Determination of ATP with a Firefly Luciferase-Based System for the Detection of Life in Mars.

Author: Jacob T. Shelley

Indiana University – Bloomington

Adviser: Dr. Gary M. Hieftje

Table of Contents

Introduction

Humans have always been enamored with the idea of finding life outside of our own planet. It is difficult to imagine the impact this finding would have on both science and society. Finding primitive forms of life on another planet could not only indicate how life started on Earth, but may also give clues to the interactions primitive life must have with its environment in order to survive and evolve. Also, if life as we know it can exist on another planet, then it could be possible for humans from Earth to inhabit that planet.

It is commonly assumed that in order for life to exist on another planet, the planet must exhibit characteristics similar to Earth. Due to the fact that Venus has an average surface temperature that is over fifteen times that of on Earth and there have been no signs on water on the planet, many researchers believe life might not be able to exist there.[1] More importantly, under such extreme environmental conditions, life from Earth would not be able to survive. Of all the planetary bodies known, Mars is the most likely to support indigenous life as well as the easiest, relatively, to explore and study. Recent findings, however, propose that liquid water once existed on Mars and solid ice exists below the surface.[1-4] Also, the Martian atmosphere is known to contain CO_2, N_2 and O_2, which are needed in all biological systems. The atmospheric pressure is between 0.7 and 0.9 kPa where 95.3%, 3.6%, 2.7%, 0.13% of that being CO_2, N_2, Ar, and O_2, respectively. Additionally, trace amounts of methane gas have been detected in the Martian atmosphere. This finding is significant because methane would not exist long in such an oxidizing environment. This fact would imply that the methane is constantly being produced by some biogenic process or geothermal activity. Because of these facts, there have been a

number of recent missions to Mars in search for life or signs that life once existed on the planet.

The first mission to Mars to look for life used four experiments to determine if life did exist. In 1975, NASA launched the two Viking landers which were designed to look for signs of life based on the detection of chemical substances released when energy is consumed by a life-form. In preliminary experiments the air directly above the soil was examined with gas chromatograph-mass spectrometry (GC-MS), but no organic molecules were detected.[1] In the second experiment, termed gas exchange, a soil sample was exposed to water and different nutrients. After some time, the gas above the soil was analyzed using GC-MS for molecules that would result from metabolic processes. A large amount of molecular oxygen was detected after the soil came into contact with the nutrient solution. Though O_2 production may indicate the presence of life, oxygen was later attributed to heavy oxidants present in the Martian soil. In the third experiment, called labeled release (LR), the soil was exposed to nutrients labeled with ^{14}C. If living organisms were present, then they would consume the nutrients and convert them into CO_2 or CH_4 which would be released and detected with GC-MS. A steady output of radioactive gases was detected coming from the soil after exposure to the nutrients, which was what had been seen in similar experiments performed on terrestrial soils. However, it was later found that exposure to H_2O_2 or the mineral limonite yielded the same results seen on the Martian surface.[1, 5, 6] The final experiment exposed the Martian soil to $^{14}CO_2$ and ^{14}CO and a light which mimicked the sunlight on the planet; this was to test for signs of photosynthesis. It was found that a there was a higher concentration of radioactive materials above the soil after treatment, however this result was still seen even when the

sample was held at 175°C for three hours.[1] There is no known organism that could survive after exposure to such a large range of temperatures. Therefore, it could not be definitively determined if either of the Viking landers had found any form of life on the planet.

More recent missions, the Odyssey orbiter and the Spirit and Opportunity rovers, utilized γ-ray spectrometry and α-particle X-ray spectrometry to look for sub-surface water and minerals indicative of liquid water, respectively. Relatively large amounts of hydrogen were found approximately one meter below the surface, which is assumed to be in the form of ice.[7, 8] Additionally, minerals known to be transported only by liquid water were found deposited in the crevasses of igneous rocks.[4] Two future Mars expeditions, the Reconnaissance orbiter and the Phoenix scout, are geared more towards looking for life itself. Reconnaissance, which launched in August 2005, has instruments which will have the capacity to both map the mineralogy and detect water up to one kilometer below the surface. One of the main experiments for Phoenix is to determine the acidity, alkalinity, and salinity of water that was mixed with Martian soil in order to discover if life could exist in such an environment.

All of the completed or planned studies to determine if life does or did exist on Mars are based on two approaches that are not necessarily correct. First, all of the samples analyzed were soil samples. Terrestrial life needs water in order to survive; therefore directly looking for signs of life in either frozen or liquid water is preferable. Second, these experiments were designed to find byproducts of life, not components that are found exclusively in living entities. The former approach is prone to false-positives because, as seen in the results of the Viking experiments, target analytes can be generated

in ways other than reduction-oxidation chemistry of life. A new approach must be developed that will be able to look for direct signs of life that currently exists or has recently existed.

Before a method to detect life can be developed, it is important to outline a set of criteria needed in order to identify life. The classical biological requirements for life are that it must have an organized structure, use metabolism as means for energy, be able to grow, and have the ability to self-replicate.[9] Under this definition, viruses and prions would not be considered living entities because they do not have the ability to metabolize on their own, nor can they replicate without the use of some other life-form. From a chemical standpoint, life is a complex process by means of which energy is used to perform oxidation and reduction reactions to sustain an internal process. Therefore from a chemical point of view life can be thought of as a flow of electrons in a carefully controlled, step-wise fashion. Adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD) are key molecules in the transfer of these electrons. ATP and NAD can, thusly, be considered as some of the most universal markers for life which are needed in all of the biological criteria listed above. More importantly, ATP and NAD are exclusive to living creatures; as far as we know, only living entities can create these molecules. As such, the detection of ATP and/or NAD is a valid and viable proof of the existence of life.

Although there is not *a priori* reasons from a biological standpoint for choosing between these two molecules, from an analytical point of view the detection of ATP at very low levels is more simple and understood. One way to detect ATP at extremely low levels is through a sensitive enzymatic assay which incorporates the firefly luciferin-

luciferase (LL) reaction.[10, 11] When firefly luciferase is combined with the substrate luciferin in the presence of ATP and two cofactors a photon of light is emitted at about 560 nm.[10, 12] The mechanism for the reaction is given below with the overall reaction given in Figure 1. Roughly, the reaction consists of the enzymatic oxydation of luciferin to oxy-luciferin, in the presence of ATP. The oxy-luciferin is formed in an excited state, and it decays through a radiative process. The amount of light produced is proportional to the amount of ATP consumed.[10-13]

1. Rapid reversible reaction: esterification of luciferin:

$$\mathbf{LH_2\text{-}COOH + ATP + E \rightleftharpoons E\text{-}LH_2\text{-}COO\text{-}AMP + PP_i}$$

2. Rate determining step: detachment of a proton and formation of a carbanion, which reacts with O_2 yielding excited oxyluciferin, which emits a photon:

$$\mathbf{E\text{-}LH_2\text{-}COO\text{-}AMP + O_2 \longrightarrow E\text{-}oxyL\text{-}AMP + CO_2 + Light\ (560\ nm)}$$

3. Slow dissociation of the enzyme-product complex:

$$\mathbf{E\text{-}oxyL\text{-}AMP \longrightarrow E\text{-}oxyL + AMP}$$

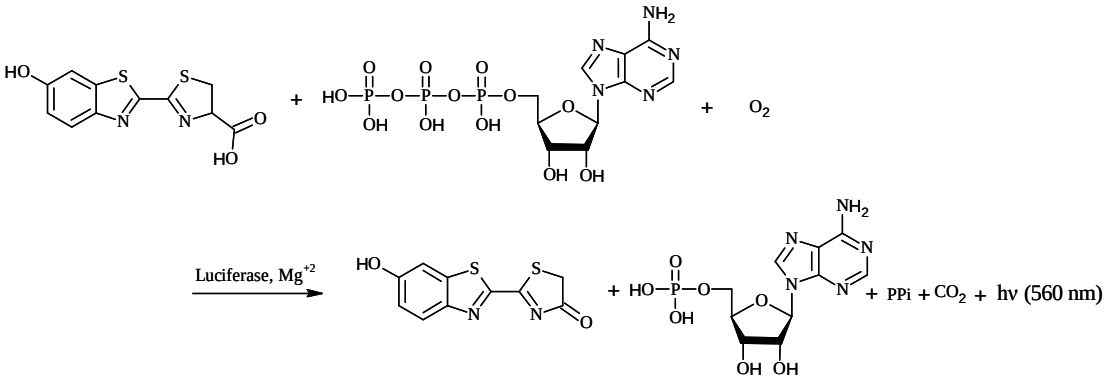

Figure 7. Overall reaction of the LL reaction where luciferin combines with ATP to yield oxyluciferin and AMP.

This reaction scheme is desirable for the detection of ATP because the quantum yield is 88%[11, 14]; therefore, approximately one photon is emitted for every molecule of ATP that reacts.[14] Under typical conditions, the reaction yields a "flash" of photons lasting less than one minute because all of the substrate binds to the enzyme.[15, 16] However, enhancers and coenzymes can be added to the buffers to create a steady output of light until all the ATP has been converted in adenosine monophosphate (AMP).[13] The Promega Corporation distributes a LL assay where the light output is amplified and changed from a quick flash of light to a steady light output. Figure 2 is a comparison between the photon emission for a typical "flash" reaction and for the Promega Enliten® kit.[17]

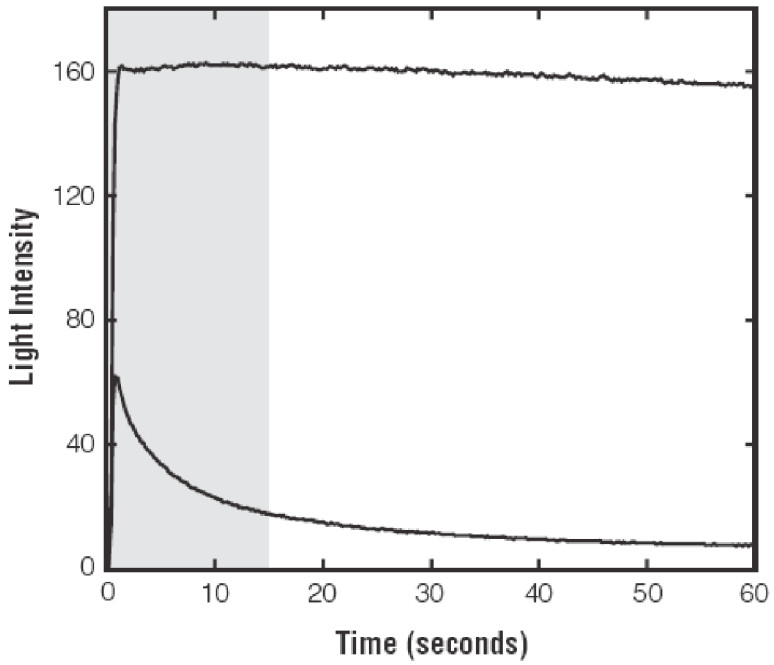

Figure 8. Light intensity versus time (s) for the typical "flash" reaction (bottom) and the Eliten® assay optimized reaction (top).[18]

Currently, the LL reaction is the basis of standard procedures for the determination of ATP in different media. Typical limits of detection in ATP-free water are in the femtomolar region, but with amplification can be reduced to sub-attomolar levels.[19]

However, the presence of Group I cations (Na+, K+, etc) elevates the detection limit to the picomolar level.[20] Several efforts have been devoted to improve the analytical performance of this detection scheme, such as the study of quenching effects of different buffers and ions in solution[20-23], or amplifying the number of photons emitted per ATP molecule to lower the detection limit.[13, 23, 24] Work to optimize reaction conditions and detection have mostly been performed independently of one another; one focus of this project will be to combine the work done by others to fully optimize the detection of ATP.

The purpose of this project is to develop the methodology and instrumentation required in order to use the LL reaction to detect ATP present in either frozen or liquid water present on Mars. Because ATP is only known to be produced by living organisms, if ATP is detected on Mars, then it is likely that life either exists or recently existed. Though minimal levels of ATP can be detected via the LL reaction, it is important to stress that these reactions are performed under very controlled experimental conditions. Using this reaction in a remotely operated, field instrument on samples with unknown composition presents several chemical and instrumental challenges. In the design and development of instrumentation to detect for the presence of life, certain assumptions had to be made. If liquid water is present on Mars it would be below the permafrost level, which would require some sort of drilling to access. Therefore, the energy consumption of the instrument can be ignored because a large energy source must be present to power the drills. Also, any water present on Mars would most likely have high salinity much like the oceans on Earth which could severely quench the emission from the LL reaction. Similarly, detergents used to extract ATP from cells could also serve as quenching agents. Finally, if life were to exist on Mars, the number of cells present would most likely be very

small and contain little ATP because of the harsh conditions present and a lack of nutrients. Ultimately the instrument must be compact, rugged, and either remotely-operated or entirely self-operating.

In this work, a manual system was developed for measuring the output generated by the LL reaction and minimizing the detection limits to a point where the sensitivity is limited by background contamination. Next, a flow system was developed in order automate the analysis of samples and reduce the possibility of background contamination. Finally, the quenching effects of different matrixes on the reaction were examined: an artificial brine that mimics water on Mars, and two detergents, Triton X-100 and Triton N-101, which are often used for the lysis of cells.

Experimental

Reagents. All studies were performed using the Enliten® ATP assay system (Promega Corporation, Madison, WI) by mixing 100 μL LL assay reagents in the supplied buffer with 100 μL of sample. All samples contained a known amount of ATP standard supplied with the Enliten® ATP assay system with different matrixes. Matrixes used were ATP-free water (included in kit), Triton® X-100 (laboratory grade, Sigma-Aldrich, Madison, WI), Triton® N-101 (laboratory grade, Sigma-Aldrich, Madison, WI), or an artificial seawater solution as per Kester et al. (Table 1). All salts used in the artificial brine were reagent grade from Sigma (Madison, WI).

Table 1. Salts and concentrations used for artificial seawater.

		Mass of Salt (g)	Salt Conc. (mM)
Anhydrous Salts	NaCl	4.2611	372.57
	Na_2SO_4	0.7262	26.13

	KCl	0.0962	6.59
	$NaHCO_3$	0.0354	2.15
	KBr	0.0200	0.859
	H_3BO_3	0.0058	0.48
	NaF	0.0005	0.06
Hydrated Salts	$MgCl_2 \cdot 6H_2O$	1.9355	48.641
	$CaCl_2 \cdot 2H_2O$	0.1440	5.005
	$SrCO_3 \cdot 6H_2O$	0.0077	0.15

Emission spectrum. An emission spectrum from 470 nm to 700 nm was collected using a Cary Eclipse fluorescence spectrophotometer equipped with a Hamamatsu R928 photomultiplier tube (PMT). The operating voltage of the PMT was 800 V. The emission spectrum was taken as the average of 16 scans from a sample containing 50 nM ATP with a total reaction volume of 400 μL. Such analyte levels were necessary to provide a sufficient S/N to get an accurate representation of the emission spectrum.

Batch detection. Two different detection systems were used for the measurement of light emission from the standard reaction. The first consisted of a cuvette holder placed in front of a Hamamatsu R446 PMT inside of a darkbox with the amplified signal recorded on an Apple Quadra 700 at 200 Hz. The second consisted of a homebuilt cuvette holder attached directly to the PMT case of a Hamamatsu R1527 PMT with the amplified signal recorded using a Dell computer equipped with a National Instruments Data Acquisition Card at 20 Hz. Both PMTs were operated at 950 V, powered with a Bertran high-voltage power supply (205B-03R), and amplified with a Stanford Research Systems low-noise current-amplifier (SR570). In order to minimize background contamination, a face mask and gloves were always worn when working with solutions. In addition, pre-sterilized, aerosol-free pipette tips and disposable, micro-volume cuvettes were used when mixing reagents. All data sets were smoothed by using a moving average with a 50 ms averaging

time. Output signal was taken as an average over a 15 s interval. Limits of detection (LOD) and limits of quantitation (LOQ) are reported as 3σ and 6σ, respectively. Experimental and instrumental LOD's are taken as 3σ of background noise and blank signal, respectively.

Flow system. A flow system was constructed with a 6-port injection valve (V-451, Upchurch Scientific) and a peristaltic pump. The output of the 6-port valve was connected to a home built flow-through cell that was mounted directly in front of a Hamamatsu R928 PMT, which was operated in the same conditions as in the manual set-up. When the valve was in the load position, spectroscopically pure water was passed through the system while the sample loop was manually filled with the reaction mixture by a syringe. In the inject position, the sample loop was inserted in the flow line allowing the reaction products to pass in front of the PMT. The sample loop volume, reaction volume, and flow-through cell volume were found to be 207 μL, 180 μL, and 191 μL, respectively. A standard concentration of 1.0 nM (200 fmol) ATP was used for all flow system experiments. Data was smoothed using a four second moving average. Signal was recorded as an average over one second at the maximum signal intensity.

Results and Discussion

Batch detection. The emission spectrum collected using the Enliten kit reagents is given in Figure 3. The wavelength of maximum intensity, λ_{max}, for the Enliten assay was

found to be 556 nm. Reported values of λ_{max} for the typical LL reaction range from about 550 nm to 570 nm. The large range of values for λ_{max} can be attributed to the influence of both the solution ion content and pH. Initially a comparison was going to be made between detection limits of the commercial fluorescence spectrophotometer and of the various detection setups described above, but the signal was too low, even with high ATP concentrations, to have reasonable S/N for comparison.

Each batch detection setup was characterized by comparing the calculated experimental and instrumental LOD. A calibration curve was generated for each system to find the linear dynamic range and lowest measurable concentration of ATP. The calibration curve for the R446 PMT setup is given is Figure 4. The calculated experimental and instrumental LOD's were 1.44 nM and 1.39 nM, or 288 fmol and 277 fmol, respectively. The calibration curve exhibits very good linearity over three orders of magnitude of concentration, however, commercial luminometers are able to detect much lower ATP concentrations using the LL reaction. It was determined that the R446 PMT was not the best detector to use for detecting low levels of light at 560 nm because not only is peak sensitivity wavelength 330 nm, but the typical dark current is 3 nA. Also, the recording system used, Apple Quadra 700, had a very low response time and needed to perform signal averaging while recording in order to operate, which would make flow injection analysis very difficult to perform.

A more sensitive PMT, R1527, and more reliable recording system were then characterized in the same fashion as with the R446 system. The calibration curve for the R1527 system (Figure 5) shows higher sensitivity, five times the photocurrent output, and larger linear dynamic range, almost five orders of magnitude, than the R446 system. The

experimental and instrumental LOD's were calculated to be 200 amol and 49.4 amol of ATP, respectively, which demonstrates that this system is currently blank limited. The background contamination is higher than our system instrument noise. The R1527 PMT was found to have about 20 dark counts/s.

Matrix analysis. With a sensitive detection system built and characterized for an ideal LL reaction, the next step was to examine the quenching effects of anions and detergents on the reaction. The possible ways in which an atom or molecule can quench luminescence are static, dynamic, and Förster quenching.[25] Static quenching occurs when the quencher and the luminescent molecule complex, which does not allow for excitation and thus no emission can occur. Dynamic quenching is a result of nonradiative energy transfer from the molecule in the excited state to the quencher; therefore the luminescent molecule and quencher must come into close proximity with each other. This means that the quenching rate is controlled by diffusion. The Stern-Volmer equation gives a relationship between the bioluminescent intensity, I_{BL}, and the quencher concentration, [Q]. This equation is given below where I_{BL}^{0} is the unquenched luminescence and K_q is the Stern-Volmer quenching constant.

$$\frac{\phi_{BL}^{0}}{\phi_{BL}} = \frac{I_{BL}^{0}}{I_{BL}} = 1 + K_q[Q]$$

If the LL reaction is experiencing dynamic quenching, then it should exhibit Stern-Volmer behavior.

First was the analysis of quenching effects of the artificial seawater on the reaction. A sample containing 1 nM ATP was analyzed with varying concentrations of brine concentrations (Figure 6). The quencher concentration was taken to be the chloride ion

concentration because it is the most abundant ion present in the artificial seawater (Table 1). When a quarter of the reaction volume was the brine solution, the signal was quenched by over 80%. This demonstrates that some sort of reaction quenching is occurring, so the inverse of the signal was plotted against the quencher concentration to yield a Stern-Volmer plot (Figure 7). This relationship is fairly linear suggesting that the reaction is being quenched dynamically with a quenching constant of 0.00387 M^{-1}. These findings demonstrate that any ions that could be present in Martian water will pose a large problem with this detection system. It will be essential to develop a way in which to significantly decrease the amount of ions present in solution.

The next set of potential quenchers that were analyzed was detergents. Detergents will be needed to extract the ATP from the cells. The effect of varying amounts of Triton N-101 and Triton X-100 detergents on signal for a sample containing 1 nM ATP is given in Figure 8. It is apparent that the presence of detergents did decrease the signal, but it was only by a maximum of about 10% and 30% for Triton N-101 and Triton X-100, respectively. One interesting feature was that there is an increase in signal between 0.05% and 0.1% Triton X-100. Fluorescence intensity is known to increase in the presence of micelles from SDS and other detergents. The increase in signal is likely due to the detergent in solution being above its critical micelle concentration (CMC). However, more experiments will have to be performed to determine if that is the reason for the increased signal.

Flow system. Another requirement for this detector is that it must be able to be remotely operated, so the sensitivity of the detection system needs to be explored with a flow system. The flow experiments were performed before the use of the R1527 PMT

and thus there was more noise and less sensitivity present in the signal. The first use of the flow system was to determine if signal could be detected and if the transport process (physical dispersion of the sample plug) decreased the signal severely. This was done by measuring a 1 nM ATP sample in a continuous flow mode and a stop-flow mode (Figure 9). Stop-flow techniques are very useful in reactions that are under kinetic control, so this approach might be used as a way of increasing the reaction time and signal averaging time, thus improving the detection limits. Next, the effect of flow rate on the signal was explored (Figure 10). Most importantly, when the flow rate is altered, the maximum signal stays constant with the exception of 0.5 mL/min. Because of this information, signal can be taken as the peak height. Finally, the same studies using the artificial seawater matrix were performed with the flow system (Figure 11, 12). As with the batch detection, the quenching exhibited a strong Stern-Volmer relationship. A quenching constant of 0.00415 M^{-1} was found using the flow system, which agrees closely with the number seen in the batch detection with only 7% difference between the values. Many aspects of the flow system need to be improved and explored in future work. Mainly, applying the flow system to the more sensitive detection setup and reducing stray light in the system.

Conclusions:

- The luciferin reaction is a promising approach for the detection of life in Mars.
- The reaction allows the detection of extremely low amounts of ATP (attomole levels) with a simple instrumental setup.
- Further work needs to be conducted in order to evaluate the robustness of this approach in a “real” (Martian expedition) situation.

- Additionally, further work is required in order to design an autonomous system able to operate in such harsh conditions while maintaining sensitivity exhibited by batch detection.

Future Work

Through this work, a sensitive instrument capable of detecting low levels of ATP (attomoles) has been built and characterized; future work will be directed toward determining the influence of matrix effects on the reaction. A calibration curve needs to be made for ATP in a constant concentration of the artificial seawater to see if the quenching effects are more severe at lower ATP concentrations. Additionally, investigating the quenching/enhancement effects of each of the salts in the brine solution is essential to definitively determine if chloride ions are in fact the predominant quencher. Also, more experiments need to be performed with the detergents to determine if the bioluminescence is being enhanced by the Triton N-101 at concentrations higher than the CMC. It is also known the cyclodextrins increase fluorescence signals, however little work has been conducted on the effects on bioluminescence. Thus, the enhancement or quenching effects of α, β, and γ-cyclodextrins on the reaction will be studied in detail. Finally, the LL reaction will be probed in conditions that would mimic those expected on Mars, i.e. by controlling temperature, oxygen pressure, and introducing high levels of oxidizing agents into the samples.

In order to lower the experimental detection limits it will be essential to minimize background contamination. Including mixing tee's in the flow system which would allow for LL reagents and sample to be mixed online just before the PMT should eliminate a

significant portion of the environmental contamination. To reduce contamination further, all solutions and materials will be sterilized to minimize the amount of internal contamination. After these considerations have been implemented, it is likely that instrument noise will be reduced further only through reducing the temperature of the PMT.

Once the detection limits and noise have been reduced, it will be necessary to quantify the amount of ATP present in actual cell samples. To do this, the cells will have to be lysed and the ATP extracted while at the same time inactivating ATPase and other enzymes that could breakdown the ATP. This process can be prevented through the use of detergents or boiling water. A commercial assay, BacTiter-Glo (Promega)[26] is also now available in which there is no need to wash or lyse the cells. Buffers used for the reaction contain substances that will extract the ATP after a five minute incubation period. The efficiency of this assay on *E. coli* cells will be compared to other ATP-extraction methods found in the literature. Once all the methods have been optimized for well-known systems, analysis can be performed on cell samples that would be similar to the types of cells expected in the harsh environment of Mars. This analysis will serve two purposes: ensure that very small numbers of unhealthy, tough cells can be detected/measured and give insight on how much the signal will be quenched. Because it was found that anions present in solution will heavily quench the signal, it will be important to develop a method to minimize the quenching effects. One idea for reducing or eliminating the quenching is to spin the cells out of solution (centrifugation), rinse away the supernatant, and extract the ATP into ATP-free water. Other ideas include filtering the cells out of solution, then extracting ATP through and using dialysis to reduce the

concentration of quenchers. Long-term goals are directed towards modifying this device to fit on a microfluidic device to permit remote-controlled operation, similar to devices currently used by Liu et al.[27]

Acknowledgements

I would like to acknowledge everyone in the Hieftje laboratory for all their guidance, especially Dr. Francisco Andrade for his expertise on fluorescence/luminescence and flow injection analysis systems. Dr. Faming Zhang's group for graciously letting me borrow small-volume pipettes and Dr. Richard DiMarchi's group for their insight on biological uses of the LL reaction. Funding is provided by a NASA grant through Dr. Lisa Pratt in the Department of Geology.

References

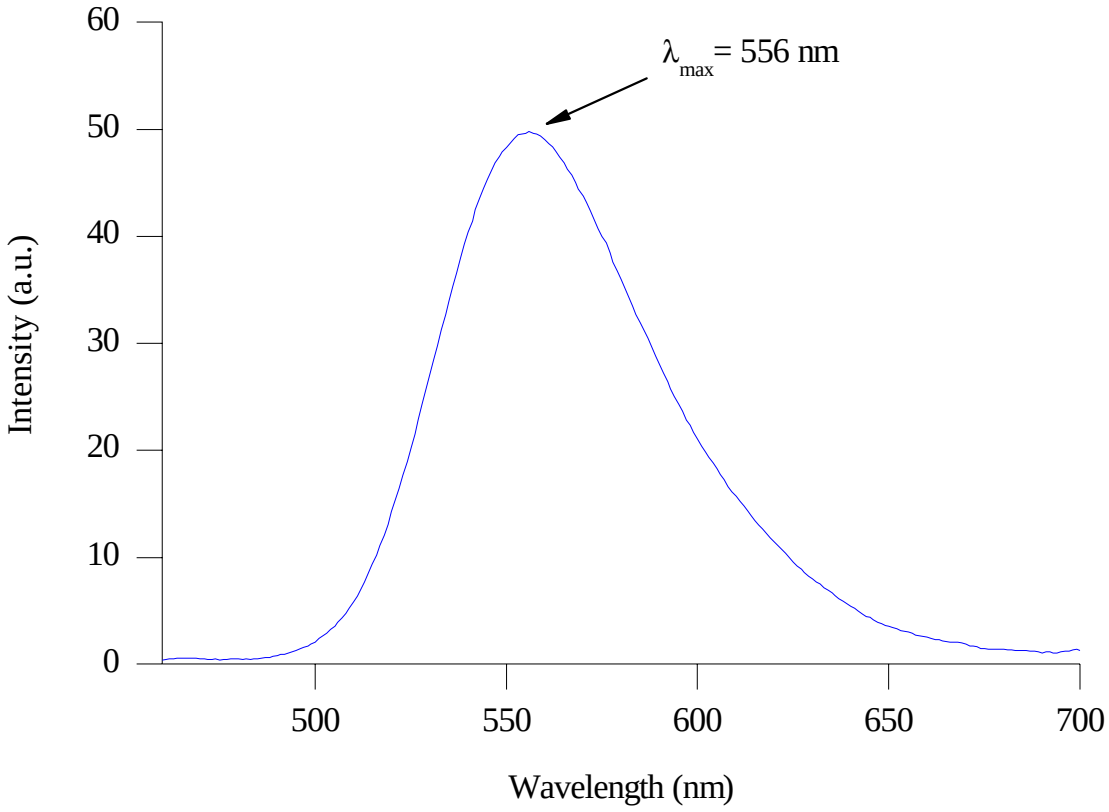

Figure 9. Emission spectrum, 450 nm to 700 nm, of firefly LL reaction using Enliten assay.

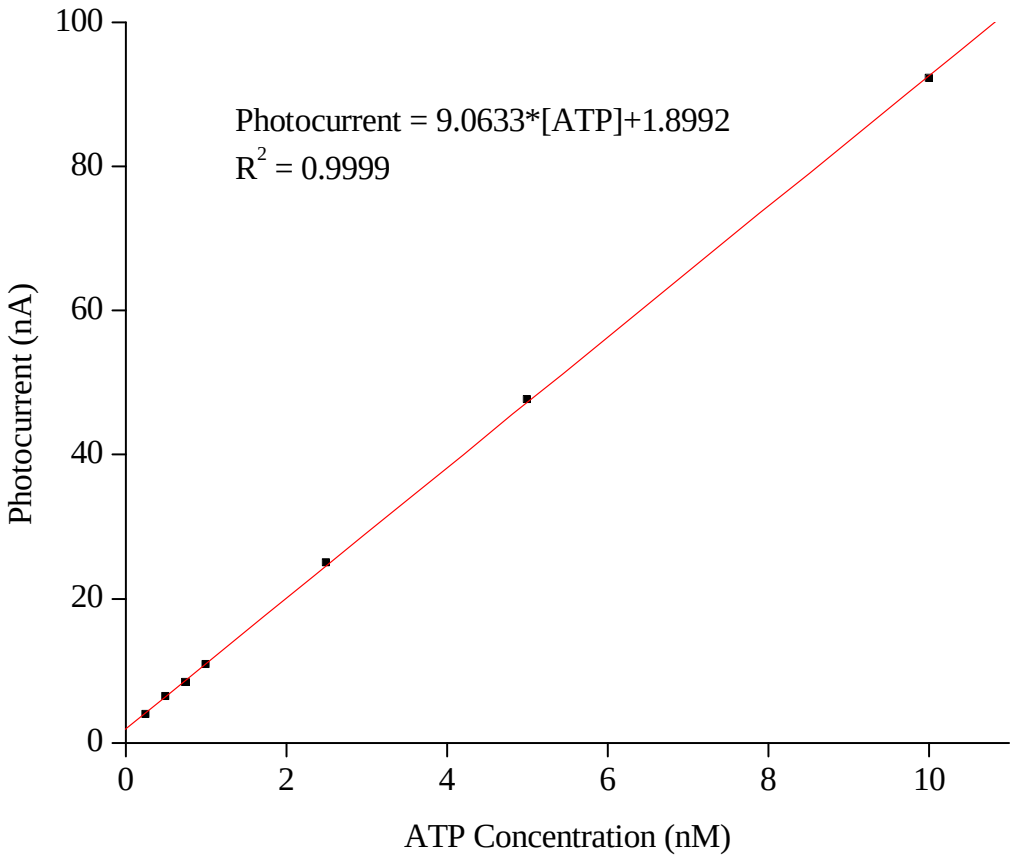

Figure 10. Calibration curve using the R446 PMT setup.

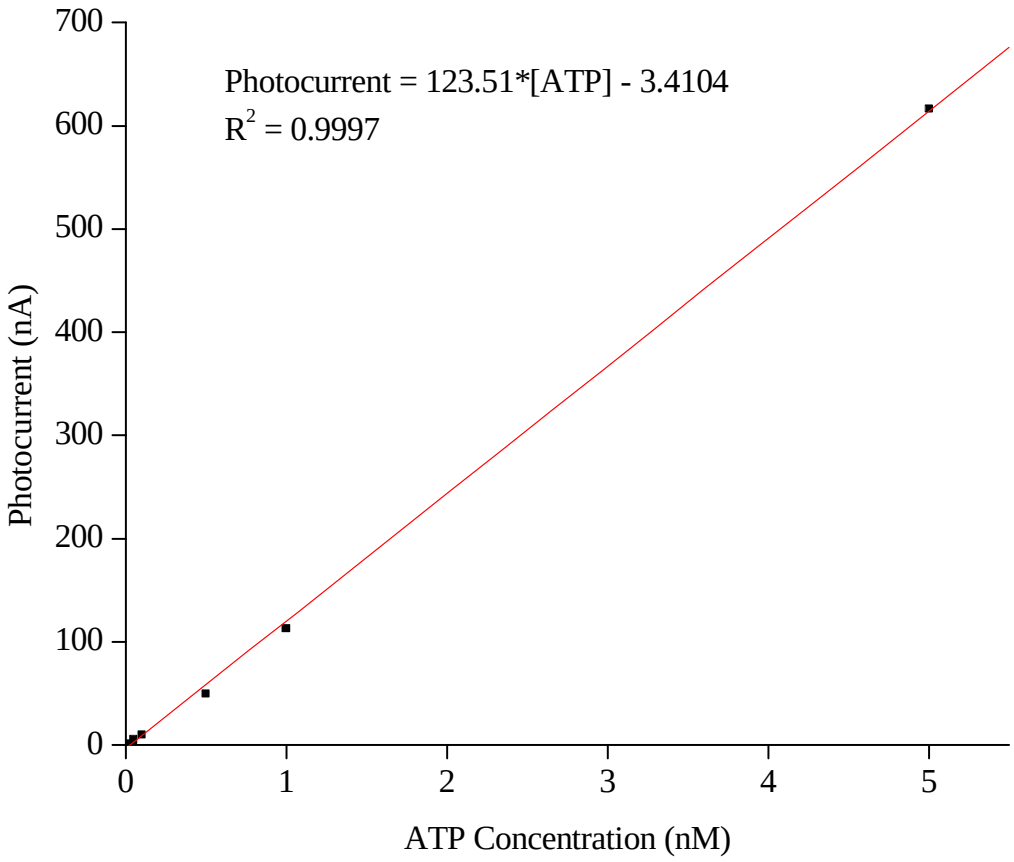

Figure 11. Calibration curve using the R1527 PMT setup.

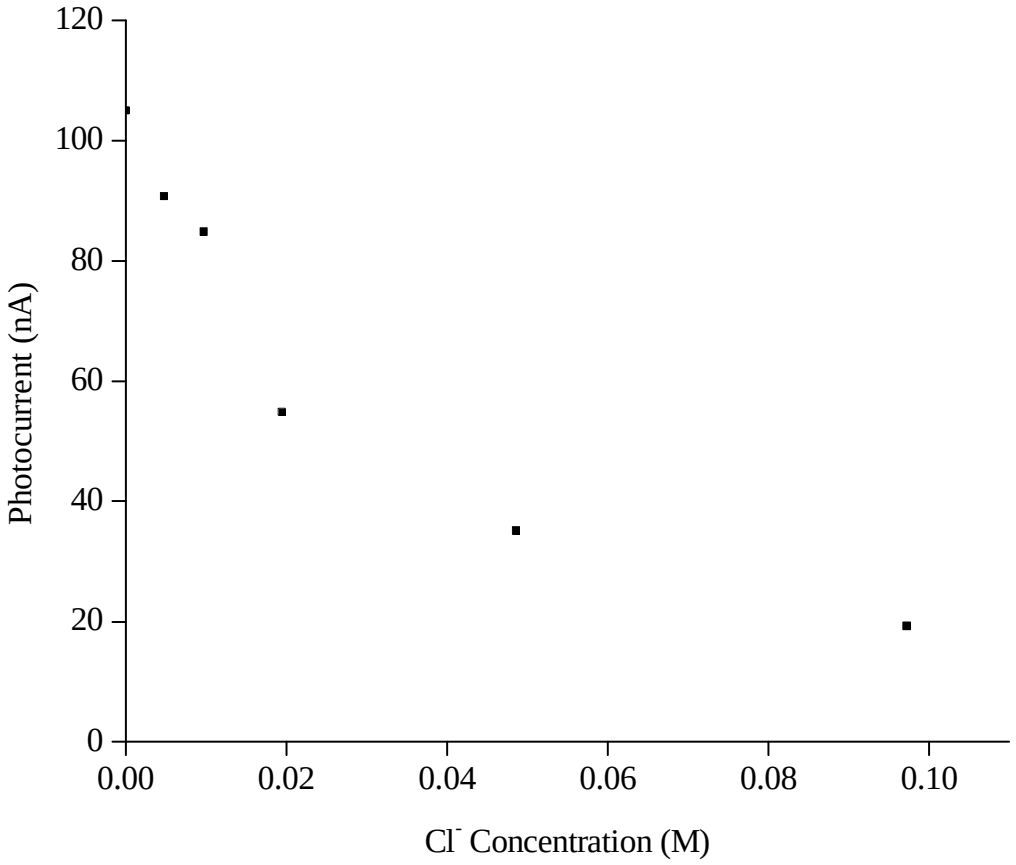

Figure 6. Quenching effects of artificial seawater, expressed as Cl^- concentration, on LL reaction signal for a 1 nM ATP sample recorded using the R1527 batch detection system.

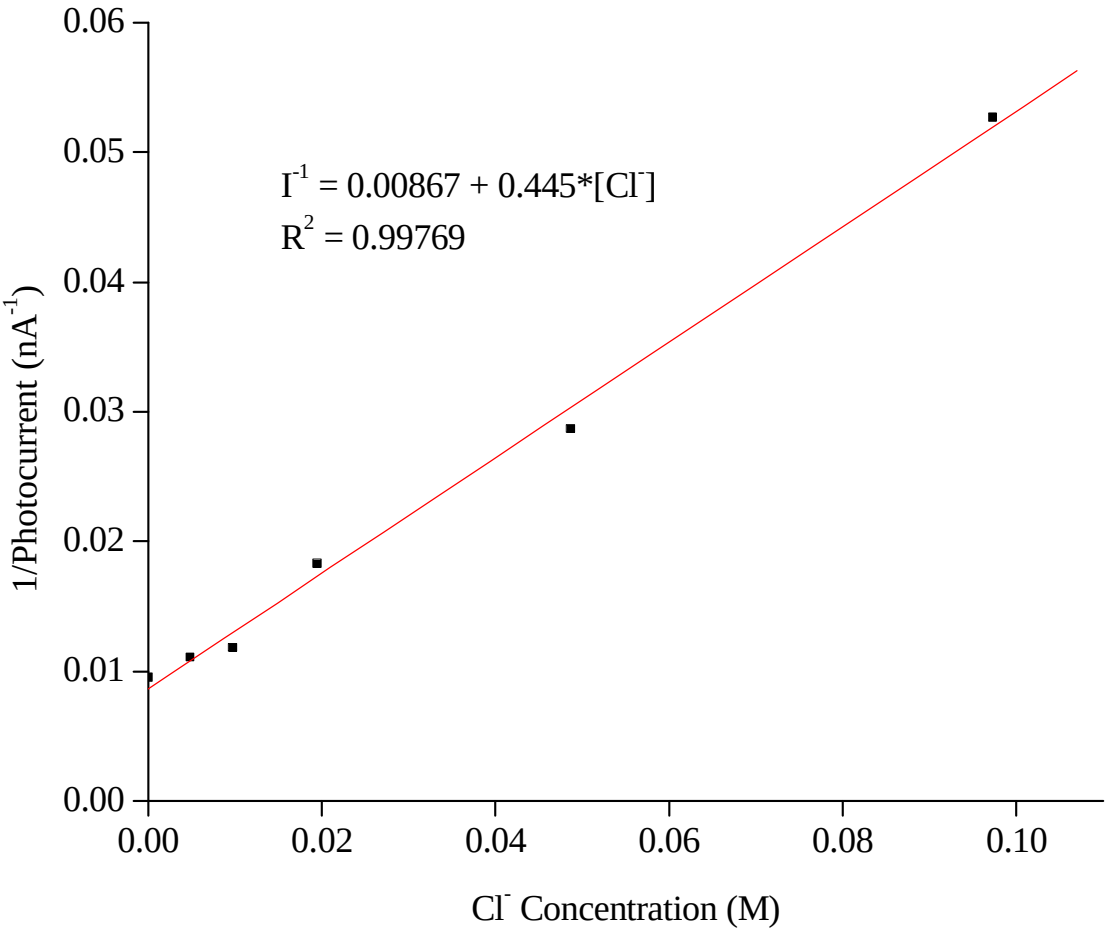

Figure 7. Stern-Volmer plot of data from Figure 6 showing dynamic quenching from artificial seatwater anions.

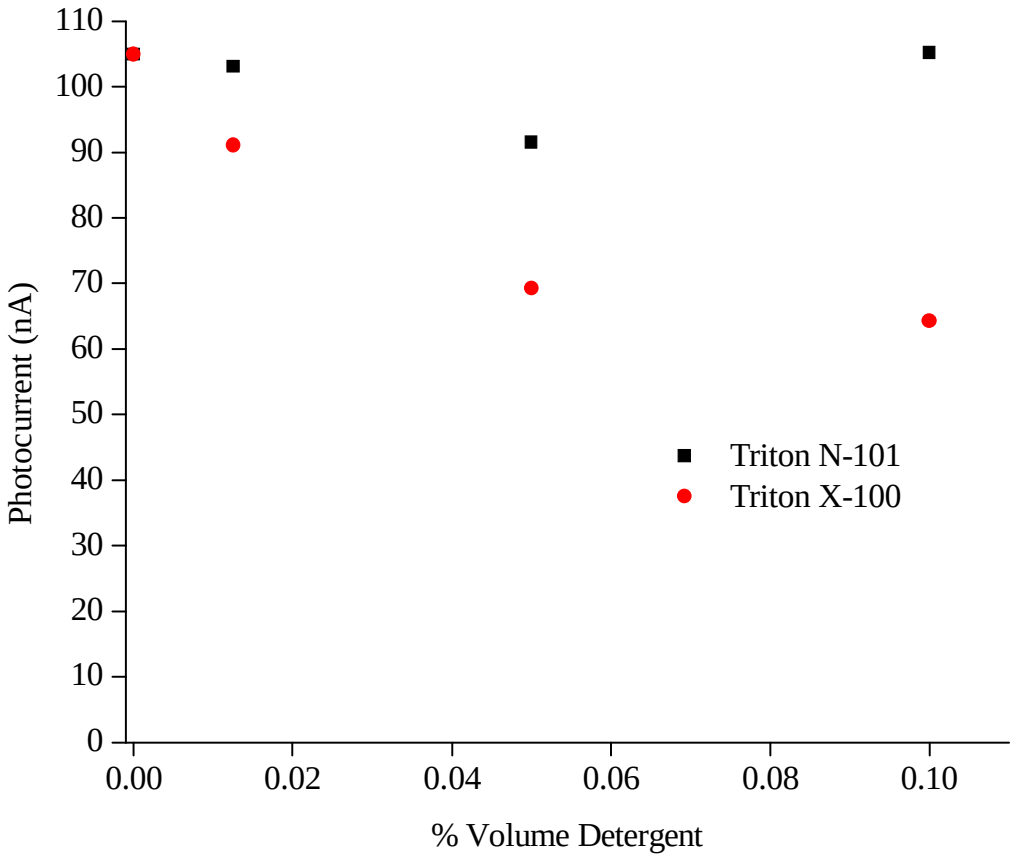

Figure 8. Effect of Triton N-101 (black) and Triton X-100 (red) detergents on signal from a 1 nM sample recorded with R1527 batch detection system.

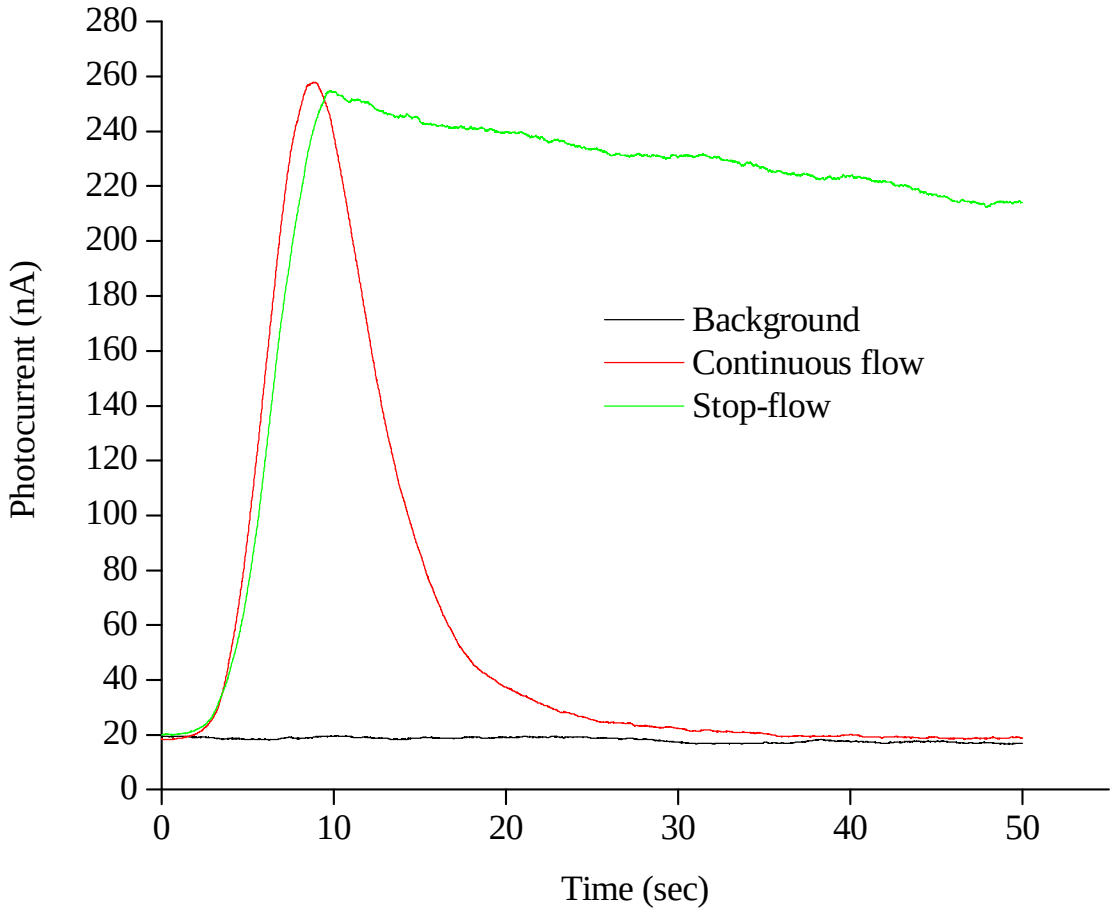

Figure 9. Comparison of background (black), continuous flow (red), and strp-flow (green) signals versus time for samples containing 1 nM ATP.

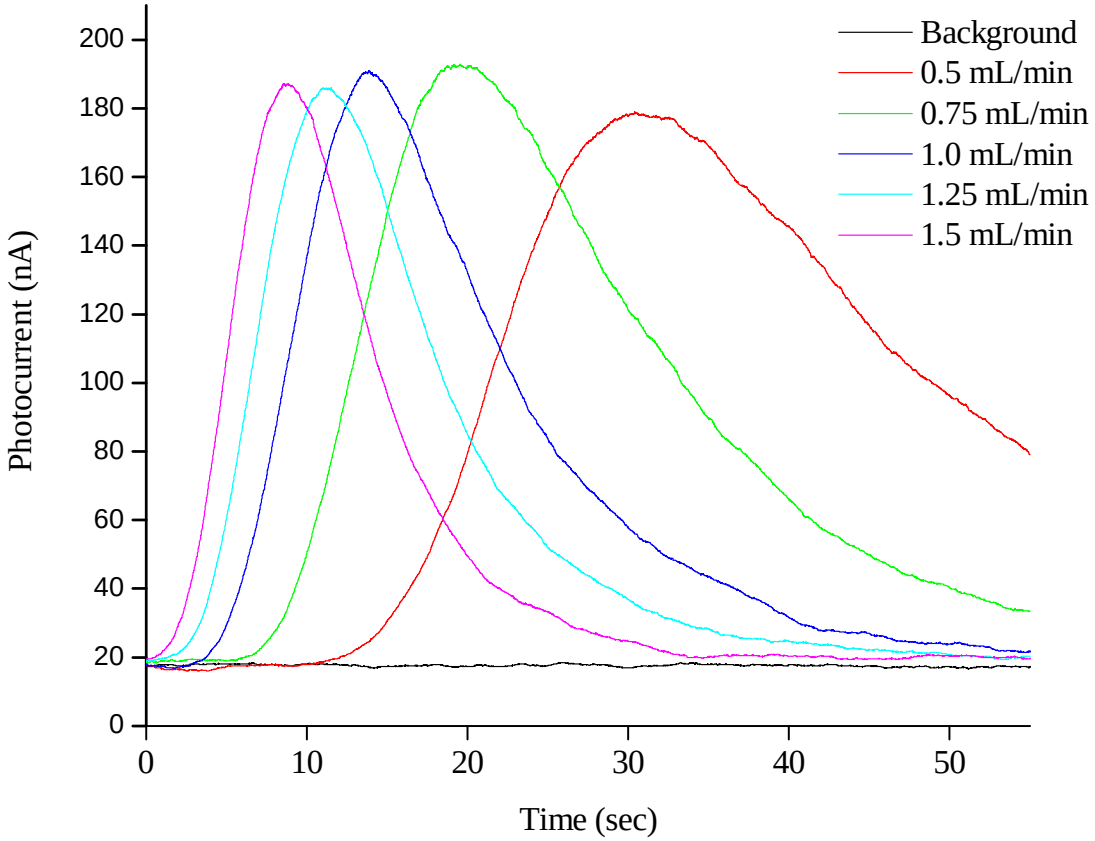

Figure 10. Comparison of time-signal traces at different flow rates for a sample containing 1 nM ATP.

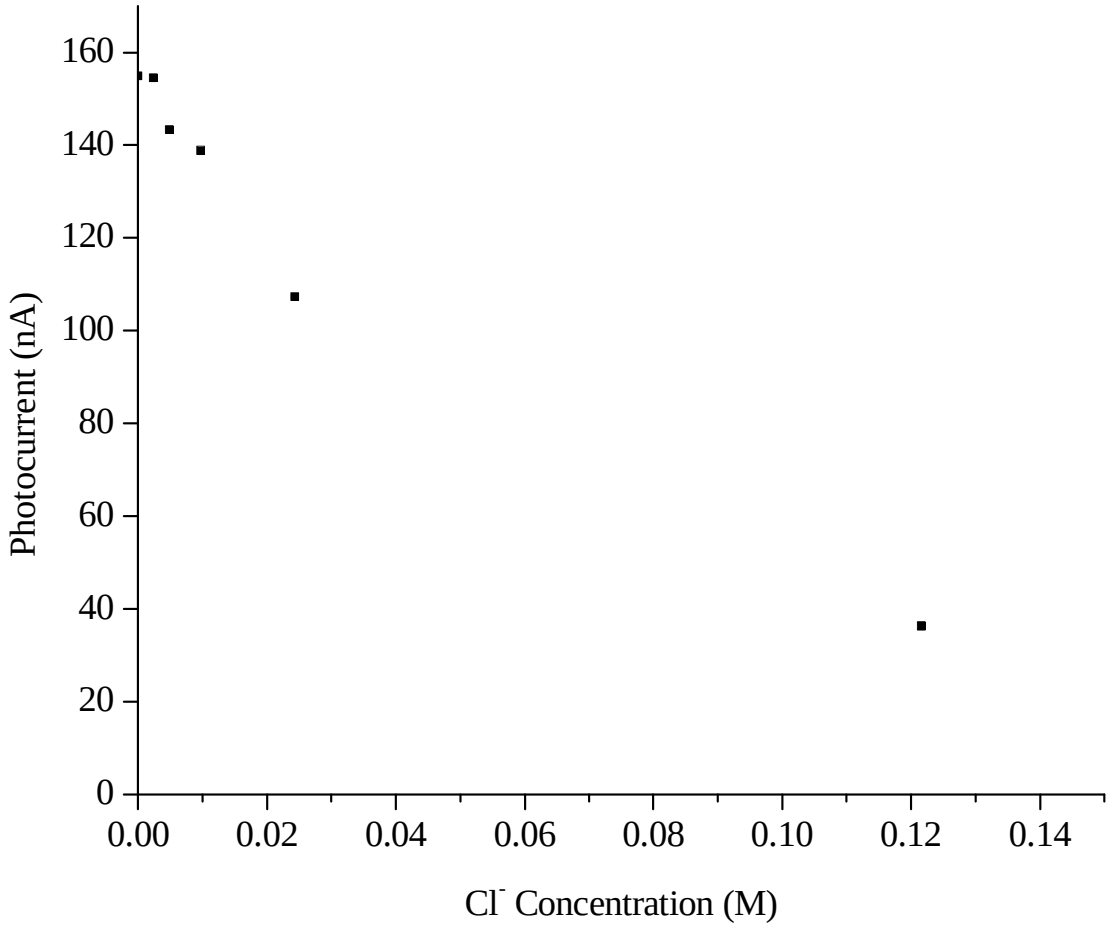

Figure 11. Quenching effects of artificial seawater, expressed as Cl^- concentration, on LL reaction signal for a 1 nM ATP sample for the R928 flow system.

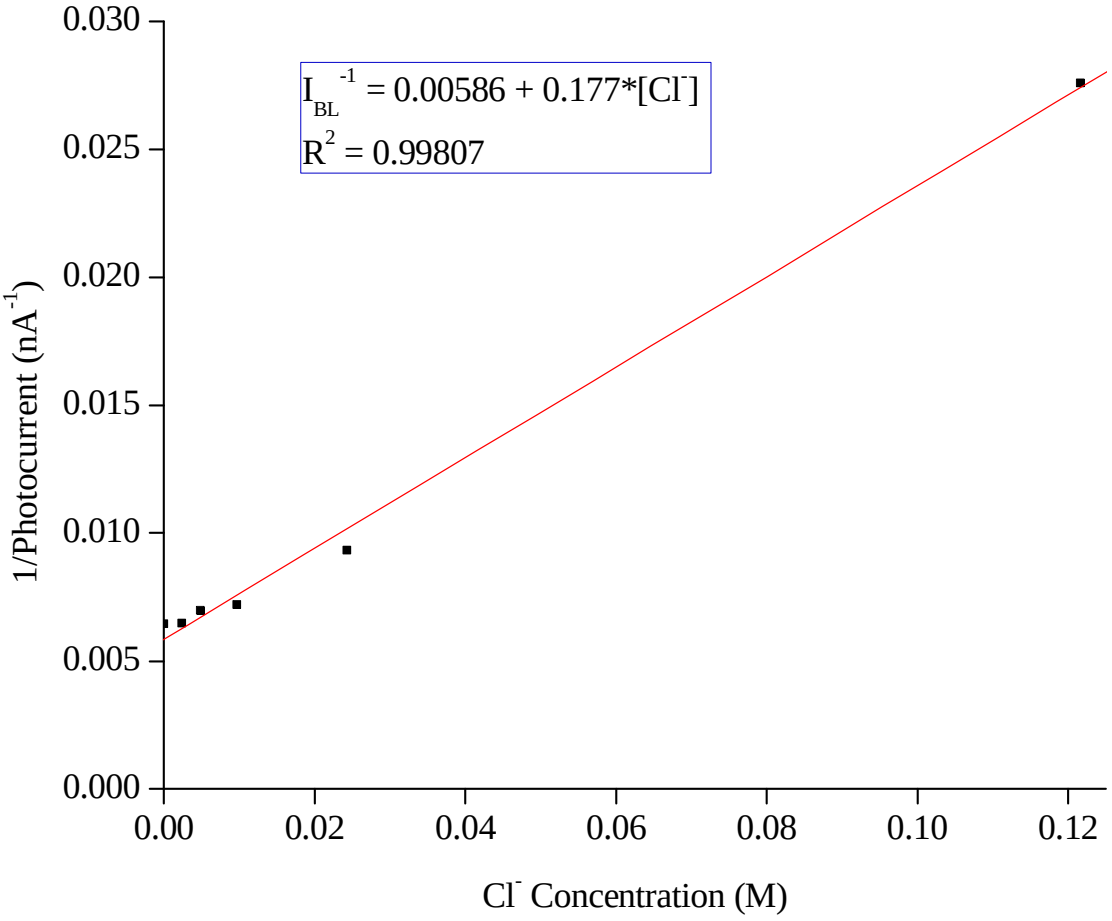

Figure 12. Stern-Volmer plot of data from Figure 11 showing dynamic quenching from artificial seatwater anions.