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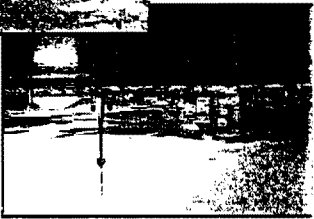
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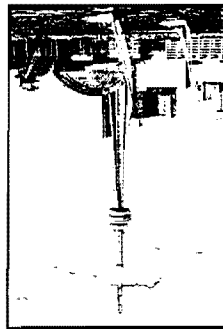
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## EMERGING TECHNIQUES FOR ON-LINE ANALYSIS OF SPECIFIC MICROBES USING THEIR NATURAL FLUORESCENCE CHARACTERISTICS

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### ABSTRACT

Research projects conducted under NASA sponsorship are developing techniques for on-line microbiological analysis in process waters and in potable water. The research is directed toward the measurement not only of total microbiological populations but also the detection and measurement of individual nuisance species of bacteria and fungi. Conventional analysis techniques are not able to provide "real time" information necessary for close control of biological process and water supplies on long term space missions where humans will rely upon artificial environments in an interactive life support system.

A newly developed version of fluorescence spectroscopy will be used for the analysis. Fluorescence is well suited for on-line microbiological analysis because all living cells contain metabolic cofactors such as reduced forms of nicotinamide adiene dinucleotides (NADPH) and adenosine triphosphate (ATP), both of which are natural fluorophores. The relative amount of these substances and other fluorescent materials such as tryptophan and tyrosine that are present in a cell will result in a unique fluorescent signature for each species. These signatures can be identified and analyzed in a multicomponent solution through the use of pattern recognition techniques and methods such as comparison of fluorescent decay times.

Experimental work with specific microorganisms include flavobacteria, trichoderma reesei, and pseudomonas. Experiments were performed showing the ability to determine the metabolic state of a facultative microbe through measurement of fluorescent intensity related to NADPH concentrations under aerobic and anaerobic conditions. Other experiments demonstrated the potential for the use of fluorescent signatures to identify specific microbes in nutrient solutions, even in solutions that contain diversified microbial populations.

### NASA MICROBIAL MONITORING REQUIREMENTS

The Controlled Ecological Life Support System (CELSS) is a long range interdisciplinary program under NASA sponsorship to assemble the knowledge to design, construct and operate a self-contained bioregenerative life support system. NASA's objective is to provide a nutritionally stable and reliable environment for a permanent human presence in space, but the research related to the accomplishment of these space ecology objectives have important immediate application to earthbound environmental problems.

Research supported by the Kennedy Space Center will provide hardware, control systems and techniques for three major CELSS subsystems:

Biomass Production Chamber. A biomass production chamber (BPC) will incorporate technology to grow plants under controlled conditions. The majority of current efforts are devoted to higher plants such as wheat, soybeans, sweet potatoes, lettuce, rice and sugar beets. Plants are now being grown by a nutrient film technique in plant growth trays contained within

sealed chambers that have rigidly controlled artificial atmospheres. Several such chambers are currently in operation at the Kennedy Space Center and at the University of Wisconsin.

Food Processing Modules. Food processing modules are being developed to extract edible content from all plant materials. This area includes microorganism based systems and technologies to convert inedible biomass into edible biomass.

Waste Management Modules. Waste management modules will recover and recycle all solids, liquids and gases necessary to support life in the controlled environment.

The relationship between these modules can be seen in Figure 1. All of these subsystems require analytical monitoring of critical operational and environmental parameters. This requirement includes continuous real time analysis of nutrient solution chemical content and nutrient solution microbial content in the biomass production chamber. Analysis of microbial populations will also be necessary for control of the waste management and food processing subsystems.

#### REASONS FOR MICROBIAL MONITORING

Certain forms of bacteria and fungi can limit the effectiveness of the biomass production systems used in the CELSS. Because of this, BPC nutrient solutions will need to be monitored to measure total microbiological populations and also for the presence of individual nuisance species of bacteria or fungi at concentrations that are harmful to plant growth. Another CELSS microbiological monitoring need includes drinking water which must be monitored to assure that it is safe for human consumption. Several CELSS bioreactors using biological processes will require a means of rapid or continuous analysis of total microbiological populations and individual microbial species for control purposes.

Conventional analysis techniques such as culturing and microscopic assays are not able to provide the real time information needed for close control of biological processes and water supplies. Conventional optical monitoring techniques, such as optical density analysis or cell mass analysis are not able to distinguish between living and dead cells, a distinct disadvantage in a "closed loop" system, where process solutions are continuously recirculated. None of these conventional analysis techniques are able to provide on-line microbial identification.

Because of the need to provide both automation of microbial monitoring tasks and close control of microbiological parameters in process liquids, NASA sponsored a Phase I research investigation during the first half of 1991 to evaluate fluorescence as a feasible technology for on-line microbiological analysis. NASA is also sponsoring a subsequent Phase II project starting at the beginning of 1992 to develop the analytical techniques and instrumentation to perform on-line analysis of microbes in complex media.

#### FEASIBILITY OF FLUORESCENCE FOR MICROBIAL ANALYSIS

Fluorescence is well suited to microbial analysis because all living cells contain both nicotinamide adiene dinucleotides (NADPH) and adenosine triphosphate (ATP), metabolic cofactors which are natural fluorophores. Since the relative amount of these and other fluorescent substances (such as tryptophan, tyrosine, amino acids, etc.) present in the cell will be different for each species, it follows that each species shows a unique fluorescent signature over unique combinations of excitation and emission

wavelengths. Each species will also show a unique fluorescent decay time profile, also over various sets of excitation and emission wavelengths. When there are a number of different species present in a solution, analysis of fluorescence spectra or decay times for selected excitation and emission sets should permit specific microbes to be identified and quantified using pattern recognition techniques. Research investigations conducted under NASA sponsorship have evaluated the feasibility of this hypothesis.

#### PHASE I FLUOROMETRIC INDICATOR CHARACTERIZATION

Two fluorometric indicators of microbial activity were analyzed in order to characterize their spectral features. Tryptophan and tyrosine were measured as isolated substances and in combination to establish their excitation and emission spectra. An example of the combined emission spectra observed for one combination of these fluorophores can be seen in Figure 2. Two fluorophores, NADPH and ATP are difficult to measure in isolation from living cells, but were evaluated in both fungi and bacteria even though such analysis could only provide a rough approximation of the pure biochemical compound. In addition, tryptophan was also evaluated in fungi and bacteria for comparison to results from isolated analysis. This analysis provided background information concerning sensitivities, relative intensities of spectra, and the location of possible excitation and emission sets for use in later experiments.

#### PHASE I METABOLIC STATE EXPERIMENTS

Experiments were conducted using a facultative microbe (flavobacteria) before and after a change in metabolism from an aerobic to an anaerobic state. Changes in metabolic state are important for control and monitoring of many biological processes. Because NAD serves as a cofactor in metabolic reactions, the increasing intercellular NADPH concentration in the aerobic condition should result in a greater fluorescent intensity for the microbe, compared to the intensity in the anaerobic condition.

Results from the experiment show a measurable (3.9 intensity unit) difference in fluorescent intensity between the aerobic and anaerobic states for the facultative microbe. A repeat of the experiment using an aerobic microbe (pseudomonas fluorescens) shows an enormous drop (39.0 intensity units) in fluorescent activity under anaerobic conditions, as would be expected.

#### PHASE I ON-LINE FLUOROMETER EXPERIMENTS

Two microbes, flavobacteria and trichoderma reesei fungus were selected for experimental analysis due to their pertinence to CELSS objectives. Both microbes were individually characterized at several concentrations using the laboratory luminescence spectrometer to select optimal excitation and emission wavebands.

Experiments were conducted using an On-Line Fluorometer, after installation of the appropriate excitation and emission filters. This instrument was calibrated using a simple two point slope-intercept model, a very unsophisticated analysis algorithm by today's chemometric analysis standards. Each microbe was individually analyzed at three concentrations in water to test the ability of on-line analysis to track changes in concentration.

Results from these experiments, reported in Figure 3, demonstrate the ability of the instrument to closely track concentration variations for these microbes.

### PHASE I ANALYSIS OF BACTERIA AND FUNGI

Flavobacteria and trichoderma reesei samples were mixed in water in varying proportions to create combined samples with several mix ratios. Each combined sample was analyzed on a laboratory luminometer with three different excitation wavelengths and emission wavebands. One scan was at an excitation and emission set considered to be optimal for the bacteria, another was at the set was considered optimal for the fungi, and the third scan set was at an intermediate region. A total of 25 samples were processed, 20 as a calibration set and 5 as a test set.

This analysis, although insightful, was hampered by the long run time required (45 seconds) and the tendency of the fungi to settle, even when a magnetic stirrer was used. Analytical models employed included regression of untransformed fluorescent values and regression of fluorescent spectra principal components.

The lowest standard error of prediction (1.42%) for flavobacteria was produced using regression of fluorescence values produced by the excitation and emission set considered optimal for bacteria, as expected. The lowest standard error of prediction (5.74%) for trichoderma reesei was produced using regression of fluorescence values produced by the intermediate excitation and emission set. Remarkably, the set considered to be optimal for analysis of fungi produced higher prediction errors (12.28%) than were produced with either the bacteria set (9.86%) or the intermediate set (5.74%) for analysis of the fungus in this experiment.

### PHASE I ANALYSIS OF TWO BACTERIA IN NUTRIENT MEDIA

These experiments were conducted in a manner similar to the two microbe experiment above. This step introduced several complexities into the experiment. First, the two bacteria (flavobacteria and pseudomonas) have overlapping spectra to a greater extent than the two microbes previously tested. Second, the media used for the solution was a nutrient solution which contains a background of many different components, including a range of bacterial and fungal species possessing their own fluorescent characteristics that can interfere with spectra for the two bacteria of interest.

A ten member learning set was processed using regression of the fluorescence values obtained using the "optimal" excitation and emission set for bacteria. The percent errors (calculated by dividing the standard error of prediction by the mean of the learning set) were 9.64% for flavobacteria and 15.6% for pseudomonas. By way of comparison, the percent error for flavobacteria in the two microbe/pure water experiment reported above was 5.0% or nearly half of the error observed in this complex condition experiment. (Note, however, that the pattern recognition techniques in use at this point are not highly developed.)

### PHASE I ANALYSIS OF THREE MICROBES IN NUTRIENT MEDIA

The final set of Phase I fluorescent analysis experiments added a third microbe (trichoderma reesei) to the analysis. Forty samples were prepared in nutrient solutions, with thirty samples used as a learning set and ten samples used as a test set. Four excitation and emission wavelength sets were used to scan each sample, each set considered to be optimal for a different analytical characteristic (bacteria, NADH, fungi, and ATP). Initial analysis of the data was performed using regression analysis of fluorescence values, although a newly developed neural network was also used to process a portion of the information.

The best regression results for all three microbes were obtained with the "optimal" bacteria excitation and emission set, which uses an excitation wavelength of 390 nm and an emission waverange of 410 nm to 610 nm. These results produced percent errors of 23.4% for flavobacteria, 10.8% for pseudomonas and 17.4% for trichoderma.

These were considered to be respectable results for this stage of research, given the fact that neither the instrumentation nor the analysis algorithms had been optimized. As an example of the improvement that may be possible, the fluorescence information that produced the above results was rerun using a newly developed genetic backpropagation neural network instead of regressions. Standard errors of prediction and percent errors for each of the microbes were substantially improved through use of the neural network. Figure 4 presents a comparison of results for each analytical method.

## PHASE II OBJECTIVES

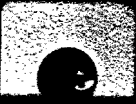
Although the Phase I results were encouraging, it was recognized that actual plant growth nutrient solutions would be composed of a great variety of microbes and contaminants. The resulting fluorescent spectra would be very complex and information extraction will be vastly more difficult. It was clear that improvements would be needed for the spectral characteristics detected and the analytical techniques used to extract information. The Phase I experiments concentrated on the use of emission spectra at a constant excitation wavelength. An objective for Phase II was to evaluate the potential for using spectral information gathered using expanded sets of emission and excitation wavelengths. Also, fluorescence lifetime measurements which were not studied experimentally in Phase I would need to be evaluated. It was equally clear that while available instrumentation would be adequate to gather experimental information, a new type of on-line fluorescence analyzer would need to be developed to implement whichever analytical technique was chosen for actual on-line use.

## PHASE II SPECTRAL INTENSITY EXPERIMENTS

An initial investigation was organized to characterize sets of excitation and emission spectra for four individual bacteria. Four species known to be present in CELSS nutrient solutions were selected (*Pseudomonas fluorescens*, *Agrobacteria tumefaciens*, *Bacillus pumilus*, and *Staphylococcus sciuri*). A matrix of excitation and emission wavelengths were selected, with spectra recorded and graphed to document the unique spectral "fingerprint" of each bacteria.

A more elaborate spectral analysis experiment was then organized. Spectral data was collected from 41 samples containing a random concentration of four microbes (*Agrobacteria tumefaciens*, *Pseudomonas paucimobilis*, *Pseudomonas fluorescens*, and *Trichoderma reesei*). Nine emission scans were collected for each sample, with successive excitations at 20 nm increments starting at 240 nm. Emission spectra was gathered in increments of 0.5 nm, starting at a point 30 nm above the excitation wavelength and continuing up to 600 nm. This produced a total of 369 emission sets for analysis.

The data sets were then analyzed using a stepwise regression technique and the same genetic neural network (NETGEN) that was used at the end of the Phase I project. An initial indication of fit and predictability using stepwise regression was made, with optimal wavelengths selected on the basis of past experience and observation.



This initial analysis was followed by a more systematic technique, using blocks of ten emission wavelengths until all wavelengths in the set had been analyzed. The wavelengths containing the best information for each microbe were identified and used for learning sets and test sets analyzed using stepwise regression and NETGEN. Learning sets and test sets analyzed using stepwise regression demonstrated good correlation and tracking in the learning set and a correspondingly good ability to predict the test set concentrations for three of the four microbes. *P. paucimobilis* produced the most encouraging fit while *P. fluorescens*, a microbe in the same genus, demonstrated a poor fit. The remaining two microbes, *A. tumefaciens* and *T. reesei*, had reasonably good learning set fits and test set predictions. Unlike the results from Phase I, analysis of the data using NETGEN did not produce significant improvements or different results. Graphs of the stepwise regression results for *Pseudomonas* data are shown in Figures 5 through 8.

#### PHASE II TIME DECAY EXPERIMENTS

An alternative technique that may be capable of the necessary specificity and sensitivity is the use of fluorescent decay time. If a fluorescent microbe is excited by a pulse of light, the emitted light will increase in intensity and then decay over its lifetime. Each fluorescent molecule and each type of bacteria (or other microbe) exhibits a characteristic lifetime that depends on its composition. Because all microbes are made up of a number of fluorophores, their fluorescent lifetimes will depend on the number and type of fluorophores that make up the microbial cell. Experimental evidence indicates that even bacteria with similar excitation/emission spectra will have different fluorescent lifetimes.

Experiments were organized to evaluate the potential of the time decay technique. Because experimental apparatus capable of collecting decay times at multiple excitation and emission wavelengths does not yet exist, a time correlated single photon counting (SPC) instrument was used. This instrument provides a laser generated excitation at 287 nm with a selectable emission wavelength. Seven bacteria from the CELSS nutrient solution were prepared for analysis. These including a strain of *A. tumefaciens* and two strains of *P. paucimobilis*. An emission wavelength of 345 nm was selected for analysis.

Fluorescence decay data were collected for each isolate at the selected excitation and emission wavelengths. Each bacterial sample exhibited a similar decay function, probably due to the fluorescence of tryptophan in this region. The data shown in Figure 9 is representative of these results.

This experiment illustrates the need to collect and analyze time decay information over variable wavelength ranges in order to increase fluorophore information.



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FIGURE 1. CELSS MODULES

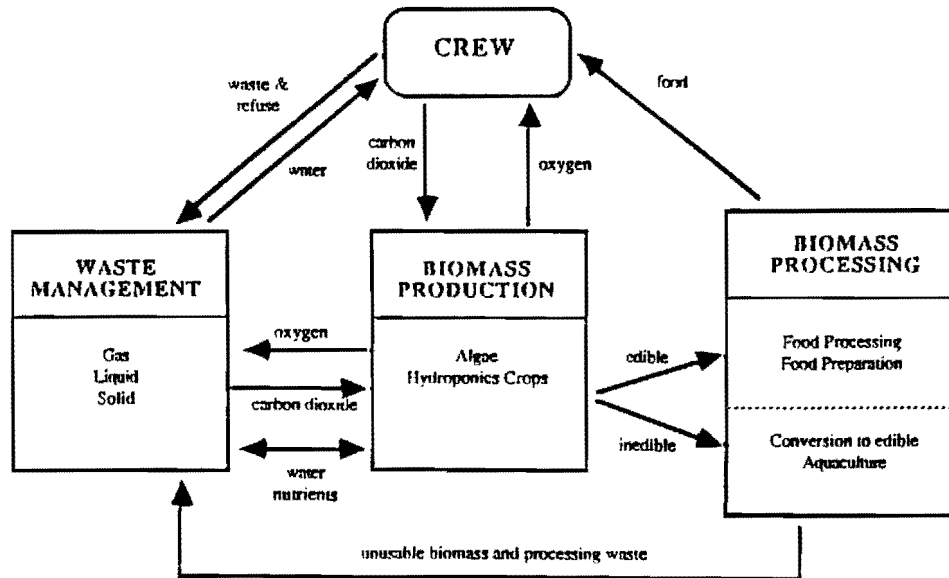


FIGURE 2. TRYPTOPHAN (.313 ppm) AND TYROSINE (.156 ppm) FLUORESCENCE EMISSION SPECTRA

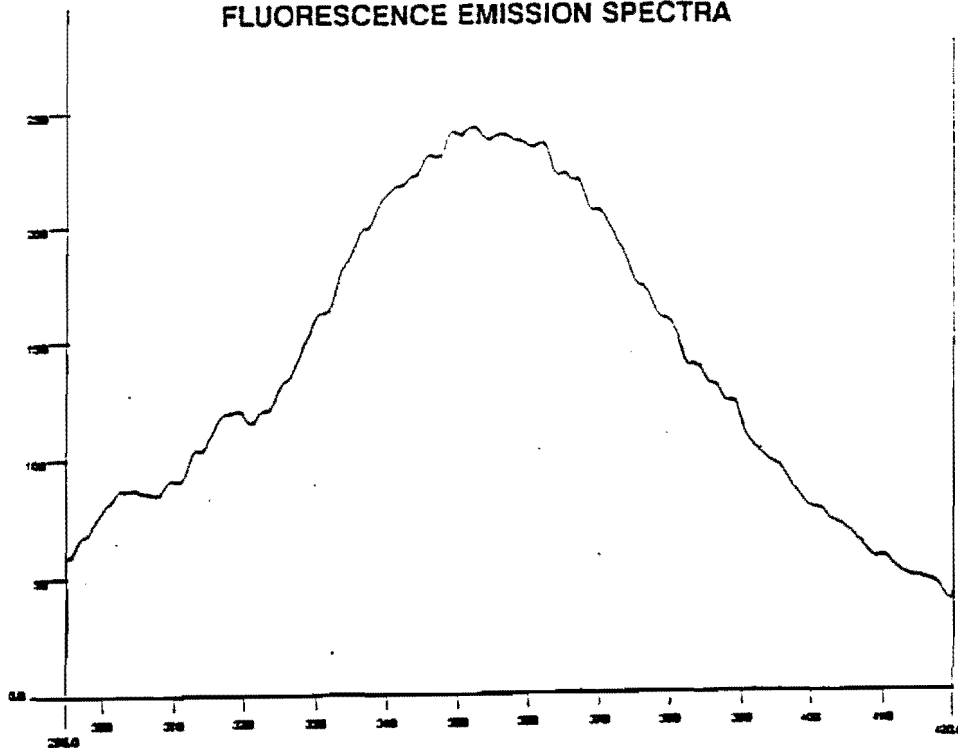


FIGURE 3. RESULTS OF ON-LINE TRACKING EXPERIMENT

<u>MICROORGANISM TESTED</u>	<u>PREDICTED CONCENTRATION FROM ON-LINE ANALYZER</u>	<u>ACTUAL CONCENTRATION BASED ON DILUTIONS</u>
Flavobacteria	25.2%	25.0%
	54.2%	50.0%
	98.6%	100.0%
Trichoderma	22.7%	25.0%
	67.0%	75.0%
	104.0%	100.0%

FIGURE 4. ANALYSIS RESULTS FOR THREE MICROBES IN NUTRIENT SOLUTIONS

	<u>FLAVOBACTERIA</u>		<u>PSEUDOMONAS</u>		<u>TRICHODERMA</u>	
	<u>S.E.P.</u>	<u>% ERROR</u>	<u>S.E.P.</u>	<u>% ERROR</u>	<u>S.E.P.</u>	<u>% ERROR</u>
MULTIVARIABLE LINEAR REGRESSION						
SET A	8.22	23.4	4.47	10.8	2.28	17.4
SET B	13.80	39.5	17.10	43.2	2.83	21.5
SET C	7.92	27.5	15.00	38.0	2.50	19.0
SET D	15.40	45.4	21.50	54.3	4.54	34.6
GENETIC BACKPROPAGATION NEURAL NETWORK						
SET A	6.91	15.3	3.54	8.71	1.25	9.27

DEFINITION OF TERMS

	<u>EXCITATION WAVELENGTH</u>	<u>EMISSION WAVEBAND</u>
SET A: Considered optimal for bacteria	390 nm	410-610 nm
SET B: Considered optimal for NADPH	340 nm	360-560 nm
SET C: Considered optimal for fungi	295 nm	310-510 nm
SET D: Considered optimal for ATP	272 nm	300-500 nm

S.E.P. (Standard Error of Prediction): stated here in E6 bacteria/ml or mg/ml  
 % ERROR: (S.E.P./Mean of Learning Set) \* 100

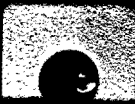


FIGURE 5.

## Actual vs Predicted Concentration *Pseudomonas paucimobilis* Learning Set

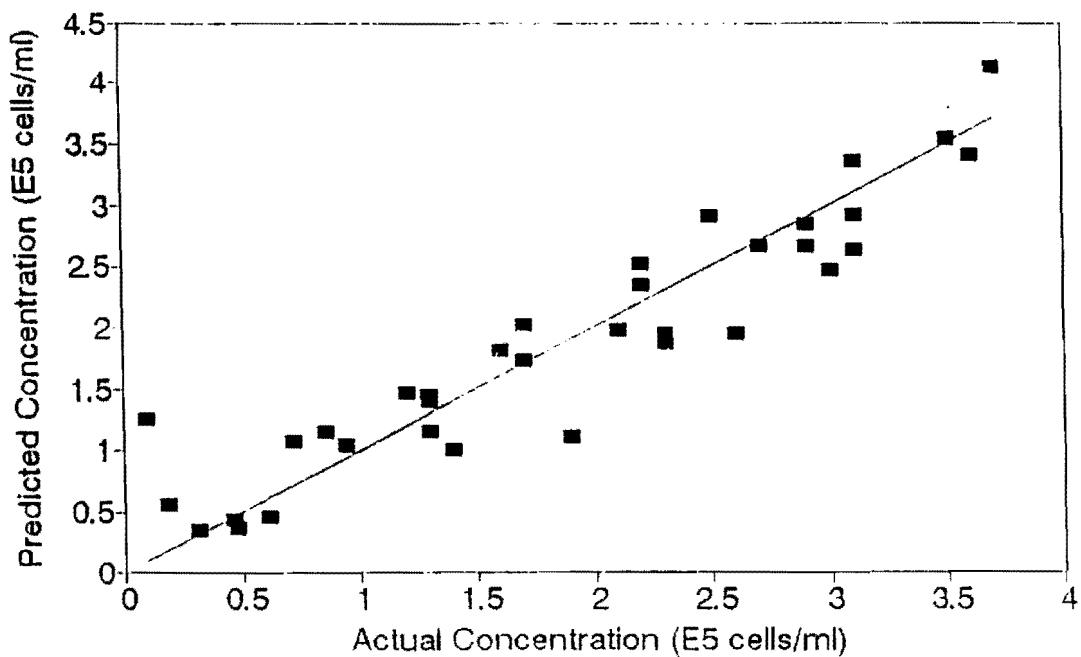
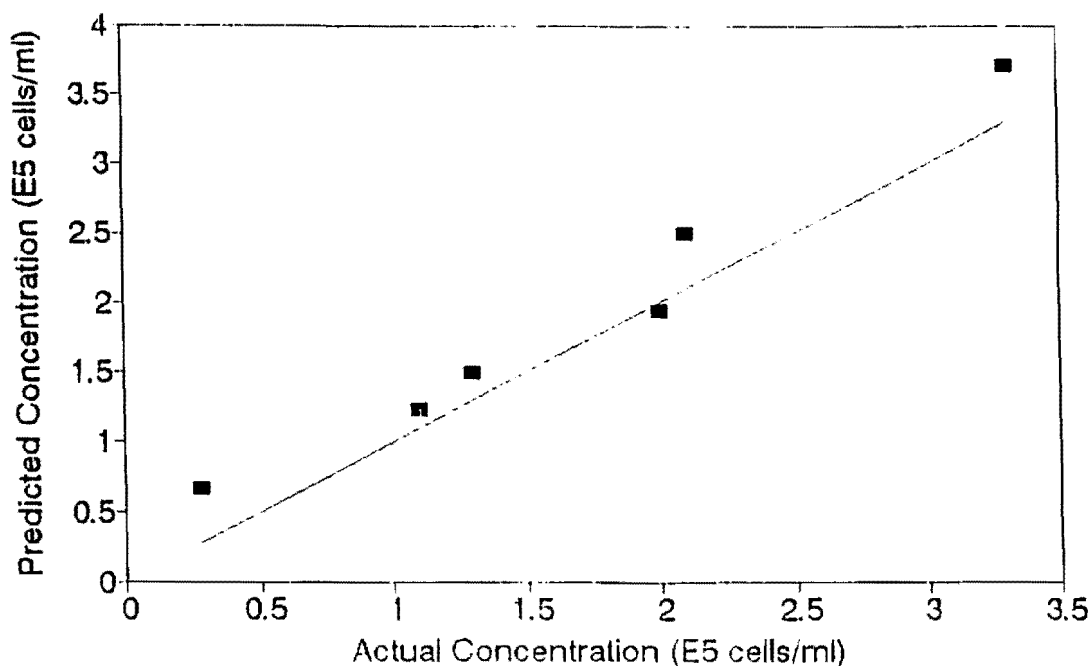


FIGURE 6.

## Actual vs Predicted Concentration *Pseudomonas paucimobilis* Test Set



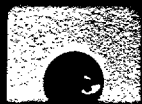


FIGURE 7.

## Actual vs Predicted Concentration Pseudomonas fluorescens Learning Set

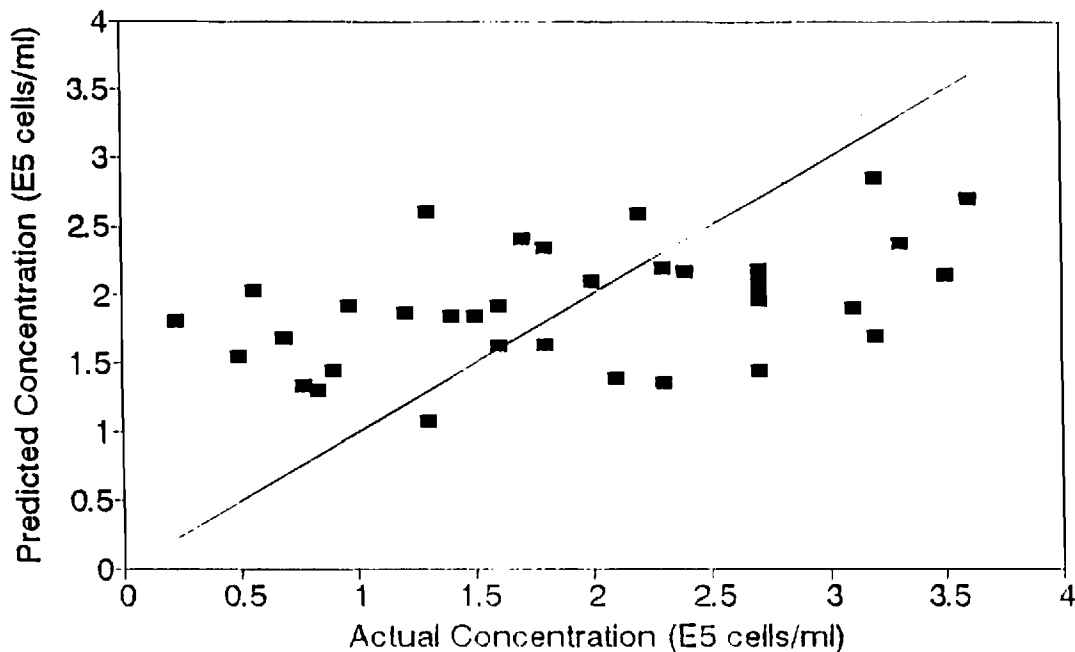
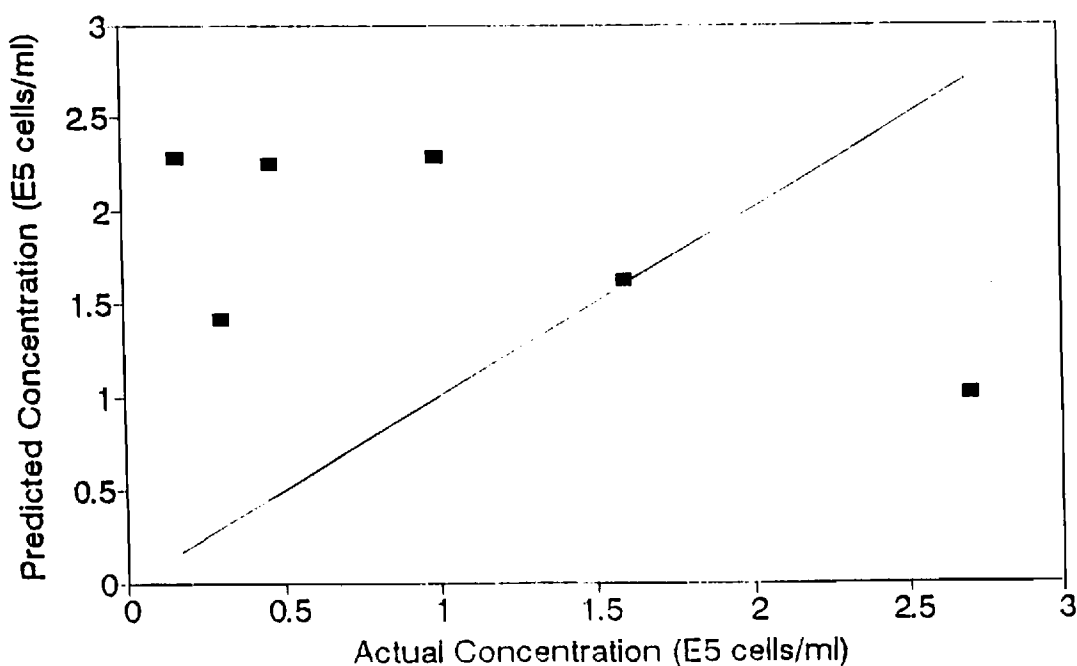


FIGURE 8.

## Actual vs Predicted Concentration Pseudomonas fluorescens Test Set



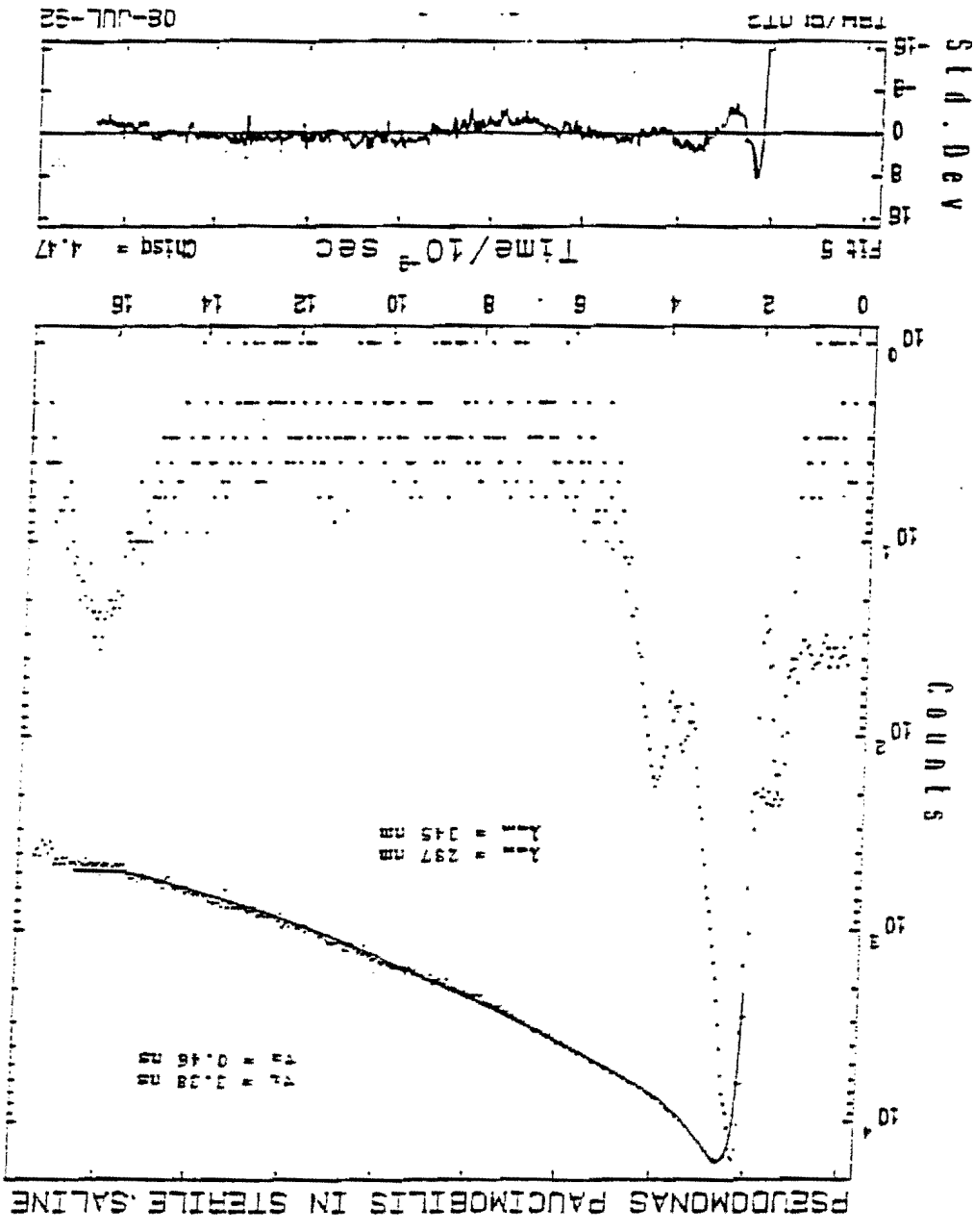


FIGURE 9.