♲EPA

ORD

US EPA Office of Research and Development

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

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

DISCLAIMER

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

ABSTRACT

This project, sponsored by EPA's Environmental Monitoring for Public Access and Community Tracking (EMPACT) program, evaluated the ability of an automated biological monitoring system that measures fish ventilatory responses (ventilatory rate, ventilatory depth, and cough rate) to detect developing toxic conditions in water. In laboratory tests, acutely toxic levels of both brevetoxin (PbTx-2) and toxic *Pfiesteria piscicida* cultures caused fish responses primarily through large increases in cough rate. In the field, the automated biomonitoring system operated continuously for 3 months on the Chicamacomico River, a tributary to the Chesapeake Bay that has had a history of intermittent toxic algal blooms. Data gathered through this effort complemented chemical monitoring data collected by the Maryland Department of Natural Resources (DNR) as part of their pfiesteria monitoring program. After evaluation by DNR personnel, the public could access the data at a DNR Internet website, (www.dnr.state.md.us/bay/pfiesteria/00results.html), or receive more detailed information at aquaticpath.umd.edu/empact. The field biomonitor identified five fish response events. Increased conductivity combined with a substantial decrease in water temperature was the likely cause of one event, while contaminants (probably surfactants) released from inadequately rinsed particle filters produced another response. The other three events, characterized by greatly increased cough rate (two events) or increased ventilation rate and depth (one event), did not have identified causes. Water quality variations did not correspond to the timing of the three events. Analyses of water taken by an automated sampler were negative for the presence of pfiesteria or chemicals that could be associated with the observed responses, and no fish kills occurred on the Chicamacomico River during the monitoring period. Continuing activities to improve the biomonitoring system include providing a change detection algorithm for fish ventilatory patterns that does not depend on a baseline monitoring period, integrating the fish biomonitor with other automated biomonitoring systems, and developing an expert system to better detect toxic events and distinguish them from fish responses to normal variations in ambient water quality conditions.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

PREFACE

The National Center for Environmental Assessment-Washington Office (NCEA-W), within EPA's Office of Research and Development, has prepared this final report of a project sponsored by EPA's Environmental Monitoring for Public Access and Community Tracking (EMPACT) program. The report describes the development and operation of a real-time automated biomonitoring system for detecting toxicity caused by harmful algal blooms and other water quality perturbations. The system was developed and evaluated over a 2-year period (March 1999 through November 2000) on the Chicamacomico and Transquaking Rivers, tributaries to the Chesapeake Bay on Maryland's Eastern Shore. Relevant literature has been reviewed through May 2001.

This project was a collaborative effort among many organizations, including NCEA-W, the U.S. Army Center for Environmental Health Research, the Maryland Department of Natural Resources, the U.S. Army Medical Research Institute for Infectious Diseases, the University of Maryland, the U.S. Food and Drug Administration, the Johns Hopkins University Applied Physics Laboratory, GEO-CENTERS, Inc., and North Carolina State University. Project results demonstrated the feasibility of incorporating real-time automated biomonitoring technology with complementary on-line chemical monitoring to provide data for use by local or state regulatory agencies and the public in a readily-available Internet format.

AUTHORS AND CONTRIBUTORS

EPA's National Center for Environmental Assessment-Washington Office (NCEA-W) was responsible for preparation of this document, but many individuals and organizations contributed to the material contained in this report.

AUTHORS

William H. van der Schalie (NCEA-W) provided overall project coordination and compiled this report.

Tom Shedd and Mark Widder (U.S. Army Center for Environmental Health Research [USACEHR]) directed all aspects of biomonitoring facility operation and provided the automated biomonitoring technology for use in Dr. Kane's and Dr. Burkholder's laboratories.

Andrew Kane (Aquatic Pathobiology Center, University of Maryland) conducted laboratory testing with the ventilatory test system involving brevetoxin, coordinated neurotoxicity and histopathologic studies, and developed the Internet website for the project.

Ellen Silbergeld (Program in Human Health and the Environment, University of Maryland) directed the neurotoxicity testing.

Renate Reimschuessel (U.S. Food and Drug Administration) conducted the histopathologic analysis of brevetoxin-exposed fish.

Mark Poli (U.S. Army Medical Research Institute for Infectious Diseases) provided the brevetoxin used in testing and conducted the analysis of water samples for brevetoxin.

Charles Sarabun (Johns Hopkins University Applied Physics Laboratory) developed advanced water quality sensors and explored improved methods for analyzing the fish ventilatory signals.

JoAnn Burkholder and Howard Glasgow (North Carolina State University) worked with Tom Shedd and Mark Widder to conduct the exposures of bluegills to *Pfiesteria piscicida* in the fish ventilatory test system at the North Carolina State University pfiesteria laboratory.

1. INTRODUCTION

Harmful algal blooms, including those associated with toxicity, have been increasing in frequency, intensity, and severity in U.S. coastal areas. Recently, the Mid-Atlantic region has experienced blooms of the dinoflagellate pfiesteria and pfiesteria-like organisms leading to fish kills that have damaged local fisheries and caused concern regarding potential effects on people exposed while engaged in sport or commercial fishing, swimming, or other water-related recreational activities. Unfortunately, the public and environmental decisionmakers may not find out that an algae-related fish kill is underway until large numbers of dead or dying fish are observed, so the availability of early warning information on developing toxic conditions in susceptible waters is critical. In this project, an automated biomonitoring system that tracks the ventilatory and movement patterns of fish was used to continuously monitor estuarine waters susceptible to toxic algal blooms and to provide rapid notification of water quality perturbations.

An automated biomonitoring approach fits well with the goals of the EPA's Environmental Monitoring for Public Access and Community Tracking (EMPACT) program. The EMPACT program seeks to provide public access to time-relevant environmental information. EMPACT-funded monitoring and communication projects emphasize partnerships among local, state, and tribal governments, research institutions, non-governmental organizations, the private sector, and the Federal government. EMPACT research projects are, "intended to research innovative time-relevant monitoring and measurement technologies with the intent of sharing these results with other EMPACT projects" (U.S. EPA, 2001). Although the communication of time-relevant information to environmental decisionmakers and the public is an important aspect of this project, emphasis is on research to further develop and evaluate automated biomonitoring technology that will facilitate detection and communication of significant water quality perturbations. The remainder of this section provides additional background information on automated biomonitoring systems and discusses the goals, objectives, and organization of this research project.

1.1. AUTOMATED BIOMONITORING SYSTEMS

Automated biomonitoring systems continuously record an organism's behavioral or physiological responses and evaluate changes that could indicate developing toxic conditions. As discussed below, these systems have several advantages relative to sole reliance on chemical monitoring and have been developed for a wide range of organisms and to fulfill diverse monitoring needs. Selection of a fish automated biomonitoring system for this project fits well with local regulatory and public concerns over the potential effects of pfiesteria blooms on fish and humans in the Chesapeake Bay and its tributaries.

1.1.1. Automated Biomonitoring System Advantages and Considerations

Because automated biomonitoring systems directly measure toxic effects, they provide an important complement to available chemical monitoring technology. Biological measures of water quality can detect unsuspected materials and evaluate the toxic action of mixtures of multiple chemicals. As noted by Cairns and Mount (1990), "No instrument has yet been devised that can measure toxicity! Chemical concentrations can be measured with an instrument, but only living material can be used to measure toxicity."

Automated biomonitoring systems are particularly useful for detecting intermittent toxic events in the environment. Continuous, real-time information on time-variable toxicity levels is important to environmental managers who need to understand point source and nonpoint source impacts in a watershed, evaluate whether surface water is of suitable quality for use in a water treatment plant, or decide if an effluent from a wastewater treatment plant is suitable for discharge. Neither traditional toxicity tests nor chemical-specific sensors can provide comprehensive, real-time information on toxic events in an aquatic system. Furthermore, automated biomonitoring systems can bolster the public's confidence in the operation of chemical monitoring systems (Shedd, 2001). In summary, important advantages of automated biomonitoring systems include:

- Detection and early warning of transient, episodic, and developing toxic events;
- Identification of potential toxicity from unsuspected chemicals;
- Integration of the effects of complex chemical mixtures;
- Acquisition of samples for detailed chemical analyses based on biological responses; and
- Increased public confidence in chemical monitoring system performance.

While these are significant advantages, it is important to realize that the toxic chemicals detected by an automated biomonitor are only one of many stressors that may affect an aquatic system. Physical stressors (such as habitat alteration or destruction), biological stressors (e.g., invasive species), and chemical stressors acting through indirect pathways (e.g., nutrients) may have greater influence on an aquatic community than toxic chemicals. In addition, van der Schalie et al. (2001) has compiled several considerations important to the successful operation of an automated biomonitoring system, including:

- Rapidity and sensitivity of response to environmental toxicants. Every automated biomonitor will show a range of sensitivity to environmental toxicants, the usefulness of the biomonitor response characteristics will depend on the intended

application. Fish ventilatory monitoring systems have been shown to respond to a wide variety of organic and inorganic chemicals (American Society for Testing and Materials, 1995), with initial responses to toxicant concentrations near the 96 h LC50 frequently occurring within an hour, but sometimes taking up to several hours (Morgan and Kuhn, 1984; Evans and Wallwork, 1988; Gruber et al., 1989; Diamond et al., 1990; Baldwin et al., 1994; Johnston et al., 1994). Sensitivity and response time are related in that response time tends to vary inversely with toxicity (e.g., Diamond et al., 1988), and different types of organisms tend to respond differentially to different classes of toxicants. For example, while algae are more sensitive to herbicides than fish, fish and other vertebrates possess the metabolic enzymes necessary to convert some chemicals into their proximal toxic form. To increase the likelihood of the rapid detection of a wide spectrum of toxic chemicals, some have advocated the simultaneous use of multiple species (Kramer and Botterweg, 1991).

- <u>Specificity of response to environmental toxicants</u>. Automated biomonitors are used as broad-based toxicity detectors. Their strength lies in their ability to detect unsuspected materials or toxicity due to interactions among mixtures of chemicals. However, automated biomonitors are not necessarily effective at identifying the cause for their response; thus, it is important to use biomonitors in conjunction with other monitoring devices with greater specificity or diagnostic capabilities, such as water quality monitoring probes or chemical-specific biosensors. Automated biomonitors have an important role in biologically directed sampling; an alarm response can be used to trigger an automated water sampling device, allowing follow-on analytical chemistry determinations. This can be an effective approach to capturing transient environmental events, such as chemical spills or nonpoint source discharges related to precipitation events.

- <u>Reliability of alarm identification (lack of false positives)</u>. One issue that any type of toxicity monitor must address is false alarms. False alarms may be caused by equipment malfunctions, but automated biomonitors may respond with "false" alarms to changes in water quality conditions. While these alarms reflect real effects on monitored organisms, they are unrelated to the presence of toxic chemicals. Another issue that may arise is masking, where non-toxic water quality changes may occur at the same time as toxic events. This might occur in a surface water when precipitation causes simultaneous changes in temperature, dissolved oxygen, and suspended particulates (which may elicit an automated biomonitor response) at the same time as toxic chemicals (e.g., pesticides) are

washed into the waterway. In this case, careful evaluation of response patterns may be the one way to help evaluate the cause of the alarm, and it is essential that common interfering water quality parameters be monitored. An acceptable frequency of false alarms will depend on the application. Baldwin and Kramer (1994) suggest no more than once per year, while Evans et al. (1986) recommend no more than once every two months. It is likely that some experience with an automated biomonitor at a given location will be essential in determining response characteristics and limiting the occurrence of false alarms.

- Suitability of environmental conditions. Like any analytical chemistry device, automated biomonitors operate within a range of environmental conditions. Just as sample preparation may be necessary before making analytical determinations in a gas chromatograph, water quality parameters such as temperature or dissolved oxygen need to be within a range appropriate for the organism being used. If necessary, variations in ambient water temperature can be controlled, and aeration can compensate for low dissolved oxygen, although volatile toxic materials may be lost. Automated biomonitors are best used for detecting transient increases in toxicity. Waters with persistent acute toxicity problems can be evaluated using traditional aquatic toxicity tests.

- Physical requirements. Especially in remote monitoring situations, size and power utilization of an automated biomonitor should be minimized. Small size and low power consumption are especially important for field configurations such as on buoys (Waller et al., 1996) or in stream-side enclosures. Communication systems that provide remote access capabilities are especially important for real-time monitoring devices. The Internet provides an excellent vehicle for delivering real-time information.

- Installation, maintenance, and training needs. Successful application of an automated biomonitor at a new location requires some understanding of the composition, variability, and water quality characteristics at the site. Once the system is operational, maintenance requirements should be minimal. Successful system operation requires that outputs be interpretable in a fashion that integrates well with site or facility operations. Should an automated biomonitor alarm occur, the primary response is usually further investigation through the evaluation of current water quality parameters, operation of the water delivery system, and, if needed, analytical chemistry evaluation.

- Cost-benefit considerations. The absolute cost of an automated biomonitor is less important than the cost-benefit aspects. In discussing the costs of early warning

systems for detecting hazardous events in water supplies, the International Life Sciences Institute's Risk Science Institute (1999) points out that local support for such systems will be crucial in determining their utility, while cost/benefit considerations will be influenced by the existence of credible threats to the water supply, the range of contaminants the early warning system can detect, and the ease of use of the system, among other factors.

1.1.2. Types of Automated Biomonitoring Systems

Over the past 30 years, the development and use of aquatic organisms as biological early warning indicators for monitoring water supplies and effluents has been extensive, and many applications of such automated biomonitoring systems have been proposed. The fundamental components, design, and operating parameters of aquatic automated biomonitoring systems have been reviewed elsewhere (Cairns and van der Schalie, 1980; Diamond et al., 1988; Kramer and Botterweg, 1991; Gerhardt, 1999).

A number of automated biomonitoring systems have made the transition from laboratory testing to field use. While examples of field testing of automated biomonitoring systems in the United States are relatively few (Smith and Bailey, 1988; Gruber et al., 1989; Shedd et al., 2001), a number of systems have been evaluated in Europe (Koeman et al., 1978; Scharf, 1979; Evans and Wallwork, 1988; Hendriks and Stouten, 1993; Borcherding, 1994; Borcherding and Jantz, 1997; International Life Sciences Risk Science Institute, 1999) and in South Africa (Morgan et al., 1982; Biomonitoring Committee, Working Group of the Federal States on Water Problems, 1996). Many of these systems use algae or invertebrates. Of the European systems that use fish, most rely on changes in rheotaxis, which may be relatively insensitive to toxicants (Biomonitoring Committee, Working Group of the Federal States on Water Problems, 1996).

There are other endpoint choices for fish automated biomonitoring systems besides rheotaxis. Possibilities include the locomotor behavior and movement patterns of fish (e.g., Korver and Sprague, 1988; Kramer and Botterweg, 1991; Steinberg et al., 1995; Vogl et al., 1999), electrical discharges from weakly electric fish (e.g., Geller, 1984), and fish ventilatory response patterns (e.g., Cairns et al., 1970; Cairns and van der Schalie, 1980; van der Schalie et al., 1988; Diamond et al., 1990; American Society for Testing and Materials, 1995).

Automated biomonitoring systems have been used for diverse reasons. In Germany and the Netherlands, many biomonitoring stations were installed in response to the Sandoz chemical spill in the Rhine River in 1986 (Gerhardt, 1999). As warning systems for accidental discharges, these surface water biomonitors protect human health by monitoring source waters for drinking water systems, while also identifying conditions potentially hazardous to ecological systems (Biomonitoring Committee, Working Group of the Federal States on Water Problems, 1996). In

addition, automated biomonitors have been used for wastewater monitoring (van der Schalie et al., 1979; Morgan et al., 1982; Wallwork and Ellison, 1983; Gruber et al., 1989; Shedd et al., 2001).

1.1.3. Automated Biomonitoring System Selection

Toxic pfiesteria-like species, including *Pfiesteria piscicida*, have been implicated in major fish kills in the mid-Atlantic region (Burkholder and Glasgow, 1997). As a result of concerns over the occurrence of pfiesteria in the Chesapeake Bay region, the Maryland Department of Natural Resources (DNR) has established an intensive sampling program to characterize fish health, water quality, habitat, and pfiesteria occurrence. Part of this program includes a rapid response capability should a reported fish health or suspicious human health problem be observed. Under the EMPACT program, the DNR has established several real-time water quality monitoring stations to help provide early indications of changing water quality. Automated biomonitoring system technology was a logical choice to complement this monitoring system.

While any of several biomonitoring systems could have been used, a fish system was most appropriate given the primary concern for fish health. The system selected can monitor multiple parameters (ventilatory rate, ventilatory depth, cough rate, and whole body movement) and has proven reliable in long-term operation (Shedd et al., 2001). A significant challenge was adapting the system from freshwater to use in an estuarine environment, where tidally influenced and highly variable water quality parameters such as temperature, dissolved oxygen, and conductivity can have a great influence on fish ventilatory behavior.

1.2. PROJECT GOAL AND OBJECTIVES

The goal of this research and development project was to evaluate the ability of an automated biomonitoring system to provide environmental decisionmakers and the public 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. Specific objectives undertaken to reach this goal include:

- Modify an existing automated biomonitoring system that measures the ventilatory responses of freshwater fish for use in estuarine waters that have had historical problems with toxic algal blooms or other intermittent water quality hazards,
- Establish the response characteristics of the fish biomonitoring system to algal toxins,

- Provide for continuous system operation and data communication and interpretation to the DNR officials and the public, and
- Recommend system improvements for real-time water quality monitoring.

1.3. PROJECT AND REPORT ORGANIZATION

Completion of the project objectives required a collaborative approach involving a number of organizations and investigators, as shown in Figure 1-1. Major elements included laboratory testing of bluegill (*Lepomis macrochirus*) responses to two algal toxins (brevetoxin and toxic *Pfiesteria piscicida* cultures), field development and testing of the automated biomonitoring system, and coordination of the testing approach with the DNR. The DNR officials are primarily responsible for tracking the occurrence of pfiesteria-related activity in the Chesapeake Bay and perform a key role with regard to actions such as issuing advisories or closing water bodies because of outbreaks of pfiesteria or other water quality problems.

This report begins with a description of automated biomonitoring system operation and interactions with potential users of the automated biomonitoring data (see Section 2). Section 3 demonstrates system response to algae-related toxicity in laboratory tests and evaluates system operation over an extended period (August through October 2000). Section 4 provides recommendations for further improvement of the fish biomonitor.
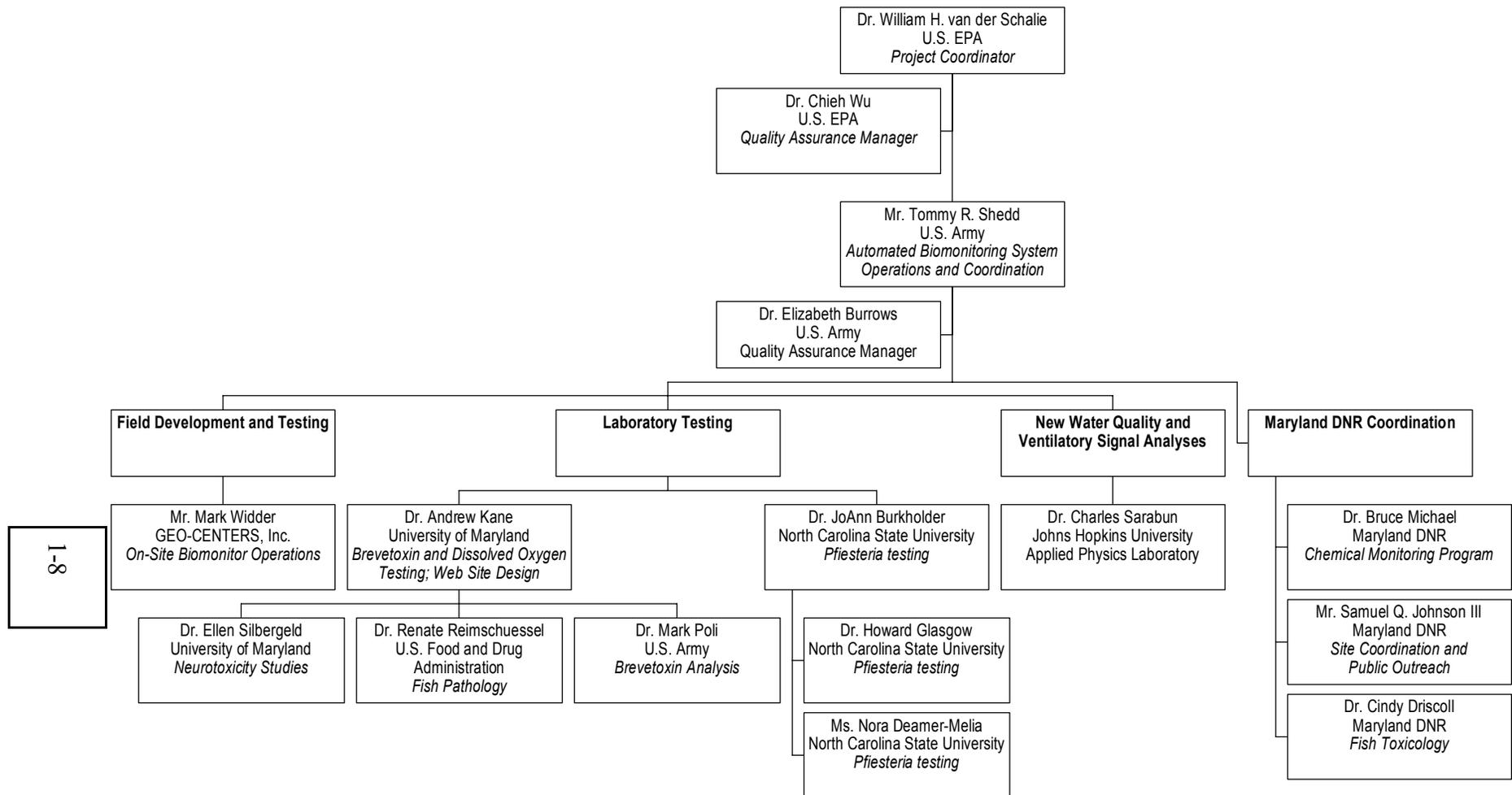
Figure 1-1. Project organizational chart.

2. AUTOMATED BIOMONITORING FIELD SYSTEM OVERVIEW

This section describes the automated biomonitoring system that was field tested on the Chicamacomico River in the summer of 2000. An overview of system operation is followed by a summary of how system monitoring information was provided to the DNR and the public. Specific adaptations of the automated biomonitoring system for laboratory testing are described in the appropriate sections of this report. Critical system modifications based on experience gained during preliminary system testing on the Transquaking River in 1999 are described in Appendix A.

2.1. THE AUTOMATED BIOMONITORING SYSTEM

The biomonitoring system is housed in a 14.6 m (48 ft) trailer that was located on Maryland's eastern shore (Figure 2-1) at sites on the Transquaking and Chicamacomico Rivers selected in coordination with DNR to complement their real-time water quality monitoring program. A diagram of the biomonitoring system is provided in Figure 2-2. Water is pumped from a dual intake system in the river through dual Hayward Simplex Basket Strainers (0.79 mm pore size) followed by parallel Kestone Bag Filters (100 μm pore size) to eliminate particles above 100 μm. Water then flows into a manifold that provides about 200 mL/min to individual acrylic fish ventilatory monitoring cells (2.5 x 9.5 x 6 cm, volume ~150 mL). Eight individual cells are part of a larger chamber (23 x 15 x 12 cm), individual cells are separated by common translucent walls. Water enters individual cells at the bottom, passes through the cell, exits the top over a spillway into a common drain, flows to a Hydrolab® water quality monitor, and then is returned to the river. A computer collects temperature, pH, dissolved oxygen, and conductivity data every 30 minutes from the Hydrolab® sensors. The Hydrolab® was calibrated every 2 weeks, and the dissolved oxygen membrane was cleaned twice per week.

To prevent potentially lethal low dissolved oxygen levels, an aeration system was included to provide the fish with a mixture of 50% aerated river water and 50% ambient river water if the ambient river water dissolved oxygen level dropped below 3 mg/L. Although low dissolved oxygen levels are known to be a frequent cause of fish kills in estuarine environments, and it is clearly preferable to minimize modifications of natural conditions in the monitoring system, it did not make sense to allow monitored fish to die (and thus lose monitoring capability) solely due to extremely low oxygen levels, since the on-line dissolved oxygen probe could identify when oxygen levels alone would be lethal to the fish. As it turned out, the aeration system was used only once (for less than 12 h) during the entire 85-day field deployment and so was not a significant factor in the interpretation of monitoring results. (The aeration system is
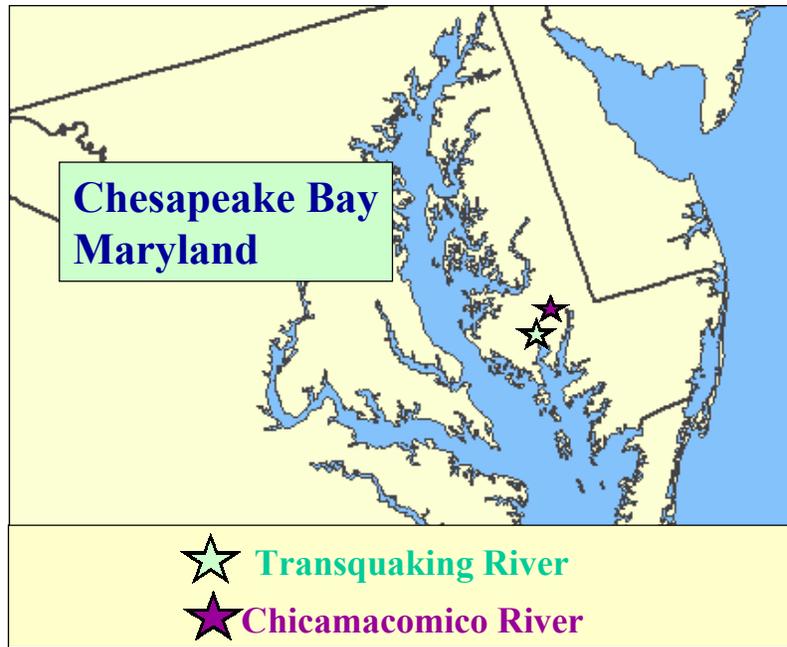
Transquaking River (1999)   Chicamacomico River (2000)

Figure 2-1. Biomonitoring system locations on the Chicamacomico and Transquaking Rivers, Maryland. These sites were selected in collaboration with the DNR based on the historical presence of pfiesteria and pfiesteria-related fish kills. Additional information can be found at: http://www.dnr.state.md.us/pfiesteria/mles.html.

Figure 2-2. Biomonitoring system overview. The solid line indicates the flow of water, the dotted line shows the flow of electronic information.

described in more detail in Appendix A, Section A.2. Other research to develop a low-level nutrient [phosphate] sensor is discussed in Appendix B.)

Fish are acquired from a local pond and held in the laboratory for at least 4 weeks before transfer to the biomonitoring site, where they are acclimated in river water for a minimum of 2 weeks. No mortality has occurred during holding. Fish are acclimated to continuous light conditions during on-site holding. Continuous light is used to eliminate the diel variability in fish ventilatory patterns. Most researchers using fish ventilatory monitoring systems elect to conduct testing under either constant light or constant darkness (American Society for Testing and Materials, 1995). Baldwin et al. (1994) note that, although fish under constant light in their automated biomonitor showed occasional random changes in activity, this extra variability was small in comparison to changes in ventilatory patterns caused by chemicals at acutely toxic levels.

During acclimation, fish are fed commercial trout chow and frozen brine shrimp. Fish are not fed while in the ventilatory cells to avoid ventilatory signal disruptions associated with feeding. To determine the effect of not feeding the fish during ventilatory testing on fish condition, Tieman and Burton (1997) computed the relative weights (a standard fishery condition factor metric) of bluegills used in a ventilatory monitoring system similar to the one used in this study, and compared them to the length/weight relationships characteristic of wild bluegill populations. They found that 375 of 378 bluegills used over a one-year period were considered to be in good condition using this metric (Moehl and Davies, 1993). Further, based on work by Swingle and Shell (1971) who used fish condition as an indicator of prolonged physiological stress on fish populations, Tieman and Burton concluded that the testing conditions in the ventilatory monitoring system were not particularly stressful to bluegills.

Bluegills selected for use in ventilatory testing are 4-8 cm total length to fit in the ventilatory test cells. Electrical signals generated by the ventilation and body movements of individual fish are monitored by graphite electrodes suspended above and below each fish in a cell. The electrical signals are amplified, filtered, and passed onto a personal computer for analysis. Amplification is performed by SCM5B30-1136 analog input modules (Dataforth Corporation, 3331 E. Hemisphere Loop, Tucson, AZ). Each input channel is independently amplified by a high-gain true differential-input instrumentation amplifier. Signal inputs of 0.05-1 mV are amplified by a factor of 1000. Signal interference by frequencies above 10 Hz is attenuated by low-pass filters. After initially being placed in a cell, the ventilatory signal from each fish is checked. Any fish with a signal less than 0.5 V is replaced.

The ventilatory parameters monitored by the computer include ventilatory rate, ventilatory depth (mean signal height), gill purge (cough) rate, and whole body movement (rapid irregular electrical signals) as shown in Figure 2-2. Each parameter is calculated at 15-second

intervals, and any interval in which whole body movement is detected is excluded from calculation of the other three parameters, since ventilatory responses cannot be computed during 15-second intervals that contain body movement. The computer ventilatory parameter accuracy was established by comparing the computer-generated values with concurrent strip chart recorder tracings (Shedd et al., 2001). Ventilatory rate accuracy was found to be 99% (R^2 0.997, slope 0.94). Since average depth is computed as the mean height of identified ventilatory peaks, ventilatory rate accuracy should be indicative of average depth accuracy. Cough rate accuracy was 118% (R^2 0.781, slope 1.27).

Continuous biomonitoring is achieved by alternating between groups of eight fish, each of which is "on-line" for 14 days. Each new group of eight entering the system is monitored for 7 days in river water before going on-line, 3 days for acclimation followed by 4 days for collection of baseline data. If a ventilatory parameter of an individual fish becomes statistically different from its baseline responses, the response is said to be "out-of-control."

If, during the on-line monitoring period, six of the eight fish exhibit statistically different responses from their baseline periods, in ventilation rate, depth, or cough rate, a group out-of-control response is said to occur. (Associated research to develop a combined parameter that considers these elements together and does not depend on a baseline period is described in Appendix B, Section B.2.) Movement data are used to indicate when ventilatory parameters cannot be calculated and are not used to compute group out-of-control responses. Fish with ventilatory rates below 9/min or depths below 0.2 V are either severely stressed or dead, and all parameters for that fish are set to be out-of-control. An absolute measurement of death is not possible because very low levels of electrical noise present in any system may cause spurious electrical peaks after fish death, and some toxicants (e.g., those that cause narcosis) can reduce ventilatory depth to the point that ventilatory peaks are indistinguishable from electrical noise.

When a group out-of-control response is detected, the biomonitoring program activates an ISCO® autosampler to take water samples for possible follow-on investigations to help determine the probable cause of the response. The system is checked daily for possible alarms using PC Anywhere® software. As described in Section 2.2, data quality are evaluated and provided to DNR for Internet website posting every 2 weeks. Further information is available concerning both the fish ventilatory signal analysis methods used in this project (Shedd et al., 2001), and general approaches for measuring and interpreting fish ventilatory patterns as early warning signals of water quality changes and toxicity (e.g., American Society for Testing and Materials, 1995).

2.2. REGULATORY AND PUBLIC COMMUNICATION AND OUTREACH

This project was oriented towards research and development activities in support of the EMPACT program's primary aim of providing time-relevant environmental information to the public. In addition, the automated biomonitoring activity provided complementary data in support of the DNR's own EMPACT project, whose goal was to provide real-time water quality data made available to the public through the DNR's Internet website. It is believed that automated biomonitoring data provide a valuable complement to on-line chemical monitoring data and can be used primarily by local or state regulatory agencies and, secondarily, by the public to increase awareness, answer questions, and track significant water quality perturbations.

Coordination with the DNR was essential to the success of this project, and United States Army Center for Enviromental Health Research (USACEHR) staff met with members of the DNR's pfiesteria study team at least monthly, with additional ad hoc meetings to address issues of specific concern to the automated biomonitoring system. DNR staff had daily access to the real-time biomonitoring data and was provided data for posting on the DNR website every two weeks, as described below. The DNR was notified when fish response events occurred and was provided with the results of investigations into the response events. In addition, a site tour was provided to Sarah Rogers, the DNR Secretary.

Making biomonitoring system data available to the public involved several considerations. First, the accuracy of the data had to be verified before being passed to the DNR. Second, the biomonitoring data needed to be posted first at the DNR's own Internet website so that it could be seen in the context of the DNR's EMPACT chemical monitoring data. Finally, an Internet website was needed that would provide more background on the automated biomonitoring system from which the data were generated. To accomplish these goals, biomonitoring system response data were sent to DNR every 2 weeks and posted on their Internet website (www.dnr.state.md.us/pfiesteria/index.html). Although the biomonitoring data could have been posted in real time, the 2-week time delay was an acceptable compromise between desirability of rapid dissemination of provisional data and the possibility of false alarms, given the research and development nature of this project and the sensitivity of the pfiesteria issue.

The DNR site provided a link to our automated biomonitoring Internet website (aquaticpath.umd.edu/empact), which provided detailed background information on this project. The home page at this site (Figure 2-3) contains a brief explanation of the projects and shows the biomonitoring location on the Chicamacomico River. The home page navigation bar links viewers to more specific information on the following topics:

- General information about EMPACT,
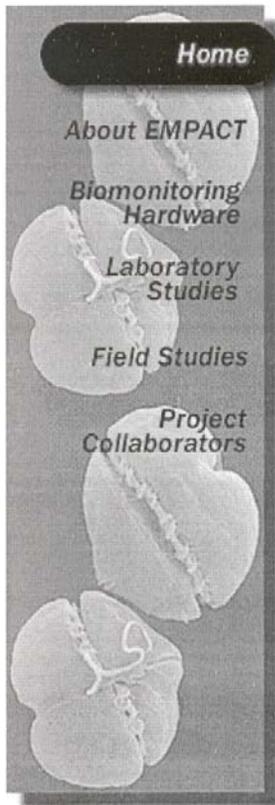
Real-Time Monitoring for Toxicity Caused by Harmful Algal Blooms and Other Water Quality Perturbations

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 waterway. This website is supported by the University of Maryland, Aquatic Pathobiology Center.

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.
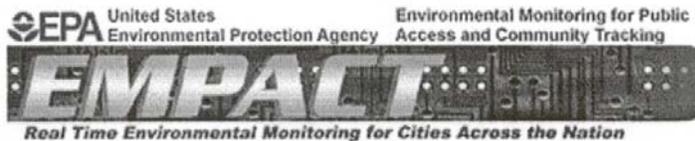
This site developed through the University of Maryland Aquatic Pathobiology Center

Figure 2-3. Biomonitoring system Internet site.
Source: http://aquaticpath.umd.edu/empact/index.html.

- Biomonitoring system hardware and fish ventilatory signal graphics,
- Results of laboratory studies,
- Results of field studies, and
- Additional information on project collaborators.

In addition to the Internet websites, public outreach efforts included direct contact with the public and local community officials. Prior to initial deployment in 1999, the local county commissioners were briefed on the biomonitoring system. They requested and were given a tour of the facility. A local newspaper, the *Dorchester Star*, featured the biomonitoring system in an article. There was considerable local public interest, and many individuals have stopped by the biomonitoring facility for a tour. On November 17, 2000, as the biomonitoring facility was about to be moved off-site, a "media day" was held and attended by local print and television media as well as one of the county commissioners. As a result of this event, the following information was provided to the public:

- Guy, C; Coan, L. (2000) Early Warning System for Bay's Aquatic Life. *Baltimore Sun* (Nov. 22, 2000),
- Dean, G. (2000) Bluegills Test Waters of Chicamacomico. *The Star Democrat* (Nov. 20, 2000),
- WBOC television interview, and
- WMDT television interview.

The high level of public interest in this project is consistent with our previous experience with a fish automated biomonitoring system at a groundwater treatment facility at a Superfund site. A major advantage of that system was the reassurance it provided to the public that acutely toxic materials would not be discharged into the Chesapeake Bay (Shedd et al., 2001). Public support helped make the fish biomonitor an integral part of the monitoring plan developed in response to the Record of Decision for the Superfund site. Even after several years of operation, the biomonitor continues to be a focal point for public involvement in the project. Automated biomonitoring can be effective both in engaging the public and, when used in conjunction with chemical monitoring, in effectively conveying environmental quality information.

3. LABORATORY AND FIELD TESTING

The automated biomonitoring system was evaluated in the field for an extended period of time (August through October 2000) at a site on the Chicamacomico River (see Section 3.2). In addition, laboratory tests of the automated biomonitoring system were conducted with the algal toxin brevetoxin and with toxic *Pfiesteria piscicida* cultures (see Section 3.1).

3.1. BIOMONITORING SYSTEM RESPONSES: LABORATORY TESTING

A major goal of this EMPACT project was to use an automated biomonitoring system to detect environmental perturbations such as the presence of harmful algal blooms, particularly those of toxic *Pfiesteria piscicida*. To confirm the response of the system to levels of algal toxins causing acute lethality, laboratory tests were conducted both with toxic *Pfiesteria piscicida* cultures and with brevetoxin, an additional algal toxin. Except as noted below, automated biomonitoring system operation followed procedures described in Section 2.1.

3.1.1. Brevetoxin Testing

Brevetoxin is biologically formed by the dinoflagellate *Gymnodinium breve*, one of the most common harmful algal bloom species on the U.S. Atlantic coast. Brevetoxin, a sodium channel modulator, is known to cause a syndrome described as neurotoxic shellfish poisoning. Symptoms in humans include gastrointestinal irritation and neurological confusion, in addition to respiratory and eye irritation in the presence of aerosols. Eating shellfish obtained from affected areas and inhalation of the toxin aerosolized by wave action are the two most common methods of exposure (Morris, 1999).

Several studies of brevetoxin toxicity to the bluegill were conducted. Acute toxicity and associated histopathology were determined. A neurotoxicity test was performed using 2-deoxyglucose (2-DG) to detect and localize alterations in central nervous system (CNS) activity of fish exposed to brevetoxin. In addition, ventilatory responses of fish to brevetoxin were determined.

3.1.1.1. *Brevetoxin Analytical Methods and Water Quality Information*

Brevetoxin (PbTx-2) was obtained as a dehydrated crystalline powder from Dr. Daniel Baden (University of North Carolina at Wilmington). The toxin was dissolved in absolute ethanol to make a super-stock solution. Subsequently, a final toxin stock solution was made by adding the super-stock to the diluent medium containing 0.0001% Emulphor-620. Actual exposure concentrations were determined using a radioimmunoassay (RIA). This RIA is specific for brevetoxins sharing the PbTx-2-type backbone and is fully described elsewhere (Poli

and Hewetson, 1992; Poli et al., 1995). Standard curves were constructed by incubating antiserum (1:7,500 dilution in phosphate-buffered saline [PBS] solution containing 0.01% emulsifier) with increasing concentrations of PbTx-2 in the presence of a constant concentration (0.1 nM) [^{3}H]PbTx-9 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. Centrifugation for 15 minutes at 1500xg sedimented the charcoal and separated bound from free label. The clear supernatant (1 mL) was transferred to scintillation vials, acidified with 50 µL glacial acetic acid, and the bound radioactivity counted on a scintillation counter. Results were quantified by comparison of unknowns to a standard curve and expressed as PbTx-2 equivalents/mL.

The brevetoxin studies were conducted at the University of Maryland Aquatic Pathobiology Center. Pond-reared bluegills were acclimated to a 24-hour lights-on photoperiod for four weeks prior to exposure. Fish were maintained in flow-through 200 L aquaria and fed fish chow (Zeigler Bros. Inc., Gardners, PA; 38% protein). The water source for holding and testing was dechlorinated Baltimore city municipal water (pH 6.8-7.0; hardness 78 mg/L (as CaCO$_3$)). General test conditions included dissolved oxygen >80% saturation and temperature 25 °C ± 1 °C.

3.1.1.2. *Acute Toxicity and Histopathology*

In order to determine an appropriate concentration of PbTx-2 for the behavioral and neurotoxicologic studies, preliminary 24h LC50 exposure and histopathologic evaluations were conducted.

3.1.1.2.1. *Specific methods*. Due to a shortage of fish, fish used in the acute toxicity and histopathology studies were obtained from a different local source than fish used in the laboratory and field ventilatory studies. Two replicates of five fish each were exposed in a series of PbTx-2 concentrations: control, solvent control, and measured (nominal) concentrations of 28 (30), 36 (40), 40 (50), and 58 (60) µg/L. After 1 hour of brevetoxin exposure to 2 L of test solution in 4 L covered glass beakers, fish were transferred to PbTx-2-free water (3.5 L) for 23 hours, during which there were three 50% water changes. Vessels receiving PbTx-2 (and the solvent control) contained the same concentration of the solvent Emulphor-620 (0.0001%). Un-ionized ammonia concentrations during the 1-hour exposure did not exceed 0.05 mg/L. 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. The LC50 was computed using probit analysis. Fish used in the pathology studies were taken at the time of death (or morbidity) from the LC50 study. Specimens were necropsied (Kane, 1996) and processed for routine histopathology

(Profet et al., 1992). Glass slides were read by Dr. Renate Reimschuessel at the U.S. Food and Drug Administration Center for Veterinary Medicine.

3.1.1.2.2. *Acute toxicity results and discussion*. The 24h LC50 (based on a 1-hour initial exposure) was 35 µg/L (95% CI: 22-42 µg/L; see Table 3-1). During the initial 3 hours after exposure there were no gross signs of intoxication in PbTx-2-exposed fish, relative to control fish. After 8 hours, some of the animals, particularly at the higher concentrations, 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-hour exposure were already dead or moribund. Because this test was conducted for range-finding purposes, it was not repeated even though control mortality (3/10) was somewhat high.

Table 3-1. Acute toxicity of PbTx-2 (1-hour exposure, 24-hour holding).

Exposure Concentration (µg/L)		Number Responding (n=10)
Nominal	**Measured**	
0	**0**	1
0 **(carrier solvent)**	**0**	2
30	**28**	5
40	**36**	4
50	**40**	8
60	**58**	10

3.1.1.2.3. *Pathology results and discussion*. 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 (mostly 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 pathologies. 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 histopathologic differences between the controls and treatment groups caused by brevetoxin exposure.

3.1.1.3. *Brevetoxin Ventilatory Test*

This test was conducted to characterize ventilatory responses of bluegills to an acutely toxic level of brevetoxin. Fish were exposed for 1-hour to a nominal brevetoxin concentration similar to that which resulted in an LC50 in the acute toxicity range-finding test.

3.1.1.3.1. *Methods*. Fish were held in the ventilatory monitoring cells for 3 days to acclimate to the cells and 4 days to collect baseline control data, as described in Section 2.1. Ventilatory signal integrity from the exposure cells was verified electronically using an oscilloscope as well as visually using a remote video camera. A gravity-fed dilutor system delivered water (35 mL/min) to each of the eight individual monitoring cells. During the acclimation and baseline periods, carrier solvent was added to the diluent flow by means of a peristaltic pump. PbTx-2 was included at the beginning of the exposure period. Toxin was pumped into the exposure cells for 60 minutes to achieve a nominal concentration of 40 µg/L PbTx-2 (measured concentration 53 µg/L). Fish ventilatory responses were monitored for 24 hours after the start of exposure.

3.1.1.3.2. *Results and discussion*. Fish ventilatory responses to brevetoxin exposure are shown in Figure 3-1. Bluegills provided a group response within 30 minutes to brevetoxin due to increased cough and ventilation rates (Figure 3-2a). The large increase in cough rates associated with exposure is worth noting in that it is not a response observed by Carlson (1984), who studied ventilatory responses of bluegills to substantial changes in several common water quality parameters (temperature, dissolved oxygen, and pH). This suggests that a cough response may help differentiate fish responses caused by normal variations in some water quality parameters from those due to other factors, including some toxicants.

There were no brevetoxin-related deaths, although two fish died during testing as a result of clogged incoming water lines. Although there are continuing ventilatory responses from individual fish after the cessation of brevetoxin exposure, the second group response at 54 hours is an artifact, since two of the six fish responding were the fish whose deaths were not related to brevetoxin exposure. The lack of brevetoxin-related mortality in the ventilatory study is inconsistent with the high mortality at comparable concentrations in the range-finding LC50 test (Table 3-1), but the range-finding test was conducted with fish from a different source than the ventilatory study, which may explain the difference in mortality.
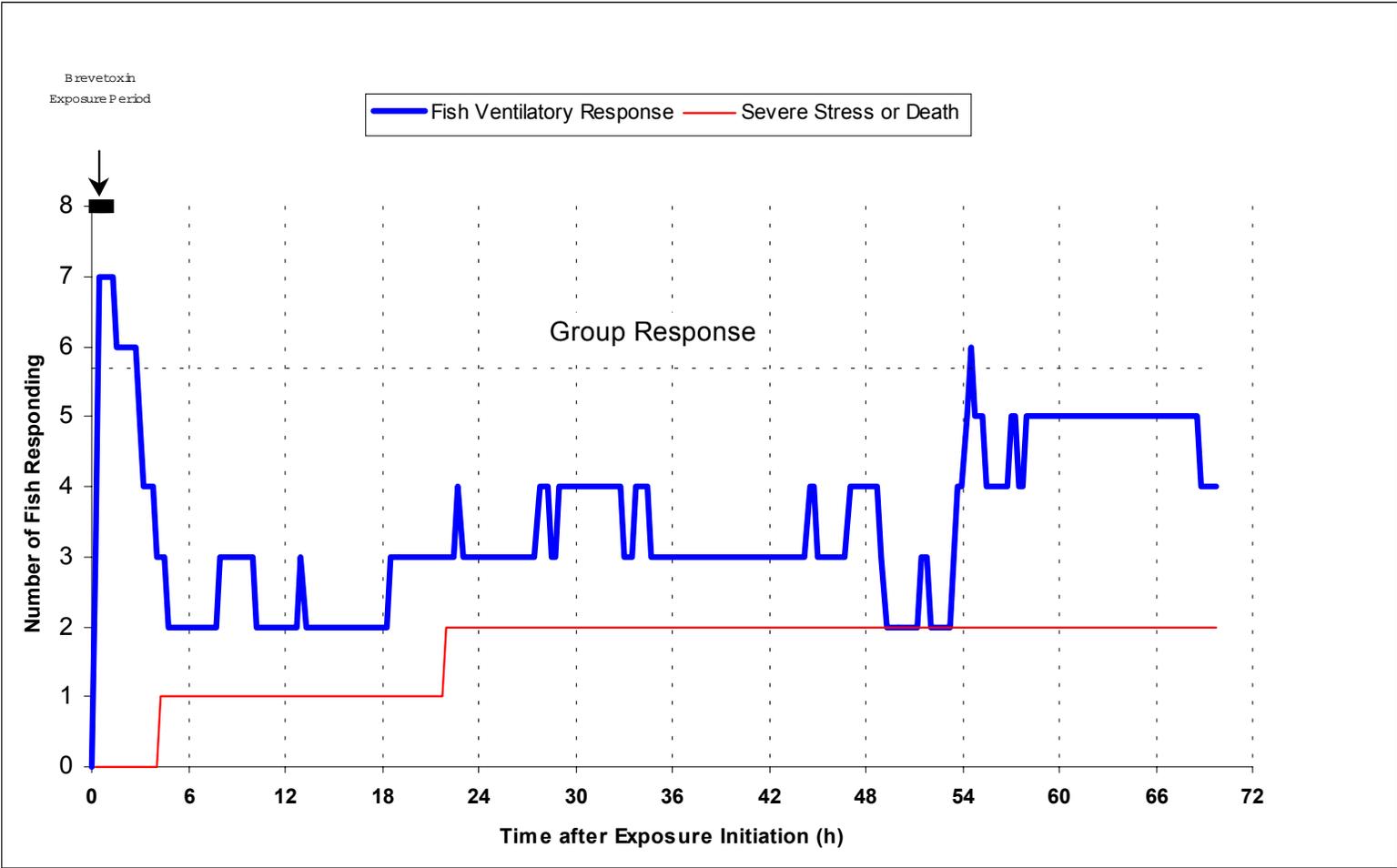
Figure 3-1. Fish ventilatory responses to brevetoxin. An individual fish response is due to a significant change in at least one of the ventilatory parameters (ventilatory rate, ventilatory depth, or cough rate). In this test, a fish group response (six or more fish responding simultaneously) occurred within 30 minutes of the initiation of brevetoxin exposure as a result of increased cough and ventilation rates.

3-5
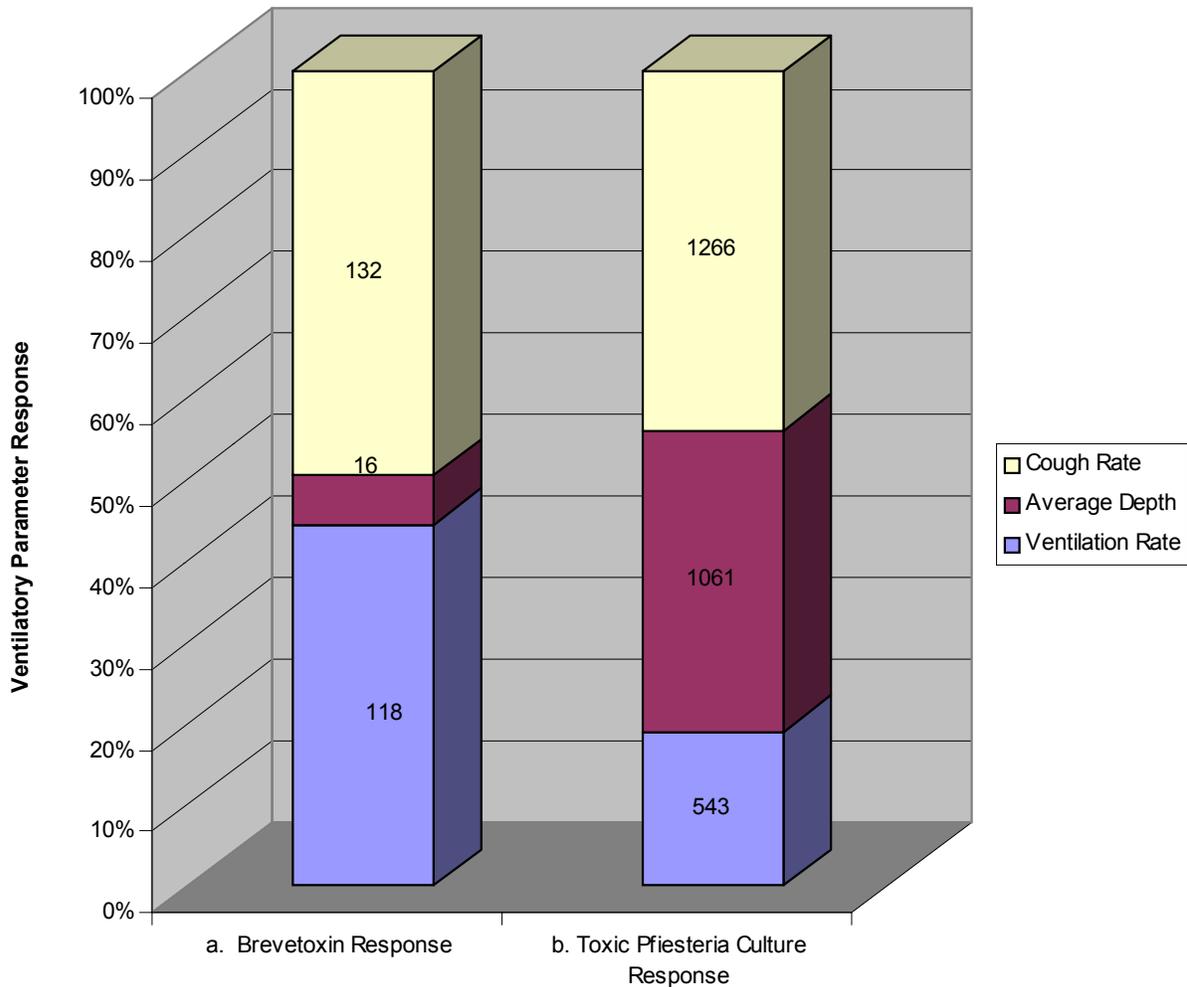
Figure 3-2. Relative occurrence of ventilatory parameter responses during fish group responses in laboratory studies. The relative percentages of individual fish responses due to cough rate, average depth, and ventilatory rate for all group out-of-control responses are shown. Numbers of individual responses are shown within each bar. A fish can respond in one, two, or all three parameters in any given 15-minute interval. a. Brevetoxin response. The data from the group response at 54 hours are not included because two of the fish responding died from causes unrelated to brevetoxin exposure. b. Toxic pfiesteria culture response.

3.1.1.4. *Brevetoxin Neurotoxicity Test*

The goal of the 2-DG portion of this research was to detect and localize alterations in CNS activity in fish exposed to brevetoxin. The method is based on the pioneering work of Sokoloff who demonstrated a direct relationship between glucose metabolism and brain activity at the regional level (Sokoloff, 1977). When a specific area of the brain is stimulated or inhibited by a stimulus, neural activity in that area increases or decreases, resulting in a relative increase or decrease in regional glucose uptake and metabolism. Sokoloff used 2-DG as a tracer of neural activity. When ^{14}C-labeled 2-DG is present in the system and taken up by the cells, the resultant breakdown product is deoxyglucose-6-phosphate. Deoxyglucose-6-phosphate lacks the necessary oxygen for further enzyme recognition by glucose-6-phosphatase and, as a result, does not metabolize further. Instead, the deoxyglucose-6 phosphate remains trapped in the tissue where it is taken up and can be visualized using autoradiography.

3.1.1.4.1. *Methods.* Fish were exposed to diluent water only, diluent water plus carrier solvent control (0.0001% Emulphor-620), or diluent water plus carrier solvent with PbTx-2 at a concentration of 45 µg/L (nominal; 47 µg/L measured). Exposures with five replicate fish were conducted in separate 4 L beakers containing 2 L of exposure media. After 1 hour in the treatment beaker, each fish was injected intramuscularly below the dorsal fin with 2 mCi of ^{14}C-2-DG (Amersham Pharmacia Biotech, Piscataway, NJ) and placed in a beaker containing freshwater for a 30-minute recovery period. Following the recovery period, fish were sacrificed by cervical dislocation and whole brains were removed.   Brains were snap frozen on aluminum foil dipped in 2-methyl butane, chilled over dry-ice, and subsequently stored at –80 °C.

Frozen whole fish brains were then horizontally cryosectioned at 15 µm and thaw-mounted directly onto frost-free microscope slides. Figure 3-3 indicates the plane of tissue sectioning. Slides were then coated with liquid emulsion (Ilford Nuclear Research, NC) in a darkroom and placed flat into light-tight desiccator 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
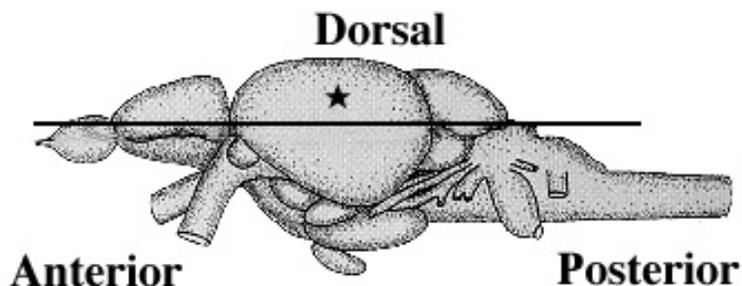
Figure 3-3. Plane of tissue section for bluegill brains.

into photographic fixative (Kodak) for 2 minutes. Slides were then washed in water, dried, and analyzed by microscopy. Developed slides were viewed using light and dark field microscopy (2x magnification). Autoradiograms were visualized with a video-based digital system (Alpha Innotech Corporation, computer software AlphaImager 2000, version 4.03) and digital images were recorded.

3.1.1.4.2. *Results and discussion*. Digital images of brain tissues taken from the three experimental groups depict visible differences in regional brain uptake of 2-DG between treatment and control groups (Figure 3-4). All treatment group fish presented regional elevations in 2-DG uptake, compared with carrier solvent and diluent controls, as a result of their exposure to brevetoxin. Multiple areas of high 2-DG uptake were observed in brevetoxin-treated fish. Glucose uptake was particularly elevated in the dorsal telencephalic region, *corpus cerebelli*, *tectum opticum*, and the *nucleus lateralis valvulae*.
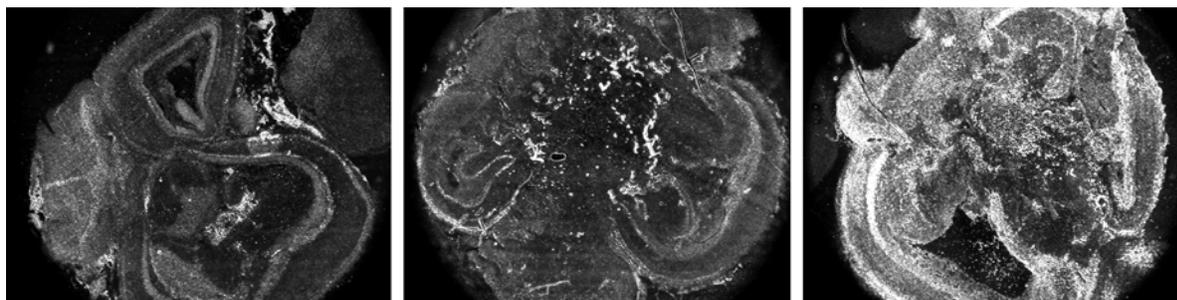
a.   b.   c.

Figure 3-4. 2-DG activity in the brains of bluegills. a. Control. b. Solvent control. c. Brevetoxin-exposed.

The telencephalon in fish is believed to be an area of sensory concentration where most sensory systems are controlled, including the mechanoreception system (lateral line system) responsible for the monitoring of local water movement. Biologically significant functions that are affected by changes in telencephalic functioning are prey localization, schooling behavior, and navigation. The cerebellum in fish dominates functions in motor learning and coordination, which would be effected by cerebral changes in neural activity (Wullimann et al., 1996). The general visceral sensory system has motor neurons located medially throughout the brain, including the area of the nucleus lateralis valvulae. These neurons innervate the gastrointestinal tract and the heart (Wullimann et al., 1996). The increased activity observed in this region is consistent with the gastrointestinal irritation reported as a symptom of neurotoxic shellfish

poisoning (Morris, 1999). High neural activity in this region, demonstrated in this report, would have an observable effect on the visceral sensory system of 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 fish exposed to pfiesteria were not tested, the technology transfer was demonstrated.

By using ^{14}C-labeled glucose, the alterations in CNS activity using autoradiographic techniques (obviously not in real time as in the human PET scans) were examined. Data clearly indicate that CNS activity is altered under conditions of PbTx-2 exposure, and that there are regional areas affected. This is the first time fish have been examined using a PET-like technique. This methodology can be applied to discern effects of exposure to pfiesteria-like dinoflagellates or other environmental stressors.

3.1.2. Toxic *Pfiesteria piscicida* Culture Testing

The objectives of this test were to: (1) demonstrate that bluegills in the automated biomonitoring system will respond to toxic *Pfiesteria piscicida* cultures, (2) determine which ventilatory parameters respond, and (3) determine the relationship between the time to ventilatory responses and deaths (if any) of *Pfiesteria piscicida*-exposed fish.

3.1.2.1. *Methods*

Testing was conducted at the pfiesteria culture facility at North Carolina State University and incorporated an adaptation of a fish bioassay system in which tilapia (*Oreochromis niloticus*) are used to evaluate toxic pfiesteria cultures (Burkholder and Glasgow, 1997). A portable bench scale fish ventilatory monitoring system was designed to provide bluegill biomonitoring data (as described in Section 2.1), while accommodating the safety and space requirements of the pfiesteria culturing facility. In this system, ventilatory signals were amplified by a factor of 2000 using a variable gain amplifier (see Appendix A, Section A.1). The portable system consisted of a chamber with cells for eight fish that received water pumped from a fish culture tank by a 750 L/h power head submersible pump through a manifold into each cell. Flow rates to each chamber averaged about 125 mL/min. The water was circulated between the fish culture tank and the biomonitoring unit. Initially, bluegills received water from a 9.4 L culture tank

containing dilution water only. Bluegills were acclimated to the ventilatory chambers for about 24 hours, then 24 hours of baseline data were taken. At the end of the baseline period, the water source for the ventilatory chambers was switched to a culture tank containing *Pfiesteria piscicida* in which three tilapia had been killed overnight. After these fish were removed and three new tilapia were added to the tank, water from the tank was pumped to the bluegill ventilatory cells. Water quality conditions differed slightly before and during exposure, due to the switch between source water aquaria, as shown in Table 3-2. Bluegills were exposed to *Pfiesteria piscicida* culture water for 24 hours. Ventilatory data were analyzed in the same manner as other laboratory and field studies, except that the baseline period was 24 hours instead of 96 hours. Counts of *Pfiesteria piscicida* zoospores were taken from the overflow of the ventilatory chamber prior to exposure at 15 minutes and at 1, 2, 4, 6, and 23 hours after the start of exposure. Cells were fixed with Lugol's solution and counts were made using a Sedgewick/Rafter slide.

Table 3-2. **Selected water quality parameter levels before and during exposure to *Pfiesteria piscicida* culture water.**

Parameter	Pre-Exposure	During Exposure
Temperature (°C)	20.9 - 22.2	21.1 - 23.1
pH	7.7 - 7.8	6.7 - 6.9
Specific Conductivity (mS/cm)	23.9 - 24.0	23.9 - 24.7
Dissolved Oxygen (mg/L)	6.5 - 6.8	5.7 - 6.6

3.1.2.2. *Results and Discussion*

Figure 3-5 shows responses of the bluegill ventilatory and movement parameters to *Pfiesteria piscicida* culture exposure. Four fish responded about 3 hours after exposure initiation by exhibiting a sharp drop in ventilation depth. However, this response did not reach the threshold for a group out-of-control response (six or more fish responding). The first group response occured about 9 hours after the start of exposure, caused by a marked increase in cough rate as well as ventilation rate and depth (Figure 3-2b). This response occured just before the majority of the fish became severely stressed or died. Six of the eight bluegills were in this condition after 11 hours. By the end of the exposure period, only one bluegill was alive, and it was moribund. All three tilapia held in the *Pfiesteria piscicida* culture tank from which the bluegills received water, died between 6 and 24 hours after exposure. No lesions were observed on either the bluegills or tilapia used in this study.
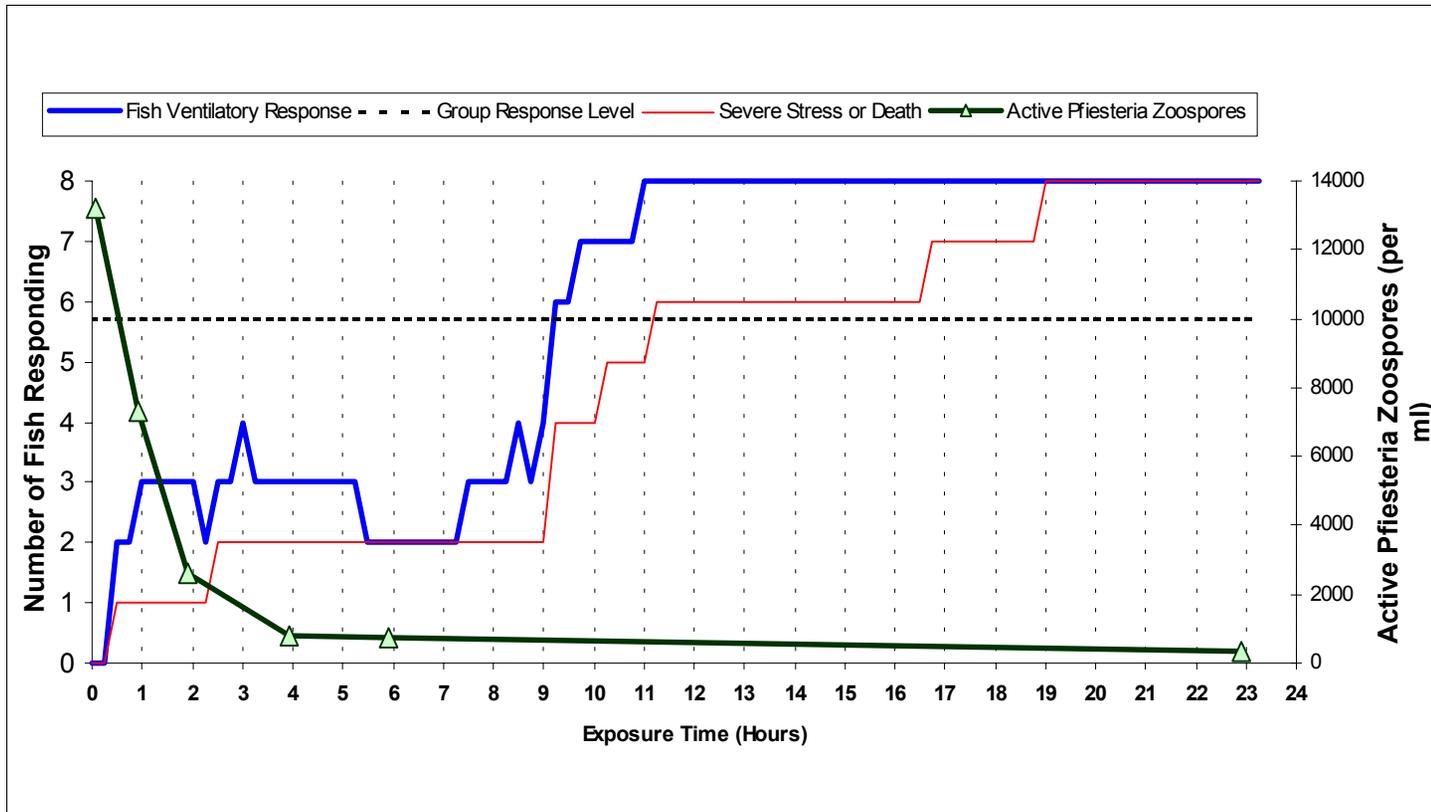
Figure 3-5. Fish ventilatory responses to *Pfiesteria piscicida* culture water. An initial fish response due to decreased ventilation depth after 3 hours (below the group response threshold) was followed by a group response after 9 hours due to increased cough rate. All but one fish died by the end of the exposure period.

Although the pH and dissolved oxygen were somewhat lower during the exposure period than during the baseline period (Table 3-2), it is unlikely that either of these parameters contributed to fish ventilatory responses. As with brevetoxin (Section 3.1.1.3.2), cough rate was a major response parameter to the *Pfiesteria piscicida* culture water. Carlson (1984) reported that coughing in bluegills tested at 21.5 °C was unchanged over a wide range of dissolved oxygen concentrations ($\leq$44 to 117% of saturation, 3.8 to 11.9 mg/L at 22 °C) and pH (4.3 to 10.1). As noted above, the increase in cough rates associated with exposure to toxic pfiesteria cultures may help distinguish a ventilatory response to pfiesteria observed in the field from responses due to ventilatory responses caused by changes in some common water quality parameters (temperature, dissolved oxygen, and pH).

The *Pfiesteria piscicida* zoospore count declined rapidly during the exposure period. It is possible that the zoospores encysted due to the turbulence created by pumping water between the tilapia culture tank and the fish ventilatory cells. Bluegill ventilatory effects and fish mortality occurred well after the zoospores were no longer in the water column. It is not known whether fish death was related to damage caused by zoospore attack or to a toxin released into the water. However, Burkholder and Glasgow (1997) have found that tilapia placed into ultrafiltered media from fish-killing cultures of *Pfiesteria piscicida* will also be killed, suggesting toxic activity even in the absence of zoospores.

3.2. BIOMONITORING SYSTEM RESPONSES: FIELD TESTING

During the summer of 2000, the mobile biomonitoring facility was located on the Chicamacomico River at Drawbridge, Maryland (Figure 2-1). This site was selected at the request of the DNR because of reported pfiesteria-related fish kills in1999, and to allow co-location with a DNR continuous chemical monitoring station. The purpose of this deployment was to demonstrate the capabilities of the automated biomonitoring system to detect water quality perturbation and, in particular, pfiesteria-associated toxicity, should it occur.

3.2.1. Methods and Materials

Overall automated biomonitoring system operation is described in Section 2.1. Routine maintenance activities are described in Table 3-3. Total time required for routine maintenance averaged 4 to 5 hours per week. System operation was continuous, except for a 1- to 2-hour period every 2 weeks when a new group of fish were switched from baseline monitoring to on-line monitoring. There was one gap of 4 days (7 to 11 September) due to a software problem. Fish response data from the biomonitoring system were reviewed every day at the USACEHR via PC Anywhere®, and DNR personnel were contacted immediately when developing system responses were observed. At the end of a 2-week monitoring period for a group of fish, data

were downloaded via PC Anywhere®, quality assured, and interpreted. Graphic response information for the 2-week interval was sent to DNR and posted on their Internet website, which also provided a link to a fish biomonitoring Internet website that included detailed background information on this project.

Whenever a group of fish in the monitoring system displayed an out-of-control (or "alarm") response, water samples were taken automatically by the ISCO® refrigerated autosampler. Follow-up evaluations were conducted on the second, third, and fourth of the five biomonitor response events encountered. Since a primary concern of the DNR was the potential presence of toxic pfiesteria, water samples were analyzed for presence of either *Pfiesteria piscicida* or *Pfiesteria shumwayae* using polymerase chain reaction (PCR) (Bowers et al., 2000; Oldach et al., 2000). Additional water chemistry analyses included metals analysis (by inductively coupled argon plasma mass spectrometry) and a qualitative analysis for a broad range of organic chemicals using capillary gas chromatography/mass spectrometry, using a Hewlett-Packard-6890 gas chromatograph and a Hewlett-Packard-5973 gas chromatograph/mass spectrometer.

3.2.2. System Operation: Results and Discussion

After an initial start-up period, the automated biomonitoring system was run continuously on-site for 85 days, from August 7 through October 30, 2000. Although there were considerable variations in water quality parameters during the monitoring period (see Section 3.2.2.1), only one of five response events detected by the biomonitor (event four) was linked to water quality variation. Toxicity was the apparent cause of event five, but a cause could not be determined for the other three events (see Section 3.2.2.2). The relationship between water quality parameter variation and fish ventilatory responses is discussed further in Section 3.2.2.3.

3.2.2.1. *Water Quality Variations*

In field testing, the automated biomonitoring system encountered wide variations in water quality parameters. Ranges of water quality parameters and their maximum rates of change are reported in Table 3-4. Temporal patterns in temperature, pH, conductivity, and dissolved oxygen followed diel and tidal cycles. As might be expected, conductivity fluctuations followed tidal cycles, while fluctuations in temperature followed a diel cycle. Dissolved oxygen and pH patterns were less distinct, but tended to follow a diel cycle. Turbidity and fluorescence data obtained from a DNR automated chemistry monitor (http://mddnr.chesapeakebay.net/empact/EmpactReform2.cf) located immediately upstream of the biomonitoring facility indicated that turbidity was relatively low, briefly exceeding 50

nephelometric turbidity units (NTUs) on only three occasions, and that fluorescence similarly showed a few sporadic high peaks during the monitoring period.

Table 3-3. Routine maintenance activities during deployment on the Chicamacomico River.

Activity	Frequency	Comments
Analyze response data	Daily	Download response data via PC Anywhere® to USACEHR and analyze for significant events.
Verify water quality readings	2/week	Check readings from Hydrolab® against instrument readings, clean Hydrolab® probes.[1]
Maintain particulate filters	2/week	Check filter pressures, clean basket filters, clean and replace bag filters as necessary.
Back flush river water intake	2/week	Back flush and switch water intakes.
Flush ISCO® autosampler lines	2/week	
Remove/add fish	1/2 weeks	Remove old fish, put new eight fish set on-line.
Clean water lines	1/2 weeks	Clean and flush manifold water delivery system.
Archive biomonitoring data	1/2 weeks	Archive data and send to DNR for Internet website posting.
Calibrate Hydrolab®	1/2 weeks	Calibrate when a new group of fish is put on-line.

[1] The Hydrolab® oxygen probe was prone to fouling. During the field test, instantaneous increases of 1-2 mg/L dissolved oxygen occurred frequently when the probe was cleaned. Research to develop a dissolved oxygen sensor less prone to fouling is described in Appendix B.

Variability in multiple water quality parameters is heightened during storms. Automated biomonitor responses may occur if changes in the parameters are of sufficient magnitude and if they exceed the levels and rates of change to which the fish were exposed during their baseline monitoring period. Further, toxic materials that might be present in the Chicamacomico River watershed (e.g., herbicides, pesticides, or hydrogen sulfide present in wetlands) may be present in nonpoint source runoff during storms. To help explain the biomonitoring system response events encountered during field testing, changing patterns of water quality parameters such as temperature, dissolved oxygen, and turbidity were considered along with available chemical analyses of water samples taken during the response events and inferences about causality based on the nature of the fish responses themselves.

3.2.2.2. *Biomonitor Responses During Field Testing*

Overall biomonitor responses for the entire field monitoring period and associated water quality data are shown in Figure 3-6. Five response events (with six or more of the eight fish responding) were noted during the monitoring period. Each response is considered in chronological order in the context of variations in water quality parameters (see Section 3.2.2.1)
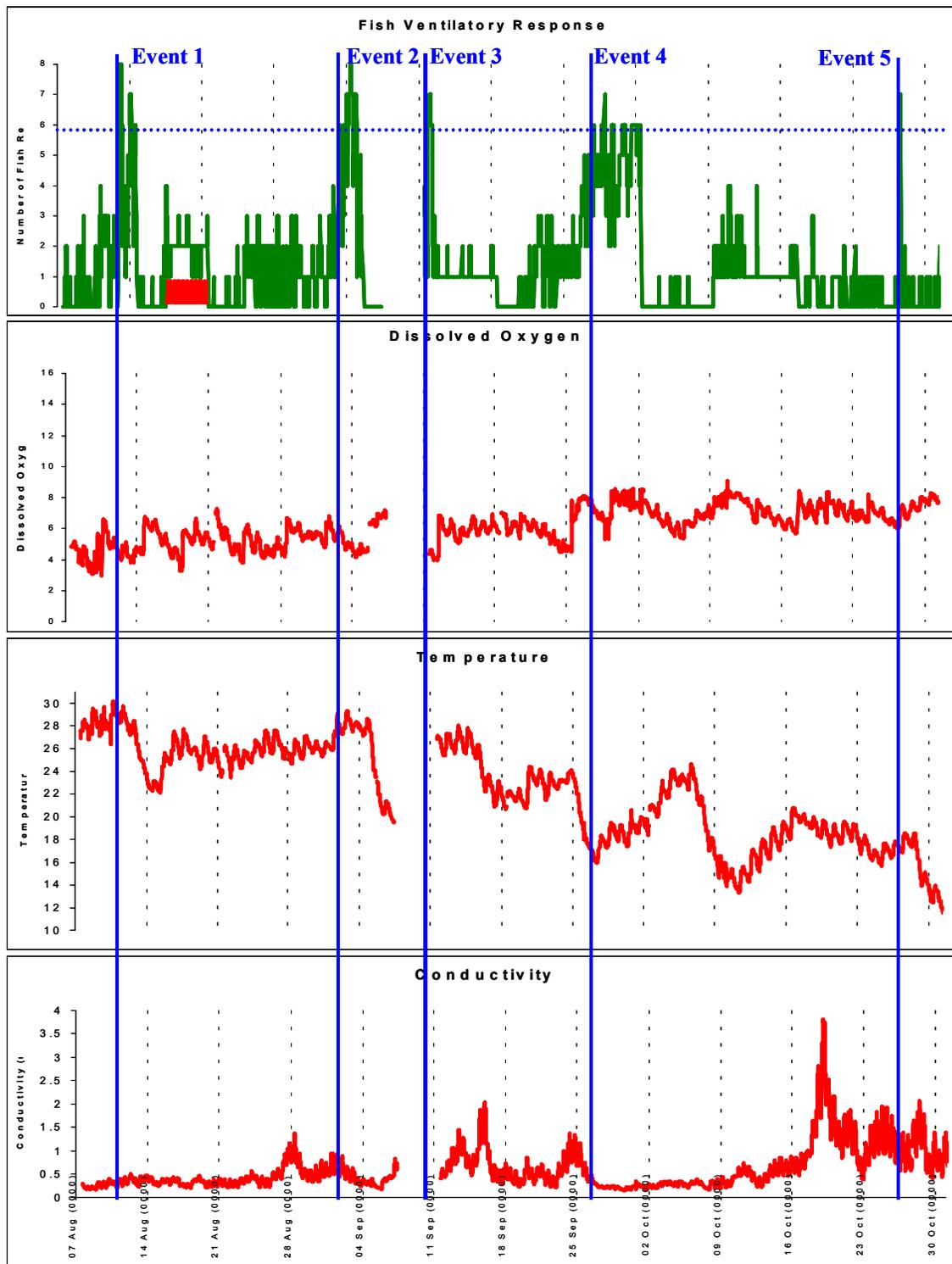
Figure 3-6. Fish ventilatory responses and water quality data during field deployment, August 7 to October 30, 2000. a. Ventilatory responses with dissolved oxygen, temperature, and conductivity data. Of the five response events, toxicity is implicated in event five, and

changing temperature and conductivity in event four. Causes could not be established for the other three responses.
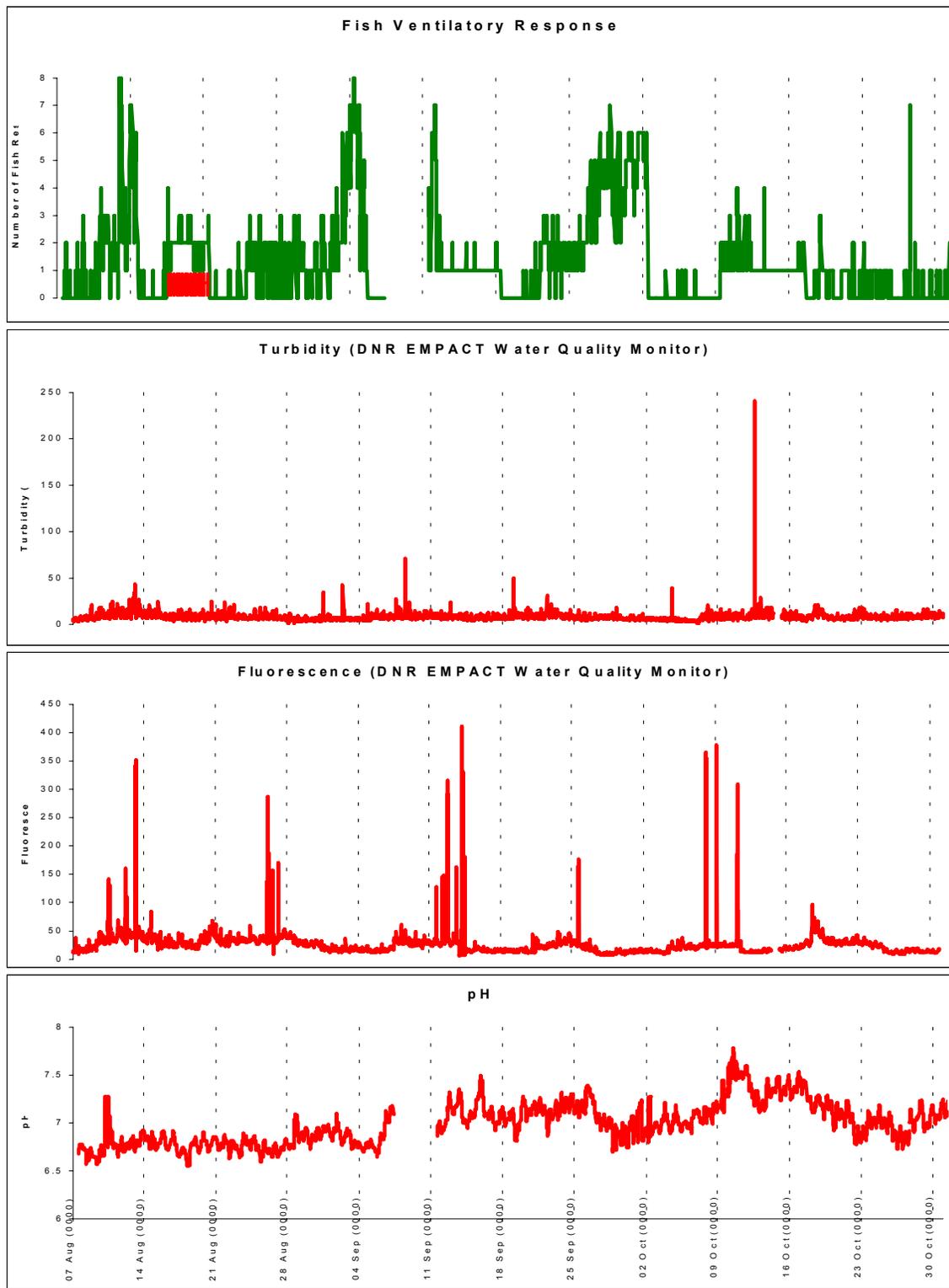
Figure 3-6 (Continued). Fish ventilatory responses and water quality data during field deployment, August 7 to October 30, 2000. b. Ventilatory responses with turbidity, fluorescence, and pH data. Of the five response events, toxicity is implicated in event five, and changing temperature and conductivity in event four. Causes could not be established for the other three responses.

3-18

Table 3-4. Ranges of selected water quality parameters during deployment on the Chicamacomico River

Parameter	Minimum	Maximum	Maximum Rate of Change (/h)
Temperature (°C)	11.6	30.2	1.9
pH	6.6	7.8	0.4
Specific Conductivity (mS/cm)	0.15	3.8	0.44
Dissolved Oxygen (mg/L)	3.1	9.0	1.4

and the potential for effects due to other natural or anthropogenic causes. Pfiesteria was not detected during the monitoring period.

The results of the first monitoring period (August 7 to 21) are shown in Figure 3-7. This was the only monitoring period during which a fish died and the only one in which the aeration system (see Section 3.3) was used because the dissolved oxygen level in the incoming river water fell below 3.0 mg/L. The rapid variation in dissolved oxygen level and pH on August 10 were associated with the aeration system switching on and off. No fish group responses were associated with the operation of this system.

The first biomonitor response associated with an increase in coughing rate (Figure 3-8) was recorded from August 13 to 14. Storms associated with the passage of a cold front occurred during this time period, as is reflected in the falling water temperatures and a pulse of turbidity. However, it is unlikely that changing temperature was the cause of the initial fish response, since the response first peaked at about midnight on August 13, and the temperature was still well within the range encountered during the prior several days when no fish group responses were encountered. Further, although both turbidity and fluorescence show peaks during this event, there are other instances during the entire monitoring period when similar or greater peaks were encountered without a corresponding fish response (Figure 3-6). Pfiesteria PCR analyses of the water samples taken on August 13, 14, and 15 were negative.

A second biomonitor response event was encountered during the next monitoring period (August 21 to September 5, Figure 3-9). In contrast with the first event, this event was caused mostly by increases in ventilatory rate and, to a lessor extent, depth, but not by increased cough rate. Changes in water quality parameters prior to the initiation of the event exhibited no remarkable changes when compared with the rest of the monitoring period. Analyses of water samples taken on September 3 and 4 did not detect pfiesteria, specific organic chemicals, or elevated metal levels.

3-19
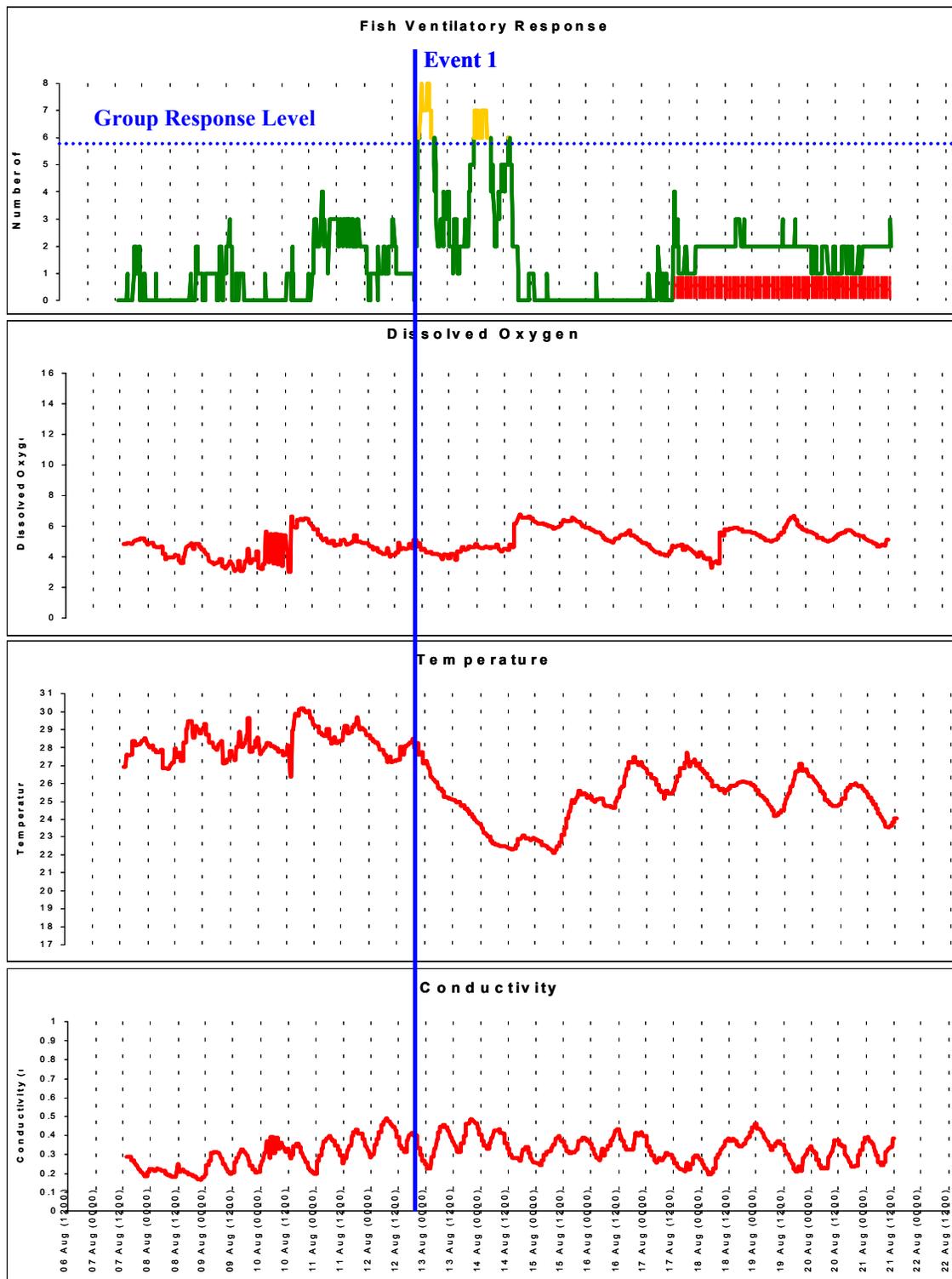
Figure 3-7. Fish ventilatory responses and water quality data during field deployment, August 7 to 21, 2000. a. Ventilatory responses with dissolved oxygen, temperature, and conductivity data. The rapid variation in dissolved oxygen on August 10 is associated with aeration system operation. Event one was associated with increased cough rates and occurred just prior to a storm (note subsequently falling temperatures). No definitive cause could be determined.
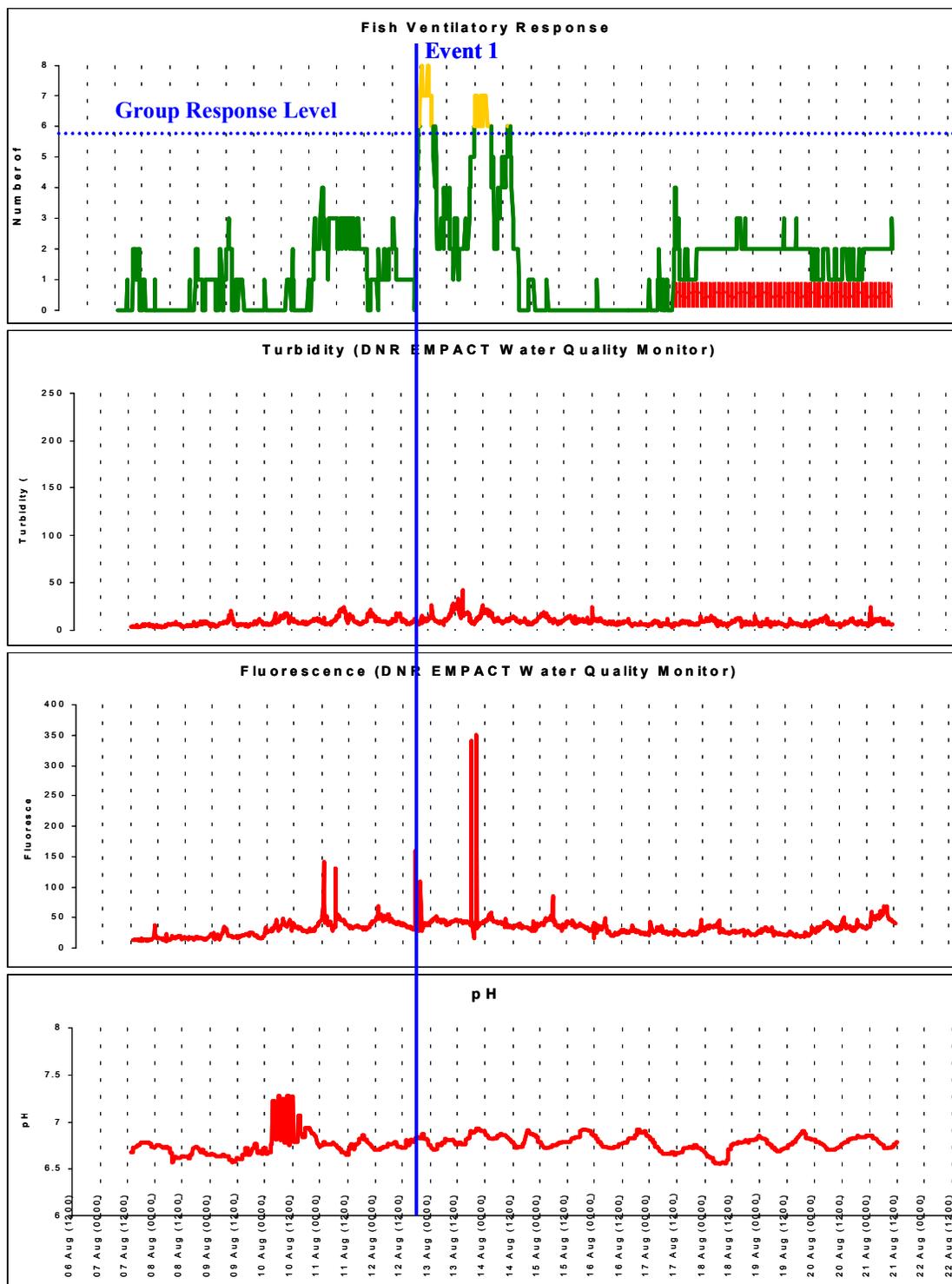
Figure 3-7 (Continued). Fish ventilatory responses and water quality data during field deployment, August 7 to 21, 2000. b. Ventilatory responses with turbidity, fluorescence, and pH data. The high variation in pH on August 10 is associated with aeration system operation. Event one was associated with increased cough rates and occurred just prior to a storm (note subsequently falling temperatures). No definitive cause could be determined.
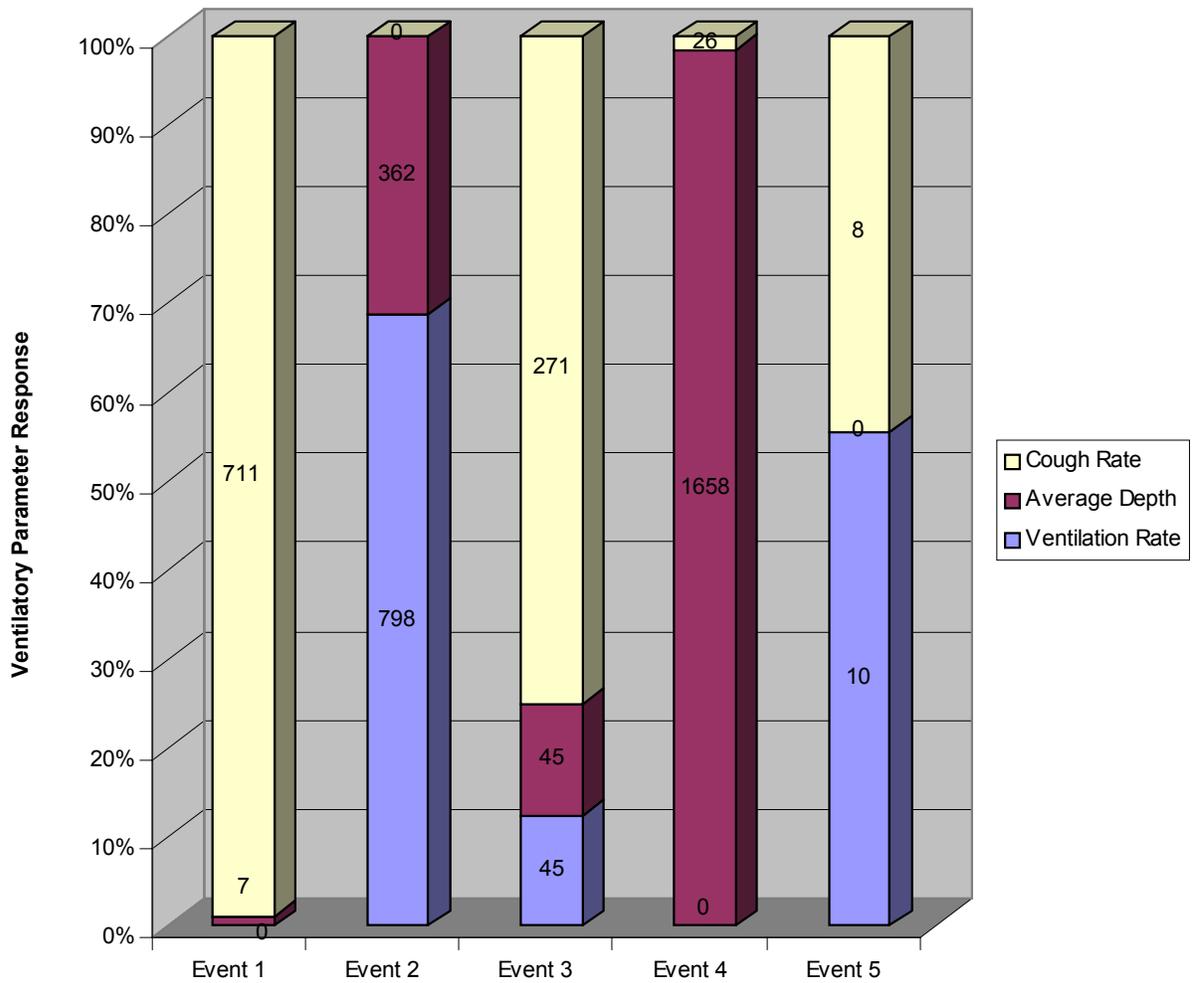
Figure 3-8. Relative occurrence of ventilatory parameter responses during fish group response events in field studies. The relative percentages of individual fish responses due to cough rate, average depth, and ventilatory rate for all group out-of-control responses are shown from the start to the end of each of the five response events. Numbers of individual responses are shown within each bar. A fish can respond in one, two, or all three parameters in any given 15-minute interval.
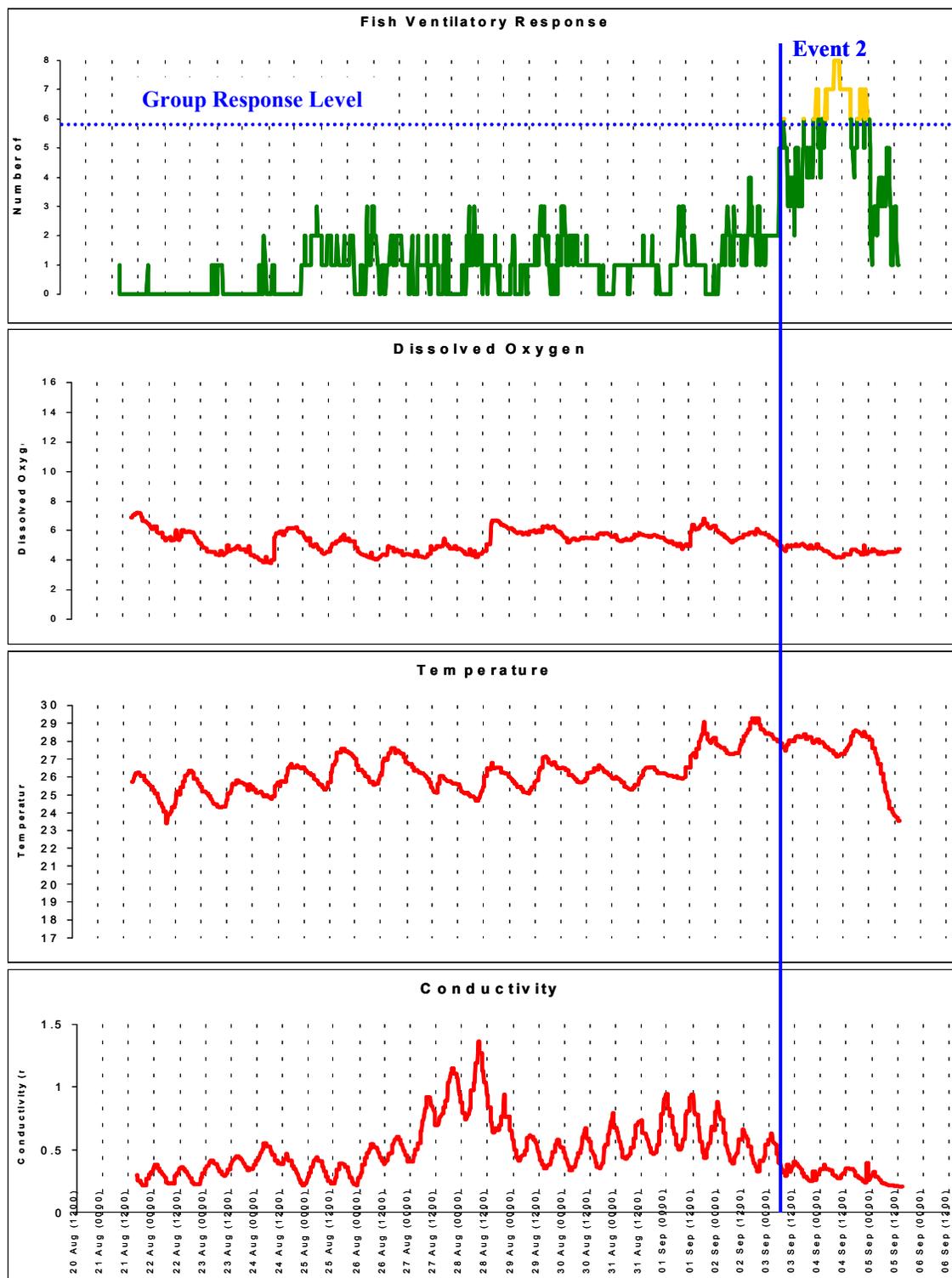
Figure 3-9. Fish ventilatory responses and water quality data during field deployment, August 21 to September 5, 2000. a. Ventilatory responses with dissolved oxygen, temperature, and conductivity data. Event two was associated with increased ventilatory rate (and depth). Neither water quality parameter changes, nor chemical analyses, suggested a cause for the event.
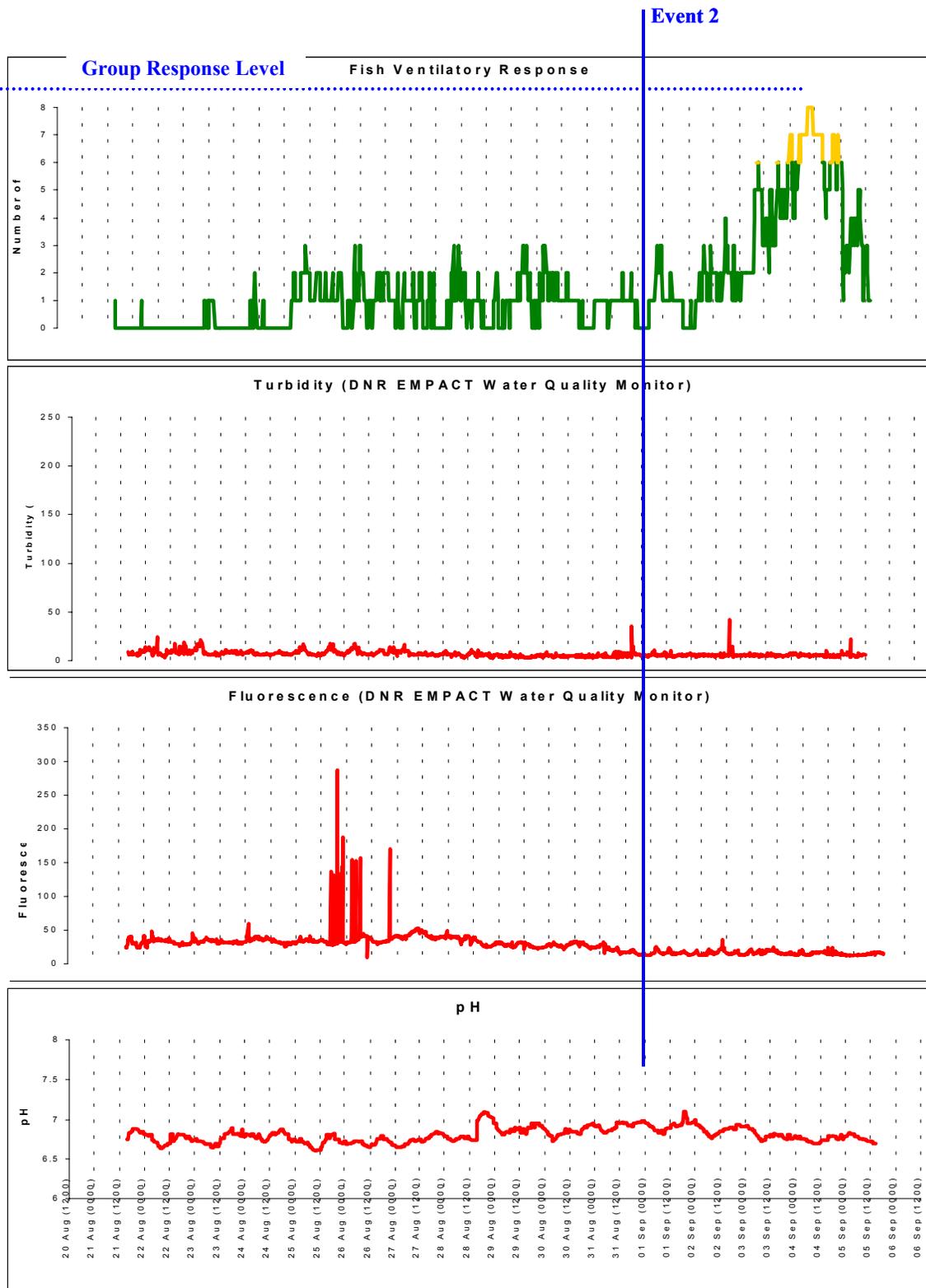
Figure 3-9 (Continued). Fish ventilatory responses and water quality data during field deployment, August 21 to September 5, 2000. b. Ventilatory responses with turbidity, fluorescence, and pH data. Event two was associated with increased ventilatory rate (and depth). Neither water quality parameter changes, nor chemical analyses suggested a cause for the event.

The next monitoring period (September 5 to 18, Figure 3-10) includes the third response event that occurred on September 11 and 12. Similar to the first response event, the third event was driven solely by increased cough rate. The most notable water quality change preceding the event was a rise in temperature of about 8 °C. The timing of the temperature change is not apparent because of a 4-day gap in the data. The gap occurred because the biomonitoring system displayed but did not store the monitored data due to a software program error that was not identified until the next on-site routine maintenance work. However, the temperature was virtually unchanged in the six hours immediately preceding the response, and there were no significant cough responses for a rapid temperature change of 4 °C in a laboratory test (see Section 3.2.2.3). Once again, analyses of water samples taken on September 11 and 12 did not detect elevated metal levels, and a September 14 pfiesteria sample was negative. However, organic chemical analysis on both September 11 and 12 detected 2-(2-butoxyethanol)ethanol acetate, a material associated with pesticide formulations. It is not known whether this material contributed to the biomonitor response, but a sample taken on September 25 when no fish response was observed also showed the presence of 2-(2-butoxyethanol)ethanol acetate.

During the next two-week period (September 18 to October 2, Figure 3-11), there was a large drop in water temperature (from about 24 to 16 °C) beginning on September 25. There were corresponding biomonitor responses due to increased ventilatory depth. Although ventilatory depth would be expected to decrease as temperature falls (Heath, 1973), conductivity also showed a substantial decrease, and it has been observed that ventilatory depth tends to increase as conductivity falls. This fourth biomonitor response appears to be primarily related to decreases in conductivity and temperature resulting from increased freshwater input from precipitation and the passage of a cold front.

No fish responses occurred during the period October 2 to 17 (Figure 3-12), in spite of a large drop in temperature. This was probably related to large variance in the baseline period for these fish due to the high temperature variability during September 18 to October 2. (A statistical technique for evaluating fish ventilatory responses that does not depend upon a baseline period is described in Appendix B, Section B.2.) The DNR data showed spikes in turbidity and fluorescence during the monitoring period that do not correspond to any fish responses.

There was only one fish group response during the last monitoring period (October 17 to 31, Figure 3-13). Conductivity was somewhat higher than the previous 2-week period, and temperature decreased greatly during the last 3 days of the period. The lone fish response,
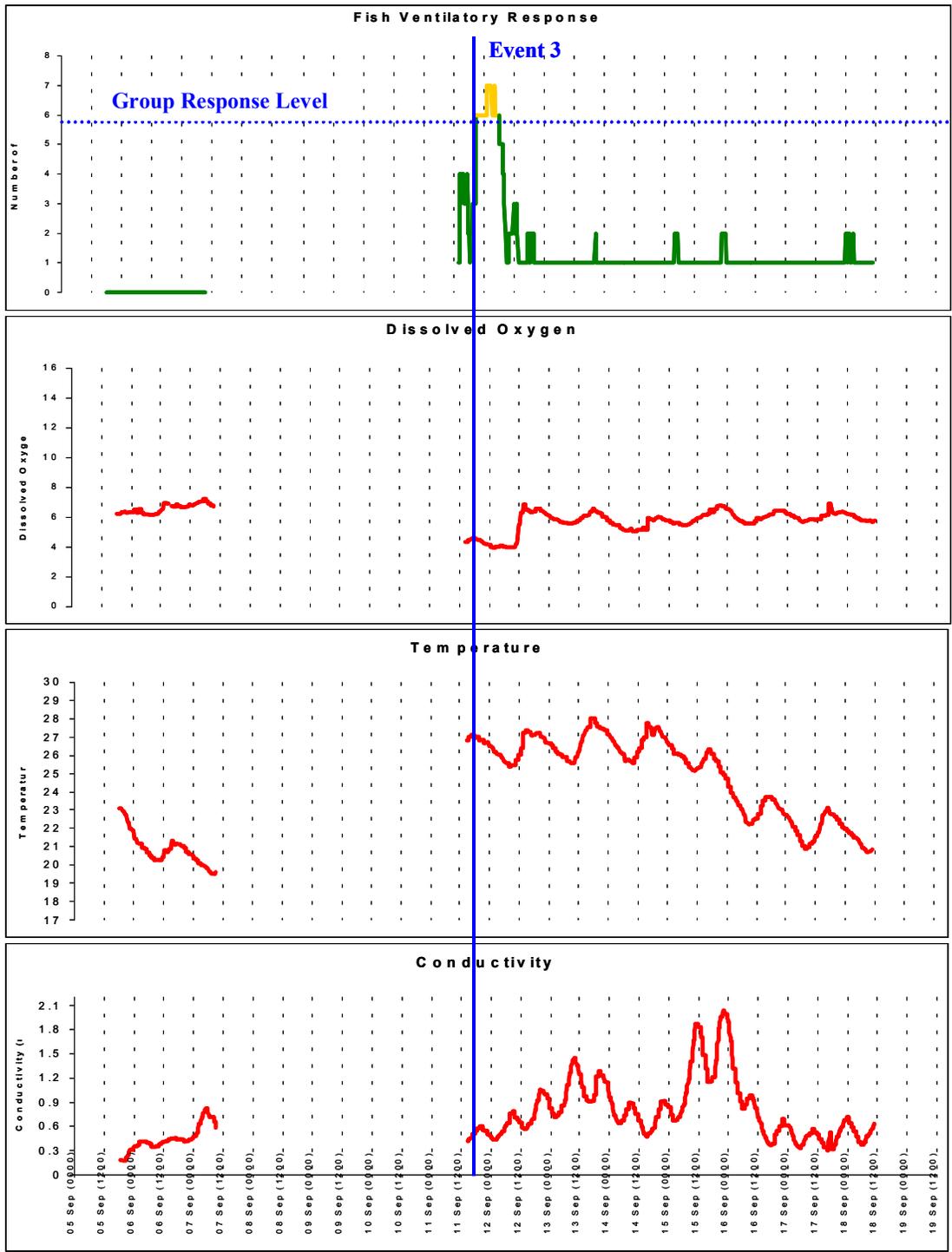
Figure 3-10. Fish ventilatory responses and water quality data during field deployment, September 5 to 18, 2000. a. Ventilatory responses with dissolved oxygen, temperature, and conductivity data. The gap in the data was caused by a software problem. The event was associated with increased cough rate. Although there was a substantial temperature rise, temperature was stable for six hours prior to the event. No definitive cause could be determined, although 2-(2-butoxyethanol)ethanol acetate was detected in water samples.
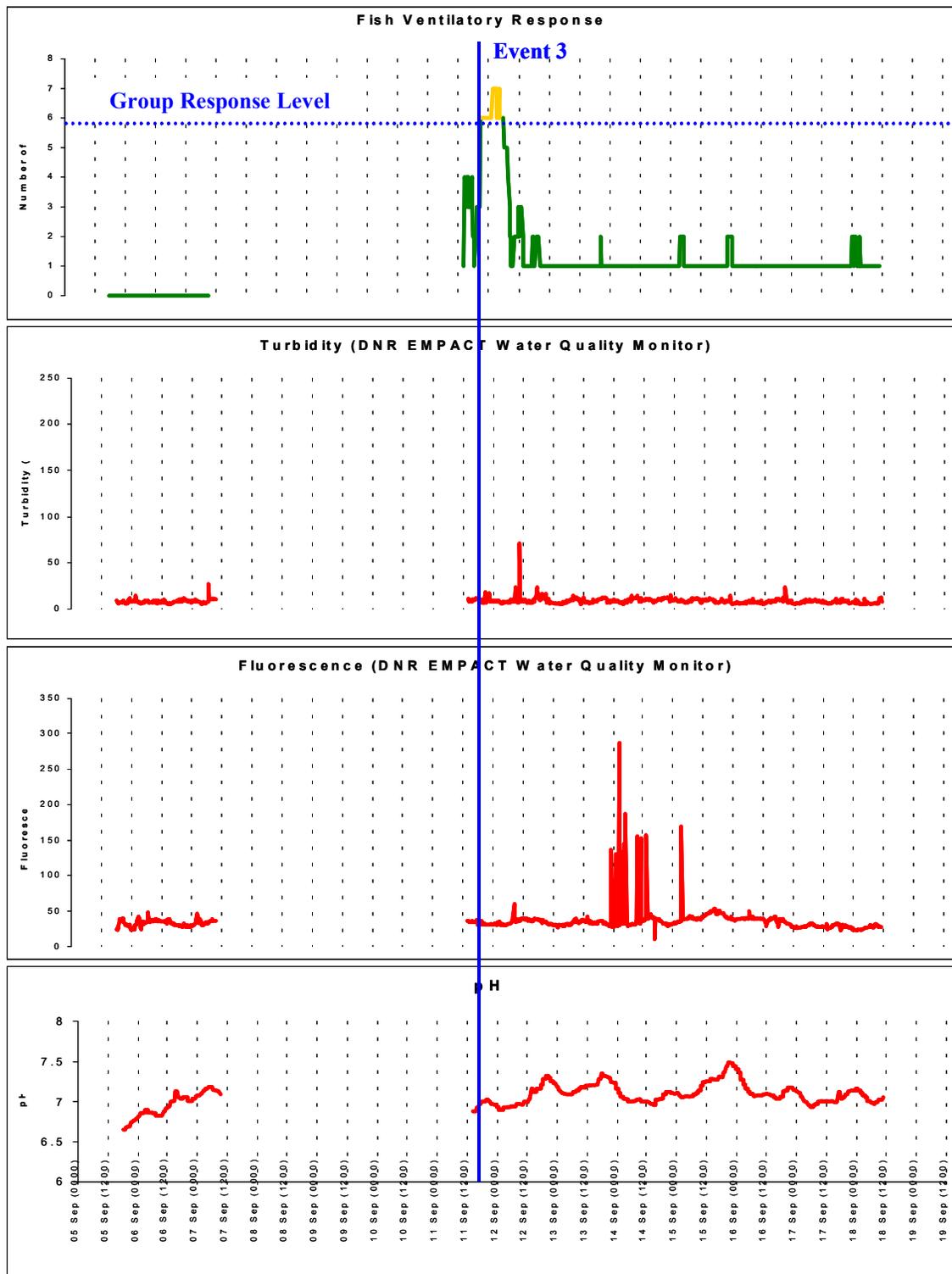
Figure 3-10 (Continued). Fish ventilatory responses and water quality data during field deployment, September 5 to 18, 2000. b. Ventilatory responses with turbidity, fluorescence, and pH data. The gap in the data was caused by a software problem. The event was associated with increased cough rate. No definitive cause could be determined, although 2-(2-butoxyethanol)ethanol acetate was detected in water samples.
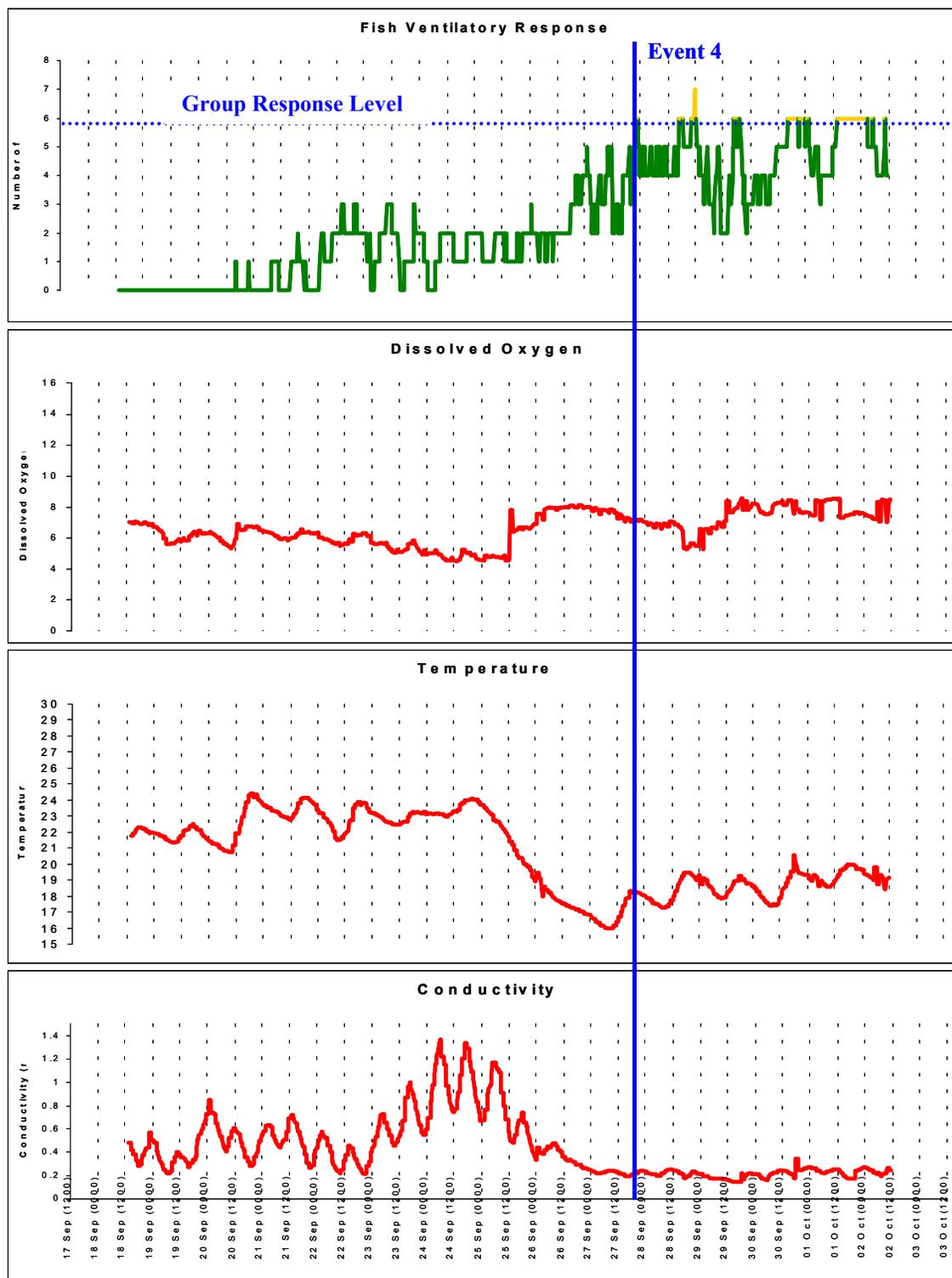
Figure 3-11. Fish ventilatory responses and water quality data during field deployment, September 18 to October 2, 2000. a. Ventilatory responses with dissolved oxygen, temperature, and conductivity data. A large temperature drop and decrease in conductivity associated with the passage of a cold front is linked to a fish response characterized by increased ventilatory depth.
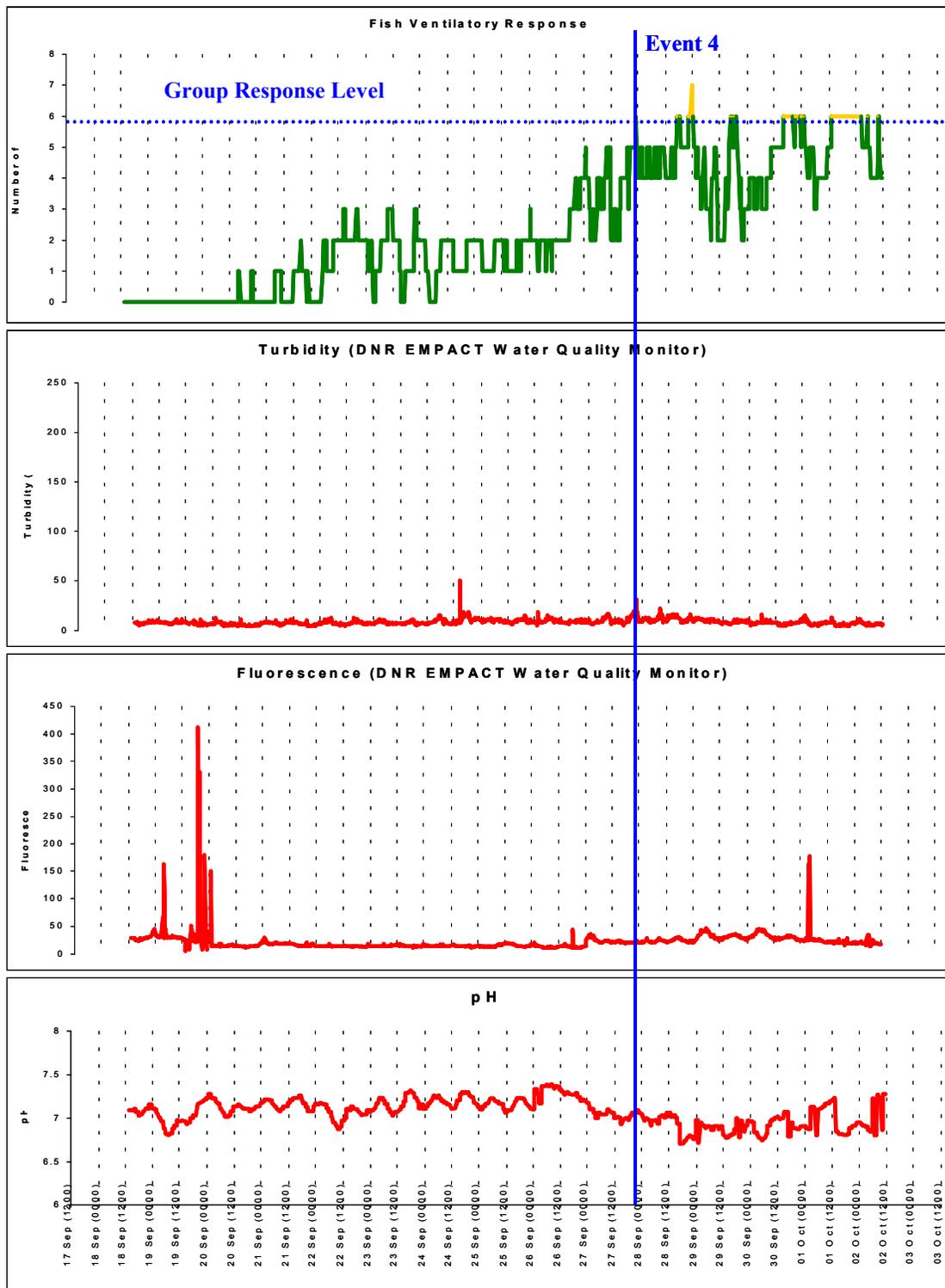
Figure 3-11 (Continued). Fish ventilatory responses and water quality data during field deployment, September 18 to October 2, 2000. b. Ventilatory responses with turbidity, fluorescence, and pH data. A large temperature drop and decrease in conductivity associated with the passage of a cold front is linked to a fish response characterized by increased ventilatory depth.
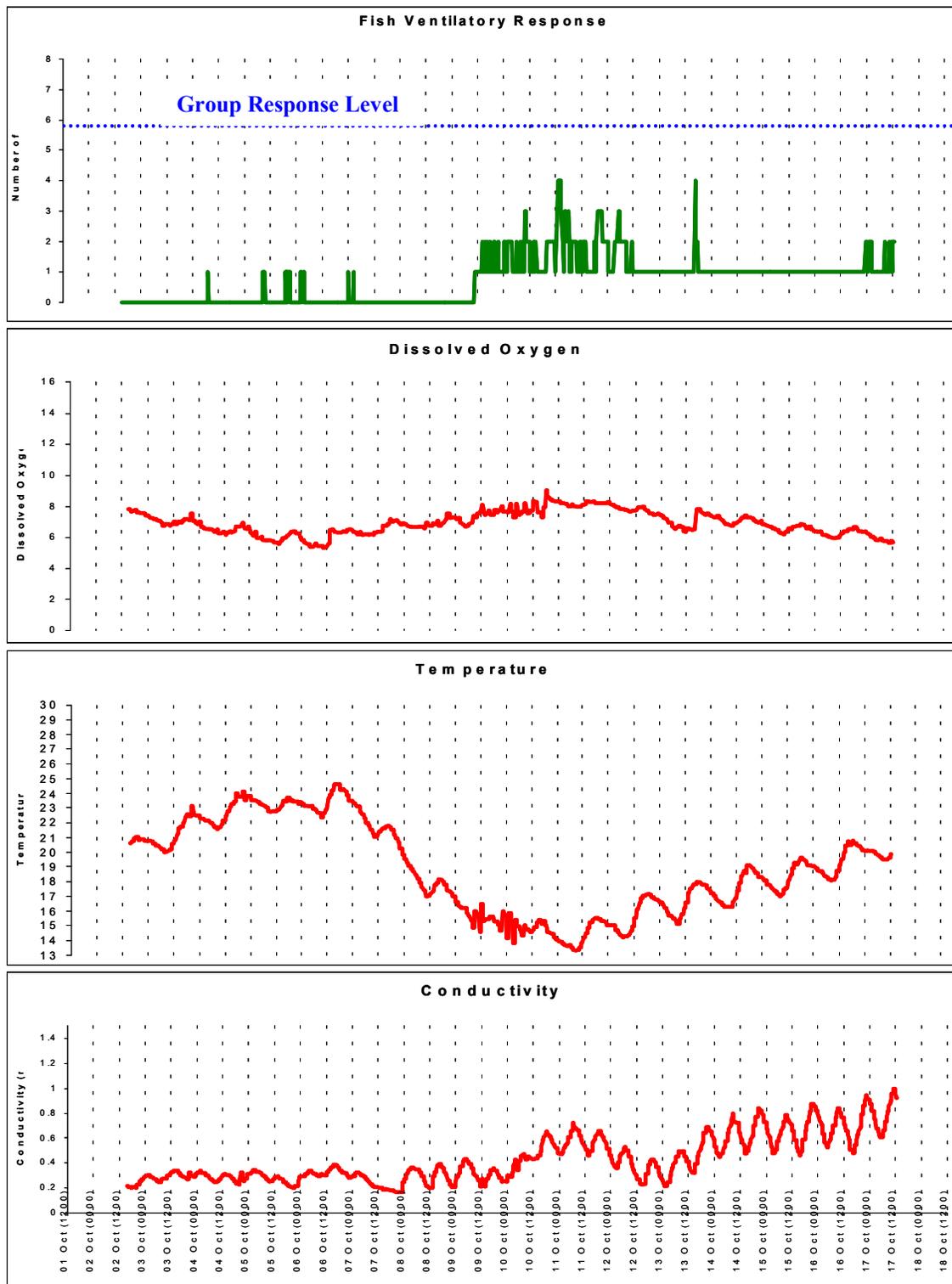
Figure 3-12. Fish ventilatory responses and water quality data during field deployment, October 2 to 17, 2000. a. Ventilatory responses with dissolved oxygen, temperature, and conductivity data. In spite of a large drop in temperature, there were no fish group responses.
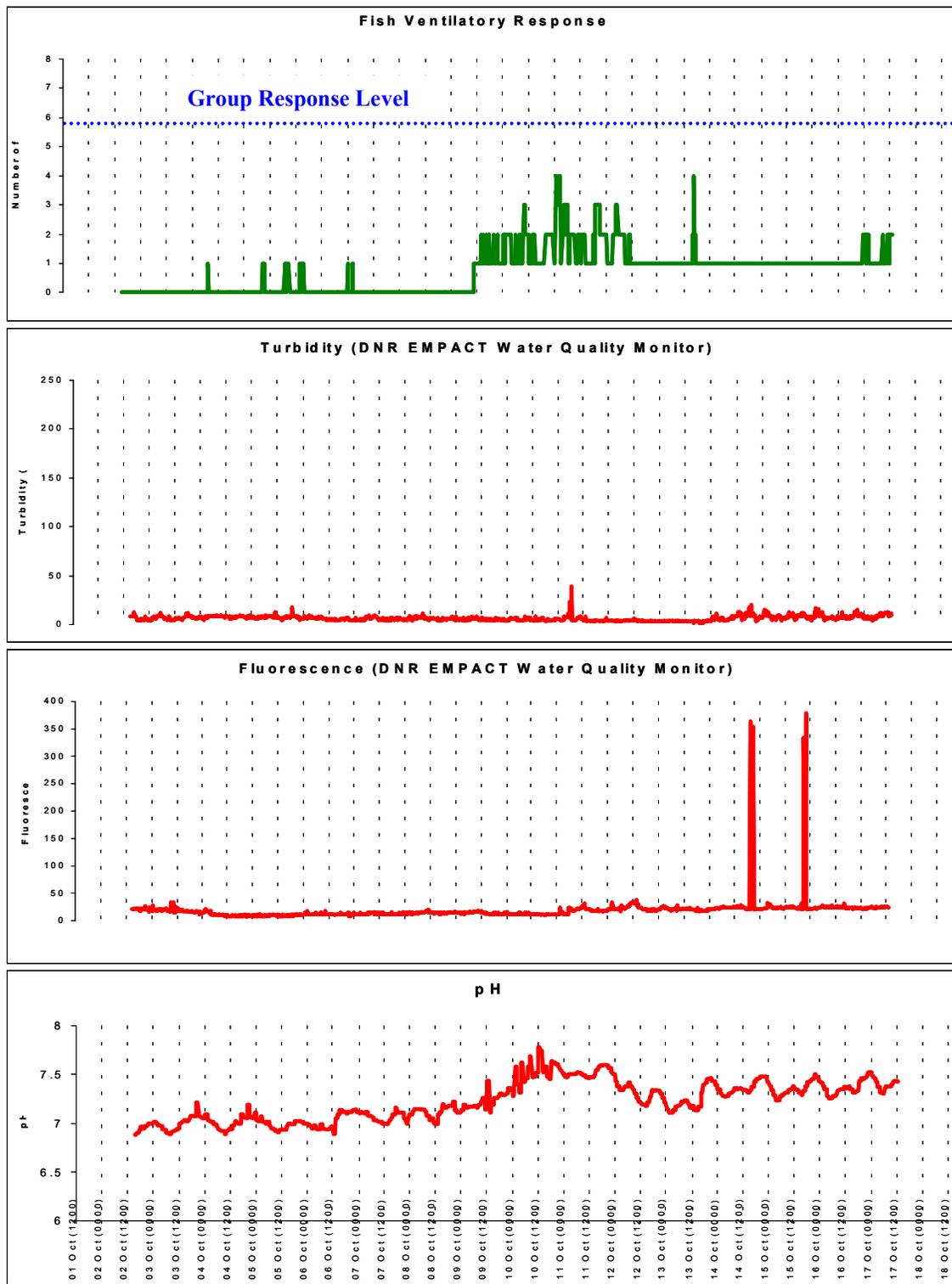
Figure 3-12 (Continued). Fish ventilatory responses and water quality data during field deployment, October 2 to 17, 2000. b. Ventilatory responses with turbidity, fluorescence, and pH data. Although there were spikes in turbidity and fluorescence, there were no fish group responses.
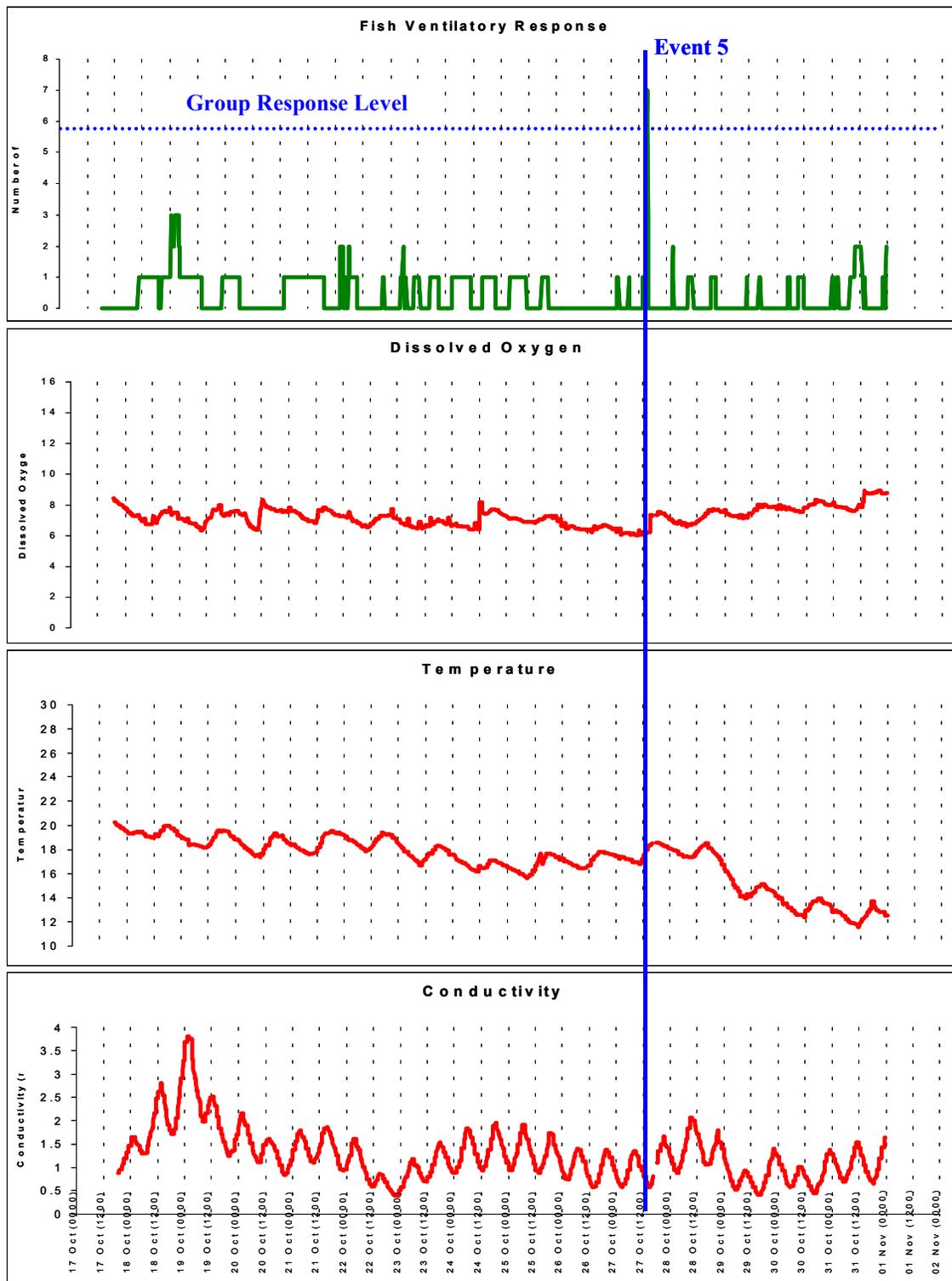
Figure 3-13. Fish ventilatory responses and water quality data during field deployment, October 17 to 31, 2000. a. Ventilatory responses with dissolved oxygen, temperature, and conductivity data. The brief fish response event characterized by increased cough and ventilation rates was associated with the use of unrinsed bag filters in the biomonitoring facility, which may have exposed the fish to contaminants.
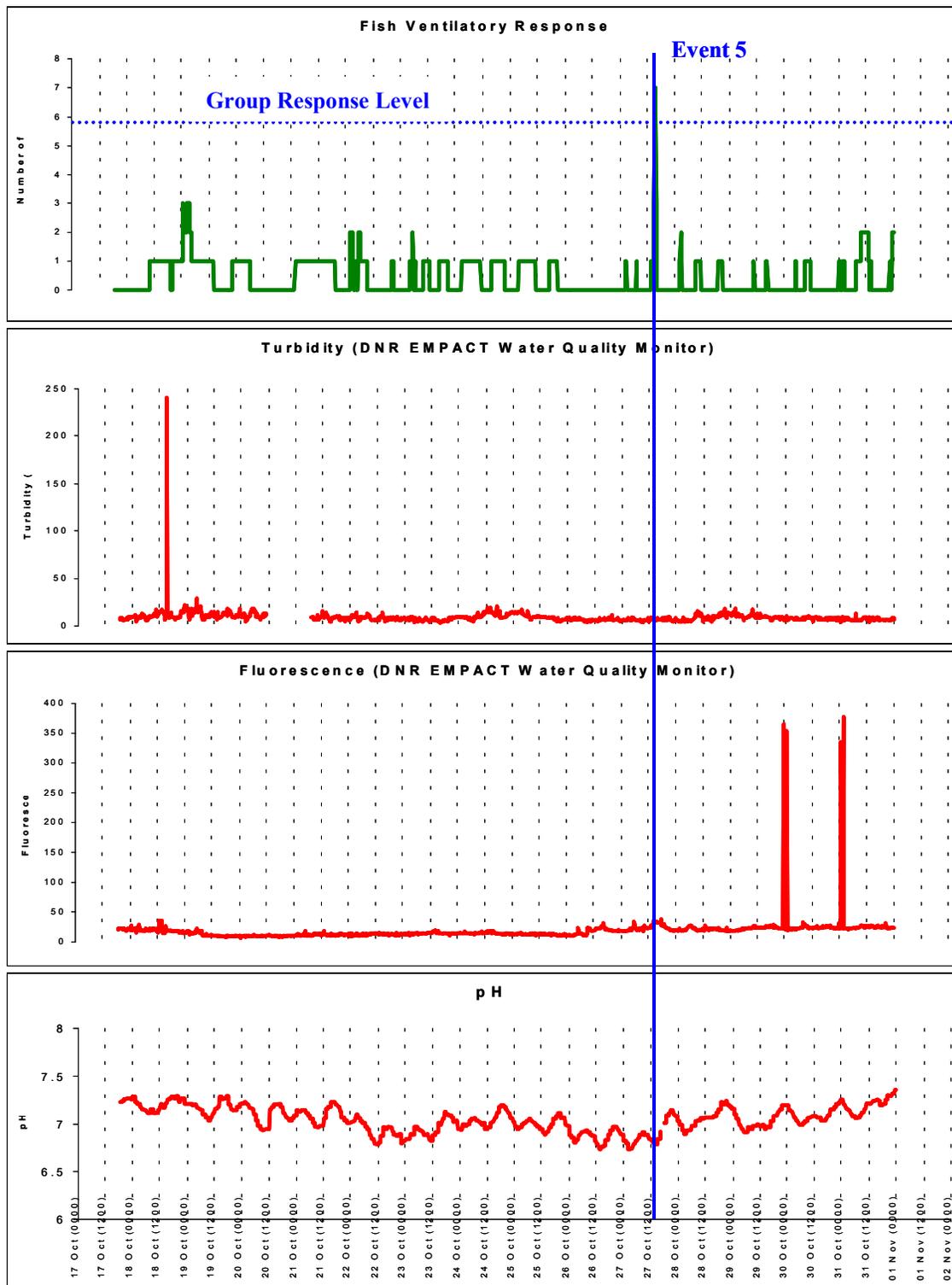
**Figure 3-13 (Continued). Fish ventilatory responses and water quality data during field deployment, October 17 to 31, 2000.**   b. Ventilatory responses with turbidity, fluorescence, and pH data. The brief fish response event characterized by increased cough and ventilation rates was associated with the use of unrinsed bag filters in the biomonitoring facility, which may have exposed the fish to contaminants.

caused by increased cough and ventilation rates, was for a 30-minute period on October 27. In this case, a clear cause can be identified. As shown in Table 3-3, one of the twice weekly maintenance activities was to wash the bag filters that remove particulate matter larger than 100 μm. New bag filters are always rinsed thoroughly, and technicians have noted a small amount of foaming at the initiation of the rinsing process. On October 27, the bag filters were installed without being rinsed. It is suspected that material leaching from the bags (possibly surfactants) caused the fish response, which subsided rapidly after the bags were placed in service.

3.2.2.3. *Discussion of Fish Ventilatory Responses to Water Quality Changes*

Substantial water quality variations were encountered during field testing, so it is important to ask how such changes affect bluegill ventilatory patterns. In this section, effects reported in the literature of changes in pH, turbidity, dissolved oxygen, and temperature on bluegill ventilatory patterns are evaluated. Given what is known from the literature and the associations observed in this field project, increased cough rate, observed in two of the three response events in this study with unknown causes, is much less likely to be associated with the changes in water quality variables found in the Chicamacomico River during the monitoring period than either ventilatory rate or depth.

It is unlikely that variations in pH influenced biomonitor response in the field evaluation. Carlson (1984) found that bluegill ventilatory and cough rates did not change significantly over the pH range encountered during the deployment on the Chicamacomico River. The potential effects of increased turbidity in this study are less clear. Carlson showed that ventilatory rates were unaffected by turbidity (from clay particles) up to 323 NTU and that cough rate was increased at 90 NTU but not 76 NTU. In this study, turbidity seldom exceeded 50 NTU, and this measure included all particulates, not just those below 100 μm in the filtered river water to which the fish were exposed. On the other hand, Carlson noted that the type, size, and shape of suspended particles may affect cough response. Clay particles used in his study were 2 μm in diameters, and Carlson believed larger particles might be more effective in eliciting a cough response. Although size distribution information on particulate matter from the Chicamacomico River is unavailable, a review of the field data (see Section 3.2.2.2) does not indicate an association between the occurrence of high turbidity and fish group responses.

Changes in temperature are known to affect bluegill ventilatory patterns. Heath (1973) evaluated the effect of increasing temperature (1.5 °C/h) on ventilatory rate and depth in bluegills. Over a temperature range approximating that encountered on the Chicamacomico River (15 to 30 °C, Table 3-4), ventilation rate and buccal and opercular pressure amplitudes (which should be proportional to ventilatory signal depth) increased by about the same amount (~2.5 fold). Consistent with this observation are data from control fish in a USACEHR test

conducted following procedures similar to the zinc test described in Appendix B, Section B.2.1. In this study, a heater malfunction caused the temperature to drop from 25 to 21 °C within a 15-minute period, where it remained for about an hour. During this period, the average control fish ventilatory rate and depth decreased substantially, causing a group response. However, cough rate was much less affected. An increase in average cough rate was within the range of variability observed during the baseline monitoring period, and only one of eight fish showed a cough response. Concerning the field data from the Chicamacomico River, temperature is most likely to cause responses in ventilatory rate and depth, especially when temperature variations exceed those experienced by the fish during their baseline monitoring period.

Although changes in temperature alone should result in fairly predictable changes in ventilatory rate and amplitude, bluegill response patterns to changing oxygen concentrations vary with temperature. Spitzer et al. (1969) reported changes in temperature-acclimated bluegill ventilation rate and amplitude when dissolved oxygen levels were reduced from saturation to about 10% of saturation over 8 hours at three temperatures (13, 25, and 30 °C). The rate of change in dissolved oxygen was about 1 mg/L/h at 25 °C, somewhat less than the maximum rate of change noted in Table 3-4. At 13 °C, ventilation rate was relatively constant to about 60% of saturation, then increased by about 50% to a maximum of about 19% of saturation, accompanied by large increases in ventilatory amplitude. At 25 °C, ventilation rate increased about 1.6 fold from saturation down to about 35% of saturation (~3 mg/L), then fell rapidly at lower oxygen levels, while ventilatory amplitude did not change. A pattern similar to that of 25 °C was seen at 30 °C, except that ventilation rate decreased slightly from saturation down to 35% of saturation, with ventilatory depth declining throughout. Although varying oxygen levels can affect ventilatory rate and depth, Carlson (1984) found coughing in bluegills to be unaffected over a wide range of dissolved oxygen concentrations (≤44 to 117% of saturation), as noted above.

3.2.2.4. *Summary*

Overall, the biomonitor operated very reliably throughout the monitoring period. No pfiesteria-related events were detected and no fish kills occurred on the Chicamacomico River during the monitoring period. Of the five group-response events observed, two (the first and third) were characterized by an increase in cough rate that could not be related to changes in the monitored water quality variables. While increases in turbidity and fluorescence showed some association with response events, similar increases occurred at other times without an associated fish response. The second event was caused by changes in ventilatory rate and depth, and also could not be linked to water quality changes. The fourth event, a change in ventilatory depth, was likely caused by changes in conductivity. The last event was most likely caused by contaminants originating from unrinsed filter bags.

4. FUTURE IMPROVEMENTS AND POTENTIAL APPLICATIONS

One of the goals of EMPACT projects is to identify ways to further implement and sustain time-relevant environmental monitoring technology. Based on the results of this project and related efforts, this section recommends follow-on activities (several of which have already been initiated) and describes possible future applications and clients.

4.1. RECOMMENDATIONS FOR SYSTEM IMPROVEMENTS

Specifically with regard to this EMPACT project, a number of key points for future improvement, evaluation, and use have been identified, and work is proceeding or planned in many of these areas.

•	Recommendation:	Improve the ability of the automated fish biomonitor to detect and identify toxic events while minimizing responses to normal variations in water quality parameters.
	Rationale:	Key approaches include testing with a range of toxic chemicals, development of an expert system, evaluating further diagnostic procedures to help establish the causes of observed group responses, and incorporation of toxicity verification procedures. Building on an extensive laboratory and field database, an expert system could help eliminate the need for a baseline monitoring period, factor out fish responses to normal water quality variations, and evaluate the use of fish responses to help identify causal factors. Baseline monitoring periods are problematic because if contaminants or excessive water quality variations are present, biomonitor sensitivity to perturbations during the subsequent on-line monitoring period may be adversely affected. It is possible that patterns of fish response may be useful for diagnostic purposes. Investigators have used ventilatory response parameters as part of classification systems in which changes in a limited number of key behavioral characteristics are used to help classify the mode of toxic action of a chemical (McKim et al., 1987a, b; Bradbury et al., 1989; Drummond and Russom, 1990; Bradbury et al., 1991; Rice et al., 1997; Russom et al., 1997). For example, Fish Acute Toxicity Syndromes use data from a limited number of physiological parameters (including cough and ventilatory-related

measurements) to differentiate among modes of toxic action for rainbow trout (*Onchorynchus mykiss*) exposed to a wide range of chemicals (McKim et al., 1987a). However, since Carlson (1990) found that changes in the ventilatory and cough rate of bluegills by themselves had only a limited ability to distinguish among chemicals with different modes of toxic action, it is likely that additional physiological parameters would have to be monitored. In any case, interpretation of responses to complex mixtures (versus single chemicals) could be difficult.

Coupling biomonitor responses with confirmatory toxicity tests could help eliminate "false alarms." Toxicity verification could be achieved by an aquatic toxicity test, perhaps using a rapid screening method (e.g., Toussaint et al., 1995; Shedd et al., 1999). If toxicity is confirmed, a Phase I Toxicity Identification Evaluation (TIE) (EPA, 1991) could be undertaken to identify the class of chemicals causing the abnormal behavior.

<u>Actions</u>:	• Conduct ventilatory biomonitor tests with chemicals having varying modes of toxic action, possibly including toxins from bloom-forming microalgae other than pfiesteria, such as red tide dinoflagellates and certain cyanobacteria, to define the sensitivity, rapidity, and pattern of biomonitor responses.

• Develop an expert system for use with the fish biomonitor. An expert system is under development through two USACEHR-initiated Small Business Incentive Research (SBIR) projects.

• Incorporate a change detection algorithm (Appendix B.2) that uses all the ventilatory response information and does not rely on a baseline monitoring period.

• When a biomonitor response is observed, develop a protocol to first evaluate whether it can be explained by water quality variations or other conditions unrelated to toxicity. If toxicity is suspected, establish follow-up procedures such as confirmatory toxicity tests and analytical evaluations.

-     Recommendation: Provide a commercially available fish automated biomonitor for use and evaluation.

  Rationale: One of the goals of EMPACT is to make time-relevant monitoring technology more broadly available. One way to do this is through commercialization.

  Action:
  - USACEHR and their commercial partner, GEO-CENTERS, Inc., have obtained a patent on the fish automated biomonitor and are evaluating opportunities for commercialization.

-     Recommendation: Integrate the fish biomonitor with other automated biomonitoring systems.

  Rationale: The use of more than one type of organism (i.e., clams and algae in addition to fish) can significantly improve system response because of the wide range of toxicant sensitivity often found between organisms of different trophic levels (State Environment Agency North Rhine-Westphalia, 1996). As noted by Kramer and Botterweg (1991) in their review of biological early warning (BEW) systems: "The most sensitive organism or monitoring system suitable for the detection of all possible toxic substances does not exist. Therefore it is recommended that when a wide range of toxicants needs to be detected, several BEW systems be incorporated, that are based on the response of different organisms."

  Actions:
  - A demonstration project is scheduled for initiation in the fall of 2001 to integrate the fish automated biomonitoring system with clam, daphnid, and algal biomonitors at a site on the Ohio River. Collaborators include individuals with EPA's National Exposure Research Laboratory, the National Risk Management Laboratory, and the Ohio River Valley Water Sanitation Commission (ORSANCO).
  - As a specific follow-on to this EMPACT project, an integration project has been proposed by the Maryland DNR and EPA's Chesapeake Bay

4-3

program to include chemical monitoring as well as fish and clam monitors from this and one other EMPACT project at a site on the Potomac River.

- Recommendation: Evaluate other types of biological sensors as components of an integrated platform.

  Rationale: Recent improvements in technology have greatly enhanced the capabilities for monitoring the physiological responses of cells and tissues. Such tissue-based biosensors may be able to provide real-time biological monitoring information without some of the logistical problems associated with maintaining higher organisms and, if engineered to respond to particular types of materials, could contribute to both detection and causal evaluation of observed responses. Biochemical sensors for specific enzymatic or metabolic activities also could be useful in specific circumstances.

  Action:
  - USACEHR has initiated discussions regarding collaboration with the Defense Advanced Research Projects Agency (DARPA) and the Naval Research Laboratory regarding field evaluation of promising candidate technologies from DARPA's tissue-based biosensor program.

This EMPACT research and development project has demonstrated both the strengths and limitations of the automated fish biomonitor. Through the actions outlined above, we believe the full potential of the biomonitor for environmental evaluation and assessment can be achieved.

4.2. FUTURE APPLICATIONS FOR AUTOMATED BIOMONITORING SYSTEMS

With further development and improvement, a possible follow-on application of this fish biomonitor is watershed monitoring. A network of automated biomonitoring units could provide state and local environmental managers with real-time information on watershed condition, as some have long advocated (e.g., Morgan et al., 1988). Although automated biomonitoring systems are important components of water quality monitoring networks in large river basins in Europe, such as the Rhine (State Environment Agency North Rhine-Westphalia, 1996; Gerhardt, 1999), this approach has not been used in the United States. A watershed-based network of automated biomonitoring systems (using fish as well as other species) combined with real-time chemical sensors, integrated with Geographical Information Systems (GIS) technology, could be

useful for managing both point and nonpoint contaminant sources. By locating biomonitoring platforms at key points on tributaries, managers can continuously track the nature and magnitude of pollutant discharges as they pass through a watershed. Specific applications of a watershed monitoring network could include:

- Detecting transient toxic events associated with chemical spills, harmful algal blooms, or precipitation events;
- Confirming major sources for pollutants and toxicity;
- Helping to prioritize control efforts in a watershed for implementation of best management practices (BMPs);
- Contributing to total maximum daily load (TMDL) development for nutrients and other pollutants (if appropriate chemical sensors are used); and
- Providing data to help evaluate long-term pollutant trends.

Clients for these watershed monitoring applications include regulatory agencies (state and federal) and watershed organizations (e.g., ORSANCO), but automated biomonitoring data also can be useful to the public. For example, a major advantage of a fish biomonitor installed at a Superfund site at Aberdeen Proving Ground has been the confidence it provided to the public that acutely toxic materials would not be discharged into the Chesapeake Bay (Shedd et al., 2001). There was considerable public interest in this EMPACT project as well (see Section 2.2), but careful data interpretation is needed to ensure that the information is understandable and meaningful to the public. Provision of automated biomonitoring data to the public is probably best implemented in conjunction with a local regulatory authority, as was done in this project with the DNR, or in cooperation with an academic institution.

Other potential applications for automated biomonitoring systems include source water protection for drinking water systems (part of the function of the European systems noted above), industrial effluent monitoring (Shedd et al., 2001), and aquaculture systems. In aquaculture systems, harmful water quality conditions may propagate rapidly, and automated biomonitoring system could provide rapid feedback on developing problems, potentially saving valuable resources. Achieving the full potential of automated biomonitoring systems will require the kind of additional research and development activities described in the preceding section.

REFERENCES

American Society for Testing and Materials (ASTM). (1995) Standard guide for ventilatory behavioral toxicology testing of freshwater fish. E 1768-95. West Conshohocken, PA: American Society for Testing and Materials.

Baldwin, IG; Kramer, KJM. (1994) Biological early warning systems (BEWS). In: Kramer, KJM, ed., Biomonitoring of Coastal Waters and Estuaries. Boca Raton, FL: CRC Press, Inc., pp. 1-25.

Baldwin, IG; Harman, MMI; Neville, DA. (1994) Performance characteristics of a fish monitor for detection of toxic substances. I. Laboratory tests. Water Res 28(10):2191-2199.

Biomonitoring Committee, Working Group of the Federal States on Water Problems. (1996) Recommendation on the deployment of continuous biomonitors for the monitoring of surface waters. Berlin, Germany: Geschaftstelle der Landerarbeitsgemeinschafft Wasser (LAWA).

Borcherding, J. (1994) The "Dreissena-Monitor" – improved evaluation of dynamic limits for the establishment of alarm-thresholds during toxicity tests and for continuous water control. In: Hill, IR; Heimbach, F, eds. Freshwater field tests for hazard assessment of chemicals. Boca Raton, FL: Lewis Publishers, pp. 477-484.

Borcherding, J; Jantz, B. (1997) Valve movement response of the mussel *Dreissena polymorpha* – the influence of pH and turbidity on the acute toxicity of pentachlorophenol under laboratory and field conditions.   Ecotoxicol 6:153-165.

Bowers HA; Tengs T; Glasgow HB, Jr; et al. (2000) Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl Environ Microbiol 66(11):4641-4648.

Bradbury, SP; Carlson, RW; Niemi, GJ; et al. (1991) Use of respiratory-cardiovascular responses of rainbow trout (*Oncorhynchus mykiss*) in identifying acute toxicity syndromes in fish: part 4. Central nervous system seizure agents. Environ Toxicol Chem 10:115-31.

Bradbury, SP; Henry, TR; Niemi, GJ; et al. (1989) Use of respiratory-cardiovascular responses of rainbow trout (*Salmo gairdneri*) in identifying acute toxicity syndromes in fish: part 3. Polar narcotics. Environ Toxicol Chem 8:247-61.

Cairns, J, Jr; Dickson, KL; Sparks, RE; et al. (1970) A preliminary report on rapid biological information systems for water pollution control. J Water Pollut Contr Fed 42:685-703.

Cairns, J, Jr; Mount, DI. (1990) Aquatic toxicology. Environ Sci Technol 24(2):154-161.

Cairns, J, Jr; van der Schalie, WH. (1980) Biological monitoring Part I: early warning systems. Water Res 14:1179-1196.

Carlson, RW. (1984) The influence of pH, dissolved oxygen, suspended solids, or dissolved solids upon ventilatory and cough frequencies in bluegill *Lepomis macrochirus* and brook trout *Salvelinus fontinalis*. Environ Pollut (Ser A): 34:149-169.

Carlson, RW. (1990) Ventilatory patterns of bluegill (*Lepomis macrochirus*) exposed to organic chemicals with different modes of toxic action. Comp Biochem Physiol 95C(2):181-196.

Civelek, AC; Villemagne, VL; Dannals, RF; et al. (1999) Assessment of changes in regional cerebral glucose metabolism by FDG PET in subjects exposed to Pfiesteria infected water. J Nuclear Medicine 40(5):454 Suppl.

Diamond J; Collins, M; Gruber, D. (1988) An overview of automated biomonitoring – past developments and future needs. In: Gruber DS; Diamond, JM. eds. Automated biomonitoring: living sensors as environmental monitors. Chichester, UK: Ellis Horwood Ltd., pp. 23-39.

Diamond, JM; Parson, MJ; Gruber, D. (1990) Rapid detection of sublethal toxicity using fish ventilatory behavior. Environ Toxicol Chem 9:3-11.

Drummond, RA; Russom CL. (1990) Behavioral toxicity syndromes: a promising tool for assessing toxicity mechanisms in juvenile fathead minnows. Environ Toxicol Chem 9:37-46.

Evans, GP; Briers, MG; Rawson, DM. (1986) Can biosensors help to protect drinking water? Biosensors 2:287-300.

Evans, GP; Wallwork, JF. (1988) The WRc fish biomonitor and other biomonitoring methods. In: Gruber, DS; Diamond, JM, eds. Automated biomonitoring: living sensors as environmental monitors. Chichester, UK: Ellis Horwood Ltd., pp. 75-90.

Geller, W. (1984) A toxicity warning monitor using weakly electric fish, Gnathonemus petrsii. Water Res 18(10): 1285-1290.

Gerhardt, A. (1999) Recent trends in online biomonitoring for water quality control. In: Gerhardt, A, ed. Biomonitoring of Polluted Water. Utikon-Zurich, Switzerland: Trans Tech Publications Ltd., pp. 95-118.

Gruber, D; Diamond, J; Johnson, D. (1989) Performance and validation of an online fish ventilatory early warning system. In: Suter, GW; Lewis, MA, eds. Aquatic toxicology and environmental fate: eleventh volume, ASTM STP 1007. Philadelphia, PA: American Society for Testing and Materials, pp. 215-230.

Hendriks, AJ; Stouten, MDA. (1993) Monitoring the response of microcontaminants by dynamic Daphnia magna and Leuciscus idus assays in the Rhine delta: biological early warning as a useful supplement.   Ecotoxicol Environ Safety 26: 265-279.

International Life Sciences Institute Risk Science Institute. (1999) Early Warning Monitoring to Detect Hazardous Events in Water Supplies. Washington, DC: International Life Sciences Institute Risk Science Institute.

Johnston, NAL; Campagna, VS; Hawkins, PR; et al. (1994) Response of the eastern rainbowfish (Melanotaenia duboulayi) to toxic Microcystis aeruginosa. Australian J Mar Freshwater Res 45:917-23.

Koeman, JH; Poels, CLM; Sloof, W. (1978) Continuous biomonitoring systems for detection of toxic levels of water pollutants. In: Lelyveld, IH; Zoeteman, BCJ, eds. Aquatic pollutants, transformation and biological effects. New York, NY: Pergamon Press, pp. 339-347.

Korver, RM; Sprague, JB. (1988) A real-time computerized video tracking system to monitor locomotor behavior. In: Gruber DS; Diamond, JM, eds. Automated biomonitoring: living sensors as environmental monitors. Chichester, UK: Ellis Horwood Ltd., pp. 157-171.

Kramer, KJM; Botterweg, J. (1991) Aquatic biological early warning systems: an overview. In: Jeffrey, DJ; Madden, B, eds. Bioindicators and environmental management. London, UK: Academic Press, pp. 95-126.

Marvin, DE; Heath, AG. (1968) Cardiac and respiratory responses to gradual hypoxia in three ecologically distinct species of fresh water fish. Comp Biochem Physiol 27:349-55.

McKim, JM; Schmieder, PK; Carlson, RW; et al. (1987a) Use of respiratory-cardiovascular responses of rainbow trout (*Salmo gairdneri*) in identifying acute toxicity syndromes in fish: part 1. pentachlorophenol, 2,4-dinitrophenol, tricaine methanesulfonate, and 1-octanol. Environ Toxicol Chem 6:295-312.

McKim, JM; Schmieder, PK; Niemi, GJ; et al. (1987b) Use of respiratory-cardiovascular responses of rainbow trout (*Salmo gairdneri*) in identifying acute toxicity syndromes in fish: part 2. malathion, carbaryl, acrolein, and benzaldehyde. Environ Toxicol Chem 6:313-28.

Moehl, JF; Davies, WD. (1993) Fishery intensification in small water bodies: A review of management and development of small water bodies for fisheries in North America. M.S. Thesis, Auburn University., Auburn, AL.

Morgan, WSG; Kuhn, PC; Allais, B; et. al. (1982) An appraisal of the performance of a continuous automatic fish biomonitoring system at an industrial site. Water Sci Technol 14:151-161.

Morgan, WSG; Kuhn, PC. (1984) Aspects of utilizing continuous fish biomonitoring systems for industrial effluent control. In: Freshwater Biological Monitoring: Proceedings of a Symposium. New York, NY: Pergamon Press, pp. 65-73

Morgan, EL; Young, RC; Wright, JR, Jr. (1988) Developing portable computer-automated biomonitoring for a regional water quality surveillance network. In: Gruber, D; Diamond, J, eds. Automated Biomonitoring. Chichester, UK: Ellis Horwood Ltd., pp. 127-141.

Morris, G J. (1999) Harmful algal blooms: An emerging public health problem with possible links to human stress on the environment. Ann Rev Energy Environ 24:367-90.

Musick, JA. (1972) Fishes of Chesapeake Bay and the adjacent coastal plain. In Waff, ML, ed., A Check List of the Biota of lower Chesapeake Bay. Special Scientific Report # 65. Gloucester Point, VA: Virginia Institute of Marine Sciences, pp. 175-212.

Oldach DW; Delwiche CF; Jakobsen KS; et al. (2000) Heteroduplex mobility assay-guided sequence discovery: elucidation of the small subunit (18S) rDNA sequences of *Pfiesteria piscicida* and related dinoflagellates from complex algal culture and environmental sample DNA pools. Proc Natl Acad Sci USA 97(8):4303-4308.

Poli, MA; Hewetson, JF. (1992) Antibody preparation and development of a radio-immunoassay for the PbTx-2-type brevetoxins. In: Tosteson, TR, ed. Proc of the Third Intl Conf on Ciguatera Quebec: Polyscience Publications, pp. 115-127.

Poli, MA; Rein, KS; Baden, DG. (1995) Radioimmunoassay for PbTx-2-type brevetoxins: epitope specificity of two anti-PbTx sera. J Assoc Off Analyt Chemists 78(2): 538-542.

Rice, PJ; Drewes, CD; Klubertanz, TM; et al. (1997) Acute toxicity and behavioral effects of chlorpyrifos, permethrin, phenol, strychnine, and 2,4-dinitrophenol to 30-day-old Japanese medaka (*Oryzias latipes*). Environ Toxicol Chem 16(4):696-704.

Russom, CL; Bradbury, SP; Broderius, SJ; et al. (1997) Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 16(5):948-67.

Scharf, BW. (1979) A fish test alarm device for the continual recording of acute toxic substances in water. Arch Hydrobiol 85:250-256.

Shedd, TR; Widder, MW; Toussaint, MW; et al. (1999) Evaluation of the annual killifish (*Nothobranchius guentheri*) as a tool for rapid acute toxicity screening. Environ Toxicol Chem 18(10):2258-2261.

Shedd, TR; van der Schalie, WH; Widder, MW; et al. (2001) Long-term operation of an automated fish biomonitoring system for continuous effluent acute toxicity surveillance. Bull Environ Contam Toxicol 66:392-399.

Smith, EH; Bailey, HC. (1988) Development of a system for continuous biomonitoring of a domestic water source for early warning of contaminants. In: Gruber, DS; Diamond, JM, eds. Automated biomonitoring: living sensors as environmental monitors. Chichester, UK: Ellis Horwood Ltd., pp. 157-171.

Sokoloff, L. (1977) The 14C deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anaesthetized rat. J Neurochem 28:897-916.

State Environment Agency North Rhine-Westphalia. (1996) Water Quality Monitoring in North Rhine-Westphalia. Essen, Germany: Landesumweltamt NRW.

Steinberg, CEW; Lorenz, R; Speiser, OH. (1995) Effects of atrazine on the swimming behavior of zebrafish, Brachydanio rerio. Water Res 29(3):981-985.

Swingle, WE; Shell, EW. (1971) Tables for computing relative conditions of some freshwater fishes. Auburn, AL: Auburn University Agricultural Experiment Station Circular No. 183.

Tieman, DM; Burton, DT. (1997) Continuous Acute Toxicity Biomonitoring of Aberdeen Proving Ground-Edgewood Area Old O-Field Groundwater Treatment Facility Effluent. University of Maryland at College Park, Agricultural Experiment Station, Wye Research and Education Center, Queenstown, MD. Contract DAMD17-92-C-2066.

Toussaint, MW; Shedd, TR; van der Schalie, WH; et al. (1995) A comparison of the sensitivity of rapid toxicity screening tests. Environ Toxiocol Chem 14: 907-915.

U.S. Environmental Protection Agency. (1991) Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures. Environmental Research Laboratory, United States Environmental Protection Agency, Duluth, MN: EPA/600/6-91/003.

U.S. Environmental Protection Agency. (2001) Environmental Monitoring for Public Access and Community Tracking (EMPACT): frequently asked questions. http://www.epa.gov/empact/faqs.htm

van der Schalie, WH; Shedd, TR; Zeeman, MG. (1988) Ventilatory and movement responses of rainbow trout exposed to 1,3,5-trinitrobenzene in an automated biomonitoring system. In: Gruber DS; Diamond, JM, eds. Automated biomonitoring: living sensors as environmental monitors. Chichester, UK: Ellis Horwood Ltd., pp. 67-74.

van der Schalie, WH; Dickson, KL; et al. (1979) Fish bioassay monitoring of waste effluents. Environ Manag 3(3):217-35.

van der Schalie, WH; Shedd, TR; et al. (2001) Using higher organisms in biological early warning systems for real-time toxicity detection. Biosensors & Bioelectronics 16:457-465.

Vlcková, B; Gu, XJ; Moskovits, M. (1997) SERS Excitation Profiles of Phthalazine Adsorbed on Single Colloidal Silver Aggregates as a Function of Cluster Size. J Phys Chem B, 101 (9):1588-1593.

Vogl, C; Grillitsch, B; Wytek, R; et al. (1999) Qualification of spontaneous undirected locomotor behavior of fish for sublethal toxicity testing –Part I–variability of measurement parameters under general test conditions. Environ Toxicol Chem 18(12):2736-2742.

Waller, WT; Acevedo, MF; Allen, HJ; Schwalm, FU. (1996) The Use of Remotely Sensed Bioelectric Action Potentials to Evaluate Episodic Toxicity Events and Ambient Toxicity. College Station, TX: Texas Water Resources Institute, Texas A&M University.

Wallwork, JF; Ellison, G. (1983) Automated sensor systems for water resource pollution warning and treatment process control. Aqua 6:313-20.

Wulliman, MF; Rupp, B; Reichert, H. (1996) Neuroanatomy of the Zebrafish Brain. Basel, Switzerland: Birkhauser Verlag.

APPENDIX A. PRELIMINARY SYSTEM FIELD EVALUATION
AND DEVELOPMENT

During the summer of 1999, the mobile biomonitoring facility, housing the automated biomonitoring system, was located on the Transquaking River near its junction with the Chicamacomico River in the Fishing Bay Wildlife Management Area on the Eastern Shore of Maryland, near the town of Bestpitch (Figure 2-1). The purpose of this deployment was to adapt the biomonitoring system for operation in an estuarine system. The biomonitoring facility was on-site and manned for 49 days, from July 29 through September 15. Although less than daily maintenance of the system was anticipated, water flow difficulties necessitated daily activity at the site. Ventilatory monitoring began on August 4 and lasted throughout the tenure at the site. Two hurricanes (Dennis and Floyd) impacted the Chesapeake Bay during the deployment period. Anticipated flooding associated with Floyd required field operations to be terminated.

One key challenge was the degree of variation in several water quality parameters (Table A-1). Temperature and conductivity varied with the tidal cycle and with storm events, as did dissolved oxygen, which frequently approached acutely toxic levels for bluegills. In addition, due to a prolonged drought, conductivity levels were much higher than normal, which became an issue for the bluegills initially used in the biomonitoring system. As a result of these water quality issues, the suitability of several alternative fish species were evaluated for use in the estuarine ventilatory monitoring system. Similarly, the electrodes and amplifiers used to monitor fish ventilatory patterns for operation in saline water were modified, and the water delivery system was changed to reduce clogging associated with excessive particulate matter in the water.

Table A-1. Ranges of selected water quality parameters during deployment on the Transquaking River.

Parameter	Minimum	Maximum	Maximum Rate of Change (/h)
Temperature (°C)	19.8	31.5	0.2
pH	6.6	7.6	-
Specific Conductivity (mS/cm)	13.9	27.0	0.3
Dissolved Oxygen (mg/L)	1.0	7.3	0.4

A.1. ADAPTATION OF FISH ELECTRODES AND AMPLIFIERS FOR OPERATION IN HIGH CONDUCTIVITY WATER

Since the conductivity variations characteristic of estuarine waters cause variations in the strength of the electrical signal generated by fish ventilatory movements, the fish monitoring

electrodes and amplifiers were modified to compensate for changing salinities. New graphite electrodes were developed to replace the stainless steel electrodes used in freshwater. Graphite material reduces the noise and signal instability associated with stainless steel electrodes at higher specific conductivities (above 4.7 mS/cm).

Initial studies demonstrated the greater efficacy of the graphite electrodes at high conductivity, and accurate signal recognition and stability were verified up to a specific conductivity of 40.9 mS/cm. Further studies evaluated signal attenuation with increasing conductivity. An attenuation of a simulated fish signal over a specific conductivity range of 0-34.0 mS/cm was used to determine a common equation of attenuation given input signals of various amplitudes (Figure A-1). A compensation equation was developed and the accuracy was compared to measured attenuation values, again at several initial amplitudes (Figure A-2). Variable gain compensation circuitry was designed and the signal accuracy of the system was verified with a simulated fish signal up to 19.5 mS/cm, the upper level of the conductivity probe used. The system was installed in the biomonitoring facility, and signal accuracy for bluegills was confirmed up to a specific conductivity of 27.0 mS/cm, meaning that accurate operation of the amplifiers could be expected for bluegills held in water with salinities of equal or lower conductivities. As it turned out, water conductivity at the Chicamacomico River site during the summer of 2000 was below the level requiring variable gain amplifiers, so only the graphite electrodes were used. Because of their greater versatility, graphite electrodes are now recommended instead of stainless steel electrodes in both freshwater and estuarine systems.

The ventilatory monitoring system was further adapted to estuarine use by replacing a pulsed water flow system with a manifold that provided a continuous flow of water. Although the pulsed water delivery system works well in freshwater, increased electrical noise was associated with the fish ventilatory signals at the beginning of each water pulse in estuarine water. Continuous water flow eliminated the additional electrical interference.

A.2. FISH SPECIES SELECTION

Since the automated biomonitoring system had been developed using bluegills (e.g., Shedd et al., 2001), bluegills were the first choice for use in this EMPACT project. Bluegills are found in the estuarine streams being monitored and are reported to occur in salinities up to 18 ppt (~29.2 mS/cm) in the Chesapeake Bay region (Musik, 1972). Bluegills were considered an acceptable model for use in the Transquaking and Chicamacomico Rivers, since the specific conductivity range in the field was estimated to be 10.0-17.0 mS/cm, based on historic records and readings taken 2 months prior to deployment at the field site. In addition, bluegills acclimated and held in well water at the USACEHR aquaculture facilities for 3 months at 25 ºC
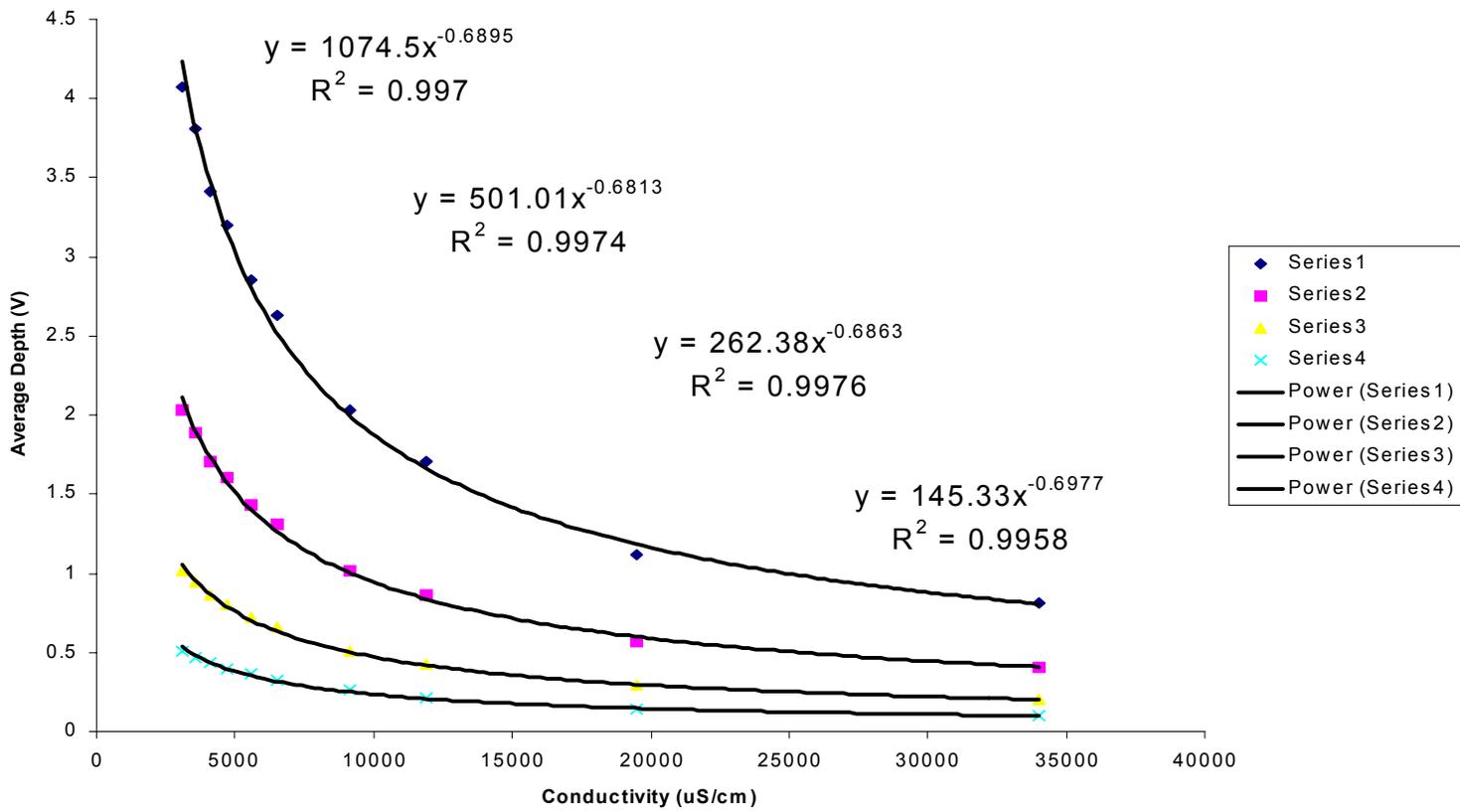
with a conductivity raised to 25.0 mS/cm using artificial sea salts (Instant Ocean™) showed no
apparent adverse effects.

**Figure A-1. Attenuation of voltage levels in a simulated fish signal
with increasing conductivity.**
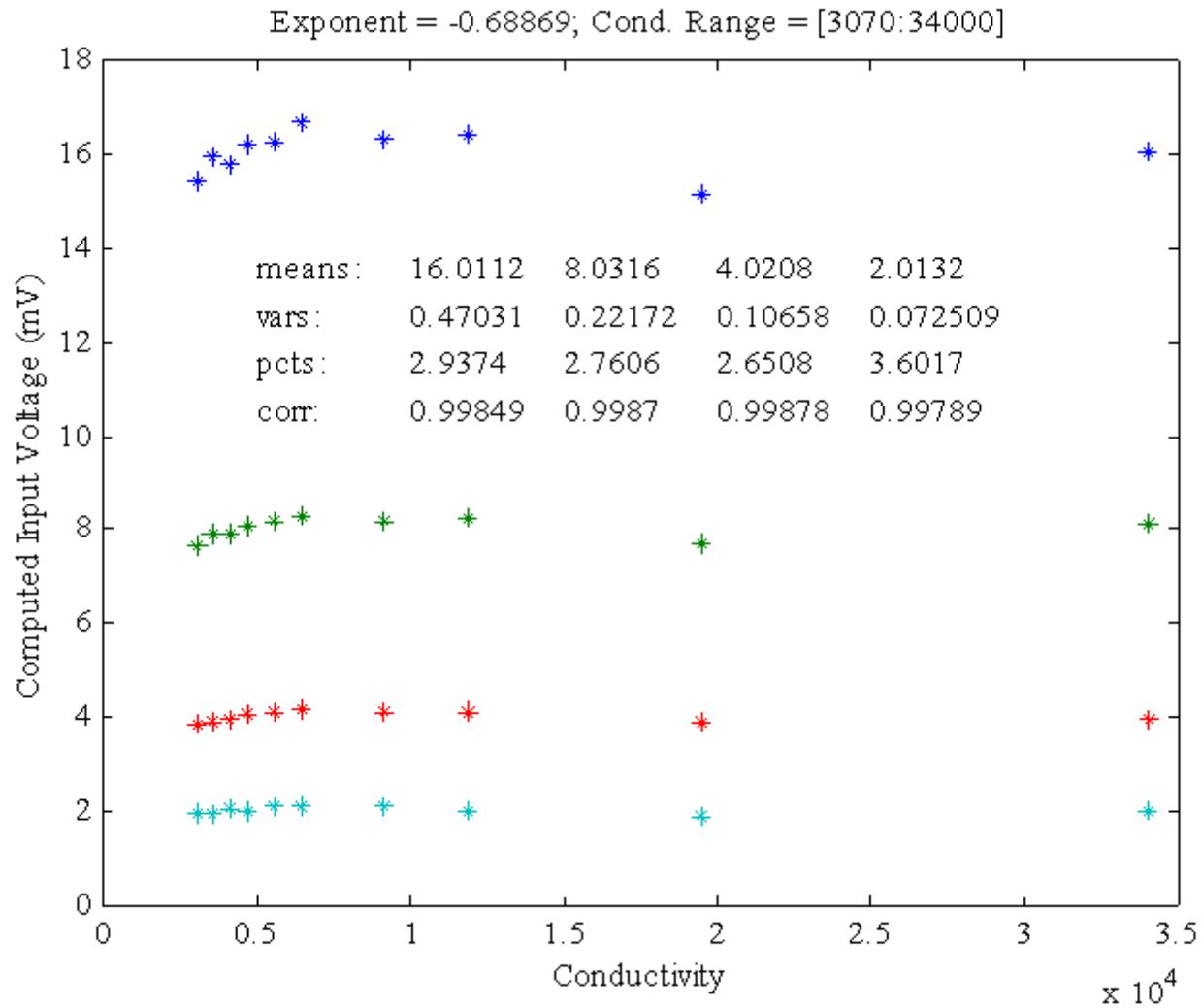
Figure A-2. Compensation for changing conductivity by a variable gain circuit.

Excessively low dissolved oxygen levels (as low as 1 mg/L, Table A-1) at the monitoring site on the Transquaking River made environmental conditions unsuitable for bluegill survival. Dissolved oxygen concentrations of 1.0 to 1.5 mg/L are reported to be lethal to bluegills at 25 °C (Marvin and Heath, 1968). During one 9-day period, there were numerous mortalities of bluegills in the monitoring cells associated with low dissolved oxygen in ambient water (< 1 mg/L) compounded by low water flows to the monitoring cells because of sediment-related blockages in water distribution-lines. High water temperatures also may have contributed to stress on the fish (Table A-1).

As a result of the difficulties encountered with using bluegills, other fish species were collected from local waters and evaluated for their suitability in the ventilatory monitoring system. White perch (*Morone americanus),* mummichog (*Fundulus heteroclitus*), and Atlantic menhaden (*Brevoortia tyrannus*) were collected in a range of sizes suitable for evaluation in the ventilatory cells. Initial evaluations demonstrated that ventilatory signals could be acquired for all three alternative species. Menhaden provided signal strengths comparable to bluegills, but they did not survive long enough to establish long-term movement rates in the ventilatory cells. The menhaden could not adjust to the confined ventilatory cells and continuously swam into the end of the cell. The white perch and mummichog survived well in the cells, but their ventilatory signal amplitudes dropped below detection levels once the fish had acclimated to the ventilatory cells. To compensate, signal strength had to be increased by coupling two ventilatory amplifiers together. Although field observations indicated that mummichog could survive at low dissolved oxygen/high temperature combinations that proved lethal to bluegills, their ventilatory signals had high variability and movement when compared to bluegill data. White perch were similar to mummichog, but their ventilatory signal strength was lower and movement higher. Given their superior overall ventilatory signal, bluegills were retained as the species of choice for the automated biomonitoring system. Water delivery system modifications to prevent bluegill mortality due to low dissolved oxygen levels are described in the next section.

A.3. WATER DELIVERY SYSTEM

In 1999, the Transquaking River had very high suspended solid levels most of the time, and the problem was made worse by boat traffic from a nearby boat ramp that continually stirred up bottom sediments. As a consequence, there was a great deal of sediment buildup in the water delivery system leading up to the biomonitoring facility and in the facility itself. Plant material in the water initially clogged pump water intakes and the large particulate filtration system in the biomonitoring facility. There also was a great deal of sediment buildup in the tubing and holding tanks inside the biomonitoring facility. Reduced or blocked water flows resulting from sediment

buildup caused inadequate water flow through the ventilatory cells, lowered dissolved oxygen, and fish mortality.

In 2000, the biomonitor was relocated to a site on the Chicamacomico River. To reduce the water delivery problems encountered during 1999, several modifications were made to the system. An integrated self-priming water intake distribution pump was installed with manual valve control to facilitate regular back flushing and purging of the dual water intake system in the river. The particulate filtration system was redesigned with redundant architecture to allow for filter maintenance while maintaining continuous water flow. The first stage of filtration consisted of dual Hayward Simplex Basket Strainers (0.79 mm pore size), followed by three parallel Kestone Bag Filters (100 μm pore size). This system removed system-clogging particulates while maintaining water flow. Twice-weekly cleaning prevented excessive particulate buildup in the filters.

To prevent potentially lethal, low, dissolved oxygen levels, an aeration system was included to provide the fish with a mixture of 50% aerated river water and 50% ambient river water, if the ambient river water dropped below 3 mg/L dissolved oxygen. The system was designed to prevent fish mortality and ventilatory responses due solely to low dissolved oxygen levels, while retaining the ability to detect toxic substances that would otherwise be masked. In this system, river water flow was directed both to the fish monitoring cells and to an aerated reservoir tank. If the dissolved oxygen level in the water flowing from the ventilatory cells exceeded 3 mg/L, water from the aerated reservoir flowed only to the culture tanks holding bluegills for future use in the ventilatory system. When water exiting the ventilatory cells dropped below 3 mg/L, an electronic controller automatically opened a series of solenoid valves, which resulted in a 50% ambient water plus 50% aerated reservoir water mixture to flow to the ventilatory cells. In this way, water reaching the ventilatory cells could never drop below 50% of saturation during a low dissolved oxygen event in the river water. Although this system was available for use at the Chicamacomico River site in 2000, it was actually used only once (for less than 12 hours) because of the higher oxygen levels at this site.

# APPENDIX B. SYSTEM COMPONENTS UNDER DEVELOPMENT

Although the primary goal of this project was to develop and demonstrate the operation of the automated biomonitoring system, research was also conducted to develop improved water quality sensors to complement the system. While prototype dissolved oxygen and nutrient sensors (see Section B.1) could not be completed in time for use with the automated biomonitor, the design, testing, and potential utility of these sensors is described here. Another promising area for improvement was ventilatory signal data processing. A change detection algorithm for improving the current monitoring system's baseline/exposure period approach to fish group- response detection (see Section B.2) showed significant promise for incorporation into future biomonitoring applications.

B.1. WATER QUALITY SENSORS

Rapid fouling was a major problem for the dissolved oxygen probes used for this EMPACT project. A new dissolved oxygen probe based on an optical sensor was evaluated that had the potential for significant reductions in fouling. The experimental dissolved oxygen sensor fielded was based on an optical sensor developed for use in air. The sensor was encased in a potting material to protect the sensor electronics from contact with the ambient water. The sensor appeared to work properly until the potting seal failed. A second sensor was also potted but the seal failed before the unit could be fielded. Although initial sensors could not be effectively protected from water damage, the limited deployment showed that the measurement technology is feasible. Figure B-1 shows the performance of the in-air sensor. Notice that the sensor is temperature dependent, especially at low-oxygen concentrations.

Based on the limited success of the sensor, a new design was developed specifically for aquatic deployment (Figure B-2). A glass prism is used as the substrate for ruthenium-doped silicon which also could include a doping agent to retard the growth of fouling organisms. The blue LED light source and the photodiode detector are placed on the opposing sides of the prism. The entire unit can then be potted, leaving only the ruthenium side of the prism exposed to water. In the adapted unit that was fielded, the photodiode detector was exposed and proved to be the point of failure for the potting. The new design obviates this problem. The Johns Hopkins University Applied Physics Laboratory (JHU/APL) is continuing development of this dissolved oxygen sensing methodology.

JHU/APL also evaluated the use of a Fourier-transform surface enhanced Raman spectrometer system (FT-SERS) for measuring nutrients (inorganic phosphorus) at high sensitivities (detection levels as low as 100 µg/L). Figure B-3 shows surface-enhanced Raman

Temperature Dependence
Sensor #9
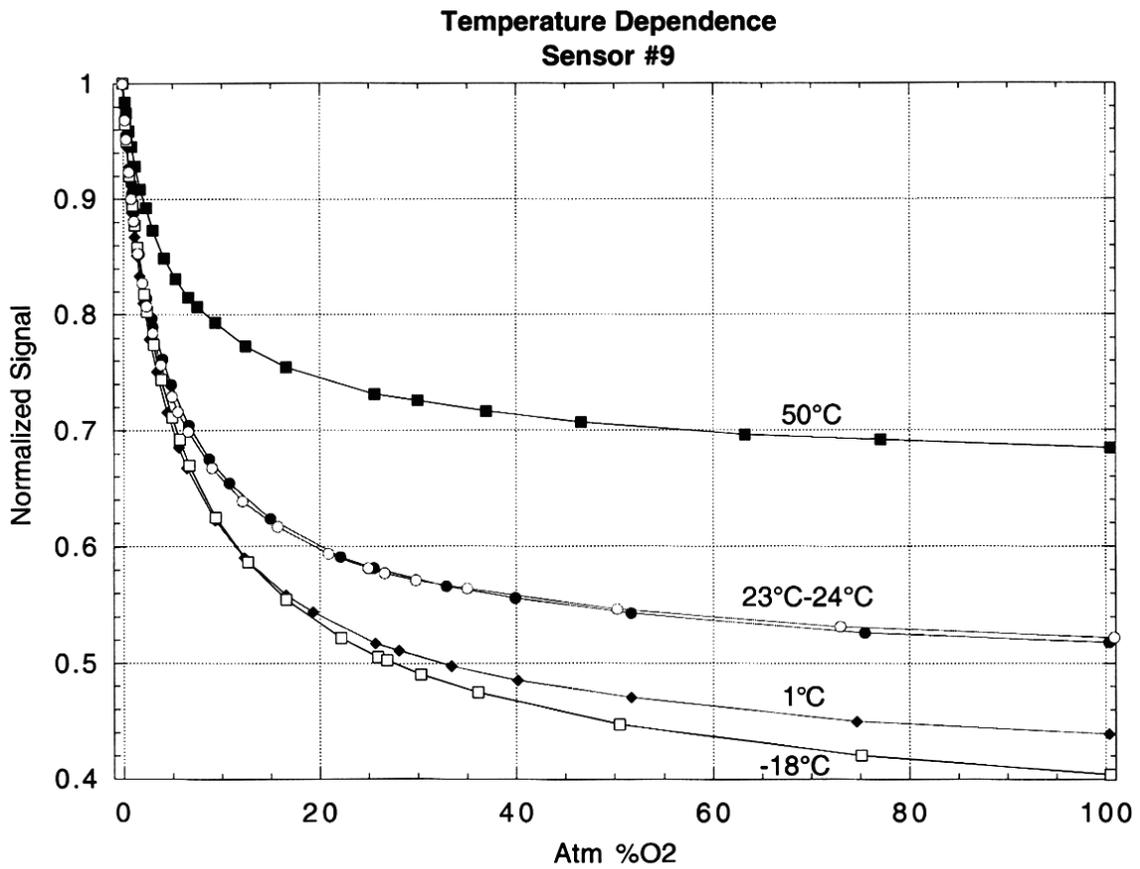
spectra (SERS) of phosphate taken with the blue laser and using silver colloid for the surface enhanced effect. The Raman peak at about 1000 wave numbers is clearly evident. The inset

Figure B-1. Ruthenium-based oxygen sensor output curves as a function of percent oxygen content and temperature.
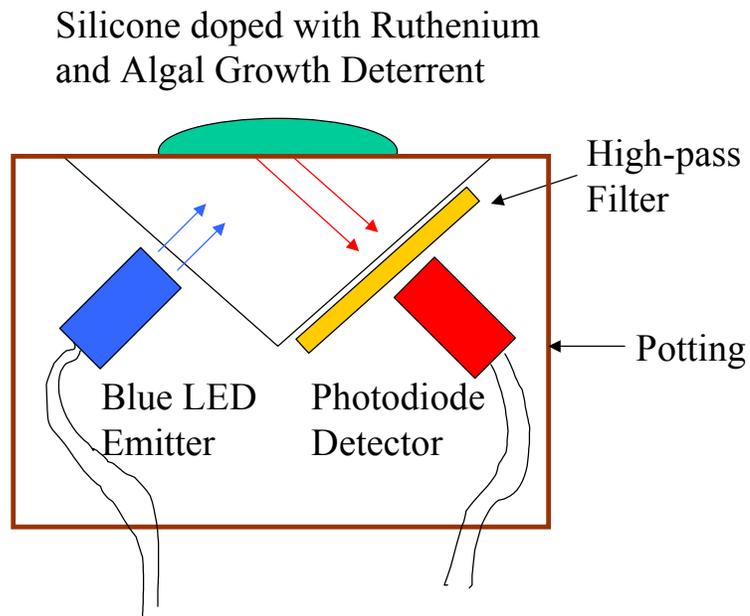
**Figure B-2. Ruthenium-based oxygen sensor designed for aquatic deployment.**
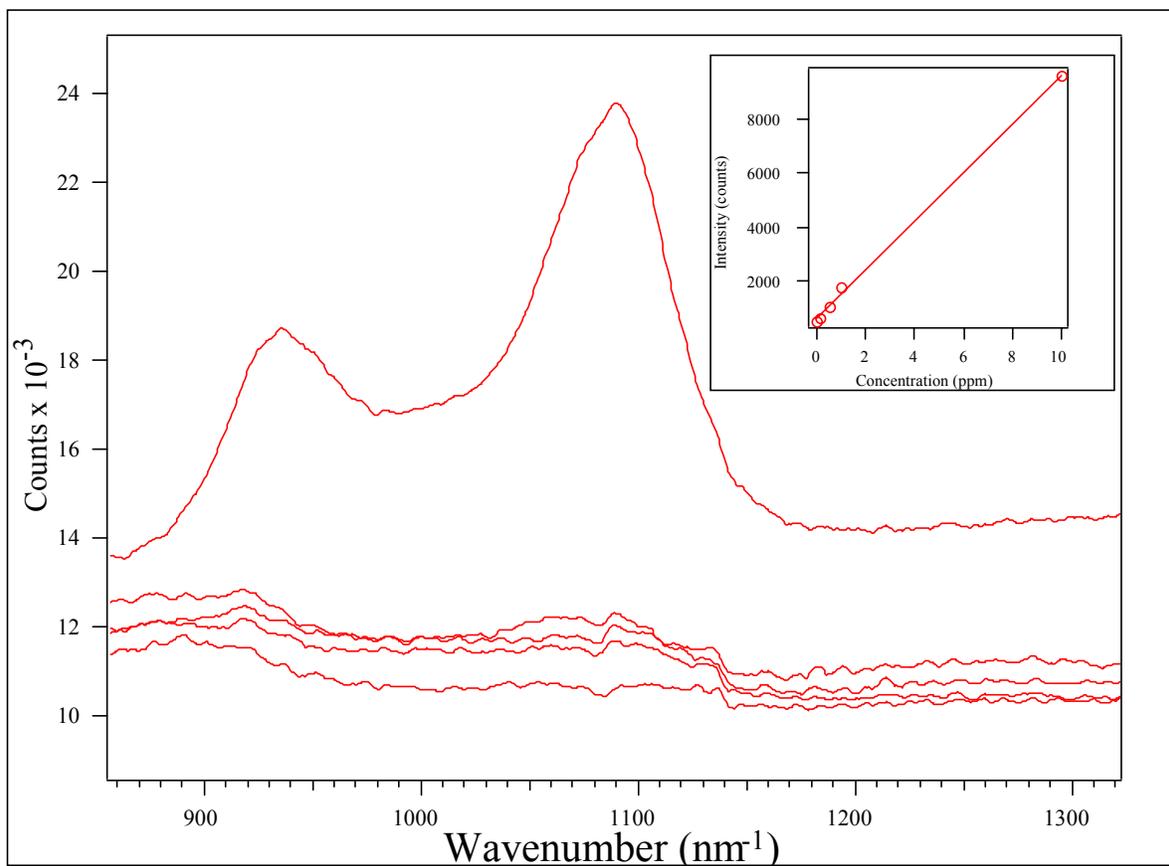
Figure B-3. Surface-enhanced Raman spectra of phosphate. The peak of the Raman spectrum for phosphate is ~1000 wavenumbers. The uppermost curve is 10 mg/L. The remaining curves are 1 mg/L, 500 µg/L, 100 µg/L, and the reference blank (lowest curve). The inset shows the conversion from counts to concentration.

shows the conversion from counts to concentration. The low-end concentrations down to at least the intended detection limit of 100 µg/L are clearly visible. Figure B-4 shows a second set of Raman spectra where we have replicated the Raman spectra for a 1 mg/L phosphate sample. These results demonstrate the repeatability of the Raman spectra obtained. Note that this conversion curve was based only on peak height. In practice, the area under the peak would be integrated to improve the detection limit. However, using the blue laser produces very broad peaks. Figure B-5 shows the change in peak character moving from blue to red laser sources. Using a red laser source results in stronger, narrower peaks that should further enhance our capability to lower detection limits. The laser used in the laboratory tests had a wavelength of 512 nm. While suitable for showing proof of the SERS approach, this wavelength would stimulate considerable fluorescence from the natural waters in the band where the Raman signal would occur. The next version of this system will have a laser with a wavelength of 830 nm, which should result in narrower, more distinct peaks. An associated system developed to deliver water samples to the SERS probe and then wash the probe after each measurement shows promise for adaptation to the automatic cleaning of water quality sensors. This should noticeably improve performance in future deployments.

B.2. VENTILATORY DATA ANALYSIS

The present approach to analysis of the ventilatory data, accumulates data for each of the parameters (ventilatory rate and depth and cough rate) for each of the eight fish for a 4-day baseline period (following a 3-day acclimation period). The mean and standard deviations are then calculated. During the subsequent 2-week monitoring period, conditions are deemed normal as long as the current values remain within a fixed number of standard deviations of the established baseline means. An alarm condition is said to exist when 70% of the fish (six or more of eight) exceed the baseline envelop.

This methodology was developed for periods when a controlled baseline could be established. However, in waterways like tidal streams, there is significant diurnal and tidal variation in water quality parameters such as temperature, dissolved oxygen, and conductivity that can cause large variations in the ventilatory parameters during the baseline period. This leads to wide tolerance boundaries that reduce sensitivity to adverse changes in water quality. Additionally, long-term trends in water quality parameters (e.g., decreasing water temperature in the fall) could, near the end of a monitoring period, drive the fish out of the previously established boundaries. The object of this ventilatory data analysis project was to develop a processing methodology that did not rely upon a baseline period but that could reliably detect significant changes in fish ventilatory behavior.
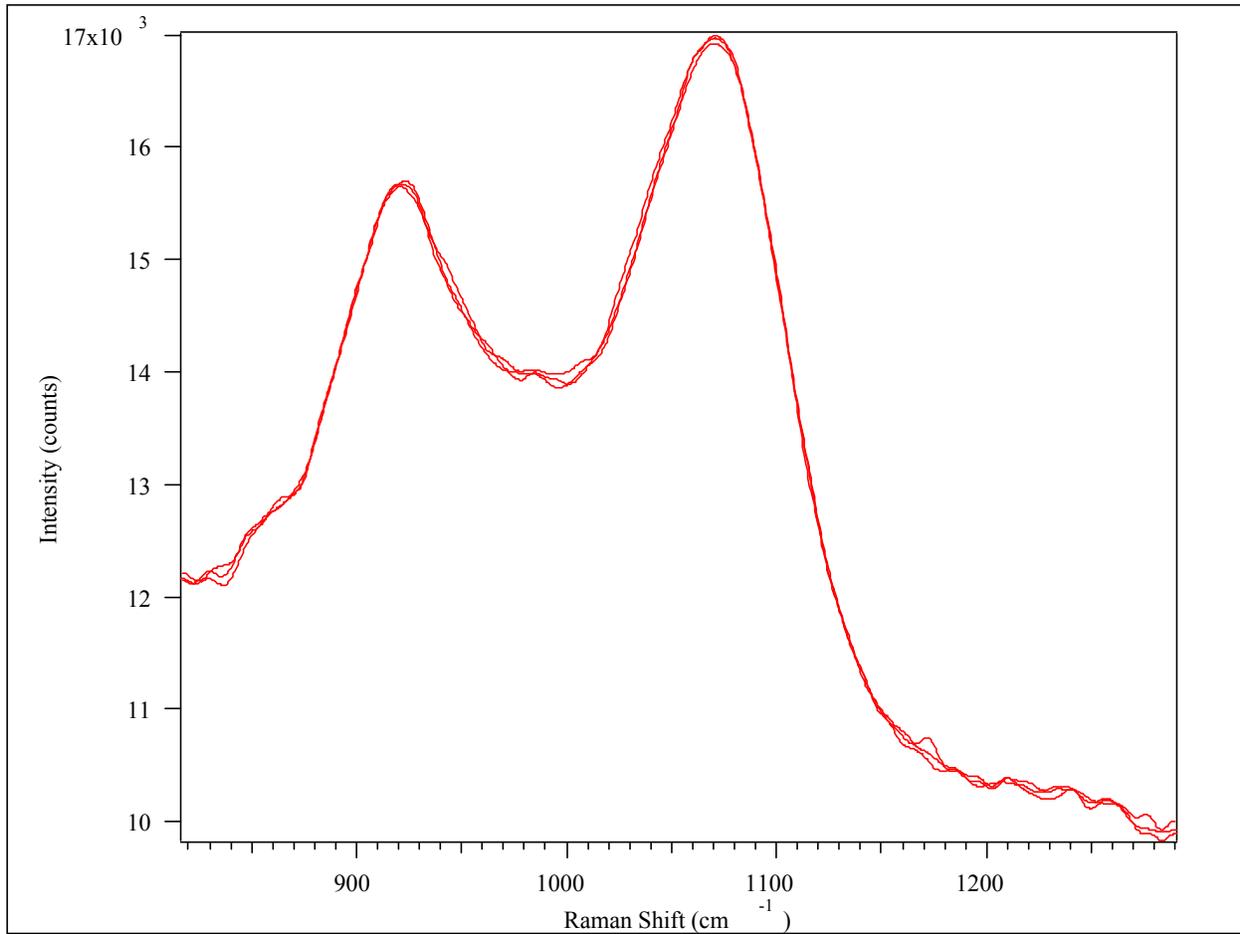
The methodology developed uses statistical tests that measure the change in position of each fish in ventilatory parameter space and tests whether or not that change in position from one

Figure B-4. Replicate RAMAN spectra (1 mg/L phosphate).
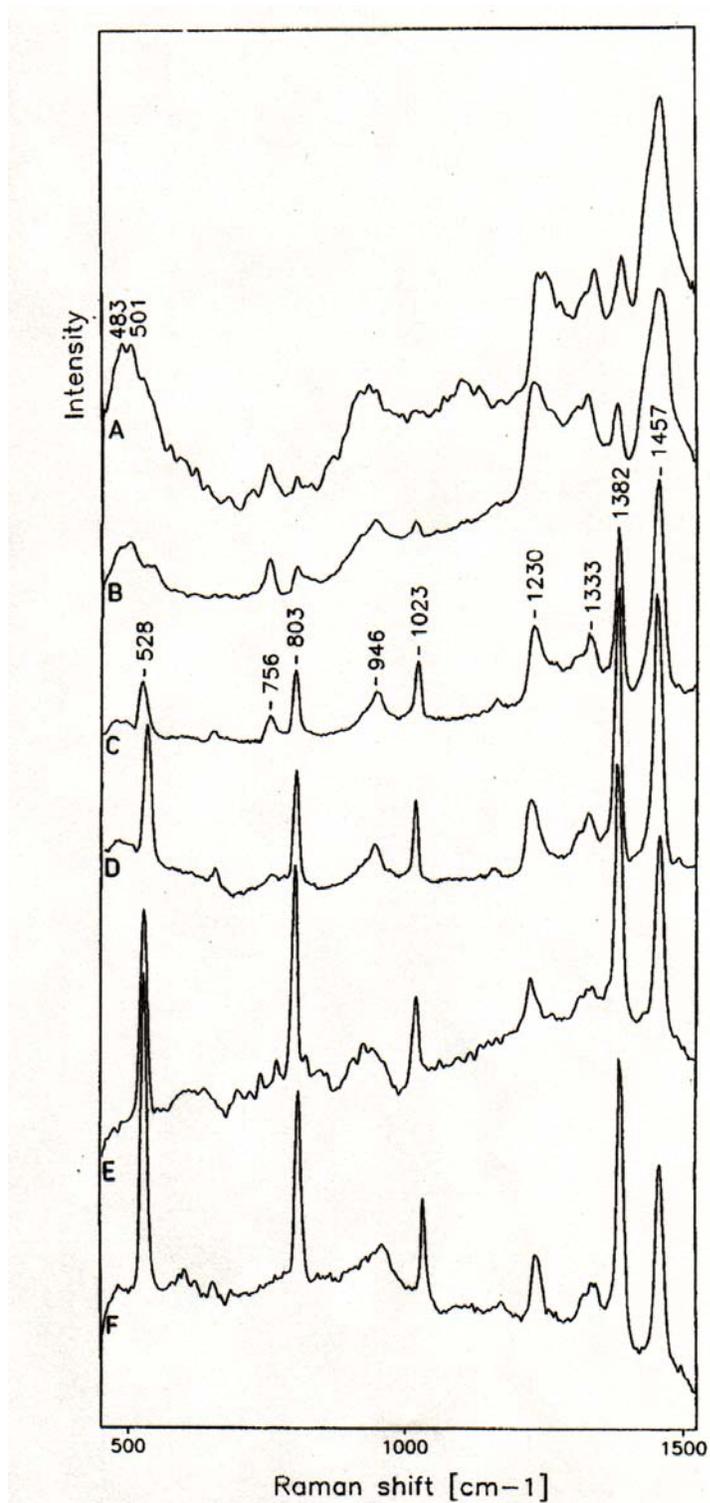
Figure B-5. Raman spectra of phosphate excited with different wavelengths of laser light.
(A) 468.0, (B) 476.2, (C) 530.9, (D) 568.2, (E) 632.8, and (F) 647.1 nm laser light.

Source: Vlcková et al., 1997.

time period to the next is statistically significant. The approach was first refined and tested using laboratory time-to-response studies to verify that the approach was effective. The refined methodology was then applied to data from the EMPACT deployment. In Section B.2.1 the methodology development is described and used as a ground truth against zinc time-to-response studies. In Section B.2.2 the results of applying the methodology to the Chicamacomico River field data are presented.

B.2.1. Methodology Development and Zinc Time-to-Response Study

Data for evaluation by the new analysis techniques were provided from a time-to-response study with zinc. The study was conducted to characterize the response time of bluegills in the ventilatory monitoring system to acutely toxic levels of zinc. Groups of eight fish were exposed for 96 hours to zinc at three concentrations: ~10%, 40%, and 100% of the 96h LC50, which was 4.5 mg/L for the fish and dilution water used in this study. The zinc ventilatory study was conducted in fresh water under laboratory test conditions following the general testing and analysis procedures described in Section 2.1, except that there were seven fish (rather than eight) at the high-and low-zinc concentrations. Water quality conditions included: temperature at $25 \pm 1\,°C$, alkalinity at 120-134 mg/L as CaCO3, hardness at 176-188 mg/L as $CaCO_3$, pH at 7.3 ± 0.5, and dissolved oxygen at > 75% of saturation. Test results using conventional ventilatory data analysis are summarized in the table below.

Table B-1. Time to response for bluegills exposed to acutely toxic concentrations of zinc.

Zinc (mg/L)	Zinc (% of 96-h LC50)	Time to First Response (h)	Earliest Mortality(h)
4.38	97	0.75	55
1.66	37	1.25	none
0.51	11	none	none
0 (control)	0	none	none

Data from fish exposed to the highest zinc concentration in this ventilatory study were plotted in feature space (an area in space that is defined by the N variables which, therefore, occupies an area in N space) with each of the three ventilatory parameters as a dimension (Figure B-6). In Figure B-6a, there is only one cluster, while in Figure B-6b, the effect of the zinc exposure is just being felt and there are two clusters. To develop the plots, the data were
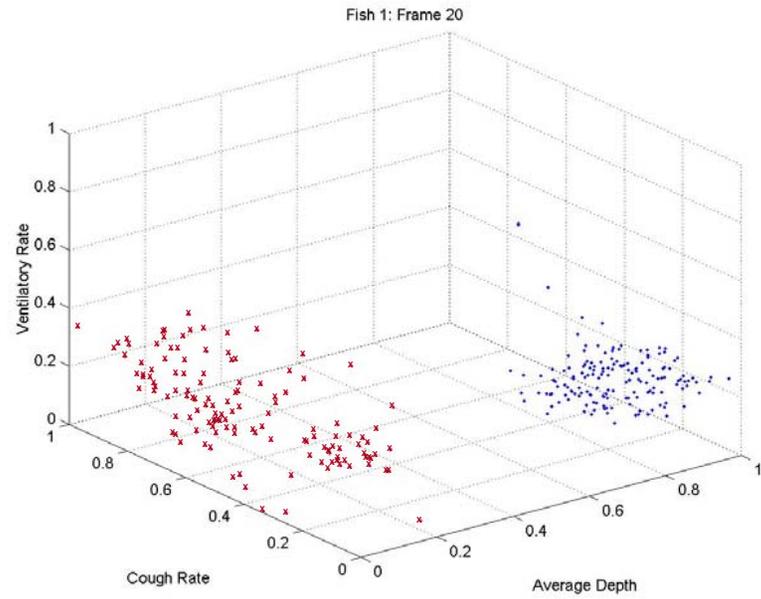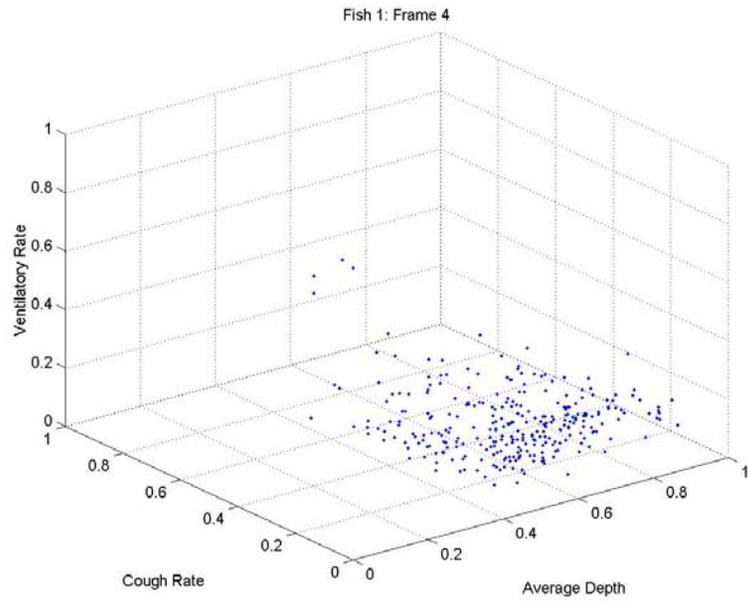
Figure B-6. Clustered ventilatory data from the zinc test, high concentration. a. Data at the beginning of zinc exposure. b. Data after exposure to zinc.

subdivided into 24-hour segments that "slid" one hour (Figure B-6). Thus, the first segment contained hours 1 to 24, while the second segment contained hours 2 to 25. The 24-hour time frame was used to minimize tidal and diurnal effects in the EMPACT field data. The clusters were developed using a measure of the distance from any given point to all other points. Figure B-7 shows an empirical distribution of those distances. Note the second peak developing in Figure B-7b. This second peak is the result of the data separating into two clusters in feature space; in this case, the beginning of a response to the introduction of zinc.

In order to use this feature of the empirical distributions to determine when an event has occurred, a statistically based test to compare the two distributions is desired. To do this, the distance matrix must be calculated. In practice, the Mahalanobis distance (MD),

$$MD_{ik}^T = \left(\bar{x}_i - \bar{x}_k \right)' S^{-1} \left(\bar{x}_i - \bar{x}_k \right)$$, where S is the covariance matrix, is used in order to account for

the covariance (variability) in the data. The empirical distribution of the distances, MD^T, for any given time period T, is formed. If the two distributions are separated in time by Δ, the hypothesis, H_o: $MD^T = MD^{T+\Delta}$, must be tested. The Kolmogorov-Smirnov test statistic is used since the underlying distributions are unknown. The object is to detect the change from unimodal to bimodal. If the hypothesis is rejected at some predetermined significance level, then the fish is deemed to have experienced an event. Figure B-8 shows a sample of the Kolmogorov-Smirnov test statistic time series for one fish at the high concentration. The horizontal lines in the figure represent different significance levels. Note that there are two peaks. The first corresponds to the initial change to a new location in feature space corresponding to a shift to a bimodal distribution, as in Figure B-7b. The distribution then stays bimodal until all points have shifted to a new locus. Thus, the second peak corresponds to a shift from bimodal back to unimodal as in Figure B-7a. The advantage of this approach is that all of the ventilatory data is used simultaneously rather than examining each ventilatory parameter separately.

A second approach to deciding if a significant event has occurred exploits a feature of the data that can be seen in Figure B-9, which shows the scatter plot from the last data segment ("•") as well as the trajectory of the cluster centroids for all of the data segments ("+"). While the "+"s are clustered closely together in some places, in others they are spaced quite far apart. The closely spaced clusters are from time periods when little is happening and the fish is experiencing no significant events. When an event occurs, the fish begins to move to another region of the feature space. At first, the change involves a few points (Figure B-6b), but eventually all the points cluster in a new region of feature space.
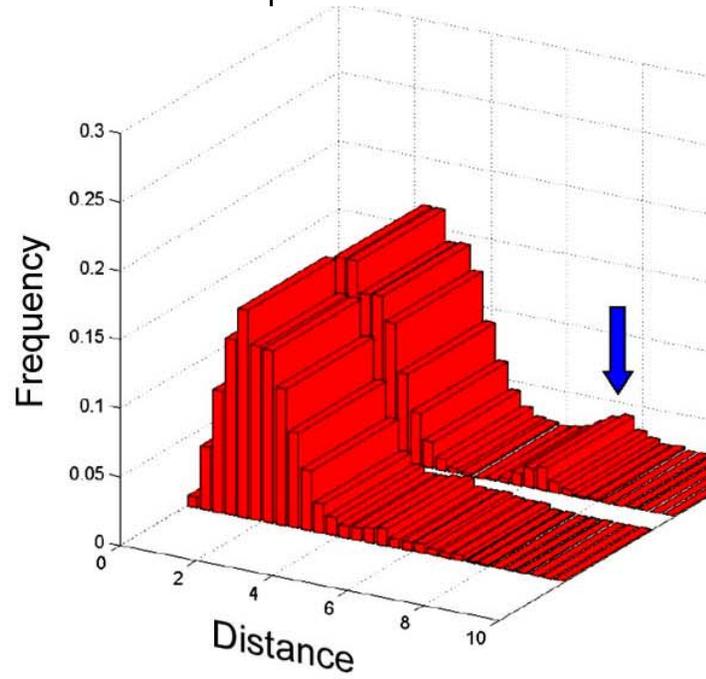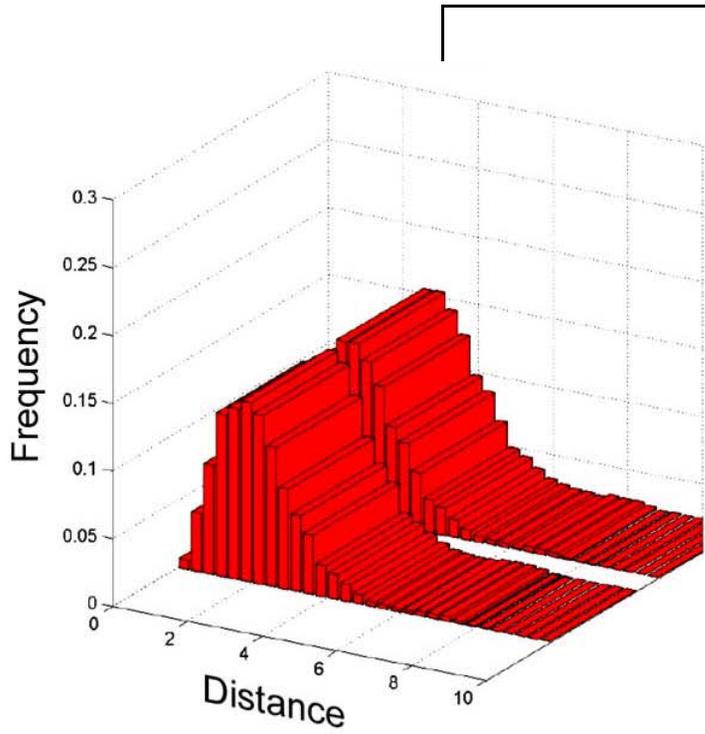
Figure B-7. Distances among data points. a. Corresponds to Figure 6-6a. b. Corresponds to Figure 6-6b. The arrow points to a second peak caused by the separation into two clusters.
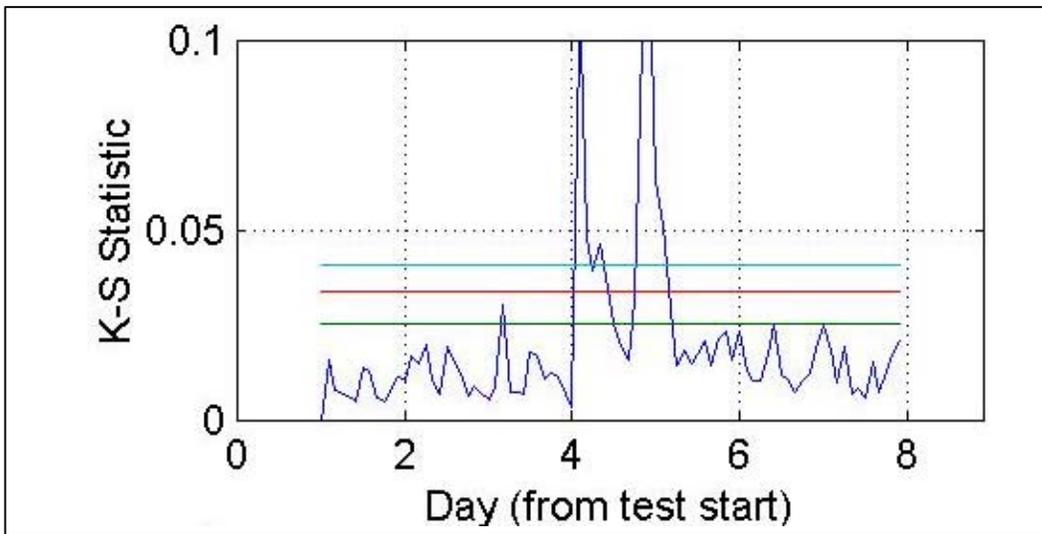
Figure B-8. Kolmogorov-Smirnov test statistic for one zinc-exposed fish (high concentration). Statistical significance levels (90, 95, and 99) are shown on the vertical axis. Exposure began on day 4.
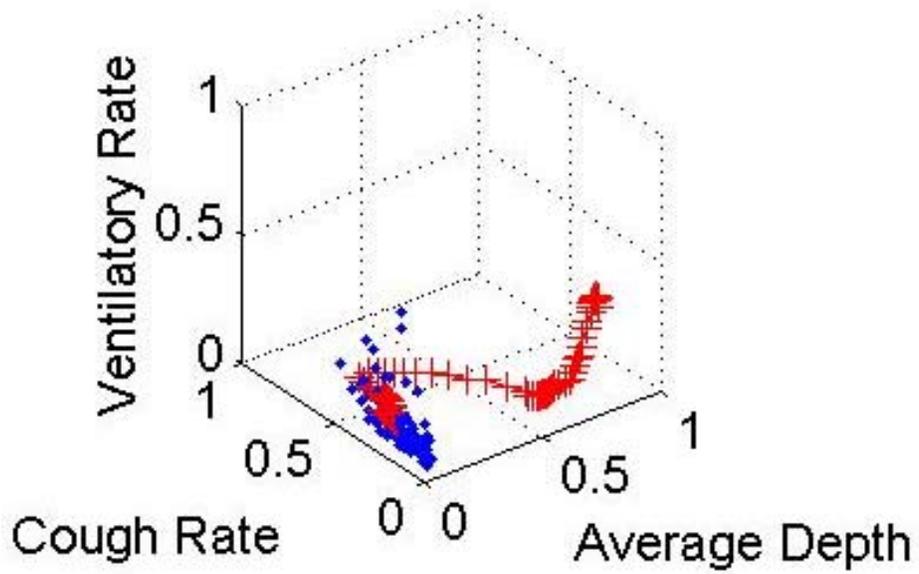
Figure B-9. Movement of cluster centroids in feature space over time. Each "+" represents the centroid of a 24-hour data segment. The "•" are the actual data points from the last 24-hour block of data.

This effect is exploited by computing the MD between the mean position, $\vec{\mathbf{X}}$, of each

cluster: $\quad MD = \left(\vec{\mathbf{x}}_o - \vec{\mathbf{x}}_{o+\Delta}\right)^T S^{-1}\left(\vec{\mathbf{x}}_o - \vec{\mathbf{x}}_{o+\Delta}\right)$. Hotelling's T^2 is formed as $\quad T^2 = \left(\dfrac{n_1 \times n_2}{n_1 + n_2}\right) MD$.

T^2 is then transformed to F-ratio as $\quad F = \dfrac{(n_1 + n_2 - p - 1)}{(n_1 + n_2 - p) \bullet 2} T^2 \approx F_{3,n_1+n_2-p}$

and the hypothesis $H_o : \vec{\mu}_o = \vec{\mu}_{o+\Delta}$ is tested. Note that for large sample size, $\vec{\mathbf{X}} \sim$ Normal. Figure B-10 shows a time series of the F statistic for the same fish as in Figure B-8. This test also has the advantage of using all three dimensions of the data vector simultaneously.

The means are now available to detect events in each fish that uses all of the data simultaneously, and the results for all eight fish can be seen by over-plotting the curves. Figure B-11 shows those results. At this point the fish can be "voted" as it is in the processing scheme currently used with the fish ventilatory system. However, by doing so, there is the possibility of missing the events. Figure B-12 illustrates this problem. As can be seen, although there seems to be response from the fish at the lowest zinc concentration, the level of the test statistics for the K-S test is quite low, and not enough fish will cross the threshold except for very low values of the test statistic, although the mean test does much better. Instead, the statistics must be combined in a way that allows the generation of a single measure of response for the whole group of fish.

Due to the large data window used, the F-distribution for the F ratio can be approximated by a χ_3^2 distribution. To do this, it is observed that the sum of the F statistic (the mean test) is χ^2 distributed so that the results can be pooled for all eight fish and arrive at a single test statistic. In practice, what has been constructed is the pooled statistic after discarding the highest and lowest scoring fish to reduce the impact of outliers. This results in a new statistic that better reflects the consensus "vote" from the grouped fish. Figure B-13 shows the "pooled" statistics. These results can be compared to the results from the existing statistical approach shown in Figure B-14. Note that in Figure B-14, the time zero point coincides with day four in Figure B-13. While the pooled statistics from the newer analysis are clearly able to call an event (the initial response to zinc exposure), the current processing scheme does not.
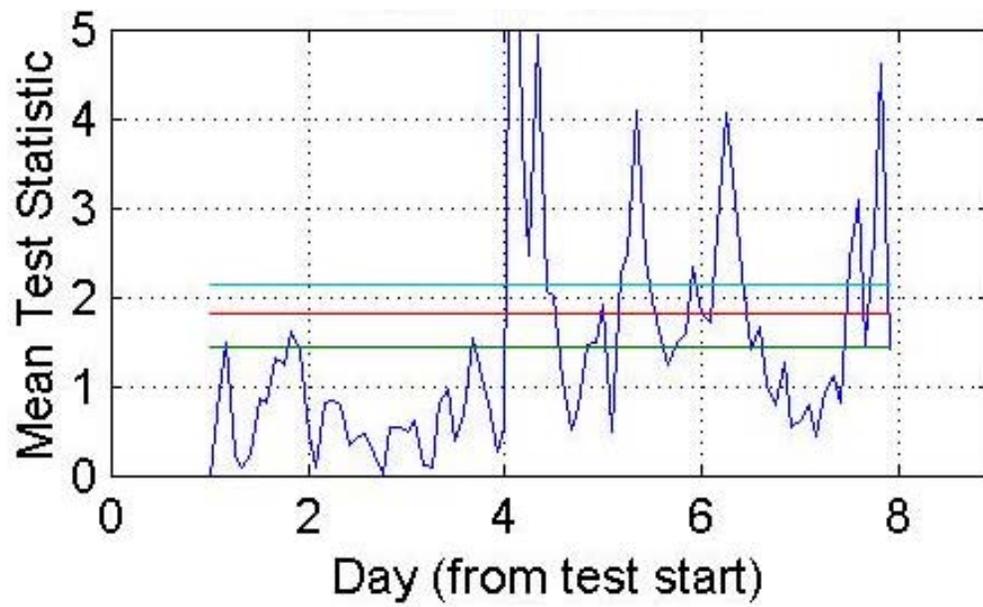
Figure B-10. Time series of mean test statistic F for one zinc-exposed fish (high concentration). The horizontal lines represent different levels of statistical significance (90, 95, and 99). Exposure began on day 4.
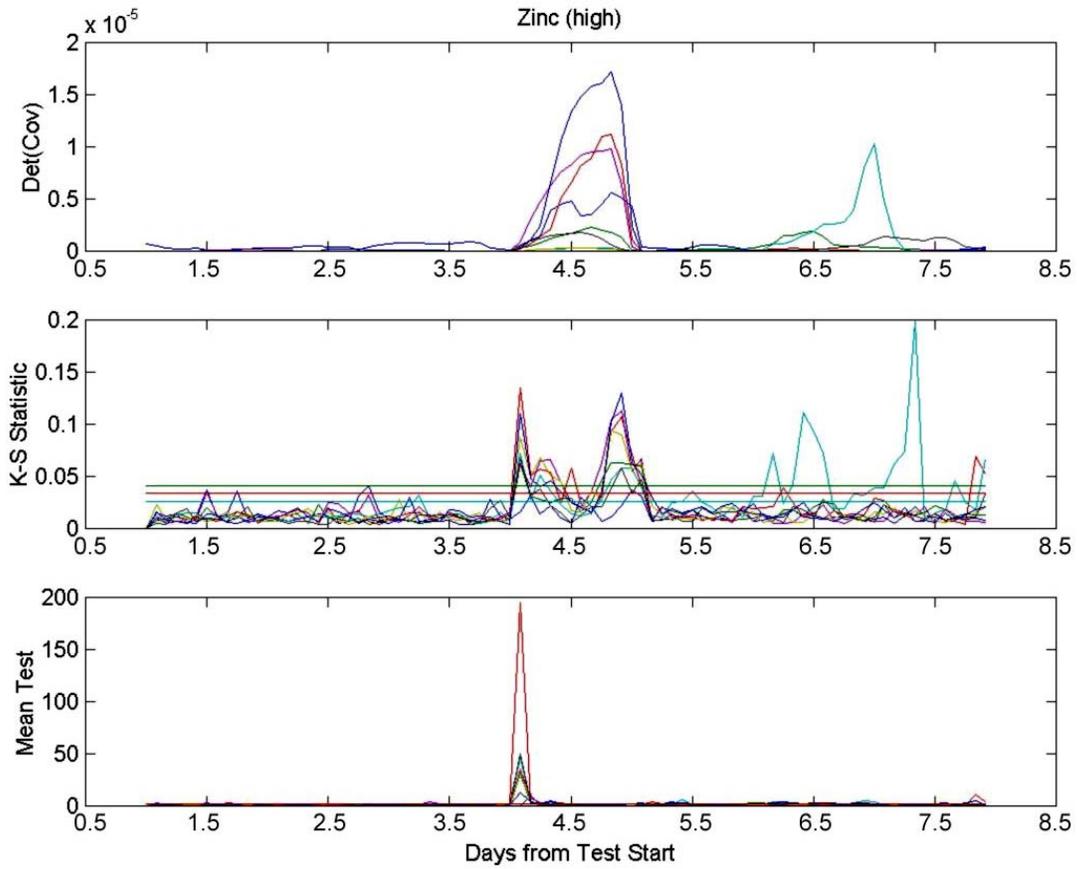
Figure B-11. Statistical tests for seven fish exposed to the high concentration of zinc. The top panel is the covariance matrix, the middle panel is the Kolmogorov-Smirnov test statistic, and the bottom panel is the Mean test statistic, F, for eight fish. Confidence limits for the K-S statistic are 90, 95, and 99. Those for the Mean test are compressed by the vertical scale and do not show.
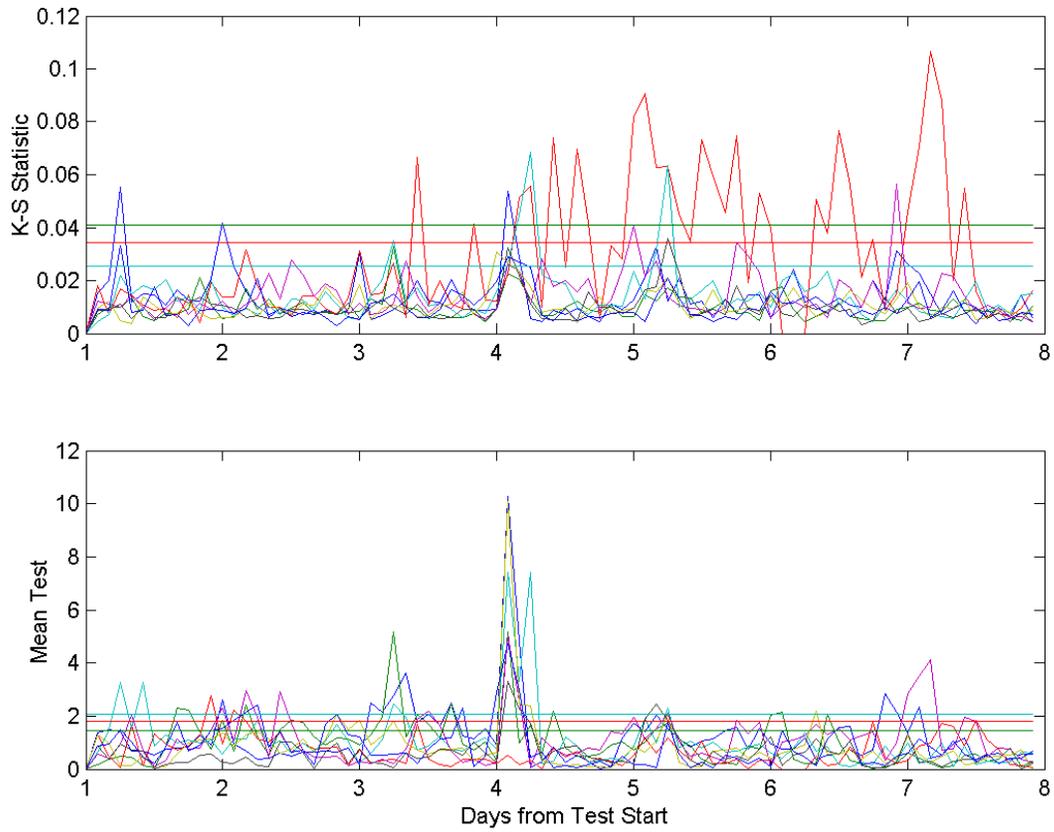
Figure B-12. Statistical tests for seven fish exposed to the low concentration of zinc. Zinc exposure began at the start of day 4. The top panel is the Kolmogorov-Smirnov test statistic and the bottom panel is the Mean test statistic, F. Confidence limits are 90, 95, and 99.
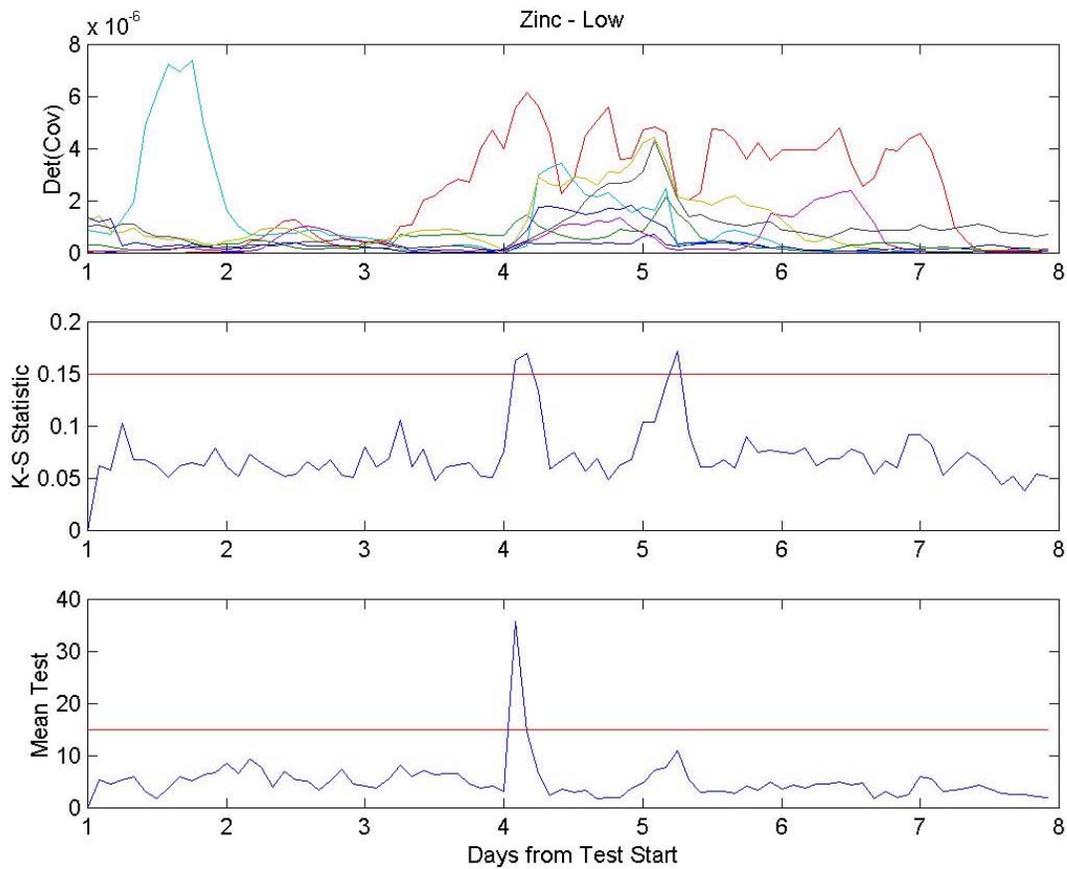
Figure B-13. Pooled statistical tests for seven fish exposed to the low concentration of zinc.
The top panel is the Kolmogorov-Smirnov test statistic and the bottom panel is the Mean test
statistic, F. The horizontal lines represent detection thresholds.
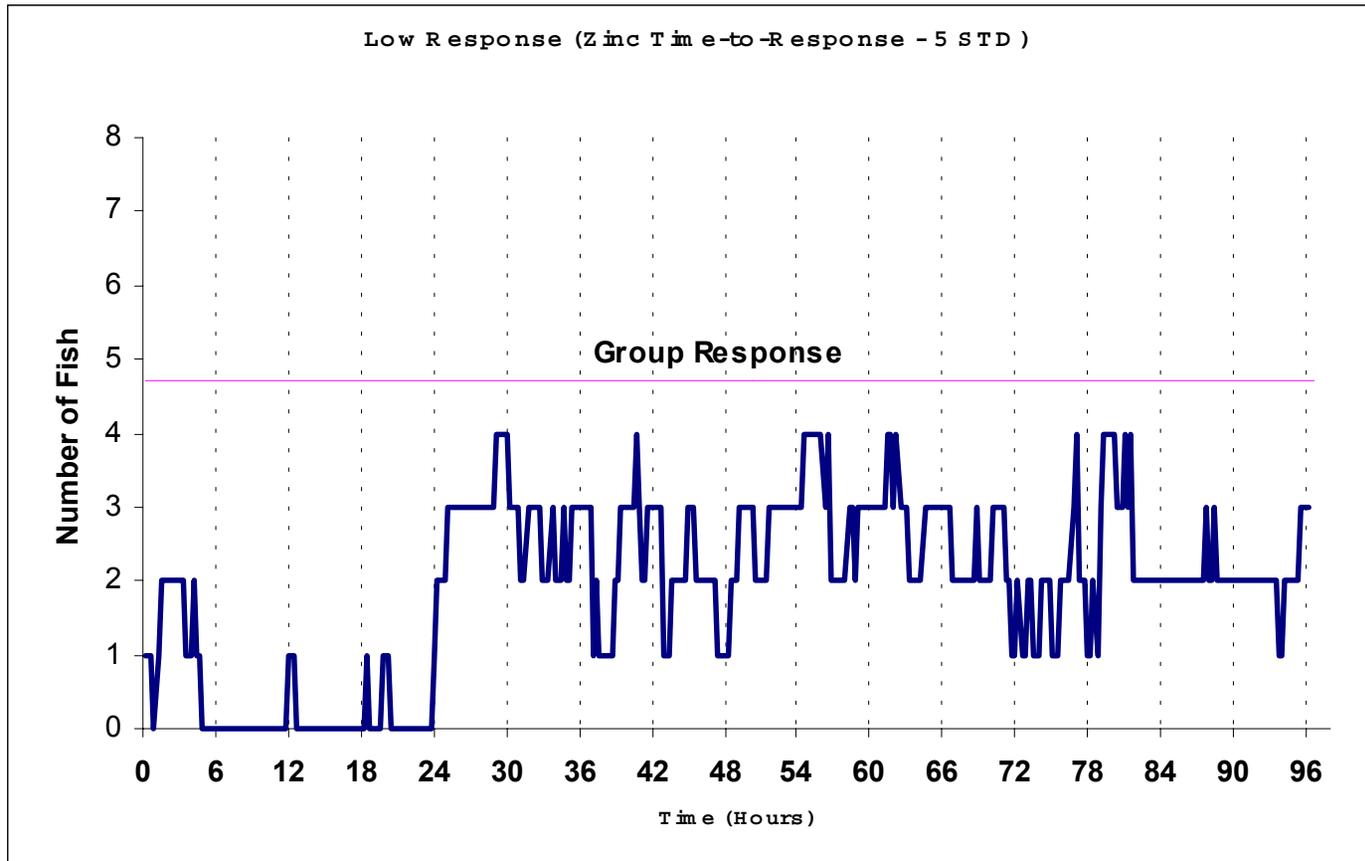
Figure B-14. Response data for seven fish exposed to the low concentration of zinc using the existing automated biomonitoring statistical analysis approach. Time zero (the start of zinc exposure) corresponds to day 4 in the preceding figures. Because there are seven fish (rather than eight) at this concentration, the simultaneous responses of only five fish are required for a group response.

B.2.2. *Application of the New Statistical Methods to Chicamacomico River Field Data*

The analysis methodology described above was applied to the data from the first event from the Chicamacomico River data for August 2000 (Figure B-15). One event was clearly detected by the existing test statistics, as well as the new test statistics. However, the new tests each show another event early in the time series, and the existing test shows another event just prior to the event detected by all three. To gain further insight into these events, the response data was compared with a subset of the water quality data (Figure B-16). None of the events detected by the new statistical techniques show any clear relationship to the water quality data, with the possible exception of the third event. There is a marked change in dissolved oxygen and a less marked change in pH just prior to the third event that may be connected to the event detected in the Kolmogorov-Smirnov and Mean tests. This does not, however, rule out other causes for response that were not recorded.

The new processing methodology clearly shows that significant events can be detected without relying on a baseline period. Based on the laboratory zinc toxicity test, the new analytical method shows a low false alarm rate for the pooled test statistics, as well as the sensitivity to detect events not seen by the existing approach. Increased ability to detect change resulting from the merging of all ventilatory data into single tests for significance, and pooling those tests for the whole group of fish, thus utilizing all of the available information in a single test rather than looking at each parameter and each fish separately and then "voting" the fish. Although the results from the field application were less clear, this new statistical approach offers a promising complement to the traditional parameters being monitored.
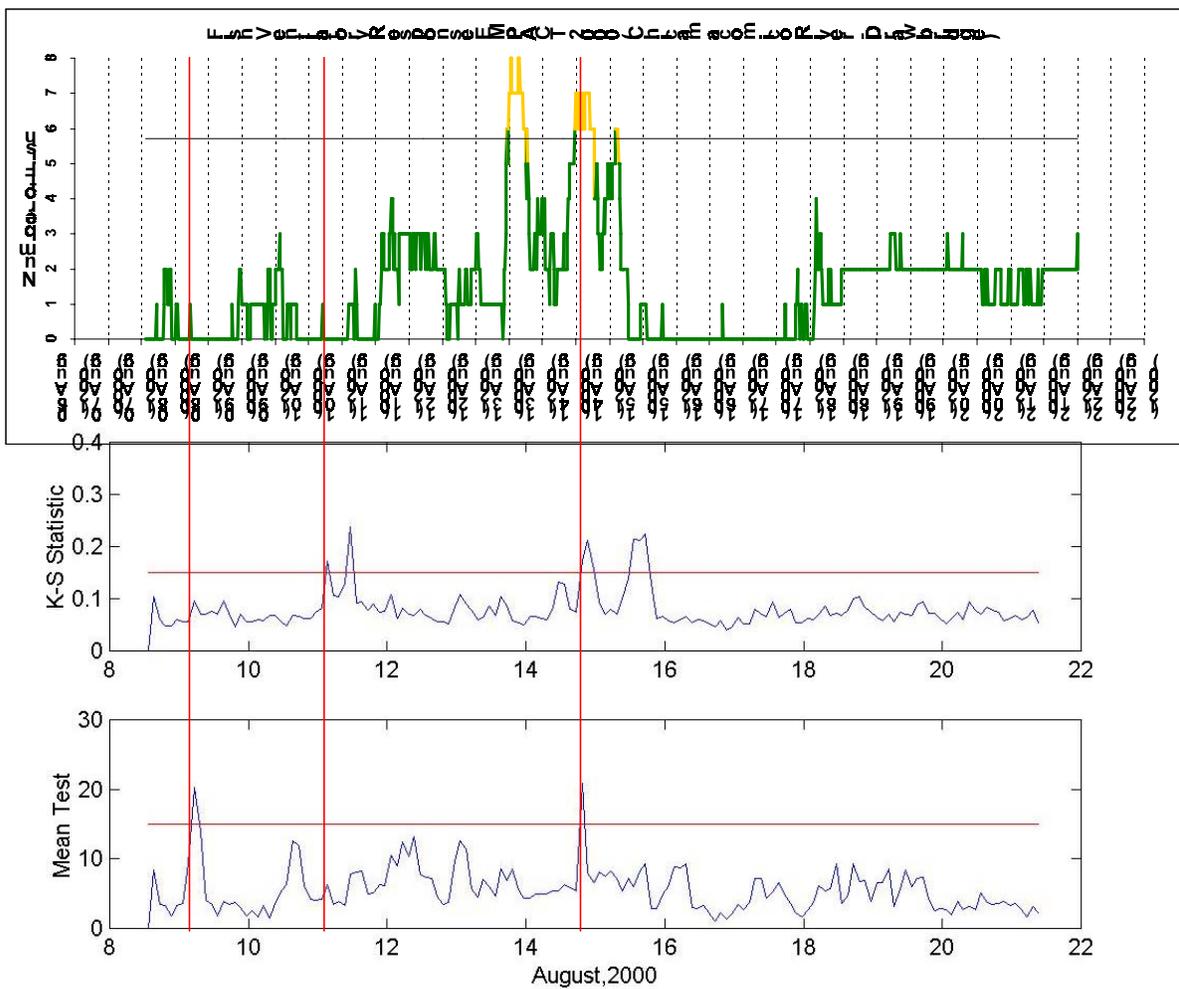
Figure B-15. Comparison of current statistical analysis (top panel) with new methodology (bottom two panels) for the time period including event one. (See Section 3.2.2.) The horizontal lines in the bottom two panels represent detection thresholds.
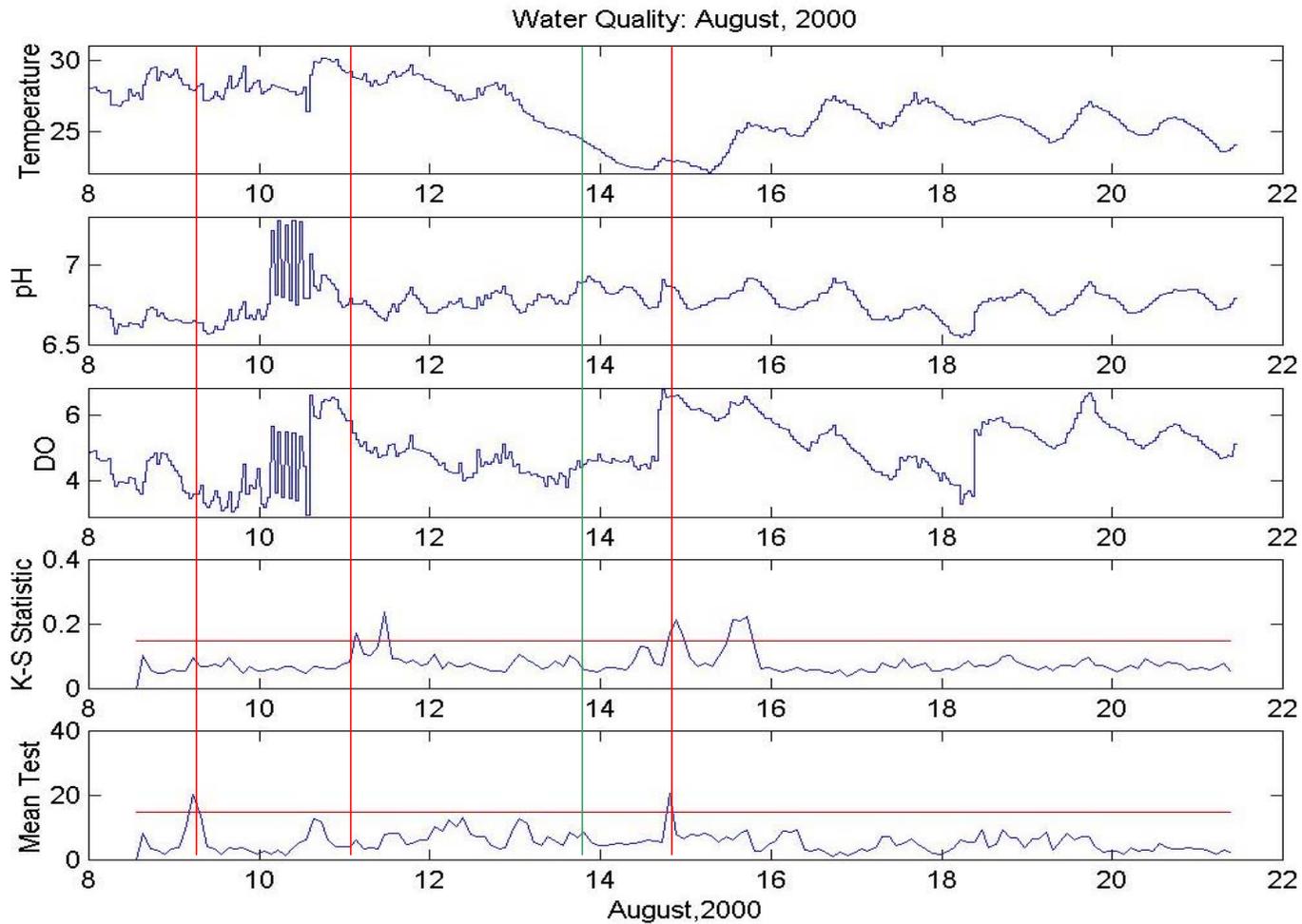
Figure B-16. Comparison of water quality data (top three panels) with fish responses found by statistical methodology (bottom two panels) for the time period including event one. (See Section 3.2.2). The third vertical line from the left corresponds to an event found by the existing statistical analyses; the other three events were found only by the new statistical methodology.

United States
Environmental Protection Agency/ORD
National Center for
    Environmental Assessment
Washington, D.C.2 0460

Official Business
Penalty for Private use
$300

NCEA-W-1089