INTERNATIONAL PACIFIC SALMON
FISHERIES COMMISSION

PROGRESS REPORT

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ABSTRACT

Sockeye, coho and pink salmon and rainbow trout were exposed to butoxyethyl ester of 2,4-D (BEE) derived from Aqua-Kleen 20 in semi-static acute and chronic bioassays. The 96 hr LC50 was approximately 0.45 mg/l BEE for sockeye fingerlings, coho fry and pink fry. Rainbow trout were slightly more sensitive to BEE than the salmon tested. Sockeye smolts and pink salmon fry survived transfer to seawater after 24 hour exposure to concentrations of BEE nearly equal to the 96 hr LC50.

Pink and coho salmon and rainbow trout were shorter than control specimens at yolk absorption following exposure to 0.220 mg/l BEE during the alevin stage.

No mortality or distress was observed among sockeye fingerlings exposed to 200 mg/l 2,4-D acid for 168 hours.

The influences of bacterial activity, temperature and pH on hydrolysis of BEE to 2,4-D acid are discussed.

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INTRODUCTION

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was first introduced to public use in 1944. Since its introduction it has become one of the most widely used herbicides in the world. In recent years the butoxyethyl ester of 2,4-D (BEE) has been applied as the product Aqua-Kleen 20 to selected sites in Okanagan Valley lakes to control Eurasian water milfoil (Myriophyllum spicatum)[1]. More recently milfoil has been identified in Cultus Lake, Sweltzer Creek and in numerous watercourses in the Fraser Valley.

In view of possible proposals to control milfoil in the Fraser River watershed by application of Aqua-Kleen 20, bioassays were conducted to measure the acute and chronic toxicity of BEE to selected salmonids.

METHODS

Dilution water and BEE

Cultus Lake water drawn from 36.6 meters was used for all bioassays except salinity tolerance tests. This water has been tested regularly for the past 15 years (IPSFC, unpublished data 1963-1978) and is stable with respect to hardness, alkalinity, pH and conductivity. During the course of this study several determinations of hardness, alkalinity, pH and conductivity were made on water drawn from bioassay aquariums. Total hardness and alkalinity were determined according to Standard Methods, 1975, and pH was determined with a Radiometer Model 29 pH meter. Conductivity was measured with an Industrial Instruments Model RC conductivity bridge.

Dissolved oxygen (D.O.) was measured in incoming water and in bioassay containers several times during the study. D.O. measurements were made with a YSI model 54 oxygen meter calibrated against the azide modified Winkler method (Standard Methods, 1975).

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1. Eurasian water milfoil, M. spicatum, will be referred to as milfoil for the sake of brevity.

Bioassay aquariums were immersed in a cooling bath of Cultus Lake water. Temperatures prevailing during the bioassay period were similar to those of the winter, spring and summer of 1978 at 36.6 meters depth in Cultus Lake.

Seawater (28^{o}/oo) for salinity tolerance tests was obtained courtesy of the Vancouver Public Aquarium and hauled by truck to Cultus Lake in a 300 gallon wooden tank.

The source of BEE for bioassays was Aqua-Kleen 20[1] consisting of bentonite clay granules impregnated with 20% acid equivalent[2] (a.e.) BEE by weight. To permit accurate weighing and more rapid dissolving of BEE, the granules were pulverized in a Sorvall Omni-Mixer before use in bioassays.

One bioassay was conducted with 2,4-D acid (98% pure).

BEE Measurement

A suitable method was not available for measuring BEE during bioassays. However, following the bioassay study, BEE was measured under simulated bioassay conditions but without fish present. Preliminary results at 1.0 mg/l nominal BEE indicated concentrations peaked between three and 12 hours after make-up and declined thereafter as BEE hydrolyzed to the 2,4-D acid form. Since BEE was more toxic than 2,4-D acid, further samples for measurement of BEE were collected at six hours after make-up.

Sub-samples of one liter were triple extracted with methylene chloride, taken to near dryness in a rotary evaporator and submitted to Pacific Environment Institute for measurement of BEE content by gas chromatography.

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1. Aqua-Kleen 20 - manufactured by Amchem Products, Inc., Windsor, Ont., Ambler, Pa., Clinton, Iowa, St. Joseph, Mo., Fremont, Calif.
2. Except where noted, all BEE concentrations are reported as equivalent 2,4-D acid (a.e.). To convert acid equivalent to BEE, multiply the acid equivalent by 1.49.

Test Fish

Sockeye salmon (Oncorhynchus nerka), pink salmon (O. gorbuscha), coho salmon (O. kisutch), and rainbow trout (Salmo gairdneri) were used in bioassays. Sockeye, pink and coho salmon eggs were collected from Pitt Lake, Sweltzer Creek and Capilano stocks, respectively. Rainbow trout eggs were obtained from Tunkwa Lake. Coho salmon and rainbow trout eggs were obtained courtesy of the Fisheries and Marine Service and the B.C. Fish and Wildlife Branch, respectively. Wild sockeye salmon smolts were collected from a counting fence at Cultus Lake.

All eggs were transferred to Sweltzer Creek Salmon Research Laboratory at Cultus Lake for incubation, rearing and bioassays.

Bioassays

Acute and chronic bioassays of eggs, alevins and fry were conducted simultaneously in glass aquariums. Bioassays were usually static replacement type with daily (Monday through Friday) renewal of BEE test solutions in each of the five BEE concentrations and the control. Exceptions to the preceeding will be noted where applicable. Each group of eggs, alevins and fry was isolated within an aquarium by confinement in one liter polyethylene bioassay incubators. The contents of each aquarium were circulated through each bioassay incubator at a flow rate of 150 to 200 ml/min. (Figure 1). Thirty liter aquariums were used initially but were replaced by 64 ℓ aquariums to create acceptable fish loading rates. Aquariums were cleaned daily to suppress bacterial growths and minimize biochemical hydrolysis of BEE to 2,4-D acid. Incubators were also cleaned periodically. Windows of the bioassay room faced north, eliminating direct sunlight. Daytime illumination was augmented by flourescent lighting.

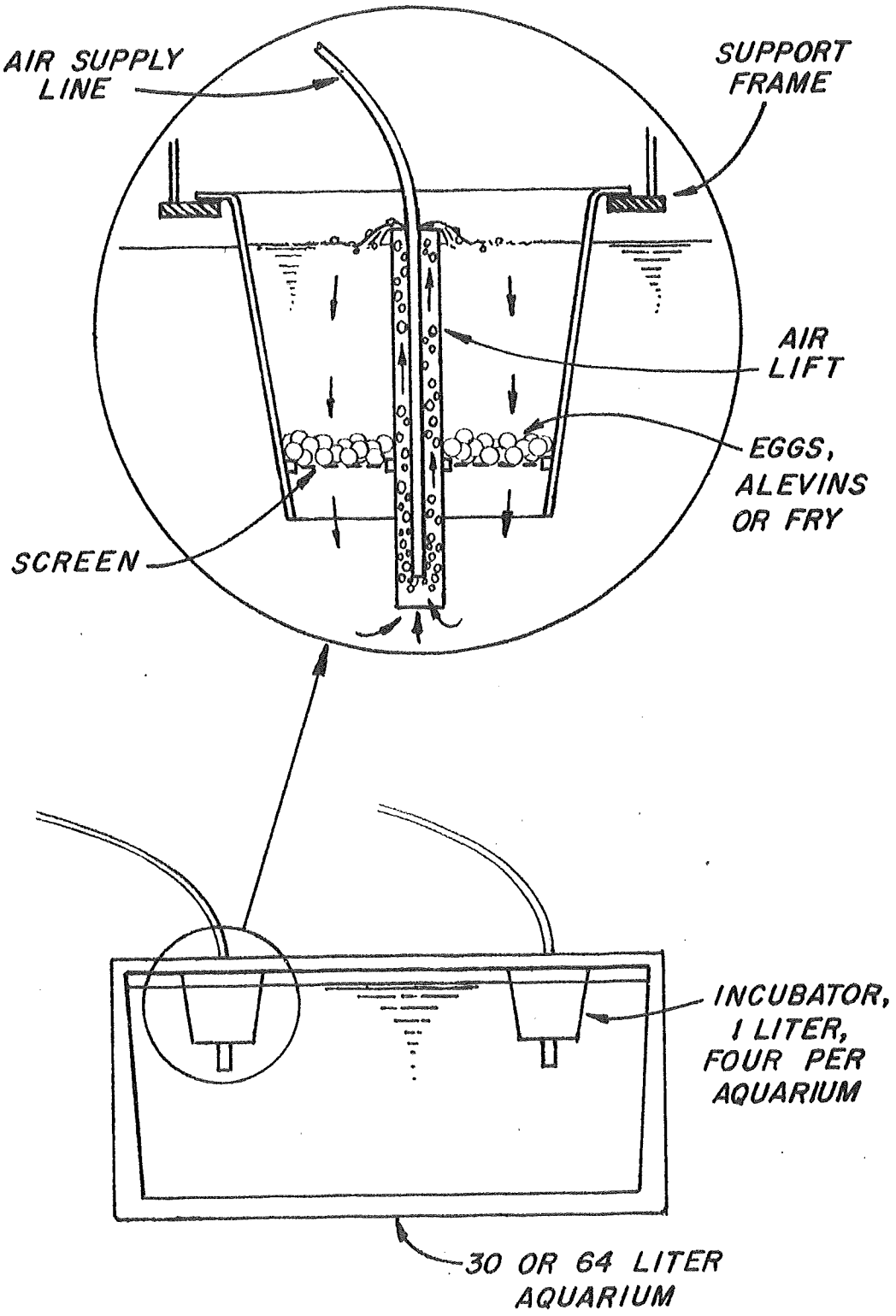

Figure 1. Incubators for bioassays using eggs, alevins or fry.

Fish loading densities were a function of the sizes of the test groups and the volumes of the test vessels. The highest density, 0.81 gm fish/l occurred when pink salmon alevins, early coho eggs and Pitt Lake sockeye fingerlings were tested simultaneously in 30 ℓ aquariums. Pink salmon fry were removed following yolk absorption, reducing fish density to 0.20 gm/l. The highest fish density during rainbow trout exposures was 0.25 gm/l. Fish density during sockeye smolt bioassays was 0.35 gm/l.

Nominal BEE concentrations (1.0, 0.7, 0.3, 0.1, 0.05 and 0.00 mg/l) were chosen following several preliminary tests and were used in all acute and chronic bioassays, except for acute bioassays of smolts.

Acute bioassays were usually of seven days duration with mortality observations on each of the first four days and the seventh day.

Ten alevins or fry were tested in each bioassay incubator during acute bioassays. Owing to their size, wild Cultus Lake sockeye smolts were bioassayed in 62 ℓ glass aquariums. Smolts were bioassayed five per aquarium in duplicate at each of three concentrations and in control water.

Acute bioassays of 2,4-D acid were conducted using 30 ℓ aquariums without polyethylene incubators. Ten Pitt Lake sockeye fingerlings were placed in each 30 ℓ aquarium at concentrations equalling 0, 20, 30, 40, 50, 65, 70, 80, 90, 100 and 200 mg/l 2,4-D acid.

All 2,4-D acid concentrations except 100 and 200 mg/l were renewed daily. Premixing of each concentration 16 hours in advance of testing or renewal was necessary owing to the slow solution rate of 2,4-D acid.

Chronic bioassays of pink and coho salmon eggs and rainbow trout eggs commenced when eggs became available and terminated when alevins reached yolk absorption. The aforementioned procedure resulted in continuous exposure from blastopore closure of eggs to fry for coho salmon and the first

group of rainbow trout but pink salmon began exposure as alevins. A second test was made with rainbow trout beginning in the late alevin stage. This latter group will be referred to as group II and the rainbow trout exposed from blastopore closure to fry as group I. Temperatures in each incubator were recorded daily and dead eggs, alevins or fry removed and recorded. Dead eggs were cleared of opaqueness using Stockard's Solution[a] and the number of infertile eggs in each test group determined. Coho alevins were sampled during chronic exposure and preserved in 10% formalin for length measurement. In addition, all surviving fry were preserved for length measurement following chronic exposure. Calipers were used to measure fork length of alevins and fry to the nearest 0.1 mm.

Salinity Tolerance

Seawater tolerance of sockeye smolts and pink salmon fry was determined following 24 hour exposure to BEE. Smolts were exposed to 0.0, 0.220, 0.416 and 0.540 mg/1 BEE (duplicate 62 ℓ aquariums; 5 smolts/aquarium) for 24 hours, transferred to freshwater overnight and then to 28°/oo seawater for the next 96 hours. Smolts were exposed to seawater in 100 ℓ polyethylene containers. Pink salmon fry were tested similarly except that exposures to BEE and seawater took place in 18 ℓ glass aquariums (10 fish/aquarium).

RESULTS

Dilution Water Characteristics and BEE Measurements

Hardness and alkalinity of Cultus Lake water averaged 78.6 and 61.6 mg/1, respectively as $CaCO_3$. Conductivity averaged 169 µmohs/cm and mean pH was 7.7. The preceeding values are in the ranges usually observed for Cultus Lake water. Dissolved oxygen in all incubators was greater than 93% of air saturation (12.8 mg/1 D.O.). Temperatures in incubators ranged from 3.3°C (38°F) during winter to 7.2°C (45°F) during summer.

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[a]Stockard's Solution: formalin, 25 ml; acetic acid, 20 ml; glycerol, 30 ml; water, 425 ml.

Analyses at Pacific Environment Institute using methylene chloride in a Soxhlet extractor to extract powdered Aqua-Kleen 20 confirmed that it contained 20% BEE (a.e.). However, the BEE content measured in aqueous solution accounted for only a portion of that added. For example, six hours after make-up,BEE increased to 59% of the nominal 0.7 mg/1 concentration then declined to 23% nominal at 96 hours (Table 1) presumably due to hydrolysis to 2,4-D acid and the corresponding alcohol (Zepp at al, 1975). Peak measured concentrations of BEE were 54, 59, 73 and 75% of nominal values in six hours at nominal concentrations equal to 1.0, 0.7, 0.3 and 0.1 mg/1, respectively (Table 2). Analyses for 2,4-D acid did not account for the difference between BEE added and the amount measured.

Since adsorption of phenoxy acids on microorganisms, organic and inorganic particles and vessel walls was cited as a reason for variable extraction recoveries (National Research Council of Canada, 1978), additional measurements were made. Extraction using methylene chloride in a separatory funnel indicated small amounts of BEE remained with the powdered Aqua-Kleen 20 and on the walls of the glass aquarium after six hours. However, these amounts were less than the difference between BEE added and that measured. Thus 25 to 46% of the BEE added as Aqua-Kleen 20 was not accounted for in analytical measurements and may not have been available to the test specimens during bioassays. Therefore, peak measured concentrations of BEE are reported with results of bioassays instead of nominal concentrations.

Acute Bioassays

All fish died in each bioassay at 0.540 mg/1 BEE but no fish died at 0.220 mg/1 or less (Table 3). Furthermore, there was no evidence of distress among fish exposed to 0.220 mg/1 BEE. Although mean mortalities of sockeye fingerlings and coho fry were greater after 168 hours than after 96 hours exposure to 0.416 mg/1 BEE, mortalities increased only in tests where fish were already moribund after 96 hours of exposure.

Table 1. Measured concentrations of BEE at a nominal concentration equal to 0.7 mg/l.

Elapsed time from make up	BEE(a.e.) measured	Measured BEE / Nominal BEE
hours	mg/l	%
3	0.382	55
6	0.416	59
24	0.360	51
48	0.302	43
72	0.219	31
96	0.160	23

Table 2. Peak BEE concentrations measured 6 hours after make-up.

BEE(a.e.)		Measured BEE
Nominal mg/l	Measured mg/l	Nominal BEE %
1.000	0.540	54
0.700	0.416	59
0.300	0.220	73
0.100	0.075	75
0.050	-	-
0.000	0.000	-

Based on mortalities at 0.416 mg/1 BEE, rainbow trout fry were most sensitive to BEE while sockeye smolts were least sensitive during acute bioassays. Sockeye fingerlings, coho fry, and pink fry were about equally sensitive to BEE.

The 96 hr LC50 derived from mean mortalities was approximately 0.45 mg/1 BEE for sockeye fingerlings, coho fry and pink fry. The 96 hr LC50 would be between 0.45 and 0.54 mg/1 BEE for sockeye smolts and was between 0.2 and 0.4 mg/1 BEE for rainbow trout.

No mortalities or distress were observed among sockeye fingerlings exposed to nominal 2,4-D acid concentrations up to 200 mg/1 for 168 hours.

Chronic Bioassays

All pink and coho salmon and rainbow trout alevins died when exposed to 0.416 mg/1 BEE (Table 4). Group I rainbow trout and coho salmon were exposed to BEE following blastopore closure of eggs but mortalities prior to hatching were generally not significant, except that 73 and 100% of group I rainbow trout eggs died at 0.416 and 0.540 mg/1 BEE, respectively. In contrast, only 1 and 11% of coho eggs died at 0.416 and 0.540 mg/1 BEE respectively.

Group I rainbow trout alevins were most sensitive of the test specimens, experiencing 44% mortality at 0.220 mg/1 BEE.

Observations of hatching among coho salmon and group I rainbow trout eggs indicated no delay in hatching. In addition, malformed alevins were absent at lethal and non-lethal test concentrations among all four experimental groups.

Measurements of fork length showed that groups exposed to 0.220 mg/1 BEE were smaller than controls at yolk absorption (Table 5).

Table 3. Mortality in 96 and 168 hour bioassays.

Test Fish	No. Tests	Mean mortality, % Peak measured BEE concentration mg/l (a.e.)						
		0.540	0.416	.416[b]	0.220	0.075	0.05[a]	0.00
Sockeye fingerlings	12	100	33 0-100[c]	51 0-100[c]	0	0	0	0
Sockeye smolts	1	100	0	0	0	0	0	0
Coho fry	3	100	30 10-40[c]	37 10-60[c]	0	0	0	0
Pink fry	1	100	20	20	0	0	0	0
Rainbow fry	2	100	100	100	0	0	0	0

[a] Nominal concentration

[b] Mean 168 hr mortality

[c] Range

Table 4. Mortality of pink and coho salmon and rainbow trout alevins during chronic exposure to BEE.

Peak Measured BEE (a.e.) mg/l	Mortality % Pink[a] Salmon	Coho[b] Salmon	Group 1[c] Rainbow Trout	Group II[d] Rainbow Trout
0.540	100	100	100	100
0.416	100	100	100	100
0.220	3	2	44	4
0.075	0	0	0	0
0.05 (nominal)	0	2	0	0
0.00	0	0	3	0
Exposure (Days)	34	102	85	17

[a] Pink salmon were exposed from the alevin stage to yolk absorption.

[b] Coho salmon were exposed from blastopore closure to yolk absorption.

[c] Group I rainbow trout were exposed from blastopore closure to yolk absorption.

[d] Group II rainbow trout were exposed from the late alevin stage to yolk absorption.

Growth was unaffected at 0.075 mg/1 BEE and less, except coho alevins were larger than controls during exposure to 0.05 mg/1 BEE. However, the difference in length between the latter two groups of coho was not significant at yolk absorption.

Salinity Tolerance

All sockeye smolts and pink salmon fry survived 96 hour exposure to 28 °/oo salt water following 24 hour exposure to 0.220, 0.416 and 0.540 mg/1 BEE. In addition, body coloration and fright response of test fish following exposure to BEE and 28 °/oo salt water was similar to controls.

Table 5. Mean length of alevins and fry following chronic exposure to BEE

Peak Measured BEE (a.e.) mg/1	Mean fork length, mm				
	Pink Fry	Coho Alevins	Coho Fry	Rainbow Fry Group I	Raibow Fry Group II
0.220	29.5[a]	25.4[b]	27.2[a]	20.9[a]	22.3[b]
0.075	31.2	25.9	27.9	22.6	23.0
0.05 (nominal)	31.1	26.9[c]	28.5	22.6	23.1
control	31.1	26.1	28.1	22.5	22.9

[a] Significantly shorter than control (p = .01).

[b] Significantly shorter than control (p = .05).

[c] Significantly longer than control (p = .05).

DISCUSSION

In this study 2,4-D acid was much less toxic than BEE to sockeye fingerlings. No mortality or distress was observed among sockeye fingerlings exposed to 200 mg/l 2,4-D acid for 168 hours while the 96 LC50 for BEE was about 0.45 mg/l. Thus the toxicity of 2,4-D acid was less than 0.2% of the toxicity of BEE. A similar result was reported for fathead minnows (Pimephales promelas), where no observable effect was seen at 500 mg/l 2,4-D acid while BEE had an LC50 of 5.6 mg/l in static bioassays, (Mount and Stephan, 1967). Somewhat similar results were reported by Meehan, Norris and Sears (1974) who reported the butyl ester of 2,4-D was lethal at 1 mg/l while 2,4-D acid was not lethal to Alaska coho fingerlings, dolly varden (Salvelinus malma) and rainbow at 50 mg/l. These authors also reported chum fry (O. keta), coho fry, sockeye smolts and Oregon coho fingerlings experienced variable mortality at 50 mg/l 2,4-D acid but none at 10 mg/l. Pink fry experienced a wide range in mortality from 0 to 20% at 1 mg/l to 0 to 90% at 10 mg/l 2,4-D acid. In another study, no mortalities were observed when yearling coho salmon were challenged with 200 mg/l of 2,4-D dimethylamine for 144 hours followed by transfer to seawater for 240 hours (Lorz et al, 1979).

A review by the National Research Council of Canada (1978) summarized the toxicity of various 2,4-D ester formulations to fish but little information was available on the toxicity of BEE. The 48 hr LC50 for bluegill (Lepomis machrochiris) was 2.1 mg/l BEE (Hughes and Davis, 1963) but a 24 hour LC50 equal to 1.5-1.6 mg/l (a.e. not specified) was reported for rainbow trout (Inglis and Davis, 1972). A recent study reports a 96 hour LC50 of 3.05 mg/l BEE for rainbow trout fingerlings (Pearce and McBride, 1978). The foregoing LC50 values are much larger than those

reported herein where the 96 hour LC50 values were 0.45 mg/1 BEE for sockeye fingerlings, coho fry and pink fry and between 0.2 and 0.4 mg/1 BEE for rainbow trout fry. Shorter exposure times may account for part or all of the differences in LC50's between the studies by Hughes and Davis, (1963) and Inglis and Davis (1972) and the present study. In addition, bluegills and sockeye salmon may not be equally sensitive to BEE. The difference in 96 hour LC50 values reported by Pearce and McBride (1978) and those reported herein may be owing in part to differing bioassay procedures. Aqua-Kleen 20 was pulverized before use in bioassays in the present study while in the study by Pearce and McBride, Aqua-Kleen granules were added directly to bioassay aquariums (B. Pearce, personal communication). Use of the larger clay granules would probably result in a slower BEE solution rate. In addition, bioassay solutions were renewed daily in the present study (Monday-Friday), but bioassay solutions in the Pearce and McBride study were not renewed. According to Table 1, BEE would decline to a fraction of the nominal amount in 96 hours owing to hydrolysis. Finally, 96 hr LC50 values reported herein were based on measured amounts of BEE whereas Pearce and McBride reported nominal concentrations.

The lowest concentration of BEE causing a significant adverse effect during egg to fry exposure was 0.220 mg/1. This concentration was highly lethal to rainbow trout eggs during an 85 day exposure while coho alevins and fry and pink fry were significantly smaller than controls. Rainbow trout exposed to 0.220 mg/1 BEE during development from alevin to fry were significantly smaller than controls after 17 days. No adverse effects were observed at 0.075 mg/1 BEE.

The lowest concentration of BEE causing an observable adverse effect on test specimens was about 50% of the 96 hr LC50 while the "no effect" concentration was about 17% of the LC50. Mount and Stephan (1967) reported the "no effect" and lowest observed effect concentrations were about 5 and 25%, respectively, of the 96 hr LC50 for fathead minnows exposed to BEE in life cycle studies.

The fate of BEE in water is essential to assessing proposals to control milfoil by applying Aqua Kleen 20. The degradation of phenoxyherbicides in water was discussed extensively in a review by the National Research Council of Canada (1978). Thus only an abbreviated summary will be given in this report. Briefly, mechanisms by which BEE decreases in water are microbial decomposition, photodecomposition, volatilization and chemical hydrolysis.

Microbial decomposition of BEE was studied by Paris et al (1975) who concluded that BEE was rapidly converted to 2,4-D acid by acclimated bacteria and fungi in basal salts medium but degradation of 2,4-D acid was much slower. After three hours, 99% of a 9.3 μ mol/l (2.99 mg/l) solution of BEE was converted to 2,4-D acid. However, 20% of the 2,4-D acid remained after 11 days. Similarly, Aly and Faust (1964) reported esters of 2,4-D were hydrolysed to 2,4-D acid and the corresponding alcohol by bacteria in aqueous solution, but only the alcohols were oxidized by the bacteria. Further tests showed 2,4-D acid persisted up to 120 days in lake waters incubated in the laboratory. However, 2,4-D acid was degraded in 65 and 35 days, respectively, in muds taken from a lake which had not been treated with 2,4-D and one which had been treated previously. Schwartz (1967) also reported 2,4-D acid resistant to biological degradation; noting at least 60% of 2,4-D acid persisted for 3 to 6 months

in spite of excellent conditions for biological activity in aqueous solution.

Photolysis of BEE in water is a slow process dependent on pH, season, time of day and latitude (Zepp et al, 1975). These investigators found the half-life of BEE under September sunlight in the southern United States was about 14 days. Photolysis half-life in Canada would be greater than 14 days owing to the more northerly latitude.

Volatilization rates of pesticides from completely mixed water bodies are proportional to their vapor pressures and inversely proportional to their solubilities (Mackay and Wolkoff, 1973). Based on the foregoing relationships, Zepp et al (1975) calculated half-lives ranging from about 1 day for the butyl ester of 2,4-D to over two years for the low-volatile BEE at a water depth of one meter.

Rapid hydrolysis rates observed in alkaline water led Zepp et al (1975) to suggest hydrolysis is often the major pathway for transformation of 2,4-D esters in natural waters. These authors determined that reaction rate for chemical hydrolysis of BEE is a function of pH and temperature; primarily pH. For example, using reaction rate constants reported by Zepp et al (1975) at 28^{o}C, half-lives of BEE owing to hydrolysis were 6.4 and 63.6 hours at pH 8 and 7, respectively. Half-lives would be longer at lower temperatures, but reaction rate constants were not reported. Precise measurements of reaction rate constants for hydrolysis of BEE could not be made from the data reported herein since the change in concentration of BEE observed (Table 1) was a function of dissolution from pulverized clay granules of Aqua Kleen 20 and hydrolysis.

It is evident from the foregoing discussion that BEE will be hydrolyzed to 2,4-D acid when applied to water. The rate of hydrolysis will be a combination of chemical and biochemical reactions dependent on temperature,

pH and presence of acclimated bacteria. Further testing is needed to define chemical hydrolysis reaction rate constants for a range of temperatures encountered in waters of British Columbia. Finally, since BEE is more toxic to aquatic life than 2,4-D acid, monitoring at sites where Aqua Kleen 20 is applied should include measurements of BEE as well as 2,4-D acid.

CONCLUSIONS

1. When pulverized Aqua-Kleen 20 was applied to water, dissolution caused an initial increase in BEE followed by decline as hydrolysis to 2,4-D acid became the dominant process.
2. The 96 hr LC50 of BEE to sockeye fingerlings, coho and pink fry was approximately 0.45 mg/1. The 96 hr LC50 would be between 0.45 and 0.54 mg/1 BEE for sockeye smolts and was between 0.2 and 0.4 mg/1 BEE for rainbow trout.
3. The lowest concentration of BEE tested which caused an observed adverse effect was 0.220 mg/1. No adverse effects were observed at 0.075 mg/1 BEE.
4. Pre-exposure of sockeye smolts and pink fry to 0.540 mg/1 BEE for 24 hours did not result in mortality during exposure to BEE or in a subsequent exposure to 28 ^{o}/oo salt water for 96 hours.
5. No mortality or distress was evident among sockeye fingerlings exposed to 200 mg/1 2,4-D acid for 168 hours.

ACKNOWLEDGEMENTS

The cooperation and assistance of the Department of Fisheries and Oceans (Pacific Environment Institute) in supplying herbicide analyses is gratefully acknowledged.

Measurements of alevin and fry length were made courtesy of Habitat Protection, Department of Fisheries and Oceans.

In addition, coho salmon eggs supplied by Department of Fisheries and Oceans (Capilano Hatchery) and rainbow trout eggs provided by B.C. Fish and Wildlife Branch (Fraser Valley Trout Hatchery) are gratefully acknowledged.

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