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Electromyogram as a Measure of Heavy Metal Toxicity in Fresh Water and Salt Water Mussels

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The response of bivalves to heavy metals and other toxins has usually been determined by observing valve position (e.g., Davenport and Manley 1958; Borcherding 1992). Since mussels close their valves to avoid noxious stimuli, experimental delivery of chemicals is uncertain. To obtain constant results, Preston (1994 and personal communication) employed plastic spacers to hold the valves apart. This obviates the observation of valve position as an index of response, and some other method is required. Electromyography of intact mussels is one such index, and is shown to be a simple, effective and quantitative measurement of activity. Experiments are reported on the effects of added mercury on salt water and fresh water species. Parts of this work have appeared in brief form (Kidder and McCoy 1995).

MATERIALS AND METHODS

A block diagram of the apparatus is shown as Figure 1. The mussel is glued to the bottom of the dish and two Ag|AgCl electrodes are inserted between the valves. After amplification (gain = 1000 to 10,000, 3 dB bandwidth 0.3 to 30 Hz.) the signal was sent to the analog-to-digital converter (ADC) operated with a gain 8, which resolves

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its ± 625 V range into $2^{12} = 4096$ digital values. The computer program samples and displays voltages 50 times per second, a speed which produces good recordings of individual muscle contractions, including heart beats. It is impractical to save all samples at such a rate (8 hr = 1.44 x 10^6 values ≈ 8 MB), but slower sample rates would miss transient events. Therefore, the maximum and minimum values of each 50 samples (one second) were calculated and saved. These smaller files fit on a 1.44 MB "floppy" disk.

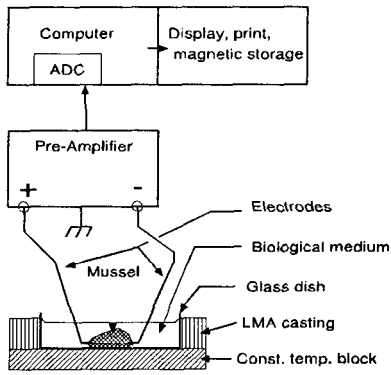

Figure 1. Block diagram of the apparatus. The mussel is mounted in a glass dish held at constant temperature. The electrodes are inserted between the valve margins, supported by a pair of micromanipulators (not shown), and connected to the preamplifier (Princeton Applied Research Mod. 113). The amplified output drives an analog-to-digital converter (Advantech PCL711) to record data for display and subsequent manipulation.

The glass chamber (a "60 mm" Petri plate bottom, 53 mm inside diameter by 10 mm deep) was placed on a brass sheet to which was soldered brass tubes carrying water held at 12° by a Haake constant temperature bath. To improve the heat transfer from the brass to the glass dish, a thin layer of thermal grease was used between them, while the sides of the dish were in contact with a casting of low melting-point alloy (Small Parts, Inc. LMA-117, 47° C). Except for the open top, the apparatus was surrounded by foam insulation. With these precautions, the temperature of the chamber fluid was never more than 0.3° different from that of the water bath.

The electrodes consisted of 5-cm lengths of silver wire (#16 AWG, 1.27 mm diameter) which was flattened at the tip to a thickness of 0.6 mm, bent appropriately to fit the apparatus, and mounted on a pair of micromanipulators. The shaft of the electrode was insulated with polyethylene tubing to confine the active surface to the flattened tip, after which the tip was electrolytically coated with AgCl. When fresh water mussels were used, the conductivity of the tissue in contact with the electrode tips was much higher than that of the surrounding medium, and electrodes recorded essentially the full EMG signal. For these experiments, an amplifier gain of 1000 was sufficient, giving a voltage resolution of 0.31 µV. With low gain, a small magnetic stirrer could be used to maintain bath circulation. In salt water, the bathing medium has a conductivity approximating that of the tissue, and therefore shunts the EMG signal, requiring a gain of 10,000. Under these conditions the magnetic stirrer caused noticeable electrical noise, and was replaced with a paddle driven by a 10-cm shaft and a remote motor.

We compared a salt water and a fresh water bivalve. The salt water species was the common blue mussel, *Mytilus edulis*, which was gathered from the shores of Frenchman Bay, Maine. We used young animals which would fit into our bath, with an average weight of 1.74 ± 0.09 g. The animals were maintained in running natural sea water until used; experiments were conducted in artificial sea water (ASW) having the composition shown in Table 1. A mussel was attached with cyanoacrylate glue to the glass bottom of the chamber and covered with 25 ml of ASW. The electrodes were positioned near the valve openings, and the mussel observed until it opened its valves. The electrodes were then simultaneously inserted between the valves by appropriate movement of the micromanipulator controls, and recording started shortly afterwards. The electrode tips were thus used both for recording and to keep the valves blocked open (0.6 mm).

Table 1. Composition of solutions

Ion	Artificial Fresh Water, m*M*	Artificial Sea Water, m*M*
Na^+	0.30	440.0
K^+	0.03	11.2
Ca^{2+}	1.05	9.3
Mg^+	0.40	49.0
Cl^-	0.50	513.0
SO_4^{2-}	0.20	26.0
CO_2/HCO_3^-	1.33	2.2

Mytilus rapidly reaches a steady rate of muscle contraction, and responds to the addition of Hg (as $HgCl_2$) in a rapid and reversible manner. Therefore, the equilibration period before the first addition of Hg was 0.5 hr, each exposure to Hg was likewise for 0.5 hr, and the washout periods following Hg removal were 1 hr each.

Zebra mussels (*Dreissena polymorpha*) were collected from the Illinois River at Peoria, and maintained and tested in artificial fresh water (AFW, Table 1.) Since these animals did not reliably open their valves in the chamber, they were manually opened and fitted with a plastic spacer before being glued down. The electrodes were inserted through the open valve margins. The mussels had an average weight of 2.68 ± 0.34 g, which for this species is a large adult.

Preliminary experiments showed that *Dreissena's* response to various agents was rather slow. Therefore, experiments with this species started with a 2-hr equilibration period, with each Hg concentration tested for a 1-hr period and washed out for 2 hr, for a total of 8 hr.

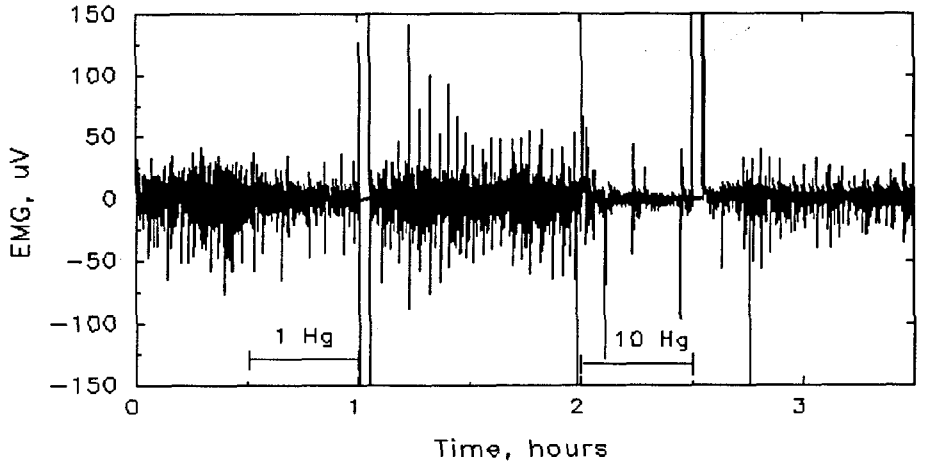

Figure 2. Recording of the EMG from a *Mytilus* in ASW, alone and in the presence of 1 or 10 µ*M* $HgCl_2$ during the periods indicated. Each second, the maximum and minimum of the SO samples collected is plotted, with these values connected by a line on the graph. The gaps following Hg periods are times during which the solution was changed with the amplifier off.

Stock solutions were prepared containing 10 m*M* $HgCl_2$ in ASW or AFW. A small addition (2.5 or 25 µL) of this stock solution was made to the 25 ml bathing solution to give a final concentration of 1 or 10 µ*M* Hg. To remove Hg, the chamber was emptied by suction and refilled with medium, with two washes of medium before resuming recording. Concentrations reported are those of the bath after addition of stock solution, calculated as total Hg (see below).

Raw data can be plotted to give an overview of the results. For summary and quantitation, the RMS (Root Mean Square) voltage for each 5-min period was calculated, averaged and plotted. Finally, a Fourier transform was used to produce the frequency

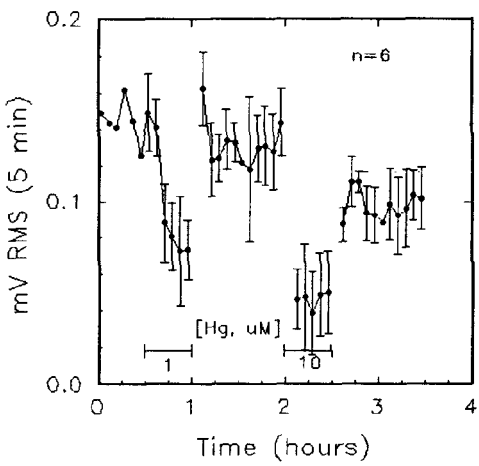

Figure 3. RMS voltage from 6 *Mytilus* before, during and after the addition of 1 and 10 µ*M* $HgCl_2$ in artificial sea water (ASW).

spectrum (μV/HZ vs. Hz), which gives additional information about the activities being altered by the inhibitor. For all means, the error bars are ± one standard error.

RESULTS AND DISCUSSION

Figure 2 shows the raw data for a a single experiment with *Mytilus.* Adding mercury to raise the concentration to 1 μ*M* $HgCl_2$ decreases muscular activity, which recovered nearly completely during the subsequent washout period. Activity nearly ceased in 10 μ*M* $HgCl_2$, but recovered at least partially after mercury removal. These impressions can be quantified by calculating the RMS voltage for each period and summing these periods for 6 mussels, to produce a graph such as Fig. 3. It is clear that 1 μ*M* $HgCl_2$ causes a marked, significant and reversible reduction in muscular activity; 10 μ*M* $HgCl_2$ produced more and faster inhibition, which was at least partially reversible. The RMS voltage in the presence of the higher mercury concentration was only slightly above the system noise level; muscular activity had virtually ceased.

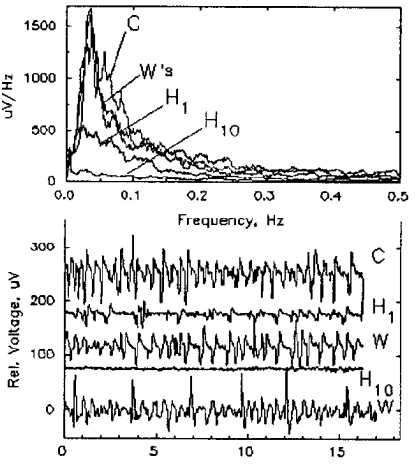

Figure 4. The terminal 1024 seconds of the 5 experimental periods from a single *Mytilus* (below) and the FFT of these data (above). The FFT data was smoothed by a these 11-point running average after transformation. C = control (pre-Hg), W = washout (recovery) from either Hg concentration, H_1, H_{10} = 1 and 10 μ*M* $HgCl_2$, respectively.

The ability of the computer to perform a fast Fourier transform (FFT) on a large number of data points in a reasonable time allows translation of these data into the frequency domain. In Figure 4, the terminal segments of the 5 experimental periods for one animal are shown below, with the FFT of segments above. The periodicity which seems apparent in the time domain data is obvious in the frequency transformation; it is also clear that the addition of mercury reduced the amplitude of the muscular activity, but did not greatly affect the major frequency, which remained about 0.04 Hz (2.4 cycles per minute).

When similar experiments were performed with *Dreissena,* the results were less satisfactory. The control period showed bursts of intense activity, but this pattern was quite variable, both between mussels and with time In some animals, 1 μ*M* $HgCl_2$

may have been slightly inhibitory, since activity increased when the mercury was removed. The addition of 10 µ*M* $HgCl_2$ seems to show inhibition, although recovery was not complete. However, the summary RMS results for 6 animals, shown as Fig. 5, demonstrate no significant difference between the various treatments. It appears that the inhibition, stimulation and recovery which seems to appear on some individual records may be random spontaneous variation in activity. The Fourier transformation (not shown) reflects this picture.

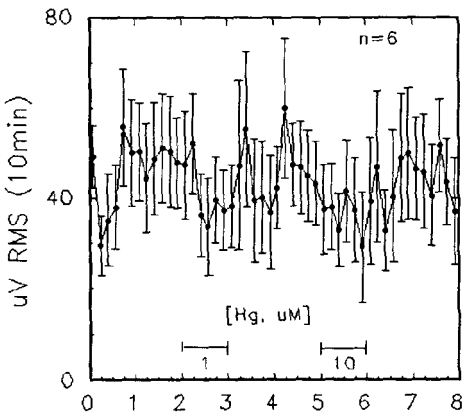

Figure 5. RMS voltage from 6 *Dreissena*, before, during and after addition of 1 and 10 µ*M* $HgCl_2$ in artificial fresh water (AFW).

The EMG changes we measure reflect the gross behavioral changes which can be observed in whole organisms. We added Hg to groups of *Mytilus* and noted whether they appeared open (foot, siphon or mantle exposed) or closed. The proportion in each state was determined every 5 min; the results are presented as Fig. 6. The EMG seems to be an accurate quantitative reflection of behavior in response to mercury.

Mercury has long been used for the identification of enzymatic -SH groups, with concentrations in the millimolar range generally used. Mercury has also been used to assess the properties of the apical water channel in various epithelia (e.g., Grosso and DeSousa 1993; Preston *et al.* 1993; Folkesson *et al. 1994;* Grosso *et al.* 1991). and other surface proteins (Preston and Chen 1987). Again, the concentrations employed (0.5 to 3 µ*M*) were well above the environmentally-relevant range. Most experiments on whole organisms in fresh water have used 0.5 to 50 µ*M* Hg for periods of hours to days (e.g., Allen *et al.* 1988; Punzo 1993a and 1993b; Pal and Nandi 1990; Mahtre and

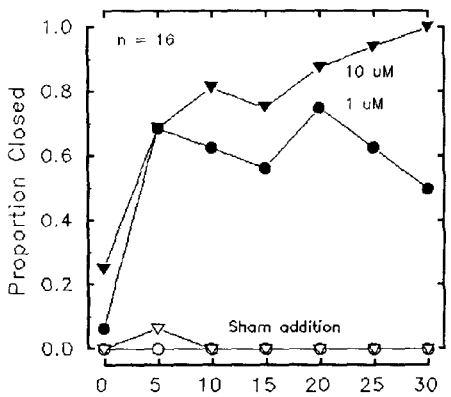

Figure 6. The behavioral response of *Mytilus* to additions of 1 and 10 µ*M* Hg. Note parallel pattern to EMG determination.

Chaphetkar 1985). Bayne *et al.* (1985) report that exposure to 5 μM Hg for 11 days inhibits growth in the marine hydroid *Campanularia fluxulosa*, but this is hardly an acute exposure. The response of *Mytilus* is reasonably sensitive for acute exposure.

There is virtually no Hg^{2+} in biological solutions; the actual species resulting from $HgCl_2$ addition are $Hg(OH)_x$ (x = 0 to 2), $HgCl_x$ (x = 0 to 4) and polyanionic forms such as HgCl(OH) (Webb 1966). Insoluble forms such as the sulfide become important if these anions are present (Björnberg *et al.* 1988). In artificial sea water (Cl^- concentration 513 mM), the predominant form is $HgCl_4^{2-}$ (99.6%). In artificial fresh water (Cl^- concentration 0.5 mM), $HgCl_2$ is 99.6% of total Hg, with minor amounts of $HgCl_3^-$ (0.35%) and $HgCl^+$ (0.06%). Experiments with natural (Passow and Rothestein 1960; Rothstein 1973) and artificial (Gutknecht 1981) lipid membranes have shown that the permeant species is probably the neutral $HgCl_2$, which is consistent with observations of diffusion equilibrium in marine cells (Kidder 1995). But since the concentration of $HgCl_2$ in AFW would be ~2 x 10^7 times higher than for the same nominal addition to ASW, while the observed inhibition is higher in salt water, one cannot explain the differences between *Mytilus* and *Dreissena* on the basis of membrane permeabilities. Moreover, the rapid reversibility of the Hg inhibition in *Mytilus* does not seem consistent with the washout of Hg from the cell cytoplasm. Binding to some surface receptor seems a more likely explanation of the acute inhibition observed.

Electromyography is thus shown to be a rapid, quantitative and inexpensive measure of the responses of bivalves to toxins, and to have the advantage that it allows holding the valves agape to permit solution access. It can readily be expanded to monitor multiple organisms simultaneously, should large numbers be important, and is not restricted to this particular toxin.

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