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Abstract approved:

James D. Ingle, Jr.

A spectrometer, here denoted a **S**imple, **L**ow-power, **I**nexpensive, **M**icrocontroller-based or **SLIM** spectrometer, was developed that exploits the small size and low cost of solid-state electronic devices. This approach results in customizable spectrometers that are considerably less expensive and smaller than traditional instrumentation. The performance of the SLIM spectrometer was evaluated and compared to that of a commercial spectrophotometer. Thionine was the analyte, and the detection limit was ~0.2 micromolar with a 1.5-mm path length flow cell.

Another device, here denoted a **S**ub-surface **P**robe for the **E**valuation of **A**naerobic **R**egions or **SPEAR**, was also developed. The SPEAR is inserted directly into the ground or sediment and automatically records depth-resolved redox information in real time. The device is based on membrane sampling devices or "peepers" that allow subsurface samples to be collected without changing the redox speciation of sensitive species. Miniature spectrometers incorporated in the probe measure the absorbance of the redox indicators immobilized on clear membranes to

track the redox status in situ. The device was tested with the redox indicator thionine in a waterlogged soil in the Willamette Valley of Oregon.

The reducing region of the pore water was observed over a two and a half week period in late March.

A device for monitoring the absorbance of an immobilized redox indicator, in direct contact with a sample inside a transparent microcosm bottle or bioreactor, through the wall of the enclosure has been developed. In this device, denoted as Spectrometer for **N**on-invasive **A**naerobic **R**eflectance **E**valuation or **SNARE**, the indicator is immobilized in a film or membrane, which is attached to a circular magnet. This disk is placed inside the laboratory vessel, and reductants or oxidants in the solution interact with the immobilized indicator causing a change in its color. Another magnet, imbedded in the external probe, attracts this disc to the side of the container and aligns the probe and disc in the proper orientation. The operation of this device was demonstrated with the redox indicator thionine to determine the redox status of a sample containing an enriched culture in which the anaerobic degradation of chlorinated ethenes was occurring.

The Development and Characterization of Miniature Spectrometers for Measuring the Redox Status of Environmental Samples

by

Kevin Cantrell

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Kevin Cantrell, Author

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In memory of Dr. Ed Piepmeier

The Development and Characterization of Miniature Spectrometers for Measuring the Redox Status of Environmental Samples

Chapter 1: Introduction

The focus of this work is on the development of new techniques and instrumentation for use in environmental monitoring, especially in the design and fabrication of miniature spectrometers for determining redox conditions. The devices described in this thesis are inexpensive, portable, and automated. They are tailored to specific exposure environments in the field and laboratory including wells, laboratory containers, and sub-surface soil samples.

1.1 Environmental analysis

By their nature, environmental samples are often difficult to analyze. In this work sub-surface samples are of primary interest and include solid, liquid, and gas phases. The components of these samples include organic and inorganic chemical species in soil and groundwater along with microorganisms, which mediate the transformation of many of the chemical components. A spectrum of analytical techniques is available for such samples, and these techniques are often quite different. In one paradigm the sample is collected, preserved, and brought back to a well-equipped laboratory for a suite of chemical analyses. The other paradigm involves

field instruments where the chemical analysis is performed on site with minimal manipulation. These approaches are not mutually exclusive and often complement each other.

With the first approach, which includes sophisticated instrumentation and well understood analytical methods, theoretically any constituent in the sample can be accurately determined. These techniques can reveal subtle nuances in the chemistry that is occurring. The primary weaknesses of this approach lie in the collecting, preserving, transporting, and storing of the sample. For example, when analytical results are delayed, the integrity of the sample can be compromised. Furthermore, unless many samples are taken at regular time intervals and under a variety of conditions (e.g., seasons, temperature), important aspects of the chemical system can be overlooked. Monitoring over time and varying conditions requires dedicated instrumentation.

In the second approach, these sample manipulation difficulties can be minimized or avoided, and real-time feedback is possible. It is sometimes difficult to implement powerful analytical techniques either in the field (where instruments such as chromatographs are cumbersome) or with minimal sample handling (where the method requires extraction or multiple reagents). Simple on-site methods cannot usually provide the selectivity, detection limits, accuracy, or multiple species capabilities of laboratory-based techniques.

For these environmental samples the analyte is often interwoven in its environment, and to remove it from this environment without changing some of its characteristics would require a level of understanding that we do not as of yet possess.

For example, many interesting sub-surface samples are anaerobic in nature and any exposure to atmospheric oxygen can dramatically alter their composition. In addition, sample preservation and handling techniques (e.g., pH adjustment), adsorption to the storage container, temperature changes, and microbial activity can all change the chemical makeup of the sample as it awaits analysis. With most current laboratory based techniques, which involve sample collection and storage, it is likely that information about redox-sensitive species is altered or lost.

The focus of this work is to develop automated means of measurement of redox status that minimize perturbation of the chemical composition of the system of interest. For example, the redox status of sub-surface pore water is determined in-situ, and a change in the speciation of redox-sensitive constituents is avoided. This approach requires that the instruments must be portable and robust so that they can be placed in or near the sample in the field or lab. To achieve this "low-impact" type of analysis, compromises are made. Design criteria lie more toward the simple, in-situ, and inexpensive end of the spectrum rather than the sophisticated, sensitive, and expensive end.

1.2 Redox status and redox indicators

Among the most important factors controlling the persistence, mobility, and biological effects of many organic and inorganic contaminants are oxidation-reduction (redox) transformations. These redox reactions involve the transfer of electrons and are the basis of energy generation in all living organisms. Determination of the redox

status of a sample is important in environmental analysis. This parameter along with temperature and pH can provide great insight into where a chemical equilibrium lies. For example, a Pourbaix diagram employs redox activity and pH as its axes and is useful in predicting which species is thermodynamically stable (1).

Assessing the dominant terminal electron acceptors (TEA) is a method of characterizing microbial systems, especially those used in a bioremedial setting. Degradation rates and pathways of organic compounds are known to depend strongly on ambient redox conditions. For example, several halogenated organic pollutants undergo redox transformations to less toxic compounds under reducing conditions (2). In the design of in-situ remediation strategies, control of the predominant redox processes and microbial metabolic processes through the proper balance of carbon substrate and the terminal electron acceptor is critical in obtaining the desired transformation of an organic contaminant (3).

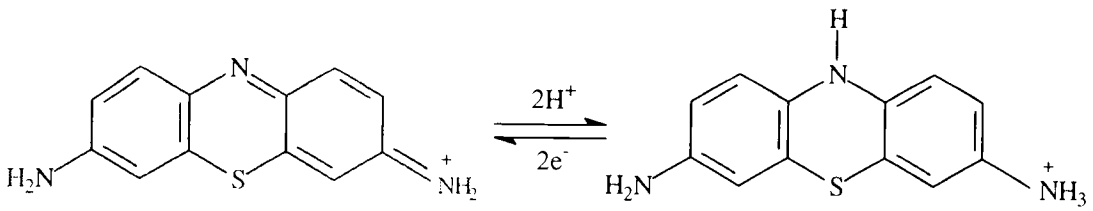
A clear understanding of the redox status of a system including the dominant redox processes and the redox state of critical species is required in virtually all aspects of hazardous substance management in ground water including: (i) assessing contaminated sites to evaluate clean-up options, (ii) monitoring the progress of in-situ bioremediation, (iii) understanding the fundamentals of microbially-mediated processes as a basis to optimize performance and develop improved methods.

Due to its ease of use, the Pt electrode is the most popular and convenient sensor for measuring the redox status of environmental systems. However, in numerous studies, the Pt electrode provides inconsistent results (4) and the absolute value of its potential cannot generally be correlated to concentrations of specific

redox-active species or the dominance of a particular microbial process (5, 6). It can delineate between oxic and anoxic conditions, but does not by itself provide an adequate evaluation of redox status.

There is no one "best method" to determine redox status for all situations, and all methods to evaluate redox conditions suffer from complications due to non-equilibrium conditions, slow kinetics, and transport processes. Different methods that have been used are summarized in Table 1-1. Redox indicators, the newest method, provide a simple, practical, and rapid method suitable for many applications (7).

In previous work (4, 8) in this laboratory, numerous redox indicators have been evaluated for their suitability for environmental monitoring. Important characteristics include reversibility with a variety of reductants/oxidants, coupling to a Pt electrode, and formal potential at pH 7. These multiple-ring, organic compounds are usually colored in their oxidized form and colorless in their reduced form, allowing monitoring of their redox speciation spectrophotometrically. For example, the reduction of blue thionine to a colorless form can be monitored at 600 nm.



blue oxidized form *colorless reduced form*

Table 1-1. Methods of Evaluating Redox Status of Environmental Samples

Sensing Method **Redox level**	E_{Pt}[a] (mV)	TEA or reduced product	H_2 conc.[b] (aq, nM)	
O_2 reducing	400 – 300	O_2, H_2O	<0.05	
NO_3^- reducing	300 – 200	NO_3^-, NO_2^-, N_2O, N_2	<0.05	
Fe(III) reducing	150 – 0	$Fe(OH)_3$ (s), Fe(II)	0.1 – 0.8	Thionine $E_7^{0'}$ = 52 mV
SO_4^{2-} reducing	-100 – -250	SO_4^{2-}, S(-II)	1 – 4	Cresyl Violet $E_7^{0'}$ = -81 mV
Methanogenic	-200 – -300	organic, CO_2 CH_4	7 – 10	Phenosafranine $E_7^{0'}$ = -267 mV

[a] references 4, 9, 10, and 11, [b] references 5 and 6, [c] references 4 and 8

E_{Pt} is the potential measured with a Pt electrode
$E_7^{0'}$ = formal potential of indicator at pH 7 (half-reduced) for immobilized indicators
Potentials here and elsewhere are reported versus a standard H_2 electrode (SHE)

Because these positively-charged indicators adsorb to minerals and soils, methods have been developed in this laboratory to immobilize them in a form that can be monitored spectrophotometrically, that does not leach out, and that is accessible to reductants and oxidants in the water sample (4). This covalent immobilization of the indicator to porous particles and thin (~50 nm) permeable films provides sensor materials that are amenable to direct contact with environmental samples. Some redox indicators that have proved useful are listed in Table 1-2 (7). Jones (4) has demonstrated that these "redox particles and films" can be placed in flow cells and used to monitor the redox status of anaerobic soil, sludge, and groundwater samples in a bioreactor. Solution can be pumped in a closed loop without exposure of the sample to O_2.

The general hypothesis of the redox indicator approach (7) is that, under different redox conditions, there is a different average composition of soluble species in the groundwater which includes reductants and oxidants which can interact with other redox-active species such as redox indicators. It is proposed that the concentrations of reduced species and stronger reductants will increase as the microbial redox level becomes lower. Thus, the redox indicator becomes a surrogate indicator for redox conditions by responding to the redox-active species present.

A given redox indicator can be reduced only if there is a sufficient concentration of a relevant reductant(s) with a suitably low redox potential. If a particular redox indicator in contact with a sample solution is oxidized, this fact provides a very clear indication that the redox level is not below a certain value (assuming that the reductants do couple with redox indicator). If an indicator is

Table 1-2. Some applications of immobilized redox indicators.[a]

Indicator	Formal Potential[b] (mV)	Application
thionine (Thi)	52	1) indicates Fe(III)-reducing conditions 2) partially reduced when [Fe(II)] ~100 mM at pH 7 3) indicates Cr(VI) is completely reduced 4) indicates microbial conditions suitable for reduction of As(V) to As(III) 5) mostly reduced before transformation of TCE to *cis*-DCE
cresyl violet (CV)	-81	1) indicates sulfate-reducing conditions 2) half-reduced as total sulfide levels of ~1 μM at pH 7 3) partially reduced when reductive dechlorination of TCE to *cis*-DCE occurred in a bioreactor
nile blue (NB)	-131	1) reduced completely in bioreactor containing a sewage sludge approaching methanogenic conditions
phenosafranine (Psaf)	-267	1) indicates methanogenic conditions 2) reduced completely by enriched methanogenic cultures before TCE dechlorination began

[a] references 4,7
[b] reference 4

reduced, the redox level is only known to be less than some value and one or more of several reductants may have been responsible. Several indicators with different formal potentials can then be used to establish that the redox level is within a certain interval.

As previously stated and established, the immobilized form of these redox indicators made them very amenable to environmental samples since direct contact with the sample is possible. The general questions addressed in this work are "how should the sample of interest be brought into contact with the sensing material without

changing its redox properties?" and "how can the changes in the absorbance of the material be transformed into useful chemical information?"

1.3 Miniature spectrometers and exposure environments

Miniature spectrometers were developed for two sample environments. The devices described here are designed around immobilized redox indicators and tailored to a specific exposure environment. They replace commercial spectrophotometers and can be distributed more widely (inexpensive), are more portable (small), and are applicable to a greater variety of situations (adaptable). The light sources are single and multiple color LED's. The photodetectors are single chip integrated circuits with their own signal processing circuitry. Embedded microcontrollers are used for control of the optical sources and detectors and data acquisition, processing and storage. The electronic features common to most of the devices include data logging, automated sampling, a computer interface, and user-selectable parameters. The devices can monitor and store redox data (e.g., absorbance vs. time data) over periods of days without external microcomputers or data loggers, and they are inexpensive enough that a device can be dedicated to a specific location.

"Exposure environment" is the term used to describe the interface between the immobilized redox indicator and the sample of interest. The first such environment is some variety of laboratory vessel that simulates or contains a sample such as soil or groundwater. Examples include microcosm bottles, bioreactors, or soil wedge models. The second environment is a field site where the "sample" has not

been removed from the site. Examples include aquifers and groundwater accessible via sampling wells or sites where the subsurface sampling zone is near the surface or accessible without a well (e.g., wetlands, lake bottoms, waterlogged soils, etc.). Each exposure environment is further divided into two sub-classes: one that requires a pumping apparatus and one that does not. If a pump is present, water or a slurry from a sampling well (field) or a bioreactor (laboratory) can be simply delivered to a flow cell in a spectrometer containing immobilized redox indicators. Where a pump is not appropriate, the sensing surface (immobilized redox indicator) must be placed directly into the sample (inside a container or driven into in the ground directly).

Three different types of miniature spectrometers are discussed in Chapters 2, 3, and 4. These spectrometers are tailored to the types of exposure environments outlined above.

The SLIM (*S*imple, *L*ow-power, *I*nexpensive, *M*icrocontroller-based) spectrometer is described in Chapter 2. It is useful for monitoring the redox status of laboratory or field samples with pumped sample solutions. The SLIM exploits the small size and low cost of solid-state electronic devices. Light emitting diodes, single-chip integrated circuit photodetectors, embedded microcontrollers, and batteries replace traditional optoelectronic components, computers, and power supplies. This approach results in customizable spectrometers that are considerably less expensive and smaller than traditional instrumentation. The performance of the SLIM spectrometer is compared to a commercial HP diode array spectrometer, and issues associated with the design of miniature spectrometers in general are discussed.

In Chapter 3 a device designed to measure redox status in sub-surface pore water directly is presented. This device, denoted a *S*ub-surface *P*robe for the *E*valuation of *A*naerobic *R*egions or SPEAR, is inserted directly into the ground or sediment and automatically records depth-resolved redox information in real time. The device was tested with the redox indicator thionine in a waterlogged soil in the Willamette Valley of Oregon. The reducing region of the pore water was observed over a two and a half week period in late March. Both the design of the device and the redox changes measured are discussed.

In Chapter 4 the design, construction, and data collected with a device designed to provide real-time, non-invasive information about the redox status inside a sealed microcosm bottle are presented. In this device, denoted as SNARE (*S*pectrometer for *N*on-invasive *A*naerobic *R*eflectance *E*valuation), all the complex parts are outside the bottle, and the sensor is configured to easily make measurements on many bottles in a batch type study. In a preliminary experiment, the color change of the redox indicator thionine is used to monitor the anaerobic transformation of polychlorinated ethenes in microcosm bottles spiked with an enriched bioremedial culture.

A number of appendices are included at the end of the thesis. Appendices A, B, and C are used to present additional details (including programs) that are relevant to some of the miniature spectrometers in chapters 2, 3, and 4, respectively. Appendices D and E are concerned with the use of immobilized redox indicators and another custom spectrometer, the STanD (*S*tand-alone *T*wo *D*etector) spectrometer, to evaluate the efficacy of an abiotic soil amendment to degrade TCE. Appendix F

details the immobilization and application of two pH indicators immobilized in the same manner as redox indicators. Appendix G concerns the design of flow cells for redox indicators immobilized on thin films. Appendices H and I provide additional details about the circuit fabrication and immobilization processes, respectively.

1.4 References

1. Shriver, D. F.; Atkins, P.; Langford, C. H., *Inorganic Chemistry*, W. H. Frreman and Company, **1994**.

2. Atlas, R. M.; Bartha, R., *Microbial Ecology: Fundamentals and Applications*, Addison Wesley, **1998**.

3. Vogel, T. M.; McCarty, P. L., *Appl. Environ. Microbiol.*, **1985**, *43*, 1080-1083.

4. Jones, Brian D., *Applications of Redox Indicators for Evaluating Redox Conditions in Environmental Samples*, **1999**, Ph.D. Thesis, Oregon State University.

5. Chapelle, F. H.; Haack, S. K.; Adriaens, P.; Henry, M. A.; Bradley, P. M, *Environ. Sci. Technol.*, **1996**, *30*, 3565 - 3569.

6. Lovley, D. R.; Goodwin, S, *Geochim. Cosmochim. Acta*, **1988**, *52*, 2993-3003.

7. Ingle, J. D., A Research Proposal submitted to the EPA, **2000.**

8. Lemmon, Teresa, *Development of Chemostats and Use of Redox Indicators for Studying Transformations in Biogeochemical Matrices*, **1995**, Ph.D. Thesis, Oregon State University.

9. Frevert, T., *Schweiz. Z. Hydrol.*, **1984**, *46*, 269-290.

10. Lindberg, R. D.; Runnells, D. D., *Science*, **1984**, *225*, 925-927.

11. Kempton, J. H., *Heterogenous Electron Transfer Kinetics*, **1987**, M.S. thesis, University of Colorado.

Chapter 2: The SLIM Spectrometer

2.1 Abstract

A new spectrometer, here denoted the SLIM (simple, low-power, inexpensive, microcontroller-based) spectrometer, was developed that exploits the small size and low cost of solid-state electronic devices. In this device, configured with a flow-cell, light emitting diodes, single-chip integrated circuit photodetectors, embedded microcontrollers, and batteries replace traditional optoelectronic components, computers, and power supplies. This approach results in customizable spectrometers that are considerably less expensive and smaller than traditional instrumentation. The performance of the SLIM spectrometer was evaluated and compared to that of a commercial spectrophotometer. Thionine was the analyte and the detection limit was ~0.2 μM with a 1.5 mm path length flow cell. Non-linearity due to the broad emission profile of the LED light sources is discussed.

2.2 Introduction

Small portable spectrophotometers are useful in field applications and process monitoring. Several companies such as Hach and Chemetrics sell battery-powered, filter -based instruments that are generally used with reagent kits tailored to a specific determination. Often in the design of these spectrometers compromises are made in the selection of the optoelectronic components to allow for reduced size and cost.

Several researchers have used LED light sources to replace traditional spectrophotometric sources in which the small size, low cost, low power consumption, and robust nature of these LEDs allow for the design of new spectrometers. Dasgupta and coworkers have provided an excellent review article of the spectrometric applications of LEDs (1), demonstrated of the use of multiple LED based spectrometers to correct errors due to refractive index and turbidity in FIA (2), and designed several novel flow cells around LED light sources (3).

Hauser (4, 5) focused on using several LEDs to gain the functionality of a multiple wavelength spectrometer. Because LED emission profiles are somewhat narrower (20 to 40 nm) than typical molecular bands (50 to 100 nm), a device that incorporates multiple LEDs can function as a multiple wavelength spectrometer that covers a large portion of the visible band. Commercially available multi-emitter LEDs (two and three color varieties), as well as individual LEDs coupled together with fiber optic hardware, have been investigated. Huang et al. (6) also explored the applications of multiple emitter LEDs and has used rapid microcomputer-controlled modulation to produce a FIA detector with low drift. Worsfold and Clinch (7, 8, 9) focused on the

applications of LED-based spectrometers coupled with simple FIA techniques in the areas of environmental monitoring and process control. These rugged spectrometers were used to determine water quality parameters such as phosphate, nitrate, and ammonia and were able to collect data unattended for periods of approximately one week.

In this work we outline the design and construction of a new type of re-configurable miniature spectrometer based on modern semiconductor technology that incorporates an array of useful features. The design philosophy de-emphasizes the complexity, optical sophistication, computational ability, accuracy, resolution, and processing power usually found in bench-top spectrometers. Instead, characteristics such as miniaturization, automation, reduced cost, low power consumption, simplicity, and re-configurablity are emphasized. Spectrometers of this design have uses in the areas of flow stream/process monitoring, field sampling, environmental monitoring, and disposable applications. This device incorporates a LED light source, photodetector, flow cell, battery, re-programmable microprocessor, clock and memory for data logging capability, and both a computer and a user interface on a single circuit board with the footprint of a credit card. The spectrometer is intended to be inexpensive, small, rugged, and versatile.

The spectrometer was evaluated in a flow cell configuration with thionine as the analyte. Calibration and noise data were obtained and compared to results obtained with a commercial spectrometer under equivalent conditions. The effect of the broad emission of the red, yellow, and blue LEDs on the calibration curves is discussed.

2.3 Instrumentation

2.3.1 General considerations

The new spectrometer, denoted the SLIM (simple, low-power, inexpensive, microcontroller-based) spectrometer, is similar to traditional spectrophotometers in many respects. Table 2-1 shows a comparison of the common functional elements of a spectrometric system. The new device was developed with environmental monitoring in mind and many of the incorporated features relate to this type of measurement. For example, it is capable of operating in an unattended data-logging mode in which the user selects a sampling interval and allows the unit to collect data. The user then returns and downloads the logged data into a computer for processing and interpretation. These devices do not contain a built in mechanism for display and rely on a PC or supplementary electronics for this function.

Table 2-1. Elements of conventional and SLIM spectrometers

Component	**Conventional**	**SLIM**
Light Source	Tungsten lamp	LED
Sample Delivery	Cuvette	Acrylic flow cell
Detector	Photodiode, PMT, or Diode Array	Photodiode IC
Data Storage	Computer disk	EEPROM
Control Unit	PC	Microcontroller

The basic function of this device is that of a spectrometer, to measure the intensity of light passing through a sample. To achieve this function, a programmed microcontroller turns on a LED light source and measures intensity as the frequency output from a photodetector chip (a radiant power-to-frequency converter). The spectrometer then stores this information and continues to collect data over time. The diagram in Figure 2-1 outlines the functional elements of the spectrometer, and the flow chart in Figure 2-2 shows the basic steps in the microcontroller's program. A sample schematic is provided in Figure 2-3.

The design for the flow cell is illustrated in Figure 2-4. This cell was designed to accommodate a reagent, such as a pH or redox indicator dye, that is immobilized on a film. For this type of application, the cell must be demountable and accommodate a replaceable disk of immobilized reagent (~2 cm in diameter). The sample solution is contained in a space defined by an O-ring compressed by two sheets of acrylic (~500 μL internal volume). The thicker of these sheets is machined with tapped holes to accept ¼*28 male fittings for connecting to tubing. The sample solution is directed into and away from the sample cavity by right-angle drilled holes. The thick side of the cell body also contains holes drilled to accommodate three LED light sources. The LED cavity in the center of the cell is pointed directly at the other side. LED cavities adjacent to the center of the cell are oriented a 30° angle so that light from the inserted LEDs illuminates the same photodetector in the center of the opposite side of the cell. The screws that compress the O-ring between the sides of the cell also affix the entire flow cell to a circuit board where the photodetector chip is soldered in the proper position. The path length of the cell varies with the degree of O-ring compression.

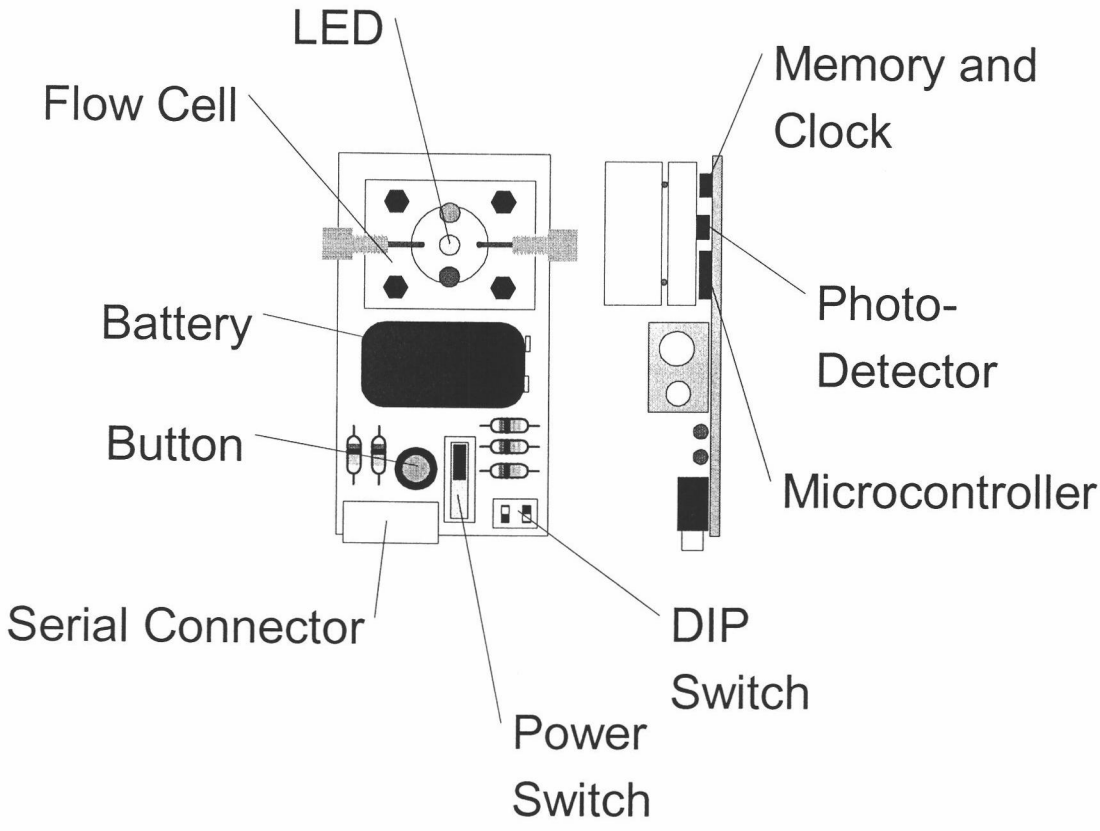


Figure 2-1. Instrumental diagram indicating the layout of the basic components of the solid-state spectrometer. These components include the LED light source, photodetector, microcontroller, clock, memory, battery, serial connector, and switches. The circuit board dimensions are 2 in by 4 in by 1.5 in.

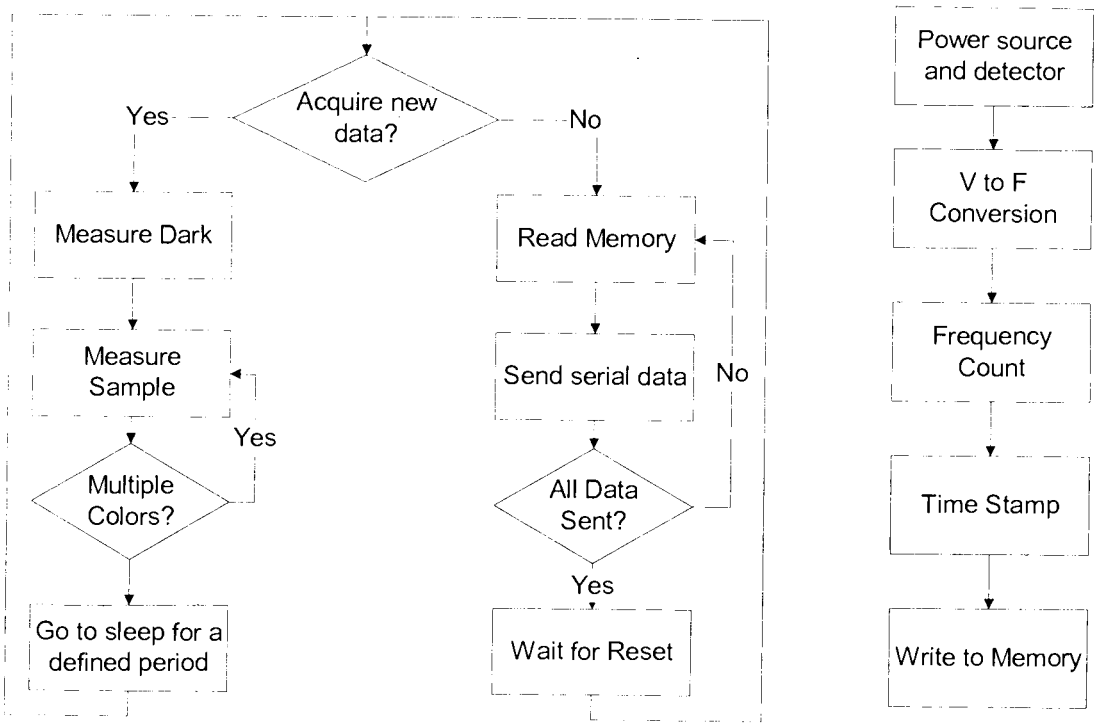
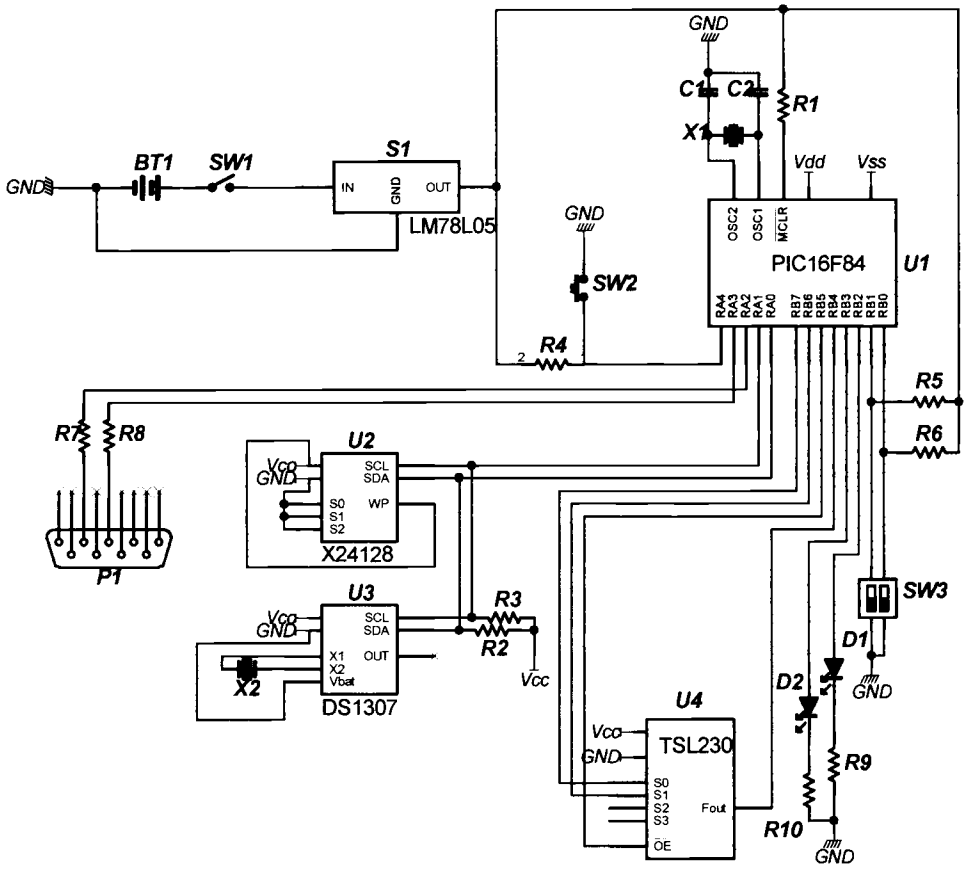


Figure 2-2. Flow diagram of the steps in the MCU program. The decision point "Acquire new data?" is determined by the state of the pushbutton switch. The sequence of events on the right is executed each time the spectrometer measures an intensity.



Reference:	Value:	Description:
U1	PIC16F84	Microcontroller
U2	X24128	Serial EEPROM
U3	DS1307	Real Time Clock
U4	TSL230	Light to Frequency Converter
S1	LM78L05	Voltage Regulator
SW1	SWITCH	Sliding Power Switch
SW2	BUTTON	Momentary Pushbutton
SW3	SWITCH	2x DIP Switch
X1	4MHz	Crystal Oscillator
X2	32kHz	Crystal for RTC
P1	DB9 FEMALE	Serial Connector
BT1	9V	Battery
C1,C2	22pF	Decoupling Capacitor
D1,D2	LED	See Text
R1,R2,R3,R4,R5,R6	4.7K	Pull Up Resistor
R7	1K	Resistor
R8, R9	22K	Resistor
R10	0.47K	Resistor

Figure 2-3. Schematic of the SLIM spectrometer

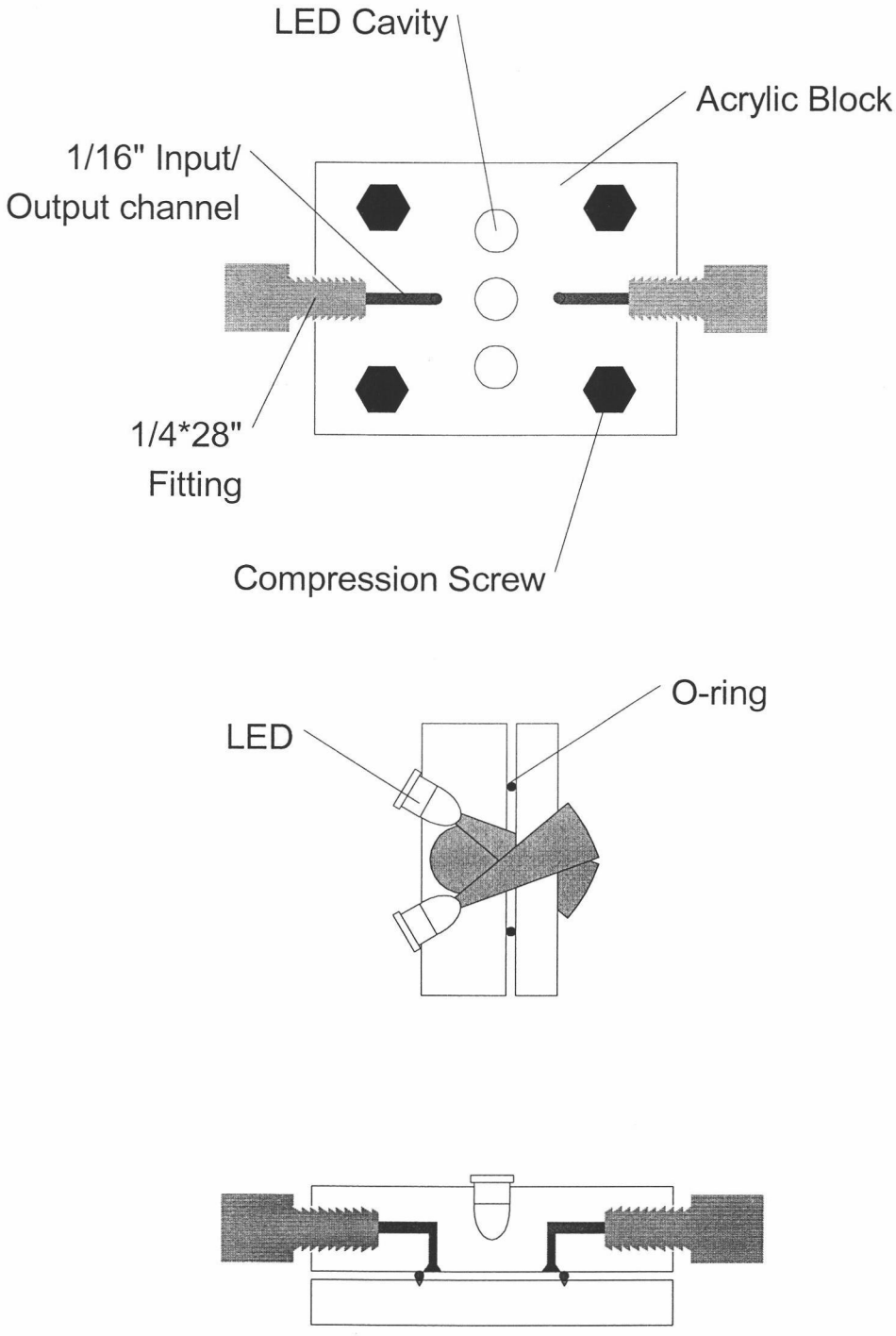


Figure 2-4. Diagrams of the flow cell. The outside dimensions of the flow cell are 2-in wide, 1-in long, and ¾-in deep. The cones in the middle figure indicate the 30° beams emitted by the two LEDs.

This variation is not critical when immobilized indicators are used, but spacers would be necessary for exact control of the path length for solution work in which the path length must be known. In this work the path length was ~1.5 mm. The flow cell mounts via the four holes and occupies the upper 1/3 of the circuit board. A layout of this circuit board is included in Appendix A.

2.3.2 Electronic components

The electronic components were selected to match the low cost and low power consumption of the light emitting diodes. The total cost of the integrated circuits and LEDs used to construct the spectrometer is ~$25. The life of the battery is determined by the duty cycle of the LEDs. An alkaline 9 V battery can power an LED with 10 mA of current for approximately 60 hours. If the data are acquired once per minute, this battery should power the instrument for two months.

The IC selected to serve as the embedded microcontroller in these devices is the PIC-16F84 (Microchip Technologies Incorporated). This chip is an 8-bit MCU (microcontroller) with 1K of program memory and 68 bytes of data RAM. It is a member of their flash family of products, so it can be electrically erased allowing for rapid prototyping and code development. It is a RISC (reduced instruction set computer) paradigm chip so there are only 35 assembly language instructions. The MCU has 13 I/O lines, and each line can source 20 mA or sink 25 mA of current. In this application a 4 MHz crystal was used as the oscillator. The MCU chip internally divides the resonant frequency of this crystal by four to derive an instruction clock rate of 1 MHz. An 8-bit MCU was selected because its cost, size, power consumption, and computational power

are well matched to this particular application. This model and manufacturer was selected because the cost of programming hardware and software were minimal and the company provides extensive literature and programming examples for smaller scale operations.

The program for the microcontroller was written in a high level BASIC programming language and compiled into assembly instruction with the PIC Basic Pro compiler (MicroEngineering Labs Inc). This high level programming language makes complex activities such as communicating with other ICs much easier to code.

LEDs from a variety of manufacturers such as Nichia, HP, and Panasonic and resellers including Jameco, Digi-Key, and Mouser were evaluated. A variety of colors are available and a graph with several profiles is included in Appendix A. For this work a red and a yellow-orange LED from Jameco, and a blue LED from Nichia were used as the light sources for the spectrometer.

The photodetector was the TSL230 programmable light-to-frequency converter manufactured by Texas Instruments. Although TI no longer manufactures this part, Texas Advanced Optoelectronic Solutions (TAOS) has been granted a license to produce and market their family of optoelectronic sensors. This chip integrates a configurable grid of photodiodes and a current-to-frequency converter in a single package. With no additional electronic components, it provides a TTL square wave with a frequency proportional to light intensity. This photodiode's sensitivity is adjustable over three orders of magnitude by manipulating two input lines to change the active readout area of the photodetector. The output frequency can be divided by 1, 2, 10, or 100. Division by

2 or more is necessary to produce a 50% duty cycle. This photodetector is ideal for this application because no separate ADC chip or domain converters are required.

A DS1307 serial real time clock (Dallas Semiconductor) was used as the timekeeping device. This IC uses a 38.768 kHz crystal to keep track of seconds, minutes, hours, day, date, month, and year. It communicates as a slave on the I^2C bus developed by Phillips (10). This communication protocol is a 2-wire synchronous serial bus that supports multiple devices connected to the same bus.

An X24128 128K-bit serial EEPROM (Xicor) serves as the memory for this device. This memory is organized in a 16K x 8-bit fashion. It is used to store the time stamp information along with light intensity information in a formatted data block. Both read and write operations are controlled via the same I^2C bus used for communication with the real time clock. This component was selected because it provides a functional amount of storage with a minimum of hardware lines and software overhead for non-volatile data storage and retrieval. With this EEPROM the spectrometer can hold over 1,000 data acquisitions. The four intensity readings require two bytes of storage each, and the time stamp requires four bytes. If data are acquired once per minute the memory will suffice for approximately one day, or if the data are acquired every hour the memory can store approximately two months of data. The memory can be easily expanded by adding more EEPROM chips (currently up to a factor of 8). The storage capacity of these ICs is constantly being increased, and storage space is not anticipated to be an issue in the future.

2.3.3 Operation

All devices are built around a simple user interface involving a single button and a power switch. Pressing the button instructs the device to collect data. When the power switch is turned on and the button is not pressed, the device sends out all of the data that it has stored in the EEPROM over its serial port. If the button is then pressed (after the download of the data), the device begins acquiring new data and appends these new data points to those already stored in memory. If the button is pressed when the power switch is initially turned on (before the download of the existing data), then the old data are discarded and the new data are stored starting from the base memory address. In either case pushing the button initiates new data acquisition. If it is pressed after the power switch is on, these data are appended to existing data. If it is pressed before the power switch is on, the new data replace the existing data.

Any time the power is switched on the real time clock is reset to zero. The time stamp is simply the number of days, hours, minutes, and seconds that have elapsed since the power was switched on. If the device is used in the append mode, the data will contain multiple time zero stamps. The operator must keep track of the times when the device was turned on so that a correspondence between the time stamp and actual time can be established.

Most of the important variables for signal acquisition such as the sensitivity of the photodiode, integration time, and the number of sample points to average are set when the device is programmed. Changing these variables requires that the user change them in the microcontroller's program, recompile the program with the new variables, remove the microcontroller, reprogram the flash memory of the chip, and return the chip

to the device. This process requires some expertise, knowledge of the structure of the program, and understanding of the programming hardware. Although it is somewhat inconvenient, most of the variables are rarely changed for a given application, and their inclusion in the program greatly simplifies the user interface.

One parameter that may change is the sampling interval (the time between sampling events). To facilitate this customization of the device, a set of two DIP switches, each with two states, provides four possible settings. In the current version of the spectrometer, two of these four positions designate a fixed sampling interval: one short and one long (e.g., 1 s and 60 s). When the switch is set to the third switch position, the instrument uses the last value it was sent via its serial connection as the sampling interval. It stores this sampling interval in a fixed location in the EEPROM memory. In the fourth position the device is under direct control via the serial connection. The PC (or other device) to which it is connected may send it a new sampling interval (which would be subsequently used if the switch were set to the third position) or it may initiate a sample event immediately. In this manner the device can operate as a tethered spectrometer with data acquisition and display managed by a spreadsheet or stand alone program running on a PC.

2.4 Experimental

The spectrometer was designed to work with immobilized reagents such as redox and pH indicators. The performance of the cell and spectrometer was characterized with simple colored solutions rather than reagent films because it is easier to prepare a series of solutions with known concentrations. The redox indicator dye thionine was selected as the analyte since it is of interest in other redox monitoring applications (11). Figure 2-5 shows the relationship between the emission profiles of the LEDs selected as light sources and the absorption profile of thionine.

The absorbance of the flowing colored solution was measured with a HP diode array spectrometer (Hewlett Packard 8452A) and the SLIM spectrometer at the same time. The HP spectrometer serves as a reference for benchmarking the SLIM spectrometer. As illustrated in Figure 2-6, a peristaltic pump (Alitea) was used to circulate the solution from a 250-mL reservoir, through a in-house flow cell (3-mm path length) positioned in the HP spectrometer, the flow cell of the SLIM spectrometer, and back into the solution reservoir. The flow rate was ~2.5 mL/min.

At the beginning of the experiment, the reservoir was filled with 150 mL of deionized water from a Millipore ion exchange system and continually stirred with a magnetic stir bar. This water was circulated for ~5 min and trapped gas bubbles were removed from both flow cells. A reference spectrum was then collected and stored on the HP spectrometer and all subsequent values measured by this instrument were stored as absorbance. The HP instrument was configured in kinetics mode and set to monitor four wavelengths (400, 552, 600, and 700 nanometers) at a 10-s interval.

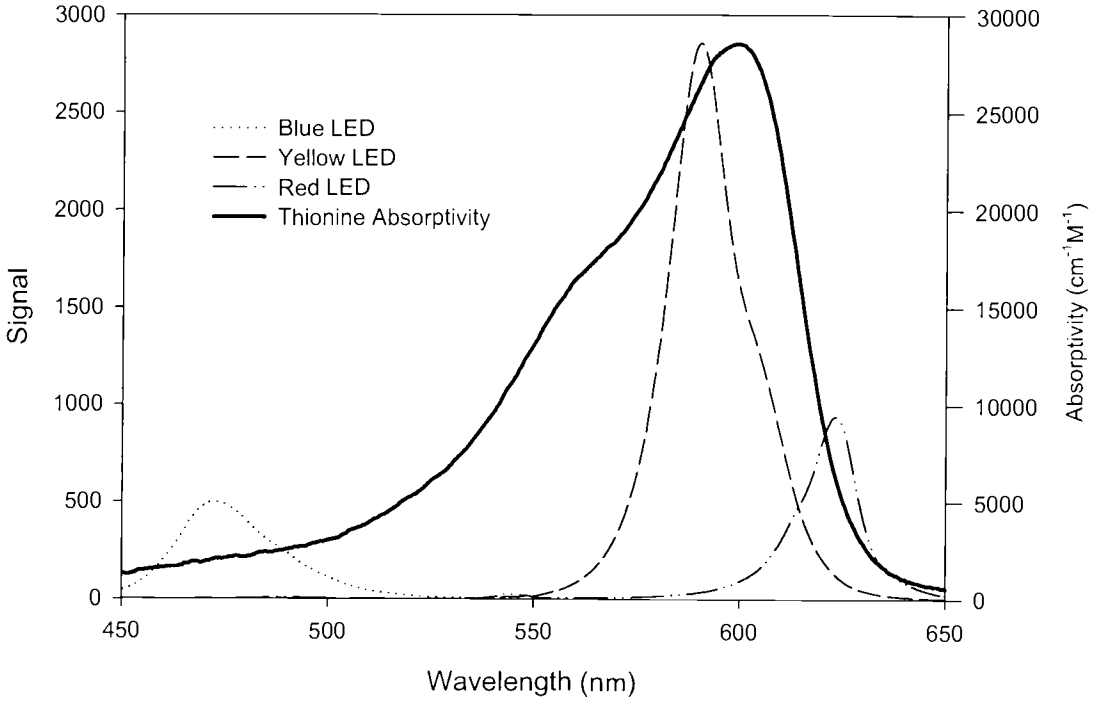


Figure 2-5. Profiles of the emission of the LEDs and of thionine absorptivity. The LED emission profiles were obtained with a CCD type spectrometer and are not corrected for the grating efficiency or the detector responsivity. All LEDs were T 1 ¾ (5 mm) size. The blue LED (λ_{max} at 473 nm) was operated with a 22 kΩ resistor at approximately 0.2 mA of current. The yellow LED (λ_{max} at 590 nm) was operated with a 470 Ω resistor at approximately 10 mA of current. The red LED (λ_{max} at 623 nm) was operated with a 2.2 kΩ resistor at approximately 2 mA of current.

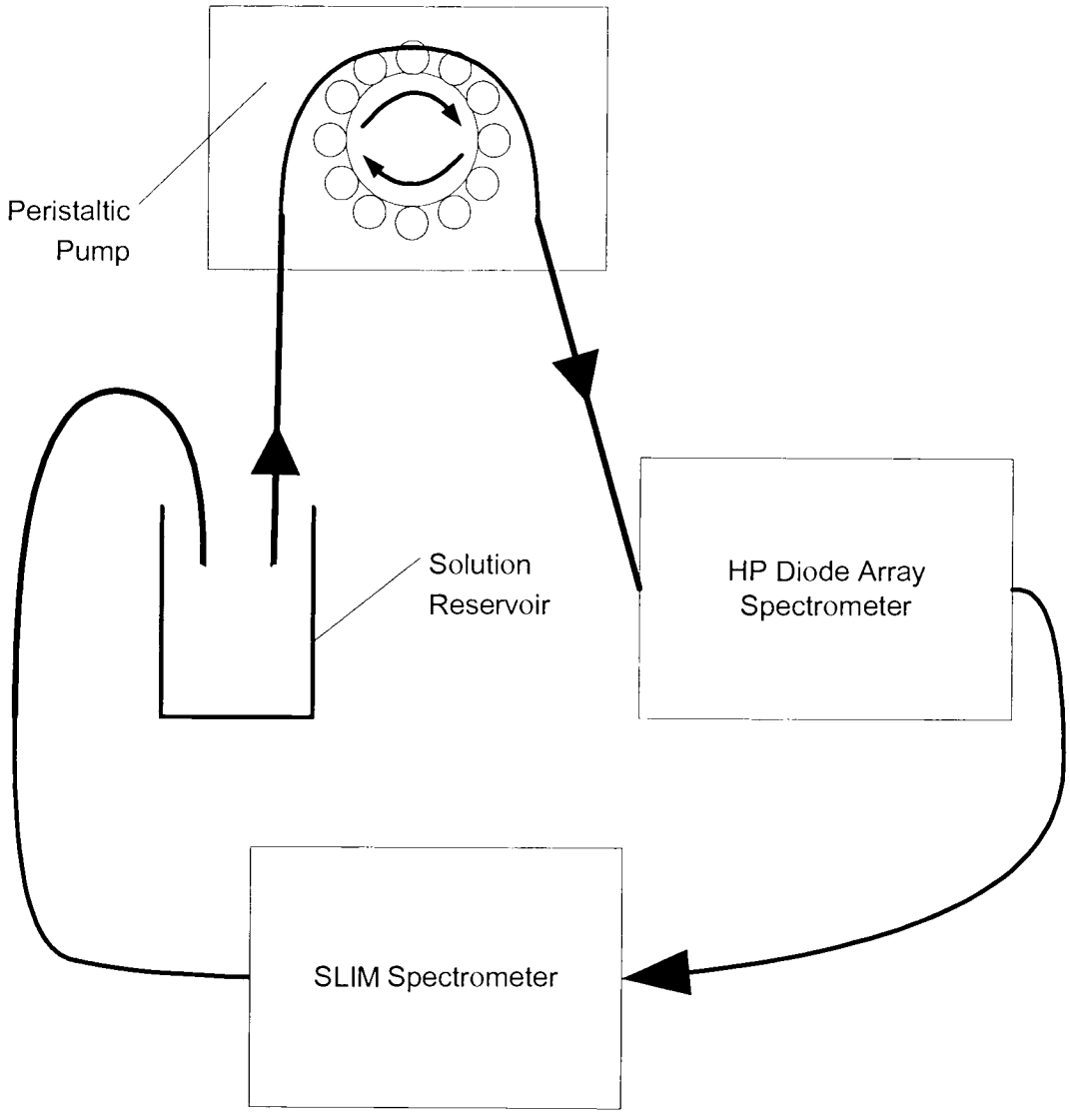


Figure 2-6. Configuration of the tandem calibration experiment

Because the SLIM spectrometer is not configured to calculate absorbance, the data were stored as signals proportional to transmitted intensities. The embedded controller simply counts the pulses output by the photodetector chip for a defined period of time. This quantity is linearly related to light intensity. From the final data set described later, a spreadsheet was used to calculate absorbance (A) from equation 2-1.

$$A = -\log\left[\frac{(Sample\ Intensity - Dark\ Intensity)}{(Blank\ Intensity - Dark\ Intensity)}\right] \qquad (2\text{-}1)$$

The average of the first five signal values with each LED were used as the blank intensity in all absorbance calculations.

The SLIM spectrometer was configured in the data-logging mode and set to monitor the transmitted intensities (counts from the photodetector) of three LEDs (blue, yellow, and red) and the dark signal (all LEDs off). The current limiting resistor for each LED was adjusted until the responses with each lamp were similar. With a 100-ms integration time, the dark signal was measured first, followed by that of the three LEDs in succession. This cycle was repeated ten times until each quantity was measured for 1 s and the total measurement time was 4 s. This period was followed by a 10-s (adjustable via the DIP switches) sleep time in which the instrument enters a low-power consumption mode. The modulation during data acquisition of the sources provides some suppression of 60-cycle noise and other non-fundamental shifts or drift in the background ambient light level. The spectrometer was also wrapped in black felt to further reduce the noise associated with fluctuations in room light.

Both spectrometers were activated simultaneously so that they marked the same initial time. Once the experiment was underway, 100-μL to 250-μL aliquots of 2 mM

thionine were added to the reservoir at approximately 2.5-min. intervals. The aliquots were added with an Eppendorf pipette to a continuously stirred reservoir. No effort was made to be precise about the concentration of the solutions because absorbance was continually measured by the HP spectrometer. This incremental addition of colored compound allowed for 40 distinct concentrations of analyte to be measured several times each by both spectrometers in an experiment with a 100-min duration. The absorbance at the absorption band maximum (as measured by the HP spectrometer) ranged from 0 to 2 AU over the course of the experiment as the thionine concentration increased from 0 to ~300 μM. At the end of the experiment, the data stored in each instrument were saved to a computer disk and imported into a spreadsheet.

2.5 Results and Discussion

Figure 2-7 shows time dependence of the measured photodetector signals with the three LEDs and the dark signal as the absorbance increased due to the periodic addition of thionine. Because it takes time to completely flush the flow path and sample cells and achieve a uniform composition throughout the system, the concentration of the analyte had not stabilized during data acquisition immediately following the addition of thionine. The derivative of the dark-corrected signal with respect to time was used as a criterion to eliminate data points in which the signal was dramatically different from the previous signal. Because fewer measurements were taken with the SLIM spectrometer and a one-to-one correspondence was desirable, only the data points from the HP spectrometer that matched up closely (5 s or less) in time with the SLIM spectrometer were included in the final data set. In summary, data points taken at times when the concentration of analyte was changing or when the HP acquisition time did not match the SLIM acquisition time were removed. After the removal of these points, approximately 250 data points remained. From these remaining values, the absorbances were then calculated with equation 1 and are shown in Figure 2-8.

Figure 2-9 shows the calibration curves based on the absorbances measured with the HP spectrometer (3-mm path length) at two wavelengths and with the SLIM spectrometer (1.5-mm path length) with three different LEDs. The absorbance at 552 nm (about one half that at the band maximum at 600 nm) measured with the HP spectrophotometer was used to estimate the thionine concentration in the flow cells at a given time. Because the absorbance at the band maximum (600 nm) exhibits non-linearity at higher absorbances, it was not used to define the concentration axis. The

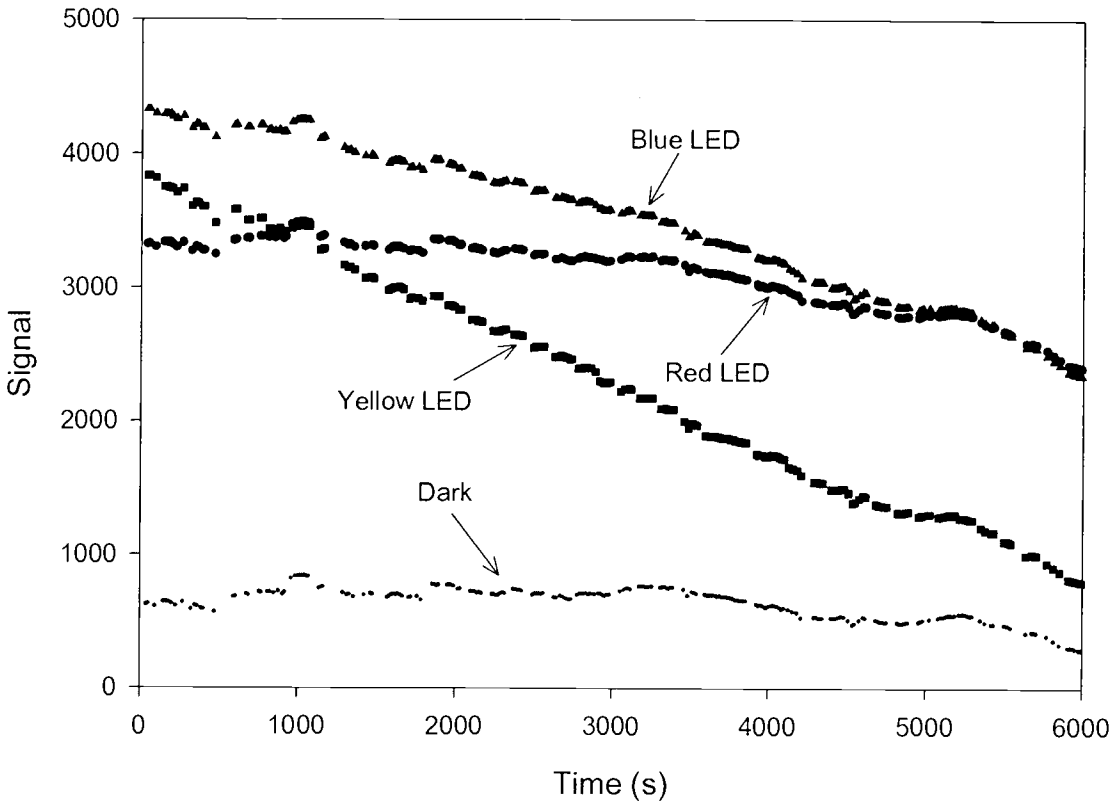


Figure 2-7. The time dependence of the uncorrected photodetector signals measured with the SLIM spectrometer.

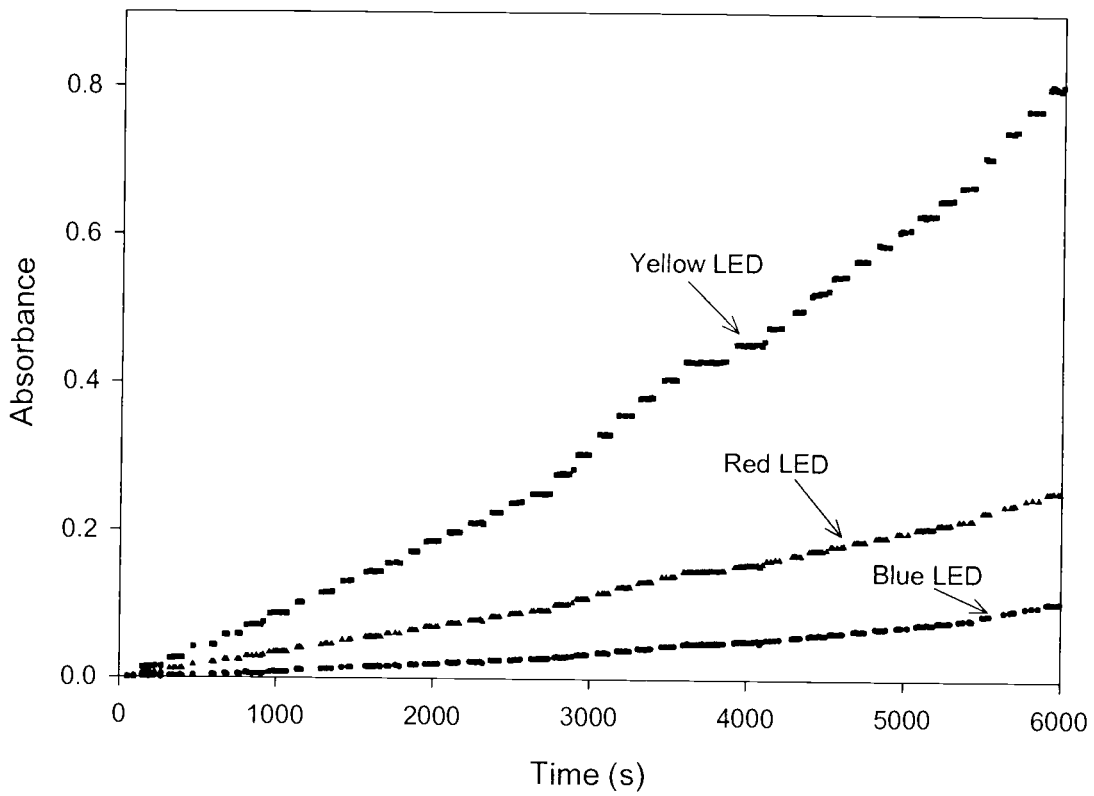


Figure 2-8. Time dependence of the absorbance measured with LED light sources.

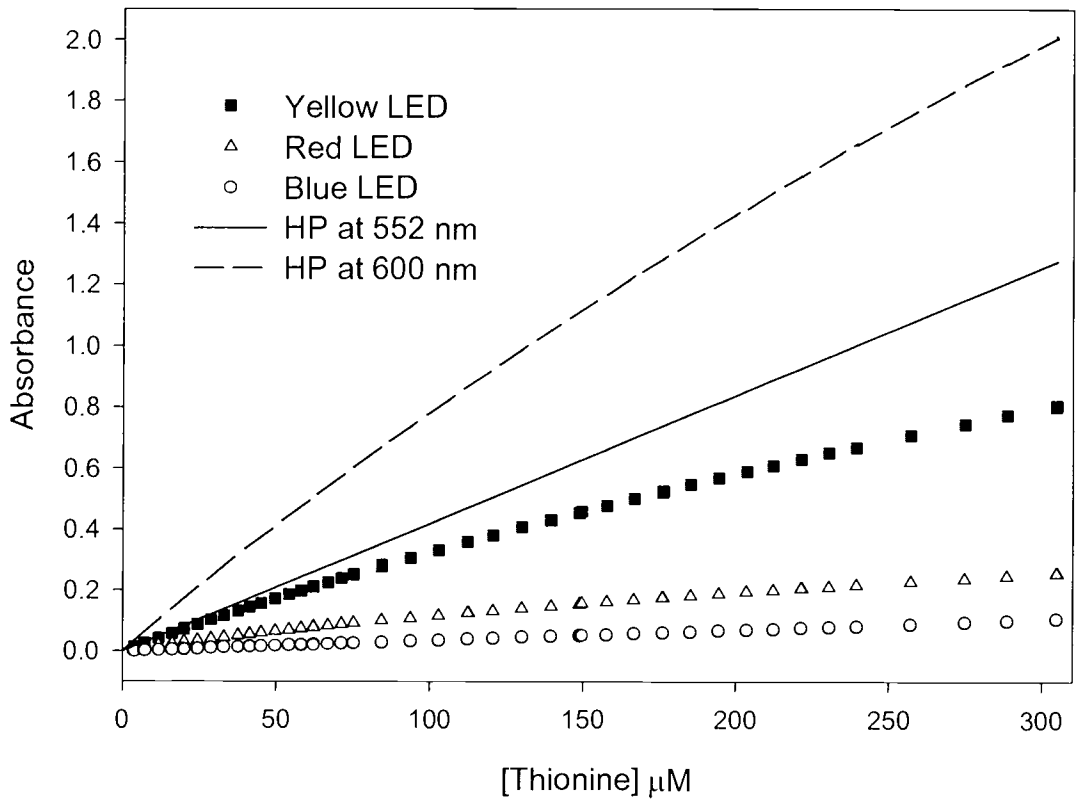


Figure 2-9. Calibration curves with the HP spectrometer monitoring different wavelengths and with the SLIM spectrometer with different LED light sources. The initial calibration slopes are as follows: HP at 600 nm, 0.0089 AU/μM; HP at 552 nm, 0.0042 AU/μM; yellow LED, 0.0036 AU/μM; red LED, 0.0016 AU/μM; blue LED, 0.0003 AU/μM. The path length of the SLIM spectrometer is half that of the HP spectrometer.

absorptivity at 552 nm was determined from the absorbance previously measured at 552 nm with the HP spectrometer, a 1-cm path length cuvette and a 20 μM solution. A separate calibration curve obtained for thionine standard solutions at 552 nm in a standard 1-cm sample cell exhibited good linearity up to an absorbance of 1 or more. The calibration curve measured with the HP spectrometer at 552 nm is linear because this wavelength was used to define the concentration axis. Due to the difference in path length and the polychromatic nature of the LEDs and their often non-optimal overlap with the absorption band of thionine (Figure 2-5), the initial slopes observed for the calibration curves are a factor of 2 to 20 smaller than the slope of the calibration curve for the HP spectrometer obtained at the band maximum. The initial slopes with the HP spectrometer (600 nm) and the SLIM spectrometer with the yellow LED light source are very similar if the path lengths are normalized.

In Figure 2-10, the differences in non-linearity can be more directly compared because each of the calibration curves is rescaled so that all slopes at low concentrations (0 to 10 μM) are identical. The non-linearity for all conditions (except 552 nm) is apparent at concentrations above approximately 50 μM. Note that the curve derived from the HP spectrometer at the band maximum (600 nm) shows a significant deviation from linearity. Because the LEDs used in the SLIM spectrometer emit over a fairly broad wavelength region (the width at half height is ~25 nm), deviations from linearity due to polychromatic radiation are expected. This non-linearity is a direct result of the nature of the shape of the absorbance profile within the envelope described by the LED's emission. Those LEDs that emit over a wavelength region where the absorptivity changes dramatically (red and yellow) give rise to more extreme deviations from

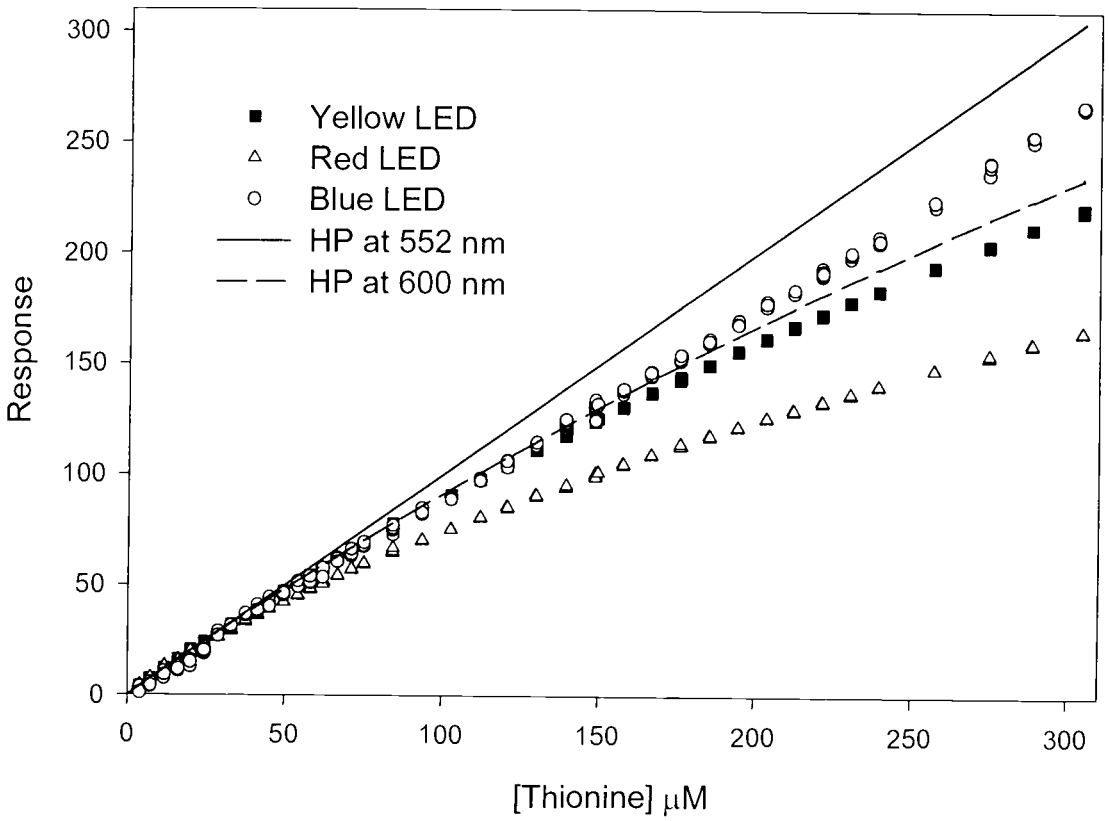


Figure 2-10. Calibration curves re-scaled to demonstrate non-linear behavior. The response of the HP spectrometer is the measured absorbance divided by the absorptivity at that wavelength (in mm^{-1} μM^{-1}) and the 3-mm path length. The LED- based absorbances are scaled so that the response at 7.5 μM equals 7.5.

linearity than the LED that emits in a region where the absorptivity is relatively constant (blue).

To model these non-linear calibration curves, equation 2-2 was employed where E_λ is the "emission-response profile" of an LED and ε_λ is the molar absorptivity profile of the absorbing species.

$$A_{predicted} = -\log\left[\frac{\sum_{\lambda} 10^{(-\varepsilon_\lambda bc)} E_\lambda}{\sum_{\lambda} E_\lambda}\right] \qquad (2\text{-}2)$$

The "emission-response profile" and molar absorptivity profile were determined with a CCD spectrometer (Ocean Optics) from 400 nm to 800 nm with each data point per pixel corresponding to approximately a 0.3-nm range. Because the photodetectors (photodiode and CCD) have different responsivities and the response of the CCD spectrometer is affected by the grating efficiency, the true "emission-response profile" of the LED spectrometer is somewhat different from that assumed for the calculation in equation 2.

These calculations were performed for the blue, red, and yellow LEDs used in this spectrometer and results are given in Figure 2-11. For the yellow and red LEDs, the experimental deviation from linearity is significantly greater than the theoretical deviation from linearity predicted by this model. These differences are attributed to incorrect values of E_λ used in equation 2. The closest match with the calculated values is observed with the blue LED, where the absorptivity of thionine is lowest and relatively flat over the emission profile. The model correctly predicts that the degree of deviation from linearity at the low concentrations occurs in the order red LED source, yellow

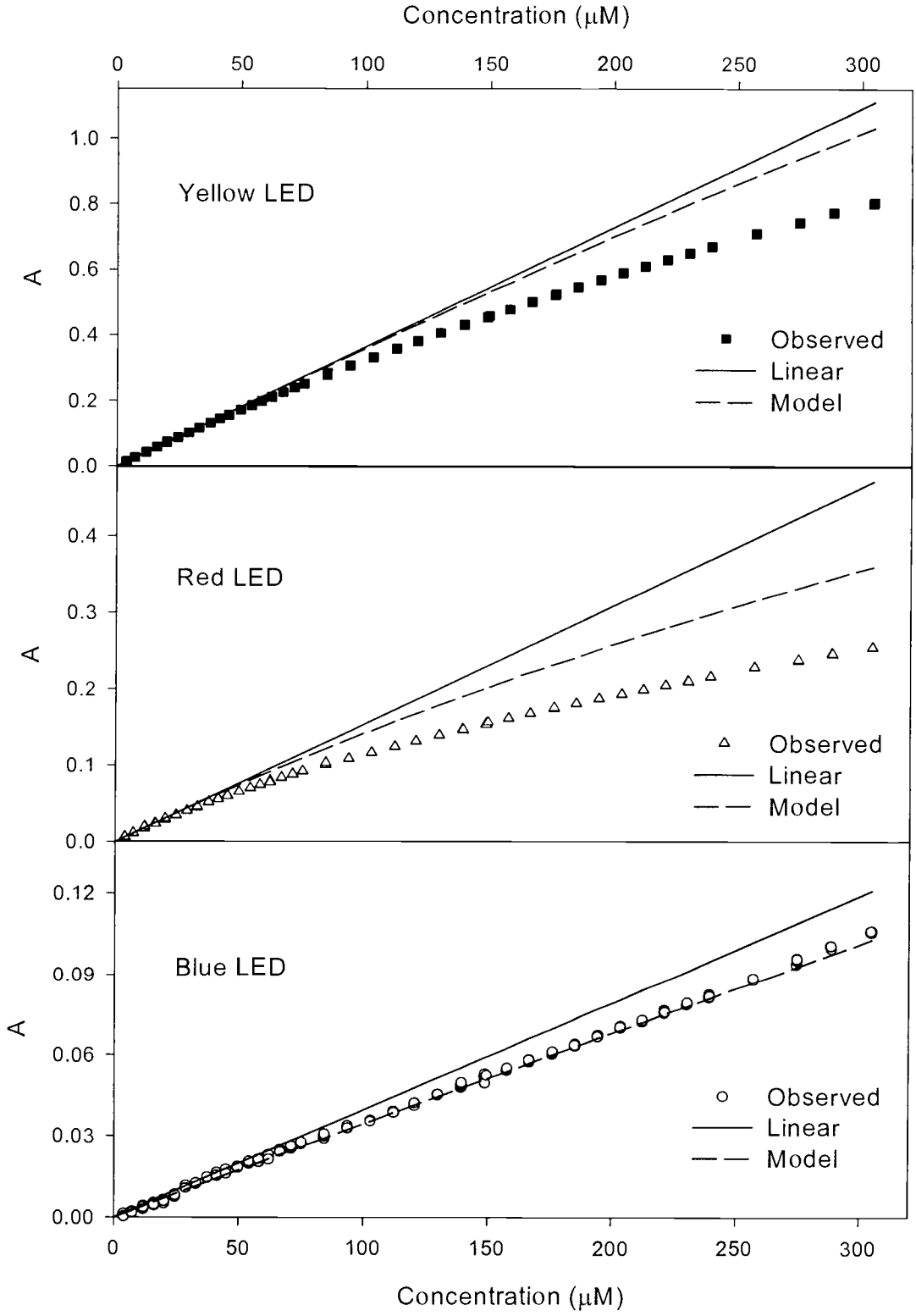


Figure 2-11. Comparison of predicted, ideal, and observed calibration curves with the SLIM spectrometer.

LED, and blue LED. Although this model is useful in understanding the performance of the spectrometer, its primary value is in the selection of a set of LEDs for a given application. Given a molar absorptivity profile of an analyte and the "emission-response profiles" of several LEDs, this model can be used to compare the predicted sensitivities and linear ranges of spectrometers configured with various LED light sources.
The absorbances calculated for a given LED were regressed against concentrations to give a calibration equation that relates the response of the instrument to concentration. Both a first-order fit (without an intercept) and a second-order fit (without an intercept) were performed with the absorbance data from the three LEDs used in this experiment. Plots of predicted concentration against a true concentration axis are presented in Figure 2-12. Because the original calibration curves are nonlinear, the first-order fit exhibits positive systematic deviations at concentrations in the lower half of those concentrations studied and negative deviations from the true value for the higher concentrations studied.

The standard calibration errors of the concentration estimates are compared in Table 2-2. This figure of merit indicates the agreement between the values predicted by substituting the SLIM spectrometer data into the respective calibration equations and the true concentration of thionine. Clearly the second-order fit is preferred because it provides a lower standard error than the first-order fit for measurements over a large range of concentrations. Note that useful calibration information can even be obtained with a very poor match between the LED emission and analyte absorption. For example, in this application the blue LED source can be used to produce a calibration curve with a standard error of 2-μM thionine in a cell with only a 1.5 mm path length.

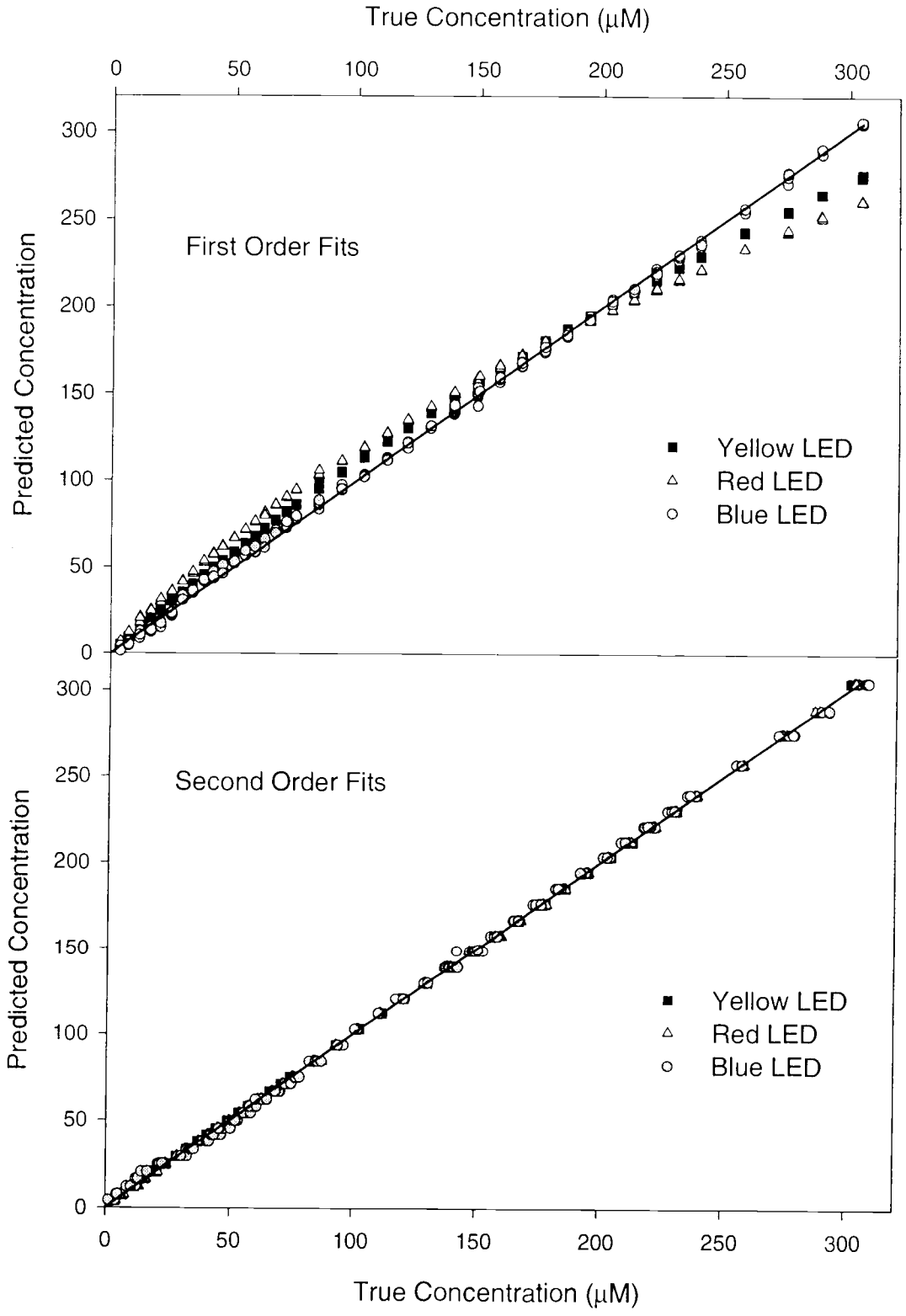


Figure 2-12. Comparison of first-order (A) and second-order (B) calibration equations.

Table 2-2. Comparison of errors associated with different fitting techniques and sources

	Standard Error (μM)		Calibration Coefficients	
Source or Wavelength	**First-Order Fit**	**Second-Order Fit**	**First-Order Fit [a]**	**Second-Order Fit [b]**
Yellow LED	9.7	0.6	343	263, 143
Red LED	16.0	0.8	1020	609, 2290
Blue LED	2.4	2.3	2880	2830, 795
HP 600 nm	8.1	0.3	140	113, 19.2

[a] based on an equation with the form

$c\ (\mu M) = \text{Coefficient} * A$

[b] based on an equation with the form

$c\ (\mu M) = (\text{First Coefficient} * A) + (\text{Second Coefficient} * A^2)$

The precision of the absorbance measurement of the LED based spectrometer was compared to the precision of the HP diode array spectrometer for this simple system. Twenty concentrations were measured multiple times (4 to 19) and the standard deviations of the measured absorbances were calculated. The standard deviation was independent of the absorbance in all cases. The pooled standard deviations obtained with both spectrometers are summarized in Table 2-3. Clearly the blank noise of the SLIM spectrometer in this application is comparable to that of a commercial diode array spectrometer. The blank noise is greater than expected with a diode array spectrometer because measurements are made on a flowing stream over a long time period.

The detection limit depends on the ratio of the blank noise to the calibration slope at low concentrations. For this application the detection limit for thionine with the yellow, red, and blue LEDs in the SLIM spectrometer is estimated to be worse by about

Table 2-3. Comparison of the precision of commercial and SLIM spectrometers [a]

Source or Wavelength	Pooled Standard Deviation (mAU)
Yellow LED	0.68
Red LED	0.46
Blue LED	0.47
HP at 400 nm	0.18
HP at 552 nm	0.33
HP at 600 nm	0.42
HP at 700 nm	0.24

[a] These standard deviations are pooled together according to Equation 2-3 where N is the number of determinations in a group and s is the standard deviation of the group

$$s_{pooled} = \left(\sum_a (N_a - 1) s_a^2 \Big/ \sum_a N_a - d \right)^{1/2} \qquad (2\text{-}3)$$

a factor of 2, 4, and 13, respectively, relative to the 0.12 μM detection limit with the HP spectrometer at the absorbance maximum of 600 nm (based on twice the pooled standard deviation of the signal at several concentrations divided by the initial calibration slope). This loss in detectability is a result of the lower calibration curve slope realized with the LED light sources emitting over a broad range of analyte absorptivities. It is possible to place a small diameter absorption filter in front of the LED to narrow the emitted wavelength range and thus increase the calibration slope and improve the detection limit.

In the design of the SLIM spectrometer, selectivity, detection limit (calibration slope), and linearity are somewhat compromised in order to gain simplicity, low cost, small size, low power consumption, remote data logging capability, and versatility. Its

performance is still quite suitable for many applications where the monitored species is a dominant absorbing species in the sample with a concentration well above the typical detection limit. The SLIM spectrometer is sufficiently sensitive to accurately measure a 0.005 AU change. Although the linear range with LEDs having broad emission profiles is limited, the data are easily calibrated with a second-order equation, and much of the dynamic range is preserved.

2.6 Conclusions

A new class of absorption spectrometer has been described which makes use of modern semiconductor technology. We have demonstrated that this suite of components can produce quantitative information and remain inexpensive, small, and adaptable. The focus of this paper is to outline the fundamental components and characterize their performance for a simple application. The device is not intended as a bench-top instrument (although its low cost makes it attractive for educational purposes) and this work only illustrates some of the advantages of the design. In other more specific applications the benefits of this approach are more clearly realized. The current physical construction is designed around a flow cell for easy laboratory bench-top evaluation, but the units are intended to be re-configurable for a particular determination and setting. For example, the same technology could be the basis for the unattended monitoring of an environmental contaminant or a dedicated industrial process monitor.

There are several significant differences between the SLIM spectrometer and LED-based spectrometers previously described in the literature. In this new design the small size, low power consumption, and simplicity of the LED light source is well matched to the other components of the spectrometer. The result is a complete, self-contained, and compact unit about the size of bath soap capable of unattended data collection over a period of weeks or months. There is no attempt to duplicate a high performance spectrometer in terms of wavelength resolution or noise. Instead, the intended application is one where a dominant absorbing species is monitored and baseline noise levels around 1 mAU are sufficient. For example, the SLIM spectrometer has been shown to work with redox and pH indicators immobilized on films (Appendices

A and F), and instruments based on the approach are now being tested for evaluating redox conditions in groundwater.

This same basic design has been used to construct a spectrometer that accepts a standard 1-cm path length cell. In another application under development, both the source and detector are moved to the same side of the sensor to create a device capable of measuring the reflectance of a sample in a sealed container (Chapter 4 of this thesis). Several of these units can be stacked on top of each other in a solid housing that can be driven into the ground or lowered into a well for depth profiling of environmental parameters (Chapter 3 of this thesis). The entire unit could be encapsulated and placed inside a closed container where the measured data are transmitted outside the container via light or radio waves. The unit is also capable of controlling a miniature pumping system, and with the proper reagents, a very small flow injection system can be constructed.

2.7 References

1. Dasgupta, P. K.; Bellamy, H. S.; Liu, H.; Lopez, J. L.; Loree, E. L.; Morris, K.; Petersen, K.; Mir, K. A., *Talanta*, **1993**, *40*, 53-74.

2. Liu, Hanghui; Dasgupta, P. K., *Anal. Chim. Acta*, **1994**, *289*, 347-353.

3. Jambunathan, Sivakumar; Dasgupta, P. K.; Wolcott, D.K.; Marshall, G. D.; Olson, D.C., *Talanta*, **1999**, *50*, 481-490.

4. Hauser, P. C.; Rupasinghe, T. W. T.; Cates, N. E., *Talanta*, **1995**, *42*, 605-612.

5. Hauser, P. C.; Rupasinghe, T. W. T.; Tan, R., *Chimia*, **1995**, *49*, 492-494.

6. Huang, J.; Liu H.; Tan, A.; Xu, J.; Zhao, X., *Talanta*, **1992**, *39*, 589-592.

7. Worsfold, P. J., Clinch, J. R., *Anal. Chim. Acta*, **1987**, *197*, 43-50.

8. Clinch, J. R., Worsfold, P. J., *Anal. Chim. Acta*, **1987**, *200*, 523-531.

9. Clinch, J. R., Worsfold, P. J., *Anal. Chim. Acta*, **1988**, *214*, 401-407.

10. Philips Semiconductor. http://wwwus.semiconductors.philips.com/acrobat/various/I2sbus.pdf (published 1995)

11. Lemmon, T. L.; Westall, J. C.; Ingle, J. D. Jr., *Anal. Chem.*, **1996**, 68, 947-953

Chapter 3: The SPEAR

3.1 Abstract

A new device, here denoted a ***S***ub-surface ***P***robe for the ***E***valuation of ***A***naerobic ***R***egions or SPEAR, was developed. The SPEAR is inserted directly into the ground or sediment and automatically records depth-resolved redox information in real time. The device is based on membrane sampling devices or "peepers" that allow subsurface samples to be collected without changing the redox speciation of sensitive species. The SPEAR further extends the capabilities of this sampling method by submerging immobilized redox indicators into the cavities behind the membranes. Miniature spectrometers incorporated in the probe measure the absorbance of the redox indicators immobilized on clear membranes to track the redox status in situ. The device was tested with the redox indicator thionine in a waterlogged soil in the Willamette Valley of Oregon. The reducing region of the pore water was observed over a two and a half week period in late March. Both depth and time resolved changes in the redox status of the soil in real time were observed with minimal perturbation of the sample.

3.2 Introduction

In this paper we introduce a sampling/measurement device that combines a unique established sampling technique with a new measurement technique for redox status. The sampling methodology is based on subsurface membrane sampling devices or "peepers" to collect environmental samples with minimal perturbation to anoxic systems. The analytical information is obtained with redox indicators immobilized in a form that allows for direct spectrometric evaluation of the redox status of aqueous samples.

3.2.1 Redox status and redox indicators

Redox transformations are among the most important environmental processes. These transformations are the basis of energy generation and the formation of ATP in all living organisms. Redox status is important in understanding the fate and transport of inorganic and organic species in the environment (1). Several methods can be used to evaluate redox status (2). Often, redox status is defined operationally according to the dominant terminal electron acceptor (TEA). Microbial levels include aerobic conditions and anaerobic conditions such as nitrate-reducing, iron-reducing, and sulfate-reducing. These microbial states are often inferred from the presence of certain TEAs or their reduction products. For example, the presence of a significant concentration of Fe(II) is indicative of Fe(III)-reducing conditions but not positive proof.

Due to its ease of use, the Pt electrode ("redox electrode") is the most popular and convenient sensor for measuring the redox status of environmental systems. However, numerous studies (2, 3, 4, 5) indicate that the Pt electrode provides inconsistent results, and the absolute value of its potential (E_{Pt}) cannot generally be correlated to concentrations of specific redox-active species or the dominance of a particular microbial process (6, 7). For example, Fe(III)-reducing conditions have been observed over a wide range of E_{Pt} values (generally +100 to -100 mV although as high as +600 mV and as low as -200 mV).

In our laboratory (2, 8), we have evaluated the suitability of numerous redox indicators for environmental monitoring (e.g., reversibility with a variety of reductants/oxidants, coupling to a Pt electrode, and formal potential at pH 7 ($E_7^{(0)}$)). These multiple-ring, organic compounds are usually colored in their oxidized form and colorless in their reduced form, allowing for monitoring of their redox speciation spectrophotometrically. For example, the reduction of blue thionine to a colorless form can be monitored at 600 nm.

These positively-charged hydrophobic indicators adsorb to minerals and soils. To make them applicable to environmental samples, we have developed methods to immobilize them in forms that can be monitored spectrophotometrically, that do not leach out, and that are accessible to reductants and oxidants in the water sample (2, 8, 9). The absorbance of the immobilized indicator is related to the fraction in the oxidized form. This ratio of fraction oxidized to fraction reduced can be related to the "redox potential" by the Nernst equation.

Results obtained in environmental samples strongly support the notion that Fe(II) is a product and the primary reductant of thionine under Fe(III)-reducing conditions, and S(-II) is a product and the primary reductant of cresyl violet under sulfate-reducing conditions (2). The relationship between indicator speciation (fraction oxidized) and reductant concentration is consistent and correlates fairly well to values calculated from equilibrium models (10). Two indicators define a redox window or zone. For example, both thionine and cresyl violet can be reduced by sulfide, but only thionine is reduced by Fe(II) under Fe(III)-reducing conditions.

3.2.2 Equilibrium diffusion chambers

The physical construction of the SPEAR device is based on equilibrium diffusion chambers or "peepers" originally described by Hesslein (11). These devices, constructed from materials and designed with a shape that allows then to be driven into the ground, typically consist of a vertical series of chambers filled with deionized water and separated from the soil or sediment by a semi-permeable membrane. Over time the solution in the chamber is allowed to equilibrate with the pore water present in sediment. After a sufficient equilibration period, the device is removed, and each chamber contains a water sample that is "representative" of the non-transient species that can diffuse thought the membrane at that sediment or soil depth.

Several researchers have taken advantage of equilibrium diffusion chambers or "peepers" as a means to collect pore water samples in situ. This in-situ technique is a preferred sampling method because it avoids the changes in concentration of pore

water species that can occur when the collection temperature or pressure is different from in situ conditions (12). Of primary concern when manipulating these samples is avoiding exposure to atmospheric oxygen and the associated changes in speciation of redox-sensitive species (13). Bandl and Hanselmann (14) prefer the equilibrium diffusion technique to centrifugation, squeeze-water, pressurized cores, and suction for microbiological and biogeochemical work. Steinmann and Shotyk (15) successfully used this method to acquire samples and perform a complete analysis of all the important inorganic species including redox-sensitive species such as Fe(II) from two peat bogs in Switzerland. They compare "peepers" with other sampling techniques and conclude the advantages of this method include: samples are not degassed or oxidized, sample treatment is not required before analysis, sampling with spatial resolution of the order of 1 cm is possible, and environmental perturbation is minimized (13).

Membrane sampling methodology has some limitations. The unsaturated zone cannot be effectively sampled. The sampling process is a one-time event and does not capture transient changes in pore water composition.

3.2.3 The SPEAR device

In a preliminary proof-of-principle experiment (unpublished), redox indicators immobilized on filter paper were placed directly in the chambers of a traditional sampling peeper. After equilibration (approximately two weeks in a water-saturated soil), the device was withdrawn into a transparent glove bag purged with N_2. As the chambers were then opened, the color of the redox indicators was observed while the samples were withdrawn for later analysis. Both thionine and cresyl violet were reduced in chambers near the surface, and thionine (but not cresyl violet) was reduced in chambers at greater depth.

Several difficulties were encountered during this initial field study. Of primary concern was the oxidation of the indicators in the glove bag during the time required for sample collection (~30 min for 16 chambers) because of atmospheric oxygen leaks or incomplete purging. Also, the degree of reduction of the indicators by simple visual inspection could not be even semi-quantitatively determined, primarily because of a lack of a reference color. In addition there was no record of the state of the indicators before the sampling device was removed. These observations provided the impetus for developing a new method for quantitatively observing the indicators while they were still in the ground.

The SPEAR (***S***ub-surface ***P***robe for the ***E***valuation of ***A***naerobic ***R***egions) device functions as a spectrometer array that can be driven into the ground to provide depth-profile information that is time-resolved. Each chamber in the probe contains a redox indicator immobilized on a clear substrate that is held between a light source

and a photodetector so that the light transmission of the indicator can be measured. The control electronics are housed in a water-resistant enclosure, connected to the probe via an interface cable, and based on a clock, memory, and an embedded microcontroller chip. The instrument is configured to perform and store transmission data for the redox indicator substrate in each chamber at a programmable data collection rate.

The SPEAR was used to evaluate the redox status of a waterlogged site at a grass seed farm in the Willamette valley of Oregon. The measurements were taken with a ~25-min time interval over a span of two weeks during March of 2000. The centers of the disks of the immobilized redox indicator thionine were spaced 1 in apart at depths of 2 to 7 in.

3.3 Instrumentation

3.3.1 Physical construction

The deployment of the SPEAR in the field is illustrated in Figure 3-1. The probe is driven into the ground and is connected to the control electronics in a watertight enclosure at the surface. The inset shows the general dimensions of the probe and the orientation of the LEDs and photodetectors in each of the six sampling cavities.

A more detailed drawing of the probe itself is provided in Figure 3-2. The body of the sampling device was constructed from a 1-in thick sheet of acrylic. The sample cavities are 0.5-in diameter holes drilled 0.6-in deep into the 1-in sheet. The cavities are connected by a vertical channel cut perpendicular to the cavities, which accommodates a strip of opaque black acrylic that holds the six indicator disks. When this indicator strip is inserted, the internal volume of each sample chamber is approximately 1.5 mL.

The indicator strip has six countersunk holes drilled 1-in apart. The indicator material is cut into disks with a 0.375-in punch and placed inside the recesses cut in the strip. Each disk is then held in place by a Delrin ring which press fits into a recess in the indicator strip. The strip is easily removed to allow for changing of the indicators. When the strip is in place, each chamber of the device is isolated (the channel that connects them is filled by the strip) and contains a 0.25-in diameter

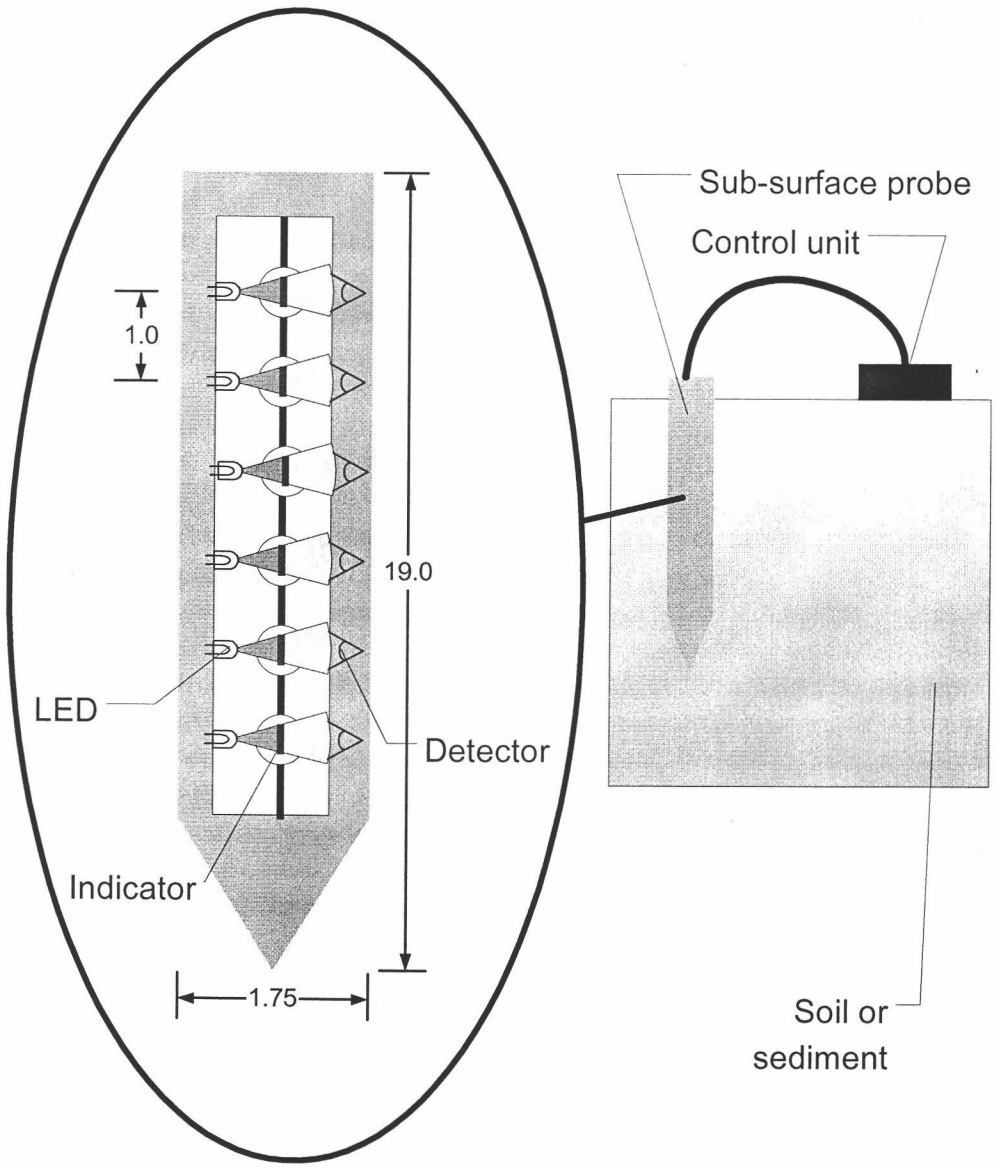


Figure 3-1. An illustration of the SPEAR device in operation. All dimensions are in inches.

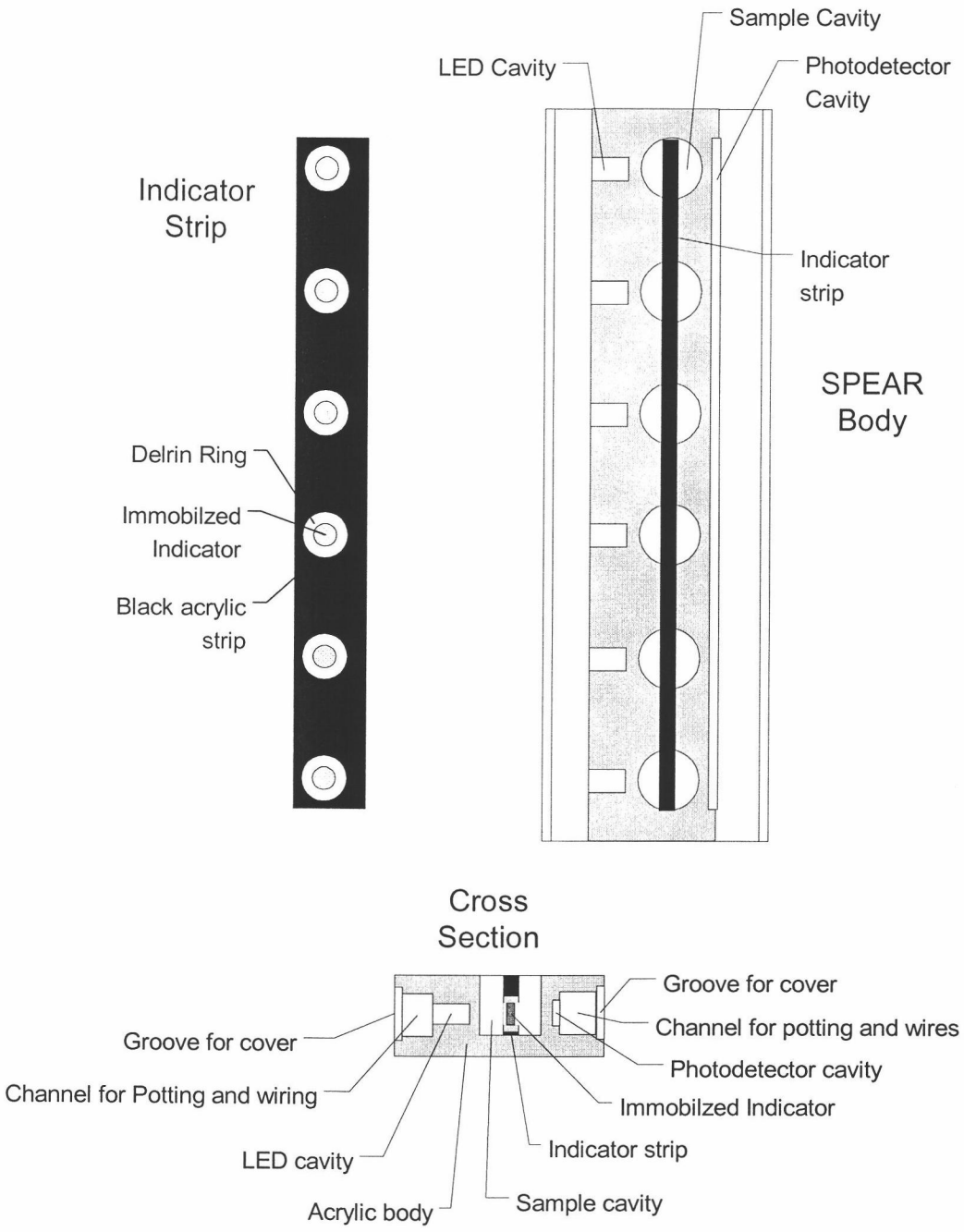


Figure 3-2. An illustration of the physical construction of the SPEAR device. The indicator strip is 5.5-in long and 0.125-in thick. The six countersunk holes are drilled with the centers 1-in apart. The holes are a 0.25-in in diameter and the countersinks are a 0.375-in in diameter and extend approximately half way through one side. The Delrin rings with a 0.375-in outer diameter and a 0.25-in inner diameter press fit into the countersinks to hold the indicator disks in place.

porous disk of immobilized indicator held perpendicular to the chamber opening and exposed to the water that diffuses into the chamber.

As illustrated in Figure 3-3, one of the sides of the sampling probe (the left side in the drawing) contains the cavities for T1 ¾ (5 mm) LED light sources. These six LEDs, spaced 1-in apart, fit into holes (0.185-in diameter and 0.3-in deep) drilled in the body of the probe. Above the holes (toward the outside edge of the body) is a groove that accommodates the wires from the LEDs. After the device is wired and tested, this chamber is filled with RTV100 series silicone rubber sealant (General Electric, Newark Electronics). The potted chamber is then closed with an acrylic cover plate that is glued in place after the silicone has cured.

The other side of the sampling device (the right side of the SPEAR body in Figure 3-3) contains the photodetector chips (8 pin DIP). These six light-to-frequency converter chips, spaced 1-in apart, are soldered to a custom circuit board. The chips are aligned in a cavity cut into the body of the sampling device. Above the chips (toward the outside edge of the body) is a groove that accommodates the wires from the circuit board. After the device is wired and tested, this groove is also filled with silicone rubber sealant and glued in place after the silicone has cured.

The chamber openings are covered with a strip of polysulfone Tuffryn membrane filter with 0.2 μm pores (Gelman Sciences HT-2000). The membrane is sealed into place by an acrylic cover strip that compresses the filter between it and the body of the probe with eighteen Nylon machine screws (Small Parts, Inc.)

The wires leading to the LED light sources and photodetector chips are brought into the sampling probe from a multiple conductor cable that enters the device

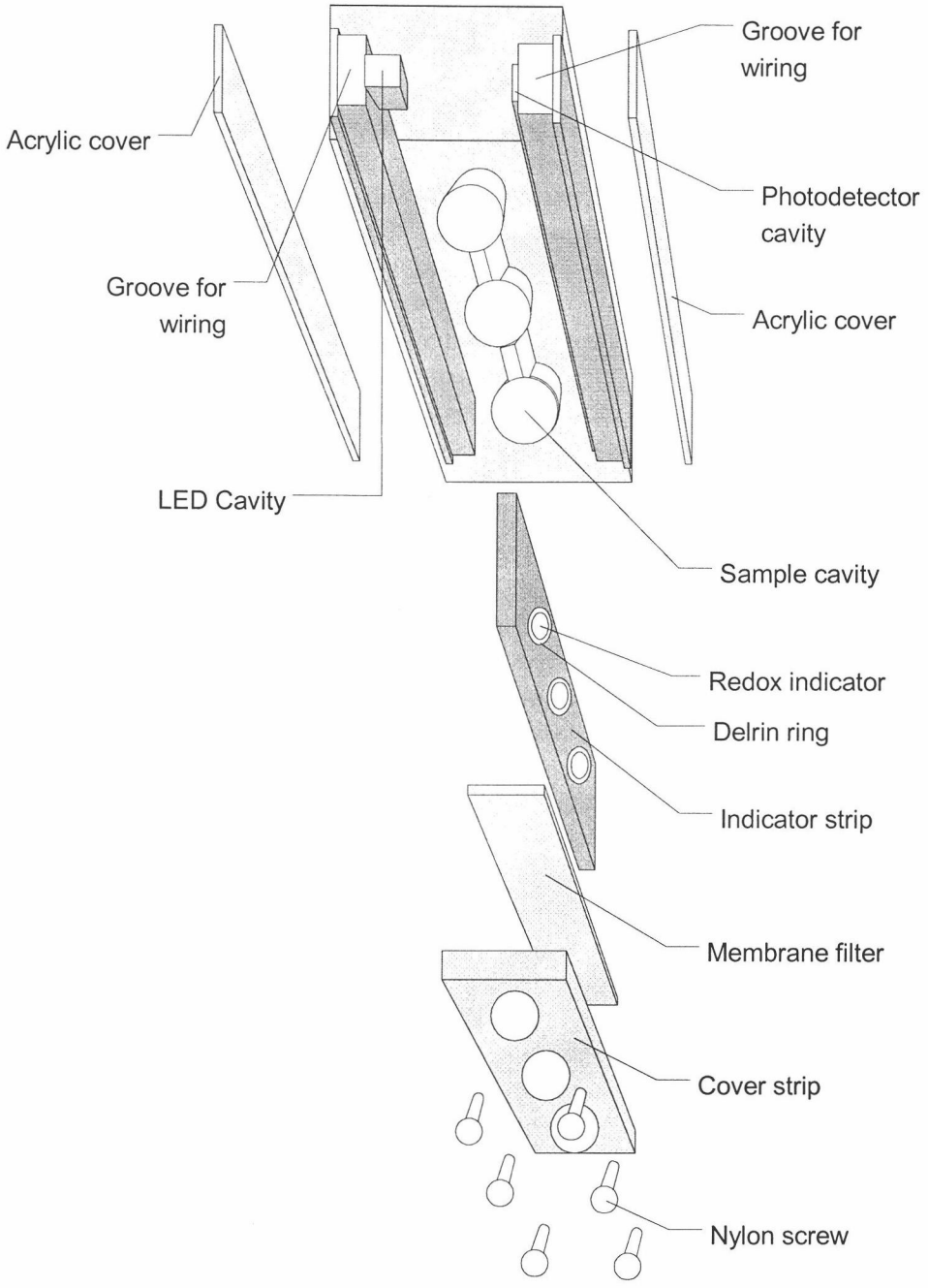


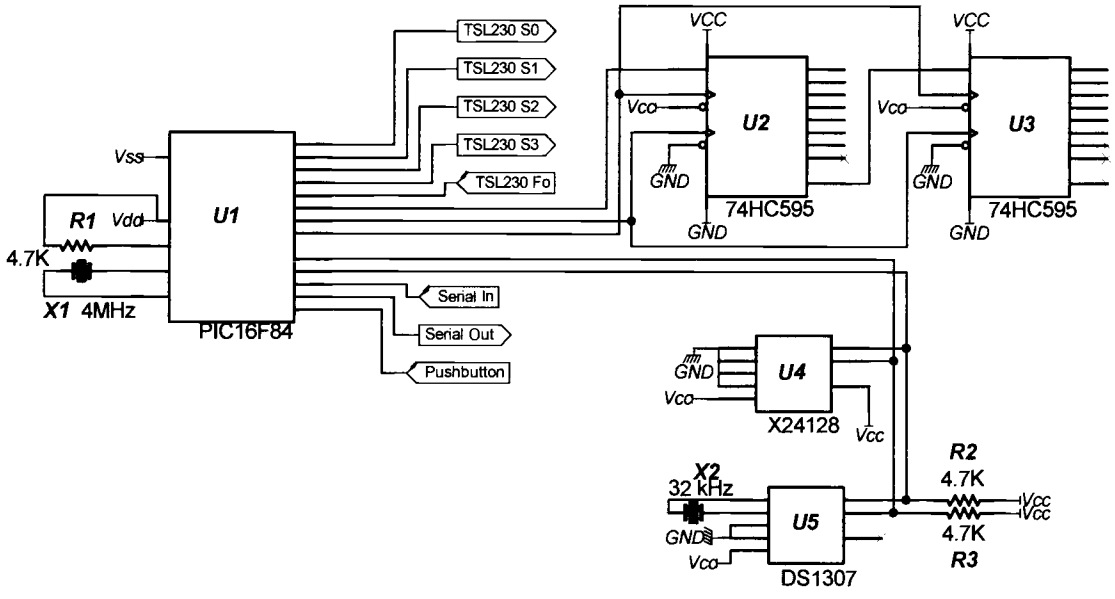
Figure 3-3. An exploded three-dimensional view of the construction of the SPEAR probe. The grooves for the wiring are 0.5-in wide, 0.3-in deep, and 10.5-in long. The membrane filter is cut to be 0.75-in wide and 7.75-in long. The cover strip is 0.75-in wide and 8.5-in long and held in place with eighteen 4*40 by 3/8-in Nylon machine screws.

probe about 1.5 inches from the top on the same surface as the sample chambers. This cable is sealed with silicone rubber and individual wires are routed to their respective connections via a "T" shaped opening in the top of the sampling device.

3.3.2 Electronics

The basic function of the SPEAR is to collect transmitted intensity data from each of the six sample chambers at a prescribed time. An embedded microcontroller controls the LED light sources, measures the output of the photodetector IC, computes a time stamp, stores the data in nonvolatile memory, and enters a low-power sleep mode for a programmed time interval. In addition to data collection and storage, the control electronics also communicate with a PC via a serial connection. All of the electronic components including the light sources, photodetectors, microcontroller, and support ICs are matched to achieve compact size, low power consumption, and reduced cost. Similar electronic components were used in the construction of a SLIM (simple, low-power, inexpensive, microcontroller-based) spectrometer which is a miniature, flow cell spectrometer described previously (16).

A schematic of the circuit is given in Figure 3-4. A custom circuit board was constructed and housed in separate plastic enclosure connected to the probe by a 25-conductor umbilical. The primary electronic components used to achieve the functionalities outlined above are an 8-bit embedded microcontroller (Microchip Technologies Incorporated PIC-16F84), a 128K-bit serial EEPROM (Xicor X24128) for nonvolatile data storage, a serial real time clock (Dallas Semiconductor DS1307)



Label	Part number	Function
U1	PIC16F84	Microcontroller
U2, U3	74HC595	Serial to parallel
U4	X24128	Serial EEPROM
U5	DS1307	Real time clock
X1	4 MHz	MCU crystal
X2	32 kHz	RTC crystal
R1, R2, R3	4.7 kΩ	Pull-up resistors

Figure 3-4. A schematic and parts list of the SPEAR control electronics. Serial to parallel IC U2 drives the LED light sources and U3 controls the photodetectors.

for timekeeping, and a photodetector (Texas Instruments TSL230). The photodetector is based on a photodiode and current-to-frequency conversion and features programmable gain control. The circuit is powered by four AA batteries with an expected service time of more than two months when data are collected every half hour.

The program for the microcontroller was written in a high level BASIC programming language and compiled into assembly instruction with the PIC Basic Pro compiler (MicroEngineering Labs Inc). This program is stored in the flash memory of the microcontroller.

The 16F84 microcontroller has only 13 I/O pins, which are not sufficient to control the six two-color LEDs along with addressing the six photodetector chips. An 8-bit serial to parallel shift register (Phillips 74HC595) was used to expand the I/O capability of the electronics. Two of these 8-bit chips were cascaded to provide 16 additional control signals. The first chip controls the LEDs and the second addresses the photodetectors.

The two color diffuse red/green LEDs used in the device (Jameco XC5491 34711) are bi-state parts with two electrical leads. This LED was selected because its emission profiles are well matched to two immobilized indicators we have found useful for immobilized monitoring as shown in Figure 3-5. The polarity must be reversed to change the color of the LED. Thus when both leads are logic 0 or logic 1 the LED is off, when lead A is logic 1 and lead B is logic 0 the LED is green, and when lead A is logic 0 and lead B is logic 1 the LED is red. To control the six LEDs

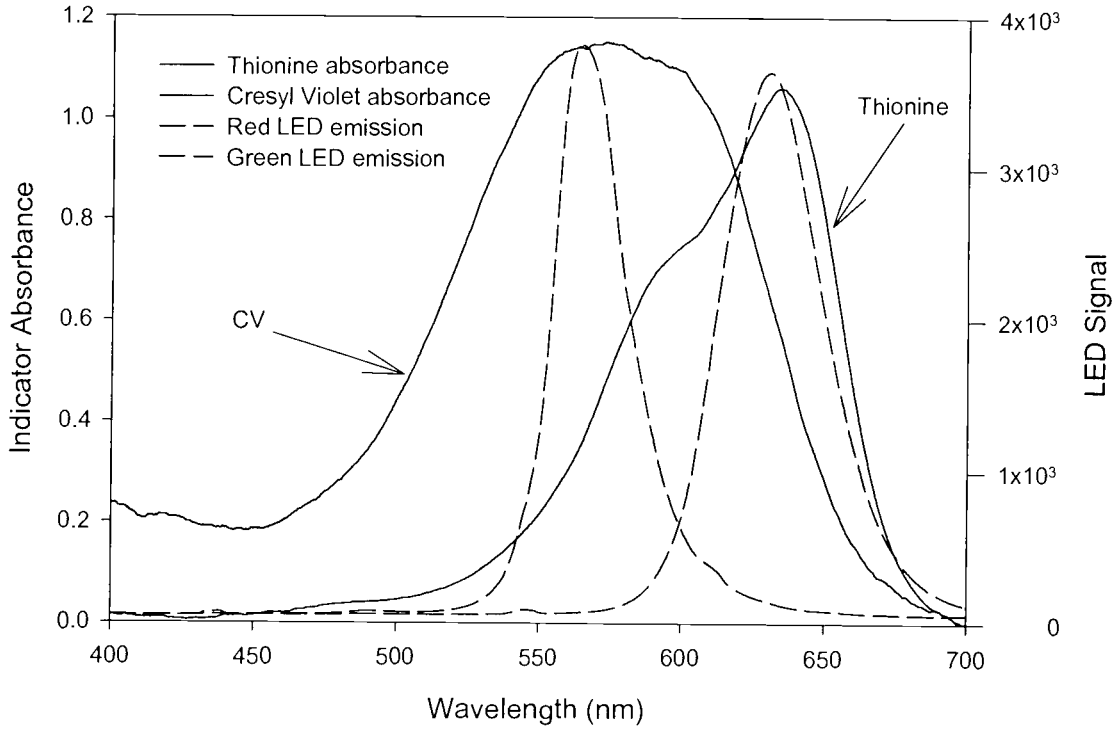


Figure 3-5. LED emission and indicator absorbance. The absorbance spectra of thionine and cresyl violet immobilized onto dialysis membranes were obtained with a HP 8452A diode array spectrometer and normalized to a maximum absorbance of ~1 AU. The LED emission signals were measured with an Ocean Optics S2000 spectrometer and are dependent on the grating efficiency and CCD responsivity of that instrument.

with seven signals, it was necessary to connect one lead of each of the LEDs together to a single I/O line. To cycle each LED through the OFF, RED, GREEN, the timing sequence shown in Figure 3-6 is employed where the individual control lines are shown at the top, and the signal shown at the bottom of the trace is the one common to all of the LEDs.

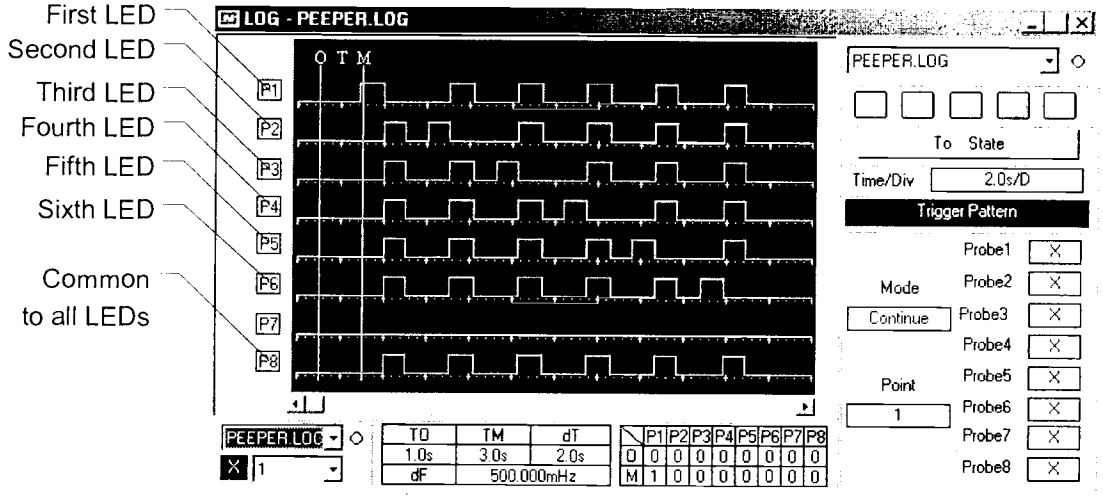


Figure 3-6. Timing diagram of the LED control signals. The pulse widths are 1 s. The red LED is powered on when its individual lead is logic one and the common lead is logic zero. The green LED is on when its individual lead is logic zero and the common lead is logic one.

3.3.3 Operation

When the SPEAR is powered on, the internal clock is reset to midnight of day 1. The operator must note the time and add this base time to all of the recorded time stamps. The control electronics sends all stored data out to the serial connection, and

it then begins polling the state of the pushbutton switch that initiates data collection. If this button is pushed and the device is simultaneously turned on, all data stored in the memory are cleared; otherwise all data are appended to the existing data.

Once in the data collection mode, the device writes the address of the next usable memory location to a fixed address in the EEPROM. It then reads and stores the number of days, minutes, hours, seconds that have elapsed since the device was powered. Each LED is then sequentially powered for 1 s and the photodetector signal is recorded (for the ambient, red LED illuminated, and green LED illuminated cases). The photodetector signal is represented by TTL frequency output of the light-to-frequency converter IC and related to the transmitted light intensity. Finally the device enters a low power consumption mode, which it exits to initiate a new round of data collection after a programmed period of time (20 min).

The ambient, red LED, and green LED signals are each stored as 2-byte values, and there are six such chambers in the spectrometer array (36 bytes total). Along with the time stamp, the data block associated with each sampling event requires 40 bytes of storage. The 16K byte EEPROM is capable of storing over 400 blocks of data, or approximately one week's worth of data with a 25-min data collection interval. This memory can easily be expanded by a factor of 4 by the addition of more ICs.

A Visual Basic program running on a laptop computer in the field that downloads and formats the information from the probe was also written. This program receives information from the device via the serial port and both displays it on the screen and converts it to a functional file format. Because the device is

programmed to send out each new data point via the serial port a laptop computer can be used to monitor the device in the field as the data are collected. The SPEAR can also be used independent of a PC in the field and later returned to the laboratory for downloading the acquired data. The Visual Basic interface program converts the time stamp stored in the device into a form (with units of days) usable by a spreadsheet. The ambient photodetector signal is subtracted from the total photodetector signal for each red or green LED data point. The ambient signal includes the dark signal and the photodetector signal due to extraneous light (e.g., room light, sun light). The data are written to a tab-delimited file that can be opened directly in a spreadsheet. The format of the data file is time, followed by signals for the green LEDs at various depths, signals for the red LEDs at various depths, and finally the ambient signals.

3.4 Experimental

3.4.1 Preparation and deployment of the SPEAR

The redox indicator thionine was covalently immobilized on a dialysis membrane (SpectraPor, Inc.) through a process described in Appendix H and cut with a punch into 0.375-in circles. To prepare the SPEAR for a field experiment, the components were immersed throughout the assembly process in Millipore water in a tray that had been purged with N_2 gas for 30-min. The indicator discs were fit into the indicator strip, and all bubbles were removed before the strip was loaded into the sampling device. The membrane filter was then affixed with the protective cover and nylon screws. The assembled probe was then transferred to a large PVC tube filled with de-aerated water and transported to the field site, which was a grass seed farm in the Willamette Valley of Oregon. The soil is classified as "fine, montmorillonitic, mesic, typic Albaqualf" (17).

At the field site the SPEAR was removed from the PVC container and immediately forced by hand into the waterlogged soil until 4.4 in remained above ground and the center of the first cell was positioned 2 in below the surface of the soil. The control box was placed on a wood stake and pedestal, which was driven into the soil, and covered with a Ziploc bag as an additional moisture barrier. Both the probe and recording electronics were covered with a five-sided wire enclosure to prevent damage from wild or domestic animals on the site.

The SPEAR probe was placed next to three other previously installed conventional membrane sampling devices that were used for sampling the field site for subsequent analysis of the pore water for soluble Fe. These sampling devices (similar to the devices described in the historical section) were left in the ground for approximately three weeks before being withdrawn into an argon filled glove bag. Once inside the glove bag, aqueous samples were withdrawn and preserved by acidification. The Fe concentrations in these samples were then determined by ICP emission spectrometry.

A preliminary download of the data, performed after approximately 72 hours, revealed that strong sunlight at midday was adversely affecting the photodetectors. This effect was most pronounced in the topmost cell where at times the photodetector was saturated. Because the device is constructed of transparent acrylic, more sunlight reached the sampling cavities near the top of the probe and the photodetectors were over or near the upper limit of their output range for the sensitivity settings chosen. An opaque tarp was then installed over the wire enclosure to reduce the amount of sunlight incident on the probe. Data collection continued for the next 14 days with a download and review of the collected data occurring approximately every five days.

3.4.2 Data processing

Because the SPEAR probe records the ambient signal (dark plus extraneous light) and a total signal related to transmitted intensity, data processing with a spreadsheet includes the following basic steps. For each chamber and LED color, any

outliers are eliminated from the data set. Then the ambient signal is subtracted from the total signal (obtained with the red or green LED on) to yield a corrected signal proportional to the transmitted LED intensity. The transmittance is then calculated as the ratio of the corrected signal to a reference signal that is determined at the end of the experiment (to be discussed later) followed by the calculation of absorbance. Finally the absorbance value at time *t* (A_t) is calculated. The maximum absorbance (A_{max}) is calculated as the average of five absorbance values measured during the night before any reduction of the indicators occurred. The absorbance at a given time is then divided by A_{max} and converted to a dimensionless measure of the indicators speciation, f_{ox}.

All downloaded data including photodetector signals and timestamps were compiled in a single spreadsheet, and the time stamps were adjusted to give a contiguous time axis. Data points for which the change in measured signal over the 25-minute interval was greater than ~25% of the previous signal were deleted.

In the laboratory, the indicator disks were removed from the strip that holds them in the probe. The strip without the indicators was reinstalled, and the sampling chambers were filled with water. The ambient and total signals with both the green and red LEDs were measured five times. The signals (corrected for the ambient signals) were averaged together and used as the reference signal for the calculation of the transmittance and absorbance over time during the deployment of the probe.

3.5 Results and Discussion

Figure 3-7 shows the corrected photodetector signals with the green LED source (ambient signal subtracted) and the red LED source (ambient signal subtracted) for all six chambers. Because at the beginning of the experiment the redox indicators are all oxidized (and colored), the signals are small. The signals increase as the indicator at a given depth becomes reduced and more transparent. The signals from the chambers at more shallow depths reverse from this trend and decrease about midway through the experiment due to exposure of the redox indicators to atmospheric oxygen because the water table dropped.

Figure 3-8 illustrates the time dependence of the ambient signal in the shallowest and the deepest sampling cells over the course of the 16-day experiment. The high exposure of the shallowest cell to sunlight is quite evident. The diurnal nature of this ambient light is also clearly discerned. At night the ambient signals are approximately the same for all chambers. The ambient signal was reduced significantly when the tarp was installed over the transparent acrylic probe and prevented photodetector saturation on sunny days.

The time dependence of the absorbance measured with the green and red LEDs at the six depths is provided in Figure 3-9. The decrease in the measured absorbance over time corresponds to reduction of the indicator or more reducing conditions. The later increase in the absorbance observed for the upper three chambers is attributed to oxidation of the indicator caused by a dropping water table. For these chambers, the absorbances at the end of the experiment are greater than at the beginning of the

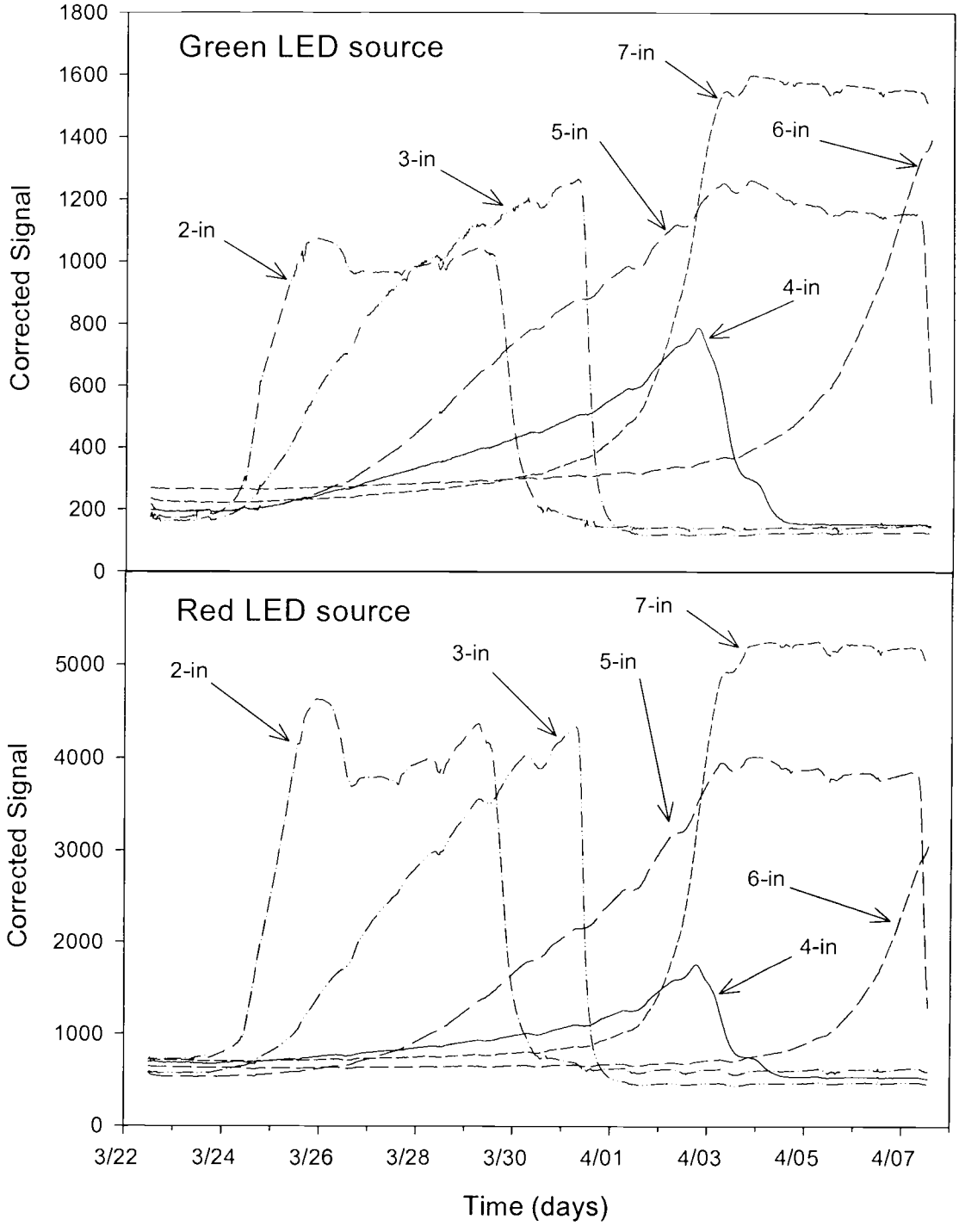


Figure 3-7. Photodetector signals acquired with the green and red LEDs corrected for ambient signal. The signal represents the number of counts accumulated in 1 s due to light from the LED source transmitted by the immobilized indicator.

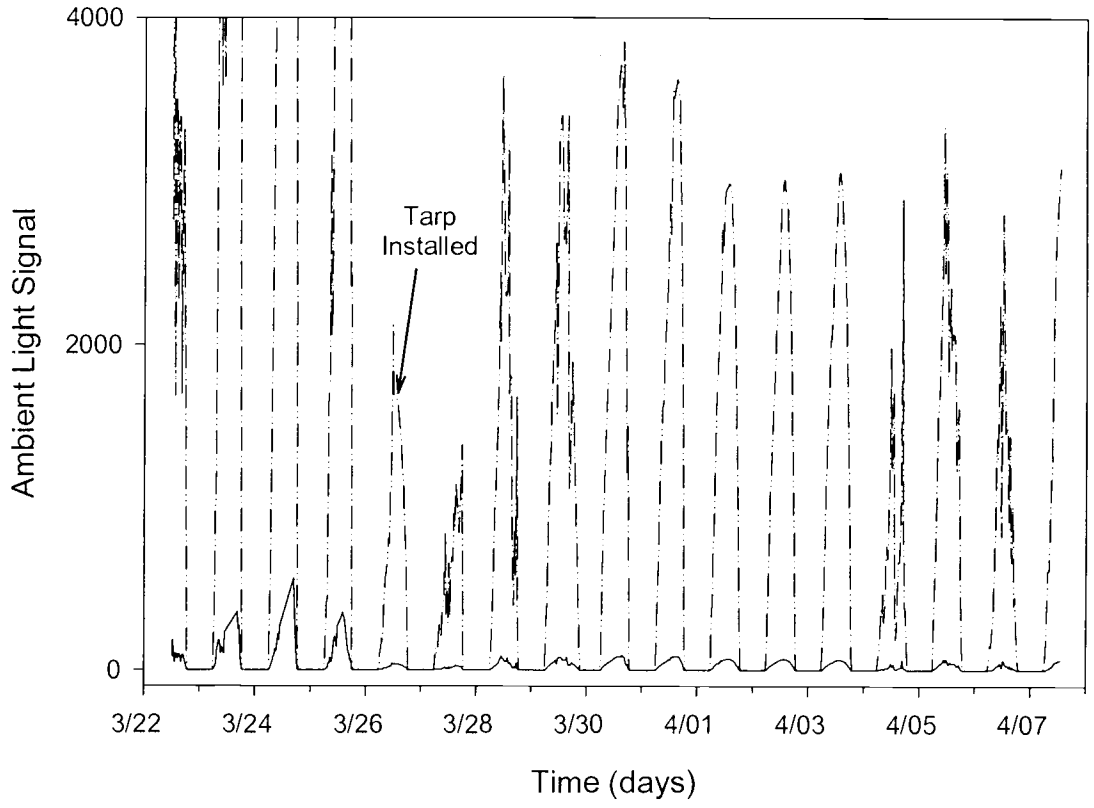


Figure 3-8. Signal acquired with the LEDs turned off. The dashed line is the ambient signal in the first cell at a 2-in depth and the solid line is the ambient signal at a 7-in depth. The diurnal cycle in the data is a result of variations in sunlight. The signal from the 2-in cell up to 3/27 is off scale due to photodetector saturation.

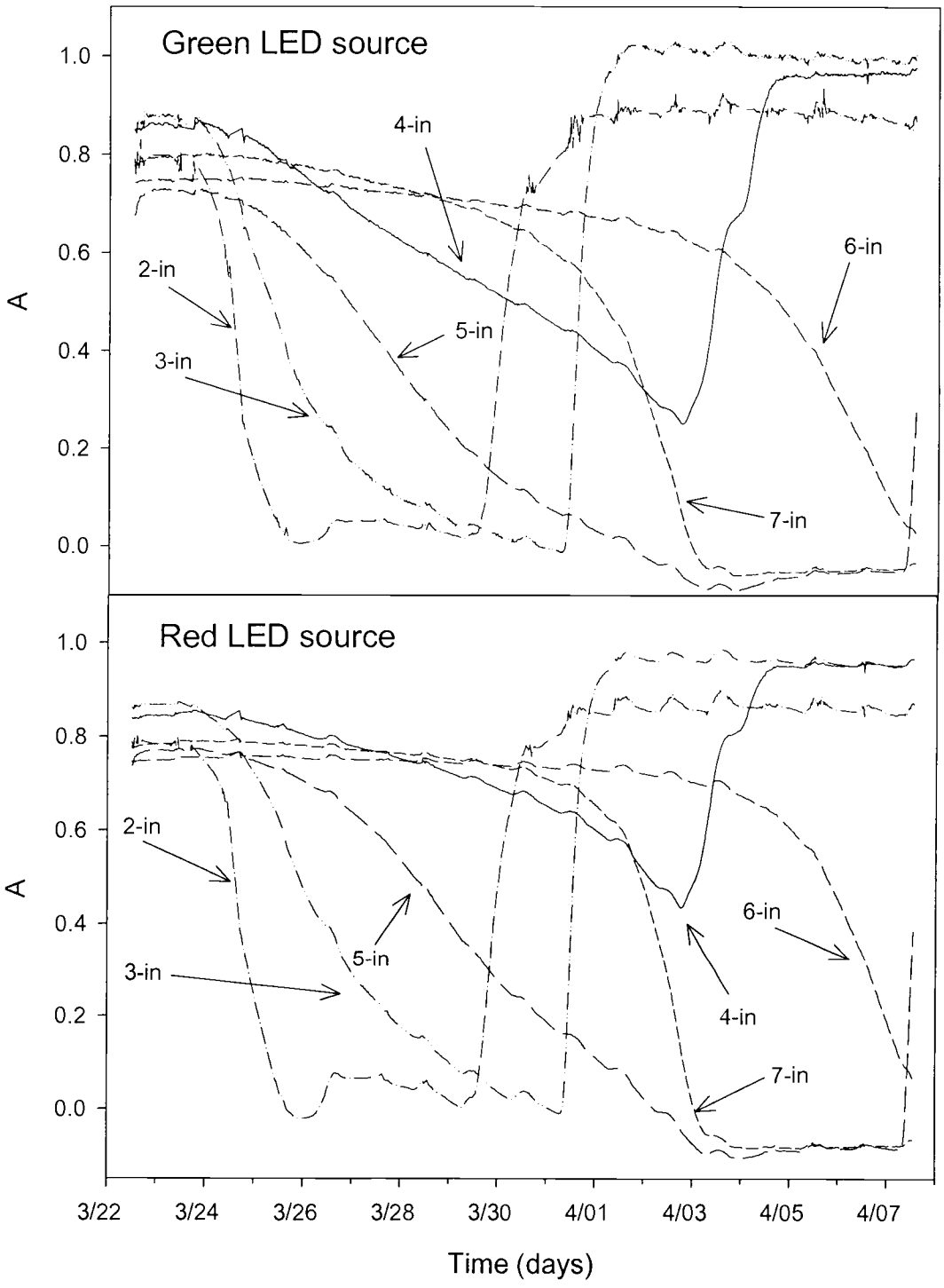


Figure 3-9. The time dependence of the absorbance of the immobilized indicators measured with the green and red LEDs.

deployment. This behavior could be due to the chamber partially draining when the water table dropped. The transmission of an empty chamber is less than that of a water-filled chamber because of increased scattering and reflection at the acrylic/air interfaces.

Note that the absorbances of the redox indicator in a given chamber are similar for both LED light sources. Calculations based on the overlap of indicator absorptivity and LED emission, as seen in Figure 3-6, predict that the absorbance with the red LED should be ~20% greater than that with the green LED (16). The colors of the LEDs were chosen to allow for changing the redox indicator in future experiments. They were not intended as sample and baseline wavelengths for this experiment.

Figure 3-10 shows a plot of the fraction of oxidized thionine at various depths throughout the course of the experiment. Fraction oxidized, or $f_{ox,}$ is the ratio of absorbance of the indicator at time *t* divided by the absorbance in the fully oxidized form. This dimensionless parameter, theoretically ranging between zero and one, is related (by a log transformation) to the "redox potential" of the redox indicator and can be indicative of the "redox status" of the sample and the types of transformations that are occurring (2). As previously noted, at the shallow depths of 3 in and less the indicators became re-oxidized from 7 to 12 days after the insertion of the probe, and f_{ox} reaches values greater than 1.0 due to exposure to atmospheric oxygen and partial draining of the chambers. The order of this re-oxidation (chambers at 2, 3, and 4 in) is consistent with a slowly dropping water table. These plots are very similar to those in Figure 3-9 because f_{ox} is based on absorbance. There are also undulations or ripples in

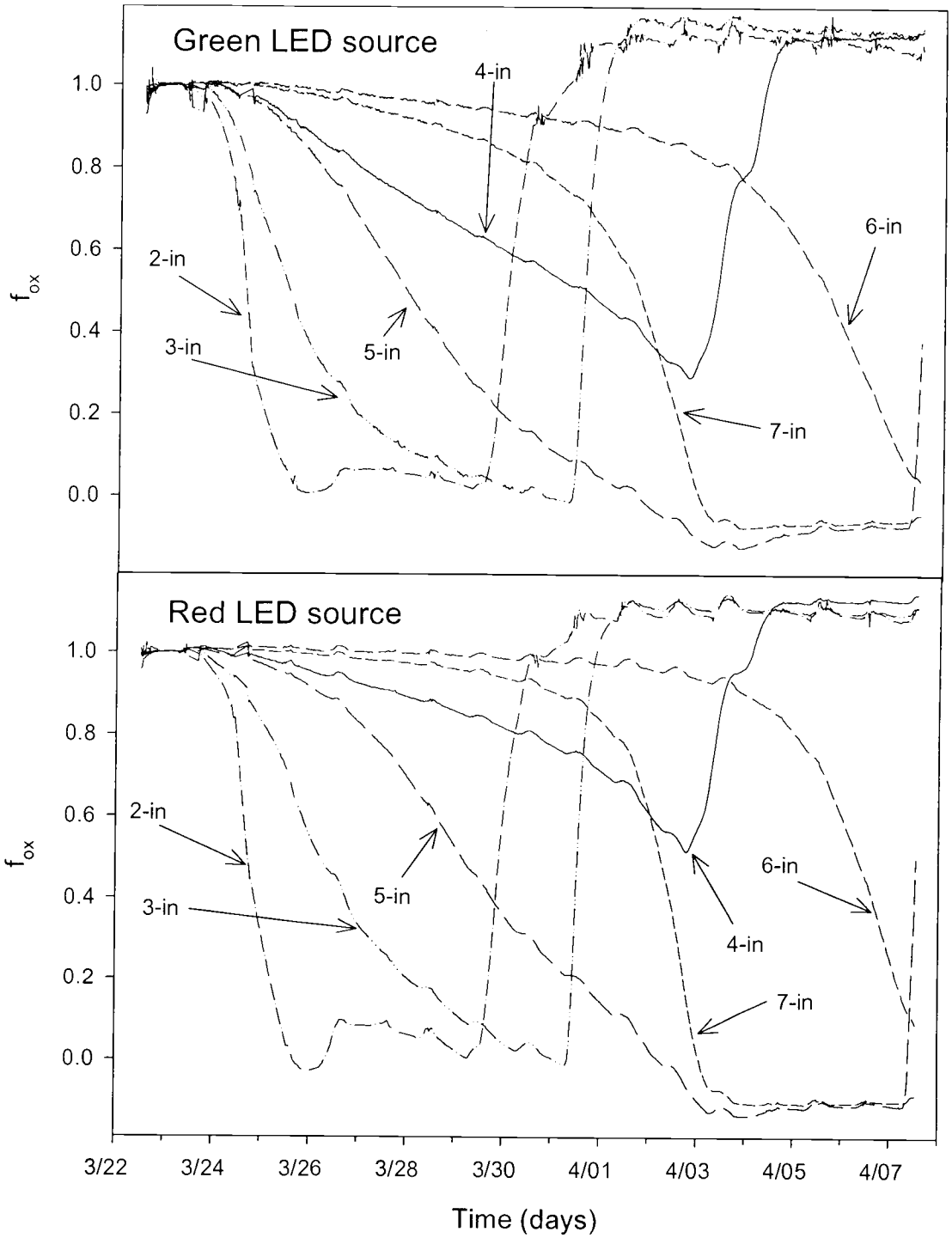


Figure 3-10. The time dependence of the fraction of oxidized indicator with the green and red LEDs.

the measured f_{ox} of thionine in many chambers that occur over time. These undulations in the measured f_{ox} coincide in time with the diurnal changes in ambient light levels.

The three-dimensional plot in Figure 3-11 provides a different perspective about how the redox states of the redox indicators varied with time and depth. The general trend in the order in which the thionine in chambers at various depths becomes reduced suggests that the most reducing region of the pore water migrates to greater depth as time passes. Thionine in chambers at 2 and 3 in was reduced before thionine in chambers at 4 and 5 in, and the indicators in the middle of the probe were reduced before those at the bottom of the probe at 6 and 7 in. In the middle and bottom of the probe, there are minor deviations from this general trend. The indicator at 5 in was reduced before that at 4 in, and the order of reduction was also in reverse order for the indicators at 6 and 7 in. These slight variations in the general trend may be an indication of heterogeneity in the soil composition and structure.

The changes in f_{ox} over time and the apparent movement of the reducing zone to lower depth are complex phenomena. Further study is needed, and only possible explanations can be presented at this time. A redox indicator becomes reduced when a soluble reductant species with a sufficiently negative redox potential reaches a threshold concentration in solution (in the chamber in this experiment). At pH 7, thionine is half reduced (f_{ox} is 0.5) when the soluble Fe(II) concentration is about 100 μM and completely reduced when the sulfide concentration is greater than 1 μM (2). The most probable reductant is Fe(II). During a study of a similar Oregon soil in a

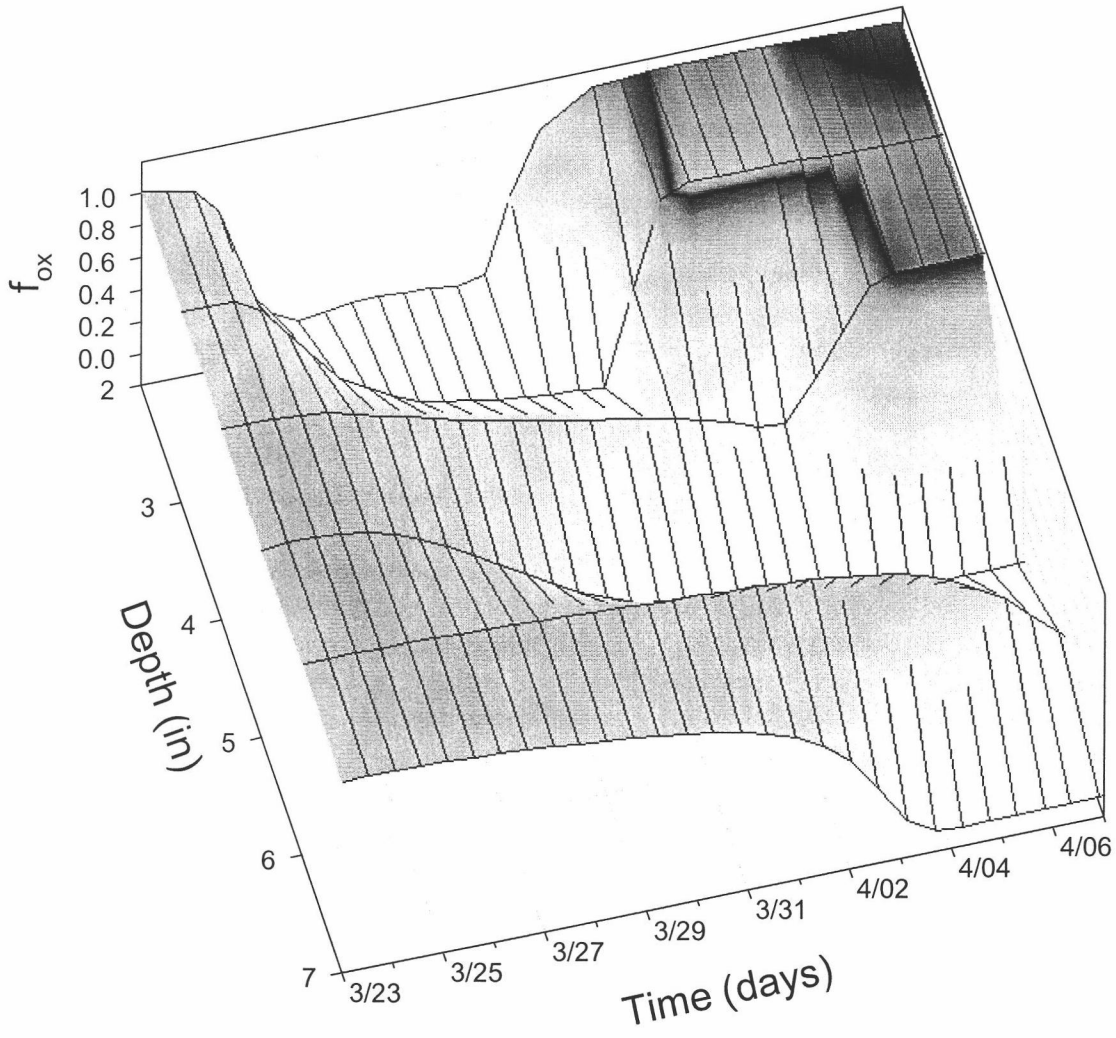


Figure 3-11. A three-dimensional contour plot of the time and depth dependence of the fraction of oxidized thionine (measured with the red LED light source). The dark zone in the upper right corner indicates the time period in which the indicators in the top three chambers became re-oxidized due to the water table dropping.

bioreactor, the reduction of thionine occurred when Fe(III)-reducing bacteria became active and the concentration of Fe(II) increased (8).

The time-dependent decrease in f_{ox} at a given depth is proposed to be due to some combination of two types of factors: i) the response time of the transport process of reductants across the membrane into the chamber, and ii) actual changes in the composition of the solution outside a given membrane and chamber. First consider the response time. If the reductant concentration for a lower depth is half that at a higher depth and the flux is controlled by the concentration gradient, it will take twice as long to reach the threshold concentration (and a particular value of f_{ox}) in the lower chamber because the concentration gradient and transport rate is half. Note that when f_{ox} approaches zero (completely reduced), it does not indicate that equilibrium has been reached, and the reductant concentration inside the chamber may still be less than the concentration outside the membrane.

Now consider changes in the chemical composition of the water outside the chamber due to the dropping water table. One possibility is that the strata of solution with the largest Fe(II) concentration (initially near the surface) descends. Another hypothesis is that the dropping water table brings substrate initially near the surface that stimulates the growth of iron-reducing bacteria at greater depths. It is also possible for redox conditions and reductant concentrations to change at any depth even without the changing water table due to factors which include: a substrate or nutrient being significantly depleted, horizontal groundwater flow, or climatic changes such as rainfall or temperature.

Several observations about the data from the SPEAR suggest that the slight oscillation in the redox state (f_{ox}) of the immobilized thionine is correlated to the diurnal cycle. These features are illustrated in Figure 3-12.

a) When the ambient light level reached a maximum, the reduced thionine became somewhat more oxidized.

b) The oscillations were not observed until some reduction of thionine had taken place (not shown in Figure 3-12).

c) The oscillations continued even after the water table had dropped and thionine became (at least partially) re-oxidized.

d) The oscillations are observed at almost all depths, and their amplitudes are similar.

e) There is no observable lag between the variation in ambient light signal (LED off) and the oscillations in f_{ox}.

Several possible explanations are proposed. A non-chemical explanation of this behavior is that the ambient light correction causes an artifact in the data, and the oscillation in f_{ox} does not reflect a true change in the redox status of thionine or the soil solution. This explanation is not consistent with observations b and d. Chemical explanations include changes in temperature, pH, microbial activity, or photosynthesis. This hypothesis is counter-indicated by observations c and e. Clearly further study is needed to better understand the data.

The distribution of soluble iron at various depths for the three sampling peepers installed at the site is illustrated in Figure 3-13. Iron is usually soluble in a reduced (+2) oxidation state, but forms insoluble $Fe(OH)_3$ complexes in its more

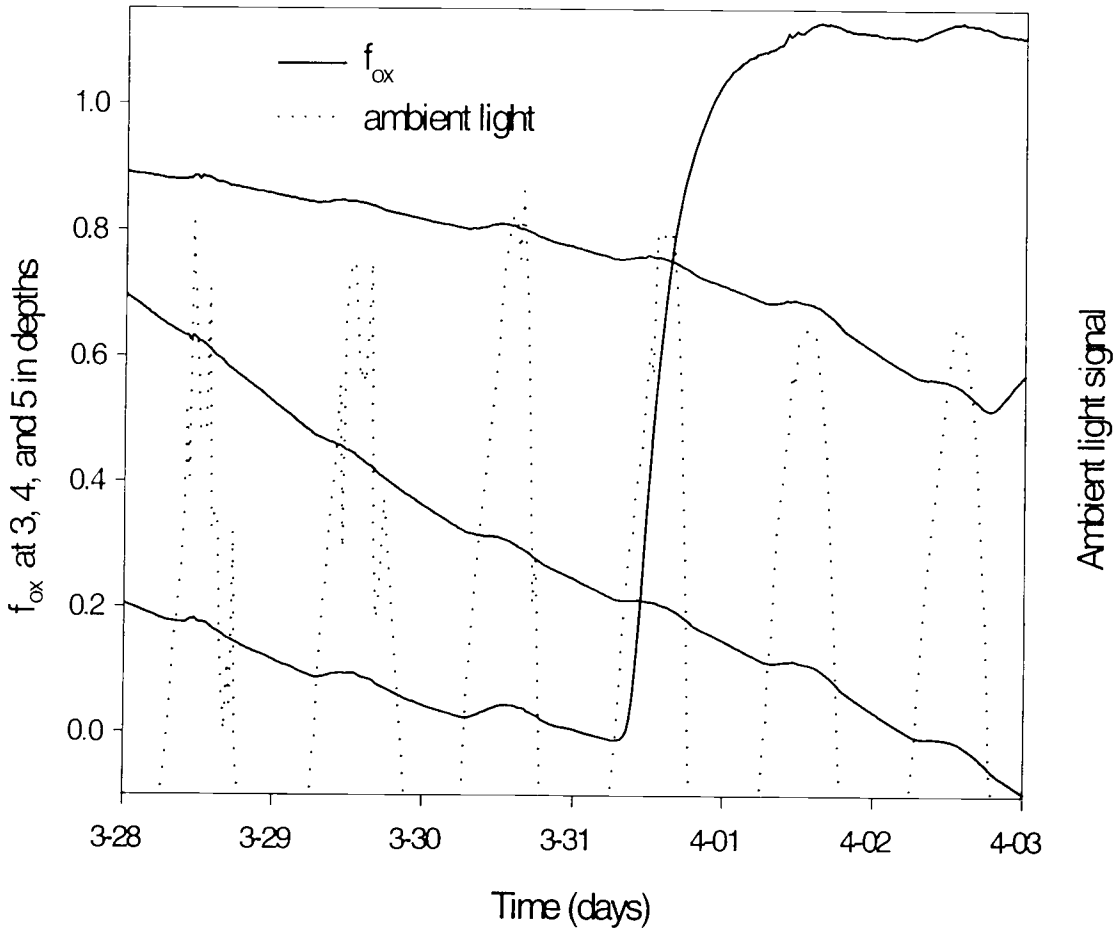


Figure 3-12. The time dependence of f_{ox} on the ambient light intensity. The fraction of oxidized indicator is shown for the chambers at depths of 3, 4 and 5 in. The ambient light intensity signal is that measured with the photodetector in the uppermost chamber when the LED light source was turned off. Data shown is extracted from Figures 3-8 and 3-10. On 3/28 the chamber at 4 in. is the uppermost line, followed by the chamber at 5 in and 3 in.

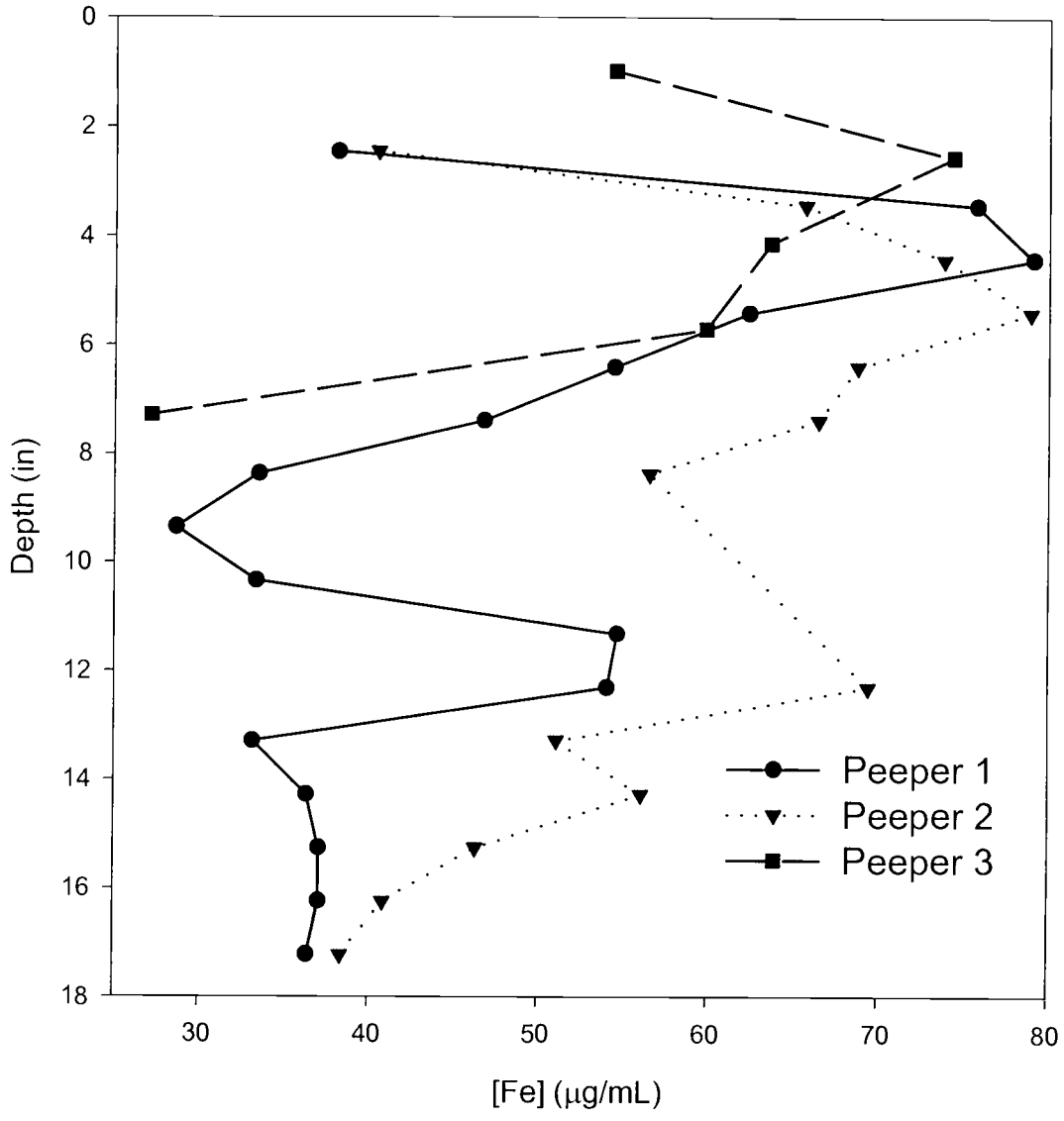


Figure 3-13. Soluble iron concentrations determined by ICP-AES after sampling with traditional membrane sampling devices.

oxidized (+3) form. Thus larger concentrations of aqueous iron indicate a more reducing environment. Although there is considerable variability in the data from the three probes, each set indicates the highest soluble iron concentrations are found between 2 and 6 in. The sensors were not placed in the ground at the same time as the SPEAR probe, and it is uncertain if equilibrium was ever achieved. All of the devices were in different physical locations, which may have a dramatic influence on the depth profile.

Other unpublished data (17) taken with the same sampling peepers at other times and locations suggest that the most reducing zone and highest observed soluble iron concentrations are found just below the water table (1 to 5 in). This observation is consistent with a model in which most of the organic matter that microbes use as an electron donor for energy generation is closer to the surface. Data from the same studies (17) suggest that sulfate reduction occurs along with iron (III) reduction in this reducing zone near the surface.

3.6 Conclusions

This first experiment with the SPEAR highlights its great potential for studying sub-surface environmental redox conditions. The key attribute of the SPEAR is the ability to provide in-situ information about the variations of the redox status of the soil or sediment with time and depth. Unexpected events, such as the water table dropping in the discussed experiment are not missed. With conventional membrane sampling probes, only the redox status at the end of the sampling period is observed, and there is no feedback about the state of equilibrium between the sampling device and the pore water.

Several improvements to future versions of the SPEAR probe are suggested based on the experience from this first experiment. These include improving the light shielding near the top of the probe to reduce the amount of ambient light reaching the photodetectors, opening the other side of the probe body to the pore water to improve the response time, and adding an LED that emits in an unabsorbed region of the indicator absorbance profile to provide a reference signal. A reference signal could be used to compensate for changes in intensity not related to indicator absorbance such as scattering due to changes in turbidity within the chambers.

In the future, a measurement of the light transmitted through a fully reduced membrane is needed before the deployment of the probe to allow for the calculation of indicator absorbance and f_{ox} in real time as the data are acquired. This measurement can either be made when the probe is assembled without the indicators in the strip or by reducing the indicators in a reservoir of some reductant (e.g., 0.5 M ascorbic acid).

The later method is preferable because it accounts for the absorbance and scattering of the membrane itself. The light transmitted through a fully reduced membrane should also be measured after the experiment is over so that any corrections for drift in the source intensity over the course of the experiment can be applied.

The probe was designed and the LEDs were chosen to accommodate a variety of immobilized indicators, and future experiments will include redox indicators with reduction potentials different from thionine. For example, cresyl violet is reduced at potentials corresponding to the microbial reduction of sulfate but not Fe(III) and should provide a means to discriminate how reduced a soil becomes. It also should be possible to incorporate immobilized pH indicators and temperature sensor in the SPEAR.

The SPEAR probe was designed to fit inside a 2-in diameter PVC well and future experiments include its use in water logged soils, river and lake bottoms, and sampling wells for ground water monitoring. The SPEAR can be used as a companion to conventional membrane sampling devices, and it may indicate when conditions are appropriate for taking a conventional sample for more comprehensive analysis. The SPEAR can also be used as a stand-alone device. For example, buffer zones (un-plowed and anaerobic) that surround plowed fields are often employed to minimize contamination of drinking water from agricultural runoff. The SPEAR might be used to continuously monitor the redox conditions in these buffer zones and ensure that they are proper for anaerobic denitrification. It will serve as a tool for understanding soil conditions, seasonal and diurnal redox cycles, the fate and transport of species in

the environment, and the extent and role of redox transformations in sub-surface environments.

3.7 References:

1. Schwarzenbach, Rene P.; Gschwend, Philip M.; Imboden, Dieter M., *Environmental Organic Chemistry,* John Wiley & Sons, Inc., **1993**.

2. Jones, Brian D., *Applications of Redox Indicators for Evaluating Redox Conditions in Environmental Samples*, **1999**, Ph.D. Thesis, Oregon State University.

3. Frevert, T., *Schweiz. Z. Hydrol.*, **1984**, *46*, 269-290.

4. Lindberg, R. D.; Runnells, D. D., *Science*, **1984**, *225*, 925-927.

5. Kempton, J. H., *Heterogenous Electron Transfer Kinetics*, **1987**, M.S. thesis.

6. Chapelle, F. H.; Haack, S. K.; Adriaens, P.; Henry, M. A.; Bradley, P. M, *Environ. Sci. Technol.*, **1996**, *30*, 3565 - 3569.

7. Lovley, D. R.; Goodwin, S, *Geochim. Cosmochim. Acta,* **1988**, *52*, 2993-3003.

8. Lemmon, Teresa, *Development of Chemostats and Use of Redox Indicators for Studying Transformations in Biogeochemical Matrices*, **1995**, Ph.D. Thesis, Oregon State University.

9. Lemmon, T. L.; Westall, J. C.; Ingle, J. D., *Anal. Chem.*, **1996**, *68*, 947-953.

10. Jones, B.; Ingle, J. D., *Talanta*, in press

11. Hesslein, R. H., *Limnol. Oceanogr.*, **1976**, *21*, 912-914

12. Mudroch, Alena (ed.); MacKnight, Scott D. (ed.); Adams, Donald, *CRC Handbook of Techniques for Aquatic Sediments Sampling*, CRC Press

13. Steinmann, Philipp; Shotyk, William, *Fres. J. Anal. Chem.*, **1996**, *354*, 709-713

14. Brandl, Helmut; Hanselmann, Kurt W., *Aquatic Sciences*, **1991**, *53*, 55-73

15. Steinmann, Philipp; Shotyk, William, *Geochimica et Cosmochimica Acta*, **1997**, *61*, 1143-1168

16. Chapter 2 of this thesis

17. Baham, John, Personal communication, Oregon State University, March, 2000.

Chapter 4: The SNARE

4.1 Abstract

A device for monitoring the reflectance (absorbance) of an immobilized redox indicator, in direct contact with a sample inside a transparent microcosm bottle or bioreactor, through the wall of the enclosure has been developed. In this device, denoted as SNARE (Spectrometer for *N*on-invasive *A*naerobic *R*eflectance *E*valuation), the indicator is immobilized in a film or membrane, which is attached to a donut-shaped magnet. This disk is placed inside the laboratory vessel (microcosm bottle, bioreactor, etc.). Reductants or oxidants in the solution interact with the immobilized indicator causing a change in its color (absorbance). Another donut-shaped magnet, imbedded in the head of the external probe, attracts the magnetic sensing disc to the side of the container and aligns the probe and disc in the proper orientation. The operation of this device was demonstrated with the redox indicator thionine to determine the redox status of a sample containing an enriched culture in which the anaerobic degradation of chlorinated ethenes was occurring. This redox sensing apparatus provides a simple and inexpensive means to monitor a critical parameter in a large number of samples that are being tested simultaneously.

4.2 Introduction

The Natural and Accelerated Bioremediation Research (NABIR) program whose mission is to "provide the scientific understanding needed to use natural processes and to develop methods to accelerate these processes" for bioremediation at Department of Energy (DOE) facilities defines bioremediation as "the process by which living organisms act to degrade hazardous organic contaminants or transform hazardous inorganic contaminants to environmentally safe levels in soils, subsurface materials, water, sludges, and residues." (1) Some strategies for applying or augmenting the bioremedial process include: (i) isolating, growing, and returning an enhanced culture to a contaminated site, (ii) introducing microorganisms from other sites where they were effective to a contaminated site, and (iii) encouraging an increase in population onsite by adding nutrients or manipulating other conditions (e.g., pH) (2)

Chlorinated hydrocarbons are of particular interest because many are commonly found, suspected carcinogens, xenobiotic, or recalcitrant. Anaerobic degradation is especially important for perchlorinated organic compounds where chlorine atoms are removed and replaced with hydrogen, apparently by a reductive dechlorination mechanism (3). The aerobic biodegradation of highly chlorinated ethenes, such as tetrachloroethene (PCE) and trichloroethene (TCE), is less efficient than anaerobic biodegradation (2). Unfortunately, many anaerobic pathways go through vinyl chloride (VC), which is a potent carcinogen. The anaerobic pathway for the transformation of PCE to ethene is provided in Figure 4-1 (4).

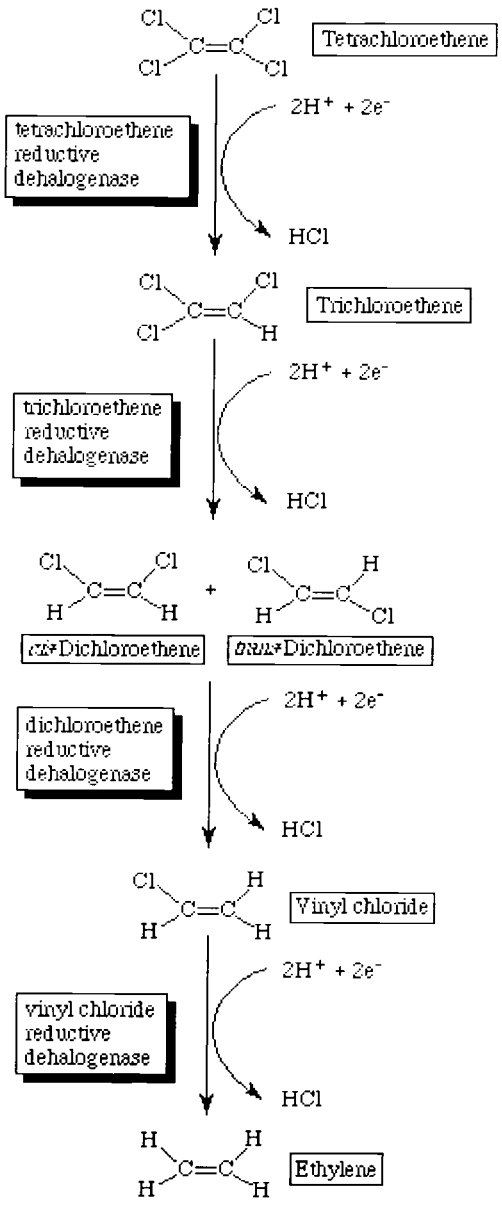


Figure 4-1. The anaerobic transformation pathway for PCE. (4)

Microcosm bottles are an important tool in understanding microbial processes. These containers are typically glass bottles ranging in size from 50 to 500 mL and are often fitted with a cap with a durable septum that allows for periodic sampling without perturbing the system. They provide a means to carefully study the effect of many important variables such as initial electron donor and acceptor concentrations, biomass, temperature, and pH on microbial activity and transformations. For anaerobic cultures, these bottles provide an inexpensive means to grow organisms on a benchtop. These microcosms also afford investigators with the ability to monitor several cultures and adjust parameters in a batch study

A clear understanding of the redox status of a system including the dominant redox processes and the redox state of critical species is required in virtually all aspects of hazardous substance management in ground water. In the laboratory, understanding the redox status of microbially mediated processes is critical to optimize performance for later field development.

In our laboratory (5, 6), we have evaluated numerous redox indicators for their suitability for environmental monitoring (e.g., reversibility with a variety of reductants/oxidants, coupling to a Pt electrode, and formal potential at pH 7 ($E_7^{0\prime}$)). These multiple-ring, organic compounds are colored in their oxidized form and colorless in their reduced form, allowing monitoring of their redox speciation spectrophotometrically. For example, the reduction of blue thionine to a colorless form can be monitored at 600 nm. Because these positively-charged indicators adsorb to minerals and soils, we have developed methods to immobilize them in a form that

can be monitored spectrophotometrically, that does not leach out, and that is accessible to reductants and oxidants in the water sample (5, 6)

Results obtained in environmental samples strongly support the notion that Fe(II), produced under Fe(III)-reducing conditions, and S(-II), a product of sulfate-reducing conditions, are the primary reductants of thionine and cresyl violet, respectively (6). The relationship between indicator speciation (fraction oxidized) and reductant concentration is consistent and correlates fairly well to values calculated from equilibrium models. Two indicators define a redox window or interval. For example, both thionine and cresyl violet are reduced by sulfide, but only thionine is reduced under Fe(III)-reducing conditions.

After a culture and amendments are transferred to a microcosm bottle, there is normally a variable lag time before anaerobic microbial activity is significant. Often some amount of oxygen is introduced even when careful handling precautions are taken. The free redox indicator resazurin is sometimes added to the mother reactor or microcosm bottle for anaerobic cultures as an "oxygen monitor" (7). Resazurin, which is blue, is irreversibly reduced to resorufin, which is pink. Resorfurin then undergoes a reversible reduction to dihydroresorufin, which is colorless (8). Typically an anaerobic culture is colorless, and the media becomes pink as the indicator is oxidized because of an oxygen leak during sample handing.

There is a need for real-time evaluation of "redox status" in these microcosm experiments, and for this purpose a redox sensor has been developed. This sensor can serve to alert the investigator when redox conditions are appropriate to start taking

samples to monitor microbial transformations (e.g., dechlorination can be observed by monitoring degradation products).

In this paper we outline the design and operation of a device designed to provide real-time, non-invasive information about the redox status of the media inside a sealed container such as a microcosm bottle or bioreactor. During operation, the complex spectrometric probe is outside the bottle; whereas, the redox sensing material itself is inside each microcosm bottle. This physical design mininizes cost especially for batch mode studies with many microcosm bottles. A suite of electronic components based on modern integrated circuits, LEDs, and embedded microcontrollers that are well matched in terms of their low cost, small size, and low power consumption are also presented.

In preliminary experiments the reflectance (absorbance) of the redox indicator thionine was monitored in a single bottle along with the headspace concentrations of selected chlorinated species, ethene, and methane. An enriched culture of organisms previously collected from the Evanite site in Corvallis, OR was selected for evaluation of the sensor device. This consortium of anaerobic organisms has been enriched for their ability to transform chlorinated hydrocarbons (PCE and its breakdown products in particular), and both TCE and VC were studied. The correlation between indicator response and the transformation of redox-active species is discussed.

4.3 Instrumentation

4.3.1 Introduction

A conceptual view of the SNARE (Spectrometer for *N*on-invasive *A*naerobic *R*eflectance *E*valuation) in operation is provided in Figure 4-2. The basic functions of the device include aligning the disk which holds the redox indicator with the sensor head, illuminating the redox indicator with light from LEDs at wavelengths in both absorbed and unabsorbed regions by the indicator, measuring the amount of reflected light, and the unattended recording of these measured values. Critical design criteria for the SNARE device include the number of sources and detectors and their orientation.

The redox indicators are immobilized by covalent bonding to transparent dialysis membrane disks and mounted on a white Teflon or Delrin disk. Calibration data suggest an "absorbance" type model for the measured "reflectance" signal. In this model, the light from the LED travels though the immobilized indicator, reflects from the white backing disk, and travels though the indicator disc again to reach the photodetector (on the same side of the disk as the light source). The effective path length is about twice the disc thickness in this model. For this reason the data from the probe are treated as transmitted intensities and the absorbance of the indicator is calculated. When the indicator is fully oxidized, the absorbance is greatest and the photodetector signal is a minimum. The photodetector signal for a completely reduced indicator disc defines the maximum value and is the reference signal in the calculation of absorbance.

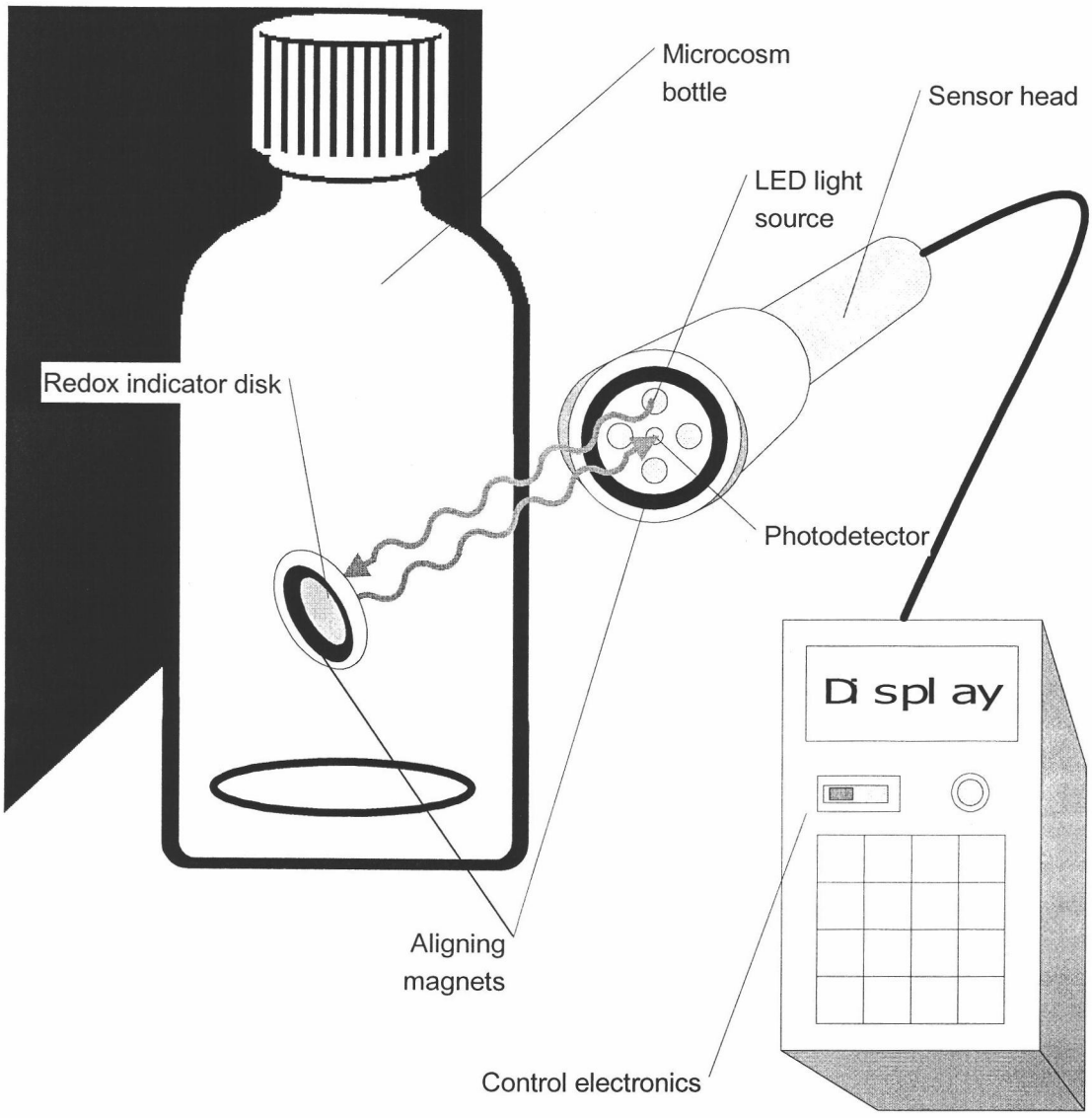


Figure 4-2. A general view of the SNARE device in operation. The magnet in the sensor head attracts and aligns the magnet in the redox indicator disk through the bottle. The indicator is press fit into the outer ring that also holds the magnet.

4.3.2 Physical construction

The SNARE consists of a sensor head containing all of the optical components connected to an enclosure containing the other electronic components along with a keypad and display. A detailed view of the device from different perspectives is provided in Figure 4-3. The immobilized redox indicator is cut with a 3/8-in punch, mounted onto the part of the white Delrin holder with the aligning magnet, and held in place when the pieces of the holder are press fit together. The sensor head is made from two black (opaque) Delrin pieces that press fit together. The radius of the head was machined so that the sensor head fits flush against the curved exterior of a 250- or 150-mL microcosm bottle (Wheaton). Inside this curved outer radius is an indentation that accommodates a donut-shaped rare earth magnet. Inside and behind the magnet four LEDs all oriented at a 30° angle. In the center of the sensor head is a channel that leads to a light-to-frequency photodetector integrated circuit.

The LEDs are oriented in a manner that minimizes reflection signals from the walls of the bottle and maximizes the amount of light collected from diffuse reflectance (off the backing disk). Several prototypes and ray tracing computer models were evaluated, and those with the photodetector in the center and light sources arranged at angles around the detector gave the best results.

The colors of the LEDs are selected so that one color (red in this application with thionine as the redox indicator) provides light in a wavelength region adsorbed by the immobilized reagent, and the other color (blue in this application) corresponds to a wavelength region that is unabsorbed by the immobilized indicator on the sensing

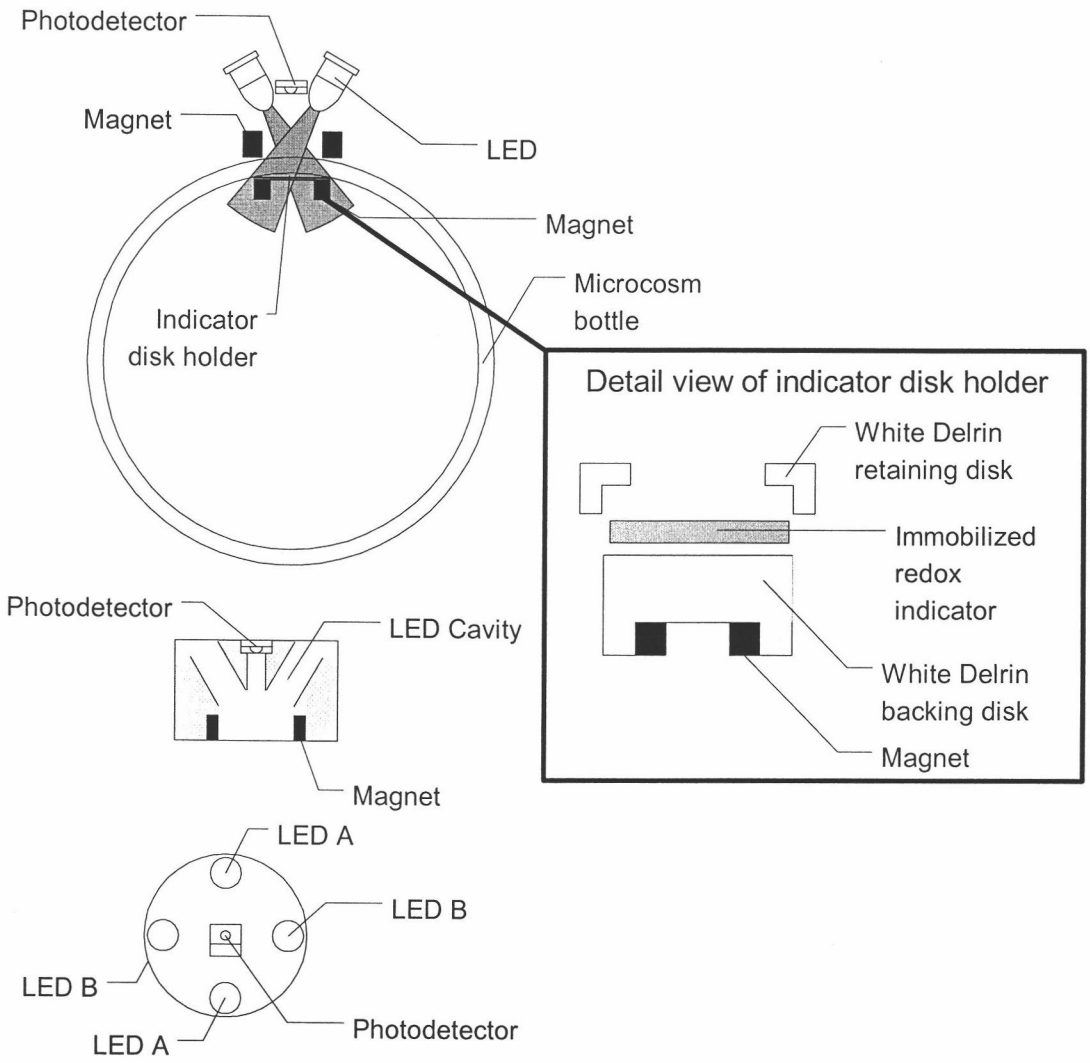


Figure 4-3. A more detailed view of the SNARE device. The uppermost drawing shows a cross section of the device in operation when viewed from above. The shaded cones represent the LED beams. The center picture illustrates a cross section of the sensor head with the cavities that orient the LED sources oriented at a 30° angle. The bottom drawing shows the surface of the sensor head and the orientation of the LEDs and the photodetector. The red LEDs are labeled LED A, and the blue LEDs are labeled LED B.

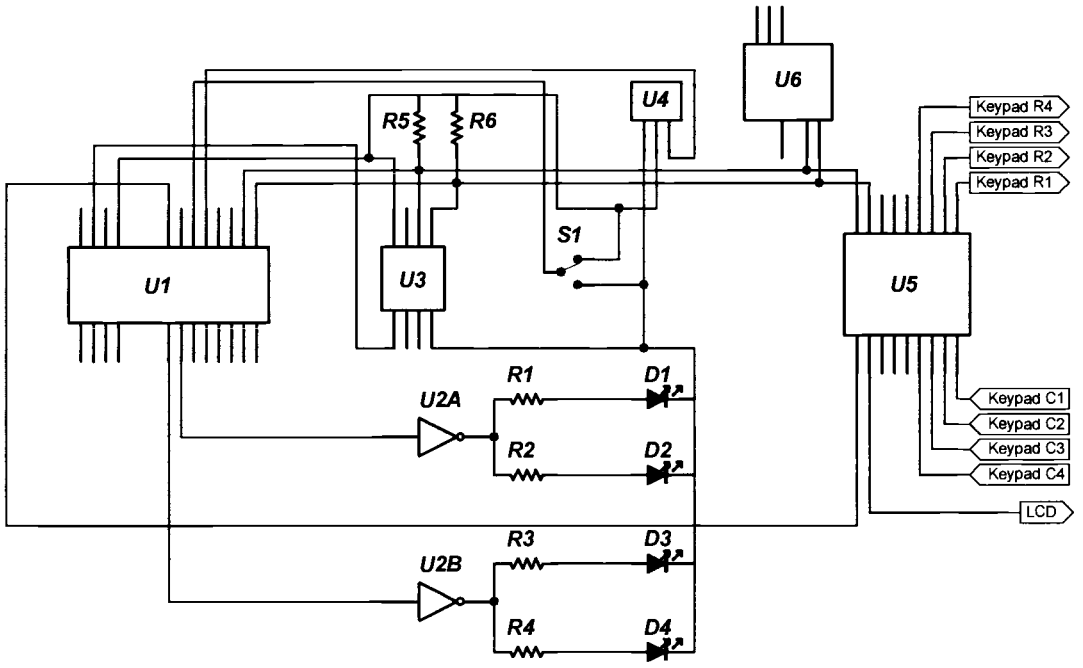
disk. Measurements with the unabsorbed light are used to correct for variations in the reflectance of the walls of the enclosure and optical alignment. Two LEDs with the same emission profile (four LEDs total) are employed to provide more even illumination of the surface of the sensing disc, thus reducing the effects of inhomogeneities and increasing the signal from the LEDs relative to ambient light levels.

4.3.3 Electronics

The electronic features and functions of the device include: data logging, time stamp, automated data acquisition, computer interface, and input of user-selected parameters. The plastic electronics enclosure contains a circuit board with two microcontrollers: a Parallax Basic Stamp II (BS II) and a PIC 16F84. A schematic and parts list is provided in Figure 4-4. The user interface is implemented with a PIC16F84 microcontroller connected to a keypad and a serial LCD display (Scott Edwards Electronics). The text of the user interface messages and prompts are stored in a 4K serial EEPROM.

The program for the BS II is written in interpreted PBasic (Parallax) and stored in the on-chip serial EEPROM memory. The program for the PIC is written and compiled into assembly instruction with the PIC Basic Pro compiler (MicroEngineering Labs Inc) and stored in the internal 1K of RAM.

The user-selected parameters are stored in the scratchpad memory of a Dallas real time clock (RTC) connected via an I2C bus. The stamp is also connected to this



U1	Stamp II	MCU
U2	7406	OC inverter
U3	X24128	Serial EEPROM
U4	TSL235	Photodetector
U5	PIC16F84	MCU
U6	DS1307	RTC
D1,D2	Blue LED	Unabsorbed
D3,D4	Red LED	Absorbed
R1,R2	330 Ω	Resistor
R3,R4	1000 Ω	Resistor
R5,R6	7200 Ω	Resistor
S1	Push Button	Momentary switch

Figure 4-4. A schematic and parts list for the electronic circuit of the SNARE device. All LEDs are 5-mm size packages. The red LED is distributed by Jameco, and the blue LED is a 30° dispersion model from Nichia.

RTC and parameters are passed from the user interface to the stamp in this manner. To ensure mutual exclusion (a single device accesses shared resources at a time) on the I2C bus, the microcontrollers (MCUs) also share a normally high logic line that they lower when a shared resource (the I2C clock or display) is in use. Either device must first check to make sure this line is high before using a shared resource. The Stamp is connected to a 16K serial EEPROM that stores all of the acquired data, the RTC, two pushbutton switches, and a serial port. Power is supplied either by 4 AA batteries or by a 9-V wall transformer. The voltage regulator built into the stamp MCU regulates the supply for the rest of the circuit at 5 V.

4.3.4 Operation

The user-set parameters are the year, month, day, hour, minutes, integration time in milliseconds, data acquisition frequency in data points per hour, and the number of data points to acquire. The user can toggle between manual and automatic mode, acquire and download mode, and "no data save" and "data logging" mode. All of these selections are made from the menu choices implemented on the PIC MCU, and any parameters are entered via the keypad. The basis of data acquisition is counting the pulses output from the TSL235 photodetector, which is a light-to-frequency conversion IC.

During the data acquisition phase, counts are accumulated first when the indicator disk or just the microcosm bottle is illuminated with light from the LEDs (S_t) and then with the LED sources turned off to determine the photodetector signal due to ambient light (S_{amb}). The difference between these measurements (S_c) yields a count

total proportional to the intensity of LED light reflected and is added to running total. This process is repeated five times, and the total of the differences (calculated as shown in equation 4-1) is displayed and stored in the EEPROM if data logging is selected.

$$S_c = S_t - S_{amb} \tag{4-1}$$

This measurement, S_c, is made with both the red (absorbed) and the blue (unabsorbed) LED light sources. The measurement of the ambient light-corrected signal from an unabsorbed light source was intended to correct for variations in optical alignment. In practice, this correction was not effective (possibly because the light from the blue LEDs is absorbed by the indicator) and the data presented are based on the measurements made with the red LEDs.

A provision for another measurement that is used to correct for the amount of light reflected back to the detector by the bottle itself (S_g, the background signal reflected from the glass container) is also included in the software of the SNARE. This correction term is determined when the sensing disk is not in front of the sensor head, and its calculation is shown in equation 4-2 where the prime denotes the absence of the sensing disk.

$$S_g = S'_c = S'_t - S'_{amb} \tag{4-2}$$

Note that S'_{amb} is different than S_{amb} because the indicator disk blocks some room light from reaching the photodetector. This measurement should be made each time a new bottle is used. When the pushbutton switch is depressed at the end of the data acquisition phase, the accumulated totals (S_g) are stored as the background signals for the red and blue light sources respectively. This term is a part of the data-block stored in EEPROM and the correction is applied during post-acquisition processing in a spreadsheet.

In summary, S_t is the total signal measured when the indicator disk is illuminated by a given pair of LEDs. This signal is comprised of three components: the useful analytical signal, which is attenuated by the redox indicator (S_{ind}), the signal due to reflection from the glass wall of the container (S_g), and the signal due to ambient light (S_{amb}). The signal from the indicator is obtained by difference as shown in Equation 4-3

$$S_{ind} = S_t - S_{amb} - S_g = S_c - S_g \tag{4-3}$$

S_c is calculated in the device, stored in memory, and displayed on the LCD readout. The values of S_g, determined in the absence of the indicator disk, are stored in memory and must be applied in a spreadsheet after data acquisition. The device also displays and stores a ratio value, which is calculated as shown in equation 4-4.

$$R = \frac{S_{ind}\ for\ the\ red\ LED}{S_{ind}\ for\ the\ blue\ LED} \tag{4-4}$$

The data from the SNARE probe are transferred to a spreadsheet via the MS Windows Terminal program. The time information is converted to units of days. The absorbance at some time during the experiment (A_t) was calculated according to equation 4-5, where S_{ind} is the corrected signal at time t in the experiment and $S_{ind\,(max)}$ is the maximum observed signal over the course of the entire experiment.

$$A_t = -\log\left(S_{ind} / S_{ind\,(\max)}\right) \tag{4-5}$$

The fraction of oxidized indicator is a dimensionless measure of the degree of oxidation of the indicator used in previous work (5, 6). This parameter is computed from equation 4-6 where f_{ox} is 1 in the fully oxidized form and 0 when indicator is completely reduced.

$$f_{ox} = A_t / A_{\max} \tag{4-6}$$

4.4 Experimental

The microbial culture was harvested from the Evanite site in Corvallis, OR (9). The enrichment culture was grown in a minimal salt media (10) within a 4-L Fill-and-Draw reactor. Sodium benzoate was added as the electron donor, and PCE was added as an electron acceptor to enhance the rate of growth (11). Every week, benzoate (1800 mg), PCE (900 mg), and 1 mL of yeast extract (10% by wt.) were added to the 4-L reactor with 400 mL of fresh media. Following the additions, 400 mL of the media was harvested from the effluent outlet.

Four 50 mL aliquots of the harvested culture were centrifuged in capped containers, and the cells were concentrated into ~35 mL of solution. In an anaerobic glove box, approximately 5 mL of this solution was combined with ~ 200 mL of fresh media (pH ~7) in a 250-mL microcosm bottle (Wheaton) capped with a butyl rubber septa to allow for temporal sampling. The headspace was allowed to equilibrate with the ~90% N_2/10% H_2 gas mixture in the glove box. The indicator disk was then loaded in its holder, placed inside the bottle, and the cap was secured.

In the first experiment, a single unstirred microcosm with diluted, unwashed Evanite culture was spiked by syringe with a solution of TCE to yield a target headspace concentration of ~1 mg/L of TCE. TCE, cis-DCE, VC, ethene, methane, and H_2 were monitored daily by using a gas-tight syringe to sample the headspace followed by GC/FID analysis. In the second experiment, a gas tight syringe was used to inject VC into the headspace of a single unstirred microcosm bottle with the

unwashed, diluted Evanite culture to yield a target headspace concentration of ~100 mg/L of VC. Ethene, methane, and H_2 were monitored daily.

In both experiments the SNARE probe was configured in automatic data logging mode and set to acquire data every 30 min. The redox indicator thionine was covalently immobilized on a dialysis membrane (SpectraPor, Inc.) through a process described in Appendix H. The immobilized redox indicator is cut with a 3/8-in punch, mounted onto the Delrin holder, and placed inside the microcosm bottle. The sensor head was held against the microcosm bottle in a ring-stand clamp. After the thionine was completely reduced for at least a day, the SNARE device was removed and the data were downloaded to a PC via a terminal program.

4.5 Results and Discussion

The data from the SNARE probe were examined in a spreadsheet. There were obvious breaks (>2% difference between contiguous points) in the S_{ind} (the analytical signal which is attenuated by the redox indicator) data due to optical realignment when the indicator holder and sensor head were separated during headspace sampling. The data acquired during these periods were removed. In the first experiment, 265 of the original 294 data points were kept, and the removed points were in 6 groups. For the second experiment, 264 of 297 points were kept, and the removed points were in 9 groups. When a group of outlying points was removed, the remaining data points were corrected by an offset so that the contiguous signals were equal. For example, in a set of ten points where points 3 and 4 are obvious outliers, points 3 and 4 represent group 1 and are removed. Points 5 through 10 are then corrected by an offset so that signals values for points 2 and 5 are the same. The error associated with optical repositioning of the indicator disk is the limiting factor in the reproducibility of the SNARE. The average of the difference in the signal (S_{ind}) before and after repositioning is ~12% of the range of the signal over the course of the experiment.

The results from the first experiment are summarized in Figure 4-5. In about three days, the TCE was almost completely reduced to cis-DCE or other products and reduction of thionine commenced. Thionine was not completely reduced until day 6. The initial reduction of thionine coincided with the production of both ethene and methane in the microcosm bottle. Vinyl chloride is observed before significant reduction of thionine. For simplicity DCE concentrations are not included on the plot.

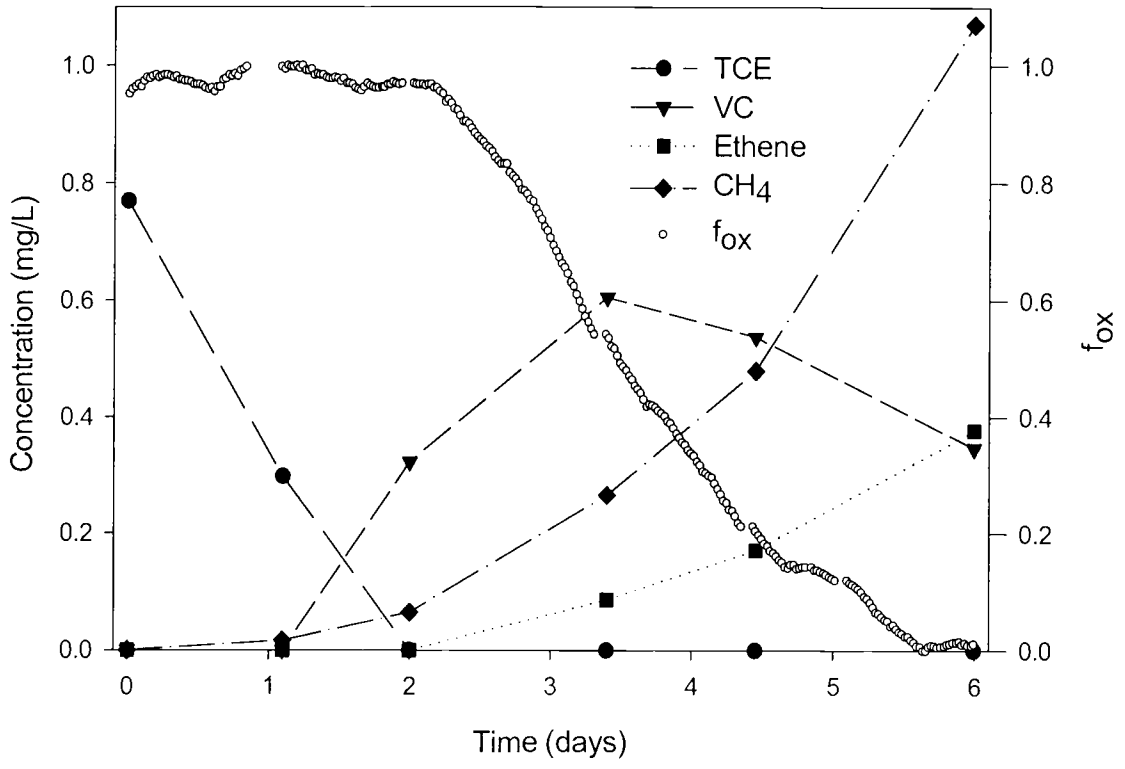


Figure 4-5. The time dependence of the concentration of headspace gasses and f_{ox} for thionine in a microcosm bottle spiked with TCE. Time 0 represents the first measurement of headspace concentration (06/17/2000). This measured concentration was ~0.8 mg/L in the headspace and ~2.3 mg/L in solution from Henry's Law. The dimensionless Henry's constant is 0.34 for TCE.

DCE is an intermediate between TCE and VC, and its concentration reached a maximum around day 1 or 2 (before reduction of thionine was observed) and decreased to below the detection limit by day 3. Measured H_2 levels in the headspace decreased from ~10% to ~5% throughout the course of the experiment.

One goal is to determine if the reduction of any one redox indicator is indicative of a dominant microbial culture or the concentration of a redox-active species. Results with the enriched Evanite culture (first experiment) suggest that the significant reduction of thionine is correlated with both methane production (possibly indicating the dominance of methanogenic organisms in the culture) and ethene production (indicating the onset of the final step in the reductive dehalogenation of TCE). In previous work (6) the indicators thionine, cresyl violet, and phenosafranine were sequentially reduced in a methanogenic activated sludge. No TCE was added and the culture had not been enriched for dechlorinating organisms. In another experiment (6), a groundwater culture known to reduce TCE to cis-DCE was spiked with TCE, and immobilized thionine was almost completely reduced before cis-DCE was observed. The partial reduction of cresyl violet occurred only after significant levels of cis-DCE were measured. Clearly, the reduction of thionine is not an absolute indication that conditions are appropriate for the dechlorination of TCE. Also, pH can greatly affect the reduction of the indicators and reductants. The speciation of thionine may have to be calibrated to different cultures and types of microbial redox transformations. The reduction of thionine is a clear indication of the absence of O_2 and a redox potential of ~50 mV or lower at pH 7.

In previous microcosm experiments with this culture (9), high levels of VC inhibited methanogenesis, and these conditions were selected for the second experiment in hopes of decoupling methanogenesis and reductive dechlorination. As shown in Figure 4-6, after one day, thionine is completely reduced and the VC concentration decreases about a factor of 2. The microcosm bottles were not continually shaken and little ethene is formed. Hence, part of the decrease is attributed to equilibration of the VC between the two phases. Note that the scale for VC is different from that of ethene, and most of the VC still remains after four days. The reduction of the redox indicator was correlated with the initial production of ethene, and methane production was suppressed. In both experiments the reduction of the redox indicator thionine appears to indicate when conditions are suitable for the last step in the dehalogenation of chlorinated ethenes, the conversion of vinyl chloride to ethene.

The indicator disk became dark after 3 days of exposure in the microcosm bottle. The f_{ox} values after this time are affected by this change in the optical properties of the immobilized indicator and are not shown in Figure 4-6. This change is attributed to microbial activity that could have degraded the cellulose acetate backbone of the dialysis film.

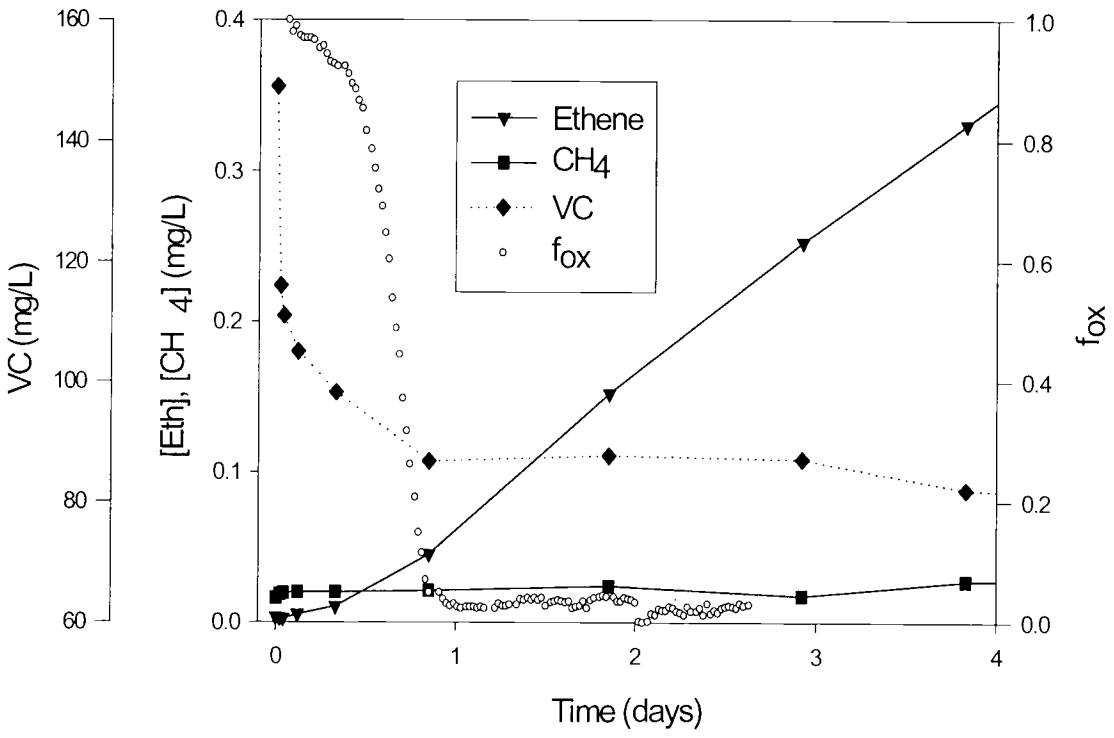


Figure 4-6. The time dependence of the concentration of headspace gasses and f_{ox} of thionine in a microcosm bottle spiked with VC (as a gas). Time 0 represents the first measurement of headspace concentration (07/19/2000). This measured concentration was ~150 mg/L in the headspace and ~140 mg/L in solution from Henry's Law. The dimensionless Henry's constant is 1.07 for VC. The initial decrease in headspace VC concentration is attributed to equilibration between the gas and liquid phases.

4.6 Conclusions

The SNARE device was designed to further enhance the capabilities of microcosm-based laboratory techniques by providing a convenient means to non-invasively and inexpensively monitor redox changes within these bottles in real time. A membrane with immobilized indicator was placed in direct contact with the media inside the bottle. The data clearly demonstrate that the color change of the redox indicator thionine coincides with the anaerobic reductive dechlorination of vinyl chloride in microcosm bottles spiked with an enriched Evanite culture.

Although the actual reductant of thionine in the studied culture is unidentified, the timing of ethene production and indicator reduction are well correlated in these experiments. Further study is necessary to understand the relationship between the steps in the reductive dechlorination pathway and the overall redox status of the culture in the microcosm bottle, the degree of reduction of thionine, and other conditions such as pH.

Several future experiments are suggested. Washing cells before transfer to the microcosm bottle would eliminate the possibility that a reductant in the mother reactor is transferred to the microcosm and reduces the indicator. Testing the SNARE device with multiple "equivalent" bottles in a batch study is needed to evaluate the reproducibility of the measurements and preparation of the bottles. The effect of stirring the cultures should be studied, as it should enhance the rate of transfer of volatile species between the solution headspace and of reductants to the indicator.

For experiments in which the microcosm is stirred with a magnetic stir bar, it has been demonstrated that the sensing disk can be affixed to a 3-in length of rigid PEEK tubing, which is longer than the diameter of the bottle. This tubing prevents the sensing disk from settling to the bottom of the bottle and becoming magnetically attracted with the stir bar.

One long-term goal is to employ redox sensors for fundamental investigations of anaerobic cultures used for bioremediation. Toward this goal, the SNARE will be used to study the rate of reductive dechlorination with a particular emphasis on the lag time. The SNARE can indicate when O_2 introduced during sample handling has been consumed and redox conditions are appropriate for dechlorination. Indicators with different formal potentials (e.g., cresyl violet) should be studied to determine what additional information they provide about the progress of the bioremedial process. Two redox indicators with different reduction potentials define a redox "interval". For example both thionine and cresyl violet are reduced under sulfate reducing conditions, but only thionine is reduced under iron reducing conditions (6). It may be possible to define similar "intervals" in dechlorinating cultures with the proper indicators.

4.7 References

1. Natural and Accelerated Bioremediation Research Bioremediation Glossary. http://www.lbl.gov/NABIR/info_5.html (accessed May, 2001)

2. Baird, Colin, *Environmental Chemistry*, W.H. Freeman and Company, **1999**.

3. Vogel, T. M.; McCarty, P. L., *Appl. Environ. Microbiol.*, **1985**, *43*, 1080-1083

4. The University of Minnesota Biocatalysis/Biodegradation Database. http://umbbd.ahc.umn.edu/tce2/tce2_map.html (accessed May, 2001)

5. Lemmon, T. L.; Westall, J. C.; Ingle, J. D., Jr. *Anal. Chem.*, **1996**, *68*, 947-953.

6. Jones, Brian D., *Applications of Redox Indicators for Evaluating Redox Conditions in Environmental Samples*, **1999**, Ph.D. Thesis, Oregon State University.

7. Madigan, Michael T.; Martinko, John M.; Parker, Jack, *Brock Biology of Microorganisms*, Prentice Hall, 2000

8. Bishop, Edmund (ed.), *Indicators*, Pergamon Press, 1972

9. Pon, George, "Complete Anaerobic Transformation of PCE to Ethylene by the Mixed Culture from Evanite Site, Corvallis" *In Situ and On-Site Bioremediation the Fifth International Symposium,* April, 19-22, **1999**

10. Shelton, D. R.; Tiedje, J. M., *Appl. Environ. Microbiol.,* **1984,** *47*, 850-857.

11. Maymo-Gatell, X.; Trndoi; Valter; Zinder, S. H., *Appl. Environ. Microbiol.,* **1995**, *61*, 3928.

Chapter 5: Conclusions

The general focus of this work has been the development and characterization of miniature spectrometers for monitoring environmental samples. The specific focus has been on evaluating redox conditions in anaerobic systems. These systems were divided into three exposure environments including flow streams (e.g., sample pumped from a bioreactor or sampling well), sub-surface field sites, and laboratory containers, and each was addressed in a chapter of this thesis. These anaerobic environments are very difficult to work with because sample collection and handling procedures that preserve the speciation of redox-sensitive constituents are cumbersome or nonexistent. Our approach involves constructing spectrometers that bring the sensing elements of an analytical determination into contact with the sample itself, rather than bringing the sample into the laboratory for analysis.

These spectrometers share a common design philosophy, and mutual characteristics include their small size, low cost, and adaptability. A suite of modern electronic components including LED light sources, embedded microcontrollers, and IC photodetectors are combined with immobilized redox indicators to create these miniature spectrometers. These spectrometers allow for exposure of the redox sensing material to the sample with minimal perturbation (specifically without O_2 exposure) and for the collection and storage of spectrometric data that relates to the redox status of the sample.

The SLIM (***S***imple, ***L***ow-power, ***I***nexpensive, ***M***icrocontroller-based) spectrometer represents a new class of absorption spectrometer that makes use of

modern semiconductor technology. It is a complete, self-contained, and compact unit about the size of bath soap that is capable of unattended data collection over a period of weeks or months with battery power. This device was designed for flow stream exposure environments where the sample solution is pumped from a laboratory bioreactor or field sampling well. Although its simple design compromises some performance characteristics, it is quite suitable for many applications where the monitored species is a dominant absorbing species in the sample with a concentration well above the typical detection limit. The SLIM spectrometer can accurately measure a 0.005 AU change, and the detection limit for the redox indicator thionine is within a factor of 2 of a commercial HP diode array spectrometer. The SLIM spectrometer is more than 100 times smaller and less expensive than the commercial spectrometer.

The SPEAR (***S***ub-surface ***P***robe for the ***E***valuation of ***A***naerobic ***R***egions) is an acrylic probe with chambers, covered with a membrane, that each have a spectrometer and immobilized redox indicator film. The key attribute of the SPEAR is the ability to provide in-situ information about the variations of the redox status of the soil or sediment with time and depth. The SPEAR was inserted in a subsurface soil in the Willamette Valley for about 3 weeks. Immobilized thionine was reduced more quickly in chambers near the surface and the effect of an unexpected event, the water table dropping, was observed. With conventional membrane sampling probes, only the redox status at the end of the sampling period is observed, and there is no feedback about the state of equilibrium between the sampling device and the pore water.

The SNARE (***S***pectrometer for ***N***on-invasive ***A***naerobic ***R***eflectance ***E***valuation) device was constructed to provide a convenient means to non-invasively and inexpensively monitor changes in redox conditions within microcosm bottles. The color change of the redox indicator thionine was found to coincide with a specific step in the anaerobic reductive dechlorination polychlorinated ethenes in microcosm bottles spiked with an enriched Evanite culture. Although the reductant of thionine is not known, the timing of the conversion of vinyl chloride to ethene and indicator reduction is well correlated in these experiments.

These spectrometers were designed to be disseminated and used by other investigators including soil scientists, environmental engineers, and microbiologists. In fact, it is critical that these spectrometers be used in various situations to determine how the response of different redox indicators is correlated to analytical information about specific species and specific types of microbial processes. Particular applications include their use as monitors for environmental barriers such as zero-valent-iron trenches or agricultural buffer zones. Preliminary data suggest that they may provide insight into the efficacy of dechlorinating microbial cultures in the bioremediation of chlorinated organic species.

Use of these spectrometers in a variety of applications will inevitably lead to refinements and improvements in their design. In particular new LED light sources and changes in the optical design could lead to more accuracy in the calculation of indicator speciation (f_{ox}). More importantly, the immobilization of redox indicators with different formal potentials would allow a greater variety of redox levels in natural systems to be investigated (e.g., de-nitrification).

This work exists as a stepping zone in our path toward understanding these complex environmental systems. In this thesis, spectrometers were designed around immobilized redox indicators and brought into contact with environmental samples. In the future the design of these spectrometers will improve, and the sensors will be used to increase our knowledge about redox transformations in the environment including bioremedial processes and the fate and transport of redox active species.

Bibliography

Atlas, R. M.; Bartha, R., *Microbial Ecology: Fundamentals and Applications*, Addison Wesley, **1998**.

Baham, John, Personal communication, Oregon State University, March, 2000.

Baird, Colin, *Environmental Chemistry*, W.H. Freeman and Company, **1999**.

Bishop, Edmund (ed.), *Indicators*, Pergamon Press, 1972

Brandl, Helmut; Hanselmann, Kurt W., *Aquatic Sciences*, **1991**, *53*, 55-73

Chapelle, F. H.; Haack, S. K.; Adriaens, P.; Henry, M. A.; Bradley, P. M, *Environ. Sci. Technol.*, **1996**, *30*, 3565 - 3569.

Clinch, J. R., Worsfold, P. J., *Anal. Chim. Acta*, **1987**, *200*, 523-531.

Clinch, J. R., Worsfold, P. J., *Anal. Chim. Acta*, **1988**, *214*, 401-407.

Dasgupta, P. K.; Bellamy, H. S.; Liu, H.; Lopez, J. L.; Loree, E. L.; Morris, K.; Petersen, K.; Mir, K. A., *Talanta*, **1993**, *40*, 53-74.

Frevert, T., *Schweiz. Z. Hydrol.*, **1984**, *46*, 269-290.

Hauser, P. C.; Rupasinghe, T. W. T.; Cates, N. E., *Talanta*, **1995**, *42*, 605-612.

Hauser, P. C.; Rupasinghe, T. W. T.; Tan, R., *Chimia*, **1995**, *49*, 492-494.

Hermanson, G. T.; Mallia, A. K.; Smith, P. K., *Immobilized Affinity Ligand Techniques*, Academic Press, **1992**

Hesslein, R. H., *Limnol. Oceanogr.*, **1976**, *21*, 912-914

Huang, J.; Liu H.; Tan, A.; Xu, J.; Zhao, X., *Talanta*, **1992**, *39*, 589-592.

Hubbard, Daisy, Laboratory notebook #1, 2000, Oregon State University

Ingle, J. D., A Research Proposal submitted to the EPA, **2000.**

Jambunathan, Sivakumar; Dasgupta, P. K.; Wolcott, D.K.; Marshall, G. D.; Olson, D.C., *Talanta*, **1999**, *50*, 481-490.

Jones, Brian D., *Applications of Redox Indicators for Evaluating Redox Conditions in Environmental Samples*, **1999**, Ph.D. Thesis, Oregon State University.

Jones, B.; Ingle, J. D., Evaluation of immobilized redox indicators as reversible, in situ redox sensors for determining Fe(III)-reducing conditions in environmental samples, *Talanta*, in press

Kempton, J. H., *Heterogenous Electron Transfer Kinetics*, **1987**, M.S. thesis.
Lemmon, T. L.; Westall, J. C.; Ingle, J. D. Jr., *Anal. Chem.*, **1996**, 68, 947-953

Lemmon, Teresa, *Development of Chemostats and Use of Redox Indicators for Studying Transformations in Biogeochemical Matrices*, **1995**, Ph.D. Thesis, Oregon State University.

Lindberg, R. D.; Runnells, D. D., *Science*, **1984**, *225*, 925-927.

Liu, Hanghui; Dasgupta, P. K., *Anal. Chim. Acta*, **1994**, *289*, 347-353.

Lovley, D. R.; Goodwin, S, *Geochim. Cosmochim. Acta,* **1988**, *52*, 2993-3003.

M.G. Chemicals Positive Photofabrication Process.
http://www.mgchemicals.com/techsupport/instruct.html (accessed May, 2001)

Madigan, Michael T.; Martinko, John M.; Parker, Jack, *Brock Biology of Microorganisms*, Prentice Hall, 2000

Maymo-Gatell, X.; Trndoi; Valter; Zinder, S. H., *Appl. Environ. Microbiol.,* **1995**, *61*, 3928.

Mudroch, Alena (ed.); MacKnight, Scott D. (ed.); Adams, Donald, *CRC Handbook of Techniques for Aquatic Sediments Sampling*, CRC Press

Natural and Accelerated Bioremediation Research Bioremediation Glossary.
http://www.lbl.gov/NABIR/info_5.html (accessed May, 2001)

Philips Semiconductor.
http://wwwus.semiconductors.philips.com/acrobat/various/I2sbus.pdf (pub. 1995)

Pon, George, "Complete Anaerobic Transformation of PCE to Ethylene by the Mixed Culture from Evanite Site, Corvallis" *In Situ and On-Site Bioremediation the Fifth International Symposium,* April, 19-22, **1999**

Schwarzenbach, Rene P.; Gschwend, Philip M.; Imboden, Dieter M., *Environmental Organic Chemistry,* John Wiley & Sons, Inc., **1993**.

Shelton, D. R.; Tiedje, J. M., *Appl. Environ. Microbiol.*, **1984,** *47*, 850-857.

Shriver, D. F.; Atkins, P.; Langford, C. H., *Inorganic Chemistry*, W. H. Frreman and Company, **1994**.

Steinmann, Philipp; Shotyk, William, *Fres. J. Anal. Chem.*, **1996**, *354*, 709-713

Steinmann, Philipp; Shotyk, William, *Geochimica et Cosmochimica Acta*, **1997**, *61*, 1143-1168

University of Minnesota Biocatalysis/Biodegradation Database. http://umbbd.ahc.umn.edu/tce2/tce2_map.html (accessed May, 2001)

Vogel, T. M.; McCarty, P. L., *Appl. Environ. Microbiol.*, **1985**, *43*, 1080-1083

Worsfold, P. J., Clinch, J. R., *Anal. Chim. Acta*, **1987**, *197*, 43-50.

Appendices

Appendix A: Supplemental information for the SLIM spectrometer

This section provides additional details regarding the SLIM spectrometer design and operation including: LED spectra, data from a tandem calibration experiment with immobilized thionine, performance data for samples in solution and immobilized on transparent films, printed circuit board artwork, microcontroller source code, an Excel macro for predicting LED performance, an Excel macro for real time acquisition an plotting of SLIM spectrometer data, and stand alone Visual Basic source code for SLIM spectrometer communication

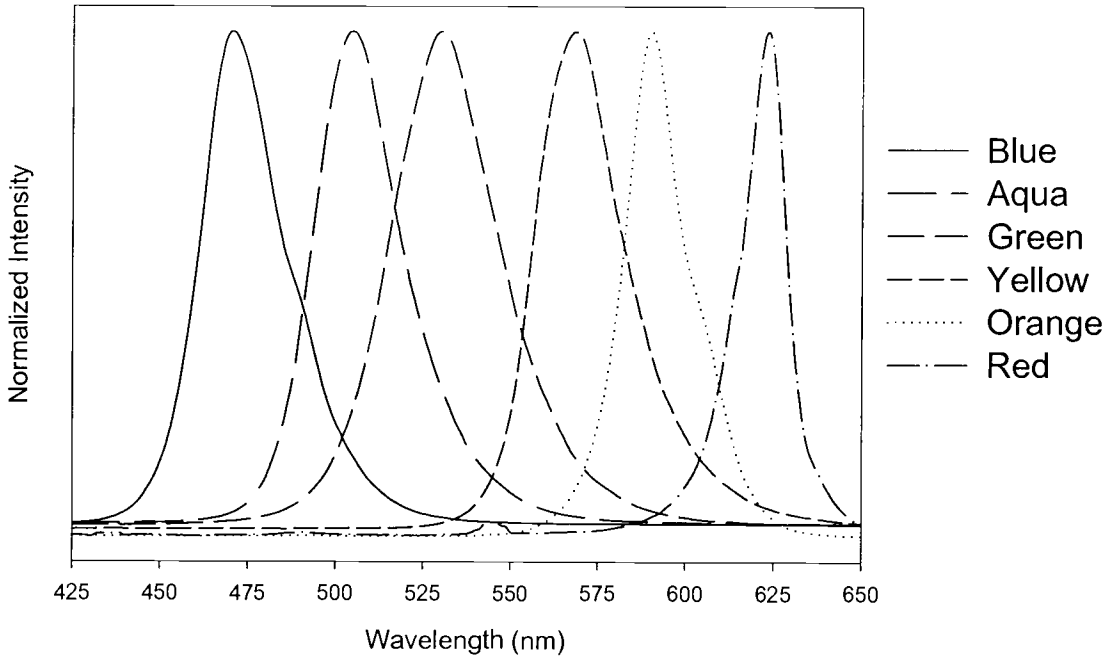


Figure A-1. Spectra of various LED light sources. The blue, blue/green, and green LEDs are manufactured by Nichia. The yellow, yellow/orange, and red LEDs are from Jameco. All intensities have been normalized to 1.0.

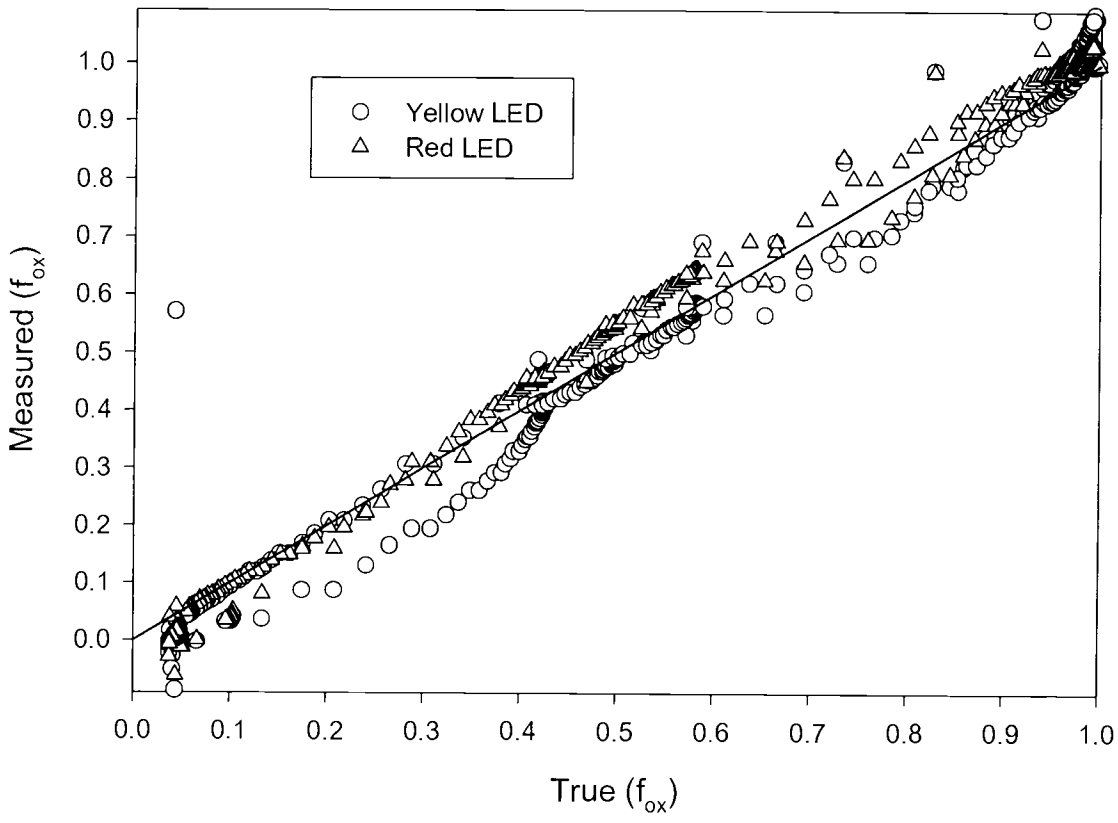


Figure A-2. Comparison of HP and SLIM spectrometer determination of f_{ox} using thionine indicator immobilized on dialysis membrane. The redox status was manipulated with solutions of ascorbic acid and oxygenated water. The X-axis is fraction oxidized as measured with the HP spectrometer, and the Y-axis is fraction oxidized as measured by the SLIM spectrometer in a tandem experiment. The line indicates a perfect correlation with the HP spectrometer.

Table A-1. The standard deviation (in AU) of measurements at various concentrations of thionine from both the HP and SLIM spectrometers.

HP 400 nm	HP 552 nm	HP 600 nm	HP 700 nm	SLIM Yellow	SLIM Red	SLIM Blue	Num. Samples
1.4E-04	2.4E-04	4.3E-04	2.3E-04	3.4E-04	3.5E-04	1.5E-04	4
1.5E-04	1.6E-04	1.3E-04	2.4E-04	2.3E-04	1.9E-04	2.2E-04	6
8.8E-05	1.4E-04	1.3E-04	8.5E-05	4.2E-04	4.3E-04	4.4E-04	7
1.0E-04	1.3E-04	1.5E-04	1.2E-04	5.5E-04	2.6E-04	5.5E-04	5
1.7E-04	2.6E-04	1.8E-04	1.7E-04	8.7E-04	6.5E-04	6.5E-04	6
6.0E-05	1.1E-04	1.7E-04	1.7E-04	2.2E-04	3.2E-04	2.9E-04	4
1.3E-04	2.0E-04	2.3E-04	2.2E-04	4.9E-04	1.9E-04	3.9E-04	5
1.9E-04	2.2E-04	9.6E-05	1.4E-04	5.8E-04	2.4E-04	1.9E-04	5
8.9E-05	1.8E-04	1.5E-04	1.8E-04	6.4E-04	3.7E-04	4.6E-04	6
1.1E-04	2.7E-04	2.2E-04	1.7E-04	4.6E-04	2.4E-04	4.4E-04	5
3.2E-04	4.4E-04	2.9E-04	4.3E-04	3.4E-04	2.6E-04	2.1E-04	6
2.1E-04	3.0E-04	2.1E-04	2.6E-04	8.1E-04	4.6E-04	5.4E-04	6
1.7E-04	3.5E-04	4.0E-04	2.6E-04	8.3E-04	5.7E-04	4.5E-04	19
1.2E-04	2.8E-04	3.3E-04	2.1E-04	8.3E-04	8.1E-04	8.6E-04	12
7.6E-05	3.8E-04	2.8E-04	1.6E-04	6.4E-04	5.1E-04	3.4E-04	4
2.7E-04	5.4E-04	7.1E-04	4.5E-04	1.0E-03	2.2E-04	3.0E-04	6
2.1E-04	5.6E-04	2.8E-04	2.6E-04	5.7E-04	3.5E-04	3.3E-04	5
3.0E-04	3.5E-04	3.8E-04	2.9E-04	4.7E-04	2.1E-04	2.5E-04	5
2.3E-04	4.7E-04	9.1E-04	2.0E-04	8.4E-04	4.2E-04	4.4E-04	8
1.9E-04	4.3E-04	8.7E-04	2.3E-04	6.3E-04	3.7E-04	3.4E-04	6

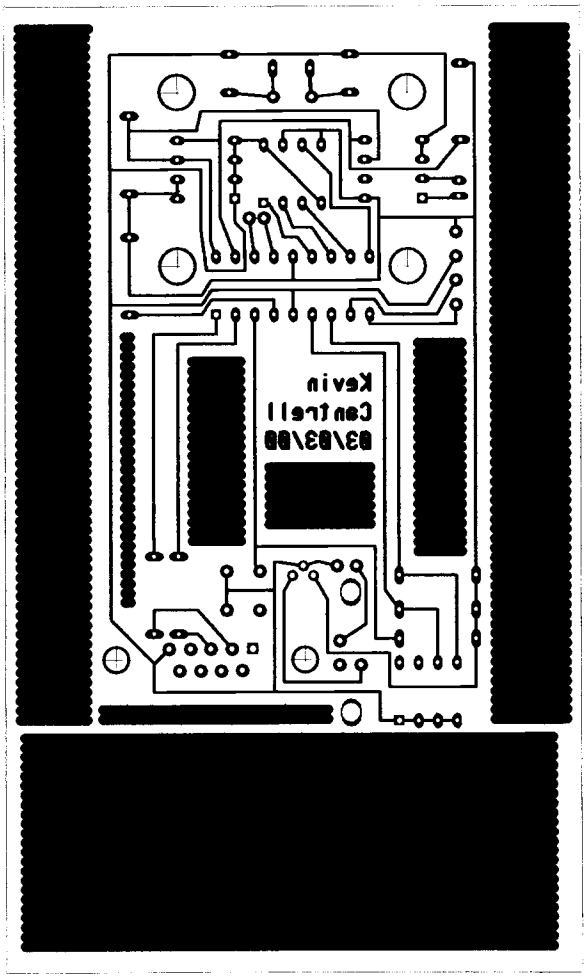


Figure A-3. The artwork for the SLIM spectrometer printed circuit board. The transparency should be placed over the sensitized board so that the text is readable and not reversed.

SLIM PIC microcontroller source code

```
'portb.0 is left switch
'portb.1 is right switch
'portb.2 is up LED
'portb.3 is down LED
'portb.4 is TSL230 6 (freq out)
'portb.5 is TSL230 3 (~oe)
'portb.6 is TSL230 2 (s1)
'portb.7 is TSL230 1 (s0)
'porta.0 is i2c 5 (SDA)
'porta.1 is i2c 6 (SCL)
'porta.2 is 1k (serout pin)
'porta.3 is 22k (serin pin)
'porta.4 is button

DEFINE      OSC                 4           '4 MHz oscillator
DEFINE      DEBUG_REG   PORTA
DEFINE      DEBUG_BIT           2
DEFINE      DEBUG_BAUD 9600
DEFINE      DEBUG_MODE1
'use porta.2 for inverted serial output at 9600,8,n,1

EEdataSize  con     12      'twelve bytes of data
DS          con     ","     'use a comma to separate data for output

newcmd      var     byte    'receives and stores the command character from the PC
sleeptime   var     word    'delay in seconds between samples
dark        var     word    'dark intensity
leda        var     word    'intensity when LED A is on
ledb        var     word    'intensity when LED B is on
ledc        var     word    'intensity when LED C is on
sample      var     word    'variable for passing intensity out of subroutine
SDA         var     porta.0 'SDA is alias for porta.0 part of I2C bus
SCL         var     porta.1 'SCL is alias for porta.1 part of I2C bus
clockCon    var     byte    'the I2C constant of the clock
EECon       var     byte    'the I2C constant of the EEPROM
i2cData     var     byte    'holds data byte for I2C I/O
i2c8Add     var     byte    '8 bit I2C address for clock
i2c16Add    var     word    '16 bit I2C address for EEPROM
i2c16ABack  var     word    'holds backup of 16 bit I2C address for EEPROM
seconds     var     byte    'holds seconds from the clock
minutes     var     byte    'holds minutes from the clock
hours       var     byte    'holds hours from the clock
day         var     byte    'holds day from the clock
avgLoop     var     byte    'number of samples to average and looping variable
```

```
porta=%11111011
'porta.0 (i2c SDA) is       1
'porta.1 (i2c SCL) is       1
'porta.2 (serout) is        0
'porta.3 (serin) is         1
'porta.4 (button) is        1

trisa=%00011000
'porta.0 (i2c SDA) is       out
'porta.1 (i2c SCL) is       out
'porta.2 (serout) is        out
'porta.3 (serin) is         in
'porta.4 (button) is        in

portb=%00100000
'portb.0 (left switch) is   0
'portb.1 (right switch) is  0
'portb.2 (LED A) is         0
'portb.3 (LED B) is         0
'portb.4 (TSL230 freq out) is       0
'portb.5 (TSL230 ~oe) is    1
'portb.6 (TSL230 s1) is     0
'portb.7 (TSL230 s0) is     0

trisb=%00010011
'portb.0 (left switch) is           in
'portb.1 (right switch) is          in
'portb.2 (LED A) is                 out
'portb.3 (LED B) is                 out
'portb.4 (TSL230 freq out) is       in
'portb.5 (TSL230 ~oe) is            out
'portb.6 (TSL230 s1) is             out
'portb.7 (TSL230 s0) is             out

pause 100

clockCon=%11010000
EECon=%10100000
i2c8add=6
i2cData=0               'year
gosub clockwrite
i2cData=1               'month
gosub clockwrite
i2cData=1               'date
gosub clockwrite
i2cData=1               'day
gosub clockwrite
i2cData=0               'hours
gosub clockwrite
i2cData=0               'minutes
gosub clockwrite
i2cData=0               'seconds
gosub clockwrite
'the above section sets the clock to Sunday 1/1/2000 12:00:00 am
```

```
i2c16add=65535
i2cdata=2
gosub EEi2c
'unlocks the EEPROM

i2c16Add=0

if porta.4=1 then download
'if button is NOT pressed then download

loop:           if (portb.0) and (portb.1) then talkPC
                'if both DIP switches are UP then listen for PC command

samplestart:    i2cread SDA,SCL,clockCon,0,[seconds,minutes,hours,day]
                'read seconds,minutes,hours, and day from the clock

                dark=0
                leda=0
                ledb=0
                ledc=0

        for avgLoop=1 to 10
        'sample each LED 10 times and add the samples together

                portb=%11000000
                'portb.2 (LED A) is             0
                'portb.3 (LED B) is             0
                'portb.5 (TSL230 ~oe) is    0
                'portb.6 (TSL230 s1) is     1
                'portb.7 (TSL230 s0) is     1
                'LEDs OFF and Detector on with maximum gain
                gosub measureLight
                dark=dark+sample

                portb=%11000100
                'portb.2 (LED A) is             1
                'portb.3 (LED B) is             0
                'portb.5 (TSL230 ~oe) is    0
                'portb.6 (TSL230 s1) is     1
                'portb.7 (TSL230 s0) is     1
                'LED A ON and Detector ON with maximum gain
                gosub measureLight
                leda=leda+sample

                portb=%11001000
                'portb.2 (LED A) is             0
                'portb.3 (LED B) is             1
                'portb.5 (TSL230 ~oe) is    0
                'portb.6 (TSL230 s1) is     1
                'portb.7 (TSL230 s0) is     1
                'LED B ON and Detector ON with maximum gain
                gosub measureLight
                ledb=ledb+sample
```

```
                portb=%11001100
                'portb.2 (LED A) is              1
                'portb.3 (LED B) is              1
                'portb.5 (TSL230 ~oe) is   0
                'portb.6 (TSL230 s1) is    1
                'portb.7 (TSL230 s0) is    1
                'LED A ON and LED B ON and Detector ON with maximum gain
                'if used with an OR chip (74LS03) this state can turn a third LED on
                gosub measureLight
                ledc=ledc+sample

        next avgLoop
        portb=%00100000
        'portb.2 (LED A) is         0
        'portb.3 (LED B) is         0
        'portb.5 (TSL230 ~oe) is    1
        'portb.6 (TSL230 s1) is     0
        'portb.7 (TSL230 s0) is     0
        'LEDs OFF and Detector OFF

        gosub DataOut
        'send data via serial port

        if (portb.0) and (portb.1) then talkPC
        'if both DIP switches are UP then listen for PC command

        gosub EEWrite
        'save data in EEPROM

        if portb.0 then cstmslp
        'if just the LEFT DIP switch is UP then use the custom sleep time

        if portb.1 then longslp
        'if just the RIGHT DIP switch is UP then use the long sleep time

                sleep 296
                'if both the DIP switches are DOWN then use this short sleep time
                goto loop

longslp: sleep 1796
                'sets the long sleep time
                goto loop

cstmslp:        i2cread SDA,SCL,EECon,16378,[sleeptime]
                sleep sleeptime
                'read the custom sleep time from EEPROM and sleep for that amount
                of seconds
                goto loop

talkPC: serin porta.3,6,["$"],newcmd,#sleeptime
                'waits for PC command that starts with "$" followed by a character and
                a number
                if newcmd="i" then
                'if the character is "i" then use the following number as the sleep time
                        i2c16ABack=i2c16Add
```

```
                'first backup current position in memory
                i2c16add=16378
                'move to the fixed address for time in memory
                i2cdata=sleeptime.byte1
                gosub EEi2c
                'write the high byte of sleep time
                i2cdata=sleeptime.byte0
                gosub EEi2c
                'write the low byte of sleep time
                i2c16add=i2c16ABack
                'restore the previous position in memory
                debug dec sleeptime,13,10
                'echo back the stored sleep time
        else
                goto samplestart
                'if the command char is not an "I" the acquire a new sample
        endif
        goto loop


download:       i2cread SDA,SCL,EECon,16380,[i2c16ABack]
                'read the address of the last data set stored in a fixed address
                for i2c16add=0 to i2c16ABack step EEdataSize
                'loop through all of the stored data blocks
                i2cread SDA,SCL,EECon,i2c16Add,
                        [minutes,hours,seconds,day,dark,leda,ledb,ledc]
                'reading minutes,hours,seconds,day,dark intensity,
                'LED A's intensity, LED B's intensity, and LED C's intensity
                gosub DataOut
                'send the data to the PC via the serial port
                next i2c16add

downloop:       if porta.4=1 then downloop
                'wait for the acquire button to be pressed
                'NOTE: THIS LOOP WASTES POWER
                goto loop

'the ClockWrite routine backs through the clock's registers setting the time parameters
ClockWrite:     pause 20
                i2cwrite SDA,SCL,clockCon,i2c8Add,[i2cData]
                i2c8Add=i2c8add-1
                pause 20
                return
'EEWrite stores a block of data in the EEPROM
EEWrite:        i2c16ABack=i2c16Add
                'backup current EEPROM position
                i2c16add=16380
                'point to the "last address written" EEPROM location
                i2cdata=i2c16ABack.byte1
                gosub EEi2c
                i2cdata=i2c16ABack.byte0
                gosub EEi2c
                'store the address of the data block you are about to write
                i2c16add=i2c16ABack
                'restore the EEPROM position
```

```
                i2cdata=minutes
                gosub EEi2c
                i2cdata=hours
                gosub EEi2c
                i2cdata=seconds
                gosub EEi2c
                i2cdata=day
                gosub EEi2c
                i2cdata=dark.byte1
                gosub EEi2c
                i2cdata=dark.byte0
                gosub EEi2c
                i2cdata=leda.byte1
                gosub EEi2c
                i2cdata=leda.byte0
                gosub EEi2c
                i2cdata=ledb.byte1
                gosub EEi2c
                i2cdata=ledb.byte0
                gosub EEi2c
                i2cdata=ledc.byte1
                gosub EEi2c
                i2cdata=ledc.byte0
                gosub EEi2c
                'the lines of code above store the minutes,hours,seconds,day,dark
                intensity,LED A intensity, LED B intensity, and LED C intensity
                return

'this routine sends the I2C data to the EEPROM and increments the address
EEi2c:          pause 20
                i2cwrite SDA,SCL,EECon,i2c16Add,[i2cdata]
                i2c16add=i2c16Add+1
                pause 20
                return

DataOut:        debug "S",dec (i2c16Add/EEdataSize),DS,dec day,DS,
                        dec hours,DS,dec minutes,DS, dec seconds,DS,
                        decdark,DS,dec leda,DS,dec ledb,DS, dec ledc,13,10
                'sends a formatted string of data out via the serial port
                return

measureLight:count portb.4,100,sample
                'count the frequency output of the detector for 100 ms
                return
```

EXCEL macro LED calculator code

This program is a macro designed to run in Microsoft Excel. It expects the worksheet to be formatted as follows: column A contains wavelength (with the first value in cell A2), column B contains the LED emission profile, column C contains the absorptivity of the species of interest, column D contains the concentrations for which the absorbance should be predicted, and cell E1 should contain the path length. Columns A through C should all have the same number of entries (up to 1024). The output is provided in column E.

```
Sub Guess()
Dim absorb(1024) As Single
Dim calcAbsorb(1024) As Single
Dim emission(1024) As Single
Dim attenuate(1024) As Single
Dim newConc(1024) As Single
Dim attenuatedSum As Single
Dim emissionSum As Single
Dim LEDAbsorb As Single
Dim pathLength As Single
Dim i%, j%
Dim entries%, concEntries%

For i = 2 To 1024
      If Sheet1.Cells(i, 3) <> "" Then
         absorb(i) = Sheet1.Cells(i, 3)
         emission(i) = Sheet1.Cells(i, 2)
      Else
         Exit For
      End If
Next i
entries = i - 1

For i = 2 To 1024
      If Sheet1.Cells(i, 4) <> "" Then
         newConc(i) = Sheet1.Cells(i, 4)
      Else
         Exit For
      End If
Next i
concEntries = i - 1

pathLength = Sheet1.Cells(1, 5)
```

```
emissionSum = 0
For i = 2 To entries
      emissionSum = emissionSum + emission(i)
Next i

For j = 2 To concEntries
   attenuatedSum = 0
   For i = 2 To entries
      calcAbsorb(i) = absorb(i) * newConc(j) * pathLength
      attenuate(i) = 10 ^ (calcAbsorb(i) * -1) * emission(i)
      attenuatedSum = attenuatedSum + attenuate(i)
   Next i
   LEDAbsorb = -Application.Log(attenuatedSum / emissionSum)
   Sheet1.Cells(j, 5) = LEDAbsorb
Next j
End Sub
```

SLIM EXCEL data acquisition code

This program is a macro designed to run in Microsoft Excel. It used the MSComm Control to acquire data from the SLIM spectrometer, which it then records and plots from within the spreadsheet. The CommForm contains the code for serial port communication and the transfer of the acquired data to the spreadsheet cells. The <ThisMacroFirst> subroutine activates the CommForm and formats the XY plot of the acquired data. On the page named "Intensity" the time appears in column A (units of days), the intensity from the green LED appears in column B, the intensity from the red LED appears in column C, and the dark intensity appears in column D. The plot of the data is on a separate page called "Redox"

```
Sub ThisMacroFirst()
CommForm.MSComm1.PortOpen = True
CommForm.Show

Sheets("Redox").Select
ActiveChart.ChartType = xlXYScatterLines
ActiveChart.SetSourceData Source:=Sheets("Intensity").Range("A1:B50"), PlotBy:= _
    xlColumns
    ActiveChart.Location Where:=xlLocationAsNewSheet
    With ActiveChart
        .HasTitle = True
        .ChartTitle.Characters.Text = "SPEAR LED Intensities"
        .Axes(xlCategory, xlPrimary).HasTitle = True
        .Axes(xlCategory, xlPrimary).AxisTitle.Characters.Text = "Time"
        .Axes(xlValue, xlPrimary).HasTitle = True
        .Axes(xlValue, xlPrimary).AxisTitle.Characters.Text = "Intensity"
    End With
    With ActiveChart.Axes(xlCategory)
        .HasMajorGridlines = False
        .HasMinorGridlines = False
    End With
    With ActiveChart.Axes(xlValue)
        .HasMajorGridlines = False
        .HasMinorGridlines = False
    End With
    ActiveChart.HasLegend = True
    ActiveChart.Legend.Select
    Selection.Position = xlBottom
    ActiveChart.ApplyDataLabels Type:=xlDataLabelsShowNone, LegendKey:=False
End Sub
```

CommForm Code

```
Dim inpstring$
Dim samNumber%
Dim nextVal%
Dim days As Long, hours As Long, minutes As Long, seconds As Long, blank1 As Long, ledA
As Long, ledB As Long, ledC As Long, blank2 As Long
Dim dataRecord(1500, 6) As Single
Dim rawInString$

Private Sub CommandButton1_Click()
CommForm.Hide
End Sub

Private Sub MSComm1_OnComm()
Dim serin$
Dim indata As Long
Dim i%

serin = MSComm1.Input
For i = 1 To Len(serin)
   Char = Mid(serin, i, 1)
   If Char = "," Or Char = Chr(13) Then
      If InStr(inpstring, "S") Then
         samNumber = Val(Right(inpstring, Len(inpstring) –
                  InStr(inpstring, "S"))) + 1
         nextVal = 1
      Else
         indata = Val(inpstring)
         If nextVal = 1 Then
            days = indata
         ElseIf nextVal = 2 Then
            hours = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 3 Then
            minutes = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 4 Then
            seconds = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 5 Then
            blank1 = indata
         ElseIf nextVal = 6 Then
            ledA = indata
         ElseIf nextVal = 7 Then
            ledB = indata
            dataRecord(samNumber, 1) = days + (hours / 24) +
                        (minutes / 24 / 60) + (seconds / 24 / 60 / 60)
            dataRecord(samNumber, 2) = blank1
            dataRecord(samNumber, 3) = ledA
            dataRecord(samNumber, 4) = ledB
         End If
         nextVal = nextVal + 1
      End If
```

```
      inpstring = ""
    Else
     inpstring = inpstring + Char
    End If
Next i

activeRange = "A" + Right(Str(samNumber), Len(Str(samNumber)) - 1)
Range(activeRange).Select
ActiveCell.FormulaR1C1 = Str(dataRecord(samNumber, 1))
activeRange = "B" + Right(Str(samNumber), Len(Str(samNumber)) - 1)
Range(activeRange).Select
ActiveCell.FormulaR1C1 = Str(dataRecord(samNumber, 3) - dataRecord(samNumber, 2))
activeRange = "C" + Right(Str(samNumber), Len(Str(samNumber)) - 1)
Range(activeRange).Select
ActiveCell.FormulaR1C1 = Str(dataRecord(samNumber, 4) - dataRecord(samNumber, 2))
activeRange = "D" + Right(Str(samNumber), Len(Str(samNumber)) - 1)
Range(activeRange).Select
ActiveCell.FormulaR1C1 = Str(dataRecord(samNumber, 2))

End Sub
```

SLIM VB communication code

This standalone Visual Basic program communicates with the SLIM spectrometer via the PCs serial port. The MSComm control in Visual Basic provides for software control of this port.

```
Dim inpstring$
Dim samNumber%
Dim nextVal%
Dim days As Long, hours As Long, minutes As Long, seconds As Long
Dim blank1 As Long, ledA As Long, ledB As Long
Dim dataRecord(1500, 6) As Single
Dim rawInString$

Private Sub cmdInterval_Click()
Dim intervalvalue As Long
Dim intervalstring$
intervalstring = InputBox("1) Turn power OFF" + Chr$(13) + "2) Set DIP switches to up
position" + Chr$(13) + "3) Turn Power ON while pressing button", "Change Interval Between
Samples", 0)
Comm2.Output = "$i" & intervalstring & Chr$(13)
End Sub

Private Sub cmdSave_Click()
Dim i%, j%
Dim fyle$
CommonDialog1.ShowSave
fyle = CommonDialog1.FileName
Open fyle For Output As #1
For i = 0 To samNumber
   For j = 1 To 4
      Print #1, Str(dataRecord(i, j)) + ",";
   Next j
   Print #1,
Next i
Close #1
End Sub
```

```
Private Sub Comm2_OnComm()
Dim serin$
Dim indata As Long
Dim i%
serin = Comm2.Input
For i = 1 To Len(serin)
   Char = Mid(serin, i, 1)
   If Char = "," Or Char = Chr(13) Then
      If InStr(inpstring, "S") Then
         samNumber = Val(Right(inpstring, Len(inpstring) –
                           InStr(inpstring, "S")))
         LblRcv.Caption = samNumber
         SpecInter.Refresh
         nextVal = 1
      Else
         indata = Val(inpstring)
         If nextVal = 1 Then
            days = indata
         ElseIf nextVal = 2 Then
            hours = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 3 Then
            minutes = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 4 Then
            seconds = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 5 Then
            blank1 = indata
         ElseIf nextVal = 6 Then
            ledA = indata
         ElseIf nextVal = 7 Then
            ledB = indata
            txtTime.Text = Str(days) + ":" + Str(hours) + ":" + Str(minutes) + ":"
                           + Str(seconds)
            txtleda.Text = Str(ledA - blank1)
            txtledb.Text = Str(ledB - blank1)
            dataRecord(samNumber, 1) = days + (hours / 24) +
                           (minutes / 24 / 60) + (seconds / 24 / 60 / 60)
            dataRecord(samNumber, 2) = ledA
            dataRecord(samNumber, 3) = ledB
            dataRecord(samNumber, 4) = blank1
         End If
         nextVal = nextVal + 1
      End If
      inpstring = ""
```

```
    Else
      inpstring = inpstring + Char
    End If
Next i
txtRawComm.Text = txtRawComm.Text + serin
'rawinsting = rawInString + serin
LblRcv.Visible = True
End Sub

Private Sub Form_Load()
Comm2.PortOpen = True
End Sub
```

Appendix B: Supplemental information for the SPEAR spectrometer

This section provides additional details regarding the SPEAR design and operation including: PIC source code, VB interface code, and circuit board artwork (control circuit and photodetectors)

SPEAR PIC microcontroller source code

```
DEFINE                  OSC             4
DEFINE                  DEBUG_REG       PORTA
DEFINE                  DEBUG_BIT       3
DEFINE                  DEBUG_BAUD      9600
DEFINE                  DEBUG_MODE      1
'0 for max 232 or 1 for serial backpack or direct

EEdataSize      con     40
DS              con     ","


i               var     byte
counter         var     byte
dark            var     word
leda            var     word
ledb            var     word
sample          var     word
ShiftData       var     byte
SDA             var     porta.0
SCL             var     porta.1
clockCon        var     byte
EECon           var     byte
i2cData         var     byte
i2c8Add                 var     byte
i2c16Add                var     word
i2c16AddBackup          var     word
seconds                 var     byte
minutes                 var     byte
hours                   var     byte
day                     var     byte

porta=%11101011
trisa=%00010100
portb=%00000000
trisb=%00010000

pause 100

clockCon=%11010000
```

```
EECon=%10100000

i2c8add=6
i2cData=0
'year
gosub clockwrite
i2cData=1
'month
gosub clockwrite
i2cData=1
'date
gosub clockwrite
i2cData=1
'day
gosub clockwrite
i2cData=0
'hours
gosub clockwrite
i2cData=0
'minutes
gosub clockwrite
i2cData=0
'seconds
gosub clockwrite

i2c16add=65535
i2cdata=2
gosub EEi2c
i2c16Add=0

if porta.4=1 then download
loop:   i2c16AddBackup=i2c16Add
                i2c16add=16380
                i2cdata=i2c16AddBackup.byte1
                gosub EEi2c
                i2cdata=i2c16AddBackup.byte0
                gosub EEi2c
                i2c16add=i2c16AddBackup
                i2cread SDA,SCL,clockCon,0,[seconds,minutes,hours,day]

                i2cdata=minutes
                gosub EEi2c
                i2cdata=hours
                gosub EEi2c
                i2cdata=seconds
                gosub EEi2c
                i2cdata=day
                gosub EEi2c

                gosub TimeDataOut

                counter=2
                for i = 0 to 5
                        portb=%00000111
                        ShiftData=0
```

```
                gosub shift595
                gosub measureLight

                ShiftData=counter
                gosub shift595
                gosub measureLight

                ShiftData=~(counter)
                gosub shift595
                gosub measureLight

                counter=counter << 1
        next i
        debug 13,10
        ShiftData=0
        gosub shift595
        portb=%00000100
        sleep 10
        goto loop

shift595:       counter=~counter
        shiftout portb.5,portb.7,1,[counter\8]
        counter=~counter
        shiftout portb.5,portb.7,1,[ShiftData\8]
        portb.6=1
        portb.6=0
        return

download:       i2cread SDA,SCL,EECon,16380,[i2c16AddBackup]
        for i2c16add=0 to i2c16AddBackup step EEdataSize
                i2cread SDA,SCL,EECon,i2c16Add,[minutes,hours,seconds,day]
                gosub TimeDataOut
                for i= 0 to 5
                        i2cread SDA,SCL,EECon,
                                ((i2c16Add+4)+(i*6)),[dark,leda,ledb]
                        gosub LightDataOut
                next i
                debug 13,10
        next i2c16add
downloop:       if porta.4=1 then downloop
        goto loop

ClockWrite:     pause 20
        i2cwrite SDA,SCL,clockCon,i2c8Add,[i2cData]
        i2c8Add=i2c8add-1
        pause 20
        return

EEi2c:          pause 20
        i2cwrite SDA,SCL,EECon,i2c16Add,[i2cdata]
        i2c16add=i2c16Add+1
        pause 20
        return

TimeDataOut:debug "S",dec (i2c16Add/EEdataSize),DS, dec day,DS,dec hours,DS,
```

```
                dec minutes,DS,dec seconds,DS
        return

LightDataOut:debug dec dark,DS,dec leda,DS,dec ledb,DS
        return

measureLight:count portb.4,1000,sample
        i2cdata=sample.byte1
        gosub EEi2c
        i2cdata=sample.byte0
        gosub EEi2c
        debug dec sample,DS
        return
```

SPEAR VB communication code

```
Dim inpstring$
Dim samNumber%
Dim nextVal%
Dim days As Long, hours As Long, minutes As Long, seconds As Long
Dim blank1 As Long, ledA1 As Long, ledB1 As Long
Dim blank2 As Long, ledA2 As Long, ledB2 As Long
Dim blank3 As Long, ledA3 As Long, ledB3 As Long
Dim blank4 As Long, ledA4 As Long, ledB4 As Long
Dim blank5 As Long, ledA5 As Long, ledB5 As Long
Dim blank6 As Long, ledA6 As Long, ledB6 As Long
Dim dataRecord(1500, 19) As Single
Dim rawInString$

Private Sub cmdProcess_Click()
Dim i%, j%
Dim fyle$
CommonDialog1.FileName = ""
CommonDialog1.ShowSave
fyle = CommonDialog1.FileName
Open fyle For Output As #1
Print #1, "Time,LEDA1,LEDA2,LEDA3,LEDA4,LEDA5,LEDA6,,LEDB1,LEDB2,LEDB3,
        LEDB4,LEDB5,LEDB6,,DARK1,DARK2,DARK3,DARK4,DARK5,DARK6"
For i = 0 To samNumber
   Print #1, Str(dataRecord(i, 1)) + ",";
   For j = 2 To 12 Step 2
      Print #1, Str(dataRecord(i, j) - dataRecord(i, (j / 2) + 13)) + ",";
   Next j
   Print #1, ",";
   For j = 3 To 13 Step 2
      Print #1, Str(dataRecord(i, j) - dataRecord(i, ((j - 1) / 2) + 13)) + ",";
   Next j
   Print #1, ",";
   For j = 14 To 19
      Print #1, Str(dataRecord(i, j)) + ",";
   Next j
   Print #1,
Next i
Close #1

End Sub

Private Sub cmdSave_Click()
Dim i%, j%
Dim fyle$
CommonDialog1.ShowSave
fyle = CommonDialog1.FileName
Open fyle For Output As #1
For i = 0 To samNumber
   For j = 1 To 19
      Print #1, Str(dataRecord(i, j)) + ",";
   Next j
   Print #1,
```

```
Next i
Close #1

End Sub

Private Sub Comm2_OnComm()
Dim serin$
Dim indata As Long
Dim i%

serin = Comm2.Input
For i = 1 To Len(serin)
   Char = Mid(serin, i, 1)
   If Char = "," Or Char = Chr(13) Then
      If InStr(inpstring, "S") Then
         samNumber = Val(Right(inpstring, Len(inpstring) - InStr(inpstring, "S")))
         LblRcv.Caption = samNumber
         SpecInter.Refresh
         nextVal = 1
      Else
         indata = Val(inpstring)
         If nextVal = 1 Then
            days = indata
         ElseIf nextVal = 2 Then
            hours = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 3 Then
            minutes = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 4 Then
            seconds = (indata And 15) + ((indata And 240) / 16 * 10)
         ElseIf nextVal = 5 Then
            blank1 = indata
         ElseIf nextVal = 6 Then
            ledA1 = indata
         ElseIf nextVal = 7 Then
            ledB1 = indata
         ElseIf nextVal = 8 Then
            blank2 = indata
         ElseIf nextVal = 9 Then
            ledA2 = indata
         ElseIf nextVal = 10 Then
            ledB2 = indata
         ElseIf nextVal = 11 Then
            blank3 = indata
         ElseIf nextVal = 12 Then
            ledA3 = indata
         ElseIf nextVal = 13 Then
            ledB3 = indata
         ElseIf nextVal = 14 Then
            blank4 = indata
         ElseIf nextVal = 15 Then
            ledA4 = indata
         ElseIf nextVal = 16 Then
            ledB4 = indata
         ElseIf nextVal = 17 Then
            blank5 = indata
```

```
            ElseIf nextVal = 18 Then
               ledA5 = indata
            ElseIf nextVal = 19 Then
               ledB5 = indata
            ElseIf nextVal = 20 Then
               blank6 = indata
            ElseIf nextVal = 21 Then
               ledA6 = indata
            ElseIf nextVal = 22 Then
               ledB6 = indata
               txtTime.Text = Str(days) + ":" + Str(hours) + ":" + Str(minutes) + ":" + Str(seconds)
               txtleda1.Text = Str(ledA1 - blank1)
               txtledb1.Text = Str(ledB1 - blank1)
               txtleda2.Text = Str(ledA2 - blank2)
               txtledb2.Text = Str(ledB2 - blank2)
               txtleda3.Text = Str(ledA3 - blank3)
               txtledb3.Text = Str(ledB3 - blank3)
               txtleda4.Text = Str(ledA4 - blank4)
               txtledb4.Text = Str(ledB4 - blank4)
               txtleda5.Text = Str(ledA5 - blank5)
               txtledb5.Text = Str(ledB5 - blank5)
               txtleda6.Text = Str(ledA6 - blank6)
               txtledb6.Text = Str(ledB6 - blank6)
               dataRecord(samNumber, 1) = days + (hours / 24) + (minutes / 24 / 60) +
                     (seconds / 24 / 60 / 60)
               dataRecord(samNumber, 2) = ledA1
               dataRecord(samNumber, 3) = ledB1
               dataRecord(samNumber, 4) = ledA2
               dataRecord(samNumber, 5) = ledB2
               dataRecord(samNumber, 6) = ledA3
               dataRecord(samNumber, 7) = ledB3
               dataRecord(samNumber, 8) = ledA4
               dataRecord(samNumber, 9) = ledB4
               dataRecord(samNumber, 10) = ledA5
               dataRecord(samNumber, 11) = ledB5
               dataRecord(samNumber, 12) = ledA6
               dataRecord(samNumber, 13) = ledB6
               dataRecord(samNumber, 14) = blank1
               dataRecord(samNumber, 15) = blank2
               dataRecord(samNumber, 16) = blank3
               dataRecord(samNumber, 17) = blank4
               dataRecord(samNumber, 18) = blank5
               dataRecord(samNumber, 19) = blank6
            End If
            nextVal = nextVal + 1
         End If
         inpstring = ""
      Else
        inpstring = inpstring + Char
      End If
   Next i
   txtRawComm.Text = txtRawComm.Text + serin
   'rawinsting = rawInString + serin
   LblRcv.Visible = True
   End Sub
```

```
Private Sub Form_Load()
Comm2.PortOpen = True
End Sub
```

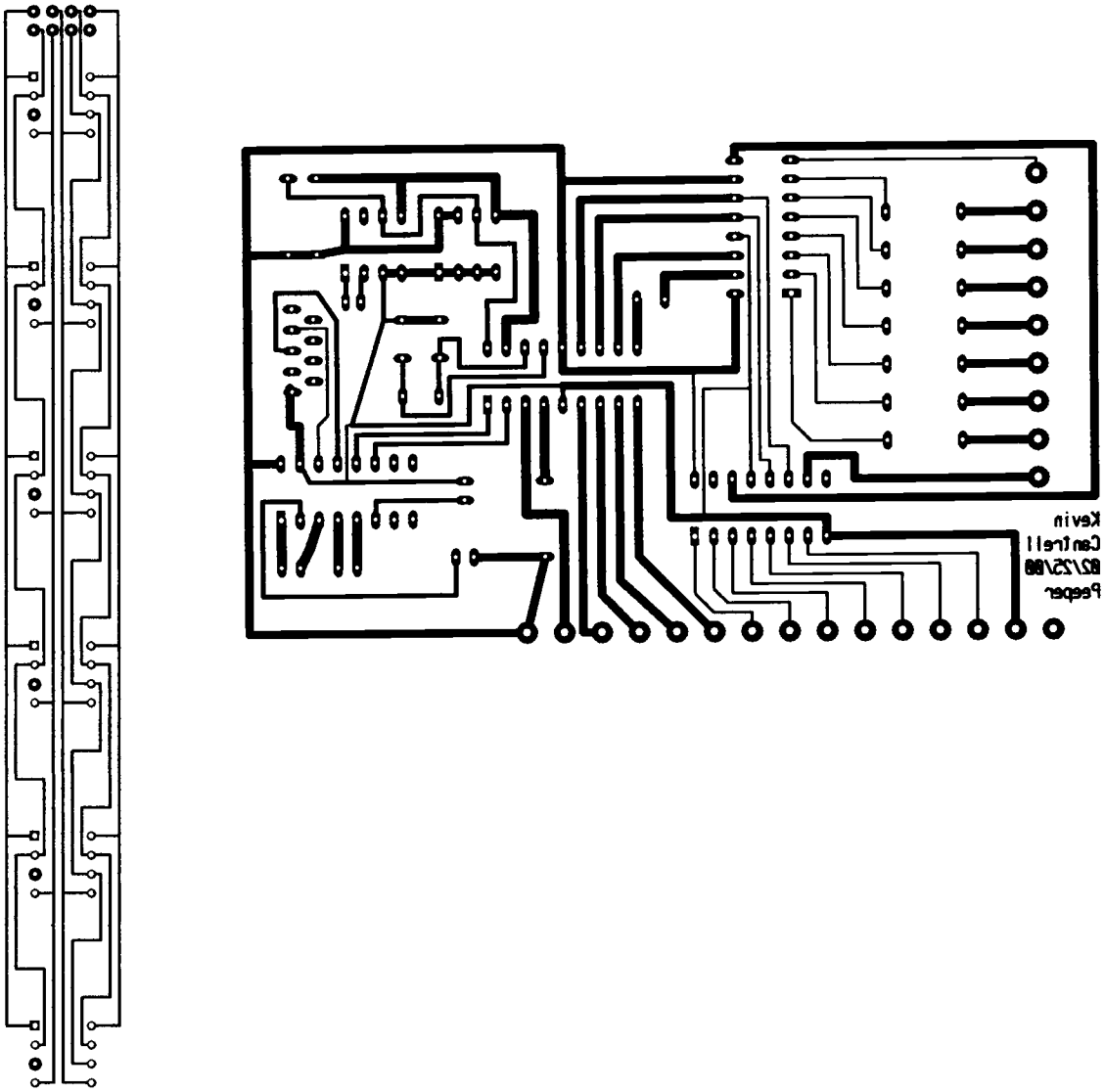


Figure B-1. Printed circuit board artwork for the SPEAR control circuit and photodetector board

Appendix C: Supplemental information for the SNARE spectrometer

This section provides additional details regarding the design and operation of the SNARE including calibration information and microcontroller source code

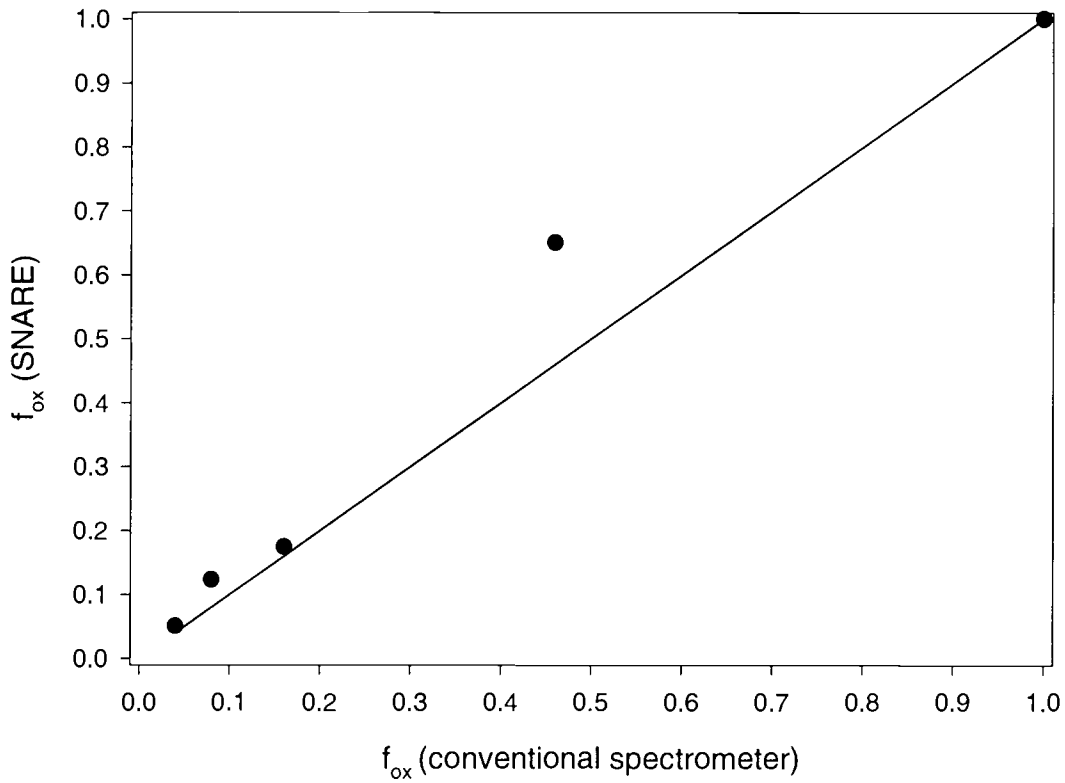


Figure C-1. Comparison of the f_{ox} determined with the SNARE to the f_{ox} determined with conventional absorbance measurements. For these measurements, different amounts of thionine were immobilized on cellulose filters by changing the concentration of thionine. The SNARE was used in a reflectance mode where f_{ox} is the ratio of the pseudo-absorbance (A') obtained for a given test membrane to that for the filter paper with the most immobilized thionine. The pseudo absorbance is defined by equation C-1.

$$A' = -\log \frac{S_{total\ (paper\ with\ indicator)} - S_{glass}}{S_{total\ (blank\ paper)} - S_{glass}} \qquad \text{(C-1)}$$

Conventional absorption measurements were taken on the same filter disks with an Ocean Optics spectrometer and white LED light source and used to calculate f_{ox}.

SNARE BSII microcontroller source code

```
redbackgrnd     var     word
redsample       var     word
bluebackgrnd    var     word
bluesample      var     word
ratio           var     word
timedta         var     word
temp            var     byte
i               var     word
freq            var     word
light           var     word
auto            var     bit
logged          var     bit
review          var     bit
EEData          var     word
WordAdd         var     word
TheData         var     byte
AckResp         var     bit
countPer        var     byte

SCL             con     9
SDA             con     8
EEBase          con     %10101110  'add 1 for read
CLOCKBase       con     %11010000
RED             con     0
BLUE            con     1
DATASIZE        con     16
HANDSHAKE       con     15
N96N            con     $4054
LCD             con     3


input HANDSHAKE
input LCD
output RED
output BLUE
low RED
low BLUE
high SCL
high SDA

gosub EEPrep
pause 2000
```

```
cycle:gosub GetConfig
        if review=1 then revdta
        if auto=1 then waitd
        if in13=0 then cycle
        goto getd
waitd:gosub getfreq
        sleep (3600/timedta)
getd:   if in14=0 then memclear
        redsample=0
        bluesample=0
        gosub getperiod
        countPer=timedta
        for i= 1 to 5
                high RED
                gosub measure
                light=freq
                low RED
                gosub measure
                redsample=(light-freq)+redsample

                high BLUE
                gosub measure
                light=freq
                low BLUE
                gosub measure
                bluesample=(light-freq)+bluesample
        next
        if in13=1 then bkg

resume:ratio=((redsample-redbackgrnd)*10)/((bluesample-bluebackgrnd)/10)
swait:  input HANDSHAKE
                if in15=0 then swait
                low HANDSHAKE
                output LCD
                serout LCD,N96N,[254,1]
                serout LCD,N96N,[254,128,"S:",sdec (redsample-redbackgrnd)]
                'debug "S:",sdec (redsample-redbackgrnd)," "
                serout LCD,N96N,[254,136,"R:",sdec (bluesample-bluebackgrnd)]
                'debug "R:",sdec (bluesample-bluebackgrnd)," ",cr 'key to 9th place
                serout LCD,N96N,[254,192,"T:",sdec ratio]
                'debug "T:",sdec ratio," "
                gosub getminutes
                temp=timedta
                gosub gethours
                timedta=temp+(timedta*100)
                serout LCD,N96N,[254,199,dec timedta dig 3,dec timedta dig 2,
                        ":",dec timedta dig 1,dec timedta dig 0]
                'debug dec timedta dig 3,dec timedta dig 2,":",dec timedta dig 1,
                        dec timedta dig 0,"  "
                gosub getconfig
                gosub getNums
                if logged=1 then willstordta
                serout LCD,N96N,[254,205,dec EEData]
                'debug dec EEData,cr
                input HANDSHAKE
```

```
                input LCD
                goto cycle
willstordta:serout LCD,N96N,[254,205,dec EEData+1]
                'debug dec EEData+1,cr
                input HANDSHAKE
                input LCD
                goto stordta


bkg:            redbackgrnd=redsample
                bluebackgrnd=bluesample
                goto resume

measure:        count 12,countPer,freq
                return

stordta:        gosub getNums
                freq=EEData
                WordAdd=freq*dataSize
                'debug ? WordAdd
                if WordAdd>(1000*dataSize) then rollAddress
queuedata:      goto gettime
gottime:        EEData=timedta
                'debug dec timedta dig 3,dec timedta dig 2,":",
                        dec timedta dig 1,dec timedta dig 0," "
                WordAdd=freq*dataSize
                gosub EEWrite

                goto getdate
gotdate:        EEData=timedta
                'debug dec timedta dig 3,dec timedta dig 2,"/",dec timedta dig 1,
                        dec timedta dig 0," "
                WordAdd=(freq*dataSize)+2
                gosub EEWrite

                gosub getyear
                EEData=timedta
                'debug dec timedta+1999,cr
                WordAdd=(freq*dataSize)+4
                gosub EEWrite

                EEData=redbackgrnd
                WordAdd=(freq*dataSize)+6
                gosub EEWrite

                EEData=bluebackgrnd
                WordAdd=(freq*dataSize)+8
                gosub EEWrite

                EEData=redsample
                WordAdd=(freq*dataSize)+10
                gosub EEWrite

                EEData=bluesample
                WordAdd=(freq*dataSize)+12
```

```
                gosub EEWrite

                EEData=ratio
                WordAdd=(freq*dataSize)+14
                gosub EEWrite

                WordAdd=(freq*dataSize)+16

                freq=freq+1
                EEData=freq
                gosub SetNums
                'debug "stored Samples :",dec freq,cr,cr
                goto cycle

rollAddress:    WordAdd=0
                debug "EEPROM full !!!!!",cr
                goto queuedata

revdta:         if in13=0 then cycle
                gosub GetNums
                freq=EEData
                WordAdd=$0000
                for i=1 to freq
                        debug "S",dec i
                        gosub EERead
                        WordAdd=WordAdd+dataSize
                        pause 10
                next
                debug "S"
                goto cycle

SendAck:        low SDA
                shiftout SDA,SCL,1,[0\1]
                return

NoAck:          high SDA
                shiftout SDA,SCL,1,[1\1]
                return

PollAck:        shiftin SDA,SCL,1,[AckResp\1]
                if AckResp=1 then HoldAck
                output SDA  'this is critical
                return

HoldAck:        pause 50
                output SDA
                debug "Ack Failure!!!!!",cr
                end
                return

EEStart:        high SDA
                high SCL
                low SDA
                return
```

```
EEStop:     high SCL
            high SDA
            pause 2
            return

SendDta:    shiftout SDA,SCL,1,[TheData\8]
            return

GetDta:     shiftin SDA,SCL,0,[TheData\8]
            return

SetAdd16:   gosub EEStart
            TheData=EEBase
            gosub SendDta
            gosub PollAck
            TheData=WordAdd.highbyte
            gosub SendDta
            gosub PollAck
            TheData=WordAdd.lowbyte
            gosub SendDta
            gosub PollAck
            'debug "Addressed ",dec WordAdd,cr
            return

SetAdd8:    gosub EEStart
            TheData=ClockBase
            gosub SendDta
            gosub PollAck
            TheData=WordAdd.lowbyte
            gosub SendDta
            gosub PollAck
            return

EEPrep:     input HANDSHAKE
            if in15=0 then EERead
            low HANDSHAKE
            WordAdd=$FFFF
            gosub SetAdd16
            TheData=%00000010
            gosub SendDta
            gosub PollAck
            gosub EEStop
            input HANDSHAKE
            return

EEWrite:    input HANDSHAKE
            if in15=0 then EERead
            low HANDSHAKE
            gosub SetAdd16
            TheData=EEData.lowbyte
            gosub SendDta
            gosub PollAck
            TheData=EEData.highbyte
            gosub SendDta
            gosub PollAck
```

```
                gosub EEStop
                'debug "Wrote ",dec EEData,cr
                input HANDSHAKE
                return

getNums:        input HANDSHAKE
                if in15=0 then getNums
                low HANDSHAKE
                WordAdd=$fffd
                gosub SetAdd16
                gosub EEStart
                TheData=EEBase+1
                gosub SendDta
                gosub PollAck
                EEData=0
                gosub GetDta
                gosub SendAck
                EEData=TheData
                gosub GetDta
                gosub NoAck
                EEData=EEData+(TheData*256)
                gosub EEStop
                input HANDSHAKE
                return

setNums:        WordAdd=$fffd
                gosub EEWrite
                return

EERead:         input HANDSHAKE
                if in15=0 then EERead
                low HANDSHAKE
                gosub SetAdd16
                gosub EEStart
                TheData=EEBase+1
                gosub SendDta
                gosub PollAck
                for temp=1 to ((dataSize-2)/2)
                        EEData=0
                        gosub GetDta
                        gosub SendAck
                        EEData=TheData
                        gosub GetDta
                        gosub SendAck
                        EEData=EEData+(TheData*256)
                        debug "E",dec EEData
                next
                EEData=0
                gosub GetDta
                gosub SendAck
                EEData=TheData
                gosub GetDta
                gosub NoAck
                EEData=EEData+(TheData*256)
                debug "E",dec EEData,cr
```

```
                gosub EEStop
                input HANDSHAKE
                return

ClockRD:        input HANDSHAKE
                if in15=0 then ClockRD
                low HANDSHAKE
                timedta=0
                gosub SetAdd8
                gosub EEStop
                gosub EEStart
                TheData=CLOCKBase+1
                gosub SendDta
                gosub PollAck
                gosub GetDta
                gosub NoAck
                gosub EEStop
                timedta=thedata.lownib
                timedta=timedta+((thedata.highnib)*10)
                input HANDSHAKE
                return

getseconds:     WordAdd=0
                gosub ClockRD
                return

getminutes:     WordAdd=1
                gosub ClockRD
                return

gethours:       WordAdd=2
                gosub ClockRD
                return

gettime:        gosub getminutes
                temp=timedta
                gosub gethours
                timedta=temp+(timedta*100)
                goto gottime

getdays:        WordAdd=4
                gosub ClockRD
                return

getmonth:       WordAdd=5
                gosub ClockRD
                return

getdate:        gosub getdays
                temp=timedta
                gosub getmonth
                timedta=temp+(timedta*100)
                goto gotdate

getyear:        WordAdd=6
```

```
                gosub ClockRD
                return

getperiod:      WordAdd=8
                gosub ClockRD
                temp=timedta
                WordAdd=9
                gosub ClockRD
                timedta=(timedta*100)+temp
                return

getfreq: WordAdd=10
                gosub ClockRD
                temp=timedta
                WordAdd=11
                gosub ClockRD
                timedta=(timedta*100)+temp
                return

'getnumsam:     WordAdd=12
'               gosub ClockRD
'               temp=timedta
'               WordAdd=13
'               gosub ClockRD
'               timedta=(timedta*100)+temp
'               return

getConfig:      WordAdd=14
                gosub ClockRD
                review=(timedta & %00000001)/1
                logged=(timedta & %00000010)/2
                auto=  (timedta & %00000100)/4
                return


memFull:        debug "Memory Full!!!",cr
                debug? light
                debug? timedta
                if in13=0 then memfull
                goto cycle

memClear:       input HANDSHAKE
                if in15=0 then memClear
                low HANDSHAKE
                output LCD
                serout LCD,N96N,[254,1]
                serout LCD,N96N,[254,128,"Hold to Clear"]
                pause 2000
                if in14=0 then contClear
                serout LCD,N96N,[254,1]
                input HANDSHAKE
                input LCD
                goto cycle

contClear:      serout LCD,N96N,[254,192,"Memory Cleared"]
```

```
'debug "Memory Cleared!!!",cr
EEData=0
gosub SetNums
input HANDSHAKE
input LCD
goto cycle
```

SNARE PIC user-interface microcontroller source code

```
define          osc     10
column          var     byte
key             var     byte
oldkey          var     byte
i               var     byte
messageAdd      var     byte
oldmessageAdd   var     byte
CONT            var     byte
EEin            var     byte
EEAdd           var     byte
EEMessage       var     byte
LCDAdd          var     byte
num_entry       var     word
thebit          var     bit
Auto            var     bit
Review          var     bit
Logged          var     bit
Sadd            var     byte
Sdata           var     byte
row1            var     portb.4
row2            var     portb.5
row3            var     portb.6
row4            var     portb.7
SDA             var     porta.0
SCL             var     porta.1
LCDpin          var     porta.3

trisa.2=1
pause 1000
Sadd=1
Sdata=0
gosub serialOut
trisa.3=1

loop:           messageAdd=16
                'gosub DisplayMessage
scrollMain:     gosub keyloop
backtomain:     if key="e" then jumpMain
                if key="u" and messageAdd>16 then decrement
                if key="d" and messageAdd<21 then increment
maindisplay:    oldMessageAdd=messageAdd
                messageAdd=0
                gosub DisplayMessage
                messageAdd=oldMessageAdd
                gosub DisplayMessage
                if messageAdd=18 then autoflip
                if messageAdd=19 then reviewflip
                if messageAdd=20 then logflip
                goto scrollMain
```

```
decrement:      messageAdd=messageAdd-1
                goto maindisplay
increment:      messageAdd=messageAdd+1
                goto maindisplay


jumpMain:       if messageAdd=16 then
                        messageAdd=1
                        gosub DisplayMessage
                        gosub get_number
                        num_entry=num_entry-1999
                        EEAdd=6
                        gosub clockWrite

                        messageAdd=2
                        gosub DisplayMessage
                        gosub get_number
                        EEAdd=4
                        gosub clockWrite
                        EEAdd=5
                        num_entry=num_entry/100
                        gosub clockWrite

                        messageAdd=3
                        gosub DisplayMessage
                        gosub get_number
                        EEAdd=1
                        gosub clockWrite
                        EEAdd=2
                        num_entry=num_entry/100
                        gosub clockWrite

                        EEAdd=0
                        num_entry=0
                        gosub clockWrite

                        messageAdd=0
                        gosub DisplayMessage
                        messageAdd=16
                        gosub DisplayMessage
                endif
                if messageAdd=17 then
                        messageAdd=4
                        gosub DisplayMessage
                        gosub get_number
                        EEAdd=8
                        gosub clockWrite
                        EEAdd=9
                        num_entry=num_entry/100
                        gosub clockWrite
                        messageAdd=0
                        gosub DisplayMessage
                        messageAdd=17
                        gosub DisplayMessage
                endif
```

```
                if messageAdd=21 then
                        Sadd=1
                        Sdata=0
                        gosub serialOut
                        trisa.2=1
                        trisa.3=1
                endif
                goto scrollMain

autoflip:       thebit=auto
                gosub bithandler
                if thebit<>auto then
                        auto=thebit
                        if auto=1 then getautoparms
autoreturn:             gosub updateConfig
                        goto autoflip
                endif
                goto backtomain


reviewflip:     thebit=review
                gosub bithandler
                if thebit<>review then
                        review=thebit
                        gosub updateConfig
                        goto reviewflip
                endif
                goto backtomain

logflip:        thebit=logged
                gosub bithandler
                if thebit<>logged then
                        logged=thebit
                        gosub updateConfig
                        goto logflip
                endif
                goto backtomain

bithandler:     Sadd=200
                Sdata=127-thebit
                gosub serialOut
                gosub keyloop
                if key="n" then
                        thebit=~thebit
                endif
                return

get_number:     messageAdd=31
                gosub DisplayMessage
                num_entry=0
                for i=0 to 5
                        gosub keyloop
                        if key="e" then decode_entry
                        if key="c" then get_number
                        if key<=57 then
```

```
                        Sadd=192+i
                        Sdata=key
                        gosub serialOut
                        key=key-48
                        num_entry=(num_entry*10)+key
                else
                        i=i-1
                endif
        next i
        return
        decode_entry:   return

keyloop:        gosub getkey
                if key=oldkey then keyloop
                oldkey=key
                if key>=16 then keyloop
                lookup key,["123u456d789nc0he"],key
                trisa.2=1
keywait:        If porta.2=0 then keywait
                trisa.2=0
                porta.2=0
                trisa.3=0
                return

getkey:         portb.0=1
                portb.1=1
                portb.2=1
                portb.3=1
                for column = 0 to 3
                        low column
                        if row1=0 then firstrow
                        if row2=0 then secondrow
                        if row3=0 then thirdrow
                        if row4=0 then fourthrow
                        high column
                next column
                key=16
                return

firstrow:       key=column
                return
secondrow:      key=column+4
                return
thirdrow:       key=column+8
                return
fourthrow:      key=column+12
                return

DisplayMessage:         EEMessage=messageAdd
                        if EEmessage<=15 then
                                CONT=%10100000
                                LCDAdd=128
                        else
                                CONT=%10100010
                                EEmessage=EEmessage-16
```

```
                LCDAdd=192
        endif
        EEmessage=EEmessage*16
        for i=0 to 15
                gosub EEDisplay
        next i
        return

EEDisplay:      EEAdd=EEmessage+i
                i2cread porta.0,porta.1,CONT,EEAdd,[EEin]
                Sadd=LCDAdd+i
                Sdata=EEin
                gosub serialOut
                return

getautoparms:           messageAdd=5
                        gosub DisplayMessage
                        gosub get_number
                        EEAdd=10
                        gosub clockWrite
                        EEAdd=11
                        num_entry=num_entry/100
                        gosub clockWrite

                        messageAdd=0
                        gosub DisplayMessage
                        messageAdd=18
                        gosub DisplayMessage
                        goto autoreturn

clockWrite:     CONT=%11010000
                EEin=( ( (num_entry dig 1) *16 ) + (num_entry dig 0) )
                i2cwrite porta.0,porta.1,CONT,EEAdd,[EEin]
                pause 10
                return

serialOut:      serout2 porta.3,$4054,[254,Sadd,Sdata]
                return

updateConfig:   num_entry=(auto*4)+(logged*2)+(review)
                EEAdd=14
                gosub clockWrite
                return
```

SNARE PIC EEPROM message transfer code

```
define      osc     10
CONT        var     byte
address     var     byte
SCL         var     porta.1
SDA         var     porta.0

pause 1000


CONT=%10100000
Address=0
i2cwrite SDA,SCL,CONT,Address,["USE ARROW KEYS  "]
pause 100
Address=16
i2cwrite SDA,SCL,CONT,Address,["YYYY          "]
pause 100
Address=32
i2cwrite SDA,SCL,CONT,Address,["MMDD          "]
pause 100
Address=48
i2cwrite SDA,SCL,CONT,Address,["HHMM          "]
pause 100
Address=64
i2cwrite SDA,SCL,CONT,Address,["COUNT TIME (MS) "]
pause 100
Address=80
i2cwrite SDA,SCL,CONT,Address,["FREQ (SAM/HOUR) "]
pause 100
Address=96
i2cwrite SDA,SCL,CONT,Address,["NUM OF SAMPLES  "]
pause 100

CONT=%10100010
Address=0
i2cwrite SDA,SCL,CONT,Address,["SET CLOCK      "]
pause 100
Address=16
i2cwrite SDA,SCL,CONT,Address,["SET PERIOD     "]
pause 100
Address=32
i2cwrite SDA,SCL,CONT,Address,["MANUAL     AUTO"]
pause 100
Address=48
i2cwrite SDA,SCL,CONT,Address,["ACQUIRE   DNLOAD"]
pause 100
Address=64
i2cwrite SDA,SCL,CONT,Address,["NO SAVE   LOGGED"]
pause 100
Address=80
i2cwrite SDA,SCL,CONT,Address,["DONE          "]
pause 100
Address=240
```

```
i2cwrite SDA,SCL,CONT,Address,["              "]
pause 100

serout2 porta.3,$4054,[254,128,"DONE"]
```

Appendix D: The determination of the redox status of the eluent of a dithionite reduced soil column

An experiment was conducted in an attempt to establish the relationship between the speciation of immobilized redox indicators and the efficiency of the abiotic transformation of TCE in a soil column amended with dithionite (a reducing agent). This experiment, conducted in collaboration with Mark Humphrey, Ralph Reed, Jack Istok, and Jennifer Field, was funded by PNNL. The overall objective of the study was to evaluate the degree of reductive dechlorination of TCE in water by soil treated with dithionite to reduce some of the iron to Fe(II). A correlation between the fraction of reduced iron and the capacity for TCE reduction was expected.

Experimental

To study this transformation, a large ported chamber was packed with soil collected from Ft. Lewis air force base. All particles greater than 2 inches in diameter were removed, and the column (approximately 2-ft wide, 4-ft tall, and 1-ft deep) was packed with the soil. A FMI pump was used to force solution from an inlet carboy reservoir up through the soil column and out into a collection reservoir. Ports were drilled into the column at three points to provide spatial sampling.

At the beginning of the experiment a dithionite solution (pH ~10) was pumped through the column and allowed to react with the soil for several days. After this amendment period, the inlet carboy was replaced with a deaerated solution of 1.8

mg/L TCE and Br^- tracer. This solution was pumped through the reduced soil column at ~1mL/min for ~3 weeks. Samples for the inlet, outlet, and three sampling ports were collected daily and analyzed (headspace) for TCE and ethene concentrations.

The redox status of the column effluent was determined by measuring the absorbance of immobilized redox indicators. For this study the redox indicators thionine and cresyl violet were covalently immobilized to cellulose acetate dialysis membranes (immobilization details are provided in Appendix H). These indicators are colored in the oxidized form and colorless in the reduced form. A 5-mm square piece of the sensor material was placed inside a flow cell (detailed in Appendix G) connected to the outlet of the column. This cell was connected in series with an electrode flow cell fitted with a combination Pt electrode which was also useful in measuring the redox potential of some systems.

Each day the flow cell was removed from the flow path and replaced by another cell with a new sensor material. The flow cell was fitted with quick connect fittings (Cole Parmer, Inc) with valves on both the male and female halves to prevent the introduction of oxygen. The removed flow cell was then transported to a HP 8452A diode array spectrometer, and the absorption spectrum of the redox indicator from 400 to 800 nm versus an air reference was calculated.

Information was extracted from the spectra as follows. For thionine, a line between the absorbances at 500 nm and 710 nm was used to establish a baseline. The difference between this baseline and the absorbance measured at the band maximum of 636 nm was then calculated. For cresyl violet, the absorbances at 450 nm and 710 nm defined the baseline, and the difference between this baseline and the absorbance

at the band maximum at 562 nm was calculated. These corrected absorbances were then normalized by dividing by the largest value of corrected absorbance for that indicator measured over the course of the experiment to give a measure of the degree of oxidation. This parameter is denoted f_{ox}' or the apparent fraction of the oxidized indicator. The value of f_{ox}' is systematically larger than actual f_{ox} values because the value used for the maximum absorbance is smaller than the true maximum due to degradation of the immobilized indicator.

The system was configured with a Pt combination redox electrode (Orion 9678BN) placed in a flow through cell at the outlet of the column. A Campbell data logger was used to measure the potential difference between the Pt electrode and the Ag/AgCl reference solution every 15 min throughout the experiment.

Results and Discussion

The calculated values of the fraction of oxidized thionine and cresyl violet, f_{ox}', are summarized for in Tables D-1 and D-2, respectively. The time dependence of f_{ox}' of both thionine and cresyl violet and the concentration of TCE at the column outlet is shown in Figure D-1. Initially the indicators were reduced, and the concentration of TCE was low. Apparently the dithionite-amended column had appreciable reductive capacity and dechlorination was reasonably efficient.

Table D-1. Degree of thionine oxidation

Time	Date	f_{ox}'
11:15 AM	06/04/99	0.06
10:55 AM	06/07/99	-0.01
11:36 AM	06/16/99	0.88
12:41 PM	06/19/99	0.55
11:28 AM	06/20/99	0.56
12:41 PM	06/21/99	1.00
10:44 AM	06/22/99	0.96
10:40 AM	06/23/99	0.61

Table D-2. Degree of cresyl violet oxidation

Time	Date	f_{ox}'
11:50 AM	06/08/99	0.43
11:20 AM	06/09/99	0.44
10:50 AM	06/10/99	0.77
09:09 AM	06/11/99	0.91
11:37 AM	06/14/99	1.00
10:16 AM	06/18/99	0.98

For a redox indicator to be reduced, some soluble reduced species with a sufficiently negative redox potential must be present in the effluent from the column. In this case this soluble reduced species was most likely Fe(II), dithionite, or some dithionite breakdown product that was slowly released from the column. It is hypothesized that the dithionite reduced iron present in the soil sample, and TCE passing through the column was reduced on the surfaces containing reduced iron. Between days 5 and 10 of the experiment, as the column apparently lost its reductive capacity, the indicators at the outlet were reduced less, and breakthrough of untransformed TCE increased.

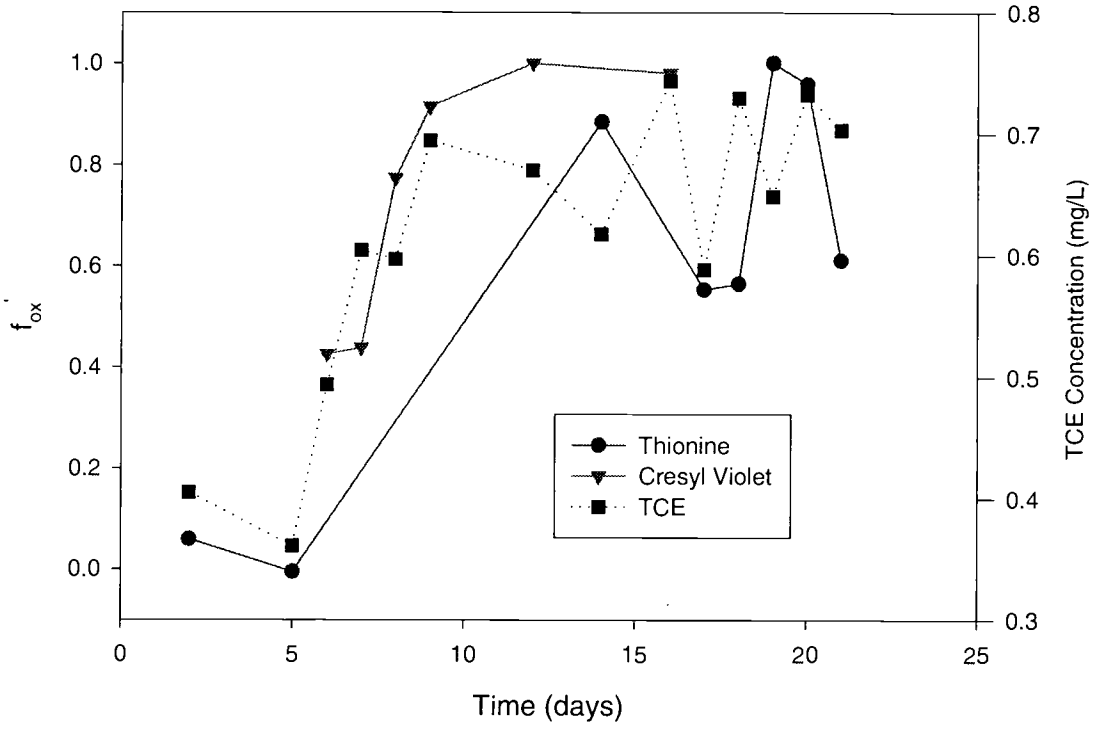


Figure D-1. The temporal relationship between degree of indicator oxidation and TCE concentration at the column outlet. Day 1 is June 4, 1999.

Figure D-2 shows there is a correlation between the concentration of TCE and the degree of oxidation of the redox indicators. A linear regression was performed on these data for each indicator and the equations D-1 and D-2 resulted where the TCE concentration is in mg/L.

$$[TCE] = 0.31 f'_{ox(thi)} + 0.42 \quad R^2 = 0.7 \tag{D-1}$$

$$[TCE] = 0.28 f'_{ox(CV)} + 0.42 \quad R^2 = 0.7 \tag{D-2}$$

Thionine is more easily reduced than cresyl violet. For a given concentration of reductant that partially reduces thionine, f_{ox}' of thionine should be less than f_{ox}' of cresyl violet. There is a limited range for which the fraction of oxidized indicator can track the concentration of a reductant, and this range typically spans one or two orders of magnitude. Values of f_{ox} near 0 or 1 provide little information when. Because the behavior of thionine and cresyl violet is similar under the conditions in this experiment (they both track [TCE]), the soluble species responsible for the reduction of the indicators is more likely a strong reductant such as dithionite rather than a mild reductant such as Fe(II). However at higher pH, Fe(II) is a much stronger reductant than near neutral pH were Fe(II) must be on the order of 10 μM to reduce cresyl violet (1).

All of the potentials measured with the Pt electrode in a single day were averaged together and corrected for the potential of the reference solution (0.242 V). The potential data plotted in Figure D-3 are not well correlated with the TCE concentration at the column outlet as were the redox indicator data.

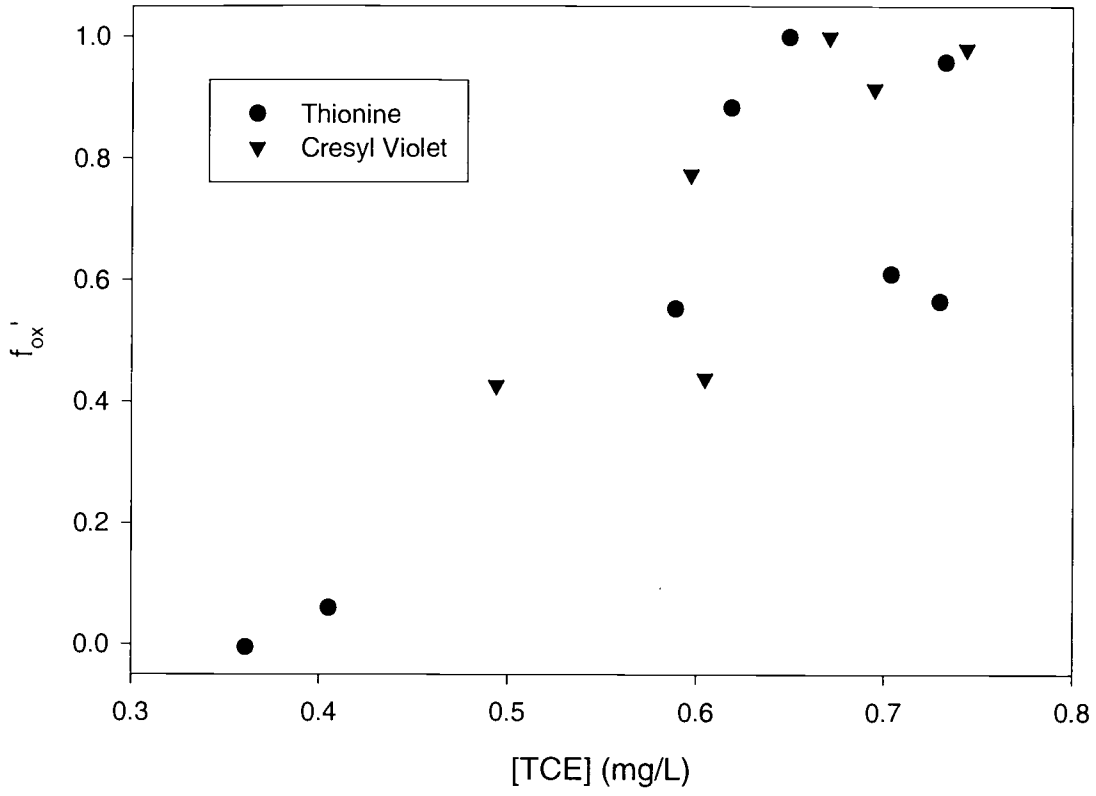


Figure D-2. Relationship between apparent fraction of oxidized indicator and TCE concentration. For thionine, the standard error of the estimate of TCE concentration is 0.09 mg/L and R^2 is 0.69. For cresyl violet, the standard error of the estimate of TCE concentration is 0.05 mg/L and R^2 is 0.72.

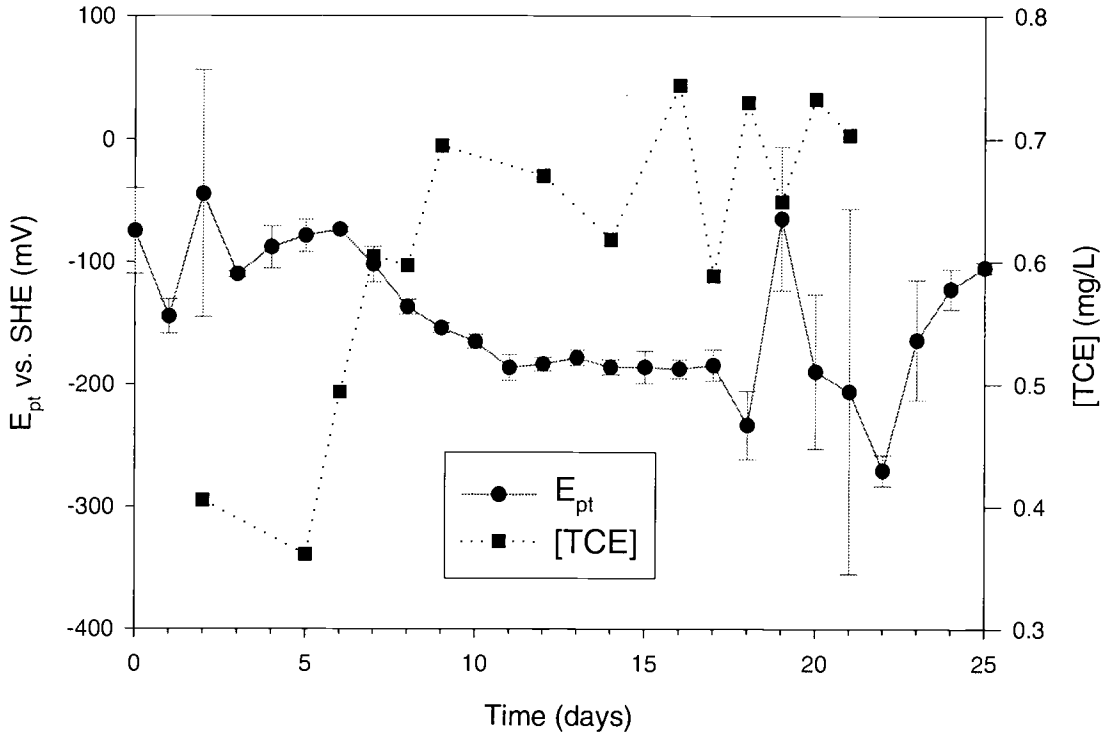


Figure D-3. Average platinum electrode readings from the column outlet. The error bars represent plus and minus the standard deviation of readings taken every 15 min for one day.

The high ionic strength and alkalinity of the dithionite solution caused the degradation of the dialysis membranes. After 24 hr of exposure to the eluent (which was pH 9 to 11 for most of the experiment), the sensor material was extremely fragile and had to be replaced and could not be returned to its fully oxidized absorbance value. This irreversibility in the sensor material makes it impossible to calculate the true fraction oxidized, which is more easily related to quantities such as Fe(II) concentration. This lack of durability in the immobilized material led to refinements in the immobilization process described in Appendix H.

Conclusions

This experiment demonstrated that monitoring the speciation of an immobilized indicator in a flow cell can provide direct information about the redox state of the effluent from a column and information about the redox state of material in a column. Furthermore, the experiment also demonstrated that immobilized redox indicators might be useful as "break-through" monitors for reductive barriers used to contain contaminant plumes. In this experiment the indicator was in its oxidized form at the beginning of each day, and the subsequent reduction of the indicator in the flow stream was a positive indication of the presence of a soluble reductant at concentrations sufficient to reduce the indicator. A potential problem with using this sensor as a break-through type monitor is insuring there is some species that will re-oxidize the indicator if becomes reduced during the course of its deployment.

The data from this experiment are difficult to interpret because the speciation of the redox indicator is not monitored during deployment. This situation made it

difficult to establish the absorbance of the indicator in its fully oxidized and reduced forms. Furthermore, the possible introduction of oxygen during sample analysis could have affected the speciation of the indicator. For these reasons it became clear that real-time monitoring would be of great benefit for this type of application. This need for a complete redox sensor, that is small, inexpensive, and adaptable, lead to the SLIM (2) discussed earlier in this thesis.

References

1. Jones, Brian D., *Applications of Redox Indicators for Evaluating Redox Conditions in Environmental Samples*, **1999**, Ph.D. Thesis, Oregon State University.

2. Chapter 1 of this thesis.

Appendix E: A stand-alone spectrometer and flow cell for use with immobilized redox indicators for the determination of the reductive capacity of a dithionite reduced soil column

Introduction

An experiment was conducted in an attempt to measure the reductive capacity of a soil column that was field treated with a dithionite (a reducing agent) solution. These experiments, conducted in collaboration with Mark Humphrey and Jack Istok, were funded by PNNL. In appendix D a laboratory evaluation is described that deals with the process in which soil was amended with dithionite to promote the abiotic transformation of TCE.

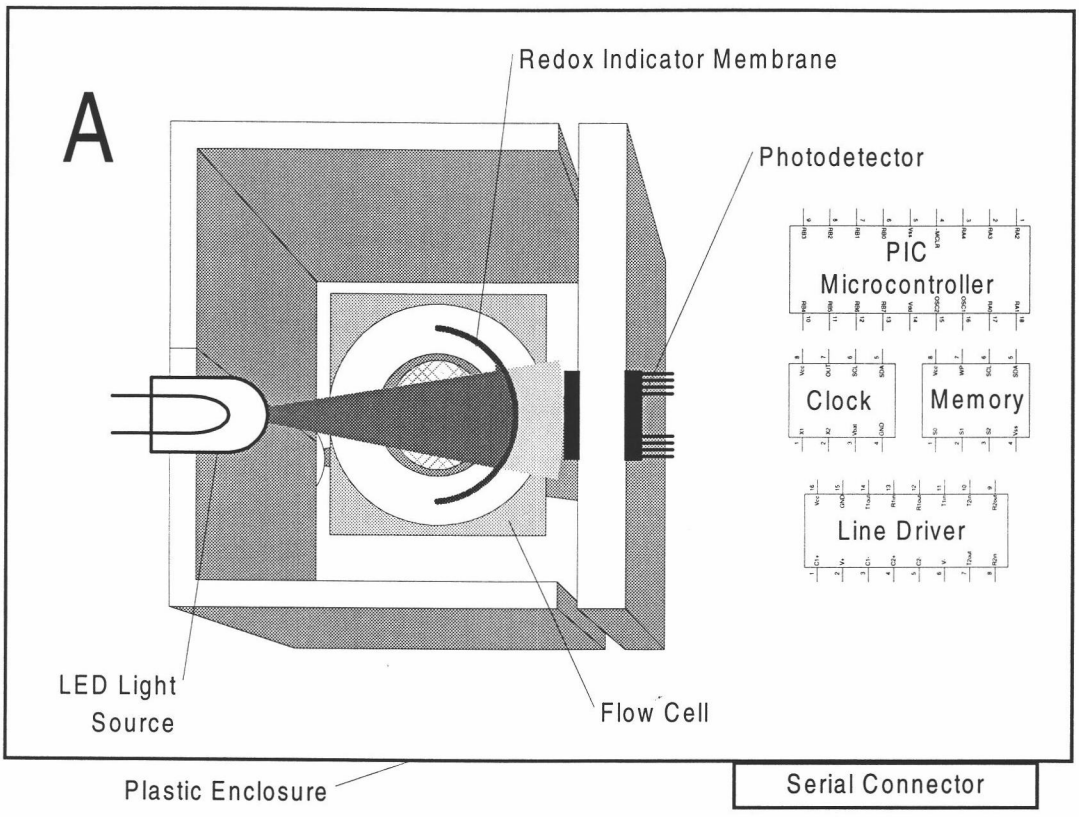
Experimental

In this work, soil samples from an area treated on site with dithionite at Ft. Lewis were provided. In an anaerobic glove box, these soil samples were opened, the large particles (>1 cm) were removed, and the remaining samples were packed into columns (~1-in diameter by 12-in long) with ports and filters on each end to accommodate tubing connectors for a flow experiment. An FMI pump was used to direct oxygenated water from a reservoir, through the column, and out to waste.

A Kloehn syringe pump with a distribution value was used to remove aliquots from the flow stream at points both before and after the soil column. These aliquots were then directed to an electrochemical dissolved oxygen probe that was connected to a data logger.

The redox monitoring system is based on the **S**tand-alone **T**wo **D**etector, or STanD, spectrometer and a custom flow cell that holds films with immobilized redox indicators. For this work, the "L" shaped flow cell containing the immobilized redox indicator thionine was installed between the soil column and the waste reservoir and is described in Appendix G. The STanD spectrometer/data logger was also designed to work with standard 12.5-mm (square on outside) cuvettes. A diagram of this spectrometer is provided in Figure E-1, and a schematic and parts list are found in Figure E-2. The artwork for the circuit board is included as Figure E-3. The source code for the PIC 16F84 microcontroller (in PIC BasicPro), the PC based software for data transfer (in Visual Basic), and a few notes about the actual operation of the device are provided at the end of this appendix.

An embedded microcontroller controls the LED light sources, measures the output of the photodetector IC, computes a time stamp, stores the data in nonvolatile memory, and enters a low-power sleep mode for a programmed time interval. In addition to data collection and storage, the control electronics also communicate with a PC via a serial connection. The primary electronic components used to achieve the functionalities outlined above are an 8-bit embedded microcontroller (Microchip Technologies Incorporated PIC-16F84), a 128K-bit serial EEPROM (Xicor X24128) for nonvolatile data storage, a serial real time clock (Dallas Semiconductor DS1307) for timekeeping, and a photodetector (Texas Instruments TSL230). The photodetector is based on a photodiode and current-to-frequency conversion and features programmable gain control. The device is housed in a plastic enclosure and the cuvette holder is constructed from Teflon.



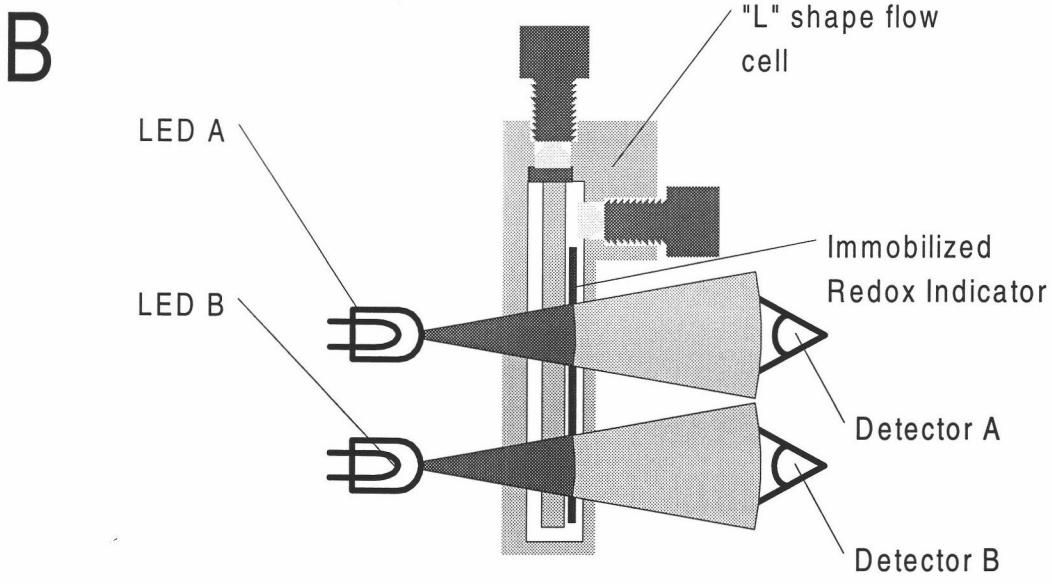
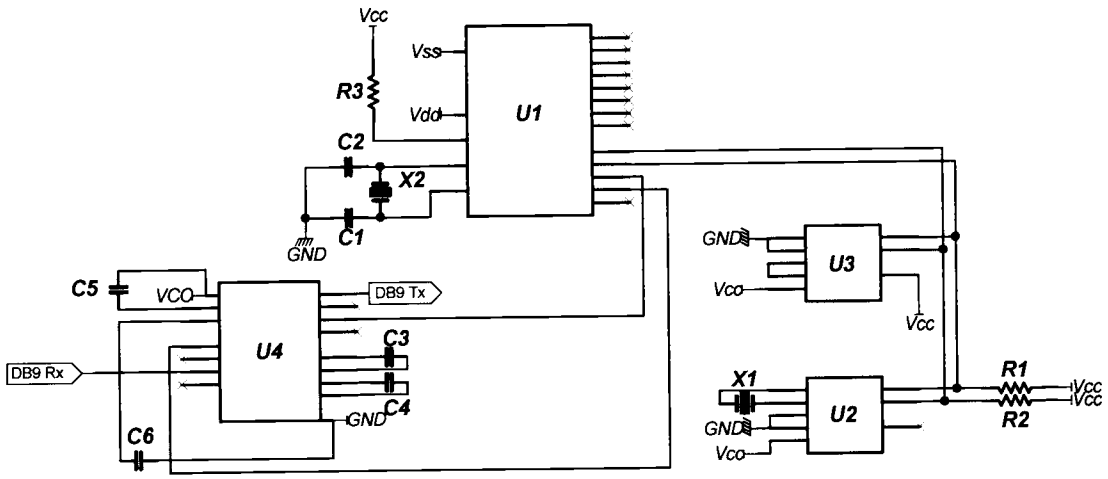


Figure E-1. A diagram of the STanD spectrometer and flow cell. Drawing A shows the entire STanD spectrometer including the housing, optical components, and electronics. Drawing B shows the two light source configuration and the "L" shaped flow cell.



Reference	Value	Type
U1	PIC16F84	Microcontroller
U2	DS1307	Real time clock
U3	X24128	Serial EEPROM
U4	MAX232	TTL to RS232 converter
X1	32 kHz	Clock crystal
X2	4 MHz	MCU crystal
C1,C2	22 pF	Capacitor
C3,C4,C5,C6	1 μF	Capacitor
R1,R2,R3	4.7 kΩ	Resistor

Figure E-2. A schematic and parts list of the STanD spectrometer

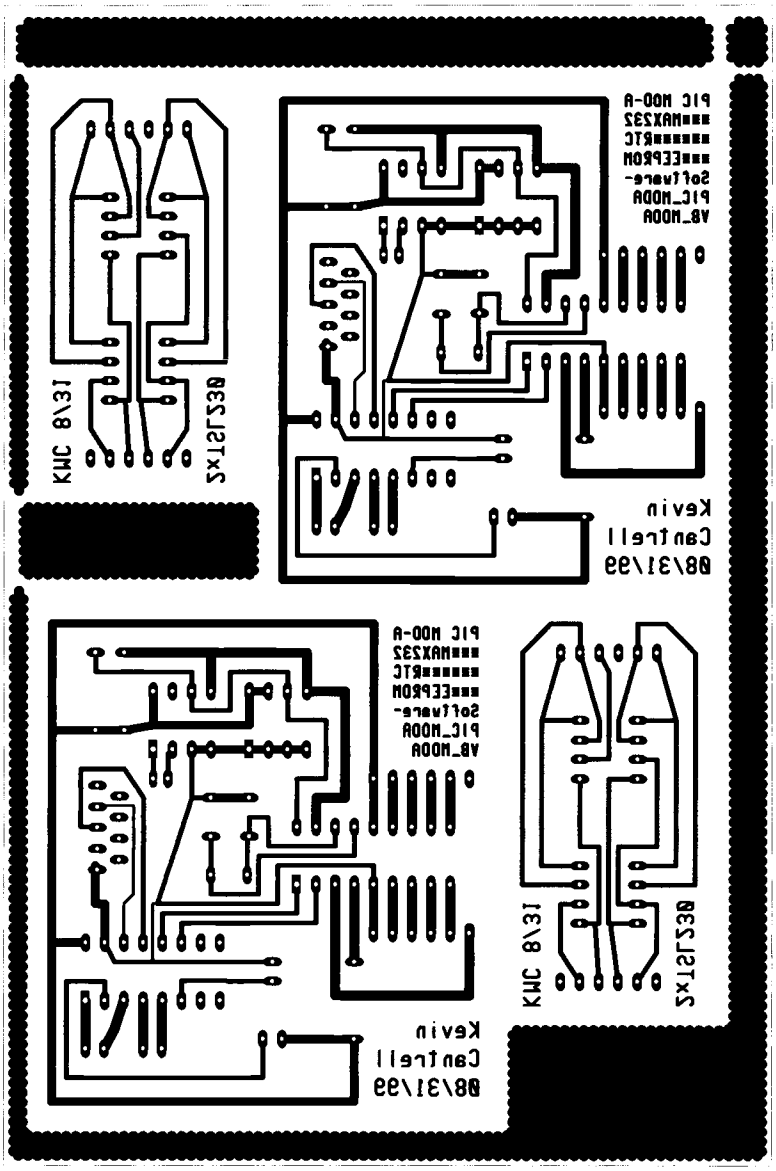


Figure E-3. Circuit board artwork for the STanD spectrometer.

When the STanD is powered on, the internal clock is reset to midnight of day 1. The operator must note the time and add this base time to all of the recorded time stamps. The control electronics send all stored data out to the serial connection, and then begins polling the state of the pushbutton switch that initiates data collection. If this button is pushed and the device is simultaneously turned on, all data stored in the memory are cleared; otherwise all data are appended to the existing data.

Once in the data collection mode, the device writes the address of the next usable memory location to a fixed address in the EEPROM. It then reads and stores the number of days, minutes, hours, seconds that have elapsed since the device was powered. Each LED is then sequentially powered for 1 s and the photodetector signal is recorded (for the ambient signal for detectors A and B, red LED illuminated, green LED illuminated, and IR illuminated cases). Finally the device enters a low power consumption mode, which it exits to initiate a new round of data collection after a programmed period of time.

This spectrometer employs a dual color (red/green) LED and an IR LED (~920 nm band maximum) as light sources. One photodetector is used for the dual color LED and another for the infrared LED. Although the pair of sources and photodetectors sample a different portion of the optical cell, the IR LED does serve as an effective reference because light at this wavelength is unabsorbed by thionine.

To determine the absorbance just due to thionine, the difference between the total absorbance of the indicator film (A_{ind}) measured with the red LED and the baseline absorbance (A_{base}) is calculated from the photodetector signals for the absorbed (S_{ind}) and unabsorbed (S_{base}) wavelengths according to equation E-1. The

ratio of the blank intensities (R) for the indicator (S_{blank}) (totally reduced) and baseline (S'_{blank}) is assumed to be constant.

$$A_{ind} - A_{base} = -\log\left[\frac{S_{ind}}{S_{base}}\right] + R \qquad where\ R = \log\left[\frac{S_{blank}}{S'_{blank}}\right] \qquad \text{(E-1)}$$

In the future, designs based on a single optical path and photodetector will be considered so that corrections for bubbles, particulate matter, and other inhomogeneities in the flow stream are possible.

The electronic components of the sensor are very similar to the SLIM device previously described in the Chapter 2. Unlike the SLIM, this circuit does include a MAX232 (Maxim, Inc.) converter chip that translates the TTL voltage output (0 and 5 V) of the microcontroller into the RS232 specified levels of the PC (~ ±12V). The power consumption of this chip (which uses several capacitors in a charge pumping technique to generate voltages in excess of the power supplies) is quite large, and it is not suitable for a battery-powered application. In this experiment, a 12-VDC unregulated wall transformer and a 5-V regulator were used to power the device.

The general approach used for this experiment is as follows. Water equilibrated with air (with a relatively constant oxygen content) is passed through the reduced soil in the column at a flow rate of ~0.4 mL/min. Dissolved O_2 is consumed as it oxidizes the reduced species (most likely Fe(II)) on the surfaces of the soil particles. The membrane with the immobilized redox indicator thionine is inserted into the flow cell and reduced before the flow cell is connected to the outlet of the soil column. After most of the reduced species on the soil particles have been oxidized, O_2

will begin to break through the soil and oxidize the redox indicator. Hence, thionine will change from its colorless reduced form to a blue oxidized form after the soil's reductive capacity is mostly exhausted.

The immobilized thionine was reduced in the flow cell with an ascorbic acid solution. This solution was prepared by dissolving ~3.5 g ascorbic acid in 40 mL DI water (~0.5 M) in an ICHEM vial. The vial was then N_2 purged via the septum (long needle into bottom, short needle for release) for ~5 min. With a luer tip syringe, 5 mL of this ascorbic acid solution was passed through the cell. The cell was then capped with two ¼*28 plugs and stored overnight until the indicator was clear.

Results and Discussion

Data from three experiments with soil reduced in the field are plotted in Figure E-4. Samples A and B had not been exposed to O_2, but sample C had already been titrated with oxygenated water. The fraction of oxidized indicator (f_{ox}) starts out near zero because the indicator was initially in its reduced form. Note that f_{ox} begins to increase after some time because the indicator was exposed to dissolved O_2 that had broken through the soil column. For the soil columns with greater reductive capacity (A and B), the delay this breakthrough is longer. The soil in column B has a greater reductive capacity than that in column A. Both columns clearly have higher reductive capacity than the soil in the control column, column C, that has already been fully oxygenated.

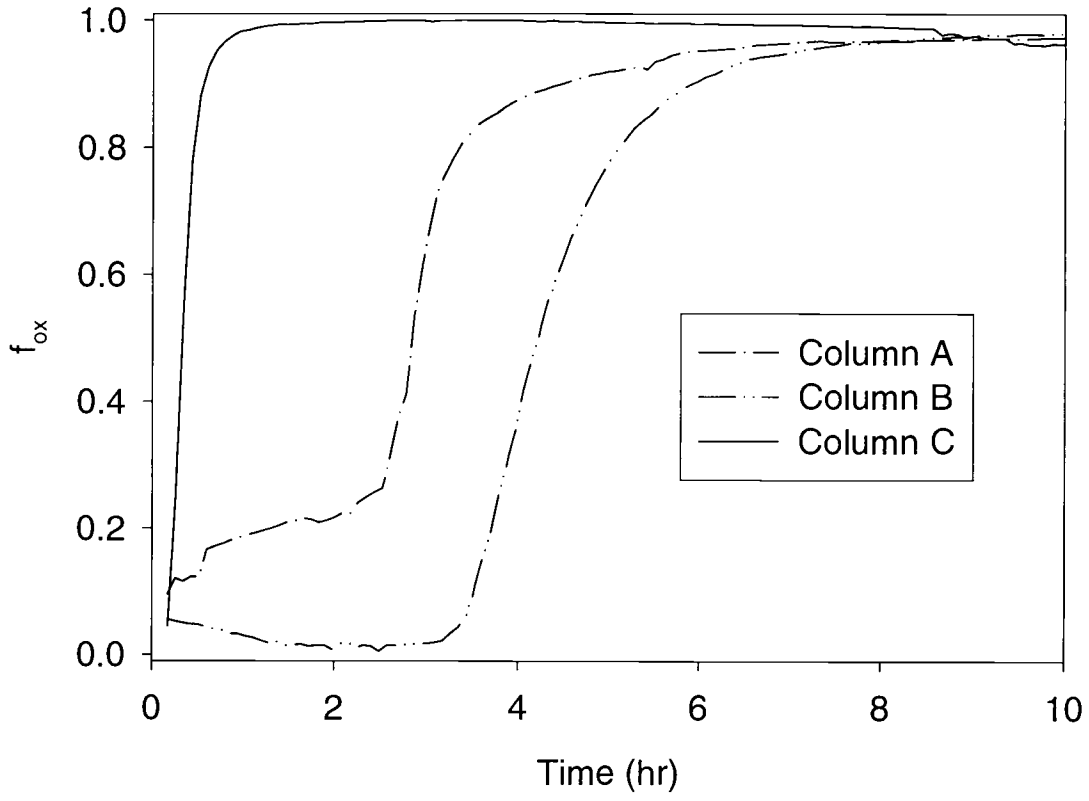


Figure E-4. The time dependence of indicator speciation for three soil cores. The fraction of oxidized indicator, f_{ox}, is calculated from the baseline corrected absorbance, where f_{ox} equals the absorbance at time *t* divided by the maximum observed absorbance over the course of the experiment. Here the absorbance is calculated according to the following relationship:

$$A = -\log\left(\frac{red\ LED\ signal}{IR\ LED\ signal}\right) + 0.23$$

Soils in columns A and B were taken from different areas of the field reduced site at Ft. Lewis. Column C was a control column with soil that had been previously exposed to O_2

Conclusions

This experiment demonstrated the use of immobilized redox indicators as a dissolved O_2 breakthrough sensor. If a convenient means to initially reduce indicators such as thionine (either chemically or electrochemically) in the flow cell can be devised, this sort of device can be used as a dissolved O_2 field sensor. Although a thorough comparison was not performed, it is hoped that this approach could detect lower levels of O_2 be more sensitive and less subject to calibration drift than conventional electrochemical dissolved oxygen probes.

STanD PIC microcontroller source code

```
DEFINE      OSC             4
DEFINE      DEBUG_REG       PORTA
DEFINE      DEBUG_BIT       3
DEFINE      DEBUG_BAUD      9600
DEFINE      DEBUG_MODE      0
'0 for max 232 or 1 for serial backpack

EEdataSize  con     14
DS          con     ","


i           var     byte
dark1       var     word
dark2       var     word
leda        var     word
ledb        var     word
ledc        var     word
sample      var     word
SDA         var     porta.0
SCL         var     porta.1
clockCon    var     byte
EECon       var     byte
i2cData     var     byte
i2c8Add     var     byte
i2c16Add    var     word
i2c16AddBackup      var     word
seconds     var     byte
minutes     var     byte
hours       var     byte
day         var     byte

porta=%11101011
trisa=%00010100
portb=%00000011
trisb=%00010000

pause 100

clockCon=%11010000
EECon=%10100000
```

```
i2c8add=6
i2cData=0               'year=   %00000000     '0
gosub clockwrite
i2cData=1               'month=%00000001     '1
gosub clockwrite
i2cData=1               'date=   %00000000     '1
gosub clockwrite
i2cData=1               'day=    %00000001     '1
gosub clockwrite
i2cData=0               'hours= %00000000     '0
gosub clockwrite
i2cData=0               'minutes=%00000000   '0
gosub clockwrite
i2cData=0               'seconds=%00000000   '0
gosub clockwrite

i2c16add=65535
i2cdata=2
gosub EEi2c
i2c16Add=0

if porta.4=1 then download
loop:           i2cread SDA,SCL,clockCon,0,[seconds,minutes,hours,day]

                portb=%00011110
                gosub measureLight
                dark1=sample

                portb=%00111110
                gosub measureLight
                leda=sample

                portb=%01011110
                gosub measureLight
                ledb=sample

                portb=%00010101
                gosub measureLight
                dark2=sample

                portb=%10010101
                gosub measureLight
                ledc=sample

                portb=%00010011

                gosub DataOut
                gosub EEWrite
                sleep 10
                goto loop
```

```
download:       i2cread SDA,SCL,EECon,16380,[i2c16AddBackup]
                for i2c16add=0 to i2c16AddBackup step EEdataSize
                        i2cread SDA,SCL,EECon,i2c16Add,
                                [minutes,hours,seconds,day,dark1,leda,ledb,dark2,ledc]
                        gosub DataOut
                next i2c16add
downloop:       if porta.4=1 then downloop
                goto loop

ClockWrite:     pause 20
                i2cwrite SDA,SCL,clockCon,i2c8Add,[i2cData]
                i2c8Add=i2c8add-1
                pause 20
                return

EEWrite:        i2c16AddBackup=i2c16Add
                i2c16add=16380
                i2cdata=i2c16AddBackup.byte1
                gosub EEi2c
                i2cdata=i2c16AddBackup.byte0
                gosub EEi2c
                i2c16add=i2c16AddBackup
                i2cdata=minutes
                gosub EEi2c
                i2cdata=hours
                gosub EEi2c
                i2cdata=seconds
                gosub EEi2c
                i2cdata=day
                gosub EEi2c
                i2cdata=dark1.byte1
                gosub EEi2c
                i2cdata=dark1.byte0
                gosub EEi2c
                i2cdata=leda.byte1
                gosub EEi2c
                i2cdata=leda.byte0
                gosub EEi2c
                i2cdata=ledb.byte1
                gosub EEi2c
                i2cdata=ledb.byte0
                gosub EEi2c
                i2cdata=dark2.byte1
                gosub EEi2c
                i2cdata=dark2.byte0
                gosub EEi2c
                i2cdata=ledc.byte1
                gosub EEi2c
                i2cdata=ledc.byte0
                gosub EEi2c
                return
```

```
EEi2c:          pause 20
                i2cwrite SDA,SCL,EECon,i2c16Add,[i2cdata]
                i2c16add=i2c16Add+1
                pause 20
                return

DataOut:        debug "S",dec (i2c16Add/EEdataSize),DS,dec day,DS,dec hours,DS,
                      dec minutes,DS,dec seconds,DS,dec dark1,DS,dec leda,DS,
                      dec ledb,DS,dec dark2,DS,dec ledc,13,10
                return

measureLight:   count portb.4,1000,sample
                return
```

STanD VB communication code

```
Dim inpstring$
Dim samNumber%
Dim nextVal%
Dim days As Long, hours As Long, minutes As Long, seconds As Long, blank1 As Long,
        ledA As Long, ledB As Long, ledC As Long, blank2 As Long
Dim dataRecord(1500, 6) As Single
Dim rawInString$
Private Sub cmdSave_Click()
Dim i%, j%
Dim fyle$

CommonDialog1.ShowSave
fyle = CommonDialog1.FileName
Open fyle For Output As #1
For i = 0 To samNumber
   For j = 1 To 6
      Print #1, Str(dataRecord(i, j)) + ",";
   Next j
   Print #1,
Next i
Close #1
End Sub

Private Sub Comm2_OnComm()
Dim serin$
Dim indata As Long
Dim i%

serin = Comm2.Input
For i = 1 To Len(serin)
   Char = Mid(serin, i, 1)
   If Char = "," Or Char = Chr(13) Then
      If InStr(inpstring, "S") Then
         samNumber = Val(Right(inpstring, Len(inpstring) - InStr(inpstring, "S")))
         LblRcv.Caption = samNumber
         SpecInter.Refresh
         nextVal = 1
      Else
         indata = Val(inpstring)
         If nextVal = 1 Then
            days = indata
         ElseIf nextVal = 2 Then
            hours = (indata And 15) + ((indata And 240)/16*10)
         ElseIf nextVal = 3 Then
            minutes = (indata And 15) + ((indata And 240)/16*10)
         ElseIf nextVal = 4 Then
            seconds = (indata And 15) + ((indata And 240)/16*10)
         ElseIf nextVal = 5 Then
            blank1 = indata
         ElseIf nextVal = 6 Then
            ledA = indata
         ElseIf nextVal = 7 Then
```

```
            ledB = indata
          ElseIf nextVal = 8 Then
            blank2 = indata
          ElseIf nextVal = 9 Then
            ledC = indata
            txtTime.Text = Str(days) + ":" + Str(hours) + ":" + Str(minutes) + ":" + Str(seconds)
            txtleda.Text = Str(ledA - blank1)
            txtledb.Text = Str(ledB - blank1)
            txtledc.Text = Str(ledC - blank2)
            dataRecord(samNumber, 1) = days + (hours / 24) + (minutes / 24 / 60) +
                  (seconds / 24 / 60 / 60)
            dataRecord(samNumber, 2) = ledA
            dataRecord(samNumber, 3) = ledB
            dataRecord(samNumber, 4) = ledC
            dataRecord(samNumber, 5) = blank1
            dataRecord(samNumber, 6) = blank2
          End If
          nextVal = nextVal + 1
        End If
        inpstring = ""
      Else
       inpstring = inpstring + Char
      End If
Next i
txtRawComm.Text = txtRawComm.Text + serin
'rawinsting = rawInString + serin
LblRcv.Visible = True
End Sub
Private Sub Form_Load()
Comm2.PortOpen = True
End Sub
```

Notes on the operation of the spectrometer

General operation:

Inlet is the top of the cell
Outlet is on the side of the cell
Orient cell so that the outlet points toward 12 o'clock
Cover cell with black cloth

To download data:

Turn power OFF on black box (slider down)
Connect 9-pin cable between black box and serial port Comm1
Run the Visual Basic executable program
Turn power ON on black box (slider up)
Wait until numbers stop increasing
Click on save data, type in filename, and click OK

To append new data: (without erasing the old data)

Follow procedure above for downloads

Note: the box does not have to be connected to the computer, but you should allow it some time to complete its download routine

Press black pushbutton until you see the red light come on

To collect new data and erase old data:

Turn unit off
Press and hold black push button while turning power on (slider up)
Release black pushbutton when you see the light come on

Appendix F: Immobilized pH indicators

This appendix contains preliminary results from work done with pH indicators, which might be used along with redox indicators in devices such as those presented in the Chapters 2, 3 and 4 of this thesis. As a part of her undergraduate research project, Daisy Hubbard (1) studied two pH indicators, neutral red and congo red, that contain primary amines. These indicators were immobilized to dialysis membranes by a scheme similar to the procedure for redox indicators described in Appendix H. The immobilized pH indicators were then evaluated for their reversibility, durability, kinetics, and useful pH range.

Neutral Red

The absorption spectra of neutral red at various pH values are provided in part A of Figure F-1. The pH was adjusted with pH buffers prepared from potassium dihydrogen phosphate, potassium acid phthalate, and borax. Like many pH indicators (but unlike redox indicators), neutral red is colored in both its acidic and basic forms. The pK_a of neutral red in the free form is ~6.7, based on an equilibrium model of the collected spectra.

Neutral red was immobilized onto a dialysis membrane that was activated by reaction for 5 min with sonication in a solution of ~40 mL of DI water containing ~0.25 g of periodate. The imine bond was formed in a solution of 10 mL of glacial acetic acid, 10 mL of ethanol, and 10 mL of ~2 mM neutral red. This solution was

allowed to react in the sonicator for ~30 min and then stored in the refrigerator overnight. The imine bond was then reduced to a 2° amine by the addition of ~0.3 g of $NaCNBH_4$ to a solution containing 10 mL of pH 4 buffer, 10 mL of ethanol, and 10 mL of ~2 mM neutral red. This process is different from the immobilization of redox indicators described in Appendix H in that the imine bond formation is separated from the subsequent reduction to a 2° amine. This modification yields a greater amount of immobilized indicator because the imine bond is formed at lower pH.

Absorption spectra of immobilized neutral red at various pH values are provided in part B of Figure F-1. Note that the absorbance maximum of both the acid and base forms of neutral red have shifted in the immobilization process. The maximum of the absorbance of the basic form occurs at a longer wavelength in the immobilized form, but the absorbance maximum of the acid form is at a shorter wavelength in the immobilized form. The baseline absorbance is approximately 0.4 AU for neutral red immobilized onto a dialysis membrane.

To determine the speciation of the pH indicator (the concentration ratio of acid to base forms), it is necessary to calculate the absorbance of the colored acid and base forms of the indicator. For neutral red, the absorbance a pH 2 was used as the baseline for the base peak and the absorbance at pH 10 was used as the baseline for the acid peak. After these baselines were subtracted, the wavelength maximum of the basic peak was found to be 478 nm and the wavelength maximum of the acid peak is 562 nm. The corrected absorbance at 478 nm (A_A) was taken as proportional to the concentration of the base form and was normalized by dividing by its maximum value to yield the fraction of the neutral red in the base form (α_A). In a similar fashion, the

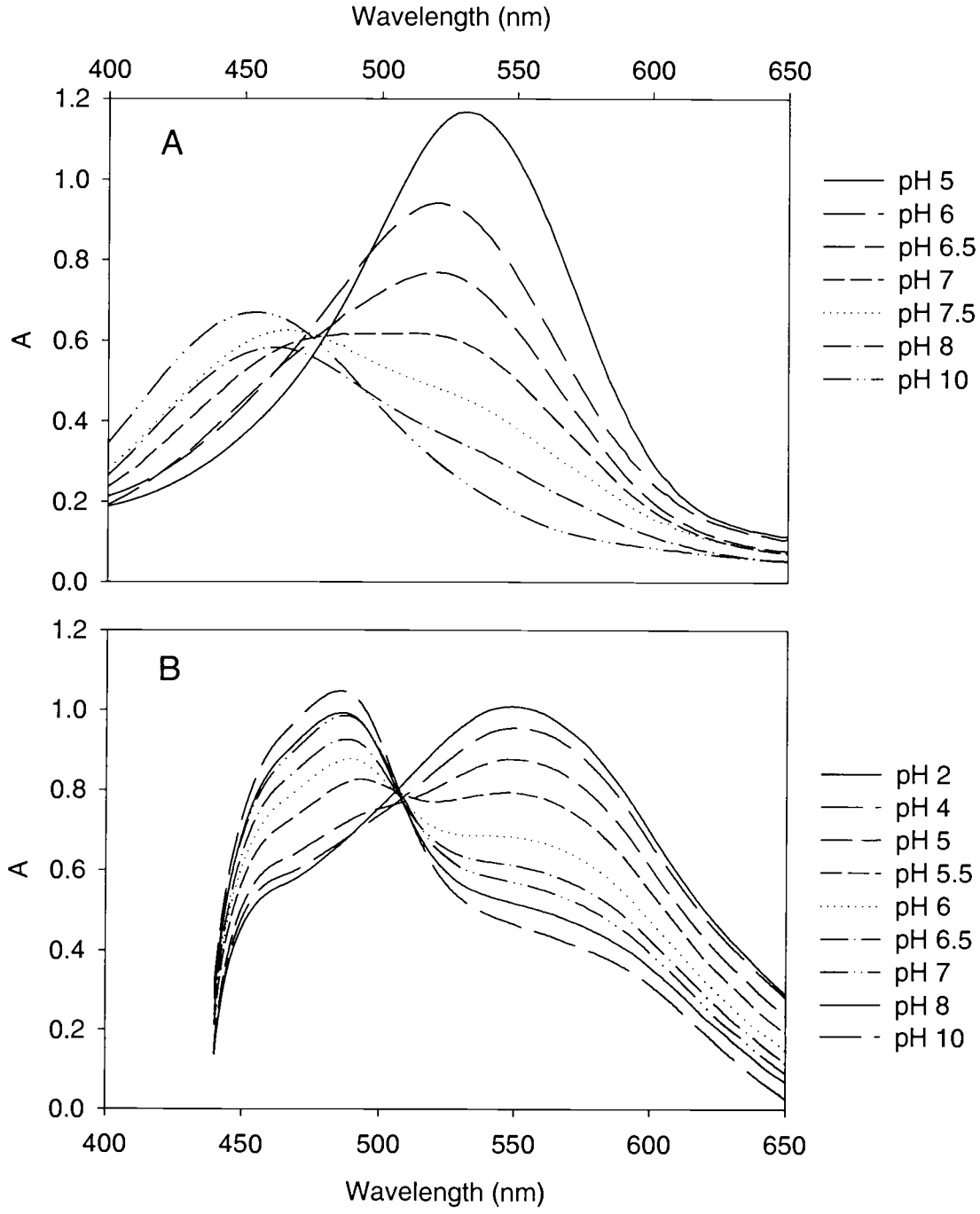


Figure F-1. Dependence of the absorption spectra of neutral red on pH. Graph A displays the spectra of neutral red in free solution taken with an HP diode array spectrometer. Graph B displays the spectra of neutral red immobilized onto dialysis membrane measured with an Ocean Optics S2000 spectrometer in a custom flow cell.

corrected absorbance at 562 nm (A_{HA}) was used to determine the fraction of indicator in acid form (α_{HA}).

In Figure F-2, the experimental and theoretical dependence of the fractions on the pH of the solution are shown. The theoretical curve is based on an equilibrium model for a monoprotic acid with a pK_a of 5.5 and the Henderson-Hasselbach equation (equation F-1) where [A] is the concentration of the base form and [HA] is the concentration of the acid form.

$$pH = pK_a + \log {[A]}/{[HA]} = pK_a + \log {A_A}/{A_{HA}} \qquad \text{(F-1)}$$

The pK_a was determined by adjusting the value to achieve the best "visual" fit and is about 1.2 pH units less than that of the free indicator form. Equation F-1 is rearranged to give the expression for the fraction of indicator in the acid form.

$$\alpha_{HA} = \frac{1}{1+10^{pH-pK_a}} \qquad \text{(F-2)}$$

The second form of equation F-1 was used to predict pH values from the corrected absorbance of the base and acid forms of the indicator, and the results are show in the Figure F-3 plotted as circles (without baseline correction). The solver tool of Excel was used to adjust the pK_a to minimize the sum of the squared residuals and a pK_a of 6 was chosen.

A second fit was based on the uncorrected absorbances (those for which baselines at the extreme pH values were not subtracted from the raw absorbance values). Both the pK_a and baseline values for the acid and base forms were adjusted

for the best fit of the predicted pH. The adjusted baseline values differ from those determined at the pH extremes by 0.03 AU and 0.06 AU for the base and acid form, respectively. The standard error with the adjusted baseline is approximately a factor of 3 better. Clearly establishing an accurate baseline value is critical for calibrating the response of the immobilized pH indicators.

The rate at which the immobilized neutral red responds to changes in pH was studied in solutions with a pH of 10 and 1.5. The absorbance of the indicator was monitored as the solution pumped into a flow cell, containing the immobilized indicator, was switched back and forth between these pH extremes. This procedure was repeated as the buffers were serially diluted by a factor of ten. The half life ($t_{½}$) is the time taken to reach half of the absorbance maximum minus the 10 s lag time for flushing the flow cell and tubing. The results are summarized in Figure F-4. Factors affecting the kinetics of the indicator response include the rate at which species diffuse into the immobilized indicator and the total amount of hydronium ion delivered to the indicator.

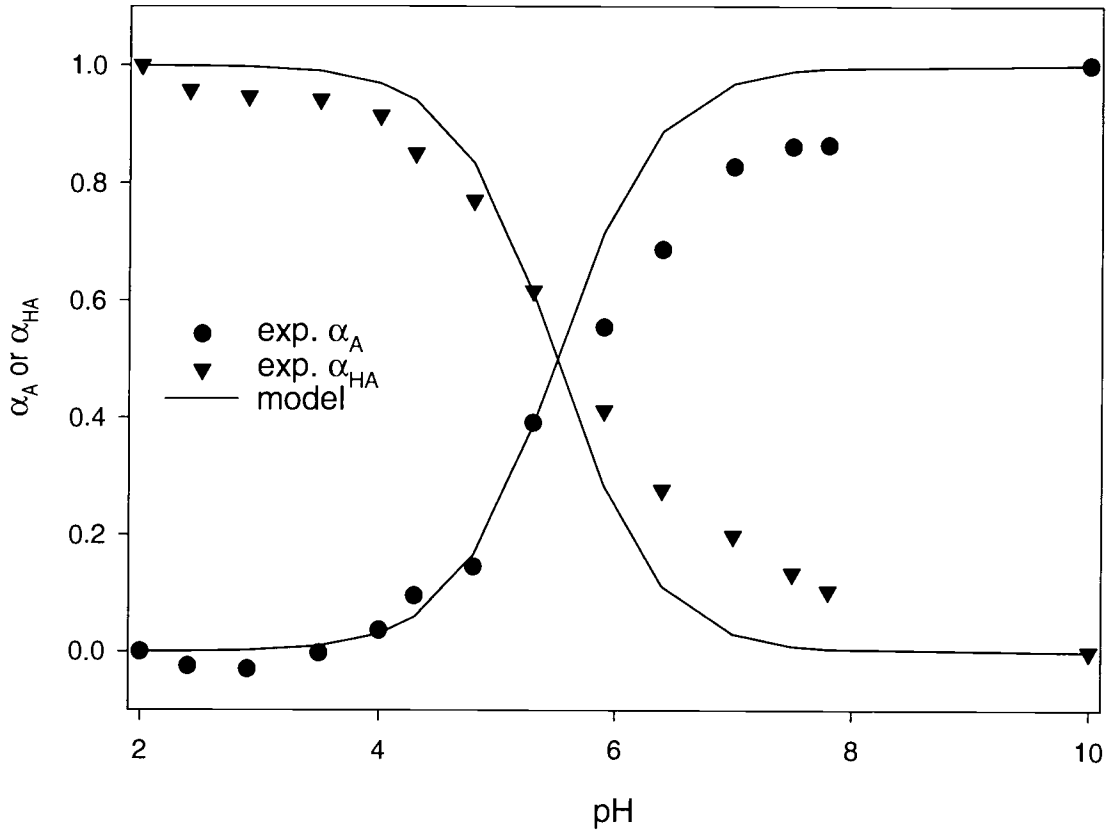


Figure F-2. Dependence of the fraction of the acid and base forms of neutral red on pH calculated from spectra of immobilized indicator ($pK_a = 5.5$). The curves are based on equation F-2.

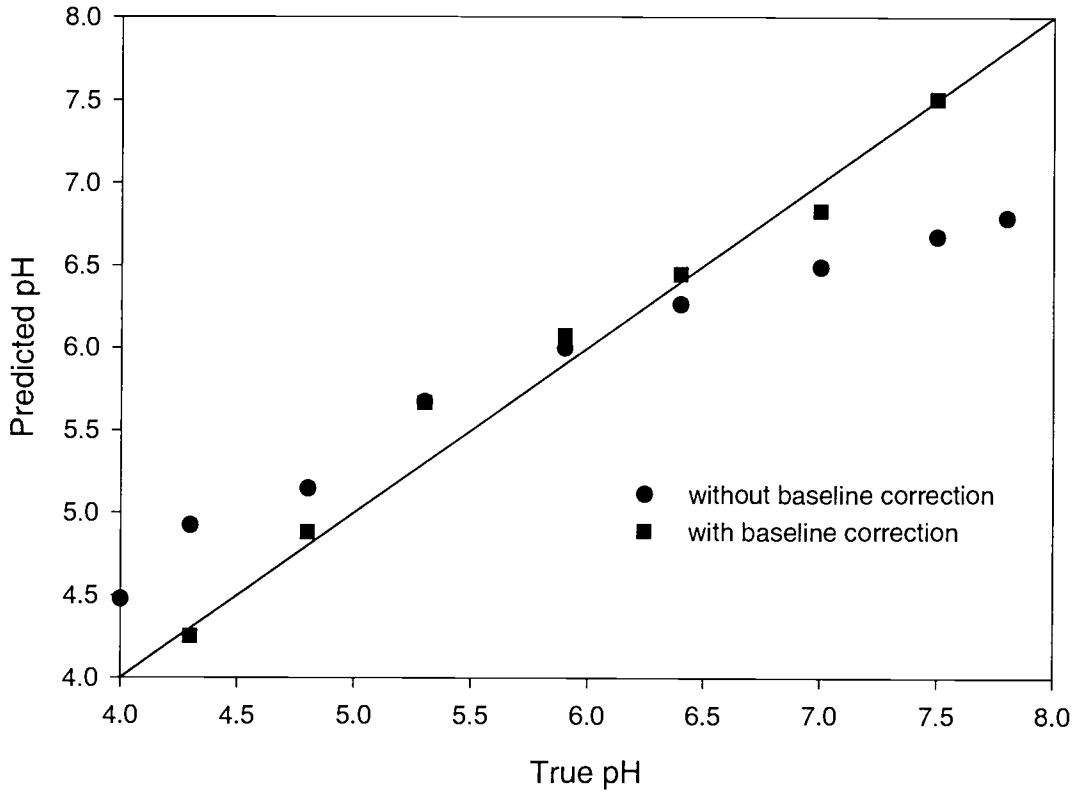


Figure F-3. Correlation plot for immobilized neutral red. The standard error of the predicted pH values without the baseline correction is 0.52 pH units, and the standard error of prediction when the baseline correction is applied is 0.19 pH units.

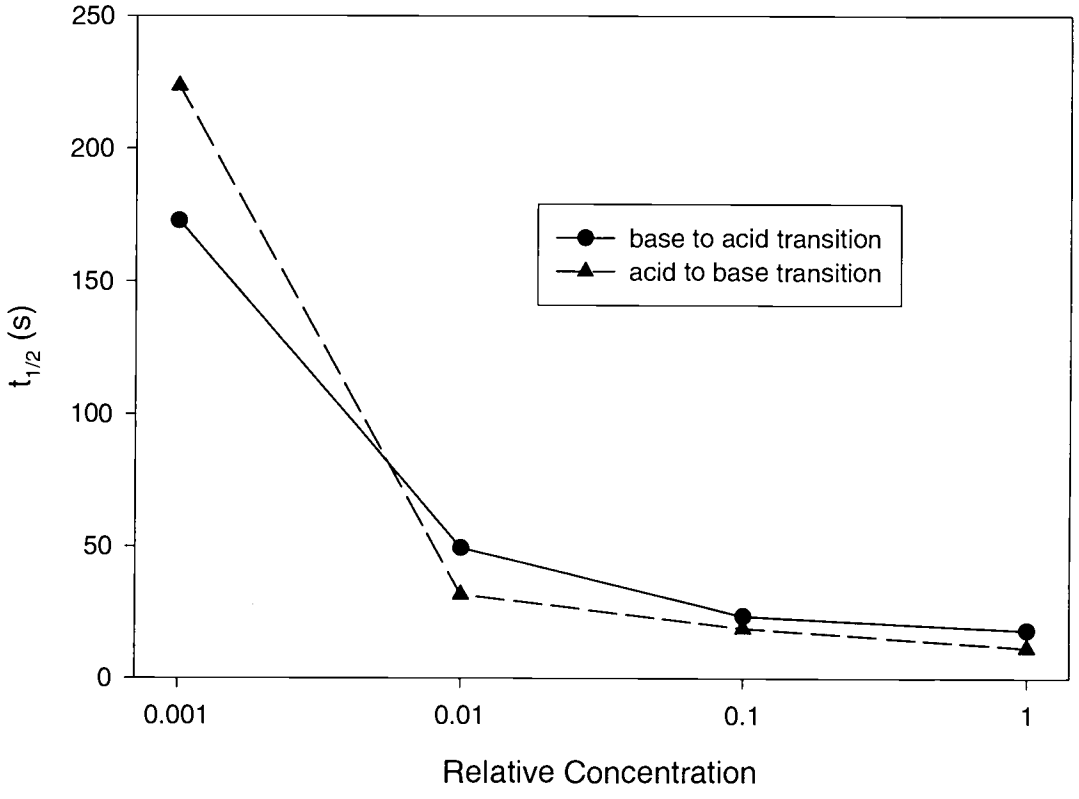


Figure F-4. Kinetics of the response of neutral red. Note that relative concentration is plotted on a logarithmic scale. A relative concentration of 1 represents the original concentration of the buffer solution. The pH 10 bicarbonate buffer was prepared from a commercial tablet (Metrepak).

Congo Red

Congo red was immobilized, experimentally evaluated, and modeled in a very similar manner to neutral red. The absorbance spectra of free and immobilized congo red at various pH values is presented in Figure F-5. The pK_a of free congo red is ~5. Similar to neutral red, the immobilization shifts the wavelength maxima of the absorbance band spectra. The maximum of the absorbance in the basic form shifts from 484 to 511 nm, and that of the acid form shifts from 604 to 617 nm.

The fractions of the acid and base forms of the indicators at various pH values calculated were from the absorbance data and are summarized in Figure F-6. For congo red, the extreme pH value of 1 serves as the baseline for the absorbance band of the basic form, and the data from the pH 8 solution is the baseline for the acid band. From the equilibrium model (equation F-1) the pK_a was optimized and a value of 2, provided the best "visual" fit to the measured values.

This pK_a of 2.0 and corrected absorbances were substituted into equation F-1 to calculate a pH. The results shown in Figure F-7 illustrate a good fit. Optimization of the baseline absorbance did not significantly improve the predicted pH values in this case.

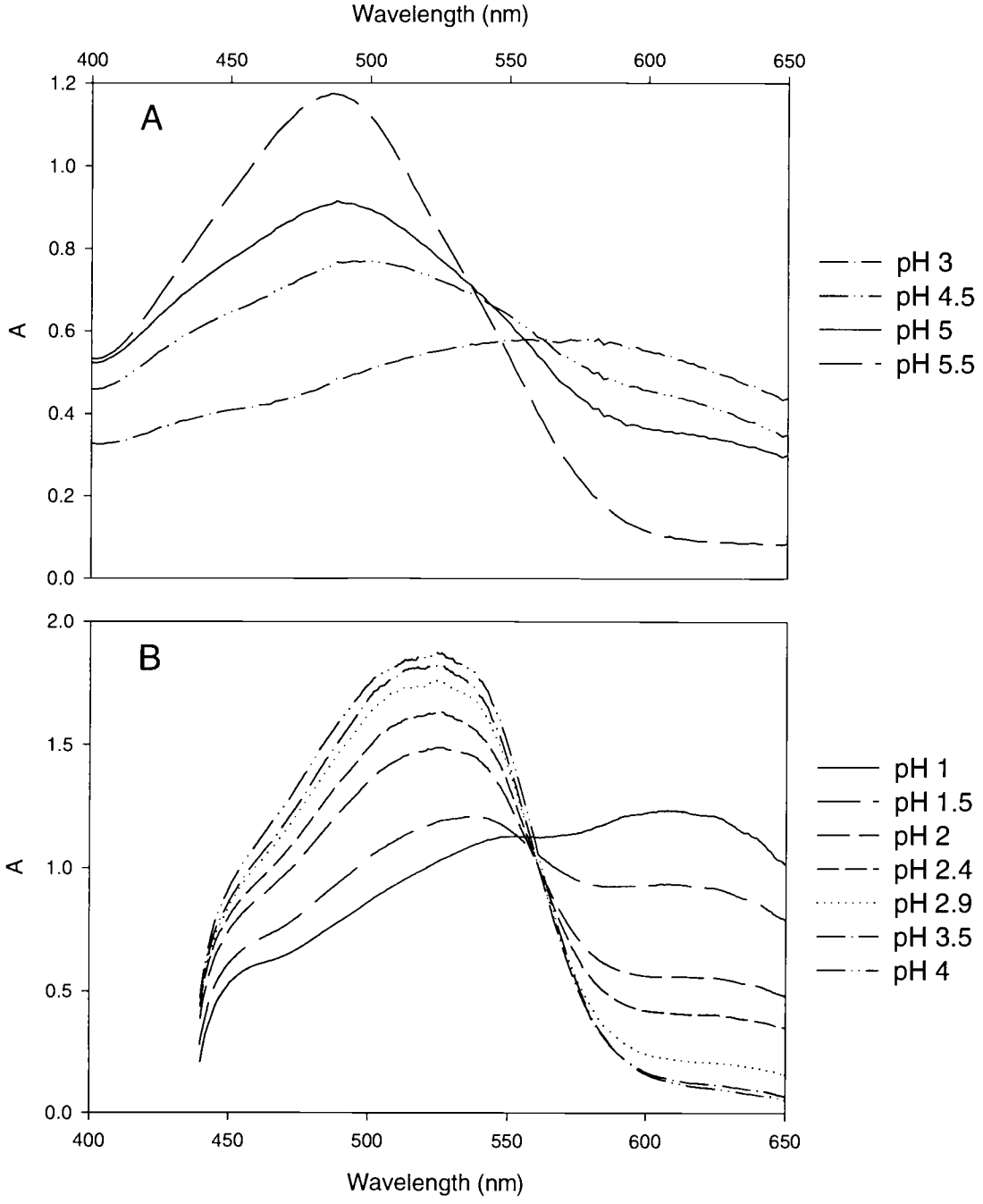


Figure F-5. Dependence of the absorption spectra of congo red on pH. Graph A displays the spectra of congo red in free solution taken with an HP diode array spectrometer. Graph B displays the spectra of congo red immobilized onto dialysis membrane measured with an Ocean Optics S2000 spectrometer in a custom flow cell.

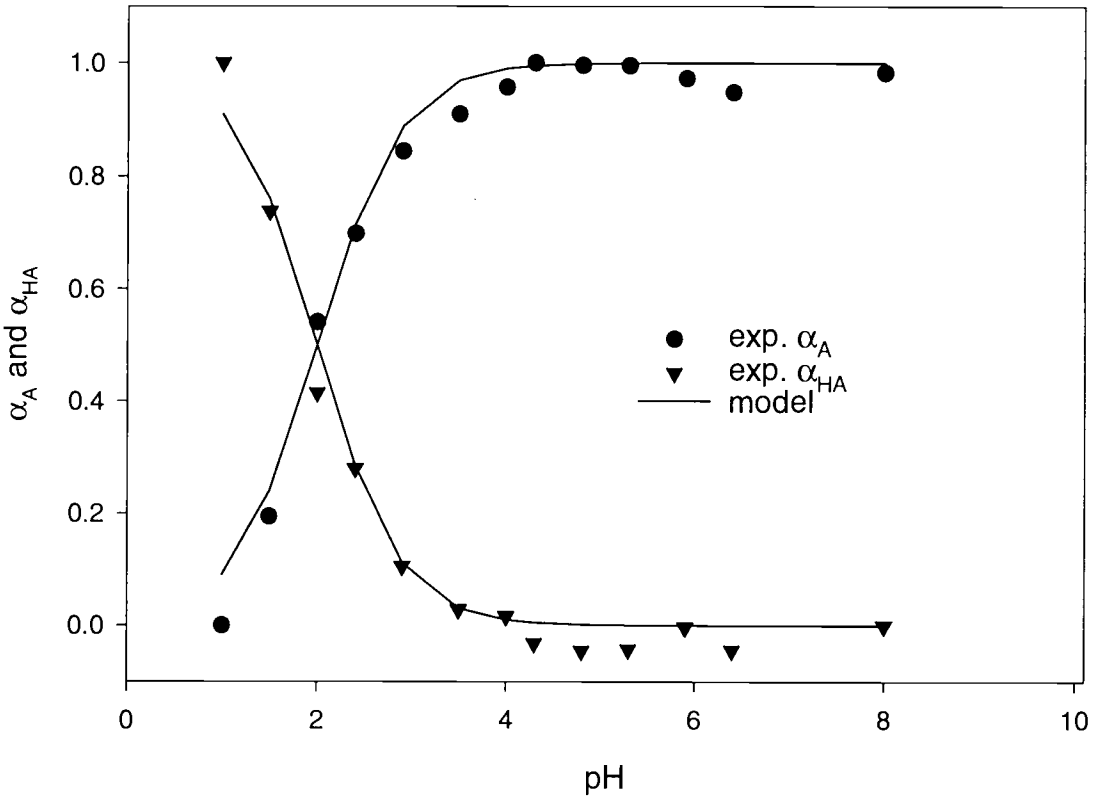


Figure F-6. Dependence of the fraction of the acid and base forms of congo red on pH calculated from spectra of immobilized indicator ($pK_a = 2.0$). The curves are based on equation F-2.

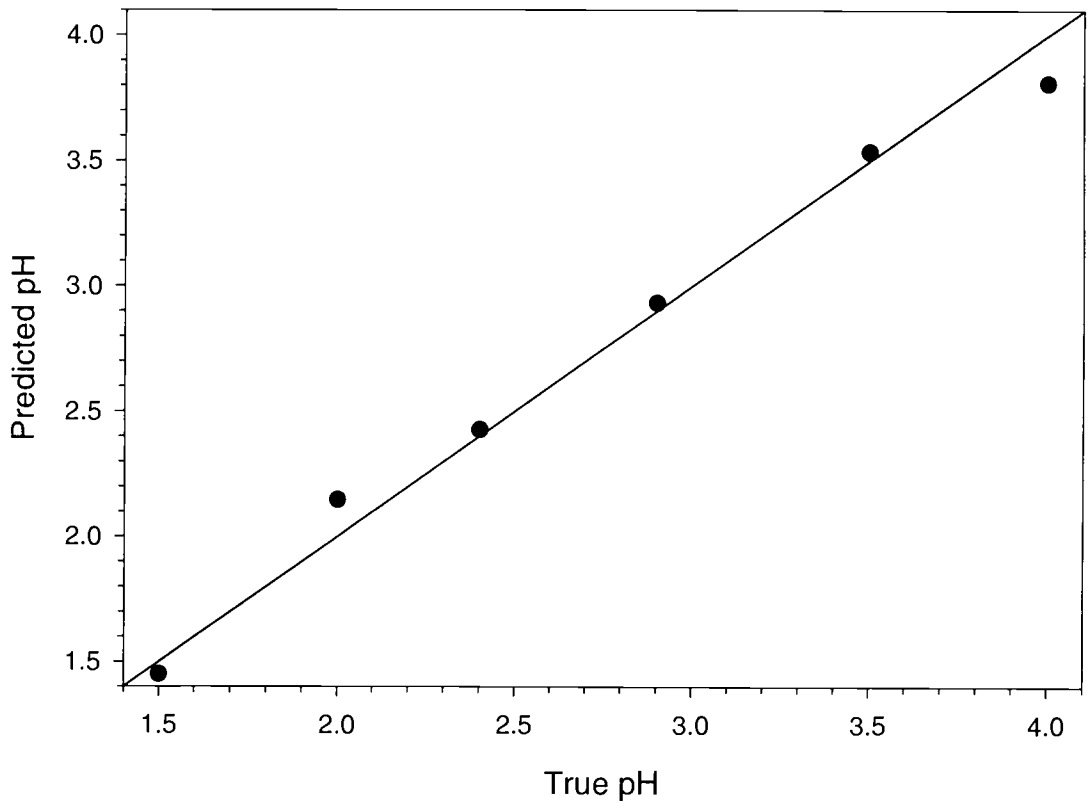


Figure F-7. Correlation plot for immobilized congo red. The standard error of the predicted pH values without the baseline correction is 0.11 pH units.

LED studies

Neutral red was evaluated for possible use in a LED based spectrometer similar to the SLIM (2). The overlap of indicator absorption spectra and the emission profiles of three LEDs manufactured by Nichia is illustrated in Figure F-8. The blue LED is a good match for the absorption band of neutral red in the basic form, and the green LED is a reasonable match for the indicator in the acidic form.

The spectra previously acquired with immobilized neutral (Figure F-2) and the emission profiles of the green and red LEDs were used to estimate an absorbance that would be measured with these light sources. Figure F-9 shows the dependence of the calculated absorbance for the acid and base forms on pH. The pH values predicted from these calculated absorbances and equation F-1 are shown on the secondary Y-axis. The baseline values for the acid and base forms and the pK_a of the indicator were optimized in this model. The prediction of pH works reasonably well from pH 4 to 7. Clearly an LED-based sensor based on immobilized neutral red could be useful in solutions with a pH between 4 and 7.

References

1. Hubbard, Daisy, Laboratory notebook #1, 2000, Oregon State University.
2. Chapter 2 of this thesis.

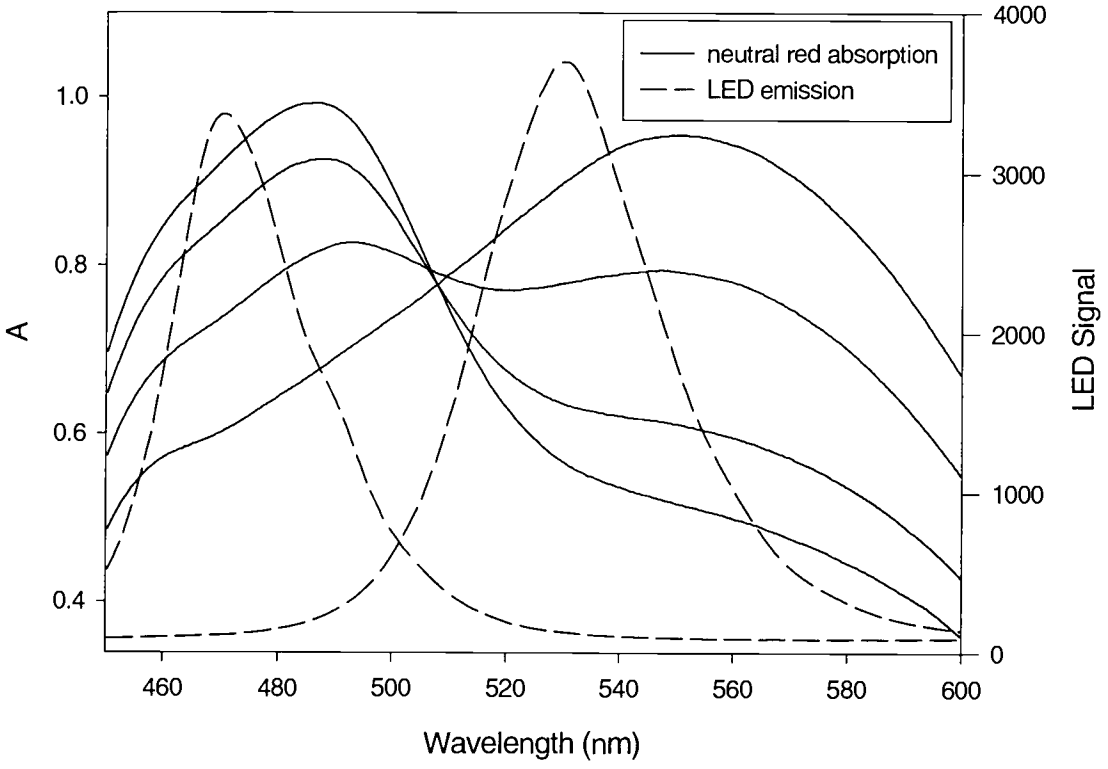


Figure F-8. The overlap of the neutral red absorption band and LED emission profiles. The solid lines indicate absorption of neutral red at pH values of 4, 5.5, 6.5, and 8.

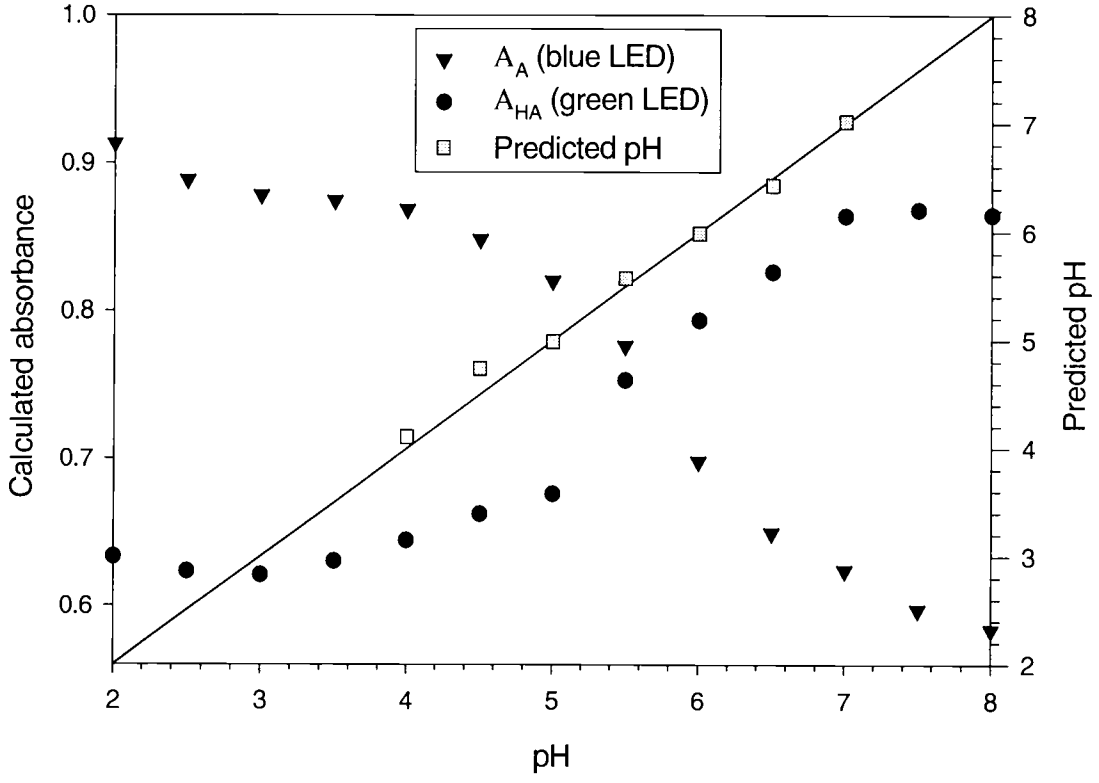


Figure F-9. The dependence of the estimated absorbance of neutral red on pH. A_A is the absorbance estimated for the blue LED and A_{HA} is the absorbance estimated for the green LED. This calculation is based on a model discussed in Chapter 2 of this thesis, and the Excel macro is given in Appendix A. The pH values calculated from these estimated absorbances (equation F-1) are shown as gray squares. The standard error of prediction is 0.12 pH units after an optimized baseline correction (minimizing the standard error of prediction) is applied to the absorbance values.

Appendix G: The design and construction of various flow cells

The design and fabrication of a variety of flows cells that are useful for monitoring immobilized indicators has been one focus of this research. The flow environment is a common exposure situation that arises when monitoring samples from wells in the field, large-scale laboratory models, and small-scale laboratory bioreactors. Two examples are illustrated in Figures G-1 and G-2.

In previous work (1), redox indicators were immobilized on agarose beads and packed in a commercial spectrometer flow cell (Helma, Inc.) designed to withstand medium pressures (200 psi). During field experiments, it became clear that packed cells are subject to clogging by particles in the sample and would not be practical for many applications. The immobilization process was refined (2) so that the indicators could be immobilized onto thin films and inserted in a flow cell as illustrated in Figure G-3. With this type of design, soluble reductants and oxidants can diffuse into the medium and interact with the redox indicator, and small particles can flow around the film. It is possible to monitor unfiltered or coarsely filtered solutions.

The first flow cell constructed to use redox indicators immobilized onto thin films (2) is shown in Figure G-4. The cell is demountable and allows for insertion and removal of the thin film or membrane. Although this flow cell design works well, the cell is somewhat complex and costly to machine. It requires sapphire windows and a custom holder that fits in some spectrometers with a large enough sample compartment.

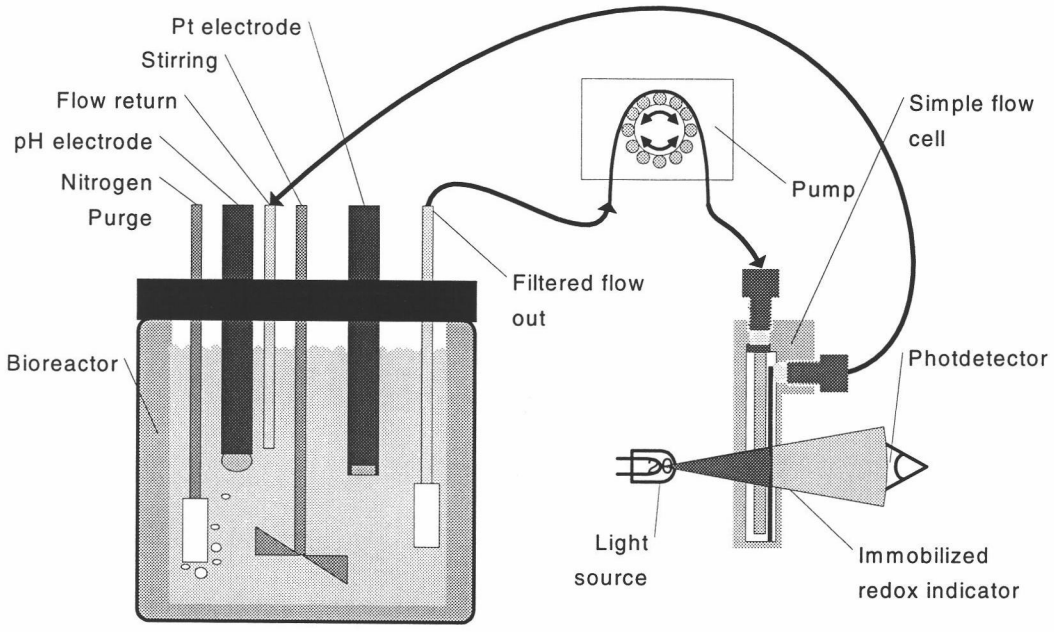


Figure G-1. A flow cell used to monitor the redox status of a bioreactor in the laboratory. In this system the solution is continuously circulated through the sample cell and back to the bioreactor.

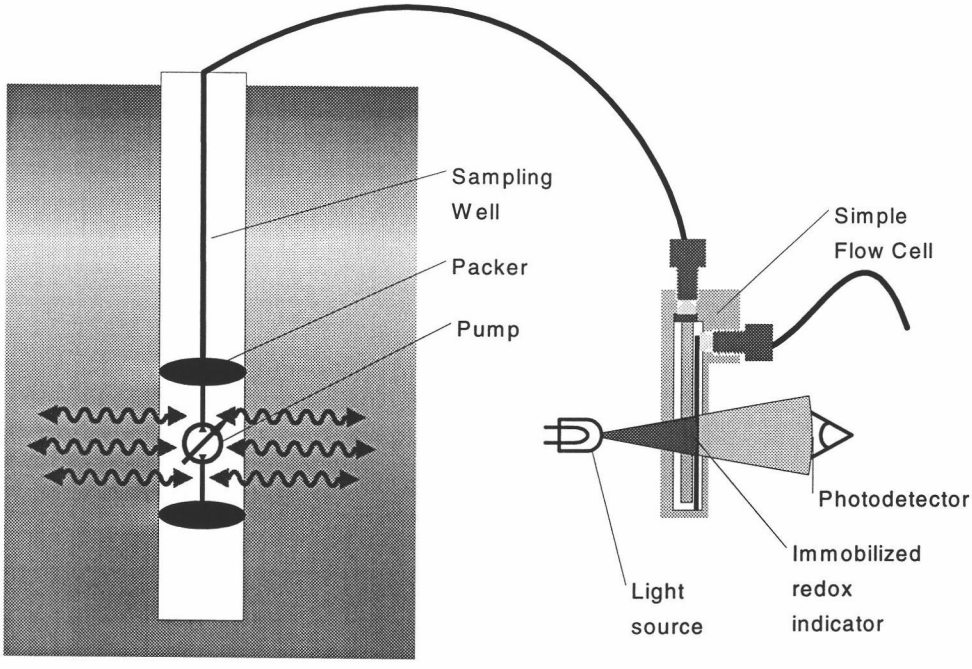


Figure G-2. A flow cell used to monitor the redox status of groundwater from a sampling well in the field. In this case, the solution is pumped from the well and through the cell, and is then available for collection in sampling bottles.

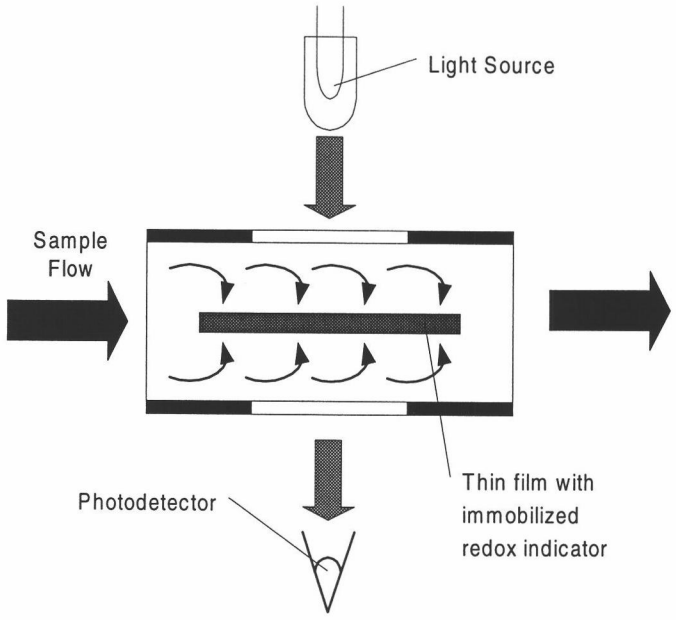


Figure G-3. General design of optical flow cells used with redox indicators immobilized on thin films.

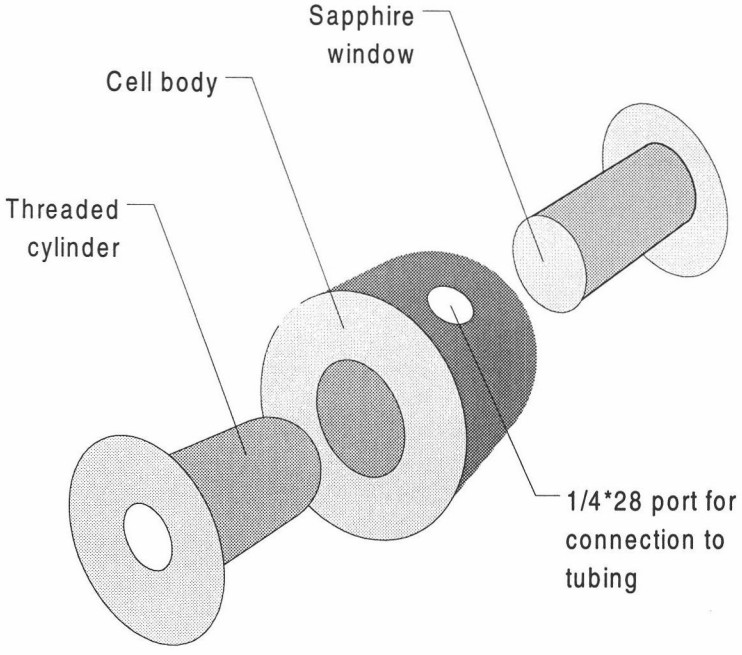


Figure G-4. A demountable flow cell for redox indicators immobilized on thin films. The cell is made from a Delrin, and two cylinders, with outside threads and sapphire windows mounted at the end, screw into the tapped cell body, which has input and exit ports for solution flow. The inside cavity has dimensions of 3 x 3 x 1 mm. The outside diameter is 38 mm and the length of the optical axis is 47 mm.

Two other types of flow cells were constructed in this research. They allow the insertion and removal of a thin redox indicator membrane but are much more economical.

The "L" shaped sample cell

A sample cell, here denoted the "L" cell, is shown in Figure G-5. The cell is cut as a single piece from inexpensive acrylic sheet with a nominal thickness of 0.5 in. The un-machined surfaces are placed perpendicular to the optical path to minimize transmission losses due to light scattering. The input and output ports of the flow cell are machined directly into the acrylic body and tapped for ¼*28 fittings. A hole ~0.25 inches in diameter is drilled approximately 2-in deep to create the cell cavity. A small adapter ring made of Delrin (0.30 in OD and 0.125 in ID) sits on top of a lip and provides enough area for a low-pressure ferrule (Upchurch) to seat and seal. The flow path is coaxial with the solution entering at the top of the cell and flowing down through a transparent length of 1/8-in high purity PFA tubing (Upchurch). The solution then flows up in the volume between the outer wall of the tubing and the acrylic body of the cell and out through the side port. The film of immobilized indicator fits between the transparent tubing and the acrylic body in the upward flow region of the cell. The cell was used with dialysis membrane cut into pieces 0.4 x 2 in and inserted before the adapter ring was installed.

There are several compromises made in this sort of design, and the optical characteristics of the cell are not ideal. The drilled hole that creates the cell cavity is

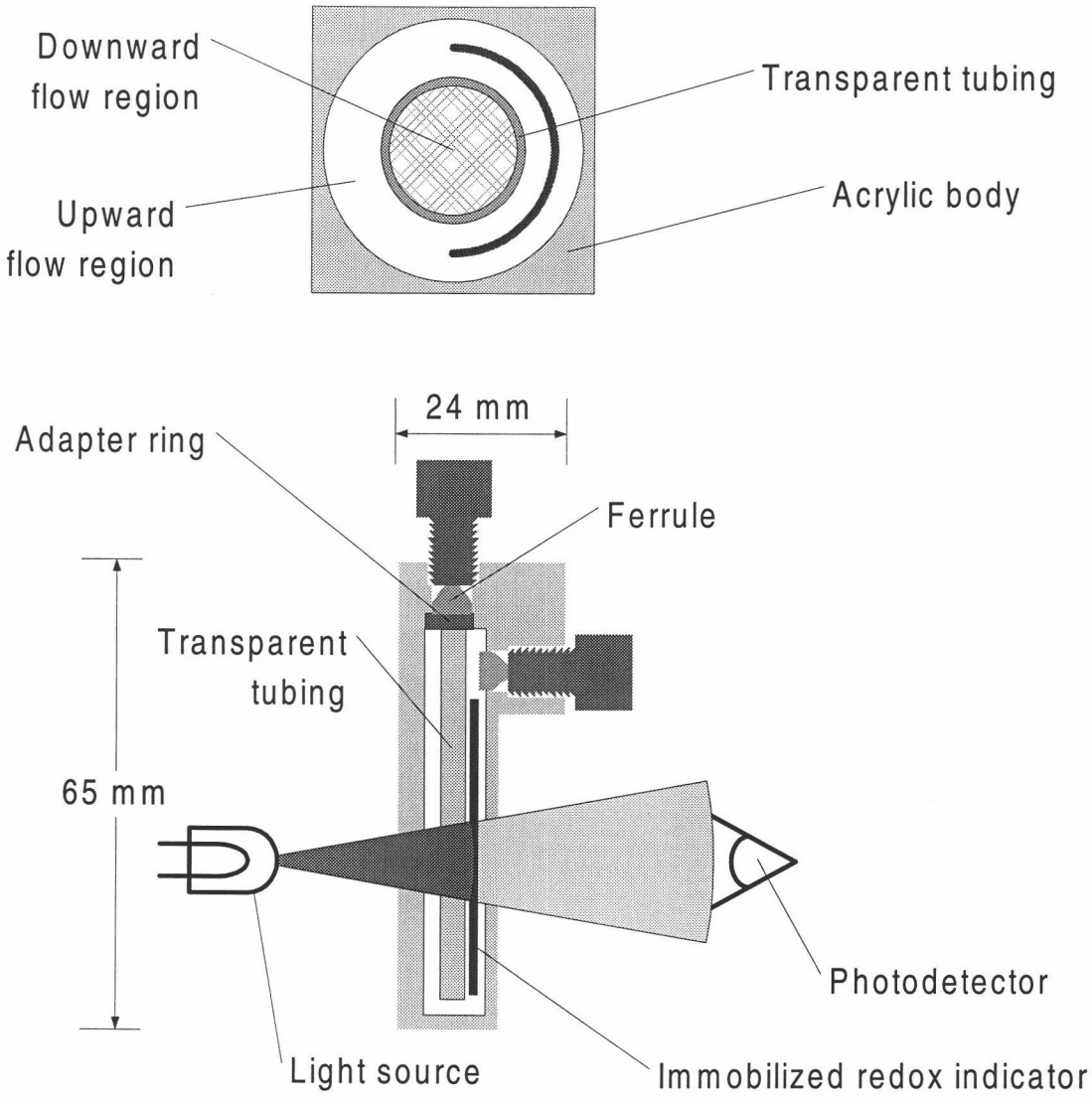


Figure G-5. A diagram of the "L" shaped cell. The upper drawing is a cross section illustrating the coaxial flow path. The lower diagram illustrates the input and output fittings along with the orientation of generalized optical components of a spectrometer.

difficult to polish, and a good deal of light scattering occurs. Other concerns are light transmission losses through the "transparent" tubing and lensing effects due to the curved surfaces in the optical path. These effects combine to reduce the amount of light transmitted through the cell (no membrane) to about 10% versus an air blank.

Although the design of this cell could be refined, its performance is acceptable for many applications. Its outside dimensions are the same as standard 1-cm path length cells which make it readily adaptable to almost any spectrometer. Custom spectrometers designed around this cell can also be used with many commercial cuvettes and flow cells. Although the redox indicator membrane is somewhat difficult to seat inside the cell, the length of transparent tubing serves as a positioning tool. Once in place the indicator does not move even in experiments with high flow rates. The cell is also relatively inexpensive and easy to manufacture.

The visual cell

To encourage widespread deployment of the redox indicators, the flow cell illustrated in Figure G-6 was designed to allow a redox indicator immobilized on a film to be observed visually without the aid of a spectrometer. The design parameters of this cell included minimal cost and construction time and ease of use. The body base of the cell is constructed from Delrin and has ports that accept standard ¼*28 male fittings for the input and output flow streams. The transparent acrylic cover press fits and seals over an o-ring in the Delrin base. This cover is constructed from a

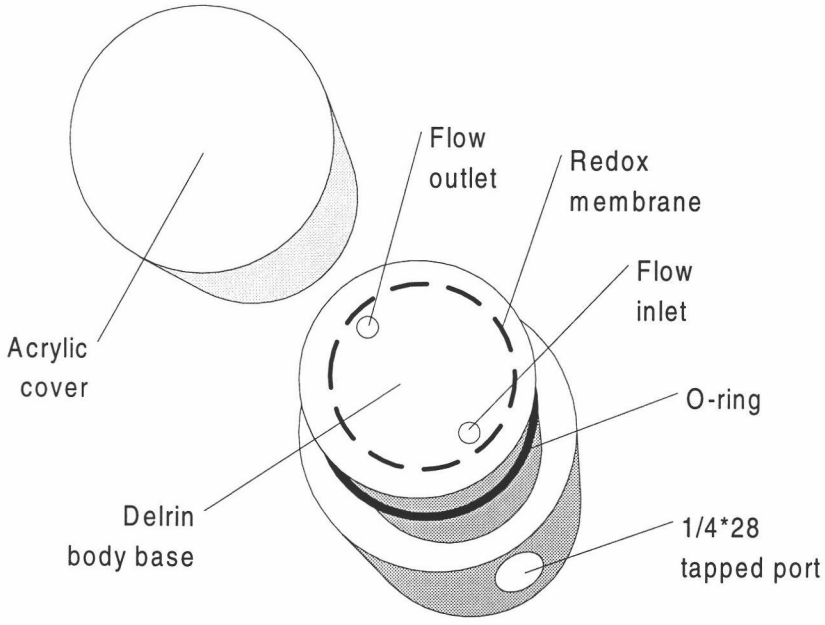


Figure G-6. A diagram of the simple visual flow cell for indicators immobilized on thin films. The indicator film is cut in a disk shape and sandwiched between the cover and base.

short length of 1-in outer diameter acrylic tubing to which a piece of 1/8-in acrylic sheet is glued with an acrylic adhesive.

The cell cavity is created in the space between the acrylic cover and the Delrin base. The diameter of this cylindrical cavity is 0.875 in and the height is 0.06 in, yielding an internal volume of ~0.6 mL. The two openings on the base are connected through a right angle flow path to the ¼*28 tapped ports.

The redox indicator membrane is cut with a 3/4-inch circular punch, and inserted inside the acrylic cover. The cells are designed so that they can be chained together in series, and the behavior of several redox indicators with different formal potentials can be observed in the same exposure environment.

Other flow cell variations

In addition to the flow cell designs described above, many variations were also explored. An o-ring cell (similar to the cell used for the SLIM described in Chapter 2) was constructed without ¼*28 fittings. Two small holes were drilled on opposite sides of the o-ring itself, and 1/16-in tubing was inserted into these holes. When the o-ring and tubing are compressed between two sheets of acrylic, the tubing is sealed, resulting in a very inexpensive flow cell.

The "SLIM" flow cell (3) was also modified to mate with an optical fiber terminated with an SMA connector (Ocean Optics) so that absorption spectra could be acquired with an Ocean Optics spectrometer and LED sources. The photodetector side of the flow cell was built up by gluing a ¼ in acrylic sheet to the cell. A SMA threaded hole was then drilled into this sheet and a bulked fitting was screwed in to allow for connection of the optical fiber.

Another integrated spectrometer/flow cell was constructed in a standard ¼*28 union (Upchurch). A 0.20-in hole was drilled through both sides of the union perpendicular to the flow path. A three-color LED was inserted into one side of this hole and glued in place. A TSL230 (Texas Instruments, Inc.) light-to-frequency converter IC was glued over the other side of the drilled hole. The light source and photodetector plug the hole. This inexpensive, miniature spectrometer is easily screwed into any flow stream with ¼*28 fittings. Although this design does not accommodate an immobilized indicator membrane, it may be useful as the detection system for a miniature flow injection system.

References

1. Lemmon, Teresa, *Development of Chemostats and Use of Redox Indicators for Studying Transformations in Biogeochemical Matrices*, **1995**, Ph.D. Thesis, Oregon State University.

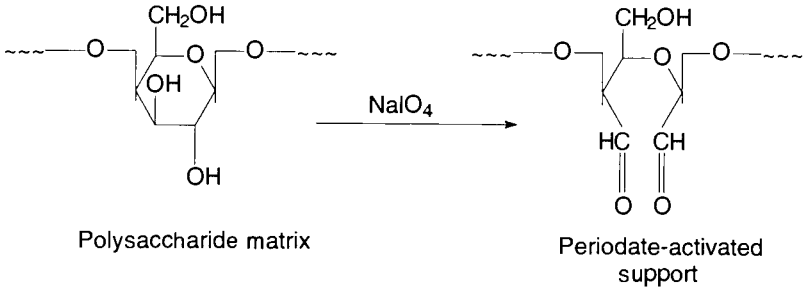
2. Jones, Brian D., *Applications of Redox Indicators for Evaluating Redox Conditions in Environmental Samples*, **1999**, Ph.D. Thesis, Oregon State University.

3. Chapter 2 of this thesis

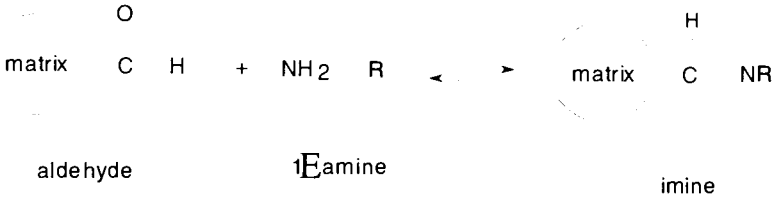
Appendix H: Immobilization process

Redox indicators with primary amine functionalities were covalently immobilized to aldehyde groups on dialysis membranes through a reductive amination reaction. These aldehyde groups were formed on the cellulose acetate dialysis membranes by oxidation with periodate (1). These steps are depicted in Figure H-1.

First step



Second step



Third step

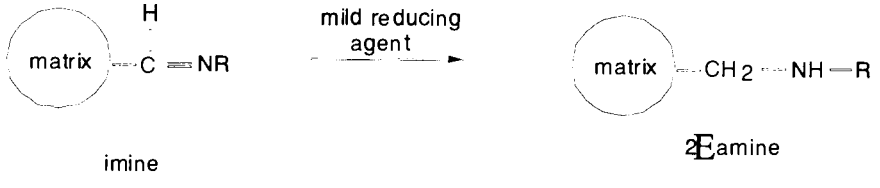


Figure H-1. The steps in the immobilization of redox indicators to cellulose acetate membranes. In the first step periodate is used to oxidize alcohol functionalities in the cellulose acetate to aldehyde. In the second step the primary amine on the indicator molecule forms a covalent bond with the aldehyde functionality on the membrane, which is then reduced to a secondary amine in the third step.

The step-by-step immobilization process follows:

1) Prepare stock solution of redox indicator in DI water (see table H-1 for concentrations)

2) Prepare pH 4 acetate buffer (~ 1 g of sodium acetate in 250 mL water, titrate to pH 4 with acetic acid)

3) Dissolve ~0.2 g of periodate in 30 mL of DI water in a 40-mL ICHEM vial

4) Cut dialysis membrane into two ~4-in^2 strips that fit inside a 40-mL ICHEM vial

5) Place the strips of dialysis membrane in the vial containing the periodate solution and sonicate (immersed in a water bath) for 5 min

6) Remove strips with tweezers from periodate solution and rinse thoroughly with water

7) In a 40-mL ICHEM vial, combine the redox indicator solution, 10 mL of 95% ethanol, the activated dialysis membrane, 0.2 to 0.3 g of $NaCNBH_3$, and enough acetate buffer to bring the final volume to ~40 mL (see table H-1 for volume and concentration of redox indicator solution)

8) Sonicate the vial for 30 min and allow time to react (see table H-1 for times)

9) Remove the membrane from the vial with tweezers, rinse thoroughly in DI water, and store in a 25% ethanol/water solution in refrigerator

Table H-1. Indicator concentrations and reaction times for dialysis membrane immobilization.

	Indicator amount and concentration	Reaction time after sonication
Thionine	2 mL of 2 mM	0 min
Cresyl Violet	10mL of 2 mM	30 min
Safranine O	10 mL of 5 mM	24 hr

The cellulose dialysis membranes used were Spectra/Por type 4 in 150 x 150 mm flat sheets (#132712). The molecular weight cutoff is 12,000 to 14,000 Daltons. The sheets are ~50-μm thick.

Both the amount of periodate and the time it is allowed to form aldehyde groups on the dialysis membrane were dramatically reduced from previous immobilization procedures (2). If the amount or time is increased, the resulting membrane degrades in basic solutions. For some redox indicators, it is difficult to bind a sufficient amount of the indicator to a membrane with this more conservative aldehyde formation procedure, and some modification may be necessary. In fact, it is difficult to immobilize a sufficient amount of phenosafranine. Another indicator, safranine O (formal potential near –250 mV), in the same family was easier to immobilize.

Greater amounts of indicator are immobilized at lower pH values, but the reducing agent, $NaCNBH_3$, is not effective below a pH of ~4. It is possible to separate the last two steps of the immobilization procedure. In this strategy, the imine bond is formed at low pH (e.g., acetic acid), and the indicator membrane is then transferred to another vial containing the reducing agent, pH 4 buffer, and additional free indicator.

This procedure was used to immobilize the pH indicators and is detailed in appendix F. It is also possible to use sulfide to reduce the imine bond at low pH, but sulfide is difficult to handle and less convenient than this two-step process.

References

1. Hermanson, G. T.; Mallia, A. K.; Smith, P. K., *Immobilized Affinity Ligand Techniques*, Academic Press, 1992

2. Jones, Brian D., *Applications of Redox Indicators for Evaluating Redox Conditions in Environmental Samples*, 1999, Ph.D. Thesis, Oregon State University.

Appendix I: Printed circuit board fabrication details

The artwork for all printed circuit boards presented in this thesis was prepared with Winboard (Ivex) software. Because a positive photofabrication process was used, the large areas of the boards that do not contain traces were shaded so that the copper remains in these areas and developing time and chemical quantities were reduced. The completed circuit design was then printed onto Inkjet Transparency Film (Hewlett Packard).

During the design process it was easier to visualize the layout of the traces as viewed from the component side of the board. The circuit was designed and printed from this perspective, and the printed transparency was reversed (by flipping it over) for the actual exposure of the board. To ensure this reversal was done properly, all of the artwork contains text that is mirrored along the X-axis. Although the text appears reversed on the PC screen and in the printed artwork, when the board was exposed the transparency was placed over the board so that it was readable and the other traces were properly reversed.

The photofabrication process used pre-sensitized boards, equipment, and chemical from M. G. Chemicals, and the following description is a summary of the guidelines provided with these products (1). The boards are coated with a photosensitive material that becomes soluble in a developing solution after exposure to UV light. The traces printed onto the transparency film block this UV light so that, after exposure and developing, the coating remains in the areas in black on the artwork. The board is then etched in ferric chloride and the conductive copper layer is

removed from areas unprotected by the coating. Thus, after the entire process is complete, only the areas that are black in the artwork have copper left on the board.

Exposure

A low wattage (40 W) incandescent bulb was used as a safelight during the exposure and development processes. The boards were packaged in heavy plastic opaque bags and protected with a white protective film that was removed just before exposure. The materials were layered with the pre-sensitized board on the bottom with the copper side of the board facing the exposure fixture, followed by the artwork, and a clear acrylic sheet. An exposure kit (M. G. 416-X) containing a single tube fluorescent light and stand, was used as the exposure light source. The boards were exposed for 5 min.

Development

The development solution was prepared by mixing one part of the alkaline developer solution (M. G. 418) with ten parts of water at room temperature. The exposed board was completely submerged in this solution. The image developed within ~5 min with gentle rocking of solution. According to the manufacturer, light brushing was acceptable to speed development, but in practice this treatment was too aggressive and breaks in fine traces resulted. After development, the board was

thoroughly rinsed with water to stop the process, and the board was etched as soon as possible.

Etching

Approximately 750 mL of the ferric chloride solution (M.G. 415) was heated to ~50 °C in a plastic container with a glass enclosed aquarium heater. The solution was agitated with air bubbles from an aquarium air pump. The ferric chloride solution was re-used until development times significantly increased. For a 5- by 7-in board the etching time was less than 10 min with new ferric chloride solution. When the etching process was complete, the board was washed thoroughly under running water. The photosensitive coating remained after the process and was not removed because it prevents oxidation.

Drilling and soldering

The circuit boards were drilled with a Dremel tool in a drill press style holder. A variety of sizes of carbide coated drill bits were used to drill holes for ICs, terminal blocks, and other connectors. All components were soldered in place using standard techniques. The heat of soldering disintegrated the photosensitive coating underneath the solder.

References

1. The M.G. Chemicals Positive Photofabrication Process. http://www.mgchemicals.com/techsupport/instruct.html (accessed May, 2001)