 -0.2 | 1.7% | 0.0033 | 7.6 | 3.3 | |
| 3 @ flow shut off | 0.0 -0.1 | 3.8% | 0.0038 | 8.0 | 6.8 | 54% |
| 3 @ flow resume | 0.1 - 0.2 | 1.6% | 0.0031 | 7.6 | 3.1 | |
| 4 @ flow shut off | 0.0 - 0.1 | 4.1% | 0.0041 | 8.0 | 6.4 | 49% |
| 4 @ flow resume | 0.1 - 0.2 | 2.1% | 0.0042 | 7.7 | 3.3 | |
| 5 @ flow shut off | 0.0 - 0.1 | 3.8% | 0.0038 | 8.0 | 6.7 | 60% |
| 5 @ flow resume | 0.3 - 0.4 | 1.6% | 0.0062 | 7.6 | 2.7 | |

**Table 1.** Water quality data taken during the 5 hypoxia exposures from the 8$^{th}$ experimental chamber (i.e., only 7 fish respiratory measurements were taken).

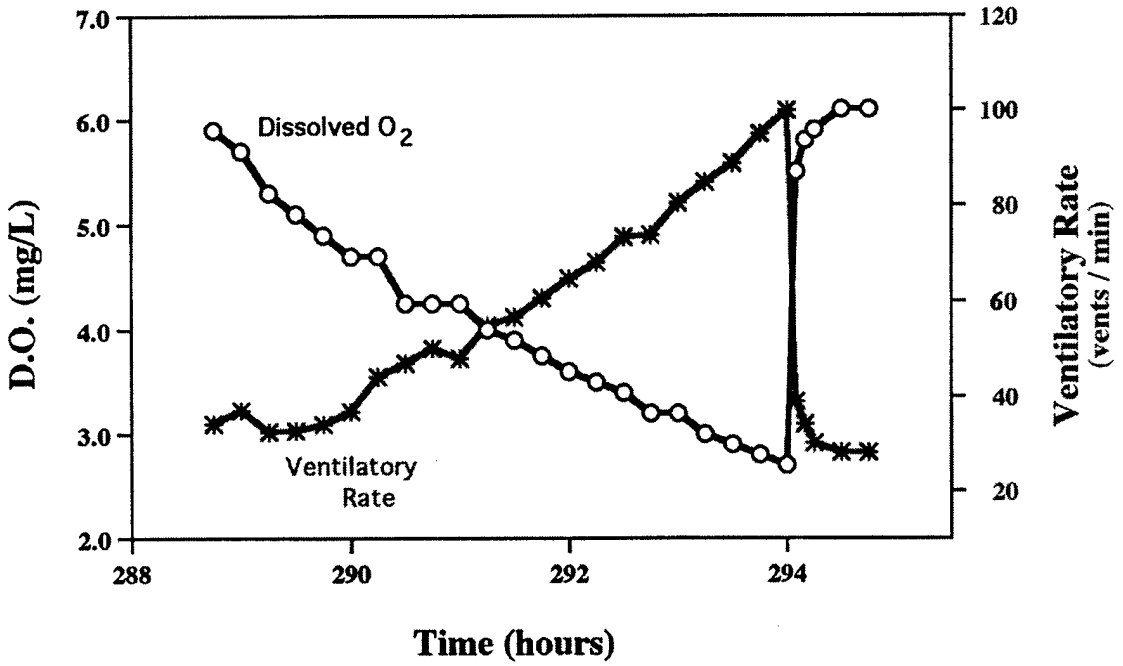


**Figure 5a**. Sample data showing reduction in dissolved oxygen in the 8th exposure chamber (typical of all 5 hypoxia trials). There is a close inverse relationship between dissolved oxygen concentration and VR. Note that recovery of VR closely mirrors the slope of the DO curve.

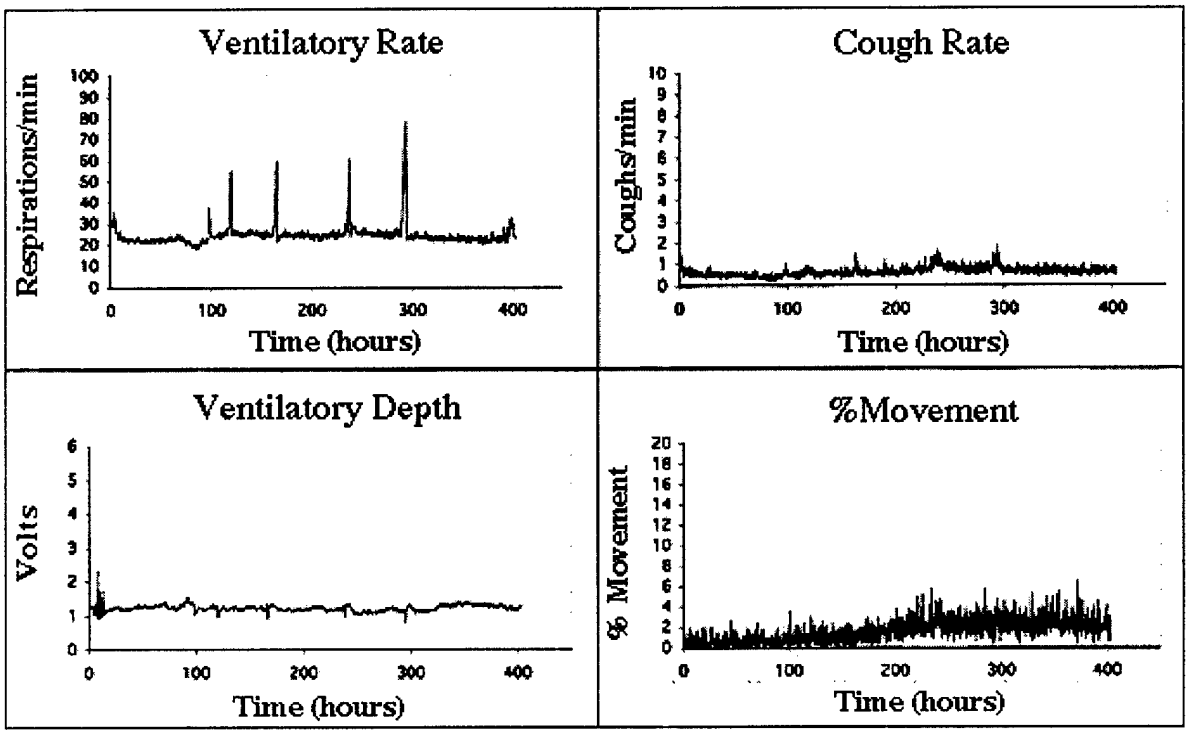


**Figure 5b.** Respiratory responses to 5 discrete hypoxia stress events at 98, 117, 162, 235 and 288 hours. Dissolved oxygen concentrations were reduced, on average, by 50% while ammonia and pH remained within acceptable levels. Biological responses indicated notable elevations in respiratory rate with associate minor depressions in ventilatory depth and minor elevations in cough response. Stress of up to 60% reduction in dissolved oxygen appears to cause reversible responses in the parameters measured in this study. Data shown are averages from 7 fish.

***Brevetoxin Studies***. Two PbTx2 exposures were conducted, one at 19°C and one at 25°C. The 19°C exposure collected data for over 120 hours of exposed fish including a 96 hour baseline without toxin. Toxin was pumped into the exposure chambers for 60 minutes to achieve a nominal concentration of 40 µg/L (49 µg/L measured) PbTx2. The exposure data indicates a minor temporal elevation of VR and suppression of AD (Figure 6). These responses, albeit minor, were brief and responses returned to baseline levels after the toxin cleared from the exposure system. No observable changes were noted in CR or %MOV.

The 25°C exposure study was conducted similarly. Toxin was pumped into the exposure chambers for 60 minutes to achieve a nominal concentration of 40 µg/L (53 µg/L measured) PbTx2. In this study there was a minor elevated spike in VR, as noted in the 19°C PbTx2 exposure. However, there was also a major elevated spike in CR and %MOV (Figure 7).

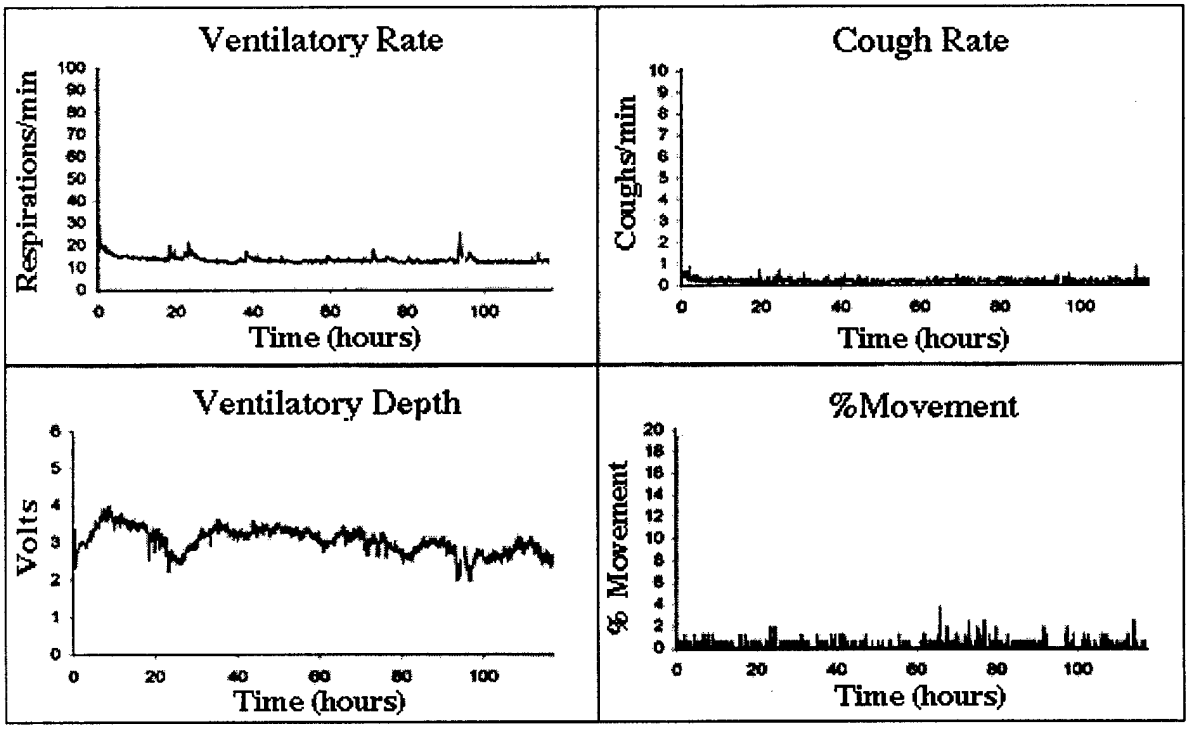


**Figure 6**. Fish response to 49 µg/L PbTx2 exposure at 19°C. Data shown are averaged from 7 fish.

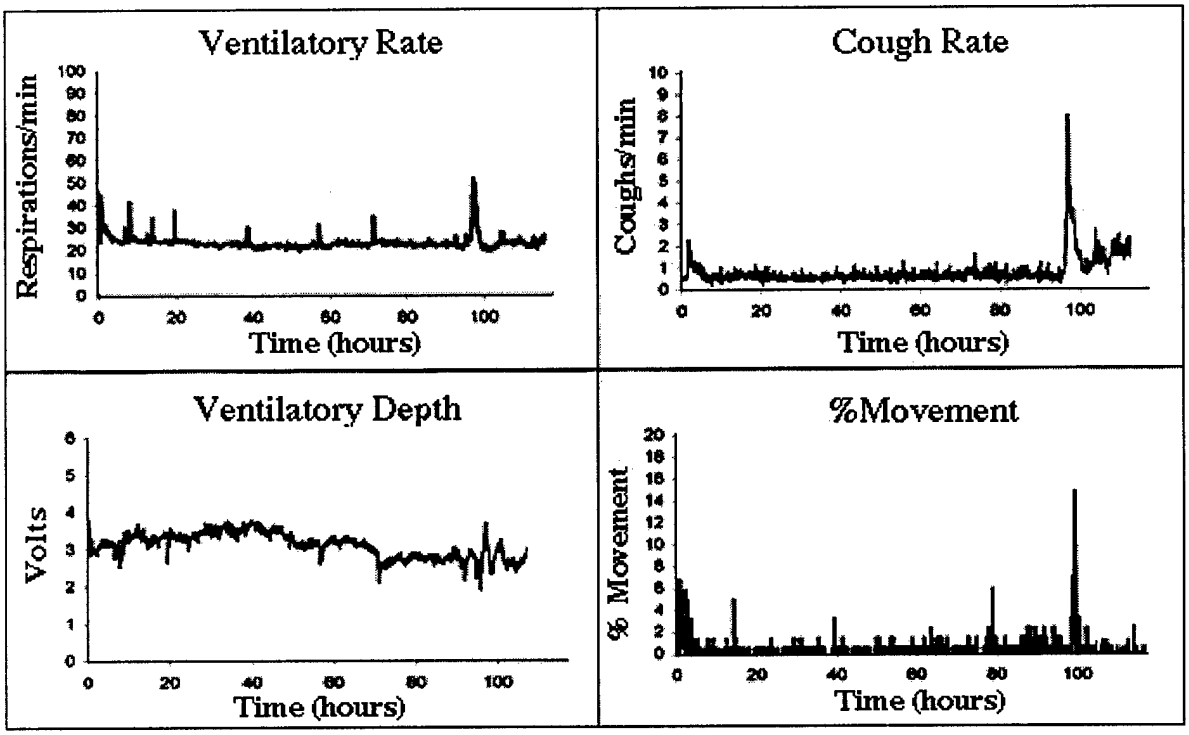


**Figure 7.** Respiratory responses to 53 µg/L PbTx2 exposure at 25°C. Data are averaged from 6 fish. There were no PbTx-related mortalities during the exposures, however 2 fish died due to clogged dilutor delivery tubes. The large increase in cough rate is notable in that it is not a response observed in fish experiencing changes over substantial ranges of several common water quality parameters (temperature, dissolved oxygen, and pH; Carlson, 1984). Thus, in field monitoring situations, when the causes of fish responses may be difficult to discern, the type of altered ventilatory response may be useful in differentiating fish responses caused by some toxicants from those resulting from normal variations in some water quality parameters.

Data averaged from 7 fish during baseline experiment demonstrated the relative steady-state of all 4 response variables. The temperature fluctuation experiment showed the sensitivity of the VR, AD and CR responses to subtle temperature change. There was a close temporal relationship between rising temperature and small increases in VR and CR, with concomitant decreases in VD. The hypoxia experiment showed notable elevations in VR with concomitant depressions in VD during each of 5 hypoxia "events." Fish appeared to recover from each of the DO depressions when normoxic conditions returned. In the 19°C brevetoxin experiment, fish responded with a minor temporal elevation of ventilatory rate and a suppression of ventilatory depth. In the 25°C brevetoxin experiment, there was also a minor elevated spike in VR. However, there was also a major elevated spike in cough rate and percent movement.

Additional data (from outside the scope of this collaborative agreement) with bluegill exposed to supernatants from *Pfiesteria* cultures was generated outside the scope of this cooperative agreement. These data were from experiments with Dr. JoAnn Burkholder et al. at North Carolina State University. The data showed that fish responded to exposure with elevations in CR and %Mov, and AD dropped as fish succumbed. Therefore, there appears to be a fairly discrete trend in response signatures to the different types of exposure stress examined in the present study and with the NCSU experiments (i.e., temperature fluctuation, hypoxia, PbTx, *Pfiesteria* culture water). Summary data from the 4 stress experiments is summarized in Table 2. These varying response signatures indicate that the algorithms used to discern VR, VD, CR and %MOV have utility for recognizing and discerning variation in biological (respiratory) responses to real-world stress phenomena.

| | Response Variable: | | | |
|---|---|---|---|---|
| **Stressor:** | **VR** | **AD** | **CR** | **%MOV** |
| **Increased temperature** | ↑ | ↓ | ↑ | ○ |
| **Decreased dissolved $O_2$** | ↑↑↑ | ↓↓ | ↑ | ○ |
| **19° PbTx2** | ↑ | ↓ | ○ | ○ |
| **25° PbTx2** | ↑ | ↓ | ↑↑↑ | ↑↑ |
| ***Pfiesteria*** | ○ | ↓ | ↑ | ↑ |
| **Visual disturbance** | ↑↑↑ | ↓↓ | ↑ | ↑↑ |

**Table 2.** Summary data from the different stress experiments. Increase (↑), decrease (↓), and no change (○) symbols indicate relative difference compared with baseline data. One, two or three symbols indicate the relative degree of change, i.e., minimal, moderate, marked, respectively. Data with *Pfiesteria* cultures taken by USACEHR at the NCSU facility (not part of this collaborative agreement) are shown for comparison; the relative degree of change from baseline in the *Pfiesteria* exposures cannot be discerned from data available at the time of this report. Observations in this table are empirical and statistical differences have not been discerned.

*Neurotoxicity Studies*

Digital images of brain tissues taken from the three experimental groups depict visible differences in brain uptake of 2-deoxyglucose between treated and control animals (Figure 8).

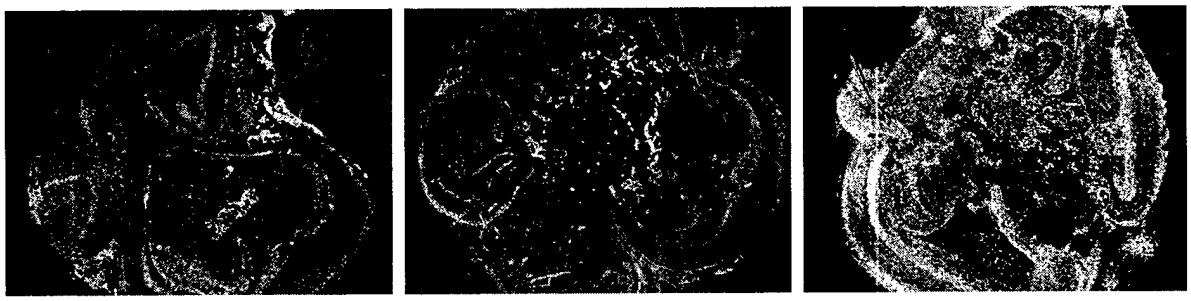

**Figure 8.** Uptake of $^{14}$C-labeled 2-deoxyglucose, a glucose analog that cannot be metabolized, is shown by the incorporation in the optic lobe of exposed control (left), vehicle control (middle) and PbTx2-exposed (right) fish. As viewed under dark field microscopy (2x magnification), there is no notable difference between either of the controls. However, there is a notable difference between the controls and the PbTx2-exposed fish. These images are slices of individual fish, typical of all 5 fish in each exposure group.

Multiple areas of high 2-DG uptake were observed in all fish treated with brevetoxin. Observations by Dr. Silbergeld indicated that glucose uptake was elevated in the dorsal telecephalic region, corpus cerebelli, tectum opticum, and the nucleus lateralis valvulae of fish treated with PbTx-2, compared with both vehicle and diluant controls. Appendix 1 provides slice image data from all exposed fish. Graphic data in this appendix depict the consistent increase in 2-DG uptake in all brevetoxin-exposed fish relative to control or vehicle control fish.

The purpose of including the 2-DG methodology in this study was to include a similar neurotoxicity endpoint as used in humans with possible exposure to *Pfiesteria*-like organisms. Recent efforts by Civelek et al. (1999) demonstrated that there was altered CNS activity in persons believed to be exposed to waterways containing *Pfiesteria*-like dinoflagellates. These authors examined regional glucose metabolism using fluorodeoxyglucose. The tagged glucose

was visualized using positron emission tomography (i.e., PET scanning). It was hoped that fish exposed to *Pfiesteria*-like organisms in this study could be similarly analyzed if the technology could be transferred to fish. Although we were unable to analyze fish exposed to *Pfiesteria*, we were able to demonstrate the technology transfer.

By using $^{14}C$-labeled deoxyglucose, we were able to examine alterations in CNS activity using autoradiographic techniques. These techniques obviously do not reveal alterations in real time as human PET scans can. However, our data clearly indicate that CNS activity is altered under conditions of PbTx2 exposure, and that regional areas may be affected. This is the first time fish have been examined using this PET-like technique. We are confident that this methodology can be applied to discern effects of exposure of fish to *Pfiesteria*-like dinoflagellates or other environmental stressors.

***Pathology Studies***

Fish were necropsied and tissues were preserved for routine histological analysis. Glass slides were read by Dr. Renate Reimschuessel at the US FDA Center for Veterinary Medicine. Data from these slides is presented in Appendix 2. Fish 6-15 were exposed to 60 ppb PbTx2; fish 16 and 17 were exposed to 50 ppb PbTx2; fish 18-23 were exposed to 40 ppb PbTx2; and fish 24-29 were exposed to 30 ppb PbTx2. Fish 30-46 were control fish.

Gross data from the time of necropsy indicate that gills were bright cherry red in most specimens, regardless of the exposure treatment regime. This indicates lack of obvious anemia or nitrite poisoning. Infestations of parasitic nodules were grossly visible in the heart, liver and posterior kidney. These observations were confirmed in the histologic examination. Parasite infestations (encysted metazoans, most likely cestodes) ranged from mild to marked. There were occasional observations of myxosporidean (marked) and nematode (mild) parasites as well. Other than parasite observations, all tissues and organ systems appeared to be within the normal range for the species and did not exhibit any notable pathology. However, mild edema was observed around CNS ganglia in one to three fish in each treatment group, including controls. This could be due to mild hypoxia prior to fixation. There were no findings that would suggest differences between the controls and treatment groups caused by brevetoxin exposure.

***Project Outreach & Website Efforts***

Outreach for this project has been in the form of poster presentations at two well-recognized scientific meetings, and a website developed and maintained by the UM Aquatic Pathobiology Center. The posters were presented at the International Harmful Algal Bloom Conference in Hobart, Tasmania (2001) and at the Harmful Algal Bloom Conference in Woods Hole, MA (2001). A version of the poster will also be presented at the Society for Environmental Toxicology and Chemistry (Baltimore, Maryland, 2001). A small-scale copy of this poster is presented in Appendix 3.

A website for this project was designed to provide laypersons insight into this Maryland EMPACT project and the overall application of the biomonitoring system. It was developed by Dr. Kane at the APC using hypertext markup language (HTML), java scripts, graphic jpeg files and hypertext links. The website was developed to be content-driven, and to be professional, stimulating, informative and show how the project worked toward its intended goals. The site was developed and reviewed by the Army prior to making it public.

The website design included the development of a project-specific header and a navigation bar that linked viewers to the different portions of the website. Both the header and the navigation bar included subtle animated gif files: The header includes a respiring bluegill with a running EKG-like output below it. The vertical navigation bar includes an animated flagellum on one of the dinoflagellates (on the homepage only). The website consists of a homepage and respective links (see below). Additional hypertext links are made to germane information outside the EMPACT site. A printout of the EMPACT portion of the website is included in Appendix 4.

➢ Homepage

(http://aquaticpath.umd.edu/empact)

➢ About EMPACT

(http://aquaticpath.umd.edu/empact/aboutempact/aboutempact.html)

➢ Biomonitoring Hardware

(http://aquaticpath.umd.edu/empact/biomonitoring/biomonitoring.html)

- ➢ Laboratory Studies

  (http://aquaticpath.umd.edu/empact/laboratorystudies/laboratorystudies.html)

- ➢ Field Studies

  (http://aquaticpath.umd.edu/empact/fieldstudies/fieldstudies.html)

- ➢ Project Collaborators

  (http://aquaticpath.umd.edu/empact/projectcollaborators/projectcollaborators.html)

The website also acknowledges all participating workgroups (EPA, Army, MD DNR, FDA, UM, GEOCENTERS, JHU) though textual representation of efforts as well as agency logos. Hypertext links permit linking to participating agency websites; reciprocal links from participating agency websites to our website has been encouraged. The website is posted on a UM server; this permits Dr. Kane to maintain the site and foster its development pending additional project growth and support).

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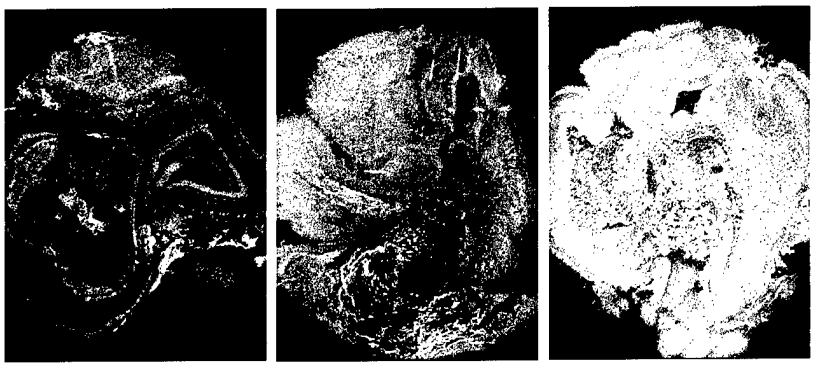
# Appendix 1.

## Neurotoxicity image data.

Images shown are dark field microscopic views of fish brains, primarily telencephalon, from animals exposed in the 2-deoxyglucose study. Each page contains data from three individual fish in columns: the left column is from a control fish; the middle column is from a vehicle control fish; and the right column is from a PbTx2-exposed fish. In each column, there are 4-5 slices from different planes of sectioning of the same fish brain. Note that the brain illustrated in the right-hand column on all pages consistently show brighter $^{14}C$-labeled 2-deoxyglucose labeling than brains the left-hand or middle columns. See text for additional detail.



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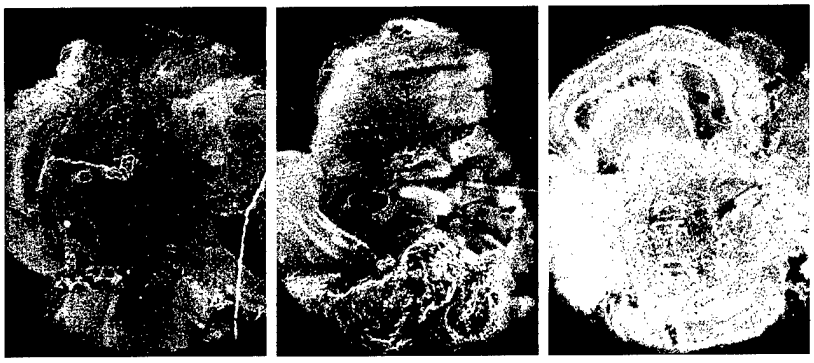

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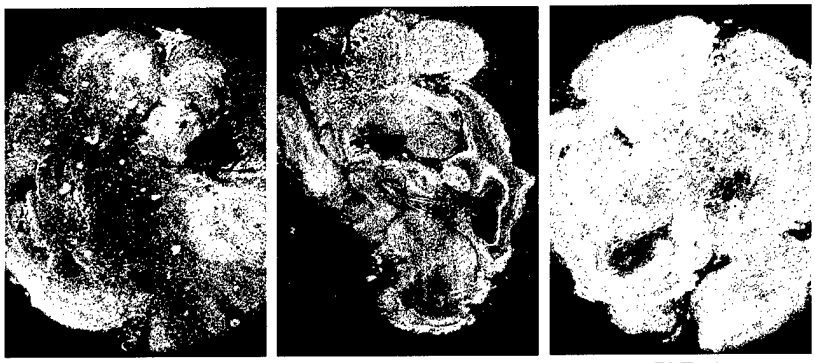

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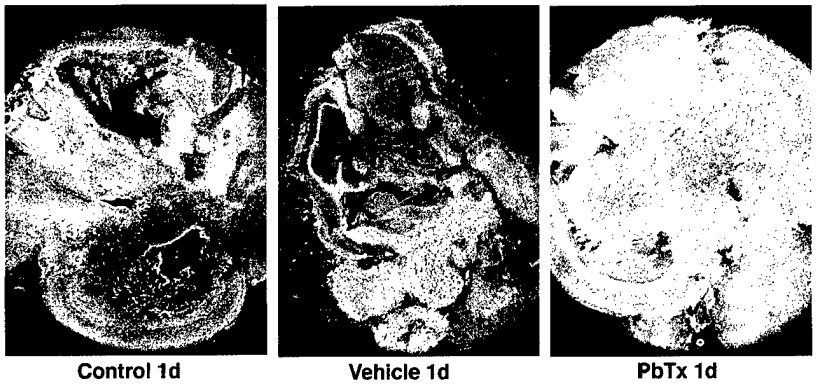

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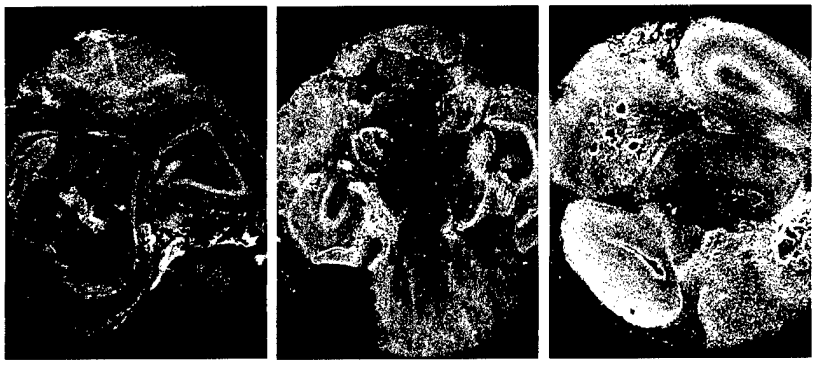

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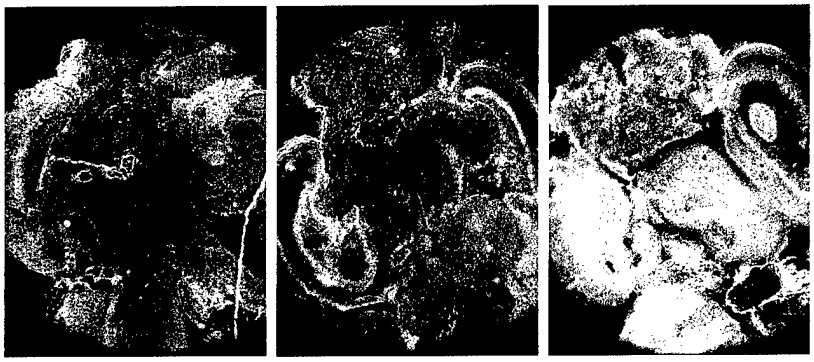

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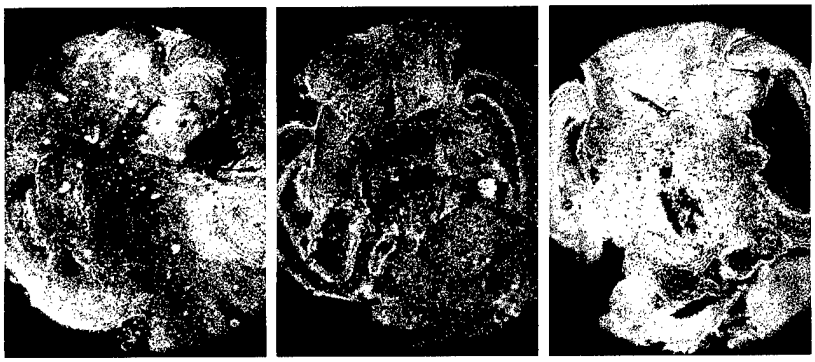

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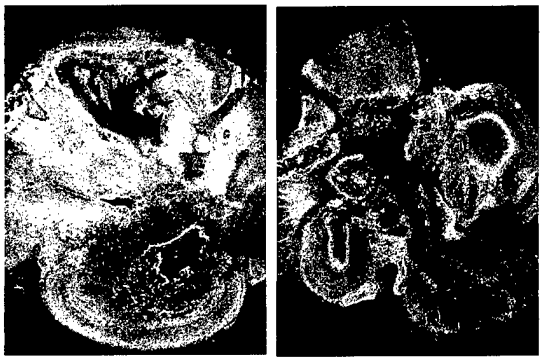

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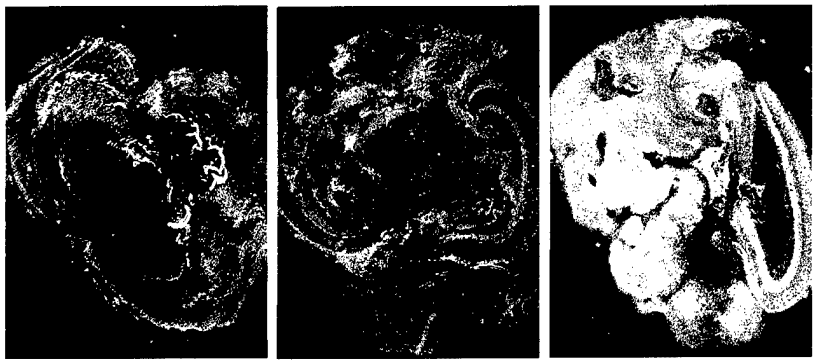

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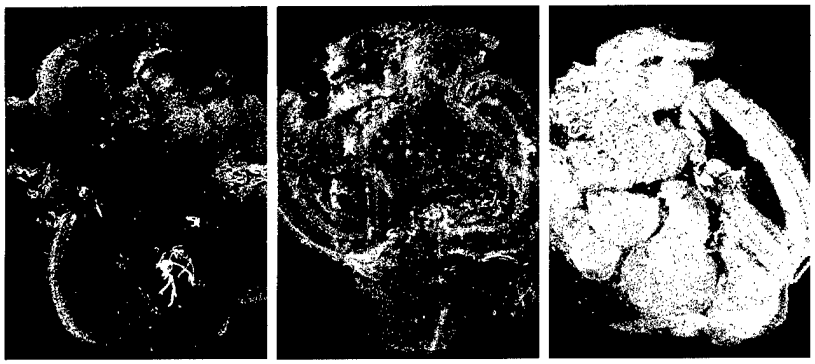

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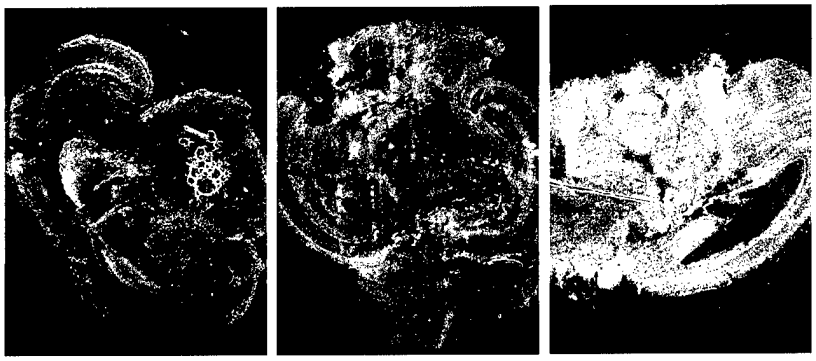
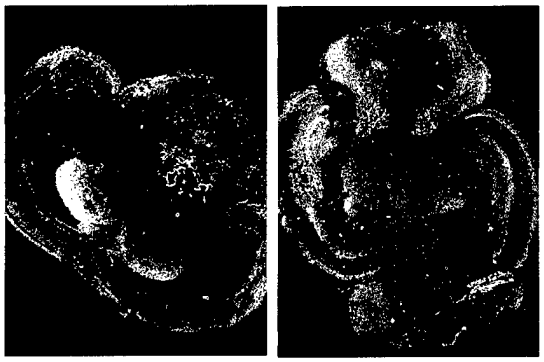
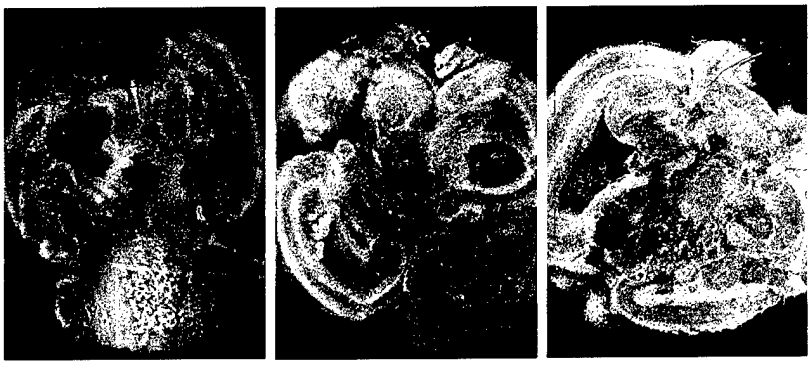


Control 3c | Vehicle 3c | PbTx 3c



Control 3d | Vehicle 3d



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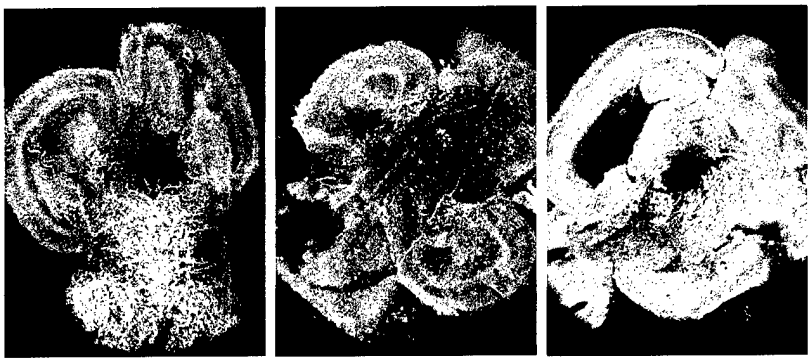
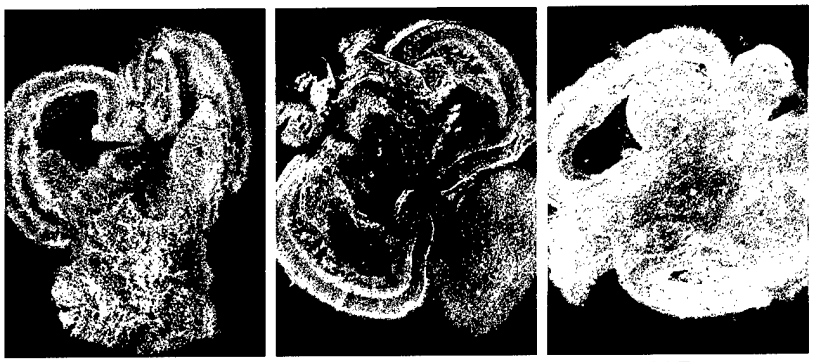
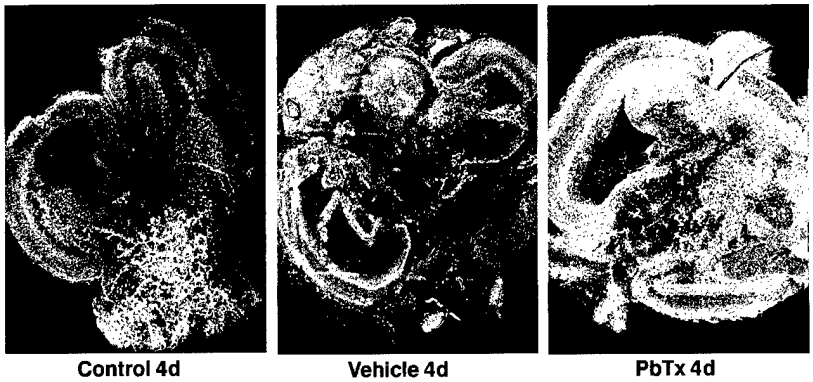


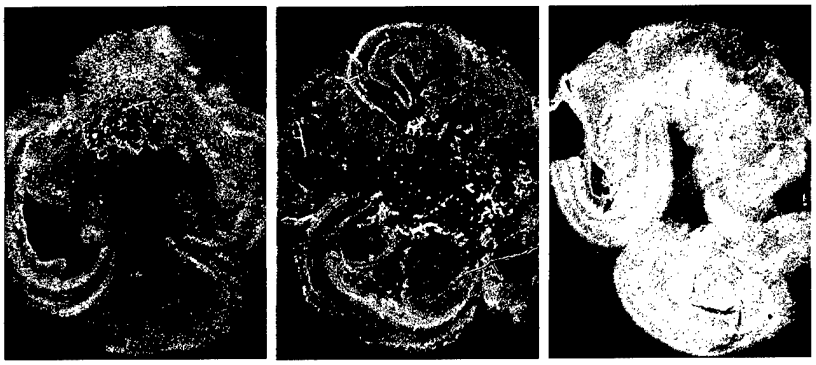
Control 4b Vehicle 4b PbTx 4b



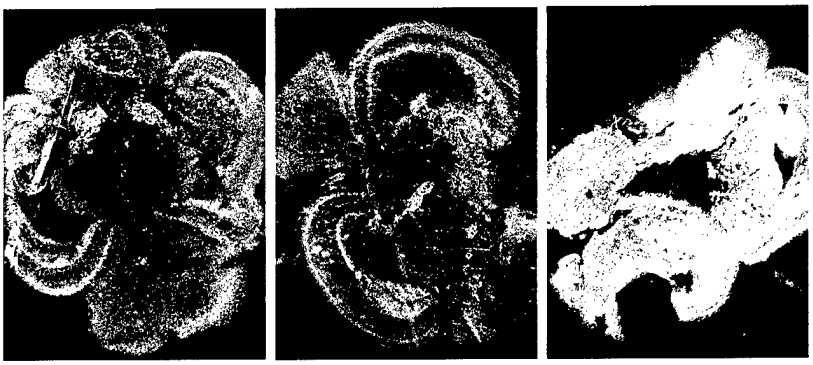
Control 4c Vehicle 4c PbTx 4c



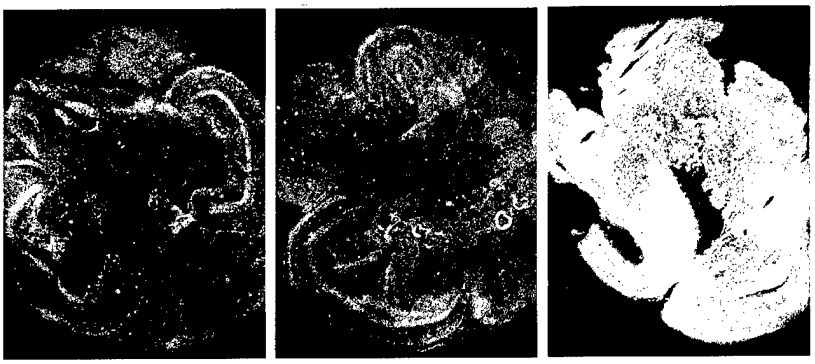
Control 4d Vehicle 4d PbTx 4d



Control 5a | Vehicle 5a | PbTx 5a



Control 5b | Vehicle 5b | PbTx 5b



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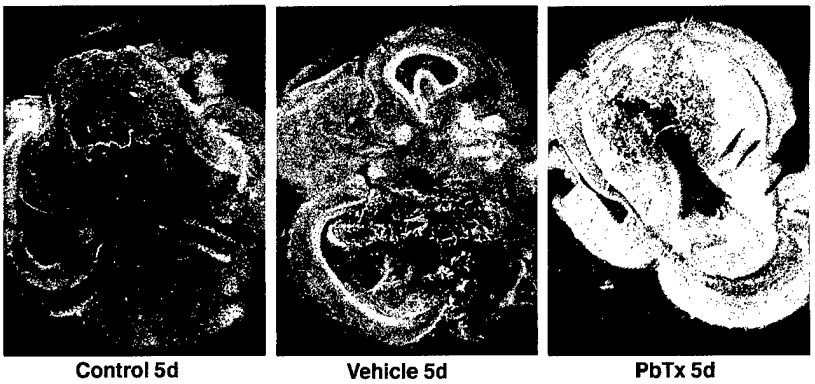

Control 5d | Vehicle 5d | PbTx 5d

## Appendix 2.

### Pathology data generated US FDA CVM.

Data within the table are observations noted for individual fish (fish number across top of table) by organ system. Some observations are normal, indicating the presence of particular structures seen on the glass slide; some indicate pathology. An "X" indicates the presence of an organ or structure on the slide without any notable pathology. Pathology observations may be focal (f), diffuse (d) or multifocal (m). Pathology observations may be ranked by their apparent severity on a scale of 1-5: 1 (minimal), 2 (mild), 3 (moderate), 4 (marked), 5 (severe). Fish 6-15 were exposed to 60 ppb PbTx2; fish 16 and 17 were exposed to 50 ppb PbTx2; fish 18-23 were exposed to 40 ppb PbTx2; and fish 24-29 were exposed to 30 ppb PbTx2. Fish 30-46 were control fish. Note that two fish exposed to 60 ppb PbTx2 were inadvertently given the same accession number, hence the notation of "7a" and "7b," instead of "7" and "8."

| Organ | 6 | 7a | 7b | 9 | 10 |
|---|---|---|---|---|---|
| | | | | | |
| Brain | X | X | X | X – edema surrounding neurons in ganglion and cranial nerve. | X |
| Eye | Optic nerve | Optic nerve | | Retina-pigment | Optic nerve |
| Nares | X | | | | |
| Heart | X | X | X | | X |
| Gills | X | X | X | X, some doublets | Small section |
| Pseudobranch | | | | | X |
| Liver | Necrosis, 2, m (possible autolysis) | Parasitism, cestode, , 3 m | Parasitism, cestode, 3 m | Parasitism, cestode 4 m | X, parasitic vacuoles |
| Esophagus | | Is | | | |
| Stomach | X | Is | X | X | X |
| Intestine | Parasitism, nematode, 3, m gravid | X | Parasitism, nematode, 3, m gravid | X | X |
| Peritoneum | Parasitism, trematode, di. 2 m | X | | | |
| Swim bladder | | | | | |
| Pancreas | | | | | |
| Spleen | X | | X | X Parasitism, cestode, 1 f | X |
| Head Kidney | | X | | X | |
| Trunk Kidney | Parasitism, cestode 3, m | Parasitism, cestode, 3, m | | Ns | X |
| Repro | Ov, de | Ov, de | | TE, Parasitism, cestode, 3. – calc. corpuscles | |
| Muscles | X | X | X | X | X |
| Skin | X | X | X | X | X |
| bone | X | X | X | X | X |
| teeth. | | | | | |
| Misc | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

| Organ | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|
| | | | | | |
| Brain | X | X, mild edema around nerves in ganglion | X | X – edema around nerves in ganglion – look autolytic | X |
| Eye | Choroid, pigment | Optic nerve | Optic nerve | | Optic nerve |
| Nares | X | | | X | X |
| Heart | | | Epicardium, Parasitism, cestode 3, m | X | Epicardium Parasitism, cestode, 3, m |
| Gills | X | X | X | | |
| Pseudobranch | X | | | | |
| Liver | Parasitism, cestode 4, m | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m Bile ducts: Parasitism, myxosporidaia n, 4 m | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m |
| Esophagus | | | | | |
| Stomach | X | X | X | X | X |
| Intestine | X | X, Parasitism, nematode 3, m | X, Parasitism, nematode, 2, f | X | X |
| Peritoneum | X | | | | |
| Swim bladder | | | | | |
| Pancreas | | | | | |
| Spleen | Parasitic vacuoles, 3 m | X | X | X | Parasitism, cestode, 3, m |
| Head Kidney | | | | X | |
| Trunk Kidney | Parasitism, cestode 3 m | Parasitism, cestode, 3 m | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m |
| Repro | te | | Ov | Ov | |
| Muscles | X | X | X | X | X – (adj. To eye) – Parasite – trem. Di,focal 1 |
| Skin | X | X | X | X | X |
| bone | X | X | X | X | X |
| Teeth. | X | | | X | X |
| Misc | | | | | |
| | | | | | |
| | | | | | |

| Organ | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|
| | | | | | |
| Brain | X | X, olfactory nerve | X, pit | X | X, saculus, good |
| Eye | Optic nerve | | | Back of eye | Optic nerve, back of eye |
| Nares | | X | | | |
| Heart | Epicardium, parasitic cysts | X | Epicardium Parasitism, cestode3 m | X | X |
| Gills | X | X | X | X | X |
| Pseudobranch | | | X | | |
| Liver | Parasitism, cestode, 3, m | Parasitism, cestode, 4, m | Parasitism, cestode 3, m | Parasitism, cestode, 3 m | |
| Esophagus | | | | | |
| Stomach | X | X | X | | X |
| Intestine | X | X | X | Parasitism, nematode 4, m | Parasitism, nematode 2, m |
| Peritoneum | Parasitism, cestode 3 m | | Parasitism, cestode 3, m | | Parasitism, cestode 3 m |
| Swim bladder | | | | | |
| Pancreas | | | | | |
| Spleen | Parasitism, cestode, 2, m | X | Parasitic vacuoles, 1 | Parasitism, cestode, 2, m | X |
| Head Kidney | | | | | |
| Trunk Kidney | Parasitism, cestode, 4, m | Parasitism, cestode, 4, m | Parasitism, cestode, 3, m | Parasitism, cestode, 4, m | X |
| Repro | Ov | Ov-de | Ov | Ov | |
| Muscles | X | X | X | X | X |
| Skin | X | X | X | X | X |
| bone | X | X | X | X | X |
| teeth. | | X | | X | IS |
| Misc | | | | | |
| | | | | | |

| Organ | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|
| | | | | | |
| Brain | X | X, saculus | X | X | Olfactory nerve |
| Eye | Optic nerve | Optic nerve | Optic nerve | Optic nerve | Optic nerve |
| Nares | | | | | |
| Heart | X | Parasitic vacuoles, 3, m | | Parasitism, cestode, 3, m | X |
| Gills | X | | X | X | X |
| Pseudobranch | | | | | |
| Liver | Parasitism, cestode, 3, m Bile ducts: Parasitism, myxosporidian, 4 m, necrosis, 3, m – associated with bile ducts above. | Parasitism, cestode, 4, m | Parasitism, cestode, 2, m | Parasitism, cestode, 3, m | Parasitism, cestode, 2, m |
| Esophagus | | | | | |
| Stomach | | X | X | X | X |
| Intestine | Parasitism, nematode, 2, m | Parasitism, nematode, 2, m | X | Parasitism, nematode, 3, m | Parasitism, nematode 2 m |
| Peritoneum | | | | | |
| Swim bladder | | | | | |
| Pancreas | | | | | |
| Spleen | X | Parasitic vacuoles, 2,f | X | Parasitic vacuoles 2 | Parasitism, cestode, on capsule |
| Head Kidney | | | X | | |
| Trunk Kidney | Parasitic vacuoles 5 m | Parasitism, cestode, 4, m | Parasitism, cestode, 2, m | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m |
| Repro | Ov | Ov | Ov | Ov is | |
| Muscles | X | X | X | X | X |
| Skin | X | X | X | X | X |
| bone | X | X | X | X | X |
| Teeth. | X | | X | | |
| Misc | | | | | |
| | | Torn sections | Torn sections | | |
| | | | | | |

| **Organ** | **26** | **27** | **28** | **29** | **30** |
|---|---|---|---|---|---|
| | | | | | |
| Brain | Perinerual edema; pituitary present, olfactory lobe | X, saculus | X | X | Mild edema Saculus present |
| Eye | Optic nerve | Optic nerve | Optic nerve | Back of eye | |
| Nares | | x | | | |
| Heart | Epicardium Parasitism, cestode, 3, m | X | X | X | |
| Gills | X | | X | X | x |
| Pseudobranch | | | | | |
| Liver | Parasitism, cestode, 3, m | Parasitism, cestode, 2, m | Parasitism, cestode, 2, m | Parasitism, cestode, 2, m | Parasitism, cestode, 4, m |
| Esophagus | | | | | |
| Stomach | X | X | X | X | X |
| Intestine | Parasitism, nematode 2 m | Parasitism, nematode 2 m | X | X | X |
| Peritoneum | Parasitism, cestode, 3, m | Parasitism, cestode, 2, m | | Parasitism, cestode, 2, m | Parasitism, nematode, 2 f |
| Swim bladder | | | | | |
| Pancreas | | | | | X- prominent islets |
| Spleen | Parasitic vacuoles, 2,f | X | Parasitic vacuoles 2 f | X | |
| Head Kidney | | | | | X |
| Trunk Kidney | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m | |
| Repro | | Ov | | Ov | Ov |
| Muscles | X | X | X | X | X |
| Skin | X | X | X | X | X |
| bone | X | X | X | X | X |
| Teeth. | | X | | X | |
| Misc | | | | | |
| | | | | | |
| | | | | | |

| **Organ** | **31** (3 slides) | **32** | **33** | **34** | **35** |
|---|---|---|---|---|---|
| | | | | | |
| Brain | | X | X | x- small section | Small section |
| Eye | | Ix | | X | X |
| Nares | | | | | X |
| Heart | inflammation, chronic, Granuloma, 1 f | Atrium | X | | |
| Gills | X | Is | X | X | X |
| Pseudobranch | | | | X | X |
| Liver | Parasitism, cestode, 3, m Gall bladder | Parasitism, cestode, 3, m Gall bladder | Parasitism, cestode, 4, m | Parasitism, cestode, 4, m | Parasitism, cestode, 3, m |
| Esophagus | | | | | |
| Stomach | X | X | X | X | |
| Intestine | X | X | X | X | Parasitism – nematode 3, m Muscularis – parasitism trem. 1 f |
| Peritoneum | X | X | Granulomata, 3 m | X | Parasitism, cestode, 3, m |
| Swim bladder | | | | | |
| Pancreas | x-prom islets | X – prom islets | x-prom islets | X | x- prom islets |
| Spleen | X | X | X- one large vacuole – possibly parasitic | | X |
| Head Kidney | | | X | | X |
| Trunk Kidney | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m | Parasitism, cestode, 3, m | Parasitism, cestode, 4, m | Parasitism, cestode, 4, m. Collecting duct – Myxosporean, 3 m |
| Repro | | | | Ov | Ov |
| Muscles | X | X | X | X | X |
| Skin | X | X | X | X | X |
| bone | X | X | X | X | X |
| Teeth. | | | | X | |
| Misc | | | | | |
| | | | | | |
| | | | | | |

| **Organ** | **36** | **37** | **38** | **39** | **40** |
|---|---|---|---|---|---|
| | | Only one slide | | | |
| Brain | X | X | x- nice section | x-torn | X |
| Eye | | Is | | X | X |
| Nares | | | | | |
| Heart | | | X | inflammation, chronic epicardium, 3, f | X |
| Gills | X | | X | X | X |
| Pseudobranch | | | | | |
| Liver | Parasitism, cestode 2, m | | Parasitism, cestode 3, m | Parasitism, cestode 1, f | Parasitism, cestode 3, m |
| Esophagus | | | | | |
| Stomach | X | | X | X | X |
| Intestine | X | | X | X | X |
| Peritoneum | Parasitism, cestode 3, m | | X | | X |
| Swim bladder | | | | | |
| Pancreas | X | | X | X | X |
| Spleen | | | X | X | Parasitism, cestode 1,f (large) |
| Head Kidney | X | | | | |
| Trunk Kidney | Parasitism, cestode 2, m | | | Parasitism, cestode 3, m | Parasitism, cestode 4, m |
| Repro | Ov-parasitism, cestode 1 f | | | Ov | |
| Muscles | X | | X | X | X |
| Skin | X | | X | X | X |
| bone | X | | X | X | X |
| Teeth. | X | | X | | |
| Misc | | | | | |
| | | | | | |
| | | | | | |

| Organ | 41 | 42 | 43 | 44 | 45 |
|---|---|---|---|---|---|
| | | | | | |
| Brain | X | X | Edema in nerves, mild | X | X |
| Eye | x-retina | X | X | | X |
| Nares | | | | | |
| Heart | | | | Parasitism, cestode 4, m | |
| Gills | X | X | X | X | |
| Pseudobranch | | | | | |
| Liver | Parasitism, cestode 2, m | Parasitism, cestode 2, m | Parasitism, cestode 2, m | Parasitism, cestode 3, m | Parasitism, cestode 3, m |
| Esophagus | | | | | |
| Stomach | | X | X | X | X |
| Intestine | X | X | X- parasitism, nematode, 3, m | - parasitism, nematode, 3, m | Parasitism, nematode, 2, m |
| Peritoneum | X | X | X | X | X Parasitism, cestode 2, m |
| Swim bladder | | | | | |
| Pancreas | X | X | X | X | X |
| Spleen | X | X | X | Parasitism, cestode 1,f | Parasitism, cestode cyst |
| Head Kidney | X | X | X | | |
| Trunk Kidney | Parasitism, cestode 2, m | Parasitism, cestode 2, m | Parasitism, cestode 2, m | Parasitism, cestode 4, m | Parasitism, cestode 5, m |
| Repro | | | | Ov | Ov |
| Muscles | X | X | X | X | X |
| Skin | X | X | X | X | X |
| bone | X | X | X | X | X |
| Teeth. | | | | | X |
| Misc | | | | | |
| | | | | | |
| | | | | | |

| **Organ** | **46** | | | | |
|---|---|---|---|---|---|
| | | | | | |
| Brain | X | | | | |
| Eye | | | | | |
| Nares | | | | | |
| Heart | Is | | | | |
| Gills | | | | | |
| Pseudobranch | | | | | |
| Liver | Parasitism, cestode 2,m | | | | |
| Esophagus | | | | | |
| Stomach | X | | | | |
| Intestine | X | | | | |
| Peritoneum | X | | | | |
| Swim bladder | | | | | |
| Pancreas | X | | | | |
| Spleen | X | | | | |
| Head Kidney | X | | | | |
| Trunk Kidney | Parasitism, cestode 3, m inflammation, chronic, near parasite Granuloma –4 f | | | | |
| Repro | | | | | |
| Muscles | X | | | | |
| Skin | X | | | | |
| bone | X | | | | |
| Teeth. | | | | | |
| Misc | | | | | |
| | | | | | |
| | | | | | |

## Appendix 3.

Poster presented at Harmful Algal Bloom Conference, Hobart, Tasmania and Woods Hole, MA.

# REAL-TIME MONITORING FOR TOXICITY CAUSED BY HARMFUL ALGAL BLOOMS AND OTHER WATER QUALITY PERTURBATIONS

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(3) University of Maryland Program on Human Health and the Environment, 10 South Pine Street, Baltimore, Maryland, 21201-1192, USA
(4) North Carolina State University, Center for Applied Aquatic Ecology, 620 Hutton Street, Suite 104, Raleigh, North Carolina 27606, USA
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## ABSTRACT

Harmful algal blooms, including those associated with toxicity, hypoxia events and other water quality perturbations, have increased in frequency and severity worldwide. An automated, remote biomonitoring system is being developed to continuously monitor waterways that are susceptible to these water quality perturbations. The system consists of a series of flow-through chambers that expose sentinel fish. Changes in ventilatory and movement patterns, as they relate to altered or toxic water quality, are monitored. Paired electrodes in each chamber non-invasively transmit an amplified electrical signal from each fish to an on-site computer. These signals and water quality data can be transmitted to remote locations for further analysis and potential management response. Freshwater laboratory trials with bluegill indicated that exposure to sublethal hypoxia produced an increase in ventilatory rate with minor associated depressions in ventilatory depth and elevations in cough rate. For the purpose of validating the system with HAB toxins, bluegill were also exposed to brevetoxin (PbTx2). One hour exposure to 40 ppb PbTx2 followed by recovery produced minor elevations in ventilatory rate with strong cough and movement responses. Brackish water (15 ppt) laboratory trials with tilapia exposed to *Pfiesteria* culture water showed a different response pattern: strong elevations in cough rate and % movement. Initial efforts to link biomonitoring signals with stress mechanisms were initiated with PbTx2-exposed bluegill; these animals showed altered patterns of glucose metabolism in their brains based on incorporation of 2-deoxyglucose (2-DG) *in vivo*. These laboratory biomonitoring studies indicate different response patterns under different stress conditions, and demonstrate utility of the biomonitoring system and the 2-DG method. This study has been supported in part by a U.S. Environmental Protection Agency EMPACT grant, #DW21938668-01-1, and the University of Maryland Aquatic Pathobiology Center.

## BACKGROUND

This is a report of a collaborative effort between University of Maryland Aquatic Pathobiology Center and the U.S. Army Center for Environmental Health Research on a U.S. EPA EMPACT project entitled "Real Time Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations." The overall EMPACT project was designed to provide near real-time monitoring of potentially toxic waterway conditions using an automated biomonitoring system. The purpose of this study was to evaluate the biomonitoring system responses under controlled laboratory conditions, and to attempt to link biomonitor signal responses to actual stress mechanism(s) in exposed fish by evaluating changes in brain glucose kinetics.

Fish respiratory response signals are non-invasively transmitted, amplified and uploaded into a personal computer, where biological response signals from exposed fish are analyzed and can be remotely transmitted. Biological responses include ventilatory rate (VR), ventilatory depth (VD), cough rate (CR) and percent movement (%Mov). The %Mov response is from data not recognized as a ventilatory signal, and is a function of the number of non-ventilatory data blocks per unit time. The datasets may be used to generate an integrated measurement of intoxication, sufficient to generate an alarm function. An alarm is generated when >70% of the fish in a test group are "out of control" (i.e., individual fish exceed 5 SD from its own baseline mean in any ventilatory parameter). This biomonitoring system was designed to generate data that health and environmental officials can use to remotely provide objective, timely advice on the safety of waters.

This project represents a multi-organizational effort between US EPA, US Army Center for Environmental Health Research, University of Maryland , Johns Hopkins University, US FDA Center for Veterinary Medicine, and the US Army Medical Research Institute for Infectious Diseases.

## METHODS

The biomonitoring system consists of a series of eight flow-through Plexiglas™ chambers that house sentinel fish (Figure 1). Chamber-acclimated juvenile bluegill (*Lepomis macrochirus*) were assessed for biological signal responses due to exposure to hypoxia and to brevetoxin (PbTx2) at 19°C and 25°C in freshwater at the UM Aquatic Pathobiology Center. Juvenile hybrid tilapia were exposed to brackish *Pfiesteria piscicida* culture water (15 ppt salinity) at the NCSU Center for Applied Aquatic Ecology. Dinoflagellate culture water contained $10^2$ - $10^3$ zoospores/mL that were derived from clonal cultures that killed fish. The identity of these cultures was confirmed by scanning EM of suture-swollen cells and cross-checked using FISH (P. Rublee) and a heteroduplex mobility assay (D. Oldach).

Biomonitoring responses included ventilatory rate (VR), average ventilatory depth (VD), cough rate (CR) and percent movement (%Mov). Six to eight unique fish were exposed simultaneously in each exposure. Five dissolved oxygen (DO) depressions were accomplished by temporarily stopping water flow into the individual exposure chambers; the biological oxygen demand of the fish to naturally reduce the DO concentration to approximately 50%. PbTx2 was reconstituted in non-chemically dechlorinated tap water with the vehicle Emulphor-620 (0.0001%). Exposure concentrations approximated the LC50, 40 µg/L. PbTx2 exposure concentrations were determined by ELISAs.

In order to monitor changes CNS activity, bluegill were also injected with $^{14}C$ labeled 2-deoxyglucose and exposed to 40 µg/L PbTx2 for 60 min. Fish were sacrificed and their brains were removed for cryosectioning and autoradiography.

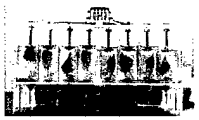
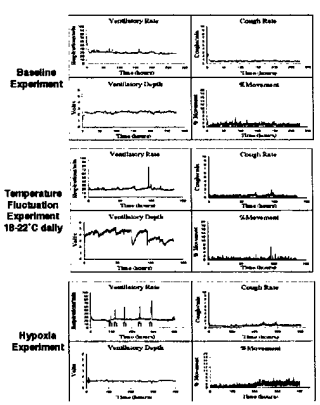


FIGURE 1. Bank of 8 flow-through biomonitoring chambers. Note the set of electrodes above and below the fish connected to their respective wiring harnesses. These electrodes non-invasively pick up weak electrical impulses and transmit them through an amplifier to a computer, where the sine wave responses are deciphered into the VR, VD, CR and %Mov endpoints by software algorithms.

## RESULTS & SUMMARY

| Exposure Type | $NH_3$ (mg/L) | pH | D.O. (mg/L) | Hardness (mg/L as CaCO3 equiv) |
|---|---|---|---|---|
| Flow-through | 0.0043 ± 0.0008 | 8.0 ± 0.1 | 6.7 ± 0.2 | 79 ± 4 |
| Intermittent hypoxia | 0.0035 ± 0.0005 | 7.8 ± 0.1 | 3.4 ± 0.6 | -- |

TABLE 1. Water quality in biomonitoring chambers from flow-through experiments (baselines, temperature fluctuations and PbTx2) and hypoxia experiments. Data are means ± SD.



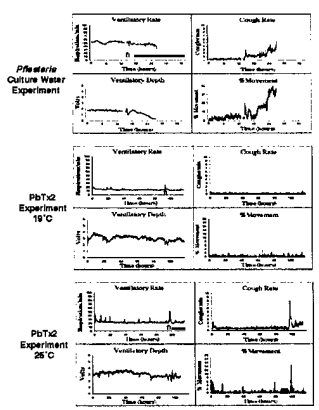




FIGURE 2. Ventilatory responses (VR, VD, CR and %Mov) from the different stress experiments (6 white quad panels on left). A denotes time of exposure shown for convenience along respective VR x-axes; a red line along an x-axis indicates the generation of an alarm signal in the toxin exposure experiments. **The baseline experiment** as well as initial data from all other exposures demonstrates the relative steady-state of all 4 response variables. **The temperature fluctuation experiment** shows the sensitivity of the VR, AD and CR responses to subtle temperature change. There was a close temporal relationship between rising temperature and small increases in VR and CR, with concomitant decreases in VD. Also, note the VR and %Mov spikes at 96 hours – these were caused by an investigator walking into the exposure room. **The hypoxia experiment** showed notable elevations in VR with concomitant depressions in AD and elevations in CR during each of 5 4-hour hypoxia "events." Fish appeared to recover from each of the DO depressions when normoxic conditions returned. **The *Pfiesteria* culture water experiment** showed elevations in CR and %Mov; AD dropped as fish succumbed. Time to response was 1.75 hours. **The PbTx2 experiment** at 25°C demonstrated a very different response signature, with temporal spikes in VR, CR and %Mov. Time to response was 0.5 hours. Interestingly these responses did not differ from baseline when PbTx2 exposure occurred at 19°C. Response data for all experiments are averages for 6 - 8 fish.

FIGURE 3. Brain autoradiographs of PbTx2-exposed bluegill. There is a notable increase in $^{14}C$ 2-deoxyglucose labeling in the midbrain of exposed fish (right panel) compared with water exposed fish (left) and vehicle water exposed fish (middle). This difference indicates altered regional brain activity in the CNS due to PbTx2 exposure. Images shown are typical of 3-5 slices per fish with 5 fish per exposure.

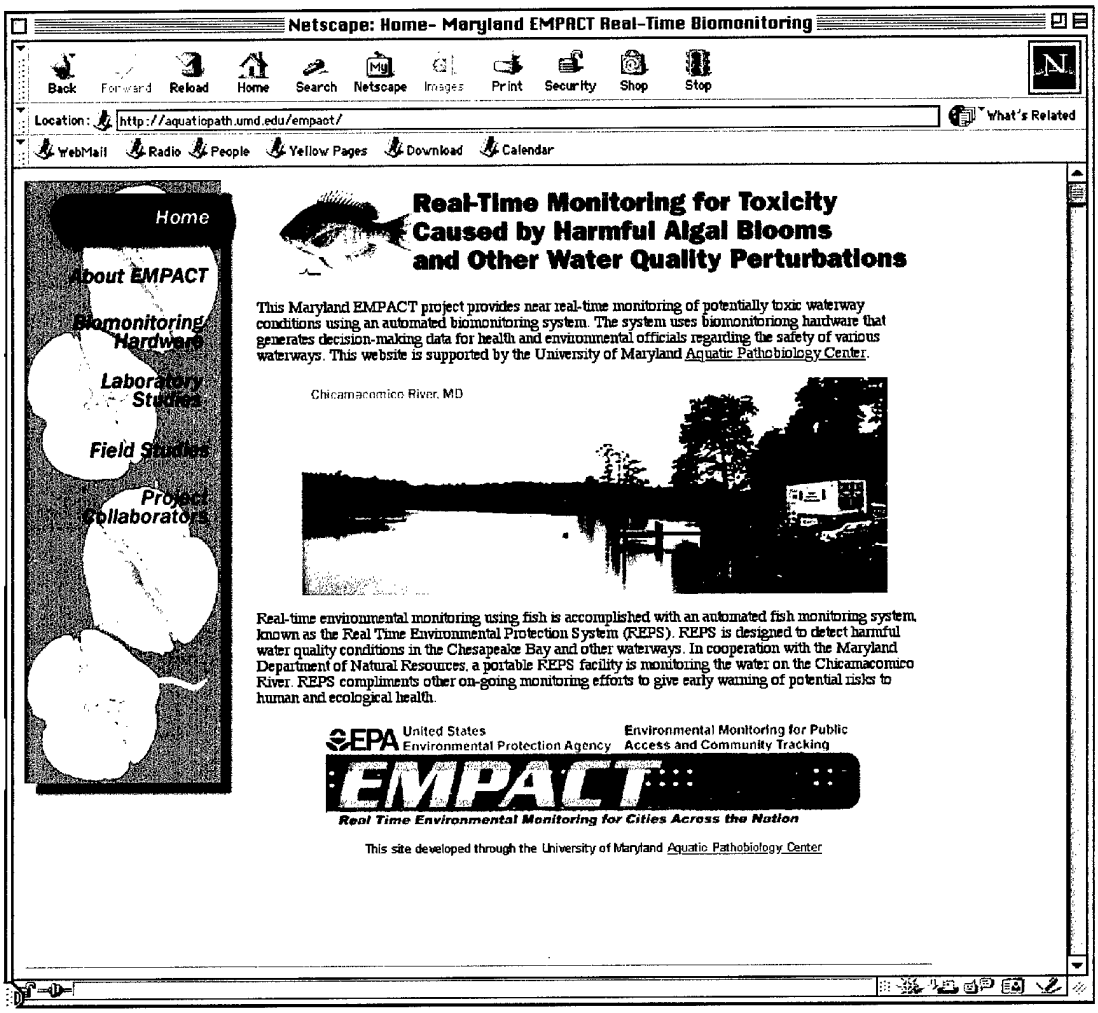
## ACKNOWLEDGEMENTS

We thank H. Glasgow, Center for Applied Aquatic Ecology, North Carolina State University, for his insights and assistance with the *Pfiesteria* culture water exposure component of this project. All other exposure efforts were conducted at the University of Maryland Aquatic Pathobiology Center. We also thank J. Sass, University of Maryland Program in Human Health and the Environment, for her development efforts with the 2-DG assay.

## Appendix 4.

### UMB EMPACT Website.

Project outreach to the public and other agencies was made available through a custom website that was developed and maintained by the UM Aquatic Pathobiology Center. At the time of this report, the unique resource location (URL) for the website is http://aquaticpath.umd.edu/empact.


Netscape: Home- Maryland EMPACT Real-Time Biomonitoring
Back
Forward
Reload
Home
Search
Netscape
Images
Print
Security
Shop
Stop
Location: http://aquatiopath.umd.edu/empact/
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Radio
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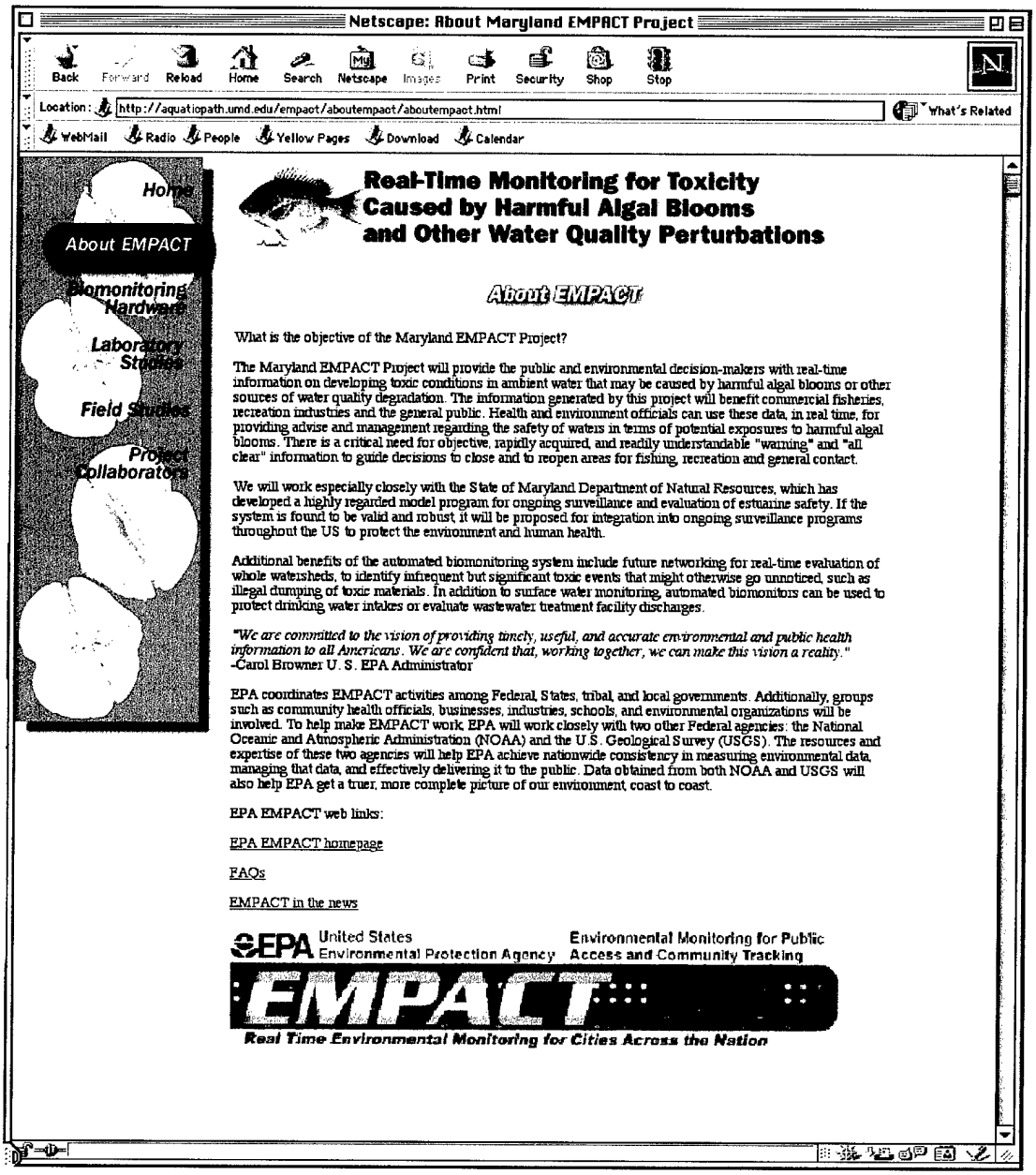
About EMPACT
Biomonitoring Hardware
Laboratory Studies
Field Studies
Project Collaborators
Real-Time Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations
This Maryland EMPACT project provides near real-time monitoring of potentially toxic waterway conditions using an automated biomonitoring system. The system uses biomonitoring hardware that generates decision-making data for health and environmental officials regarding the safety of various waterways. This website is supported by the University of Maryland Aquatic Pathobiology Center.
Chicamacomico River, MD
Real-time environmental monitoring using fish is accomplished with an automated fish monitoring system, known as the Real Time Environmental Protection System (REPS). REPS is designed to detect harmful water quality conditions in the Chesapeake Bay and other waterways. In cooperation with the Maryland Department of Natural Resources, a portable REPS facility is monitoring the water on the Chicamacomico River. REPS compliments other on-going monitoring efforts to give early warning of potential risks to human and ecological health.
EPA United States Environmental Protection Agency
Environmental Monitoring for Public Access and Community Tracking
EMPACT
Real Time Environmental Monitoring for Cities Across the Nation
This site developed through the University of Maryland Aquatic Pathobiology Center


Netscape: About Maryland EMPACT Project
Back
Forward
Reload
Home
Search
Netscape
Images
Print
Security
Shop
Stop
Location: http://aquaticpath.umd.edu/empact/aboutempact/aboutempact.html
What's Related
WebMail
Radio
People
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About EMPACT
Biomonitoring Hardware
Laboratory Studies
Field Studies
Project Collaborators
Real-Time Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations
About EMPACT
What is the objective of the Maryland EMPACT Project?
The Maryland EMPACT Project will provide the public and environmental decision-makers with real-time information on developing toxic conditions in ambient water that may be caused by harmful algal blooms or other sources of water quality degradation. The information generated by this project will benefit commercial fisheries, recreation industries and the general public. Health and environment officials can use these data, in real time, for providing advise and management regarding the safety of waters in terms of potential exposures to harmful algal blooms. There is a critical need for objective, rapidly acquired, and readily understandable "warning" and "all clear" information to guide decisions to close and to reopen areas for fishing, recreation and general contact.
We will work especially closely with the State of Maryland Department of Natural Resources, which has developed a highly regarded model program for ongoing surveillance and evaluation of estuarine safety. If the system is found to be valid and robust, it will be proposed for integration into ongoing surveillance programs throughout the US to protect the environment and human health.
Additional benefits of the automated biomonitoring system include future networking for real-time evaluation of whole watersheds, to identify infrequent but significant toxic events that might otherwise go unnoticed, such as illegal dumping of toxic materials. In addition to surface water monitoring, automated biomonitors can be used to protect drinking water intakes or evaluate wastewater treatment facility discharges.
"We are committed to the vision of providing timely, useful, and accurate environmental and public health information to all Americans. We are confident that, working together, we can make this vision a reality."
-Carol Browner U. S. EPA Administrator
EPA coordinates EMPACT activities among Federal, States, tribal, and local governments. Additionally, groups such as community health officials, businesses, industries, schools, and environmental organizations will be involved. To help make EMPACT work, EPA will work closely with two other Federal agencies: the National Oceanic and Atmospheric Administration (NOAA) and the U.S. Geological Survey (USGS). The resources and expertise of these two agencies will help EPA achieve nationwide consistency in measuring environmental data, managing that data, and effectively delivering it to the public. Data obtained from both NOAA and USGS will also help EPA get a truer, more complete picture of our environment, coast to coast.
EPA EMPACT web links:
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EMPACT in the news
EPA United States Environmental Protection Agency
Environmental Monitoring for Public Access and Community Tracking
EMPACT
Real Time Environmental Monitoring for Cities Across the Nation

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Location: http://aquaticpath.umd.edu/empact/biomonitoring/biomonitoring.html

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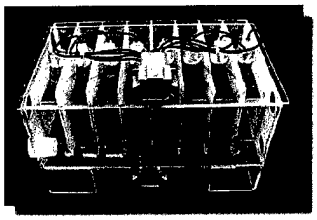
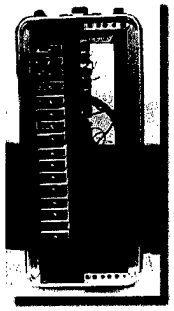
# Real-Time Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations

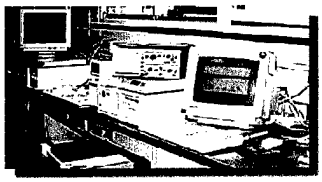
## Biomonitoring Hardware



River water is filtered to remove large particles, and then pumped through an 8-chamber exposure system. Each flow-through chamber houses a single fish. An upper and a lower electrode are above and below the fish. The electrodes do not emit any electricity but do pick up weak electrical impulses generated by the fish respiratory movements. The black wires from the upper electrodes are seen on the top of the chamber.



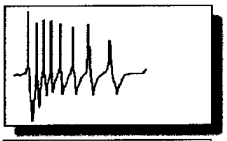
Signals from the 8 fish are transmitted via the electrode wires to an amplifier system. The amplifier enhances the signals approximately 10,000 times before they are relayed to a computer.



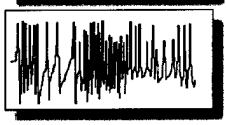
The computer stores thousands of data points from each fish over the exposure period. Specially developed software detects four types of responses:

- Ventilatory rate (number of breaths per minute)
- Ventilatory depth (the force of the respiratory muscles, respiratory tracing height)
- Cough rate (number of coughs per minute)
- Total movement (amount of fish movement within the chamber).

This electrical tracing shows a series of (10) ventilatory breaths. If that tracing were from 20 seconds of data, the ventilatory rate (i.e. frequency of peaks per 20 second interval) would be 30. Note that the height (ventilatory depth) of the peaks is relatively constant.

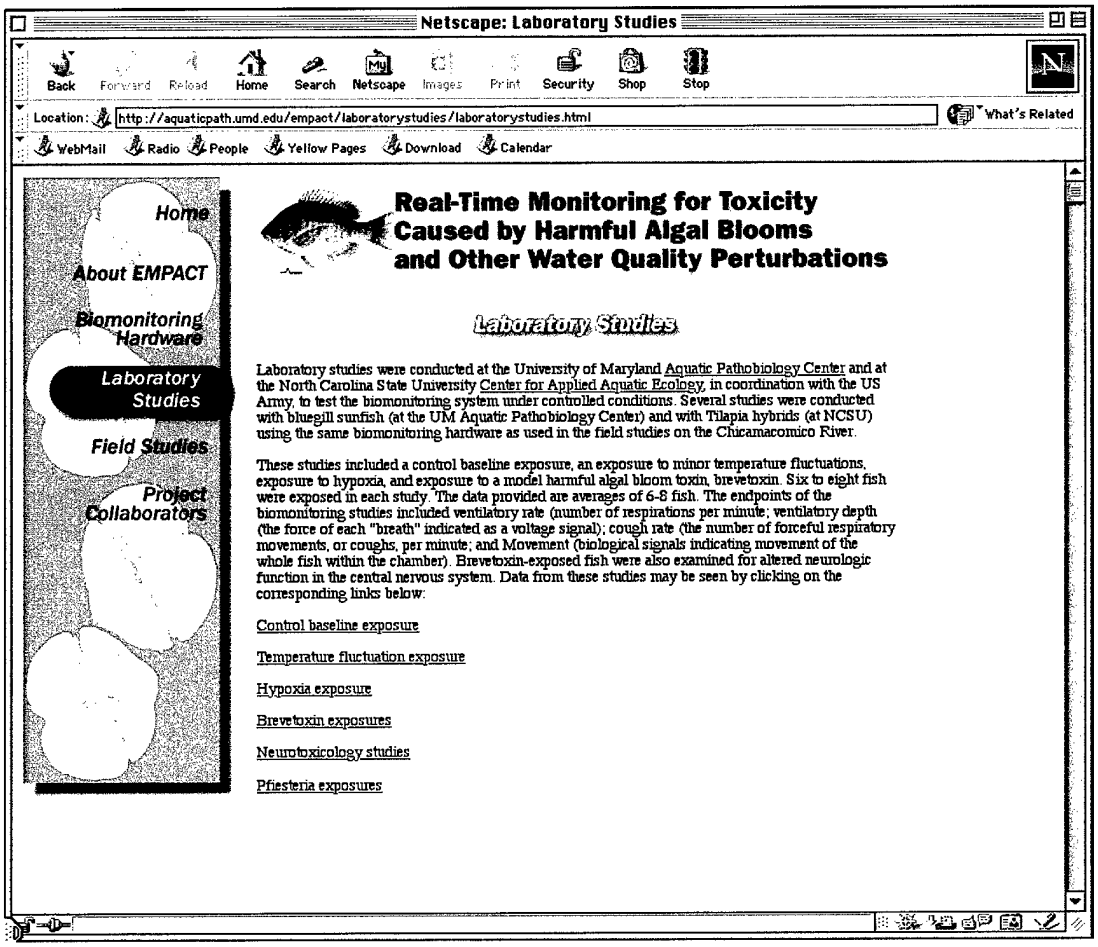


In the middle tracing, the peak height is notably greater. This set of peaks shows a cough response. There are actually two types of cough responses. The first (leftmost) peak is a "spike cough," followed by 5 several normal ventilatory breaths. The last peak in this trace shows a "high frequency cough." A high frequency cough is a rapid reversal of water through the fish gills.



The bottom tracing shows momentary whole body movement of a fish inside a chamber. The electrodes are picking up muscle movement much greater than would be generated from respiratory muscles alone.

If ventilatory signals from at least 6 out of 8 fish fall outside a normal range, than an "alarm" signal is generated. This signal can trigger an automated phone call to managers, indicating some suboptimal water quality. The alarm also triggers an automated water sampling device to make collections for later analysis. A series of in-line electrodes (not shown) measures dissolved oxygen, temperature, pH and salinity). This additional water quality monitoring helps define why the fish fall outside the range of normal ventilatory activity.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Laboratory Studies
Laboratory studies were conducted at the University of Maryland Aquatic Pathobiology Center and at the North Carolina State University Center for Applied Aquatic Ecology, in coordination with the US Army, to test the biomonitoring system under controlled conditions. Several studies were conducted with bluegill sunfish (at the UM Aquatic Pathobiology Center) and with Tilapia hybrids (at NCSU) using the same biomonitoring hardware as used in the field studies on the Chicamacomico River.
These studies included a control baseline exposure, an exposure to minor temperature fluctuations, exposure to hypoxia, and exposure to a model harmful algal bloom toxin, brevetoxin. Six to eight fish were exposed in each study. The data provided are averages of 6-8 fish. The endpoints of the biomonitoring studies included ventilatory rate (number of respirations per minute; ventilatory depth (the force of each "breath" indicated as a voltage signal); cough rate (the number of forceful respiratory movements, or coughs, per minute; and Movement (biological signals indicating movement of the whole fish within the chamber). Brevetoxin-exposed fish were also examined for altered neurologic function in the central nervous system. Data from these studies may be seen by clicking on the corresponding links below:
Control baseline exposure
Temperature fluctuation exposure
Hypoxia exposure
Brevetoxin exposures
Neurotoxicology studies
Pfiesteria exposures

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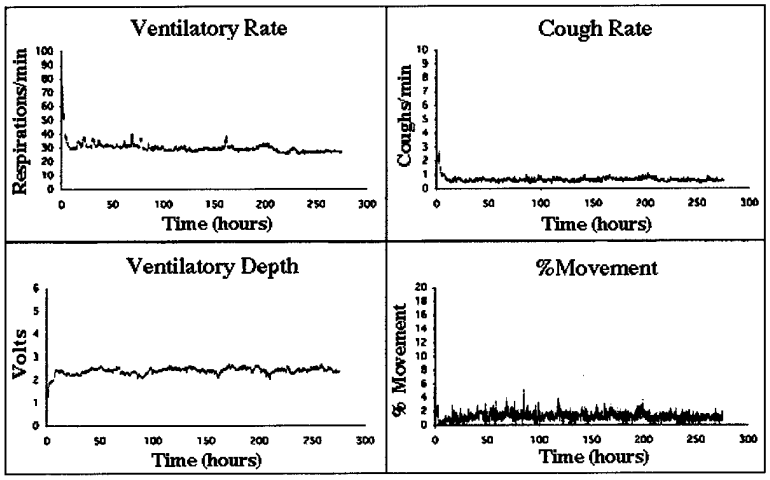
**Control Baseline Study**. Fish were maintained within the exposure chambers for over 250 hours. Although there are some minor fluctuations in ventilatory rate and ventilatory depth, the fish did not exhibit any "out of control" behavior, as might set of a biomonitoring system alarm.

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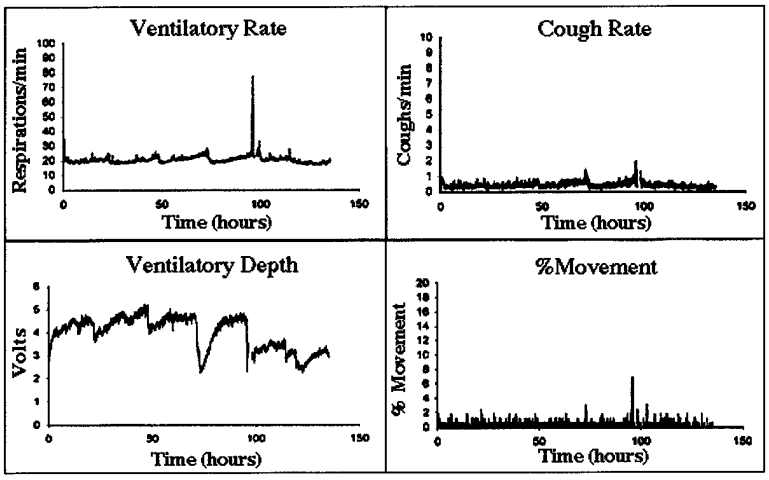
**Temperature Fluctuation Study**. The temperature fluctuation study collected chamber data for over 120 hours. We used a water delivery system that caused daily 4°C changes in water temperature (from 19°C rising slowly to 24°C, then relatively more abruptly back down to 19°C). With each daily rise in water temperature there was an associated minor elevation in ventilatory rate and depression in ventilatory depth. Cough rate and %Movement did not appear notably effected. Note the major ventilatory rate and ventilatory depth spikes at 98 hours; this was caused by one of the investigators simply walking into the exposure room!

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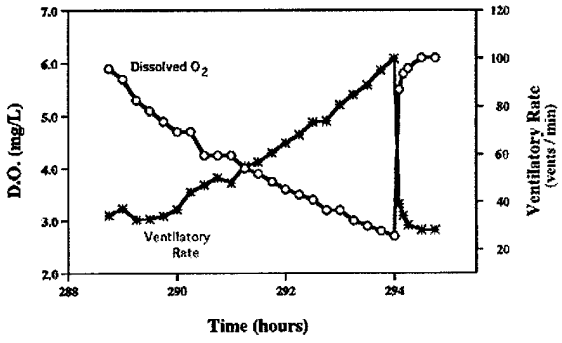
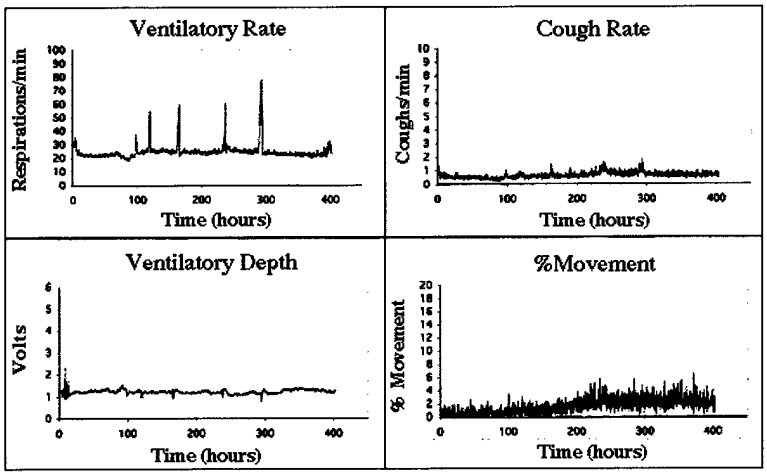
**Hypoxia Studies.** Hypoxia was chosen as a stressor since it is a common cause of aquatic animal stress and fish kills along the U.S. Atlantic coast, and it is relatively easy to experimentally execute. The experimental design utilized 7 out of 8 chambers of the system for fish exposure and collection of biological data. The 8th chamber was used for monitoring dissolved oxygen, pH and ammonia. The figure below exemplifies a reduction of dissolved oxygen over several hours. Note the inverse relationship between dissolved oxygen in the water and the ventilatory response of the fish, and that this response is rapidly reversible.




We induced 5 discrete dissolved oxygen (DO) depressions (i.e., hypoxic events) at 98, 117, 162, 235 and 288 hours after test initiation. This was accomplished by stopping water flow to the exposure chambers. Reduction in DO, therefore, was caused by the biological oxygen demand of the fish respiring in the chambers. DO concentrations in the exposure chambers were reduced by approximately 50% while ammonia and pH remained within acceptable limits. Biological responses (see immediately below) indicated significant elevations in ventilatory rates with minor associated depressions in ventilatory depth and elevations in cough rate. In a field application these discrete responses would have triggered an alarm status from the system's computer. Note that the stress of up to 60% reduction in DO appears to cause reversible responses in the parameters measured in this study.

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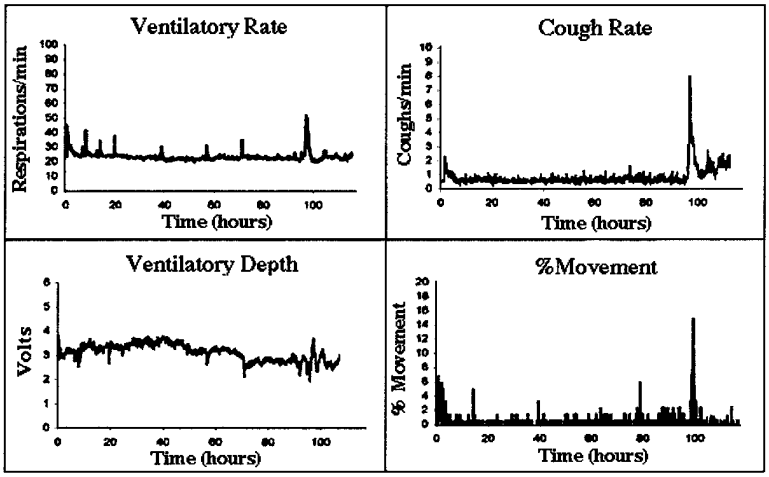
**Brevetoxin Studies.** A 60 minute brevetoxin (PbTx2) exposure was conducted after 4 days of baseline acclimation at 25 degrees C. The figure below shows an elevated spike in ventilatory rate with a major concomitant elevation in cough response and %Movement. In a field application these responses would have triggered the system's alarm function.

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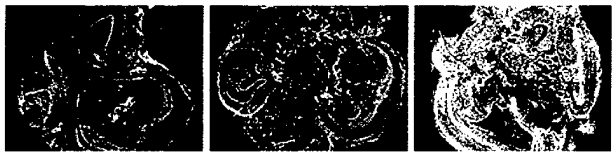
**Neurotoxicology Studies**. This aspect of the project was done in collaboration with the Program for Human Health and the Environment at the University of Maryland School of Medicine. We developed a novel system to better understand the mechanisms of environmental neurotoxins and to evaluate brevetoxin as a model biotoxin. This was accomplished by detecting radiolabeled 2-deoxyglucose, a non-metabolizable form of glucose (glucose is an important energy source used by brain cells). Control and brevetoxin-exposed fish were exposed to 2-deoxyglucose and then were humanely put to sleep. The brains of these fish were examined for the radiolabel of the alternate form of glucose. The figure below shows three bluegill brain cross sections. The image on the left and in the middle are control fish (no brevetoxin). The image on the right is from a fish exposed to brevetoxin. These images indicate that there is some alteration of energy utilization in brevetoxin-exposed fish. By analyzing the areas of the brain that are most affected, we can better understand the mechanism by which various neurotoxins act.



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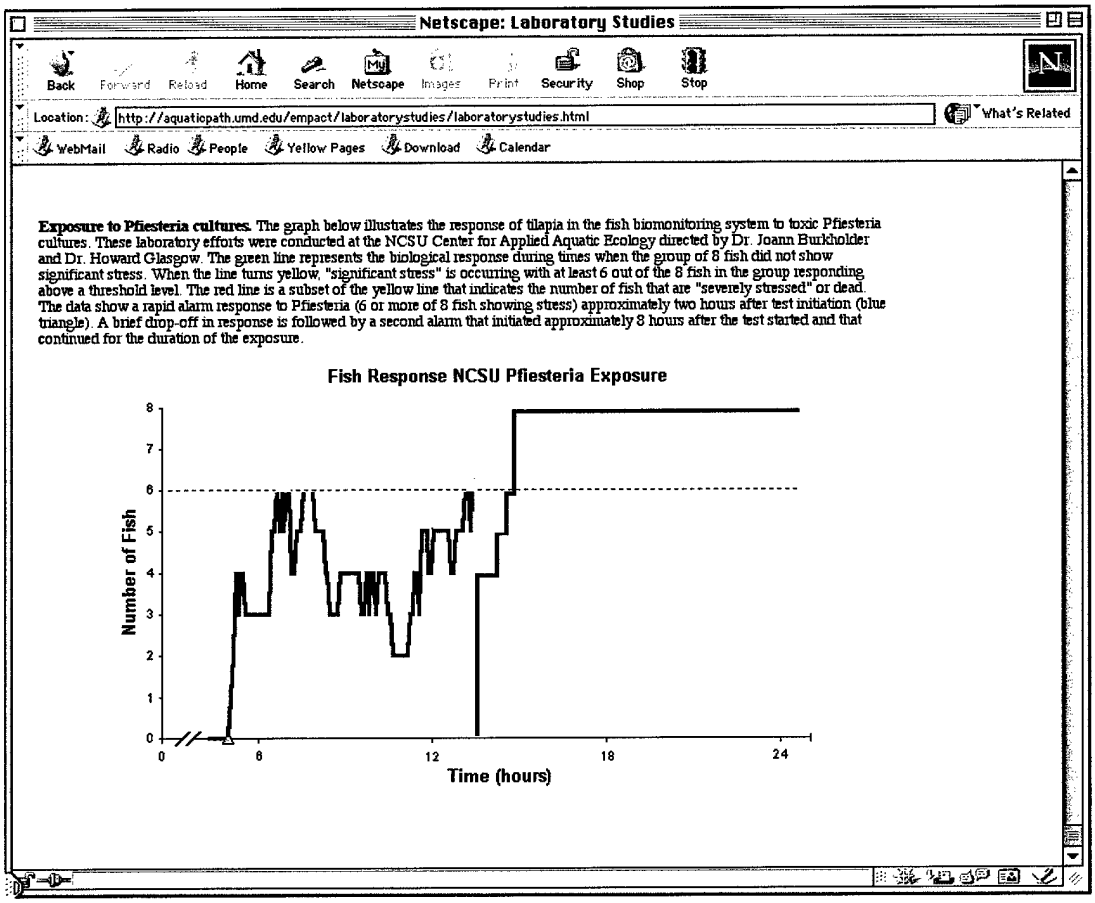
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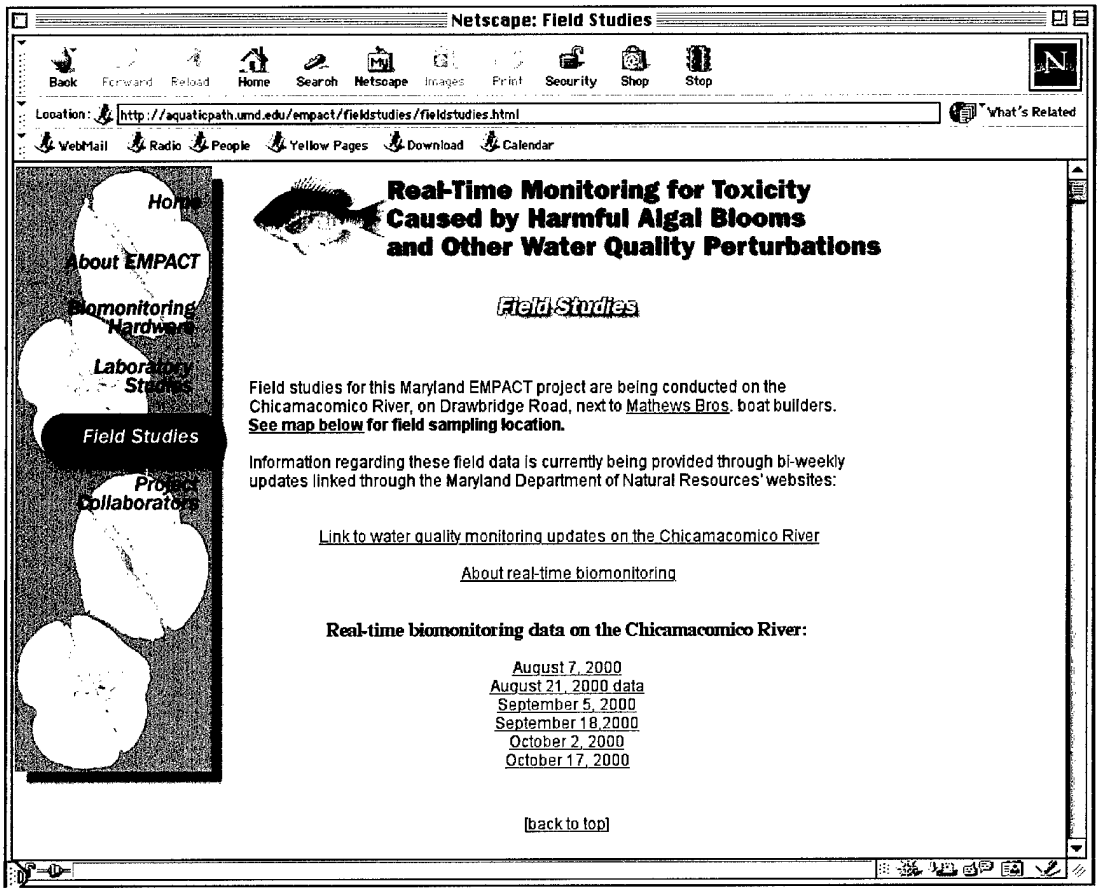
Exposure to Pfiesteria cultures. The graph below illustrates the response of tilapia in the fish biomonitoring system to toxic Pfiesteria cultures. These laboratory efforts were conducted at the NCSU Center for Applied Aquatic Ecology directed by Dr. Joann Burkholder and Dr. Howard Glasgow. The green line represents the biological response during times when the group of 8 fish did not show significant stress. When the line turns yellow, "significant stress" is occurring with at least 6 out of the 8 fish in the group responding above a threshold level. The red line is a subset of the yellow line that indicates the number of fish that are "severely stressed" or dead. The data show a rapid alarm response to Pfiesteria (6 or more of 8 fish showing stress) approximately two hours after test initiation (blue triangle). A brief drop-off in response is followed by a second alarm that initiated approximately 8 hours after the test started and that continued for the duration of the exposure.
Fish Response NCSU Pfiesteria Exposure
Number of Fish
0
1
2
3
4
5
6
7
8
0
6
12
18
24
Time (hours)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Field Studies
Field studies for this Maryland EMPACT project are being conducted on the Chicamacomico River, on Drawbridge Road, next to Mathews Bros. boat builders.
See map below for field sampling location.
Information regarding these field data is currently being provided through bi-weekly updates linked through the Maryland Department of Natural Resources' websites:
Link to water quality monitoring updates on the Chicamacomico River
About real-time biomonitoring
Real-time biomonitoring data on the Chicamacomico River:
August 7, 2000
August 21, 2000 data
September 5, 2000
September 18,2000
October 2, 2000
October 17, 2000
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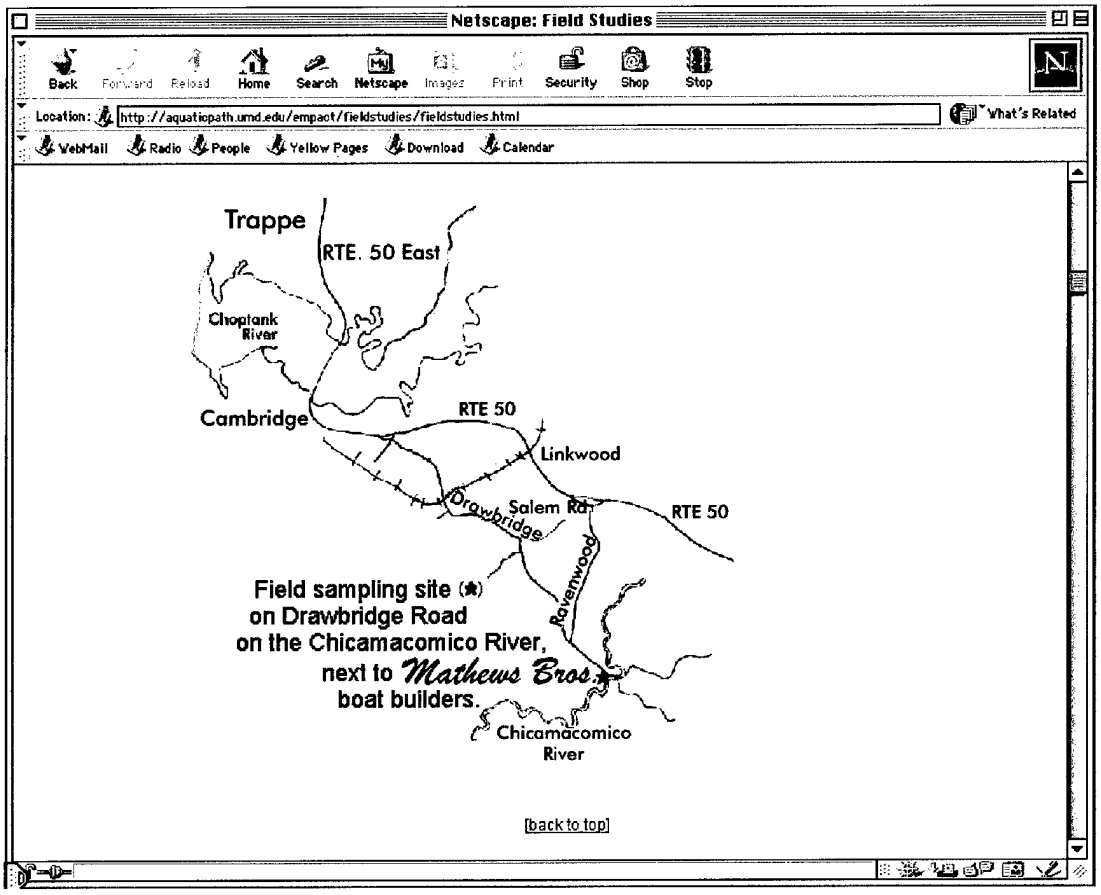
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Ravenwood
Field sampling site (*)
on Drawbridge Road
on the Chicamacomico River,
next to Mathews Bros.
boat builders.
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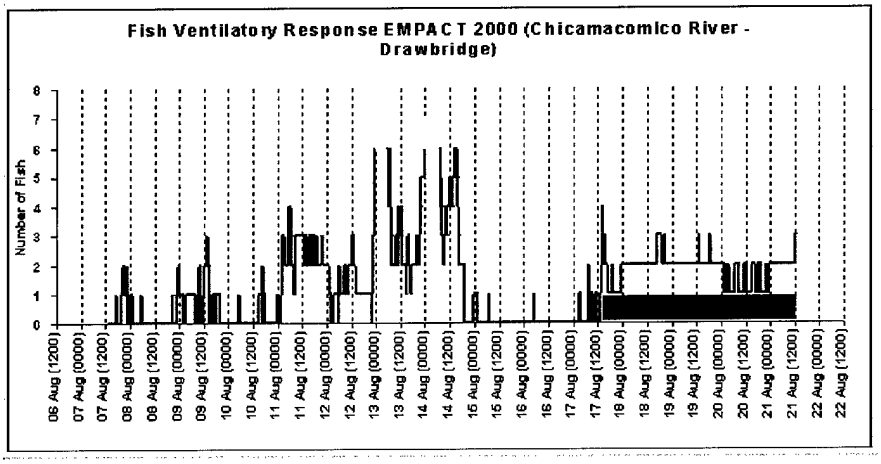
**Real-time environmental monitoring using fish.**

The U.S. Army Center for Environmental Health Research (USACEHR) has developed an automated fish monitoring system, known as the Real Time Environmental Protection System (REPS). REPS is designed to detect harmful water quality conditions in the Chesapeake Bay and other waterways. In cooperation with the Maryland Department of Natural Resources, a portable REPS facility is monitoring the water at a potential site of toxic Pfiesteria activity on the Chicamacomico River. REPS complements other on-going monitoring efforts to give early warning of potential risks to human and ecological health.

**August 7 through August 21, 2000 Biomonitoring on the Chicamacomico River**

The figure below shows the responses for the fish monitoring system from August 7-21, 2000 on the Chicamacomico River. For most of the two-week period, the on-line group of fish was not showing significant stress to the water quality conditions. On the 13 and 14 of August, the fish group became stressed due to a water quality change associated with rain events. Previous rain events did not cause the group of fish to show significant stress. The fish then recovered and there was no additional significant stress to the fish to the end of the two-week period. One fish did become severely stressed by the end of the two-week period and later identified to be dead. The fish events on the 13 and 14 of August signaled an automated water sampler to pull river water samples during the stress events. Follow-up testing of the water samples by Dr. Oldach's lab at University of Maryland School of Medicine was negative for Pfiesteria using the Pfiesteria gene probe. The stress event at the Chicamacomico River, although significant, was not severe enough to cause the whole group to remain severely stressed or die as did the exposure to water from toxic-Pfiesteria cultures at NCSU.

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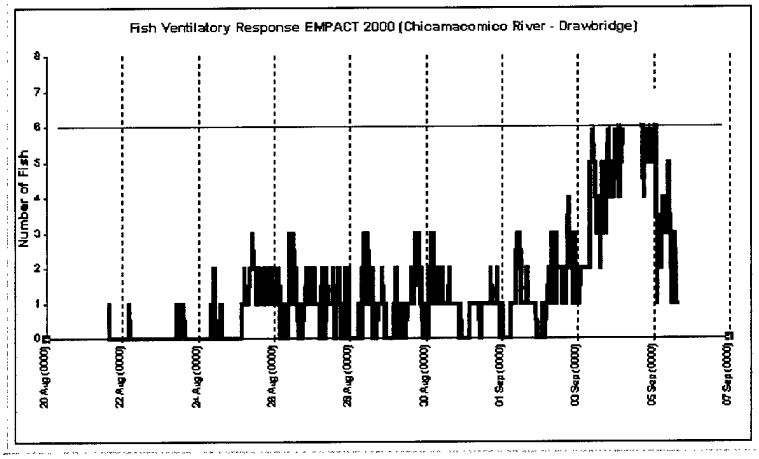
**August 21 through September 5, 2000 Biomonitoring on the Chicamacomico River**

The figure below shows the responses for the fish monitoring system from 21 August to 5 September 2000 on the Chicamacomico River. For most of the two-week period, the on-line group of fish was not showing significant stress to the water quality conditions. On the 3 through 5 September, the fish group became stressed due to a water quality change associated with rain events. The fish then recovered and there was no additional significant stress to the fish to the end of the two-week period. The fish events on 3 through 5 September signaled an automated water sampler to pull river water samples during the stress events. Follow-up testing of the water samples by Dr. Oldach's lab at University of Maryland School of Medicine was negative for Pfiesteria using the Pfiesteria gene probe. The stress event at the Chicamacomico River, although significant, was not severe enough to cause the whole group to remain severely stressed or die as did the exposure to water from toxic-Pfiesteria cultures at NCSU.

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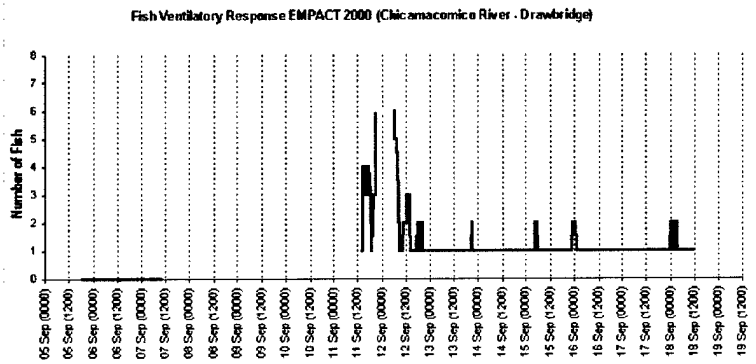
**September 5 through September 18, 2000 Biomonitoring on the Chicamacomico River**

The figure below shows the responses for the fish monitoring system from 5-18 September 2000 on the Chicamacomico River. For most of the two-week period, the on-line group of fish was not showing significant stress to the water quality conditions. On 11 September, for a brief time (~10 hours) the fish group became stressed. The fish then recovered and there was no additional significant stress to the fish to the end of the two-week period. The fish event on 11 September signaled an automated water sampler to pull river water samples during the stress event. Follow-up testing of the water samples by Dr. Oldach's lab at University of Maryland School of Medicine was negative for Pfiesteria using the Pfiesteria gene probe. The stress event at the Chicamacomico River, although significant, was not severe enough to cause the whole group to remain severely stressed or die as did the exposure to water from toxic-Pfiesteria cultures at NCSU. The missing data in the graph below (7 through 11 September) was associated with an undefined program interruption. Remote data review identified a potential problem with data acquisition and MD DNR Vienna Field Office (Mr. Samuel Q. Johnson) provided on-site support to restart data collection. The timely response to the problem allowed for rapid data recovery and prevented the loss of the fish stress event identified in the graphic below.

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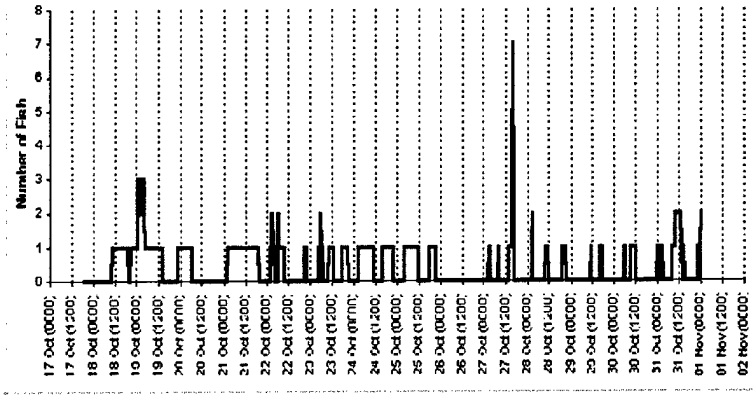
**October 17 through November 1, 2000 Biomonitoring on the Chicamacomico River**

The figure below shows the responses for the fish monitoring system from 17 October - 1 November 2000 on the Chicamacomico River. During this two-week monitoring period, the fish continue to show little signs of stress. The two elevated response times on 19 and 27 October are related to on-site maintenance activities. The shorter days and the drop in water temperature through the month of October have reduced the risk of rapid water quality shifts as a result of algal blooms. There is no need for continued monitoring through the cold weather months as is apparent by the lack of fish response during October.

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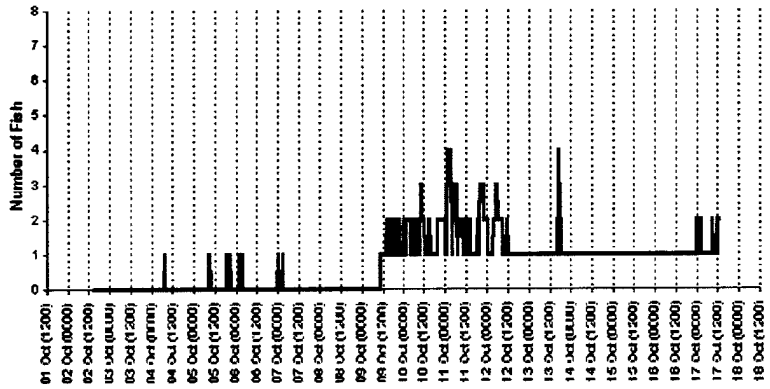
**October 2 through October 17, 2000 Biomonitoring on the Chicamacomico River**

The figure below shows the responses for the fish monitoring system from 2-17 October 2000 on the Chicamacomico River. During this two-week monitoring period no significant fish stress events occurred.

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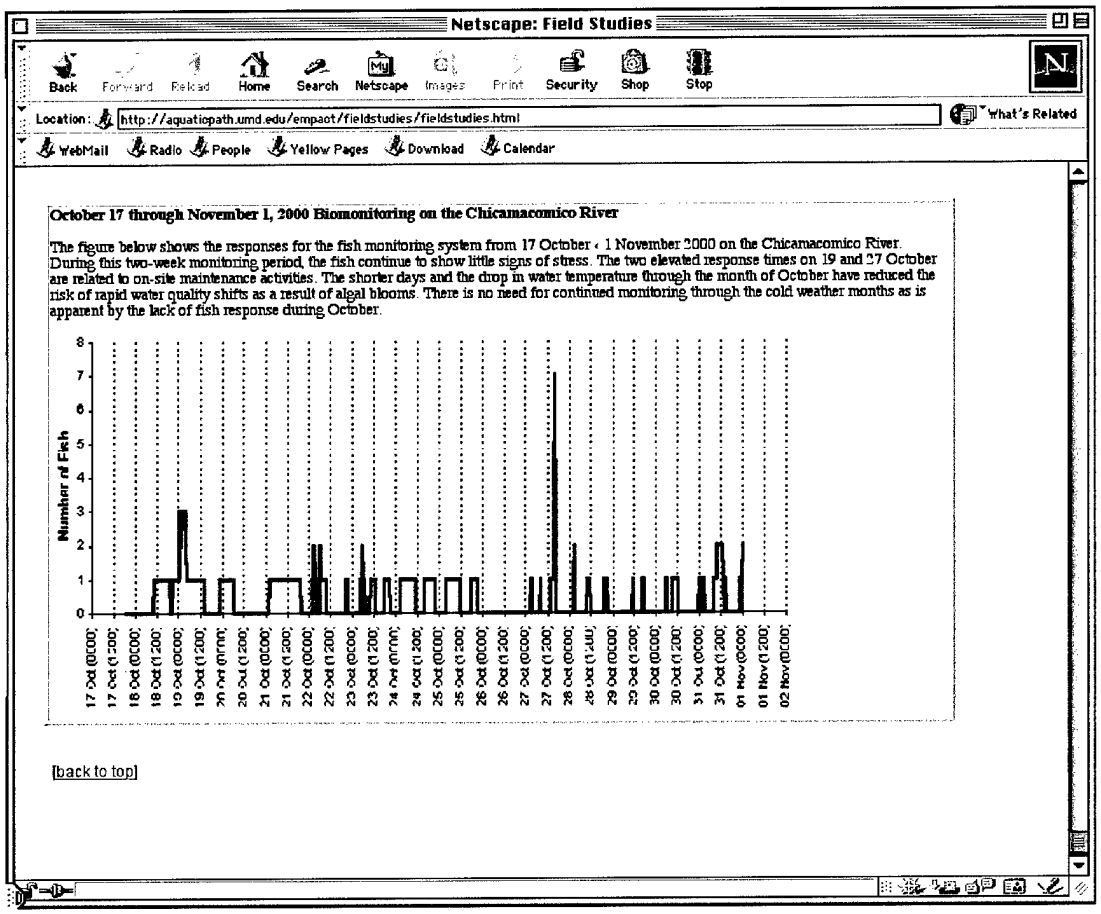
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October 17 through November 1, 2000 Biomonitoring on the Chicamacomico River
The figure below shows the responses for the fish monitoring system from 17 October - 1 November 2000 on the Chicamacomico River. During this two-week monitoring period, the fish continue to show little signs of stress. The two elevated response times on 19 and 27 October are related to on-site maintenance activities. The shorter days and the drop in water temperature through the month of October have reduced the risk of rapid water quality shifts as a result of algal blooms. There is no need for continued monitoring through the cold weather months as is apparent by the lack of fish response during October.
Number of Fish
0
1
2
3
4
5
6
7
8
17 Oct (0000)
17 Oct (1200)
18 Oct (0000)
18 Oct (1200)
19 Oct (0000)
19 Oct (1200)
20 Oct (0000)
20 Oct (1200)
21 Oct (0000)
21 Oct (1200)
22 Oct (0000)
22 Oct (1200)
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27 Oct (0000)
27 Oct (1200)
28 Oct (0000)
28 Oct (1200)
29 Oct (0000)
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31 Oct (0000)
31 Oct (1200)
01 Nov (0000)
01 Nov (1200)
02 Nov (0000)
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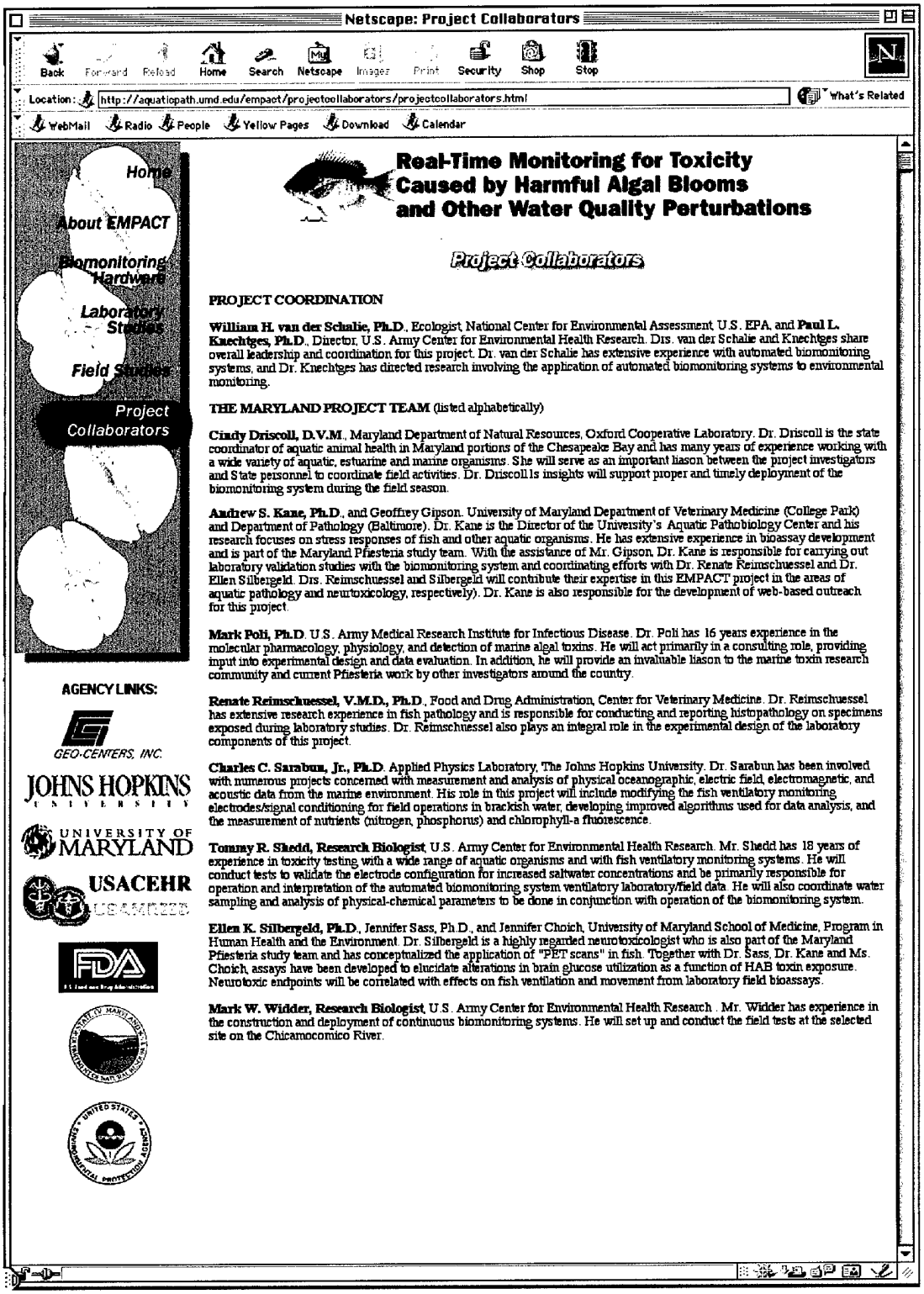
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PROJECT COORDINATION
William H. van der Schalie, Ph.D., Ecologist, National Center for Environmental Assessment, U.S. EPA, and Paul L. Knechtges, Ph.D., Director, U.S. Army Center for Environmental Health Research. Drs. van der Schalie and Knechtges share overall leadership and coordination for this project. Dr. van der Schalie has extensive experience with automated biomonitoring systems, and Dr. Knechtges has directed research involving the application of automated biomonitoring systems to environmental monitoring.
THE MARYLAND PROJECT TEAM (listed alphabetically)
Cindy Driscoll, D.V.M., Maryland Department of Natural Resources, Oxford Cooperative Laboratory. Dr. Driscoll is the state coordinator of aquatic animal health in Maryland portions of the Chesapeake Bay and has many years of experience working with a wide variety of aquatic, estuarine and marine organisms. She will serve as an important liason between the project investigators and State personnel to coordinate field activities. Dr. Driscoll's insights will support proper and timely deployment of the biomonitoring system during the field season.
Andrew S. Kane, Ph.D., and Geoffrey Gipson. University of Maryland Department of Veterinary Medicine (College Park) and Department of Pathology (Baltimore). Dr. Kane is the Director of the University's Aquatic Pathobiology Center and his research focuses on stress responses of fish and other aquatic organisms. He has extensive experience in bioassay development and is part of the Maryland Pfiesteria study team. With the assistance of Mr. Gipson, Dr. Kane is responsible for carrying out laboratory validation studies with the biomonitoring system and coordinating efforts with Dr. Renate Reimschuessel and Dr. Ellen Silbergeld. Drs. Reimschuessel and Silbergeld will contribute their expertise in this EMPACT project in the areas of aquatic pathology and neurtoxicology, respectively). Dr. Kane is also responsible for the development of web-based outreach for this project.
Mark Poli, Ph.D. U.S. Army Medical Research Institute for Infectious Disease. Dr. Poli has 16 years experience in the molecular pharmacology, physiology, and detection of marine algal toxins. He will act primarily in a consulting role, providing input into experimental design and data evaluation. In addition, he will provide an invaluable liason to the marine toxin research community and current Pfiesteria work by other investigators around the country.
Renate Reimschuessel, V.M.D., Ph.D., Food and Drug Administration, Center for Veterinary Medicine. Dr. Reimschuessel has extensive research experience in fish pathology and is responsible for conducting and reporting histopathology on specimens exposed during laboratory studies. Dr. Reimschuessel also plays an integral role in the experimental design of the laboratory components of this project.
Charles C. Sarabun, Jr., Ph.D. Applied Physics Laboratory, The Johns Hopkins University. Dr. Sarabun has been involved with numerous projects concerned with measurement and analysis of physical oceanographic, electric field, electromagnetic, and acoustic data from the marine environment. His role in this project will include modifying the fish ventilatory monitoring electrodes/signal conditioning for field operations in brackish water, developing improved algorithms used for data analysis, and the measurement of nutrients (nitrogen, phosphorus) and chlorophyll-a fluorescence.
Tommy R. Shedd, Research Biologist, U.S. Army Center for Environmental Health Research. Mr. Shedd has 18 years of experience in toxicity testing with a wide range of aquatic organisms and with fish ventilatory monitoring systems. He will conduct tests to validate the electrode configuration for increased saltwater concentrations and be primarily responsible for operation and interpretation of the automated biomonitoring system ventilatory laboratory/field data. He will also coordinate water sampling and analysis of physical-chemical parameters to be done in conjunction with operation of the biomonitoring system.
Ellen K. Silbergeld, Ph.D., Jennifer Sass, Ph.D., and Jennifer Choich, University of Maryland School of Medicine, Program in Human Health and the Environment. Dr. Silbergeld is a highly regarded neurotoxicologist who is also part of the Maryland Pfiesteria study team and has conceptualized the application of "PET scans" in fish. Together with Dr. Sass, Dr. Kane and Ms. Choich, assays have been developed to elucidate alterations in brain glucose utilization as a function of HAB toxin exposure. Neurotoxic endpoints will be correlated with effects on fish ventilation and movement from laboratory field bioassays.
Mark W. Widder, Research Biologist, U.S. Army Center for Environmental Health Research . Mr. Widder has experience in the construction and deployment of continuous biomonitoring systems. He will set up and conduct the field tests at the selected site on the Chicamocomico River.