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Cobalt sorption studies on a bacterium and a natural organic material and On-line beehives: Counter design and software

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Cobalt Sorption Studies on a Bacterium & a Natural Organic Material

and

On-line Beehives: Counter Design and Software

by

Bruce E. King

B.S., Drexel University, 1992

presented in partial fulfillment of the requirements
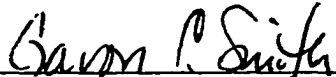
for the degree of

Doctor of Philosophy

The University of Montana

1998

Approved by:

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Dean, Graduate School

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King, Bruce E. Ph.D., December 1998   Chemistry

Cobalt Sorption Studies on a Bacterium & a Natural Organic Material and,
On-line Beehives: Counter Design and Software

Director: Garon C. Smith   *Garon C. Smith*

The biosorption of metal cations was investigated with a sub-surface bacterium, *Bacillus simplex*, isolated from the vadose zone of the Hanford Site in Richland, WA. Gibbs-Donnan theory is used to describe the ionic strength dependence of exopolymer phase metal cation sorption. Previous experiments showed that heat treated *B. simplex* cells have greater metal ion sorption capacity than untreated cells. Metabolic inhibition studies employing sodium azide revealed trivial contributions from active intracellular uptake of metal ions. Metal ion sorption by cells was greatest at lower ionic strength, which agrees well with the Gibbs-Donnan equilibrium description for components diffusing into a polymer matrix. The significance of this result in comparison to a model based solely on ion exchange is discussed.

NMR spectroscopy was used as an investigative tool for describing the interaction of metal ions with organic material. Experiments were conducted to show that the presence of metal ions near functional groups affected the ^{13}C NMR linewidth and chemical shift. This marks a vast improvement over titration based methods in that the interaction between the metal ion and functional groups in the organic material can be directly observed.

Flight activity of honey bees (*Apis mellifera*) was studied for biomonitoring applications at Aberdeen Proving Ground, MD and Missoula, MT. Bee-counters were designed and constructed for measuring passage of bees in and out of specialized shells (condos) holding colonies of bees, seven of which were located at each of four sites. Custom software was written to process and evaluate data obtained from bee-counters at each site. This software provides an extensive set of data manipulation and analysis tools for: 1) the display of data from seven colonies at each site, 2) site by site comparisons, and 3) interspecies (honey bee and bumble bee) comparisons. Complex numerical analysis routines that are sensitive to unusual flight activity behavior are used to characterize short term events affecting flight activity. Ultimately, a software based tool for analyzing flight activity behavior was produced.

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Acknowledgments

Many people have helped me along the most recent path of my education, some professionally by teaching, providing advice or helping out with the 'grunt work' and others whose actions and attitude toward life provided me with a model for self improvement. This degree would not have been attempted without the prompting of Sandeep Patel, whose hard work and sense of humor I will always strive to match. My advisor, Dr. Garon Smith, allowed me the freedom to explore scientific avenues that most others would have declared off-limits and provided me with initial direction and funding that set me on a course toward the Ph.D. Dr. Jerry Bromenshenk has been a close mentor since my first summer at PNNL. He has honed my abilities as a researcher by involving me in all aspects of a research project, from proposal and report writing to all the daily minutia in between. Working with Dr. Bromenshenk has taught me to step back from a problem and examine all reasonable options before proceeding.

Dr. John Zachara for providing me a crash-course in geochemical modeling. Both he and Dr. Harvey Bolton, Jr. guided me in my work during the summers of 1993 and 1994 at PNNL. Dr. James Gannon for allowing me to use his lab for preparing cells for titration experiments. I would also like to thank my committee for insightful comments.

I need to thank the DOE-EPSCoR program for funding during 1993 and 1994, the United States Army Center for Environmental Health Research for funding the bee project, and the Chemistry Department for making a TA position available when research funding was slim.

My experience at The University of Montana has been heightened by interactions with other graduate students. I owe many thanks to Ted Christian for insisting that I use the right tools and being a role model. Dr. Lisa McKenzie for dragging me out to run and providing advice when I needed it, all the while being another excellent role model. Chris Wrobel for dragging me out to go hiking just because I was spending too much time working and for keeping my standards high. Dr. Tim Looper for being the first person to take my advice and incorporate it into a thesis project, and in the process providing a sounding board for ideas about the NMR project. Bob Seccomb for showing me how a computer works and for keeping a cool head during the highly stressful past three years. Matt Loeser, Lenny Hahn and Kenny Morris for breaking the work-study mold in keeping the field sites running and doing most of the grunt work for the bee project. Rachelle Adams for wanting to work with bumble bees and for pushing me to make the bumble bee counters work. Gayle Zachariasen, Bonnie Gatewood and Ronnie Patrick for making paperwork fun and insisting that I follow all the pertinent university rules.

Last but not least, my parents, for without whom I wouldn't exist. My mother for asking when this would be done and my father for always knowing that she had already had asked. The steady flow of chocolate chip cookies and other support from the east coast kept me nourished and motivated.

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Chapter 1

1.1   Statement of the Problems

This thesis describes work done on two distinctly different research projects. The first project deals with determining, describing and modeling the mechanism of metal ion sorption to organic material, including bacterial cell surfaces. The second project involves a) the design, construction, testing, and field implementation of electronic devices that were used to provide a real-time metric of insect behavior for an environmental monitoring study, b) development of an extensive set of numerical analysis and visualization tools to efficiently review the data collected, and c) the verification and application of these data analysis tools.

1.1.a   Cobalt Sorption Study

Gaining a better understanding of the processes behind metal ion sorption to organic materials will improve our understanding of subsurface contaminant transport. It was commonly believed that metal ions chelated to or bound to organic material were destined to travel through the subsurface unhindered (1). Recent studies, however, have revealed that organic material plays an important role in the mineral coating and sediment formation process (2). Binding of organic material to mineral surfaces in combination with metal ions suggests that it may be possible to allow contaminated sites to undergo a natural attenuation process - one that is much less expensive and destructive than some of today's harsher remediation methods.

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The metal ion sorption study was designed to study a problem from the ground up. The intent was to determine the fundamental processes occurring and to model these processes with methods based on first principles. Through a combination of well controlled experiments, it was possible to obtain a clear picture of the system under study.

1.1.b Bee-counter/Numerical Analysis

The second project, design and construction of bidirectional bee-counters, uses a top-down approach to study a problem. The flight behavior of a colony of bees can be affected by other nearby colonies, weather conditions, maintenance by beekeepers, strength of the colony, time in the foraging season (phenology), time of day, nearby forage resources, water availability and the presence of environmental contaminants, to name a few. It is difficult to assign flight activity characteristics to any one of these parameters without knowledge of how each affects flight activity. Before this disturbance can be sorted out, it is important to obtain normal flight activity in order to establish a baseline.

1.2 Rationale for Two Separate Projects

Although it is unusual for a thesis to contain two such widely disparate topics, the scientific approach I have taken to each project has broadened my horizons as a scientist. Both projects are linked by a central component of environmental monitoring and characterization.

1.2.a   Discussion of What an Analytical Chemistry Ph.D. Entails

Because modern analytical instrumentation is primarily based on electronic circuits, an analytical chemist requires a fundamental understanding of electronics. Prior to using data acquired from modern instruments, one must consider all possible sources of error. These can be as simple as 60Hz noise due to AC line voltage or more complex, such as having unmatched resistors in the comparator circuits contained in a dual detector instrument. Other contributions could be due to the electronic and physical limitations of the instrument used for analysis. At the very least, the analytical chemist should be capable of working with technical support personnel when troubleshooting an instrument.

Another quality that today's analytical chemist should possess is some level of computer programming ability. This may be limited to simply reformatting large sets of data files prior to interpretation, or extended to building, controlling, and extracting data from a custom made instrument. This requires some knowledge of a programming language and how that language can be used for instrumental control, data acquisition, and data analysis.

Finally, the most important thing that I have learned in the past several years is that the accomplishment of the Ph.D. is not nearly as important as how the accomplishment was achieved. This knowledge applies not only to the core of this thesis, but to future research and lifelong activities.

1.2.b Why Each Project?

Part 1 of this thesis includes work that was done at Pacific Northwest National Laboratory (PNNL), while I received funding as a DOE-EPSCoR Trainee. This work initially dealt with real-time, *in-situ* monitoring of aqueous geochemical systems. I soon became fascinated with how complex the underlying processes were. I challenged myself to gain a better understanding of these processes even if it meant veering away from the original project. During my first summer at PNNL, I was taught the fundamentals of geochemical modeling with a system that contained a well characterized mineral surface, an organic chelating agent and multiple metal-ions. This system was devised to mimic the environment that metal ions and chelating agents would encounter in the subsurface. Being a novice, I thought that incorporating the influence of anthropogenic microbial material and organic matter into the geochemical models would be a trivial task. I was gravely mistaken, and the first part of this thesis is proof. I eventually resigned myself to finding a more reliable method than the traditional pH titration/sorption edge approach for determining the interactions between metal-ions and naturally occurring organic material.

Part two of this thesis describes a real-time, *in-situ* device used to monitor honey bee flight activity for biomonitoring applications. As part of the USA-BRDL (now USA-CEHR) project directed by Bromenshenk and Smith, I was asked to produce a bee-counter to monitor the flight activity of bees in relation to environmental conditions. While other researchers have constructed and used bee-counters, their experiments dealt with individual colonies, not groups of colonies deployed across a region for biomonitoring.

Most of these researchers were interested in temporal resolution from five to thirty minutes. This work is based on flight activity summed over 30-second intervals. Although collecting more data presented storage, processing and interpretation concerns, I decided that post-processing and advanced data analysis techniques could help sift through the data. The number of colonies being monitored and the amount of data being collected from each colony required that an extensive set of data analysis tools be compiled to further understand the factors that affect honey bee flight activity for individual colonies and for colonies across different geographic regions. These tools were compiled as I reviewed data arriving from the field. Ultimately, an advanced analysis tool was fine-tuned to target anomalous flight activity.

1.2.c How the Two Projects Complement Each Other

What makes the two projects in this thesis so compelling is the different approach used for problem solving in each one. The work with metal ions and organic materials uses a theory driven approach based on data from well characterized and controlled experiments. One can use the results of these experiments to model the behavior of metal ions in an environment containing organic material. The work with the bee-counters uses a data driven approach that can only show specific effects (flight activity) of a known or unknown (or difficult to characterize) stimulus. The challenge with the bee-counter research was to process and present the data in a way such that the stimulus was detectable to even the untrained eye.

The two topics studied in this thesis provided me with the opportunity to implement two distinctly different approaches to scientific study, one from the ground-up (Part 1) the other from top-down (Part 2). This allowed me the freedom to experience the benefits of both approaches in designing experiments and understanding their outcome. The combination of these two studies and the learning process therein enhanced my scientific abilities in a way that few graduate students experience.

1.3   Overall Organization of Thesis Parts

This thesis is comprised of two substantive chapters, one summarizing the research performed using pH titrations and NMR spectroscopy with organic material; the other detailing analysis performed on flight activity data produced by the bee-counters. There are several appendices that include previously reviewed documents, experimental procedures, manuals for using hardware and software, and source code. Data from the bee-counters exist in CD-ROM format for DOS or Windows based computers and can be requested from the author. Because of the length of some of the source code, it is included in a CD-ROM only.

Chapter 2

2.1 Introduction

This chapter describes laboratory experiments exploring the protonation and metal ion sorption characteristics of a microbial strain. *Bacillus simplex* was isolated from the uncontaminated Yakima Barricade area on the Hanford site in Richland, Washington. Isolation and characterization of the bacterium was performed by the Environmental Microbiology group at Battelle, Pacific Northwest National Laboratory (PNNL). *Bacillus simplex* has been shown in an earlier study to exhibit a larger metal ion exchange capacity than similar species (3). Experiments were designed to distinguish between sorption and uptake of metal ions. Sorption and uptake of metal ions was examined using pH controlled sorption edges and diffusion studies. In the process we also explored the permeability of the exopolymer phase of this subsurface bacterium.

Over the past fifty years, the Hanford Site in Richland, Washington was heavily used as a plutonium production facility for nuclear weapons programs. The site is set on 558 square miles of semiarid terrain which is bordered by the Columbia River to the north and east, with Rattlesnake Mountain to the west and the city of Richland to the south. The site consists of three physically isolated areas, where different parts of the plutonium production process were carried out. Facilities for fabrication of reactor fuel and pilot-scale evaluation of extraction processes are in the southeastern corner of the site, closest to Richland. In the northernmost portion of the site, reactors for plutonium production were located along the Columbia River to take advantage of the large volume of water needed for reactor cooling purposes. Separation and extraction of plutonium from the reactor fuel was performed in the

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central part of the site, six miles south of the reactor areas. Because the general flow of groundwater is from the northwest corner to southeast corner, there has been concern over possibility of contaminant transport into the Richland water supply.

There is an urgent need to remediate the site in a cost effective manner because of the wide array of waste produced over such a large area. The Hanford Site has experienced an interesting history of radioactive waste storage and disposal practices. Some examples include trenches, injection wells, airborne releases, single and double-walled underground storage tanks, storage cribs, and solar evaporation ponds. These practices have led to contaminated groundwater such as in a tritium plume that originated from the separation and extraction facilities and has expanded underground across the southeast portion of the site. In contrast to tritium, which is highly mobile in the aquifer, there are several localized plumes containing less mobile radioactive metal ions in all of the formerly active areas. Some sites show evidence of contamination involving various metal ions plus chelating agents that may enhance contaminant transport (4-5).

Ideally, large scale remediation should be performed with renewable materials that are inexpensive to produce. Properly harvested and prepared bacteria or fast growing organic material, such as seaweed, could be used as an economical substrate for sorbing and concentrating metal ions prior to vitrification, as well as in pump and treat methods (6-7). Natural organic material could provide a more economical and innocuous medium than synthetic sorbants. By using a ubiquitous microbial strain isolated from the Hanford site, we can develop a better an understanding of biological contributions to contaminant transport occurring on the site. A mechanistic understanding of bacterial and natural organic material

metal ion sorption characteristics will facilitate implementation of field remediation technologies by providing a basis for comparison of sorbant materials.

2.2 Background

The initial focus of this work was to explore the effects of cell treatment on protonation and cobalt sorption properties of a subsurface bacteria. Subsequently, this was augmented to examine the physiological processes that may be controlling these properties. Since intact cells were used to better simulate a natural sub-surface environment, it was necessary to differentiate between uptake (metals transported into the cells, active or passive mechanism) and biosorption (binding of metals to the cell exterior with no uptake) (8). Although this study was not intended to investigate uptake processes, minimization of their influence was essential in determining sorption characteristics. Heat treated cells were also examined to represent sporulated and non-viable cell characteristics.

Uptake can be actively controlled by the cell or proceed as a diffusion process. The active transport mechanism can be eliminated via use of agents such as sodium azide or 2,4-dinitrophenol which ultimately hinder ATP synthesis by blocking electron transport or destroying proton gradients (resulting in leakage of protons across the cell membrane), respectively (9). The passive transport mechanism (diffusion) cannot be controlled by chemical agents, but it can be monitored via long term sorption studies. Passive transport becomes the time limiting factor in equilibrium-based titration and metal sorption experiments. If uptake due to passive transport is shown to contribute little to the total metal ion sorption, data obtained in experiments utilizing ATP synthesis inhibitors can be ascribed

to metal ion biosorption behavior of the microbial extracellular surface. Additionally, one can extend the tests to determine if biosorption is a dominant process by comparing ATP inhibited cells to untreated cells. If inhibited cells show similar sorption characteristics to untreated cells and passive transport is an insignificant contributor to sorption, one can then attribute sorption characteristics of the bacterium to biosorption.

In addition to the cobalt diffusion study, we used potentiometric studies following an analytical method developed by Marinsky and coworkers (10-17) for polymers and humic acids. Their method determines ion transport into a polymer phase from potentiometric titration data obtained over a range of ionic strengths (10-11,14). The Donnan equilibrium expression can be used to describe a polymer phase invaded by both protons and sodium ions from the bulk solution:

$$pH_s - pNa_s = pH_g - pNa_g \tag{1}$$

where the subscript (s) represents the bulk solution phase and the subscript (g) denotes the gel phase. The Donnan equilibrium condition implies that the difference in pH and ionic strength of the bulk solution is equal to a corresponding difference in the polymer phase. Combining this with the Henderson-Hasselbalch equation:

$$pH - \log\frac{\alpha}{1-\alpha} = pK^{app}_{(HA)_v} \tag{2}$$

where α is the degree of neutralization, one can derive information regarding the physical state of the polymer phase. This relation can be used to determine flexibility and ion permeability of the polymer phase from bulk solution ionic strength and acid/base titration

data. Plotting pK^{app} vs. α will result in curves that are displaced upward for decreasing ionic strength (18).

Polymer phase flexibility can be enhanced with changes in ionic strength. At low ionic strength, few cation exchangable sites on a polymer will be occupied. The resulting negatively charged sites will repel each other. If the polymer is flexible, conformations will yield maximum distance between all negatively charged sites in order to minimize free energy. For high ionic strengths, most cation exchangable sites will be occupied. The resulting site charge neutralization will produce a more compact and rigid polymer conformation.

Structural characteristics determine permeability and diffusion of cations into the polymer phase. Cation diffusion into a polymer phase may be hindered by the size of the cation (including solvation sphere) as well as rigidity and packing of the polymer phase. For most polymer systems, permeability is dependent upon ionic strength in the most extreme sense. High ionic strength produces a polymer phase that is so rigid and compact that cation diffusion will be severely restricted. Thus, high ionic strength can impede cation diffusion into a flexible and permeable polymer by first occupying all of the cation exchangable sites within the polymer phase and then compacting the polymer in such a way that further diffusion is severely restricted. This was shown by Ephraim as the point at which different ionic strengths converge in plots of pK^{app} vs. α (18).

In addition to the pK^{app} vs. α curves, one can also apply the Donnan equilibrium relation in equation (1) to show that a plot of pK^{app} vs. pH gives a straight line for systems in which ionic strength effects are minimal. For polymer systems, these would correspond to salt

impermeable matrices. The simple plots in Figure 2.1 help illustrate this concept. The plot in Figure 2.1a represents a system in which a unique function describes the pK^{app} vs. pH relation for several ionic strengths. Because there is no ionic strength correction, this would correspond to a polymer phase that is impermeable to sodium ion invasion. Figure 2.1b describes an ionic strength dependent polymer system with individual functions for each bulk solution ionic strength. For a polymer matrix to have potentiometric properties dependent upon ionic strength, electrolyte diffusion into the gel phase must be occurring. This can be accounted for via the Donnan equilibrium term by correcting for the ionic strength of the bulk solution.
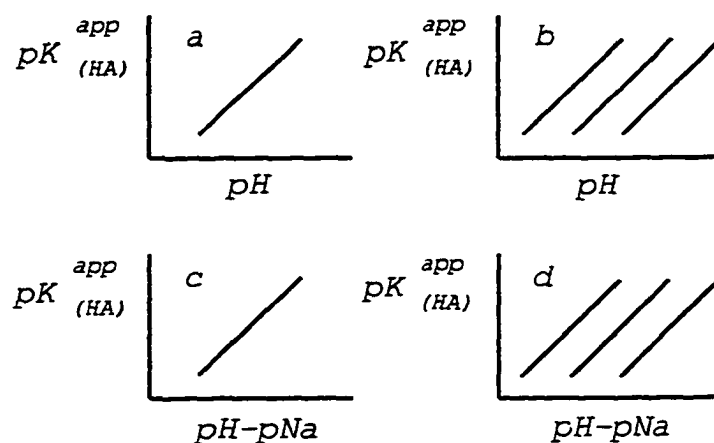
Figure 2.1. Sample plots describing potentiometric behavior of polymer matrices. a.) Impermeable b.) Permeable c.) Na-ion diffusion d.) No Na-ion diffusion

Figure 2.1c represents an ionic strength corrected system fitting a unique function for the pK^{app} vs. pH-pNa relation. Using a salt correction such as this describes a system in which sodium ions have diffused into the polymer phase. In Figure 2.1d, the ionic strength correction resulted in three functions, each one corresponding to a bulk solution of different ionic strength. The assumption that invasion of sodium ions into the polymer phase is a

function of ionic strength is not valid here as shown by the discrete solutions acquired.

Donnan theory can be applied in determining whether cations are participating in exopolymer phase ion exchange or sorption mechanisms. If cations are able to diffuse into the exopolymer phase, both sorption and ion exchange must be explored as potential contributors to biosorption. On the other hand, if the driving mechanism is exclusively sorption or ion exchange on the external surface of the polymer, cations will not penetrate the exopolymer phase, thus Donnan theory will not apply. This would be the case if no ionic strength correction were required in pK^{app} vs. pH plots. These concepts have been applied to characterize diffusion of cations into the *Bacillus simplex* exopolymer phase.

2.2.a   Spectroscopic Investigations of Metal Ion Binding

Many researchers have relied on sorption 'edge' and/or sorption kinetics experiments to establish a mechanism for the interaction of metal ions and mineral surfaces or natural organic material. Unfortunately, these experiments can only be used to quantify the macroscopic accumulation process. At best a guess can be made about the mechanism involved. A long-term trend is the use of sorption data and the proposed mechanisms derived by these experiments in mathematical models that attempt to describe the processes occurring in the aqueous phase as well as the solid/liquid interface. These studies attempted to describe a molecular process with macroscopic data. This approach often produced nothing more than elegant methods of curve fitting.

Previous research based on sorption experiments (edges and/or kinetics) have one major flaw: lack of direct evidence describing the accumulation process. It is impossible to obtain

structural data from sorption experiments that do no more than provide quantitative results. The exclusive use of sorption experiments to determine the mechanism of ion species accumulation at the solid/liquid interface is merely speculative.

Molecular and surface spectroscopic techniques provide a means to determine the nature of the accumulation process. Surface accumulation that occurs on the molecular scale can only be examined with techniques that operate on the molecular scale. X-ray diffraction (XRD) can provide information about the crystal structure of the surface that has formed as a result of the accumulation process. If precipitation has occurred, it will be indicated by sharp lines in the diffraction pattern. If there are no sharp lines, it can be assumed that no ordered surface structure exists. Electron spin resonance (ESR) can probe the speciation of certain metals in solution and sorbed to surfaces permitting the addition of a new dimension of analytical capability for use in sorption/kinetics experiments.

Spectroscopic techniques have instrumentation dependent weaknesses in the detection of chemical species. ESR, for example, is only sensitive to chemical species that contain unpaired electrons, thus species that lack unpaired electrons will not produce a signal in an ESR spectrum. Some techniques analyze dried powder or liquid samples, but not a combination of both, which would be required for observing accumulation processes at the solid/ liquid interface. Finally, there are techniques that use high energy radiation that could damage or destroy a sample.

Solid state samples can be analyzed via *ex-situ* (19) techniques such as X-ray photoelectron, X-ray diffraction (XRD), Auger electron spectroscopy, and secondary ion mass spectrometry. These techniques use extremely high energies in analyzing a sample

which could result in altering the physical characteristics of the sample during analysis or render the sample useless for further investigation (destructive techniques). Electron spin resonance (ESR) and cross polarization-magic angle spinning NMR (CP-MAS NMR) both use low frequency radiation to alter the electron (ESR) or nuclear (NMR) spin of the sample. Methods are available for the analysis of solid state samples for ESR and NMR spectroscopy that are usually non-destructive, although some sample heating can occur during analysis. Samples that consist of a mixture of solid and liquid phases can be examined using *in-situ* (19) techniques such as XRD, ESR, NMR, Raman and Fourier transform infrared (FTIR) spectroscopy. Some of these also qualify for use as *ex-situ* techniques. X-ray diffraction uses the highest energy, observing the crystal structure of the solid surface in the sample. Raman and FTIR spectroscopy use energy in the visible and infrared regions to vibrate the molecular bonds of solution and solid material. ESR and NMR are explained above and are normally considered solution phase techniques.

2.2.b   Use of NMR in Geochemical Applications

Current research in evaluating metal ion sorption to natural organic material uses titration curves and pK_a values for assignment of the primary functional groups active in the sorption process. Unfortunately, titrations are an indirect method for assigning sorption sites. Because of the time consuming nature of titrations, a faster, more efficient and direct spectroscopic technique is highly desirable to explore these interactions. NMR can be used to provide information about metal ion sorption to specific functional groups present in natural organic material.

This study determined the utility of NMR spectroscopy as a direct method of assigning functional groups active in the metal ion sorption process. Exposing a natural organic material to metal ions that exhibit significant paramagnetic properties decreases relaxation times. Peaks corresponding to functional groups influenced by the paramagnetic metal ions broaden as metal ion concentration is increased. Following the change in line broadening caused by presence of metal ions allows more accurate and reliable assignment of active functional groups in the sorption process.

2.2.c   Interaction of Co^{2+} Ions with Sodium Alginate

Preliminary experiments demonstrated that obtaining ^{13}C NMR spectra of microbial material for use in metal ion sorption experiments would not be successful. It was decided that a similar but less complex natural organic material should be used for the NMR investigations of metal-ion binding. The first choice was humic substances because of an in depth reference study previously performed by Thorn and coworkers (20). They compiled the ^1H and ^{13}C NMR of humic and fulvic acids in solution. Alginic acid was eventually chosen because of its low cost and availability. Alginic acids are widely distributed in nature, found both in seaweeds and bacteria (21), thus making them an excellent model for natural organic matter. A ^{13}C NMR investigation of purified alginic acid in the presence of heavy metal ions was presented in a paper by Wang and coworkers (22).

Highly purified sodium alginate samples allow resolution of carbon signals from the mannuronate and guluronate monomer units. NMR peak assignments obtained via a multi-step cleanup procedure provide information about the functional groups present in sodium

alginate. This resolution is informative, but not required to determine metal ion sorption properties. The sodium alginate ^{13}C NMR spectrum consists of three important regions. The most downfield one, at about 175 ppm, corresponds to the carboxylic acid group (C-6) on both the mannuronate and guluronate monomer units. The second group, at about 100 ppm, represents the C-1 linkage carbons, and the third group, upfield at 65-80 ppm represents the remaining ring-carbons. It would be expected that the carboxylic acid group would show the strongest interaction with Co^{2+} ions.

2.3 Materials and Methods

2.3.a   Cell Preparation

Bacillus simplex cells were incubated for 16 hours in PTYG broth (23) at 25°C, shaking with an orbital shaker at 140 rpm so that late log phase growth was attained. Harvested cells were immediately washed using either 0.001, 0.01, or 0.1M $NaNO_3$ for potentiometric titrations and 0.00033, 0.0033 and 0.033M $Ca(NO_3)_2$ for ^{60}Co sorption experiments. Cells were pelleted by centrifuging at 6000 rpm for 10 minutes. The supernatent solution was decanted and the pellets were washed two more times with the sodium nitrate or calcium nitrate solution. From the final cell solution, 3 to 5 mL aliquots were filtered with 0.2 μm polyethersulfone filters (Gelman Supor®-200) to obtain cell dry weights. It was later determined that filtering 5mL aliquots achieved higher levels of precision, thus the greater volume was used in subsequent experiments. Typical dry weights of 300 $\mu g/mL$ were obtained using this method. Data from solutions containing less than 240 $\mu g/mL$ were rejected in order to maintain late log phase growth conditions. At this stage the cell solution

were prepared for either metal ion sorption experiments or potentiometric titrations.

2.3.b   Cell Treatment

Sodium azide (NaN_3) was used as an ATP synthesis inhibitor in metal sorption experiments. In potentiometric titrations, both sodium azide and 2,4-dinitrophenol (DNP) were used in concentrations of 10^{-4} and $10^{-5}M$, respectively. Cells were heat treated by submerging cell solutions in an 80°C water bath for 15 minutes.

2.3.c   Wet Chemical Analysis

Potentiometric titrations were performed in a reduced CO_2 atmosphere to minimize carbonate buffering effects. Solution pH was monitored with an Orion Model 290A pH/ISE meter using a Cole-Parmer® Red-Rod™ combination electrode capable of operating under high sodium concentrations. The pH was adjusted with 0.1M HNO_3 (Baker Instra-Analyzed Reagent) and 0.0975N NaOH volumetric standard (Aldrich). Electrode maintenance was performed after every experiment to reduce the risk of electrode junction clogging. Maintenance consisted of soaking the electode, N_2 sparger and thermometer in a 1% pepsin/0.1M HCl solution for 30 minutes followed by draining and refilling the reference chamber. Cleaned electrodes were refreshed by soaking in storage solution for at least 1 hour. After removal from storage solution, the electrode was equilibrated in pH 7.00 buffer for at least 3 hours prior to calibration. Calibration was performed using VWR calibration solutions of pH 4.00, 7.00 and 10.00, with calibration curve slope ranging between 92% and 100% of Nernstian for all experiments. A DC-motor driven magnetic stirrer was used with

teflon stir bars to aid equilibration of solution and eliminate heat transfer effects typical of AC motors. Routine monitoring of solution temperature showed a fluctuation of less than 1 °C over the course of each experiment.

In metal sorption experiments, cell solution pH was lowered to a predetermined initial value using nitric acid. After acid addition, $Co(ClO_4)_2$ spiked with ^{60}Co as $^{60}CoCl_2$ was added to the cell solution to yield a final concentration of $10^{-5}M$ $Co(ClO_4)_2$ and 9000 cpm/mL ^{60}Co. For metal sorption experiments and potentiometric titrations, the cell solution was allowed to equilibrate for 30 minutes prior to starting base addition. When the solution had equilibrated, triplicate 1.00 mL samples were removed from the cell solution at each 0.5 pH unit and centrifuged at 8000 rpm to achieve phase separation. A 0.50 mL aliquot was extracted from the liquid phase and added to 4.5 mL of Ultima Gold scintillation cocktail (Packard, Downer Grove, IL) for liquid scintillation analysis.

During titrations, the cell solution was allowed to equilibrate at each half pH unit for an additional five minutes. Solutions were equilibrated for up to fifteen minutes during sorption experiments. These equilibration times were later shown to be sufficient in data from sorption experiments. A BASIC program was written for automated acquisition and recording of titration data using the RS-232C port of the Orion pH meter. See Appendix B for the instruction manual and program listing.

2.3.d   Preparation and Characterization of Sodium Alginate Samples

This study was based on work previously performed by Wang et al. (22) on the effect of several different metal ion concentrations on ^{13}C NMR spectra of sodium alginate solutions. Because their description of NMR parameters and sample preparation was so vague, the purpose of this study was to repeat their experiments with facilities currently available at UM. Sodium alginate is available in different viscosities and biological origins, and was initially purchased as the ICN high viscosity sodium alginate that originates from *Macrocystis pyrifera*. This has a nominal viscosity of 14,000 cps. The low viscosity product has an advertised viscosity of approximately 250 cps. These values correspond to 2% solutions of the polymer in water. The low viscosity sodium alginate was purchased after the high viscosity material produced and unacceptable signal to noise ratio on the NMR.

The goal was to use the procedure outlined in the paper by Wang and coworkers to analyze alginate samples using NMR spectroscopy. The samples they used originated from *Lessonia nigresens* and were supplied by Kimitsu Chemical Industries. They characterized their samples using GPC to obtain values for weight average molecular weight and polydispersity. They also used ^{13}C NMR of an acid hydrolyzed sample to obtain the ratio of mannuronate to guluronate residues. The hydrolysis step was based on work done previously by Haug and coworkers in the 1960's and is summarized below.

A series of papers starting in 1959 represents the efforts of Haug and coworkers (24-25) to explore solubility, viscosity, and electrophoresis of sodium alginate. In their initial studies, they obtained the alginate from dried seaweed using a series of extractions. This started with overnight extraction in 0.2 N sulfuric acid followed by shaking in 3% sodium

carbonate solution, filtration and finally precipitation with addition of alcohol to about 30% by volume. The final alginate sample was obtained after several centrifugation and filtration steps. A later paper investigating the viscosity of different alginates salts described how to obtain lower molecular weight alginates. They suggest an additional extraction using 1 N H_2SO_4 at 60°C for "a certain time" after the acid extraction and before the usual base extraction. This paper produced a 0.125% solution of sodium alginate with a relative viscosity of 31.99 in DI water.

A comparison of alginic acid intrinsic viscosities for *Laminaria digitata* and *Macrocystis pyrifera* shows that the two are similar. Alginic acid from *Laminaria digitata* has an intrinsic viscosity of 27.7 compared to 10.0 for *Macrocystis pyrifera*. The *Macrocystis pyrifera* Haug and coworkers used was from Kelco. Using the equation given for intrinsic viscosity:

$$[\eta] = \lim_{c \to 0} \left(\frac{\eta r - 1}{c} \right)$$

where c is a concentration, gives one results with units of ml/g. A two percent solution of *Macrocystis pyrifera* would have a relative viscosity of 10*100*.02 = 20. This is in agreement with the low viscosity sodium alginate that was used for this study.

Wang and coworkers (22) started with a 5% solution of sodium alginate, dialysed it twice for three hours in DI water using 12000 MWCO cellulose tubular membranes, and then once more for three hours in DI water. After dialysis, they centrifuged the sample at 20,000 rpm (38,460g) for 20 minutes to remove water insoluble substances. The sample was then freeze-dried. The absolute viscosity of a 5 wt% solution of the final sample was 2.93×10^{-2} Pa•s (29.3 cps).

The alginic acid samples used in the current study were purchased from ICN Biomedicals Inc. as the low viscosity sodium salt. In order to more closely mimic a natural organic material, no cleanup was performed on the sodium alginate. Samples were prepared by weighing out the appropriate weight percent and gently shaking in a closed tube for several days to produce a homogenous mixture. The sharp NMR peaks reported in earlier papers were not expected in this study due to the greater range of molecular weights present in the solution.

2.4 Results and Discussion

2.4.a Ionic Strength Contribution to Sorption Properties

Isotopic dilution sorption experiments were performed with ^{60}Co and the bacterial isolate to determine metal binding as a function of bulk solution ionic strength (Figure 2.2). These experiments utilized calcium nitrate solutions of ionic strength 0.1 and 0.001 (concentrations of 0.033 and 0.00033M). The significance of these data is the dependance of cobalt sorption on bulk solution ionic strength. The DeBye-Huckel treatment predicts that the higher ionic strength solution would sorb 47% that of the lower ionic strength solution. For these experiments, the data reveal higher ionic strength solutions sorbing only 23% as much of the cobalt ions as the lower ionic strength solution.
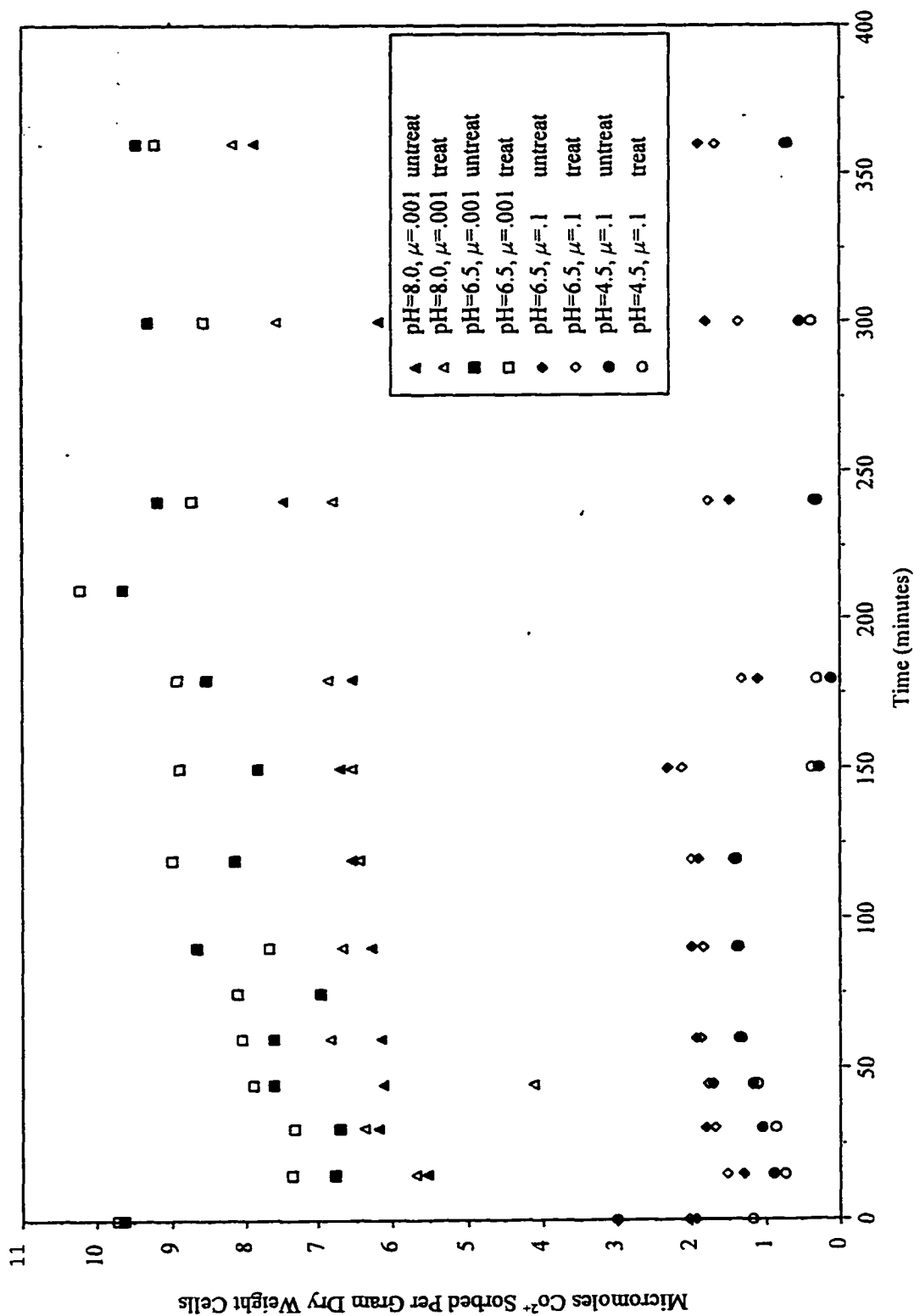
Figure 2.2. Sorption of cobalt by *Bacillus simplex* at different solution pH and ionic strength. Open symbols denote samples that were treated with 10^{-4}M sodium azide.

2.4.b Determination of Sorption Mechanism

A first set of titration experiments was used to determine a starting pH. Unlike humic substances, which can be protonated by passing through an ion exchanger, live cells cannot endure such treatment without suffering deleterious effects. This prompted the assumption that all available sites were fully protonated at an established low pH (26). Since the ionic strength effects in solutions as dilute as 1 mM were being investigated, the initial pH could not be extended below 3 without overwhelming maintenance of ionic strength. Thus, full protonation was assumed to occur at pH 3.

Three experiments were performed using different intital pH values for the start of the titrations. Figure 2.3 was produced by truncating data sets to start at pH 4.5 so that congruent curves indicate lack of contribution from initial pH. From this plot, the similarity of titration curves led to the conclusion that intitial pH was not a factor. The two outlier curves were later identified as pre-late log phase, hence this technique was also sensitive to cell growth phase.

Potentiometric titrations were performed on washed cells to determine protonation characteristics of different cell treatment methods. Earlier studies indicated that heat treated cells of this *B. simplex* strain showed a higher sorption capacity than untreated cells (3). This prompted the exploration of the potentiometric properties of heat treated cells. Heat treated cells (Figure 2.4) exhibit significantly different protonation properties than untreated cells. Inflection points from second derivative plots (Figure 2.5) show heat treated cells having 30% greater capacity for proton exchange (1.96×10^{-3} moles per gram dry weight cells) than untreated cells (2.79×10^{-3} moles per gram dry weight cells). The skewed proton exchange
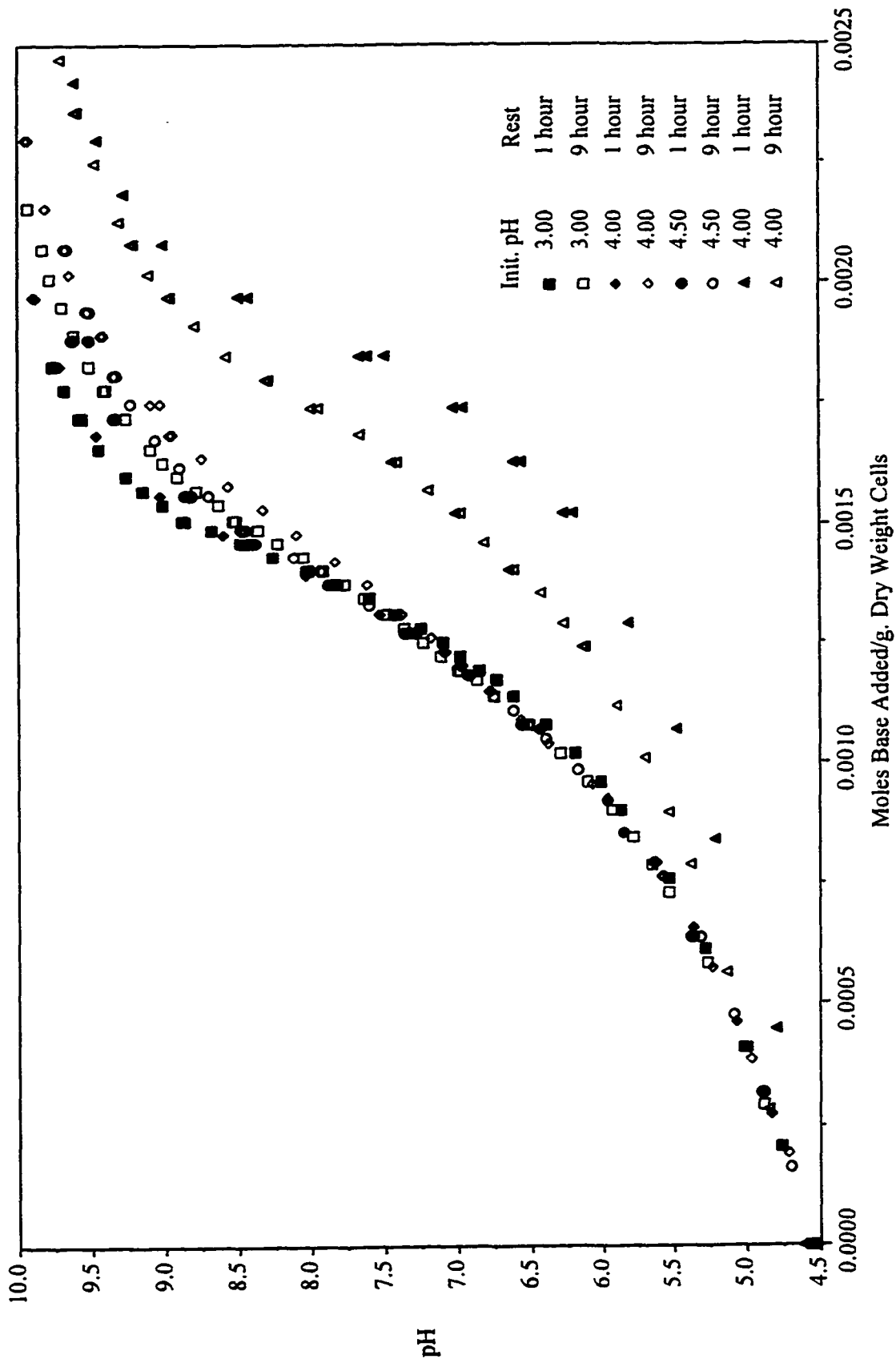
Figure 2.3. Initial pH corrected titration curves. Triangles (open and closed) represent systems with non-late log phase cells.

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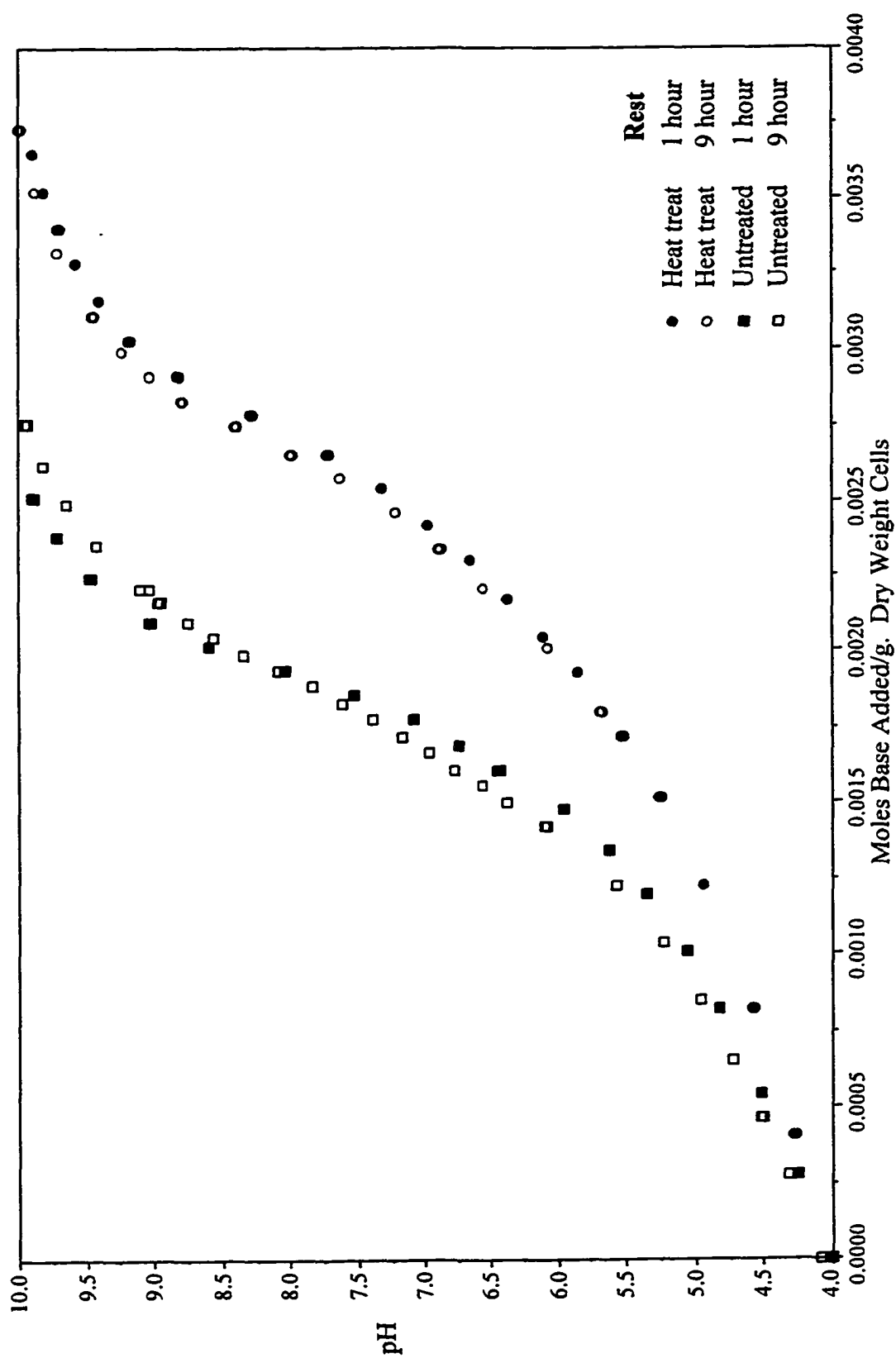
Figure 2.4. Effect of heat treatment on cell potentiometric titration properties. Circles indicate heat treated cells.
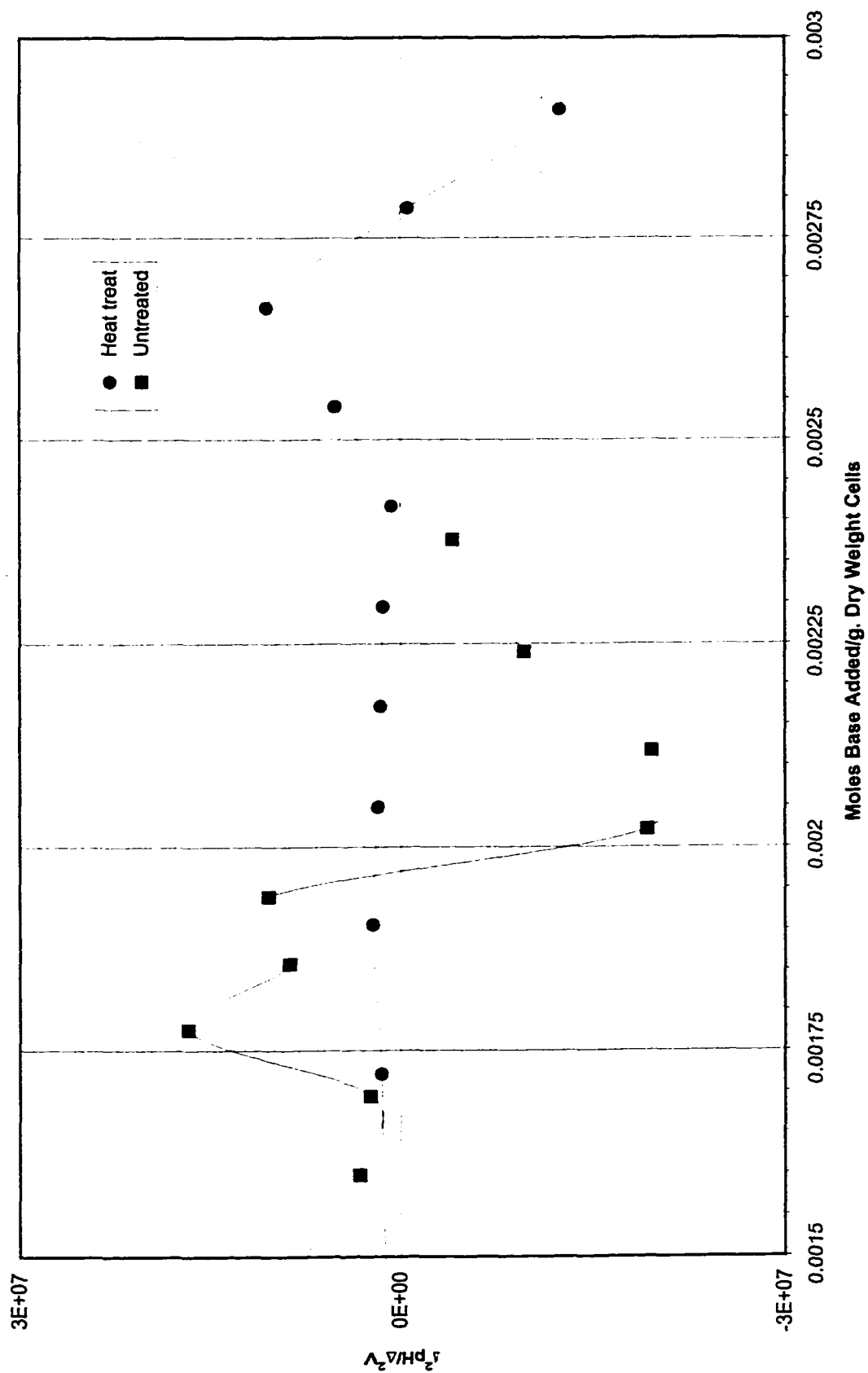
Figure 2.5. Second derivative titration plot of heat treated cells.

capacity could be due to heat induced exopolymer conformational changes or spore formation. In either case, non-viable cell and viable (as spores) cell material are measured simultaneously, and distinction is not possible.

In order to address the active transport problem, we compared the proton and cobalt exchange capacity of untreated cells to cells treated with sodium azide and 2,4-dinitrophenol. If active transport were a significant process, one would anticipate untreated cells to have a greater exchange capacity than cells treated with ATP synthesis inhibitors. On the contrary, second derivative plots of acid titrations (Figure 2.6) show that NaN_3 treated cells exhibited slightly higher (15-18%) exchange capacities than untreated cells. DNP treated cells demonstrated a trivial increase in proton exchange capacity over untreated cells. These increases could be due to inhibitor induced complexation, but this study could provide no data to support this. It is also possible that destruction of proton gradients by DNP allowed protons to move freely across the cell membrane. These data suggest that active transport is not a significant proton exchange process.

The cobalt sorption experiments served a dual purpose by providing information on metabolic inhibitor contributions to sorption characteristics. Figure 2.2 represents cobalt sorption data for cells that were exposed for up to 7 hours. From this plot one can see that cells treated with sodium azide exhibit cobalt ion sorption properties similar to untreated cells. This indicates there is little cobalt uptake by the cells via an active transport process. By combining titration data with cobalt sorption data we are able to determine that there is little hydrogen or cobalt ion uptake due to the active transport mechanism for washed cells.
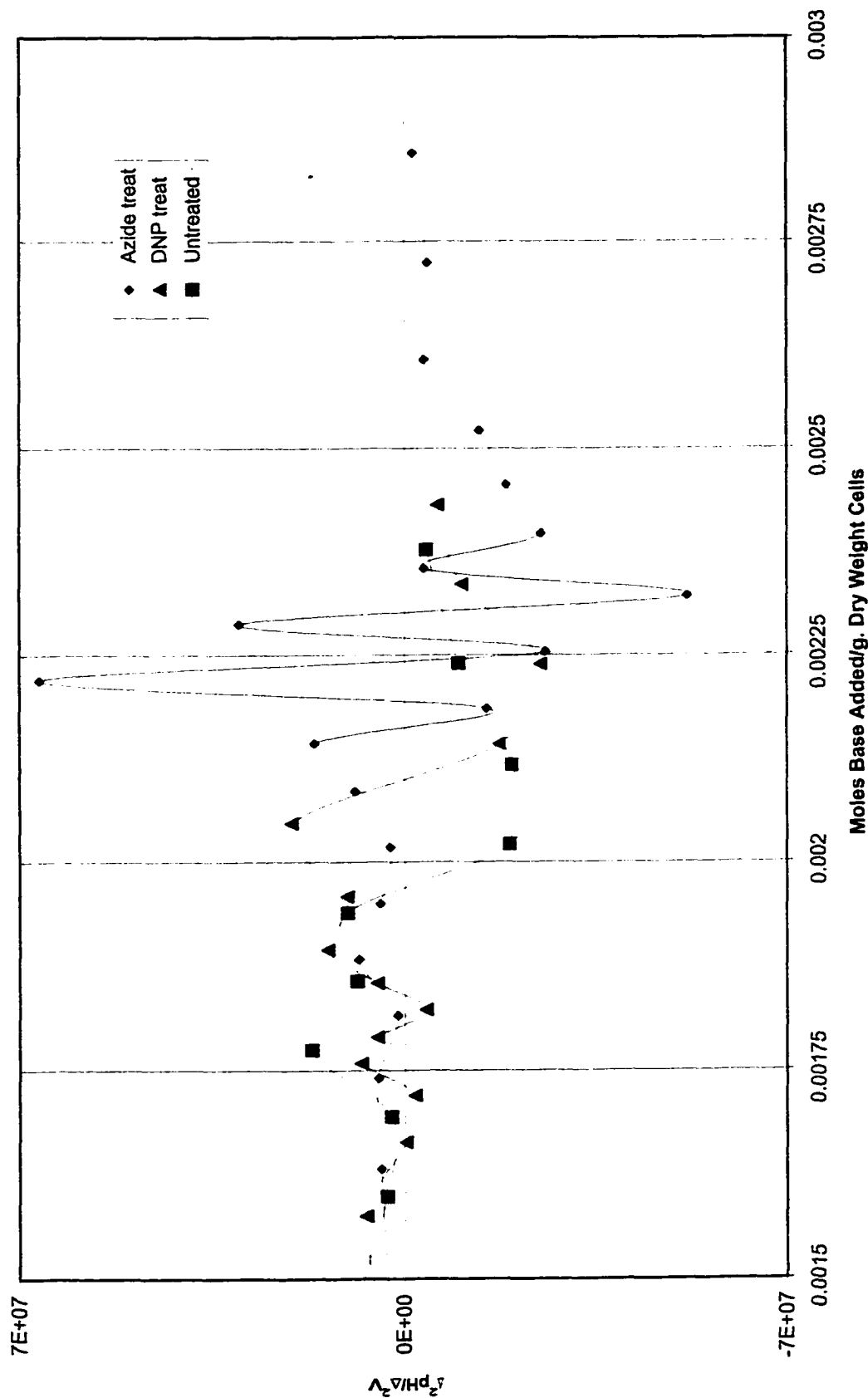
Figure 2.6. Second derivative titration plot of metabolic inhibitor treated cells.

Passive transport was monitored using data from cobalt sorption experiments run over an 7-hour period. The sorption profile of untreated and NaN_3 treated cells in Figure 2 demonstrates that no significant sorption occurs after the first 15 minutes of exposure. If passive transport was a factor, it would appear as a steadily increasing trend in sorption behavior. These results suggest that the passive transport mechanism contributes little to overall sorption characteristics and that biosorption is most likely not occuring on the interior of the cells.

2.4.c   Physicochemical Description of Ion Diffusion

Following Marinsky's treatment of polymers (10-11,14), an ionic strength correction was applied to the pK^{app} vs. pH plots of potentiometric data from *Bacillus simplex* cells. Figure 2.7 displays ionic strength corrected data from the *B. simplex* potentiometric titrations. Each ionic strength has a different curve but the same slope and range of pK^{app}, suggesting that salt invasion or diffusion is an insignificant factor with this species. Because the ionic strength corrected data suggest an impermeable polymer phase, the next step was to look at the uncorrected titration data. In Figure 2.8, one finds a unique function for all bulk solution ionic strengths, thus confirming that the exopolymer phase is impermeable to sodium ion diffusion according to the Donnan approach.
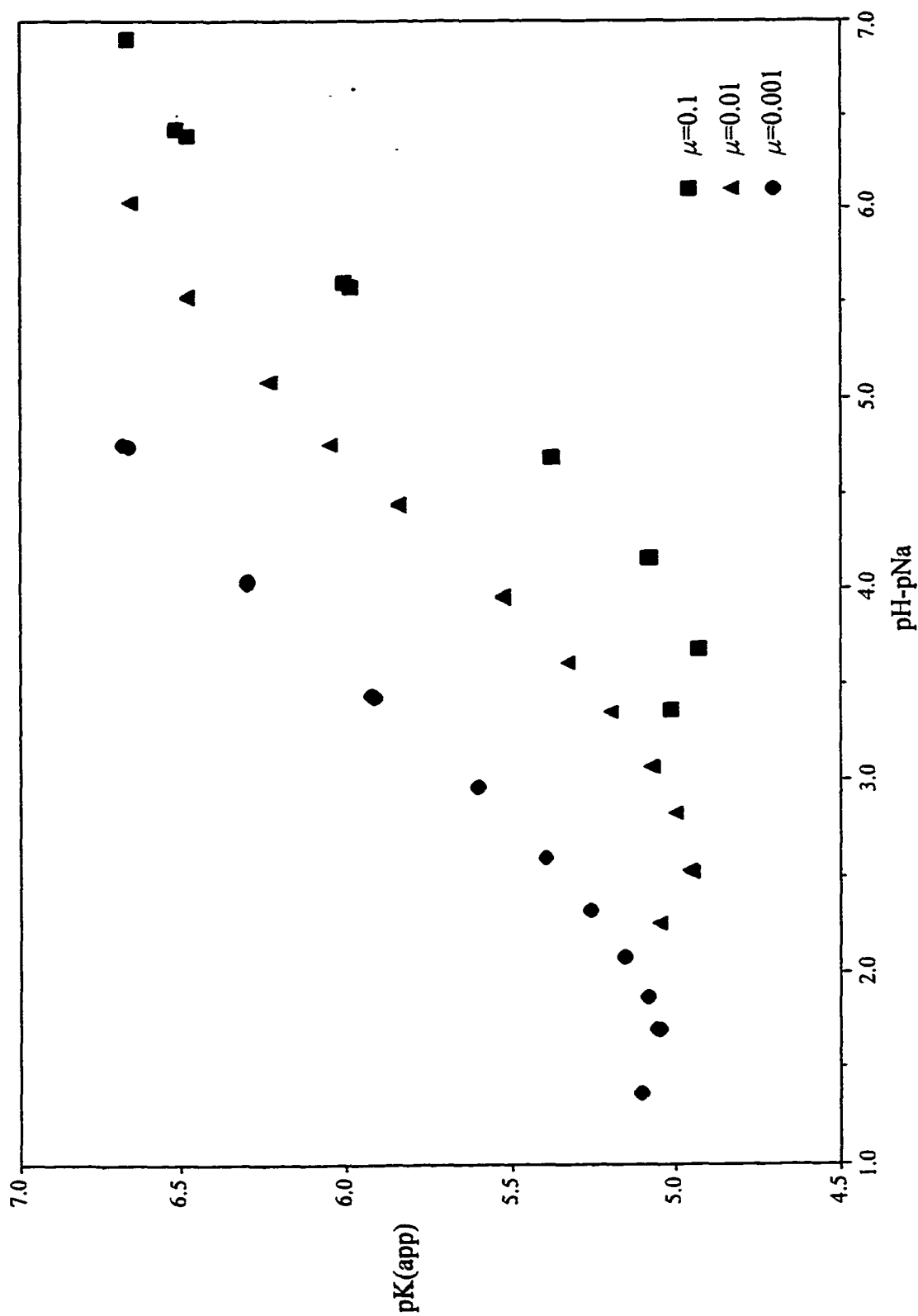
Figure 2.7. Ionic strength corrected potentiometric titration data from washed *Bacillus simplex* cells. The separation of the curves indicate that a salt correction is not required to achieve uniqueness.
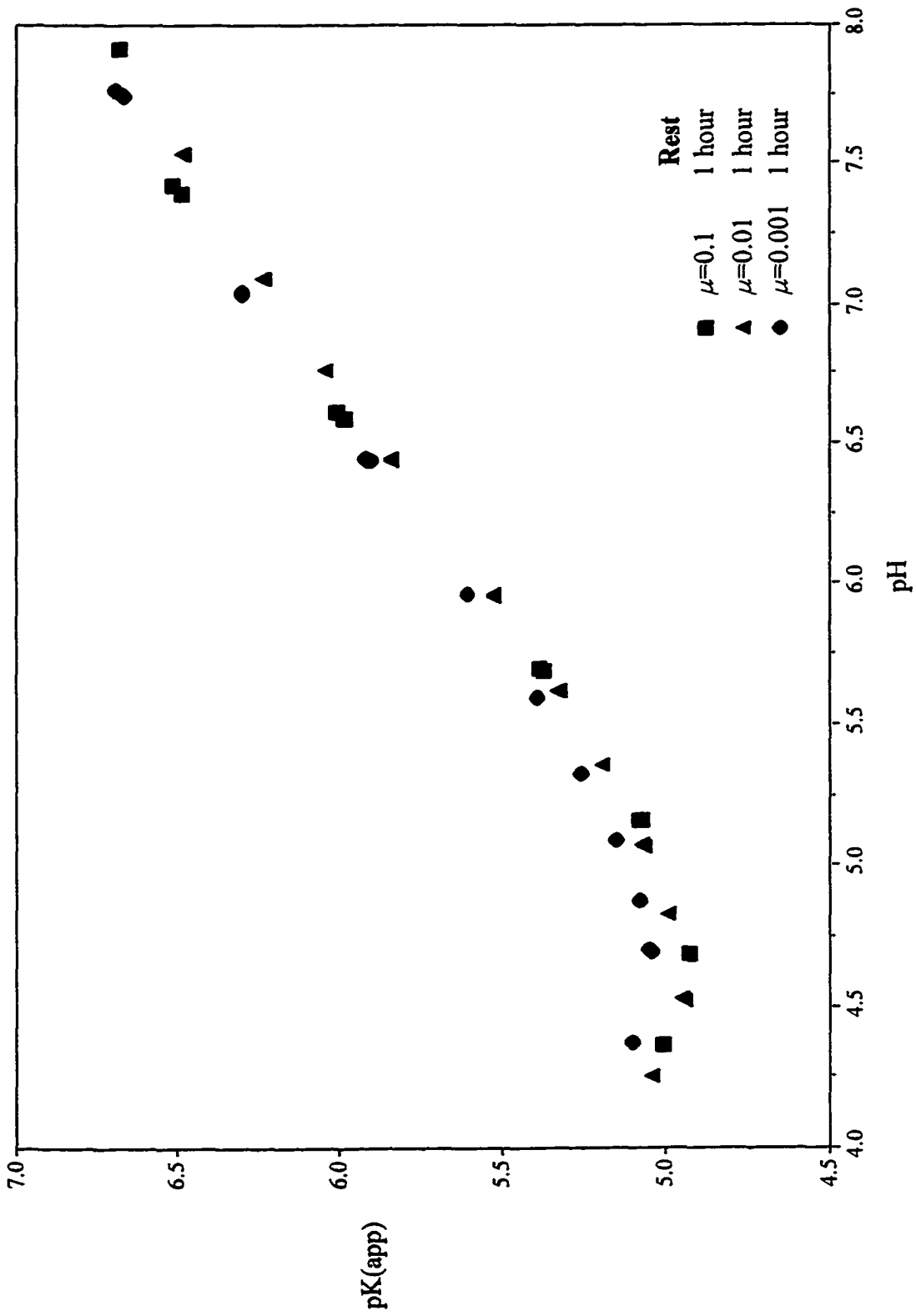
Figure 2.8. Potentiometric titration data from washed *Bacillus simplex* cells. The unique function represents a system with a salt impermeable matrix.

2.4.d Description of Spectra Obtained with Varian 400 MHz NMR

Several samples of sodium alginate with varying weight percent and temperature were run to optimize spectral features. Weight percent was explored first because the concentration change would have a greater effect on the spectra than the viscosity decrease that would accompany a temperature increase. Samples run were at 1%, 3% and 5% wt/vol. Temperature for all NMR experiments was 30°C except for the second run of the 5% solution sample which was performed at 40°C.

The natural polymeric material used in these experiments was very heterogeneous and, thus, had extremely fast relaxation times. Fast relaxation produces FID's with the majority of important spectral information occurring early, while the latter part consists mostly of noise. Line broadening is an apodization function that applies an exponential weighting factor of the form $\exp^{(-t^*\pi^* lb)}$ to enhance the early, signal-rich portion of the FID and minimizes the influence of the later, noise-rich portion (27). Spectra were all processed with line broadening set to 30 (lb=30) to enhance the broad peaks encountered. Figure 2.9 represents a typical spectrum obtained for the sodium alginate in solution.
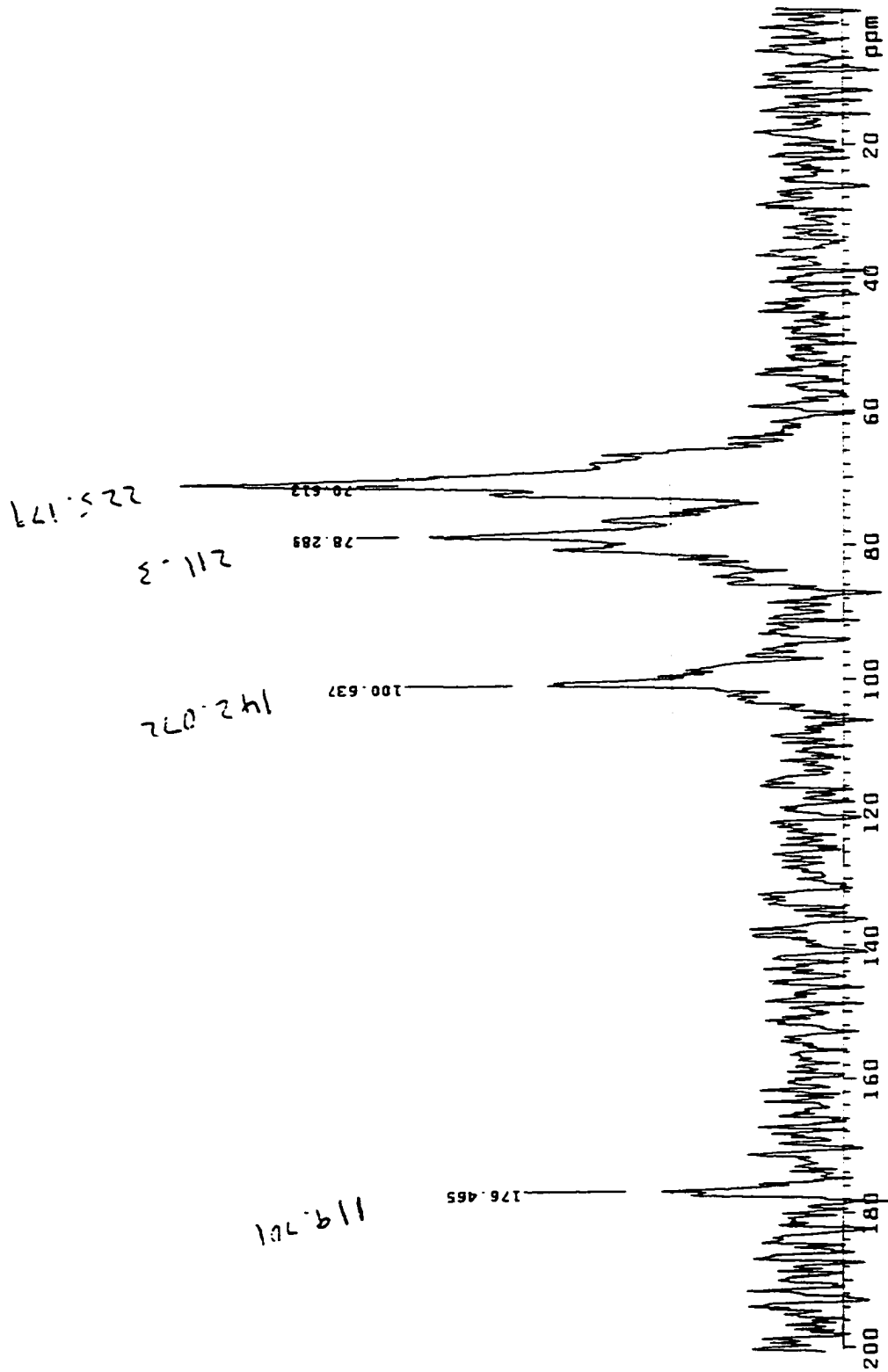
Figure 2.9. NMR spectrum of sodium alginate solution.

Table 2.1 Compilation of Peak Linewidths

	Chemical Shift				
Sample Name	176	100	78	75	70
1%lowvis30	111.975	252.431	129.023	120.217	330.913
3%lowvis30	126.975	240.928	385.474	364.826	329.438
3%lv30	107.867	151.978	348.527	330.049	324.807
5%lowvis30	123.117	214.896	438.410	194.292	366.247
5%lowvis40	93.654	197.076	347.721	145.277	301.779
5%lowvis30Co001	174.193	285.439	320.511	2019.7	306.427

Table 2.2 Compilation of Chemical Shifts

	Chemical Shift				
Sample Name	176	100	78	75	70
1%lowvis30	175.213	99.932		75.794	69.095
3%lowvis30	175.213	99.970	77.849	75.809	70.082
3%lv30	175.183	99.879		75.771	70.051
5%lowvis30	175.206	99.894	77.804	75.801	70.066
5%lowvis40	175.237	100.081	78.060	76.084	70.262
5%lowvis30Co001	176.837	100.341	78.267		70.468

2.5 Conclusions

Combining the cation diffusion data with previous sorption experiments exhibiting little difference in cobalt sorption for metabolic inhibitor treated cells and untreated cells, one can infer that diffusion of cations into the cell is not a major factor in metal ion sorption. It was concluded that the surface of washed *Bacillus simplex* cells is impermeable to sodium and cobalt ions and that sorption is due to surface charges in contrast to isolated sites inside the polymer matrix.

Sample preparation methods, sample concentrations and NMR experiments have all been optimized for the sodium alginate system. From line broadening data of the 5% w/vol samples, it is apparent that there is some advantage to heating the sample to 40°C during the NMR experiment. It is possible that the line broadening observed when the alginic acid was exposed to Co^{2+} ions is due to increased relaxation rates produced by the contraction of the polymer as the ionic strength increased. While this is a result of the metal ions binding to the polymer, it is not specific to where the metal ions are bound to the polymer. The contraction of the polymer could interfere with assignment of functional groups involved in metal ion binding. The cobalt induced chemical shifts for functional groups involved in metal ion binding are independent of the contraction of the polymer.

Chapter 3

3.1   On-line Beehives: Counter Design and Software

This chapter describes the design of the UM electronic bee counters and the software written to visualize and interpret flight activity data collected from these devices. This system provides a means of monitoring an important metric of honey bee population dynamics— foraging behavior. The research was funded by the United States Army Center for Environmental Health Research (USA-CEHR). The UM bee counters have collected data for three years at three field sites in Maryland, for two years at the Fort Missoula reference site, and for nine months from UM's on-line observation hive in the Health Science Building.

3.2   Design and Fabrication of the Bee Counters:

The design, fabrication, and development of the bee counters has been thoroughly described in a series of manuals and publications, which are included in the Appendices attached to this dissertation. The design of the bee counters can be found in Appendix E. The circuit diagrams used for the bee counters are located in Appendix N.

The bee-counter system (Figure 3.1) consists of: 1) a counting unit built into a porch that attach directly to the front of the protective boxes (condos) enclosing each of the nucleus beehives, 2) a modular digital interface that transfers the data to a computer, and 3) an IBM-compatible, 486 or Pentium-grade Personal Computer (PC) that collects and analyzes flight activity data. Each counting unit has 14 passageways through which bees can enter or leave

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the hive. Each passageway has an infrared emitter in the ceiling and two detectors in the floor under a clear Lexan® floor.
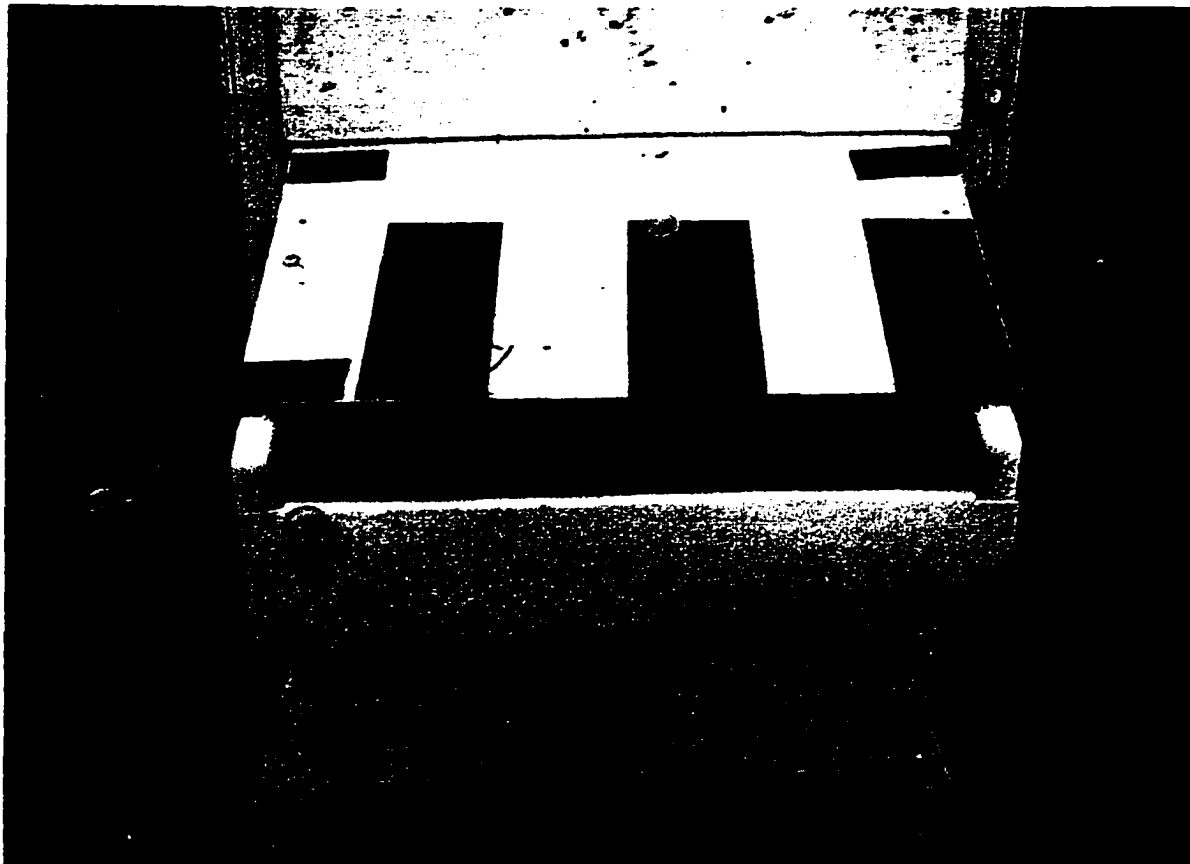
Figure 3.1. Bee-counter attached to bee-condo.

This design differs greatly from the counter described by Liu *et al.* (28), who used a microprocessor-based computer with a keyboard and display for each hive. Struye *et al.* (29) also used a hive-mounted, single central processing unit (CPU) which had a serial port connection that could collect data from more than one hive. Like Lui *et al.* (28) and Struye *et al.* (29), we used infrared photo-emitters and detectors to track bee activity. Struye *et al.* (29) used a custom dual-detector diode with 0.1 mm between the detectors to decrease the chance of counting two bees as one bee. The UM counter relies on rapid counts (i.e., each

detector is read at 200 times per second) and data acquisition and processing software to provide this resolving power. In addition, as many as ten of the UM counters can be monitored by a single PC computer. By taking this approach, the cost of monitoring multiple hives is greatly reduced. A single computer and digital interface is needed for each site, rather than a processor and interface for each hive. The software, not the hardware provides the required resolution. As evidenced by over three years of field applications described in Appendices F and G, these system have proven to be rugged and reliable. The initial counters are still working after three field seasons and multiple shipments back and forth across the United States each spring and fall.

3.3   Data Acquisition Software

Having developed and tested the counter systems, the next step was to provide the software for acquiring and storing the data collected at each field site. Stryue *et al.* (29) used a hardware based system to determine the direction of bees through their counter, while the UM system relies on data acquisition software for this task. Our software buffers snapshots of the detector states at the rate of 200 Hz over 30 second intervals. It then analyzes the 30 second data summaries to determine the direction of bee passage. Appendices F and G detail the software development, while appendix H explains the features and operation of the data acquisition software.

3.4 SITEVIEW Numerical Analysis and Visualization Software

As more and more beehives were outfitted with counters, the data files rapidly grew in size. It soon became apparent that simply collecting and storing the data in a text-based format did not address the problem of data processing and evaluation. Early attempts at using a spreadsheet or generalized advanced analysis software (MathCAD) did not allow the rapid processing and viewing of flight activity data required for the amount of data we were collecting. Most spreadsheets do not have enough rows to contain more than a few days of counter data, which can generate up to 1920 data collection periods per day.

Stryue *et al.* (29) used a spreadsheet and plotting software to generate their cumulative loss results, but they were looking at data from one counter, collected at 5 minute intervals. They calibrated their system using short-term intervention events such as inducing swarming or exposing a hive to pesticide spraying. Their software simply calculated the cumulative increase or loss in numbers of bees over a single day.

The SITEVIEW software was developed in response to the need to process and view the large amount of data (approximately 38 Gigabytes after three years) being continuously collected over long periods of time at several sites, each with seven or more counters per site. The raw data, a compiled version of the data acquisition program, and data files processed with the SITEVIEW program for the past three years are included in the accompanying CD-ROM. A user's manual for SITEVIEW appears in Appendix J. The manual explains the use of the software and provides guidelines and examples based on the accompanying raw data sets. Applications of SITEVIEW to actual field studies in Maryland and Montana, the results

of the data processing, visualization, and interpretation, and the conclusions derived from the field trials are described in Appendices F and G.

. The raw data from the bee counters are very similar to data collected from modern instrumentation used in analytical chemistry. The first problem to overcome was the noise associated with integrating the data over thirty second periods. Figure 3.2 provides an example of the raw data obtained by the bee-counters. This was accomplished using boxcar smoothing and high-pass filtering techniques. One of the caveats of boxcar smoothing is that brief events can be masked by using too large a boxcar. For example, if a sharp peak in activity occurred over a 2 minute period, using a boxcar of 16 (each point represents a thirty second period) would average the peak to the level of the normal activity. Figure 3.3 displays the effect of boxcar smoothing on the data used in Figure 3.2. In general, a boxcar of 4 to 6 proved to be most appropriate for evaluating bee flight data. Fourier analysis (using MathCAD) was attempted to determine if cyclic patterns would emerge in the data. The data proved to be relatively free of such patterns.

It soon became apparent that an important metric was the loss of bees due to events such as predation, exposure to toxics, death from old age, or drifting into the wrong hive. Drift into a hive and robbing of a weak hive by a strong hive was evidenced by a gain of bees. Thus, the number of bees entering the counter compared to the number of bees exiting the counter was calculated as the *Percent Returned*. This provided information as to the short term loss or gain of bees throughout the day or over more extended periods such as weeks or months.
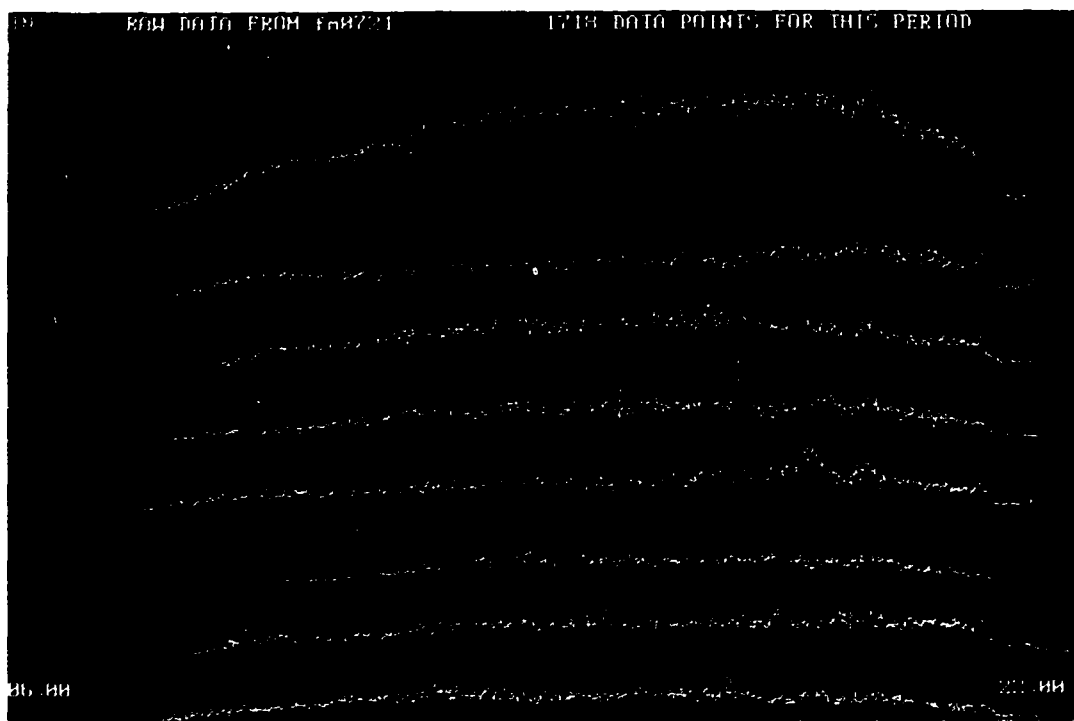
Figure 3.2. Screen capture of SITEVIEW displaying raw flight activity data.
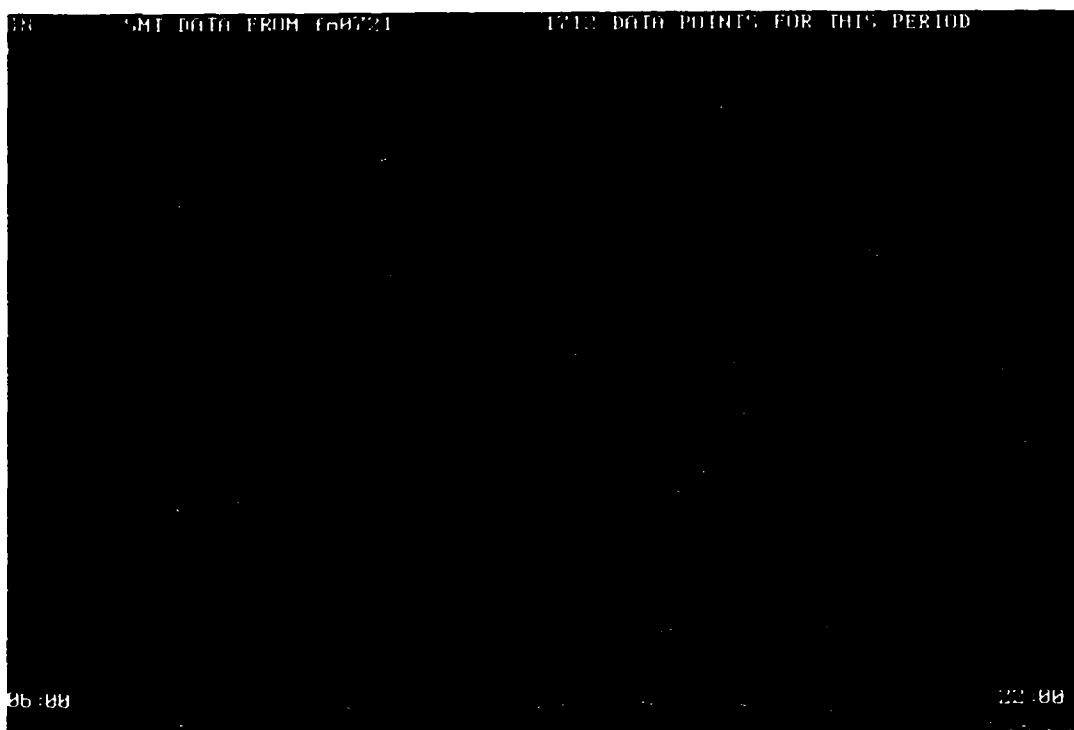
Figure 3.3. Screen capture of SITEVIEW displaying boxcar smoothed flight activity data.

Many short-term events were difficult to extract from a visual display of the data. The derivative of flight activity, (entering or exiting bees) proved to be useful in determining whether a sharp change in activity occurred. Both *Percent Returned* and the derivatives displayed peaks if unusual activity was detected, but these two techniques often produced a poor signal to noise ratio.

The DER*DIFF algorithm arose from the need to produce a sharp peak when changes in flight activity occurred and to improve the signal to noise ratio. This algorithm is optimized for the 'phase shift' that occurs between exiting and entering bees when an event, such as a chemical exposure, affects their flight activity. For example, puffs of smoke directed into the hive produced a sharp drop in the number of bees exiting the counter and then, after a brief period, a gradual drop in the number of entering bees. A similar phase shift was also found on days where methanol and Pine-Sol were released into the atmosphere of the Fort Missoula Apiary. Figure 3.4 provides an example of the high signal to noise ratio of the DER*DIFF algorithm.
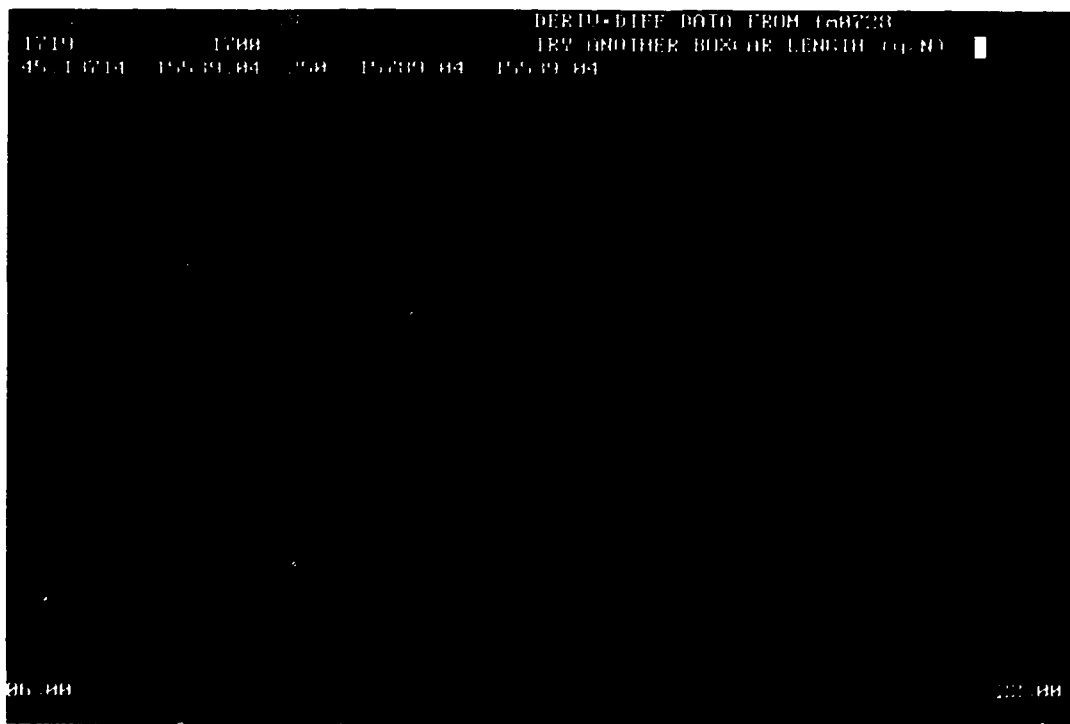
Figure 3.4. Screen capture of SITEVIEW displaying results of DER*DIFF
algorithm.

The SITEVIEW software provides a tool for researchers to process and view the

flight activity data collected from the bee counters. This tool aids further investigation of

honey bee flight activity behavior by making the data more accessible for viewing and for

saving the results into a format (comma delimited) that can be read by most spreadsheets and

graphing packages. Advanced analysis techniques developed into SITEVIEW can also be

used to detect real-time changes in flight activity.

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Appendix A

Contents of CD-ROM

The attached CD-ROM is compatible with MS-DOS or Windows based computers. It contains six directories that are described below.

ORION   This directory contains QuickBasic source code and executable files for the Orion pH Meter interface described in Appendix B. The original manual is included as a WordPerfect 8.0 document.

QDIG   This directory contains QuickBasic source code and executable files for the IoTech bee-counter interface in four different directories. The **1HV130KM** directory houses files for the single counter system for use with the Metrabyte digital interface. The **7HV130** directory houses data acquisition files for use with the standard seven counter field system. The **7HV130BB** directory contains a version of the data acquisition files for standard seven hive field system that has been modified to monitor three bumblebee counters as well. The **7FLD105** directory contains files for the testing and alignment of the emitters and detectors in the counters.

PDIG   This directory houses PowerBasic source code and executable files for the ComputerBoards bee-counter interface in two directories and the data processing and analysis software in another directory. The **1HV130KM** directory houses files for the single counter system for use with the ComputerBoards interface. The **10HV130K** directory houses files for use with the 10 counter field system. This directory also houses files for testing and aligning counters connected to the ComputerBoards interface. Neither of the files in the above directories have been tested in field use as these system are currently being brought online. The **MANIP6** directory houses three directories, **MANIP6, STVU6**, and **CHVU6**. The **MANIP6** directory contains the FILEFORM program used to process raw data files into a format that the SITEVIEW program can read. The **STVU6** directory contains the SITEVIEW program. The **CHVU6** directory contains the CHANNELVIEW program, used to view the individual channels from selected hive. The CHANNELVIEW program has not proven to be as useful as the SITEVIEW program, and thus is not as user-friendly.

LICORR   This directory houses PowerBasic source code and executable files for processing data collected from the LiCor Solar Radiation Datalogger. The COMM.EXE file was obtained to communicate with the datalogger, while the LCFRM.EXE file transforms the data into a format readable by most spreadsheet programs.

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BDATA   This directory houses compressed data files collected at the field sites included in the biomonitoring study. See Chapter 3 for a description of the site codes. The names of the directories for each site are constructed from the site code, the year and appended with the suffix 'data'. For example, all data from the Fort Missoula (FM) site during 1998 (98) is stored in a directory titled 'FM98DATA'. Inside of the site directories the daily datasets are stored in individual directories named with the site code and the date. Data collected from Fort Missoula (FM) on April 1, 1998 would be stored in a directory titled 'FM980401'.

BRES   This directory houses the SITEVIEW program and the flight activity data files that are accessed by SITEVIEW. Running the SITEVIEW.EXE file from the desktop or command line opens the SITEVIEW program. The naming system for the data files is similar to that used for the **BDATA** directory, but the suffix 'res' is appended to the site code and year code.

Operating instructions for Orion pH meter/data acquisition system

B.1.0 Introduction

This interface has been constructed because of problems reading the display on the Orion 290A pH meter. This meter is used for potentiometric titrations inside the glovebox, thus capabilities must include general data acquisition (saving and display), calibration, and display of meter information (calibration information, temperature, plotting parameters, length of data acquisition period). The computer currently used is an 80286 equipped with a 20M hard drive and 1.4M 3.5 inch floppy drive running Microsoft DOS 5.00. This system facilitates data collection and verification of calibration conditions during repeated titrations. Test files that can be read across many platforms are saved during experiments and include information about calibration parameters and titration data. The CPU fits beneath the glovebox table, a monitor can be placed to the side of the glovebox and the keyboard can be placed under the glove-ports to ease control of the meter during experiments. The RS-232C cable is manufactured onsite (pinouts provided below) and requires drilling another hole in the side of the glovebox. Moving the Orion pH meter to the back wall of the glovebox addresses 'real estate' problems due to housing the centrifuge required for metal sorption experiments.

The Orion 290A pH meter experiences some difficulty with reliable transfer of data through its serial (RS-232C) port. An earlier attempt to interface this meter was abandoned because of the communications problem. The program 'PH10.EXE' uses the structured nature of QuickBASIC 4.5 to overcome the serial data transfer problem. It does this by simply calling a data acquisition subprogram repeatedly until the variables returned fit the criteria for a reasonable pH measurement ($2.0 < pH < 14.0$). If the data acquisition subprogram detects a problem with incoming data, it empties the RS-232C buffer, sets the pH to an unreasonable value (1.00) and exits to be called again by the main program. The rest of the program features commands that are normally available to the user via the Orion 290A pH meter. This manual will display screen-prompts in *ITALIC* text and the responses to be entered in **BOLD**, any extra explanations will be in BLACK.

B.2.0 Starting the Program 'PH10.EXE'

The program is started by typing '**PH10**' at the DOS prompt in the C:\ORN\DISP\ directory. The first screen that appears should look like this:

WELCOME TO THE ORION 290A METER WITH NO SCREEN
(1) START A NEW EXPERIMENT WITH NEW FILENAMES
(2) ADJUST METER TEMPERATURE READING
(3) CALIBRATE pH ELECTRODE
(4) CHECK CURRENT CALIBRATION PARAMETERS
(5) ACQUIRE, DISPLAY AND ARCHIVE pH DATA
(6) COMPRESS AND TRANSFER DATA FILES TO FLOPPY DISK
(10) END PROGRAM AND EXIT TO DOS

CURRENT EXPERIMENT IS -->
PLEASE SELECT ONE OF THE ABOVE OPTIONS:

The user can select any of the above options to calibrate the electrode, acquire titration data, compress and transfer files to floppy disk, or exit the program. If a number appears in the top left corner of the screen, the user can press the *2nd-Print* keys on the meter to unlock the serial port. If this screen does not appear within thirty seconds, the user can attempt the *2nd-Print* key sequence on the meter, but most likely a system error has occurred and the user must enter the magic key sequence (control-alt-del) to reboot the system and start over.

B.3.0 Data File Nomenclature

At the beginning of each experiment the user must choose the first option to generate a filename for data files. Choosing option (1) will prompt the user with the following screen.

THE FOLLOWING IS A LIST OF EXPERIMENT CODES:
CODE   DESCRIPTION
A   FIRST EXPERIMENT OF THE DAY
B   SECOND EXPERIMENT OF THE DAY
C   THIRD EXPERIMENT OF THE DAY
D   FOURTH EXPERIMENT OF THE DAY
E   FIFTH EXPERIMENT OF THE DAY

PLEASE ENTER AN EXPERIMENT CODE? A
FILES FOR THIS EXPERIMENT WILL BE LABELLED AS 970114A

Filenames are formatted in six characters describing the date the experiment is performed as year-month-day. An additional one letter suffix is required so that multiple titrations could be performed on the same day. The user must provide a one letter suffix that will adequately describe the experiment being performed. Overwriting experimental data is not in accordance with Good Laboratory Practices (GLP), thus each experiment must have a unique filename. The one letter suffix is combined with the 6 letter date code prefix in constructing the filename (FLNAM) used to identify all files related to this experiment. Four files are associated with each experiment. The calibration routines will generate files named

FLNAM.CAL and FLNAM.SLP and the data acquisition routines will generate files titled FLNAM.BIG and FLNAM.DAT. The content and uses of these files will be discussed when the specific routines are described. Every time a data point is collected the files are opened and appended to minimize data loss due to power outages. After the user has entered an appropriate filename the previously selected procedure will be initiated.

B.4.0 Adjusting Meter Temperature

Temperature correction is performed by the Orion meter before data are transferred to the computer. It is important to adjust the temperature displayed on the meter to obtain accurate pH readings. Calibration on the Orion meter requires an accurate temperature because the meter automatically performs a temperature correction on the buffer pH value. The user can select the second option in the main menu to check and adjust the temperature displayed by the meter. If this is not in agreement with the sample or buffer temperature, the user can press + or - to adjust accordingly. The following is an example of the prompts displayed on the screen:

TEMPERATURE VALUE IN METER IS
PRESS + OR - TO ADJUST THIS VALUE
PRESS Q WHEN TEMPERATURE VALUE IS CORRECT

When satisfied with the temperature displayed, press **Q** to return to the main menu.

B.5.0 Calibrating pH Electrode

Calibration should be performed at the beginning of each day that experiments are conducted. The Orion meter gives the user the option of selecting a calibration curve consisting of up to five standard buffers. The current version of the interface will only support a three point buffer calibration. There is no support for a one-point 'adjustment calibration', and as this would not follow GLP, there is no intention to support this option. If necessary, the user could start a new experiment to record the value of the pH=7.00 buffer. This would give an indication of the level of electrode drift and whether the electrode is clean enough for another experiment.

Calibration with three buffers starts with the pH=7.00 buffer, moves to the pH=4.00 buffer and then to the pH=10.00 buffer. Entering and adjusting these values for temperature will be discussed below. The calibration data will be plotted in real-time with 10 second updates. The y-axis scale is adjustable from the keyboard by pressing the 'E' of 'C' keys. Pressing 'E' will expand the limits of the y-axis and pressing 'C' will compress the limits. The user can enlarge the data-stream plot to allow visual verification that the buffer pH reading is stable. A calibration data file will be written to record the progress of the calibration. The real-time display will allow up to ninety points to be plotted on the screen before a screen redraw will take place. A screen redraw will clear the screen, take the latest two-thirds of the data and plot it on the left-most two-thirds of the screen to allow use of the right-most third for plotting new data.

When the user is finished calibrating the electrode, a summary file is written that will be used to display calibration parameters. This can be accessed from the main menu by entering the third option or during titration data acquisition by pressing 'S'. After the summary file is written, the program automatically returns to the main menu.

B.5.1 Calibration Commands

Several keyboard entered commands are available to the user during calibration. These include **Y, N, +, -,** and **M**. The **Y** and **N** commands allow the user to accept (Yes) or reject (No) a buffer value. The **+** and **-** commands allow the user to raise (+) or lower (-) the buffer pH value. The **M** command tells the meter to exit the calibration procedure and begin measuring the solution pH. The E and C commands can be used as described above to control the y-axis limits on the real-time display.

B.5.2 Calibration Procedure

After the calibration option is selected in the main menu, the user can begin calibration of the pH electrode. The electrode must first be clean, filled with reference solution and equilibrated in storage solution. Next, place electrode in pH=7.00 wash buffer for at least 30 minutes to equilibrate. Rinse electrode by gently lifting up and down in the wash buffer for several seconds. Remove electrode from wash buffer (patiently waiting for the last drop of solution from the electrode) and place in the pH=7.00 calibration buffer. The status of electrode equilibration in the calibration buffer can be monitored by expanding (**E**) or contracting (**C**) the y-axis limits. When the y-axis range approaches 0.1, the ultimate resolution of the meter can be detected. A straight line for a significant number of data points indicates equilibrium has been achieved. When satisfied with equilibrium conditions, the user can press **Y** (yes) to accept this as buffer one. When the display changes to '*Buffer number 2*', the electrode can be removed from the calibration buffer and rinsed thoroughly with DI water. After washing electrode with DI water, insert into the second (pH=4.00) wash buffer and treat as above. Equilibration can usually be reached within five minutes because of the small change in ionic strength between buffers in comparison to the large change between storage solution and buffers. The third buffer (pH=10.00) can follow the same procedure as the second one. After pressing **Y** to accept the third buffer, the user must then press **M** to end the calibration.

B.5.3 Calibration Data File Formats

Three data files are produced as a result of the calibration procedure. The first file records the data collected from the pH meter at ten second intervals. This file is labelled FLNAM.CAL. The data format for FLNAM.CAL is space delimited as follows:

Time   pH   Temperature

The second and third files are duplicates of each other. One is saved as FLNAM.SLP and the other as SLOPE.DAT. The first of these files is used to maintain a record of the calibration parameters for the named experiment while the program uses the SLOPE.DAT file to display calibration parameters. If the user wanted to monitor electrode and buffer conditions using the slope of the calibration curve, the data could be retrieved from the file FLNAM.SLP. If it is necessary to read the FLNAM.SLP file, the full text appears below.

(blank line)
meas
(blank line)
CALIBRATION
P1 pH=4.007
178.7mV 22.3C
P2 pH=7.005
9.9mV 22.3C
P3 pH=10.040
-159.2mV 22.3C
(blank line)
SLP=95.5%
ISO=7.000
14:03 01-16-97

This file is created directly from what is sent through the serial interface from the Orion meter following the end of a calibration. Blank lines are not filtered out. A QuickBASIC subprogram called CALIBPARAM has been written to read and format data from this file. This code can be used to generate reports that track electrode performance.

B.6.0 Verification of Calibration Parameters

Calibration parameters can be verified from the Main Menu by choosing option 4. This option prints out the following data:

TIME AND DATE OF LAST CALIBRATION WAS 14:03 01-16-97
SLOPE = 95.5 PERCENT OF NERNSTIAN   IEP = 7.000

BUFFER	pH	mV	TEMP
P1	4.007	178.7	22.3
P2	7.005	9.9	22.3
P3	10.040	-159.2	22.3

PRESS ENTER TO RETURN TO MAIN MENU

This allows the user to determine whether the slope of the calibration curve was within an acceptable range. If the slope is unacceptable, a new calibration can be performed by returning to the main menu and starting a new experiment. Do not delete the calibration information, it can be accumulated as a record of electrode performance.

B.7.0 Description of the Data Acquisition Routine

The data acquisition routine is designed to collect, plot and save data at discrete time intervals (approximately 10 seconds) to facilitate monitoring of equilibrium conditions during a titration. It features real-time plotting with automatic baseline correction, automatic screen updates, temperature correction (user entered), and y-axis (pH) scaling options. The user is given the freedom to 'focus-in' the plot by pressing keyboard characters. Information about the current experiment and latest calibration can be displayed at the user's request.

To exit the data acquisition routine, press '**Q**'. This will close all files and return to the main menu.

B.7.1 Display Controls

When the data acquisition routine is activated, the first prompt that appears is as follows:

PLEASE ENTER THE DESIRED X-AXIS WIDTH (DEFAULT = 300)

This is the maximum number of discrete data points that can be drawn on the x-axis before a screen redraw occurs. A screen redraw takes the latest two-thirds of the screen and replots it on the first two-thirds of the screen. Real-time plotting will resume as before, but new data are only plotted in the last one-third of the screen. In order to minimize redraw times and to limit memory usage, the maximum value that can be entered for the x-axis length is 1000. After the user enters a value for the x-axis limits, the following prompt appears:

PLEASE ENTER THE DESIRED Y-AXIS WIDTH (DEFAULT = 1.0)

This controls how wide a range of pH is to be plotted on the y-axis. The program sets the first pH value obtained to be the initial baseline and plots that point at 40% of the screen height. 40% is chosen to reduce clutter at the top of the screen. Once data acquisition is in progress, the user can enter '**E**' or '**C**' to expand the pH range viewed or contract the pH range viewed, respectively. After the user enters this value, data acquisition begins.

While data are being acquired, the program will display, in the top right corner of the screen, the date and time in the first line, the pH and temperature in the second line and the number of samples plotted on the third line. Time is updated on the screen while the computer is not busy acquiring data or sending commands to the meter. The sampling interval is set to 10 seconds by the program (Shorter sampling intervals could allow multiple transfer errors to affect discreteness of data sampling). After each sampling interval, the new pH is displayed and the plot is updated.

B.7.2 Temperature Correction

If a temperature probe is connected to the Orion 290A meter, automatic temperature compensation will be used by the meter when determining the pH value. If no temperature probe is available, the user can adjust the current temperature (which is displayed on the screen) in 0.1°C increments by pressing '+' or '-' on the computer's keyboard. As the temperature is adjusted, the meter will make the appropriate pH corrections and new temperature data will be displayed on the computer screen. When the temperature is correct, the user can return to the main menu.

B.7.3 Data File Formats

Data will be stored in two files, one for monitoring equilibrium conditions and the other for addition of titrant. These files are labelled FLNAM.BIG and FLNAM.DAT, respectively. FLNAM.BIG saves data in the following format:

Time   pH   Temperature

This allows the user to import data as 'space delimited' into Mathcad, Excel or Deltagraph for further data analysis. During data acquisition, when the user adds titrant to the sample, a record is made of how much titrant is added and what time it was added. This is done by pressing '<enter>' and following the prompts. The first prompt asks if the user has added any titrant. This is done to allow the user to exit by pressing 'N' if the '<enter>' key was mistakenly pressed. If the user enters 'Y' at this prompt, the next prompt will ask what volume of titrant was added (in μL). If none was added, but the data point is considered significant, the user can enter zero (0). When the user has entered the volume of titrant added, the data will be recorded to the file FLNAM.DAT as:

Time   pH   Temperature μL added

These data can then be imported as 'space delimited' data as above for further processing.

B.7.4 Current Experiment and Calibration Information

If the user would like to see more information, a subprogram was written to display the current operating conditions. This subprogram is activated by pressing 'S' while data are being acquired. An example of the screen displayed is shown below:

TIME AND DATE OF LAST CALIBRATION WAS 15:00 10-22-96
SLOPE = 96.2 PERCENT OF NERNSTIAN   IEP = 7.000

BUFFER	pH	mV	TEMP
P1	4.005	190.4	20.4
P2	7.012	23.3	20.4
P3	10.068	-148.9	20.4

SAMPLES TAKE DURING THIS RUN = 24352
DATA TRANSFER ERRORS ACCUMULATED DURING THIS RUN = 1051
ERROR PERCENTAGE = 2.05
DURATION OF RUN = 243576.00 SECONDS
DURATION OF RUN IN MINUTES = 4059.60
DURATION OF RUN IN HOURS = 67.66
AVERAGE SAMPLING INTERVAL = 10.00 SECONDS
X-AXIS RANGE IS FROM 0 TO 1000
Y AXIS RANGE IS FROM 6.431 TO 6.531

PRESS ENTER TO RETURN TO DATA ACQUISITION

The screen is designed to inform the user of the date and results of the last calibration performed. It also include information about the length of the current run, the total number of samples collected, the number of data transfer errors through the RS-232C port, and the size of the plot window used to display current data. The error percentage corresponds to the number of data transfer errors that occurred in the sampling period.

B.8.0 Transferring Data Files to Floppy Disk

If the user has completed an experiment and would like to transfer data files to a computer suitable for data analysis, a **6** is entered in the main menu. The next screen will remind the user of the current experiment and ask if the user would like to transfer a different experiment to floppy disk. If the user wants to save a different experiment a prompt appears to provide the experiment name. After the experiment is transferred, the main menu appears again.

B.9.0 Additional Features

The program 'PH10.EXE' has some features in addition to those described above that aid in the reliable acquisition of data. First and foremost, is the power outage data saver. This is a subprogram that saves all data by opening data files, saving the data and then immediately closing the files. This minimizes the chances of a power outage or system crash leaving unclosed files. Some titrations last for several hours and loss of these data would be devastating.

Another feature is the user indicator. This indicator resides in the top right corner of the screen and is updated during titrations and calibration. When the indicator is not turning, the program is communicating with the serial port or replotting data. If the indicator does not move for over twenty seconds, the system has probably crashed and needs to be rebooted.

Fifteen seconds before midnight, the program will go into a timer update mode. This is to recalibrate the program with the computer's clock. The program relies on the TIMER command in BASIC to determine the length of wait periods for transferring data. If the TIMER is not updated properly, the program will be stuck in a loop until the following night. There is a seventeen second period where data are not being collected. This loss of data is considered insignificant in comparison to the consequences of not updating the TIMER function.

Appendix B.1 Available Commands

```
Press:     Result:
    Q      Quit sampling and return to main menu
<enter>  Save data to condensed log (pH titration file)
    +      Raise temp in 'pH' mode, raise pH in 'cal' mode
    -      Lower temp in 'pH' mode, lower pH in 'cal' mode
    E      Expand y-axis (pH)
    C      Compress y-axis (pH)
    M      Send 'meas' command to meter to end calibration
    Y      Accept pH value in 'cal' mode
```

Appendix B.2 Pinouts for RS-232C DB9 Connectors

```
          Meter Pin              Computer Pin

            1  _____         _____ 1

            2  _____         _____ 2

            3                            3

            4  _____         _____ 4

            5  _____ 5

            6                            6

            7                            7

            8  _____         _____ 8

            9  _____         _____ 9
```

The RS-232C cable uses two DB9 female connectors. This cable can be manufactured on site from any 8-conductor cable with two DB9F connectors.

For More Information:

Orion Laboratory Products Group, Portable pH/ISE Meters Instruction Manual; 1991; Orion Research Incorporated, Boston MA, 02129.

Appendix C
Analytical Procedures for University of Montana Experiments

U of M microbiology method:
Preparation of cells for titration experiments:

This method is used to determine the effect of cell treatment on titration curves of *B. simplex* cells. We are interested in changes in the cell surface due to factors such as inhibitor treatment, pH, ionic strength, lysis, freeze-drying, heat treatment and UV treatment. We will probably run three different ionic strength levels, but only after a run with only the middle ionic strength to confirm that the titration method is sensitive enough to see the effects of the treatments. The cell preparation and washing method will mimic that used at PNL for consistency.

Growth, prep, and pretreatment of cells:

Cell growth:
1) Prepare 10% PTYG broth from the 20X stock solution.
   a) Contents per liter:
      0.5g peptone
      0.5g tryptone
      1.0g yeast extract
      1.0g glucose
      0.6g $MgSO_4 \cdot 7H_2O$
      0.07g $CaCl_2 \cdot 2H_2O$
   b) Prep enough needed for each run.
      Minimum of 4x125 gives about 500 mL for cell growth, thus 250mL final volume.
      This will require four 250mL erlenmeyer flasks for each batch of cells.
      Add about 125mL of the 10% PTYG to each flask, then autoclave 'sterile' for 15 minutes.
2) Remove G8013 from -80°C freezer.
   Found on top shelf in rightmost styrofoam cooler (Gannon's).
   Remove the vial marked 'STOCK' and let thaw for approximately 20 minutes.
3) Add $10\mu L$ of G8013 from freezer vial to each of the 250mL erlenmeyer flasks using sterile pipette tips.
   Return vial to -80°C freezer immediately.
4) Shake at ≈140rpm for 16 hours at room temperature to reach stationary phase culture.

Cleanup and washing:
1) Transfer cell solutions from two 250mL flasks into eight 35mL Oak Ridge polypropylene centrifuge tubes.
   30mL into each tube works best.
2) Weigh each tube and match up to balance the rotor.
3) Centrifuge at 6000 rpm for 10 minutes at 6°C.
4) Remove from centrifuge and decant supernatant.
5) Add cell solution from remaining 250mL flasks to the centrifuge tubes and follow steps 2-4.
6) Wash each pellet with 20 mL of selected ionic strength $NaNO_3$ electrolyte (pH will be ≈6.5).
7) Weigh each tube and match up to balance the rotor.
8) Vortex for 20 seconds.
9) Centrifuge at 6000 rpm for 10 minutes at 6°C.
10) Remove from centrifuge and decant supernatant.
11) Repeat steps 6-10.
12) Add 30 mL of selected ionic strength $NaNO_3$ electrolyte (pH will be ≈6.5) into each tube.
13) Vortex tubes and combine into 500mL erlenmeyer flask for transport to Chemistry for analysis.

Dry weight determination:
1) Pre-weigh Gelman SUPOR-200 $0.2\mu m$ 25 mm filter paper and aluminum dishes.
   Use the five digit balance in Wood-Chemistry. Best to do this prior to growing cells.
2) Place filters in the Gelman 25mm Easy Pressure Syringe filter holder and close.
3) Attach 10mL syringe reservoir to holder and place on Cole-Parmer manifold.
4) Add a 5mL aliquot of cell solution to three of the filters, and 5mL of $NaNO_3$ electrolyte to one as a blank.
5) Use rotary vacuum pump to pull the liquid through the filters.
6) Within 6 hours, remove the filters from the holders and dry for one minute at 150° C.
4) Measure dry weights of filters.

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Freeze-drying process:
1) Pre-weigh VWR polystyrene vials using the five digit balance in Wood-Chem. Do not include cap weight.
2) Follow 'Cleanup and Washing' procedure up to step 11. Repeat step 11 for final wash.
3) Isolate pellets from centrifuge tubes and combine into pre-weighed VWR polystyrene vial.
4) Immediately place vial into the ultra-low freezer to pre-freeze sample for 1 hour.
5) Transport sample to Chemistry for placement in freeze-drier.
6) Follow instructions below for freeze-drier use.
7) When samples are dry, obtain dry weight of the cells.
8) To compare dry weights to previous experiments, divide by 240mL to get GDW/mL solution.
9) Add cells to 100g of selected ionic strength NaNO₃ electrolyte and reweigh vial to determine mass of cells added.

U of M Chemistry methods:
Operation of glovebox:
Daily maintenance:
1) Cycle main chamber with at least three glove volumes of N_2.
2) Check level of filling solution in pH electrode.
    If low, fill with 4M KCl using 100μL pipette.
Weekly maintenance:
1) Redistribute Ascarite in tray. Add another layer.
2) Cycle with He for 1 hour to refurbish atmosphere.
3) Standardize acid and base solutions using KHP.
4) Take inventory of solutions needed to be replaced. (DI, acid or base, buffers)
5) Every two weeks, replace the calibration buffer solutions.
6) Every two months, replace the bubbler solution. (Fieser's solution, Albert and Serjeant, Det. of Ion. Constants)
    16g sodium dithionite
    0.8g sodium anthraquinone-2-sulphonate
    15g sodium hydroxide
    in 100mL DI H_2O

Cycling main chamber:
1) Turn on N_2 tank by turning main valve 3/4 turn. Pressure should be between 40 and 60 psi.
2) Plug in vacuum pump connected to box.
3) Open the round valve on the right of the port 1/3 turn. This vacuum valve remains open during recycling.
4) Open valve directly above outer port door 1/2 turn to allow N_2 to enter the main chamber to fill gloves up.
    The is the 'recycle' valve.
5) When gloves are erect, close the valve above the outer port door.
6) Leave the vacuum valve open to empty gloves.
7) Repeat steps (4) to (6) four more times.
8) After the last cycle, close the vacuum valve and open 'recycle' valve to fill gloves.
9) When solution in 2 liter erlenmeyer flask bubbles, close the 'recycle' valve immediately.
10) Unplug vacuum pump and close the N_2 tank valve.

Using the port: (If unsure about plumbing, always trace lines first)
1) Set valves so that port is isolated from main chamber and close the flip-valve on top of chamber.
2) Open outside door using socket wrench.
3) Place materials in the port making sure that all lids are open to avoid air 'traps'.
4) Close outside door using socket wrench.
5) Open flip-valve on top of chamber and cycle repeatedly for 15 to 20 minutes.
    Adjust N_2 pressure to 40psi on regulator.
    Pull vacuum to no more than 30mm of Hg. Otherwise, you may catastrophically damage the box.
6) Switch valves over to the main chamber.
7) Remove clamping device from inner door.
7) Place clamping device securely on top of the plastic shelves.
    Be extremely careful here. Edges of the clamping device could rip the gloves. **Work precisely.**
8) Remove inner door to access materials.
9) Remove any waste accumulated inside of box. Always attempt to reduce clutter.
10) Close inner door being careful around sharp edges. Again, work precisely.

Use of carbon dioxide scrubber solution:
1) Adjust the pressure on the N$_2$ tank to less than 10psi.
2) Open the exit valve from the bubbler.
3) Slowly, open the valve into the bubbler. Allow a continuous stream to run through the bubbler.
4) Attach copper tube to the front N$_2$ inlet in the front of the glovebox. Place bubbler tube into sample.
5) Closing the bubbler is the opposite of opening.
> Be sure not to open the main valve while bubbler is open. Back pressure creates a mess.

Calibration of pH electrode: (Orion 290A meter)
1) Electrode must be clean, filled with reference solution and equilibrated in the storage solution.
2) Place electrode in the pH=7.00 wash buffer for at least 30 minutes to equilibrate.
3) Adjust the temperature on the meter to match the thermometer in the glovebox to ±0.5°C.
4) Press '2nd' then 'cal' on the meter to start the calibration.
5) Rinse electrode by gently lifting up and down in the wash buffer until the meter flashes continuously.
> Remove from wash buffer (get that last drop) and place in the pH=7.00 calibration buffer.
6) When the calibration buffer is equilibrated, the meter will flash the temperature corrected pH value.
> Press 'yes' to accept this as buffer one (P1).
7) Remove electrode from the calibration buffer and rinse thoroughly with DI H$_2$O.
8) Insert electrode into the pH=4.00 wash buffer (P2) and repeat steps 5-7.
> Wait at least five minutes for electrode to equilibrate with the new buffer.
9) Repeat steps 5-7 for the pH=10.00 buffer (P3), again waiting 5 minutes for the electrode to equilibrate.
10) Press 'measure' to accept the three-point calibration and record the slope.
> Slope should be between 92 and 102 for acceptable performance. If not, recalibrate or pepsin wash.

Standardization of NaOH solution:
1) Dry approx. 5 grams of potassium acid phthalate (KHP) at 110°C overnight. Let cool in desiccator.
2) Weigh out the KHP and dilute to 100mL in a volumetric flask. Gives ≈0.245M stock solution.
3) Dilute exactly 1mL into approx. 50 mL of DI H$_2$O into a wide-mouth polypropylene bottle.
4) This will require about 2.5mL of the 0.0975N NaOH solution to reach an endpoint.

Standardization of HCl solution:
1) Add exactly 2.5mL of standardized NaOH and approx. 50mL DI H$_2$O into a wide-mouth polypropylene bottle.
2) This will require about 2.5mL of the 0.1M HCl solution to reach an endpoint.

Titrations:
1) Place 1 inch stir bar in sample to be titrated.
2) Remove electrode from buffer solution and rinse thoroughly with DI H$_2$O.
3) Begin stirring sample at slow speed and insert electrode so that tip is in the center of the sample.
> The ammeter on the HP power supply should read ≈145 mA. Yes, it is supposed to bounce like that. (DC motor)
4) Insert thermometer and N$_2$ bubbler tube into sample.
5) When pH reading is stabilized, record pH and begin by adding about 100μL of 0.1M HNO$_3$.
6) After five minutes, record pH and add more acid if the pH reading is stable.
> If pH is near an integer value or a half integer value, record the pH but do not add for another five minutes.
> A stable pH reading will drift less than 0.005 units per 5 minutes.
7) Continue adding acid until pH≈4.00
8) When pH≈4.00, allow to equilibrate for at least 20 minutes, recording pH every five minutes.
9) When pH reading has stabilized, begin adding 0.0975N NaOH following the procedure for acid addition.
10) When titration is finished, thoroughly rinse pH electrode, thermometer, and N$_2$ bubbler tube with DI H$_2$O.
11) Place electrode in appropriate solution for next experiment:
> Unsterile pH=7.00 • If another titration is to be done within 6 hours.
> Unsterile storage soln. • If washing the electrode in pepsin the following morning.

Pepsin wash of pH electrode:
1) Remove electrode from unsterile storage solution and rinse with DI H_2O.
2) Immerse in 5% pepsin in 0.1M HCl solution (or HNO_3) for ≈ 30 minutes.
3) Remove electrode from pepsin solution and rinse with DI H_2O.
4) Using the glass pipette, <u>carefully</u> drain the KCl filling solution from the electrode.
5) Rinse with fresh KCl, drain and refill again.
6) Cover filling solution hole and rinse entire electrode with DI H_2O to remove excess KCl.
7) Place in sterile storage solution for at least one hour.

Pepsin wash of filter holders:
1) After removing filters for weighing, immerse tops of filter holders in 5% pepsin in 0.1M HCl bath for ≈ 30 minutes.
2) Rinse the holders in warm water and then DI H_2O.
3) Place in drawer to dry until next run.

Performance testing calibrated pipettes:
1) Transport pipettes, tips, vial of DI water and empty vial to a room housing a calibrated 5-digit analytical balance.
2) Allow to equilibrate to room temperature overnight.
3) Perform autocalibration on balance.
4) Tare a vial half-filled with DI water.
5) Pre-rinse the pipette tip several times with DI water.
6) Add designated volume to vial in balance by dispensing along the side of the vial.
7) Record the weight of each dispensing.
8) Repeat steps (6) and (7) 15 times.
9) Calculate accuracy and precision as shown below:
   Adjust weighed values for density changes due to temperature, barometric pressure and evaporation rate.
   Accuracy(% error) = 100* (adjusted observed reading - expected reading)/expected reading
   Precision(coefficient of variation): CV = 100*s/X
      where: X=mean; s=standard deviation

$$\text{Mean}(X) = \frac{1}{N}\sum_{i=1}^{N} X_i \quad \text{and Standard deviation}(s) = \sqrt{\frac{\sum_{i=1}^{N}(X_i - X)^2}{N-1}}$$

      where: N=number of pipettings; X_i=adjusted sample weight

Glassware washing:
1) Wash all glassware in warm soapy water and rinse in DI H_2O.
2) All titration vessels must be acid washed and then autoclaved prior to use.

Operation of freeze-drier:
1) This apparatus belongs to Jerry Bromenshenk, permission from Jerry or Bruce King is required prior to access.
2) Read manual attached to freeze-drier.
3) Prepare samples by pre-freezing if possible.
4) Place covered samples inside the drying chamber to prevent spray from contaminating the walls.
5) Check the silicon vacuum grease on the top plate seal, if necessary, add more from tube on bench.
6) Close drying chamber and start freeze-drier.
7) When temperature reaches -50°C, start vacuum pump and turn vacuum inlet to 'vac'.
8) When done, release vacuum and turn off freeze drier.

Appendix D
Analytical Procedures for PNNL Experiments

PNL microbiology method:

Micro-scale approach to conc vs. time experiments: *(Centrifuge for phase separation)*

This method is used to determine the rate of accumulation of metal ions by the microbial surfaces. This experiment is designed to take advantage of the facilities available at PNL's Environmental Microbiology (Bolton) and Interfacial Processes (Zachara) groups.

Growth, prep, and pretreatment of cells:

Cell growth:
1) Find 10% PTYG broth or make up fresh batch.
   a) Contents per liter:
      0.5g peptone
      0.5g tryptone
      1.0g yeast extract
      1.0g glucose
      0.6g $MgSO_4 \cdot 7H_2O$
      0.07g $CaCl_2 \cdot 2H_2O$
   b) Prep enough needed for each run.
      Minimum of 3x120 gives about 400 mL for cell growth, thus 200mL final volume.
      The Whatman $0.2\mu m$ sterile filter has a 1000mL reservoir.
2) Remove G8013 from -80°C freezer.
   Found on second shelf in the middle towards the front of the first rack.
   Place in opposite freezer to thaw. Takes about twenty minutes.
3) Filter 10% PTYG broth using $0.2\mu m$ sterile Whatman vacuum filter with 1000mL reservoir.
4) Transfer 120mL of sterilized 10% PTYG broth into each autoclaved 250mL screw-top erlenmeyer flask.
5) Add $10\mu L$ of G8013 from freezer vial to each of the 250mL screw-top erlenmeyers using sterile pipette tips.
   Return vial to -80°C freezer immediately.
6) Shake at 140 rpm for 14 hours at room temperature to reach stationary phase culture.

Cleanup and washing:
1) Transfer cell solutions from 250mL flasks into 35mL polypropylene centrifuge tubes.
2) Centrifuge at 22,000xg (14000 rpm) for 15 minutes at 6°C.
3) Remove from centrifuge and decant supernatant.
4) Wash pellet with 30 mL of 0.001 M (or selected ionic strength solution) $NaNO_3$ electrolyte at selected pH.
   Combine sets of pellets from the first centrifuge run, but no more than 2 pellets in one tube.
5) Vortex for 20 seconds.
6) Repeat steps 2 to 5 two more times. The final solutions will then be combined in a 250mL erlenmeyer.
7) Obtain dry weight of a 3mL aliquot of cell solution using tared filter paper on the multi-filter system.
   Place filters in oven at 50°C for 24 hours to evaporate any residual liquid.
8) Measure dry weights of filters.
9) Transport cells to RTL for analysis.

Work done at RTL

Concentration vs. time method:

Centrifuge and scintillation vial preparation: (Done day prior to run)
1) Label vials with a three letter code as follows:
   First letter indicates the time interval for sample exposure.
      Use 1, 2, 3, 4, 5... for 15 min., 30 min., 1 hour, 1.5 hour, 2 hour.
   Second letter indicates treated vs untreated.
      Use T for treated and U for untreated.
   Third letter indicates sample number or type.
      C for control, and 1 or 2 for the exposed cell containing samples.
2) Weigh each centrifuge and scintillation vial empty.
3) Cover vials to eliminate dust collection.

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Cell pretreatment:
1) Split the cell solution into two flasks of 50mL each.
        Add 50μL of the 0.05M NaN₃ solution to obtain 10⁻⁴M azide in the 50mL cell solution.
2) Verify pH of both solutions and adjust with HNO₃ or NaOH if necessary.

Cell exposure and extraction:
1) Add 1.0mL of cell solution to each centrifuge vial.
2) Weigh and subtract the vial weight. This gives the cell solution weight.
3) Add 10μL of metal solution to each centrifuge vial.
4) Weigh and subtract the vial weight. This gives the spike weight.
5) Shake centrifuge vials for allotted time intervals at setting 15 on the Blue M shaker.
6) Centrifuge at 10,000 rcf for 8 min. at 20°C on the Sorvall centrifuge using GSA rotor and custom sleeves.
7) Extract 0.5mL solution from each centrifuge tube and add to corresponding weighed scintillation vials.
8) Immediately measure the pH of the remaining solution in the centrifuge vial.
9) Weigh and subtract the scintillation vial weight. This gives the scintillation aliquot weight.
10) Add Ultima Gold scintillation cocktail and count with liquid scintillation counter.
        Use Protocol #29. This is the test protocol and thus will give cpm instead of dpm.

EDTA treatment:
1) At the latest time periods sampled, treat 3 sets of samples with EDTA.
        Samples are: zero time, 15 min, and 30 min.
        Each sample treated receives 50μL of the 10⁻⁴M EDTA solution, giving 5 X 10⁻⁶M EDTA.
2) Samples are then shaken and processed as above.

Sorption edges:
*Microbiology method was modified by using a tared 250 mL polypropylene container to transport the cells. 200mL of cell solution was placed into this container for sorption studies.
Centrifuge and scintillation vial preparation: (Done day prior to run)
1) Label caps of scintillation vials for triplicate samples at each pH.
2) Weigh each scintillation vial empty.
3) Cover vials to eliminate dust collection.

Cell exposure and extraction:
1) Weigh polypropylene bottle containing the cell solution.
2) Weigh bottle to obtain the amount of spike solution added.
3) Add acid (HNO₃) in small increments until pH=4.0.
4) Let solution equilibrate for 30 minutes.
5) Spike the cell solution with 2mL of the ⁶⁰Co solution at 10⁻³M total Co to obtain 10⁻⁵M solution.
6) Allow solution to equilibrate again for 30 minutes.
7) Collect triplicate 1mL samples into labeled microcentrifuge vials.
8) Centrifuge at 10,000 rcf for 8 min. at 20°C on the Sorvall centrifuge using GSA rotor and custom sleeves.
9) While samples are in centrifuge, increment the pH by adding base (NaOH) and equilibrate for 15 minutes.
10) Extract 0.5mL solution from each centrifuge tube and add to corresponding weighed scintillation vials.
11) Weigh and subtract the scintillation vial weight. This gives the scintillation aliquot weight.
12) Add Ultima Gold scintillation cocktail and count with liquid scintillation counter.
        Use Protocol #29. This is the test protocol and thus will give cpm instead of dpm.

Design and Fabrication of Electronic Bee-counters

E.1.0 Introduction to Bee-counter Design and Theory

The bee-counters provide a means of passively monitoring the flight activity behavior of bees by measuring the number of bees that traverse an artificial entrance/exit passageway. The physical design of the bee-counter serves several purposes, including:

- Prevent drifting of bees to other hives using takeoff and landing areas for forager bees with unique color and shape markings on each counter.

- Force bees to traverse an artificial passageway to enter/exit the hive.

- House the electronics used to count the passage of bees through the counter. Includes emitter and detector boards with a ruggedized connector to the central computer interface.

- Protect the electronics from damage due to weather and small animal intrusion.

- Protect the hive and electrical components from water damage by setting the counter platform on a slight downward angle.

- Minimize effect on chemical sampling by constructing of plastic materials similar to other parts of the hive housing.

- Build to withstand shipping to remote field sites.

- Construct to be easy to clean, troubleshoot and maintain.

The bee-counters are constructed from plastic materials (polyethylene) that are easy to machine. A higher density polyethylene is used for housing the emitter and detector components so that cross-talk between channels is minimized. The high density polyethylene was also less susceptible to melting during machining. Early experiments showed that the low density polyethylene allowed penetration of the infrared beams used in the counting apparatus.

The bee-counter frame consists of two side plates (one is slightly larger than the other to hold the ruggedized connector), a landing porch, a detector holding unit, a clear Lexan® sheet, and a roof to block out sunlight. The landing porch serves to connect the two side plates and provide a slightly enlarged area for the bees to enter and exit the counter. The detector holding unit also connects the two side plates and is leveled with the landing porch.

Detector circuits are attached to the detector holding unit. The emitter holder is attached to the detector holding unit during final assembly to aid alignment of the emitters and detectors. The Lexan® sheet provides a surface for the bees to walk on and also allows the infrared beams to pass from the emitter to the detector. The roof is placed so that late afternoon sunlight (low angle) is prevented from activating the detectors. The ruggedized connector withstands harsh field treatment (dropping, tugging), secures the cable to the counter and provides a weather proof seal.

The bee-counters are connected to the interface unit by means of shielded 30 conductor cables. These cables are equipped with ruggedized connectors to match the connectors on the bee-counters. The interface unit is housed in a weather proof shed or tool-box with the central computer and other electronic monitoring interfaces.

E.2.0 Theory of Operation

The bee-counters rely on bees to block the path of an infrared light beam from an infrared emitter photodiode to a wavelength matched infrared photodetector. In order to determine the direction of the bee traversing the counter, a single emitter (photodiode) illuminates two detectors (photodetectors) as shown in Figure E.1.

Emitter

■

■ ■

Detector 1   Detector 2
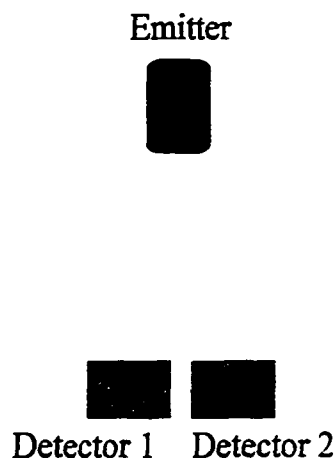
Figure E.1.   Alignment of infrared emitter and photodetectors used in bee-counters.

A dual detector design can determine the direction of the object blocking the detectors by decoupling the sequence in which the detectors are blocked. For example, if the object first blocks detector 1 (on the left), then blocks both detectors, and finally blocks only detector 2 (on the right), it will have moved from left to right. This sequence is shown in Figure E.2.
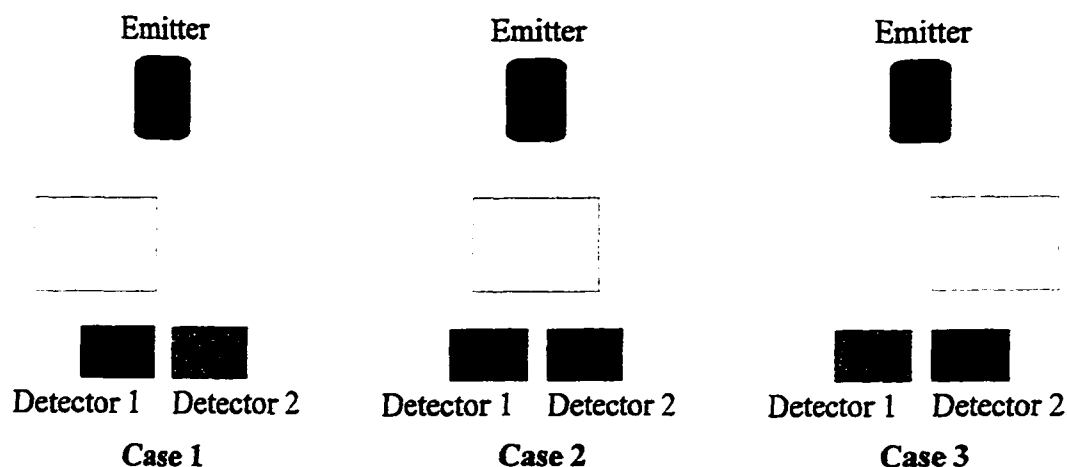
Figure E.2.   Direction of object determines sequence of detector responses.

In Figure E.2, the object is properly detected as moving from left to right. The detection of the object depends on several conditions:

a)   IR transmissivitty of the object, i.e., does it block the infrared beam?
b)   Size of the object.
c)   Spacing of the detectors.
d)   Reception angle of the detectors.
e)   Rate at which the object travels past the detectors.
f)   Rate at which the detectors are monitored.
g)   Response time of the detectors and all electronic components in the detector circuits, including the computer interface.

Condition (a) is independent of all other conditions, but crucial for detection of the object. Conditions (b), (c), and (d) are dependent upon one another. The size of the object affects the spacing of the detectors. A small object requires smaller detector spacing than a large object. Minimizing the reception angle of the detector helps to eliminate crosstalk, but also makes alignment more difficult. A wide detector spacing could require a detector with a large reception angle.

Conditions (e), (f) and (g) are interdependent and all depend upon conditions (b), (c), and (d). A small object that travels past the detectors at a fast rate will require that the detectors be monitored faster than a slow moving object. The rate at which the detectors are monitored depends on the response time of the detectors. Monitoring a detector faster than the rise or fall time of the detector will produce a delayed response to the object blocking the detector.

E.3.0 Description of Electronic Components
E.3.1 Infrared Emitters and Emitter Circuits

The infrared emitters used were manufactured by QT Optoelectronics (DigiKey part# F2D2QT-ND). These emitters operated at 880 nm and provided a beam angle of ±10 degrees. Emitters were placed in a circuit that would provide a light source for all fourteen passageways on the counter. A simple circuit diagram for one emitter is shown in Figure E.3.

$$+5 \; VDC \qquad R_I \qquad LED \qquad GND$$
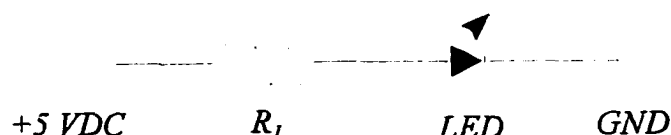
Figure E.3.   Emitter circuit diagram.

The value of resistor R_1 is 100 Ω. This resistor is used to limit the current through the LED to a safe value (Mims, 1986). The equation used to calculate the value of resistor R_1 is:

$$R_1 = \frac{V_I - V_{LED}}{I_{LED}}$$

The F2D2QT-ND infrared emitter has a LED voltage drop (V_{LED}) of 1.7 volts and a forward current (I_{LED}) of 100 mA. This, coupled with a 5 VDC power supply, requires a resistor value of at least 33 Ω. In order to limit the current to some degree, the 100 Ω resistor was used. Because of the parallel nature of the final circuit used for the emitters, a larger value for R_1 could be used to minimized power consumption. If a different value for R_1 is used, an appropriate change to the detector circuit would be required to guarantee compatibility. The heat generated by the emitters during normal operation provides evidence that 100 Ω may be too small a value for R_1.

E.3.2 Emitter Power Supplies

The emitter circuits described in section E.3.1 draw more power than can be supplied by the computer interface without affecting data acquisition. There is also a limitation to the number of wires that can be used for the 30 conductor cables that connect the bee-counters with the computer interface (these wires are reserved for data acquisition to maximize the number of passageways available for the bees). Because each condo unit was equipped with AC power, it was possible to supply the required 5 VDC from a voltage regulated wall transformer. The 12 VDC wall transformer (part # T502-ND) was purchased from DigiKey Corporation (Thief River Falls, MN). Each set of fourteen emitters required two 5.0 V, 1.5 A voltage regulators (part # LM340T-5.0-ND). These voltage regulators are equivalent to

the more common 7805 ICs. The voltage regulator circuits are simple to build and can be found in many basic electronics books (Mims, 1983).

E.3.3 Infrared Photodiodes and Detector Circuits

The infrared photodiodes (QT Optoelectronics, DigiKey part # QSE113QT-ND) were chosen to match the wavelength of the infrared emitters and because of their fast response time (8 μs). The reception angle of this detector is ±8 degrees. The photodiode was combined with a 5.1 V Zener diode (DigiKey part # 1N4733ACT-ND) and a 2.2 KΩ resistor (DigiKey part # 2.2KQBK-ND) as illustrated in Figure E.4.
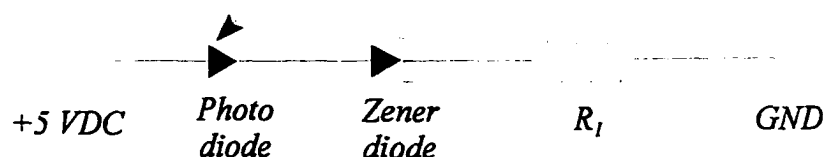
Figure E.4.   Detector circuit diagram.

In Figure E.4, the photodiode is intended to only allow current flow if exposed to 880 nm radiation. A combination of dark current from the photodiode and crosstalk from any of the 27 other detector circuits included on each board requires the use of a Zener diode. The Zener diode will only allow current to flow if the voltage that accompanies the current is at least as large as the Zener voltage (Diefenderfer). The resistor, R_1 provides the voltage drop required in the digital I/O circuit used by the computer interface. When the photodiode is exposed to 880 nm radiation, current flows through both diodes and a digital signal of 'high' or '1' is recorded. If the photodiode is not exposed to sufficient 880 nm radiation to induce a current flow, the voltage across the resistor R_1 is zero and a digital signal of 'low' or '0' is recorded.

The maximum practical sampling frequency of the computer interface is dictated by the rise and fall time of the photodiodes. The photodiodes used for this study are capable of being monitored at 125 KHz, well beyond the 200 Hz sampling rate used to collect data from the bee-counters.

E.4.0 Honey Bee Counter Design

The honey bee counters were manufactured from high and low density polyethylene. Cutting and machining of plastic parts was performed by Dr. Jerry Bromenshenk. Final assembly included attaching the pre-cut plastic parts and attaching the completed circuit boards. Emitters and detectors were then aligned prior to shipping to field sites.

The frame of the counter, shown in Figure E.5, was constructed using polyethylene and deck screws. Once this basic frame was assembled, the detector holder assembly was attached to complete the counter framework. The detector holder assembly was then removed to position and attach the completed detector electronics (circuit boards, components and signal cables). After re-attaching the detector assembly, the ruggedized 37-pin connector was wired and fastened to the left side of the counter frame. Appendix L details the wiring sequence used for the counters and the IoTech interface.
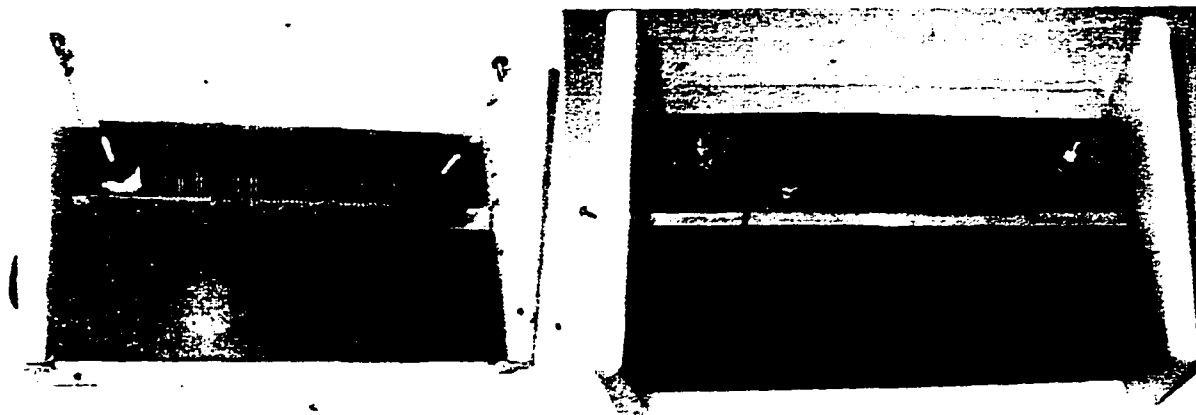
Figure E.5. Plastic frame of used (left) and unused (right) bee-counters.

Each counter had 14 passageways for the bees to enter and exit. Each passageway was machined into the emitter holder as a tall arch with the bottom and sides flat. Early observations suggested that bees were more likely to walk on a flat surface. The arched passageway provided a means to control the orientation of bees traversing the bee-counter. The flat bottom surface served as a floor for the bees to walk on, while the arched shape of the passageway guided the orientation of the bees. The size of the passageway (5mm x 7mm x 47mm) constricted movement of the bees so that the light path from the infrared emitter to the infrared detector was sufficiently blocked to trigger the detector electronics. The emitters were placed in small holes in the middle of the passageway at the top of the arch. Two additional holes were drilled in the emitter holder for attachment to the detector assembly.

The emitter circuit board was connected to the emitter holder by carefully pushing the emitters into 14 holes drilled in the emitter holder. The holes were drilled with tight fit that would hold the emitters in place during shipping and hive inspections. Hanger bolts were used to attach the emitter holders to the detector assembly.

E.5.0 Bumble Bee Counter Design

Bumble bee counters were built for a field trial at Fort Missoula during Summer 1997. The electronics and bee passageways were similar to the honey bee counter, but used only one passageway. The small population of bumble bees (50 to 500 per hive) allowed the use of only one passageway without creating bottlenecks.

Bumble bee counters were constructed out of ABS plastic plumbing materials. A downward angled ABS pipe was connected to the bumble bee counter for weather protection. This same pipe was used to limit the bumble bees from accessing the counter electronics and to force the bees through the counter passageway. The counting device used for the bumble bees had only one passageway available for bee traversal. The circuit boards used were capable of operating two channels and required four digital input wires each.

Literature Cited:

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DESIGN AND FIELD APPLICATION OF ELECTRONIC FLIGHT ACTIVITY MONITORS FOR DETERMINING BEE COLONY DYNAMICS

F.1 Introduction

Electronic bee-counters were designed and tested to monitor honey bee forager flights. These devices were optimized for simplicity in construction and ease of use compared to other published designs (1-4). The electronic components were inexpensive, durable, and required little maintenance. The counters measured flight activity, a critical behavior that reflects colony strength and condition. It also responds quickly to weather and to other factors such as exposures to environmental contaminants.

The bee-counter system consisted of: 1) a counting unit built into a porch that attached directly to the front of the protective boxes (condos) enclosing each of the nucleus beehives, 2) a modular digital interface that transferred the data to a computer, and 3) an IBM-compatible, 486 or Pentium grade Personal Computer (PC) that collected and analyzed flight activity data. Up to ten condos could be connected to the central digital interface via 10 m cables. This provided for colony replication at a site as well as flexibility in layout and spacing among sample hives.

This design differed greatly from the counter described by Liu *et al.* (1), who used a microprocessor-based computer with a keyboard and display for each hive. Struye *et al.* (2) also used a hive-mounted, single central processing unit (CPU) which had a serial port connection that could collect data from more than one hive. In both of these designs, the data processing and storage unit was mounted to the front of the hive, where it was exposed to heat, cold and moisture as well as being vulnerable to theft, vandalism, or accidental damage. Because this complex circuitry was part of the counter, building more than one system was neither inexpensive nor simple to assemble. For maintenance and repair the entire counter assembly had to be removed from the hive.

Like Lui *et al.* (1) and Struye *et al.* (2), we used infrared photo-emitters and detectors to track bee activity. The bee-counters were built into a polyethylene bee-landing and take-off porch affixed to the entrance of each condo. The porch caused the bees to enter and exit the hive in an upright position. Each porch had fourteen side-by-side, 5mm x 7mm x 47mm long, entrance tubes for individual bee passage. Each tube had an infrared emitter inset into the ceiling and two photo-detectors mounted front-to-back underneath a clear, Lexan® floor. Using paired detectors allowed differentiation of incoming from outgoing bees, depending on which detector was first blocked by the bee. The spacing between detectors was adjusted to resolve the passage of a single bee from that of two, head-to-tail bees (1-2). Because these counters were used with small (nucleus) bee colonies, fourteen passageways proved to be sufficient to avoid queuing at the hive entrance.

With this system, a 486 or Pentium grade notebook computer can simultaneously monitor up to ten colonies. A bi-directional, parallel port, digital interface system connected to a square-wave generator ensured fast and consistent sampling rates. Monitoring each passageway permitted investigation of preferential channel use (e.g., end or center passageways) and detection of any tube blockage by dead bees (2) or pollen buildup. The custom software summarized and stored the data from each counting tube at 30 second intervals. This system allowed data comparisons among the entrance tube counts for each hive, the overall flight activity among hives at each site, and ultimately, flight activity levels among several sites. Data from individual channels was also used to align the detector units for each passageway.

The software was written to record and display flight activity in real-time during daytime hours and to save the day's flight data to a user-accessible floppy disk at night when no bees were flying. In addition, off-line analysis software was developed that could detect abrupt changes in flight activity due to external (e.g., weather, food resource availability, environmental contaminants) and internal (e.g., queen condition, hive maintenance, smoke in the hive, chemical treatment to control mites) events. This software was recently expanded to detect long-term changes in overall flight activity on a site-wide basis. The strengths of this system include simplicity of the electronics attached to the hive, use of a single computer for data acquisition from up to ten hives, and real-time analysis and display, at relatively low cost.

F.2 Evolution of Counter Design

Prototype counters used during the summer of 1995 employed a W-shaped detector holder that reflected light from the emitters to the detectors. This system proved useful for assessing patterns of overall flight activity but did not provide accurate counts of individual bees. The system's counting resolution was lower than expected for several reasons: 1) the W-shaped holder allowed stray light reflected from other tubes to create spurious counts, 2) detector alignment was difficult, and 3) resistors used in the voltage drop part of the detector circuit also reduced detector sensitivity to bee passage.

Replacing the W-shaped detector mount with one that placed the detectors head to head in flat grooves improved overall resolution, but the counters still miscounted some of the bees due to insensitivity of the detector circuit. Lowering circuit resistance increased detector sensitivity and improved counter accuracy, which was tested with paper bee models. With the lower resistance (1KΩ), even a thin sheet of paper was sufficient to block a passageway and trigger a count. With the higher resistance (4.7MΩ), only thick cardboard blocked the signal to the detectors. Because detector alignment also affected counter resolution, the emitter and detector holders were redesigned to permit a broader range of adjustment.

These improved counters were tested on condos at The University of Montana-Missoula (UM) apiary at the start of seasonal flight activity in the spring of 1996. During the early summer, the emitter and detector holders went through another stage of improvement. Detector alignment continued to be difficult due to small variations in the machining of the polyethylene holders. To improve tolerances, a custom built milling jig was fabricated. A steel guiderail on the jig provided consistent spacing of the detector and emitter grooves. The jig produced detector and emitter holders that could be interchanged among counters.

The new counters included weather-resistant, plastic skirts to protect the wiring and electronic circuits from rain and snow. Earlier tests revealed that on cold nights, bees were attracted to the warmth produced by the emitters and sometimes got into the counter circuitry. The skirts also prevented damage to the electronics by small animals such as mice and skunks. Flexible plastic seals above and below the entrance tubes kept bees from by-passing or getting inside the counters.

F.3   Data Acquisition Hardware and Software Description

The original interface system collected data for three hives at approximately 1 kilobyte per second. This was the maximum data transfer rate for the serial (RS-232), A-bus® interface system (Alpha Products, Fairfield, Connecticut) that was employed. This interface could not be expanded to collect data from ten hives. Several other interface systems were tested, but only the Digibook/72 parallel port interface system (IOTech, Inc., Cleveland, Ohio) achieved transfer rates greater than 150 kilobytes per second. This interface takes advantage of the high rates of data transfer achieved with bi-directional parallel ports. The Digibook interface allowed more hives to be connected to the computer and dramatically increased sampling rates, improving the ability to differentiate between bees closely following each other through the counters.

A single IOTech DigiBook/72 parallel port interface can be used with three, five, seven, or ten hives. To optimize data collection accuracy, a square-wave generator was built that provided consistently spaced reference pulses. Software was written that controlled sampling rates by referencing the generated pulses. This enhanced the ability to detect bees traversing the tunnels at high rates of speed. The computer-controlled sampling rate was optimized at 200 observations per second for good resolution and to reduce the time required for data analysis.

Two custom software programs were written to interface the flight activity counters with a computer. One provided a set of diagnostic tools for testing counter performance. The other conducted data collection, processing, and graphical display at the field site. Colonies were monitored and analyzed for flight activity from 6:00 AM to 9:00 PM, after which the program made multiple backups of the data on the central computer's hard disk. Daily data summaries were automatically stored in compressed form on a floppy disk. The software also tested the operation of the detector/emitter pairs and generated a daily systems audit report for each hive.

F.4 Data Analysis Software Description

Data collected from the interface system was processed using code written in BASIC. Originally, three algorithms were evaluated for counting bee flights. Using three different algorithms provided a method for comparing analytical consistency and accuracy. All three algorithms searched each detector pair to find when both detectors were blocked. One of these algorithms tested only the immediately preceding data point for detector blockage; whereas another examined only the succeeding data point. The most rigorous algorithm tested the preceding data point to decide which detector was first blocked and then searched the succeeding data points to find when only a single detector was again blocked. If this algorithm failed to detect complete passage through a detector pair, no count was recorded. For all three algorithms, the sequence of detector blockage indicated the direction of bee movement. Because bees sometimes balked and backed out of a counter passageway, only the most rigorous analysis method provided reliable counts. This algorithm was used to create the daily summaries of flight activity at each of the field sites.

F.5 Real-time Graphical Data Display

Because a major project goal is to use changes in bee behavior to provide a warning of the presence of environmental contaminants, real-time measuring of flight activity was needed to provide a feedback system for enacting the chemical sampling program. By the summer of 1996, a software program was added that provided a real-time, monitor-based, graphical display of counter data for each colony. The on-screen plots of flight activity facilitated assessment of the operational status of hardware, revealed departures from normal flight activity, and provided a means of examining the correlation of flight activity with colony population size and weather events.

The real-time monitoring display was accomplished by splitting the computer screen into two parts. The left half of the computer monitor displayed condensed data from each of the previous five days, and the right half displayed all of the current day's data (Fig. F.1). In the evening, the program condensed the day's data by averaging flight activity over 2.5 minute intervals. The condensed data set was then added to the display of previous days, and the oldest day's data was removed. Plots of total flight activity for all seven colonies at each site and of the mean flight activity for all of the colonies at each site were simultaneously displayed.

F.6 Off-line Data Analysis Methods

A three-tier visualization system used color-keyed charts to facilitate comparisons of different aspects of flight activity data. Tier 1 charts focused on seasonal (i.e., long-term) contrasts among sites; while Tier 2 charts examined seasonal dynamics among the colonies within each site. Tier 1 (Fig. F.2) provided time series assessments of the total number of flights for each of the three field sites. Tier 2 (Figs. F.3-F.5) displayed the daily total flight activity for each of the colonies at a single site. Tier 3 charts (Fig. F.6) evaluated shorter-term data by depicting for each day the 30 second summaries of all flight activity variables (bees in, out, and total) for each colony within a site. In addition, the numbers of bees
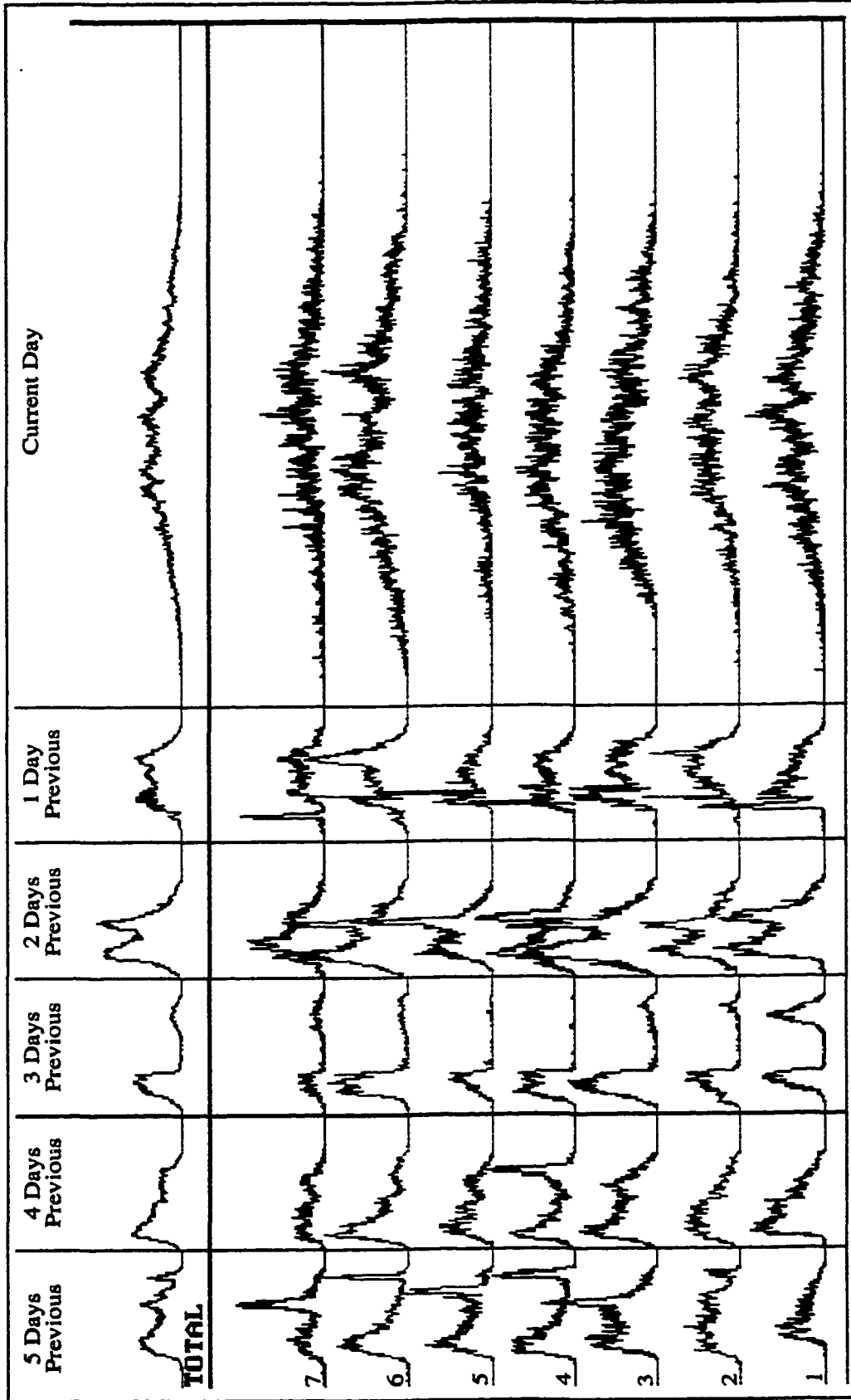
Figure F.1. Screen-Capture of the Real-Time Graphical Display for Colonies at West Branch Canal Creek Displaying Bee-Counter Data from August 7, 1996 to August 12, 1996. In this figure the current day is August 12, 1996, and the remaining days fill from right to left on the left half of the display. The numbers along the left side indicate the number of the colony, with the top trace, labelled 'TOTAL' representing the mean activity for all seven colonies.
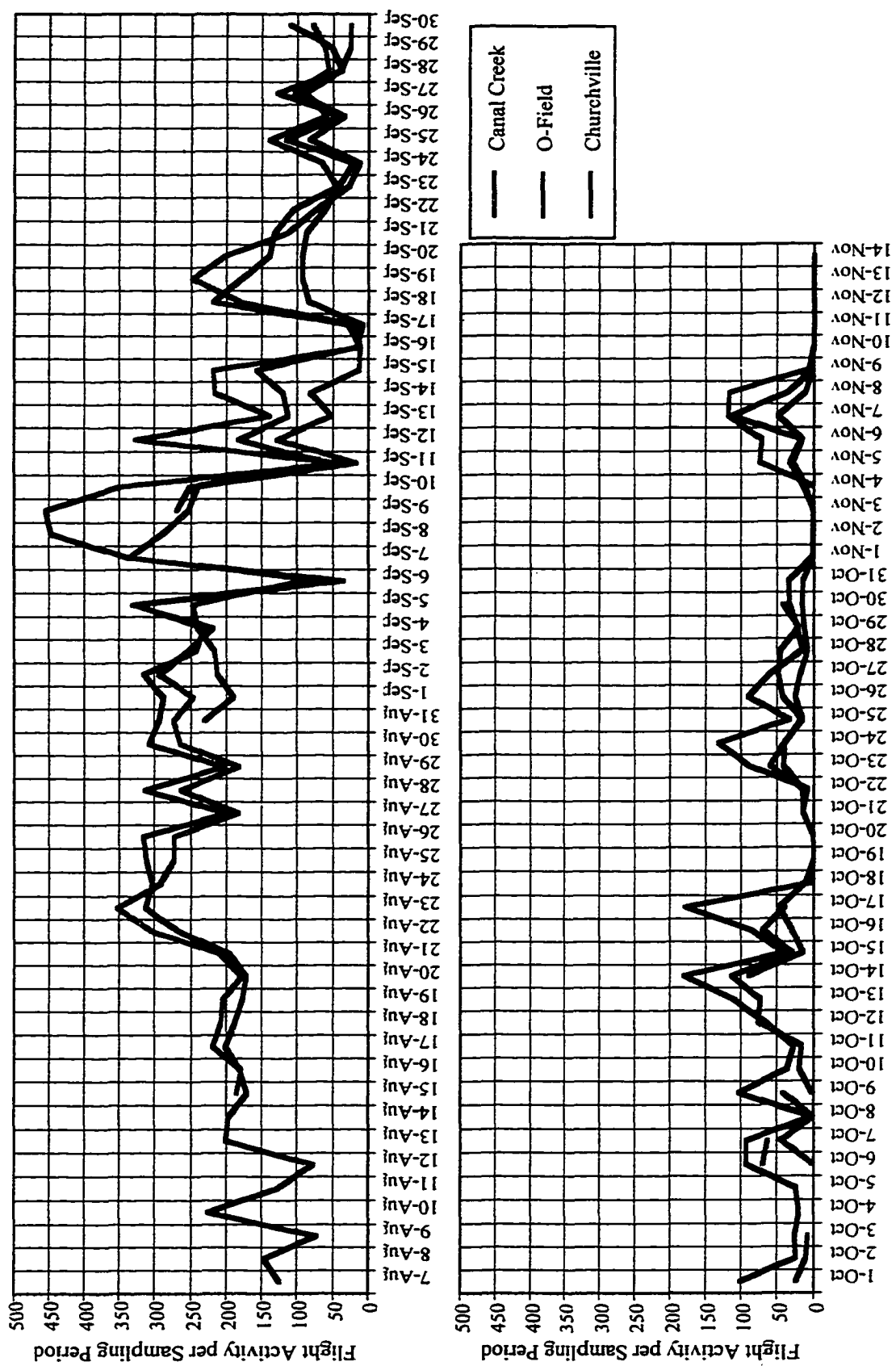
Figure F.2 APG site by site comparison of total daily flight activity (entering + exiting) corrected for number of 30 second sampling periods collected per day. The x-axis contains dates and the y-axis is corrected for total flight activity.

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Hive 1   Hive 2   Hive 3   Hive 4   Hive 5   Hive 6   Hive 7

Figure F.3. Total daily flight activity for Canal Creek hives. Hotter colors denote higher activity.
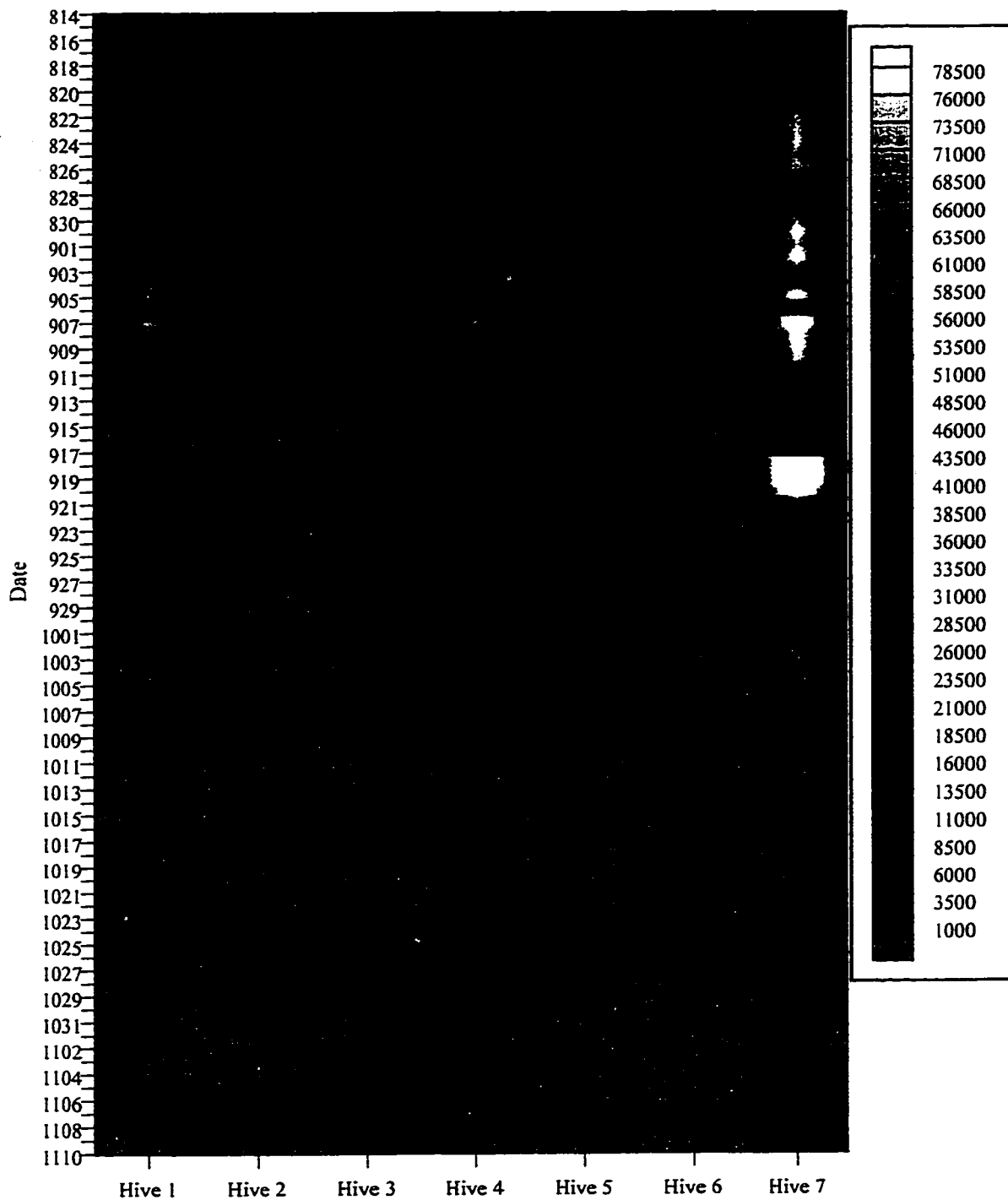
Figure F.4. Total daily flight activity for O-Field hives. Hotter colors denote higher activity.
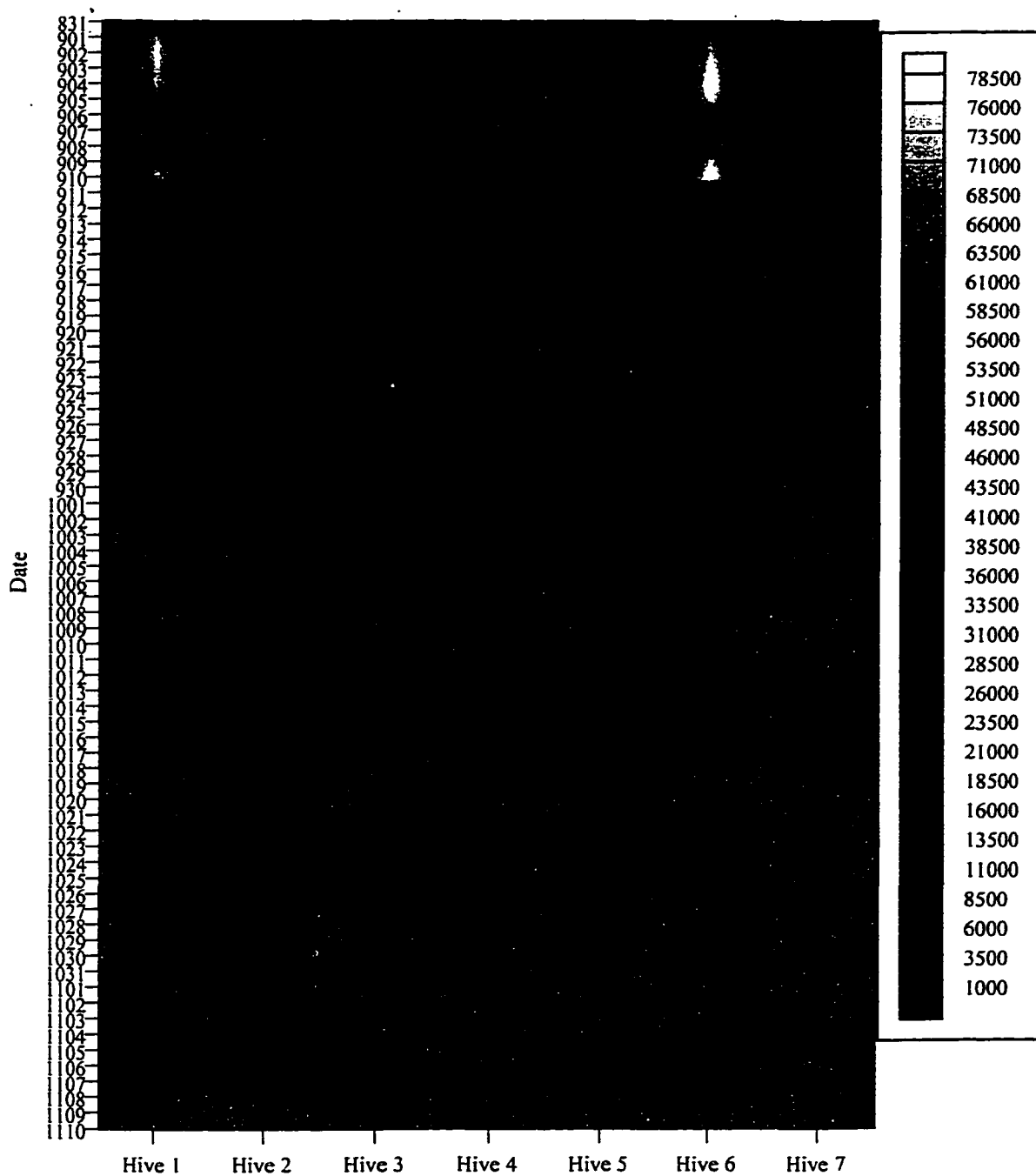
Figure F.5. Total daily flight activity for Churchville hives. Hotter colors denote higher activity.
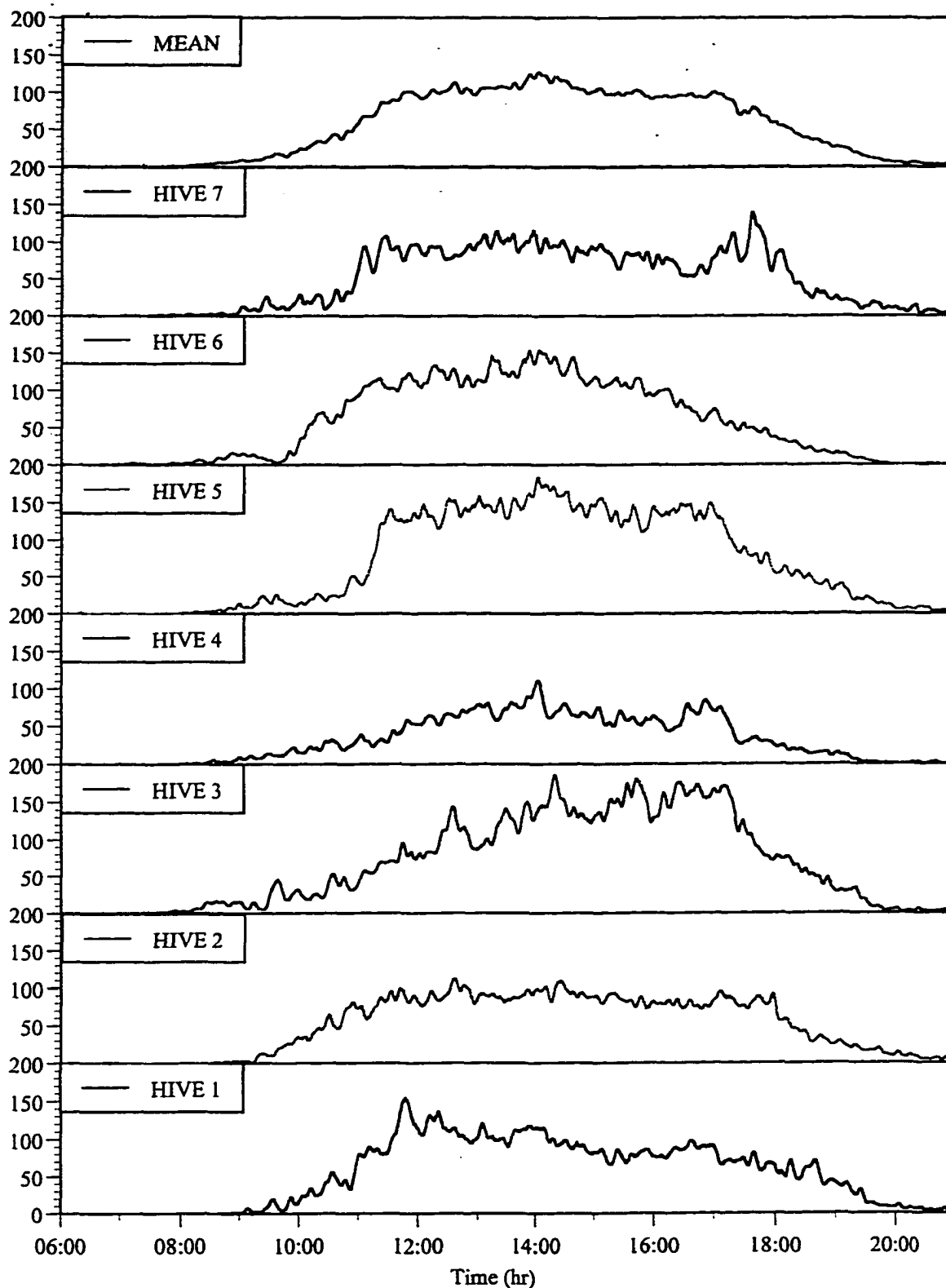
Figure F.6. Total Flight Activity for West Branch Canal Creek Colonies on Sunny Day in August 1996. The combination of flight activity data from hives 1 to 7 produces the broad continuum exhibited in the mean activity trace.

passing through each counter tube for each hive could be displayed. This provided a means of checking counter performance (e.g., whether all of the counting tubes were clear and the detectors working) as well as more closely inspecting the movements of bees in and out of each colony.

Tier 1 flight data was computed as the total number of flights per 30 second count period multiplied by the number of count periods that were recorded for a given day. The among colony coefficient of variation (C.V.) for total activity (bees entering the hive + bees leaving the hive) was used to further assess Tier 1 data for long term trends (Fig. F.7) in flight behavior as well as differences among the field sites. The C.V. provided a benchmark on which to compare the relative strength and vigor of the colonies at a site.

If all the colonies at a location are alive, actively foraging, and queenright (i.e., a queen is present and laying eggs), the C.V. should be relatively low and constant from day to day (e.g., below 40% as indicated by Fig. F.7a). If the total foraging activity of one or more colonies changes relative to the other colonies at a site, the C.V. should increase. The C.V. for total foraging should go to zero at night when bees don't fly, or if all of the colonies die.

The strength of the Tier 2 visualization of flight activity for each of the hives at a given site is that it provides a means of determining the source of variation in Tier 1 data. A quick glance at Tier 2 data will reveal any colony or colonies that differs from the others in foraging levels. With this information in hand, closer inspection of the weak colonies by an apiculturist and chemical sampling can be enacted to help identify the cause of the problem.

Tier 3 charts provided a means of displaying rapid changes in the incoming, outgoing, or total flight activity of any colony. Tabulating activity counts every thirty seconds yielded extremely dense data plots. The high frequency of data sampling tended to conceal short-term changes in bee flight. In other words, the sampling frequency noise concealed the event signal. To separate the colony response signal from the sampling noise, data smoothing techniques such as boxcar averaging described by Savitsky and Golay (5) for spectroscopic data were employed.

High frequency data smoothing was accomplished by first averaging consecutive data points within a selected range (usually 6 data points) using a two-point boxcar. The same smoothing technique was then applied to the resultant average. In order to enhance sensitivity to changes in flight activity, absolute derivatives of smoothed raw data were used to produce peaks that indicated the time and magnitude of an activity change. Data used for derivatives was smoothed using a two-part consecutive boxcar average with each boxcar containing six samples.

A QuickBASIC program was written to prepare data files for analysis using Deltagraph© plotting software, Excel© spreadsheets and Mathcad©workbooks. This program was capable of simultaneously displaying raw data for all colonies at one site for each day, and it included
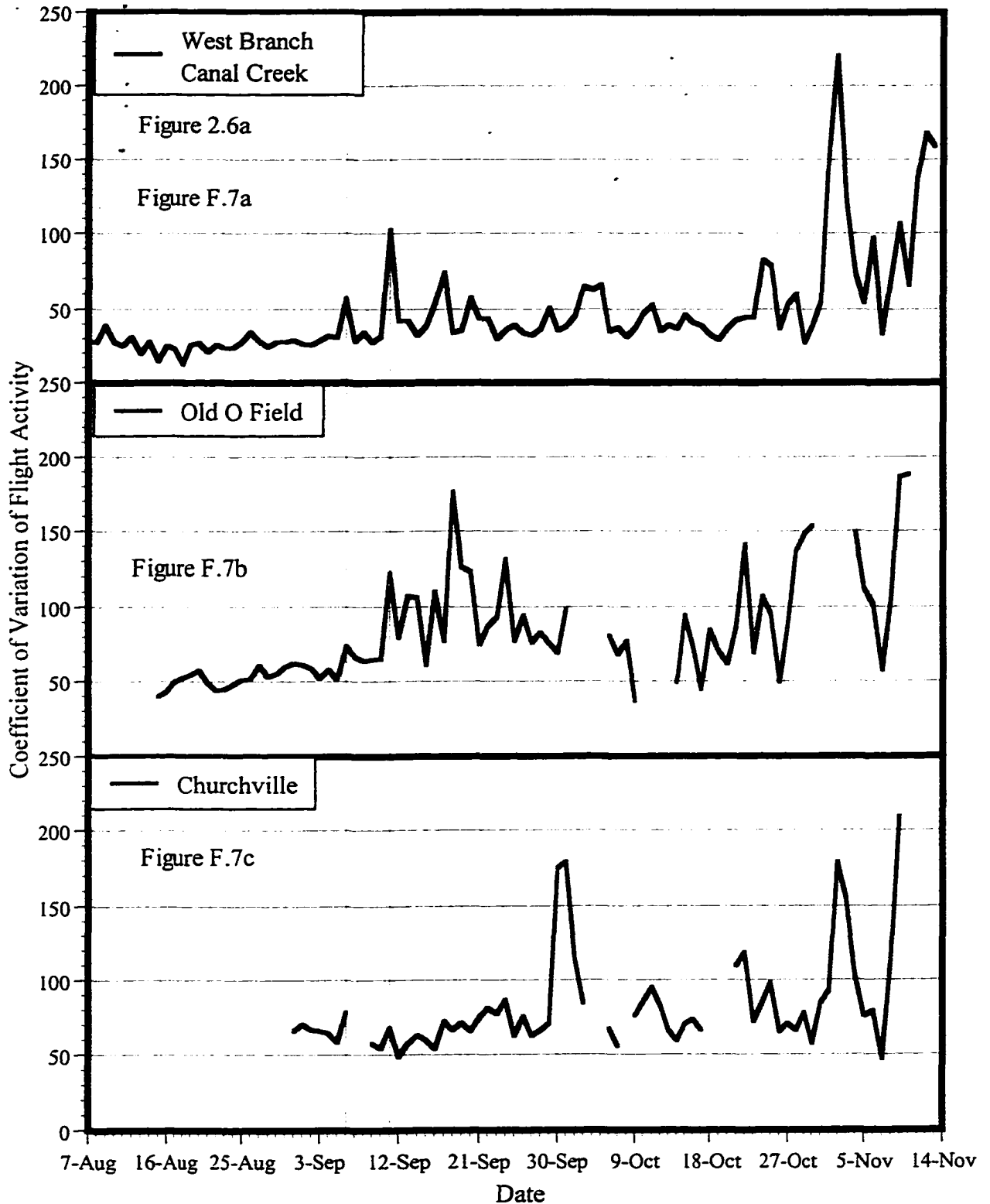
Figure F.7 a, b, and c. Coefficient of Variation of Flight Activity Among Colonies at APG Sites During Late Summer 1996. The two solid lines common to all plots represent days of heavy rainfall, and thus, low flight activity producing high variability among colonies.

data smoothing, derivative analysis, and relative return ratio (i.e., numbers of incoming versus outgoing bees) analyses routines. An important feature of the software is the ability to retrieve a previously stored data file and to display the data in the same graphical format as seen in the field when the counters are operating (see Fig. F.1). Another useful feature of this program is the ability to patch together broken data files that result from restarting the computer in the middle of the day. Recently, this program was ported to PowerBASIC for a substantial increase in speed.

F.7 Field Trials Conducted

Four sets of field trials were conducted. The first trial was conducted with six hives in Maryland in August of 1995 as a preliminary test and demonstration of the technology. The second trial at the UM apiary took place in the fall of 1995 using counters with redesigned emitter and detector holders. Over the winter, the detector circuits were modified and tested. The third field trial began in the Spring of 1996 at the UM apiary with three hives. These trials included the new parallel port interface, vastly improved emitter and detector alignment, enhanced detector sensitivity, and refined analysis software. The fourth trial was conducted from August to November of 1996 using 21 hives at three different sites in Maryland. Seven colonies at each site were continuously monitored for flight and other indicators of colony performance. The Maryland field trials included real-time analysis and display software, the finalized design of the counter emitter/detector system, and improved weather protection for the counters.

F.8 Results of the Final Methods Trials

The Three-Tiered Data Visualization system provided an organized approach in identifying changes in flight behavior as affected by acute and chronic exposure to internal (inside of hive) and external (outside of hive) stimuli. Internal stimuli consisted of factors such as hive manipulation (including smoke injected into the hive to calm bees, adding brood frames or bees to strengthen or equalize bee populations, and inserting Mite-A-Thol Menthol™ and Fluvalinate™ to control bee mites), egg-laying by the queen, brood rearing, and concentrations of environmental contaminants inside the hive. External stimuli primarily consisted of weather events, resource availability (e.g., nectar, pollen, resin, water), and bioavailability or release of environmental contaminants, including acute episodes such as crop spraying . Acute responses were characterized by a sudden change in flight activity, while chronic effects appeared as more gradual or longer-term changes (e.g., days, weeks) in flight activity.

The 1996 flight data revealed that flight activity can undergo drastic changes in less than one minute. Data collected with the original serial interface (Summer/Fall 1995) was collected at 5 minute intervals. During the Spring of 1996, the sampling interval was reduced from 2 minutes to 1 minute with the parallel port interface, and eventually to 30 seconds when real-time analysis was brought on-line. This increase in sampling capacity allowed characterization of events that previously would have been disregarded. Earlier work done with bee-counters by Struye *et al.* integrated data on a 15 minute interval. Data

from the present study indicated that such a long integration interval can mask the effects of short-term internal or external events making it more difficult to assign potential causes.

Boxcar averaging and filtering techniques eliminated the high frequency sampling noise, but retained the important fine features used to detect changes in flight activity. The derivative technique accurately identified short-term responses to acute events, whether biotic or abiotic. Events that induced a simultaneous response in most or all of the colonies at a given site were particularly evident when derivative analysis was applied (Fig. F.8). This method was less useful for detecting longer-term or gradual changes in flight behavior.

For distinguishing the effects of chronic events, the coefficient of variability among colonies for total flight activity proved to be more effective, especially for site comparisons (discussed in Section F.9.2). Other methods being evaluated for detecting colony behavioral responses to chronic stressors include applications of advanced computer learning methods such as the use of an artificial neural network (ANN) to detect patterns in colony flight activity as affected by weather conditions and modified by other factors such as food availability and colony growth which are measured by other electronic hive sensors.

In addition to the BASIC data analysis software, Mathcad© and Excel© proved to be the most useful software for viewing data files and for conducting exploratory data analysis. They allowed direct comparisons of smoothing techniques and also helped determine the minimum length of time necessary for a sampling interval.

F.9   Results of Field Trials of Equipment and Methods

During the August 1995 field trial at APG, the prototype bi-directional bee counters proved adequate for identifying general flight activity patterns. Bee activity was highest between 11:00 and 17:00 hours, except on the 20th when activity remained high until 20:00. All days showed some bee activity until 20:00 hrs.

The prototype counters exhibited some spurious nighttime counts. However, the general activity patterns appeared to be reasonable. During a second set of field trials in Montana, additional tests of the positioning of the infrared detectors were conducted. It was found that detector spacing affects counter resolution. For example, if one bee closely follows another through a pair of detectors, the counter has to be able to resolve whether one or two bees passed through within a fraction of a second. Decreasing the gap between counters and increasing sampling frequency (i.e., the number of times each detector was sampled per second) improved sensitivity. Bees sometimes investigated, hesitated or backed out of counter tunnels, resulting in spurious counts, especially with closely set detectors. Slightly wider spacing of the counters combined with a higher sampling frequency reduced the number of spurious counts without sacrificing resolution.
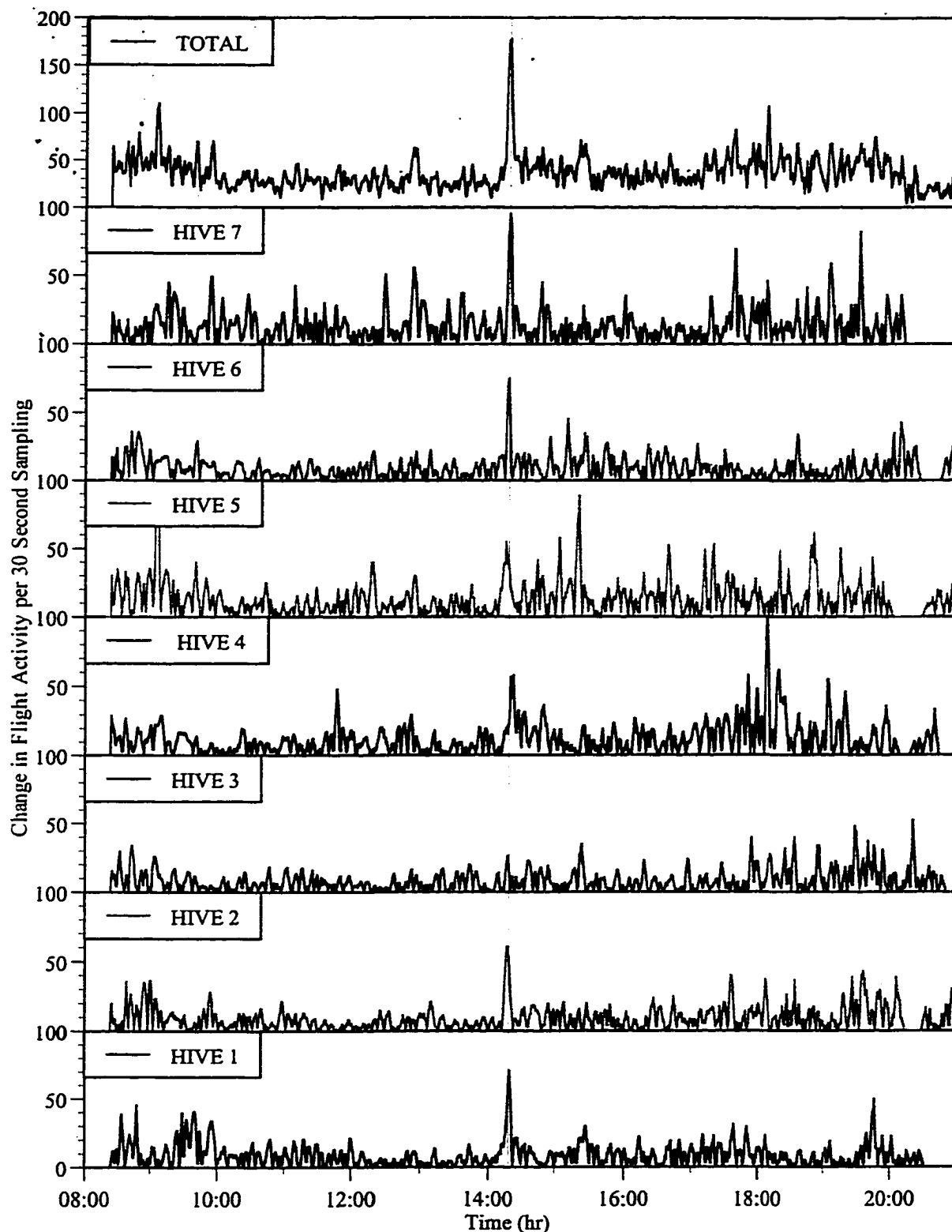
Figure F.8 Derivative of Departing Bees on August 8, 1996 at West Branch Canal Creek During 'Smoke Event'. Hives 1, 5 and 6 were exposed to smoke puffs at 14:30. The solid line indicates a synchronous response for all colonies.

Further improvements made to the counter hardware and software (described in 2.4-2.7) eliminated spurious counts, increased sensitivity to bees passing through the tubes, provided better alignment of detectors with emitters, and resulted in faster and more accurate sampling rates. Whereas the original serial interface sampled the hive at approximately 12 Hz, the parallel port interface and more rigorous analysis software permitted reliable, user-adjustable, sampling rates of up to 200 Hz. If sampling is done too slowly, bees pass through undetected which probably happened in the initial field study. On the other hand, overly fast sampling produced an enormous amount of redundant data that pressed the memory and data storage capacity of the notebook computer. Field trials conducted in Montana during Spring 1996 established that 150 Hz is the minimum sampling rate from which reliable bee passage data can be obtained.

Following additional testing and finalization of hardware and software design in Spring 1996, two hives were monitored through early July in Montana and 21 hives were deployed in late July in Maryland for monitoring through mid-November. Flight activity during late spring and early summer in Montana was similar to that of mid-summer through autumn in Maryland. Total daily flight activity per hive of about 70,000 trips per day in Montana. In Maryland, flight activity tended to be somewhat lower, with the most populous colonies only occasionally achieving the maximum observed in Montana. The high Montana counts appeared to be due to the intense foraging that occurred during the period of maximum population growth (i.e., May-June) combined with abundant resources of both nectar and pollen. Also, the number of hours that bees could forage was longer in Montana than in Maryland due to the increased day length (i.e., period of daylight).

In Maryland, the major nectar flows occurred before the colonies were deployed at the test sites. By August, the Maryland bees primarily foraged for pollen (as evidenced by pollen trapping), brought back water for evaporative cooling in the afternoon, and collected small amounts of nectar. As evidenced by pulses of incoming bees that exceeded the numbers of outgoing bees on several occasions, Hive 7 at Old O Field recruited bees from nearby colonies and as such was characterized by very high levels of flight activity. Several queenless colonies at Old O Field declined in population size, while the colony in Hive 7 increased in size. This robust colony probably benefitted from the tendency of returning bees to drift toward the outermost colony in a long row of hives.

For both years and in each state, the rigorous data analysis algorithm consistently gave the most accurate results. The less stringent algorithms were useful for aligning detectors and improving counter sensitivity, but these analysis programs were susceptible to errors induced by electronic noise or by bees backing out of entrance passageways. Only the more rigorous method could reliably distinguish between bees that fully entered or exited the mini-hives and bees that stopped and reversed direction in the counting tubes. Consequently, this method was utilized for the field applications in Maryland during 1996.

As the counter technology improved throughout the spring and early summer of 1996, the count accuracy and resolution also improved. Before being shipped to Maryland in the summer of 1996, the final improvements to the counter technology were completed and the sampling performance remained consistent throughout the 1996 Maryland field study.

F.9.1 Results of the 1996 Tests of Equipment and Methods at APG

In mid-September of 1996 a fault in the data analysis code that caused data from counting tubes 1 and 2 (out of 14 total) on even-numbered hives to be deleted prior to analysis was discovered and fixed. This problem was resolved by renaming the arrays passed to subprograms used to analyze flight data and by only deleting arrays after all data had been analyzed. Another bug was discovered at the same time that caused Hives 5 and 7 to repeat data for some counting channels. Despite these software problems, in each case sufficient numbers of count tubes recorded accurate count numbers to provide representative estimates of total flight activity, although not every bee was counted.

The real-time monitoring display made it easy to compare counter output graphs to actual bee flight at the front of each hive. By standing next to the computer and watching both the monitor and the bees at the front of the hives, the visually observed and computer recorded levels of flight activity could be easily assessed with respect to known colony population size and current weather conditions (e.g., sunny, windy, rainy, or advancing storm fronts). In all cases, the relative levels of flight activity seen at the front of the seven hives at each site were consistent with the recorded levels seen on the computer screen. Colony size was periodically addressed by opening the hive and inspecting the colony to determine overall colony condition and strength. Total colony biomass which provided an indirect measure of population size was continuously measured by strain gauges. These examinations confirmed the relative colony strength and status (such as presence or absence of a queen) as indicated by the flight counters.

The software faults only became evident in Maryland, when the number of hives connected to the central interface was increased from three to seven, apparently inducing memory allocation problems that reflected inherent limitations of QuickBasic. Lowering the sampling rate to ease the demands on memory was considered, but not pursued in order to maintain data consistency throughout the year. Eliminating these memory problems and producing the next generation of software for networked bee counters has been a major component of the ongoing development of this technology.

Porting the QuickBASIC code to Visual BASIC was recently attempted. However, Visual BASIC allocated so many user interface resources that it was not capable of maintaining a consistent sampling rate. PowerBASIC offers good array management, access to upper memory, and faster compiled code. The entire QuickBASIC data analysis and manipulation program was ported to PowerBASIC in less than 4 hours. Additional code has been written in PowerBASIC to expand the capabilities of the data analysis and manipulation program. Currently, work is being done in collaboration with IOTech technicians to create

a software driver for the Digibook/72 that is compatible with PowerBASIC. When this is done, memory allocation problems should be resolved and both IOTech and the bee counters will benefit. IOTech will be able to offer PowerBASIC compatibility with their product, and the bee counters will have a faster and more stable software platform on which to run.

F.9.2 Results of 1996 Tier 1 and 2 Evaluations of Flight Activity at APG

The flight activity from the Summer and Fall of 1996 was summarized using the Tier-based approach. Fig. F.2 presents a Tier 1 summary comparing corrected (i.e., adjusted for the number of sampling periods per day) total daily flight activity throughout the season for each of the three primary test sites. West Branch Canal Creek was the first site to be put on-line, followed by Old O Field, and the Churchville reference site. Overall, each of the sites displayed similar day to day seasonal trends. At all sites, activity leveled-off from the third week of August through mid-September and then gradually dropped off as the foraging season ended.

As can be seen in Fig. F.2, most of the major weather events affected all three sites. For example, greatly reduced flight activity at all three sites on September 6 and 11 coincided with heavy rain showers throughout the region. More localized weather events produced site specific differences in total flight activity. West Branch Canal Creek and Old O Field were in close proximity, both being located at APG—Edgewood on a peninsula extending into Chesapeake Bay. By comparison, the Churchville reference site was inland from the Bay and far from the APG sites. Weather conditions at the APG sites should more closely resemble each other than those at the more distant Churchville site. Preliminary analysis of the weather data suggests that this was a correct assumption.

Tier 1 graphical summaries indicate that through early September total flight activity at West Branch Canal Creek and Old O Field was similar. As the season progressed, the colonies at West Branch Canal Creek usually displayed the highest flight activity, while those at Old O Field tended to have the lowest flight activity and often more closely resembled the colonies at Churchville than those at Old O Field. This suggested a response that was not simply a consequence of differing weather conditions.

The differences in flight activities at these three sites were further investigated by plotting the coefficient of variation (C.V.) of flight activity among the seven colonies at each site (Fig. F.7a-c). As seen in Fig. F.7, the colonies at West Branch Canal Creek established a baseline level below 40% for variation in flight activity, which stayed below 50% as the season progressed. There were several brief periods where the C.V. exceeded this baseline, but most periods corresponded with inclement weather. At Churchville, the variation in flight activity hovered around 65% for most of the season. Colonies at Old O Field established a C.V. baseline at approximately 55% until September 6th, but were unable to make a consistent return to that level thereafter. On September 6, 1996, hurricane Fran swept through the east coast. Whether the change observed at Old O Field was a direct response to the storm or in response to some other factor is still under investigation.

The coefficient of variations for flight activity data were indicative of the site-wide colony condition. If all colonies at one site were thriving and showing similar activity patterns, the C.V. values remained at a relatively low, constant level (<40%, as was the case for West Branch Canal Creek). The same also could be true if all of the colonies at a site were in poor condition and behaving similarly. Therefore, the C.V. data must be used in conjunction with total flight activity data and other measures of hive condition such as biomass in order to determine the source of the low variability. If the C.V. begins to deviate from a baseline level characteristic of the colonies at a site (<40% for West Branch Canal Creek, 55% for Old O Field and 65% for Churchville), then one or more of the colonies must be exhibiting a different flight behavior. This deviation could be due to a colony with a much increased number of flights compared to the other colonies as well as to a colony with a depressed activity level.

Tier 1 plots of total flight activity and the associated C.V. revealed site specific trends, but these plots did not reveal the strength or weaknesses of individual colonies. Tier 2 analysis (Fig. F.3-F.5) provided the means of distinguishing weak colonies from strong colonies. Tier 1 analysis revealed when one or more colonies at a site were displaying atypical flight characteristics. Tier 2 analysis identified the specific colonies that were the source of this variation. Having identified these colonies, each colony could then be inspected to determine if it was queenright, whether it had brood and adequate food stores, or whether it showed any symptoms of disease or mite infestation. If the change in foraging could not be assigned to a difference in colony structure, a chemical sampling program could be initiated to look for the presence or an increase in exposure to chemical contaminants.

Tier 2 analysis provided additional insights regarding colony condition and equipment performance. From August 14 through August 22, one half of the counter channels on Hive 7 at Old O Field were out due to a defective power circuit. The fault can be easily seen in Fig. F.4 by the abrupt change in colors (indicating flight numbers) on August 23 on the contour plot for Hive 7, following repair of the power supply.

The repaired counter clearly demonstrated that throughout the season the colony in Hive 7 made more foraging trips than any of the other colonies at Old O Field. Observations of dense populations of bees inside the hive with large masses (beards) of bees hanging outside the hive at night confirmed that this hive contained the strongest colony at O Field. Fig. F.4 not only identified the strongest colony at Old O Field, but it also showed that colonies 2, 5, and especially 6 had the lowest flight activity. Upon inspection, these three colonies were found to be queenless, and two were broodless.

Tier 1 analysis showed that flight activity was most consistent among the colonies at West Branch Canal Creek. As at Old O Field, Tier 2 plots identified which of the hives differed from the others. Hive 4 displayed the least flight activity. When opened it was found to contain the smallest population of bees, although it had a queen and brood. Similarly, the plots show that at the Churchville reference site (Fig. F.5), flight activity

throughout the season was lowest in Hives 4 and 7. Hive 4 became queenless and was replaced mid-test with a colony that had a small bee population. Hive 7 contained only drone brood (indicating queen failure and possible replacement by a laying worker).

F.9.3 Results of the 1996 Tier 3 Evaluations of Flight Activity at APG

Whereas Tier 1 and 2 plots proved to be most useful for detecting site specific and long-term changes in colony activities, Tier 3 analysis had the advantage of plotting flight activity data for a single day for each colony. This provided information about the effects of sudden events such as storms, food resource availability, opening the hive, or sudden release of environmental contaminants. The strength of Tier 3 was to identify rapid changes in a colony's flight behavior and to compare the number of bees entering the hive to the number of bees exiting the hive during a given sampling period, resulting in a measure of net loss of forager bees.

Fig. F.6 displays the total flight activity typical of a sunny day in August at West Branch Canal Creek. As mentioned earlier, boxcar averaging and other filtering techniques provided data smoothing to reduce the high frequency sampling noise. For each plot, the bottom line represents Hive 1, ascending to Hive 7, with the uppermost red line the mean of the total flight numbers for all seven hives. As can be seen from the plot, the overall flight activity for the day for each hive appears as a more or less continuous, broad continuum.

By comparison, Fig. F.9 displays a large peak in the number of bees returning to the hive before a thunderstorm. Few bees left the hive until after the rain. Bees avoid heavy rain, which can knock them out of the air while flying. Many beekeepers believe that bees avoid rainstorms in response to changes in barometric pressure, but our bee-counter graphs in combination with digital weather data show that bees probably use multiple meteorological clues, not simply barometric pressure drops to determine when to return to the hive to avoid a storm. In this case, foraging activity was clearly affected by an external factor that first influenced the behavior of foragers in the field, causing them to return to the hive, and then influenced the bees in the colony by inhibiting flight.

Fig. F.10 shows the smoothed flight activity traces from a day on which six small puffs of cool smoke (from a bee smoker using burlap as fuel) were directed over a three-minute period into the entrances of three of the seven hives at West Branch Canal Creek. The smoke event can best be seen as a drop in activity for the bees leaving the hive. Fig. F.8 shows derivative plots of the flight activity data for bees entering each hive at the site. The peaks corresponding to the smoke event are discernable in the total activity and the bee-exit data, but they are absent from the incoming bee data. Because the data smoothing and derivative procedures enhance the signal, one can not only see the response evidenced by the three smoked colonies (Hives 1, 5, and 6) but also by three other nearby colonies. We suspect that these colonies were responding to smoke drift due to gusty winds. Only one colony (Hive 3) did not demonstrate the response.
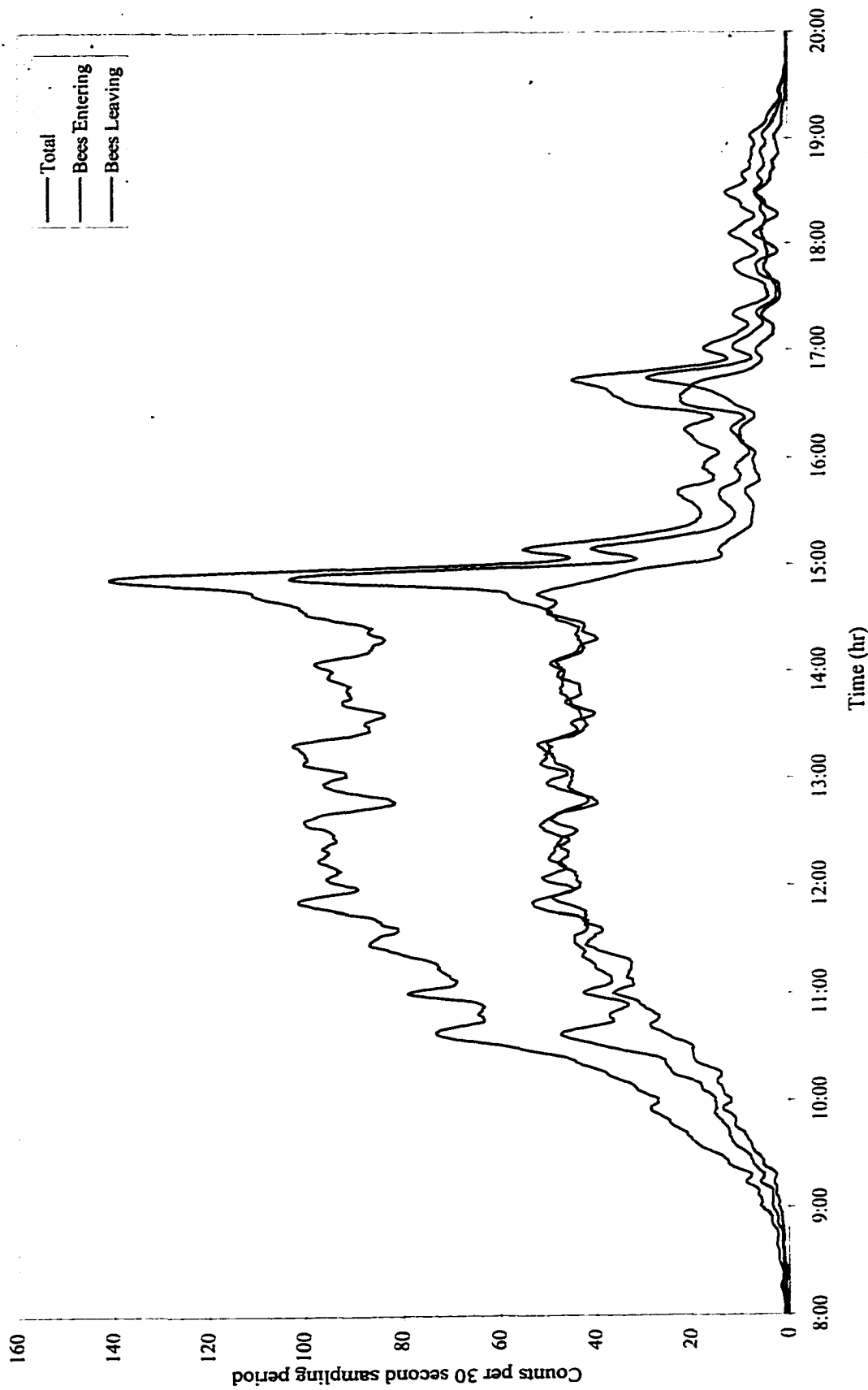
Figure F.9. Expanded Flight Activity Data for West Branch Canal Creek Colonies on August 27, 1996 During Rainstorm. Sharp drop in activity at 15:00 represents beginning of rain.

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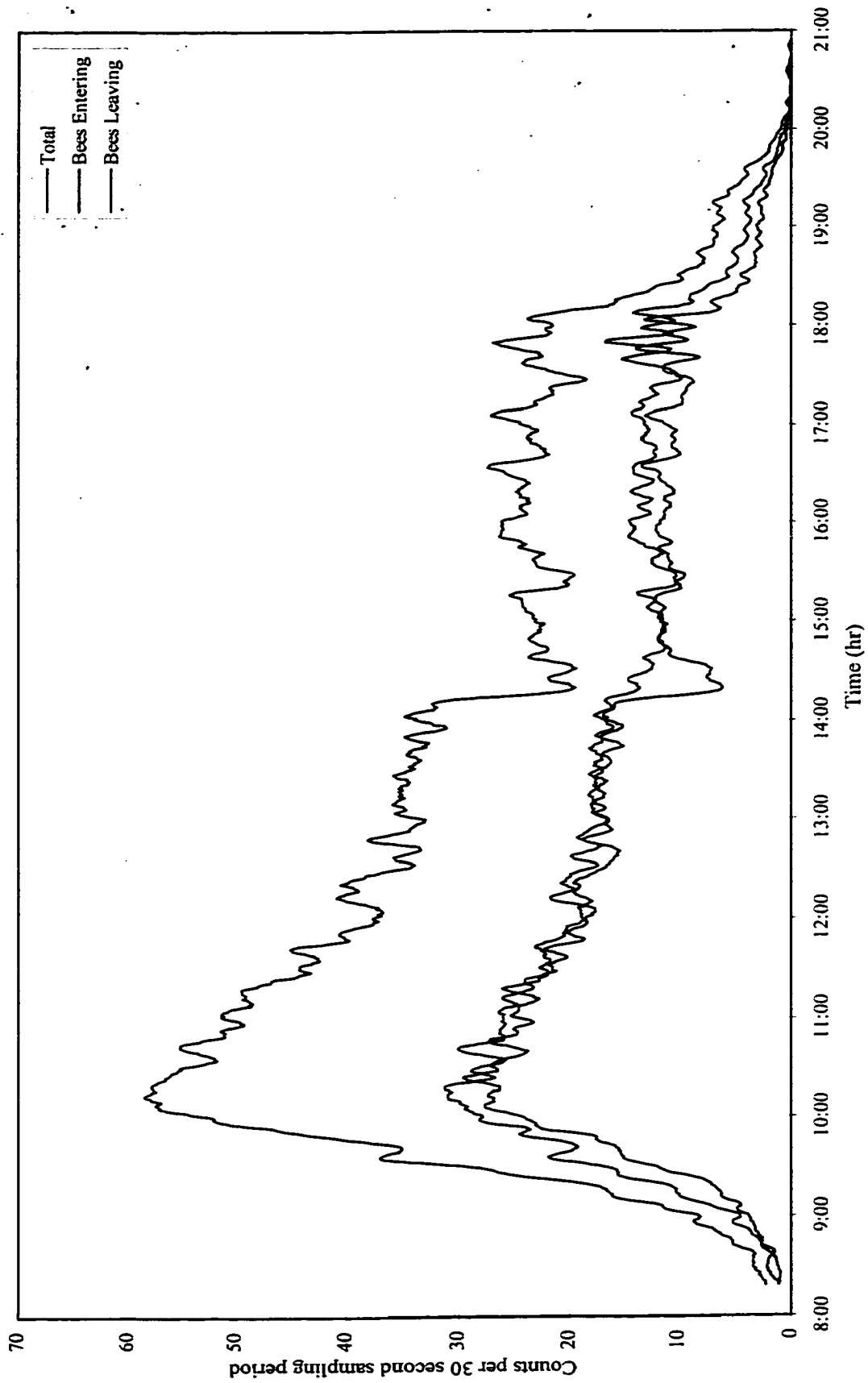
Figure F.10. Expanded Flight Activity Data for West Branch Canal Creek Colonies on August 8, 1996 During 'Smoke Event'. Sharp drop in bees leaving the hive at 14:30 is due to smoke.

As evident in these plots, following injection of smoke into the hive, bees continued to return at the same rate, but the rate of outgoing bees decreased within a few minutes. Activity levels remained low for 20 minutes or more, possibly for several hours as indicated by the response of Hive 1. Beekeepers use smoke to calm bees while performing maintenance on hives. Apparently, the bees are fooled into believing the hive is threatened by fire. Instead of rushing out to fight an intruder, some of the bees gather up as much nectar as possible and prepare to leave the hive. Others may fan to remove smoke from the hive. Since the smoke was injected directly into the hive, it should have been sensed primarily by the bees within the hive rather than bees outside the hive.

In the fall and early spring, a colony's foraging habits vary depending on the presence of brood in the colony and the availability of floral resources. At these times, daily flight activity was often inconsistent, characterized by peaks and valleys, rather than the broad continuum typically seen on days of high foraging activity. In order to be able to distinguish responses to external events of particular interest for environmental monitoring, such as exposure to contaminants, from normal variations in flight activity, the subsequent data analysis will have to consider relevant ancillary data for weather, food resource availability, and other factors that alter colony dynamics. To address these needs, the honey bee condos have been equipped with electronic systems for continuously measuring colony core temperature, time and rate of pollen gathering, and hive weight.

F.10 General Discussion

By continuously measuring colony responses (e.g., total flight activity, net loss of forager bees, colony biomass, thermoregulation in the broodnest) to a variety of internal and external stressors, an extensive database is being compiled. The goal is to associate colony responses to intrinsic and extrinsic factors as they occur or very soon thereafter. By combining the knowledge gained from this database with continuous monitoring of weather and food resource availability (e.g., clock driven pollen traps) monitoring, it should be possible to determine when chemical sampling is warranted to examine possible contaminant exposure. Based on the information obtained over the past two years, it is apparent that flight activity can respond quickly to a variety of stressors.

With sufficient data, various external and internal events can be characterized by unique behavioral responses. For example, an external event such as an approaching storm often induced a large positive change in the rate of bees entering the hive with a negative change in the rate of bees exiting the hive. One would expect bees to return to the safety of the hive and, once inside, stay until the weather improved. An internal event such as smoke in the hive was characterized by a large negative change in the rate of bees exiting the hive with little or no change in the rate of bees entering the hive. Again, one would expect bees in the field to be unaffected, because they were not exposed to the smoke. Similarly, swarming produced a rapid increase in the number of bees exiting the hive with some a increase in rate of bee returns after the main swarm had departed.

The bee counters have some inherent limitations with respect to identifying the presence of bioavailable toxins. First, bees normally only fly during daylight hours. This raises a concern about detecting toxins that are emitted at night, during storms, or when the bees are clustered inside the hive in the winter, especially in northern climates. Although chemical releases may occur at night from industrial sources, dumps, and various media, some potential sources of harmful chemicals normally occur only during the daytime. For example, crop-dusters don't fly, nor do most clean up crews work at night.

In addition, bees can monitor air borne chemicals even when confined to the hive box. Bees switch from their daytime routine of sampling multiple media and wide areas to a point sampling system for air quality at night. They accomplish this by drawing air through the hive while fanning with their wings. Thus, while the bees are enclosed in the hive they exhibit a sampling behavior that is similar to that of the carbon sorption tube traps used in more conventional sampling of air quality. During the day, they change to an area sampling mode that is less point specific than traditional methods. Taking into consideration the seasonal nature of foraging, the spatial averaging and sampling potential of bees is limited to the active foraging season, but the point specific sampling potential continues year-round. The advantage that the bees offer over traditional chemical sampling methods is their ability to indicate when to begin sampling for the presence of bioavailable, hazardous substances. This helps reduce the amount of chemical analyses being performed, thus reducing costs.

Our counters provided an accurate means of indicating both acute toxicity and colony condition. Traditional methods of determining acute toxicity (dead bee traps) have been limited to counting bees that died after returning to the hive and do not adequately evaluate the number of bees dying in the field. The bee counters provided a measure of bees that failed to return from the field (i.e., net loss for each hive for each day). Occasionally, bees are unable to return to the hive due to abrupt changes in weather at the end of a day, and these bees usually returned to the hive the next morning. Colony condition has traditionally been monitored by periodic inspections and estimates of population size that rely upon the observer's experience with colonies. The bee counters provide an unbiased metric (i.e., C.V. among hives at a site) that can not only evaluate the vigor of the colony (due to trends in total flight activity), but also indicate changes in colony function. Problems in the colony, ranging from exposures to toxins to the loss of the queen or lack of brood, will be reflected by changes in foraging levels.

Both raw and smoothed flight data, as illustrated by the Figures, clearly shows the influence of weather and of at least one complex chemical mixture smoke. Struye et al.(2) demonstrated that flight activity changed when bees were exposed to toxic chemicals (pesticides) and that these changes are different than those induced by weather. By monitoring multiple hives simultaneously at three sites, this study demonstrated a correlation in flight activity among the hives at these locations that can be attributed to meteorological conditions. Although some colony specific differences occur at each site, the overall patterns are remarkably similar.

Ultimately, the ability to accurately interpret the movement of bees into and out of a hive in conjunction with similar data for weather conditions and food resource availability depends on the use of artificial neural networks, advanced statistics or other advanced software tools. The goal of the portion of the study described in this section is to provide the tools needed to assess the normal foraging patterns of bees as influenced by natural factors such as weather and abiotic stressors such as exposure to pollutants.

This work is beginning to produce an extensive data base that will be invaluable in determining the sensitivity of bee flight to environmental stressors. Currently, two colony performance parameters have proven to be useful: net loss of forager bees and the coefficient of variation for flight activity at a given site. Net loss can quickly identify even small decreases in the number of bees returning to the hive. Because exposure to a toxic chemical is a likely cause of this response, a sudden or increasing rate of loss may warrant additional investigation and sampling to determine the underlying cause of the change. First steps would include examining weather conditions and the reproductive status of the colony (e.g., as indicated by the presence or absence of brood). If these actions fail to identify the likely source of the loss, then chemical sampling can be initiated. In combination with the use of the more traditional dead bee traps, samples can be taken for body burden analysis. Also, exposure to a toxic substance should increase the numbers of dead and dying bees removed by housekeeping bees from the colony and dropped into the traps.

The coefficient of variation addresses differences among colonies and appears to be a more useful metric for assessing overall colony condition. This statistic also provides a link between the Tier 1 (comparison of locations) and Tier 2 (comparison of hives at a location) graphical summaries. The latter helps identify the specific hives that are the source of the observed variability. Thus, higher C.V.s at Old O Field were in part due to the disappearance of the queen from three of the seven colonies. The sudden increase in C.V. values at this site following hurricane Fran, and a failure to return to the levels seen before the upheaval suggest that something changed at this site that had a long-term impact on the colonies. Whether this was due to a change in forage availability, exposure to toxins in surface waters, or some other factor is unknown. However, this is the type of response that could be used as part of a feedback system to provide a real-time alert of an alteration in the environment. Consequently, the change may signify a reason for initiating chemical sampling.

F.11 References

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Appendix G

MONITORING OF COLONY BEHAVIORAL RESPONSES TO CONTAMINANTS, WEATHER, AND OTHER ENVIRONMENTAL FACTORS

G.1 Colony Dynamics and Behavioral Responses

For this project, colony response metrics are guided by two overall objectives:

> 1) Developing real-time monitoring of colony population dynamics to establish the relationship between acute exposures to specific chemical agents and measured behavioral endpoints; and

> 2) Assessing the responses of honey bee colony populations for site-to-site comparisons with respect to the effects of chronic as well as acute ecosystem exposures to bioavailable chemical agents.

We have made considerable progress toward accomplishing automated, real-time monitoring of honey bee colony performance. During the summer of 1996, 21 electronically-equipped, mini-hives containing nucleus (small) colonies of bees were deployed at two Aberdeen Proving Ground (APG) and a rural Maryland reference site. Another set of seven electronic hives were established in 1997 at a Montana reference site. All of these hives were equipped with: (1) sensors that continuously measured several colony performance parameters, (2) sorption traps that sampled hive atmospheres over 8-10 hour periods, and (3) in-hive traps that continuously collected pollen and dead bees. The field trials produced an extensive data set needed to: (1) determine the sensitivity, variability, and usefulness of several population-level, toxicity assessment endpoints, (2) further develop and refine models of honey bee population dynamics, and (3) conduct ongoing hazard assessments at APG.

These automated systems provided real-time monitoring capability for a select set of colony responses, as well as a wealth of data and experience from applications at the APG field sites. Colony response data was continuously displayed on computer screens in real-time at the field locations. However, the data could not be easily accessed from a distance, nor could it be simply retrieved from the resultant databases that increased daily in size by several Mbs. Also, bee flight data, collected at a rate of 200 counts per second, produced enormous, data-rich files. The data then needed to be reduced and simplified via averaging, smoothing, and other numerical processing procedures. With appropriate data processing, it was possible to isolate response signals from a background of rapidly changing bee activity and to interpret bee movements as reactions to a variety of internal and external stressors.

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Two new aspects of behavioral monitoring are presented in this year's report. First, we produced an extensive array of software tools for the display, inspection, interpretation, and automated numerical analysis of colony behavioral data, with an emphasis on the bi-directional flight data. These software routines have been bundled as a set and can be distributed on a CD-ROM along with the colony response and meteorological data from 1996 and 1997. Second, we developed the software required to deliver colony response data in real time via Internet or Intranet connections.

All of the custom and commercial interface software that had been used until the fall of 1997 has been replaced with custom software that we wrote. The new software offers several improvements. Automatic updates and aggressive error correction routines are more robust than those in the "commercial software" and have virtually eliminated occasional data losses by the interface hardware used for the hives and for the meteorological stations. The new software ports the data to Linux (a Unix operating clone) for a more stable multi-tasking environment. JAVA and CGI scripts can post data summaries in text format every 15 minutes and provide interactive graphical displays of the data. An on-line weather station updates every 15 seconds. The on-line weather station and two weeks of archived data from August, 1997, at the Missoula reference locations can be accessed and interactively displayed via our web site *(http://www.umt.edu/ biology/bees)*.

G.2 Materials and Methods

Design and construction of the electronic bee-counters and other hive sensors was discussed in detail in the previous report submitted to the Army (Bromenshenk *et al.*, 1997). Because all of the real-time monitoring devices, software, and data acquisition software were developed during the first two years of this project, materials and methods development continues to be an important aspect of this project. The 1996 annual report discussed our prototype off-line data analysis tools used to characterize honey bee forager flight activity as applied to the three Maryland study sites. In 1997, four field sites were monitored for *Apis mellifera* flight activity, three in Maryland in a biomonitoring application mode, and one in Montana used as a research and development station for further identification and characterization of flight activity disturbances. Because the 1995 and 1996 trials indicated that flight activity and core temperature of the brood nest were critical assessment and measurement endpoints, we focused the 1997 investigations on real-time monitoring of these two metrics and upgrading of the software from prototypical to robust, finalized versions. Real-time monitoring was continued from the previous year with stations set up at West Branch Canal Creek, Old O Field, and a rural Maryland reference site at Churchville. J Field was added September 1997, using electronic hives that had been at West Branch Canal Creek earlier in the season.

Software bugs encountered during the 1996 field season were addressed prior to field deployment in 1997. A duplicate channel problem described in the 1996 annual report (Bromenshenk *et. al.*, 1997) was solved by correcting errors in the data acquisition sub-

program. In this case, the program assigned incorrect channel inputs from the interface to arrays designated to other channels. Another problem was addressed by reformatting the daily compressed file and directory naming system. The new system labels files and directories with a two letter code for each site followed by six numbers representing the date (i.e., YY/MM/DD). Thus the files and directories are labeled as SSYYMMDD. Table G.1 provides examples of the new filenaming scheme, which anticipates the year 2000 changeover.

Table G.1
Explanation of 1997 File Naming System.

Name of Site	Site-Code	Date and Year	File Name
West Branch Canal Creek	CC	August 7, 1996	CC960807
J Field	JF	September 5, 1997	JF970905
Old O Field	OF	June 22, 1997	OF970622
Churchville Reference	CV	September 1, 1997	CV970901
Fort Missoula	FM	July 21, 1997	FM970721

The basic structure of the flight activity data files has remained unchanged. Originally, stopping data acquisition to service the counter system posed a problem to the new file-naming format. This was overcome by automatically appending previously opened files.

A final software fix implemented prior to the start of 1997 field sampling addressed problems encountered with temperature dependence of the oscillation frequency of the square-wave generator used to trigger sampling. The square wave generator was based on a 555 oscillator circuit that included a temperature sensitive capacitor. The thirty second sampling interval used in data acquisition is based on the number of samples collected and the sampling rate (6000 samples for a 30 second period at 200 Hz). Typical operating frequencies to trigger sampling at 200 Hz were in the range of 2000 to 3000 Hz. The thirty second period was found to range from 28 to 39 seconds, because temperature variations affected the oscillation frequency of the 555 based square-wave generator circuit. During the 1996 field season, the 555 square-wave generator was recalibrated every 15 to 30 minutes to account for these weather induced temperature changes. In addition to the temperature variations, transients were discovered when the raw output of the square-wave generator was saved to a disk file. The transients sometimes caused premature triggering of sampling.

A more rigorous sampling algorithm was tested that eliminated problems caused by the transients but was unable to remedy the temperature dependence of the square wave generator. The problem was finally resolved by abandoning the 555 square-wave generator

circuit and instead using the 8254 timer circuit used for sound generation in computers. The revised sampling algorithm operating at 200 Hz was able to generate sampling intervals to within ±0.07 seconds of the desired thirty second period on computers ranging from 120 MHZ Pentiums to 80286's. The increase in triggering accuracy guaranteed consistent thirty-second sampling periods throughout the year regardless of temperature or the computer used to acquire data.

Several software enhancements were implemented after field placement of bee-counter systems during the 1997 flight season. First, the data analysis code was optimized to decrease the length of time required for processing raw data collected during each thirty second sampling interval. To aid the code-optimization process, a test version of the field data acquisition program was written that accessed a simulated data file instead of the IOTech interface. The simulated data file was created from raw data collected during the Spring of 1996 at the U of M Prescott House apiary. This program allowed improvements to be tested and benchmarked as the code was written. Next, disk caching via the DOS utility SMARTDRV.EXE was used to improve the hard drive access speed. For new features and code optimization described below, this program proved a reliable testing ground prior to field deployment of software.

1920 thirty second intervals exist during a 16 hour sampling period (6:00 to 22:00). The computer processes raw data for approximately 1 to 5 seconds at the end of each thirty second interval, during which time data acquisition does not occur. Using the Fort Missoula site as an example, the typical number of thirty second sampling intervals collected during a day was 1680 before the code was optimized and 1830 after the code was optimized. This increased the sampling coverage from 85.5% to 95.3% of the total flight population. This 9% increase in number of samples collected over the course of a day did not affect the daily percent return rates in any way, indicating that the unoptimized code had adequately characterized flight activity.

Real-time delivery of data over the Internet, via a World Wide Web (WWW) home page, was a principal focus of the 1997 Methods Development. The bee-counter data acquisition software was modified to communicate via the RS-232 (serial) port to a computer equipped to receive data on fifteen minute intervals. The fifteen minute interval was used to minimize the impact of data transfer on the bandwidth required for data collection and transmission. The time interval can be further reduced to fit specific real-time application demands. The field data acquisition and display software was modified to transfer data to a computer running the Linux operating system. For field-based delivery of real-time data, the bee-counter computer will connect to the WWW via a Linux based computer (described later in this section). Flight counts for bees entering and leaving each hive are updated throughout the day. A compressed data file that includes count information for each of the 14 individual bee passageways in each hive is transferred across the network at the end of the sampling day.

Finally, the field data acquisition and display program was updated to address problems encountered during brief power outages in which the computer interface would shut-down and fail to restart. This was fixed by adding a command to restart the interface on fifteen minute intervals throughout the day. In other words, within 15 minutes of restoration of power, the system will be back on-line.

The Fort Missoula Research and Development Apiary also was the home to three colonies of *Bombus occidentalis* (bumble bees) that were placed in a man-made burrow with specially designed bee-counting devices attached. *B. occidentalis* was chosen as a model native species for use in environmental biomonitoring. The counters were housed in ABS plumbing material and constructed with 1 large bee-passageway to accommodate the larger sized bumble bees. These colonies housed less than 100 bees each. A single passageway proved to be sufficient for counting bumble bees. There were never more than 4 bees passing through the counter in 1 thirty-second interval. By comparison, honey bee counters averaged 34 bees passing through each channel per thirty second interval during a swarm event. Comparisons were made between bumble bee and honey bee flight activity. Both species responded to changes in weather in a similar manner. The bumble bee prototype systems and tests were funded by the National Science Foundation (NSF), The University of Montana, and the Montana Beekeepers Association. There was no cost to the army. Costs to these other sponsors were relatively low because we were able to apply lessons learned from designing the equipment and software already developed under this DOD project.

Correlation of forager flight activity with weather events was aided by the introduction of a solar radiation data-logger at the Fort Missoula apiary (loaned to this project by Dr. V. Watson, UM Environmental Studies Program). Data containing mean, high and low solar radiation values were collected every 10 minutes. The amount of sunlight present each day was correlated with flight activity data to further characterize daily flight activity patterns.

G.3 Off-line Data Tier Analysis Methods

As mentioned, electronic bee-counters were deployed at five locations during the 1997 field monitoring season. Analysis of flight activity data by site followed the Tier-based approach established during the 1996 monitoring season. The Tier system for flight activity is based on a biological organization (i.e., among populations, between populations, among and between individual bees by site and year) and is summarized in Table G.2.

Table G.2
Overview of the Flight Activity Data Tier System.

Tier	Capabilities and applications
1	Site-wide daily flight activity indicators compared between sites Site-wide daily flight activity indicators compared from year to year for each site
2	Flight activity indicators compared among hives at a site Can be used to compare seasonal changes at a site
3	Monitor short-term response of single counter or all counters at a site Site-wide short-term flight activity response compared between sites
4	Site-wide counter diagnostics using data from 14 bee passageways on each counter

G.3.1 Tier 1

Tier 1 flight activity analysis includes total flight activity (TFA), inter-colony coefficient of variation (C.V.) of total activity, the percentage of bees returning to the colonies at each site at the end of each day (PRC), the net loss (NL) of bees at the end of each day, and the adjusted net loss (ANL) of bees at the end of each day. The PRC for each colony at each site at the end of the day was calculated as the total number of bees that entered the counters divided by the total number of bees that exited the counters at each site for a given day. Net loss (NL) was calculated by subtracting the total number of bees that entered the counters from the total number of bees that exited the counters at each site for a given day. Adjusted net loss (ANL) was calculated by dividing the net loss of bees for the site by the total activity for the site.

Although similar, the flight activity analysis methods have individual strengths that determine their function. The percentage of bees returning (PRC) to the hive at the end of each day usually remained in the range of 92% to 98%. Deviations outside this range were due to events such as rainshowers at the beginning or end of the day, natural colony dynamics (swarms), exposures to acutely toxic chemical events, or equipment malfunction (power outages). The daily net loss (NL) of bees was affected by the level of total flight activity for each day. As such, it was used as a secondary indicator in conjunction with adjusted net loss (ANL). Net loss must be corrected for the total flight activity to be a reliable indicator. For example, on a warm sunny day total flight activity may exhibit a net loss of bees (by count) that exceeds the total flight activity on a low activity day. Recruitment (or drift) of bees from other nearby colonies could result in a net gain of bees for an individual colony, but no change when averaged across all of the hives at a site.

Adjusted net loss normally ranged from -4 to +6, depending on whether there was a net loss (positive numbers) or net gain (negative numbers) of bees for each hive/day. If a large net gain or loss occurred, the net loss and total flight activity data were then reviewed to investigate the nature of the event. Based on the results, appropriate action could be taken, such as initiating chemical sampling or inspecting the colonies. All Tier 1 results were represented using 2-dimensional X-Y plots.

G.3.2 Tier 2

Tier 2 analysis applied Tier 1 analysis methods to individually compare the colonies at a site. The PRC, NL and ANL methods were all expected to demonstrate greater fluctuations for individual colonies than observed in the Tier 1 results. Normalized Total Activity (NTA) was calculated as the total activity for each colony divided by the total activity for the seven colonies at the site. This indicator provided an easy method of determining the strength of individual colonies in relation to one another. With seven colonies at a site, the optimal NTA value for colonies of equal strength was about 14.3%, larger values indicated a stronger than average colony and lower values indicated a weaker colony. All Tier 2 results were represented using color maps for formal analysis and 2-dimensional X-Y plots using Option 10 of the SITEVIEW program on the included CD-ROM. A SITEVIEW Manual is included in Appendix J.

G.3.3 Tier 3

Tier 3 flight activity analyses included all data analysis methods that operate on short-term flight activity data. SITEVIEW offers many analysis options for Tier 3 data. Each option is a custom software utility program written at UM. These utilities offer data processing and visualization capabilities ranging from: (1) data file formatting and storage, (2) simultaneous display of raw or smoothed data from seven colonies at each site, (3) site by site comparisons, (4) honey bee and bumblebee species comparisons, and (5) complex numerical analysis methods that are highly sensitive to unusual flight activity behavior. The following text is taken from the main menu of the SITEVIEW program.

WELCOME TO THE BEE-COUNT DATA MANIPULATOR AND VISUALIZATION

THERE ARE SEVERAL SELECTIONS AVAILABLE FOR PROCESSING AND
VIEWING DATA OBTAINED FROM SITES CONTAINING SEVEN BEE COUNTERS.

(1)   PLOT TOTAL ACTIVITY FOR 7 HIVES (NOISY DATA)
(2)   PLOT SMOOTHED TOTAL ACTIVITY DATA FOR 7 HIVES
(3)   PLOT DIFFERENCE BETWEEN IN AND OUT ACTIVITY FOR 7 HIVES
(4)   PLOT IN AND OUT DATA SIMULTANEOUSLY TO SEE OVERLAP
(5)   PLOT SMOOTHED PERCENT RETURNED FOR 7 HIVES
(6)   CALCULATE AND PLOT SMOOTHED DERIVATIVES OF FLIGHT ACTIVITY
(7)   PLOT COMPONENTS OF THE DER*DIFF ALGORITHM USED IN OPTION 8
(8)   PLOT DERIV*DIFF RATIO TO SEE PHASE SHIFT SENSITIVE ANALYSIS
(9)   DISPLAY AN HOURLY OR USER SELECTED INTEGRATION PERIOD
(10) PLOT SITEWIDE DAILY ACTIVITY FOR ENTIRE SEASON
(11) SIMULATE THE FIELD DISPLAY
(12) PLOT FOUR DAYS/SITES SIMULTANEOUSLY
(15) REVERSE BACKGROUND / FOREGROUND COLOR
(19) DELETE OPENED DATA FILES FROM DISK, LEAVES COMPRESSED FILES
ALONE
(20) END SITE/STUDY VIEWER MENU AND RETURN TO MAIN MENU
      PLEASE ENTER A NUMBER FROM THE ABOVE LIST:

Each of these options are described in detail in the SITEVIEW Program Description and
Trouble Shooting Manual found in Appendix A and included on the CD-ROM disk. The
SITEVIEW Manual includes instructions and suggested parameters for use of each option.
The CD-ROM contains a complete archive of the 1996 and 1997 field data for this project.
Significant aspects of the flight activity data are described to aid the user in interpretation of
the results from each option. Specific data sets are used to illustrate visualization and
interpretation of the output of many of the SITEVIEW routines. Equations are described to
clarify the numerical methods used to process data from the bee-counters.

The options available in the SITEVIEW program provide rapid viewing of the flight
activity data using a PC based computer. Some of these options illustrate the daily flight
activity patterns for the colonies at a single site, while others allow comparisons of the site-
wide total flight activity pattern for several sites simultaneously. For example, tracking
events such as a storm moving from one site to another can be accomplished by concurrently
displaying the total flight activity throughout a given day for two or more sites (Option 12
of the SITEVIEW program). Established metrics of flight activity such as TFA, C.V., PRC,
ANL, and NL can be plotted to rapidly provide information about changes in colony
behavior.

G.3.4 Tier 4

Tier 4 analyses used count data from each of the 14 bee-passageways of each bee-counter as a diagnostic tool. Tier 4 diagnostics used the coefficient of variation among passageways on a single bee-counter, as well as among the similar passageways for each of the seven bee-counters at a site. Large values for the C.V. among passageways on a single bee-counter alerts the operator to the presence of defective channel-counters but does not provide information about which passageways were blocked. Large values of the C.V. among similar passageways for bee-counters at a site helped pinpoint which passageways were blocked or defective. When an unusually large C.V. was observed, direct observation of the data from each bee passageway identified the defective or blocked units that required replacement. Several options of the SITEVIEW program can be used to perform these Tier 4 analyses. Application of Tier 4 diagnostics allowed quick evaluation for detection and assignment of hardware problems.

G.4 Results of Tier 1 Evaluations of Flight Activity Data

Flight activity data collected during the Summer and Fall of 1997 were summarized and compared with data from the 1996 field monitoring season. Figure G.1 presents Tier 1 summaries comparing the corrected (i.e., adjusted for the number of sampling periods and number of bee-counters being monitored) total daily flight activity throughout the season for each of the test sites. A detailed summary of events that occurred during the 1996 field monitoring season was presented in the previous annual report (Bromenshenk et al., 1997). In general, flight activity at all of the APG sites displayed similar day to day seasonal trends, probably as a consequence of colony responses to local weather conditions. All sites showed a similar seasonal trend characterized by high activity during the summer, a slow decline in activity as summer progressed into fall, and then very little activity after the middle of October.

Weather events had a similar effect on all APG sites during both the 1997 and 1996 monitoring programs. In 1997, large drops in activity were visible at all APG sites on July 23-24, August 20 and September 10, indicating the presence of rain. Unlike the 1996 season, in 1997 both the West Branch Canal Creek site and the Old O Field site displayed a general drop in activity from July 29 to August 14. This decrease was not mirrored by the bees at the Churchville reference site. During the same period bees at the Fort Missoula site were busy foraging nearby knapweed and were increasing activity. The drop in activity at Old O Field was similar to that seen in the previous year after a hurricane passed through the area. The drop in activity for West Branch Canal Creek was not as severe as that seen for Old O Field. This response could be due to a lack of available foraging materials on the APG peninsula. Because the Old O Field change in activity again corresponded with an increase in the levels of PCE and other organics in the air inside the hives, it is possible that greater change at Old O Field might have been due to exposure to these chemicals. In 1996, a severe drop in activity and loss of the queen from 50% of the hives at Old O Field corresponded with a period of high exposures to organic solvents (relative to other APG sites).
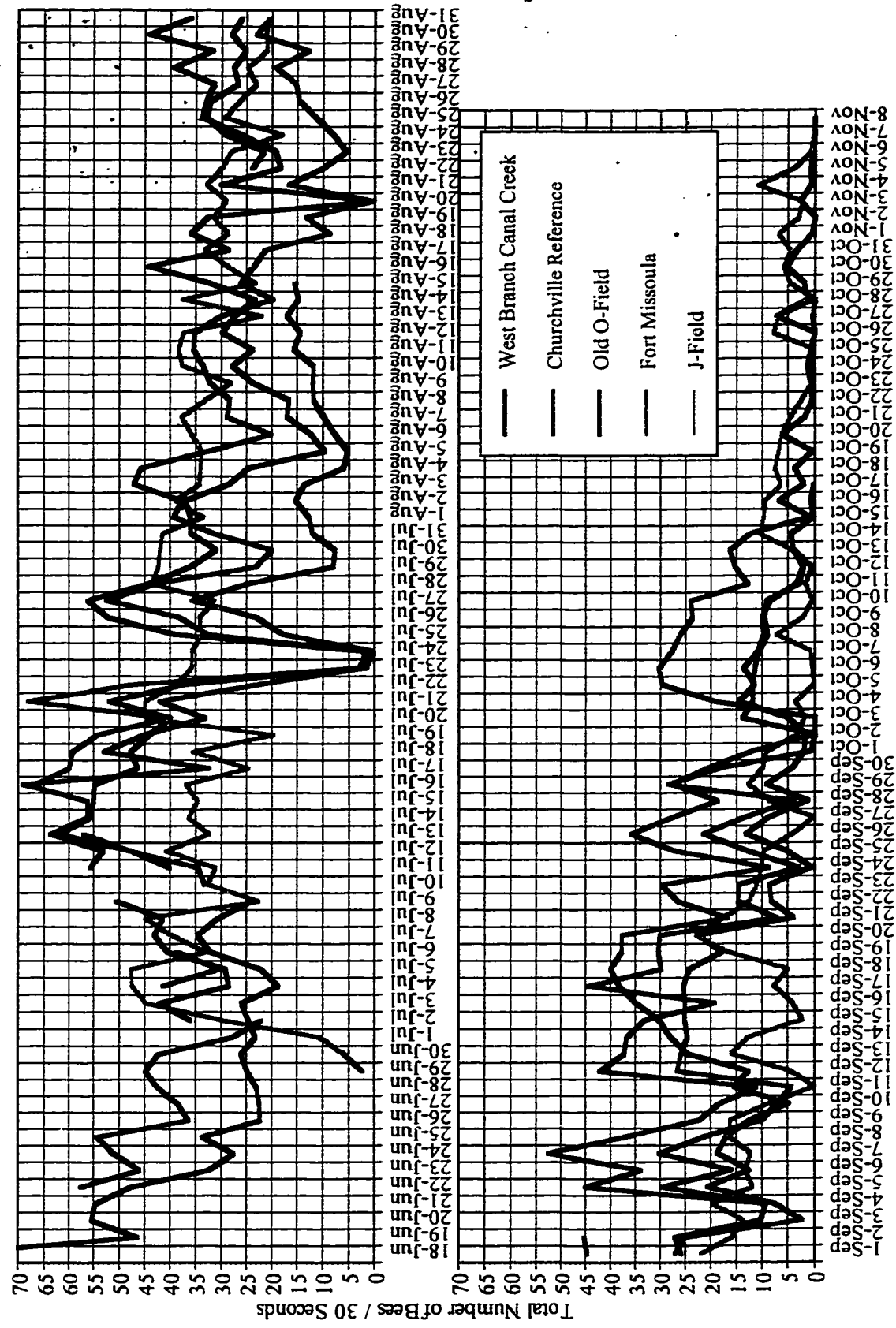
Figure G.1. Cumulative daily flight activity by site, 1997. Total number of bees entering and leaving the hives every 30 seconds. Each site had seven colonies of bees.

West Branch Canal Creek
Churchville Reference
Old O-Field
Fort Missoula
J-Field

Total Number of Bees / 30 Seconds

Coefficients of variation of flight activity among the colonies at a site for the 1997 monitoring season also were plotted and compared. As seen in Figure G.2, the C.V. for all sites remained near or below 50% throughout the summer and into October, with a few notable exceptions. A two day long rain-event during July 23 and 24 produced the large peak found at West Branch Canal Creek and the Churchville reference site. Oddly enough, this peak is barely discernable for only the first day at Old O Field. The swarm at one of the Churchville colonies raised the C.V. from around 30% to nearly 50% until the colony was reconstituted.

Power failures, usually following thunderstorms, continued to plague all of the Maryland sites as evidenced by gaps in data, resulting in high C.V. peaks for these days. The Fort Missoula site was brought on-line in stages, with the last four colonies installed on July 13, explaining the unusually high C.V. at the beginning of its season. The increase in C.V. at Fort Missoula from the beginning of October until the end of the monitoring season was attributed to a combination of low flight activity, and experiments testing the effects of a misaligned counter.

Extremely low flight activity or incomplete data sets have a detrimental effect on the calculation of C.V. Low flight activity increased the C.V., if even one colony had modest activity, while other colonies had little or none. As expected, missing data from the daytime period increased the C.V., although missing data during the night had no effect since bees don't fly at night. If total daily flight activity was lower than 3000 bees entering and exiting the counter, or an incomplete data set was collected, the C.V. was not analyzed.

Sister queens were used during the 1997 season to test whether reducing genetic variability would improve the correlation of responses between colonies and across sites. All of the Maryland colonies were established with sister queens. At Fort Missoula in Montana, colonies 1, 2, and 3 received sister queens; colonies 4–7 had queens from different genetic stocks. The presence of sister queens appeared to lower the C.V.s for 1997 compared to 1996 at Old O Field and the Churchville reference site (Table G.3). At West Branch Canal Creek, the C.V. remained around 50% until the end of July when it dropped to levels similar to the previous year. The higher C.V. at the beginning of the season was probably a result of a strong colony swarming. Although the C.V. at the Old O Field site improved dramatically over the previous year, it remained higher than both West Branch Canal Creek and the Churchville reference site.

The percentage of bees returning to all of the hives at a site at the end of the day was calculated by the site-wide PRC analysis (Table G.3). This metric provided an indication of the general health of the colonies at each site. Figure G.3 illustrates the percent returned to the hive at the end of the day for sites monitored in 1997. Generally, for West Branch Canal Creek and the Churchville reference site, the sitewide PRC results stayed within the range of 94% to 98% during the foraging season. A rain event on July 23 and 24 produced PRC peaks for West Branch Canal Creek, Old O Field and the Churchville reference site.
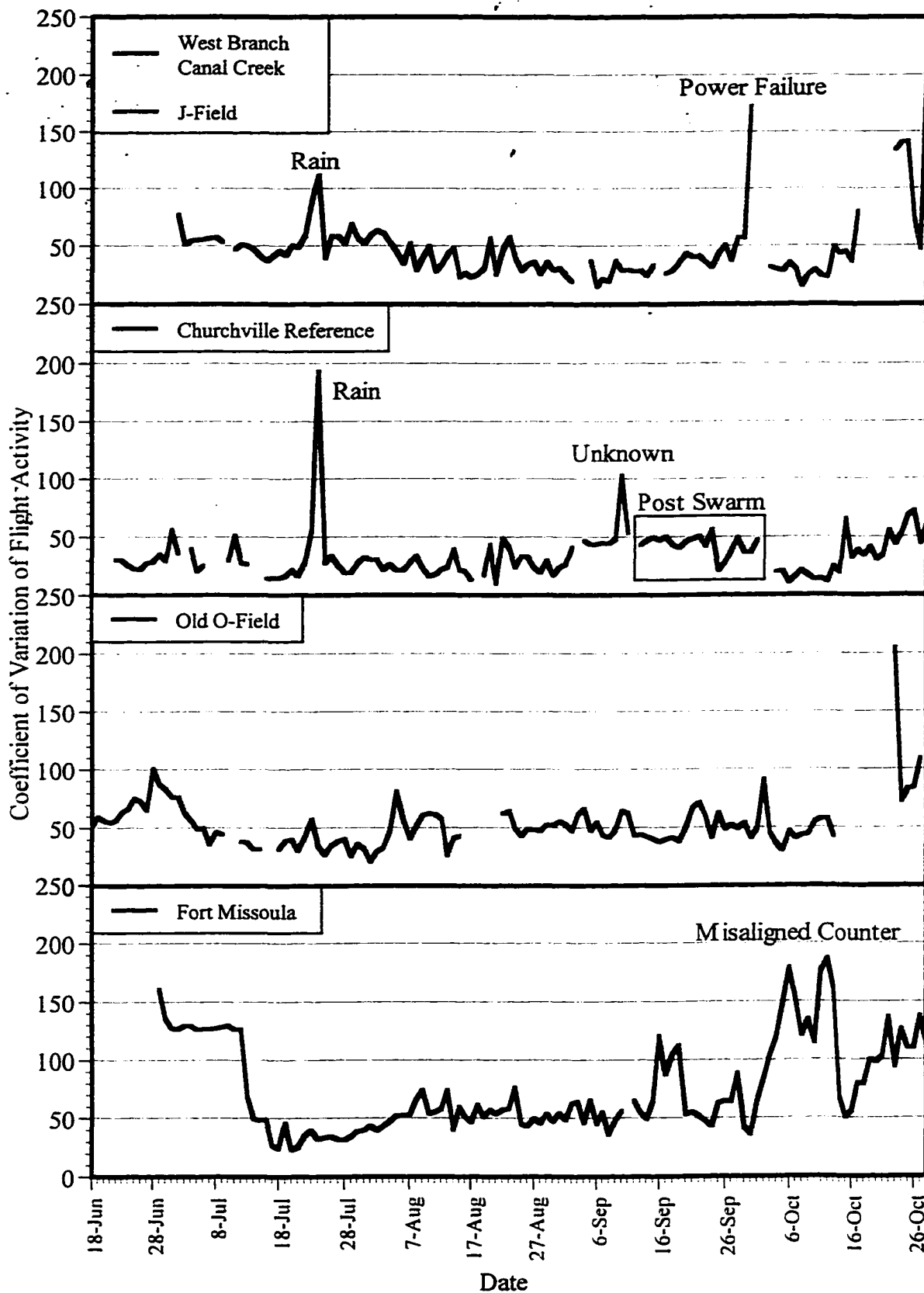
Figure G.2. Coefficient of variation of flight activity by site, 1997.
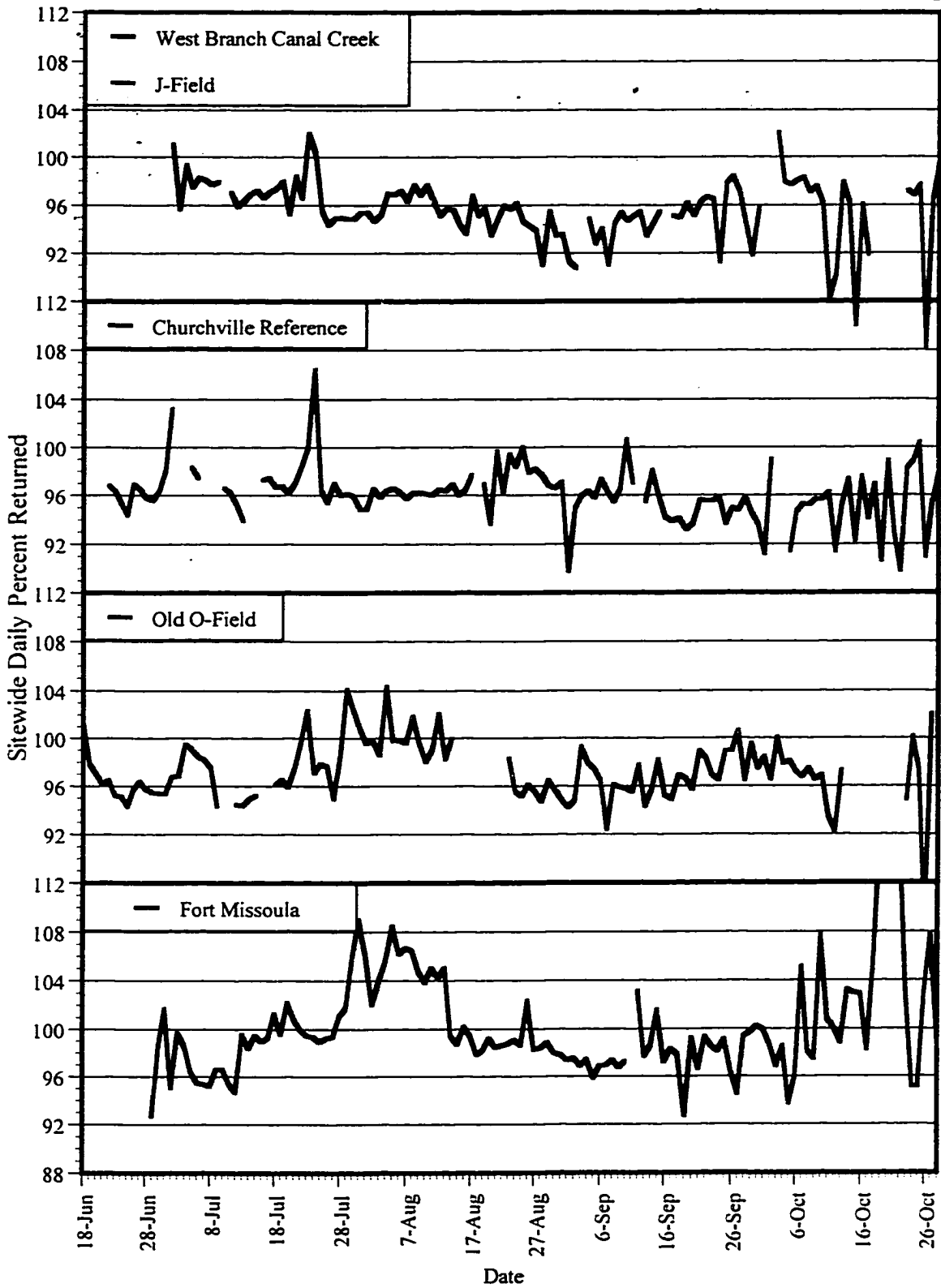
Figure G.3. Cumulative percent return of bees by site, 1997.

A swarm that occurred on September 1 at the Churchville reference site yielded a PRC of less than 90% for the entire site, due to a 43% PRC for the colony that swarmed. More information on the swarm is included in Tier 2 and 3 analysis. The site-wide PRC at Old O Field and Fort Missoula varied from 94% to 104% and 94% to 108%, respectively, during the foraging season. Both sites experienced occasional days with PRCs greater than 100%, especially from late July to mid-August. At the Fort Missoula site this correlated with the knapweed bloom occurring in adjacent fields. The increase in PRC at the Old O Field site is still unexplained. We suspect that during heavy nectar flows, some of the bees may stay in the field overnight, returning the next day. Tests of the flight counters indicated that the PRC in excess of 100% were real and not an artifact of some equipment malfunction.

Table G.3
Comparison of Tier 1 (Site-Wide) Indicators.
Percent values in this table represent "normal" colony variability and do not include maximums induced by events such as severe rainstorms, swarming, or power outages.

Site	C.V.		PRC		ANL	
	1996	1997	1996	1997	1996	1997
CC	15%-60%	20%-50%	90%-108%	94%-98%	-4 to +4	0 to +5
OF	50%-130%	25%-75%	90%-102%	94%-104%	-2 to +6	-2 to +3
CV	50%-90%	15%-50%	92%-100%	94%-98%	0 to +4	0 to +3
JF	-	15%-50%	-	92%-98%	-	+1 to +5
FM	-	30%-140%	-	94%-108%	-	-4 to +3

Adjusted net loss and corrected PRC results for total flight activity were calculated so that a quality control window could be established for daily forager flight behavior. Values of ANL between 0 and 4 are considered to be acceptable, while values outside of that range require closer inspection. As seen in Table G.3, variation of ANL improved dramatically from to 1996 to 1997.

As seen in Table G.3, ANL variation improved dramatically in 1997 compared to 1996. PRC results from the 1997 monitoring season also showed lower day to day variations and a smaller range for the sites under study, compared to 1996. In 1996, West Branch Canal Creek colonies had a PRC that ranged from 90% to 108%. In 1997, the West Branch Canal Creek/J Field colony mean return rates varied from 94% to 98%. The Churchville reference site saw a similar change, with the range being from 92% to 100% in 1996 and then from 94% to 98% (with the exception of the day of the swarm) in 1997. Old O Field varied from less than 90% to 102% in 1996 compared to 94% to 104% in 1997. During both years, we

observed drift from weak to strong colonies at Old O Field, contributing to PRCs in excess of 100%. Table G.3 summarizes the C.V., PRC and ANL results for all of the sites under study.

Large day to day variations in C.V., PRC and ANL at the end of each year indicated the close of the productive forager flight season. The queens stop laying eggs and foraging drops off with as nectar and pollen floral resources disappear due to frost and plant senescence. The onset of increased variability correlated with the time when NL consistently remained below 2000 for each of the sites. In 1997 the end of the productive forager flight season for J Field colonies occurred on October 3. At the Churchville reference site the season ended on October 1, while the forager flight season ended on September 21 at Old O Field. Assignment of the exact end of the productive forager flight season for Fort Missoula colonies was hindered by the tests documenting the effects of a misaligned bee-counter. September 24 appeared to be the end of the forage period for Fort Missoula.

In 1996, a high degree of variability evidenced in the late season data made determination of the exact end of the productive foraging season difficult. Because naturally colonies suspend foraging in the fall, variability for all of the flight metrics is expected increase. The time when this occurs effectively determines how the end of the period when these metrics can be reliably used as bioassessment indicators. Obviously, the non-foraging period will be longest in northern states with cold winters, and short or non-existent in warmer southern states.

G.5 Results of Tier 2 Evaluations of Flight Activity Data

Daily flight activity data from individual colonies were compared with data from other colonies at each site. Sites that were part of a continuing study were compared to results from the previous year using Tier 2 analysis methods. Figures G.4 - G.8 present total daily flight activity for the sites under study. Colonies at West Branch Canal Creek were moved to J Field on September 4. Therefore, the initial behaviors of the colonies when placed at J Field should reflect patterns while still residing at West Branch Canal Creek.

With the exception of the Churchville reference locations, one or more colonies at each site exhibited unusually high flight activity when compared to the other colonies at the same site. Colony (#1) at West Branch Canal Creek was found to have 2 queens in early-August. One of these queens was removed on August 10. Colonies #4 and #5 exhibited flight activity levels of 75,000-80,000 round trips per day. Figures G.5 and G.7 show that the transfer of colonies from West Branch Canal Creek to J Field did not affect the relative flight activity levels in any way.

Figure G.4. Cumulative daily flight activity, Churchville, 1997. Hotter colors denote higher activity.

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Z-axis

Figure G.5. Cumulative daily flight activity, West Branch Canal Creek, 1997. Hotter colors denote higher activity.

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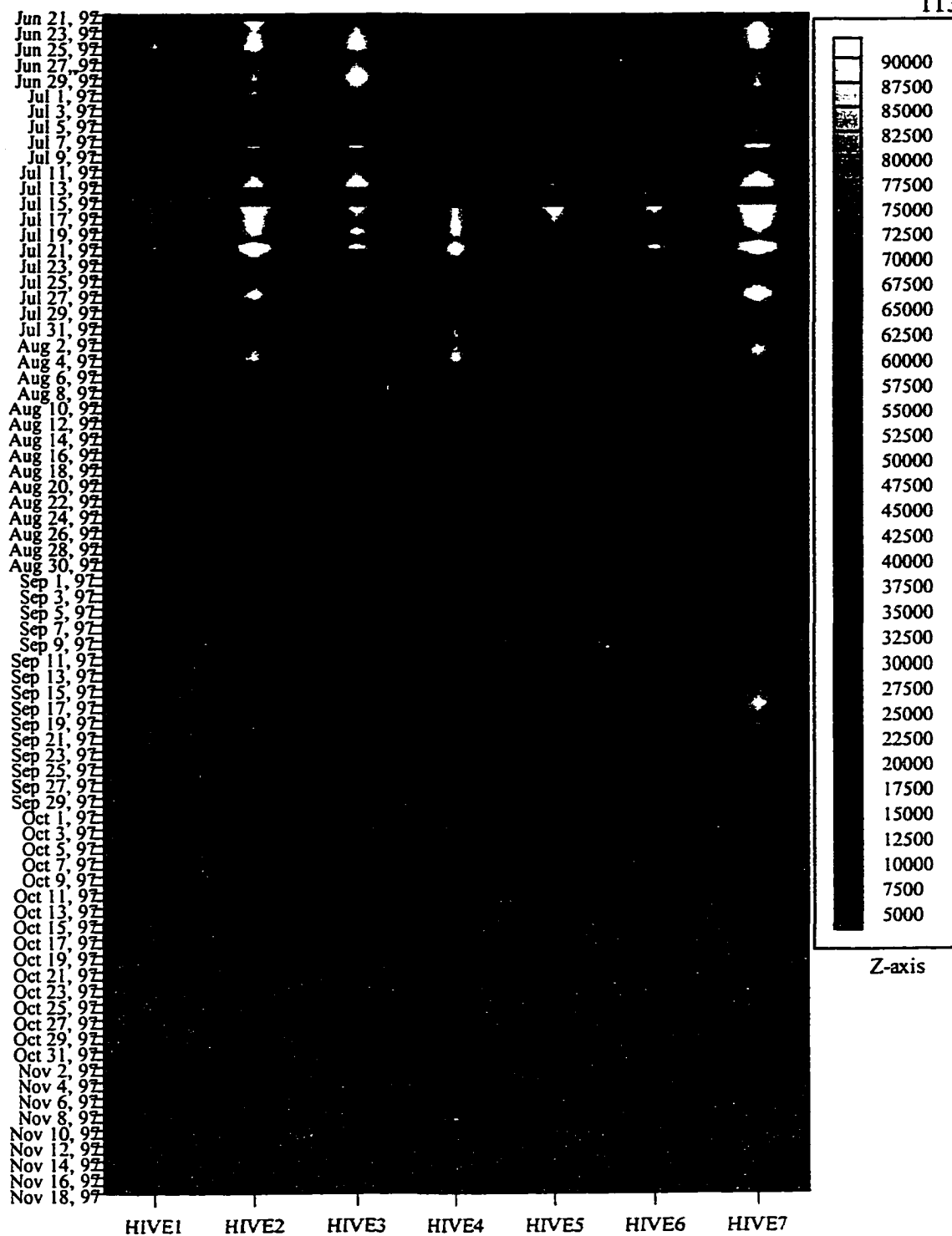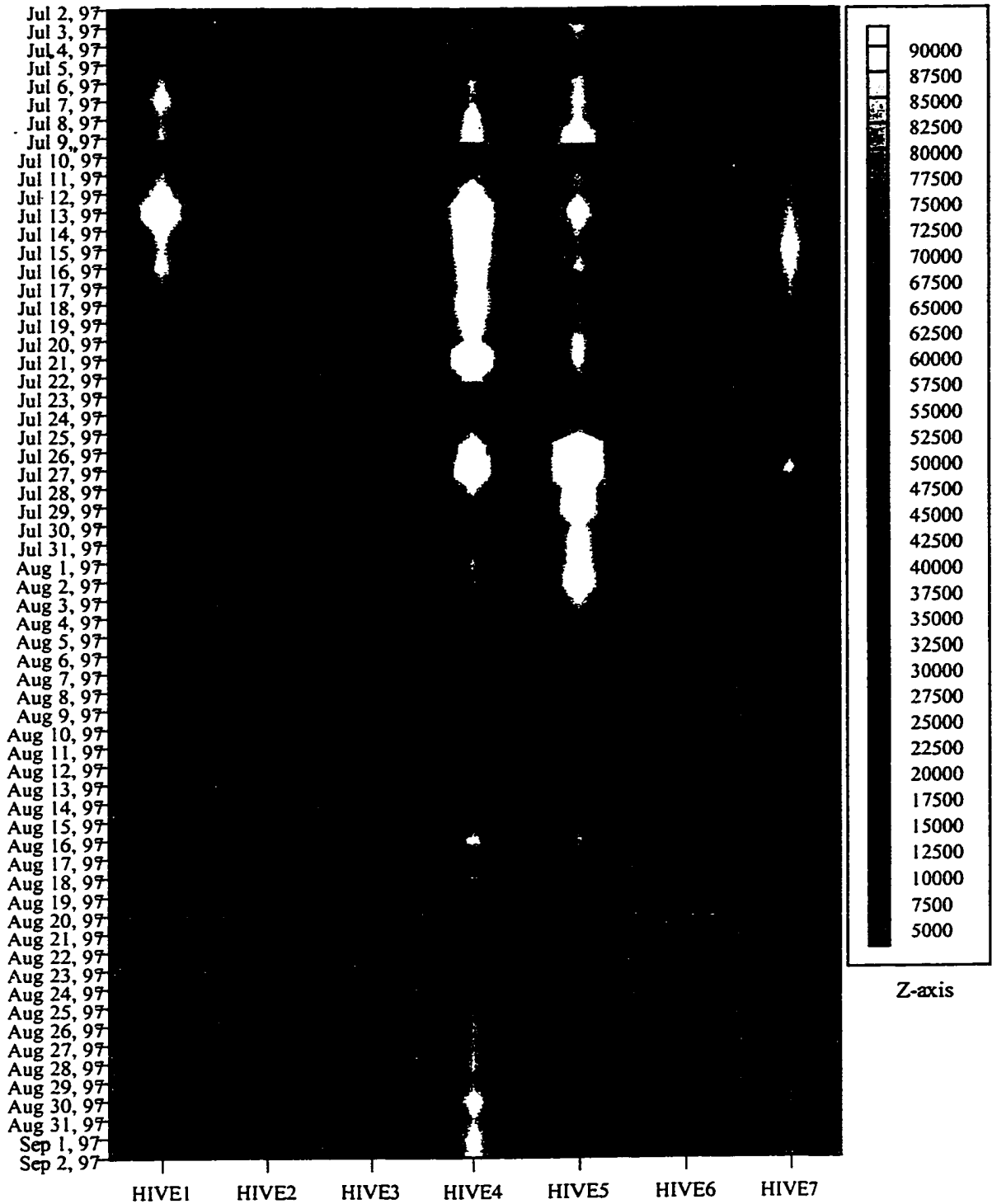**Figure G.6.** Cumulative daily flight activity, Old O-Field, 1997. Hotter colors denote higher activity.

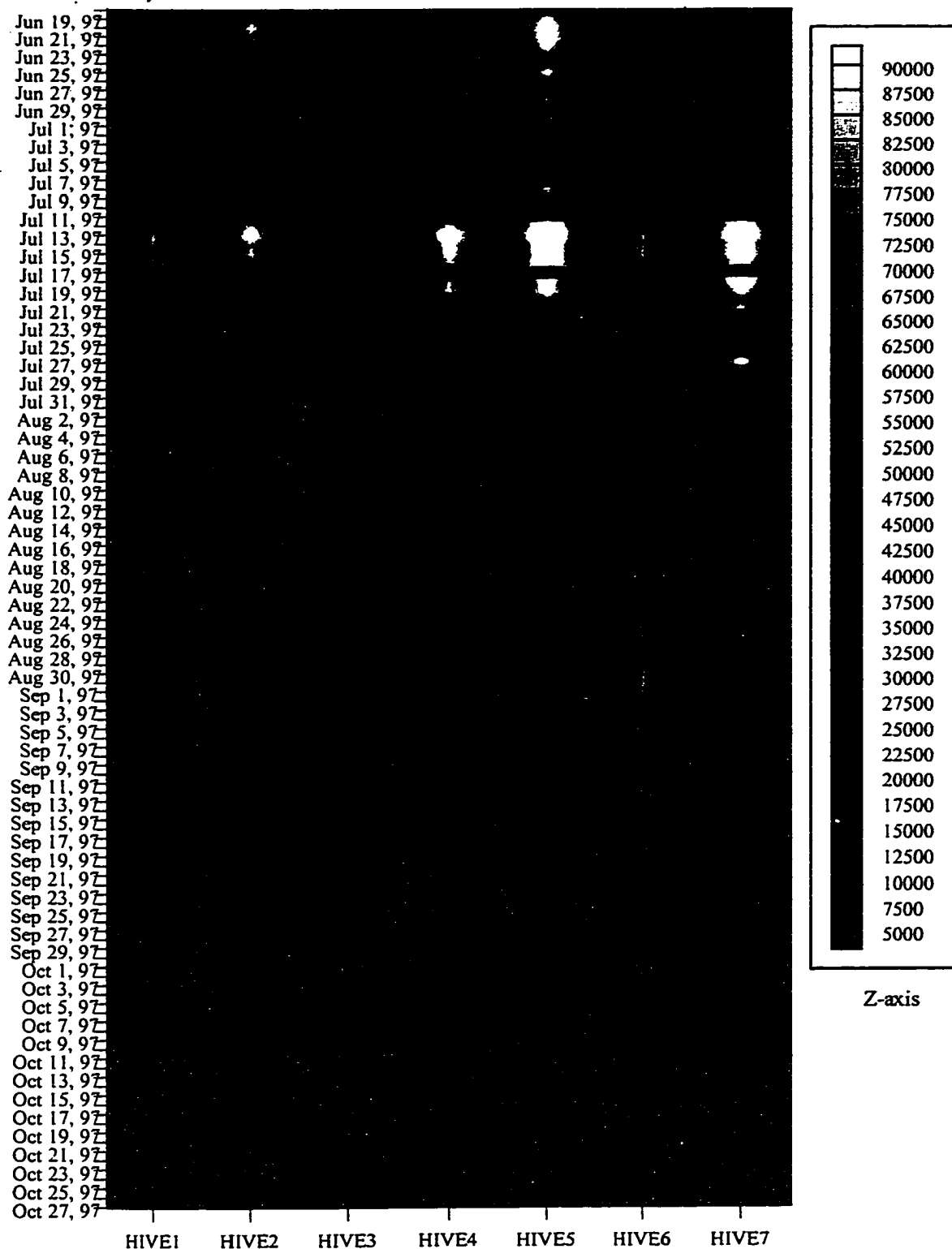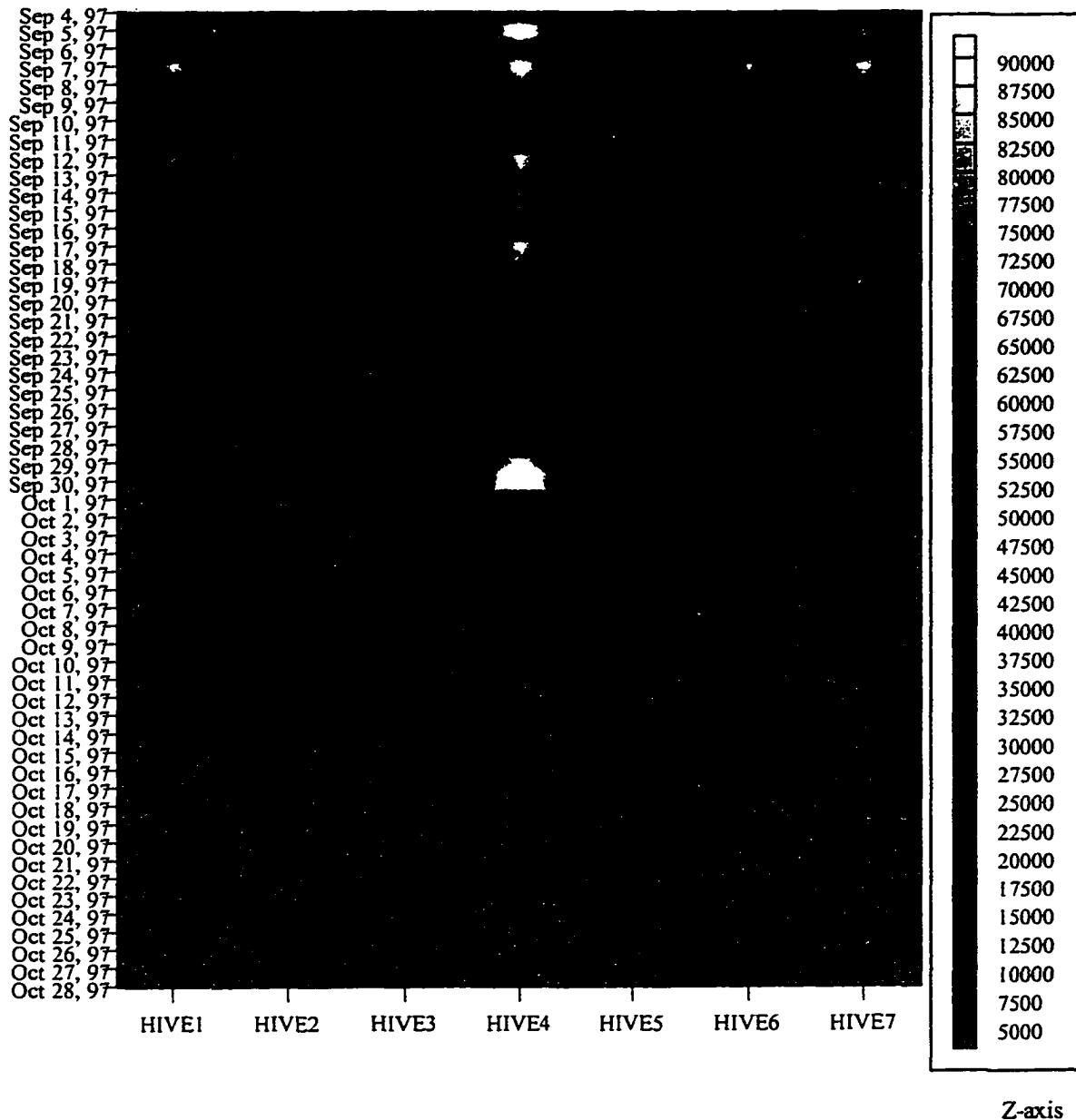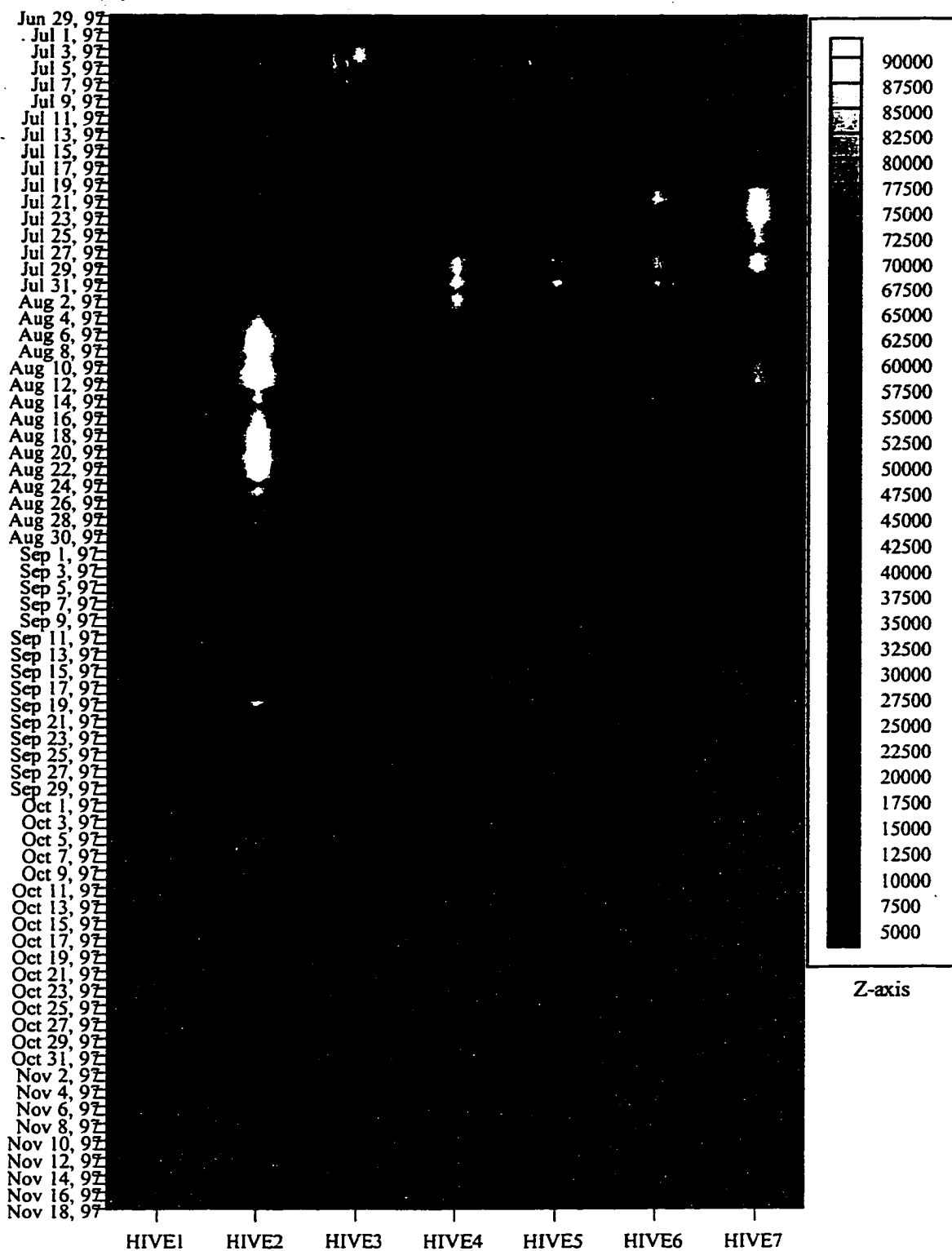**Figure G.7.** Cumulative daily flight activity, J-Field, 1997. Hotter colors denote higher activity.

Figure G.8. Cumulative daily flight activity, Fort Missoula, 1997.
Hotter colors denote higher activity.

Figure G.4 shows the flight activity levels for the Churchville reference site during 1997. In comparison to the combined West Branch Canal Creek and J Field plots, flight activity trends were similar for all colonies except for #1 which swarmed on September 9. This colony was re-queened on September 27, and returned to normal flight activity in less than 10 days.

Flight activity maps for Old O Field are plotted in Figure G.6. On August 24, the queen in colony #4 was discovered to be laying drones, indicating that she had exhausted her supply of spermatozoa. This colony was re-queened with a fertilized queen and supplied with frames of brood on September 2. It returned to normal flight activity levels by mid-September.

Figure G.8 is a flight activity map for the Fort Missoula site. Colonies 1-3 were brought on-line early in the summer. Colonies 4-7 were brought on-line as more equipment became available for use. Colonies 1-3 were re-queened with sister queens, while 4-7 had queens of mixed genetic origin. Initially, colony #2 exhibited extremely high flight activity levels. At the same time, colony #3 exhibited unusually low flight activity. A colony check revealed that a brood break had occurred, but that a healthy, laying queen was present, so no corrective action was taken. The brood break probably resulted because this queen took longer than the other sisters to start laying. This colony eventually exhibited high forager flight activity levels, but peaked later in the summer than the other colonies.

Variations in flight activity can be quantified as C.V.s among hives (Tier 1) or graphically visualized for individual hives (Tier 2) by using normalized flight activity color maps for each site as displayed in Figures G.9-G.13. This analysis provides a visual benchmark on which to compare colonies based on the assumption that all colonies contribute an equal amount to site-wide flight activity if conditions are optimal.

In general, colonies at all sites displayed higher levels of activity during the 1997 flight season than the 1996 flight season. Decreased variability in total flight activity among the hives can be seen at most sites during the 1997 flight season, compared to 1996. Colonies at West Branch Canal Creek displayed similar colony to colony variability, but higher levels of flight activity than at the same time during the previous year. Colonies at the Churchville reference site showed a much higher level of flight activity, but lower degree of variability (with the exception of the colony that swarmed) than the previous year. In 1996, the Churchville site was the last to be established. In 1997, it was set up earlier in the summer and had more time to become established. At Old O Field, the flight activity was much higher and the degree of variability among hives was much lower than the previous year.

In Figure G.10, two colonies (#4 and #5) at West Branch Canal Creek displayed a higher than average contribution to the site-wide total flight activity, while two other colonies exhibited somewhat decreased levels of flight activity. Prior to transferring the West Branch Canal Creek colonies to J Field, the flight activity at colony #5 returned to more normal
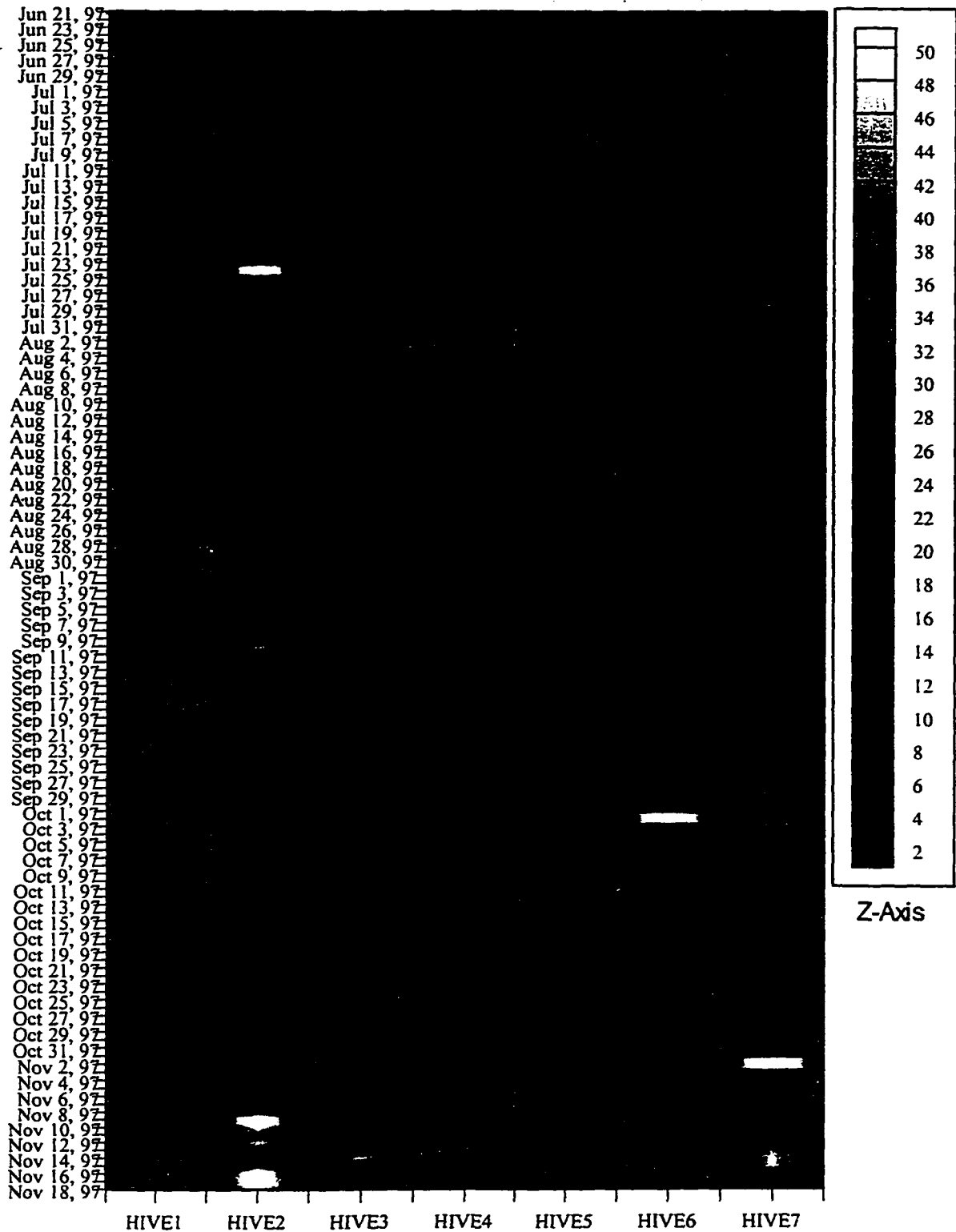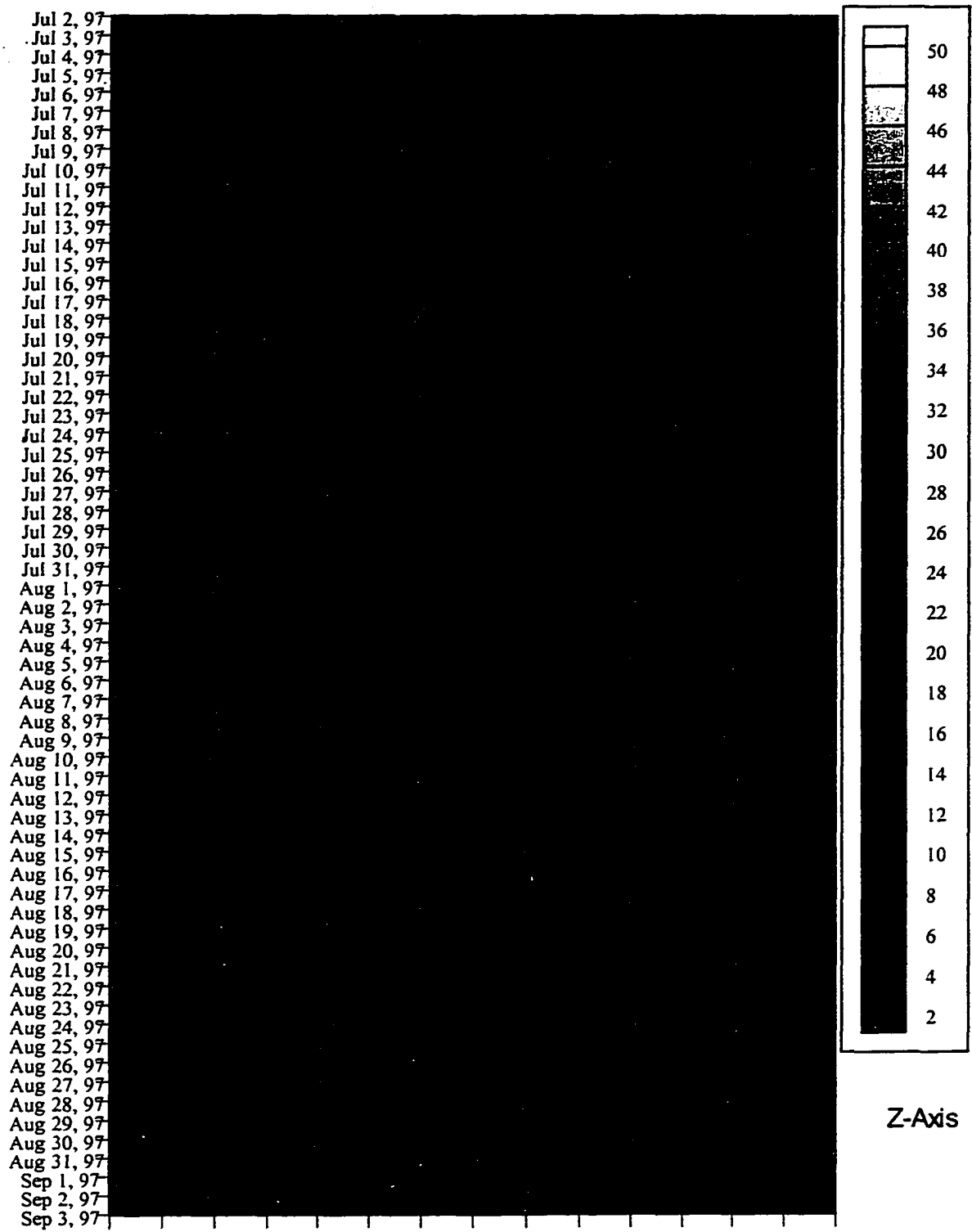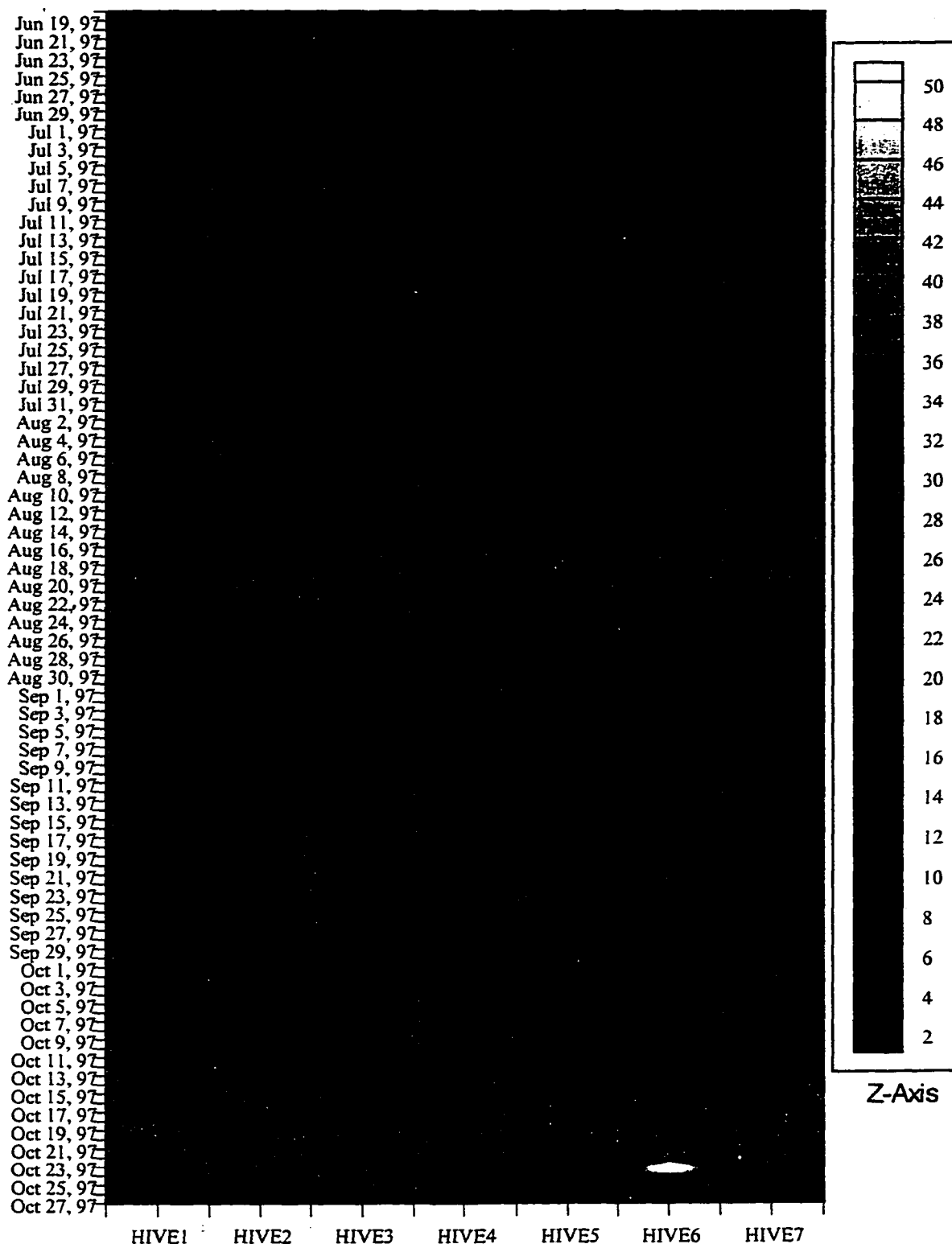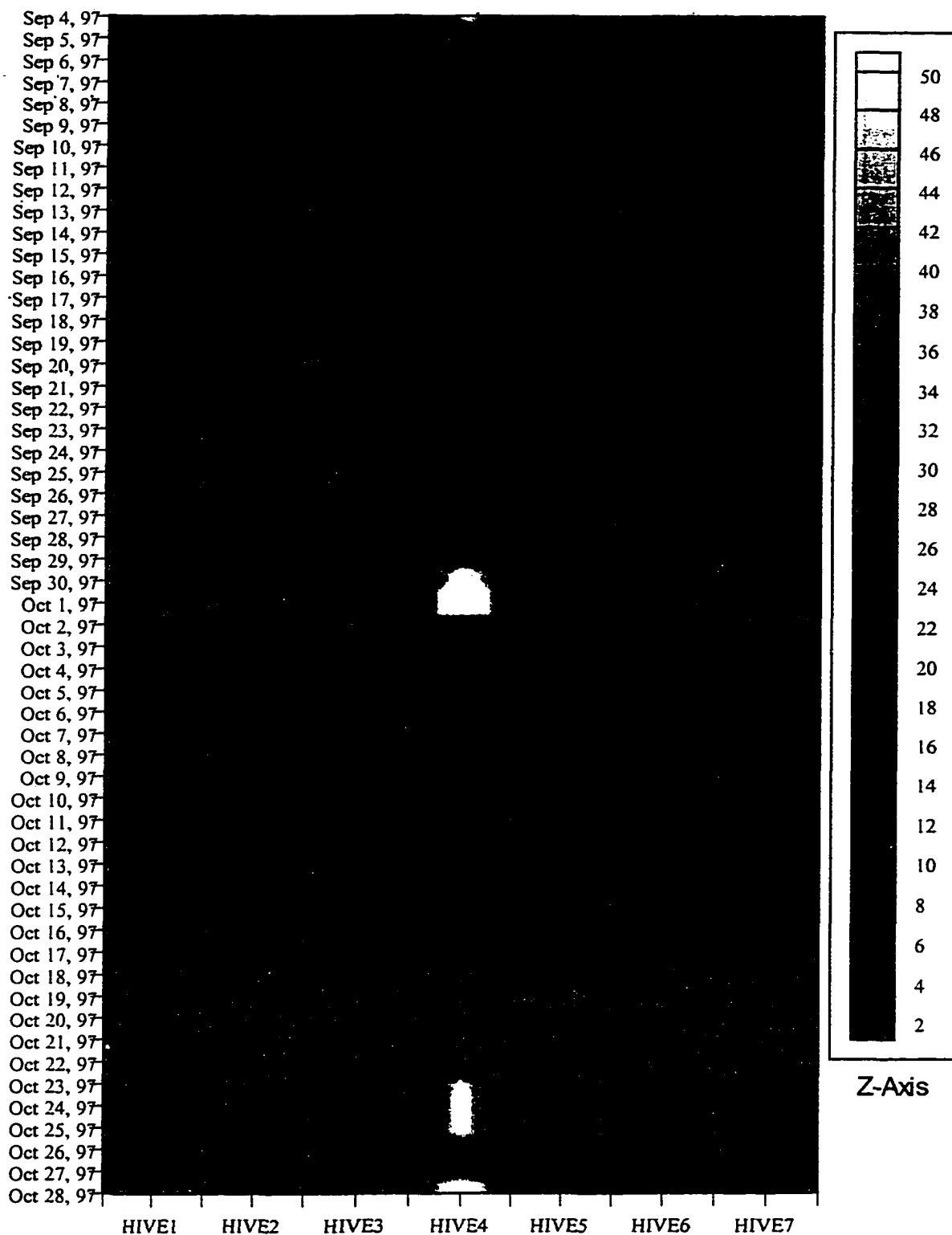
Figure G.9. Normalized cumulative daily flight activity, Churchville, 1997. Blue to purple colors indicate a weak colony, while red to yellow indicate a strong colony.

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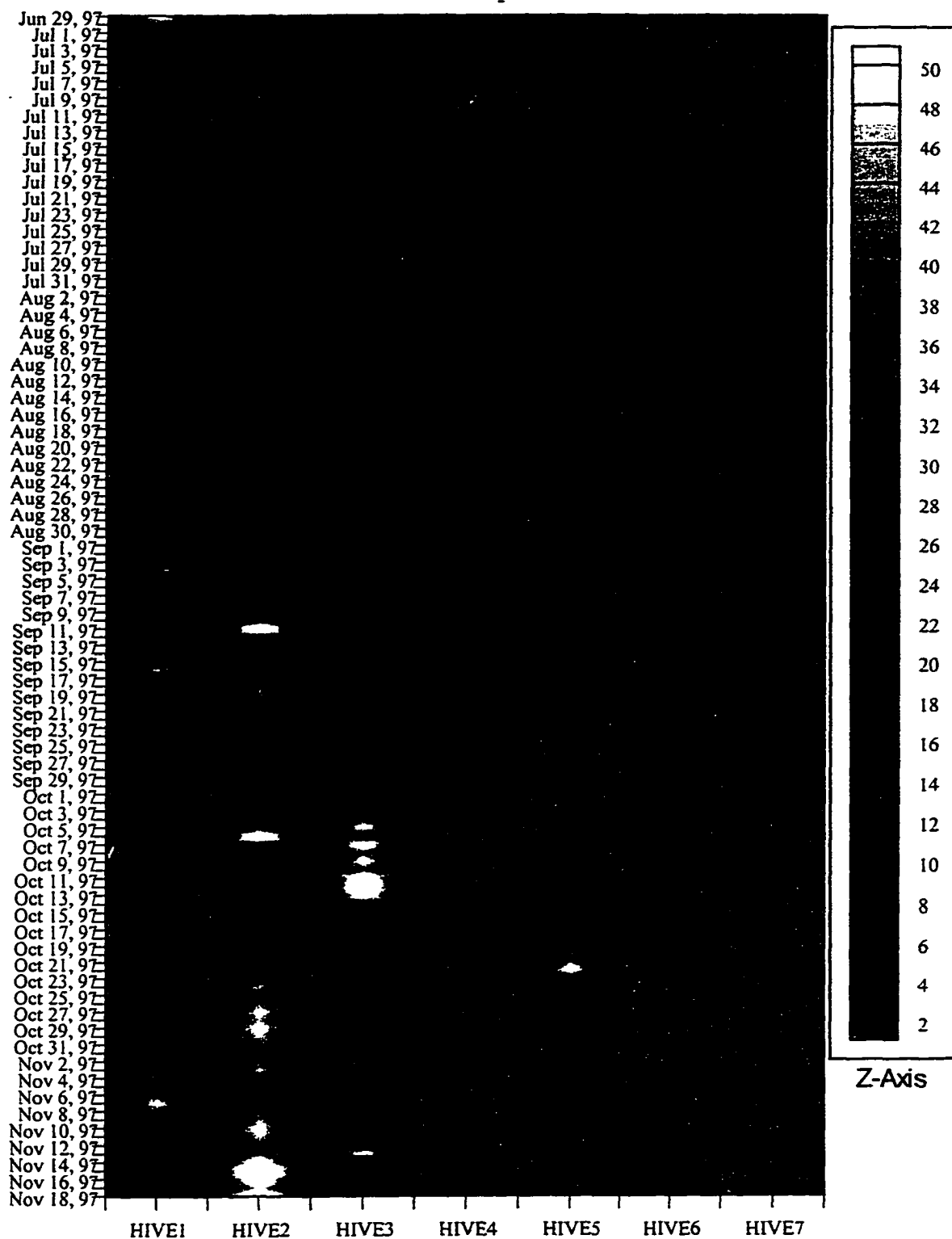18
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Z-Axis

Figure G.10. Normalized cumulative daily flight activity, West Branch Canal Creek, 1997. Blue to purple colors indicate a weak colony, while red to yellow indicate a strong colony.

Figure G.11. Normalized cumulative daily flight activity, Old O-Field, 1997. Blue to purple colors indicate a weak colony, while red to yellow indicate a strong colony.

Figure G.12. Normalized cumulative daily flight activity, J-Field, 1997. Blue to purple colors indicate a weak colony, while red to yellow indicate a strong colony.

Figure G.13. Normalized cumulative daily flight activity, Fort Missoula, 1997. Blue to purple colors indicate a weak colony, while red to yellow indicate a strong colony.

levels. This trend continued for the remainder of the flight 1997 season at J Field, as shown in Figure G.12. During 1996, all but two colonies at West Branch Canal Creek showed slightly higher than expected contributions to site-wide total flight activity. In other words, strong, vigorously flying colonies were common at this site both in 1996 and 1997.

At Old O Field in 1997, as shown in Figure G.11, colonies #5, #6 and #7 exhibited high contributions to the site-wide total flight activity for the entire season. Colony #4 demonstrated similar behavior until early-August, after which the queen was found to be laying drone cells.

Colonies at the Churchville reference site displayed little variance in total flight activity during 1997 as shown in Figure G.9. Flight activity for colony #1 dropped significantly for a brief period after the swarm occurred during September. In general, no one colony demonstrated higher than average flight activity levels in comparison to the others.

Both Old O Field and the Churchville reference site showed a high degree of variability in total flight activity among the colonies at each site during 1996. The Churchville reference site exhibited much less colony to colony variability in total flight activity in 1997, compared to 1996. However, again in 1997, Old O Field continued to exhibit higher levels of difference in flight activity among the colonies at the site, compared to other sites.

Figure G.13 displays the normalized flight activity map for the Fort Missoula site. This Figure demonstrates the importance of using related queens for environmental monitoring applications. Colonies #1-3 used sister queens, while the queens in colonies 4-7 were of mixed origins. Clearly, the sister queens were able to establish and maintain more uniform flight activity levels than the mixed queens.

G.6 Results of Tier 3 Evaluations of Flight Activity Data

A main focus of this year's data analysis efforts was the continued development of the data processing software (Section G.3.3) and exploration of: (1) the data collected at thirty second intervals, and (2) of the results of daily sums used to provide event indicators. This was done in conjunction with field simulations and experiments such as weather events, hive maintenance activities, and chemical events/exposure performed at the Fort Missoula research and development site, with some parallel experimentation occurring at the Churchville reference site.

Flight activity response patterns documented for events at the reference sites can then be used to assess the probable cause of events occurring at other sites. For example, a swarm at one of the colonies at the Churchville reference site was used to demonstrate the capabilities of the bee-counter system under extremely high flight activity conditions.

Weather events such as approaching thunderstorms affected short-term flight activity patterns by causing bees to return to the hive en masse before the storm. The fast return of bees to the hive produced peaks in the thirty second flight activity plots that were indicative of the type of event that occurred. Rain events were characterized by a peak in bees returning to the hive and a sharp drop in the bees leaving the hive (Bromenshenk et al., 1997).

With two years of data from multiple sites, we were able to track the passage of weather events across sites located within the same region. Figure G.14 illustrates the passage of a rain event between two monitoring sites at APG and then on to the more distant Churchville reference site on 3 September, 1996. The ability to follow the progress of weather changes across a region suggests that a similar trend analysis could also be applied to monitoring the release of chemical contaminants from point and non-point sources, where gradients of exposure to the chemicals should be evident in the responses of a network of monitored colonies.

Honey-bee forager flight activity depends upon colony size and structure, ambient temperature, sunlight intensity, precipitation, other weather events, and water and forage resource availability. To the degree possible, all of the colonies in Maryland were started with sister queens, similar size populations, and housed in identical hive bodies.

Not unexpectedly, the sites at Maryland did not have exactly the same flight activity patterns under similar weather conditions. Whereas total flight activity is a function of colony size and status (e.g., presence of a laying queen and brood) and overall flight activity for a specific day is strongly influenced by weather conditions, between site seasonal differences in flight activity also are a function of different needs and availability of forage resources and water (e.g., for cooling during hot periods).

Changes in forage resources and local temperatures can dramatically alter flight activity patterns within a few days. This is demonstrated in Figure G.15 by comparing the daily flight activity patterns for bees at West Branch Canal Creek between August 14 and August 17. On August 14 flight activity reached a steady level by 6:30 and maintained this level until a broad peak at 17:00. Flight didn't begin until 7:30 the next morning, and never reached a stable level for the rest of the day. August 16 began with a unusually large, broad flight activity peak that leveled off by 11:30 that morning. The large peak is not evident in the flight activity pattern for August 17. The peak occurring on August 16 could be due to a brief hot-spell beginning on August 15 and ending by August 17. Because flight activity depends on multiple variables, advanced methods of assessing complex data are required, such as the application of Artificial Neural Networks to discern response patterns and predict "expected" flight.
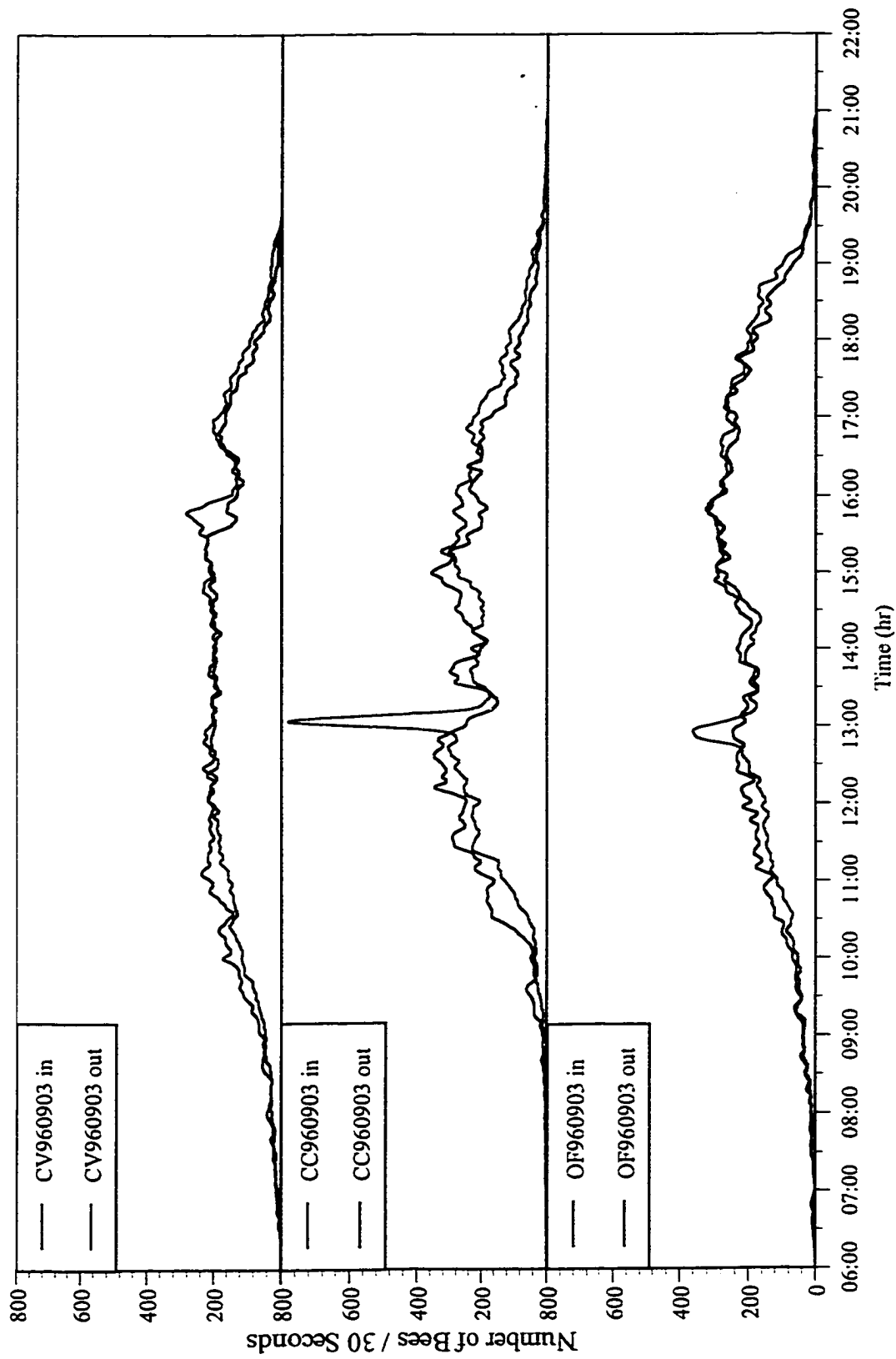
Figure G.14. Total flight activity / 30 second at APG sites, 09/03/96. A rainstorm passed from Old O-Field to West Branch Canal Creek and then to the Churchville reference site.
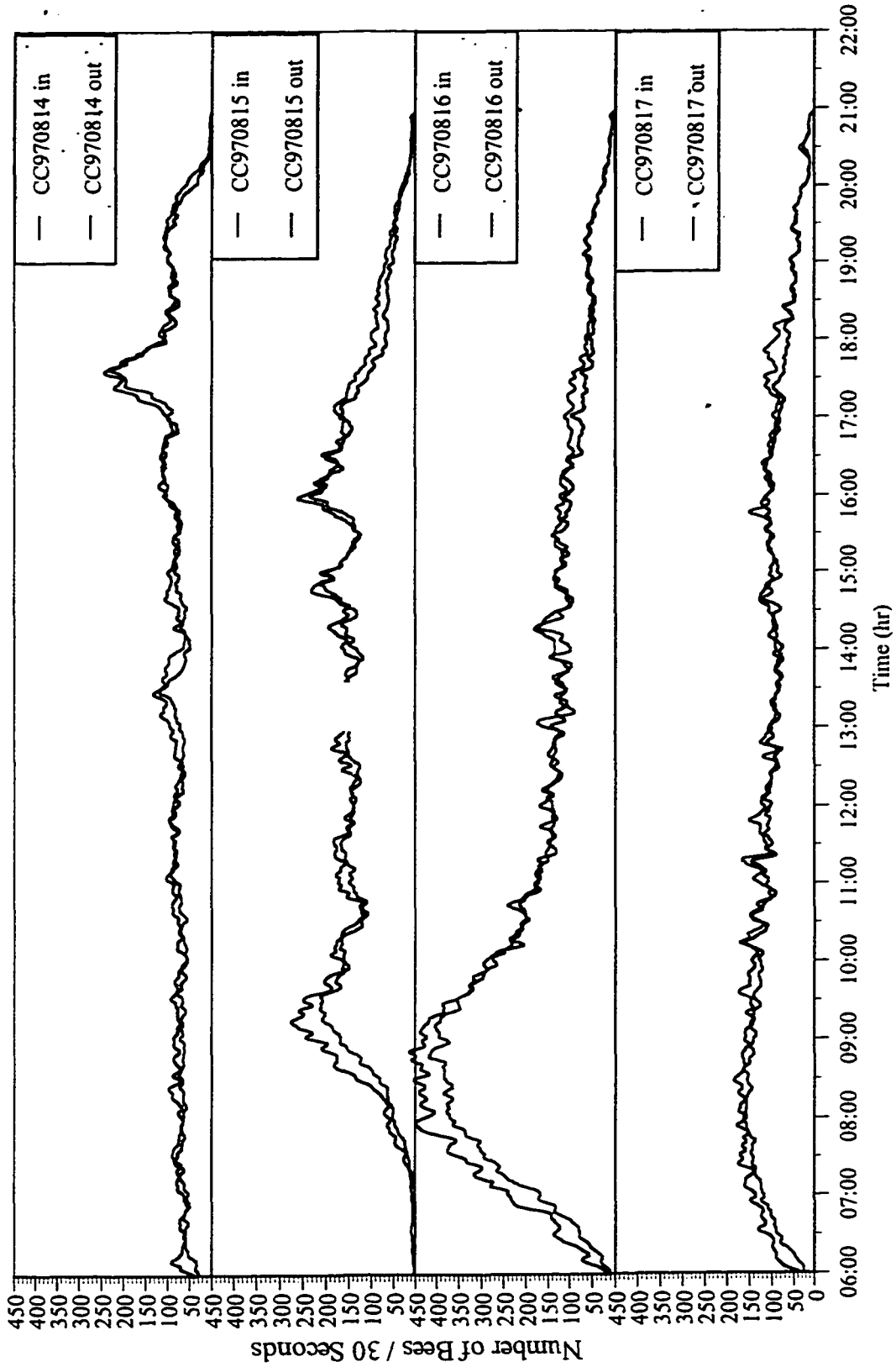
126

Figure G.15. Total flight activity / 30 seconds at West Branch Canal Creek over a four day period.

However, the suite of tools provided with the numerical analysis and graphical display programs included on the CD-ROM (and described in Appendix A) proved to be sufficient to flag short-term disturbances. For example, on August 2, at Churchville, we found a consistent interval between the response peaks extracted by the DER*DIFF utility as shown in Figure G.16. The algorithm is described in the SITEVIEW program manual on the CD-ROM. Checking the log books, we found that one of our technicians was inspecting the hives. As this person moved from hive to hive, the plot reveals that the colony check disturbed colonies in order from #1 to #7, having the greatest impact on colonies #5 and #7.

On September 1, 1997 at the Churchville reference site, colony #1 swarmed. Strong colonies reproduce by swarming, although this swarm left the hive rather late in the season. While this was not the first time a swarm had been captured by bee-counters, it was unique in that the colony had been monitored over a long period of time, and that the swarm was a natural event, rather than one produced by beekeeper intervention. This colony's flight activity was monitored for several months both before and after the swarm occurred. Colony #1 began to reduce its flight activity five days prior to the swarm. The change could be seen both in an increased C.V. for flight activity among the colonies and by the Tier 2 graphical display of the data. The site-wide PRC dropped to 90% because the PRC for colony #1 dropped to 43% the day of the swarm. Normally, PRC is expected to be in the 94% to 98% range. The day after the swarm occurred, flight activity dropped below 5000 round trips per day and stayed at this level for the next 3 weeks until the hive was supplemented with more bees. The contribution to total site-wide flight activity from colony #1 remained below 4% for a three week period following the swarm.

The time resolution at which data is captured and integrated by the bee-counter system allowed close inspection of flight activity patterns during the swarm. Figure G.17 demonstrates that extremely high levels of flight activity occurred during the swarm of colony #1. Over a 35 minute period, 10,068 bees left the colony, while the total flight activity for that time was 13,782 (i.e., while most bees were leaving the hive, others re-entered it). Over the next two hours, 438 bees returned to the colony with most returning within a period of only a few minutes. This return probably was due to bees that failed to move on when the swarm finally left the apiary. Typically, a swarm will leave a hive, regroup nearby, then later move to another site. Any bees left behind would be expected to return to the hive of origin.

The high level of flight activity attained during the swarm presented an excellent test to the capabilities of the electronics system and the physical limitations of bees traversing the counters. For a 35 minute period, a single counter is theoretically capable of detecting 1,176,000 bees. The number of bees traversing the counter during the swarm represented only 1.2% of the counting capability of the electronics. It is possible that the number and size of the bee-passageways could limit the amount of bees exiting at any given time. However, inspection of the counter passageway flight activity indicated that during the swarm all passageways were used at similar levels. A maximum of 469 bees traversed the
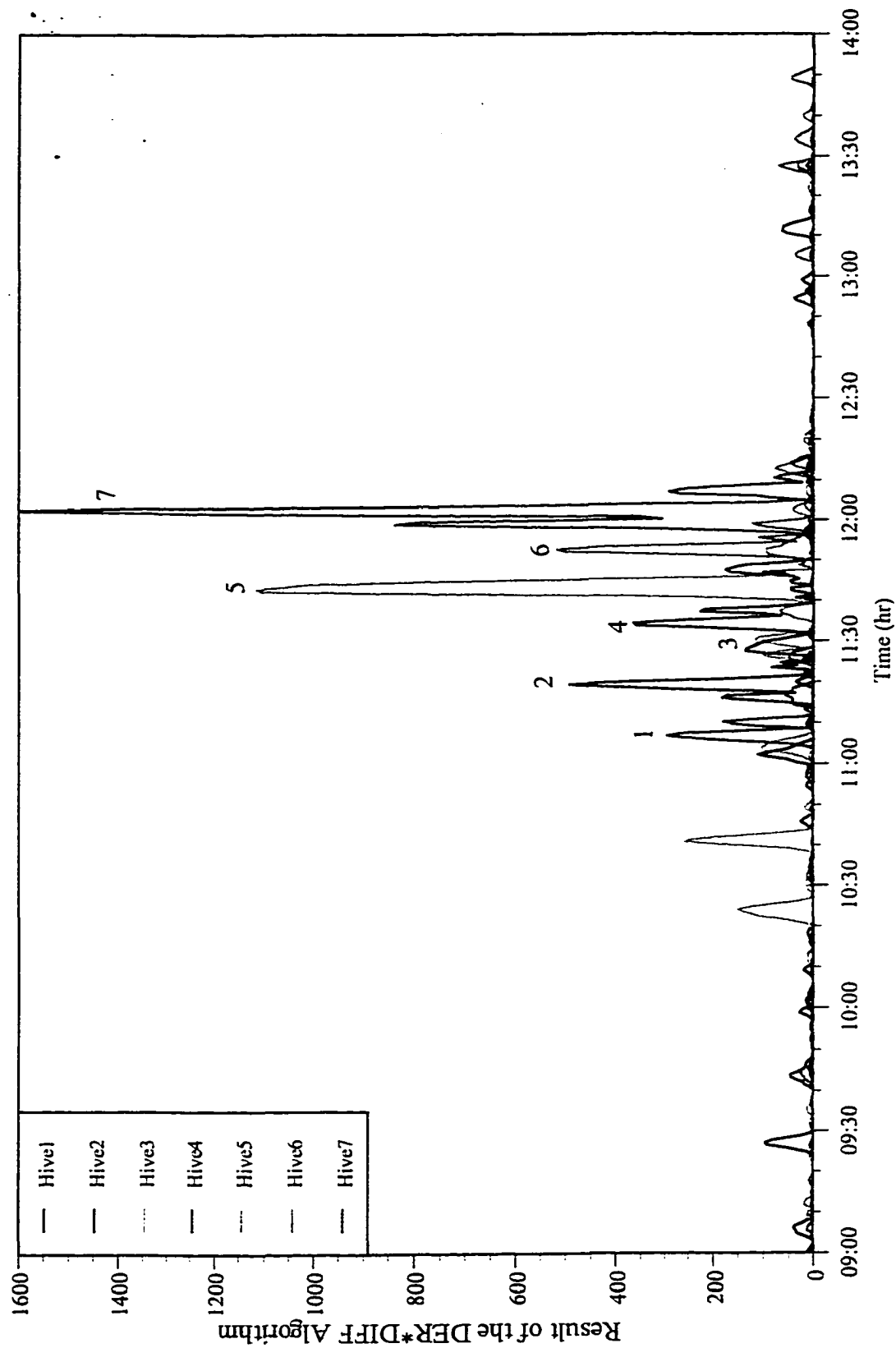
Figure G.16. Sensitivity of flight activity to hive maintenance. Peaks indicate periods of hive maintenance as the beekeeper's colony check progressed from hive 1 to hive 7.
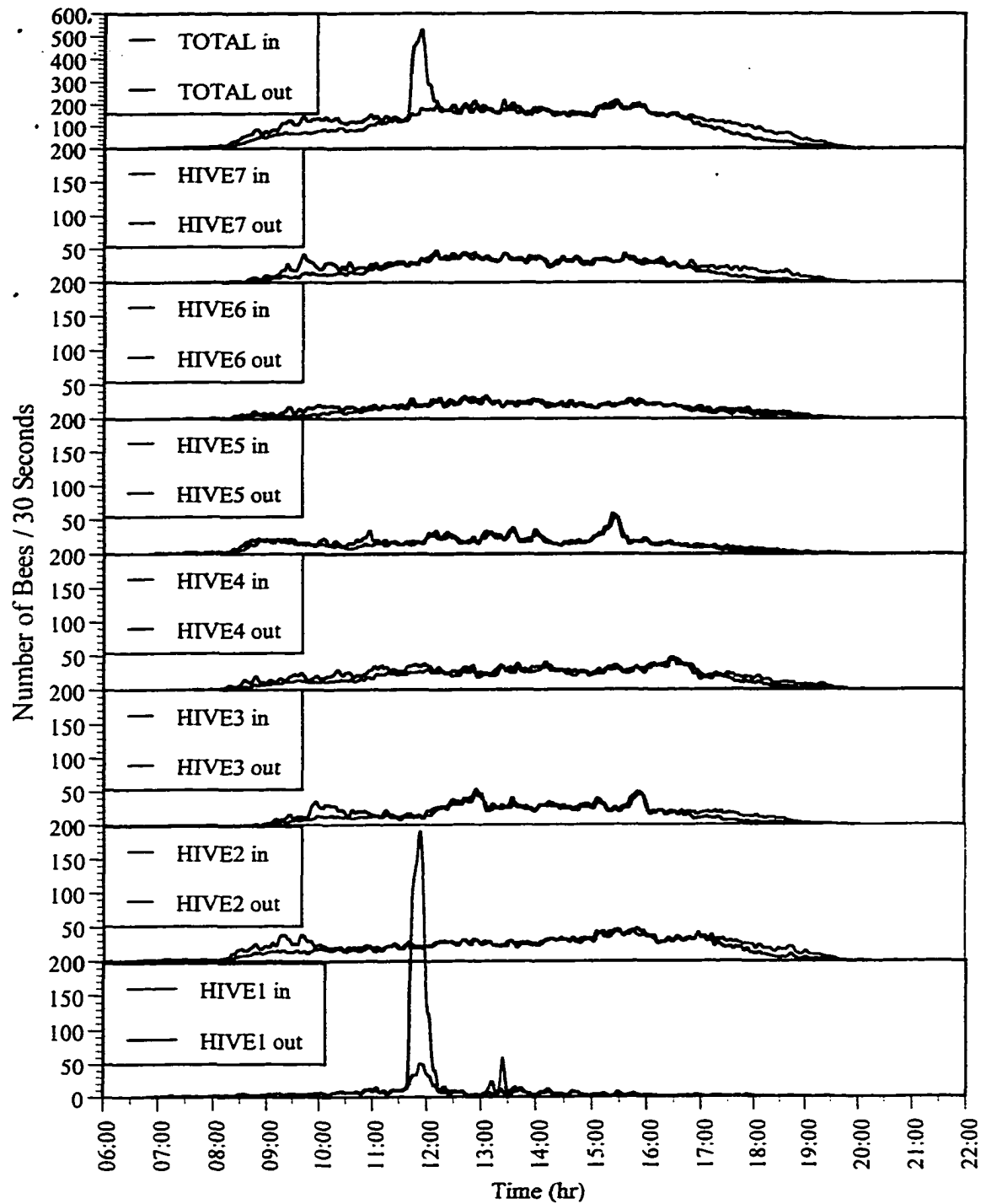
Figure G.17. Total flight activity / 30 seconds, Churchville, 09/01/97. Colony 1 swarmed at 11:30. More than 10,000 bees departed, about 400 returned by 13:25.

counter during a 30 second period during the swarm. This indicated that each bee required just less than one second to traverse a counter passageway. Based on this rate of passage, the maximum number of bees that could traverse a bee-counter during a 15 hour monitoring period is 844,200. This number is far less than the theoretical limit of the device.

Because day length, weather conditions, and the water and floral resource needs of colonies vary greatly between different geographical locations, we have included Figure G.18 to demonstrate the differences between the start, duration, and end of forager flight on the same day at Fort Missoula, in Montana, and the APG sites in Maryland. Forager activity at Fort Missoula did not begin until about 9:00 hr in Montana, whereas all of the Maryland sites exhibited some flight activity by 6:30 hr. Flight activity continued at a high rate until 21:30 hr at Fort Missoula, while the Maryland sites shutdown between 18:00 and 20:30 hr. A sudden increase in bees returning to the colonies late in the day at the Churchville reference site was due to evening rainshowers. Typically, light levels remain higher much later in the day in Montana than in Maryland, which probably accounts for bees still flying at 21:30.

The shape and variance of the Fort Missoula flight activity pattern in Figure G.18 differs from that of the Maryland sites. The Fort Missoula site is adjacent to a field of knapweed, which the bees foraged extensively during the month of August. Figure G.19 demonstrates the seasonal change in flight activity pattern brought about by the knapweed blossom. On July 23, bees were not foraging on knapweed. The flight activity pattern was characterized by a broad hump that covers the entire day. Less than two weeks later, when knapweed was in blossom, the flight activity pattern had a double-hump shape with little variance throughout the day. One month later, while knapweed resources were still sparingly available, the double-hump shape was barely discernable. Also, the flight activity variance had returned to the level observed before the onset of the blossom. The low variance in flight pattern during August and early-September is the result of results of bees being stimulated to actively forage plentiful resources in close proximity to the hives. Diminishing floral resources, more sparely distributed, would be expected to result in reduced and more variable flight patterns, such as were seen by the beginning of September.

An earlier example demonstrated that our analysis routines could track the disturbance of a beekeeper opening hives for inspection. A much less invasive experiment was designed to determine the impact, if any, of someone standing in front of the hive, blocking the entrance. A beekeeper, dressed in white coveralls, a hat and veil, blocked the flight path of incoming bees by standing 5 feet to the front of the counter entrance for 5 minutes and then stepping away.

Figure G.20 demonstrates the impact of this experiment on normal flight activity patterns. Blocking the flight path was clearly characterized by a decrease in bees entering the counter, although it had little or no effect on the number of bees leaving the hive. When the beekeeper moved away from the flight path, a sharp increase in bees entering the counter

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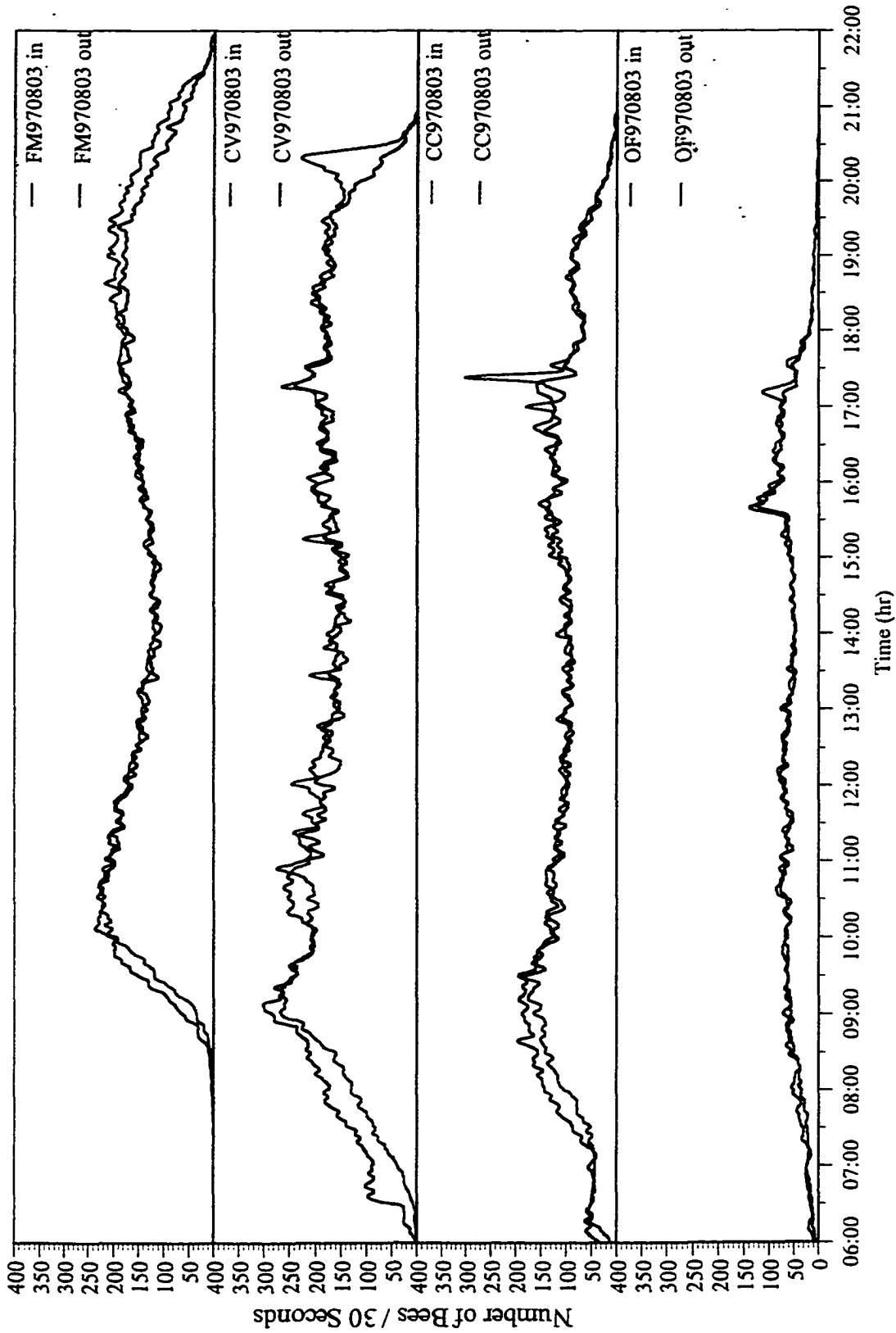
Figure G.18. Comparison of Maryland and Montana forage patterns. Bees fly later in Montana.
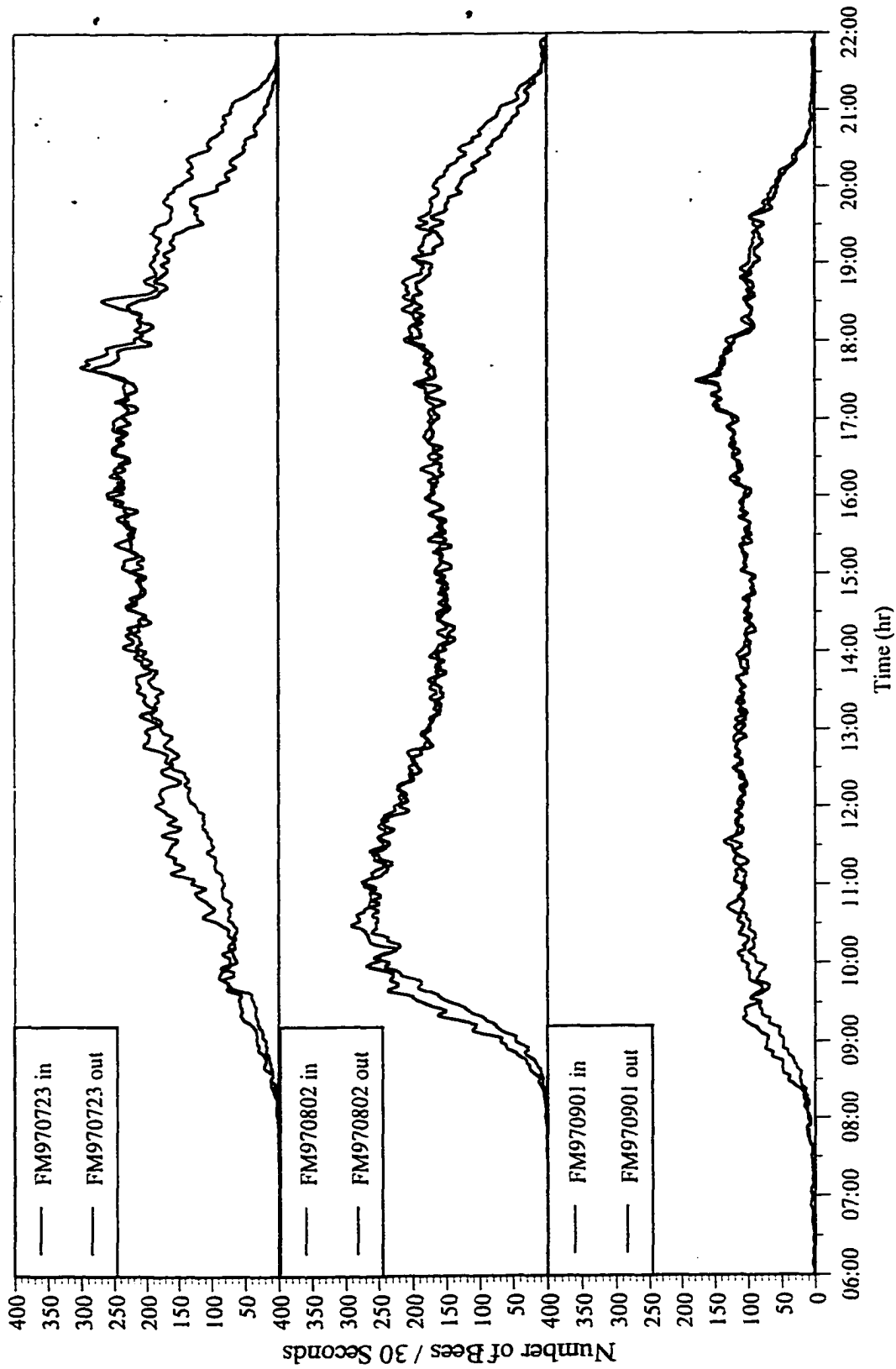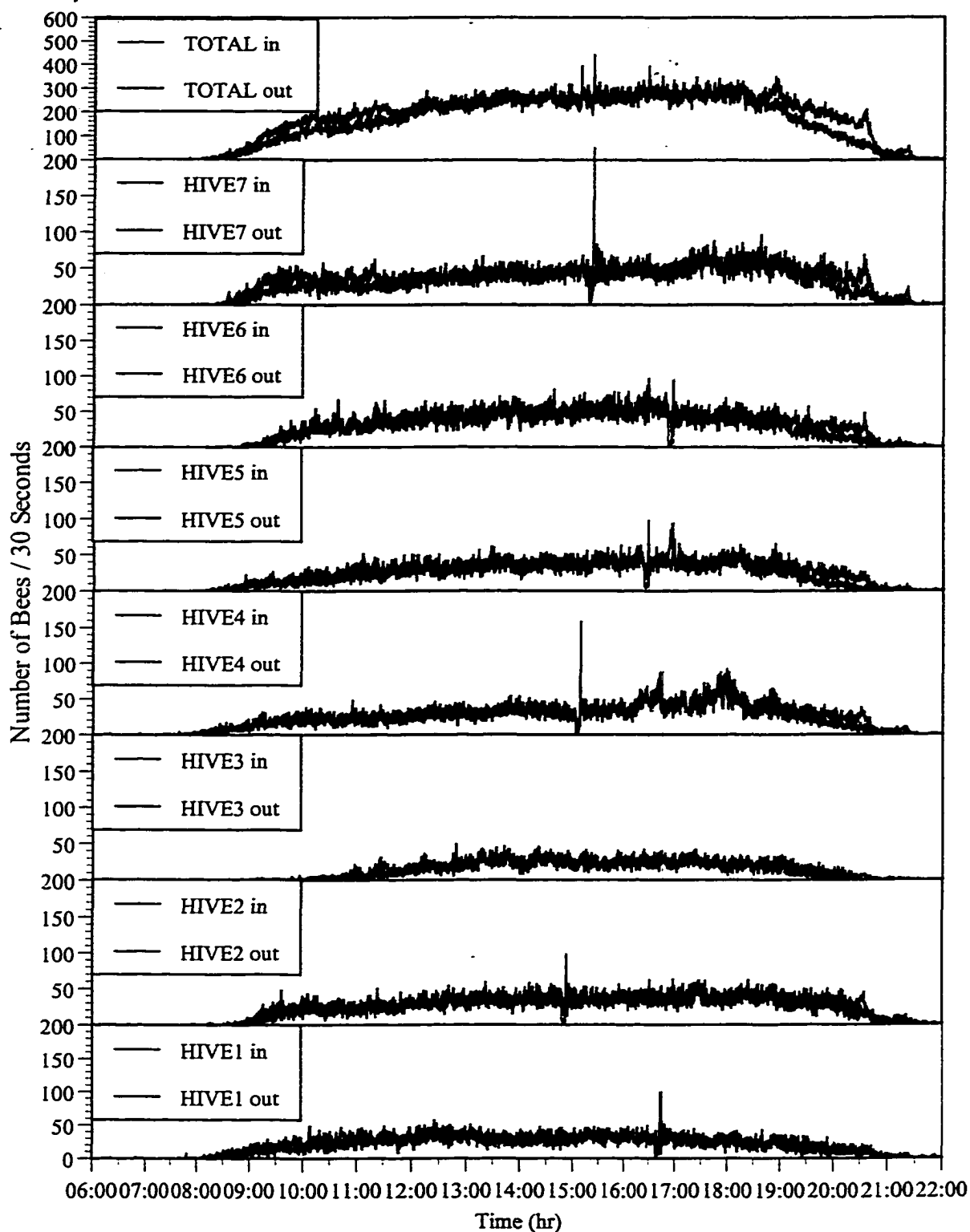
Figure G.19. Comparison of Montana forage patterns relative to floral resource availability.

Figure G.20. Total flight activity / 30 seconds, Fort Missoula, 07/21/97. Beekeepers blocked the entrance to selected hives during the afternoon causing spikes in activity. Thin lines denote drift between adjacent colonies 1 and 4, and 5 and 6.

occurred. Visual observations confirmed that a cloud of bees built up behind the beekeeper, then landed when the person moved away. In some cases, such as between hives #5 and #6, and #1 and #4, drifting occurred into adjacent hives.

Derivatives of flight activity patterns were used to better examine drifting. Drifting is defined as instances where bees leave one hive and return to another hive. The results of examining the filtered derivatives of flight activity patterns are shown in Figure G.21. It is clear (indicated by a thin line) that drifting occurred from hive #6 to hive #5 as well as from hive #1 to hive #4. The total derivative (top plot) is calculated as the sum of the derivative from each hive. The derivative of the total activity (not shown) produced barely discernable peaks because the contribution to total flight activity from each hive was offset by the others. The DER*DIFF algorithm also was applied to this data to test its ability to discriminate short term events. Figure G.22 indicates that drifting also occurred from hive #5 to hive #6 and that the drifting from hive #1 to hive #4 was not as intense as suggested by derivative analysis.

Total flight activity failed to produce readily discernible peaks via the DER*DIFF analysis, The brief changes in flight activity created by blocking the flight path of bees to single hives had little effect on the total site-wide flight activity. The top plot in Figure G.22 is based on the DER*DIFF analysis of the total flight activity, not the sum of the DER*DIFF analysis for each of the hives.

The most striking difference between Figure G.21 and G.22 is the lack of noise in Figure G.22. Filtered derivative analysis produces noisy results because of the variability of short term changes in flight activity. The DER*DIFF algorithm minimizes this noise by enhancing sensitivity to differences in bees entering and exiting the counter for a given period. The noise minimization is what makes the DER*DIFF analysis powerful for detection of short-term events.

Anomalous flight activity patterns were detected in 1997 on June 22 at Old O Field, July 28 at Fort Missoula, and August 5 at Fort Missoula. These patterns consisted of a sharp drop in the rate of bees leaving the counter, followed by a decrease in bees entering the counter. The lack of a sudden increase in the number of bees returning to the hive distinguishes these activity patterns from those induced by the approach of a rain storm. Because none of the regularly scheduled chemical sampling dates coincided with these unanticipated responses, these changes could not be evaluated with respect to potential exposures to chemicals.

Anomalous flight patterns were first detected on 28 July, 1997 at the Fort Missoula site as shown in Figure G.23. This was the same day in which methanol was sprayed at the entrance of six of the seven counters, although the application of methanol preceded the response by several hours. A response was detected from all seven colonies, even though only 6 were treated with methanol.
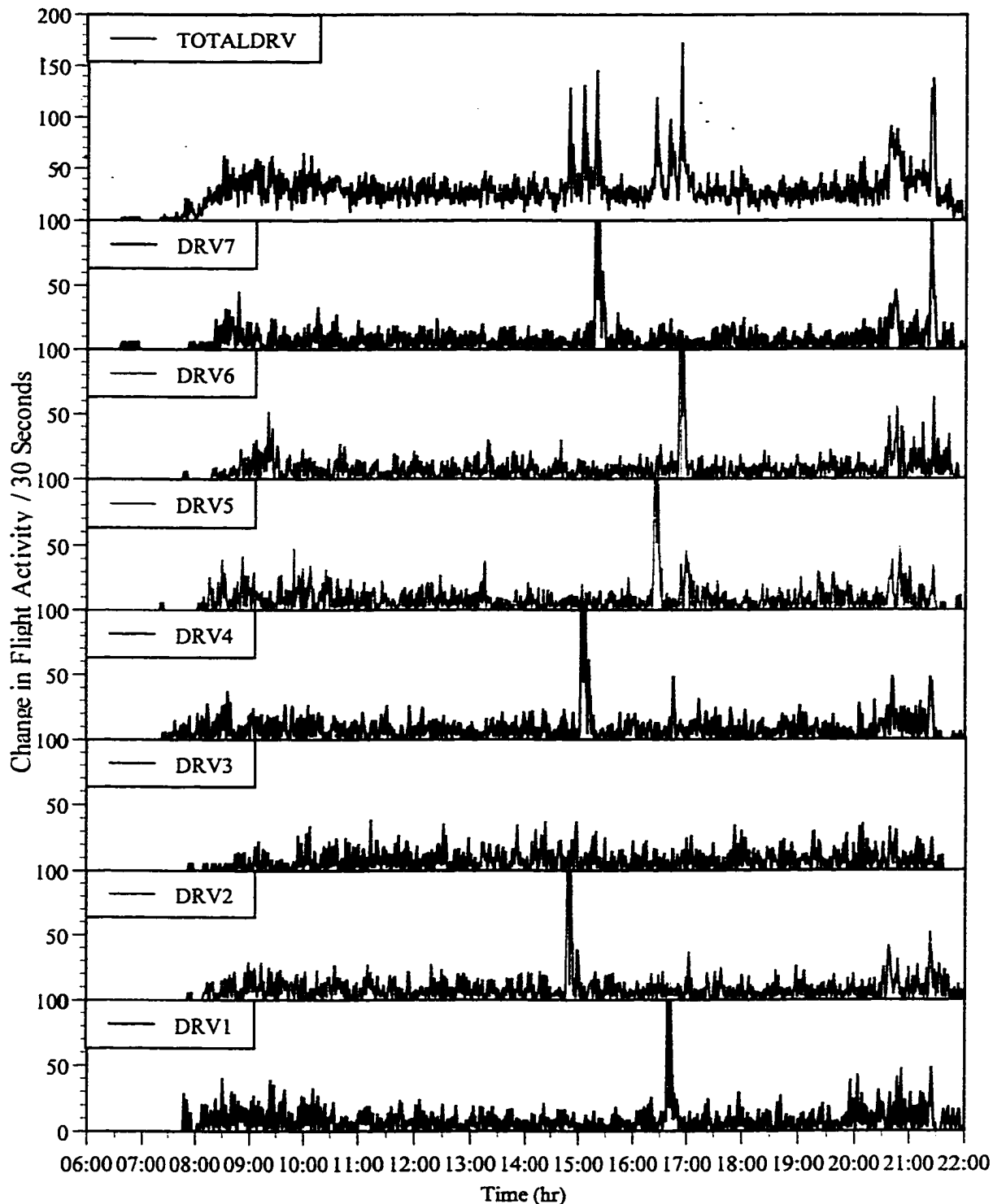
Figure G.21. Derivative of bees entering counters for colonies at Fort Missoula, 7/21/97. Beekeepers blocked entrances by standing in front of selected hives at different times. The solid line indicates potential drift from colony 6 to 5 which are approx. 10 feet apart. The second vertical line indicates potential drift from colony 1 to 4. Colony 4 is approx. 20 feet behind colony 1. Sharp peak at end of day indicates rain event.
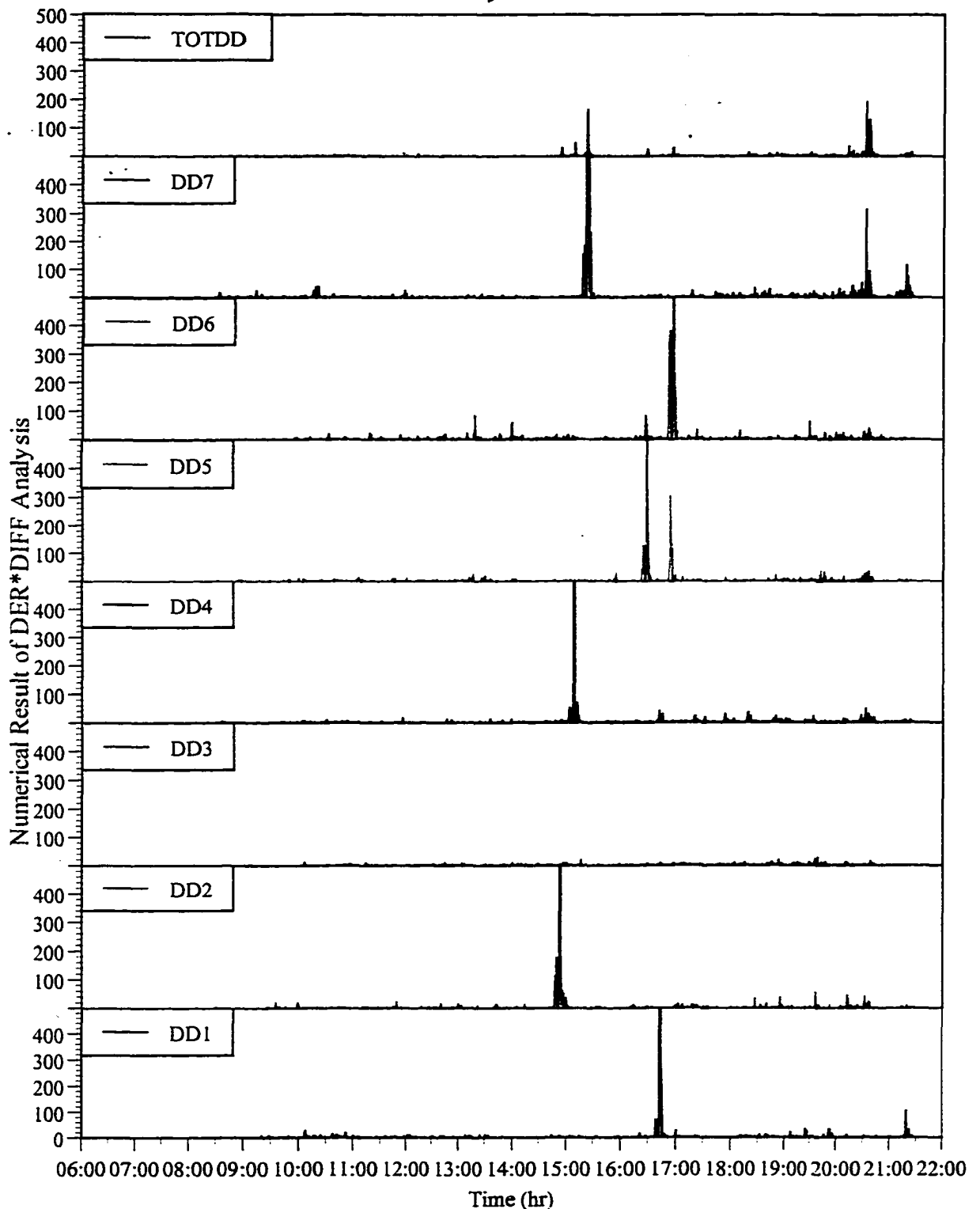
Figure G.22. DER*DIFF analysis, Fort Missoula, 07/21/97. Large spikes are due to beekeepers standing in front of hive entrances. Note drifting between colonies 1 and 4 and 5 and 6.
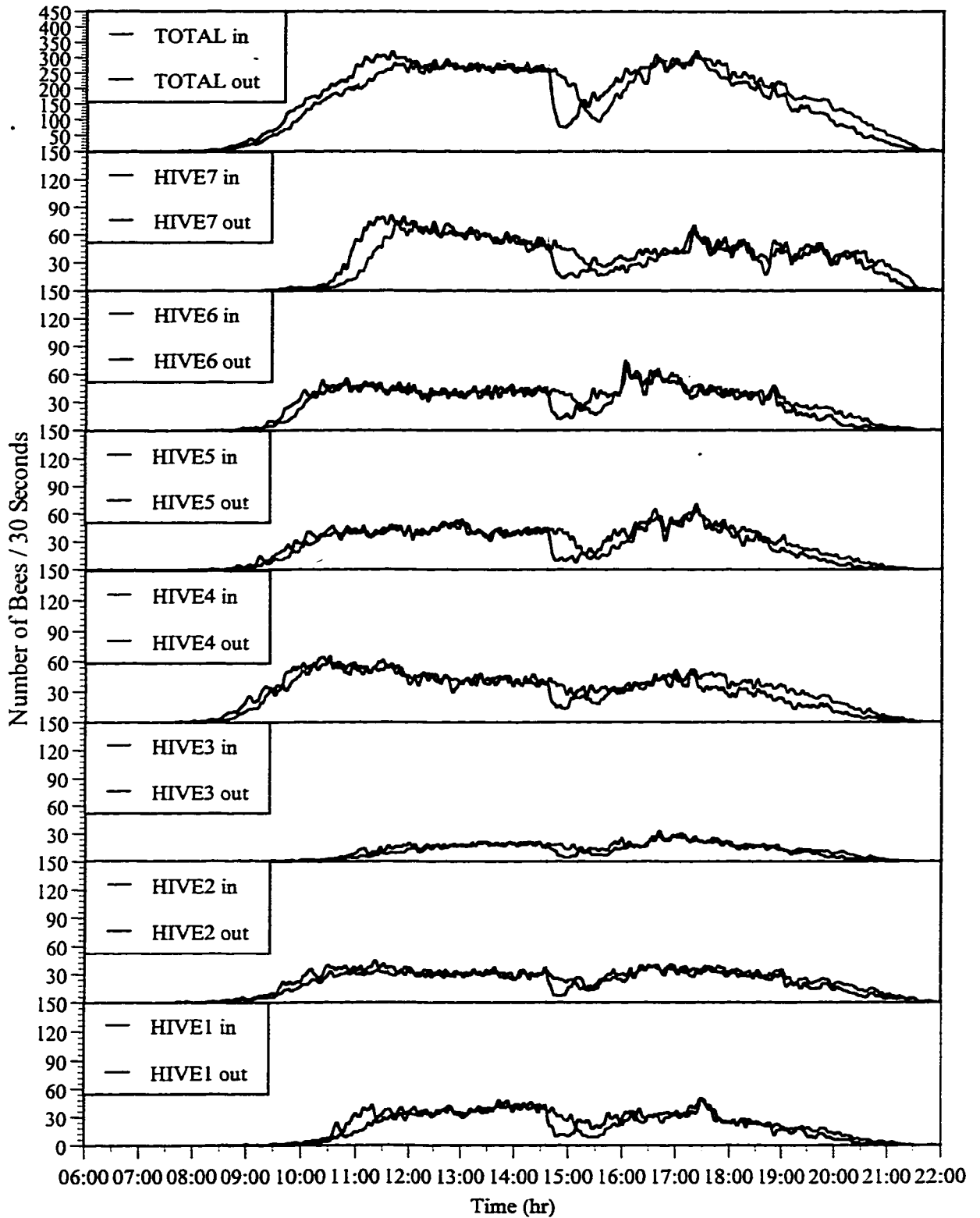
Figure G.23. Total flight activity / 30 seconds, Fort Missoula, 07/28/97. Post-methanol treatment response.

Another event was detected on 5 August, 1997 at Fort Missoula, as shown in Figure G.24. This event occurred after a cleaning agent Pine Sol™ was used near the apiary to clean materials. Neither of these changes in flight activity occurred at the time when the contaminant (i.e., methanol or Pine Sol™ was first introduced into the air in the apiary. A search of a USGS earthquake database indicated no seismic activity in the region. Also, there was no detectable change in weather patterns that should have produced the observed response.

These anomalous patterns were compared to the rest of the flight activity archives, and two similar events were found for APG sites. The first was the smoke event of 8 August, 1996 at the West Branch Canal Creek site, where beekeepers used smoke to calm the bees before working them. A plot of this data was included in the 1996 Annual Report. The second match occurred 22 June, 1997 at the Old O Field site as shown in Figure G.25.

Figure G.26 compares the responses garnered from events of known and unknown origin. The first plot shows the sitewide response to injection of smoke into hive entrances performed 8 August, 1996 at West Branch Canal Creek. The second plot demonstrates a response to an unknown stressor on June 22, 1997 at Old O Field. The third and fourth plots are from events that may have been caused by exposure to methanol (28 July, 1997) and Pine Sol™ (5 August, 1997), respectively. It is clear from the data that these responses are similar in nature.

With 3 of the 4 days following known releases of contaminants into the apiary, we believe that although the initial exposure may not have induced an immediate response, the exposures due to buildup of contaminants in the hive atmosphere caused some stress to the bees, which was manifested in the data. Without hive atmosphere chemical data for these subsequent days, it is difficult to assign the exact cause of the anomalous flight patterns. The potential for external stressors to induce a delayed response will be an important focus of our 1998 studies.

G.7 Results of Tier 4 Diagnostic Evaluations of Flight Activity Data

Tier 4 analysis of flight activity is a diagnostic tool. It is used to trouble shoot the counter equipment. The analysis uses suboptions 10-15 of Option 10 of the program SITEVIEW program (on the CD-ROM). This program allowed quick evaluation of counters and channels to find counters that were either defective or needed re-alignment. This provides the tools needed for systems and performance audits of the counters.

G.8 Summary of APG Results

The Tiered Analysis approach was applied to the monitoring trials at APG sites in 1996 and 1997. The results are discussed here in the context of other measures of colony performance and of the chemical exposure data. There was a general decrease in variability
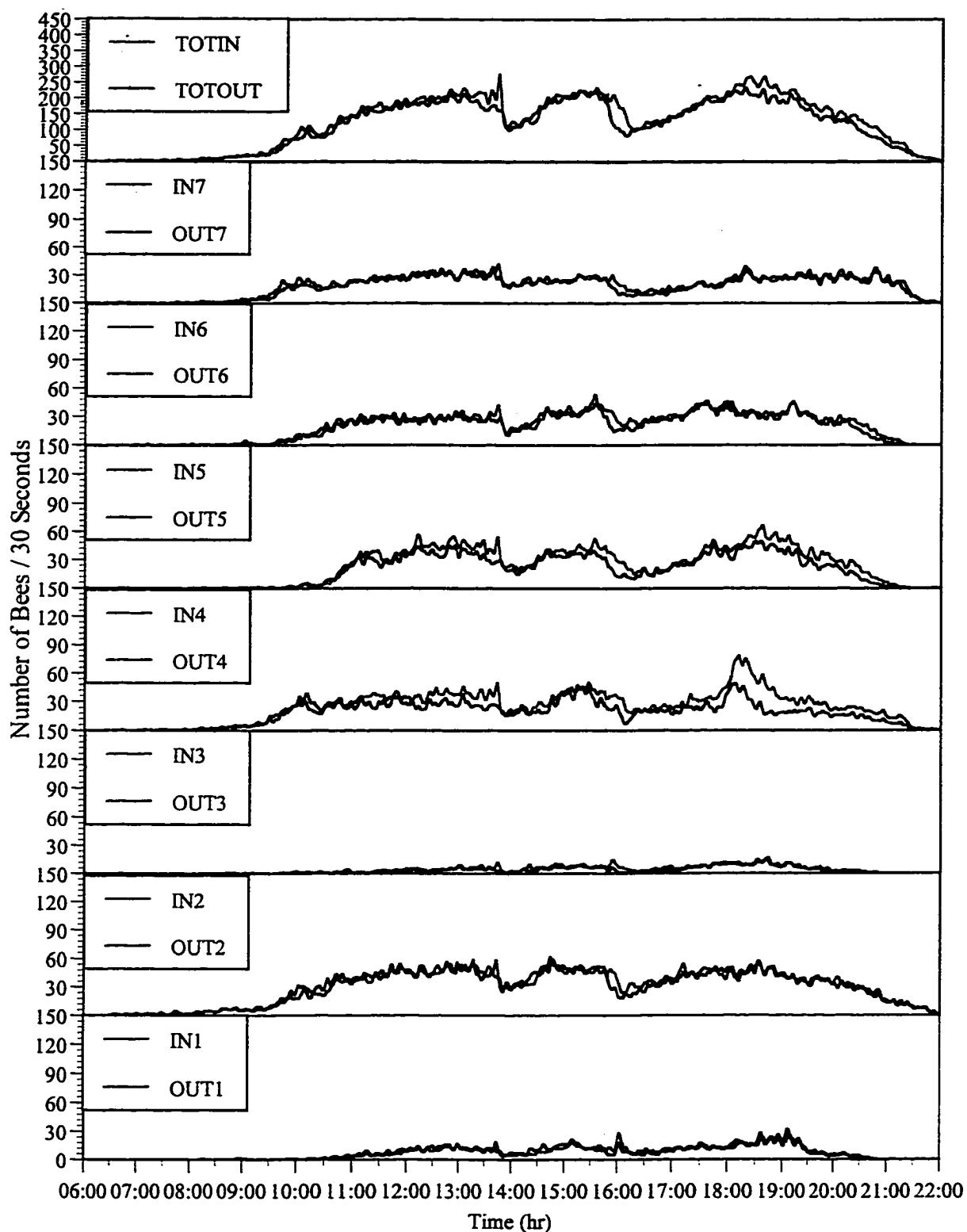
Figure G.24. Total flight activity / 30 seconds, Fort Missoula, 08/05/97. Colony response to exposure to Pine Sol™.
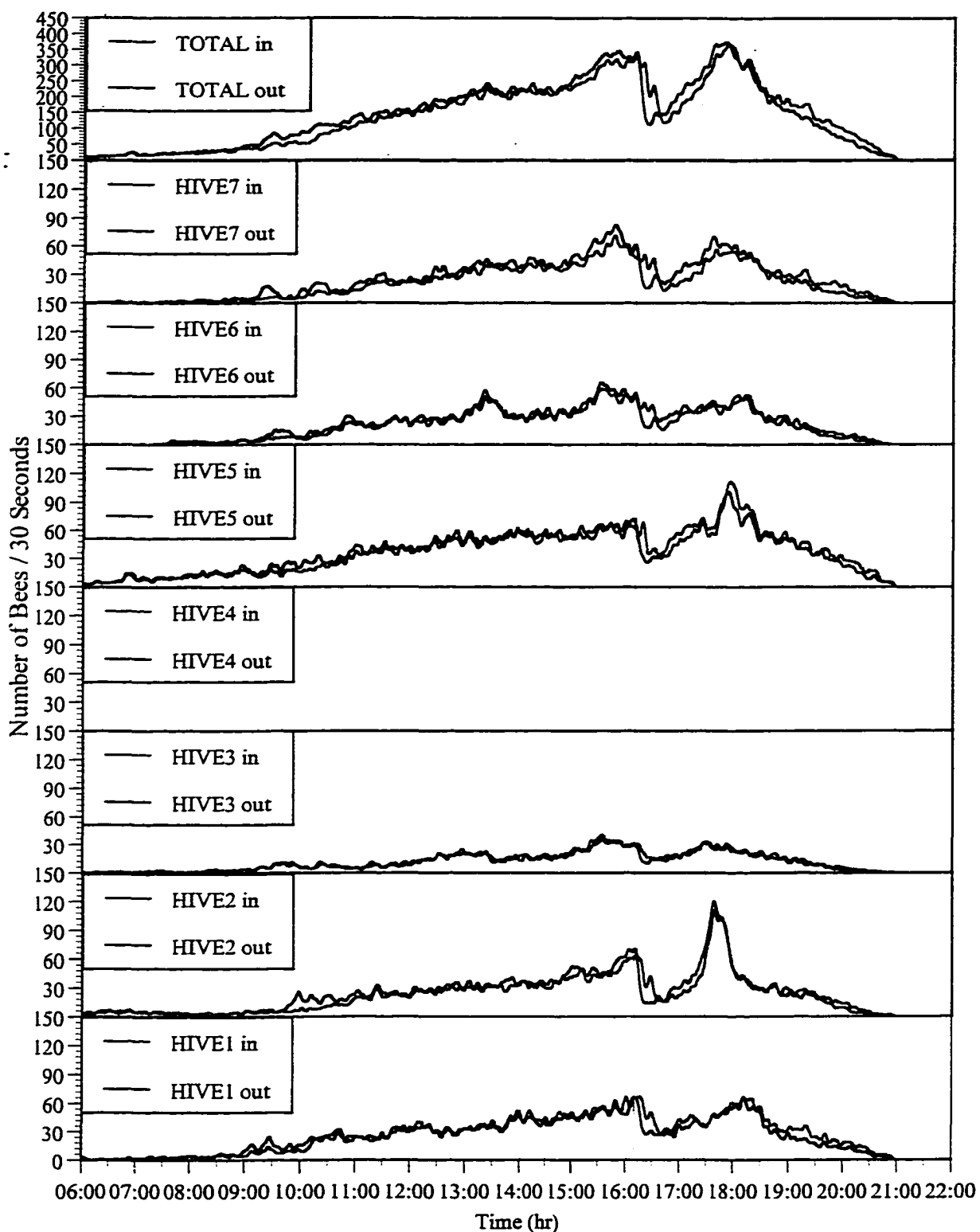
Figure G.25. Total flight activity / 30 seconds, Old O-Field, 06/22/97. Anomalous event at 16:20 matches the response pattern found at Fort Missoula hives on 07/28/97 and 08/05/97 when elevated levels of contaminants where present in the apiary.
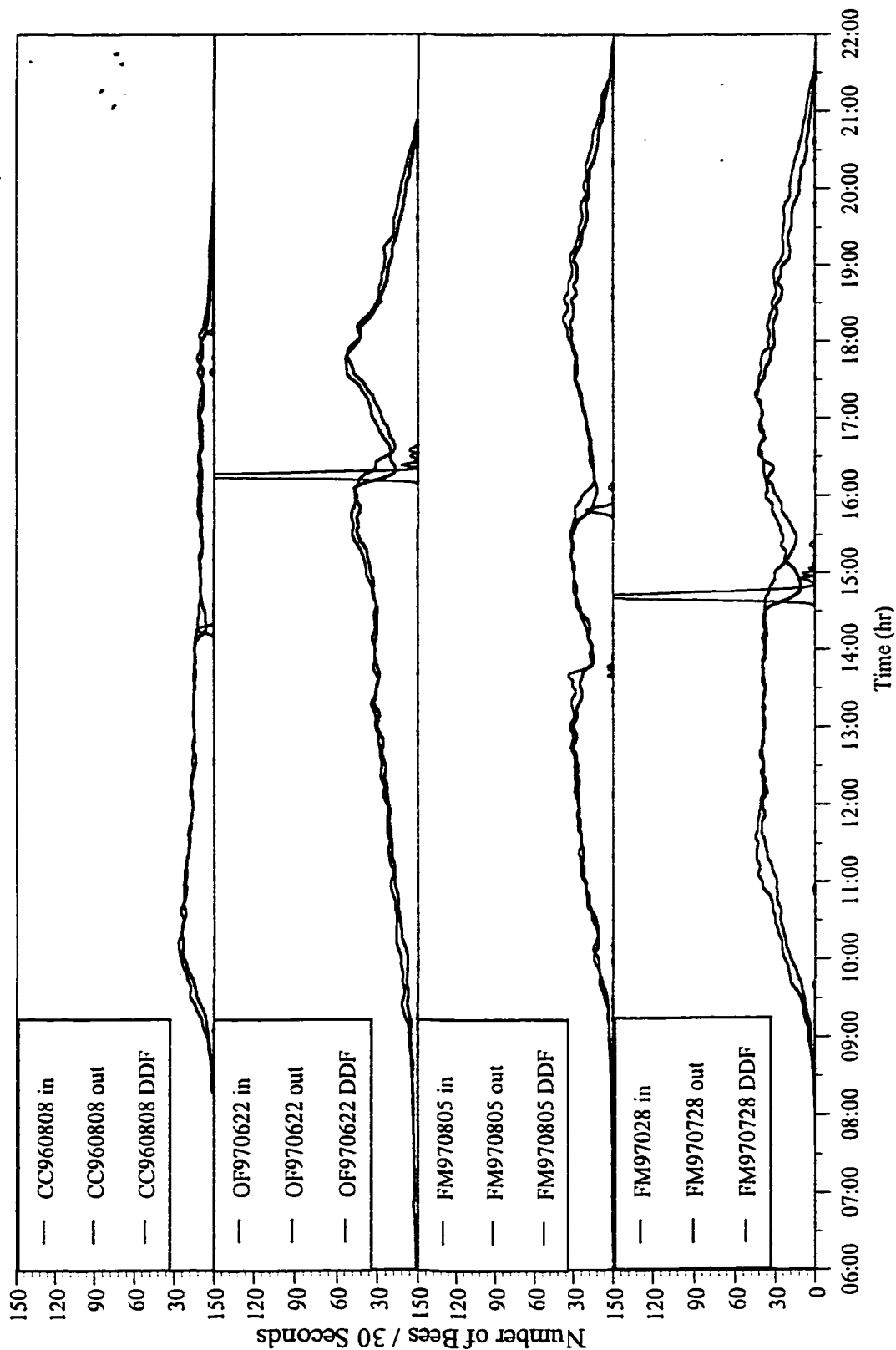
Number of Bees / 30 Seconds

—	CC960808 in
—	CC960808 out
—	CC960808 DDF

—	OF970622 in
—	OF970622 out
—	OF970622 DDF

—	FM970805 in
—	FM970805 out
—	FM970805 DDF

—	FM97028 in
—	FM970728 out
—	FM970728 DDF

Time (hr)

Figure G.26. Detection of anomalous events using the DER*DIFF algorithm.

in the flight activity among hives at each of the Maryland sites in 1997 compared to 1996. Because of the presence of sister queens in all of the colonies in 1997, it is possible that the genetic makeup of the queen was partially responsible for this decrease in variability in flight activity. In a Fort Missoula (FM) test, some of bee colonies had sister queens and others had queens of mixed origin. The FM results indicated that the use of closely related queens reduces variability among the colonies at a given site. Based on these results, we believe that the use of sister queens in all of the Maryland colonies was partly responsible for reducing within site variability at APG sites in 1997. Also, we deployed the Maryland colonies in May and June of 1997, compared to July and August in 1996. By placing the colonies on-site earlier in the season, the bees were able to benefit from the early season nectar flows that are characteristic of this geographical area. This allowed the populations to increase in size as well as to harvest and store more nectar and pollen.

In early September 1996 a hurricane swept through APG. Immediately after the storm, a sharp drop in activity occurred at Old O Field. A two day rain in late July of 1997 produced similar results, with decreased flight activity levels occurring at both West Branch Canal Creek and Old O Field. The duration and severity of the decrease in flight activity at Old O Field was much greater than that observed at West Branch Canal Creek. Flight activity at Churchville also dropped during the rain event, but quickly returned to normal. Section 3 of this report describes the exposures to chemical agents at all of the bee monitoring sites. In both 1996 and 1997, increased levels of volatile organics were found in the Old O Field hives during the periods of decreased flight activity. The levels of these chemical agents were about an order of magnitude higher in 1996. Sudden increases in the levels of chemicals inside the hives following rain storms suggest that the bees may be exposed to these agents via water, possibly as an oily film on the surface of puddles. Chemical uptake experiments conducted in Missoula in 1997 confirmed that this is a route of entry into the hive for organic solvents.

The Tier 1 indicators, TFA, C.V., PRC, and ANL (Table G.3), were all used to provide metrics for comparing sites to one another. In 1996, all of these metrics showed Old O Field to be highly stressed. Although the colonies at Old O Field showed a marked improvement in C.V.s values in 1997 compared to 1996 (50-130%), the C.V. values for 1997 at Old O Field were still higher (25-75%) than those at West Branch Canal Creek (20-50%) and at the Churchville reference site (15-50%). The PRC metric shows improvement in 1997, compared to 1996, for both West Branch Canal Creek and Churchville. The changes were similar in nature to changes in the C.V. metric. In 1997 the percent of bees returning (PRC) each day to their hives ranged from 94% to 98% for both Churchville and West Branch Canal Creek. These levels were similar to those also observed for the sister queen colonies in Montana. We consider these to be acceptable levels of attrition in a healthy bee hive. The PRC for Old O Field in 1996 ranged from 90% to 102%. The 90% return levels were considered to be somewhat low. This metric improved to 94% to 104% in 1997. All of the Maryland hives exhibited return rates of 94% or better in 1997, compared to values as low as 90% in 1996. Percent return values were observed at Old O Field in both years, and at

Old O Field and West Branch Canal Creek in 1996. Only Old O Field, among the Maryland sites, demonstrated values in excess of 98% in 1997. Values in excess of 100% indicate drifting among the hives at Old O Field. Our data suggests that drifting becomes more evident when population size and flight activity varies greatly among hives. The general decline of the bee populations at Old O Field, severe in 1996, and moderate in 1997, reflected a wider range of activity levels and colony sizes at Old O Field than at any of the other sites. The ANL results for Old O Field in both years were among the most variable for all of the APG area sites. Those for the Churchville reference site were the lowest.

Basically, the trends listed in Table G.3 indicate improvements for colonies at West Branch Canal Creek and the Churchville reference site in 1996 compared to 1997. At Old O Field, some improvement occurred, but not to the extent as the other sites. Over both years, bees at West Branch Canal Creek did nearly as well or as well as the colonies at the Churchville reference site, based on all of the Tier metrics, colony core temperatures (which address the colony's ability to thermoregulate), and levels of exposure to bioavailable environmental contaminants. Two of the four survey colonies were lost at J Field. These colonies were deployed in early June. The electronic hives were moved onto J Field toward the end of the season. This trial provided information about end of season variability and established that the electronic hives could be operated at this site. The trial was not of a long enough duration to adequately assess colony performance as affected by chemical agents at the site. However, the data provided a good basis for resuming the study at J Field in 1997. The loss of two of the four survey colonies in 1996 warranted deployment of the real-time monitoring system at J Field throughout the 1997 season.

The combination of abnormally high C.V., elevated PRC, and low flight activity in August, 1996, following a heavy rain event in late July, indicate that the colonies at Old O Field were exposed to external stressors. Exceeding any of these individual metrics is usually cause for a colony check. The colonies at Old O Field managed to exceed several metrics simultaneously, indicating that the hives were displaying symptoms of stress that could affect colony health.

G.9 Summary of Colony Responses to External Stimuli

Most of the biological responses to external stimuli were detected by the Tier 3 analysis. It can be concluded that most of the events that occurred in response to a biological stressor did so quickly. The timing of these responses varied from immediately after the stressor was introduced to several hours later, as was the case with methanol exposure.

Six different abiotic and biotic contributions to flight activity behavior were explored. These included weather events, seasonal changes, geographical differences, chemical exposure, hive maintenance, and swarming. Rain events have been characterized as having a large peak in the number of bees returning to the hive followed by an immediate drop to almost no bees entering or leaving the hive. Seasonal changes are primarily due to changes

in temperature, rainfall, and forage resource material. Typically, flight activity increases during the warmest months and when forage material is abundantly available. Geographical differences are evident in the length of the forage period and the general daily patterns of flight activity; i.e., whether the activity remains at a constant level throughout the day, or variations in the activity occur.

Behavioral responses to chemical exposures have been characterized by smoking the hives to calm the bees, by experimental exposure to methanol, and the fortuitous Pine Sol™ incident. All of these events produced a drop in the bees leaving the hive followed by a matching drop in bees entering the hive. The DER*DIFF analysis method was devised to seek out these events by producing a large signal whenever the characteristic 'phase-shift' occurred. Hive maintenance affects flight activity by producing sharp, brief peaks in flight activity immediately after the event is concluded. Swarming produced a large enough change in flight activity for the swarm hive to have been detected by almost all of the analysis methods. In fact, the swarm produced the most extreme conditions of flight activity over a 40 minute period.

Because many external events induce rapid responses, detection of these responses and appropriate action such as initiating colony inspection or chemical sampling needs to be accomplished in real-time. To date, our systems have proven to be capable of monitoring colony behavior in real-time with the computers at each field site. However, transmission, access, and evaluation of the data at a remote site has relied upon physical exchanges of data storage devices such as shipping a data cartridge from Maryland to Montana. The ability to deliver data in real-time for rapid detection of colony reactions and for cause and effect analysis is crucial to the success of the concept. That capability was realized in our laboratory during the last six months.

G.10 REFERENCES

Bromenshenk, J.J.; Smith, G.C.; King, B.E.; Seccomb, R.A.; Alnasser, G; Henderson, C.B.; Loeser, M.R.; and C.L. Wrobel. 1997. New and Improved Methods for Monitoring Air Quality and the Terrestrial Environment; Applications at Aberdeen Proving Ground—Edgewood Area. Contract No: DAMD17-95-C-5072. Performance Organization Report # GESL-96-01-ANN/DEF for U.S. Army Medical Research and Materials Command, Fort Detrick, Frederick, MD 21702-5012. 176 pp.

Appendix H

7HV130.exe   Manual 1.5bw for field use, 5/26/98

H.1   Definitions:

Toolbox - this is the large box that houses the computers and the interfaces

Interface box - the black box beneath the computer running the counter software. It has a label on it that says "Iotech Digibook/72"

Interface lights - the interface box has 4 LEDs that could light up. When the interface box is first powered up, the first one labeled "POWER" will light up. When the computer prepares to communicate with the interface box, the second light (labeled "EXPANDED I/O MODE") will light up. When the computer is getting data from the interface the third light (labeled "P1-P2-P3 ACTIVE") will light up. This is normal operation. The fourth light should not light up, if it does, don't worry about it.

Counter - The device that counts the bees.

Condo - the bees are in here, each condo has a counter

PCMCIA - this is how the computer (the TI) talks to the interface box.

TI - the manufacturer (Texas Instruments) of one of the computers used for the counters. This is a 75 MHZ Pentium. This is the only computer that requires the PCMCIA card interface to connect to the Iotech Digibook/72 interface. AST recently bought out TI's laptop lines.

TI Hard Drive - The TI has a hard drive that can occasionally fails to boot up after the computer has been moved. If this happens (you don't need to move it, so it probably won't), shut down the computer, remove all the connectors from the computer and gently shake the computer. Reattach all the connectors and try to start it up again. This usually works. Recently (August 1997), this drive has produced corrupt data sets.

AST - Another computer used for the counter interface system. This is a 100 MHZ Pentium. It has a history of display problems but it should be OK after the last fix (Spring 1997).

Compaq - This computer has a hard drive and driver card replaced in June of 1997, working fine now.

I will display the prompts here in `Courier` text and the responses you need to enter in **BOLD**, any extra explanations will be in BLACK.

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H.2   Preliminary Setup and Installation of Software Updates:

1)   Get all the connectors set up properly:

The TI connects to the interface box via the PCMCIA card and cable. Make sure that the cable is connected properly to the PCMCIA card, it is keyed to only go in one way. All other computers connect to the interface box via a DB25 parallel port cable. Seven cables from 30 to 60 feet in length are used to connect the interface box to the condo units. It is strongly recommended that all cables be anchored at each end to prevent accidental disconnection. The number taped to each 37 pin connector on the interface box is the number that the connected condo will be designated in the data files. All power connecters used for the interface and computer should be identified with tape to prevent unnecessary data loss.

2)   Turn on the Iotech interface and the computer.

It should be pretty easy to find the power button on the computer. Providing detailed instructions for that would be difficult considering the number of computers being used. The Iotech interface switch is on the top "black box" on the same panel as the interface lights (see above). The last thing the TI should do is look for the PCMCIA card, once this is done, you will see the "c:\" prompt. The AST seems to be working OK for now.

3)   Check the computer's clock. Set it to the local time. This is important for analysis. Think ahead.

4)   If the computer has been shipped from Montana (CP005), everything is installed and you are ready to go. Enter the 7HV130 directory by typing **CD \QDIG\7HV130**.

Skip the next two steps to begin collecting flight data.

5)   If the computer does not have the software installed you must follow these instructions:

a)   Now you are ready to copy the data acquisition and field testing software.

Enter the working directory and prepare to copy the software from floppy:

Enter the QDIG directory by typing **CD QDIG**. Now create a directory called 7HV130 by typing:

MKDIR 7HV130

Enter the 7HV130 directory by typing **CD 7HV130**. Insert the floppy disk you received into the floppy drive. Copy the contents of the floppy drive into the 7HV130 directory by typing:

COPY A:*.*

If you have collected data on this computer before, there will be files titled "BEEPLOT1.DAT", "BEEPLOT2.DAT",...,"BEEPLOT5.DAT". These file should be copied into the 7HV130 directory to display the previous data. To do this enter the following command at the C:\QDIG\7HV130> prompt:

COPY C:\QDIG\7HV130\BEEPL*.DAT

6)   Start the SMARTDRV.EXE program to improve analysis speed.

I'm not sure yet what the RAM requirements for 7HV129.EXE are, but I do know that having greater than 590 Kbytes of RAM available will work. To start the SMARTDRV.EXE program, type:

SMARTDRV /E:4096

This will improve analysis times by using disk caching when saving data. The slowest operation in the analysis process is saving data to disk.

H.3   7HV130 Configuration and Operation:

The beecounter software is now configured using a file called BCNT.CNF. Pre-xHV129 versions of the counter software collected operational parameters by asking the user a series of questions. Since the counter software will eventually be used in a multitasking environment, a configuration file will aid changing parameters on the fly. Below is a listing of the BCNT.CNF text file that can be modified using the EDIT program in DOS.

```
0      'using PCMCIA parallel port?  1 if yes, 0 if no
CP     'two letter site code, see manual for examples
06     'default sampling start hour
00     'default sampling start minutes
21     'default sampling end hour
00     'default sampling end minutes
200    'samples collected per second, 200 is default
0.5    'sampling interval in minutes, 0.5 is default
       'do not collect more than 6000 samples per sampling period
Y      'plot previously collected data?  Y for yes, N for no
1.0    'scaling factor, 1 is normal operation, 0.1 for tst, 2.0 for winter
N      'save to floppy?  Y for yes, N for no
N      'communicate with Linux box through serial port?  Y for yes, N for
no
900    'seconds between serial port dumps to the Linux box
C      'data send format, T for total activity, C for both in and out
N      'counter test result format, Y for detailed, N for errors only
19200  'baud rate, 19200 for slow computers, 38400 for fast computers
```

Each line represents a different operational parameter. The comments seen above (right of the apostrophe) are present in the BCNT.CNF file, but are not read during input. The number of characters read during input is equal to the number of characters used above for each line. For example, for line 1, only one character is read and it must be either a 1 or a 0. One exception is line 15 (seconds between serial...), where 4 characters are read to accommodate times greater than 999 seconds.

The first line should be set to 0 unless the computer being used is the TI. The TI uses a PCMCIA card for a parallel port because it lacks a bi-directional parallel port. If using the TI, set the first line to 1.

The second line is the two letter site code used for naming files. The following is a list of current site codes:

CODE	SITE
UM	U of Montana, Prescot House Apiary / Bromenshenk Office Observation Hive
FM	U of Montana, Fort Missoula Apiary
CP	U of Montana, CP005 Software Testing Lab
CC	APG, West Branch Canal Creek
OF	APG, Old O Field
CV	APG, Churchville Reference
JF	APG, J Field Site
BR	APG, Bush River Site
DF	APG, D Field Site
UD	U of Delaware, Ag Station

The program will automatically create a directory structure in the root directory for housing data files. In addition, directories for the following year will be created in case monitoring continues unattended through the winter.

Lines 3 to 6 set up the starting and ending times for sampling. Sampling will begin when the computer's clock reaches the time entered by combining lines 3 and 4. For the sample above, the starting time will be 06:00 hours. If the program is started before the starting time, it will wait until the starting time to begin sampling. If the program is started after the starting time, it will immediately begin sampling. **It is very important for the computer's clock to agree with local time.** Lines 5 and 6 are combined to build the time that denotes the end of sampling. Sampling will only occur between the times entered for the start of sampling and the end of sampling.

Lines 7 to 9 determine the sampling rate and the length of sampling interval. Theses lines should not be changed under normal operating conditions at any field site. Line nine is left blank so that the extra message can be included. If 6000 samples per sampling period is exceeded, the program will exit and not run until lines 7 and 8 are changed. In the above example, 200 samples are collected per second for 30 seconds (0.5 minutes) to give exactly 6000 samples.

Lines 10 and 11 deal with parameters affecting the real-time, on-screen display. Line ten determines what data will be used for the left half of the field display. If data has been collected previously, files titled BEEPLOTx.DAT (where x = 1 to 5) will be present in the C:\QDIG\7HV130 directory. If the BEEPLOTx.DAT (where x = 1 to 5) files are present, enter Y in line ten, otherwise, enter N to create blank files. Line eleven determines the y-axis scaling factor to be used in plotting data on the screen. The default y-axis scaling factor of 1.0 is suitable for normal flight activity conditions. When flight activity is low (such as during the winter months), it may be necessary to increase the scaling factor to 2.0. A similar version of this software for the single counter equipped observation hive will only plot 1 trace on the screen. For this application a scaling factor of greater than 2.0 could be used to enhance the display of flight activity. The 7HV1xxTS.EXE program repeatedly accesses a data set with extremely high flight activity, so the scaling factor should be set to 0.1 to allow easier viewing.

Line 12 allows the saving of compressed data to floppy disks at the end of each day. At the moment this program cannot detect defective floppies. Please check your floppies before trying this option. A floppy error will put the system into a hold state until a good floppy is inserted. It is **not** recommended to save data to floppy disks because of the potential for data collection interruptions. Choosing 'N' is recommended.

Lines 13 to 15 address parameters affecting communication with another computer through the serial port. Line 13 determines if data will be sent through the serial port. Entering 'Y' here will send data to a computer communicating through the serial port at intervals selected in line 14. The program will detect the presence of a physical connection between the two computers, but will not detect if the other computer is capable of receiving data. It is up to the user to guarantee that the other computer is ready to receive data. If the receiving machine is not prepared to receive data, a timeout error will occur in the counter software and the program will need to be restarted. See below (**Communicating via the serial port**) for details of how the data is sent.

Line 14 determines the interval (in seconds) during which the program will buffer data before sending it through the serial port to the other computer. Each time data is sent to the other computer, the program is not monitoring flight activity. It is recommended that a 15 minute interval (900 seconds) be used to minimize the impact of serial port communications on flight activity monitoring. Any number up to 9999 is valid on this line, although it is strongly recommended that 900 seconds be used to minimize the data collection downtime due to communications. Line 14 also determines the interval between restarts of the Iotech interface. Restarting the interface decreases the amount of lost data due to brief power outages.

Line 15 determines the format in which data is transferred to the serial port during the selected intervals throughout the day. Entering 'C' sends data for bees leaving and entering each counter, while selecting 'T' only sends the total number of bees passing through the counter (total = leaving + entering).

Line 16 dictates which format the counter alignment testing subprogram will use for its output files. Selecting 'Y' will result in the output file detailing the status (working or not) of each passageway on each counter at the site. That is 98 lines (7 counters x 14 passageways) everyday. Selecting 'N' will print only the passageways or detectors that failed the counter alignment test. Since the counter alignment problems have been addressed (hopefully!) In the Spring of 1998, most passageways should pass the alignment test. See below (**Counter alignment test subprogram**) for how to interpret the output file.

Line 17 determine the baud rate for communications with the Linux computer. 19200 baud is the default, if the computers are working well, the baud rate can be increased to 38400. Increasing the communication rate of the counter computer must be accompanied by a similar increase on the Linux computer. See the Linux documentation for how to increase the communication rate of the data receiving software.

While BCNT.CNF determines operating parameters for the counter software, another file called SYSTAT.DAT stores information about the status of the data files being accessed by the program. The 21 data files are compressed using PKZip at the end of each day's sampling period. During the sampling day, these files are left in text format and appended at the end of each thirty-second interval. If a power outage occurs, the program will be automatically restarted (if the computer is set up for this, currently only the AST, the 386 in the office and Drone are setup to autorestart) and the files appended. If a power outage occurs outside of the sampling period (22:00 to 6:00), thefiles will not be affected. By having the program automatically record the state it was in before it shutdown, I am avoiding having to overcome QuickBasic's inability to reliably detect the presence of files.

There should be a single numberin the SYSTAT.DAT file, either -1, 0 or 1 depending on the following conditions:

1   Proper shutdown and restart in middle of day
0   Clean start with no data recorded previously for the day
0   Proper shutdown and restart outside of data acquisition hours
0   Power outage and reboot outside of data acquisition hours
-1 Power outage with reboot in middle of day

Once the BCNT.CNF file is configured properly, you may start the program by typing:
7HV130
The screen should turn white and begin collecting data if you have started within the sampling period.

At this point you can walk away from the system until you press e or E. It will save all files to the data and backup directories corresponding to the site you chose.

If you encounter (or cause) a brief power outage, resetting the Iotech interface by pressing "R" will prevent data loss. It is not necessary to do this since 7HV130 will automatically reset the interface throughout the day at intervals selected on line 14 of the BCNT.CNF file.

If you are having difficulty seeing the display due to excess sunlight, it is possible to change the background color when data is being acquired. The default background is white with black lines separating the days. The optional background is black with white lines separating the days. To select the black background, press "B" and wait for the clock to update. To return to the white background, press "W" and again, wait for the clock to update.

H.4   Extra Precautions:

Because of the limited lifetime of backlights (about 2500 hours) used in laptop displays it is recommended that the laptop video mode be switched to external if possible. There is no need to leave the display running if nobody is around to see it. Try switching the video mode before collecting data to verify that it doesn't put the computer into a power saving mode. When the data acquisition program starts, switch the video mode to external and then switch it back of the laptop display to verify it works. The TI and AST seem to work fine, the Compaq automatically shuts its screen off after a brief period, any others have not been tested to date.

H.5 Known Bugs or Conflicts:

This software **must** be run under DOS until further notice is received (in writing from BK only). A very serious conflict with the WIN95 DOS shell has been found. I'm working on it. If a computer has WIN95 installed on it, you must restart in DOS mode to run the software. It will work fine then and only then. If Windows 3.xx is installed I would prefer that it also still be run under DOS only. I will be performing reliability testing with Windows over the Summer of 1997 at Fort Missoula.

H.6 Notes and Details:
H.6.a Communicating Via the Serial Port:

Real-time monitoring is a goal that can be accomplished by having a means of communicating from field sites to a central location. It has been decided that in the future both the WeatherMax weather station and the Alpha data acquisition and control system will be operated on a computer running the Linux OS equipped for multiple serial ports. The counter software integrates flight activity over thirty second intervals and plots this data directly on the computer's screen. Transferring data at thirty second intervals to a remote computer would prove to be a communication intensive and error prone task. Therefore, it has been decided that data should be transferred to a remote computer at fifteen minute intervals. This corresponds to selecting 900 seconds in line 14 of the BCNT.CNF file. The final compressed data file will be transferred at the end of the sampling day.

Data is transmitted at 38400 baud with 8 data bits, no parity, and one stop bit. 7HV130 is able to detect whether or not a live RS-232 connection is in place, but cannot at present determine whether software is operating to receive the data. If software is not running to receive this data, the counter system could experience delays in acquiring flight activity data. In other words, if the Linux box isn't up, stable, and running the right communications software, the counter system will fill its communication buffers and hang, resulting in a loss of data.

In order to enable the counter system to communicate via the serial port enter 'Y' in line 13 of the BCNT.CNF file. If sending data to through the serial port, line 15 must have either a 'T' or a 'C'. Enter 'T' to send only total (sum of in and out) flight activity data for each hive to the serial port. Enter 'C' to send both data representing bees entering and exiting the hive to the serial port. If 'T' is selected, the following will be sent to the serial port for each thirty second interval collected in the user selected serial port communication interval:

Time Total1 Total2 Total3 Total4 Total5 Total6 Total7

No delimiters are used, and each total represents a single counter. If 'C' is selected, the following will be sent to the serial port for each thirty second interval collected in the user selected serial port communication interval:

Time In1 Out1 In2 Out2 In3 Out3 In4 Out4 In5 Out5 In6 Out6 In7 Out7

When done sending counter data, the program then sends the end of data stream delimiter 'BNB' to the Linux box. The serial port is then closed and data acquisition resumes for another serial port communication interval.

The compressed file created at the end of the day will then be transferred to the Linux server via the serial port using communication protocols that are the same as for the previous user selected serial port communication intervals (38400 baud, 8 data bits, no parity, one stop bit). This time the binary PkZip compressed file is translated to ASCII code prior to transfer. Before transferring any data, the starting signal 'START' is sent to the Linux box to initiate communications. After sending the converted binary file, the end of file code 'FINISH' is sent to indicate the end of data transfer. Software on the receiving computer must be equipped to store or decode this data. The original compressed data file can be restored by decoding the transferred data from ASCII to binary format.

H.6.b Counter Alignment Test Subprogram:

The counter alignment test program performs two tasks; determining the alignment of the counter detectors and emitters, and reporting the key daily flight activity metrics for quick analysis via the DETSUM.DAT file. Counter alignment is determined by checking the status of the two detectors that make up each passageway. If the detector is blocked by an insect or out of alignment it is reported as being blocked. A rigorous testing algorithm is used to help distinguish passageways blocked by insects and passageways with failed emitters. A snapshot of each counter is taken every second over a 5 second period. This is long enough for insects to traverse the counter passageway. The snapshots are then analyzed to determine which detectors were blocked. If 'Y' is entered in line 16 of the BCNT.CNF file, the following will be printed to the DETSUM.DAT file if both detectors in passageway 8 of the counter on condo #1 are working properly:

```
HIVE 1 , PASSAGEWAY 08 PASSED
```

If 'N' is entered on line 16 of the BCNT.CNF file, the above statement would not be present in the DETSUM.DAT file. This option provides a condensed summary of detector alignment test by only printing data for detectors that are out of alignment. If the outside (the detector furthest from the condo) detector in passageway 3 of the counter on condo #1 was blocked for all five of the snapshots, the following would be printed in the DETSUM.DAT file:

```
HIVE 1 HAS OUTSIDE DETECTOR IN PASSAGEWAY 03 BLOCKED WITH A SCORE OF 5
```

If both detectors in passageway 7 of the counter on condo #7 were blocked the following would be printed:

```
HIVE 7 HAS PASSAGEWAY 07 BLOCKED WITH A SCORE OF 5
```

The score provides a means of determining the cause of the detector blockage. If the detector is blocked for all five snapshots, the score will be five. In some cases a flashing detector indicates an alignment problem. This should produce a score of less than five but greater than one. If both detectors in a passageway are blocked, even for a single snapshot, the passageway will be recorded as blocked but no indication of the number of single detector blocks will appear. If a passageway is recorded as being blocked with a score of less than 5, opening the DETTST.DAT file will allow viewing of the number of detector failures. A sample from the DETTST.DAT file is shown below:

```
1
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
OUT,, 1 , 1 ,,IN
```

The first line indicates the number of the condo that the counter is attached to. The next fourteen lines correspond to the detectors in each passageway. Passageways are numbered 1 to 14 from left to right when

facing the counter as it is attached to the condo. The detector nearest the 'OUT' label is the detector in the passageway that is furthest from the condo (on the outside part of the counter). The detector nearest the 'IN' label is the detector in the passageway that is nearest the condo. This pattern is repeated for each snapshot that is taken of the detectors on each counter.

If you stop the program during normal flight activity hours, many detectors will fail the detector alignment test because of bees traversing the passageways. Even if one detector is blocked it could simply mean that a bee is blocking the passageway. A blocked detector that only occurs for one day is probably just a bee in the passageway. If the detector or passageway is blocked for several days, then there is an alignment problem or a hardware failure (emitter or detector). An example of this is shown below:

```
HIVE 2 HAS INSIDE DETECTOR IN PASSAGEWAY 02 BLOCKED WITH A SCORE OF 5
HIVE 4 HAS INSIDE DETECTOR IN PASSAGEWAY 05 BLOCKED WITH A SCORE OF 5
```

In this case hive 2 has shown the same message for the previous 2 days. There is a good possibility that either an alignment problem or a bad detector is causing the problem. For hive 4, this is the first time the problem has appeared, and a quick check confirmed that a bee was blocking the passageway.

The second part of the DETSUM.DAT file is the summary of flight activity metrics. These metrics are explained in detail in the annual reports. The file is self-explanatory as it appears below:

```
FLIGHT ACTIVITY ANALYSIS FOR FM980322
TOTAL ACTIVITY
HIVE 1 =  4530
HIVE 2 =  10892
HIVE 3 =  14050
HIVE 4 =  5649
HIVE 5 =  5501
HIVE 6 =  12387
HIVE 7 =  34547
SITEWIDE TOTAL =  87556


PERCENT RETURNED
HIVE 1 =  97.64355
HIVE 2 =  98.43305
HIVE 3 =  98.36214
HIVE 4 =  97.37911
HIVE 5 =  104.1184
HIVE 6 =  97.21366
HIVE 7 =  96.31203
SITEWIDE PERCENT RETURNED =  97.64332


NET LOSS
HIVE 1 =  54
HIVE 2 =  86
HIVE 3 =  116
HIVE 4 =  75
HIVE 5 =  -111
HIVE 6 =  175
HIVE 7 =  649
SITEWIDE NET LOSS =  1044
```

```
ADJUSTED NET LOSS
HIVE 1 =  1.192075
HIVE 2 =   .7895795
HIVE 3 =   .8256298
HIVE 4 =  1.327686
HIVE 5 = -2.017797
HIVE 6 =  1.412779
HIVE 7 =  1.878603
SITEWIDE ADJUSTED NET LOSS =  1.192381


NORMALIZED FLIGHT ACTIVITY
HIVE 1 =  5.173831
HIVE 2 = 12.44004
HIVE 3 = 16.04687
HIVE 4 =  6.451871
HIVE 5 =  6.282836
HIVE 6 = 14.14752
HIVE 7 = 39.45703


AMONG COLONY COEFFICIENT OF VARIATION
SITEWIDE C.V. =  83.24627
```

H.7   Version History:
H.7.a   7HV130.exe

The addition of the alignment and summary subprograms in 7HV129 caused some sporadic crashes due to the excessive amount of memory taken up by the program. 7HV130 addresses these problems by making the alignment and summary subprograms into a standalone program that is called by 7HV130. This keeps memory usage to a minimum and should prevent the crashing. The cause of crashing was a variable scope error in QuickBASIC. The code used to calculate the daily summary was taken directly from PowerBASIC and didn't agree with QuickBASIC.

Spring cleaning on unused variables was done to streamline the program some. Line 13 of the BCNT.CNF file was eliminated. The program is now 'smart' enough to restart without user assistance if a power failure occurs. (For this to work properly I will be testing the restart options, this includes the possibiliy of running under WIN95 or WIN3.11) The restart option uses the SYSTAT.DAT file to identify the status of the program when it was last active. The divider line loss problem with the white background active has been addressed.

H.7.b   7HV129.exe

7HV129 includes several new features that will make the counter software much easier to use. The user interrogation used in previous versions has been replaced by a configuration file (BCNT.CNF) that is read when the program begins, but not at the beginning of each sampling day. Future network based versions will include the ability to switch parameters in the BCNT.CNF file on a day to day basis. 7HV129 is (hopefully) the final version of the counter software to be written in BASIC.

Directories for the following year are created when the program is started. This allows the program to be run through the winter without interruption or maintenance.

A new subprogram was added that tests the alignment of the counters and summarizes daily flight activity metrics into a data file called DETSUM.DAT. This file can be extracted by the Linux computer and emailed to key personnel to provide a summary of counter hardware operating conditions and daily flight activity for each colony at the site. Another file, DETTST.DAT, stores the digital snapshots taken of each of the counter's detectors before the DETSUM.DAT file is created. If detectors or passageways are blocked the DETTST.DAT file will help determine the cause of blockage.

H.7.c 7HV128.exe

7HV128 is being released to provide a brighter background for the field display. Any comments on the colors used are welcome. Configuration is aided by a white background. The user is now able to toggle between white and black backgrounds to aid viewing of the display. If these color changes fail to create a solution, contact Bruce King. The field display has been modified somewhat in that the current day displays "in" (green) and "out" (red) data for bees entering and exiting the counter. This enables immediate detection of the results of experiments being performed on the bees. The previous days are plotted as before with slight color changes.

Another improvement included in 7HV128 is the ability to communicate via the serial port on 15 minute intervals to a Linux box. This feature will be used on future real-time access systems. Finally, the software is ahead of the hardware!

A safety feature was added to minimize data loss from power outages. 7HV128 automatically resets the Iotech interface on fifteen minutes intervals throughout the day. This feature will not prevent data loss when power to the emitters is lost (plugs inside the condo).

H.7.d 7HV127.exe

7HV127 was released to implement the work done to speed up the analysis time. 7HV126 accurately provides a 30 second interval while 7HV127 tries to minimize the analysis time to fit more 30 second intervals into a sampling day. The optimization of the analysis code should cut the analysis time by a little more than a third. This is pretty good. At Fort Missoula I have tested the application of the SMARTDRV.EXE disk caching feature in DOS. This combined with the code optimization has cut the analysis time in half (actually a little better than that). Although disk caching is risky, the problems we have had with losing data due to power outages have had the same effect as the results of losing power while the disk cache is not empty. Another problem with using SMARTDRV.EXE is that it uses up RAM.

H.7.e 7HV126.exe

7HV126 accurately provides a 30 second interval by accessing the 8254 timer. 7HV126.exe does not require the 555 timer that all previous versions have. If you encounter an Iotech interface that does not have a 555 timer box attached to it, you must be running this version of the counter software. I have done an extremely detailed analysis to determine the problems (there were many) with the 555 timer. I have implemented a sampling trigger that relies of the status of the 8254 chip that is commonly used for sound generation in computers. This sampling trigger has worked on computers ranging from 80286, 80386, Pentium... and it even works on the TI. The biggest problem with the 555 timer was that it could not provide an accurate (or precise for that matter) sampling period. For example, a 30 second sampling interval actually ranged from 28 to 39 seconds. This is obviously unacceptable. The 8254 timer provides a 30 second sampling interval that ranges from 29.995 to 30.5 seconds. The only difference that will exist between sites from now on will be the length of the data analysis period required for each 30 second sampling interval. On the Pentiums tested in the lab (75 MHZ and 120 MHZ) the analysis time has been less than 4 seconds. A file called RATETST.DAT is saved with each sampling period to track the length of the analysis period required. This might sound like a lot of technical jibberish, but I put a lot of time into it and it makes a huge improvement in the consistency of sampling among sites. Aside from coming up with a new file naming system, fixing the 'repeating channel' problem and allowing the restarting of data collection in the middle of the day by appending previous files, this was probably the most important improvement since last fall. *I (BK) reserve the bragging rights for this one.* Further testing at Fort Missoula will determine the reliability of using this software in a DOS shell under Windoze.

H.7.f 7FLD105D.exe

Users can now reset the interface by pressing 'R' if the channels look weird. No need to toggle the Iotech interface power or restart the program. Pressing 'E' will exit the program and return to DOS. It is required that 100 samples per second be collected during sampling.

Appendix I

7HV130.exe Field Reference Sheet

Enter the counter software directory by typing:
cd \qdig\7hv130

check to see if the current day's data exists by typing:
dir c:\xxyydata\xxyymmdd

where:
      xx   sitecode, CV, FM, JF, OF, or UM
      yy   two digit year (1998 = 98)
      mm   two digit for month (May = 05)
      dd   two digit for day (5 = 05)

If the current day's data exists, type:
pkzip c:\xxyydata\xxyymmdd\xxyymmdd.zip c:\xxyydata\xxyymmdd*.*

to save the zipped file.
Now check the status of the SYSTAT.DAT file by typing:
edit systat.dat

There should be a single number, either -1, 0 or 1 depending on the following conditions:

1	Proper shutdown and restart in middle of day
0	Clean start with no data recorded previously for the day
0	Proper shutdown and restart outside of data acquisition hours
0	Power outage and reboot outside of data acquisition hours
-1	Power outage with reboot in middle of day

If data exists for the current day and you have pkzipped it properly, replace the character in the SYSTAT.DAT file with a "1". If it is after 22:00 hours (outside of data acquisition hours), use a "0". Data acquisition occurs from 06:00 to 22:00.
Exit and save the bcnt.cnf file by typeing ALT-F-X
To start the software, type:

7HV130

Wait until the screen updates the date and time in the upper right hand corner. If starting the program after 21:00 hours and before 06:00 hours, the screen may stay blank, this is normal.

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Appendix J

SITEVIEW DATA VIEWING, ANALYSIS, PRESENTATION, AND PROCESSING SOFTWARE

J.1 Introduction to Honeybee Behavioral Response Monitoring

This chapter describes software written to visualize and interpret flight activity data collected from bee-counters located at several sites in Maryland and a reference site in Missoula, MT. Electronic devices (bee-counters) were constructed to provide a metric of honey bee forager behavior for a project funded by the United States Army Center for Environmental Health Research (USA-CEHR). The bee-counters have collected data for three years at the Maryland field sites and 2 years at the Fort Missoula reference site. The raw data the data acquisition program and the processed data files used with the SITEVIEW program are included in the accompanying CD-ROM.

J.2 Introduction to the SITEVIEW© Program

SITEVIEW© was written to ease the numerical processing and viewing of data recorded and archived by our bi-directional honey bee counters. The **FILEFORM©**program first converts field data files into a database format for use by **SITEVIEW©**. Data files are stored in a BRES directory on a hard drive, removable drive, or CD-ROM. Field data files, data formatting routines, and data processing and viewing software can be distributed on a CD-ROM. The CD-ROM installs a BRES directory in the root directory of the user's computer. This directory provides for the temporary storage of decompressed data files. **SITEVIEW©** provides the user with an array of viewing and secondary data processing options for data from both honey bee and bumble bee flight activity. All of the menu selections provide choices in parentheses, with the defaults capitalized.

Site codes and dates are checked by the program to verify existence of files, examples of site and date codes are provided in Table 3.1. Data was collected from five different sites, using a maximum of four field systems. While some sites have consistent data collection, relocation of the field systems caused discontinuous data sets at others. For example, studies at West Branch Canal Creek were completed in September of 1997, and that field system was relocated to J Field. Table 3.2 outlines periods that data were collected at each of the sites.

Table J.1. Date and Site Codes for the SITEVIEW Program.

Site codes for our USA CEHR study sites are:		Date Codes use the DD/MM format:	
CC	West Branch Canal Creek	0808	August 8
CV	Churchville Reference	0929	September 29
OF	Old O-Field		
FM	Fort Missoula	The program asks for the	
JF	J-Field ·	YR (e.g., 96, 97)	

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Table J.2.   Data Collection Coverage for Biomonitoring Study.

Site	Data coverage
West Branch Canal Creek	8/7/96 to 11/14/96, 7/2/97 to 9/3/97
Old O Field	8/14/96 to 11/10/96, 6/18/97 to 10/27/97, 4/29/98 to 8/13/98
J Field	9/4/97 to 10/28/97, 5/5/98 to 8/18/98
Churchville Reference	8/31/96 to 11/10/96, 6/21/97 to 9/13/98
Fort Missoula	6/29/97 to 10/6/98
University of Delaware	11/23/96 to 3/1/97

Each **SITEVIEW**© option is a custom software utility program written at UM. These utilities offer data processing and visualization capabilities ranging from: (1) data file formatting and storage, (2) simultaneous display of raw or smoothed data from seven colonies at each site, (3) site by site comparisons, (4) honey bee and bumblebee species comparisons, and (5) complex numerical analysis methods that are highly sensitive to unusual flight activity behavior. The following text is taken from the main menu of the **SITEVIEW**© program.

WELCOME TO THE BEE-COUNT DATA MANIPULATOR AND VISUALIZATION
THERE ARE SEVERAL SELECTIONS AVAILABLE FOR PROCESSING AND VIEWING
DATA OBTAINED FROM SITES CONTAINING SEVEN BEE COUNTERS.

(1)   PLOT TOTAL ACTIVITY FOR 7 HIVES (NOISY DATA)
(2)   PLOT SMOOTHED TOTAL ACTIVITY DATA FOR 7 HIVES
(3)   PLOT DIFFERENCE BETWEEN IN AND OUT ACTIVITY FOR 7 HIVES
(4)   PLOT IN AND OUT DATA SIMULTANEOUSLY TO SEE OVERLAP
(5)   PLOT SMOOTHED PERCENT RETURNED FOR 7 HIVES
(6)   CALCULATE AND PLOT SMOOTHED DERIVATIVES OF FLIGHT ACTIVITY
(7)   PLOT COMPONENTS OF THE DER*DIFF ALGORITHM USED IN OPTION 8
(8)   PLOT DERIV*DIFF RATIO TO SEE PHASE SHIFT SENSITIVE ANALYSIS
(9)   DISPLAY A HOURLY OR USER SELECTED INTEGRATION PERIOD
(10) PLOT SITEWIDE DAILY ACTIVITY FOR ENTIRE SEASON
(11) SIMULATE THE FIELD DISPLAY
(12) PLOT FOUR DAYS/SITES SIMULTANEOUSLY
(13) STRUYE TYPE ACTIVITY PLOT
(15) REVERSE BACKGROUND / FOREGROUND COLOR
(19) DELETE OPENED DATA FILES FROM DISK, LEAVES COMPRESSED FILES ALONE
(20) END SITE/STUDY VIEWER MENU AND RETURN TO MAIN MENU
*   PLEASE ENTER A NUMBER FROM THE ABOVE LIST:*

J.3   Individual Options for the SITEVIEW© Program

J.3.a   (1) Plot Total Activity for 7 Hives (Noisy Data)

This option plots the data for all of the hives for one day at one site with no smoothing. Once the date, year, and site code have been entered, another prompt allows the user to select Total (incoming plus outgoing bees), In (incoming bees) or Out (outgoing) for the bee flight data. These options can be selected by typing **T**, **I**, or **O** respectively. The plot displayed to the screen has eight traces. The traces are numbered from bottom to top as COUNTERS 1 through 7, where 1 shows the data from the counter on hive #1, 7 displays the data from hive counter #7. The eighth trace presents the average flight activity for all seven colonies. At the bottom of the screen is an x-axis grid with time labels. Since most flight activity occurs between 06:00 and 22:00 hr, these values represent the beginning and end of the data collected and plotted for each day.

The plotting algorithm identifies the time corresponding to each data point before displaying it to the screen. Data is plotted by drawing a line from the current point to the next point in the array. Breaks in the sequence of data collection will cause the last point plotted before the data break to be the first point after the data break occurs.

J.3.b   (2) Plot Smoothed Total Activity for 7 Hives

This option is similar to option 1, but allows the user to select smoothing parameters. Four types of smoothing are available, as shown by the screen prompt below:
4 TYPES OF OVERLAPPING BOXCAR SMOOTHING ARE AVAILABLE. DESCRIPTIONS FOLLOW:

OPTION	*SMOOTHING TYPE*
1	*SINGLE PASS*
2	*DOUBLE PASS*
3	*SINGLE PASS, 2 POINT INNER BOXCAR*
4	*DOUBLE PASS, 2 POINT INNER BOXCAR*

DEFAULT IS 2

The first option is a simple overlapping boxcar average, while the second option repeats the overlapping boxcar average on the smoothed data (also called double smoothing). Options 3 and 4 follow the same sequence, but include a 2 point high frequency filter inside each of the user selected boxcars. The user may not choose a boxcar length of less than 2 for options 1 and 2, or less than 3 for options 3 and 4. Pressing RETURN will run the default double filtering (highly recommended) mode. For most cases a boxcar length of 6 is sufficient. Choosing 6 will smooth the data over a 3 minute period for each point plotted. This applies to all subsequent choices that use any form of smoothing. Output to the screen consists of eight traces as before.

J.3.c   (3) Plot Difference Between In and Out Activity for 7 Hives

This option plots the difference between the bees entering and exiting a counter for each thirty second interval through the selected day. Eight traces are displayed on the screen

with the lowest one representing counter 1 and the topmost trace the total difference between bees entering and bees exiting the counter for all of the hives.

The general flight pattern for a sunny day with strong flight activity should start with a long dip as the bees begin foraging. This dip soon returns to zero as foraging bees return to the hive in appreciable numbers. As the end of the day approaches, the difference between incoming and outgoing bees reaches a maximum when foragers return in greater numbers than those that leave. Any unusual peaks or valleys should be investigated further. Viewing the following or previous day is facilitated by entering "X" and following the prompts. If "X" is entered a second time the program returns to the main menu.

J.3.d   (4) Plot In and Out Simultaneously to See Overlap

This option allows the user to plot data simultaneously from bees both entering and exiting the counter throughout a single day. This option is highly recommended for data interpretation and is very useful for identifying and assigning the cause of flight activity events. The user can choose between raw (raw data) and smoothed (filtered) data by entering "R" or "S". Data for bees entering the counter are plotted in green; bees exiting the counter are plotted in red. The screen display shows eight pairs (in/out) of traces. As in other displays, the counter data for hive #1 appears at the bottom of the screen, the average for all seven hives at the top. As before, viewing the data for the following or previous day is facilitated by entering "X" and following the prompts. If "X" is entered a second time the program returns to the main menu.

J.3.e   (5) Plot Smoothed Percent Returned for 7 Hives

This option provides smoothing parameters for the percentage of bees returning to the hive over each thirty second interval. Again we have found that entering the number 6 usually works best for each smoothing parameter. However, the user may wish to try other values. This produces a complex screen display. We suggest that the user view one of these plots while reading the following description. One or two sets of traces can be displayed on the screen. The default plot is a single set of traces, containing 14 lines, two for each counter. Each data trace is laid over a horizontal, straight line that is color coded to match the traces of interest. These horizontal lines represent a 100% return rate for the corresponding traces. The actual percent returned is calculated as:

$$100 * (\text{bees entering counter} / (\text{bees leaving counter} + 1))$$

Adding 1 to the denominator eliminates 'divide by zero' errors encountered at run time. The resultant raw data plots are summarized for 30 second periods and tend to be data rich, "noisy", and visually difficult to interpret. The smoothing routines can greatly facilitate data visualization.

A second set of traces can be displayed at the top of the screen to show either: (1) the smoothed total flight activity for each counter (7 traces), or (2) the total percent returned for the site (2 traces, one of which is the 100% level). Before the data is plotted, the program will ask whether the user wants to display total activity or the total percent returned. Note that the percent returned for each counter should start out below 100% in the morning as

more bees are leaving than entering the hive. Slowly the traces should rise to the 100% level during mid-day. At the end of the day, the plots may exhibit more than a 100% return, as more bees come home to the hive than go out. Any unusual drops or peaks in the percent return data during the day should be investigated.

J.3.f   (6) Calculate and Plot Smoothed Derivatives of Flight Activity

This option plots derivatives based on smoothed flight activity data. A derivative can help isolate events by displaying them as distinct peaks. The user can plot total activity, or data from bees entering (in data) or exiting (out data) the counter or choose to plot smoothed data with the derivatives. In the latter case, the derivative peak(s) should stand out against the smoothed data traces. Again, we suggest using 6 and 6 for the boxcar lengths, 60 or 100 (or whatever makes it look best) for the derivative multiplier, and 6 for the derivative span. The derivative multiplier is used to adjust the scaling of the derivative data. Because some modifications of the derivative are calculated using the average (AVE) of each half of the derivative spans (half above and half below the middle of the derivative span) an even number must be chosen for the derivative span. There are four options available for the derivative calculation as shown below:

STANDARD DERIVATIVE CALC USES THE AVE OF EACH HALF OF THE DERIVATIVE SPAN. YOU CAN MODIFY THIS BY USING THE ABSOLUTE DERIVATIVE AS WELL AS USING THE EXTREME DATA POINTS TO CALC THE DERIVATIVE (POINT TO POINT DERIVATIVE):

OPTION   MODIFICATION
N   NO MODIFICATION, AVE OF EACH HALF OF THE DERIVATIVE
1   POINT TO POINT DERIVATIVE CALCULATION
2   ABSOLUTE VALUE OF THE AVE OF EACH HALF OF THE DERIVATIVE
3   ABSOLUTE VALUE OF THE POINT TO POINT DERIVATIVE
CALCULATION
DEFAULT IS N

*NOTE: Option 3 is highly recommended for most purposes. The point to point derivative calculation is in the form of:

$$MULT * (SM(D) - SM(D - DERIVSPN)) / (DERIVSPN * H1)$$

where SM is the array holding smoothed flight activity data, D is the current data point in the array, DERIVSPN is the user entered derivative span, and MULT is the user entered derivative multiplier. H1 is the average of the upper half of the derivative span, which is used here to aid presentation by eliminating large peaks due to low flight activity at the beginning and end of the day. The H1 correction is automatically removed in options 7 and 8 (see below) in order to maintain true derivative peak height consistency.

Smoothing can be used to filter out short term events and enhance long term events. A large (>10) boxcar enhances long term events, while long term events can be filtered and short term events enhanced by using a small (<10) boxcar. A large derivative span can hide

short term events, and conversely, small derivative span may be insensitive to long term events. Choosing a derivative span that is greater than the chosen boxcar smoothing lengths increases sensitivity. For example, if the user is attempting to detect changes in flight activity during a single day, and the event to be detected lasts for approximately five minutes, a boxcar length of 6 should be chosen to smooth over 3 minute intervals.

A simple method of determining how quickly an event occurred is to vary the derivative span. Rapid, short duration changes in flight activity will result in a peak if a small enough derivative span is used. Long term changes in flight activity will not be detected by short derivative spans. Note the rise and fall in activity at the beginning and end of the day. A short derivative span will not see this as a change in activity. Successively longer derivative spans will increase as the length of the event is approached, then eventually begin to decrease as the duration of the event is overtaken. Thus, when the result of changing the derivative span is decreasing peak heights, the duration of the event has been passed, but can be approximated by dividing the derivative span by 2.

J.3.g (7) Plot Components of the DER*DIFF Algorithm Used in Option 8

The previous options all display on-screen either the raw flight activity data or some combination of smoothed flight activity data and secondary data processing. It was obvious from the raw data that some type of smoothing was necessary. Once smoothing was performed, patterns of flight activity could be studied. For example, it is easy to see that flight activity changed slowly throughout a warm, sunny day. Sudden deviations from this pattern usually indicated a response to weather, beekeeping activities, housekeeping by the bees, or other externally induced events. However, retrospective evaluation of real-time data is time consuming. More importantly, it fails to fully realize our objective of being able to conduct not only real-time acquisition, but also real-time reporting and analysis of colony behavioral data. Our goal is to not only detect these events, but to assign the observed responses to potential causes in real-time. SITEVIEW© is a testing ground for algorithms that can be implemented to detect these events and discern their causes.

One of these algorithms is DER*DIFF for calculating phase shift sensitive analysis of parameters. The process is straightforward. Data from the counters is smoothed according to user selected boxcar lengths using methods from option 2. This smoothed data is then collected into arrays called SMI and SMO. SMI contains smoothed data for bees entering the counter and SMO contains smoothed data for bees leaving the counter. Next, the difference between SMI and SMO is calculated. Derivatives are taken of the smoothed data following the method used in option 6, but excluding the H1 activity correction factor. We recommend a derivative multiplier of 10 to align the y-axis scaling of all of the traces. These derivatives look back from the current smoothed data point to a point that is as far back in the array as the user selected derivative span (DERIVSPN). The derivatives are collected into arrays called DVI and DVO to yield the derivative of in data and out data, and the difference between DVI and DVO is calculated. The absolute difference in the percent returns are calculated as shown below:

$$ABS((SMI(x) / (SMO(x) + .1)) - (SMI(y) / (SMO(y) + .1)))$$

where x is the number of the current data point in the array and y is calculated as the number of the current data point minus the user selected derivative span. The addition of .1 in each denominator prevents 'divide by zero' errors while exerting little influence on the calculated result. Artificially induced phase changes are eliminated because the derivative and the difference in percent return are calculated by looking back in the array by the same user selected derivative span.

The are two options for calculating the components of the DER*DIFF algorithm. A screen prompt will allow selection of the desired calculation method. Because of the number of traces being displayed, we recommended using the A option.

YOU CAN PLOT THE INDIVIDUAL ELEMENTS OF THE DER*DIFF ALGORITHM TO VIEW THE PROCESS BY WHICH THEY COMBINE. THE FIRST PART IS THE DIFFERENCE BETWEEN THE SMOOTHED IN AND OUT DATA, AND THE SECOND PART IS THE DIFFERENCE BETWEEN THE SMOOTHED DERIVATIVE OF THE IN AND OUT DATA

YOU CAN SELECT AN OPTION BELOW TO PLOT THE ELEMENTS:

OPTION   DISPLAYS
N   PLOT THE DIFF OF THE SMOOTHED DATA AND THE DERIVATIVE
A   PLOT THE ABSOLUTE VALUE OF THE DIFFERENCES
DEFAULT IS N

The y-axis is scaled according to the minimum and maximum values of the site-wide total data in the analysis. This is not done for individual counters because of the high level of variability displayed among the counters. If the user would like to display data for all of the counters simultaneously, it is recommended that the y-axis be manually adjusted and the maximum value multiplied by 2 to 3. If a dataset is encountered where the phase shift analysis is not providing a reasonable y-axis autoscale, write it down and report it to us. The smallest y-axis span available is currently 65, resulting from y-axis values of -40 and 25. The largest y-axis span produced via autoscaling is 101000, with y-values of -1000 and 100000.

After the user has entered the appropriate parameters a prompt will appear to display results from all seven counters or from the total of all seven counters. Although the option to display the components of the DER*DIFF calculation for all seven counters is available, it is not recommended because a large number of overlapping traces will be drawn on the screen. If the user is interested in an event occurring at one of the counters, enter "Y", but don't be surprised by a very cluttered screen. We recommend entering "N" to display only the analysis and the combined activity for the site. This will produce a single line for each component of the phase shift analysis and two lines representing in and out activity for the site throughout the day. If the peaks do go off scale, the Y-axis can be adjusted or, in extreme cases, a smaller derivative multiplier can be selected.

The colored numbers displayed at the top left corner of the screen identify the individual counter traces. Directly below the colored numbers are the labels for each of the traces normally on the display. SMT is the difference between the smoothed in and out data,

DRV is the difference between the derivatives of the smoothed in and out data, PRC is the percent returned bees for the smoothed data, DRPRC is the derivative of the percent returned of the smoothed data, IN is the smoothed in data, and OUT is the smoothed out data. The bottom line of numbers is the maximum smoothed activity per sampling period, the y-axis maximum, the y-axis minimum and the y-axis span. The in and out activity are plotted so that the bottom of the screen is zero on the y-axis, while the phase-shift analysis automatically adjusts.

J.3.h   (8) Plot DER*DIFF Ratio to See Phase Shift Sensitive Analysis

This is an ultra-sensitive phase shift analysis that can detect smoke events and possibly, what we believe may be acute chemical events (although they often can only be reported as anomalous events). A smoke event falls under the characterization of an acute chemical event. Events such as this can be used to provide sensitive flight activity-based analysis for comparison with weather and colony temperature to initiate chemical sampling. Currently, we are conducting dose-response trials to build a library of colony responses to external events. Until a database of real-time chemical data can be produced, many of the changes revealed by phase shift analysis must be listed as anomalous events.

To achieve the highest sensitivity and best false alarm rejection, the difference between the smoothed in and out activity, the difference between the derivatives of the in and out activity and the derivative of the percent returned are employed. Each of these methods provides a detectable peak or valley when a flight activity event occurs. They also have individual limitations such as: (1) the derivative is overly sensitive to change in flight activity during periods of low activity, and (2) the percent return is relatively insensitive in the morning and overly sensitive in the afternoon. The derivative and percent returned are multiplied together to minimize the impact of early and late day differences in bees entering and leaving the counter while enhancing the differences in mid-day events.

Setting this up to run is very simple. The user must first select the date and site of interest. Next the user will be prompted to plot smoothed flight data. We recommend entering "Y" because it will help correlate flight activity patterns with DER*DIFF calculated peaks throughout the day. Next, the user will be prompted for smoothing and derivative parameters. Because this analysis compares differences between in and out activity, it can be overly sensitive (noisy) to boxcar lengths of less than 6. We recommend using a boxcar length of 6 to 14, a derivative multiplier of 100 and a derivative span of 8 to 14 (even numbers only).

Enter the appropriate derivative method (N or 1 for maximum sensitivity and 2 or 3 for noise and error rejection) and select a phase sensitive analysis method from the list below:

THE STANDARD DER*DIFF CALCULATION MULTIPLIES THE DIFFERENCE OF THE SMOOTHED IN AND OUT DATA BY THE DIFFERENCE OF THEIR RESPECTIVE DERIVATIVES. YOU CAN SELECT AN OPTION BELOW TO MODIFY THE CALCULATION:

OPTION   MODIFICATION
    N   NONE
    S   ABSOLUTE VALUE OF THE DIFFERENCE BETWEEN SMOOTHED IN AND OUT DATA
    D   ABSOLUTE VALUE OF THE DIFFERENCE BETWEEN DERIV OF IN AND OUT DATA
    B   ABSOLUTE VALUE OF DIFF FOR BOTH SMOOTHED AND DERIVATIVE DATA
DEFAULT IS N

Choosing option B produces all positive peaks and a display that is easy to interpret. Thus it is the preferred method.

The y-axis is scaled according to the minimum and maximum values of the site-wide total data in the analysis. This is not done for individual counters because of the high level of variability displayed among the counters. If the user would like to display data for all of the counters simultaneously, we recommend manual adjustment of the y-axis and multiplying the maximum value by 2 to 3. If a dataset is encountered where the phase shift analysis is not providing a reasonable y-axis autoscale, write it down and report it to us. The smallest y-axis span available is currently 500, resulting from y-axis values of -250 and 250. The largest y-axis span produced via autoscaling is 104000, with y-values of -4000 and 100000.

After the user has entered the appropriate parameters a prompt will appear to display results from all seven counters or from the total of all seven counters. If the user is interested in an event occurring at one of the counters, enter "Y", but don't be surprised by a very cluttered screen. We recommend entering "N" to display only the analysis and the combined activity for the site. This will give one line that represents the phase shift analysis and two lines representing in and out activity for the site throughout the day. If the peaks do go off scale, the Y-axis can be adjusted or, in extreme cases, a smaller derivative multiplier can be selected.

The colored numbers displayed in the top left corner of the screen identify the individual traces. Directly below the colored numbers are two lines of numbers, the first line being the number of samples collected that day. The bottom line of numbers is the maximum smoothed activity per sampling period, the y-axis maximum, the y-axis minimum and the y-axis span. The in and out activity are plotted so that the bottom of the screen is zero on the y-axis, while the phase-shift analysis automatically adjusts. If the x-axis tick marks are not visible, it is because the y-axis maximum is too large. Try lowering the y-axis maximum value if the tick marks are necessary for identification of event sequence.

If all colonies are foraging normally, the analysis will produce a flat line. If a rain event occurs, peaks will be present as the bees recognize the onset of rain. A good example of a rain event is 7/19/97 at Fort Missoula. If an anomalous event occurs, the resulting phase shift (bees inside the hive stop leaving while bees outside the hive continue to return) will be detected as a sharp peak. Some excellent examples of this are 7/28/97 and 8/5/97 at Fort Missoula (methanol and Pine Sol respectively), 8/8/96 at Canal Creek (smoke event), and 6/22/97 at Old O-field (unassigned event). On 8/5/97 at Fort Missoula the second set of triplet shaped peaks looks like a possible cloud cover event or light rain, while the sharp, tall peak is the peak of interest.

An interesting example of the results of changing smoothing and derivative parameters applies to an experiment conducted on 7/21/97 at Fort Missoula. This was the "stand in front of the hive" experiment. Sharp bursts of bees entering hives occurred after the beekeeper moves from the front of the hive. The peaks do not show up in the total activity, but instead separate out when all hives are displayed. Eliminating the peaks at the end of the day without eliminating the peaks of interest by changing the length of the smoothing boxcar and/or the derivative span is an interesting challenge. Another example is the combination of the weather event and possible chemical event on 8/5/97. Shortening the smoothing boxcar and derivative span will enhance the short lived weather event, while lengthening these same parameters will favor the anomalous events (possible chemical event).

J.3.i   (9) Plot Hourly and User Entered Sums for Honeybee and Bumblebee Data

All of the Maryland sites have honey bee flight data processed for hourly or 2, 5, 10, and 15 minute sums, while only Fort Missoula has bumble bee data processed for hourly or 2, 5, 10, and 15 minute sums. No option exists to mix sites. When entering the user selected sum, a 2 digit number must be entered, thus, for 2 minute sums the appropriate entry will be "02". The same is true for 5 minute sums, while 10 and 15 minute sums are entered as 10 or 15. Not all sites have data for 365 days of the year. **There are missing days in the bumble bee data.** This subprogram does not detect days missing from the bumble bee experiments, and will crash out if a missing day is selected. The bumble bees were brought online in Missoula on July 31 1997 and taken off-line about September 11. This option was written to demonstrate the utility of integrating data at different intervals and the comparison of flight activity of *Apis mellifera* and *Bombus occidentalis* under identical environmental conditions.

Selecting option 9 will provide another list of options that can be used to display either integrated honey bee or bumble bee activity. The options in this list are described below:

(1)   Plot Hourly Sum of Honeybee Flight Data

This option plots the hourly sum of honey bee flight activity for total, in, or out data. The traces are set up as before, with the trace for counter 1 being at the bottom of the screen and the total (in, out, or total) activity for the seven counters at the top.

(2)   Plot Hourly Sum of Bumblebee Flight Data

This option allows plotting of the hourly sum of bumble bee flight data for Fort Missoula. Even though it asks for a site code, only Fort Missoula had bumble bees. The bottom trace is colony number 1, and the top trace is the total activity for all three colonies.

(3)   Plot User Entered Sum of Honeybee Flight Activity Data

This option allows the user to enter a number corresponding to the number of minutes in the integration period. The traces displayed follow the pattern of option 1. The intervals available, as described above, are 2, 5, 10 and 15 minutes, and must be entered as a two digit sequence. The two minute interval has a high frequency component that is the result of the integration algorithm relying on the actual sampling time instead of the number of samples.

(4)   Plot User Entered Sum of Bumblebee Flight Activity Data

This option follows that of option 3, but is used with the bumble bee flight activity data. The traces displayed are similar to that of option 2. Again, bumble bees have only been monitored at Fort Missoula during the 1997 season.

(5)   Plot Honeybee and Bumblebee Hourly Sum Flight Data Together

This option allows direct comparison of the hourly sums of flight activity for the honey bees and the bumble bees. The top trace is the honey bee total activity divided by a number that makes it fit on the screen with the bumble bee data.

(6)   Plot Honeybee and Bummblebee User Entered Sum Flight Data Together

This option allows direct comparison of the user entered sums (2, 5, 10 and 15 minute intervals) of flight activity for the honey bees and the bumble bees. The top trace is the honey bee total activity divided by an arbitrary number that makes it fit on the screen with the bumble bee data.

(7)   Sum and Plot a User Selected Amount of 30 Second Sampling Intervals

This option sums a user selected number of consecutive sampling periods. Since each field sampling period is 30 seconds in duration, summing 10 sampling periods will be roughly the equivalent of a five minute sampling period. This is another demonstration of the utility of 30 second sampling intervals. This option was added to eliminate the high frequency component of the 2, 5 and 10 minute sampling intervals.

J.3.j   (10) Plot Site-wide Daily Activity for the Entire Season

This option allows the user to plot site-wide activity for an entire flight season. All of the data plotted with the following options are available in a comma delimited form in the SUM directory for the site of interest. The menu is pretty descriptive, just enter the number of the desired option. In some cases, two scaling options are available. The first option plots all traces on the same y-axis, while the second option offsets the traces so that individual counters can be easily identified. The first display option is the default. The first five

options are self-explanatory, as the plot of the daily activity totals over the entire sampling season. Option six plots the daily net loss (bees out - bees in). See below for a detailed description and interpretation of the significance of the available options:

(1) **Daily Sum Of Total Activity**
(2) **Daily Sum of Bee In Data**
(3) **Daily Sum of Bee Out Data**
(4) **Daily Sum of In-Out Data**
(5) **Daily Sum of Out-In Data**

Each of the above options reads daily sum data from the *.SUM file. The eight traces displayed on the screen represent the 7 counters at the site and the site-wide total for each option. An option is presented to display the traces in a spread out or condensed form. The default condensed format creates a common y-axis for all of the counters. The spread format creates a unique y-axis for each counter, with counter 1 being the lowest on the screen and the total being the highest.

(6) Daily Net Loss

This option displays the net loss, also displayed in option 5 as the daily sum of out-in data. Net loss is calculated as bee leaving the counter minus bees entering the counter. Positive values represent a net loss and negative values a net gain of bees for the day.

(7) Daily Adjusted Net Loss

This option corrects the net loss for the total activity of the hive by dividing the net loss by the total flight activity of the counter. This is useful for detecting any unusual behavior resulting from either a colony or a counter malfunction. Adjusted net loss typically varies between -3 and +4, any outliers should first be investigated for weather events, power outages, or counter failure. If none of these factors are involved, then the number of bees returning at the end of the day was less than expected.

(8) Daily Percent Returned Data

This option will plot the percent return data for a single site throughout the season. It is also useful for identifying unusual colony or counter behavior. Daily percent returns typically range from 94% to 100% due to natural attrition, any outliers should be investigated.

(9) Daily Normalized Percent Returned Data

This option corrects the percent return for the normalized (against the total activity of the site) activity of each hive. This can help identify strong and weak colonies and how well their return rate is functioning. It could be used to help identify hives being robbed at the end of the season. An unusually high normalized percent return (NPC) may indicate a strong colony robbing from other colonies.

(10) Daily CV Among Channels Of Each Counter   **IN**
(11) Daily CV Among Channels Of Each Counter   **OUT**
(12) Daily CV Among Channels Of Each Counter   **TOTAL**

Options 10-12 display the daily coefficient of variation (CV) among channels of each counter at the selected site. Option 10 displays the CV for bees entering the counter, option 11 displays the CV for bees exiting the counter and option 12 displays the CV for bees both entering and exiting the counter. This plot has 7 traces, each representing the CV of the channels for a single counter on the site. This can help identify which counters have alignment problems or defective channels. If alignment is perfect, one would expect the CV for that counter to have a low value on a high activity day. If the alignment is not optimal, or if some of the emitters are not operating, the CV will be high because most channels will have normal activity while others will be 0 for the day. Occasionally, counters may have a higher CV for bees entering than exiting the counter. If this is the case, it may indicate more serious alignment problems.

(13) Daily CV Among Counter Channels at a Site   **IN**
(14) Daily CV Among Counter Channels at a Site   **OUT**
(15) Daily CV Among Counter Channels at a Site   **TOTAL**

Options 13-15 display the CV among counter channels at a site. This plot has 14 traces, with each trace representing a single counter channel among all of the counters at a site. For example, the CV of channel 1 from all the counters is calculated, same for channel 2... This provides a method of determining which channels are out of alignment or malfunctioning. As with the previous set of options, a high CV values indicates a defective channel.

Combining options 10-12 and 13-15 enables the detection of malfunctioning counters and helps identify the problem therein. Once a malfunctioning counter has been identified, further diagnosis with another program, **CHANVIEW**© (about to be upgraded) can be performed. Alignment or replacement recommendations can be based on the final analysis.

(16) Daily Max of the DER*DIFF Algorithm   **6 POINT BOXCAR**
(17) Daily Max of the DER*DIFF Algorithm   **10 POINT BOXCAR**
(18) Daily Max of the DER*DIFF Algorithm   **14 POINT BOXCAR**

Options 16-18 display the results of applying the DER*DIFF algorithm (see option 8 for information regarding the algorithm) to the flight activity for each hive at a site. These options plot the maximum values of the DER*DIFF algorithm obtained by scanning the entire day using a predetermined boxcar length. The length of the boxcar dictates the length and severity of the change in flight activity detected by the DER*DIFF algorithm. Peaks composed of all seven hives indicate an event that affected the entire site equally. Peaks composed of one or two hives may indicate an event that provoked a change in activity for those hives.

J.3.k (11) Simulate the Field Display

This option allows demonstration and testing of the field display using previously collected data. This is a fully functional field display. All dates and sites are included and trapped. If data does not exist for the chosen date and site, the user will be forced to re-enter a new date. The user can choose between a format that displays the total flight activity for all days including the current day, and a format that plots both in and out flight data for the current day and for the previous five days.

J.3.l (12) Plot Four Days/Sites Simultaneously (For One or More Sites)

This option allows plotting of up to four days of raw or smoothed site-wide flight activity data. The days selected could be from the same site or from different sites. The user only needs to know the site and year for which data exist to produce a date selection table. A date from this table must be chosen and verified. It is possible to select one day from four sites if there are four sites collecting data during that day. It is also possible to select any combination of days and sites. The purpose of this option is to compare activity at different sites. An excellent example of this is the comparison between Fort Missoula and the Maryland sites. Fort Missoula typically has a long steady forage period, while the Maryland bees forage with more intensity over a shorter period during the day. Another comparison can be made about the location of forage materials. When foraging begins, there is a lag time difference between the outgoing bees and the incoming bees. This difference should be smaller if forage materials are available within a short distance of the hives.

J.3.m (15) Struye Type Activity Plot

This option stems from the paper published by Struye and coworkers (1). In their paper they present data from their flight activity counters in a plot containing three traces. The first two traces represent the number of bees entering the hive as positive numbers, and the number of bees leaving the hive as negative numbers. The third trace is the cumulative number of bees remaining outside of the hive. As seen in their paper, the third trace usually exceeds the y-axis scale of the first two traces. For this reason, the y-axis scale for the first two traces plotted in this option are automatically adjusted according to a fraction of the maximum range of the third trace. When this option is selected the following screen appears:

WELCOME TO THE STRUYE TYPE ANALYSIS SUBPROGRAM

Please refer to the paper by Struye, Mortier, Arnold, Miniggio, and Borneck in Apidologie (1994), 25, 384-395 for an explanation of these plots. These options will plot the flight activity data on a separate axis from the cumulative number of bees outside the hive.

(1)   PLOT 7 HIVES AT A SITE

(2)   PLOT SITEWIDE TOTAL ONLY

(3)   PLOT SINGLE HIVE

(15)   REVERSE BACKGROUND / FOREGROUND COLOR

(19)   DELETE OPENED DATA FILES FROM DISK, LEAVES COMPRESSED FILES ALONE

(20)   END DATA INTEGRATOR VIEWER MENU AND RETURN TO MAIN MENU

PLEASE ENTER A NUMBER FROM THE ABOVE LIST:

Options 15 to 20 are the same as those used for the main menu and thus are explained below. Each of the 3 options will allow the user to save the cumulative return data to a text file. Data is saved as the cumulative number of bees remaining outside of the hive for each of the seven hives and the sitewide total. Raw data, smoothed data and sums of consecutive 30 second intervals can be plotted for each of options 1 to 3 by typing "R", "S" or "I", respectively. The y-axis autoscale is a little rough around the edges, so it may be necessary to manually adjust the y-axis in some cases.

For option 1, there are 8 groups of traces plotted in the same order as used for other plots (counter 1-7 and then the sitewide total). Each group has three traces with similar colors. The darkest colors represent the bees entering (positive) and leaving (negative) the counter. The lightest trace represents the cumulative number of bees that have left the counter and not returned.

For option 2, the sitewide total will be plotted on the screen using green and red traces for bees entering and leaving the counter and a magenta trace for the cumulative number of bees remaining outside of the hive. Option 3 will ask for the number of the hive (1-7) that the user wishes to plot on the screen. The plot will be similar to option 2, but now only include data from one hive.

J.3.n (15) Reverse Background / Foreground Color

If the user dislikes the default black background, another color can be chosen. The only caveat is that there are approximately 256,000 colors available with color codes ranging from 0 to over 4,000,000. Here's a hint, black is 0 and white is 4144959. The others are in between. We strongly suggest using the defaults displayed when you are asked to provide the color numbers. Even the lightest colors I've tried are too dark and/or conflict with other colors chosen for the data traces. **Pressing ENTER at both prompts will change the background from black to white and the text from white to black.** To return to the black background/white lettering, simply enter this option again and press ENTER for the two prompts.

J.3.o (19) Delete Opened Data Files from Disk, Leave Compressed Files Alone

This option will clean up the decompressed data files in the TEMP and PLOT directories. It will detect whether the CD-ROM is being used to determine which directories should have extraneous data files deleted. Only decompressed data files and plot files will be deleted.

J.3.p (20) End Site/Study Viewer Menu and Exit Program

This ends the session and exits the program.

Appendix K

Counter Cleaning and Maintenance Manual

Bees passing through the counters deposit dust, dirt and propalis. In addition, wax moth cells have been found between the plastic plates of the counter. The infrared emitter LEDs, the black plastic bee-passageway plate, and the thin Lexan© sheet covering the photodiode detectors need to be cleaned to ensure proper counter performance for the next field season.

K.1   Parts and Supplies:

    7FLD105D.exe program for testing alignment and performance of counters
    Digibook/72 interface system assembled for use with bee-counter system
    Computer with parallel port connection available
    Emitter power supply with wall transformer
    30 conductor cable with 37 pin female connectors on each end (30' length is preferred)
    Philips head screwdriver
    Needlenose pliers
    Methanol in spray bottle
    Soft cotton material for cleaning Lexan© sheets
    Staple gun for repairs
    Spare plastic materials to replace broken parts
    Special counter alignment tool

K.2   Testing of Counters Prior to Cleaning:

Attach the counter to the Digibook/72 interface system using the 30 conductor cable. Note which 37 pin male connector is used when attaching the 30 conductor cable to the interface, this is important for determining which area of the screen display to observe during performance testing. Turn on the emitters by attaching the emitter power supply to the 4 conductor cable on the counter (this is a keyed connector, make sure you put it in the right way). Start the 7FLD105D.exe program by typing 7FLD105D at the command prompt. The first prompt will ask if you are using any bumble bee counters, if so, enter 1. After that a prompt will appear asking for the desired number of samples to collect per second, enter 100. Another prompt will appear asking how many seconds you would like the program to collect samples, enter whatever length you feel is necessary. Sampling can be terminated at any time by pressing 'E", so 10000 seconds is recommended (the program resets the second count at midnight, so it may be necessary to exit manually if you are working that late). If the screen display doesn't seem to be updating properly, pressing 'R' will reset the interface and resume sampling. The screen display should appear similar to Figure K.1.

If the counter is performing properly, the detectors in each passageway of the counter will be represented by a 1. If a detector is blocked, it will be displayed as a zero on the screen. Columns representing counters that are not attached to the interface may have a combination of 1's and 0's on the display. This is caused by the connectors on the

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Digibook/72 interface not being properly terminated unless a counter is attached. Fortunately, when a counter is attached there is no cross-talk between data input channels. If any detectors are blocked (represented by 0's on the display), record as follows (the counter number can be found on the bottom of the detector holder):

Counter	Passageway	Detector	Comments
1	13	in	ripped skirt, needs replaced

Detectors that are not blocked do not need to be noted, this is the expected condition. If no detectors are blocked enter 'OK' in the **Passageway** column.
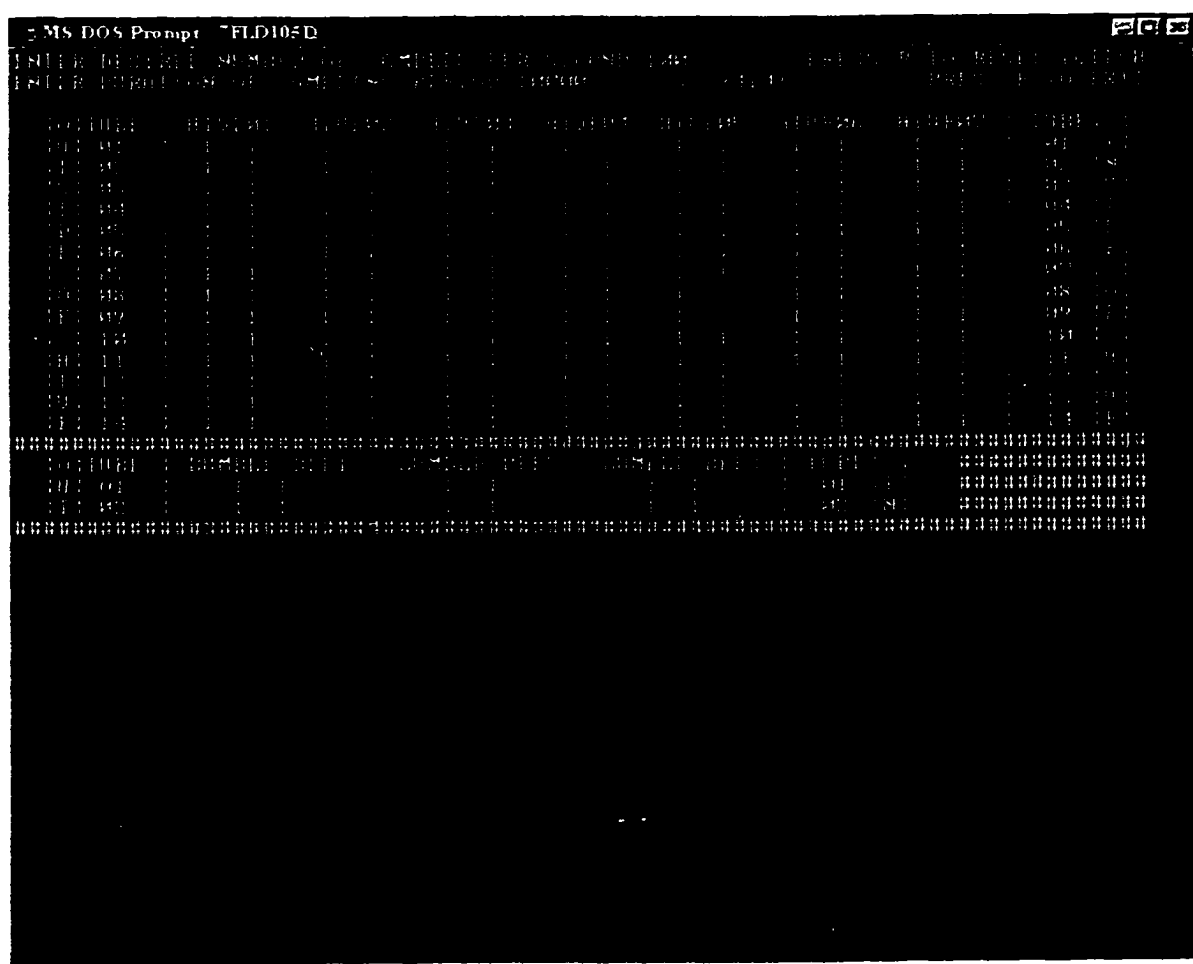
Figure K.1. Screen capture of the 7FLD105D.exe program in action. Seven counters are located in adjacent columns and the 14 bee-passageways are located in rows. The right side represents the detector nearest the landing porch and the left side represents the detector nearest the back of the counter which attaches to the condo. Bumble bee counters are displayed at the bottom of the screen following similar formats as the honeybee counters.

K.3   Dis-assembly and Cleaning of Counters

The roof of the counter consists of a 1/4" thick piece of plastic attached to the side supports with two philips-head screws. Remove the two screws from the roof. The emitter circuit board and emitter holder / bee-passageway plate should be accessible. Use a 90° philips screwdriver to remove the two screws holding the bee-passageway plate to the detector holder plate. A gray cable on left side of the emitter circuit may interfere with accessing the screws, gently move it aside (DO NOT REMOVE IT!). Once the roof and the emitter holder plate are freed from the base of the counter, the clear Lexan© sheet covering the detectors can be accessed. The Lexan© sheet may be stuck to the emitter holder plate or the base of the counter, gently remove and place on soft cotton to prevent scratching.

Methanol or isopropanol is used to clean the propalis from the Lexan© sheet. Place the Lexan© sheet onto a white, soft cotton material and coat the top of the Lexan© with methanol. The bottom should not be very dirty, so it will receive less attention than the top. Cover the methanol coated Lexan© sheet with the soft cotton material and let sit for about ten minutes. Once the solvent has been allowed to loosen the propalis, a soft cotton cloth should be used to clean the Lexan©. During the ten minute period the following steps should be performed.

At this point, the base of the counter can be set aside. It should not need cleaning, but may need some new staples for attaching the plastic skirt on the bottom of the counter. If the skirt is ripped, note this in the **Comments** column in the inspection checklist. Do not attempt to inspect the wiring inside the base of the counter. This will be performed during the counter alignment procedure.

The roof of the counter is still attached to the emitter holder plate by a thin green (or blue, etc.) piece of plastic that has a smaller opening and the emitter circuit board. It is possible to remove this thin sheet of plastic without removing the circuit board from the emitter holder plate. Gently slide one side of the thin plastic sheet over the emitter circuit board until the other side falls out. It's difficult to describe the process, but it's similar to a puzzle. Once the roof and the emitter holder plate are separated, cleaning of the emitter holder plate and the emitters should commence.

Prepare a flat, shallow tub of methanol so that the methanol level is about 3/8" deep. Place the emitter holder plate in the methanol, making sure that the cables and circuit boards are not submerged. It is not necessary to remove the emitters from the emitter holder plates during this procedure. After the emitter holder plate has soaked for approx. 10 minutes, use a brush to remove the propalis. After soaking a brushing until the yellow propalis is removed, carefully rinse the bee passageways with solvent and allow to dry.

When the emitter holder plate and the Lexan© sheet are clean the counter is ready for to be re-assembled. The 7FLD105.exe or the 10FLD200.exe program should be run depending on the type of interface used to test and align the counter.

The counter should be aligned using the special alignment tool. This tool is a piece of Lexan© with 14 bee passageway sized fingers. At the tip of each finger rest two thin strips of wood. (The wood must be thick enough to block the light from the emitter to the detector similarly to the body of a bee.) Figures K.2 to K.4 show a sequence of detector blockages during alignment using the special alignment tool. The special alignment tool is inserted in the back of the counter.

Figure K.2. Screen capture of the 7FLD105D.exe program during the insertion of the special alignment tool into the bee passageways (inside). Thirteen of the detectors on the inside and two of the detectors on the outside of counter number one have been blocked.

Figure K.3. Screen capture of the 7FLD105D.exe program during the insertion of the special alignment tool into the bee passageways (all). All of the detectors in counter number one have been blocked.

Figure K.4. Screen capture of the 7FLD105D.exe program during the insertion of the special alignment tool into the bee passageways (outside). The detectors on the outside of counter number one have been blocked.

IOTech Interface Wiring Key

30-conductor wire key				Inside (I) Outside (O)	Channel Number	Cntr1	Cntr2	Cntr3	Cntr4	Cntr5	Cntr6	Cntr7
	stripe 1	stripe 2	Pin#									
white			1	I	1	A0	A4	B0	B4	C0	C4	A0
black			2	O	1	A1	A5	B1	B5	C1	C5	A1
red			3	I	2	A2	A6	B2	B6	C2	C6	A2
blue			4	O	2	A3	A7	B3	B7	C3	C7	A3
green			5	I	3	A4	B0	B4	C0	C4	A0	A4
orange			6	O	3	A5	B1	B5	C1	C5	A1	A5
white	black		7	I	4	A6	B2	B6	C2	C6	A2	A6
red	black		8	O	4	A7	B3	B7	C3	C7	A3	A7
blue	black		9	I	5	B0	B4	C0	C4	A0	A4	B0
green	black		10	O	5	B1	B5	C1	C5	A1	A5	B1
orange	black		11	I	6	B2	B6	C2	C6	A2	A6	B2
grey/black	white		12	O	6	B3	B7	C3	C7	A3	A7	B3
red	white		13	I	7	B4	C0	C4	A0	A4	B0	B4
blue	white		14	O	7	B5	C1	C5	A1	A5	B1	B5
green	white		15	I	8	B6	C2	C6	A2	A6	B2	B6
white	red		16	O	8	B7	C3	C7	A3	A7	B3	B7
black	red		17	I	9	C0	C4	A0	A4	B0	B4	C0
blue	red		18	O	9	C1	C5	A1	A5	B1	B5	C1
orange	red		19	I	10	C2	C6	A2	A6	B2	B6	C2
red	green		20	O	10	C3	C7	A3	A7	B3	B7	C3
orange	green		21	I	11	C4	A0	A4	B0	B4	C0	C4
red	black	white	22	O	11	C5	A1	A5	B1	B5	C1	C5
blue	black	white	23	I	12	C6	A2	A6	B2	B6	C2	C6
green	black	white	24	O	12	C7	A3	A7	B3	B7	C3	C7
orange	black	white	25	I	13	A0	A4	B0	B4	C0	C4	A0
white	black	red	26	O	13	A1	A5	B1	B5	C1	C5	A1
red	black	green	27	I	14	A2	A6	B2	B6	C2	C6	A2
black	red	white	28	O	14	A3	A7	B3	B7	C3	C7	A3
black	red	green	29	-	gnd	gnd	gnd	gnd	gnd	gnd	gnd	gnd
white	red	green	30	-	+5	Vcc	Vcc	Vcc	Vcc	Vcc	Vcc	Vcc
						Brd 1	Board 2		Board 3		Brd 4	Brd 5

Emitter Power Supply	
Pin #	value
1	gnd
2	gnd
3	+5
4	+5

Bumble bee counter wiring			
cable	value	board	Pin#
red	+5	emitter	28
black	gnd	emitter	27
orange	+5	counter	30
brown	gnd	counter	29
blue	1 inside	counter	1*
green	1 outside	counter	2*
yellow	2 inside	counter	3*
lt. brown	2 outside	counter	4*

* Pattern repeats for each additional counter (3 max)

Appendix M

Operating Instructions for Circuit Board Preparation and Etching
Version 1.0, 970814

Circuit boards are prepared via a process known as 'Positive Etch'. In this process a positive image of the circuit is drawn on a plastic transparency sheet. The transparency sheet is placed on top of a photosensitized copper clad circuit board with the copper side up. A high intensity light (halogen works well) is directed at the transparency sheet/circuit board which will activate the exposed photosensitive coating. The circuit board is then placed in a developer solution that strips the activated photosensitizer material, but leaves the unexposed portion of the photosensitizer coating. The final step is to strip the exposed copper clad material from the circuit board.

M.1   Parts and Supplies:
    1/16" thick single sided, 1 oz., 18"x18" copper clad circuit board (DigiKey #PC8-E)
    GC Electronics Positive Etch Resist Sensitizer (Radio Shack)
    GC Electronics Positive Etch Developer Solution (Radio Shack)
    GC Electronics Ferric Chloride Etching Solution (Radio Shack)
    Darkroom facilities similar to HS205
    Yellow Halogen lamp
    Trays for developer and etching solutions
    Timer or stopwatch

M.2   Preparation of the Transparency Sheet:
    The circuit image to be placed on the transparency sheet can be drawn using several methods ranging from freehand to very expensive CAD software. For most purposes, most commonly available drawing software (e.g. Corel Draw, MacDraw) will suffice. When a suitable image of the circuit is drawn, printing on the transparency sheet can be performed using a Laser printer. Only use transparency sheets that are certified for use with the Laser printer being used otherwise they could melt due to the high temperatures used in the printing process. Inkjet printers will not work as the light will pass directly through the image with little or no attenuation. Wax printers have not been tested. Create the darkest image possible, making sure that there are no breaks in the circuit traces.

M.3   Cutting of Printed Circuit Board (PCB) Material:
    All cutting of PCB material should be done with a fiber disk on a table saw. Dr. Bromenshenk will do this when the board dimensions are supplied. This ensures straight edges and square corners. Sloppy circuit boards reflect poorly on the project and are difficult to work with during final assembly.

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M.4   Photosensitizing PCB Material:

Spaying the PCB material with photoresist requires a dark room and light-tight containers for transporting or storing of the sensitized circuit boards. Red or blue lamps are recommended for use during the spaying procedure. these lights should only be turned on when it is necessary to observe the progress of the spraying or drying of the photoresist. All extraneous light should be blocked from the room in which spraying and drying occurs. It is recommended that the room being used have a fume hood or an adequate ventilation system to disperse the fumes generated during the spraying process.

Clean the copper side of the circuit board with very fine steel wool immediately prior to spraying to remove any oxidation from the surface. Be very careful not to touch the copper side of the circuit board with fingers. Oil residue from skin can undermine the photoresist coating. Position the circuit boards vertically against a protected wall (newspaper works well) prior to spraying. Direct the photosensitizer spray away from the circuit boards to test the spray pattern. Hold the photosensitizer approx. 8 inches from the circuit boards spraying in a horizontal pattern from bottom to top for 3 to 4 quick passes. Immediately after spraying the circuit boards, remove them from the vertical position and place them on a horizontal surface to dry overnight.

Inspect the sprayed circuit boards under blue or red light for uneven spray patterns or runs. If any are found, the defective boards must be cleaned with very fine steel wool and resprayed.

M.5   Preparation of the Darkroom for Exposure and Etching:

Attach the yellow halogen lamp to the overhead light assembly and plug into the circuit with the halogen side-lamps. All exposure lamps should be controlled from one switch to ensure even exposure. Prepare the developer solution by adding 3 parts of warm water to one part concentrated developer solution. Prepare enough solution so that a circuit board will be evenly covered. This solution will last approx. 4 hours, less if a high throughput is achieved. As the developer solution turns darker red its effectiveness diminishes.

Prepare the ferric chloride etching solution in a plastic tray according to the directions on the bottle. Usually, it is recommended that the solution be used at full strength. Again, use enough solution to evenly cover a circuit board. As this solution turns green its effectiveness diminishes. Warming this solution to 50°-60°C will hasten the etching process.

M.6   Exposure of Circuit Boards:

In subdued light, place the circuit board on a level surface and the transparency sheet on top of it. Use a sheet of 1/8" or 1/4" glass to prevent the transparency sheet from moving. Align the image of the circuit board on the transparency sheet onto the circuit board. When the alignment is completed, exposure can begin. Adjust the yellow halogen lamp so that it is approx. 8 inches from the surface of the transparency sheet. Remember: Each time the distance from the light source is doubled, the exposure time must be tripled. Here is a list of alternative light sources and sample exposure times:

1) Sunlight (noon on a clear day) for 3-8 minutes
2) Ordinary fluorescent light source at 3" for 20 minutes.

Turn the lamps on for 2 to 5 minutes depending on the life of the developer and intensity of the lamps. Do not allow the lamps to overheat the transparency sheet. When exposure is complete and the lamps turned off, wait 30 seconds to allow the lamps to cool before removing the transparency sheet from the circuit board.

M.7   Developing the Circuit Image:

In subdued light, place the exposed circuit board into the developer solution and agitate until the image of the circuit appears. Continue to agitate until all of the background material is dissolved, down to the bare copper. This should take no more than 6 minutes depending on the life of the developer solution. After the image appears, rinse with tap water and prepare for etching. Repair and breaks or skips in the circuit image with a oil type signature pen.

M.8   Etching:

Place the developed circuit board into the ferric chloride etch solution and agitate to begin the etching process. once the process has started occasional agitation may be used to remove the background copper from the circuit board. Continue until only the circuit pattern remains. This process should take from 15 to 60 minutes, any period longer than that hint that the etch solution should be renewed. When etching is complete, rinse the circuit board thoroughly and dry with a paper towel.

M.9   Preparation for Soldering:

Clean the circuit board image with very fine steel wool to remove the remaining photosensitizer spray. Use a drill press to rill holes through the board with the circuit drawing as a guide. After drilling is complete, clean all the burrs from the board and check for continuity.
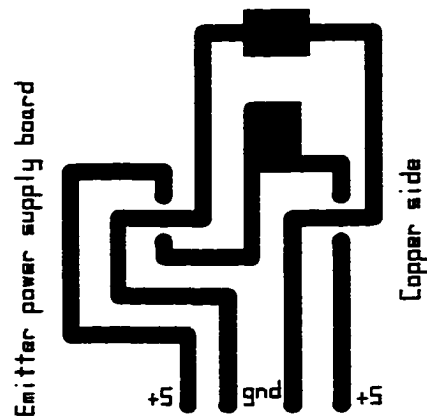
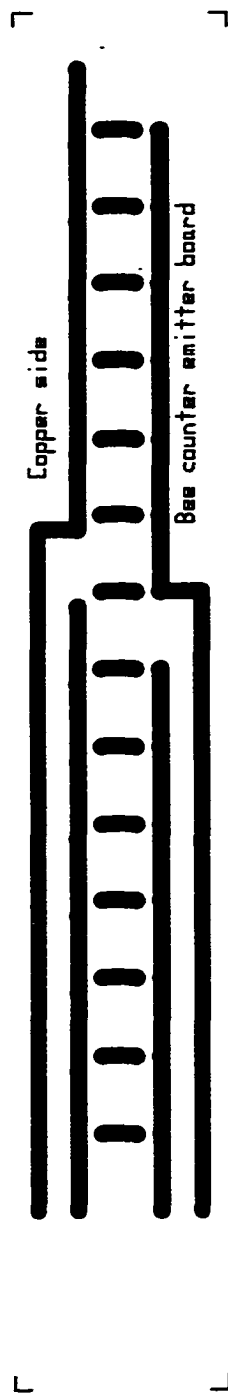Figure N.1.   Emitter power supply board circuit diagram.

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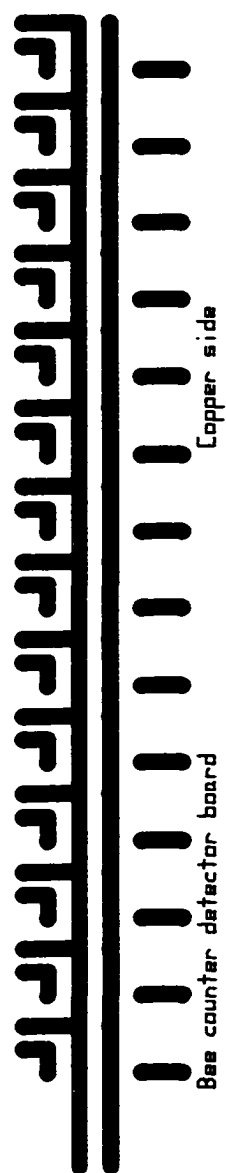Figure N.2.   Bee-counter emitter board circuit diagram.

Figure N.3.   Bee-counter detector board circuit diagram.

Appendix O

Operating Instructions for the Li-Cor LI1000 DataLogger
Version 1.0   970830

The Li-Cor LI1000 datalogger is on loan from Vicki Watson. It is capable of logging solar radiation data at 10 minute intervals for 7 days before filling its buffers. A computer program allows downloading of data via a 4800 baud serial serial port. Another computer program has been written in-house that converts the downloaded data into a format readable by most spreadsheet programs (comma delimited?). The solar radiation sensor is attached to a 25 foot cable that connects to the datalogger with a BNC connector. The cable and sensor are weatherprrof and submersible. A manual is provided, but not all of the features described in the manual are available on the datalogger, thus the manual can only be used as a rough guide for operation.

O.1 Parts and Supplies:
Li-Cor LI1000 DataLogger
Solar Radiation Sensor and Cable
Li-Cor Software disk with LCFRM.EXE included
DB25F to DB9F serial cable
4 "D" size batteries (Rechargables operate at too low of a voltage to work)
Li-Cor LI-1000 DataLogger Instruction Manual
Li-Cor Carrying Case

O.2 Power Considerations:
The LI1000 Instruction Manual provides a detailed section on battery life. For solar radiation only one channel is monitored every 10 minutes. This should provide about 55 days of battery life. To date (970830) this has not been verified. When the battery is low on power, the symbol $"L_o"$ will be displayed (again, this has not been confirmed yet). If the datalogger shuts off due to insufficient voltage, data in memory will be maintained by the remaining power in the batteries and by a lithium battery backup. It is recommended that operation of the datalogger be verified every 4 days to prevent data loss.

O.3 Configuration of the LI1000 DataLogger for Field Data Collection:
Turn the datalogger on pressing the "FCT/ON" key on the data logger. To view the amount of buffer remaining, press the "VIEW" key followed by pressing the "." key twice and then the "ENTER" key. The display will prompt for a (y/N) response, press the "Y" key to reset the buffer, otherwise press the "ENTER" key. To exit the view mode, press "VIEW" followed by "ENTER".

Set the time and date by pressing the "TIME" key and filling in the appropriate time and date. It is recommended that this time and date be synchronized with that of the other data collection systems (Alpha, Weather, Counters) to maintain consistency.

Configure the datalogger's parameters by pressing the "CFG" key. Configuration mode allows selection of the input channel, correction coeficients, sampling interval, starting time and data format. If the datalogger has been used previously, then the only option that may need adjusted is the starting time.

The configuration sequence for solar radiation measurements is as follows:
mode is **LOG**
ch1 is **ON**
range = **A**
c0 = **.0000E00**   (C stands for coefficient, and accompanying number is order of the coefficient)
c1 = **.1887E03**
c2 = **.0000E00**
c3 = **.0000E00**
c4 = **.0000E00**

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```
c5 = .0000E00
label = UM
per = 10                    (sampling interval)
reset = 0000 HHMM           (set to the next even 10 minute time increment from the current time)
thr = -1.000E10             (threshold value, we use the default)
store = MEAN                (stores the mean value of the solar radiation for the chosen sampling period)
min/max = YES               (storing the min/max values helps to identify cloudy days)
ch2 is OFF
ch3 is OFF
ch4 is OFF
ch5 is OFF
ch6 is OFF
ch7 is OFF
ch8 is OFF
chM is OFF
chA is OFF
chB is OFF
```

O.4 Downloading Data with the COMM.EXE Program:

Connect the serial cable to the computer and the LI1000 datalogger. From the DOS prompt in the \LICORR directory type "COMM" to start the data transfer program. Once the program is started press "RETURN" to close the startup screen and "F6" to name the data file to receive data from the LI1000 datalogger. It is recommended that the files be named as follows: 970828.txt for August 28, 1997 raw data file. At this point to computer is ready to receive data from the datalogger. On the Datalogger press the "OUT" key. Prompts will appear as follows (press "ENTER" to accept values):

```
baud = 48
form = H
len = 120
```

After the length of the line is entered the datalogger will commence sending data to the computer at the leisurely rate of 4800 baud. Seven days (90% of the buffer) will take approximately 45 minutes to transfer. Plan accordingly.

O.5 Reformatting Data with the LCFRM.EXE Program:

After the data is transferred to the computer, run the LCFRM.EXE program to convert the data into a format (comma delimited) readable by spreadsheet and plotting software. Type "LCFRM" to start to reformatting program. A prompt will appear to name the data file to be processed, enter the name of the data file with the 3 letter extension. Another prompt will appear to name the file to receive the reformatted data. Type the name of the date as *YYMMDD* followed by ".SR". After the data scrolls through the screen the conversion is complete and the datalogger restarted. Remember to clear the buffer after successfully downloading data from the datalogger.

IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"
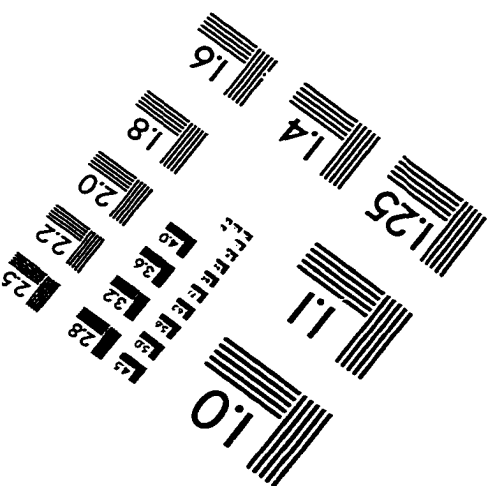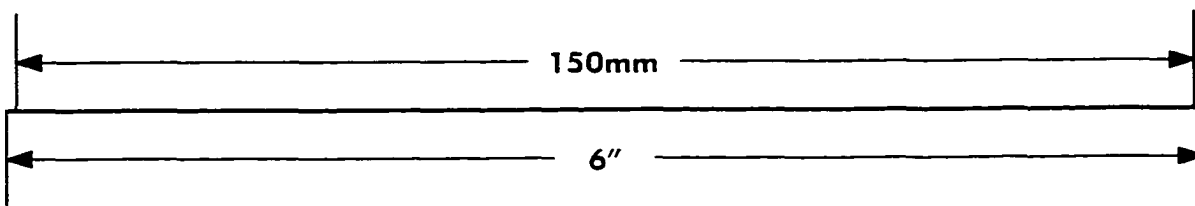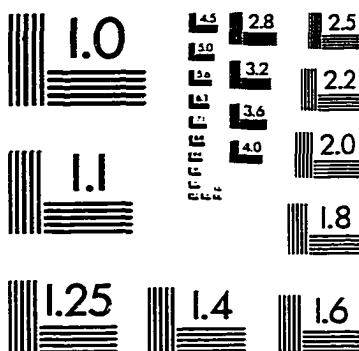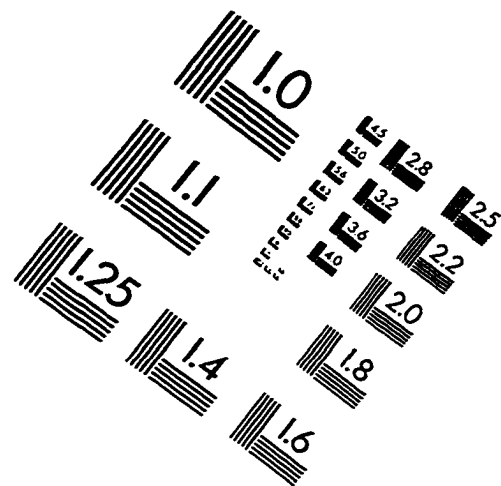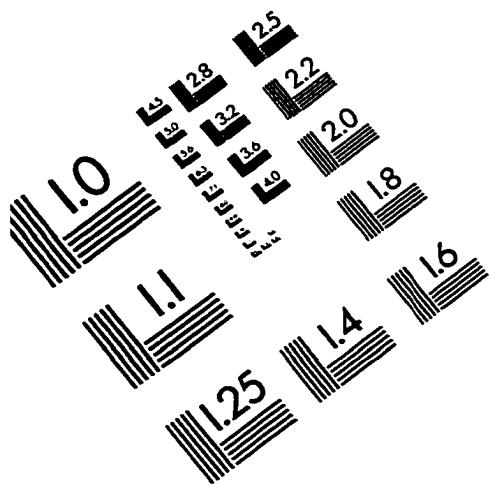
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