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In Vitro And In Vivo Measurments Of Serotonin For Ecological And Mental Health Via Fast Scan Cyclic Voltammetry

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IN VITRO AND *IN VIVO* MEASURMENTS OF SEROTONIN FOR ECOLOGICAL AND MENTAL HEALTH VIA FAST SCAN CYCLIC VOLTAMMETRY

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ABBREVIATIONS

5-HT – 5-Hydroxytryptamine

5-HIAA – 5-Hydroxyindole Acetic Acid

FSCV – Fast Scan Cyclic Voltammetry

CFME – Carbon Fiber Microelectrode

SSRI – Selective Serotonin Reuptake Inhibitor

CV – Cyclic Voltammogram

IT – Current vs. Time

EPA – Environmental Protection Agency

ADC – Analogue to Digital Converter

DAC – Digital to Analogue Converter

NIST – National Institute of Standards and Technology

FSCAV – Fast Scan Controlled Adsorption Voltammetry

Chapter 1 – Introduction

1.1 Neurotransmission

Neurotransmission is one of the most important and fundamental processes of life. Every action, though, movement, emotion, internal life sustaining and homeostatic mechanism depends on neurotransmission. The process of neurotransmission relies on neurons to carry electrical signals to and from the brain in response to stimuli.

Neurons are comprised of three components: dendrites, the cell body, and axon (Figure 1). When stimulus is applied to a neuron; ion channels open up to allow the flow of sodium ions into the cell causing a partial depolarization. This depolarization generates an electrical signal which is known as an action potential. The action potential travels along the axon until it reaches the pre-synaptic terminal on the far end of the axon. Once at the terminal, the action potential triggers neurotransmitter-containing vesicles to fuse with the pre-synaptic membrane and release their contents from the pre-synapse into the synaptic cleft.

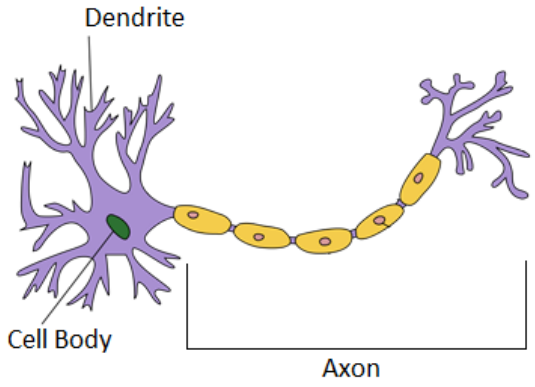


Figure 1. Basic overview of a neuron.[1]

The neurotransmitters then diffuse into the synaptic cleft, where some neurotransmitters will drift onto the post-synaptic neuron (located on the dendrites of the subsequent neuron), binding with their respective receptor. The binding of neurotransmitters to the post-synapse will trigger an excitatory or inhibitory response from the post-synaptic neuron to either fire or stop firing, respectively. The bound neurotransmitter is then released to diffuse back into the synaptic cleft. Any neurotransmitters remaining in the synaptic cleft that is not bound are quickly reabsorbed by the presynaptic neuron by one of several mechanisms to be repackaged or degraded. Alternatively enzymes in the cytoplasm will rapidly degrade the neurotransmitter in the extracellular fluid.[2.3]

1.2 Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is considered one of the most important neurotransmitters for life. *In vivo* synthesis of serotonin involves the conversion of tryptophan into 5-hydroxytryptophan by tryptophan hydroxylase which is then decarboxylated by L-amino acid decarboxylase (Figure 2).[4]

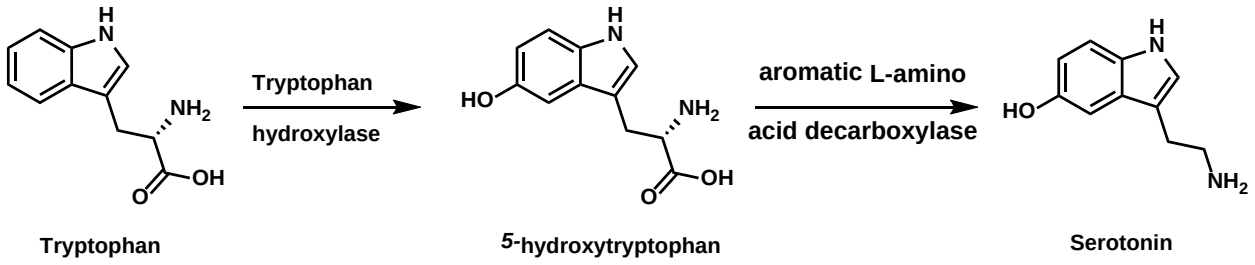


Figure 2. Biosynthesis of Serotonin

Serotonin's primary role in the human body is designated to the gut and central nervous system, is to forces smooth muscle, arteries, and veins to contract. As a neurotransmitter, serotonin plays a key role in immunity functions, survival behaviors, and maintaining

homeostasis. Notably, serotonin is typically associated with emotional balance, appetite, and sleeping patterns, and it is known colloquially as the “feel good” molecule.[5]

Mental illnesses such as depression, anxiety, obsessive compulsive disorder, aggression, and behavioral instability, eating and sleeping disorders, schizophrenia, autism, and Parkinson’s disease are associated with serotonin imbalance. Between 2006 and 2008 over 9.7 million Americans have been diagnosed with depression and of those afflicted 1 million commit suicide, yearly, due to depression issues.[6]

There are several theories proposed to explain their pathophysiological basis of depression. The most popular attempt at an explanation comes from the monoamine hypothesis of depression. This hypothesis proposes that the physiological basis of depression stems from a depletion of the amount of monoamine neurotransmitters such as serotonin, norepinephrine, and dopamine in the brain.[2] In order to combat depression, pharmaceutical industries have developed antidepressants to increase monoamine concentrations in the brain.

A common family of antidepressants used to combat depression is Selective Serotonin Reuptake Inhibitors (SSRIs). SSRIs work by blocking the reuptake transport channels on presynaptic neurons, thereby preventing the presynaptic neurons from clearing excess serotonin from the cytoplasm. As a result, higher concentrations of serotonin are observed in the synaptic cleft, ultimately resulting in increased binding to receptors.

Over the last decade, global usage of SSRIs has increased and through several mechanisms, including low adsorption in the body or discarding unwanted medication in general refuse, low but significant levels of several SSRIs are now found in many natural water systems.[7-9] As a result, many aquatic species are inadvertently ingesting notable quantities of

antidepressants leading to severe changes in survivability.[8] The increase of serotonin in aquatic species can lead to erratic behaviors, such as a loss of predator avoidance behavior or a lack of autonomic responses.[9] This contamination of natural waters can lead to ecological catastrophes, including complete collapse of ecologic systems if left unchecked. Furthermore, the ingestion of SSRI by water-dwelling animals has the potential to bioaccumulate.

1.3 Fast Scan Cyclic Voltammetry

Fast Scan Cyclic Voltammetry (FSCV) is a relatively new technique allowing for real-time analysis and sub-second electrochemical analysis of an analyte.[10-12] Since its inception in the early 1980s, FSCV has been rapidly gaining popularity due to its high temporal resolution, ease of use, and range of applications, namely quantification of electrochemically active species.[11.12] FSCV improves upon traditional cyclic voltammetry by increasing the capable scan rate to above 100 $V \cdot s^{-1}$ and applied at frequencies between 10-1000 Hz. This process allows for the collection of hundreds of voltammograms that can be assembled into a single 3-dimensional false color plot for sub-second temporal resolution.

Until recently, FSCV not commonly used due to limitations in hardware and software. A large, non-Faradaic current is generated at the electrode's surface because of the high scan rates, lead to the charging of a double layer as the potential ramp switches direction. This can obscure the signal generated by the analyte.[11-13] With the recent increase in computing power and improved software incorporating the technique of background subtraction, FSCAV has become a practical analytical technique. This integration of background subtraction with FSCV has made the technique a widely accepted electroanalytical technique throughout the neuroanalytical community providing a rapid response and high sensitivity.

Fundamentally, FSCV applies a potential waveform to a CFME. This waveform (Figure 3) is a rapid change of potential, typically in a triangular fashion, in regular intervals. Between scans, the electrode is held at a specified potential that allows for the adsorption of analyte. The sweeping of a potential voltage window induces oxidation and reduction of the adsorbed analytes. These oxidation cycles generate a flow of electrons to and from the electrode which generates a small (nA) Faradaic current.

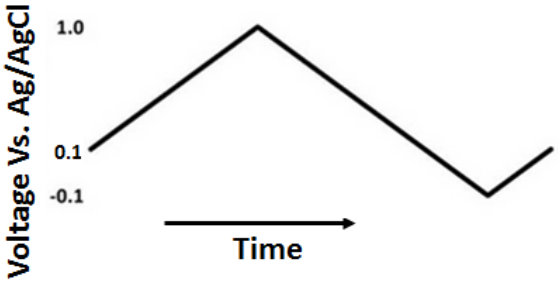


Figure 3. Visualization of a FSCV Waveform

Conversion of the x-axis from voltage to the time domain (Figure 4) generates a voltammogram that can be stacked together with numerous scans taken over the course of a run. Stacking of the current vs. time plots yields a 3-dimensional plot that can relate current, voltage, and time, known as a false color plot. Analysis of the false color plot in the y-axis generates information about the current vs. voltage. Analysis in the x-axis generates the current generated vs. time (Figure 5).

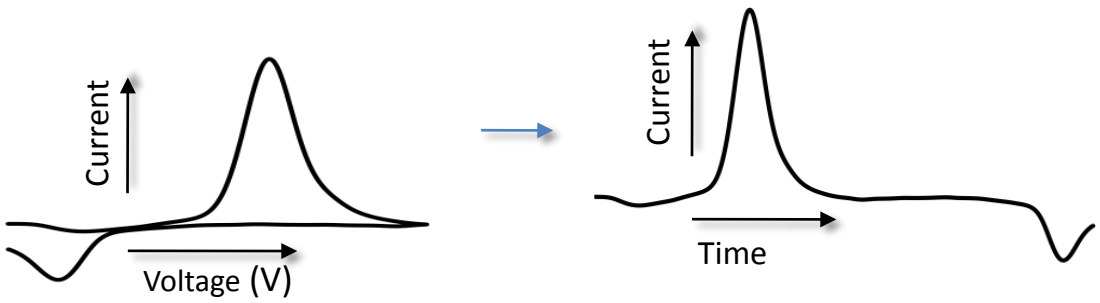
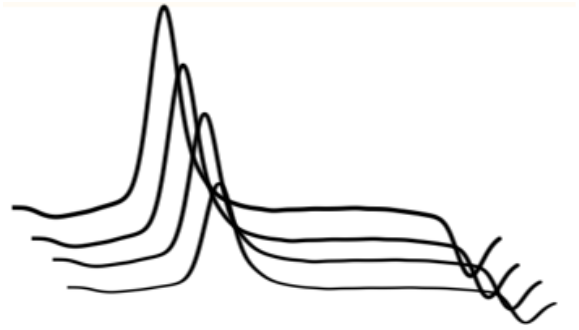


Figure 4. Conversion of voltage to time domain of a Current vs. Voltage plot to yield a Current vs. Time plot.



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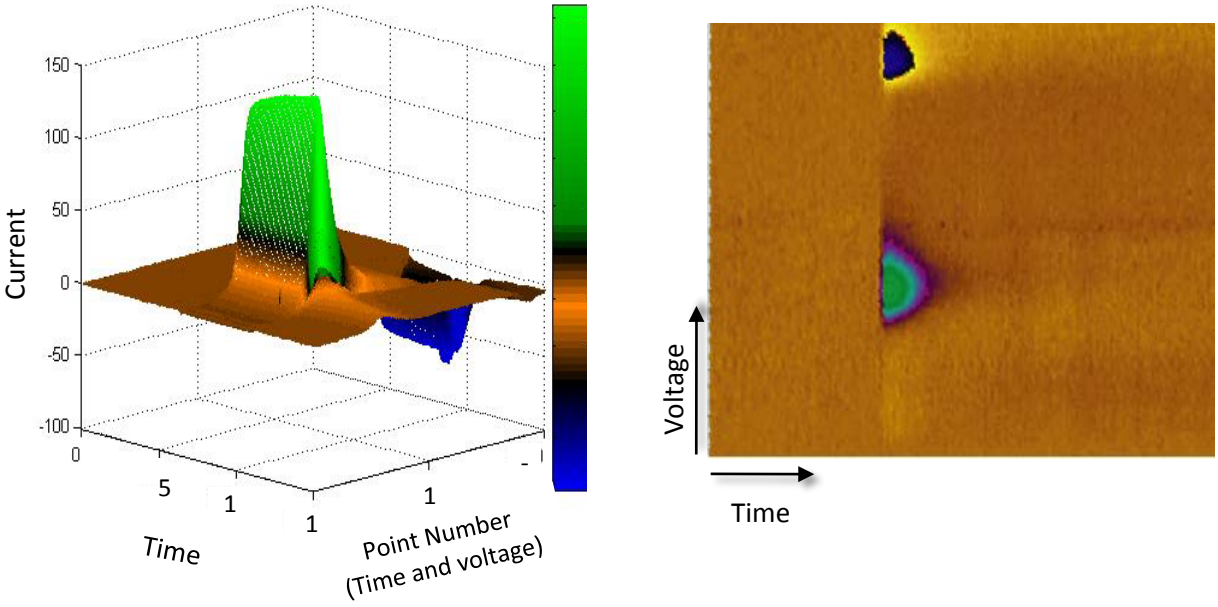


B C

Figure 5. A) Construction of 3-dimensional false color plot by stacking consecutive current vs. time plots. B) False color plot. Green coloring indicates an increase in current (oxidation) and blue indicated a decrease in current (reduction). C) Top-down view.

Chapter 2 - The Role of 5-Hydroxytryptamine in the Phototactic Dive Reflex of *Daphnia*

2.1 Introduction

2.1.1 *Daphnia magna*

Cladocera (*Daphnia magna*), as seen in Figure, are small (1-5 mm), robust planktonic crustaceans that are well established model organisms for measuring the ecological effects of contaminants, or environmental stressors.[10] *Daphnia* have an innate ability to adapt to changes in their environment physiologically, behaviorally, and/or morphologically.[14]

Figure 6. Female *Daphnia magna*. [15]

These daphnids demonstrate a phototactic response to light stimuli which induces an involuntary "dive reflex" in which the *Daphnia* instinctively moves away from the source of light. As their predators typically live in shallower waters and are mostly active during the day their dive reflex is accepted to be a defense mechanism developed by the *Daphnia* to avoid predation and remain in the safety of deeper and darker waters.[15, 16] The neurochemistry and physiology behind their dive reflex is not fully known, but it has been speculated that the chemistry underlying this mechanism is mediated mostly by histamine.[12]

2.1.2 Preliminary Data

Initially, our efforts in studying *Daphnia* were focused on the detection and quantification of the release of histamine upon exposure to light. As such, electrodes of 150 µm length were implanted into the brain of *Daphnia*, and analysis was carried out using the histamine-selective waveform (HSW): -0.7 to +1.1 V at 600 V s^{-1} [17]. Using this waveform, the expected oxidation peak would occur at +0.3 V vs. Ag/AgCl.

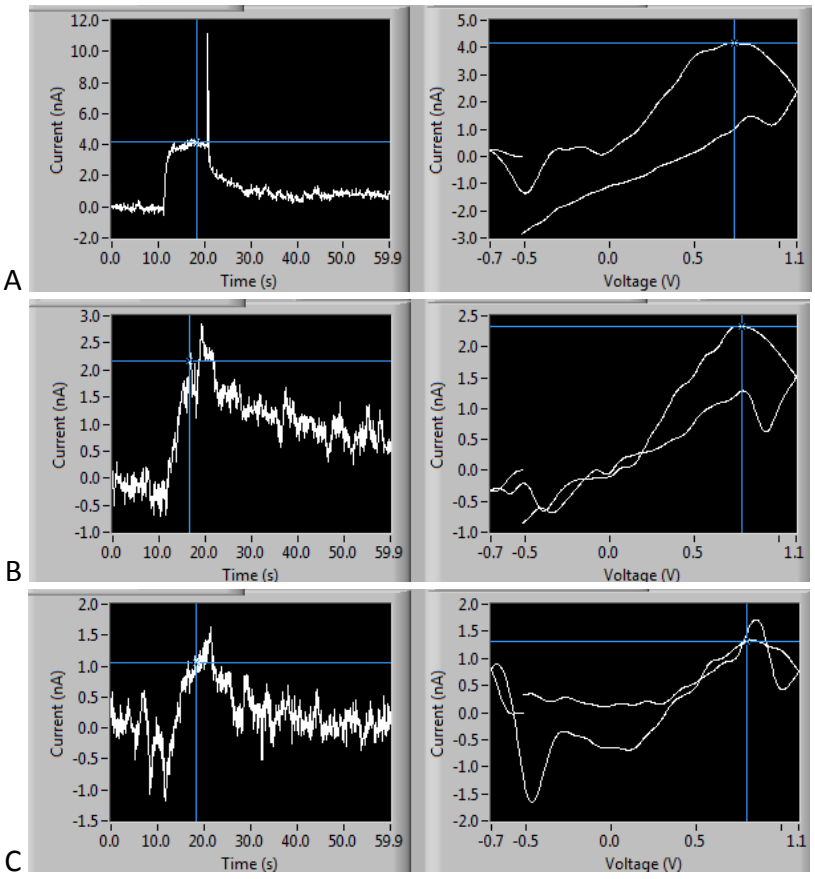


Figure 7. Preliminary current vs. time and current vs. voltage data of *Daphnia*'s response to light stimulation using HSW at A) high intensity (1.0 mW/cm^2) B) medium intensity (0.54 mW/cm^2) and C) low intensity (0.31 mW/cm^2).

As seen in Figure 7, the expected histamine oxidation peak at +0.3 V is not very prevalent in the votammagram, but a relatively strong current was generated at a potential of +0.6 V. Furthermore, the current generated appeared to be directly proportional in amplitude with increasing amounts of light intensity and the timing of the spikes in current appeared to be dependent on the application of light stimulations. This preliminary data indicates that serotonin, which oxidizes at +0.6 V, may play a larger role in the phototactic response. As such, we re-evaluated the neurotransmission using the serotonin waveform: -0.1 to 1.0 V at 1000 V s^{-1} with a 0.2 V holding potential.

2.2 Behavioral Studies

To further determine if serotonin is a factor in the dive reflex of the *Daphnia*, we began with behavioral studies where *Daphnia* were stimulated with light, in the presence and absence of SSRIs. 5 *Daphnia* were placed in a column with two regions, Region 1 and Region 2. They were allowed to aggregate without stimuli. Light was then applied and the number of *Daphnia* in each area was manually counted. The index number (ratio of Daphnia in region 1 vs. region 2) was then calculated for the intensity of light applied. As the intensity of light increased a direct correlation to the number of *Daphnia* that retreated to the deeper waters of region 2 was observed (Figure 8).

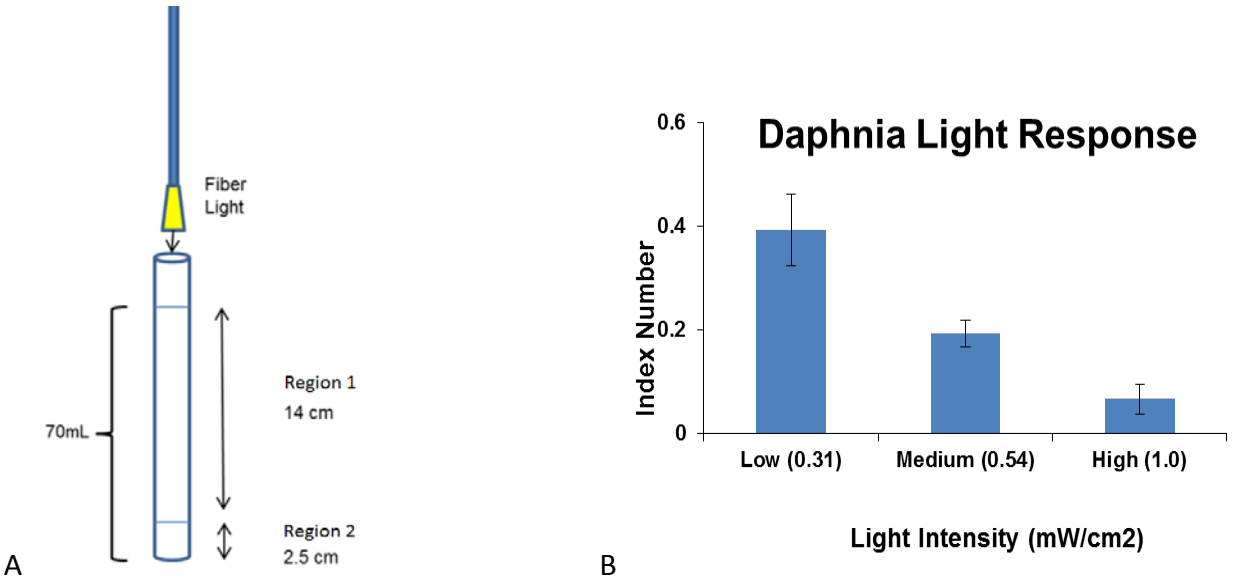


Figure 8. A) Schematic of light stimulus apparatus for behavioral trials. B) *Daphnia* response to stimulus without application of SSRI.

After these baseline measurements, *Daphnia* were subjected to water containing 5 µM and 10 µM Fluoxetine, a commonly used SSRI, for 18 and 30 hours. The *Daphnia* were then placed in the stimulus apparatus and exposed to the same levels of light. The Fluoxetine at 5 µM appeared to have had no effect on the dive reflex of the *Daphnia*. However, at 10 µM the dive reflex seemed to diminish as a large portion of *Daphnia* remained in region 1. While this data gives some insight to serotonins role in the phototactic reflex, this behavioral study was only carried out twice. As such, more testing needs to be done in order to state the significance of this data,

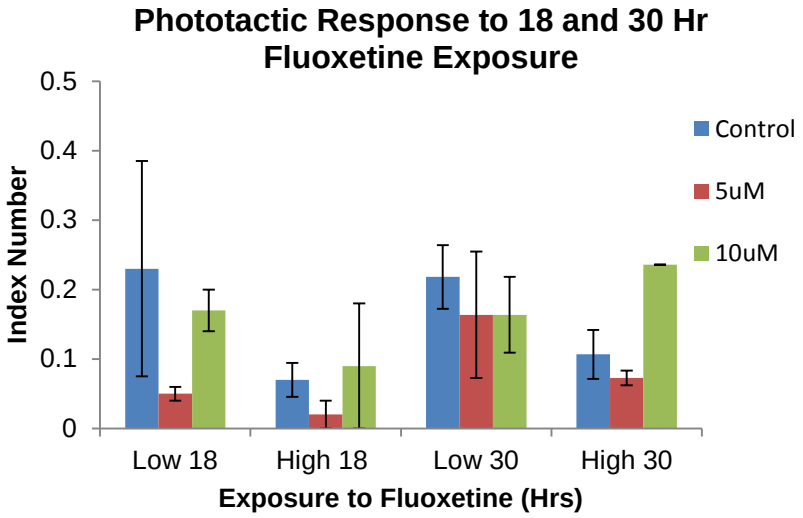


Figure 9. Phototactic dive reflex response for *Daphnia* after exposure to 5 µM and 10 µM Fluoxetine at low and high light stimulus.

These preliminary findings lend credence to the assumption that serotonin might influence the phototactic dive mechanism present in the *Daphnia*. At this point, our attention directed toward two objectives: calibration and characterication of electrodes *in vitro* and measurement of serotonin release during the stimulation of the dive reflex *in vivo*.

2.3 *In vitro* calibrations

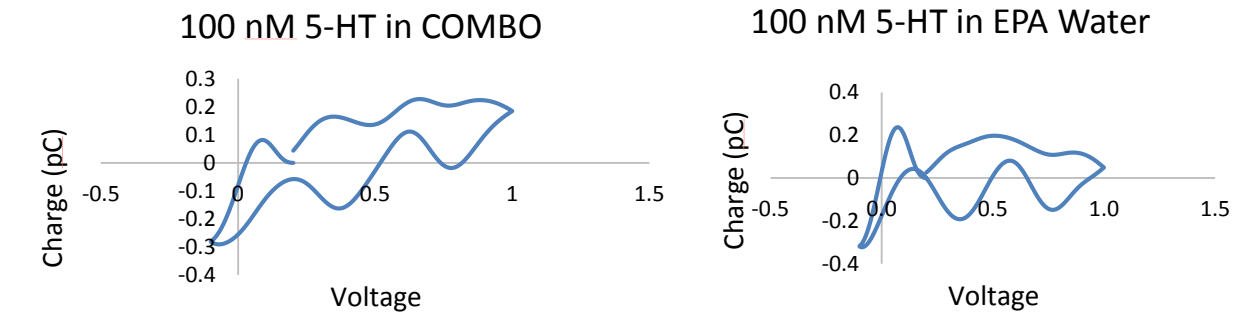
2.3.1 Chemical Environment

In vitro measurements began with fabrication of 150 nm electrode. Next, we considered ideal composition of the buffer for these experiments. Because the *Daphnia* used in these experiments were raised in COMBO buffer solution, we initially focused on executing FSCV in this buffer. COMBO buffer solution is EPA synthetic water (see Experimental Method) with the addition of a vitamin package of $4x10^{-4}$ µM B_{12}, $2x10^{-3}$ µM Biotin, and 0.3 µM Thiamine. Physiological ranges of serotonin typically fall between 20-100 nM [18] therefore, 20-mL aliquots

of 100 nM, 50 nM, and 20 nM solutions were created by diluting 100 µL, 50 µL, and 20 µL of 20 µM of serotonin to 20 mL, respectively.

COMBO buffer solution was injected into the flow cell at a rate of 2 mL/min for approximately 5 minutes to allow for the equilibration the electrode. Once at equilibrium, the serotonin/COMBO solutions were injected, one at a time, for 10 seconds per injection. After each injection, COMBO solution was again applied to re-equilibrate the electrode.

Analysis proved difficult because the oxidation peak for serotonin at physiological concentrations was virtually undetectable in this medium. To further complicate the analysis, the electrodes were prone to fouling even after application of a Nafion® coating. We hypothesize that the vitamins in COMBO were interfering with serotonin's detection. Therefore, EPA Synthetic water was tested as a replacement. Unfortunately, the same shortcomings seen in the COMBO buffer solution were present with EPA synthetic water (Figure 10).



A B

Figure 10. 5-HT signal detection in A) COMBO and B) synthetic EPA water.

Ultimately, 1X Tris buffered saline was selected. The Tris buffer range falls between pH 7-9, which matches pH levels found inside biological mediums and has been extensively used in neuroanalytical measurements.[18]

Although the use of COMBO and EPA synthetic water were not viable for *in vitro* experimentation, *Daphnia* used for *in vivo* trials depend on COMBO buffer as a habitat for survival. Therefore, once secured to the pedestal, COMBO buffer solution was allowed to flow through the chamber as a measure to prolong the life of the subject for a complete analysis.

As we saw before, the serotonin signal was undetectable in COMBO and EPA synthetic water while *in vivo* analysis depended on COMBO to keep the *Daphnia* alive. The electrode length for *in vitro* measurements had to be adjusted to compensate for the length of carbon fiber that is not exposed to the *in vivo* location. High resolution imaging showed that approximately 70% of the 150 µm electrode is inserted into the *Daphnia*. Therefore, electrodes fabricated for *in vitro* analysis were cut to approximately 100 µm in order to reflect the amount of electrode surface exposed in the site of analysis (Figure 11).

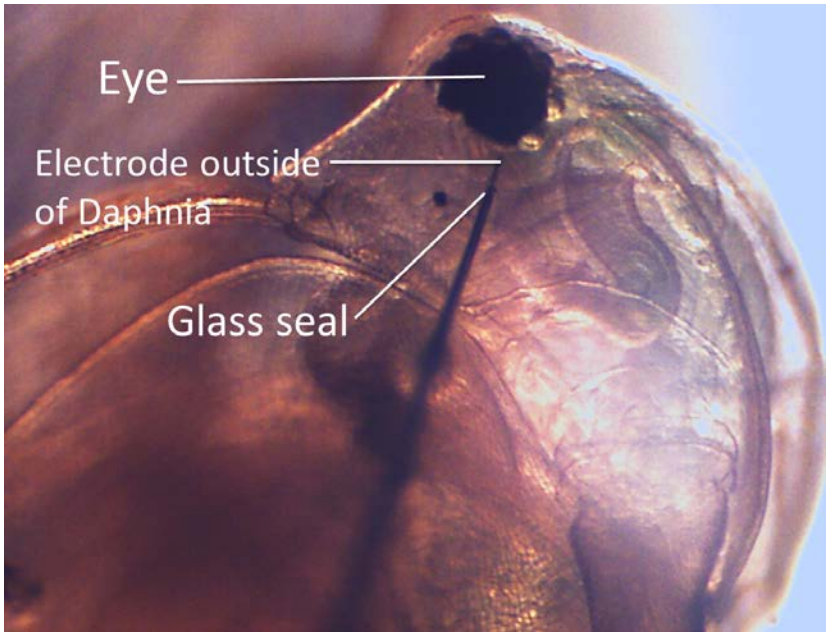


Figure 11. Electrode insertion in *Daphnia* detailing the length of carbon fiber not exposed to internal environment vs the length inserted into the experimental site.

2.3.2 Sample Introduction

Measurements in the flow cell apparatus were inconsistent. Analysis of the same sample measured in triplicate yielded three very different results (Figure 12). These large deviations could be attributed to many of different factors, including flow rate, electrode placement in the cell, and contamination. With these large deviations for a single concentration as well as a related shifting in the serotonin oxidation peak, this technique was deemed to be too problematic and the method of analysis used needed to be reevaluated. In order to successfully quantify the release of serotonin, the *in vitro* method needs to mimic neurotransmission events that occur in the brain.

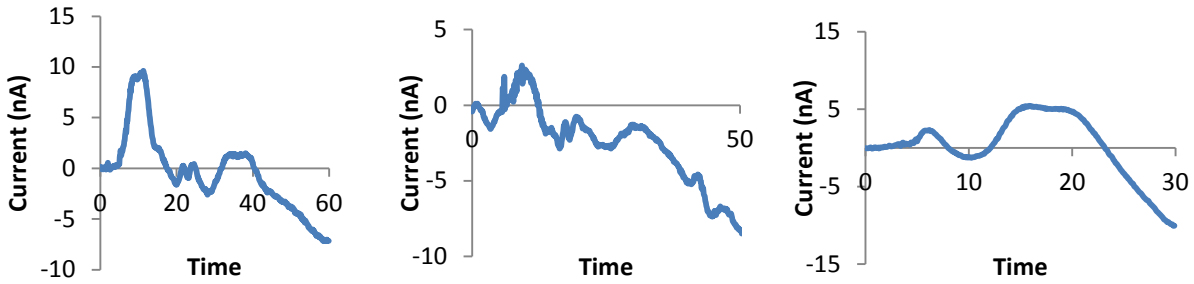


Figure 12. Current vs. Time plots for three successive scans of 100 nM 5-HT in COMBO buffer in flow cell. Stimulation applied at 5 seconds and removed at 15 seconds for the first two plots and 10 to 20 seconds for the final plot.

Neurotransmitters are released from the presynaptic membrane into the synaptic cleft, then diffuse across the cleft and bind with the receptor on the postsynaptic membrane.[19] This process occurs without the use of any mixing or flow. Since the release of serotonin in the brain is not analogous to a flow cell, the experimental setup was redesigned. The new experimental setup consisted of manual injection of serotonin solutions into a beaker filled with 20 mL of pure Tris buffer. This method allowed for the electrode to stabilize in the buffer solution which then allowed for changes in the chemical environment to be detected. To compensate for dilution of the analyte via injection, the concentration of serotonin injected was calculated by determining the final desired concentration in 20 mL buffer. Once the injection volume was determined, an equal amount of Tris buffer was removed from the beaker to prevent over dilution of the analyte.

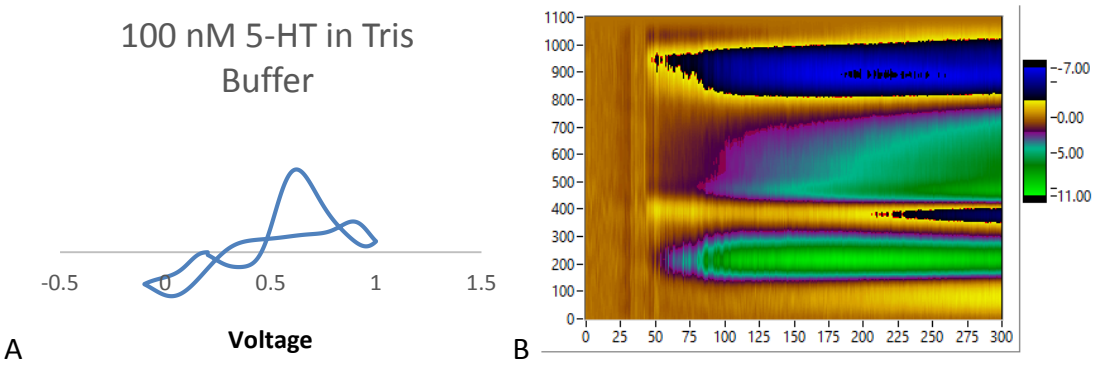


Figure 13. A) Average waveform generated from 100 nM 5-HT in Tris buffer solution.

Initial results showed higher signal, stability, and reproducibility of the 5-HT signal under these conditions (Figure 13). Once stability was determined, selectivity to the analyte was established against several potential interferents known to be present.

2.3.3 Potential Interference from Other Neurotransmitters

Octopamine, tyrosine, and histamine are all electrochemically active neurotransmitters that have previously been reported to be present in *Daphnia*.[20] Their presence could potentially cause interference with 5-HT measurements leading to higher signals, or other forms of obfuscation. To determine the effects of these interferents on serotonin, 50 nM 5-HT signals were compared to the signals of potential interferents at concentrations within physiological ranges. The individual neurotransmitters that could cause interference of the serotonin signal were analyzed using the serotonin waveform. Their voltammograms were the overlaid on the 5-HT voltammagram to prove that they did not overlap with serotonin's signal (Figure 14). Once selectivity was determined, *in vitro* measurements were conducted to determine sensitivity to 5-HT as well as to construct calibrations to establish the repeatability of measurements.

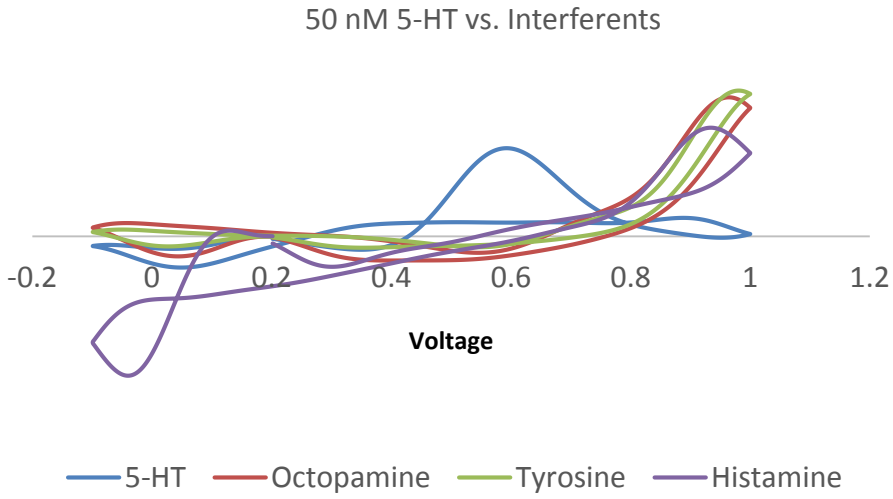


Figure 14. Averaged Serotonin CV vs Octopamine, Tyrosine, and Histamine.

2.3.4 Serotonin Calibration Curves

Solutions of 5, 10, 20, 50, and 100 nM serotonin were tested by injecting 5, 10, 20, 50, and 100 µL of 20 µM serotonin into a beaker of Tris buffer to make a total of 20 mL for each solution. Each concentration was measured four times and the maximum current generated was recorded for each trial and then averaged. The average currents were then normalized to allow for comparisons to other electrodes.

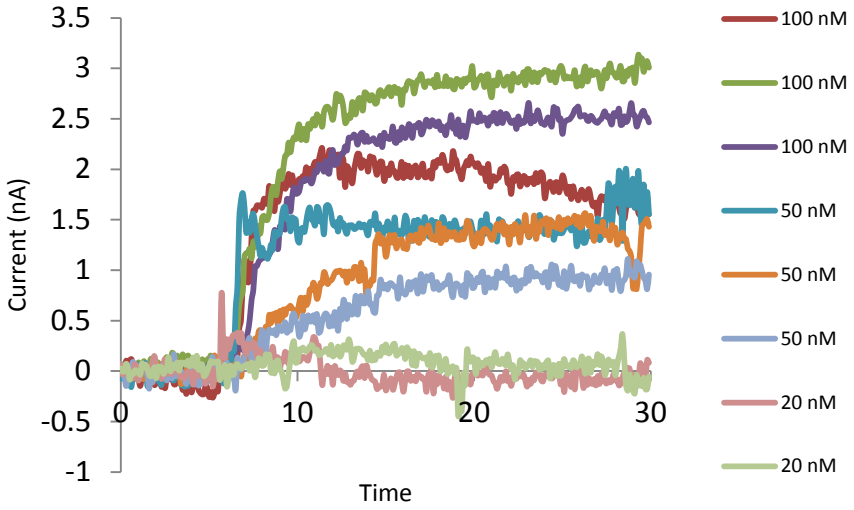


Figure 15. Response of an electrode through consecutive scans.

During these trials, stability and sensitivity of the electrodes diminished greatly (Figure 15). As such, electrodes were visually inspected after conducting trials, and it was observed that substantial fouling has occurred on the electrode surface (Figure 16). The fouling of the electrode accounts for the wide range of signals generated from repetitious sampling of a single analyte concentration. We hypothesize that the biomass accumulated on the surface can act as a trapping layer preventing the movement of serotonin to and from the bulk solution and the surface of the electrode.

Figure 16. 500x image of fouling of CFME submerged in Tris buffer solution for 1 hour (taken though optical lens with a cellular phone.)

Previous studies have shown that Nafion® coated carbon fiber micro electrodes are highly stable for the detection of serotonin in Tris buffer. This indicates that the fouling may have come from contamination of any reagents or equipment used during solution preparation. Furthermore, prepared 1X Tris buffer became cloudy if left overnight while the 10X Tris remained clear.

Since Tris buffer solutions are highly stable and are fairly resistant to microbial attack, they should remain viable for use for up to two weeks after.[21. 22] The clear appearance of the 10X Tris buffered saline stock and cloudy appearance of 1X Tris buffered saline indicates that the contaminant was appearing in the preparation of the 1X buffer solution. Inspection of the pH probe used to adjust the buffer solution showed contaminant growth on the surface of the electrode which could leach into the Tris solution when submerged in the buffer.

The pH electrode was then cleaned and decontaminated. Fresh 1X Tris buffer was then prepared and allowed to sit overnight to determine if the contamination from the pH probe was completely removed. As expected, the 1X Tris buffer solution remained clear the next day.

Data collection began again. New data started to show the expected linearity of the calibration curve as seen in Figure 17. While exploring the source of contamination, another setback occurred. The headstage used for all experimentation up to this point failed to operate. A suitable replacement could not be found in the time frame for analysis to be completed. As such, *in vitro* experimentation has ceased for the immediate future.

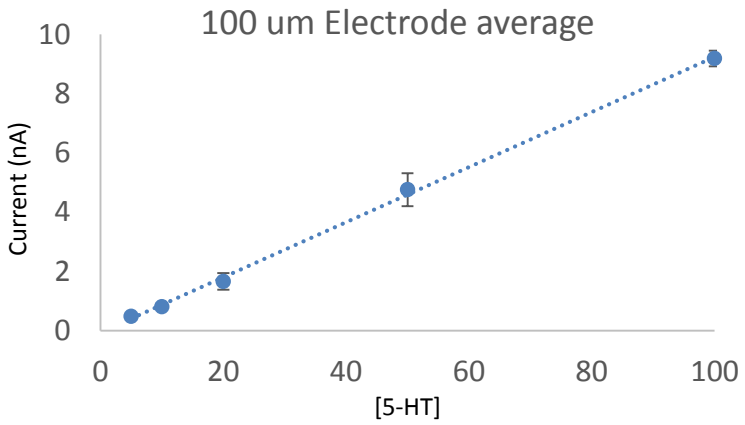


Figure 17. Normalized calibration curve for 5-HT detection (n=2).

2.4 Future Outlook

Both systems designed for this experiment were built identically. In order to continue to compare data between the two systems, the calibrations for the serotonin will need to be completed with a new 5 MΩ headstage. Solutions for the physiological concentration of serotonin, 10-100 nm, will need to be re-made and run. This method of analysis has been validated by previous and ongoing studies from the Hashemi group.[23-25] Although the linearity of serotonin has been demonstrated previously, the change in length of the CFME will have an

effect on the current generated as the signal is proportional to exposed surface area of the electrode. The change in electrode length reduces the amount of surface area exposed to the chemical environment and new calibrations are required to reflect the change in surface area.

Once linearity is re-established, the method needs to validate that the potential interferents and metabolite of serotonin, 5-hydroxyindoleacetic acid (HIAA), at physiological levels, does not affect the serotonin signal and that linearity is preserved. Since each analyte, excluding HIAA, has been independently tested using this method the next step in this process would be to make mixtures of a set concentration of 5-HT with varying levels of octopamine, tyrosine, histamine, and 5-HIAA at the extreme high and low concentration of their respective physiological ranges. Once these measurements have been completed, focus can be shifted towards *in vivo* experiments.

2.5 *In Vitro* Experimental Method

T-650 carbon fibers (Goodfellow, Coraopolis, PA) were aspirated into glass capillaries of 0.4 mm ID and pulled on a vertical pipette puller (Narishige Group, Tokyo, Japan). Electrodes were then cut to a length of 100 ± 5 µM. Next, electrodes were dipped in a 5% v/v Nafion® solution (Liquion-1105-MeOH, Ion Power, DE) by completely submerging exposed fiber and applying a constant potential of -1.0 V vs Ag/AgCl for 10 seconds.[24] The newly coated electrodes were then air dried for 10 min at room temperature, then cured for 10 min. at 80 °C.

EPA synthetic water was prepared from µM 250 $CaCl_2 \cdot 2H_2O$, MgSO4·$7H_2O$, 150 µM $MgSO_4 \cdot 7H_2O$, 50 µM K_2HPO_4, 1000 µM NaNO3, 150 µM NaHCO3, 100 µM $Na_2SiO_3 \cdot 9H_2O$, 388 µM H_3BO_3 100 µM KCl, 11.7 µM $Na_2EDTA \cdot 2H_2O$, 3.7 µM $FeCl_3 \cdot H_2O$, 0.9 µM $MnCl_2 \cdot 4H_2O$, 0.004 µM $CuSO_4 \cdot 5H_2O$, 0.08

μM $ZnSO_4 \cdot 7H_2O$, 0.05 μM $CoCl_2 \cdot 6H_2O$, 0.09 μM $NaMoO_4 \cdot 2H_2O$, 0.012 μM H_2SeO_3 0.01 μM Na_3VO_4, 7.3 μM LiCl, 0.6 μM RbCl 0.07, 0.57 μM $SrCl_2 \cdot 6H_2O$, 0.16 μM NaBr, and 0.02 μM KI.

Tris buffer was prepared in 10X concentration (0.15 M Tris Hydrogen chloride, 1.41M NaCl, 32.6 mM KCl, 13 mM anhydrous $CaCl_2$, 12.5 mM sodium phosphate monobasic monohydrate, 12.2 mM $MgCl_2$, and 20 mM anhydrous Na_2SO_4) then diluted to 1X concentration and buffered to a pH of 7.4. The 200 μM stock serotonin solutions were prepared by addition of 2.2 mg of serotonin hydrochloride (Sigma-Aldrich St. Louis, MO, USA) to 50 mL of the pH buffered 1X Tris buffer solution. From this stock, a 20 μM solution was created for use in analysis.

An electrochemical potential was applied from -0.1 to 1.0 V at a rate of 1000 V s^{-1} at 10 Hz. Application of the waveform and monitoring were carried out using WCCV (Knowmad Technologies LLC, Tucson, AZ) written in LabVIEW (National Instruments, Austin, TX) with a Chem-Clamp potentiostat fitted with a 5 MΩ headstage (Dagan Corporation, Minneapolis, MN). To interface the software with the instrument, a DAC/ADC card was employed (NI USB-6341, National Instruments).

2.6 *In Vivo* Analysis

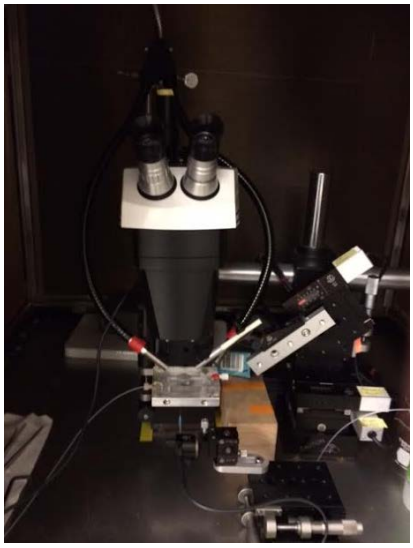
A  B 

Figure 18. A) Experimental setup for in vivo analysis. B) Placement of CFME in *Daphnia*'s brain.

During stimulation with high, medium, and low intensity light an oxidation current was observed at +0.6 V proportional to the strength of light emitted. To determine if this current was a result of noise generated from operation of the light source, trials were conducted in which the light source was directed away from the subject. During the latter trials, no current was generated, indicating that the current generated in the light exposure experiments is a result of serotonin release and not a product of interference from the mechanical switch to turn on the light.

Once confident that there was no interference from the electrical and mechanical devices operated during the trials, stimulation trials were carried out to determine the refractory period of the dive reflex. Simulation patterns were developed by applying maximum intensity light stimulus in 1 and 5 minute intervals as seen in Figure 19. Preliminary data shows that at after 5 minutes the signal generated is mostly stable, but further optimization is required.

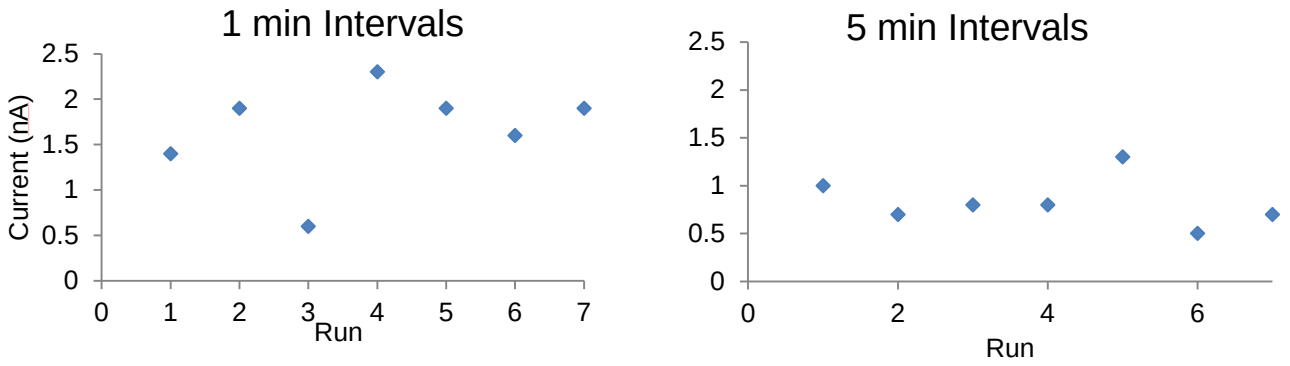


Figure 19. Refractory period of phototactic response.

High intensity stimulus of the phototactic response yielded positive results with an increase in current and an oxidation peak around +0.5 V. Although 5-HT oxidizes at +0.6 V it is known that the redox peak can shift relative to the Ag/AgCl reference due to the unique changes chemical microenvironment.[24] Stimulation with low intensity light was more difficult to quantify as apparent interferences prevented the stabilization of the baseline. Although an oxidation peak was present around +0.6 V, the drifting baseline obfuscated the signal, resulting in an apparent decrease in current from the peak (Figure 20). As this side of the project was working with animal subjects, the issue of electrode placement becomes critical. In order to more accurately quantify the release of 5-HT at low intensity stimulation levels, the placement of the electrode needs to be more precise.

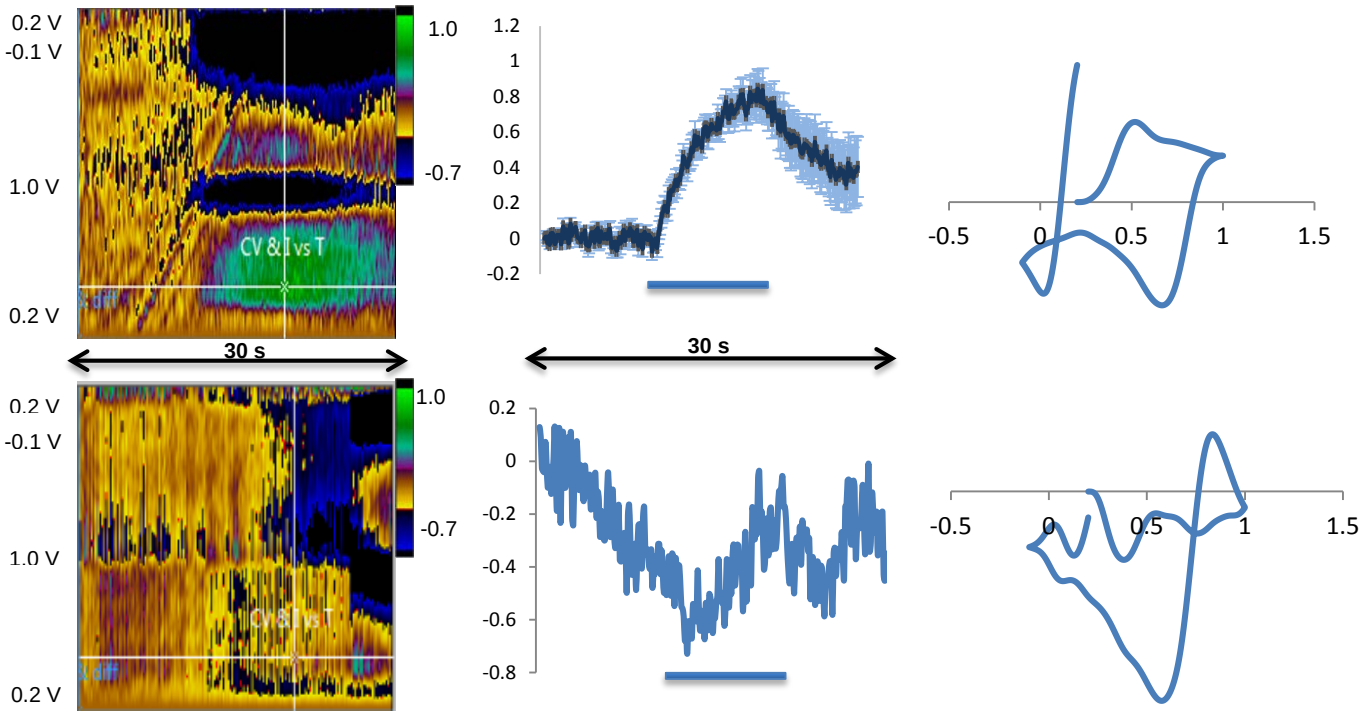


Figure 20. Representative color plot and averaged IT and CV plots for high (top) and low (bottom) intensity stimulation.

2.7 Future Work

As *in vivo* testing is still in its infancy there is much more work to be done. To successfully determine if serotonin is responsible for the phototactic response, more behavioral trials should be carried out. Since the preliminary data gathered relied on 2 trials, more data needs to be collected in order to prove serotonin's involvement. To successfully quantitate the amount of 5-HT released, there are several tasks that need to be completed. First, the placement of the electrode will need to be optimized. Currently, the operation of implantation involves placement through visualization using a microscope. As the *Daphnia* brains are very small, it is difficult to confirm the exact placement of the electrode. Use of a stereotaxic device might improve consistency in placement of the electrode.

To further improve the accuracy of the measurements, electrode length will need to be reduced in order to calibrate post-animal analysis. *In vitro* research has shown that 5-HT signals cannot be generated in COMBO solutions. Therefore, any electrode surface not inside the *Daphnia* does not contribute to the analysis. This is acceptable for *in vivo* work but in order to quantify the amount of 5-HT released, each electrode will need to be calibrated after analysis.

During the post-calibration of the electrode. The surface which was outside of the *Daphnia* did not contribute to analysis. When submerged into Tris buffered saline it will then become active and have the ability to interact with 5-HT. By reducing length of the electrode to match *in vitro* experimentation, this should ensure proper fit of the electrode in the *Daphnia* with minimal unexposed surface, further improving the accuracy of future measurements.

2.8 *In Vivo* Experimental Methods

T-650 carbon fibers (Goodfellow, Coraopolis, PA) were aspirated into glass capillaries of 0.4 mm ID and pulled on a vertical pipette puller (Narishige Group, Tokyo, Japan). Electrodes were then cut to a length of 150 ± 10 µM. Electrodes were dipped in a 5% Nafion® solution (Liquion-1105-MeOH, Ion Power, DE) by completely submerging exposed fiber and applying a constant potential of -1.0 V vs Ag/AgCl for 10 seconds.[24] The newly coated electrodes then were air dried for 10 min at room temperature, then cured for 10 min. at 80 °C.

Daphnia were extracted from a combo buffer solution via pipette and mounted laterally on a custom made pedestal by applying a small amount of polyacrylate glue. The pedestal was then placed in the experimental apparatus flow cell with COMBO medium flowing at a rate of 2 mL/min. A lancet was mounted onto an actuator and used to pierce a hole in the carapace. This process was visualized under a microscope. Once through the carapace the lancet was

retracted and replaced by a CFME, which was inserted into the hole. The CFME was left to equilibrate for 10 minutes using the Serotonin Selective Waveform with a frequency of 60 Hz. The frequency was then adjusted to 10 Hz for data collection. To ensure proper placement, several files were taken to detect the release of serotonin.

The *Daphnia's* dive reflex was stimulated using a Fiber-Lite High Intensity Illuminator Model 170D (Dolan-Jenner, Boxborough, MA). Light intensities were calibrated to NIST traceable light measurement via ILT-1400A Radiometer Photometer (International Light Technologies, Peabody, MA).

Chapter 3 - Pretreatment of Carbon Fiber Microelectrodes for Serotonin Selectivity

3.1 Introduction

3.1.1 Fast Scan Controlled Adsorption Voltammetry

While Fast Scan Cyclic Voltammetry has widely been accepted as an excellent method for analysis of neurotransmitters, it has limitations. FSCV can only measure changes in the amount of analyte present. That is, while excelling in measurements of analyte as a function of change in concentration, basal level measurements are not possible. To address this issue the method of Fast Scan Controlled Adsorption Voltammetry (FSCAV) was developed by Heien and colleagues in 2013 to directly measure the basal concentration of analytes.[26] This method employs a technique which allows for controlled adsorption by holding the electrode at a constant potential without the need to cycle the waveform.

FSCAV can be broken down into three fundamental steps: minimized adsorption (stripping), adsorption of analyte, and FSCV analysis. During the minimized adsorption phase, the electrode is cycled with the analyte specific waveform applied at an over-oxidizing potential with frequency of 100 Hz. This step strips the electrode's surface of any analyte, effectively refreshing the electrode's surface and preventing substantial adsorption. The second step involves application of a constant potential for a specified amount of time, allowing for adsorption of analyte onto the surface of the electrode. In the final step, the analyte-specific waveform is applied, resulting in the oxidation and reduction of the analyte, as in FSCV. The oxidation/reduction peak can then be integrated with respect to time using Faraday's Law to calculate the concentration of analyte on the electrode's surface.

3.1.2 Electrochemical Modification of Carbon Fiber Microelectrode

The technique of electrode modification to increase selectivity has widely been used for many different applications. The most common technique for neurotransmitter detection is electrodeposition of a thin layer of Nafion® on the surface. This technique allows for precise control over the thickness of the coating, rapid modification, and ease of use.[27]

When serotonin is cleared from the synaptic cleft, it happens in one of two ways. The remaining serotonin is either uptaken by the presynaptic cell to be packaged back into vesicles, or enzymes in the synaptic cleft metabolize the serotonin to 5-hydroxyindoleacetic Acid (5-HIAA). 5-HIAA is a negatively charged compound in at physiological conditions (carboxylic acid pKa=4.54) with a high affinity to carbon fiber, is present at 200 to 1000 times the concentration of serotonin, and has nearly identical electrochemical properties to serotonin.

As a waveform is applied to a bare electrode, 5-HIAA will irreversibly bind to the surface, causing a fouling effect. With these characteristics, analysis of serotonin becomes impossible without electrode modification. Nafion® is a negatively charged compound which when deposited on the surface of the electrode can repel 5-HIAA, and increase the selectivity to 5-HT. In our analysis of serotonin via FSCV, the need for a new modification technique arose when analysis of mixtures of 5-HT and concentrations of 5-HIAA near the upper extreme of the physiological range appeared to be skewed by the increasing amounts of 5-HIAA.

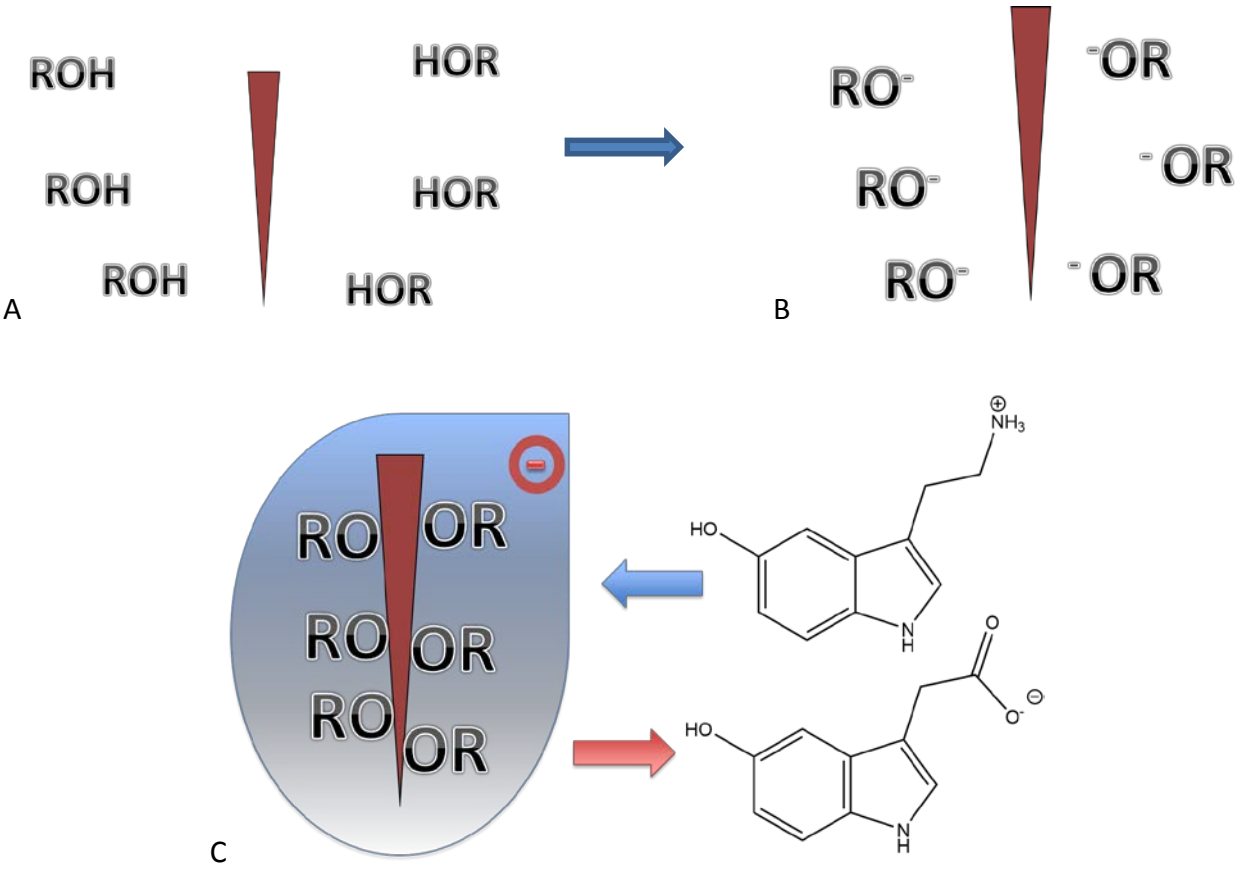


Figure 21. A) Submerged CFME before application of ECO waveform. B) Attraction of oxygen functional groups to the CFME during ECO waveform. C) Increased sensitivity to serotonin due to the oxygen functional groups.

3.2 Electrochemical Modification of Carbon Fiber Microelectrode

3.2.1 Overview

Bare CFME were submerged into Tris buffer solution with pH adjusted to 7.40. Once submerged and checked for good capacitance, the electrochemical oxidation waveform (-0.5 to +1.8 V, 400 V s^{-1}) was applied at a frequency of 10 Hz for two minutes. By applying this large of a potential, compounds in solution are decomposed and generate oxygen functionnal groups which then randomly and irreversibly is adsorbed to the surface of the electrode (Figure 21). Once the oxygen functional groups are bound, the electrode becomes slightly negatively

charged. Like Nafion®, this negative charge further increases selectivity by simultaneously attracting the positively charged 5-HT and repelling the negatively charged 5-HIAA.

The electrode was then placed in a solution of 100 nM 5-HT and 100 µM 5-HIAA. The electrode was allowed to equilibrate, with no waveform, for 5 minutes. Using a modified Selective Serotonin Waveform, with a 0.1 V holding potential, the FSCAV waveform was applied at a rate of 5 minutes and a "cleaning" waveform (-0.1 to 1.3, 0.1 holding potential) was applied at 100 Hz for 30 seconds immediately after each scan was completed to ensure the surface was refreshed.[28] The addition of the oxygen functional groups greatly increased the capacitance of the electrode; which slowly decreased with each successive trial. This led to the need of an ageing process in which the FSCAV sequence was applied until the capacitance and resulting signal stabilized as seen in Figure 22. While the charge is stabilized, this process greatly increases the length of the calibration process.

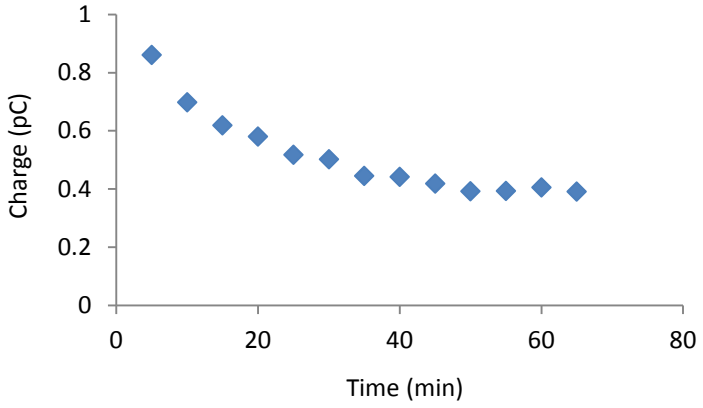


Figure 22. Response to 5-HT during ageing of the electrode.

3.2.2 Characterization

As the electrochemical pretreatment relies on the random nature of adsorption of oxygen functionality, signals vary from electrode to electrode. The normalized signal for the response generated by 5-HT, seen in Figure 23, proved linear within the physiological ranges of the specimen. As such the next step of characterization was to measure the signal generated from 5-HIAA to observe how the electrode responded to the metabolite.

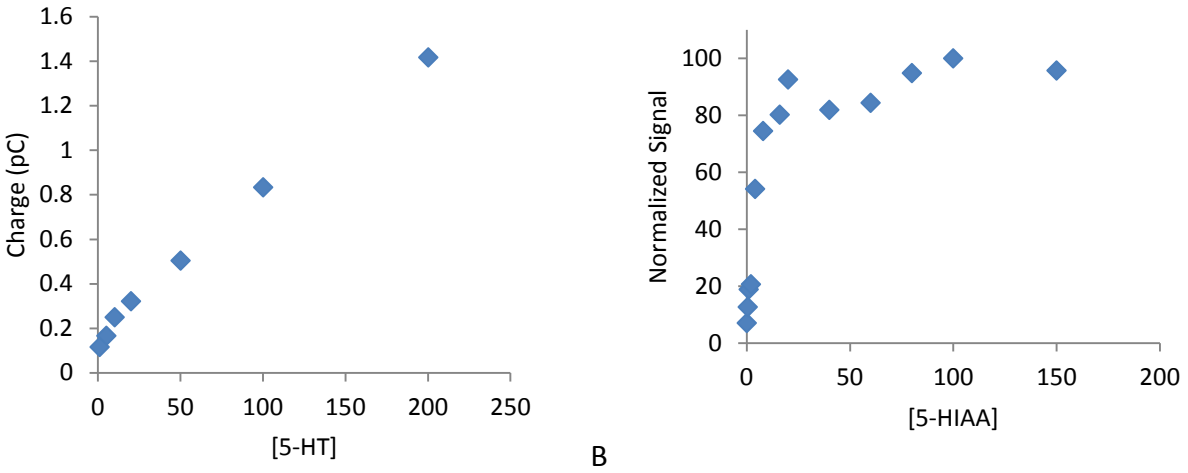


Figure 23. A) Linear range of 5-HT. B) Normalized signal for 5-HIAA.

As 5-HIAA ranges from 200 to 1000 times the amount of 5-HT[18] the performed analysis extended beyond this range in order to obtain a more complete picture. Below a concentration of 20 nM there is a large spike in sensitivity to the metabolite. The response to 5-HIAA appears to plateau from the bottom-end of the physiological level and extend beyond the upper limit. Finally, mixtures of 5-HT at selected physiological levels and 5-HIAA at physiological extremes were analyzed to prove the minimal effects of 5-HIAA on analysis. As suspected, the amount of 5-HIAA had little to no effect on the generated 5-HT signal as the data points for each 5-HIAA concentration with a static amount of 5-HT appeared to be virtually identical (Figure 24).

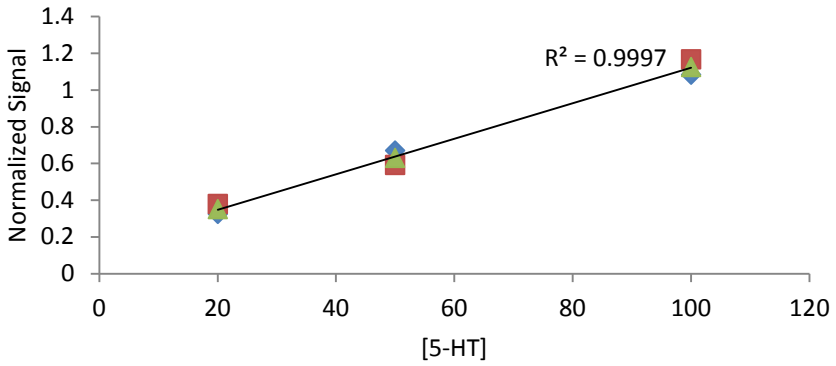


Figure 24. Representative calibration curve. Red squares and blue diamonds represent the extreme lower and upper physiological ranges of 5-HIAA, respectively. Green diamonds are the averaged response between the extremes.

3.2.3 Calibration of Electrodes

In order for *in vivo* analysis of serotonin to work properly, each electrode needs to be calibrated and characterized individually, due to the random nature of the electrochemical modification. Calibrations were carried out in a similar manner to the 5-HIAA interference on 5-HT as mixtures of serotonin, within physiological range, were mixed with the extreme limits of physiological 5-HIAA. The signals were checked to ensure a cohesive response between the varying 5-HIAA mixtures against static 5-HT levels, the linear response from 5-HT was consistent, and the signal generated was sensitive enough to discern minute changes in concentration. Electrodes that did not meet the aforementioned criteria were rejected as the electrochemical modification's random nature made re-treating the electrodes impossible.

3.3 Evaluation of the Electrochemical Modification Method

After the initial method for the electrochemical modification was completed, the technique was moved to the University of South Carolina with the Hashemi group to continue to be evaluated as a viable method for analysis. During this time it was discovered that the integration parameters were improperly selected, as the 5-HT and 5-HIAA peaks were not completely resolved. As the signals were not fully separated, the technique did not account for information that was buried within the 5-HIAA signal. As such, the viability of this method came into question.

Comparing the electrochemical modification to the Nafion® technique showed several issues. First, the electrochemical modification technique required each electrode to be individually characterized before *in vivo* analysis could begin. This process took upwards of 6 hours to fully test and characterize a single electrode. In the event of successful *in vivo* analysis, the electrode then had to be calibrated after the analysis to prove the data generated was accurate. In comparison, Nafion® modification required only 11 minutes to prepare and, as the Nafion® forms a uniform surface, only require post-calibrations to ensure accuracy. With this data in hand, the technique was determined to be unsuccessful and subsequently abandoned.

Chapter 4 – Conclusion

Fast Scan Cyclic Voltammetry has proven to be a highly viable method for *in vivo* quantification of neurotransmitters. Its ability to be modified in a multitude of ways can increase the capability of CFMEs to sense and differentiate between a variety of different chemical species. Although *in vitro* testing in "The Role of 5-hydroxytryptamine in the Phototactic Dive Reflex of *Daphnia*" project has stopped, research is ongoing and the outlook is optimistic. Application of the aforementioned future work could definitely pave the way for a novel method for detection of antidepressants in natural water sources and open up a new area of research to explore.

Although the "Pretreatment of Carbon Fiber Microelectrodes for Serotonin Selectivity" project failed it further narrows the list of acceptable methodologies to chemically separate serotonin from 5-HIAA. As the project involved a lengthy process, the continuation for a new analysis has shifted back to Nafion® pretreatment due to time constraints.

APPENDIX

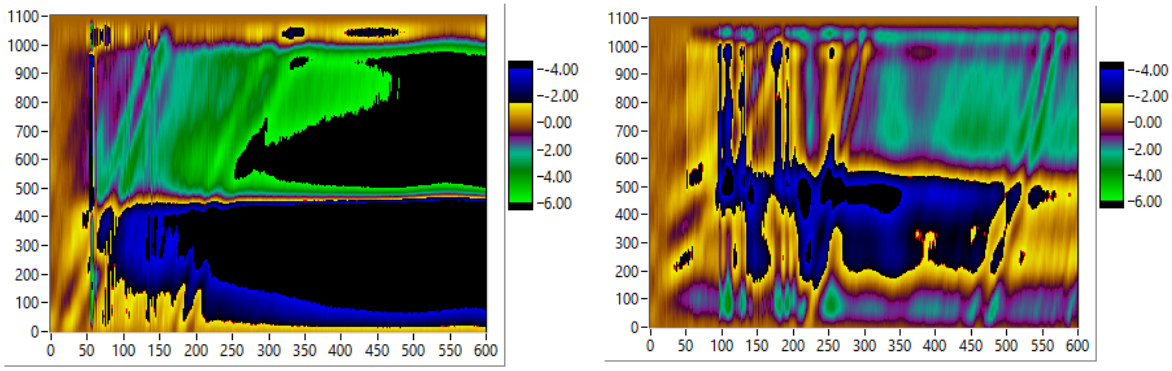


Figure S1. Representative False Color Plots of 100 nM 5-HT in COMBO buffer solution

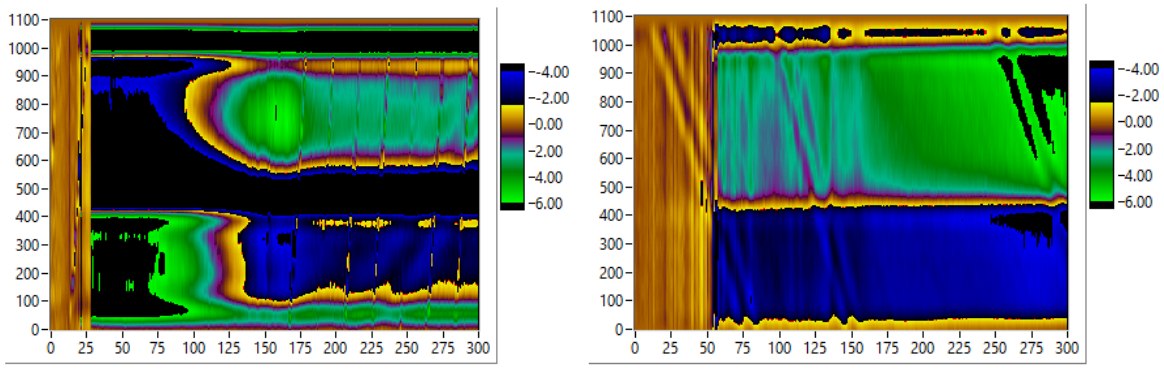


Figure S2. Representative False Color Plots of 100 nM 5-HT in EPA Synthetic Water

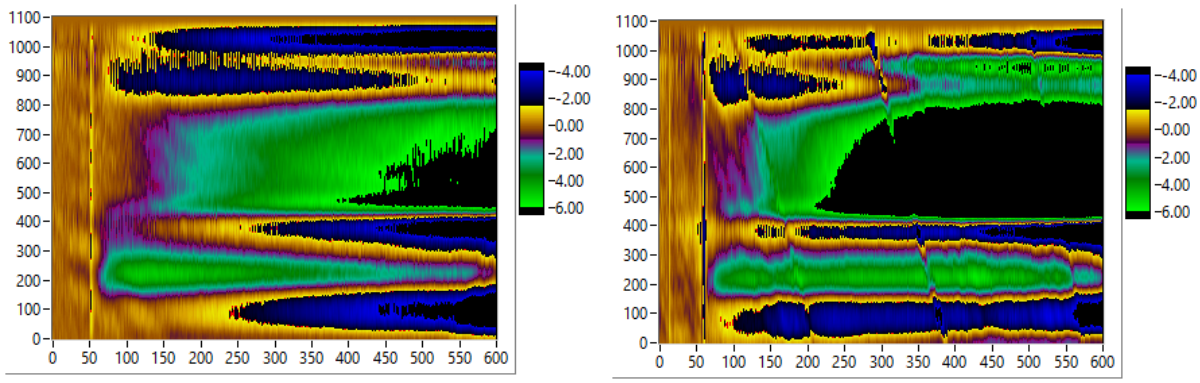


Figure S3. Representative False Color Plots of 100 nM 5-HT in Tris Buffered Saline.

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ABSTRACT

IN VITRO AND _IN VIVO_ MEASURMENTS OF SEROTONIN FOR ECOLOGICAL AND MENTAL HEALTH VIA FAST SCAN CYCLIC VOLTAMMETRY

by

MATTHEW JACKSON

December 2016

Advisor: Dr. Jennifer Stockdill

Major: Chemistry (Analytical)

Degree: Master of Science

Fast Scan Cyclic Voltammetry has been proven to be a highly valuable technique in analysis and detection of neurotransmitters. With this technique, two novel applications are being explored to protect natural water sources as well as furthering the efficacy of antidepressants. Despite setbacks, promising data has been collected to further understand the mechanisms involved in the phototactic response observed in *Daphnia* Magna. Electrochemical modification of carbon fiber microelectrodes has proven to be inefficient as a means to effectively differentiate between serotonin and its metabolite. As such, this unsuccessful attempt has further narrowed down the list of candidates to electrochemically differentiate between 5-HT and 5-HIAA.

Autobiographical Statement

Matt Jackson received his B.S. in chemistry from the University of Michigan – Flint in 2014. While at U of M Flint Matt's research focused on the green synthesis of tertiary alkyl amines. Before starting at Wayne State, Matt interned at Corrigan Oil where he developed methods of analysis for fuel and oil samples. At Wayne State he started with Professor Parastoo Hashemi, focusing on *in vivo* detection and quantification of neurotransmitters. When the Hashemi group left for South Carolina, Matt joined the Stockdill lab, continuing to work on the detection of serotonin in *Daphnia*. Matt started working for Corrigan Oil after departure from Wayne State in July 2016 where he continues to develop new methods of analysis and environmentally friendly metalworking oils.