Draft Final Report

Evaluation of a Rapid Test for Quantifying Assimilable Organic Carbon (AOC) in Membrane Desalination Feedwaters

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Executive Summary

Introduction

Biodegradable organic carbon (BDOC) is a fraction of the dissolved organic carbon (DOC) in water that can be mineralized by heterotrophic bacteria. Assimilable organic carbon (AOC) is a fraction of the total organic carbon that can be utilized by heterotrophic bacteria to increase their biomass. AOC concentration serves as an indicator of the nutrient level and a measure of the potential of microbial regrowth in water. AOC can impact taste, water quality and can influence biological fouling of reverse osmosis (RO) and microfiltration membranes (MF). High levels of AOC are associated with rapid biofilm formation and loss of membrane performance. Currently, a standard method that is laborious, costly and requires weeks to complete is used to assess AOC concentrations. This study was designed to evaluate new methods that are inexpensive and rapid that can be used for monitoring AOC concentrations to prevent biofilm formation, loss of membrane performance and improve water quality.

Objective

The aim of the study was to develop and test a rapid bioassay for determining AOC concentrations, which can be used as an alternative to the standard method currently employed by many of the water utilities. Originally, genetic modifications were going to be performed to *Pseudomonas fluorescens* P17, a standard AOC bacterium. The genetically modified P17 was going to be evaluated for its ability to produce fluorescence that would relate to known concentrations of AOC. This work was redirected to evaluate a Checklight AOC bioassay that was recently marketed in the United States. This bioassay employs *Vibrio fischeri* that naturally harbors a luminescence gene that produces light when organic compounds are metabolized by the organism. The level of luminescence indicates the concentration of AOC metabolized by the organism. This bioassay has been used with surface water samples but has never been tested for wastewater application. In addition, an effort was going to be made to improve the Standard Method currently in use. The standard assay involves inoculation of test water samples with two known strains of bacteria. After some period of time, between 5-14 days, the growth yield of the bacteria is determined which is related to the concentration of AOC in the test water. This approach is laborious and costly mainly due to the time required for growth. A means of improving this assay would be to monitor growth yield by using a Coulter Multizer, which is essentially an automated cell counter.

Approach

A series of evaluations were conducted under well-defined and controlled conditions to determine if AOC concentrations could be monitored using the Checklight bioassay. Several assays were performed to evaluate the method for sensitivity, speed, reliability, accuracy and ease of use. Several organic compounds were selected to determine the nutritional profile of *Vibrio fischeri*, the organism used in the assay. The sensitivity

range at which these compounds were utilized were determined and compared with the organisms used in Standard Methods. Various wastewater sources were selected to determine the sensitivity range and the field application of this assay. Accuracy was determined by comparing the range obtained from the Checklight bioassay with Standard Methods. In addition, an effort was made to improve the rate at which Standard Method calculated growth; this involved the use of the Coulter Multizer to determine the increase in cell volume in the presence of a carbon source. For this study, acetate and OCWD secondary municipal wastewater were used as the carbon sources.

Results

Results from several assays performed indicate that the Checklight bioassay as shipped could not reliably be employed to monitor AOC concentrations due to the physiology of *Vibrio fischeri* cells. Light production from these cells is highly variable and can be reduced by the presence of chlorine, acidic pH, and other unidentified inhibitors. Several modifications were made to the manufacturer's protocol to improve the stability of the organism and consequently the light produced by the organism. These modifications included using glass assay vials that were acid washed and stabilizing the cells prior to use in an assay. Once these modifications were implemented, it was determined that *Vibrio fischeri* are nutritionally as versatile as the organisms used for the Standard Method. It was resolved that Acetate carbon, the standard carbon source used in Standard Methods can also be used as a standard for the Checklight bioassay. The sensitivity range of the assay was determined to between 5-100ppb for several organic compounds, but the assay required longer than 2 hours to complete, as stated by the manufacturer. Since light production needed to be stabilized before the cells could be used, assays could take up to 8 hours to complete. The AOC concentration for OCWD secondary municipal wastewater was tested twice using Checklight bioassay and Standard Method, a range of 274-317ppb; 540-549ppb and 332ppb; 642 ppb was determined by the respective assays. Using the Checklight bioassay, AOC concentration for reverse osmosis product water was in the range of 0-0.891 ppb; Fountain Valley potable drinking water was in the range of 34-55 ppb, and a concentration could not be determined using Deep well injection water or Santa Clara secondary municipal wastewater.

To improve the rates at which growth yields could be calculated using the Standard Method, a Coulter Multizer was used to calculate cell volume. Several cultures were grown that contain different concentrations of carbon and OCWD secondary municipal wastewater as an AOC source. Data following 48 hours of incubation was used as representative data that showed cell volume increase in cultures that contained no carbon, 5 and 10ppm of carbon. However, at 50ppm total volume of cells did not relate to the concentration of carbon provided in the culture. Using OCWD secondary municipal wastewater as a carbon source, decreased cell volumes were observed than at time zero. These inconsistencies could not be explained.

Conclusions

Several modifications of the protocol provided by Checklight were tested to improve the stability, sensitivity and accuracy of the assay. The light produced by *V. fischeri* cells in response to a carbon source was affected by the presence of chlorine and acidic pH. Therefore, all waters that were analyzed were dechlorinated and pH was monitored. The sensitivity range of the assay using the carbon cocktail solution provided by the manufacturer was calculated between 3-100ppb, the reported sensitivity using the carbon cocktail was between 50-400ppb. However, numerous inconsistencies were observed with the assay, suggesting that the physiology of the cells was highly variable and that all viable cells may not be producing light.

Since the amount of light produced did not relate to the concentration of carbon provided, numerous modifications were made to the protocol. Plastic assay vials were replaced with acid-washed, heat sterilized glass vials and cells that were hydrated were stabilized for up to 250 minutes before use. These modifications were used to determine the nutritional profile of *V. fischeri* cells that were able to metabolize a variety of compounds. Bioluminescence produced was greater with Glucose and Fructose-carbon than with Glycerol, sodium acetate and the carbon cocktail. The sensitivity range for most organic compounds was between 5-100ppb.

A toxicity test was performed when different waters were tested for their concentration of AOC to determine if inhibitors are present. OCWD secondary municipal wastewater was tested twice and the values were compared to the Standard Methods; it was calculated that both values were within 20% of each other. The level of AOC could not be calculated when deep well injection water and Santa Clara secondary municipal wastewater was used as a carbon source. A range between 34-55ppb was calculated for Fountain Valley potable drinking water. RO product water contained a low concentration of AOC, it was calculated that this water contained a range of 0-0.891 ppb of acetate-C.

The Coulter Multizer was used to observe an increase in cell biomass by measuring cell volume. This method was tested to determine if the Coulter Multizer could be used to calculate growth rates. Inconsistencies were observed with several of the controlled samples and the OCWD secondary municipal wastewater at different time intervals. The assay was not developed further since the Coulter Multizer is an extremely sensitive instrument that requires prefiltered solutions to obtain reliable results.

Recommendations

The Checklight bioassay was evaluated under defined and controlled conditions and it is suggested that the bioassay needs to be developed further before it can be used to routinely monitor AOC levels in treated or filtered water. This assay has shown some promising results, but needs to be perfected for field applications. The physiology of *V. fischeri* cells varies within each assay and from one kit to another, resulting in light production that is highly variable. Therefore, glass vials and stabilizing the cells before an assay is performed are highly recommended to curtail the variability observed. It is

also suggested that the cells should be grown in mass using a chemostat, which would result in cells that are physiologically stable and in the same growth phase when they harvested for the development of the kit.

Benefits to California

Any new rapid methods that can provide measurements to calculate AOC levels that can predict microbial growth can be used to prevent biofilm formation on membranes, predict loss of membrane performance and can be used to optimize disinfection protocols. These rapid methods can equate to considerable cost saving to the water utilities in California and at the same time improve water quality.

Abstract

This study was designed to evaluate new methods for rapid determination of assimilable organic carbon (AOC). The AOC represents a fraction of the total organic carbon (TOC) in water that bacteria can use for growth and other metabolic processes. High levels of AOC are associated with rapid biofilm formation, loss of membrane performance and poor water quality. Currently there is no rapid test available that can be used to monitor the levels of AOC. A Standard Method to determine the AOC concentrations is available, but requires 5-14 days to complete. A rapid bioassay made available by Checklight was evaluated for its speed, sensitivity, accuracy and complexity to perform the assay.

A number of defined tests were run using the Checklight bioassay and it was determined that the sensitivity ranged from 3-100ppb using a mixed carbon solution. Whereas, the sensitivity range using simple and complex carbon compounds ranged from 5-100ppb. Light produced by *Vibrio fischeri* cells used in the assay was greater with Glucose and Fructose-carbon than with Glycerol, sodium acetate and the mixed carbon solution. OCWD secondary municipal wastewater was tested using the Checklight bioassay and the values were compared to Standard Methods that uses different organisms. The two values were within 20% of each other. The AOC concentrations could not be calculated when deep well injection water and Santa Clara secondary municipal wastewater were evaluated. A range between 34-55ppb was calculated for Fountain Valley potable drinking water.

The Coulter Multizer was used to observe an increase in cell biomass by measuring cell volume. This method was tested to determine if the Coulter Multizer could be used to calculate growth rates. Several inconsistencies, which easily could not be explained, were observed with controlled samples and the OCWD secondary municipal wastewater at different time intervals.

The Checklight assay was evaluated under defined and controlled conditions and it was determined that the assay is highly variable due to the physiology of *V. fischeri* cells. Several modifications are outlined in this study that improved the variability, sensitivity and accuracy of the assay.

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1.0. Introduction

Assimilable organic carbon (AOC) is the fraction of the total organic carbon present in water that is most readily used by bacteria for regrowth and therefore, is of greatest interest to water utilities. An increase in the bacterial population can have several deleterious effects such as regrowth of coliforms, generation of undesired color and taste, corrosion of pipes, possible depletion of disinfectant residual, and biological fouling of reverse osmosis (RO) and microfiltration membranes (MF). A salient factor influencing how rapidly membrane biofouling occurs is the available nutrient content of the feed water. Reduction or removal of AOC is necessary to prevent these problems, which may be accomplished by appropriate filtration methods. Most of the standard or proposed methodologies that measure the concentration of AOC in water are laborious, costly and can require up to two weeks to complete. Results obtained after such a long time frame have little practical value to control water quality and for monitoring purposes. Testing for chemical parameters such as total or dissolved organic carbon has also proven inadequate for monitoring bacterial regrowth because it has been shown that the fraction of the total carbon pool available in water for use by microorganisms is very small and is generally highly variable. Demonstrating that standard chemical methods for monitoring are not sensitive enough to detect such low concentrations, as those that are present in AOC. Consequently, the objective of the proposed project was to evaluate and compare new methods that are rapid for monitoring AOC in treated and or filtered water. This was accomplished by evaluating a commercial bioassay kit that was recently made available in the United States. The manufacturer of the assay has used this kit to monitor AOC concentrations from different raw drinking water sources along the Israeli Water Carrier system (http://www.checklight.co.il/pdf/case_studies/aoc-case-study.pdf)
We evaluated this kit for its sensitivity, accuracy, reliability and cost for monitoring AOC in treated or filtered water. Concomitantly, modifications to the standard AOC assay as described by Van der Kooij were made using the Coulter counter to improve the rate and the cost at which these assays could be carried out.

1.1. Background

The presence and growth of microorganisms in distribution systems is an economic and health issue that is of concern to water utilities in the United States, especially due to the potential appearance of pathogens in distribution systems. The increase in population and drought conditions in Southern California, and other arid regions of the United States, has resulted in limited water supplies. Shortage of potable water has created the need for treatment of alternative water sources such as recycled wastewater, ground and surface waters. The need for new water sources has initiated the search for innovative treatment methods and solutions. Water utilities are striving to reduce the presence of microbiological growth, especially pathogens in treated drinking water to improve water quality and to improve the fouling potential of RO and MF feed waters. It has been

illustrated that water quality deterioration in drinking water distribution systems may be the result of microbiological replication (i.e., regrowth) within the systems (Volk and LeChevallier, 1999; Frias, et. al., 1994). For bacterial growth to occur, various nutrients sources must be present. In particular organic compounds either dissolved or particulate that can be used as energy and carbon sources by heterotrophic bacteria to produce new cellular material, or biofilms on membrane surfaces. It has been well documented that bacterial regrowth can depend in part, on the concentration of the organic carbon available in a water system (Van der Kooij, 1992). This organic material has been defined using several terms such as biological organic material and assimilable organic carbon (AOC). The term AOC refers to a fraction (typically 0.1-0.9%) of total organic carbon (TOC) that is in a form that can be utilized by bacteria for metabolic activity and can result in an increase in their biomass (Escobar et. al, 2000). In most cases, drinking water that has been treated has been exposed to biological activity either during movement through the ground or during the treatment process. As a result, only a small portion of the organic carbon remaining in drinking water is expected to be available as a source of carbon and energy for microorganisms to use for regrowth. Generally, compounds that serve as a nitrogen (N) source are present from a few tenths of a milligram to a milligram per liter; phosphates (P) are present between few tenths to few hundred micrograms per liter (Van der Kooij et. al., 1982). Many biodegradable compounds of natural origin, such as amino acids, peptides, fatty acids, hydroxycarboxylic acids, carbohydrates, humic and fulvic acids are present in extremely low concentrations, but combined, these organic compounds can be assimilated by heterotrophic microorganisms to support regrowth (Van der Kooij et. al., 1982). Another indicator of bacterial regrowth potential is biodegradable dissolved organic carbon (BDOC). The BDOC content of the organic carbon represents the fraction of dissolved organic carbon that is mineralized by bacteria, but does not necessarily result in an increase in their biomass. The presence of AOC and BDOC are often measured separately but can be measured together as indicators of bacterial regrowth and disinfection by-product formation potential, respectively.

One of the major issues with bacterial regrowth is the multiplication of potentially pathogenic bacteria (e.g., *Legionella spp*., *Aeromonas*, *Pseudomonas*, *Flavobacterium*, etc.) that can affect the taste, odor, color, corrosion and possibly the proliferation of macroinvertebrates (Volk and LeChevallier, 1999; Frias et. al., 1994; Allen et. al., 1980). Prevention and control of bacterial regrowth in distribution systems and the removal of pathogenic bacteria are essential water quality issues which are often addressed by utilities. Chlorine has been used routinely to meet the microbiological standards for water quality. However, it has been reported that chlorine is not completely effective in controlling bacterial regrowth; to restrict bacterial regrowth potential it is necessary to either remove or maintain a low concentration of AOC. It has been demonstrated that bacterial levels in nonchlorinated systems did not increase when AOC levels were lower than 10 µg/L (Van der Kooij, 1992). Additionally, it was shown that regrowth of potentially pathogenic bacteria was limited when AOC levels were less than 50-100 µg/L (LeChevallier, et. al., 1991).

Bacteria assimilate a small fraction of the total TOC, since other inorganic and organic compounds are present in sufficient quantity for bacterial growth. The amount of organic compound that is available, as sources of energy for bacterial growth cannot be measured directly by a single chemical method, since many compounds (e.g., amino acids, peptides, carbohydrates) are routinely found at very low concentrations (Frias et. al., 1994). Inorganic compounds, such as ammonia, can be determined by specific chemical analysis, but determining the concentration of organic compounds has proven to be more complicated. Not only due to the number of different classes of compounds that AOC may be composed of, but also the concentrations of these compounds are very low in drinking water, typically micrograms per liter. It has been demonstrated that many different types of bacteria are capable of dividing and growing in water that contains 2-5 part per billion (ppb) of variable organic carbon (Van der Kooij, 1988). For these reasons, several different methods have been developed to measure AOC.

1.2. Overview

Several bioassay methods have been developed to determine the concentration of AOC available for regrowth. Most of these techniques can be divided into two groups: (a) those that indirectly measure cell biomass linked to some type of metabolic or physiological activity (i.e., cell metabolism or adenosine triphosphate [ATP] production), (b) those that directly measure biomass production; a technique which can directly measure the number of specific type of bacteria present or an increase in the number of cells (biomass). Many of the bioassay methods that are used to measure AOC concentrations employ a combination of microscopic, classical microbiology and genetic techniques. These techniques can provide valuable information about the concentration of available assimilable carbon that can stimulate bacterial regrowth potential in water. However, most methods that directly measure biomass production require up to two weeks to complete, making them impractical for monitoring water quality in an operational time-frame, or for rapid implementation of treatment changes to control bacterial regrowth in water treatment facilities and distribution systems.

A widely accepted and standard technique used to measure bacterial regrowth potential is the Van der Kooij method developed in 1982 (Van der Kooij et. al., 1982). This technique uses two microorganisms, *Pseudomonas fluorescens* strain P17 and *Spirillum* strain NOX in a bioassay to measure AOC. The concentration of AOC is measured by relating the number of these organisms to substrate concentration using a standard growth curve. These two organisms were isolated for the explicit purpose of determining the concentration of AOC from drinking water. *Pseudomonas fluorescens* strain P17 was chosen from other fluorescent Pseudomonads that were isolated from drinking water because of its higher growth yields, is nutritionally versatile, growth in the presence of a simple nitrogen source, does not require special growth factors, grows rapidly on non-selective media and produces clearly visible colonies (Van der Kooij et. al., 1982, Frias et. al., 1994, Kaplan et. al., 1993). A list of the various organic compounds used by *Pseudomonas fluorescens* strain P17 for regrowth is listed in (Table 1).

Spirillum species strain NOX is used in conjunction with *Pseudomonas fluorescens* strain P17 in most bioassays. *Spirillum* NOX was isolated from drinking water after being plated on enrichment medium. This organism utilizes additional organic acids that are not utilized by *Pseudomonas fluorescens* strain P17. Table 2 lists organic compounds most commonly utilized by *Spirillum* species strain NOX.

The standard Van der Kooij AOC bioassay uses these two strains, which are separately inoculated into a water sample. These organisms assimilate the AOC in a water sample as a carbon source to grow or to increase their numbers (increase biomass). Therefore, the AOC is converted to biomass that is measured by standard plate counts at regular intervals. The increase in biomass is proportional to the concentration of AOC and is generally determined at the stationary growth phase, which is measured 3 to 5 days after inoculation. The cell yields in the test water samples are related to an equivalent yield of these organisms grown in a known concentration of acetate carbon. AOC values are reported as the sum of P-17 and NOX with acetate carbon equivalents measured in micrograms per liter. Since this bioassay directly measures colony-forming units (CFU), a measurement of their growth yield, it can take days to weeks before data can become available. This assay is best used as an indicator of the growth potential of the water and not as a direct measurement of biodegradable carbon; it is also used as a tool for predicting regrowth of coliform and heterotrophic plate count bacteria in water (Huck, 1990). At present, Van der Kooij AOC bioassay is used as the standard method according to Standard methods (1998).

In order to reduce the time needed to execute the standard AOC bioassay, ATP bioluminescence based assay was developed. This method uses ATP concentrations to determine the total number of viable bacteria present in a water sample. ATP occurs in all living cells and is not associated with nonliving particulate material. ATP concentrations are obtained by an enzymatic reaction using the luciferine-luciferase assay. The amount of light produced is determined by luciferase, which is shown to be proportional to the number of cells present. The concentration of ATP is determined empirically from a linear relationship between light production and reference ATP concentrations (standards). The ratio of ATP to cell number varies from species to species, but is constant enough to reliably estimate the number of cells from ATP measurements (Vrouwenvelder, 2001). In the ATP-based assay, the concentration of the test organisms (P17 and NOX) are evaluated based on the release of ATP from these bacteria after placing them on membrane filters (LeChevallier et. al., 1993). In this specific study, AOC concentrations were compared to the standard assay. It was demonstrated that in some samples the ATP-based assay produced results that could be related to the standard method, but with other samples resulted in higher AOC values (LeChevallier et. al., 1993). In some samples, the background levels due to non-cell associated ATP was too high which obscured the AOC concentrations. Likewise, intensely colored water interfered with the bioluminescence produced in the assay and the determinations of ATP concentration could been biased towards growth of P17 rather than NOX, since P17 contained approximately 9 times more ATP per cell than NOX (LeChevallier et. al., 1993).

Recently, the ATP-based bioluminescence assay was redesigned using genetically modified P17 and NOX strains (Haddix et. al., 2004). In this assay, *Pseudomonas fluorescens* strain P17 and *Spirillum* species strain NOX were mutagenized to harbor the *luxCDABE* operon as part of their genes to produce luminescence under induced conditions, following the addition of *p*-aminosalicylate (PAS). Due to low concentrations of AOC in most test waters, which resulted in low cell densities, an additional substrate (*n*-decylaldehyde) was directly added to the cell suspensions to achieve adequate luminescence to complete the test (Haddix et. al., 2004). In this assay bioluminescence intensity was directly related to cell metabolic activity and the to the standard AOC assay. It was determined that following the addition of the inducer and the additional substrate, there was a linear relationship between the bioluminescence assay and the standard AOC growth-based assay. To date, this bioassay can be used to obtain results within 3 days following inoculation with the test strains, representing a significant time reduction from 5 days using the ATP-based assay. This method is still in development to improve accuracy, sensitivity, reproducibility, and it still remains to be determined if the method can be automated using a microplate and a reader (Haddix et. al., 2004).

Traditional methods for assessment of microbial growth and activity generally lack specificity, are laborious and impractical for water quality monitoring. Increasingly, genetically engineered microorganisms (GEMS) are being constructed for environmental applications. Molecular-based techniques polymerase chain reaction (PCR), DNA probing and marker gene tagging have been modified to identify and quantitate microorganisms directly in the environment. One of the most widely used markers is the *gfp* gene, which encodes the information for green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Bloemberg et. al., 1997). This is a fluorescent marker that does not require any additional substrate or cofactor in order to fluoresce (Chalfie et. al., 1994), does not interfere with bacterial growth and only requires oxygen to express. In addition, cells tagged with *gfp* can be studied nondestructively and can be easily visualized by microscopy with commonly available fluorescent filters; in some cases where signal is weak; a charged-coupled device (CCD) camera can be used to enhance signals.

In many cases, to fully understand the dynamics of the microbial population with its environment, both the total bacterial biomass and the metabolic activity of the cells have to be assessed. As a result, two markers, *gfp* and *lux* were genetically combined into a marker system, *gfp-luxAB*, (Unge et. al., 1999). This system was designed for dual monitoring of metabolic activity and determining the cell number of the microbial population in a complex environment, such as soil. Using this system, microbial populations can be identified, quantified and more importantly, their metabolic status can be determined directly in the environment. This approach involves the use of luminometry and flow cytometry, which do not depend on cell cultivability, since it is not measuring the increase in cell biomass. Currently, this system is under development in several labs, its stability and reproducibility need to be evaluated; the cost of the equipment and expertise of personnel may become prohibitive for this method to be adopted as a standard method.

Traditional techniques for assessment of metabolic activity and microbial regrowth lack specificity, speed, and accuracy for monitoring water quality or for rapid implementation of treatment changes to control bacterial regrowth. Due to speed and cost, application of these techniques usually results in limited frequency of sampling and or limited number of sites sampled. As a result, water utilities may either over or under disinfect which can result in augmented costs or poor water quality. Currently, to ensure water quality, most water utilities are seeking rapid and simple methods that do not depend on cell culturability to determine the concentration of AOC. New methods are constantly being developed and evaluated by utilities that could provide rapid measurements of AOC so that they can prevent bacterial regrowth, optimize disinfection regimes and reduce the presence of excess disinfectant and by-products, which could result in considerable cost savings.

1.3. Projective Objective

The objective of this study was to evaluate a new method for rapid determination of AOC in treated and or filtered waters, this includes, but is not limited to reverse osmosis (RO) and microfiltration (MF) feed waters. Originally, the Orange County Water District (OCWD) proposed to use the *gfp-luxAB* marker system as a means of rapidly detecting the concentration of AOC in filtered source waters. This planned approach was an extension of the work performed by Haddix (2004) and Unge (1999), outlined above. Due to adjustments in the personnel involved and time constraints, the scope of the project was re-directed to evaluate a commercially marketed kit by Checklight Ltd. (Qiryat-Tiv-on, Israel) and concurrently, to develop a rapid method to directly measure biomass accumulation using the Coulter Multisizer II (Beckman Coulter, Inc., Miami, Fl.). As outlined in the work agreement, genetic modification of standard AOC bacteria were not performed since the Checklight assay used a naturally occurring luminescent bacterium, *Vibrio fischeri*. Task 2 of the agreement called for broad laboratory based evaluation and validation of the genetically modified bacteria. Instead, extensive evaluation of the Checklight bioassay was performed. Task 3 involved the use of different feedwaters to assess the performance of the genetically modified bacteria; instead, various feed waters were evaluated using the Checklight assay and one using the Coulter counter method.

During the course of the study, it was determined that Checklight Ltd. was marketing a bioluminescence AOC assay for drinking water which employs lyophilized (freeze-dried) preparations of *Vibrio fischeri* bacteria. It was reported that this organism harbors a naturally occurring luminescence gene, which correlates luminescence with the metabolism of AOC. The genes are induced in the presence of organic compounds and produce light; the intensity of luminescence is proportional to the concentration of the organic compound catabolized by *Vibrio fischeri* (http://www.checklight.co.il/pdf/case_studies/aoc-case-study.pdf; see Figure 1). It has been reported that the assay can be completed in approximately two (2) hours at minimal cost and has a sensitivity range of sub-parts per million (ppm) for a variety of organic

compounds. The manufacturer used acetate carbon and the carbon cocktail to verify the reliability of the assay (http://www.checklight.co.il/pdf/case_studies/aoc-case-study.pdf; see Figure 2).

For this study, a series of validation assays were conducted to evaluate the Checklight bioassay for the wastewater application. Specific organic compounds (Table 3) and various water sources (Table 4) were used as test sources to assess the performance of the assay as it relates to sensitivity, accuracy and reliability for wastewater. When water sources were examined, standard Van der Kooij method was run in parallel with the same water source to determine the performance and accuracy of the Checklight bioassay. Series of dilutions of organic compounds (Table 3) and water sources (Table 4) were used to evaluate the sensitivity, inhibition (due to chlorine, pH, etc.) and the time it takes to complete the assay. Specific organic compounds were used to determine the nutritional requirements for *Vibrio fischeri*, a non-standard AOC bacterium. The nutritional requirements of this organism are not well characterized (Haddix et. al., 2004) and needed to be identified before the test water (Table 4), which contains a variety of these organic compounds, could be analyzed. The advantage of the Checklight bioassay is speed and ease of use, which does not require highly trained personnel or equipment and ultimately can translate to cost savings.

In conjunction with validation of the Checklight bioassay, a series of runs were made in an attempt to improve the standard assay as described in Standard Methods using a Coulter Multizer or Coulter particle counter to determine growth. This instrument is an automated cell counter that can be used to relate biomass from measurements of volume. The Coulter counter uses the Coulter principle to measure volume. In this device, particles such as bacterial cells are suspended in a weak electrolyte solution and drawn through a small aperture that separates two electrodes between which an electric current can flow. The voltage applied across the aperture creates a sensing zone. As each bacterial cell passes through the aperture, it displaces its own volume of conducting liquid, thereby increasing the impedance of the aperture. This change in impedance produces a tiny but proportional current flow into an amplifier that converts the current fluctuation into a voltage pulse large enough to measure accurately. The Coulter principle states that the amplitude of this pulse is directly proportional to the volume of the particle that produced it. Scaling these pulse heights in volume units enables a size distribution to be acquired and displayed. In addition, metering device is used to draw a known volume of bacterial suspension through the aperture; a count of the number of pulses then yields the concentration of bacteria in the sample (http://www.beckman.com/products/instrument/partChar/pc_multisizer3.asp). One immediate advantage of using this approach to count bacterial cells, if successful, is the rate at which the Standard assays could be performed (Salzman et. al., 1990; Robertson et. al., 1998) which could equate to cost savings due to the speed of the assay.

1.4. Report Organization

The remainder of this report contains detailed information regarding the test procedure used to validate the Checklight bioassay, the modifications performed to improve sensitivity and reliability of the bioassay (section 2.0). Included in section 2 is the outline of the approach used to determine biomass increase using the Coulter counter. This is followed by a list of graphs and findings from both types of assays (section 3.0). Section 4.0 offers an interpretation and discussion of the findings from both assays as well as recommendations for future direction with respect to use of bioluminescence based assays currently being investigated.

2.0. Project Approach

2.1. AOC Multi-Shot Test (Checklight, Ltd., Tivon, Isreal):

The concentration of AOC was determined using the Checklight AOC Multi-Shot Test Kit and method as outlined by the manufacturer. The appropriate numbers of assay vials provided were labeled accordingly (1-15). The first nine vials were used for the test water analysis; the remaining vials were used as controls (negative and positive controls).

Preparation of diluted assay buffer (DAB): The DAB was prepared via a 1/10 dilution, using the concentrated assay buffer (CAB) provided with the kit and nutrient-free clean water (HPLC Grade Water) (Burdick & Jackson; Muskegon, MI; cat. no. 365-4).

Preparation of diluted carbon cocktail solution, 5ppm (DCS): DCS was prepared fresh for each experiment using the concentrated carbon cocktail solution (CCCS, 5mg/mL) provided with the kit at a final concentration of 5ppm. A 1mL total volume DCS was prepared via a 1/1000 dilution (CCCS/CAB). Reference and positive control samples of carbon cocktail solution (CCS) used in many of the assays were made by further diluting DCS to the appropriate concentration.

Preparation of negative controls: Two vials (#10 & #11) served as negative controls, containing no carbon source. Both vials contained 1mL of DAB only.

Preparation of positive controls: From four vials, each containing 1mL of DAB 10, 20, 40, and 80µl were removed and replaced with DCS respectively to prepare the 50, 100, 200 and 400ppb carbon cocktail vials (#2-15).

Preparation of test samples: Dispensed into the first vial were 1.8mL of the test sample and 0.2mL CAB, the volumes were mixed thoroughly. Test sample was diluted by serial dilution from vial #1 to #9 (final dilution ~270 fold). Finally, 1mL diluted test solution was discarded from vial #9.

Hydration of *Vibrio fischeri (V. fischeri):* The lyophilized *V. fischeri* hydration entailed the rapid addition of cold 0.5mL Checklight hydration buffer (HB) into the lyophil vial

and vortexing the volume for approximately 1minute with no stopper atop the vial. Finally, 20µl of *V. fischeri* was dispensed into each assay vial (#1-15). The assay vials were incubated at 26°C +/- 2°C with orbital spinning at approximately 1200 RPM in an Environ-shaker (Lab-Line Instruments, Inc.; Melrose Park, IL). The TD-20e Luminometer (Turner Designs; Sunnyvale, CA; model serial no. 0515) was used to measure the luminescence after 60-150 minutes using 5second (sec.) delay with 60sec. integrations.

2.1.1. Test sensitivity using carbon cocktail standard:

Additional positive controls were added to determine the sensitivity of the assay and to compare the range with the standard Van der Kooij assay, which has a reported range of approximately 1- 126ppb of acetate carbon (Van der Kooij, 1988). Positive controls were added to the above-described protocol, increasing the total number of vials from 15 to 19. Each additional vial was filled with 1mL DAB. The additional positive controls were prepared by dispensing 1, 2, 4 and 8µl DCS to yield 5, 10, 20 and 40ppb carbon cocktail, respectively.

2.1.2. Comparison of normal concentration of *V. fischeri* to diluted cells:

High background readings were observed within a short time span with negative controls (tubes that contained DAB only), it was postulated that by diluting the cells by 10 fold, we could lower the luminescence during the assay (120min) and possibly improve the sensitivity range of the assay.

Two sets of assay vials were prepared following Project Approach (PA)-2.1.1. A specific concentration of CCS (2mL) was prepared as the test sample in the first vial.

Preparation of Test Sample: To the first vial, 2mL DAB was added. The appropriate volume of DCS was used to prepare the specific concentration of CCS.
From the same lyophil, both sets of assay vials were inoculated. The first set of vials was inoculated as outlined above in Project Approach (PA)-2.1 using the recommended concentration of *V. fischeri*. The second set of assay vials was inoculated with 1/10 dilution of *V. fischeri* hydrated in HB.

2.1.3. Reproducibility test:

To test the reproducibility and stability of *V. fischeri* cells once hydrated but from a single lyophil, two sets of assay vials with the same CCS concentrations (100ppb and 200ppb CCS) were prepared as described below.

Both the DAB and the DCS were prepared following PA-2.1. Into the two negative controls vials, 1mL of DAB was dispensed. Then, 6mL of 100ppb and 200ppb CCS were

prepared separately. The total volumes of both 100ppb and 200ppb were dispensed; 1mL separately into assay vials labeled a-f for each concentration. Into all 14 vials, 20µl *V. fischeri* was added following PA-2.1.

2.1.4. Test sensitivity using a mixture of complex organic compounds:

To resolve the sensitivity and nutritional versatility of *V. fischeri*, a mixture of Casamino acids (CA; DIFCO Laboratories; Detroit, MI; cat. No. 0288-15-6) and CCS was used as a standard carbon source. The final concentration of the CCS/CA mixture was 5ppm (50/50 concentration of CCS/CA). The mixed carbon standard solution was prepared by mixing 2.5µl DCS (5ppm CCS) and 2.5µl CA (5ppm CA) in 5mL CAB. The new carbon mixture was used to prepare six positive controls (20, 40, 50, 100, 200, and 400ppb). The luminescence response by the *V. fischeri* was measured after 175 minutes.

2.1.5. *V. fischeri* starvation:

In an attempt to lower background readings and to obtain a liner response that is directly proportional to the concentration of AOC present in the sample, *V. fischeri* cells were starved (no carbon was provided) following hydration. Project Approach-2.1.1 was followed using CCS as the carbon source for positive controls and test sample at 200ppb CCS. The lyophil contents were hydrated following PA- 2.1, pelleted via centrifugation at 12000RPM, the HB removed, and re-hydrated with a new volume of HB, containing no carbon. The washed *V. fischeri* cells were starved for an hour at 26°C with orbital shaking. The starved cells were used to inoculate assay vials (#1-18).

2.1.6. Test response of *V. fischeri* on simple and complex organic compounds:

The luminescence response of *V. fischeri* was highly variable with the carbon standard provided (CCCS), which contained a mixture of two carbon sources, Glucose (Glu) and Yeast extract (YE). To resolve whether the variability was due to the mixed carbon source, a simple organic compound (Acetate) and a complex organic compound (Casamino acids) were tested separately as sole carbon sources. These compounds were also used to test the nutritional versatility and sensitivity of *V. fischeri*.

Preparation of Sodium Acetate (NaOAc): A 5mg-Carbon/mL (5mg-C/mL) solution of NaOAc (Sigma; St. Louis, MO; cat. no. S-8625) was prepared.

Preparation of Casamino Acids (CA): A 5mg/mL solution of CA was prepared.

Two sets of negative and positive control vials were prepared following PA-2.1.1. One set of positive controls was prepared using the 5mg-C/mL NaOAc and the other set using 5mg/mL CA. Both sets of assay vials containing either NaOAc or CA were inoculated with Vibrio from the same lyophil, prepared as outlined above in PA-2.1.

2.2. AOC-II Test Kit (Checklight, Ltd. Tivon, Isreal; cat. no. AOC1520):

An AOC-II kit was purchased; through communications with the manufacturer, it was determined that modifications were being performed to the original kit to improve the stability of the organism and to lower the background readings.

The concentration of AOC was determined using the Checklight AOC-II Test Kit. The assay vials were labeled appropriately. The first seven vials were used for test samples; the remaining vials were used as controls (negative and positive controls).

Preparation of Diluted Assay Buffer (DAB): A 1/8 dilution of DAB was prepared using Concentrated Assay Buffer (CAB) and nutrient-free clean water (HPLC Grade Water) (Burdick & Jackson; Muskegon, MI; cat. no. 365-4).

Preparation of DCS (5ppm): DCS was prepared fresh for each experiment (test sample and standard curve) using the Concentrated Carbon Cocktail Solution (CCCS, 5µl/mL). A 1mL total volume DCS was prepared via a 1/1000 dilution of CCCS in CAB.

Preparation of Negative Controls: Three vials served as negative controls (no carbon source). All three vials contained 1mL DAB only.

Preparation of Positive Controls: To eight vials, 1mL of DAB was added; 1, 2, 4, 8, 10, 20, 40, and 80µl DCS respectively was added to prepare the 5, 10, 20, 40, 50, 100, 200 and 400ppb carbon cocktail respectively.

Preparation of Test Samples: Dispensed into the first vial was 1.75mL of the test sample and 0.25mL CAB, the volume was mixed thoroughly. The dilution of the test sample was performed by serial dilution from the first vial to the seventh vial (final dilution ~90 fold). Finally, 1mL diluted test solution was discarded from vial #7.

Hydration of *V. fischeri*: *V. fischeri* was hydrated by the rapid addition of cold 0.5mL HB into the lyophil vial; the total volume was vortexed for approximately 1min. with no stopper atop the vial. Finally, 20µl of *V. fischeri* was dispensed into each assay vial. The assay vials were incubated at 26-28°C with orbital spinning at approximately 1200RPM. The luminescence was measured after 60-120 minutes using 5sec. delay with 60sec. integrations. Readings at 120 minutes were reported and used to estimate the concentration of AOC.

2.2.1. Test diversity using simple organic compounds:

To test the diversity of organic compounds that can be metabolized by *V. fischeri* as sole carbon and energy source, several different organic compounds were tested as alternative carbon sources (Table 3).

Preparation of substitute carbon sources: A 5mg-Carbon/mL (5mg-C/mL) solution was prepared for Glycerol (Gly, Sigma; St. Louis, MO; cat. no. G-7893), D-(+)-Glucose (Glu, Sigma; St. Louis, MO; cat. no. G-7528) and Sodium Acetate (NaOAc, Sigma Chemical Co.; St. Louis, MO; cat. no. S-8625). A 5mg/mL solution of CA was prepared. Following PA-2.2, positive controls were prepared using the individual carbon sources; negative controls, incubation and luminescence parameters were followed as outlined in PA-2.2.

2.2.2. Evaluation of Checklight assay vials (plastic):

Following PA-2.2, negative controls and positive controls (5-200ppb) were prepared using DCS (5ppm). The CCS concentration range of the positive controls was changed to 2.5-250ppb from 5-400ppb. This was done to determine the sensitivity of the assay and to compare the sensitivity of the kit to the standard assay.

Preparation of 2.5ppb CCS control: In an appropriate assay vial, 2mL DAB was dispensed: 1µl was replaced with 1µl DCS (5ppm). The volume was mixed; 1mL 2.5ppb CCS was discarded to reduce the volume to 1mL total volume.

Preparation of 250ppb CCS control: Into an appropriate test vial, 1mL DAB was dispensed; 50µl DAB was replaced with 50µl DCS (5ppm).
Three separate starting concentrations (300, 250 and 200ppb) of CCS were prepared as "test samples" by serial dilution.

Preparation of 300ppb CCS: In 2mL DAB, 120µl replaced with 5ppm DCS. This concentration was serially diluted to down to 9.38ppb. The concentrations used in the assay were 300, 150, 75, 37.5 and 9.38ppb.

Preparation of 250ppb CCS: Into the appropriate assay vial, 1mL DAB was added; 50µl was replaced with DCS (5ppm).

Preparation of 200ppb CCS: Into the appropriate assay vial, 2mL DAB was added; 80µl was replaced with DCS (5ppm). This concentration was serially diluted to down to 6.25ppb. The concentrations used in the assay were 200, 100, 50, 25, 12.5 and 6.25. The assay vials were inoculated sequentially from vials #1-19. The luminescence parameters were 60sec. integration with a 5sec. delay.

2.2.3. Comparison of "Unwashed" and "Washed" assay vials:

It has been shown that all glassware for sample collection and analyses has to be washed and AOC-free due to the sensitivity of the various assays (LeChevallier, 1993;

http://www.checklight.co.il/pdf/case_studies/aoc-case-study.pdf). In an attempt to lower the background readings obtained from negative control vials (no carbon source; only DAB and *V. fischeri* cells); all vials were "washed" since all assay vials provided with the kit were shipped in bulk and uncapped.

Preparation of "Washed" assay vials: The appropriate numbers of assay vials were rinsed three times each with 2mL clean nutrient-free water. The washed vials were allowed to dry overnight covered with aluminum foil.

Preparation of "Washed" pipet tips: Before a pipet tip was used for transferring volume into vials, "washed" or "unwashed", the tip was rinsed three times with a volume of nutrient-free water equal to the volume of transfer; each rinse volume then was discarded. Two sets of positive controls were prepared following PA-2.2 with CCS as the standard carbon source. "Set 1" was prepared in "washed" assay vials using "washed" pipet tips; "set 2" was prepared in "unwashed" assay vials using "washed" pipet tips.

Inoculation of "Unwashed" and "Washed" assay vials: The *V. fischeri* was hydrated as per PA-2.2, next the entire volume was kept on ice during the inoculation period. The inoculation procedure was altered such that a 65 second delay was observed between each inoculation. The delayed inoculation was performed so that the time frame of inoculation and luminescence readings corresponded after 120 minutes. The two sets of test vials were incubated as per PA-2.2.

2.2.4. Acid-washed assay vials:

In an attempt to lower background readings, all assay vials were acid-washed and heat sterilized at 250°C (American Scientific Products, DS-64) to remove any residual acid and AOC.

Preparation of acid-washed assay vials: Assay vials were soaked in a 10% Hydrochloric Acid solution (J.T. Baker, Phillipsburg, NJ; cat. no. 9535-05) for approximately 5 hours, rinsed thoroughly with 18MOhm water and finally baked at 250°C for approximately 18h. The assay vials were prepared and inoculated as described in PA-2.2.3. Acid-washed tubes were used for all of the following assays and tests.

2.2.5. Examination of integration times:

It was highly recommended by the manufacturer that the integration and delay times be shortened to improve the response from the negative control samples.

Preparation of Glycerol carbon sample: From a 1mg-C/mL Gly working solution, a 200ppb Gly-Carbon solution was prepared in the first vial (total volume, 2mL).

Positive and negative controls were prepared as per PA-2.2 using CCS as the carbon standard. The Glycerol carbon sample was then serially diluted to the next nine vials. Finally, to each assay vial (control and test), 20µl *V. fischeri* was added with a 60 second delay between each inoculation. The set of assay vials was incubated as per PA-2.2. The luminescence of each vial was measured two times. First, using a 5sec integration time with a 5sec. delay; second, using a 30sec. integration time 5sec. delay

2.2.6. *Vibrio fischeri* stabilization:

To remove the affects of residual carbon present in the lyophil and to stabilize the luminescence to obtain a linear response from *V. fischeri*; cells were allowed to establish without any carbon before they were used for the assay.

In acid-washed assay vials, negative controls (no CCS) and positive controls with CCS ranging from 2.5-250ppb CCS were prepared; both 100 and 150ppb CCS as "test carbon samples" were prepared and serially diluted. The CCS "test carbon samples" were diluted serially in the following manner: 150, 100, 50, 37.5, 25, 18.75, 12.5, 9.38, 6.25, 4.69, 3.13 and 2.43ppb in CCS.

***Vibrio fischeri* stabilization:** A new lyophil of *V. fischeri* was hydrated as per PA-2.2. The luminescence was monitored until the *V. fischeri* approached the stationary phase or the luminescence leveled off (stabilized). After the *V. fischeri* stabilized the assay vials were inoculated and the luminescence was measured as per PA-2.2.

2.2.7. Assessment of stabilized *V. fischeri* cells:

A new lyophil of *V. fischeri* was hydrated and stabilized as outlined above (PA-2.2.6). Negative controls containing no CCS were prepared; positive controls ranging from 2.5-200ppb CCS were prepared and four "test carbon sample" of 200ppb, 175ppb, 150ppb and 125ppb CCS were prepared. All vials were inoculated with 20µl stabilized *V. fischeri*. The luminescence was measured using 5sec. integration and a 3sec. delay.

2.2.8. Metabolism of simple organic compounds by stabilized *V. fischeri* cells:

To resolve the sensitivity range of stabilized cells, various organic compounds were tested to determine how well these compounds could be metabolized. The various organic compounds ("test carbon samples") were run as the sole carbon source for the cells. Compounds tested were Glu, Gly, NaOAc and D- (-)-Fructose (Fru, Sigma Chemical Co.; St. Louis, MO; cat. no. F2543; Table 3). Two negative controls (no CCS) and six positive controls, 10, 20, 40, 50, 100 and 200ppb of CCS were prepared as standard carbon source following PA-2.2.

Preparation of "test carbon sample": A 5 mg-C/mL stock solution was prepared separately for Glu, Gly, NaOAc and Fru. Then, a 1mg-C/mL working solution was prepared by drawing 1µl of the stock "test carbon sample" into 1mL of CAB. It was utilized to prepare three concentrations (150, 125, 100ppb Carbon) for each "test carbon sample".

Preparation of 150ppb "test carbon sample": In 2mL DAB, 60µl was replaced with 5ppm working solutions.

Preparation of 125ppb "test carbon sample": In 2mL DAB, 50µl was replaced with 5ppm working solutions.

Preparation of 100ppb "test carbon sample": In 2mL DAB, 40µl was replaced with 5ppm working solutions.

The assay vials were inoculated with 20µl stabilized *V. fischeri* sequentially as per PA-2.2. The luminescence parameters used were 5s integration and 3s delay.

2.2.9. Use of Sodium acetate as a standard curve:

To compare methods and the concentration of AOC using this assay with Standard Methods (1998) and other published methods, Sodium acetate was used to develop the standard curve. Acetate-C is used in Standard Methods and other published assays.

Instead of CCS, Sodium acetate was utilized as a carbon source for the standard curve. A 5mg-C/mL NaOAc stock solution was prepared using nutrient-free water. A working solution (5ppm) was prepared by drawing 1µl of stock NaOAc into 1mL of CAB, it was used next to prepare the NaOAc standards vials. The standards were prepared as per PA-2.2 contained 2.5, 5, 10, 20, 50, 100, 150 and 200ppb NaOAc-C.

2.2.10. Assessment of water samples for AOC concentration:

OCWD secondary municipal wastewater (SMW; Table 4) was analyzed for AOC concentration. The water sample was collected using AOC-free glassware. It was empirically determined that the luminous bacteria *V. fischeri* were sensitive to chlorine treatment. Therefore, 20ppm of Sodium thiosulfate (NaThio) was routinely added to the water sample to neutralize any chlorine that may have been present. OCWD SMW sample was filter sterilized using a 0.22µm Nylon sterile filter system (Corning Costar, Corning, NY; cat. no. 25944), then refrigerated for storage. As per PA-2.2, NaOAc-C standards 2.5, 5, 10, 20, 50, 100, 150, and 200ppb were prepared by replacing the following: a volume of DAB with the same volume of 1ppm NaOAc solution, respectively.

A new lyophil of *V. fischeri* was hydrated and stabilized as per PA-2.2.6. Once the cells were stabilized, 20µl *V. fischeri* was added to each assay vial, which were incubated as per PA-2.2.

2.3. AOC-II Test Kit (Checklight, Ltd., Tivon, Isreal; cat. no. AOCV1510):

A second Checklight AOC-II Test kit was purchased due to the manufacturer making the following adjustments to the AOC-II kit: The number of lyophils of *V. fischeri* supplied were reduced by 10; subsequently, the inoculation volume for each assay vial was abridged by 10µl of *V. fischeri.* This increased the number of assay vials per lyophil from 25 to 50. An additional practice that was continued was the use of acid-washed assay vials. The vials used for the test water samples were prepared as outlined in PA-2.2 with modifications listed above. The parameters for incubation and luminescence measurement were 26-28°C with orbital spinning at approximately 1200RPM and the luminescence read by the TD-20e luminometer after 60-120 minutes; 5sec integrations with 3sec delay. A number of carbon sources were evaluated using the AOC-II test kit (Table 3).

2.3.1. Toxicity Evaluation:

Some compounds may interfere with luminescence produced by *V. fischeri* cells, resulting in diminished light levels in samples that contain a low concentration of AOC. In other cases, where AOC concentrations are high, the more diluted samples may exhibit higher luminescence than the concentrated samples, due to dilution of the toxic agent.

Toxicity testing was performed when the luminescence response was lower for the first vials compared to the response of the more diluted samples. Testing for toxicity involved the preparation of four additional vials. Taking 3.5mL of the test water sample and placing it into 0.5mL of CAB prepared an 87.5% mixture of the test water sample. Following that, 1mL aliquots were dispensed into 4 separate vials. The putative "toxic" vials (#1-4) were spiked with 10, 20, 40 and 80µl DCS, respectively. The vials were inoculated following PA-2.3.

2.3.2. Metabolism of several organic compounds as an energy source:

While developing the standard AOC assay, Van der Kooij tested several organic compounds to determine the nutritional requirements, versatility and sensitivity of the AOC organisms (P17 and NOX). To test these parameters for *V. fischeri* several organic compounds (Sodium acetate, Casamino acids, Yeast extract, Sodium citrate, Starch, Glucose, Glycerol, Benzoic acid, Lysine, Maltose, Phenylalanine, and Pyruvic acid; Table 3) were tested. Two negative controls (no carbon source) and six positive controls, 5, 10, 20, 50, 100 and 200ppb of CCS were prepared as "test carbon sources". For each

"test carbon source", either a 5mg-C/mL or a 5mg/mL (for Casamino acid and Yeast extract) volume was prepared. It was diluted to a 5ppm concentration of sample. In each appropriate assay vial, 1, 2, 4, 10, 20, 40µl of DAB was replaced with an equal volume of the appropriate 5ppm working "test carbon source" solution following PA-2.3.

2.3.3. Analysis of test water samples for AOC concentrations:

Several test water samples (RO-P, OCWD SMW, EC, PD, and SC SMW; Table 4) were analyzed for AOC concentration. The water samples were collected using AOC-free glassware. As reported in PA-2.2.10, it was determined empirically that the luminous bacteria *V. fischeri* were sensitive to chlorine treatment. Therefore, 10ppm of Sodium thiosulfate (NaThio) was added routinely to each water sample to neutralize any chlorine present. All water samples were filter sterilized using a 0.22µm Nylon sterile filter system (Corning Costar, Corning, NY; cat. No. 25944), then refrigerated for storage. The test water samples were prepared and ran as outlined in PA-2.3.

2.4. Detection of cell volume increase using Standard AOC bacteria (P17) and (NOX)

A different approach was applied for measuring biomass increase in an attempt to improve the time required to measure growth for the Standard AOC method (1998). It was hypothesized that growth could be measured using an automated cell counter, such as the Beckman Coulter Multisizer II (CC; Beckman Coulter, Inc., Miami, Fl.), which would allow one to measure growth in a time frame of 12-24 hours. Currently, as stated in the Standard AOC method, biomass increase is monitored using plate counts that can require up to two weeks. The objective was to develop this method and then apply it to the OCWD SMW sample several times to validate the approach with wastewater.

2.4.1. P17 and NOX culture preparation

A lyophil of *Pseudomonas fluorescens,* P17 (ATTC no. 49642) and *Aquasprillum sp.,* NOX (ATTC no. 49643) was obtained from the American Type Culture Collection (Manassa, VA). Each organism was grown at room temperature in Nutrient broth (NB; Beckton Dickinson and Co.; Sparks, MD; no. 234000), which was prepared as per the manufacturers instructions. After approximately 24 hours and when turbidity was observed, 1mL aliquot was removed and placed in 1/4 dilution of NB at room temperature. Typically, turbidity was observed in 1/4NB after 12-24h for P17 and 24-48h for NOX. A 1mL aliquot was removed and placed in 1/10 HCMM2 media, which was used as a defined water source.

Preparation of HCMM2: In a 1L Erlenmeyer flask, 939mL of 18Mohm deionized water was added; followed by 20mL of 1M Phosphate buffer stock (28.4g Sodium phosphate, dibasic; 27.2g Potassium phosphate, monobasic in 300mL 18Mohm water),

10mL of 1.8M Ammonium sulfate stock (47.6g Ammonium sulfate in 200mL 18Mohm water), 10mL of 49.5mM Potassium nitrate stock (10.0g Potassium nitrate in 200mL 18Mohm water), 10mL of 20mM Magnesium sulfate stock (1.0g Magnesium sulfate in 200mL 18Mohm water), 10mL of 6.8mM Calcium Chloride stock (0.2g Calcium chloride in 200mL 18Mohm water) and 1mL of Trace metals mix which contained 2.86g of H_3BO_4; 1.53g of MnSO4; 3.52g $Fe(NH_4)2(SO_4)_2$; 0.0392g $CuSO_4$; 0.0209g $ZnCl_2$; 0.0406g of $CoCl_2$; and 0.0252g Na_2MoO_4 in one liter. The HCMM2 media was appropriately diluted to a 1/10 concentration; the media was sterilized by heat and pressure (121°C at 15psi) for 20 minutes.

Preparation of P17 and NOX stock cultures (1ppm NaOAc-C): In separate 125mL Erlenmeyer flasks, 49mL of 1/10 HCMM2 media was added, and 10µl of media was replaced with an equal volume of stock NaOAc (5mg-C/ml) resulting in a 1ppm NaOAc-C solution. Each stock culture was inoculated separately with 1ml of P17 and NOX grown in 1/4NB at room temperature until turbidity was observed, and then cultures were stored at 4°C.

Preparation of P17 and NOX test cultures: In separate 125ml Erlenmeyer flasks, 49ml of 1/10 HCMM2 media was added. To prepare each culture with 5, 10, and 50ppm NaOAc-C, respectively, 50, 100 and 500µl volumes of media were replaced with equal volumes of NaOAc stock (5mg-C/ml). Each test culture was inoculated with 1ml of the respective stock culture and grown at room temperature.

Preparation of OCWD SMW test culture: In separate 125ml Erlenmeyer flasks, 49ml of OCWD SMW test water was added. Each test culture, P17 and NOX, was inoculated with 1ml of the respective stock culture and grown at room temperature.

2.4.2. Coulter Multisizer evaluation of cell volume:

The evaluation and determination of volume increase for P17 and NOX cultures were performed using the CC to measure the increase in cell volume through lag phase, log phase and stationary phase.

For each culture, multiple volume measurements were taken over a period of time using constant device parameters: Siphon volume at 100µl; orifice diameter was set at 20µm; range at full; coincidence correction is on; total sample volume was 10mL; instrument background was set generally for less than 10%. The ionic solution, Isoton Diluent II (Beckman Coulter, Inc., Fullerton, CA), used for the study was filter sterilized (0.22µm Nylon sterile filter system (Corning Costar, Corning, NY; 430769) and degassed for 15+ hours before use in sample preparation.

Preparation of CC samples: Into a 20mL plastic CC cuvette, a 10mL aliquot of Isoton II was placed. A 200µl aliquot of the appropriate test culture (5, 10, 50 ppm of NaOAc or OCWD SMW) replaced an equal volume of Isoton II, which prepared a 1/50 diluted sample for CC. As the turbidity of the culture increased, the dilution factor used for the

CC samples increased to 1/100, 1/500 and 1/1000, and appropriately the culture aliquots of the 10ml CC sample volume that were replaced, decreased to 100, 20, and 10µl, respectively. Each prepared CC sample was run on the instrument within 15 minutes of preparation. This was done to prevent crenation of the cells in the hypertonic environment. The test cultures were measured many times for a period of time ranging from 0-335 hours. The raw cell volume determined by the CC instrument was converted into total cell volume/mL and examined against time.

3.0. Project Outcome

All readings were performed at 120 minutes to estimate the concentration of AOC for organic compounds and source waters as per PA-2.2. Negative controls were run and the average readings were calculated (Ave. value) along with the standard deviations (SD). Consequently, as stated in the manual supplied by the manufacturer, the Ave. values were subtracted from all assay readings and graphed. In addition, three times the standard deviation (3XSD) was determined from negative controls (run in triplicate). Generally, only the luminescence values that exhibited greater than 3XSD and were graphically linear were used to calculate a range of AOC values. It was strongly recommended by the manufacturer that values greater than 3XSD be used to calculate the range of AOC present in the water samples. The manufacturer considered values that were less than 3XSD as background readings, which resulted from noise in the assay.

3.1. Evaluation of the AOC Multi-Shot Test:

The Checklight bioassay was initially performed as specified by the manufacturer, using the number of vials and buffers provided with the assay (Figure 3). The assay was performed using the Carbon cocktail solution (CCS) provided (Figure 4; Table 5) to determine its sensitivity range. The response to Carbon cocktail standards (after correction, in which values from the negative control samples were subtracted from all readings) showed negative values, was non-linear (Figure 4) and furthermore, none of the values were three times the standard deviation or greater (Table 5). As a result, the sensitivity range could not be determined, this experiment showed that the luminescence response of *V. fischeri* cells was very weak and that it did not correlate to the CCS concentration. Therefore, the cells did not metabolize the carbon provided.

The cells from the same lyophil were used to test RO-I feedwater (Table 4) that was diluted in assay buffer (CAB) from 90% to 0.35% concentrate (Table 6). As shown in Figure 5, no response was observed, even when the sample was diluted to 0.35% of the concentrate. As observed in Table 6, the relative luminescence was never greater than the values obtained from the negative control samples. This suggested that some aspect of RO-I feedwater might have inhibited bioluminescence. After much investigation, it was determined that RO-I feedwater contained some chlorine residual and acidic pH (pH 5.5-5.9) and that the presence of both chlorine and acidic pH can inhibit bioluminescence, even when samples are diluted. Therefore, all samples analyzed from this point that were

suspected to contain a chlorine residual were dechlorinated by adding 5ppm of sodium thiosulfate to the sample at the time of collection. Sample RO-I feedwater was not analyzed any further due to chlorine and acidic pH. Instead, OCWD SMW was used, which could be dechlorinated and the pH was neutral.

3.1.1. Test sensitivity using carbon cocktail standard

Few additional controls were added to the assay to determine the sensitivity range and to compare results with the standard Van der Kooij AOC bioassay. The standard AOC bioassay has a reported sensitivity range of 1-126ppb of acetate carbon (Van der Kooij, 1988), whereas the Checklight assay recommended range, as reported by manufacturer was between 50-400ppb. Additional positive control samples were prepared for each assay to test a range from 5-400ppb. A new lyophil was used to test the sensitivity at this range. As shown in Table 7, the luminescence response from this lyophil was greater and more consistent (low 3XSD value) than the cells in the previous lyophil. A linear response that was greater than 3xSD was observed in the range of 10-100ppb (Figure 7) using CCS as the standard, showing that a correlation can be demonstrated between CCS metabolism and bioluminescence by *V. fischeri* in that range. The cells did not correlate at higher concentrations of CCS, ranging from 200-400ppb (Figure 6). This experiment needed to be repeated to confirm the sensitivity range and to determine the maximum threshold of carbon that can be metabolized by these cells.

3.1.2. Comparison of normal concentration of *V. fischeri* to diluted cells

Initially the assay was performed using the concentration of cells recommended by Checklight (Figure 3). As shown in Table 8, the overall bioluminescence of the cells in response to CCS was stable (a very low value for 3XSD with negative controls) and much greater than observed from previous lyophils. Overall, a linear response that was greater than 3XSD was seen between 5-200ppb (Figure 8). The relative luminescence value observed at 50ppb was believed to be an anomaly that may have resulted from cells sticking or improper mixing. Overall, the response that was observed from the previous lyophil to CCS concentrations was repeated in this experiment (Figure 8). In an additional experiment, using the same cells, the concentration of CCS was serially diluted from 100 ppb to 0.39 ppb to determine the sensitivity range (Table 9) and to repeat the previous observations using the same lyophil of *V. fischeri* cells. As seen in Figure 9, a linear response was observed between 3.13 –100 ppb of CCS. This repeated the range that was deciphered in Figure 8, suggesting that the sensitivity range of the assay using CCS as a carbon source is between 3-100ppb. This experiment strongly suggested that variability in the response observed in previous experiments might be a result of different lyophils used in the various experiments. Suggesting that the physiology of the *V. fischeri* cells in the lyophils may be highly variable.

In most cases, high background readings were observed with negative controls within a very short time period. Therefore, it was proposed that diluting the cells by 1/10

concentration would result in lowering the overall luminescence values, which could improve the sensitivity range of the assay. In Figure 10, the sensitivity range of the assay was determined to be between 50-100 ppb, a very tight range in which a linear response that was greater than 3XSD was observed. This observation was also repeated in Figure 11, where CCS was used as a test carbon sample and then serially diluted from 100 ppb to 0.39ppb and 1/10 concentration of cells were used for the assay. The overall luminescence value for the negative control samples was extremely low, as was the overall response to the carbon cocktail standard (Table 10 and Table 11). As expected, overall luminescence values were approximately 1/10 of the concentrated values observed in Tables 8 and 9, respectively. This strongly suggested that a minimum concentration of cells was required to perform the assay and the number of cells could not be varied to improve the bioluminescence response.

3.1.3. Reproducibility test:

To test the reproducibility of the bioluminescence response to carbon cocktail from a single vial, several sets of assays were run with the same concentration of CCS (Figure 12 and Figure 13; Table 12 and Table 13). Both 100 and 200 ppb concentration of CCS resulted in a response that was reasonably constant, a projected result. The bioluminescence response to 200ppb of CCS was constant as with the 100ppb. As expected, luminescence values were twice as high as 100ppb values. This experiment was considered important because it demonstrated that the variability observed was putatively due to the physiology of the cells that varied in each new lyophil.

3.1.4. Test sensitivity using a mixture of complex organic compounds

The standard Van der Kooij assay uses P17 and NOX as standard AOC organisms. These organisms can nutritionally assimilate a variety of compounds for the purpose of increasing their biomass (Table 1 and Table 2). To determine a profile of compounds that can be metabolized by *V. fischeri* as a carbon source, complex carbon sources were chosen to start with. A mixture of carbon cocktail and Casamino acids (CA) was selected as a standard. All luminescence readings were read at 175 minutes as oppose to 120 minutes due to laboratory conditions. A linear response that was 3XSD was observed between 20 and 200ppb with this mixture that leveled off between 200 and 400 ppb (Figure 14; Table 14). Providing additional complex carbon did not improve the resolution of the assay or improve the relative luminescence readings. The relative luminescence readings were considered low (Table 14), suggesting that the carbon source might be too complex to be metabolized by *V. fischeri* cells. These cells may not be adapted to utilize complex carbons, since their natural environments normally do not contain complex carbon at these concentrations.

3.1.5. *V. fischeri* starvation:

It was hypothesized that if the *V. fischeri* cells were starved, and not provided any carbon for a period of time, a linear response would result that was directly proportional to the amount of carbon provided. In addition, starving the cells could result in lower background readings, and in doing so improve the sensitivity of the assay. In this experiment, cells were starved for approximately an hour and that resulted in a response that was weak (Figure 15) with luminescence values that were less than 3XSD of the negative controls (Table 15). It was suspected that the cells were injured without the carbon source. Another possible explanation that could not be eliminated was that the cells present in this lyophil were physiologically inactive and could not respond to the carbon provided. This experiment was repeated using 200 ppb of carbon cocktail that was diluted to 0.75ppb of carbon cocktail (Figure 16) a weak response was observed that was linear at the higher dilutions of CCS (50-200ppb), but the response was too weak to be discernable to correlate to the concentration of carbon (Table 16).

3.1.6. Test response of *V. fischeri* on simple and complex organic compounds

To determine the nutritional requirements of *V. fischeri*, simple and complex organic compounds were tested for metabolism. Casamino acid (CA) was repeated as a complex carbon source. As shown in Figure 17, a linear response was observed between 20-400ppb of Casamino acid-Carbon (CA-C), but the luminescence response was too weak to correlate it to the concentration of carbon present, even at 200ppb (Table 17). Using cells from the same lyophil, sodium acetate (NaOAc) was used as an example of a simple organic compound to develop a linear relationship between carbon metabolized and luminescence readings. A linear response was observed between 20-200ppb, but values greater than 3xSD were only observed between 50-200ppb of NaOAc-C (Figure 18). As seen in Figure 18, the response with NaOAc was even weaker than that observed with CA (Figure 17). Initially, it was speculated that the response was due to the residual carbon present in the media used in the lyophil to sustain the cells in a freeze-dried state. Another possibly explanation was that not all the cells in the lyophil were viable, resulting in relatively poor response to both CA-C and NaOAc-C. To test this possibility, cells were grown on recommended medium (Marine agar) for *V. fischeri*. It was determined that all of the cells that grew on the media did not illuminate in the dark (data not shown). Even after subsequent transfer on the growth medium, most of the cells remained dark. Suggesting that the *lux* gene naturally harbored in *V. fischeri* was unstable and could not be recovered even after repeated plating during which the cells had gone through various growth stages.

It is thought that a combination of the two explanations described above may be occurring. The relative luminescence that was recorded was in response to the residual carbon present in the lyophil and not all the cells that are viable are able to express bioluminescence. This may explain the weak response and the variability observed in 3xSD values from the two sets of negative controls prepared (Table 17 and Table 18). The values for 3xSD for the two sets of negative controls were significantly different

even though the cells were removed from the same lyophil, suggesting that the response was not uniform and not related to the carbon sources provided.

3.2. AOC-II Test Kit

It was communicated by the manufacturer that alterations were being performed to the bioassay to improve stability of the organism and to lower background readings. Therefore, additional AOC bioassay kit was purchased and assays were performed using CCS as per manufacturer recommendations. Figure 19 shows that the bioluminescence response to CCS was higher than previously observed and all values were greater than 3XSD and linear between 5-200ppb (Figure 20). The bioluminescence response to CCS was saturated and leveled off after 200ppb (Figure 19). In most cases the level of response observed correlated to the concentration of CCS-C provided to the cells (Table 19). The relative luminescence reading for 50ppb of CCS-C did not correlate with the other concentrations tested, and seem to be an anomaly that may have resulted from sticky cells or improper mixing in that particular vial.

3.2.1. Testing diversity using simple organic compounds

To test the diversity of organic compounds that can be metabolized by *V. fischeri* cells as a sole carbon and energy source, several simple organic compounds were tested. The carbon cocktail standard (CCS) provided with the kit was determined to be a complex carbon source that was not well defined (the exact concentration of Yeast extract and Glucose varied in each kit); therefore, was not used as a carbon source to develop the standard curve for each of the assays. Instead, a defined complex carbon such as Casamino acids (CA) was used as a putative carbon standard to develop a standard curve.

Sodium acetate (NaOAc)-C was used as an example of a simple organic compound. This compound was used by Van der Kooij to test P17 and NOX, the standard AOC organisms (Table 1 and Table 2) and to develop a standard curve for his assays. As seen in Figure 21, the relative luminescence values were linear between 5-50ppb NaOAc-C but none of the values were greater than 3XSD (Table 20), suggesting that NaOAc was not metabolized by the cells and the relative luminescence readings were a result of either residual carbon or contaminating carbon that may be present in the plastic vials provided with the kit. Using the cells from the same lyophil, Casamino acids was used to develop the standard curve and the result was a response that was highly variable (Table 21; Figure 22) and did not compare well to the concentration of carbon provided (the relative luminescence values were almost identical between 50 and 400ppb CA-C; Table 21). In addition, glucose-carbon (Glu-C) was tested as an energy source with the cells from this specific lyophil. The response was variable as with the compounds tested above (Figure 23) and did not correlate to the concentration of Glu-C provided (Table 22).

Glycerol was used as a simple carbon source that is easily metabolized by the cells to test variability in the physiology of the cells from one lyophil to another. Cells from three

different lyophils were run in parallel using Glycerol (Gly) as a carbon source. As seen in Figure 24 and Table 23, the response was highly inconsistent from one lyophil to another, even for the negative control and 3XSD values (Table 23). Lyophils A and B showed linear regions but the relative luminescence values did not compare to the concentration of Gly-C provided.

As suggested above, a combination of conditions may be occurring that result in a highly inconsistent response by the cells. It was speculated that residual carbon, variability in the physiology of the cells and/or contaminating carbon might all contribute to the inconsistencies observed in the relative luminescence values. Each of these possibilities was explored starting with contaminating carbon in the plastic assay vials provided.

3.2.2. Evaluation of Checklight assay vials (plastic)

As established by Van der Kooij, the standard AOC assay is a fastidious and sensitive assay that requires AOC-free glassware (LeChevallier, et.al., 1993), therefore, Checklight plastic assay vials were tested for the presence of contaminating AOC-carbon since they were shipped unsealed.

Four different lyophils were tested using CCS as a carbon source and plastic vials were used as provided with the kit. As seen in Table 24, the 3XSD values for the negative control are highly inconsistent but both lyophils provided a linear response with lyophil B displaying a liner response that can directly relate to the concentration of CCS (2.5-250ppb), since the values were greater than 3XSD (Figure 25). Lyophil A corresponded to CCS concentration that ranged from 20-200ppb. These observations were repeated when CCS was serially diluted from 300-6.25ppb (Figure 26; Table 25), where lyophil B showed a linear response from 6.25-200ppb, whereas lyophil A was linear from 25-200ppb.

3.2.3. Comparison of "unwashed" and "washed" assay vials

Cells were hydrated and used to inoculate "washed" vials that were compared with "unwashed" vials. It was shown that both sets of vials displayed similar negative control values but 3XSD values were considerably lower with "washed" vials and showed significantly higher relative luminescence values than "unwashed" vials (Figure 27 and Figure 28). When the sensitivity ranges were compared between the "washed" set and unwashed, it was determined to be between 40-200ppb and 50-200ppb, respectively (Table 26 and Table 27). The similarity in the ranges could not be explained.

3.2.4. Acid-washed assay vials

As outlined in Standard Methods (1998), all AOC assays are performed using glassware that is acid-washed and heat sterilized to remove any residual AOC. This was tested using glass assay vials that were prepared as per Standard Methods.

Two individual lyophils were run separately using CCS as a standard carbon source using acid-washed assay vials. The 3XSD values were relatively low for both lyophils (Table 28) and the relative luminescence response was linear from 2.5-250ppb, suggesting that the cells responded to the carbon source provided (Figure 29). At the same time, CCS was serially diluted and used as a test sample to repeat the experiment. As seen in Figure 30, a linear response was observed from approximately 35-300ppb and 12.5-250ppb for lyophil A and B, respectively (Figure 30; Table 29). Consequently, greater homogeny was observed in the relative luminescence values with the acid-washed tubes. Consequently, acid-washed tubes were used for all the assays performed from this point.

3.2.5. Examination of integration times

It was highly recommended by the manufacturer that the integration and delay times be shortened to reduce the noise in the assay. A simple carbon source such as Gly-C was used to test this using an integration time of 5 and 30 seconds. As seen in Figure 31, a linear response that is greater than 3XSD was observed between 25-200ppb Gly-C with both integration times (Table 30); since no significant difference was observed, a 5 second integration time was incorporated into the protocol along with using acid-washed glass vials. Some anomalies were observed relating to the relative luminescence values (Table 30) from 0.78-12.5ppb. One explanation for such irregularity is that the cells clumped during the assay and therefore were improperly mixed before inoculation.

3.2.6. *Vibrio fischeri* stabilization

In order to further stabilize the relative luminescence values and to remove the affects of residual carbon from the media used in the lyophils, cells were monitored and allowed to establish before the assay vials were inoculated. During the stabilization process, cells were hydrated in hydration buffer that contains no carbon, and the relative luminescence was monitored until the luminescence values leveled off. Therefore, all prior values were considered background noise in response to the carbon present in the media used for the lyophil. This was tested using CCS-C ranging from 2.5-100ppb (Figure 32). It took approximately 125 minutes before the relative luminescence values stabilized (Figure 32). Essentially, the cells remained dark for approximately 50 minutes, even though lingering carbon may be present. Stabilized cells were tested with CCS-C for metabolism and sensitivity (Figure 33; Table 31). The relative luminescence values were greater than observed before and linear even though the 3XSD values very relatively high (Figure 33; Table 31). The results were reproduced when CCS was diluted from 150-2.34 ppb and

used as a test carbon sample (Table 32; Figure 34). The relative luminescence was higher and linear suggesting that the cells metabolized the CCS-C to produce light. This demonstrates that by starving the cells for some time, all the residual carbon is utilized by the cells so that when carbon is provided, light production is in response to the supplied carbon.

3.2.7. Assessment of stabilized *V. fischeri* cells

Cells were hydrated from a new lyophil and then stabilized for approximately 115 minutes before they were provided CCS as a carbon and energy source (Figure 35). Seen in Table 33, 3XSD values were low and the relative luminescence was significantly higher (Figure 36). A linear response that is greater than 3XSD was observed from 5-200ppb of CCS-C (Figure 36). This was repeated by serially diluting CCS-C from 200-2.34ppb and a linear response was observed from approximately 9-200ppb (Figure 37 and Table 34). An erroneous reading was observed at approximately 20ppb of CCS-C (Figure 37 and Table 34); one explanation is that the cells had clumped during the assay, resulting in such a high value.

3.2.8. Metabolism of simple organic compounds by stabilized *V. fischeri*

To characterize the nutritional profile of stabilized cells, various simple organic compounds were tested to determine if they could be metabolized as sole carbon sources. It was also important to determine the sensitivity ranges of these compounds since a variety of these are present at extremely low concentrations in wastewater but can still support growth. *V. fischeri* cells were hydrated and then stabilized for approximately 177 minutes prior to use in the assay (Figure 38). As seen in Figure 38, cells remained dark (virtually dark) for approximately 50 minutes following hydration even though some residual carbon is present in the system. In this experiment, CCS was used as a standard carbon to compare the response of the organic compounds. As seen in Figure 39, a linear response that was greater than 3XSD was observed from 10-200ppb of CCS-C (Table 35). This essentially repeated the observations in Figure 36; strongly suggesting that stabilizing the cells reproducibly reduced the variability (low 3XSD values and liner response).

To determine if the stabilized cells could produce light in response to glucose (Glu) and glycerol (Gly), several dilutions of Glu and Gly were prepared as test carbon samples (Table 36). Both carbon sources produced a linear response between 9-150ppb of carbon (Figure 40; Table 36), in which all luminescence values were greater than 3XSD. It is important to note that light production correlated better with Glu-C than with Gly-C, suggesting that these cells preferred Glu-C as an energy source (Figure 40; Table 36). A separate lyophil was used to test metabolism of Sodium acetate (NaOAc) and Fructose (Fru). These cells were stabilized for approximately 300 minutes and remained fairly dark for approximately 75 minutes (Figure 41). CCS-C was used as a standard that produced a linear response between 10-200ppb of CCS-C with exceedingly high relative

luminescence values (Figure 42; Table 37). These values were compared with of NaOAc and Fru, which resulted in a linear response between 9-150ppm (Figure 43; Table 38) and all values were greater than 3XSD. This demonstrates that the light production was a result of metabolizing NaOAc-C and Fru-C carbon and not due to noise. The overall light production was greater and linear with Fru-C, suggesting that Fru-C is preferred over NaOAc (Figure 43; Table 38).

3.2.9. and 3.2.10. Use of Sodium acetate as a standard curve and assessment of water samples for AOC concentration

Many of the AOC assays published, including the Standard Method (1998) use acetate as a standard. To compare methods, NaOAc-C was tested to determine if it could be used as a standard with stabilized *V. fischeri* cells. This standard curve was then applied to estimate the range of AOC present in OCWD secondary municipal wastewater (OCWD SMW). In addition, this sample was also analyzed using the Standard Method (assay was performed at Metropolitan Water District), in triplicate, using AOC bacteria P17 and NOX. For the Checklight assay, the cells were stabilized for approximately 200 minutes (Figure 44) and then inoculated with in NaOAc-C carbon that ranged from 2.5-200ppb. The response was relatively high, linear and greater than 3XSD between 10-100ppb (Figure 45; Table 39). The stabilized cells were used to approximate the range of AOC concentration in OCWD SMW sample that was serially diluted from 87.5 to 1.36% of concentrate (Table 40). All relative luminescence values were greater than 3XSD (Table 40) and linear in that range with an R^2 of approximately 0.99 (Figure 46). It was calculated (as per manufacturer recommendations) that this converted to a range of 274.7 - 317.3 ppb of acetate-C (units normally used to monitor AOC concentrations). The expected range for this water source is between 1,000-1,500 ppb, which is based on empirical TOC values determined by OCWD. It has been reported that characteristically AOC concentrations are expected to be approximately 10% of TOC (LeChevallier, et. al., 1993), depending on the treatment process. The range observed with this bioassay does not reflect 10% of the TOC present in the water sample. This water sample was also analyzed using Standard Methods, which determined that OCWD SMW contained 332.33 ppb of Acetate-C. Therefore, it was speculated that the range obtained by Checklight might be within the normal variation for this water source due to alterations in the treatment process. To model this variation, AOC concentrations need to be monitored for OCWD SMW over a long period of time and determined using both the Standard Method and the Checklight bioassay.

3.3.2. Metabolism of several organic compounds as an energy source

An additional AOC-II kit was purchased to evaluate the performance of this assay with a variety of feedwaters. First, several organic compounds were tested to describe the nutritional versatility of *V. fischeri* cells and to determine if the bioassay could be used with a variety of water sources that contain a mixture of these compounds at extremely low concentrations. It is known that standard AOC bacteria use these compounds for

growth. Second, it was also necessary to determine if *V. fischeri* cells could metabolize these compounds without having to become acclimated to them. For each of the assays performed, acid-washed glass vials were used and the cells were stabilized following hydration. Three different lyophils were used to complete the test; time required to stabilize the lyophils are presented in Figure 47. The light output from lyophil C did not stabilize until after 120 minutes, but cells were used to inoculate vials at 120 minutes (Figure 47). As shown in Figure 48, the following compounds were metabolized by *V. fischeri* cells within the respective range: Pyruvic acid (20-200ppb); Starch (20-200ppb); Acetate (10-200ppb); Lysine (5-200ppb); Maltose (5-200ppb); Casamino acids (20-200ppb); Yeast extract (20-200ppb); Glucose (10-200ppb); Phenylalanine (50-200ppb); Citrate (5-200ppb); Benzoic acid (20-200ppb) Glycerol (5-200ppb). Following this characterization, stabilized *V. fischeri* cells are nutritionally comparable with the standard AOC bacteria (P17 and NOX), but the sensitivity range with the standard AOC bacteria is enhanced. It was observed that with certain organic compounds (i.e. acetate) the sensitivity range for this AOC-II kit was not as low as with previous kits purchased. The manufacturer could not explain this in detail, other than that physiology of the cells can vary from one kit to another.

3.3.3. Analysis of test water samples for AOC concentrations

Several test wastewater samples were analyzed for AOC concentrations using the Checklight bioassay. OCWD SMW samples were split and were also analyzed using the Standard Method (assay was performed at Metropolitan Water District of Southern California), and the values were compared. When both methods were employed, samples were prepared similarly, except samples analyzed using the Checklight assay contained 10ppm of NaThio; both methods used sodium acetate as a standard. All water samples were evaluated for interfering compounds (toxicity; see 2.3.1), therefore, all Acetate-C carbon standards were prepared between 50-400ppb since a limited number of lyophils with *V. fischeri* cells were available. It was alleged that if Acetate-C was linear and above 3XSD between 50-400, then it should be linear at the lower concentrations.

AOC concentrations in OCWD secondary municipal wastewater (OCWD SMW) and Santa Clara secondary municipal wastewater (SC SMW) were determined. OCWD SMW was monitored using both the Checklight bioassay and Standard Method. Once hydrated, the cells were stabilized (Figure 49); as observed in the tests above, the cells remained fairly dark for approximately 50 minutes and light production stabilized after approximately 125 minutes. A NaOAc-C standard was run along with the assays that resulted in a relatively high and linear response between 50-400ppb of NaOAc-C (Figure 50; Table 41). The standard was used to determine the concentration of Acetate-C in the water samples. In addition, extra vials were run to determine if the water samples contained inhibitors that may interfere with light production; no toxicity affects were observed with either one of the water sources (Figure 51). As shown in Table 42, a linear and high response that was greater than 3XSD value (3.818) was observed between 87.5-5.46% of the concentrated OCWD SMW sample (Figure 52), suggesting that the cells metabolized AOC from the conventionally treated wastewater. Using the Acetate-C as

standard, it was calculated that the range of AOC present in the sample was between 540.45-549.2 ppb of Acetate-C. This range was compared with the Standard Method that resulted in 624.2 ppb of Acetate-C. It is hypothesized that if OCWD SMW was monitored for a period of time, a correlative coefficient factor to convert values to Standard assay could be modeled that may be applied to the Checklight assay for future tests.

In contrast, Santa Clara secondary municipal wastewater (SC SMW) failed to produce response from *V. fischeri* cells (Figure 53). A linear response was not observed with this sample, and more importantly, none of the relative luminescence values were greater than 3XSD (Table 42), therefore, none of the readings could be used to compare to the Acetate standard. No further opportunities were made available to repeat the sample or to have the sample analyzed using Standard methods. Since inhibitors were not present, it could not be determined if the sensitivity range of the assay was not low enough to detect low concentrations of AOC in the sample.

A new lyophil was used to analyze RO product water (RO-P) and to determine if inhibitors were present due to the treatment process. The *V. fischeri* cells were stabilized for 120 minutes (Figure 54) prior to use in the assay. NaOAc-C was used as a standard, which was linear and all values were greater than 3XSD between 50-400ppb (Figure 55; Table 43). The presence of inhibitors was determined by adding 50-400ppb of NaOAc-C to RO-P water. A linear response that was comparable to the acetate standard was observed (Figure 56), demonstrating that inhibitors (if any present) did not impact light production. RO-P water was diluted from 87-1.36% (Table 44) and a fairly linear response was observed between 5.46-43.75% (Figure 57). The relative range of luminescence was much lower than that observed with acetate, suggesting that the water contained very low concentrations of AOC. Using Acetate-C as a standard, it was calculated that the range of AOC present in the sample was between 0 – 0.891 ppb of Acetate-C, suggesting that this RO product water has very little biofouling potential during distribution and storage.

Fountain Valley potable drinking water (PD) was examined for AOC. Stabilized cells were used to execute the assay (Figure 58) and NaOAc-C was used as a standard (Figure 59; Table 45). The samples were tested to determine if any inhibitors were present that may interfere with the assay resulting in artificially low luminescence values. As demonstrated in Figure 60, the overall luminescence developed was analogous to the luminescence developed with acetate. Suggesting that luminescence would not be impacted by inhibitors. However, bioluminescence readings from diluted PD water were extremely low (Figure 61) and highly variable, such that only 43.75% and 87.5% of the concentrated water resulted in values that were greater than 3XSD (Table 46). Luminescence values mostly leveled out between 21.87-87.5% (Table 46), therefore, a linear region that can be used to calculate the concentration of AOC could not be determined, suggesting that the concentration of AOC was low and below the detection limit of the assay. Similar results were experienced when the assay was repeated (data not shown). This was communicated to the manufacturer and the results were offered to them for interpretation. The manufacturer calculated a range between 34.60-55.27ppb of

Acetate-C using luminescence values from 5.46% and 87.5% (Figure 62), with an R^2 of 0.8609.

In addition, deep well injection water (EC) was examined using stabilized cells (Figure 63) and NaOAc-C standard (Figure 65), which resulted in a low 3XSD value (Table 47). Figure 64, demonstrates that the EC water was tested for the presence of inhibitors in the presence of NaOAc-C. A linear response was observed between 50-400ppb of carbon, suggesting that inhibitors should not influence light production. When the water source was evaluated, relatively a low luminescence response was seen (Figure 66) that leveled out between 10.93-87.5% (Table 48), but all values were greater than 3XSD. Similar observations were observed when the test was repeated at a later time. This indicated that the concentration of AOC was too low to detect with this kit and perhaps the Standard method would be better suited to reliably monitor AOC concentrations in water sources such as colored groundwater (EC) and Fountain Valley potable drinking water (PD).

3.4.2. Coulter Multizer evaluation of cell volume

To determine an increase in cell volume, which would be converted to an increase in biomass, P17 and NOX were analyzed using the Coulter Multizer. Several cultures were started at time 0 hours and the volume of P17 and NOX was measured at 1, 3, 20, 24, 27, 32, and 48 hours. Each contained either no carbon, 5, 10 or 50ppm of NaoAc-C or filter sterilized OCWD SMW, as a carbon source. Each of these was separately inoculated with P17 or NOX and cell volume in um^3/mL was read. As shown in Table 49, between 10^4-10^6 cells were used to inoculate the various sample types and time zero represents the inoculum size. These values were then subtracted from the values obtained at the various time points. Table 50, lists the total volume of cells that were present after 48 hours. This time frame was chosen as a representative time because some turbidity was observed in the cultures containing P17 and NOX. Therefore, data at 48 hours was used as representative measurements to illustrate the inconsistencies observed using this approach. At 48h, a volume increase was observed with the no carbon source sample (Table 49 and Table 50), greater increase was observed with P17 than with NOX. This suggests that the cells utilized some of the indigenous carbon present to increase their volume. Similarly, 5ppm of carbon resulted in an increase in cell volume, suggesting the cells metabolized acetate-C. Total volume of P17 increased even further with 10ppm of carbon, as expected, but the total volume of NOX at 10ppm did not increase in proportion to the amount of carbon supplied. At 50ppm, the total volume of P17 and NOX did not correlate to the concentration of carbon supplied. In addition, OCWD SMW was used as a representative sample to test this approach, since this sample contained the greatest amount of AOC when last analyzed. As seen in Table 50, inconsistencies were observed with a decrease in volume after 48h, which could not be explained. Volume measurements taken at other times with these samples resulted in similar inconsistencies so the approach was not taken any further. In addition, it was determined that the Coulter Multizer is a device that is extremely sensitive to vibrations, air bubbles, or anything else that might clog the aperture. Therefore, all solutions were prefiltered and required a

trained technician to operate the instrument, suggesting that the approach would not be applicable for most water utilities.

4.0. Project Conclusions and Recommendations

4.1. Conclusions

The objective of this study was to evaluate a new method for rapid determination of AOC in treated and or filtered waters, this includes, but is not limited to reverse osmosis (RO) and microfiltration (MF) feedwaters. AOC measurements are important because it represents the fraction of total organic carbon that is most readily used by microorganisms for growth and other metabolic processes. Therefore, the AOC component is of the greatest interest to water utilities, since high levels of AOC are associated with loss of water quality, rapid biofilm formation and loss of membrane performance. An impressive amount of literature has been published describing different methods that can be used to determine the concentration of AOC, including a Standard method. Currently, Standard Method is the only method available in which increase of biomass of test organisms is directly related to the concentration of AOC. Other methods that have been developed or are currently under development, relate the concentration of AOC to metabolism of organic compounds. Most methods that are fully developed are laborious and costly, which means that when applied, a limited number of samples are analyzed and at a low frequency. Therefore, these methods cannot be used by water utilities for monitoring water quality.

Recently, Checklight was able to demonstrate that the AOC kit made available could provide a rapid assay that could be used to monitor the level of AOC in surface waters. This assay was not tested with wastewater and the relationship of this test to the Standard Method was never determined. Therefore, the Checklight bioassay was systematically evaluated to determine if this approach could be used by water utilities to monitor the level of AOC. Several well-defined tests were performed to determine the sensitivity, effectiveness and reliability of the assay.

The Checklight bioassay was initially performed as outlined by the manufacturer, using the carbon provided with the assay and using RO feedwater (RO-I). Light production in response to the carbon or RO feedwater was not observed. However, it was determined that bioluminescence was inhibited by chlorine and acidic pH. Consequently, all samples that were further analyzed were dechlorinated and their pH was monitored. The sensitivity range of the assay using the carbon cocktail solution provided was determined between 3-100ppb that is greater than the suggested range by the manufacturer (50-400ppb). However, significant inconsistencies were observed with the lyophils provided within each kit and further test demonstrated that physiology of the cells is exceedingly variable. This explained the lack of reproducibility observed when tests were performed at different times. It was also determined that not all the viable cells were able to produce

light, demonstrating that the physiology of the cells provided was variable and therefore, requiring a new standard curve with each assay executed.

In many of the controlled tests, it was observed that the bioluminescence response did not correlate to the amount of carbon provided to the cells. It was hypnotized that contaminating carbon or inhibitors may be present in the vials provided with the kit. Therefore, as outlined in Standard Methods, glass-vials that were acid washed and heat sterilized were used. An improvement was observed in the luminescence values as well as the sensitivity range of the assay. All subsequent tests were performed using the acid-washed, heat sterilized vials.

To further remove the affects of any indigenous carbon that may be present, cells were stabilized following hydration and monitored until the light production leveled out before the cells were utilized in the assay. This approach allowed the *lux* genes to be induced by the carbon provided in the sample and resulted in a linear correlation between the concentration of carbon and light production. In many of the stability tests performed, it took up to 250 minutes for the light production to level out and the cells remained dark, or showed relatively low bioluminescence for up to 60 minutes. As per manufacturer protocol and prior to stabilization, all assays were completed in 120 minutes. This suggests that full bioluminescence potential was not reached by the time the assay was completed in 120 minutes, explaining the low level of light produced. In most of the assays where the cells were allowed to stabilize, the overall light produced was much higher than prior to stabilization. Therefore, stabilization was incorporated into the protocol when all assays were performed. The time for all assays was extended up to 8 hours due to stabilization.

Since a variety of organic compounds are present in wastewater, the nutritional versatility and the sensitivity was determined. Sensitivity of the assay is important because many microorganisms are able to utilize organic compounds as an energy source for growth and other metabolic processes at very low concentrations. Therefore, several different organic compounds most that were metabolized by the standard AOC bacteria were used as test carbon source for *V. fischeri* cells. The nutritional profile confirmed that *V. fischeri* cells were able to metabolize a variety of compounds and produce light in response. Since overall light production was greater with Glucose and Fructose-carbon over Glycerol, sodium acetate and the carbon cocktail, it was determined that Glucose and Fructose were preferred as a carbon source. The sensitivity range for most compounds was between 5-100ppb. The reported sensitivity of the Standard assay is between 1-126ppb of acetate carbon, it is suggested that the sensitivity of the Standard assay is greater since it is a growth-based assay that uses cells that have been acclimated to the acetate carbon used as a standard.

The Checklight assay was used to examine several different waters; a toxicity test was performed in conjunction to determine the presence of unknown inhibitors. The presence of inhibitors needed to be determined to evaluate the outcome of the assay. If toxicity is observed, the relative bioluminescence response is reduced then the concentration of AOC can be determined by diluting the water sample further. Generally, when toxicity

was not observed, and a linear response was observed, the level of AOC in the source water was calculated. In some instances, like deep well injection water, inhibitors were not observed and a liner response that was greater than 3XSD was not obtained, suggesting that the concentration of AOC was below the detection limit of this assay. An approach that could further improve the sensitivity of the assay would be to either concentrate or perhaps pasteurize the sample. However, this would further increase the time required to complete the assay.

The concentration of AOC in OCWD secondary municipal wastewater was evaluated using both the Checklight bioassay and Standard Method. The values obtained by both methods were within approximately 20% of each other. Suggesting, that if OCWD secondary municipal wastewater was monitored for a period of time, a correlative coefficient could be modeled that can be applied to both methods. Santa Clara secondary municipal waste water and deep well injection water was also analyzed using Checklight, and a value for AOC concentration could not be determined due to the sensitivity of the assay. When RO product was used, a range of 0-0.891 ppb of acetate-C was calculated, suggesting that this water has a low potential for biofilm formation in its storage or distribution systems. For Fountain Valley, potable drinking water a range of 34-55 ppb of acetate-C was calculated.

The Coulter Multizer was used to observe an increase in cell biomass by measuring cell volume. This method was tested to determine if the Coulter Multizer could be used to calculate growth, which could then be used in combination with Standard Method to measure AOC. The inconsistencies observed with several of the controlled samples and OCWD secondary municipal wastewater at different time intervals suggests that the device, as used, could not reduce the rate or the cost at which the Standard Method is performed. The Coulter Multizer is an extremely sensitive instrument that requires prefiltered solutions to obtain reliable results. In addition, a trained technician that is dedicated to the instrument would be required to perform the assay.

4.2. Recommendations

Based on the tests performed and the findings presented, the Checklight bioassay is a promising assay under controlled conditions, provided some of the modification outlined in this study are implemented. But this assay needs to be developed further before it can be used to monitor AOC levels in treated or filtered water. Currently, the physiology of *V. fischeri* cells varies within each assay and from one kit to another, resulting in light production that is highly variable. It is recommended that the manufacturer use glass vials and containers for the buffers provided. As published by Van der Kooij, this is a fastidious assay that is sensitive to low concentrations of carbon; therefore, all glassware used in the assay needs to be AOC free.

Based on the findings presented, it is suggested that the *V. fischeri* cells are grown in a chemostat, which is a bioreactor in which constant growth conditions for microorganisms

are maintained over prolonged period of time. This would produce a mass of cells that are physiologically stable and then can be provided with the kit. In addition, *V. fischeri* cells would still need to be stabilized prior to use in an assay to eliminate the impact of indigenous carbon on the amount of light produced by the cells. This could improve the sensitivity of the assay. In addition, it is suggested that the cells are grown in a defined carbon source such as acetate, which is used in the Standard Methods. This could also improve the sensitivity of the assay, since acetate is used in most as a standard in most assays. Finally, it is suggested that if some of the recommendations are implemented and developed into the protocol, then this bioassay can be tested by water utilities to monitor treated or filtered water to obtain a relative level of AOC. Once fully developed, this assay would be easy to use, does not require expensive equipment or highly trained personnel and can be completed in a single workday.

4.3. Benefits to California

The increase in population and drought conditions in California has resulted in limited water supplies. Shortage of potable water has created the need for treatment of alternative water sources such as recycled wastewater, ground and surface waters. The need for new water sources has initiated the search for innovative treatment methods. Water utilities are continuously striving to reduce the presence of microbiological growth; especially pathogens, to supply water that is reliable safe.

Traditional techniques which can require up to two weeks to complete for assessment of microbial growth lack speed and therefore have limited use for water utilities in California. To ensure water quality, water utilities may over disinfect resulting in augmented costs. Currently, most water utilities are seeking rapid and simple methods to predict microbial growth. Any new method that can provide rapid measurements to calculate AOC levels that can predict microbial growth can be used to prevent biofilm formation on membranes; predict loss of membrane performance and can be used to optimize disinfection protocols. These rapid methods can equate to considerable cost saving to the water utilities and at the same time improve water quality.

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Glossary

AOC	assimilable organic carbon
ATP	adenosine triphosphate
ATCC	American Type Culture Collection
Ave. value	average value of negative control samples
BDOC	biodegradable dissolved organic carbon
C	carbon
CA	casamino acids
CAB	concentrated assay buffer
CC	Coulter Counter
CCS	carbon cocktail solution
CCCS	concentrated carbon cocktail solution
CCD	charged-coupled device
CFU	colony forming units
DAB	diluted assay buffer
DCS	diluted carbon cocktail solution
EC	colored ground water
Fru	fructose
g	grams
GEMS	genetically engineered microorganisms
GFP	green fluorescent protein
Glu	glucose
Gly	glycerol
h	hours
HB	hydration buffer
HCMM2	HCMM2 media used as a defined water source
HPLC	high performance liquid chromatography
lux	*lux* bioluminescence operon in symbiotic bacterium *Vibrio fischeri*
LuxCDABE Operon	bioluminescence operon from *Photorhabdus luminescens*
MF	microfiltration
µl	microliter
mL	milliliter
μm^3	micrometers cubed
MMF	mixed media filtration water
Mohm	mega ohm
MSDS	material safety data sheet
N	nitrogen
NaOAc	sodium acetate
NaThio	sodium thiosulfate
NOX	*Spirillum* species strain NOX
P17	*Pseudomonas* fluorescens strain P17
P	phosphates

PA	project approach
PAS	*p*-aminosalicylate
PD	potable drinking water
ppb	parts per billion
RO	reverse osmosis
RO-I	reverse osmosis feedwater
RO-P	reverse osmosis product water
RPM	revolutions per minute
SC SMW	Santa Clara secondary municipal wastewater
sec	seconds
SMW	OCWD secondary municipal waster water
SMW-Cl	OCWD secondary municipal waste water, chlorinated
SD	standard deviations of negative control samples
TOC	total organic carbon
V. fischeri	*Vibrio fischeri*
YE	yeast extract

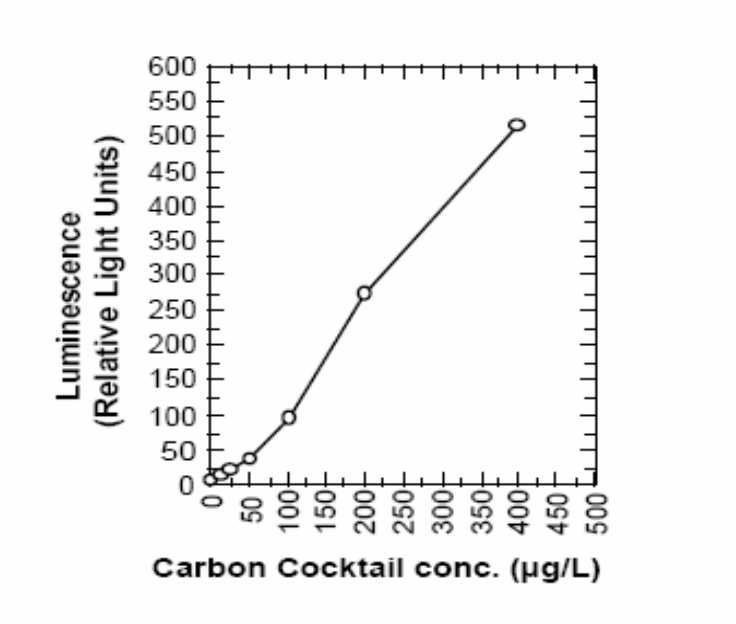

Figure 1: Carbon cocktail as a reference carbon source demonstrating luminescence is proportional to the concentration of available organic carbon (http://www.checklight.co.il/pdf/case_studies/aoc-case-study.pdf).

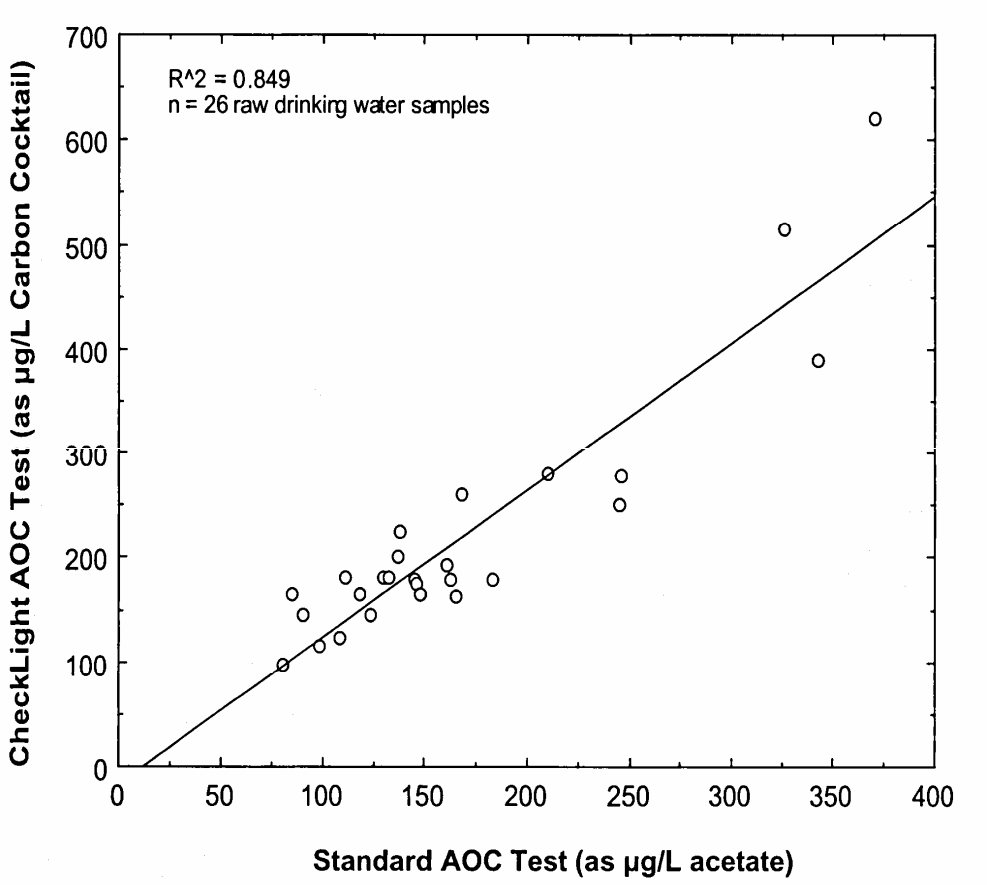

Figure 2: Accuracy and reliability was established by testing 26 different samples of raw drinking water sources along the Israeli Water Carrier system. AOC concentrations were measured using both Checklight AOC test and Standard 7 day test, which measured AOC concentration using acetate (http://www.checklight.co.il/pdf/case_studies/aoc-case-study.pdf).

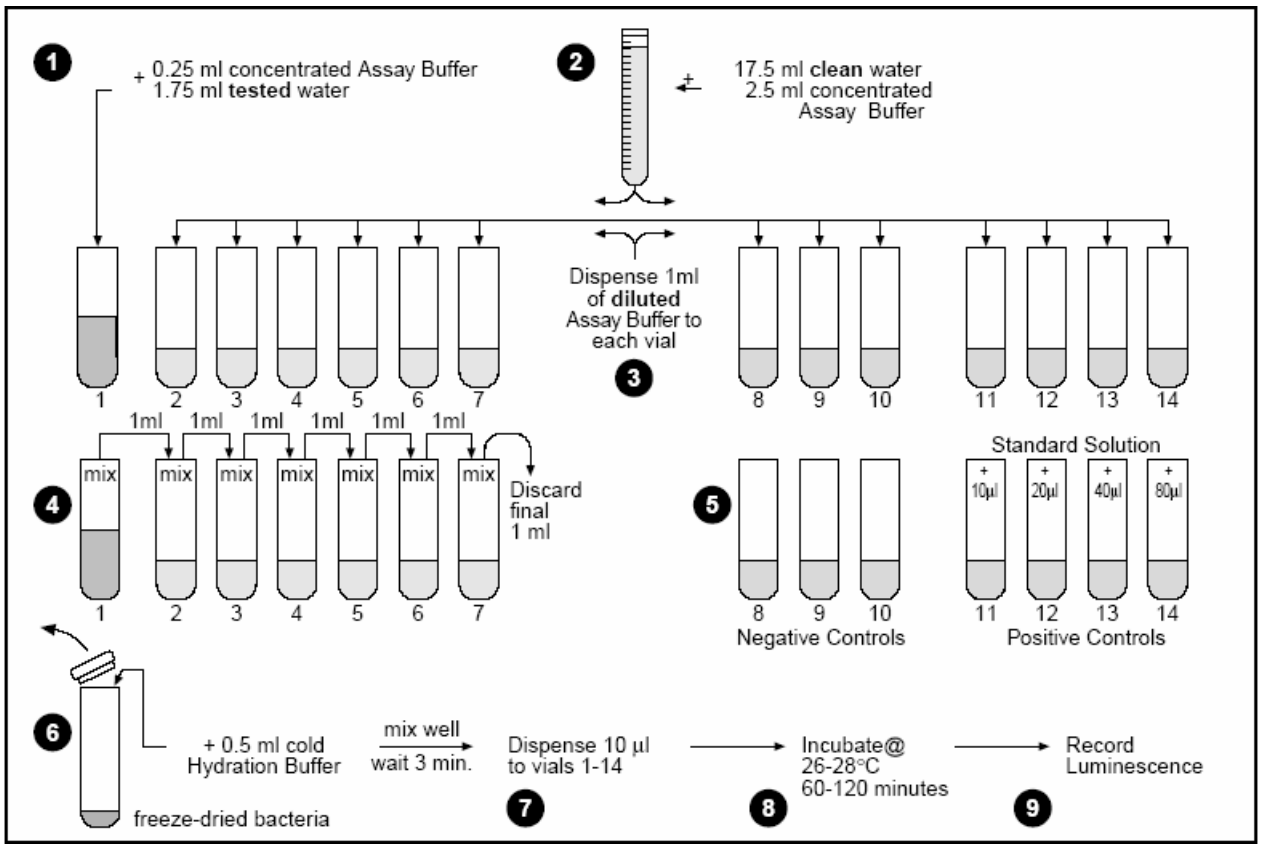

Figure 3: AOC test procedure as outlined by the manufacturer (http://www.checklight.co.il/new_pdf/AOC-II_manual_2004_copy.pdf).

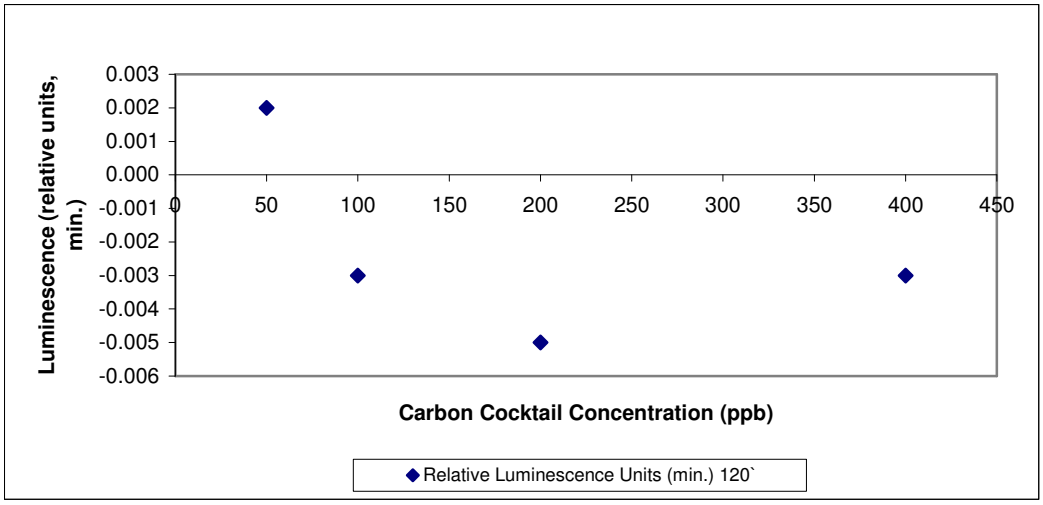

Figure 4: Bioluminescence readings from *V. fischeri* of carbon cocktail standard (CCS) ranging from 50-400ppb @ 120 minutes.

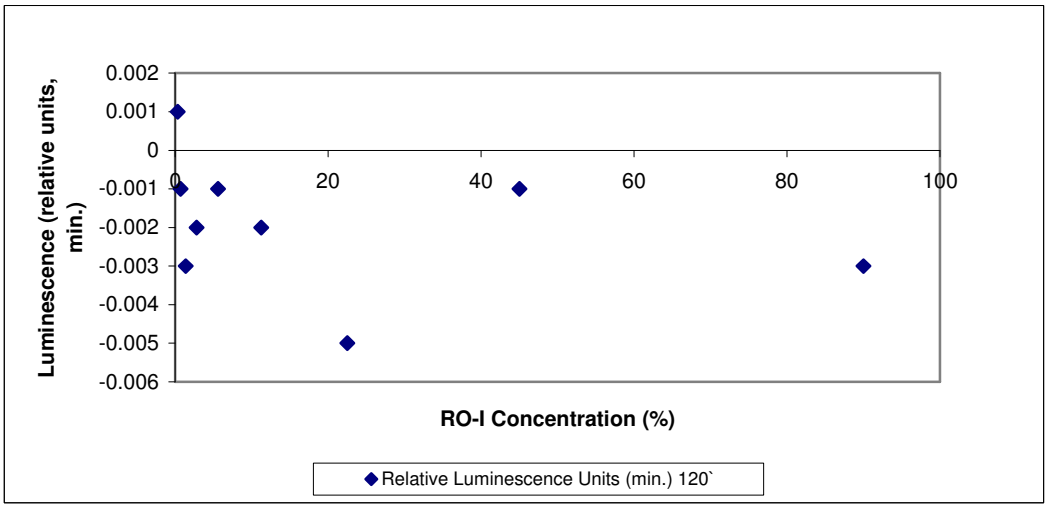

Figure 5: Bioluminescence readings using RO feedwater (RO-I) diluted from 90% to 0.35% of concentrate by *V. fischeri*.

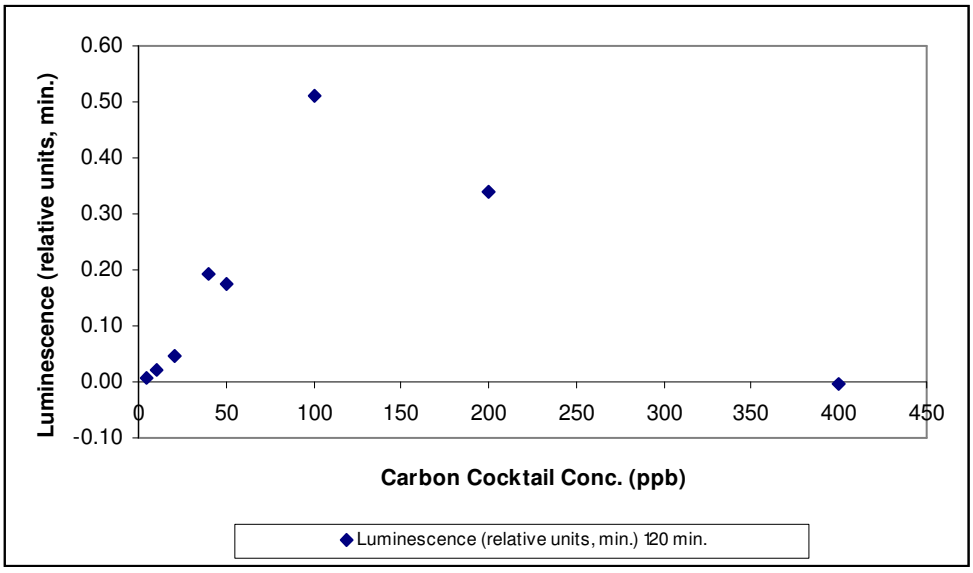

Figure 6: Bioluminescence readings of carbon cocktail standard (CCS) ranging from 50-400ppb @ 120 minutes by *V. fischeri*.

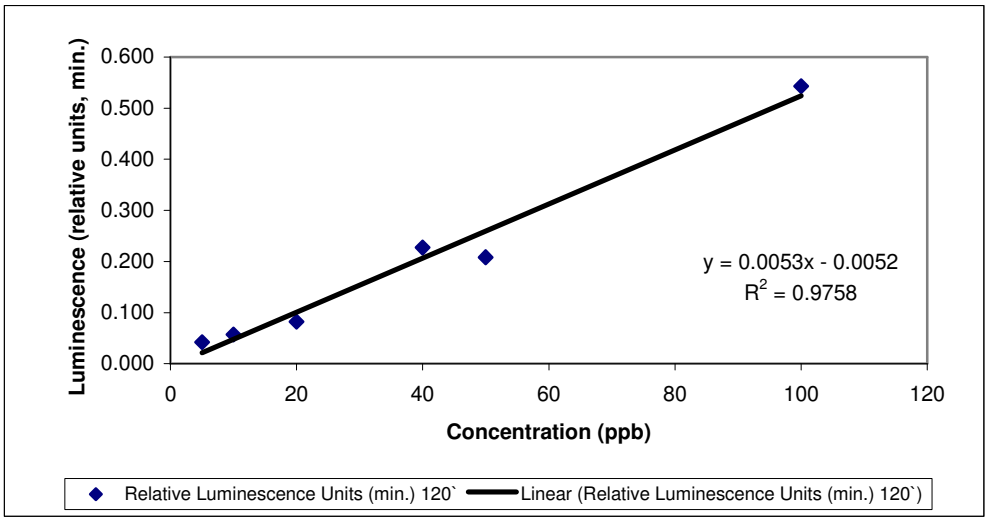

Figure 7: A linear response by *V. fischeri* between 5-100ppb Carbon Cocktail (CCS-C) from Figure 6. Greatest luminescence response was at 100ppb, the cells did show a slightly positive response down to 5ppb.

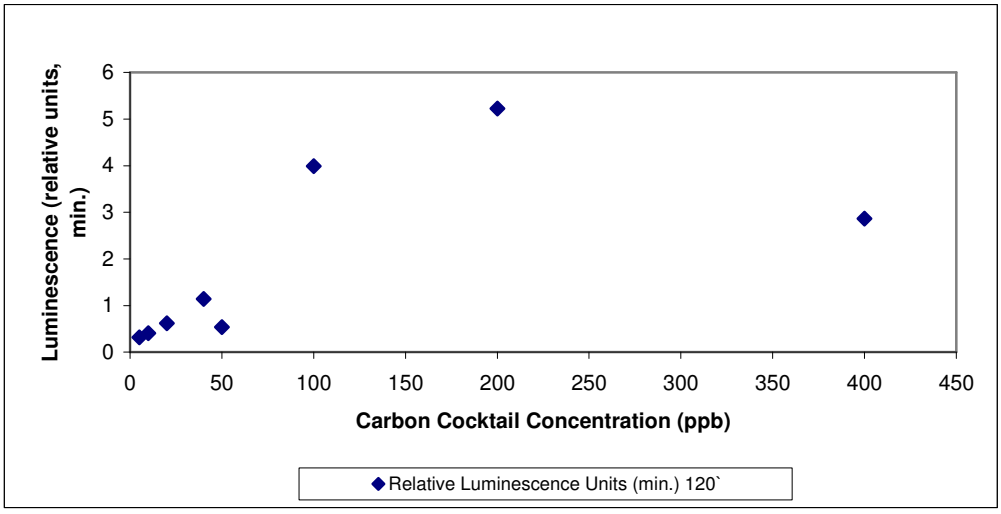

Figure 8: Bioluminescence readings of carbon cocktail standards from 5-400ppb@ 120 minutes with the normal concentration of *V. fischeri* cells.

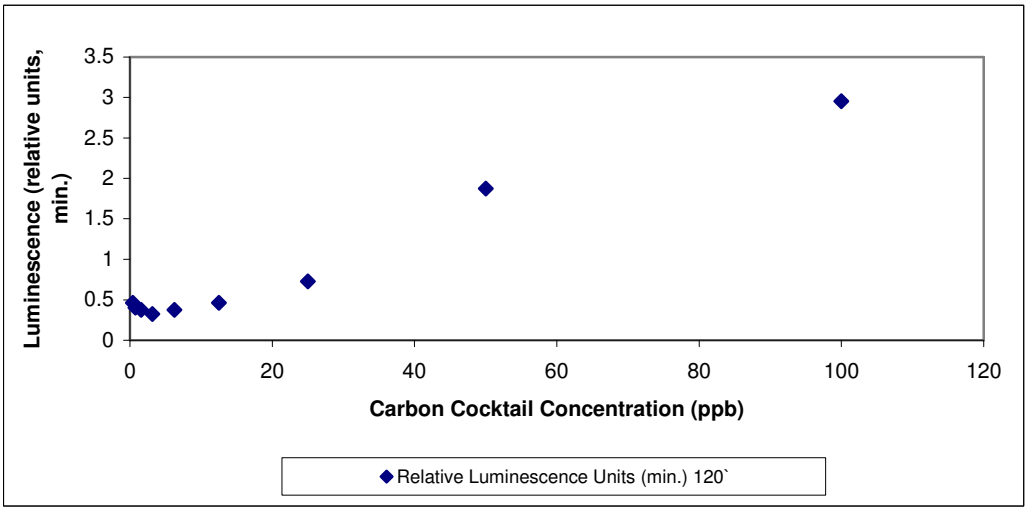

Figure 9: Test sample of 100ppb of Carbon Cocktail (CCS) serially diluted from 100-0.39ppb and inoculated with normal concentration of *V. fischeri* cells; luminescence readings at 120 minutes.

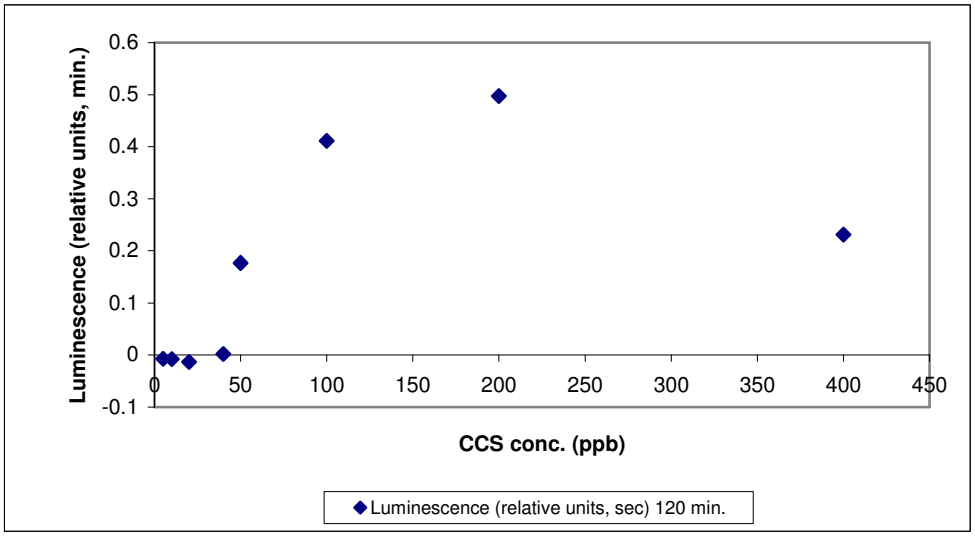

Figure 10: Bioluminescence readings of carbon cocktail standards from 5-400ppb with diluted *V. fischeri* (1/10) cells @ 120 minutes.

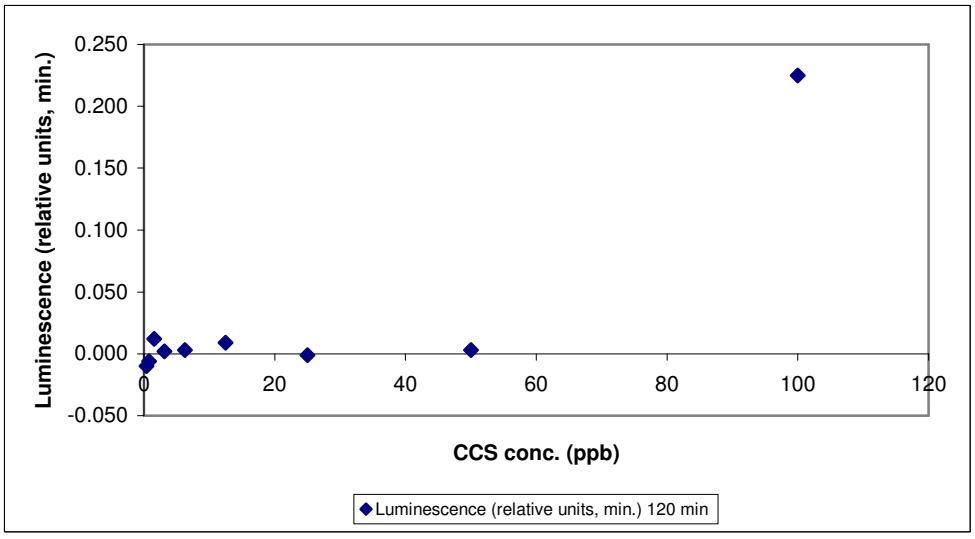

Figure 11: Test sample 100ppb of CCS serially diluted from 100-0.39ppb; inoculated with diluted *V. fischeri* (1/10); luminescence readings @ 120 minutes.

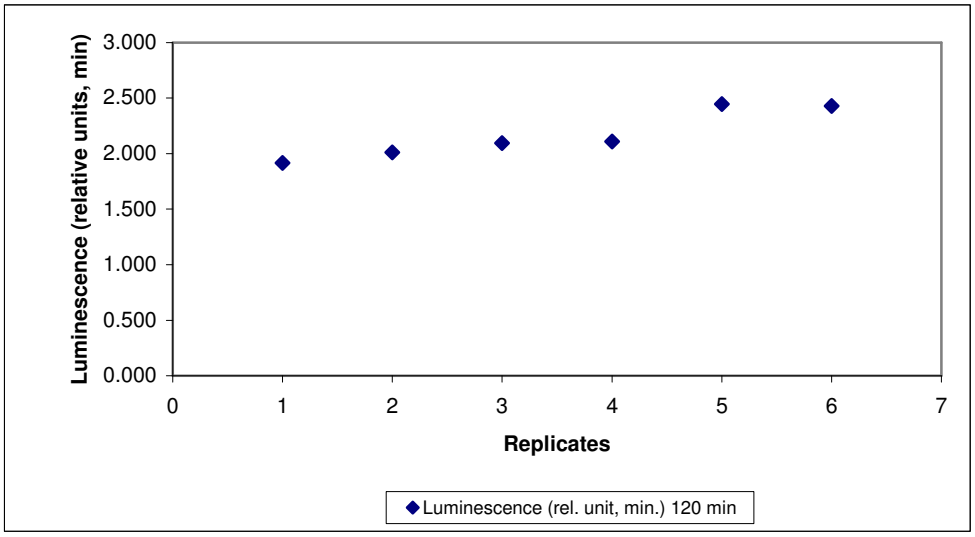

Figure 12: Replicates of 100ppb Carbon Cocktail solution @ 120 minutes. The standard deviation value is 0.220 indicating a reproducible *V. fischeri* response to carbon sources.

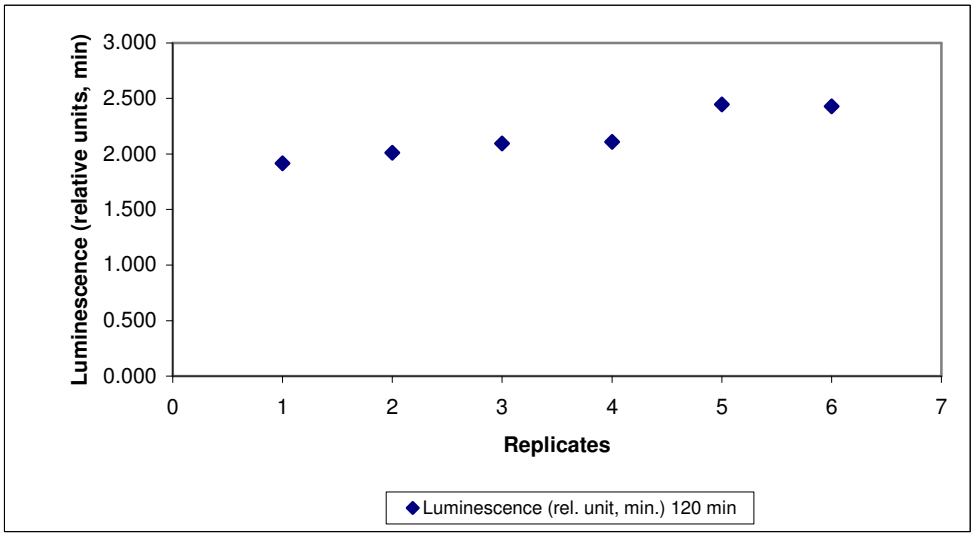

Figure 13: Replicates of 200ppb Carbon Cocktail solution @ 120 minutes. The standard deviation value is 0.273 indicating a reproducible *V. fischeri* response to carbon sources

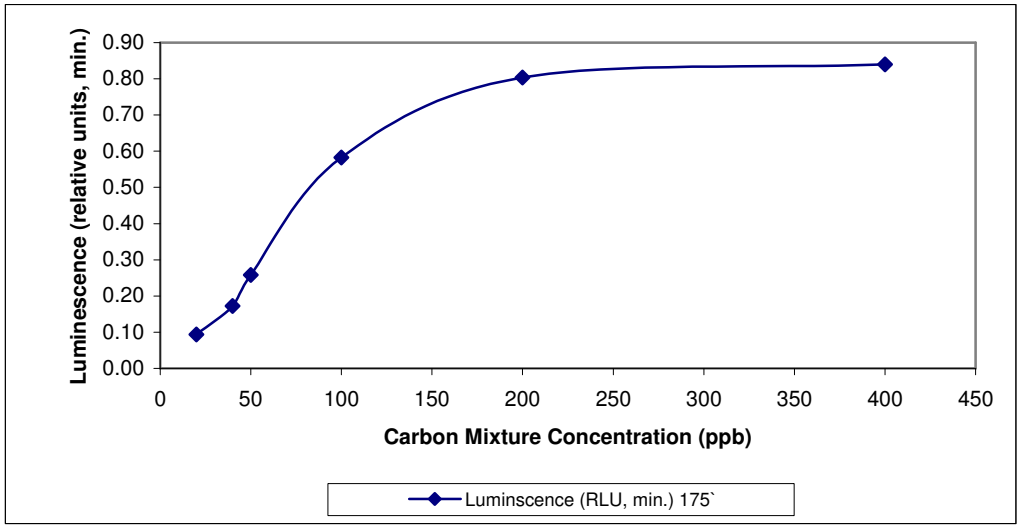

Figure 14: The bioluminescence response @ 175 minutes of *V. fischeri* to a 50/50 CCS/CA mixture as a carbon standard. The *V. fischeri* cells showed a linear response up to 200ppb carbon mixture, but values greater than 3XSD were only observed between 100 and 200ppb carbon mixture.

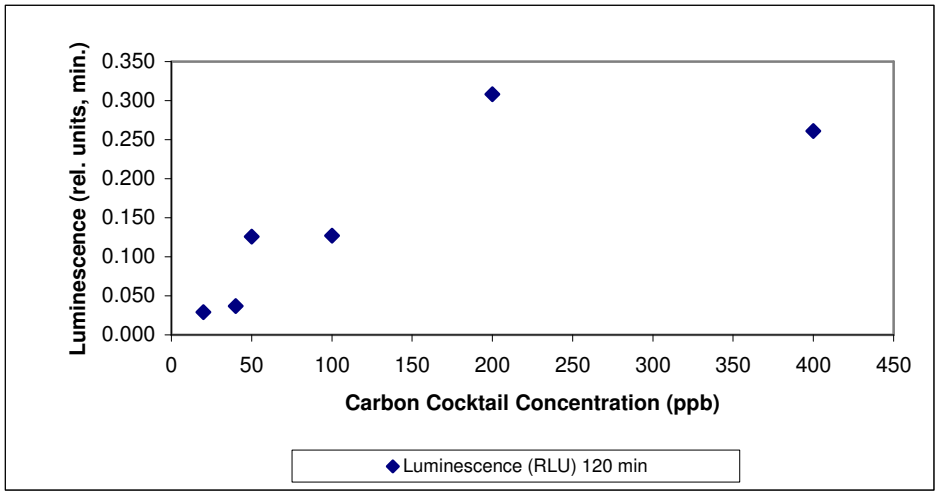

Figure 15: The bioluminescence response to CCS ranging from 20-400ppb by starved *V. fischeri* cells.

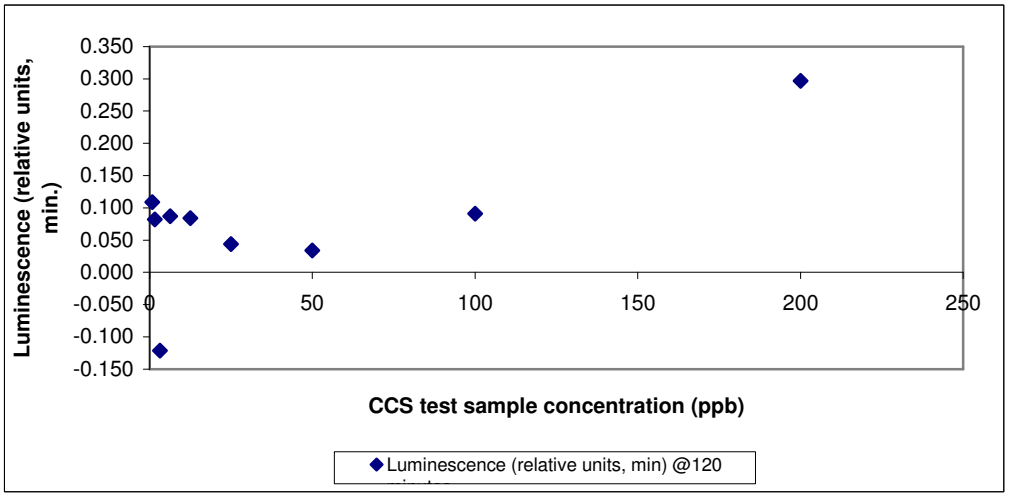

Figure 16: The bioluminescence response to a 200ppb CCS test sample serially diluted from 200-0.78ppb by starved *V. fischeri* cells.

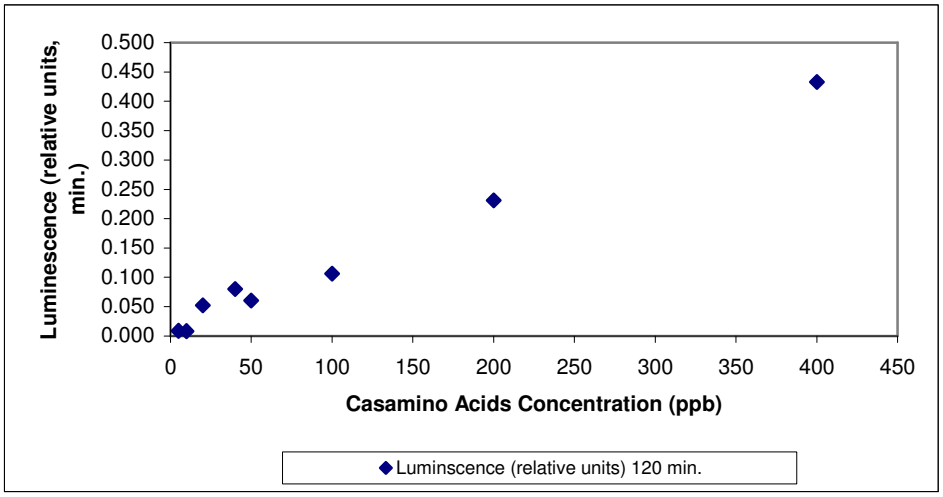

Figure 17: Casamino Acids (CA) as a substitute carbon source. A linear response by *V. fischeri* cells was observed from 20ppb to 400ppb with CA, a higher response than with Carbon Cocktail as the standard carbon source.

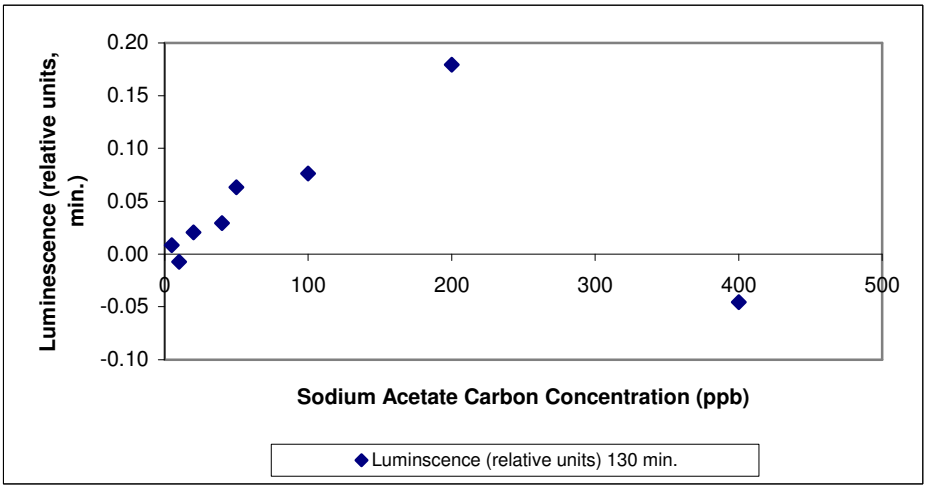

Figure 18: Sodium Acetate (NaOAc) as a substitute standard carbon source. The response by *V. fischeri* cells was linear from 20ppb – 200ppb. Compared to CA, the luminescence response to NaOAc was less.

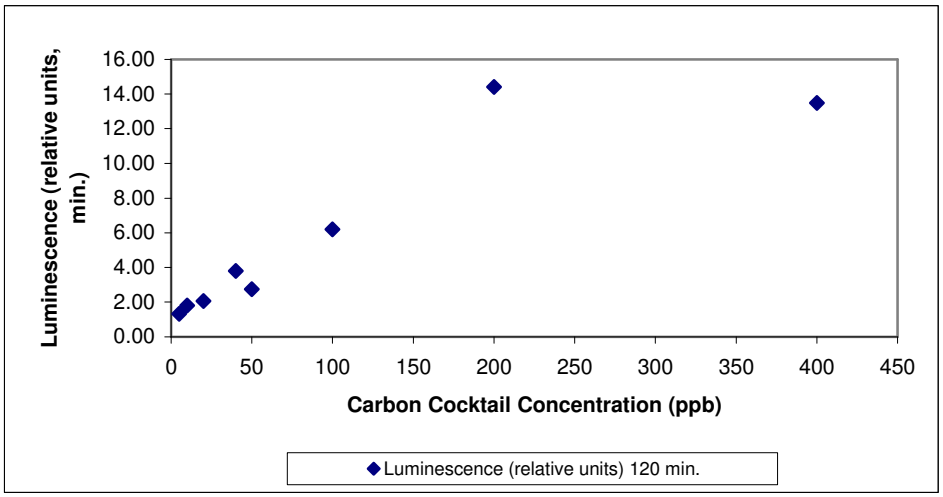

Figure 19: The bioluminescence response @ 120 minutes to the Carbon Cocktail standards from 5-400ppb inoculated with *V. fischeri*.

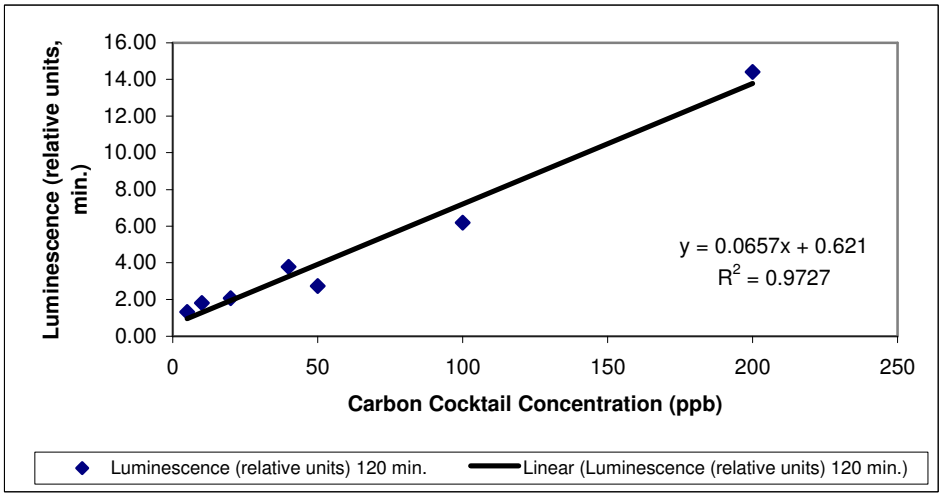

Figure 20: Linear segment from Figure 19. The bioluminescence response by *V. fischeri* was linear from 5-200ppb of Carbon Cocktail with intensity greater than observed with AOC Multi-Shot kit.

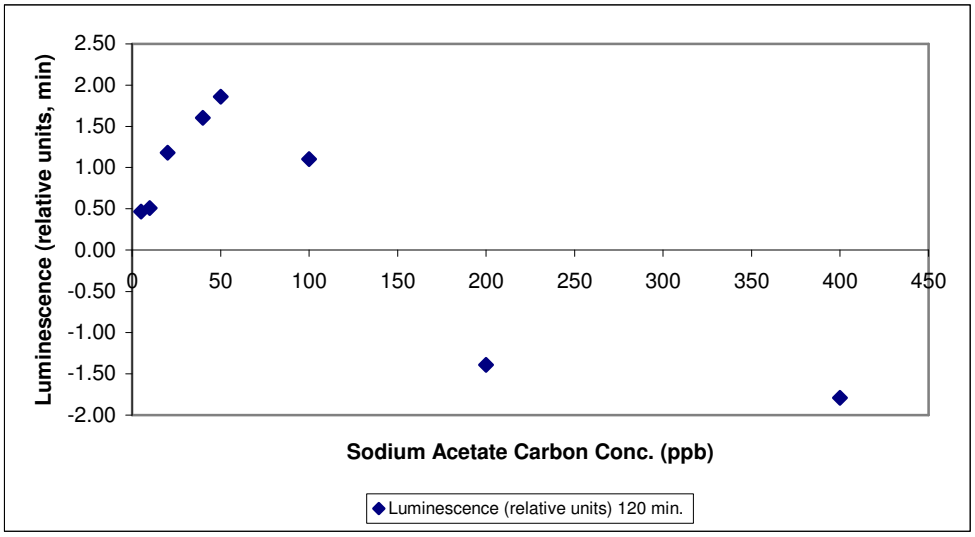

Figure 21: The bioluminescence response at 120 minutes to Sodium Acetate Carbon (5-400ppb) inoculated with *V. fischeri*.

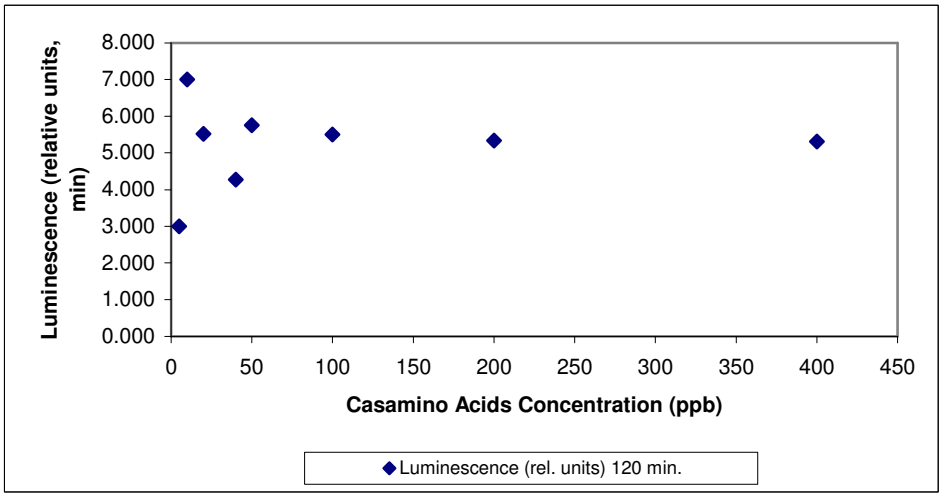

Figure 22: The bioluminescence response at 120 minutes to Casamino Acids as a carbon source (5-400ppb CA) inoculated with *V. fischeri.* The *V. fischeri* response was too scattered to use as a standard curve. Bioluminescence values greater than 3XSD were observed at all CA concentrations.

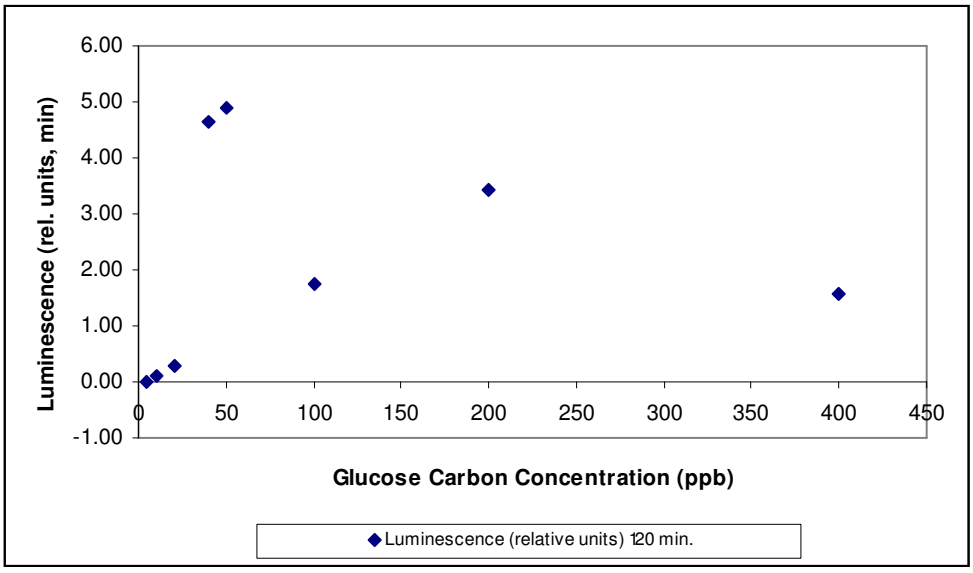

Figure 23: The bioluminescence response @ 120 minutes to Glucose as a substitute carbon source (5-400ppb Glu-C) by *V. fischeri.*

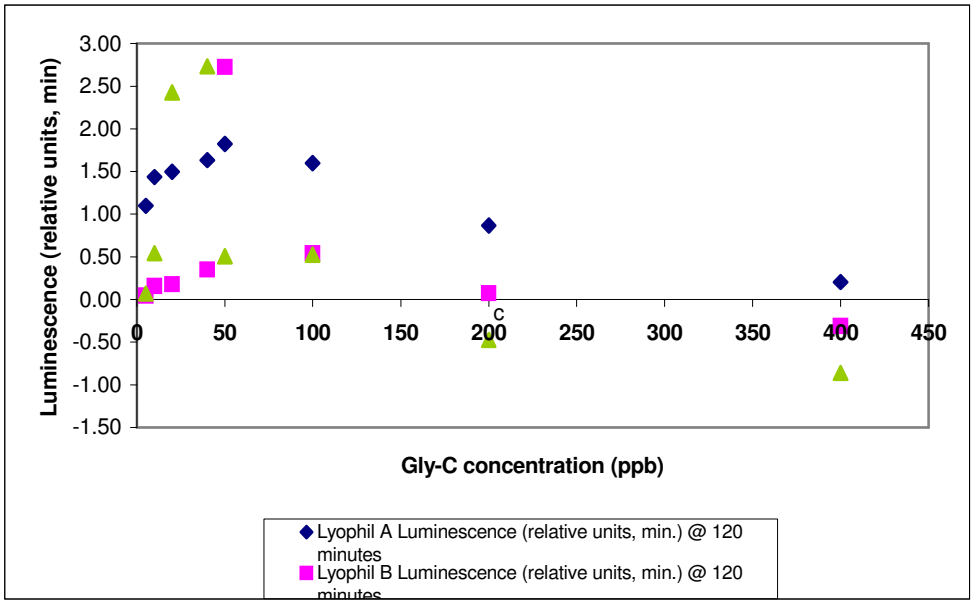

Figure 24: The bioluminescence response @ 120 minutes to Glycerol (Gly) as a substitute carbon source (5-400ppb Gly-C) inoculated with *V. fischeri* cells from three different lyophils.

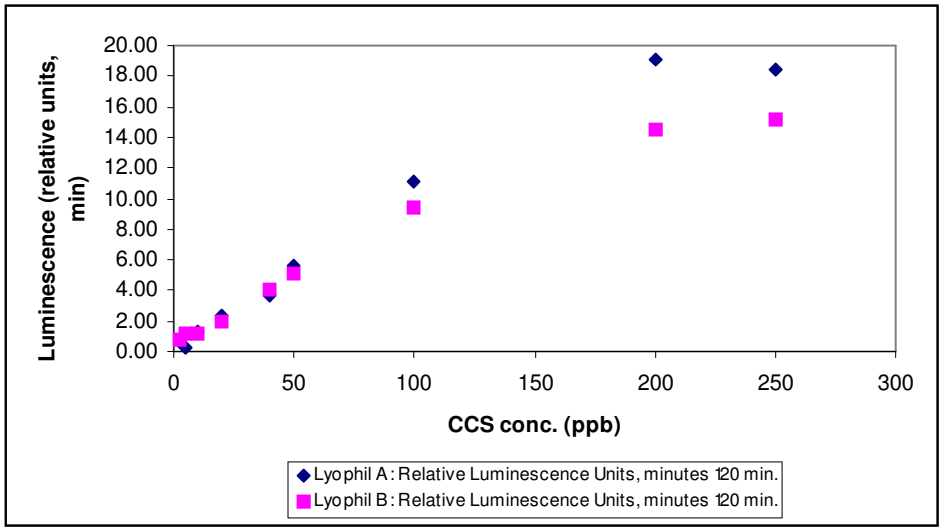

Figure 25: The bioluminescence response at 120 minutes to the CCS from 2.5-250ppb inoculated with *V. fischeri* in plastic assay vials.

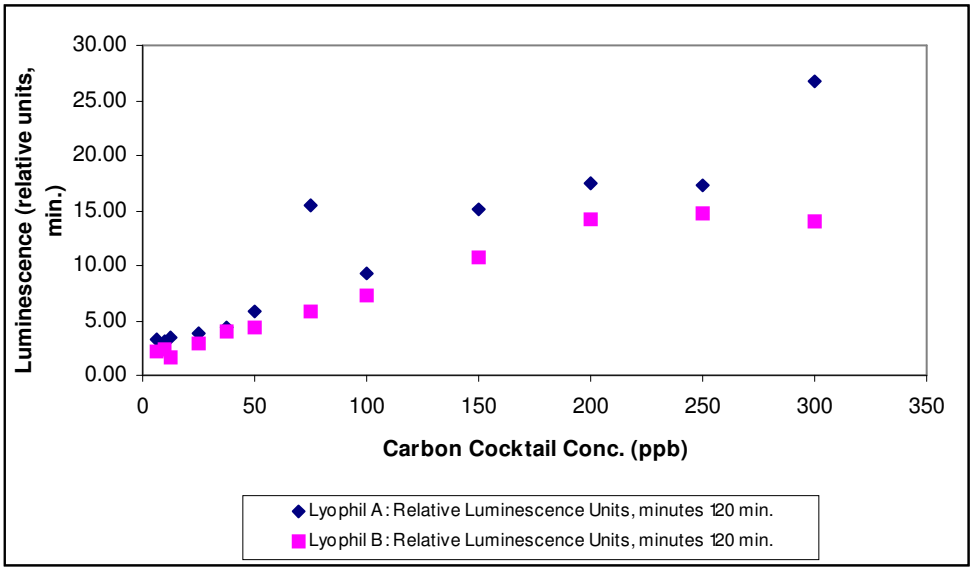

Figure 26: The luminescence response @ 120 minutes to CCS in plastic assay vials inoculated with *V. fischeri*.

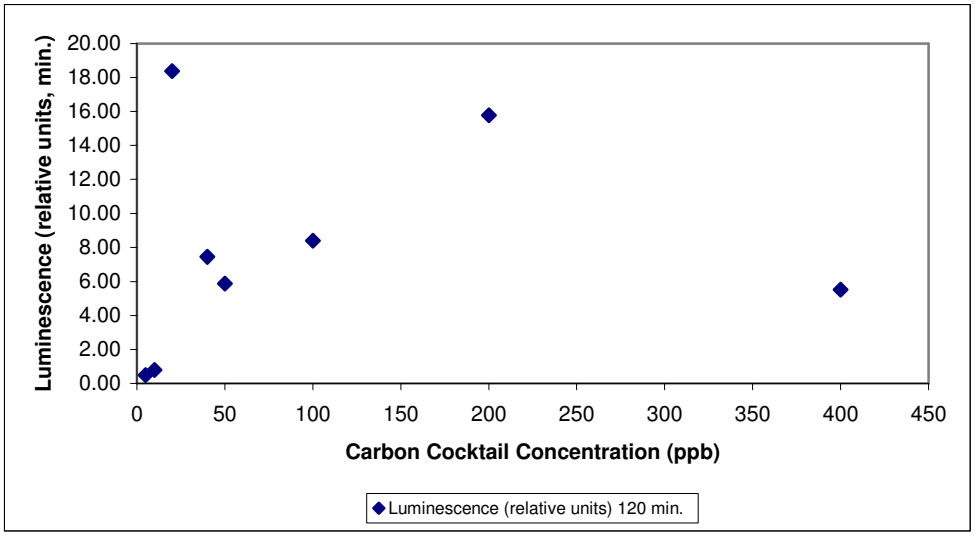

Figure 27: The bioluminescence response @ 120 minutes to CCS (5-400ppb) in "unwashed" assay vials inoculated with *V. fischeri*. Note: response at least 3X standard deviation of the negative controls began at 20ppb.

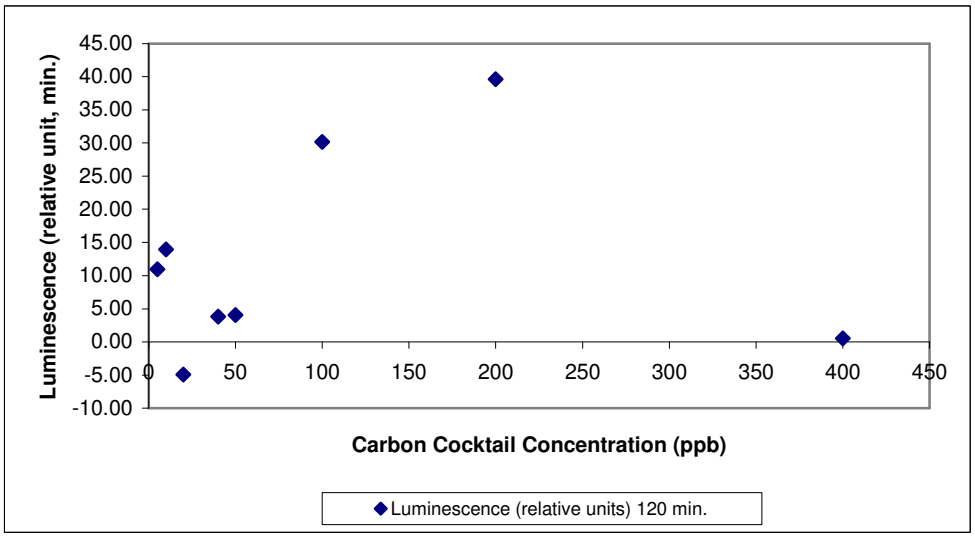

Figure 28: The bioluminescence response @ 120 minutes to CCS (5-400ppb) in "washed" assay vials inoculated with *V. fischeri*. Although the response was not as linear with the "washed" vials compared to the 'unwashed' vials, a 3X standard deviation luminescence response was exhibited in the 'washed' vials from 5ppb to 200ppb CCS.

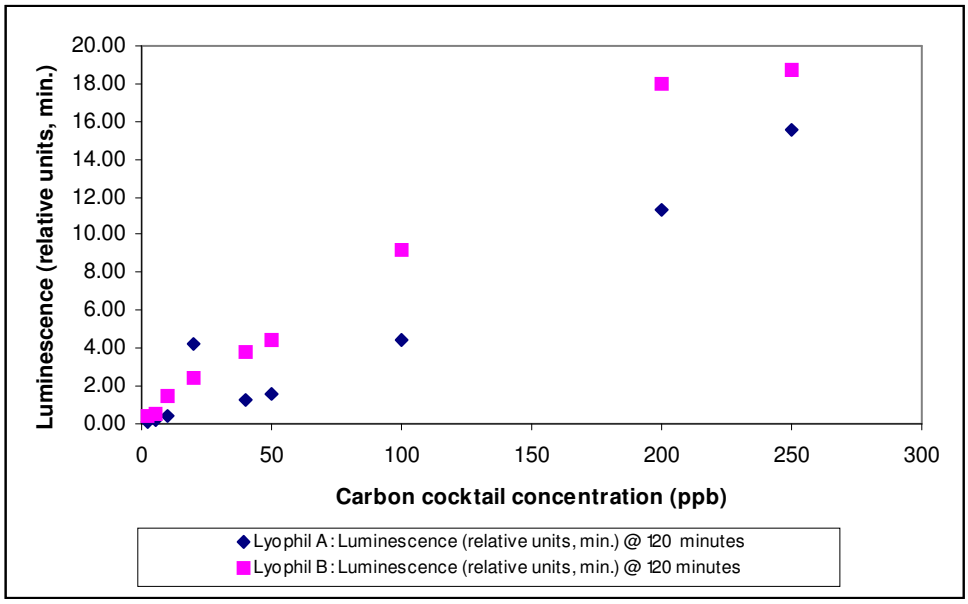

Figure 29: Bioluminescence readings of CCS ranging from 2.5-250ppb in acid-washed assay vials @ 120 minutes by *V. fischeri.*

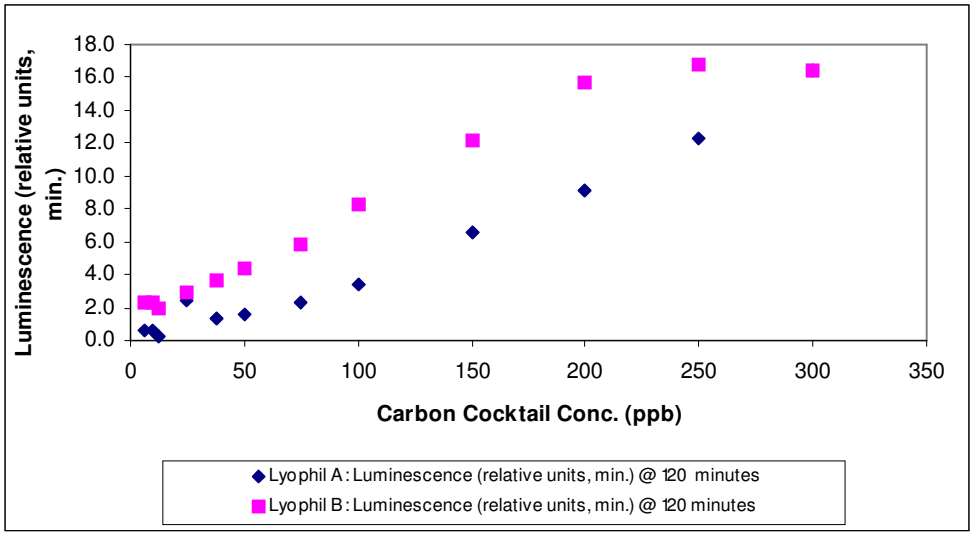

Figure 30: In Acid-washed glass vials, 300ppb CCS used as test samples were evaluated using Lyophils A and B @ 120 minutes. The bioluminescence response by *V. fischeri* (Lyophil A) was linear from approximately 25ppb to 300ppb, while the bioluminescence response by Lyophil B was linear from 12.5ppb to 200ppb. The 3XSD value for Lyophils A and B were 0.206 and 0.452, respectively; therefore, *V. fischeri* had a better and more consistent response in the acid-washed vials.

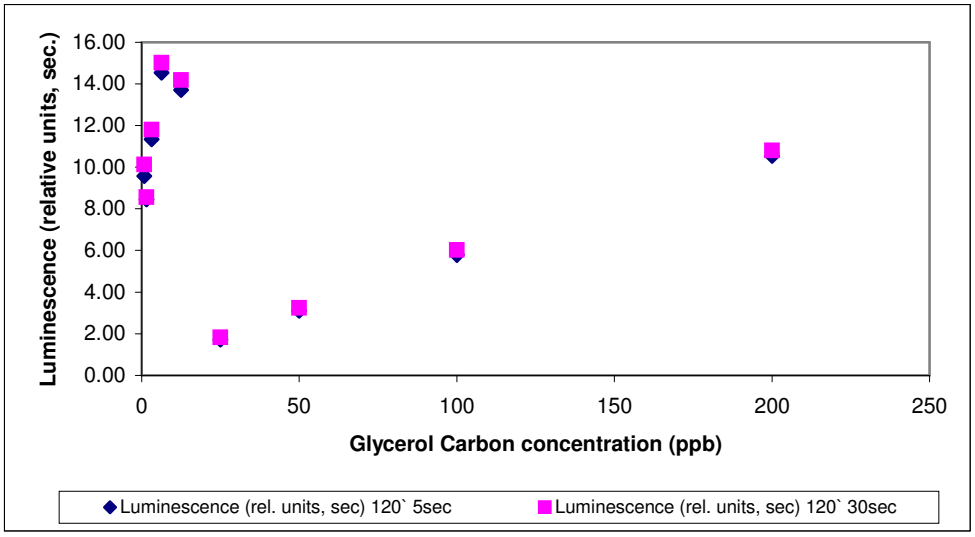

Figure 31: The bioluminescence response @120 minutes to Glycerol as a carbon test sample (400-0.78ppb) in vials inoculated with *V. fischeri*. A linear response to Glycerol carbon was observed from 200 – 25ppb. The luminescence measurements between 5 and 30-second integration times exhibited no significant difference in response. Therefore, a 5 second integration time was incorporated into the protocol.

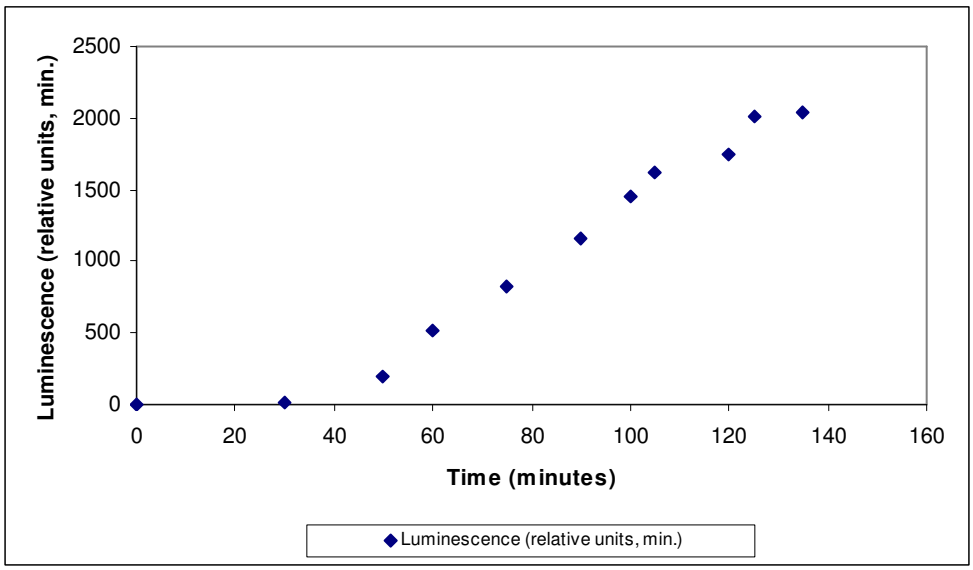

Figure 32: A lyophil of *V. fischeri* was observed until the luminescence leveled off. The *V. fischeri* cells did not begin to give off large amounts of light until approximately 50 minutes after the lyophil was hydrated.

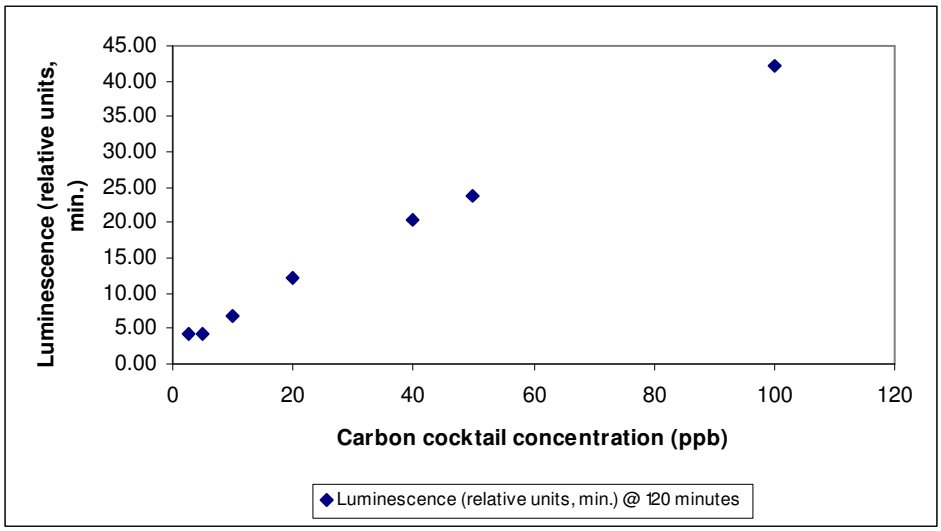

Figure 33: The bioluminescence readings of CCS ranging from 2.5-100ppb using stabilized *V. fischeri* @ 120 minutes.

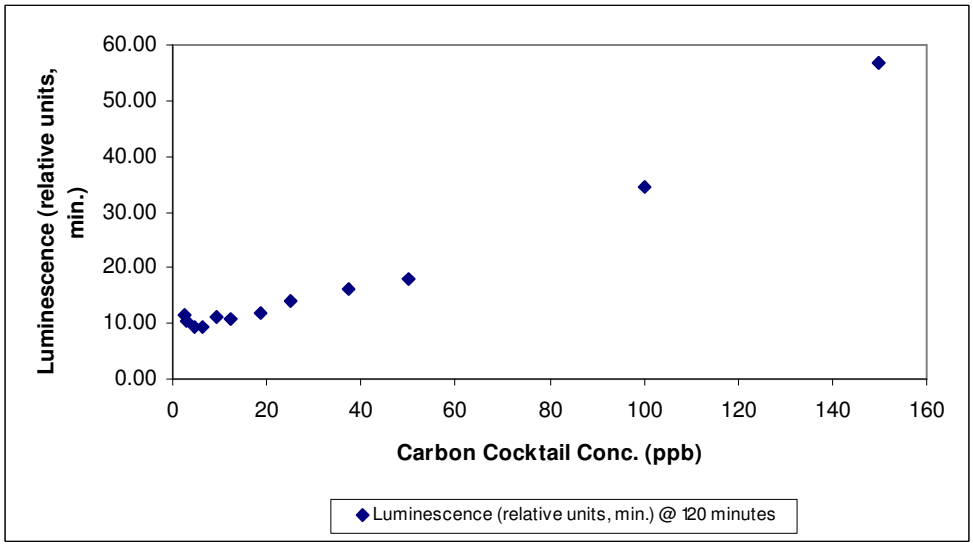

Figure 34: The bioluminescence readings of CCS test sample ranging from 150-2.34ppb using stabilized *V. fischeri* @ 120 minutes.

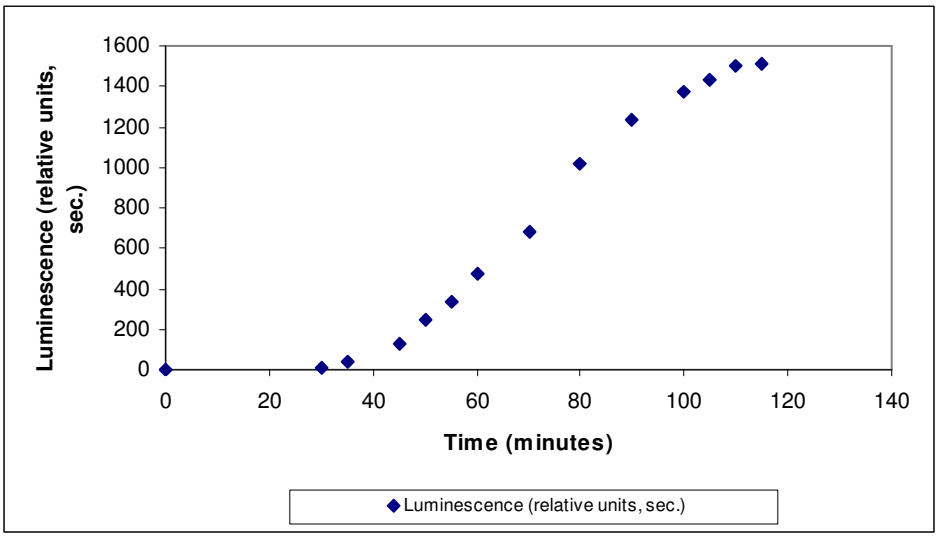

Figure 35: The bioluminescence readings for a lyophil of *V. fischeri* was observed until the luminescence leveled off.

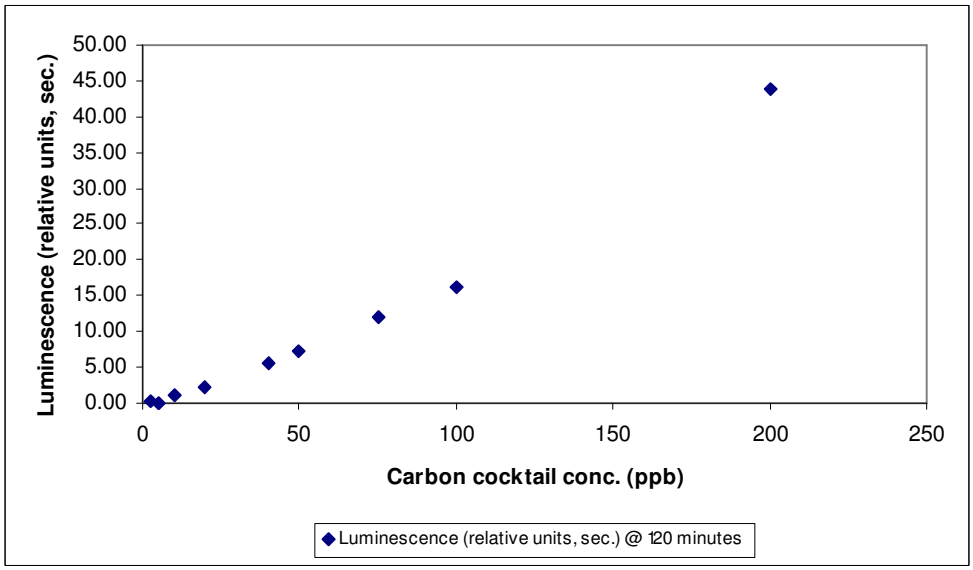

Figure 36: The bioluminescence readings of CCS ranging from 2.5-200ppb using stabilized *V. fischeri* @ 120 minutes.

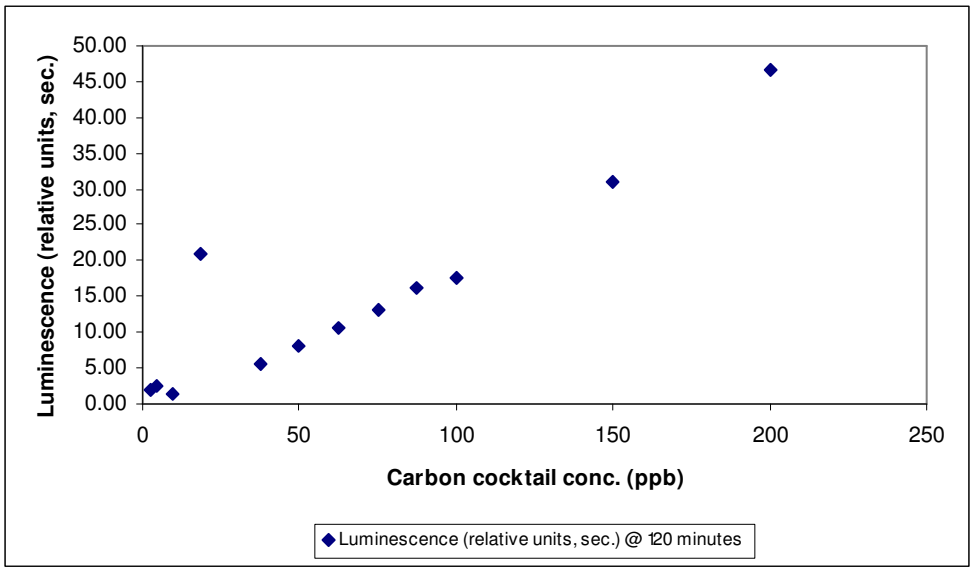

Figure 37: The bioluminescence readings of CCS test sample ranging from 200-2.34ppb using stabilized *V. fischeri* @ 120 minutes.

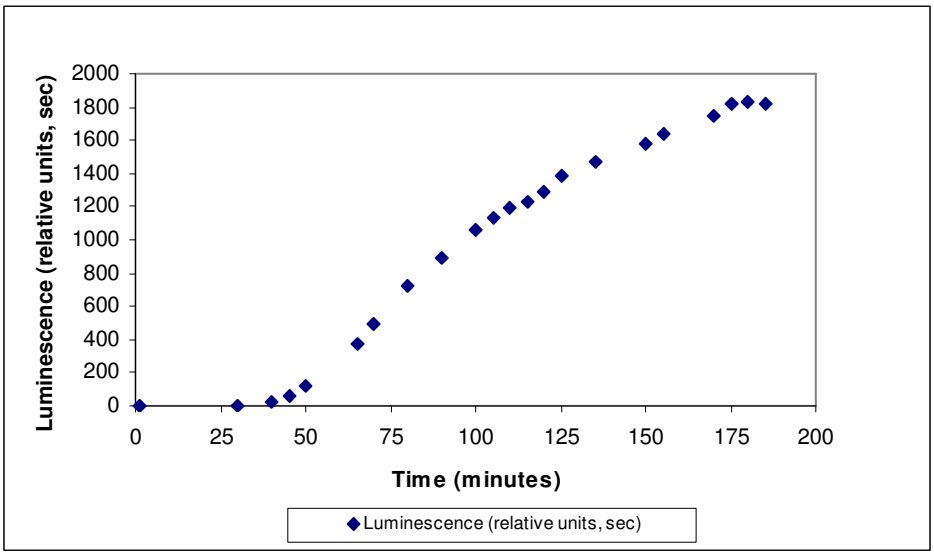

Figure 38: The stabilization of hydrated *V. fischeri* used to determine the metabolism of CCS-C, Glucose-C and Glycerol-C.

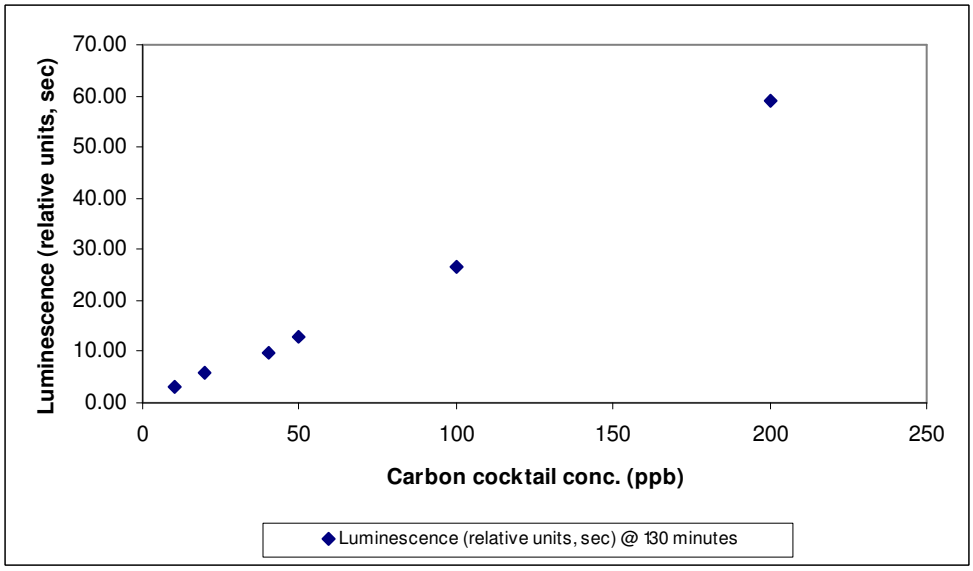

Figure 39: The bioluminescence response by stabilized *V. fischeri* cells to CCS ranging from 10-200ppb @ 120 minutes. Linear responses greater than 3XSD value for the negative controls were observed from 10-200ppb CCS.

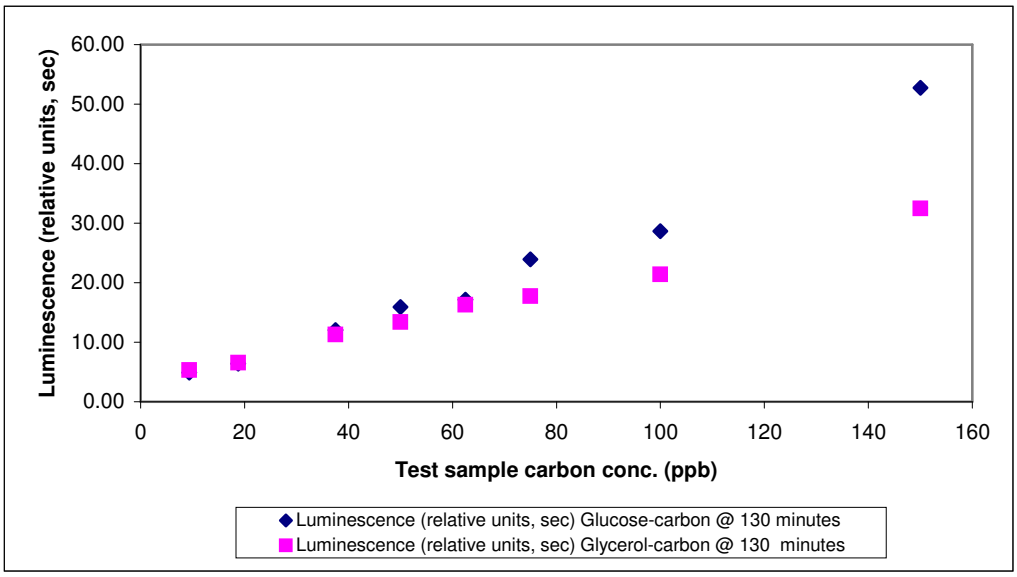

Figure 40: The bioluminescence response @ 130 minutes by stabilized *V. fischeri* to Glu-C and Gly-C (test carbon samples serially diluted from 150 to 9.38ppb). Although the response by the cells was linear for both the Glu-C and the Gly-C, the cells expressed greater luminescence from the metabolism of the Glu-C as opposed to the Gly-C. All concentrations of carbon for both test carbon samples exhibited a response greater than the 3XSD value.

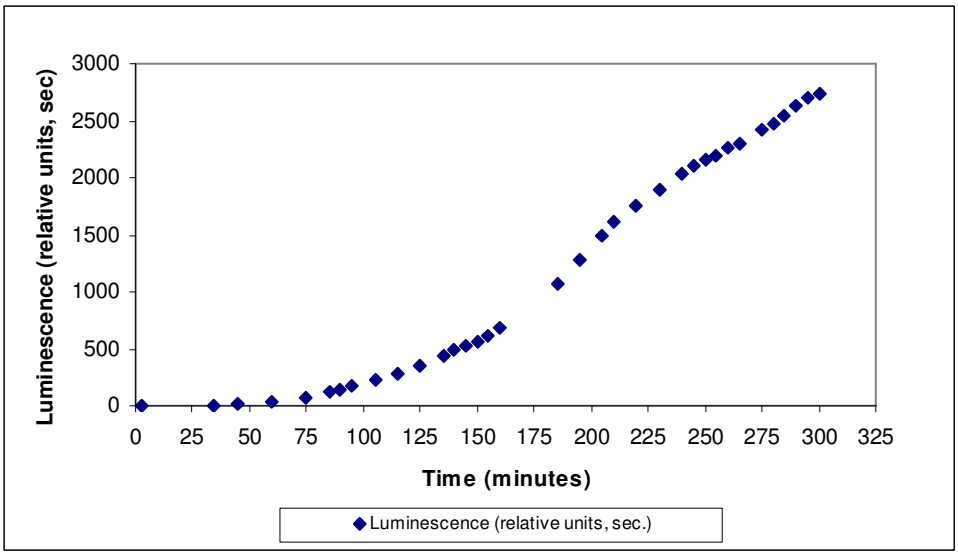

Figure 41: The stabilization of hydrated *V. fischeri* used in the assay vials that tested the metabolism of CCS-C, Sodium acetate-C and Fructose-C.

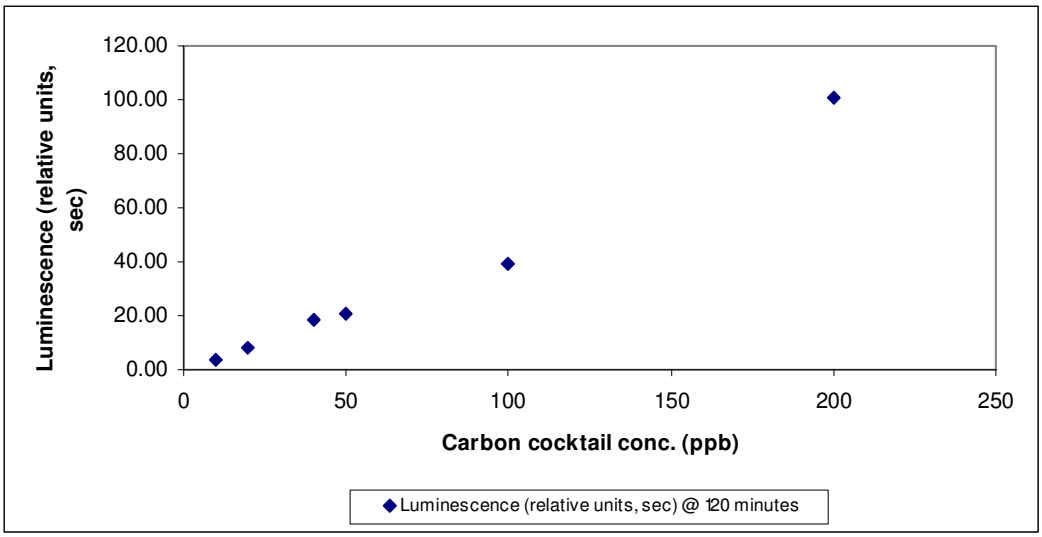

Figure 42: The bioluminescence readings by stabilized *V. fischeri* cells to CCS-C ranging from 10-200ppb @120 minutes. Linear responses with values greater than 3XSD for the negative controls were observed from 10-200ppb CCS.

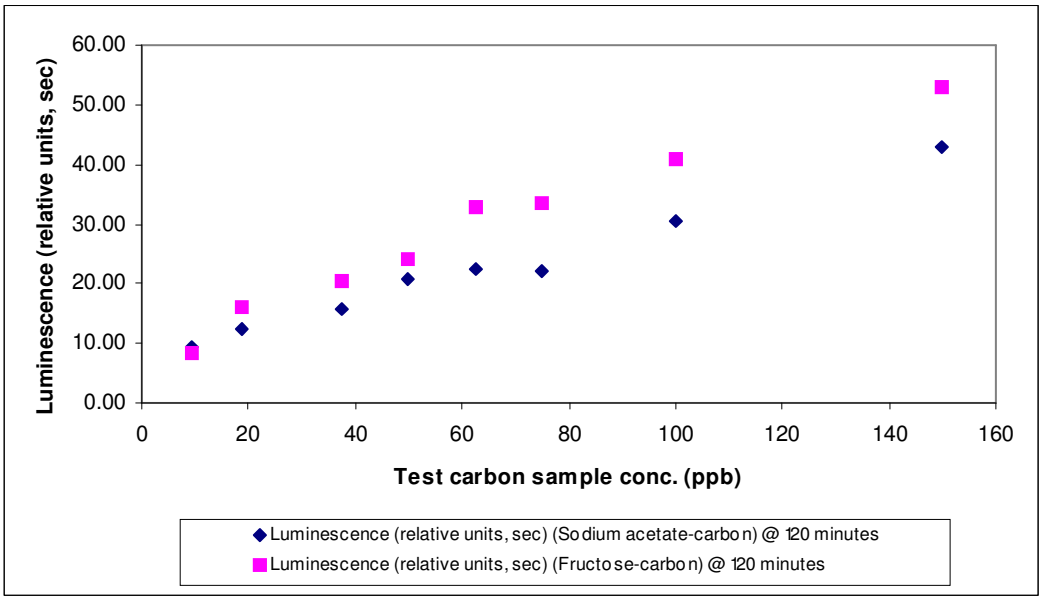

Figure 43: The bioluminescence readings @ 120 minutes by stabilized *V. fischeri* to NaOAc-C and Fru-C (test carbon samples serially diluted from 150ppb to 9.38pp). Although the response by the cells was linear for both the NaOAc-C and the Fru-C, the cells expressed greater luminescence from the metabolism of the NaOAc-C as opposed to the Fru-C. All concentrations of carbon for both test samples exhibited a response greater than the 3XSD value.

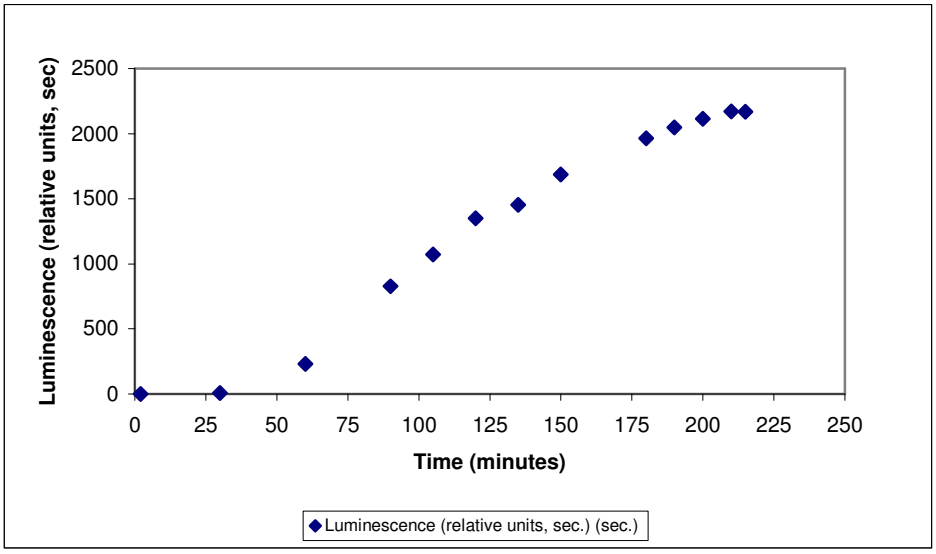

Figure 44: The stabilization of *V. fischeri* used in the assay vials that tested the AOC concentration in OCWD SMW sample and the corresponding Sodium acetate standards. As shown above, it took approximately 200 minutes for the luminescence of the hydrated cells to stabilize.

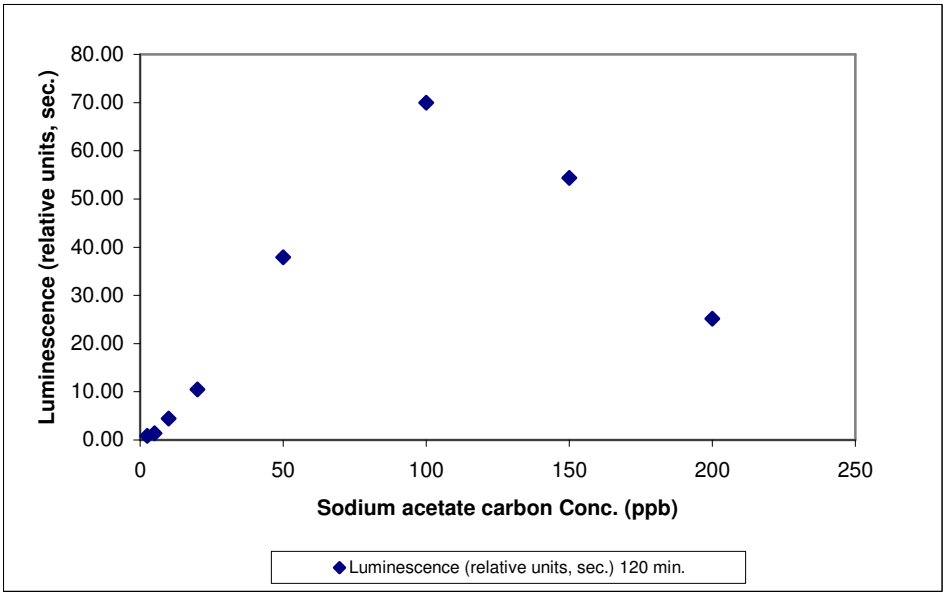

Figure 45: The bioluminescence response by stabilized *V. fischeri* to the Sodium acetate-carbon. Although a positive response was observed from 5-100ppb NaOAc-C, the 10ppb NaOAc concentration was the lowest concentration that exhibited a response greater than the 3XSD value for the corresponding negative controls.

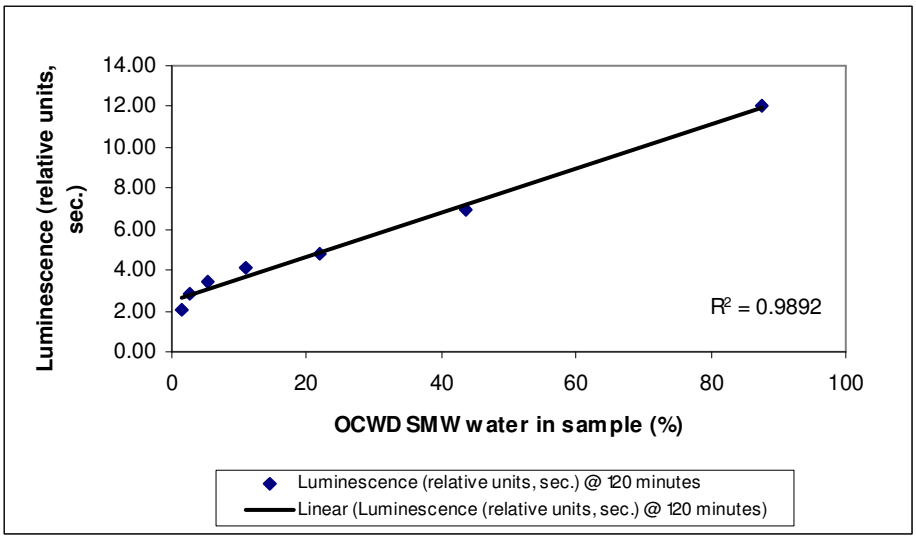

Figure 46: The bioluminescence response by the stabilized *V. fischeri* to OCWD SMW sample @ 120 minutes was linear from 87.5% to 1.36%.

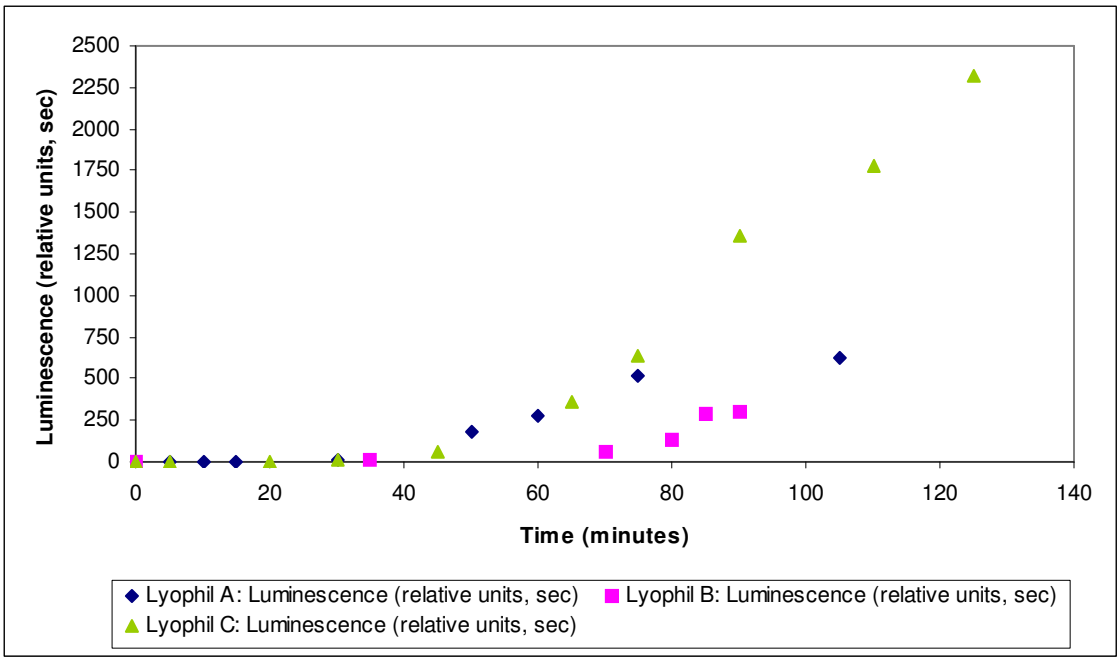

Figure 47: The bioluminescence readings by *V. fischeri* for lyophils used to inoculate the assays ran to determine the metabolism of organic compounds.

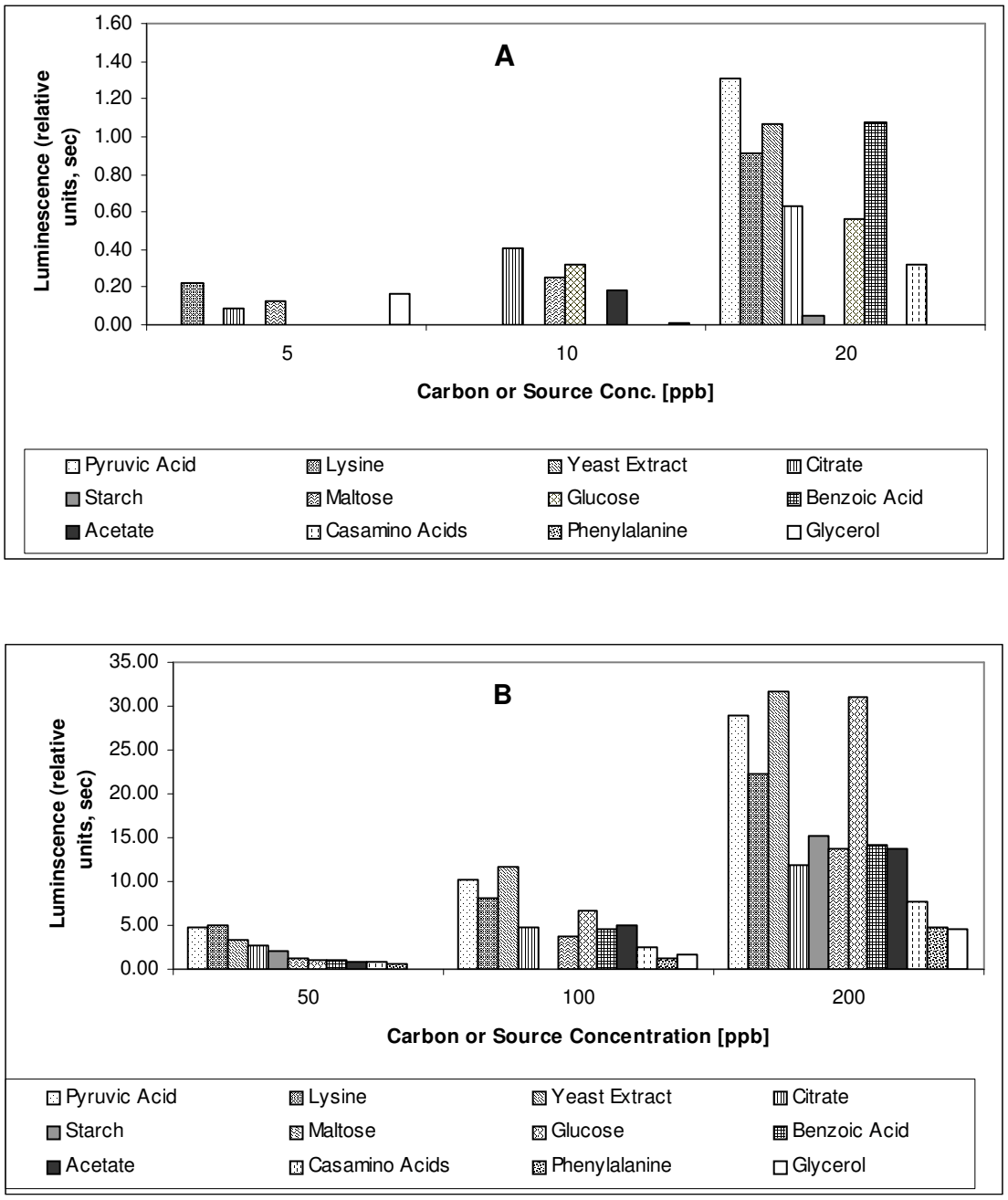

Figure 48: Bioluminescence readings of organic compounds ranging from 5-20ppb (Figure A) and 50-200ppb (Figure B) @ 120 minutes by *V. fischeri*.

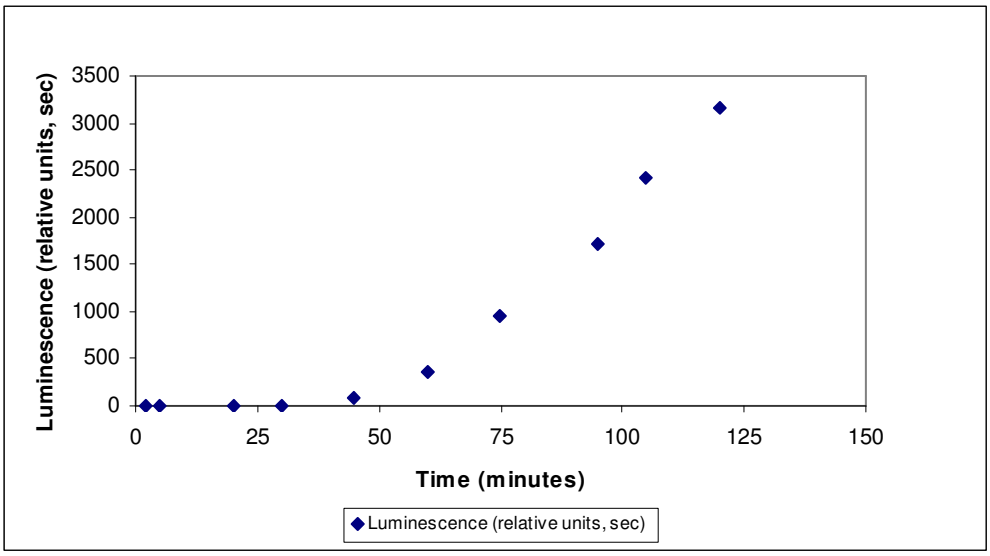

Figure 49: Bioluminescence readings for the stabilization of a lyophil of *V. fischeri*.

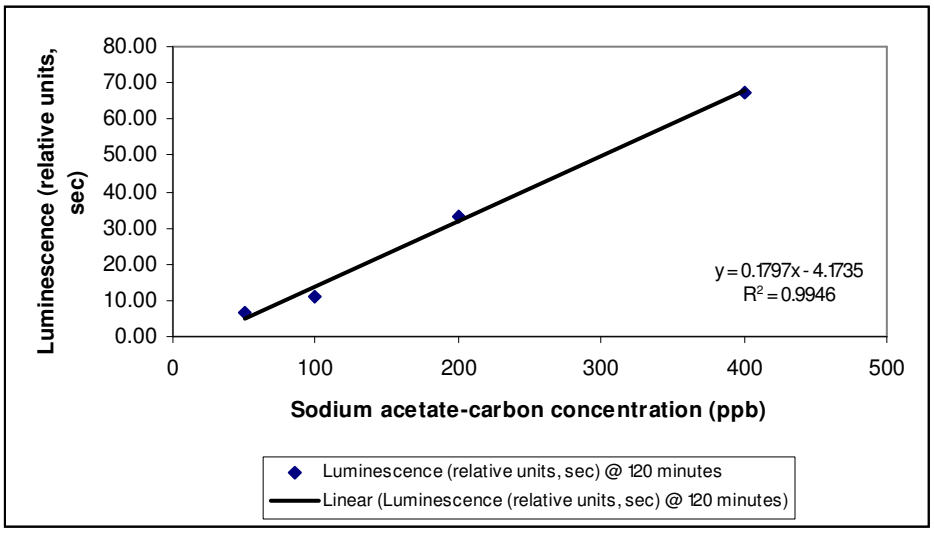

Figure 50: The bioluminescence readings of NaOAc-C ranging from 50-400ppb by *V. fischeri* @ 120 minutes

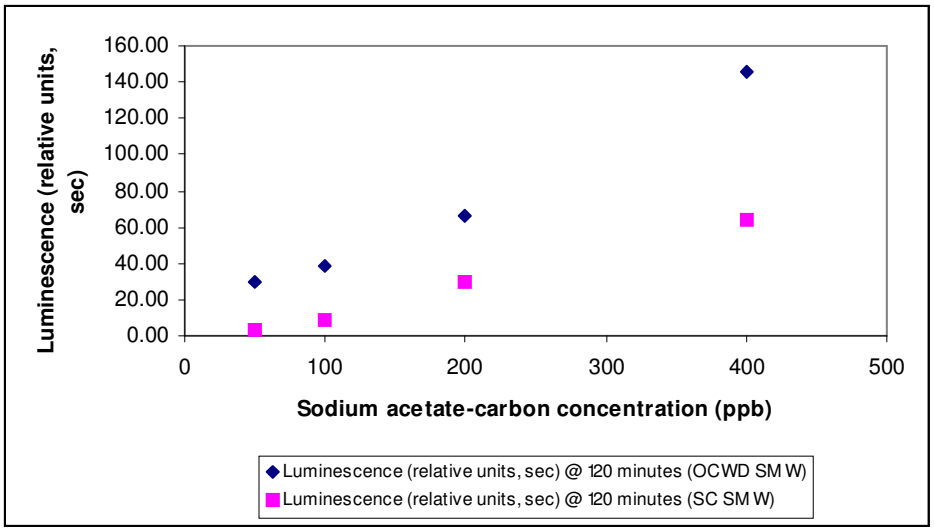

Figure 51: The bioluminescence readings for toxicity tests ran on OCWD SMW and SC SMW water samples @ 120 minutes by *V. fischeri*.

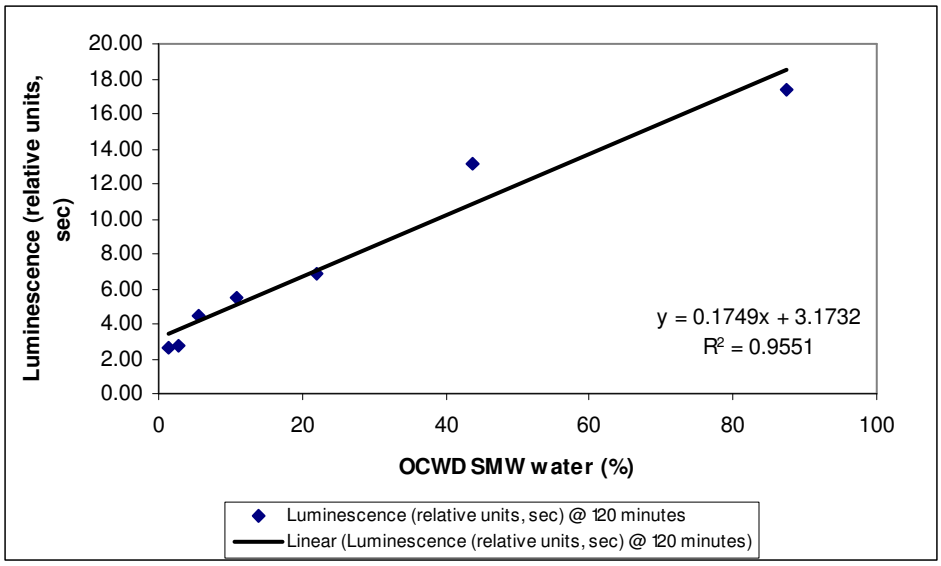

Figure 52: Bioluminescence readings in response to OCWD SMW water sample @ 120 minutes by *V. fischeri*.

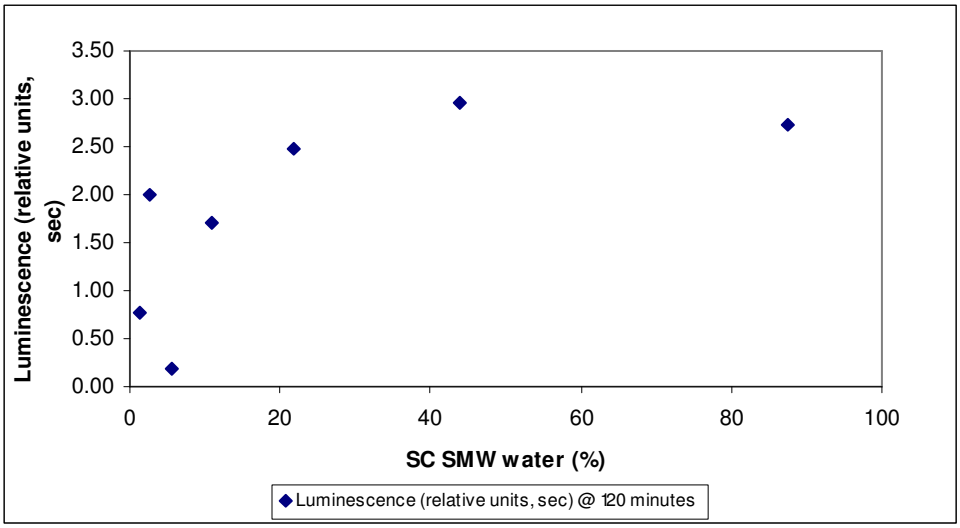

Figure 53: Bioluminescence readings in response to SC SMW water sample @ 120 minutes by *V. fischeri*

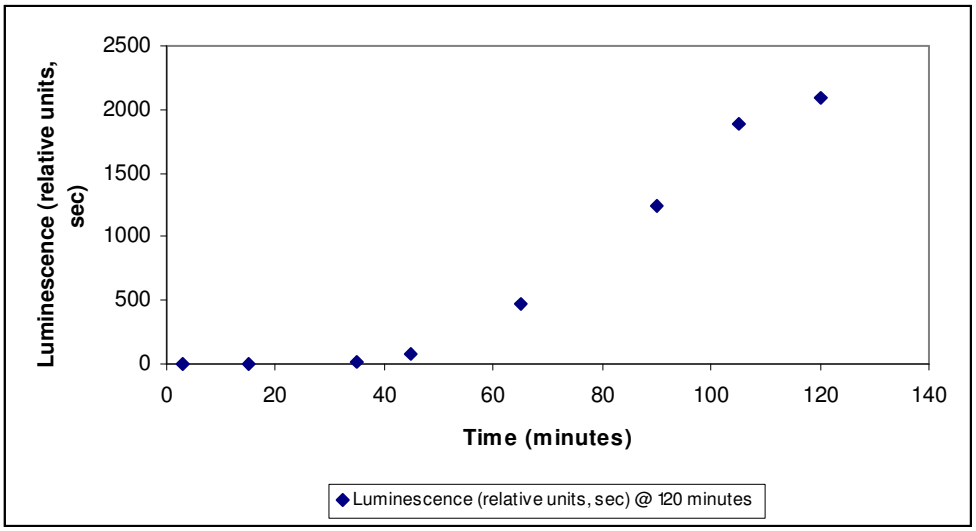

Figure 54: Bioluminescence readings for the stabilization of a *V. fischeri* lyophil.

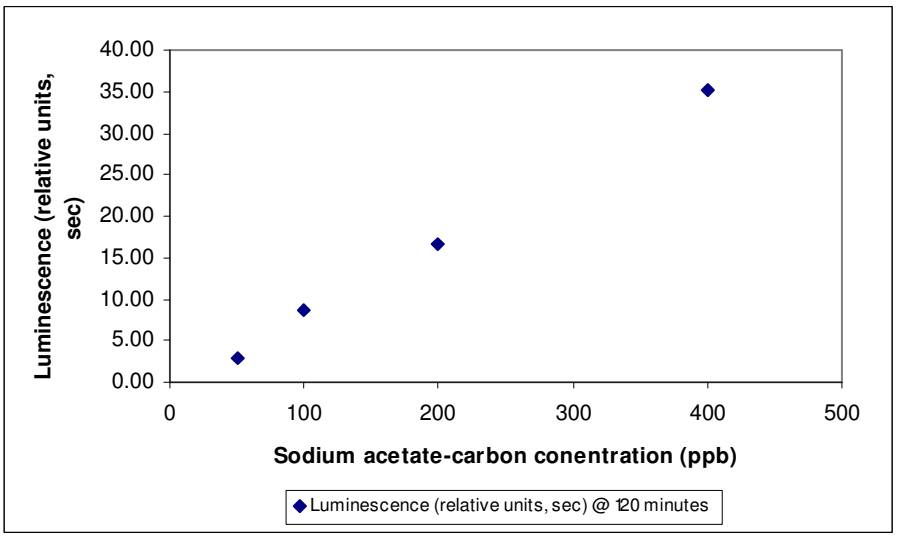

Figure 55: The bioluminescence readings from NaOAc-C as a standard of RO-P water @ 120 minutes by *V. fischeri*

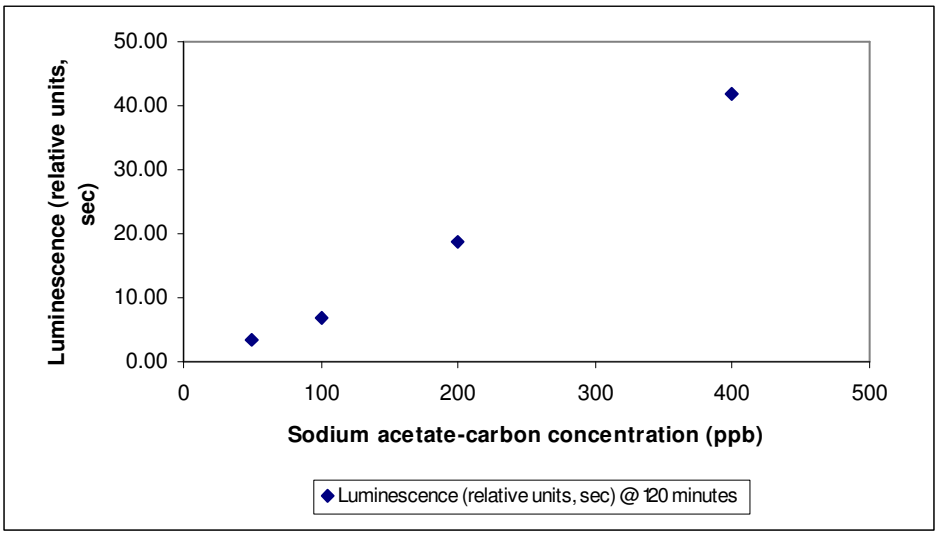

Figure 56: Bioluminescence readings of NaOAc-C ranging from 50-400ppb in RO-P water (toxicity test) @ 120 minutes by *V. fischeri*

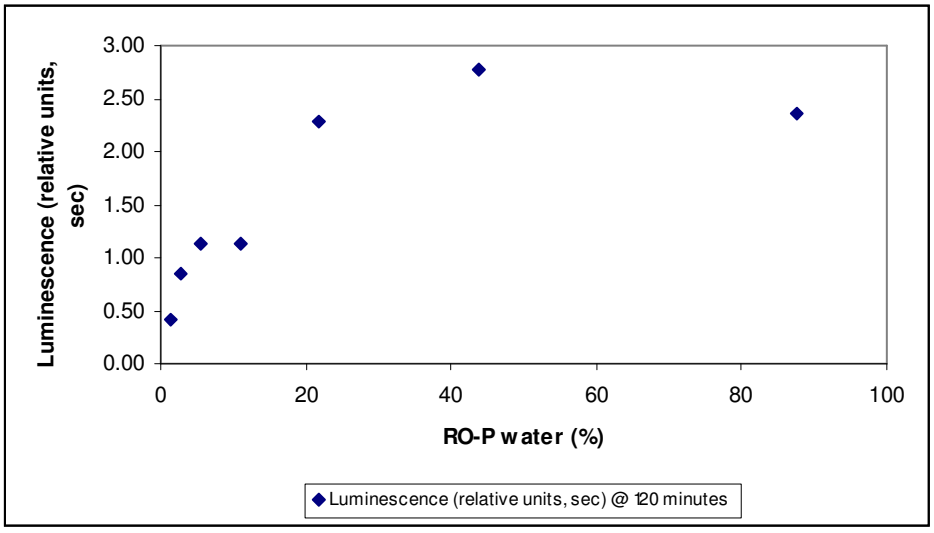

Figure 57: The bioluminescence readings for RO-P water @ 120 minutes by *V. fischeri*.

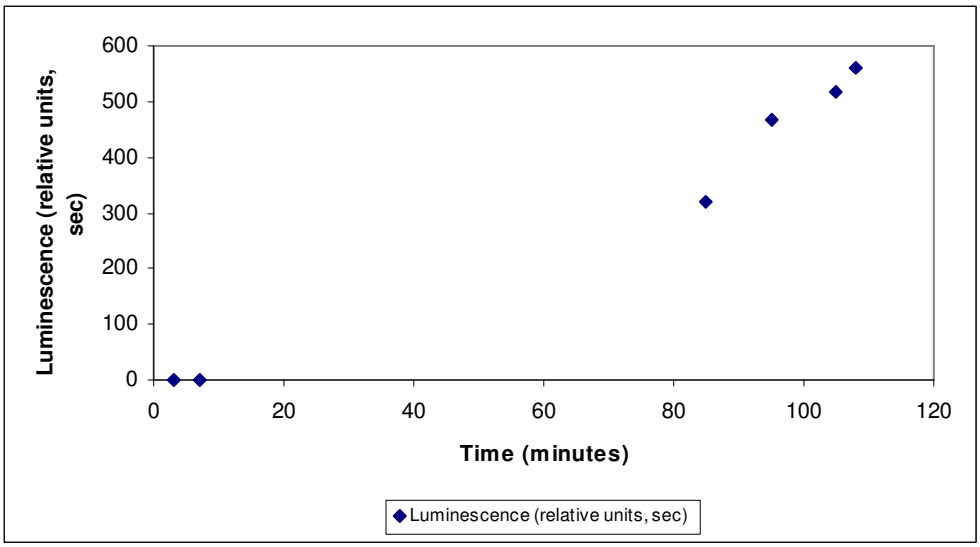

Figure 58: The bioluminescence readings for the stabilization of a *V. fischeri* lyophil.

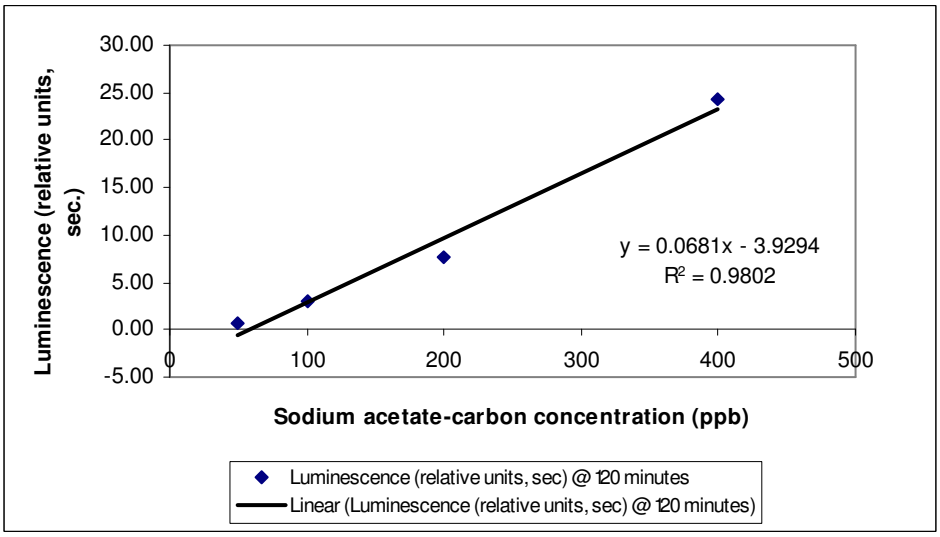

Figure 59: The bioluminescence readings for NaOAc-C ranging from 50-400ppb @ 120 minutes by *V. fischeri*

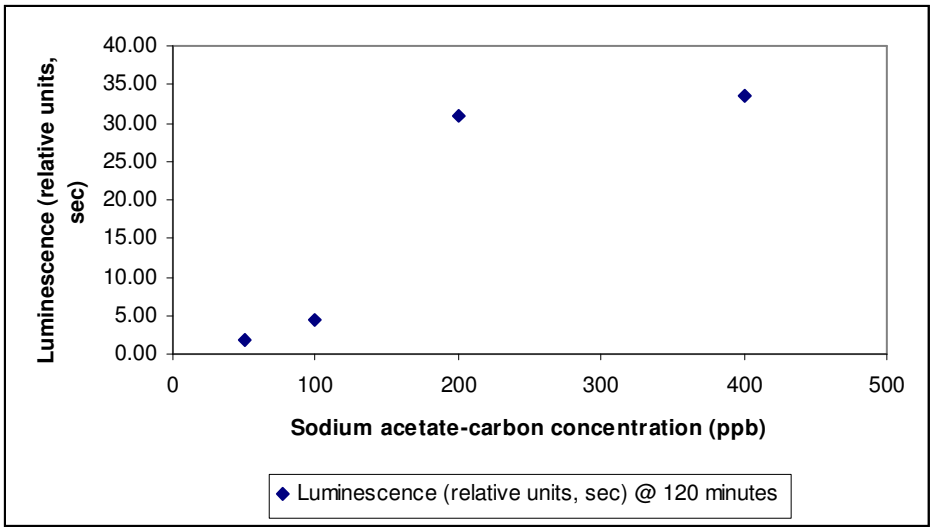

Figure 60: The bioluminescence readings of NaOAc-C ranging from 50-400ppb in PD water (toxicity test) @ 120 minutes by *V. fischeri*.

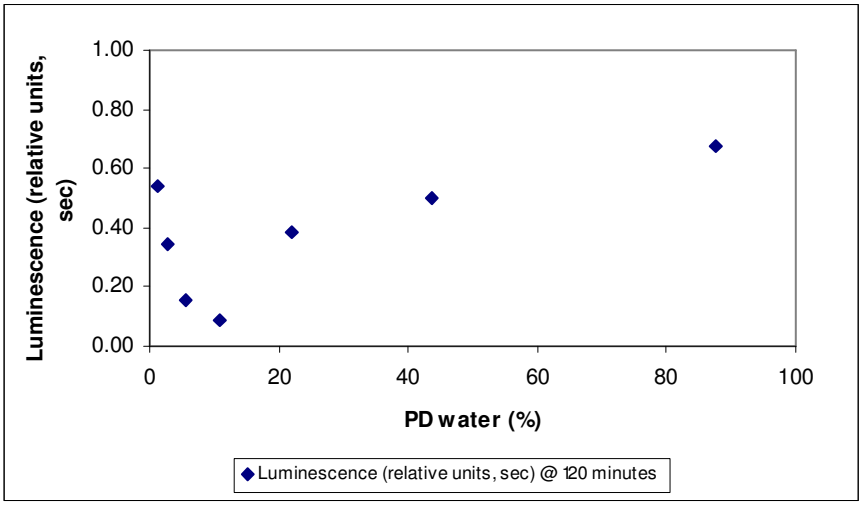

Figure 61: The bioluminescence readings of PD water @ 120 minutes by *V. fischeri*

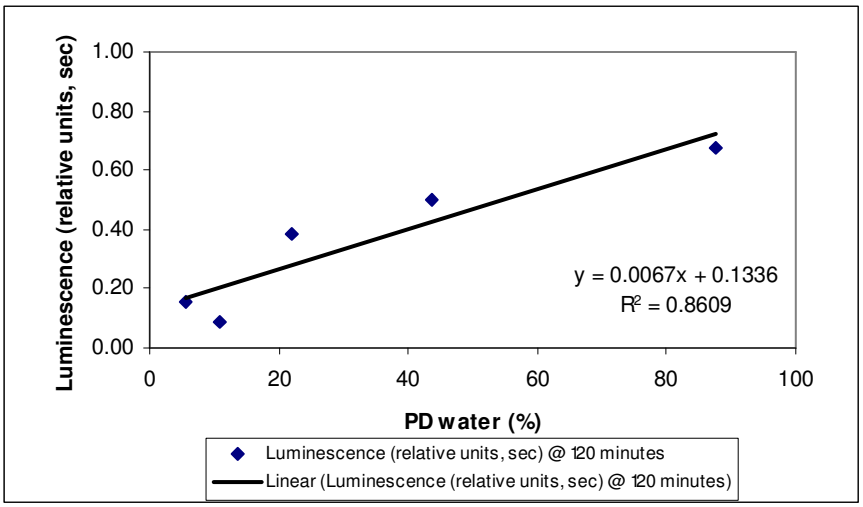

Figure 62: Bioluminescence values used by Checklight Ltd. to calculate AOC concentration present in PD water.

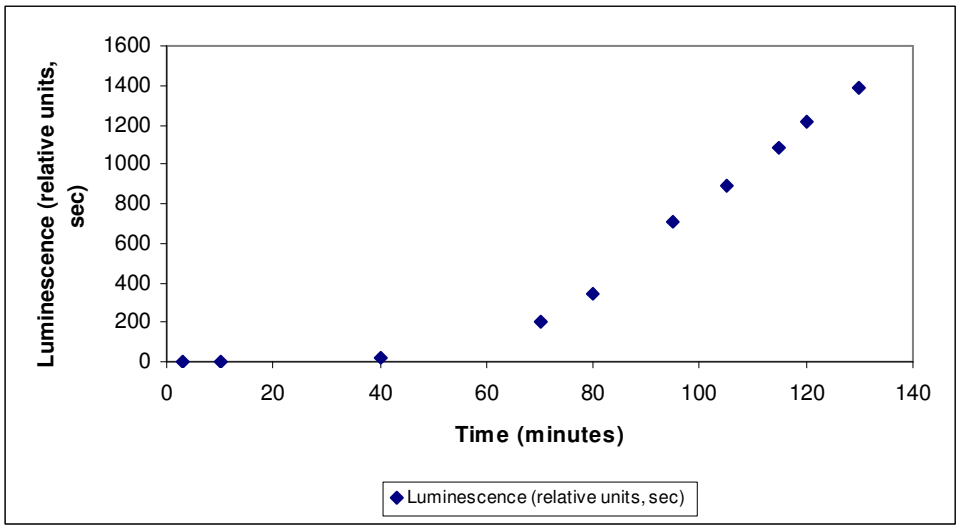

Figure 63: Bioluminescence readings for the stabilization of a lyophil of *V. fischeri*

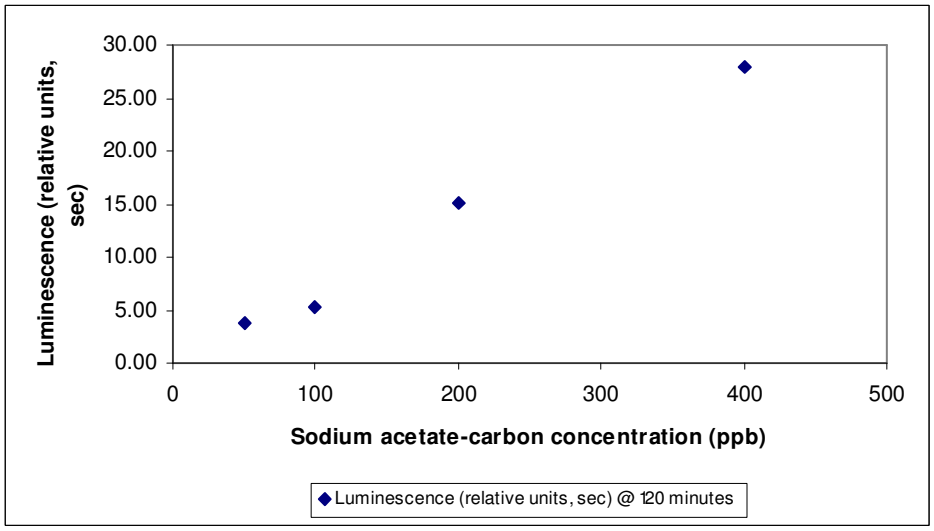

Figure 64: The bioluminescence readings for NaOAc-C ranging from 50-400ppb in EC water (toxicity test) @ 120 minutes by *V. fischeri*.

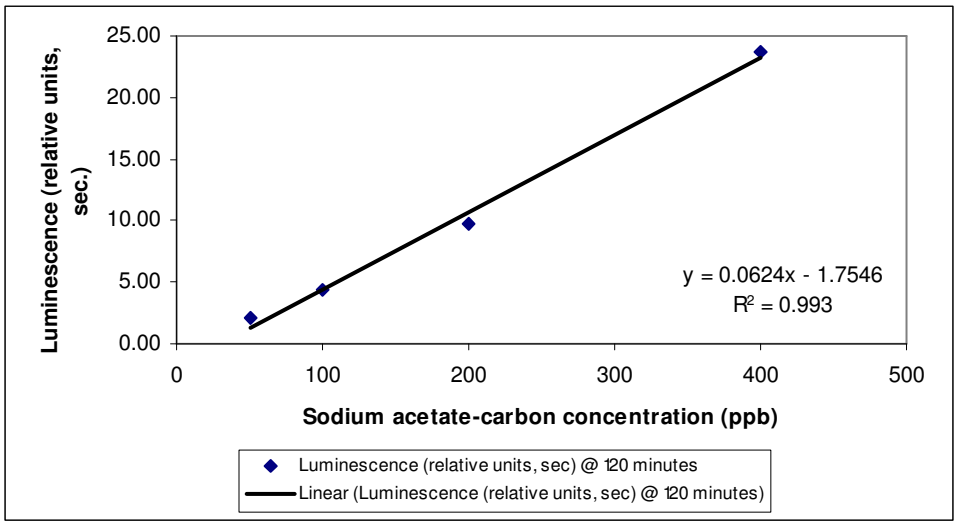

Figure 65: The bioluminescence readings for NaOAc-C ranging from 50-400ppb as the standard @ 120 minutes by *V. fischeri*

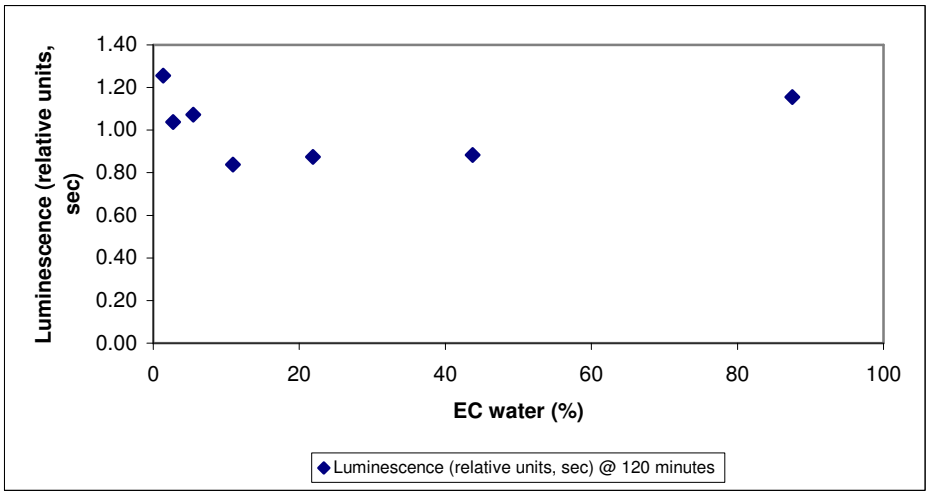

Figure 66: Bioluminescence readings for EC water sample @ 120 minutes by *V. fischeri*

Table 1: Compounds used by *Pseudomonas fluorescens* strain P17 as sole carbon and energy source for growth (Van der Kooij et. al., 1982).

Amino acids	Carboxylic acids	Carbohydrates and Alcohols	Aromatic acids
alanine	acetate	glucose	benzoate
valine	propionate	galactose	hydroxybenzoate
leucine	butyrate	fructose	anthranilate
isoleucine	valerate	mannose	
serine	capronate	rhamnose	
threonine	lactate	mannitol	
lysine	pyruvate	inositol	
arginine	malonate	adonitol	
aspartate	fumarate	ethanol	
asparagine	succinate	glycerol	
glutamate	adipate	propylene	
proline	citrate	glycol	
histidine		gluconate	
tyrosine		maltose	
phenylalanine		starch	
tryptophan			
citrulline			
ornithine			

Table 2: List of organic compounds used by *Spirillum* species strain NOX as a carbon source for growth (Van der Kooij et. al., 1982, Frias, et. al., 1994).

Carboxylic acids	Amino acids
acetate*	**alanine***
fumarate*	Glycine
glycolate	
propionate*	
glyoxylate	
lactate*	
ketoglutarate	
malate	
malonate	
succinate*	
oxalate	

*Compounds assimilated by both *Spirillum* species strain NOX and *Pseudomonas fluorescens* strain P17 in the Van der Kooij method (Van der Kooij et. al., 1982).

Table 3: Organic compounds used to evaluate the diversity of *V. fischeri* in the Checklight bioassay.

Amino acids	Carboxylic acids	Carbohydrates and Alcohols	Aromatic acids	Others
Lysine	Sodium acetate	Glucose	Benzoic acid	yeast extract
phenylalanine	Pyruvic acid	[a] fructose		carbon cocktail
Casamino acids	Citrate	glycerol		
		maltose		
		Starch		

*Note: Carbon cocktail provided with Checklight AOC kit is a mixed organic carbon source and used as a reference and positive control for many of the assays performed.
[a]Note: Organic compound not evaluated by the second AOC-II kit.

Table 4: Water sources examined for AOC concentration to access performance of Checklight bioassay.

Source water	Description
RO-I	RO feedwater
RO – P	RO product water
OCWD SMW	OCWD secondary municipal waste water
EC	Deep well injection water (colored ground water)
PD	Potable drinking water; Fountain Valley: blend of EC and Metropolitan water
SC SMW	Santa Clara secondary municipal waste water

Table 5: Bioluminescence readings of carbon cocktail solution (CCS) ranging from 50-400ppb @ 120 minutes by *V. fischeri.*

Ave. Value for negative controls	0.008
3XSD for negative controls	0.024

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
50	0.002
100	-0.003
200	-0.005
400	-0.003

Table 6: Bioluminescence readings of RO-I test sample from 90% to 0.35% @ 120 minutes by *V. fischeri.*

RO-I concentration (%)	Luminescence (relative units, min.) @ 120 minutes
90	-0.003
45	-0.001
22.5	-0.005
11.25	-0.002
5.62	-0.001
2.81	-0.002
1.4	-0.003
0.7	-0.001
0.35	0.001

Table 7: Bioluminescence readings of CCS ranging from 5-400ppb @ 120 minutes by *V. fischeri.*

Ave. value for negative controls	0.034
3XSD for negative controls	0.012

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
(ppb)	**120 min.**
5	0.008
10	0.023
20	0.048
40	0.193
50	0.174
100	0.509
200	0.339
400	-0.004

Table 8: Bioluminescence readings of CCS ranging from 5-400ppb with normal concentration of *V. fischeri* @ 120 minutes.

Ave. value for negative controls	0.208
3XSD for negative controls	0.003

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
(ppb)	120 min.
5	0.321
10	0.408
20	0.625
40	1.147
50	0.543
100	3.993
200	5.226
400	2.860

Table 9: Bioluminescence readings of a 100ppb CCS sample diluted to 0.39 pbb with normal concentration *V. fischeri* @ 120 minutes.

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
(ppb)	120 min.
100	2.954
50	1.874
25	0.729
12.5	0.463
6.25	0.378
3.13	0.325
1.56	0.376
0.78	0.403
0.39	0.465

Table 10: Bioluminescence readings of CCS ranging from 5-400ppb @ 120 minutes by *V. fischeri.*

Ave. value for negative controls	0.018
3XSD for negative controls	0.003

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
(ppb)	**120 min.**
5	-0.007
10	-0.008
20	-0.013
40	0.002
50	0.177
100	0.411
200	0.497
400	0.231

Table 11: Bioluminescence readings of a 100ppb CCS test sample diluted to 0.39ppb with 1/10 diluted *V. fischeri* @ 120 minutes.

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
(ppb)	**120 min.**
100	0.225
50	0.003
25	-0.001
12.5	0.009
6.25	0.003
3.13	0.002
1.56	0.012
0.78	-0.006
0.39	-0.010

Table 12: Bioluminescence readings by *V. fischeri* of 100ppb CCS replicates @ 120 minutes.

Ave. value for negative controls	0.179
3XSD for negative controls	0.036

Replicate	Luminescence (relative units, min.) @ 120 minutes
1	1.915
2	2.012
3	2.096
4	2.110
5	2.446
6	2.430
Replicate Ave. value	2.168
Replicate SD	0.220

Table 13: Bioluminescence readings by *V. fischeri* of 200ppb CCS replicates @ 120 minutes.

Replicate	Luminescence (relative units, min.) @ 120 minutes
1	4.389
2	4.046
3	3.934
4	4.023
5	4.521
6	4.543
Replicate Ave. value	4.242
Replicate SD	0.273

Table 14: Bioluminescence readings by *V. fischeri* of a carbon mixture ranging from 20-400ppb @ 175 minutes.

Ave. value for negative controls	0.155
3XSD for negative controls	0.027

Carbon mixture concentration (ppb)	Luminescence (relative units, min.) @ 175 minutes
20	0.094
40	0.173
50	0.259
100	0.583
200	0.804
400	0.840

Table 15: Bioluminescence readings of CCS ranging from 20-400ppb with starved *V. fischeri* @ 120 minutes.

Ave. value for negative controls	0.126
3XSD for negative controls	0.003

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
20	0.029
40	0.037
50	0.126
100	0.127
200	0.308
400	0.261

Table 16: Bioluminescence readings of a 200ppb CCS test sample with starved *V. fischeri* @ 120 minutes.

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
200	0.297
100	0.091
50	0.034
25	0.044
12.5	0.084
6.25	0.087
3.13	-0.121
1.56	0.082
0.78	0.109

Table 17: Bioluminescence readings of Casamino acids (CA) ranging from 5-400ppb @ 120 minutes by *V. fischeri.*

Ave. value for negative controls	0.083
3XSD for negative controls	0.51

CA concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
5	0.009
10	0.008
20	0.052
40	0.080
50	0.060
100	0.106
200	0.231
400	0.433

Table 18: Bioluminescence readings of Sodium acetate-carbon (NaOAc-C) ranging from 5-400ppb @ 130 minutes by *V. fischeri.*

Ave. value for negative controls	**0.095**
3XSD for negative controls	**0.018**

NaOAc-C concentration (ppb)	Luminescence (relative units, min.) @ 130 minutes
5	0.008
10	-0.008
20	0.021
40	0.030
50	0.064
100	0.077
200	0.180
400	-0.046

Table 19: Bioluminescence readings by *V. fischeri* of CCS ranging from 5-400ppb @ 120 minutes

Ave. value for negative controls	3.968
3XSD for negative controls	0.802

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
5	1.310
10	1.797
20	2.060
40	3.784
50	2.735
100	6.192
200	14.41
400	13.49

Table 20: Bioluminescence readings by *V. fischeri* of Sodium acetate-carbon as a substitute carbon source ranging from 5-400ppb @ 120 minutes

Ave. value for negative controls	1.835
3XSD for negative controls	2.206

NaOAc-C concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
5	0.466
10	0.510
20	1.179
40	1.603
50	1.860
100	1.101
200	-1.389
400	-1.790

Table 21: Bioluminescence readings by *V. fischeri* of Casamino acids as a substitute carbon source ranging from 5-400ppb @ 120 minutes

Ave. value for negative controls	1.843
3XSD for negative controls	0.266

CA concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
5	3.003
10	7.001
20	5.521
40	4.278
50	5.755
100	5.502
200	5.330
400	5.305

Table 22: Bioluminescence readings by *V. fischeri* of Glucose-carbon (Glu-C) as a substitute carbon source ranging from 5-400ppb @ 120 minutes

Ave. value for negative controls	1.030
3XSD for negative controls	0.406

Glu-C concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
5	-0.008
10	0.102
20	0.280
40	4.625
50	4.889
100	1.749
200	3.439
400	1.571

Table 23: Bioluminescence readings by *V. fischeri* of Glycerol-carbon (Gly-C) as a substitute carbon source ranging from 5-400ppb @ 120 minutes (three different lyophils)

	Lyophil A	Lyophil B	Lyophil C
Ave. value for negative controls	2.096	0.969	0.881
3XSD for negative controls	0.028	0.371	0.110

Gly-C concentration (ppb)	Lyophil A Luminescence (relative units, min.) @ 120 minutes	Lyophil B Luminescence (relative units, min.) @ 120 minutes	Lyophil C Luminescence (relative units, min.) @ 120 minutes
5	1.098	0.046	0.069
10	1.437	0.156	0.541
20	1.497	0.175	2.427
40	1.632	0.350	2.733
50	1.823	2.724	0.504
100	1.597	0.540	0.524
200	0.867	0.072	-0.475
400	0.203	-0.311	-0.863

Table 24: Bioluminescence readings by *V. fischeri* of CCS ranging from 2.5-250ppb @ 120 minutes ran in plastic assay vials; Lyophils A and B

	Lyophil A	Lyophil B
Ave. value for negative controls	4.922	3.076
3XSD for negative controls	1.928	0.239

CCS concentration (ppb)	Lyophil A: Luminescence (relative units, min.) @ 120 minutes	Lyophil B: Luminescence (relative units, min.) @ 120 minutes
2.5	0.629	0.741
5	0.214	1.136
10	1.354	1.192
20	2.301	2.003
40	3.710	4.030
50	5.659	5.040
100	11.05	9.464
200	19.04	14.57
250	18.43	15.14

Table 25: Bioluminescence readings by *V. fischeri* of a 300ppb CCS test sample @ 120 minutes ran in the plastic assay vials; Lyophils A and B

CCS concentration (ppb)	Lyophil A: Luminescence (relative units, min.) @ 120 minutes	Lyophil B: Luminescence (relative units, min.) @ 120 minutes
(ppb)	**120 min.**	**120 min.**
300	26.65	13.99
250	17.32	14.67
200	17.45	14.16
150	15.07	10.79
100	9.329	7.284
75	15.44	5.816
50	5.729	4.330
37.5	4.339	3.957
25	3.823	2.947
12.5	3.383	1.628
9.38	3.005	2.349
6.25	3.215	2.160

Table 26: Bioluminescence readings by *V. fischeri* of CCS ranging from 5-400ppb in "unwashed" assay vials @ 120 minutes

Ave. value for negative controls	5.120
3XSD for negative controls	2.938

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
5	0.497
10	0.797
20	18.37
40	7.450
50	5.880
100	8.380
200	15.78
400	5.530

Table 27: Bioluminescence readings by *V. fischeri* of CCS ranging from 5-400ppb in "washed" vials @ 120 minutes

Ave. value for negative controls	5.297
3XSD for negative controls	0.743

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
5	10.97
10	13.93
20	-4.933
40	3.803
50	4.083
100	30.14
200	39.64
400	0.523

Table 28: Bioluminescence readings by *V. fischeri* of CCS ranging from 2.5-250ppb ran in acid-washed glass assay vials @ 120 minutes; Lyophils A and B

	Lyophil A	Lyophil B
Ave. value for negative controls	0.652	2.837
3XSD for negative controls	0.206	0.452

CCS concentration (ppb)	Lyophil A: Luminescence (relative units, min.) @ 120 minutes	Lyophil B: Luminescence (relative units, min.) @ 120 minutes
2.5	0.153	0.372
5	0.236	0.502
10	0.444	1.477
20	4.231	2.466
40	1.250	3.759
50	1.569	4.439
100	4.436	9.253
200	11.29	17.98
250	15.58	18.68

Table 29: Bioluminescence readings by *V. fischeri* of a 300ppb CCS test sample @ 120 minutes ran in acid-washed glass vials; Lyophils A and B

CCS concentration (ppb)	Lyophil A: Luminescence (relative units, min.) @ 120 minutes	Lyophil B: Luminescence (relative units, min.) @ 120 minutes
300	16.36	16.39
250	12.27	16.82
200	9.12	15.68
150	6.51	12.20
100	3.401	8.253
75	2.33	5.887
50	1.521	4.429
37.5	1.325	3.671
25	2.478	2.897
12.5	0.227	2.004
9.38	0.585	2.311
6.25	0.657	2.258

Table 30: Bioluminescence readings by *V. fischeri* of a 200ppb Glycerol-carbon test sample @ 120 minutes integrated for 5 and 30 seconds

	5sec Integration	30sec Integration
Ave. value for negative controls	5.908	5.927
3XSD for negative controls	1.191	0.866

Gly-C concentration (ppb)	Luminescence (relative units, sec) @ 120 minutes	
	5sec integration	30sec integration
200	10.53	10.79
100	5.782	6.013
50	3.102	3.243
25	1.729	1.835
12.5	13.71	14.18
6.25	14.54	15.02
3.13	11.33	11.80
1.56	8.442	8.553
0.78	9.562	10.12

Table 31: Bioluminescence readings of CCS ranging from 2.5-100ppb @ 120 minutes using stabilized *V. fischeri*.

Ave. value for negative controls	30.82
3XSD for negative controls	5.234

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
2.5	4.183
5	4.203
10	6.743
20	12.03
40	20.43
50	23.91
100	42.10

Table 32: Bioluminescence readings of a 150ppb CCS test sample @ 120 minutes using stabilized *V. fischeri.*

CCS concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
150	56.75
100	34.59
50	18.11
37.5	16.20
25	14.10
18.75	11.88
12.5	10.66
9.38	11.21
6.25	9.18
4.69	9.25
3.13	10.42
2.34	11.43

Table 33: Bioluminescence readings of CCS ranging from 2.5-200ppb @ 120 minutes using stabilized *V. fischeri*.

Ave. value for negative controls	6.381
3XSD for negative controls	0.703

CCS concentration (ppb)	Luminescence (relative units, sec.) @ 120 minutes
2.5	0.293
5	0.103
10	1.181
20	2.110
40	5.549
50	7.309
75	11.88
100	16.14
200	43.84

Table 34: Bioluminescence readings of a 200ppb CCS as a test sample @ 120 minutes using stabilized *V. fischeri.*

CCS concentration (ppb)	Luminescence (relative units, sec.) @ 120 minutes
200	46.74
150	30.92
100	17.59
87.5	16.20
75	13.08
62.5	10.55
50	8.10
37.5	5.54
18.75	20.98
9.38	1.34
4.69	2.43
2.34	1.95

Table 35: CCS standards @ 130 minutes with stabilized *V. fischeri* cells.

Ave. value for negative controls	10.98
3XSD for negative controls	0.679

CCS concentration (ppb)	Luminescence (relative units, sec) @ 130 minutes
10	3.230
20	5.820
40	9.960
50	12.92
100	26.68
200	59.22

Table 36: Glucose-C and Glycerol-C as test carbon @ 130 minutes ran with stabilized *V. fischeri*.

Test carbon concentration (ppb)	Luminescence (relative units, sec) (Glucose-Carbon) @ 130 minutes	Luminescence (relative units, sec) (Glycerol-Carbon) @ 130 minutes
150	52.75	32.48
100	28.66	21.42
75	23.95	17.73
62.5	17.16	16.28
50	15.91	13.38
37.5	12.03	11.28
18.75	6.42	6.58
9.38	4.89	5.31

Table 37: CCS standard @ 120 minutes with stabilized *V. fischeri*.

Ave. value for negative controls	28.08
3XSD for negative controls	3.203

CCS concentration (ppb)	Luminescence (relative units, sec) @ 120 minutes
10	3.755
20	8.185
40	18.43
50	20.81
100	39.58
200	100.4

Table 38: Sodium acetate-C and Fructose-C as test samples @120 minutes ran with stabilized *V. fischeri*.

Test carbon concentration (ppb)	Luminescence (relative units, sec) (Sodium acetate-carbon) @ 120 minutes	Luminescence (relative units, sec) (Fructose-carbon) @ 120 minutes
150	42.87	53.07
100	30.41	40.91
75	21.98	33.50
62.5	22.62	32.79
50	20.87	24.03
37.5	15.70	20.60
18.75	12.47	16.05
9.38	9.37	8.53

Table 39: Bioluminescence readings of Sodium acetate-carbon ranging from 2.5-200ppb as a standard carbon source for an OCWD SMW sample @ 120 minutes using stabilized *V. fischeri*

Ave. value for negative controls	7.08
3XSD for negative controls	1.59

NaOAc-C concentration (ppb)	Luminescence (relative units, min.) @ 120 minutes
2.5	0.832
5	1.409
10	4.460
20	10.51
50	37.90
100	69.90
150	54.38
200	25.19

Table 40: Bioluminescence readings of an OCWD SMW test sample ranging from 87.5%-1.36% @ 120 minutes using stabilized *V. fischeri*

OCWD SMW water (%)	Luminescence (relative units, sec.) @ 120 minutes
87.5	12.03
43.75	6.94
21.87	4.80
10.93	4.08
5.46	3.43
2.73	2.86
1.36	2.03

Table 41: Bioluminescence readings for NaOAc-C ranging from 50-400ppb (standard curve) by V. fischeri @ 120 minutes.

Ave. value for negative controls	10.07
3XSD for negative controls	3.818

NaOAc-C concentation (ppb)	Luminescence (relative units, sec) @ 120 minutes
50	6.400
100	10.97
200	33.22
400	67.48

Table 42: Bioluminescence readings for water sources SC SMW and OCWD SMW by stabilized *V. fischeri* @ 120 minutes

Test water (%)	Luminescence (relative units, sec) SC SMW water @ 120 minutes	Luminescence (relative units, sec) OCWD SMW water @ 120 minutes
87.5	2.72	17.33
43.75	2.95	13.17
21.87	2.48	6.80
10.93	1.71	5.48
5.46	0.19	4.41
2.73	2.00	2.79
1.36	0.78	2.60

Table 43: The bioluminescence readings by *V. fischeri* for NaOAc-C ranging from 50-400ppb @ 120 minutes

Ave. value for negative controls	2.068
3XSD for negative controls	1.025

NaOAc-C (ppb)	Luminescence (relative units, sec) @ 120 minutes
50	2.504
100	8.573
200	16.643
400	35.07

Table 44: The bioluminescence readings by *V. fischeri* for RO-P water ranging from 87.5-1.36% @ 120 minutes.

RO-P water (%)	Luminescence (relative units, sec) @ 120 minutes
87.5	2.350
43.75	2.775
21.87	2.276
10.93	1.134
5.46	1.130
2.73	0.850
1.36	0.414

Table 45: Bioluminescence readings by *V. fischeri* for NaOAc-C ranging from 50-400ppb (standard) @ 120 minutes

Ave. value for negative controls	1.402
3XSD for negative controls	0.492

NaOAc-C (ppb)	Luminescence (relative units, sec) @ 120 minutes
50	0.659
100	2.918
200	7.565
400	24.22

Table 46: Bioluminescence readings by *V. fischeri* for PD water sample @ 120 minutes

PD water (%)	Luminescence (relative units, sec) @ 120 minutes
87.5	0.673
43.75	0.503
21.87	0.383
10.93	0.090
5.46	0.157
2.73	0.343
1.36	0.543

Table 47: Bioluminescence readings by *V. fischeri* for NaOAc-C ranging from 50-400ppb as the standard carbon source @ 120 minutes

Ave. value for negative controls	2.529
3XSD for negative controls	0.066

NaOAc-C (ppb)	Luminescence (relative units, sec) @ 120 minutes
50	2.127
100	4.312
200	9.672
400	23.70

Table 48: Bioluminescence readings by *V. fischeri* for EC water sample @ 120 minutes

EC water (%)	Luminescence (relative units, sec) @ 120 minutes
87.5	1.156
43.75	0.884
21.87	0.874
10.93	0.838
5.46	1.073
2.73	1.038
1.36	1.256

Table 49: Cell volume determined by Coulter Counter (CC) @ 0 hours

Sample Type	Total Volume of P17 (um^3/mL) @ 0 hours	Total Volume of NOX (um^3/mL) @ 0 hours
No carbon source	$1.21X10^6$	$1.26X10^6$
5ppm of NaOAc-C	$4.2X10^6$	$6.45X10^5$
10ppm of NaOAc-C	$3.66X10^6$	$2.10X10^5$
50ppm of NaOAc-C	$7.9X10^6$	$2.7X10^5$
OCWD SMW	$1.48X10^7$	$1.2X10^7$

Table 50: Cell volume determined by Coulter Counter (CC) @ 48 hours

Sample Type	Total Volume of P17 (um^3/mL) @ 48 hours	Total Volume of NOX (um^3/mL) @ 48 hours
No carbon source	$1.48X10^7$	$1.72X10^6$
5ppm of NaOAc-C	$2.98X10^8$	$4.85X10^6$
10ppm of NaOAc-C	$1.86X10^9$	$2.12X10^6$
50ppm of NaOAc-C	$1.99X10^8$	$9.9X10^5$
OCWD SMW	$7.25X10^6$	$7.2X10^6$