BIODEGRADATION OF METALWORKING FLUIDS

by

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CHAPTER 1

INTRODUCTION

Straight oils and soluble oil in water emulsions were the primary metalworking fluids in the automotive industry until the late 1960s. Use of these materials creates oil mist in a manufacturing plant and presents a fire hazard, and causes a significant housekeeping burden. These factors and the oil crisis of the early 1970s led to use of semi-synthetic and synthetic metalworking fluids (Sutton et al., 1985). Semi-synthetics are chemically preformed micro-emulsions containing some oils, whereas synthetics, by definition, contain no oil and are true solutions of complex organics in water. Chemical agents contained in these fluids include amines, nitrites, borates, and carboxylates for rust protection; nitrates for stabilization; phosphates and borates for water softening; soaps and wetting agents for lubricity and reduction of surface tension; chlorine and sulfur compounds for chemical extreme-pressure lubrication; glycols, blending agents, humectants, and germicides for control of the bacterial growth (Hunz, 1983).

The main impetus behind the use of synthetics is the substantial productivity and tool life increases that result from their use. The metalworking fluid also has a longer life in the system. These factors alone can represent substantial cost savings. In addition, oil mist is virtually eliminated, thereby reducing, substantially, housekeeping

tasks. In apite of theee benefits, there are serioue concerns about the uee of synthetics. First of all, moat aynthetics are incompatible with existing waste treatment eysteme. Also, the complex organics in the eynthetics may release harmful vapors in the workplace environment directly or by thermal degradation. In addition, aynthetic metalworking fluide may contain toxic and hazardous eubstancee.

In the past, waatewater treatment eystems were designed for the removal of oils from oil-based metalworking fluids. The process generally conaiated of flow equalization, gravity aeperation of free oil, chemical emulsion breek, flocculation, diesolved air flotation, and in eome cases, clarification and filtration. Since the synthetic metalworking fluids contain little or no oil and consist primarily of water soluble organic compounds, their removal from the wastewater etream cannot be accompliahed by the processes used for removing oila. This leada to increaaed levela of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) in the plant wastewater effluent. Biological treatment using adapted culture can be one of the coet-effective alternativea for the treatment of euch a waetewater for compliance with effluent diecharge regulations.

The biodegradation of natural and synthetic organic compounde is important for the design of biological waste treatment proceesee; in many cases biodegradation studies

serve to illustrate the futility of experimental design that fails to recognize the heterogeneity of natural habitats.

The first objective of this research was to study the biodegradation of synthetic metalworking fluids by using open flasks on a shaker. Experiments were carried out with heterogeneous microbial populations of sewage origin and with synthetic metalworking fluids containing synthetic medium as inorganic sources. The system parameters that were measured include the biomass concentration in terms of dry weight, chemical oxygen demand (COD), biochemical oxygen demand (BOD), temperature, pH, dissolved oxygen, chemical elements detectable through elemental analysis, and specific chemical components detectable through high performance liquid chromatography (HPLC).

The second objective of this research was to investigate the adaptation and dilution effects with developed mixed culture. Dry weight and COD were measured parameters. The effect of pH was studied also with fermenters which were provided with temperature control, pH control, and metered aeration. Biomass concentration was measured by the dry weight method. Finally, several biochemical tests were performed to identify three species, Pseudomonas aeruginosa, Proteus mirabilis, and Klebsiella pneumoniae, which were found in the metalworking fluid samples.

CHAPTER 2

BATCH KINETICS AND BIODEGRADATION WITH MIXED POPULATIONS

2.1 BATCH KINETICS

The classical growth curve of microorganisms in batch culture consists of four different phases. They are, namely, a lag phase, an exponential phase, a stationary phase, and an endogenous metabolism phase. The kinetics are related to characteristics of the microbial population and the environmental conditions in the system. Among the important environmental conditions are pH; temperature; dissolved oxygen concentration; types and amounts of carbon source, nitrogen, phosphorous, and other nutrients; presence of inhibitory substances; and changes in all these conditions that may occur during growth of the population.

Each phase is of potential importance in a microbiological process. For example, the general objective of a good process design may be to minimize the duration of the lag phase and to maximize the rate and duration of the exponential phase; the last objective is achieved by retarding the onset of the transition to stationary growth. To achieve such goals, we should understand the variables which influence each phase of batch growth.

The duration of the lag phase observed when a fresh medium is inoculated depends on both the change in the nutrient composition experienced by the cells and the age and size of the inoculum. At the end of the lag phase, the

population of microorganisms is well adjusted to its new environment. The cells can then multiply rapidly, and cell mass, or the number of living cells, doubles regularly with time. Deviations from exponential growth eventually arise when a significant variable, e.g., nutrient level or toxin concentration, achieves a value which can no longer support the maximum growth rate. Eventually, due to nutrient depletion and toxic-product build up, the population cannot sustain itself, and the endogenous phase begins (Bailey, 1977).

Mathematically, the microbial growth rate is often expressed as follows:

$$\frac{dX}{dt} = \mu X \qquad (2.1)$$

where

$\frac{dX}{dt}$ = the growth rate

μ = the specific growth rate

X = the concentration of microorganisms

Upon integration, Eq. (2.1) can be transformed into conventional straight-line form for easy determination of specific growth rate, μ:

$$\mu_i = \frac{\ln X_{i+1} - \ln X_{i-1}}{t_{i+1} - t_{i-1}} \qquad (2.2)$$

If the time required for X to double, t_d, is chosen as the time interval, Eq. (2.2) can be written as follows:

$$\mu = \frac{\ln 2}{t_d} = \frac{0.693}{t_d} \qquad (2.3)$$

μ is calculated by fitting this expression to the experimental data. An exponential growth phase may extend through several doubling times or it may not even last through one. The doubling time of cells in the exponential growth phase is constant.

It is usually assumed that, for a given organism and limiting substrate, the mass of bacterial cells produced per unit mass of nutrient utilized is constant under the same environmental conditions, that is,

$$Y = \frac{\text{mass of organisms formed}}{\text{mass of the limiting substrate utilized}} \qquad (2.4)$$

The factor Y is usually termed the yield constant.

With the synthesis of organisms during the growth process, the substrate is simultaneously consumed. The stoichiometric relationship between the amount of cells produced and substrate utilized is usually expressed as a yield coefficient as shown below:

$$\frac{dX}{dt} = -Y\frac{dS}{dt} \qquad (2.5)$$

where S is the limiting substrate concentration.

Monod (1949) has proposed a quantitative description covering both the exponential and declining growth phases, which is similar to the Michaelis-Menten equation used for describing enzymatic reactions. With this model, the growth rate can be expressed as

$$\frac{dX}{dt} = \frac{\mu_m SX}{K_s + S} \qquad (2.6)$$

Where μ_m is the maximum specific growth rate and K_s is the saturation constant which is numerically equal to the substrate concentration at $\mu = \mu_m/2$.

Combination of Eqs. (2.5) and (2.6) yields the rate expression for substrate consumption.

$$\frac{dS}{dt} = - \frac{\mu_m SX}{Y(K_s+S)} \qquad (2.7)$$

The yield coefficient, Y, varies from case to case. However, as a first approximation, it is usually assumed to be constant for a given biological process treating a specific waste.

In most biologocal waste-water treatment processes, the microorganisms are retained in the reactors for a considerable length of time. In such processes endogenous metabolism or organism decay becomes significant. The decay rate is usually taken to be a function of the organism concentration, even though it is probably a function of the substrate concentration as well. In the present formulation,

the decay rate is approximated by a first order term with respect to the biomass concentration. Under this assumption, Eq. (2.6) is modified to

$$\frac{dX}{dt} = \frac{\mu_m SX}{K_s + S} - k_d X \qquad (2.8)$$

It is important to note that, although the Monod model is basically empirical, it contains some mechanistic implications relative to the adsorption, transport, and enzymatic consumption of substrate. This model is commonly used to show the relationship between the specific growth rate and substrate concentration, especially for pure culture systems. The Monod model was originally developed to describe the behavior of a pure culture. In mixed cultures, the use of models in which differences in organism behavior are considered is sometimes desirable (Chiu et al., 1972).

The lag portion of the classic growth curve has received relatively little attention in the study of biological wastewater treatment processes. It is obvious that the Monod model does not suitably express the lag phase of the growth curve. Therefore, in fitting the data, the lag phase was not considered. Relatively few studies have been made on the endogenous phase of cell cultures, perhaps because many industrial batch microbial processes are terminated before this phase begins.

2.2 BIODEGRADATION

Before man started large scale industrial activities the concentration of organic chemicals on the surface of this planet remained more or less constant with biosynthesis and biodegradation being held in balance by the integrated activities of plants, animals and microbes. Today we are faced with numerous industrial chemicals that do not readily biodegrade. Such chemicals cause problems of disposal and may, if they escape containment, lead to adverse effects on the environment.

To assess the pollution potential of a particular chemical, we have to consider not only the quantity in which it is released into the environment but also its chemical and toxicological properties. A chemical's structure is of primarily importance in determining whether it accumulates or not, while its concentration and its toxicity determine the environmental impact of accumulation.

2.2.1 Xenobiotics as Pollutants

Since mineralization or complete biodegradation of organic chemicals in natural ecosystems is primarily due to microorganisms (Alexander, 1981), any structural feature of a chemical precluding or retarding its attack by microbes will lead to its accumulation in the environment, i.e., to persistence or recalcitrance of the chemical. Recalcitrance of a chemical may be caused by insolubility as in the case

of a synthetic polymers, e.g., polystyrene, polyethylene or polyvinylchloride (Faber, 1979), or it may be due to its novel chemical structure, to which microorganisms have not been exposed in evolutionary history. In the latter case the chemical is a xenobiotic aubatance (Hutzinger, 1981).

The challenge to the evolutionary potential of microbes presented by xenobiotics has fascinated microbiologists and has stimulated basic research on experimental enzyme evolution (Wu, 1978: Clarke, 1980). Studies performed with pure culturea, using known segments of microbial metabolism aa model syatema, have given insight into the genetics and the biochemiatry of enzyme acquisition and thereby have provided guidelines for adaptation experiments with xenobiotics. It has indeed been possible, uaing continuous culture techniques, to select bacteria capable of degrading previously nondegradable xenobiotics. Succeasful adaptationa in utilization of recalcitrant xenobiotics have been performed. Since these adaptations require extended periods of cultivation in continuous culture, they have been performed with mixed cultures under non-aterile conditions. The systems are thus extremely complex to analyze, and the molecular events having led to the novel pathways remain obscure in moat cases.

The strongly selective environment of continuous culture haa proven effective for obtaining bacteria with novel biodegradative capacitiea. However, txe relative contributions to evolutionary process of novel genetic

information generated by mutation and of preexisting genetic information put together by gene transfer are hard to assess.

2.2.2 Microbiological Basis of Biodegradation

The fate of xenobiotics in nature is an area of great importance for assessing the environmental impact of chemicals. The scheme presented in Figure 2.1 gives an overview of the types of reactions a xenobiotic can undergo in the environment. Total absence of degradation of a chemical in nature has not been demonstrated so far; even TCDD (2,3,6,8-tetrachlorodibenzo-p-dioxin), one of the most persistent chemicals known, has been shown to be metabolized at a low rate by microbial cultures (Philippi et al., 1982). Indeed, as pointed out by Alexander (1981), microorganisms are of primary importance for the changes in the structures of xenobiotics introduced into soil or water.

The various types of microbial transformations of xenobiotics listed in Figure 2.1 have been discussed by Bollag (1979). Complete mineralization of a compound is the most desirable of the processes. It generates carbon and energy for microbial growth and leads to the disappearance of the xenobiotic compound. Cometabolism (Horvath, 1972: Alexander, 1979: Grady, 1985) is a process by which microorganisms, in the obligate presence of a growth substrate, transform a non-growth substrate. Although

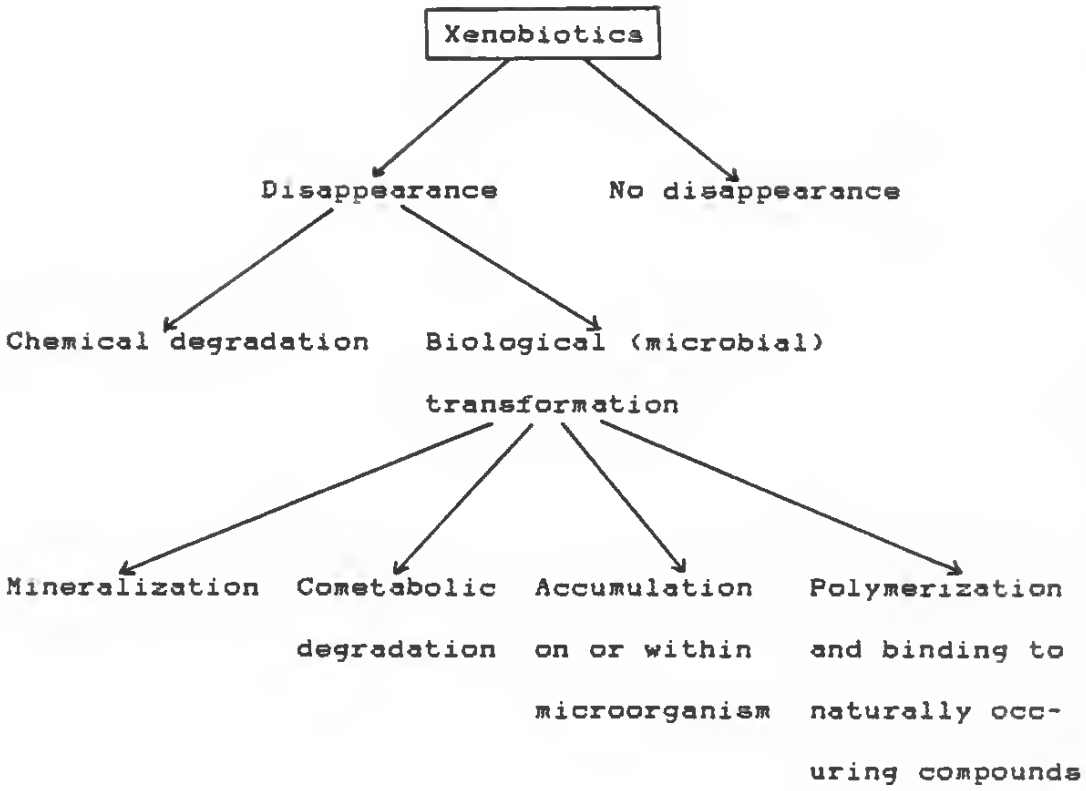

Fig. 2.1. Fate of xenobiotics in environment (Leisinger, 1983).

cometabolizing organisms do not derive benifits from the metaboliam of non-growth substrates, cometabolism is thought to play a significant role in the degradation of xenobiotics in nature. As pointed out by Harder (1981), this assumption is difficult to verify. Cometabolic formations in the environment do not necessarily result in the complete oxidation of xenobiotics but may lead to the accumulation of transformation products with increased or decreased toxicity as compared to the original compound (Alexander, 1981). Cellular accumulation represents another type of interaction of microorganisms with xenobiotics (Grady, 1985). The process that may be the result of active uptake or of adsorption phenomens has usually adverse effects. It may lead to bioconcentration of hazardous chemicals and to their entry into the food chain (Lal, 1982). Microorganisms can favor and microbial enzymes can catalyze the binding of xenobiotics to soil humus. Under some conditions the xenobiotics bound to humic substances are persistent while other conditions lead to their release from soil. The question whether or not humus-bound xenobiotics represent a danger to health, and other questions on the complex behavior of soil-bound residues have to be resolved by further research.

2.2.3 Importance of Microbial Communities

The significance of mixed substrates, mixed cultures and microbial communities has received relatively little

attention both theoretically and experimentally. Almost without exception, natural environments are heterogeneous. These environments supply a diveraity of substrates for microbial growth and a spectrum of conditions at the interfaces between aolid, liquid and gaaeous regions. These environments also supply the characteristic physicochemical conditions of the macrohabitat, a range of gradients of solutes, solvents and gases, and a population of different microorganisms. Even a so-called extreme environment maintains considerable species diversity. For instance, the Dead Sea, with a salinity of at least 10 times that of the oceans, contains more than 10 bacterial species (Brock, 1969). Low grade orea containing copper sulphide and copper oxides in a metal leaching dump usually establish regimes of relatively high temperature or very low pH, aupporting a diverse microbial flora.

Bull (1980) has discussed varioua conceptual and experimental limitations of analyses based on the notions of pure-culture sytems, single-substrate systems, batch (closed) culture systems, purely aerobic systems, constant environment syndrome, and culture and system homogeneity. He has concluded that these limitations are the consequence of the Koch (1881) pure-culture tradition; it is well estabiiahed that the mechanisms and rates of biodegradation of most compounds vary according to the types and characteristics of mixed cultures, multi-substrate systems,

differant growth syatems, open culture systams, environments tending towarda complete anaerobiosia, transiant environmantal systams, and cultura and systam heterogeneity.

Theae problema are important not only in terms of detailed underatandinga of the biochemistry of biodegradation of a particular compound but also the phyaiology and ganetics of microorganisms involved in ita degradation; there is alao a practical connotation since mora pragmatic atudies concerned with tha fate of organic compounds, particularly synthetic and xenobiotic compounds, often fail to appraciate the divarsity and heterogeneity of natural environments (Slater, 1982).

Grady (1985) demonstrated distinct advantages of biodegradation atudiea performed with mixed microbial communities over atudiea performed with pure cultures. First and foramoat, this is because the biodegradation capacity of a community is much greater, both quantitativaly and qualitatively, particularly where xenobiotic components are involved. Furthermore, the resiatance of a community to toxic aubatancea may be much graatar bacauae thare ia a greater likelihood that an organiam that can detoxify them will be present. Finally, minaralization of xenobiotic compounds somatimea requires the combined activity of multiple species.

To provide microbial strains exhibiting improved biodegradation capacitiea is one of tha moat challenging fialds of microbiological resaarch related to pollution

control. Strains with degradative capacities for heretofore persistent compounds have to be enriched from nature or generated in the laboratory by continuous culture techniques as described in the article of Cook et al. (1983). Strains with improved degradation rates or with a widened range of degradative ability may be constructed by in vivo or in vitro genetic manipulation (Chakrabarty, 1982: Ghosel et al., 1985). Such an approach requires extensive knowledge of the biochemistry of the microbial pathway under investigation. Information on the rate limiting steps in the degradative pathways, on the substrate specificities of the relevant enzymes, and on the types of regulatory mechanisms involved in gene expression are prerequisites for a rational approach in strain construction.

For strain constructions to become feasible, biochemical research has to be complemented by studies on the genetics of degradative pathways. The development of genetic techniques for Pseudomonas described by Haas (1983) provides the tools for manipulating genes among members of the one bacterial genus that exhibits the most varied biodegradative capacities. Considering a large amount of information necessary before rational and reproducible experiments in strain construction can be performed, it is not surprising that this approach, with the exception of the examples dicussed by Haas (1983), has not yet been applied on a wide scale in biodegradation research.

Comparad to enrichmant from nature and to strain davelopment in the chemoatat, aasembling dagradative pathwaya from differant bacterial straina in one cell by genetic manipulation is a laborious technique by which to obtain organisms with novel degradative capacities. It will find its application in casea where a precise stratagy can be formulated and where obstacles to dagradation, as for axample the intracellular accumulation of dead-end metabolitea from problem compounds (Knackmuss, 1981), cannot be overcome by cocultivation of strains with diffarant degradative pathwaya.

2.2.4 Biodegradation of Synthetic Metalworking Fluids

Today'a water-baaed metalworking lubricants, referred to also as cutting or grinding fluids, are far different from thosa conaiderad to be the ultimate in performance only 10 years ago. They are complex aolutions or emulsions of agenta deaignad to minimize friction and remove heat; to protect against corroaion; to maintain the aolution stable; to lubricate and reduce aurface tension; to provide extreme-pressure lubrication; to control growth of bacteria; and to protact the anvironment in which operatora work.

Synthetic, or chamical, cutting fluids contain no mineral oil. Inataad, thay rely on water-soluble compounds for lubricity. Semi-aynthatic fluida contain soma minaral oil in addition to chamical additivaa. Tha axact chemical composition of aynthetic and aami-aynthetic metalworking

fluids is held to be proprietary by the manufacturers of these materials. In general, they contain complex glycols, aminea, amides, esters, fatty acida, etc.. Material safety data sheets are often inadequate for assessing the impact on the inplant environment and the waste treatment system. Alao, quality control checka by uaera are a very difficult problem (Sutton et al., 1985).

Moat automotive metalworking planta have an oily wastewater treatment ayatem where four operations are performed, namely, removal of free oil, breaking of chemical emulaion, flotation of oil floc, and clarification. Some planta reproceas and reuse the aeperated oil in some of their operations. Introducing synthetica into an oily wastewater treatment aystem has cauaed aeveral difficultiea. Although breaking of emulaions ia poasible at the very least in some casea, chemical usage goes up tremendously along with increased aludge production. Recovery of oil becomes difficult and the quality of the recovered oil ia not as good. All of theae factors imply higher operating coats. Unless additional treatment facilities are provided, the complex aoluble organica go largely untreated and cauae higher BOD and COD loadings in the effluent leading to possibly higher surcharge coats. In some cases, the effluent can become unacceptable to the municipal treatment system.

Numerous researchers investigated biodegradation of metalworking fluids and identification of a defined

microbial inoculum in metalworking fluid. Rossmoore (1980) found that three bacterial species Pseudomonas aeruginosa, Proteus mirabilis and Klebsiella pneumoniae were dominant in more than 100 field samples of water-based metalworking fluids. Those species survived well together in 12 of 15 fluids marketed; those were recommended as candidate inocula for evaluating biocides. Even though the point of view differs from that of an environmental engineer, the results are very helpful, e.g. providing microbial strains which have degradative capacities.

Davis (1979) studied methods of decomposing waste metalworking fluids into carbon dioxide, water, and biomass using an activated-sludge biological process. After 478 days of operation, he recommended some operational guidelines about substrate concentration, nitrate and phosphate concentrations, liquid residence time in the reactor, DO level, maintenance of pH, temperature, etc.. Young (1981) demonstrated that waste metalworking fluids used in metal-cutting operations could be biologically degraded after operation of a pilot aerobic digester for 2 years. Sutton et al.(1985) applied fluidized-bed technology for biological treatment of metalworking fluids. In their pilot plant study, single and two-stage Oxitron fluidized bed reactors were operated to determine information for process design of a system to achieve carbonaceous BOD removal and nitrification of an industrial wastewater from machining operations in an automotive parts manufacturing plant.

CHAPTER 3

MATERIAL AND METHODS

3.1 BIODEGRADATION

Thirteen synthetic metalworking fluids from five different manufacturers were selected as the growth-limiting subatrates. Each substrate was fed to the growth flasks at a concentration of 1% (v/v). Experiments were started at substrate concentrations ranging from 5990 to 29360 mg/liter of COD (Table 3.1). Sample numbers listed in Table 3.1 will be used in this thesis without manufacturer's or product names.

3.1.1 Experimental Procedure

For all experiments, BOD dilution water (APHA, 1985), as shown in Table 3.2, was used as the inorganic nutrient. Components of Manhattan tap water are liated in Table 3.3. Seed microorganiams were obtained from the settled influent of the municipal wastewater treatment plant at Manhattan, Kanaas. The aeed was stored at 20 °C for 24 to 36 hrs prior to inoculation. In the biodegradation experiments, 6 ml of the raw sewage waa placed in each of the growth flaska (500 ml, Erlenmeyer), and the volume waa made up to 300 ml with growth medium containing varioua concentrations of the carbon aource.

Aeration and mixing in the flaska were accomplished by ahaking the flaaka at a rate of 175 rpm, leaving open

Table 3.1. Initial Substrate Concentration of Samples

Sample No.	Manufacturer's Name	Product Name	Color*	COD (mg/L)
1	Cincinnati Milacon**[a]	CM A	colorless	8940
2	"	CM B	colorless	5990
3	"	CM C	white	29360
4	2V Industries**[b]	Mach-3 Synthetic	colorless	8900
5	E.F. Houghton**[c]	Product A	blue	13450
6	"	Product B	green	11580
7	"	Product C	yellow	7130
8	"	Product D	yellow	15150
9	Int.Refining&Mfg.**[d]	IRMCO 141	colorless	9150
10	"	IRMCO 156	colorless	8670
11	Van Straaten Chem.**[e]	826	green	9920
12	"	902	blue	8210
13	"	930	blue	9150

Note:

* After 1 % (v/v) dilution and pH adjustment

** Address of the manufacturers

a. 4701 Marburg Ave., Cincinnati, OH 45209

b. 48553 West Road, Wixon, MI 48096

c. Unknown

d. 2117 Greenleaf St., Evanston, IL 60202

e. 630 W. Washington Blvd., Chicago, IL 60606

Table 3.2 Composition of Growth Medium

Costituent	Concentration (per L)
Carbon Source (Metalworking fluid)	10 ml
Magnesium Sulfate, $MgSO_4.7H_2O$	22.5 mg
Calcium Chloride, $CaCl_2$	27.5 mg
Ferric Chloride, $FeCl_3.6H_2O$	0.25 mg
0.025N Sodium Sulfate, Na_2SO_3	1.575 mg
Phosphate Buffer, pH 7.2	1 ml
KH_2PO_4	8.5 mg
K_2HPO_4	21.75 mg
$Na_2HPO_4.7H_2O$	33.4 mg
NH_4Cl	1.7 mg
Tap Water *	100 ml
Deionized Water	to volume

Note:

* The components of Manhattan tap water are listed in Table 3.3.

Table 3.3. Components of Manhattan Tap Water

Constituent	Concentration (per L)
Ca	28 mg
Mg	10 mg
Fe	0.23 mg
Mn	0.01 mg
Na	40 mg
Cl	47 mg
SO_4	79 mg
N	0.15 mg
F	1.1 mg
Total Hardness	111
Total Alkalinity	63
Total Phosphate	0.19 mg

Note:

The source of these data is Water Plant at Manhattan, KS.

mouth. The G24 Environmental Incubator Shaker and Lab-Line Orbit Environ-Shaker, manufactured by New Brunswick Scientific and Lab-Line Instruments, were the shakers used in the basic experiments.

The growth of microorganisms along the side walls of the flasks caused difficulty in obtaining the exact values of biomass concentrations; it was found that this could affect the extent of reaction. To minimize the effect, the wall of the flask was cleaned as often as possible every sampling day.

Since the flasks were open during the operation, evaporation was large enough to cause error in measuring the substrate concentration. Deionized water was added in an amount equal to that evaporated on every sampling day.

Each experiment was carried out for 40 to 55 days so that the final constant substrate concentration could be accurately estimated. Dry weight, COD, temperature, pH, and DO were analyzed every sampling day. Each sample was analyzed twice with HPLC, once before biodegradation and once after biodegradation. Biodegradation experiments continued until substrate concentration would be constant.

In addition, BOD was measured at the 5th day and the 20th day after incubation (APHA, 1985). Nitrifying bacteria usually are not considered a problem in 5 day BOD determination. Because nitrification usually occurs between the fifth and seventh days of incubation, it causes only a

minor error in the normal 5 day BOD. However, an abnormally high uptake of oxygen is evidence that nitrifying bacteria are adding appreciably to the oxygen demand. This problem could be alleviated by chemically inhibiting nitrification with the Hach Nitrification Inhibitor. Approximately 0.16 g of this inhibitor was added directly to the wastewater sample by dispending the powder into the empty 300 ml BOD bottle before the sample is poured in.

Temperature was maintained at 24 ± 1 °C through room temperature control. The range of pH was between 7 and 8 for most of the experiments. DO was maintained above 5 mg/liter.

3.1.2 Analytical Procedure

Samples were analyzed for cell concentration in terms of dry weight, organic concentration in terms of chemical oxygen demand (COD), biochemical oxygen demand (BOD), chemical elements detectable through elemental analysis, and specific chemical components detectable through high performance liquid chromatography (HPLC). Temperature, pH, and dissolved oxygen concentration (DO) were also measured.

Determination of biomass concentration was accomplished by the dry weight method. Biomass measurements were made directly by weighing the suspended solids retained upon filtration of samples through 0.45 μm pore size Millipore HA membrane filters with the Millipore 1225 Sampling Manifold. Each filter was placed in an air oven to dry for 24 hrs at 105 °C and then weighed (APHA,

1985). Assessment of biological solids in several colorless samples was made by taking optical density readings of samples at a wavelength of 540 nm (Gaudy, 1971: Peil, 1971) in a Spectronic 20 spectrophotometer (Bausch and Lomb). Nevertheless, it is known that the optical density provides a reliable estimate of biological solids only for these cases for which the biomass concentration in terms of the dry weight, X, is no greater than 300 mg/liter and where cells are not flocculated. (Gaudy, 1980). In the present work, therefore, when the biomass concentration in terms of dry weight was greater than 300 mg/liter, the optical density was ignored. The dry weight and photometric methods were recorrelated frequently because of changes in cell populations and flocculation conditions. Linear regression analyses were performed and correlation coefficients were calculated (Draper, 1981).

Determination of the existence and extent of an exponential phase as well as the value of its numerical descripter μ are directly related to the quantity and quality of the growth data obtained. The estimation was carried out by choosing the first exponential growth phase. A systematic selection of the experimental data was made to eliminate the effect of the lag phase on the parameter estimation. The values of the specific growth rate, μ, and the biomass yield, Y, were obtained from the batch data by using Eqs. (2.2) and (2.4), respectively.

The filtrate collected from dry weight analysis was used for COD determination which was carried out by EPA approved Hach system using premixed reagents in ready to use, screw capped vials and compact reator heater. After digestion results were measured photometically right in the vial with Bausch & Lomb Spectronic 20 spectrophotometer. BOD was measured with the dilution method (APHA, 1985). The pH was monitored with a model 7 Corning pH meter. DO was determined by a YSI model 51A oxygen meter with a model 5739 oxygen probe (Yellow Springs Instrument).

The HPLC procedure (Environmental Research Group, 1983) includes two columns, TSK 1000 PW and TSK 2000 PW, connected in series, in a Varian Model 5000 liquid chromatograph equipped with a Varian VARI-CHRM UV-Vis detector (at 283 nm), a Varian Model 9176 recorder, and a ISIS Auto Sampler manufactured by ISCO. Liquid chromatography sorts mixture according to size, with larger molecules moving more rapidly than smaller ones and eluting sooner. The method not only resolves a mixture but also gives information about the size of the components of mixture. All samples were filtered through 0.45 μm pore size Millipore HA membrane filters before injection. All parameters are listed in Table 3.4.

Table 3.4. HPLC Parameters

Columns	:	TSK 1000 PW, TSK 2000 PW
		7.5 mm * 30 cm (Varian)
Guard Column	:	TSK GPWPC 75 mm * 7.5 mm (Varian)
Detector	:	Ultraviolet (Varian VARI-CHRM)
Solvent	:	HPLC grade water (Fisher Scientific Co.)
Flow Rate	:	1.0 ml/min
Injection size	:	20 µl
% B	:	0
Column Pressure	:	34 atm
Column Temp.	:	30 °C
Chart Speed	:	1.0 cm/min

Note:

The injector and detector were at the ambient temperature (23-25 °C).

All samples were diluted in deionized water to 1% (v/v).

3.2 ADAPTATION AND DILUTION EFFECTS

One (Sample No. 4 in Table 3.1) of the thirteen synthetic metalworking fluids was selected as the growth-limiting substrate, and fed to the growth flasks at the concentration of 1 %, 0.8 %, 0.5 %, and 0.2 % (v/v). Experiment was started at the different substrate concentration ranges 8900, 7120, 4450, and 1780 mg/liter.

For this experiment, BOD dilution water, as shown in Table 3.2, was used as the inorganic nutrient. At the end of the biodegradation experiment (55th day), a portion of the mixed culture was harvested and employed as the inoculum. In this experiment, 0.3 ml of the cell suspension (0.82 mg/liter) was placed in each of the growth flasks (500 ml, Erlenmeyer), and the volume was made up to 300 ml with growth medium. Aeration and mixing in the flasks were accomplished by shaking the flasks at a rate of 175 rpm, leaving open mouth.

The growth of microorganisms along the side walls of the shakers caused difficulty in obtaining the exact values of biomass concentrations; it was found that this could affect the extent of reaction. To minimize the effect, the shaker wall was cleaned as often as possible every sampling day. Since the flasks were open during the operation, evaporation was large enough to cause error in measuring the substrate concentration. Deionized water was added in an amount equal to that evaporated on every sampling day.

Growth was measured as optical density at 540 nm by using Bausch & Lomb Spectronic 20 spectrophotometer. COD determination for the substrate concentration was carried out by EPA approved Hach system using premixed reagents in ready to use, screw capped viala and compact reactor heater. After digestion results were measured photometrically right in the vial with Bauach & Lomb Spectronic 20 apectrophotometer.

3.3 PH EFFECT

One (sample No. 2 in Table 3.1) of the thirteen synthetic metalworking fluids waa selected as the growth-limiting substrate, and fed to the fermentors at the concentration of 1 % (v/v). For this experiment, BOD dilution water, as shown in Table 3.2, waa used as the inorganic nutrient. Seed microorganisms were obtained from the settled influent of the municipal wastewater treatment plant at Manhattan, Kansas. In this experiment 14 ml of the raw sewage was placed in three of the fermentors, and the volume was made up to 700 ml with growth medium.

The basic experimental unit was the LH fermentor 500 series, manufactured by LH fermentation. Thia unit provided a reactor with a working volume of 1 liter, a speed adjustable baffle which has two impellers, temperature control, pH control, and metered aeration. The temperature of the reactor was maintained constant by means of water circulation from the cold water tap through the heat

exchanger. The pH waa controlled by pumping 0.1 N sulfuric acid for acid and 0.1 N sodium hydroxide for alkali to the culture veaael uaing peristalitic pumps.

Agitation and mixing in the reactors were accomplished by the combination of a mechanical atirrer and aeration. Throughout the present atudy, the atirrer speed was maintained at a rate of 700 rpm, and the air flow rate to the reactor was at least at 1.0 vvm. The temperature of the circulation was controlled at 24 ± 0.5 °C. The pH was controlled at 6.0, 7.5, 8.0, and 9.0 in the four reactors, respectively. Growth was measured aa optical denaity at 540 nm by uaing Bauach & Lomb Spectronic 20 apectrophotometer.

3.4 IDENTIFICATION OF THREE SPECIES

Sample No. 3 (Table 3.1) was aelected aa the growth limiting aubatrate, and fed to a ahaker at the concentration of 1 % (v/v). BOD dilution water was used for the inorganic nutrient. Seed microorganiams were obtained from the settled influent of the municipal wastewater treatment plant at Manhattan, Kanaas. Six ml of raw sewage was placed in the growth flask (500 ml, Erlenmeyer), and the volume was made up to 300 ml with growth medium. Aeration and mixing in the flaak were accomplished by ahaking the flask at a rate of 175 rpm, leaving open mouth.

After 7 days, when it was in exponential phaae, the most turbid portion waa chosen and atreaked on five

different agars, Pseudomonas F agar, Desoxycholate agar, MacConkey agar, Endo agar, and Eosin Methylene Blue (EMB) agar (Skinner, 1980: Collins, 1984). After 24 hrs, a representative colony was picked and streaked again on nutrient agar plates to obtain pure cultures, and incubated at 37 °C for 24 hrs. After 24 hrs again, each colony was observed under oil immersion objective and inoculated into nutrient broth for biochemical tests.

KIA, Glucose, Lactose, Mannitol, Urea, Indol, MR-VP, and Citrate Tests were used to identify three microorganisms (Breed et al., 1957: Urban, 1977). A loopful of each nutrient broth was inoculated to each biochemical test media and incubated at 37 °C for 24 hrs.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 BIODEGRADATION

Thirteen sets of batch experimental data are tabulated in Appendix I. All experiments were carried out at a temperature range between 23 and 25 °C. Ranges of pH and DO were recorded for each experiment. All batch runs lasted at least 40 days or more.

The results of BOD measurements are listed in Table 4.1. Some difficulties were encountered in completing the BOD test. The first difficulty stemmed from the inhomogeniety of seed; the observed oxygen depletions in the blank fluctuated widely. The second difficulty arose from the fact that comparision of the BOD values were exceedingly low in relation to the corresponding COD values in numerous samples. At least 3 replicates were required to obtain reliable data. The fact that, generally, 20 day BOD is much higher than 5 day BOD shows most of the samples are slow to be biodegraded.

COD removal efficiency data in Table 4.2 are more consistent with BOD_{20}/COD than BOD_5/COD in Table 4.1. Table 4.2 indicates that samples Nos. 8, 9, 10, 12, and 13 were not readily biodegraded. It appears that this result does not correlate with the values of the specific growth rate, μ, and the biomass yield, Y, in Table 4.3. Table 4.3 lists the kinetic and yield values estimated from the batch data

Table 4.1. BOD Data of Samples

Sample No.	BOD_5 (mg/L)	BOD_{20} (mg/L)	COD (mg/L)	BOD_5/COD (-)	BOD_{20}/COD (-)
1	660	3180	8940	0.07	0.36
2	1020	2030	5990	0.17	0.34
3	6840	12300	29360	0.23	0.42
4	590	2160	8900	0.07	0.24
5	660	3480	13450	0.05	0.26
6	1200	3450	11580	0.10	0.30
7	420	1920	7130	0.06	0.27
8	840	2820	15150	0.06	0.19
9	660	2030	9150	0.07	0.22
10	120	840	8670	0.01	0.10
11	1500	3310	9920	0.15	0.33
12	720	1080	8210	0.09	0.13
13	1140	1620	9150	0.12	0.18

Table 4.2. COD Reduction of Samples

Sample No.	Initial COD(mg/L)	Final COD(mg/L)	Sampling Time(days)	Reduction (%)
1	8940	2770	40	69
2	5990	1770	40	70
3	29360	3030	40	90
4	8900	3170	55	64
5	13450	3030	40	77
6	11580	4790	55	59
7	7130	1770	55	75
8	15150	11600	40	23
9	9150	5450	50	40
10	8670	6270	40	28
11	9920	2130	40	79
12	8210	6600	40	20
13	9150	7790	40	15

Table 4.3. Kinetic and Yield Data of Samples

Sample No.*	Product Name	Specific Growth Rate (1/hr)	Biomass Yield (g cells/g subs.)
1	CM A	0.065	0.33
2	CM B	0.043	0.29
3	CM C	0.083	0.16
4	MACH-3 SYNTHETIC	0.026	0.40
5	PRODUCT A	0.040	0.21
6	PRODUCT B	0.022	0.17
7	PRODUCT C	0.046	0.36
8	PRODUCT D	0.072	0.47
9	IRMCO 141	0.039	0.20
10	IRMCO 156	0.021	0.49
11	826	0.073	0.26
12	902	0.009	0.20
13	930	0.008	0.18

Note:

* Numbers and product names are the same as in Table 3.1.

given in Appendix I. The method for calculation of the specific growth rate has been described in detail in section 3.1. The number of experimental data obtained in the present work was insufficient. Some more data points were needed during the first exponential phase for calculating the valid estimate of the specific growth rate, μ.

Table 4.4 lists the chemical elemental compositions of the samples. The carbon concentrations are consistent with the initial substrate COD concentrations listed in Table 3.1. The nitrogen concentrations do not correlate well with the values of COD reduction in Table 4.2. Table 4.5 lists analysis of oxidation state and nitrogen availability of the samples. The fourth column in this table is obtained by dividing the second column with the third column. The fifth column is obtained by dividing initial nitrogen listed in Table 4.4 with biomass produced; its values have been calculated as

Biomass produced=(final COD-initial COD)(biomass yield)

The necessary data for this calculation are given in Tables 4.2 and 4.3. Note that the ratios of nitrogen present to biomass produced exceed 0.10 for all samples except samples Nos. 3 and 5. The results summrized in Tables 4.4 and 4.5 indicate that nitrogen depletion is not the primary reason why COD reduction ceased before reduction of the initial substrate concentration reached 50 percent in several

Table 4.4. Elemental Compositions of Samples

Sample No.	N (mg/L)	C (mg/L)	H (mg/L)
1	359	2080	1076
2	436	1435	1065
3	226	6995	1151
4	234	2070	1073
5	186	3220	1113
6	314	2752	1060
7	262	2272	1076
8	282	3607	1025
9	300	2200	1085
10	130	2149	1032
11	353	2326	1052
12	334	1950	1101
13	355	2118	1047

Note:

Concentrations of elements were determined by assuming specific gravities of samples = 1.0.

All samples were diluted in deionized water to 1 % (v/v).

Table 4.5. Analysis of Oxidation State and Nitrogen Avalability of Samples

Sample No.	Initial COD (equiv./L)	Initial C (moles/L)	Equiv. COD per Mole C	Ratio of N to Biomass Produced
1	1118	173	6.46	0.176
2	749	120	6.24	0.646
3	3670	583	6.30	0.054
4	1112	173	6.43	0.124
5	1681	268	6.27	0.085
6	1148	229	5.01	0.272
7	891	189	4.71	0.122
8	1894	301	6.29	0.169
9	1144	183	6.25	0.405
10	1084	179	6.06	0.111
11	1240	194	6.39	0.174
12	1026	163	6.29	1.040
13	1144	176	6.50	1.450

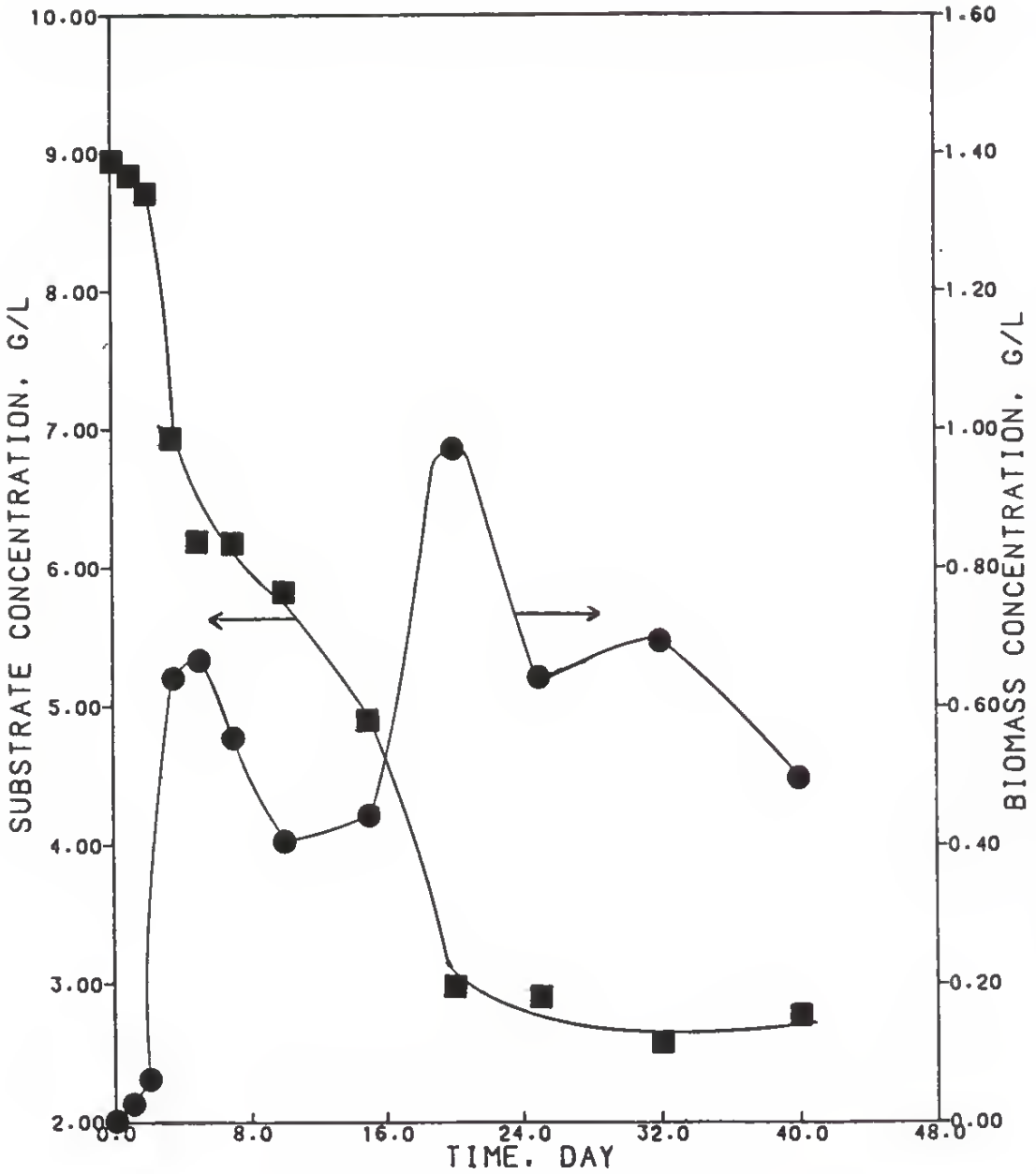

Fig. 4.1. Growth and substrate removal for mixed population growing on sample No. 1.

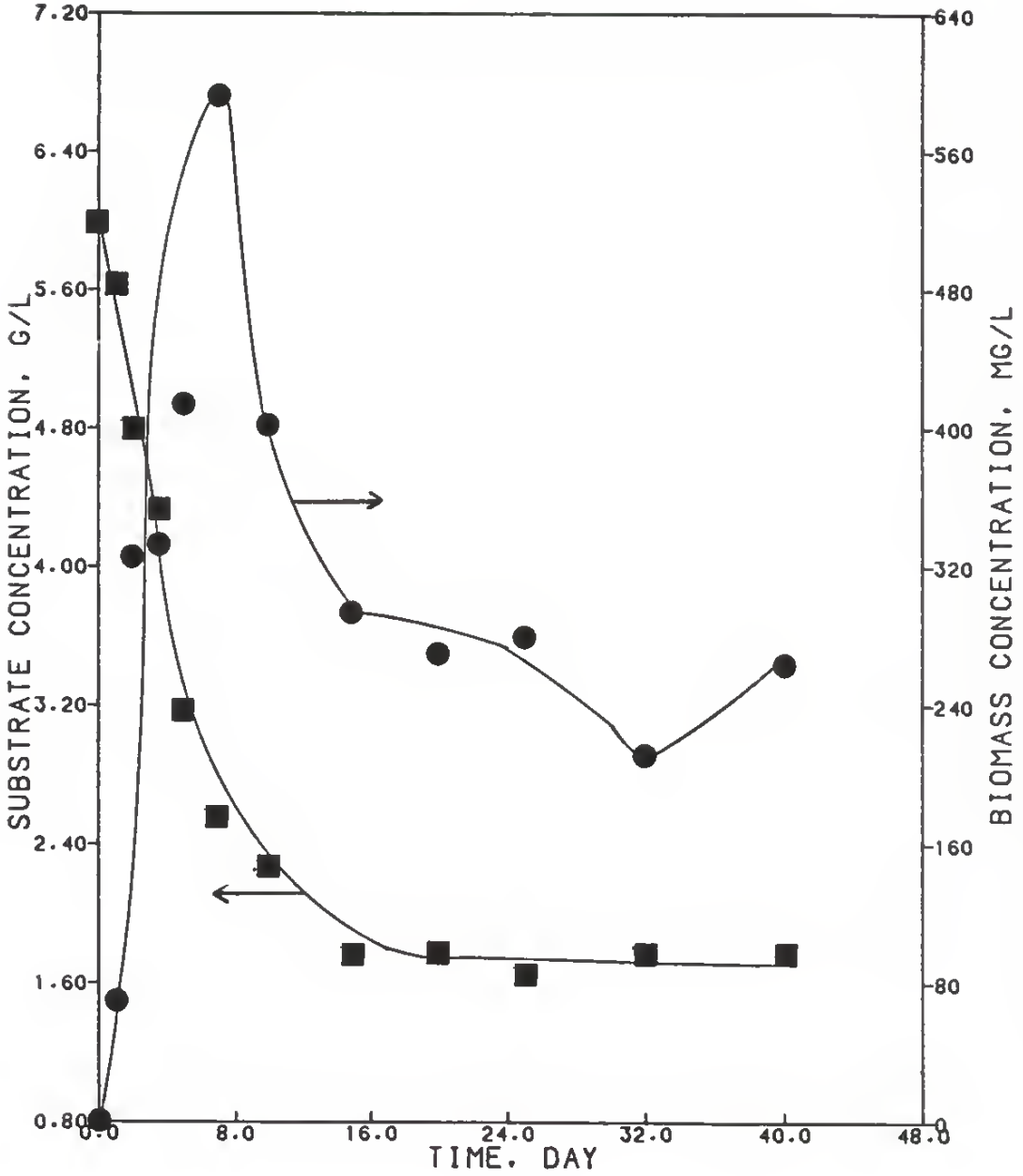

Fig. 4.2. Growth and substrate removal for mixed population growing on sample No. 2.

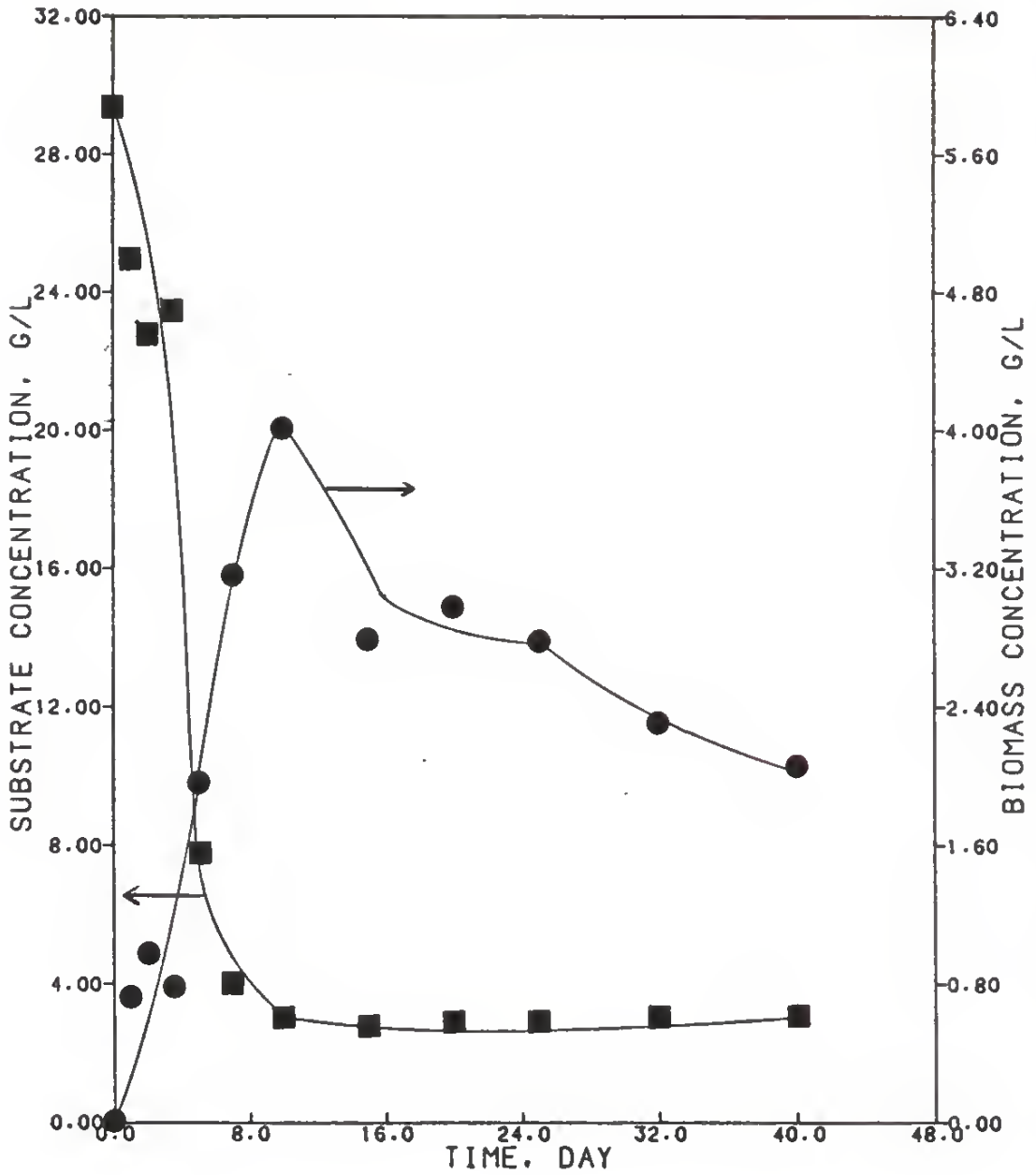

Fig. 4.3. Growth and substrate removal for mixed population growing on sample No. 3.

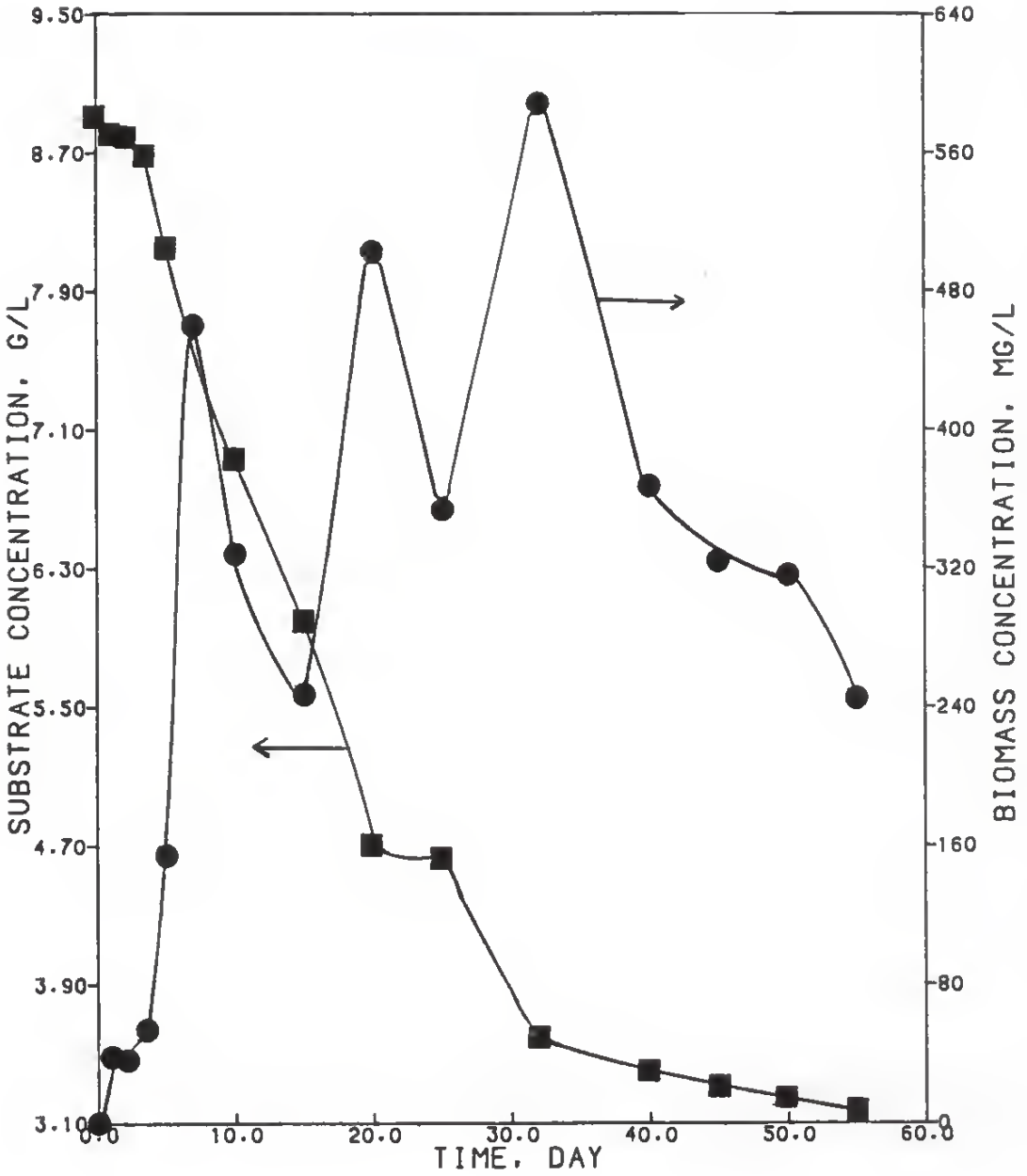

Fig. 4.4. Growth and substrate removal for mixed population growing on sample No. 4.

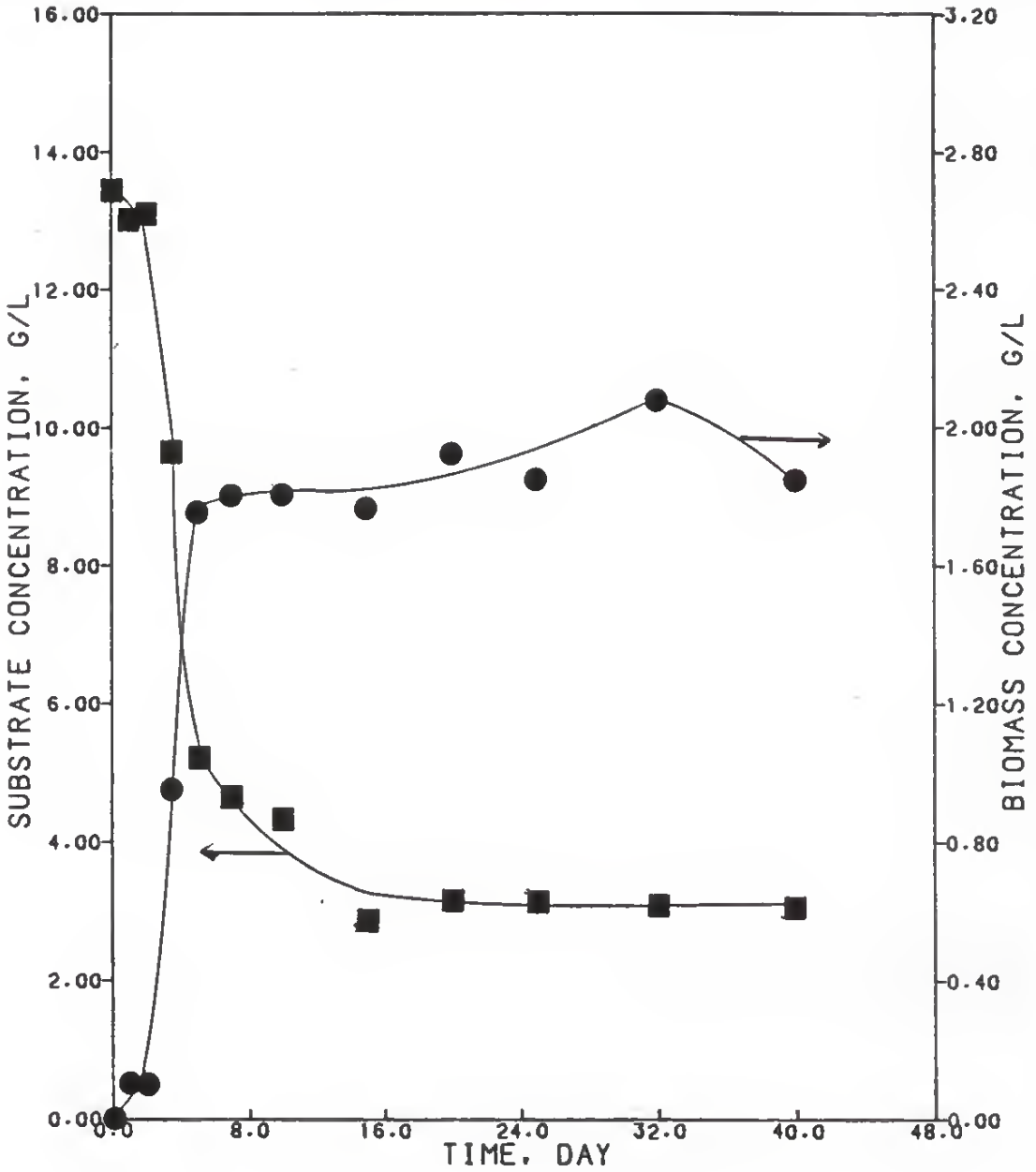

Fig. 4.5. Growth and substrate removal for mixed population growing on sample No. 5.

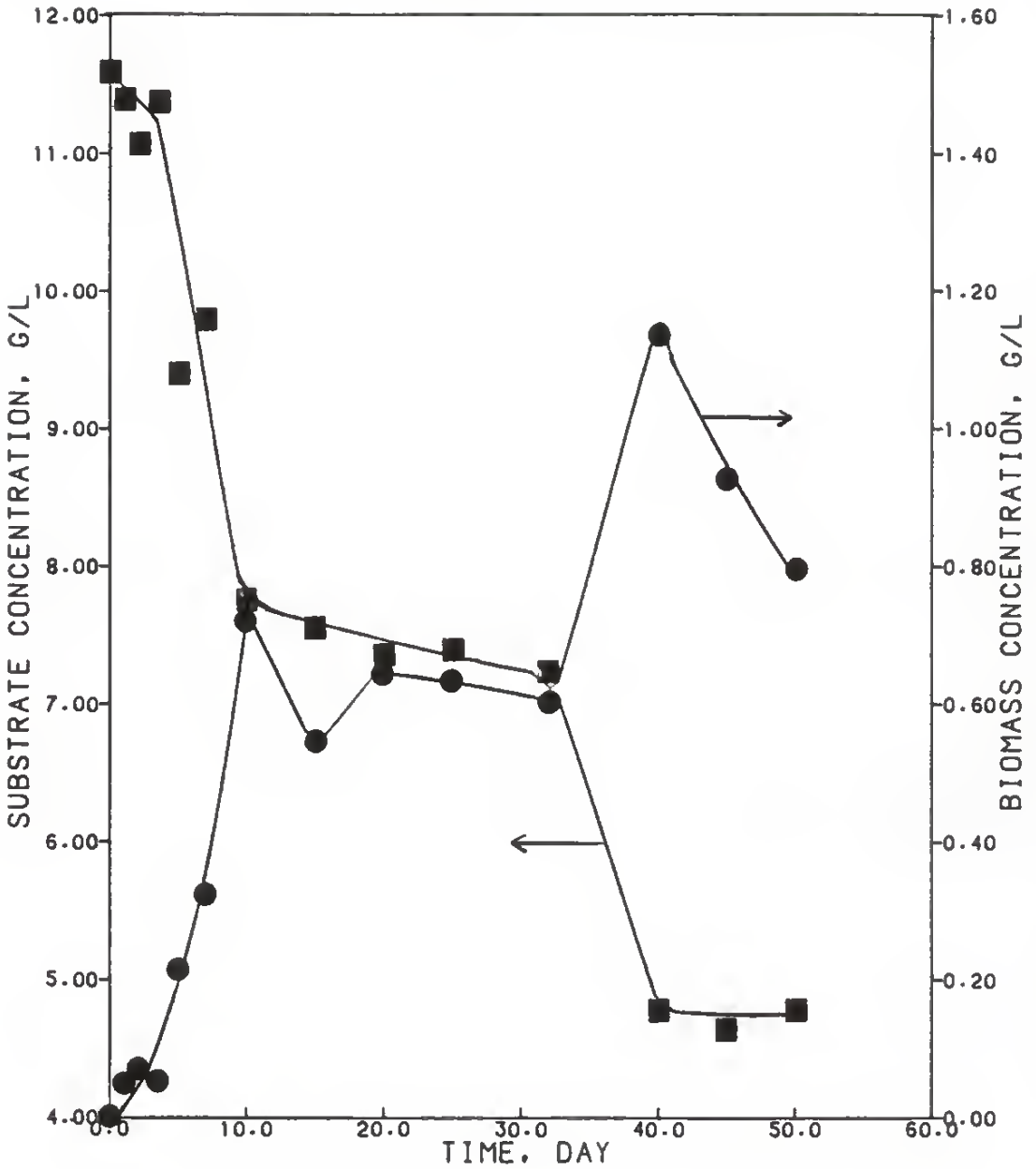

Fig. 4.6, Growth and substrate removal for mixed population growing on sample No. 6.

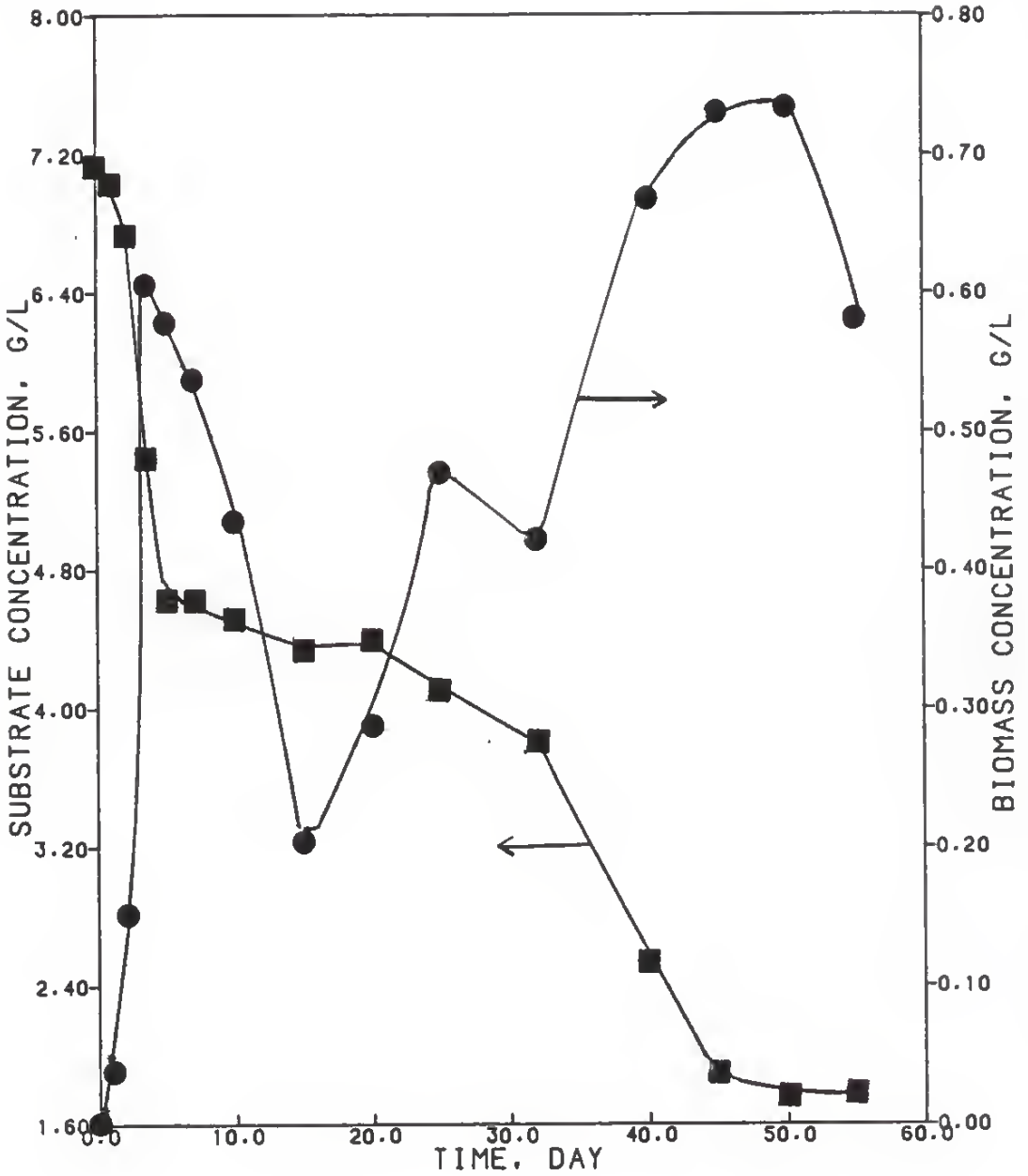

Fig. 4.7. Growth and substrate removal for mixed population growing on sample No. 7.

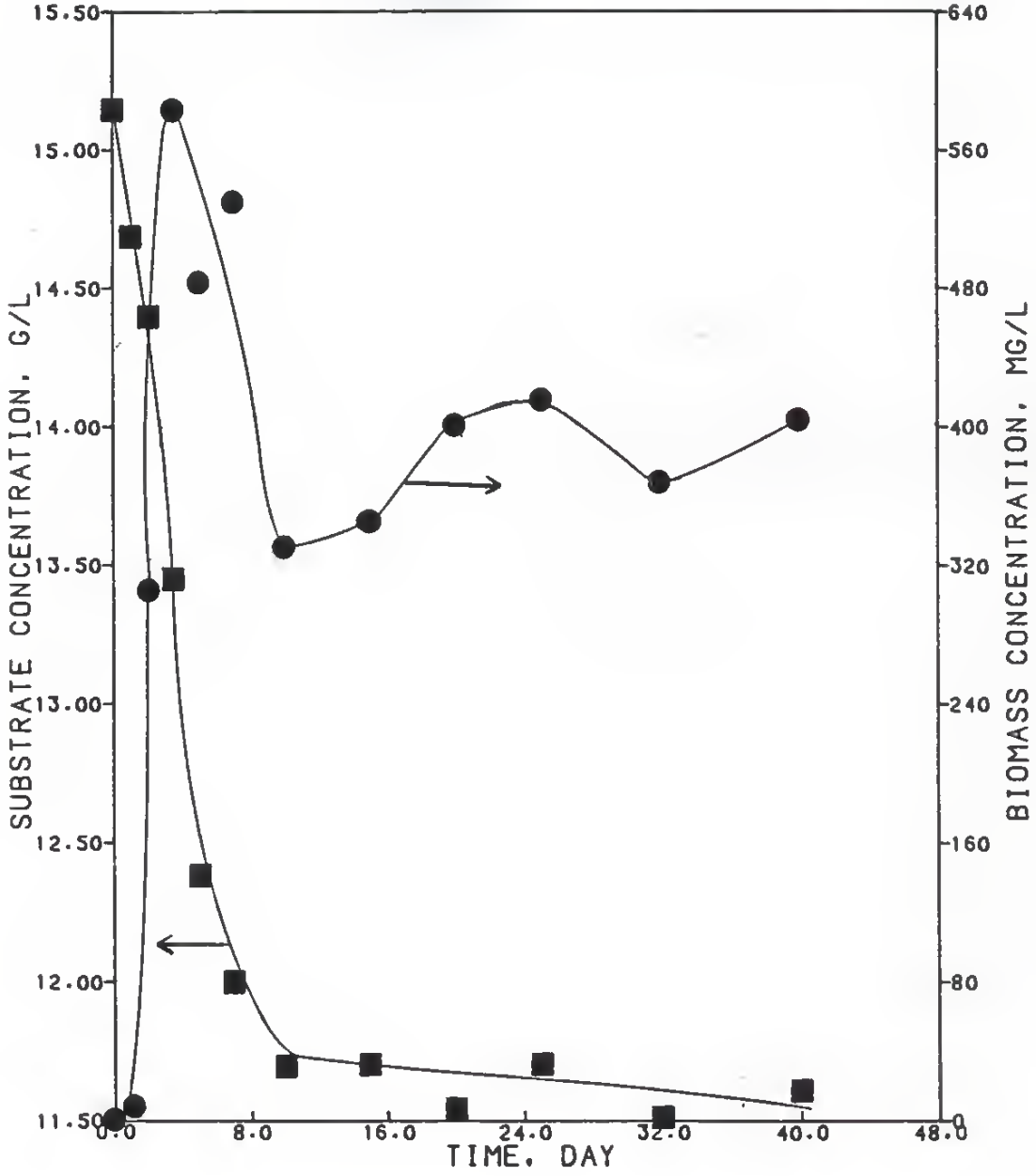

Fig. 4.8. Growth and substrate removal for mixed population growing on sample No. 8.

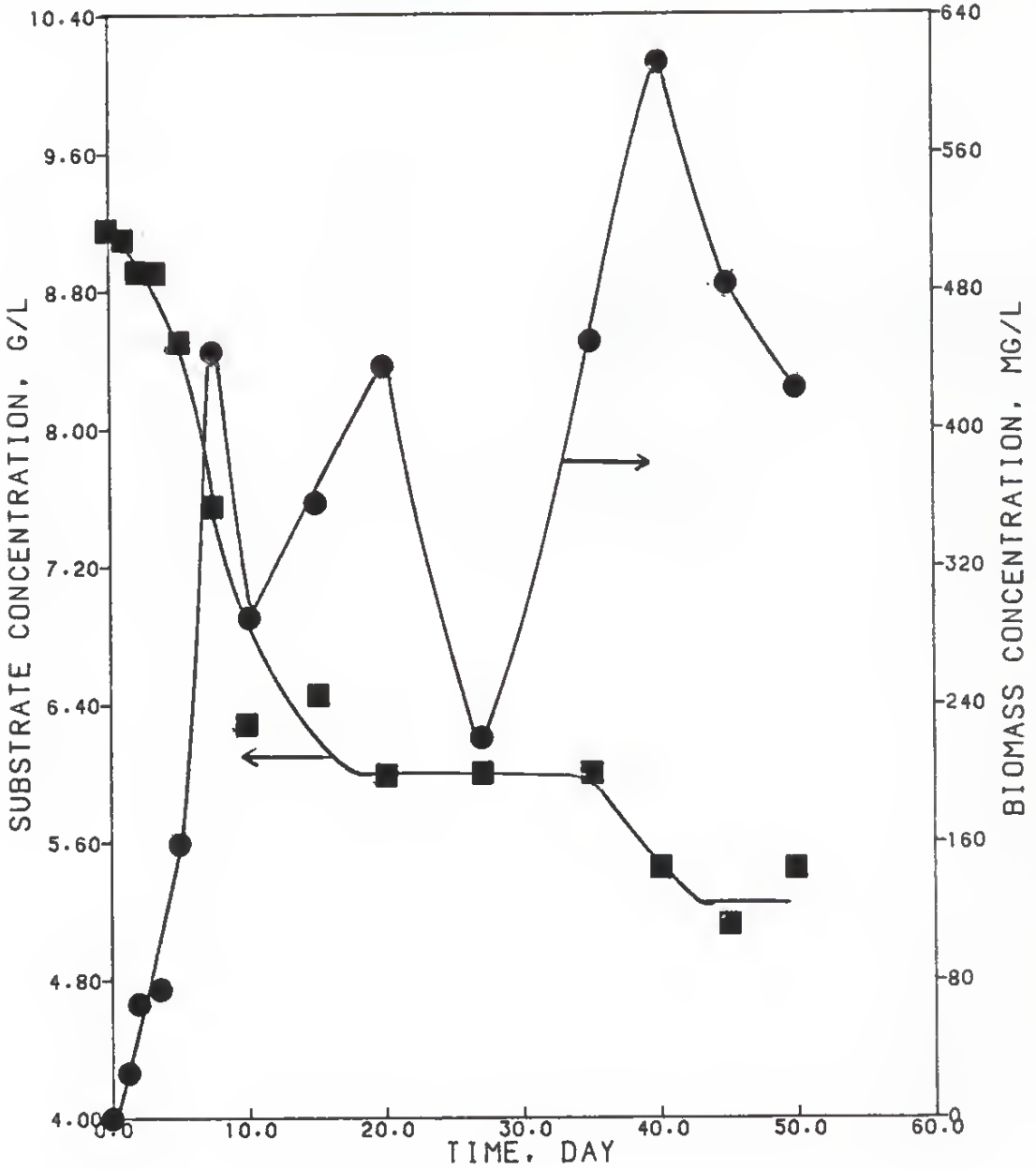

Fig. 4.9. Growth and substrate removal for mixed population growing on sample No. 9.

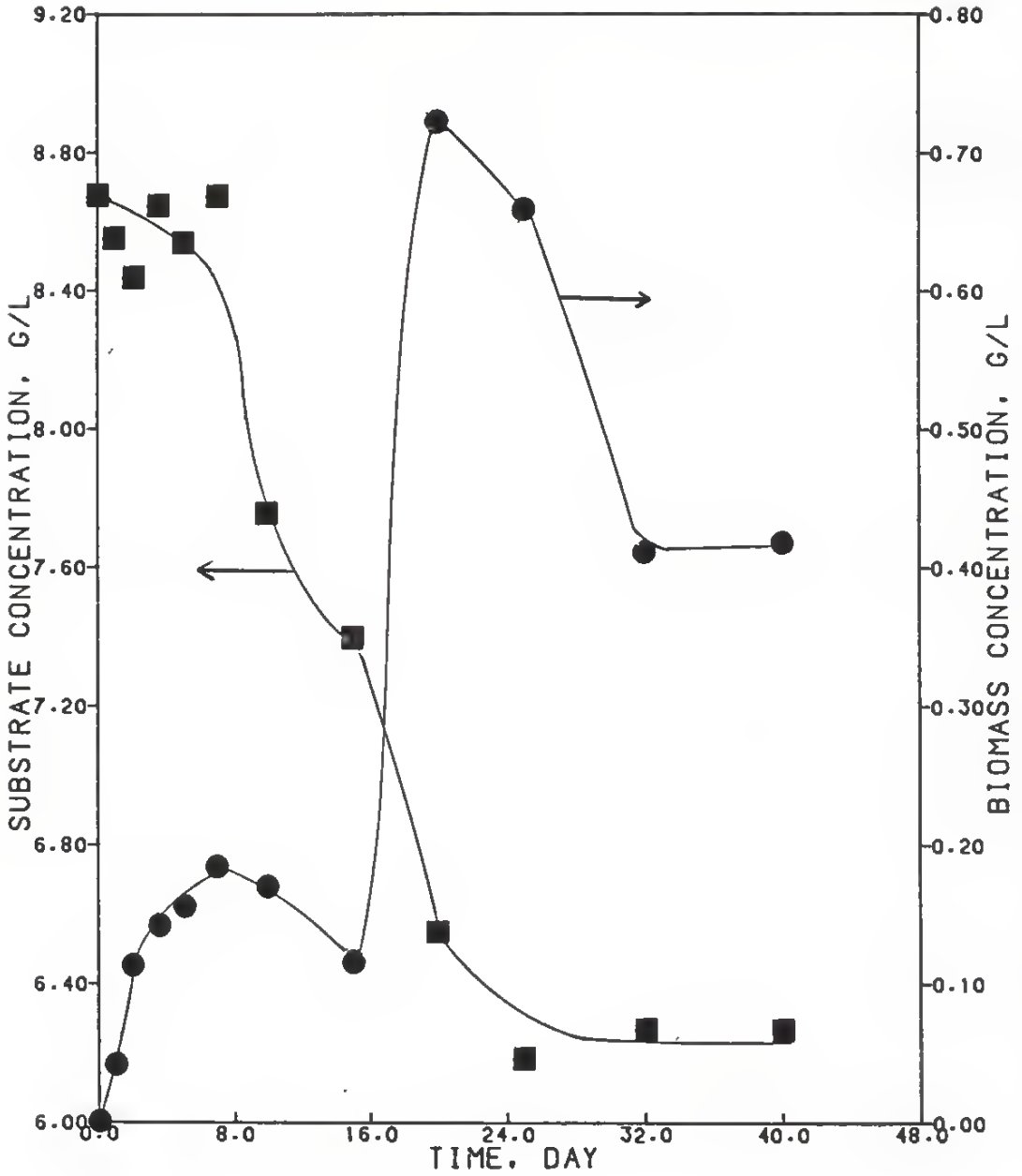

Fig. 4.10. Growth and substrate removal for mixed population growing on sample No. 10.

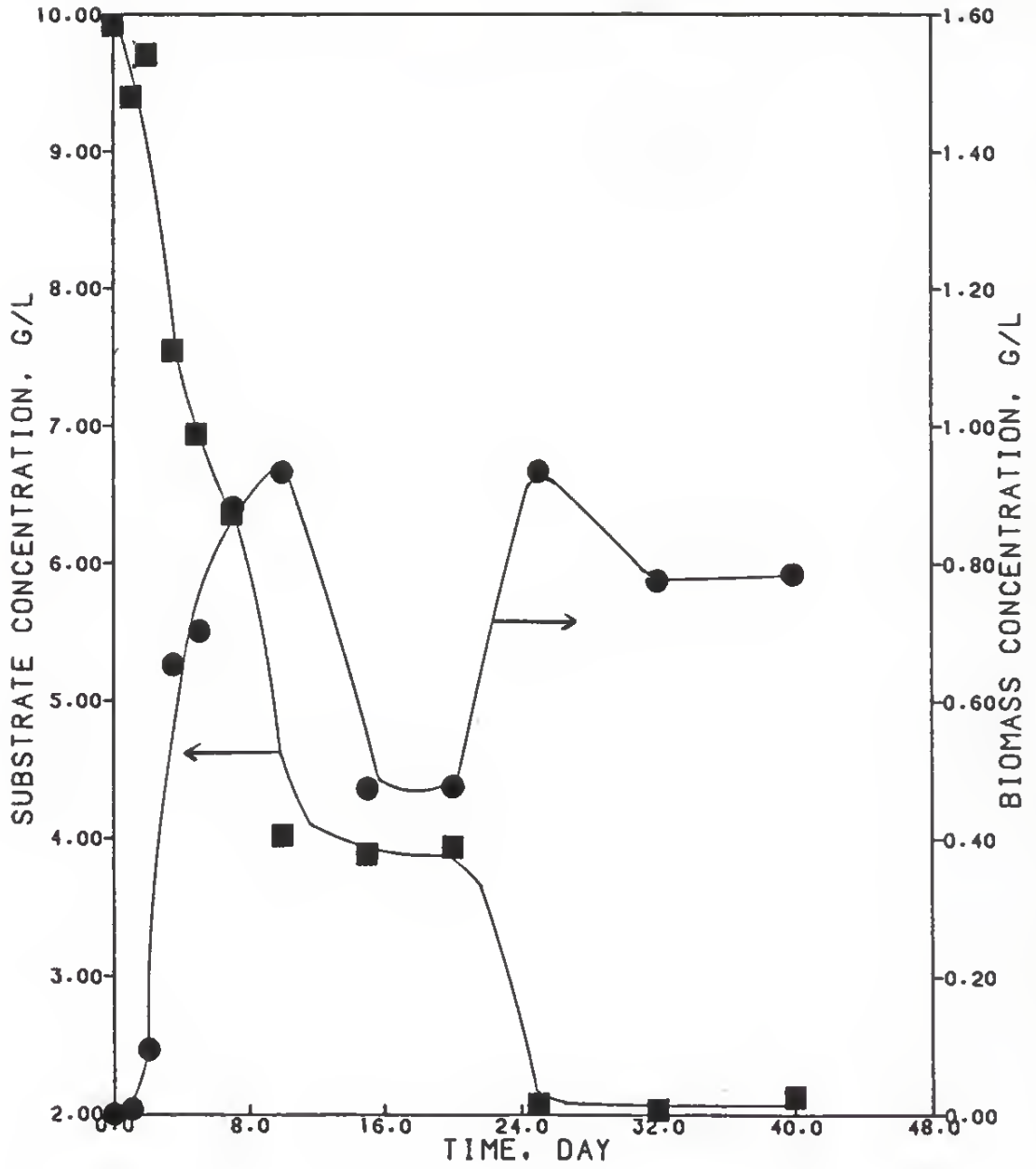

Fig. 4.11. Growth and substrate removal for mixed population growing on sample No. 11.

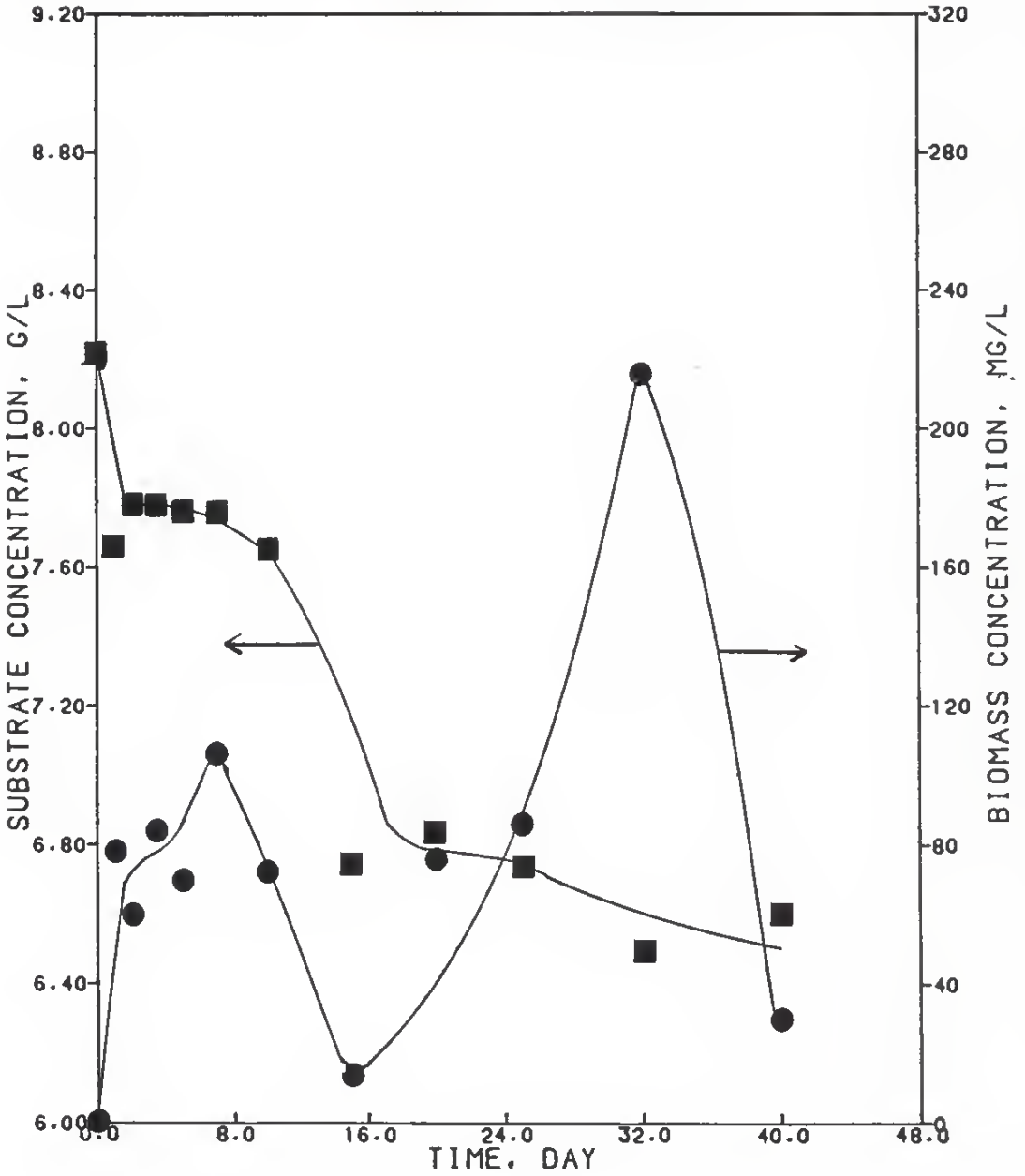

Fig. 4.12. Growth and substrate removal for mixed population growing on sample No. 12.

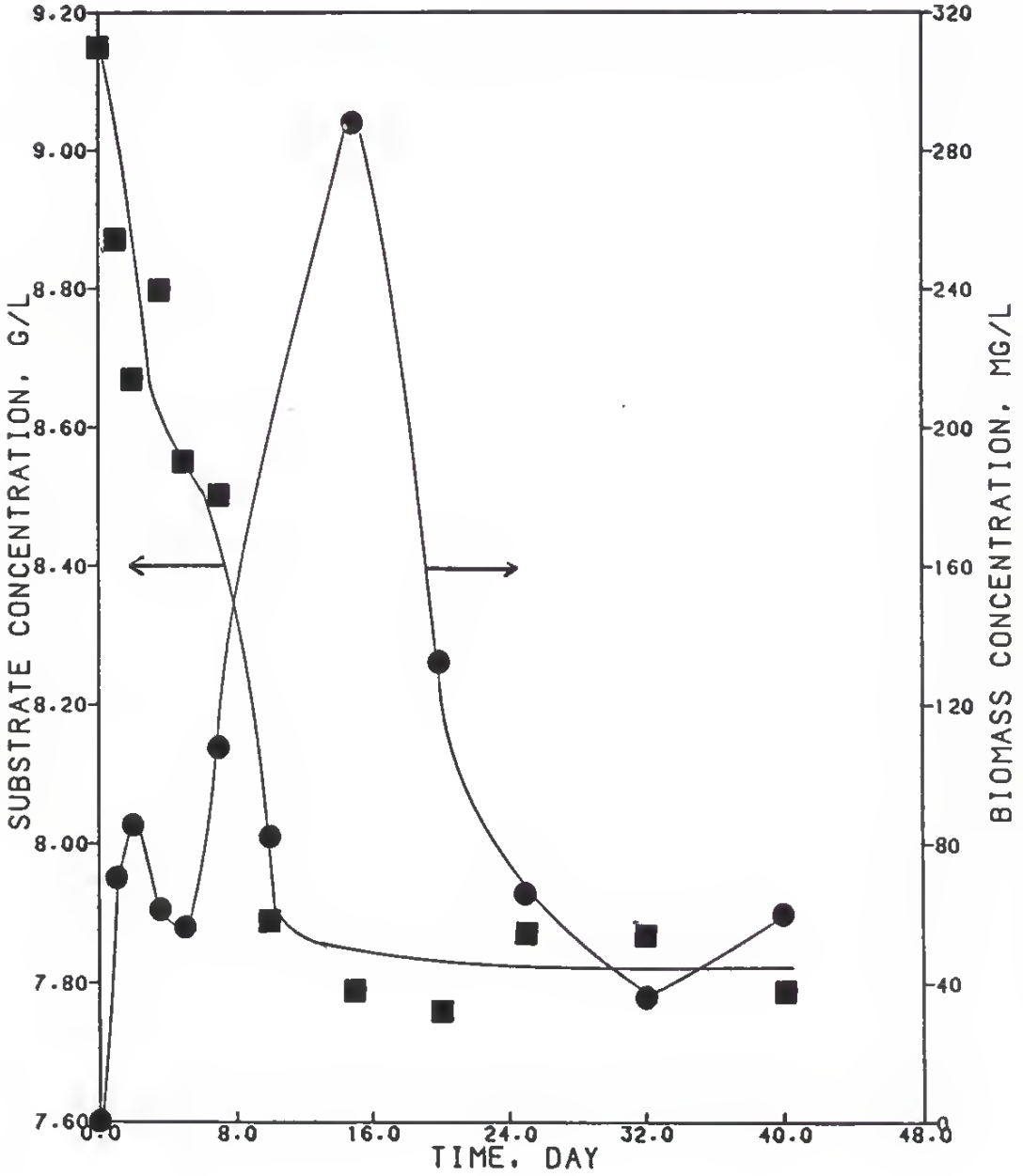

Fig. 4.13. Growth and substrate removal for mixed population growing on sample No. 13.

samples.

Figures 4.1 through 4.13 illustrate the courses of substrate removal and biomass change for each experiment. All of curves in the figures were made by following the eyeball approximation. The initial biomass concentration was approximately zero (0.08 mg/liter).

Most of the samples exhibited several lag phases. This is unusual for batch growth. Efforts were made to analyze samples as accurately as possible. Each COD datum presented is the average of two independent measurements. Each point of biomass concentration was the combined result of at least two dry weight measurements and an optical density determination. The fact that these samples exhibited similar trends appear to indicate that occurrence of a couple of lag phases is natural phenomenon for a system with the mixed substrates and populations.

Multiple lag phases may sometimes be observed when the substrate contains multiple carbon and energy sources. This phenomenon, known as diauxic growth, is caused by a shift in metabolic patterns in the midst of growth. The most biodegradable substrate is used first. While growing on this substrate, microorganisms exhibit the normal or first lag, exponential, and endogeneous phases of growth. Exhaustion of the easily metabolized substrate results in induced synthesis of the appropriate enzymes to metabolize the second substrate. This leads to the second lag phase during which the cell activates the production of the second

substrate metabolizing enzymes; this eventually gives rise to a second exponential phase.

For example, cultures of E. Coli exhibit biphasic growth when inoculated into a medium containing both glucose and lactose; glucose is preferentially metabolized as a substrate. While growing on glucose, E. Coli exhibits the normal lag, exponential, and stationary phases of growth. Rather than exhibiting a prolonged stationary phase, E. Coli enters a secondary lag phase when the glucose has been completely exhaused. During this second lag phase allolactose acts as an inducer to derepress the lac operon system. The enzymes that are necessary for lactose metabolism are synthesized and the bacteria begin to grow exponentially by using the lactose substrate. When the lactose is completely consumed, the bacteria enter the second stationary phase.

Growth curves obtained in the present experiments are not exactly the same as other curves of diauxic growth (Atlas, 1984). Considering the difference in experimental conditions, some of the phenomena may be associated with diauxic growth while other variations may be associated with mixed culture population dynamics.

It took longer time (50-55 days) for samples Nos. 4, 6, 7, and 9 to arrive at respective constant substrate concentrations. For samples Nos. 2, 3, 5, or 11, approximately 50 percent of the substrate was removed during

the first exponential phase.

All of the HPLC results are listed in Appendix II. Figures II-1 through II-13 are chromatograms of samples taken before biodegradation, and Figures II-14 through Figure II-26 are chromatograms of samples taken after biodegradation. It was difficult to adjust to zero when the absorbance was at the most sensitive range (0.01-0.005). Using higher concentrations of solutions is needed for more resonable chromatograms. Except samples Nos. 2, 3, 5, and 10, two components were detected by Ultraviolet detector. Only one component was detected with samples Nos. 2, 3, and 10, while 3 components were detected with sample No. 5. In each figure exhibiting two peaks, a small peak correspond to the component with a larger molecular weight, and the other to that with a smaller molecular weight. In Figure II.1, the minor component with a larger molecular weight appears after 12 minutes, and the major component appears almost immediately after appearance of the minor component. In samples Nos. 1, 4, 5, 6, 7, 9, or 11, after biodegradation the minor component which has a larger molecular weight almost disappeared. This result was consistent with the fact that the COD reduction ratio of each of these samples was more than 60 % except sample No. 9. After the biodegradation experiment, several new peaks were detected in sample No. 6, and one additional peak in sample No. 7. The chromatograms exhibited interesting responses in the region of low molecular weights. All these peaks corresponded to the

components with small molecular weights. In sample No. 8, 12, or 13, a small peak for the component with a larger molecular weight remained even after biodegradation. This observation is consistent with the fact that the COD removal efficiency for each sample was less than 20 %. These results indicate that a minor component with a larger molecular weight can not be ignored in a biodegradation study.

The results for samples Nos. 2, 3, and 10 are somewhat difficult to explain. For sample No. 2, the amount of the only one component determined with the UV detector increased even though the COD removal efficiency was as high as 70 %. For sample NO. 3, a new component with a larger molecular weight appeared even though COD removal was 90 %. The COD reduction for this sample was 77 %. For sample No. 10, the amount of the component determined with the UV detector was larger after biodegradation. To further clarify these results, exact chemical compositions of metalworking fluids need be known.

4.2 ADAPTATION AND DILUTION EFFECTS

Figure 4.14 compares the results of the growth experiments with a developed culture with those of mixed population of sewage organisms; sample No. 4 in Table 3.1 was the carbon source. The solid lines indicate the growth curves with mixed population of sewage organisms while the dotted line shows the result with adapted microorganisms in

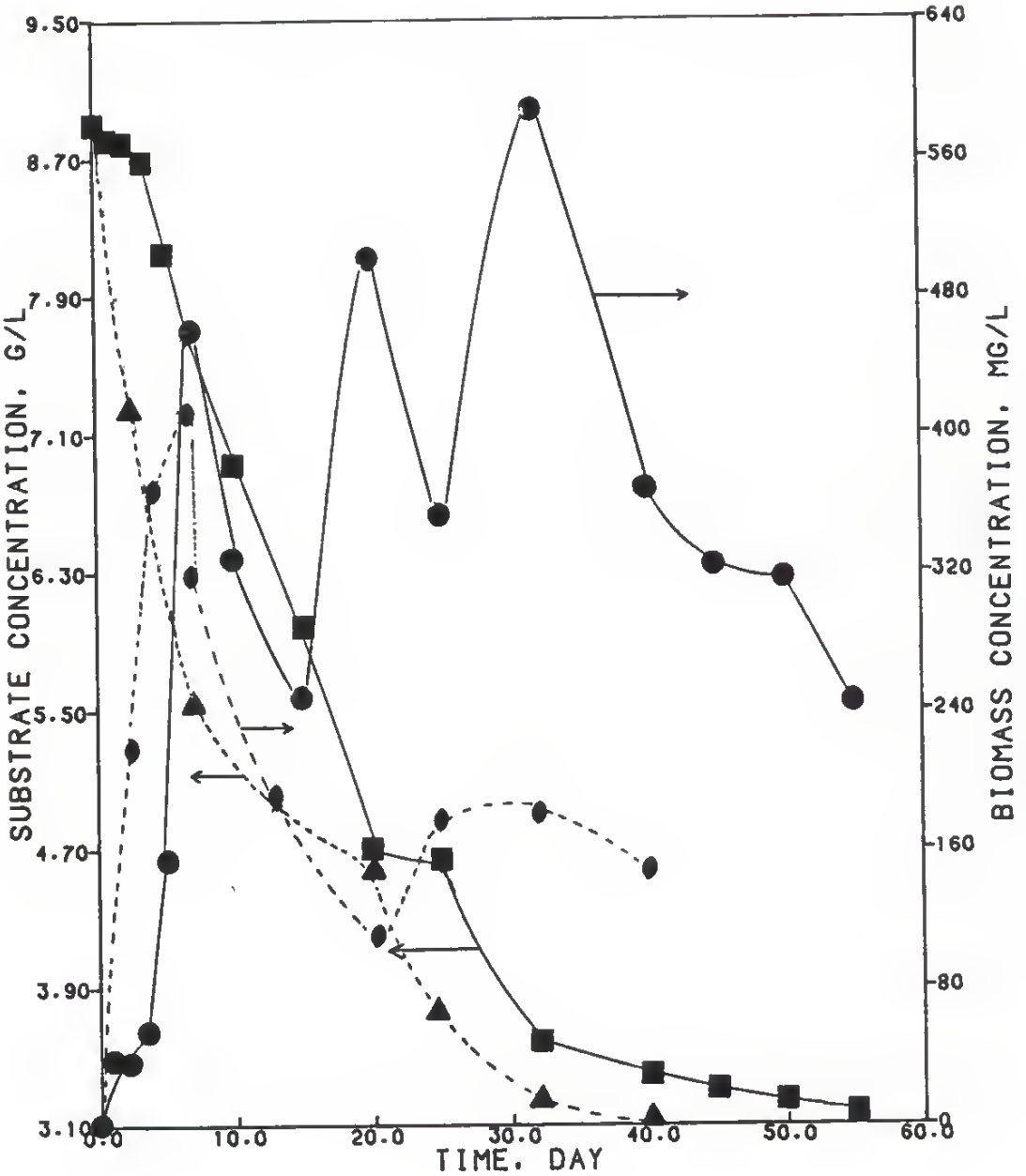

Fig. 4.14. Growth and substrate removal for mixed population in raw sewage (solid lines) and developed culture (dotted lines) growing on sample No. 4.

developed culture. The figure indicstes thst the developed culture hss less initisl lag than the mixed populstion. Nevertheless, the former hss sbout the same ability to degrade the synthetic metslworking fluid ss the lstter.

The specific growth rste of microorgsnisms in the developed culture wss 0.095 while the growth rste of a mixed populstion in rsw sewsge wss 0.026. For the first seversl dsys, growth snd substrste removsl for the adapted microorgsnisms in batch culture wss fsster thsn those in the original mixed populstion. After 5 to 7 dsys, growth snd substrate removal for the developed microorgsnisms decressed. It took 21 dsys to srrive st spproximstely 50 percent reduction in substrste with the developed culture while it took 25 days with sewsge microorgsnisms. Also, the final COD removsl efficiency with the sdspted culture wss 65 percent while that with the originsl mixed populstion wss 64 percents. These results suggest thst microbial strains with degradative capscities should be enriched from nsture or genersted in the laboratory by continuous culture techniques as described in the srticle of Cook et al. (1983).

Dependence of the specific growth rate on the initisl concentrstion of substrate is shown in Figure 4.15. Experimental data for Figure 4.15 sre listed in Tsble I.14. In Figure 4.15, the data points sre simply connected by the strsight lines. The method for cslculation of the specific growth rste hss been described in detail in section 3.1. The

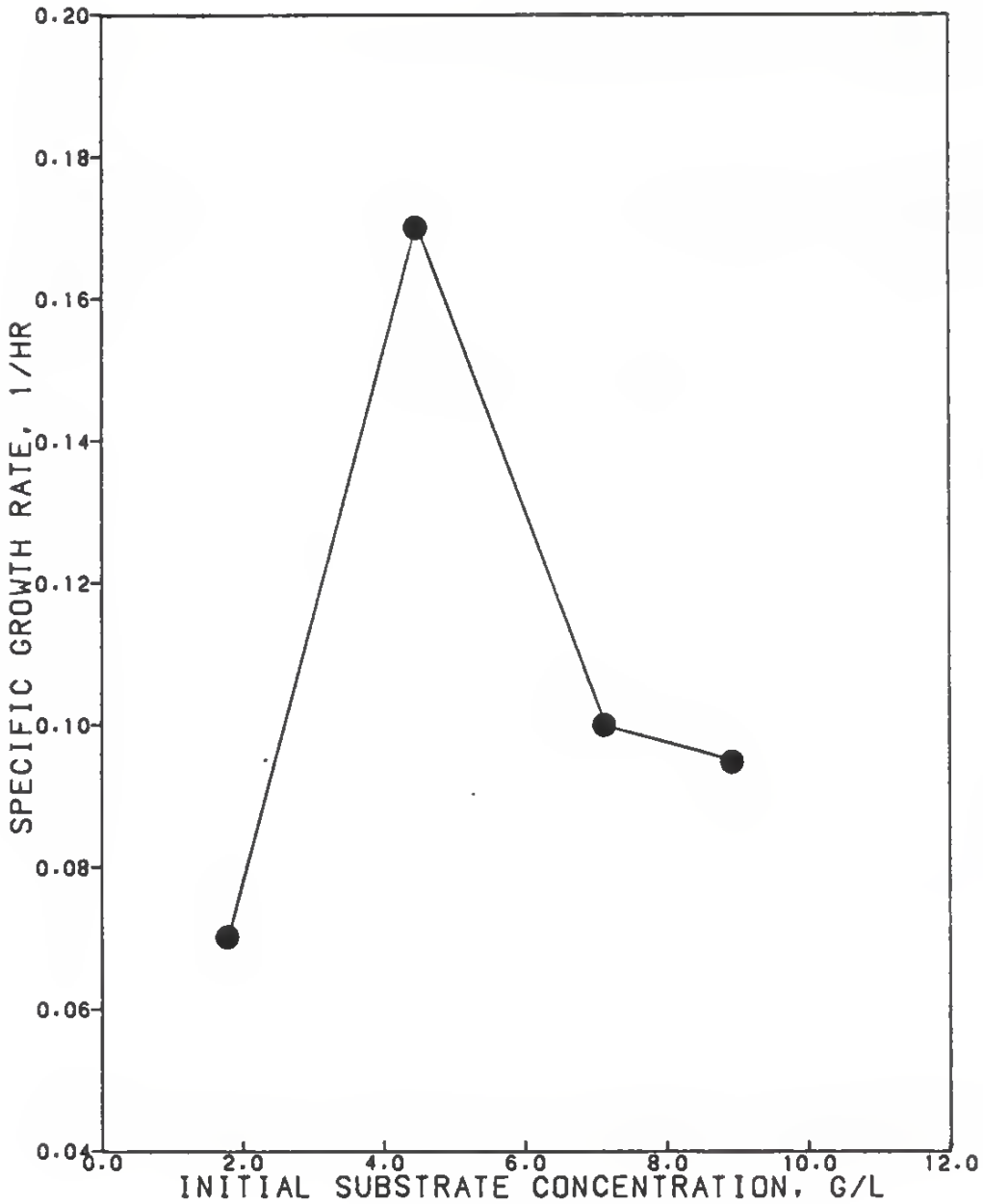

Fig. 4.15. Effect of dilution on the specific growth rate of mixed populations in batch culture with sample No. 4 as the carbon source.

figure demonstrates clearly that the specific growth rate increases drastically with the increase in the initial concentraton between 1780 and 4500 mg/liter. The latter appears to be the optimum concentration for the maximum growth. At a higher than the optimum concentration, the specific growth rate decreases drastically until the initial concentration reaches approximately 7000 mg/liter. Thereafter, it remains essentially constant. This indicates that the concentration of substrate should be maintained at 3000-6000 mg/liter of COD. This may not be practical in a present wastewater treatment plant without increased flow and increased system size.

4.3 PH EFFECT

In batch fermentors, the effect of pH was observed at 24 °C in a pH range from 6.0 to 9.0. The dependence of the growth rate on pH is shown in Figure 4.16. The curve in Figure 4.16 was made by connecting the data points with straight lines. The method of calculating the specific growth rate has been described in detail in section 3.1. There was a pronounced shift in the region of the maximum growth rate. The optimum value of pH value was between 7.5 and 8.0. The initial pH of the 1 % (v/v) synthetic metalworking fluid was between 8.5 and 9.5. Unfortunately, growth of the mixed population with the minimum rate occurred in this pH range. Therefore, for optimum operation,

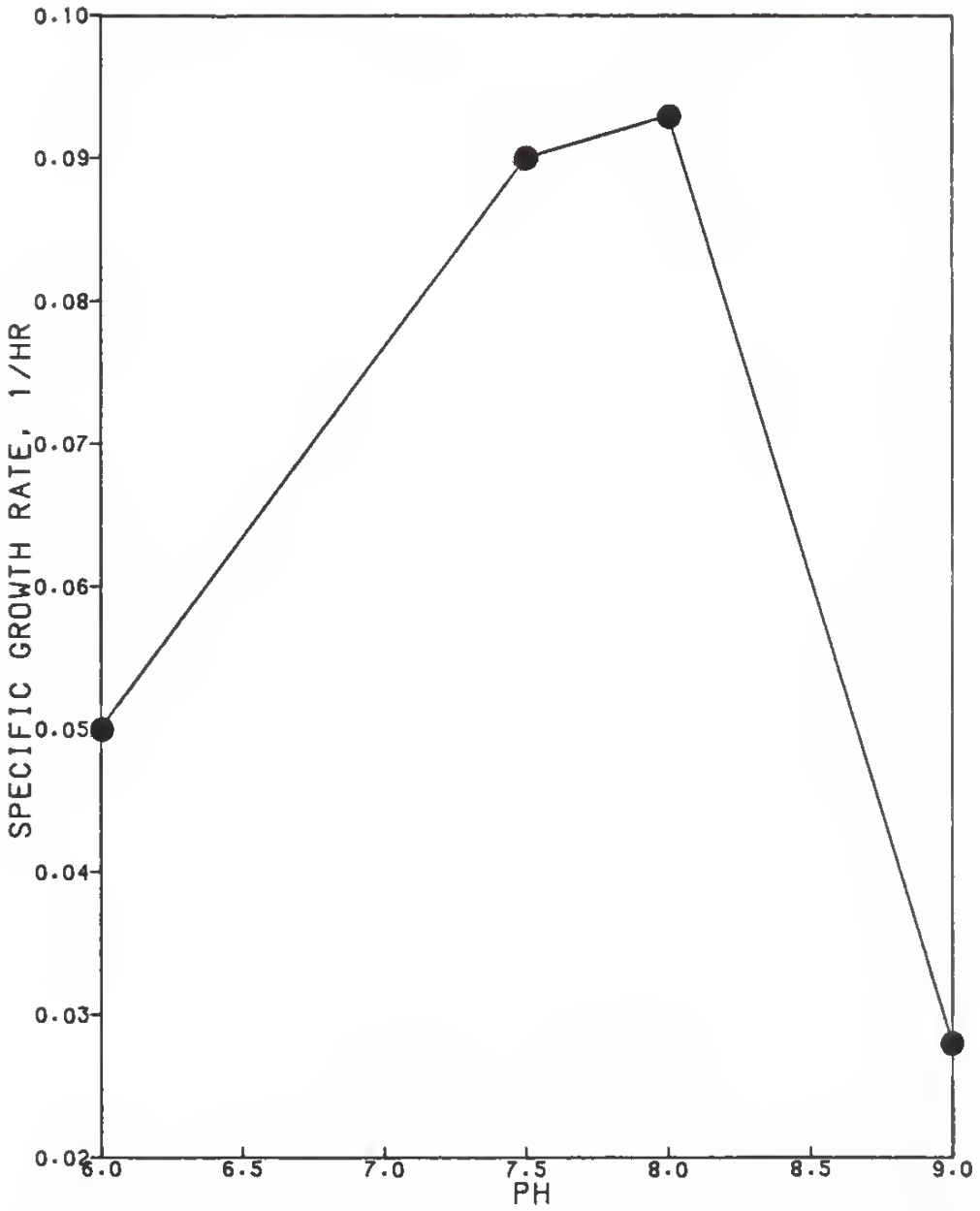

Fig. 4.16. Effect of pH on the specific growth rate of mixed populations with sample No. 2 as the carbon source.

the pH should be adjusted to 7.5-8.0 with any inorganic acid or base.

4.4 IDENTIFICATION OF THREE SPECIES

Pseudomonas F agar is selective for Pseudomonas species. Desoxycholate agar is selective for Gram (-) enteric bacilli and differential for lactose fermentation organisms. MacConkey agar is selective for coliform bacilli. Endo agar is a solid medium for the detection of coliform and other enteric organisms. Eosin Methylene Blue (EMB) agar is used for the isolation and differentiation of Gram negative enteric bacilli. All three microorganisms are Gram (-) for Gram reaction and single or pair rod forms for cell morphology.

Colonies of Pseudomonas aeruginosa on the agar medium are large, flat spreading and irregular, greyish green in color. The greenish pigment diffuses into the medium. Broth cultures are blue-green in color. Colonies of Proteus mirabilis are slightly raised yellow often with black center on the Desoxycholate agar. Colonies of Klebsiella are large, mucoid, slimy and white on the MacConkey agar, large, brownish, convex and mucoid and tend to coalesce on the EMB agar; the color is deep red, and this deep red diffuses the surrounding medium, may have a golden yellow sheen on Endo agar (Skinner, 1980: Collins, 1984). In the present work microorganisms were identified by shape, color, and size of colonies.

Table 4.6. Results of Biochemical Tests

Biochemical media	Pseudomonas aeruginosa	Proteus mirabilis	Klebsiella pneumoniae
KIA slant	NR	K	A
butt	NR	A gas	A gas
H_2S	-	+	-
Glucose	-	+	+ gas
Lactose	-	-	+ gas
Mannitol	-	-	+
Urea	+	+	+
Indol	-	-	-
M.R.	-	+	+
V.P.	-	-	-
Citrate	+	+	+

A representative colony on each agar plate was selected for biochemical tests. The results of these tests were compared with the standard results of the biochemical tests with three species (Breed et al., 1957: Skinner, 1980: Collins, 1984). These results are listed in Table 4.4. Representative colonies on Pseudomonas F agar were Pseudomonas aeruginosa, and representative colonies on Desoxycholate agar and those on EMB agar were Proteus mirabilis. Also representative colonies on MacConkey agar and Endo agar were Klebsiella pneumoniae. For convenience, visual clues for identifying the reactions in the biochemical tests are summerized in Table 4.5 (Urban, 1977). Those findings imply that Pseudomonas aeruginosa, Proteus mirabilis, and Klebsiella pneumoniae were present in large numbers during the batch cultivation with a synthetic metalworking fluid as the carbon source.

Table 4.7. Identification of Biochemical Reactions

Biochemical Media	How to read
Kligler's Iron Agar (KIA)	Orangish alant = no reaction (NR) Pink slant = alkaline (K) Yellow slant = acid (A)
	Orangish butt = no reaction (NR) Yellow butt=acid (A), Cracked agar=gas Black butt=H_2S production + (when butt haa H_2S assume acid production also)
Glucose Broth Lactose Broth Mannitol Broth	No color change = - Yellow = acid (+) Gas in Durham tube = gas
Urea	Hot pink color = + No change in medium = -
Indol	Add 8-10 drops Kovac's reagent Red ring = +, Yellow ring = -
Clark and Lube (MR-VP Broth)	MR - Add 5-6 drops Methyl Red indicator Red color = + (acid) No color change = - (no acid)
	VP - Add 3-5 dropa α-Naphthol and 15 drops 40% KOH. Let stand 20-30 min. Pink color = +, No color change = -
Simon's Citrate Agar	Intenae blue color = + No color change = -

CHAPTER 5

CONCLUSIONS

The significant conclusions from the present work are listed below.

1. The results from the biodegradation study show that more than half of synthetic metalworking fluids (8 of 13) are readily biodegradable with mixed populations of sewage origin. COD removal efficiencies of those metalworking fluids ranged from 59 to 90 % in the experiments conducted.

2. Samples Nos. 8, 9, 10, 12, and 13 are not easily biodegradable. COD reduction of those samples ranged between 15 and 40 % in the experiments. A further study need be performed to elucidate the causes for the low extent of substrate removal for these samples.

3. Occurrence of multiple lag phases is fairly common in a system with multiple carbon sources and mixed populations.

4. The results of analyses with HPLC are useful as indicators of the biodegradability of synthetic metalworking fluids except samples Nos. 2, 3, and 10. To further clarify these results, exact chemical compositions of metalworking fluids need be known.

5. The studies of adaptation have revealed that the extent of initial lag for developed microorganisms in batch culture is less than that for mixed population in wastewater plant, but about the same ability to degrade the synthetic metalworking fluid. This suggests that microbial strains

with degradative capacities will be generated in the laboratory by continuous culture techniques. It is also recommended that a further study be carried out for the development of genetically improved microorganisms which can more easily degrade the metalworking fluid.

6. The study of dilution effect suggests that the concentration of substrate should be kept lower than the present wastewater treatment concentration of 1 % (v/v), i.e., between 3000 and 6000 mg/liter of COD. This may not be practical in a conventional wastewater treatment plant without increasing its size.

7. The studies of pH effect indicates that the pH should be adjusted to 7.5-8.0 with any inorganic acid or base for optimum biodegradation operation.

8. In the present research, three species, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, were identified in the batch culture of a metalworking fluid through several biochemical tests. Each species can serve as pure culture in the future biodegradation studies of metalworking fluids for the development of genetically improved microorganisms.

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APPEDIX I

EXPERIMENTAL DATA FOR BATCH RUNS

Table I.1. Experimental Data for Sample No. 1;

April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	8940	7.2	7.4	23
1	26	8840	7.3	7.3	23
2	62	8700	7.4	7.4	25
3.5	640	6930	7.1	7.5	24
5	666	6180	7.5	7.4	24
7	554	6180	7.7	7.7	24
10	406	5810	7.7	7.6	24
15	442	4880	7.9	7.4	24
20	972	2970	7.2	6.4	24
25	644	2900	8.1	7.4	24
32	694	2580	7.9	8.2	23
40	496	2770	7.7	7.3	24

* 0.08 mg/L

Table I.2. Experimental Data for Sample No. 2:

April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	5990	7.6	7.4	23
1	70	5630	7.5	6.9	23
2	326	4790	7.5	7.0	25
3.5	333	4320	7.5	7.2	24
5	414	3170	6.2	7.5	24
7	592	2550	5.2	7.7	24
10	402	2260	5.5	5.2	24
15	294	1770	7.3	7.4	24
20	270	1770	7.1	7.8	24
25	280	1650	7.0	7.5	24
32	212	1770	6.9	8.0	23
40	264	1770	6.9	7.3	24

* 0.08 mg/L

Table I.3. Experimantal Data for Sample No. 3;

April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	29360	8.0	7.4	23
1	723	24960	8.0	7.5	23
2	972	22760	8.2	5.3	25
3.5	788	23400	8.2	5.4	24
5	1958	7760	7.2	5.3	24
7	3164	4020	6.2	5.2	24
10	4008	3030	5.5	5.2	24
15	2794	2770	5.1	5.5	24
20	2978	2900	4.9	6.7	24
25	2784	2900	4.1	6.4	24
32	2312	3030	4.5	8.0	23
40	2056	3030	5.4	7.2	24

* 0.08 mg/L

Table I.4. Experimental Data for Sample No. 4:

April 15 - June 9, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	8900	7.3	7.5	23
1	38	8810	7.3	7.5	23
2	37	8800	7.4	7.4	25
3.5	54	8670	7.4	7.5	24
5	154	8150	7.2	7.4	24
7	460	7650	7.4	7.6	24
10	328	6930	7.4	7.6	24
15	248	5990	7.3	7.2	24
20	502	4700	6.9	7.7	24
25	354	4630	7.3	7.4	24
32	588	3590	7.1	8.0	23
40	368	3400	7.7	7.2	24
45	324	3310	7.4	7.5	24
50	316	3240	7.3	7.7	25
55	245	3170	7.6	7.1	24

* 0.08 mg/L

Table I.5. Experimental Data for Sample No. 5;

April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	13450	7.2	7.4	23
1	102	13030	7.2	7.2	23
2	99	13080	7.4	7.4	25
3.5	951	9650	6.9	7.5	24
5	1754	5200	6.6	7.4	24
7	1804	4630	6.3	7.4	24
10	1808	4320	7.3	6.5	24
15	1766	2840	7.4	7.1	24
20	1924	3170	7.9	7.5	24
25	1852	3140	7.8	7.3	24
32	2080	3100	8.1	8.0	23
40	1850	3030	8.0	7.1	24

* 0.08 mg/L

Table I.6. Experimental Data for Sample No. 6: April 15 - June 4, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	11580	7.2	7.4	23
1	49	11380	7.2	7.3	23
2	70	11070	7.3	7.5	25
3.5	52	11380	7.2	7.4	24
5	214	9400	7.0	7.5	24
7	324	9790	7.1	7.7	24
10	720	7760	7.8	7.5	24
15	546	7540	7.9	7.5	24
20	644	7330	7.9	7.7	24
25	634	7400	7.9	7.0	24
32	604	7230	7.8	6.4	23
40	1136	4790	8.0	7.2	24
45	930	4630	7.8	7.5	24
50	798	4790	7.6	7.5	25

* 0.08 mg/L

Table I.7. Experimental Data for Sample No. 7;

April 15 - June 9, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	7130	7.6	7.4	23
1	39	7030	7.6	7.2	23
2	151	6740	7.5	7.5	25
3.5	606	5450	7.9	7.4	24
5	578	4630	7.7	7.5	24
7	538	4630	7.7	7.6	24
10	434	4500	7.8	7.6	24
15	204	4320	7.9	7.5	24
20	288	4390	7.9	7.6	24
25	470	4100	7.9	7.1	24
32	422	3810	7.8	7.5	23
40	668	2520	8.0	7.0	24
45	730	1880	7.8	7.5	24
50	734	1770	7.7	7.6	25
55	582	1770	7.7	7.0	24

* 0.08 mg/L

Table I.8. Experimental Data for Sample No. 8;

April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	15150	7.4	7.4	23
1	8	14680	7.4	7.3	23
2	305	14400	7.4	7.5	25
3.5	582	13450	7.7	7.4	24
5	484	12380	7.7	7.5	24
7	530	12000	7.7	7.6	24
10	331	11700	7.8	7.7	24
15	346	11700	7.9	7.4	24
20	402	11540	7.9	7.9	24
25	416	11700	7.9	7.4	24
32	368	11500	7.8	8.1	23
40	404	11600	7.8	7.2	24

* 0.08 mg/L

Table I.9. Experimental Data for Sample No. 9;

April 20 - June 4, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	9150	7.8	7.4	24
1	26	9100	7.8	7.4	24
2	66	8900	7.5	7.6	24
3.5	75	8900	6.8	8.1	24
5	160	8500	7.6	6.5	24
7.5	445	7540	7.7	7.5	24
10	290	6280	7.7	7.5	24
15	358	6460	7.7	7.6	24
20	436	5990	7.7	7.4	24
27	222	5990	7.7	7.6	23
35	450	5990	7.7	7.2	24
40	612	5450	7.7	7.5	24
45	484	5120	7.7	7.6	25
50	424	5450	7.7	6.9	24

* 0.08 mg/L

Table I.10. Experimental Data for Sample No. 10; April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	8670	7.2	7.4	23
1	42	8550	7.2	7.4	23
2	113	8440	7.3	7.5	25
3.5	147	8650	7.4	7.4	24
5	156	8540	7.2	7.5	24
7	184	8670	7.2	7.7	24
10	170	7760	7.2	7.4	24
15	116	7400	7.2	7.5	24
20	722	6550	7.2	7.5	24
25	660	6180	5.8	7.3	24
32	412	6270	6.2	8.0	23
40	418	6270	6.7	7.1	24

* 0.08 mg/L

Table I.11. Experimental Data for Sample No. 11: April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	9920	7.7	7.4	23
1	8	9400	7.7	7.5	23
2	93	9700	7.7	7.4	25
3.5	653	7540	7.6	7.5	24
5	700	6930	7.8	7.4	24
7	880	6370	7.7	7.2	24
10	930	4020	7.7	7.2	24
15	474	3880	8.0	7.3	24
20	478	3950	7.6	7.7	24
25	934	2070	7.3	6.7	24
32	776	2010	7.9	8.1	23
40	784	2130	7.8	6.9	24

* 0.08 mg/L

Table I.12. Experimental Data for Sample No. 12;

April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	8210	7.2	7.4	23
1	78	7760	7.2	7.6	23
2	60	7780	7.3	7.5	25
3.5	84	7780	7.4	7.5	24
5	70	7760	7.3	7.4	24
7	106	7760	7.3	7.8	24
10	72	7650	7.4	7.6	24
15	14	6740	7.6	7.6	24
20	76	6830	7.6	7.7	24
25	86	6740	7.7	7.3	24
32	216	6500	7.8	8.1	23
40	30	6600	7.9	7.0	24

* 0.08 mg/L

Table I.13. Experimental Data for Sample No. 13; April 15 - May 25, 1986

Sampling Time(day)	Biomass (mg/L)	COD (mg/L)	pH	DO (mg/L)	Temp. (°C)
0	0*	9150	7.3	7.4	23
1	70	8870	7.3	7.4	23
2	85	8670	7.4	7.5	25
3.5	61	8800	7.5	7.4	24
5	56	8550	7.2	7.5	24
7	108	8500	7.3	7.8	24
10	82	7890	7.4	7.6	24
15	288	7790	7.5	7.5	24
20	132	7760	7.5	7.5	24
25	66	7870	7.7	7.4	24
32	36	7870	7.6	8.0	23
40	60	7790	7.8	7.0	24

* 0.08 mg/L

Table I.14. Experimental Data for Dilution Effect

Sampling Time (hour)	Biomass Concentration (O.D.) 8900* (mg/L)	7120* (mg/L)	4450* (mg/L)	1780* (mg/L)
0	0.0	0.0	0.0	0.0
18	0.005	0.000	0.000	0.030
24	0.015	0.014	0.050	0.230
27	0.020	0.025	0.072	0.300
29	0.040	0.050	0.105	0.320
36	0.150	0.155	0.360	0.450
39	0.200	0.215	0.495	0.465
42	0.260	0.290	0.450	0.455
45	0.310	0.390	0.410	0.440
48	0.365	0.480	0.400	0.440
51	0.460	0.520	0.410	0.425
60	0.480	0.485	0.420	0.430
63	0.500	0.470	0.440	0.435
69	0.500	0.440	0.460	0.430
72	0.430	0.430	0.510	0.430

Note:

* Initial substrate concetration

Standard curve for sample No. 4 is

Y(O.D. at 540 nm) = 0.06896+0.00189X (biomass conc..mg/L).

Correlation coefficient r^2 = 0.98682

APPENDIX II

CHROMATOGRAMS OF HPLC

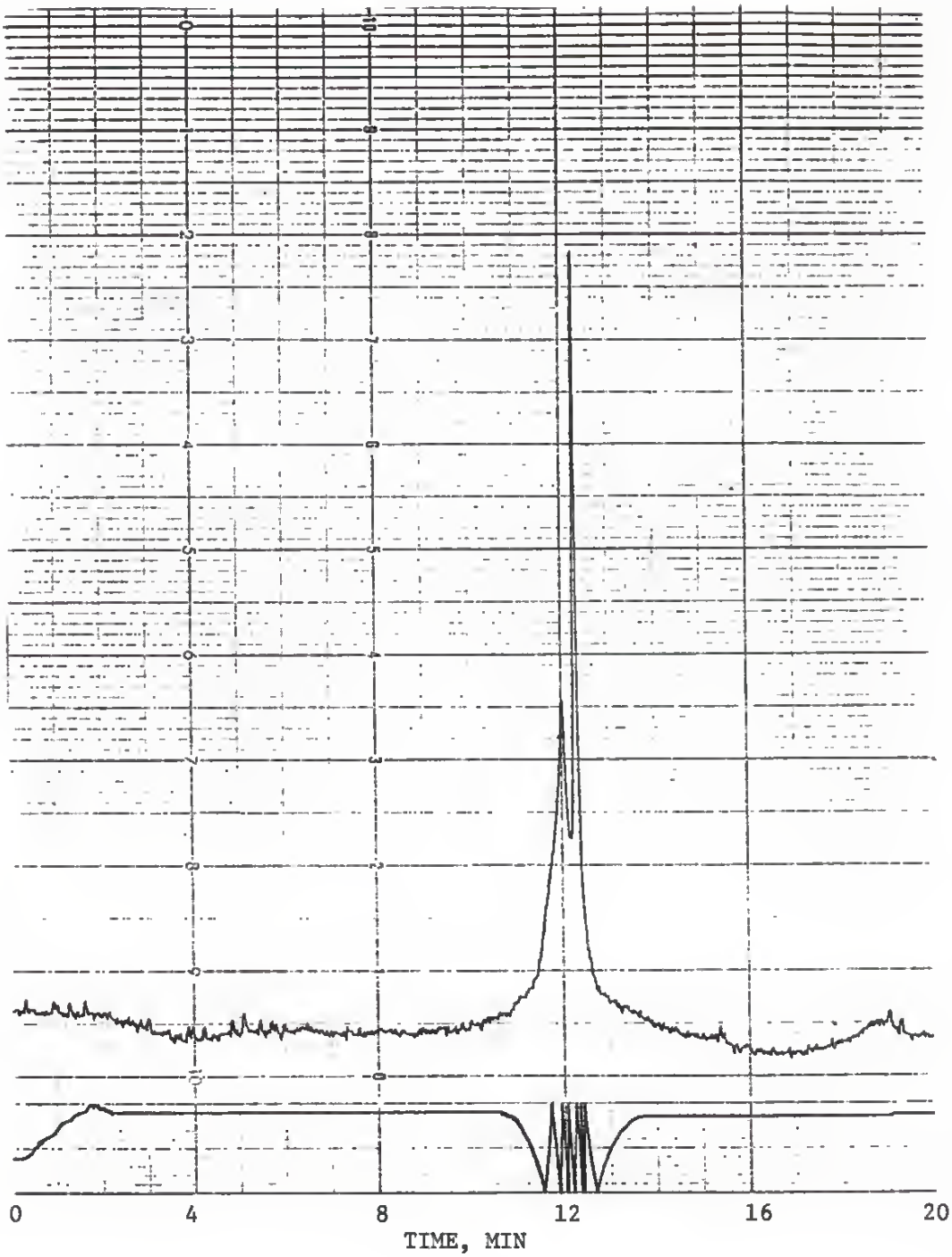

Fig. II.1. Result of HPLC analysis before biodegradation for sample No. 1 at absorbance = 0.01.

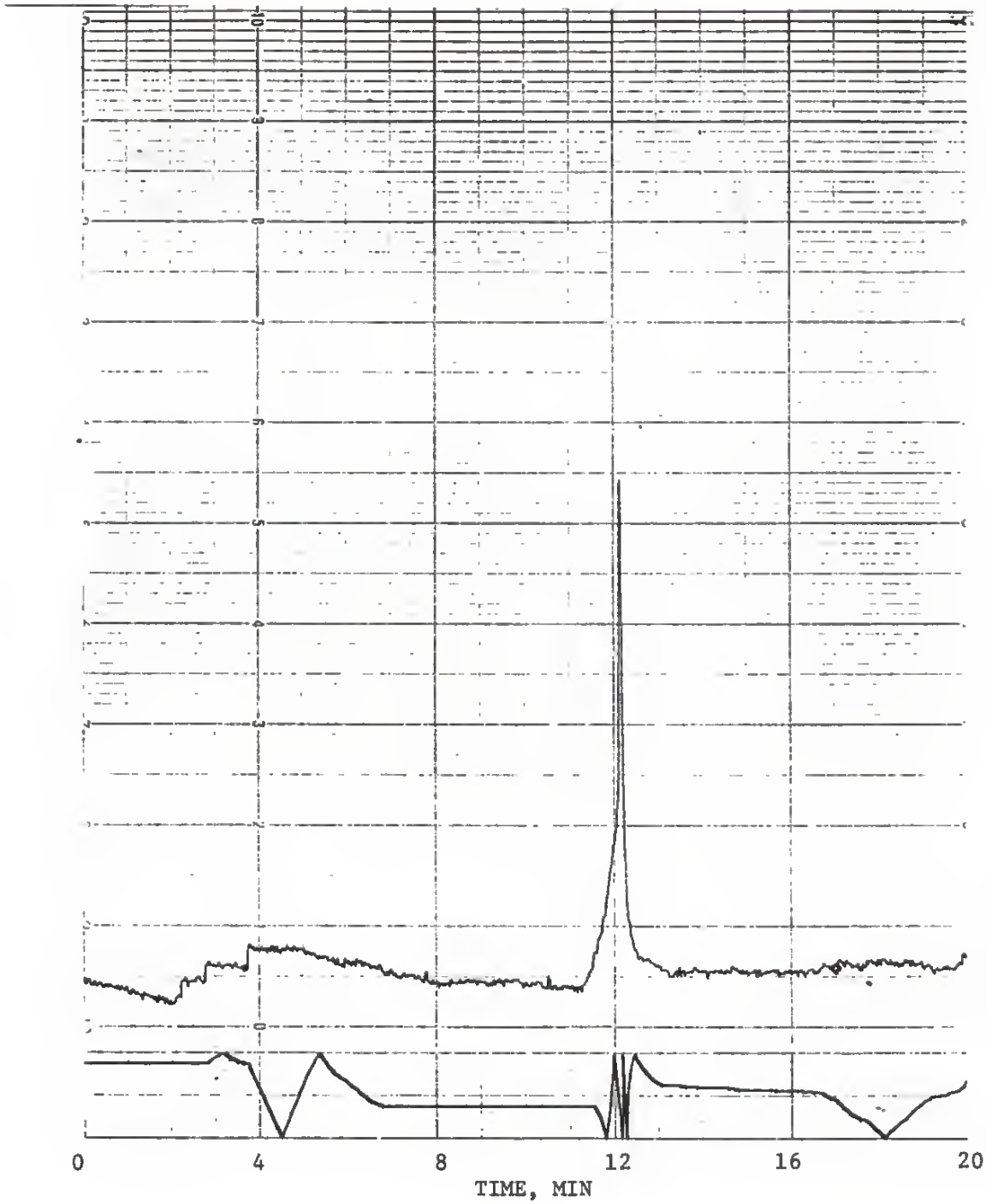

Fig. II.2. Result of HPLC analysis before biodegradation for sample No. 2 at absorbance = 0.01.

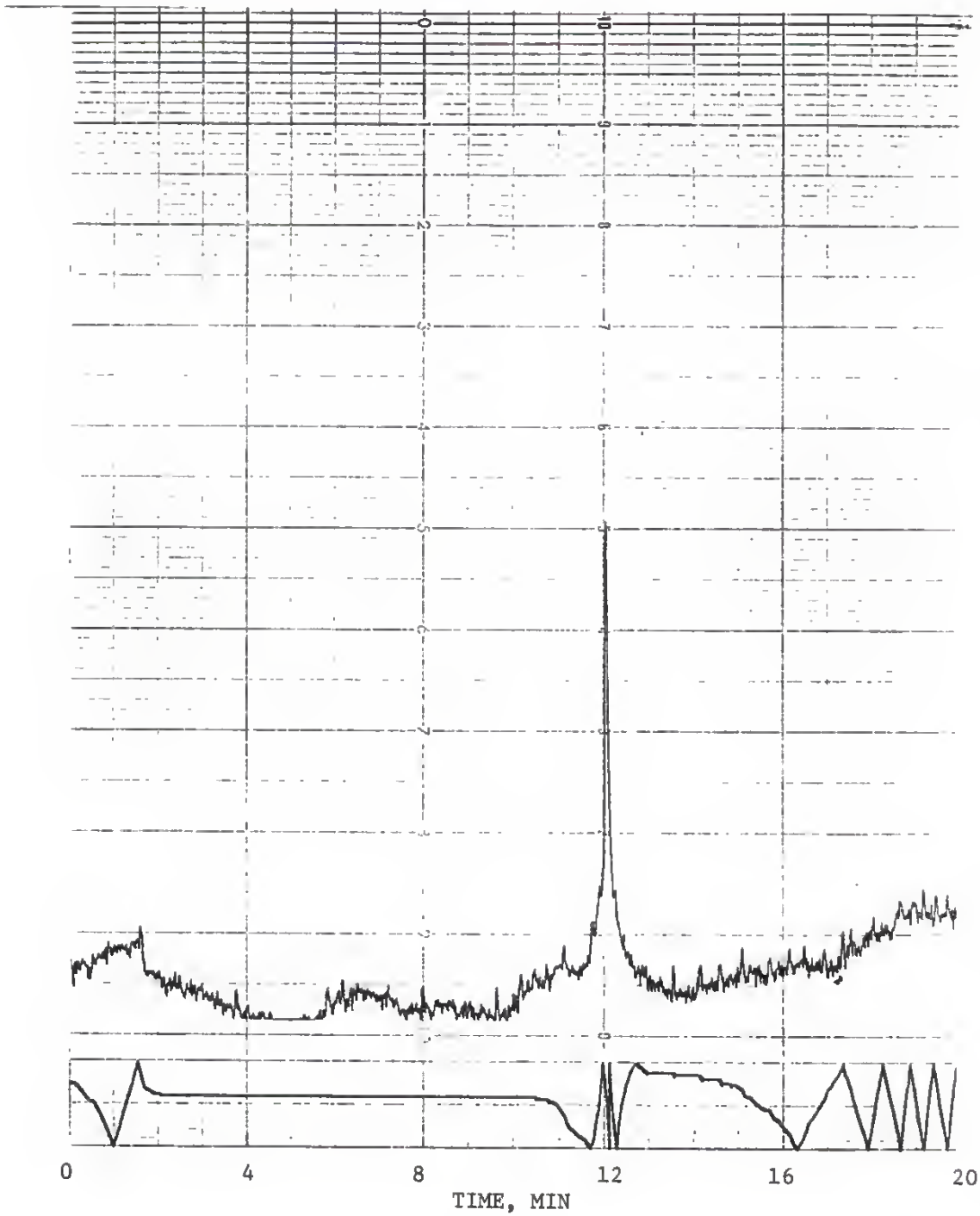

Fig. II.3. Result of HPLC analysis before biodegradation for sample No. 3 at absorbance = 0.005.

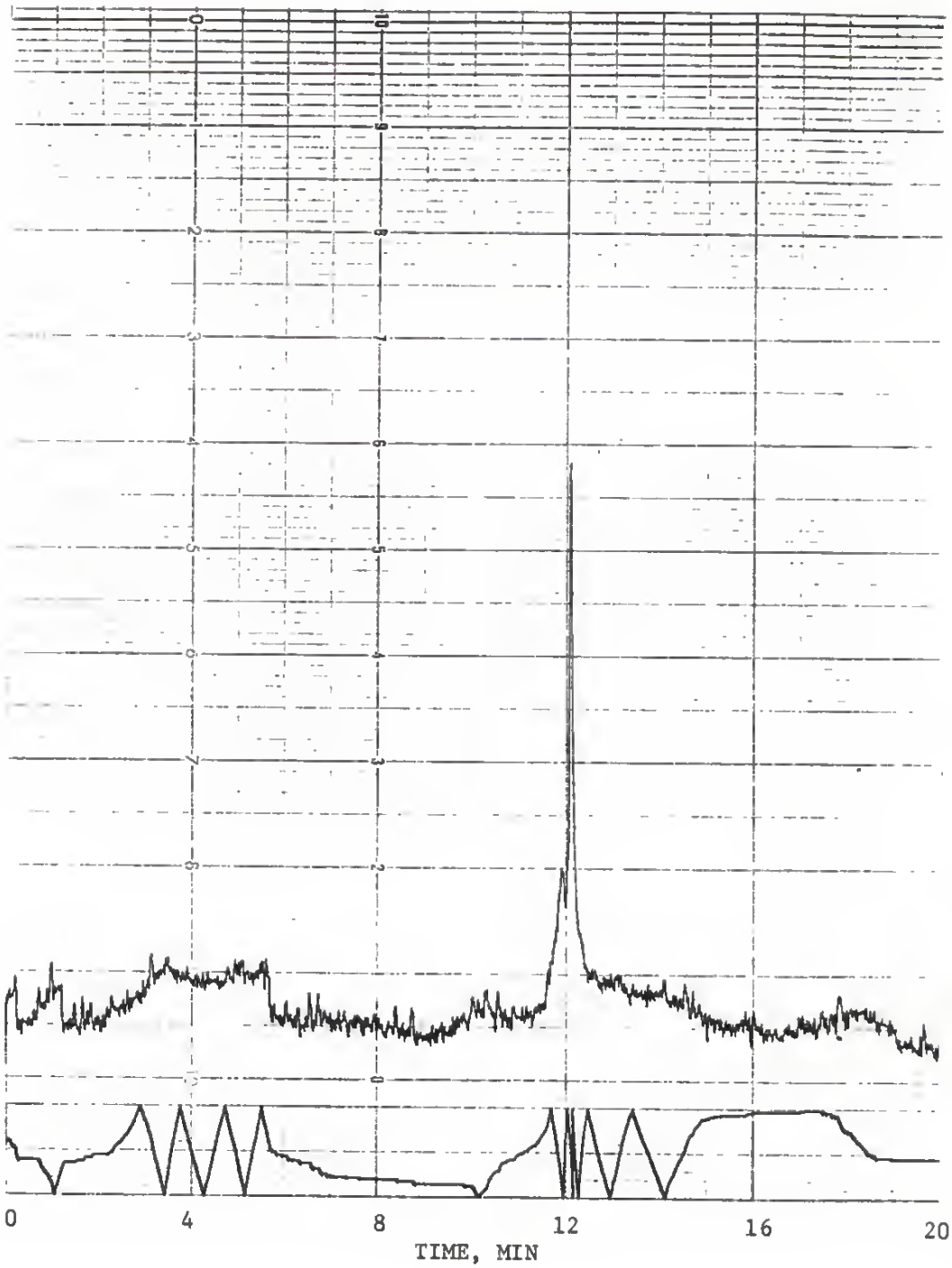

Fig. II.4. Result of HPLC analysis before biodegradation for sample No. 4 at absorbance = 0.005.

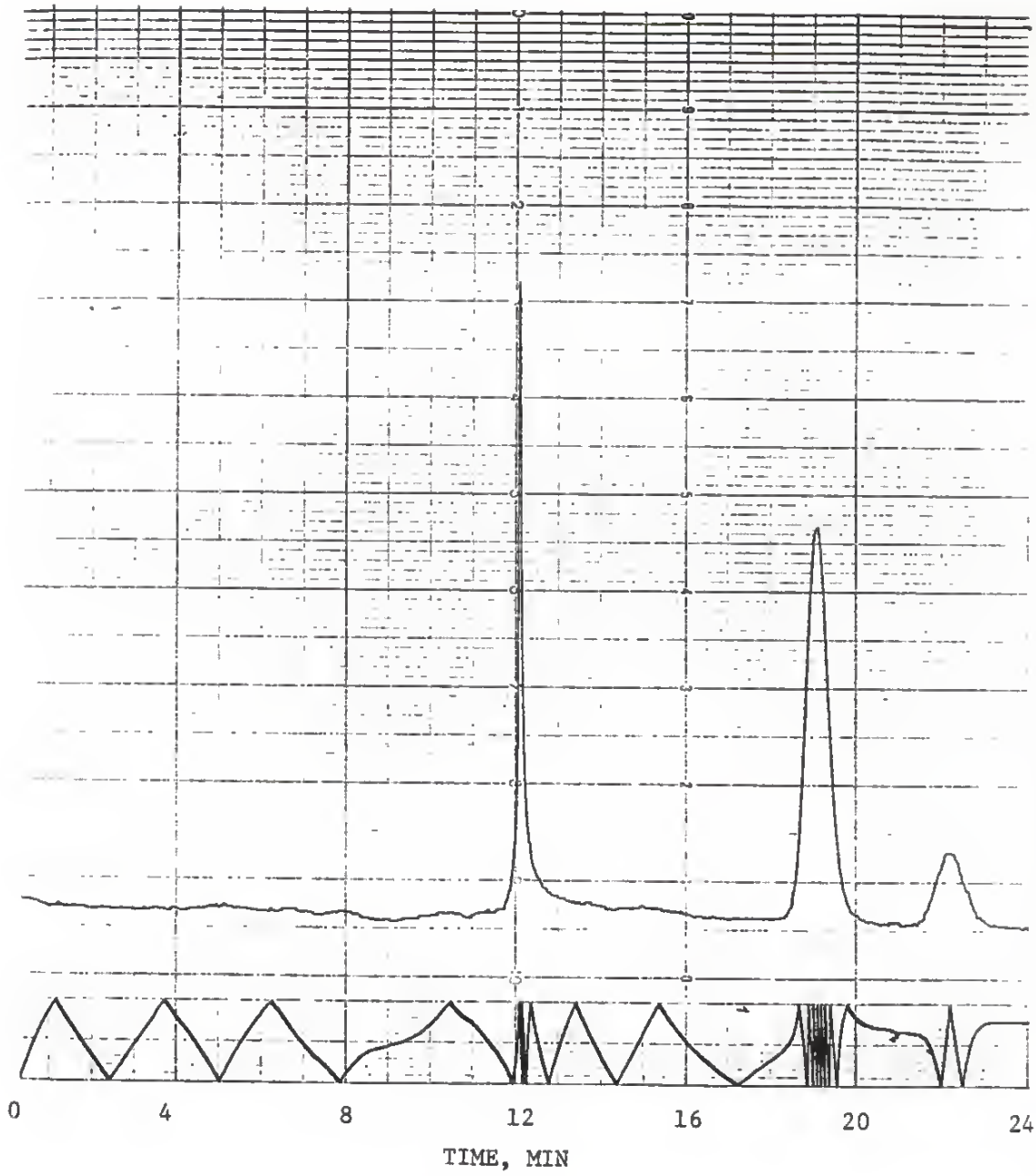

Fig. II.5. Result of HPLC analysis before biodegradation for sample No. 5 at absorbance = 0.02.

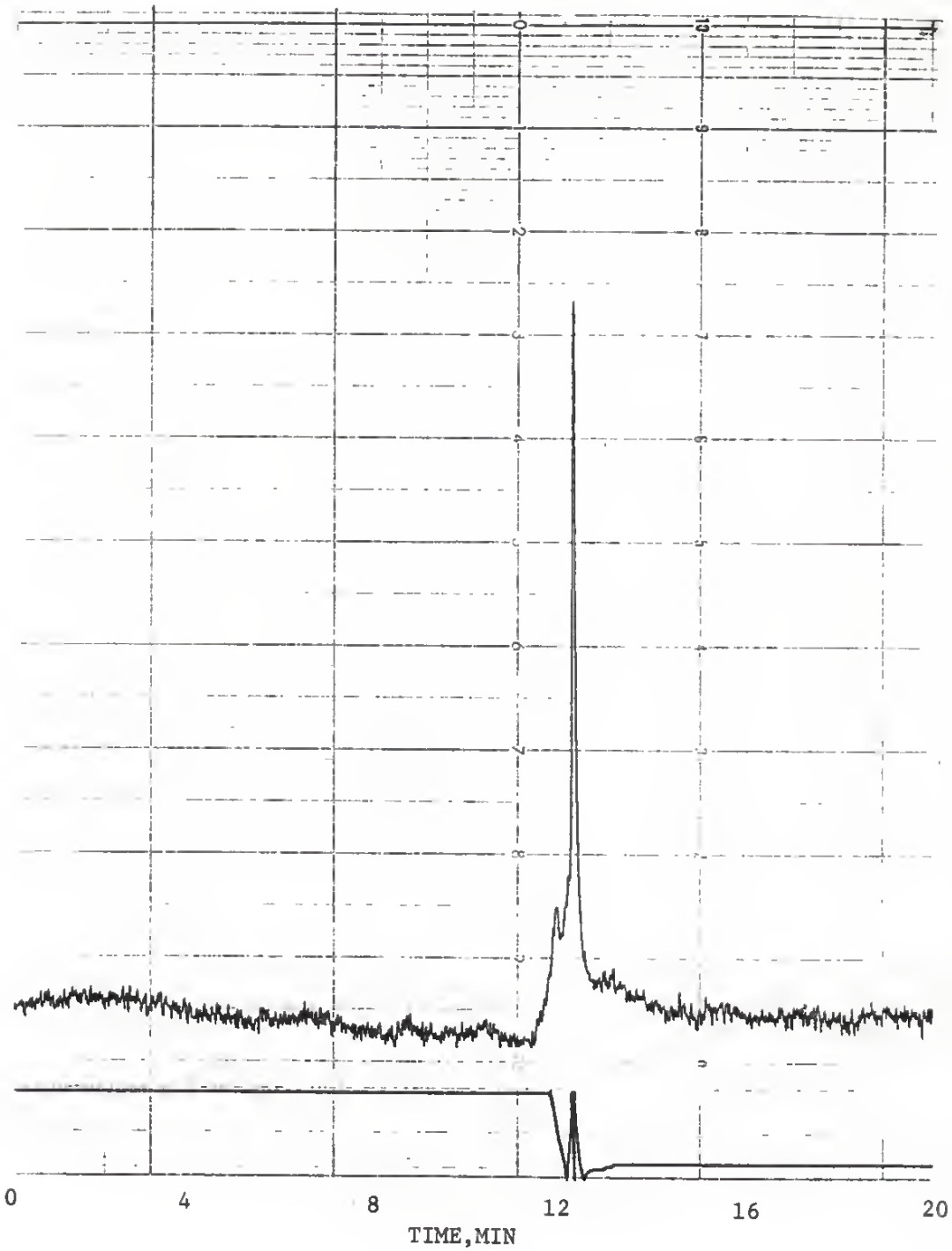

Fig. II.6. Result of HPLC analysis before biodegradation for sample No. 6 at absorbance = 0.005.

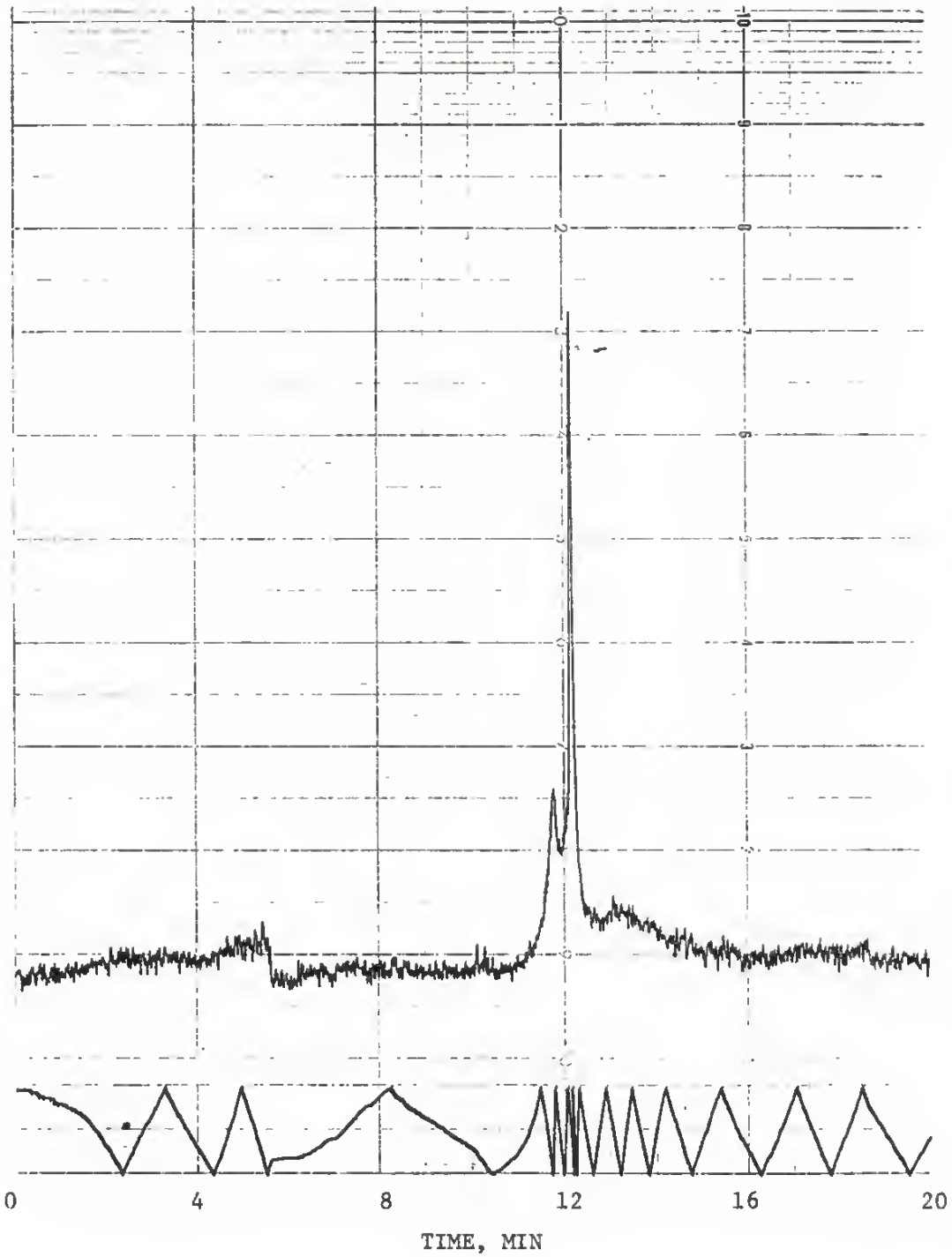

Fig. II.7. Result of HPLC analysis before biodegradation for sample No. 7 at absorbance = 0.005.

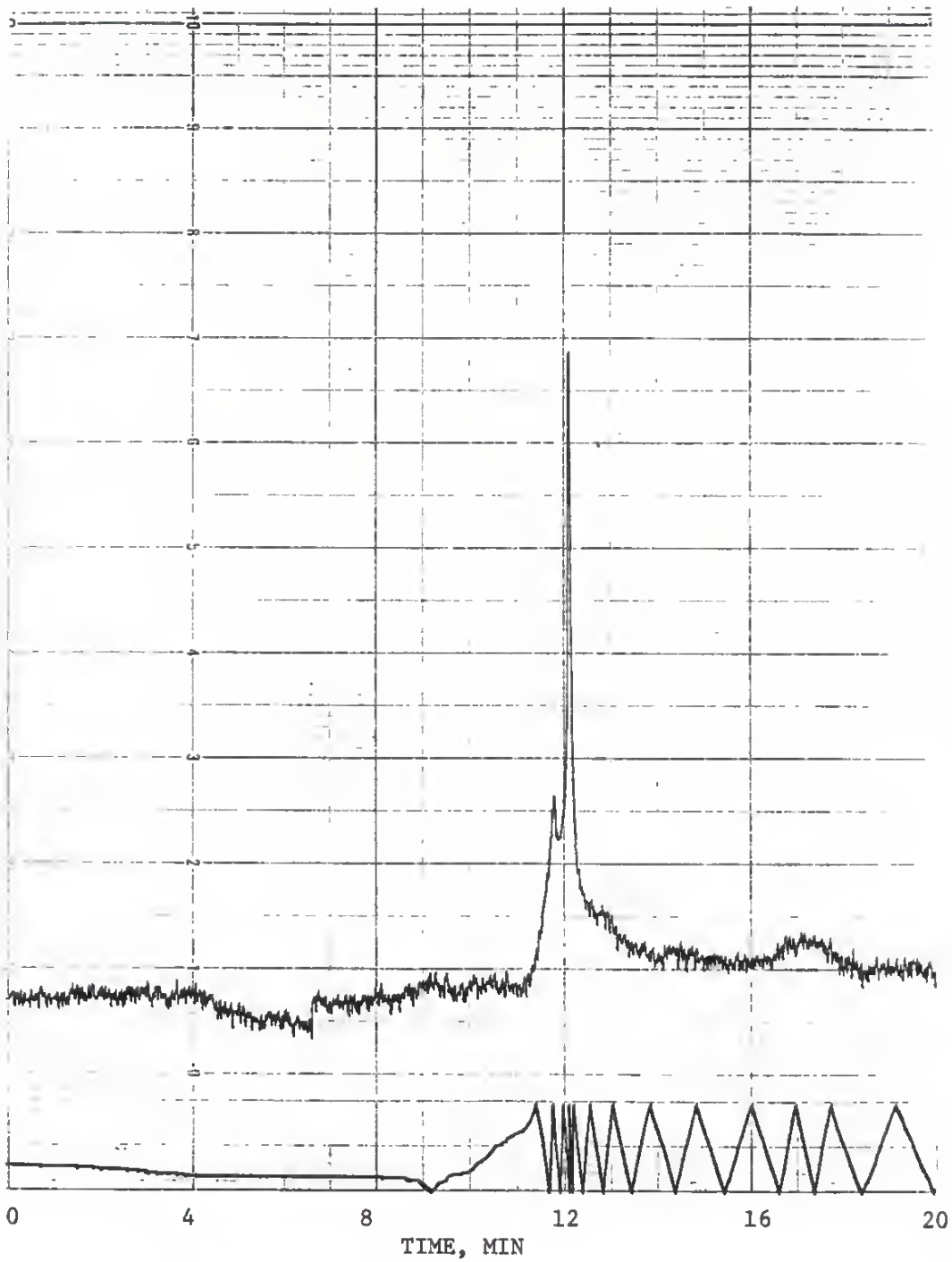

Fig. II.8. Result of HPLC analysis before biodegradation for sample No. 8 at absorbance = 0.005.

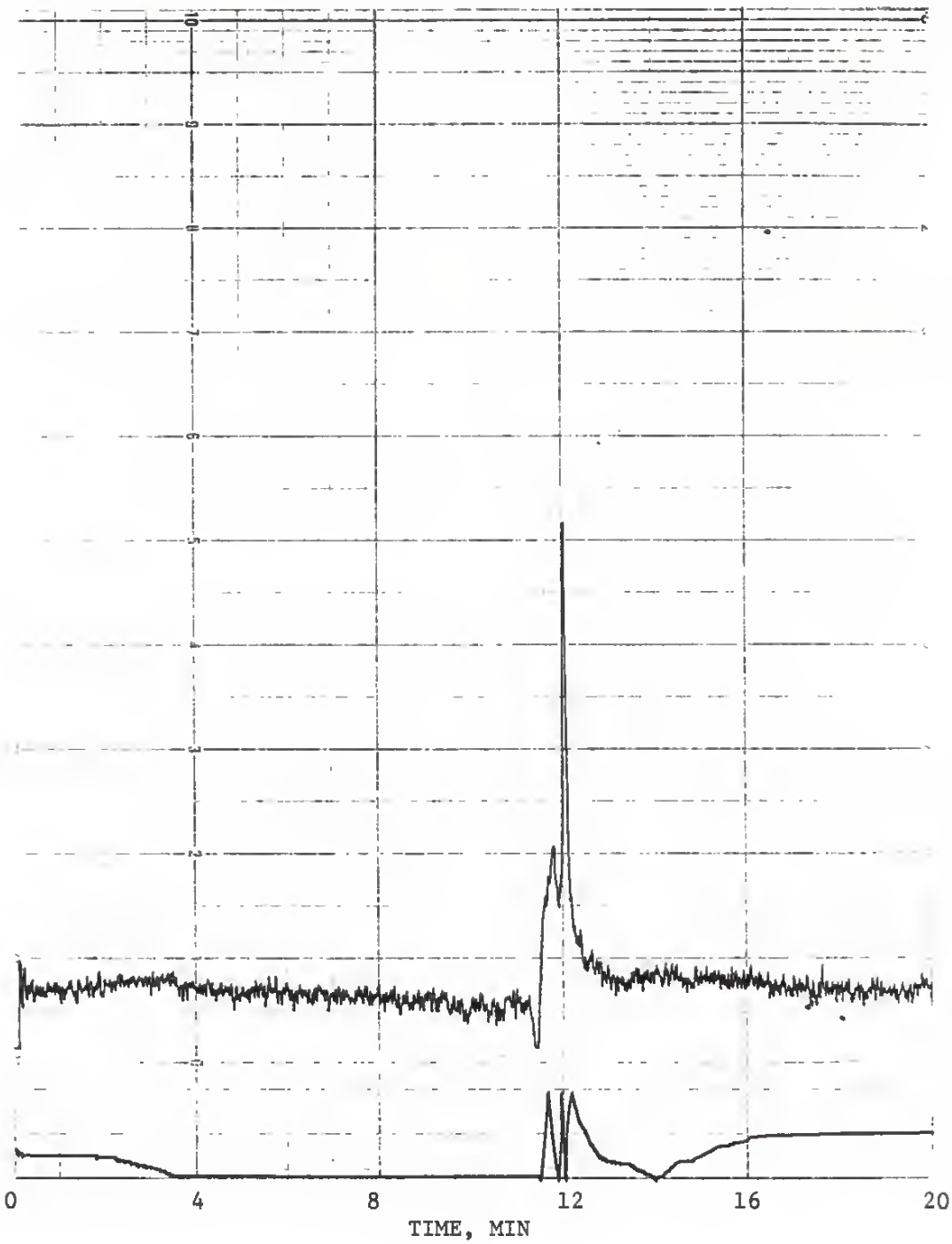

Fig. II.9. Result of HPLC analysis before biodegradation for sample No. 9 at absorbance = 0.005.

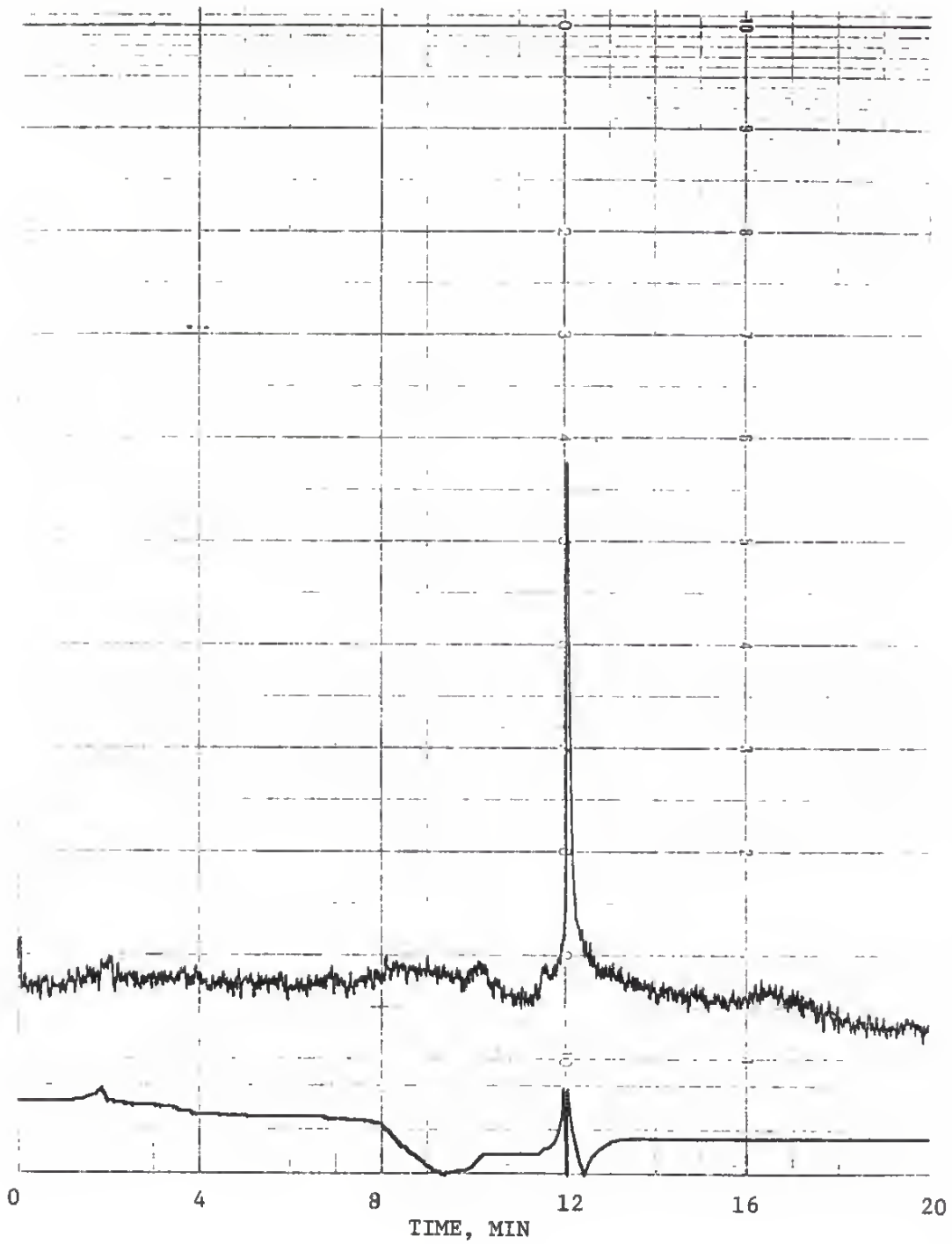

Fig. II.10. Result of HPLC analysis before biodegradation for sample No. 10 at absorbance = 0.005.

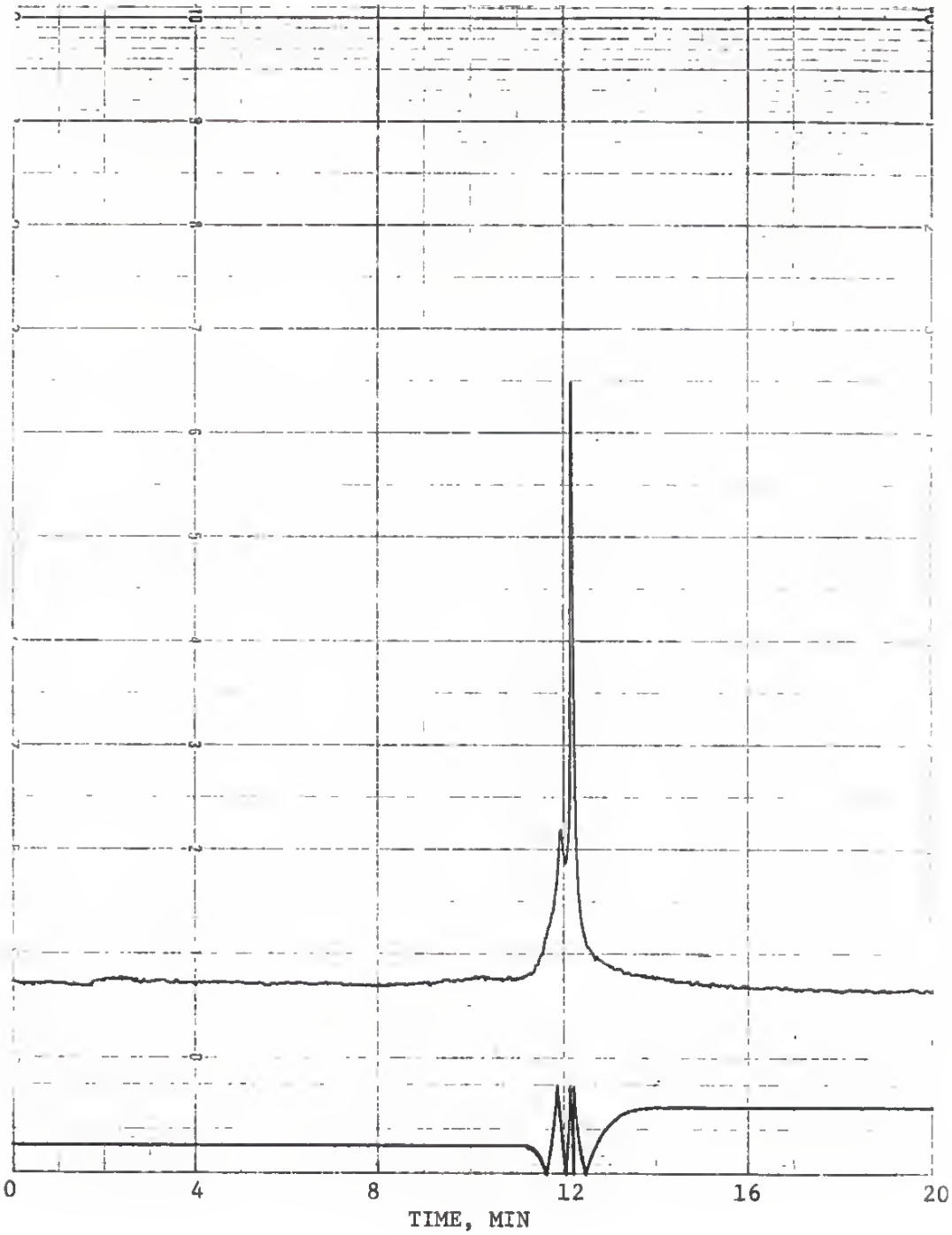

Fig. II.11. Result of HPLC analysis before biodegradation for sample No. 11 at absorbance = 0.01.

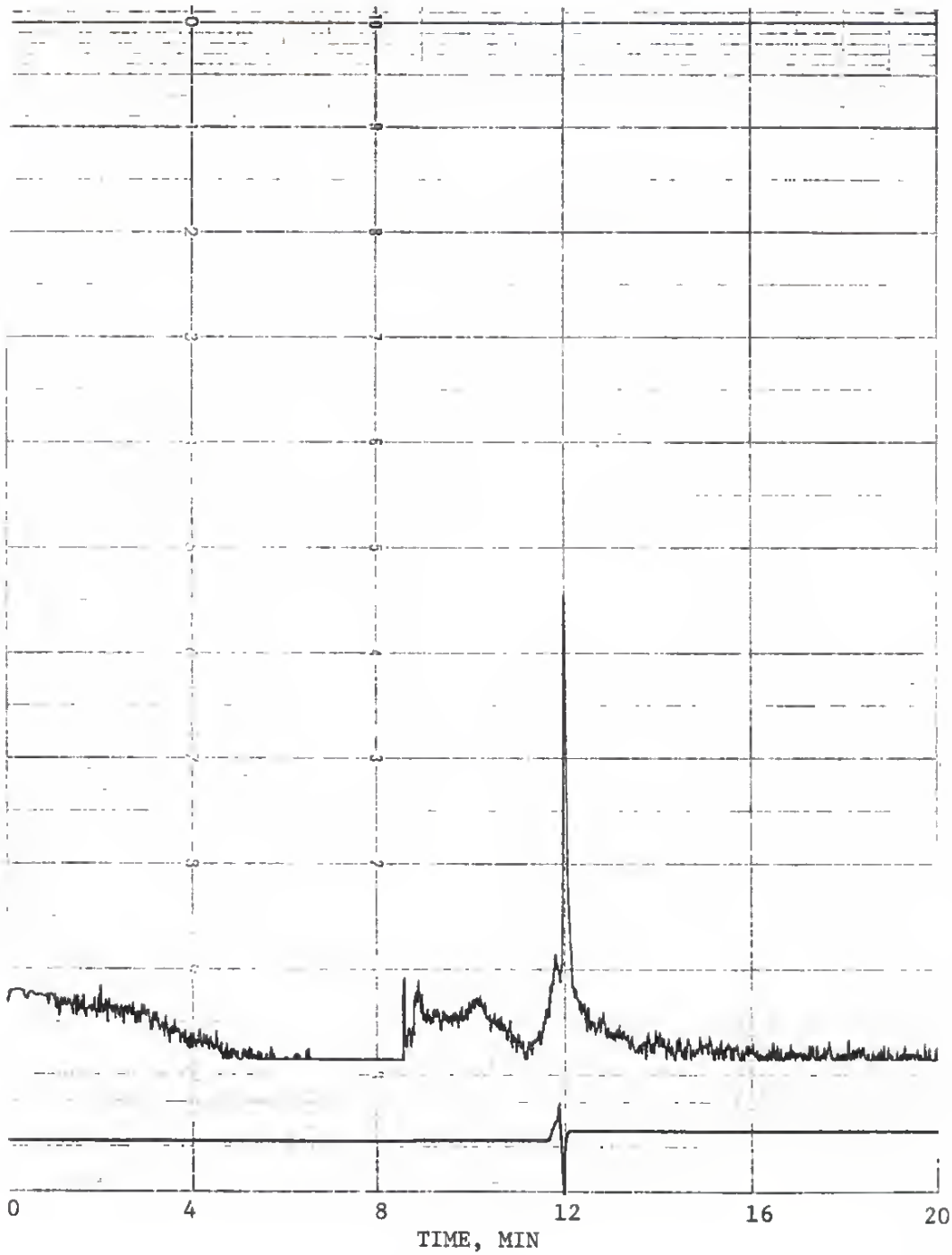

Fig. II.12. Result of HPLC analysis before biodegradation for sample No. 12 at absorbance = 0.005.

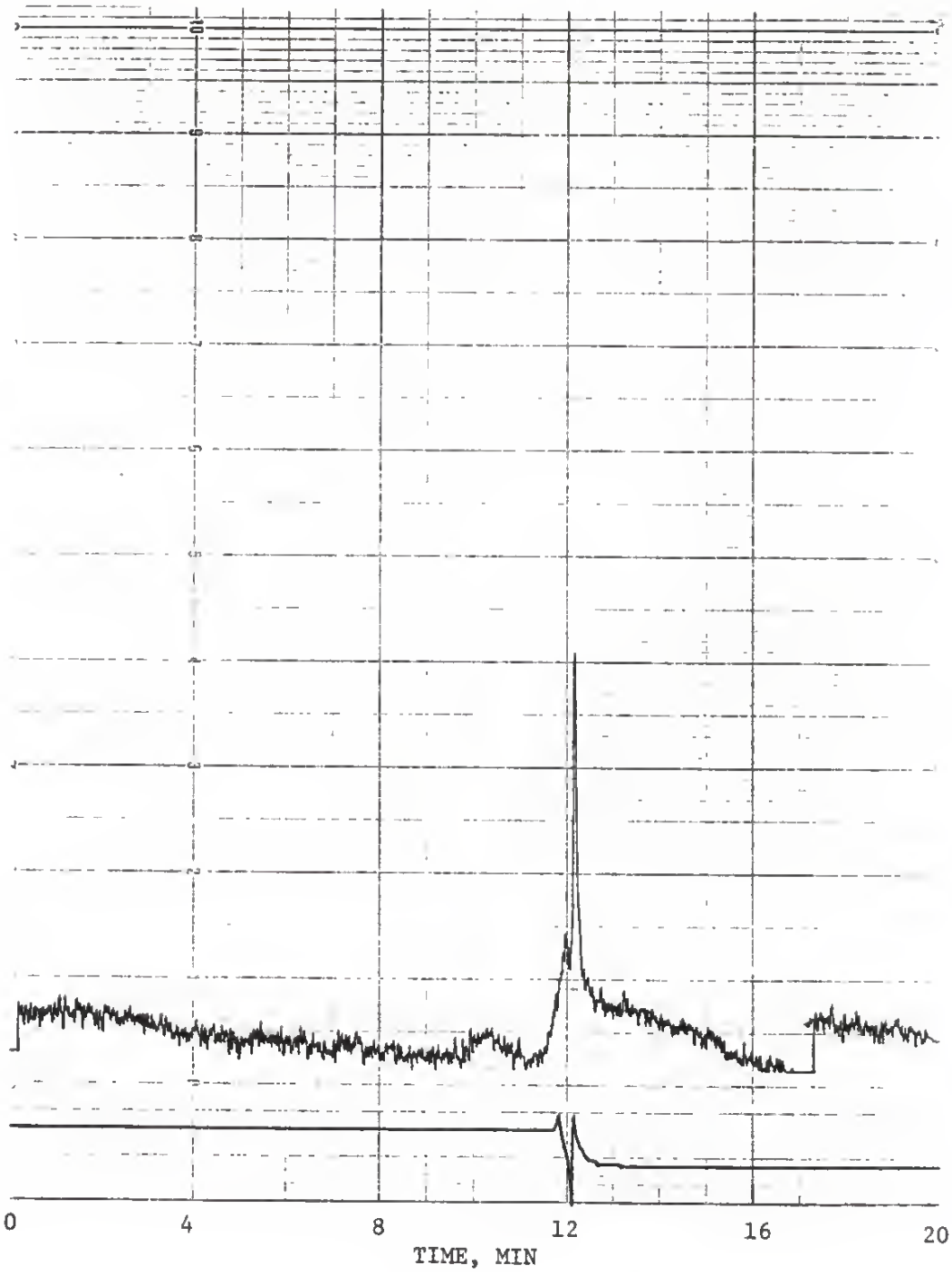

Fig. II.13. Result of HPLC analysis before biodegradation for sample No. 13 at absorbance = 0.005.

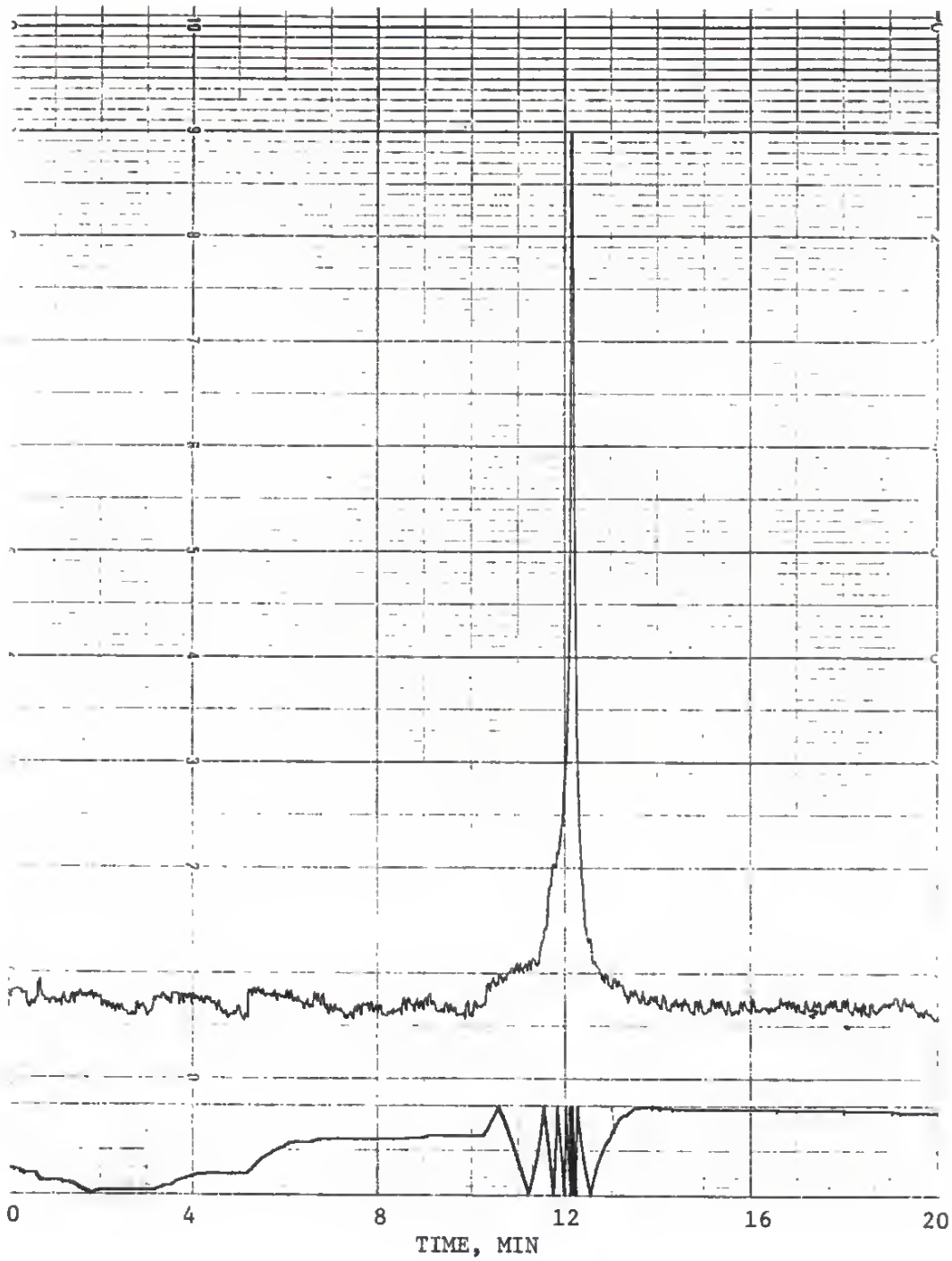

Fig. II.14. Result of HPLC analysis after biodegradation for sample No. 1 at absorbance = 0.01.

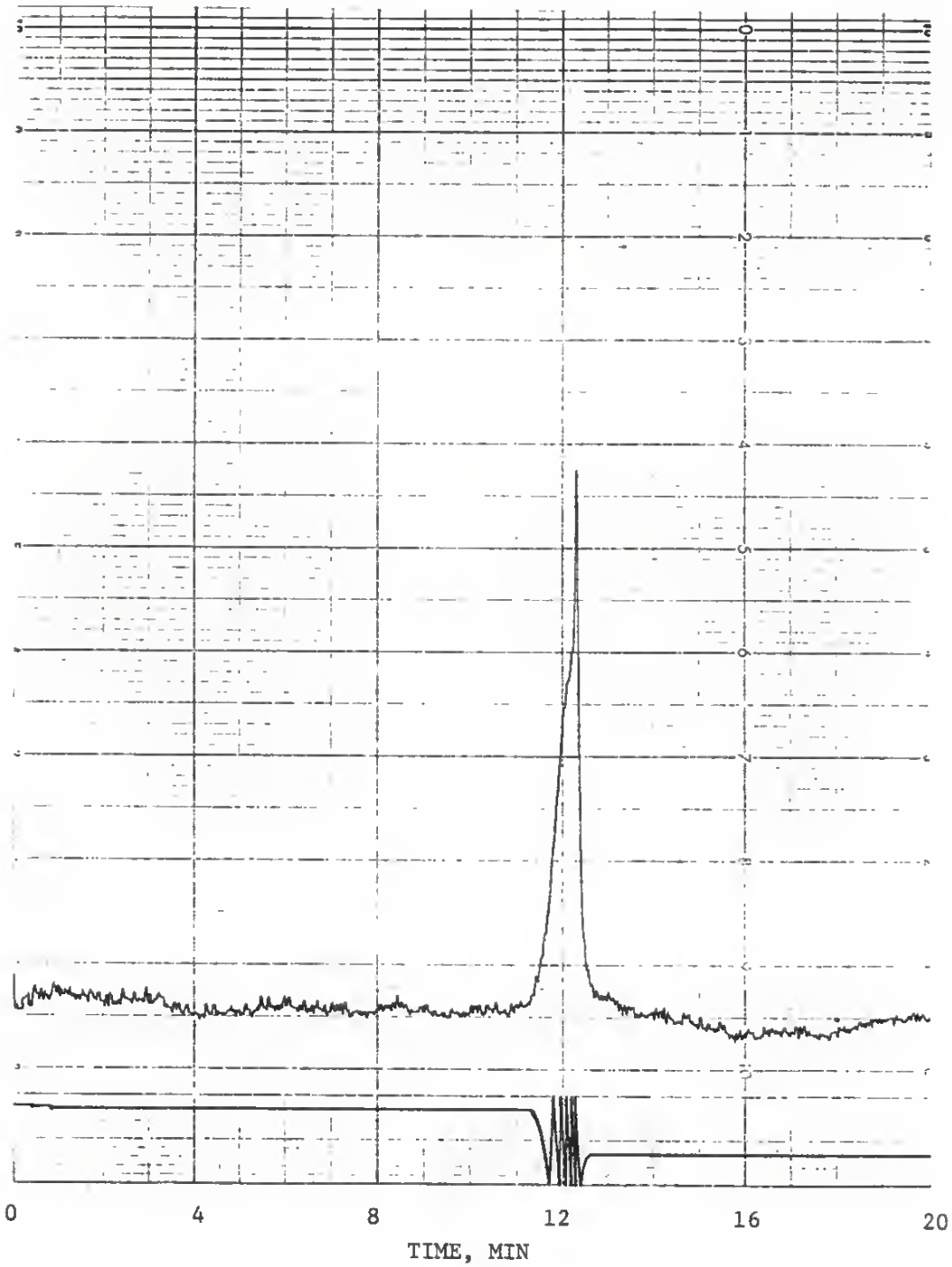

Fig. II.15. Result of HPLC analysis after biodegradation for sample No. 2 at absorbance = 0.01.

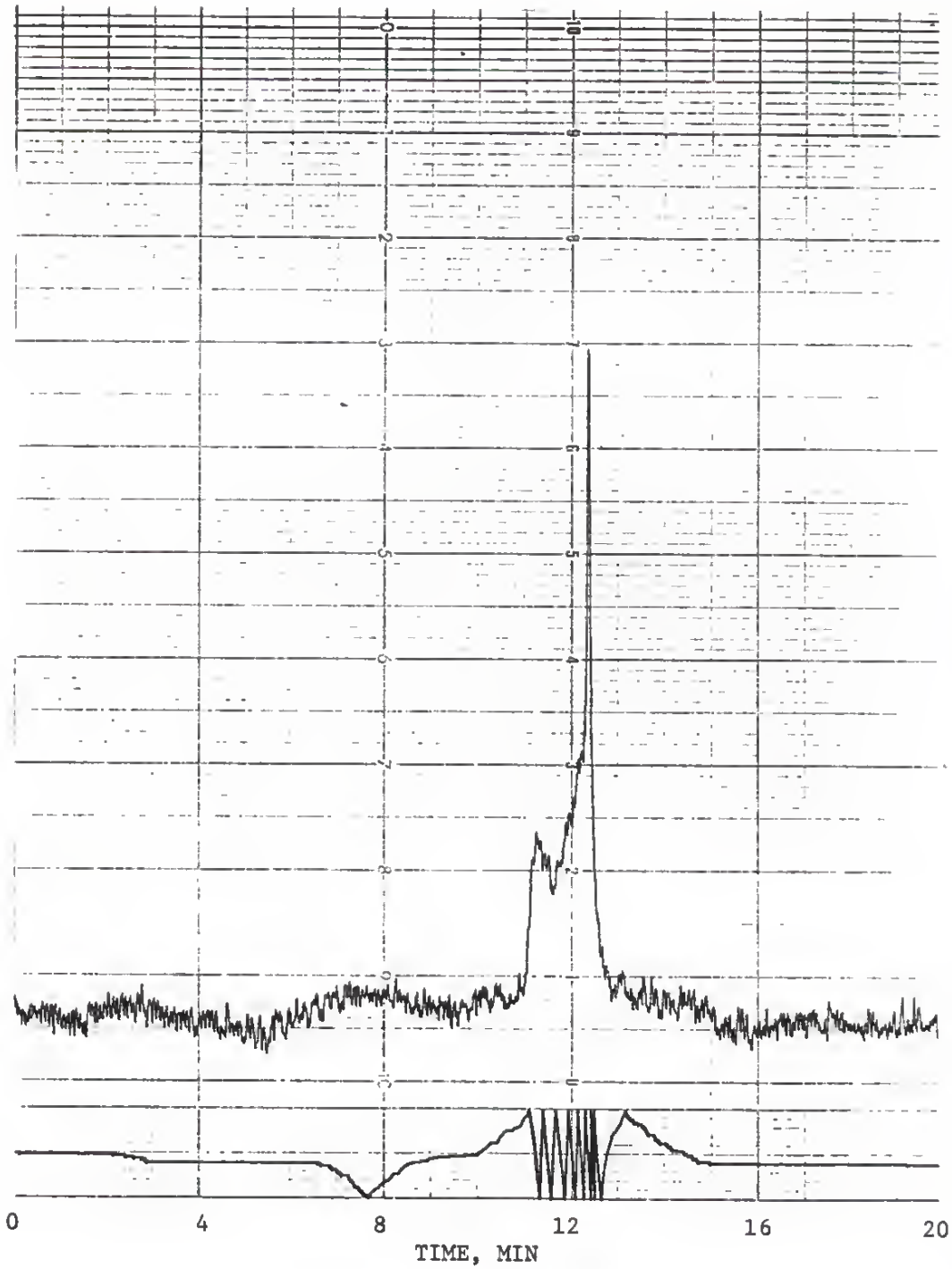

Fig. II.16. Result of HPLC analysis after biodegradation for sample No. 3 at absorbance = 0.005.

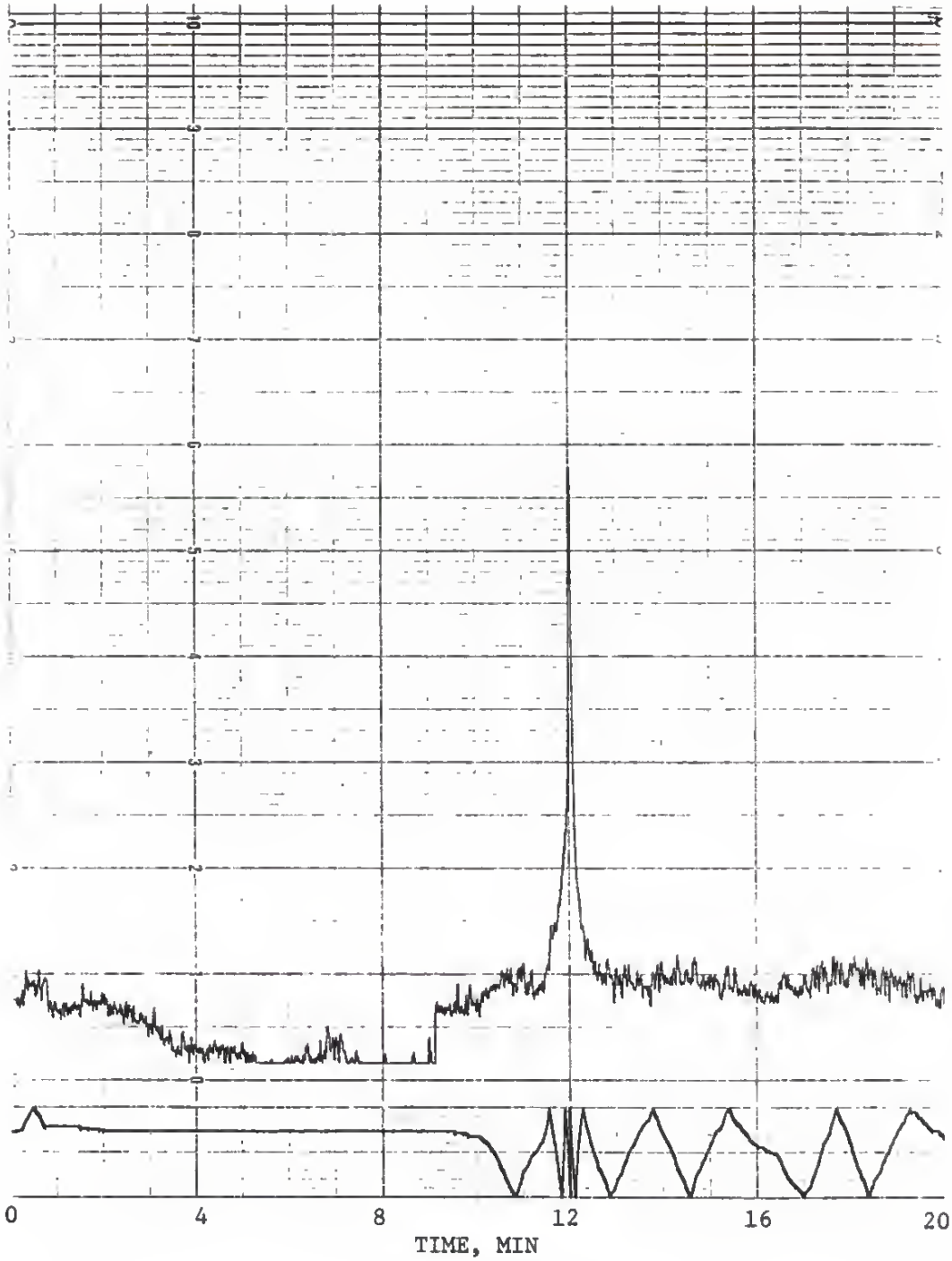

Fig. II.17. Result of HPLC analysis after biodegradation for sample No. 4 at absorbance = 0.005.

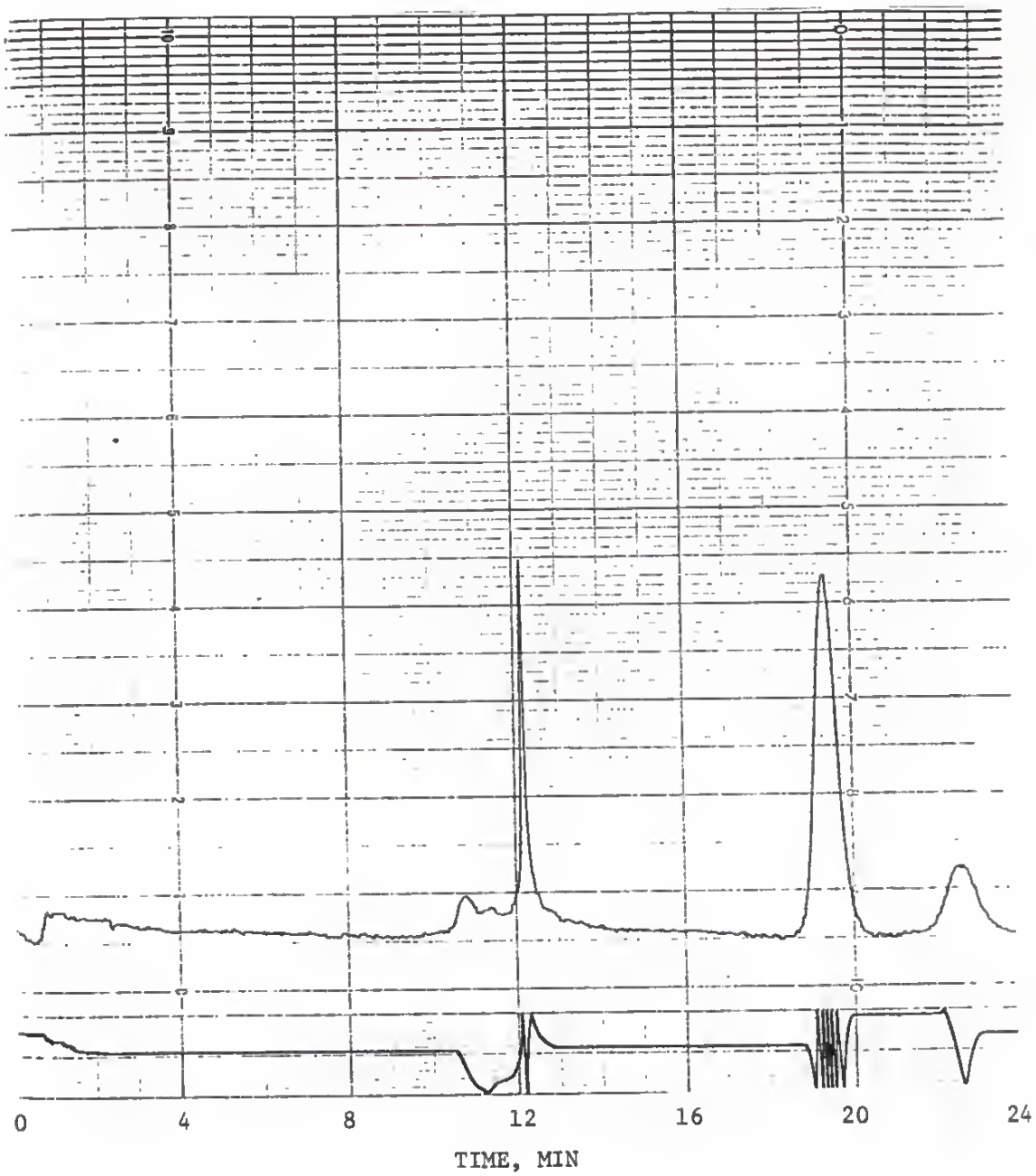

Fig. II.18. Result of HPLC analysis after biodegradation for sample No. 5 at absorbance = 0.02.

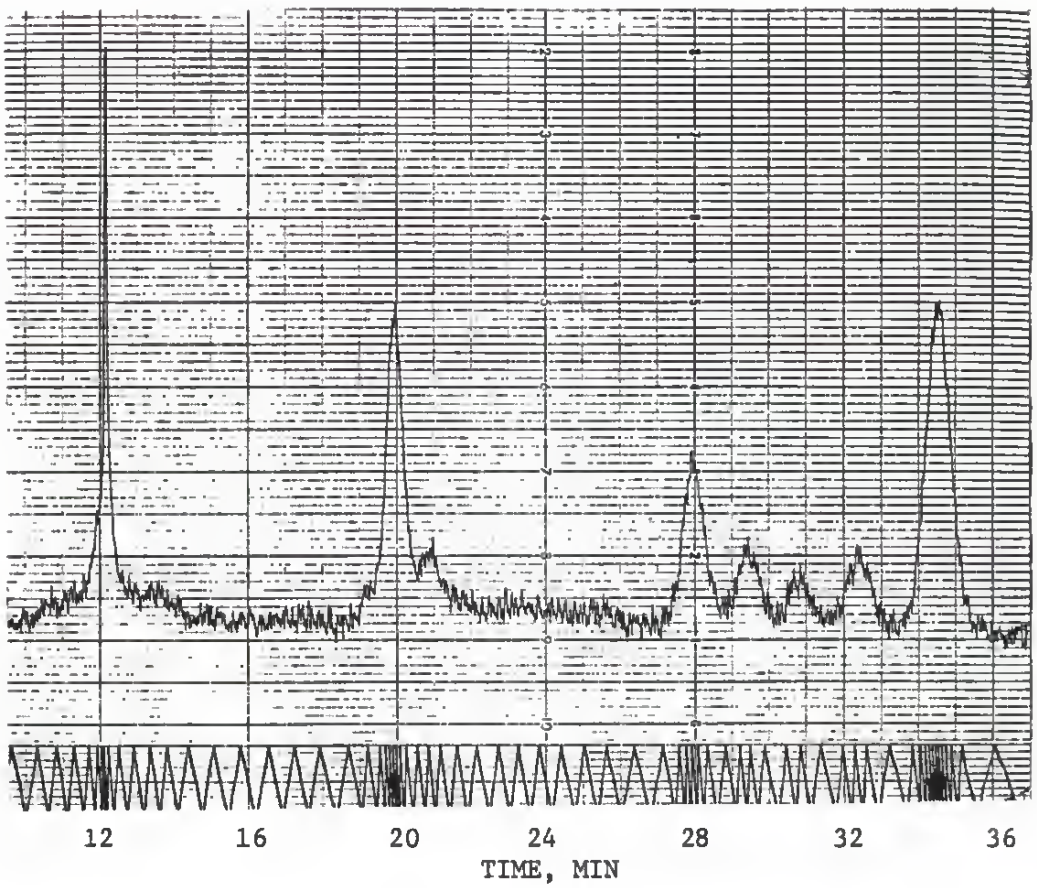

Fig. II.19. Result of HPLC analysis after biodegradation for sample No. 6 at absorbance = 0.005.

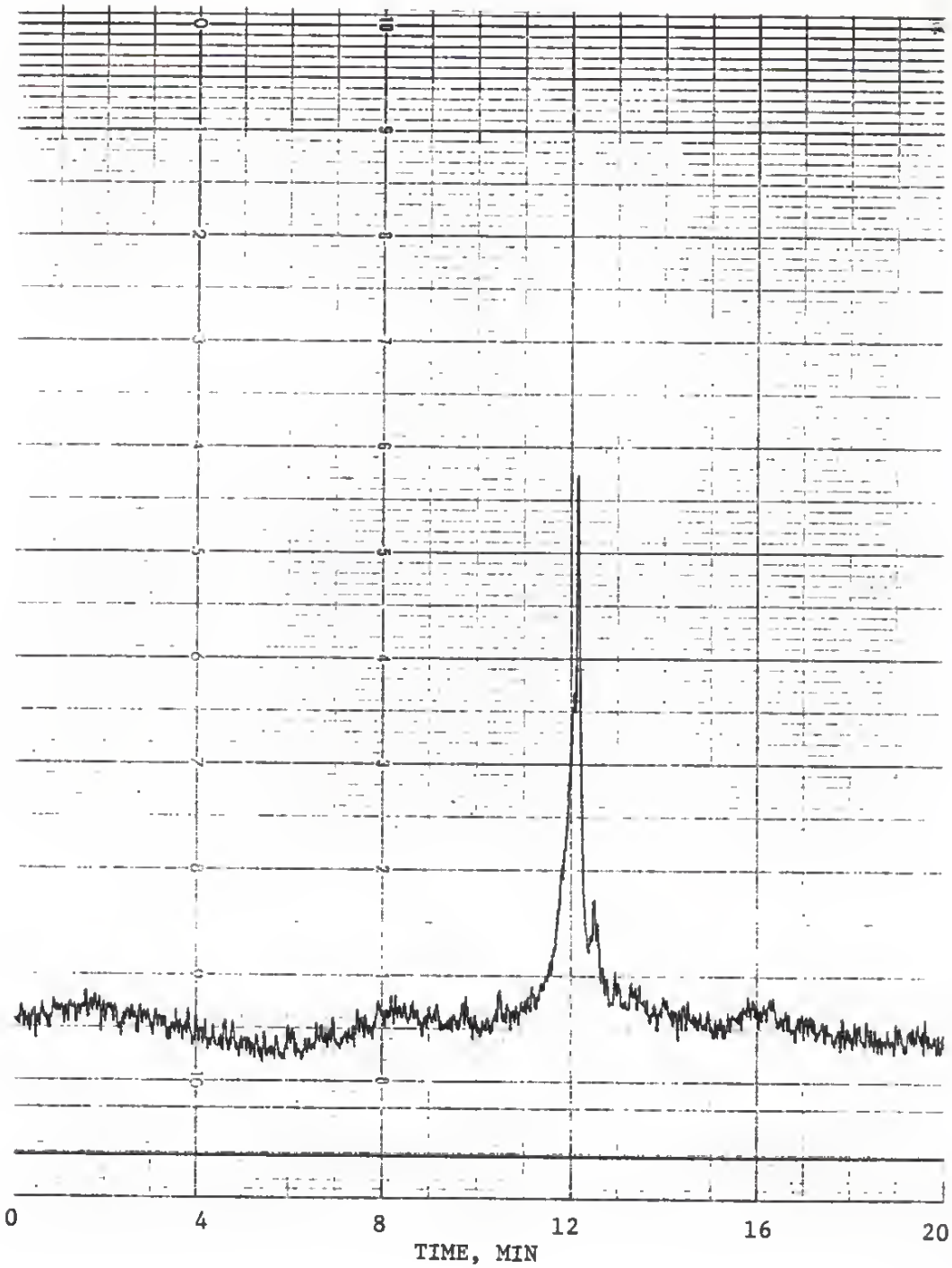

Fig. II.20. Result of HPLC analysis after biodegradation for sample No. 7 at absorbance = 0.005.

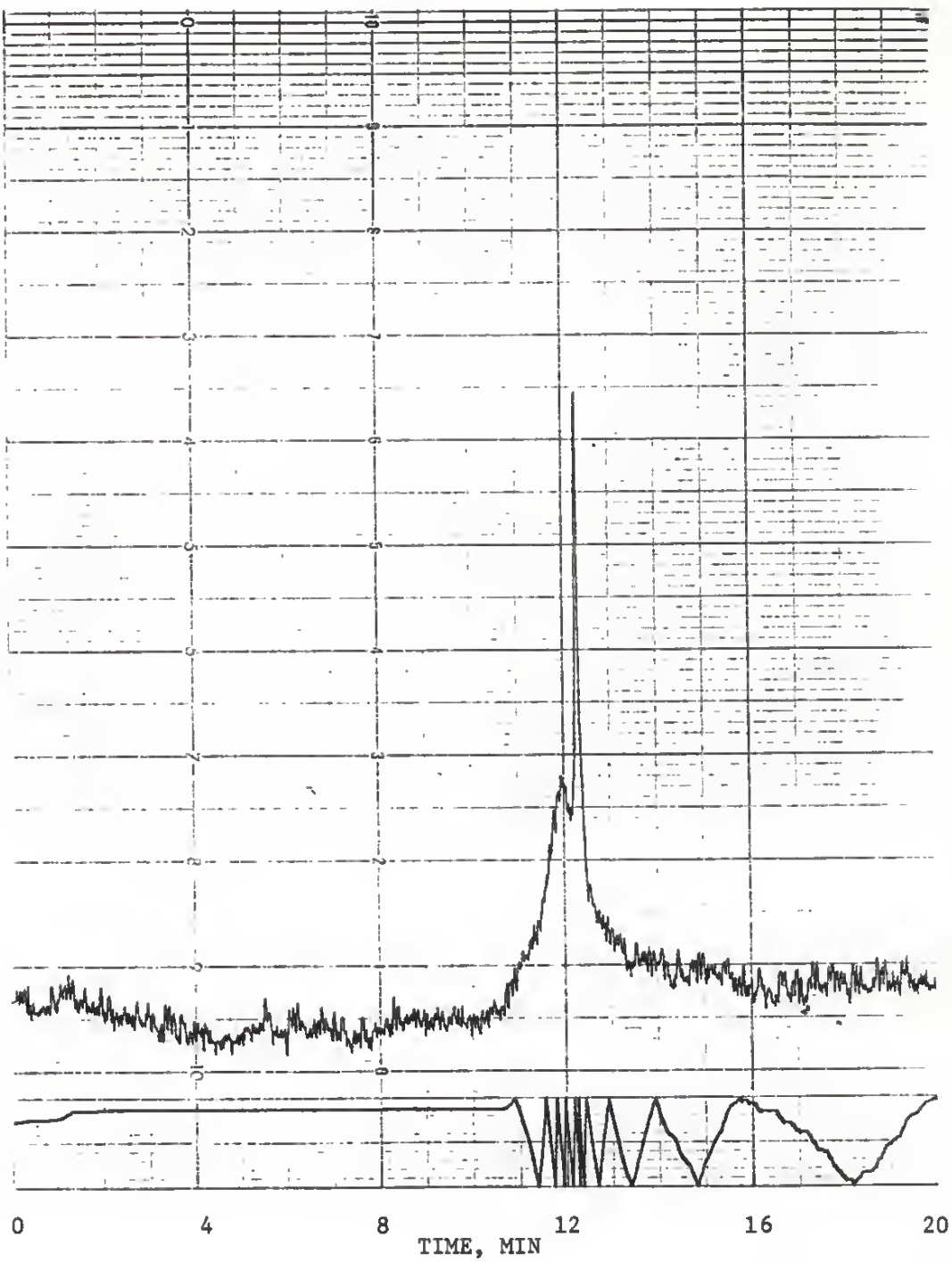

Fig. II.21. Result of HPLC analysis after biodegradation for sample No. 8 at absorbance = 0.005.

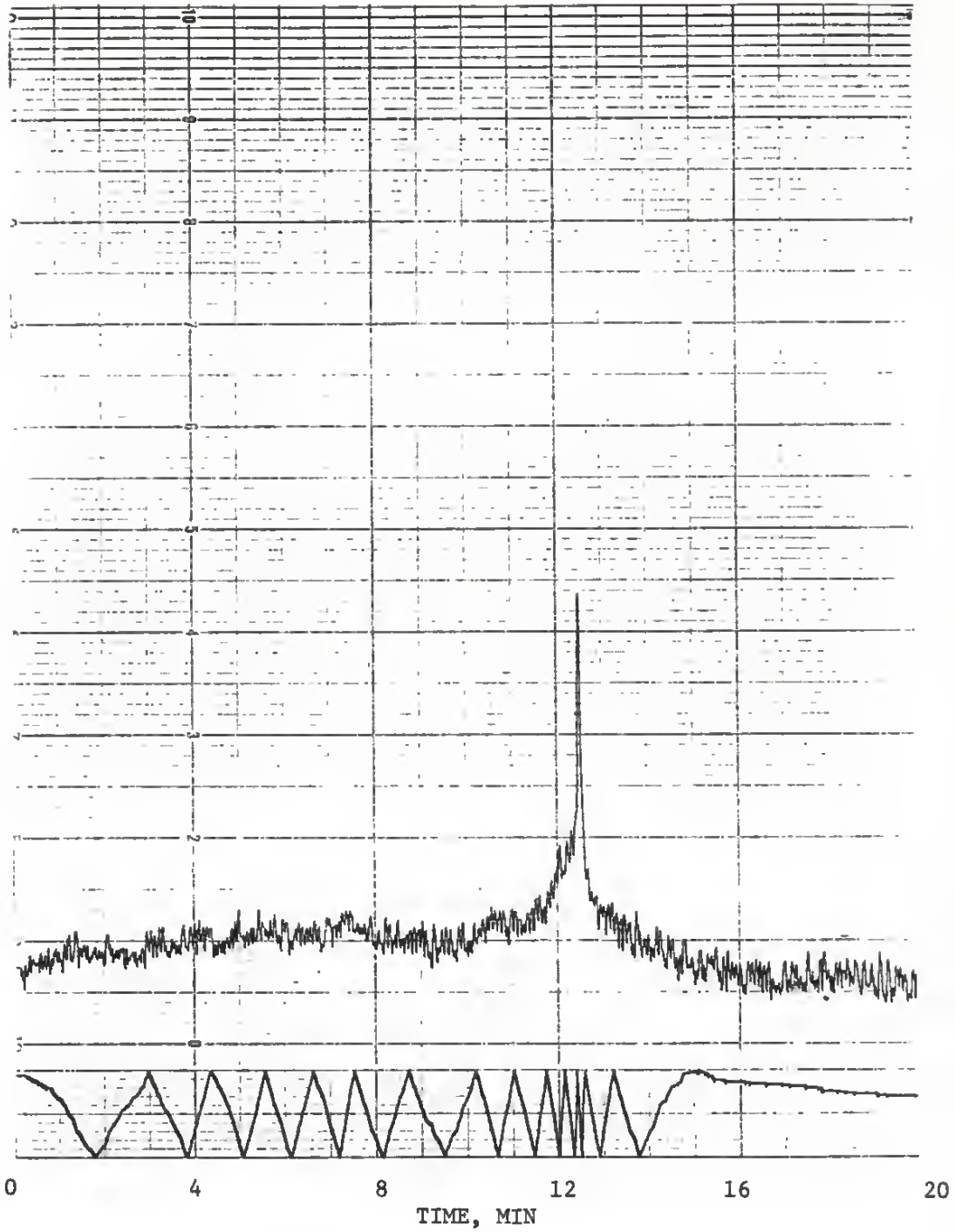

Fig. II.22. Result of HPLC analysis after biodegradation for sample No. 9 at absorbance = 0.005.

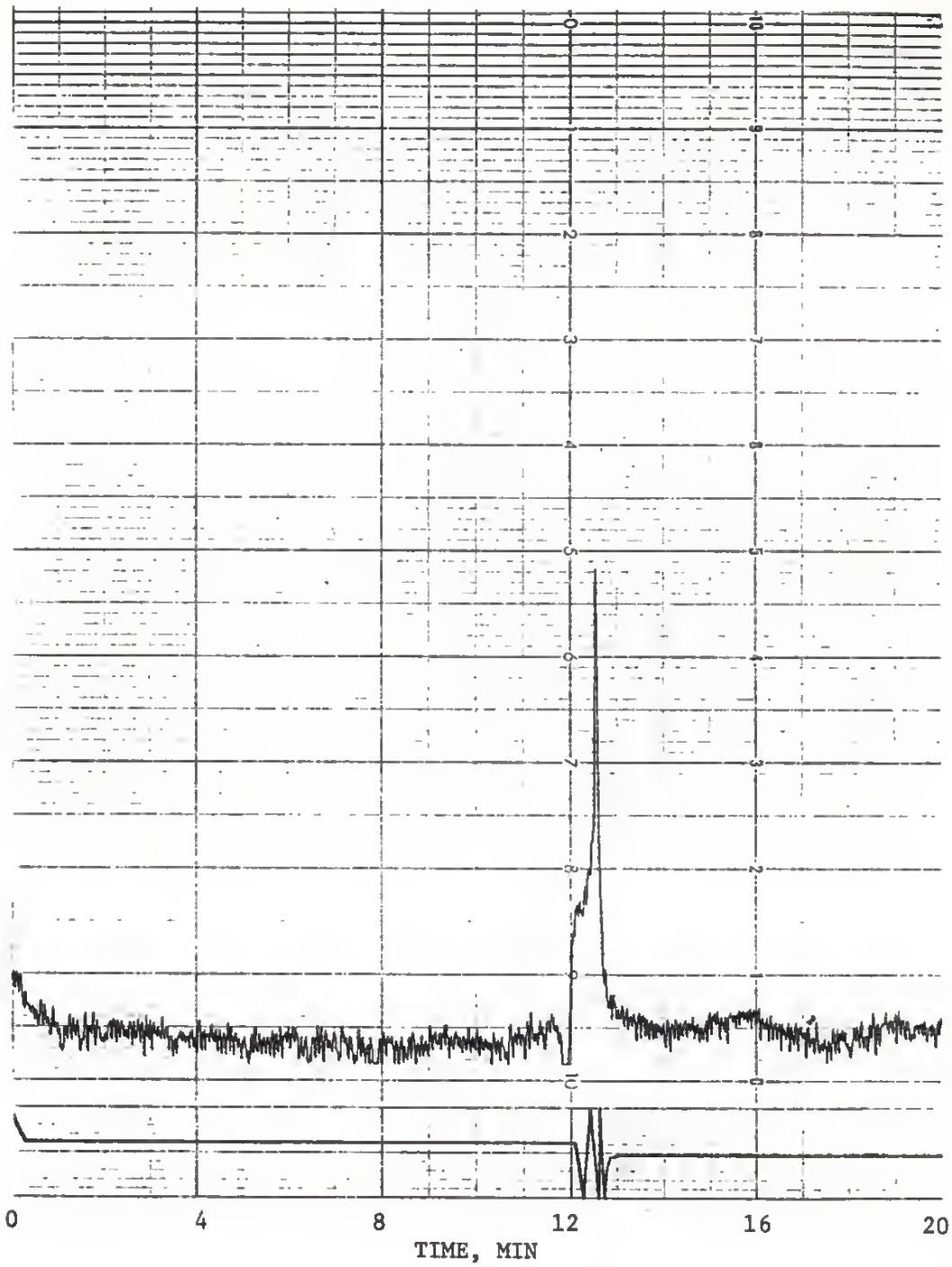

Fig. II.23. Result of HPLC analysis after biodegradation for sample No. 10 at absorbance = 0.005.

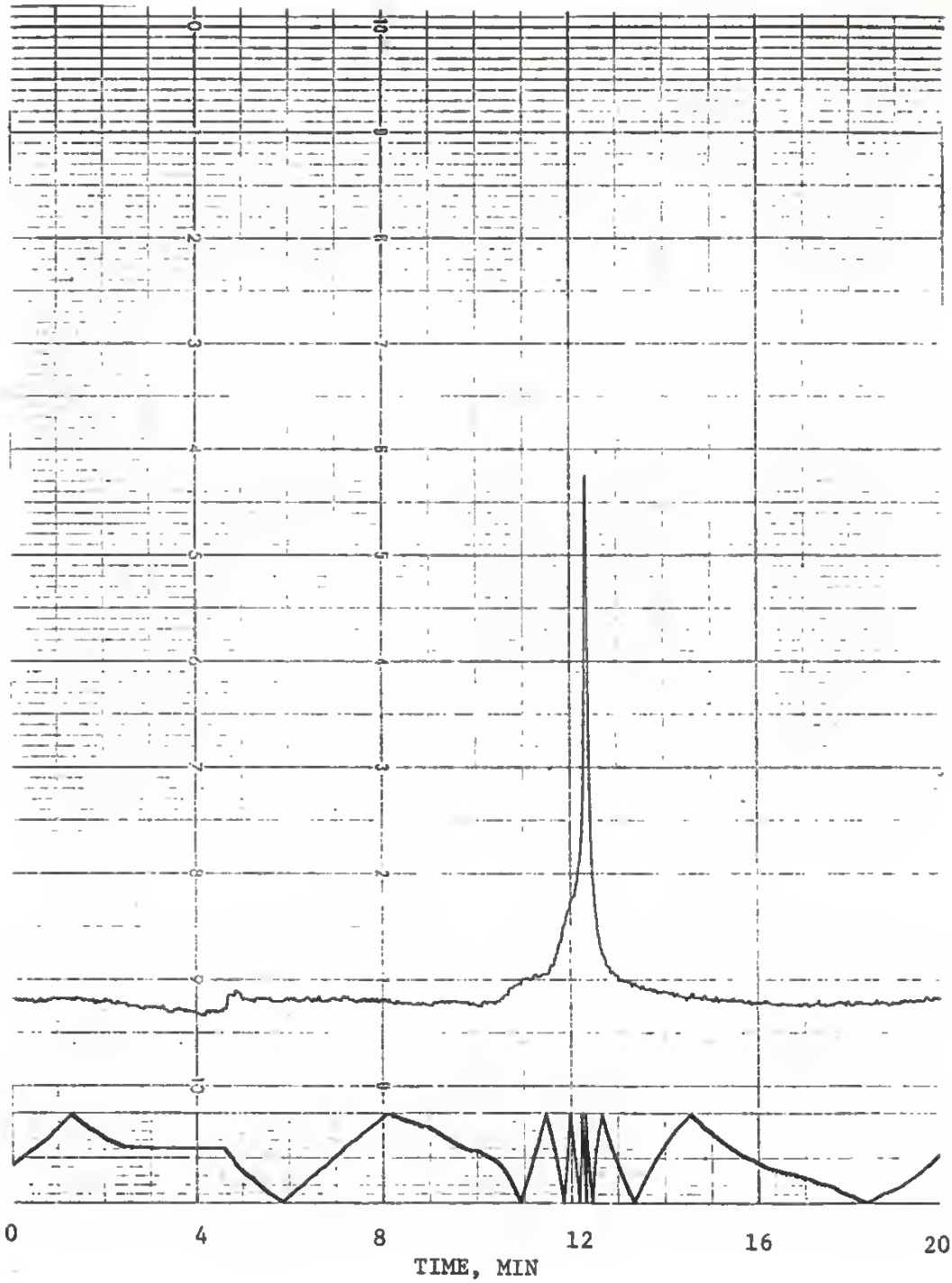

Fig. II.24. Result of HPLC analysis after biodegradation for sample No. 11 at absorbance = 0.02.

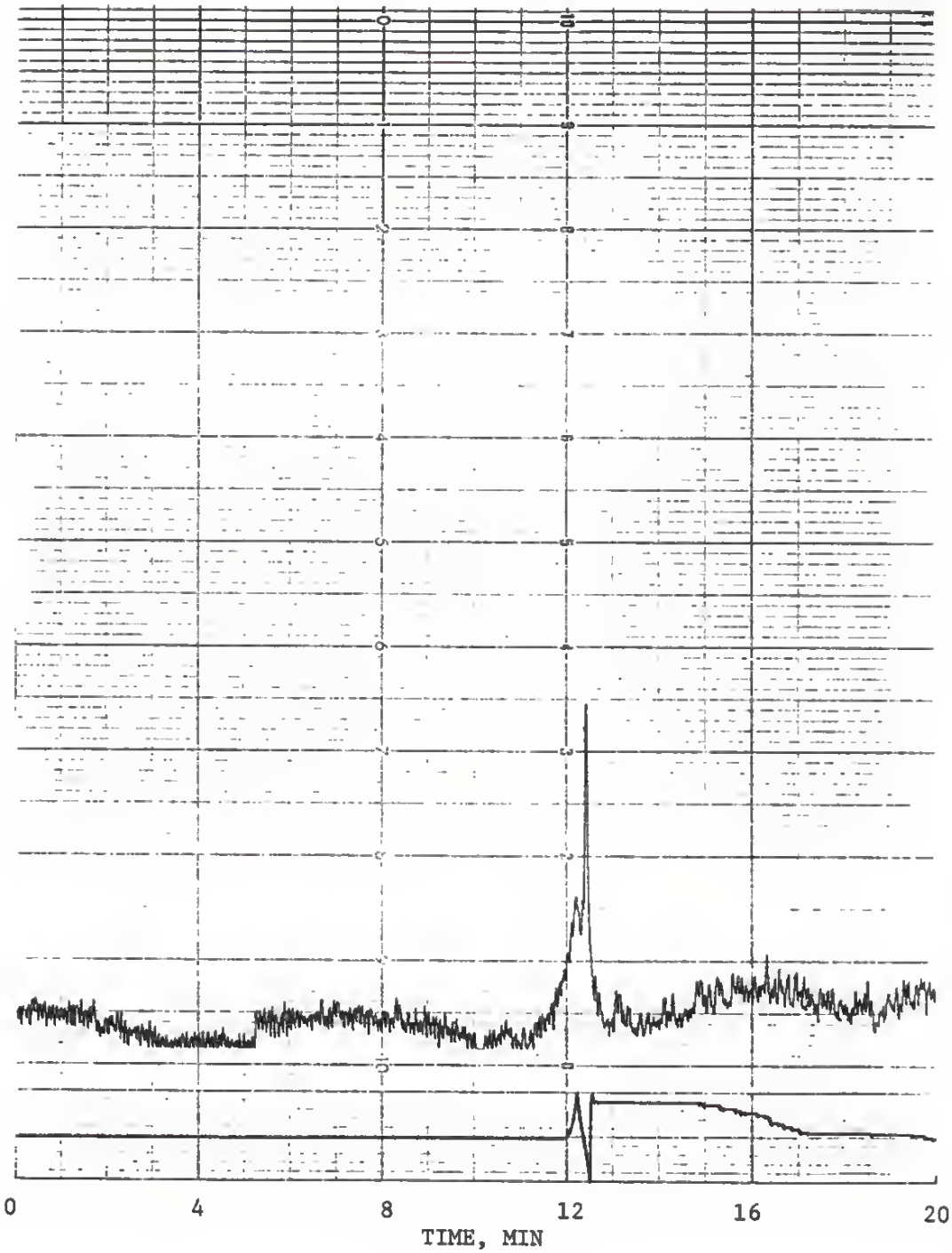

Fig. II.25. Result of HPLC analysis after biodegradation for sample No. 12 at absorbance = 0.005.

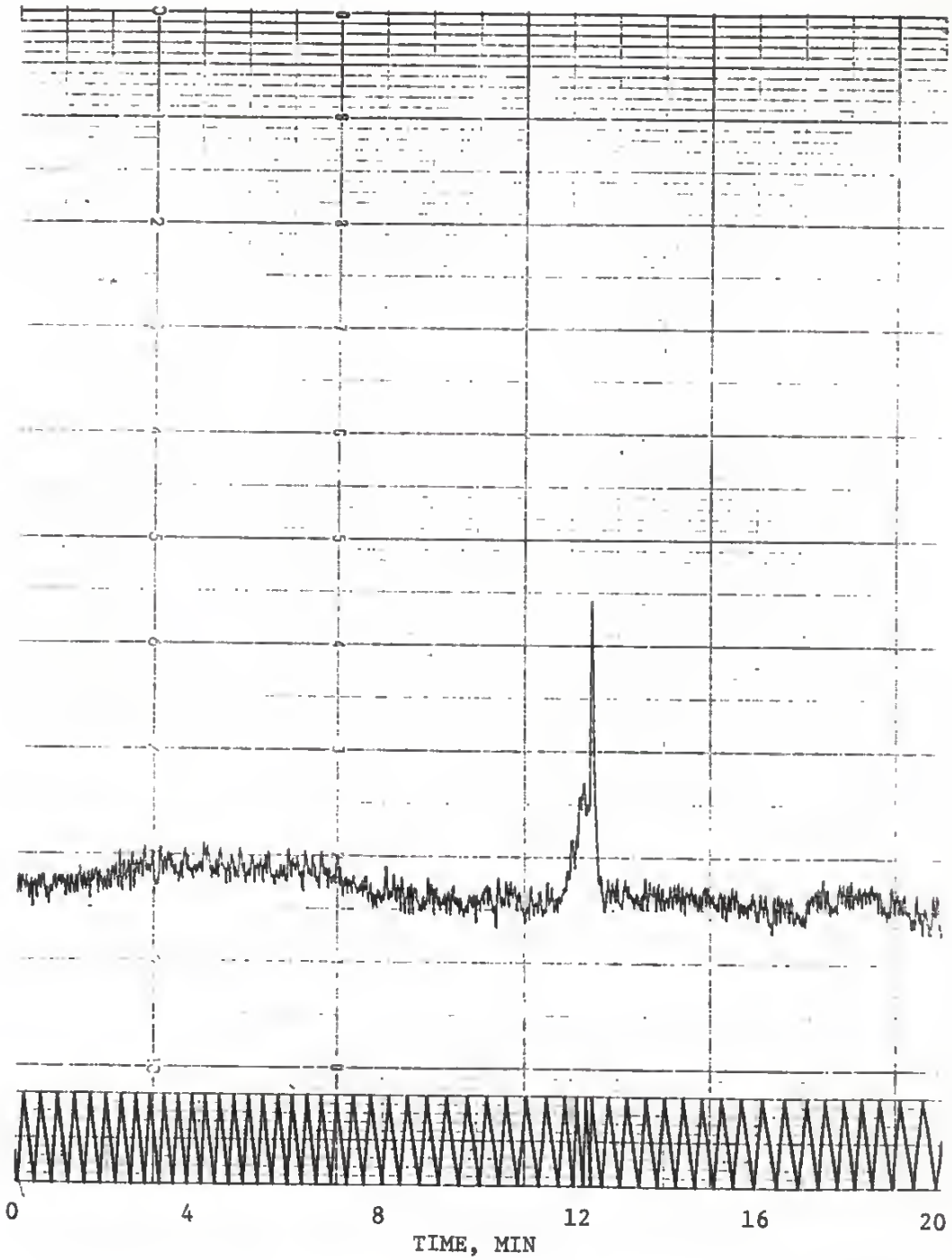

Fig. II.26. Result of HPLC analysis after biodegradation for sample No. 13 at absorbance = 0.005.

BIODEGRADATION OF METALWORKING FLUIDS

by

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

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1986

The use of synthetic and semi-synthetic metalworking fluids in the automotive industry is accelerating at a fast pace. Along with some of the benefits, the use of synthetic metalworking fluids has raised some concerns as well. One of these concerns is the fact that synthetic metalworking fluids are generally incompatible with existing wastewater treatment systems at automotive manufacturing plants.

The first objective of this research was to study the biodegradation of synthetic metalworking fluids. The results show that, in general, metalworking fluids are readily biodegraded with mixed populations of sewage origin.

The second objective of this research was to investigate the adaptation and dilution effects with developed mixed culture, and the pH effect with mixed population of sewage origin. The lag time was reduced using adapted culture. For optimal treatment of the substrate concentration of metalworking fluid wastewater should be maintained at 3000-6000 mg/liter of COD, and pH should be adjusted to 7.5-8.0. The three major species, Pseudomonas aeruginosa, Proteus mirabilis, and Klebsiella pneumoniae. were identified during the batch runs.