## ABSTRACT

DAVID W. ROBINETTE, Jr. Biodegradation of Surfactant Chemicals in Estuarine and Near Shore Marine Environments of North Carolina. (Under the direction of Dr. Frederic K. Pfaender)

Estuaries are among the most diverse and productive ecosystems on earth. Estuarine and near shore marine environments receive nearly one-third of anthropogenic discharges to surface waters; however, very little is known about the fate of these chemicals in estuarine environments. The fate of cationic, anionic, and nonionic surfactants was studied in estuaries of coastal North Carolina. Radiolabeled chemicals were used to assess respiration, cellular uptake, and residual material in sewage impacted and unimpacted estuaries, and along the horizontal salinity gradient of the estuary. Respiration was the major fate of all compounds, with little incorporation into biomass. Adaptation of the microbial community through previous exposure to the chemicals appears important only for Linear Alkylbenzene Sulfonate; a chemical which also appears not to degrade significantly in marine waters. In some samples, metabolism resulted in a plateau in degradation extent instead of complete or near complete mineralization, which may be the result of formation of recalcitrant metabolic intermediates.

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# I. INTRODUCTION AND OBJECTIVES

The aquatic environment constitutes one of the major sinks for the discharge of anthropogenic chemicals, and consequently, pollution of aquatic habitats is of growing public concern. Within the United States, there are over 85 thousand miles of tidal shoreline which border estuarine environments. A large and growing segment of the population resides in cities along this shoreline, and millions of people use these areas for recreation. The estuarine environment not only receives pollutants from direct wastewater discharge, but also indirectly from discharges into upstream waters which eventually reach the estuary. The potential impact of wastewater constituents on the estuarine environment is enormous, especially when one considers the significant commercial and recreational importance of estuarine and near shore ocean waters.

Estuaries are complex, highly variable ecosystems which represent transition zones between freshwater and marine environments. Estuaries typically exibit a horizontal gradient in salinity as freshwater mixes with and dilutes seawater. This gradient may range from zero to 35-37ppt from the freshwater to oceanic portion of the estuary, and may also vary vertically by several ppt (Barnes, 1984; Ketchum, 1983 ). These salinity variations, as well as sediment and circulation patterns often result in estuaries acting through precipitation and sedimentation, to remove and retain nutrients and other materials (Knox,1986; Schubel and Kennedy, 1984; Hobbie et al., 1975). These physical and chemical properties of estuaries contribute to pollution problems, as estuaries may accumulate anthropogenic contaminants such as petroleum products , pesticides and other agricultural products, detergent materials, and thousands of other organic chemicals which may be discharged each year (Schubel and Kennedy, 1984; Hobbie et al., 1975; Larson

and Wentler, 1982).

The pollution of estuarine and near shore waters in the U.S. may have serious commercial implications, as it has been estimated that nearly two-thirds of all fish and shellfish taken from waters off the U.S. coast are species which are dependent upon estuaries (Knox, 1986; Alexander, 1981). In addition, estuarine environments and their adjacent salt marshes offer an enormous diversity in plant and animal life, and provide many recreational opportunities, all of which are vulnerable to human activity (Knox, 1986).

Of the thousands of synthetic organic chemicals discharged into the aquatic environment each year, a number are used and disposed of in such large quantities that their presence in receiving waters is of concern when assessing their environmental safety. Synthetic detergent chemicals are one such group of highly used, commercially important chemicals. The detergent chemicals of interest from an environmental perspective are the surfactants, which generally compose from 10-30% of modern detergent formulations (Swisher, 1987). Discharge of surfactants into estuarine environments is typically preceded by wastewater treatment, and concentrations in receiving waters usually fall into the ppb range, depending on such factors as the effectiveness of wastewater treatment, level of dilution by the receiving water, and human population density (Swisher, 1987; Larson et al., 1983).

In order to properly assess the potential impact on estuarine ecosystems associated with the discharge of consumer chemicals such as surfactants, more needs to be known about the fate of these chemicals and their transport in the environment. A number of chemical and biological transformation mechanisms may interact to determine the fate of a chemical in the aquatic environment. However, chemical transformation mechanisms rarely result in the complete conversion of an organic chemical to its inorganic components. It is generally recognized that in

aquatic environments, complete conversion of a compound to its inorganic components is almost always the result of degradation by indigenous microbial populations (Larson and Wentler, 1982; Alexander, 1981; 1983). Microbial biodegradation processes may lead to small changes in the parent structure so that characteristic chemical or physical properties are lost (primary biodegradation), or the parent structure may be completely broken down to $CO_2$ (Ultimate biodegration or mineralization). With regard to detergent chemicals in particular, biodegradation processes are thought to be the most important means of their transformation in both wastewater treatment and aquatic environments (Larson and Wentler, 1982; Larson et al., 1983; Cain, 1976).

Relatively little information is available on the biodegradation of anthropogenic chemicals in estuarine environments, as only a few studies have addressed this area. Estuarine biodegradation of consumer chemicals such as surfactants and related compounds has received even less attention than other pollutants (Larson et al. 1983; Palumbo et al. 1988; Pfaender et al. 1985; Larson and Wentler, 1982; Pritchard et al. 1987; Shimp, 1989; Walker et al. 1984 and 1988.)

The overall objective of this study is to determine the extent to which a number of common consumer chemicals can be degraded by microbial communities from estuarine and near shore ocean waters, and how characteristics of the environment influence these transformations. The following specific approaches were used to achieve this objective:

-- Assess the biodegradation of compounds representing the three main classes of detergent chemicals (anionic, nonionic, and cationic surfactants) and several reference compounds, in North Carolina estuarine environments.

-- Evaluate the importance of adaptation (necessity for prior exposure of

the microbial community to the chemical before appreciable degradation occurs) to the biodegradation of the surfactants.

-- Examine the effects of salinity variations on the biodegradation of surfactants by comparing biodegradation along the geographical salinity gradient from freshwater to marine environments.

# MATERIALS AND METHODS

## SAMPLE SITES

The sample sites used in this study are located near Morehead City, North Carolina. These sites were chosen because they are representative of typical southeastern United States estuaries. Since all of the sites are located in the same geographic region of North Carolina, they will be exposed to similar climatic and hydrological factors. Characteristics of each of the study sites are discussed below.

### Pollution Impacted and Unimpacted Sites

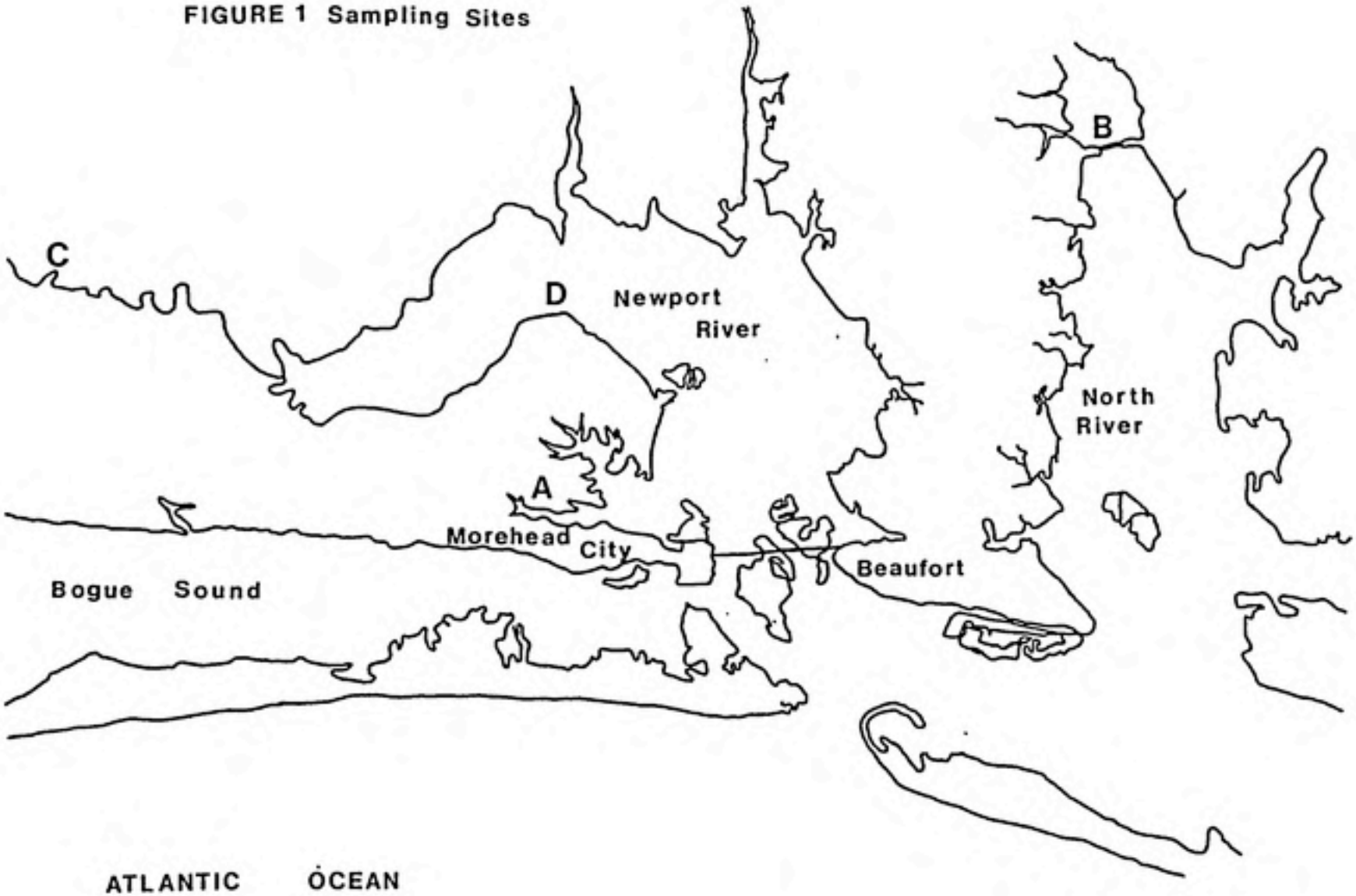
Calico Creek represents a domestic wastewater impacted site (Fig. 1-A). The sample site is located approximately a half mile downstream from the Morehead City wastewater treatment plant. This plant is a 1 MGD(million gallons per day) trickling filter secondary treatment facility that receives mostly domestic wastewater. At low tide the flow of the creek is approximately 70% wastewater effluent.

The site not impacted by wastewater is located on the North River Estuary (Fig. 1-B). This site was chosen because it has no treatment plants that discharge into it, and it has the fewest inputs of anthropogenic pollutants in the area. The water here is open to shellfishing, and levels of coliform bacteria (an indicator of potential fecal contamination) are usually within regulatory limits.

### Salinity Gradient Sites

Salinity gradient experiments were carried out in the Newport River Estuary system. These sites were used to document the effect on biodegradation of the transition from freshwater to seawater. The totally freshwater site is located near the town of Newport (Fig. 1-C). Salinity values for this part of the Newport River

FIGURE 1 Sampling Sites



are typically at or near zero parts per thousand.

Penn Point (Fig. 1-D ) is a typical mid-estuary site with salinity values ranging from 10 to >30 ppt, depending on factors such as river flow and tide.

Marine waters used in this study were collected from a site beyond Beaufort Inlet approximately 3 miles offshore (Fig. 1-E). Salinity of waters from this site was typically near 35 ppt.

## SAMPLE COLLECTION AND HANDLING

Water samples were collected in a site water washed galvanized bucket and transported in site water washed 20 liter polyethylene containers. Sediment samples were collected in the same way and transported in previously autoclaved mason jars. Samples were returned to the laboratory within 5 hours of collection and processed on the same day when possible. When same day processing was not possible, samples were stored not longer than overnight at 4 degrees C, and processed on the following day.

## BIODEGRADATION METHODOLOGY

The basic procedure followed was a modification of that developed by the Procter and Gamble Co. Environmental Safety Dept., as specified in their Standard Test Method for Biodegradation. Radiolabeled surfactants and reference chemicals (see Table 1) were provided by Procter and Gamble and were used to quantify the percentage of chemical metabolized to $^{14}CO_2$, the amount incorporated into cell biomass, and the amount of radiolabel remaining in solution.

Site water was mixed by pooling into a 10 gal. carbouy and then distributed into modified Gledhill type flasks (Fig. 2), each receiving one liter of water and 25 ug/liter of the appropriate radiolabeled substrate. In cases where sediment was to

TABLE 1. Characteristics of the chemicals used in the study

| Chemical | Label Position | Specific Activity | Purity | Source |
|---|---|---|---|---|
| LAS | [U-ring]$^{14}$C | 23.6mCi/mmol | ND | P&G |
| C18-TMAC | $^{14}$C | 12.5uCi/mg | ND | P&G |
| C12-TMAC | $^{14}$C | 95.0uCi/mg | ND | P&G |
| LAE | U-ethox.$^{14}$C | 13.4uCi/mg | ND | P&G |
| Phenol | [U-ring]$^{14}$C | 11.4mCi/mmol | >99% | Sigma |
| Stearic acid | $^{14}$C | 111 dpm/ng | ND | P&G |
| Cellulose | U-$^{14}$C | 3.4uCi/mg | >99% | ICN |
| Thymidine | $^{3}$H[methyl] | 87 Ci/mmol | 99.3% | Amer. |
| Amino Acids | $^{3}$H-mixture | 0.1695mg/mCi | ND | NEN |

ND-No Data Available P&G-Procter & Gamble Co. Cincinatti,OH

LAS- LINEAR ALKYLBENZENE SULPHONATE
TMAC- TRIMETHYL AMMONIUM COMPOUND
LAE- LINEAR ALCOHOL ETHOXYLATE

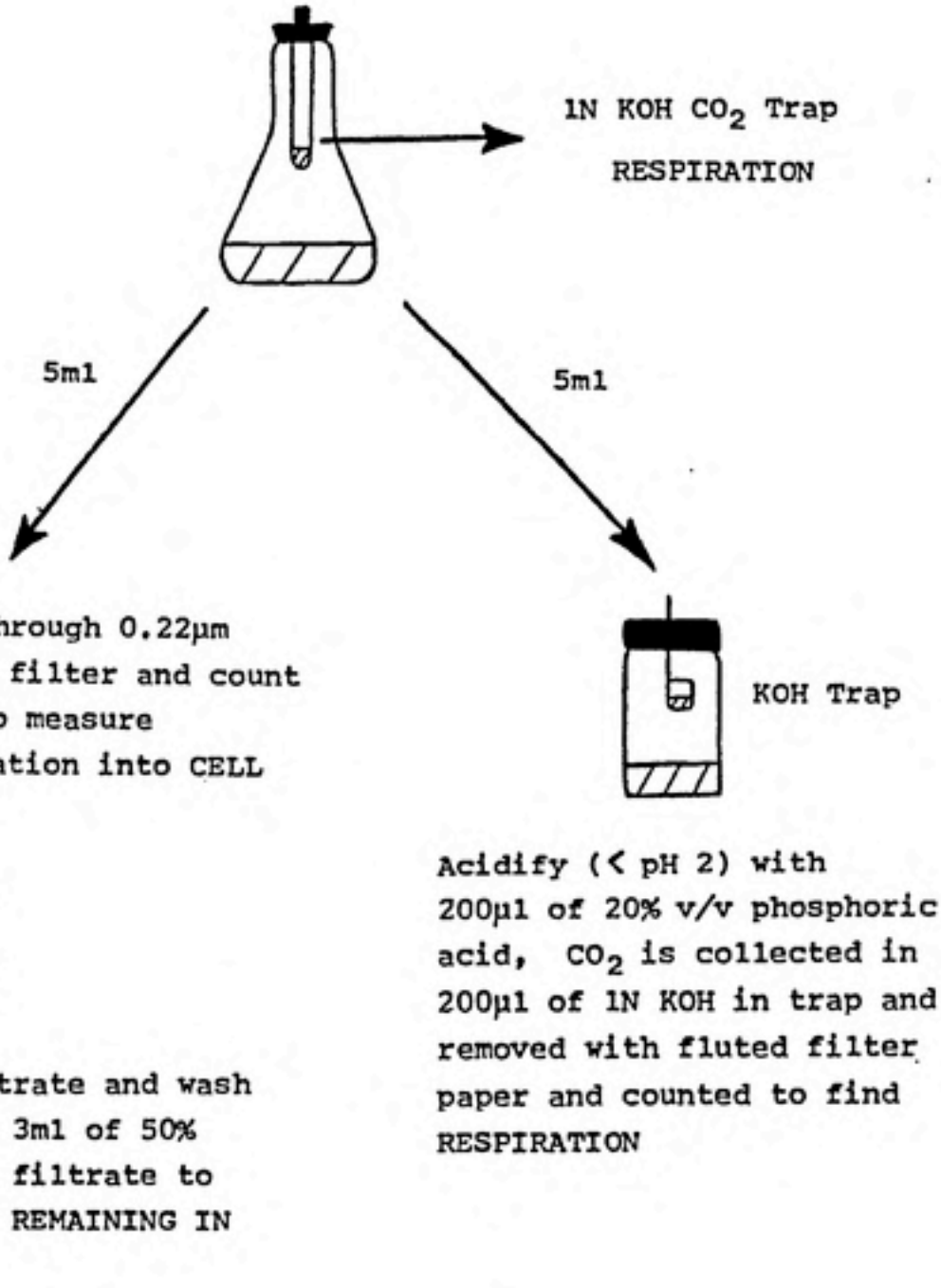



FIGURE 2. Schematic of Biodegradation Methodology

be incorporated into the experiment, one g/liter (100$^0$C air dry weight) of site sediment was also added to each sample flask. All incubations had three live replicates and one control killed with 1ml of 36% formaldehyde in order to assess abiotic metabolism of the compounds. Sample flasks were mixed by swirling at least five times per week.

Incorporation of the compounds into cell biomass was measured by filtering a 5ml aliquot of sample water through a 0.22 micron membrane filter (Gelman Sciences,Inc. Ann Arbor,Mi.) and counting the filter in 10ml of scintillation cocktail (ScintiVerse II, Fisher Scientific Co.) for $^{14}C$ activity in a Packard model 300CD liquid scintillation counter with internal standards for counting efficiency.

Respiration of the compounds to $CO_2$ was determined by counting $^{14}CO_2$ trapped in 1N KOH suspended in traps within the sample flasks. The KOH was removed from the traps and placed in 10ml of scintillation cocktail and counted as before.

The amount of $^{14}CO_2$ remaining in solution was determined by acidifying a 5ml aliquot of sample water to pH <2 with 0.2ml of 20% v/v phosphoric acid and collecting the $^{14}CO_2$ driven off in 0.2ml of 1N KOH in suspended traps as before. The KOH was recovered from the traps by absorbing it on fluted filter paper (Whatman #1 ) which was then placed in 10ml of scintillation cocktail and analyzed for $^{14}C$ activity.

To calculate a mass balance of added label, the amount of chemical remaining was determined by counting the filtrate from the sample flask for $^{14}C$ activity after accounting for incorporation into biomass.

These parameters were normally measured at time 0(20 minutes after addition of substrate), 1 day, 3 days, 5 days, 7 days, and approximately weekly to biweekly thereafter for the remainder of experiments.

## SUPPORTING MEASUREMENTS

Since estuarine environments are often quite variable both temporally and spatially with regards to physical, chemical, and biological characteristics, it is necessary to define this variability in order to permit comparisons between data from different estuaries, or the same site over time. A number of parameters are useful for assessing estuarine variability, and those used in this study are shown in Table 2, along with a description of the methods used to collect the data.

Temperature, salinity, and dissolved oxygen data were determined in the field as samples were collected. The remaining supplementary measurements were obtained when the samples were returned to the laboratory before the biodegradation experiments were set up.

### $^{3}$H Amino Acid Uptake Method

Amino acid turnover time is an indication of the heterotrophic activity of a community of microorganisms. The turnover time is the time required by the community to take up an amount of substrate equal to the ambient concentration. The amino acid turnover time calculated in this study is after that of Gocke (1977).

Site water samples were distributed into four 20ml subsamples, one of which was killed with 0.1ml of 37% formaldehyde. Approximately 20000 dpm of $^{3}$H-L amino acid mixture(specific activity 1mCi/0.1695mg; New England Nuclear, Boston, MA.) were then added to each vial, which was then capped, mixed by inverting, and incubated at room temperature on a rotary shaker for exactly 30 minutes. At the end of the incubation, the reaction was stopped by adding 0.1ml of 37% formaldehyde and each replicate was then filtered through a 0.22um membrane filter. The filter was then placed in 10ml of scintillation cocktail and analyzed for $^{3}$H activity. Amino acid turnover time ($T_t$) was calculated as follows:

| CHARACTERISTIC | PARAMETER | METHOD |
|---|---|---|
| Temperature | °C | Thermometer |
| Salinity | ppt NaCl | Refractometer |
| Oxygen | dissolved $O_2$ | DO meter |
| Community Activity | amino acid turnover time | $^3$H-amino acid turnover |
| | growth rate | $^3$H-thymidine uptake |
| Total Microbial Cells | Acridine Orange Direct Counts | Hobbie et al., 1977 |
| Sewage Pollution | Coliform bacteria | Colilert assay |

TABLE 2. Environmental Supporting Measurements

$T_t$ = incubation time(hr)/dpm counted/dpm added

**$^3$H Thymidine Uptake Method**

The $^3$H Thymidine uptake measurement gives an estimate of the relative growth rate of heterotrophic aquatic microorganisms. The method used in this study was adapted from that of Fuhrman and Azam (1980) and Riemann et al. (1982). The following is an outline of the method used:

1.) Site water samples are divided into 10ml subsamples, one of which is killed with 0.1ml 37% formaldehyde. Add $^3$H-[methyl]thymidine (specific activity 87 Ci/mmol; Amersham, Inc.) to a final concentration of approx. 5nM, and incubate at room temperature on a rotary shaker for 30 min.

2.) At end of incubation, immerse samples in ice water for 5 min.

3.) Add equal volume of ice cold 10% trichloroacetic acid (TCA) to precipitate insoluble material.

4.) Filter through 0.22um membrane filter and rinse filter with 5 X 1ml of ice cold 5% TCA and place filter in 10ml of scintillation cocktail.

5.) Count $^3$H activity in liquid scintillation counter.

The number of moles of thymidine incorporated into new cells is calculated as follows:

$$\#\ \text{moles incorporated} = \text{dpm}\ (SA)^{-1}(4.5 \times 10^{-13})$$

where dpm= dpm's on filter

SA= specific activity in Ci/mol

$.5 \times 10^{-13}$= # Ci/dpm

**Coliform Bacteria Enumeration by the Colilert System**

Numbers of total coliform bacteria were determined using the Colilert system (Access Analytical Systems, Branford,CT) and comparing to tables of Most Probable Number of total coliforms in Standard Methods for the Examination of Water and Wastewater.

Colilert is a reagent formulation of salts, nitrogen, and carbon sources specific only to total coliforms. As specific indicator nutrients are metabolized by total coliforms and E. coli, yellow color and flourescence are released confirming the presence of total coliforms and E. coli respectively.

**Enumeration of Bacteria by Acridine Orange Direct Counts**

The method followed was taken after that described by Hobbie et al.(1977). Samples were held in 20ml scintillation vials which had been rinsed with particle free water (filtered through 0.22um Nuclepore filters 3x) and dried at 200 °C. Particle free 37% formaldehyde was then added to a final concentration of approximately 2%.

A filter flask assembly fitted with tower and base which have been soaked in 75% ethanol and flamed followed by two rinses with particle free water is assembled with a 25mm 0.22um Nuclepore filter. The filter are presoaked at least 45 min. in Iragalan Black solution (2gm Iragalan Black dye/l 2% acetic acid). One-tenth of the sqmple volume of Acridine Orange is added to the tower with a sterile serological pipet, followed by 1 to 5 ml of sample also added with a sterile serological pipet. The sample is stained for 2-4 minutes , at which time vacuum is applied to filter the sample. Filter is placed on a clean microscope slide and topped with 1 drop of Cargille type A immersion oil. A coverslip is placed atop the filter and examined under 1250x oil immersion magnification. Bacterial cells will

flouresce green, yellow, or red and cell shapes are not distorted. Four fields are counted and averaged. Number of cells/ml is determined by the following formula:

cells/ml= (C)(F)(1.055)/(V)(A) where

C= mean # counts/ grid field

F= area of filter(201.66mm$^2$ for 25mm filter

V= sample vol.(ml)

A= area of grid field (0.00308mm$^2$)

## CALCULATION OF FIRST ORDER RATE CONSTANTS

Determination of the average rate of degradation was done by estimating the first order rate constants following the method outlined by Larson (1984). Biodegradation data were fitted to the following empirical first order model:

$y= A(1-\exp^{-K1t})$ where

y= % $^{14}CO_2$ at time t(days)

A= extent of degradation (%)

K1= first order rate constant

The rate constants in this study were estimated directly by nonlinear regression analysis using the data for counts obtained as $^{14}CO_2$ representing mineralization of the compounds.

## $^{14}$C MOST PROBABLE NUMBER METHOD FOR DETERMINING NUMBER OF DEGRADERS

Determination of the number of LAS degraders present in a sample followed the procedure of Lehmicke et al.(1979). The method estimates the number of degraders of a radiolabeled chemical by scoring for $^{14}CO_2$ production from a dilution series.

Five replicates of successive 10-fold dilutions were prepared for each dilution level ($10^0$-$10^4$). Abiotic controls were used as blanks for scoring replicates as + or - . For each replicate set, 2ml of dilution sample was pipetted into each of 5 4ml sterile mini vials (Fisher Sci.), and then radiolabeled substrate was added to each vial to give a final activity of 4500dpm. The mini vials were placed uncapped into 20 ml scintillation vials containing 1ml of 1N KOH to trap $CO_2$. The scintillation vials were capped and incubated at 22°C for 75 days, at which time 150 ul of 20% $H_3PO_4$ was added to each inner vial to drive off $CO_2$ in solution. The scintillation vials were recapped and incubated overnight on a rotary shaker. At this time, the inner vials were discarded, and 10ml of scintillation cocktail was added to the scintillation vials and counted. The dilutions were scored as + if the response was 3x that of controls. The MPN of degraders was determined from tables in Standard Methods for the Examination of Water and Wastewater.

## CHARACTERIZATION OF RESIDUAL RADIOLABEL

A number of experiments showed a plateau in $CO_2$ production while significant counts remained in solution. These results raised questions about the nature of the residual material; ie. was the material parent compound or products. Several experiments were conducted to address the metabolism of these residuals. $^3H$ amino acid turnover time and $^{14}C$ Most Probable Number of C12 LAS degraders were determined for the microbial communities remaining in the C12 LAS and Calico Creek samples of Oct. 1988 containing no sediment in order to determine if an active community of C12-LAS degraders was still present. The remaining volume in the LAS and LAE live sample flasks was pooled and distributed into 250ml glass bottles equipped with a KOH trap. Eight bottles for C12 LAS and twelve for C13-E6 LAE were set up in this way, each receiving approximately 200ml

of appropriate Calico Creek sample water. The C12 LAS samples were divided into two groups of four bottles each, with one bottle in each group serving as the abiotic control. One group was amended with unlabeled C12 LAS to restore the original total LAS concentration of 25mcg/liter in order to assess if the plateau was due to a concentration threshold being reached. The amount of LAS needed to reach this point was determined by estimating the amount of LAS remaining at the last time point of the degradation experiment. The other group of C12 LAS bottles received an addition of fresh Calico Creek microorganisms to see if a fresh pool of organisms would stimulate metabolism. The organisms were obtained by centrifuging fresh Calico Creek water at 5000 x g for 30 minutes and resuspending the pellet in 5ml of fresh Calico Creek water which had been filtered through 0.22 micron membrane filters. Each bottle received 1ml of the organism concentrate. The twelve C13-E6 LAE bottles were divided into three groups of four with one bottle in each group serving as the abiotic control. One group received no additional treatment and served as a control to assess the possible effects associated with setting up the experimental procedure. The second group received fresh organisms as discussed above. The bottles of the third group were drained of half of their contents and to them was added an equal amount of 0.22 micron filtered fresh Calico Creek water to determine if the plateau could be related to a depletion of nutrients or other factors from the sample water.

The same parameters used in the biodegradation experiments were measured in these experements on days 0, 1, 4, 7, 10, 14, 21, and 28.

## ISOLATION OF C12 LAS DEGRADING ORGANISMS

Microorganisms presumably capable of utilizing C12 LAS as a sole carbon and energy source were isolated on solid plate media consisting of :

| | |
|---|---|
| C12 LAS | 250 ug/l* |
| Yeast extract | 10 mg/l |
| Agar | 10 g/l |
| deionized H2O pH adjusted to 7.5 | |

* Filter sterilized through 0.22 micron membrane filter, added after autoclaving other media constituents.

One ml of fresh Calico Creek water was inoculated onto the plates, and after growth individual colonies were picked and transferred to liquid media of the same composition as the solid media. Growth of the organisms was verified by the AODC method. It can only be assumed that the organisms isolated were truly growing on LAS alone, as the possibility exists that growth could also result from metabolism of components in yeast extract and agar.

# LITERATURE REVIEW

## Estuarine Biodegradation

Biodegradation of synthetic chemicals in estuarine environments has received relatively little attention to date, primarily due to the complexity of generalizing phenomena in environments which have significant temporal and spatial variation, such as estuaries, and because of difficulties accessing study sites which may be located many hours from the laboratory. The advent of radioisotope methods for studying evironmental systems (Pfaender and Bartholomew, 1982; Button et al.; and Shimp and Young, 1987) has allowed more researchers to direct their attention toward estuarine biodegradation with greater predictive abilities than in the past. This is due in part to the sensitivity and unequivocal nature of chemical fate data generated using radioisotopes in model environmental systems.

The majority of esutarine biodegradation studies have focused on chemicals of agricultural and industrial significance, as these chemicals are commonly implicated in posing health and environmental risks. The biodegradation of pesticides by estuarine water and sediment microorganisms has received the most attention. Using $^{14}C$ labeled methyl parathion, Pritchard et al. (1987) found that mineralization of this compound in estuarine water/sediment systems significantly exceeded that in systems containing water only. It was concluded that disappearance of methyl parathion was due to microbial activity since the compound did not irreversibly sorb to sediments or degrade in abiotic controls. The more substantial degradation of pesticides and other organic chemicals by organisms associated with estuarine sediments in comparison to suspended bacteria has also been noted by several other studies (Lee and Ryan, 1979; Walker et al., 1984; 1988; Jones et al., 1982). However, this is not true for all chemicals, as

degradation of some compounds such as hexachloraphene, DDE, and endosulfan has been shown to be very slow or absent in sediment systems (Lee and Ryan, 1979; Walker et al., 1988). Whether this is due to toxicity of some compounds to sediment bacteria, absence of degrading organisms, or some environmental variable is unclear. The role of environmental conditions on the extent of degradation in estuarine sediments has been examined by Hambrick et al. (1980), who found that microbial mineralization of petroleum hydrocarbons was greatest in the slightly basic pH range and at high oxidation-reduction potentials (i.e. aerobic sediments).

Several studies have addressed the adaptation of estuarine microbial communities to pollutants. Adaptation is often functionally defined as the observed increase in biodegradation rate as a result of prior exposure of the community to the chemical (Pfaender et al., 1985). Adaptation has been demonstrated with a number of chemicals in geographically varied estuaries. It has been noted that lengthly adaptive periods may be necessary before some chemicals begin to be appreciably degraded. Pfaender et al. (1985) studied the degradation of the detergent builder nitrilotriacetic acid (NTA) in estuaries both exposed and unexposed to NTA discharges. An adaptation period of 50 days was required before the onset of degradation at one unexposed site, while the exposed community rapidly degraded NTA with no lag period and could adapt to degrade increased levels of NTA with only short lag periods. Chronic exposure of estuarine microbial communities to low(ng/g) levels of aromatic hydrocarbons has been shown to greatly enhance their degradation (Heitkamp and Cerniglia, 1987) compared to pristine sites with nonadapted populations. The results of Spain et al. (1980) and Walker et al. (1984) show that adaptation of estuarine microbial communities may be site specific and/or chemical specific. Spain et al. (1980; 1983) also report that microbial populations in some systems do not adapt to degrade certain compounds,

and concluded that this is due to the absence of specific bacteria capable of metabolizing these compounds. However, these studies were conducted over relatively short time periods of a few days to several weeks, and other evidence indicates that adaptation may take considerably longer than a few weeks (Pfaender et al., 1985). It is clear from these reports that sufficient time must be allowed for an environmental community to adapt before concluding that no degraders are present or that a chemical will not degrade at a given concentration in a particular estuary.

The influence of environmental variables on the biodegradation of organic pollutants in estuaries has been examined by very few laboratories. Bartholomew and Pfaender (1983) looked at the spatial variation associated with the transition from freshwater to estuarine to marine sites and its effect on biodegradation rates for m-cresol, NTA, chlorobenzene, and trichlorobenzene. In general, degradation rates decreased going up the salinity gradient, and tended to dramatically decrease during winter periods, with the exception of the marine site, which tended to exhibit low and constant rates year round. Similar findings are reported by Palumbo et al. (1988), who also studied the degradation kinetics of m-cresol and NTA across a salinity gradient in the same ecosystem as Bartholomew and Pfaender, and additionally from the Mississippi River and adjacent coastal waters. The authors found that m-cresol uptake rates were greatest in the freshwater sites, and NTA uptake was greatest in the estuarine site where bacterial numbers and activity were highest. Both compounds were slower to degrade across all sites in the colder months when bacterial activity is lowest. Interestingly, m-cresol degradation was more sensitive to environmental variation in temperature and salinity than was NTA. These findings suggest that physical/chemical characteristics of the environment and the chemical itself may play a significant role in the results obtained from a

biodegradation study using natural samples. Factors such as salinity, temperature, concentration of the chemical, adaptive capacity of the microbial population, and many other elements interact to determine pollutant fate and must be considered when evaluating biodegradation in environmental samples.

**Surfactant Biodegradation**

Interest in the biodegradation of surfactants originated in the 1950's and 60's when these compounds began to be noticeable in wastewaters and receiving waters as foam buildup occurred (Swisher, 1987; and Cain, 1976). The buildup of surfactants was particularly troublesome to operators of wastewater treatment plants, who noted that excessive foaming created unsightly and unsafe conditions, as well as interfering with the rate of oxygen transfer in water, thus decreasing the efficiency of aerobic treatment processes (Cain, 1976). The foaming problem was found to be mainly due to the major surfactant in use at that time -- Alkylbenzene sulfonate (ABS). ABS was found to be exceeding resistant to microbial attack, primarily because the highly branched alkyl moiety physically interferes with enzymatic breakdown of the molecule (Swisher, 1987; Alexander, 1965). Development of surfactants which were more easily degraded by microorganisms soon began, and resulted in the development of linear alkylbenzene sulfonate (LAS), which has an unbranched alkyl moiety. LAS was found to be more degradable and also functionally superior to ABS, and thus became one of the most widely accepted synthetic surfactants (Swisher, 1987). Excellent reviews of the biochemistry, genetics, and methods of studying surfactant biodegradation by pure cultures of microorganisms are provided by Swisher (1987), Cain (1976), and Willets (1973).

With the development of LAS and other surfactants, investigators began to

examine the fate of these compounds initially in wastewater treatment processes, under the assumption that if the chemicals were readily and extensively degraded, they should not pose a threat to the environment. The literature on surfactant biodegradation in wastewater treatment generally falls into one of two categories: that dealing with anionic compounds (primarily LAS), and that dealing with cationic compounds (primarily the Quaternary Ammonium Compounds or QAC's). Wastewater biodegradation of nonionics such as the linear alcohol ethoxylates used in this study has not been significantly researched to date, although a great deal is known about the chemistry of LAE degradation by pure cultures (Swisher, 1987).

Because of the importance of LAS, researchers began to study its fate in wastewater treatment models in order to classify it with regards to biodegradability. A number of methods are available for these analyses, and Swisher (1987) has reviewed each in detail. One of the most widely used analytical methods for monitoring LAS degradation in waste treatment is the Methylene Blue Activated Substance Test (MBAS). In the MBAS test, anionic surfactants form one to one ion pairs with the dye methylene blue; the amount of undegraded surfactant remaining is determined by extracting the ion pairs into organic solvent and measuring the dye colorimetrically (Swisher, 1987). By comparing MBAS and TOC values for a laboratory activated sludge unit, Janicke (1971) proposed that LAS should be classed as only of medium biodegradability because resistant intermediates formed during the course of degradation. The intermediates subsequently did not react with methylene blue, leading to an overestimate of degradation, but TOC and UV absorption (detects the aromatic nucleus) values indicated LAS was not highly mineralized. The presence of recalcitrant intermediates was later substantiated by Leidner et al. (1976) who identified the intermediates as various sulfophenylcarboxylic acids. The ultimate biodegradability of LAS has also been

questioned by Pitter and Fuka (1979), who reported an average of 64% degradation in laboratory activated sludge, based on DOC disappearance and COD measurements.

In contrast to the previous studies on LAS are those of Janicke and Hilge (1979), Steber (1979), and Swisher (1981), who find LAS to be extensively degradable. Janicke and Hilge found that LAS was degraded to the extent of 95-97% in activated sludge when it was complexed with the cationic surfactant dialkyl dimethyl ammonium chloride. Also, Steber demonstrated 80% ring mineralization in activated sludge sewage plants with 3hr. retention times using $^{14}C$ ring labeled LAS. Undegraded LAS represented 2-3% of the original radioactivity, and 15-25% was in the form of intermediates composed mainly of sulfophenylcarboxylic acids. It has been suggested that the reason for the low estimates of LAS ultimate degradation proposed by some, and the wide variation reported in the literature is due mainly to the selection of test method (Swisher, 1981). Chemical Oxygen Demand (COD) and Dissolved Organic Carbon (DOC) analytical methods are somewhat nonspecific, and will respond not only to LAS biodegradation intermediates but also to any other organics which may present, leading to conservative estimates of ultimate degradability.

Because cationic surfactants have a rather strong germicidal activity (Swisher, 1987), their fate in biological treatment is of obvious concern. Several laboratories have reported DSDAC (distearyl dimethyl ammonium chloride) degradation of >90% (May and Neufarht, 1976) in model sewage treatment models with no adverse effects on biological activity (May and Neufarht, 1976; Gericke et al., 1978). Extensive mineralization of C18-TMAC (Games et al., 1982) and ditallowdimethylammonium chloride (DTDMAC) (Sullivan, 1983) by wastewater microorganisms has also been reported. These studies also revealed the strong

tendency of QAC's to adsorb to wastewater solids; sometimes to the extent of almost 100% within 30 minutes of initial exposure. Thus, removal of at least some cationic surfactants in activated sludge appears to be the result of both a sorptive process and biodegradation. However, the sorptive tendency must be controlled for in a biodegradation study of QAC's in order to distinguish between removal due to biodegradation and that due to sorption. From the work done to date on surfactant biodegradation in wastewater treatment systems, it can be concluded that anionic and cationic surfactants are readily degraded by wastewater microorganisms, and available evidence also suggests that nonionic surfactants are extensively removed during wastewater treatment (Swisher, 1987).

Although synthetic surfactants typically undergo degradation and are extensively removed in treatment systems, small quantities of the compounds still reach the aquatic environment through treatment effluents (Larson et al., 1983). Few studies have assessed the biodegradability of surfactants in the post waste treatment aquatic environment, and only a small proportion of these studies deal with the estuarine environment. Aquatic research has primarily focused on freshwater ecosystems, although some work has diverged into such areas as the role of aquatic plants in harboring degrading microbes (Federle and Schwab, 1989).

Several studies have examined the fate of the benzene ring carbon of LAS. Using environmentally realstic concentrations (ug/l), Larson and Payne found 70-80% of the LAS ring mineralized by indigenous river water microorganisms from Rapid Creek, SD. Similar results are reported by Nielson and Huddleston (1981) in experiments exposing LAS from simulated secondary waste treatment effluents to additional microbial oxidation in a simulated receiving water. After 190 days, 98.5% of the original LAS had been mineralized and the remaining label was determined to be associated with the biomass fraction. Both of these studies are in contrast to

earlier reports which suggested that recalcitrant aromatic intermediates impeded complete LAS degradation (Leidner et al., 1976). This apparent conflict is likely due in part to the concentrations of LAS used in the studies. Early studies which questioned LAS degradability in wastewater treatment were conducted at mg/l concentration ranges, while Larson and Payne, and Neilsen and Huddleston used more realistic environmental concentrations. Larson and Payne have even suggested that LAS degradation by natural communities is hampered at concentrations greater than 20mg/l, apparently due to microbial toxicity. As yet, there is no evidence in the literature which points to the presence or accumulation in the environment of recalcitrant LAS intermediates produced by aquatic microbial assemblages.

The fate of quaternary ammonium compounds has been studied in lake and river ecosystems by Ventullo and Larson (1986) and Larson and Vashon (1983), respectively. Chronic exposure of lake microorganisms to ug/l levels of long chain (C12-C18) QAC's resulted in a significant adaptive response by the bacteria, which exibited an increase in number of cells and biodegradation capability. Although bacterial heterotrophic activity (as measured by glucose turnover time) was hindered by short term chronic exposure to mg/l levels of QAC; the activity of cells exposed to ug/l levels over long terms was not adversely affected, indicating the selection of more tolerant bacteria within the community. River water microorganisms respond similarly to low levels of QAC's. Larson and Vashon report that degradation of C16-C18 QAC's follows first order kinetics, and that although the QAC's readily sorb to river sediments, the sorbed material was apparently quite available to degradative organisms.

Larson and Games (1981) examined the kinetics of LAE degradation by Ohio River organisms. At concentrations of $^{14}C$ labeled C12E9 and C16E3 LAE up to

100ug/l, degradation was first order with respect to concentration and as much as 90% was respired in a matter of one to two weeks. Neither the ethoxylate nor alkyl chain lengths had a significant effect on the extent or rate of degradation.

A comparison of biodegradation rates in natural river water samples was compared to those predicted from laboratory screening tests by Larson (1983). Biodegradation of C18-TMAC, LAS, NTA, and dioctadecyldimethylammonium chloride (DODMAC) was examined in two standard biodegradability screening tests using activated sludge as a source of microbes and 20-100mg/l test material, and also in environmental samples using ug/l levels of $^{14}C$ labeled substrate. In general, screening tests tended to underestimate the potential for degradation in the environment, especially for the QAC's. Biodegradation rates in environmental samples using realistic environmental concentrations were generally greater than corresponding rates in screening tests using high (mg/l) concentrations. Similar conservative estimates of QAC degradation rates based on screening tests have been reported by Larson and Vashon (1983),and Larson and Wentler (1982), indicating that care should be used when extrapolating from screening tests to environmental situations.

One of the only reports of ultimate degradation of some LAE's by indigenous estuarine microorganisms was made by Vashon and Schwab (1982). They found rapid (alkyl chain carbon half life of 2.3 days) and extensive (>75%) mineralization of $^{14}C$ labeled C16E3 and C12E9 LAE in waters from Escambia Bay, Florida.

The literature dealing with biodegradation of detergent chemicals in estuarine environments is obviously quite limited. There is a pressing need for more research in the area of microbial degradation of these and other compounds in estuaries and in near shore marine environments.

# RESULTS AND DISCUSSION

## Background Environmental Data

Background environmental data for Calico Creek and North River at each sampling date are shown in Table 3. This data was collected to characterize the physical, chemical, and microbiological nature of the sampling sites, and to provide reference points for comparing data collected over time.

As shown by the data, the North River site appears relatively less polluted; there are somewhat fewer bacteria, and the activity of the cells as measured by amino acid turnover time and $^{3}$H-Thymidine uptake appears substantially lower, suggesting an absence of significant anthropogenic contamination. Additionally, measures of total coliforms and E. coli point to domestic wastewater contamination of the Calico Creek site, but not the North River site. The environmental characteristics measured at Calico Creek vary only slightly over time, as might be expected at a site subject to significant constant anthropogenic inputs. Calico Creek samples collected in October 1988 are quite similar to those collected in December 1988 when the weather was substantially colder. Microbial activity and numbers remained relatively constant, while dissolved oxygen and pH showed slight increases in the December samples. At the North River site however, microbial activity and numbers decreased somewhat in the December samples, as shown by the amino acid turnover and thymidine uptake data. With the exception of temperature, the other parameters remained essentially stable at the North River site.

Environmental data for the salinity gradient study sites (Table 4) again shows that although the salinity values for the estuarine and marine sites are fairly close; the two environments are distinguished well by the other parameters. The absence

TABLE 3. Environmental supporting Data: Calico Creek and North River

| Parameter | CC (10/88) | NR | CC (12/88) | NR |
|---|---|---|---|---|
| Temp. (°C) | 20.5 | 17 | 8 | 6 |
| Salinity (‰NaCl) | 26 | 31 | 29 | 30 |
| Dissolved $O_2$ (mg/l) | 4.0 | 7.7 | 7.7 | 7.3 |
| pH | 7.6 | 8.1 | 8.2 | 8.2 |
| AODC (x $10^6$ cells/ml) | 5.1 | 3.5 | 5.4 | 1.6 |
| $^3$H-amino acid turnover (hr) | 2.9 | 50 | 2.4 | 16.7 |
| $^3$H-thymidine uptake (nmol/l/hr) | 0.50 | 0.21 | 0.52 | 0.14 |
| Coliform bacteria(#/100ml) | 23 | 6.9 | >23 | 9.2 |
| E. coli (total #/100ml) | 2.2 | <1 | <1 | 2.2 |

TABLE 4. Environmental Supporting Data: Salinity Gradient Sites

| Parameter | FW | EST | MAR |
|---|---|---|---|
| Temperature (°C) | 28 | 30 | 27 |
| Salinity (‰NaCl) | 2 | 32 | 36 |
| Dissolved $O_2$ (mg/l) | 5.5 | 4.6 | 5.0 |
| pH | 7.5 | 8.0 | 8.3 |
| AODC (x $10^6$ cells/ml) | 3.1 | 5.1 | 2.5 |
| $^3$H-amino acid turnover (hr) | 5.9 | 5.9 | 3.2 |
| $^3$H-thymidine uptake (nmol/l/hr) | 0.41 | 0.50 | 0.21 |
| Coliform bacteria (total#/100ml) | ND | <1 | <1 |
| E. coli (total#/100ml) | ND | <1 | <1 |

FW- freshwater EST- midestuary MAR- marine ND-no data

of detectable coliform bacteria at these sites suggests that they have not been contaminated by domestic wastewater, and therefore should not have been exposed to synthetic detergent compounds. Bacterial numbers as measured by AODC are lowest at the marine site and highest at the midestuary site. Amino acid turnover time and thymidine uptake indicate that the midestuary site also has the most metabolically active microbial community, while the marine environment has the least active community. The physical-chemical parameters are comparable between sites. Dissolved $O_2$ and temperature are similar, while pH increases as expected in the transition from freshwater to seawater.

**Mass Balance of Added Radiolabel**

The mass balance data were collected in order to establish the reliability of the biodegradation method used by accounting for as many potential fates of the radiolabeled substrate as possible. Table 5 contains example mass balance data for samples from the North River and Calico Creek taken in October 1988 for the compounds LAS and C18 TMAC. Data for other compounds and sample dates is presented in Appendix A.

It is clear from Table 5 that a high percent of labeled LAS can be accounted for as either material respired to $CO_2$, incorporated into cell biomass, or remaining in solution. These data were typical of that obtained for the other compounds, except C18 TMAC, which showed a roughly 60% decrease in solution counts after one day in Calico Creek-sediment samples. The label missing from the mass balance was revealed to be sorbed to the sediment when sediment was retrieved from the samples and counted for radioactivity. In general, the biodegradation procedure was able to account for a high proportion of the added labeled material (Appendix A).

TABLE 5. Example mass balance data for LAS and C18-TMAC from impacted vs. unimpacted sites experiment of October 1988. Mass balance data for all chemicals and experiments are found in Appendix A.

| | Average % of Initial label Accounted For | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | LAS | | | | C18-TMAC | | | |
| Day | NR | NRS | CC | CCS | NR | NRS | CC | CCS |
| 1 | 159 | 109.6 | 203 | 133 | 121.6 | 115.2 | 111.9 | 114.9* |
| 7 | 101.4 | 91.3 | 102.3 | 90.4 | 84.8 | 75.2 | 73.3 | 41.0 |
| 23 | 90.6 | 94.7 | 78.1 | 87.3 | 102.4 | 93.9 | 92.4 | 41.1 |
| 46 | 87.2 | 99.1 | 95.1 | 99.3 | 117.8 | 104.2 | 104.6 | 40.1 |
| 78 | 100.3 | 97.1 | 85.9 | 94.8 | 106.5 | 99.1 | 96.3 | 42.0 |
| 120 | 101 | 98.1 | 95.1 | 95.5 | 100.3 | 96.2 | 89.4 | 49.8 |

NR-North River
NRS-NR with sediment
CC-Calico Creek
CCS-CC with sediment
*-data for day 0

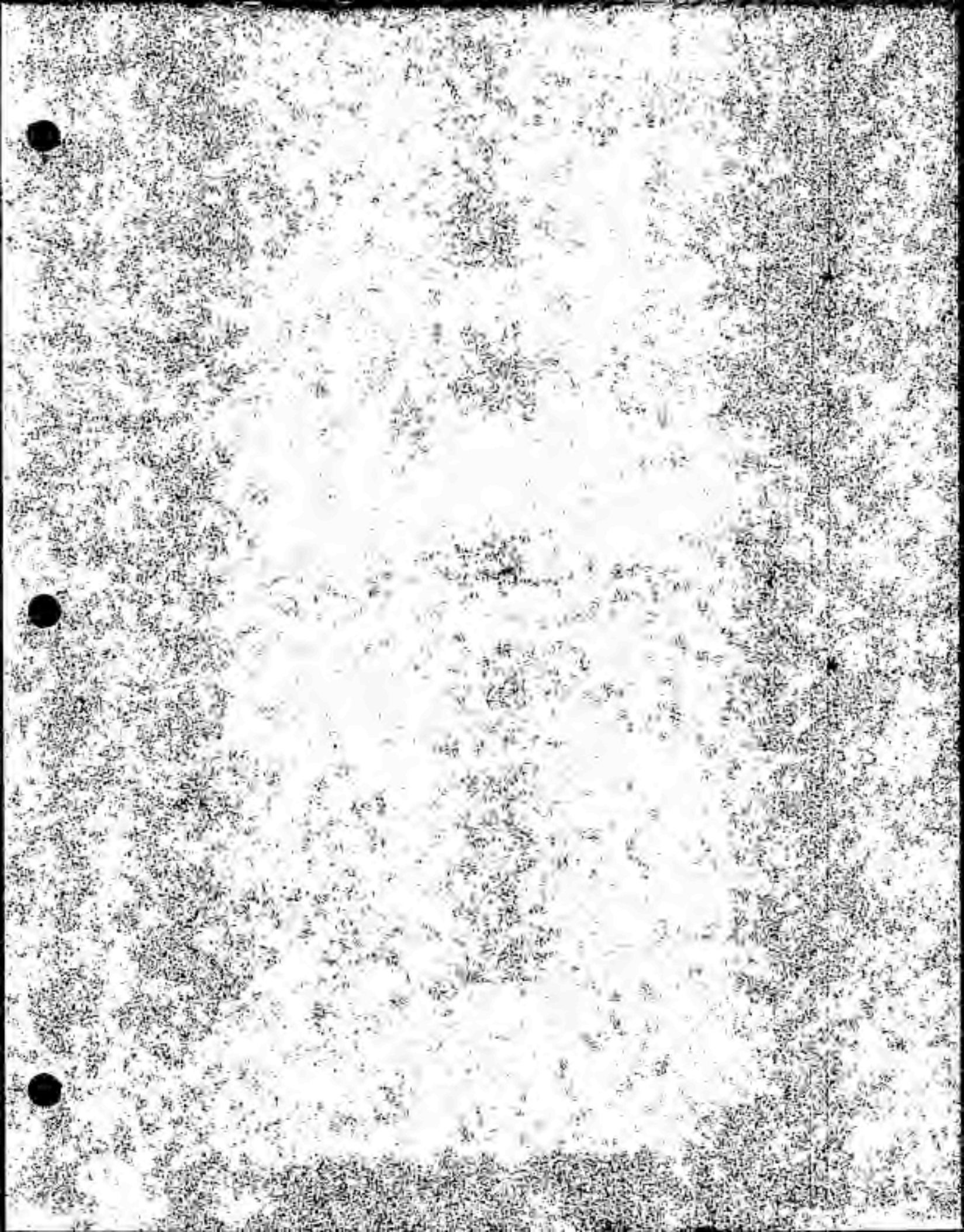
**Biodegradation Experiments: Impacted vs. Unimpacted Sites**

A basic question to be addressed by this research was whether exposure to detergent chemicals through domestic wastewater effluents would result in an adapted community of degrader microorganisms in the sewage impacted Calico Creek environment compared to the North River, where the community should have been minimally exposed.

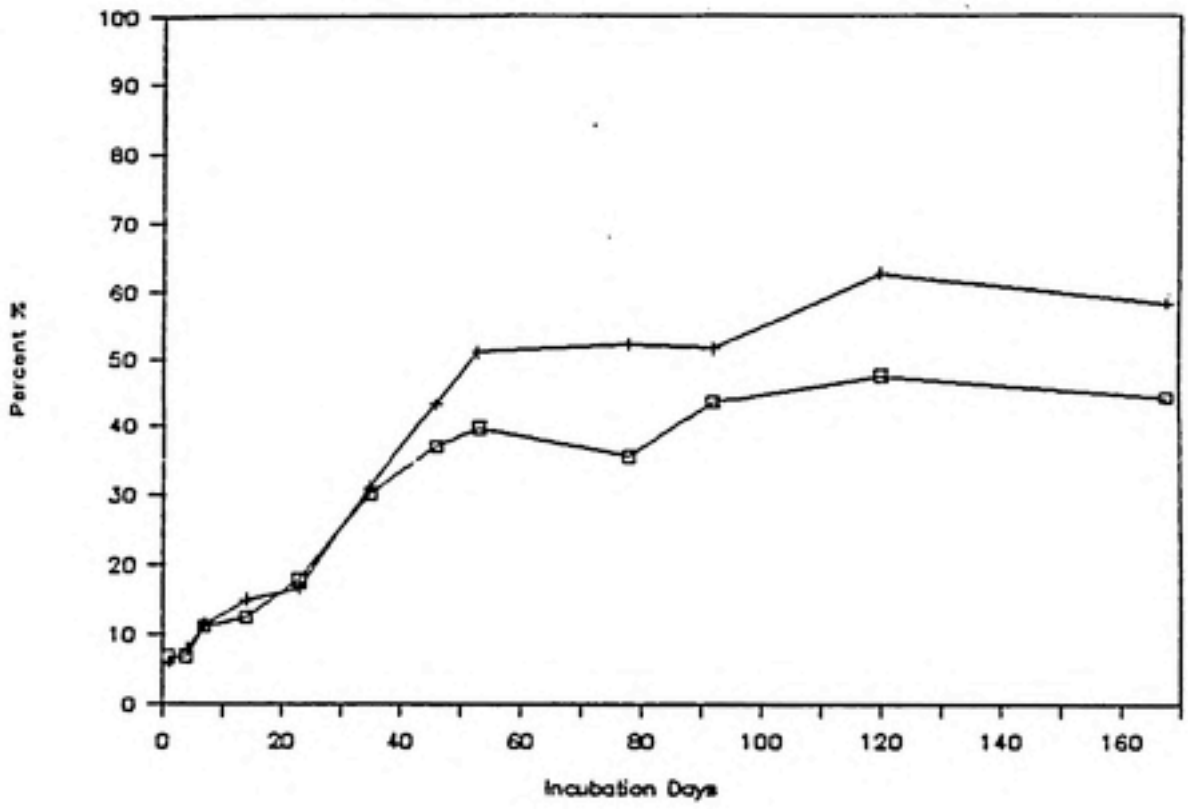
Seven compounds were tested for their extent of biodegradation at the Calico Creek and North River sites during the fall of 1988. The compounds tested represented four surfactants (LAS, C12-TMAC, C18-TMAC, and C13E6-LAE) and three reference compounds (phenol, cellulose, and stearic acid). The data are presented as percent respiration for samples with and without sediment for each site and each chemical. Data regarding percent cellular uptake and percent remaining in solution are found in Appendix A. Sediment addition allows determination of the effect on biodegradation of such factors as adsorption to sediment particles, contribution of organic matter or nutrients from the sediment, and effect of additional microbial biomass introduced by the sediment.

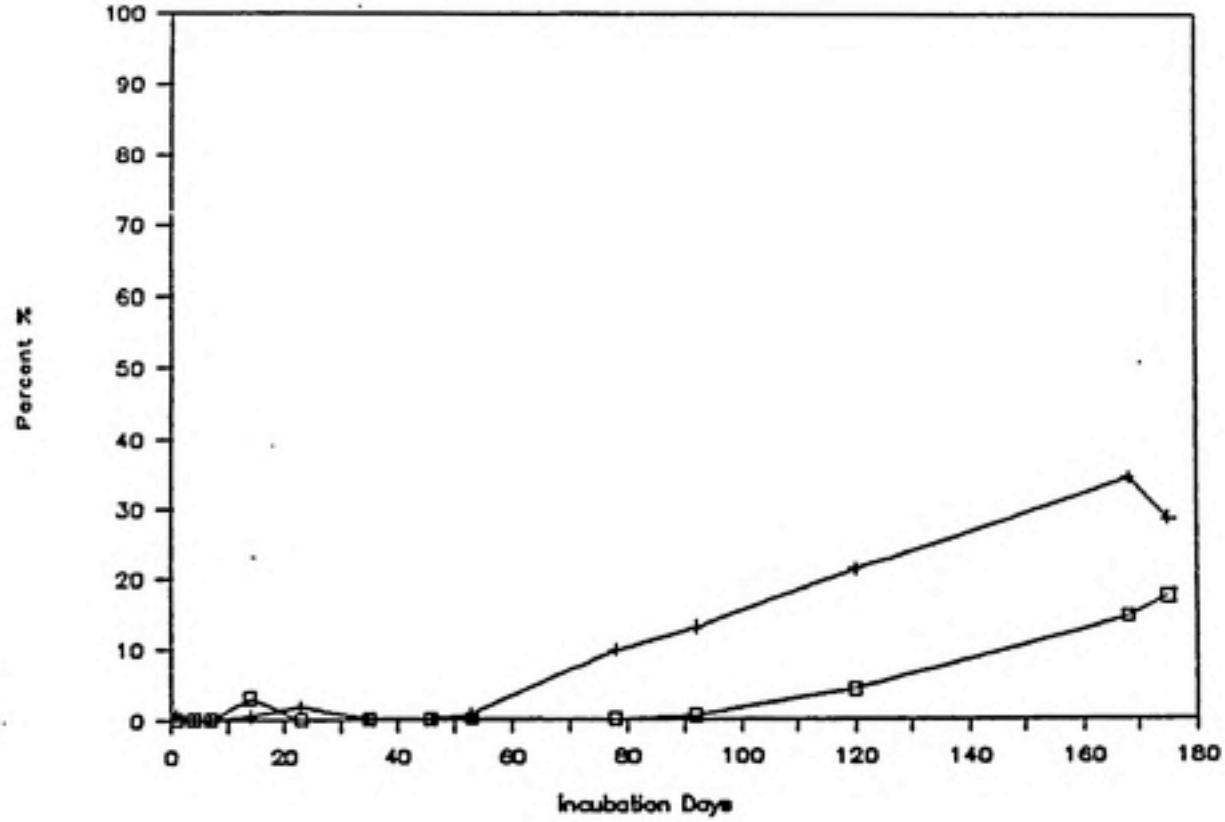
Data for LAS degradation in Calico Creek and North River samples taken in October 1988 are shown in Fig. 3. It is apparent that LAS is degraded in Calico Creek with no discernable lag period, and no significant difference in rate between the system with sediment and that without. However, a slightly greater percent is respired in the sediment amended samples, perhaps due to organisms introduced from the sediment.

The North River samples show an apperent adaptation period of almost 50 days before appreciable degradation begins with sediment, and even longer in the samples without sediment. This suggests that LAS degradation depends on the presence of an adapted population of microbes, and that this community is present



# C12LAS DEGRADATION



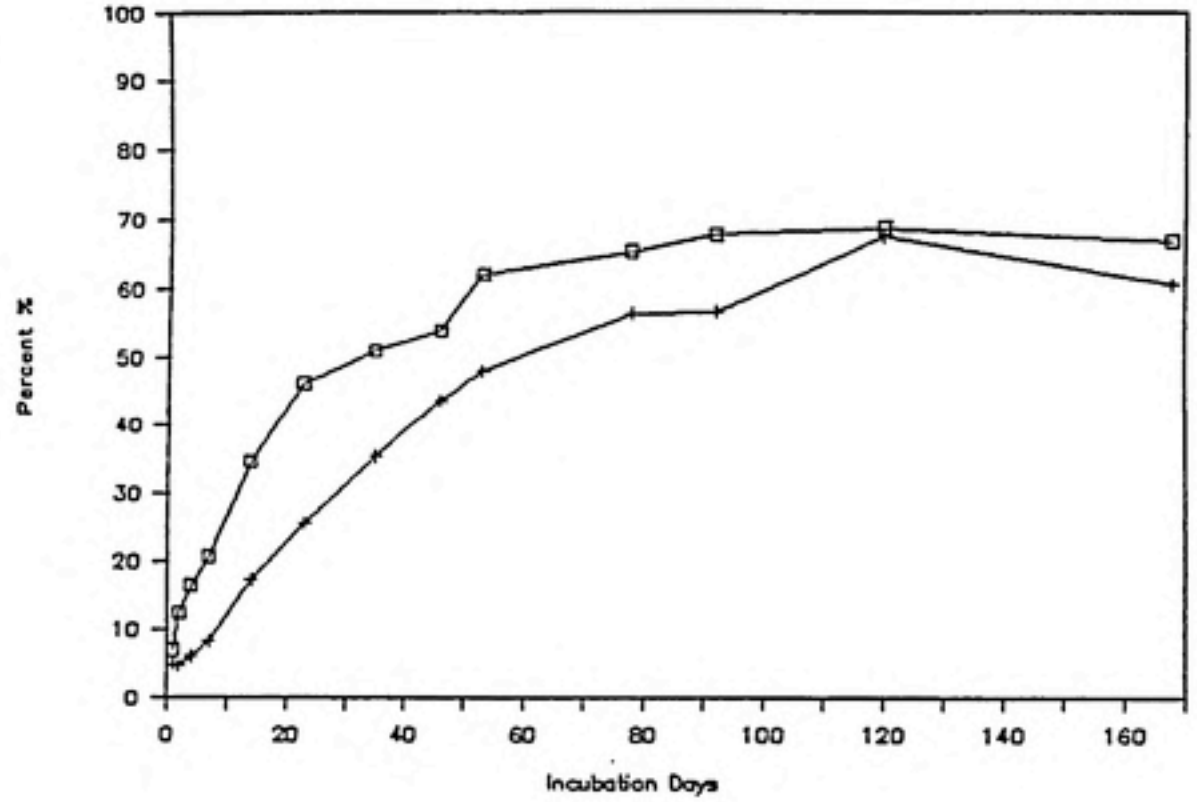
and active in the Calico Creek, but is induced only after exposure to LAS in the North River.

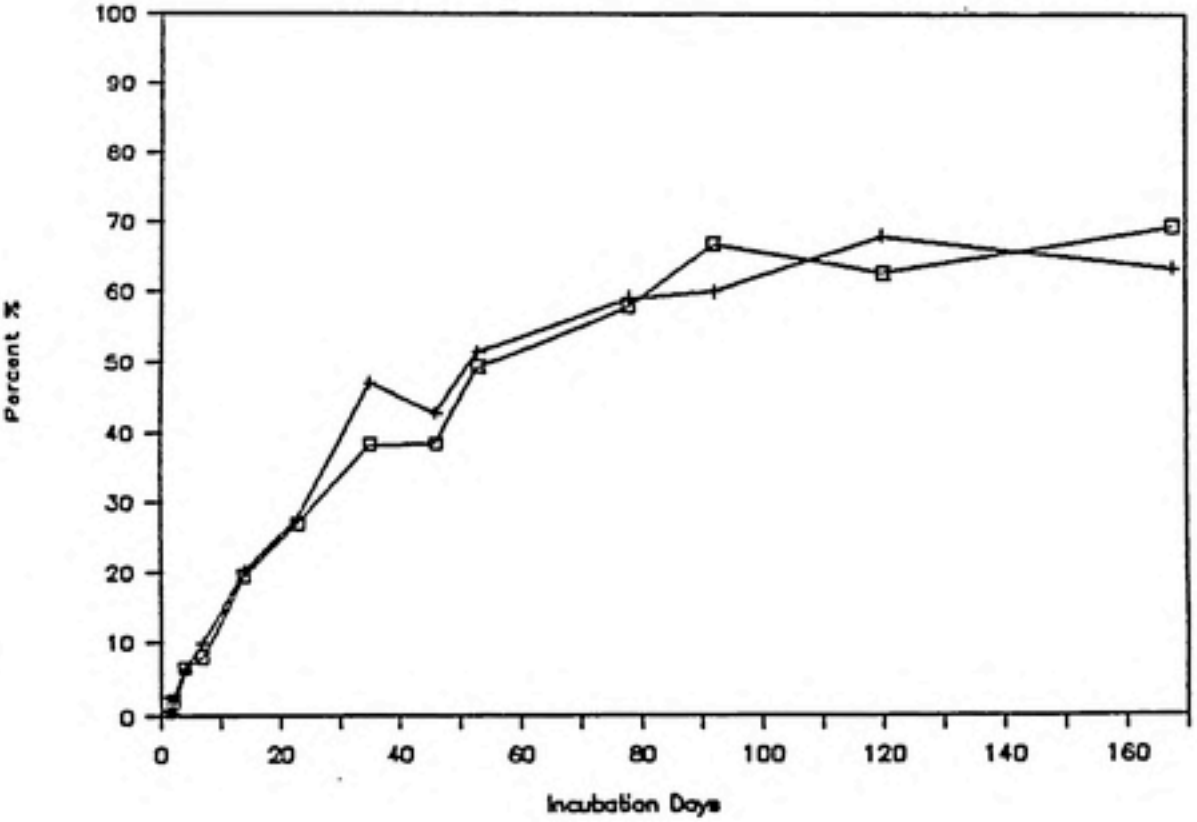
The major fate of the compound in all Calico Creek samples is respiration; very little (generally less than 5%) is taken up into cell biomass. Similar data are found for North River samples, and again, respiration appears to be the major fate, with almost no cellular incorporation. Uptake of the compounds into cell biomass was minimal for all the chemicals tested in these experiments on each date (see Appendix A).

An interesting phenomenon appears in the results from these samples, which will be seen with the other compounds as well, and that is a plateauing of metabolism of the compounds to $^{14}CO_2$ before a major portion of the chemical has been respired. This effect was unexpected, in that many researchers studying biodegradation in other environments have recorded upwards of 90% respiration (Vashon and Schwab, 1982; Larson and Games, 1981; Larson and Wentler, 1982). A similar plateau at between 60-70% respired can be seen in the data of Larson (1983), and Larson and Payne (1981), although no explanation for this effect was proposed. Further experiments on this phenomenon and the nature of the unrespired material were done in this study and will be discussed later.

Percent respiration data for C12-TMAC degradation in October 1988 samples are provided in Fig. 4. There is no apparent adaptation necessary in either environment. Sediment addition appears to result in less of the compound being respired in Calico Creek samples, perhaps due to some sorption, but makes no difference in North River samples. The major fate of this compound is also respiration, with little uptake into biomass. The initial peak of biomass incorporation which decreases over time and is seen also in the data of other compounds may represent material sorbed to the outside of cells which is released as the solution

# C12-TMAC DEGRADATION



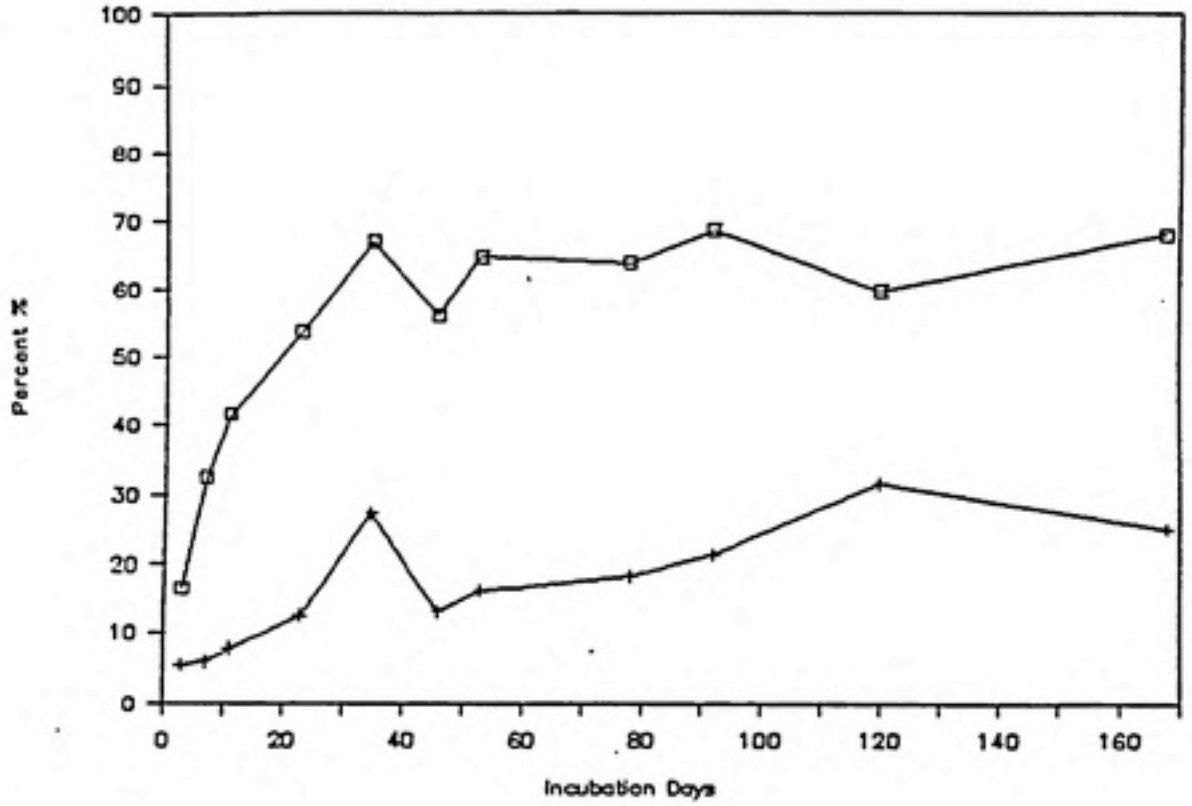
concentration declines due to metabolism. The patterns of C18-TMAC degradation in samples from October 1988 differ from C12 in Calico Creek systems with sediment (Fig. 5). Here, less degradation is observed, due to C18 sorption to sediments and reduced availability to the organisms in the water column. The mass balance presented in Table 3 suggests almost 60% of the C18-TMAC was bound to the organic rich sediments. Assuming that the rest of the C18 is available, then the microbial community respired approximately 60-75% of that, which is similar to the samples without sediment and to previous patterns based on the amount of substrate available. No discernable sediment sorption occurs in the North River samples and degradation patterns here closely resemble Calico Creek without sediment. As before, respiration is the primary fate, and biomass accumulation is insignificant.

Data for biodegradation of C13E6-LAE in Calico Creek and North River samples taken in October 1988 are shown in Fig. 6. There is little variation in LAE degradation between samples with and without sediment and between sites. There is also no need for adaptation with C13E6-LAE in either environment. The one apparent difference between sites is that the plateau effect is more prominent in the North River, with only 40-50% respired, compared to 60-70% in Calico Creek. This may be related to the metabolic activity or numbers of organisms being lower in the North River, as compared to Calico Creek.

The reference compounds used in this study are included for comparing detergent chemical biodegradation data to that for chemicals that are common pollutants,for which a large data base exists, or are naturally occurring compounds. Data for the chemicals used in this study in the fall of 1988: phenol, stearic acid, and cellulose, are shown in Figs. 7-9.

Phenol degradation (Fig.7) is quite comparable in both environments, with no

# C18-TMAC DEGRADATION



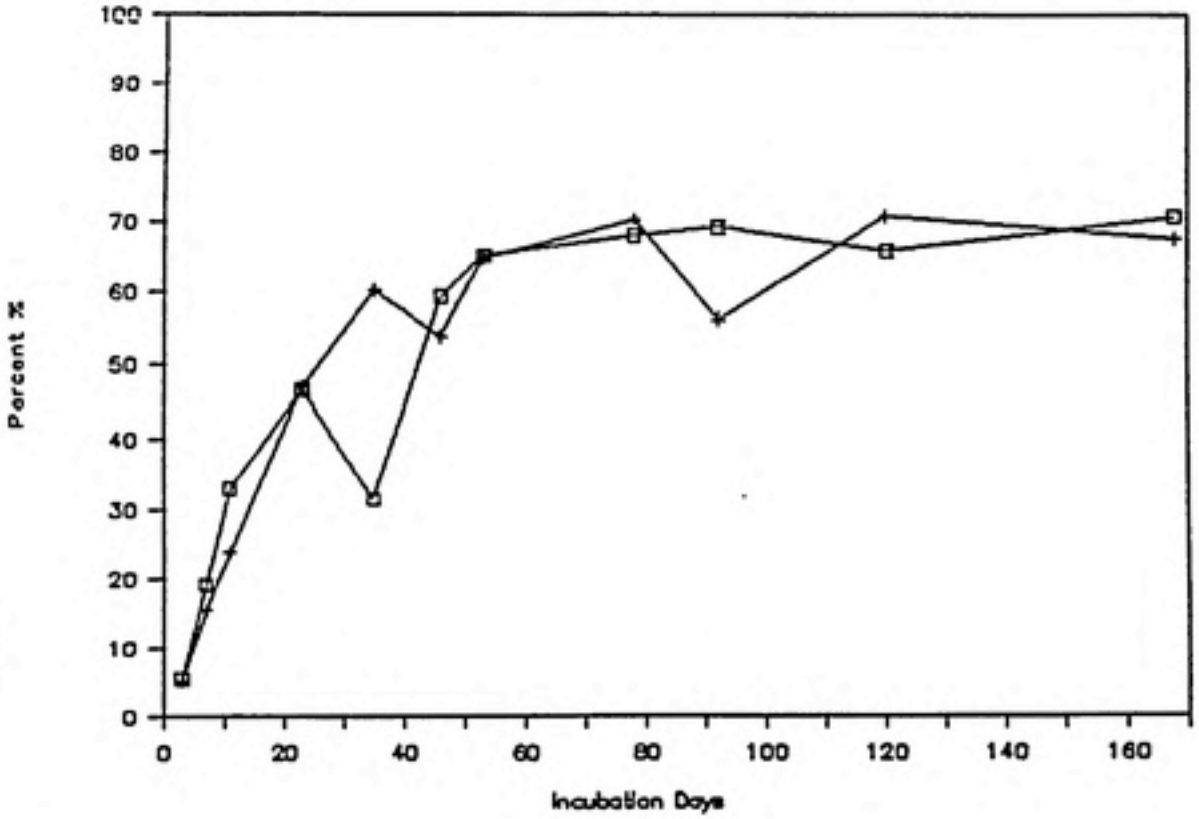
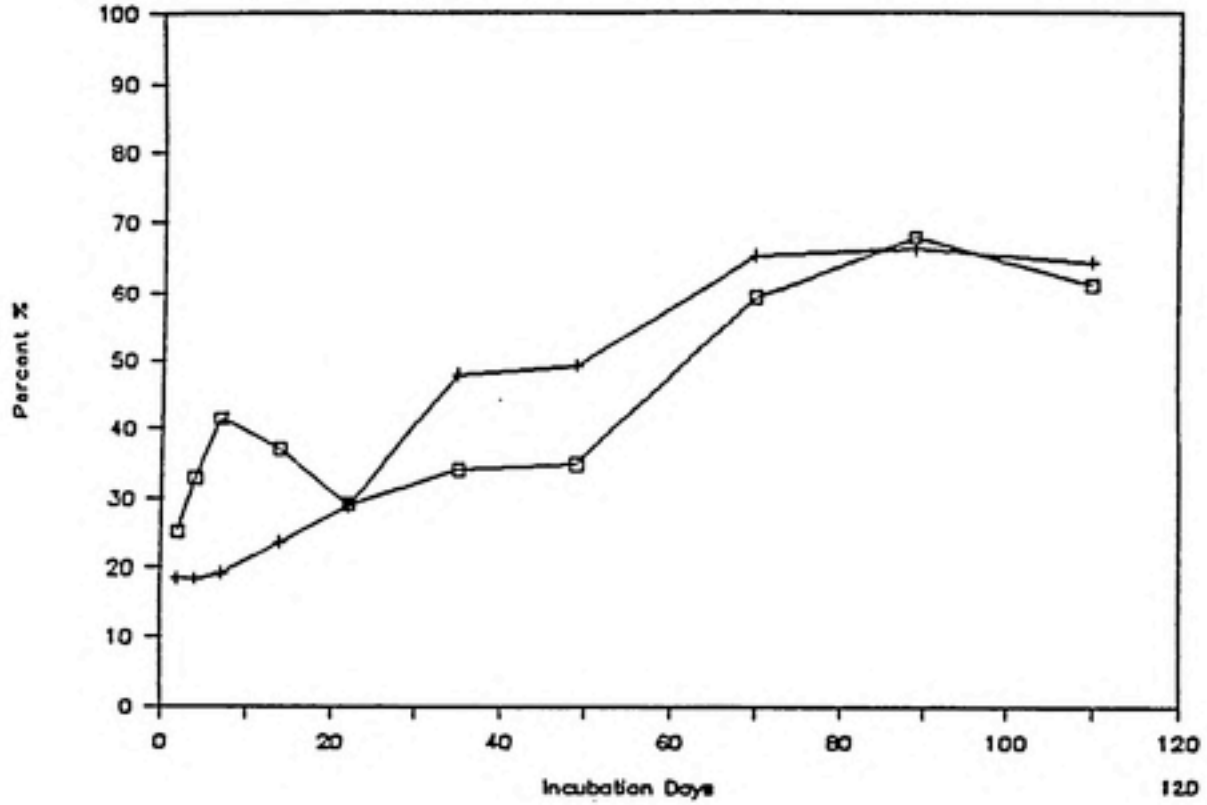
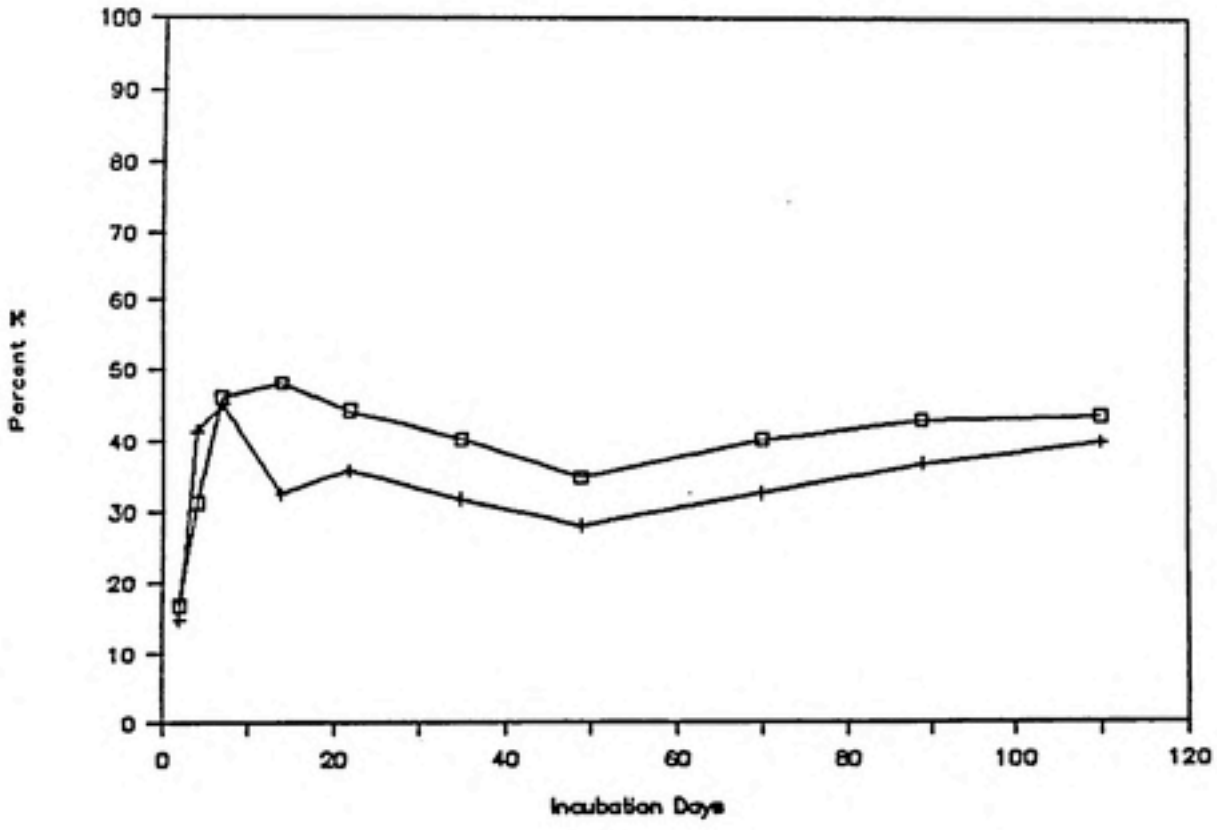



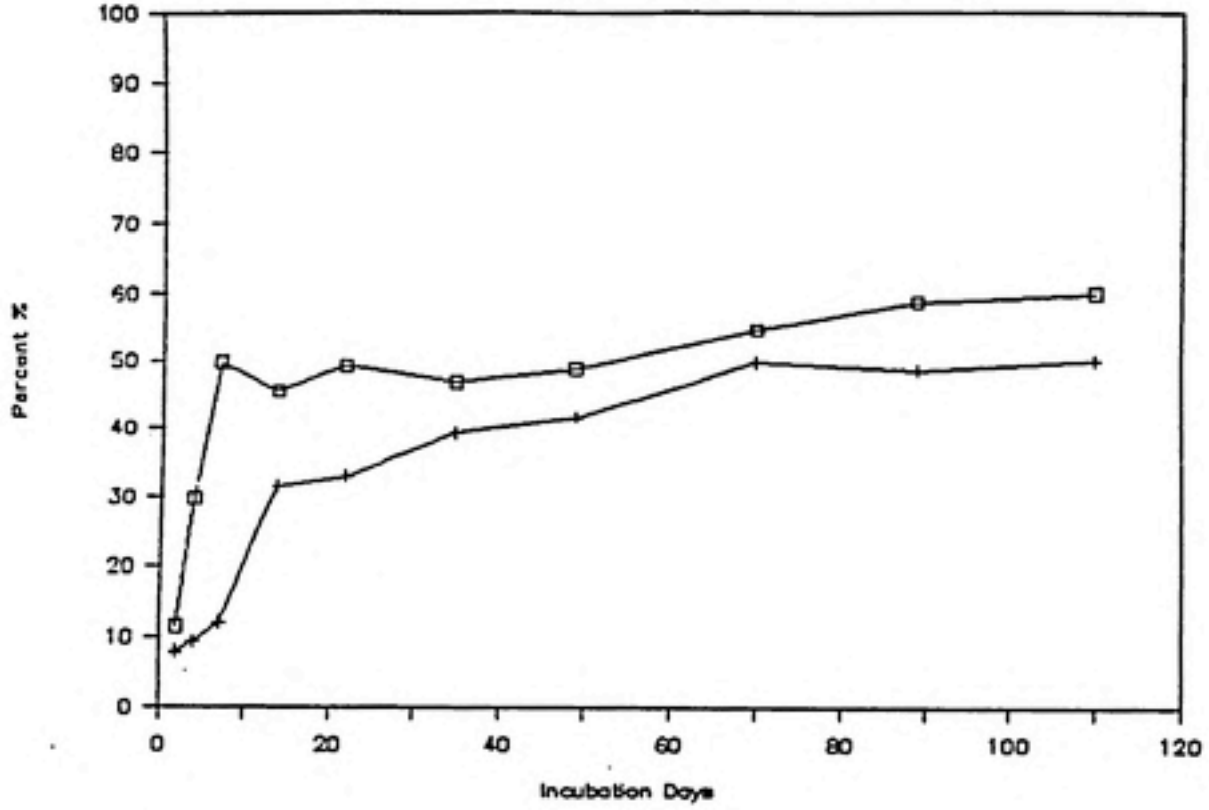
FIGURE 6. Respiration of C13E6 LAE in Calico Creek (top panel) and North River (bottom panel). Expressed as % respired as $CO_2$.
+ sediment □ no sediment

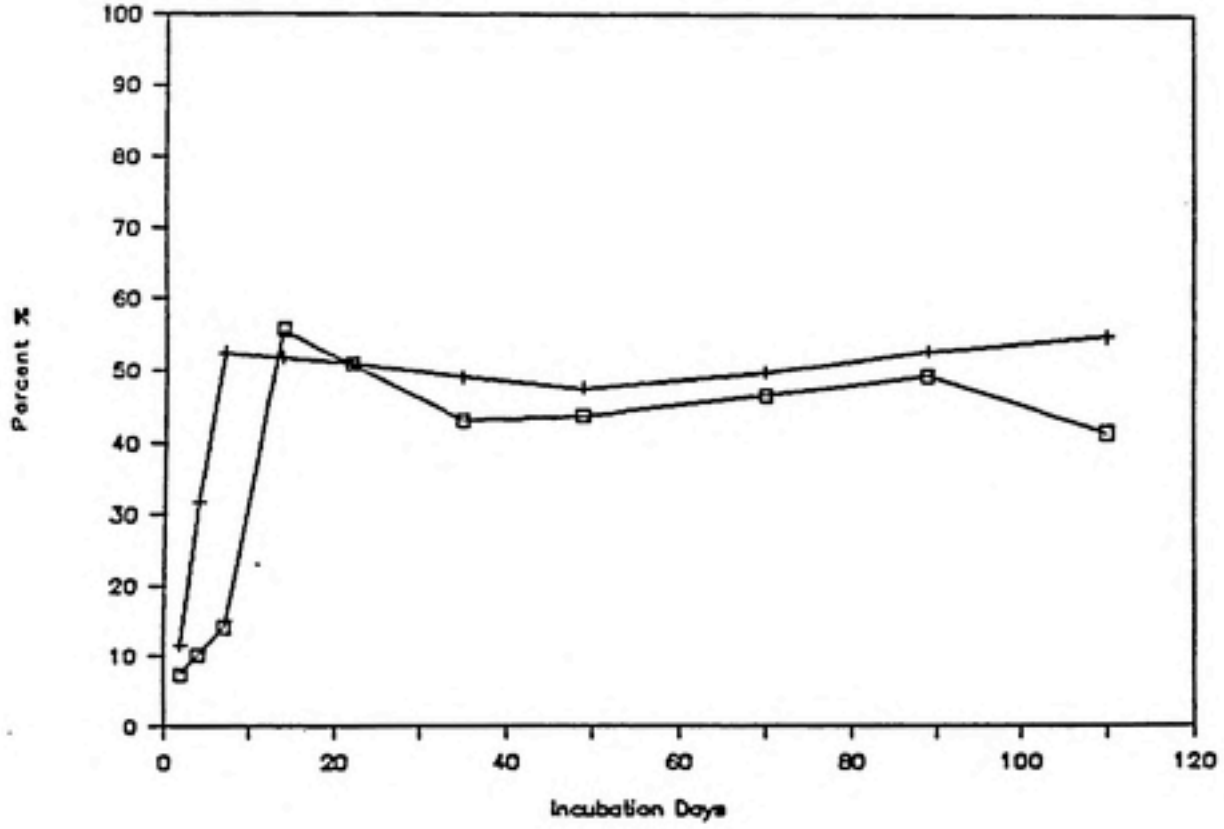
# C13-E6 DEGRADATION



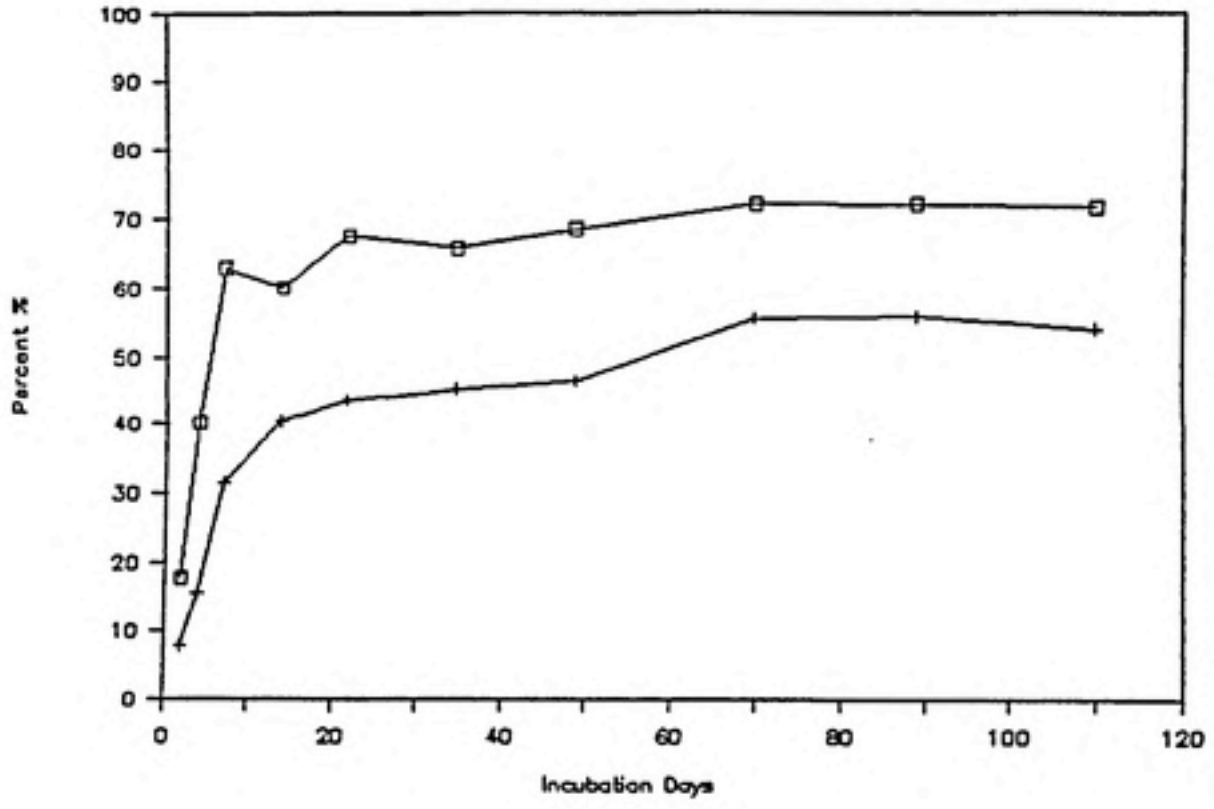



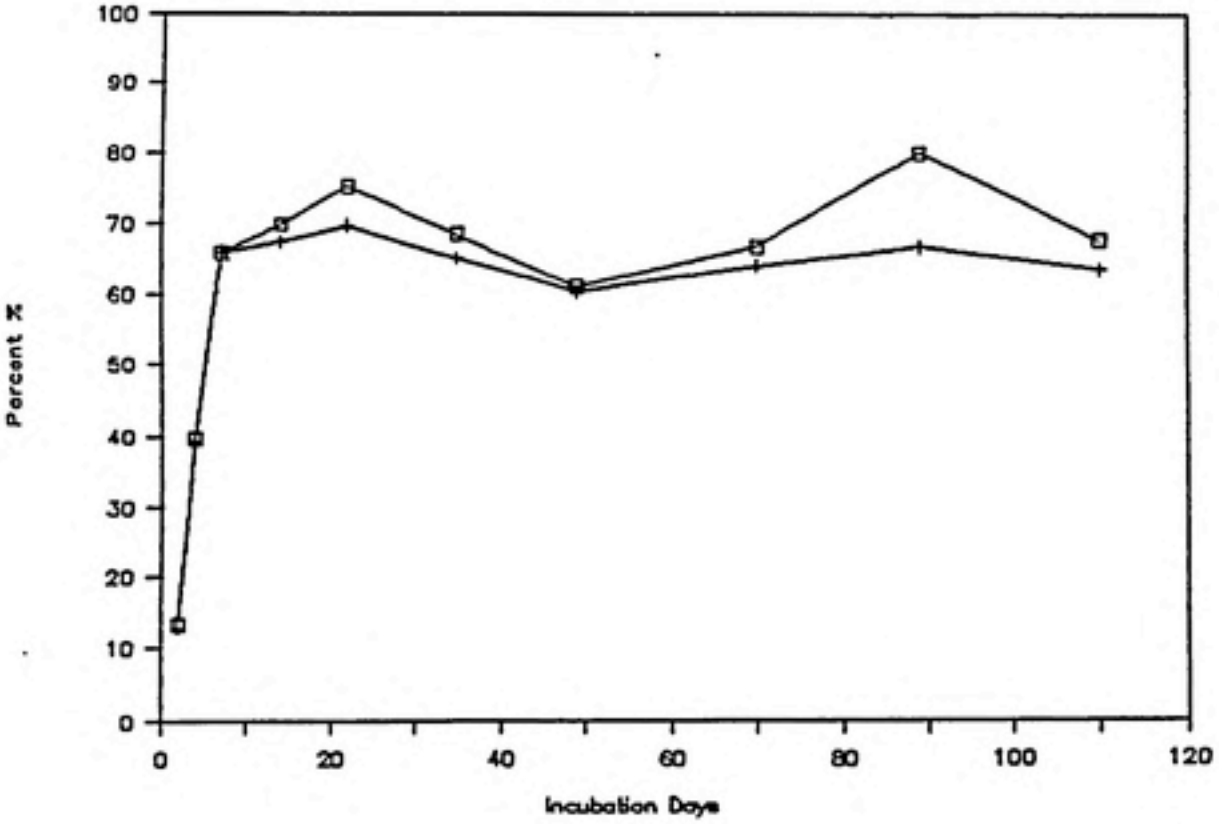
# PHENOL DEGRADATION



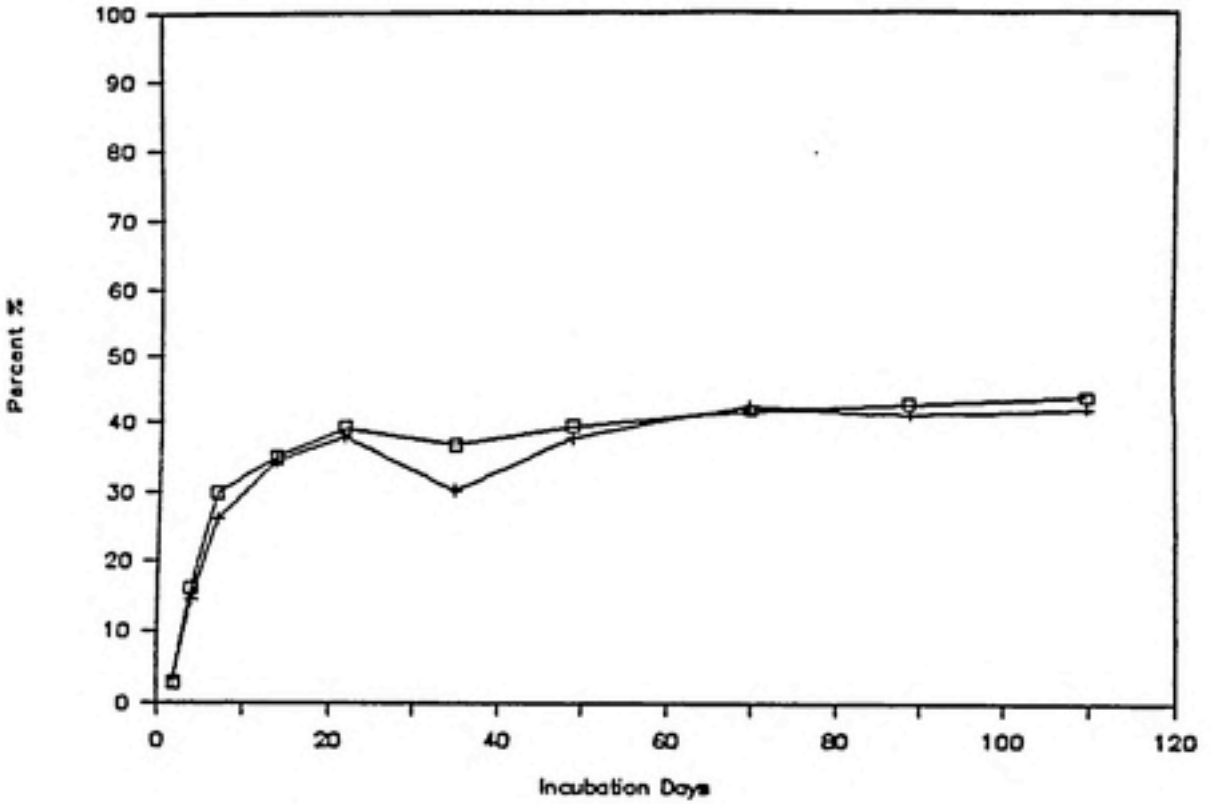



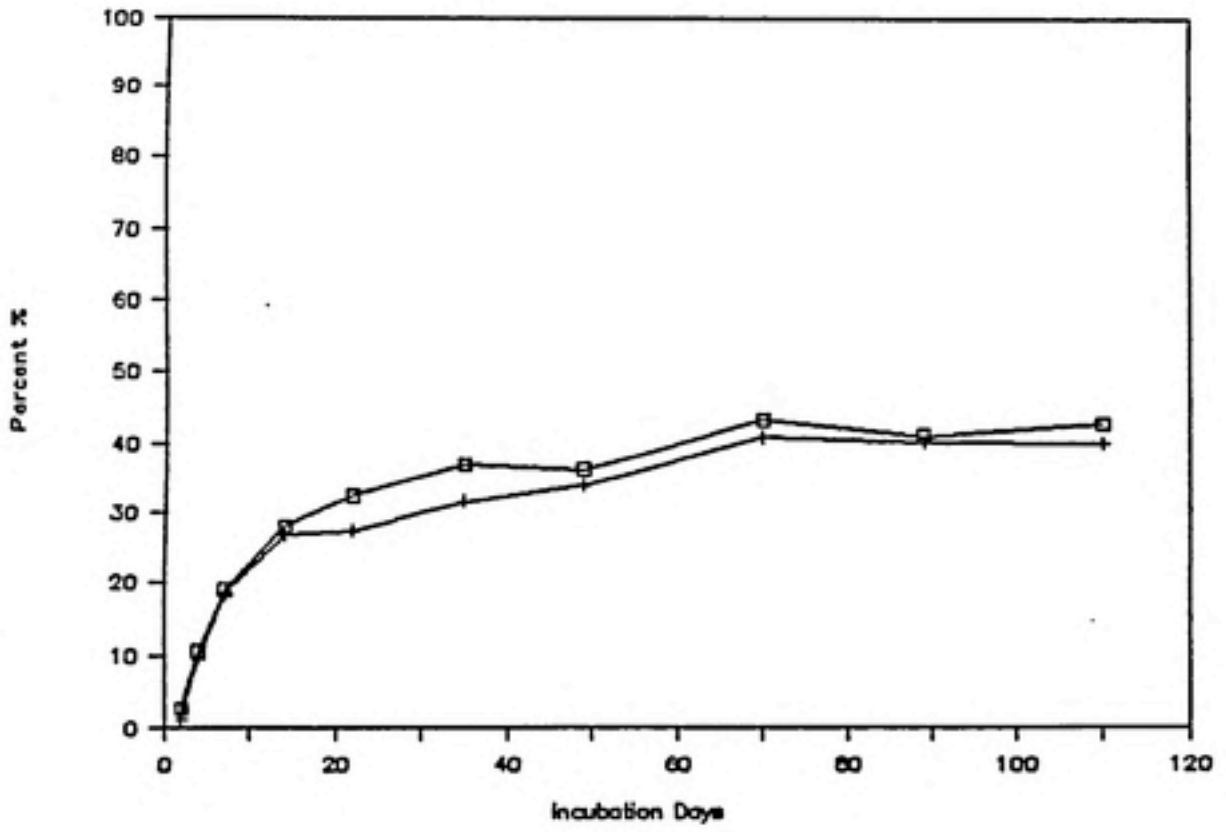
# STEARIC ACID DEGRADATION



# CELLULOSE DEGRADATION



need for adaptation, and most of the metabolism being respiration. The plateau, however, is very pronounced in the North River, with degradation approaching only 50%.

Stearic acid, on the other hand, is rapidly and extensively mineralized to almost 80% in the North River, but in Calico Creek metabolism is lower, especially in sediment containing samples (Fig.8). The Calico Creek sediment may tend to sorb the stearic acid due to the sediment's organic rich nature.

Degradation of cellulose is shown in Fig. 9. Although metabolism is rapid and there appears to be no difference between sites and presence of sediment; degradation plateaus at less than 50%. Since the cellulose used was a microcrystalline variety, the degradation may have been limited by the ability of the organisms to have access to the nonsoluble cellulose.

In general, degradation of the reference compounds required no adaptation, and respiration to $CO_2$ was the major result of metabolism. Biodegradation of these chemicals exibited the same overall trends as the detergent chemicals.

**Assessment of Biodegradation Variability with Time**

In order to assess variability in biodegradation of the detergent compounds over time, these experiments were repeated with LAS, LAE, C18-TMAC, and phenol at 25 ug/l concentrations at the Calico Creek and North River sites in October of 1989. Rather than present the complete figure for each compound again, the data is presented as biodegradation rates. First order biodegradation rate constants ($K_1$) were calculated for LAS, LAE, C12-TMAC, C18-TMAC, and phenol for both the October 1988 and 1989 experiments, and this data is presented in Table 6. The first order rate constant is a useful summary parameter for comparing biodegradation variability over time and site. As shown in the data, with the

| Site(date) | LAS | LAE | C12-TMAC | C18-TMAC |
|---|---|---|---|---|
| CC(10/88) | 0.029 (.02-.037) | 0.299 (.092-.506) | 0.050 (.04-.061) | 0.095 (.075-.114) |
| NR | 0.012 (-.033-.057) | 0.346 (.213-.479) | 0.021 (.017-.024) | 0.038 (.028-.050) |
| NRS | 0.014 (-.0002-.028) | 0.542 (.199-.884) | 0.026 (.022-.031) | 0.046 (.035-.058) |
| CCS | 0.022 (.017-.027) | 0.033 (.023-.044) | 0.023 (.018-.028) | 0.033 (.013-.052) |
| CC(10/89) | 0.027 (.009-.045) | 0.153 (.089-.216) | -- | 0.108 (.076-.139) |
| NR | 0.017 (-.002-.037) | 0.093 (.053-.132) | -- | 0.049 (.042-.057) |
| FW(10/89) | -- | 0.067 (.046-.088) | -- | 0.067 (.042-.091) |
| EST | -- | 0.159 (.111-.207) | -- | 0.076 (.038-.114) |
| MRN | -- | 0.167 (.086-.248) | -- | 0.046 (.011-.082) |

| | CELLULOSE | PHENOL | STEARIC ACID |
|---|---|---|---|
| CC(10/88) | 0.133 (.094-.172) | 0.191 (.144-.237) | 0.236 (.197-.274) |
| NR | 0.075 (.064-.087) | 0.101 (.064-.139) | 0.248 (.188-.308) |
| NRS | 0.075 (.060-.089) | 0.252 (.192-.311) | 0.258 (.184-.332) |
| CCS | 0.125 (.094-.156) | 0.053 (.043-.064) | 0.100 (.079-.122) |
| CC(10/89) | -- | 0.182 (.129-.235) | -- |
| NR | -- | 0.205 (.118-.290) | -- |
| FW(10/89) | -- | 0.141 (.101-.180) | -- |
| EST | -- | 0.183 (.106-.261) | -- |
| MRN | -- | 0.112 (.055-.170) | -- |

TABLE 6. Biodegradation first order rate constants (K1). Units are /day. 95% Confidence Intervals are indicated in parentheses.

exception of LAE and phenol, the rates for the chemicals remain relatively constant at each site over time.

Previous exposure of the community to the chemicals results in a consistently higher rate (NR vs. CC) only in the case of LAS, and this trend is consistent from one sampling date to the next. The increased rates in the Calico Creek samples are probably the result of higher numbers of organisms and nutrient concentrations in the sewage enriched waters.

**Biodegradation Along a Salinity Gradient**

In order to determine the effect on detergent chemicals of the many changing physical, chemical, and biological properties of estuaries as the salinity increases, experiments were carried out along a horizontal salinity gradient within the Newport River estuary system. The sites were chosen to represent freshwater, mid-salinity estuarine, and marine waters.

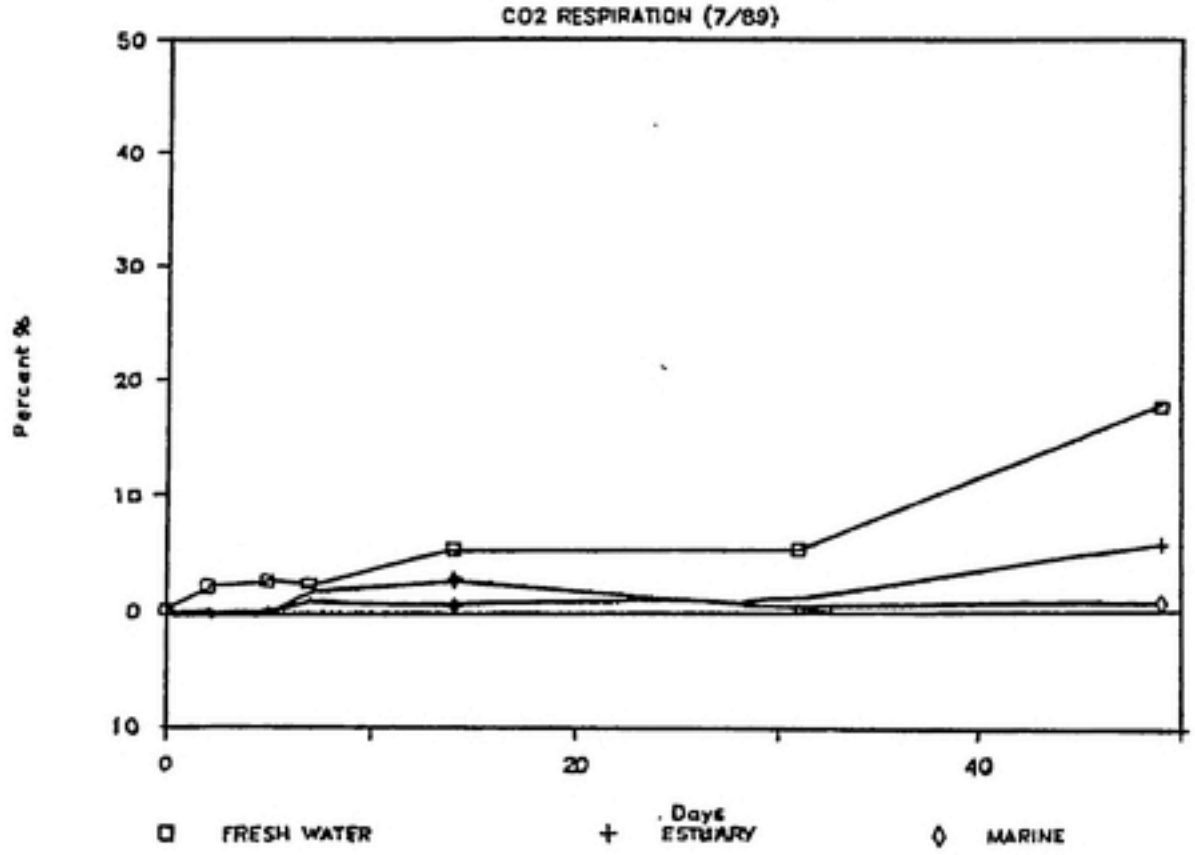
Background environmental data taken in June 1989 for the three sites of the salinity gradient experiment are presented in Table 3. From the data it is apparent that the sites chosen represent a reasonable gradient from freshwater to marine salinity. Amino acid turnover and thymidine uptake data indicate that the marine community is very active heterotrophically, but the freshwater and estuarine communities are more actively dividing to form new cells. Palumbo and Ferguson (1978), in a study using the Newport River Estuary, noted that marine waters tend to have significantly fewer cells than do fresher waters. Temperature remains relatively constant across the gradient, while pH and salinity increase across the gradient.

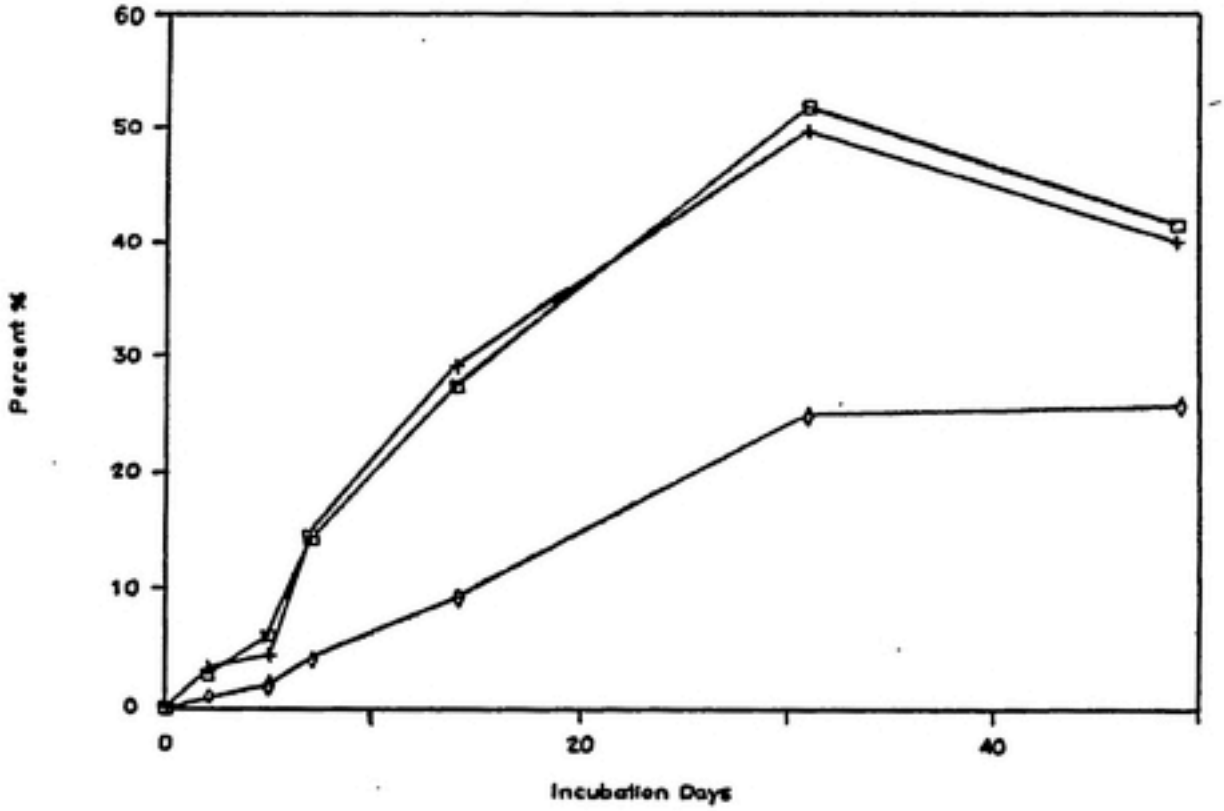
Data for the respiration of three detergent chemicals (LAS, LAE, C18-TMAC) across the salinity gradient was determined for samples from June 1989 and the

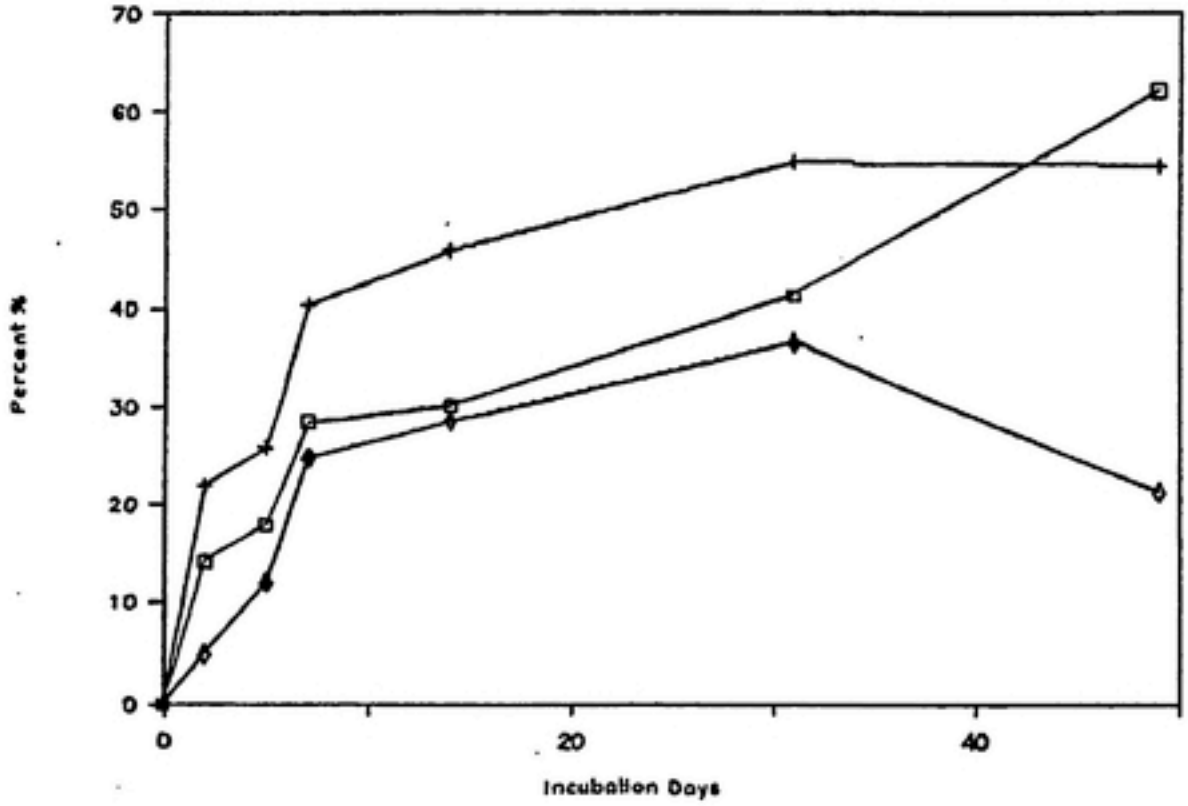
results are shown in Figs. 10-12. Degradation of all three chemicals was greatest in freshwater and estuarine samples. At the marine site, degradation was much less extensive for C18-TMAC and LAE, and insignificant for LAS. The almost total absence of LAS degradation in marine waters is interesting. LAS is not mineralized in these samples even after more than four months of incubation. There are several possible explanations for the absence of LAS degradation in the marine samples. The marine microbial community may not have been induced to degrade LAS by the low concentration used in this study. Alternatively, LAS' mineralization in marine waters may require a concerted attack by a consortium of more than one organism. Removal of the alkyl portion of LAS may be carried out by one or more organisms, while ring cleavage and utilization may be due to other organism(s). If either part of the consortium were absent or inactive, it could result in no observed LAS mineralization. The alkyl moiety of the LAS could have been partially degraded in the marine samples, but would not have been detected here due to the fact that the LAS used was labeled in the aromatic ring. Adaptation for the other compounds shows a patternsimilar to Calico Creek and North River in not being necessary at any of the sites. There appears to be a lag of about a month before the estuarine community begins to significantly mineralize LAS, which is somewhat shorter than the period observed in North River. It may also be possible that the high ionic strength of estuarine and marine waters affects LAS' degradability; ie. complexation with $Ca^{++}$ and $Mg^{++}$ in seawater may hinder biodegradation at low substrate concentrations (Shimp, 1989).

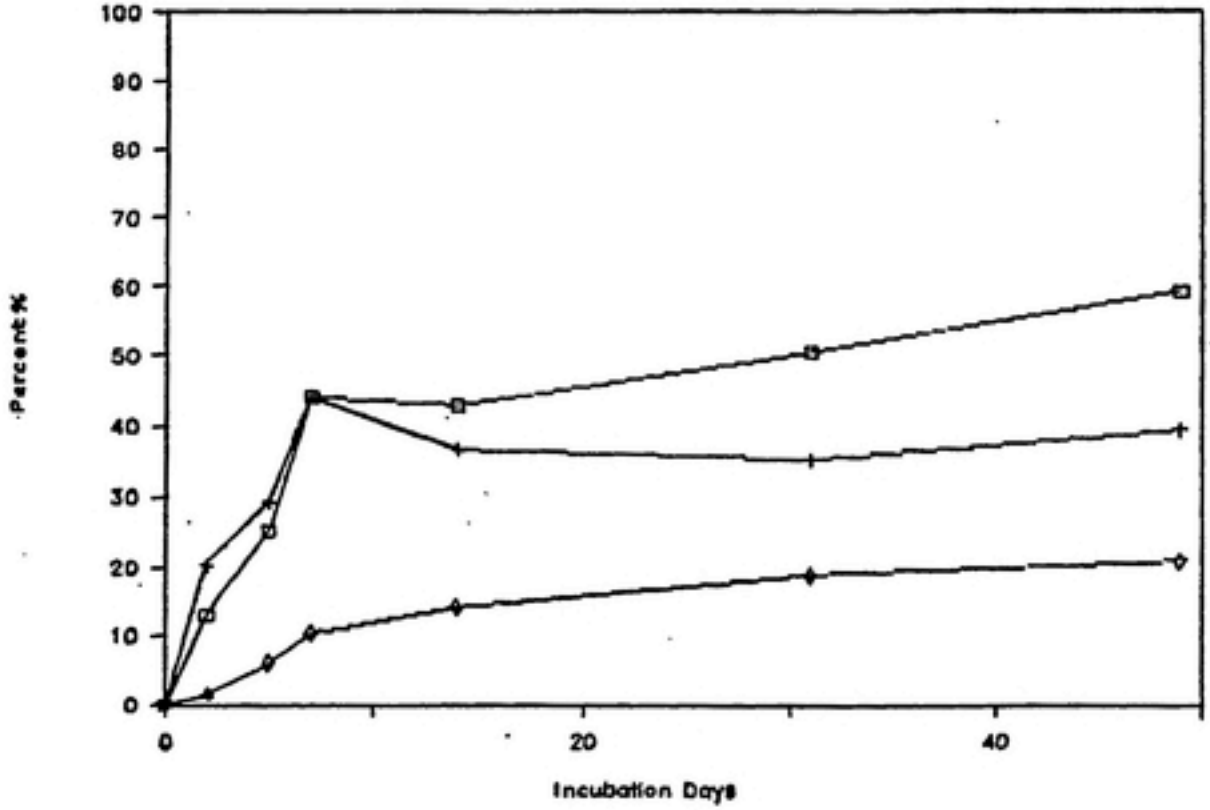
Phenol was used in this experiment as the sole reference compound. Its mineralization pattern (Fig. 13) is not unlike that for the detergent chemicals, with much greater degradation occurring in the freshwater and estuarine environments than in the marine.

# LAS DEGRADATION




60
50
40
30
20
10
0
Percent %
0
20
40
Incubation Days


70
60
50
40
30
20
10
0
Percent %
0
20
40
Incubation Days


100
90
80
70
60
50
40
30
20
10
0
Percent%
0
20
40
Incubation Days

First order rate constants for LAE, C18-TMAC, and phenol in freshwater, midestuarine, and marine samples are shown in Table 6. The mideatuarine samples tend to show the highest rates, and all three communities are slower to degrade C18-TMAC than the other compounds. For LAE, the marine samples show the most rapid rate of degradation, although typically marine communities exibit slower rates compared to fresher waters. Swisher (1987) notes that some bacterial enzymes may be more active at higher salt concentrations, but this has not been studied in conjunction with surfactant biodegradation.

The plateau effect first noted in the impacted vs. unimpacted study is again evident in the salinity gradient results. The plateau is seen with all compounds except LAS, which shows an almost linear increase in percent respired even after 130 days of incubation. Perhaps the communities in the LAS fresh water and estuarine samples have exibited an extremely long adaptaion period and would have leveled off eventually. However, this would mean that incubation would probably extend to six months or more, and the data would have to be viewed with caution due to perturbations caused by enclosing the community for such long periods.

## Nature of the Degradation Plateau and Residual Material

The observed plateau in biodegradation of the chemicals in this study raises several questions about the cause of the plateau and the nature of the material left in solution. Several experiments were designed to address the following questions regarding the degradation plateau: 1. Is it due to prolonged confinement of the organisms (bottle effects); 2. Has the material in solution been metabolized to more recalcitrant products; and 3. Has community metabolism simply stopped? and if so, why?

Since most of the incubations in this report were carried out over at least several months duration, one obvious explanation for the observed plateaus is that the microbial communities in the containers have few active or viable organisms. Prolonged confinement may bias results because it terminates exchange of cells, nutrients, etc., with the surrounding water.

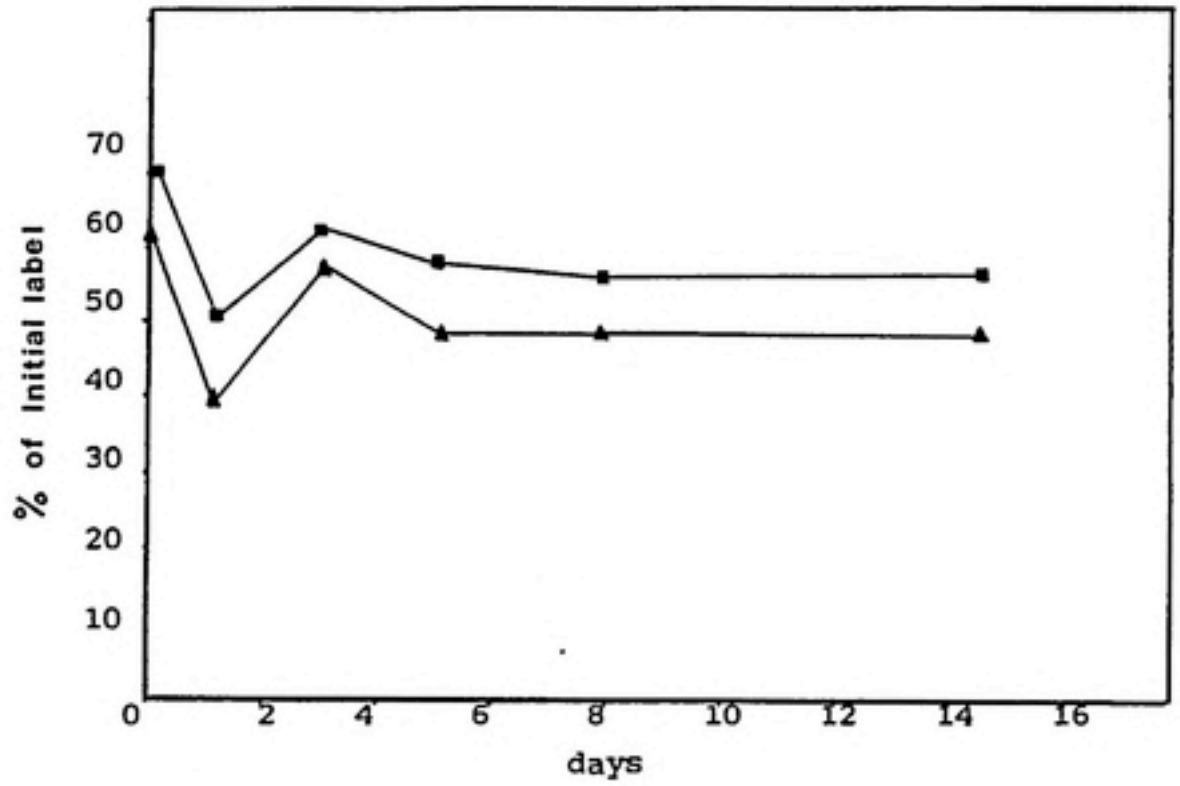
Comparison of Calico Creek community activity after 180 days of incubation with C12 LAS (Table 7) shows that although amino acid turnover time had more than doubled, the organisms are still active. Calculation of $^{14}C$ MPN of LAS degraders suggest that LAS degrading organisms are still present and have not been eliminated from the samples.

Results of experiments to determine the nature of the unmetabolized residual material are presented in Figs. 14-15 for LAS and LAE from Calico Creek samples. Several possible alternative explanations for the plateau were considered: a concentration threshold had been reached, below which no degradation occurs; too few degrading organisms remained to significantly remove the compounds; and confinement had resulted in the depletion of some necessary constituent(s) found in the site water.

For LAS, concentration thresholds were apparently not a factor, as addition of unlabeled LAS failed to stimulate $^{14}CO_2$ production. Addition of fresh organisms from Calico Creek or filtered fresh Calico Creek water also failed to stimulate metabolism. These results tend to suggest that the radiolabel remaining in solution is no longer LAS but is instead a stable degradation product(s). This was tentatively confirmed by experiments in which organisms capable of presumably growing with LAS as a sole carbon source were added to plateaued samples (Fig. 16). Addition failed to stimulate metabolism relative to controls, suggesting that the plateau is not due to a lack of degrading organisms, but is instead due to some

| Day | Amino Acid Turnover (hr) | MPN of LAS Degraders |
|---|---|---|
| 0 | 2.5 | -- |
| 180 | 6.7 | 150/100ml (50-550) |

TABLE 7. Activity of Microbial Community From Calico Creek after 180 days of Incubation With LAS; 95% Confidence Interval for LAS Degrader MPN is given in parentheses.


% of Initial label
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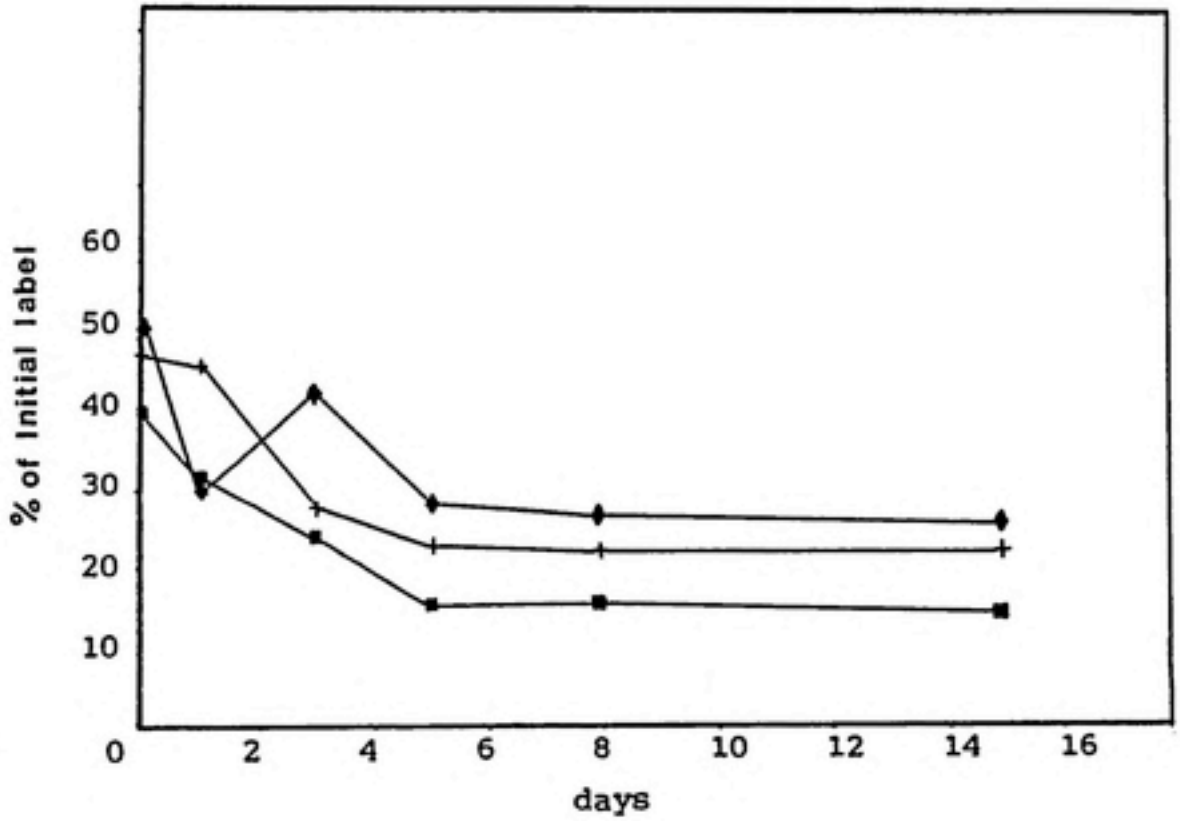
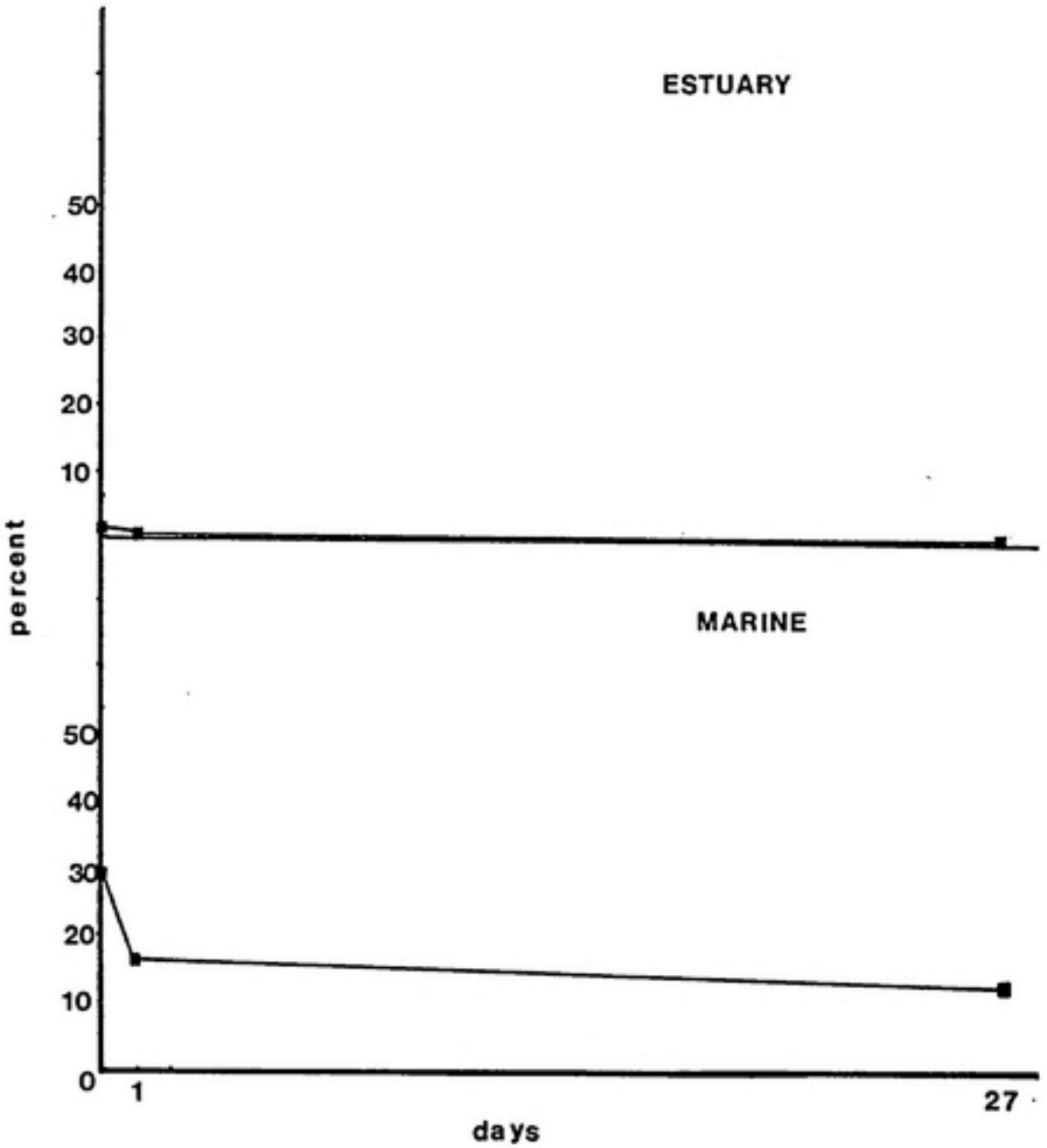
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
days


% of Initial label
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
days


ESTUARY
50
40
30
20
10
percent
MARINE
50
40
30
20
10
0
1
27
days

alteration of the parent material.

For LAE, results (Fig. 15) suggest that the compound declines in solution, but this does not result in $CO_2$ formation, even in samples which received no amendment. The lack of $CO_2$ recovery may have been an experimental artifact. The LAE samples were incubated in a place subject to periodic sunlight, resulting in algal growth which may have consumed the $CO_2$ produced by degradation. Filtration of the sample bottles revealed that the counts were filterable, and that therefore respiration could have been the fate of label leaving solution. Apparently the mixing associated with transferring the samples to smaller containers was sufficient to stimulate metabolism. This suggests that at least for LAE, the plateau could be an artifact of long confinement, and that periodic mixing of sample bottles may not be sufficient. Confinement has been shown to affect the community characteristics of aquatic microbes (Ferguson et al., 1983). Changes in total cell numbers and metabolic activity, as well as shifts from one dominant species to another may occur after confinement. The role of carbon, mineral nutrient, or some trace constituent depletion following long incubations has not been clearly defined, but should not be discounted.

The possibility also exists that recalcitrant byproducts of metabolism are formed with some of the other chemicals in this study, as is postulated here for LAS.

The results shown here for ug/l initial levels of LAS are very similar to those reported by Shimp (1989) for C13 LAS in the Calico Creek system. Shimp found 42% mineralization of LAS in Calico Creek water alone; while addition of 1g/l sediment increased mineralization to 60%. These results closely resemble those of this study for C12 LAS (Figure 3).

Results for C13E6 LAE (Figs. 6&12 and Table 6) show variable agreement

with other studies. Larson et al. (1983), found K1's for C12E9 LAE of approximately 0.22/d in estuarine waters of Florida, which is similar to the K1 of 0.159/d reported here for LAE at the estuarine site(Table 6). However, Larson and Wentler (1982) repot a K1 for C12E9 LAE in freshwater of approximately 0.5/d, which is not in agreement with the freshwater K1 of 0.067/d for the LAE in this study. Unfortunately, there is no data in the literature for C13E6 LAE, so it is not possible to make direct comparisons between data for different LAE's in different estuaries, but it appears that LAE biodegradation may be more variable between estuaries than LAS.

The Newport River Estuary system has also been studied by Palumbo et al. (1988). They reported the biodegradation of NTA and m-cresol across the same salinity gradient sites used in this study. Their results are similar to this study in that uptake of the compounds tended to be greatest in the biologically active midestuary waters and lowest in the offshore marine waters.

## SUMMARY AND CONCLUSIONS

This study has assessed the impact of degradation by natural estuarine and marine microbial communities on the environmental fate of examples of a major class of consumer chemicals, the surfactants. A number of conclusions emerge concerning the fate of these compounds in estuarine and near shore marine environments of North Carolina:

-- Previous exposure of microbial communities to detergent chemicals through treated domestic wastewater appears important only for metabolism of LAS.

-- Biodegradation of these detergent chemicals is more extensive in freshwater and estuarine environments than in marine environments. The reasons for the difference need to be addressed by additional research, but may be due to lack of sufficient available nutrients or growth substrates in marine waters, or some alteration of the chemical or its availability due to the different chemical matrix of seawater

-- LAS does not appear to degrade significantly in marine environments

-- Biodegradation rates and adaptation patterns of the detergent chemicals are not appreciably different than the naturally occurring reference compounds

-- For some compounds and samples, metabolism resulted in a plateau and not in complete or near complete mineralization of the chemicals. At least for LAS, it is postulated to be the result of recalcitrant metabolic intermediates, such as the sulfophenylcarboxylic acids seen in wastewater studies

-- The major fate of all chemicals studied was respiration, significant accumulation into biomass was not observed

-- In general, these detergent chemicals should not be expected to accumulate in estuarine or marine environments, with the possible exception of LAS, which appears not to degrade in marine waters

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# APPENDIX A

The followimg tables represent the percent of each compound which was mineralized to CO2 (CO2 Accum.), incorporated into cell biomass (Net % Filter), and that remained in solution (Ave % Filtrate) for each chemical at each site as discussed in this study.

| Incubation Days | Net % CO2 Accum. | Net % Filter | Ave. % Filtrate | Mass Balance Ave. % |
|---|---|---|---|---|
| C18-TMAC-CC Oct. 1988 | | | | |
| 0 | 0 | 7.5 | 59.2 | 111.9 |
| 3 | 16.6 | 16.3 | 37.1 | 82.7 |
| 7 | 32.5 | 7.5 | 24.9 | 73.3 |
| 11 | 41.5 | 4.4 | 41.9 | 96.5 |
| 23 | 53.7 | 0.4 | 27.6 | 92.4 |
| 35 | 67.0 | 0.3 | 38.1 | 115.7 |
| 46 | 56.4 | 5.2 | 32.2 | 104.6 |
| 53 | 64.7 | 0.6 | 31.0 | 107.3 |
| 78 | 63.9 | 2.0 | 23.7 | 96.3 |
| 92 | 68.8 | 0.1 | 8.9 | 84.4 |
| 120 | 59.9 | 0.0 | 22.6 | 89.4 |
| 168 | 68.1 | 0.0 | 14.0 | 89.4 |
| C18-TMAC-NR Oct. 1988 | | | | |
| 0 | 0 | 2.4 | 48.3 | 121.6 |
| 3 | 5.5 | 11.7 | 33.2 | 65.6 |
| 7 | 19.2 | 18.7 | 38.7 | 84.8 |
| 11 | 33.0 | 1.9 | 43.7 | 88.8 |
| 23 | 46.6 | 2.5 | 37.9 | 102.4 |
| 35 | 31.5 | 0.9 | 43.7 | 88.7 |
| 46 | 59.3 | 6.2 | 39.5 | 117.8 |
| 53 | 65.2 | 1.1 | 42.4 | 121.5 |
| 78 | 68.0 | 0.6 | 29.3 | 106.5 |
| 92 | 69.2 | 0.3 | 12.8 | 90.8 |
| 120 | 65.9 | 0.1 | 25.6 | 100.3 |
| 168 | 70.7 | -0.1 | 16.9 | 96.7 |
| C18-TMAC-CCS Oct. 1988 | | | | |
| 0 | 0 | 9.5 | 70.3 | 114.9 |
| 3 | 5.6 | 13.6 | 18.4 | 50.0 |
| 7 | 6.1 | 7.9 | 14.3 | 41.0 |
| 11 | 7.7 | 0.3 | 16.1 | 35.0 |
| 23 | 12.3 | 2.4 | 14.6 | 41.1 |
| 35 | 27.2 | 0.9 | 15.8 | 80.4 |
| 46 | 13.0 | 1.2 | 13.4 | 40.1 |
| 53 | 16.2 | 0.5 | 13.7 | 43.3 |
| 78 | 18.4 | 0.5 | 11.8 | 41.8 |
| 92 | 21.3 | 0.0 | 5.5 | 35.1 |
| 120 | 31.5 | 0.2 | 9.9 | 49.8 |
| 168 | 25.0 | 0.0 | 7.2 | 40.7 |
| C18-TMAC-NRS Oct. 1988 | | | | |
| 0 | 0 | 4.6 | 50.3 | 115.2 |
| 3 | 5.0 | 6.8 | 32.7 | 55.1 |
| 7 | 15.6 | 17.6 | 33.4 | 75.2 |
| 11 | 24.0 | 10.4 | 37.1 | 79.3 |
| 23 | 46.9 | 2.2 | 35.0 | 93.9 |
| 35 | 60.1 | 1.2 | 42.5 | 113.6 |
| 46 | 53.8 | 4.4 | 36.2 | 104.2 |

| | | | | | |
|---|---|---|---|---|---|
| 53 | 64.8 | 0.6 | 35.2 | 110.3 | 79 |
| 78 | 70.2 | 0.2 | 22.9 | 99.1 | |
| 92 | 56.2 | 0.0 | 11.1 | 73.0 | |
| 120 | 70.7 | 0.1 | 19.9 | 96.2 | |
| 168 | 67.7 | 0.0 | 10.4 | 84.0 | |

| Incubation Days | Net % CO2 Accum | Net % Filter | Ave. % Filtrate | Mass Balance Ave. % |
|---|---|---|---|---|
| C12-TMAC-CC Oct. 1988 | | | | |
| 0 | 0.0 | -0.3 | 73.2 | 112.0 |
| 1 | 7.1 | 21.7 | 44.6 | 89.2 |
| 2 | 12.4 | 70.3 | 34.8 | 126.4 |
| 4 | 16.3 | 15.6 | 30.6 | 74.2 |
| 7 | 20.7 | 9.9 | 23.5 | 62.2 |
| 14 | 34.6 | 4.2 | 38.3 | 89.4 |
| 23 | 46.3 | 1.3 | 27.6 | 84.0 |
| 35 | 51.0 | 0.1 | 29.3 | 91.7 |
| 46 | 54.1 | 3.4 | 24.6 | 93.5 |
| 53 | 62.1 | 0.8 | 24.6 | 99.1 |
| 78 | 65.5 | 0.5 | 19.5 | 94.1 |
| 92 | 67.9 | 0.2 | 9.0 | 85.8 |
| 120 | 68.8 | 0.0 | 17.9 | 95.5 |
| 168 | 66.8 | -0.1 | 11.4 | 87.3 |
| C12-TMAC-NR Oct. 1988 | | | | |
| 0 | 0.0 | 151.1 | 87.3 | 308.4 |
| 1 | 1.8 | 66.8 | 28.7 | 111.7 |
| 2 | 2.1 | 75.0 | 21.5 | 106.9 |
| 4 | 6.3 | 37.6 | 27.2 | 82.0 |
| 7 | 7.8 | 28.3 | 36.4 | 80.8 |
| 14 | 19.4 | 11.2 | 49.0 | 92.0 |
| 23 | 27.0 | 5.7 | 30.3 | 73.2 |
| 35 | 38.3 | 4.4 | 36.8 | 90.8 |
| 46 | 38.4 | 10.7 | 29.7 | 90.3 |
| 53 | 49.4 | 3.9 | 30.9 | 95.8 |
| 78 | 58.0 | 1.1 | 24.7 | 91.6 |
| 92 | 66.8 | 0.2 | 13.9 | 88.8 |
| 120 | 62.7 | 0.0 | 23.7 | 94.1 |
| 168 | 69.3 | 0.0 | 16.1 | 93.4 |
| C12-TMAC-CCS Oct. 1988 | | | | |
| 0 | 0.0 | 11.8 | 44.8 | 101.6 |
| 1 | 4.9 | 17.6 | 31.2 | 66.9 |
| 2 | 4.7 | 35.8 | 20.0 | 69.4 |
| 4 | 6.1 | 8.8 | 22.9 | 50.2 |
| 7 | 8.2 | 7.3 | 13.5 | 37.4 |
| 14 | 17.2 | 1.5 | 27.3 | 57.5 |
| 23 | 25.5 | 0.1 | 30.2 | 66.9 |
| 35 | 35.2 | 0.6 | 24.4 | 70.1 |
| 46 | 43.6 | 1.2 | 19.9 | 76.5 |
| 53 | 48.2 | 0.6 | 20.2 | 79.2 |
| 78 | 56.6 | 0.5 | 14.2 | 79.4 |

| | | | | |
|---|---|---|---|---|
| 92 | 56.8 | 0.4 | 6.5 | 72.1 |
| 120 | 67.5 | 0.0 | 11.0 | 87.0 |
| 168 | 60.7 | -0.1 | 7.3 | 81.0 |

C12-TMAC-NRS Oct. 1988

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | -19.5 | 75.5 | 131.8 |
| 1 | 0.5 | 57.8 | 23.0 | 94.9 |
| 2 | 0.5 | 70.3 | 23.3 | 104.1 |
| 4 | 6.3 | 25.3 | 26.9 | 69.9 |
| 7 | 9.7 | 19.3 | 31.5 | 68.6 |
| 14 | 20.2 | 10.7 | 37.1 | 80.2 |
| 23 | 27.8 | 3.4 | 25.2 | 65.5 |
| 35 | 46.9 | 1.6 | 32.6 | 92.6 |
| 46 | 42.6 | 3.7 | 29.0 | 86.9 |
| 53 | 51.6 | 0.2 | 30.6 | 94.1 |
| 78 | 59.2 | 0.5 | 21.5 | 88.8 |
| 92 | 60.0 | 0.2 | 10.9 | 78.8 |
| 120 | 68.0 | 0.1 | 17.3 | 93.2 |
| 168 | 63.4 | -0.1 | 14.5 | 86.1 |

C13E6-LAE-CC Oct. 1988

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | 0.8 | 77.6 | 81.3 |
| 2 | 25.1 | 2.3 | 101.3 | 137.3 |
| 4 | 32.9 | 6.6 | 46.9 | 94.5 |
| 7 | 41.4 | 4.8 | 66.6 | 118.3 |
| 14 | 37.0 | 0.3 | 66.5 | 110.6 |
| 22 | 29.0 | 0.1 | 49.7 | 86.2 |
| 35 | 34.1 | 0.3 | 48.9 | 95.1 |
| 49 | 34.9 | 0.1 | 20.9 | 63.1 |
| 70 | 59.5 | 0.2 | 23.0 | 87.2 |
| 89 | 67.9 | 0.4 | 13.3 | 86.7 |
| 110 | 61.4 | 0.1 | 28.0 | 95.1 |

C13E6-LAE-NR-Oct.1988

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | 2.4 | 74.0 | 79.9 |
| 2 | 16.8 | 0.5 | 82.5 | 108.3 |
| 4 | 31.1 | 2.7 | 61.2 | 112.5 |
| 7 | 46.0 | 0.2 | 72.6 | 123.8 |
| 14 | 48.0 | 0.0 | 60.8 | 115.4 |
| 22 | 44.2 | 0.3 | 52.5 | 103.8 |
| 35 | 40.2 | 0.2 | 46.1 | 93.3 |
| 49 | 34.7 | 0.0 | 45.1 | 85.8 |
| 70 | 40.2 | 0.7 | 49.5 | 94.9 |
| 89 | 42.9 | 0.6 | 40.4 | 90.0 |
| 110 | 43.5 | -0.2 | 42.7 | 93.0 |

C13E6-LAE-CCS Oct. 1988

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | 0.6 | 61.9 | 66.9 |
| 2 | 18.6 | 1.3 | 60.2 | 90.1 |
| 4 | 18.4 | 3.0 | 53.9 | 87.6 |
| 7 | 19.2 | 1.4 | 54.7 | 89.0 |
| 14 | 23.5 | 0.1 | 61.3 | 99.6 |
| 22 | 28.7 | 0.1 | 47.4 | 89.7 |
| 35 | 48.0 | 0.2 | 12.8 | 73.4 |
| 49 | 49.4 | 0.1 | 6.1 | 66.8 |

| | | | | |
|---|---|---|---|---|
| 70 | 65.4 | 0.1 | 23.5 | 96.9 |
| 89 | 66.4 | 0.3 | 6.1 | 81.0 |
| 110 | 64.5 | 0.1 | 17.8 | 90.1 |
| C13E6-LAE-NRS Oct. 1988 | | | | |
| 0 | 0.0 | 1.2 | 49.0 | 53.7 |
| 2 | 14.7 | 0.2 | 73.8 | 98.0 |
| 4 | 41.2 | 1.2 | 53.4 | 105.5 |
| 7 | 45.2 | 0.7 | 65.8 | 117.1 |
| 14 | 32.3 | 0.3 | 56.0 | 103.1 |
| 22 | 35.6 | 0.3 | 44.3 | 95.0 |
| 35 | 31.8 | 0.1 | 34.5 | 79.0 |
| 49 | 28.2 | 0.1 | 36.0 | 77.5 |
| 70 | 32.5 | 0.1 | 39.7 | 81.8 |
| 89 | 36.7 | 0.2 | 26.6 | 75.3 |
| 110 | 39.9 | 0.3 | 27.2 | 79.1 |

| Incubation Days | Net % CO2 Accum. | Net % Filter | Ave.% Filtrate | Mass Balance Ave.% |
|---|---|---|---|---|
| LAS-CC Oct. 1988 | | | | |
| 0 | 0.0 | 15.6 | 5122.6 | 5182.0 |
| 1 | 6.9 | 29.3 | 153.1 | 203.9 |
| 4 | 6.9 | 8.6 | 70.7 | 96.1 |
| 7 | 11.1 | 4.0 | 79.1 | 102.3 |
| 14 | 12.5 | 1.3 | 59.9 | 90.9 |
| 23 | 18.0 | 0.1 | 48.4 | 78.1 |
| 35 | 30.1 | 0.6 | 46.8 | 86.5 |
| 46 | 36.8 | 4.6 | 45.1 | 95.1 |
| 53 | 39.6 | 8.4 | 48.3 | 105.6 |
| 78 | 35.5 | 0.6 | 41.3 | 85.9 |
| 92 | 43.8 | 0.0 | 29.0 | 81.6 |
| 120 | 47.8 | 0.0 | 38.5 | 95.1 |
| 168 | 44.5 | 1.0 | 30.9 | 85.3 |
| LAS-NR-Oct. 1988 | | | | |
| 0 | 0.0 | -393.1 | 4299.5 | 4623.0 |
| 1 | 0.1 | 41.8 | 103.1 | 159.3 |
| 4 | 0.2 | 13.0 | 70.0 | 94.1 |
| 7 | 0.1 | 7.9 | 85.7 | 101.4 |
| 14 | 3.0 | 1.4 | 94.6 | 112.2 |
| 23 | 0.0 | 1.2 | 79.4 | 90.6 |
| 35 | 0.1 | 0.3 | 80.8 | 89.5 |
| 46 | 0.0 | 0.7 | 79.7 | 87.2 |
| 53 | 0.2 | 0.6 | 84.1 | 92.0 |
| 78 | 0.1 | 0.7 | 94.4 | 100.3 |
| 92 | 0.6 | 0.4 | 91.5 | 97.6 |
| 120 | 4.1 | 0.3 | 91.2 | 101.0 |
| 168 | 14.6 | 0.1 | 75.1 | 97.0 |
| 175 | 17.4 | 0.0 | 40.4 | 68.8 |
| LAS-CCS-Oct. 1988 | | | | |
| 0 | 0.0 | 4.4 | 260.1 | 299.0 |

| | | | | |
|---|---|---|---|---|
| 1 | 6.2 | 13.1 | 99.1 | 133.1 |
| 4 | 8.0 | 11.4 | 65.7 | 99.3 |
| 7 | 11.4 | 2.3 | 68.8 | 90.4 |
| 14 | 15.0 | 1.0 | 72.3 | 101.5 |
| 23 | 16.8 | 3.2 | 55.5 | 87.3 |
| 35 | 31.1 | 0.5 | 55.7 | 98.9 |
| 46 | 43.4 | 1.1 | 41.5 | 99.3 |
| 53 | 51.2 | 1.1 | 38.9 | 103.0 |
| 78 | 52.4 | 0.6 | 32.4 | 94.8 |
| 92 | 51.8 | 0.2 | 21.4 | 83.0 |
| 120 | 62.8 | 0.1 | 22.8 | 95.5 |
| 168 | 58.3 | -0.1 | 14.4 | 82.6 |

LAS-NRS-Oct. 1988

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | 5.9 | 326.0 | 386.3 |
| 1 | 0.7 | 30.3 | 64.5 | 109.6 |
| 4 | 0.0 | 15.1 | 29.0 | 54.5 |
| 7 | 0.1 | 6.2 | 77.3 | 91.3 |
| 14 | 0.5 | 1.1 | 91.2 | 105.4 |
| 23 | 1.7 | 9.6 | 73.4 | 94.7 |
| 35 | 0.1 | 0.1 | 86.8 | 95.5 |
| 46 | 0.2 | 0.2 | 92.0 | 99.1 |
| 53 | 0.8 | 0.0 | 86.5 | 94.7 |
| 78 | 9.8 | 0.8 | 81.1 | 97.1 |
| 92 | 13.1 | 0.1 | 71.8 | 90.8 |
| 120 | 21.3 | -0.1 | 71.0 | 98.1 |
| 168 | 34.4 | 0.2 | 46.9 | 87.2 |
| 175 | 28.7 | 0.0 | 21.0 | 67.2 |

| Incubation Days | Net % CO2 Accum. | Net % Filter | Ave. % Filtrate | Mass Balance Ave % |
|---|---|---|---|---|
| Cellulose-CC- Oct. 1988 | | | | |
| 0 | 0.0 | -7.2 | 42.0 | 86.6 |
| 2 | 2.8 | 7.6 | 42.5 | 64.6 |
| 4 | 16.1 | 5.3 | 32.2 | 65.0 |
| 7 | 29.9 | 0.5 | 40.4 | 79.2 |
| 14 | 34.9 | 0.8 | 26.7 | 73.0 |
| 22 | 39.0 | 0.3 | 22.1 | 68.7 |
| 35 | 36.6 | 0.3 | 15.1 | 60.1 |
| 49 | 39.2 | 0.1 | 13.4 | 60.5 |
| 70 | 41.6 | 0.2 | 26.2 | 75.7 |
| 89 | 42.6 | 2.5 | 12.8 | 66.0 |
| 110 | 43.8 | 0.8 | 14.9 | 68.8 |
| Celloluse-NR- Oct. 1988 | | | | |
| 0 | 0.0 | -14.6 | 44.4 | 90.8 |
| 2 | 2.6 | 3.1 | 39.3 | 57.0 |
| 4 | 10.6 | 1.8 | 33.2 | 56.6 |
| 7 | 18.9 | 0.2 | 32.5 | 59.8 |
| 14 | 28.0 | 0.5 | 26.1 | 64.7 |
| 22 | 32.4 | 0.1 | 22.5 | 62.4 |
| 35 | 36.9 | 0.1 | 15.1 | 59.4 |
| 49 | 36.1 | 0.1 | 14.6 | 57.6 |

| | | | | |
|---|---|---|---|---|
| 70 | 43.1 | 0.0 | 25.9 | 75.0 |
| 89 | 40.9 | 0.3 | 12.9 | 61.2 |
| 110 | 42.6 | 0.3 | 14.7 | 66.9 |
| Celloluse-CCS- Oct 1988 | | | | |
| 0 | 0.0 | -30.6 | 44.0 | 89.3 |
| 2 | 3.2 | 0.0 | 32.7 | 52.0 |
| 4 | 14.3 | 0.5 | 28.2 | 54.5 |
| 7 | 26.0 | 0.7 | 33.2 | 66.5 |
| 14 | 34.4 | 0.2 | 34.0 | 75.7 |
| 22 | 37.9 | 0.2 | 17.0 | 62.3 |
| 35 | 30.2 | 0.2 | 11.3 | 50.1 |
| 49 | 37.5 | 0.4 | 11.1 | 55.6 |
| 70 | 42.4 | 0.2 | 21.9 | 70.8 |
| 89 | 40.9 | 0.0 | 10.1 | 58.6 |
| 110 | 41.9 | 0.0 | 17.8 | 67.4 |
| Cellulose-NRS-Oct. 1988 | | | | |
| 0 | 0.0 | 15.2 | 58.7 | 107.8 |
| 2 | 1.2 | 3.6 | 37.7 | 53.9 |
| 4 | 9.6 | 1.6 | 30.4 | 52.5 |
| 7 | 18.4 | 0.3 | 33.9 | 59.7 |
| 14 | 26.8 | 0.3 | 32.1 | 68.2 |
| 22 | 27.3 | 0.3 | 22.0 | 57.2 |
| 35 | 31.5 | 0.3 | 14.8 | 54.4 |
| 49 | 33.8 | 0.3 | 14.1 | 54.8 |
| 70 | 40.8 | 0.2 | 27.6 | 74.7 |
| 89 | 40.2 | 0.1 | 13.2 | 60.6 |
| 110 | 39.9 | 0.1 | 19.7 | 66.4 |

| Incubation Days | Net % $CO_2$ Accum. | Net % Filter | Ave.% Filtrate | Mass Balance Ave. % |
|---|---|---|---|---|
| Phenol-CC- Oct. 1988 | | | | |
| 0 | 0.0 | 1.4 | 110.9 | 119.5 |
| 2 | 11.4 | 7.3 | 52.8 | 87.1 |
| 4 | 29.6 | 36.5 | 47.5 | 126.6 |
| 7 | 49.9 | 6.7 | 52.0 | 119.9 |
| 14 | 45.7 | 0.4 | 42.8 | 105.5 |
| 22 | 49.2 | -0.2 | 21.8 | 82.0 |
| 35 | 47.0 | 0.5 | 22.8 | 81.5 |
| 49 | 48.8 | 0.1 | 17.8 | 77.4 |
| 70 | 54.7 | 0.0 | 20.7 | 84.7 |
| 89 | 58.9 | 0.1 | 11.2 | 78.6 |
| 110 | 60.1 | 0.3 | 11.7 | 81.6 |
| Phenol-NR- Oct. 1988 | | | | |
| 0 | 0.0 | 1.6 | 120.1 | 126.7 |
| 2 | 7.3 | 3.3 | 61.3 | 83.0 |
| 4 | 10.1 | 9.7 | 43.3 | 86.4 |
| 7 | 14.1 | 2.0 | 46.0 | 99.7 |
| 14 | 55.7 | 0.2 | 32.9 | 100.8 |
| 22 | 50.9 | 0.1 | 27.5 | 87.8 |
| 35 | 42.9 | 0.4 | 15.1 | 66.8 |

| | | | | |
|---|---|---|---|---|
| 49 | 43.5 | 0.2 | 14.8 | 69.2 |
| 70 | 46.3 | 0.5 | 23.2 | 78.1 |
| 89 | 49.3 | 0.1 | 13.4 | 70.6 |
| 110 | 41.3 | 0.1 | 10.5 | 61.3 |
| Phenol-CCS- Oct. 1988 | | | | |
| 0 | 0.0 | -0.4 | 60.1 | 67.5 |
| 2 | 7.9 | 2.2 | 49.9 | 83.2 |
| 4 | 9.3 | 0.6 | 31.5 | 72.3 |
| 7 | 11.9 | 1.0 | 34.0 | 80.6 |
| 14 | 31.4 | 0.2 | 37.1 | 89.8 |
| 22 | 32.9 | 0.2 | 21.2 | 73.6 |
| 35 | 39.1 | 0.1 | 14.1 | 71.3 |
| 49 | 41.5 | 0.8 | 7.9 | 66.0 |
| 70 | 49.8 | 0.0 | 18.0 | 80.5 |
| 89 | 48.6 | 0.0 | 7.1 | 69.7 |
| 110 | 50.0 | 0.0 | 15.5 | 78.8 |
| Phenol-NRS- Oct. 1988 | | | | |
| 0 | 0.0 | 2.0 | 75.7 | 82.5 |
| 2 | 11.6 | 3.4 | 51.5 | 77.8 |
| 4 | 31.7 | 8.9 | 41.8 | 91.0 |
| 7 | 52.6 | 0.5 | 47.1 | 109.0 |
| 14 | 51.9 | 0.1 | 26.3 | 86.3 |
| 22 | 51.0 | 0.1 | 16.3 | 74.4 |
| 35 | 49.0 | 0.1 | 11.6 | 68.7 |
| 49 | 47.4 | 0.3 | 11.8 | 68.8 |
| 70 | 49.7 | 0.1 | 13.4 | 72.5 |
| 89 | 52.8 | 0.2 | 9.2 | 70.4 |
| 110 | 54.8 | -0.2 | 11.8 | 73.2 |
| LAS-NR- Oct. 1989 | | | | |
| 0 | 0.0 | -4.5 | 40.8 | 60.0 |
| 1 | 0.0 | -0.3 | 64.5 | 112.3 |
| 4 | 0.3 | -6.5 | 48.8 | 89.4 |
| 9 | 0.8 | 21.4 | 30.7 | 101.9 |
| 30 | 2.9 | -0.1 | 57.8 | 98.9 |
| 57 | 18.0 | -0.5 | 36.8 | 96.7 |
| 118 | 19.2 | 18.6 | 53.5 | 138.3 |

| Incubation Days | Net % CO2 Accum. | Net % Filter | Ave. % Filtrate | Mass Balance Ave.% |
|---|---|---|---|---|
| LAS-CC- Oct 1989 | | | | |
| 0 | 0.0 | -8.0 | 46.6 | 66.5 |
| 1 | 2.0 | -5.1 | 60.3 | 106.3 |
| 4 | 4.5 | 0.0 | 40.2 | 87.3 |
| 9 | 7.4 | 13.3 | 44.7 | 113.0 |
| 30 | 9.4 | 3.4 | 73.6 | 130.4 |
| 57 | 17.9 | 12.5 | 47.0 | 143.9 |
| 118 | 21.3 | 0.7 | 55.4 | 125.5 |
| C18-TMAC-NR- Oct. 1989 | | | | |
| 0 | 0.0 | -16.2 | 19.3 | 68.1 |

| | | | | | |
|---|---|---|---|---|---|
| 1 | 1.3 | 1.6 | 18.7 | 59.7 | 85 |
| 4 | 10.4 | 0.8 | 17.3 | 35.4 | |
| 9 | 17.2 | -0.1 | 19.4 | 46.5 | |
| 30 | 46.9 | 0.4 | 8.1 | 60.5 | |
| 57 | 63.2 | 0.8 | 39.5 | 111.5 | |
| 118 | 61.0 | -1.3 | 26.5 | 94.7 | |
| C18-TMAC-CC- Oct. 1989 | | | | | |
| 0 | 0.0 | 0.5 | 14.5 | 67.9 | |
| 1 | 4.0 | 0.0 | 12.0 | 32.9 | |
| 4 | 9.7 | -2.1 | 6.6 | 21.4 | |
| 9 | 14.4 | -1.0 | 12.7 | 34.3 | |
| 30 | 21.0 | 0.2 | 6.8 | 33.0 | |
| 57 | 28.1 | 0.1 | 21.7 | 57.8 | |
| 118 | 22.6 | -0.1 | 12.3 | 50.0 | |
| LAE-NR- Oct. 1989 | | | | | |
| 0 | 0.0 | -1.2 | 43.5 | 53.9 | |
| 1 | 20.5 | -8.2 | 45.2 | 75.3 | |
| 4 | 26.9 | 1.1 | 45.4 | 76.8 | |
| 9 | 31.7 | 0.0 | 48.4 | 85.2 | |
| 30 | 49.9 | 1.1 | 18.9 | 75.2 | |
| 57 | 68.9 | -1.4 | 26.7 | 104.6 | |
| 118 | 66.3 | -1.2 | 20.1 | 98.6 | |
| LAE-CC- Oct. 1989 | | | | | |
| 0 | 0.0 | 1.9 | 28.9 | 43.4 | |
| 1 | 28.5 | 2.4 | 18.5 | 60.2 | |
| 4 | 34.7 | 0.9 | 15.2 | 58.4 | |
| 9 | 40.8 | 0.5 | 17.0 | 63.4 | |
| 30 | 60.8 | 0.1 | 4.8 | 70.2 | |
| 57 | 74.5 | 0.6 | 16.2 | 96.7 | |
| 118 | 70.4 | -1.7 | 5.7 | 85.3 | |
| Phenol- NR- Oct. 1989 | | | | | |
| 0 | 0.0 | -1.6 | 86.4 | 95.4 | |
| 1 | -0.9 | -1.7 | 78.0 | 97.2 | |
| 4 | 39.4 | -1.4 | 13.1 | 68.2 | |
| 9 | 46.9 | 1.3 | 20.6 | 82.1 | |
| 30 | 47.3 | 0.2 | 15.5 | 76.8 | |
| 57 | 61.8 | 0.0 | 29.9 | 107.7 | |
| 118 | 63.7 | -1.3 | 27.8 | 113.1 | |

| Incubation Days | Net % $CO_2$ Accum. | Net % Filter | Ave. % Filtrate | Mass Balance Ave. % |
|---|---|---|---|---|
| Phenol-CC- Oct. 1989 | | | | |
| 0 | 0.0 | 0.4 | 87.0 | 95.6 |
| 1 | 4.3 | 0.6 | 74.3 | 94.4 |
| 4 | 36.3 | 0.2 | 10.6 | 63.5 |
| 9 | 44.4 | -0.4 | 15.8 | 71.4 |
| 30 | 52.8 | -0.1 | 7.7 | 73.5 |
| 57 | 58.8 | -1.0 | 15.2 | 89.9 |
| 118 | 66.0 | 0.2 | 10.0 | 85.3 |

LAS-FW- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | -0.9 | 73.0 | 86.6 |
| 2 | 2.2 | -0.8 | 82.6 | 112.3 |
| 5 | 2.7 | -0.4 | 86.2 | 115.9 |
| 7 | 2.4 | -1.2 | 75.9 | 100.7 |
| 14 | 5.4 | -0.1 | 82.4 | 109.2 |
| 31 | 5.3 | 0.1 | 104.9 | 136.9 |
| 49 | 17.8 | -1.0 | 57.8 | 111.4 |
| 82 | 38.7 | -3.9 | 30.9 | 91.5 |
| 133 | 67.0 | 0.8 | 30.7 | 119.6 |

LAS-EST- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | -3.0 | 68.9 | 82.7 |
| 2 | -0.2 | -0.9 | 80.5 | 125.6 |
| 5 | 0.2 | 0.7 | 107.2 | 134.8 |
| 7 | 0.9 | 0.2 | 93.3 | 117.6 |
| 14 | 0.6 | 0.6 | 76.8 | 99.4 |
| 31 | 1.1 | -1.2 | 114.7 | 140.3 |
| 49 | 6.6 | -0.4 | 79.7 | 114.8 |
| 82 | 15.6 | -5.0 | 43.4 | 79.9 |
| 133 | 51.4 | -0.1 | 55.1 | 128.2 |

LAS-MRN- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | -0.2 | 58.5 | 71.5 |
| 2 | -0.5 | 1.0 | 67.2 | 95.2 |
| 5 | -0.2 | 0.1 | 63.9 | 90.9 |
| 7 | 1.6 | -0.8 | 76.2 | 100.3 |
| 14 | 2.8 | -0.7 | 81.0 | 106.2 |
| 31 | 0.4 | -2.4 | 106.0 | 129.4 |
| 49 | 1.0 | 0.5 | 90.7 | 120.9 |
| 82 | -0.3 | -4.9 | 91.8 | 112.8 |
| 133 | 1.1 | -0.4 | 97.3 | 120.1 |

LAE-FW- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | 1.6 | 43.7 | 50.1 |
| 2 | 14.2 | 0.1 | 42.0 | 66.5 |
| 5 | 18.0 | -1.5 | 47.5 | 70.4 |
| 7 | 28.4 | -1.3 | 35.7 | 68.9 |
| 14 | 30.6 | -1.3 | 45.6 | 81.5 |
| 31 | 41.3 | 0.1 | 29.4 | 76.1 |
| 49 | 61.4 | 0.2 | 7.2 | 75.1 |
| 82 | 58.1 | 0.9 | 3.4 | 65.8 |

| Incubation Days | Net % $CO_2$ Accum. | Net % Filter | Ave.% Filtrate | Mass Balance Ave. % |
|---|---|---|---|---|
| LAE-EST- June 1989 | | | | |
| 0 | 0.0 | -0.2 | 39.8 | 43.2 |
| 2 | 21.9 | -1.1 | 35.3 | 66.1 |

| | | | | | |
|---|---|---|---|---|---|
| 5 | 25.8 | -1.0 | 37.6 | 69.5 | 87 |
| 7 | 40.3 | -0.1 | 29.6 | 76.8 | |
| 14 | 46.0 | -1.9 | 40.7 | 92.9 | |
| 31 | 54.4 | -0.2 | 33.4 | 92.6 | |
| 49 | 54.8 | 0.1 | 10.9 | 70.9 | |
| 82 | 59.5 | -0.8 | 9.7 | 73.3 | |

LAE-MRN- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | 0.1 | 43.4 | 46.6 |
| 2 | 4.8 | 2.2 | 44.2 | 63.2 |
| 5 | 12.2 | 0.7 | 53.3 | 72.2 |
| 7 | 24.3 | -0.7 | 47.1 | 79.8 |
| 14 | 28.5 | -1.2 | 67.7 | 102.6 |
| 31 | 35.8 | 0.1 | 60.2 | 101.5 |
| 49 | 21.2 | -0.5 | 35.7 | 63.2 |
| 82 | 27.6 | 1.2 | 22.7 | 55.7 |

C18-TMAC-FW- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | 5.7 | 21.1 | 68.5 |
| 2 | 2.8 | -1.0 | 23.3 | 56.4 |
| 5 | 6.0 | 0.5 | 18.1 | 31.7 |
| 7 | 14.1 | 0.2 | 28.5 | 51.7 |
| 14 | 27.5 | -0.4 | 42.8 | 77.2 |
| 31 | 52.1 | 0.1 | 26.1 | 84.1 |
| 49 | 41.9 | 0.8 | 12.1 | 61.8 |
| 82 | 38.0 | 0.1 | 6.3 | 48.6 |

C18-TMAC-EST- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | -4.5 | 28.0 | 61.5 |
| 2 | 3.2 | 0.8 | 29.9 | 60.0 |
| 5 | 4.4 | 1.4 | 21.6 | 33.7 |
| 7 | 14.7 | -1.2 | 22.5 | 58.6 |
| 14 | 29.2 | 0.1 | 36.7 | 73.9 |
| 31 | 47.8 | 0.0 | 36.7 | 89.8 |
| 49 | 40.2 | 0.4 | 12.1 | 58.2 |
| 82 | 28.7 | 0.6 | 8.0 | 40.8 |

C18-TMAC-MRN- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | -7.4 | 31.2 | 53.9 |
| 2 | 0.7 | 2.7 | 25.4 | 56.6 |
| 5 | 1.8 | -0.2 | 77.3 | 86.0 |
| 7 | 3.9 | -0.6 | 23.8 | 47.8 |
| 14 | 9.3 | 0.0 | 45.2 | 74.7 |
| 31 | 25.2 | -2.7 | 41.8 | 84.9 |
| 49 | 26.0 | 0.0 | 18.9 | 58.7 |
| 82 | 16.6 | -9.6 | 12.4 | 34.4 |

Phenol-FW- June 1989

| | | | | |
|---|---|---|---|---|
| 0 | 0.0 | 0.6 | 58.3 | 77.2 |
| 2 | 13.0 | 10.4 | 25.6 | 61.6 |
| 5 | 25.3 | -1.5 | 34.0 | 68.9 |
| 7 | 44.2 | 1.5 | 14.6 | 68.4 |
| 14 | 42.9 | 0.0 | 19.9 | 73.9 |
| 31 | 50.5 | 0.1 | 17.1 | 75.6 |

| | | | | |
|---|---|---|---|---|
| 49 | 59.4 | -0.1 | 13.0 | 80.3 |
| 82 | 60.6 | -2.5 | 7.8 | 75.2 |

| Incubation Days | Net % CO2 Accum. | Net % Filter | Ave. % Filtrate | Mass Balance Ave. % |
|---|---|---|---|---|
| Phenol-EST- June 1989 | | | | |
| 0 | 0.0 | -3.2 | 52.7 | 73.5 |
| 2 | 20.3 | 2.4 | 23.1 | 60.3 |
| 5 | 29.2 | 4.9 | 28.7 | 81.8 |
| 7 | 44.1 | 1.2 | 16.2 | 72.8 |
| 14 | 36.6 | -0.3 | 33.3 | 87.8 |
| 31 | 55.2 | 0.4 | 29.0 | 92.4 |
| 49 | 61.0 | -0.2 | 17.2 | 86.5 |
| 82 | 53.3 | -2.8 | 11.2 | 72.6 |
| Phenol-MRN- June 1989 | | | | |
| 0 | 0.0 | -0.5 | 100.7 | 107.4 |
| 2 | 1.6 | 0.0 | 85.2 | 101.4 |
| 5 | 5.9 | 2.0 | 36.5 | 59.0 |
| 7 | 10.2 | 1.2 | 78.6 | 102.9 |
| 14 | 14.1 | -0.6 | 84.7 | 119.2 |
| 31 | 18.8 | 0.4 | 76.4 | 104.5 |
| 49 | 20.8 | 0.0 | 66.7 | 96.6 |
| 82 | 11.8 | -2.2 | 43.4 | 71.7 |