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**Advanced Technology Section
Semiannual Progress Report
for the Period April 1, 1977,
to September 30, 1977**

**Volume 1: Biotechnology
and Environmental Programs**

W. W. Pitt, Jr.
J. E. Mrochek

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ADVANCED TECHNOLOGY SECTION SEMIANNUAL PROGRESS REPORT
FOR THE PERIOD APRIL 1, 1977, TO SEPTEMBER 30, 1977
VOLUME 1: BIOTECHNOLOGY AND ENVIRONMENTAL PROGRAMS

Compiled by:

W. W. Pitt, Jr.

J. E. Mrochek

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SUMMARY

This volume contains the progress report of the Biotechnology and Environmental Programs in the Advanced Technology Section of the Chemical Technology Division. Research efforts in these programs during this period have been in six areas:

1. centrifugal analyzer development,
2. advanced analytical systems,
3. environmental research,
4. bioengineering research,
5. bioprocess development and demonstration,
6. environmental control technology.

Centrifugal Analyzer Development

Development efforts on the centrifugal analyzer concept have centered on the portable Centrifugal Fast Analyzer (CFA) and on applications which take advantage of the centrifugal analyzer's unique capabilities and versatility. The development of the portable CFA resulted in its selection for one of the IR-100 awards for 1977. During the reporting period, emphasis was on the reduction of electronic noise and improvement of temperature control. Additional software capabilities were added to the microcomputer, and the first production model was delivered to the Environmental Sciences Division. Application studies resulted in the development of an enzyme amplification reaction which yielded a part-per-billion sensitivity to high-energy phosphates and a kinetic assay which, with the CFA's capability for a high-sample throughput, helped map a structural gene on a mouse chromosome.

Advanced Analytical Systems

Development efforts in advanced analytical systems were concentrated in two areas: application of high-resolution liquid chromatography (HRLC) to the measurement of enzyme activity, and a new concept for automated gel electrophoresis. The application of HRLC to measurement of enzyme activity, as illustrated by the separation and assay of arylsulfatase

isoenzymes developed during this report period, has a potential for improved clinical diagnostic procedures for noninvasive diagnosis of organic damage and disease. An automated gel electrophoresis system which has the potential for a large sample throughput yielding additional data for recognizing mutant proteins was conceptualized and its feasibility established.

Environmental Research

Environmental research efforts during this report period have been in four areas: (1) identification of dissolved organics in polluted waters, (2) environmental effects of antifoulants, (3) chemical effects of disinfectants, and (4) environmental monitoring.

Pollutant identification, data processing, and mass spectra interpretation were greatly facilitated by the installation of a Systems Industries System 150 to accumulate and process data from the Finnigan 3000 mass spectrometer. The use of a computer to collect and massage the data from the GC/MS will permit examination of mass spectra for specific indicator masses and assist in evaluation and elimination of background or noise peaks. Reconstructed gas chromatogram and mass spectra printouts will provide permanent records of mass spectral analyses. To complement anion exchange separations on aqueous effluents from coal hydrocarbonization bench-scale experiments, a "trace enrichment" method using reverse-phase chromatography has been initiated. The trihalomethanes, chloroform, bromodichloromethane, and dibromochloromethane are formed during the chlorination of cooling waters from both once-through and closed-cycle (cooling towers) systems. Based on values determined at the local industrial sites, the local production of the carcinogen chloroform is in excess of a ton per year. Planning for the Second Conference on Water Chlorination: Environmental Impact and Health Effects was completed and the program finalized. Equipment for laboratory uv irradiation of wastewater effluents was assembled to allow the comparison of the chemical effects of uv irradiation, ozonation, and chlorination. Several wastewater samples were collected and processed

for chemical and mutagenic studies, and collection of field samples was initiated. The development of a multicellular scintillation counter for use in environmental monitoring of ultralow concentrations (less than 1 nCi/ml) of radioactive pollutants in aqueous streams was begun.

Bioengineering Research

Efforts in the bioengineering research program were concerned with the utilization of microorganisms or materials derived from microorganisms (e.g., enzymes, organelles) to carry out useful process functions for energy production and conservation, resource recovery, and environmental protection. Studies related to enzyme catalysis were focused on the enzyme-catalyzed reduction of water to produce hydrogen. Techniques were developed for enzyme separation, purification, stabilization, and immobilization. Factors affecting the rate of reduction of the electron carrier, ferredoxin, by sodium dithionite were studied. Investigations specific to problems in nuclear waste management which included the removal of radionuclides and chemical contaminants from aqueous process and effluent streams were made. Bioreactor component studies which addressed the construction and operation of a large fluidized-bed reactor with tapered entrance and fluid-particle disengaging sections were carried out, and a description of fluid and particle behavior in a two-phase (liquid-solid) fluidized bed was obtained from an analysis of data taken during the operation.

Bioprocess Development and Demonstration

Bioprocess development and demonstration activities during this report period included the operation of bench-scale and pilot-plant ANFLOW (aerobic, upflow biofilter) units and long-term operation of a large fluidized-bed denitrification bioreactor. The ANFLOW demonstration pilot plant was operated essentially continuously at feed rates up to 15.1 m³/day (4000 gal/day) with steadily improving performance. The bench-scale unit was also operated under conditions similar to the pilot plant, using raw sewage as feed. Results for the smaller unit compared

closely with those from the large unit except for a much greater rate of methane production by the smaller unit. A preliminary cost estimate comparing ANFLOW with conventional waste treatment indicated that for waste treatment plants, 1 million gallon per day or less in size, the capital costs of ANFLOW would be less and consume much less energy than would conventional methods.

Environmental Control Technology

The available and potential technologies for environmental control of the aqueous wastes from the hydrocarbonization process for coal liquefaction are being assessed. It has been concluded that the aqueous contaminants of most concern are phenolics, trace elements, and polycyclic aromatic hydrocarbons and that these groups will have the greatest impact on the types of control technologies used. Efforts during this report period have concentrated on six wastewater treatment methods, assessing their applicability for treating hydrocarbonization wastewater. The assessment led to several screening tests, including removal of polycyclic aromatic hydrocarbons with charcoal and polymeric resins and phenol removal with hydrocarbonization char. In addition, the tapered fluidized-bed bioreactor was operated on actual coal hydrocarbonization wastewater, effectively reducing the phenol level to less than 1 ppm.

1. CENTRIFUGAL ANALYZER DEVELOPMENT

M. L. Bauer, W. D. Bostick, R. B. Cumming, R. K. Genung,
J. Jackson, J. B. Overton, J. E. Mrochek

Development efforts on the centrifugal analyzer concept resulted in the receipt of a coveted I·R 100 award for the new portable Centrifugal Fast Analyzer (CFA) in September. Sponsored by Industrial Research, this award is given in recognition of selection as one of the 100 most significant new technical developments of 1977.

1.1 Development of Portable Centrifugal Fast Analyzer

Development efforts were continued on the portable CFA, with primary emphasis on the reduction of electronic noise and the improvement in heat pump capacity and temperature control. Additional software capabilities were added to the microcomputer, and a preliminary version of electronic self-check was implemented. The first production model of the portable CFA was delivered to the Environmental Sciences Division for their evaluation and use.

1.1.1 Modifications of system to decrease noise

Most of the important electronic signal paths for the portable CFA are outlined schematically in Fig. 1.1. Illustrated is the careful routing of ground interconnections between power supplies and signal sources and processing electronics to eliminate troublesome ground loops as sources of noise.

A signal current generated in the photomultiplier tube (PMT, Fig. 1.1) is converted to a voltage, conditioned by the preamplifier (AMP), amplified and sampled by the sample and hold circuitry (S&H), and converted to a digital signal by the analog-to-digital converted (ADC). The first production model of the portable CFA was designed with each of these three components (AMP, S&H, and ADC) on separate printed-circuit (PC) cards. The ADC was physically located within the microcomputer bin, and analog signals were transmitted to it from the S&H PC board, which

Fig. 1.1. Ground interconnections to reduce noise on the portable CFA.

was located on the opposite end of the analyzer. Thus, analog signals were transmitted over some distance within the analyzer and subject to noise pickup before being converted to a digital signal which is inherently less subject to noise problems. A new analog signal processing board was designed and fabricated to attempt to decrease some of the noise problems observed with the original design.¹ The changes involved placing the preamplifier, amplifier, sample and hold circuitry, and analog-to-digital converter on the same PC board. With proper power supply isolation and elimination of ground loops, an order of magnitude improvement in the signal-to-noise ratio was anticipated. The reduction in noise actually achieved with this modification is shown in Fig. 1.2 by comparing noise data obtained using the multiple-board configuration. The data in Fig. 1.2 were obtained at 1000 rpm, and improvement was even better at higher rotor speeds, where the motor generates more noise. The use of separate power supplies for this analog signal processing board would probably further reduce noise; however, these were not implemented at this time.

1.1.2 Additions to microprocessor software

A number of additions have been made to the software package for the microcomputer. Modifications include the printing of reaction rate per minute in time-based data acquisition, and rate per second in revolution-based acquisition. This format enables activities to be printed out in meaningful units by use of an appropriate enzyme factor (1-9999) entered on the last four thumbwheel switches, using program 14.

An additional program has been added to analyze the data from an equilibrium-type substrate assay. This program performs a self-blanking of the sample by extrapolating back to time equals zero by linear regression, using the first six points of the reaction. Then using a specified final measurement (at equilibrium) and an internal conversion factor, the program prints out a substrate analysis by multiplying the change in absorbance times the factor. Entry to this program is gained by entering 15 as the program number (first two thumbwheel switches).

Fig. 1.2. Reduced intracuvet signal noise demonstrated for the second portable CFA by improved electronic design.

The initial point used may be specified; if it is not specified, the program uses the first six points. Either the last data point taken (if 0 is entered) or the data point designated as the final point is used as the end-point absorbance. The correlation coefficient for the initial linear regression is printed as an evaluation of that extrapolation. The program assumes that at least seven data points have been taken; if they have not been taken, an error message is printed.

Work has been started on a program to perform internal checking of the portable CFA for diagnostic purposes. The initial program implemented checks operation of the motor, filter bars, and the optical system. Certain key values are printed out, but at this time there is no internal verification of these numbers. Gross mechanical faults would be detected, and the numbers should enable an operator to predict and thus forestall failure in various parts of the analyzer. The program is selected by dialing 30 on the first two thumbwheel switches; none of the other switches are used. The motor is monitored at three speeds for the actual speed and for the stability of that speed over ten revolutions. The speed in revolutions per minute and the coefficient of variations of that speed are printed for a nominal 50, 1000, and 2000 rpm. The motor and rotor are halted for a short time period, after which they are accelerated to 2000 rpm. The time required to attain 1800 rpm is printed and is a good representation of motor and motor power supply health. The top and side filter bars are then each moved from filter No. 1 to No. 6 and back to No. 1, and a time is printed for each bar individually in the forward direction and a composite time for the reverse traverse. Jamming of the bars or slippage of the cord drive and abnormal positional location of a filter are diagnosed by this procedure. Jamming of the filter bar results in abnormally long times for transit, while missed positional information results in unrealistically short transit time. Finally, the program goes into an automatic PMT voltage adjustment sequence and a calibration of the base plate. This brings instrument noise and optical alignment problems to the attention of the operator. Other factors to be included in this self-checking sequence are more

exact measurements of clutch and brake operation, lamp intensity, PMT power supply, and heat pump operation.

1.1.3 Evaluation of temperature control on the portable analyzer for the Environmental Sciences Division

Work on temperature control for the portable CFA has continued in two general areas. The first area involved modifications of the heating system components to extend the range of temperature control. The second area involved evaluation of the instrument in an environmental chamber to determine the effect of varying ambient temperature on cuvet temperature control.

The initial arrangement of heating system components for the portable CFA is shown in Fig. 1.3. The heat sources and sinks affecting the temperature control in this system are identified in Fig. 1.4. Previous work² had identified thermal coupling between the heat transfer plate and the heat sink as causing control problems. Additional investigation with the system shown in Fig. 1.4 identified changing air temperatures around the heat sink as being a potentially major disturbance to the temperature control system. These changes were primarily related to rotational speeds. Rotational motion produced a centrifugal pumping of air, as shown in Fig. 1.4, which produced an updraft of instrument heat. To monitor this effect, a thermistor was positioned in the air at point A, again as shown in Fig. 1.4. The results are shown in Fig. 1.5. The thermal steady state achieved under control at 1000 rpm was obviously disturbed by sudden acceleration to 3500 rpm, for instance. The rpm effectively determines the ambient temperature which the heat sink experiences. Larger changes in rpm result in larger changes in this ambient atmosphere, and thus require more response from the control system to return to the desired set point.

In order to minimize problems associated with either thermal coupling or rapidly changing ambient temperatures, the system shown in Fig. 1.6 was constructed. In this system the heat transfer plate is isolated from the heat sink by a Lexan insulating shield. This shield also increases

ORNL DWG 77-935

Fig. 1.3. Components in the initial design of the heating system for the portable CFA.

ORNL DWG. 77-983

Fig. 1.4. Schematic representation of heat sources and sinks external to the temperature control system of the portable CFA.

Fig. 1.5. Effect of rotor speed on ambient air temperatures near the heat sink in the portable CFA.

Fig. 1.6. Alternate design for the temperature control system of the portable CFA.

the isolation of both the plate and the sink from ambient changes previously experienced in the air gap between the two components. The additional support provided by the Lexan was also intended to prevent bowing of the plate and was thus intended to reduce contact problems between a rotor and the plate. In addition, the mass of the heat sink was concentrated near the heat pump in an effort to get faster response and greater capacity for heat transfer (by favoring conductive over convective effects, and by minimizing local temperature changes near the heat pump).

This alternate design was tested in an environmental chamber to determine the effect of varying ambient temperature on cuvet temperature control. The results are given in Table 1.1. In general, the present instrument has the capacity to control at temperatures higher than the prevailing ambient temperature; the most accurate control was obtained with test ambient corresponding to the conditions at which the instrument controller was tuned. For comparison with the previous instrument, it should be noted that at ambients of 15°C, control could not be achieved at even 30°C. Although further improvements are anticipated, the present instrument is thought to be adequate for most laboratory applications. For any given ambient environment, it can be tuned to give good control at desired points above that ambient.

Table 1.1. Temperature control points with varying ambient temperatures^a

Desired control point (°C)	Ambient temperature (°C)				
	15	20	25	30	35
25	23.5	24.7	26.0	b	b
30	29.0	29.7	30.5	30.4	b
37	36.3	36.8	37.4	36.9	38.2

^aInstrument temperature controller tuned for 22°C ambient.

^bUnstable temperature control.

1.1.4 Evaluation of the improved portable CFA

After implementing the new analog signal board (see Sect. 1.1.1) and improved design for the heat sink and heat transfer plate (see Sect. 1.1.3), the portable CFA destined for use by the Environmental Sciences Division was tested. Kinetic assays of two different concentrations of lactate dehydrogenase (LDH) were performed at 30 and 37°C. Previous studies had indicated that cuvet-to-cuvet temperature variations were somewhat higher than desirable and probably contributed to some of the assay variability reported for γ -glutamyltransferase and leucine aminopeptidase.³ The results obtained for LDH (see Table 1.2) after the modifications were implemented show very good precision and seem to confirm acceptable cuvet-to-cuvet temperature variations observed by optical thermometry.

Table 1.2. Kinetic assay of LDH on the portable CFA at 30 and 37°C

Enzyme level	Activity (U/liter)	
	30°	37°
Low	23.75	38.18
CV, ^a %	1.09	2.09
High	39.34	63.63
CV, %	0.45	1.15

^a Coefficient of variation.

The performance of the portable CFA for a substrate analysis (glucose) was compared with that obtained on the miniature CFA, which incorporates a PDP/8E subroutine to automatically estimate the absorbance of the reaction blank, as described previously for the GeMSAEC fast analyzer.⁴ In this routine the initial portion of the reaction progress curve is assumed to fit a polynomial,

$$\text{absorbance } (t) = A_0 + A_1 t + A_2 t^2, \quad (1)$$

where A_1 and A_2 are the coefficients, and A_0 is the intercept at $t = 0$. The intercept is used as an estimate of the reaction blank, obviating the necessity of running separate sample blanks with each analysis (variable contribution of the sample absorbance to the reaction blank). Other authors have suggested that extrapolation using a cubic equation yields better estimates of the reaction blank in the analysis of glucose;⁵ however, our experience has shown the quadratic form to be more than adequate.

Two approaches may be used on the portable CFA in assays where variable sample absorbances are a problem in substrate analysis. The simplest approach is to use a two-point procedure: the first measurement is obtained as early as possible in the reaction sequence (~ 0.1 sec for revolution-based data acquisition or ~ 0.5 sec for time-based acquisition), and the final measurement is obtained after the reaction has reached equilibrium. The difference in absorbance is multiplied by an appropriate factor to obtain substrate concentration. Results obtained for the analysis of glucose by this procedure are shown in Table 1.3. This method is adequate for relatively slow reactions in which little transformation of substrate has occurred prior to the first measurement, and it seems apparent that the glucose assay by the hexokinase reaction meets this criterion. The other approach, using the new program for the microcomputer (see Sect. 1.1.2), is based on extrapolation of a first-order polynomial and is shown tabulated under program 15 in Table 1.3. Data in the interval 0.5 to 18.5 sec (delay = 0.5 sec, 6 points taken, each 3 sec apart) were fitted to obtain the intercept shown in the table.

The standards used in this test were one year old, and the nominal concentrations (Table 1.3) may be less than the actual due to solvent evaporation. The estimate of the absorbance at time zero (A_0) derived from the second-order polynomial expression by the computer in the miniature CFA system is not statistically different from the expected reaction blank absorbance. The average estimate of A_0 derived from the first-order polynomial expression by the microcomputer of the portable

Table 1.3. Substrate analysis, with automatic reaction blank estimation, by use of the CFA

Nominal glucose concentration (mg/dl)	Glucose concentration found (mg/dl)		
	mCFA ^{a,b}	pCFA ^c	
		Program 11 ^d	Program 15 ^e
50	55.59 ± 2.11 ^f	53.16 ± 1.23 ^f	54.50 ± 1.12 ^f
100	106.99 ± 0.85	104.26 ± 0.87	105.69 ± 0.82
200	208.38 ± 2.25	206.80 ± 1.89	208.74 ± 1.90
300	289.83 ± 1.66	289.41 ± 3.31	292.05 ± 2.90

^amCFA = miniature CFA.

^bA0 computed by extrapolation to $t = 0$ of absorbance vs time data for the interval 5–28 sec. Extrapolated value is computed by nonlinear regression analysis of second-order polynomial expression. Average extrapolated intercept = 0.0003 AU (theoretical = 0.0000).

^cpCFA = portable CFA.

^dA0 taken as measured absorbance at 0.5 sec.

^eA0 computed by extrapolation to $t = 0$ of absorbance vs time data for the interval 0.5–18.5 sec. Extrapolated value is computed by linear regression analysis of first-order polynomial expression. Average extrapolated intercept = -0.0053 AU (theoretical = 0.0000).

^f±σ.

CFA is lower than expected (by 0.005 AU), thus, in principle, over-estimating the true absorbance change (and thus substrate concentration). The simple two-point approach gave the lowest estimates of substrate concentration, probably due to some substrate conversion prior to the first reading (at 0.5 sec). However, as seen from Table 1.3, there is little practical difference among the results obtained by the three computational approaches (see Table 1.4), and thus any of the previously mentioned approaches may be used for relatively slow reactions. If, however, equilibrium is approached in only a few seconds, the fast data acquisition capability of the portable CFA together with the absorbance-extrapolation subroutine would be expected to give the most accurate estimates of substrate concentration.

Table 1.4. Comparison of computational approaches to estimated glucose concentration^a

x	y	Linear least-squares regression equation	Correlation coefficient
mCFA ^b	pCFA ^c (program 11)	$y = (1.009 \pm 0.002) x - (3.32 \pm 0.62)$	0.999995
mCFA	pCFA (program 15)	$y = (1.015 \pm 0.003) x - (2.41 \pm 0.87)$	0.999990

^aSee Table 1.3.

^bmCFA = miniature CFA.

^cpCFA = portable CFA.

1.1.5 Evaluations of solution transfer and mixing

The time required to complete the transfer and mixing of sample-reagent pairs defines the fast-kinetics monitoring capability of a centrifugal fast analyzer. However, the rapid completion of fluid transfer is no guarantee that the binary solutions have been effectively mixed, and this must be evaluated separately.

An experimental program to determine the effects of rotor design on transfer and mixing is being conducted using the portable CFA. During the first phase of the program the geometric parameters of the rotor transfer channels were studied; however, dye loading procedures and dye concentrations were varied to ensure that observed effects are attributable only to the geometric variations. Later phases of the program will involve investigation of the effects of rotor speed, using selected rotor geometries; investigation of the effects of fluid properties on transfer and mixing; and detailed examination of transfer and mixing in rotors selected on the basis of initial comparative work.

Two basic types of rotors were used in the first phase of the program: the parallel-channel and the sequential-channel (see Fig. 1.7). There were two variations of the parallel-channel rotor, one with side-by-side transfer channels (as shown in Fig. 1.7, right) and the other with over-and-under transfer channels (Fig. 1.8, right). There were six variations of the sequential-channel rotor, all of which resulted from the presently used normal standard rotor. The six rotors were labeled normal sequential, inertia enhanced (Fig. 1.8, left), inertia impeded and flow splitting (Fig. 1.9), extended width, and extended width and depth (Fig. 1.10). The purpose of the many modifications to the normal sequential rotor was to enable the automated rotor loading station to be used to load the rotors for studies of kinetically fast reactions. The side-by-side parallel-channel rotor cannot be loaded on this automated station.

The tests conducted with these rotors were based on earlier work involving the mixing of homogeneous dyes.⁶ Blue Dextran dye solutions [0.5 mg/ml and 2.0 mg/ml in 0.1 M tris (hydroxymethyl) methylamine buffer at pH 8.4] were used as substitutes for the sample-reagent pairs. The time required to reach a constant absorbance measurement for a pair subjected to transfer and mixing was defined as the transfer and mixing time, τ_m . The comparison of this constant absorbance measurement with that expected for a completely homogeneous solution of the two dye concentrations (computed by application of the Beer-Lambert law and also experimentally measured for externally mixed solutions of the two dyes) gave a measure of the completeness of mixing.

Fig. 1.7. Normal sequential channel (left) and parallel-channel (right) rotors.

Fig. 1.8. Inertia-enhanced (left) and parallel over-under channel (right) rotors.

Fig. 1.9. Inertia-impeded (left) and flow-splitting (right) rotors.

Fig. 1.10. Extended width (left) and extended width and depth (right) rotors.

Experiments were performed using internal standard solutions within each rotor. These internal standards consisted of premixed and premixed-pretransferred dye solutions. Each of the two types of internal standards was spaced at 90° increments around the periphery of the rotor. Time limitations did not permit evaluation of all eight variations in rotor design; however, six designs were evaluated, with the results shown in Table 1.5. These results indicate little difference in the time required to complete solution transfer for the parallel-channel and four modified rotors. As expected, solution transfer within the normal sequential rotor was slowest. The parallel-channel rotor appeared to produce slightly more effective mixing; however, the differences between the best three rotors in this category were only slight. Further experiments to confirm the effectiveness of mixing in the absence of a braking step are planned.

Table 1.5. Comparison of transfer and mixing for six rotor designs

Rotor type	τ_m^a (ms)	ϵ_Δ^b (%)
Parallel channel		
(side-by-side channels)	55.7	0.59
Inertia impeded	57.0	0.88
Flow splitting	61.7	0.94
Extended width and depth	56.2	1.53
Inertia enhanced	55.9	2.56
Normal sequential	73.4	1.37

^a Mean steady-state settling time minus time of first observable absorbance measurement.

^b $100(\text{absorbance of premixed-pretransferred dye minus absorbance of binary dye solutions after transfer})/\text{premixed-pretransferred absorbance}.$

1.1.6 Mechanical and electronic documentation

Mechanical documentation of the portable CFA has proceeded through three distinct stages. First, working sketches and notes were developed

with the prototype instrument. Second, using these sketches and notes, a second instrument was made; using parts and assemblies from this instrument, preliminary engineering drawings were made. These drawings were used to make the third instrument; during this process, any errors in individual part drawings were detected and corrected. During the assembly and checkout of this instrument, any functional errors associated with the parts and their interaction will be detected. The final corrections will then be made to the engineering drawings, and the mechanical documentation will be complete. Assembly of the third instrument is under way.

Electronic documentation is also proceeding. Preliminary engineering drawings documenting the wire-wrap connections for the analog, main logic, and main analog bins have been completed. Engineering drawings detailing the power supply, top panel interconnections, top panel wiring, and chassis-mounted components have been completed. They are also being checked during the operational tests of the third instrument described above. Necessary corrections in the documentation will be made after these tests are complete.

1.1.7 Fabrication

During this period, final modifications were made to the portable CFA fabricated for the Environmental Sciences Division; the instrument and a semiautomated washing station were delivered to the Division. The mechanical and electronic fabrication of a portable CFA to be delivered to the Research Triangle in Raleigh, N.C., has been completed. The instrument is being integrated with a minicomputer system; delivery of the final system is scheduled for early 1978.

Three portable analyzers are being fabricated. These are to be delivered to the University of Michigan, NASA, and the Bioanalytical Technology Group of ORNL's Chemical Technology Division. The chassis and sheet-metal panel fabrication for the main housings and power pack housings are being conducted in parallel on these instruments. This work is approximately 90% completed. The power supply housings have been completed and delivered to an instrument technician for wiring.

The analyzer mechanical parts for the University of Michigan instrument are approximately 90% completed. This instrument will be completed, assembled, and delivered for wiring and testing during early October 1977. Analyzer parts for which machine setup time was large were made in triplicate. Additional parts for the other two analyzers (NASA and ORNL) will be fabricated in parallel, with mechanical completion of these instruments tentatively scheduled for May 1978. The University of Michigan system will include an automated loading station and a wash station in addition to the portable CFA. Construction of these items is tentatively scheduled to begin in late 1977, with mechanical completion scheduled for early March 1978.

1.2 Applications

The versatility of centrifugal analyzers continues to play an important role in the further development of this technology. An enzyme amplification reaction is described which yields a parts-per-billion sensitivity for high-energy phosphates. The capabilities of the instrument to provide high sample throughput for kinetic enzyme assays are utilized in a unique study which helped to map the structural gene for brain formamidase to a locus on chromosome 14 of the mouse.

1.2.1 Genetics of formamidase-5 in the mouse

Formamidase (kynurenine formamidase, aryl-formylamine amidohydrolase, E.C.3.5.1.9) is assayed by its ability to catalyze the reaction

The study of the genetics of formamidase has been hampered by the absence of satisfactory specific stains for this enzyme. Thus, electrophoretic analysis, historically the most powerful tool in biochemical genetic studies, has had only a very limited usefulness in the study of this enzyme. However, kinetic assays of formamidase which make use of the high molar absorptivity of some of the products do offer an attractive alternative approach.

The small sample capability of the CFA was utilized to assay the individual brains from freshly killed mice. Formamidase was isolated by ammonium sulfate precipitation from homogenized brain preparations.⁷ Chromatographic separation of multiple brain preparations on DEAE cellulose revealed the presence of only a single formamidase isoenzyme (Fig. 1.11A). This was in direct contrast to other tissues (Fig. 1.11B, kidney; Fig. 1.11C, liver), where several separable isoenzymes were detected.

To study the genetics of brain formamidase in the mouse, sixteen 100- μ l aliquots of individual brain preparations were subjected to heat inactivation at 51°C. Eight sets of duplicate samples for each brain were heated at 51°C for 0, 5, 10, 15, 20, 25, 30, and 40 min. An aluminum heating block, maintained at 51°C, held 320 samples in 20 rows of 16 samples each. To ease the tremendous bookkeeping task, samples were tracked by block location only, with the 16 samples from a single brain sample (forming one column in the block) being assayed within a single CFA rotor. After completion of their individual heating periods, samples were removed by rows and placed in an identical block maintained at dry-ice temperatures. In this manner, two people could assay samples on the CFA at the rate of $\sim$ 120/hr.

Two alleles of brain formamidase were unequivocally identified by their very different heat lability at 51°C. Four purebred mice (C57BL/10 R ℓ) manifested a rather stable enzyme ($T_{1/2}$ = 18.2 min), while four animals from a different inbred strain (C3Hf/R ℓ) had a more heat-labile enzyme ($T_{1/2}$ = 5 min). F_1 hybrids derived by crosses between these two inbred strains displayed a brain formamidase intermediate in heat lability ($T_{1/2}$ = 11.4 min) and clearly distinguishable from either parent strain. Linear regression of pooled or individual mouse brain samples subjected to heat inactivation for 5 to 40 min at 51°C yielded high correlation coefficients, indicating the expected linear kinetics of heat inactivation. The variance among different animals of the same inbred strain was small, and the procedure was judged to be a reliable method for determining animal genotypes. Forty-six other inbred strains yielded brain formamidase with heat inactivation

B10-34180

Fig. 1.11. Chromatographic separation of tissue isoenzymes of kynurenine formamidase from (A) mouse brain, (B) mouse kidney, and (C) mouse liver, using kinetic assay on the CFA as a column monitoring device. Separation was performed on DEAE cellulose; the substrate in the kinetic assay was $\text{N}^1, \text{N}^\alpha$ -diformyl-L-kynurenine.

kinetics identical to one or the other of the two alleles found in C57BL/10 R ℓ and C3Hf/R ℓ . Twenty-six strains have the same alleles as C57BL/10 R ℓ , while 19 strains had the other allele. One strain (HRS/J) maintained with forced heterozygosity is also a heterozygote for brain formamidase.

If these differences are genetic and based on differences at a single locus, they should segregate in crosses, giving Mendelian ratios for the various genotypes among the progeny. That this is the case is shown in Fig. 1.12, where the heat inactivation profiles of 27 F₂ animals resulting from crosses between C57BL/10R ℓ are presented. The animals fall into three discrete classes corresponding to homozygotes for the heat-stable allele, homozygotes for the heat-labile allele, and heterozygotes. The expected ratios are 1:2:1, or for 27 animals, 6.75:13.5:6.75. The observed numbers were 5:13:9, which does not differ significantly from a 1:2:1 ratio ($\chi^2 = 1.22$).

Recombinant inbred strains, first described by Bailey,⁸ have now been established as an important investigative technique in mammalian genetics. Using heat inactivation coupled with kinetic measurement of the rate of enzyme inactivation, brain formamidase (For-5) genotypes were determined in two series of recombinant inbred strains of mice developed at the Jackson Laboratory (Bar Harbor, Maine). One series of strains was derived from crosses between strains C57BL/6J and DBA/2J (BxD strains), and the other series was derived from crosses between C57BL/6J and C3H/HeJ (BxH strains). Twenty-three BxD and 13 BxH strains were tested, and they segregated cleanly into the two parental alleles for For-5. Other work performed on these same recombinant inbred strains has resulted in the naming of esterase 10 (Es-10), alleles, the description of its variants, and the mapping of its gene locus on chromosome 14 of the mouse.⁹⁻¹⁴ Within these 36 inbred strains, a comparison of the two For-5 alleles and the two Es-10 alleles showed no recombination between their loci. This suggests, along with the inbred strain distribution pattern, that For-5 is very closely linked to Es-10 and is therefore on the proximal end of chromosome 14.

B10-34183

Fig. 1.12. Heat inactivation profiles (at 51°C) of brain formamidase from F₂ crosses between C3Hf/R ℓ and C57BL/10R ℓ mice. The approximate 1:2:1 ratios for these three genotypes suggest differences related to the existence of a single genetic locus for this enzyme.

In the past, the method of choice in biochemical genetic studies with higher eukaryotes has been gel electrophoresis. It is useful with small unpurified samples and, with suitable enzyme-specific stains, has good resolution. However, electrophoresis has a number of limitations, among which are the lack of suitable stains for some enzymes and its ability to detect only a fraction of the protein variants. It is well documented that some enzyme variants which cannot be resolved by electrophoresis can be easily detected using heat inactivation techniques. Bernstein et al.¹³ and Singh et al.,^{14,15} studying allozymes of octanol dehydrogenase in Drosophila, demonstrated that heat inactivation techniques yielded 2 to 3 times more alleles than were obtained by electrophoresis.

In additional studies of formamidase in the mouse, we have identified six isoenzymes in different tissues of inbred strains. The methods used to distinguish among them involved standard methods of protein purification, genetic techniques, and automated kinetic assays coupled with enzyme-perturbing treatments such as heat inactivation, limited proteolysis, and inactivation by specific enzyme inhibition. Of these three methods, the most useful technique was heat inactivation coupled with automated kinetic determination of enzyme activity. Alleles in different strains of mice have been observed at all six of these formamidase loci. Five of the six can be resolved by heat inactivation and the other by differences in the Michaelis-Menton constant.

This study has demonstrated the utility of fast kinetic assays coupled with enzyme-perturbing and genetic techniques (such as recombinant inbred strains) in the future progress of biochemical genetics. Likewise, these techniques can play an important role in the study of chemically or environmentally induced mutation in mammals.

1.2.2 Environmental analysis

Applications of centrifugal analyzer technology to environmental problems are a high-sensitivity assay for the high-energy phosphates ADP plus ATP (adenosine diphosphate and adenosine triphosphate) and an evaluation of an extraction procedure for ATP.

Enzyme amplification method for ATP and ADP. In a previous report¹⁶ we introduced a "recycle" kinetic procedure for the estimation of the two high-energy adenylate moieties, ADP and ATP. The enzymatic processes involved in the assay are as follows:

where

HK = hexokinase (EC 2.7.1.1),

G-6-PDH = glucose-6-phosphate dehydrogenase,

NADP^+ = β -nicotinamide-adenine dinucleotide phosphate
(oxidized form),

PK = pyruvate kinase (EC 2.7.1.40).

If the enzymes HK and PK and the substrates glucose and phosphoenol pyruvate are present in the proper proportions, any ADP present in the sample or produced by the reaction with ATP very rapidly will be recycled to form ATP. By this scheme, ATP is not depleted, and the monitored chromophore, NADPH, will continue to accumulate during the reaction. The rate of production of NADPH will be constant and proportional to the amount of ADP + ATP originally present in the sample.

In Table 1.6 the performance of the recycle kinetic procedure is compared for ATP, ATP + ADP, and ADP alone. Since the measured parameter, reaction rate, is a relative parameter, concentrations by this method are computed by comparison with the reaction rate of an ATP sample standardized by an absolute method (reactions 1 and 2 above, no PK present). As seen from the table, agreement between the recycle procedure and specific assay of individual components is excellent (1-2%). This demonstrates that ATP and ADP are determined equivalently by the recycle procedure. This procedure has the advantage, relative

Table 1.6. Comparison of assay results by specific assay and by recycle kinetic procedure for ATP and ADP

Standard solution	Sample concentration ($\text{M} \times 10^4$)	
	Specific assay ^a	Recycle kinetic ^b
ATP	5.44 ^c	≈ 5.44 ^d
ADP	2.09 ^e	2.11 ^f
Equivolume mixture of ATP and ADP		
Observed	3.78 ^g	3.86 ⁺
Expected	3.77 ^h	3.78 ^h

^a Sample volume 10 μ l (total volume 130 μ l); 10 min reaction time.

^b Sample volume 2 μ l (total volume 130 μ l); 2 min reaction time.

^c Hexokinase-glucose-6-phosphate dehydrogenase procedure.

^d Measured parameter is relative rate. Rate data converted to concentration units by assigning ATP standard a value of $5.44 \times 10^{-4} \text{ M}$, as determined by specific assay calibration (see footnote c).

^e H. U. Bergmeyer, ed., Methods of Enzymatic Analysis, p. 573 (1965).

^f Computed as apparent ATP concentration (see footnote d).

^g ATP concentration (specific assay): $2.75 \times 10^{-4} \text{ M}$;
ADP concentration (specific assay): $1.03 \times 10^{-4} \text{ M}$.

^h Computed from observed concentration of components prior to mixing.

to the specific assays, of being much more sensitive; with half the analysis time required for the stoichiometric procedures, the sensitivity is enhanced by an order of magnitude.¹⁷ A longer analysis time permits detection of yet smaller amounts of substrate.

In a preliminary study, activity determinations by the recycle procedure and the reference bioluminescence procedure were compared for an anaerobic denitrification culture. Environmental Sciences Division personnel had noticed a severe decline in apparent ATP recovery when the culture suspension was exposed to an oxygen-containing atmosphere, even for the short time necessary to harvest and wash the cells prior to extraction. Therefore, a grab sample of the suspension was used for analysis. The culture medium contained large amounts of nitrate ion, a known inhibitor for the bioluminescence assay.¹⁸

Although the ATP concentration in the extract was very low due to the anaerobic culture and the small number of cells in the grab samples, the recycle kinetic procedure was found to yield higher estimates of activity than those of the bioluminescence procedure. This is consistent with the expectation that the recycle kinetic procedure measures ADP as well as ATP, and is very much less affected by the nitrate ion in the culture medium extract.

Enzyme denaturation in ATP extraction with chloroform-carbon tetrachloride. In a previous report,¹⁹ several different extraction procedures were compared for efficiency of isolation of ATP from various substrate media. Recovery of ATP was judged superior by a new chloroform extraction procedure, as compared with extraction by sulfuric acid or butanol. The latter two extraction procedures had been previously considered the most efficient in an intercomparison of various procedures reported in the literature.¹⁶ Although the chloroform extraction procedure is relatively neat and efficient, it was necessary to demonstrate that cellular ATP-degrading enzymes were inactivated as well.

In the extraction procedure the substrate is sonicated and vortexed with buffer and chloroform. Protein should be denatured at the aqueous-organic interface. After discarding the chloroform layer, residual

dissolved chloroform (v1% wt %) is back-extracted with water-immisible carbon tetrachloride (see Table 1.7, footnote c). To test the effectiveness of the procedure for protein denaturation, we examined the extraction of apyrase enzyme (catalog No. A-6132, Sigma Chemical Co.). Enzyme activity was monitored as ATPase by analysis of residual ATP:

Table 1.7. Recovery of ATP after incubation in various media at 37°C for 30 min

Incubation medium	Residual ATP found (ppm) ^a	% recovery of ATP
Buffer ^b	236.2 ± 0.5	≡ 100
Buffer (extracted) ^c	240.1 ± 0.5	101.6
Buffer + apyrase ^d	-1.2 ± 0.4	-0.5
Buffer + apyrase ^d (extracted) ^c	211.6 ± 1.6	89.6

^a As determined by Calbiochem ATP Stat-Pack procedure.

^b 0.167 M tris, pH 7.4.

^c Extraction procedure: 6-ml buffered sample (see footnote b) + 3 ml chloroform; shake 30 sec in 10-ml separatory funnel; remove lower organic phase; add 2-ml carbon tetrachloride; discard lower organic phase.

^d Apyrase added: ~2 U/ml as ATPase.

As seen in Table 1.7, buffer treated by the extraction procedure does not contain any residual inhibitory species. In the presence of apyrase, ATP is commonly degraded. However, apyrase treated by the extraction techniques retains only a trace of activity. In the

experiment described in Table 1.7, a quantity of apyrase sufficient to hydrolyze 60,000 micromoles of ATP during the incubation period could only hydrolyze 32 micromoles of ATP after being subjected to the extraction procedure, for a residual activity of only 0.05%. Large quantities of enzyme and long incubation at elevated temperature were required to demonstrate any residual activity, and normal amounts of cellular ATPase released during extraction would, in effect, be totally inactivated.

Determination of organic acids. The Environmental Sciences Division, prior to receipt of a portable CFA unit, provided us with a list of analytical development priorities for future interaction with our section. High on the list of needed analytical capabilities are assays for several organic acid species, including oxalic, malic, oxaloacetic, and glycolic acids. We selected malic acid for our initial attention.

Bergmeyer¹⁷ describes a method for the determination of L (-) malate with the enzyme malate dehydrogenase (MDH):

The equilibrium of the reaction favors production rather than consumption of malate. Oxaloacetate may be determined with MDH by use of excess NADH and near-neutral pH medium. In order to drive the reaction to stoichiometric consumption of malate, it is necessary to remove the reaction product, oxaloacetate. This may be done by use of a ketone-trapping reagent (e.g., hydrazine) in basic medium (pH 9.5):

The initial reaction conditions for the analysis of L (-) malate are given in Table 1.8. The buffer pH is adjusted just prior to use. The enzyme MDH and cofactor NAD are then added to the buffer (both of these components are relatively less storage stable in high-pH medium). Initial studies with D,L-malic acid gave quantitative results that were 47% of the value expected with 100% L (-) isomer. With L-malic acid the results were 90% of that expected for a stoichiometric reaction as

Table 1.8. Reaction conditions for kinetic assay of L-malate

Constituent	Initial reaction concentration
L-malate dehydrogenase	67 U/ml
NAD	2 mg/ml
Buffer, pH 9.5	
Hydrazine sulfate	0.2 M
Glycine	0.5 M
EDTA	1 mg/ml

indicated in Eq. (5). Under the assay conditions used, MDH has a high affinity for L-malate ($K_m = 0.16 \text{ mM}$).

There is a peculiarity of the assay of L-malate by the above reaction scheme. Analysis by a two-point stoichiometric procedure,¹⁸ with a 5-sec decay interval, results in an underestimate of substrate concentration by about 20%. This is apparently due to an initial fast-kinetic reaction phase (prior to the 5-sec delay time), before a much slower approach to equilibrium. The measured parameter, ΔA , is, however, directly proportional to L (-) malate concentration, and a control sample could be used to correct for this phenomenon. When the analysis procedure is transferred to the portable CFA, with its fast-kinetic capabilities, this problem may be obviated. Again, it must be remembered that the enzyme is specific for the L-isomer and will underestimate total malate in a racemic mixture (as indicated previously).

1.2.3 Assay of specific proteins on the CFA

The feasibility of performing inert-carrier-based assays on the CFA has been previously discussed.¹⁹ The development of such assays ultimately depends upon defining a parameter which characterizes the sedimentation of a carrier particle coated with a protein mass. For a given hybrid particle (carrier particle with protein mass), the measurement of this

parameter must be reproducible before the attached mass can be quantified. Initial work in this area has been performed with hybrid particles developed by incubating latex beads ($0.11\ \mu$ in diameter; density of $1.05\ \text{g/cm}^3$; approximately $50\ \mu\text{g/ml}$) with a purified sheep IgG (approximately $2000\ \mu\text{g/ml}$) in a total volume of 1 ml. Samples (approximately $10\ \mu\text{l}$) of the resulting suspension of hybrid particles were placed in two of the sample wells of a prototype rotor developed for these assays.¹⁷ Sucrose solutions (approximately 11% wt %) were used to fill the two cuvetts at the end of the sedimentation channels aligned with the loaded sample wells. All solutions were loaded statically; the rotor was then placed in the CFA and accelerated to 200 rpm to transfer the samples to the cuvet edges. The rotor was then accelerated to a final speed of 2500 rpm. The cuvetts were monitored continuously to determine the absorbance curves generated by the passage of the hybrid particles through the optically monitored zones of the cuvetts. The two resulting absorbance curves are shown superimposed in Fig. 1.13. The difference in total absorbance at any time is probably caused by errors in pipetting the 10-ml samples into the sample wells, or by incomplete transfer from the wells. The potential for using the time associated with the appearance of the zone peak is obvious from this figure. However, both the rate at which particles enter the monitored zone (slope of curve at early time) and the rate at which particles leave the monitored zone (slope of curve at late time) appear to be reproducible characteristic parameters. A detailed curve analysis to extract sedimentation and diffusion coefficients by the method of moments could also be performed if required, particularly when small differences in characteristic parameters between two curves are being examined.

This initial feasibility work will be expanded in future developments. The total development will ultimately demonstrate the limits of quantitation associated with dynamic loading of the sedimentation channels and transfer of samples from sample wells to sedimentation channels by rotor acceleration. The increase in sensitivity possible with the use of fluorescent carriers will also be considered.

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Fig. 1.13. Absorbance curves generated by the passage, under centrifugal force, of hybrid latex particles [0.11- μ m latex spheres equilibrated with purified sheep (gG) through the optically monitored zone of a prototype CFA rotor].

1.2.4 Determination of arylsulfatase in human body fluids

The type II arylsulfatase enzymes (ASA and ASB) have been proposed as potentially useful biochemical markers for several types of cancer. Increased sulfatase activity in urine has been reported in myeloid leukemia; diseases of the bladder, testes, and uterus; and in cancer of the breast and of the prostatic gland.²⁰ Recent reports have indicated an elevation of urinary ASB isoenzyme in colorectal cancer²¹ and ASA isoenzyme in malignant melanoma.²² A congenital deficiency of ASA occurs in metachromatic leucodystrophy, and ASB is defective in the Maroteaux-Lamb syndrome.

In a previous report,²³ we discussed some of the methodological difficulties in the assay of arylsulfatase and in the independent estimation of the two type II isoenzymes. A primary concern is the susceptibility of the enzymes to inhibitors of human arylsulfatase enzymes. Table 1.9 gives the normal range of some of the inhibitory species in human blood serum and urine. A comparison of this table with Tables 1.10 and 1.11 indicates that the assay of sulfatase in untreated body fluids is of dubious value. For example, it has been reported that in several types of cancer, sulfatase in blood serum did not exceed normal, although the activity in tumors and in dialyzed urine was elevated above normal.²⁰ However, the serum was not dialyzed or otherwise treated prior to analysis, and any elevation of sulfatase activity would be obscured by the endogenous anionic inhibitors.

We have recently reported²³ a simple anion exchange chromatographic procedure to separate ASA and ASB for subsequent assay, using the substrate p-nitrocatechol sulfate (NCS). At pH values exceeding the isoelectric pH (pI) of a macromolecule, the molecule assumes a net negative charge and thus will be retarded on an anion exchange resin. Table 1.12 lists pI values reported for human arylsulfatase enzymes. It should be possible to separate ASA and ASB by anion exchange at $5 < \text{pH} < 7.6$. We have used anion exchange at pH 7.5 to separate ASA and ASB from limpet extract and human serum and urine.²³ In addition, the potent inhibitor phosphate ion is separated from ASB, although it is

Table 1.9. Occurrence of arylsulfatase (type II) — inhibiting anions in body fluids of man^a

Anion	Normal concentrations (mM)	
	Plasma or serum	Urine
Cl ⁻	104 (95-113)	141 (56-254)
PO ₄ ³⁻	1.0 (0.8-1.4)	19 (16-24)
SO ₄ ²⁻	0.1 (0.09-0.15)	17.3 (5.5-27)

^a Data from P. L. Altman, Blood and Other Body Fluids, Fed. Amer. Soc. Exper. Biol., Washington, D.C. (1961). Data reported as average value (range given in parentheses).

Table 1.10. Effect of selected salts upon the activity of purified human arylsulfatase A

Source	Assay conditions	Salt added	Concentration (mM)	Residual activity (%)	Reference
Placenta	10 mM NCS pH 5.0	KH ₂ PO ₄	0.005	98	20
			0.5	51	
			5	7	
		Na ₂ SO ₄	0.5	100	20
			5	89	
			100	13	
		NaCl	5	100	20
			100	106	
		Ag(CH ₃ COO)	1	2	26
Brain	10 mM MUS pH 5.7	KH ₂ PO ₄	0.25	4	27
		AgNO ₃	0.25	4	27

Table 1.11. Effect of selected salts upon the activity
of purified human arylsulfatase B

Source	Assay conditions	Salt added	Concentration (mM)	Residual activity (%)	Reference
Liver	10 mM NCS pH 6.0	NaH_2PO_4	2.5	14	32
		Na_2SO_4	100	39	32
		KCl	80	52	32
	10 mM NCS pH 6.1	BaCl_2	25	59	33
Brain	10 mM MUS pH 5.7	KH_2PO_4	0.25	9	27
		AgNO_3	0.25	133	27

Table 1.12. Some properties of purified human arylsulfatase

Isoenzyme	Source	pI	Substrate	Km ^a (mM)
ASB	Placenta	α : 7.6	NCS	1.04
		β : 7.9	NCS	1.96
		γ : 8.1	NCS	0.65
	Placenta	8.2	1	
	Brain	6.8-7.2 (3 bands)		
	Brain		MUG	8.3
	Liver		NCS	2.9
	Liver		NCS	0.8
ASA	Placenta	4.7	NCS	1.4
	Urine	α : 4.7		
		β : 4.8		
		γ : 4.9		
	Liver	4.4-4.9 (6 bands)		
	Brain		MUS	12.5

^aKinetic constant from Michaelis-Menten expression.

subsequently coeluted with ASA by an NaCl gradient. Thus, ASB isoenzyme can be determined in body fluids without the necessity of prior dialysis. Dialysis, although an effective means of removing inorganic ions, is too slow and requires too large a sample size to be very useful in screening numbers of specimens for sulfatase activity.

Table 1.13 compares arylsulfatase assay with exopeptidase assay for samples of urine and blood from colorectal cancer patients. Arylsulfatase in urine was determined from dialyzed specimens; we have not yet established a normal range for urinary samples by our procedure of chromatographic separation followed by kinetic monitoring, using NCS as substrate. Using literature estimates of the normal range of ASB in urine, it appears that the enzyme is perhaps elevated in 62% (8/13, correcting for low creatinine in 660) of the samples. In several cases (e.g., samples 660 and 1135), apparently subnormal arylsulfatase activities are accompanied by subnormal urinary creatinine excretion. Urinary creatinine excretion is often used as a check for the completeness of 24-hr urine collection. A very low creatinine concentration may reflect an abnormal dilution of constituents due to excessive urine volume. Urine samples from cancer patients were provided by the National Cancer Institute, and we do not have data on 24-hr urine volume. For the three samples (705, 708, and 1043) with normal urinary creatinine excretion, elevated ASB was measured. In serum, ASB is elevated in 50% (6/12) of the samples tested, γ -glutamyl transferase (GGT) in 67% (6/9), leucine aminopeptidase (LAP) in 56% (5/9), alkaline phosphatase (AP) in 71% (5/7), and 5'-nucleotidase (5'-Nase) in 60% (3/5) of the cases. The high incidence of exopeptidase elevation probably reflects a similar incidence of liver metastasis. Again, the demonstration of elevated arylsulfatase activity in treated sera from cancer patients is in contrast to the findings of other workers who had not taken precautions to eliminate the effects of endogenous inhibitors.²⁰ Figure 1.14 illustrates the linear hydrolysis of NCS by chromatographically separated ASB from human serum; none of the "anomalous kinetics," as reported for the ASA isoenzyme,^{24,25} were observed in the ASB fraction.

The sulfatase isoenzymes are easily separated by ion exchange chromatography at near-neutral pH values (pH 6.8, Gniot-Szulzycka;²⁶ pH 7.4, Harinath and Robins;²⁷ pH 7.5, Stevens;²⁸ pH 7.55, Humbel²⁹). However,

Table 1.13. Enzyme screening in patients with colorectal cancer

Patient ID	Urine sample		Creatinine (ng/dl)	Serum sample				
	ASA	ASB		ASB	GGT	LAP	AP	5'-Nase
	(NC/ml/hr)			(NC/ml/hr)		(IU/liter at 30°C)		
Normal range								
Literature	3.8-22.2 ^{a,b}	0.3-2.8 ^{a,b} 1.8-4.8 ^e	81-162 ^{t,c}	0.5-2.0 ^d				
This study	ND	ND	ND	1.9-5.9	0-38 ^c	15.0-28.6	10-100	<14
Colorectal cancer								
705	44.0 (E?)	19.7 (E?)	150.4	7.4 (E)	435.8 (E)	79.5 (E)	385.5 (E)	15.3 (E)
321	8.1	7.2 (E?)	41.1	13.2 (E)	355.4 (E)	87.5 (E)	449.2 (E)	19.1 (E)
660	2.9	3.7	37.9	4.9	9.9	23.6	67.9	ND
661	22.4 (E?)	14.3 (E?)	ND	6.3 (E)	204.8 (E)	49.9 (E)	160.0 (E)	8.0
755	21.1	21.1	ND	4.6	339.7 (E)	66.4 (E)	550.0 (E)	53.6 (E)
1131	ND	0.9	ND	8.4 (E)	28.0	18.9	42.0	<5
318	ND	ND	ND	4.4	398.2 (E)	104.5 (E)	320.0 (E)	9.4
708	9.3	21.5 (E?)	97.3	2.8	ND	ND	ND	ND
951	0.2	1.7	ND	5.8	25.6	19.3	ND	ND
388	0.1	0.5	ND	4.3	40.9 (E)	22.4	ND	ND
1043	14.7	22.6 (E?)	140.6	6.3 (E)	ND	ND	ND	ND
1085	0.9	0.5	44.3	8.9 (E)	ND	ND	ND	ND
871	52.7 (E?)	16.0 (E?)	ND	ND	ND	ND	ND	ND
1135	10.1	0.7	ND	ND	ND	ND	ND	ND

^aH. Baum, K. S. Dodgson, and B. Spencer, Clin. Chim. Acta 4, 453 (1959).^bAssumed mean volume for 24-hr urine specimen = 1500 ml.^cP. L. Altman, Blood and Other Body Fluids, F.A.S.E.B., Washington, D.C. (1961).^dJ. Singh, D. Tavella, and N. DiFerrante, J. Pediatr. 86, 574 (1975).

Fig. 1.14. Linear rate of hydrolysis is illustrated for the reaction of chromatographically separated (from serum) arylsulfatase B isoenzyme with nitrocatechol sulfate in contrast to the nonlinear kinetics reported for arylsulfatase A isoenzyme.

we found that storage stability of urinary sulfatase eluent fractions at pH 7.5 was not particularly good. Two possible explanations for loss of enzymatic activity in the fractions are low protein content in urine (nonspecific protein could help stabilize the enzyme) and possible denaturation of enzyme at higher pH values (both isoenzymes have activity maxima in the pH range 5-6).

The chromatographic separation of dilute purified limpet sulfatase in buffer plus 0.2% bovine serum albumin (BSA) is illustrated in Fig. 1.15. Under the conditions used (pH 7.5), BSA coelutes predominantly with ASA. The activity is apparently enhanced by the presence of nonspecific protein. Stevens et al.³⁰ report that ASA in crude preparations is stable, whereas purified, dilute solutions (<0.1 mg of protein/ml) are quite unstable.

As mentioned previously, a second factor likely to influence the stability of eluate fractions is the pH of the eluate. Figure 1.16 compares the activity of sulfatase eluent fractions separated on ion exchange columns equilibrated at pH 5.5 and at pH 7.5. The dilute limpet sulfatase applied to the columns was prepared in the appropriate starting buffer (0.05 M acetate, pH 5.5, or 0.05 M tris, pH 7.5). A large enhancement of recovered sulfatase activity was observed in the pH 5.5 eluate fractions, as opposed to the same amount of enzyme eluted at pH 7.5 (the activity of all fractions was measured with substrate buffered with 0.5 M acetate, pH 5.6). The recovery of ASB was especially enhanced. Also illustrated in this figure is the fact that phosphate ion is retained on the pH 5.5 column until it is eluted with salt, just as in the case for the pH 7.5 eluent.²³

Rinderknecht et al. reported a rapid method for removing interfering anions from human biological fluids by applying the sample to an anion exchange column and eluting with acetate at pH 5.6. The eluate from serum was reported to be stable for at least two weeks at +4°C, whereas the urine eluate was stable for only about two days;³¹ the enhanced stability for the serum eluate is probably due to a greater concentration of nonspecific protein. Since these workers did not use concentrated

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Fig. 1.15. Separation of limpet arylsulfatase isoenzymes by ion exchange chromatography on fibrous DEAE cellulose. Virtually quantitative recovery of enzyme activity was ensured by the presence of a small amount of bovine serum albumin.

Fig. 1.16. Dramatically improved recovery of arylsulfatase iso-enzyme activity was demonstrated when separations were performed at pH 5.5 instead of pH 7.5.

salts in their sample elution, one would expect (from Fig. 1.15) that only ASB would be eluted; indeed, from their results the authors concluded that ASB was the preponderant enzyme in blood.

From these studies we have decided to separate the soluble sulfatase isoenzymes in human body fluids on anion exchange media, equilibrated at pH 5.5. If data on ASA activity are required, urine samples will be dialyzed vs dilute buffer at pH 5.5 to 6.0. We will also investigate the effect of adding a small amount of nonspecific protein to the dialysis tube containing the urine sample prior to dialysis, in an attempt to stabilize enzyme activity. As reported previously,²³ we found the greatest sulfatase activities in those urine samples with large amounts of cell debris and evidence of hemolysis. It is possible that one contributing factor to the higher enzyme activities in these samples was the greater concentration of protective nonspecific protein.

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2. ADVANCED ANALYTICAL SYSTEMS

J. E. Mrochek

Investigations into rapid chromatographic separation of isoenzymes with on-line monitoring of enzyme activity are continuing, with current emphasis on the isoenzymes of arylsulfatase. A new concept in rapid, automated electrophoresis is described with the capability of not only increasing sample throughput, but also uncovering the "hidden heterogeneity" of proteins by improved resolution.

2.1 High-Resolution Liquid Chromatographic Systems

The expansion of liquid chromatographic technology is continuing into new and exciting areas. One of the more recent developments is its application to the separation and continuous on-line monitoring of enzyme activity. Such applications hold the promise of not only better separations, but also improved quantitation compared with conventional electrophoretic methods. In the long run, these may result in improved clinical diagnostic procedures for noninvasive diagnosis of organ damage and disease.

2.1.1 Continuous separation and assay of arylsulfatase isoenzymes

As indicated previously,¹ p-nitrocatechol sulfate (NCS) is the usual analytical substrate used to measure the activities of the two isoenzymes of arylsulfatase (type II). Both isoenzymes demonstrate high affinity for this substrate; however, kinetic differentiation of the individual isoenzymes without prior physical separation is "problematic."² Baum et al.³ introduced a system of differential inhibitors and activators to discriminate between the isoenzymes, and this system has been utilized by almost all researchers investigating the roles of the isoenzymes in disease states. However, these inhibitors and activators are not exclusive in their reactivity with the individual isoenzymes, and thus their actual utility is questionable.³⁻⁶

We have adopted the approach of physical separation of the sulfatase isoenzymes, followed by analysis of the enzyme fractions by either specific or nonspecific reagents. The use of discontinuous chromatography on anion exchange minicolumns, as described in Sect. 1.2.4, is relatively slow, and activity determination of dilute eluate fractions by use of NCS substrate often required long incubation times. We became interested in the possibility of continuous chromatography of sulfatase isoenzymes with on-line column effluent monitoring. As an alternative to the use of NCS as a substrate, we first investigated the use of 4-methylumbelliferone sulfate (4-MUS).

The human arylsulfatase enzymes have a lower affinity for 4-MUS than for NCS (see Table 2.1) and a lower specific rate for hydrolysis of MUS (typically 5 to 10% of the specific rate for NCS substrate). However, assay of the liberated reaction product, 4-methylumbelliferone (4-MU), is much more sensitive than for nitrocatechol by a factor of 2 to 3 orders of magnitude,⁷ and, in contrast to nitrocatechol, 4-MU can be monitored directly in the reaction medium without addition of reaction-quenching chemicals. Figure 2.1 illustrates the relative fluorescence spectra of fluorogenic 4-MUS and the product 4-MU. In terms of absolute fluorescence intensity, our 4-MUS substrate (Sigma Chemical Company) has a response (relative to 4-MU) of only 0.002% at 440 nm (the emission maximum for 4-MU). The availability of a Schoeffel FS 970 LC flow fluorometer made it possible to detect even minute amounts of 4-MU product (Fig. 2.2). Another attractive feature of the use of 4-MUS as a substrate is that it yields a linear rate of hydrolysis for ASA (arylsulfatase), without the "anomalous kinetics" noted for NCS substrate.⁸ It was also hoped that by use of a continuous salt gradient, we could detect minor anionic forms of ASB found in fibroblasts⁹ and brain,¹⁰ if not perhaps the microheterogeneity of the major isoenzymes.

Figure 2.3 illustrates the fluorometric response for limpet arylsulfatase isoenzymes separated on glycopase DEAE-CPG with activity monitored by the 4-MU liberated from 4-MUS. Sensitivity is limited to some extent

Table 2.1 Some properties of purified human arylsulfatase

Isoenzyme	Source	pI	Substrate	K _m (mm)	Reference
ASB	Placenta	α : 7.6	NCS	1.04	Gniot-Szulzycka (1972)
		β : 7.9	NCS	1.96	
		γ : 8.1	NCS	0.65	
	Placenta	8.2	MUS		Stevens (1977)
	Brain	6.8-7.2 (3 bands)			
	Brain		MUS	8.3	
ASA	Liver		NCS	2.9	Shapira and Nadler (1975)
	Liver		NCS	0.8	Agobna and Wynne (1975)
	Placenta	4.7	NCS	1.4	Gniot-Szulzycka (1974)
	Urine	α : 4.7 β : 4.8 γ : 4.9			Stevens (1976)
	Liver	4.4-4.9 (6 bands)			
	Brain		MUS	12.5	
					Harinath and Robins (1970)

Fig. 2.1. Fluorescence excitation and emission spectra for 4-methylumbelliferone sulfate (4-MUS) and its hydrolysis product, 4-methylumbelliferone (4-MU).

Fig. 2.2. Sensitive detection of and linear response to 4-methylumbelliferone was demonstrated using a fluorescence flow monitor.

Fig. 2.3. Continuous monitoring of arylsulfatase isoenzyme activities after chromatographic separation. Activity is detected by monitoring the hydrolytic release of 4-methylumbelliferone.

by the poor solubility of the substrate, but is more than adequate for easy analysis. Note, however, that even highly dilute purified enzyme gave a slight background response coincident with the elution volume of ASB. When the system was used with human urine, the endogenous fluorescence was simply overwhelming (Fig. 2.4). The urine illustrated in Fig. 2.4 is from a colorectal cancer patient and had endogenous fluorescent compounds which coeluted with both ASB and ASA. The corresponding eluent fractions of raw urine (no substrate) were collected and found to have both excitation and emission spectra which overlapped those for 4-MU. As shown in Fig. 2.4, the endogenous fluorescent compounds could largely be removed by dialysis; thus, in contrast to the procedure described in Sect. 1.2.4, even the analysis of ASB in urine would require exhaustive dialysis before the detector response would be specific for arylsulfatase activity.

These difficulties caused us to investigate the possibilities of using NCS substrate for photometric on-line monitoring of sulfatase activity eluted from the chromatographic system. The problems anticipated were the reduced sensitivity (relative to fluorescence) and possible interference from background absorbance. A unique detection scheme, shown schematically in Fig. 2.5, was designed to produce what is essentially a double-beam photometric detector for column effluents. The sample (including reaction product) first passes through the reference beam of a flow-through photometer, and then a small volume of color developer (NaOH) is added immediately before the stream reenters the photometer through the sample beam. Since it is unlikely that any constituent other than nitrocatechol will give a change in absorbance at 510 nm upon addition of base, the detector response is very highly selective for sulfatase activity. An integrating time constant for the signal between reference and sample beams makes the system unresponsive to slugs of nonspecific chromophores eluted from the column. Figure 2.6 shows the detector response to chromatographically separated sulfatases in raw (nondialyzed) normal urine spiked with purified limpet sulfatase. Also shown is the response of the system to

Fig. 2.4. Chromatographic elution conditions used to separate arylsulfatase A and B result in the detection of interfering endogenous fluorescent compounds in urine from a colorectal cancer patient. The endogenous fluorescent compounds may be removed by dialysis.

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Fig. 2.5. Schematic representation of a continuous monitoring system for chromatographically separated arylsulfatase isoenzymes. Time delay between reference and sample channels (due to liquid transport) should be corrected electronically.

Fig. 2.6. Continuous monitoring of arylsulfatase isoenzyme activity in urine supplemented with exogenous enzyme activity. In the absence of substrate, no interferences were detected.

raw urine in the absence of sulfatase substrate; in contrast to the fluorescence detection results shown in Fig. 2.4, no false peaks were obtained. Again, for contrast, the urine sample used in Fig. 2.6 was diluted tenfold, and its excitation and emission spectra were scanned (see Fig. 2.7). Comparison of Figs. 2.1 and 2.7 indicates that endogenous fluorophores in urine would overwhelm the response of small amounts of 4-MU in an on-line fluorimeter. We are working to improve the sensitivity of the on-line photometric monitoring system, using NCS substrate. The "anomalous kinetics"³ of ASA isoenzyme will not be pronounced if incubation times can be reduced to 20 to 40 min; alternatively, pyrophosphate may be added to buffer A. In addition, we will investigate the utility of a short column containing gel filtration medium to be located directly after the ion exchange column in Fig. 2.5 (prior to reagent addition). It is hoped that by this means, ASA and anionic inhibitors coeluted from the ion exchange column may thus be separated.

The on-line, pseudo double-beam reactor-detector concept illustrated for sulfatase isoenzymes in Fig. 2.5 will be extended to other systems. Previously,¹ we reported the on-line column effluent monitoring of creatine kinase (CK) isoenzymes. Activity determination is based upon the production of NADPH (β -nicotinamide-adenine dinucleotide phosphate hydrogenase) by a coupled reaction:

The reagent in this scheme was rather expensive to use in a continuous-flow detection system, primarily due to the use of soluble enzymes hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH). With on-line fluorometric detection, endogenous fluorophores in serum were detected which coeluted with the CK isoenzymes.

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Fig. 2.7. Emission and excitation spectra for diluted normal urine reveal endogenous fluorescence which should mask enzymatically released 4-methylumbelliferone in a continuous detection system.

The use of a reaction loop containing coimmobilized HK and G-6-DPH on agarose beads (commercially available from Sigma Chemical Company) between reference and sample beams in a system similar to that illustrated in Fig. 2.5 would accomplish several goals. First, reagent cost would be drastically reduced, since the expensive enzyme components will be continually reused for hundreds of analyses. Second, the system will respond specifically to NADPH produced in the reactor and not to NAD(P)H produced by side reactions during the incubation of sample and reagent. Again, nonspecific endogenous chromophores will be effectively blanked out and will not produce false peaks. The reactor concept may be used to advantage in other detection schemes as well.

2.2 Automated Gel Electrophoresis System

An interesting new concept in automated gel electrophoresis is described. It offers the potential for large sample throughput, but, more importantly, the concept promises to yield additional data by which mutant proteins may be recognized in genetic studies.

2.2.1 Background and conceptual design

In the study of population biology, one of the more important screening methods relies on electrophoresis to discriminate mutant proteins by the appearance of substrate-specific bands with abnormal electrophoretic mobility. Because it permits the direct visualization of a broad array of single gene products, electrophoresis has been used by population geneticists to "count" genetic variants in nature. However, over the past several years, it has become apparent that a considerable amount of genetic variability exists in natural populations which is not detected by routine electrophoretic screening.¹¹⁻¹⁴

The power of electrophoretic methods is in providing direct evidence of some difference between normal and variant proteins, by showing that the mutant moves to a different band position when electrophoresed under

identical conditions. However, when two proteins band at the same position on a gel, the conclusion that they are identical is much weaker, and its validity depends on the amount of variation undetected by the technique. The use of electrophoresis to document protein similarity is now becoming widespread despite this apparent weakness in the method when used under a single set of conditions, as is the practice in routine screening.

Although the initial report of electrophoretically cryptic variation appeared only a few years ago,¹¹ current data suggest the result is a general one, and the universal occurrence of large amounts of previously undetected variability seems likely.¹⁵ The existence of such "hidden heterogeneity" constitutes a serious problem in the interpretation of many of the comparative electrophoretic approaches now used in population biology, because most researchers have relied on assessing protein homology from their electrophoretic data. Such proof of identity may not be valid if the elements being compared are from heterogeneous groups!¹⁴

Comparative recent discoveries of large amounts of "hidden heterogeneity" suggest rather strongly that new approaches are mandated in comparative electrophoretic studies. Johnson¹⁶ has suggested that any new approach should meet, at a minimum, two requirements: (1) It should be capable of resolving all variation which is, in principle, detectable; (2) characterizations of variants should be directly comparable from one study to another. He suggests that four physical parameters of a protein — size, asymmetry or conformation, isoelectric point, and net charge or free mobility — represent the characteristics which determine its mobility and empirically are sufficient to describe it.

The migration of a protein during polyacrylamide electrophoresis has been expressed by Rodbard and Chrambach^{1,17} as

$$R_f = M_o / U_f e^{\frac{K T}{r}},$$

where

R_f = mobility of the protein relative to a solvent front,

U_f = apparent mobility of a moving boundary in front of the resolving phase (a constant known for most common buffer systems),

M_o = free electrophoretic mobility of the protein,

K_R = retardation (frictional/hydrodynamic) coefficient
($K_R = K_r/2.303$),

T = % acrylamide (determines pore size and is inversely proportional to it).

The degree to which a protein interacts with gel fibers while migrating under the influence of an electrophoretic field may be studied by varying the pore size (% acrylamide) of the gel. By examining R_f at several values of T , the free electrophoretic mobility (M_o) and the retardation coefficient (K_R) may be estimated for any protein which can be discretely detected in a heterogeneous mixture. In experimental practice, a linear regression of $\log R_f$ on T yields a line with slope K_R and intercept $\log M_o/U_f$. The retardation coefficient is directly related to the size and shape of the protein.¹⁷ This provides a foundation for a powerful comparative electrophoretic technique, capable of meeting the first of Johnson's two criteria. The second criterion is met by including two internal standard proteins in each gel migration path. The inclusion of internal standard proteins serves to standardize individual determinations of R_f on different gels by making all measurements on migration of desired proteins relative to that of an internal standard with similar properties. The ratios of the mobilities of the two internal standards provide a sensitive measure of experimental error which can be used as weighting factors in the linear regression analysis to obtain M_o and K_R .

To implement this technique in the study of population biology requires that each sample be electrophoresed on from four to six different

concentrations of acrylamide with two internal standard proteins on each gel. Just the logistics of such analyses demands the use of automated techniques in casting the gels, electrophoresing, staining, and scanning the stained gels for measurement of protein mobilities.

The conceptual design of an electrophoresis system to accomplish large sample throughput while maintaining the necessary high resolution is based on a modular design. Four modules are envisioned, with each dedicated to one of the above tasks.

The fundamental unit of the prototype system will be a circular disk approximately 7 in. in diameter, consisting of a black body (1/8-in. plastic) with 12 radial channels (2.5 x 0.25 in.) and a clear plastic disk glued to its bottom. To cast the gels, a stack of rotors (six in the prototype unit) would be assembled with a removable plastic top for each rotor. The stack would be placed in the casting module where it would be rotated at a speed sufficient to ensure even splitting of the acrylamide solution among the 12 channels in each rotor. The acrylamide solution would be metered through six individual openings (one for each rotor) in a nozzle extending downward into the rotor stack through its open center. Here, rotation of the stack would serve not only to distribute the acrylamide equally, but also to minimize the meniscus at the end of the gel column during polymerization. After casting the gel columns, a stacking gel section would be dynamically loaded at the end of each gel column, using the same nozzle. This gel would be cast with an integral 10- μ l sample well. After completion of the casting step, the rotor stack would be disassembled and the top plate removed, replaced with a different top plate through which a coolant could be circulated, and the stack reassembled.

Electrophoresis of a stack of rotors would be performed in an enclosed vessel with a single center electrode and 12 electrodes around the periphery of the chamber, opposite each gel column in a rotor. The open center of the rotor stack would serve as one electrode chamber, while the vessel itself would serve as the other chamber. A coolant

fluid would be circulated through the rotor stack, with sealing between rotors and coolant chambers accomplished by means of "O" rings.

After completion of the electrophoresis, the stack of rotors would be stained and then disassembled for scanning. Individual rotors would be placed on a holder which would be rotated through a stationary optical head and detector. Each rotation of the rotor holder would move the optical head a precise distance in toward the center of the rotor. Scanning of all 12 gels would be completed in 1 min at a resolution of 100 points/in. Data would be acquired and stored by microcomputer. All peak locations would be ratioed to their respective internal standards to yield data with which to perform the regression analysis of $\log R_f$ vs percent acrylamide for individual samples.

2.2.2 Dynamic loading of gels

Dynamic loading of gels into rotors offers two primary advantages: first, air is eliminated from the gel during the polymerization process; and second, the interface developed for sample application approximates a plane perpendicular to the direction of electrophoretic migration. Notably, these advantages can be realized simultaneously in all electrophoretic channels of the rotor. Also, the location of the sample interface can be standardized from channel to channel if the fluid mixture introduced into the rotor for gel formation can be equally apportioned among the channels.

Three approaches to dynamically apportioning a total volume (desired volume per channel multiplied by number of channels) among a selected number of channels (12 in the prototype rotor) were considered. The first approach involved premeasuring 12 aliquots and delivering each aliquot through a separate tube and nozzle outlet into its designated channel, the nozzle assembly motion being mechanically coupled with the rotor motion. The second approach involved controlled dispensing of the total volume by syringe into the rotor center onto the bottom rotor plate and allowing the fluid to move outward as an annular wave formed under the centrifugal force field; the volume of this wave would

then be divided equally among the 12 channels if the wave was uniform and the channels had equal opening widths. The third approach involved forcing the fluid outward from a single nozzle outlet so that it jetted perpendicularly toward the channel openings. At uniform angular velocity, each channel opening is exposed to the jet for an equal total time, and therefore each channel receives an equal fluid volume if the jet discharge rate is regular.

The first approach was not experimentally investigated, since it was anticipated that it would become excessively difficult mechanically, even for dynamic loading of a single rotor, and especially for simultaneous loading of two or more rotors arranged in a vertical array as was required for the final system being developed. The second approach was attempted experimentally at speeds of 200 to 1000 rpm, using manual control of the syringe injection. The gels were successfully cast in this manner, with little measurable difference between those cast at the low speeds and those cast at the higher speeds. The polymerization process required approximately 40 min at 20°C; using an overhead heat lamp to increase the rotor temperature to 40°C decreased the polymerization time to 20 min. The standardization of the sample interface positions by this technique was not acceptable, probably due to the difficulty of producing a uniform annular wave. In an effort to improve this standardization, the third approach was experimentally investigated. The desired nozzle characteristics were simulated using a syringe needle with its tip bent at a right angle to the needle body; fluid was forced through the needle at a controlled rate, using a peristaltic pump. Gels could be cast at 200 rpm by this technique, with significant improvement in the standardization of the interface positions, which were considered acceptable for prototype development. It was also realized that this technique could easily be adapted into a nozzle configuration to be used in simultaneously loading several rotors positioned in a vertical array.

2.2.3 Electrophoresis in an experimental configuration

To demonstrate the feasibility of performing electrophoresis in the proposed geometry, the apparatus shown in Fig. 2.8 was constructed. In

Fig. 2.8. Details of the experimental setup to verify electrophoresis in the horizontal plane as designed for in the conceptual electrophoresis system.

preparation for experimental electrophoresis studies, the top cover plate, the rotor body, and the bracing plate were aligned with nut and bolt fasteners; the bracing plate was then coupled to the centrifugal drive system. Removable Teflon plugs were positioned in the top plate to create sample wells in the gels during the polymerization process. Gels were cast using the jet-forming nozzle described in Sect. 2.2.2. The rotor body was then removed from the bracing plate assembly, and the Teflon plugs were removed from the gels. Serum samples were then introduced into the sample wells. Plastic nut and bolt fasteners were used to join the outer electrode plate to the rotor body. This assembly was then positioned in the separation annulus as shown in Fig. 2.9; vacuum grease was used to provide a liquid seal at points of contact between the electrode plate and the rotor body, and at points of contact between the rotor body and the separation annulus. The rotor lift device was then positioned as shown in Fig. 2.9. Use of vacuum grease on its contact surfaces allowed it to serve as a secondary seal between outer electrode fluids and inner electrode fluids; the seal was also strong enough to allow this device to be used in removing the rotor from the annulus. The inner electrode plate was then positioned, again using vacuum grease, over the center of the rotor body. This final assembly was positioned in the system containment vessel as shown in Fig. 2.9; the containment vessel, with a magnetic stir bar placed beneath the rotor center, was placed on a magnetic stir plate to allow circulation of the inner electrode fluid during electrophoresis. Buffer solution at 5°C was introduced into the containment vessel until the level in the inner electrode chamber was above the top edge of the rotor body; an identical buffer solution was introduced into the separation annulus until the level was just below the upper edge of the inner electrode plate. The electrodes were then connected to a dc power supply to complete the experimental configuration.

In a typical feasibility study, running gels of 8% polyacrylamide were cast; in some cases, stacking gels of approximately 5% polyacrylamide were cast over the running gels. Approximately 10 μ l of serum was introduced into each sample well. The buffer used was a 0.065 M tris-borate

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Fig. 2.9. Assembled experimental setup to verify electrophoresis in the horizontal plane, showing containment vessel and electrodes.

solution with a standard 0.1% bromphenol blue tracing dye in the buffer solution contacting the gels in the inner electrode well. This dye was used to determine experimental run times in feasibility studies. Electrophoresis was usually performed at 200 V and 100 mA for 1 hr. No cooling was used during these studies.

After electrophoresis the rotor body was removed from the assembly and a stain specific for LDH (lactate dehydrogenase) was applied to the gels for approximately 30 min at 37°C. Results of a typical run with stained gels in place in a prototype rotor are shown in Fig. 2.10. Although neither the serum nor the staining solutions used in these studies were fresh, as many as three LDH isoenzymes were detected in many gels. The primary isoenzyme was detectable in each gel, indicating that a significant separation does occur simultaneously in each of the gels. The potential for uniform separations from gel to gel was also observed, although it was noted that the use of internal standards in each gel could be used to obviate the need for gel-to-gel separation uniformity. These results were used to establish the initial feasibility of the proposed geometry.

2.2.4 Automated gel scanning system

The conceptual design for the gel scanner is based to some extent on the monitoring concept of the CFA, namely, rotation of a series of samples by a single monitoring point. The plastic rotor with 12 horizontal gel columns is placed on a rotor holder which will be rotated at 300 rpm. Mechanically coupled to the rotation of this rotor holder and geared so that one complete rotation of the rotor holder will move the optical system 0.01 in. inward toward the center of the rotor is a fork-shaped optical assembly. At a rotational rate of 300 rpm, scanning of all 12 gel columns will be completed in 1 min, with the data acquired by a micro-computer. After completion of scanning, the drive motor is reversed and run at 5000 rpm to return the fork-shaped optical head back to its starting point ready to scan another rotor in less than 20 sec. An over-run clutch keeps the rotor holder from spinning during this time.

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Fig. 2.10. Serum electrophoresed in the horizontal plane and stained for LDH. As many as three isoenzymes were observed in this crude experiment.

The fork-shaped optical head contains a lamp in its upper element and two interference filters and two photodetectors in the lower element. The lamp is focused on a 0.01-in. pinhole used to define a spot diameter. This spot is then focused on the gels in the rotor. The portions of incident light transmitted through the gel columns are passed through an interference filter and focused on two photodetectors. The analog signal is digitized and sent to a data acquisition system. Data are acquired simultaneously for two wavelengths, requiring an acquisition rate of approximately 120 points/sec.

Several options are being considered with regard to data storage and analysis: the data can be stored on some accompanying storage device (diskette, etc.) for later analysis; stored on magnetic tape for later analysis on another computer; analyzed on-line in a foreground-background mode. The option selected will depend upon the work load and the amount of computing power in the data acquisition system.

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3. ENVIRONMENTAL RESEARCH

W. W. Pitt, Jr., M. S. Denton, R. L. Jolley, D. E. Schuresko,
S. J. Hartmann, G. Jones, Jr., J. E. Thompson

Environmental research efforts during this report period have been involved in four areas: (1) identification of dissolved organics in polluted waters, (2) environmental effects of antifoulants, (3) chemical effects of disinfectants, and (4) environmental monitoring.

In the area of pollutant identification, data processing and mass spectra interpretation have been greatly facilitated by the installation of a Systems Industries system 150 to accumulate and process data from the Finnigan 3000 mass spectrometer. The use of a computer to collect and massage the data from the GC/MS (gas chromatograph/mass spectrometer) will permit examination of mass spectra for specific indicator masses and assist in evaluation and elimination of background or noise peaks. Reconstructed gas chromatogram and mass spectra printout will provide permanent records of mass spectral analyses.

To complement anion exchange separations on aqueous effluents from coal hydrocarbonization bench-scale experiments, a "trace enrichment" method using reverse-phase chromatography has been initiated.

The trihalomethanes, chloroform, bromodichloromethane, and dibromochloromethane, are formed during the chlorination of cooling waters from both once-through and closed-cycle (cooling towers) systems. Based on values determined at the local industrial sites, the local production of the carcinogen chloroform is in excess of a ton per year. Planning for the Second Conference on Water Chlorination: Environmental Impact and Health Effects is complete and the program has been finalized.

Experimental equipment for the uv irradiation of wastewater effluents has been tested. The chemical effects of uv irradiation, ozonation, and chlorination will be compared in this EPA-funded program. Several wastewater samples have been collected and processed for chemical and mutagenic

studies. Collection of field samples has been initiated by sampling the wastewater ozonation pilot-plant effluent from the Advanced Waste Treatment Research Laboratory at Cincinnati, Ohio.

The development of a multicellular scintillation counter for use in environmental monitoring of ultralow concentrations (less than 1 nCi/ml) of radioactive pollutants in aqueous streams has been initiated.

3.1 Pollutant Identification

This program has two principal objectives: (1) development of methodology for analysis of organic constituents present at microgram-per-liter concentrations in complex mixtures in aqueous solutions, and (2) identify and quantitatively measure organic pollutants in waters of environmental concern. High-resolution liquid chromatography is the principal separations method used in this research. A multicomponent identification scheme is used for identification and quantitation of the separated constituents.^{1,2} Quantitative data concerning pollutants in various waters will permit risk-benefit analysis of industrial and water treatment processes and will facilitate the determination of possible health effects.

3.1.1 Gas chromatography/mass spectrometry system

GC/MS is fundamentally important in the multicomponent identification scheme used in this program. Data processing and spectra interpretation have been enhanced by the installation of a data processing system.

Computer hardware. Several modifications were made to an existing Varian Aerograph series 1400 GC/Finnigan 3000 Peak Identifier Mass Spectrometer, including interfacing to a Systems Industries system 150. The quadrupole GC/MS is linked to a PDP 8/e (Digital Equipment Corp.) central processing unit (CPU) with 12K of core memory (12,288 words of core storage and 12-bit word length) via a Systems Industries remote interface. This interface consists of an ADC (analog-to-digital converter)

with 12-bit resolution and 133-kHz conversion rate, and its counterpart DAC (digital-to-analog converter) with 15-bit resolution and 100-kHz conversion rate. All executive programs controlling automated zeroing of dc offset values, calibration, data acquisition, and data analysis are stored on disk. The disk is in turn driven by a movable-head, Series 31 Diablo disk drive, 3090 power supply module, and 3021 disk controller. Interaction with the minicomputer is accomplished by commands entered on a Tektronix 4010 computer display terminal, while permanent records are subsequently obtained using a Tektronix 4631 hard copy unit.

Software capabilities. The software capabilities of the system 150 being quite extensive, only a few of the major executive programs will be mentioned.

1. Data acquisition. Each day's run is preceded by a check of baseline drift by an automated routine called ZERO. Here one simply calls the program and adjusts the dc offset values while observing the least significant bit of the accumulator. It was found that very little baseline or calibration peak drift occurred from day to day as long as the remote interface was left on. Power is also maintained at certain circuits of the quadrupole electronic module during periods of heavy usage for further stabilization.

Once a satisfactory zero is obtained, an automated calibration routine, CONTROL, is called. This program utilizes a standard compound (perfluorotributylamine, FC-43) to create a file of mass number relative to mass set voltage. The advantages to automatically storing such a file and referencing subsequent spectra to it are straightforward when related to the former method of visual comparison of spectra.

Furthermore, any two calibration files can be analyzed, as have 10/5 and 10/5A in Fig. 3.1, where

AMP = amplitude,
 DEL N = difference in DAC units between files,
 T,MS = integration time required (in milliseconds),
 N = absolute DAC value,
 AMU = mass number.

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SYSTEM 150 IS ON SELECT MODE: CONT
 CALIBRATE?: Y
 CALIBRATION FILE NAME: 10/5
 MASS RANGE: 10-489
 MS RANGE SETTING?: P
 NON STND CAL?
 DO CALIB DIAGNOSTIC?

SYSTEM 150 IS ON SELECT MODE: CONT
 CALIBRATE?: Y
 CALIBRATION FILE NAME: 10/5A
 MASS RANGE: 10-489
 MS RANGE SETTING?: P
 NON STND CAL?
 DO CALIB DIAGNOSTIC? Y
 PREV CALIB FILE NAME: 10/5

MONITOR REPORT:

AMP	0	58	112	2756	88	619	119	215	157	68
DEL N	-1	0	1	2	-1	-1	0	1	-1	2
T.MS	0	8	8	1	16	1	2	1	1	16
N	1247	1660	2272	3513	6360	10726	14744	20767	34364	65760
AMU	10	14	18	28	50	69	100	131	219	414

SYSTEM 150 IS ON SELECT MODE:

Fig. 3.1. Analysis of MS calibration files.

In this notation, 64 DAC units are equivalent to 1 amu. The delta values reported are therefore quite good.

Finally, acquiring the raw data itself is under complete computer control once parameters have been set. Of definite advantage is the capability of viewing real-time data acquisition via the CRT terminal with manual attenuation settings on the CPU. An average of every three mass scans is plotted on the screen as well as a continually developing reconstructed gas chromatogram (RGC). This allows the operator to make note of spectrum numbers and GC temperatures attained at peaks of interest during a thermal gradient.

Several distinct modes of acquiring data are available. These are CONTROL (normal data acquisition), IFSS (integration as a function of signal strength), and SIM (selected ion monitoring). CONTROL simply scans any range of mass units, with 0 to 500 amu being the maximum. IFSS, like SIM, is a more specialized signal optimization mode particularly efficacious for samples with extremely strong or weak peaks. By setting upper or lower threshold values and sampling times, one can avoid saturating peaks or lengthy sampling periods in areas devoid of peaks. Samples where the components of interest are in trace amounts or where there are considerable interferences can be very effectively handled using SIM. In this single-ion mode, eight ions can be monitored simultaneously. Individual integration times can be specified for each of these masses, ranging from 1 to 4096 msec. In displaying spectra taken in this mode, the raw data are digitally smoothed by a 13-point least-squares polynomial.

2. Data display. In addition to real-time display, any file can be recalled and viewed at the operator's convenience. Several illustrations will best demonstrate this capability. Figure 3.2 illustrates several "limited mass range" RGCs of a synthetic mixture of 0.024 g of the following hydrocarbons: toluene, 92 amu; xylenes, 106 amu; naphthalene, 128 amu; 2-methylnaphthalene, 142 amu; and biphenyl, 154 amu. The mass number used for each of the RGCs is printed in the upper right-hand corner of the plot. Any scan number can then be designated for a

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AHC1
AHC.M

Fig. 3.2. Limited-range reconstructed gas chromatograms.

mass spectrum plot (see Fig. 3.3) with various expansion and background subtraction capabilities.

As a further example, Fig. 3.4 illustrates an RGC of a thermal-gradient 95 to 180°C run on a Tenax-GC column with only helium introduced. This chromatogram was then expanded, and four scan numbers (X axis) were chosen to investigate this column "bleed." It can be seen from Fig. 3.5 that scan 142 (minus a background spectrum, 80) revealed a strong peak at 207. This was thought to be a characteristic silicon peak and was verified when other silicon triplets and peaks such as 119, 133, 193, 281, 355, and 429 amu were identified.^{3,4} Like silicon, chlorine in an ion gives rise to "isotopic clusters" evident in Fig. 3.6.⁵ This figure represents a 10-ml tap-water sample adsorbed onto a Tenax-GC cartridge and subsequently desorbed and passed to the GC/MS column of the Tenax, undergoing a thermal program from 95 to 180°C. With the background subtracted and, especially, the selected-ion monitor, the analysis of very dilute samples should be feasible.

3. Data analysis. QUAN and QNTATE are the two system 150 routines available for peak quantitation. This can be accomplished by either an internal or external standard. Figure 3.7 illustrates the output of such a quantitative routine. The three RGCs represent the masses for naphthalene, 2-methylnaphthalene, and biphenyl of the hydrocarbon mixture of Figs. 3.2 and 3.3. Finally, a variety of statistical programs has been added to the GC/MS disk. This includes a focal "least-squares fitting routine" originally designed for the GeMSAEC program.

Hardware modifications. Several modifications made in the GC/MS hardware should be briefly mentioned at this point. As the result of a lengthy system downtime due to a power failure to the forepump, it was deemed necessary to provide the system with emergency power. This includes pumps, quadrupole electronics, remote interface, and disk drive. A sequential manual was compiled of SX-70 photographs of each component of the ionization source and electron multiplier sections of the quadrupole housing during cleaning for use in future maintenance. Further, due to frequent breakage and leakage, a new design of molecular jet separation

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AHC1 SCAN 50 SIGMA=35 RT=0:59 100%= 151200
AHCJM

Fig. 3.3. Mass spectrum plot for MS scan.

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Fig. 3.4. Reconstructed gas chromatogram of thermal gradient run.

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THERMAL GRADIENT TENAX GC/HE FLOW
SPECTRUM NUMBER 0142 - 0000

T

Fig. 3.5. Mass spectrum plot with background subtracted.

ORNL DWG. 78-17952

Fig. 3.6. Isotopic clusters of chlorine in mass spectrum of chloroform.

ORNL DWG. 78-17953

AHC1
AHCUM

Fig. 3.7. Computer printout for QUAN program.

(Gohlke) was obtained which utilizes high-vacuum Glaslok connectors rather than Swagelok fittings. Again for convenience, and to avoid breakage, a permanent calibration flask (FC-43) and valve were mounted after the separator. With the vacuum divert on and FC-43 entering the mass spectrometer at only 10^{-6} torr, sufficient sample is obtained for computer calibration. This greatly simplifies the daily removal of the column and addition of an injector and valve on the GC side of the separator for calibration. Future developments for the system include achieving simultaneous MS scope and computer terminal display during real-time data acquisition. Also, to be installed is a bypass of the internal-external system, which will allow the computer to gain control of the mass spectrometer from the manual mode by terminal commands alone. Eventually, the necessary modifications will also be made to the GC to accommodate support-coated open tubular columns.

3.1.2 Coal hydrocarbonization process

Characterization studies of coal conversion products are under way involving preparative- and analytical-scale HPLC (high-pressure liquid chromatography), GC, and computerized GC/MS.⁵⁻⁸ Specifically, samples are being obtained from the aqueous process stream of the product scrubber of the Chemical Technology Division bench-scale hydrocarbonization unit. These 1- to 2-liter samples were frozen in acetone-washed polyethylene bottles.

A comparison study of several types of treated and untreated coals is being made. The following two samples, particularly, are being investigated:

I. HC-21-10 Wyodak (Wyoming-Dakota)

Wyodak Resources Corp., Gillette, Wyoming
Roland and Smith seam
Scrubber aqueous phase:
Organic C 8300 ppm
Inorganic C 760 ppm

II. HC-23-10 Illinois No. 6

1.81 kg of coal treated with 0.24 kg CaO
 Scrubber aqueous phase: 1.27 kg NaOH and
 7.28 kg H₂O to
 prevent caking

Organic C 16,400 ppm
 Inorganic C 2,800 ppm

Sample I was collected during hydrocarbonization experiment HC-21, in which the following experimental conditions were used: average fluidized-bed temperature, 1035°F; total pressure, 280 psig; hydrogen partial pressure, 270 psig; coal feed rate, 8.0 lb/hr on a moisture- and ash-free basis (maf); hydrogen feed rate, 96.7 scf/lb maf coal.

Sample II, on the other hand, was collected under these conditions: average fluidized bed temperature, 1060°F; total pressure, 280 psig; coal feed rate, 2.9 lb/hr (maf); hydrogen feed rate, 418 scf/lb (maf).

Each sample was filtered for particulates through Whatman No. 2 filter paper, with 10- and 1.2-ml samples being used for preparative and analytical HRLC runs respectively. Fractions were collected during the preparative runs, frozen, concentrated via lyophilization, and redissolved for GC and GC/MS characterization.

To complement comparison studies of preconcentration techniques already done on vacuum distillation, lyophilization, ion exchange resins, and XAD macroreticular resin (Rohm and Hass Co., Philadelphia, PA), a "trace enrichment" method using reverse-phase chromatography (RPC) has been initialized. This has involved preparing an analytical-scale HPLC unit and ordering the appropriate RPC packings, which are to be supplied by Waters Associates. Bondapak Porosil B, C-18 (37-75 μ) packing will be used for a 4-in.-long x 3.9-mm-ID precolumn and, perhaps, for a complete prep-column. The pre-column will be followed by a 30-cm x 3.9-mm-ID, 10- μ micro-Bondapak C-18. The 10- μ packing material will involve one of the several types of slurry or slam-packing techniques available.

These RPC columns will undergo initial testing with several polycyclic aromatic hydrocarbon (PAH) standards obtained from the Analytical

Chemistry Division, including the following:

National Bureau of Standards "surrogate PAH standard"
[2-methyl-naphthalene to benz(a)pyrene (B(a)P)]

Alkyl naphthalene standard (13 PAHs, decahydronaphthalene to fluorene)

3-7 ring PAH standard (26 PAHs, fluorene to coronene)

Concentration efficiencies will be studied on secondary sewage effluents and coal conversion products, as well as on these standards. It is felt that such a trace enrichment technique has the potential for greatly simplifying and condensing the existing vacuum distillation and lyophilization methods (one week minimum for a normal-sized sample).

3.2 Environmental Effects of Antifoulants

Biofouling of condenser surfaces reduces the efficiency of electric power-generating plants. Therefore, antifoulant treatment of the cooling systems is often necessary for maintaining high production efficiencies. Chlorine is the principal biocide used for antifoulant treatment. A major objective of this program is to identify and quantify halogenated organic constituents formed during the chlorination of cooling waters. We have previously reported the formation of nonvolatile chloro-organics during the chlorination of once-through cooling waters.⁹⁻¹¹ However, a literature survey revealed no additional information concerning the formation of nonvolatile halogenated by-products during the chlorination of closed-cycle (cooling towers) cooling waters, nor information concerning formation of volatile halogenated by-products during the chlorination of either closed-cycle or once-through systems. The following data represent the first experimental assessment of volatile halogenated organics in both cooling towers and once-through cooling systems.

3.2.1 Once-through cooling systems

The Kingston Steam Plant, Kingston, Tennessee, operated by the Tennessee Valley Authority, is a coal-fired electric power-generating

plant rated at 1723 MW(e).¹² The once-through cooling system uses 100,000 to 125,000 gpm cooling water for each of its nine condensers. Each is chlorinated 30 min daily with 0.2 ppm free chlorine residual (0.5 ppm total residual). The condenser tubes are chlorinated individually, in a sequential manner, so that the discharged chlorinated coolant is diluted with unchlorinated water in the discharge canal.¹³

Analyses were conducted on grab samples from the once-through system and the waterways adjacent to the cooling system. Samples for analysis of volatile organics were collected in a manner that prevented formation of head space and resultant loss of volatiles. Five-milliliter aliquots were analyzed for the volatile halogenated organics, using a modification of the Bellar and Lichtenberg method.¹⁴

The trihalomethanes, chloroform (CHCl_3), bromodichloromethane (CHBrCl_2), and dibromochloromethane (CHBr_2Cl), are formed during the chlorination of cooling water at the Kingston Steam Plant. Figure 3.8 identifies the sampling points for this once-through system and the adjacent waterways. Table 3.1 presents the analytical results from a series of corresponding samples. Chlorination with even the low concentrations used at this plant (0.2 ppm of free chlorine, 0.5 ppm total residual) produced significant concentrations of chloroform. We can only conclude that the formation of chloroform continues in the discharged effluent, since dilution should have decreased the concentrations more than that shown at sites 4, 6, and 7. The low value for site 5 cannot be explained at this time, although it may be an analytical error due to a bubble in the head space. The enrichment of brominated trihalomethanes, presumably due to their lower volatility, is interesting. Site 8 is several miles downstream at the confluence of the Tennessee and the Clinch Rivers. Even with the considerable dilution involved and the high volatility of chloroform, it is detected at the 1-ppb level; and, surprisingly, bromoform is present at higher concentrations. Such results may represent input from the Tennessee River system and the large industrial city of Knoxville. Samples were taken of Walker Branch, which is a pristine spring-fed stream on the DOE-controlled area, and of the Clinch River

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⑧

Fig. 3.8. Sampling points for Kingston Steam Plant and adjacent waterways.

Table 3.1. Analyses of water samples taken at designated points for the Kingston Steam Plant^a and adjacent waterways

No.	Site Description	Concentration (ppb)			
		CHCl ₃	CHBrCl ₂ ^b	CHBr ₂ Cl ^b	CHBr ₃ ^b
1	Skimmer Dam (Emory River)	1.0	ND ^c	ND ^c	ND ^c
2	Pump Station	1.6	ND ^c	ND ^c	ND ^c
3	Condenser No. 2	20.5	1.3	0.4	ND ^c
4	Discharge (50 yd)	5.2	0.2	ND ^c	ND ^c
5	Discharge Channel	0.6	0.2	ND ^c	ND ^c
6	Discharge Embayment	5.0	0.3	ND ^c	ND ^c
7	Clinch River	2.5	0.2	ND ^c	ND ^c
8	Confluence Clinch and Tennessee Rivers	1.0	0.2	ND ^c	ND ^c

^aOnce-through system.

^bTentative identification.

^cNot detected.

at Melton Hill Dam, approximately 20 miles upstream, to determine whether the low concentrations of chloroform in the Emory River and at site 8 might indicate a general level of contamination. Both the Clinch River sample and the Walker Branch sample contained only 0.06 ppb chloroform. Thus the presence of chloroform at the ppb level in the Tennessee River may be a result of discharge of chlorinated effluents of several types, that is, the chlorinated cooling waters from the Kingston Steam Plant and chlorinated effluents from cities on the Tennessee River. The 1.0 ppb chloroform concentration in the Emory River cannot be explained at this time.

The chloroform production for the Kingston Steam Plant was calculated to be 4.5 to 5.4 lb/day, or nearly a ton per year. This occurs despite the high-quality water used in the cooling system and the minimal use of chlorine for antifoulant treatment.

3.2.2 Cooling tower systems

Two closed-cycle systems were studied, namely, the cooling tower systems at the Oak Ridge Gaseous Diffusion Plant and the High Flux Isotope Reactor (HFIR).

The HFIR, which is operated by the Oak Ridge National Laboratory as a nuclear facility for producing transuranic elements, uses a 121-MW(e) cooling tower. Municipal drinking water from Oak Ridge, initially chlorinated to 2-ppm chlorine residual, serves as the feed and makeup water. In addition, the cooling tower water is chlorinated on a weekly basis to an approximate 2-ppm level. The coolant system, which has a total capacity of 600,000 gal, operates with a flow rate of 26,000 gpm, a makeup or feed rate of 700 gpm, and a blowdown rate of 130 to 150 gpm to a lagoon.¹⁵

The Oak Ridge Gaseous Diffusion Plant, which is operated by Union Carbide Corporation for the Department of Energy, is a uranium enrichment facility using a 2060-MW(e) cooling tower system. The feed or makeup water is obtained from the Clinch River after sequential treatment steps of prechlorination with 1 ppm chlorine, addition of lime and polymer, settling to remove the floc, and pH adjustment with sulfuric acid. The cooling waters are chlorinated continuously to 0.3 to 0.5 ppm chlorine residual. One tower system or loop contains 13 ppm Cr(VI) as sodium dichromate, which is added to prevent corrosion. The coolant capacities of the several tower systems range from 2.5 to 13.5 million gallons. The average total flow rate is 320 million gal/day, and the makeup is 9 million gal/day. The blowdown of 0.01% is treated for Cr(VI) removal and recycled.¹⁶

Analyses were conducted on grab samples as for the once-through system (Sect. 3.2.1). In addition, concentrates of the water samples were prepared by lyophilization and then chromatographed using high-pressure, high-resolution anion exchange chromatography. The nonvolatile constituents separated by this means are being processed through a multicomponent identification procedure. Details of the chromatographic

and identification procedures are presented elsewhere.^{1,2,17} The water samples examined had pH values of 7.3 to 7.6, and all chlorine residuals were measured amperometrically.

Nonvolatile organics. The presence of low-volatility chloro-organics in the once-through cooling waters of the Kingston Steam Plant was reported previously.⁹⁻¹¹ Chromatograms of concentrates prepared from chlorinated water samples of both the HFIR and the ORGDP cooling tower basins reveal numerous nonvolatile uv-absorbing and cerate-oxidizable constituents (Figs. 3.9 and 3.10). By analogy with the chlorinated Kingston Steam Plant waters,⁹⁻¹¹ some of the nonvolatile compounds separated from the concentrates are probably chloro-organics, although this has not yet been confirmed by mass spectral evidence. Eluate fractions from preparative-scale chromatographic runs on the chlorinated cooling tower waters are being examined by gas chromatography-mass spectrometry.

Volatile organics. The trihalomethanes, chloroform (CHCl_3), bromodichloromethane (CHBrCl_2), and dibromochloromethane (CHBr_2Cl), are formed during the chlorination of cooling waters collected from the cooling tower systems. Analytical data in Table 3.2 indicate that the feedwater for the HFIR cooling tower contains over 100 ppb of chloroform, which is apparently lost to the atmosphere during cycling. The chloroform content of the tower basin water is about 1 ppb, except on the day (once per week) when sufficient chlorine is added to increase the chlorine residual to a calculated 2 ppm. The chloroform content of samples collected from the tower basin immediately after chlorine addition measured 37 to 38 ppb; however, analyses of samples collected 2 hr later showed only 6.2 ppb. Thus the major portion of the chloroform is lost rapidly to the atmosphere. Presumably the other trihalomethanes were also volatilized, since they were not detected at lower concentrations in the cooling tower basin water. Similarly, most of the chloroform formed in the ORGDP cooling waters during chlorination was lost to the atmosphere. In the ORGDP system, the bromine-substituted trihalomethanes were detected in the samples from the tower basins. It is interesting to note that

Fig. 3.9. Chromatograms of the uv-absorbing constituents (top) and of the cerate-oxidizable constituents in a 0.27-ml aliquot of a 5000-fold concentrate of the chlorinated water from a cooling tower at the Oak Ridge Gaseous Diffusion Plant.

Fig. 3.10. Chromatograms of the uv-absorbing constituents (top) and of the cerate-oxidizable constituents in a 0.27-ml aliquot of a 3300-fold concentrate of the chlorinated water from a cooling tower at the High Flux Intensity Reactor (HFIR), Oak Ridge, Tennessee.

Table 3.2. Volatile halogenated organics in chlorinated cooling tower waters

	Concentration (ppb)	
	HFIR	ORGDP
Feed or makeup water		
Chloroform	120-137	86
Bromodichloromethane	5-15	7
Dibromochloromethane	0.2-0.4	1
Bromoform	0.1	ND ^a
Tower basin water		
Chloroform	1-38 ^b	12-31 ^c
Bromodichloromethane	0.2-1.6	0.9-3
Dibromochloromethane	ND	1
Bromoform	ND	ND

^aNot detected.

^bChlorinated weekly to 2 ppm calculated chlorine dose.

^cChlorinated continuously to 0.5 ppm chlorine residual.

bromodichloromethane was volatilized to a greater extent than the other brominated trihalomethanes, apparently because of its higher volatility.

Table 3.3 presents the calculated chloroform production in and loss from the two cooling tower systems. As indicated, the amount of chloroform lost from these two plants totals more than a ton annually.

Estimate of chloroform production from electric power production.

The Federal Power Commission estimates the current U.S. power production to be about 340,000 MW(e).¹⁸ Table 3.4, which summarizes the estimated annual chloroform production in the United States from cooling water systems used in electric power generation, was prepared by using the Federal Power Commission data as a basis and assuming all the cooling systems to be of the same quality and efficiency as those cited as examples here. The total shown, 100 to 200 tons per year, is probably

Table 3.3. Chloroform production in and loss from HFIR and ORGDP cooling tower systems

	HFIR	ORGDP
Cooling capacity, MW(e)	121	2060
Cooling water		
Flow rate, gal/day	37×10^6	324×10^6
Makeup, gal/day	1.0×10^6	9.0×10^6
Chlorine, feed, ppm	0.5	1.0
Chlorine, continuous, ppm		0.3-0.5
Chlorine, weekly, ppm	2	
Chloroform production, lb/day	~ 1.1	~ 6.5
Chloroform, loss, lb/day	~ 0.8	~ 4.9
Bromodichloromethane production	Trace	Trace
Dibromochloromethane production	Trace	Trace
Bromoform production	Trace	

Table 3.4. Estimated chloroform production in the United States from chlorination of cooling systems for electric power generation^a

Cooling system	Percent of power	Chloroform production (tons/year)
Once-through, fresh water	43	77
Once-through, saline water	20-24	(40) ^b
Spray ponds	19-23	?
Cooling towers	14	24-84

^a Estimated power production: 340,000 MW.

^b Probably a mixture of chloroform and bromoform would be produced, with the bromoform increasingly in predominance with increasing salinity (see ref. 20).

conservative (i.e., low). When we consider the amount of electrical energy required to carry out the operations at ORGDP, which is a user rather than a producer facility, and at the HFIR, which is designed for purposes unrelated to electric power production, we can assume that chloroform production from chlorination of all cooling systems should be much higher. In addition, it is projected that new fossil and nuclear power plants that come on-stream during 1977-1986 will increase the annual production of electric power by 285,000 MW(e) in the next decade.¹⁹ The condenser systems of most of these plants will require antifoulant treatment.

3.2.3 Water chlorination conference

Sixty-seven technical papers will be presented at the Second Conference on Water Chlorination: Environmental Impact and Health Effects to be held October 31-November 4, 1977. Planning is complete and details have been presented previously.⁸ The following program includes most of the principal investigators in the field of water chlorination. The proceedings will be published as the second volume in a series.

*CONFERENCE ON
WATER CHLORINATION: ENVIRONMENTAL IMPACT AND HEALTH EFFECTS*

October 31–November 4, 1977

Riverside Motor Lodge
Gatlinburg, Tennessee

SPONSORED BY
Oak Ridge National Laboratory,
Energy Research and Development Administration,
and
U.S. Environmental Protection Agency

COCHAIRMEN

Robert L. Jolley, Oak Ridge National Laboratory
D. Heyward Hamilton, Jr., Energy Research and Development
Administration
Hend Gorchev, U.S. Environmental Protection Agency

PROGRAMMONDAY MORNING, OCTOBER 31

10:00 Conference registration desk opens. Personnel will be available for assistance and to provide information throughout the conference time.

MONDAY AFTERNOON, OCTOBER 31

1:00 CONFERENCE OPENING ... D. H. Hamilton, Jr.

SESSION 1. FRESHWATER SYSTEMS

Session Cochairmen ... W. A. Brungs and J. S. Mattice

1:15 Introduction

1:20 Soluble Organic Constituents of Natural Waters and Wastewaters Before and After Chlorination

W. H. Glaze and G. R. Peyton
North Texas State University

1:50 Natural and Model Aquatic Humics: Reactions with Chlorine

R. F. Christman, J. D. Johnson,
F. K. Pfaender, P. C. Singer,
H. J. Alexander, W. T. Liao,
and D. T. Norwood
University of North Carolina at
Chapel Hill

2:10 Precursors and Mechanisms of Haloform Formation in the Chlorination of Water Supplies

J. C. Morris and B. Baum
Harvard University

2:30 Brominated Compounds Found in Waste-Treatment Effluent and Their Capacity to Bioaccumulate

D. W. Kuehl, C. D. Veith, and
E. N. Leonard
U.S. Environmental Protection
Agency

3:00 Break

- 3:30 Chlorination of Polynuclear Aromatic Hydrocarbons
R. M. Carlson
University of Minnesota at Duluth
- 3:45 Investigation of the Effects of Halogenated Organic Compounds
Produced in Cooling Systems and Process Effluents on Aquatic
Organisms
J. R. Trabalka and M. B. Burch
Oak Ridge National Laboratory
- 4:00 A Programme to Introduce Site Specific Chlorination Regimes
at Ontario Hydro Generating Stations
J. A. Grieve and L. E. Johnston
Ontario Hydro
- 4:20 Toward an Understanding of the Toxicity of Intermittent Exposures
of Total Residual Chlorine to Freshwater Fishes
G. Larson
Oregon State University
- 4:40 The Effects of Intermittent Chlorination on Selected Warmwater
Fishes
A. S. Brooks and G. L. Seegert
The University of Wisconsin at
Milwaukee
- 5:00 Session Adjournment

MONDAY EVENING, OCTOBER 31

- 7:00 Mixer
- 8:00 FRESHWATER SYSTEMS WORKSHOP
Workshop Discussion Leaders ... W. A. Brungs and J. S. Mattice

TUESDAY MORNING, NOVEMBER 1

SESSION 2. MARINE SYSTEMS

Session Cochairmen ... W. P. Davis and W. F. McIlhenny

- 8:15 Introduction
- 8:20 Reactions in Chlorinated Seawater
J. H. Carpenter and C. A. Smith
University of Miami

- 9:00 Chlorinated Hydrocarbon Inputs, Distribution, Fate and Effects
in the Marine Ecosystem Off Southern California
D. R. Young and T. C. Heesen
Southern California Coastal
Water Research Project
- 9:40 Break
- 10:10 Biodegradation of Halo-Hydrocarbons in Marine Environments
D. T. Gibson and A. W. Bourquin*
University of Texas at Austin
*U.S. Environmental Protection
Agency
- 10:50 Chlorine Degradation and Volatile Halocarbon Generation in
Estuarine Waters
G. R. Helz, R. Sugam, and R. Y. Hsu
University of Maryland
- 11:15 Investigation of Halogenated Components Formed From Chlorination
of Estuarine Water
R. M. Bean, R. G. Riley, and
P. W. Ryan
Battelle Pacific Northwest
Laboratories
- 11:30 The Effect of Ammonium Ion Concentration on the Chemistry of
Chlorinated Seawater
G. W. Inman, Jr., and J. D. Johnson
University of North Carolina at
Chapel Hill
- 11:45 Determination of Halogens in Seawater With An Amperometric
Membrane Electrode
N. A. Dimmock and D. Midgley
Central Electricity Research
Laboratories
- 12:00 Session Adjournment

TUESDAY AFTERNOON, NOVEMBER 1

SESSION 3. TOXICITY IN AQUATIC SYSTEMS

Session Cochairmen ... W. P. Davis and W. A. Brungs

- 1:30 Introduction

- 1:35 Biological and Chemical Effects of Chlorination at Coastal Power Plants
J. C. Goldman, J. M. Capuzzo,
and G. T. F. Wong*
Woods Hole Oceanographic Institution
*Old Dominion University
- 1:55 Effects of Chlorinated Seawater on Decapod Crustaceans and Comparative Toxicity of Chlorinated and Bromochlorinated Treated Sewage
M. H. Roberts, Jr.
Virginia Institute of Marine Science
- 2:15 The Relative Sensitivity of Twenty Pacific Northwest Fishes and Invertebrates to Chlorinated Seawater
T. O. Thatcher
Battelle Pacific Northwest
Laboratories
- 2:35 Continuous Low-Level Chlorination for Marine Fouling Control at Power Stations in the United Kingdom
J. W. Whitehouse
Central Electricity Research
Laboratories
- 2:55 Toxicity Screening of Fifteen Chlorinated and Brominated Compounds to Four Species of Marine Phytoplankton
S. J. Erickson
U.S. Environmental Protection
Agency
- 3:10 Break
- 3:40 Seasonal Chronic Toxicity of Chlorination to the American Oyster, Crassostrea virginica
G. I. Scott and D. P. Middaugh
U.S. Environmental Protection
Agency
- 3:55 Physiological Response of White Perch (Morone americana) Exposed to Chlorine and Ozone in Estuarine Waters
R. M. Block, D. T. Burton,*
S. R. Gullans, and L. B. Richardson*
University of Maryland
*Academy of Natural Sciences of
Philadelphia

- 4:10 Response of Entrained Plankton to Low-Level Chlorination at a Coastal Power Station
M. H. Davis and J. Coughlan
Central Electricity Research Laboratories
- 4:25 The Behavioral Response of the Golden Shiner (Notemigonus crysoleucas) to Various Avoidance Indices and Chlorine Components of Total Residual Chlorine
S. R. Larrick, D. S. Cherry,
K. L. Dickson, and J. Cairns, Jr.
Virginia Polytechnic Institute and State University
- 4:40 Avoidance of Monochloramine
T. C. Teppen, R. B. Bogardus,
and D. B. Boies
Wapora, Inc.
- 4:55 Toxicity of Intermittent Chlorination to Freshwater Fish: Influence of Temperature and Chlorine Form
A. G. Heath
Virginia Polytechnic Institute and State University
- 5:10 Session Adjournment

SESSION 4. TRIHALOMETHANES

Session Chairman ... D. J. MacGregor

- 1:30 Introduction
- 1:35 Formation and Occurrence of Haloforms in Drinking Water in the Federal Republic of Germany
M. Sonneborn
Institut für Wasser-, Boden-, and
Lufthygiene des Bundesgesund-
heitsamtes
- 1:50 Chlorination and Water Treatment for Minimizing Trihalomethanes in Drinking Water
R. C. Hoehn, R. P. Goode, C. W. Randall, and P. T. B. Shaffer*
Virginia Polytechnic Institute and State University
*The Carborundum Company

- 2:05 Evaluation of the Capability of Granular Activated Carbon and Resin to Remove Trace Organics from Treated Drinking Water

I. H. Suffet, J. V. Radziul,*
P. R. Cairo,* and J. T. Coyle*
Drexel University
*Philadelphia Water Department

- 2:20 Chlorine and Activated Carbon Treatment for Removal of Toxic Substances from Water

R. G. Howland and C. J. Wallace
Jet Propulsion Laboratory

- 2:35 Organic Removal from Washington, D.C. Municipal Water by a Laboratory Water Purification System Using Activated Carbon

H. C. Rechen and J. V. Wetzel*
Hydro Service and Supplies, Inc.
*Calgon Corporation

- 2:50 The Effect of Preozonation on Chloroform Production in the Chlorine Disinfection Process

T. Riley
University of Michigan

- 3:05 Trihalomethane Reduction at the Louisville Water Company

S. A. Hubbs and J. Zogorski*
Louisville Water Company
*University of Louisville

- 3:20 Session Adjournment

SESSION 5. WASTEWATER DISINFECTION

Session Chairman ... G. C. Becking

- 3:50 Introduction

- 3:55 Bromine Chloride as an Alternative Wastewater Disinfectant to Chlorine

W. L. Woodfin, Jr., R. B. Taylor,*
and R. C. Hoehn**
Virginia State Water Control Board
*Virginia State Department of Health
**Virginia Polytechnic Institute
and State University

- 4:10 A Comparative Analysis of Chlorine and Bromine Chloride as
Wastewater Disinfectants
N. E. LeBlanc and M. J. McEuen*
Hampton Roads Sanitation District
*Ethyl Corporation
- 4:25 Chlorine Dioxide Destruction of Viruses and Bacteria in Water
S. Cronier, P. V. Scarpino, and
M. L. Zink
University of Cincinnati
- 4:40 The Feasibility of Chlorine Dioxide in Wastewater Disinfection
P. V. Roberts
Stanford University
- 4:55 Environmental Aspects of Sewage Effluent Disinfection - A Review
J. D. Ellis
Ontario Ministry of the Environment
- 5:10 Session Adjournment

TUESDAY EVENING, NOVEMBER 1

- 7:00 Mixer
- 8:00 MARINE SYSTEMS WORKSHOP
Workshop Discussion Leaders ... W. P. Davis and W. F. McIlhenny

WEDNESDAY MORNING, NOVEMBER 2

SESSION 6. HEALTH EFFECTS

Session Cochairmen ... R. B. Cumming and H. Gorchev

- 8:00 Introduction
- 8:05 The Epidemiological Approach to the Evaluation of the Health
Effects of Water Chlorination
K. P. Cantor
U.S. Environmental Protection Agency
- 8:45 A Case Control Study of Gastrointestinal and Urinary Tract
Cancer in Relation to Drinking Water Chlorination
M. Alavanja, I. Goldstein,* and
M. Susser*
City University of New York
*Columbia University

- 9:05 Epidemic Problems of Chlorination of Drinking Water and Sewage
in the Federal Republic of Germany
G. Müller
Institut für Wasser-, Boden-, und
Lufthygiene des Bundesgesundheitsamtes
- 9:25 A Priori Predictive Methods of Assessing Health Effects of
Potential Chemical Toxicants and Suspect Carcinogens
M. J. Kland
Lawrence Berkeley Laboratory
- 9:45 Break
- 10:15 Mutagenicity and Water Chlorination: Prospect and Perspective
R. B. Cumming
Oak Ridge National Laboratory
- 10:30 The Mutagenicity of Compounds Formed by Water Chlorination
V. F. Simmon and R. G. Tardiff*
Stanford Research Institute
*U.S. Environmental Protection
Agency
- 10:50 Development of Poliovirus with Resistance to Chlorine
Inactivation
R. C. Bates, P. T. B. Shaffer,*
and S. M. Sutherland
Virginia Polytechnic Institute and
State University
*The Carborundum Company
- 11:20 Risk Assessment of the Health Effects of Water Chlorination
M. Schneiderman
National Cancer Institute
- 12:00 Session Adjournment

WEDNESDAY AFTERNOON, NOVEMBER 2

Free time with bus transportation available to several areas
in the Great Smoky Mountains National Park. A nominal fee
will be charged for transportation.

WEDNESDAY EVENING, NOVEMBER 2

- 7:00 Reception

8:00 Dinner

After-Dinner Address:

"Worldwide Aspects of Water Chlorination"

Luis Orihuela, Chief
Community Water Supply Unit
Division of Environmental Health
World Health Organization
Geneva, Switzerland

THURSDAY MORNING, NOVEMBER 3

SESSION 7. INDUSTRIAL EFFLUENTS

Session Cochairmen ... W. W. Pitt, Jr., and K. Jakobsen

8:00 Introduction

8:05 Chlorination Practices in the Pulp and Paper Industry

H. K. Willard
U.S. Environmental Protection
Agency

8:45 Chlorination Practices in the Textile Industry

W. C. Tincher
Georgia Institute of Technology

9:25 Chlorination Effects on Typical Organic Chemical Plant
Effluents

R. D. Fox and J. H. Exner
Hydroscience Environmental Systems

10:05 Break

10:35 Chlorination Practices in the Iron and Steel Industry

N. Plaks
U.S. Environmental Protection
Agency

11:15 A Kinetic Model for Predicting the Composition of Chlorinated
Water Discharged from Power Plant Cooling Systems

M. H. Lietzke
Oak Ridge National Laboratory

11:30 Aqueous Chlorination of Some Phenolic Acids

A. L. Rockwell and R. A. Larson
Stroud Water Research Center

- 11:45 An Experimental Assessment of the Chlorine-Containing Compounds
in Cooling Tower Drift and Blowdown

R. L. Jolley, W. W. Pitt, Jr.,
F. G. Taylor, S. J. Hartman,
G. Jones, Jr., and J. E. Thompson
Oak Ridge National Laboratory

- 12:00 Session Adjournment

THURSDAY AFTERNOON, NOVEMBER 3

SESSION 8. ALTERNATIVES

Session Cochairmen ... G. C. Becking and D. J. MacGregor

- 1:30 Introduction

- 1:35 Biofouling Control Alternatives for Power Plants with Once-
Through Cooling Systems

D. T. Burton and L. H. Liden
The Academy of Natural Sciences
of Philadelphia

- 2:15 A Study of Alternatives to Chlorination for Disinfection of
Wastewater

A. D. Venosa and R. W. Ward*
U.S. Environmental Protection
Agency
*Grand Valley State Colleges

- 2:55 Break

- 3:25 Ozone, Chlorine Dioxide, and Chloramines for Disinfection

J. M. Symons, A. A. Stevens,
J. K. Carswell, and O. T. Love, Jr.
U.S. Environmental Protection
Agency

- 4:05 Ozonation of Organics in Wastewater

R. E. Sievers, R. M. Barkley,
G. A. Eiceman, R. H. Shapiro,
H. F. Walton, and K. J. Kolonko
University of Colorado

- 4:20 Trihalomethane Formation from Iodine and Chlorine Disinfection
of Ohio River Water

R. N. Kinman and J. Rickabaugh
University of Cincinnati

4:35 Bromine Chloride -- An Alternative Biofouling Control Agent
for Cooling Water Treatment

L. H. Bongers, D. T. Burton,*
L. H. Liden,* and T. P. O'Connor
Martin Marietta Environmental
Technology Center
*Academy of Natural Sciences of
Philadelphia

4:50 Reactions of Chlorine, Chlorine Dioxide, and Mixtures Thereof
with Humic Acid

M. C. Noack and R. L. Doerr
Olin Research Center

5:05 Ozonation Versus Chlorination in Water Disinfection

D. Z. Diamant
Ahmadu Bello University

5:20 Session Adjournment

THURSDAY EVENING, NOVEMBER 3

7:00 Mixer

8:00 HEALTH EFFECTS WORKSHOP

Workshop Discussion Leaders ... R. B. Cumming and H. Gorchev

8:00 INDUSTRIAL EFFLUENTS WORKSHOP

Workshop Discussion Leaders ... W. W. Pitt, Jr., and K. Jakobson

8:00 ALTERNATIVES WORKSHOP

Workshop Discussion Leaders ... G. C. Becking and D. J. MacGregor

Discussants:

Alternatives to Chlorination for Control of Biofouling in
Power Plant Systems

R. Kawaratani, T. D. Waite,* and
R. M. Jorden
Electric Power Research Institute
*Northwestern University

UV Irradiation

A. D. Venosa
U.S. Environmental Protection
Agency

FRIDAY MORNING, NOVEMBER 4

SESSION 9. REGULATIONS, PROGRESS, AND PROBLEMS

Session Chairman ... D. H. Hamilton, Jr.

8:15 Introduction

8:20 Regulatory Aspects of Potable Water Disinfection

J. A. Cotruvo

U.S. Environmental Protection Agency

8:50 Status of Regulation Development for Cooling Water Discharges
from Power Plants

J. W. Lum

U.S. Environmental Protection Agency

9:20 Municipal Wastewater Disinfection Policy

A. B. Hais

U.S. Environmental Protection Agency

9:50 Break

10:20 Progress and Problems: Discussion of Present Status and Future
Research and Technological Needs

Workshop Leaders

11:45 Session Adjournment

11:45 CONFERENCE CLOSING ... H. Gorchev

12:00 Conference Adjournment

3.3 Chemical Effects of Disinfectants

The objectives of this program, funded by the U.S. Environmental Protection Agency, are the following: (1) characterize the nonvolatile organics in wastewater effluents that have been disinfected with either chlorine, ozone, or uv light, and (2) determine the mutagenic properties of the organic constituents in the disinfected wastewaters. The mutagenicity determinations are to be conducted by R. B. Cumming and co-workers of the Biology Division. Table 3.5 gives the samples prepared to this date for chromatographic analysis and mutagenesis testing.

With uv irradiation gaining popularity as a viable alternative disinfection method to chlorination and ozonolysis, studies are under way to determine the effects, if any, on nonvolatile organics as a result of treating wastewater effluents. This study involves irradiating untreated as well as chlorinated secondary sewage at a set optical irradiance ($\mu\text{W}/\text{cm}^2$) for different flow rates (i.e., different retention times within the uv flow cell). Portions of the resulting samples are used in plotting "kill curves" by standard plate counts, doing routine analysis (e.g., pH, TOC, COD, BOD, and TKN), and concentrating 2000 to 2500 X for preparative HRLC runs.

A model H-1.0 uv sterilizer with sight port (Pure Water Systems, New Jersey) has been purchased and coupled with an LFE pump (Eastern Industries Div., 1/4 gpm max). Maximum flow capabilities of this 22.58-in.³ (370.09-ml) quartz flow-through cell is 1 gpm of deionized water. While much larger units are in operation (50-7000 gpm), this purifier is sufficient for obtaining a suitable range of disinfection (from near control to total kill). According to the following equation,

$$\frac{V_{\text{cell}} (\text{ml})}{V (\text{ml})/t (\text{sec})} = \frac{V_c}{F} = t_R (\text{sec}) ,$$

one can calculate the retention time of a sample for each flow rate. For instance, t_R for distilled water (DW) at $F = 1$ gpm is 5.87 sec. This value can then be used with the aid of an IL 700 research radiometer

Table 3.5. Wastewater concentrated and chromatographic fraction prepared for chromatographic analysis mutagenic activity testing^a

Sample	Sample date	Concentration factor
HRLC blank (4 fractions)	6/13/77	
ORSE ozonated ^b (7 fractions)	5/16/77	
ORSE unchlorinated	1/31/77	330
ORSE unchlorinated	2/7/77	1000
ORSE chlorinated ^b (0.5 ppm chlorine residual)	8/2/77	3100
ORSE unchlorinated	9/27/77	1000
ORSE chlorinated (0.2 ppm chlorine residual)	9/27/77	1000
ORPE unchlorinated	9/27/77	Dry
ORPE chlorinated (1.0 ppm chlorine residual)	9/27/77	Dry
CSE unozonated ^b	9/12/77	1500
CSE ozonated ^b (8.4 mg O ₃ /liter effluent)	9/12/77	1600

^aOR = Oak Ridge, C = Cincinnati, EP = Estes Park, SE = secondary effluent, PE = primary effluent, dry = stored as lyophilized powder at -60°C.

^bPreparative-scale HRLC also run on sample.

and photometer to determine the actual amount of energy impinging on the sample during this time:

Range photodetector: 190–300 nm

Optical irradiance (I): W/cm^2 or $\mu W/cm^2$

Photodetector response (R): A

Sensitivity factor

of phototube (S): $3.210 \times 10^{-3} A \cdot cm^2/W$

Energy term (E): $\mu W \cdot sec/cm^2$

$$\frac{R(A)}{S(A \cdot cm^2/W)} = I \left(\frac{W}{cm^2} \right) \text{ or } (\mu W/cm^2)$$

$$[I(\mu W/cm^2)][t_R(sec)] = E (\mu W \cdot sec/cm^2)$$

The first study carried out was to determine what flow rates and irradiance levels would be needed to accomplish various levels of purification, and thus plot a disinfection curve. Samples of chlorinated and unchlorinated secondary effluent were obtained from the Oak Ridge East-End Sewage Treatment Plant in 5-gal carboys and delivered immediately to the laboratory with no refrigeration. All samples were filtered for algae and gelatinous materials by Whatman No. 2 filter paper or a pump-driven Millipore filtering system. The latter used a Millipore 8- μ prefilter and an MF 8- μ filter (with 4 μ being sufficient to let most bacteria pass). Samples were then fed through the uv sterilizer at the appropriate flow rates. These flows and the corresponding optical irradiance value at that time (a slight upward drift was experienced with this photometer) were recorded. Samples were collected in 50-ml vials with the exception of those to be analyzed for BOD, COD, etc., where at least 1-liter samples were needed.

One milliliter of each sample was placed in a presumptive test vial containing an inverted culture tube and 10 ml of Bacto-lactose broth (B4). Gas production within 24 to 48 hr is a necessary, but not sufficient, evidence of gram-negative members of the coliform group (Escherichia coli and Aerobacter aerogenes).²¹⁻²³ Appropriate dilutions

from 1:10 to 1:10,000 were then made up of samples in autoclaved, distilled water; carefully mixed, 1/10 ml of each plated out on Bacto-Agar plates; and placed in an incubator at 35°C for a minimum of 39 hr. A total colony count was done at 39 and 120 hr. Tables 3.6 and 3.7 represent the findings of this initial uv irradiation study for control and irradiated groups respectively.

From this data, sufficient information was obtained to determine appropriate parameters for varying degrees of disinfection. A second, larger batch of samples was then collected from the Oak Ridge East-End Sewage Treatment Plant which was to be irradiated (with the exception of a control) at a level producing total disinfection. A comparison of control, chlorination, ozonolysis, and uv disinfection was also carried out. From Tables 3.6 and 3.7 and the standard uv disinfection rates of 15,000 to 30,000 $\mu\text{W}\cdot\text{sec}/\text{cm}^2$, a value of $\sim 21,000 \mu\text{W}\cdot\text{sec}/\text{cm}^2$ was chosen (see Table 3.8). Table 3.9 represents the results of analyses done on this batch. Microbiological tests were conducted in the same manner as the initial study. In both studies, uv irradiation was an extremely effective disinfection method, in fact, the most effective of the three methods compared. In the latter study, 20 liters each of control and uv irradiated secondary effluent were lyophilized in preparation for an analytical HRLC run (1-2 ml). Should major differences exist in chromatograms of these two samples, further characterization of peaks of interest will be carried out after a preparative HRLC run, utilizing GC and computerized GC/MS. Furthermore, additional uv irradiation energies will be carried out on batch samples.

Additional information is hoped to be obtained from uv-irradiating chlorinated secondary sewage to see whether this has any additional effects on products, such as chlorinated hydrocarbons, formed during either treatment.

A change in format in microbiological testing from total colony counts to the membrane filter technique has recently been undertaken.²³ This method, which is a standard total coliform procedure, is a more specific measure of disinfection effectiveness without an extravagant

Table 3.6. Controls

Sample	Dilution	(°C)	Total colony count (counts/ml), 48 hr	Presumptive test		pH	mg/liter (ppm)			
				39 hr	120 hr		TOD	COD	BOD	TKN
ORSE 9/7/77	1:10	35	1760							
	1:100		390	12 mm of gas	15 mm of gas	7.5	34	25	14	9.8
	1:1000		70							
ORSE-C1 9/7/77	1:10		530	5 mm of gas	Vial full	7.6	27	29	<5	11.0
	1:100		170							
	1:1000		20							

Table 3.7 UV-irradiated samples

								Total colony count (counts/ml)								
								1:100		1:1000						
								dilution		dilution		ppm				
Rotometer	F (ml/min)	F (gal/min)	t _R (sec)	I (10 ⁻⁴ W/cm ²)	(μ W sec) cm ²	Presumptive test (1 ml)	39 hr	120 hr	39 hr	120 hr	pH	TOC	COD	BOD	TKN	
ORSE																
9/7/77																
1	1.75	980	0.2589	22.70	4.40	9,988	(-)	160	340	100	130	7.4	34	35	<5	10.3
2	1.50	837.5	0.2212	26.44	4.42	11,686	(+)	20	170	0	40					
3	1.30	732.5	0.1935	30.34	4.44	13,471	(-)	0	20	0	30					
4	1.10	630	0.1664	35.25	4.44	15,651	(-)	0	0	0	20					
5	0.80	480	0.1268	46.26	4.43	20,493	(-)	0	0	0	20	7.5	32	25	<5	10.0
6	0.50	341	0.0901	64.93	4.42	28,699	(-)	0	0	0	10					
7	0.30	241	0.0637	92.52	4.43	40,986	(-)	0	50	0	30					
8	0.20	185.5	0.0490	119.38	4.42	42,766	(-)			0						
9	0.10	126	0.0333	176.23	4.42	77,894	(-)	10	40	0	70	7.5	33	27	<5	10.0
ORSE-2																
9/7/77																
1	1.75	980	0.2589	22.70	5.60	12,712	(-)	380	410	20	130	7.6	33	30	<5	10.2
2	1.50	837.5	0.2212	26.44	5.71	15,097	(-)	0	0	∞	∞					
3	1.30	732.5	0.1935	30.34	5.76	17,476	(-)	0	10	0	70					
4	1.10	630	0.1664	35.25	5.80	20,445	(-)	0	10	10	20					
5	0.80	480	0.1268	46.26	5.82	26,923	(-)	0	30	30	60	7.6	48	40	<5	10.8
6	0.50	341	0.0901	64.93	8.87	38,114	(-)	0	0	0	10					
7	0.30	241	0.0637	92.52	5.90	54,587	(-)	0	10							
8	0.20	185.5	0.0490	119.38	5.90	70,434	(-)	0	40	0	0					
9	0.10	126	0.0333	176.23	5.89	103,799	(-)	0	30	0	20	7.6	32	27	<5	10.8

Table 3.8. ORSE 10/5/77 (nonchlorinated); 20 liters uv-irradiated

Sample	Rotameter	F	F	t _R	I	E	pH	ppm			
		(ml/min)	(ml/sec)	(sec)	($\mu\text{W}/\text{cm}^2$)	($\mu\text{W}\cdot\text{sec}/\text{cm}^2$)		COD	BOD	TOC	TKN
ORSE 10/5/77 uv	1.50	837.5	13.96	26.51	800	21,208	7.4	39	<5	9.62	7.9
Control							7.3	45	17	11.5	3.8

Table 3.9. ORSE 10/5/77 total colony count
 [1/10 ml of each dilution was plated on Bacto-Agar
 and counted at 28- and 48-hr (no change)
 intervals]

Dilution	Counts/ml ^a control (w/o chlorine)		Counts/ml ORSE 10/5/77 uv	
	Plate 1	Plate 2	Plate 1	Plate 2
1:10	∞	∞	0	20
1:100	2170	∞	0	0
1:1000	340	330	0	0
1:10,000	10	0		

^a∞ indicates that the total colony count was too high to ensure accuracy.

investment of time and funds. With a stainless steel Millipore sampling manifold already available, the additional necessary equipment has been ordered (e.g., sterile dishes, pads, and filters; and fecal coliform media).

Two 6-liter aliquots of Oak Ridge East Wastewater Plant secondary effluent were ozonated in the laboratory to dosages of 3.5 and 20.0 mg ozone per liter of wastewater. Reaction conditions were: 25°C; ozone gas concentration, 19.4 mg ozone per liter of gas (oxygen); and use of a 12-liter glass reaction flask, a magnetic stirrer, and a porous glass frit gas diffuser.

From the chromatograms of the uv-absorbing constituents, it was deduced that most of the destruction of uv-absorbing compounds occurs rapidly and within the first 3.5-mg/liter effluent dosage of ozone. With respect to the oxidizable constituents, apparently there is initial destruction of some oxidizable constituents, but also an increase in the number and concentration of many constituents, with increasing ozone dosage. For example, the number and size of peaks in the 5- to 15-hr elution period increased. The chemical and disinfection data for the

control and ozonated wastewater samples are given in Table 3.10. The usual ozone dosage for disinfection is 6 to 10 mg of ozone per liter of effluent; thus, the chemical effects of ozonation for disinfection may be considered intermediate between the two ozonated samples. The wastewater samples are representative of effluent from an activated sludge secondary treatment after settling. The wastewater was principally domestic, containing very little industrial waste. An effort was made to determine the consumption of ozone during the ozonation process. The total dosage during the entire ozonation of the second sample was 120.3 mg ozone, of which 7.7 mg was trapped in a gas wash bottle (KI solution) following the ozonation reaction gas train. However, since the headspace gas of the reaction vessel was not flushed into the gas wash bottle, this amount of ozone represents a minimum value. The consumption or efficiency of ozone utilization based on the trapped ozone is 94%. This number must be considered a maximum, and the actual consumption is probably somewhat less. A more accurate determination of the utilization will be made in a subsequent ozonation.

The first field collection of wastewater samples was conducted at the wastewater ozonation pilot plant at the Advanced Waste Treatment Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio. A 40-liter grab sample of control (unozonated) secondary wastewater was collected on September 12, 1977. After waiting the approximate residence time (to ensure that the disinfected samples were essentially of the same composition as the control sample), a 40-liter grab sample of ozonated (~ 8 mg O_3 /liter) effluent was collected.

The samples were immediately transported by air to ORNL without refrigeration, filtered through Whatman No. 2 filter paper, frozen in lyophilization pans, and placed in the lyophilizer. This operation was completed within about 9 hr elapsed time from the initial sampling operation. Concentrates were prepared for analytical and preparative-scale chromatography and for testing for mutagenic activity.

Table 3.10. Ozonation of wastewater samples from the secondary stage of the Oak Ridge East Wastewater Treatment Plant: Chemical and disinfection data. The treatment plant uses activated sludge process for secondary treatment

Reaction ^a time (min)	Total ozone dose (mg ozone per liter effluent)	Bacteria count ^b (number/ml)	pH	COD	BOD	TKN	TOC
				(mg/liter)			
Control	0.0	9,500	7.61	30	6	18.2	10.8
4.3	3.5	18,000	7.69	28	7	17.4	9.8
24.8	20.0	0	7.84	24	<5	17.2	10.5

^a Room temperature (about 25°C); ozone-oxygen mixture at 250 ml/min containing 19.4 mg ozone per liter gas; 6-liter effluent sample; glass frit sparger with magnetic stirrer.

^b Probably statistically not meaningful because samples were not run in duplicate.

The analytical-scale chromatograms of the ozonated and control secondary effluent from the pilot plant from the Advanced Waste Treatment Laboratory, EPA, Cincinnati, Ohio, are compared in Figs. 3.11 and 3.12. It is obvious that many constituents are destroyed by ozonations. Because of the complexity of the cerate-oxidizable chromatogram, it is not clear whether there is an increase of constituents in the 5- to 15-hr elution range. Fractions from the preparative-scale separations have been lyophilized and are being processed through the multicomponent identification scheme. Both concentrates and eluate fractions have been stored at -60°C for mutagenic activity testing.

Because several of the field sampling sites are more remote, geographically, from ORNL than the Cincinnati site, a field sampling protocol was developed. For air freight shipment, an insulated box (approximate 22-in. cube) was constructed of sufficient size to contain five 2-gal and four 1-liter polyethylene containers; that is, the box is capable of containing a 41-liter water sample. Total weight of the shipping box and 41-liter water sample was 141 lb, well within air-freight shipment limitations. The water samples were frozen rapidly in the box by addition of 75 lb of crushed dry ice. The samples were partially frozen within 1 hr and solidly frozen in 3 hr. After three days had elapsed, the dry ice had sublimed completely. The water samples were still completely frozen five days after initial freezing. Thus this method would be satisfactory for air shipment, particularly if sufficient manpower were available in the field for manipulating the heavy boxes, etc.

Because some sample sites are remote and insufficient manpower is available for handling large containers, smaller containers were designed. These insulated boxes were about 12 x 22 x 22 in. and will contain two 2-gal polyethylene containers. Thus, six containers would be required for the expected two 40-liter wastewater samples. Each container filled with samples and dry ice weighs 98 lb and can be manipulated easily by one person. The containers with 7 lb or less dry ice can be conveniently shipped by Federal Express (70 lb limitation)

Fig. 3.11. Comparison of the HRLC chromatograms of the uv-absorbing constituents in unozonated and ozonated effluents from the Advanced Waste Treatment Laboratory (EPA) pilot plant in Cincinnati, Ohio.

Fig. 3.12. Comparison of the HPLC chromatograms of the cerate-oxidizable constituents in unozonated and ozonated effluents from the Advanced Waste Treatment Laboratory (EPA) pilot plant in Cincinnati, Ohio.

and thus arrive at ORNL within 24 to 30 hr of collection time. Freezing tests with water samples indicated that 25 lb of dry ice will freeze the water samples within 3 hr; the samples remained frozen for three days. This method will be used for the field sampling at Estes Park, Colorado.

3.4 Environmental Monitoring

The development of monitoring systems for sensitive and timely detection of various pollutants constitutes a very practical, necessary approach to our growing concern for the perturbations in our environment caused by various anthropogenic energy sources. The objective of this program is the development of such monitoring systems through the design, fabrication, and evaluation of prototype instruments. Three instruments are under various stages of development: (1) a continuous aquatic environmental monitor being evaluated for monitoring of treated coal conversion plant effluent; (2) a multicellular scintillation counter, fabricated and undergoing "proof of principle" testing; and (3) a remote fluorescence monitor being designed to detect fluorogenic surface contaminants.

3.4.1 Continuous monitors for aqueous effluents

During this report period, an aquatic environmental monitor which incorporates a continuous chemical oxygen demand monitor and a continuous-flow fluorimeter has been evaluated as a monitor for a treated aqueous effluent from a coal hydrocarbonization process. Details of the aquatic environmental monitor and its evaluation are presented in Continuous Environmental Monitoring for Aqueous Effluents, ORNL/TM-6118.

3.4.2 Multicellular scintillation counter for monitoring chromatographic eluents

Development of a multicellular scintillation counter for use in monitoring ultralow concentrations (less than 1 nCi/ml) of highly radioactive elements in liquid streams is being initially reported here. This instrument is to be used for dynamic continuous monitoring of

radioactive elements in process streams, such as chromatographic eluents, where process throughput and/or composition vary rapidly with time.

Referring to Fig. 3.13, it is seen that with conventional unit-cell detection, chromatographic resolution and peak signal to noise vary in opposite fashion with the detector residence time: long detector residence times yield good counting statistics but poor chromatographic resolution, and vice versa. Our multicellular counter is designed to overcome these limitations by following fluid sample segments through an array of short-residence-time detectors, while accumulating the total counts from each fluid segment during the entire time of passage through the array (Fig. 3.14).

This multielement detector is conceptually based upon the position-sensitive scintillation detector shown in Fig. 3.15. Scintillation photopulses produced by beta or gamma emission from the source propagate axially down the scintillation tube due to internal reflection. These propagating photopulses are logarithmically attenuated by axial absorbance or radial surface scattering as shown in Fig. 3.15:

$$\begin{aligned} I_{\text{left}} &= I_0 e^{-\alpha r}, \\ I_{\text{right}} &= I_0 e^{-\alpha(d-r)}, \end{aligned} \quad (1)$$

where

α = attenuation coefficient.

Hence the logarithmic difference of the left and right photopulse amplitudes,

$$\log(I_{\text{left}}) - \log(I_{\text{right}}) = -2\alpha r + \alpha d,$$

varies linearly with the location of the radioactive source r and can therefore be used to locate the source's position along the tube.

The coincident pulse ratio,

$$I_{\text{left}} \times I_{\text{right}} = (I_0)^2 e^{-\alpha d},$$

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1. SHORT RESIDENCE TIME GIVES POOR COUNTING STATISTICS
2. LONG RESIDENCE TIME GIVES POOR RESOLUTION

Fig. 3.13. Limitations of scintillation monitoring in typical flow cells.

ORNL DWG 77-1720-IM

1. GOOD COUNTING STATISTICS FROM LONG RESIDENCE TIMES
2. HIGH RESOLUTION FROM SMALL SAMPLE VOLUME

Fig. 3.14. Multi-cell scintillation monitor for flowing systems.

ORNL DWG 77-1721-1M

Fig. 3.15. Concept for position-sensitive multi-cell scintillation monitor.

is proportional to the square of the energy of the emitted beta or gamma and thus can be used to discriminate counts from different isotopes.

In this manner one can use coincident photopulses to assign counts to specific segments of a fluid moving through a flow-cell scintillation detector such as illustrated in Fig. 3.23. The multicellular detector "elements" therefore are the maximum position resolution segments of the scintillation pipe.

Test of principle was accomplished with the apparatus of Fig. 3.16. Photopulse spectra produced from scattered LED (GaP, $\lambda_{\text{max}} \approx 564 \text{ nm}$) radiation inside the taped quartz rod* were recorded on a Victoreen multichannel analyzer. Figures 3.17a and 3.17b show plots of such spectra, while Fig. 3.18 displays the logarithmic difference of the left and right photopeaks. One sees that 1% position resolution is easily attainable for LED pulses of energy equal to 10^5 photons.

Subsequent testing involved locating an encapsulated (^{144}Ce , ^{144}Pr) source, which emits a 2.98-MeV beta ray, inside a tube filled with liquid scintillator** (Fig. 3.19). Typical pulse-height spectra are shown in Figs. 3.20a and 3.20b. The capsule's position was calculated from the mean centers of the left and right detector spectra:

$$\text{center} = \frac{\sum_{j=1}^{200} JxY(J)}{\sum_{j=1}^{200} Y(J)},$$

using the PDP-10 FORTRAN program center .F4 listed in Fig. 3.21. The logarithmic difference of the left and right spectral centers is plotted vs the capsule's location in Fig. 3.22.

*The scotch tape serves two purposes: (1) to produce large scattered LED photopulses, and (2) to attenuate the propagating pulses, since it effectively frosts part of the tube surface.

**Amersham Searle Spectra-Fluor, 6 g PPO, 75 mg POPOP per liter in toluene base.

Fig. 3.16. Mock-up position sensitive scintillation monitor.

ORNL DWG. 77-1847

Fig. 3.17a. Typical pulse height spectrum of left channel LED photopulses.

ORNL DWG 77-1846

Fig. 3.17b. Typical pulse height spectrum of right channel LED photopulses.

ORNL DWG 77-1564A-1M

Fig. 3.18. Response of scintillation detector to pulsed LED beam.

ORNL DWG 77-1844

Fig. 3.19. Version 1 position sensitive scintillation detector.

Fig. 3.20a. Left channel scintillation pulse height spectrum.

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Fig. 3.20b. Right channel scintillation pulse height spectrum.

ORNL DWG 77-1841

```

REAL Y(400)
REAL*8 FILNAM
TYPE 100
100  FORMAT(' ENTER DATA FILNAM.DAT',/)
    READ(5,120)FILNAM
120  FORMAT(A10)
    OPEN(UNIT=1,FILE=FILNAM)
    READ(1)Y
    CLOSE(UNIT=1,FILE=FILNAM)
    ARL=0.
    ARR=0.
    CTRL=0.
    CTRR=0.
    TYPE 333
333  FORMAT(' ENTER START CHANNEL NO.',/,)
    READ(5,334)JIN
334  FORMAT(I3)
    DO 300 J=JIN,200
        ARL=ARL+Y(J)
        CTRL=CTRL+J*Y(J)
        ARR=ARR+Y(J+200)
        CTRR=CTRR+(J)*Y(J+200)
300  CONTINUE
        CHL=CTRL/ARL
        CHR=CTRR/ARR
        WRITE(5,400)CHL
400  FORMAT(' LEFT PMT MEAN CHAN. ',F8.3,/)
        WRITE(5,420)CHR
420  FORMAT(' RIGHT PMT MEAN CHAN. ',F8.3,/)
    STOP
    END

```

Fig. 3.21. Center.F4 source program.

ORNL DWG. 77-1845

Fig. 3.22. Pulse amplitude logarithmic difference vs sample position.

The prototype multicellular detector itself will be constructed as schematized in Fig. 3.23. Coincidence gating will be used to discriminate photopulses arising from the same disintegration; the pulse pair amplitudes will be digitized and stored on-line together with the time of the event, using the Intellec-8 microcomputer. Data analysis (i.e., developing the chromatogram) will be carried out on the ORNL PDP-10 computer.

3.4.3 Portable surface scanner for fluorogenic contaminants

Design and construction of a portable optical spotter for fluorogenic compounds is initially reported here. Detection of PAHs and other fluorescent hazardous substances, such as uranium compounds, on surfaces in work environments is the principal anticipated use for this development.

The detector head is schematically shown in Fig. 3.24; it consists of two separate compartments, one for producing a monochromatic cone of uv-visible fluorescence exciting light, and another for detecting the fluorescence emitted from the illuminated surface (see Fig. 3.25).

Discrimination against background room light, whose frequency spectrum is 120 Hz plus harmonics superimposed upon "white noise," is accomplished by modulating the exciting beam with the tuning-fork-type chopper and detecting the ac fluorescent signal with a high-Q heterodyning lock-in amplifier (not shown, Ithaco model Dynatrac 3). It is anticipated that fluorescent signals 20 dB below ambient lighting levels will be easily detectable.

The head is being constructed by the fabrication departments under W. A. Walker's supervision. Satisfactory demonstration of the sensitivity and usefulness of this instrument will predicate an in-house design of a portable power supply and electronics package.

3.5 References for Section 3

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Fig. 3.23. Prototype multi-cellular scintillation detector.

Fig. 3.24. Fluorescence scanner optics head.

Fig. 3.25. Scanner operating principle.

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4. BIOENGINEERING RESEARCH

S. E. Shumate II

The Bioengineering Research Program is concerned with the utilization of microorganisms or materials derived from microorganisms (e.g., enzymes and organelles) to carry out useful process functions for energy production and conservation, resource recovery, and environmental protection. Studies related to enzyme catalysis were focused on the enzyme-catalyzed reduction of water to produce hydrogen. Techniques were developed for enzyme separation, purification, stabilization, and immobilization. Factors affecting the rate of reduction of the electron carrier, ferredoxin, by sodium dithionite were studied. Investigations specific to problems in nuclear waste management included the removal of radionuclides and chemical contaminants from aqueous process and effluent streams and are discussed in Sects. 4.2, 4.3, and 4.4. Bioreactor component studies (Sect. 4.5) addressed the construction and operation of a large fluidized-bed reactor with tapered entrance and fluid-particle disengaging sections. A description of fluid and particle behavior in a two-phase (liquid-solid) fluidized bed was obtained from an analysis of data taken during operation of the large reactor.

4.1 Enzyme Catalysis

B. Z. Egan and J. P. Eubanks

During this report period, studies related to the enzyme-catalyzed reduction of water to produce hydrogen were continued. Two areas of interest were: (1) the rate of reduction of ferredoxin with sodium dithionite, and (2) the rate of oxidation of ferredoxin with oxygen.

4.1.1 Reduction of ferredoxin

Use of CFA parallel-channel rotor. We previously reported¹ kinetic results on the rate of ferredoxin reduction with sodium dithionite. The difference in absorbance of oxidized and reduced ferredoxin at 415 nm was utilized to measure the rate of the reaction. Since the reaction is

complete in a few seconds, the Centrifugal Fast Analyzer (CFA) was used to measure the rapid absorbance changes during the reaction. A sequential-type rotor was used in which the reactant solutions were successively transferred into the cuvetts, mixed, and the absorbance at 415 nm was measured. This rotor required about 0.4 sec for transfer of solutions, mixing, and acceleration before data acquisition began. The reaction has now been carried out in a parallel-channel rotor in which the reactant solutions were mixed as they entered the cuvet through separate parallel channels in the rotor. This rotor allowed the collection of data during the first 0.5 sec of reaction at approximately 50-msec intervals, with the initial measurement at about 100 msec after initiation of the reaction.

Data obtained during the first second of the reaction are shown in Fig. 4.1 for different concentrations of ferredoxin. The total reaction volume in each case was 135 μl : 55 μl of sodium dithionite solution containing 4.0 mg of $\text{Na}_2\text{S}_2\text{O}_4$ per milliliter of 0.05 M phosphate buffer, pH 6.6, and 80 μl of ferredoxin solution containing different amounts of ferredoxin in the same buffer solution. Buffer solutions were purged with argon before use. Sodium dithionite was stored in a glove box under an argon atmosphere, and solutions were prepared just prior to use. The ferredoxin was oxidized with air prior to reaction with the dithionite reductant. Reactant solutions were pipetted into the rotor in a glove box containing an argon atmosphere; the rotor was sealed before removal from the glove box. The reaction temperature was approximately 22°C. The series of curves shown in Fig. 4.1 were obtained by reacting different concentrations of ferredoxin with excess sodium dithionite. As shown, the absorbance of oxidized ferredoxin at 415 nm decreases as it is reduced by the dithionite. The initial rate of reduction was obtained from the slope of a linear fit of the data for the first 0.4 sec.

A plot of the initial rate of reduction as a function of the ferredoxin concentration (Fig. 4.2) indicates a linear correlation. As expected, the initial rates obtained using the parallel-channel rotor were higher than those obtained previously using the sequential-type rotor.¹

ORNL-DWG 77-1957

Fig. 4.1. Decrease in absorbance of oxidized ferredoxin on reduction with sodium dithionite. The different curves represent different concentrations of ferredoxin. Data were obtained using a parallel-channel rotor on the Centrifugal Fast Analyzer.

ORNL DWG 77-1955

Fig. 4.2. Rate of reduction of ferredoxin with sodium dithionite as a function of ferredoxin concentration in the range 10^{-5} to 10^{-4} M.

The order of addition and mixing of the reagents in the parallel-channel rotor appeared to have some effect on the reliability and scatter of the data obtained during the first 0.4 sec. Some change in absorbance of ferredoxin solutions alone was observed, either due to incomplete mixing or removal of material from the ferredoxin solution during centrifugation. Incomplete mixing during the initial 0.4 sec may result from differences in viscosity or density of the reactant solutions.

These results substantiate the conclusion that the reduction of ferredoxin by sodium dithionite is not the rate-limiting step in the production of hydrogen from the reduction of water by sodium dithionite, catalyzed by ferredoxin and hydrogenase.

Effect of pH. The effect of pH on the rate of reduction of ferredoxin with sodium dithionite was measured in the pH range 6.6 to 8.2, using the CFA. Potassium phosphate and tris-HCl solutions (0.15 M) were used to obtain buffer solutions with pH values of 6.6, 7.0, 7.4, 7.8, and 8.2. Two different concentrations of ferredoxin were studied ($A_{415} = 0.5$ and 0.9); an excess of sodium dithionite was used. Similar rates of reduction of ferredoxin were obtained throughout the pH range studied.

Spectrophotometric titration of ferredoxin with sodium dithionite. Although the reduction of ferredoxin with sodium dithionite has usually been carried out in the presence of an excess of dithionite, it was of interest to determine more closely the stoichiometry of the reaction, and to determine empirically the actual amount of dithionite required for the reduction under typical reaction conditions. Also, in order to study the oxidation of reduced ferredoxin (see Sect. 4.1.2), it was first necessary to obtain reduced ferredoxin in the absence of an excess of reducing agent. The amount of sodium dithionite required to reduce a given amount of ferredoxin was determined by spectrophotometric titration of solutions of oxidized ferredoxin, using the CFA.

Stock solutions of sodium dithionite were freshly prepared in a glove box just prior to use. Sodium dithionite was dissolved in 0.05 M phosphate buffer, pH 6.6, which had been purged with argon to remove oxygen. The ferredoxin was oxidized with air. The initial ferredoxin

concentration was the same for each experiment, and the amount of dithionite was varied by diluting the stock solution. A constant reaction volume of 135 μ l was maintained; the reaction temperature was 30°C.

The extent of reduction was measured by the decrease in absorbance of the ferredoxin at 415 nm. Complete reduction was assumed when the minimum absorbance was reached, and no further decrease in absorbance occurred with additional dithionite. Under the conditions chosen, complete reduction of the ferredoxin resulted in a decrease in absorbance of 35 to 38% (Fig. 4.3). Under other conditions, larger decreases were observed with some ferredoxin samples. In Fig. 4.3, the intersection of the extrapolated lines defines the end point, that is, the amount of dithionite required for complete reduction of the ferredoxin. The mole ratio of dithionite to ferredoxin at the end point was calculated to be about 28:1. The concentration of ferredoxin was calculated using the average initial absorbance at 415 nm and a molar absorptivity of 27,600 for oxidized ferredoxin.² The mole ratio of 28:1 is considered to be a maximum value. The concentration of dithionite in solution could be less than calculated because of reaction with dissolved oxygen in the ferredoxin solution and from disproportionation of the dithionite.

4.1.2 Oxidation of ferredoxin

Molecular oxygen exerts various inhibitory effects on enzyme-catalyzed hydrogen-producing reactions; oxidation or oxygenation may occur. Ferredoxin is also autooxidizable. The oxidation of ferredoxin by oxygen is postulated to be one of the causes for low rates and rapid decay of hydrogen production in the cell-free chloroplast-ferredoxin-hydrogenase system.³ It has also been stated that "the limiting factor in hydrogen evolution by the basis sytem is the rate at which ferredoxin reduction counterbalances ferredoxin oxidation by the oxygen produced during the reaction."³ And yet these rates are not well defined. More information is needed on the rate of oxidation of ferredoxin to better define the problem so that economical solutions can be devised.

ORNL DWG 77-1956

Fig. 4.3. Spectrophotometric titration of oxidized ferredoxin with sodium dithionite.

Several methods are being considered for measuring the oxidation rate. Methods that depend on measurement of oxygen concentration are limited somewhat by the response time of oxygen electrodes and require larger amounts of ferredoxin to produce a measurable change in oxygen concentration. Spectral methods based on the difference in absorbance of oxidized and reduced ferredoxin appear more promising at present. Requirements of spectral methods include: (1) maintaining a constant oxygen concentration, (2) mixing the solutions while simultaneously measuring absorbance, and (3) obtaining a sample of reduced ferredoxin which does not contain any extraneous oxidizable reductant. The third requirement becomes important if the rate of oxidation of the reductant is similar to the rate of oxidation of ferredoxin.

Results obtained by mixing oxygen-saturated buffer solutions with reduced ferredoxin solutions in the CFA and measuring the change in absorbance were unsatisfactory. Absorbance changes were not measurable. Injection of reduced ferredoxin into a sealed cuvet containing oxygen-saturated buffer solution was also unsatisfactory. Several seconds were required for mixing and attaining a stable absorbance reading. Best results were obtained by bubbling air or oxygen into a solution of reduced ferredoxin and measuring the increase in absorbance at 415 nm. It was also felt that this mode of reaction more closely resembled conditions which might occur in an actual process.

Reduced ferredoxin was produced by reaction of ferredoxin with sodium dithionite in an argon atmosphere in a glove box. The reduced ferredoxin solution was placed in a cuvet especially modified to reduce foaming and overflow. The cuvet was sealed with a Teflon cap with an outlet port and a small Teflon tube extending down the side to the bottom of the cuvet. The absorbance was continuously measured while air or oxygen was bubbled through the solution. The turbulence created by the gas stream served to mix the solution. The oxygen supply consisted of a cylinder of oxygen, a needle-valve flow controller, a rotameter, a sparger for saturating the gas with water, and a three-way stopcock for immediately starting and stopping the gas supply to the cuvet.

Oxidation rates were measured at different flow rates of air and oxygen, with samples of ferredoxin from both spinach and Clostridium pasteurianum, and at different concentrations of ferredoxin. Flow rates of oxygen were varied from 2.5 to 25 cm³/min; low flow rates gave insufficient mixing of the solution; high flow rates interfered with absorbance measurements. Once the flow rate was high enough to achieve good mixing (8-10 cm³/min), changing the flow rate did not appear to have an appreciable effect on the rate of oxidation.

Figure 4.4 shows representative curves obtained by oxidation of ferredoxin from spinach (curve 1) and Clostridium pasteurianum (curve 2). The dashed lines indicate the initial absorbance of the oxidized ferredoxin samples before reduction with dithionite. The amounts of dithionite and ferredoxin were chosen in each case to give a mole ratio less than 28:1, taking into account the higher molecular weight of spinach ferredoxin. Each sample was reduced with sodium dithionite (325 mg) just prior to oxidation. The spinach ferredoxin sample contained 1.20 mg of ferredoxin in a total volume of 3.6 ml of 0.05 M phosphate buffer, pH 6.6. The Clostridium pasteurianum ferredoxin sample contained 0.60 mg of ferredoxin in a total volume of 3.0 ml. The oxygen flow rate was about 12 cm³/min; in both cases, maximum oxidation was achieved in about 25 sec. The ferredoxin was not completely reoxidized. This incomplete reoxidation also occurred on each cycle when the same sample was repeatedly reduced and oxidized. Oxidation was more rapid in oxygen than in air. The time required for maximum oxidation in air ranged from 30 to 40 sec, while in oxygen the range was 8 to 25 sec.

The rate of autooxidation of ferredoxin was estimated by measuring the absorbance increase of reduced ferredoxin in a closed cuvet (argon atmosphere). The absorbance showed a gradual increase over a period of 35 to 45 min before reaching a constant value near that expected for oxidized ferredoxin. While the rate of autooxidation must be recognized in processes utilizing ferredoxin, the autooxidation rate is much less than the rate of oxidation by molecular oxygen. Potential methods for alleviating the oxidation problem are chemical or biochemical removal of oxygen as it is formed or rapid displacement of the oxygen with an inert gas.

Fig. 4.4. Oxidation of ferredoxin from spinach (curve 1) and from *Clostridium pasteurianum* (curve 2) with molecular oxygen. The dashed lines indicate the initial absorbance of the oxidized ferredoxin samples before reduction with dithionite. See text for details.

4.2 Concentration of Tritium by Microorganisms

G. W. Strandberg and J. R. Parrott, Jr.

That microorganisms might be used in a bioprocess to concentrate tritium in fuel reprocessing effluent streams was suggested by the report of a marine bacterium which could fractionate hydrogen and deuterium. This organism evolved hydrogen gas depleted in deuterium as compared with normal background levels.

The ability of this organism, as well as other species of bacteria, to fractionate hydrogen and tritium was evaluated by determining the distribution of tritium between microbial cells, hydrogen gas, and the aqueous, tritiated medium in which they were grown. Tritium was assayed by scintillation counting; the aqueous medium was assayed directly. Microbial cells were recovered by filtration or centrifugation, dried, combusted in an oxygen atmosphere (Searle analytic combustion system), and the water of combustion assayed. When sufficient quantities of hydrogen gas were recovered (by water displacement), the gas was oxidized over a 600°C copper oxide bed, and the resulting water was analyzed for tritium.

We obtained a culture of the marine bacterium (strain G4Ab) which had during storage lost its ability to produce significant levels of hydrogen. However, we proceeded to culture the organism in a complex organic medium with tritiated water. The cells showed discrimination against tritium during growth as evidenced by a tritium distribution factor of 0.12 (Ci per gH cells/Ci per gH medium) (Table 4.1).

It was possible, but unlikely, that the observed discrimination by strain G4Ab was due to incorporation of much of its hydrogen from organic components of the medium, rather than tritiated water. Selection of other organisms for study was therefore based on (1) maximum utility of environmental water for hydrogen, that is, growth on mineral salts medium with minimal organics, and (2) ability to produce hydrogen. Conditions for optimum growth and/or hydrogen evolution were used. Anabaena flos-aquae, a blue-green alga, and Clostridium pasteurianum, an

Table 4.1. Distribution coefficients for cellular and gaseous (HT) tritium from microorganisms cultured in tritiated water

Organism	Growth conditions	Initial tritium concentration in growth medium (mCi/liter)	Distribution coefficient	
			Cells ^a	H ₂ ^b
Strain G4A(b)	Aerobic, complex organic medium	2	0.12	
<u>Anabaena flos-aquae</u>	Photosynthetic, nitrogen-fixing mineral salts medium	2	0.28 (growth phase) 0.28 (H ₂ evol. phase) ^c	Insufficient for analysis
		10	0.52 (growth phase) 0.51 (H ₂ evol. phase) ^c	Insufficient for analysis
<u>Thiobacillus ferrooxidans</u>	Aerobic, mineral salts medium	10	0.002	
<u>Clostridium pasteurianum</u>	Anaerobic, nitrogen-fixing, mineral salts medium plus glucose	10	0.06	0.07

^aDistribution coefficient = Ci per gH cells/Ci per gH medium.

^bDistribution coefficient = Ci per gH gas/Ci per gH medium.

^cNitrogen-fixing cells deprived of N₂ by flushing with argon.

anaerobic, spore-forming bacterium, were grown under nitrogen-fixing conditions to promote H_2 evolution. Photosynthetic H_2 evolution by A. flos-aquae occurs when nitrogen-fixing cells (growth phase) are deprived of gaseous nitrogen (H_2 evolution phase). Although both cultures evolved hydrogen, conditions were such that only C. pasteurianum produced recoverable quantities of H_2 for analysis of tritium content. The results of the tritium distribution studies are summarized in Table 4.1. Invariably, discrimination against tritium occurred in the microbial cells and during H_2 evolution by C. pasteurianum. Thiobacillus ferrooxidans, an organism which grows on mineral salts in a highly acid medium (pH 2.5-3.0), exhibited the most discrimination.

The discrimination against tritium observed in these microorganisms is consistent with numerous literature reports of discrimination against heavier isotopes of hydrogen (deuterium and tritium) by intact biological systems as well as isolated enzymes. Discrimination against heavier isotopes of other elements has also been reported.

4.3 Isolation of Trace Metals from Aqueous Streams by Microorganisms

S. E. Shumate II, G. W. Strandberg, T. V. Dinsmore,
J. R. Parrott, Jr.

In the previous progress report,¹ it was pointed out that a number of the processing operations in the nuclear fuel cycle and special DOE facilities generate aqueous waste streams which contain trace quantities of dissolved heavy metals, including radionuclides. There are a number of physical and/or chemical methods for isolating heavy metals from aqueous streams, and these include chemical precipitation, chemical oxidation or reduction, ion exchange, filtration or ultrafiltration, electrochemical treatment, and evaporative recovery. However, when the initial heavy-metal concentration is in the range of 10 to 100 mg/liter and a reduction to less than 1 mg/liter must be achieved, such methods may be ineffective or uneconomic. In such situations the adsorption of dissolved metal species by microorganisms offers a means of achieving

a reduction in dissolved metal concentration to less than 1 mg/liter. Studies of metal sorption by microorganisms in both mixed culture and monoculture have shown very high affinities for trace levels of dissolved metals. In many cases, solid-phase (biomass) metal concentrations of 10 to 20 wt % were attained.⁴

The previous report¹ described studies of continuous isolation of strontium from a feed stream containing 10 mg/liter by an alga (Chlorella pyrenoidosa) growing in an illuminated columnar bioreactor. These studies were continued, but the feed-stream strontium concentration was lowered to 0.10 mg/liter, and ⁸⁵Sr was added as a tracer for determination of dissolved and cell-bound strontium. Glass surfaces were treated with Siliclad, a silicone cladding agent, to prevent interference by metal sorption onto the reactor and feed vessels. The algal cell concentration reached a steady-state value of 0.30 g/liter (dry weight basis) before switching to a feed containing a strontium concentration of 0.10 mg/liter. Dissolved strontium concentration at the reactor outlet was periodically determined. After processing 1, 2, and 3 reactor volumes of feed, the degree of strontium removal from solution was 40, 20, and 10%.

A continuing part of the total program effort is the screening of microorganisms for the capability to isolate and concentrate heavy metals. Uranium is being used as a model element typical of the transuranics, while strontium is being used as an element typical of the fission products.

In the screening tests for strontium isolation, strontium was added to the appropriate growth medium prior to addition of the inoculum. In the uranium isolation screening tests, partial precipitation of uranium by the medium constituents necessitated a modification to the experimental procedure. The inoculum was added to 100 ml of medium in a 250-ml culture flask. The cells were then grown to the desired cell concentration, harvested, washed repeatedly with distilled water, and resuspended in solutions containing the desired concentrations of uranium. Flasks were pretreated with Siliclad. In each case, 2-ml samples were periodically withdrawn, and the cells were removed by centrifugation or by filtration

through glass wool in the case of Penicillium chrysogenum. The supernatant (cell-free) fraction was then analyzed for strontium or uranium content. Controls were run in each case to assure that metals removed from solution were associated with biomass. The results of preliminary tests are given in Table 4.2. Metal distribution coefficients were calculated on the basis that metal removed from solution was sorbed onto biomass.

4.4 Removal of Special Organic Compounds by Microorganisms

D. D. Lee and D. A. McWhirter

The removal of trace special organic contaminants from certain aqueous process streams in nuclear fuel processing and reprocessing is important in preparing the stream for discharge or recycle. The identified organic contaminants are generally 2-, 3-, and 4-carbon alcohols and carboxylic acids, kerosene, vacuum-pump oil, tributyl phosphate, HDEHP [di(2-ethyl-hexyl)phosphoric acid], dodecane, DIPB, and possibly their degradation products. These compounds result generally from solvent-extraction steps in the cycle.

The previous report¹ presented data on the degradation of 2-, 3-, and 4-carbon alcohols and carboxylic acids in both a continuous stirred-tank reactor (CSTR) and a tapered fluidized-bed bioreactor (TFBBR). As described, the CSTR generally achieved a rate of 0.4 to 0.6 kg/cm³·day organic carbon degraded, and the TFBBR, rates of 5 to 7 kg/m³·day organic carbon degraded, using a synthetic feed mixture containing 2-, 3-, and 4-carbon alcohols and carboxylic acids. Additional tests were performed to determine organic carbon degradation rates, using individual compounds listed above.

Ethanol was tested as the sole carbon source in both the CSTR and the TFBBR. Results for the TFBBR (Table 4.3) showed that bacterial growth with a seed of Polybac (Polybac Corporation, Berlin, New Jersey) was slower when grown on ethanol than when grown on a mixture of carbon sources; that is, it took about 1-1/2 times as long to reach growth visible on the coal particles. Carbon degradation rates for ethanol were generally

Table 4.2. Isolation of uranium and strontium from solution by microorganisms during batch culture or resuspended contact

Microorganism	Metal	Initial concentration (mg/liter)	Removal (%)	Cell concentration, dry basis (g/liter)	Metal distribution ^a coefficient
<i>Pseudomonas aeruginosa</i> (bacterium)	U	20	92	1.2	9600
	Sr	0.15	60	1.5	Not determined
<i>Paecilomyces marquandii</i> (fungus)	U	20	94	1.2	13100
	Sr	0.25	19	5.0	50
<i>Penicillium chrysogenum</i> (fungus)	U	20	97	3.1	10300
<i>Ashbya gossypii</i> (yeast)	U	20	73	7.0	390
<i>Saccharomyces cerevisiae</i> (yeast)	Sr	0.25	12	5.6	24

^aMetal distribution coefficient = $\frac{\text{g metal/g cells (dry)}}{\text{g metal/g solvent}}$.

Table 4.3. Tapered fluidized-bed bioreactor operation with ethanol as the sole carbon source^a

Time after start (days)	Concentrate feed rate ^b (cm ³ /min)	Reactor carbon concentration (μg/cm ³)		Carbon reduction rate (kg/m ³ ·day)	Nitrate (μg/cm ³)		Ammonia (μg/cm ³)		Reduction rate (kg/m ³ ·day)	
		Inlet	Exit		In	Out	In	Out	Nitrate	Ammonia
3	32.7	220.8	191.0	3.17	42.9	<1.0	337.8	329	4.64	0.94
4	30.4	79.4	38.0	4.38						
7		<u>Bacteria-coated coal was removed and fresh coal was added</u>								
10	21.3	288.3	278.0	1.06	237.8	282.0	389	389	0.59	0
15	19.1	172.3	156.0	1.66	123.3	107	348.3	346	1.66	0.238
17	29.9	222.6	195.0	2.91						
20	20.3	96.2	67.0	2.99						
23	13.7	45.8	31.0	1.49	18.6	<1.0	560.3	537	1.87	2.34
25	14.8	30.7	15.0	1.59	214.0	200	313.0	305	1.41	0.81
28	17.0	46.5	23.0	2.39						

^aPolybac bacterial seed.

^bConcentrated feed-effluent recycle-recycle rate, 300 cm³/min.

about one-half (1.5 to $3.0 \text{ kg/m}^3 \cdot \text{day}$) of the value achieved with a mixed carbon source, but virtually all of the ethanol was degraded. The feed carbon to the reactor was 30 to $100 \text{ } \mu\text{g/cm}^3$, as determined using a Dohrman model DC-50 T.O.C. analyzer. The reactor was run with a concentrated feed recycle makeup to achieve adequate fluidization.

During this test, nitrate reduction averaged 1 to $2 \text{ kg/m}^3 \cdot \text{day}$ and ammonia oxidation 0.8 to $2.0 \text{ kg/m}^3 \cdot \text{day}$ on an ammonia basis. Total nitrogen reduction rate was $0.5 \text{ kg/m}^3 \cdot \text{day}$ and was determined using a Dohrman DN-10 total nitrogen analyzer. Biomass carbon production averaged $0.45 \text{ kg/m}^3 \cdot \text{day}$.

One CSTR was operated with ethanol as the sole carbon source (Table 4.4). The carbon degradation rate was 0.7 to $1.1 \text{ kg/m}^3 \cdot \text{day}$. The retention time was 0.17 to 0.39 days (4.0 to 9.4 hr) at a flow rate of 5.35 to $12.1 \text{ cm}^3/\text{min}$. The feed carbon was $340 \text{ } \mu\text{g/cm}^3$ and effluent carbon 30 to $200 \text{ } \mu\text{g/cm}^3$, depending on retention time. The amount of biomass carbon produced averaged 0.32 g per gram of carbon degraded. Nitrate and ammonia were not actively degraded to a large extent. The nitrate removal rate was a maximum of $1 \text{ kg/day} \cdot \text{m}^3$ of reactor volume at the end of the 25-day test.

A second CSTR was operated with acetate as the sole carbon source (Table 4.4). The carbon degradation rate was between 0.4 and $1.3 \text{ kg/day} \cdot \text{m}^3$ of reactor volume. Retention time was 0.19 to 0.39 day (4.5 to 9.4 hr) at feed rates of 5.3 to $11.1 \text{ cm}^3/\text{min}$ to the 3000-cm^3 reactor. Nitrate and ammonia were not degraded extensively in the reactor.

The feed composition for the tests is shown in Table 4.5 and results in Tables 4.3 and 4.4.

A series of shake flask tests was conducted on several organics typical of those to be found in the weak-acid recycle stream of nuclear fuel processing schemes. These materials are shown in Table 4.6. The first tests were conducted using three different bacterial systems to ascertain the biological seed or seed combination best able to degrade the organics. These were obtained in dry form from Polybac Corporation, Berlin, New Jersey, under the trade names Polybac, Petrobac, and Phenobac. The results of this test series are shown in Table 4.6: Polybac

Table 4.4. Continuous stirred-tank bioreactor operation with ethanol or acetate as the sole carbon source

Time after start (days)	Feed rate (cm ³ /min)	Retention time (days)	Feed carbon concentration (μg/cm ³)	Effluent carbon concentration (μg/cm ³)	Carbon degradation rate (kg/day·m ³)	Biomass carbon (μg/cm ³)	Biomass production rate (kg/day·m ³)
<u>Ethanol as the carbon source</u>							
5	7.4	0.282	351	190	0.48		
6	7.4	0.282	343	206	0.46	12	0.0426
7	9.4	0.222	343	181	0.73	30	0.135
8	10.6	0.197	343	190	0.78	48	0.244
12	5.35	0.390	357	31	0.84	53	0.136
14	10.0	0.208	345	153	0.84	37	0.178
19	10.2	0.204	325	143	0.77	72	0.325
20	8.33	0.205	340	66	1.096	36	0.144
21	9.37	0.222	311	67	1.092	35	0.157
24	5.67	0.368	188	26	0.441	66	0.180
27	10.80	0.193	315	97	1.13	70	0.363
28	12.10	0.172	332	158	1.010	12	0.070
<u>Acetate as the carbon source</u>							
6	10.10	0.206	393	198	0.945	14	0.068
7	8.27	0.252	421	237	0.730	10	0.040
8	10.30	0.202	416	158	1.276	8	0.040
12	5.32	0.392	310	54	0.653	15	0.038
14	5.57	0.374	370	220	0.382	25	0.067
19	10.00	0.208	306	85	1.061	105	0.504
20	9.83	0.212	332	58	1.293	37	0.175
21	10.40	0.200	96	16	0.400	64	0.319
27	11.00	0.189	378	245	0.702	27	0.143
28	11.10	0.188	393	173	1.172	30	0.160
29	9.50	0.219	383	100	1.290	32	0.146

Table 4.5. Feed composition for TFBBR and CSTR tests

Component	Concentration
Ethanol or acetate	~600 ppm
H_3PO_4 (85%)	$0.1 \text{ cm}^3/\text{cm}^3$ or $140 \text{ }\mu\text{g}/\text{cm}^3 \text{ PO}_4^{-3}$
$(\text{NH}_4)_2\text{SO}_4$	$500 \text{ }\mu\text{g}/\text{cm}^3$
NH_4NO_3	$250 \text{ }\mu\text{g}/\text{cm}^3$
Antifoam	$0.1 \text{ cm}^3/1000 \text{ cm}^3$
Boron	$0.01 \text{ }\mu\text{g}/\text{cm}^3$
Zinc	$0.01 \text{ }\mu\text{g}/\text{cm}^3$
Molybdenum	$0.01 \text{ }\mu\text{g}/\text{cm}^3$
Manganese	$0.01 \text{ }\mu\text{g}/\text{cm}^3$
Copper	$0.01 \text{ }\mu\text{g}/\text{cm}^3$
Iron	$0.01 \text{ }\mu\text{g}/\text{cm}^3$

Table 4.6. Effectiveness of bacterial preparations in degrading organic compounds associated with aqueous streams in the nuclear fuel cycle

Conditions: 5-day incubation period at 30°C, pH 6.8 buffer,
150 rpm shaker
Starting concentration of organic: $500 \mu\text{g}/\text{cm}^3 = \sim 200 \mu\text{g}/\text{cm}^3$
TOC
Total volume each: 100 cm^3
Bacterial seed: 0.50-cm^3 solution made by dissolving 5 g of
dry bacterial seed in 50 cm^3 warm water

Component	Carbon reduction (%)	Bacterial system
TBP 100% (tributyl phosphate) ^a	50 14 31	Polybac Phenobac Petrobac
30% TBP in dodecane (used) ^a	46 12 42	Polybac Phenobac Petrobac
30% TBP in dodecane (virgin) ^a	38 19 38	Polybac Phenobac Petrobac
HDEHP [di(2-ethyl hexyl) phosphoric acid] ^a	8.8 8.8 16.4	Polybac Phenobac Petrobac
0.8 M HDEHP in DIPB ^a	42 46 35	Polybac Phenobac Petrobac
DIPB [di(isopropyl) benzene] ^a	65 46 50	Polybac Phenobac Petrobac
Kerosene	76 49 58	Polybac Phenobac Polybac
Vacuum pump oil	78 0 0	Polybac Phenobac Petrobac

^aObtained from F. A. Kappelmann, Experimental Engineering Section, Chemical Technology Division.

is the most versatile in degrading the variety of organics tested. Petrobac was able to degrade most of the materials, and Phenobac much less.

A second series of tests was conducted using a combination of Polybac and Petrobac at a total initial $400 \mu\text{g}/\text{cm}^3$ concentration with the previously tested solvents. Results are shown in Table 4.7: the organic carbon was broken down to some extent, usually greater than 50%, and bacterial growth was evident; in several cases, the liquid was very turbid. By centrifuging the samples and measuring total organic carbon before and after centrifugation, the amount of biomass carbon was determined; it ranged from 11 to $107 \mu\text{g}/\text{cm}^3$ after 28 days of incubation.

A flask of 4000-cm^3 capacity containing 3000 cm^3 of liquid and an aerator and stirrer was operated for several months on a 70 to 85% aqueous media (Table 4.5) and 15 to 30% vacuum pump oil. The bacterial seed was initially Polybac, with subsequent seedings taken from the flask before cleaning and filling with fresh feed. In this test, there were two distinct phases present — a water phase and the vacuum pump oil phase. Up to 15 days were required by the bacteria to form a uniform emulsion, after which bacterial growth greatly accelerated. Twenty-five days after seeding, no vacuum pump oil separated out upon centrifugation. This test was repeated three times, and the results were the same. No attempt was made to operate the system on a continuous basis.

4.5 Biological Treatment of Aqueous Effluents from Coal Conversion Processes

D. D. Lee and D. A. McWhirter

4.5.1 Biotreatment of scrubber water produced by the ORNL bench-scale coal hydrocarbonization facility

This report covers experimental work conducted since the previous report¹ in which the TFBFR for treating phenolic wastes produced by coal conversion processes was described. The previous report summarized data obtained for treatment of the scrubber water produced in HC experimental runs HC-6, -8, -5, -10, and -9, -11, and -12. Treatment was in

Table 4.7. Shake-flask tests on organics with a Polybac-Petrobac bacterial seed

Flask No.	Solvent	Initial concentration ($\mu\text{g}/\text{cm}^3$)				
1	100% TBP	1600				
2	30% TBP in dodecane	1600				
3	HDEHP	800				
4	DIPB	1600				
5	50% kerosene; 50% vacuum pump oil	1600				
6	33-1/3% kerosene; 33-1/3% vacuum pump oil; 33-1/3% TBP	2400				
7	33-1/3% kerosene; 33-1/3% vacuum pump oil; 33-1/3% DIPB	2400				
8	33-1/3% kerosene; 33-1/3% vacuum pump oil; 33-1/3% (30% TBP in dodecane)	2400				

Flask No.	Initial measured carbon ($\mu\text{g}/\text{cm}^3$)	14 days	19 days	28 days	Supernate after centrifuge	Biomass carbon
1	475	240		340	310	30
2	250	224		225	185	40
3	688	567		532	502	30
4	195	58		Flask broken		
5	330	277	268/112	121	83	38
6	370	212		163	152	11
7	455	116		65	46	19
8	600	489	480/180	268	161	107

a recirculation mode of operation in which a concentrated feed is diluted with a portion of the reactor effluent to yield the proper concentration and fluidization velocity for proper operation. During the current reporting period, the reactor was run using a dilute feed, with no recycle makeup, on scrubber water from HC-9, -11, -12; -13, -14; and -3 and -4. The scrubber water batches were mixed together to provide enough volume for at least seven days of operation, using one composite feed solution. Table 4.8 shows an analysis of the feeds.

In operation, the scrubber water was diluted to the desired concentration (after acidification and 0.45- μ m filtration to remove particulates), which was 20 to 160 $\mu\text{g}/\text{cm}^3$ phenols, as determined by the standard 4-amino antipyrine method.⁵ The dilution water was process water. The feed was adjusted to a pH of 6.0 to 6.9 with NaOH, and 10 $\mu\text{g}/\text{cm}^3$ antifoam and trace metals were added to assure adequate nutrients for the bacteria. Tables 4.9 and 4.10 show the results of the tests. Figure 4.5 shows graphically the day-to-day operation of the bioreactor. The figure shows that the TFBRR is able to accommodate a range of feed conditions and will quickly adapt to new conditions. As noted in the previous report,¹ some nitrification and denitrification were observed while the reactor was degrading phenol and thiocyanate.

After concluding the dilute hydrocarbonization scrubber water biodegradation test, a series of tests was begun to determine the growth parameters of the bacteria on the surface of the coal particles to which they are attached. These tests have been conducted using a synthetic feed made up with a commercial phenol instead of actual scrubber water to better understand the reactions taking place and to reduce the variability of the reactor feed.

In addition, a clarifier was added downstream of the reactor to reduce the biomass load in the reactor effluent. The clarifier consists of a cylinder with a volume of $62.55 \times 10^3 \text{ cm}^3$, dimensions of 22.86 cm in diameter x 152.4 cm long, and made of Pyrex. The cylinder is fitted with an inlet at the bottom and an exit at the top and is fed by gravity. At a feed rate of 300 cm^3/min the retention time is 208.5 min ($\sim 1\frac{1}{2}$ hr),

Table 4.8. Analysis of coal hydrocarbonization scrubber solution

Component	Concentration ($\mu\text{g}/\text{cm}^3$)		
	Average	High	Low
Phenols	6900	10,000	3000
Thiocyanate	380	600	200
Ammonia	3600	7,000	1000
Nitrate	5700	11,600	3000
Phosphate	20	50	3
Total carbon	9300	20,000	3800
Carbon dioxide	1250	2,000	1000
Organic carbon	8150	18,000	2800
pH	9.5	10.1	8.5

Table 4.9. Hydrocarbonization bioreactor dilute feed,^a feed and effluent, typical results

Component	Feed concentration ($\mu\text{g}/\text{cm}^3$)	Effluent concentration ($\mu\text{g}/\text{cm}^3$)
Phenol	50	<1
Thiocyanate	8	2
Ammonia	25	22
Nitrate	30	2
Phosphate	40	35
Total carbon	55	25
Carbon dioxide		
Organic carbon	55	10
Biomass		15
pH	6.8	6.1

^a Feed rate, $300 \text{ cm}^3/\text{min}$; phenol rate, $10 \text{ mg}/(\text{cm}^3)(\text{day})$; scrubber water dilution, 70:1.

Table 4.10. Phenol degradation in a tapered fluidized-bed bioreactor using dilute feed

Elapsed time (days)	Feed pH	Reactor pH	Column temperature (°C)	Feed rate (cm ³ /min)	Reactor space time (min)	Feed batch in use (HC-)	Concentration (µg/cm ³)										Rate (kg/day.m ³) ^a						
							Feed phenol	Effluent phenol	Feed thiocyanate	Effluent thiocyanate	Feed nitrate	Effluent nitrate	Feed ammonia	Effluent ammonia	Feed total organic carbon	Effluent total organic carbon (cell-free)	Effluent biomass total organic carbon	Phenol degradation rate ^b	Thiocyanate degradation rate	Denitrification rate	Nitrification rate	Total organic carbon degradation rate	Biomass carbon production rate
1	6.8	6.2	20	300	15.0	(9,11,12)	50.9	19.2	4.1	2.3	31.7	15.6	26.7	24.9	54	28	5	3.04	0.173	1.74	0.173	2.50	0.48
2	6.5	6.0	21	300	15.0		44.8	9.6	6.0	1.5	11.3	7.4	22.0	22.0	56	23	5	3.52	0.432	0.37	0.0	3.17	0.48
3	6.3	5.9	19	315	14.3		32.9	1.5	8.2	2.1	20.7	2.1			43	15	10	3.17	0.615	1.87		2.82	1.01
4	6.5	5.9	19	310	14.5		23.0	2.3										2.05					
5	6.9	6.1	17.5	310	14.5		39.7	1.1										3.83					
6	6.6	6.4	18	300	15.0		39.3	3.0										3.60					
7	6.0	5.6	20	300	15.0		35.9	1.3	4.3	4.0	25.6	10.2	30.0	30.0	44	16	6	3.32	0.317	1.48		2.69	0.576
8	5.7	4.6	19	310	14.5	(9,11,13,14)	59.7	2.9	3.9	4.0	35.1	15.9	32.0	32.0	57	19	26	5.63	0.288	1.90		3.77	2.58
9	5.7	4.6	20	300	15.0	13,14	70.0	2.9	4.9	<1.0	43.8	24.3	31.6	29.4	61	29	26	6.44	0.374	2.35	0.202	3.07	2.50
10	6.2	5.0	21	300	15.0		74.8	2.7	6.2	2.5	52.0	11.5	32.2	29.0	68	17	33	6.92	0.355	3.89	0.307	4.89	3.17
11	6.6	5.8	18	305	14.8		76.3	6.0	7.3	4.0	53.9	13.3			73	25	5	6.86	0.322	3.96		4.68	0.49
12	6.4	5.8	20	310	14.5		113.1	28.4	10.2	3.8	84.4	36.3			102	42	29	8.40	0.635	4.77		5.95	2.88
13	6.5	5.6	20	310	14.5		106.4	8.9	12.3	2.6					107	36	17	9.67	0.962			7.04	1.69
14	6.5	5.7	19	315	14.3		112.4	8.8	10.2	1.9	76.4	25.0	48.5	37.5	110	43	15	10.44	0.837	5.18	1.109	6.75	1.51
15	6.4	5.6	22	305	14.8	3,4	114.0	16.1	10.1	2.1	80.3	27.1			132	58	5	9.56	0.781	5.19		7.22	0.49
16	6.5	5.7	22	300	15.0		135.6	29.2	7.2	2.2	83.0	28.0			133	60	11	10.21	0.480	5.28		7.01	1.06
17	6.9	6.1	19	305	14.8		136.5	36.0	10.2	2.2	91.0	38.0			144	64	8	9.81	0.781	5.17		7.81	0.78
18	6.8	5.9	19	310	14.5		133.6	29.3	14.3	1.8	98.5	24.2	75.6	57.7	142	48	25	10.35	1.240	7.35	1.776	9.32	2.48
19	6.8	5.9	19	300	15.0		160.0	41.2	4.5	1.1	82.1	29.1			123	49	16	11.40	0.326	5.09		7.10	1.54
20	6.9	6.3	19	300	15.0		72.2	1.29	5.3	1.0	36.0	8.25			55	15	15	6.81	0.413	2.66		3.84	1.44

^a Reactor volume (4.5 x 10³ cm³).^b Maximum phenol rate = 11.40 kg/day.m³.

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Fig. 4.5. Coal hydrocarbonization aqueous waste treatment in a tapered fluidized-bed bioreactor.

and at $725 \text{ cm}^3/\text{min}$ the retention time is 86.28 min (1.44 hr). The clarifier has been successful at removing up to 95% of the biomass entering at low flow rates ($300 \text{ cm}^3/\text{min}$) and up to 90% at high flow rates ($725 \text{ cm}^3/\text{min}$). Turbidity of the bioprocess effluent was greatly reduced and approached that of tap water ($\text{abs}_{550 \text{ nm}} 0.005$ vs 0.000 for water, while column effluent was 0.025-0.075 vs 0.000).

The TFBRR used to treat the aqueous waste from the coal hydrocarbonization facility has been kept in operation using synthetic feeds. A series of tests has been performed to help determine the characteristics of the bacterial growth on the coal surface. The tests have all used a feed stream made by combining a concentrated ($12,000\text{--}20,000 \text{ }\mu\text{g}/\text{cm}^3$) phenol stream with a process water stream in various proportions to achieve the desired reactor inlet concentration. The concentrated phenol feed was prepared as shown in Table 4.11.

Table 4.11. Concentrated synthetic phenol feed for TFBRR tests

Component	Concentration ($\mu\text{g}/\text{cm}^3$)	Concentration after dilution ($\mu\text{g}/\text{cm}^3$)
Phenol	12,000 – 20,000	27 – 660
Thiocyanate	5,975	10 – 180
Ammonium nitrate	18,000	(Nitrate) 60 – 1000
Ammonium sulfate	30,000	(Ammonia) 20 – 700
Trace metals	3,000	8 – 100
Antifoam	3,000	8 – 100
Phosphoric acid	2,472	(Phosphate) 7 – 85
Glucose	3,000	8 – 100
pH (NaOH)	7.5	7.0 – 7.5

Several different methods were tried to control the biomass inventory on the coal in the reactor. These included: periodically passing all coal particles in the reactor through a centrifugal pump followed by a venturi to remove a majority of the biomass; periodically removing

a portion of the bed coated with biomass, cleaning with caustic, and returning coal particles to the bed; and letting the particles with excess biomass float overhead and be removed by sedimentation in the reactor catch tank or the clarifier. The first method results in a uniform bed for a short period of time, but during the cleaning in the pump and venturi, some of the cells are ruptured, resulting in a very sticky gluelike material that starts to clog up the reactor by sticking to walls and other reactor internals. This provides a very good surface for biomass growth, which must be removed by shutting down the reactor and cleaning.

The second method — periodic removal of coated coal with addition of fresh coal — appears to be the best long-term method of maintaining control over the biomass inventory in the reactor. In several experiments, after a stable biomass population was reached (3–5 days after seeding), an amount of coated coal ($200\text{--}1000\text{ cm}^3$ settled volume) was removed daily and replaced with a volume of fresh coal one-third that of the removed volume. In this manner the number of particles with very large amounts of biomass — 2 to 5 mm in diameter compared with clean coal of 0.2 to 0.4 mm in diameter — was kept to a minimum.

The third method — letting the large biomass-coated particles go overhead — was very unsuccessful. In about 7 to 10 days, depending on the degradation rates, the reaction zone contained very little biomass because 95% had gone overhead. The addition of fresh coal each day to prevent the loss of all biomass was not a good remedy, because the large coated particles going overhead eventually plugged up lines and other parts of the reactor system, including the clarifier. The sloughing of the attached biomass was not evident at even the maximum liquid and gas flow rates to the reactor. Table 4.12 gives some general results for the biomass loading studies conducted to date. The table shows that at the beginning of the experiment, total settled volume of the coal changes very little as bacteria begin to grow; but the change in volume becomes very rapid, starting about five days after seeding. The settled bed volume can easily double in one day, even though a portion is removed,

Table 4.12. Biomass loading and control in a TFBFR treating phenol
(2.54- to 7.62-cm taper, 4500-cm³ volume reaction zone)

Days after seeding	Liquid flow rate (cm ³ /min)	Phenol degradation rate (kg/day·m ³)	Settled volume of reactor (cm ³)	Settled volume of removed coal (cm ³)	Wet density of biomass coal (g/cm ³)	Dry density of biomass (g/cm ³)	Volume of added coal (cm ³)	Settled volume of clean coal removed (cm ³)
3	500		1220					
4	600	6.76	1240					
5	615	3.46	1255					
6	610		1380					
7	610	3.02	1610					
8	620	3.57	1710	1000	0.816		250	700
9	600	3.09	1085					
10	625	3.27	1153	860	0.723		750	560
11	620	8.53	1270	445	0.800	0.0814	500	230
14	630	8.63	5060	880	0.509	0.183		650
15	615	9.35	5400	800	0.379	0.0375		320
16	630	10.64	5521	1000	0.178	0.0111	500	190
17	615	9.31	3320	540	0.482	0.0774	60	75
18	620	9.76	3090	730	0.269	0.246		600
3	725		700					
4	725	5.21	708					
5	720	6.60	762					
6	725	6.60	885					
7	730	6.55	1028	485	0.750	0.090	300	385
8	720		1335	500	0.850	0.150	300	460
9	730		2557	500	0.493	0.053	300	425
10	720	5.75	5375	650	0.138	0.0143	200	180
11	740	6.56	5260	850	0.007	0.002	200	160
12	725	5.02	1335	450				

cleaned, and replaced. The largest particles often reach 1/2 cm in diameter, and very few of this size particle are needed to fill the reactor.

One test was conducted on a bed several days old and operating routinely at high efficiency. This involved a simulated power outage in which the oxygen flow and feed flow were shut down for three days. The bed was left in the reactor, and the temperature in the room was 22°C. The column was left in this condition for three days, at which time feed flow and oxygen sparging were resumed. The reactor recovered from this shock condition within two days after restarting feed and oxygen, at which time the rates had returned to 85% of the rates before the shutdown. This test shows that the biomass, when attached to a solid support, can survive some severe shock conditions with no apparent irreversible effects.

The facility in Building 2528 is a relatively small reactor, but still has a capacity much greater than the available wastewater produced by the coal hydrocarbonization facility. During periods of time when no wastewater from the coal facility is available, commercial phenol synthetic feeds can be used to keep the reactor in operation. The reactor routinely can handle the synthetic feed at 30 to 60 μg phenol per cm^3 and discharge the waste at less than 50 ng/cm^3 at phenol degradation rates up to reactor volumes of 12 $\text{kg}/\text{m}^3 \cdot \text{day}$.

4.6 Bioreactor Component Studies

A larger-scale tapered fluidized bioreactor has been fabricated for testing and evaluation. The major areas of concern are: (1) taper angle and length, (2) hydraulic pressure drop and fluidization range as functions of the larger diameter and height, and (3) oxygen transfer scale-up data for three-phase aerobic operation.

The test bioreactor was fabricated in 4-ft-long modular sections which can be interchanged. The materials of construction were mild steel with epoxy paint covering. The top section consists of 12- to 16-in.-diam tapered sections 48 in. long with a 3-ft-long, 16-ft-diam top

disengaging section. The other sections are sized as follows and can be interchanged: (1) 8- to 12-in.-diam tapered section 48 in. long; (2) 12-in.-diam cylindrical section 48 in. long; (3) 8-in.-diam cylindrical section 48 in. long (see Figs. 4.6 and 4.7). Each section was equipped with viewing windows and liquid and/or pressure-drop sample ports. Coal particles (-30 +60 mesh) can be loaded into the large bioreactor by using a water jet and water-slurried coal.

4.6.1 Preliminary operation

The large bioreactor fluidized very easily. Even at a high flow rate of 76 liters/min (3.9 cm/sec bottom velocity), the fluidizing appeared to be even and stable throughout the unit. A small tapered fluidized-bed bioreactor 2.54 to 7.62 cm in diameter by 100 cm long, having about 1/100 the volume of the larger bioreactor, was also hydraulically tested.

Figure 4.8 graphically presents (1) pressure drop (cm H₂O), (2) void volume percent, and (3) liquid velocity at the top of the fluidized bed vs liquid velocity at the bioreactor inlet (based on reactor bed area at the bottom) for two tapered fluidized-bed bioreactors. This is a normalized flow rate. Reactor A is 20.3 cm in diameter at the bottom by 40.6 cm in diameter at the top, 366 cm long, and 26,000 cm³ in volume. Reactor B is 2.54 cm in diameter at the bottom by 7.62 cm in diameter at the top, 100 cm long, and 2600 cm³ in volume. Both reactors were loaded with -30 +60 mesh coal. The coal-settled void volume was 50%. Both reactors were loaded with settled coal to about 20% of this operating volume. Lines A1 and B1 show the velocity of water at the top of the fluidized coal zone in the bioreactor. The larger bioreactor "A" velocity is a constant ~1.0 cm/sec for void volume percentages (line A-2) greater than 83%. For void volume percentages greater than 83%, the wall and intraparticle friction appears to be ineffective; therefore, the particles fluidize freely at a constant velocity. The wall area-to-volume ratio is smaller in the larger reactor, therefore decreasing frictional drag forces at equal flow velocities. Since the smaller bioreactor "B" flow rate or inlet velocity is never large enough to expand the bed to an 83%

Fig. 4.6. Hydraulic test tapered fluidized-bed bioreactor (diagram).

Fig. 4.7. Photograph of Bioreactor A (see legend of Fig. 4.8 for description of system).

Fig. 4.8. Hydraulic pressure drop, void volume percent, and liquid velocity at the top of the fluidized bed vs liquid velocity at the bottom of the fluidized bed for two sizes of bioreactors. Bioreactor A: 20.3-cm-diam bottom to 40.6-cm-diam top tapered bioreactor, 366 cm long, 26,000 cm³ in volume; bioreactor B: 2.5-cm-diam bottom to 7.6-cm-diam top tapered bioreactor, 100 cm long, 2600 cm³ in volume.

void volume (line B-2), the velocity at the top of the fluidized bed is always increasing (line B-1) due to wall and particle friction forces. Lines A-3 and B-3 are the pressure-drag curves. The small bioreactor line (B-3) is straight; however, the pressure-drop curve for the larger bioreactor (A-3) has deflection at or near the inlet velocity corresponding to the 83% void volume, the starting point of the constant velocity at the top of the fluidized-particle bed.

4.7 References for Section 4

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5. BIOPROCESS DEVELOPMENT AND DEMONSTRATION

W. W. Pitt, Jr.

The bioprocess development and demonstration section of this report gives the experimental details, results, and conclusions for the ANFLOW and biological denitrification programs. The ANFLOW pilot plant is operating at a flow rate of 4000 g/day. The off-gas rate is 94 liters/day at 70% methane. The reduction of BOD_5 is from 160 mg/liter to 60 mg/liter. The 4-in.-diam x 16-ft-high semitapered fluidized-bed denitrification bioreactor feed of 200 ppm NO_3^- has attained nitrate removal rates of up to 10 g $N(NO_3)/day/liter$.

5.1 ANFLOW Demonstration Pilot Plant

5.1.1 Summary

The ANFLOW Demonstration Pilot Plant continued to operate throughout the reporting period at feed rates of 4.0 m³/day (1100 gal/day), 7.5 m³/day (2000 gal/day), and finally 15.1 m³/day (4000 gal/day). Performance steadily improved, with off-gas rising from 6.0 to as high as 93.8 standard liters/day, and methane content rising from 12% to 72%. Biochemical oxygen demand removal averaged 50%, and total organic carbon removal averaged 70%.

The bench-scale ANFLOW column, started in February from laboratory-produced inoculum, operated throughout the reporting period at feed rates of 86.4 to 108.0 liters/day of domestic sewage. Maximum gas output was 7.0 standard liters/day. Results compared closely with those from the pilot plant except in off-gas volume, in which the bench-scale column did much better.

The ANFLOW parametric metals inhibition test was started up in early September. Phase 1 of the test will consist of a factorial experiment using nine combinations of three feed rates and three feed concentrations to determine optimum start-up parameters. This test was based on mathematical models previously reported for anaerobic packed beds.

Other activities included a preliminary cost estimate on a 1 million-gal/day ANFLOW treatment plant and a comparison with other conventional plants. The estimate showed that ANFLOW could cost 45% less than activated sludge for capital items and consume only 10% as much electrical power. Also, a computer program was developed for systematic storage, retrieval, and manipulation of all ANFLOW data.

5.1.2 Pilot plant operations

The ANFLOW Demonstration Pilot Plant was operated continuously, with a downtime of less than 5% from April to September 1977. Several major modifications were made in the physical facilities and operating procedures. The performance of the pilot plant showed marked improvement throughout the spring and summer months, primarily because of these modifications, the higher ambient temperature, and the higher biochemical oxygen demand (BOD) of the feed sewage.

Operation and maintenance. The pilot plant operated at the original feed rate of $4.0 \text{ m}^3/\text{day}$ (1100 gal/day) until April 27, when the rate was raised to $7.5 \text{ m}^3/\text{day}$ (2000 gal/day). On September 20 the rate was again raised to $15.1 \text{ m}^3/\text{day}$ (4000 gal/day).

Problems with liquid and gas flow persisted until mid-July. During late April, several changes were undertaken. The 1.7-m (66-in.) top flange on the column was removed for a short time and modified to fill up some of the dead gas space in the column. A Styrofoam plug 1.4 m (56 in.) in diameter and 0.4 m (16 in.) high was attached to the underside of the flange and covered with fiberglass (see Fig. 5.1). The flange was resealed with a new 6.3-mm (1/4-in.) neoprene gasket and RTV silicone rubber, and all flange bolts were torqued evenly to 13.6 N-m (10 ft-lb_f). The internal gas volume of the column was thus reduced from 0.83 m^3 (29.4 ft³) to 0.19 m^3 (6.7 ft³).

On April 14 a new U-tube trap was installed on the liquid effluent line near the column overflow trough. This was meant to keep 10 to 15 cm of liquid in the trough at all times, eliminating the cyclic "gurgling"

Fig. 5.1. ANFLOW column top flange with added styrofoam.

effect at the trough drain hole. The gurgling had caused problems by withdrawing entrained off-gas and causing flow measurement upsets.

In mid-June, further problems developed with the effluent line. Several clogs caused the column to fill with liquid, which also filled the off-gas measurement and sample lines. On June 20 the 25-mm effluent line was removed entirely and replaced with 75-mm-diam PVC pipe, which minimized effluent flow fluctuations.

The remote weir box installed last January proved to give poor feed flow control. On June 29 a Foxboro magnetic flow tube was installed in the feed line directly downstream of the flow control valve. After some initial problems, the flow tube was made operational on July 11 and has controlled the feed rate since. The flow tube was disturbed at first by particulates and bubbles, but a high proportional band and small integral time on the controller settings damped out the transient disturbances and provided satisfactory control.

After the top flange was replaced and sealed on April 22, off-gas production increased, but not to the magnitude expected. On July 19, gas leak testing was performed by bleeding helium into the gas headspace and going over the column with a helium leak detector. Several leaks were detected, most notably around the outlet trough view plate and the off-gas pipeline. The view plate was resealed with RTV silicone rubber. Insulation was stripped from part of the off-gas line, and it was found that the electric heat tracing had partially melted through the wall of the PVC pipe. Presumably, either the heat tracing had completely melted through the pipe at some point, or the pipe had cracked during the temporary pipe freezeup last January.

In any case, to eliminate the off-gas line from consideration as a leak spot, the line was removed and the gas outlet was covered with a blind flange. The wet-test meter was reconnected to an auxiliary gas port via rubber tubing, and the gas output jumped sharply upward immediately. Replacement of the temporary gas line with a new stainless steel pipe has been scheduled.

Finally, several minor items deserve mentioning. An anionic polyelectrolyte, Nalco 8172, was added to the feed stream beginning April 29, in concentrations of 1 to 5 mg/liter. The polyelectrolyte was intended to aid retention of bacteria on the column packing and clarification of the effluent. However, the polyelectrolyte had no apparent effect, and its use was discontinued on August 11. During the week of July 10 to 16, 0.2 m^3 (55 gal) of digester sludge from the Third Creek Treatment Plant in Knoxville and 0.15 m^3 (40 gal) of cattle rumen were pumped into the column to fortify the population of methanogens. A new air compressor with twice the capacity of the former unit ($0.4 \text{ m}^3/\text{min}$ compared with $0.2 \text{ m}^3/\text{min}$) was installed in early April and has provided excellent service since. On April 15, a differential pressure cell and recorder were installed to record the pressure in the column headspace.

Computer program development. As the amount of data from the ANFLOW pilot-plant program increased, it became necessary to systematize the storage and manipulation of the numbers. T. H. Dunagin, of ORGDP Computer Services, and D. L. Million developed a FORTRAN program for implementation on a PDP-11/40 computer, which made possible easy storage, recall, and manipulation of ANFLOW data.

All programs and data files are stored on disk packs dedicated solely to ANFLOW. The programs are constructed so that technicians unfamiliar with computers may still run the programs and enter the data correctly with a minimum of training. Routines have been developed which compute data averages over time periods selected by the user, and which type data tables for use in reports.

Results and discussion. Table 5.1 lists averages of liquid composition and flow-rate data. Total organic carbon (TOC), five-day biochemical oxygen demand (BOD_5), total suspended solids (TSS), volatile suspended solids (VSS), pH, and temperatures are listed for feed and effluent samples. TOC and BOD_5 reaction rates are derived by calculating the total reduction in TOC or BOD_5 for the daily volume of feed, per unit volume of reactor. No reasonable gas flow-rate data were collected until the resealing of the column top flange on April 22.

Table 5.1. Summary of ANFLOW pilot-plant data, weekly averages from April 1, 1977, to September 30, 1977.

Averaging Periods	Feed						Effluent						Reaction Rates		Flow Rates	
	TOC ms/l	BOD5 ms/l	TSS ms/l	VSS ms/l	pH	Temp ds.C	TOC ms/l	BOD5 ms/l	TSS ms/l	VSS ms/l	pH	Temp ds.C	TOC Rate ks/m ³ ·d	BOD Rate ks/m ³ ·d	Feed m ³ /d	Offgas stdl/d
4/ 1/77 to 4/ 9/77	57	36	42	19	7.2	14.0	41	21	12	0	7.0	23.5	0.0115	0.0108	4.0	
4/10/77 to 4/16/77	46	89	92	75	7.5	14.9	30	25	27	24	7.0	23.3	0.0119	0.0457	4.0	
4/17/77 to 4/23/77	69	129	139	127	7.7	16.5	36	65	37	34	6.8	25.9 ^a	0.0237	0.0464	4.0	2.8 ^b
4/24/77 to 4/30/77	111	128	131	118	7.6	16.9	41	53	31	24	6.9	21.3	0.0763	0.0809	6.0 ^c	7.0
5/ 1/77 to 5/ 7/77	97	120	190	171	7.4	17.6	46	51	48	38	6.8	17.9	0.0688	0.0931	7.5	8.7
5/ 8/77 to 5/14/77	108	183	234	205	7.4	18.2	49	18	36	34	6.9	18.6	0.0796	0.2226	7.5	11.9
5/15/77 to 5/21/77	135	170	172	150	7.4 ^d	19.3	66	120	52	44	6.9	19.8	0.0937	0.0668	7.5	11.0
5/22/77 to 5/28/77	125	191	250	242	7.6 ^e	20.1	59	85	51	44	6.9	20.5	0.0890	0.1430	7.5	22.6
5/29/77 to 6/ 4/77	65	72	60	55	7.4	21.1	48	49	40	39	7.2	21.6	0.0229	0.0310	7.5	51.7
6/ 5/77 to 6/11/77	99	78	150	132	7.2	20.9	33	30	45	41	6.8	21.5	0.0881	0.0647	7.5	3.1
6/12/77 to 6/18/77	91	135	108	105	6.9	21.3	50	70	42	38	6.7	21.7	0.0553	0.0877	7.5	15.5
6/19/77 to 6/25/77	43	105	102	89	7.3	21.4	36	56	27	24	6.8	22.0	0.0101	0.0668	7.5	2.2
6/26/77 to 7/ 2/77	86	135	106	92	7.4	20.9	56	48	23	20	6.8	22.1	0.0398	0.1174	7.5	7.3
7/ 3/77 to 7/ 9/77	70	108	142	118	7.3	22.3	35	30	20	17	6.8	22.7	0.0465	0.1052	7.5	17.0
7/10/77 to 7/16/77	85	189	193	172	7.1	23.2	30	137	33	30	6.8	24.0	0.0742	0.0701	7.5	5.3
7/17/77 to 7/23/77	144	253	190	165	7.2	23.6	64	137	34	31	6.8	24.1	0.1086	0.1565	7.5	3.5
7/24/77 to 7/30/77	138	190	139	123	7.1	23.7	32	119	26	23	6.8	24.3	0.1421	0.0951	7.5	124.2 ^f
7/31/77 to 8/ 6/77	103	197	179	156	7.0	24.2	35	98	33	29	6.7	24.4	0.0908	0.1325	7.5	101.1
8/ 7/77 to 8/13/77	93	141	161	142	6.9	24.6	49	103	29	27	6.6	25.0	0.0590	0.0516	7.5	88.4
8/14/77 to 8/20/77	74	188	142	120	6.9	24.4	21	94	25	23	6.6	24.8	0.0715	0.1275	7.5	121.3
8/17/77	Alum settled effluent ^g						9	15	19	10						
8/21/77 to 8/27/77	91	195	166	145	6.8	25.0	21	98	26	25	6.7	24.8	0.0944	0.1315	7.5	99.7
8/28/77 to 9/ 3/77	92	178	193	164	7.0	25.0	22	57	23	21	6.7	25.3	0.0941	0.1644	7.5	75.4
9/ 4/77 to 9/10/77	87	162	137	117	7.2	24.9	20	54	27	25	6.8	24.9	0.0897	0.1457	7.5	115.2
9/11/77 to 9/17/77	142	348	465	386	7.3	23.8	54	107	119	104	6.8	23.9	0.1195	0.3258	7.5	85.2
9/18/77 to 9/24/77	84	96	74	65	6.9	23.1	19	69	18	18	6.9	23.1	0.1496	0.0619	12.9	46.4
9/25/77 to 9/30/77	44	76	83	76	7.2	22.5	20	37	19	18	7.0	22.7	0.0638	0.1059	15.1	59.5

^aFeed warmer turned off, 4/22/77.

^bTop flange resealed, 4/22/77.

^cFeed rate changed to 7.5 m³/d, 4/27/77.

^dAll pulse to 1.8, 5/21/77.

^eHigh pH to 10.4, 5/24/77-5/25/77.

^fGas leaks discovered and fixed, 7/17/77.

^gEffluent flocculated with 1 gm/l alum and settled for 2 hours.

Table 5.2 lists monthly averages of gas production data and off-gas compositions. The percent of methane in the gas began increasing soon after the flange resealing operation in April. The amount of gas measured increased sharply after the leaky gas line was removed in July. The remaining air in the column is probably derived from dissolved air in the liquid, since it resists all attempts to be reduced further.

Table 5.2. Off-gas production and composition for ANFLOW pilot plant, monthly averages

Month	Gas (std. liters/day)	Percent by volume		
		CH ₄	CO ₂	Air
April 1977	6.0	12.1	1.6	86.3
May 1977	16.0	15.4	2.9	81.7
June 1977	14.8	35.5	3.8	60.7
July 1977	40.3	68.9	3.7	27.4
August 1977	93.8	72.3	4.1	23.6
September 1977	79.3	65.3	4.4	30.3

The percent of TOC, BOD₅, TSS, and VSS consumed in the ANFLOW pilot-plant column is listed in Table 5.3 for each week. TOC removal increased from 50% and below to over 70%. Performance decreased in the latter half of September because of the upsetting effects of doubling the liquid flow rate. Removal of BOD was more erratic, but maintained a general level of 50%. Solids removal, both TSS and VSS, reached and maintained a level of over 80%, except for late September, again because of the flow-rate upset.

In August it became of interest to determine how the pilot-plant effluent could be further improved by secondary treatment, that is, clarification possibly coupled with flocculation. It became apparent that simple settling would not work because of the fineness of the suspended matter in the effluent. A rough flocculation test was done

Table 5.3. Percent reduction in pollutants resulting from ANFLOW processing, April 1, 1977, to September 30, 1977

Averaging Periods	TOC	BOD ₅	TSS	VSS
4/ 1/77 to 4/ 9/77	28.1	41.7	70.2	100.0
4/10/77 to 4/16/77	35.9	71.8	70.8	68.0
4/17/77 to 4/23/77	47.6	50.0	66.2	73.1
4/24/77 to 4/30/77	63.5	58.8	76.2	80.0
5/ 1/77 to 5/ 7/77	52.6	57.5	75.0	78.0
5/ 8/77 to 5/14/77	54.5	90.2	84.6	83.7
5/15/77 to 5/21/77	51.4	29.2	69.9	70.5
5/22/77 to 5/28/77	52.9	55.6	79.8	81.8
5/29/77 to 6/ 4/77	26.2	31.9	33.3	29.1
6/ 5/77 to 6/11/77	66.2	61.5	70.4	68.7
6/12/77 to 6/18/77	45.3	48.1	61.4	63.6
6/19/77 to 6/25/77	17.4	47.1	73.7	72.8
6/26/77 to 7/ 2/77	34.5	64.4	78.6	78.0
7/ 3/77 to 7/ 9/77	49.6	72.2	86.3	85.8
7/10/77 to 7/16/77	64.7	27.5	82.8	82.6
7/17/77 to 7/23/77	55.9	45.9	82.0	81.1
7/24/77 to 7/30/77	76.5	37.2	81.4	81.1
7/31/77 to 8/ 6/77	65.6	50.0	81.7	81.1
8/ 7/77 to 8/13/77	47.3	27.1	82.1	81.3
8/14/77 to 8/20/77	71.9	50.2	82.1	80.8
8/21/77 to 8/27/77	76.7	50.0	84.1	83.1
8/28/77 to 9/ 3/77	76.0	68.3	88.0	87.3
9/ 4/77 to 9/10/77	76.7	66.7	80.0	78.8
9/11/77 to 9/17/77	62.3	69.4	74.4	72.9
9/18/77 to 9/24/77	76.9	27.7	75.6	71.7
9/25/77 to 9/30/77	54.0	51.5	78.2	76.6

by adding approximately 1 g of alum (aluminum sulfate dodecahydrate) to a liter of effluent, gently stirring, and then settling for 2 hr. The result, compared with the unflocculated effluent, is seen in Fig. 5.2. The standard analyses were done on the settled effluent; the results (see Table 5.1) are 9 mg/liter TOC, 15 mg/liter BOD₅, 19 mg/liter TSS, and 10 mg/liter VSS. These values are well under EPA discharge limits. A more rigorous series of flocculation tests, including jar stirring tests and weekly analysis, is scheduled to begin in October.

Studies of TOC, BOD, etc., based on time have to this point provided little useful information because of the extremely erratic quality of the raw sewage used as the column feed. The reaction rates from Table 5.1, plotted as functions of concentration, do, however, display definite trends. Figure 5.3 shows a plot of TOC reaction rate as a function of feed TOC concentration, and Fig. 5.4 is a similar plot for BOD₅.

Linear regression analysis was done on each set of points on both plots. The calculated intercepts, slopes, and correlation coefficients are in the figures. It is apparent that the feed rate increase improved reaction rate performance considerably. The feed rate increased by a factor of 1.875, but the slope of the TOC line (analogous to a first-order reaction rate constant) increased by a factor of 1.996, and the slope of the BOD₅ line increased by a factor of 2.261.

5.1.3 Supporting research

The ANFLOW supporting research program consisted of two parts. The first was the continuing operation of the bench-scale ANFLOW column, which was started up in February 1977. The second was the start-up of the ANFLOW parametric metals inhibition test (APMIT), consisting of nine bench-scale columns operating at unique feed rate-feed concentration combinations.

Bench-scale column. The bench-scale column was started up in February 1977. Its purpose was to see whether an ANFLOW column could be started up without using the usual bovine rumen and digester sludge starting material, and to compare laboratory performance with pilot-plant

Fig. 5.2. Comparison of ANFLOW effluent with flocculated, settled effluent.

Fig. 5.3. TOC removal rate as a function of concentration.

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Fig. 5.4. BOD₅ removal rate as a function of concentration.

performance. The column was started up by inoculating with liquid drained from a previously operating ANFLOW column. It started up with little difficulty and continued to run through September. The column feed was raw sewage obtained from the pilot-plant site in Oak Ridge.

Table 5.4 lists weekly averages of some data and results from April 1, 1977, to September 30, 1977. Not shown in Table 5.4 are the temperatures and pH's, since feed and effluent temperatures deviated only slightly from 25°C, and the pH remained near 7.0 in both streams. The off-gas methane was 17% in April and had risen to 68% by August.

It is clear that the column improved steadily, especially in the areas of gas production and reaction rate. The TOC and BOD₅ reaction rate data are plotted as functions of concentration in Fig. 5.5 for the feed rate of 86.4 liters/day, along with least-squares-fit straight lines.

Results for the pilot plant and the bench-scale column are compared in Table 5.5. The feed rates for the respective columns were chosen to give matching residence times. Rate slopes are taken from Figs. 5.3, 5.4, and 5.5. Off-gas and methane figures are averages from the last full month of operation, at the chosen feed rate, for each respective column. It is apparent that the columns match very closely in all areas except gas production. The pilot-plant off-gas was lower because of lower feed concentrations, more erratic temperature and feed rate, and some remaining gas leaks in the column itself.

APMIT start-up. The ANFLOW parametric metals inhibition test was begun in September 1977. The purposes of the test are (1) to start up nine bench-scale columns under different feed rate and concentration conditions to determine optimum start-up parameters, and (2) once all nine columns are running at steady state, to perform toxicity tests with various substances to determine ANFLOW's resistance to shock loadings of toxic wastes. The toxicity test portion of the experiment will not be considered for several months, until part 1 has been completed.

Table 5.4. Summary of ANFLOW bench-scale column data, weekly averages
from April 1, 1977, to September 30, 1977

	IOC (mg/liter)	BOD ₅ (mg/liter)	TSS (mg/liter)	VSS (mg/liter)	TOC (mg/liter)	BOD (mg/liter)	TSS (mg/liter)	VSS (mg/liter)	Reaction rates		Flow rates	
									TCC (kg/m ³ ·day)	BOD (kg/m ³ ·day)	Feed (liters/day)	Off-gas (liters/day)
4/1/77-4/9/77	104				80				0.0350		86.4	0.2
4/10/77-4/16/77	130				113				0.0248		86.4	0.8
4/17/77-4/23/77	105				81				0.0350		86.4	0.5
4/23/77-4/30/77	127				76				0.0743		86.4	0.5
5/1/77-5/1/77	125				94				0.0452		86.4	0.7
5/8/77-5/14/77	101				76				0.0372		86.4	0.8
5/15/77-5/21/77	126				96				0.0437		86.4	1.1
5/22/77-5/28/77	88				39				0.0723		86.4	1.9
5/29/77-6/4/77	113				86				0.0392		86.4	4.1
6/5/77-6/11/77	181	99	196	180	103	79	68	59	0.1156	0.0291	86.4	5.3
6/12/77-6/18/77	109				54				0.0301		86.4	4.4
6/19/77-6/25/77											86.4	4.8
6/26/77-7/2/77											86.4	3.6
7/3/77-7/9/77	110				52				0.0352		86.4	2.9
7/10/77-7/16/77	170	426			54	144			0.1690	0.4109	86.4	3.9
7/17/77-7/23/77	425	263			181	145			0.3548	0.1712	86.4	2.1
7/24/77-7/30/77	185	269	83	72	103	249	28	24	0.1190	0.0284	86.4	3.4
7/31/77-8/6/77	127	166	200	164	72	74	37	31	0.0804	0.1344	86.4	5.3
8/7/77-8/13/77	188	237	252	202	60	76	51	44	0.1871	0.2349	86.4	2.1
8/14/77-8/20/77	139	274	177	155	73	152	66	55	0.0962	0.1781	86.4	4.2
8/21/77-8/27/77	235	436	236	203	75	120	39	34	0.2752	0.5437	102.0	5.2
8/28/77-9/3/77	193	347	221	181	71	103	47	40	0.2236	0.4438	108.0	5.8
9/4/77-9/10/77	394	469	274	234	131	231	99	89	0.4781	0.4323	108.0	6.0
9/11/77-9/17/77	261	377	206	164	118	158	58	49	0.2186	0.3655	98.0	6.0
9/18/77-9/24/77	201	473	223	190	83	85	22	19	0.1290	0.4220	64.5	4.6
9/25/77-9/30/77	82	135	189	105	57	94	56	36	0.0455	0.0751	108.0	7.0

Fig. 5.5. TOC and BOD₅ reaction rates as function of concentration for ANFLOW bench-scale column. Feed = 86.4 liters/day.

Table 5.5. Comparison of results for ANFLOW pilot plant and ANFLOW bench-scale column

	Pilot plant	Bench scale
Feed rate	7.5 m ³ /day	86.4 liters/day
Reactor volume	5.56 m ³	59.3 liters
Residence time, days	0.74	0.69
Monthly averages used	August 1977	July 1977
TOC rate slope	9.90 x 10 ⁻⁴	9.78 x 10 ⁻⁴
BOD ₅ rate slope	8.66 x 10 ⁻⁴	10.9 x 10 ⁻⁴
Off-gas, liters(m ³ of reactor)/day	16.9	52.3
Methane percent	72.3	67.9

The start-up test was designed as a 3 x 3 factorial experiment, using nine bench-scale columns with the operating conditions shown in Table 5.6. The experimental design was based on calculations done with a mathematical model of an anaerobic packed-bed reactor. Feed concentrations in the model were based on chemical oxygen demand (COD).

Table 5.6. Operating parameters for APMIT start-up phase

Column No.	Feed rate (liters/day)	COD (mg/liter)
1	86.4	100
2	86.4	350
3	86.4	600
4	259.2	100
5	259.2	350
6	259.2	600
7	518.4	100
8	518.4	350
9	518.4	600

Calculations were performed using the mathematical model of the anaerobic fixed-film bioreactor developed by Young and McCarty.¹ The differential equations used to describe the conversion of the influent waste to methane are solved using finite-difference techniques. The algorithm used divides the packed bioreactor lengthwise into 75-mm (0.25-ft) sections. The influent stream is assumed to consist of acetic acid, propionic acid, and a complex waste. Starting with the inlet (bottom) of the reactor, the fraction of the complex waste converted to microbial mass, acetic acid, and propionic acid in the bottom section during a small time increment is first calculated. Since part of the propionic acid is first converted to acetic acid, this split is calculated, and the finite-difference expressions for conversion of the acetic and propionic acids are solved for the time increment used. This determines the amount of active microbial mass, complex waste, acetic acid, and propionic acid remaining at the end of the time increment in this section. The exit concentrations are thus determined by material balance from the first section. Since the exit concentrations from the first section are inlet concentrations to the second section, this procedure is continued until the top of the column is reached. The total amount of methane produced during this time period is calculated, and the entire procedure is repeated for the number of time increments to span the time period needed. The kinetic parameters were based on the research of Lawrence and McCarty² to describe the kinetics of methane fermentation in anaerobic stirred-tank digesters.

Parametric calculations were performed for three feed strengths (100, 350, and 600 mg/liter COD) and three hydraulic flow rates (75, 150, and 225 liters/day) over a time period of 200 days. The total gas production and the amount of COD in the effluent stream were plotted as functions of time. In addition, the waste profile as a function of column height was plotted at the end of the 200-day period. Calculated results for a flow rate of 150 liters/day and a waste feed strength of 350 mg/liter COD are presented in Fig. 5.6. Calculated results for all nine conditions are summarized in Table 5.7. The calculations

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Fig. 5.6. Plot obtained with Mathemod for a feed rate of 150 liters/day and a feed strength of 350 mg/liter COD. Upper left: gas production vs elapsed time. Upper right: effluent COD vs elapsed time. Lower left: effluent COD vs column height after 200 days of operation.

Table 5.7. Summary of calculated values after 150 days

Flow (liters/day)	Waste strength (mg/liter COD)	Total gas produced (liters/day)	Effluent strength (mg/liter COD)	Conversion efficiency (%)
75	100	2	73	27
	350	19	18	97
	600	34	8	99
150	100	3	76	24
	350	30	93	73
	600	47	195	68
225	100	3	83	17
	350	22	221	37
	600	28	436	27

indicate that slow microbial growth can be expected at a 100-mg/liter feed strength. Thus a slow start-up at this feed concentration should be expected. At the 600-mg/liter feed strength, overloading occurred and calculated performance was poor.

Since the kinetic parameters used to make these calculations are not well known, these results should only be used as an indication of the expected results of the experiment under way.

The nine columns were fabricated, loaded with packing, and installed during August. Figure 5.7 shows the columns mounted on their rack under a ventilation hood. Figure 5.8 is an end view of the arrangement, showing wet-test meters and feed pumps.

A complex system of pumps was assembled to permit all the columns to be fed from one tank of feed concentrate (see Table 5.8 for the feed concentrate composition). Cole Parmer Masterflex peristaltic pumps were used, with small feed heads pumping into larger dilution heads to produce correct feed concentration. Figure 5.9 is a schematic of the pumping and

Fig. 5.7. APMIT columns after installation.

Fig. 5.8. APMIT columns showing wet-test meters.

Table 5.8. APMIT synthetic feed concentrate (kg)

For 200 liters of concentrate

Sodium acetate	8
Mannitol	8
Lactose	8
Glycine	8
Sodium bicarbonate	2
Calcium chloride	2
Magnesium sulfate	1.2
Glacial acetic acid	4
Propionic acid	1.2
n-Butyric acid	1.2
n-Valeric acid	1.2
Disodium phosphate	8

Water to make 200 liters

Concentration ~60,000 mg/liter TOC

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Fig. 5.9. Schematic of APMIT pump system.

flow arrangement. Pump No. 1 runs at 90 liters/day from each No. 18 head, pump No. 2 at 260 liters/day, and pump No. 3 at 520 liters/day. Figure 5.10 is a closeup photograph of part of the pump system.

In late August, each column was seeded with 1 liter of liquid drained from a previously operating ANFLOW column and filled 3/4 full with synthetic feed liquid at the correct concentration. After one week, another liter of inoculum was added to each column, and the columns were filled to the top with feed solution. On September 7, continuous feeding was begun.

Thus far, no one column has outstripped all others in performance. Feed concentrations in some columns have been erratic because of clogged feed lines, which are being corrected. Visible microorganism growth has appeared in some of the columns, and small amounts of gas have been recorded. However, the test has been running for too short a time to have produced any meaningful results.

5.1.4 Cost estimates

In order to estimate cost effectiveness and comparative energy requirements of the ANFLOW process, comparative estimates have been made for a 1-MGD ($3800\text{-m}^3/\text{day}$) treatment plant for municipal sewage with a 225-mg/liter average dry-weather BOD. Three process alternatives were considered as shown in Fig. 5.11. Alternative 1, a conventional activated-sludge process, was selected as the standard to compare the aerobic fixed-film (trickling filter) processes and the anaerobic fixed-film (ANFLOW) process because it is the preferred process using proven technology. As may be seen in Fig. 5.11, many of the corresponding process steps in the three processes are the same or are simply missing. These similarities between the processes simplify a preliminary comparison of the cost and energy requirements because there is extensive experience with the conventional activated-sludge process which can be used to estimate most of the requirements of the other alternatives.

Electrical energy requirements for conventional activated-sludge processes were reported by Smith³ and by Mills and Tchobanoglous⁴ for a

Fig. 5.10. Peristaltic pumps in APMIT pump system.

Fig. 5.11. Flowsheets for alternatives used in cost comparison.

1-MGD treatment plant as presented in Table 5.9. Over 85% of the electrical energy requirements are consumed in supplying oxygen to the microorganisms and mixing the biological reactor. The electrical energy requirements for the aerobic fixed-film bioreactor step were supplied by the Norton Company. Electrical energy requirements for the ANFLOW process step were estimated from the design parameters used in the 5000-gpd ($19\text{-m}^3/\text{day}$) pilot plant. No credit was made for fuel gas produced in the ANFLOW process. Comparative estimates of the electrical energy consumption for each of the process alternatives are summarized in Table 5.9.

The preliminary cost estimate for the conventional activated-sludge alternative was prepared from recent sewage plant costs. The cost of the aerobic fixed-film reactor was supplied by the Norton Company. The preliminary estimate for the ANFLOW bioreactor was prepared by UCNC Construction Engineering. These costs are summarized in Table 5.9.

As may be seen from Table 5.9, the aerobic fixed-film alternative was estimated to cost 10% less than the activated-sludge process and to consume 35% less electricity. The ANFLOW process alternative was estimated to cost 45% less than the activated-sludge alternative and to consume only about 10% as much electricity.

5.2 Biological Denitrification

5.2.1 Summary

Biological denitrification is task II of the "Removal and Segregation of Radioactive Nuclides from Liquid Waste Streams" program. The nuclear fuel cycle has many waste streams which contain biologically degradable nitrates. This section will report three phases in the development program:

1. Supportive microbiological research, work performed by Environmental Sciences Division, ORNL;
2. Process development studies, Chemical Technology Division;
3. Process feasibility studies, work performed by Y-12 Development Division.

Table 5.9. Preliminary cost comparison
(plant capacity 1, MGD)

Operation	Activated sludge		Trickling filter		ANFLOW	
	Cost	kWhr/day	Cost	kWhr/day	Cost	kWhr/day
Control house + site prep.	\$150,000	60	\$150,000	60	\$100,000	60
Preliminary treatment	60,000	17	60,000	17	60,000	17
Primary sedimentation	400,000	41	450,000	41		
Aerator, pumps, and blowers	300,000	580				
Sludge handling	600,000	230	600,000	230		
Biofilter			120,000 ^a	240	900,000 ^b	
Final clarifier	400,000	31	400,000	31		
Disinfection	40,000	4	40,000	4	40,000	4
Microscreen					40,000 ^c	24
Total	\$2,000,000	963	\$1,820,000	623	\$1,140,000 ^d	105

^aOne Norton trickling filter 38-ft diameter x 22-ft packed bed. Media cost, \$2.50/ft³.

^bTwo ANFLOW units 50-ft diameter x 10-ft packed bed. Media cost, \$10/ft³.

^cIf required.

5.2.2 Applied microbiological research relative to factors influencing rates of biological denitrification (C. W. Francis, F. S. Brinkley, B. M. Ross, C. M. DeSha)

Research was oriented toward solving possible problems in the biological denitrification of various nitrate-containing waste streams associated with the nuclear industry, namely, evaluating the influence of radiation and the concentrations of sodium, calcium, nitrate, ammonium, and nickel on denitrification rates.

Radiation effects. Low- and high-level radioactive wastes contain from 0.2 to 6.4 M concentration of nitrate.^{5,6} Removal of nitrates from such wastes will enhance the waste vitrification properties, reduce waste solids, and decrease nitrate stress-corrosion cracking of waste supernates stored in steel tanks. To test the influence of acute radiation on the efficiency of denitrifying microorganisms, 10 ml of an actively denitrifying culture was exposed to the following radiation doses: 10^3 , 10^4 , 10^5 , and 10^6 rads. After the accumulated dose from a ^{60}Co source (approximately 3×10^5 rads/hr), the 10-ml suspension of denitrifying microorganisms was mixed with 25 ml of a nitrate solution composed of 0.016 M NH_4NO_3 , 0.027 M NH_4OAc , 1 mM MgSO_4 , 0.32 mM KH_2PO_4 , and 2 mg of Fe/liter as sodium ferric diethylenetriamine pentaacetate (Fe-DTPA). The solutions were purged with N_2 , and nitrate concentrations were monitored over time (Table 5.10). It appears from these data that radiation doses greater than 10^5 rads will be required to decrease denitrification rates. A statistically significant (5% level) higher nitrate concentration on the fourth day in the 10^5 -rad treatment indicates that denitrification may have been hindered; however, after seven days there was no statistical difference (5% level) among nitrate levels in the cultures receiving 10^5 rads and the cultures not exposed to ^{60}Co radiation. Thus, if a mean lifetime of a denitrifying culture is taken to be 24 hr, a dose rate of 10^3 rads/hr would be required to deliver 10^5 rads. These data indicate that radiation per se (as levels up to 10^3 rads/hr) will not inhibit denitrification rates; however, exposure to chronic radiation doses at these high levels will likely produce chemical effects such as the production of H_2O_2 and hydrolysis products that may inhibit denitrification more than any direct effect of radiation.

Table 5.10. Influence of acute radiation on biological denitrification rates

Radiation (rads)	mg/liter of NO ₃ -N ^a at intervals (days) of:							First-order ^b rate constant	
	0	1	3	4	6	7	10	days ⁻¹	r ²
0	166	141*		22*		15*		0.38	0.92
10 ³	166	142*		28*		20*		0.33	0.92
10 ⁴	166	155*		18*		10*		0.44	0.93
10 ⁵	166	146*		53		12*		0.38	0.98
10 ⁶	166		161		100		20	0.21	0.48
Control ^c	230			232	235				

^aNumbers in the same column with an asterisk are not significantly different at the 5% probability level. All treatments except the 10⁶-rad dose were repeated three times.

^b $\hat{y} = ae^{-\lambda t}$, where $\hat{y}$ is estimated NO₃-N in mg/liter, a is initial NO₃-N concentration, λ is 1st-order rate constant in days⁻¹, and t is time in days.

^cNitrate solutions were similar to other treatments except no denitrification microorganisms were added.

Influence of nitrate concentration on denitrification rates. To operate a biological denitrification facility most effectively, one must be aware of the maximum and minimum nitrate substrate concentrations required for the engineering design. In a single-stage continuous-flow stirred reactor, influent nitrate concentrations are not important, since the nitrate concentration in the reactor is usually kept low enough for discharge to the environment. However, in multiple-stage reactors and particularly in the case of columnar denitrification (stationary or fluidized beds), it is advantageous to use the maximum nitrate concentrations in the influent stream without inhibiting the biological denitrification rate. Because most nitrate wastes in the nuclear industry are highly concentrated (often greater than 50,000 mg NO₃-N/liter), these waste streams require dilution before direct exposure

to denitrifying microorganisms. Thus, nitrate substrate levels that are toxic or inhibitory to biological denitrification should be avoided. Earlier work using NH_4NO_3 solutions in continuous-flow stirred reactors indicated that nitrate concentrations greater than 1700 mg $\text{NO}_3\text{-N/liter}$ inhibited denitrification rates.⁶

To test the influence of nitrate on denitrification, using $\text{Ca}(\text{NO}_3)_2$, six treatments in triplicate were made of varying nitrate concentrations (Table 5.11). Carbon was provided as calcium acetate and remained constant for each treatment (2080 mg C/liter). Phosphorus (10 mg P/liter as KH_2PO_4), magnesium (10 mg Mg/liter as MgSO_4), and iron (2 mg Fe/liter as Fe-DTPA) were added as nutrient amendments. An actively denitrifying culture (103 ml from a continuous-flow stirred reactor) was added, total volume brought to 250 ml, and each solution purged with N_2 for 30 sec. The nitrate and nitrite concentrations were monitored for the next six days (Table 5.11 and Fig. 5.12) to determine the respective denitrification rates.

Total nitrate removal in treatment 1 (initial nitrate concentration, 5030 mg $\text{NO}_3\text{-N/liter}$) was approximately one-half that removed in the other treatments (Table 5.11). Thus, nitrate concentrations of 5000 mg $\text{NO}_3\text{-N/liter}$ inhibited denitrification as compared with the nitrate concentration <5000 mg $\text{NO}_3\text{-N/liter}$. Rates at the lower nitrate concentrations appeared to be similar.

To evaluate the effect of initial nitrate concentrations, zero-order rates were determined based on the first two days of the experiment. All coefficients of determination (r^2) were greater than 0.970 at initial nitrate concentrations <2280 mg $\text{NO}_3\text{-N/liter}$ (Table 5.12). These rates were used with nitrate substrate concentrations after one day, namely, the average nitrate concentration over the first two days to determine any inhibition, using a conventional Lineweaver-Burk plot (Fig. 5.13). These data indicated that inhibition in denitrification occurred between substrate nitrate levels of 1700 and 2100 mg $\text{NO}_3\text{-N/liter}$ and were very similar to those levels found to decrease denitrification rates in continuous-flow stirred reactors, using NH_4NO_3 as a nitrate source.⁷

Table 5.11. Influence of nitrate concentration on denitrification rates^a

Treatment	mg NO ₃ -N/liter at intervals (days) of:					Nitrate removed (mg NO ₃ -N/liter)
	0	1	2	3	6	
1	5030 (110)	5110 (45)	4830 (100)	4900 (120)	4630 (170)	400
2	2280 (130)	2180 (50)	2000 (40)	1910 (20)	1420 (40)	860
3	1900 (90)	1660 (40)	1450 (15)	1370 (40)	930 (40)	970
4	1150 (40)	920 (10)	710 (15)	600 (35)	280 (30)	870
5	950 (40)	690 (20)	500 (15)	360 (25)	110 (5)	840
6	720 (25)	450 (5)	270 (10)	155 (20)	<10	>710

^aThe number in parentheses is the standard deviation determined from three replicates of that treatment.

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Fig. 5.12. First-order denitrification rates as influenced by nitrate concentration ($\hat{Y} = \lambda a e^{-\lambda t}$, where $\hat{Y}$ is estimated $\text{NO}_3\text{-N/liter}$, a is initial $\text{NO}_3\text{-N}$ concentration, λ is 1st-order rate constant in days^{-1} , and t is time in days).

Table 5.12. Zero-order denitrification rates as a function of initial nitrate concentration

Initial nitrate conc. (mg NO ₃ -N/liter)	Zero-order rate based on first 2 days ^a		
	a	-b	r ²
5030	5090	100	0.481
2280	2293	140	0.974
1900	1895	225	0.999
1150	1147	220	0.999
950	938	225	0.992
720	705	225	0.987

^a Model, $\hat{Y} = a - bt$, where t is time in days,
 r^2 = coefficient of determination.

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Fig. 5.13. Lineweaver-Burk plot at various nitrate substrate concentrations.

Influence of acetate as a carbon source on denitrification rates.

Excessive levels of the carbon source, as well as the nitrate, might inhibit denitrification. A similar experiment was designed as described above to evaluate the influence of acetate levels on denitrification rate. Acetate levels ranged from 800 to 13,000 mg C/liter, while initial nitrate levels were kept below 1000 mg $\text{NO}_3\text{-N}$ /liter (Table 5.13). The rate of denitrification appears to be more sensitive to increasing levels of acetate than nitrate. For example, nitrate removal at 6650 mg C/liter of acetate was less than one-half the removal rate at 840 mg C/liter. Using the zero-order rates over the first two days (Table 5.14) and initial acetate concentrations in a Lineweaver-Burk plot, it is shown that acetate concentrations greater than 6500 mg C/liter are highly inhibitory (Fig. 5.14). The negative intercept (-3.89) of the linear regression between S/R and S ($r^2 = 0.985$) implies that even at concentrations between 840 and 5390 mg C/liter, acetate inhibits rates of denitrification. Methanol at 3750 mg C/liter did not appear to inhibit denitrification; thus, these data indicate that if high-nitrate substances are desired, care should be taken with regard to quantity and type of carbon source selected.

Biological denitrification of $\text{Ca}(\text{NO}_3)_2$ as compared with NH_4NO_3 .

The two major forms of nitrate in waste streams of the nuclear industry are $\text{Ca}(\text{NO}_3)_2$ and NH_4NO_3 . Ammonium nitrate is generated predominantly in uranium recovery operations which use the ammonium diuranate process. Calcium nitrate is often associated with the milling of uranium and other operations where limestone has been used to neutralize excess nitric acid wastes.

The denitrification rates of nitrate supplied as these two forms [$\text{Ca}(\text{NO}_3)_2$ and NH_4NO_3] were compared under identical conditions, using an initial concentration of 1200 mg $\text{NO}_3\text{-N}$ /liter (Table 5.15). Carbon was supplied as the acetate salt of either calcium or ammonium (2320 mg C/liter) respectively. Phosphorus (10 mg P/liter as KH_2PO_4), magnesium (10 mg Mg/liter as MgSO_4), and iron (2 mg Fe/liter as Fe-DTPA) were added as nutrient amendments.

Table 5.13. Nitrate loss at various levels of acetate^a

Treatment	C (mg/liter)	mg of NO ₃ -N/liter at intervals (days) of:			Nitrate removed (mg NO ₃ -N/liter)
		0	1	2	
1	13,100	733 (18)	682 (14)	704 (16)	29
2	6,650	641 (23)	575 (23)	417 (14)	224
3	5,360	690 (12)	561 (37)	385 (13)	305
4	3,400	643 (77)	472 (25)	328 (27)	315
5	2,780	626 (6)	445 (14)	293 (16)	333
6	2,130	735 (31)	436 (23)	269 (14)	466
7	840	750 (88)	414 (70)	217 (41)	533

^aNumber in parentheses is standard deviation of three replicates of that treatment.

Table 5.14. Influence of acetate on denitrification rate

Treatment	C (mg/liter)	Zero-order model ^a		
		a	-b	r ²
1	13,100	721	14.5	0.321
2	6,650	656	112	0.947
3	5,360	698	153	0.992
4	3,400	638	157	0.998
5	2,780	621	166	0.997
6	2,130	713	233	0.974
7	840	727	266	0.978

$\hat{Y} = a - bt$, where $\hat{Y}$ is estimated nitrate concentration; a, initial nitrate concentration, mg NO₃-N/liter; b, the denitrification rate, mg NO₃-N/liter/day; t, the time in days.

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Fig. 5.14. Lineweaver-Burk plot at various acetate substrate concentrations.

Table 5.15. Biological denitrification rates
of $\text{Ca}(\text{NO}_3)_2$ and NH_4NO_3 ^a

Day	$\text{Ca}(\text{NO}_3)_2$			NH_4NO_3		
	$\text{NO}_3\text{-N}$	$\text{NO}_2\text{-N}$	pH	$\text{NO}_3\text{-N}$	$\text{NO}_2\text{-N}$	pH
0	1200 (50)	0.71 (0.30)	6.53 (0.09)	1200 (30)	1.59 (0.11)	8.49 (0.01)
1	870 (30)	0.76 (0.19)	6.55 (0.01)	1055 (10)	3.65 (0.12)	8.62 (0.01)
2	690 (55)	2.63 (0.68)	6.60 (0.04)	850 (30)	3.80 (0.35)	8.75 (0.01)
Zero-order model ^b						
a	1175			1210		
-b	255			175		
r^2	0.972			0.990		

^aNumber in parentheses is standard deviation of three replicates of that treatment.

^b $\hat{\gamma} = a - bt$, where $\hat{\gamma}$ is estimated nitrate concentration; a, initial nitrate concentration in mg $\text{NO}_3\text{-N/liter}$; b, the denitrification rate in mg $\text{NO}_3\text{-N/liter/day}$; t, the time in days.

The biological denitrification rate of nitrate supplied in the form of $\text{Ca}(\text{NO}_3)_2$ was approximately 1.5 times faster than that supplied as NH_4NO_3 . Three factors might be responsible for the slower rate with NH_4NO_3 , namely, excessive ammonium concentration (maximum of 2000 $\text{NH}_4\text{-N}$), higher ambient pH levels, and higher levels of soluble carbonate and bicarbonate than in solutions made of calcium nitrate. With calcium nitrate, the calcium carbonate solubility product is exceeded on the biological conversion of acetate to CO_2 , which in turn keeps the pH less than 7.2. On the other hand, in the NH_4NO_3 solutions, bicarbonate and carbonate accumulate, each of which could possibly be a competitive inhibitor.

Influence of ammonium on rates of denitrification. Because denitrification rates were faster using $\text{Ca}(\text{NO}_3)_2$ rather than NH_4NO_3 , the influence of ammonium on denitrification was investigated. Two ammonium salts, NH_4Cl and $(\text{NH}_4)_2\text{CO}_3$, were used at four levels of $\text{NH}_4\text{-N}$ (1000, 2000, 3500, and 5000 mg $\text{NH}_4\text{-N/liter}$) in nitrate solutions [1365 mg $\text{NO}_3\text{-N/liter}$ as $\text{Ca}(\text{NO}_3)_2$]. The carbon source was calcium acetate (2320 mg C/liter). Phosphorus (10 mg P/liter as KH_2PO_4), magnesium (10 mg Mg/liter as MgSO_4), and iron (2 mg Fe/liter as Fe-DTPA) were added as nutrient amendments. The solutions were seeded with an active culture of denitrifying microorganisms, purged with N_2 , and monitored for nitrate, nitrite, and pH the following four days.

Using NH_4Cl as the source of ammonium, the denitrification rates decreased on increasing levels of ammonium (Table 5.16). Using $(\text{NH}_4)_2\text{CO}_3$, on the other hand, the relationship was not at all similar. For example, ammonium concentrations of 5000 mg $\text{NH}_4\text{-N/liter}$ decreased denitrification rates as compared with ammonium levels of 1000 and 2000 mg $\text{NH}_4\text{-N/liter}$; however, the highest rate was observed at 3500 mg $\text{NH}_4\text{-N/liter}$. Thus the data are not easy to interpret.

A Dixon plot of the NH_4Cl additions indicates the presence of a non-competitive inhibitor (Fig. 5.14); the slope of competitive inhibitor would likely be nearer to zero than that observed in Fig. 5.15, considering the excess of nitrate and carbon present. Thus, because of the absence

Table 5.16. Influence of two ammonium salts on denitrification^a

Salt	mg NH ₄ -N/liter	mg of NO ₃ -N/liter at intervals (days) of:					Rate ^b -b mg NO ₃ -N/liter/day)	r ²
		0.25	1	2	3	4		
NH ₄ Cl	1000	1365	1220 (65)	850 (15)	310 (50)	<5	384	0.983
	2000	1365	1210 (10)	860 (40)	520 (35)	80 (60)	345	0.989
	3500	1365	1230 (15)	1200 (15)	960 (60)	720 (100)	165	0.943
	5000	1365	1280 (15)	1270 (30)	1070 (20)	900 (30)	121	0.925
(NH ₄) ₂ CO ₃	1000	1365	1155 (20)	960 (40)	575 (60)	270 (50)	292	0.989
	2000	1365	1125 (125)	960* (135)	605* (130)	355* (145)	267	0.991
	3500	1365	1060 (50)	765* (70)	405* (75)	145* (101)	324	0.996
	5000	1365	1120 (20)	990 (65)	790 (90)	630 (100)	188	0.982

^aNumber in parentheses is standard deviation of three replicates of that treatment, except where marked with an asterisk (*). The asterisk indicates that the treatment was repeated twice.

^b $\hat{Y} = a - bt$, where $\hat{Y}$ is estimated nitrate concentration; a, initial nitrate concentration in mg NO₃-N/liter; b, the denitrification rate in mg NO₃-N/liter/day; t, the time in days.

Fig. 5.15. Dixon plot of the influence of added ammonium on denitrification rate.

of inhibition with $(\text{NH}_4)_2\text{CO}_3$ between $\text{NH}_4\text{-N}$ concentrations of 1000 and 3500 mg/liter, it appears that the inhibitor in NH_4Cl is the chloride ion rather than NH_4 . Ammonium carbonate at 5000 mg $\text{NH}_4\text{-N}$ /liter appeared to inhibit denitrification, but the Dixon plot over the concentration range (1000 to 5000 mg $\text{NH}_4\text{-N}$ /liter) is not linear. Incidentally, if the data for 3500 mg $\text{NH}_4\text{-N}$ /liter are removed from the data set, a very good linear relationship is observed ($r^2 = 0.994$).

Ammonia, rather than ammonium, may be the inhibitor. From pH values of the solutions (Table 5.17), ammonia concentrations were calculated. In the initial solutions the ammonia concentration (192 mg $\text{NH}_3\text{-N}$ /liter) in the 5000-mg $\text{NH}_4\text{-N}$ /liter treatment was approximately eight times higher than the ammonia concentration (25 mg $\text{NH}_3\text{-N}$ /liter) in the 3500-mg $\text{NH}_4\text{-N}$ /liter treatment, indicating that initial $\text{NH}_3\text{-N}$ concentrations inhibited denitrification. However, if ammonia concentrations are calculated over the duration of the experiment (four days), one notes that ammonia concentrations in the 3500-mg $\text{NH}_4\text{-N}$ /liter treatment exceeded the ammonia concentrations in the 5000-mg $\text{NH}_4\text{-N}$ /liter treatment at the end of the experiment (Fig. 5.16). Thus, any inhibitor effect of either ammonium or ammonia on denitrification cannot be clearly evaluated from these data.

The presence of carbonate or bicarbonate in solution may be responsible for the observed inhibition. Calcium carbonate was precipitated in all treatments $[\text{Ca}(\text{NO}_3)_2 + (\text{NH}_4)_2\text{CO}_3]$; in the two lower treatment levels, calcium was in excess of carbonate, while in the higher treatments (3500 and 5000 mg $\text{NH}_4\text{-N}$ /liter) the carbonate was in excess of calcium.

Influence of sodium on denitrification. Sodium concentrations in low- and high-level radioactive waste may be as high as 12 M.^{5,6} Transient exposure to sodium concentrations as low as 0.1 M has been observed to either halt biological denitrification or drastically lower the denitrification rate. Denitrification will proceed at concentrations greater than 0.1 M; however, time is required for population selection. Studies on evaluating the influence of sodium were divided into (1) an experiment designed to determine the time required for acclimation of an active

Table 5.17. Denitrification in presence of two ammonium salts - pH values^a

Salt	mg NH ₄ -N/liter	pH at intervals (days) of:				
		0.25	1	2	3	4
NH ₄ Cl	1000	6.89 (0.07)	6.47 (0.02)	6.67 (0.02)	7.50 (0.16)	8.37 (0.02)
	2000	7.04 (0.16)	6.48 (0.01)	6.63 (0.01)	6.95 (0.05)	8.08 (0.10)
	2500	7.02 (0.02)	6.47 (0.02)	6.59 (0.01)	6.61 (0.02)	6.80 (0.08)
	5000	7.04 (0.03)	6.47 (0.01)	6.66 (0.14)	6.61 (0.01)	6.73 (0.02)
(NH ₄) ₂ CO ₃	1000	6.73 (0.02)	6.50 (0.01)	6.75 (0.01)	7.73 (0.21)	8.54 (0.07)
	2000	6.74 (0.05)	6.90 (0.36)	7.59* (0.34)	8.39* (0.12)	8.71* (0.12)
	3500	7.12 (0.04)	7.77 (0.09)	8.49* (0.05)	8.68* (0.04)	8.94* (0.26)
	5000	7.86 (0.03)	8.11 (0.01)	8.44 (0.01)	8.54 (0.01)	8.63 (0.01)

^aNumber in parentheses is standard deviation of three replicates of that treatment, except where marked with an asterisk (*). An asterisk indicates that the treatment was repeated twice.

Fig. 5.16. Calculated concentrations of ammonia in $(\text{NH}_4)_2\text{CO}_3$ -treated solutions.

denitrifying culture (population selection) to various levels of sodium (as NaCl) and their subsequent denitrification rates, (2) feasibility studies of biologically denitrifying a sodium-containing radioactive waste by selecting sodium-tolerant microorganisms from three different mixed cultures, and (3) an experiment on the effects of transient sodium perturbation of an active denitrifying reactor.

Acclimation time requirements and denitrification rates for nitrate-NaCl solutions. Duplicate solutions [226 mg $\text{NO}_3\text{-N/liter}$ as NH_4NO_3 and 17 as $\text{Ca}(\text{NO}_3)_2$] varying in sodium concentrations (0, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, and 2.5 M as NaCl) were used to estimate those concentrations that are inhibitory to denitrification, acclimation time requirements, and denitrification rates of the surviving microbial populations. Carbon was supplied as NH_4OAc (790 mg C/liter). Phosphorus (10 mg P/liter as KH_2PO_4), magnesium (10 mg Mg/liter as MgSO_4), calcium [25 mg Ca/liter as $\text{Ca}(\text{NO}_3)_2$], and iron (2 mg Fe/liter as Fe-DTPA) were added as nutrient amendments. A sodium chloride concentration of 0.25 M did not require a detectable length of time for acclimation; however, the rate of denitrification was approximately one-half the rate where no sodium chloride was added (Table 5.18 and Fig. 5.17). Increasing salt concentrations required increasing time for population selection, and the subsequent denitrification rates were considerably lower.

Table 5.18. Influence of NaCl on denitrification rates

Na (M)	Acclimation period (days)	Denitrification rate (mg $\text{NO}_3\text{-N/liter/day}$)	r^2
0	0	248	
0.25	0	123	
0.50	4-6	i.d. ^a	
0.75	9	106	
1.00	12	28	0.985
1.50	20	9	0.989

^aInsufficient data to calculate a rate.

Fig. 5.17. Influence of NaCl on denitrification.

Biological denitrification of simulated radioactive waste. Sodium simulated radioactive waste was made using sodium nitrate and sodium hydroxide. The solution was neutralized with glacial acetic acid to pH 5.6. The resulting solution was 4.5 M Na, 31,000 mg NO₃-N/liter, and 81,200 mg C/liter. Phosphorus (10 mg P/liter as KH₂PO₄), magnesium (10 mg Mg/liter as MgSO₄), and iron (2 mg Fe/liter as Fe-DTPA) were added as nutrient amendments. This simulated waste was added in 200-ml increments (after neutralizing to pH 7.5 with NaOH) to three denitrifying reactors. Each reactor contained a mixed microbial culture from one of three sources: (1) a biological denitrifying reactor from Y-12, (2) a highly productive agricultural soil from the Midwest, and (3) an estuarine sediment from the coast of California. Denitrification rates were measured at sodium concentrations greater than 0.25 M (Tables 5.19, 5.20, and 5.21, and Figs. 5.18, 5.19, and 5.20). Rates of denitrification were highly variable for each reactor. There does not appear to be a significant difference in denitrification rates among the reactors at sodium concentrations above 1.0 M (soil culture 122 ± 45, estuarine sediment culture 89 ± 50, and Y-12 culture 52 ± 30 mg NO₃-N/liter/day). The highest denitrification rates were observed using the estuarine sediment culture at sodium concentrations of 0.50 and 0.68 M (the respective rates were 569 and 612 mg NO₃-N/liter/day). These rates are similar to the daily average denitrification rate (605 mg NO₃-N/liter/day) observed in Y-12's pilot plant during April 1974, but was one-fourth the maximum denitrification rate observed in their laboratory studies.⁴ Rates can likely be increased appreciably using a constant sodium concentration into a continuous-flow stirred reactor or a fluidized-bed tapered column. These reactors tend to be biologically more stable than batch stirred reactors. Possibly one of the reasons the denitrification rates were low is the accumulation of excessive amounts of acetate in the reactor over the 90 to 100 days the reactors were operated (2.6 g of acetate carbon were added for each gram of NO₃-N). Work is continuous, using a fluidized-bed tapered column inoculated with an estuarine microbial population to determine maximum denitrification rates at sodium concentrations up to 0.5 M sodium.

Table 5.19. Denitrification using Y-12 denitrifying microorganisms as seed culture

Time (days)	pH ^a	Na (M)	Zero-order model ^b			
			a	-b	r ²	n
0-10	7.53 (0.22)	0.54	1098	90.7	0.945	7
11-22	7.50 (0.15)	0.74	928	71.2	0.973	10
23-26	7.47	0.88	598	94.1	1.000	2
27-37	7.54 (0.16)	0.86	822	110	0.989	5
38-45	8.21 (0.33)		886	112	0.858	6
46-53	8.77 (0.07)	0.85	730	91.4	0.979	5
54-59	8.90 (0.03)	0.94	513	72.1	0.740	4
60-67	8.96 (0.04)	1.12	660	80.2	0.838	4
68-90	8.87 (0.04)	0.97	694	26.4	0.848	14
91-101	8.08 (0.03)	1.12	499	50.0	0.547	7

^aThe number in parentheses under pH is the standard deviation among pH measurements.

$\hat{y} = a - bt$, where $\hat{y}$ is estimated nitrate concentration (mg NO₃ = N/liter), a is the initial nitrate concentration, and b the denitrification rate (mg NO₃-N/liter/day).
 r^2 = coefficient of determination.
 n = number of nitrate determinations.

Table 5.20. Denitrification using soil microorganisms as seeding culture

Time (days)	pH ^a	Na (M)	Zero-order model ^b			n
			a	-b	r ²	
0-11	8.60 (0.81)	0.47	3810	315	0.916	10
12-19	9.29 (0.03)	0.65	1580	191	0.911	6
20-25	9.23 (0.07)	0.67	1310	226	0.955	5
25-32	9.27 (0.07)	1.04	1450	196	0.979	5
33-50	9.20 (0.08)	1.17	1594	95	0.940	12
51-59	9.24 (0.06)	1.45	1715	153	0.855	6
60-70	9.24 (0.05)	1.54	2027	117	0.944	5
71-85	9.21 (0.06)	1.78	1494	67	0.664	8
86-96	9.31 (0.04)	1.60	976	104	0.556	7

^aThe number in parentheses under pH is the standard deviation among pH measurements.

^b $\hat{Y} = a - bt$, where $\hat{Y}$ is the estimate nitrate concentration (mg NO₃-N/liter), a is initial nitrate concentration, and b the denitrification rate (mg NO₃-N/liter/day).
 r^2 = coefficient of determination.
n = number of nitrate determinations.

Table 5.21. Denitrification using estuarine microorganisms as seeding culture

Time (days)	pH ^a	Na (M)	Zero-order model ^b			
			a	-b	r ²	n
0-6	9.04 (0.22)	0.34	2048	325	0.995	5
7-8	9.30	0.50	757	569	1.000	2
9-11	9.17	0.68	1340	612	0.920	3
12-17	9.45	0.84	1120	256	0.923	3
18-41	9.37 (0.07)	0.83	678	31.1	0.923	16
42-47	9.35 (0.02)	0.93	767	177	0.989	3
48-55	9.42 (0.08)	1.11	671	81	0.801	6
56-69	9.48 (0.06)	1.17	781	56	0.875	10
70-78	9.41 (0.17)	1.17	515	72	0.979	4
70-89	9.49 (0.04)	1.24	488	62	0.629	7

^aThe number in parentheses under pH is the standard deviation among pH measurements.

^b $\hat{\gamma} = a - bt$, where $\hat{\gamma}$ is estimated nitrate concentration (mg NO₃-N/liter), a is initial nitrate concentration, and b the denitrification rate (mg NO₃-N/liter/day).

r² = coefficient of determination,

n = number of nitrate determinations.

Fig. 5.18. Nitrate loss in denitrifying reactor seeded with soil microorganisms.

Fig. 5.19. Nitrogen losses in denitrifying reactor seeded with estuarine microorganisms as seeding culture.

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Fig. 5.20. Nitrogen losses in denitrifying reactor seeded with microorganisms from a Y-12 denitrifying reactor.

Transient sodium perturbation of an active denitrifying reactor. To illustrate the effect of an impulse of sodium chloride into an active denitrifying reactor, the influent to a fluidized-bed tapered column that was removing 77% of the influent nitrate was made 2 M Na, using NaCl (influent solution was identical to nonsodium solution with respect to $\text{NO}_3\text{-N}$, carbon, etc.). The efficiency of the reactor was immediately lowered; in fact, denitrification ceased (Fig. 5.21). After 20 hr, when the sodium concentration in the effluent was equal to the sodium concentration in the influent, the original influent solution (without the NaCl) was started through the column. Denitrification began again about 7 hr after starting the sodium-free influent (ambient sodium concentration between 0.6 and 0.7 M). Thereafter, the efficiency of the unit increased linearly with time until the sodium concentration was less than 0.01 M, at which time the efficiency dramatically increased (from 20 to 40% in less than 10 hr).

Perturbation of an active denitrifying reactor with NaCl illustrated a number of important points.

1. Denitrification immediately stopped on perturbation.
2. Denitrification commenced when NaCl concentration was lowered to approximately 0.6 M.
3. Denitrifying efficiency increased on lowering of the NaCl concentration.
4. After removal of NaCl, denitrifying efficiency increased dramatically.
5. The new equilibrium denitrification efficiency was lower than the efficiency before perturbation.

Effect of nickel on biological denitrification. "Poisoning" of a biological reaction by trace levels of certain heavy metals is a major concern regarding the feasibility of using biological denitrification as a waste treatment process. In the nuclear industry, nitric acid is used to clean or "decontaminate" equipment. Much of this equipment is made of various grades of steel, which contains rather high concentrations of nickel. Because nickel is known to be toxic to many biological systems,

Fig. 5.21. Influence of NaCl on denitrifying reactor (fluidized-bed tapered column).

an experiment was conducted to evaluate the toxicity of nickel on biological denitrification.

Four levels of nickel were added to nitrate solutions [1310 mg $\text{NO}_3\text{-N/liter}$ as $\text{Ca}(\text{NO}_3)_2$] containing calcium acetate as a carbon source (2200 mg C/liter). Phosphorus (10 mg P/liter as KH_2PO_4), magnesium (10 mg Mg/liter as MgSO_4), and iron (2 mg Fe/liter as Fe-DTPA) were added as nutrient amendments. An active culture of denitrifiers was added (final volume 175 ml), purged with N_2 , and monitored for nitrate, nitrite, pH, and soluble nickel and calcium, using ^{63}Ni and ^{45}Ca .

Data indicated that initially very low concentrations of nickel significantly inhibited denitrification rates. For example, 0.5 mg Ni/liter was found to reduce denitrification by nearly 50% (Table 5.22). The added nickel did not become sorbed to the microbial mass nor did it precipitate either as the carbonate or become occluded to CaCO_3 (note the soluble nickel concentrations in Table 5.22). After eight days the levels of nickel (as high as 3.9 mg/liter soluble nickel) were not inhibitory to denitrification, indicating that a nickel-tolerant microbial population had survived. Investigations are continuing on the mechanisms involved in selecting nickel-tolerant denitrifying cultures.

5.2.3 Process development (C. W. Hancher and G. B. Dinsmore)

The biodenitrification process development program will report two areas of interest: (1) denitrification test at 200 to 1000 mg/liter NO_3 , using the 2-in.-diam x 14-ft-high bioreactor, and (2) bacteria loading attachment to solid-support particle tests.

The denitrification tests using the 2-in.-diam by 14-ft-high semi-tapered fluidized-bed bioreactor have been continued. This 2-in.-diam bioreactor reported in detail in the last semiannual (September 1976–March 1977) was equipped with sample ports at 2-ft intervals. The feed was NH_4NO_3 with ethanol as a carbon source. The feed carbon-to-nitrate/nitrogen ratio (C/N) was 1.8 to 2.0. The carbon-to-nitrogen ratio that was used was about 1.5. The bioreactor was operated at two nitrate feed concentration areas, 600 ± 100 mg/liter NO_3 and 200 ± 50 mg/liter NO_3 . The denitrification rates were 22 and 8 mg $\text{NO}_3\text{-N/day}\cdot\text{cm}^3$ (Table 5.23).

Table 5.22. Effect of nickel on denitrification^a

Treatment Ni (mg/liter)	mg NO ₃ -N at intervals (days) of:						
	0	2	3	8	9	10	13
0	1310	860	280	<2	1160	1020	650
0.5	1310 (0.51)	930	400 (0.49)	<2 (0.48)	1160	1000 (0.47)	650 (0.44)
1.0	1310 (0.97)	1030	480 (0.97)	<2 (0.96)	1160	980 (0.91)	610 (0.93)
5.0	1310 (5.0)	1310	1110 (4.9)	<2 (3.7)	1160	930 (3.9)	590 (3.6)

^aNumbers in parentheses are soluble nickel concentrations after centrifugation at 20,000 rpm for 20 min (34,880_{av} RCF); ⁶³Ni used as a tracer to determine nickel concentrations. Nitrate concentration is an average of three treatment replications. On day 3, additional Ca(NO₃)₂ and Ca(OAc)₂ were added.

Table 5.23. Bioreactor, 2 in. in diameter by 14 ft high

Feed flow rate (cm ³ /min)	Feed NO ₃ (mg/liter)	Effluent NO ₃ (mg/liter)	Effluent NO ₂ (mg/liter)	Denitrification rate (mg NO ₃ -N/day·cm ³)
900	620	120	270	19
940	250	0	5	7

The profiles of NO₃, NO₂, and C are shown in Fig. 5.22 for the 200-mg/liter nitrate feed. The nitrite went through a maximum at about 140 mg/liter and then decreased to nearly zero before being discharged from the bioreactor.

The 2-in.-diam bioreactor operating on 150 to 200 mg NO₃/liter was shut down on August 18, 1977, by stopping the feed pump and allowing the bacteria-loaded solid-support particles to settle. The bioreactor remained unoperable at room temperature (75°F) until September 20, 1977, 33 days later. Feed at 250 mg/liter NO₃ was fed to the bioreactor. The denitrification rate before stopping was 5 g NO₃-N/day·liter. The denitrification rate after restarting reached 6 g NO₃-N/day·liter on the eighth day. This shutdown experiment demonstrated the ability of this bacterial system to reactivate to normal activity in eight days after storage at room temperature for 33 days. This means the bioreactor contents can be stored while bioreactor maintenance or cleaning is performed without extended restarting delays.

This is the conclusion of the extended ethanol-for-carbon substrate tests. Feed concentrations from 7500 to 200 mg/liter of NO₃ have been used. The denitrification rate appears to be dependent on nitrate concentrations.

J. J. Perona, University of Tennessee Chemical Engineering Department consultant, prepared the following analysis of biological denitrification kinetics in 2-in.-diam fluidized-bed bioreactors.

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Fig. 5.22. Denitrification test at a nitrate concentration of 213 g/m³.

What follows is an account of my attempts to interpret concentration profile data in the (untapered) fluidized-bed reactor. First of all, the rate of mass transfer does not control, the mass transfer coefficient being of the order of 800 times larger than the apparent overall rate constant. Of the four runs for which Chuck Hancher provided me with data, three had concave upward profiles, and one a concave downward profile (see Fig. 5.23). Assuming that the concave downward run was anomalous, I tried to interpret the concave upward profile by various models. I found that (1) axial dispersion or backmixing cannot cause such a profile with a positive-order reaction, (2) an equation of the Michaelis and Menton form with positive k_s cannot cause such a profile, and (3) to account for such a profile through a kinetic equation of the form $r = ac^b$ requires that b be negative. In fact, the profiles for run 2/14/77 can be reproduced pretty well with $b = -2$. My inclination is to consider a negative-order reaction as something to be used only if all else fails.

Upon discussing the properties of the reactor in more detail with Hancher, I learned that the biomass is less dense than the coal particles, and that, as the biomass grows on a particle and it becomes more active, it tends to float upward in the column. This raises the possibility of developing a refined model, taking into account the coupled reaction kinetics and fluid-particle mechanics. Do you think this would be worthwhile?

In the meantime we can use a crude model in which the bottom or inlet 25% of the reactor is taken as inactive. The concentration profile data for the rest of the reactor can be represented by a straight line, implying zero-order kinetics. The Michaelis and Menton equation approaches a zero-order equation when k_s is small, which agrees with values of k_s measured by Francis and Malone. The data from all three runs can be modeled very well with a single equation of the Michaelis and Menton type with a significantly large value for k_s .

The details of these considerations follow.

Fig. 5.23. Concentration profiles from fluidized-bed reactor.

Mass transfer properties. A recent article of interest in "Mass Transfer in Liquid-Fluidized Beds at Low Reynolds Numbers," Koloini, Sopcic, and Zumer, *Chem. Eng. Science*, 1977, p. 637. However, this article contains a fundamental error, as shown by the following development:

Writing a mass balance over a differential volume of the bed,

$$LdC = k_L a_p (C - C^*) d(SZ), \quad (1)$$

where

L = liquid flow rate, cm^3/sec ;

C = concentration, g/cm^3 (NO_3);

k_L = mass transfer coefficient, cm/sec ;

a_p = surface area of solid particles, cm^2/cm^3 of bed;

C^* = equilibrium concentration at solid surface, g/cm^3 (NO_3);

S = cross-sectional area of column, cm^2 ;

Z = height of column, cm .

$$L \int_1^2 \frac{dC}{C - C^*} = k_L a_p \int_1^2 d(SZ), \quad (2)$$

$$k_L = \frac{L}{a_p V} \ln \left(\frac{C_2 - C^*}{C_1 - C^*} \right), \quad (3)$$

where $(SZ)_2 - (SZ)_1 = \text{bed volume} = V$. The article by Koloini et al. uses an equation of the form above to calculate mass transfer coefficients from their data, except that instead of $a_p V$, the total surface area of the particles in the bed, Koloini et al. used S , the cross-sectional area of the bed. Hence, their values of k_L appear to be much too high. Furthermore, their article does not provide the bed size, which would allow the recalculation of their data.

An estimate of k_L can be obtained from "Mass Transfer from Benzoic Acid Granules to Water in Fixed and Fluidized Beds at Low Reynolds Numbers," Evans and Gerald, *Chem. Eng. Progress*, March 1953, p. 135. Their correlation, in English units, is

$$k_L = \frac{1.15 \times 10^{-10} g_C \rho (\rho_S - \rho)}{\mu^{1.33} N_{Sc}^{2/3}} \frac{G^{0.33}}{D_p^{0.27}} \quad (4)$$

For run 2/14/77, with $\rho_S = 96.1 \text{ lb/ft}^3$, $N_{Sc} = 315$, $G = 3880 \text{ lb/hr}\cdot\text{ft}^2$, and $D_p = 0.0014 \text{ ft}$, this correlation gives $k_L = 60.3 \text{ lb/hr}\cdot\text{ft}^2$, or $k_L = 0.008 \text{ cm/sec}$. If we assume that the run is mass-transfer-controlled, we can calculate k_L for the run with Eq. (3), taking C^* to be very small. To obtain a lower limit on the surface area of the bed, assume the particles are spherical. Then the surface-to-volume ratio of the particles is 140 cm^{-1} , and for a total bed volume of 7.4 liters with a void volume of 0.5, the surface area of the bed is $522,000 \text{ cm}^2$. Using these values in Eq. (3), we estimate k_L at 10^{-5} cm/sec . Since this value is less by a factor of 800 than the literature value, the reaction must be controlled by the chemical kinetics.

Effect of axial dispersion. Concentration profiles in tubular reactors with varying amounts of axial dispersion were calculated by Fan and Bailie, *Chem. Eng. Science*, 1960, p. 63. They presented concentration profiles for reactors with reaction orders of 1/4, 1/2, 2, and 3 with dispersion conditions varying over the complete range. In no case was a profile obtained which was concave upward.

Michaelis-Menton equation. According to Francis and Malone, *Prog. Wat. Tech.*, 1977, p. 687, the kinetics of the denitrification reaction in a tapered fluidized bed can be represented by an equation of the form

$$R = R_{\max} C / [k_S + C] \quad (5)$$

for nitrate concentrations less than 1 g/liter. If k_S is small with respect to C , the equation becomes

$$R \cong R_{\max} \quad (6)$$

Substituting the Michaelis-Menton form into the plug flow reactor equation,

$$\frac{V}{v} = \tau = - \int_{\text{in}}^{\text{out}} \frac{dC}{R} \quad (7)$$

$$\tau = - \int_{in}^{out} \frac{[k_S + C]dC}{R_{max} C},$$

$$\tau = - \frac{1}{R_{max}} \left[\int_{in}^{out} \frac{k_S dC}{C} + \int_{in}^{out} dC \right],$$

$$\tau = \frac{1}{R_{max}} \left[k_S \ln \frac{C_{in}}{C_{out}} + C_{in} - C_{out} \right]. \quad (8)$$

From Eq. (8) we see that if k_S is large, the concentration profile is concave downward. In Fig. 5.24 the Michaelis-Menton equation is applied to run 2/14/77 with k_S values of 0, 10, and 100; R_{max} is adjusted so that the concentration at a volume of 5 liters agrees with the experimental values. It is clear that the experimental profile cannot be reproduced by this equation. If we take the data in Fig. 5.23 and make up a composite concentration profile by placing the "active" reactor regions in sequence, we obtain the profile shown in Fig. 5.25. These data can be well represented by Eq. (8) with $R_{max} = 1140 \text{ g (NO}_3\text{)}/\text{day}\cdot\text{liter}$ and $k_S = 5.3 \text{ g (NO}_3\text{)}/\text{liter}$. As the nitrate concentration decreases, the log term becomes more predominant as shown in Table 5.24. Thus the log terms give the decreasing slope as concentration becomes small, which is observed in Fig. 5.23.

Table 5.24. Relative contribution of Michaelis-Menton constants to required space time (τ)

C g (NO ₃)/liter	5.3 ln $\frac{7.5}{C}$	7.5 - C	τ (days)
7.5	0	0	0
6	1.18	1.50	0.0023
4.5	2.71	3.0	0.0050
3	4.85	4.5	0.0082
1	10.68	6.5	0.015
0.3	17.06	7.2	0.021
0.1	22.8	7.4	0.026

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Fig. 5.24. Fit of Michaelis and Menten equation to run 2/11/77.

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Fig. 5.25. Composite concentration profile.

Equation (8) was developed assuming that the reactor was in plug flow. It would be nice to account for backmixing, and this should be attempted in the future. Meanwhile, we can observe that the dispersion coefficients reported in MIT-218 were fairly small, and so perhaps the assumption of plug flow is reasonable. The relationship between the space time and concentration is given by

$$\tau = \frac{V}{v} = \frac{1}{R_{\max}} \left[C_{\text{in}} - C + k_s \ln \frac{C_{\text{in}}}{C} \right], \quad (9)$$

where

- τ = space time, days;
- V = reactor volume, liters;
- v = volumetric flow rate, liters/day;
- $R_{\max}$ = 1140 g (NO_3^-)/liter·day;
- C = concentration, g (NO_3^-)/liter;
- k_s = 5.3 g/liter.

Bacteria attachment to coal loading tests. A bacteria-attachment-to-coal procedure has been needed for considerable time. There has been a small number of loading failures. The following procedure was the first and only one tested to date. It was very successful; therefore, testing of alternate procedures will receive little attention.

A clean bioreactor, 3-liter capacity, was equipped with a liquid circulation pump (50–1000 ml/min range), feed pump (5–100 range), and off-gas system to water bubbles. The attachment procedure is as follows. (1) Add 3 liters of standard feed (Table 5.25) diluted to 1000 mg (NO_3^-)/liter and pH adjusted to 7.3. (2) Add denitrification bacteria, about 50 ml of 1×10^9 cells/ml concentration. Circulate bacteria cells and feed solution at a rate of about 300 ml/min. Check pH and NO_3^- levels daily and add NH_4OH (5 M) and/or 20 g (NO_3^-)/liter concentrated feed as needed to maintain starting concentration. After 4 to 6 days the N_2 gas evolution should be measurable. (3) When the gas rate is visible, add 500 g of washed (–30 +60) mesh coal. Continue circulation pump for 1 hr, then stop circulation. (4) When gas bubbles between the coal particles become very concentrated, resume solution circulation at a low

underfluidized rate (about 200 ml/min). (5) Start feeding 2000 mg (NO_3^-)/liter at an appropriate rate to maintain the 1000 mg (NO_3^-)/liter bath liquid concentration. About 12 days after inoculation the coal particles will be covered with a gray-white bacteria coating, and they will then be ready for use in a tapered fluidized bioreactor.

Table 5.25. Standard NH_4NO_3 feed mixtures

NH_4NO_3	6.8 g/liter (4600 ppm NO_3^-)
ETOH	6.0 ml/liter
MgSO_4	0.1 g/liter
KH_2PO_4	0.05 g/liter
Trace mineral mix	0.1 ml/liter

5.2.4 Process demonstration

The purpose of the process demonstration work is to define scale-up parameters and to identify and resolve possible difficulties associated with the operation of large-scale denitrification reactors. Two different reactor configurations are being studied: a stirred-bed reactor, where the bacteria are in suspension, and a fluidized-bed reactor, where the bacteria are attached to a suitable support media.

Feed solution. The feed solution being used for the current experiments is a mixture of ammonium nitrate and ethanol. The ethanol is used by the bacteria as their energy source, and the ammonia is just a soluble, nontoxic cation for the nitrate anion. The feed solution also contains about 50 ppm phosphate, sulfate, and magnesium, and much lower amounts of other trace metals. These trace metals are known to be present in various bacterial enzymes, but the actual amount needed has not been clearly defined. Most of them should be present in sufficient quantity in normal process water. Phosphate and possibly magnesium sulfate will probably need to be added to most waste streams before biological denitrification can take place.

Stirred-bed reactor. There are two possible scale-up parameters that could be needed to design a stirred-bed reactor for a given waste stream. For a stream with a high nitrate concentration (>3 wt % NO_3^-), a reactor can be sized by using the denitrification rate alone. For a low nitrate concentration stream, the minimum residence time will determine the size of reactor needed. Both of these parameters will depend upon the organic carbon source, the temperature, and possibly other factors. It will be necessary to measure them for each waste stream before designing a full-scale reactor. However, since stirred-bed reactors usually operate very close to ideal CSTR performance, the parameters can be measured using a small reactor and scaled up directly.

This has been demonstrated during the development and pilot-plant work for the Y-12 Plant denitrification reactor. The initial measurements were made in 60-liter stirred-bed reactors. These results were checked in a 15,000-liter pilot plant and were found to scale-up directly.⁸ The full-scale reactors are 150,000 liters, and the reactor performance was conformed to the small-scale test results.⁹ The maximum denitrification rate for this waste stream was $2.9 \text{ g NO}_3^- \text{-N/liter} \cdot \text{day}$, using calcium acetate as the organic carbon source. The reactors were sized using $1.4 \text{ g NO}_3^- \text{-N/liter} \cdot \text{day}$ as the design rate in order to give flexibility in case of overloads. These reactors have proved to be very stable in operation and to require a low amount of operator time, most of which is spent in taking samples and recording results.

In a stirred-bed reactor without bacteria recycle, the hydraulic and bacterial residence times are directly related. In order to have stable operation, the bacteria in the reactor must be able to reproduce fast enough to replace the bacteria washed out of the reactor. If the residence time is too low, the bacteria population will become depleted and denitrification will stop.

The bacterial and hydraulic residence times can be decoupled by recycling bacteria, as is commonly done in commercial sewer plants. However, the denitrification bacteria in our experiments have not generally flocculated, so a centrifuge or filter would be needed to concentrate the

bacteria rather than a simple gravity clarifier. If bacteria recycle is used, the denitrification rate will be the only scale-up parameter needed, even for a low nitrate concentration stream. For a reactor operating without bacteria recycle, the minimum residence time must be measured.

For a nominal 1 wt % nitrate feed, the minimum residence time was found to be four days, using a 67-liter stirred-bed reactor. The organic carbon source was ethanol for these experiments. The denitrification rate was $0.70 \text{ g NO}_3^- \text{-N/liter day}$ with a nitrate concentration of $1150 \text{ mg NO}_3^- \text{/liter}$ in the reactor. Increasing the residence time to 4.2 days will cause the nitrate concentration to drop to below $5 \text{ mg NO}_3^- \text{/liter}$. The boundary of the minimum residence time is very unstable, so a full-scale reactor should be designed to operate substantially back from this boundary.

The minimum residence time for a given waste stream will depend on the organic carbon source, the temperature, etc., so it must be measured at the projected operating conditions of the full-scale reactor. However, the actual value can be measured in a laboratory-sized reactor and scaled up directly. Another method for decoupling the bacterial and hydraulic residence times is to attach the bacteria to a suitable support media. This is the approach used for the fluidized-bed reactors.

Fluidized-bed reactor. A 10-cm-ID column has been operated to measure the scale-up performance of the fluidized-bed system. The column is the same height but twice the diameter of the column used in the process development work. The support media used for both of these experiments is 30- to 60-mesh coal particles. The operating conditions used for the 10-cm-ID column were designed to duplicate one set of the measurements made in the smaller column. The temperature and inlet nitrate concentration were the same, and the total flow rate was four times larger to maintain the same residence time. The inlet concentration was $1000 \text{ mg NO}_3^- \text{/liter}$, and the temperature was 22°C . The denitrification rate was $24 \text{ g NO}_3^- \text{-N/liter}\cdot\text{day}$ in the 10-cm-ID column as compared with $30 \text{ g NO}_3^- \text{-N/liter}\cdot\text{day}$ in the smaller column. Both rates were measured through the straight section of the columns, with the empty column volume used to calculate the residence time.

The lower denitrification rate in the 10-cm-ID column was expected, since there is more axial mixing in the bigger column. Since the flow pattern in these columns does not approach plug flow, these reactors can not be scaled up by simply increasing the diameter for a given height. More work will be needed in this area to further define the scale-up performance.

As can be seen from the experimental data, the denitrification rate is much higher in the fluidized-bed columns than in the stirred-bed reactors. The reason for this can be easily seen by comparing the nitrate concentration profiles in the two reactors. In the stirred-bed reactor the feed is immediately diluted to the nitrate concentration in the reactor, usually less than $5 \text{ mg NO}_3^-/\text{liter}$. The column can take advantage of the high nitrate concentration in the feed, since the bacteria will actually see this concentration. Denitrification rates can be approximated by first-order reaction kinetics. However, the nitrate concentration in the column feed must be below the toxic limit of the bacteria (about $8000 \text{ mg NO}_3^-/\text{liter}$). This restraint is not present in stirred-bed reactors.

The column has been somewhat harder to operate than the stirred-bed reactors. One of the main problems has been excessive biomass growth in the column. If the bacterial layer on the coal particles becomes too thick, the inner layer turns septic, causing the whole particle to float. Biomass control has been achieved by removing about 10% of the coated coal particles each day and replacing it with an equivalent amount of new coal. This method has been found to be effective, but it requires a certain amount of foresight on the part of the operator. It may be possible to control the biomass internally by limiting the nitrate concentration in the column and increasing the flow rate to scrub off excess bacteria. This method still needs to be tested.

At present the stirred bed is the only reactor that has proven to be operational on a large scale, using an actual waste stream with the inevitable changes in flow rates and concentrations. However, the much higher denitrification rates found in the fluidized-bed reactor justify continued work on this system.

5.3 References for Section 5

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6. ENVIRONMENTAL CONTROL TECHNOLOGY

6.1 Assessment of Environmental Control Technology for Hydrocarbonization

From recent work¹⁻³ describing the wastewater streams to be found in a hydrocarbonization facility, it is concluded that the contaminants of most concern, from a wastewater treatment viewpoint, are phenolics, trace elements, and polycyclic aromatic hydrocarbons (PAHs). These three groups will have the greatest impact on the type, or types, of control technology to be utilized. Although it is obvious that little more than generalizations can be made about what contaminants will be present without actually analyzing the wastewater, the types and quantities of these materials do need to be investigated.

Recent work has concentrated on assessing a number of wastewater treatment methods for their applicability to the cleanup of hydrocarbonization wastewater. The complexity of the various aqueous streams from a coal hydrocarbonization plant has led to some doubt as to the "best" process for wastewater cleanup. At the same time the sure enactment of stricter effluent standards has put pressure on new coal conversion processes to adequately dispose of their wastes.

A previous report² has described recent assessment of biological oxidation, activated carbon, and ozonation. Biological oxidation appears capable of economically removing large quantities of phenolics from hydrocarbonization wastewater with, however, only marginal removal of trace metals and PAHs. Costs should approximate \$0.15 to \$0.20 per lb of phenol or \$6000 to \$10,000 per million gallons of effluent.^{4,5}

Activated carbon is generally more expensive, with estimates ranging from \$0.12 to \$6 per lb of phenol removed.⁴ However, most costs for carbon adsorption are in the \$0.50 to \$1 range. Although it is too expensive to be a likely candidate for the primary organic removal step, it could compete as a final polishing treatment. The fate of many of the trace metals is uncertain.

Ozonation is an even more uneconomical way of removing large amounts of phenol. Present indications are that the complete oxidation of

hydrocarbonization wastewater cannot be achieved economically with ozone.^{4,6} If either a very small amount of hydrocarbons needs to be treated, as in a final polishing step, or if only a small amount of degradation is needed, as would be the case when only the phenolic structure is to be cleaved and not completely oxidized to carbon dioxide and water, then ozonation may have some advantage. At an ozone requirement of 2.6 lb of O_3 per lb of phenol and \$0.25/lb for ozone, this is equivalent to a treatment cost of \$0.65 per lb of phenol.

Some investigators have shown that as much as 80% of the chemical oxygen demand of some wastewater streams may be contained in particles, colloidal or larger in size, and up to 50% of the total organic carbon in the effluent of biological treatment plants may be colloidal.⁷ The coagulation and flocculation of these particles can dramatically lower the demand on downstream steps.

Frequently, coagulation can occur naturally. In biological treatment processes, the microorganisms aggregate themselves to a point where they will settle out. Under some conditions the agglomerated sludge can even absorb colloidal organic substances in wastewater in a shorter time than is required to remove those materials by biological oxidation.

Traditionally, lime, alum, iron salts, or more recently, polyelectrolytes have been used for this coagulation process. Lime reacts with the bicarbonate in the water to produce the insoluble calcium carbonate. In addition, the increasing alkalinity will tend to produce insoluble hydroxides of many metals, with lead, copper, chromium, zinc, nickel, cadmium, and magnesium being readily precipitated out. Due to this effect, lime is usually the material of choice, with alum, iron salts, and polyelectrolytes being used as coagulation or flocculation aids.

Costs for coagulation and flocculation vary tremendously, depending on the wastewater to be treated. But assuming a representative dose of 3.3 lb per thousand gallons of effluent and a price of \$50 per ton of lime (including capital equipment costs), a cost of \$83 per million gallons of effluent is obtained.^{5,8}

Solvent extraction has attracted the attention of investigators for numerous years. A variety of solvents for phenol removal in both typical solvent extraction systems and dual solvent processes (polar solvent-volatile solvent) have been investigated.^{9,10} Although high distribution coefficients have been reported for many of these systems, Lurgi's Phenolsolvan process appears to be the only one that has been commercialized. Even though the solvent for this type of contactor is chosen for its high distribution coefficient for phenol, other types of organics will also be stripped out of the aqueous phase. The Phenolsolvan process should remove 99.5% of the monohydric phenols (phenol, cresol, xylenol, etc.) but only 15 to 60% of other organics and phenols. Thus, this process may produce a crude phenol product that may have a composition of 80% monohydric phenols, 15% polyhydric phenols, and 5% other organics.¹¹

Solvent extraction would seem to offer an advantage only where a moderate amount of reduction in phenol content is needed in order to allow an additional waste treatment to operate or when a saleable crude phenol can be produced. One study indicated that if the value of the recovered phenol is greater than \$3000 per million gallons of wastewater, then recovery is promising.⁹ This works out to \$0.07 per lb of phenol compared with bulk prices of \$0.26 per lb of phenol.⁸ Product upgrading may, however, prove to be quite expensive.

Another area of interest includes membrane processes such as reverse osmosis, ultrafiltration, and electrodialysis. In reverse osmosis and ultrafiltration the separation is achieved by passing the water through a membrane with the concentrated solute left behind; conversely, in electrodialysis the solute passes through the membrane (via an electric potential driving force), leaving the purified solvent behind. These membrane processes, although considered expensive, may be useful in upgrading wastewater for in-plant reuse.

Although ultrafiltration and reverse osmosis have shown phenol reductions of from 50 to 95%,¹²⁻¹⁴ these values will still leave phenol concentrations in excess of anticipated regulations. In addition, a concentrated stream will be produced and will need to be disposed of.

Economical considerations of the various membrane processes have not been made. Considering the high operating costs (membranes, 250- to 1000-psig pressure, etc.) and the need for additional treatment, application does not seem practical.

A summary of this assessment is shown in Table 6.1. Conclusions are that the major sources of pollutants are phenols, trace elements, and PAHs. Processes that show the most promise of economically treating hydrocarbonization wastewater include coagulation and flocculation, biological treatment, and adsorption as a final polishing step. Experimental screening tests will be performed to determine the applicability of these processes to actual coal conversion wastewater.

6.2 Laboratory Evaluation of Environmental Control Technology for Coal Conversion Process

A variety of screening tests have been recently completed to determine the applicability of several environmental control processes to coal conversion processes. These tests include PAH removal with charcoal and polymeric resins, and phenol removal with hydrocarbonization char. In addition, a tapered fluidized-bed bioreactor (TFBBR) has been operated with phenolic wastewater similar to actual hydrocarbonization aqueous wastes. Highlights of this work are included here, with a detailed description given in Sect. 4.5.

Operating with a synthetic feed of 40 to 60 ppm phenol and a feed rate of 10 cc/sec, the TFBBR has achieved phenol reductions of from 99.8 to 99.9%, resulting in exit levels below 50 ppb. When actual coal hydrocarbonization wastewater was used along with an effluent recycle-concentrated feed configuration, that is, phenol concentrations of 1200 to 2500 ppm, effluent phenol levels of 3 to 6 ppm were achieved. When a dilute feed (no recycle) mode of 50 ppm phenol was used, effluent concentrations of slightly under 1 ppm phenol were obtained. One test was performed to indicate the performance of the TFBBR under unfavorable conditions. Both the feed flow and the oxygen supply were shut off for three days and then restarted in order to determine the recovery time

Table 6.1. Assessment of environmental control technology for hydrocarbonization

	Biological oxidation	Adsorption	Ozonation	Coagulation- flocculation	Solvent extraction	Membrane processes
Potential applicability	High	High	Moderate	High	Moderate	Low
Economics	\$0.15-\$0.20/lb phenol \$6000-\$10,000/10 ⁵ gal	\$0.50-\$1/lb phenol	\$0.65/lb phenol	\$83/10 ⁶ gal	\$0.07/lb phenol \$3000/10 ⁶ gal	
Raw materials	None to little	Charcoal or other sorbers	None	Lime or other agents	Solvent	Membrane material
Process compatibility	Moderate to high	High	High	High	Moderate	Low to moderate
Operating conditions	Moderate temperature	Moderate, except for regeneration	Little known	Moderate	Moderate	Moderate
State of development	High	Moderate to high	Fair	High	Moderate to high	Moderate
Additional environmental problems	Disposal of sludges	Disposal of spent sorber	No trace element removal	Disposal of sludges - heavy-metal sludges	Little to none if product saleable ^a	Concentrated aqueous waste stream
Energy requirements	Low	Low (except for regeneration)	High	Low to moderate	Moderate to high	Moderate high
Process availability	High	High	Moderate	High	Moderate to high	Moderate high

^a An aqueous stream with contaminants in excess of regulations will require additional treatment; product stream will need to be disposed of if not saleable.

after a simulated outage. After start-up, phenol began to be degraded in less than 24 hr, and within 48 hr the rates were back to preshock levels.

Two ion exchange resins and activated charcoal were tested for their ability to remove PAHs from aqueous solutions. Naphthalene, anthracene, phenanthrene, and benzantracene were agitated with either activated carbon or resin. Results (Table 6.2) indicated that these two resins have marginal ability to remove PAHs, while activated carbon removes a majority of the PAHs present.

Table 6.2. Fraction of PAHs removed

Concentration (ppm)	4 hr, activated carbon	Activated carbon	16 hr	
			Resins	
			IRA-400	IR-120
Naphthalene				
0.3	0.79		0.83	
3.0	0.78	0.13 ^a	0.92	0.58
Anthracene				
0.0375	0.73	0.87		
0.075	0.50	0.90		
Benzantracene				
0.002	0.88	0.94		
0.01	0.90	0.95		
Phenanthrene				
0.232	0.91	0.89	1.00	0.78
1.16	0.43	0.65	1.00	0.93

^aQuestionable data; see 4-hr experiments.

Tests were run with 40 ml of PAH solution and 1 mg of carbon or 1 g of resin and agitated for 4 or 16 hr. Concentrations were determined by an Aminco-Bowman spectrophotofluorometer. Concentrations of the PAH tested varied from 0.002 ppm for benzantracene to 0.3 ppm for naphthalene. Concentrations used were roughly determined by their respective solubility in water.

The sulfonated polystyrene resin Amberlite IR-120 appears to be a poor adsorber of PAHs, absorbing only phenanthrene. The trimethylammonium chloride resin Amberlite IRC-400 removed 100% of the phenanthrene and most of the naphthalene. Although adsorption levels indicate an interest in the IRA-400 resin, the 4-hr results were not listed in Table 6.2 due to insignificant adsorption in that time. A microporous version of IRA-400 has been received and will be tested.

Several samples of hydrocarbonization char produced from Wyodak subbituminous coal, hydrotreated at 560°C and 260 to 300 psig for 30 min, have been tested for their ability to remove phenolics from aqueous streams. This char is approximately 80% carbon and 12% ash. In a 50-cc column with flow rates of 6 cc/min containing 200 ppm phenol, breakthrough times for the phenol occurred in less than 10 min as compared with 50 hr for activated charcoal. Future studies will involve determining phenol adsorption as a function of surface area for char and treating char by steam stripping, pyrolysis, or acid treating to improve adsorption.

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