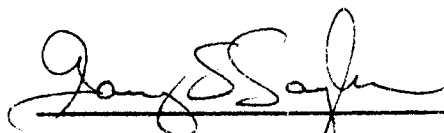


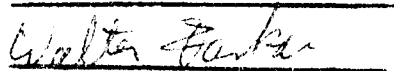
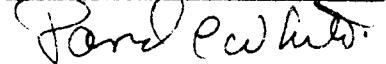
To the Graduate Council:

I am submitting herewith a dissertation written by Alexander W. Breen entitled "Isolation and Characterization of Linear Alkylbenzene Sulfonate (LAS) Mineralizing Bacterial Consortia". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.



Dr. Gary S. Sayler, Major Professor

We have read this dissertation
and we recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor
and Dean of the Graduate School

Isolation and Characterization of
Linear Alkylbenzene Sulfonate Mineralizing
Bacterial Consortia

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Alexander W. Breen

August, 1992

DEDICATION

This thesis is dedicated to my parents,

Thelma and Alexander Breen

for their encouragement, support and love.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Gary Sayler, my major professor, for his guidance and encouragement. It has been an honor to serve in his laboratory in various roles for the past ten years. I would like to thank my committee members, Dr. Walter Farkas, Dr. Gerald Strandberg, Dr. Barbara Walton and Dr. David White for their assistance, input and encouragement throughout this project. I would also like to thank Dr. Luis Jimenez whose collaboration on this project was invaluable, and Dr. Tom Federle of Procter and Gamble for advice and insights. I would also like to express my gratitude to Dr. Henry King for many helpful suggestions on this project and to Drs. John Bowman and Jim Flemming for reviewing this manuscript and Mary James for WordPerfect assistance.

Lastly, I would like to acknowledge the support and friendship of the people at the Center for Environmental Biotechnology at the University of Tennessee. This work was supported by the Procter and Gamble Company and the Waste Management Research and Education Institute of the University of Tennessee.

ABSTRACT

Linear alkylbenzene sulfonate (LAS), an anionic surfactant widely used in the detergent industry, is in most circumstances a readily degradable compound. It is of environmental concern because of its widespread and high volume usage. Though much effort has been expended studying the fate and effects of LAS in the environment not much is known about the specific bacteria responsible for its removal. The initial phase of this study focused on a field study which compared LAS impacted and pristine freshwater ponds. The objectives of this research were to: i) compare bacterial populations in two ponds; ii) determine frequency of plasmids in bacteria from these sites; iii) compare frequency of DNA sequences coding for catabolism of aromatic compounds in isolates from these two sites. Plate counts indicated that exposure to LAS resulted in higher levels of heterotrophic bacteria and of bacteria capable of growing on LAS containing medium. Mineralization of LAS in any habitat within the pond system directly correlated with the presence of bacteria able to grow in the presence of LAS. No pond isolate was capable of complete LAS mineralization. Though a higher incidence of plasmids was found in bacteria from the LAS impacted site and among bacteria able to grow on LAS containing medium, the presence of a plasmid did not necessarily confer the ability to degrade LAS nor was ability to degrade LAS dependent on plasmid occurrence. The incidence of the selected genotypes for aromatic catabolism was similar at both sites suggesting that LAS catabolism was encoded by alternative sequences.

This second phase of this study characterizes and compares two consortia, designated KJB and PG, capable of complete LAS mineralization. Both consortia are derived from activated sludge and contain four members. There is an absolute requirement for the

presence of all four members to observe $^{14}\text{CO}_2$ from U-ring labeled LAS. In pure culture only one member of each consortium was capable of transforming LAS to polar products. No member of either consortium grew on LAS as a sole carbon source or was capable of LAS mineralization as a pure culture. Consortium PG is a more proficient culture for LAS oxidation than KJB. While KJB requires greater than 10 days for 25% conversion of LAS to CO_2 , PG can convert 30% of added LAS to CO_2 in 2 days. Gene probe analysis of consortium KJB with *xylE*, *nahA*, *alkB* and *astA* (two dioxygenases, an alkane hydroxylase and an arylsulfatase gene probes) showed no homology with these genes.

TABLE OF CONTENTS

1. Introduction	1
LAS Biodegradation	2
2. Literature Review	8
What are detergents?	8
LAS in the Environment - Toxicology	17
LAS in the Environment - Fate	23
Biodegradation of LAS and Sulfonated Aromatic Compounds	29
Model Compounds for LAS Biodegradation	33
Sulfonated Aromatic non-LAS Compounds	36
Plasmids and LAS Catabolism	45
Microbial Consortia and LAS Degradation	47
3. Objectives	54
4. Materials and Methods	55
Test Chemicals	55
Study Sites	55
Bacterial Enumeration and Isolation	56
Plasmid Analysis	57
Gene Probe Analysis	57
Methylene Blue Active Substance (MBAS) Assay	59
Mineralization Experiments	59
Thin-Layer Chromatography	60
Continuous Culture	62
5. Results	64
Wisconsin Laundromat Pond Study	64
Enrichment Cultures	72
Consortium KJB	77
Consortium PG	89
Gene Probe Analysis	118
6. Discussion	124
Laundromat Pond Study	124
LAS Enrichment Cultures	127
LAS mineralizing consortia	128
Gene Probe Study of Defined Consortia	131
Literature Cited	135
Appendix	148
Vita	151

LIST OF TABLES

Table 1.	Laundry detergent components.	15
Table 2.	Acute toxicity of LAS and related compounds (LC ₅₀ mg/l).	19
Table 3.	LAS concentrations in various environmental compartments	30
Table 4.	Enumeration of bacteria (CFU/g) on LAS/YEPG and YEPG media in various habitats of the wastewater and control pond ecosystems in July and November.	65
Table 5.	Mineralization of LAS in various habitats of the wastewater and control ecosystems in July.	66
Table 6.	Percentage of bacteria isolated on LAS/YEPG and YEPG agars from wastewater and control pond ecosystems that contained plasmids.	68
Table 7.	Gene probes used to screen bacteria isolated on LAS/YEPG agar.	70
Table 8.	Percentage of bacteria on LAS/YEPG agar from wastewater and control systems that contained plasmids, exhibited MBAS positive reactions, mineralized ¹⁴ C-ring labeled LAS and hybridized with specific gene probes.	71
Table 9.	Fatty acid methyl ester (FAME) analysis and tentative identification of members of LAS mineralizing consortia KJB and PG (analysis conducted by MicroCheck Inc., Burlington VT).	78
Table 10.	Biochemical analysis of members of consortium KJB.	79
Table 11.	Carbon assimilation screening of consortium KJB.	80
Table 12.	Biochemical analysis of consortium PG.	90
Table 13.	Carbon assimilation analysis of consortium PG.	91
Table 14.	LAS mineralization by consortium PG at high LAS concentrations (5-day incubation).	97
Table 15.	Population composition of maintenance culture	103
Table 16.	Population composition over time - 0 mg/l LAS	105
Table 17.	Population composition over time - 5 mg/l LAS	106
Table 18.	Population composition over time - 10 mg/l LAS	107
Table 19.	Population composition over time - 20 mg/l LAS	108
Table 20.	Population composition over time - 50 mg/l LAS	109
Table 21.	Population composition over time - 100 mg/l LAS	110
Table 22.	Population composition over time - 200 mg/l LAS	111
Table 23.	Population composition of consortium PG upon alternate substrate exposure	117
Table 24.	Gene probes used for screening consortia KJB and PG.	122

LIST OF FIGURES

Figure 1.	The hypothetical biochemical pathway for LAS degradation adapted from reference 118 depicting both early and late desulfonation. . . .	4
Figure 2.	Schematic diagram of Wisconsin LAS contaminated laundromat pond.	7
Figure 3.	Chemical structures of surfactants from various surfactant classes . .	10
Figure 4.	The effect of alkyl chain length on surface tension reduction of LAS	12
Figure 5.	A. The effect of water hardness on detergent efficiency in the presence and absence of 1 g/l sodium tripolyphosphate. B. The effect of builder (sodium tripolyphosphate) concentration on detergent efficiency	13
Figure 6.	The biochemical pathway for the degradation of catechol	34
Figure 7.	The biochemical pathways for the biodegradation of protocatechuate and gentisate	35
Figure 8.	The proposed biochemical pathway for the degradation of naphthalene and naphthalene-2-sulfonic acid ($X = SO_3$) by <i>P. testosteroni</i> A3 and of naphthalene-1-sulfonic acid by <i>Pseudomonas</i> sp. C22	38
Figure 9.	The proposed biochemical pathways for the degradation of 1 and 2 naphthalene disulfonic acids by <i>Moraxella</i> strain ASL4	39
Figure 10.	The Biochemical pathway for the degradation of alkylbenzene sulfonates proposed by Bird and Cain (22).	41
Figure 11.	Schemes for the oxidation of mononuclear sulfonated aromatic compounds.	43
Figure 12.	Scheme for processing LAS catabolic cultures for TLC analysis. . .	61
Figure 13.	The fate of LAS in shake flask batch enrichment cultures.	73
Figure 14.	Fate of LAS in continuous culture enrichment.	75
Figure 15.	Mineralization of LAS by activated sludge cultures acclimated to ethylbenzene sulfonate (EBS), benzene sulfonate (BSA), and <i>p</i> -toluene sulfonate (pTS) in shake flasks.	76
Figure 16.	LAS removal by individual members of consortium KJB using the MBAS assay. This experiment was conducted in shake flasks at an LAS concentration of 10 mg/l.	82
Figure 17.	TLC analysis of LAS catabolism by consortium KJB following a two-week incubation with ^{14}C -ring labeled LAS.	83
Figure 18.	TLC analysis of LAS catabolism by <i>P. aeruginosa</i> KJB1 as a pure culture following a two-week incubation with ^{14}C -ring labeled LAS.	84
Figure 19.	TLC analysis of LAS catabolism by <i>X. maltophilia</i> KJB2 following a two-week incubation with ^{14}C -ring labeled LAS.	85
Figure 20.	TLC analysis of LAS catabolism by <i>E. cloacae</i> KJB3 following a two-week incubation with ^{14}C -ring labeled LAS.	86
Figure 21.	TLC analysis of LAS catabolism by unidentified bacterium KJB4 following a two-week incubation with ^{14}C -ring labeled LAS.	87

Figure 22.	Effect of LAS on growth of members of consortium KJB.	88
Figure 23.	Time course LAS mineralization experiment with consortium PG (LAS concentration = 0.05 mg/l).	93
Figure 24.	TLC analysis of LAS mineralizing Consortium PG in mineral salts medium after a 48-hour incubation with ¹⁴ C-ring labeled LAS. ...	94
Figure 25.	Mineralization of LAS by consortium PG at high LAS concentrations.	95
Figure 26.	Time course study of LAS mineralization by consortium PG at 0.05 mg/l, 80.0 mg/l and 100 mg/l LAS.	98
Figure 27.	TLC analysis of LAS catabolism by a pure culture of <i>Alcaligenes</i> sp. PG1 following a two-week incubation with ¹⁴ C-ring labeled LAS in 100 th strength YEPG medium.	99
Figure 28.	TLC analysis of LAS catabolism by a pure culture of <i>P.</i> <i>aeruginosa</i> PG2 following a two-week incubation with ¹⁴ C-ring labeled LAS in 100 th strength YEPG medium.	100
Figure 29.	TLC analysis of LAS catabolism by a pure culture of <i>E. cloacae</i> PG3 following a two-week incubation with ¹⁴ C-ring labeled LAS in a 100 th strength YEPG medium.	101
Figure 30.	TLC analysis of LAS catabolism by a pure culture of <i>X.</i> <i>campesterus</i> PG4 following a two-week incubation with ¹⁴ C-ring labeled LAS in a 100 th strength YEPG medium.	102
Figure 31.	TLC analysis of LAS catabolism by consortium PG at an LAS concentration of 80 mg/l following a 5-day incubation.	112
Figure 32.	TLC analysis of LAS catabolism by consortium PG at 100 mg/l LAS concentration following a 5 day incubation.	113
Figure 33.	TLC analysis of LAS catabolism by consortium PG at 200 mg/l LAS concentration following a 5-day incubation.	114
Figure 34.	The effect of acclimation to alternate substrates on LAS mineralization by consortium PG.	116
Figure 35.	TLC analysis of LAS catabolism by consortium PG following acclimation to <i>p</i> -toluene sulfonate.	119
Figure 36.	TLC analysis of LAS catabolism by consortium PG following acclimation to <i>n</i> -hexadecane.	120
Figure 37.	TLC analysis of LAS catabolism by consortium PG following acclimation to <i>para</i> -hydroxybenzoate.	121

1. Introduction

The post World War II rise of the petrochemical industry resulted in the introduction of a vast array of new products which would eventually make their way into everyday use in the United States. These synthetic chemical products include such things as plastics, paints and pesticides. The detergent industry was greatly affected by the petrochemical industry which sought new markets for non-fuel products. The result was a shift to new cleaning products as soaps were replaced by synthetic non-soap detergents. The availability of cheap tetrapropylene-based alkylbenzenes provided a stimulus for the production of branched alkylbenzene sulfonates (BAS) as anionic surfactants (32). These compounds, although ideal surface active agents, persisted in the environment and resulted in foaming. They also hindered sewage treatment plant operation by causing a decrease in dissolved oxygen content. Environmental persistence of BAS was due to the recalcitrance of the branched alkyl chain to biotic and abiotic degradation. The industry was able to generate an environmentally acceptable compound by replacing the branched alkyl chain with a straight chain compound to yield linear alkylbenzene sulfonate (LAS). The rise of LAS to the dominant position that it has attained in the detergent industry today was largely driven by environmental concerns. As a result of this LAS began to replace BAS by the 1960's (4, 24, 52). Other LAS properties, such as its low critical micelle concentration (<5 mg/l), permit the use of less compound to function effectively as a surface active agent. Moreover, LAS can be formulated as a powder or liquid, it is compatible with other surface active agents, and it is easily manufactured as a super concentrate (super concentrates require less packing material and are therefore considered

more environmentally friendly).

New product lines breaking into the market are expected to equal the performance of LAS and be as environmentally safe a compound as LAS. Roughly 3-4 million metric tons of synthetic surfactants are produced yearly in the United States, Western Europe and Japan with LAS accounting for 28% of this total production (137). Monsanto projects the demand for LAS to grow 1.5 to 2% per year through the early 1990s (52).

Surfactant sales totaled approximately \$11 billion in 1989 (52). The industry, though relatively recession proof, is fiercely competitive and all major manufacturers have huge advertising expenditures. Advertising strategies are often built around the consumer's heightened environmental consciousness. "Green marketing is an angle our competitors are using now, and we don't want it to be forgotten that LAS is the original green product used to replace BAS, which was slow to degrade and caused a lot of visual and toxic problems." says J. C. Ledvina, director of environmental activities at Vista Chemical Company (4).

Unlike other xenobiotics such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and trichloroethylene (TCE), LAS is not a hazardous compound. However, concern exists over the vast quantities which are discharged and the unregulated distribution of this product resulting the exposure of the general public to chronic LAS contact.

LAS Biodegradation

As is true with the vast majority of synthetic detergents, LAS has been in the biosphere less than 50 years; yet, in most situations, LAS is rapidly converted into CO₂

and H₂O in aerobic sewage treatment (32, 118, 127). The efficiency of treatment varies with environmental conditions and sorption can be a major route for LAS disappearance from wastewater. Water hardness (elevated CaCO₃) will cause LAS to precipitate. Thus, under conditions of high water hardness, LAS will settle, sorb to solids, and not be available for biodegradation until the sludge is land filled or applied to agricultural soils. Another concern in underdeveloped nations, which often do not treat domestic wastes, is LAS discharge directly into freshwater or marine environments.

Despite a considerable amount of research into the fate of LAS in the environment, the mechanisms of LAS biodegradation, such as the biochemical pathway and the organisms mediating the process, are not well understood. A number of factors can contribute to the biological recalcitrance of a compound. Some of these factors are availability of appropriate catabolic enzymes, substrate solubility, toxicity and bioavailability (31). As mentioned above LAS replaced BAS and differs from BAS only in the non-branched structure of the alkyl chain. The non-branched alkyl chain renders the molecule susceptible to beta oxidation, the process mediating routine catabolism of fatty acids. Thus, the introduction of the non-branched alkylchain into the molecule represents a logical means of altering the molecular structure in a way that maintains functionality and imparts biodegradability (10).

While imparting high solubility to LAS, the sulfone group is rare in nature and a sulfone group attached to an aromatic ring is extremely rare and imparts biological recalcitrance to the molecule (34). Figure 1 depicts the hypothetical pathways for LAS biodegradation. Though alkyl chain oxidation is almost universally accepted as the initial

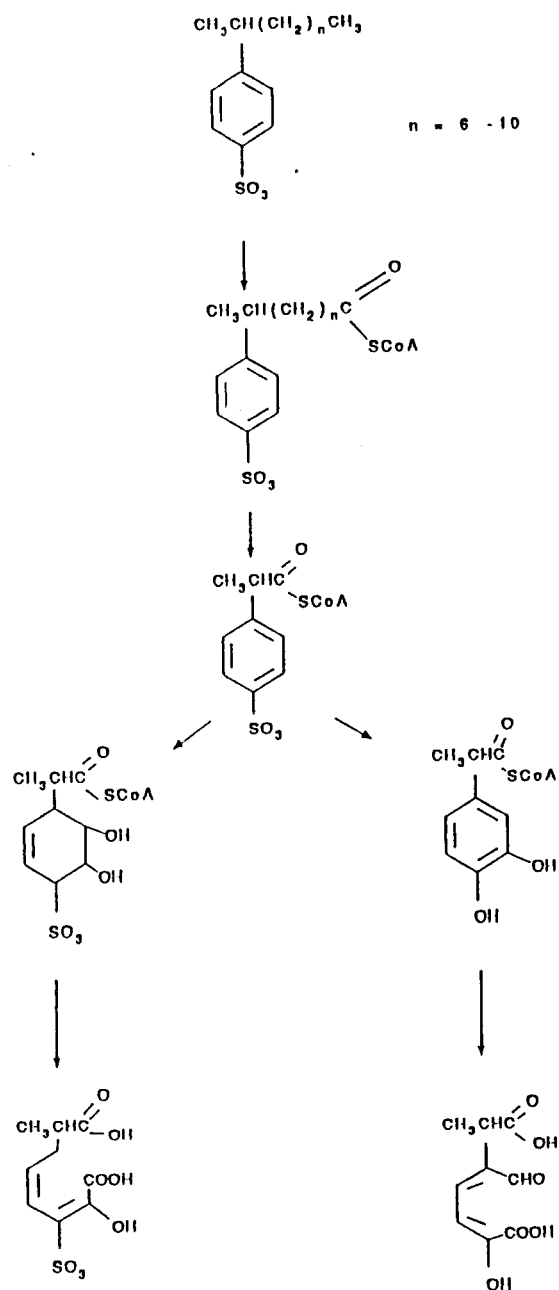


Figure 1. Hypothetical biochemical pathway for LAS degradation adapted from reference 118 depicting both early and late desulfonation.

step in LAS catabolism, the timing of desulfonation is not known and the occurrence of one scheme does not rule out the possibility of the other. Biochemical studies implicate desulfonation before ring cleavage, however, lab scale activated sludge studies have demonstrated the occurrence of sulfonated carboxylic acids, which could only have arisen from ring cleavage of LAS with the sulfone group intact. It is somewhat surprising that a definitive LAS degradative pathway has not been established. This may be due to the fact that LAS has not been shown to accumulate in any environmental compartment; and, therefore, does not pose the environmental threat that more persistent compounds present. Another explanation could be that LAS catabolism is mediated by consortia rather than individual bacterial populations, thus complicating most biochemical analyses. Cain (32) makes the statement that consortia are "...inherently unsuitable systems for investigating metabolic pathways...". This mind set has resulted in the extrapolation of data derived from pure cultures and model compounds to predictions of LAS catabolism.

Unfortunately, the data derived from these studies, although interesting in their own right, is of limited relevance to LAS catabolism. The notion that LAS metabolism is encoded by plasmid DNA originated from studies with surrogate compounds. Nevertheless, this highly speculative hypothesis, it is often cited in review papers (32, 60). The biodegradation of many xenobiotics has often been shown to be plasmid encoded (9, 116). These plasmids are often self transmissible and functional in a number of bacterial species. These "catabolic" plasmids are widely distributed in nature and their occurrence appears to increase in chemically perturbed environments (55, 96, 138, 142).

The present study was undertaken to develop new knowledge of the mechanisms

of LAS biodegradation. There are two major research objectives: (i) a survey of bacterial populations in a heavily LAS impacted freshwater pond site and (ii) the isolation and characterization of defined LAS degrading bacterial consortia. Work on this first objective was conducted on a pond in Wisconsin formed by the effluent of a laundromat operation. This field site is pictured schematically in Figure 2. In many ways this site represents a worst case scenario for LAS contamination. Undiluted, untreated wastewater is discharged into a pond overlying a shallow aquifer, separated only by a sandy zone beneath the sediment. Despite these circumstances the presence of LAS has been confined to the pond and not migrated into the groundwater (41). This provides an ideal arena to test hypotheses concerning plasmid mediated LAS catabolism as well as to see if characterized genes for aromatic compound degradation are more abundant in an LAS impacted environment. The second objective involves defining and characterizing pertinent consortia responsible for LAS removal from the environment. These studies are intended to determine which bacteria are responsible for primary LAS oxidation and which mediate degradation of LAS transformation products. Understanding of these processes would contribute to predictive fate assessments concerning LAS as well as provide the foundations for studying the genetic determinants underlying LAS catabolism.

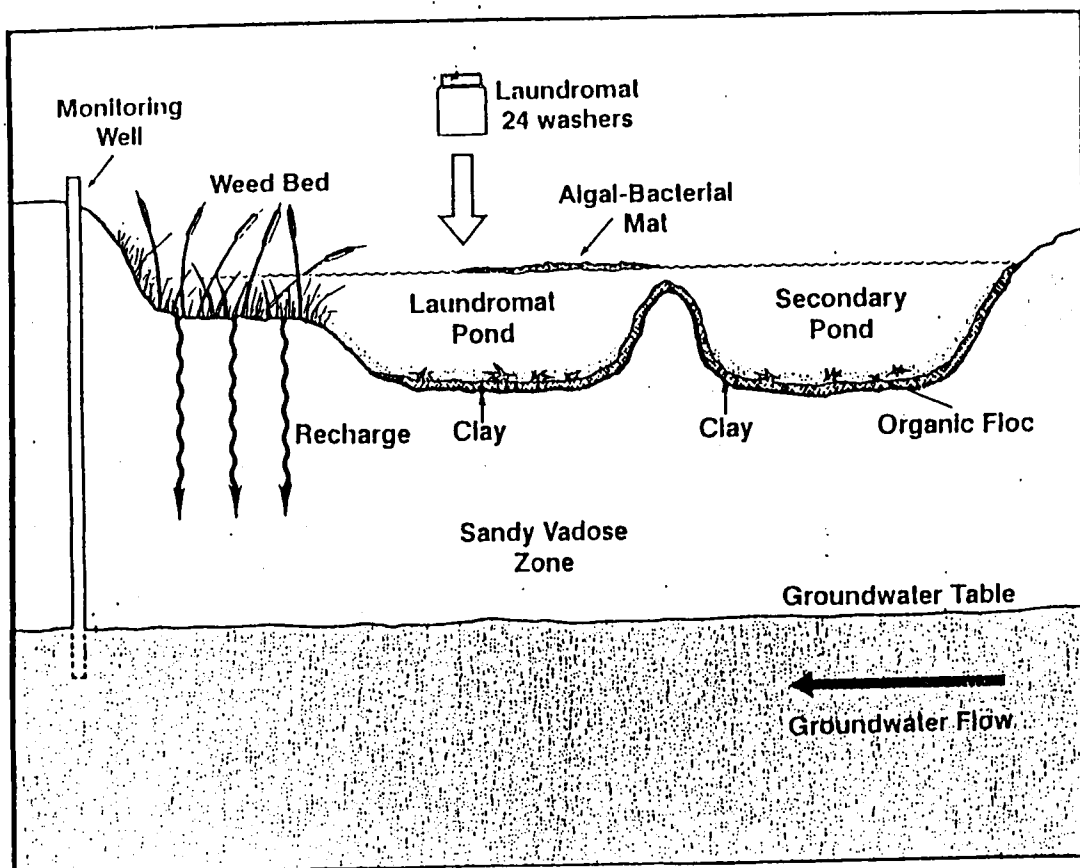


Figure 2. Schematic diagram of Wisconsin LAS contaminated laundromat pond.

2. Literature Review

Microorganisms play a central role in the cycling of carbon in the biosphere. The catabolic activities of bacteria are primarily responsible for the turnover of organic carbon in the environment. Anthropogenic compounds make a significant contribution to the environmental load of organic carbon (10, 136). Detergents are of concern, not because of their especially hazardous properties but because of their high volume usage and the fact that they are readily released into the environment. The degradation of these compounds in the environment is dependent on the activities and abundance of catabolic bacteria.

What are detergents?

Common detergents are the result of years of research into the complex and not fully understood theories of detergency. Originally, natural products derived from plants or animals were found to have cleaning or emulsifying properties. The term detergent encompasses both soap and non-soap surfactants (as well as inorganic detergents which are not surfactants) although the term is more often applied to non-soap detergents. A soap is an anionic surfactant usually composed of a mixture of sodium salts of long chain fatty acids. The name is derived from Mount Sapo where, according to Roman legend, a mixture of ashes and animal tallow washed down by rain was helpful in cleaning garments (24). It was the early soap making industry, that developed in the middle ages, that gave rise to today's massive detergent producing industry. It was the French chemist Michel Cheverul who established the scientific basis for soap production by elucidating the saponification reaction (24, 136). Saponification is the reaction in which animal fat

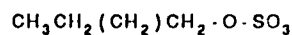
is combined with caustic soda (NaOH) to yield glycerol and soap (24, 140). The reaction is a substitution of the sodium in the alkali for the glycerin in fat. A shortage of fats and oils during World War I spurred the development of nonsoap detergents.

The non-soap surfactants fall into four main groups (some examples of which are shown in Figure 3):

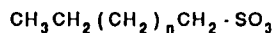
- *anionic* - most widely used, this group includes soaps, alkylsulfates and alkylbenzene sulfonates.
- *cationic* - which includes quaternary ammonium salts such as alkyl trimethyl ammonium chloride and alkyl pyridinium salts such as cetyl pyridinium bromide. These compounds are relatively expensive and have inferior deterative properties to the anionics.
- *nonionic* - do not ionize but contain hydrophilic and hydrophobic regions, this group includes alkylethoxylates and alkylphenoethoxylates.
- *amphoteric* - which can ionize as either anionic or cationic compounds depending on the alkalinity of the solution in which they are present. Products in this group, which includes alkyldimethylbetaine, are usually expensive and used for specialized purposes.

The function of surfactants is to remove deposits from the substrate and prevent redeposition. Detergents have two general properties by which surfactant action is accomplished. These are:

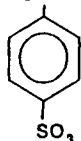
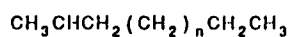
- saturation of liquid surfaces and maintenance of solution surface tension at a minimum.
- solubilization of oily matter.



alkylsulfate

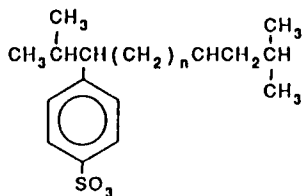


alkylsulfonate

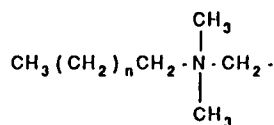


Anionic

linear alkylbenzene sulfonate (LAS)

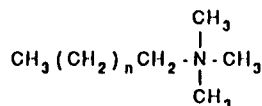


tetrapropylene-derived alkylbenzene sulfonate

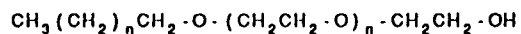


alkyldimethylbenzylammonium

Cationic

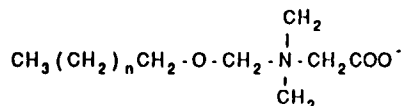


alkyltrimethylammonium



Non-ionic

linear alkylethoxylate



Amphoteric

alkyldimethylbetaine

Figure 3. Chemical structures of surfactants from various surfactant classes (32).

The surface tension of a solution can be lowered until a concentration is reached beyond which it cannot be further lowered. This varies from surfactant to surfactant and is referred to as the critical micelle concentration.

Since about 1965 LAS has been the major surfactant used in today's laundry detergent formulations (24, 140). Its precursor, branched alkylbenzene sulfonate (BAS) was first introduced on a large scale in 1946. The surfactancy of an LAS molecule is dependent on alkyl chain length. Alkyl chains shorter than 8 carbon atoms do not exhibit detergent properties. Alkyl chain lengths of C_{12} to C_{14} have the desired surfactant properties and remain readily water soluble (140). The position of the phenyl group is also important. The best detergency is obtained when the phenyl group is located at the C_1 atom, however, this form is ultra sensitive to precipitation by Ca^{2+} ions. Consequently, commercial LAS has phenyl groups attached primarily to the C_2 through C_4 atoms (140). Figure 4, from the Unilever Corporation, shows the effect of LAS alkyl chain length on surface tension.

Surfactants are not the sole components in laundry detergent formulations. Water hardness (constituted by the concentration of magnesium and calcium salts) dramatically affects the efficiency of anionic detergents, hence compounds called "builders" are commonly added. The function of builder components is the chelation of cations thus preventing their association with LAS. Anionic surfactants are precipitated and thus inactivated by Ca^{2+} and Mg^{2+} ions. Although at one time it was thought that nonsoap anionic surfactants would be less likely to be inactivated, it has been shown that this is not the case. Figure 5 shows the effect of builder concentration and water hardness on

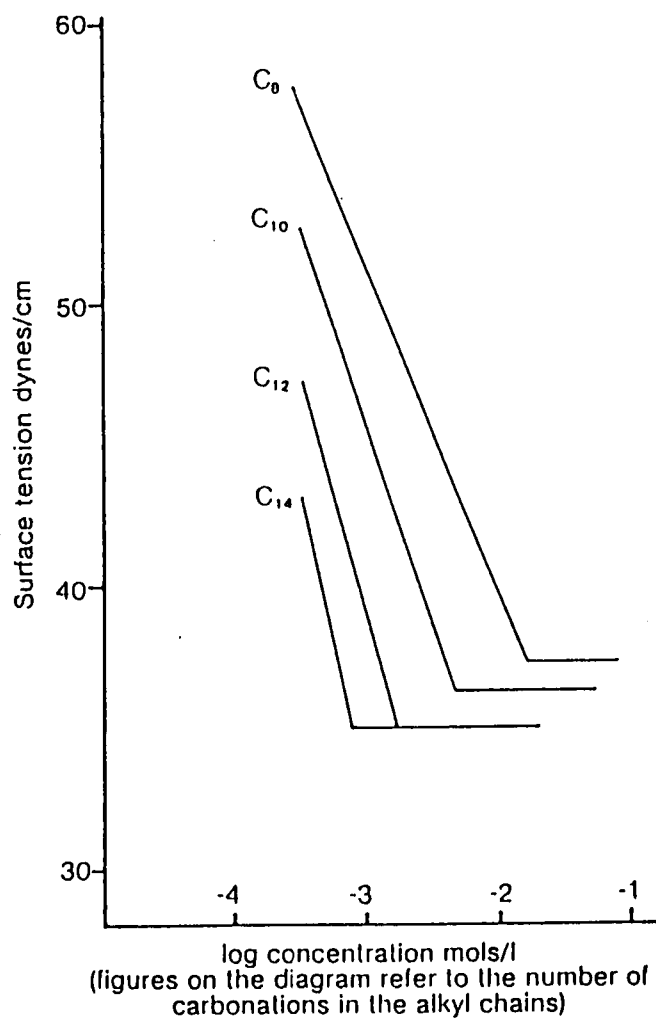


Figure 4. The effect of alkyl chain length on surface tension reduction of LAS (140).

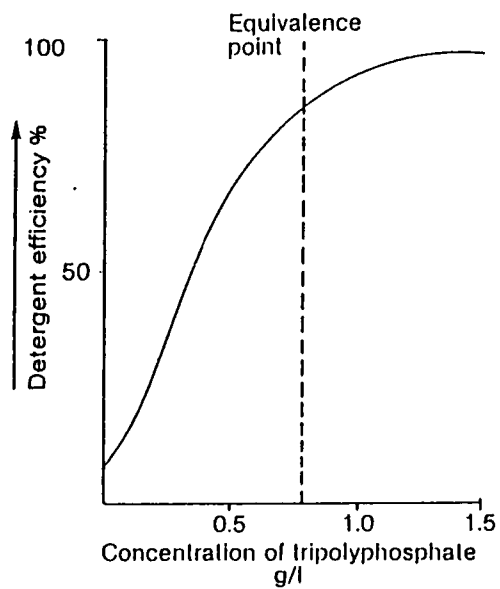
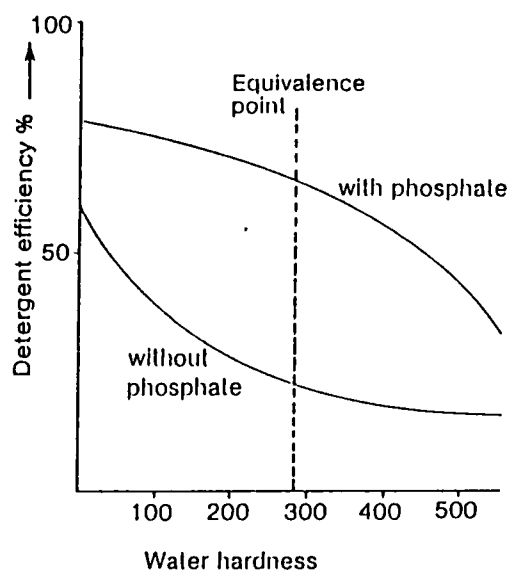


Figure 5. A. The effect of water hardness on detergent efficiency in the presence and absence of 1 g/l sodium tripolyphosphate. B. The effect of builder (sodium tripolyphosphate) concentration on detergent efficiency (140).

detergent efficiency. Detergent efficiency is measured as the amount of surfactant required to reach the critical micelle concentration. This relevance of water hardness to biodegradation of LAS will be addressed later. The following Table 1 lists other compounds and their function commonly found in laundry detergent formulations.

LAS and the Detergent Industry

As stated above LAS is the major anionic surfactant in use today with a worldwide usage of roughly 1.2 billion lb/yr (122). The rise of LAS to dominance paralleled the post World War II rise of the petrochemical industry which, at the time, was looking for new outlets for non-fuel by products (31). Initially, a paraffin fraction yielded a highly branched material which was produced from propylene polymerization to tetrapropylene (140). This provided a source for alkyl-side chain material in the production of the LAS precursors termed BAS. This material, which also included a phosphate builder, was introduced in 1946 (24). In the United States, widespread detergent use became common by the 1950s. This coincided with a number of foaming incidents in receiving waters which were traced back to detergents. BAS became termed a "hard" detergent due to its resistance to biodegradation. This resulted in a massive research effort to develop environmentally safe or "soft" detergents culminating in the appearance of LAS. By 1965 LAS had replaced BAS and became the workhorse detergent that it remains today.

Table 1. Laundry detergent components.

Function	Compounds
Bleach	sodium hypochlorite
Builders	nitriloacetic acid (NTA) ethylenedinitrilo tetraacetic acid (EDTA)
Anti-redeposition agents	sodium carboxymethyl cellulose
Optical brighteners	ditriazinyl aminostilbene disulfonates
Enzymes	amylases, proteases
Anti-caking agents	calcium silicates

The study of the fate and effects of LAS has been, and remains an area of intense interest to detergent manufacturers due to the massive use of LAS and the subsequent massive discharge of these chemicals into waterways. Among the major LAS producers today are; The Alcolac Chemical Company, The Colgate-Palmolive Company, DeSoto Chemical Company, The Dow Chemical Company, E. I. du Pont de Nemours Company, Unilever LTD., The Procter and Gamble Company, The Stepan Chemical Company, The Vista Chemical Company and The Witco Chemical Company. The major marketing theme in this extremely competitive industry has been to demonstrate that the products are environmentally sound. This has resulted in a number of well publicized campaigns such as; (i.) detergent formulations to allow for cooler wash temperatures, (ii.) the elimination of phosphates, (iii.) superconcentrates which require less packing material and eliminate filler ingredients thus reducing environmental impact (52).

Ainsworth (4) states "Ironically, although the recession and increasing public concern for the environment are pinching companies involved in the soap and detergent industry, these two factors are also bolstering certain surfactants". Among these detergents is LAS. It has proved particularly useful in the superconcentrate liquids where it allows enzymes to remain stable without the addition of stabilizers (24).

By the 1970s LAS comprised a large share of the surfactants in household use. From 1970-1985 LAS production was in the 600 million to 700 million lb range. The introduction of superconcentrates in 1988 resulted in an increase to over 819 million lb (an 11 % increase from 1987) (24). The Monsanto Company projected increased LAS production on the level of 3-4 % per year in the early nineteen nineties. The prospect of

an increasing load of LAS reaching the environment has sparked the concern of producers for environmental as well as public relations concerns. Joseph Ledvina, director of environmental studies at The Vista Chemical Company states, "As number one in its field, LAS is the target everyone is shooting at to move into the same market" (4).

LAS in the Environment - Toxicology

LAS is a xenobiotic compound with an overall reputation as being environmentally safe. The sulfone structure is not often encountered in nature; but, as pointed out by Cook and Leisinger (33), its occurrence is widespread throughout the biological world being found in archaeobacteria, eubacteria, fungi, plants and animals. Generally, naturally occurring sulfonated compounds are aliphatic (taurine, coenzyme M and 6-sulfoquinavose); the only known exception is the aromatic compound aeruginosin B. Interestingly, with the notable exception of LAS, a toxicological evaluation of sulfonates as a chemical class in the environment has not yet been done. Despite the fact that low molecular weight sulfonates are believed to be a major contributor to the pollutant load in the Rhine River they remain a relatively understudied class of pollutants (34).

LAS is a comparatively non-toxic compound. Its mammalian acute oral LD_{50} is 1000-2000 mg/Kg body weight which is in the same range as sodium chloride and sodium bicarbonate (127). The breakdown products of LAS are even less toxic than the parent compound. The major breakdown products, sulfophenylalkanoates, have an LC_{50} above 5000 mg/KG in rats and mice (127). In mammals the physiological path of LAS, following oral ingestion, is as follows; most is absorbed through the intestine and transported by the blood to the liver where early stages of oxidation, similar to bacterially

mediated transformations (omega and beta oxidation) occur, generating hydrophilic intermediates which are excreted primarily in the urine.

Although its low mammalian toxicity and rapid removal in sewage treatment systems are well documented, LAS can have a detrimental effect in the environment. The most notable detrimental effect of LAS is foaming, which can occur at concentrations in the 1 to 5 mg/l range. Though primarily an aesthetic problem, foaming causes a decrease in dissolved oxygen. LAS has an affinity for biological membranes, which can produce toxic effects on fish at concentrations of 1 to 5 mg/l (126). Table 3, adapted from Kimerle and Swisher (71), presents aquatic toxicity data on commercial LAS, individual LAS homologs and model biodegradation products (commercial LAS will show some variation in both alkyl-chain length and position of the phenyl group). The data show that homologs with the longer alkyl-chain length are more toxic than those with shorter alkyl-chains and that minnows appear to be slightly more sensitive than *Daphnia magna*.

The effects of LAS on fish vary greatly among species, salmonidae and cod being particularly sensitive (126). In general the first response of LAS exposed fish is increased activity, followed by inactivation, immobilization and death. Another generality is that larval stages are more sensitive than adults. Chronic low LAS concentrations will eventually damage gill tissue epithelium. Chronic sub-lethal concentrations were also shown to reduce the growth rate of the blue gill *Lepomis macrochirus* (126). Fertility was adversely affected by LAS; 10 mg/l caused reduced activity in the movements of spermatozoa and 5 mg/l caused a decrease in the percentage of fertilized eggs (50). Organisms at lower trophic levels have also demonstrated sensitivity to LAS. Arthur (8)

Table 2. Acute toxicity of LAS and related compounds (LC₅₀ mg/l). Adapted from reference 68.

	Average chain length	<i>Daphnia magna</i>		Fathead minnow	
		24 hr	48 hr	24 hr	48 hr
Commercial LAS	13.3	2.6 $\pm$ 0.1	2.3 $\pm$ 0.1	1.9	1.7
Individual LAS homologs					
C ₁₀	10	53.1 $\pm$ 0.4	12.3 $\pm$ 2.6	48.0	43.0
C ₁₁	11	15.8 $\pm$ 3.0	5.7 $\pm$ 0.6	17.0	16.0
C ₁₂	12	10.7 $\pm$ 1.6	3.5 $\pm$ 1.0	4.7	4.7
C ₁₃	13	2.7 $\pm$ 0.4	2.0 $\pm$ 0.3	1.7	0.4
C ₁₄	14	1.2 $\pm$ 0.2	0.7 $\pm$ 0.2	0.6	0.4
Non-linear LAS components					
C ₁₀	10	106.0 $\pm$ 27.0	98.0 $\pm$ 21.3	87.0 $\pm$ 7.5	86.1 $\pm$ 15.0
C ₁₂	12	55.1 $\pm$ 9.1	34.1 $\pm$ 5.1	24.8 $\pm$ 5.8	21.5 $\pm$ 5.5
C ₁₄	14	12.4 $\pm$ 1.4	10.0 $\pm$ 1.0	8.1 $\pm$ 5.1	5.3 $\pm$ 3.9
Model Intermediates					
C ₄ SO butyrate	4	12,000	6,000	10,000	10,000
C ₅ SO valerate	5	12,000	5,000	10,000	10,000

showed the maximum acceptable concentrations for an amphipod (*Gammarus pseudolimanaeus*) and snail (*Physa integra*) were 0.2 - 0.4 and 0.4 - 1.0 mg/l respectively.

Although solubilization of cell membranes is the most commonly cited LAS mechanism of toxicity, another mechanism of LAS toxicity was reported by Bressan et al. (25) who demonstrated the effects of LAS on the development of sea urchin embryos. They demonstrated that the skeletal development of *Paracentrotus lividus* LMK was completely inhibited at an LAS concentration of 0.45 mg/l and significantly retarded at 0.30 mg/l. The mechanism of toxicity was hypothesized to be an inhibition of Ca^{2+} uptake during skeletal calcification. Ca^{2+} normally available in sea water, is transformed in the presence of LAS into insoluble calcium sulfonates. The authors note that an increase in environmental calcium concentration does not mitigate LAS toxicity, in fact it worsened the effect. Consequently, sequestration of Ca^{2+} does not solely explain the basis of LAS toxicity.

Survival and growth rates of two benthic marine filter feeders *Botryllus schlosseri* and *B. leachi* (sea squirts) were shown to be adversely affected by LAS concentrations under 1 mg/l (88). The authors state that areas such as the lagoon of Venice (LAS concentration as high as 3.8 mg/l), Rovinj Harbor, Yugoslavia (LAS concentrations as high as 0.62 mg/l) and Izir Bay, Turkey (LAS concentrations as high as 4.8 mg/l) are areas where benthic communities could be in jeopardy. Their data must be put in perspective, however, in that LAS is far less toxic than other chemical contaminants found at these locations.

The effect of LAS on the photosynthetic activity of freshwater plankton has been

shown to vary greatly and the sensitivity of these organisms to LAS has been shown to sensitive to seasonal change (83). Surfactant toxicity increases with increasing temperature for example, Lewis and Hamm (83) noted a decrease in the EC_{50} value from 8.0 mg/l at 14°C to 0.25 mg/l at 24°C. Lewis (81, 82) conducted an evaluation of the effects of LAS (concentration range 0.43 to 108 mg/l) on density and diversity of freshwater phytoplankton. No significant effect was noted until LAS concentration reached 108 mg/l at which point the mean species number dropped from 21 to 16. This study also showed the green algae to be most LAS resistant followed by cyanobacteria and diatom species. Periphyton communities in a freshwater river, sampled above and below a municipal treatment plant (Xenia, Ohio) outfall showed no difference in numbers of taxa or photosynthetic activity under laboratory exposure to LAS, suggesting that these communities were relatively LAS tolerant (91). Conversely, Yamane et al. (143) noted a wide range of sensitivities in a survey of freshwater planktonic algae with some species inhibited at 4 - 8 mg/l concentrations and others tolerant up to the 50 -100 mg/l range. A study of the Rhone River showed toxicity of LAS to planktonic communities to be extremely site specific with variables such as particulates, nutrient levels and salinity affecting toxicity.

Terrestrial effects of LAS have been poorly studied however, this is a concern in the area of disposal of municipal sludge, due to the sorptive properties of LAS (the alkyl-chain of the molecule has tendency to sorb to biological materials) (91). The primary pathway of LAS transport to terrestrial systems is by the application of sludge amended-soil to agricultural lands or the disposal of sludge in landfills (16-18, 71, 108). The

available data on animal toxicity in terrestrial systems has focused on two species of earth worms, *Eisenia foetida* and *Lumbricus terrestris*. Studies showed that at the highest concentrations tested (1000 ug/g for *E. foetida* and 1333 ug/g for *L. terrestris*) the LC₅₀ was not reached (92). The *E. foetida* showed a 10- 20 % reduction in body weight relative to controls while *L. terrestris* showed no effect in any aspect tested including burrowing behavior. Plants showed a wide range of sensitivities when subjected to irrigation water containing LAS; peas were inhibited at 50 mg/l, paddy rice production was inhibited at 50 mg/l, Norfolk island pine growth was inhibited at 100 mg/l and cucumber growth was retarded at 100 mg/l. LAS-amended sludge showed no toxicity or had a stimulatory effect on plant growth when LAS concentration was as high as 407 mg/g sludge (92).

Some studies have attempted to develop quantitative structure-activity relationships (QSARs) for surfactants. Despite the substantial amount of data available on the aquatic toxicity of surfactants, not much effort has been devoted to correlating this data with QSARs. Most studies currently underway are at the early stage of determining whether QSARs be can be applied by comparing toxicity to calculated octanol/water partition values (octanol/water partition values are used to predict affinities of compounds for biological materials and have been a reliable predictive parameter for QSAR analysis - the higher the octanol/water partition value the greater the tendency for the compound to bioconcentrate). To date such studies have shown LogP (octanol/water) values have been successfully used in regression analysis of acute lethal toxicity data with such test species as goldfish and *Daphnia* (111). More recently attempts have been made to demonstrate

the applicability of QSARs for assessing LAS biodegradability (110). This has been used to correlate longer chain length and terminal position of the phenyl group with rapid biodegradability.

LAS in the Environment - Fate

The fate of LAS in the environment has been the subject of extensive studies and reviews, the most comprehensive being that of Swisher (127). As a common household product most LAS is disposed of "down the drain" and reaches the environment as a component of municipal waste. Aerobic sewage treatment is an extremely effective means of removing LAS from the environment (17-19, 118, 127, 133). Most industrialized countries treat 50 - 70% of their waste water but less developed countries may treat as little as 5% of their wastewater. Due to the quantity of household chemicals readily available in industrialized, as well as under developed countries (countries such as Nigeria are particularly problematic due to their tradition of environmental ambivalence) the persistence of LAS under various conditions is still being actively investigated. There is considerable concern about the kinetics of LAS removal and which particular environmental compartments could accumulate LAS.

Sewage treatment plants in general remove 97 - 99% of incoming LAS. In fact Berna (19) estimates that 50% of LAS is biologically removed in sewer lines before reaching the treatment stage. Removal of LAS during treatment is due to both biodegradation (generally accounting for 99 - 89 % of LAS disappearance) and sorption contributing 10 - 20 % to removal (19). Removal efficiencies have been reported to be 27% for primary clarification and 80% for trickling filters (108). It must be noted that "removal" is

determined by assay of the aqueous effluent and does not take into account the sludge which in fact accumulates LAS. The amount of LAS under going biodegradation is proportional to water hardness (mg/l CaCO_3). Hard water results in LAS precipitation and settling in the primary stage of sewage treatment. From that point precipitated LAS is carried along with settled materials through anaerobic digestion from which it emerges intact and awaiting sludge disposal. To date no accumulation of LAS has been observed in sludge amended soils. The half lives for LAS on sludge amended soils range from 25 - 35 days (18). It should be noted that the methylene blue active substance (MBAS) test is the most frequently used method for the assessment of LAS removal and that its limitations can often tend to over estimate LAS destruction because neither sorption nor partially degraded LAS are taken into account. The assay is based on the affinity of LAS to complex with the cationic dye methylene blue. When acidified the LAS/methylene blue complex can be extracted into chloroform and the amount of LAS present determined spectrophotometrically.

As stated above, the primary route of LAS into the soil environment is by the spreading of sewage sludge, usually following anaerobic digestion (107). A second major source of LAS in soils is septic tank discharge, which usually contains <1 to 10 mg/l LAS (102). Most studies concerning the fate of LAS in soils have dealt with sludge-amended soils. Rappaport and Eckhoff (108) conducted studies on sludge amended soils used to grow crops for silage. Concentrations of LAS averaged 28 mg/kg and was confined to the upper 25 cm of soil. LAS did not appear to migrate beyond the cultivation depth thus staying near the surface where biodegradation is most rapid. A study on 75 sludge-

amended soil sites in Great Britain showed 98% LAS disappearance per year after application; half-lives ranged from 7 to 22 days (108). As might be expected, a seasonal variation has been noted in soils such that LAS soil half-lives ranged from 5 to 25 days in summer then increased to 70 to 120 days during the winter.

Knaebel *et al.* (72) conducted LAS mineralization studies (as opposed to disappearance based tests) on 11 different soils ranging from a loamy sand impacted by sewage effluent to an alpine soil with a high organic content. All soils demonstrated LAS destruction without a lag period when LAS was applied at a concentration of 50 ng/g soil. No effort was made to ascertain the fate of unmineralized LAS. The data demonstrated the ability of indigenous soil bacteria to catabolize LAS.

The fate of LAS in freshwater aquatic environments was studied as part of an extensive survey (108). The half-life of LAS in a riverine setting below a trickling filter outfall in Rapid Creek S. D. was 0.25 days between outfall and 12 km down stream and 1.5 days between 12 km and 89 km downstream. These values were comparable to those reported by Larson and Payne (79) for acclimated freshwater systems. Using river water and sediment samples, they observed 80% conversion of LAS to CO₂ below a sewage outfall with no lag period and a 70% conversion to CO₂ above the sewage outfall which was preceded by a 5-day lag period. They also noted the inhibitory effects of high LAS concentrations (10 to 20 ppm) on mineralization capabilities. The stimulatory effect of substrate acclimation was also apparent from these studies. Seasonal changes will also effect degradation in freshwater systems. A freshwater lake estuary was shown to exhibit considerable LAS degradation in the summer when degradation was 100%, however, this

fell to 28% over a 10-day period in the winter months (7).

Federle and Pastwa (41) examined LAS degradation in subsurface sediments beneath a Wisconsin freshwater pond. The pond was formed in a natural depression as the result of discharge from a laundromat operation (the site is described in detail in the Materials and Methods section). The subsurface bacteria rapidly mineralized LAS (3.2 to 16.5 day half-life) without a lag period. In general, 33% of radiolabeled LAS was recovered as $^{14}\text{CO}_2$. It was noted that low oxygen levels correlated with decreased LAS catabolism. A proximal control pond which has never been exposed to LAS demonstrated an LAS half life of 5.2 to 1540 days and a lag period of 2 to 40 days. Indicating that acclimation dramatically enhances the rate of biodegradation.

Federle and Ventullo (42) conducted further studies on the same site examining submerged detrital material in the pond. The rationale for this study was that, among other things, decaying plant materials possess large surface areas for xenobiotic sorption and thus is a possible entry point for those compounds into the foodchain. In addition to this plant decay materials are readily colonized by diverse microbial communities. LAS demonstrated a tendency to sorb to detrital material yet the detritus was also a site of active LAS mineralization by the colonized detrital communities. Once again an adaptational response was noted as mineralization rates from control pond detritus lagged behind the laundromat pond material. The investigators cite the Wisconsin study as an extreme case in which household chemicals could pose a threat to groundwater as the pond lies above an unconfined aquifer separated from the pond by a porous sand sediment. Despite the fact that the pond water consists solely of undiluted wash and rinse

water, neither LAS or any other surfactants had migrated deeply into the sediments.

Anionic surfactants can be introduced to marine environments as a result of municipal sewage reaching the sea. In sea water at room temperature and 35% salinity the half-life of LAS was 15 days (25). The consensus appears to be that, although LAS degradation occurs in marine environments, it occurs at a slower rate relative to river water. Factors such as acclimation and temperature are likely limiting factors. Though the situation is not clear yet there is some evidence that bacteria of marine origin are capable of LAS catabolism (106, 120, 121). The situation is complicated by the fact that catabolic organisms from sludge may also be transported to the marine environment. Sigoillot and Nguyen (121) describe some ill-defined bacterial communities isolated from coastal sea water capable of complete LAS mineralization. They were complex communities composed of bacteria of sludge as well as of marine origin. A requirement for sodium was used as a means of distinguishing indigenous marine bacteria from bacteria of non-marine origin. The communities had a number of members capable of initiating LAS degradation and the investigators speculated that members not transforming LAS were mediating ring fission.

Two recurring aspects concerning the behavior of LAS in the environment are; (i.) the sorptive properties of LAS, (ii.) acclimation of the indigenous bacterial community. These factors must be taken into account when evaluating the fate of the compound. The sorptive properties of LAS are responsible for the limitations of the MBAS test in the environment. This short coming was recognized as early as 1975 and the use of ^{14}C -LAS tracer studies have since allowed for a more accurate assessment of the fate of LAS (44).

Acclimation of LAS impacted communities greatly enhances LAS destruction (42, 77, 102). This phenomenon has often been reported for xenobiotic compounds including other surfactants (119). Acclimation periods resulted in enhanced biodegradation in sediment/water microcosms as well as natural assemblages such as epilithic communities which demonstrated a 20-fold increase in LAS degradation (102). This evidence is used by LAS manufacturers to make the case that LAS is an inherently biodegradable product if sufficient time is allowed.

Swisher (127) expounds on acclimation stating that early LAS metabolites must accumulate until the bacteria are able to degrade them. The learning process is speculated to be; (i.) induction of necessary enzymes in bacterial species already present in significant numbers or (ii.) preferential multiplication of critical organisms present in low numbers. Shake flask experiments using activated sludge inoculum show that while after one year of maintenance on LAS using a 7-day cycle no removal of aromatic rings was observed; however, a 14-day cycle allowed for aromatic ring degradation. In both cases, primary oxidation was rapid (ie. 2-6 days) (127). This suggests that either the short cycle did not allow for sufficient metabolites to build up or it promoted preferential growth of the primary degraders at the expense of the secondary degraders. The fact that rapid primary degradation occurs in both cases indicates that LAS toxicity was not an inhibiting factor.

The bulk of evidence to date suggests that although LAS biodegradation in sewage treatment plants or in natural settings may have been over estimated due to sole reliance on the MBAS test, LAS does not appear to accumulate to any great extent in any

environmental compartment. Table 4 presents a survey of LAS concentrations in various settings. LAS, which accumulates on sludge for subsequent land application, is rapidly degraded following application to soils (72, 108). LAS sorbed to decaying plant material is still bioavailable to degradative bacteria (42). Pristine sites do, over time, mineralize LAS (41).

Biodegradation of LAS and Sulfonated Aromatic Compounds

Although much time and effort has been devoted to the fate and effects of LAS in the environment the biochemical pathway of LAS degradation is not known with certainty (118). The degradation of LAS requires three diverse biochemical reactions:

- oxidation of the alkyl chain; omega oxidation followed by beta oxidation.
- desulfonation of the aromatic ring or ring cleavage products.
- fission of the aromatic ring.

The exact sequence of these events is unknown; the preponderance of evidence suggests that oxidation of the alkyl chain is the first step in LAS catabolism (118, 127, 133). The order of the following steps is unclear and there is evidence support both desulfonation prior to ring fission as well as after ring fission (32, 118). The different schemes are not mutually exclusive but it is likely that one mechanism is predominant in specific environmental settings and that elucidation of this mechanism would aid in more accurate assessments of the fate of LAS.

Table 3. LAS concentrations in various environmental compartments (from ref. 18).

Compartment	Concentration
Sewers	6 - 8 mg/l
Raw sewage	4 - 24 mg/l
Sewage treatment plants (effluent)	0.05 - 0.9 mg/l
Sewage treatment plants (sludge)	0.2 - 30 g/kg (dry matter)
Sludge amended soils:	
before amendment	0.1 - 0.5 ug/g
after amendment	10 - 50 ug/g
River waters	0.01 - 0.01 mg/l
River sediments	1 -25 mg/kg
Surface soils	2 - 200 mg/g

There are some definitive statements which can be made concerning the biodegradation of LAS. The position of the phenyl group on the alkyl chain greatly affects biodegradation of the molecule (127). This is referred to as the "distance principle", formulated by Swisher, which states that primary degradation of LAS increases with the length of the alkyl chain, or as the phenyl group assumes a more terminal position (78). It is assumed that degradation occurs by the same mechanisms that are responsible for the degradation of aliphatic hydrocarbons, a mono-oxygenase attack on the terminal carbon resulting in an alcohol, which is further oxidized to an aldehyde and then a fatty acid which is then degraded by beta-oxidation (14, 127, 136). The mono-oxygenase will initiate LAS degradation at the carbon unit most distal to the phenyl group. The requirement for molecular O_2 in this initial step is believed to account for the fact that no anaerobic transformation of LAS has been definitively reported. Some investigators (4) have claimed LAS degradation in the absence of oxygen, but the preponderance of evidence shows this to be very unlikely and that disappearance was confused with degradation. There were hypothesis that linear alkylbenzene (LAB) in the environment was being generated by hydrolytic desulfonation of LAS in anaerobic environments but this could not be demonstrated experimentally and the LAB was shown to be a by product of the LAS manufacturing process (102). Cain has gone so far as to predict the likelihood that an OCT (octane catabolic) or OCT-like plasmid mediates primary LAS degradation (32). The following steps of beta oxidation of the alkyl chain is believed to be mediated by the same enzymes mediating the oxidation of fatty acids. Genetic studies with mutants deficient in the ability to oxidize fatty acids were also

deficient in the ability to oxidize LAS (32). One mutant was shown to be defective for acyl-CoA dehydrogenase, whereas another was lacking acyl-CoA synthase. Revertants simultaneously regained the ability to grow on fatty acids and LAS. The resulting products of beta-oxidation are sulfophenyl alkanoates and it is at this point where uncertainty about further metabolism arises.

Many theories on LAS biochemistry have sprung up from the extensive knowledge base which has accumulated on the degradation of aromatic compounds (reviewed 112). Six aromatic electrons, delocalized on the aromatic ring confer a reactive stability on the benzene ring. Bacteria as well as eukaryotes have a number of mechanisms for cleaving this structure and many of the associated biochemical pathways have been elucidated. There are two modes of aerobic aromatic ring fission, *ortho* or intradiol cleavage and *meta* or extradiol fission (12, 112). The products of *ortho* cleavage are metabolized by the *ortho* or beta-ketoadipate pathway. A catechol 1,2-dioxygenase is responsible for ring cleavage of catechol and a 3,4-dioxygenase is responsible for ring fission of protocatechuate (12). The *meta* pathway uses a catechol 2,3-dioxygenase and a protocatechuate 4,5-dioxygenase or a protocatechuate 2,3-dioxygenase for *ortho* dihydroxy compounds, which are then carried through the *meta* pathway (12). When hydroxy groups are *para* to one another 1,2-gentisate dioxygenase is used and products are metabolized by the gentisate pathway (gentisate = 2,5 dihydroxybenzoate) (12).

Bacterial dioxygenases (enzymes adding 2 atoms of molecular oxygen from the same atmospheric oxygen molecule) are typical entities in bacterial aromatic (e.g. benzene, toluene, naphthalene) catabolic pathways (86, 112). These dioxygenases generally have

three components; an NADH ferredoxin reductase, an iron sulfur protein, and an iron sulfur protein that adds O₂ to the substrate (43). A "typical reaction" entails deoxygenation by the multicomponent dioxygenase yielding a stable dihydrodiol. The dihydrodiol is re-aromatized by electron movement which leads to the release of a hydride ion. The latter is a poor leaving group, the elimination of which requires an NAD⁺ linked dehydrogenase.

In general the *meta* and gentisate pathways are believed to metabolize a wider range of compounds than the *ortho* pathway due to low substrate specificities of a number of enzymes in the pathways. It is a *meta* cleavage pathway that has been observed for a number of sulfonated aromatic compounds (132). Figures 6 and 7 depict these pathways. Both the *meta* and gentisate pathways are often encoded on transmissible plasmids.

Model Compounds for LAS Biodegradation

Linear alkylbenzene (LAB) has been used as a model compound for LAS biochemistry. It is of environmental concern because it is a by-product in the manufacture of LAS (49). Studies have been conducted on C₁₂ linear alkylbenzene and have shown that the "distance principle" appears to hold true for these compounds as well (129). While most studies have been done on alkylbenzenes of considerably shorter length, C₃ to C₆, thus limiting their relevance to LAS catabolism, some interesting observations were made. Baggi *et al.* (11) isolated two pseudomonads growing on 2-phenylbutane, 3-phenylpentane and 4-phenylheptane, as well as two *Nocardia* sp. growing on 3-phenyldodecane. No strain could utilize the corresponding *p*-sulfonated compound.

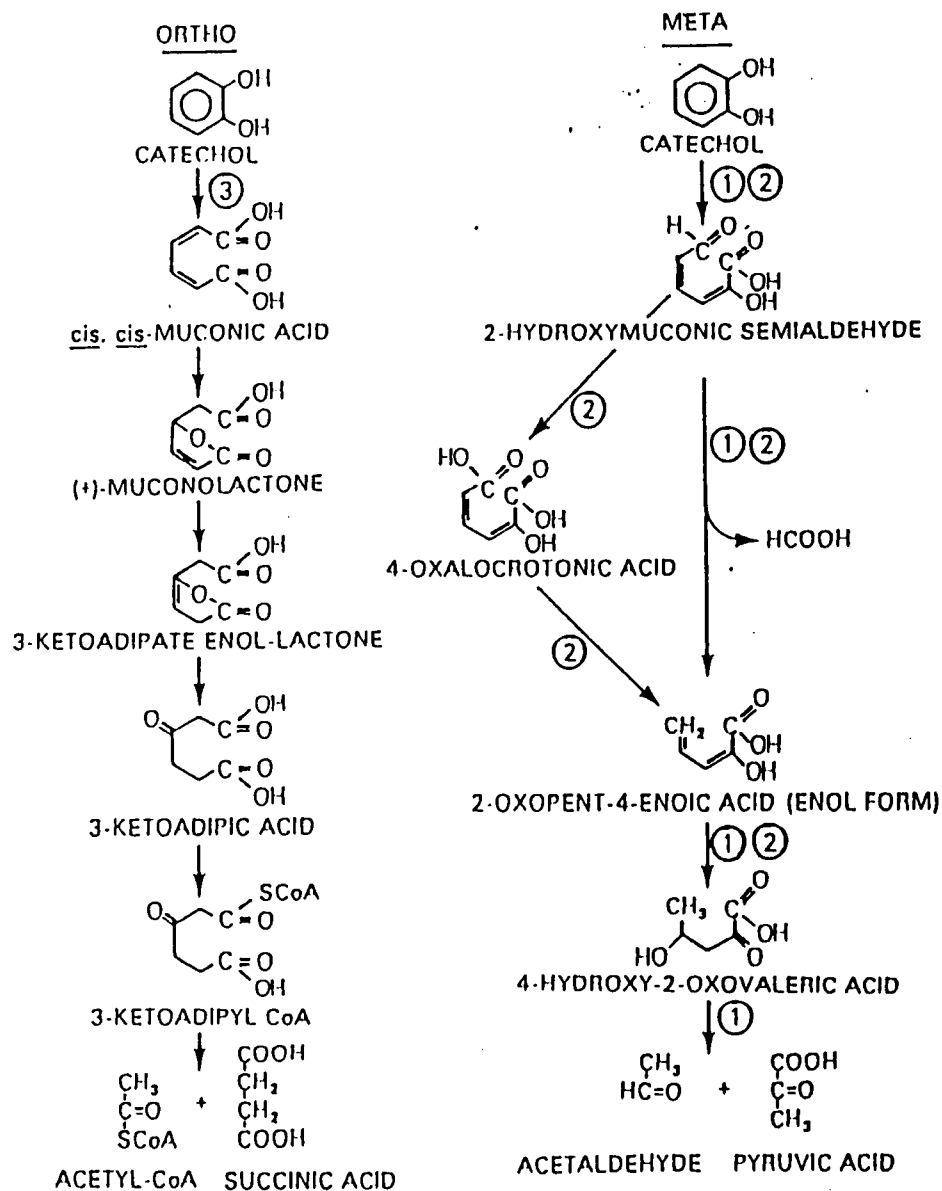


Figure 6. The biochemical pathway for the degradation of catechol (112).

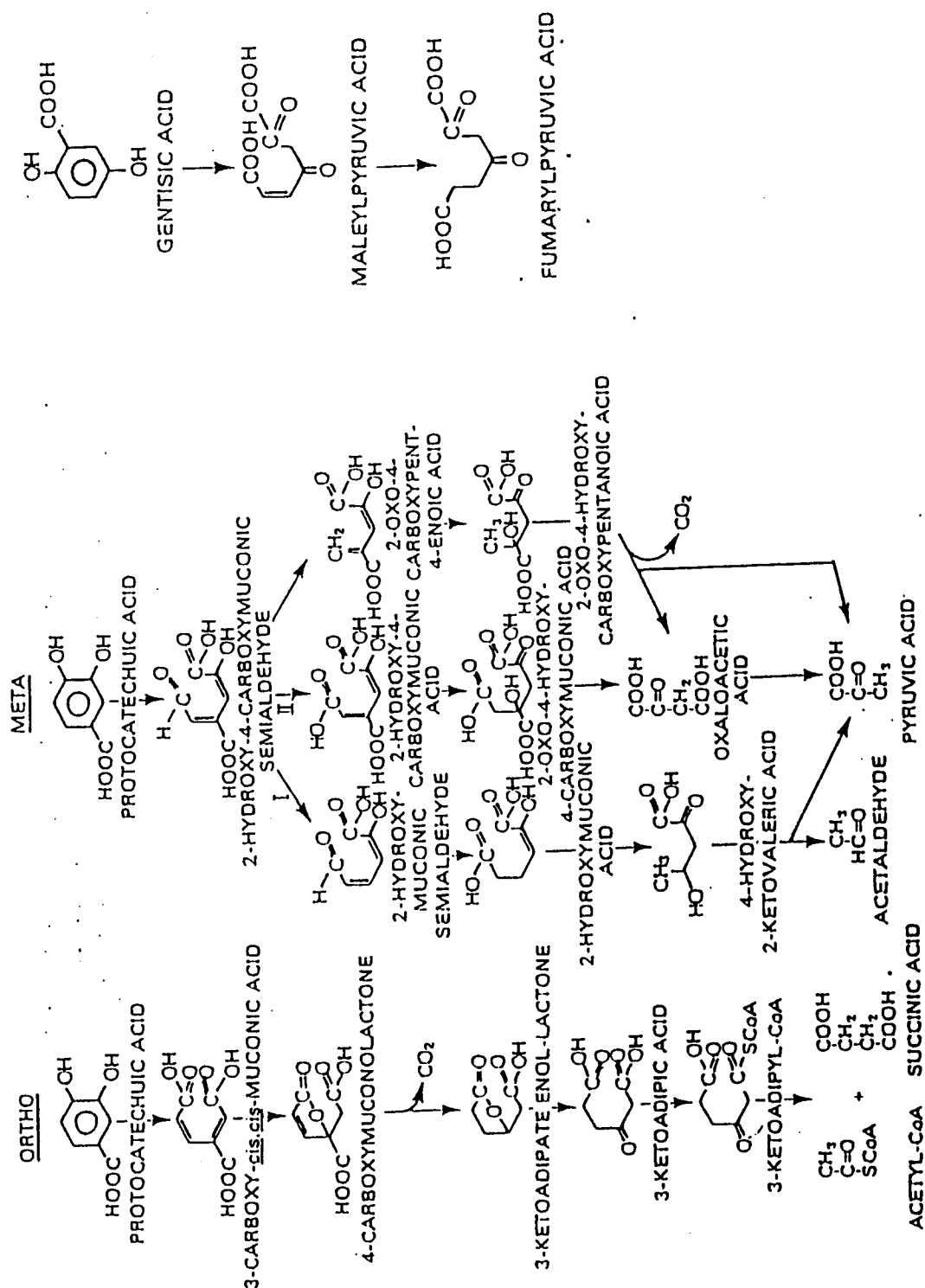


Figure 7. The biochemical pathways for the biodegradation of protocatechuate and gentisate (112).

The pseudomonads appeared to utilize a *meta* cleavage pathway but this did not appear to be true for the *Nocardia* sp. (which may well have ignored the aromatic ring). Sariaslani *et al.* (115) confirmed Baggi's *meta* cleavage pathway for alkylbenzene in studies with 3-phenylbutyric acid and suggests that in the case of alkyl chains of C₄ or less degradation will initiate at the aromatic ring. Jigami *et al.* (68) had previously demonstrated ring hydroxylation to be the first step in the catabolism of isopropylbenzene and isobutylbenzene. In a separate study Jigami (67) also showed that alkane utilizing strains of *Candida* were capable of partial and, in some cases, complete LAB oxidation.

Sulfonated Aromatic non-LAS Compounds

It is only recently that sulfonated aromatic compounds have been recognized as a class of environmentally significant compounds. As stated earlier, sulfonated compounds are rare in nature and only one naturally occurring sulfonated aromatic compound has been discovered (34). These compounds are found not only in detergents but in dyestuffs, herbicides and motor oil additives (34). To date two schools of thought concerning biological desulfonation have emerged; one from studies on sulfonated naphthalenes and the second from studies of mononuclear aromatics.

The first mechanism involves a spontaneous elimination of the sulfone group as sulfite following a regioselective oxygenation of the aromatic ring (95, 139). Naphthalene sulfonates (the building blocks of many azodyes) resist degradation or are incompletely degraded in the conventional sewage treatment process (27). Sulfonated naphthalene degraders, which used a gentisate pathway for naphthalene degradation, used the same dioxygenase used in the initial step of naphthalene oxidation to attack a sulfonated

naphthalene ring. Although the sulfone group was eliminated CH_3 , OCH_3 , Cl and NO_2 could not be removed. Wittich *et al.* (139), also working on naphthalene sulfonates, demonstrated a regioselective 1,2-deoxygenation of 5-sulfosalicylic acid. The conclusions from these studies were that naphthalene-degrading bacteria with low substrate specificities but high regioselectivities will labilize the C-S bond. Figures 8 and 9 show the metabolism of sulfonated and disulfonated naphthalenes.

Norteman *et al.* (95) isolated strain BN6 from a two-member consortium degrading 6-amino-2-naphthalene sulfonate (6A2NS). BN6 converted 6A2NS to 5-aminosalicylic acid. A more detailed study of strain BN6 revealed some unique properties (74). BN6, which did oxidize naphthalene sulfonic acid, did not oxidize naphthalene. Instead it demonstrated a specificity for naphthalene sulfonic acid, a unique property for all studied naphthalene dioxygenases. NH_2 -terminal amino acid sequence analysis showed a high level of homology (22 of 29 amino acids) with 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas paucimobilis* Q1.

Other groups of investigators have focused on single-ring sulfonated aromatic compounds. In 1975 Rippin *et al.* (109) described the abilities of a *P. testosteronii* strain to oxidize benzene sulfonate (BS), *para*-toluene sulfonate (pTS) and ethylbenzene sulfonate (EBS) but not longer chain length homologs. These compounds were metabolized via a *meta* pathway featuring a constitutive 2,3-catechol dioxygenase. This study pointed out an inverse relationship between oxidation rates and size of the alkyl substituent. A yellow *meta*-cleavage product tended to persist in cultures as the size of

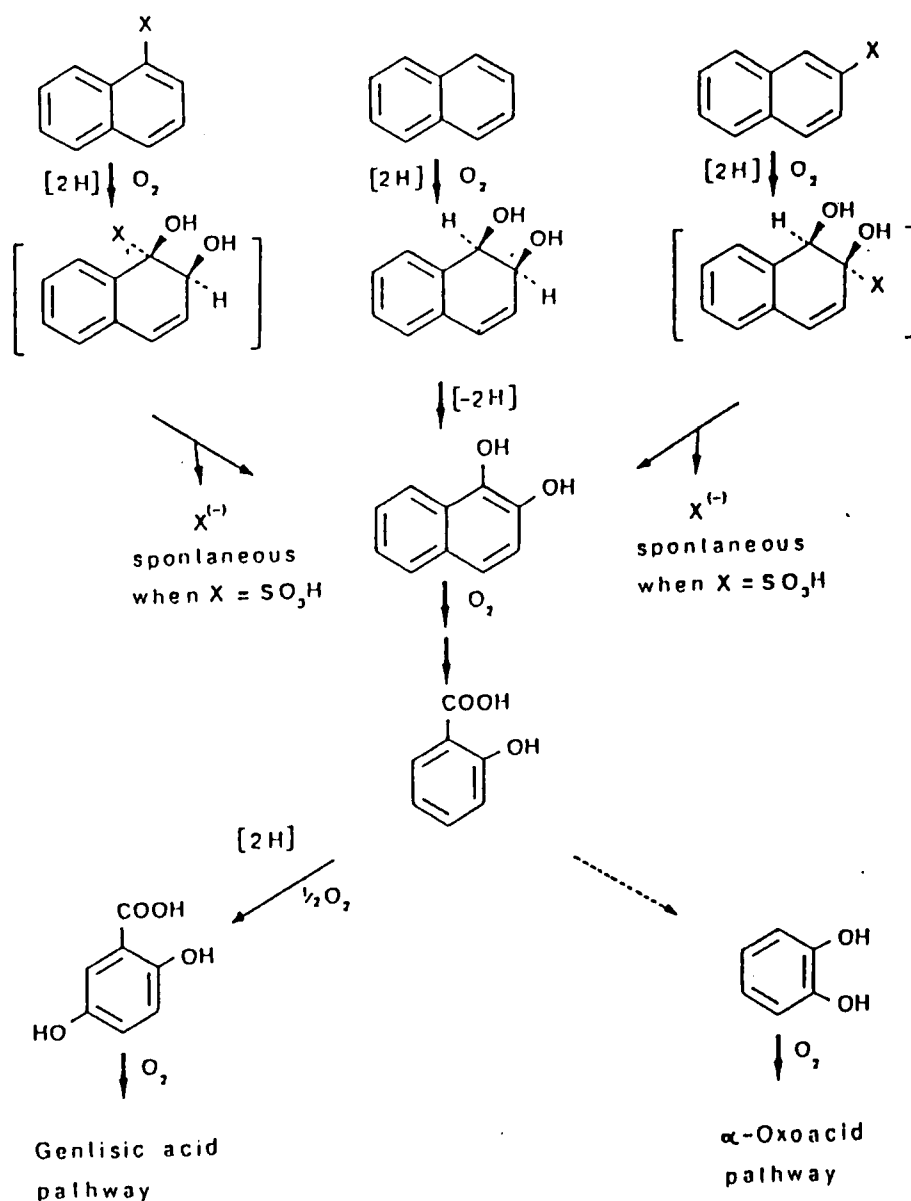


Figure 8. The proposed biochemical pathway for the degradation of naphthalene and naphthalene-2-sulfonic acid ($X = SO_3$) by *P. testosteroni* A3 and of naphthalene-1-sulfonic acid by *Pseudomonas* sp. C22 (26).

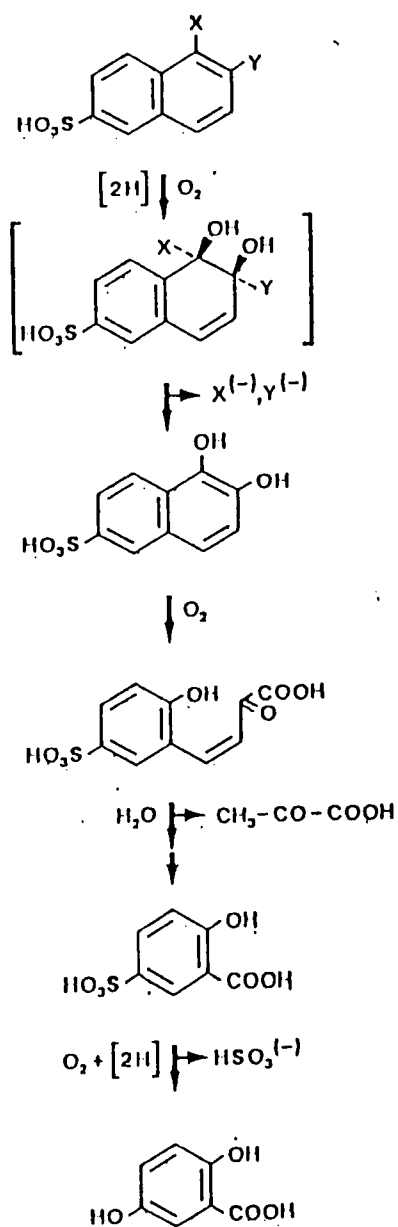


Figure 9. The proposed biochemical pathways for the degradation of 1 and 2 naphthalene disulfonic acids by *Moraxella* strain ASL4 (139).

the alkyl substituent increased leading the investigators to suggest that the hydrolase for 2-muconic acid semialdehyde has difficulty catalyzing the breakdown of the alkyl catechol hypothesized to be generated (see Figure 10). These results are similar to those of Bird and Cain (22), who studied an *Alcaligenes* sp. capable of using benzene sulfonate, *p*-toluene sulfonate and phenylethane-*p*-sulfonate showed that the organism employed a *meta* pathway and the initial step in degradation was a ring hydroxylation. The organism would not degrade alkylbenzene sulfonates with an alkyl chain length greater than C₄. The investigators speculate that such organisms mediate the ultimate destruction of LAS metabolites. More recent studies have been conducted by Thurneer *et al.* (130-132), showing that organisms utilizing sulfonated aromatic compounds tend to exhibit a relatively narrow substrate range. Seventeen different bacteria were obtained from enrichment culture on mononuclear sulfonated aromatic compounds (130). These isolates were then tested on 8 different sulfonated aromatic compounds with only one strain, *Alcaligenes* sp. O-1, able to utilize 3 compounds (benzene sulfonate, *p*-toluene sulfonate and 2-aminobenzene sulfonate). The other strains were able to utilize only one or two substrates. Ability to degrade benzene sulfonate did not necessarily correspond with ability to use *p*-toluene sulfonate or vice versa. Thurneer was also able to demonstrate desulfonation in cell-free extracts and demonstrate that growth on non-sulfonate carbon sources, such as succinate or benzoate, greatly reduced enzyme activity. *Alcaligenes* sp. O-1 was chosen for further studies because of its relative proficiency at sulfonated aromatic catabolism and it was shown that a catechol 2,3-dioxygenase and a *meta* cleavage pathway were mediating degradation. In addition, it was discovered that enzyme

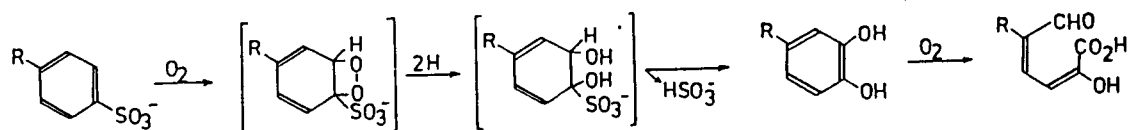


Figure 10. Biochemical pathway for the degradation of alkylbenzene sulfonates proposed by Bird and Cain (22).

extracts were capable of desulfonating 3- and 4- aminobenzene sulfonate whereas intact cells could not. This led the investigators to speculate that a selective permeability barrier to sulfonates exists and that the first step in the metabolism of these compounds is transport into the cells. This also suggests that narrow substrate usages could be attributed to transport factors rather than catabolic properties.

Locher *et al.* (84-86) focused their studies on *Comamonas testosteroni* T-2, a strain originally isolated by Thurneer *et al.* (130), which was capable of growth on *p*-toluene sulfonate *p*-carboxybenzene sulfonate but not benzene sulfonate. The pathway of pTS degradation by *C. testosteroni* differs from that of *Alcaligenes* sp. O-1. The contrasting pTS degradative pathways are shown in Figure 11. The initial catabolic step for *C. testosteroni* T-2 is oxidation of the methyl group to form 4-sulfobenzyl alcohol by an inducible monooxygenase, designated TSMOS. The oxygenase was shown to have two components consisting of an iron sulfur protein, which acts as a reductase (interestingly, it also serves as a reductase in the 4-sulfobenzoate dioxygenase system as well), and an oxygenase. This is the first monooxygenase system isolated that initiates degradation at the methyl substituent of a methyl aromatic compound (*xylA* and *xylM*, which code for components of the xylene oxygenase from the TOL plasmid have not been biochemically characterized) (85). This monooxygenase bears little resemblance to previously described monooxygenases such as the three-component alkane monooxygenase of *P. oleovorans*, the three component methane monooxygenase from *Methylosinus trichosporium* or microsomal P-450 containing monooxygenases. Comparison with the TOL plasmid encoded methyl monooxygenase shows that it too, is a two-component system. It

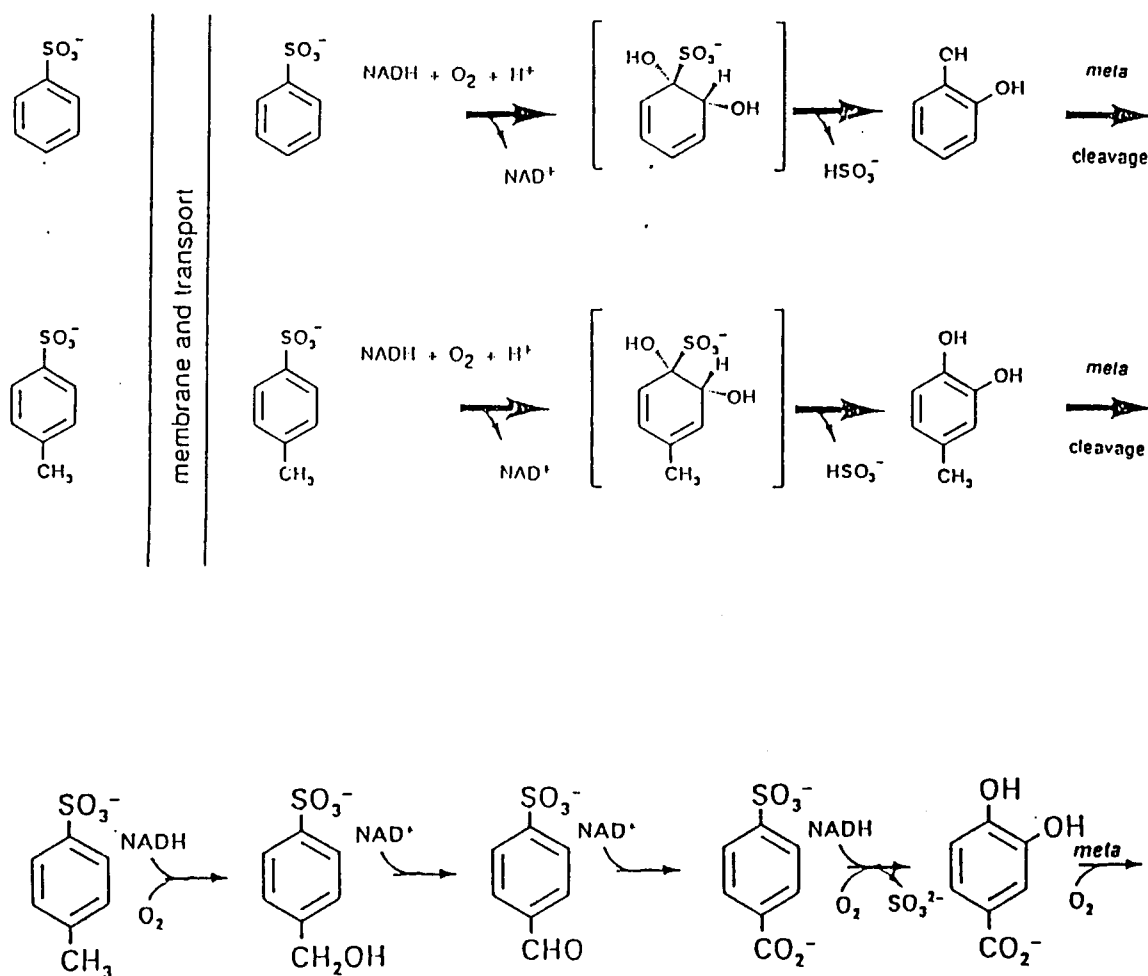


Figure 11. Schemes for the oxidation of mononuclear sulfonated aromatic compounds.

A. degradative pathway of benzene sulfonate and p-toluene sulfonate including membrane transport by *Alcaligenes* sp. O-1 (132). B. degradative pathway for p-toluene sulfonate by *Comamonas testosteroni* T-2 (86).

can transform not only toluene but its oxidation product, benzylalcohol, to benzaldehyde. The monooxygenase from *C. testosteroni* T-2 could not transform the alcohol. The TOL plasmid encoded enzyme is believed to be membrane associated while components of TSMOS are soluble. The TSMOS most closely resembled the 4-methoxybenzoate O-demethylase from *P. putida* sharing similar structure and susceptibility to uncouplers. In addition to these common properties both enzymes possess the ability to monooxygenate methyl aromatic compounds and to carry out O-demethylation. Methoxy aromatic compounds are widely distributed in nature as lignin components. This led Locher *et al.* (85) to hypothesize that TSMOS is a naturally occurring monooxygenase recruited into the degradation of sulfonated aromatic xenobiotics.

Locher *et al.* (86) also described the 4-sulfobenzoate 3,4-dioxygenase system (PSBOS) of *C. testosteroni* T-2 which converts 4-sulfobenzoate to protocatechuate. PSBDOS had an extremely narrow substrate range, displaying activity against none of 18 sulfonated and non-sulfonated analogs of 4-sulfobenzoate. PSBDOS is a two-component dioxygenase system consisting of an oxygenase and a reductase. The reductase is apparently the same one that is part of the TSMOS and the authors note that it is difficult to explain how a single reductase could generate enough reducing power for two oxygenases (81). The possibility of a primarily different enzyme fortuitously attacking 4-sulfobenzoate is not addressed. There does not appear to be much similarity between this dioxygenase and other described dioxygenases. The authors feel that the high specificity of this enzyme, if typical of desulfonative systems, could explain the narrow substrate range in bacteria utilizing sulfonated compounds.

Despite the intensive studies on the biochemistry of compounds such as benzene sulfonate and *p*-toluene sulfonate, the seemingly entrenched idea that desulfonation proceeds ring cleavage is by no means assured where LAS is concerned. Studies by Schoberl (118) and others, using laboratory-scale activated sludge tests, demonstrated the occurrence of sulfonated aliphatic LAS catabolites. Their experimental design represents more of a "real-world" picture of what probably happens to LAS in the environment. They used authentic LAS and were able to demonstrate rapid removal of LAS analogous to activated sludge LAS removal performance.

The catabolism of sulfonated aromatic compounds has a diverse genetic basis that currently is only poorly understood. Some desulfonative strategies appear to have diverged from systems mediating naphthalene catabolism (26, 27). Other strategies appear to have arisen by an enzyme recruitment process from gene products mediating the catabolism of polar aromatic compounds (85). Another under studied area concerning the fate of these compounds is the occurrence of a transport system - a logical possibility considering the polar nature of these compounds. Though knowledge of the genetic basis for the degradation of these compounds is minimal, speculation concerning the existence of LAS plasmids has been widespread (32, 60).

Plasmids and LAS catabolism

The notion that LAS catabolism is, at least in some cases, plasmid mediated stems entirely from Cain's review on surfactants and builder components (32). Though this has become somewhat fixed in the literature (reviews commonly cite plasmid mediated LAS catabolism ref. 60) the evidence for it is scanty and confined, for the most part, to non-

peer reviewed literature. There is a considerable amount of circumstantial evidence suggesting plasmid mediated degradation of LAS but it relies on studies done with non-LAS substrates. The earliest information came from Brilon *et al.* (26, 27) who noticed the irreversible loss of the ability to degrade sulfonated naphthalenes by cultures maintained under non-selective conditions and the transfer of the phenotype to non-degrading strains. No follow up work on the genetics of these organisms has been done despite the considerable knowledge gained about their catabolic capabilities.

More circumstantial evidence to suggest that plasmids mediate LAS degradation comes from the biochemical data which demonstrated the occurrence of a *meta* pathway of aromatic catabolism whenever this aspect of degradation was investigated (22, 84, 131, 132, 139). *Meta* pathways, which can be said to function as non-specific routes for the degradation of aromatic rings with alkyl substituents without modification of the substituents, are often plasmid encoded (9, 116). These factors, combined with some conjugation experiments in his own laboratory, led Cain to propose that TOL-like plasmids mediate LAS degradation. However, no molecular weight or restriction profile of a "TOL-like" plasmid has appeared. In addition to this, the test substrate of Cain's study was a C₆ alkylbenzene sulfonate not LAS. Bayly and Barbour (12) suggest that it would not be prudent to "project information concerning the biochemistry and enzymology of "simple" aromatic compounds onto compounds with more complex substituents". This statement could not be more true for LAS.

Only the work of Jahnke *et al.* (65) has unequivocally demonstrated plasmid mediated degradation of a sulfonated compound (ornthanilic acid). Using Thurneer's strain

Alcaligenes sp. O-1, the authors transferred a 175.5 kb plasmid to *P. putida* which then acquired the ability to use ornithanilate. Southern blot analysis of the plasmid, designated pSAH, did not show any homology with the archetypical TOL plasmid pWWO. As mentioned above, the work of Thurneer *et al.* (132) suggests that a permease may be the key factor in sulfonated aromatic compound degradation. It would be interesting to find that pSAH is found to encode a permease.

In summary, there is both biochemical and genetic evidence for plasmid-mediated degradation of some sulfonated aromatic compounds. These plasmids would appear to be unique relative to most plasmids described in the literature thus far. A flaw in the argument of those suggesting an authentic LAS plasmid is that degradation appears to be a process mediated by consortia rather than pure cultures (obviously this does not rule out the possibility that portions of LAS degradation could be plasmid encoded).

Microbial Consortia and LAS Degradation

That multiple organisms appear to be required for complete LAS degradation is not surprising given the three diverse chemical reactions mentioned above which are required for its complete destruction. Although pure culture technique has been a corner stone of microbiology, it has also been known that certain compounds that are recalcitrant to degradation by single organisms might be degraded by multiple organisms (20, 95). Another reason for examining microbial communities rather than strictly adhering to pure culture techniques is that these techniques (typified by serial dilution and spread plating on laboratory media) may eliminate pertinent organisms from study. In addition to this, laboratory monoculture studies of the fate of a particular substrate may differ greatly from

the real-world mixed-culture fate of the same substrate (122). In recent years an appreciation for the role of consortia has developed and a number of stable communities capable of biodegradation of recalcitrant compounds have been isolated (3, 4, 20, 95, 104).

Consortia can be loose associations or very tight and specific associations. The understanding of consortial activity was advanced considerably by the discovery of the "bacterium", *Methanobacillus omelianskii*, which was in fact two organisms in a tight association (76). One member of this consortium was a methanogen, *Methanobacterium bryantii*, which requires hydrogen to reduce CO_2 to methane. *M. bryantii* is limited in that it can employ only hydrogen to reduce CO_2 . The second bacterium, organism S, oxidizes ethanol to acetate and hydrogen. The hydrogen severely inhibits the growth of organism S and only minimal growth can be achieved in pure culture; however, when grown with *M. bryantii*, the methanogen will remove the hydrogen from the environment. Thus, through this association the growth of the methanogen is spurred and the inhibitory factor to organism S is removed.

Slater and Lovatt (123) list seven types of microbial communities:

1. Interactions based on the provision of specific nutrients.
2. Interactions based on the removal of growth-inhibitory compounds.
3. Interactions based on the modification of individual organism basic growth parameters.
4. Interactions based on combined metabolic attack.
5. Interactions based on cometabolism.
6. Interactions based on hydrogen or electron transfer.

7. Interactions based on the presence of more than one primary-substrate utilizer.

The type of interaction relevant to LAS catabolism is believed to be a combined or concerted attack on the substrate (123, 127).

The isolation of bacterial consortia is a more complicated task than pure culture isolation. Traditionally, enrichment culture isolation involves selection of a single organism growing on a particular substrate. This methodology is based on the physical separation (serial dilution and spread plating) of a population to individual organisms, then examining them as individuals for the phenotype of interest. The goal of isolating a pertinent community is to separate a "core" or minimal number of organisms needed to carry out the function of interest. This is most often accomplished by enrichment under continuous culture conditions using the compound of interest as a sole carbon source. Over time most non-pertinent bacteria should be washed out of the culture. Inoculum source and enrichment conditions such as flow rate can affect the structure and complexity of the core community isolated.

There may be two scenarios by which a core community is obtained. Parkes (103) states that key communities arise under chemostat conditions due to a "unique coming together of ... biochemical potentials". The community ultimately obtained in the chemostat may not be a functional community in the environment but a result of the enrichment process. It has been speculated that such communities would be subject to small changes in the enrichment process and consequently the reproducibility of obtaining the same core community would be low. Alternatively, the core community obtained may be one that is, in fact, viable in the environment. In this case it should be possible to

repeatedly isolate the community. The same complex, seven-membered community degrading the herbicide Dalapon could be repeatedly isolated from four different soils by two different enrichment conditions strongly suggesting that this group mediated Dalapon degradation in the environment (103).

As stated above a number of compounds resistant to complete degradation by monocultures can be completely mineralized by a combined or sequential attack by a mixed culture. Pettigrew *et al.* (104) used a chemostat to obtain a 4,4'-dichlorobiphenyl degrading consortium with an inoculum derived from PCB-contaminated freshwater sediment. The consortium consisted of 3 members; *P. testosteronii* LPS10A, which could convert 4-chlorobiphenyl and 4,4'-dichlorobiphenyl to 4-chlorobenzoate; *Arthrobacter* sp. designated LPS10B, which mineralized 4-chlorobenzoate to CO₂; *P. putida* LPS10C whose role in the consortium was undetermined but whose presence accelerated 4-chlorobiphenyl metabolism. Although at first glance the need for LPS10C seemed superfluous it did enhance catabolic activities. This is an often observed phenomenon when isolating consortia from the environment. The communities seem to be more complex than would be required for the task at hand. "Protocooperation" is a term coined by Hammer and Heitzer (56) to describe roles for ancillary organisms in defined mixed cultures. Adrians *et al.* (3) generated a 2-member consortium capable of the degradation of 4,4'-dichlorobiphenyl. The fact that this compound has chlorine atoms on both aromatic rings makes it especially recalcitrant. The members were both *Acinetobacter* strains, one designated *Acinetobacter* sp. 4CB1, and the second designated *Acinetobacter* sp. P6. The initial reaction, which was mediated by *Acinetobacter* P6, involved a

dioxygenase attack on one of the rings followed by a second attack on the same ring. A hydrogenase then released a 5 carbon acid and *p*-chlorobenzoate. Strain 4CB1 then initiated a hydrolytic ring cleavage converting the chlorobenzoate to *p*-hydroxybenzoate. This consortium was not obtained by enrichment but rather by combining two strains of known catabolic potential. When a catabolic pathway is known and intermediates in degradation have been established then an effective consortium can be established.

The role of consortia in the degradation of sulfonated compounds has already been alluded to. Nortemann *et al.* (95) were able to isolate a 6-aminonaphthalene-2-sulfonic acid (6-A2NS) degrading consortium from activated sludge. A coculture of two *Pseudomonas* sp. were able to completely oxidize the compound by virtue of interspecies transfer of 5-aminosalicylate. It was shown that strain BN6, which mediated the initial attack on the substrate, lacked a salicylate dehydrogenase. This was provided by the second strain, BN9, which could utilize 5-aminosalicylate (itself a problem to the dye industry where this compound will autooxidize into a bactericidal brown substance).

The degradation of LAS by mixed cultures has long been noted, but not much effort has been expended to define the groups and ascertain the basis for cooperation. Swisher (127) had provided preliminary evidence for a cooperative attack on LAS in his experiments mentioned above concerning long and short retention times. A possible explanation for loss of mineralization at short retention times is the wash out of organisms mediating secondary LAS metabolism. Both Slater and Lovatt (123) and Painter and Zabel (102) cite unpublished work by W. K. Phillips and G. Hollis that describes a four-membered LAS-mineralizing consortium consisting of a *P. putida*, a *P. alcaligenes*, an

Arthrobacter globiformis and a *Serratia* sp. The consortium was obtained from activated sludge by using low LAS concentrations (20 mg/l) in a chemostat.

Painter and Zabel (102) discuss a 9-membered LAS mineralizing consortium consisting of 5 pseudomonads, 2 *Achromobacter* sp. and 2 *Acinetobacter* sp. The consortium was placed in a chemostat supplemented with phosphate and synthetic sewage and six experiments were conducted at mean cell residency times of 40 hrs and 10 hrs using 20 to 50 mg/l LAS. An *Acinetobacter* sp. was predominant in four experiments and a *P. fluorescens* and another pseudomonad in two other experiments. At the 10-hr residency time only 64% of the MBAS extractable material was removed, whereas at the 40-hr residency, 96% was removed. No removal of aromatic compounds was seen at the 10 hr cell residency however, 13 to 23% of the aromatic material was removed at the longer time. The *Acinetobacter* required nutrient supplements to carry out primary LAS oxidation and rapidly lost this ability when cultured without LAS. The predominant pseudomonad carried out primary LAS oxidation and could use LAS as a primary carbon and energy source. The other seven members of the group were all incapable of primary LAS oxidation and their role(s) in metabolism is unknown.

The reasons for the complexity of LAS-degrading communities are speculative. They could be providing growth factors or removing toxicants. There could be reasons that are peculiar to LAS. It is believed that the sulfone group is released as sulfite, a toxic, reactive, substance. Sulfite detoxication could be a role for a consortia participant. Floc formation has been proposed as an important aspect of LAS degradation (97). Flocs could protect bacteria from the deterative properties of LAS and maintain the community

members in close proximity to one another. Russell *et al.* (113) demonstrated the propensity of degraders of the detergent sodium dodecyl sulfate (SDS) to form biofilms that enhance degradation. Currently G. Hammer's explanation for these ancillary organisms, "They like their friends", is as good as most others.

3. Objectives

The goal of this study is to isolate and characterize bacterial communities capable of complete biodegradation of LAS. The knowledge gained will aid in predictive fate assessments concerning LAS and provide insight into the means by which bacterial consortia combine to cooperatively remove a xenobiotic that is resistant to degradation by pure cultures. Specific objectives are:

- I. Compare bacterial populations in LAS-impacted and pristine freshwater ponds.
- II. Determine occurrence of plasmid DNA in bacteria from LAS-contaminated and pristine sites.
- III. Screen LAS-degradative bacteria with gene probes to determine relatedness between previously described catabolic genes and LAS degraders.
- IV. Isolation of core communities capable of LAS mineralization.
- V. Identify the mechanism by which bacterial consortia synergistically mineralize LAS.

4. Materials and Methods

Test Chemicals

All chemicals, unless otherwise specified, were purchased from Mallinkrodt Inc. (Paris, KY). A commercial mixture of LAS with an average alkyl chain-length of C_{12} was obtained from Stepan Company (Northfield, IL). This commercial preparation was then extracted with hexane and the aqueous phase used in experiments. [U- ^{14}C ring] sodium tridecylbenzene sulfonate (LAS) with a specific activity of 8.69 mCi/mmol was obtained from New England Nuclear (Boston, MA). Purity was 98% based upon thin-layer chromatography (TLC) on silica gel G with chloroform/methanol/water/formic acid (80/25/3/1). Ethylbenzene sulfonate was obtained from the Aldrich Chemical Company (Milwaukee, WI.), *p*-hydroxybenzoate, benzene sulfonate and *n*-hexadecane were all obtained from the Sigma Chemical Company (St. Louis, MO), and Na benzoate was purchased from Fisher Scientific (Fairlawn, N.J.).

Study Sites

The laundromat wastewater pond system consisted of two ponds that were located approximately 0.5 km north of the community of Summit Lake in north central Wisconsin. It was formed by wastewater discharged since 1962, into a natural depression. Average monthly discharge, based upon the number of loads, varied from 160,000 l in the winter to 468,000 l during the summer. One pond (Laundromat Pond) is formed by wastewater, which is discharged to a sluiceway and then into a distribution tank located in the pond's center. This pond was approximately 22 by 66 m with an average depth of 1.5 m and is characterized by low levels of dissolved oxygen (0.5-1 mg/l) and the

presence of a floating cyanobacterial mat. Approximately one-third of the total area of the pond has been colonized by emergent vascular plants. A second pond (Secondary Pond) is formed from Laundromat Pond overflow and is characterized by a continual algal bloom and supersaturated levels of oxygen. Both ponds are surrounded by large deciduous trees, and precipitation and non-point run off are the only other sources of water to the ponds. The average LAS concentration has been determined by interference limited methylene blue active substances (IL-MBAS) response was found to be 32 mg/l in the Laundromat Pond and 7 mg/l in the Secondary Pond. The MBAS test is sometimes referred to as interference limited when used in environmental settings because a number of non-LAS anionic substances could give a false positive reaction.

The Control Pond is naturally formed and located approximately 2 km south of the Laundromat Pond. It is comparable in size and shape to the Laundromat Pond (LMP) but is not impacted by laundromat operation or any other apparent point source of pollution. It also contains a wetland area and is surrounded by deciduous trees. No IL-MBAS was detected in this pond.

Activated sludge samples were obtained from the Knoxville municipal wastewater treatment facility (Knoxville, TN). Lake Loudoun sediment was obtained from the Sequoia Hills (Knoxville, TN) boat ramp from a depth of approximately 15 cm below the water surface.

Bacterial Enumeration and Isolation

Pond water samples were serially diluted in phosphate-buffered saline (1.2 g Na_2HPO_4 , 0.8 g NaH_2PO_4 and 8.5 g NaCl per liter of distilled water, final pH 7.6), and

dilutions were plated onto YEPG agar (dextrose 1.0 g/l, polypeptone 2.0 g/l, yeast extract 0.2 g/l ammonium nitrate 0.2 g/l and agar 16 g/l) [21] and YEPG agar supplemented with 0.01 % LAS/YEPG. These media served to distinguish total heterotrophic bacteria and bacteria that were growing on or tolerant of LAS. Sediment and detrital (1.0 g) material were placed in 20.0 ml of 1% sodium pyrophosphate and vortexed for one minute prior to dilution and plating. Plates were incubated at 28 °C for 10 days.

Plasmid Analysis

All bacterial isolates were tested for the presence of plasmids. Since the isolates were not characterized, two plasmid DNA isolation protocols were used to ensure the detection of plasmid DNA. The methods of Kado and Liu (20), a rapid procedure for isolation of plasmid DNA from Gram negative organisms, and of Anderson and McKay (6), a procedure optimized for Gram positive organisms, were used. Plasmid DNA bands were electrophoresed in 0.7% agarose gels and visualized under ultraviolet light. Plasmid DNA for subsequent use in gene probe analyses was prepared on a large scale using the Anderson and McKay extraction method with cesium chloride/ethidium bromide equilibrium centrifugation (29).

Gene Probe Analysis

Wisconsin pond isolates were screened for the presence of pertinent genotypic traits by colony hybridization with the gene probes described in Table 9 (15, 34). Gene probes were prepared by labeling the DNA of interest with ³²P-dCTP (ICN, Irvine, Ca.) by nick translation using a kit purchased from GIBCO BRL (Grand Island N.Y.) (82). Probe DNA was separated from unincorporated nucleotide on a G-50 sephadex column. The

isolates were grown on agar plates for 5 days and then transferred to nylon membranes (ICN Biomedicals, Irvine Ca.). The colonies were lysed and the nucleic acids were heat fixed (80°C) for 1 h. The membranes were incubated with probe DNA for 14 h at 65°C in a hybridization solution composed as follows; 0.02% (w/v) ficol, 0.02% (w/v) polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.18M NaCl, 0.01M NaPhosphate, pH 8.3, 0.1mM EDTA, 0.2% SDS and 500 ug/ml non-homologous DNA. Unbound probe DNA was washed from the membranes with buffer composed of 10 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA and 0.1 % SDS. Two 2-h washes were done at 65°C. Autoradiography was then carried out on air-dried filters for 24 h at 80 °C using X-OMAT AR X-ray film (Eastman Kodak, Rochester, N.Y), and processed with an automated X-ray film processor from AFP Imaging (Elmsford, N.Y.)

The consortia isolates were screened by slot blot analysis of genomic DNA preparations (87). The DNA was checked for purity and quantitated spectrophotometrically using a DU-70 Beckman spectrophotometer. The gene probes used are described in Table 25. The gene probes were isolated using a large-scale plasmid isolation procedure followed by restriction endonuclease digestion of the purified plasmid DNA (82). The restriction endonuclease fragments were excised from agarose gels following electrophoresis and treated with agarase to remove agarose (87, 90). The probes were labeled with ³²P-dCTP by random priming using a kit from GIBCO BRL. The slot blot apparatus was purchased from Bio-Rad (Richmond, CA.). The slot blot membranes were processed in the same fashion as the colony hybridization filters.

Methylene Blue Active Substance (MBAS) Assay

Organisms that attack LAS were detected on plates with a methylene blue reagent (King, J.M.H.1987. Ph.D. thesis, University of York). Methylene blue is a cationic dye, which will complex with LAS. The reagent is prepared as follows; 120 ml of a 0.1% (w/v) aqueous solution of methylene blue is added to 500 ml of a 10.0% (w/v) solution of Na_2HPO_4 which contains 6.8 ml of concentrated H_2SO_4 . The resulting mixture is brought up to 1 l with distilled water. LAS containing agar plates were flooded with this mixture and stained for 10 min. The plates were then washed and incubated at 37°C for 20 to 30 minutes. A clear zone surrounding a colony is indicative of LAS degradation. The MBAS solution assay was performed by acidifying the culture medium with H_2SO_4 adding methylene blue and chloroform, then monitoring absorbance of the chloroform phase at 652 nm on a Beckman DU-70 spectrophotometer.

Mineralization Experiments

Pond site - (done by The Procter and Gamble Company)

Mineralization of LAS by microbes present in water, aerobic sediments and associated with plant detritus was determined. Water was collected from the center of each pond using aseptic technique. Sediments were collected only from aerobic areas of the Laundromat Pond (LMP) and Control Pond and were found to be colonized by aquatic macrophytes. The Secondary Pond contained no such colonized sediments. Plant detritus (decaying oak leaves) was obtained from the margins of each pond. Water samples (5.0 ml), sediment samples (10 g), or pieces of leaf matter (1.9 cm diameter disks) in sterile water (1 ml) were placed into triplicate vials (40 ml) and amended with approximately

50 ng of the uniformly ring radiolabeled LAS. The vials were sealed with teflon-backed silicon septa equipped with plastic wells containing a fluted filter paper soaked with 0.2 ml of 1.5 N KOH to trap the evolved $^{14}\text{CO}_2$. The sealed vials were incubated statically for 7 days. Following acidification of the samples, the filter papers were removed and placed in a scintillation vial with cocktail, and radioactivity was determined using a liquid scintillation counter. Counts were corrected to 100% efficiency based upon external standardization. Data were expressed as the percentage of the radiolabeled LAS recovered as $^{14}\text{CO}_2$ after 7 days. The data was corrected utilizing abiotic controls amended with 0.1 ml formalin or with the detrital samples controls containing sterile water without a leaf disk.

(Activated sludge, Lake Loudoun and consortia - done at CEB University of Tennessee)

Mineralization assays were conducted using 20-ml glass vials sealed with a teflon-lined silicon septa (Pierce Chemical, Rockford IL). A 7 ml glass vial containing 1 ml of 0.5 N NaOH was used inside the 20 ml vial to trap $^{14}\text{CO}_2$. The culture volume was 2 ml which was acidified with 0.3 ml of 2 N H_2SO_4 to drive off CO_2 . Scintillation was done in *Ready Safe* scintillation fluor (Beckman Instruments, Norcross GA) using a Beckman LS3801 scintillation counter. All mineralization experiments were conducted using triplicate samples.

Thin-Layer Chromatography

The procedure initially used to prepare cultures for thin-layer chromatography is summarized in Figure 12. At the termination of mineralization experiments the acidified mineralization reactions were centrifuged to separate biomass from the medium.

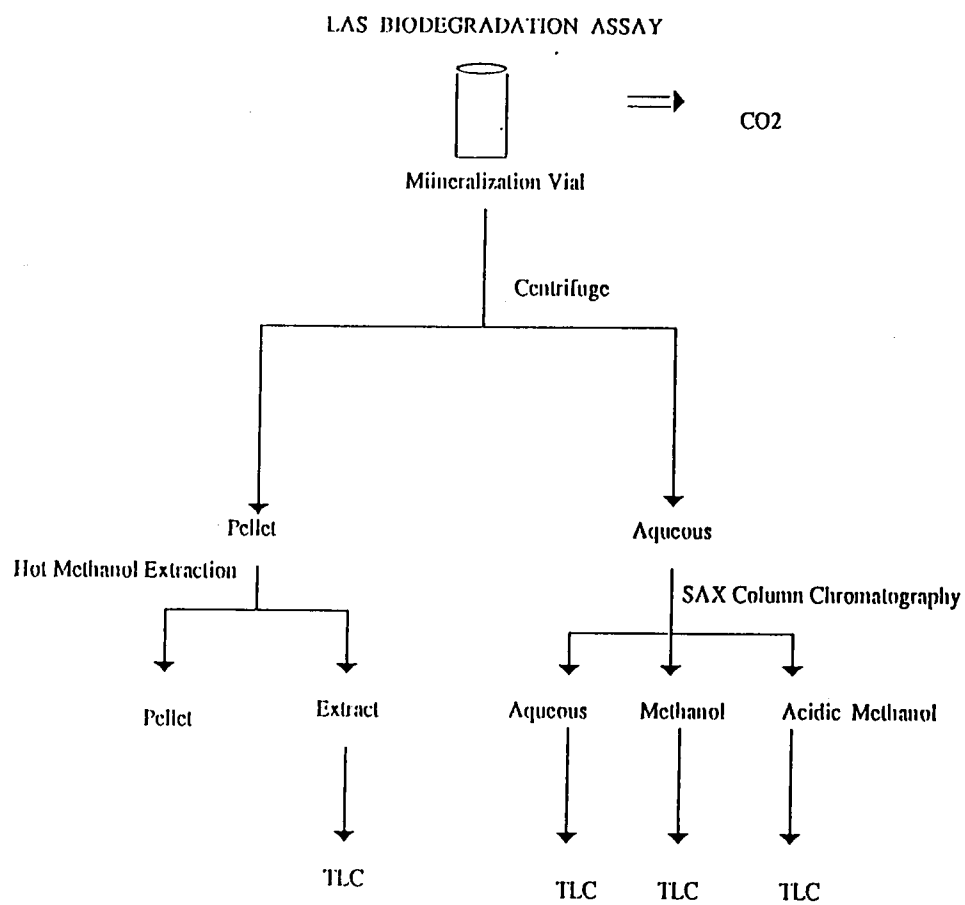


Figure 12. Scheme for processing LAS catabolic cultures for TLC analysis. Parent compound and metabolites were detected using a BioScan imaging system to detect ^{14}C .

The medium or aqueous fraction was dried under nitrogen to a volume suitable for spotting on TLC plates. The pellet was suspended in 2 ml of methanol and subjected to methanolysis at 95°C for 1 hour. The sample was then centrifuged to separate cellular material from the methanol. The methanol was then applied to a strong anionic exchange (SAX) column (*Sep-Pak* cartridge, Millipore, Milford, MA). Following the application of methanol, 2 mls of acidic methanol (1 N HCL in methanol) was applied to the SAX column. The methanolic fractions were also dried under nitrogen for subsequent TLC analysis. The samples were spotted onto K6 silica gel 60A TLC plates (Whatman International Ltd., Maidstone, England). Metabolites and parent compound were detected using a Bioscan Imaging System 200 (Bioscan Inc., Washington, D.C.). The counting efficiency for ^{14}C of this instrument was 5%. The solvent system used in the analysis was chloroform-methanol-water-formic acid (80:25:3:1) (v:v). In order to conserve radiolabeled material the procedure was modified by the omission of the SAX chromatography. This resulted in two fractions being used for analysis, an aqueous fraction and a methanol extracted biomass fraction. This modification was implemented due to the fact that 65-70% of the radioactivity was lost during SAX chromatography.

Continuous Culture

A New Brunswick Scientific (Edison, N.J.) BIOFLO chemostat with a 1 liter reactor volume was used for continuous culture experiments. The routine operating conditions for LAS degradative culture enrichment were as follows; a flow rate of 250 mls per day and a feed concentration of LAS of 10 mg/l. Due to the low flow rate required for these experiments a *Master Flex* pump (Cole-Parmer, Chicago, IL) was used rather than the

chemostat pump.

5. Results

Wisconsin Laundromat Pond Study

Sampling was conducted in July and November 1987. Microorganisms were enumerated on YEPG agar plates (total heterotrophs) and on YEPG supplemented with LAS (LAS/YEPG). Overall, bacterial densities on both media were higher in November than in July (Table 4). In addition, densities on both media were consistently higher in the wastewater system than in the control system. With one exception, densities on LAS/YEPG, expressed as a percentage of those on YEPG for sediment and detritus, were four to five times higher in the wastewater than in the control system. Furthermore, water from the control system harbored no organisms that grew on LAS/YEPG, whereas water samples from the wastewater pond contained 1.7×10^3 to 4.6×10^6 CFU/ml.

The mineralization of LAS in various habitats of the two pond ecosystems is shown in Table 5. In all habitats, mineralization levels were greater in the wastewater pond system than in the control pond system. Mineralization data were also consistent with bacterial population data. Sediments from control and contaminated sites exhibited similar levels of LAS mineralization (19.3 and 21.7% respectively) and comparable to the numbers of organisms that grew on LAS/YEPG media (2.6 and 4.5×10^3) in July. Detritus from the control pond exhibited LAS mineralization levels approximately 6-7 times lower than those exhibited by detritus from the laundromat pond in July. Densities on LAS/YEPG agar were likewise 5-fold lower with the control pond detritus. No mineralization of LAS was observed in the control pond water and no microorganisms from this habitat grew on LAS/YEPG. In contrast, in the wastewater pond there were 10^3

Table 4. Enumeration of bacteria (CFU/g) on LAS/YEPG and YEPG media in various habitats of the wastewater and control pond ecosystems in July and November.

Habitat ecosystem	July 1988		November 1988	
	LAS/YEPG	YEPG	LAS/YEPG	YEPG
Pond water				
Laundromat pond	1.8×10^4	8.1×10^7	4.6×10^6	3.2×10^8
Secondary pond	1.7×10^3	2.1×10^6	1.3×10^5	5.0×10^7
Control pond	NG*	1.2×10^4	NG	2.4×10^6
Sediment				
Laundromat pond	4.5×10^3	1.0×10^9	1.9×10^6	3.5×10^7
Control pond	2.6×10^3	6.5×10^6	2.0×10^4	1.4×10^7
Detritus				
Laundromat pond	1.0×10^4	2.6×10^5	2.8×10^6	4.3×10^8
Control pond	2.8×10^3	2.6×10^5	2.0×10^4	1.4×10^7

* - NG, no growth

Table 5. Mineralization of LAS in various habitats of the wastewater and control ecosystems in July.

Habitat ecosystem	% label converted to CO ₂
Pond water	
Laundromat pond	16.7±2.7
Secondary pond	11.4±2.2
Control pond	NM*
Sediment	
Laundromat pond	21.7±2.5
Control pond	19.3±1.6
Detritus	
Laundromat pond	25.6±3.0
Control pond	3.7±0.2

* - NM, no mineralization.

to 10^4 CFU/ml were recovered on LAS/YEPG, LAS was mineralized at a level of 41.9%

Plasmid incidence in bacteria isolated on non-selective (YEPG) medium and LAS containing medium was compared from the laundromat pond (LMP) site and the control sites (Table 6). A significant fraction of the isolates from both sites contained plasmids. Plasmid density was higher in November than in July and was higher in bacteria isolated on LAS/YEPG agar than those isolated on YEPG agar. The incidence of plasmids among total heterotrophs was 18% in the control system and 36% in the wastewater system. The incidence of plasmids in total isolates on LAS/YEPG was notably similar in both sites.

A subset of isolates from LAS/YEPG medium from both the contaminated and control sites were further characterized. The MBAS plate assay was used to examine the ability of isolates to carry out primary (alkyl chain) oxidation of the LAS molecule. Table 8 shows that most of the bacteria isolated on LAS-containing media appear to be LAS tolerant rather than capable of LAS oxidation. Only 23% of the selected isolates from the wastewater pond that grew on LAS/YEPG medium were able to degrade LAS based upon the MBAS assay. A smaller percentage (8%) of the isolates from the control pond were MBAS positive. Only a small percentage (8-23%) of plasmid bearing isolates on LAS/YEPG from the control and wastewater systems exhibited positive MBAS reactions. In the control site, all MBAS positive isolates contained plasmids. However in the wastewater site, 12% of the MBAS positive isolates carried plasmids. Thus, many plasmid containing isolates did not metabolize LAS and not all isolates that metabolized LAS contained plasmids. Notably, none of the isolates were able to mineralize LAS in

Table 6. Percentage of bacteria isolated on LAS/YEPG and YEPG agars from wastewater and control pond ecosystems that contained plasmids.

Ecosystem	Time	No of isolates tested		% plasmid bearing	
		LAS/YEPG	YEPG	LAS/YEPG	YEPG
Wastewater system	July	36	44	22	30
	November	177	173	51	37
	Total	213	217	46	36
Control system	July	20	26	35	12
	November	37	36	49	22
	Total	57	62	44	18

yes 8?

pure culture.

The same subset of LAS/YEPG positive colonies were screened by probe hybridization with genes involved in the oxidation of aromatic hydrocarbons. Table 7 lists the probes used and Table 9 presents the results. The NAH7 probe, which serves as a broad specificity indicator for aromatic hydrocarbon degradation, exhibited some homology with several isolates from the three sites surveyed. This was true to a lesser extent for the pDTG113 probe, a more specific probe consisting of the catabolic genes for the upper portion of the canonical naphthalene degradative pathway. Both probes were used to test the hypothesis that NAH or related catabolic gene sequences mediate LAS biodegradation and would be more abundant at sites exposed to LAS. NAH7 homologous sequences were found in isolates from both control and contaminated sites as were the pDTG113 genes. No organisms hybridizing with these gene probes were capable of LAS-ring mineralization in pure culture, and these genes were not more preponderant at the contaminated site relative to the control site; however, both contaminated and uncontaminated sites harbored similar levels of these genes. The whole plasmid pSS50, which has been of use in the isolation of 4-chlorobiphenyl degrading organisms from aquatic environments was also used as a probe (104). The results with pSS50 showed 5% of the wastewater isolates hybridized with pSS50, but none of the control pond isolates had any homology with this probe. A plasmid of a cryptic nature, from an uncharacterized isolate from the contaminated site, designated pP+G13, was used to assess the abundance of this indigenous plasmid in these environments. The rationale for using this plasmid as a probe was that it might have contained unique sequences relative

Table 7. Gene probes used to screen bacteria isolated on LAS/YEPG agar.

Probe DNA	Source	Phenotype
NAH7	<i>Pseudomonas putida</i>	Naphthalene degradation
pDTG113	<i>E. coli</i>	Upper pathway of naphthalene degradation
pSS50	<i>Alcaligenes</i> sp.	Chlorobiphenyl degradation
pP + G13	unknown	Cryptic

Table 8. Percentage of bacteria on LAS/YEPG agar from wastewater and control systems that contained plasmids, exhibited MBAS positive reactions, mineralized ^{14}C -ring labeled LAS and hybridized with specific gene probes.

Characteristic	Wastewater system ¹	Control system ²
Plasmid containing	46	68
MBAS positive	23	8
Mineralized ^{14}C -Ring LAS	0	0
MBAS positive with plasmid	12	8
Positive hybridizations*		
NAH7	10	12
pDTG113	6	4
pSS50	5	0
pP + G13	1	16

* - see Table 7 for description of gene probes.

to LAS degradation or survival in an unusual environment. In fact, the pP+G13 sequences proved to be more abundant among control site LAS/YEPG isolates, where 16% of the isolates hybridized, than in the LMP sediment, where 1.0% of the isolates hybridized. In summary, none of the gene probe sequences chosen for this analysis were more common in the isolates from the contaminated site relative to the control site. These data suggest that either the genetic potential for aromatic hydrocarbon oxidation as assessed with these gene probes is similar at both sites or the organisms that mineralize LAS do not grow on LAS/YEPG medium. However, a more likely conclusion is that the genes mediating LAS catabolism are unique and appropriate gene probes were not used.

Enrichment Cultures

In an effort to isolate a defined mixed culture capable of complete LAS degradation, enrichment cultures were inoculated with activated sludge from the Knoxville municipal wastewater treatment plant. In these experiments LAS was provided as the sole source of carbon and sulfur. Initial enrichment strategies involved shake flasks maintained in a "fill and draw mode" at an LAS concentration of 100 mg/l. This concentration appeared to be inhibitory to LAS mineralization, although they did maintain primary LAS oxidation capability as assayed by the MBAS method. Attempts to maintain mineralization were made by lowering the LAS concentration to 20 mg/l and using a two stage shake flask process in which a second flask was maintained on the effluent from the first. Figure 13 shows the fate of LAS in these flasks. Though LAS is rapidly removed in the first stage, as assessed by the MBAS assay, mineralization capabilities were again lost from these cultures after a two-week period. An LAS transformation

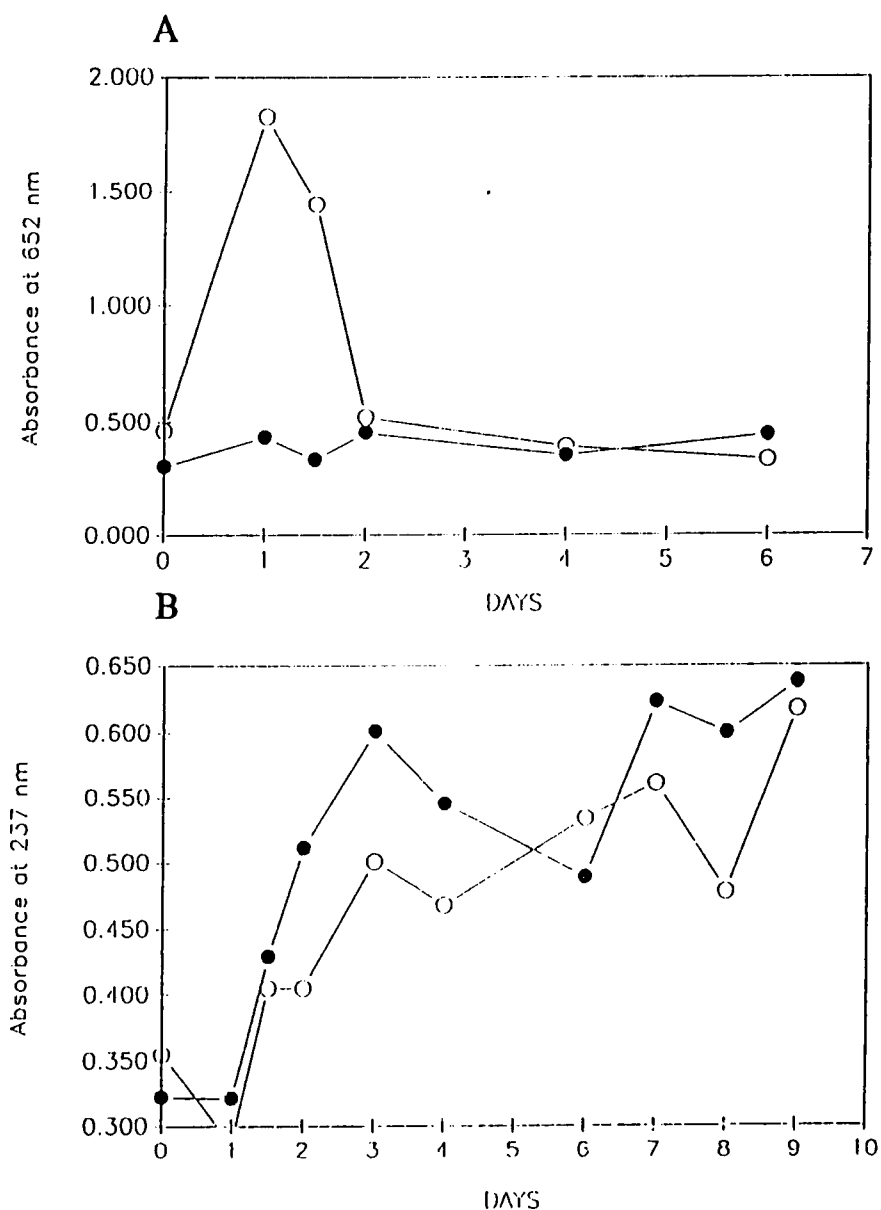


Figure 13. The fate of LAS in shake flask batch enrichment cultures. Open circles represent the first stage flask, closed circles represent the second stage flask. A. LAS remaining in solution as assayed by the MBAS. B. Absorbance of putative LAS metabolite.

product was monitored by absorbance at 236 nm. This product accumulated in both the first and second stage flasks.

Continuous culture experiments were again initiated using LAS as a sole source of carbon and sulfur at a feed concentration of 20 mg/l and a flow rate of 1 l per day. Again, the inoculum used was activated sludge. As was true with the batch cultures, the mineralization capabilities of this culture were rapidly lost. Also, as was observed with the shake flask experiments, a putative LAS metabolite, with an absorbance at 236 nm, was shown to accumulate when the chemostat pump was turned off (Figure 14).

Due to the repeated loss of LAS mineralization by enrichment cultures maintained on LAS, experiments were conducted using sulfonated aromatic compounds other than LAS as sole sources of carbon and sulfur. These experiments were conducted by using LAS acclimated and actively mineralizing cultures as inoculum for batch enrichment cultures using ethylbenzene sulfonate (EBS), *p*-toluene sulfonate (pTS) and benzene sulfonate (BSA) at a concentration of 100 mg/l. In addition to these schemes, a chemostat was maintained on LAS. These enrichment cultures appeared to maintain mineralization capabilities longer than the batch LAS enrichments but levels were not greatly enhanced (after 50 days only the EBS culture was mineralizing above 20% of the added LAS in a two-week incubation period and it had lost mineralization capability completely after 70 days - Figure 15).

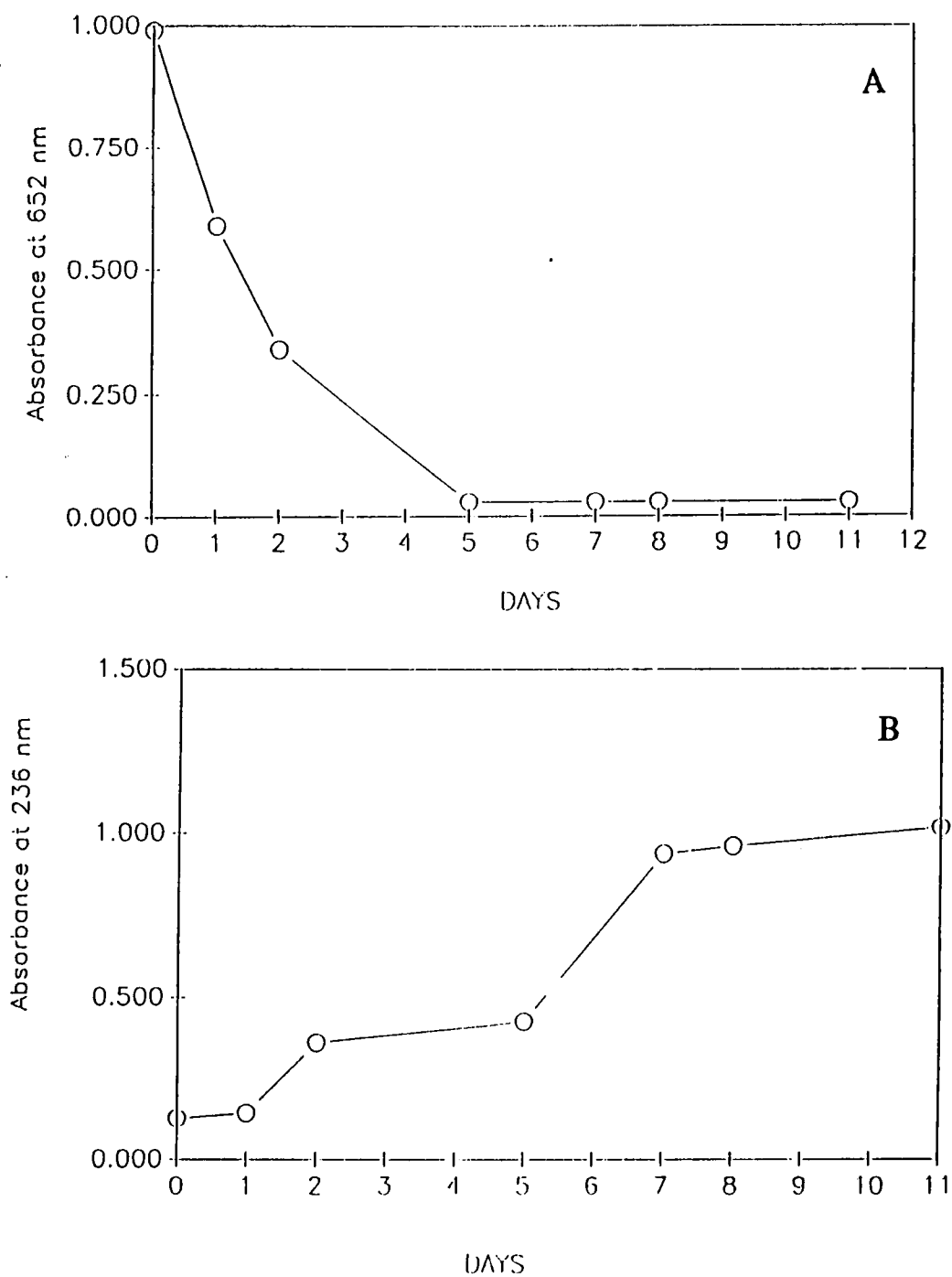


Figure 14. Fate of LAS in continuous culture enrichment. Disappearance of LAS was monitored after turning off the feed pump. A. LAS remaining in solution as determined by the MBAS assay. B. Putative LAS metabolite.

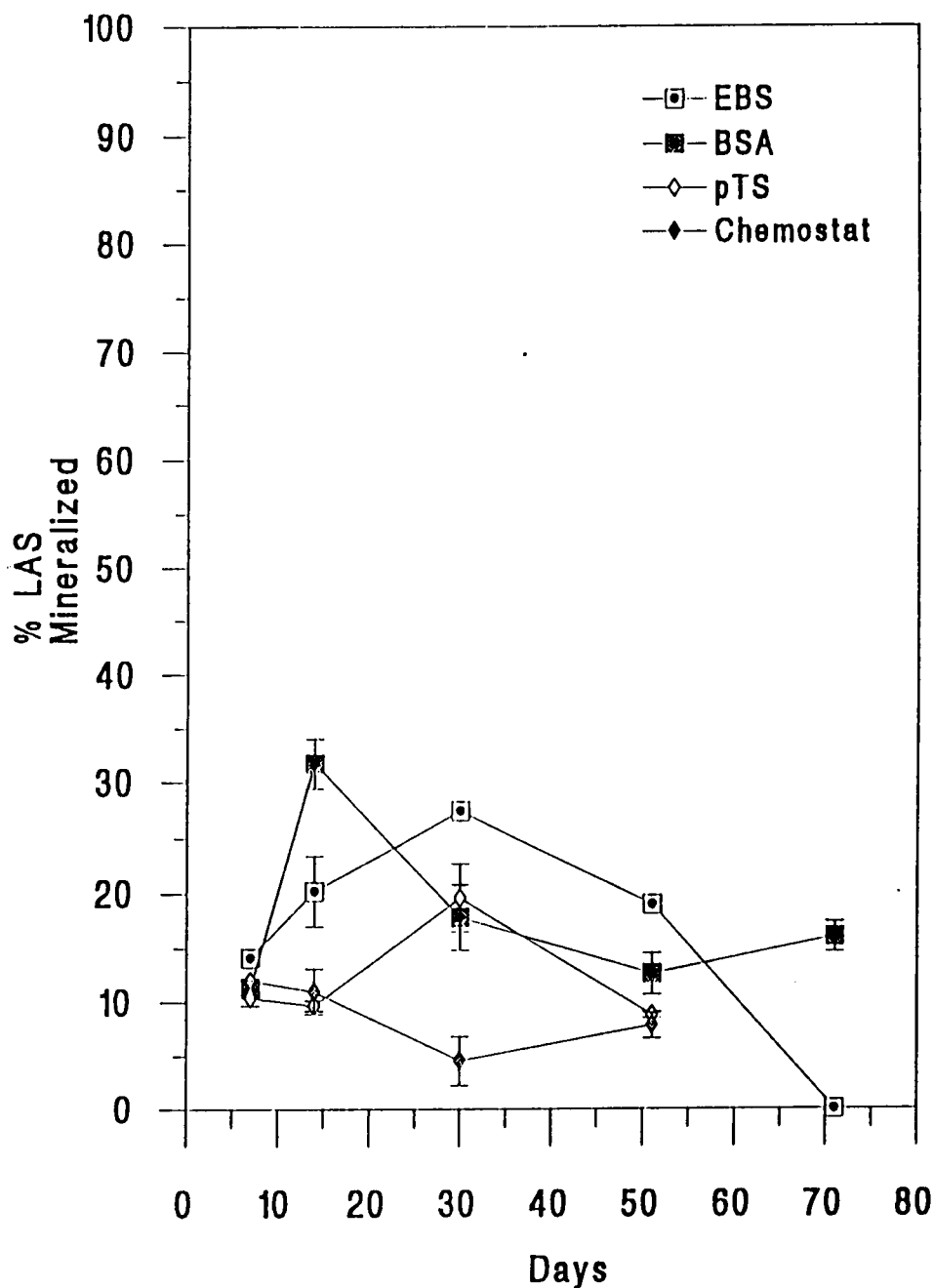


Figure 15. Mineralization of LAS by activated sludge cultures acclimated to ethylbenzene sulfonate (EBS), benzene sulfonate (BSA), and *p*-toluene sulfonate (pTS) in shake flasks. A chemostat culture derived from activated sludge was maintained on EBS.

Using continuous culture with a low (10 mg/l) LAS concentration and a low-flow rate (250 ml per day which corresponds to a four-day hydraulic retention time) mineralization was maintained above the 25% level for six weeks. During this time the chemostat culture, unlike the shake flask enrichments, did not maintain a sludge-like appearance but rather appeared to select for a low density planktonic population. This suggests that a low substrate concentration and a low flow rate appeared to be critical factors in maintaining a high level of LAS mineralization. This strategy allowed for the isolation of a defined mixed culture or consortium capable of complete LAS mineralization.

Consortium KJB

Four gram-negative bacteria were selected from dilution spread plates from the chemostat maintained on a low LAS concentration (10 mg/l) at a low flow rate (250 ml/day). When the four were incubated with LAS, they were able to convert 25% of the added label to CO₂ in a two-week period. This consortium, which was designated KJB, consisted of *Pseudomonas aeruginosa* KJB1, *Xanthomonas maltophilia* KJB2, *Enterobacter cloacae* KJB3 and unknown strain KJB4, as tentatively identified by fatty acid methyl ester (FAME) analysis (Table 9). Although similarity index values lower than 0.5 are not high enough to categorize organisms reliably into specific genera, this analysis does reveal a gross likeness among the two consortia analyzed. Table 10 shows some of the biochemical characteristics of this group and Table 11 shows results of carbon assimilation tests. The requirement for all four members was absolute. No single member could produce CO₂ from LAS nor could any combination of three organisms omitting one member of the group.

Table 9. Fatty acid methyl ester (FAME) analysis and tentative identification of members of LAS mineralizing consortia KJB and PG (analysis conducted by MicroCheck Inc., Burlington VT).

Organism	Similarity Index
KJB	
<i>P. aeruginosa</i> KJB1	.406
KJB4 (unknown)	no match
<i>E. cloacae</i> KJB3	.870
<i>X. maltophilia</i> KJB2	.316
PG	
<i>Alcaligenes</i> sp. PG1	.672
<i>P. aeruginosa</i> PG2	.575
<i>E. cloacae</i> PG3	.845
<i>X. campestris</i> PG4	.224

Table 10. Biochemical analysis of members of consortium KJB.

	KJB1	KJB2	KJB3	KJB4
Nitrate reductase	+	+	+	+
Tryptophanase	-	-	-	-
Glucose fermentation	-	-	-	-
Arginine dihydrolase	+	-	+	-
Urease	+	-	-	-
Esculin hydrolysis	+	-	+	-
Beta-galactosidase	+	-	+	-
Oxidase	+	+	+	+
Arylsulfatase	+	-	-	+
Gelatinase	+	-	+	-
LAS TTC:NBT*	red	-	-	-

* - Reduction of tertazolium dyes 2,3,5,-triphenyl-2H-tetrazolium chloride, nitroblue tetrazolium.

Table 11. Carbon assimilation screening of consortium KJB.

	KJB1	KJB2	KJB3	KJB4
Glucose	+	-	+	-
Arabinose	-	-	-	-
Mannose	+	-	+	-
Mannitol	+	-	+	-
N-acetyl glucosamine	+	-	+	-
Maltose	+	+	+	-
Gluconate	+	+	+	-
Caprate	+	-	+	-
Adipate	+	-	+	-
Malate	+	-	+	+
Citrate	+	-	+	+
Phenylacetate	+	-	+	-

The MBAS assay showed that three of the four members were able to remove LAS from solution suggesting that three of the four bacteria participate in primary oxidation of LAS with only *E. cloacae* KJB3 lacking this ability (Figure 16). Follow up work on this observation using TLC analysis showed that primary oxidation of LAS was in fact mediated by KJB4. Figures 17-21 show the TLC profiles of the consortium as a whole and individual members as pure cultures following a two week incubation with ^{14}C -LAS. These data show that although a number of consortium members were removing LAS from solution, only KJB4 was transforming it to polar products. In most cases the ^{14}C was recovered as material sorbed to the biomass.

One hypothesis for the necessity of the complex communities involved in LAS degradation is that organisms may serve as biosorbents or mediate floc formation thereby protecting more sensitive organisms from the deterative properties of LAS. The KJB members were assayed as individuals for their ability to grow in the presence of LAS. Cultures were inoculated into YEPG medium and growth monitored by optical density. Overall the KJB members were relatively resistant to LAS (Figure 22). The response of strains KJB1 and KJB3 were virtually unaffected by LAS concentrations up to 200 mg/l (the highest concentration tested). Strain KJB2 appeared to be stimulated by increasing LAS concentration but this is, in fact, not the case. KJB2 normally grows in a non-turbid floc forming mode. High LAS concentrations result in floc disruption causing a higher optical density. The dry weights of the KJB2 cultures were unchanged from 0 to 200 mg/l LAS demonstrating that LAS did not quantitatively affect growth. KJB4 appeared to be inhibited by LAS at high concentrations (100 to 200 mg/l) once the stationary phase

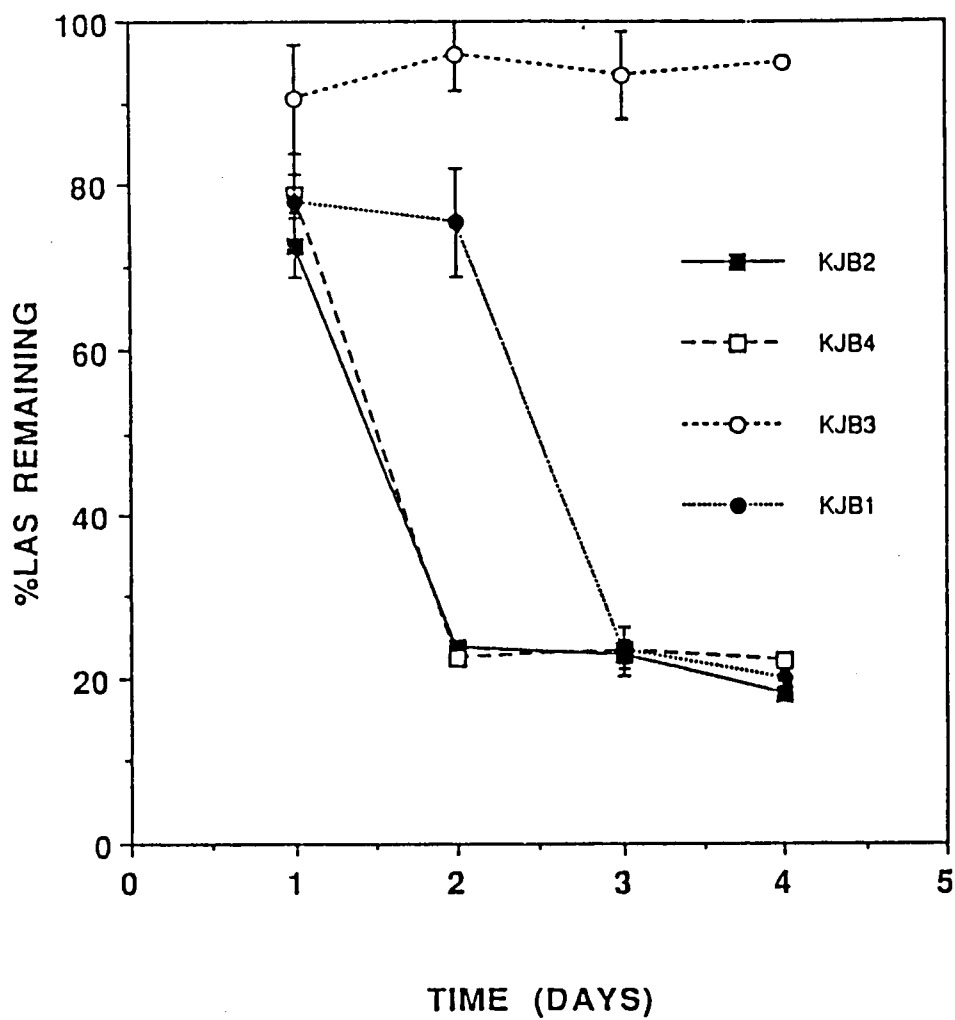


Figure 16. LAS removal by individual members of consortium KJB using the MBAS assay. This experiment was conducted in shake flasks at an LAS concentration of 10 mg/l. The results indicate that three of the four KJB consortium members are capable of primary LAS oxidation.

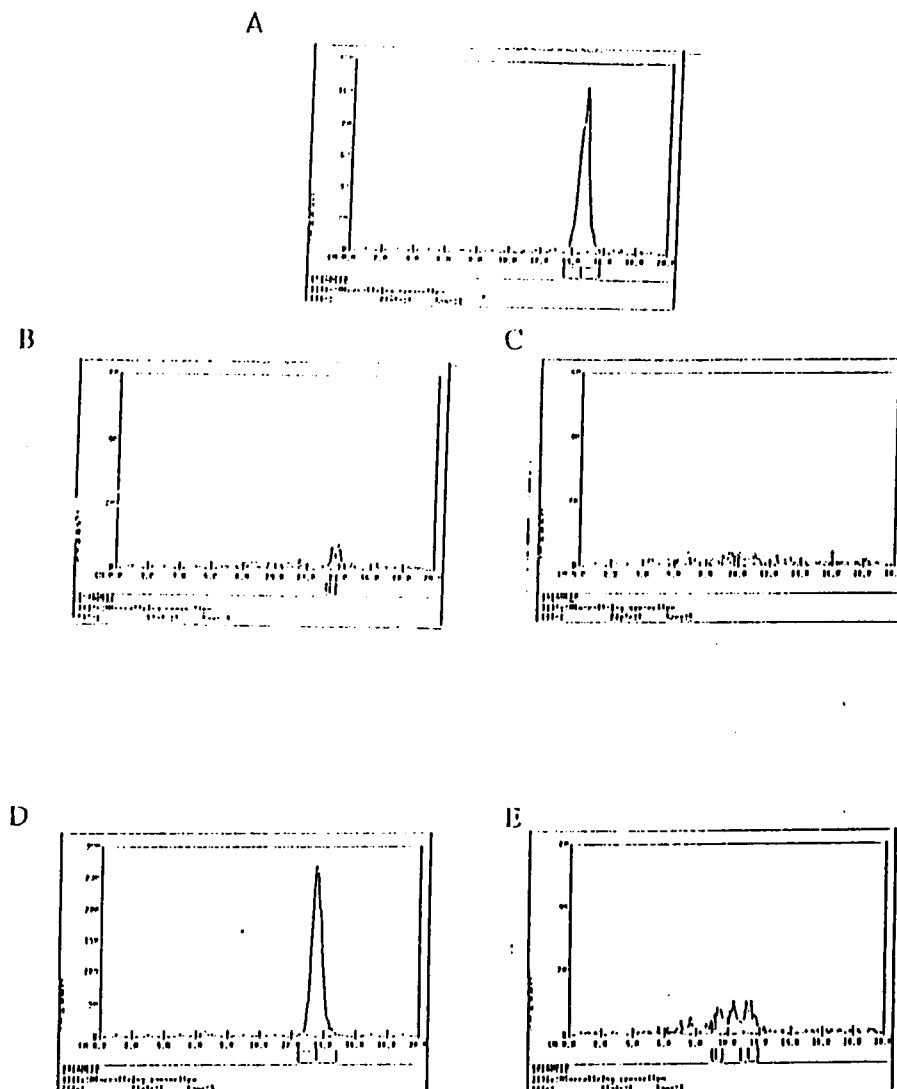


Figure 17. TLC analysis of LAS catabolism by consortium KJB following a two-week incubation with ^{14}C -ring labeled LAS. The culture was prepared in the manner depicted in figure 4. A. standard, B. cell pellet, C. methanol, D. acidic methanol, E. aqueous. The X axis is distance migrated and the Y axis is counts per minute (cpm). This culture was actively mineralizing LAS, although as seen in panel D, a considerable amount of LAS remained untransformed.

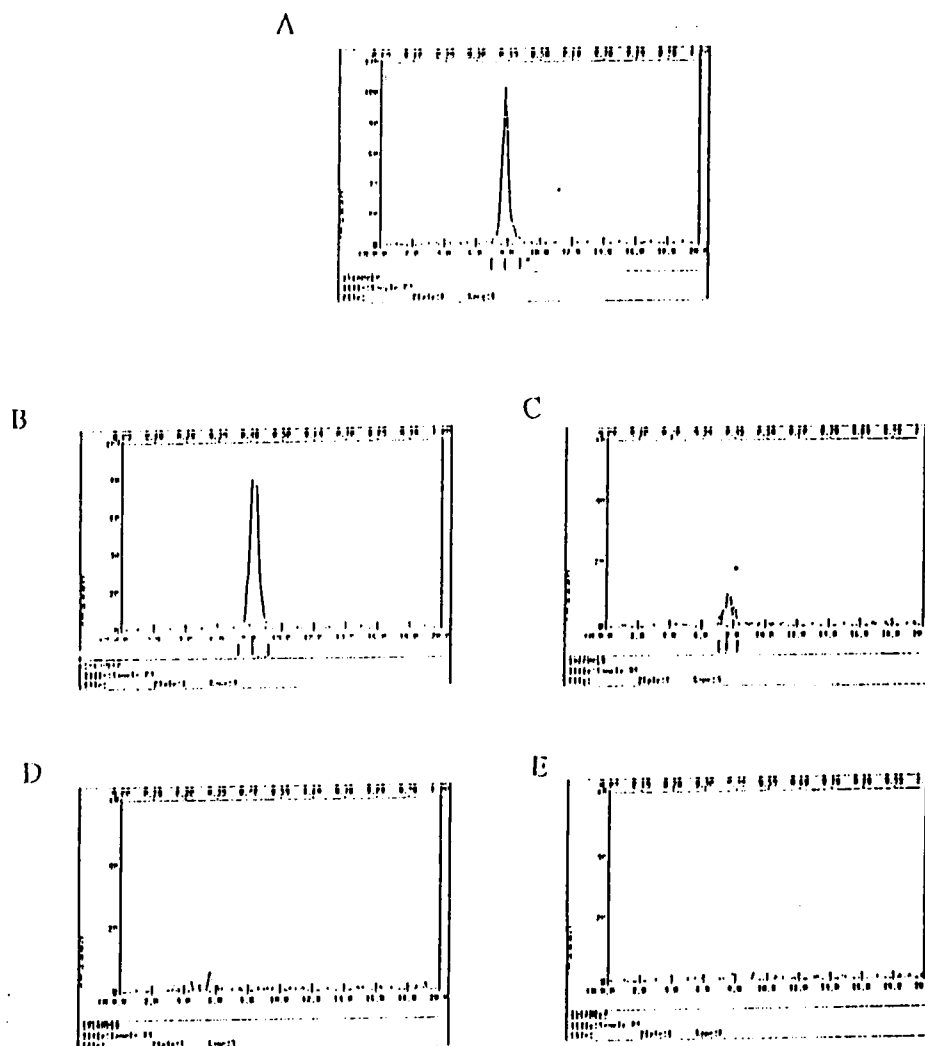


Figure 18. TLC analysis of LAS catabolism by *P. aeruginosa* KJB1 as a pure culture following a two-week incubation with ^{14}C -ring labeled LAS. The culture was processed in the manner depicted in figure 4. A. standard, B. cell pellet C. methanol, D. acidic methanol, E. aqueous (X axis = distance migrated, Y axis = cpm). No mineralization was observed, and a large fraction of the label was sorbed to biomass rather than transformed (panel B).

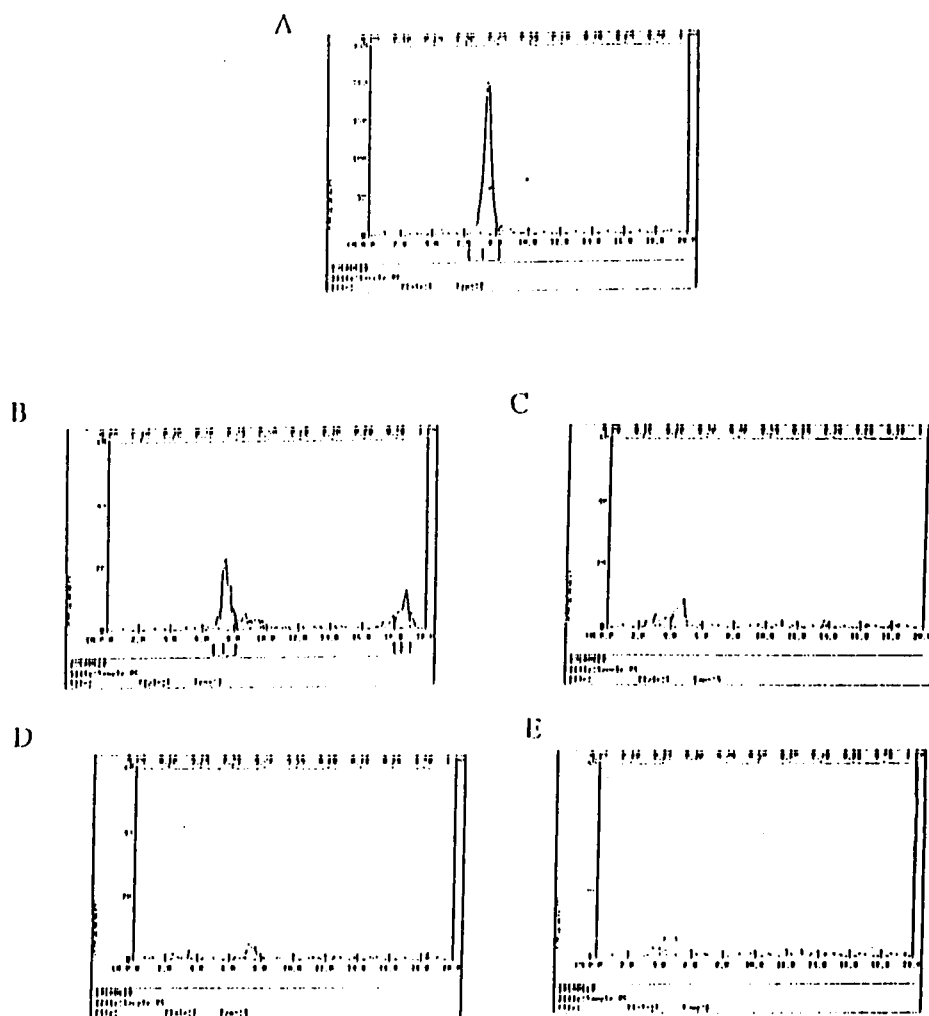


Figure 19. TLC analysis of LAS catabolism by *X. maltophilia* KJB2 following a two-week incubation with ^{14}C -ring labeled LAS. The culture was processed in the same manner depicted in figure 4. A. standard, B. cell pellet, C. methanol, D. acidic methanol, E. aqueous (X axis = distance migrated, Y axis = cpm). Some transformation of LAS is evident in pannel C; an unidentified non-polar prduct appears in panel B.

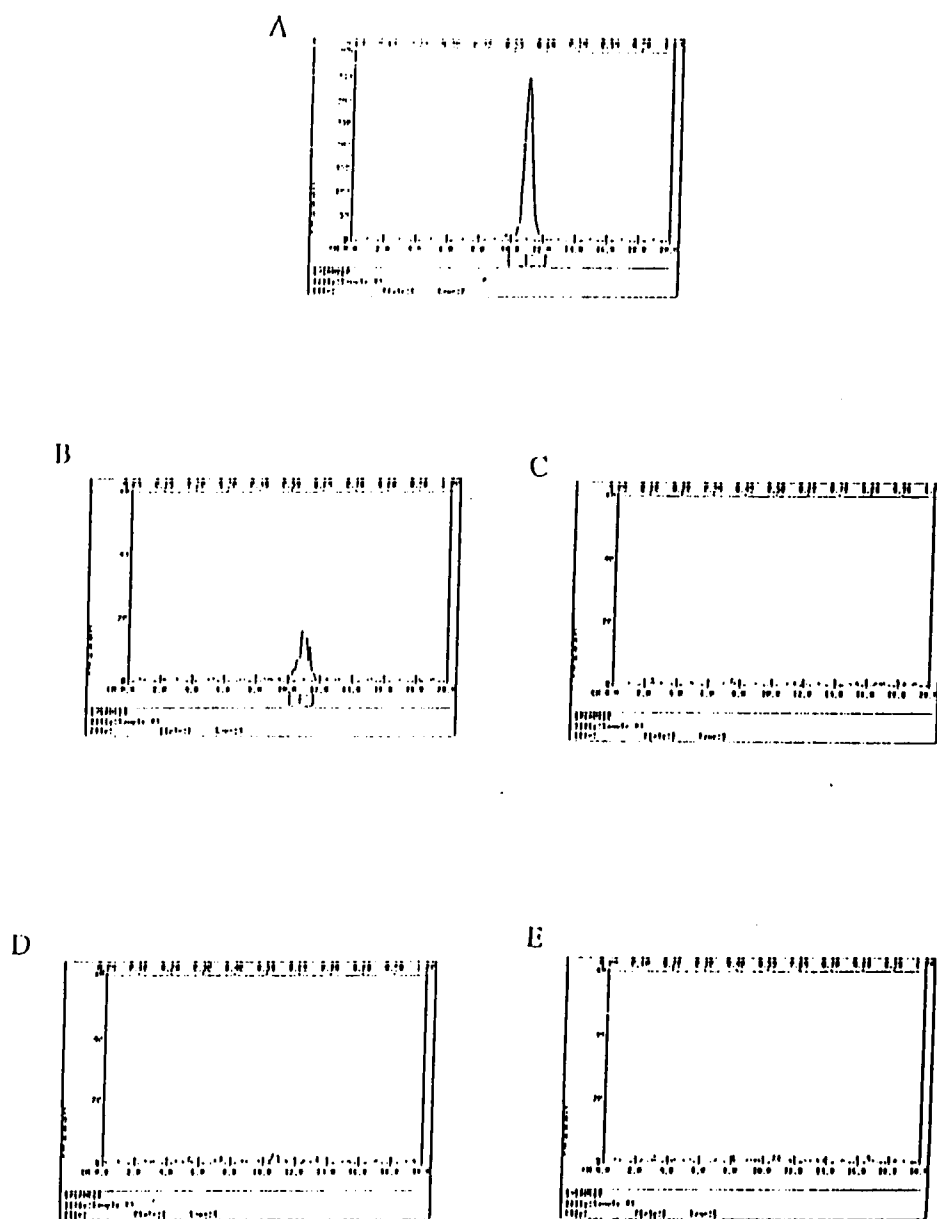


Figure 20. TLC analysis of LAS catabolism by *E. cloacae* KJB3 following a two-week incubation with ^{14}C -ring labeled LAS. The culture was processed in the manner depicted in figure 4. A. standard, B. cell pellet, C. methanol, D. acidic methanol, E. aqueous (X axis = distance migrated, Y = cpm). No transformation products are evident.

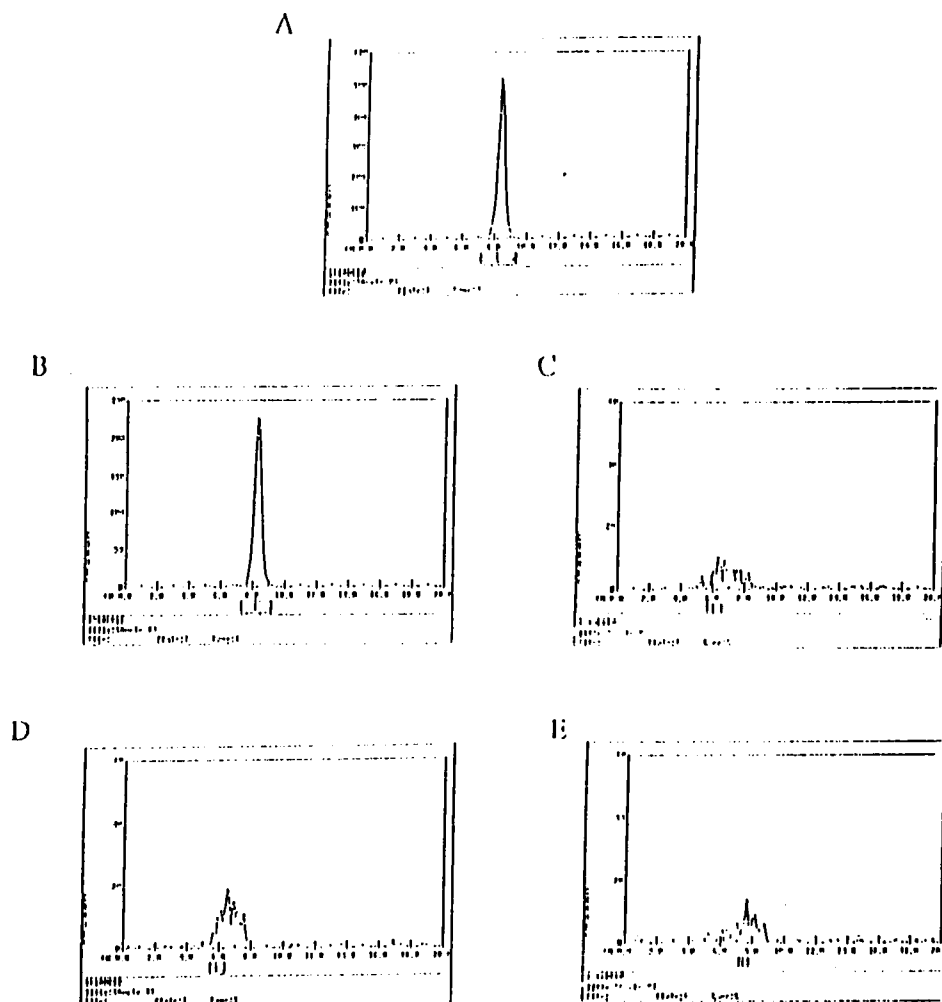


Figure 21. TLC analysis of LAS catabolism by unidentified bacterium KJB4 following a two-week incubation with ^{14}C -ring labeled LAS. The culture was processed in the manner depicted in figure 4. A. standard, B. cell pellet, C. methanol, D. acidic methanol, E. aqueous (X axis = distance migrated, Y axis = cpm). Transformation products can be observed in panels C, D and E the bulk of LAS remained untransformed and associated with the cell pellet.

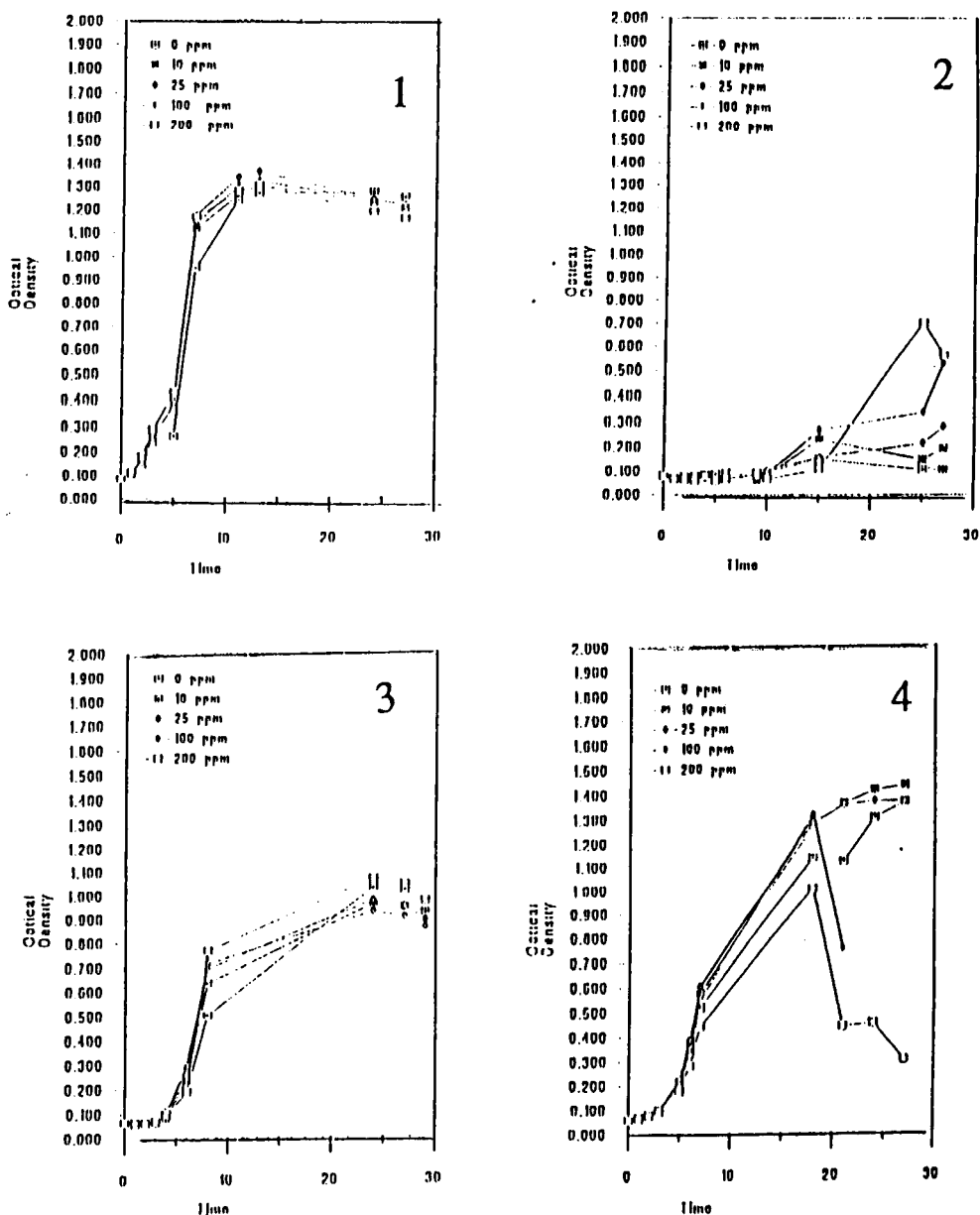


Figure 22. Effect of LAS on growth of members of consortium KJB. Shake flasks with various LAS concentrations (from 0 to 200 mg/l) were inoculated with individual members of consortium KJB and growth monitored by absorbance at 595 nm. 1. *P.aeruginosa* KJB1, 2. *X. maltophilia* KJB2, 3. *E. cloacae* KJB3, 4. unidentified isolate KJB4.

had been reached. These experiments seemed to contradict the fact that mineralization of LAS by KJB was inhibited at concentrations above 20 mg/l. It has been noted in the literature that 20 mg/l LAS concentrations were detrimental to LAS-degrading cultures yet the data generated suggest that these bacteria are resilient to the toxic properties of LAS (122).

Although an LAS-mineralizing community had been isolated and defined, its mineralizing capabilities were considerably lower than had been observed in a number of natural environments tested. For example, in a comparable time frame Lake Loudoun sediment (Knoxville, TN) or control pond sediment from the Wisconsin pond study were more adept at LAS mineralization (Lake Loudoun sediment mineralized 28.5% and the control pond 19.3% of the added LAS). Though one might expect the consortium to be primed and more proficient at LAS mineralization, this was not the case. It seemed logical that other assemblages existed that would be more proficient at LAS mineralization and thus more amenable to laboratory study.

Biomass from the original chemostat culture which yielded KJB was used to inoculate a batch flask culture which was maintained on 20-30 mg/l LAS. From this enrichment culture a second LAS mineralizing consortium (PG) was isolated which, like KJB, contained four members and, like KJB, required all four members for complete LAS mineralization.

Consortium PG

Tentative strain identification was derived from FAME profiles, carbon assimilation and biochemical tests the results of which are shown in Tables 9, 12 and 13. The PG

Table 12. Biochemical analysis of consortium PG.

	PG1	PG2	PG3	PG4
Nitrate reductase	+	+	+	+
Tryptophanase	+	+	+	+
Glucose fermentation	-	-	-	-
Arginine dihydrolase	-	-	-	-
Urease	-	-	-	-
Esculin hydrolysis	-	-	+	+
Beta-galactosidase	-	-	+	+
Oxidase	+	+	+	+
Arylsulfatase	+	-	-	-
Gelatinase	-	-	-	+
LAS TTC:NBT	-	Red	-	-

Table 13. Carbon assimilation analysis of consortium PG.

	PG1	PG2	PG3	PG4
Glucose	-	+	+	-
Arabinose	-	-	+	-
Mannose	-	-	+	+
Mannitol	-	-	+	-
N-acetyl glucosamine	-	-	+	+
Maltose	-	-	+	+
Gluconate	+	+	+	-
Caprate	-	+	-	-
Adipate	+	+	-	-
Malate	+	+	+	+
Citrate	+	+	+	+
Phenylacetate	+	+	+	-

consortium contained an *E. cloacae* and a *P. aeruginosa* as did consortium KJB, but the strains were distinct from one another as shown by carbon assimilation and biochemical analysis. Furthermore, replacement experiments in which one *P. aeruginosa* or *E. cloacae* was replaced by another did not result in LAS mineralization. This suggested that these organisms do not play analogous roles in the different consortia. The PG consortium demonstrated a proficiency for LAS catabolism superior to that of KJB. A number of observations support this fact:

- (i.) consortium PG converts over 30% of added LAS to CO₂ in 48 hrs. KJB requires 13 days for a similar level of conversion (Figure 23 shows a time course experiment of CO₂ evolution over time).
- (ii.) A comparison of metabolite production for KJB and PG show very different profiles (Figures 17 and 24). The KJB consortium shows a substantial amount of untransformed LAS remaining in solution as opposed to consortium PG where no untransformed LAS is detectable.
- (iii.) Unlike KJB, consortium PG is capable of LAS mineralization at high concentrations as shown in Figure 25 and Table 14.

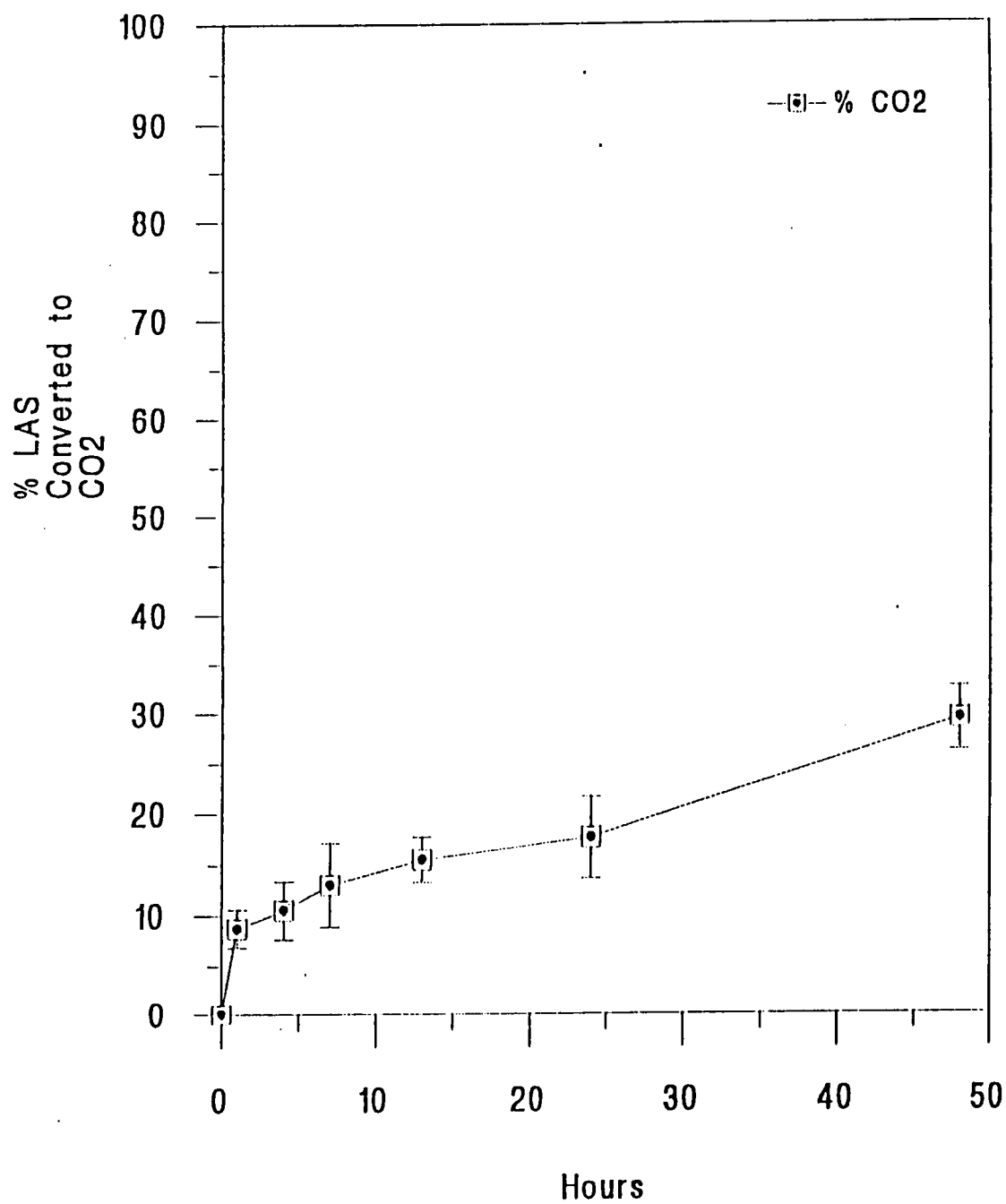


Figure 23. Time course LAS mineralization experiment with consortium PG (LAS concentration = 0.05 mg/l).

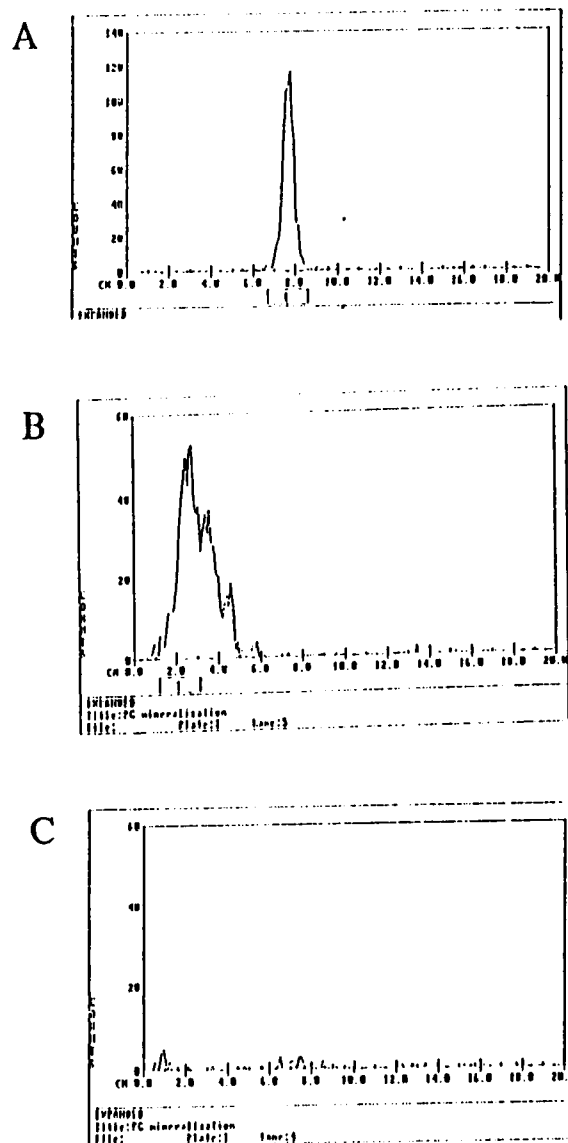


Figure 24. TLC analysis of LAS mineralizing Consortium PG in mineral salts medium after a 48-hour incubation with ^{14}C -ring labeled LAS. No detectable LAS was seen in the aqueous or cell pellet fractions. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). Panel B shows the appearance of a number of polar transformation products.

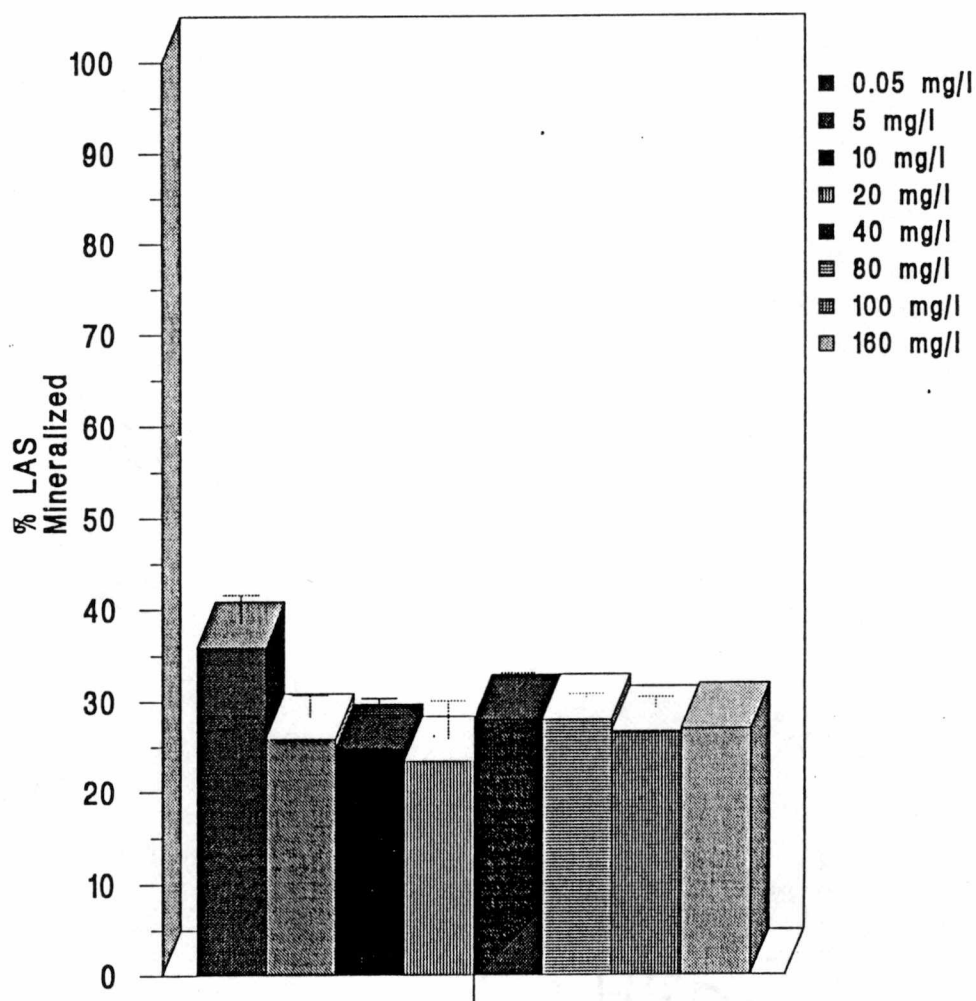


Figure 25. Mineralization of LAS by consortium PG at high LAS concentrations. Triplicate 2-ml batch assay mineralization vials were incubated with increasing LAS concentrations up to 200 mg/l. Following a 5-day incubation period the cultures were acidified and $^{14}\text{CO}_2$ trapped. No mineralization was observed at 200 mg/l.

Time-course experiments at 80 and 100 mg/l LAS demonstrated LAS mineralization almost as rapid as that observed at low LAS concentrations (Figure 26). Significant mineralization of LAS was observed at 160 mg/l, although at 200 mg/l no mineralization was seen. Despite this proficiency for LAS oxidation, when maintained in mineral salts LAS medium, the turbidity of the culture decreased.

Consortium individuals in mineral salts LAS medium showed no transformation of LAS. When incubated with LAS in 100th strength YEPG *E. cloacae* PG3 was able to generate polar transformation products from LAS. While PG2 did show a small amount of LAS transformation, PG1 and PG4 demonstrated no transformation. As shown in Figures 27-30, a considerable amount of LAS remained associated with the biomass and untransformed in these cultures.

Experiments were undertaken to determine whether changes in the composition of the consortium would correlate with changes in LAS metabolism. One strategy involved growing the consortium in 100th strength YEPG at various LAS concentrations, then correlating loss of mineralization or metabolite production with a change in consortium composition. It is important to note that an acclimated culture was maintained throughout these studies on approximately 10 mg/l LAS with trace amounts of yeast extract. This culture showed some population variation over time as shown in Table 15. Despite its role as the most adept organism for the primary oxidation of LAS, *E. cloacae* PG3, is in general the least abundant organism in the maintenance culture. This may be due to the low LAS concentrations at which this culture is kept. When population dynamics

Table 14. LAS mineralization by consortium PG at high LAS concentrations (5-day incubation).

Initial Concentration	% Mineralized	Total ug Mineralized
5 mg/l	25.9	1.3
10 mg/l	24.8	2.48
20 mg/l	23.4	4.68
40 mg/l	28.0	11.2
80 mg/l	22.4	22.4
100 mg/l	26.7	26.7
160 mg/l	27.0	43.2

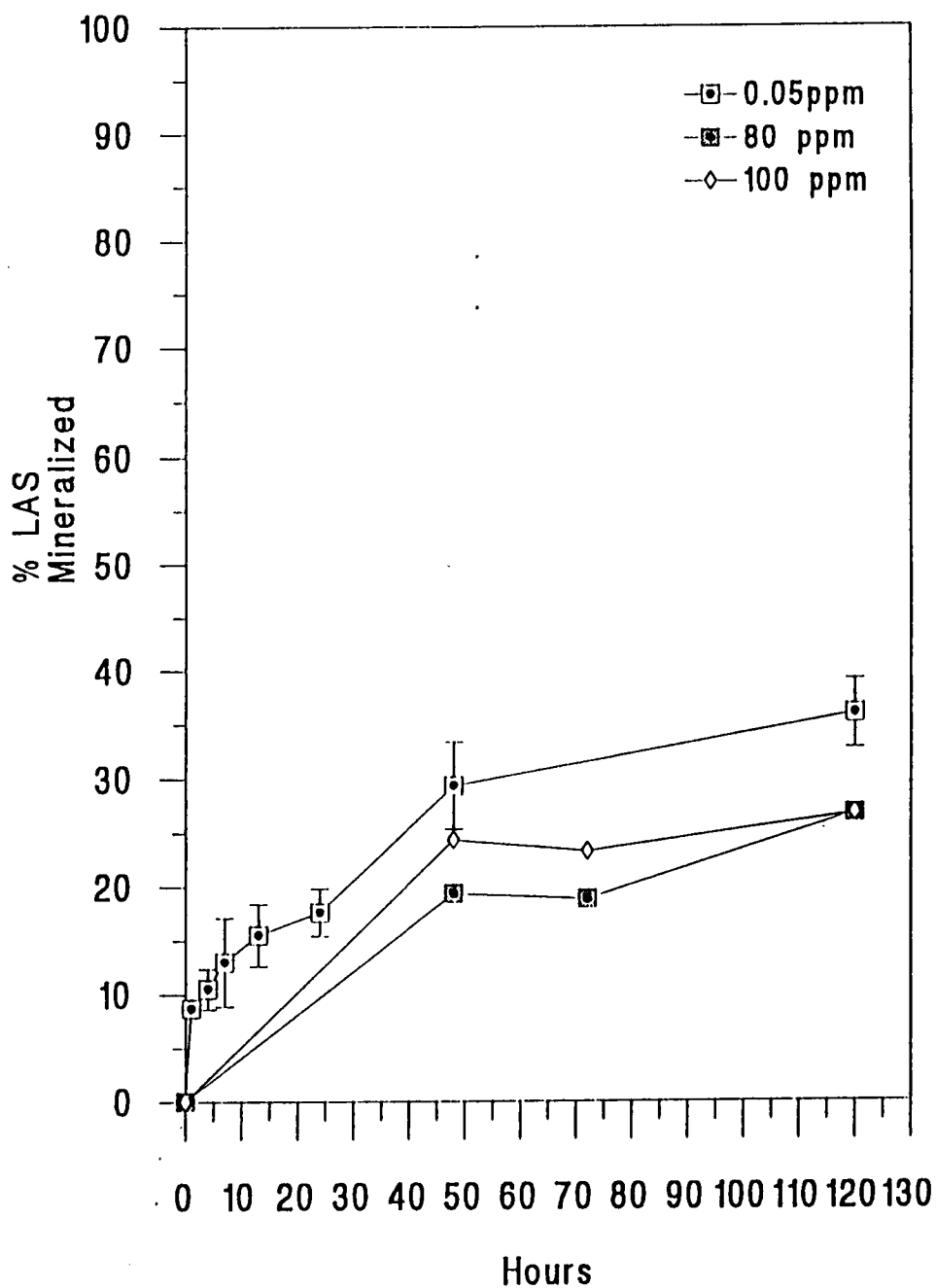


Figure 26. Time course study of LAS mineralization by consortium PG at 0.05 mg/l, 80.0 mg/l and 100 mg/l LAS. Assays were carried out in triplicate 2 ml mineralization vials and acidified to drive off $^{14}\text{CO}_2$ at appropriate time intervals.

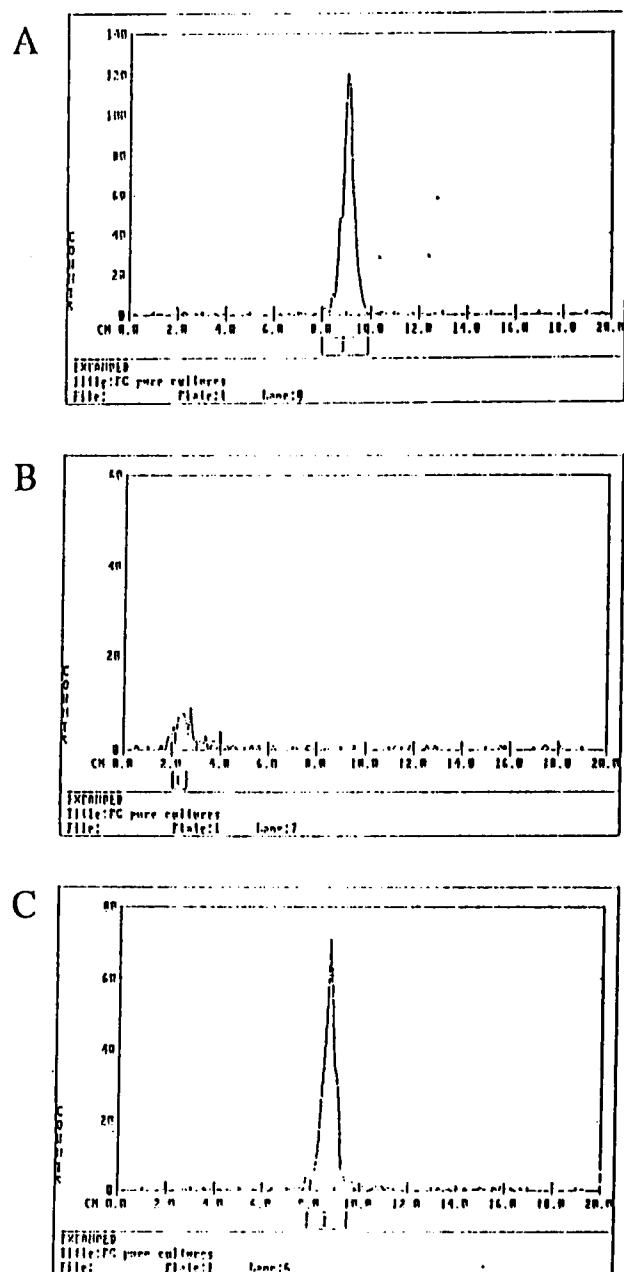


Figure 27. TLC analysis of LAS catabolism by a pure culture of *Alcaligenes* sp. PG1 following a two-week incubation with ^{14}C -ring labeled LAS in 100th strength YEPG medium. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). Some minor transformation products are visible in panel B.

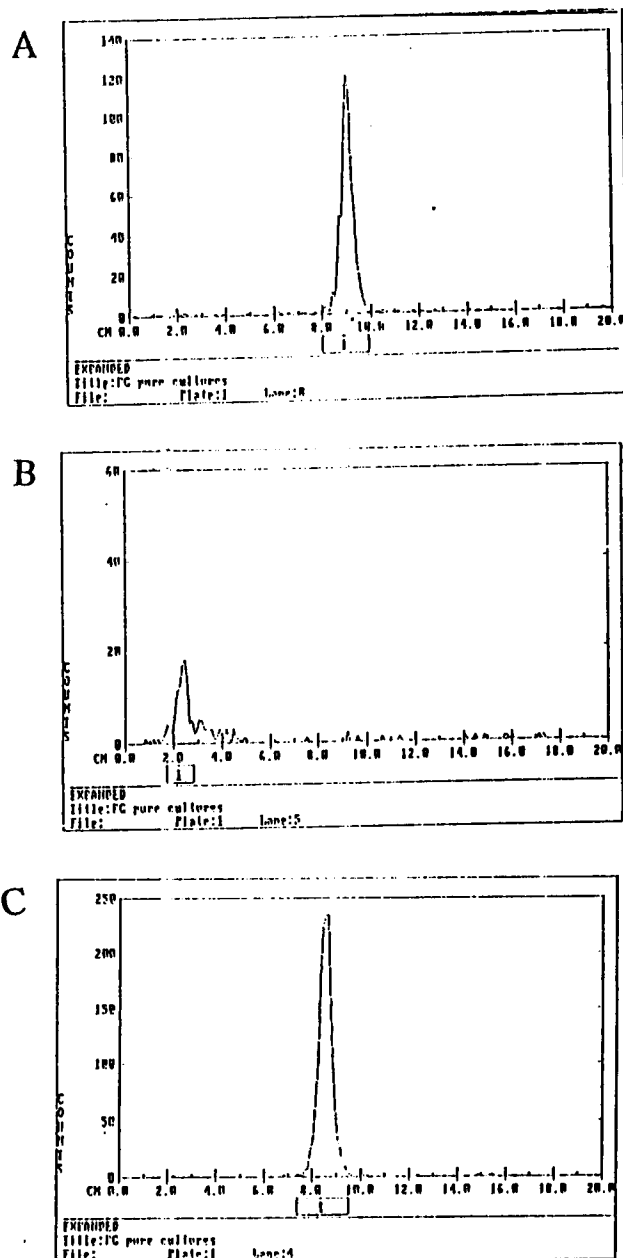


Figure 28. TLC analysis of LAS catabolism by a pure culture of *P. aeruginosa* PG2 following a two-week incubation with ^{14}C -ring labeled LAS in 100th strength YEPG medium. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). Some minor transformation products are visible in panel B.

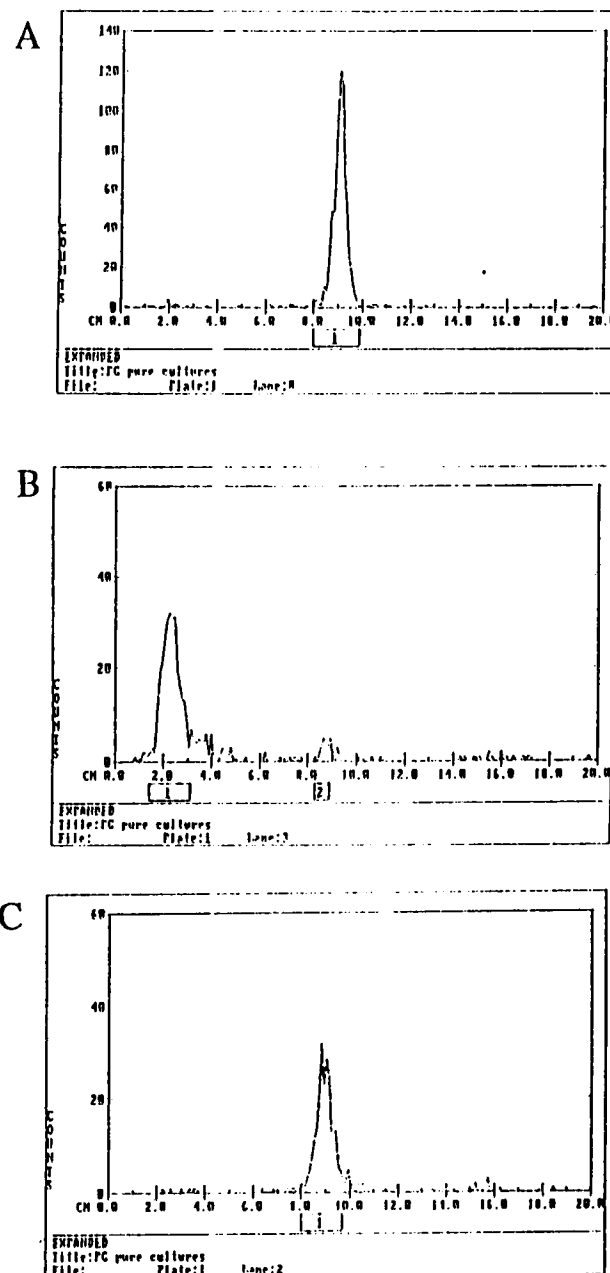


Figure 29. TLC analysis of LAS catabolism by a pure culture of *E. cloacae* PG3 following a two-week incubation with ^{14}C -ring labeled LAS in a 100th strength YEPG medium. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). Panel B shows a considerable portion of LAS being transformed to polar products.

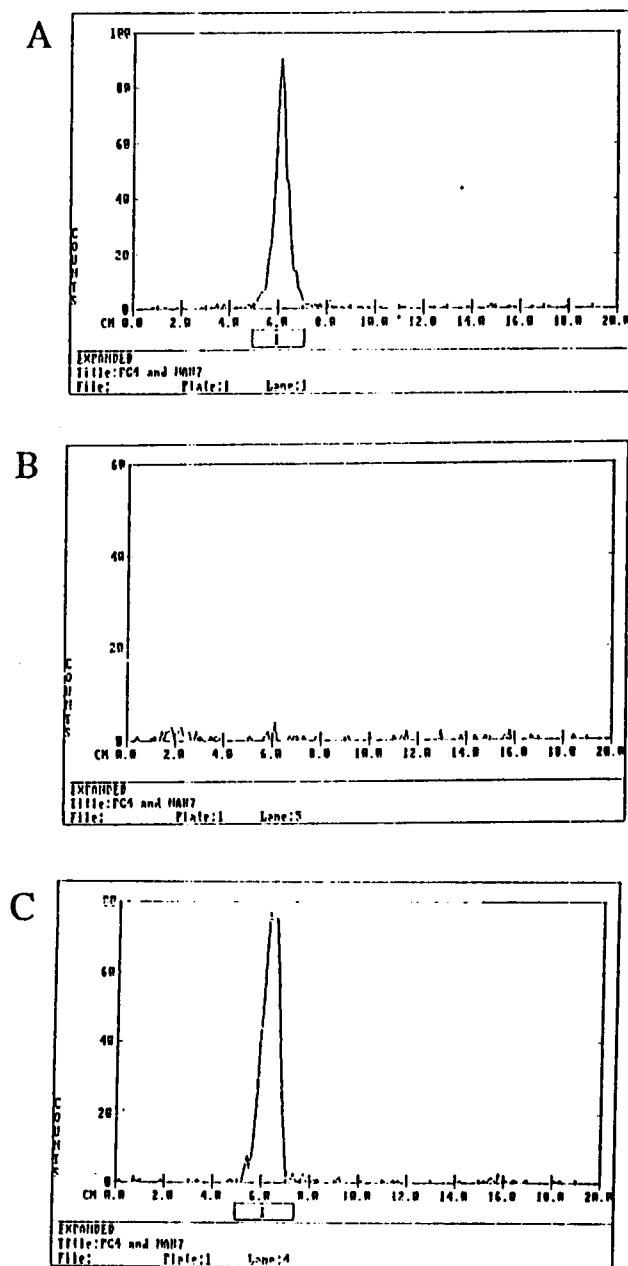


Figure 30. TLC analysis of LAS catabolism by a pure culture of *X. campestris* PG4 following a two-week incubation with ^{14}C -ring labeled LAS in a 100th strength YEPG medium. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). Virtually all the label remains as untransformed LAS associated with the cell pellet (panel C).

Table 15. Population composition of maintenance culture (% of total population).

Organism	10/91	2/92	4/92
<i>Alcaligenes</i> sp. PG1	12.0	23.1	29.6
<i>P. aeruginosa</i> PG2	3.0	37.6	35.1
<i>E. cloacae</i> PG3	3.8	6.0	11.5
<i>X. campestris</i> PG4	80.3	32.7	22.9

studies were initiated, PG3 was the least abundant organism in the maintenance culture while the other three were present at roughly similar amounts.

In these experiments PG3 was shown to grow more rapidly than the other consortium members in non-LAS supplemented 100th strength YEPG medium (Table 16). As LAS concentration increased, the predominance of this organism also increased (Tables 17-23). For example, as shown in Table 23, after 1 day PG3 was the only consortium member detected. The general trend showed that PG3 predominated during the early time points while PG4 would usually catch up as time progressed. PG1 and PG2 were more sensitive to LAS; their population levels were lessened at 50 mg/l LAS. As mentioned earlier, 200 mg/l was the concentration at which mineralization ceased. At this concentration PG1 was not detected for the duration of the experiment. PG1 also could not be detected at 100 mg/l but was presumably present at a level sufficient to play its role in LAS catabolism. The outright omission of PG1 from the consortium, as stated above, resulted in the loss of LAS mineralization. Figures 31-33 show TLC analysis of the consortium following a five-day incubation with LAS demonstrating metabolite production at 80, 100 and 200 mg/l LAS. These data suggest that PG1 may be mediating secondary LAS metabolism since metabolites are produced despite the absence of parent compound.

A second strategy used to ascertain the roles that individual organisms might play in the cooperative oxidation of LAS was to acclimate the consortium to alternate carbon sources and examine the effect on both composition of the consortium and LAS catabolic properties. The substrates were supplied at a concentration of 100 mg/l; they

Table 16. Population composition over time - 0 mg/l LAS (1/100th YEPG)

	1 Day	2 Days	4 Days	5 Days
Total cells	2.6×10^9	3.2×10^9	2.6×10^9	2.7×10^9
Organism	% of population			
PG1	10.4 ± 4.6	12.5 ± 5.0	16.2 ± 4.5	14.6 ± 5.8
PG2	19.2 ± 10.8	15.6 ± 2.5	4.9 ± 1.9	12.1 ± 6.2
PG3	69.2 ± 17.3	33.4 ± 11.9	36.5 ± 7.9	34.1 ± 6.2
PG4	2.7 ± 3.5	37.5 ± 9.1	42.5 ± 3.5	39.1 ± 4.4

Table 17. Population composition over time - 5 mg/l LAS (1/100th YEPG)

	3 Days	5 Days
Total cells	1.3 x 10 ⁹	3.0 x 10 ⁹
Organism	% of population	
PG1	17.7 +/- 6.2	10.0 +/- 7.0
PG2	20.0 +/- 6.9	6.7 +/- 2.7
PG3	36.2 +/- 16.2	37.7 +/- 5.7
PG4	25.4 +/- 14.6	46.7 +/- 9.7

Table 18. Population composition over time - 10 mg/l LAS (1/100th YEPG)

	3 Days	5 Days
Total cells	2.5×10^9	2.9×10^9
Organism	% of population	
PG1	13.2 +/- 4.8	14.3 +/- 5.7
PG2	16.0 +/- 5.6	4.6 +/- 1.7
PG3	33.2 +/- 18.8	39.3 +/- 10.4
PG4	37.2 +/- 3.6	42.9 +/- 19.3

Table 19. Population composition over time - 20 mg/l LAS (1/100th YEPG)

	3 Days	5 Days
Total cells	5.0 x 10 ⁹	3.9 X10 ⁹
Organism	% of population	
PG1	10.0 +/- 3.2	7.2 +/- 3.3
PG2	9.4 +/- 2.4	1.9 +/- 1.2
PG3	36.6 +/- 9.0	49.2 +/- 5.8
PG4	44.0 +/- 11.4	40.8 +/- 1.3

Table 20. Population composition over time - 50 mg/l LAS (1/100th YEPG)

	1 Day	2 Days	4 Days	5 Days
Total cells	2.6×10^9	3.5×10^9	4.4×10^9	3.3×10^9
Organism	% of population			
PG1	6.5 ± 1.9	2.8	5.2 ± 3.8	3.0
PG2	8.8 ± 1.9	ND*	ND	3.0
PG3	83.5 ± 17.1	91.4	47.8 ± 9.8	48.5
PG4	ND	5.7	23.5 ± 5.5	45.5

ND* - not detected

Table 21. Population composition over time - 100 mg/l LAS (1/100th YEPG)

	1 Day	2 Days	5 Days
Total cells	2.3×10^9	3.9×10^9	3.6×10^9
Organism	% of	population	
PG1	ND*	ND	ND
PG2	3.0 ± 3.9	1.7 ± 1.2	ND
PG3	97.0 ± 19.5	95.6 ± 11.0	72.2 ± 17.2
PG4	ND	ND	27.8 ± 8.1

* - not detected

Table 22. Population composition over time - 200 mg/l LAS (1/100th YEPG)

	1 Day	2 Days	4 Days	5 Days
Total cells	9.3 x 10 ⁸	1.3 x 10 ⁹	2.9 x 10 ⁸	3.3 x 10 ⁹
Organism	% of population			
PG1	ND*	ND	ND	ND
PG2	ND	12.8 ± 2.0	10.5 ± 5.6	7.0 ± 2.7
PG3	100	86.9 ± 2.2	38.3 ± 7.7	48.5± 11.2
PG4	ND	ND	51.2 ±12.9	44.5± 16.2

* ND- not detected

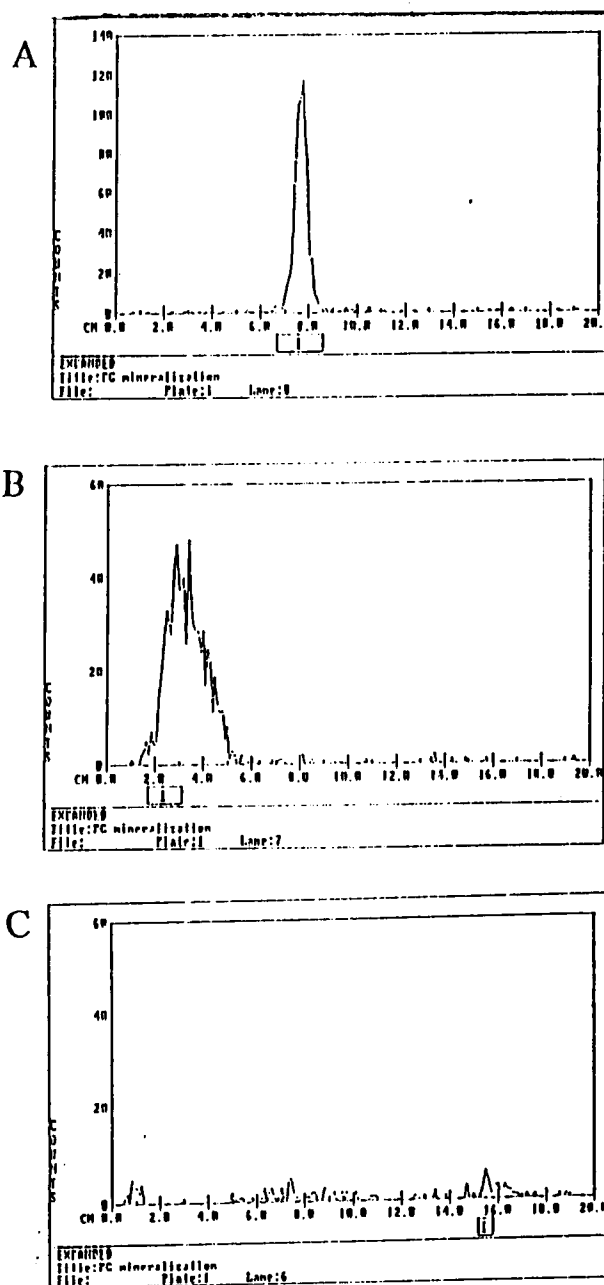


Figure 31.

TLC analysis of LAS catabolism by consortium PG at an LAS concentration of 80 mg/l following a 5-day incubation. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y = cpm). At this concentration consortium PG actively mineralized LAS and left no detectable intact LAS.

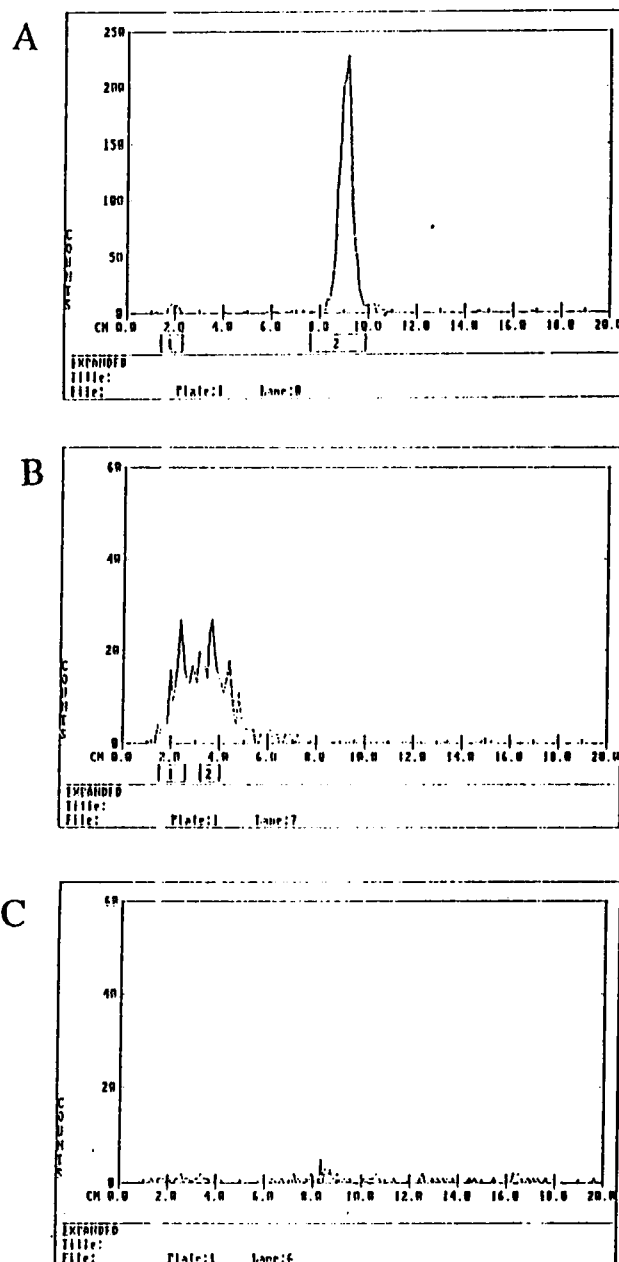


Figure 32.

TLC analysis of LAS catabolism by consortium PG at 100 mg/l LAS concentration following a 5 day incubation. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). At this concentration consortium PG actively mineralized LAS and left no detectable intact LAS.

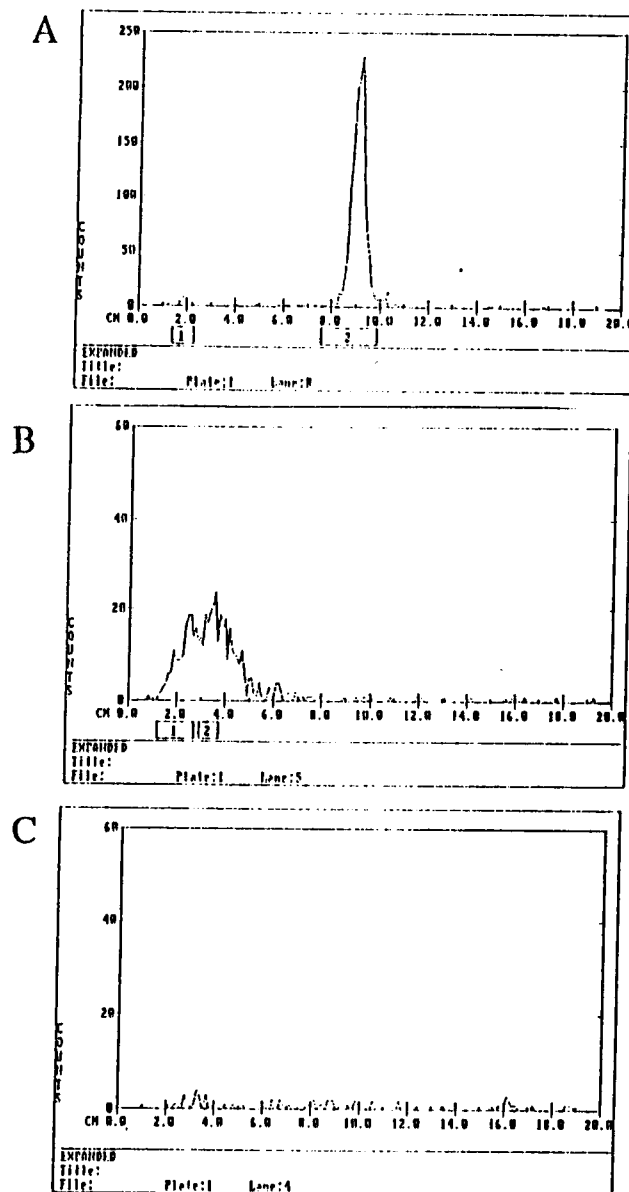


Figure 33.

TLC analysis of LAS catabolism by consortium PG at 200 mg/l LAS concentration following a 5-day incubation. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). At this concentration, no mineralization of LAS was observed, however, no intact LAS could be detected suggesting that primary oxidation of LAS was not inhibited at this concentration.

were ethylbenzene sulfonate (EBS), *p*-toluene sulfonate (pTS), benzene sulfonate (BSA), *p*-hydroxybenzoate (pHB), benzoate and *n*-hexadecane. Sub-samples of the consortium maintenance culture were washed and resuspended in mineral salts supplemented with the appropriate substrate and allowed to grow for 7 days, after which they were tested for LAS mineralization capability. As a control, a culture was incubated only in mineral salts without the addition of any carbon source. Disappearance of the aromatic compounds was monitored by ultra-violet absorbance.

Figure 34 shows the effect of alternative substrates on LAS mineralization by the consortium. The data show that the least inhibitory treatments were the control, where no carbon source was present and EBS acclimation. Both the control and the EBS acclimated cultures converted over 15% of the added LAS to CO₂. When treated with two other sulfonated aromatic substrates, pTS and BSA, both cultures mineralized slightly over 10% of the added LAS (LAS was added at a concentration of 0.05 mg/l). Acclimation to non-sulfonated aromatic compounds, pHB and benzoate, had a greatly inhibitory effect on LAS mineralization. The mineralization level following acclimation to benzoate fell below 5%. The effect was more pronounced with pHB where no ¹⁴CO₂ could be detected. The straight chain alkane, *n*-hexadecane, also inhibited LAS mineralization to a level of less than 5%. No substrate enhanced mineralization nor did substrate addition approach the 30% of mineralization levels which were observed with LAS acclimated cultures.

The changes in mineralization response appear to be the result of alterations in community structure of the consortium. The composition of the maintenance culture at

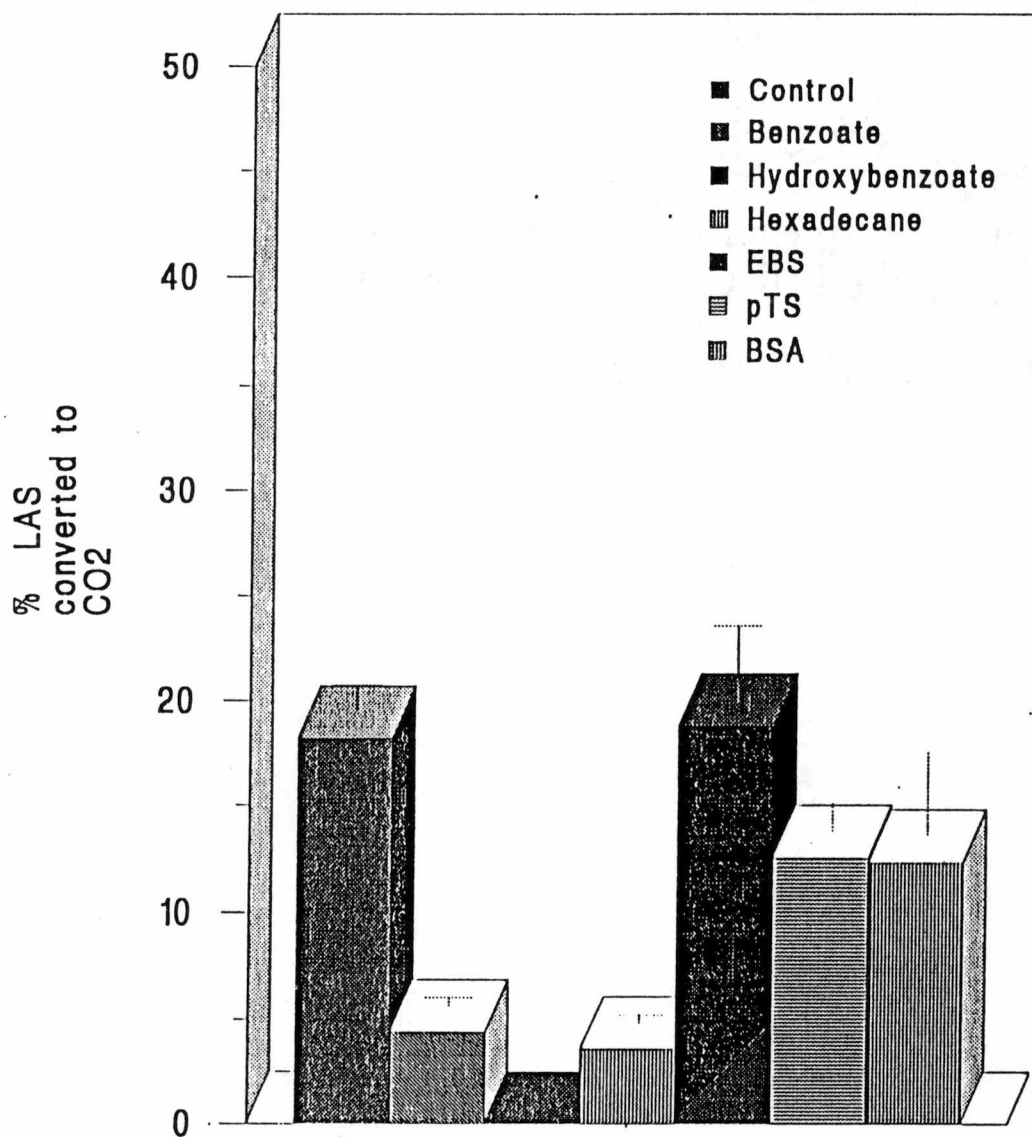


Figure 34. The effect of acclimation to alternate substrates on LAS mineralization by consortium PG. Following a week long exposure period cultures acclimated to non-LAS carbon sources were acidified and assayed for production of $^{14}\text{CO}_2$ from ^{14}C -ring labeled LAS. Results are reported as percent of added radiolabel recovered as $^{14}\text{CO}_2$.

Table 23. Population composition of consortium PG upon alternate substrate exposure (% of total population based on plate counts).

Organism	LAS	hex ¹	benzoate	pHB ²	EBS ³	pTS ⁴	BSA ⁵
PG1	23.1	23.0	6.7	4.0	55.0	13.1	8.5
PG2	37.6	ND*	86.7	85.9	23.6	8.0	7.1
PG3	6.0	ND	ND	ND	9.3	5.0	1.4
PG4	32.7	76.9	6.7	10.1	12.1	74.0	82.0

¹ *n*-hexadecane

² *p*-hydroxybenzoate

³ ethylbenzene sulfonate

⁴ *p*-toluene sulfonate

⁵ benzene sulfonate

*ND = not detected

the start of these experiments is shown in Table 16 under 2/92. It seems that acclimation to sulfonated aromatic compounds had the least effects on community structure. The EBS acclimated culture, which was the most proficient culture at LAS mineralization, had the community structure least divergent from that of the maintenance culture (Table 24). Although *n*-hexadecane caused the most dramatic change, causing two members, PG2 and PG3, to drop below the detection level, a low level of LAS mineralization was achieved. The benzoate acclimated culture, which also showed greatly depressed mineralization, was shown to be dominated by strain PG2, whereas strain PG3 could not be detected. A similar result was obtained with pHB where mineralization disappeared entirely.

Following incubation with LAS and alternate substrates some of these cultures were extracted and analyzed by TLC. As Figure 35 shows the pTS acclimated culture converted LAS to polar metabolites. Figure 36 shows similar results from the *n*-hexadecane acclimated culture. The culture acclimated to pHB, which was negative for LAS mineralization demonstrated no transformation of LAS. All the added label was associated with the biomass and was intact (Figure 37). The fact that strain PG3 was not detectable in this culture and that no LAS degradation was observed suggests that PG3 is responsible for primary LAS oxidation in the PG consortium.

Gene Probe Analysis

Both consortium KJB and PG were screened by slot blot analysis for the presence of genes hypothesized to be relevant in LAS or sulfonated aromatic compound catabolism. The gene probes used are listed in Table 25. No organism from either consortium had homology with any of the probes used. Despite the fact that no consortium member

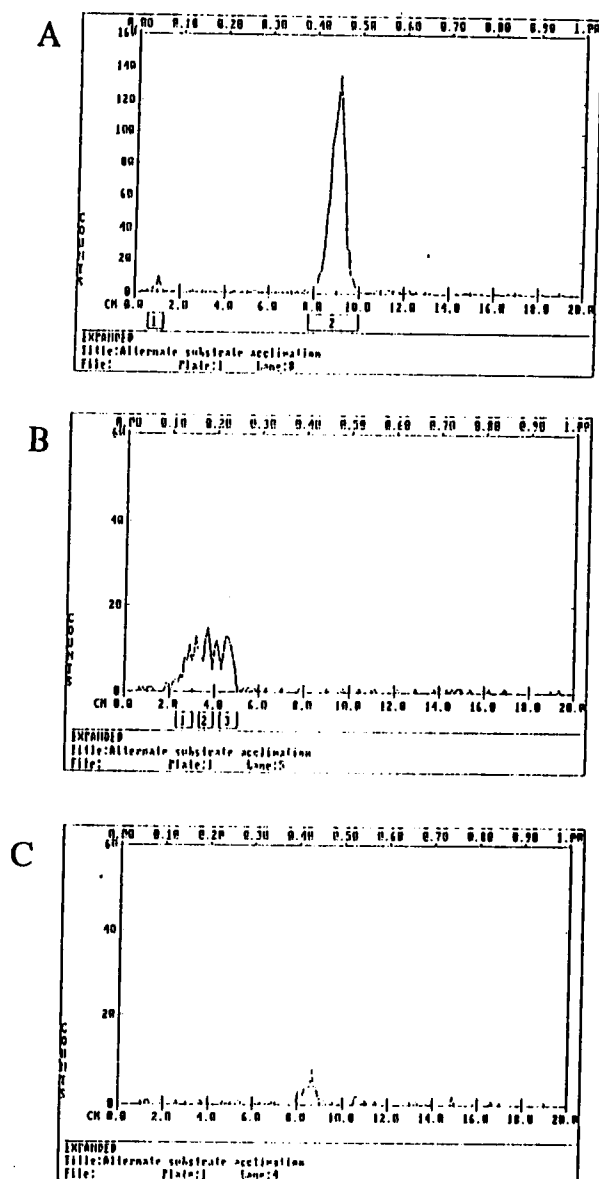


Figure 35. TLC analysis of LAS catabolism by consortium PG following acclimation to *p*-toluene sulfonate. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). In this experiment consortium PG was still capable of LAS mineralization following a week long acclimation to *para*-toluene sulfonate. Panel B shows the appearance of polar transformation products.

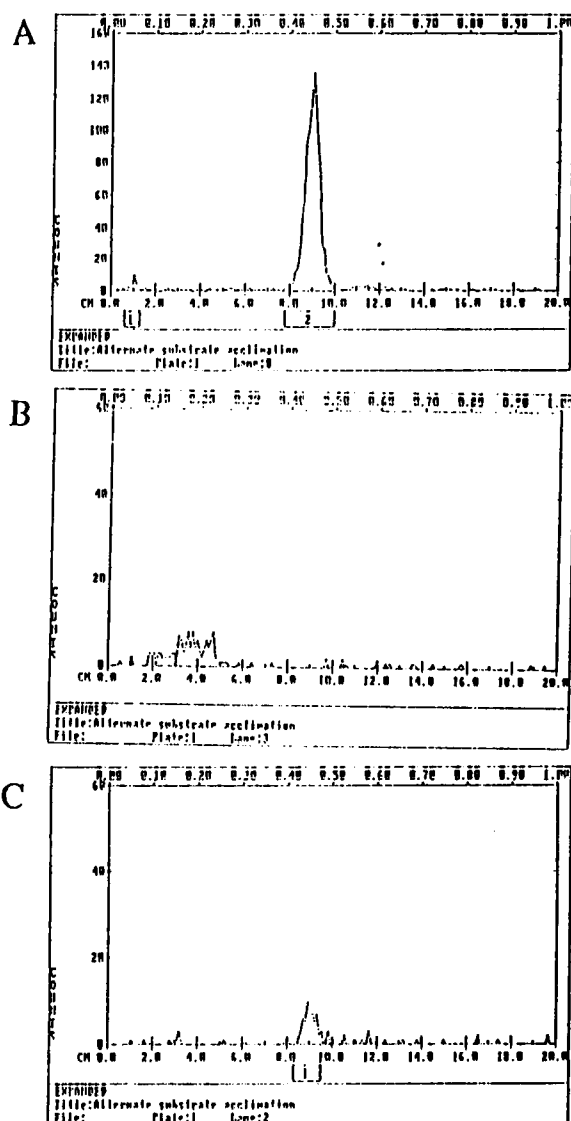


Figure 36. TLC analysis of LAS catabolism by consortium PG following acclimation to *n*-hexadecane. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). Although some mineralization was observed following a week long acclimation to *n*-hexadecane this substrate appears to inhibit rather than stimulate mineralization. Some minor transformation products are detectable in panel B.

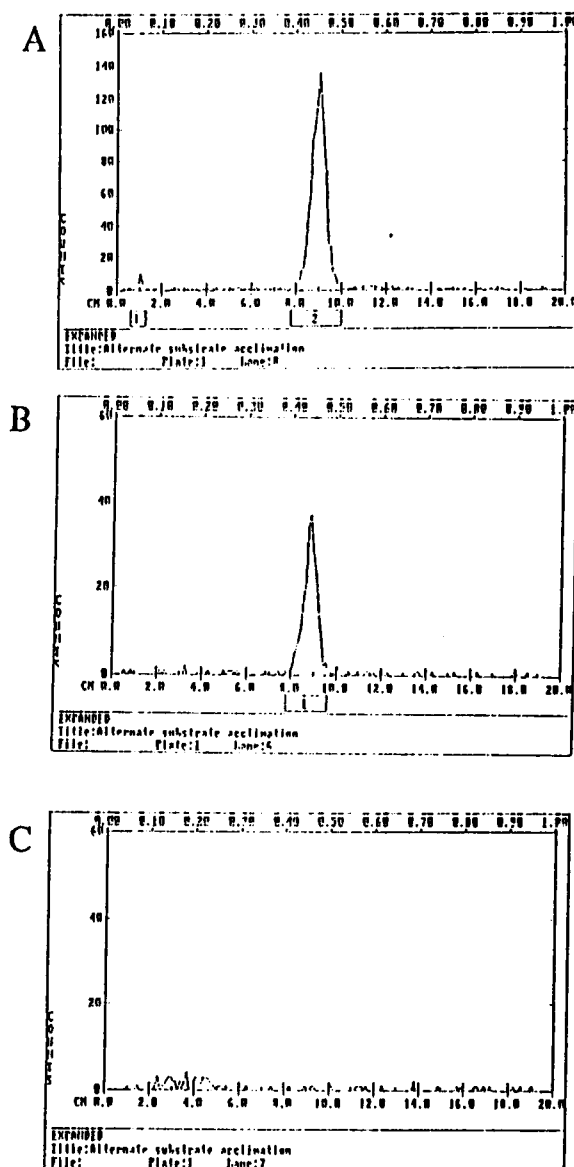


Figure 37. TLC analysis of LAS catabolism by consortium PG following acclimation to *para*-hydroxybenzoate. A. standard, B. aqueous, C. cell pellet (X axis = distance migrated, Y axis = cpm). Acclimation to this substrate completely inhibited both mineralization and primary LAS oxidation. Pannel B shows all ^{14}C remaining as untransformed LAS.

Table 24. Gene probes used for screening consortia KJB and PG.

Probe DNA	Organism	Phenotype	Source or Reference
<i>xylE</i>	<i>P. putida</i>	catechol 2,3 dioxygenase	30
<i>astA</i>	<i>Klebsiella pneumoniae</i>	arylsulfatase	88
<i>nahA</i>	<i>P. putida</i>	naphthalene dioxygenase	40
<i>alkB</i>	<i>P. putida</i>	alkane hydroxylase	96

hybridized with the *astA* (arylsulfatase structural gene) probe, strains KJB1, KJB4, PG1 and PG4 were shown by biochemical analysis to possess this activity as indicated in Table 11 and 13.

6. Discussion

Laundromat Pond Study

Although LAS degradation occurs in many habitats, relatively little is known about the degradation process. Furthermore, the population and genetic factors that control LAS degradation in the environment have not been well investigated. In first phase of this study LAS degradation in wastewater and pristine pond ecosystems were examined and an attempt was made to relate mineralization of LAS to the presence of bacteria that grew on LAS containing medium (LAS/YEPG). In addition, the relationship between LAS contamination and the occurrence of bacteria, which harbor plasmid DNA and gene sequences coding for the catabolism of aromatic compounds was investigated.

Information on bacterial population densities (cfu/ml) indicated that exposure to wastewater resulted in higher levels of both heterotrophic bacteria and bacteria capable of growing on LAS containing medium. In addition to higher numbers, a higher proportion of heterotrophs from the wastewater were capable of growing on the LAS medium. Thus, chronic exposure to high levels of LAS selected for organisms that tolerate and/or utilize LAS as a carbon source. Mineralization of LAS related directly to the presence of these bacteria. Mineralization was observed in all habitats that contained bacteria that grew on LAS/YEPG medium. Conversely, no mineralization was observed in habitats from which no organisms could be cultured on LAS/YEPG medium. In addition, high LAS/YEPG counts tended to correspond with high levels of LAS mineralization.

Although growth of organisms on LAS medium and mineralization were directly

related, most bacteria that grew on LAS medium tolerated LAS rather than degraded it. Only 23% of the isolates on LAS/YEPG from the wastewater pond could degrade LAS. From the control system, 8% of the isolates were MBAS positive. While many isolates partially degraded LAS, no isolate from either system was able to completely mineralize LAS in pure culture. Thus it appears that complete LAS mineralization involves multiple organisms acting in concert. While one can hypothesize that some populations mediate oxidation of the alkyl chain whereas others mediate oxidation of the sulfonated ring; however, as further studies with defined consortia indicate this could be an overly simplistic interpretation. An LAS/YEPG medium would select for the first population but not the second.

Plasmid DNA has been shown to confer drug and heavy metal resistance as well as catabolic properties to bacterial populations. Although plasmids are virtually ubiquitous in bacteria from natural environments, several studies have shown that the proportion of bacteria harboring plasmids is significantly greater in environments exposed to chemical contamination (31, 47, 55). For example, more than 40% of the bacteria recovered from a chemically contaminated aquifer contained plasmids compared with 0.9% from a pristine aquifer (96). Such results suggest that plasmid bearing strains are selected for and that the plasmids may confer resistance of the organisms to the contaminants or the ability to biodegrade them. In the present study, heterotrophs from the wastewater system exhibited twice the incidence of plasmids (36%) as the control system (18%). Furthermore, isolates on LAS/YEPG from both systems had a higher incidence of plasmids (44-46%) than the overall heterotrophic populations. The function of these

plasmids is unknown. There was no direct correspondence between presence of a plasmid and the ability to tolerate or degrade LAS. In the control system, 8% of the isolates on LAS/YEPG were MBAS positive. However, all MBAS positive isolates contained a plasmid. In the wastewater system, 46% of the isolates on LAS/YEPG contained a plasmid but only 12% were MBAS positive and contained a plasmid. Whether plasmids confer any ability to degrade LAS remains an open question.

In this study, the NAH7 plasmid was used as a broad specificity probe to detect aromatic catabolic genes. This plasmid is widely disseminated and commonly found at chemically impacted sites. Work by Cain suggested that TOL-like plasmids (which include NAH7) may be involved in LAS oxidation (32). Many of the aromatic catabolic pathways are evolutionarily related and share gene order and sequence similarities (53). Gene probe analysis with NAH7 as well as pDTG113 (a more specific probe containing only the genes responsible for conversion of naphthalene to salicylate) indicated no significant difference in the frequency of these sequences between contaminated and control sites. Furthermore, LAS exposure did not result in an increase in the abundance of any of the sequences tested. This finding suggests that in this setting the degradation of LAS is probably mediated by uncharacterized catabolic genes.

In summary, the wastewater site supported higher numbers of heterotrophic bacteria as well as bacteria capable of growing on LAS-containing medium. Mineralization of ^{14}C -ring labeled LAS in any habitat was related to the presence of LAS-tolerant organisms in that habitat. Although a higher incidence of plasmids was found in bacteria from the wastewater pond and among bacteria that grew on LAS containing medium, an ability to

degrade LAS is not solely dependent on the presence of plasmid DNA.

LAS Enrichment Cultures

The isolation of defined LAS-mineralizing consortia was not a straight forward task. It was complicated by the need to maintain LAS at a low concentration during enrichment and the complexity of the communities required for LAS catabolism. In most enrichment cultures, a substrate is provided in sufficient amounts as to allow the organisms using that substrate to out compete the other organisms present. However, when LAS was supplied at concentrations above 20 mg/l, the effect on LAS mineralization was inhibitory. Concentrations lower than 20 mg/l are not very effective at promoting an outgrowth of LAS degradative organisms unless the enrichment is conducted over an extended time period. Results obtained in this study suggest that earlier experiments resulted in a washing out of bacteria mediating secondary LAS catabolism. Although no LAS was detectable in the effluent from early shake flask and chemostat experiments, the sulfonated ring mineralization capability had been lost. These observations are similar to those reported by Swisher (127), who noted the loss of mineralization capabilities of activated sludge in cultures maintained on LAS using a 7-day retention time, whereas cultures maintained on a 14-day retention time did not lose the mineralization capabilities. Swisher (127) hypothesized that acclimation to degradative intermediates was essential to promoting complete LAS degradation. The fact that LAS mineralization requires organisms whose activity in regard to LAS catabolism is unknown also complicates the enrichment process. Enrichment strategies must not only maintain the bacteria mediating catabolic activities but these "ancillary" organisms as well. The theory that sulfonated

aromatic compounds, such as benzene sulfonate, might select for organisms catabolizing LAS (or at least metabolism of LAS transformation products) was not borne out by this study. As shown in Figure 15, no enhancement of LAS mineralization was observed during the test period.

LAS mineralizing consortia

The isolation of two four-membered communities, capable of complete LAS degradation has some commonalities with previous studies on LAS catabolic consortia. These consortia contain bacterial species commonly reported to be involved in LAS biodegradation such as *Pseudomonas*, *Enterobacter* and *Alcaligenes* (102). Unfortunately most of these are discussed in review papers and based on personal communications (102, 123). Slater and Lovatt (123) site a loosely defined community having as the major species *Pseudomonas* and *Alcaligenes* (it is not mentioned how many other species are present or how many *Pseudomonas* and *Alcaligenes* species are present). They note that at high LAS concentrations, the *Pseudomonas* species predominated; and at low LAS concentrations, the *Alcaligenes* species was predominant. This is suggestive of the *Alcaligenes* mediating secondary LAS degradation but specifics were not discussed.

Recent reports have shown that the catabolic properties of consortia are often superior to those of pure cultures. Fava and Marchetti (40) report that the degradation of 3-chlorobiphenyl was "faster and more intense" when two species of 3-chlorobiphenyl *Pseudomonas* are grown as a mixed culture than when they are grown as pure cultures. Pettigrew *et al.* (104) demonstrated the ability of mixed communities mediating 4-chlorobiphenyl mineralization to out compete individual bacterial populations capable of

degradation as pure cultures. These reports are suggestive of a process of "subcontracting" occurring in natural settings. This allows certain tasks to be accomplished by specialists resulting in an increased process efficiency. In some cases such as LAS degradation these arrangements may be obligatory. These studies are examples of the fact that insistence on pure culture methodology when examining a degradative process can be both naive and frustrating.

Recovery of consortia from the environment is most often accomplished using continuous culture technique. The key element in this process is to allow for interactions among pertinent organisms to be maintained while superfluous populations are eliminated. The resemblance of isolated "core" communities to the communities in the environment is an open question.

This study generated two taxonomically similar LAS-mineralizing consortia. They share as members *Enterobacter cloacae* and *Pseudomonas aeruginosa* as well as members of the genus *Xanthomonas*. Neither consortium contained a member which would grow to turbidity on LAS as sole carbon source. Each consortium contained only one member capable of transforming LAS to polar products - unidentified strain KJB4 and *E. cloacae* PG3. In spite of this similarity, like members of either consortium could not functionally replace each other in LAS degradation. These consortia are unique in that there is an absolute requirement for the presence of all four organisms to observe LAS mineralization. In general, studies on xenobiotic degradation by consortia report a decrease in catabolic efficiency rather than a total loss of mineralization upon removal of organisms whose role in degradation is not readily apparent. Slater and Lovatt (123)

believe that ancillary organisms, involved in LAS catabolism, aid in floc formation. Floc formation serves to concentrate the substrate and keeps the catabolic organisms in close proximity to one another.

The PG consortium was subjected to the most complete study in this investigation due to its superior LAS oxidative properties. As mentioned earlier, under most conditions, little to no LAS persisted following incubation with PG as opposed to KJB where LAS could be found in solution and sorbed to biomass. This made analysis easier and more rapid. It was also felt that this community may be more representative of naturally occurring assemblages where LAS degradation has been shown to be rapid. It must be noted that any laboratory enrichment may result in a community that is an artifact of the enrichment process rather than a functional community in the environment.

The fact that none of the sulfonated compounds tested, or LAS, served as growth substrates for consortia members as pure cultures complicated the task of determining the roles of individuals in LAS degradation. A picture of how the consortium functions was derived by growing the consortium under different conditions and examining the effect on mineralization and metabolite production. These effects were then correlated with alterations in the composition of the consortium.

In a set of experiments conducted at increasing LAS concentrations it was noted that mineralization was completely eliminated at 200 mg/l LAS. TLC analysis demonstrated that metabolites were still being produced at this concentration (Figure 33). The population data showed that *Alcaligenes* sp. PG1 was not detectable in the consortium for the duration of the experiment and it was the only member below detection limits. This

suggests a role for *Alcaligenes* sp. PG1 in the conversion of LAS transformation products to CO₂. It should be noted that *Alcaligenes* species have been implicated by Bird and Cain (22) and Thurneer *et al.* (132) in the catabolism of sulfonated aromatic compounds.

In another series of experiments the consortium was acclimated to various substrates believed to be relevant to LAS metabolism. The effects of these growth conditions on LAS metabolism and population composition were then compared. Three substrates could be described as severely inhibitory to LAS mineralization. These were *n*-hexadecane, benzoate and *p*-hydroxybenzoate, all of which reduced mineralization to below 5%. Of these substrates *p*-hydroxybenzoate was the most inhibitory, completely eliminating LAS mineralization. TLC analysis of this culture revealed no transformation products, as the radiolabel was recovered as untransformed LAS. The composition of the consortium showed that strain *E. cloacae* PG3 was not detected. This suggests that *E. cloacae* PG3 was responsible for the initial oxidation of LAS, an assertion supported by TLC data from this organism in pure culture which showed metabolite production (Figure 29).

Gene Probe Study of Defined Consortia

Gene probe studies did not reveal relationships between LAS-degradative organisms and the catabolic genes used as probes. In retrospect this result should not be surprising. With the exception of the arylsulfatase gene probe, the catabolic probes tested are involved in the metabolism of non-polar, non-surfactant compounds. The broad specificity of these enzymes is theorized to be due to hydrophobic pockets in the enzyme structure extracting non-polar compounds from solution and positioning them in the vicinity of activated oxygen (L. Wackett, personnel communication). This mechanism of

fortuitous metabolism may not be available to polar compounds such as LAS.

This fact supports Locher's theory that the enzymes responsible for sulfonated aromatic compound degradation may be related to those mediating transformations of methoxy aromatic compounds, which are widely distributed in nature as components of lignin (85). This offers an explanation for the lack of homology of consortia members with any of the probes used. Locher *et al.* (85) compared the TOL plasmid encoded xylene methylmonooxygenase (a two component system, the gene products of *xylA* and *xylM*) with the toluene methylmonooxygenase system (TSMOS). Although both systems mediate biochemically similar reactions on structurally similar compounds, their amino acid content is quite different and imparts a notably hydrophobic nature on the TOL plasmid encoded oxygenase. This is consistent with its presumed membrane associated location whereas the more soluble TSMOS is not membrane associated. The view of Locher *et al.* (85) is that TSMOS did not evolve to specifically transform sulfonated compounds, but is rather a broad specificity oxygenase for O-methyl or methyl aromatic compounds.

Though studies by Brilon *et al.* (26, 27) suggested that a plasmid encoded, naphthalene dioxygenase was responsible for desulfonation of sulfonated naphthalenes, this does not appear to be the case for catabolism of LAS by the consortia. The *nahA* encoded dioxygenase from the NAH7 plasmid, a widely distributed genetic element, was not present in the consortia. This was consistent with the laundromat pond study which did not show an increase in NAH7 homologous sequences in the LAS-impacted site relative to the control site. The *astA* probe for arylsulfatase did not hybridize to any

consortium member even though two KJB members and one PG member possessed this activity. This is suggestive of some diversity of the enzymes mediating this process.

Given the uncertainty of the biochemical pathway of LAS degradation, the lack of appropriate gene probes is not surprising. Interestingly, genetic studies on the response of bacteria to detergents have been largely confined to detergent shock genes in *E.coli* rather than catabolic genes (2, 136). A greater obstacle to genetic studies of LAS catabolism is the mixed culture nature of LAS degradation. The defining of specific roles for consortia members in LAS biodegradation provides a starting point for initiating genetic studies. The relevance of this line of research is apparent when considering that gene probe methods are a powerful and relatively easy way of monitoring a community's response to xenobiotics (13, 117). The ultimate goal of assessing an ecosystem's genetic potential for LAS removal with genes probes is by no means an unattainable goal.

In summary, this study demonstrated that LAS degradation is mediated by relatively complex bacterial consortia. LAS perturbation did not appear to select for plasmid bearing bacteria at the impacted laundromat pond site and both consortia lacked catabolic plasmids. The pond study did not generate any single organism capable of complete LAS mineralization. In two enrichment experiments the simplest communities capable of complete conversion of LAS to CO₂ both contained 4 members. Neither consortium KJB nor consortium PG demonstrated growth on LAS as a sole carbon source. LAS mineralization by consortium PG is mediated by the synergistic actions of *E. cloacae* PG3, which mediates primary oxidation, and *Alcaligenes* sp. PG1, which oxidizes LAS metabolites. No genetic homology was observed between consortium members and the

gene probes used.

The characterization of LAS catabolic consortia provides a basis for further research into the specifics of LAS biochemistry. For example, *Alcalignes* sp. PG1 should be a useful organism for the study of the degradation of LAS metabolites. This organism mediates secondary LAS metabolism, which is the rate-limiting step in the complete removal of LAS from the environment. The long-term goal of the development of gene probes to aid LAS fate assessments is also within reach. Microcosm as well as field studies could be employed to determine if consortium PG is an active entity in the environment. These types of studies may reveal fundamental paradigms common to consortia mediating transformations of more hazardous contaminants.

Literature Cited

Literature Cited

1. Adachi, T., Y. Murooka and T. Harada. 1975. Regulation of arylsulfatase synthesis by sulfur compounds in *Klebsiella aerogenes*. J. Bact. 121:29-35.
2. Adamowicz, M., P. M. Kelly and K. W. Nickerson. 1991. Detergent (sodium dodecylsulfate) shock proteins in *E. coli*. J. Bact. 173:229-233.
3. Adrians, P., H.-P. Kohler, D. Kohler-Staub and D.D.Focht. 1989. Bacterial dehalogenation of chlorobenzoates and coculture biodegradation of 4,4' dichlorobiphenyl by a two member bacterial consortium. Appl. Environ. Microbiol. 55:887-892.
4. Ainsworth, S. J. 1992. Soaps and detergent. Chem. and Eng. News. Jan. pp. 27 - 63.
5. Alvarez-Cohen, L. and P. L. McCarty. 1990. TCE transformation by a mixed methanotrophic culture - effects of toxicity, aeration and reductant supply. Appl. Environ. Microbiol. 57:228-235.
6. Anderson, D. G. and L. L. McKay. 1983. Simple and rapid procedure for the isolation of large plasmid DNA from lactic Streptococci. Appl. Environ. Microbiol. 46:549-552.
7. Amano, K., T. Fukushima and O. Nakasugi. 1991. Fate of linear alkylbenzene sulfonate in a lake estuary. Wat. Sci. Tech. 23:497-506.
8. Arthur, J. W. 1970. Chronic effects of linear alkylbenzene sulfonate detergent on *Gammarus pseudolimnaeus*, *Campelema decisum*, and *Physa integra*. Wat. Res. 4:251-257.
9. Assinder, S. J. and P. A. Williams. 1990. The TOL plasmids: Determinants of the catabolism of toluene and the xylenes. Ad. Microb. Phys. 31:1-69.
10. Atlas, R. M. and R. Bartha. 1981. Microbial Ecology: Fundamentals and applications. Addison Wesley, Reading, Ma.
11. Baggi, G., D. Catelani, E. Galli and V. Treccani. 1972. The microbial degradation of phenylalkanes. Biochem. J. 126:1091-1097.
12. Bayly, R. C. and M. G. Barbour. 19 . The degradation of aromatic compounds by the meta and gentisate pathways. In pp. 253-294.
13. Barkay, T., R. R. Turner, A. VandenBrook and C. Liebert. 1991. The relationships of Hg(II) volatilization from a freshwater pond to the abundance of *mer* genes in the gene pool of the indigenous microbial community. Microb. Ecol. 21:151-161.

14. Bartha, R. 1986. Biotechnology of petroleum pollutant biodegradation. *Microb. Ecol.* 12:155-172.
15. Bechard, G., J.-G. Bisallon, R. Beaudet and M. Sylvestre. 1990. Degradation of phenol by a bacterial consortium under mixed methanogenic conditions. *Can. J. Microbiol.* 36:573-578.
16. Benarde, M. A., B. W. Koft, R. Horvath and L. Shaulis. 1965. Microbial degradation of the sulfonate of dodecylbenzene sulfonate. *Appl. Environ. Microbiol.* 13:103-105.
17. Berna, J. L. 1991. The behavior of LAS in the environment. Abstract #453 SEATAC An. Meeting, Arlington, VA.
18. Berna, J. L., A. Moreno and J. Ferrer. 1991. The behaviour of LAS in the environment. *J. Chem. Tech. Biotechnol.* 50:387-398.
19. Berna, J. L., A. Moreno and J. Ferrer. 1991. Monitoring of LAS fate in the environment. Petresa: Una compnaia del grupo cepsa.
20. Beunink, J. and H.-J. Rehm. 1988. Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system. *Appl. Microbiol. and Biotech.* 29:72-80.
21. Bhatnagar, L. and B. Fathepure, B. Z. 1991. Mixed cultures in the detoxification of hazardous waste. In Mixed cultures in Biotechnology. G. Zeikus and E. A. Johnson eds. pp.293-340.
22. Bird, J. A. and R. B. Cain. 1974. Microbial degradation of alkylbenzenesulfonates. *Biochem. J.* 140:121-134.
23. Bisson, L. F. and R. E. Kunkee. 1991. Microbial interactions during wine production. In Mixed Cultures in Biotechnology. G. Zeikus and E. A. Johnson eds. McGraw Hill, New York, N.Y. pp. 37-68.
24. Bradshaw, M. L. 1991. Today's Chemist. April pp. 19-21.
25. Bressan, M., M. G. Marin and R. Brunetti. 1991. Effects of linear alkylbenzene sulfonate on skeletal development of sea urchin embryos (*Paracentrotus lividus*). *Wat. Res.* 25:613-616.
26. Brilon, C., W. Beckman and H. -J. Knackmuss. 1981. Catabolism of naphthalene sulfonic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22. *Appl. Environ. Microbiol.* 42:44-55.

27. Brilon, C., W. Beckman, M. Hellwig and H. -J. Knackmus. 1981. Enrichment and isolation of naphthalene sulfonic acid-utilizing *Pseudomonads*. *Appl. Environ. Microbiol.* 42:39-43.
28. Broda, P. 1979. *Plasmids*. W.H. Freeman and Company Limited. San Francisco.
29. Brunner, P. H., S. Capri, A. Marcomini and W. Giger. 1988. Occurrence and behaviour of linear alkybenzene sulphonates, nonylphenol, nonylphenol mono- and nonylphenol diethoxylates in sewage and sewage sludge treatment. *Wat. Res.* 22:1465-1472.
30. Bull, A. T. and J. H. Slater. 1982. Historical perspectives on mixed cultures and microbial communities. p.1-44. In A. T. Bull and J. H. Slater (eds.) *Microbial Interactions and Communities*. Academic Press, New York.
31. Burton, N. F., M. S. Day and T. Bull. 1982. Distribution of bacterial plasmids in clean and polluted sites in South Wales River. *Appl. Environ. Microbiol.* 44:1026-1209.
32. Cain, R.B. 1981. Microbial degradation of surfactants and "builder" components. *Microbial Degradation of Xenobiotics and Recalcitrant Compounds*. T. Leisinger, A. M. Cook, R. Hutter and J. Nuesch. eds. Academic Press pp. 326-370.
33. Chakrabarty, A. M. 1972. Genetic basis of the biodegradation of salicylate in *Pseudomonas*. *J. Bact.* 112:815-823.
34. Cook, A.M. and T. Leisinger. 1991. Desulfonation of aromatic compounds. *International Symposium on Environmental biotechnology*. Royal Flemish Society of Engineers. Ostend, Belgium. H. Verachest and W. Verstraete (eds.). pp. 115-122.
35. Cuskey, S. M. and A. B. Sprenkle. 1988. Benzoate-dependent induction from the OP2 operator-promoter region of the TOL plasmid pWWO in the absence of known plasmid regulatory genes. *J. Bact.* 170:3742-3746.
36. Davis, J. W. 1985. Biologically mediated removal of dissolved organic carbon from coal slurry wastewater. U. of Tennessee Ph.D. dissertation
37. Don, R. H. and J. M. Pemberton. 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *Appl. Environ. Microbiol.* 145:681-686.
38. Dowling, J. E., M. C. Mittleman and D. C. White. 1991. The role of consortia in microbially influenced corrosion. In *Mixed cultures in Biotechnology*. McGraw Hill New York N.Y. G. Zeikus and E. A. Johnson eds. pp. 341-372.
39. Egli, T., M. Bally and T. Uetz. 1990. Microbial degradation degradation of

chelating agents used in detergents with special reference to nitrilotriacetic acid (NTA). *Biodeg.* 1:121-132.

40. Fava, F. and L. Marchetti. 1991. Degradation and mineralization of 3-chlorobiphenyl by a mixed aerobic bacterial culture. *Appl. Microbiol. and Biotech.* 36:240-245.

41. Federle, T. W. and G. M. Pastwa. 1988. Biodegradation of surfactants in saturated subsurface sediments. *Groundwater* pp. 761-770.

42. Federle, T. W. and R. M. Ventullo. 1990. Mineralization of surfactants by the microbiota of submerged plant detritus. *Appl. Environ. Microbiol.* 56:333-339.

43. Finette, B. A., V. Subramanian and D. T. Gibson. 1984. Isolation and characterization of *Pseudomonas putida* PpF1 mutants defective in the toluene dioxygenase enzyme system. *J. Bact.* 160:1003-1009.

44. Fredrickson, J. K., R. J. Hicks, S. W. Li and F. J. Brockman. 1988. Plasmid incidence in bacteria from deep subsurface sediments. *Appl. Environ. Microbiol.* 54:2916-2923.

45. Ghosal, D., I.-S. You and I. C. Gunsalus. 1985. Nucleotide sequence and expression of gene *nahH* of plasmid NAH7 and homology with gene *xylE* of TOL pWWO. *Gene* 55:19-28.

46. Gibson, D. T., G. J. Zylstra and S. Chauhan. 1990. Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1. *IN Pseudomonas: Biotransformations, pathogenesis and evolving biotechnology.* American Society for Microbiology. Eds. S. Silver, A. M. Chakrabarty, B. Iglewski and S. Kaplan. pp. 121-131.

47. Glassman, L. L. and L. A. McNichol. 1981. Plasmid frequency in natural populations of estuarine microorganisms. *Plasmid* 5:231.

48. Gledhill, W. E., 1975. Screening test of ultimate biodegradability: Linear alkylbenzene sulfonates. *Appl. Environ. Microbiol.* 30:922-929.

49. Gledhill, W. E., V. W. Saeger and M. L. Trehy. 1991. An aquatic environmental safety assessment of linear alkylbenzene. *Environ. Tox. and Chem.* 10:169-178.

50. Granmo, A. 1972. Development and growth of eggs and larvae of *Mytilus edulis* exposed to a linear dodecylbenzenesulfonate. *Mar. Biol.* 15:356-358.

51. Grbic-Galic, D. and T. M. Vogel. 1987. Transformation of toluene and benzene by mixed methanogenic cultures. *Appl. Environ. Microbiol.* 53:254-260.

52. Greek, B. F. 1990. Detergent industry ponders products for a new decade. *Chem. and*

Eng. News 1/90:37-60.

53. Griffiths, E. T., S. G. Hales. N. J. Russell, G. K. Watson and G. F. White. 1986. Metabolite production during the biodegradation of the surfactant sodium dodecyltriethoxy sulfate under mixed culture die-away conditions. J. Gen. Microbiol. 132:963-972.

54. Gunner, H.B. and B.M. Zuckerman. 1968. Microbial degradation of diazinon by synergistic microbial action. Nature 217:1183.

55. Hada, H. S. and R. K. Sizemore. 1981. Incidence of plasmids in marine *Vibrio* sp. isolated from an oil field in the northwestern Gulf of Mexico. Appl. Environ. Microbiol. 41:199-202.

56. Hamer, G. and A. Heitzer. 1991. Polluted heterogeneous environments: Macro-scale fluxes, micro-scale mechanisms and molecular scale control. In Environmental Biotechnology for waste treatment. G. S. Sayler, R. Fox and J. W. Blackburn eds. pp. 233-248.

57. Hanahan, D. and M. Meselson. 1981. Plasmid screening at high colony density. Gene 10:63-67.

58. Harayama, S., M. Rekik, A. Wasserfallen and A. Bairoch. 1987. Evolutionary relationships between catabolic pathways for aromatics: Conservation of gene order and nucleotide sequence of catechol oxidation genes of pWWO and NAH7 plasmids. Mol. Gen. Genet. 210:241-247.

59. Hardman, D. J. and P. C. Gowland. 1985. Large plasmids in bacteria. Part 2. Genetics and evolution. Microbiol. Sci. 2:184-190.

60. Hardy, K. 1983. Bacterial Plasmids. American Society for Microbiology, Washington D.C.

61. Haug, W., A. Schmidt, B. Nortemann, D. C. Hempel, A. Stolz and H. -J. Knackmuss. 1991. Mineralization of the sulfonated azo dye mordant yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. Appl. Environ. Microbiol. 57:3144-3149.

62. Hegeman, G. 1984. The mineralization of organic materials under aerobic conditions. p.97-112. In E.R. Leadbetter and J. S. Poindexter (eds). Bacteria in Nature, Plenum Press, New York.

63. Hermanson, M. G., G. W. Jones and S. Kjellberg. 1987. Frequency of antibiotic and heavy metal resistance, pigmentation and plasmids in bacteria of the marine water interface. Appl. Environ. Microbiol. 53:2338-2342.

64. Humphreys, P.G. M., D. J. Shaw, K. S. Dodgson and G. F. White. 1986. Concerted induction of the S3 alkylsulfatase of *Pseudomonas* C12B by combinations of alkylsulfates and alcohols. J. Gen. Microbiol. 132:727-736.
65. Jahnke, M., T. E. Bana, R. Klintworth and G. Auling. 1990. Mineralization of ornithanilic acid is a plasmid associated trait in *Alcaligenes* sp. O-1. J. Gen. Microbiol. 136:2241-2249.
66. Jenal-Wanner, U. 1990. Anaerobic degradation of nitrilotriacetate in a denitrifying bacterium: Purification and characterization of the nitriloacetate dehydrogenase / nitrate reductase enzyme complex. Doctoral thesis, Swiss Fed. Instit. Tech.
67. Jigami, Y., T. Omori, Y. Minoda and K. Yamada. 1974. Screening of *n*-alkylbenzenes assimilating yeasts and identification of oxidation products from *n*-alkylbenzenes. Agr. Biol. Chem. 38:401-408.
68. Jigami, Y., T. Omori and Y. Minoda. 1975. The degradation of isopropylbenzene and isobutylbenzene by *Pseudomonas* sp. Agr. Biol. Chem. 39:1781-1788.
69. Kado, C. I. and S. T. Liu. 1981. Rapid procedure for the detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.
70. Kilpi, S., K. Humberg, K. Yrjala and V. Backstrom. 1988. The degradation of biphenyl and chlorobiphenyl by mixed bacterial cultures. FEMS Microbial. Ecol. 53:19-26.
71. Kimerle, R. A. and R. D. Swisher. 1977. Reduction of aquatic toxicity of linear alkylbenzene sulfonate by biodegradation. Wat. Res. 11:31-37.
72. Knaebel, D. B., T. W. Federle and J. R. Vestal. 1990. Mineralization of linear alkylbenzenesulfonate and linear alkylethoxylate in 11 contrasting soils. Environ. Tox. Chem. 9:981-988.
73. Kok, M., R. Oldenhuis, M. P. G. van der Linden, P. Raatjes, J. Kingma, P. H. van Lelyveld and B. Witholt. 1988. The *Pseudomonas oleovorans* alkane hydroxylase gene. J. Biol. Chem. 264:5435-5441.
74. Kuhm, A. E., A. Stolz, K.-L. Ngai and H.-J. Knackmuss. 1991. Purification and characterization of a 1,2-dihydroxynaphthalene dioxygenase from a bacterium that degrades naphthalenesulfonic acids. J. Bact. 173:3795-3802.
75. LaPoint, T. W. and J. F. Fairchild. 1991. Confirmation of laboratory generated NOECs for linear alkylbenzene sulfonate in outdoor experimental streams. Abstract # 456

An. Meeting SEATAC, Arlington Va.

76. Large, P. 1983. Methylophony and methanogenesis. Amer. Soc. Microbiol.

77. Larson, R.J. 1983. Comparison of biodegradation rates in laboratory screening studies with rates in natural waters. Resid. Rev. 85:159-171.

78. Larson, R.J. 1990. Structure activity relationships for biodegradation of linear alkylbenzenesulfonates. Environ. Sci. Tech. 24:1241-1246.

79. Larson, R. J., and A.G. Payne. 1981. Fate of benzene ring of linear alkylbenzene sulfonate in natural waters. Appl. Environ. Microbiol. 41:621-627.

80. Leahy, J. G., C. C. Somerville, K. A. Cunningham, G. A. Adamatiades, J. J. Byrd and R. R. Colwell. 1990. Hydrocarbon mineralization in sediments and plasmid incidence in sediment from the Campeche Bank. Appl. Environ. Microbiol. 56:1565-1570.

81. Lewis, M. A. 1991. Field investigations of an anionic surfactant to a natural periphyton community. 1991. Abstract # 454 An. Meeting SEATA, Arlington, Va.

82. Lewis, M. A. 1986. Comparison of the effects of surfactants on freshwater phytoplankton communities in experimental enclosures and on algal population growth in the environment. Environ. Toxicol. Chem. 5:319-332.

83. Lewis, M. A. and B. G. Hamm. 1986. Environmental modification of the photosynthetic response of lake plankton to surfactants and significance to laboratory-field comparison. Wat. Res. 20:1575-1582.

84. Locher, H. H., T. Leisinger and A. M. Cook. 1989. Degradation of *p*-toluenesulfonic acid via side chain oxidation, desulfonation and *meta*-ring cleavage in *Pseudomonas testosteroni* T-2. J. Gen. Microbiol. 135:1969-1978.

85. Locher, H. H., T. Leisinger and A. M. Cook. 1991. 4-Toluene sulfonate methylmonooxygenase from *Comamonas testosteronii* T-2: Purification and some properties of the oxygenase component. J. Bact. 173:3741-3748.

86. Locher, H. H., T. Leisinger and A. M. Cook. 1991. 4-Sulfobenzoate 3,4-dioxygenase. 1991. Biochem. J. 274:833-842.

87. Maniatis, T., E. F. Fritsch and J. Sambrook. Molecular Cloning. Cold Spring Harbor Press, Cold Spring Harbor N.Y.

88. Marin, M. G., M. Bressan and R. Brunetti. 1991. Effects of linear alkylbenzene sulfonate on two marine benthic organisms. Aquat. Toxicol. 19:241-248.

89. Martinez, J., Y. Soto, J. Vives-Rego and M. Bianchi. 1991. Toxicity of CU, NI and alkylbenzene sulfonate on the naturally occurring bacteria in the Rhone River plume. *Environ. Toxicol. and Chem.* 10:641-647.
90. Michiels, F., M. Burnmeister and H. Lehrach. 1987. *Science*. 236:1305-1308.
91. Mieure, J. P. 1991. Terrestrial safety assessment of linear alkylbenzene sulfonate. Abstract # 451 An. Meeting SEATAC, Arlington Va.
92. Mieure, J. P., J. Waters, M. S. Holt and E. Matthijs. 1990. Terrestrial safety assessment of linear alkylbenzene sulfonate. *Chemosphere* 21:251-262.
93. Murooka, Y., K. Ishibashi, M. Yasumoto, M. Sasaki, H. Sugino, H. Azakami and M. Yamashita. 1990. A sulfur- and tyramine-regulated *Klebsiella aerogenes* operon containing arylsulfatase (*astA*) gene and the *astB* gene. *J. Bact.* 172:2131-2140.
94. Murooka, Y., M.-H. Yim, and T. Harada. 1980. Formation and purification of *Serratia marcescens* arylsulfatase. *J. Bacteriol.* 39:812-817.
95. Nortemann, B., J. Baumgarten, H.G. Rast and H.-J. Knackmuss. 1986. Bacterial communities degrading amino- and hydroxynaphthalene-2-sulfonates. *Appl. Environ. Microbiol.* 52:1195-1202.
96. Ogunseitan, O. A., E. T. Tedford, D. Pacia, K. M. Sirotkin and G. S. Sayler. 1987. Distribution of plasmids in groundwater bacteria. *J. Indust. Microbiol.* 1:311-317.
97. Ohe, T., T. Ohmoto, Y. Kobayashi, A. Sato and Y. Wanatabe. 1990. Metabolism of naphthalenesulfonic acids by *Pseudomonas* sp. TA-2. *Agric. Biol. Chem.* 54:669-675
98. Ohwada, K. 1975. Agar plate method for detection and enumeration of alkylbenzenesulfonate-degrading microorganisms. *Appl. Environ. Microbiol.* 29:40-43.
99. Okpokwasili, G. C. and A. O. Olisa. 1991. River water biodegradation of surfactants in liquid detergents and shampoos. *Wat. Res.* 25:1425-1429.
100. Ornston, L. N. and W. K. Yen. 1982. Recurring themes and repeated sequences in metabolic evolution. In Biodegradation and detoxification of environmental pollutants. CRC Press, Boca Raton Florida, A. M. Chakrabarty ed. pp. 105-126.
101. Owen, D. J., G. Eggink, B. Hauer, M. Kok, D. L. McBeth, Y.-L. Yang and J. A. Shapiro. 1984. Physical structure, genetic content and expression of the *alkBAC* operon. *Mol. Gen. Genet.* 197:373-383.
102. Painter, H. A. and T. F. Zabel. 1988. Review of the environmental impact of LAS.

103. Parkes, R. J. 1982. Methods for enriching, isolating and analyzing microbial communities in laboratory systems. p. 45-102. In A. T. Bull and J. H. Slater (eds.) *Microbial Interactions and Communities*. Academic Press, New York.
104. Pettigrew, C. A., A. Breen, C. Corcoran and G. S. Sayler. 1990. Chlorinated biphenyl mineralization by individual populations and consortia of freshwater bacteria. *Appl. Environ. Microbiol.* 56:2036-2045.
105. Pittenger, C. A., D. M. Woltering and J. A. Masters. 1989. Bioavailability of sediment-sorbed and aqueous surfactants to *Chironomus riparius* (midge). *Environ. Toxicol. and Chem.* 8:1023-1033.
106. Quiroga, J.M. and D. Sales. 1990. Experimental variables in biodegradation of surfactant in marine environment. *Bull. Environ. Cont. and Toxicol.* 44:851-858.
107. Ramos, J. L., A. Wasserfallen, K. Rose and K. N. Timmis. 1987. Redesigning metabolic routes: Manipulation of the TOL plasmid for catabolism of alkylbenzoates. *Science* 235: 593-596.
108. Rappaport, R. A., and W. S. Eckhoff. 1990. Monitoring linear alkyl benzene sulfonate in the environment: 1973-1986. *Environ. Toxicol. Chem.* 9:1245-1257.
109. Rippin, M. J., T. M. Cook, K. F. Noon and L. E. Stark. 1975. Bacterial metabolism of arylsulfonates: Role of *meta*-cleavage in benzene sulfonate oxidation by *Pseudomonas testosteroni*. *Appl. Environ. Microbiol.* 29:382-387.
110. Roberts, D. W. 1991. Application of QSAR to biodegradation of linear alkylbenzene sulfonate isomers and homologs. *Sci. Total Environ.* 109/110:301-306.
111. Roberts, D.W. 1991. QSAR issues in aquatic toxicity of surfactants. *Sci. Total Environ.* 109/110:557-568.
112. Rochkind, M. L., J. W. Blackburn and G. S. Sayler. 1986. Microbial decomposition of chlorinated aromatic compounds. EPA/600/2-86/090.
113. Russell, N. J., D. J. Anderson, M. J. Day and G. F. White. 1991. Colonization of biofilms by bacteria capable of degrading sodium dodecyl sulfate (SDS) at clean and polluted riverine sites. *Microb. Ecol.* 22:85-98.
114. Sanders, M. E. 1991. Mixed cultures in dairy fermentations. In *Mixed Cultures in Biotechnology*. G. Zeikus and E. A. Johnson eds. McGraw Hill, New York, N.Y. pp. 105-133.

115. Sariaslani, F. S., J. L. Sudmeier and D. D. Focht. 1982. Degradation of 3-phenylbutyric acid by *Pseudomonas* sp. J. Bact. 152:411-421.
116. Sayler, G. S., S. W. Hooper, A. C. Layton and J. M. H. King. 1990. Catabolic plasmids of environmental and ecological significance. Microb. Ecol. 19:1-20.
117. Sayler, G. S., M. S. Shields, E. T. Tedford, A. Breen, S. W. Hooper, K. M. Sirotkin and J. W. Davis. 1985. Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples. Appl. Environ. Microbiol. 49:1295-1303.
118. Schoberl, P. 1989. Basic principles of LAS biodegradation. Tenside Surfactants Detergents 26:86-94.
119. Shimp, R. J. 1989. Adaptation to quaternary ammonium surfactant in aquatic sediment microcosms. Environ. Tox. and Chem. 8:201-208.
120. Sigoillot, J.-C., M.-H. Nguyen. 1990. Isolation and characterization of surfactant degrading bacteria in a marine environment. FEMS Microb. Ecol. 73:59-68.
121. Sigoillot, J.-C., M.-H. Nguyen. 1992. Complete oxidation of linear alkylbenzene sulfonate by bacterial communities selected from coastal seawater. Appl. Environ. Microbiol. 58:1308-1312.
122. Slater, J. H. 1981. Mixed cultures and microbial communities. In Mixed culture fermentations. Academic Press, New York. M. E. Bushell and J. H. Slater (eds). pp. 1-24.
123. Slater, J. H. and D. Lovatt. 1984. Biodegradation and the significance of microbial communities. p. 439-485. In D.T.Gibson (ed.) Microbial degradation of organic compounds. Marcel Dekker Inc. New York.
124. Smith, M. R. 1991. The biodegradation of aromatic hydrocarbons by bacteria. Biodeg. 1:177-190.
125. Swedmark, M., B. Braaten, E. Emanuelsson and A. Granmo. 1971. Biological effects of surface active agents on marine animals. Mar. Biol. 9:183-201.
126. Sweeney, W. A., and R. G. Anderson. 1989. Biodegradability of alkylbenzene sulfonates. J. Am. Oil Chem. Soc. 66:1844-1849.
127. Swisher, R.W. 1987. Surfactant Biodegradation. Marcel Dekker Inc. New York, N.Y.

128. Sylvestre, M., R. Masse, C. Ayotte, F. Messrer and J. Futeaux. 1985. Total bidegradation of 4-chlorobiphenyl by a two member bacterial culture. *Appl. Microbiol. and Biotechnol.* 21:192-195.
129. Takada, H, and R. Ishiwatari. 1990. Biodegradation experiments of Linear alkylbenzenes (LABs): Isomeric composition of C₁₂ LABs as an indicator of the degree of LAB degradation in the aquatic environment. *Environ. Sci. Technol.* 24:86-91.
130. Thurneer, T., T. Kohler, A. M. Cook and T. Leisinger. 1986. Ornthanilic acid and analogues as carbon sources for bacteria: Growth physiology and enzymic desulfonation. *J. Gen. Microbiol.* 132:1215-1220.
132. Thurneer, T., A.M.Cook and T.Leisinger. 1988. Co-culture of defined bacteria to degrade seven sulfonated aromatic compounds: efficiency rates and phenotypic variations. *Appl. Microbiol.Biotech.* 29:605-609.
133. Thurneer, T., D. Zurrer, O. Holinger, T. Leisinger and A. M. Cook. 1991. Initial steps in the degradation of benzene sulfonate, 4-toluene sulfonic acid and ornthanilic acid in *Alcaligenes* sp. *Biodegradation* 1:55-64.
134. Trehy, M. L. and W. E. Gledhill. 1990. Determination of linear alkylbenzene sulfonates and diakyltetralin sulfonates in water and sediments. Abstr. # P327 Ann. Meeting SEATAC Arlington Va.
135. Tuovinen, O. H., B. C. Kelly, and S. N. Groudev. 1991. Mixed cultures in biological leaching processes and mineral biotechnology. In Mixed Cultures in Biotechnology. G. Zeikus and E. A. Johnson eds. pp. 373-472.
136. Wakayama, Y., M. Takagi and K. Yano. 1984. Gene responsible for protecting *Escherichia coli* from sodium dodecylsulfate and toluidine blue plus light. *J. Bact.* 159:527-532.
137. Watkinson, R.J. and P. Morgan. Physiology of aliphatic hydrocarbon degrading microorganisms. *Biodegradation* 1:79-92.
138. Werdelman, B. W. 1984. Tenside in unserer Welte-heute und morgen. pp. 3-21. In *Proceedings of the Second World Surfactants Congress*, vol. 1 Syndicat National des Fabricants d'Agents de Surface et de Produits Auxiliaires Industriels, Paris.
139. Wickham, G. S., and R. M. Atlas. 1988. Plasmid frequency fluctuations in bacterial populations from chemically stressed soil communities. *Appl. Environ. Microbiol.* 54:2192-2196.
140. Wittich, R. M., H.G. Rast and H.-J.Knackmus. 1988. Degradation of naphthalene-

2,6- and naphthalene disulfonic acid by a *Moraxella* sp. Appl. Environ. Microbiol. 54:1842-1847.

141. Woollatt, E. 1985. The manufacture of soaps, other detergents and glycerine. John Wiley and Sons, New York N.Y.

42. Wortman, A. T. and R. R. Colewell. 1988. Frequency and characteristics of plasmids in bacteria isolated from deep-sea amphipods. Appl. Environ. Microbiol. 54:1284-1288.

143. Yamane, A. N., M. Okada and R. Sudo. 1984. The growth inhibition of planktonic algae due to surfactants used in washing agents. Wat. Res. 18:1101-1105.

Appendix

Appendix

List of Abbreviations

BAS	branched alkylbenzene sulfonate
BN6	<i>Pseudomonas</i> sp., oxidizes 6-amino-naphthalene-2-sulfonate
BN9	<i>Pseudomonas</i> sp., oxidizes 5-aminosalicylate
BSA	benzene sulfonate
CFU	colony forming unit
DDT	dichlorodiphenyldichloroethane
DNA	deoxyribonucleic acid
EDTA	ethylenedinitrilotetraacetic acid
EBS	ethylbenzene sulfonate
FAME	fatty acid methylester
IL-MBAS	interference limited methylene blue active substance assay
KJB	four-membered LAS mineralizing consortium
KJB1	<i>Pseudomonas aeruginosa</i> (member of KJB)
KJB2	<i>Xanthomonas maltophilia</i> (member of KJB)
KJB3	<i>Enterobacter cloacae</i> (member of KJB)
KJB4	unidentified member of KJB
LAB	linear alkylbenzene
LAE	linear alkylethoxylate
LAS	linear alkylbenzene sulfonate
LMP	laundromat pond
LPS10C	4-chlorobiphenyl mineralizing-consortium
MBAS	methylene blue active substance
NADH	nicotinamide adenine dinucleotide
NAH7	naphthalene catabolic plasmid
OCT	octane catabolic plasmid
PCB	polychlorinated biphenyl
pDTG113	upper naphthalene pathway clone from NAH7
PG	4-member LAS-mineralizing consortium
PG1	<i>Alcaligenes</i> sp. (member of PG)
PG2	<i>P. aeruginosa</i> (member of PG)
PG3	<i>E. cloacae</i> (member of PG)
PG4	<i>X. campestris</i> (member of PG)
pHB	<i>p</i> -hydroxybenzoate
pSAH	pTS catabolic plasmid
PSBDOS	<i>p</i> -sulfobenzoate dioxygenase system
pSS50	plasmid associated with chlorobiphenyl mineralization
pTS	<i>p</i> -toluene sulfonate
pWWO	genetically characterized TOL plasmid
QSAR	quantitative structure activity relationship
SDS	sodium dodecylsulfate
TCE	trichloroethylene

TTC:NBT	trichlorotetrazolium chloride: nitroblue tetrazolium
TLC	thin layer chromatography
TSMOS	toluene sulfonate monooxygenase system
YEPG	yeast extract peptone glucose

Vita

Alexander W. Breen was born in Chicago, IL. He attended St. Anastasia grammar school in Teaneck, N.J. and St. Joseph Regional High School in Montvale, N. J. He received an undergraduate degree in microbiology from the University of Tennessee in 1982. From 1982 until 1984 he worked as a research technician in Dr. Gary Sayler's laboratory focusing on degradative kinetic of priority pollutants in activated sludge and the applications of molecular biology to microbial ecology. In 1987 he received an M.S. degree from U.T. under Dr. Sayler's direction (thesis title "Molecular characterization of the groundwater bacterium ABS10"). He served as Dr. Sayler's laboratory manager from 1987 to 1988. From 1987 to 1991 he was a summer workshop instructor at the Gray Freshwater Institute of the University of Minnesota. He has accepted a post-doctoral position at the EPA Risk Reduction Engineering Laboratory in Cincinnati, Ohio.