ABSTRACT

CLAIRE CAUGHEY MOST. Metabolite Accumulation during m-Cresol Degradation by Aquatic Bacteria. (Under the Direction of DR. FREDERIC K. PFAENDER)

The accumulation of metabolites in the aqueous phase was measured during studies of m-cresol biodegradation with natural water samples. 14C-m-cresol was separated from radiolabeled products remaining in the aqueous filtrates using HPLC. In heterotrophic activity studies, which involve short-term incubations (< 24 h), metabolite accumulation accounted for only a small fraction of the radiolabeled m-cresol originally added. After longer incubations characteristic of die-away studies, metabolite accumulation accounted for as much as 30% of the label originally added. The nature of metabolite accumulation varied, and appeared to reflect differences in the initial environmental samples. Sometimes no metabolite accumulation was detected. One time, a few products accumulated and they were less polar than m-cresol. At other times, many different products accumulated and they were more polar than m-cresol. When metabolite accumulation occurred, it was greater in samples receiving 960 ug/l m-cresol than 120 ug/l m-cresol. No one metabolite accumulated consistantly to a level greater than 5% of the radiolabel originally injected.

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INTRODUCTION

Hazard assessments for chemicals released into the environment involve two types of studies: toxicity studies and environmental fate studies (Gledhill and Saeger, 1979; Lee and Jones, 1980). Environmental fate studies are an integral part of any hazard assessment scheme because they may reveal the ultimate modes and concentrations of exposure to the compound. The transport and persistance of any compound will be influenced by a complex web of physical, chemical and biological factors. One factor especially important in the disappearance of compounds in the environment is microbial degradation (Gledhill and Saeger, 1979; Alexander, 1980).

Several methods are available for assessing the biodegradation of potentially hazardous chemicals in the environment. The techniques available have been reviewed by Larson (1984). One of the newer and most sensitive methods, the heterotrophic bacterial activity method, involves the use of radiolabeled substrates to assess biodegradation kinetics. Experimental conditions are designed to simulate those existing in the natural environment as much as possible. Natural water samples are amended with low concentrations of radiolabeled substrate and allowed to incubate until a small proportion of the label has been metabolized. The incubations are short

(less than 24 hours), and as such, are advantageous because they minimize the effects of confinement on the aquatic sample. Following sample incubation, the radioactivity evolved as carbon dioxide and/or incorporated into cells is measured. This radioactivity is then used to compute rates of biodegradation, and when appropriate, analyze the data according to an enzyme-saturation kinetic model.

Heterotrophic activity studies are concerned with only two possible metabolic fates of the radioactive label, uptake into cells and production of carbon dioxide. One potential limitation of these studies is that they do not measure a third possible metabolic fate, the release of metabolites of the parent compound from cells back into the surrounding water. If such metabolites were to accumulate in the aqueous phase, then the heterotrophic activity method would underestimate the extent of biodegradation.

Little is known about which products in a metabolic pathway are excreted and accumulated outside the microbial cell (Alexander, 1980; Fletcher, 1979). Furthermore, most metabolic pathways are established under experimental conditions so different from those in the natural environment that it is difficult to extrapolate from the results of these studies to the natural environment. For example, degradation pathways are typically established using pure cultures of microorganisms grown in artificial media with the substrate of interest present at high concentrations (Alexander, 1980; Dagley, 1975). In

contrast, the heterotrophic activity studies involve mixed microbial populations, low substrate concentrations, and a natural water medium. The effect of these conditions on the mechanisms of degradation and the accumulation of metabolites is largely unknown and difficult to predict (Alexander, 1980; Bollag, 1974).

In this project, metabolite accumulation was explored using m-cresol, which has been identified as a priority pollutant under the Toxic Substances Control Act. The compound is produced from the high-temperature carbonization of coal and as a by-product in the petroleum and petrochemical industries (Chapman, 1972). It is used in the manufacture of disinfectants, fumigants, photographic developers, and explosives (Merck Index, 1983). m-Cresol was chosen for this particular project because it has been studied extensively in this laboratory using the radiolabel method described above. The project objectives were threefold: (1) to determine the extent of metabolite accumulation in biodegradation studies using short term heterotrophic uptake assays, (2) to look at metabolic products under close to real world conditions, and (3) if products do accumulate, to determine the number, amount and possibly identification of the metabolites.

LITERATURE REVIEW

Microbial Degradation

When an organic chemical is released into the environment, biological, chemical and physical factors will influence its fate. Major structural changes in the compound, however, are mainly accomplished by microorganisms (Alexander, 1980). Two types of microbial metabolism are involved in the biodegradation of organic compounds (Harder, 1981). In the first form of metabolism, the compound serves as a substrate for growth and energy. Microbial enzymes degrade the compound into other structures involved in intermediary metabolism. Some of the compound's carbon may then be converted into cell constituents, while the rest may be channeled into oxidative cycles. Complete oxidation produces carbon dioxide and water, while providing energy for biosynthesis (Chapman, 1979). Thus, this form of metabolism may lead to the mineralization of an organic substrate into inorganic products (Bollag, 1974; Rubin et al., 1982; Subba-Rao et al., 1982). In a recent study, Chesney et al. (1985) found that microorganisms in freshwater samples completely removed phenol present at trace concentrations. About eighty percent of the parent phenol was mineralized to carbon dioxide and about twenty percent was incorporated

into trichloroacetic acid-precipitable biomass (cellular macromolecules such as nucleic acids, proteins, or polysaccharides). This study suggests that when a hazardous compound serves as a substrate for growth and energy, microbial metabolism may be instrumental in its complete removal from the environment.

In the second form of metabolism, a chemical is transformed by a microorganism without deriving carbon or energy from that transformation to support growth (Horvath, 1972). This phenomenon is termed cometabolism and has been demonstrated in the laboratory for compounds such as DDT, 2,3,6-trichlorobenzoate (2,3,6-TBA), 2,4,5-trichlorophenoxyacetate (2,4,5-T) and 3-methylcatechol (Horvath, 1972). From an environmental standpoint, one of the major features of such cometabolic transformations is that they only lead to partial degradation of the parent compound. Cometabolism of 2,3,6-TBA by a Brevibacterium sp. results in the accumulation of its transformation product 3,5-dichlorocatechol (Horvath, 1971). Similarly, p,p-dichlorodiphenyl methane accumulates from the cometabolism of DDT by Aerobacter aerogenes (Wedemeyer, 1967). Such cometabolic products are often structurally similar to the parent compound and can be less toxic, as toxic, or even more toxic than the parent compound (Alexander, 1980; Bollag, 1974). Thus, partial degradation by cometabolism may not remove the hazard posed by the release of the parent compound into the environment.

The extent of biodegradation is especially difficult to predict because different organisms may transform a compound differently. The insecticide carbaryl, for example, is subject to hydrolysis by some organisms and to hydroxylation by others (Bollag, 1979). Moreover, hydroxylation of carbaryl by Aspergillus terreus produces 1-Naphthyl N-hydroxy-methylcarbamate as the major metabolic product. Hydroxylation by Penicillium sp., in contrast, produces 4-hydroxy 1-napthyl methyl carbamate as the major metabolic product (Bollag, 1979). Formation of the former involves a side chain hydroxylation, whereas the latter involves a ring hydroxylation. Hence, even a similar initial reaction can produce products differing in the subsequent transformations required and in the products which ultimately accumulate.

A further complication are population interactions among microbes living in a community. Gunner and Zuckerman (1968) isolated from soil an Arthrobacter sp. and a Streptomyces sp. which together could synergistically degrade the ring structure of the organophosphate insecticide diazinon. When incubated alone, neither organism could degrade the ring structure. Similarly, Beam and Perry (1974) reported a commensal relationship involved in the degradation of cyclohexane. Mycobacterium vaccae cometabolicly degraded cyclohexane to cyclohexanone while growing on propane. A second organism, which was unable to grow on either cyclohexane or propane, then used the

cyclohexanone as a growth substrate. Such population interactions may enhance the degradation of many organic compounds, especially those only partially degraded or cometabolized. Negative interactions such as competition, predation, or parasitism may also influence compound degradation.

A host of physical and chemical factors affect biodegradation in the natural environment. Factors of importance in freshwater habitats include temperature, pH, dissolved oxygen, and the presence of organic and inorganic compounds (Atlas and Bartha, 1981; Fewson, 1981). Such environmental factors tend to influence overall microbial metabolic activity rather than biodegradation specifically (Scow, 1982). These factors also may determine which microbial populations persist within an ecological niche or are active at particular times. Consequently, a chemical susceptible to microbial attack under one set of conditions may not be degraded under other environmental conditions.

Substrate concentration is one factor that may specifically influence the rate and extent of microbial attack. Some studies suggest that at least some organisms may have dual enzyme systems, one which functions at low concentrations and another at high concentrations. Geesey and Morita (1979) found that a single marine bacterium exhibited bimodel kinetics in the uptake of arginine. At low concentration, uptake of arginine involved a high-affinity transport system, whereas at high concentrations,

a lower-affinity transport system was involved. Rubin et al. (1982) found that for microbial communities from three lakes, "the rate of phenol mineralization as a function of concentration was linear below 1 ug/ml, fell off between 1 and 100 ug/ml, and was again high at levels above 100 ug/ml." They hypothesized that this reflected the activity of oligotrophs at the lower concentrations and eutrophs at the higher concentrations. In a more recent study, Wang et al. (1984) found that the microbial community in lake water samples mineralized N-phenylcarbamate (IPC) at a concentration of 400 pg/ml. At the higher concentration of 1 ug/ml, however, IPC was not mineralized but was converted to organic products. The partial degradation at the higher concentration may reflect the activity of different organisms or enzyme systems. All together, the above studies suggest that concentration may influence the mode, extent and kinetics of biodegradation, and thus, the persistance of a chemical and/or its metabolites in the environment.

Measures of Biodegradation

A wide variety of methods are used to study biodegradation. Methods typically include three parts: the analytical test, the chemical to be studied, and the biological sample containing microorganisms (Johnson, 1979). The analytical technique and testing conditions

will influence not only the type of information obtained, but also the extent of biodegradation observed (Gilbert and Lee, 1979). Consequently, the methods chosen will depend on the nature of chemical and where it is released into the environment.

Most analyses follow the accumulation of biomass, the disappearance of reactants, or the appearance of degradation products (Raymond, 1979). Some of these analyses only follow biodegradation indirectly, as in the measurement of oxygen uptake and carbon dioxide evolution. These analyses tend to be simple and inexpensive because a specific analytical technique does not have to be developed for each chemical tested (Gilbert and Lee, 1979). They are typically limited to screening tests, however, because of their low sensitivity and limiting test conditions (Gilbert and Lee, 1979; Gledhill et al, 1979).

Biodegradation is followed directly by analyses which monitor the disappearance of parent compound. Common techniques include gas chromatography, high pressure liquid chromatograpy, and ultraviolet spectrophotometry. In contrast to the nonspecific analyses discussed above, direct methods are applicable under a wide range of test conditions (Gibert and Lee, 1979). Complete degradation is not distinguished from partial degradation, however, unless degradation products are also monitored. The development of such procedures to detect parent compound and all significant degradation products is expensive and difficult

because the identity of products is rarely known (Gilbert and Lee, 1979).

The most reliable methods for measuring biodegradation involve radiolabeled compounds (Johnson, 1979). These compounds not only provide a sensitive and specific means for assessing degradative activity, but they are applicable to nearly all types of tests (Brock, 1979; Gilbert and Lee, 1979). The overall fate of the compound may be established by monitoring the radioactivity present as the parent compound, metabolites or in different environmental compartments. Marinucci and Bartha (1979), for example, used radioactive labeling to follow the biodegradation of 1,2,3- and 1,2,4-trichlorobenzene in soil. They measured the radioactivity evolved as carbon dioxide, volatilized products, and other products, as well as the radioactivity bound to and extracted from soil. The primary disadvantage of such studies is the expense of the radiolabeled compound, but the development of specific analytical techniques to monitor the fate of the compound may be more expensive and more complicated (Lee and Jones, 1979).

In the aquatic environment, radiolabeled compounds are frequently used in two types of biodegradation studies: (1) river die-away studies, and (2) heterotrophic bacterial activity studies. In an effort to simulate environmental conditions as much as possible, both methods often use natural water samples which are amended with low concentrations of substrate and incubated under

environmentally relevant conditions. The methods differ, however, in their length of incubation and in the kinetic models usually applied to the generated data.

In die-away studies, the disappearance of compound is monitored as a function of time. These studies typically involve long incubations because they continue until a significant fraction of the compound has disappeared (Larson, 1984; Scow, 1982). Radioactivity evolved as carbon dioxide, incorporated into cells, and/or removed from solution may be monitored as an indication of compound disappearance. Lee and Ryan (1979), for example, monitored only $^{14}CO_2$ production in a die-away study of surface waters amended with radiolabeled benzene, phenol, chlorobenzene, and other organochlorine compounds. They found that the mineralization of many of the compounds could be described by first-order kinetics i.e. the rate of biodegradation was directly proportional to the concentration of the chemical present (Larson, 1984). The first-order kinetic model has been applied in many other die-away studies (Larson, 1984; Larson and Payne, 1981; Paris et al. 1981). Other investigators have used a second-order kinetic model (Paris et al., 1981), which includes a term for biomass.

Heterotrophic activity studies, in contrast, are based on the idea introduced by Parsons and Strickland (1962) that radiolabeled compounds can be used to measure substrate uptake by heterotrophic bacteria in natural waters. They observed that the substrate uptake activity

followed enzyme saturation kinetics and could be analyzed by the Michaelis-Menten kinetic model (Wright, 1974). The Parsons and Strickland method has subsequently been modified by other investigators (Hobbie and Crawford, 1969; Wright and Hobbie, 1965), and more recently, has been applied to the biodegradation of pollutants in natural waters (Bartholomew and Pfaender, 1983; Larson, 1984; Pfaender and Bartholomew, 1982; Shimp and Pfaender, 1985 a,b). Pfaender and Bartholomew (1982), for example, measured radioactivity mineralized and incorporated into cells in natural water samples amended with m-cresol, chlorobenzene, nitrilotriacetic acid, and 1,2,4-trichlorobenzene. They observed saturation kinetics with each pollutant tested. Because of assumptions inherent to the kinetic model, these studies involve short incubations which are stopped after a very small proportion of the label has been metabolized (Larson, 1984; Wright, 1973). Short term incubations minimize the effect of bottle confinement on the community composition and metabolic activity of aquatic samples (Ferguson et al, 1984). Consequently, the biodegradation rates observed in heterotrophic activity studies may more closely approximate rates which would occur in the natural environment.

General Features of Aromatic Degradation

The microbial metabolism of aromatic compounds has

been studied extensively using pure culture methods. Fewson (1981) suggests that the degradation of an aromatic may be divided into five phases. In the first phase, an aromatic compound must gain entry to the microbial cell. Some compounds enter by diffusion, whereas others enter by facilitated diffusion or active transport processes. In the second phase, the aromatic compound is modified to form a substrate appropriate for ring fission. This modification may involve the manipulation of side chains and the introduction of hydroxyl groups into the ring. A ring fission substrate must have at least two hydroxyl groups located ortho or para to each other, and the placement of these groups will determine the one pathway used by the organism to degrade the compound (Dagley, 1978).

The third phase of degradation involves enzymatic cleavage of the modified substrate ring. The enzymes responsible for ring cleavage are dioxygenases, and in the process of ring fission, these enzymes incorporate both atoms of molecular oxygen into the substrate molecule (Fewson, 1981; Dagley, 1978). When the substrate contains hydroxyl groups on adjacent carbon atoms, ring cleavage may occur either between the two carbon atoms bearing the hydroxyl groups or between only one carbon bearing a hydroxyl group and a different carbon. The former mode of cleavage is termed an "ortho" cleavage, while the latter is termed a "meta" cleavage (Chapman, 1972).

In the fourth phase of degradation, the products of ring fission are converted to tricarboxylic acid (TCA) cycle intermediates. This conversion occurs by one of several catabolic pathways, each involving separate suites of enzymes (Dagley, 1978). The enzymes include hydrolases, hydrolyases, aldolases, and thiolases. Although the enzymes in different pathways catalyze similar reactions, they are specific for their substrates and usually do not attack the metabolites of other pathways (Dagley, 1978). Final products of the pathways include TCA intermediates such as pyruvate, fumarate, and oxaloacetate, and other structurally related compounds (Fewson, 1981).

In the fifth phase of aromatic degradation, these final products are catabolized as a source of energy, used as building blocks for other compounds, or excreted. How the compound is used will depend on the current environment within the cell i.e. endogenous metabolite pools and alternate substrates, enzyme induction and repression, and other factors governing the integration of metabolism within the organism. Thus, the exact fate of the end products of aromatic degradation pathways may be complex to determine and variable. The likelihood that the ultimate catabolic or anabolic products would pose an environmental hazard, however, is small.

Metabolism of m-Cresol

M-cresol is metabolized by three known pathways. In each pathway, the m-cresol is modified to form a different substrate for ring fission (Figure 1). While all three ring fission substrates appear to be central intermediates in the degradation of aromatic compounds, the particular substrate formed from m-cresol depends on the genus or species of the organism involved. A study by Bayly et al. (1966) revealed that a fluorescent Pseudomonas modified m-cresol to form 3-methylcatechol. They found that (1) whole cells of the organism oxidized 3-methylcatechol when grown on m-cresol, (2) heat-treated extracts of the organism oxidized 3-methylcatechol to a ring-fission product, which they characterized by spectrophotometry, and (3) this same ring-fission product was isolated from whole cells grown on m-cresol. Hence, they concluded that 3-methylcatechol was the ring fission substrate for that Pseudomonas species.

Hopper and Chapman (1970), in contrast, found that some pseudomonads first oxidize the methyl group of m-cresol and then introduce a hydroxl group to form the ring-fission substrate. When m-cresol was metabolized in the presence of an inhibitor of bacterial oxygenases, the intermediates formed prior to ring cleavage accumulated in the culture medium. This enabled them to isolate m-hydroxybenzoic acid, the oxidized intermediate, by thin-layer chromatography.

Previous investigators (Yano and Arima, 1958; Wheelis et al., 1967) had shown that m-hydroxybenzoic acid may be

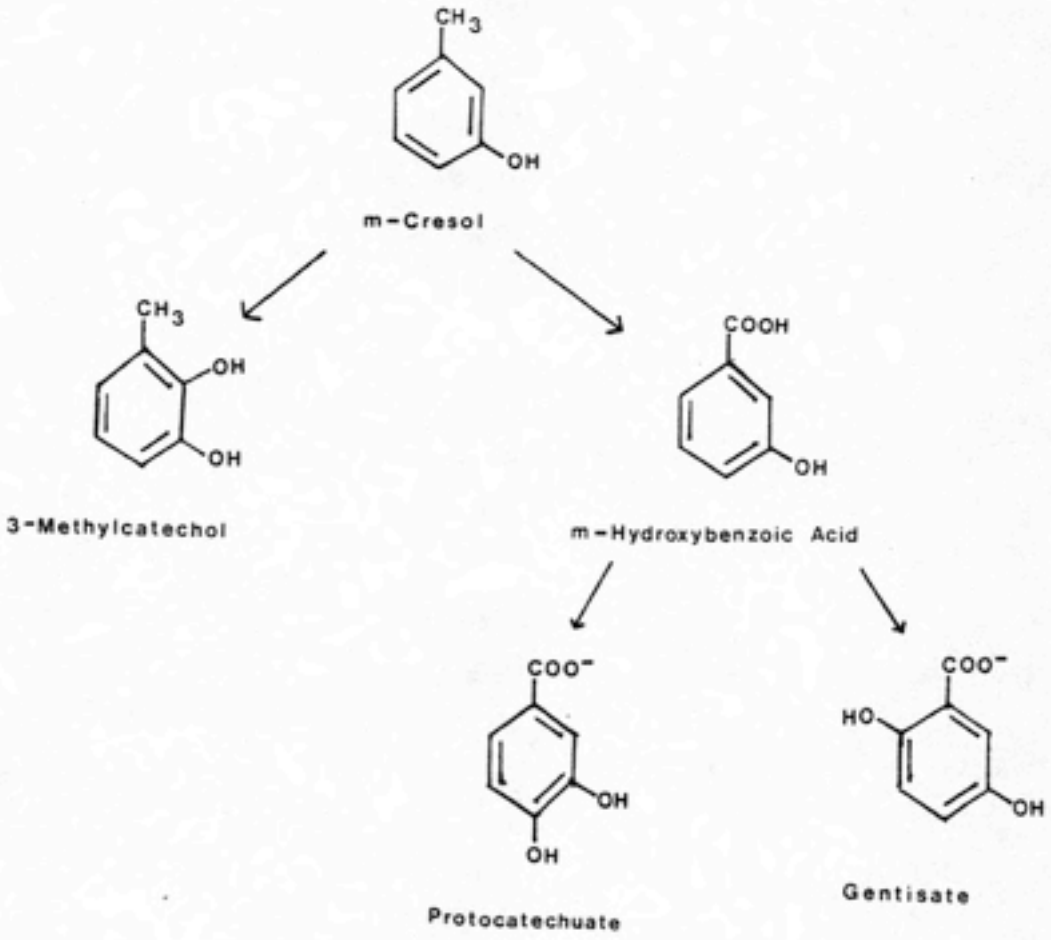


Figure 1: Degradation of m-cresol to ring-fission substrates.

hydroxylated at two different locations, thus forming two different ring-fission substrates. Yano and Arima (1958) isolated numerous bacterial strains from soil and sewage, and found that some strains metabolized m-hydroxybenzoic acid through the gentisic acid pathway and others through the protocatechuic acid pathway. They extracted and partially characterized the m-hydroxybenzoate hydrolases from strains using different pathways. One hydrolase introduces a hydroxl group at the C-4 position, thus forming protocatechuic acid, while the other introduces a hydroxyl at the C-6 position, thus forming gentisic acid.

Gentisic acid, protocatechuic acid, and 3-methylcatechol are each cleaved by dioxygenases. The mode of cleavage determines which pathway is used to metabolize the fission product to TCA intermediates or related compounds (Bayly and Barbour, 1984; Dagley, 1971). In the case of protocatechuic acid, whose hydroxyl groups are located on adjacent carbon atoms, an ortho or a meta cleavage may occur. The subsequent pathways of metabolism are thus termed the ortho-fission pathway and the meta-fission pathway, respectively. The latter pathway is also involved in the metabolism of 3-methylcatechol; although the specific enzymes involved are different, the sequence of reactions are the same (Stanier and Ornston, 1973). In the case of gentisic acid, whose hydroxyl groups are para to one another, cleavage occurs between the carbon bearing one hydroxyl group and the carbon bearing the carboxylic

group, and the subsequent pathway of metabolism is termed the gentisate pathway (Bayly and Barbour, 1984).

Metabolism of protocatechuic acid by the ortho-fission pathway is shown in Figure 2 (Stanier and Ornston, 1973). Ring fission by protocatechuate 3,4-dioxygenase produces beta-carboxy-cis,cis-muconate, and subsequent metabolism yields the two end products of the pathway, succinate and acetyl-CoA. Metabolism of protocatechuic acid and 3-methyl catechol by the meta-fission pathway is shown in Figure 3 (Chapman, 1972; Stanier and Ornston, 1973; Bayly and Barbour, 1984). The meta cleavage of protocatechuate by protocatechuate 4,5-dioxygenase produces alpha-hydroxy-gamma-carboxymuconic semialdehyde. Subsequent metabolism produces two molecules of pyruvate and one molecule of formate. Cleavage of 3-methylcatechol by catechol 2,3-dioxygenase produces 2-hydroxy-6-ketohepta-2,4-dienoate. End products of this pathway are acetate, acetaldehyde and pyruvate.

The gentisate pathway is shown in Figure 4 (Bayly and Barbour, 1984). Gentisic acid is cleaved by gentisate 1,2-dioxygenase to form maleylpyruvate. In some organisms maleylpyruvate is isomerized to form fumarylpyruvate, which is then hydrolysed to form fumarate and pyruvate. Hydration of fumarate produces L-malate, which is an intermediate in the TCA cycle. In other organisms, the isomerization step is omitted. Maleylpyruvate is directly hydrolysed to maleic acid and pyruvate. Hydration of

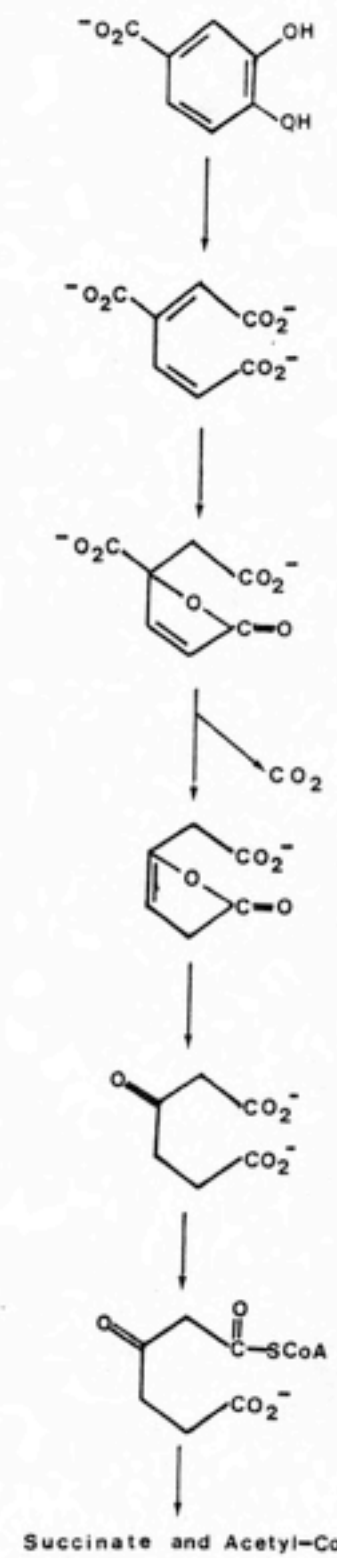


Figure 2: The ortho fission pathway of protocatechuic acid (Stanier and Ornston, 1973).

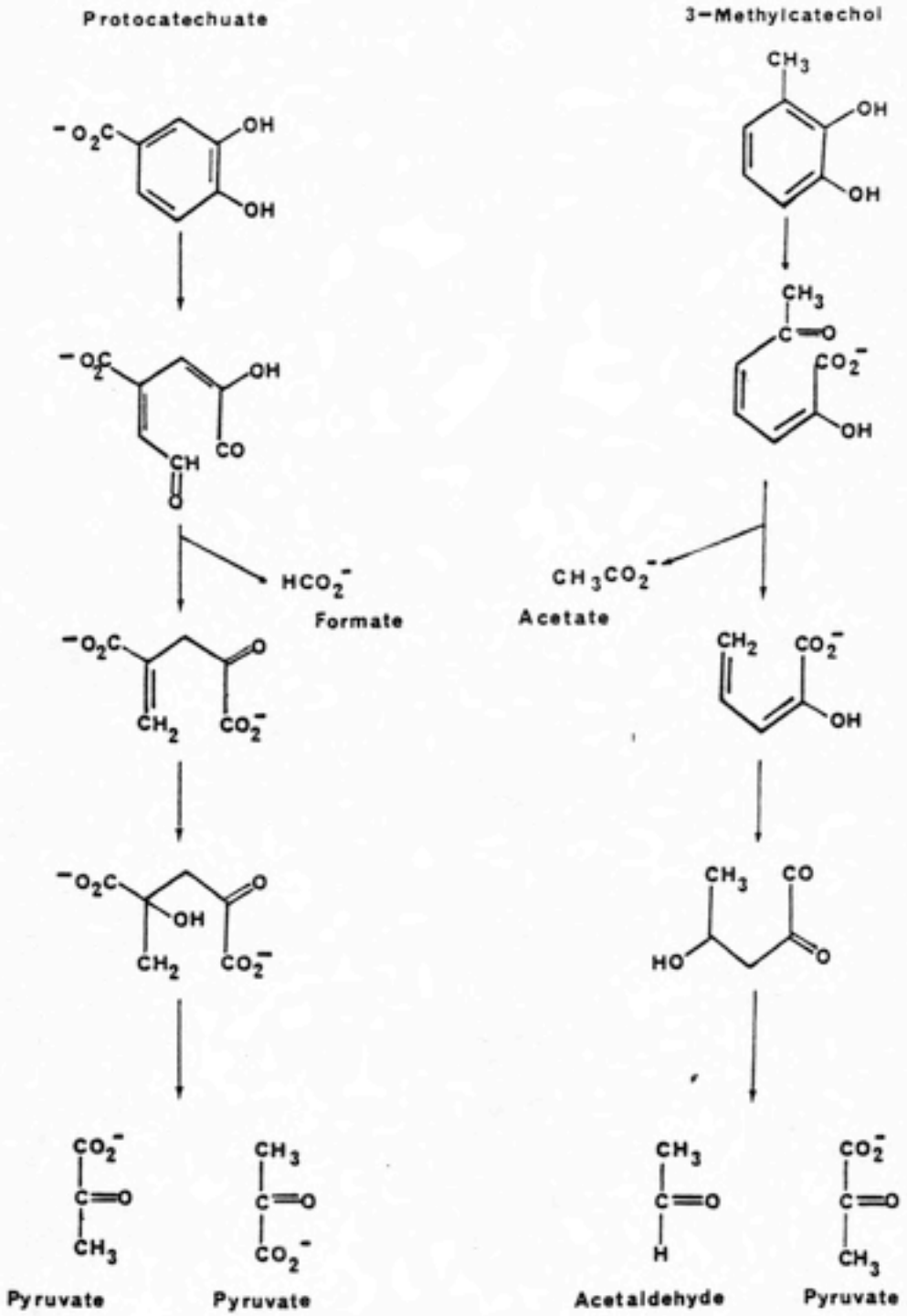


Figure 3: The meta fission pathways of protocatechuic acid and 3-methylcatechol (Chapman, 1972; Stanier and Ornston, 1973).

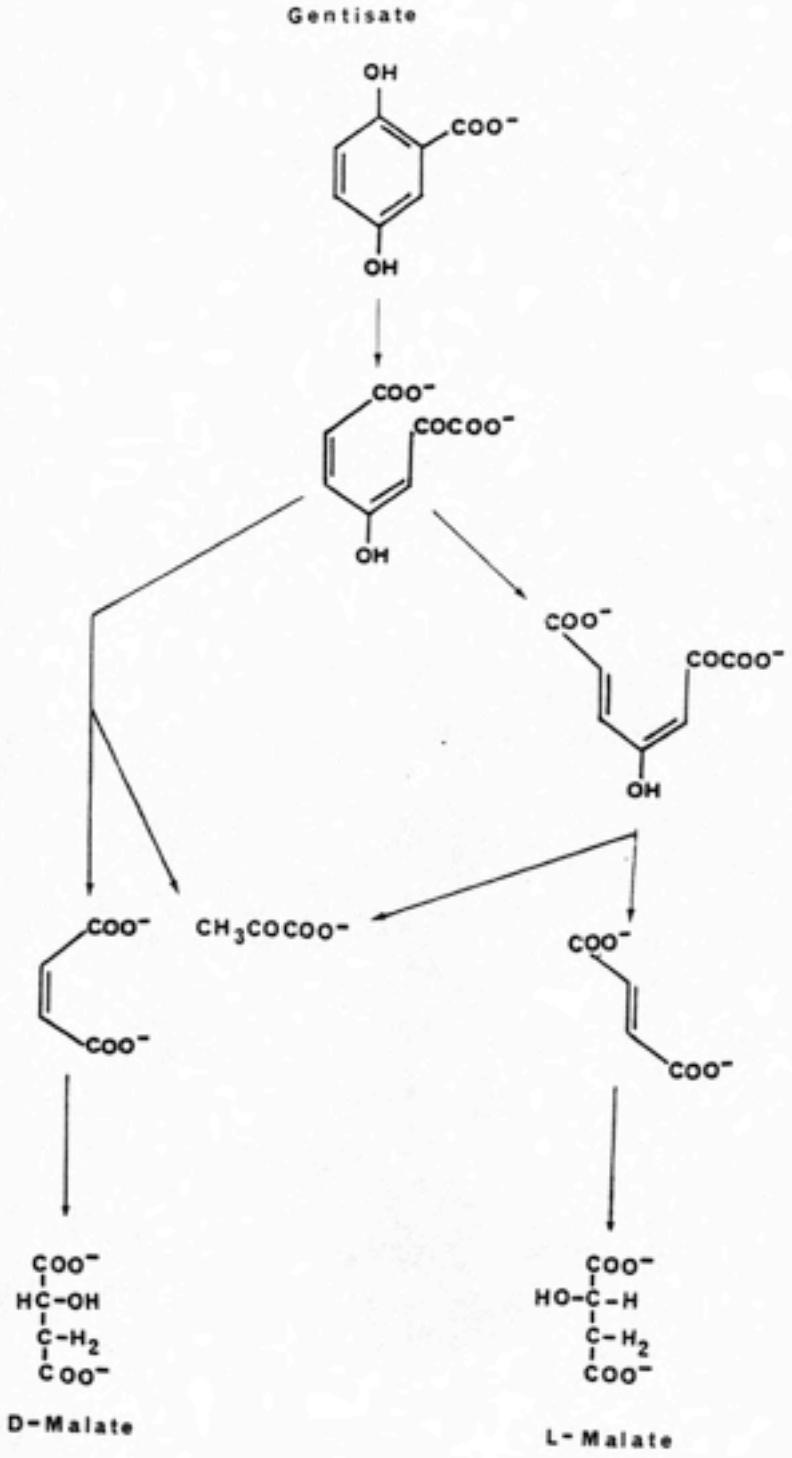


Figure 4: The gentisic acid pathway (Bayly and Barbour, 1984).

maleic acid produces D-malate, which cannot enter directly into the TCA cycle. The latter form of metabolism has been observed in the degradation of m-cresol by two species of Pseudomonas (Hopper and Chapman, 1970).

As was discussed in the previous section, the end products of the ortho, meta and gentisate pathways may be excreted, further oxidized, or channeled into anabolic pathways. It must be emphasized, however, that ATP is not generated from m-cresol during the couse of its degradation via the ortho, meta or gentisate pathways. ATP is generated by the oxidation of their respective end products to CO_2 through the TCA cycle, and the re-oxidation of NADH in the electron-transport system.

Pure Culture Studies, the Natural Environment, and Metabolite Accumulation

The mechanisms of m-cresol degradation have been revealed by studies using pure cultures of microoganisms. These cultures are usually isolated by their ability to grow in artificial media with the substrate of interest present at high concentration and as the sole source of carbon (Alexander, 1980; Dagley, 1975). The strains, mutants of the strains, or isolated enzymes are then used in an effort to discern the mechanism of interest. Though these methods have been valuable in the elucidation of degradation pathways, the experimental conditions are so

different from those in the natural environment that it is difficult to extrapolate from the results of these studies to the natural environment (Alexander, 1980).

The effects of mixed populations, low substrate concentrations, alternate carbon sources, and physical conditions on degrading mechanisms are all difficult to predict (Alexander, 1980). Little is known about which pathways predominate in the environment, whether the organisms isolated by enrichment cultures contribute to a compound's degradation in nature, or whether other degradation pathways exist (Alexander, 1980; Bollag, 1974). Furthermore, little is known about which products in a metabolic pathway are excreted and accumulated outside the microbial cell (Alexander, 1980). Even if a product is not directly toxic to humans, its accumulation may inhibit microbial activity and prevent further degradation of the toxic primary substrate.

In the case of m-cresol, studies with pure cultures suggest that few of the metabolites formed after ring-fission would accumulate because of the regulation of enzyme induction. In two Pseudomonas putida isolates, for example, m-cresol induces simultaneously all the enzymes of the meta-cleavage pathway (Bayly and Barbour, 1984). Coincident induction of enzymes has also been observed in two species which degrade protocatechuate via the ortho-cleavage pathway. In Pseudomonas putida and P. aeruginosa four enzymes are induced coincidently by a product, beta-

ketoadipate, whereas in Acinetobacter calcoaceticus, nearly all enzymes of the pathway are coincidently induced by the substrate, protocatechuate (Stanier and Ornston, 1973). Thus, in the organisms studied so far, few metabolites in the pathway have an inductive function, and with coincident enzyme induction, most metabolites along the sequence will not accumulate within the cell. Furthermore, most of these metabolites are chemically unstable in aqueous solution and are usually unable to permeate the membrane of intact cells (Dagley, 1975; Stanier and Ornston, 1973). These factors suggest that the intermediary metabolites are unlikely to accumulate outside the cell.

In contrast, pure culture studies suggest that the aromatic metabolites (before ring fission), end products, and intermediates of central oxidative pathways may accumulate. Ring-fission substrates, for example, have been detected in culture fluids (Dagley and Chapman, 1971). Dagley (1978) suggests that when the supply of dissoved oxygen is limited, the "rate of formation of a dihydric phenol may sometimes exceed its rate of oxidation", thus leading to the accumulation and excretion of the ring-fission substrate.

Classic microbial and biochemical studies with pure cultures have often revealed that overall metabolism is a finely tuned process exhibiting such phenomena as feedback inhibition and allosteric control mechanisms. Batch studies have shown that the rate of glucose uptake is

closely balanced with the rate of glucose metabolism. Similarly, feedback inhibition of meta pathway enzymes by the end product acetate has been observed in Pseudomonas putida (Bayly and Barbour, 1984). In this case, the continued accumulation of acetate and other end products would not be expected.

Neijssel and Tempest (1979) argue that metabolic processes are not always so finely tuned, and that overproduction of metabolites can and does occur. In chemostat cultures of Klebsiella aerogenes, these investigators studied the effect of nutrient limitation on the use of four carbon substrates: glucose, glycerol, manitol and lactate (Tempest and Neijssel, 1978; Neijssel and Tempest, 1979). They found that when the carbon substrate was the limiting nutrient the only growth products were organisms and carbon dioxide. When a different nutrient was limiting, however, the non-limiting carbon substrate was used less efficiently and certain intermediary metabolites were excreted into the culture fluids.

Nearly all these metabolites were intermediates of central oxidative pathways. They included the TCA intermediates pyruvate and acetate which, it is important to note, were not necessarily the end products of the carbon substrate's degradation pathway. The particular metabolites excreted depended not only on the carbon source, but on the nature of the nutrient limitation. A

similar phenomenon was observed with the organisms Escherichia coli and Bacillus subtilis. Thus, this study suggests that the overproduction and excretion of central metabolites may occur in response to stressful environmental conditions. Whether such excretion and accumulation would occur in batch studies with a mixed microbial community is unclear.

MATERIALS AND METHODS

Sample Source and Collection

Natural water samples were collected from two water sources: the B. Everett Jordan Lake and the Haw River. Surface water from Jordan Lake was used in the first phase of the research project. Jordan Lake is a eutrophic reservoir located in the Piedmont region of North Carolina. The sampling site was located on a bridge where State Road 1008 crosses the reservoir. The water temperature was recorded at the time of collection. Samples were then stored in polyethylene containers, returned to the laboratory, and processed for experiments within two hours of collection.

Degradative activity in Jordan Lake water had been studied in numerous short-term kinetic studies and in three long-term die-away studies during the past two years. A kinetic study on May 24, 1984 found unusually low degradation rates for that time of year. Similarly, the first cresol disappearance study (May 29) was terminated on its seventh day because almost no degradation had occurred. In the previous die-away studies at least 45 percent of the m-cresol was degraded by the seventh day. Comparable activity was observed in the second cresol disappearance experiment (June 6). Moreover, results from

this experiment suggested that at these levels of activity, metabolites did accumulate in the water. In the first two metabolite accumulation experiments, however, little degradative activity and no metabolite accumulation was observed. It became apparent that the Jordan Lake environment had changed, precipitating changes in microbial activities and/or community composition.

In view of time constraints, a scarce supply of radioactive m-cresol, a desire to have degradative activity comparable to the levels observed in the time course studies over the last two years, and the need to have m-cresol degraded in order to study whether its metabolites accumulate in the aqueous phase, we decided to try a different water source, the Haw River. The Haw River is a polluted, nutrient-rich river located in the Piedmont region of North Carolina and which flows into Jordan Lake. Samples were collected from a bridge where State Road 1545 crosses the river about 10 miles southeast of Saxapahaw, North Carolina. Samples were collected in the same manner as those from Jordan Lake. Because sufficient degradative activity was consistently observed, this water source was used for the rest of the project.

Sample Preparation, Incubation and Termination

Although certain details varied depending on the particular circumstances involved with each experiment, the

basic method used in the die-away studies is outlined below.

25 ml aliquots of the collected water sample were dispensed into autoclaved 250 ml flint-glass bottles. Typically, three bottles were used for each concentration and time period. Two served as sample replicates and the other received 50 ul of 6.8% mercuric chloride or was autoclaved, and served as an abiotic control. After allowing the autoclaved bottles to cool, the appropriate volumes of ^{12}C m-cresol and ^{14}C m-cresol delivery solutions were added to each bottle to give the desired final concentration (120 - 1000 ug/l) and level of radioactivity (typically 1 uCi/bottle). Delivery solutions were prepared just before the experiment from the stock ^{12}C and ^{14}C m-cresol solutions. Diluent for the ^{12}C was distilled water, and for the ^{14}C, 50% EtOH. Duplicate injections of the radioactive delivery solutions were added directly to scintillation vials. In this way, the radioactivity added initially to each bottle could be determined.

All bottles were then capped with screw caps and silicone septa lined with Teflon (Pierce Chemical Co., Rockford, IL) and incubated. In some experiments, evolution of $^{14}CO_2$ was measured. In this case, duplicate sets of samples and controls were inoculated at each concentration. Suspended from each septumof the respiration vials was a small plastic cup. After capping, all bottles were incubated at room temperature (22°C + 2

°C). The incubation period depended on the experiment and ranged from 0 days to 16 days. The typical incubation period was 8 days.

For the respiration samples, incubation was stopped by the injection of 50 ul of 2 N H_2SO_4 through the septum and into the water sample. Then 150 ul of 1 N KOH, a CO_2 trapping agent, was injected into the suspended cup. Bottles were placed on an rotary platform shaker for 5-6 hours. The caps were subsequently removed and a folded piece of filter paper (Whatman #1) was used to absorb the KOH from the trap. Filter papers were then added to scintillation vials containing cocktail, and subsequently counted for radioactivity. Measured respiration was corrected for the CO_2 recovery efficiency, as determined by the radioactive $^{14}CO_2$ recovered from a 25 ml water sample amended with $Ba^{14}CO_3$ (New England Nuclear) and processed identically to the other respiration samples.

For the uptake/filtrate samples, incubation was stopped by filtering the water sample through a 47 mm, 0.45 um polycarbonate filter (Nuclepore Corp., Pleasanton, CA). The vacuum apparatus included a glass funnel and a glass filter holder equipped with a stainless steel screen (Millipore Corp., Bedford, MA). This apparatus was autoclaved prior to each experiment. After each filtration, the apparatus was rinsed with 300-400 ml of sterile distilled, deionized water.

Each filtrate was collected in an autoclaved side-arm

Chromatographic separations were performed with a Waters high pressure liquid chromatograph (Model 660) equipped with a loop injector (Waters Associates, Model U6K), a 254 nm fixed wavelength UV absorbance detector (Waters Associates, Model 440), a fluorescence spectrophotometer (Perkin-Elmer, Model 650-10S), a recorder (Linear Instruments, Model 300), and a prepacked uBondapack-C_{18} column (Waters Associates; 3.9 cm x 30 cm). Fractions were collected with a LKB 2112 RediRac fraction collector (LKB-Prodkter AB, Bromma, Sweden). An Uptight precolumn

(Upchurch Scientific, Inc.) packed with C_{18} material was placed between the injector and analytical column. Another precolumn (identical to the analytical column) was placed between pumps to clean up the water mobile phase (Randy Goodman, personal communication). This column was cleaned following each day of use by backflushing with acetonitrile.

The mobile phase consisted of two solvents, A and B. Solvent A was water or water with 0.2% (v/v) glacial acetic acid (J.T. Baker Chemical Co., Phillipsburg, N.J., 99.9% pure). The water had been purified with a Corning Mega-Pure system. Solvent B was acetonitrile (Fisher Scientific Co., Fair Lawn, N.J., HPLC grade). Each solvent was degassed prior to use. Though the flow rate was almost always 2 ml/min, the elution conditions depended on the particular experiment.

Separation Procedure for Cresol Disappearance Experiment:

In this experiment, samples were eluted isocraticly using a 20% acetonitrile/80% water mobile phase. The injection volume for all samples and standards was 10 ul. m-Cresol was detected using the fluorescence detector with emission and excitation wavelengths set as follows: excitation λ = 275 nm, slit width = 5 nm; emission λ = 310 nm, slit width = 5 nm. Neither the UV detector nor the fraction collector were used.

Under these conditions, m-cresol eluted in 5.25 minutes

sample mixture was achieved. Detection was monitored by the UV detector set at 254 nm and a sensitivity of 0.1. The sample mixture was an aqueous solution of m-cresol, protocatechuic acid, m-hydroxybenzoic acid, 4-methylcatechol, and when available, gentisic acid. The m-cresol was prepared from the stock ^{12}C solution, whereas aqueous solutions of the other compounds were all prepared fresh the day of the experiment. Typically 2 ug of each

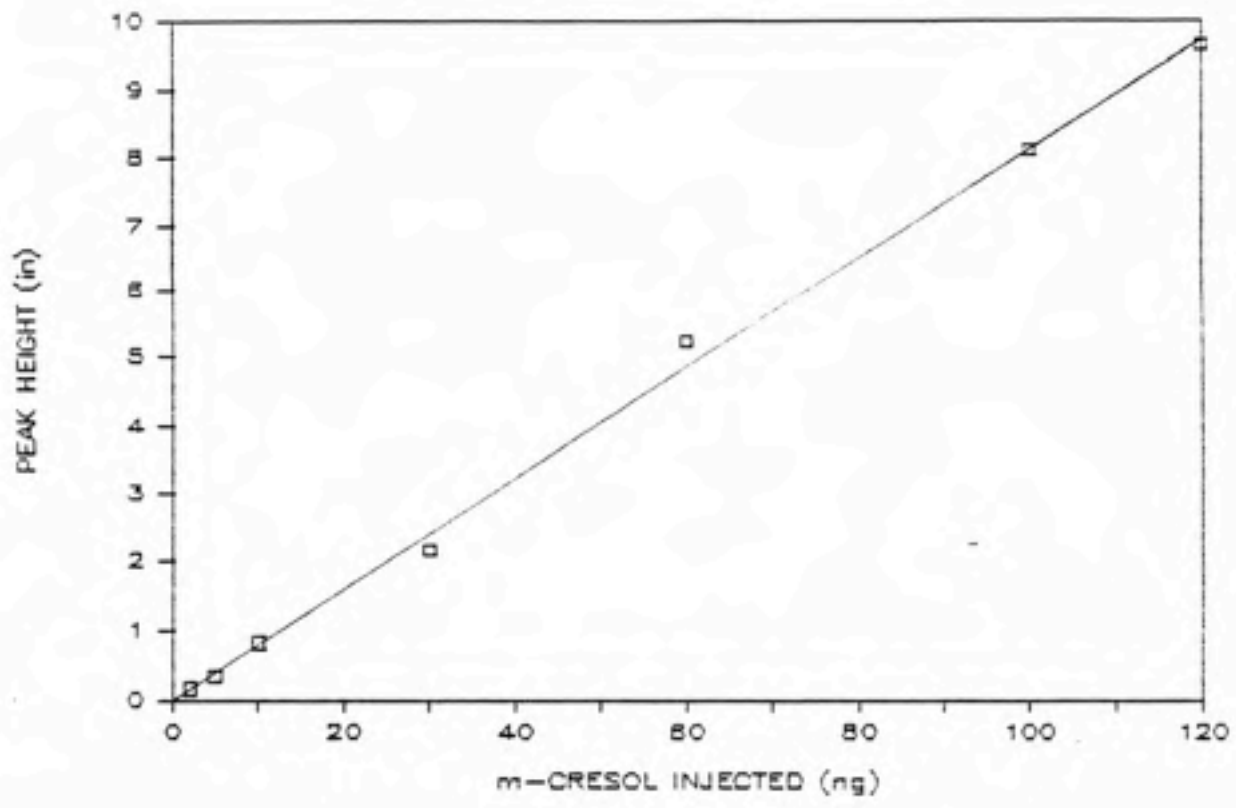


Figure 5: Calibration Curve.

aliquots of each sample were also injected directly into scintillation vials so that the recovery efficiency could be determined. All scintillation vials were counted on a Packard Tri-Carb 300CD scintillation counter.

Sep-Pak Concentration Procedure

In the cresol disappearance study, reverse-phase Sep-Pak C_{18} cartridges (Waters Associates) were used to concentrate m-cresol from the aqueous samples. The cartridge was first prepared by passing 2 ml of acetonitrile through the cartridge, followed by 5 ml of water (the same as that used for the HPLC mobile phase). Then 20 ml of sample filtrate were passed through the cartridge at a flow rate of 6 ml/min. Sample elution was with 2 ml of acetonitrile, and the exact eluent volume was measured and recorded.

A preliminary study was performed using the same water source and initial concentration as the subsequent cresol disappearance study. Four replicate samples were passed through Sep-Pak cartridges as described above and then analyzed by HPLC. The average m-cresol recovery efficiency was 89%, with a standard deviation of 2%. HPLC data in the cresol disappearance experiment was then corrected for this recovery efficiency.

RESULTS AND DISCUSSION

Cresol Disappearance Experiments

In the Environmental Microbiology laboratory of the UNC Department of Environmental Science and Engineering, the biodegradation of m-cresol has been studied in numerous heterotrophic activity experiments and in several die-away experiments during the past two years. In these experiments, water samples were amended with radiolabeled m-cresol and biodegradation was measured by the evolution of $^{14}CO_2$ and the uptake of ^{14}C into cells. When radioactivity in the aqueous phase was monitored, it was assumed that all radioactivity remaining in this phase was in the form of the unmetabolized parent compound, m-cresol. In other words, the phenomenom of metabolite excretion was assumed to be insignificant in the degradation of m-cresol. Two questions were thus posed: (1) With regard to the past experimental methods, has this assumption been valid?, and (2) if metabolites are formed, what conditions favor their production, and what might they be? The cresol disappearance experiment was designed to test the validity of this assumption and to determine whether there was metabolite accumulation to study in future experiments.

This experiment was set up just like a typical die-away study, with incubation periods ranging from 0 to 336 hours.

For each incubation period, however, two sample sets were inoculated. One set received a m-cresol solution containing ^{14}C m-cresol, whereas the other received a solution containing only ^{12}C m-cresol. The final concentration of both sets was 1000 ug/l. The sample set containing ^{14}C m-cresol was used to follow the disappearance in radioactive label in sample filtrates. The ^{12}C samples, in contrast, were used to specifically follow the disappearance of m-cresol in the sample filtrates. m-Cresol in these filtrates was concentrated using a C_{18} Sep-Pak cartridge and then analysed by HPLC. The concentration chosen for this experiment was about 2x-4x higher than the maximum concentration typically used in earlier experiments, but was chosen so metabolism observed over the experiment would be within the detection limits of the procedure.

Results from this experiment are shown in Figure 6. After 48 and 96 hours of incubation, the percent of radioactivity and m-cresol remaining in the aqueous phase is very similar. Thus, most radioactivity remaining in solution is in the form of the parent compound, m-cresol, rather than its metabolites. After longer incubation periods, a discrepancy appears between the percent of radioactivity remaining and the percent of m-cresol remaining. This discrepancy suggests that radioactivity remains in solution in the form of compounds other than m-cresol.

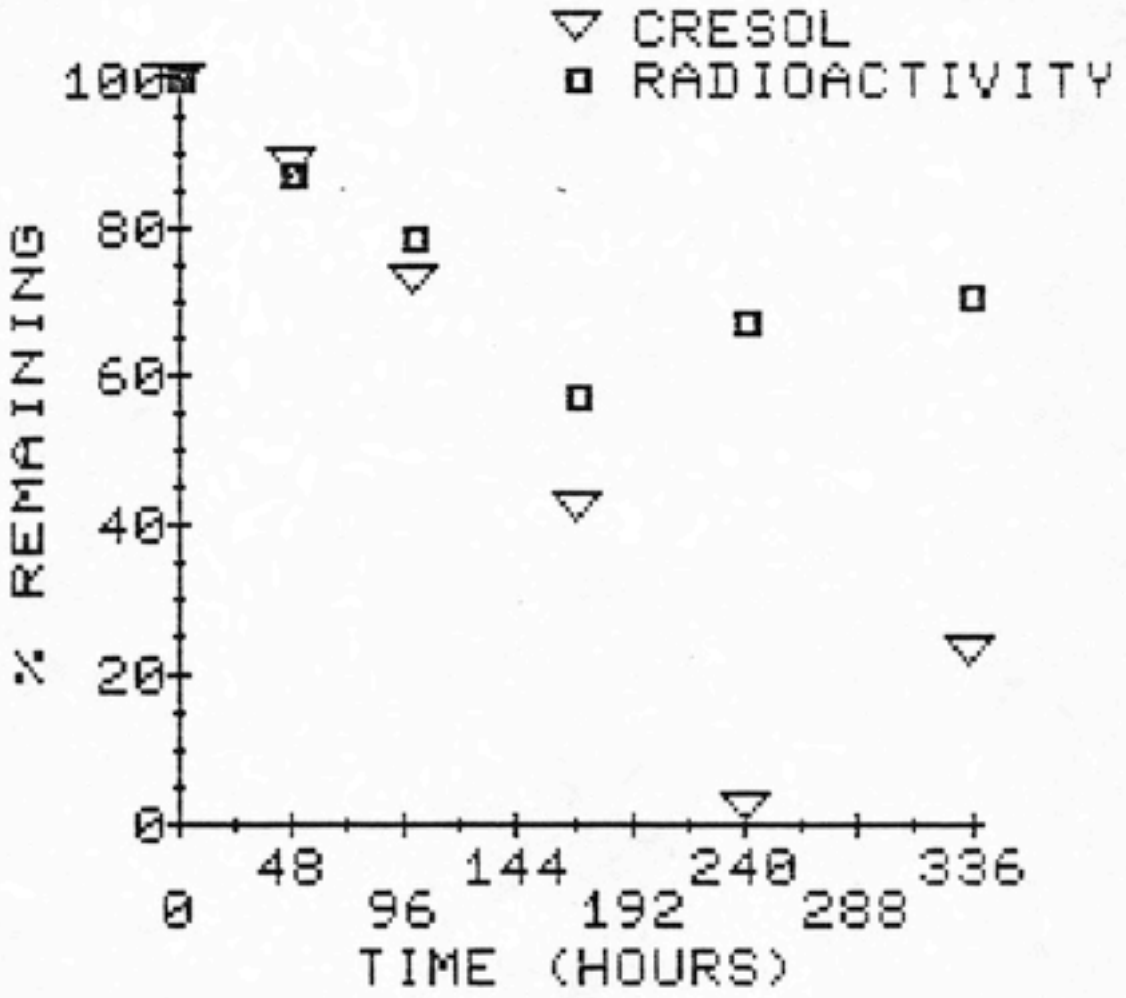


Figure 6: Percent of m-Cresol and Radioactivity Remaining in Samples vs. Time.

These results have obvious implications for the previous studies with m-cresol. For the heterotrophic activity experiments, which involve short incubation periods (less than 24 h.), the results support the assumption that metabolite accumulation (in the water medium) accounts for only a small fraction of degradative activity. For the die-away studies, which involve much longer incubations and are similar to most procedures used to assess pollutant biodegradation, the results do not support the assumption of no product formation. Rather, the method underestimates the extent of degradation by 15% at 168 hours (7 days) and nearly 50% at 336 hours (14 days).

These results also have implications for future studies. They suggest that there may indeed be metabolites accumulating during the incubation, and that they begin to appear after approximately four days of incubation. Future experiments were designed to confirm the results obtained in this study while yielding more information about the quantity of metabolites.

An appreciable amount of degradation is apparent in the $HgCl_2$ controls, particularly after the longer incubation periods (Figure 7). This activity could be due to abiotic removal mechanisms, biodegradation by resistant microorganisms, or microbial contamination. In subsequent experiments, all controls were autoclaved, and almost no degradation was observed.

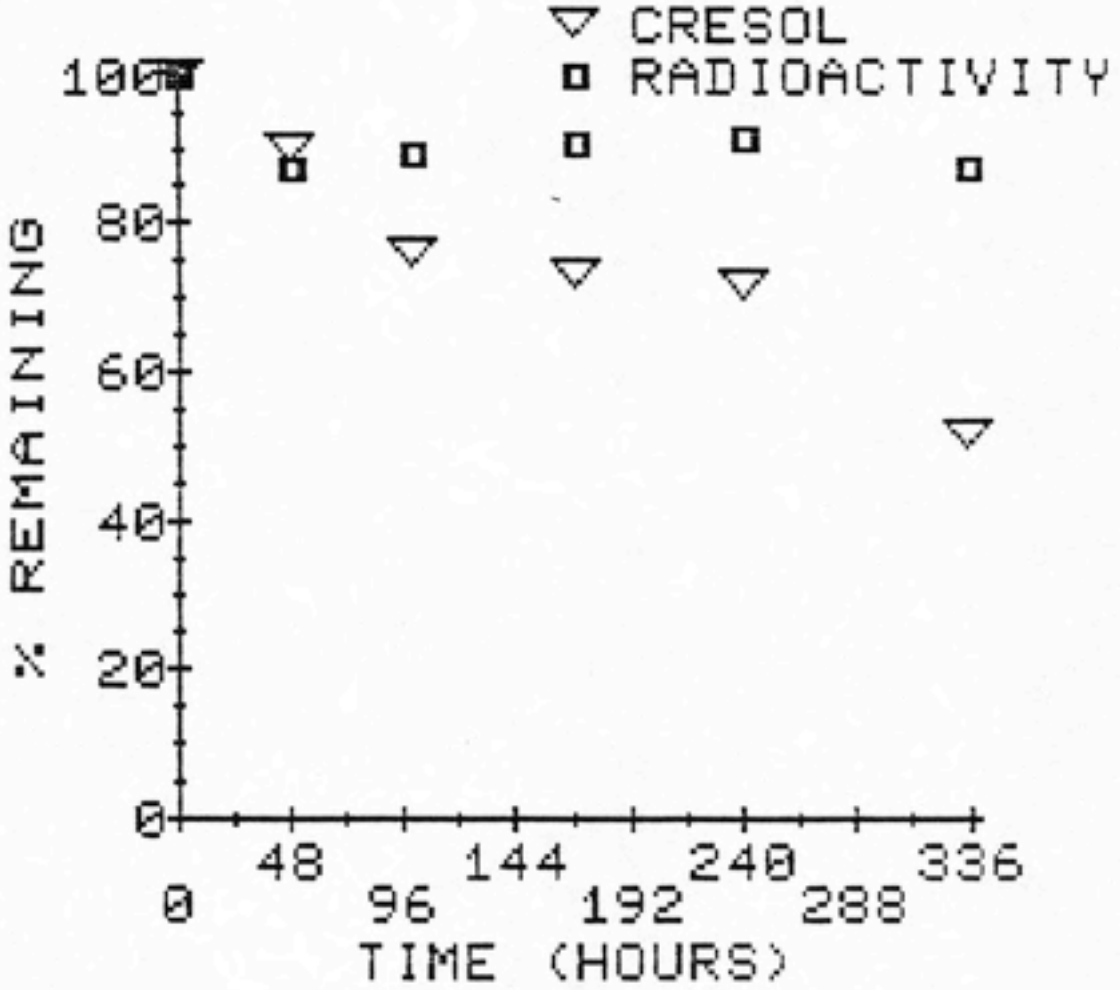


Figure 7: Percent of m-Cresol and Radioactivity Remaining in Controls vs. Time.

Cresol-Metabolite Separation Experiments

The cresol disappearance experiment suggested that metabolites may accumulate in the aqueous phase under certain conditions. The next step was to develop a procedure to separate m-cresol from its metabolites. The number, quantity and identity of metabolites generated under such conditions was unknown. Therefore, the first step was to develop a separation procedure using some known metabolites. This procedure could then be refined later, as needed.

The metabolites used in the procedure development experiments were m-hydroxybenzoic acid, protocatechuic acid, 4-methylcatechol and gentisic acid. These compounds were chosen because (1) they were all detectable with the UV detector at 254 nm, (2) they were readily available, and (3) the latter three are ring-fission substrates, which have been known to accumulate in some pure culture fluids (Dagley and Chapman, 1971). 4-methylcatechol was used as a substitute for 3-methylcatechol, which was unavailable. It was subsequently discovered that the 4-methylcatechol was impure, and thus, of little value as a substitute anyway.

Other than the ring-fission substrates, the compounds most likely to accumulate were the end products of the degradation pathway(s) or other TCA intermediates. Most of these compounds are dicarboxylic acids or similar

compounds, and being very polar, are likly to elute before the aromatic metabolites on a reverse-phase column. Therefore, the primary objective was to find elution conditions that would separate m-cresol from the ring-fission substrates, the ring-fission substrates from more polar compounds and, as much as possible, the ring-fission substrates from each other.

The final elution conditions are described in Materials and Methods. The separation achieved under such conditions is shown in Figure 8. The 4-methylcatechol eluted in two small peaks, one of which coeluted with m-hydroxybenzoic acid. Because the 4-methylcatechol was only being used as a substitute for 3-methylcatechol (and would not necessarily have the same elution characteristics as that compound anyway), the impurity problem was not pursued further.

The detention time of each compound under the final elution conditions is shown in Table 1. The detention times were corrected for the 0.6 minute time lag between the UV detector and fraction collector. From the time of arrival at the fraction collector, the fraction containing the compound peak maximum was estimated. In this way, peaks detected with the UV detector could be correlated with radioactive peaks detected in the metabolite accumulation experiments.

Metabolite Accumulation Experiments

1. protocatechuic acid
2. gentisic acid
3. 4-methylcatechol
4. m-hydroxybenzoic acid
5. m-cresol

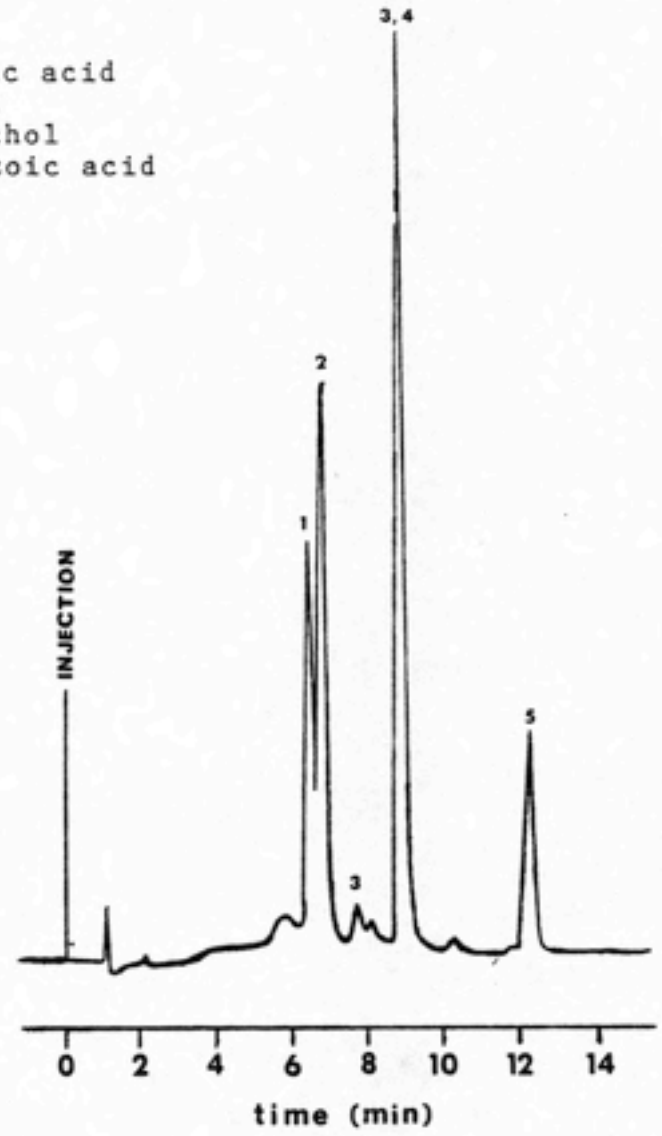


Figure 8: Sample Chromatogram of m-Cresol and Aromatic Metabolites.

Table 1: HPLC Detention Times and Fraction Numbers of m-Cresol and Metabolite Standards.

Compound	Detention Time (min)		Time at Collector	Postulated Fraction No.	
	$\bar{x}$*	s_x	(min)	1 min	0.5 min
protocatechuic acid	6.32	0.07	6.92	7	14
gentisic acid	6.62	0.08	7.22	8	15
4-methylcatechol	7.6	0.04	8.2	9	17
	8.71	0.08	9.31	10	19
m-hydroxybenzoic acid	8.68	0.05	9.28	10	19
m-cresol	12.22	0.04	12.82	13	26

* mean of 5 or 6 measurements, depending on the compound

After development of the metabolite separation procedure, the next step was to perform a series of die-away studies. The focus of these die-away studies was not measuring mineralization, radioactive uptake, or degradation rates, but assessing the number and relative proportions of metabolites present in the aqueous phase. A rigorous attempt to identify a particular metabolite would only proceed if it were produced in significant quantities (greater then 5% of the label).

The first two experiments were performed with Jordan Lake water, and as for two other degradation studies that summer, unusually low degradative activity was observed. The percent m-cresol metabolized by the seventh day was about 1/6 the level observed in previous die-away studies, and under these conditions, no metabolite accumulation was detected. Because of the low levels of activity, we decided to try a different water source, the Haw River.

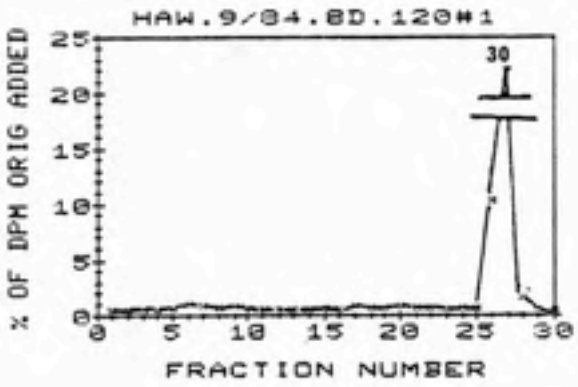
In light of the problems with the Jordan Lake samples, the first metabolite accumulation experiment with Haw River water was a small study. Samples were incubated for 8 days at two m-cresol concentrations, 120 ug/l and 960 ug/l. No uptake or respiration measurements were made; only the radioactivity remaining in the sample filtrate was monitored.

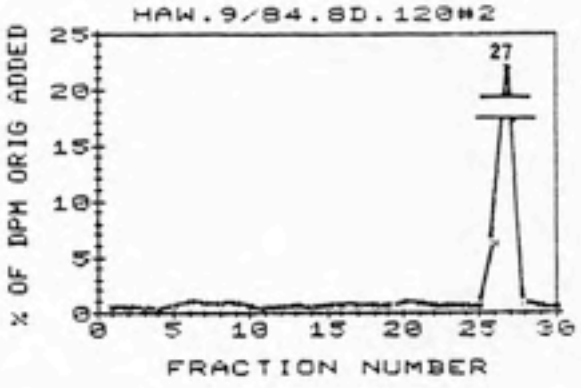
HPLC fractionations of the sample filtrates are shown in Figure 9. Fractions were each collected for 0.5 minutes

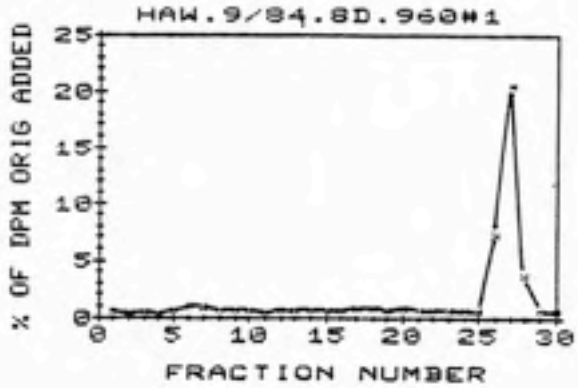
and the radioactivity per fraction is expressed as a percent of the radioactivity added to the water sample at day 0. The peak eluting at fractions 26, 27 and 28 is m-cresol. Most samples show little radioactivity coming off before the m-cresol peak. The exception is one 960 ug/l sample, which has radioactivity eluting in fractions 5-10 and 16-17. Together these peaks represent 16% of the radioactivity originally added (Table 2). Only a trace of radioactivity is apparent in these same fractions in the other samples.

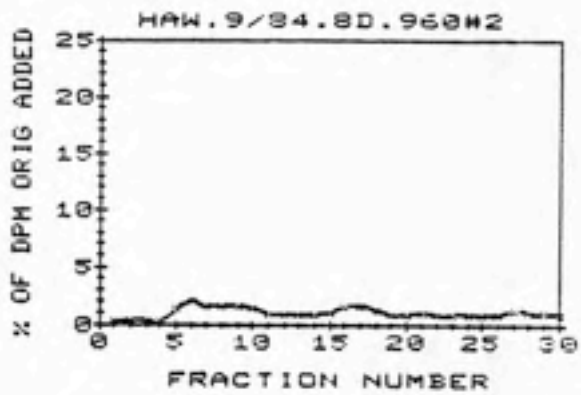
Further examination of the data, however, revealed that the fractionation procedure failed to recover all the radioactivity present in the sample filtrates (Table 2). Only 41%, 58%, 81% and 76% of the injected label was recovered from the 960#2, 960#1, 120#1, and 120#2 samples, respectively. This suggested two possibilities: (1) that the radioactivity was binding irreversibly to the HPLC column, or (2) that it was eluting after, rather than before, m-cresol.

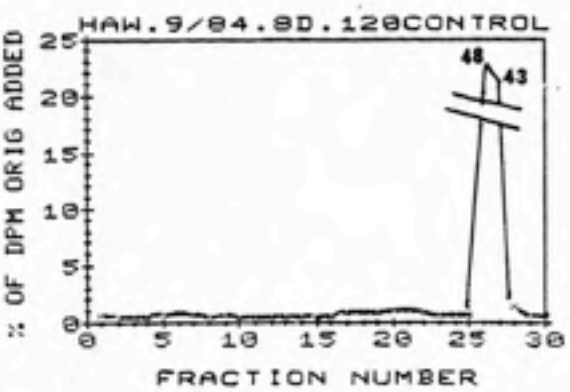
To test these possibilities, the recovery efficiency of each sample was determined when eluted with acetonitrile (100%). Under these conditions, radioactive compounds eluting after m-cresol should be recovered, whereas those binding irreversibly to the column should not. The high recovery efficiencies (Table 3) suggest that most of the "missing" label was eluting after the m-cresol. This was confirmed for the 960 ug/l #1 sample. When the linear











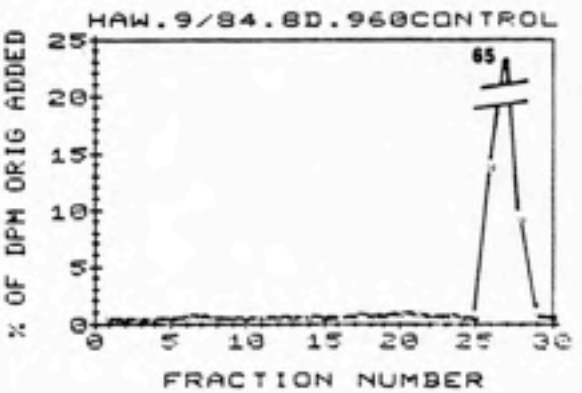


Figure 9: HPLC Fractionations of Sample Filtrates. September, 1984.

Table 2: Recovery in Aqueous Filtrates of the Radioactive Label Originally Injected. September, 1984.

Concentration (ug/l)	Replicate No.	% Label Remaining	% m-Cresol Recovered	% Other Recovered
120	1	58	41	6
120	2	51	33	6
120	control	104	92	6
960	1	66	31	7
960	2	44	2	16
960	control	101	87	4

Table 3: Recovery Efficiencies of Radioactive Label in Sample Filtrates when Eluted with an Acetonitrile Mobile Phase. September, 1984.

Sample	Replicate No.	Recovery Efficiency (%)*
120 ug/l	1	94
120 ug/l	2	99
120 ug/l	control	102
960 ug/l	1	93
960 ug/l	2	82
960 ug/l	control	98
m-cresol standard		100

* (Radioactivity Recovered/Radioactivity Injected) x 100%

gradient was increased from 15 minutes to 45 minutes, 3 radioactive peaks eluted after the m-cresol (Figure 10). To accomodate such a long gradient, however, fractions of 1 minute had to be collected. Consequently, in this figure, m-cresol elutes in fractions 13 and 14.

The identity of peaks a, b, and c is unknown, but their elution after m-cresol indicates that they are less polar compounds than m-cresol, and that they are not metabolites in its degradation pathway. One possibility is that the compounds are growth products. In this case, m-cresol is degraded to pathway end products (Figures 2-4) which enter the central pathways of metabolism. Intermediates in the central pathways may enter a diverse and diverging series of biosynthetic pathways to form proteins, polysaccharides, lipids, nucleic acids, or their respective precursors. Thus, if peak a,b, and c are in fact growth products, they could be many different compounds.

Another possibility is that peak a, b and c are polymerization products of m-cresol. Bollag et al. (1979) isolated an extracellular enzyme from the soil fungus Rhizoctonia practicola that catalyzed the formation of polymerized products from phenolic compounds. The enzyme polymerized p-methylphenol to a dimer, o-methoxyphenol to dimers and trimers, and phenol to dimers, trimers and tetramers. Fungi producing such enzymes are active in the formation of humic compounds in soil (Atlas and Bartha, 1981; Bollag, 1979). It is possible, therefore, that

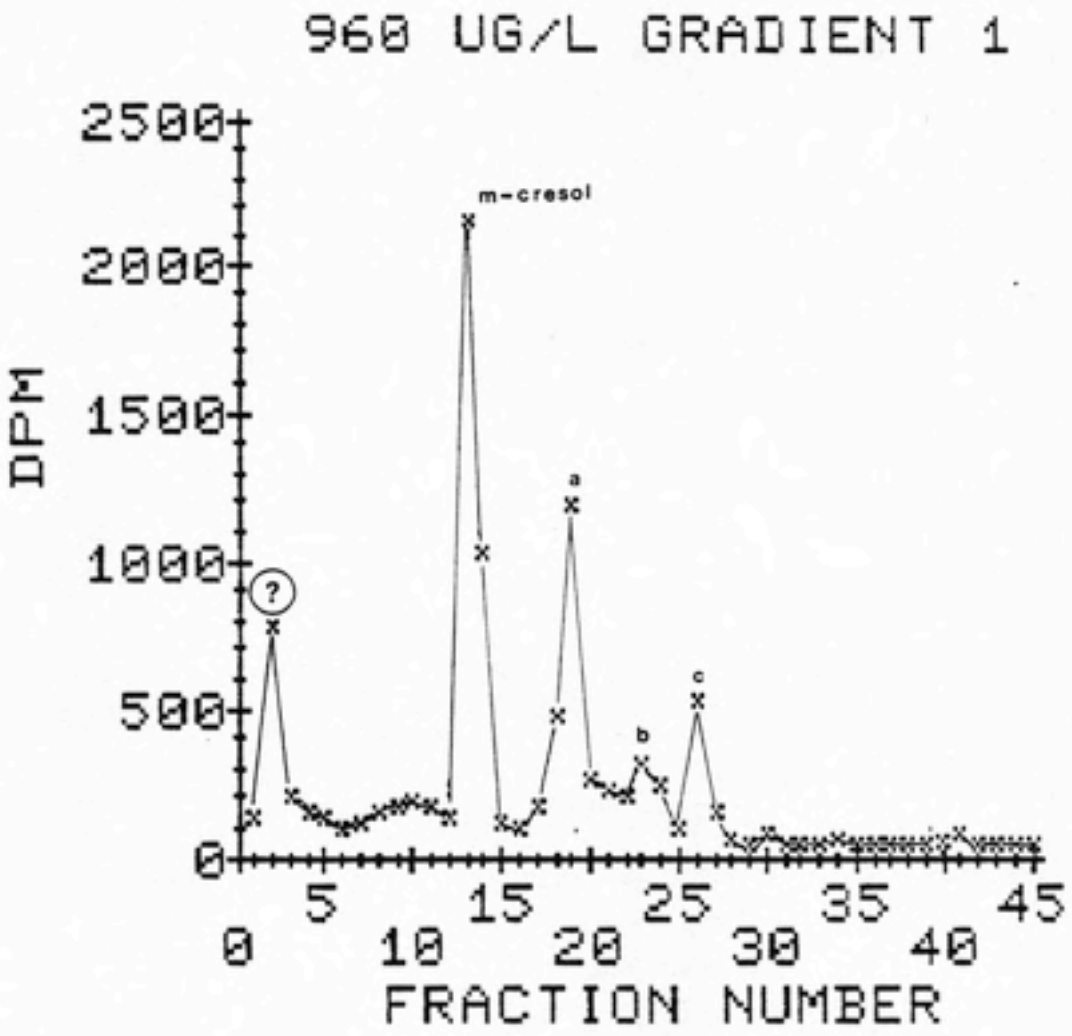


Figure 10: HPLC Fractionation of One Sample Filtrate under Extended Gradient Elution Conditions (see text). September, 1984.

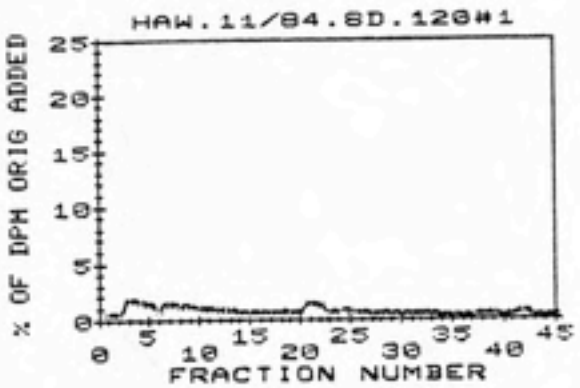
recent rains caused such a fungus to be carried with soil runoff into the river. This possibility is further supported by the fact that peaks a, b, and c were not observed in any subsequent or previous experiments. Thus, their production may reflect a change in the microbial populations present.

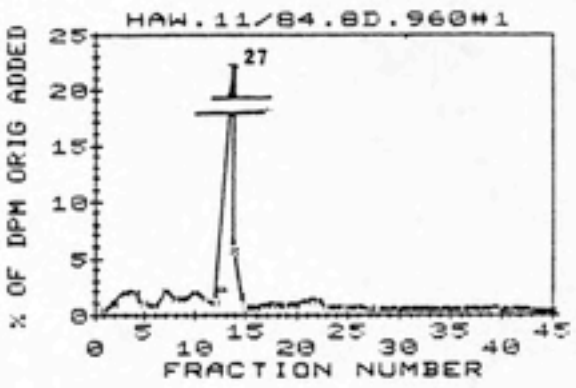
One peak appeared to elute about 55 minutes after an injection, leading to an extraneous peak in the following chromatogram (see peak with "?" in Figure 10). To correct this problem and to accomodate the possibility of peaks eluting after m-cresol, the gradients in all subsequent experiments were 45 minutes, followed by a 15 minute wash with acetonitrile, and 1 minute fractions were collected. In all other respects, the gradients were identical.

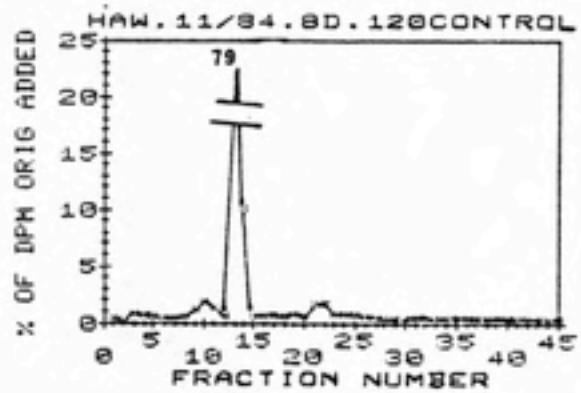
A second metabolite accumulation experiment was initiated in November. This experiment was a repeat of the September metabolite accumulation experiment. Fractionation results are shown in Figure 11 and Table 4. Clearly, these results are entirely different from those observed in the September metabolite accumulation study. Nearly all radioactivity was recovered with the expanded fractionation procedure, but aside from the m-cresol impurity which eluted in fractions 20 and 21, most radioactivity eluted before m-cresol (which elutes in fractions 13 and 14). Thus, the products which accumulate in this experiment may be metabolites in the degradation pathway of m-cresol. A more detailed discussion of their

Table 4: Recovery in Aqueous Filtrates of the Radioactive Label Originally Injected. November, 1984.

Concentration (ug/l)	Replicate No.	% Label Remaining	% m-Cresol Recovered	% Other Recovered
120	1	17	<1	15
120	2	16	<1	17
120	control	102	89	12
960	1	49	32	17
960	2	42	23	21
960	control	101	87	14







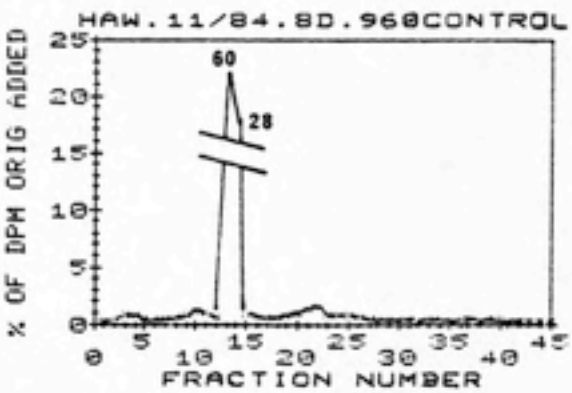


Figure 11: HPLC Fractionation of Sample Filtrates. November, 1984.

identity will follow later.

Concentration differences were also clearly apparent. In the 120 ug/l samples, only 17% of the radioactivity initially injected remained in the aqueous phase after eight days, and almost none of this was present as m-cresol. In contrast, about 45% of the initial radioactivity remained in the 960 ug/l samples, but about 60% of this was present as m-cresol. Curiously, the % metabolites of label initially added was about the same at both concentrations. This is in distinct contrast from the September experiment, when the label theoretically present as metabolites at 960 ug/l was twice that at 120 ug/l. Given that the type of metabolites which accumulate in the two experiments are so different, the factors which govern their formation and release are bound to differ. Different enzyme systems, and possibly, different organisms are involved. Therefore, they would not be expected to respond to concentration in the same way.

Given the conflicting results of the September and November experiments, a third metabolite accumulation experiment was initiated in January, 1985. The objectives of this experiment were twofold: (1) to confirm the results of one, or both, of the previous experiments, and (2) to follow cresol disappearance and metabolite accumulation in the filtrate at several different periods. The experiment was similar to the two previous studies, except that duplicate sets of samples were incubated for 0,

3, 8 and 16 days. Sample uptake and respiration were also measured.

Time study results are shown in Table 5. m-Cresol disappears more quickly from the aqueous phase than the radioactive label. Moreover, as the incubation time increases, the recovered metabolites account for an increasing proportion of the radioactive label remaining in the aqueous phase (Figure 12). These results confirm those observed in the cresol disappearance experiment and demonstrate that metabolite accumulation does occur with longer incubations.

Metabolite recovery and fractionation results at day 8 are nearly identical to those observed in the November metabolite accumulation experiment (Table 5, Figure 13-14). A similar fractionation pattern is observed at each time and concentration. As the percent of label in the form of metabolites increases, the radioactivity in most fractions eluting before m-cresol also increases. The increase occurs after and including fraction 7, where aromatic metabolites would elute, and before fraction 7, when nonaromatic metabolites would elute. Thus, both aromatic metabolites and other products may accumulate.

No one fraction accounts for a significant percentage (greater than 5%) of the label initially added, but small radioactive peaks do consistantly appear in fraction 7 and fractions 3-5. Fraction 7 may be tentatively identified as protocatechuic acid on the basis of coelution (Table 1).

Table 5: Recovery in Aqueous Filtrates of the Radioactive Label Originally Injected after Different Periods of Incubation. January, 1985.

Incubation Time (days)		120 UG/L SAMPLES % Label Remaining	% m-Cresol Recovered	% Other Recovered	960 UG/L SAMPLES % Label Remaining	% m-Cresol Recovered	% Other Recovered
0	(20 min)*	91	82	9	85	76	8
3	sample*	33	14	15	69	53	13
	control	109	97	9	104	95	8
8	sample*	22	3	15	54	36	18
	control	101	86	11	102	89	10
16	sample*	21	1	19	21	1	18
	control	103	91	12	100	88	11

* average of 2 sample replicates

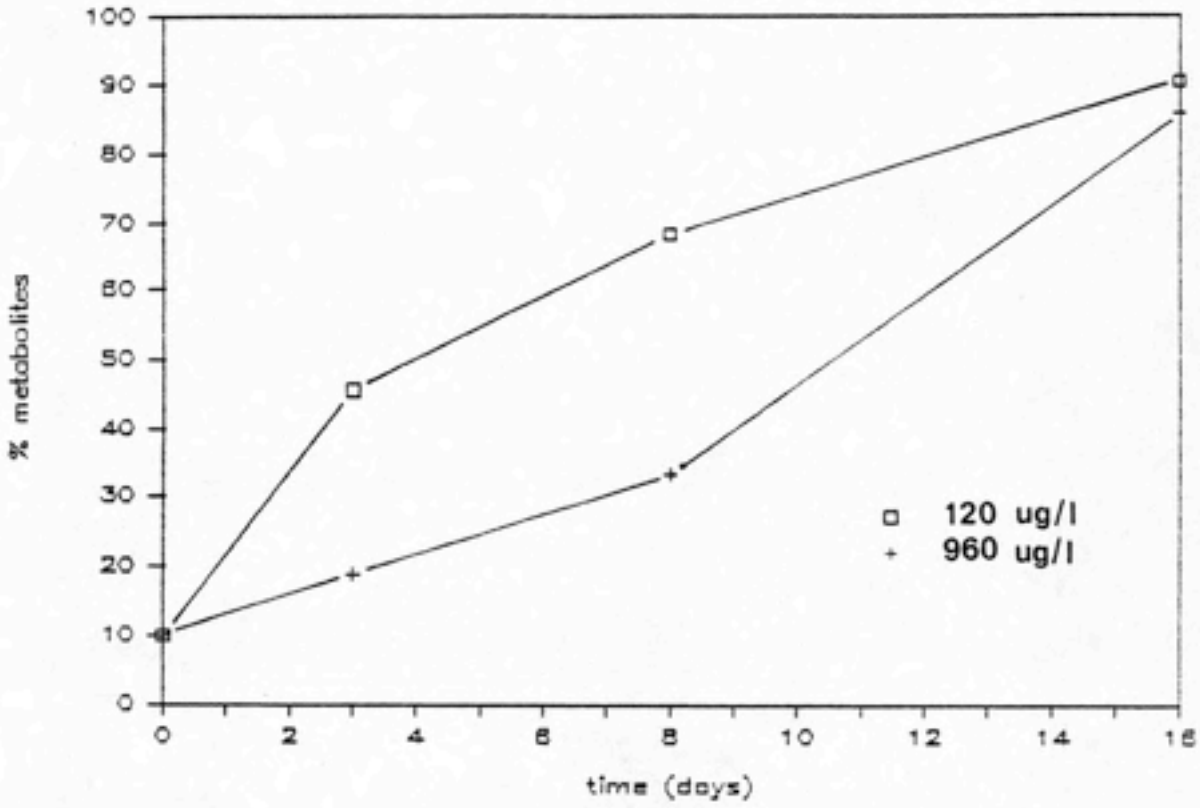


Figure 12: Proportion of Radioactive Label Remaining in the Sample Filtrates Which is Recovered as Metabolites vs. Incubation Time. January, 1985.

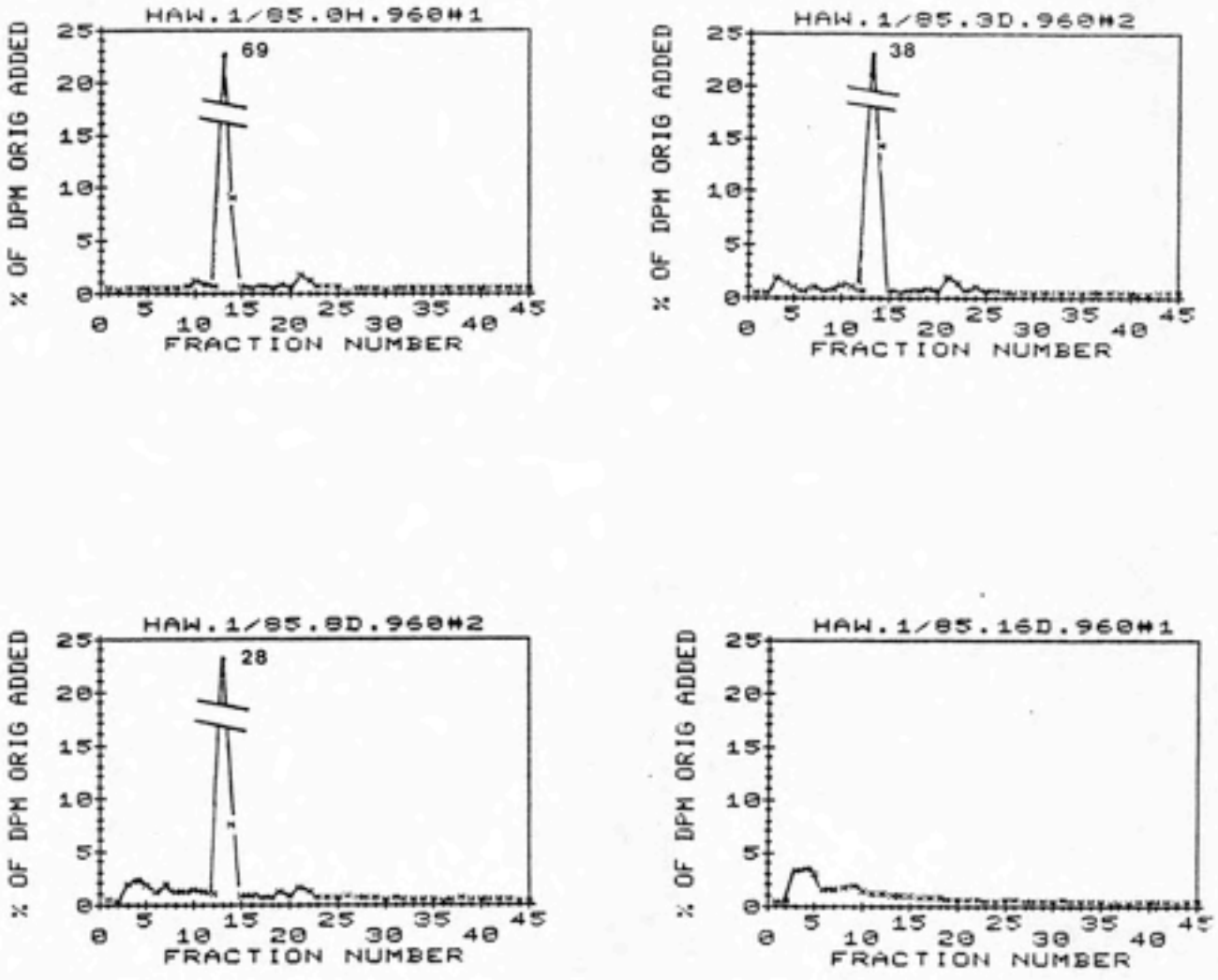


Figure 13: HPLC Fractionations of 960 ug/l Sample Filtrates at 0 hours, 3 days, 8 days and 16 days. January, 1985.

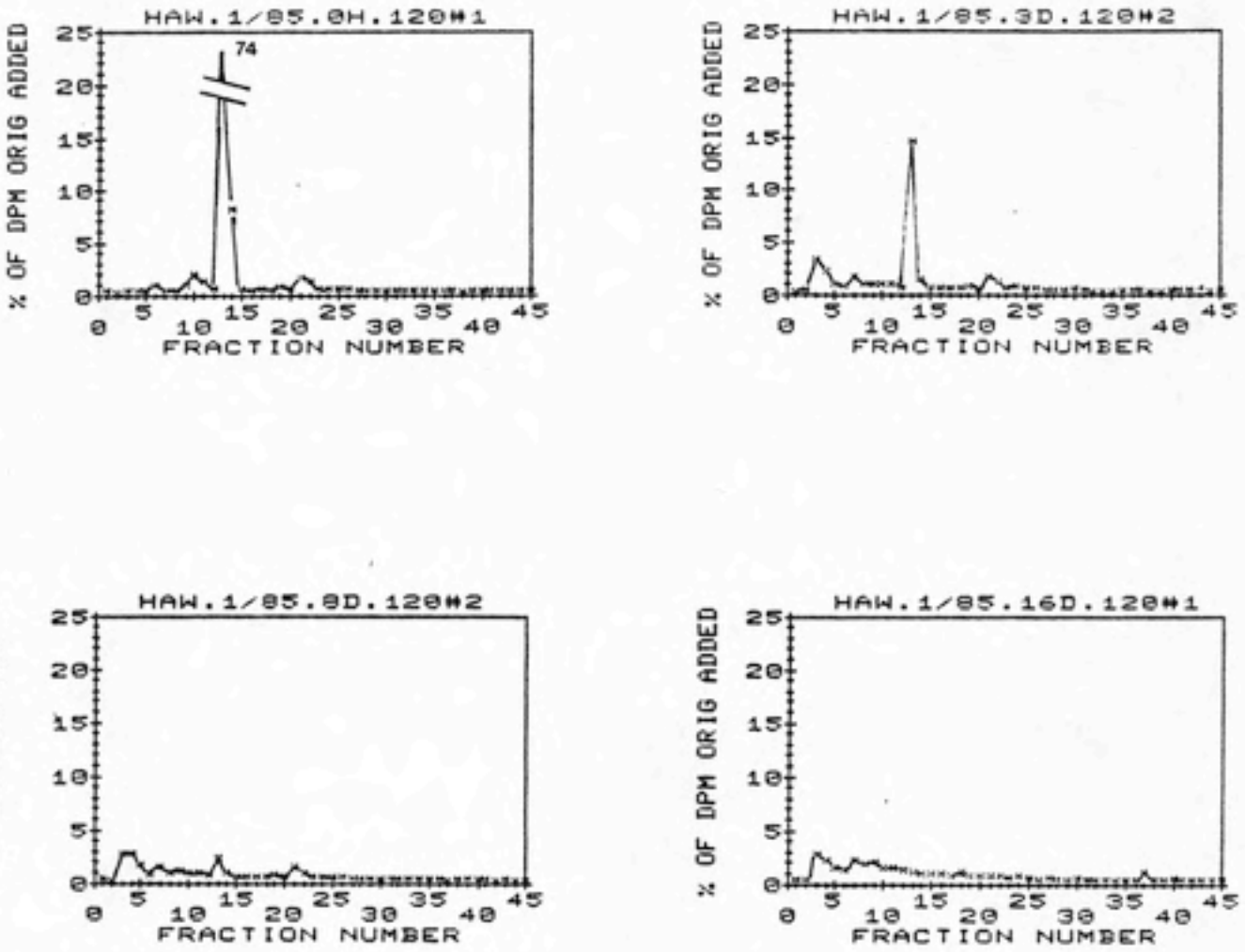


Figure 14: HPLC Fractionations of 120 ug/l Sample Filtrates at 0 hours, 3 days, 8 days and 16 days. January, 1985.

This compound has been known to accumulate in culture fluids during studies with pure cultures (Dagley, 1971). More rigorous methods of identification were not pursued, however, because the fraction typically contains less than 2% of the label initially added.

The radioactivity in fractions 3-5 together ranges from about 4 to 9 percent of the label initially added, though they typically contain about 6 percent. Compounds eluting in these fractions are polar and poorly retained by the nonpolar C_{18} column. A major consequence of this is that the compounds will also be poorly separated on the column. Thus, the higher radioactivity in these fractions could easily be due to the coelution of a number of different compounds. Indeed, when smaller fractions of a 960 ug/l sample were collected, the radioactive peak in fractions 3-5 was separated into two smaller peaks (Figure 15). Even if each of the two smaller peaks were to represent a single compound, they would each only account for about 3% of the label initially added. A rigorous attempt to identify compounds present in such small amounts was outside the scope of this project.

The only information gleaned from the chromatograms is that the compounds are polar and probably nonaromatic. Thus, fractions 3-5 may contain both nonaromatic intermediates and end products of the m-cresol degradation pathways. Products formed from m-cresol, such as intermediates of oxidative metabolism, may also accumulate

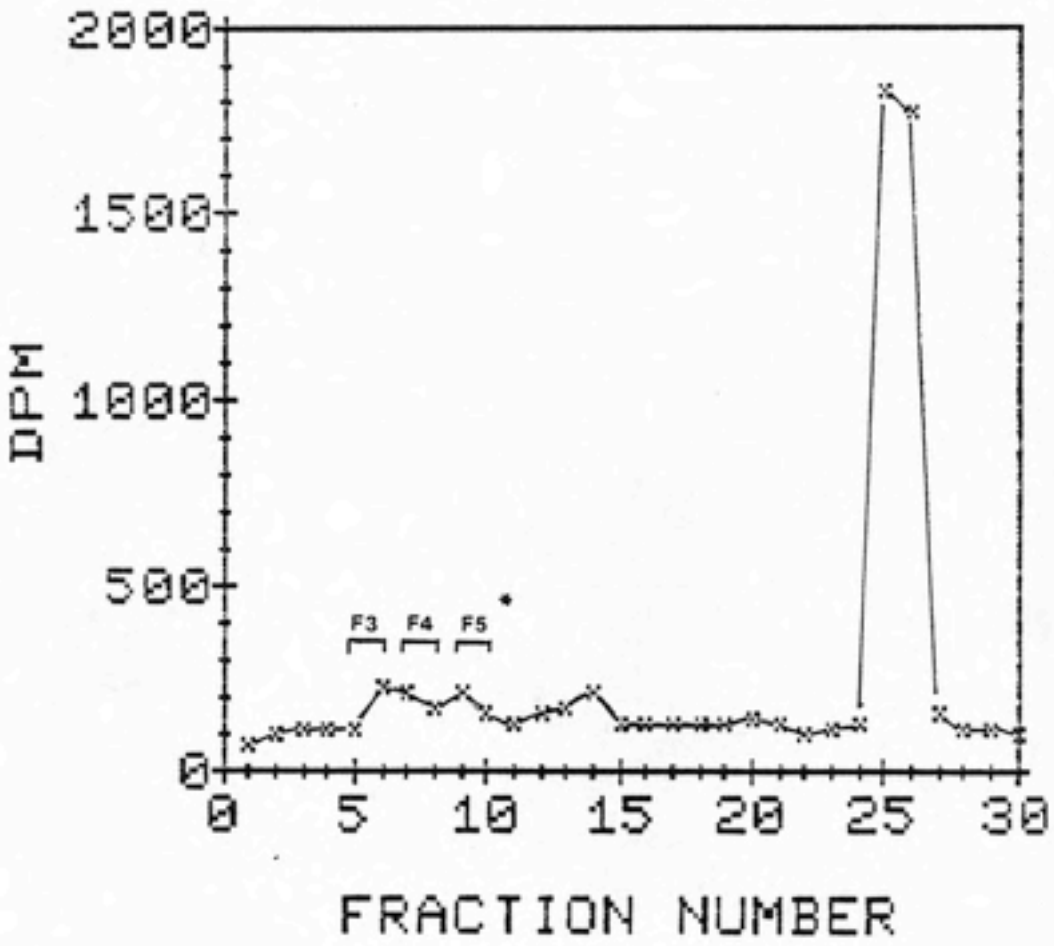


Figure 15: HPLC Fractionation when Collect 1 ml Fractions instead of 2 ml Fractions. 960 ug/l Sample Filtrate after 8 Days of Incubation.

* 2 ml fractions which elute forming one peak, rather than two

and elute in these fractions (Neijssel and Tempest, 1979). Pure culture studies suggest that the nonaromatic intermediates of m-cresol degradation are unlikely to accumulate. Most of these compounds are chemically unstable in aqueous solution and are unable to permeate the membrane of intact cells (Dagley, 1975; Stanier and Ornston, 1973). Which products are actually present cannot be distinguished by these methods. The fact that they are labeled, however, clearly shows that they arose from m-cresol.

Concentration does not appear to influence which metabolites accumulate because the fractionation patterns are similar at both concentrations. Concentration differences are apparent, however, in the quantity of metabolites accumulate. The % metabolites of the label originally injected is similar at both concentrations (Table 5). In 120 ug/l samples, it ranges from 15 to 19 %, and in the 960 ug/l samples, it ranges from 13 to 18 %. But the ratio of ^{12}C to ^{14}C differs at the two concentrations, so metabolite accumulation in the 960 samples is roughly seven times that at 120 ug/l. Degradation rates were also higher in the 960 samples (Appendix 1). That the degradation rates of m-cresol and other phenols may increase with an increase in concentration has been shown in numerous studies (Boethling and Alexander, 1979; Pfaender and Bartholomew, 1982; Rubin et al., 1982). But why more metabolites would accumulate

at the higher concentration is less clear.

One possibility is that the rate of m-cresol degradation exceeds the capacity of the cell to use pathway end products to form cellular constituents or to produce energy. For example, in studies with pure cultures, Neijssel and Tempest (1979) found that the overproduction of metabolites can occur in response to non-carbon nutrient limitation. They hypothesized that microorganisms "will overproduce metabolites only when forced by particular environmetnal conditions to do so, and that they do this in order to resolve physiological problems." They suggested that one factor important for metabolite overproduction is that there is no extensive regulation of the carbon source. This is known to be true for certain organisms which degrade m-cresol. In two Pseudomonas putida isolates, for example, m-cresol induces simultaneously all enzymes of the meta-cleavage pathway (Bayly and Barbour, 1984). In this case, the degradation rate would be controlled by the concentration of m-cresol, rather than an end product or intermediate. Thus, even if the cell were unable to use the metabolites, they would continue to be produced. This may explain why more metabolites would accumulate at the higher m-cresol concentration.

Another possiblity is that certain organisms in the sample are cometabolizing m-cresol. In this case, partial degradation products of m-cresol may accumulate (Alexander, 1980; Horvath, 1972). Such products may subsequently be

cometabolized, mineralized, or left unaltered by other microorganisms. This would explain why so many different products accumulate and in relatively small amounts.

One hypothesis used to explain cometablism is related to enzyme specificity (Alexander, 1980; Horvath, 1972). Enzymes falling earlier in a metabolic sequence may have less stringent substrate specificities than an enzyme occurring later in the sequence. Hence, a compound degraded by the former enzymes may produce an unsuitable substrate for the latter enzyme. This may lead to the accumulation of the partially degraded substrate (Alexander, 1980; Horvath, 1972). Because only a few steps in the metabolic sequence may be involved, the usual mechanisms for pathway regulation may not function. Thus, the nonspecific enzymes may continue to degrade m-cresol at a rate proportional to its concentration, and consequently, more product(s) would accumulate at the higher concentration.

Similar results were observed in a study with lake water by Wang, et al.. At a concentration of 1 ug/ml, isopropyl N-phenylcarbamate (IPC) was converted only to organic products, presumably by cometabolism. At 400 pg/ml, IPC was mineralized and converted to organic products, but fewer organic products were produced at the lower concentration. This suggests that cometabolic activity is influenced by concentration. An important difference between the two studies, however, is that in the

Wang study, organic products inside and outside microbial cells were measured, whereas in the metabolite accumulation experiments, only products which accumulate outside the cell were measured.

In this context, it is interesting to note that the fractionation pattern remains similar throughout the time course experiment. The unchanging fractionation pattern could reflect the actual pattern of compounds released. This pattern may remain the same despite all the changes occurring in the bottle. Another possibility is that microbial or abiotic removal mechanisms obscure changes in the quantity and quality of metabolites released. Some compounds, for example, may volatilize into the sample headspace. In other cases, compounds excreted by one population may be taken up by other microbial populations within the community. Inducible uptake systems for certain end products, C_4-dicarboxylic acids, and other intermediates have been demonstrated (Ghei and Kay, 1972; Kay and Kornberg, 1970; Ornston and Parke, 1976).

It is difficult to extrapolate the results of the metabolite accumulation experiments to the natural environment or other experimental methods. The experimental system is complex and poorly defined. As the sample incubates, the characteristics of the microbial community change in response to sample confinement in a bottle. Ferguson et al. (1984) found that confinement of a natural community of marine bacterioplankton caused

increases in the number of culturable bacteria, the total number of cells, the average cell volume, and the metabolic activity. Changes in the species composition of the microbial community were also observed. In this laboratory, similar bottle effects were observed previously in two m-cresol time-course studies. Bottles with and without m-cresol showed similar responses to confinement, though numbers of culturable bacteria increased more dramatically in bottles receiving m-cresol. Due to such bottle effects, the environmental conditions causing metabolite accumulation within the bottle are largely unknown. The results only show what can happen, not necessarily what would happen, in the natural environment.

Neither can the mechanisms by which the metabolites accumulate be determined. Only label which accumulates is measured, but accumulation itself will depend upon the rates of compound release and the rates of compound disappearance. Compound release may occur by excretion or cell lysis, and compound disappearance may occur by microbial uptake or abiotic mechanisms.

Despite their limitations, the metabolite accumulation experiments have demonstrated that metabolite accumulation does occur with m-cresol, but after long-term incubations which are characteristic of die-away studies. The nature of the metabolite accumulation can vary dramatically, and because sample replicates within an experiment typically

correlate closely, this variation may reflect differences in the initial environmental samples. The extent of metabolite accumulation appears to vary with the length of incubation, the sample of water and the concentration of m-cresol. Moreover, no one metabolite accumulates consistantly to significant levels.

These results have several implications for biodegradation studies with radiolabeled cresol. First, with the longer incubations of die-away studies, the extent of degradation may be underestimated when only mineralization and uptake are measured. Second, no specific metabolite which accumulates in the aqueous phase appears to be appropriate as a third measure of biodegradation. And third, the monitoring of radioactivity in the unfractionated aqueous phase is only useful as part of an effort to make a mass-balance of the label, rather than as an indication of the concentration of m-cresol remaining.

CONCLUSIONS

1. The accumulation of metabolites can occur in the aqueous phase during studies of m-cresol biodegradation with natural water samples.

2. Most metabolite accumulation occurs after several days of incubation. In heterotrophic activity studies, which involve short-term incubations (less than 24 h.), metabolite accumulation accounts for only a small fraction of the radiolabel originally added. Thus, this fate may be insignificant in these studies. After longer incubations, which are characteristic of many other methods used to study biodegradation, metabolite accumulation accounted for as much as 30 % of the label originally added. Thus, studies which only measure uptake and mineralization may underestimate the extent of m-cresol degradation.

3. The nature of metabolite accumulation varied, and appeared to reflect differences in the initial environmental samples. Sometimes no metabolite accumulation was detected. One time, a few products accumulated and they were less polar than m-cresol. At other times, many different products accumulated and they were more polar than m-cresol. No one metabolite accumulated consistantly to a level greater than 5% of the

radiolabel originally injected.

4. When metabolite accumulation occurred, it was greater in samples recieving a higher concentration of m-cresol.

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APPENDIX A

Degradation Rates - Metabolite Accumulation Experiment.
January, 1985.

conc. (ug/l)	time (d)	respiration rate (ug/l/h)	total uptake rate (ug/l/h)
120	3	0.59	0.83
120	8	0.21	0.24
120	16	0.05	0.08
960	3	2.25	2.89
960	8	0.93	1.16
960	16	0.24	0.41