REFERENCES

1. Swisher, R. D., JAOCS *40*, 648–656 (1963).
2. Vath, C. A., Soap Chem. Specialties *40*, No. 2, 56–58, 182; No. 3, 55–58, 108.
3. Myerly, R. C., "Straight Chain Secondary Alcohol Ethoxylates, A New Class of Biodegradable Detergent Intermediates," presented at the 37th Annual Convention of the Soap and Detergent Assoc., New York, N.Y., 1964.
4. Lobo, P. A., D. C. Coldiron, L. D. Vernon and A. T. Ashton, Chem. Eng. Prog. *58*, No. 5, 85–88 (1961).
5. Liddicoet, T. H., JAOCS *40*, 631–636 (1963).
6. Jackman, V., "Special Techniques Used in the Study of Material Properties," Instron Application Series, October 28, 1960.
7. Newman, S. B., and W. M. Lee, "Surface Tension Measurements with a Strain Gauge Type Testing Machine," Review of Scientific Instruments *29*, No. 9, September, 1958.
8. Draves, C. Z., and G. R. Clarkson, American Dyestuff Reporter *20*, 201 (1931); A.A.T.C.C. Test Method, 17-1952.
9. Ross, J., and G. Miles, Oil Soap *18*, 99–102 (1941).

[Received June 17, 1964—Accepted September 9, 1964]

Fish Bioassays of Linear Alkylate Sulfonates (LAS) and Intermediate Biodegradation Products

R. D. SWISHER, Inorganic Chemicals Division, Research Department, Monsanto Company, St. Louis, Missouri; and J. T. O'ROURKE and H. D. TOMLINSON, Department of Environmental and Sanitary Engineering, Washington University, St. Louis, Missouri

Abstract

Linear alkylate sulfonates (LAS) are relatively toxic to fish when tested under static conditions by standard bioassay techniques, the median tolerance limit (TL_m) being around 3 mg/liter and 0.6 mg/liter for the C_{12} and C_{14} homologs, respectively. However, these materials are so readily degraded by bacterial attack that bluegill fingerlings live with no trouble in effluents from laboratory continuous flow activated sludge units being fed 100 mg/liter or more of either product. No effects on the fish were noticeable in exposures of 96 hr or more, except for slight alterations in the microscopic appearance of the gill tissue. Thus, the removal of the LAS by biodegradation is paralleled by the removal of toxicity and there is no indication that toxic intermediates accumulate during the biodegradation process. This conclusion is substantiated by the observation of a much lower degree of toxicity (TL_m 75 mg/liter) for sulfophenylundecanoic acid disodium salt (mixed isomers), synthesized as a model of an intermediate degradation product. Characterization, gas chromatography and methylene blue analysis of this product are also discussed.

Introduction

THE DETERGENT INDUSTRY of the U.S. has announced that it is discontinuing the production of tetrapropylene derived alkylbenzene sulfonates (ABS) and replacing them with the more biodegradable linear alkylate sulfonate (LAS) (1). This new product is ABS in which the alkyl groups are straight chains, readily attacked by bacteria. In contrast, tetrapropylene ABS is a mixture characterized by highly branched alkyl groups, and about one-quarter to one-third of it is quite resistant to bacterial attack because of unfavorably compact alkyl structure (2). This has sometimes resulted in objectionable foaming in rivers and ground waters upon contamination with sewage (3), which has in turn provided the incentive for the expenditure of a large amount of capital in the construction of the new manfacturing facilities required for the LAS raw materials.

LAS, like its predecessor tetrapropylene ABS, exhibits a very low degree of toxicity to mammals (4). Again like tetrapropylene ABS, LAS is significantly toxic to fish, with median tolerance limit (TL_m, 50% survival) values lying in the region of 1–10 mg/liter for 24–96 hr exposure. The comprehensive studies of Hirsch have shown very interesting correlations between the fish TL_m and the structure of the LAS components, and that some of these components are significantly more toxic than tetrapropylene ABS when tested under the same conditions (5).

Nevertheless, the use of LAS in detergent formulations need not constitute any added threat to the nation's fish life; in fact, it may be that a still greater margin of safety will result. The present concn of ABS found in our rivers are for the most part below 1 mg/liter (6) and have been judged to be no particular hazard to fish life (7). Since LAS is destroyed more rapidly and more completely by biodegradation than the current tetrapropylene ABS (8), it is to be expected that the concn in rivers will be much lower than at present, with a correspondingly lower hazard.

This expected lower hazard in contingent not only upon more rapid degradation of LAS, but also upon intermediate degradation products of LAS being no more toxic than LAS itself. The relatively low toxicity of the intermediates is implicit in the work of Herbert et al. (9) and of Niemitz and Pestlin (10), who found that the ABS remaining after partial degradation of either tetrapropylene or straight chain ABS was less toxic than the original. The solutions which they examined would of course also contain intermediate products originating from that portion of the ABS which had already degraded.

The present experiments involved determination of a) fish TL_m of C_{12} and C_{14} LAS in standard reference water, b) effects on the fish of effluents from continuous activated sludge units fed up to several hundred times these TL_m concn, c) toxicity of C_{14} LAS in activated sludge effluents, and d) TL_m of sulfophenylundecanoic acid disodium salt (mixed isomers), a probable intermediate biodegradation product of LAS, in standard reference water. Our results suggest that no complications relating to fish toxicity should arise when the detergent industry changes from tetrapropylene ABS to LAS.

Materials and Methods

C_{12} and C_{14} Linear Alkylate Sulfonate (C_{12} LAS and C_{14} LAS). These samples were prepared by alkylation of benzene with alpha-dodecene (Aldrich D22160) and alpha-tetradecene (Aldrich T980) respectively, using $AlCl_3$ catalyst. The monoalkylbenzenes were isolated by fractional distillation and comprised mixtures of all possible secondary isomers: 2-,3-,4-,5- and 6-phenyldodecane in one case and 2-,3-, 4-,5-,6- and 7-phenyltetradecane in the other, in proportions decreasing somewhat from the 2-phenyl to the

more internal isomers (11). These two alkylbenzene mixtures were sulfonated with H_2SO_4, neutralized with sodium hydroxide in 80% isopropyl alcohol, filtered to remove sodium sulfate and the final products were recovered in substantially quantitative yields upon drying the filtrates. In simulation of commercial practice no attempt was made to separate the small amounts of ortho sulfonates which were probably present along with the major products, the para sulfonates (12).

Sulfophenylundecanoic Acid Disodium Salt Mixed Isomers (SΦU). This product was made in the same manner as the two LAS samples (above) by sulfonation of phenylundecanoic acid (Eastman 5352).

The phenylundecanoic acid had been prepared by reaction of benzene with 10-undecenoic acid (13) and was a mixture of isomers, presumably with the phenyl attached to any of the internal carbons along the chain. The methyl ester was prepared in substantially quantitative yield by saturating a methanol solution with anhydrous hydrogen chloride (14) and capillary gas chromatography showed the presence of at least five components (Fig. 1). The free acid gave only a slight response in the capillary instrument, which was equipped with a strontinum 90 detector; in a temp programmed instrument with a two-foot packed column and flame ionization detector it gave a poorly resolved but characteristic peak with a shape which again suggested that several components were present. The methyl ester gave a similar peak under these conditions, but with a somewhat shorter retention time.

The sulfonated product was freed from a small amount of unsulfonated phenylundecanoic acid by extracting the acidic aqueous solution (pH 1.5) with *n*-hexane prior to complete neutralization in aqueous isopropyl alcohol for removal of sodium sulfate as outlined in the preceeding section. The final dried product was obtained as a friable powder which upon pH titration gave a curve breaking between pH 9.5 and 3.5, characteristic of a carboxylate group. The equivalent wt observed for this weak acidity was 391.5 compared with the value of 386.4 calculated for the disodium salt.

The nature of this sulfonate was further verified by desulfonation of a 95-mg sample using a micro modification of the Knight-House procedure, by refluxing with 90–95% phosphoric at 200–210C for two hr (15). The product isolated (55 mg) had a neutralization equivalent wt of 273 compared with 255 determined on the original acid before sulfonation (theoretical value 262.4). Its gas chromatogram in the temp programmed unit was very similar to that of the original phenylundecanoic acid. It was concluded that sulfonation and desulfonation did not cause any great change in the carbon structure.

Thus the product is probably a mixture of at least five isomeric sulfophenylundecanoic acids, differing in the position of linkage of the phenyl group to the chain. In analogy with the formation of phenyldodecanes from α-dodecene (11), probably no primary phenyl isomer (ω-phenyl) is present. Presumably the proportion of each individual secondary isomer diminishes for increasing distance between the phenyl group and the ω-carbon, which was the position of the original double bond. Probably most of the sulfonate group is at the para position on the ring, with a smaller proportion at the ortho (12).

Methylene Blue Analysis of Sulfonates. Three procedures were used, the Longwell-Maniece (16) occasionally, the Standard Methods procedure (17) more often and the Hellige method (18) was used for routine daily analyses. The latter is a shortened procedure involving a single extraction and visual comparison of color intensity with glass color standards ranging from 0–2 mg/liter of ABS; we used the methylene blue solution described in Standard Methods (17) and the Hellige color disk for tetrapropylene ABS. No corrections were made for variation in the type or mol wt of surfactant since these would only amount to ca. one or two tenths of a mg/liter in the dilutions being measured. Checks by the two more precise methods validated the use of this procedure within the necessary limits of accuracy. There is a possibility that some of the apparent LAS measured in the effluents may actually have been early-stage degradation products, noticeable because of the extraordinarily high LAS concn being fed.

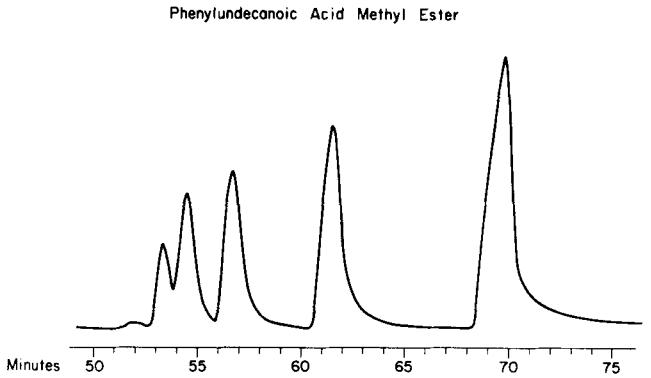

FIG. 1. Gas chromatogram of phenylundecanoic acid methyl ester. Capillary stainless steel column 150 feet x 0.02 in (46 meters x 0.05 cm); silicone rubber substrate; strontium-90 detector; column temp 187C; argon pressure 4 psi (0.3 kg/sq cm).

Methylene Blue Response of the Sulfonates. If the methylene blue salt of an ABS is a stoichiometric salt, if it is quantitatively extracted into the chloroform phase and if it obeys Beer's Law, the color of the chloroform phase should be proportional to the molar amount of ABS present in the sample, so that application of a mol wt factor should give the amount by wt. The data reported by Laws and Hancock suggest that these assumptions are correct for sulfonates in the detergent range (19). In the present work we found this to be true for the C_{12} and C_{14} LAS by comparison of the Standard Methods calibration curves for the two samples.

On the other hand, the SΦU calibration curve, corrected for the ratio of mol wt, was significantly different from the LAS curves, corresponding to only ca. 85% of the theoretical. This might arise from differences in the stoichiometry of the methylene blue salts, from incomplete extraction in the SΦU system, or (we feel less likely) from the products being significantly different in content of impurities. Response of the SΦU with the Hellige procedure was only ca. 70% of the calculated amount.

Using the Longwell-Maniece method the C_{12} and C_{14} LAS were again in good agreement, while the response of the SΦU was only ca. 1% of the calculated amount. This behavior demonstrates the increased selectivity which has been designed into the Longwell-Maniece method: the SΦU is held back in the preliminary extraction step, presumably because of the presence of two ionic centers in the molecule in the alkaline system used at that stage. When the preliminary alkaline extraction step was omitted, the response of the SΦU was 80%, in reasonable agreement with the Standard Method.

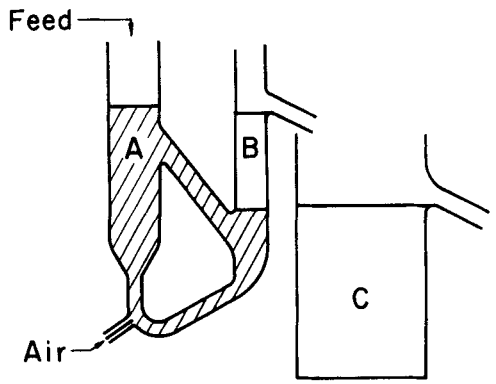

FIG. 2. Activated sludge system. A = Aerator, working volume 300 ml. B = Settler, working volume 75 ml. C = Fish Tank, working volume 3 liters.

Substantially quantitative response was attained by titration of SΦU with Hyamine 1622 by the Weatherburn-Epton procedure using methylene blue indicator (20). A sample of the crude sulfonic acid before neutralization was titrated with NaOH for carboxyl group (weak acidity between breaks at pH 3.5 and 8.8) and then for anionic surfactant with Hyamine. A second sample was titrated for sulfonic acid content using a variant of Critchfield and Johnson's morpholine-acetonitrile technique (21). All three values agreed closely:

carboxyl group	1.857 meq/gm
anionic surfactant	1.819, 1.829
sulfonic group	1.857

Unfortunately, the Weatherburn-Epton titration as it stands requires mg quantities of surfactant instead of μg quantities and is thus not directly applicable at the concn of the present work, although it may be possible to develop a micro modification.

The response of the SΦU in the various methylene blue methods may be summarized as follows:

Standard Methods	85%
Hellige	70
Longwell-Maniece	0
L-M without alkali	80
Weatherburn-Epton	100

Determination of Median Tolerance Limits (TL_m). Median tolerance limit (TL_m) by definition is the concn of a material in which 50% of the test fish survice for a specified time period. It is not a concn that one would consider to be safe in the natural stream environment for the species of fish used in the bioassay. The procedure of Standard Methods (22) was followed by using bluegill fingerlings (Lepomis macrochirus, 5–7.5 cm in length). Some supplementary details were incorporated with the standard procedure to ensure the highest degree of uniformity and reliability of the results, particularly since the compounds being studied were biodegradable. The medium was the standard reference water of Freeman (23), made up from deionized water and inorganic salts to be representative of the average surface water of the U.S., with the following composition:

Component	Range mg/liter
M.O. Alkalinity (as $CaCO_3$)	94–104
EDTA Hardness (as $CaCO_3$)	100–111
Sulfate	30.6–39.4
Chlorides	8.5–14.4
Total dissolved solids	165–188
pH	8.2–8.5

Test fish were acclimated to laboratory conditions, i.e., temp 22C, and standard reference water prior to the screening tests. The final bioassays using test solutions were made at the same temp as the screening tests. The test fish were not fed for two days before being exposed to the test solutions, nor during the period of exposure.

Each test solution was prepared by diluting a quantity of the stock solution of the compound being investigated with standard reference water to a given volume. A 3-liter final volume was used for screening tests and a 10-liter final volume for full-scale tests.

To locate the approx toxic range or "critical concn range" for each bioassay study, screening tests were conducted using a relatively wide range of concn. Three test fish were placed in each vessel and data obtained were used to establish the concn for the final bioassay study.

The experimental vessels used for the final bioassay tests were wide-mouth, five-gallon containers fitted with suitable glass and rubber tubing to provide continuous aeration. Ten test animals were placed in 10 liters of solution in each container. A control of 10 fish in 10 liters of standard reference water was used with each set of bioassay tests to validate the water and the stock of fish used in the study. During both the exploratory and final tests, continuous aeration was provided to hold the dissolved oxygen level above the critical level. The rate of aeration was controlled to maintain adequate dissolved oxygen levels without excessive turbulence in the container which would physically affect the fish when exposed to it over the test period.

Periodic observations of the test fish were made during the bioassay to establish the time of death, and also to detect any other physiological effects on their over-all appearance, color, activity and equilibrium. In particular, any unusual exudations from the fish were also noted for possible correlation with subsequent histological examinations. The TL_m values were determined by straight-line interpolation from a semi-logarithmic plot of the concn which bracket the 50% survival point vs. an arithmetic plot of the corresponding percentages of survival.

Activated Sludge Units and Fish Tanks (Figure 2). The sludge units comprised a glass aeration chamber of 300 ml working volume (A) and a glass settling chamber of 75 ml (B). The design provided for rapid and complete mixing of the aerator contents with the feed, (introduced below the surface to minimize foaming) and for rapid circulation to and from the bottom of the settler, maintained by the airlift action of ca. 200 ml/min of filtered air introduced at the bottom of the aerator.

The activated sludge cultures originated in a municipal sewage treatment plant, but had been well acclimated to the laboratory regime by several months' operation on synthetic sewage and LAS of various types. Mixed liquor suspended solids were in the range of 3500–4500 mg/liter during the fish tests. Feed flow rates were either 100 ml/hr (average residence time 3 hr in aerator) or 40 ml/hr (7 hr residence). The feed to the control activated sludge unit was that of Huddleston and Allred (29), made up from city tap water by adding:

Soy peptone	150 mg/liter
K_2HPO_4	50
NaCl	30
$MgSO_4$	10

The LAS sludge units were fed this same mixture to which the surfactant was added at the desired concn. No foaming has been experienced even with LAS concn as high as 200 mg/liter in the feed over periods of several months, proving the sludge has been acclimated to the general conditions previously and to the LAS by gradual increase of the concn in increments of say 50 mg/liter/week. However, upsets may occasionally occur due to operating irregularities and at these high concn the resultant foaming will carry the sludge up out of the aerator. Smooth operation can usually be restored by temporarily adding an antifoaming agent (e.g. Dow Corning Antifoam A) or by temporary reduction of the feed rate or of the surfactant concn in the feed.

The substantially clear effluent from the top of the settler (B) flowed into a vertical cylindrical fish tank (C) made of acrylic plastic (Lucite) with a working volume of three liters which provided average residence times of 30 hr or 70 hr at the two feed rates used. Although the tap water fed to the sludge units showed a positive test for free chlorine by the orthotolidine method (24) this was removed by the sludge, and the effluent passing into the fish tank gave a negative test. A slow stream of air bubbles in the fish tank maintained the dissolved oxygen at 5.8–7.7 mg/liter. The pH ranged from 6.5–7.7 and temp from 21–25C. No food was added.

Procedure for Histological Studies. In order to obtain a reasonable comparison of the effects of the various LAS compounds and intermediate degradation products on the gill tissue of the test fish, a laboratory procedure was developed and adhered to throughout the course of these bioassays. It included preservation of the test specimens, preparation of the gill tissue sections, microscopic examination and photomicrography.

The fish were removed from the bioassay vessel as soon as possible after death, and placed in approx 7% formaldehyde solution (1:4 Formalin : H_2O) for preservation. Typical surviving fish were removed upon completion of the exposure period, and were preserved in the same manner.

Upon completion of each bioassay, sections of gill tissue from the preserved fish were prepared by carefully cutting the gill cover plate from the test specimen with a sharp scalpel to expose the respiratory folds of the gills. An entire section of the exposed gill structure was then cut from the fish and washed in ethanol, placed on a 1 x 3-in glass slide and the excess ethanol was allowed to evaporate. The sections were then coated with an acrylic plastic to affix the tissue to the slide and to prevent complete drying of the tissue.

After gill tissue sections for each bioassay were prepared they were examined microscopically at 100 X. Photomicrographs were taken of each section using high-speed 35-mm color film.

TABLE I
Standard Median Tolerance Limits (mg/liter, bluegill fingerlings)

	C_{12} LAS	C_{14} LAS	SΦU
TL_m 24 hr	3.1	0.64	120
TL_m 48 hr	3.0	0.64	75
TL_m 96 hr	3.0	0.64	75
100% kill 24 hr	8.0	0.80	150+
0 kill 96 hr	2.0	0.40	50

C_{12} LAS: Alkylbenzene sodium sulfonate derived from alpha-dodecene.
C_{14} LAS: Alkylbenzene sodium sulfonate derived from alpha-tetradecene.
SΦU: Sulfophenylundecanoic acid disodium salt (mixed isomers).

Results and Discussion

Median Tolerance Limit (TL_m). The TL_m values shown in Table I for the C_{12} and C_{14} LAS (3.1 and 0.64 mg/liter, respectively) are in reasonable agreement with LD_{50} values of 2.6 and 0.26 reported by Hirsch who used goldorfen, a different variety of fish (5).

In contrast, Table I shows that the SΦU is much less toxic than LAS. This material (or derivatives thereof) is probably formed at an early stage in the biodegradation of C_{11} LAS by oxidation of a terminal methyl group on the alkyl chain to a carboxylate. It also is probably formed at somewhat later stages in the biodegradation of C_{13} and C_{15} LAS, resulting from terminal carboxylation followed by one or two steps of β-oxidation (25). Hirsch reported the goldorfen LD_{50} of C_{11}, C_{13} and C_{15} LAS to be 6.5, 0.57 and 0.25 mg/liter, respectively (5). Our TL_m values of 75–120 mg/liter for the carboxylated product correspond to a toxicity one or two orders of magnitude lower. Thus it is to be expected that the fish toxicity of a system containing LAS will be markedly lowered upon biodegradation.

Effects of Continuous Activated Sludge Effluents. In these experiments the activated sludge unit was first acclimated to the desired operating conditions for a week or two, then the fish tank was added to the system. When it had filled to the 3-liter overflow level, three bluegill fingerlings were introduced for a 96-hr exposure. The results are shown in Table II, Runs A–D.

Feeding the C_{12} LAS to the sludge unit at 100 mg/liter, 7-hr residence time, gave an effluent containing 0.2–0.4 mg/liter apparent LAS. Even though the initial LAS concn was over 30 times the TL_m concn, all three fish used in Run A survived the 4-day exposure in the effluent, and two out of three survived in Run B. During and after their exposure the surviving fish gave no indications of distress and they looked and behaved the same as those in the control unit, fed no surfactant.

Similar results were obtained in Runs C and D, feeding C_{14} LAS to a 3-hour sludge unit at 100 times the TL_m concn. All six fish survived with no difficulty.

In the final run, E, the procedure was modified so that the three fish were exposed for a total of 11 days: first for four days feeding the sludge unit at 100 mg/

TABLE II
Continuous Activated Sludge Experiments

Run	LAS	Sludge aerator detention time, hr	mg/liter LAS in			Fish kill	Exposure, days
			Sludge feed (actual)	Sludge effluent (analyzed)	Fish tank (analyzed)		
Control	none	7	0	0.1–0.2	0.1–0.2	0/3	4
A	C_{12}	7	100	0.2–0.4	0.2–0.4	0/3	4
B	C_{12}	7	100	0.2–0.3	0.2–0.3	1/3	4
C	C_{14}	3	65	0.2–0.6	0.2	0/3	4
D	C_{14}	3	65	0.2–4.0	0.3–0.9	0/3	4
E-1	C_{14}	3	100	0.5–1.9	0.4–0.9	0/3	4
E-2	C_{14}	3	150	0.2–2.0	0.4–0.9	0/3	7 (4 + 3)
E-3	C_{14}	3	200	3.2–35.0	0.6–2.0	0/3	9 (7 + 2)
E-4	C_{14}	(E-3 held batchwise)			0.3	0/3	11 (9 + 2)

TABLE III
Toxicity of C_{14} LAS in Acclimated Sludge Effluent

Time, days	C_{14} LAS, mg/liter			Number of fish surviving
	Before addition (analyzed)	Increment added (actual)	After addition (analyzed)	
0	0.28	0.64	0.38	3
1	0.15	0.32	0.33	3
2.25	.....	1.00	0.87	3
3	0.35	3.00	1.71	3
4.2	1.18	2.00	2.08	1
5	1.36	.....	.....	0

liter of C_{14} LAS, after which the feed concn was raised to 150 mg/liter for three days and then to 200 mg/liter. As a result of this rapid increase, the sludge experienced an upset on the ninth day (Run E-3) so that the effluent was entering the fish tank at 35 mg/liter apparent LAS and the fish tank contents were up to 2 mg/liter. At this point the fish tank was isolated from the sludge unit and held for two more days, the apparent LAS content dropping rapidly to 0.3 mg/liter. All three fish survived with no difficulty.

In these experiments the LAS concn in the feed in addition to being many times greater than the TL_m concn were ca. 10 times the concn of tetrapropylene ABS currently present in sewage entering municipal treatment plants (3). The absence of toxic effects in our experimental effluents indicates that a) the biodegradation rate of the LAS is rapid enough in an acclimated system to proved a great margin of safety, and b) toxic intermediates are not formed in any significant amounts.

Fish Bioassays in Activated Sludge Effluents. Activated sludge effluents contain substantial quantities

FIG. 3a. Gill tissue section from fish utilized in TL_m determination of C_{14} LAS. Surviving control fish.

TABLE IV
Toxicity of C_{14} LAS in Unacclimated Sludge Effluent

Run	C_{14} LAS, mg/liter			Fish surviving	
	Added (actual)	Analyzed			
		0 hr	24 hr	0 hr	24 hr
1	0	0.26	.....	2	2
2	1.0	0.92	1.07	3	3
3	2.0	1.57	.....	3	0
4	3.0	2.58	1.96	3	0

of organic compounds whether or not they are fed LAS. For example, these laboratory effluents contained 50–75 mg/liter of chemical oxygen demand as determined by the dichromate method (26). Screening studies were undertaken to determine what effect these components had on the toxicity of the C_{14} LAS in comparison with the TL_m values determined in the standard water. In these experiments the apparent LAS concn were determined by the Standard Methods methylene blue procedure (17).

In the first experiment (Table III) three bluegill fingerlings were placed in the three liters of effluent remaining from Run E-4 (Table II), which contained 0.28 mg/liter apparent C_{14} LAS by analysis. The TL_m amount of fresh C_{14} LAS was then added, but the analysis immediately after addition was only 0.38 mg/liter, and this had dropped to 0.15 one day later. A second increment of 0.32 mg/liter was added at that time, and on the next day a third increment of 1.00 mg/liter with similar results, the solution analyzing as 0.87 mg/liter immediately after the third increment, dropping to 0.35 by the next day. All three fish survived, whereas some fatalities might be expected at these concn in standard dilution water. Two further increments of 3.00 and 2.00 mg/liter were subsequently added, maintaining the apparent LAS concn between 1.18 and 2.08 during the next two days; none of the fish survived at these higher concn.

The rapid disappearance of the C_{14} LAS when added to this effluent is the result of biodegradation. The results indicate that even in the absence of the activated sludge process, the biodegradation rate is high enough in an acclimated system to provide a certain amount of protection when amounts of LAS above the calculated toxic limits are added.

A second set of experiments was made using effluent from the control activated sludge unit to minimize the effects of biodegradation (Table IV). The disappearance of added C_{14} LAS was slower in this unacclimated system, and the addition of 1.0 mg/liter gave a solution analyzing as 0.92 at time zero and 1.07 at 24 hr. The three fish survived in this solution for the 24 hr of the test with no difficulty, and their behavior and appearance was identical with the fish in the control experiment to which no LAS had been added. Two parallel experiments using three fish each at 2.0 and 3.0 mg/liter of C_{14} LAS resulted in death of all fish within 24 hr.

The survival of the three fish in the 1.0 mg/liter experiment indicates that the activated sludge effluent may exert some protective action against the LAS toxicity, since this same C_{14} LAS in the TL_m determinations using the standard water caused 100% kill at 0.8 mg/liter within 24 hr.

Physiological Observation. There was no significant change in the over-all appearance of the surviving fish even in the higher concn of LAS. They were indistinguishable from those in the control solution.

All of the fish that expired during exposure to the LAS solution seemed to follow the same pattern prior to death. The initial distress signs were inflammation

of the area around the gills, fins and tail, and were generally followed by a loss of activity and equilibrium, by excretion of a gelatinous material from the gills, and by varying degrees of bulging of the eyes. Shortly before death these fish underwent a general loss of coloration, and after death generally appeared very white and slimy.

This death cycle or pattern was followed quite closely by all the fish that expired during exposure to the LAS solutions used in these studies.

In addition to these physiological observations the physical characteristics of the test solutions were noted throughout the bioassays. There was a noticeable increase in the turbidity of the test water with increasing concn of LAS and with time. In the test water which contained the SΦU at concn of 100 or more mg/liter the turbidity was so great that it was difficult to observe the fish in the test vessel. There was no increase in turbidity noticed in the control vessel containing only standard reference water and fish.

In most of the LAS test solutions there was a noticeable increase in particulate material in suspension during testing. This was probably a result of the gelatinous material which was observed exuding from the gills of the test animals described in the following section.

Histological Examinations. Detailed microscopic examination of the sections revealed no noticeable damage to the gill tissue of the control fish. There was also no significance difference in gill tissue between the controls and test fish surviving sublethal concn of LAS below two-thirds of the TL_m value. Those surviving 96 hr exposure to LAS concn near the TL_m exhibited only isolated damage to the mucous layers and soft tissues of the gills while the fish expiring in the same solutions had suffered extensive damage.

This difference was dramatic. The fish that expired first suffered a severe hematomas of the respiratory folds of the gills. This symptom corresponds to the inflammation noticed in the early stages of the death cycle or pattern. This condition was also accompanied by a matting or sticking together of the respiratory folds, and was followed by a stripping of their mucous layers. Once this protective layer was removed, the soft tissue beneath was severely damaged, and in most cases was completely destroyed, leaving only a hard, skeleton-like structure. Throughout this investigation the damage to the gill tissue, as revealed by histological examinations, was generally the same for all fish that expired under test conditions. The extensive or complete stripping of the soft, outer mucous layer from the gill structure was undoubtedly the source of streamers of gelatinous material which characteristically exuded from the gill openings before death.

This extensive damage is demonstrated in the photomicrographs presented in Figure 3. These show gill tissue sections from a) a surviving control fish, b) a surviving fish after 96 hr exposure to C_{14} LAS concn near the TL_m value, and c) a fish which expired in the same solution. It can be readily seen that there is only slight isolated damage evident in gill section (b), compared with extensive damage in gill section (c).

Similar histological effects were evident in the ex-

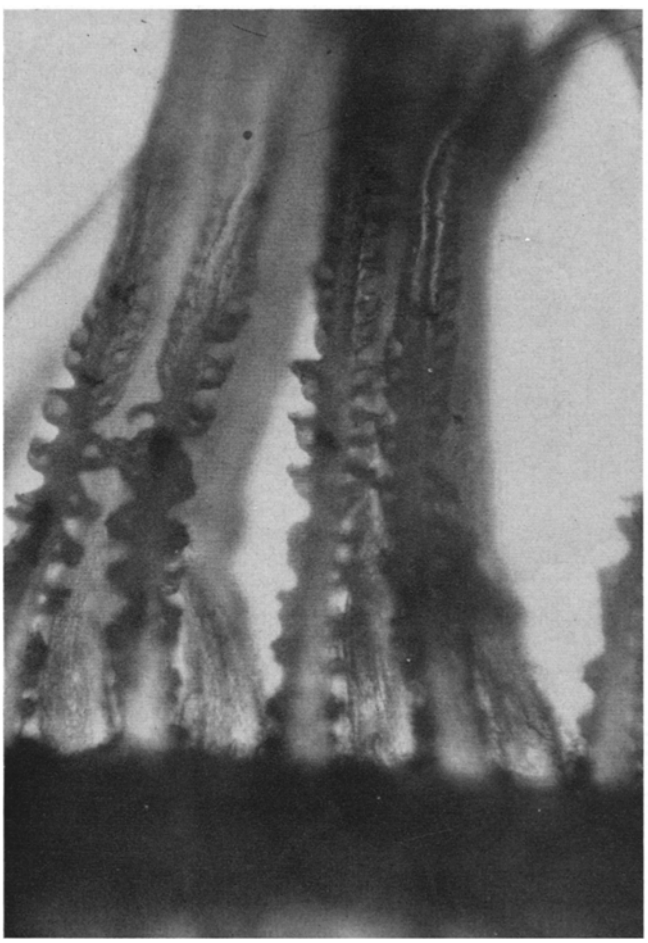

FIG. 3b. Gill tissue section from fish utilized in TL_m determination of C_{14} LAS. Surviving fish after exposure to 0.6 mg/liter.

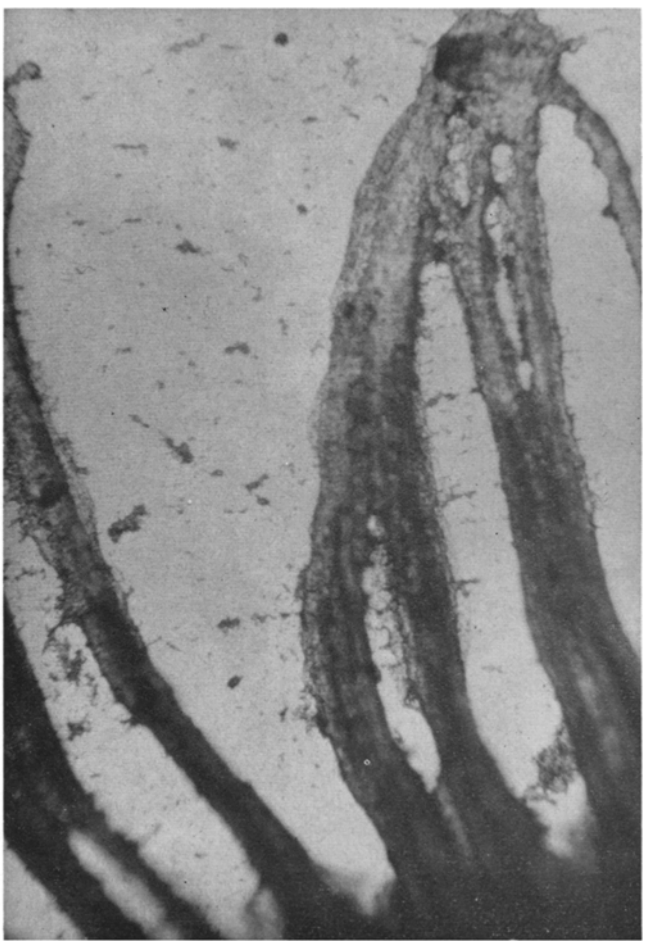

FIG. 3c. Gill tissue section from fish utilized in TL_m determination of C_{14} LAS. Fish expired during exposure to 0.6 mg/liter.

posure to SΦU: stripping of the gill tissue in the fish which died, little change in the fish which survived.

The fish exposed to the continuous activated sludge effluents (Table II) showed a similar pattern. Those from the control run fed no LAS exhibited a slight roughening of the gill tissue as compared with control fish in standard water. The survivors from Runs A–D showed a further slight loss of smoothness and some indication of isolated points of damage. The 11-day exposure received by the three fish in Run E resulted in a further incremental change in microscopic appearance, but with damage less severe than illustrated in Figure 3 b. Macroscopic appearance and behavior still could not be distinguished from the controls. The one casualty, in Run B, showed complete loss of the mucous layer and soft tissue of the gill structure, similar to those which had died in the TL_m determinations.

In the static tests using unacclimated sludge effluent, no difference in the microscopic appearance could be detected between the control fish (Table IV, Run 1) and those exposed to 1 mg/liter of C_{14} LAS (Run 2), which was significantly higher than the standard TL_m value. Each group showed a very slight loss of smoothness in texture of the gill tissue, while those which died (Table IV, Runs 3 and 4) suffered near complete loss of color and soft tissues.

Thus in all cases the attack on the gill tissue appears to be related not only to the LAS concn, but also to the sensitivity of the individual fish. The susceptible individual suffers rapid loss of gill mucosa with resultant death, while the resistant individual survives under the same conditions with little if any detectable damage. Such differences in physiological response between individuals of any species are not uncommon.

Schmid and Mann have reported that sodium dodecylbenzene sulfonate attacks the mucous cells on the top of the gill lamina of trout starting at a concn of 5 mg/liter (27). Cairns and Scheier noted similar effects on pumpkinseed and bluegill sunfish after several months' exposure to 3.1 mg/liter of tetrapropylene ABS, ca. one-fourth to one-third of the TL_m concn (28). The present work shows that LAS likewise attacks the gill mucous membranes at concn near or above the TL_m value, but with much less damage to the fish that survive than to those that die at the same concn, and that no damage at all is observed in four days at concn below two-thirds of the TL_m.

ACKNOWLEDGMENTS

Design of the activated sludge units by W. M. Davis; development of operational techniques by R. J. Day; and development of operational techniques and supervision of the daily operation of the activated sludge units by J. C. Gromada.

REFERENCES

1. Anon., Chem. Eng. News, June 24, 1963, pp. 36–37.
2. Swisher, R. D., JAOCS *40*, 648–656 (1963).
3. Ohio River Valley Water Sanitation Commission Detergent Subcommittee, J. Am. Water Works Assoc. *55*, 369–402 (1963).
4. The Soap and Detergent Association, New York, unpublished results (1964), see also Bornmann, G., and A. Loeser, Fette, Seifen, Anstrichmittel *65*, 818–824 (1963).
5. Hirsch, E., *Ibid.* *65*, 814–818 (1963).
6. Reference 3, pp. 377–379.
7. Reference 3, pp. 387–388.
8. Borstlap, C., and P. L. Kooijman, JAOCS *40*, 78–80 (1963).
9. Herbert, D. W. M., G. H. J. Elkins, H. T. Mann and J. Hemens, Water Waste Treat. J. *6*, 394–398 (1957).
10. Niemitz, W., and W. Pestlin, Städtehygiene *13*, 231–233 (1962).
11. Swisher, R. D., E. F. Kaelble and S. K. Liu, J. Org. Chem. *26*, 4066–4069 (1961).
12. Gray, F. W., J. F. Gerecht and I. J. Krems, *Ibid.* *20*, 511–524 (1955).
13. Pulver, M. R., Eastman Kodak Co., private communication (1958).
14. Meyer, V., Ber. *27*, 510–512 (1894).
15. Swisher, R. D., J. Water Pollution Control Federation *35*, 877–892 (1963).
16. ABCM-SAC Committee, The Analyst *82*, 826–827 (1957).
17. "Standard Methods for Examination of Water and Wastewater," 11th ed., American Public Health Association, New York, 1960, pp. 246–248.
18. Hellige Inc., Garden City, N.Y., ABS Testing Outfit No. 367-DO.
19. Laws, E. Q., and W. Hancock, Nature *183*, 1473–1474 (1959).
20. Weatherburn, A. S., JAOCS *28*, 233–235 (1951).
21. Critchfield, F. E., and J. B. Johnson, Anal. Chem. *26*, 1803–1806 (1954).
22. Reference 17, pp. 457–473.
23. Freeman, L. A., Sewage Ind. Wastes *25*, 845–848 (1953).
24. Reference 17, pp. 91–92.
25. Swisher, R. D., J. Water Pollution Control Federation *35*, 1557–1564 (1963).
26. Reference 17, pp. 399–402.
27. Schmid, O. J., and H. Mann, Nature *192*, 675 (1961); Archiv für Fischereiwissenschaft *13*, 41–51 (1962).
28. Cairns, J., Jr., and A. Scheier, Purdue Univ., Eng. Bull., Ext. Ser. No. *112*, 14–28 (1962); Ind. Water Wastes, *9*, 22–28 (1964).
29. Huddleston, R. L., and R. C. Allred, JAOCS *41*, 732–735 (1964).

[Received June 17, 1964—Accepted September 11, 1964]

The Determination of Polyoxyethylene Nonionic Surfactants in Water at the Parts per Million Level

N. T. CRABB and H. E. PERSINGER, Research and Development Department, Union Carbide Corporation, Chemicals Division, South Charleston, West Virginia

Abstract

A method has been developed to determine the concn of polyoxyethylene nonionic surfactant (PNS) in the parts/million concn range in a water-bacteria medium. The method has successfully determined the concn of PNS during the course of biodegradation studies using either activated sludge or river water as the bacterial source. The nonionic surfactant was removed from the water solution by continuous ether extraction. Detection and measurement of the PNS was accomplished using cobalt thiocyanate and measuring the absorbance of the blue cobalt-PNS complex at 620 mμ in a five-cm absorption cell.

Optimum extraction conditions required a neutral pH and a low ionic strength. The colorimetric step required that each molecule of PNS contain at least six ethylene oxide units for color development, and since the absorbance varies with the length of the polyoxyethylene chain, the method must be standardized using the particular compound under investigation.

Introduction

AN IMPORTANT MEMBER of the detergent family is the nonionic surfactant prepared by adding ethylene oxide to an alcohol, amine or alkylphenol starter. This variety of detergent is known as a polyoxyethylene nonionic surfactant which henceforth will be designated as nonionic surfactant or PNS. Bifunctional solubility is built into the molecule as is evidenced by the organic soluble, hydrophobic nature of the