

## ***SMALL SCALE WASTE MANAGEMENT PROJECT***

### **Laboratory Studies on Stimulation of Biological Denitrification**

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## LABORATORY STUDIES ON STIMULATION OF BIOLOGICAL DENITRIFICATION

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Introduction

The efficiency of properly designed, well managed, septic tank-soil seepage bed systems to remove pathogens, organic carbon, and in most cases, phosphorus, has been well established (Magdoff et al., 1974; Walker et al., 1973a, b; Dudley and Stephenson, 1973). Nitrogen removal is much less efficient in these systems because quantitative nitrification in the aerobic subcrust portion of the bed results in nitrate which moves nearly uninhibited with percolating effluent to the water table (Walker et al., 1973b). Research in our laboratory has indicated that some denitrification may occur in fine-textured, well-structured subsoils (Magdoff et al., 1974), but that in general the nitrate concentration of the percolate beneath a seepage bed approximates the total N concentration of the septic tank effluent (Walker et al., 1973b).

Calculations of the N input by septic tank systems indicate that this source would not supply significant N to ground water of typical rural watersheds when the density of residences is less than one per 0.2 hectare (Walker et al., 1973b). However, in areas of higher population density, septic tank systems may supply significant quantities of nitrate (Dudley and Stephenson, 1973; Smith and Baier, 1969; Walker et al., 1973b). This can be particularly significant in areas with sandy soils or fractured bedrock close to the soil surface. In Wisconsin these situations exist in the sandy soils of the central and northwest parts of the state, and in the dolomite bedrock areas of Door County and southwest Wisconsin. These areas are currently undergoing expansion of non-sewered resort and second home site developments.

Widespread concern over the presence of high nitrate concentration in water as well as food has been noted in recent reports (Nat. Acad. Sci., 1972; EPA, 1973). This concern centers on: a) the toxicology of nitrate to infants drinking water with  $\text{NO}_3^-$ -N levels higher than 10 ppm (USPHS, 1962); b) toxicology of nitrate in waters given to livestock; and c) eventual contribution of N to surface waters resulting in increases in productivity, and hence, eutrophication of these waters.

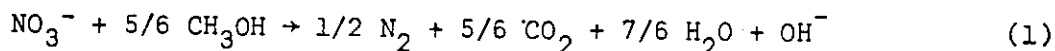
The work reported in this paper represents the initial developmental studies to evaluate the feasibility of a denitrification system for individual home sewage systems. The requirements for a system are that it is simple in design, economical to construct and maintain, has an adequate life expectancy, and is effective in removing nitrate without adding another pollutant. The initial studies have involved a survey of possible energy sources and continuous flow column experiments using certain of these sources.

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## Materials and Methods

Batch studies

Organic materials as energy sources for denitrification were evaluated by incubation of the material in a 250 ml square bottle equipped with a rubber stopper which allowed for gas flushing. Two to twenty g of solid material along with a nitrate solution (50 ug  $\text{NO}_3^-$ -N/ml) were added to bottles containing 50 g of approximately 1 cm diameter  $\text{CaCO}_3$  chips. The  $\text{CaCO}_3$  stabilized the pH of the system and also served as a support in column studies. Deionized water was added so that the solids were covered by 1 cm of liquid and the systems were flushed with He for 30 minutes with intermittent shaking to remove the air and ensure anaerobic conditions. Plainfield sand (50 g) was used as the inoculum in the experiments to evaluate  $\text{CH}_3\text{OH}$ . The sand was wetted with 10 ml deionized water and preincubated at 30 C for one day after which the nitrate and methanol solutions were added. The  $\text{CH}_3\text{OH}$  concentration chosen for the standard system equaled two times the stoichiometric weight needed to reduce the added  $\text{NO}_3^-$  to  $\text{N}_2$  as determined by the following equation:



Energy sources were chosen on a basis of varying C content and probable rate of decomposition. They included: a) a peat sample from a marsh at the west end of Lake Wingra in Madison; b) coniferous forest litter from the University of Wisconsin Arboretum, Madison; c) paper mill sludges from the Kimberly-Clark plants in Appleton and Lakeview, Wisconsin; d) oat straw; and e) molasses from a local supermarket. All dry materials were ground to < 2 mm.

The mucky peat sample was obtained at the site reported in Isirimah et al. (1970, sample 2A). This material has about 67% organic matter and a total N content of about 3% (C/N ratio of about 12). The paper mill sludges are similar to the material used by Dolar et al. (1972). They are classified as primary sludges with the Kimberly sludge containing more sulfur due to alum [ $\text{Al}_2(\text{SO}_4)_3$ ] addition. Molasses composition may vary widely in different areas of the country, but probably contains at least 50% sugar (Casida, 1968). Straw and forest litter can be expected to have a high C/N ratio (100 or greater) with much of the C being refractory.

Attempts were made to determine the soluble C levels for each energy source. One g of each of the solid energy materials was shaken with 100 ml of deionized water for 1 hr after which the extract was filtered through a Whatman no. 2 filter paper and a 0.45  $\mu$  Millipore filter. The filtrate was analyzed for total organic carbon using a Beckman Model 915 Total Organic Carbon Analyzer. A similar procedure was used for molasses.

Denitrifiers were determined by the most probable number method using nitrate broth medium (Focht and Joseph, 1973). The tubes were incubated at 30 C for 7 days and analyzed for nitrate. Inorganic N estimations involved extraction of the sample with 2M KCl. Nitrite plus nitrate -N and  $\text{NH}_4^+$ -N were determined by steam distillation (Bremner and Keeney, 1965). Nitrite -N was estimated by a modified Griess-Ilosvay method (Bremner, 1965).

Continuous Flow Columns Dosed with Methanol

Plexiglas columns (10.2 cm I.D. x 64 cm) were filled with  $\text{CaCO}_3$  (1 cm diam.) and sealed. Sampling ports and Pt black electrodes were placed at 14, 34, and 54 cm from the top of the columns (Fig. 1). The electrodes were

prepared according to Magdoff et al. (1974) and checked according to Quispel (1946). Readings were adjusted to pH 7 by using the  $-0.059/\text{pH}$  correction factor (Bohn, 1971). Residence times were determined by continuous addition of chloride and analysis of the effluent using a Buchler-Cotlove chloridometer (Cotlove et al., 1958). Influent and effluent from the columns were controlled by a peristaltic pump. Methanol addition to the influent was also controlled by the pump and mixing was accomplished by using a mixing coil. Inorganic N was estimated as described previously.

← FIG. 1

Gas samples were collected by a water displacement method (Fig. 1). Prior to sampling the gas collection bottle, the syringe was flushed several times with He and samples were taken through the rubber septum by first injecting a volume of He equal to the sampling volume (0.5 cc). Samples were immediately analyzed for  $\text{N}_2$ ,  $\text{O}_2$ ,  $\text{NO}$ ,  $\text{N}_2\text{O}$ ,  $\text{CO}_2$ ,  $\text{CH}_4$ , and  $\text{H}_2\text{S}$ . A 406 Series Packard dual column gas chromatograph equipped with Molecular Sieve 5A column (0.64 x 200 cm) for analyses of  $\text{N}_2$ ,  $\text{O}_2$  and  $\text{NO}$  and Porapak Q column (0.64 x 366 cm) for analyses of  $\text{CO}_2$ ,  $\text{N}_2\text{O}$ , and  $\text{H}_2\text{S}$  was used in connection with an Autolab 6300 digital integrator. Methane was determined on a 407 Series Packard gas chromatograph with a flame ionization detector (Macgregor et al., 1973).

## Results and Discussion

The most difficult problem in designing a denitrification system to be placed under seepage beds is choosing the energy source. One approach is to use an easily obtainable, slowly decomposable, solid energy source, with sufficient material being available for the planned life of the system (e.g., 10 years might be an economical life time). The release of energy material ideally would be enough for denitrification but would not add excess C to the effluent. The other approach is to supply the energy in a readily available form such as methanol ( $\text{CH}_3\text{OH}$ ). The latter would have the disadvantage that the  $\text{CH}_3\text{OH}$  must be metered with the influent at the required rate. This would require periodic maintenance. Even distribution of the liquid energy source for proper mixing would pose another problem. The solid energy material has the distinct disadvantage that it is difficult to envisage a material capable of sustaining denitrification for such long periods. Also, should the system malfunction, corrective action would require extensive renovation. Therefore, research was necessary to determine the method which will be best suited for home sewage disposal.

The denitrification rates (Table 1) are based on 100 mg soluble C added and varying incubation times due to the rates of various energy sources to reduce nitrate a significant amount, e.g., 4 days for peat and 0.75 days for molasses. Methanol and the paper mill sludges gave rapid denitrification rates while with straw, forest litter and peat, nitrate was denitrified much more slowly. The molasses value is misleading because far more molasses was added than necessary, and thus, the resulting rate is unrealistically low. A rate similar to  $\text{CH}_3\text{OH}$  would be expected because molasses should be an almost completely available energy source. The data show that the sludges are far superior to the other solid energy sources. Sources which yield rapid rates would be preferable because denitrification rates are inversely proportional to the residence time and accordingly the size of the bed or reactor needed for denitrification. Inasmuch as these data are expressed on the basis of soluble C which would generally be available for denitrification, the variations observed in rates must be for other reasons. One was stated previously, that being that more soluble C was added than necessary for reduction. Another reason may be the availability of the soluble C present. Peat gave a low denitrification rate even though sufficient C was available. Other constituents such as the particulates (Zobell, 1943) found

in the paper mill sludges may affect the rate. Organism number is another variable. Therefore, several explanations could be advanced to partially explain the data. However, in designing a long-term denitrification system, the interaction of all factors as well as the rate of release and immobilization of C must be evaluated. Therefore, long-term continuous flow studies are presently underway using the energy sources such as the paper mill sludges which showed promise in the batch studies.

Table 1. Mean denitrification rates, soluble carbon levels and MPN of denitrifiers in bottles incubated with various energy materials at 30 C.

Energy Material	MPN per bottle	mg soluble C per bottle	Denitrification Rate*
Peat	$2.4 \times 10^7$	84	72
Coniferous Forest Litter	$6.7 \times 10^6$	63	471
Kimberly Paper Mill Sludge	$1.7 \times 10^8$	14	7140
Lakeview Paper Mill Sludge	$2.5 \times 10^5$	17	7820
Oat Straw	$2.4 \times 10^9$	122	763
Sand-Molasses	$1.3 \times 10^6$	930	30
Sand-Methanol	$4.9 \times 10^6$	2	16250

\*  $\mu\text{g NO}_3^- \text{-N decrease/day/100 mg soluble C added.}$

#### Continuous Flow Column Studies

Preliminary investigations to evaluate the feasibility of continuous flow denitrification systems were conducted using  $\text{CH}_3\text{OH}$  as the energy source. Mechanically aerated septic tank effluent, which contained typical  $\text{NO}_3^- \text{-N}$  concentrations (40-50 ppm) and  $\text{CH}_3\text{OH}$  at about twice the stoichiometric concentration (eq. 1), was passed through columns at a rate to give a total residence time of 8.33 hr (as determined by chloride tracer). Data from Table 2 show that denitrification during non-steady state conditions was essentially complete at 14 cm, equivalent to a 1.8 hr residence time. Steady-state conditions were defined as those times when inorganic N levels at sampling ports did not vary by more than 10% over a three-day period. Non-steady-state would usually be indicated by consistent decreases in  $\text{NO}_3^- \text{-N}$  or variances greater than 10%. These results (Table 2) support other studies

Table 2. Inorganic N levels ( $\mu\text{g N/ml}$ ) in methanol continuous flow studies (20 C, non-steady state).

Sampling port	$\text{NH}_4^+ \text{-N}$	$\text{NO}_3^- \text{-NO}_2^- \text{-N}$	$\text{NO}_2^- \text{-N}$
Top	0	42.4	0.2
14 cm	0.1	0.4	0.2
34 cm	0	0.3	0.2
54 cm *	0	0.1	0.1
Bottom	0	0.4	0.1

\* Residence time = 8.33 hr.

(Requa and Schroeder, 1973) indicating that methanol is a good energy source for continuous flow denitrification and that a column system is a feasible method for evaluating denitrification.

← FIG. 2

When a higher concentration (180 to 200 ppm) of  $\text{NO}_3^-$ -N (obtained by addition of  $\text{KNO}_3$  to the mechanically treated effluent) along with proportionally higher concentration of  $\text{CH}_3\text{OH}$  and steady-state conditions were evaluated, essentially complete disappearance of nitrate occurred in 4.2 hr (Fig. 2). Thus, even high nitrate effluents can be denitrified in a relatively short time.

One disturbing event occurred during this experiment. After approximately four months of continuous flow, clogging occurred due to accumulation of a crust formed at the top of each duplicate column. Drying the columns out in the air for one day was sufficient to re-establish the original flow rate. Therefore, it would appear that field systems should have some means for alternate dosing and resting of the denitrification system for a short period of time (see Bouma et al., 1974). Another critical point in designing a denitrification system would be allowance for adequate gas release. Reduction of the flow rate due to gas build-up has been noted by Requa and Schroeder (1973) in their column which was not equipped for gas release. Resting the bed would also relieve this problem and therefore, it is possible that denitrification systems will have to be designed as dosing and resting systems.

The curvilinear relationship between decreases in  $\text{NO}_3^-$ - $\text{NO}_2^-$ -N and residence time was tested for a first order kinetic fit and was found not to apply along the entire distance of the columns. Monod kinetics (Monod, 1950) would appear to be a more accurate representation of denitrification under the continuous flow system and research is presently underway to obtain cell growth parameters necessary for evaluating such kinetics. One question which needs to be answered is whether the greater accuracy of Monod kinetics is necessary for denitrification modeling or whether the more simple first order kinetic assumption such as in Mann et al. (1973) and Focht (1973) can be used.

Nitrite-N increased and then decreased with time and never reached high concentrations. Thus, nitrite accumulation should not present any problems in this system.

#### Eh readings

The initial  $\text{Eh}_7$  readings taken when columns had undergone one week of continuous flow studies ranged from +275 - 300 mv. Once denitrification began and had continued for a number of days,  $\text{Eh}_7$  levels dropped steadily to the values shown in Fig. 3. These readings were taken just prior to and during the period designated as steady-state. The 34 cm electrode commonly read about +225 mv, the redox potential commonly observed during denitrification (Bohn, 1971). At 14 and 54 cm, lower values (+120 to +200 mv) were obtained. The lower values at 14 cm may be due to the higher rate of decomposition occurring in this portion of the column. This is also substantiated by the amount of denitrification occurring at the 14 cm level which indirectly is a measure of electrons passed to nitrate as a result of  $\text{CH}_3\text{OH}$  decomposition. Accumulation of reduced compounds may occur which would lower the observed  $\text{Eh}_7$  readings. It is likely that the soluble C which reached 54 cm was undergoing further decomposition without the poisoning effect of nitrate.

← FIG. 3

The electrodes responded rapidly to changing conditions. For example, if the columns were refilled completely with fresh aerated effluent, electrodes' readings would raise to +300 mv in a few minutes. As denitrification ensued, the readings decreased to those given in Fig. 3. Also, when flow was continued with effluent containing no nitrate, redox readings fell to near zero. However, they quickly returned when effluent containing nitrate was placed in the columns. Thus, Eh measurements are a good qualitative means of evaluating denitrification systems such as were used here.

### Gas Production

Under steady-state conditions,  $N_2$  consistently constituted almost all of the gas produced (Table 3). The very low  $O_2$  levels are probably the result of sample contamination, while the low  $CO_2$  levels are due to the fact that the  $CaCO_3$  support acts as a  $CO_2$  sink. Trace concentrations of  $CH_4$  were also detected during steady-state conditions. This was unexpected because it is generally considered that  $CH_4$  formation does not occur when nitrate is present (Macgregor and Keeney, 1973; Bell, 1969). Also,  $CH_4$  formation usually occurs at redox readings much lower than those recorded in these columns. A possible reason for the production of  $CH_4$  under these conditions may be the formation of highly anaerobic microenvironments within the porous limestone. Another possible source of  $CH_4$  may be from the energy source ( $CH_3OH$ ) itself. It has been reported that  $CH_4$  can be a metabolite of  $CH_3OH$  oxidation under anaerobic conditions (McCarty, 1964). But if this were the case, much larger quantities of  $CH_4$ , corresponding to the amount of  $CH_3OH$  used, should have occurred. Nitrous oxide ( $N_2O$ ) was detected only occasionally in concentrations of less than  $0.1 \mu M/cc$  gas and not detected at all during that portion of the study designated as steady-state. This was expected since  $N_2O$  is soluble in water (ca. 130 cc/100 ml; CRC Hndbk, 1970-71) and would remain in the liquid phase. At no time prior to or during steady-state conditions was nitric oxide (NO) detected in gas emanating from the methanol columns.

Table 3. Gas concentrations in methanol continuous flow studies (steady-state conditions).

Gas	Mean	Range
$O_2$	0.19*	0.02- 0.30
$N_2$	40.00	26.02-50.50
$CO_2$	0.12	trace- 0.60
$N_2O$	---	---
$CH_4$	0.07	0.02- 0.10

\*

$\mu$  moles/cc gas.

### Conclusions

The results show that a number of energy sources can be used for a denitrification system but that sources such as the easily degradable  $CH_3OH$  yielded the most rapid rates. Paper mill sludges also were a good energy source and studies to evaluate these materials are continuing as an energy source for denitrification.



The continuous flow studies showed that  $\text{CH}_3\text{OH}$  is a suitable energy source for systems where rapid denitrification is desired. Nitrate in mechanically aerated septic tank effluent was removed in less than two hr. However, techniques for gas release and removal of cell accumulation must be designed into the system and a dosing-resting procedure may be needed. The decrease in nitrate versus time is curvilinear resembling Monod more closely than first order kinetics.

These studies have demonstrated some of the practical problems which may occur with a denitrification system. Other variables which remain to be evaluated include the effects of low temperature and shock loading on the efficiency and longevity of the system.

#### References

- Bell, R. G. (1969). Studies on the decomposition of organic matter in flooded soil. *Soil Biol. Biochem.* 1:105-116.
- Bohn, H. L. (1971). Redox potentials. *Soil Sci.* 112:39-44.
- Bouma, J., J. C. Converse, and F. R. Magdoff (1974). Dosing and resting to improve soil adsorption beds. *Trans. ASAE* 2:295-298.
- Bremner, J. M. (1965). Inorganic forms of nitrogen. In C. A. Black et al. (ed.). *Methods of soil analysis, Part 2.* Agronomy 9:1221. Amer. Soc. of Agron., Madison, Wisconsin.
- Bremner, J. M. and D. R. Keeney (1965). Steam distillation methods for determination of ammonium, nitrate and nitrite. *Anal. Chim. Acta.* 32:485-495.
- Casida, L. E., Jr. (1968). *Industrial Microbiology.* John Wiley and Sons. Inc. New York.
- Cotlove, E., V. Trantham, and R. L. Bowman (1958). An instrument for and method for automatic, rapid, accurate and sensitive titration of chloride in biological samples. *J. Lab. Clin. Med.* 50:358-371.
- CRC Handbook of Chemistry and Physics (1971). R. G. Weast (ed.). The Chemical Rubber Co., Cleveland, Ohio, p. B115.
- Dolar, S. G., J. R. Boyle, and D. R. Keeney (1972). Paper mill sludge disposal on soils. Effects on the yield and mineral nutrition of oats (*Avena sativa* L.) *J. Environ. Qual.* 1:405-409.
- Dudley, J. G. and D. A. Stephenson (1973). Nutrient enrichment of ground water from septic tank disposal systems. Inland lake renewal and shoreland management demonstration project report. Upper Great Lakes Regional Commission.
- Environmental Protection Agency (1973). Nitrogenous compounds in the environment. Hazardous Materials Advisory Committee. U.S. Environmental Protection Agency. Washington, D.C.
- Focht, D. D. (1973). Isotope fractionation of  $^{15}\text{N}$  and  $^{14}\text{N}$  in microbiological nitrogen transformations: A theoretical model.
- Focht, D. D. and H. Joseph (1973). An improved method for the enumeration of denitrifying bacteria. *Soil Sci. Soc. Amer. Proc.* 37:698-699.



- Isirimah, N. O., D. R. Keeney, and G. B. Lee (1970). Chemical differentiation of selected Wisconsin histosols. *Soil Sci. Soc. Amer. Proc.* 478-482.
- Macgregor, A. N. and D. R. Keeney (1973). Methane formation by lake sediments during in vitro incubation. *Water Res. Bull.* 9:1153-1158.
- Macgregor, A. N., D. R. Keeney, and K. L. Chen (1973). Nitrogen fixation in lake sediments: Contribution to nitrogen budget of Lake Mendota. *Environ. Lett.*, 4:21-26.
- Magdoff, F. R., D. R. Keeney, J. Bouma, and W. A. Ziebell (1974). Columns representing mound-type disposal systems for septic tank effluent. II. Nutrient transformations and bacterial populations. *J. Env. Qual.* 3:228-234.
- Mann, L. D., D. D. Focht, H. A. Joseph, and L. H. Stolzy (1972). Increased denitrification in soils by additions of sulfur as an energy source. *J. Environ. Qual.* 1:329-332.
- McCarty, P. L. (1964). The methane fermentation. In H. Heukelekian and N. C. Dondero (eds.). *Principles and Application in Aquatic Microbiology*. John Wiley and Sons, New York.
- Monod, J. (1950). La technique de culture continue theorie et applications. *Ann. Inst. Pasteur* 79:390-410.
- National Academy of Science. (1972). Committee on Nitrate Accumulation. *Nat. Acad. Sci.*, Washington, D.C.
- Quispel, A. 1946. Measurement of the oxidation-reduction potentials of normal and inundated soils. *Soil Sci.* 63:265-275.
- Requa, D. A. and E. D. Schroeder (1973). Kinetics of packed-bed denitrification. *J. Water Poll. Control Fed.* 45:1696-1707.
- Smith, S. O. and J. H. Baier (1969). Report on nitrate pollution of ground water, Nassau County, Long Island. Bureau of Water Resources, Nassau County Department of Health, Mineola, New York.
- U.S. Public Health Service Drinking Water Standards. P.H.S. Publ. No. 956 (1962).
- Walker, W. G., J. Bouma, D. R. Keeney, and F. R. Magdoff (1973a). Nitrogen transformations during subsurface disposal of septic tank effluent in sands: I. Soil transformations. *J. Environ. Qual.* 2:475-480.
- Walker, W. G., J. Bouma, D. R. Keeney, and P. G. Olcott (1973b). Nitrogen transformations during subsurface disposal of septic tank effluent in sands: II. Ground water quality. *J. Environ. Qual.* 2:521-525.
- Zobell, C. E. (1943). The effect of solid surfaces upon bacterial activity. *J. Bacteriol.* 46:39-56.

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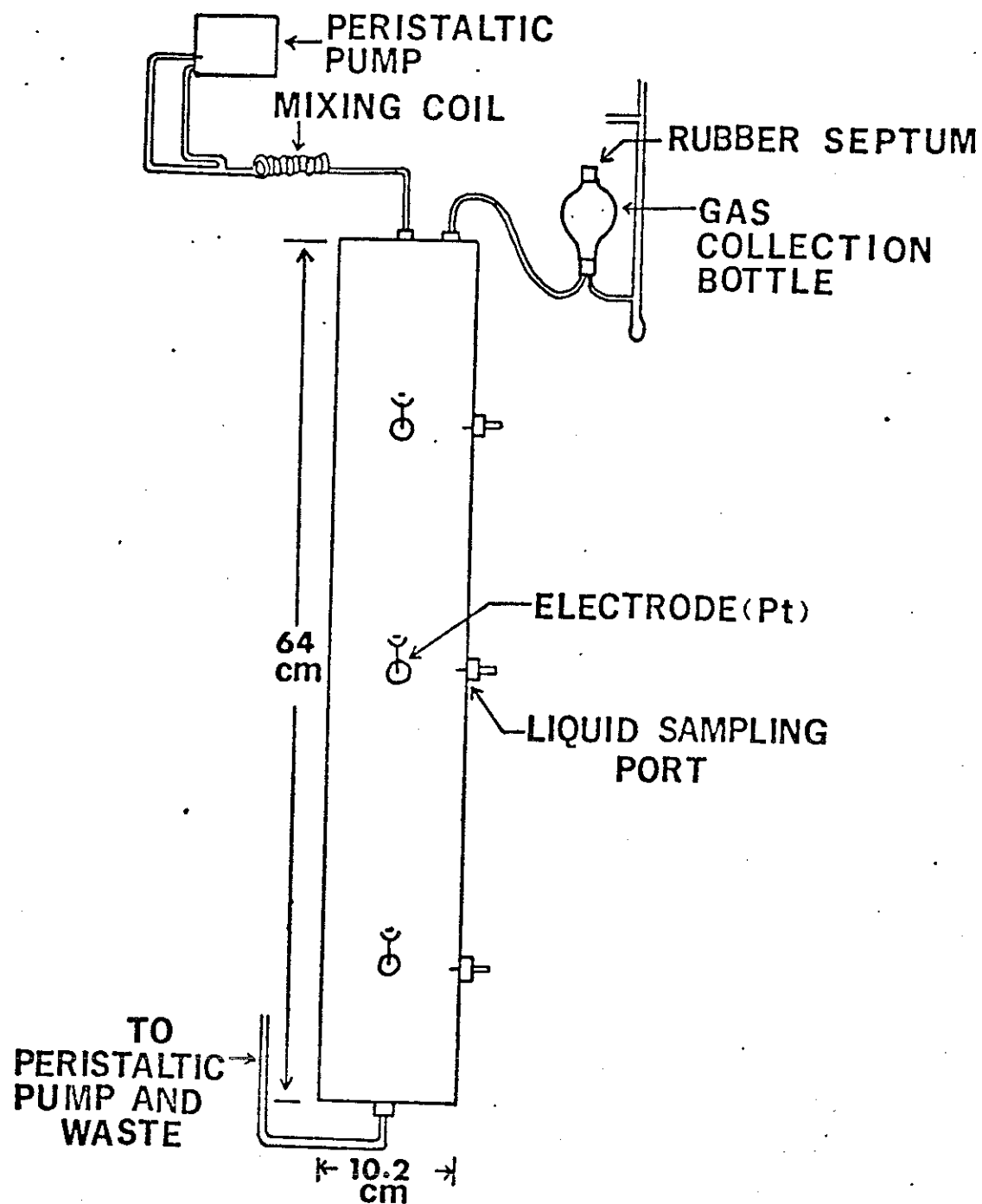


Fig. 1. Diagram of continuous flow column apparatus.

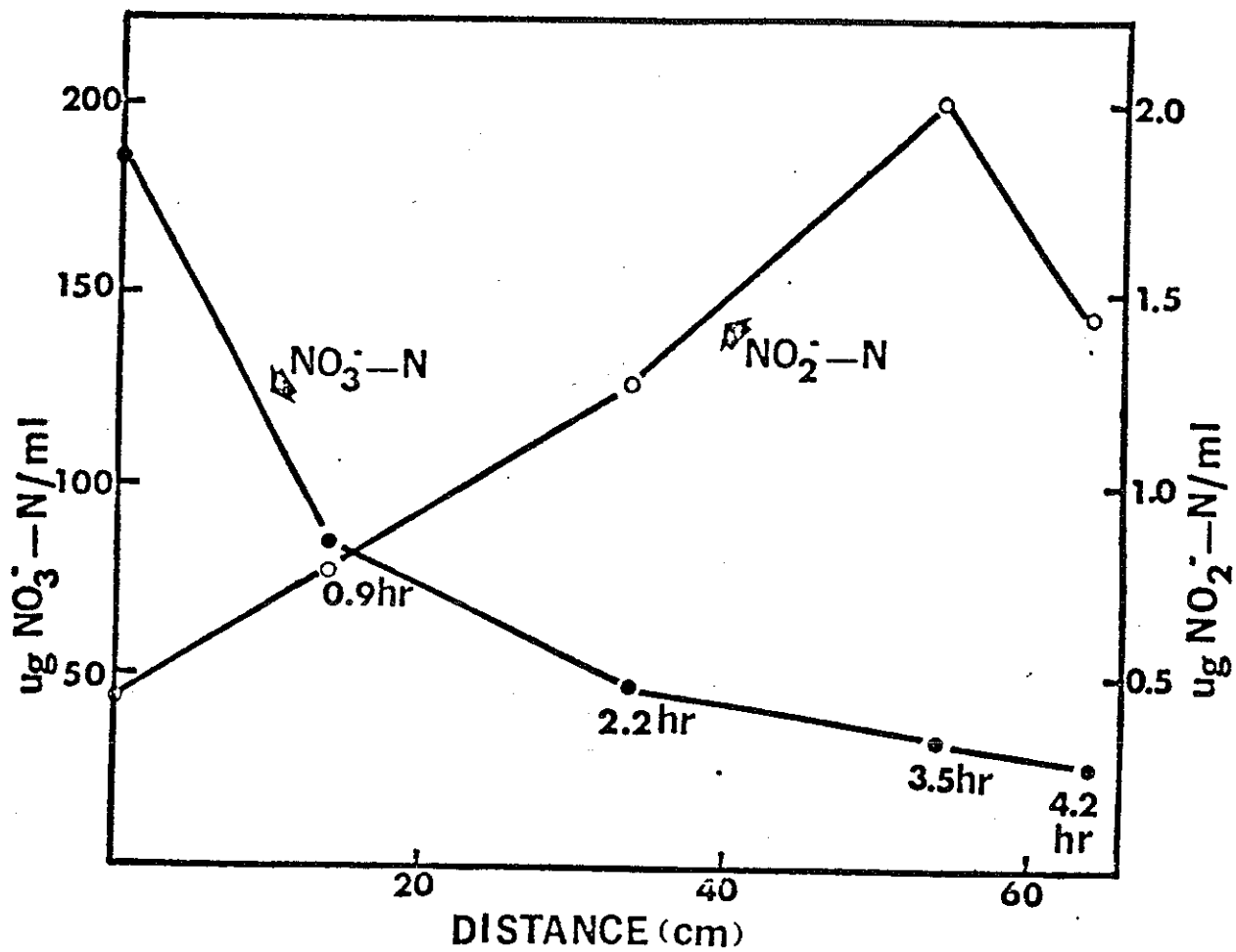


Fig. 2. Inorganic N levels and residence time in continuous flow columns at steady state using methanol as an energy source.

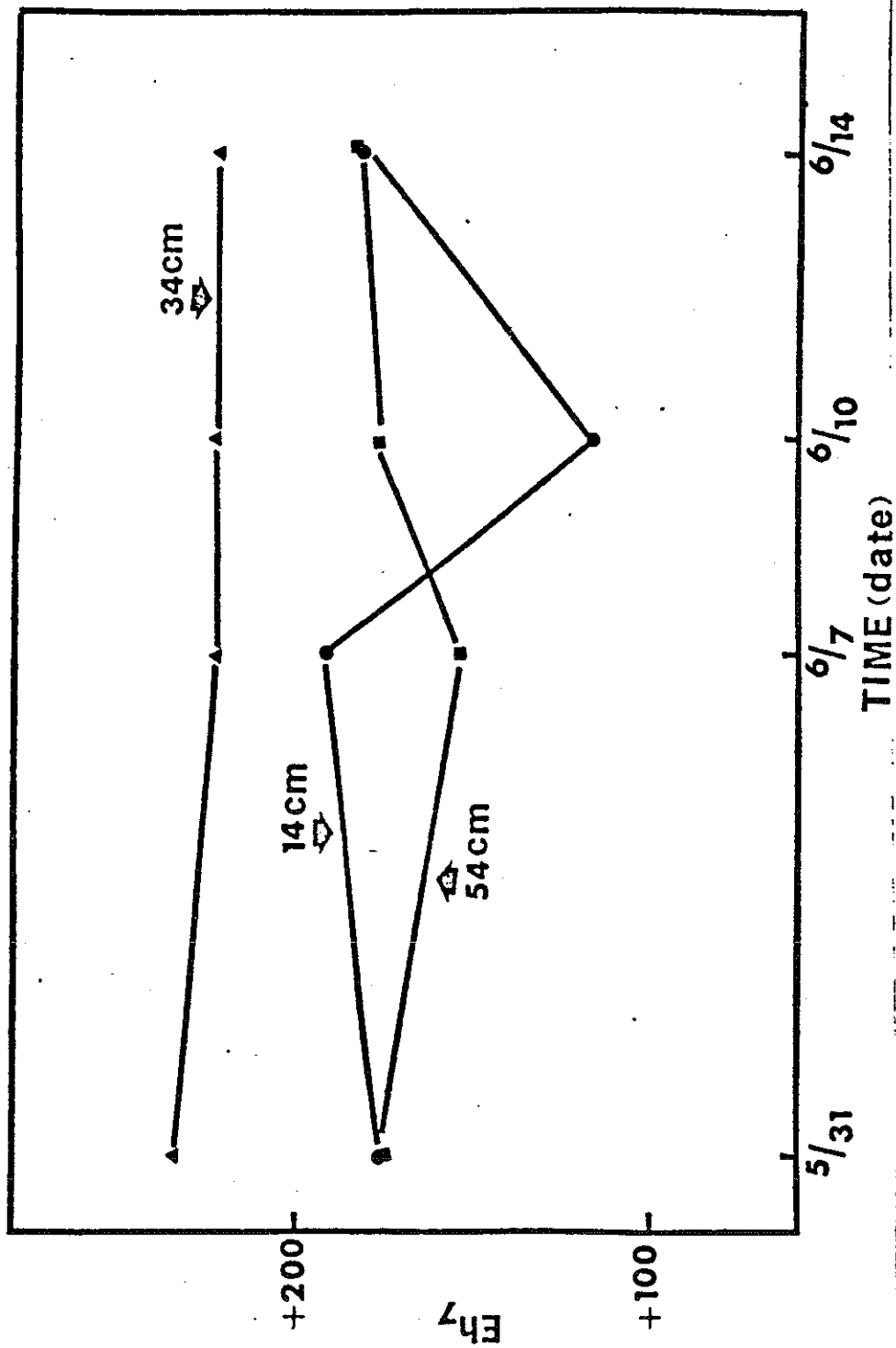


Fig. 3. Oxidation-reduction potentials at three levels in methanol columns during steady-state conditions.

