INVESTIGATIONS IN FISH CONTROL

36. **A Method for Rating Chemicals for Potency Against Fish and Other Organisms**

37. **Comparative Toxicity of 29 Nitrosalicylanilides and Related Compounds to Eight Species of Fish**

38. **Toxicity of 33NCS to Freshwater Fish and Sea Lampreys**

United States Department of the Interior
Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife

INVESTIGATIONS IN FISH CONTROL

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Continued on inside back cover--

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Washington, D.C. · July 1970

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IN THIS COVER

INVESTIGATIONS IN FISH CONTROL

36. A Method for Rating Chemicals for Potency Against Fish and Other Organisms

By Leif L. Marking

UNITED STATES DEPARTMENT OF THE INTERIOR, WALTER J. HICKEL, *SECRETARY*
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CONTENTS

A METHOD FOR RATING CHEMICALS FOR POTENCY AGAINST FISH AND OTHER ORGANISMS

By Leif L. Marking, Chemist
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ABSTRACT.--A potency rating is presented by which the toxicity of chemicals to organisms can be assessed with a minimum of data from preliminary bioassays. This method permits effective and rapid evaluation of toxicity when data from preliminary tests are inadequate for statistical analysis. In bioassays of chemicals against fish, the mortality is recorded in 3- to 96-hour tests when exposed to three concentrations. The potency rating of a bioassayed chemical, when compared with the maximum value on the rating scale, indicates the relative toxicity of the chemical. Concentrations and exposure times can be changed to fit a specific bioassay program. Results of 24- or 48-hour bioassays can be compared with 96-hour bioassays because the maximum values of the rating scales are approximately equal at all time intervals. The potency rating is more applicable to toxicants which have similar dose-effect curves.

Data from preliminary bioassays are usually tedious and difficult to analyze since the toxicity is not defined by statistical parameters such as those described by Litchfield and Wilcoxon (1949). A minimum number of organisms and concentrations limit statistical evaluations for median tolerance limits. Preliminary bioassay data usually indicate toxic ranges of concentrations and are followed by more definitive tests. However, a greater number of chemicals can be tested in preliminary trials such as those described by Lennon and Walker (1964), but a mathematical method is needed for analyzing and summarizing these data in a form that is easily stored and retrieved. In programs in which many chemicals are not tested beyond preliminary screening, the potency rating of a large number of compounds is useful in making comparisons and selecting those having potential. It also reduces the bulk of data to easily comprehended terms.

POTENCY RATING

Potency is necessarily stated in absolute units for therapeutic applications, but for comparison of drugs relative potency is a more convenient term, as Fingl and Woodbury (1965) point out. They also state that dose-effect curves which are similar in slope permit comparisons of relative potencies, while those with dissimilar dose-effect curves do not. Relative potency is more applicable to bioassay data derived from related chemical structures or from chemicals producing similar biological effects on the test organisms.

Relative potency is described by Finney (1962) and is calculated as a constant difference between the dosage-response regressions of two stimuli. This method does not apply to preliminary type data which cannot be analyzed by probit regression methods.

I have therefore derived an equation expressing relative activities of chemicals to fish or other organisms. This equation defines potency rating.

The potency rating effectively separates toxic from nontoxic chemicals. It also indicates the degree of general toxicity and the differential toxicity among species tested. The values obtained are compared with a maximum potency rating calculated by assuming complete mortality at all concentrations and time intervals.

Preliminary screening yields data on numbers of organisms killed at selected concentrations in multiples of 10 that are easily analyzed by comparing their potency ratings. Mortality is dependent upon the concentration and exposure time. Potency rating (Pr) is dependent on the mortality at each concentration and each exposure time. In addition, the number of tests must be considered, since Pr is the accumulation of values throughout a bioassay period. The relation of Pr to the variables involved is suggested by formula 1 where mortality is a function of concentration and time (table 1). In essence, the mortality (M) is multiplied by the reciprocal of the concentration (C) times the reciprocal of the log of time (T) at each concentration and time interval. The sum of these factors is divided by the log of the number of observations (N). The arrangement of the values used in deriving this index of activity does not follow any particular mathematical relations, and the values do not express units of measurement. The Pr merely indicates the activity of one test substance in comparison with others.

Reciprocals for concentration and the log of time are necessary to emphasize the effects of lower concentrations and shorter exposure periods (figs. 1 and 2). If the reciprocal relations are neglected, the accumulated potency rating increases drastically in longer exposures even though none of the organisms die during this period, and the effects of higher concentrations are weighted more heavily. The effects of the reciprocals are best seen in table 2, where maximum potencies (Pmax) are calculated for five time intervals and three concentrations. I have assumed that 10 organisms die among 10 tested in each case. The contributions of potency ratings are listed at each time interval under each concentration of 0.1, 1.0, and 10 ppm. Pmax contribution at 3 hours at 0.1 ppm is 209.60, whereas that for 96 hours at 0.1 ppm is 50.45, and subsequent contributions at higher concentrations are less by factors 0.1 and 0.01. Therefore, chemicals which are

TABLE 1.--Formulas for potency rating

(1) $$Pr = \frac{\sum_{j=1}^{5} \sum_{i=1}^{3} \frac{M(C_i, T_j)}{C_i \log T_j}}{\log N}$$

Where M = mortality among the total number tested
C = concentration
T = time of exposures in hours, $T > 1$
N = number of observations, $N > 1$
j = finite variables of T: $j_1 = 3$, $j_2 = 6$, $j_3 = 24$, $j_4 = 48$, $j_5 = 96$
i = finite variables of C: $i_1 = 0.1$, $i_2 = 1.0$, $i_3 = 10$

When the individual concentrations are considered, formula 1 expands to:

(2) $$Pr = \frac{\sum_{j=1}^{5} \left[\left(M \times \frac{1}{0.1} \times \frac{1}{\log T_j}\right) + \left(M \times \frac{1}{1.0} \times \frac{1}{\log T_j}\right) + \left(M \times \frac{1}{10} \times \frac{1}{\log T_j}\right) \right]}{\log N}$$

When the observation times are considered also, the formula expands further to:

(3) $$Pr = \frac{\left(M \times \frac{1}{0.1} \times \frac{1}{\log 3}\right) + \left(M \times \frac{1}{1.0} \times \frac{1}{\log 3}\right) + \left(M \times \frac{1}{10} \times \frac{1}{\log 3}\right) + - - - \left(M \times \frac{1}{10} \times \frac{1}{\log 96}\right)}{\log N}$$

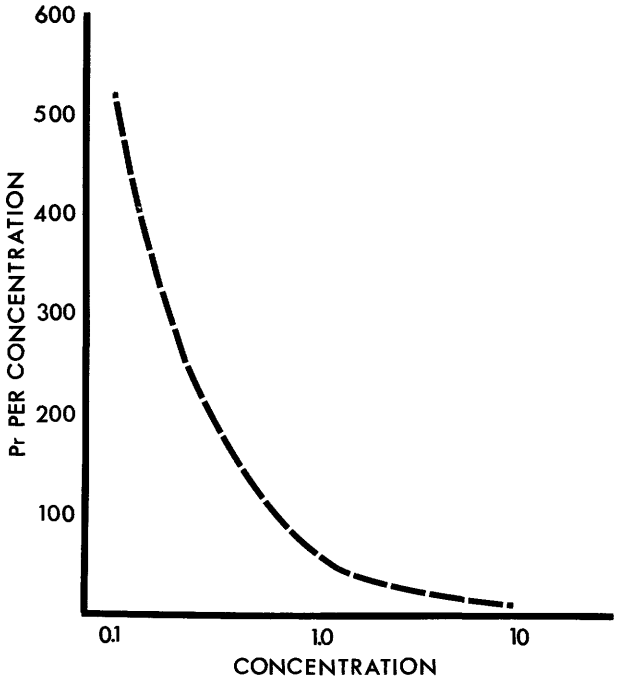

Figure 1.--The potency rating is larger at lower concentrations through the reciprocal relationship.

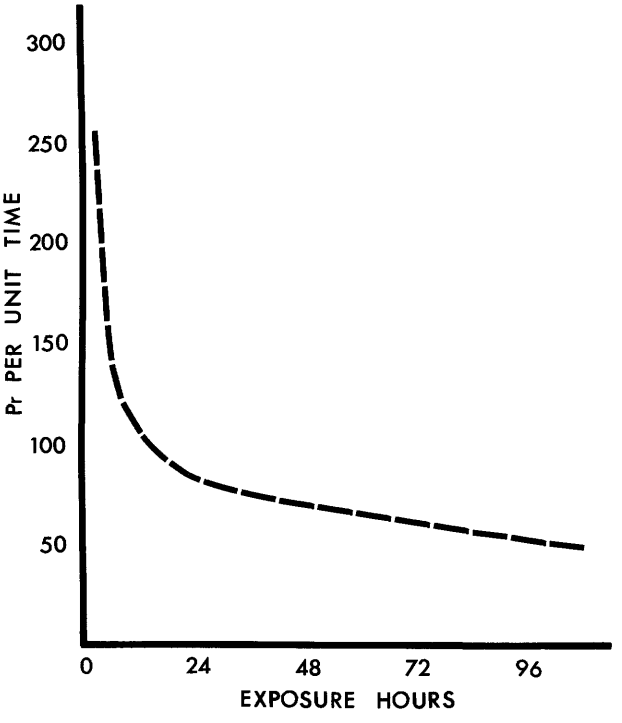

Figure 2.--The potency rating is larger at shorter exposure periods through the reciprocal relationship.

TABLE 2.--Maximum potencies at three concentrations and five time intervals

Exposure	log T	$\frac{1}{\log T}$	N	log N	Concentration (ppm)			Sum at T	Pmax
					0.1	1.0	10.0		
3 hours...	0.4771	2.0960	3	0.4771	209.60	20.96	2.10	232.66	487.65
6 hours...	0.7782	1.2850	6	0.7782	128.50	12.85	1.29	142.64	482.27
24 hours...	1.3802	0.7245	9	0.9542	72.45	7.25	0.72	80.42	467.11
48 hours...	1.6812	0.5948	12	1.0792	59.48	5.95	0.59	66.02	483.45
96 hours...	1.9823	0.5045	15	1.1761	50.45	5.04	0.50	55.99	491.23
Total....	--	--	--	--	520.48	52.05	5.20	577.73	--

highly toxic in short exposure periods indicate greater potency than chemicals which are effective only in longer exposures. Chemicals which are toxic only at higher concentrations will definitely yield lower potency ratings regardless of exposure time. The values at 1.0 and 10 ppm are rounded off to two decimal places.

The divisor in the formula, log N, accounts for the number of observations being analyzed. This arbitrary factor also reduces the additive effect of potency once the fish are recorded dead. Pmax in table 2 indicates little change at any time interval, and data from 24- or 48-hour tests could be compared with data from 96-hour tests. The maximum potency in a 96-hour bioassay is 491.23; thus it becomes the standard for comparison of the calculated experimental potencies. If the potencies were merely summed at the various time intervals, the maximum potency would be 577.73 (table 2), but these effects are cumulative, and the sum depends on the number of time intervals in the testing program. This cumulative potency increases even though no more fish die.

The size of the number describing Pr and the number of digits the Pr contains adequately indicate the toxicity with respect to time. Since Pmax is 467.11 to 491.23 depending on T, and the contribution at 0.1 ppm is a 3-digit whole number, I have assumed that 3-digit whole-number Pr's indicate mortality at 0.1 ppm. Corresponding, 2-digit whole numbers indicate mortality at 1.0 ppm (maximum contribution = 52.05) and values less than 10 indicate mortality at 10 ppm (maximum contribution = 5.20). If none of the specimens die at the highest concentration, the value becomes zero. The Pr values can vary from

0 to 491.23, and their proportion of the maximum potency with respect to T defines the relative activity. Values greater than 300 indicate mortality of 10 organisms at 0.1 ppm in 6 hours or less.

The virtues of the potency rating concept are more comprehensible through example. Table 3 lists results of a preliminary fish bioassay and defines the individual potencies at each concentration and time interval. The value at 1.0 ppm is found by using the part of formula 2 pertaining to 1.0 ppm and 3 hours exposure time, or the value may be obtained from the appendix.

$$Pr = M \times \frac{1}{1.0} \times \frac{1}{\log T} = 5 \times \frac{1}{0.4771} = 10.48$$

The sum of the individual values, 284.46, is divided by log N (1.1761) according to formula 3, and the Pr of this chemical to fish is 241.87. The Pr of 241.87 is then compared with Pmax of 491.23 in table 2, and since this 3-digit whole number is a large proportion of Pmax but not 300, mortality was assumed to have occurred at 0.1 ppm in exposures less than 24 hours.

If the chemical produced no mortality at 0.1 ppm, Pr becomes the sum of potencies at 1.0 and 10 ppm divided by log N, or 45.07/1.1761, which equals 38.32. This 2-digit whole-number Pr indicates mortality at 1.0 ppm in the shorter exposures but not complete mortality in 3 hours, since the maximum in this case is 57.25/1.1761 or 48.68. The 2-digit whole-number value also indicates no mortality at 0.1 ppm.

TABLE 3.--Potency rating determined from mortality among 10 fish

Exposure	Mortality at concentrations (ppm) of--			Contributions to Pr at concentrations (ppm) of--			Sum
	0.1	1.0	10.0	0.1	1.0	10.0	
3 hours....	0	5	8	0.00	10.48	1.68	12.16
6 hours....	5	9	10	64.25	11.57	1.29	77.11
24 hours....	9	10	10	65.21	7.25	0.72	73.18
48 hours....	10	10	10	59.48	5.95	0.59	66.02
96 hours....	10	10	10	50.45	5.04	0.50	55.99

Since the concentrations are in multiples of 10, the contribution to Pr can be calculated for one concentration, such as 0.1 ppm, for total mortality among the 10 fish. These figures can then be used for the contributions to Pr at 1.0 and 10 ppm by multiplying them by 0.1 and 0.01 and rounding off to two decimals. Once the values for mortalities are established, the procedure becomes simple and efficient.

DISCUSSION AND CONCLUSIONS

The potency rating concept is more applicable to data derived from toxicants similar in nature or ones with similar modes of action. The concept is therefore limited to materials producing dose-effect curves similar in slope. Chemicals which produce rapid effects cannot be compared accurately with ones which require longer time for results.

The potency rating concept is especially applicable to data such as those reported by Walker, Starkey, and Marking (1966) and Marking and Willford (1970). The compounds in these investigations are related to structure and activity against fish and produce similar slopes on dose-effect curves.

The formula and concept are general and can be used to include different concentrations and time exposures depending on the program. In each case, the maximum potency must be calculated and the Pr values compared with it. If larger or smaller figures are sought, the values can be multiplied by a constant to yield the desired number of digits.

Pmax is uniform at all exposure times (table 2), but Pr changes to some extent with exposure time. Pr increases with exposure time when the mortality is delayed, but the increase is much less than the additive effects of the potencies at the different exposures. If mortality occurs at 3 hours, the Pr values approximate more closely the uniformity of Pmax found in table 2.

Equal numbers of test animals must be used in trials in order to compare Pr values with a single maximum potency. Data containing

different numbers of test animals must be grouped and compared with a maximum potency for each group.

SUMMARY

Potency ratings effectively estimate the toxicity of chemicals to organisms from preliminary data which otherwise are often considered inadequate. The concept is especially useful in comparing results of numerous toxins tested against a wide variety of organisms. The values derived indicate the general and differential toxicity to the species tested.

The formula is versatile in that different concentrations and exposures can be selected to better fit a specific testing program. Mortality is a function of concentration and time, and the potency rating does not change in relation to the maximum potency if different concentrations or time intervals are selected. Data from 24- or 48-hour tests can be compared with those from 96-hour tests since the maximum potency is approximately equal for the three exposure times.

Shorter exposures and lower concentrations are emphasized in the formula by reciprocating the concentrations and the log of the time exposures. The effect is greater Pr's for highly toxic materials which kill organisms rapidly.

The potency rating concept is more applicable to data obtained from chemicals producing similar dose-effect curves. Chemicals of similar structure and chemicals which produce similar response to organisms can be readily evaluated and compared.

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Pr contributions at 0.1, 1.0, and 10.0 ppm for 10 test organisms

Exposure and concentration	Pr contributions calculated from death of--									
	1	2	3	4	5	6	7	8	9	10
3 hours:										
0.1 ppm.....	20.96	42.92	62.88	83.84	104.80	125.76	146.72	167.68	188.64	209.60
1.0 ppm.....	2.10	4.29	6.29	8.38	10.48	12.58	14.67	16.77	18.86	20.96
10.0 ppm.....	0.21	0.43	0.63	0.84	1.05	1.26	1.47	1.68	1.89	2.10
6 hours:										
0.1 ppm.....	12.85	25.70	38.55	51.40	64.25	77.10	89.95	102.80	115.65	128.50
1.0 ppm.....	1.29	2.57	3.86	5.14	6.43	7.71	9.00	10.28	11.57	12.85
10.0 ppm.....	0.13	0.26	0.39	0.51	0.64	0.77	0.90	1.03	1.16	1.29
24 hours:										
0.1 ppm.....	7.25	14.49	21.74	28.98	36.23	43.47	50.72	57.96	65.21	72.45
1.0 ppm.....	0.73	1.45	2.17	2.90	3.62	4.35	5.07	5.80	6.52	7.25
10.0 ppm.....	0.07	0.15	0.22	0.29	0.36	0.44	0.51	0.58	0.65	0.73
48 hours:										
0.1 ppm.....	5.95	11.90	17.84	23.79	29.74	35.69	41.64	47.58	53.53	59.48
1.0 ppm.....	0.60	1.19	1.78	2.38	2.97	3.57	4.16	4.76	5.35	5.95
10.0 ppm.....	0.06	0.12	0.18	0.24	0.30	0.36	0.42	0.48	0.54	0.60
96 hours:										
0.1 ppm.....	5.05	10.09	15.14	20.18	25.23	30.27	35.32	40.36	45.43	50.45
1.0 ppm.....	0.51	1.01	1.51	2.02	2.52	3.03	3.53	4.04	4.54	5.05
10.0 ppm.....	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.51

INVESTIGATIONS IN FISH CONTROL

37. Comparative Toxicity of 29 Nitrosalicylanilides and Related Compounds to Eight Species of Fish

By Leif L. Marking and Wayne A. Willford

UNITED STATES DEPARTMENT OF THE INTERIOR, WALTER J. HICKEL, *SECRETARY*
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CONTENTS

COMPARATIVE TOXICITY OF 29 NITROSALICYLANILIDES AND RELATED COMPOUNDS TO EIGHT SPECIES OF FISH

By Leif L. Marking and Wayne A. Willford, Chemists
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ABSTRACT.--The relative potencies of 29 nitrosalicylanilides and related structures against rainbow trout, goldfish, carp, fathead minnows, black bullheads, green sunfish, bluegills, and yellow perch were determined in 96-hour static bioassays. They varied from zero to the maximum potency of the system, depending on the type and position of substitutions. The 4'- substitutions on 3-nitrosalicylanilide are essential in producing high toxicity and selectivity to fish. The toxic activity of nitrosalicylanilides increases as halogen substitutions are shifted from the 2' to the 3' and to the 4' positions on the aniline moiety. Activity increases as molecular weight of the substituent increases. 4'-azophenyl-3-nitrosalicylanilide and 4'-iodo-3-nitrosalicylanilide are more toxic than 4'-bromo- or 4'-chloro-3-nitrosalicylanilide. The dihalo-substituted 3-nitrosalicylanilides are more toxic than monohalo-substituted 3-nitrosalicylanilides provided one of the halo- substitutions is at the 4'- position on the aniline moiety. 4'-bromo-3-nitrosalicylanilide is more toxic to carp, fathead minnows, bluegills, and yellow perch than to rainbow trout. Several compounds are selective to yellow perch. Goldfish are the most resistant species to the salicylanilides.

The nitrosalicylanilides are among the more biologically active chemicals and are under intensive investigation by scientists in many fields. Gönnert (1962) and Schräufstatter (1962) defined the molluscicidal activity of salicylanilides and reported reduction of Schistosomiasis through control of the vector snail. Vinson, Dineen, and Schneider (1961) point out that salicylanilides are efficacious as hospital detergent sanitizers. Molnar and Baron (1964) stated that certain polybrominated salicylanilides possess the desirable properties of germicides to the maximum extent. Molnar (1965) reported synergistic activity against bacteria when two polybrominated salicylanilides were combined.

The salicylanilide structure is also of interest in sea lamprey control. Bayluscide, chemically known as 2',5-dichloro-4'-nitrosalicylanilide and better known as Bayer 73, is presently used to synergize 3-trifluoromethyl-4-nitrophenol (TFM) (Howell et al., 1964). Bayer 73 is highly toxic to fish, and Marking and Hogan (1967) reported 96-hour LC50's of 0.043 to 0.230 ppm. They found that the toxicity of the compound is reduced in waters of extremely high or low pH, but it is not reduced or enhanced greatly by water temperatures between 7° and 22° C.

Starkey and Howell (1965) investigated the activity of a number of salicylanilides and

related compounds against rainbow trout and larval sea lampreys and reported greater toxicity to larval lampreys in many instances.

The biological activity of salicylanilides may be enhanced or diminished by specific substitution on the molecule. Taborsky, Darker, and Kaye (1959) and Taborsky and Starkey (1962, 1963) demonstrated various antimicrobial activities of salicylanilides with selected halogens at certain positions.

Walker, Starkey, and Marking (1966) showed that the piscicidal activity of salicylanilides and related compounds is governed by the nature and location of substitutions on the molecule. The halo- substitutions on the nitrosalicylanilides, in particular, had greater influence on toxicities to fish than other substituents.

Twenty-nine nitrosalicylanilides and related structures, many having halo- substitutions at selected positions, were obtained for preliminary bioassays against eight species of fish. These tests, conducted at the Fish Control Laboratories at La Crosse, Wis. and Warm Springs, Ga., in facilities described by Lennon and Walker (1964), provide comparative data on the toxicity of the chemicals to selected freshwater fish.

MATERIALS AND METHODS

The nitrosalicylanilides and related structures were furnished by Ben Venue Laboratories, Bedford, Ohio. Each structure was chosen according to its toxicity determined previously on rainbow trout and goldfish. The general structure of the compounds is illustrated in figure 1.

The fish were obtained from National Fish Hatcheries (table 1). They were introduced to the bioassay following a routine procedure of 10-day quarantine, removal from feed, and acclimation to water chemistry and temperature as outlined by Lennon and Walker (1964).

The tests were conducted in 15 liters of standard bioassay medium at 12° C. at La Crosse and at 17° C. at Warm Springs.

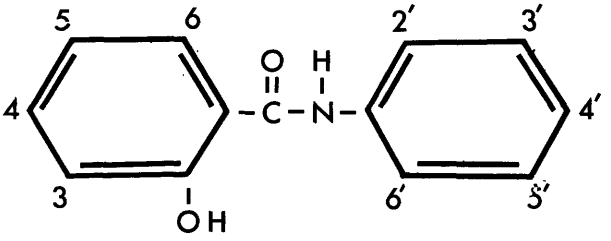

Figure 1.--Basic structure of salicylanilides. Benzanilides lack the OH^- at the 2 position.

TABLE 1.--Sizes and sources of test fishes

Species and lot	Average length (inches)	Average weight (grams)	Source
Rainbow trout (Salmo gairdneri):			
Lot 17	2.0	1.2	Manchester NFH, Iowa
Lot 180	1.2	0.2	La Crosse FCL, Wis.
Lot 192	1.9	1.1	Manchester NFH, Iowa
Lot 287	1.9	1.1	Do.
Goldfish (Carassius auratus):			
Lot 19	--	2.4	Lake Mills NFH, Wis.
Lot W54	1.6	1.1	Marion NFH, Ala.
Lot 245	1.7	2.3	Lake Mills NFH, Wis.
Carp (Cyprinus carpio):			
Lot W56	1.3	0.7	Marion NFH, Ala.
Lot W76	1.5	0.6	Do.
Lot 148a	1.7	1.0	Lake Mills NFH, Wis.
Lot 289	1.6	0.8	Do.
Fathead minnow (Pimephales promelas):			
Lot 257	1.6	0.7	Do.
Lot 296	1.8	1.0	Do.
Black bullhead (Ictalurus melas):			
Lot 153	2.0	1.6	Necedah NWR, Wis.
Lot 237	1.3	0.5	Genoa NFH, Wis.
Lot 275	2.1	2.0	Guttenberg NFH, Iowa
Lot 290	2.2	2.2	Do.
Green sunfish (Lepomis cyanellus):			
Lot 145	1.4	0.8	Lake Mills NFH, Wis.
Lot 190	1.7	1.7	Do.
Lot 251	1.5	1.1	Do.
Lot 272	1.5	0.8	Do.
Bluegill (Lepomis macrochirus):			
Lot W84	1.5	0.9	Marion NFH, Ala.
Lot 266	1.2	0.4	Lake Mills NFH, Wis.
Lot 288	1.5	0.9	Do.
Yellow perch (Perca flavescens):			
Lot 151	2.2	1.6	Do.
Lot 267	2.3	1.9	Do.

Ten specimens were exposed to each concentration of 0.1, 1.0, and 10.0 ppm of the candidate compounds. A stock solution of each compound, using acetone as the solvent, was prepared immediately before each test. Test concentrations of 0.1, 1.0, and 10.0 mg/l were obtained by adding an appropriate aliquot of a stock to 15 liters of reconstituted water. Observations on the effects of the compounds were recorded at 15 and 30 minutes and at 1, 3, 24, 48, 72, and 96 hours.

The data were analyzed according to the method of Marking (1970) in which the potency rating (Pr) is defined for each chemical to

each species of fish. The values derived indicate the toxicity as a proportion of the predetermined maximum potency with respect to concentrations and observation periods. Since the observations were taken at 3, 24, 48 and 96 hours in these experiments, the Pr is found as follows:

$$Pr = \frac{\sum_{j=3,24}^{48,96} \sum_{i=0.1,}^{1.0,10.0} \frac{M(C_i, T_j)}{C_i \log T_j}}{\log N}.$$

where M = mortality among 10 fish.
C = concentration in ppm.
T = time of exposure in hours, T>1.
N = number of observations, N>1.
j = finite variables of T.
i = finite variables of C.

When the individual concentrations of 0.1, 1.0, and 10.0 ppm at each observation period are considered, the formula becomes

$$Pr = \frac{\left(M \times \frac{1}{0.1} \times \frac{1}{\log 3}\right) + \left(M \times \frac{1}{1.0} \times \frac{1}{\log 3}\right)}{\log N} + \frac{\left(M \times \frac{1}{10} \times \frac{1}{\log 3}\right) + - - - \left(M \times \frac{1}{10} \times \frac{1}{\log 96}\right)}{\log N}.$$

Pmax is obtained by assuming that all of the fish died at each concentration and observation interval, and the values are given in table 2. Pmax is 403.16 in a 96-hour bioassay, and Pr values for experimental chemicals are compared with it. The number of digits in the Pr and size of the number effectively define the potency. Three-digit whole numbers indicate mortality at 0.1 ppm. Three-digit whole numbers over 200 indicate mortality at 0.1 ppm in 3 hours, and as Pr approaches the value of Pmax a greater number of fish die at this concentration and time interval.

Two-digit whole numbers indicate mortality at 1.0 ppm, and 1-digit whole numbers indicate mortality at 10 ppm. If none of the fish die at any concentration and time interval, Pr becomes zero.

TABLE 2.--Maximum potency at four time intervals

Time	log T	$\frac{1}{\log T}$	N	log N	At concentration (ppm) of--			Sum	Pmax
					0.1	1.0	10		
3 hours	0.4771	2.0960	3	0.4771	209.60	20.96	2.10	232.66	487.65
24 hours	1.3802	0.7245	6	0.7782	72.45	7.25	0.72	80.42	402.31
48 hours	1.6812	0.5948	9	0.9542	59.48	5.95	0.59	66.02	397.30
96 hours	1.9823	0.5045	12	1.0792	50.45	5.04	0.50	55.99	403.16
Total	--	--	--	--	--	--	--	435.09	--

RESULTS

The behavioral response of fish on contact with the nitrosalicylanilides is immediate at 10 ppm, and with some at 1.0 ppm. The response usually involves irritation, which is revealed by intermittent opercular flipping that simulates a coughing action. Swimming becomes irregular and is characterized by surfacing and diving. Loss of equilibrium and orientation are usually followed by a period of slow movement and quiescence. The opercular flipping becomes infrequent just before death.

The gill covers of dead fish are often distended, and a proliferation of mucuous is noticeable about the gill area. In some cases, long strings of mucous were observed in the anal area and on the ends of the ventral fins. Microscopic examination indicates that the bodies of the dead fish are devoid of mucous. Hemorrhage of the gills occurs frequently at the higher concentrations.

The iodo- substitutions on the nitrosalicylanilide structure (table 3, compounds 1, 2, 3, and 4) are toxic to all the species tested. The degree of toxicity is progressively enhanced for most species by moving the iodo- from the 2' to the 3', and from the 3' to the 4' positions. Also, the rate of toxic action is progressive. Survival is almost complete at 1.0 ppm in 3 hours with the 2'-iodo- substitution, whereas complete mortality occurs at this concentration and exposure with the 4'-iodo- substitution. By shifting the nitro-group from the 3 position, as in compounds 1 to 3, to the 5 position (compound 4) the general activity decreases.

TABLE 3.--The potency rating of 29 chemicals to fish in bioassays at 12° C.

[Rating calculated from data in the appendix]

Chemical	Rainbow trout	Gold-fish	Carp	Fathead minnow	Black bullhead	Green sunfish	Blue-gill	Yellow perch
(1) 2'-iodo-3-nitrosalicylanilide...	39.95	1.68	22.47	20.52	20.52	6.79	7.01	20.52
(2) 3'-iodo-3-nitrosalicylanilide...	39.37	19.61	4.09	26.35	39.95	20.52	20.52	73.75
(3) 4'-iodo-3-nitrosalicylanilide...	241.08	39.95	208.94	208.94	208.94	112.82	202.23	247.79
(4) 4'-iodo-5-nitrosalicylanilide...	21.80	19.84	19.18	20.52	20.52	13.81	20.52	92.23
(5) 2'-bromo-3-nitrosalicylanilide..	39.95	2.26	20.52	39.95	20.52	6.33	9.65	20.52
(6) 3'-bromo-3-nitrosalicylanilide..	39.95	10.22	38.00	39.95	39.95	13.73	20.52	56.85
(7) 4'-bromo-3-nitrosalicylanilide..	39.37	20.52	403.16	128.51	87.26	39.95	99.87	228.36
(8) 4',5-dibromo-3-nitrosalicyl-anilide.....	208.94	38.30	67.27	186.69	158.52	83.80	169.38	208.94
(9) 4'-bromo-2'-methyl-3-nitro-salicylanilide.....	67.03	26.19	190.00	85.46	39.95	110.18	25.19	202.23
(10) 2',5'-dibromo-3-nitrosalicyl-anilide.....	39.95	6.07	19.85	36.06	28.29	26.35	8.40	39.95
(11) 4'-chloro-3-nitrosalicylanilide.	48.18	41.53	44.30	104.90	58.65	44.62	31.82	73.75
(12) 2'-chloro-4'-methyl-3-nitro-salicylanilide.....	53.11	53.11	38.00	39.95	90.65	38.00	39.95	235.56
(13) 4'-chloro-2'-methyl-3-nitro-salicylanilide.....	39.37	19.61	133.39	45.41	39.95	22.47	20.20	175.14
(14) 2'-chloro-4'-nitro-3-nitro-salicylanilide.....	39.37	67.03	37.33	68.52	54.81	19.85	25.19	52.97
(15) 5'-chloro-2'-methoxy-3-nitro-salicylanilide.....	56.85	3.66	41.17	26.35	20.52	20.52	20.52	32.18
(16) 2',5'-dimethoxy-4'-chloro-3-nitrosalicylanilide.....	39.37	52.37	74.92	26.35	20.52	17.53	29.87	95.70
(17) 2'-chloro-5'-trifluoromethyl-3-nitrosalicylanilide.....	175.14	39.95	175.14	165.20	168.66	38.00	114.49	127.92
(18) 3',4'-dichloro-3-nitrosalicyl-anilide.....	53.11	42.22	38.00	148.53	208.94	30.24	149.72	209.42
(19) 4'-chloro-5-bromo-3-nitro-salicylanilide.....	208.94	29.88	173.27	202.23	63.32	116.87	152.79	228.36
(20) 2'-chloro-5-nitrosalicylanilide.	39.95	2.84	11.40	16.94	17.84	18.77	3.62	11.10
(21) 3'-chloro-5-nitrosalicylanilide.	15.94	1.68	10.67	--	3.62	5.02	3.62	19.18
(22) 4'-chloro-5-nitrosalicylanilide.	21.80	20.60	15.26	20.52	32.65	20.52	20.52	46.25
(23) 2'-methoxy-4'-nitro-3-nitro-salicylanilide.....	7.01	1.68	5.85	6.60	3.43	3.35	3.08	7.06
(24) 4'-methoxy-2'-nitro-3-nitro-salicylanilide.....	20.52	18.16	4.56	15.25	20.52	4.44	3.50	20.52
(25) 2',4'-dimethyl-3-nitrosalicyl-anilide.....	39.95	3.62	19.85	15.39	20.52	4.56	4.56	22.47
(26) 4'-azophenyl-3-nitrosalicyl-anilide.....	228.36	39.37	176.58	208.94	182.09	137.50	208.94	208.94
(27) 3'-chloro-3-nitrobenzanilide....	7.47	3.62	5.30	3.62	3.23	3.62	3.43	5.30
(28) 2',6'-diethyl-3,5-dinitrobenz-anilide.....	0	0	0	1.01	0	0	0	0
(29) 5-bromo-3-nitrosalicylic acid...	39.95	20.52	20.52	20.52	20.52	27.14	85.48	205.06

The 2'-, 3'-, and 4'-bromo- substitutions on the 3-nitrosalicylanilide structures (compounds 5, 6, and 7) are slightly less toxic than the equivalent iodo- substitutions with the exception of compound 7 to carp. Of the mono-bromo- substitutions, 4'-bromo-3-nitrosalicylanilide is the most toxic, killing all species at 1.0 ppm and all carp at 0.1 ppm in 3 hours. The Pr of this chemical to carp is 403.16 and equals the Pmax of the system. Its Pr values suggest specificity for carp. All carp and yellow perch and 20 percent of the fathead minnows were killed at 0.1 ppm within 24 hours, but no mortality occurred

among the other species. At 96 hours, 90 percent of the fathead minnows and bluegills and 70 percent of the black bullheads died.

In most instances, the toxicity of the 4'-bromo- substitution is further enhanced by addition of a second bromo- on the acid portion of the molecule at the 5 position (compound 8). This congener produced 100-percent mortality in all species at 0.1 ppm within 96 hours. The rate of toxic action, however, decreases. This is evidenced by less mortality of fish at 1.0 ppm after 3 hours, and longer survival of carp at 0.1 ppm than with the mono- 4'-bromo- substitution (appendix).

Substitution of a methyl group in 2' position on 4'-bromo-3-nitrosalicylanilide does not increase the activity of the compound (compound 9). All carp and yellow perch, and 60 percent of the green sunfish died when exposed to 0.1 ppm for 96 hours (appendix). The 2',5'-dibromo- substitution (compound 10) produces approximately the same toxicity as the mono-, 2'-bromo- substitution (compound 5).

Corresponding chloro- substitutions on the 3-nitrosalicylanilide are usually less toxic than the bromo- or iodo- substitutions (compounds 11-19). This is illustrated with 4'- substitutions of the three halogens. Toxicity increases as the substitution is changed from chloro- to bromo- to iodo- (compounds 3, 7, and 11).

Methyl-, nitro-, methoxy-, or dimethoxy- substitutions on mono-chloro-3-nitrosalicylanilide fail to exhibit outstanding toxicity (compounds 11-16). However, the 2'-chloro-4'-methyl and 4'-chloro-2'-methyl structures are relatively toxic to yellow perch (compounds 12 and 13) and may have potential as selective toxicants. The Pr of cpd 12 is 235.56 and is 2 to 6 times as great as the Pr values for other species.

The 5'-trifluoromethyl substitution (compound 17) on 2'-chloro-3-nitrosalicylanilide is more toxic than mono-substituted 4'-chloro-3-nitrosalicylanilide to six of the eight species.

The 3'-chloro- or 5-bromo- substitutions (compounds 18 and 19) on 4'-chloro-3-nitrosalicylanilide also enhance the general toxicity of the molecule. The dichloro- substitution, however, failed to kill carp or green sunfish at 0.1 ppm, while the bromo- substitution killed all carp and green sunfish at 0.1 ppm in 96 hours. Both compounds killed all of the fathead minnows, bluegills, and yellow perch at 0.1 ppm in 96 hours (appendix).

The chloro-5-nitrosalicylanilides (compounds 20-22) are less toxic than the chloro-3-nitrosalicylanilides. Activity increases by relocating the chloro- from the 2' or the 3' to the 4' positions in much the same manner as demonstrated by the iodo- and bromo-3-nitrosalicylanilides.

Nonhalo- substitutions on the 3-nitrosalicylanilide produce variable results. The 2'-methoxy-4'-nitro- substitution (compound 23) appears less toxic than the 4'-methoxy-2'-nitro- and the 2',4'-dimethyl- substitutions (compounds 24 and 25). None of these are as toxic as mono- 4'-halo- substitutions.

Substitution of the larger azophenyl- radical at the 4' position (compound 26) produces greater toxicity than any of the nonhalo-, and in most cases greater than the halo- substitutions. All fish tested at 0.1 ppm died within 96 hours (appendix).

The 3'-chloro-3-nitrobenzanilide (compound 27) is far less toxic than the halo- substitutions on 3- or 5-nitrosalicylanilides. The 3'-chloro-3-nitrobenzanilide produces little mortality of any species at 1.0 ppm in 96 hours, and Pr values range from 3.23 to 7.47 for all species.

The toxicity of 2',6'-diethyl-3,5-dinitrobenzanilide (compound 28) is considerably less than that of 3'-chloro-3-nitrobenzanilide. This compound, containing multiple substitutions, fails to kill any species at 10.0 ppm in 96 hours, and consequently the Pr values are zero. One fathead minnow died unnaturally at 1.0 ppm in 48 hours, and this produces a Pr of 1.01 at 96 hours.

The 5-bromo-3-nitrosalicylic acid (compound 29), although composed of only a portion of the nitrosalicylanilide molecule, contains the vital hydroxy- in the 2 position and the nitro- in the 3 position. Its toxicity is approximately equal to or greater than that of the 2' and 3' bromo- or iodo- on 3-nitrosalicylanilides (compounds 1, 2, 5, and 6) but less than that of the 4' bromo- or iodo- (compounds 3 and 7).

DISCUSSION

The highly toxic nitrosalicylanilides are active in shorter periods than the tables indicate. In 15 to 30 minutes, 10 ppm of the 4'-halo-3-nitrosalicylanilides kill rainbow trout, fathead minnows, and yellow perch. The more potent 4'-azophenyl-3-nitrosalicylanilide kills all species tested including the relatively resistant carp, black bullheads, green sunfish, and bluegills in 30 minutes at 10 ppm. Several of these species are killed in one hour at 1.0 ppm. All of the nitrosalicylanilides at 10 ppm produce stress in trout and yellow perch within 15 minutes.

We found that the activity of the halo- substitutions increases as the atomic weight of the substituted halogen increases. This agrees with the results of Walker, Starkey, and Marking (1966). However, this order of activity does not correspond to that for fungi and bacteria. Taborsky and Starkey (1962) reported that 4'-chloro- and 4'-bromo- substitutions on the 3- and 5-nitrosalicylanilides are among the more active antimicrobials. They also observed that 4'-bromo-5-nitrosalicylanilide was a more potent antimicrobial than 4'-chloro-3-nitrosalicylanilide. Our data indicate that the 3-nitrosalicylanilides are more toxic to fish than 5-nitrosalicylanilides. The inverse antimicrobial activity of these compounds suggests their possible use in treating fish diseases of bacterial and fungal origin.

The 4'- position appears to be a key position on the molecule. The halo- substitutions at this position are toxic to all species tested. The azophenyl- substitution in this position enhances the compound even further and suggests that bulkier unsaturated substitutions may increase the activity.

The potency ratings indicate that several chemicals are selective to target species of fish. Compound 7 (4'-bromo-3-nitrosalicylanilide) appears more toxic to carp, fathead minnows, black bullheads, bluegills, and yellow perch than to rainbow trout. Compound 12 (2'-chloro-4'-methyl-3-nitrosalicylanilide) and compound 29 (5-bromo-3-nitrosalicylic acid) are considerably more toxic to yellow perch than to any of the other species tested.

CONCLUSIONS AND SUMMARY

Nitrosalicylanilides continue to be of interest in fish control. The versatility of the parent structure permits a wide variety of substitutions enabling a critical review of the structure-activity relationships. The potency ratings on the three chemicals tested against eight species of fish indicate desirable selective toxicities. Of the 29 chemicals, 28 were toxic to fish at concentrations of 10 ppm or less.

Nitrosalicylanilides are more toxic to the eight species of fish tested than nitrobenzanilides with respective substitutions. This may be attributed to the hydroxy- at the 2 position on the benzoic acid moiety of the nitrosalicylanilides. Also, the activity of nitrosalicylanilides increases as the nitro- is shifted from the 5 to the 3 position.

The activity of a structure increases as the halogen is shifted from the 2' to the 3' and to the 4' positions on the aniline moiety. In addition, activity increases as the molecular weight of the substituent increases. Greater toxicity is observed with the heavier azophenyl- substitution, followed by iodo-, bromo-, and chloro- in that order. The activity of 4'-bromo-3-nitrosalicylanilide is enhanced further with the addition of another bromo-, providing that a bromo- remains at the 4' position.

The 5-bromo-3-nitrosalicylic acid produces toxicity which is approximately equal to

or greater than that of 2' and 3' bromo- or iodo- substitutions on the 3-nitrosalicylanilide. This molecule, although smaller and simpler, contains the hydroxy- at the 2 position which is necessary to produce maximum biological activity.

The results indicate that 4' halo- and azophenyl- substitutions on 3-nitrosalicylanilide produce the greatest toxicity against fish. These compounds deserve further consideration as fish control agents.

The relative potency concept expedited the critical analysis of these preliminary data. It permitted rapid comparison to distinguish highly toxic from less toxic chemicals and aided in distinguishing selectivity.

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APPENDIX

Toxicity of 0.1, 1.0, and 10 ppm of experimental chemicals to fish at 12° C.

[Numbers indicate mortalities among 10 fish in each bioassay]

Chemical and exposure time	Rainbow trout			Goldfish			Carp			Fathead minnow			Black bullhead			Green sunfish			Bluegill			Yellow perch		
	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10
(1) 2'-iodo-3-nitrosalicylanilide:																								
3 hours	0	10	10	0	0	0	0	1	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10
24 hours	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	1	10	0	2	10	0	10	10
48 hours	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	2	10	0	2	10	0	10	10
96 hours	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	3	10	0	2	10	0	10	10
(2) 3'-iodo-3-nitrosalicylanilide:																								
3 hours	0	10	10	0	1	10	0	0	10	0	3	10	0	10	10	0	0	10	0	0	10	0	10	10
24 hours	0	10	10	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
48 hours	0	10	10	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
96 hours	--	--	--	--	--	--	0	1	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
(3) 4'-iodo-3-nitrosalicylanilide:																								
3 hours	2	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
24 hours	9	10	10	0	10	10	10	10	10	10	10	10	10	10	10	2	10	10	9	10	10	10	10	10
48 hours	10	10	10	0	10	10	10	10	10	10	10	10	10	10	10	4	10	10	10	10	10	10	10	10
96 hours	10	10	10	0	10	10	10	10	10	10	10	10	10	10	10	8	10	10	10	10	10	10	10	10
(4) 4'-iodo-5-nitrosalicylanilide:																								
3 hours	0	2	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	6	10
24 hours	0	10	10	0	5	10	0	8	10	0	10	10	0	10	10	0	0	10	0	10	10	3	10	10
48 hours	0	10	10	1	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	3	10	10
96 hours	--	--	--	--	--	--	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	5	10	10
(5) 2'-bromo-3-nitrosalicyl-anilide:																								
3 hours	0	10	10	0	0	3	0	0	10	0	10	10	0	0	10	0	0	10	0	0	10	0	0	10
24 hours	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	1	10	0	0	10	0	10	10
48 hours	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	2	10	0	5	10	0	10	10
96 hours	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	2	10	0	7	10	0	10	10
(6) 3'-bromo-3-nitrosalicyl-anilide:																								
3 hours	0	10	10	0	1	10	0	9	10	0	10	10	0	10	10	0	0	10	0	0	10	0	10	10
24 hours	0	10	10	0	1	10	0	10	10	0	10	10	0	10	10	0	4	10	0	10	10	1	10	10
48 hours	0	10	10	0	3	10	0	10	10	0	10	10	0	10	10	0	6	10	0	10	10	1	10	10
96 hours	0	10	10	0	5	10	0	10	10	0	10	10	0	10	10	0	9	10	0	10	10	1	10	10
(7) 4'-bromo-3-nitrosalicyl-anilide:																								
3 hours	0	10	10	0	0	10	10	10	10	0	10	10	0	9	10	0	10	10	0	5	10	1	10	10
24 hours	0	10	10	0	10	10	10	10	10	2	10	10	0	10	10	0	10	10	0	10	10	10	10	10
48 hours	0	10	10	0	10	10	10	10	10	6	10	10	3	10	10	0	10	10	5	10	10	10	10	10
96 hours	--	--	--	0	10	10	10	10	10	9	10	10	7	10	10	0	10	10	9	10	10	10	10	10
(8) 4'-bromo-5-bromo-3-nitro-salicylanilide:																								
3 hours	0	10	10	0	1	10	0	0	10	0	2	10	0	7	10	0	0	10	0	0	10	0	10	10
24 hours	10	10	10	0	10	10	0	10	10	9	10	10	5	10	10	0	10	10	7	10	10	10	10	10
48 hours	10	10	10	3	10	10	0	10	10	10	10	10	8	10	10	3	10	10	10	10	10	10	10	10
96 hours	10	10	10	--	--	--	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
(9) 4'-bromo-2'-methyl-3-nitro-salicylanilide:																								
3 hours	0	10	10	0	4	10	0	10	10	0	9	10	0	10	10	0	10	10	0	0	10	0	10	10
24 hours	2	10	10	0	10	10	8	10	10	1	10	10	0	10	10	3	10	10	0	10	10	9	10	10
48 hours	2	10	10	0	10	10	9	10	10	4	10	10	0	10	10	4	10	10	0	10	10	10	10	10
96 hours	--	--	--	--	--	--	10	10	10	4	10	10	0	10	10	6	10	10	1	10	10	10	10	10
(10) 2',5'-dibromo-3-nitrosalicyl-anilide:																								
3 hours	0	10	10	0	0	0	0	0	10	0	8	10	0	4	10	0	3	10	0	0	10	0	10	10
24 hours	0	10	10	0	2	10	0	9	10	0	10	10	0	10	10	0	10	10	0	2	10	0	10	10
48 hours	0	10	10	0	3	10	0	10	10	0	10	10	0	10	10	0	10	10	0	2	10	0	10	10
96 hours	0	10	10	0	3	10	0	10	10	0	10	10	0	10	10	0	10	10	0	5	10	0	10	10
(11) 4'-chloro-3-nitrosalicyl-anilide:																								
3 hours	0	9	10	0	6	10	0	7	10	0	10	10	0	10	10	0	10	10	0	1	10	0	10	10
24 hours	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	2	10	10
48 hours	1	10	10	0	10	10	1	10	10	5	10	10	0	10	10	0	10	10	0	10	10	2	10	10
96 hours	1	10	10	2	10	10	1	10	10	8	10	10	4	10	10	1	10	10	2	10	10	2	10	10
(12) 2'-chloro-4'-methyl-3-nitro-salicylanilide:																								
3 hours	0	10	10	0	10	10	0	9	10	0	10	10	0	10	10	0	9	10	0	10	10	2	10	10
24 hours	1	10	10	1	10	10	0	10	10	0	10	10	3	10	10	0	10	10	0	10	10	9	10	10
48 hours	1	10	10	1	10	10	0	10	10	0	10	10	3	10	10	0	10	10	0	10	10	9	10	10
96 hours	--	--	--	--	--	--	0	10	10	0	10	10	3	10	10	0	10	10	0	10	10	10	10	10
(13) 4'-chloro-2'-methyl-3-nitro-salicylanilide:																								
3 hours	0	10	10	0	1	10	0	8	10	0	8	10	0	10	10	0	1	10	0	0	--	0	10	10
24 hours	0	10	10	0	10	10	4	10	10	0	10	10	0	10	10	0	10	10	0	10	--	8	10	10
48 hours	0	10	10	0	10	10	6	10	10	0	10	10	0	10	10	0	10	10	0	10	--	8	10	10
96 hours	--	--	--	--	--	--	8	10	10	2	10	10	0	10	10	0	10	10	0	10	--	8	10	10
(14) 2'-chloro-4'-nitro-3-nitro-salicylanilide:																								
3 hours	0	10	10	0	0	10	0	1	10	0	7	10	0	10	10	0	0	10	0	0	10	0	8	10
24 hours	0	10	10	2	10	10	0	10	10	0	10	10	0	10	10	0	9	10	0	10	10	1	10	10
48 hours	0	10	10	2	10	10	1	10	10	2	10	10	1	10	10	0	10	10	0	10	10	1	10	10
96 hours	--	--	--	--	--	--	2	10	10	5	10	10	2	10	10	0	10	10	1	10	10	1	10	10

Toxicity of 0.1, 1.0, and 10 ppm of experimental chemicals to fish at 12° C.--Continued

Chemical and exposure time	Rainbow trout			Goldfish			Carp			Fathead minnow			Black bullhead			Green sunfish			Bluegill			Yellow perch		
	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10
(15) 5'-chloro-2'-methoxy-3-nitrosalicylanilide:																								
3 hours	0	10	10	0	0	5	0	1	10	0	3	10	0	0	10	0	0	10	0	0	10	0	6	10
24 hours	1	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10
48 hours	1	10	10	0	1	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10
96 hours	1	10	10	0	1	10	4	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10
(16) 2',5'-dimethoxy-4'-chloro-3-nitrosalicylanilide:																								
3 hours	0	10	10	1	1	10	0	6	10	0	3	10	0	0	10	0	0	5	0	0	10	0	6	10
24 hours	0	10	10	1	6	10	0	6	10	0	10	10	0	10	10	0	7	10	0	10	10	2	10	10
48 hours	0	10	10	1	10	10	4	10	10	0	10	10	0	10	10	0	10	10	0	10	10	4	10	10
96 hours	--	--	--	--	--	--	5	10	10	0	10	10	0	10	10	0	10	10	2	10	10	6	10	10
(17) 2'-chloro-5'-trifluoromethyl-3-nitrosalicylanilide:																								
3 hours	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	9	10	0	10	10	0	10	10
24 hours	8	10	10	0	10	10	8	10	10	5	10	10	4	10	10	0	10	10	2	10	10	4	10	10
48 hours	8	10	10	0	10	10	8	10	10	9	10	10	10	10	10	0	10	10	6	10	10	6	10	10
96 hours	8	10	10	0	10	10	8	10	10	9	10	10	10	10	10	0	10	10	6	10	10	6	10	10
(18) 3',4'-dichloro-3-nitrosalicylanilide:																								
3 hours	0	10	10	0	5	10	0	9	10	0	10	10	0	10	10	0	5	10	0	10	10	1	10	10
24 hours	1	10	10	1	10	10	0	10	10	1	10	10	10	10	10	0	10	10	2	10	10	8	10	10
48 hours	1	10	10	1	10	10	0	10	10	10	10	10	10	10	10	0	10	10	9	10	10	9	10	10
96 hours	--	--	--	--	--	--	0	10	10	10	10	10	10	10	10	0	10	10	10	10	10	10	10	10
(19) 4'-chloro-5-bromo-3-nitrosalicylanilide:																								
3 hours	0	10	10	0	0	10	0	2	10	0	10	10	0	10	10	0	0	10	0	2	10	1	10	10
24 hours	10	10	10	0	10	10	7	10	10	9	10	10	0	10	10	0	10	10	4	10	10	10	10	10
48 hours	10	10	10	2	10	10	10	10	10	10	10	10	0	10	10	9	10	10	10	10	10	10	10	10
96 hours	10	10	10	--	--	--	10	10	10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10
(20) 2'-chloro-5-nitrosalicylanilide:																								
3 hours	0	10	10	0	0	6	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	1	10
24 hours	0	10	10	0	0	10	0	4	10	0	7	10	0	6	10	1	0	10	0	0	10	0	3	10
48 hours	0	10	10	0	0	10	0	5	10	0	8	10	0	10	10	1	0	10	0	0	10	0	3	10
96 hours	0	10	10	0	0	10	0	5	10	0	9	10	0	10	10	1	0	10	0	0	10	0	4	10
(21) 3'-chloro-5-nitrosalicylanilide:																								
3 hours	0	0	10	0	0	0	0	0	10	--	--	--	0	0	10	0	0	10	0	0	10	0	0	10
24 hours	0	4	10	0	0	10	0	3	10	--	--	--	0	0	10	0	0	10	0	0	10	0	8	10
48 hours	0	9	10	0	0	10	0	4	10	--	--	--	0	0	10	0	0	10	0	0	10	0	10	10
96 hours	0	10	10	0	0	10	0	6	10	--	--	--	0	0	10	0	3	10	0	0	10	0	10	10
(22) 4'-chloro-5-nitrosalicylanilide:																								
3 hours	0	2	10	0	0	10	0	0	10	0	0	10	0	1	10	0	0	10	0	0	10	0	8	10
24 hours	0	10	10	0	6	10	0	3	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10
48 hours	0	10	10	1	10	10	0	9	10	0	10	10	1	10	10	0	10	10	0	10	10	1	10	10
96 hours	--	--	--	--	--	--	0	10	10	0	10	10	1	10	10	0	10	10	0	10	10	1	10	10
(23) 2'-methoxy-4'-nitro-3-nitrosalicylanilide:																								
3 hours	0	0	10	0	0	0	0	0	9	0	0	9	0	0	9	0	0	0	0	0	0	0	0	10
24 hours	0	2	10	0	0	10	0	0	10	0	1	10	0	0	10	0	1	10	0	0	10	0	0	10
48 hours	0	2	10	0	0	10	0	1	10	0	2	10	0	0	10	0	1	10	0	0	10	0	2	10
96 hours	0	2	10	0	0	10	0	4	10	0	3	10	0	0	10	0	1	10	0	3	10	0	5	10
(24) 4'-methoxy-2'-nitro-3-nitrosalicylanilide:																								
3 hours	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	9	0	0	7	0	0	10
24 hours	0	10	10	0	8	10	0	0	10	0	6	10	0	10	10	0	0	10	0	0	10	0	10	10
48 hours	0	10	10	0	9	10	0	0	10	0	7	10	0	10	10	0	1	10	0	0	10	0	10	10
96 hours	0	10	10	0	9	10	0	2	10	0	8	10	0	10	10	0	1	10	0	1	10	0	10	10
(25) 2',4'-dimethyl-3-nitrosalicylanilide:																								
3 hours	0	10	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	1	10
24 hours	0	10	10	0	0	10	0	9	10	0	4	10	0	10	10	0	0	10	0	0	10	0	10	10
48 hours	0	10	10	0	0	10	0	10	10	0	8	10	0	10	10	0	0	10	0	0	10	0	10	10
96 hours	0	10	10	0	0	10	0	10	10	0	10	10	0	10	10	0	2	10	0	2	10	0	10	10
(26) 4'-azophenyl-3-nitrosalicylanilide:																								
3 hours	1	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10
24 hours	10	10	10	0	10	10	6	10	10	10	10	10	6	10	10	1	10	10	10	10	10	10	10	10
48 hours	10	10	10	0	10	10	9	10	10	10	10	10	10	10	10	8	10	10	10	10	10	10	10	10
96 hours	10	10	10	--	--	--	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
(27) 3'-chloro-3-nitrobenzanilide:																								
3 hours	0	0	10	0	0	10	0	0	10	0	0	10	0	0	8	0	0	10	0	0	9	0	0	10
24 hours	0	2	10	0	0	10	0	1	10	0	0	10	0	0	10	0	0	10	0	0	10	0	1	10
48 hours	0	2	10	0	0	10	0	1	10	0	0	10	0	0	10	0	0	10	0	0	10	0	1	10
96 hours	0	3	10	0	0	10	0	1	10	0	0	10	0	0	10	0	0	10	0	0	10	0	1	10
(28) 2',6'-diethyl-3,5-dinitrobenzanilide:																								
3 hours	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24 hours	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
48 hours	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
96 hours	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
(29) 5-bromo-3-nitrosalicylic acid:																								
3 hours	0	10	10	0	0	10	0	0	10	0	0	10	0	0	10	0	1	10	0	0	10	0	8	10
24 hours	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	10	10	10
48 hours	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	5	10	10	10	10	10
96 hours	0	10	10	0	10	10	0	10	10	0	10	10	0	10	10	1	10	10	8	10	10	10	10	10

INVESTIGATIONS IN FISH CONTROL

38. Toxicity of 33NCS to Freshwater Fish and Sea Lampreys

By Leif L. Marking, Everett L. King,
Charles R. Walker, and John H. Howell

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CONTENTS

TOXICITY OF 33NCS (3'-CHLORO-3-NITROSALICYLANILIDE) TO FRESHWATER FISH AND SEA LAMPREY

By Leif L. Marking, Everett L. King,
Charles R. Walker, and John H. Howell

ABSTRACT.--The chemical 33NCS (3'-chloro-3-nitrosalicylanilide) was evaluated as a fish control agent and as a larvicide for sea lampreys at the Fish Control Laboratories of the Bureau of Sport Fisheries and Wildlife and the Hammond Bay Biological Station of the Bureau of Commercial Fisheries. The chemical is rapidly toxic to many species. Sea lampreys, bowfin, and channel catfish are the most sensitive species. Carp are more sensitive than trouts or sunfishes. Use of 33NCS in selective control of freshwater fishes or sea lampreys requires precise control because its toxicity is strongly influenced by variations in water quality.

The salicylanilides are of great interest in fishery research because of their promise as selective controls for freshwater fish and sea lamprey larvae (Walker, Starkey, and Marking, 1966; Starkey and Howell, 1966). Among them, 33NCS (3'-chloro-3-nitrosalicylanilide) showed the greatest potential for development as a sea lamprey larvicide during early testing at the Hammond Bay Biological Station. It also showed properties of interest during preliminary screening at the Fish Control Laboratories. A cooperative investigation was initiated to evaluate 33NCS as a selective toxicant under a variety of conditions.

The importance of studying the biological activity of a compound in different environments was demonstrated repeatedly during the development of the sea lamprey larvicide TFM (3-trifluoromethyl-4-nitrophenol) and its synergist, Bayer 73 (2',5-dichloro-4'-nitrosalicylanilide) (Applegate et al., 1961; Howell et al., 1964). Walker, Lennon, and Berger (1964) found that environmental conditions strongly influence the toxicity of antimycin. Strufe and Gönnert (1962) observed that the molluscicidal activity of Bayer 73 is affected by chemical and physical factors. Later, Fox, Ritchie, and Frick (1963) determined the effect of pH on the efficacy of Bayer 73 and postulated that the inconsistencies in published lethal concentrations are the result of differences in water quality.

Recognizing that the biological activity of 33NCS may be influenced by chemical or physical factors, our bioassays were conducted in reconstituted and natural waters of different qualities. A variety of organisms including invertebrates and fish were exposed to the toxicant in laboratory or simulated field tests.

MATERIALS AND METHODS

Pharmaceutical grade 33NCS was used in all tests. Organic solvents employed in formulating the toxicant were not in excess of amounts tolerated by the test animals.

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Everett L. King and John H. Howell are Fishery Biologists at the Hammond Bay Biological Station of the Bureau of Commercial Fisheries, Millersburg, Mich.

The common and scientific names of the six kinds of invertebrates and 30 species of fish used in the bioassays are given in table 1.

Fish Control Laboratories

The facilities and procedures for routine bioassays were described by Lennon and Walker (1964).

Reconstituted deionized water was used in most of the bioassays of 33NCS, and hard and soft water was formulated by varying the quantity of salts added (table 2). Desired pH levels were obtained with buffer systems of potassium hydrogen phthalate ($KHC_8H_4O_4$), boric acid (H_3BO_3), and sodium hydroxide (NaOH). The bioassay solutions were aerated prior to introduction of fish.

TABLE.1.--Common and scientific names of test organisms

Common name	Scientific name
Mollusks:	
Snail	Physa spp.
Freshwater clam	Elliptio spp.
Crustaceans:	
Crayfish	Cambarus spp.
Insects:	
Stonefly nymph	Togoperia spp.
Mayfly nymph	Hexagenia spp.
Dragonfly nymph	Ophiogomphus spp.
Fish:	
Sea lamprey	Petromyzon marinus
Bowfin	Amia calva
Rainbow trout	Salmo gairdneri
Brown trout	Salmo trutta
Brook trout	Salvelinus fontinalis
Lake trout	Salvelinus namaycush
Northern pike	Esox lucius
Goldfish	Carassius auratus
Carp	Cyprinus carpio
Lake chub	Hybopsis plumbea
Common shiner	Notropis cornutus
Sand shiner	Notropis stramineus
Fathead minnow	Pimephales promelas
Longnose dace	Rhinichthys cataractae
Longnose sucker	Catostomus catostomus
White sucker	Catostomus commersoni
Black bullhead	Ictalurus melas
Yellow bullhead	Ictalurus natalis
Brown bullhead	Ictalurus nebulosus
Channel catfish	Ictalurus punctatus
Trout-perch	Percopsis omiscomaycus
Rock bass	Ambloplites rupestris
Green sunfish	Lepomis cyanellus
Pumpkinseed	Lepomis gibbosus
Bluegill	Lepomis macrochirus
Smallmouth bass	Micropterus dolomieui
Largemouth bass	Micropterus salmoides
Yellow perch	Perca flavescens
Logperch	Percina caprodes
Walleye	Stizostedion vitreum

Most of the bioassays were carried out in 5-gallon glass jars containing 15 liters of water each. Bioassays with larger fish were made in plastic tanks which held up to 45 liters of water. The toxicant, 33NCS, was dissolved in acetone, and aliquots of stock solutions were pipetted directly into bioassay vessels to produce desired concentrations.

The fish in routine bioassays ranged from 1.0 to 2.5 inches in length and were obtained from National, State, and private fish hatcheries in the vicinity of La Crosse, Wis., and Warm Springs, Ga. Larger fish used in special tests were also obtained from these sources. All fish were held in quarantine for at least 10 days. Three days before testing, the fish were taken off feed and acclimated to the water chemistry and temperatures of the bioassay.

The preliminary bioassays took place in standard reconstituted water at concentrations of 0.1, 1.0, and 10 ppm of 33NCS and at 12° and 17° C. Ten fish of each species were exposed to each concentration and their responses were observed at 0.5, 1, 3, 6, 24, 48, 72, and 96 hours.

Delineative bioassays were performed at 7°, 12°, 17°, and 22° C. in waters of various qualities. Ten fish were exposed to each of ten concentrations. The results were used to delineate concentrations which produced 50-percent mortality (LC50 values) at selected observation periods. The LC50's, confidence

TABLE 2.--Quality and composition of reconstituted water used at the Fish Control Laboratories

Classification of water	Salt added in mg/l				pH range	Total hardness[1]	Total alkalinity[1]
	$NaHCO_3$	$CaSO_4$	$MgSO_4$	KCl			
Soft	12	7.5	7.5	0.75	6.4-6.8	10- 13	10- 13
Medium[2]	48	30.0	30.0	3.00	7.2-7.6	40- 48	30- 35
Hard	192	120.0	120.0	12.00	7.6-8.0	160-180	110-120

[1]As ppm $CaCO_3$.

[2]Standard reconstituted water used in routine bioassays.

intervals (C.I.), and slope functions were derived according to methods of Litchfield and Wilcoxon (1949).

Hammond Bay Biological Station

Closed system bioassays.--The water used in most tests and for holding test specimens was pumped directly from Lake Huron at Hammond Bay. The chemical and physical characteristics of the lake water fluctuate seasonally but are considered to be generally intermediate to the extremes found among tributaries of the upper Great Lakes: total alkalinity (expressed as ppm $CaCO_3$) is 85 to 118, pH is 7.4 to 8.3, and specific conductance is 148 to 203 micromhos at 18° C.

Special tests were made to evaluate the effect of water quality on the toxic action of 33NCS, with natural water from representative tributaries in which sea lampreys spawn.

In standard bioassays, 5 liters of water were added to 10-liter glass battery jars which were then placed in constant-temperature troughs maintained at 12.8° C. The troughs were similar to those described by Lagler (1953). The effect of temperature was determined by tests at 1.7°, 7.2°, 12.8°, 18.3°, and 23.9° C.

Two larval sea lampreys 3.5 to 5.0 inches long and two rainbow trout 3.0 to 5.0 inches long were placed in each jar and were acclimated for 1 to 2 hours before introduction of the toxicant. At least four replications were made simultaneously at each concentration.

Aliquots of a 5-percent stock solution of 33NCS in dimethyl formamide (DMF) were diluted to 1 liter and added to the test jars to establish the final 6-liter test volume and desired concentrations. All test solutions were aerated to maintain oxygen at or near saturation.

Bioassays provided data on minimum lethal (MLC100) and maximum allowable (MAC25) concentrations (Howell and Marquette, 1962). The MLC100 is the lowest concentration which produces a complete kill of larval lampreys. The highest concentration causing no greater than a 25-percent kill among other test fish is the MAC25. The working range of 33NCS falls between these values. The ratio of working range to the MLC100 gives the permissible additional flow (PAF) and represents the amount of dilution before the MAC25 will be reduced to the MLC100. In sea lamprey control applications, a PAF less than 1 is considered to limit utility (Howell and Marquette, 1962).

Hourly observations on specimens were recorded through the first 12 hours and at the end of each assay period, usually 21 to 24 hours. The bioassays at Hammond Bay were not conducted for longer periods, since the period of exposure in sea lamprey control is seldom greater than 24 hours.

Flowing water bioassay.--Experiments with 33NCS were conducted in raceways with flowing water to simulate natural stream conditions. These also permitted the simultaneous exposure of a variety of aquatic species and larger fish than could be accommodated in the laboratory. Simulated stream tests also provided information on the physical properties of 33NCS when applied to flowing water, the type of formulations required, and the adequacy of the chemical metering devices for stream application.

Raceway tests were made in the early fall of 1964 and again in the late spring of 1965. Chemical and physical analyses of the water were made daily during the two test periods. Ranges of values for the 1964 tests were as follows: pH, 7.8 to 8.3; conductivity, 176 to 197 micromhos; total alkalinity, 97 to 106 ppm; and water temperature 5.5° to 11.1° (mean 8.2° C.). The 1965 test characteristics were: pH, 7.6 to 8.2; conductivity, 127 to 180 micromhos; total alkalinity, 96 to 108 ppm, and water temperature, between 11.1° and 19.4° C. (mean 15.6°).

The concrete raceways were 65 feet long, 6 feet wide and 30 inches deep. Water from Lake Huron was delivered to them from a surge tank which stabilized the flow. The flow was held constant at 0.34 cfs by a "V" weir. Water depth throughout the length of the raceway was maintained at 14 inches by a stopboard.

Trout were provided by State Fish Hatcheries (Michigan Department of Conservation) at Grayling and Oden, Mich., except for one lot of brown trout which was obtained in the fall of 1964 from the Rifle River, Ogemaw County, Mich. Most of the other specimens used in the raceway tests were collected from the Ocqueoc River and Bullhead and Lost lakes, Presque Isle County, Mich. Several species were from the Cheboygan River, Cheboygan County, and from a commercial bait dealer at Traverse City, Mich. All specimens were held in flowing Lake Huron water for at least 7 days before exposure to 33NCS.

Larger fish were allowed to move freely within the raceway. Smaller specimens were confined in cylindrical screen cages. Larval lampreys were placed in cages containing 3 to 5 inches of clean beach sand at least 24 hours before each test to allow sufficient time for them to establish burrows. The confinement of larval lampreys and the other smaller specimens in cages facilitated rapid and accurate observations of mortality and prevented predation by larger fish.

Stock solutions of 33NCS were prepared using DMF as the solvent. An electric fuel pump was used to apply solutions of various strengths depending on the concentration desired (Anderson, 1962).

RESULTS

Fish Control Laboratories

Preliminary investigations.--The results of early trials with 33NCS indicate a high degree of toxicity to the fish at 12° and 17° C. (table 3). The fish respond to the toxicant immediately after its introduction into the water. At lower concentrations they become mottled in appearance, are quiescent, and swim slowly to the surface or bottom. At higher concentrations they become disoriented, irritated, and excited. Erratic swimming and surfacing occurs within 15 minutes at less than 1 ppm. Close examination shows that the entire integument is devoid of mucous. Respiration is usually rapid and

TABLE 3.--Toxicity of 0.1, 1.0, and 10.0 ppm of 33NCS to fish at two temperatures

[expressed as the number of mortalities in a bioassay of 10 fish]

Temperature and species	Average length (in.)	Mortality at--											
		3 hours			24 hours			48 hours			96 hours		
		0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10
At 12° C.													
Bowfin	2.2	0	10	10	10	10	10	10	10	10	10	10	10
Rainbow trout	1.2	0	10	10	0	10	10	0	10	10	0	10	10
Brown trout	1.9	0	10	10	0	10	10	0	10	10	0	10	10
Lake trout	2.1	0	9	10	0	10	10	0	10	10	0	10	10
Northern pike	2.1	0	10	10	0	10	10	--	--	--	--	--	--
Goldfish	1.8	0	0	10	0	9	10	0	9	10	0	10	10
Carp	1.5	0	9	10	7	10	10	7	10	10	8	10	10
Fathead minnow	1.6	0	2	10	0	10	10	0	10	10	0	10	10
Black bullhead	1.3	0	8	10	0	10	10	0	10	10	0	10	10
Channel catfish	1.7	0	10	10	10	10	10	10	10	10	10	10	10
Green sunfish	1.5	0	0	10	0	10	10	0	10	10	1	10	10
Bluegill	1.0	1	1	10	1	10	10	1	10	10	3	10	10
Yellow perch	2.0	0	10	10	8	10	10	8	10	10	9	10	10
Walleye	1.3	0	10	10	5	10	10	8	10	10	10	10	10
At 17° C.													
Goldfish	1.6	1	0	10	1	3	10	1	7	10	1	7	10
Carp	1.2	0	2	10	0	9	10	0	10	10	0	10	10
Fathead minnow	2.1	0	0	10	0	10	10	0	10	10	0	10	10
Green sunfish	1.9	0	3	10	0	9	10	0	10	10	0	10	10
Smallmouth bass	2.0	0	9	10	0	10	10	0	10	10	0	10	10

irregular, and the operculums are distended and retracted abruptly in a coughlike action in all species. The respiratory rate slows preceding death, accompanied by spastic gulps, twitching, and tetany. The response to touch and sound progressively decreases and the fish soon lose equilibrium. Death is accompanied also by the occurrence of mucous about the gills and anus. The fish have open mouths and extended operculums at the time of death.

33NCS is rapidly as well as highly toxic to fish. Ten ppm kills all species in 3 hours. One ppm kills all bowfin, rainbow trout, brown trout, northern pike, channel catfish, yellow perch, and walleye within 3 hours, and it produces partial mortality among lake trout, carp, fathead minnow, black bullhead, green sunfish, bluegill, and smallmouth bass within the same time (table 3). Goldfish, carp, and green sunfish are the only species among which mortality was not complete at 1.0 ppm in 24 hours. Even 0.1 ppm was highly toxic, and bowfin, channel catfish, and walleye died within 96 hours.

Delineative investigations.--The LC50's of 33NCS for 11 species of fish range from 0.068 to 0.830 ppm in 96 hours at 12° C. (table 4). Channel catfish are by far the most sensitive species. Carp, yellow perch, brown trout, lake trout, rainbow trout, fathead minnow, and black bullhead are intermediate in sensitivity to 33NCS. Goldfish, green sunfish, and bluegill are more resistant.

33NCS is unique because it kills most species in a matter of minutes. Statistical expressions of LC50 are possible in 1 to 6 hours at concentrations only slightly higher than those required to kill all fish in 24 hours. Also, concentrations necessary to produce mortality are within a very narrow range at each time interval. Minute increments in concentrations delineate all-or-none effects as denoted by extremely low slope functions. This is illustrated by a very low mean slope function which ranges from 1.13 to 1.67 (table 4). In contrast, Marking (1966) found that the slope function for goldfish exposed to p,p'-DDT was 6.02.

The trout respond considerably faster to 33NCS than warmwater species, and LC50 values were established in a 1-hour exposure (fig. 1). Higher concentrations are required to kill warmwater fish in short-term exposures. For instance, it requires less than twice as much 33NCS to kill rainbow trout in 3 hours

TABLE 4.--Toxicity of 33NCS for selected fishes at 12° C.

Species	Average length (inches)	At 24 hours		At 48 hours		At 96 hours		Mean slope function
		LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.	
Rainbow trout.....	1.9	0.233	0.216-0.252	0.203	0.190-0.217	0.203	0.190-0.217	1.13
Do.....	1.9	0.300	0.275-0.327	0.260	0.245-0.276	0.240	0.214-0.269	1.15
Brown trout.....	3.3	0.173	0.142-0.210	0.167	-- --	0.167	-- --	1.25
Lake trout.....	2.1	0.325	0.304-0.348	0.207	0.190-0.226	0.200	0.176-0.228	1.19
Goldfish.....	1.8	1.400	1.240-1.580	1.030	0.840-1.270	0.830	0.690-1.000	1.47
Carp.....	1.5	0.155	0.119-0.202	0.130	0.104-0.162	0.123	0.098-0.154	1.67
Fathead minnow.....	1.6	0.385	0.324-0.458	0.250	0.172-0.362	0.245	0.194-0.309	1.40
Black bullhead.....	2.4	0.440	0.350-0.550	0.255	0.203-0.307	0.255	0.203-0.307	1.39
Do.....	1.3	0.380	0.339-0.426	0.328	0.285-0.377	0.260	-- --	1.19
Channel catfish....	1.7	0.085	0.075-0.096	0.068	0.050-0.071	0.068	0.050-0.071	1.27
Green sunfish.....	1.5	0.707	0.637-0.785	0.600	0.484-0.737	0.490	0.422-0.568	1.28
Do.....	1.7	0.840	0.750-0.900	0.500	0.465-0.537	0.320	0.270-0.380	1.16
Bluegill.....	1.3	0.625	0.521-0.750	0.530	0.465-0.604	0.428	0.382-0.479	1.42
Yellow perch.....	2.2	0.195	0.156-0.244	0.172	0.146-0.203	0.155	0.135-0.178	1.39

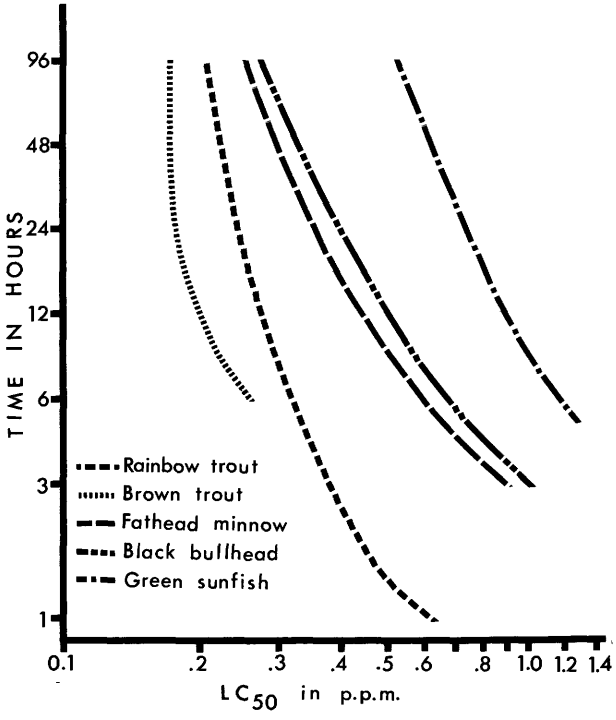

Figure 1.--Rate of response for several species of fish to 33NCS.

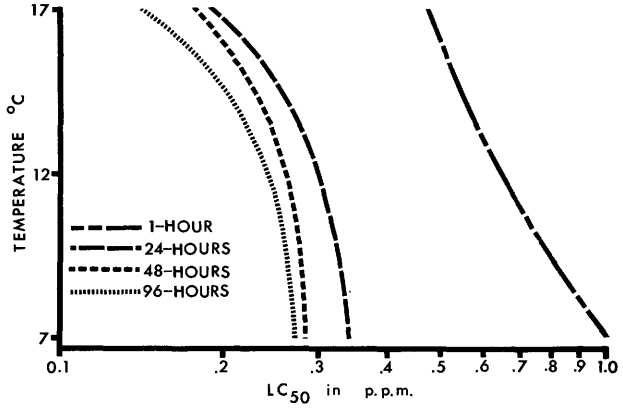

Figure 2.--Effects of temperature on the toxicity of 33NCS to rainbow trout.

as in 96 hours whereas it takes three times as much or more for carp, fathead minnow, black bullhead, and channel catfish.

Effects of temperature.--The 1-hour LC50 for rainbow trout at 7° is 2.5 times that at 17° C. (fig. 2 and table 5); at 24 and 96 hours, the LC50's at 7° are less than twice those at 17°. Although the same trend prevails with green sunfish, the difference in LC50 values for 12°, 17°, and 22° is not as great. There is, however, a very similar decline in the toxic concentrations calculated at 1 to 5 hours as opposed to the 24- and 96-hour values. The data also indicate that 33NCS is more effective at warmer temperatures, and much less material is required to produce lethal effects. Examination of the slope functions at all temperatures reveals consistent values, and the narrow range of concentrations necessary to permit survival and produce mortality (all-or-none effect) prevails at all temperatures.

Effects of water quality.--The toxicity of 33NCS to rainbow trout is much greater in soft water than in hard water (table 6). The LC50's in hard water are approximately 5 times as great as those for soft water at 6, 24, 48, and 96 hours. The LC50's in water of medium hardness are about twice those for soft water. The range of pH is 6.4-6.8 for soft water and 7.6-8.0 for the hard water. Thus, pH and water hardness influence toxicity of 33NCS.

The toxicity of 33NCS to rainbow trout at all water hardnesses manifests itself within a short period of time. The LC50's at 96 hours are only slightly less than those at 6 hours. Decreases in LC50 values of 28, 19, and 22 percent are noted for soft, medium, and hard water respectively.

Recognizing the variable responses in waters of different pH and hardness, we sought to identify the constituents of the reconstituted water which may affect toxicity. The toxicity of 33NCS to brook trout is considerably less when four times as much sodium bicarbonate is added to the reconstituted formula (table 7). Four times the regular amounts of potassium chloride, magnesium sulfate, or calcium sulfate had no appreciable effect on toxicity of the chemical. The addition of more sodium bicarbonate increases the pH from 7.2 to 8.2, whereas adding more of the other constituents has less effect on pH. Further, the slight increase in pH from 7.7 to 8.2 accounts for complete survival of fish at 0.5 ppm.

The specific effect of pH is demonstrated by using buffer systems at pH 5.8 and 8.5 with the standard reconstituted water (table 8). We

TABLE 5.--The toxicity of 33NCS to fish at selected temperatures

Species and exposure time	At 7° C.		At 12° C.		At 17° C.		At 22° C.	
	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.
Rainbow trout:								
1 hour.....	1.080	-- --	0.642	0.500-0.686	0.380	0.331-0.437	--	-- --
24 hours.....	0.331	0.302-0.360	0.300	0.275-0.327	0.195	0.177-0.214	--	-- --
48 hours.....	0.281	0.264-0.296	0.260	0.245-0.276	0.180	0.172-0.187	--	-- --
96 hours.....	0.275	0.262-0.288	0.240	0.214-0.269	0.142	0.123-0.163	--	-- --
Green sunfish:								
5 hours.....	--	-- --	1.220	-- --	0.870	0.820-0.920	0.715	0.660-0.775
24 hours.....	--	-- --	0.730	0.665-0.800	0.710	0.635-0.795	0.600	0.550-0.655
48 hours.....	--	-- --	0.610	0.565-0.660	0.500	0.437-0.634	0.445	0.364-0.542
96 hours.....	--	-- --	0.511	0.464-0.552	0.361	0.302-0.432	0.271	0.208-0.352

TABLE 6.--The toxicity of 33NCS to rainbow trout in various water qualities at 12° C.

Water hardness	At 6 hours		At 24 hours		At 48 hours		At 96 hours	
	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.
Soft.....	0.118	0.092-0.152	0.104	0.078-0.138	0.102	0.083-0.125	0.092	0.069-0.123
Medium.....	0.250	0.242-0.258	0.233	0.216-0.252	0.203	0.190-0.217	0.203	0.190-0.217
Hard.....	0.580	0.560-0.603	0.520	0.481-0.562	0.500	0.463-0.540	0.420	0.385-0.458

TABLE 7.--The toxicity of 0.5 ppm of 33NCS to 7.5-inch brook trout in selected water qualities

[Ten fish per test in 45-liter tanks with aeration]

Observation time	Percent mortality				
	pH 7.2 (standard bioassay water)	pH 7.5 (4 times KCl)	pH 7.6 (4 times $MgSO_4$)	pH 7.7 (4 times $CaSO_4$)	pH 8.2 (4 times $NaHCO_3$)
1 hour.....	30	10	0	20	0
3 hours.....	30	20	20	30	0
6 hours.....	40	40	40	60	0
24 hours....	40	40	40	60	0
96 hours....	40	40	40	60	0

TABLE 8.--Mortality of 2.5-inch rainbow trout resulting from exposure to 33NCS at selected pH values

pH	Buffer applied	33NCS (ppm)	Percent mortality at given time
5.8.....	KHP - NaOH	0.33	100 at 30 minutes
5.8.....	KHP - NaOH	0.35	100 at 15 minutes
7.4.....	None	0.35	100 at 1 to 24 hours
8.5.....	NaOH - H_3BO_3	0.35	None at 96 hours
8.5.....	NaOH - H_3BO_3	0.37	30 at 96 hours

observed that 0.35 ppm of 33NCS killed all fish within 15 minutes at pH 5.8; it killed all fish in 24 hours at pH 7.4; but none died in 96 hours at pH 8.5. Thus, pH governs effectiveness and rate at which the chemical kills fish.

Brook trout which averaged 7.2 inches long were exposed in aerated vessels containing 45 liters of test solution. Erratic results such as mortality at lower concentrations and survival at higher concentrations suggested inconsistent test media. At 0.5 ppm all of the trout were dead in 1 hour, whereas 30 percent of the fish survived exposure to 0.6 ppm for 96 hours. At 0.7 ppm, 40 percent of the fish survived 1 hour, and 10 percent survived 3 hours. The swift and complete mortality at 0.5 ppm cannot be explained. However, in a subsequent bioassay using 2.5-inch rainbow trout in 15 liters of aerated test solution, the pH changed slightly owing to aeration rates. Here, 0.34 and 0.36 ppm produced 100-percent mortality in 3 hours, while 0.4 ppm produced no mortality in 48 hours and only 10 percent mortality in 96 hours. A measure of pH revealed that the values for the lower concentrations were 7.6 and 7.5 but that of the bigger concentration was 8.0. Thus, a small increment in pH of only 0.4 or 0.5 unit was responsible for the decreased activity of 33NCS.

The effects of pH and alkalinity were differentiated in bioassays conducted with 2.5-inch rainbow trout. The LC50 for 96 hours using standard reconstituted water was 0.24 ppm (C.I.= 0.21-0.27). An increase of pH from 7.2 to 8.2 through the use of buffers increases the LC50

value to 0.35 ppm (C.I.=0.32-0.37). The toxicant in hard water, pH 8.2, had an LC50 value of 0.44 ppm (C.I.=0.41-0.47), whereas in hard water buffered to pH 7.2 the value was 0.32 ppm (C.I.=0.29-0.35) in 96 hours. These results indicate that pH apparently has slightly greater effect on the toxicity of 33NCS than does hardness.

Larger fish appear slightly more resistant to 33NCS than smaller fish. Although LC50 values are not available, 8-inch brown trout and 7.2-inch brook trout survived 0.4 ppm for 96 hours in an aerated bioassay. Smaller trout tested under regular bioassay conditions are more sensitive, and LC50's range from 0.20 to 0.24 ppm in 96 hours. Aeration increases pH, and this factor may contribute to these differences.

Several of the larger fish which had completely lost equilibrium were placed in fresh water to see whether they would recover. Two brown trout exposed to 0.6 ppm for 15 minutes regained equilibrium in 45 minutes. One of the fish died, and the other apparently recovered fully only after 24 hours in fresh water. Brook trout exposed to 0.5 ppm for 1 hour died within 10 minutes after being placed in fresh water. Thus, it appears that fish do not recover after critical exposure periods.

Effects of preexposure.--The rate of toxic action is reduced by preexposure to a sublethal level of 33NCS (table 9). Rainbow trout preexposed to 0.2 ppm of 33 NCS for 15 minutes are more resistant at 3 hours than unexposed trout. The difference in resistances at 24 hours is reduced considerably and at 48 hours it is insignificant.

TABLE 9.--The response of rainbow trout to 33NCS after preexposure to sublethal concentration

	At 3 hours		At 24 hours		At 48 hours	
	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.
Unexposed.....	0.368	0.350-0.390	0.290	0.245-0.342	0.237	0.214-0.257
Preexposed[1]....	0.625	([2])	0.325	0.290-0.364	0.239	0.204-0.283

[1]Preexposed to 0.2 ppm for 15 minutes.
[2]95-percent confidence intervals not available.

Hammond Bay Biological Station

Preliminary testing.--The toxicity of 33NCS to larval lampreys varied little from April 28, 1964, to April 22, 1965 (table 10). The MLC_{100} values ranged from 0.7 to 0.3 ppm (average 0.4 ppm) and exceeded 0.5 ppm only three times in the 42 assays performed.

Toxicity to fingerling rainbow trout was more variable. The MAC_{25} ranged from 0.8 to 2.1 ppm (average 1.3 ppm). Again the

TABLE 10.--Biological activity of a 5-percent-by-weight formulation of 33NCS in Lake Huron water in 21-hour assays at 12.8° C.

Date	Lamprey larvae MLC_{100} (ppm)	Rainbow trout MAC_{25} (ppm)	PAF[1]	Properties of test water		
				Conductivity (μmhos/18° C.)	Total alkalinity (ppm $CaCO_3$)	pH
1964						
Apr. 28	0.3	0.9	2.0	169.0	90.0	8.0
June 8	0.4	1.4	2.5	184.0	104.0	8.1
15	0.4	1.0	1.5	163.0	99.0	8.0
July 20	0.3	1.6	4.3	171.0	94.0	8.0
21	0.4	1.4	2.5	173.0	93.0	8.1
22	0.5	1.5	2.0	174.0	93.0	8.1
23	0.4	1.7	3.3	174.0	96.0	8.0
24	0.5	2.1	3.0	160.0	86.0	8.0
27	0.3	2.0	5.7	158.0	93.0	8.1
Aug. 4	0.6	1.6	1.7	149.0	93.0	8.2
5	0.7	1.8	1.6	172.0	96.0	8.3
6	0.3	1.8	5.0	172.0	96.0	8.1
17	0.3	1.6	4.3	186.0	103.0	8.0
18	0.4	1.4	2.5	181.0	96.0	8.3
19	0.5	1.6	2.2	181.0	96.0	8.3
20	0.3	1.4	3.7	177.0	98.0	8.3
Sept. 1	0.5	1.2	1.4	175.0	97.0	8.2
Oct. 12	0.3	1.2	3.0	-	-	-
19	0.4	1.0	1.5	-	-	-
20	0.4	1.0	1.5	-	-	-
Nov. 9	0.3	1.0	2.3	192.0	105.0	8.2
10	0.3	1.0	2.3	185.0	100.0	8.2
Dec. 2	0.4	1.0	1.5	179.0	96.0	8.1
7	0.4	0.8	1.0	156.0	85.0	7.6
14	0.5	1.2	1.4	179.0	103.0	8.0
15	0.5	0.9	0.8	159.0	96.0	7.9
17	0.6	1.2	1.0	203.0	118.0	7.8
1965						
Jan. 4	0.4	0.9	1.3	180.0	106.0	7.8
8	0.4	0.8	1.0	170.0	91.0	7.7
11	0.4	1.4	2.5	172.0	94.0	7.4
12	0.4	1.2	2.0	186.0	100.0	7.8
13	0.4	1.2	2.0	173.0	94.0	8.0
14	0.4	1.4	2.5	183.0	101.0	7.8
18	0.5	1.4	1.8	201.0	110.0	7.9
Feb. 26	0.3	1.2	3.0	190.0	98.0	7.9
Mar. 5	0.5	0.9	0.8	186.0	94.0	7.8
12	0.3	1.4	3.7	179.0	94.0	7.8
19	0.4	1.0	1.5	179.0	99.0	7.8
29	0.5	1.0	1.0	177.0	94.0	7.8
Apr. 6	0.4	0.9	1.3	175.0	95.0	7.8
13	0.5	1.0	1.0	182.0	106.0	7.8
22	0.4	0.8	1.0	148.0	85.0	7.9
Average value	0.4	1.3	2.2	175.7	97.1	8.0

[1] Permissible additional flow expressed as cubic feet per second is the ratio of working range to MLC_{100} (see text for explanation).

higher values occurred infrequently; only twice did the MLC25 exceed 1.8 ppm.

The PAF values were highly variable, ranging from 0.8 to 5.0 and averaging 2.2. A PAF of less than 1.0, which is considered below usefulness for stream treatment, occurred only twice.

The data from all 42 assays show that there is some seasonal variation in toxicity of 33NCS in Lake Huron water, at least for rainbow trout. The results are therefore divided into two groups, June through October (summer) and November through April (winter).

The average ML100 of 0.42 ppm for summer and 0.41 ppm for winter assays did not differ significantly ($P>0.04$), but the average MAC25 levels for rainbow trout of 1.49 ppm for summer and 1.07 ppm for winter assays were highly significant ($P<0.001$). Because PAF values reflect the relation between MLC100 and MAC25, they also differed significantly ($P<0.005$) between the two seasons (averages were 2.80 in the summer and 1.68 in the winter).

Only pH showed a significant variation ($P<0.001$) between the two seasons (fig. 3).

TFM was more selective than 33NCS in 6 out of 9 tests in Lake Huron water (table 11), but 33NCS was 5 to 10 times as potent on larval lamprey (MLC100) and 6 to 12 times as potent on rainbow trout (MAC25) as TFM. The average PAF value was 3.0 for TFM and 2.2 for 33NCS; the MAC25's of TFM and 33NCS for rainbow trout were 2.5 to 6 times the MLC100's for lamprey larvae.

Effects of temperature.--The toxicity of 33NCS, expressed as LC50, becomes greater with each increment of test temperature (table 12). But, an examination of the 95-percent confidence limits on those values indicates that the variations are slight between 7.2° and 18.3° C. The difference observed between 1.7° and 7.2° is greater but even more so between 18.3° and 23.9° C. The slope function for each species is essentially the same and did not vary with temperature. These slope functions (1.20-1.25 for rainbow

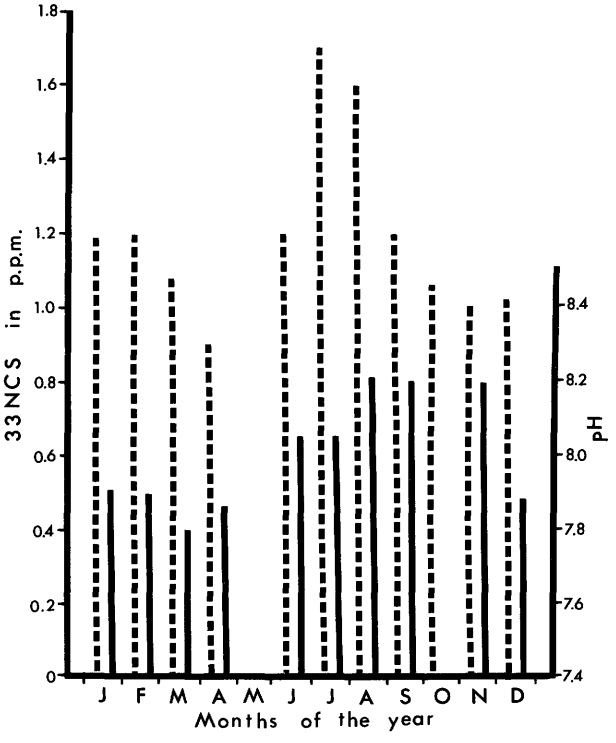

Figure 3.--Seasonal variations in pH (solid line) and concentrations of 33NCS (broken line) required to kill 25 percent (MAC25) of the rainbow trout.

TABLE 11.--Relative toxicity and selectivity of TFM and 33NCS in Lake Huron water conducted at 12.8° C. for 21 hours

Lamprey larvae			Rainbow trout			PAF	
MCL_{100} (ppm) TFM	33NCS	Potency ratio (TFM/33NCS)	MAC_{25} (ppm) TFM	33NCS	Potency ratio (TFM/33NCS)	TFM	33NCS
3.0	0.3	10	12.0	1.8	7	3.0	5.0
3.0	0.3	10	12.0	1.4	9	3.0	3.7
2.0	0.4	5	12.0	1.0	12	5.0	1.5
3.0	0.5	6	18.0	1.6	11	5.0	2.2
3.0	0.5	6	12.0	1.2	10	3.0	1.4
3.0	0.5	6	10.0	1.4	7	2.3	1.8
4.0	0.5	8	12.0	1.0	12	2.0	1.0
4.0	0.6	7	10.0	1.6	6	1.5	1.7
4.0	0.7	6	14.0	1.8	8	2.5	1.6
Average values							
3.2	0.5	7	12.4	1.4	9	3.0	2.2

TABLE 12.--Effect of temperature on toxicity of 33NCS to larval lamprey and rainbow trout in 21-hour bioassays

Temperature (C.)	Lamprey larvae		Rainbow trout		Slope function	
	LC_{50} (ppm)	95-percent C.I.	LC_{50} (ppm)	95-percent C.I.	Lamprey larvae	Rainbow trout
1.7°.....	0.80	0.70-0.92	1.72	1.47-2.01	1.20	1.25
7.2°.....	0.28	0.25-0.32	1.36	1.17-1.58	1.13	1.25
12.8°.....	0.26	0.22-0.30	1.20	1.07-1.34	1.25	1.22
18.3°.....	0.25	0.21-0.29	1.20	1.07-1.34	1.28	1.22
23.9°.....	0.17	0.14-0.12	0.25	0.21-0.30	1.20	1.20

TABLE 13.--Effect of temperature on toxicity, selectivity, and utility of 33NCS in 21-hour bioassays

Temperature (C.)	Lamprey larvae MLC_{100} (ppm)	Rainbow trout MAC_{25} (ppm)	PAF
1.7°.....	1.2	1.4	0.2
7.2°.....	0.4	1.0	1.5
12.8°.....	0.4	1.0	1.5
18.3°.....	0.4	1.0	1.5
23.9°.....	0.3	0.2	-0.3

trout and 1.13-1.28 for larval lampreys) indicate that the narrow range of concentrations required to produce an "all-or-none" response held throughout the series of temperatures used.

The MLC100, MAC25, and PAF values obtained between 7.2° and 18.3° C. are the same and indicate a usable degree of selectivity (table 13). The complete loss of selectivity observed at 23.9° C. is probably a result of unnatural stress placed on the rainbow trout rather than a true reflection of toxicity, since this temperature is near the lethal threshold for the species.

Effects of water quality.--The toxicity of 33NCS and TFM decreases with an increase in alkalinity and conductivity (table 14). The MAC25's increase from 0.6 to 3.5 ppm for 33NCS and from 5.0 to 26.0 ppm for TFM in waters of increasing alkalinity. MLC100 values show a parallel increase as best illustrated in figure 4. The potency ratios of 33NCS to TFM range from 4.0 to 8.5 ppm for MLC100 and 7.1 to 10.0 ppm for MAC25 in waters of various alkalinities. Therefore, alkalinity, conductivity, and pH and their interactions profoundly influence the toxicity of 33NCS and TFM to larval lamprey and rainbow trout.

Flowing water bioassay.--Of the fish used, the sea lamprey was by far the most sensitive species to 33NCS in "simulated" stream tests in 1964 and 1965 (tables 15 and 16). In contrast, the trouts are among the most resistant. The LC50's for various life stages of the sea lamprey range from 0.37 to 0.61 ppm. The calculated LC50's for other fish ranged from 0.66 to 1.75 ppm. Consistent with the laboratory tests, the narrow range of concentrations which delineate "all-or-none effects" are demonstrated by these low slope function values. Equally important is the narrow range of the confidence intervals about the LC50 values.

Data on several species of fish were insufficient to calculate the LC50 values, but we were able to determine the highest concentration which did not kill at least 25 percent of the test fish (table 17).

TABLE 14.--Variation in the toxicity of TFM and 33NCS in waters of various alkalinities in Michigan

[Tests conducted for 21 hours at 12.8° C]

Source of test water	Properties of test water		pH	Toxicant	Lamprey larvae MLC_{100} (ppm)	Rainbow trout MAC_{25} (ppm)	PAF
	Alkalinity (ppm $CaCO_3$)	Conductivity (μmhos/18°C.)					
Pendills Creek, Chippewa County.....	43.0	74.0	7.6	TFM	2.0	5.0	1.5
				33NCS	0.3	0.6	1.0
Lake Huron, Presque Isle County.....	97.0	175.0	8.2	TFM	3.0	12.0	3.0
				33NCS	0.5	1.2	1.4
Little Billies Creek, Cheboygan County.....	148.0	225.0	8.1	TFM	7.0	18.0	1.6
				33NCS	0.8	2.2	1.8
Ocequeoc River, Presque Isle County..	163.0	250.0	8.3	TFM	5.0	16.0	2.2
				33NCS	0.8	2.2	1.8
Pere Marquette River, Mason County...	175.0	274.0	7.9	TFM	5.0	18.0	2.6
				33NCS	1.3	2.5	1.0
Au Gres River, Iosco County.....	184.0	345.0	8.2	TFM	7.0	25.0	2.6
				33NCS	1.3	3.5	1.8
Jordan River, Charlevoix County.....	189.0	304.0	8.3	TFM	8.0	24.0	2.0
				33NCS	1.6	2.8	0.8
Trout River, Presque Isle County.....	200.0	310.0	8.1	TFM	8.0	26.0	2.3
				33NCS	1.1	3.0	1.8

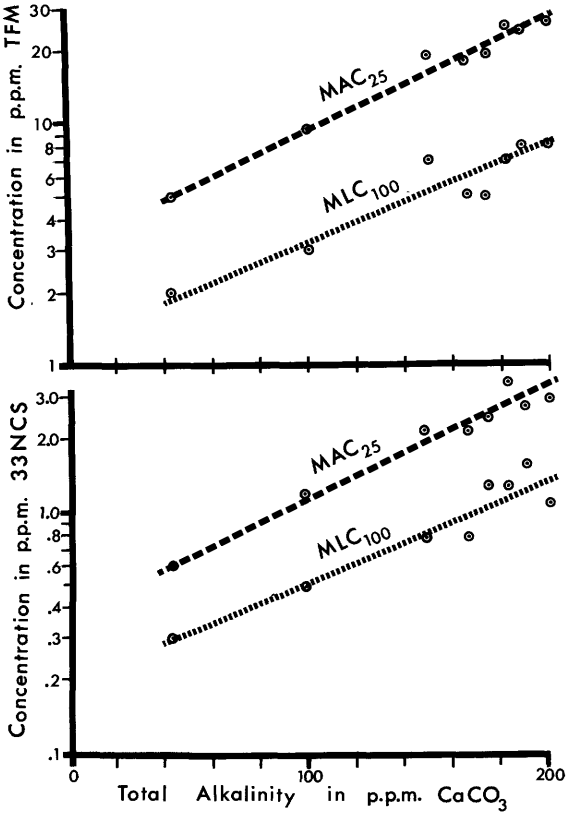

Figure 4.--The effect of alkalinity on the toxicity of 33NCS and TFM to rainbow trout and larval lamprey.

TABLE 15.--Order of toxicity of 33NCS to mature fish in 21 hours (unless otherwise specified) in simulated stream tests in fall, 1964

Test organism	LC_{50} (ppm)	95-percent C.I.	Slope function
Sea lamprey (transformer).	0.37	0.32-0.43	1.11
Sea lamprey (larvae).....	0.43	0.41-0.45	1.28
Sand shiner.....	0.66	0.60-0.73	1.35
Common shiner.....	0.84	0.82-0.86	1.03
Yellow perch.....	0.89	0.79-1.00	1.15
Northern pike.....	1.00	0.83-1.20	1.08
White sucker (immature)...	1.05	0.96-1.16	1.43
Fathead minnow.....	1.09	1.03-1.16	1.10
White sucker.....	1.13	0.86-1.49	1.35
Rainbow trout (immature)..	1.18	1.02-1.37	1.35
Brook trout.....	1.30	1.16-1.46	1.25
Brown trout.....	1.30	1.17-1.44	1.28
Rock bass.....	1.48	1.37-1.60	1.06
Rainbow trout.....	1.50	1.35-1.67	1.22
Brown bullhead.....	1.60	1.43-1.79	1.14
Brown trout[1].....	1.65	1.29-2.11	1.48

[1] Stream-dwelling fish collected from the Rifle River, Ogemaw County, Mich.

33NCS is toxic to invertebrates, and LC50's range from 0.78 to 1.8 ppm. The LC50 value for 33NCS on stonefly nymphs was 0.78 ppm (0.64 to 0.95) in 1964 with a slope function of 1.25. The LC50 for burrowing mayfly nymphs in 1965 was 1.26 ppm (1.07 to 1.49) with a slope function of 1.34. Estimates of the

TABLE 16.--Order of toxicity of 33NCS to mature fish in 21 hours (unless otherwise specified) in simulated stream tests in spring, 1965

Test organism	LC_{50} (ppm)	95-percent C.I.	Slope function
Sea lamprey (larvae).....	0.38	0.37-0.40	1.22
Sea lamprey.....	0.61	0.55-0.68	1.08
White sucker.....	0.85	-	1.29
White sucker (immature)....	0.88	0.80-0.97	1.27
Yellow perch.....	0.90	0.80-1.02	1.22
Brown bullhead.....	1.14	0.94-1.38	1.20
Longnose sucker.....	1.19	1.02-1.39	1.35
Longnose dace.....	1.20	1.09-1.32	1.35
Yellow bullhead.....	1.24	1.08-1.43	1.08
Northern pike.....	1.25	0.96-1.60	1.08
Brook trout.....	1.30	1.15-1.47	1.25
Rainbow trout.....	1.31	1.16-1.48	1.15
Brown trout.....	1.50	1.32-1.71	1.23
Common shiner.....	1.75	1.59-1.94	1.20

TABLE 17.--Highest concentrations of 33NCS in ppm which did not kill more than 25 percent of the fish in simulated stream tests

Species	1964	1965
Rainbow trout (immature).....	--	1.59
Lake chub.....	--	0.40
Rock bass.....	--	1.59
Pumpkinseed sunfish (immature)..	1.80	1.59
Pumpkinseed sunfish.....	1.80	1.59
Bluegill (immature).....	1.80	1.59
Bluegill.....	1.80	1.59
Smallmouth bass.....	1.60	1.59
Largemouth bass (immature).....	1.80	1.59
Largemouth bass.....	1.80	1.00
Logperch.....	--	0.50
Walleye.....	1.20	--

LC50 values in 1964 and 1965 tests for other organisms were 1.4 and 1.26 ppm for snails, 1.4 and 1.59 for clams, and 1.8 and 1.59 for crayfish. We also estimated the LC50 to be 1.0 ppm for stoneflies and 1.59 for dragonfly nymphs in 1965.

Although the LC50 values of 33NCS varied among some species of fish from one raceway test to the other, a general pattern is evident. 33NCS is almost uniformly nontoxic to centrarchids at the levels tested. In descending order of resistance, after the Centrarchidae, are the Salmonidae, Ictaluridae, Catostomidae, Cyprindae, Percidae, Percopsidae, and Petromyzonidae (tables 18 and 19). This listing of relative resistance to 33NCS is similar to one given by Howell (1966) for TFM.

Among the many species tested, both vertebrates and invertebrates, none is as sensitive to the toxic effect of 33NCS as the larval lamprey (LC50 = 0.38 to 0.43 ppm). The compound is also highly toxic to the lamprey at

TABLE 18.--Order of selectivity of 33NCS calculated from simulated stream tests in 1964 based on curves plotted using the data from table 15

Species	MAC_{25} (ppm)	PAF[1]
Sand shiner	0.54	-0.10
Common shiner	0.70	0.17
Yellow perch	0.82	0.37
White sucker (immature)	0.85	0.42
White sucker	0.92	0.53
Northern pike	0.96	0.60
Rainbow trout (immature)	0.98	0.63
Fathead minnow	1.02	0.70
Brown trout	1.09	0.82
Brook trout	1.14	0.90
Brown trout (stream dwelling)	1.25	1.08
Rainbow trout	1.30	1.17
Rock bass	1.40	1.33
Brown bullhead	1.47	1.45

[1] Based on a MCL_{100} of 0.60 ppm.

TABLE 19.--Order of selectivity of 33NCS calculated from simulated stream tests in 1965 based on curves plotted using the data from table 16

Species	MAC_{25} (ppm)	PAF[1]
White sucker	0.72	0.14
White sucker (immature)	0.76	0.21
Yellow perch	0.79	0.25
Longnose sucker	0.96	0.52
Longnose dace	0.98	0.56
Brown bullhead	1.00	0.59
Brook trout	1.12	0.78
Yellow bullhead	1.16	0.84
Northern pike	1.20	0.90
Rainbow trout	1.20	0.90
Brown trout	1.30	1.06
Common shiner	1.55	1.46

[1] Based on a MLC_{100} of 0.63 ppm.

other stages in the life cycle (LC50 = 0.37 to 0.61 ppm). The trout-perch may be as susceptible as the adult lamprey to 33NCS, but the data on trout-perch are limited, and we merely state that the LC50 is less than 0.50 ppm.

The LC50 varied somewhat among the trouts in the two tests. The LC50 for immature (3- to 5-inch) rainbow trout was 1.18 in 1964 and greater than 1.59 in 1965. The reason for this difference is unknown, but both values are within the limits observed during laboratory studies. In the 1964 raceway series, mature brown trout (7- to 14-inch) from two sources were compared. The LC50 was 1.30 for hatchery stocks, whereas it was 1.65 for fish collected from a stream. The two values, noted during the same test, illustrate that toxicity may vary among fish from different sources. In both tests, brook trout were more sensitive to 33NCS than brown or rainbow trout.

The brown bullhead was one of the most resistant species during the 1964 test (LC50 = 1.60). In the second series, brown and yellow bullhead were slightly less tolerant than the trouts. the LC50 values for these two species are in close agreement (1.14 for brown bullhead and 1.24 for yellow bullhead during the 1965 tests).

There were some differences in the toxicity of 33NCS to suckers and minnows in the two series of tests. These differences were small except for the common shiner. The LC50 value in 1964 was 0.84 ppm, whereas the value in 1965 was 1.75. It is difficult to account for these differences on the basis of water quality, temperature, or source of test animals, and it seems more likely to be a reflection of some basic physiological difference between the specimens used in each test.

DISCUSSION

At Hammond Bay, the 21-hour LC50 of 33NCS to rainbow trout in Lake Huron water was 1.2 ppm. The 24-hour LC50 in standard reconstituted water at the Fish Control Laboratories was 0.3 ppm, four times the toxicity at approximately the same temperature and exposure time. The contrast is even greater in softer waters.

Bioassays on rainbow trout in harder water at La Crosse yielded an LC50 of 0.52 ppm in 24 hours. Water of similar alkalinities from Pendills Creek and Lake Huron produced LC50 values of 0.74 and 1.42 ppm, respectively, in aerated bioassays in 21 hours.

The apparent differences in results of bioassays at Hammond Bay and the Fish Control Laboratories are attributable to variations in the quality of aerated and nonaerated waters. Data in table 9 indicate that an increase in the bicarbonate of bioassay water, with corresponding increase in pH and

alkalinity, reduces the toxicity of 33NCS considerably. Seasonal variations in pH and alkalinity in Lake Huron water show a similar effect (table 10). For a specific concentration of toxicant, the pH alone can effect either 100 percent mortality or survival (table 8).

Aeration is a major influence on the toxicity of 33NCS; trout survive 0.5 ppm of 33NCS in reconstituted water only when the bioassay is aerated (table 7). This observation is substantiated further by the decreased toxicity in aerated bioassays at Hammond Bay.

Another factor influencing the toxicity of 33NCS is the volume of fish per volume of test solution. Heavier loads of fish in test vessels are possible at Hammond Bay because of aeration. At the heavier loadings, the ratio of toxicant per unit of biomass is considerably less than with lighter loadings. The size of test fish also is a factor. In general, larger fish are more tolerant to 33NCS.

The toxicity of 33NCS at temperatures between 7° and 18° C. showed little variation at Hammond Bay and at the Fish Control Laboratories. Toxicity to larval lamprey, rainbow trout, and green sunfish tends to increase at the higher temperatures. The extreme temperatures of 1.7° and 23.9° C. produce more drastic variations in toxicity, and such variations are more noticeable in rainbow trout than in lamprey larvae.

PAF values for 33NCS were usually less than those calculated for TFM on rainbow trout in laboratory and simulated stream trials. Lower PAF values for other species in simulated stream trials were in the descending order of resistance to 33NCS: Centrarchidae, Salmonidae, Ictaluridae, Catastomidae, Cyprinidae, and Percopsidae. These PAF values suggest that 33NCS may be limited as a selective larvicide to situations involving only trouts and sunfishes.

CONCLUSIONS

1. 33NCS is rapidly toxic to a wide variety of fishes and invertebrates.
2. The toxicity and selectivity of 33NCS are comparable under different test conditions even though absolute values may vary. Sea lamprey, bowfin, carp, and channel catfish are more sensitive than trouts or sunfishes.
3. 33NCS was more toxic to fish in bioassays at the Fish Control Laboratories than at Hammond Bay. Water quality, aeration, loading, and size and source of test fish contributed to the differences in results in flowing and static bioassays.
4. The toxicity of 33NCS decreases with increasing pH, alkalinity, and hardness of water. A change in pH from 7.8 to 8.2 is very critical, and it may cause 100-percent mortality or permit survival of fish exposed to a given concentration.
5. The toxicity of 33NCS increases with temperature. At extreme temperatures the effect is more profound.
6. The regressions for 33NCS are nearly parallel for all species tested, even under different test conditions. Minute increments in concentrations delineate all-or-none effects as denoted by extremely low slope functions.
7. The toxicity of 33NCS varies with seasons. The bioassays indicate greater toxicity to rainbow trout from November through April.
8. Preexposure does not affect the ultimate lethal level but does appear to retard the rate of acute toxic action.
9. Trout did not recover after critical exposures to 33NCS.
10. 33NCS is approximately 6 times as toxic to larval lamprey as TFM in most waters tested, but 33NCS does not yield as high PAF values as TFM under the same conditions.
11. PAF values are greater than 1.0 for trouts and sunfishes in simulated stream trials. However, MLC100 values for sea lamprey and MAC25 values for other species of fish in these studies produce PAF values which may seriously limit

use of 33NCS for selective control of freshwater fish and sea lamprey larvae.

12. In field use, 33NCS would require rigid control since its performance is highly dependent on water quality.

13. The results of this study form a base for further investigation of the selective activities of 33NCS and closely related nitrosalicylanilides

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