A PRELIMINARY ASSESSMENT FOR THE USE OF METABOLIC INHIBITORS TO EVALUATE THE BIODEGRADATION POTENTIAL OF SOIL

by

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(ABSTRACT)

The widespread contamination of our nation's groundwater has become a major threat to public health. The magnitude of this threat is escalated by our reliance upon groundwater. Groundwater is the primary drinking water source for over 50% of the U.S. population. Groundwater comprises more than 95% of all available freshwater in the United States.

The objectives of this study were: (1) to make a preliminary assessment of the use of inhibitors to evaluate the biodegradation potential of soil, and (2) to evaluate the significance of each microbial group in the biodegradation process.

Static microcosms containing Newport News and Blacksburg soil were used as the experimental environments. Butyrate and propionate were used as substrates. The metabolic inhibitors used were sodium molybdate (Na_2MoO_4) and 2-Bromoethane sulfonic acid (BESA), which inhibit sulfate reduction and methanogenesis, respectively. Denitrification was suppressed by the absence of sodium nitrate ($NaNO_3$) in the microcosm dilution water.

Both Newport News and Blacksburg soils were found to be dominated by denitrfying bacteria. In nitrate-amended Newport News microcosms, 300 mg/L butyrate degraded to zero concentration in 5.5 days. This microcosm's degradation rate was 255% greater than non-nitrate amended microcosms, where sulfate reduction was predominant. Little or no methanogenic activity was observed in both the Newport News or Blacksburg soils.

Advantages of this approach are its simplicity and directness in obtaining approximately the same information as more complicated enumeration procedures. A disadvantage of this approach is its reliance on metabolic inhibitors to suppress the microbial activity of specific microbial groups. Little is known on the effect of Na_2MoO_4 or BESA on the activities of denitrifiers, fermenters, or acetogens.

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CHAPTER 1

INTRODUCTION

The widespread contamination of our nation's groundwater by organic compounds has become a major threat to public health. The U.S. EPA estimates that 11 million gallons of gasoline alone may be leaking from underground storage tanks each year (O'Brien and Gere, 1988). A gallon of gasoline is capable of making up to a million gallons of groundwater unsafe to drink.

This problem is exacerbated by the reliance of our nation on groundwater. Groundwater is the primary source of drinking water for over 50% of the U.S. population (Buower *et al.*, 1988). It also provides 40% of the water used for agricultural irrigation (O'Brien and Gere, 1988). Forty million people are served by unregulated private wells (Buower *et al.*, 1988). Nearly 1.8 million Virginians rely completely on groundwater as their source of water for drinking (VWQA, 1988).

Virginia's major sources of groundwater contamination were listed in the 1988 Virginia Water Quality Assessment. Leaking underground storage tanks were listed as the greatest contributor, followed by municipal and industrial landfills and abandoned hazardous waste sites.

Once a groundwater system has been contaminated, several response actions may be taken. These include physical containment, *in situ* treatment with chemicals or microorganisms, and withdrawal and treatment via various forms of physical, chemical or biological processes. In some cases, the system must be abandoned in favor of alternative water supplies.

In recent years, *in situ* tretment with microorganisms has received increasing attention. Recent findings have indicated that subsurface microorganisms are plentiful (Novak and Hickman, 1989), metabolically active (Balkwill and Ghiorse, 1985; Webster *et al.*, 1985), and often nutritionally diverse (Hirsh and Rade-Rohkohl, 1983; Novak *et al.*, 1984; Balkwill and Ghiorse, 1985; Wilson *et al.*, 1985). In such an environment, it is possible that a population of bacteria in the subsurface would become acclimated and eventually degrade many organic compounds.

Unfortunately, there remains much to learn about this emerging technology. One question which research has not confronted is, how to quickly assess the biodegradation potential of a soil. Current biological enumeration procedures are time-consuming and often give misleading results (Novak and Hickman, 1989). Therefore, the objectives of this study were: (1) to make a preliminary assessment as to the use of a multiple inhibitor approach to evaluate the biodegradation potential of soil, and (2) to evaluate the significance of each microbial group in the biodegradation process. It was expected that this inhibitor approach would provide approximately the same information as an enumeration study in only a fraction of the time.

CHAPTER 2

LITERATURE REVIEW

2.1 The Anaerobic Sediment Environment

Anaerobic conditions in sediments occur when an environment's supply of oxygen is exceeded by its biological oxygen demand. These conditions may occur in anaerobic microsites in otherwise aerobic environments, as well as, permanently anaerobic regions. Under these conditions, the biologically-mediated reactions of greatest importance are: denitrification, fermentation, sulfate reduction and methanogenesis. Energy produced from these reactions is shown in Table 1.

Denitrifying bacteria are found in anerobic environments where nitrate is present. Nitrate respiration and denitrification occur at a redox potential (Eh) which precludes fermentation, sulfate reduction and methanogenesis.

Fermentation is another important reaction which occurs in the subsurface under anaerobic conditions. This reaction accounts for the breakdown of many organics, such as: polysaccharides, monosaccharides, amino acids, sugars and alcohols. Fermentation bacteria, such as acetogens, are unable to use butyrate or propionate as an energy source, and would die unless other carbon and energy sources are available (Ferry, personal communication, February, 1989).

Sulfate reduction and methanogenesis occur in deeply anaerobic sediments. Sulfate reducing and methane producing bacteria are incapable of hydrolizing proteins and polysaccharides (Pfennig *et al.*, 1981; Mah and Smith, 1981), and are therefore dependent on fermentative and denitrifying bacteria which initialize the mineralization process (Bryant, 1979; Laanbroek and Veldekamp, 1982). Acetate, H_2 and CO_2 are the major products of the first and second stages of anaerobic mineralization (McCarty and Smith, 1986; see Figure 4). Acetate and H_2 are known substrates of both sulfate reducing and methanogenic bacteria. Carbon dioxide (CO_2) is a substrate of methanogenic bacteria.

Table 1. Electron Acceptance Reactions (reduction reactions) (energy-producing reactions) in Aerobic and Anaerobic Systems (Snoeyink and Jenkins, 1980).

	Redox Potential	Name of Reaction
AEROBIC		
$O_{2(g)} + 4H^+ + 4e^- = 2H_2O$	+20.8	Aerobic respiration
ANAEROBIC		
$2NO_3^- + 12H^+ + 10e^- = N_{2(g)} + 6H_2O$	+21.0	Denitrfication
$NO_3^- + 10H^+ + 8e^- = NH_4^+ + 3H_2O$	+14.9	Nitrate Reduction
$CH_2O + 9H^+ + 2e^- = CH_3OH$ (Formaldehyde) (methanol)	+3.99	Fermentation
$SO_4^{2-} + 9H^+ + 8e^- = HS^- + 4H_2O$	+4.13	Sulfate Reduction
$CO_{2(g)} + 8H^+ + 8e^- = CH_{4(g)} + 2H_2O$	+2.87	Methanogenesis

It has been shown that sulfate reducers have a higher affinity for both acetate and H_2 than methanogens (Kristjansson *et al.*, 1982; Lovely *et al.*, 1982; Schonheit *et al.*, 1982). Therefore, methanogenic bacteria can dominate only in anaerobic environments in which the activities of sulfate reducers are limited, either by low sulfate concentrations or by an an inhibitory agent (Smith and Klug, 1981a). Some methanogenic activity has been reported in environments containing high sulfate concentrations, when substrates have not been limiting (Oremland, 1975; Oremland and Taylor, 1978; Mountfort *et al.*, 1980; Winfrey and Ward, 1981).

2.2 Subsurface Microbial Activity

As recently as a decade ago, the idea that an active and diverse subsurface microbiological community existed had not been widely accepted. Microbiologists were skeptical about biological activity below the rhizosphere because of its oligotrophic conditions (Leenhauer *et al.*, 1974), and because of an early study which indicated that microbial numbers declined precipitously with depth (Waksman, 1916). However, because of the use of new microscopic methods and modified sampling techniques (Wilson *et al.*, 1983), we are finding that subsurface microorganisms are plentiful (Novak and Hickman, 1989), metabolically active (Balkwill and Ghiorse, 1985; Webster *et al.*, 1985), and nutritionally diverse (Novak *et al.*, 1984; Balkwill and Ghiorse, 1985; Wilson *et al.*, 1985).

Subsurface environments are inhabited by a great number of bacteria. Direct microscopical counts of microorganisms in the subsurface often measure 10^6-10^7 total cells per gram of soil, and viable counts are commonly 10^4-10^6 colony-forming units per gram of soil (Wilson, 1983; Novak *et al.*, 1985; White, 1986; Thorn and Ventullo, 1988). The discrepancy between direct counts and viable counts may be attributed to two facts: (1) direct counts are made up of both viable and dead cells, (2) viable counts are limited to those organisms which can grow on the growth medium and under the conditions of incubation. Direct counts are highest in surface soils and decrease rapidly to a depth of approximately 2 meters. However, below 2 meters, direct counts show little variation with depth (Goldsmith, 1985; Novak *et al.*, 1985; Webster *et al.*, 1985; Bone and Balkwill, 1988).

Subsurface microorganisms are metabolically active. Stimulation of the indigenous microbial population by the addition of nutrients and electron acceptors has been shown to

be effective in restoring hydrocarbon contaminated aquifers (American Petroleum Institute report, 1980). Wilson (1981, 1983) found several halogenated volatile organics which were degraded by an indigenous population of microorganisms. Kuhn *et al.* (1985) found that both dimethyl and dichlorobenzenes were biotransformed under aerobic conditions. There has also been reported evidence of anaerobic degradation of phenolics under aquifer conditions (Ehrlich *et al.*, 1982). Several halogenated aliphatic organic compounds have been degraded by an indigenous population of microorganisms under anaerobic conditions that were not degraded under aerobic conditions (Buower and McCarty, 1983a, 1983b).

The subsurface is characterized by microbial diversity. Hirsh and Rades-Rohkohl (1983) observed 90 morphologically diverse organisms in groundwater obtained from a 10 meter well. Of these, 72 organisms were bacteria, 10 were protozoa, and 8 were fungi. Both gram-positive and gram-negative bacteria occur in the subsurface (Wilson *et al.*, 1983; Ghiorse and Balkwill, 1985; Bone and Balkwill, 1988). Bone and Balkwill (1988) found that gram-negative bacteria predominated in the top soil, while gram-positive bacteria predominated the deeper sediments.

The subsurface environment is often nutritionally poor. Because of this, viable counts of subsurface bacteria, grown on nutrient-rich media, are often significantly less than viable counts on nutrient-poor media (Balkwill and Ghiorse, 1985; Bone and Balkwill, 1988). However, studies have found that these organisms have an ability to adapt readily to high nutrient conditions (Kobayashi and Rittman, 1982).

2.3 Denitrification

The microbial transformation of nitrate to gaseous nitrogen (N_2), is known as denitrification. The sequence of products most often observed in soil denitrification studies include NO_2, N_2O and N_2, in that order (Kluyver and Verhoeven, 1954; Wijler and Delwiche, 1954; Cady and Bartholomew, 1960, 1961; Schwartzbeck *et al.*, 1961; Cooper and Smith, 1963; Fillery, 1979). This sequence of intermediate production and conversion is presented in Figure 1.

This reaction can be broken down into two groups: nitrate respiration and denitrification. Nitrate respiration refers to the reduction of nitrate to nitrite. Denitrification

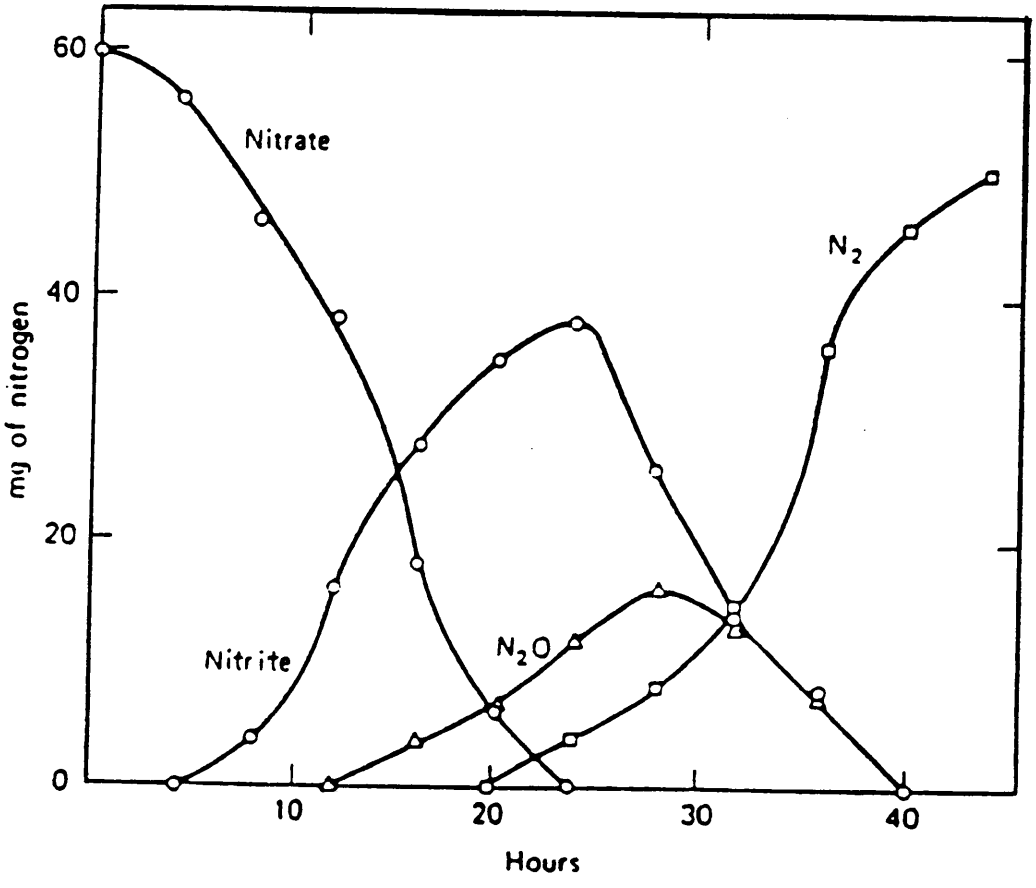

Figure 1. Products commonly formed during denitrification in a soil with near-neutral pH (Alexander, 1977).

refers to the conversion of nitrite to gaseous nitrogenous compounds (i.e. N_2O and N_2). However, commonly the term denitrification refers to any or all of these reactions.

A large group of bacteria, known as nitrate respirers, can reduce nitrate to nitrite (Payne, 1973; Ingraham, 1981). A much smaller group of bacteria, known as denitrifiers, can convert nitrite to gaseous nitrogenous compounds (Payne, 1981; Ingraham, 1981; Fillery, 1983). Genera of bacteria that include nitrate-respiring and denitrifying strains are presented in Tables 2 and 3, respectively. By comparing Table 2 with Table 3, it is evident that the vast majority of denitrifying bacteria are also capable of nitrate respiration; notable exceptions are as follows: *Achromobacter*, *Acinetobacter*, *Aquaspirillum*, *Azospirillum*, *Gluconobacter*, *Kingella*, *Lysobacter*, *Rhodopseudomonas*, *Thermotrix*, and *Xanthomonas*.

2.3.2 Intermediates of the Denitrification Reaction

Nitrite is the immediate product of nitrate respiring and denitrifying bacteria. With the degradation of nitrite, small quantities of nitric oxide (NO) have also been detected during denitrification (Nommick, 1956; Cady and Bartholomew, 1961, 1963; Fillery, 1979). There has been much debate over whether or not NO is an obligatory intermediate in the denitrification dissimilatory pathway.

St. John and Hollocher (1977) were unable to trap ^{15}N-labelled NO in an exogenous pool of ^{14}N-labelled NO during dissimilation of ^{15}N-labelled NO_2. Conversely, in a similiar experiment, Firestone *et al.* (1979b) found that as much as 54% of ^{13}N-labelled NO_2 appeared in a pool of non-labelled NO in *Pseudomonas aureofaciens*. Averill and Tiedje (1982) suggested that NO could arise from the decomposition of a labile ferrous-nitrosyl complex, which they thought could be formed during nitrite reduction to N_2O (see Figure 2).

The role of N_2O as an obligatory intermediate in dissimilatory denitrification has been demonstrated repeatedly. Nitrogen from labelled NO_2 has been readily trapped in pools of non-labelled N_2O, while labelled N_20 has been shown to be rapidly reduced to N_2 (St. John and Hollocher, 1977; Firestone *et al.*, 1979a). Many pure culture studies involving

Table 2. Genera of bacteria that include nitrate-respiring strains (Ingraham, 1981).

Actinobacillus
Actinomyces
Aeromonas
Agrobacterium
Alcaligenes
Arachnia
Arthrobacter
Bacillus
Bacterionema
Bacteroides
Beneckea
Bordetella
Branhamella
Brucella
Campylobacter
Cellulomonas
Chromobacterium
Citrobacter
Clostridium
Corynebacterium
Cytophaga
Dactylsporangium
Enterobacter
Erwinia
Eschericia
Eubacterium
Flavobacterium
Fusobacterium
Geodermatophilus
Haemophilus
Halobacterium
Halococcus
Hyphomicrobium
Hyphomonas
Klebsiella
Lactobacillus
Leptothrix
Listeria
Lucibacterium
Microbispora
Micrococcus
Micromonospora
Moraxella
Mycobacterium
Neisseria
Nocardia
Paracoccus
Pasteurella
Peptococcus
Photobacterium
Planobispora
Planomonospora
Plesiomonas
Propionibacterium
Proteus
Pseudomonas
Rhizobium
Rothia
Salmonella
Selenomonas
Serratia
Shigella
Simonsiella
Spirillum
Sporosarcina
Streptomyces
Streptosporangium
Thiobacillus
Thiomicrospira
Veillonella
Vibrio

Table 3. Genera of bacteria that include denitrifying strains (Payne, 1981; Ingraham, 1981; Fillery, 1983).

Achromobacter	*Kingella*
Acinetobacter	*Lysobactera*
Agrobacterium	*Micrococcus*
Alcaligenes	*Moraxella*
Aquaspirillum	*Neisseria*
Arthrobacter	*Paracoccus*
Azospirillum	*Propionibacterium*
Bacillus	*Pseudomonas*
Branhamella	*Rhizobium*
Campylobacter	*Rhodopseudomonas*
Chromobacterium	*Simonsiella*
Corynebacterium	*Spirillum*
Cytophaga	*Thermotrix*
Flavobacterium	*Thiobacillus*
Gluconobacter	*Thiomicrospira*
Hyphomicrobium	*Vibrio*
Halobacterium	*Xanthomonas*

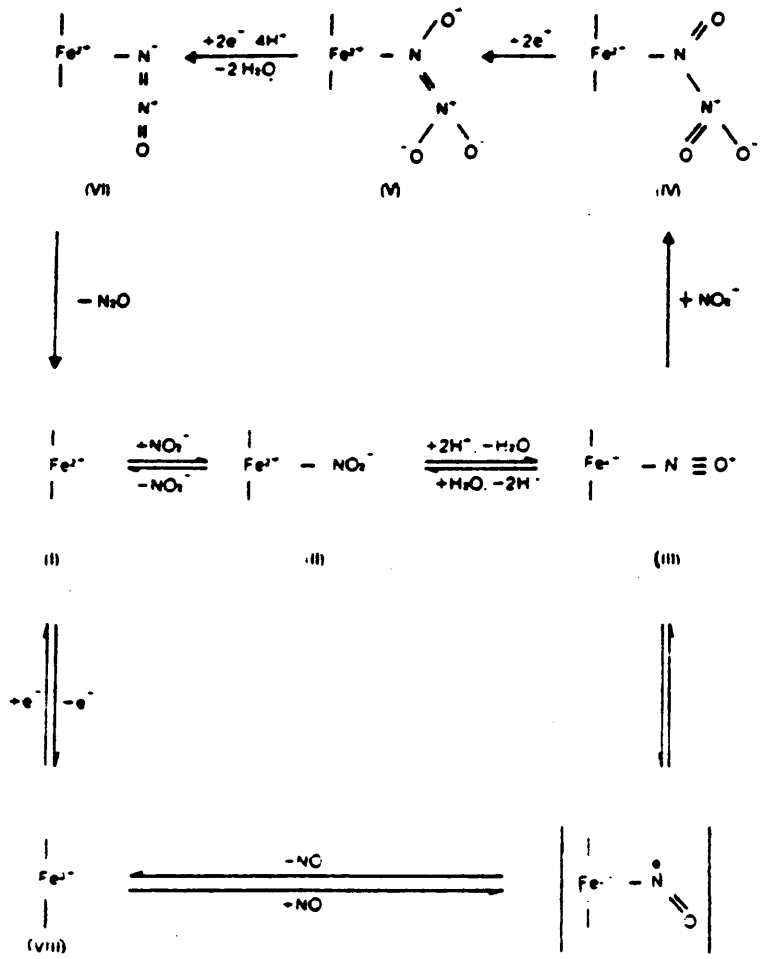

Figure 2. Proposed pathway of nitrite reduction to nitrous oxide (Averill and Tiedje, 1982).

denitrifying bacteria are also in support of N_2O as an intermediate in denitrification. For example, cultures of *Pseudomonas denitrifications* (Matsubara and Garcia, 1975) and *Thiobacillus denitrificans* (Baldensperger and Garcia, 1975) have been shown to form N_2O during nitrate reduction. In addition, *Paracoccus denitrificans* has been grown anaerobically, in the absence of oxygen, nitrate and nitrite, using N_2O as an electron involving denitrifying bacteria are also in support of N_2O as an intermediate in denitrification. For example, cultures of *Pseudomonas denitrifications* (Matsubara and Garcia, 1975) and *Thiobacillus denitrificans* (Baldensperger and Garcia, 1975) have been shown to form N_2O during nitrate reduction. In addition, *Paracoccus denitrificans* has been grown anaerobically, in the absence of oxygen, nitrate and nitrite, using N_2O as an electron acceptor (Matsubara, 1971).

2.3.3 Environmental Factors which Affect Denitrification

Many environmental factors affect bilogical denitrification in soils, including: soluble oxygen concentration, soil moisture content, organic matter content, temperature, pH, and nitrate concentration (Fillery, 1983).

The sediment's soluble oxygen concentration may restrict denitrification by repressing the synthesis and activity of dissimilatory nitrate reductase, the enzyme which catalyzes nitrate respiration (Payne, 1973). Also, when oxygen is present many denitifying bacteria will use this in favor of nitrate by using aerobic respiration. The critical factor determining whether anaerobic or aerobic metabolism occurs at a particular point, is a soil's soluble oxygen content, not its average gaseous O_2 content (Focht and Verstraete, 1977). Anaerobic microsites can arise, where the biological demand for oxygen exceeds the environments rate of reaeration. Thus, many investigators report the occurrence of denitrification in otherwise, well-aerated soil (Allison *et al.*, 1960; Cady and Bartholomew, 1961; Greenland, 1962).

The soil's moisture content also affects denitrification. Smith (1978) stressed the importance of soil moisture content on the development of anaerobic microsites in soil aggregates. The saturation oxygen concentration in air is 20,000 times that in water (Wark and Warner, 1981). Therefore, oxygen is transferred much more quickly in dry soil than in flooded soils.

Organic matter content and nitrate concentration in soil affect biological denitrification. Starr and Parlange (1975) reported that denitrification followed zero-order kinetics when carbonaceous sustrate was limiting or when nitrate is present in concentrations above 40 mg/L. Nitrate and nitrite concentrations have been reported as having an inhibiting effect on N_2O reductase activity (Wijler and Delwiche, 1954; Blackmer and Bremner, 1978; Firestone *et al.*, 1979a; Fillery, 1979).

The rate of denitrification is strongly affected by pH. Denitrification activity is optimal at pH values of 7.0-8.0 (Knowles, 1982), and decreases with decreasing pH (Bremner and Shaw, 1958). These results are surprising since the denitrifying bacteria are represented in many genera and that the activity of the soil flora remains largely unaffected over wide ranges of pHs (Fillery, 1983). This pH effect on denitrification activity may be due to the inhibitory effect of pH on denitrification intermediates. Fillery (1979) reported increased NO_2 accumulation in soils during denitrification in acidic soils, and N_2O was found to accumulate in soils during denitrification at pHs below 6.0 (Wijler and Delwiche, 1954; Nommick, 1956).

2.3.4 Additional Information on Denitrification

A long list of organic compounds used as electron donors for denitrification, has been compiled in the literature. Table 4 presents a list of organic compounds found to be biodegraded by nitrate respiring and denitrifying bacteria.

Biological denitrification has been observed to influence other reduction processes. In many studies, the presence of nitrate was inhibitory to sulfate reduction and methanogenesis (Bell, 1969; Cappenburg and Patrick, 1969; MacGregor and Keeney, 1973; Bollag and Czlonkowski, 1973). Far more energy per electron transferred is yielded via nitrate respiration and denitrification than either sulfate reduction or methanogenesis. Table 1 shows energy-producing reactions in aerobic and anaerobic systems and their corresponding redox potentials. Nitrate respiration and denitrification maintain an Eh at a level inhibitory to sulfate reduction and methanogenic bacteria.

Table 4. Organics degraded by nitrate respiring and denitrifying bacteria consortia (Hickman, 1988).

COMPOUND	REFERENCE
p-cresol	Bossert & Young, 1986; Bossert *et al.*, 1986
o-phthalic acid	Aftring *et al.*, 1981; Aftring and Taylor, 1981
2-fluorobenzoate	Schennen *et al.*, 1985
vanillic acid and other monochlorobenzoate	Taylor, 1983
monofluorobenzoate and monochlorobenzoate	Taylor *et al.*, 1979
benzoate	Williams & Evans, 1975
benzene	Taylor *et al.*, 1970
mono-, di- and trimethylamine	Meiberg & Harder, 1978
dibromochloropropane, dibromochloromethane, ethylene dibromide, 1,1,1,-trichloroethane, bromodichloroethane, bromoform, carbon tetrachloride	Buower & McCarty, 1983b; Buower *et al.*, 1986
protocatechuate, phenylalanine, *p*-hydroxybenzoate, benzoate, tyrosine, succinate	Oshima, 1965
benzoate, 2-aminobenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-carboxylbenzoate (phthalate), 1-cyclo hexenecarboxylate, adipate, pimelate, acetate, propionate, butyrate, caprolate, lactate, malate, fumarate, succinate, glucose, fructose, sucrose, maltose, acetoin, acetone	Braun & Gibson, 1984
benzene, toluene, xylene	Major *et al.*, 1988
toluene, m-xylene, 3-ethyltoluene, *p*- and *m*-cresol, *p*-hydroxybenzoic acid, benzaldehyde, benzoate, *m*-toluylaldehyde, *m*-toluate, cyclohexanecarboxylic acid	Kuhn *et al.*, 1988
naphthol, naphthalene, acenaphthene	Mihelic & Luthy, 1988a,b
phenol, benzoate, 3-, and 4-hydroxy-benzoate, 3,4-dihydroxybenzoate, *o*-, *m*-, and *p*-cresol	Bakker, 1977

2.4 Sulfate Reduction

Sulfate reduction occurs in both assimilatory and dissimilatory biological processes. In the assimilatory process, sulfate is reduced and incorporated into sulfur-containing amino acids and then into proteins. In the dissimilatory process, sulfate serves as the electron acceptor for the oxidation of organic acids by anaerobic sulfate reducing bacteria (SRBs).

2.4.1 Dissimilatory Sulfate Reduction

Sulfate reduction occurs in nitrate-free anaerobic sediments, having redox potentials of approximately -220 mV (Buower, 1978). All sulfate reducers are obligate anaerobes. The dissimilatory sulfate reduction of organic acids can result in either incomplete oxidation to acetate (Postgate, 1979; Widdel, 1980; Pfennig and Widdel, 1981), or complete oxidation to carbon dioxide (Pfennig and Widdel, 1977; Pfennig and Widdel, 1981).

Pfennig and Widdel (1982) have suggested that SRBs utilize beta-oxidation to break down the carbon chains of fatty acids. Beta-oxidation is a process, whereby, fatty acids are oxidized by the successive removal of 2-carbon fragments in the form of acetyl-CoA. Incomplete oxidation of C-even fatty acids by SRBs yield stoichiometric quantities of acetate. Incomplete oxidation of C-odd fatty acids are converted to stoichiometric quantities of acetate plus one propionate molecule.

2.4.2 Acetate-Oxidizing Sulfate Reducing Bacteria

In 1928, Rubentschik first reported an SRB which used acetate as its electron donor. Baars (1930) observed the same process and described a bacterium, *Vibrio rubentschikii*. Almost 50 years passed before Friedrich Widdel and Norbert Pfennig reported a second bacterium, *Desulfotomaculum acetoxidans*, which used acetate as its sole carbon source and electron donor (Widdel and Pfennig, 1977). Since then, acetate-oxidizing SRBs belonging to the genera: *Desulfovibrio*, *Desulftomaculum*, *Desulfobacter*, *Desulfococcus*, *Desulfocarcina*, and *Desulfonema*, have been reported in the literature (Laanbroek and Pfennig, 1981; Pfennig and Widdel, 1981; Postgate, 1984).

These acetate-oxidizing SRBs may account for the majority of sulfate utilization during biodegradation of butyrate and propionate. Sorensen (1981) reported that if propionate and

Table 5. Organics degraded by sulfate reducing bacterial consortia (Hickman, 1988).

COMPOUND	REFERENCE
C_1-C_{18} fatty acids, benzoate, phenylacetate, 3-phenylpropionate	Pfennig & Widdel, 1981
H_2/CO_2	Badziong *et al.*, 1978
cyclohexane, carboxylate, hydrooxylbenzoate, hippurate	Widdel, 1980, cited by Peck, 1984
methanol, ethanol, propanol, butanol, lactate, pyruvate, malate, benzoate, succinate, oxamate, fumarate, citrate, cysteine, choline, formate, acetate, glucose, glycerol	Postgate, 1984
benzoate	Balba & Evans, 1980
phenol, benzoate	Suflita & Miller, 1985
	Gibson & Suflita, 1986
o-, *m*-, and *p*-cresol	Smolenski & Suflita, 1987
H_2, formate, propionate, butyrate, valerate, caproate, heptoanate, caprylate, pelargonate, methanol, ethanol, 1-propanol, 1-butanol, glycerol, lactate, pyruvate, succinate, fumarate, malate, oxaloacetate, choline	Nanninga & Gottschal, 1987

butyrate were completely oxidized by SRBs, the stoichiometry of the reactions indicate that H_2, acetate, propionate and butyrate account for 5 to 10, 40 to 50, 10 to 20, and 10 to 20%, respectively, of the electron donors for the SRBs.

2.4.3 Inhibition of Methanogenesis by Sulfate Reduction

Inhibition of methanogenesis by sulfate reduction has been observed repeatedly in the literature (Cappenburg, 1975; Winfrey and Zeikus, 1977; Oremland and Taylor, 1978; Abram and Nedwell, 1978a; Abram and Nedwell 1978b). To explain this, arguments have been proposed on both thermodynamic and kinetic grounds.

Decker *et al.*(1970) argued that sulfate reducers should be able to outcompete methanogens for available acetate and H_2, on thermodynamic grounds. They calculated that the reduction of CO_2 by H_2, to methane, yields only 88% of the energy obtained by the reduction of sulfate to hydrogen sulfide by H_2. Similiarly, the conversion of acetate to CO_2 and methane yields only 60% of the energy by the reduction of sulfate to hydrogen sulfide by acetate.

Another argument proposed, which favors SRBs over MPBs for available H_2 and acetate, is based on kinetic factors. The half-saturation constants for H_2 and acetate are lower for SRBs than MPBs (Kristjansson *et al.*, 1982; Lovely *et al.*, 1982; Schonheit, 1982). Monod (1949) defined the half-saturation constant (k_s), as the substrate concentration at which the specific growth rate of a microorganism is equal to half the maximum rate. This condition is represented graphically in Figure 3. Lovely *et al.*(1982) and Kristjansson *et al.*(1982) estimated the saturation constant for SRBs as 17 and 24% of that for MPBs, respectively, when using H_2 as an electron donor. Schonheit *et al.*(1982) estimated the saturation constant for SRBs to be approximately 7% that of MPBs, when using acetate as an electron donor. These results suggest that at low acetate and H_2 concentrations, SRBs have an overwhelming advantage over MPBs, in the competition for available substrate.

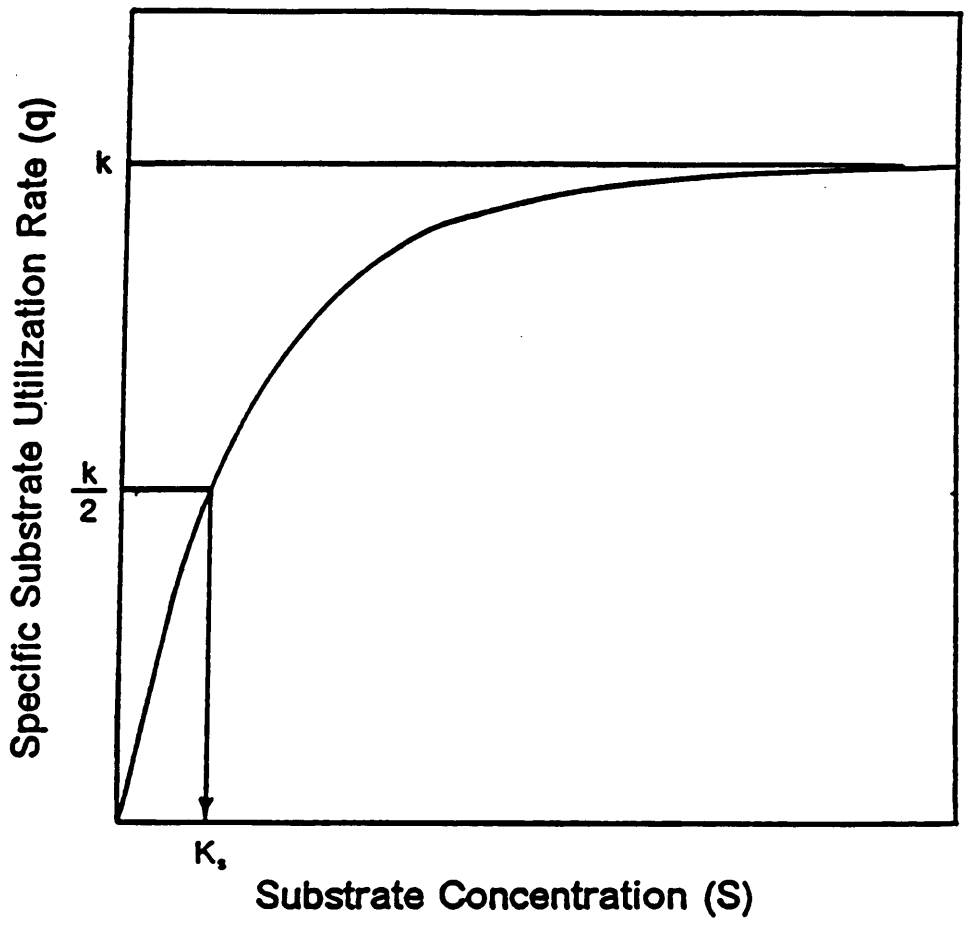

Figure 3. Relationship between specific growth rate and growth-limiting substrate concentration (Monod, 1949).

2.4.4 Metabolic Capabilities of Dissimilatory Sulfate Reducing Bacteria

The metabolic capabilities of the dissimilatory sulfate reducing bacteria show extensive variation. Propionate, butyrate and odd- and even- fatty acids up to C_{18} are oxidized to completion, to acetate, or to propionate by sulfate reducing bacteria (Widdel, 1980). Table 5 lists a wide variety of organics reported to have been biodegraded by SRBs.

Dissimilatory SRBs are capable of chemoautotrophic growth. Chemoautotrophic growth is the growth of bacteria via chemical reactions with inorganic compounds. *Desulfonema magnum*, *Desulfosarcina variabilis*, *Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans* and *Desulfovibrio gigas* grew on hydrogen and CO_2 plus sulfate (Widdel, 1980; Brandis and Thauer, 1981; Pfennig and Widdel, 1981).

Dissimilatory SRBs can grow in the absence of sulfate, by fermentative means. Postgate (1952, 1963) showed that *Desulfovibrio desulfuricans* and *Desulfotomaculum nigrificans* had the ability to grow in the absence of detectable sulfate on pyruvate. However, Postgate (1979) reported that not all bacteria in the genera *Desulfovibrio* and *Desulfotomaculum* possess this capability.

2.5 Methanogenesis

Methanogensis is the production of methane gas through the oxidation of various organic compounds, and reduction of CO_2. Methanogenic bacteria are obligate anaerobes that require redox potentials of -240 mV (Buower, 1978), and the absence of nitrate and sulfate, to be active (Bell, 1969; Cappenburg, 1974). The seven known genera of methane producing bacteria (MPBs) are: *Methanobacterium*, *Methanobrevibacter*, *Methanococcus*, *Methanomicrobium*, *Methanospirillum*, *Methanogenium* and *Methanosarcina* . Table 6 lists a wide variety of organics reported to have been biodegraded by MPBs.

2.5.1 Methanogenic Decomposition Process

McCarty and Smith (1986) presented a three stage flowchart which depicts the methanogenic decomposition process. This diagram is presented in Figure 4. The three stages which describe the decomposition of complex organic compounds to form methane,

Table 6. Various Substrates Capable of Being Oxidized by Methane Producing Bacteria (Hickman, 1988).

Compound	Reference
acetaldehyde, acetic anhydride, acetone, acrylic acid, adipic acid, aniline, 1-amino-2-propanol, 4-amino butyric acid, benzoic acid, butanol, butyraldehyde, butylene glycerol, catechol, cresol, crotonaldehyde, crotonic acid, diacetone, gulusonic acid, dimethoxy benzoic acid, ethanol, ethyl acetate, ethyl acrylate, ferulic acid, formaldehyde, formic acid, fumaric acid, glutamic acid, glutaric acid, glycerol, hexanoic acid, hydroquinone, isobutyric acid, isopropanol, lactic acid, maleic acid methanol, methyl acetate, methyl acrylate, methyl ethyl ketone, methyl formate, nitrobenzene, pentaerythritol, pentanol, phenol, phthalic acid, propanal, propanol, isopropyl alcohol, propionate, propylene glycol, protocatechuic acid, resorcinol, *s*-butanol, *s*-butylamine, sorbic acid, syringaldehyde, syringic acid, succinic acid, *t*-butanol, vanillic acid, vinyl acetate	Speece, 1983
tetrachloroethylene, chloroform, 1,1,2,2-tetrachloroethane, 1,1,1-trichloroethane, tetrachloride, dibromochloropropane, bromodichloromethane, dibromochloromethane, bromoform, ethylene dibromide	Buower 1983c, 1985 Buower *et al.*, 1986 carbon
trichloroethylene, 1,1-dichloroethylene, 1,2-dichloroethylene, ethylene dibromide, benzene, toluene, ethylbenzene, *o*-xylene	Wilson *et al.*, 1986a
phenoxyacetate, 2,4-dichlorophenoxyacetate (2,4-D), 2,4,5-trichlorophenoxyacetate (2,4,5-T), 3-chlorobenzoate,3,4-dichlorobenzoate, 3,5-dichlorobenzoate, 3-bromobenzoate, 3-iodobenzoate, 4-amino-3,5-dichlorobenzoate, phenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol	Horowitz *et al.*, 1983 Suflita & Miller,1985 Gibson & Suflita, 1986
1,1,1-trichloroethane	Vogel & McCarty, 1987
dimethyl selenide	Oremland & Zehr, 1986

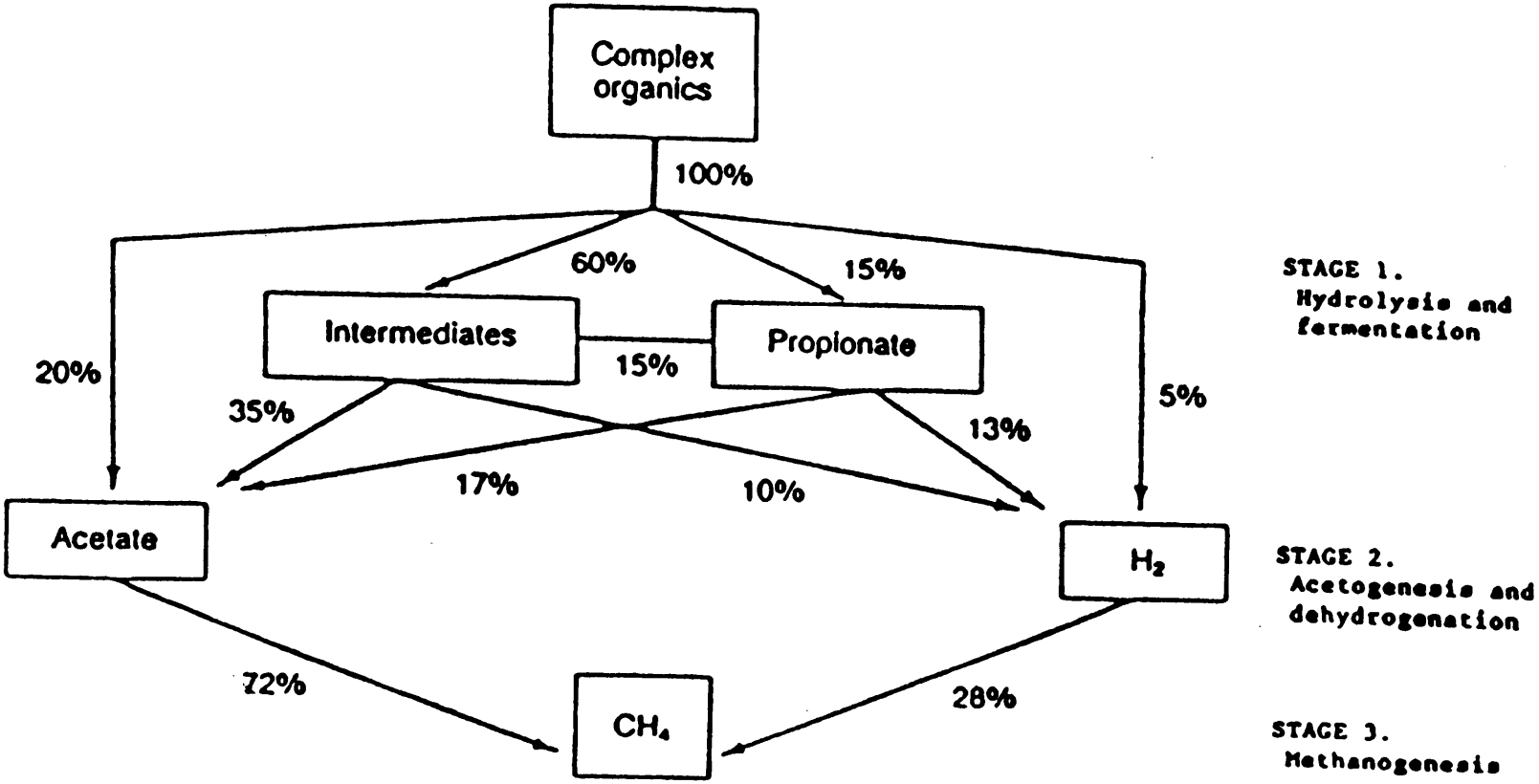

Figure 4. Methanogenic decomposition process (McCarty and Smith, 1986).

are: (1) Hydrolysis and Fermentation, (2) Acetogenesis and Dehydrogenation, and (3) Methanogenesis. These first two stages occur through the association of many microorganisms. The process is terminated by the production of methane by methanogenic bacteria.

Figure 4 illustrates the dependency of methanogenic bacteria on those microorganisms which carry out the first two stages of methanogenic decomposition for their supply of a carbon and energy source. However, it is important to note, that these higher organisms are likewise dependent upon the action of methanogenic bacteria, in order to successfully carry out the reactions involved in the first stage of decomposition. This interdependency between fermentative and methanogenic bacteria, is known as "interspecies hydrogen transfer".

2.5.2 Interspecies Hydrogen Transfer

Inter-species hydrogen transfer refers to the mutualistic relationship which exists between fermentative bacteria, which dispose of electrons via proton reduction to H_2, and H_2-oxidizing methanogens.

Bryant (1967) reported a mixed culture of two species, existing in a symbiotic association. The first species oxidized ethanol to acetic acid. This oxidation reaction liberated two protons which were then disposed of by reduction to H_2, by a second bacterial species. This disposal of electrons through the reduction of proteins, was established through a nicotinamide dinucleotide dependent reaction (Reddy *et al.*, 1972). These reactions are as follows:

$$CH_3CH_2OH + NAD^+ = CH_2CHO + NADH + H^+ \qquad G^o=+5.7kcal \quad (2.1)$$

$$NADH + 2H^+ = NAD^+ + H_2 \qquad G^o=+4.3kcal \quad (2.2)$$

These reactions, under standard conditions, are thermodynamically unfavorable, as indicated by the net positive value of free energy. However, as the second organism continually disposes of electrons by the reduction to H_2, the hydrogen partial pressure decreases, and the free energy change of reactions 2.1 and 2.2 decreases, until finally the net free energy of the reaction is negative, making the reaction thermodynamically

favorable. Without this organism's continual disposal of electrons, the biodegradation of ethanol would not occur. Methanogenic bacteria take on the function of electron disposal, and make possible the efficient degradation of complex organic compounds.

2.6 Microbial Metabolic Inhibitors

In mixed microbial communities, one way of examining the role of a particular part of the community is to selectively inhibit that part, and then to observe how the inhibition affects the rest of the community, or community activity (Banat and Nedwell, 1984). In this study, two metabolic microbial inhibitors were used: sodium molybdate (Na_2MoO_4) and 2-bromoethane sulfonic acid (BESA). These chemicals inhibit sulfate reduction and methanogenesis, respectively.

2.6.1 Molybdate as an Inhibitor of Sulfate Reduction

Postgate (1949) first showed that selenate inhibited sulfate reduction in *Desulfovibrio desulfuricans*. A decade later, Peck (1959) found that anions of group VI, such as, molybdate, selenate, and tungstate, share many similiarities to sulfate in their chemistry, and have been shown to be inhibitory to sulfate reduction. The degree of inhibition was generally in the order: molybdate, selenate, and tungstate (Banat and Nedwell, 1984). Smith and Klug (1981) reported that sulfate reduction was inhibited at concentrations from 0.2 to 200 mM. They also reported that methane production was inhibited at concentrations of sodium molybdate greater than 20 mM. Banat and Nedwell (1984) reported that addition of sulfate to systems containing molybdate, did not result in proportional increases in sulfate reduction. This result suggests that molybdate inhibits sulfate reduction by a non-competitive mechanism.

2.6.2 BESA as an Inhibitor of Methanogenesis

A coenzyme which is unique to methanogenic bacteria is called Coenzyme-M (i.e. 2-mercapto ethane sulfonic acid). A coenzyme is the non-protein portion of an enzyme. The protein portion of an enzyme is referred to as the apoenzyme. When united, the two form a single, complete and active enzyme, the haloenzyme.

Coenzyme-M aids in the conversion of a carboxyl group to CO_2, in the conversion of acetate to methane and carbon dioxide (Smith and Mah, 1978). Addition of 70 uM BESA resulted in 75% inhibition of methanogenesis, compared with untreated controls (Smith and Mah, 1978). Zehnder and Brock (1979) reported 100% inhibition of methane formation from acetate, in the presence of 1 mM BESA.

Chapter 3

Materials and Methods

3.1 Experimental Approach

In this study, the biodegradation potential of a soil was assumed to be approximated by the diversity and number of a soil's microbial population. It seems reasonable, therefore, that enumeration of specific organism groups could be used to determine the biodegradation potential of soil under specific oxidation-reduction conditions. Unfortunately, the enumeration of specific organism populations is both a slow process and subject to difficulties in interpretation of organism counts (Tiedje, 1982; Goldsmith, 1985; White, 1986; Hickman, 1988). Therefore, a simple, more direct approach was sought.

The proposed method of approach was to add an easily degradable substrate, capable of being degraded by many organism groups, and to monitor the resulting degradation. By placing this substrate in different aqueous environments, it was thought possible to determine which group of microorganisms would be most active in the biodegradation process. For example, if this substrate was placed in a soil-dilution water microcosm system which contained no nitrate or nitrite, denitrification would not occur, and sulfate reducers and methanogens might be favored. If we then compared the biodegradation rate of this system, to that of a system where denitrifiers were active, conclusions could then be made as to the relative importance of the denitrifying population in this system. If the biodegradation rate of the system containing no nitrate, was similiar to that of the system containing nitrate, one might conclude that the denitrifying population of that soil was either not viable, or was not capable of biodegrading the substrate used. In a similiar manner, we could select or alter soil-dilution water microcosm environments to favor either sulfate reducers or methanogens.

Denitrifiers were suppressed by placing the soil in an aqueous environment which contained no nitrate. Sulfate reducers were inhibited by placing the soil in an aqueous environment containing 206 mg/L sodium molybdate (Na_2MoO_4). Methanogens were inhibited by placing the soil in an aqueous environment containing 211 mg/L

2-Bromoethane sulfonic acid (BESA). If we compared the biodegradation rates of these microcosms to that of a microcosm which contained no inhibitors, one could determine the effect of denitrifiers, sulfate reducers and methanogens on the biodegradation of a simple substrate.

Two series of experiments were performed in this study: single inhibition experiments and multiple inhibition experiments. The single inhibition expeiments were performed to describe the biodegradation of butyrate and propionate, in Newport News and Blacksburg soils, under conditions in which a single microorganism group per microcosm was inhibited or suppressed. To accomplish this, four microcosms for each soil were prepared, as discussed in 3.4, and each was subjected to a specific microcosm dilution water condition. The following microcosm dilution water conditions were used: (A) allowed denitrifiers, sulfate reducers, and methanogens to have unimpeded biological activity, (B) allowed denitrifiers and methanogens to have unimpeded biological activity, but impeded the activity of sulfate reducers, (C) allowed denitrifiers and sulfate reducers to have unimpeded biological activity, but impeded the activity of methanogens, and (D) allowed sulfate reducers and methanogens to have unimpeded biological activity, but impeded the activity of denitrifiers. Specific microcosm dilution water additives to create these conditions is provided in Table 7 and in Section 4.1.

The multiple inhibition experiments were performed to describe the biodegradation of butyrate and propionate, in Newport News and Blacksburg soils, under conditions in which two or more inhibitors were present in each microcosm system. To accomplish this, four microcosms for each soil were prepared, as discussed in 3.4, and each was subjected to a specific microcosm dilution water condition. The following microcosm dilution water conditions were used: (E) allowed denitrifiers to have unimpeded biological activity, but impeded the activity of sulfate reducers and methanogens, (F) allowed sulfate reducers to have unimpeded biological activity, but impeded the activity of denitrifiers and methanogens, (G) allowed methanogens to have unimpeded biological activity, but impeded the activity of denitrifiers and sulfate reducers, and (H) impeded the biological activity of denitrifiers, sulfate reducers, and methanogens. Specific microcosm dilution water additives to create these conditions is provided in Table 7 and in Section 4.1.

Along with the single and multiple inhibition experiments, control experiments were also performed. The control experiments were used to estimate the loss of substrate due to

bacterial activity and various non-biological processes, such as, adsorption and volatilization. Preparation of the control microcosms is discussed in greater detail in section 3.9.

Although there are other microbial groups present in soil, such as, iron reducers, fermentative bacteria and acetogenic bacteria, the growth of these was impeded by the microcosm environments. The iron content of both Newport News and Blacksburg soil was negligible (Hickman, 1988; Morris, 1988). Butyrate and propionate can not be used as substrates by bacteria with fermentative metabolisms (eg. fermentative and acetogenic bacteria).

3.2 General Methods

The following general methods were used during the course of this study. All glassware was acid washed in a 10% HCl acid bath for 24 hours, rinsed thoroughly with deionized-distilled water, and autoclaved for 20 minutes at 121 C and 15 psig pressure. All utensils used in handling the soils were flame-sterilized prior to each use.

3.3 Site Location and Sample Collection

The soils used were obtained from two previously uncontaminated sites in Newport News, Virginia and Blacksburg, Virginia. The locations of these sites are shown in Figure 5. The Newport News site was located at the Harwood's Mill Reservoir. This soil was saturated just below the surface and was composed of sand and silt. The Blacksburg site was located on the dairy farm at the Virginia Polytechnic and State University campus. The soil was unsaturated, and consisted primarily of sand. Both sites were sampled at depths 2-3 meters below the surface.

3.4 Microcosms

Static microcosms were constructed using one liter reagent bottles sealed with a rubber stopper. A single glass tube ran through the rubber stopper, well below the dilution water's surface. By placing the tube well below the dilution water's surface, the volume of air which would have to be purged by nitrogen gas was limited to that in the glass tube. Each microcosm contained approximately 600 g of soil and 800 mL of dilution water.

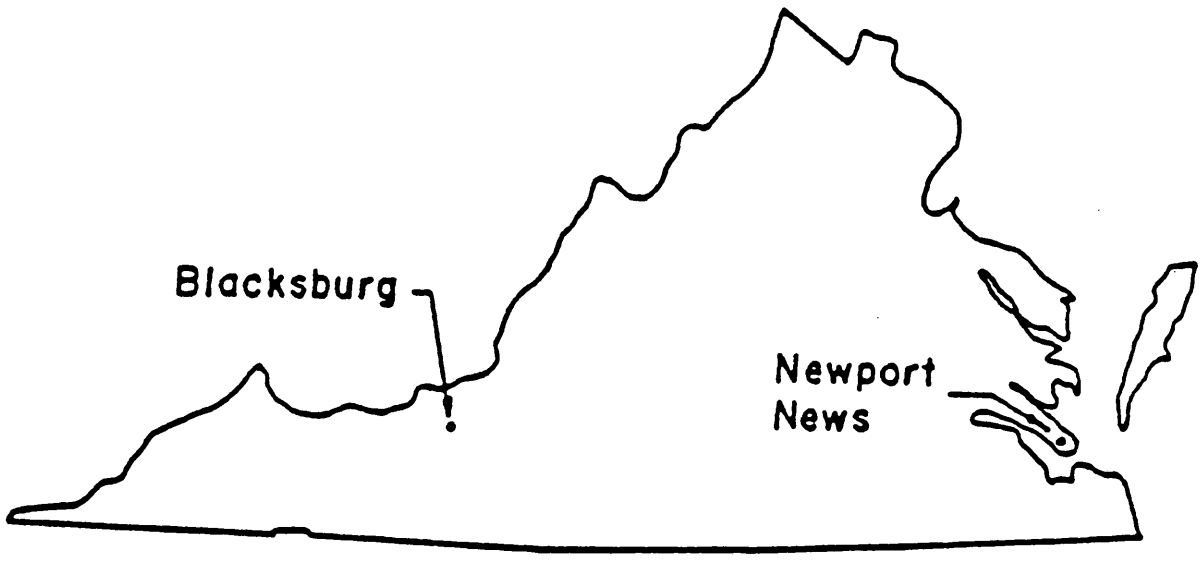

Figure 5. Soil sampling site locations. Blacksburg soil sampled at the Dairy Farm on Virginia Polytechnic and State University campus, Blacksburg, Virginia. Newport News soil sampled at Harwood's Mill Reservoir, Newport News, Virginia.

The dilution water was obtained from a Milli-Q Reagent Water System and autoclaved for 20 minutes at 121 C and 15 psig pressure. Nitrogen (N_2) gas was then bubbled into the dilution water for 10 minutes to reduce the oxygen level to near 0 mg/L. Added to the dilution water were nutrients, inhibitors, and a substrate. The substrates added were either butyrate or propionate. The nutrients added were ammonium chloride (NH_4Cl), sodium sulfate (Na_2SO_4) and potassium mono-phosphate (KH_2PO_4). The inhibitors added were sodium molybdate (Na_2MoO_4) and 2-bromoethane sulfonic acid (BESA). Sodium nitrate ($NaNO_3$) was added to enhance denitrification. In those cases where denitrification was suppressed, sodium nitrate was excluded from the dilution water. Volumes of the substrates, and masses of the nutrients and inhibitors added to each microcosm are listed in Table 7.

All microcosms were stored in a dark cabinet at a constant temperature of 20 C.

3.5 Addition of Nutrients, Inhibitors and Substrates

Before adding nutrients, inhibitors and substrates, the microcosms were prepared. The Newport News and Blacksburg soils were weighed out to individual 600 g portions, and placed in acid-washed, autoclaved microcosms. Nitrogen (N_2) gas was bubbled through a volume of 800 mL of autoclaved dilution water for 10 minutes which was then poured into microcosms.

The nutrients and inhibitors were then weighed out to the nearest 0.1 mg, according to the mass' listed in Table 8, dissolved in 3 mL of boiling dilution water, added to the soil-dilution water microcosm system, and vigorously stirred with a flame-sterilized, "glass-L" petri-plate spreader. The microcosms were then placed in a dark closet at 20 C for 5 days, in order to allow the nutrients and inhibitors to reach equilibrium, and for any suspended soil particles to settle. This waiting period also allowed for the degradation of trace organics before the addition of butyrate.

After 5 days, the microcosms were taken out of the cabinet. Butyrate or propionate were then added to the microcosms by discharging the appropriate volume, listed in Table 7, from an automatic pipette. The microcosms were then vigorously stirred with a flame-sterilized "glass-L" petri-plate spreader, and a sample was immediately taken, as

Table 7. Microcosm Dilution Water Additives.

	Dilution Water Additives (g/mol)	Molecular Weight	Concentration (mg/L)	Weight (g) added to 800 mL
Nutrients				
	$NaNO_3$	85	30 mg/L - N*	0.1821
			100 mg/L - N**	0.6070
			150 mg/L - N***	0.9105
	Na_2SO_4	142	200 mg/L - SO4	0.2366
	NH_4Cl	53.5	30 mg/L - N	0.0918
	KH_2PO_4	136	5 mg/L - P	0.0175
Inhibitors				
	Na_2MoO_4	206	206 mg/L	0.1648
	BESA	211	211 mg/L	0.1688
				Volume (mL) added to 800 mL
Substrates				
	Butyrate (s.c. 11.0 M)	88	300 mg/L+	0.248
			200 mg/L++	0.165
			150 mg/L+++	0.124
	Propionate (s.c. 13.4 M)	74	150 mg/L	0.121

*added to butyrate microcosms: A(NN), B(BB).
**added to butyrate microcosms: A(BB), B(NN), C(NN,BB).
***added to butyrate microcosms: E(NN), E(BB), and all propionate microcosms.
+added to microcosms: A(NN,BB), B(NN,BB), C(NN,BB), and controls.
++added to microcosms: D(NN,BB).
+++added to micrcosms: E(NN,BB), F(NN,BB), G(NN,BB), and H(NN,BB).

Where: A(NN,BB) = microcosm dilution water condition A for both Newport News and Blacksburg soils.

s.c. = stock concentration (mol/L).

described in 3.7.

During the course of either the single or multiple inhibition experiment, several microcosms became depleted of either sulfate or nitrate. When this occurred, the microcosms were respiked with these anions to their original concentrations, so that sulfate reduction and denitrification would not be impeded by the lack of electron acceptors.

3.6 Butyrate and Propionate Study Sequence

In this study, the microcosms were first spiked with butyrate, and the resulting degradation was analyzed. After all of the butyrate and its resulting intermediate, acetate, was degraded, the microcosms were respiked with $NaNO_3$ (when needed), Na_2SO_4, NH_4Cl, and KH_2PO_4. After this, the microcosms were spiked with propionate, and the resulting degradation was analyzed.

The initial concentration of butyrate used for microcosm dilution water conditions A, B, and C, and control, was 300 mg/L. The initial concentration of butyrate for microcosm dilution water condition D, was 200 mg/L. The initial concentration of butyrate for microcosm dilution conditions E, F, G, and H, was 150 mg/L. The initial concentration of propionate for microcosm dilution water conditions A, B, C, D, E, F, G, and H, was 150 mg/L.

For each of the dilution water conditions: A, B, C, D, E, F, G, and H, two microcosms were prepared; one containing Newport News soil, the other containing Blacksburg soil.

3.7 Sampling Procedure

A closed 5 mL pipette was inserted into the center of the microcosm and then opened. A 3 mL sample was then drawn and discharged into two centrifuge tubes. Into the first centrifuge tube, 2 mL of sample was discharged. This sample was used for analysis of fatty acid concentrations. Into the second centrifuge tube, 1 mL of sample was discharged. This sample was used for the analysis of anion concentrations. One 3

mL sample was taken for each microcosm. Once the sample was taken, the microcosm was purged with nitrogen (N_2) gas for 2 minutes, sealed, and placed in a dark cabinet at 20 C. The samples were then stored at 0 C until they were analyzed. Samples were typically stored for 7 days before analysis.

3.8 Analysis Procedure

Fatty acid and anion concentrations were determined using a Dionex 2010i Ion Chromatagraph. The machine was equipped with two carbon columns. The first column was used to detect fatty acids and the second column was used to detect anions. The machine was only able to operate with one column at a time. Therefore, all fatty acid samples for a given day were analyzed using the one column, then all of the anion samples for a given day were analyzed using the other column.

Samples for fatty acid analysis were removed from the freezer. While they thawed, three fatty acid standards, containing known concentrations of acetate and either butyrate or propionate, were passed through the ion chromatograph. The results of these injections were then compared. When these standards were within 5% of each other, the ion chromatograph was assumed to be equilibrated, and experimental samples were then analyzed. All samples were then analyzed for fatty acids. After this, another three standards were passed through the machine an compared with the first three standards. From the ion chromatograph output, concentrations of acetate, and either butyrate or propionate, were determined.

Samples for anion analysis were then removed from the freezer. Three anion standards of known concentrations of nitrate, nitrite, and sulfate, were passed through the machine and compared, as were the fatty acid standards. After all samples were passed through the machine, another three standards were analyzed and compared with the first three anion standards.

All standard solutions were stored in a 4 C refrigerator, in glass bottles with teflon-sealed, screw-caps. At the beginning of the study a nitrate-nitrite-sulfate standard was made. Based on the Dionex output, this standard did not change significantly over the course of this study. Fatty acid standards were prepared as they were being used (ie. the standard was made immediately prior to the injection).

3.9 Control Microcosms

Control microcosms to determine the loss of butyrate due to bacterial activity, and various non-biological processes, such as , adsorption and volatilization. The biological activity may result from bacteria introduced during the sampling process and bacteria which were not destroyed during the soil sterilization process. These microcsms contained soil which was weighed out to 600 g portions, dried at 350 F for 45 minutes, pulverized using a ceramic mortar and pestle, and then autoclaved once a day for 5 consecutive days at 121 C and 15 psig pressure. The soil was then placed in acid-washed, autoclaved, one-liter reagent bottles. A volume of 800 mL of sterilized, anoxic dilution water was added to each of the microcosms and the soil-dilution water systems were then autoclaved a sixth time to destroy microorganisms introduced during the transfer operation. Butyrate (11.0 M stock concentration) was then added to the microcosms via automatic pipette, to attain an initial concentration of 300 mg/L. The control microcosms were sampled at 1, 4, 7, 8, 14, 24, 39, and 63 days, using the procedure discussed in 3.7. These samples were then analyzed for butyrate concentration, using the procedure discussed in 3.8. A graph illustrating time vs. butyrate concentration for these microcosms is presented in Figure 15. Control microcosms did not contain $NaNO_3$, Na_2SO_4, NH_4Cl, KH_2PO_4, Na_2MoO_4, or BESA.

3.10 Evidence to Support the Control Experiments

Although control microcosms were prepared and analyzed to examine the fate of butyrate, no control experiments were performed to investigate the existence of a viable denitrifier, sulfate reducer, or methanogen population, in either the Newport News or Blacksburg soils. However, both Hickman (1988) and Morris (1988) analyzed these soils and provided information concerning each of these points.

The presence of a large denitrifier population in the Newport News and Blacksburg soils was confirmed based on enumeration studies by Hickman (1988). In the Newport News and Blacksburg soils, denitrifiers were present at concentrations of $100x10^3$ and

$48.9x10^3$ MPN/g, respectively.

The possibility of nitrate leaching from the soil and resulting in denitrification, is small, based on soil constituent studies performed by Hickman (1988) and Morris (1988). Morris (1988) reported concentrations of nitrate, in both soils, below detectable limits. Hickman (1988) reported concentrations of nitrate expected in the aqueous-phase of distilled-water-dosed microcosms of not detectable and 1.2 mg/L for the Newport News and Blacksburg soils, respectively.

The presence of a large sulfate reducer population in the Newport News and Blacksburg soils was confirmed based on enumeration studies performed by Hickman (1988). Hickman (1988) reported sulfate reducer populations in the Newport News and Blacksburg soils of $84.3x10^3$ and $5.9x10^3$ MPN/g, respectively.

There is also evidence which may indicate that methanogens were not present in either the Newport News or Blacksburg soils. Hickman (1988) reported no methane production from these soils during his enumeration studies.

3.11 Biodegradation Rate Calculations

Butyrate and propionate biodegradation rates were determined solely for the purpose of comparing the relative biodegradation rate of one microcosm system to another. In general, the biodegradation of butyrate and propionate, presented in Appendices A and B, occurred as a zero-order, linearly-decreasing reaction. Therefore, in most cases it was convenient to determine the biodegradation rate of butyrate or propionate by dividing the initial substrate concentration by the time to reach zero (butyrate or propionate) concentration. However, in Figures A12, A16, B9, B10, B12, B14 and B16, the substrate may have reached a zero concentration long before the microcosm was sampled. If that was the case, using the above method to determine the biodegradation rate would yield a rate much lower than the actual rate. In these situations, the biodegradation rate was computed by subtracting the initial substrate concentration from the last measurable substrate concentration, and dividing this difference by the time to reach this last measurable concentration.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results of the Single Inhibition Experiments

A series of experiments were performed to describe the biodegradation of butyrate and propionate under conditions in which a single microorganism group per microcosm was inhibited or suppressed. This single inhibition experiment was performed on both Newport News and Blacksburg soils. Figures illustrating butyrate and propionate biodegradation under these conditions are presented in Appendix A.

In Figures 6-9, the disappearance of butyrate and propionate in both the Newport News and Blacksburg soils, under several microcosm dilution water conditions, are shown. The microcosm dilution water conditions are: (A) 100 mg/L sodium nitrate added to the dilution water, (B) 206 mg/L sodium molybdate and 100 mg/L sodium nitrate added to the dilution water, (C) 211 mg/L BESA and 100 mg/L sodium nitrate added to the dilution water, and (D) 0 mg/L sodium nitrate added to the dilution water. The addition of sodium molybdate and BESA in (B) and (C), inhibited sulfate reduction and methanogenesis, respectively. The addition of 100 mg/L sodium nitrate in (A), (B), and (C), allowed for the occurence of denitrification. The absence of sodium nitrate in (D), suppressed the occurrence of denitrification.

In Figures 6 and 7, the disappearance of 300 mg/L butyrate in both the Newport News and Blacksburg soils, under conditions (A), (B), and (C), occurred in 5.5 days. The disappearance of 200 mg/L butyrate in both Newport News and Blacksburg soils, under condition (D), occurred in 8.0 days.

In Figures 8 and 9, the disappearance of 150 mg/L propionate in both the Newport News and Blacksburg soils, under conditions (A), (B), and (C), occurred in 1.2 days. The disappearance of 150 to 45 mg/L propionate in Newport News soil, and the disappearance of 150 to 35 mg/L propionate in Blacksburg soil, under condition (D), occurred in 7.0 days.

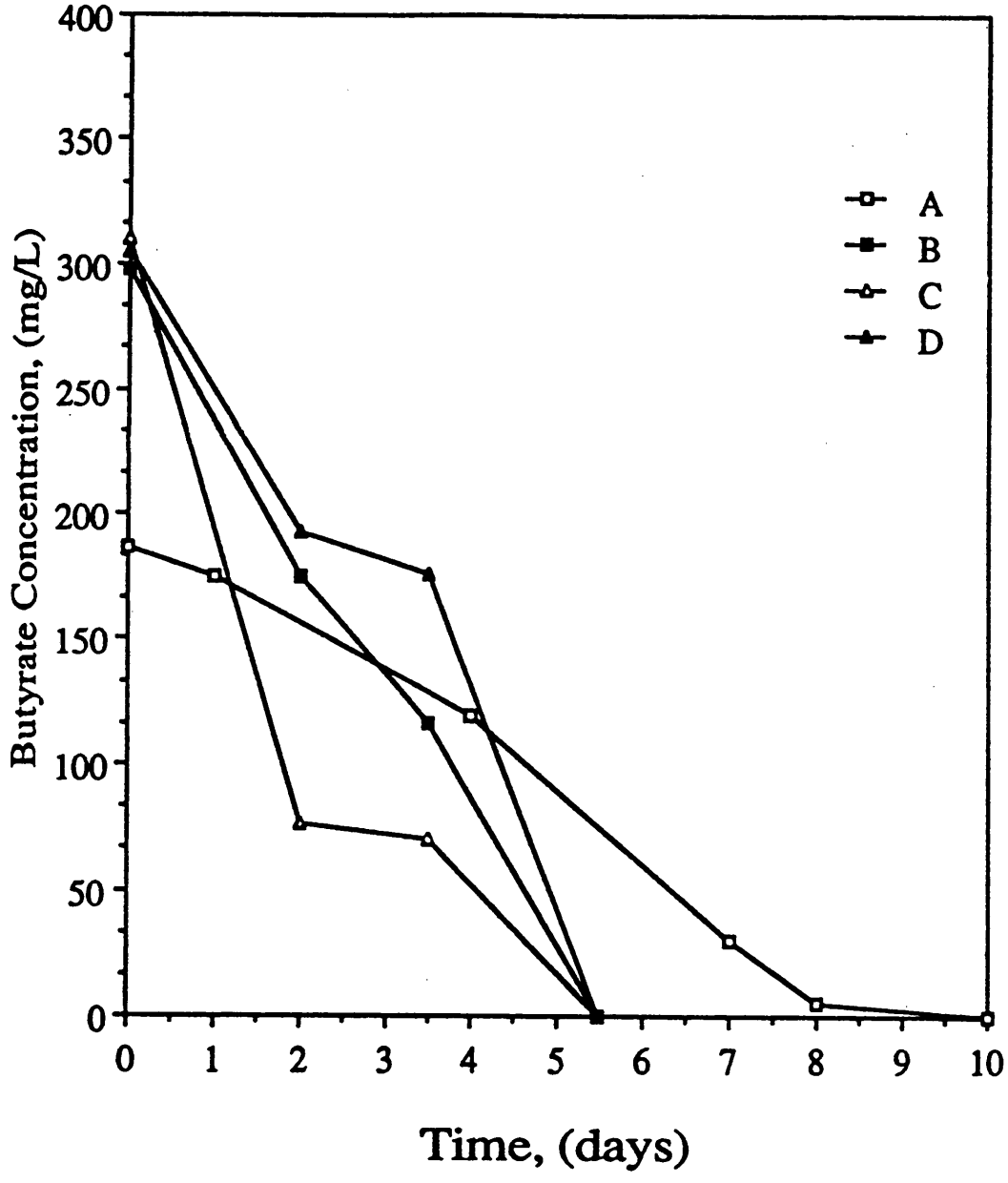

Figure 6. Butyrate biodegradation in the Newport News soil under several dilution water conditions: (A) 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (B) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (C) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, and (D) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water.

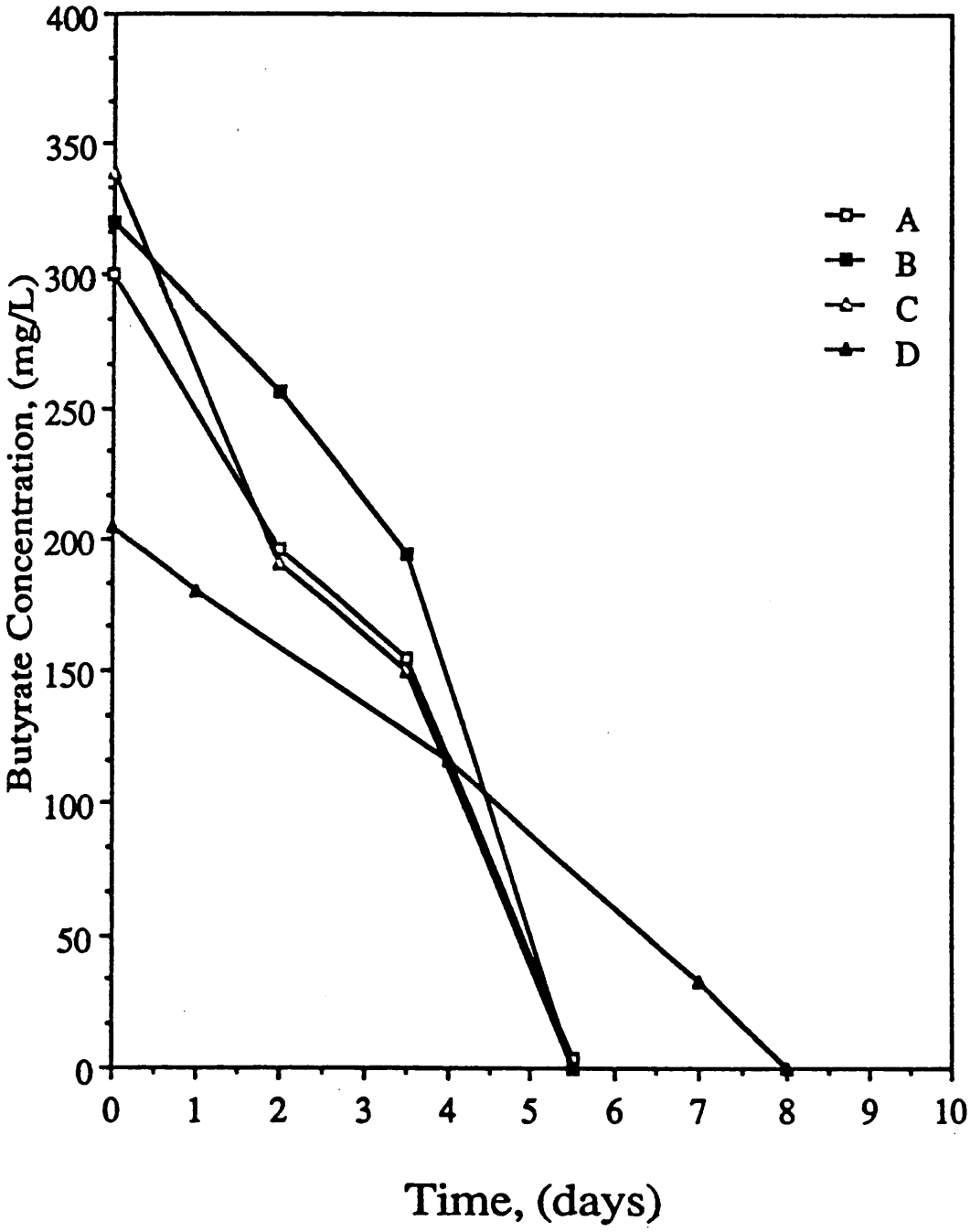

Figure 7. Butyrate biodegradation in the Blacksburg soil under several dilution water conditions: (A) 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (B) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (C) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, and (D) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water.

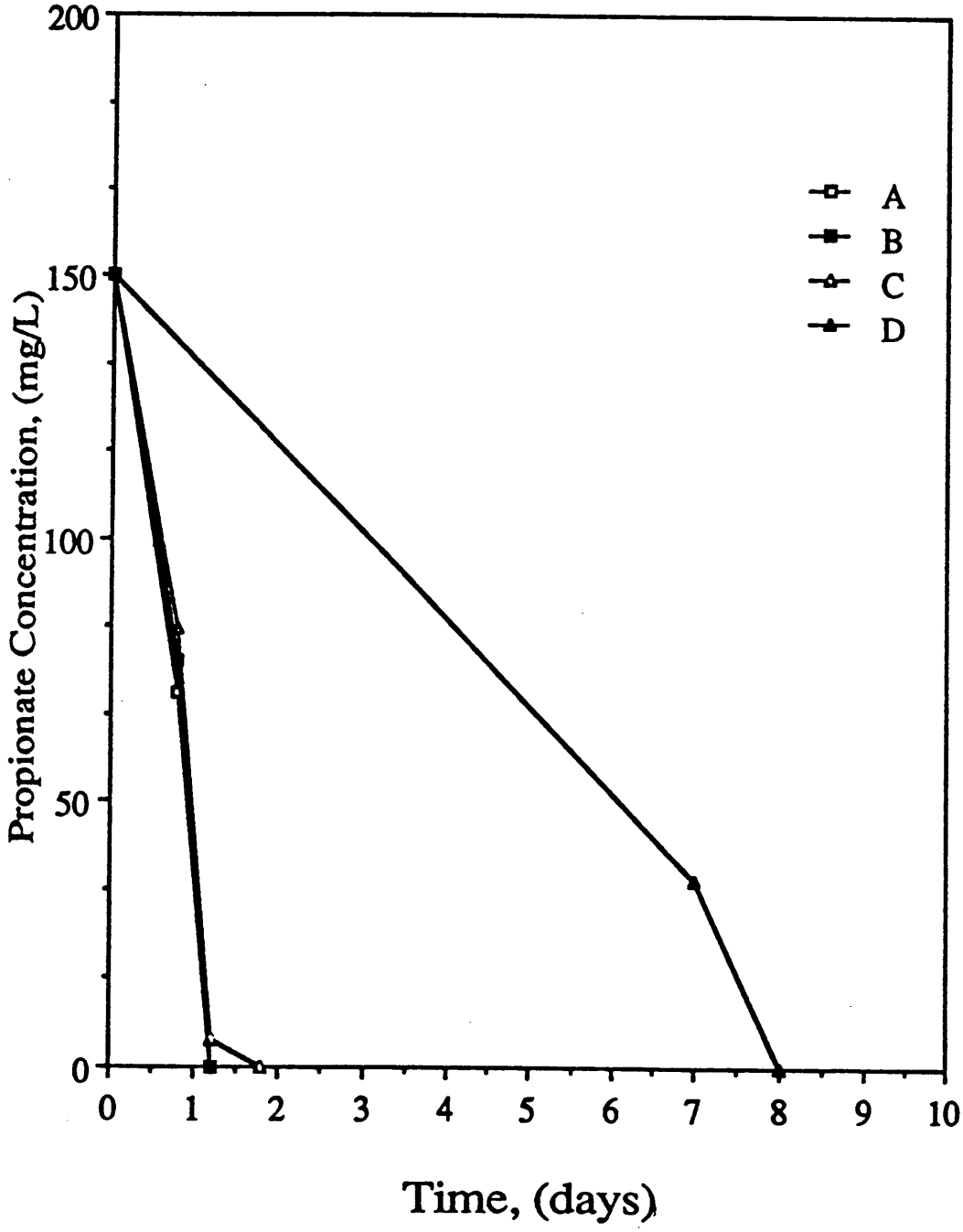

Figure 8. Propionate biodegradation in the Newport News soil under several dilution water conditions: (A) 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (B) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (C) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, and (D) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water.

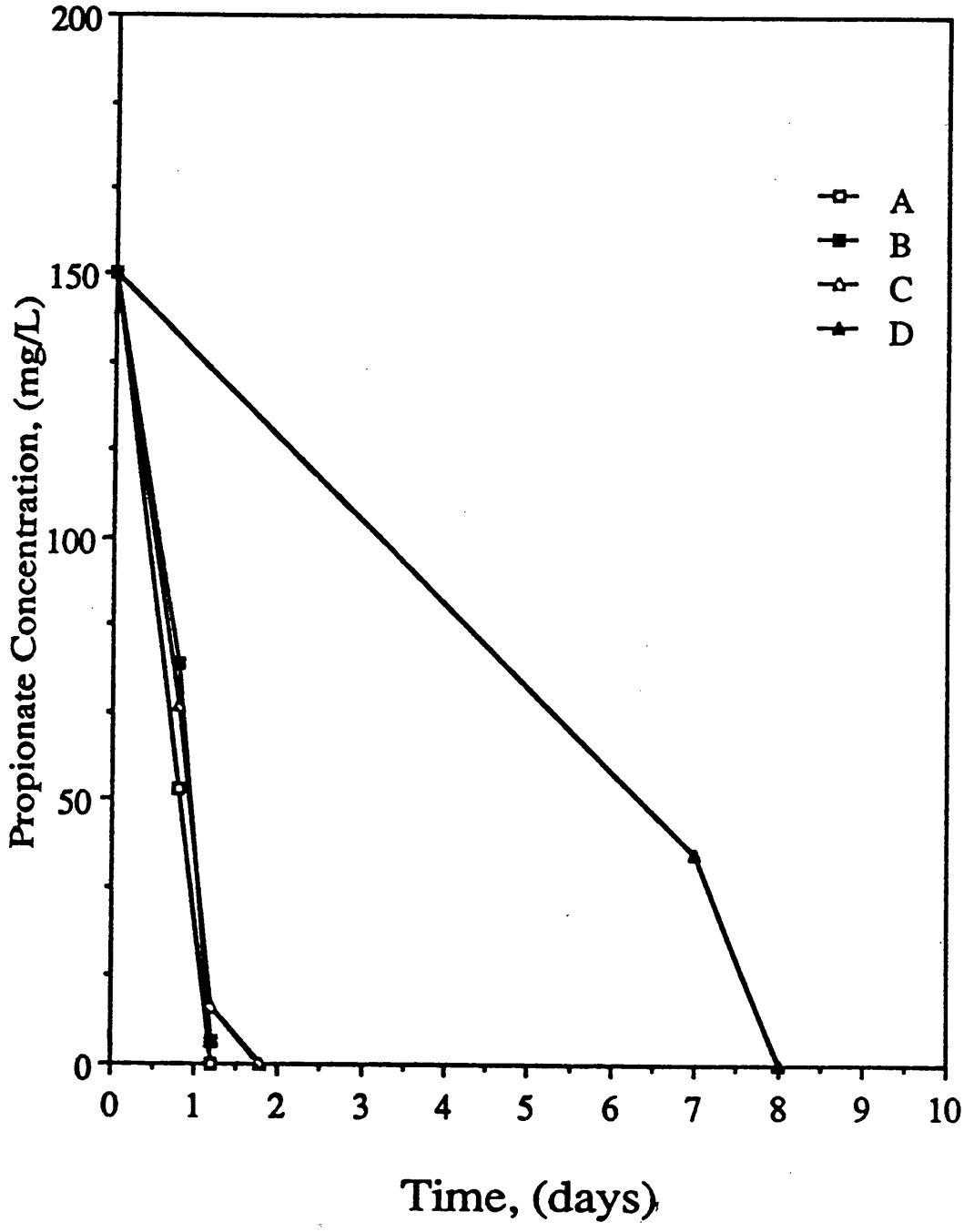

Figure 9. Propionate biodegradation in the Blacksburg soil under several dilution water conditions: (A) 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (B) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (C) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, and (D) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water.

Table 8. Butyrate and propionate biodegradation rate data for the Newport News and Blacksburg soils under several microcosm dilution water conditions.

Soil Type	Microcosm Dilution Water Conditions	Substrate Biodegradation Rates (mg/L·d)	
		Butyrate	Propionate
Newport News	A	55	125
"	B	55	125
"	C	55	125
"	D	25	16
Blacksburg	A	55	125
"	B	55	125
"	C	55	125
"	D	25	16

Where:

A = 100 mg/L sodium nitrate, 0 mg/L sodium molybdate and 0 mg/L BESA were added to the dilution water.

B = 100 mg/L sodium nitrate, 206 mg/L sodium molybdate and 0 mg/L BESA were added to the dilution water.

C = 100 mg/L sodium nitrate, 0 mg/L sodium molybdate and 211 mg/L BESA were added to the dilution water.

D = 0 mg/L sodium nitrate, 0 mg/L sodium molybdate and 0 mg/L BESA were added to the dilution water.

Butyrate and propionate biodegradation rate data for the Newport News and Blacksburg soils, under microcosm dilution water conditions (A), (B), (C), and (D), were determined according to the method outlined in section 3.11. These data are presented in Table 8.

The butyrate biodegradation rates in the Newport News and Blacksburg soils were 55 mg/L·d, under conditions (A), (B), and (C), and 25 mg/L·d, under condition (D). The propionate biodegradation rates in the Newport News and Blacksburg soils were 125 mg/L·d, under conditions (A), (B), and (C), and 16 mg/L·d, under condition (D).

The presence of sodium molybdate and BESA, in microcosm dilution water conditions (B), and (C), had no apparent affect on the biodegradation rate of butyrate or propionate. Thus, the biodegradation rates for the nitrate-amended dilution water conditions, (A), (B), and (C), were identical. However, the absence of sodium nitrate in microcosm dilution water condition (D), caused a 55 and 87% decrease in the butyrate and propionate biodegradation rates, respectively. These results indicate that both the Newport News and Blacksburg soils contained denitrifying bacteria. The denitrification reaction may have precluded the activity of sulfate reducers and methanogens, because it occurs at a redox potential much higher than that for the latter two reactions.

The absence of nitrate in the biodegradation of propionate seemed to be more important than that in the biodegradation of butyrate. The absence of nitrate caused a 55% decrease in the butyrate biodegradation rate, and an 87% decrease in the propionate biodegradation rate. This result is interesting because the propionate experiment was conducted in the same microcosm as the butyrate experiment, after all the butyrate had been depleted. Microbial populations should have increased, and became acclimated to the microcosm conditions throughout the butyrate test. Higher rates were therefore expected during the propionate test. However, the degradation rates for propionate were 36% less than those for butyrate. This may support Sandberg's conclusion that butyrate and propionate were degraded using different biological mechanisms (Sandberg, 1988).

Unfortunately, these single inhibition experiments possibly had two or three microorganism groups active at a time, and the degradation due to a single group was not determined. Therefore, a second series of experiments was performed.

4.2 Results of the Multiple Inhibition Experiment

A second series of experiments was performed to describe the biodegradation of butyrate and propionate under conditions in which two or more inhibitors were present in each microcosm system, and to obtain insight into the individual activity of the denitrifiers, sulfate reducers, and methanogens. Figures illustrating butyrate and propionate biodegradation under multiple inhibition conditions, for the Newport News and Blacksburg soils, are presented in Appendix B.

In Figures 10-13, the disappearance of butyrate and propionate in both the Newport News and Blacksburg soils, under several microcosm dilution water conditions, are shown. The microcosm dilution water conditionsare: (E) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA were added to the dilution water, (F) 0 mg/L sodium nitrate, and 206 mg/L sodium molybdate and 0 mg/L BESA were added to the dilution water, (G) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate and 211 mg/L BESA were added to the dilution water, and (H) 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA were added to the dilution water. The microcosm dilution water conditions (E), (F), and (G), were designed to allow only one microorganism group per microcosm to have unimpeded metabolic activity; denitrifiers, methanogens, and sulfate reducers, respectively. The microcosm dilution water condition (H) was designed to impede the metabolic activity of denitrifiers, sulfate reducers, and methanogens.

In Figures 10 and 11, the disappearance of 150 mg/L butyrate, in both the Newport News and Blacksburg soils, under condition (E), occurred in 7.0 days. The disappearance of 150 mg/L butyrate in the Newport News soil, under conditions (F) and (H), occurred in 53.0 days. The disappearance of 150 mg/L butyrate in Blacksburg soil, under conditions (F) and (H), occurred in 46.0 days. The disappearance of 150 mg/L butyrate in the Newport News and Blacksburg soils, under condition (G), occurred in 14.0 and 18.0 days, respectively.

In Figures 12 and 13, the disappearance of 150 mg/L propionate in the Newport News and Blacksburg soils, under condition (E), occurred in 1.2 and 1.8 days, respectively. The disappearance of 150 mg/L propionate in the Newport News soil, under

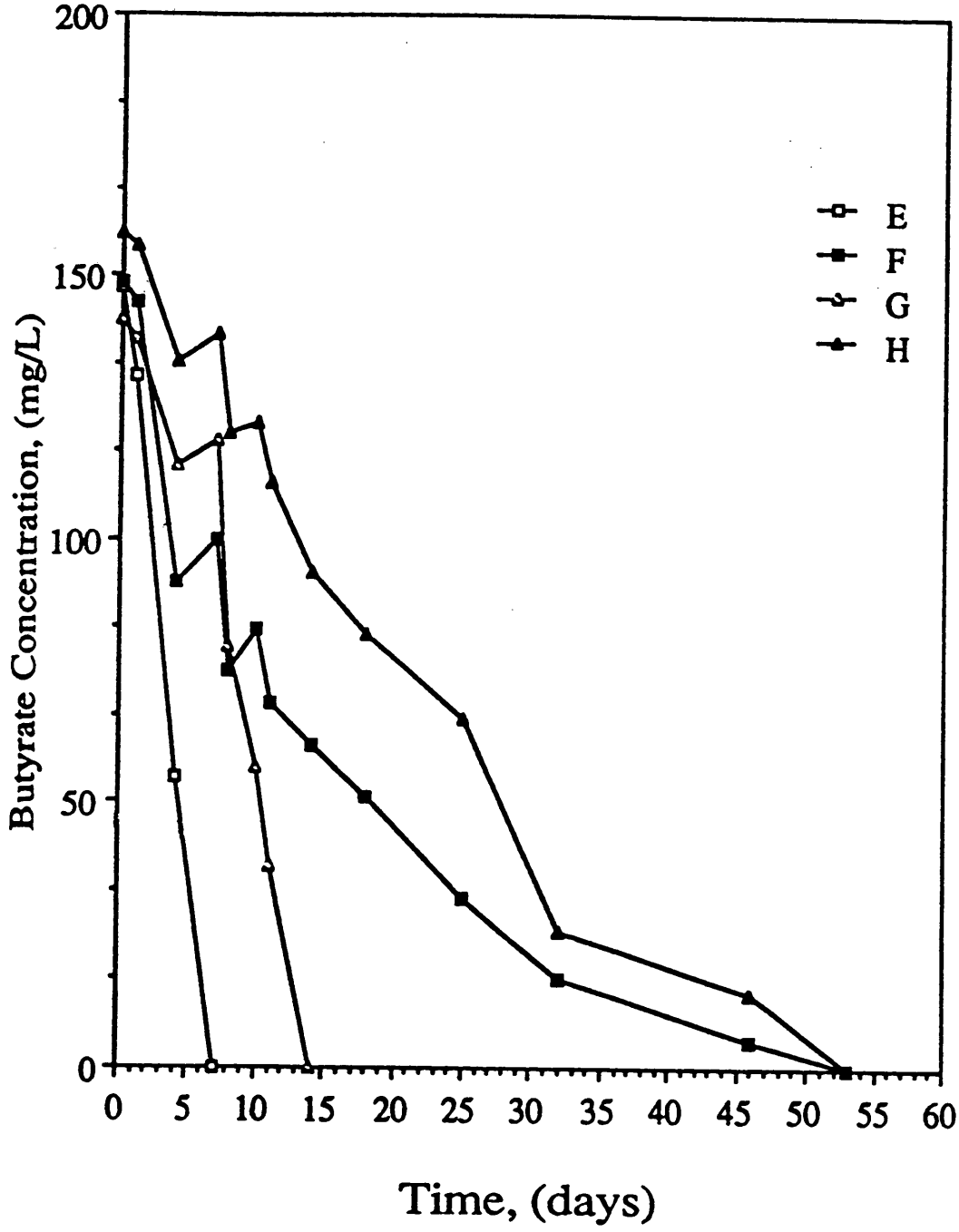

Figure 10. Butyrate biodegradation in the Newport News soil under several dilution water conditions: (E) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, (F) 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (G) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, and (H) 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water.

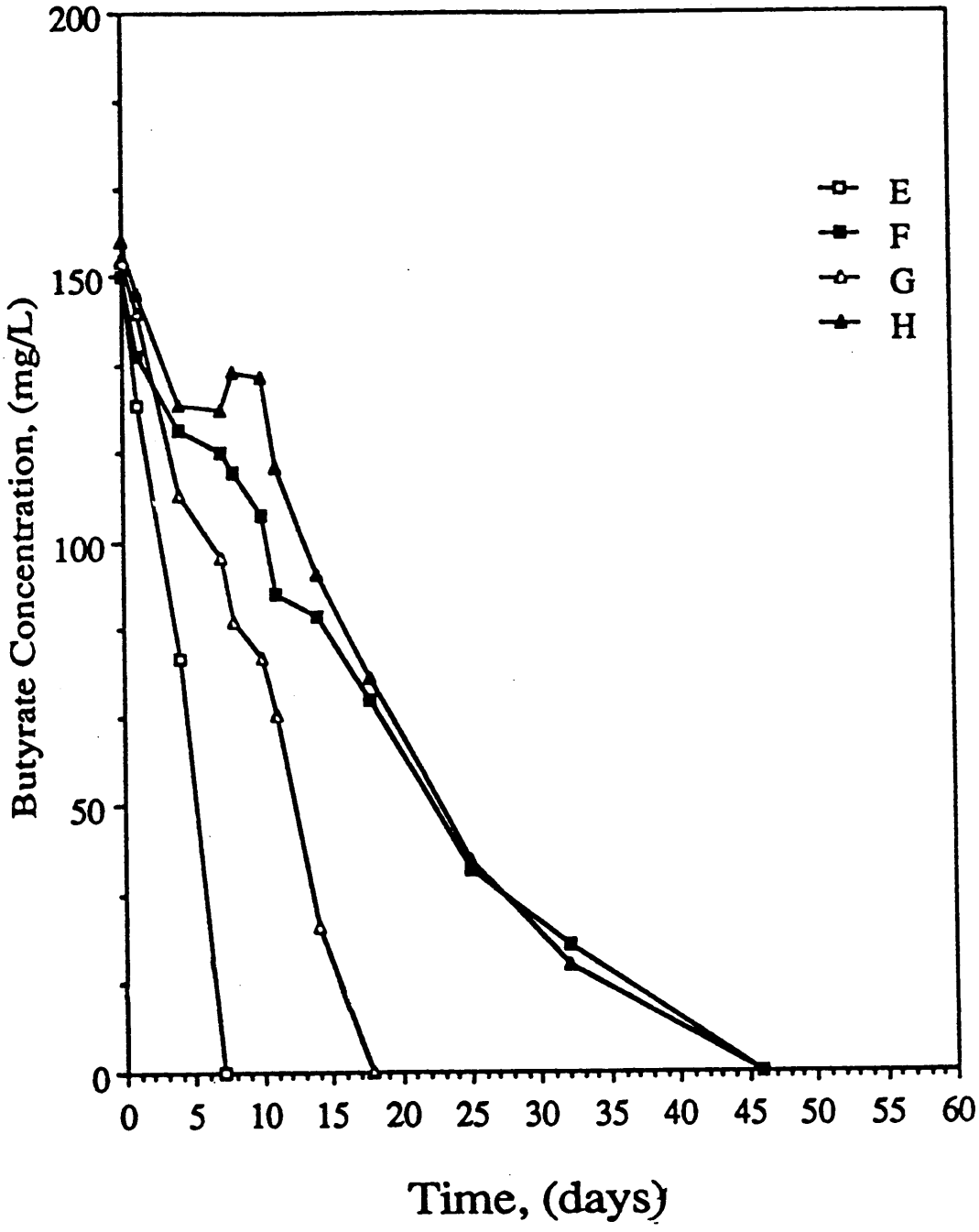

Figure 11. Butyrate biodegradation in the Blacksburg soil under several dilution waterconditions: (E) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, (F) 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (G) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, and (H) 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water.

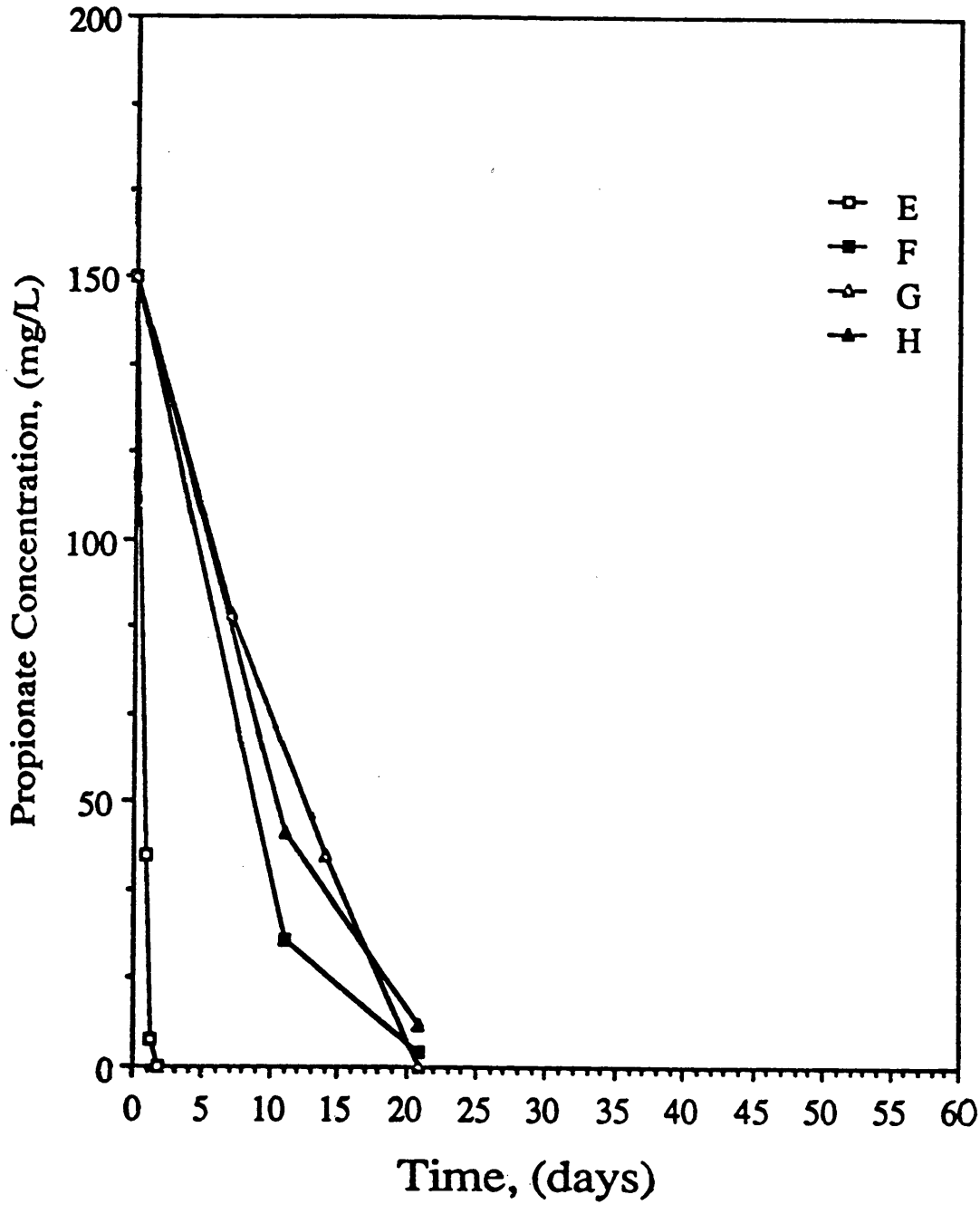

Figure 12. Propionate biodegradation in the Newport News soil under several dilution waterconditions: (E) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, (F) 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (G) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, and (H) 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water.

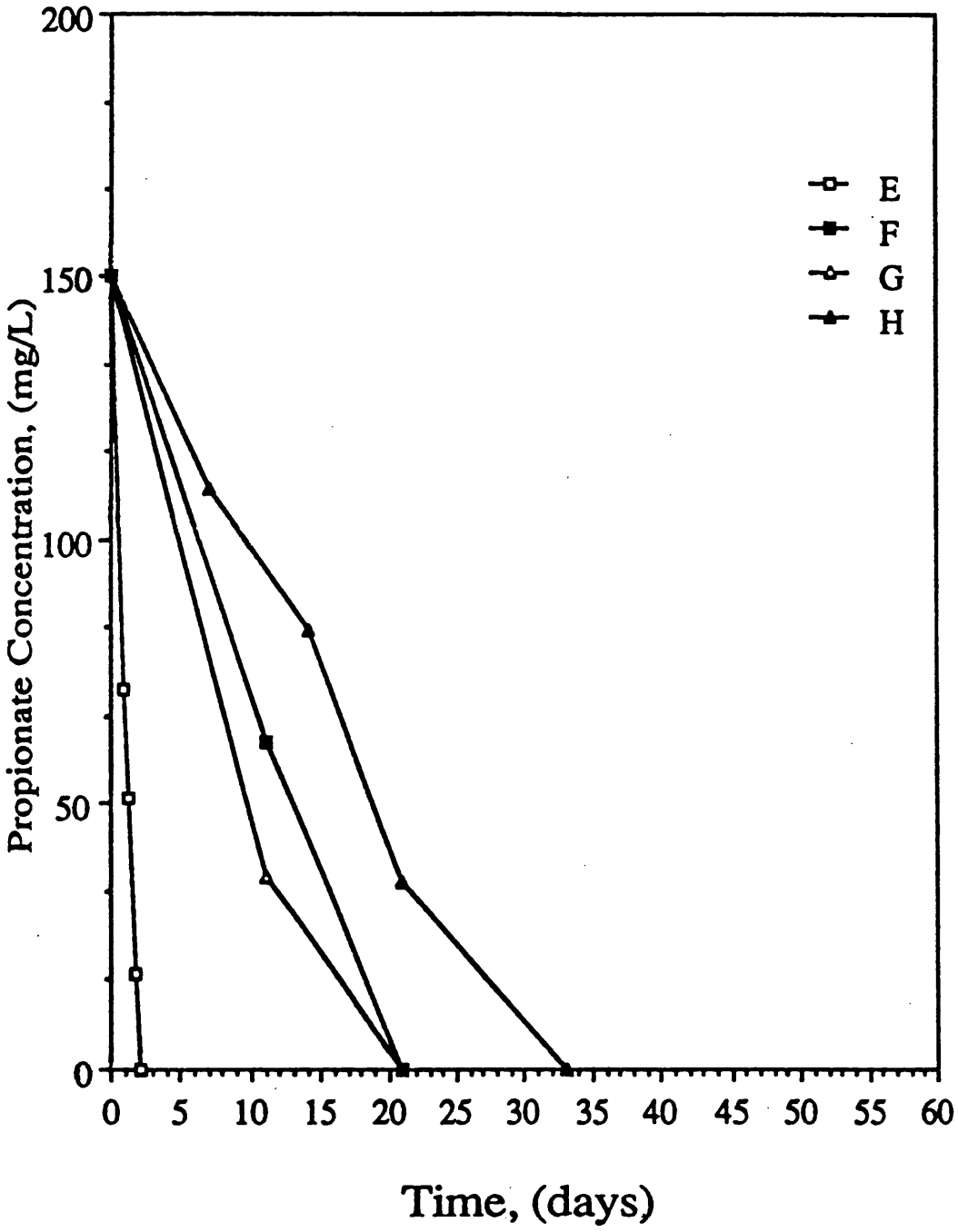

Figure 13. Propionate biodegradation in the Blacksburg soil under several dilution waterconditions: (E) 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, (F) 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water, (G) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 211 mg/L BESA added to the dilution water, and (H) 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, and 0 mg/L BESA added to the dilution water.

Table 9. Butyrate and propionate biodegradation rate data for the Newport News and Blacksburg soils under several microcosm dilution water conditions.

Soil Type	Microcosm Dilution Water Conditions	Substrate Biodegradation Rates (mg/L·d)	
		Butyrate	Propionate
Newport News	E	21	125
"	F	3	4
"	G	11	7
"	H	3	4
Blacksburg	E	21	83
"	F	3	8
"	G	8	5
"	H	3	10

Where:

E = 100 mg/L sodium nitrate, 206 mg/L sodium molybdate and 211 mg/L BESA were added to the dilution water.

F = 0 mg/L sodium nitrate, 206 mg/L sodium molybdate and 0 mg/L BESA were added to the dilution water.

G = 0 mg/L sodium nitrate, 0 mg/L sodium molybdate and 211 mg/L BESA were added to the dilution water.

H = 0 mg/L sodium nitrate, 206 mg/L sodium molybdate and 211 mg/L BESA were added to the dilution water.

conditions (F) and (H), occurred in 37.0 days. The disappearance of 150 to 62 and 36 mg/L, in the Newport News and Blacksburg soils, occurred in 11.0 days, respectively. The disappearance of 150 mg/L propionate in the Newport News and Blacksburg soils, under condition (G), occurred in 21.0 and 33.0 days, respectively.

Butyrate and propionate biodegradation rate data for the Newport News and Blacksburg soils, under conditions (E), (F), (G), and (H), were determined according to the method outlined in section 3.11. These data are presented in Table 9.

The butyrate biodegradation rates in Newport News soil, under conditions (E), (F), (G), and (H), were 21, 3, 11, and 3 mg/L·d, respectively. The butyrate biodegradation rates in Blacksburg soil, under conditions (E), (F), (G), and (H), were 21, 3, 8, and 3 mg/L·d, respectively.

The propionate biodegradation rates in Newport News soil, under conditions (E), (F), (G), and (H), were 125, 4, 7, and 4, mg/L·d, respectively. The propionate biodegradation rates in Blacksburg soil, under conditions (E), (F), (G), and (H), were 83, 8, 5, and 10 mg/L·d, respectively.

The butyrate and propionate biodegradation rates for the nitrate-amended dilution water condition (E), were substantially higher than those for the non-nitrate amended conditions (F), (G), and (H). The butyrate biodegradtion rate for condition (E) in the Newport News soil was 700, 191, and 700% higher than that for conditions (F), (G), and (H), respectively. Similiarly, the butyrate biodegradation rate in the Blacksburg soil for condition (E) was 700, 263, and 700% higher than those for conditions (F), (G), and (H), respectively. The propionate biodegradation rate in the Newport News soil for condition (E) was 3125, 1785, and 3125% higher than those for conditions (F), (G), and (H), respectively. The propionate biodegradation rate in the Blacksburg soil for condition (E) was 1038, 1660, and 830% higher than those for conditions (F), (G), and (H), respectively. These results indicate that denitrifying bacteria are able to biodegrade these substrates at a much higher rate than either sulfate reducing or methanogenic bacteria, assuming all groups are present.

The butyrate and propionate biodegradation rates in the Newport News and Blacksburg soils, under condition (G), where sulfate reducer activity was unimpeded, was

unimpeded. The butyrate biodegradation rates in the Newport News and Blacksburg soils for condition (G) were 367 and 267% higher than those for condition (F), respectively. The propionate biodegradation rate in the Newport News soil for condition (G) was 175% higher than that for condition (F). These results may occur because of the sulfate reducers' ability to outcompete with methanogens for these substrates, as mentioned in section 2.4.3.

Alternatively, the propionate biodegradation rate in the Blacksburg soil for condition (G) was actually 160% less than that for condition (F). This result is peculiar and may have resulted from the partial loss of the sulfate reducer population between the butyrate and propionate experiments. The propionate biodegradation rates in both the Newport News and Blacksburg soils, under condition (G), were 37% less than the butyrate biodegradation rates.

There was little or no difference between the butyrate biodegradation rates in the Newport News and Blacksburg soils, under condition (F), where methanogenic activity was unimpeded, and those under condition (H), where the activity of each microorganism group was impeded. This result may indicate that little or no methanogenic activity occurred.

Sodium molybdate was observed as being less than 100% efficient in inhibiting sulfate reducer activity. In Figure 14, the disappearance of butyrate in Newport News soil, under conditions (F) and (H), is shown. In Figures 14(a) and 14(b), the loss of sulfate and concomitant production of both a black precipitate and H_2S gas, were observed after 25 and 21 days, respectively. Both of these microcosms contained 206 mg/L (1mM) sodium molybdate whicha at this concentration, has been found to be 100% effective in inhibiting sulfate reduction (Winfrey and Ward, 1977; Oremland and Taylor, 1979).

4.3 Comparisons Between the Single Inhibitor and Multiple Inhibitor Experiments

Comparisons between the single inhibiton and multiple inhibition experiments, suggest that there were important interactions between the active microbial group, and the other inhibited groups, which aid in the biodegradation of these substrates.

The biodegradation of butyrate and propionate by denitrifying bacteria may be influenced by the activity of sulfate reducers and methanogens. The propionate

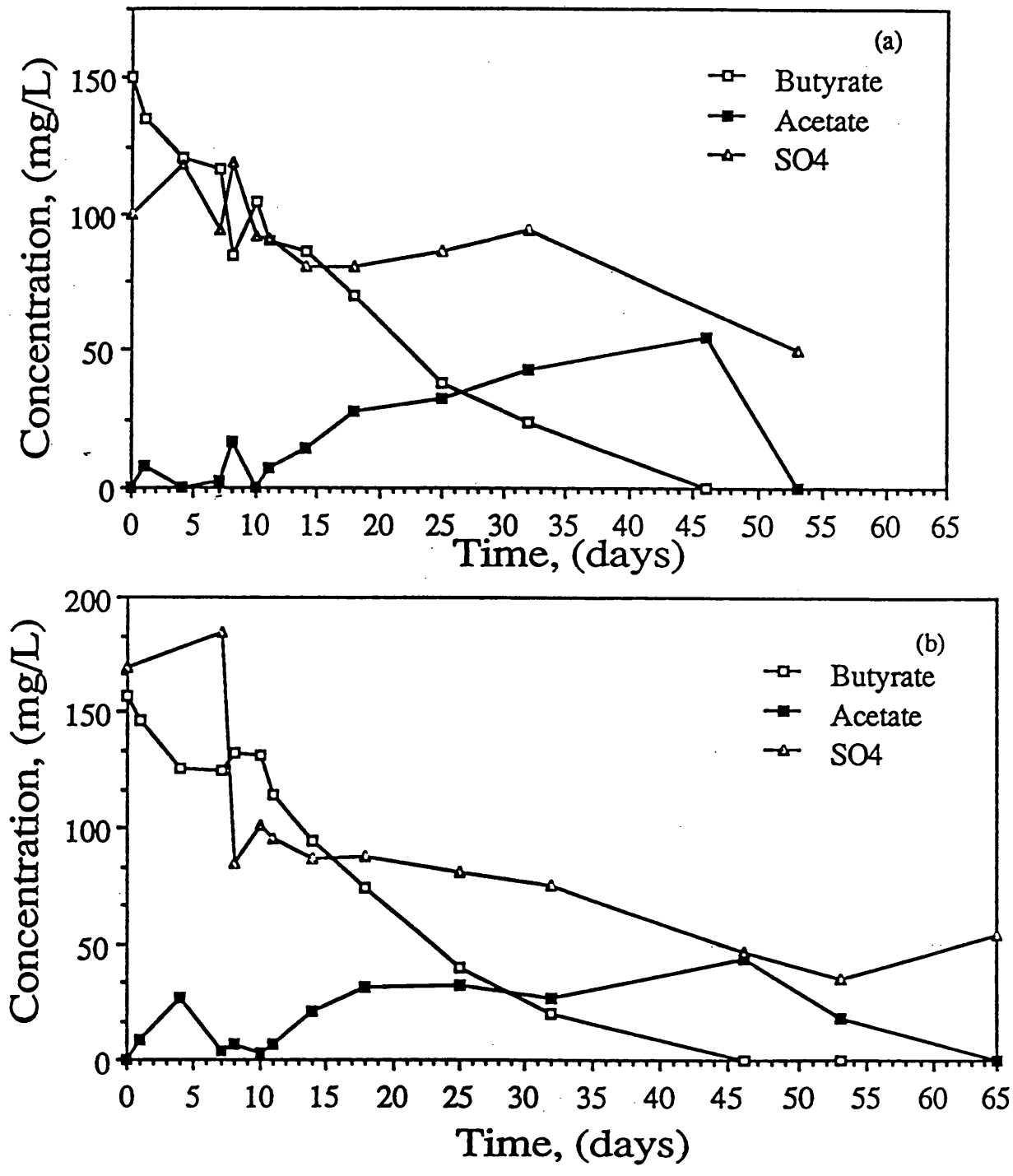

Figure 14. Illustrations of sulfate loss in sodium molybdate-amended systems: (a) butyrate biodegradation in Blacksburg soil with 0 mg/L sodium nitrate and 206 mg/L sodium molybdate added to the microcosm dilution water, and (b) butyrate biodegradation in Blacksburg soil with 0 mg/L sodium nitrate, 206 mg/L sodium molybdate and 211 mg/L BESA added to the dilution water.

biodegradation rate in Blacksburg soil under microcosm dilution water condition (F), which allowed for the occurrence of denitrification and impeded the activity of sulfate reducers and methanogens, was 34% less than that under condition (A), which allowed for denitrification, sulfate reduction, and methanogenesis.

Similiarly, the propionate biodegradation rate in the Newport News and Blacksburg soils, under microcosm dilution water conditions (G), which allowed for sulfate reduction but impeded denitrification and methanogenesis, were 56 and 69% less than that for condition (D), which allowed for both sulfate reduction and methanogenesis but impeded denitrification.

4.4 Comparisons Between the Newport News and Blacksburg Soil Results

The results in both the Newport News and Blacksburg soils, for the single inhibition and multiple inhibition experiments, were surprisingly similiar. There may be several reasons for this similiarity.

Both soils were sampled near the surface, at a depth of between 1 and 3 meters. Near-surface soils are often characterized by high microbial populations and organic matter (Novak *et al.*, 1985; Bone and Balkwill, 1988). Both soils used in the single inhibition experiment, underwent a preliminary test with butyrate, before this study began. This preliminary test may have allowed enough microorganisms to grow in their corresponding soils, that the new populations tested for in the single inhibition experiment, were no longer representative of *in situ* conditions.

The sampling frequency during this test may have been too low. If the Newport News and the Blacksburg soils biodegraded 300 mg/L butyrate to a zero concentration within a day of each other, and the frequency of sampling was every other day, there is a strong likelihood that one would not detect a difference in the biodegradation rates of these soils.

4.5 Results of the Control Experiment

Control microcosms were prepared to determine the loss of butyrate due to bacterial activity and various non-biological processes, as described in section 3.9. The biological

activity could have resulted from microorganisms introduced during the sampling process or microorganisms which were not destroyed during the soil sterilization process.

In Figure 15, the disappearance of butyrate over time for two (2) replicate, "sterilized" Newport News soil microcosms, is presented. The two microcosms, which began with an initial butyrate concentration of 300 mg/L, contained 185 and 32 mg/L butyrate after 63 days, respectively. After 25 days, the replicates contained 98 and 90%, respectively, of their original substrate concentration.

4.6 Experimental Evidence to Support the Control Experiments

Although control microcosms were prepared and analyzed to examine the fate of butyrate, no control experiments were performed to investigate the suppression of denitrifiers by the absence of sodium nitrate, the inhibition of sulfate reduction by the presence of sodium molybdate, or the inhibition of methanogenesis by the presence of BESA. However, there was indirect evidence to support these assumptions.

The assumption that denitrification was suppressed by the absence of sodium nitrate is supported by the production of both a black precipitate, and H_2S gas (indicated by its characteristic "rotten egg" odor). This black precipitate was indicative of sulfate reduction, which occurs at a redox potential lower than that for denitrification. In microcosms which did not contain sodium nitrate or sodium molybdate, a sulfate loss was observed, along with concomitant production of both a black precipitate and H_2S gas. However, in microcosms which contained sodium nitrate, biodegradation rates were 40-50% higher, nitrate loss was observed, and no black precipitate was formed; all indications of denitrification.

Similiarly, there was evidence to support the assumption that the presence of sodium molybdate inhibited sulfate reduction. As stated above, it was observed that in microcosms which did not contain sodium nitrate, or sodium molybdate, sulfate reduction occurred. Whereas, in microcosms which contained sodium molybdate, but did not contain sodium nitrate, no black precipitate was observed during the biodegradation process; in some cases, some sulfate reduction occurred after 20 days, as discussed in section 4.2.

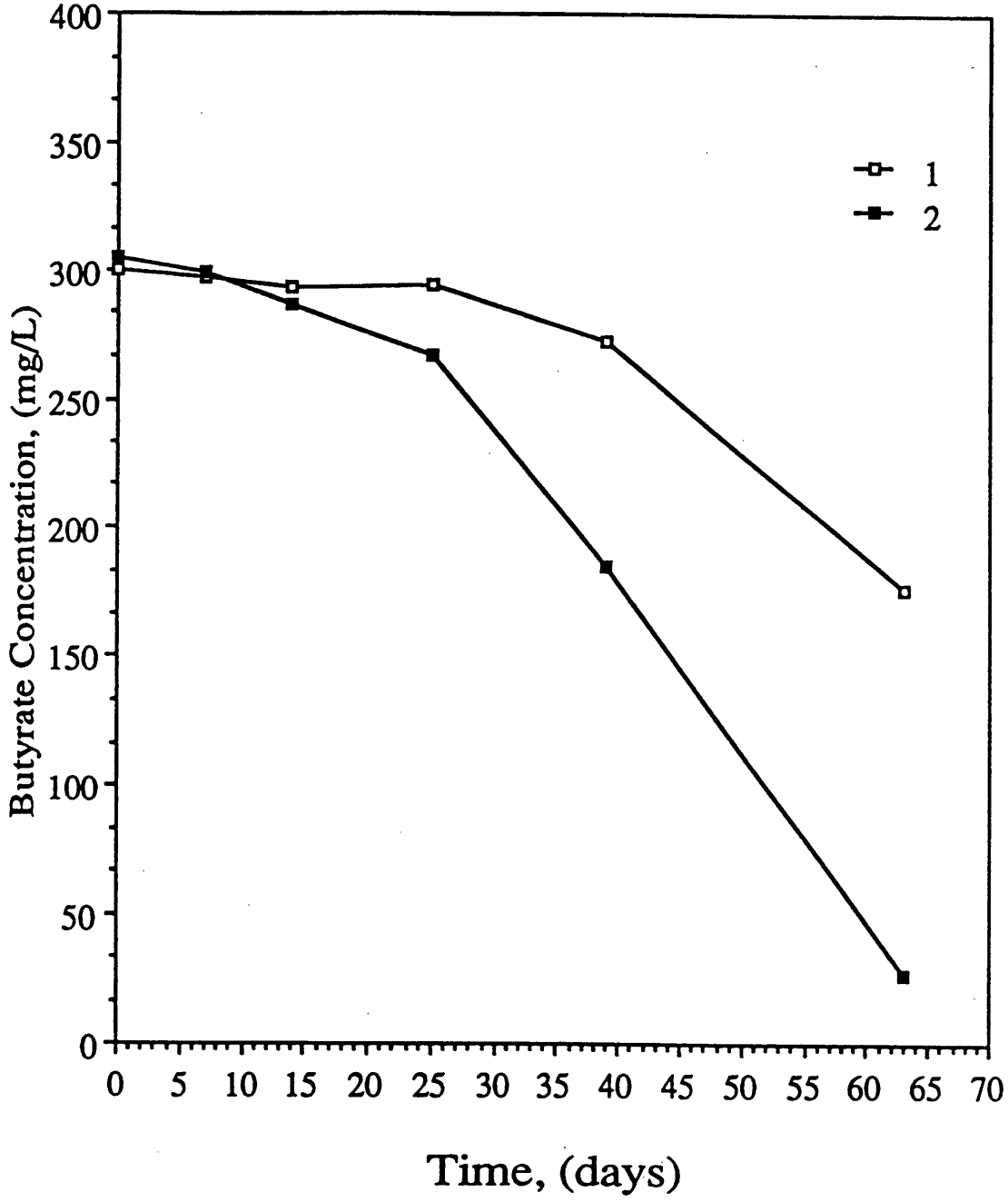

Figure 15. Replicate experiments for butyrate biodegradation in "sterilized" Newport News soil (microcosm contained an initial 300 mg/L butyrate, but did not contain $NaNO_3$, Na_2SO_4, NH_4Cl, KH_2PO_4, Na_2MoO_4, or BESA; stored in a dark cabinet at 20 C).

There was evidence which may support Hickman's (1988) observation (discussed in section 3.10) that methanogens were not present in either the Newport News or Blacksburg soils. The biodegradation rates under microcosm dilution water conditions (F), which allowed methanogen activity to occur unimpeded, and (H), which impeded denitrification, sulfate reduction and methanogenesis, were nearly identical in all cases (see Table 9). This indicates that either no methanogenic activity occurred or that methanogenic activity had little influence in the biodegradation of these substrates.

The assumption that butyrate disappeared by microbiological activity, in the single and multiple inhibition experiments, is supported by the production of acetate. Pfennig and Widdel (1982) suggested that SRBs utilize beta-oxidation to break down the carbon chains of fatty acids. Therefore, C-even fatty acids, like butyrate, would yield stoichimetric amounts of acetate during the biodegradation process. This acetate production is illustrated in Figure 14. Acetate was not added to the system, so the only mechanism by which acetate could have appeared was through the metabolism of butyrate.

4.7 Assessment of the Multiple Inhibitor Approach for Evaluating the Biodegradation Potential of Soil

A primary objective of this research was to make a preliminary assessment of the use of inhibitors to determine the biodegradation potential of soils. While this method holds promise, some major limitations exist. Some of these limitations can be overcome with additional studies, while others appear to be limited by this procedure.

The following are limitations of this procedure:

1. Inhibition of sulfate reduction by sodium molybdate appeared to be only partially successful, as discussed in section 4.2. During butyrate biodegradation in Blacksburg soil, sulfate loss and the concomitant formation of a black precipitate were observed after 20 days indicating that sodium molybdate did not entirely inhibit sulfate reduction.

2. Methanogenic activity was not detectable. Monitoring of methane generation would be required to quantify the activity of MPBs. Monitoring would complicate the test procedure and require the use of more elaborate microcosms.

3. The two soils studied did not differ substantially. Therefore, the ability of this method to characterize systems where soil microbial populations differ, was not

determined.

4. The influence of sodium molybdate and BESA on the many microbial reactions which take place during the biodegradation process are not fully understood, which places a degree of uncertainty in the evaluation of experimental data.

The following are potential benefits of this approach:

A. It is a simple, direct approach for the possible evaluation of biodegradation potential of soils.

B. By using the support data discussed in section 4.6, one can make inferences as to the significance of each microbial group in the biodegradation process.

C. This procedure may yield approximately the same information as more complicated and time-consuming microbial enumeration procedures.

Microbial enumeration studies have the same limitations as 2, 3, and 4, above. However, this multiple inhibitor approach appears to be promising enough to warrant further study. Coupling these tests with specific microcosm studies on individual organic contaminants, one may evaluate the biodegradation potential of a variety of soil systems.

CHAPTER 5

CONCLUSIONS

From this study the following conclusions can be made:

1) The Newport News and Blacksburg soils were characterized by a highly active denitrifier population. Butyrate and propionate biodegradation rates were 21 and 125 mg/L·d, respectively.

2) The Newport News and Blacksburg soils also showed an active sulfate reducer population. When nitrate was absent from the microcosm dilution water, significant losses in aqueous sulfate concentrations, along with concomitant production of H_2S gas and a black precipitate, indicated active biological sulfate reduction. Under these conditions, butyrate and propionate biodegradation rates in the Newport News soil were 8 and 5 mg/L·d, respectively.

3) The Newport News and Blacksburg soils did not respond to BESA addition, indicating little or no methanogenic activity.

4) The results obtained from this study on the Newport News and Blacksburg soils, using a multiple inhibitor approach, agreed with those from Hickman's (1988) and Morris' (1988) enumeration studies. That is, both soils had active denitrifier and sulfate reducer populations, and displayed no methanogenic activity.

CHAPTER 6

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APPENDIX A

SINGLE INHIBITION BIODEGRADATION FIGURES

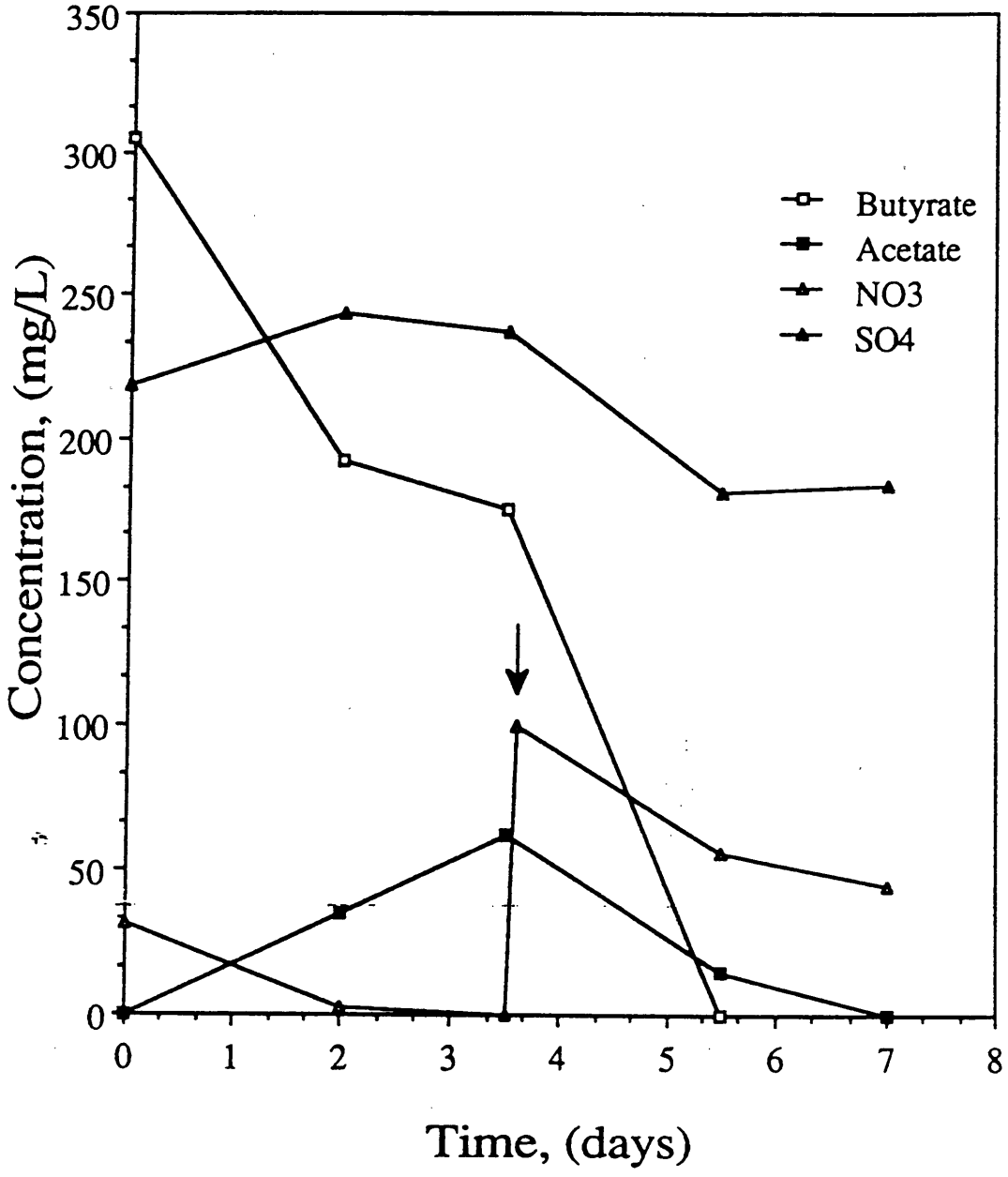

Figure A1. Butyrate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA (arrow denotes respiking of sodium nitrate).

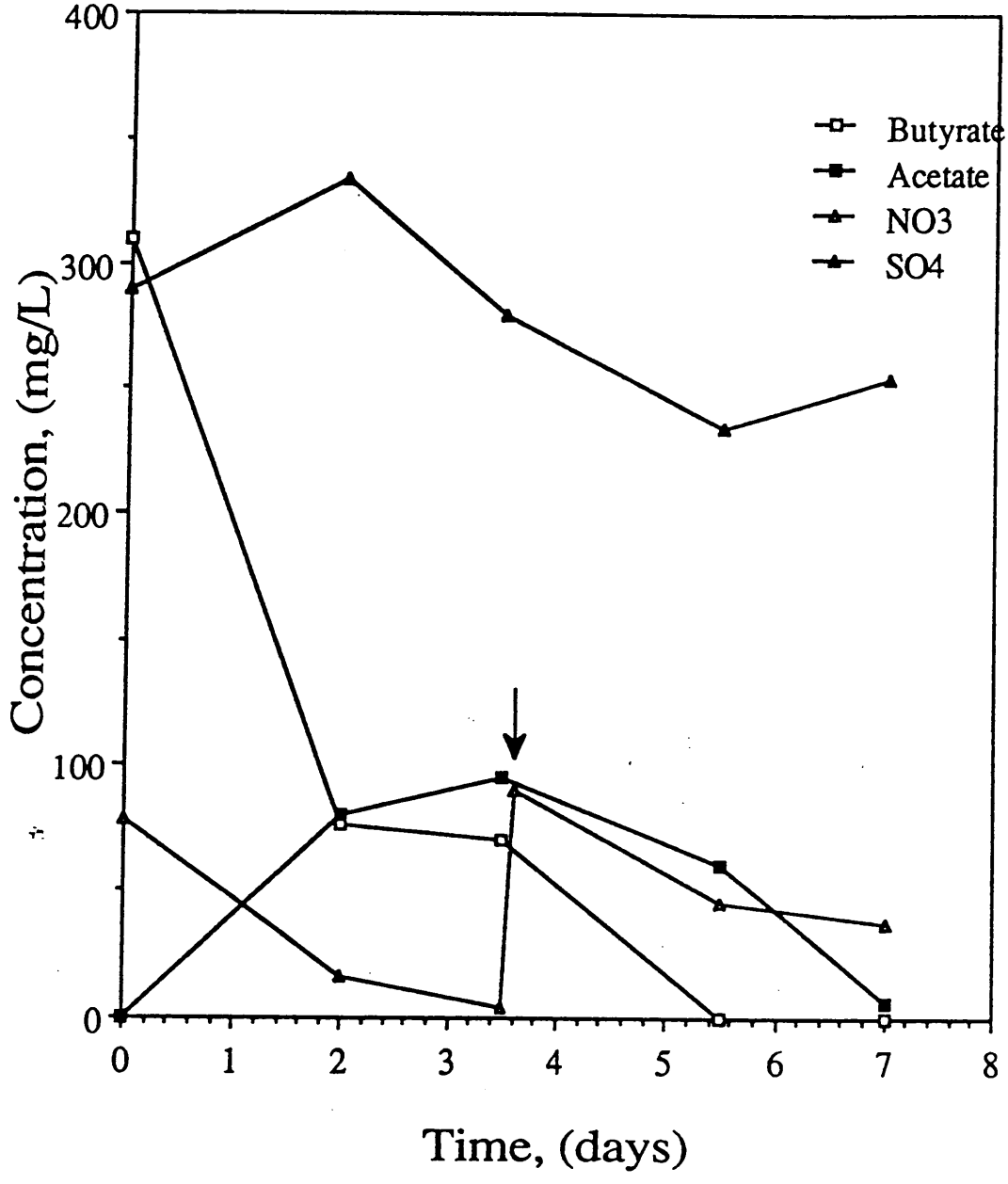

Figure A2. Butyrate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (arrow denotes respiking of sodium nitrate).

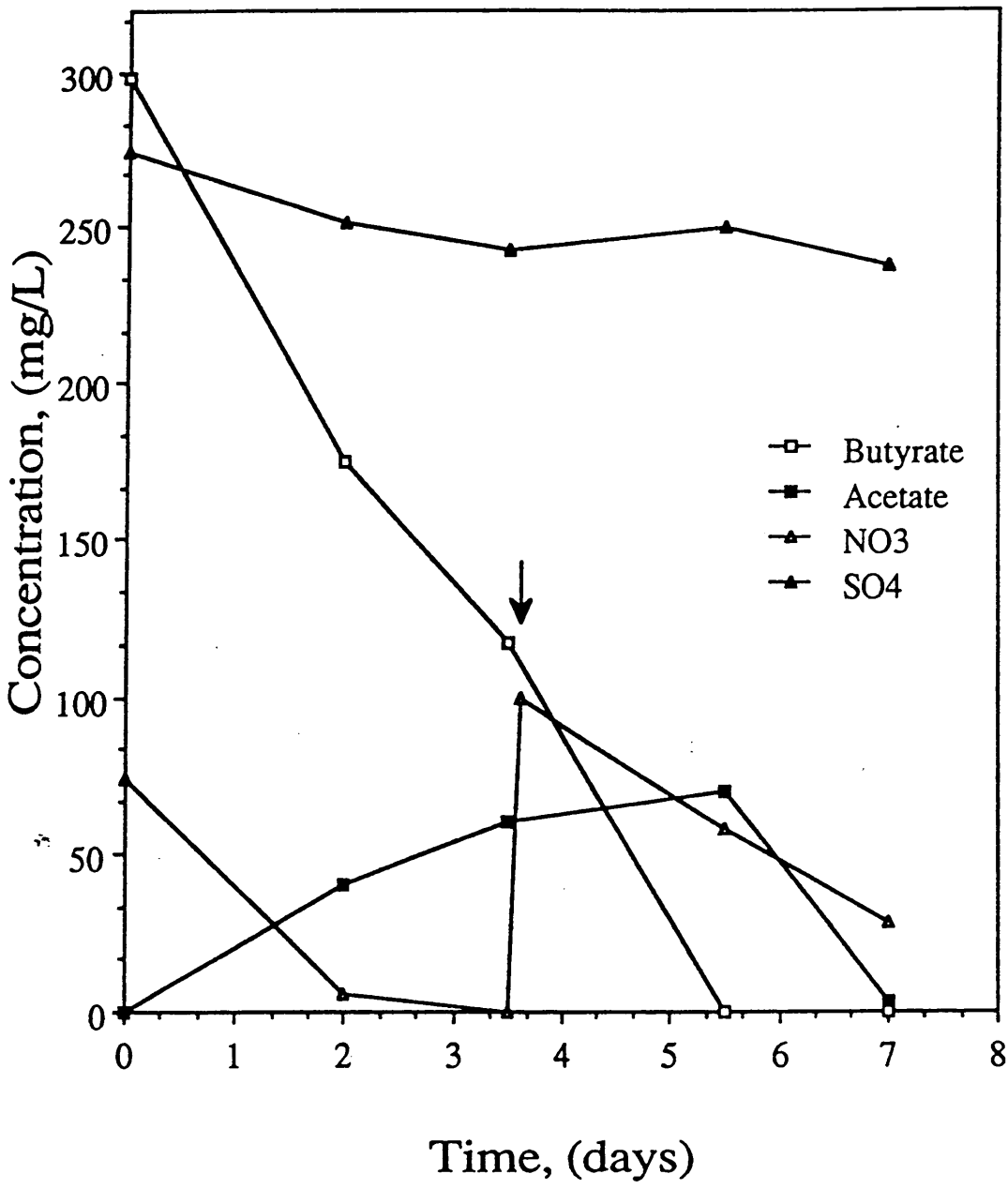

Figure A3. Butyrate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (arrow denotes respiking of sodium nitrate).

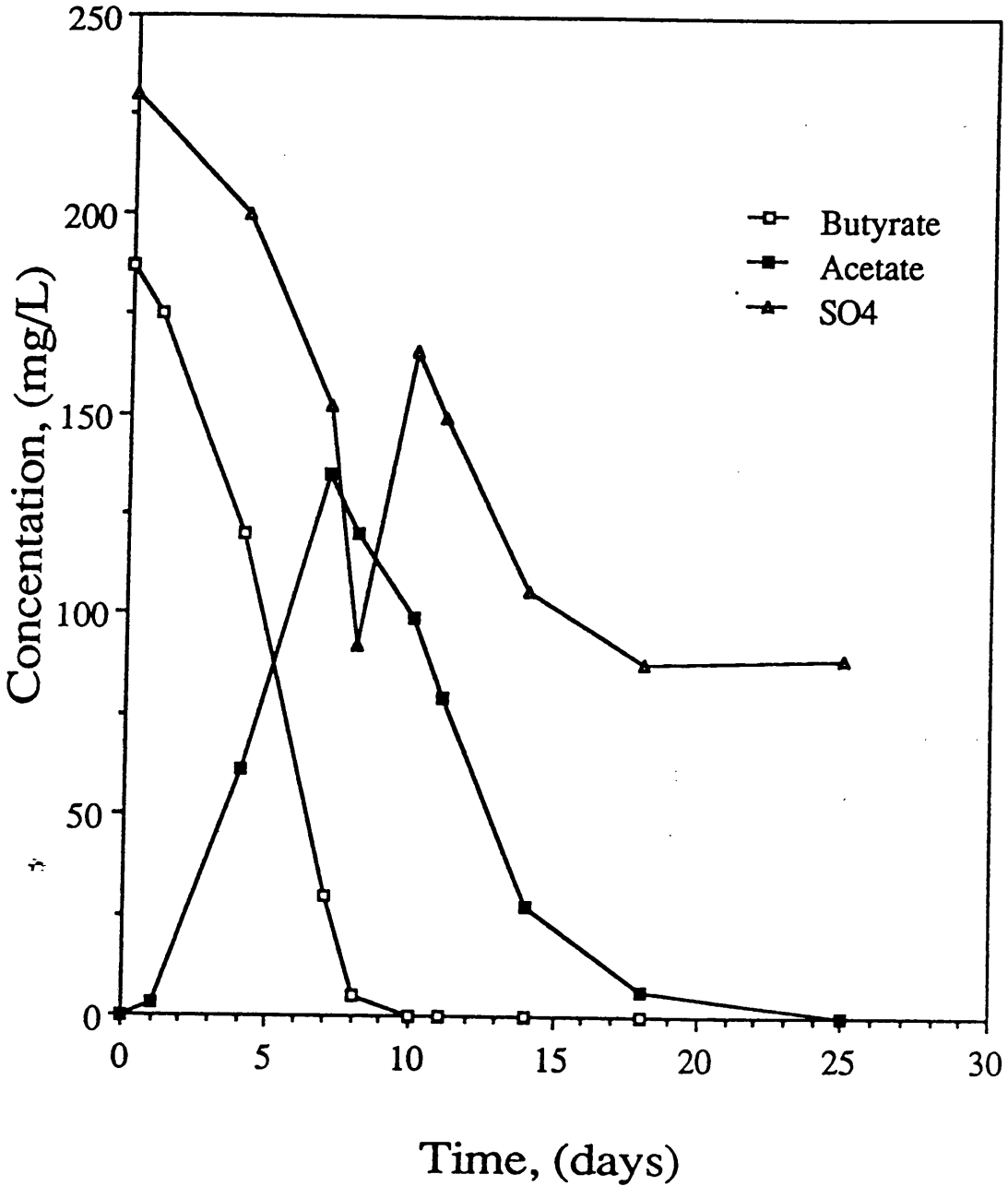

Figure A4. Butyrate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA.

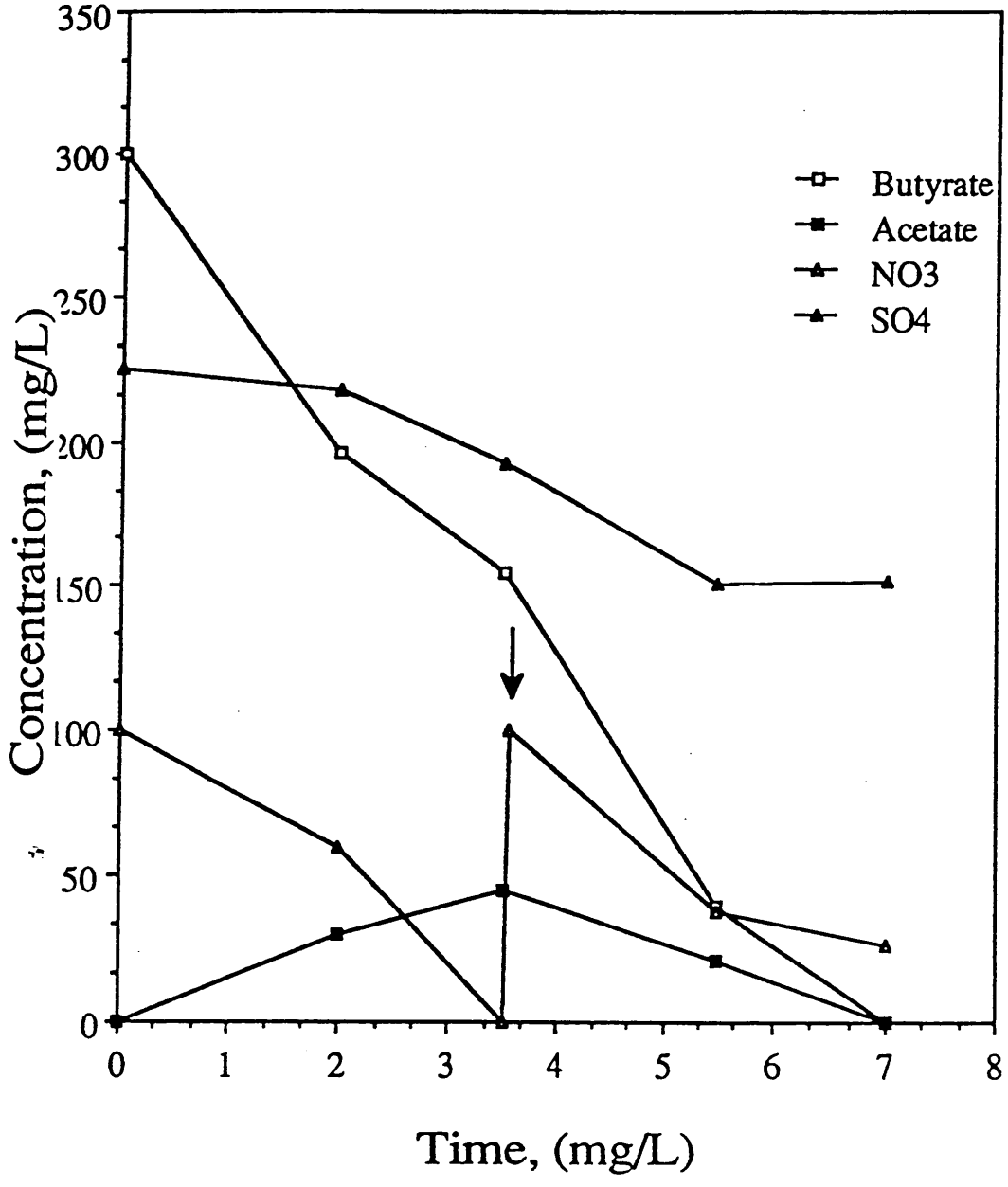

Figure A5. Butyrate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA (arrow denotes respiking of sodium nitrate).

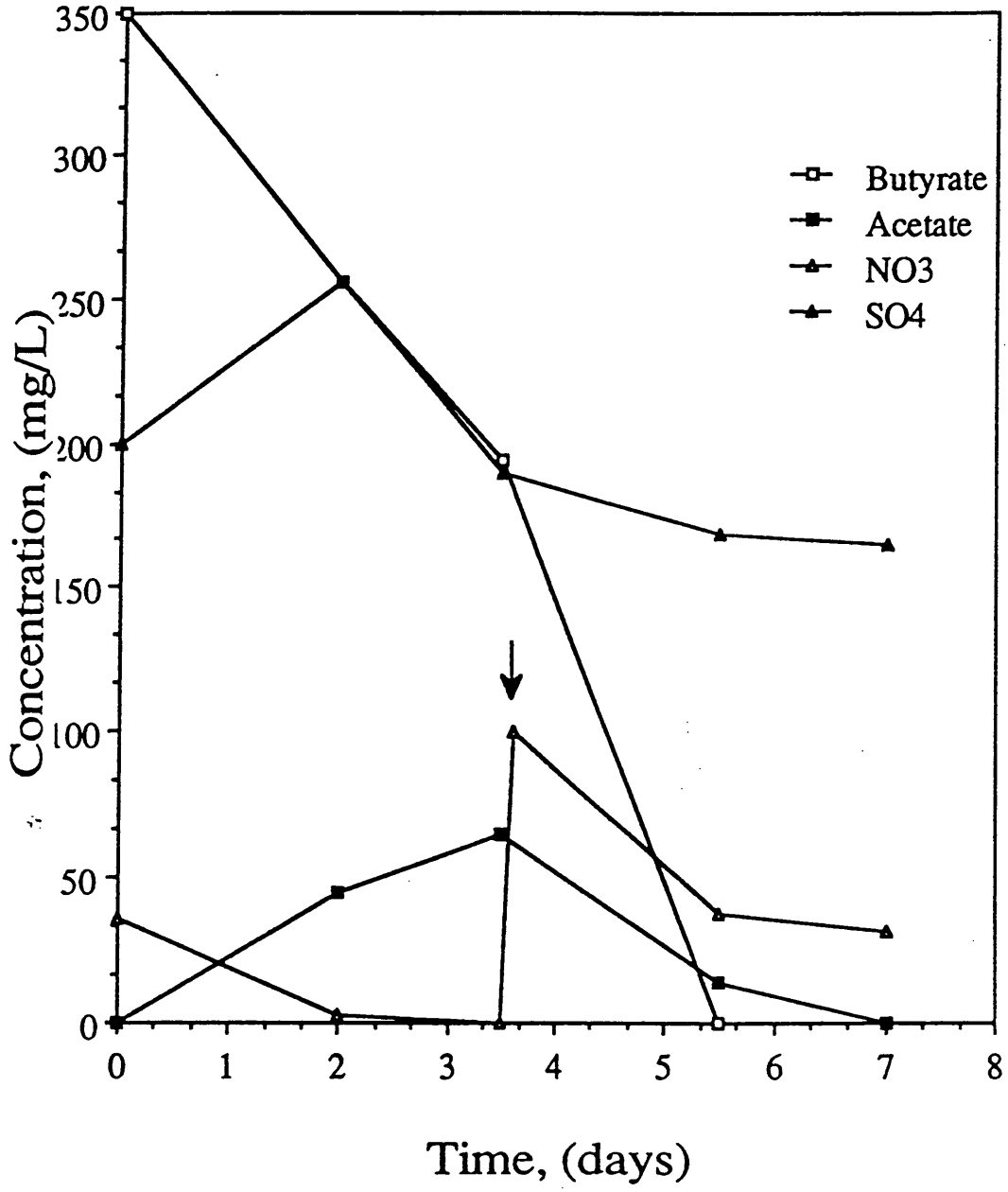

Figure A6 Butyrate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (arrow denotes respiking of sodium nitrate).

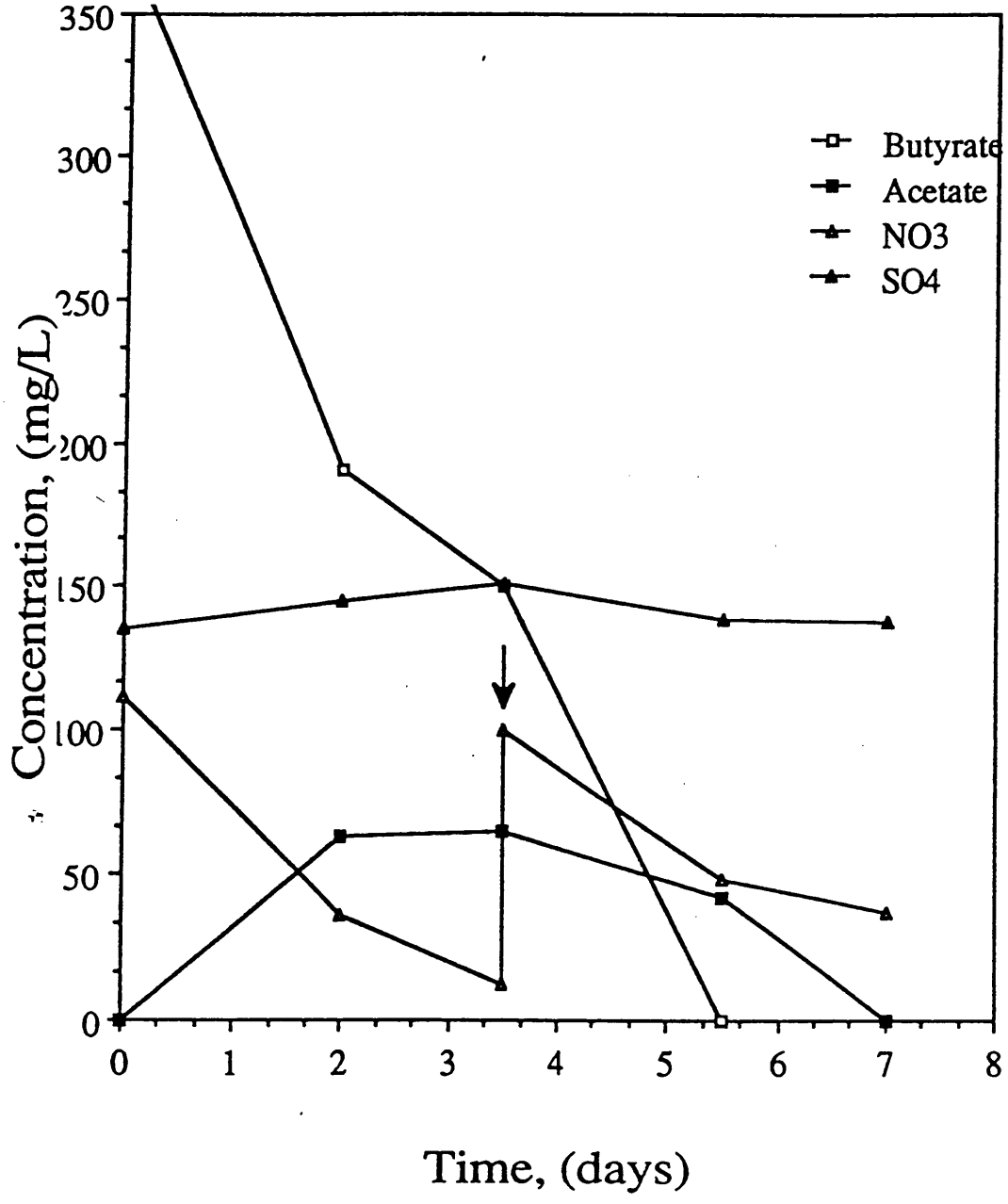

Figure A7. Butyrate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (arrow denotes respiking of sodium nitrate).

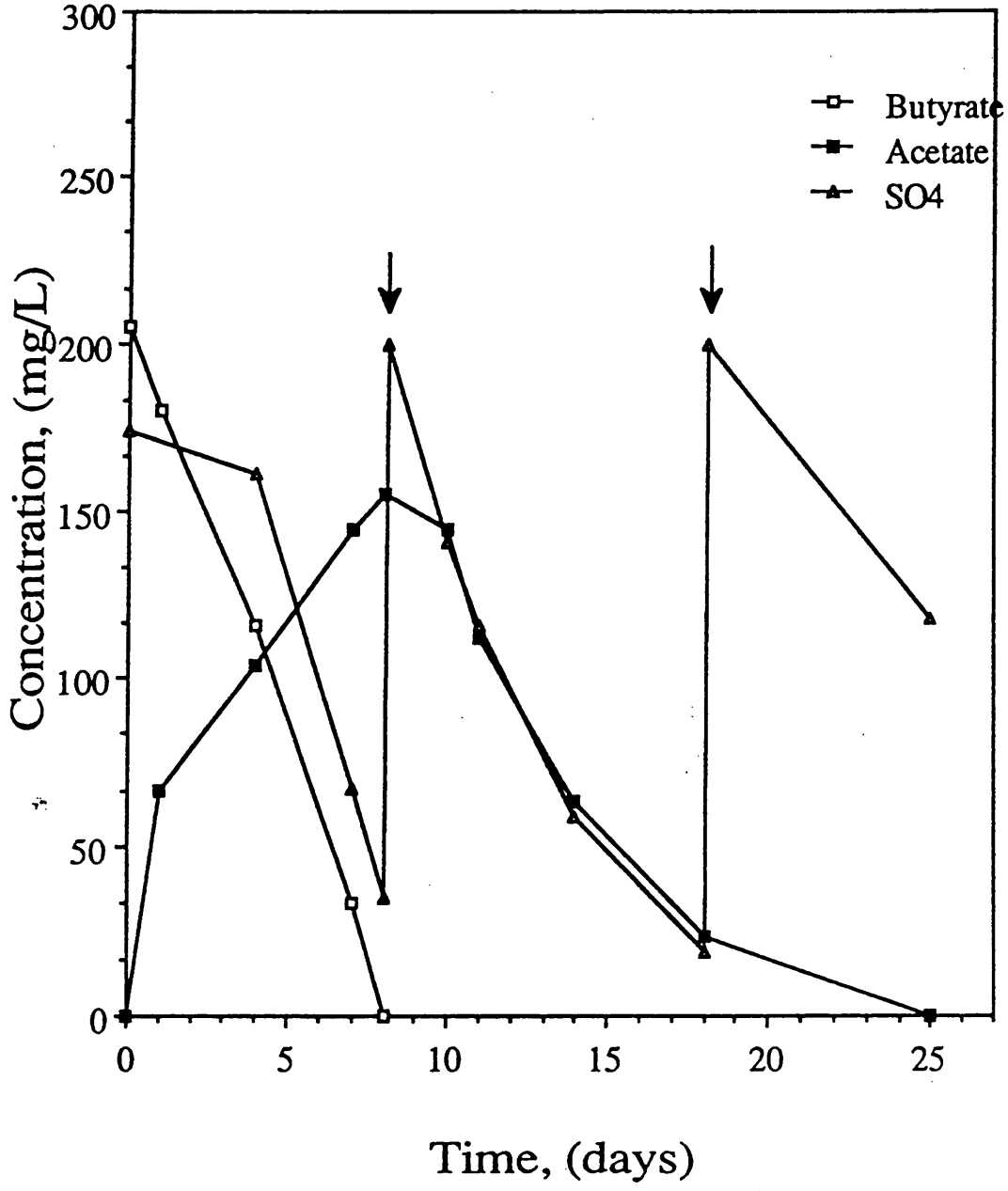

Figure A8. Butyrate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA.

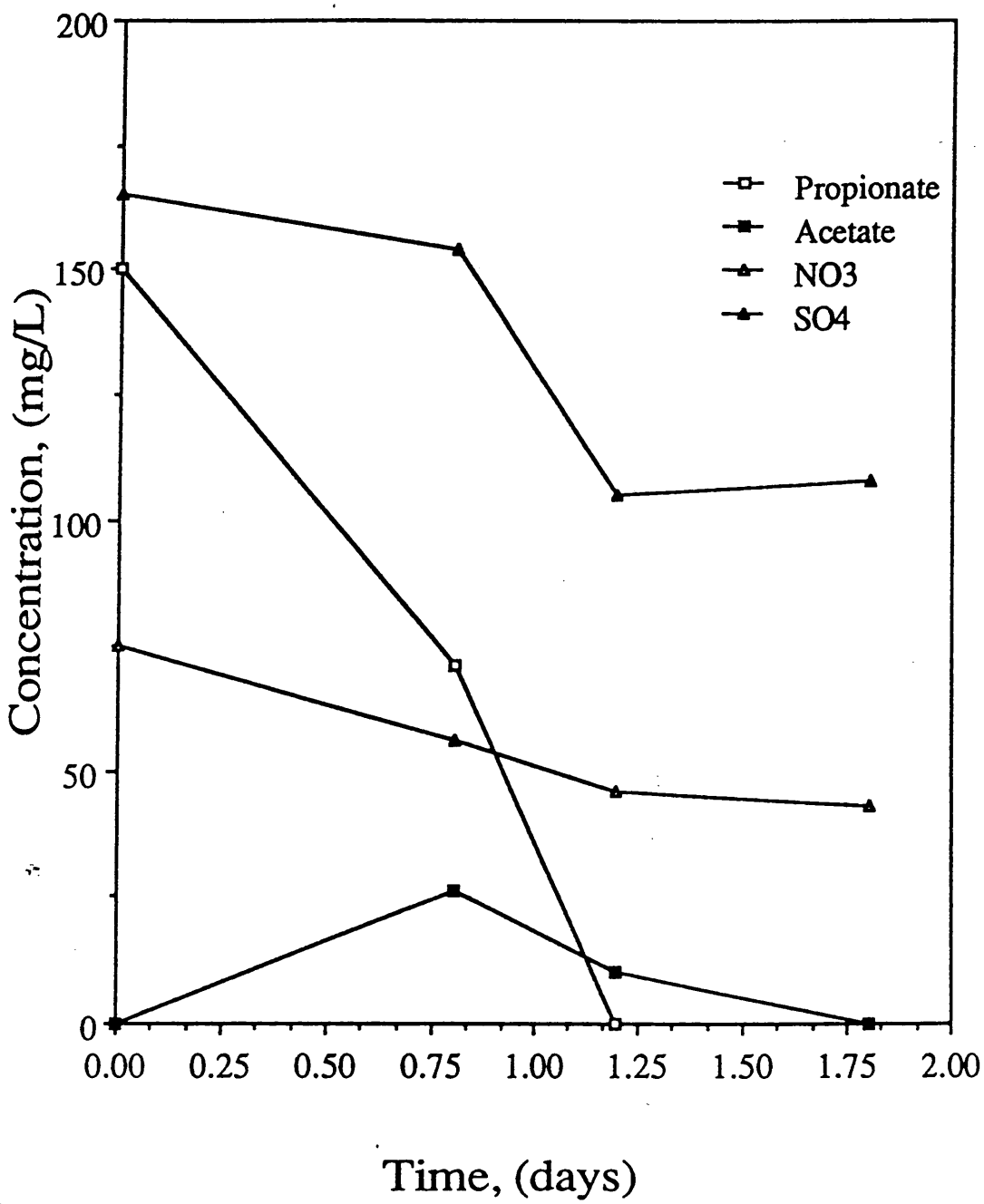

Figure A9. Propionate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA (arrow denotes respiking of sodium nitrate).

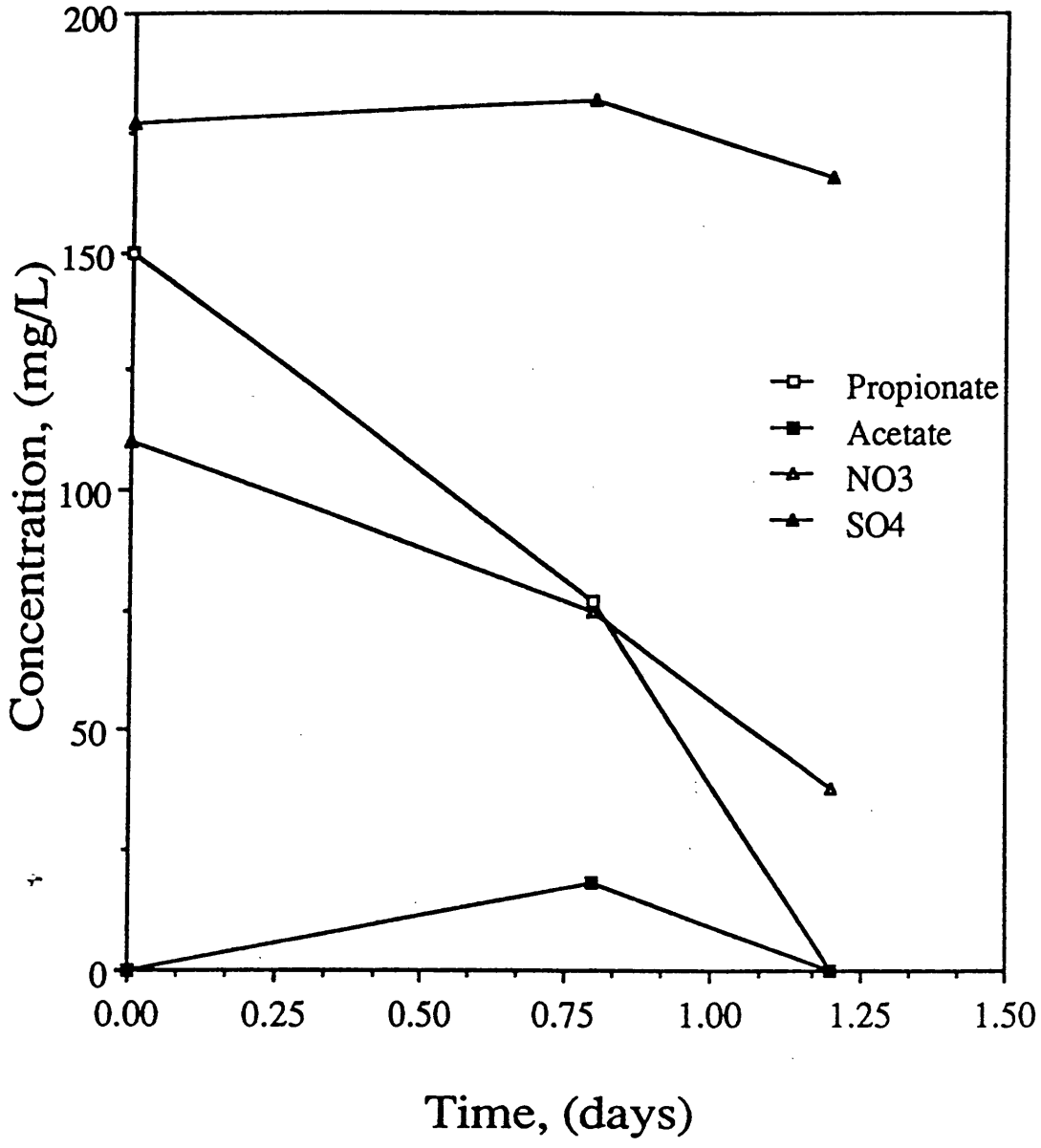

Figure A10. Propionate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (arrow denotes respiking of sodium nitrate).

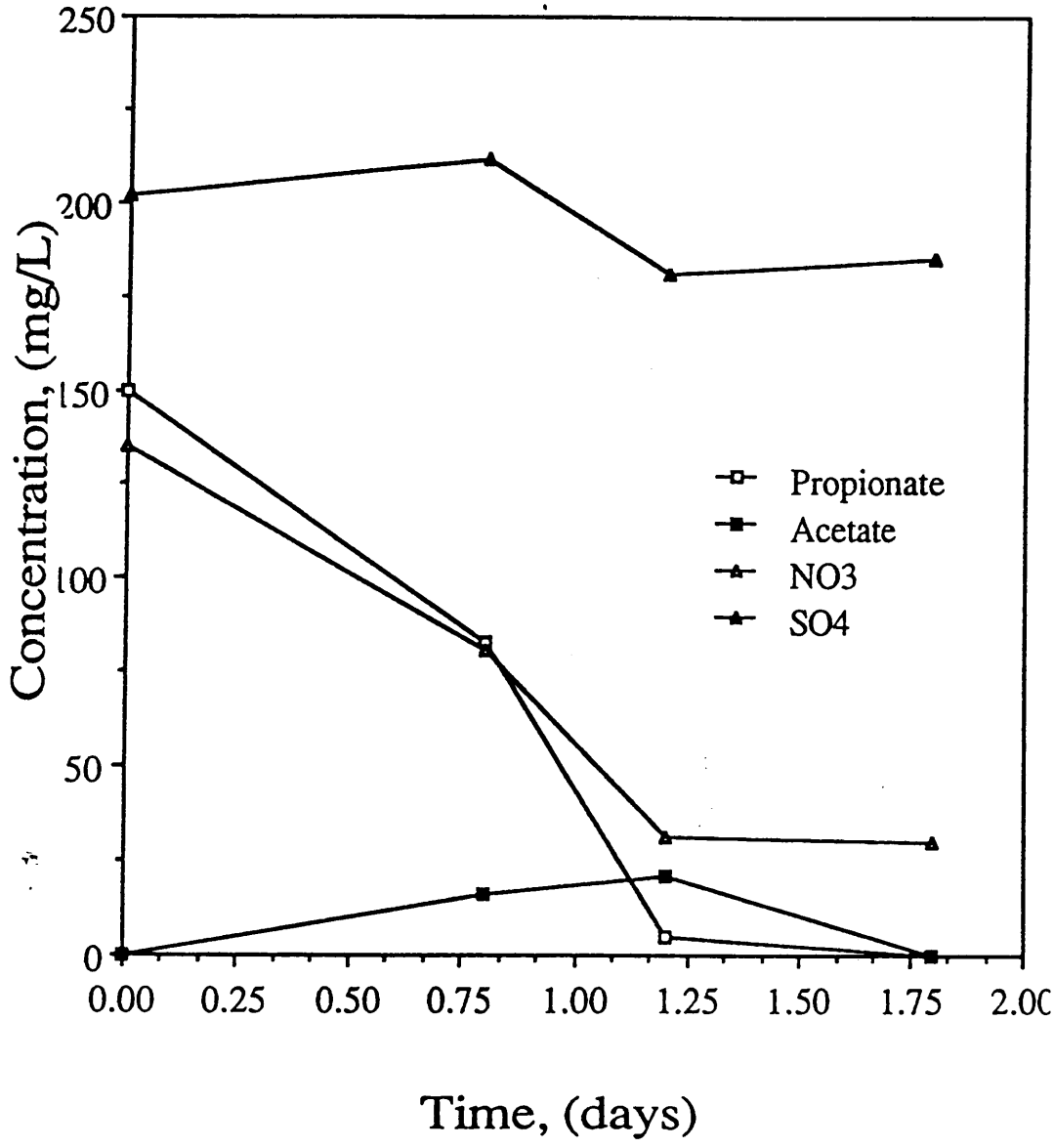

Figure A11. Propionate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (arrow denotes respiking of sodium nitrate).

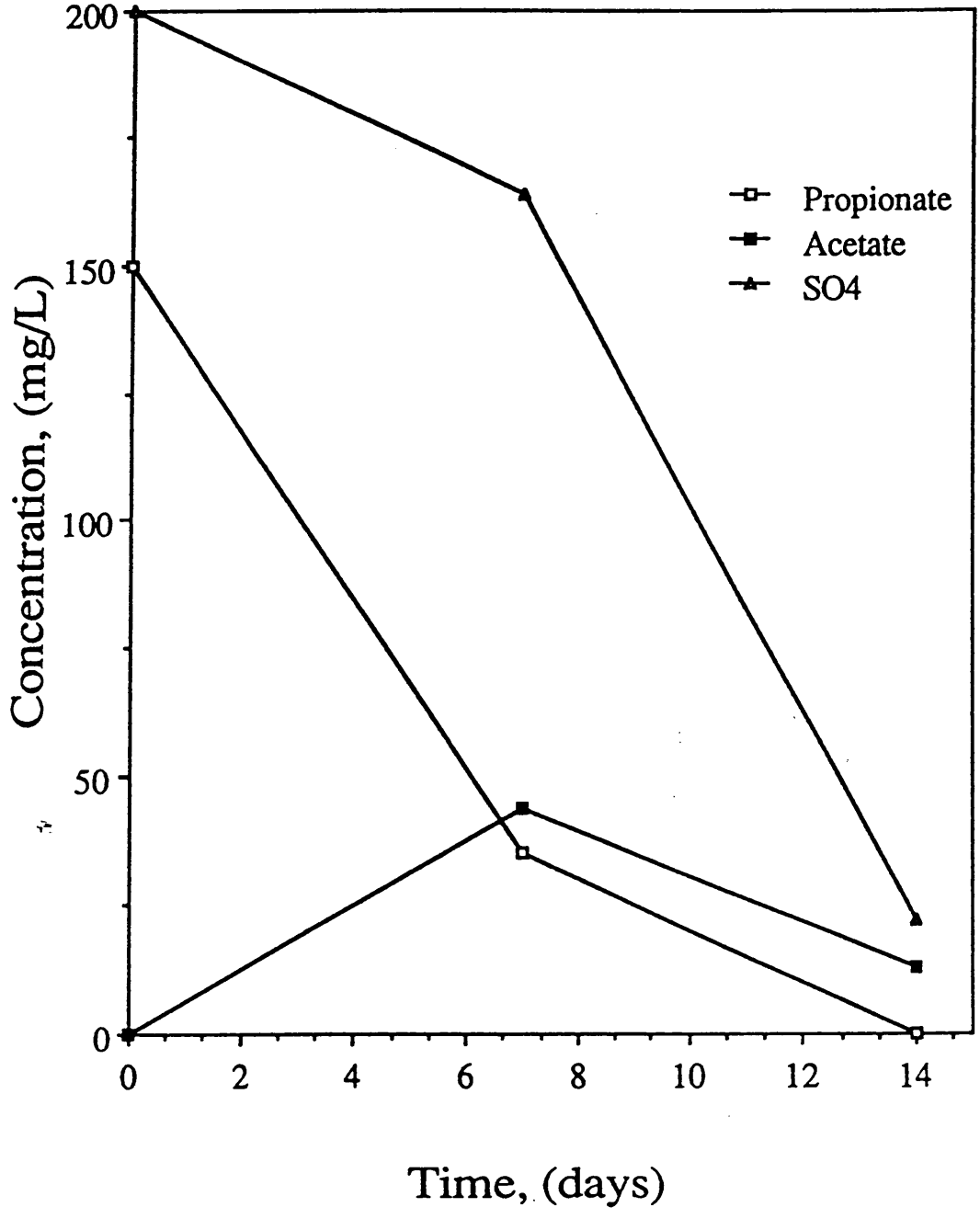

Figure A12. Propionate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA.

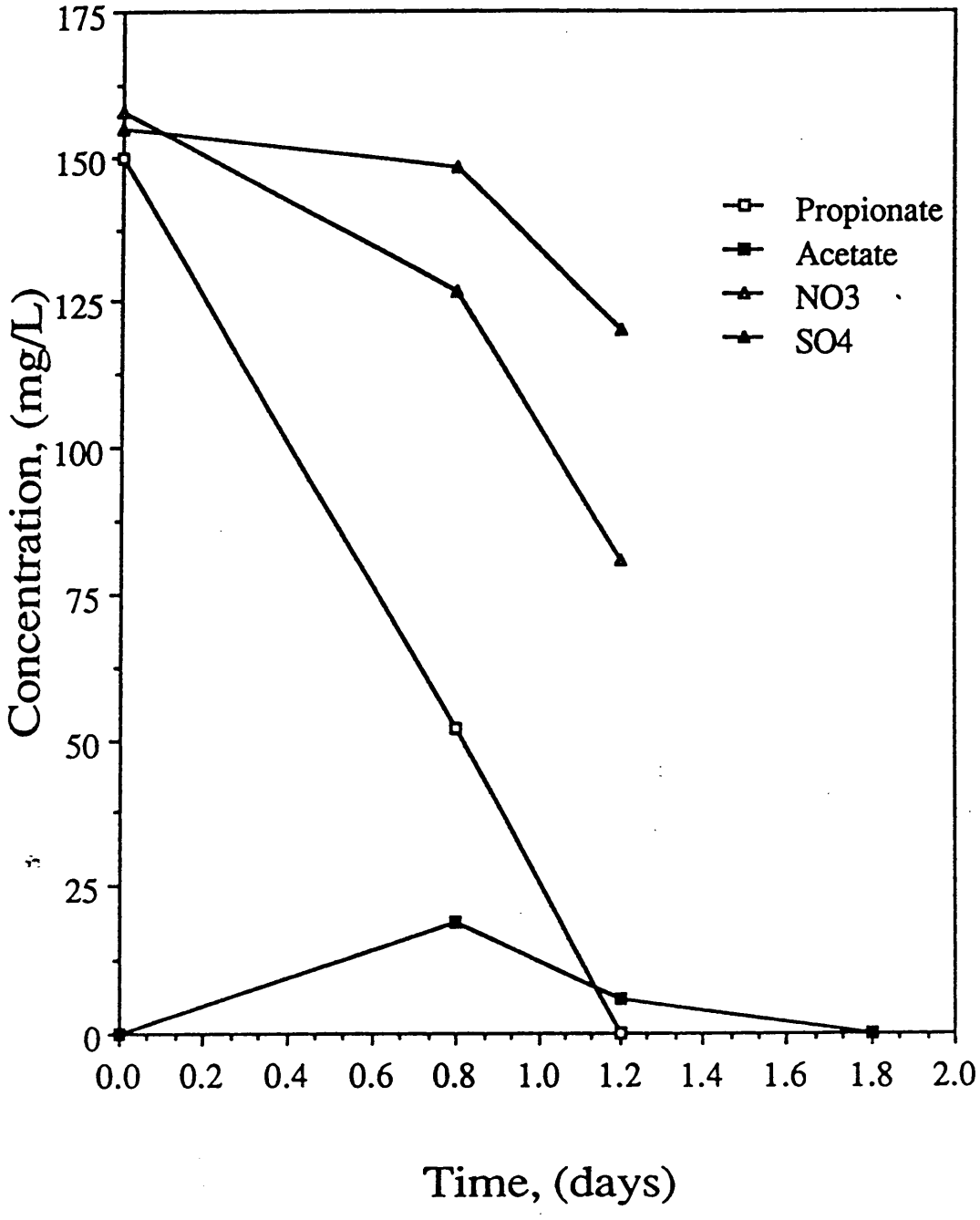

Figure A13. Propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA (arrow denotes respiking of sodium nitrate).

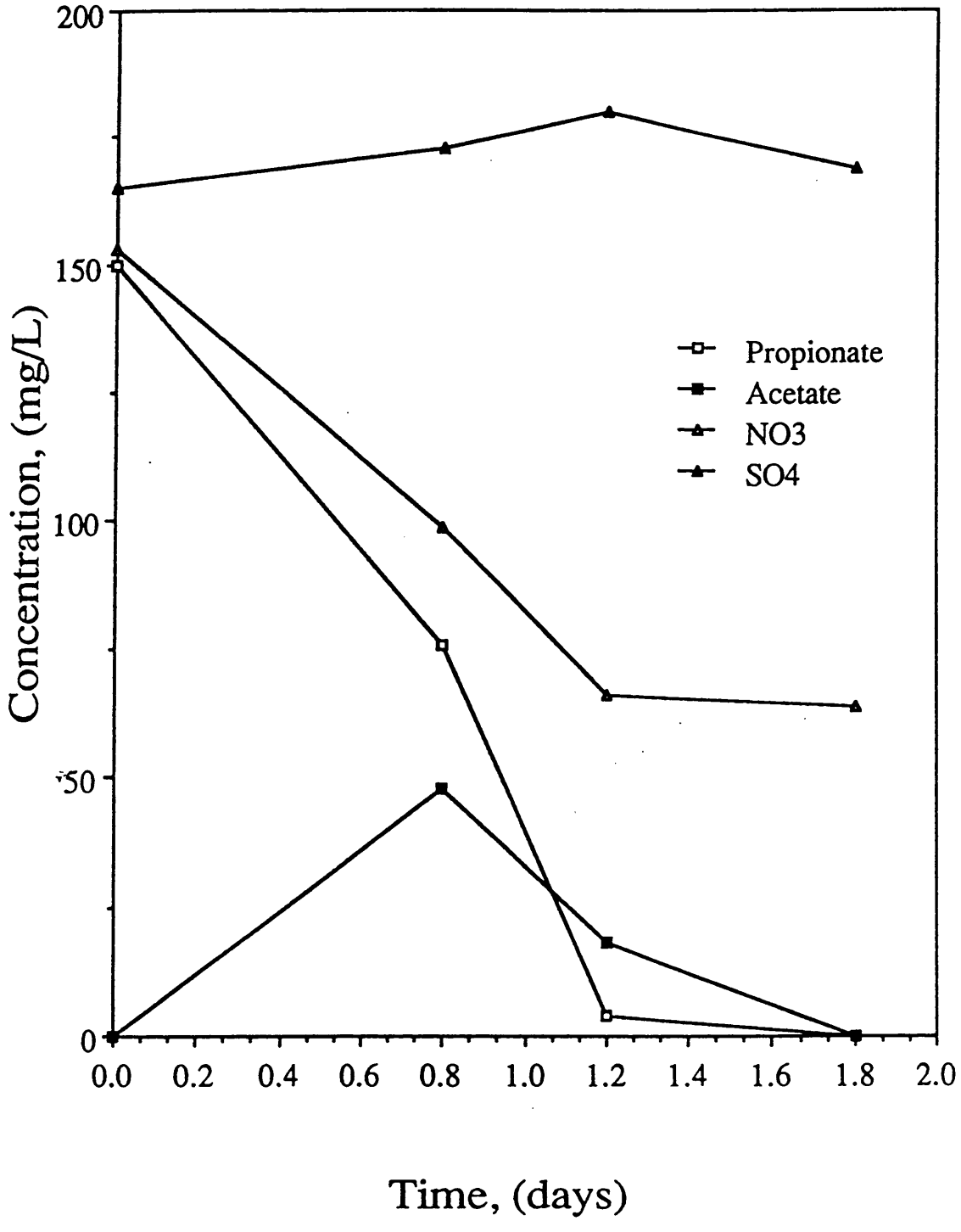

Figure A14. Propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (arrow denotes respiking of sodium nitrate).

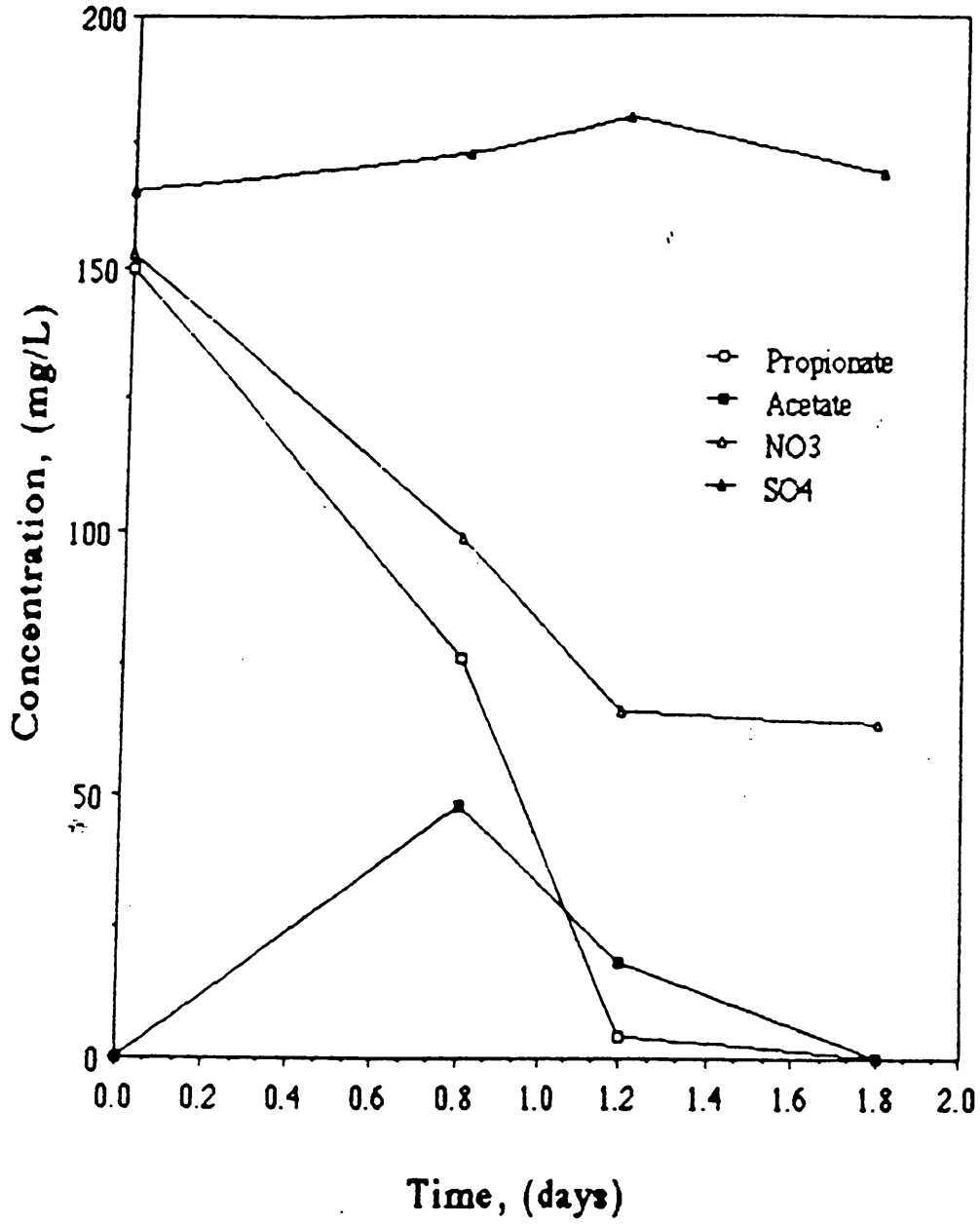

Figure A15. Propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (arrow denotes respiking of sodium nitrate).

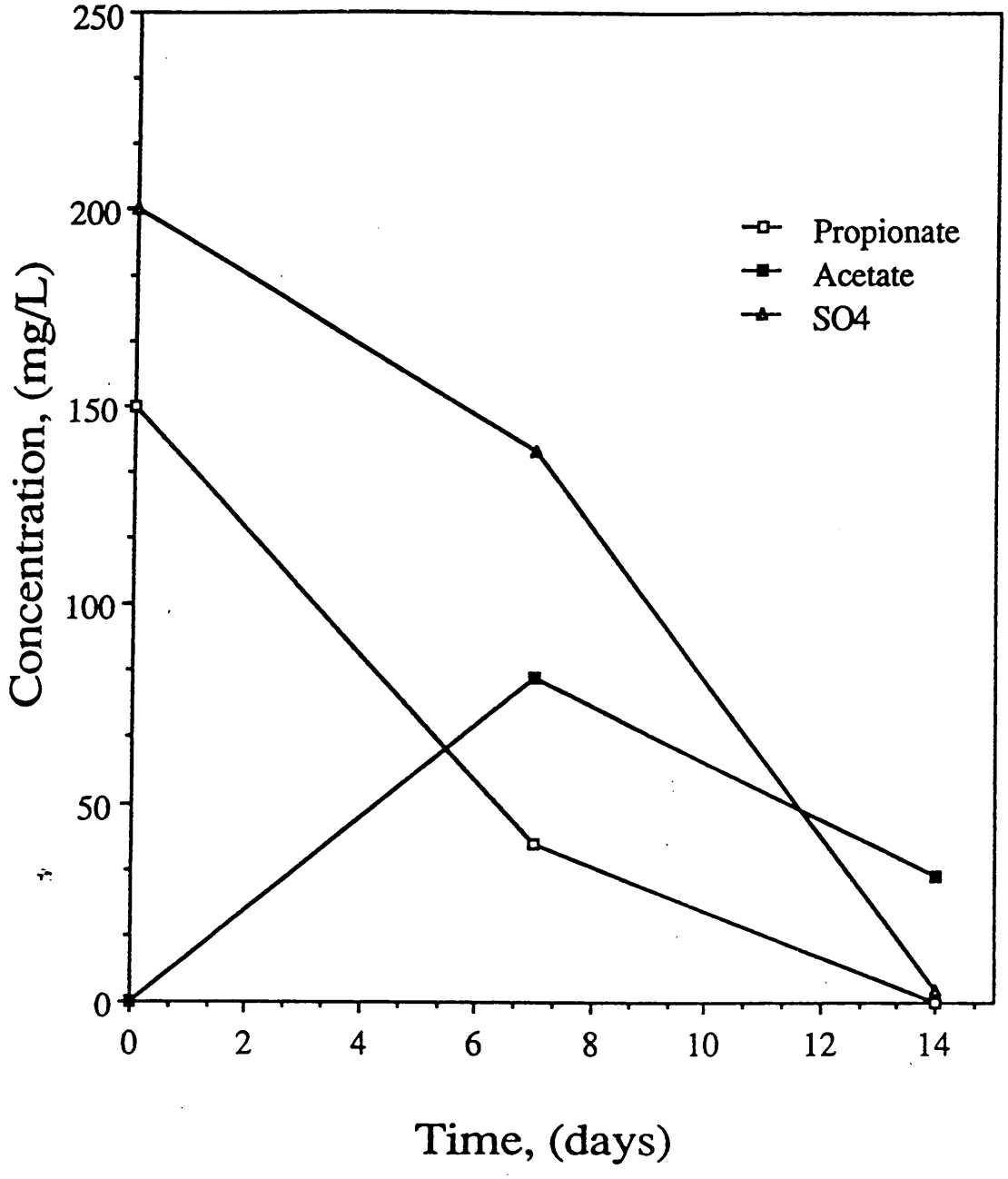

Figure A16. Propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA.

APPENDIX B

MULTIPLE INHIBITION BIODEGRADATION FIGURES

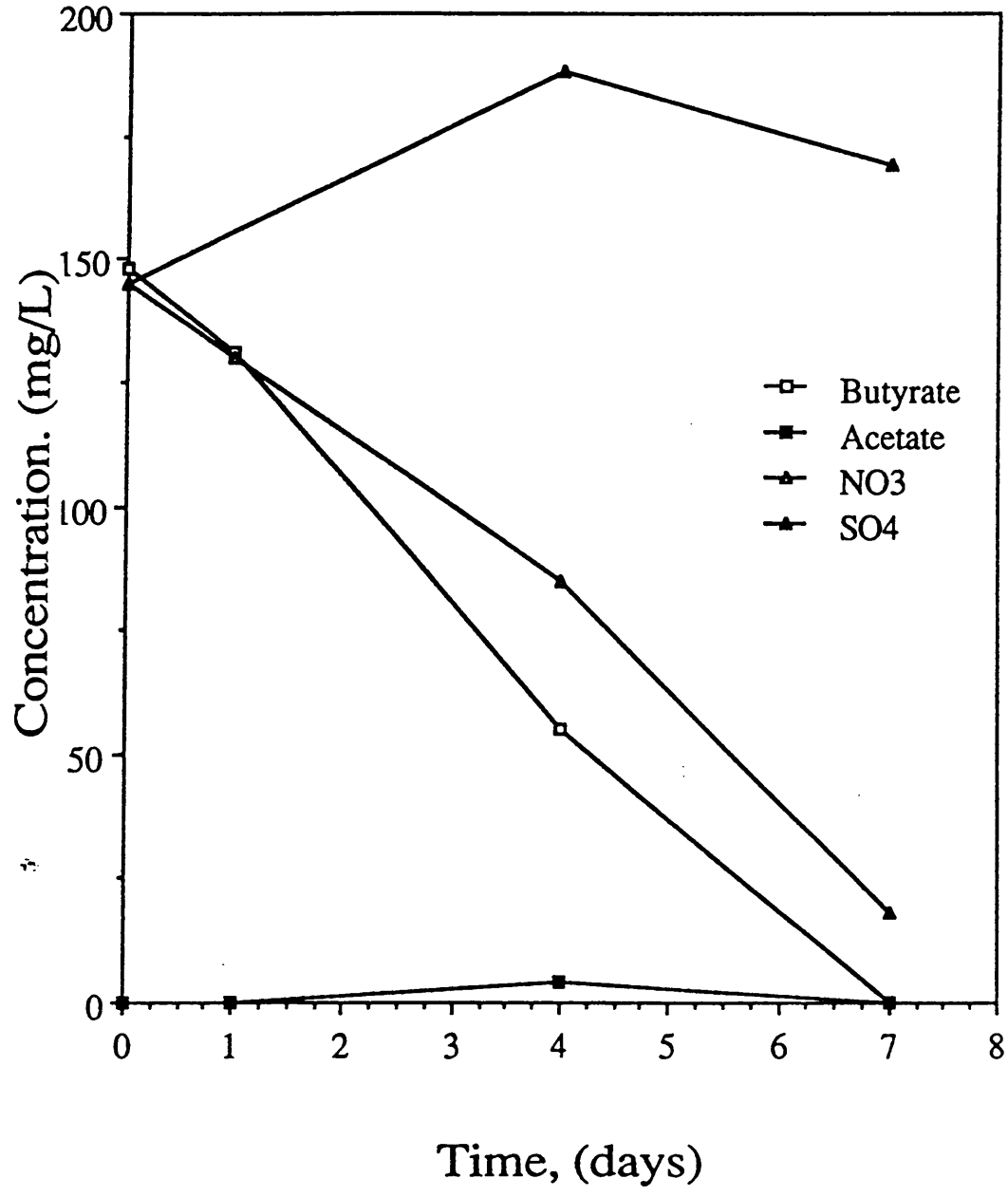

Figure B1. Butyrate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (denitrifiers active).

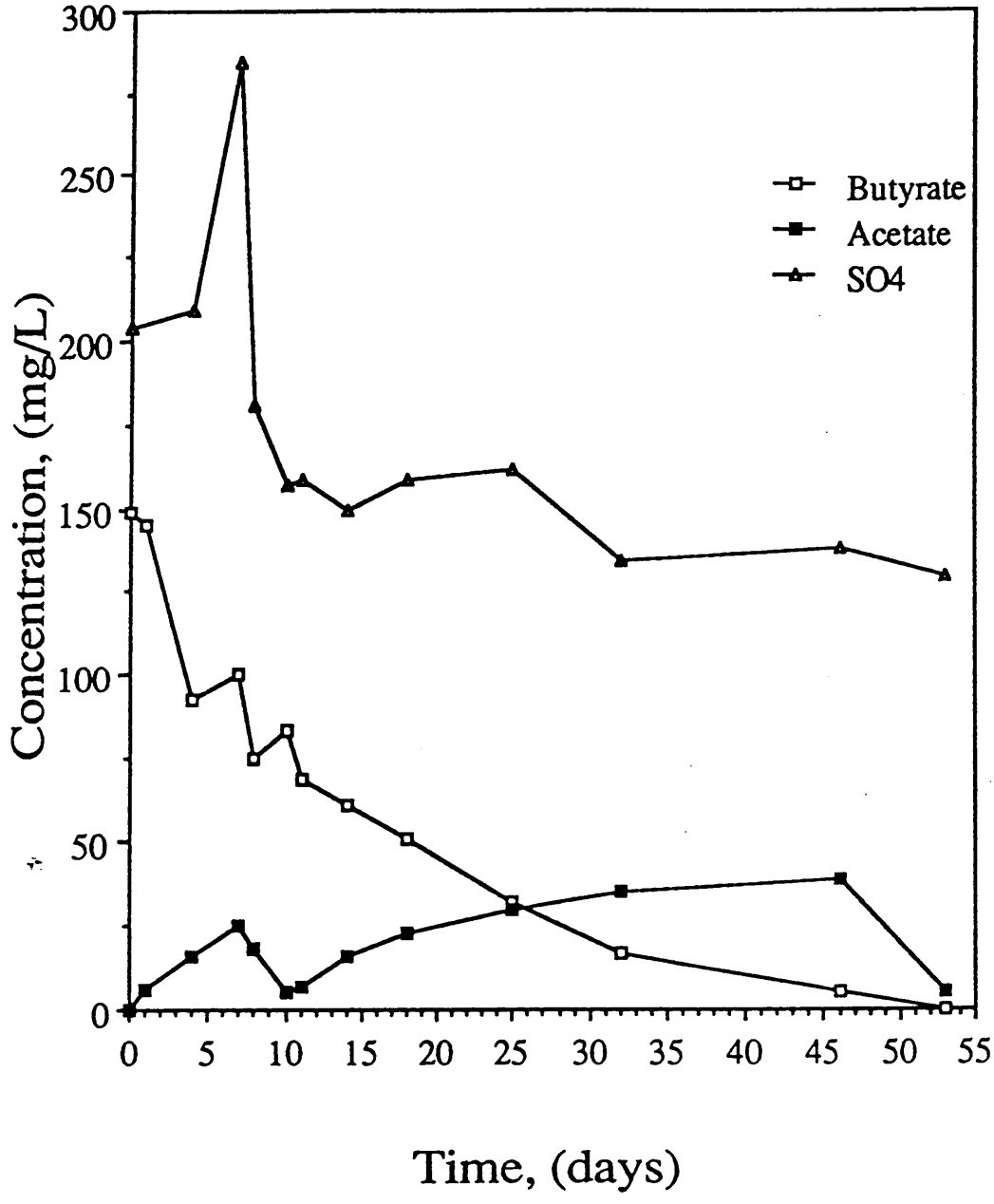

Figure B2. Butyrate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (methanogens active).

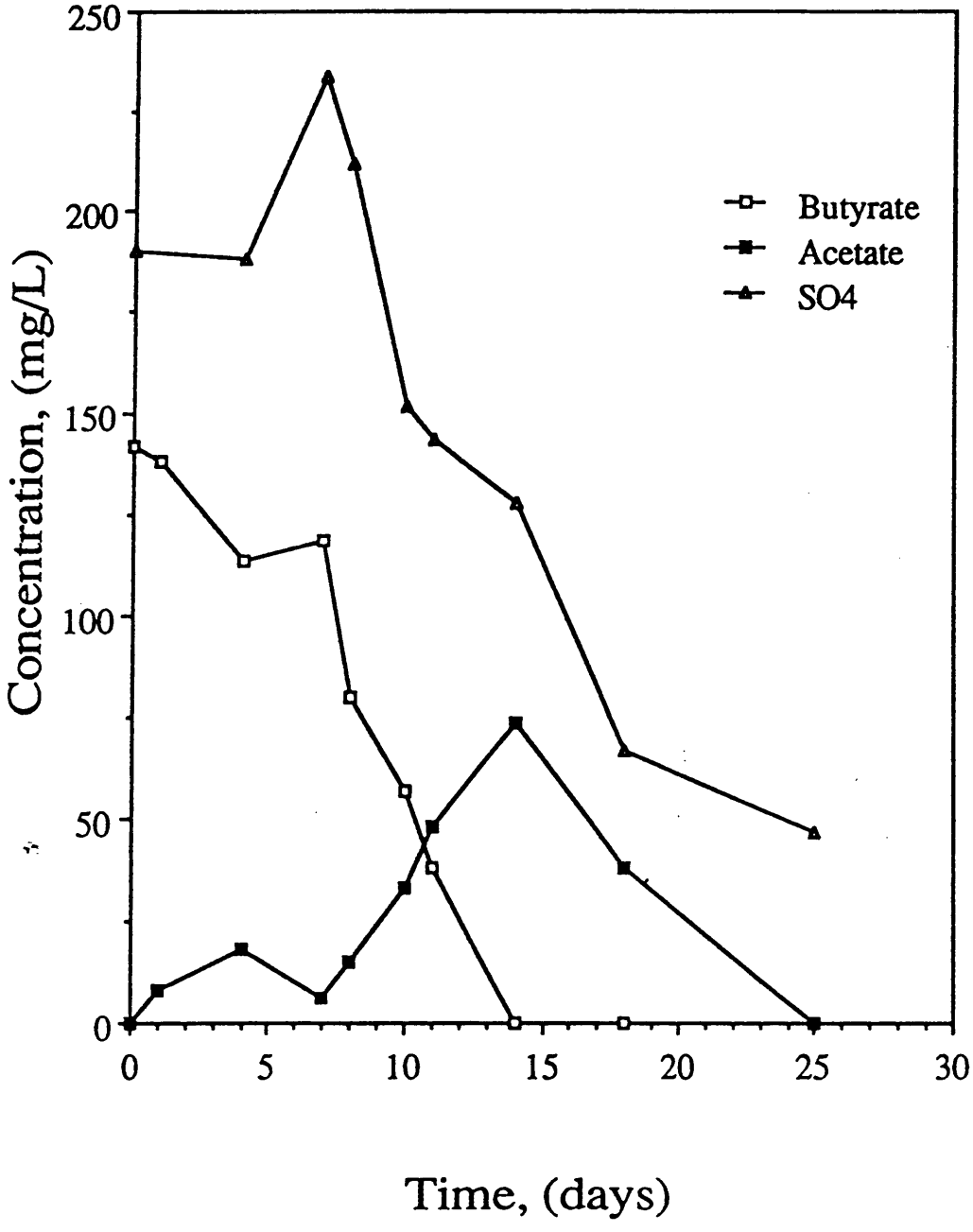

Figure B3. Butyrate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (sulfate reducers active).

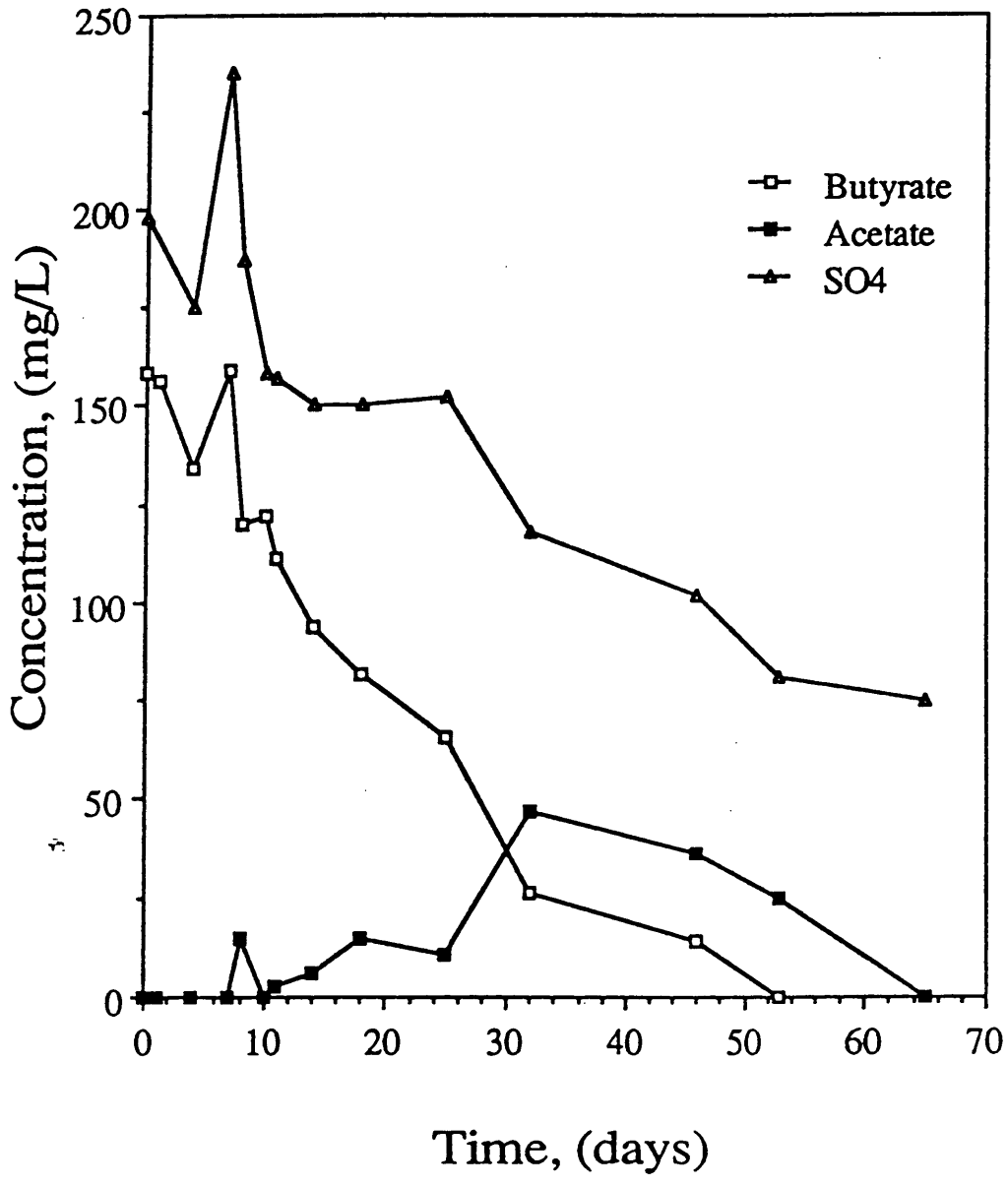

Figure B4 Butyrate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (no microbial groups assumed active).

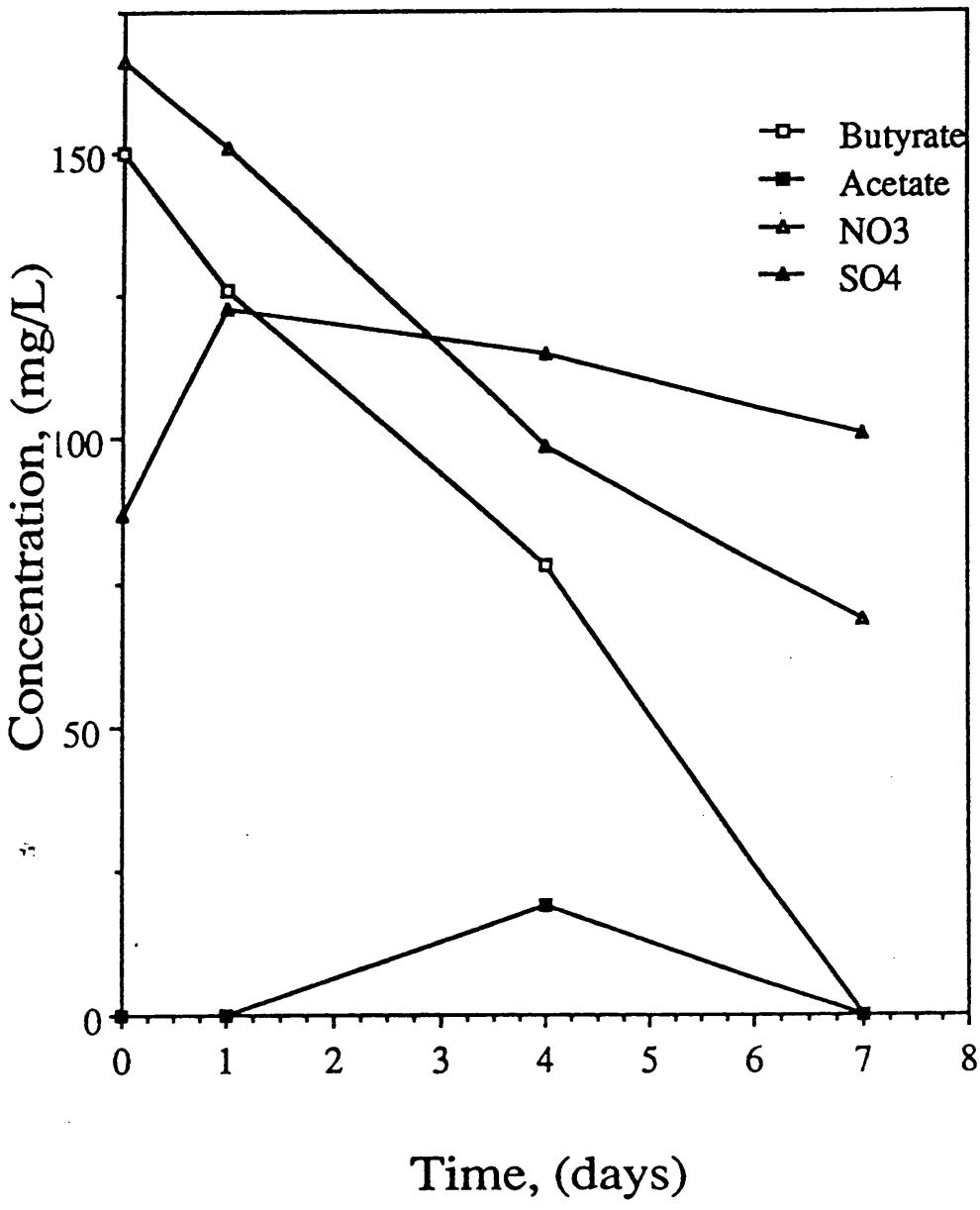

Figure B5. Butyrate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (denitrifiers active).

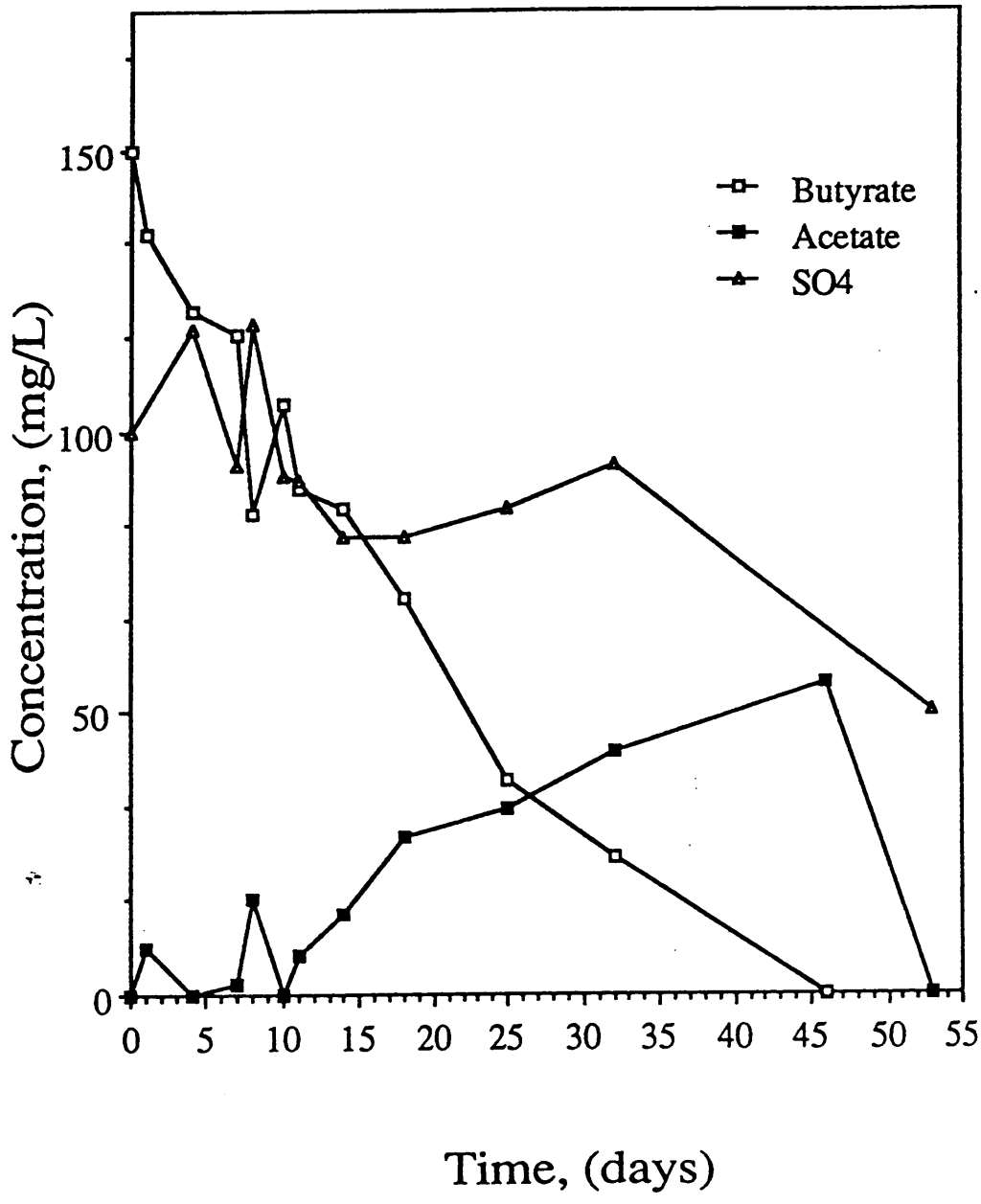

Figure B6. Butyrate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (methanogens active).

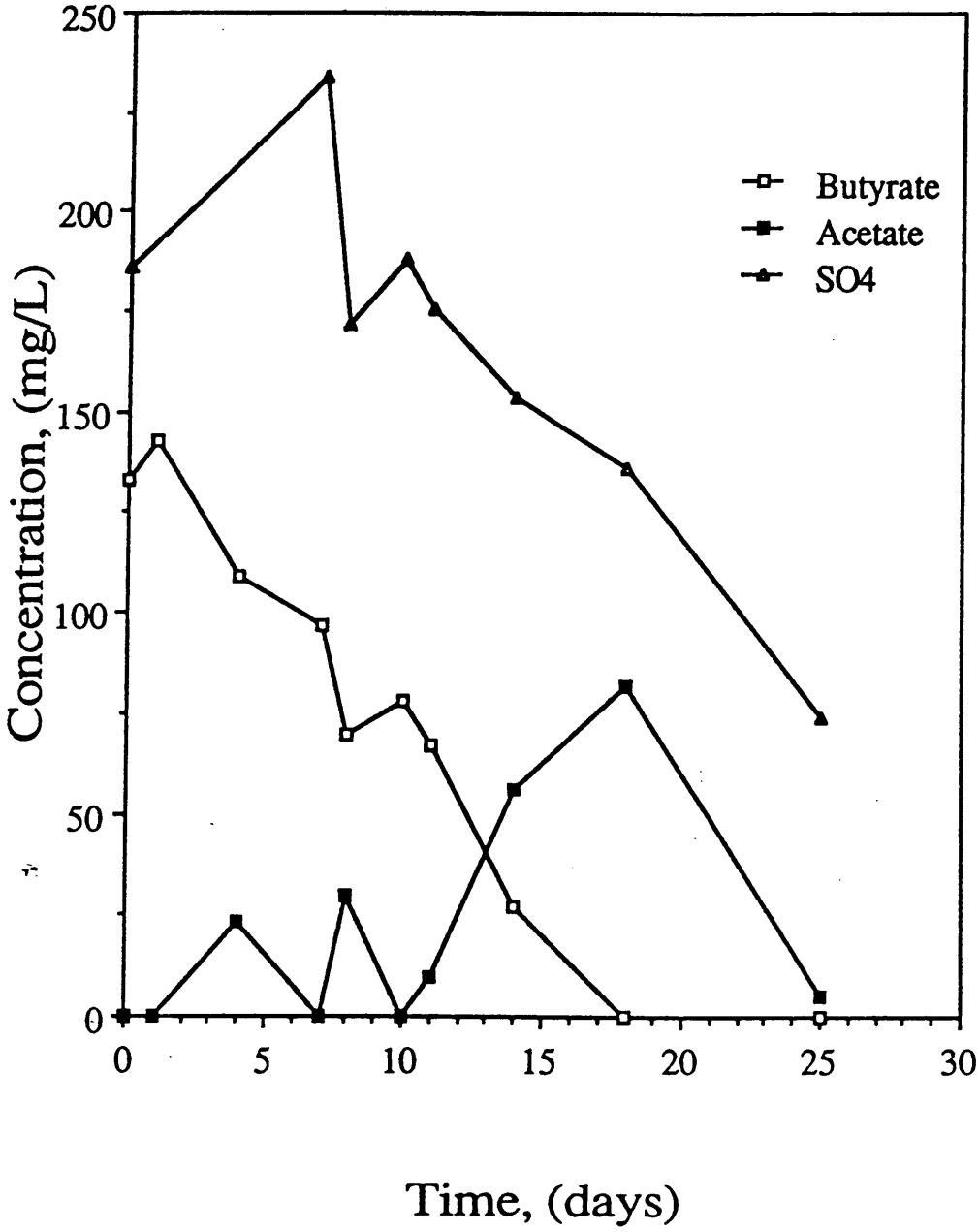

Figure B7. Butyrate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (sulfate reducers active).

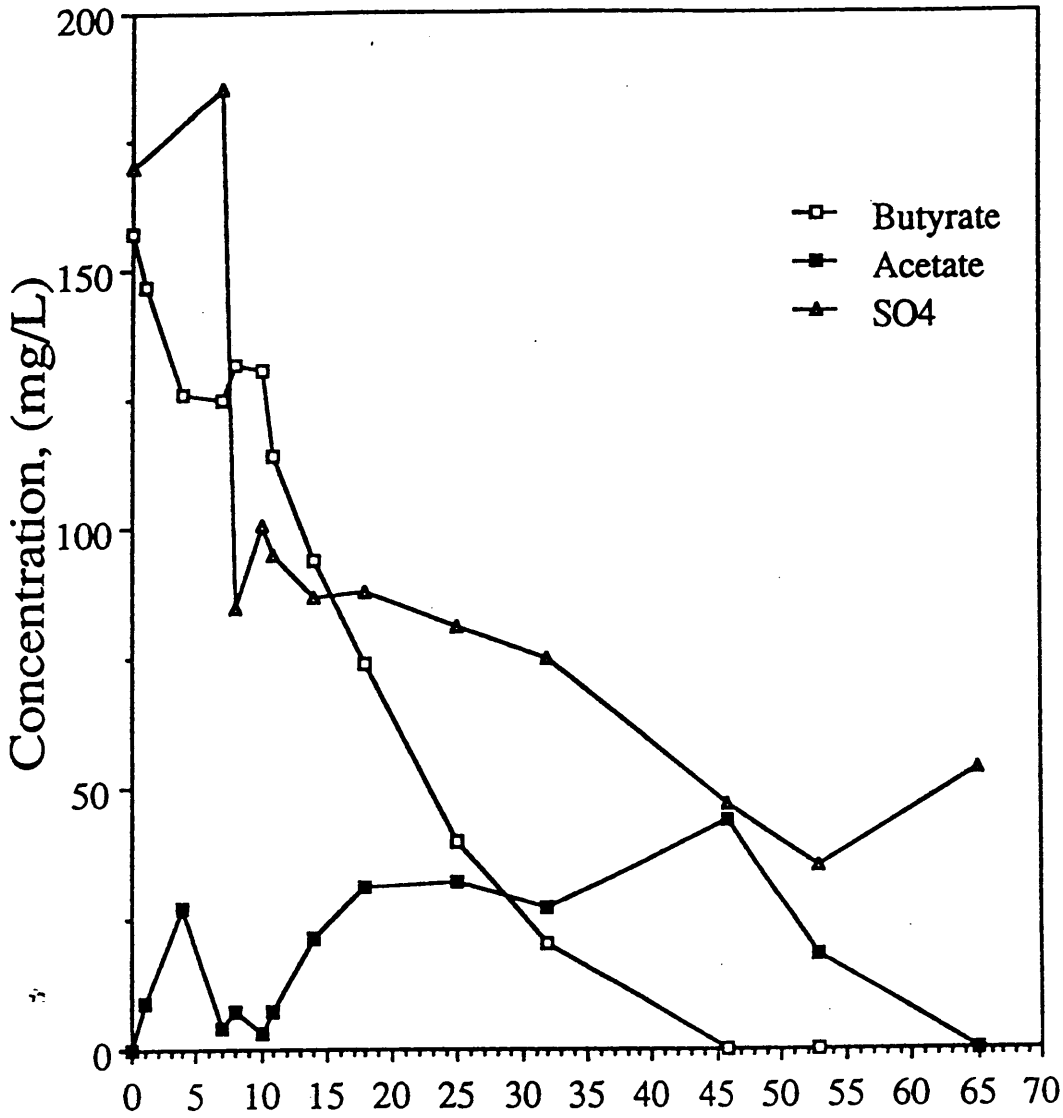

Figure B8 Butyrate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (no microbial groups assumed active).

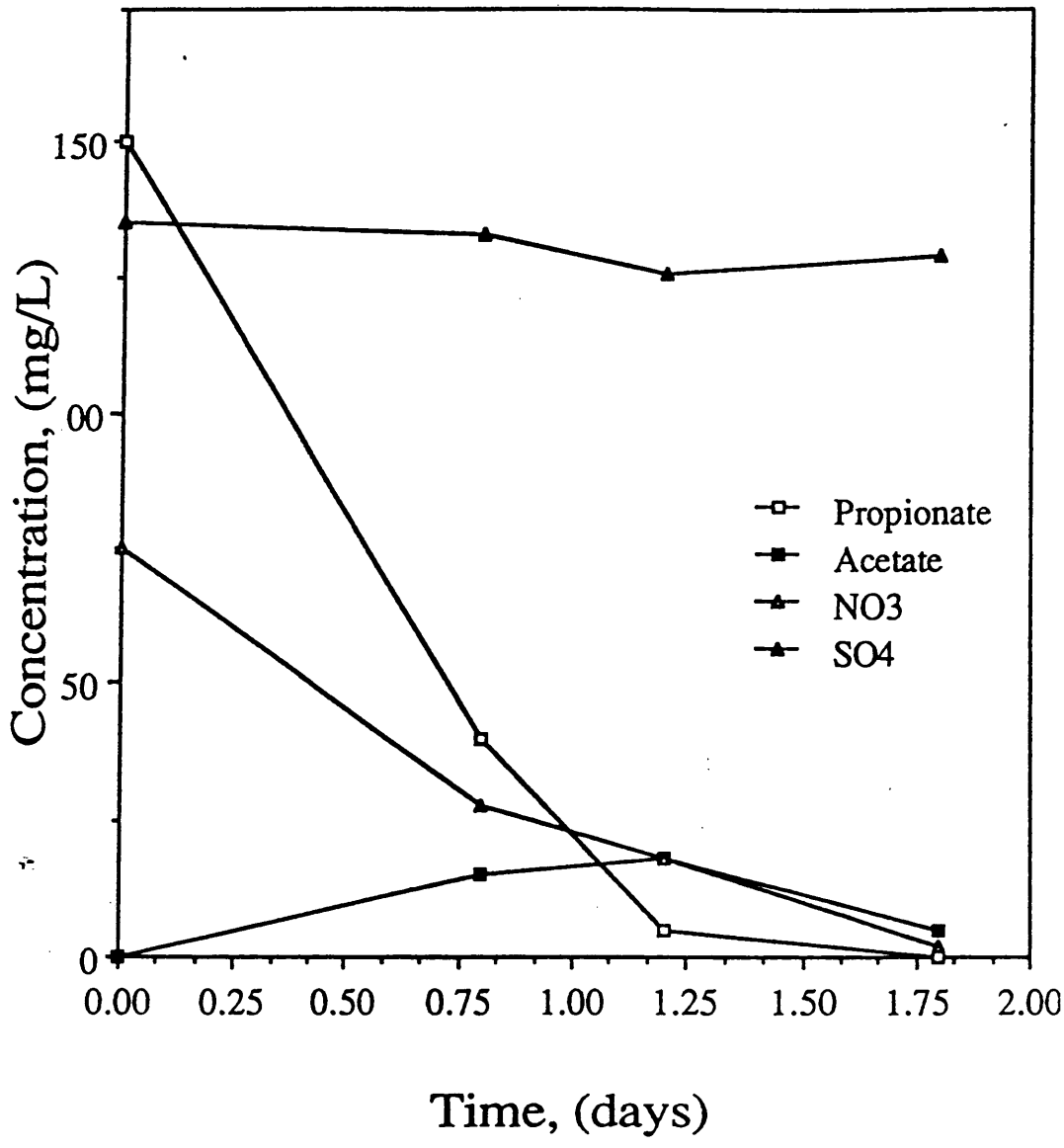

Figure B9. Propionate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (denitrifiers active).

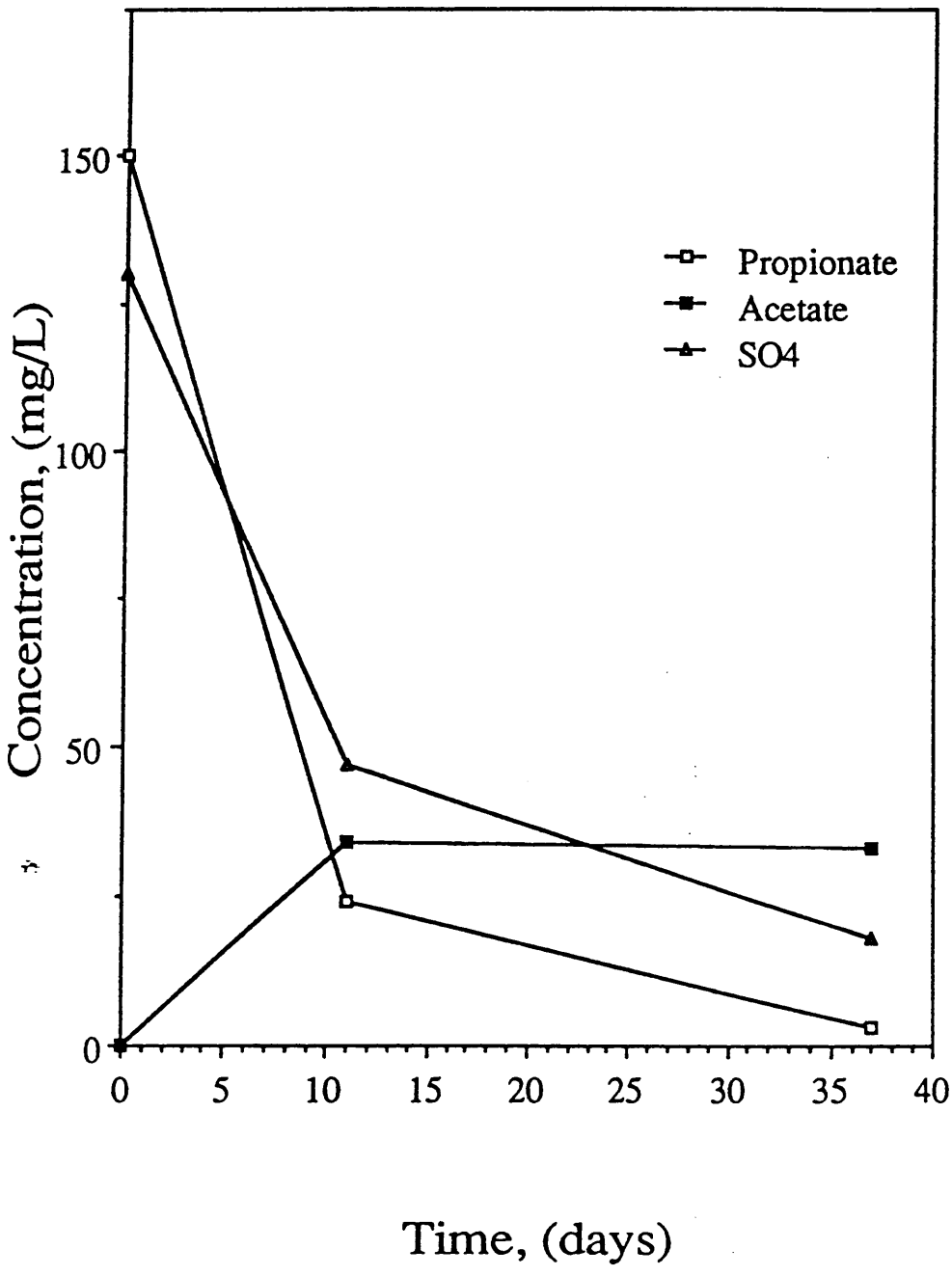

Figure B10. Propionate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (methanogens active).

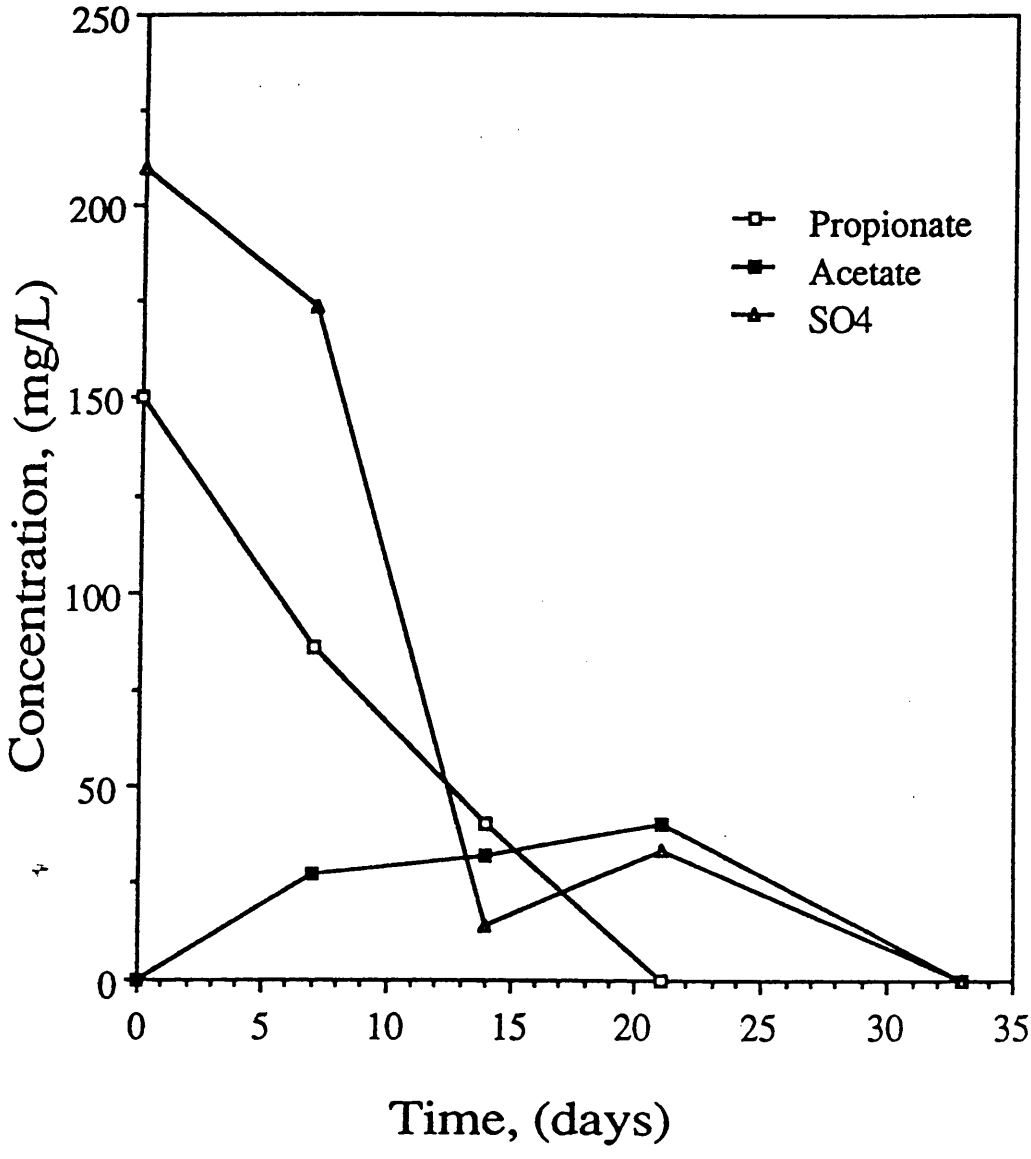

Figure B11. Propionate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (sulfate reducers active).

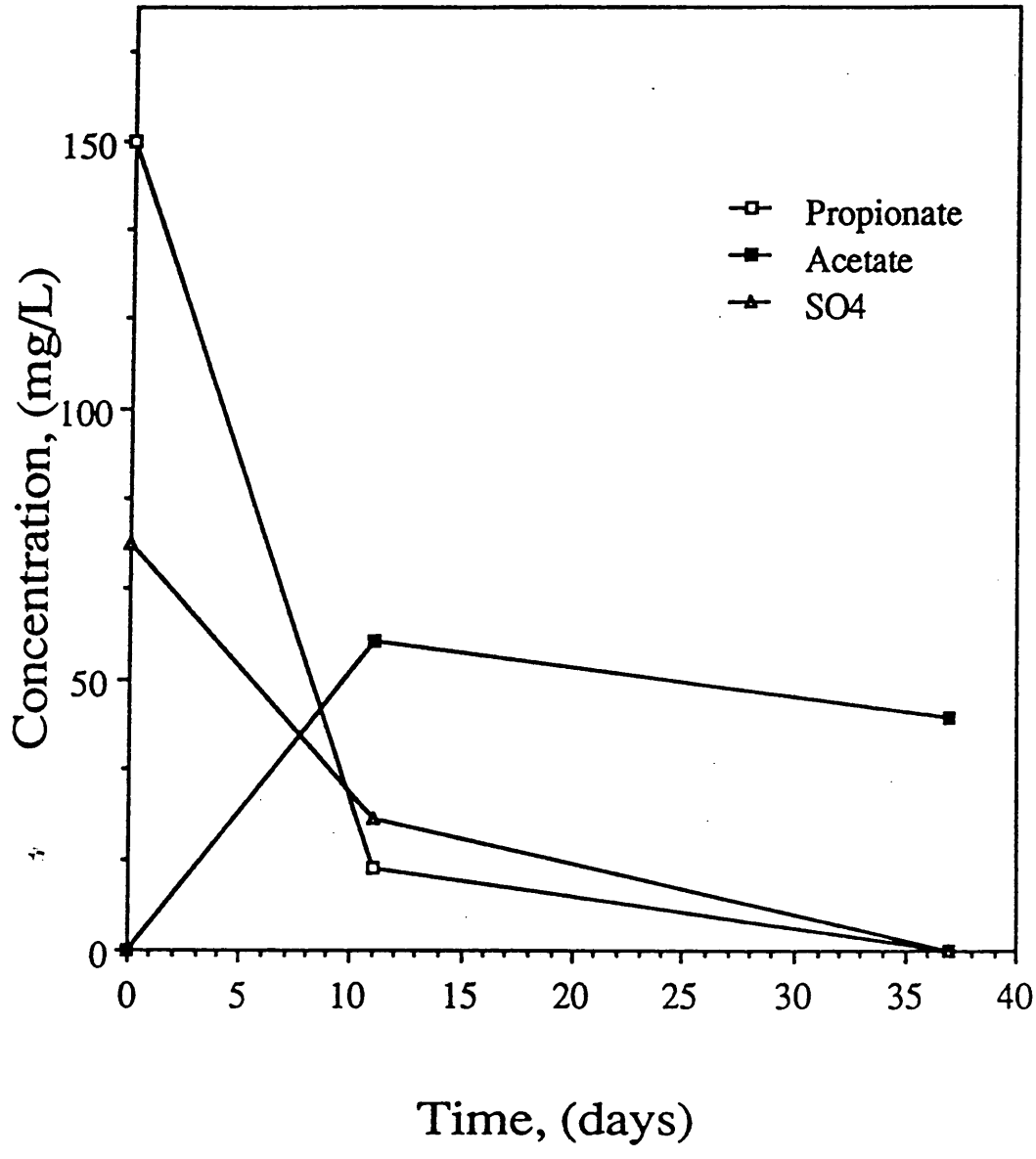

Figure B12. Propionate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (no microbial groups assumed active).

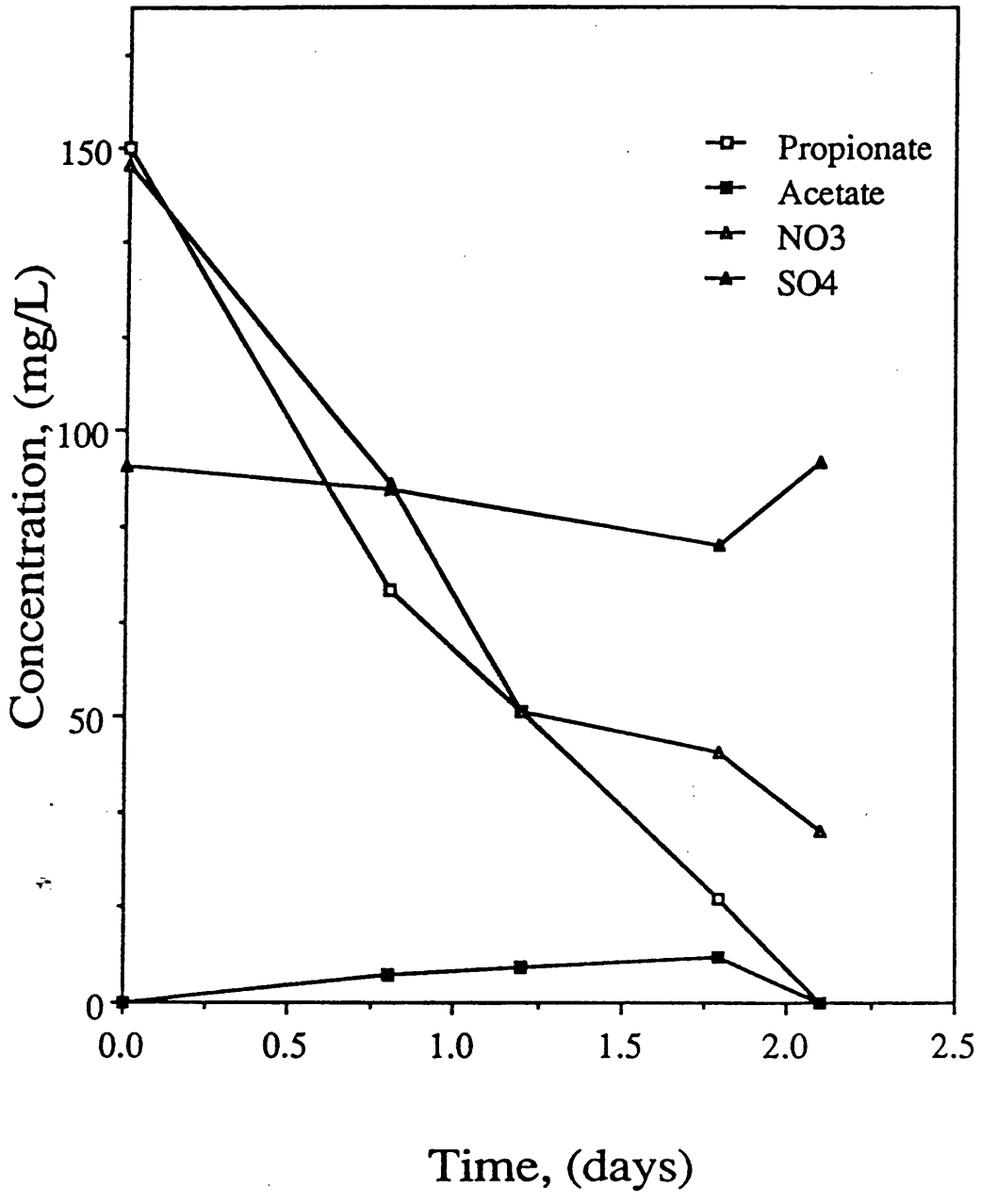

Figure B13. Propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (denitrifiers active).

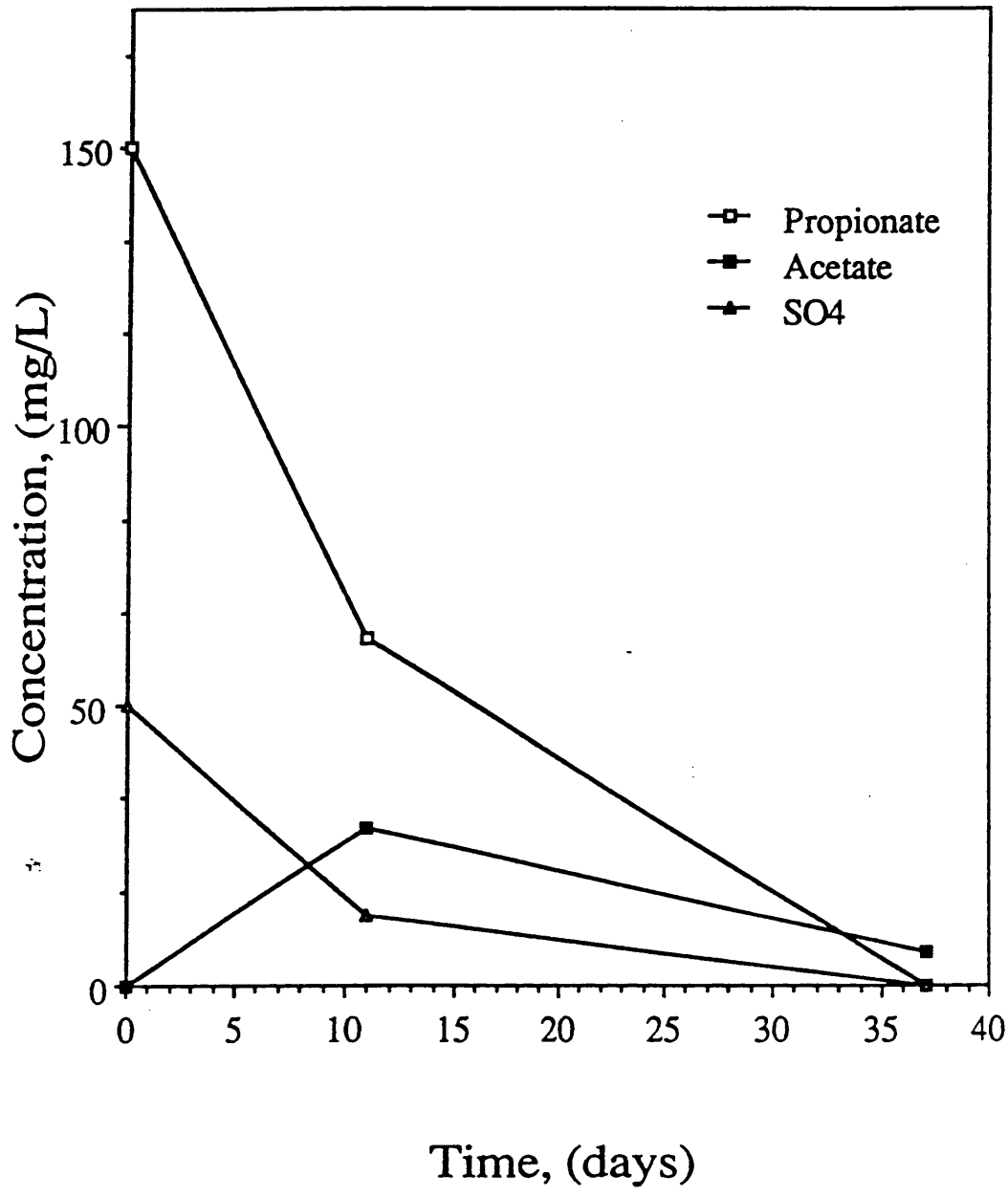

Figure B14. Propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (methanogens active).

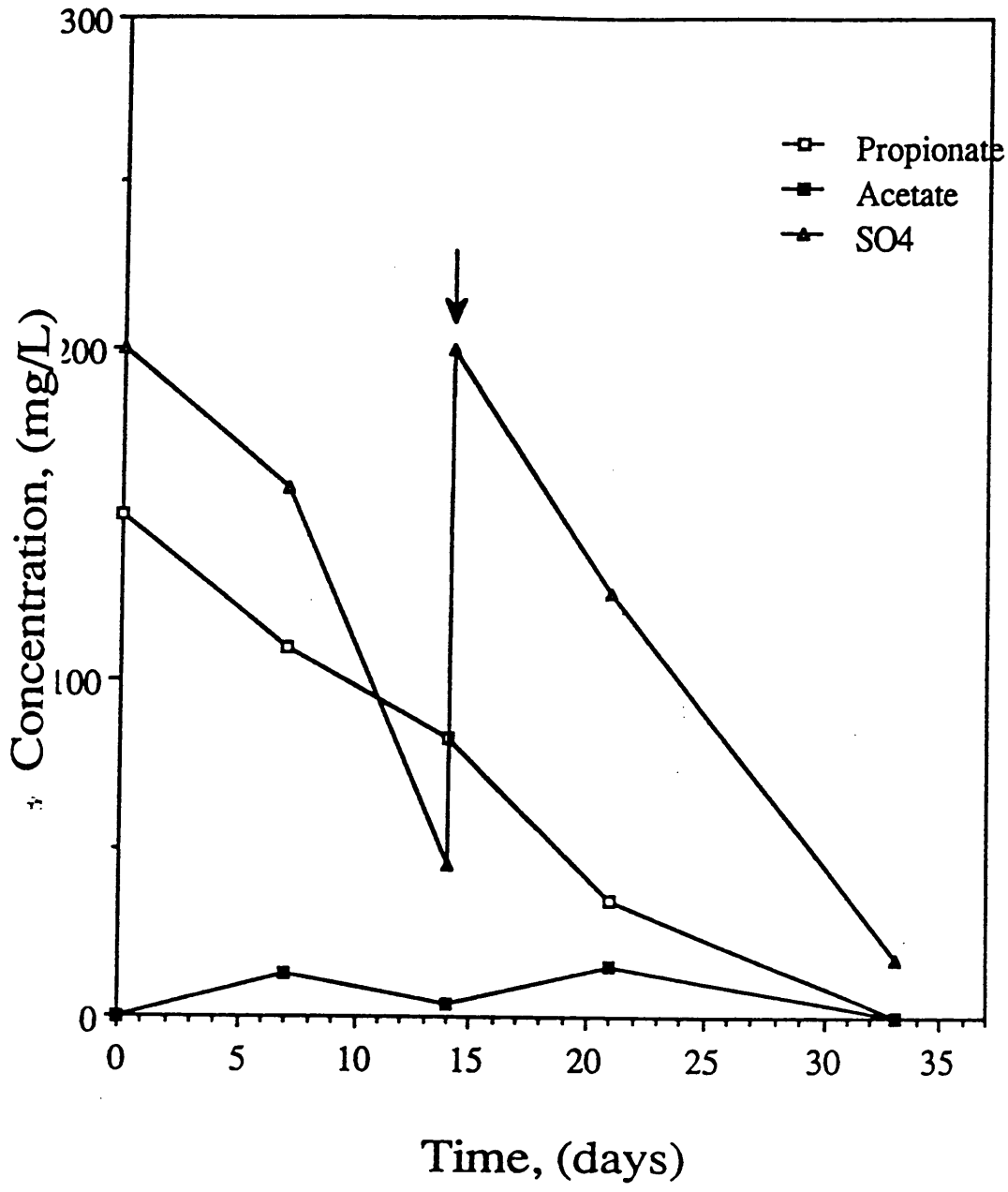

Figure B15. Propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (sulfate reducers active; arrow denotes respiking of sodium sulfate).

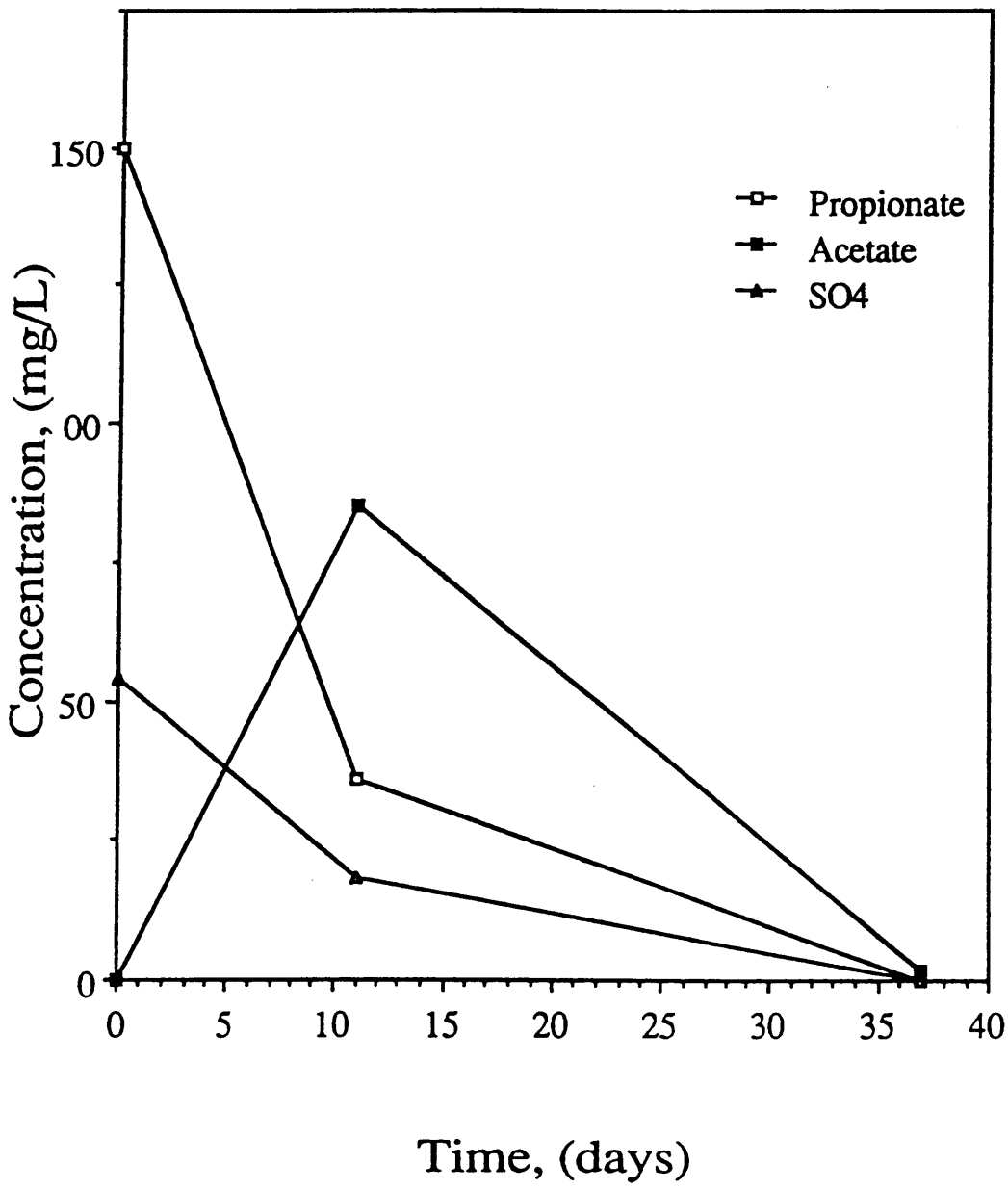

Figure B16. Propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (no microbial groups assumed active).

APPENDIX C

SINGLE INHIBITION EXPERIMENTAL DATA

Table C1. Butyrate and propionate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	305	35	0	32	218
2.0	193	45	8	3	244
3.5	176	62	0	0	237
5.5	0	15	6	56	182
7.0	0	0	0	29	185

Time (days)	Concentration (mg/L)				
	Propinate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	75	165
0.8	71	26	0	56	154
1.2	0	10	0	46	105
1.8	0	0	0	43	108

Table C2. Butyrate and propionate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	310	0	0	78	289
2.0	76	80	0	16	334
3.5	70	95	22	4	279
5.5	0	60	0	76	234
7.0	0	6	0	68	254

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	110	177
0.8	77	18	0	75	182
1.2	0	0	0	38	166

Table C3. Butyrate and propionate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	298	0	0	74	274
2.0	175	40	0	6	252
3.5	117	60	0	0	243
5.5	0	70	0	100	250
7.0	0	3	0	50	238

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	135	202
0.8	83	16	0	81	212
1.2	5	21	0	31	181
1.8	0	0	0	30	185

Table C4. Butyrate and propionate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	187	0	0	0	230
1.0	175	3	0	0	--
4.0	120	61		0	200
7.0	30	135	0	0	152
8.0	5	120	0	0	92
10.0	0	99	0	0	166
11.0	0	79	0	0	149
14.0	0	27	0	0	106
18.0	0	6	0	0	88
25.0	0	0	0	0	89

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	200
7.0	35	44	0	0	164
14.0	0	13	0	0	22

Table C5. Butyrate and propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	300	0	0	100	225
2.0	196	30	2	60	218
3.5	155	45	1	0	193
5.5	40	21	3	100	151
7.0	0	0	3	68	152

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	158	155
0.8	52	19	0	127	148
1.2	0	6	0	81	120
1.8	0	0	0	--	--

Table C6. Butyrate and propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	320	0	0	36	200
2.0	256	45	0	3	256
3.5	194	65	0	0	190
5.5	0	14	9	100	169
7.0	0	0	0	38	165

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	153	165
0.8	76	48	0	99	173
1.2	4	18	10	66	180
1.8	0	0	0	64	169

Table C7. Blacsburg and propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	369	0	0	112	135
2.0	191	63	0	36	145
3.5	150	65	0	12*	151*
5.5	0	42	15	100	139
7.0	0	0	0	48	138*

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	145*	195*
0.8	68	5	15	117	188
1.2	11	8	10	51	173
1.8	0	0	0	46*	178*

Table C8. Blacksburg and propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 0 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	205	0	0	0	174
1.0	180	66	0	0	–
4.0	116	104	0	0	161
7.0	33	145	0	0	67
8.0	0	155	0	0	35
10.0	0	145	0	0	141
11.0	0	112	0	0	116
14.0	0	63	0	0	59
18.0	0	13	0	0	19
25.0	0	0	0	0	118

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	200
7.0	40	82	0	0	139
14.0	0	32	0	0	3

APPENDIX D

MULTIPLE INHIBITION EXPERIMENTAL DATA

Table D1. Butyrate and propionate biodegradation in the Newport News soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (denitrifiers active).

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	148	0	0	145	145
1.0	131	0	0	130	–
4.0	55	4	52	85	188
7.0	0	0	54	18	169

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	75	135
0.8	40	15	0	28	133
1.2	5	18	0	18	126
1.8	0	5	0	2	129

Table D2. Butyrate and propionate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (methanogens active).

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	149	0	0	0	204
1.0	145	6	0	0	–
4.0	92	16	0	0	209
7.0	100	25	5	0	285
8.0	75	18	0	0	181
10.0	83	5	0	0	157
11.0	69	7	0	0	159
14.0	61	16	0	0	150
18.0	51	23	0	0	159
25.0	32	30	0	0	162
32.0	17	35	0	0	134
46.0	5	39	0	0	138
53.0	0	5	0	0	130

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	130
11.0	24	34	0	0	47
37.0	3	33	0	0	18

Table D3. Butyrate and propionate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (sulfate reducers active).

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	142	0	0	0	190
1.0	138	8	0	0	–
4.0	114	18	0	0	188
7.0	119	6	0	0	234
8.0	80	15	0	0	212
10.0	57	33	0	0	152
11.0	38	48	0	0	144
14.0	0	74	0	0	128
18.0	0	38	0	0	67
25.0	0	0	0	0	47

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	210
7.0	86	27	0	0	174
14.0	40	32	0	0	14
21.0	0	40	0	0	33
33.0	0	0	0	0	0

Table D4. Butyrate and propionate biodegradation in the Newport News soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (no microbial groups assumed active).

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	158	0	0	0	198
1.0	156	0	0	0	--
4.0	134	0	0	0	175
7.0	159	0	0	0	235
8.0	120	15	0	0	187
10.0	122	0	0	0	158
11.0	111	3	0	0	157
14.0	94	6	0	0	150
18.0	82	15	0	0	150
25.0	66	11	0	0	152
32.0	26	47	0	0	118
46.0	14	36	0	0	102
53.0	0	25	0	0	81
65.0	0	0	0	0	75

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	130
11.0	24	34	0	0	47
37.0	3	33	0	0	18

Table D5. Butyrate and propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 100 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA (denitrifiers active).

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	166	87
1.0	126	0	0	151	123
4.0	78	19	0	99	115
7.0	0	0	14	69	101

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150		0	147	94
0.8	72		0	91	90
1.2	51		0	51	--
1.8	18		0	44	80
2.1	0		0	30	95

Table D6. Butyrate and propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 0 mg/L BESA (methanogens active).

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	100
1.0	135	8	0	0	--
4.0	121	0	0	0	118
7.0	117	2	0	0	94
8.0	85	17	0	0	119
10.0	105	0	0	0	92
11.0	90	7	0	0	91
14.0	86	14	0	0	81
18.0	70	28	0	0	81
25.0	38	33	0	0	86
32.0	24	43	0	0	94
46.0	0	55	0	0	--
53.0	0	0	0	0	50

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	50
11.0	62	28	0	0	12.5
37.0	0	6	0	0	0

Table D7. Butyrate and propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 0 mg/L sodium molybdate, 211 mg/L BESA (sulfate reducers active).

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	133	0	0	0	186
1.0	143	0	0	0	--
4.0	109	23	0	0	--
7.0	97	0	0	0	234
8.0	70	30	0	0	172
10.0	78	0	0	0	188
11.0	67	10	0	0	176
14.0	27	56	0	0	154
18.0	0	82	0	0	136
25.0	0	5	0	0	74

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	200
7.0	110	13	0	0	158
14.0	83	4	0	0	45
21.0	35	15	0	0	127
33.0	0	0	0	0	17

Table D8. Butyrate and propionate biodegradation in the Blacksburg soil with microcosm dilution water containing 0 mg/L sodium nitrate, 206 mg/L sodium molybdate, 211 mg/L BESA.

Time (days)	Concentration (mg/L)				
	Butyrate	Acetate	NO_2	NO_3	SO_4
0.0	157	0	0	0	170
1.0	147	9	0	0	--
4.0	126	27	0	0	--
7.0	125	4	0	0	185
8.0	132	7	0	0	85
10.0	131	3	0	0	101
11.0	114	7	0	0	95
14.0	94	21	0	0	87
18.0	74	31	0	0	88
25.0	40	32	0	0	81
32.0	20	27	0	0	75
46.0	0	44	0	0	47
53.0	0	18	0	0	35
65.0	0	0	0	0	54

Time (days)	Concentration (mg/L)				
	Propionate	Acetate	NO_2	NO_3	SO_4
0.0	150	0	0	0	54
11.0	36	85	0	0	18
37.0	0	2	0	0	0

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