Live Organism Toxicity Monitoring: Signal Analysis

Charles C. Sarabun, Tommy R. Shedd, C. Scott Hayek, and Amir-Homayoon Najmi

Live organisms offer the opportunity to monitor water resources for toxic conditions by measuring changes in their established behavioral and physiological responses. The U.S. Army Center for Environmental Health Research uses fish ventilatory pattern analysis to monitor aquatic environments for toxic conditions. The Center and APL have initiated an exploratory analysis of data from a series of controlled, single-substance validation tests being conducted at Ft. Detrick, Maryland. This article presents the results of some preliminary analyses and outlines future directions for follow-on studies. (Keywords: Live organisms, Signal analysis, Toxicity monitoring.)

INTRODUCTION

The U.S. Army Center for Environmental Health Research (USACEHR) is working on a program to evaluate environmental hazards in anticipated troop deployment areas and, upon deployment, to monitor the area for existing or emerging environmental hazards. An integral part of the program is the use of live organisms to observe toxic conditions. Live organisms respond to the complex mixtures of toxins encountered in a real environment and provide a good indicator of the total toxicity of that environment.

The USACEHR has developed a fish ventilatory system that uses bluegills (*L. Macrochirus*) to monitor aquatic conditions. The system has been deployed at Aberdeen Proving Ground, Maryland, for 3 years to examine remediated groundwater. Although it has been shown to be effective, USACEHR is continually improving the system hardware and software to increase response time and, potentially, to discriminate between classes of toxins. As part of this effort, USACEHR and APL have collaborated on a study of the signal time series from the system. This article describes the fish ventilatory system and some preliminary analyses directed at discovering additional information that can be extracted from the system's data.

LIVE ORGANISM TOXICITY MONITORING

Over the past 25 years, the development and use of aquatic organisms as biological early warning indicators for monitoring water supplies and effluents has been extensive, but reports of the application of such organisms to provide continuous, automatic observation over extended periods have been few, and even fewer of the systems have been available commercially.[1] Fish were the organisms originally selected, and they continue to be a popular choice. Other classes include bivalves,[2-4] crustacea,[5] daphnia,[6,7] bacteria,[1,8] protozoa,[9] and algae.[10-12]

Automated early warning systems using fish as biomonitors are designed to record continuously certain established behavioral or physiological parameters so that changes possibly indicative of developing toxic conditions can be evaluated. For example, changes in movement patterns and loss of positive rheotaxis (the ability to maintain position in a stream) have been observed by video tracking and image processing.[1,13] Changes in fish ventilatory response patterns have long been studied and used to detect a variety of environmental pollutants and toxicants.[14-17] Ventilatory parameters known to be sensitive to toxicity and monitored by the USACEHR are ventilatory rate (i.e., opercular movement over time; the operculum is the gill cover), depth or amplitude of ventilation (mean signal height), coughing or gill purge rate, and whole body movement (rapid, irregular electrical signals). Electrical signals generated by ventilatory movements are received by electrodes inside a test chamber (Fig. 1), conditioned, and interfaced to a strip chart recorder and/or a computer for continuous, automatic evaluation.

FISH VENTILATORY SYSTEM

The USACEHR acquires bluegills from local sources and acclimates them in control water under continuous light for at least 2 weeks. During acclimation, the fish are fed commercial trout chow and frozen brine shrimp. Once placed in the ventilatory chambers for testing, however, they are not fed because the feeding process causes alterations in behavior that can be mistaken for toxic response. Deleterious effects from lack of food are not apparent until after 6 weeks, but the monitoring period for ventilatory toxic response is only 3 weeks.

The electrical signals, which were continuously detected by two opposing stainless steel electrodes inside each test chamber, were amplified, filtered, and transduced to a DOS-based computer system that ran the USACEHR-developed automated biomonitoring software. In field applications, the system continuously monitored the ventilatory and whole body movements of 32 fish; two groups of 8 fish received effluent water and two groups of 8 served as controls. Each new group of 16 fish entering the system was monitored for 7 days in control water before introduction of effluent, the first 3 days for acclimation and the subsequent 4 days for collection of baseline data. On day 8, 8 fish in this group started to receive effluent, replacing a similar test/control group that had received effluent for the previous 14 days.

In laboratory single-substance validation tests, the 32 fish were divided into four groups of 8. Testing began after acclimatization and baselining. During the testing period, one group received no test substance (the control group), while the other three groups of 8 received high, medium, and low concentrations of the substance under test.

Examples of the parameters measured by the USACEHR ventilatory system are shown in Fig. 2. Each parameter was calculated at 15-s intervals, and any interval in which whole body movement was detected was excluded from the calculation of the other three parameters. During exposure, the ventilatory and whole body responses of each fish were continuously compared with its own baseline or pre-exposure limits. If a ventilatory or body movement parameter of a fish became statistically different from its normal or pre-exposure baseline response, the response was said to be "out of control." If six of the eight fish exposed to effluent exhibited statistically different responses, the group response was said to be out of control, and the program sounded an alarm and activated an ISCO autosampler to investigate the probable causes of the responses.

VENTILATORY SIGNAL ANALYSIS

In June 1997, USACEHR began a series of controlled, single-substance validation tests wherein
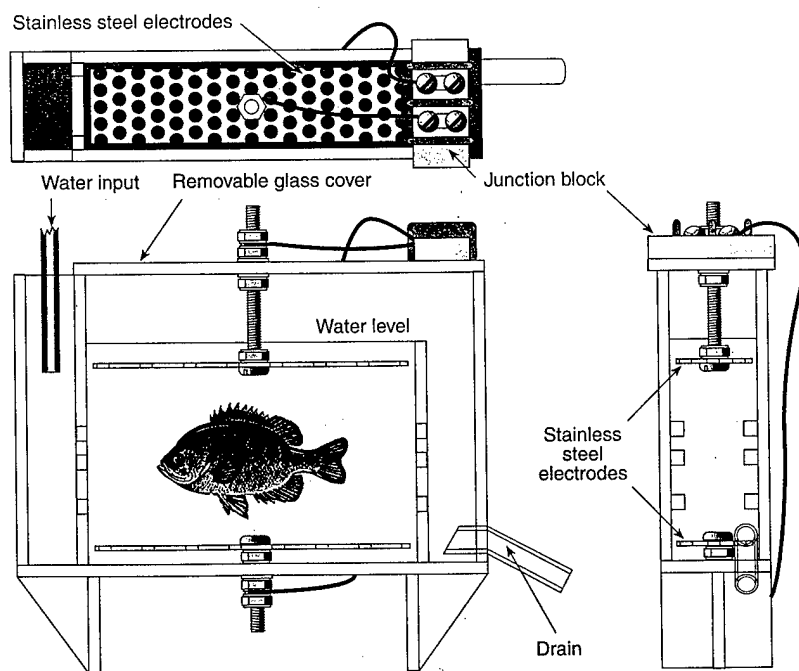

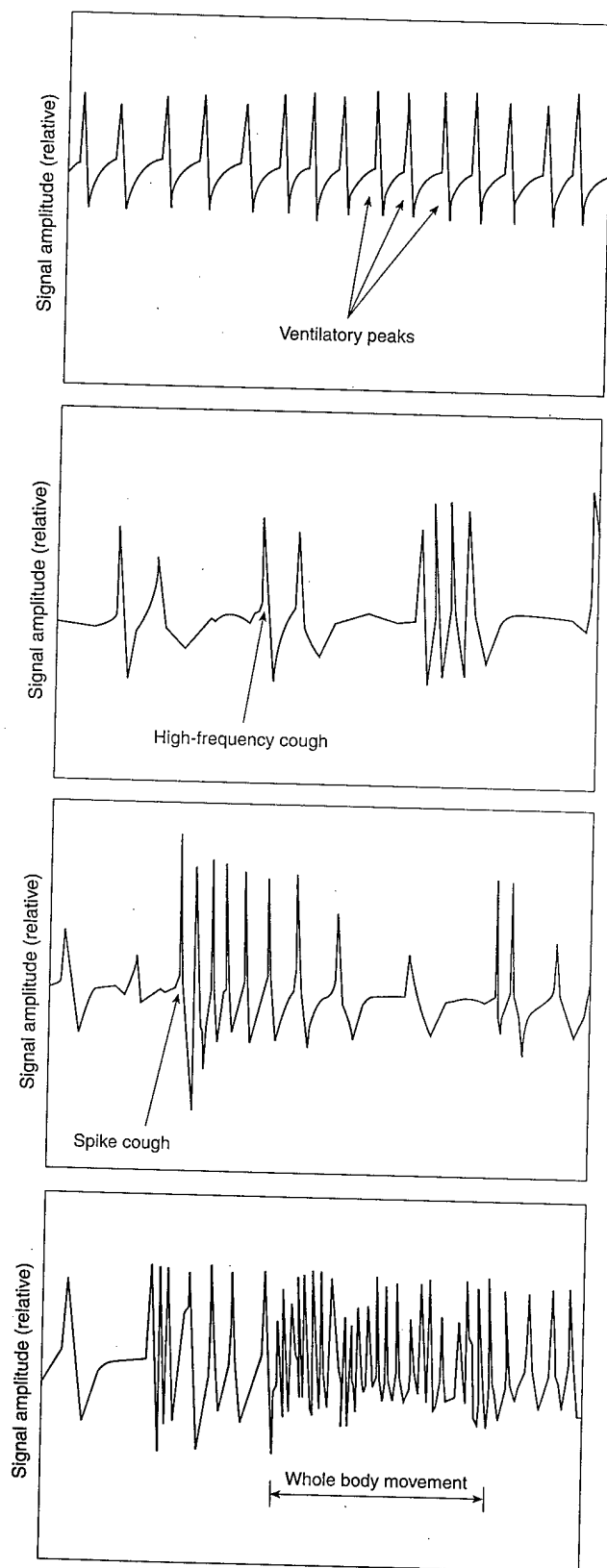**Figure 1.** Schematic of the fish ventilatory test chamber.

Figure 2. Examples of ventilatory waveforms used to monitor fish response. Each parameter was calculated at 15-s intervals, and any interval in which whole body movement was detected was excluded from the calculation of the other parameters. Arrows indicate locations of peaks.

the toxicant was added to the diluent water in which the fish swam and then was pumped into a flow-through diluter using a peristaltic pump. From the mixing chamber, the toxicant solution flowed into the respective ventilatory chambers at 50 ± 3 mL/min.

APL fielded a PC-based data acquisition system to record the output for subsequent analysis. Data from 32 fish were digitized at a 64-Hz rate and stored in binary form on Jaz drive cartridges (a single cartridge holds 48 h worth of data). The cartridges were then returned to APL, where the data were transferred to CD-ROMs for archiving and analysis. APL concentrated its exploratory analysis on the MS-222 test, as this was the first test for which a complete, uninterrupted data stream was acquired by the Laboratory's data acquisition system. (MS-222 is an anesthetic used by fisheries that can be toxic in higher dosages.)

MS-222 Data Set

For the MS-222 sensitivity study, USACEHR initiated toxicant administration at 10:06 on 21 July 1997. First responses (i.e., times at which six out of eight fish per group exceeded 5 standard deviations from the mean baseline value) were as follows: control group, 03:21 on 26 July; low-dosed group, 13:21 on 3 August; medium-dosed group, none; high-dosed group, 07:51 on 25 July. APL conducted both time and frequency domain analyses of the data. The remainder of this article examines processing and analysis of the data with a summary of results as well as areas for follow-on work.

Results

Time Domain Analysis

The various ventilatory anomalies can be distinguished by their interpeak time intervals, amplitudes, and waveshapes (ranked from most to least apparent relevance for classification). Our goal was to determine whether MS-222 had any effect on the bluegill ventilatory pattern, and if so, how quickly that pattern shift could be detected. Our hypothesis was that the simplified set consisting only of interpeak intervals and peak amplitudes would preserve the stress indication while simplifying data presentation and analysis. The data series from APL instrumentation was imported to a Pentium 166-MHz PC for low-pass filtering, peak (positive and negative) identification, and recording of amplitudes and times of the identified peaks.

Table 1 presents the excerpts chosen to sample the data before toxicant administration through a week after toxicant dosing. Each data set started at the time indicated and contained approximately 16 h of time series from all 32 fish. The sequence of peak amplitudes and clock times was converted to peak amplitudes and

Table 1. Data sampling excerpts.

Excerpt	Time, date
1	10:00, 19 July
2	17:00, 20 July
3	09:00, 21 July
4	01:00, 22 July
5	17:00, 22 July
6	09:00, 23 July
7	01:00, 24 July
8	17:00, 24 July

are natural variation over time and, in some cases, changes in the ambient environment.)

Fish receiving a high dose of MS-222 at 10:06 on 21 July produced the patterns shown in Fig. 4. Figure 4a shows the consistency of the cloud patterns before dosing the fish. A significant change in ventilatory behavior,
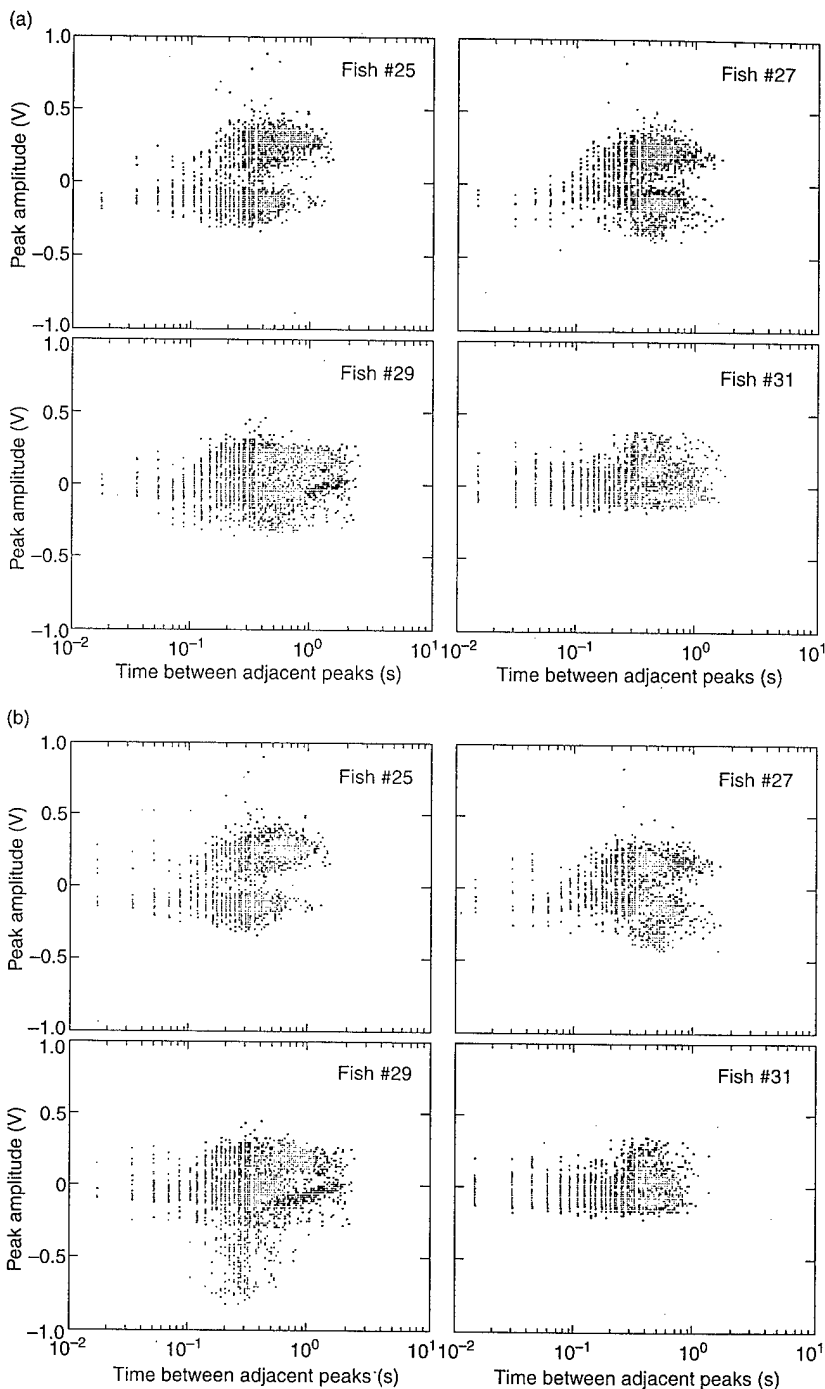
interpeak intervals. The first 2500 {interval, amplitude} pairs were then plotted for each start time listed and each fish. These "ventilatory pattern plots" conveniently captured the full range of interpeak intervals on a log scale, together with the corresponding peak amplitudes on a linear scale.

The ventilatory pattern plots immediately revealed the unique and relatively stable ventilation habits of each fish. Plots of four odd-numbered fish in the control group, which received no MS-222, are shown in Fig. 3a at 10:00 on 19 July and 17:00 on 20 July. The format of each plot is a presentation of the "cloud" of all 2500 {interval, amplitude} pairs analyzed (approximately 25 min of experiment time). The preceding day's data are shown in blue, and the later day's in red. A shift in ventilatory pattern would have been indicated by separation of the red and blue clouds. The general shape of each pattern clearly persisted from 19 to 20 July, albeit with some variation of the details.

Figure 3b shows the patterns of the same fish at 10:00 on 19 July (blue) and 01:00 on 24 July (red). One observes that the patterns are consistent even over this extended period. The stability of the control group's amplitude versus interpeak interval pattern is thus established. (In the absence of toxic stresses, the causes of the ventilatory changes

Figure 3. Peak amplitude versus peak interval plots for four odd-numbered fish in the control group (a) before (17:00 on 20 July) and (b) after (01:00 on 24 July) MS-222 was administered to the noncontrol groups of fish. In each plot the data are compared to reference data (in blue) taken at 10:00 on 19 July. Note the consistency of the red "cloud" patterns.
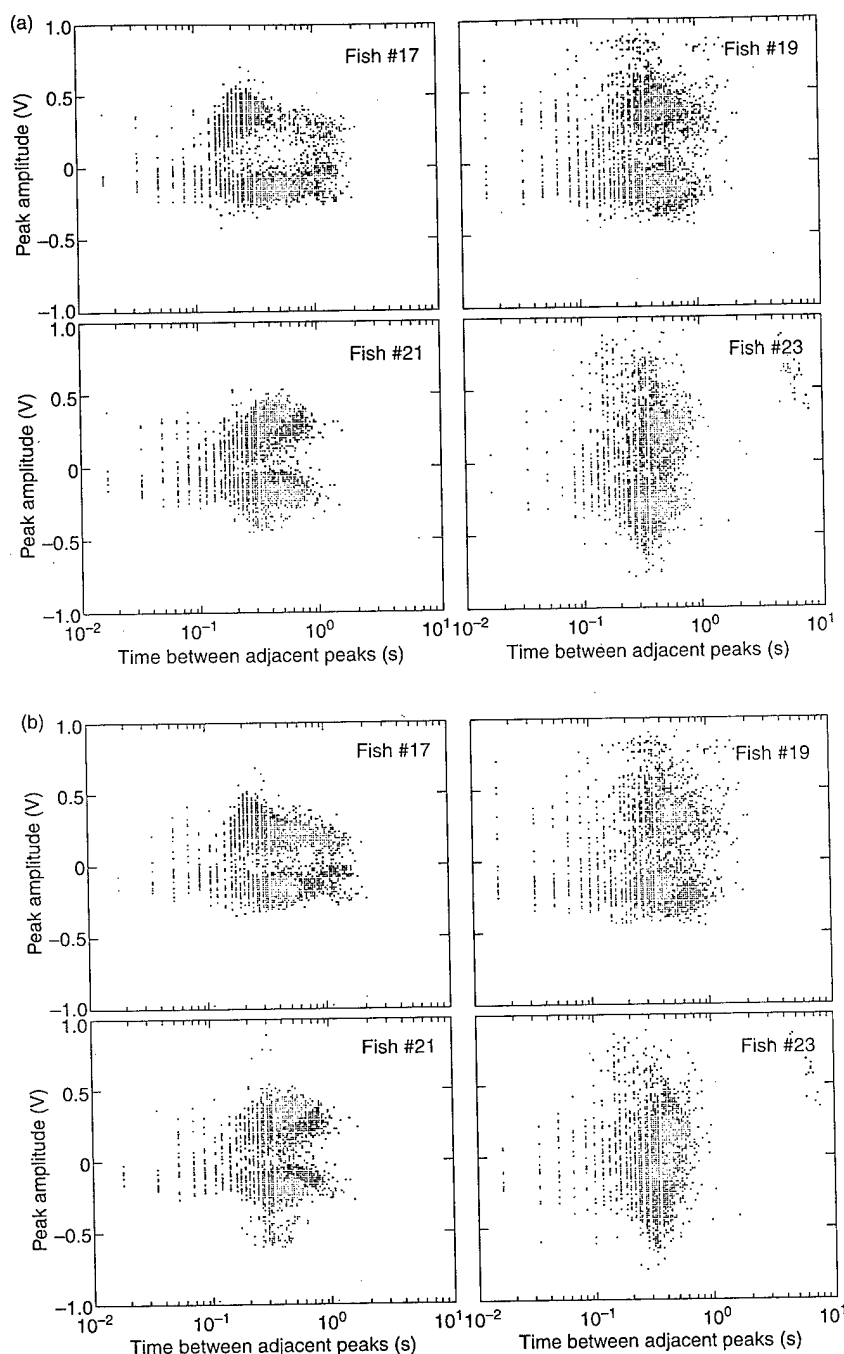
Figure 4. Peak amplitude versus peak interval plots for four odd-numbered fish in the high-dosed group (a) before (17:00 on 20 July) and (b) after (01:00 on 24 July) MS-222 administration. In each plot the data are compared to reference data (in blue) taken at 10:00 on 19 July. Note the greater shift in the cloud patterns with time relative to Figs. 3a and b. This is indicative of the change in ventilatory behavior of the high-dosed group.

intervals, relative to the controls. Figures 3 and 4 illustrate that the change in fish ventilatory behavior with time and administration of MS-222 is captured graphically by the cloud plots.

We next tested the hypothesis that cloud similarity would distinguish the control, high, medium, and low dosed groups by their ventilatory habits. Our measure of similarity was the degree of overlap area maintained by a given cloud pattern over time. We therefore examined the overlap area time histories over the duration of the MS-222 experiment. One "common area" value (reference cloud at 10:00 on 19 July) for each fish was computed for each 23 min of elapsed time. The initial common area over approximately 14 h of the earliest data (beginning at 10:00 on 19 July) was subtracted, giving all plots a starting value of zero and increasingly negative common area values as the commonality diminished.* The resulting common area time series were low pass–filtered using an 18-point finite impulse response filter to effect a smoothing over a period of about 5.5 h.

Overlap area as a function of time averaged over control fish versus overlap area of high-dosed fish appears in Fig. 5a. The control fish maintained a reasonably high overlap through roughly mid-day on 24 July. The high-dosed fish lost overlap area early, and their ventilatory patterns became even more severely shifted toward the end of the data series. This reflects the pattern shifts seen when looking at the cloud plots. One does observe that the degradation of pattern similarity in the high-dosed group begins prior to the

signaled by a reduction in cloud pattern overlap, is seen by 01:00 on 24 July (Fig. 4b). Comparison of the data from the high-dosed group and control group taken on 28 July showed that the former exhibited a shift in time between breaths, predominantly toward shorter time

*Two fish in the control group were removed from the common area average because of relatively large shifts in cloud patterns by 20 July (within the "baseline" period). One fish was removed from the high group, one from the medium group, and two from the low group for the same reason. This mirrors the USACEHR procedure for eliminating highly variable fish before performing data analysis.
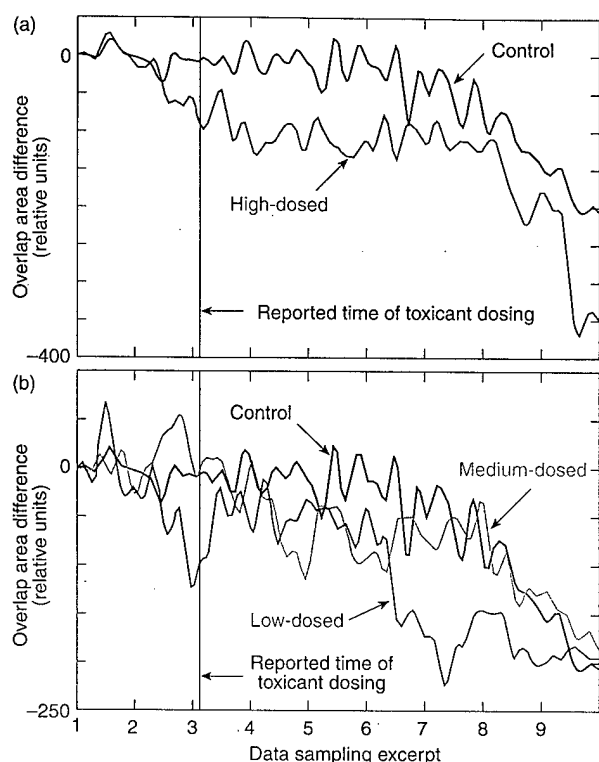
Figure 5. Plot of overlap area difference between reference (excerpt #1, 10:00 on 19 July) cloud and subsequent clouds. (a) High-dosed fish show a clear indication of ventilatory shift relative to controls. (b) Low- and medium-dosed fish show some indication of ventilatory shift relative to controls. (Excerpt times and dates are listed in Table 1.)

reported time of toxin administration, which may indicate another effect influencing that group or uncertainty in the reported time. Comparing the control and the low- and medium-dosed groups (Fig. 5b), one sees a tendency toward a lower overlap area in the dosed groups after exposure to the toxin. The pattern shift becomes dramatic in the low-dosed group on 23 July (excerpt #6).

When similar processing was completed on the even-numbered fish, both control and high-dosed groups showed dramatic pattern shifts from the outset. Discussions with USACEHR experimenters indicated that a change in conditions between the odd bank of fish and the even bank of fish could have caused the difference.

In summary, significant information about each fish's ventilatory pattern was captured in the interval versus amplitude plots. These plots appear to be unique to each fish, yet have been observed to be stable for 5 days in the absence of detrimental change in environment. In addition, ventilatory pattern changes are readily discerned by changes in these plots. The plotted values are directly related to the fish's actions, i.e., an increase or decrease in its ventilation amplitude and/or frequency.

The eye can readily detect the pattern change between the control and high-dosed groups in the MS-222 study as early as 24 July. The challenge is to detect the change via software. The limited scope of this investigation constrained our approach to the computation of a simple pattern feature: area overlap between a reference time and a later time. The low- and high-dosed groups exhibited obvious overlap shifts on and before 23 July, respectively. This was well before the 25 July call of "first response" using the existing software at USACEHR. The success of the simple overlap measure in showing the onset of ventilatory changes bodes well for further improvements using more sophisticated pattern discrimination algorithms. When fish showing spontaneous, significant pattern shifts were excluded, the overlap area versus time curve served to separate the control fish from the dosed fish.

Frequency Domain Analysis

The data analyzed here comprised 8 days beginning 18 July and ending 25 July, totaling ≈4 GB of data. Based on preliminary Fourier spectrogram analysis of the data (for example, Fig. 6a), one low-dosed fish, two high-dosed fish, and two controls were chosen for final analysis. These fish showed the most stability in their spectra before and after toxin administration. The fish that were excluded showed exceedingly noisy spectra such as the one shown in Fig. 6b. Fourier spectra in the 0.5- to 4.0-Hz range were computed using 16-s fast Fourier transforms (i.e., 4096 points for our data) and averaging 15 min of data using an overlap of 50% and a Bessel window. Three quantities were then saved: the maximum value of the spectrum (in decibels), the frequency at which the maximum occurred, and the width of the spectrum based on the difference between the two local minima occurring before and after the maximum frequency. For example, using Fig. 6a, the peak frequency occurred at 1 Hz and the local minima at 0.4 and 1.5 Hz, respectively.

The three "time series" thus computed where then smoothed using a Gaussian window and a smoothing length of about 30 min. The smoothed data were fitted using various polynomials because the fluctuations in these time series were still high. The best fits resulted from using two regression lines, denoting the time series of maximum frequency values by f and its length by n, and using the notation $f(0:n-1)$ to refer to the n data points. Then, for each arbitrary sample point m, where $1 \leq m \leq n - 2$, a regression line for $f(0:m)$ and another regression line for $f(m:n-1)$ were computed. The total combined error was a function of the sample point m. Finally, the point m was chosen to be the one that minimized the total error.

The fitted data were then used to generate time series of scatter plots of two of the three variables at a
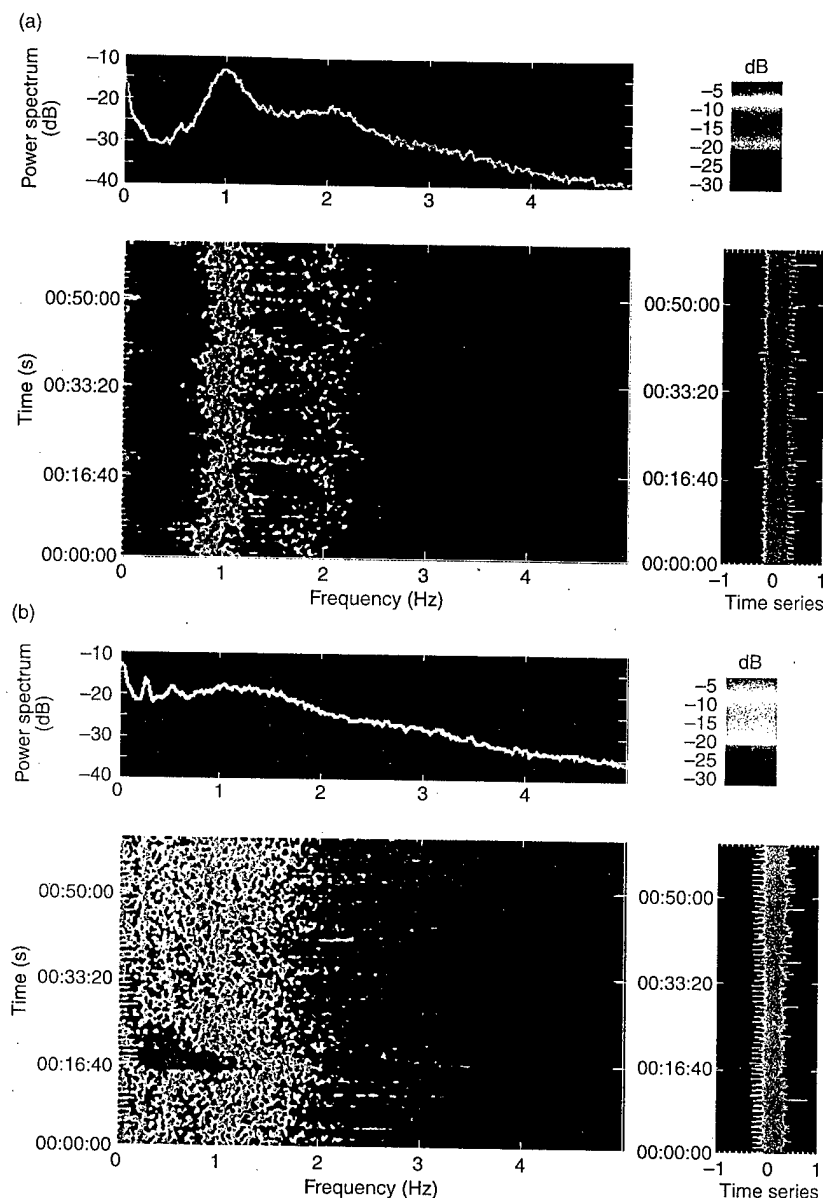
Figure 6. Time series, time–frequency, and power spectrum for (a) one fish in the high-dosed group (note the spectral peak at 1 Hz), and (b) the fish excluded from analysis because of excessive noise and lack of structure in the spectra.

FUTURE ANALYSIS DIRECTIONS

The results presented in the preceding sections represent initial analyses in the time series measurements of fish ventilatory activity. These results show promise for application to the early detection of toxins. Follow-on analyses may take several directions.

Additional effort needs to be directed at quantifying the results of the time-domain work to develop a best measure of "overlap" in the scatter plots of time series parameters. For example, Fig. 8 shows a cloud plot with density contours overlaid. Figure 9 shows a time series of such contours for the same fish represented in Fig. 6a. This figure clearly shows that the densest grouping of points in the amplitude–time interval space moves away from the baseline as the time after toxic introduction increases. These preliminary results suggest that there are potentially useful parameters that could be incorporated into the existing software to aid in earlier "calls" of toxic response.

In the frequency-domain work, a response by the fish is clearly indicated by the movement of the location of plotted values of peak frequency and amplitude. As with the time domain analyses, further cases need to be examined to develop reliable statistically based decision criteria for calling a toxic response. Also, it should be possible to develop parameter measures that could be incorporated into the existing software.

The analyses described earlier clearly have potential as indicators of toxic stress on the fish. They also suggest that some exploration of the reliability of individual fish should be undertaken. Both the time- and frequency-domain studies have shown that some fish are more stable during the baseline period than others. This information ought to be part of the decision-making process in calling a toxic event. Thus, an additional area of investigation should look at the "voting" process. In the processing system implemented by USACEHR, the fish vote in an unweighted way; that is, individual fish vote equally without consideration of the stability of the measured
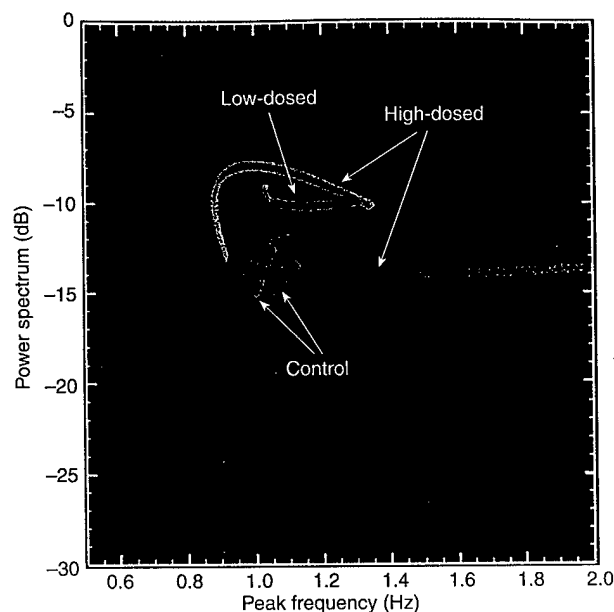
time. Figure 7 shows the last frame of the time series. (For an MPEG-format movie version of the time series, see http://www.jhuapl.edu/digest/td2003.) The time series suggests a fairly early reaction in the high-dosed fish after the administration of the toxin. This result is most easily seen in the time series scatter plots of spectrum amplitude versus frequency. In the affected fish, the aftermath of exposure to MS-222 is seen as an ongoing change in peak frequency, where the peak frequency increases with time. The result is most dramatic in the high-dosed fish. If the peak frequency is connected to the ventilatory rate, then this result is consistent with the results observed by USACEHR.

Figure 7. Final frame in the time series of plots of peak frequency versus amplitude. Note that the increases in curve thickness coincide with administration of MS-222.
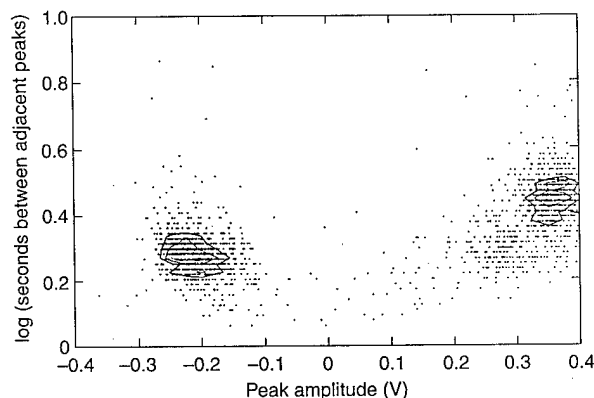

Figure 8. Scatter plot of peak amplitude versus log (seconds between adjacent peaks), with contours of point density overlaid.
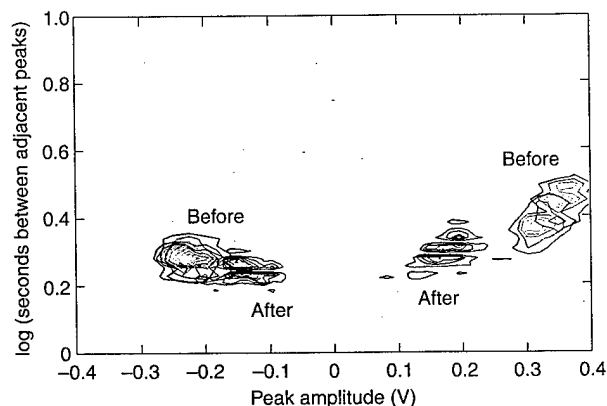

Figure 9. Overplots of scatter density contours before and after administration of MS-222. Note the clear movement of the contour centers after administration away from the contour centers before administration.

parameters during the baseline period. It would seem that more weight ought to be accorded to fish that react to a toxicant if their baseline parameters are more stable than those whose parameters vary more widely. The time-domain studies clearly show promise in this area.

In addition to the areas explored in the initial study, other approaches will be investigated in an upcoming project involving USACEHR, the Environmental Protection Agency, and APL. These will include modeling both the time series using an autoregressive model and dynamical system using delay differential equations.

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THE AUTHORS

CHARLES C. SARABUN, received a B.S. in mathematics from Rensselaer Polytechnic Institute in 1969, an M.S. in applied mathematics and computer science from the University of North Dakota in 1973, and a Ph.D. in marine science from the University of Delaware in 1980. Dr. Sarabun is a member of the Principal Professional Staff in the APL Submarine Technology Department. His interests include application of advanced sensor and signal processing technology to human environmental health problems. His e-mail address is charles.sarabun@jhuapl.edu.

TOMMY R. SHEDD received his M.A. in environmental science from Hood College, Frederick, Maryland, in 1986. He is a research biologist and has been the manager of the U.S. Army Center for Environmental Health Research's Aquatic Toxicology Laboratory since 1989. Much of his work has involved the development of acute and chronic aquatic toxicity test methods. Recent research has focused on the development of test methods that provide real-time data for rapid aquatic hazard detection and evaluation. Mr. Shedd's efforts have led to the development of intricate mobile facilities capable of on-site hazard assessment. His e-mail address is tommy_r_shedd@ftdetrck-ccmail.army.mil.

C. SCOTT HAYEK is a member of APL's Principal Professional Staff. He received B.S. and M.S. degrees in physics from the University of Maryland. He joined APL's Submarine Technology Department in 1978, working in ocean physics and signal processing, and also worked for 3 years in the Naval Warfare Analysis Department in radar signal intelligence. Mr. Hayek has been the principal investigator and test scientist for various sea tests studying the performance limits of passive and active sonar. His current work is split between ocean acoustic surveillance and counterproliferaton R&D. He is a member of the Acoustical Society of America. His e-mail address is scott.hayek@jhuapl.edu.
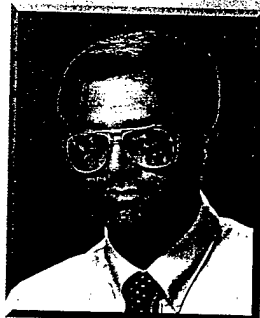
AMIR-HOMAYOON NAJMI received B.A. and M.A. degrees in mathematics from the University of Cambridge in 1977, where he completed the MathematicalTripos, and a D.Phil. degree in theoretical physics from the University of Oxford in 1982. He was a Fulbright scholar at the Relativity Center of the University of Texas at Austin during 1977–1978, a research associate and instructor at the University of Utah from 1982 to 1985, and a research physicist at the Shell Oil Company Bellaire Geophysical Research Center in Houston from 1985 to 1990. Dr. Najmi joined APL in December 1990. His e-mail address is najmi@jhuapl.edu.