RECEIVED BY DTIC JUN 7 1971

ACCUMULATION AND RETENTION OF TRITIUM (TRITIATED WATER)
IN EMBRYONIC AND LARVAL FISH, AND RADIATION EFFECT¹

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by

J. A. Strand, W. L. Templeton, and E. G. Tangen
Ecosystems Department, Battelle Memorial Institute
Pacific Northwest Laboratory, Richland, Wash. 99352

For: Third National Symposium on Radioecology, Oak Ridge, Tennessee,
10-12 May, 1971

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¹Support for Thermal Effects Studies in the Columbia River at Hanford, Washington is provided by the U.S. Atomic Energy Commission under Contract AT(45-1)-1830 with Battelle Memorial Institute, Richland, Washington.

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Abstract

Eggs of rainbow trout, Salmo gairdneri, were spawned artificially in the laboratory and exposed to 0.01, 0.1, 1.0 and 10.0 $\mu\text{C}/\text{ml}$ tritium- H_3 (biological grade) contaminated spring water. Rearing throughout embryogenesis, 25 days at $10.5 \pm .5^\circ \text{C}$, was facilitated within a recirculating drip incubation system. Water and embryos were sampled at intervals to ascertain the time necessary to reach equilibrium. After 20 days exposure, selected lots of embryos were removed to inactive free-flowing water to study retention and turnover components. Combustion of samples for liquid scintillation counting was accomplished using Model 300 Tri-Carb Sample Oxidizer. Distinction between volatile or "body-water" tritium- H_3 and non-volatile or "bound" tritium- H_3 was emphasized.

A rapid uptake of tritium- H_3 during the first 1 to 2 days was indicated for all three concentrations, followed during the ensuing 27 to 28 days of exposure by maintenance of equilibrium levels. The "bound" tritium- H_3 fraction accounted for approximately 20 percent of the total label in each egg, a level not exceeded as measured over the duration of exposure. Both volatile and "bound" fractions, after transfer to free-flowing water, elute rapidly with estimated half-times of ~ 1.0 and ~ 2 hours, respectively. A slower component of the volatile fractions persists for ~ 17 days, as determined in hatched fry. The slow component of the "bound" fraction, for comparison, persists at approximately 0.6 percent of the initial "bound" equilibrium level at 17 days. Even after six months, in juveniles, a trace but measurable level of this component was detected.

The effects of each treatment on embryogenesis was assessed in terms of the proportions of eggs hatching and the numbers of obviously abnormal larvae produced. Selected behavioral and physiological tests were applied to detect "sub-lethal" effect. Preliminary consideration of all data indicated that no significant degree of detriment resulted from exposure at these levels during embryogenesis.

Introduction

The biological consequences of low level irradiation has become an important consideration in all environments. Knowledge in the aquatic areas is, however extremely limited, and is mainly concerned with somatic effects. Since tritium will be a major contributor to the radioactivity in the effluents from nuclear power plants and cannot be removed by conventional technology, increasing concern has been expressed as to the ultimate concentration in and the effect upon segments of the aquatic food web.

Accumulation and Incorporation

Most studies on the accumulation of tritium have emphasized acute exposures carried out over short durations of time and have generally reflected rapid biological turnover or half-life. Such investigations using aquatic organisms have demonstrated the incorporation of tritium into body water occurs at a rate corresponding to a half-time of approximately 15 minutes (Foster 1955). After several hours exposure, the tritium concentration amounted to only 95 per cent of that of the environment (Foster 1955). However, it is known from studies of mammalian systems, slower turnover components are characteristic of tritium elimination and presumably incorporation. Certain tissue components have demonstrated half-times of 100 to 300 days (Thompson 1954). As well, under chronic exposure conditions, there may occur greater organic binding in remote body pools which could result in an overall slower turnover rate. Under equilibrium conditions, this could result in higher concentration and hence radiation exposures, than would be predicted from water concentrations alone.

Radiation Effects

Most available experimental data is concerned with the effects of acute exposures of radiation. While these data indicate that fish and other aquatic organisms are relatively radioresistant in comparison with terrestrial mammals, little pertinent data is available for assessment of the effects of low-level and chronic exposure regimens.

In Soviet literature, which is extensive in the area of radioecology, there is indication that embryonic development in fish is seriously impaired at radioactivity concentrations of 10^{-10} to 10^{-12} Ci/liter; that is of ^{90}Sr , ^{90}Y (Polikarpov 1966). However, similar studies conducted in the United Kingdom and United States in no way substantiate effects at such low levels (Templeton et al 1970). Over all, it would appear that an activity concentration of 10^{-4} Ci/liter is the level at which most pronounced effect will likely occur, and which is obviously many orders of magnitude greater than that can be expected in the environment due to current practices of waste disposal. Although studies in the United States and United Kingdom demonstrate relatively high concentrations of radioactivity are required to cause mortality and even abnormality, it is clear that embryonic and larval life stages are more radiosensitive than other ontogenetic forms. To date, experimental studies on chronic and "sub-lethal" effects in fish and other aquatic life forms have been limited to studies on fish embryos and larval forms of shellfish for restricted developmental periods and have produced conflicting results. Furthermore, in no known situations have similar studies with tritium been conducted with aquatic species.

Preliminary investigations recently made into this subject at this laboratory in an attempt to resolve some of the conflicting data are reported herein.

Methods

Eggs of rainbow trout, Salmo gairdneri, were spawned artificially in the laboratory, water hardened; and at 6 hr after fertilization were immersed in 0.01, 0.1, 1.0, and 10.0 $\mu\text{Ci/ml}$ tritium (biological grade) contaminated spring water (essentially pathogen free). Rearing throughout embryogenesis, 28.5 days at $10.6 \pm 0.5^\circ \text{C}$ was facilitated within a recirculating drip incubation system of 150-liter capacity, Figure 1.

Accumulation and Incorporation

Eggs used for tritium analyses were rapidly frozen and stored at -40°C . Combustion of samples for liquid scintillation counting was accomplished using the Model 300 Tri-carb Sample Oxidizer. Eggs in which volatile and "bound" fractions were separated were removed from the freezer and placed in an open paper cup. The membranes of each egg were ruptured by puncture with a sharp scalpel blade. The egg was then immediately placed within the oxidizer unit and subjected to an instrument heat of 200°C . A low flow of N_2 gas was used to sweep the evolved volatile tritium fraction into the scintillant solution. This procedure was carried out for a duration of 45 min at which time a fresh scintillant vial was emplaced and the residue combusted in the oxygen stream to obtain the "bound" tritium fraction. A naphthalene, PPO, POPOP, dioxane, toluene, and methanol scintillant was used for all determinations.

To ascertain the distribution of beta particles within the embryo and larvae, autoradiographs were prepared by dipping slides in Ilford K.5 liquid emulsion, exposing for 12 weeks, and processing in Kodak D-19b developer. These slides were stained through the emulsion with

hematoxylin and eosin.

Radiation Effects

The effects of each treatment were assessed in terms of the proportions of eggs hatching and the numbers of obviously abnormal larvae produced. Anomalies included major malformations of the eyes and of the body. Additionally, length frequencies after hatch were determined. The eggs were inspected each day and all dead eggs removed. Infertile eggs were not removed until 16 days after fertilization or when embryos were firmly eyed. The experiment was terminated at hatching.

Selected behavioral and physiological tests were applied to detect "sub-lethal" effect. The relative performance of tritium-treated (1.0 $\mu\text{Ci/ml}$ exposure for 20 days during embryogenesis) and untreated (control) juvenile fish to predation was determined using the method of Bams (1967). For other groups of tritium-stressed fish (1.0 $\mu\text{Ci/ml}$ exposure for 20 days during embryogenesis), equilibrium loss and thermal death times were determined.

Results

Accumulation and Incorporation

A rapid uptake of tritium during the first 1 to 2 days was demonstrated at all concentration levels; this was followed during the ensuing 27 to 28 days of exposure by maintenance of equilibrium levels. The patterns of uptake for both volatile and "bound" fractions at the 0.1 $\mu\text{Ci/ml}$ level are illustrated in Figure 2. The "bound" tritium fraction accounts for approximately 20% of the total label in each egg, a level not exceeded as determined over the duration of exposure.

The loss of tritium upon transfer to inactive free-flowing water after exposure for 20 days at an initial level of 1.0 $\mu\text{Ci/ml}$ is shown in Figure 3. For both volatile and "bound" fractions, fast and slow components of turnover are indicated. Initially both volatile and "bound" fractions elute rapidly. A slower component of the volatile fraction persists for ~ 17 days, as determined in hatched fry. The slow component of the "bound" fraction, for comparison, persists at $\sim 0.6\%$ of the initial bound equilibrium level at 17 days.

From autoradiographs it was evident that exposure to tritiated water resulted in essentially a uniform distribution of the isotope in all tissues of the embryo and subsequently all tissues and organs of the larvae. No localization within nuclear organelles was demonstrated.

Radiation Effects

The results of exposure to the four concentrations of tritiated water as determined throughout embryogenesis are illustrated in Tables I and II. In experiment No. 1, the 95% Confidence Limits for each treatment compared with the control indicate that under the described experimental conditions, a statistically significant reduction in hatching percentage could not be demonstrated. In fact, a slight enhancement in hatching efficiency occurred in the 0.01 $\mu\text{Ci/ml}$ and 0.1 $\mu\text{Ci/ml}$ levels. No demonstrable delay in time of hatching for any of the three treatment levels as compared with the control was observed. A statistically significant increase in the incidence of abnormal larvae as compared with the control was encountered. The embryos in each treatment produced larvae that were of significantly different size than those of the control, as determined at 35 days, although the high concentration produced larvae of greater length.

In experiment No. 2, the lack of rigid temperature control during the last week of development unavoidably resulted in terminating the tritium treated lots before the greater proportion of the embryos had hatched. The control lot hatched under near normal experimental conditions. However, as in Table II if mortality is normalized to a period in development where environmental parameters were adequate, 21 days, then a comparison between control and each treatment level is possible. In this case, a significant increase in the incidence of mortality as compared with the control is encountered, and which may be correlated to increasing exposure level. From Table I, the embryos in the 0.1 $\mu\text{Ci/ml}$ and 1.0 $\mu\text{Ci/ml}$ levels produced significantly more abnormal larvae than the control.

For each of the procedures applied to detect "sub-lethal" effect as a result of exposure during embryogenesis and inclusive of determinations of growth rate (Table III), relative performance to predation (Table IV, and equilibrium loss and thermal death (Table V), no consistent statistically significant degree of impairment was demonstrated.

Discussion

Accumulation and Incorporation

By inspection of the uptake curves in Figure 2, and with the understanding that the level of isotope in the treatment bath also decreases with time, it would appear that no concentration of tritium above the water equilibrium level had occurred. However, to adequately prove this contention, further statistical analyses normalized on an embryonic weight or available water volume basis must be performed. Preliminary findings in support of this effort disclose an estimated concentration factor of only 0.92 to 1.1.

The curves for elution of volatile and "bound" fractions, Figure 3, indicate, as would be expected, an exponential loss of activities over the first several days. The slopes of the curves correspond to estimated half times of ~ 1.0 and 2.0 hr respectively. As expected, the rate of loss of tritium activity in the volatile fraction becomes equal to the rate of loss of the "bound" fraction. This may be attributed to the fact that after sufficient time all essentially volatile or "body water" tritium will be derived from the breakdown or turnover of "bound" tritium-containing organic compounds. The "bound" curve can be resolved fairly well into two major exponential components with slopes corresponding to biological half lives of ~ 2.0 hours and ~ 8 days. Such resolution can only be considered as an approximation of tritium-binding capacities. Undoubtedly, there are as many more components of the curves as there are compounds in the embryo that are capable of binding tritium. Further uncertainty of the half life of the longer lived components is realized because of the lack of information as to the course of the curve beyond 17 days.

Radiation Effects

A review of all experimental results to date reveal considerable variation between similar treatments, and as great as those differences arising within treatments. Statistically significant reduction in the proportions of embryos attaining a particular stage in development or hatching could not be demonstrated consistently. Although a statistically significant increase in the incidence of abnormal larvae as compared with controls is encountered in most all treatments; the degree of increase could not be correlated to increasing exposure level.

These data, in degree, conflict with those reported elsewhere (Polikarpov 1966), although rearing within essentially a pathogen free environment may have afforded some level of protection. There is, however, the possibility that impairment at the higher exposure levels cannot be detected with the criteria presently applied; that is embryonic death, larval abnormality and such "sub-lethal" indices as growth, predation, equilibrium loss and thermal death. However, the present effort treats only a portion of the early life stages. Longer term studies will be necessary to investigate the problem fully, to examine first embryonic mitosis (four-cell) blastomere stage, to examine the time of reabsorption of yolk sac, and to examine the production of chromosome aberrations.

Acknowledgements

The authors wish to acknowledge the assistance of Mr. C.W. Apts, Mr. R.G. Genoway, and Mr. E.W. Lusty in the experimental work, and of Mr. A.C. Case for tritium analyses, and of Mr. R.J. Olson and Mr. J.M. Thomas for statistical analyses.

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Table I. Effect of Tritiated Water on Embryogenesis of Salmo gairdneri

Experiment 1

<u>Treatment</u>	<u>No. Eggs</u>	<u>% Hatch</u>	<u>95% Binomial Confidence Limits</u>	<u>No. Larvae Sampled</u>	<u>% Abnormal</u>	<u>95% Binomial Confidence Limits</u>	<u>35 Day Larval Length mm + S.D.</u>	<u>+ 95% Confidence Value</u>
Control	3743	79.2	77.5-80.4	2298	1.0	0.6-1.4	17.00 $\pm$ 0.909	0.06
0.01 μ Ci/ml	7985	83.1	82.2-83.7	6240	5.5	4.9-6.1	16.89 $\pm$ 1.04	0.04
0.1 μ Ci/ml	8123	81.9	81.2-82.8	6352	3.5	3.0-4.0	17.60 $\pm$ 0.892	0.04
1.0 μ Ci/ml	4239	80.2	78.9-81.1	3338	1.8	1.4-2.3	17.36 $\pm$ 0.886	0.05

Experiment 2

Control	3182	89.6	88.9-91.0	2849	0.7	0.4-1.1		
0.1 μ Ci/ml	6953	66.8	65.7-68.3	2882	1.6	1.2-2.1		
1.0 μ Ci/ml	3434	57.0	55.4-58.5	1918	3.5	2.8-4.4		
10.0 μ Ci/ml	4413	72.8	71.6-74.4	3203	0.5	0.3-0.8		

Table II. Effect of Tritiated Water on Embryogenesis of Salmo gairdneri

Experiment 2

<u>Treatment</u>	<u>No. Fertile Eggs</u>	<u>No. Deaths Through 21 Days</u>	<u>% Mortality Through 25 Days</u>	<u>95% Binomial Confidence Limits</u>
Control	3166	270	8.5	7.5-9.5
0.1 $\mu\text{Ci/ml}$	6946	747	10.7	9.9-11.6
1.0 $\mu\text{Ci/ml}$	3431	681	19.8	18.4-21.4
10.0 $\mu\text{Ci/ml}$	4411	618	14.0	12.9-15.1

Table III. Growth of Tritium-treated (1.0 $\mu\text{Ci/ml}$ - 20 days During Embryogenesis) and Untreated (Control) Salmo gairdneri

<u>Treatment</u>	<u>Larval Age Days</u>	<u>No. Observations</u>	<u>Mean Length mm $\pm$ S.D.</u>	<u>95% Confidence Limits</u>	<u>95% Confiden Value</u>
Control	35	800	17.36 $\pm$ 0.886	17.30-17.43	0.06
1.0 $\mu\text{Ci/ml}$	35	1050	17.60 $\pm$ 0.892	17.55-17.66	0.05
Control	76	100	28.34 $\pm$ 2.811	27.78 $\pm$ 28.90	0.55
1.0 $\mu\text{Ci/ml}$	76	100	28.43 $\pm$ 2.408	27.95 $\pm$ 28.91	0.47
Control	125	106	45.71 $\pm$ 6.604	44.47 $\pm$ 46.98	1.26
1.0 $\mu\text{Ci/ml}$	125	203	50.55 $\pm$ 6.706	49.62-51.47	0.92
Control	149	75	60.25 $\pm$ 8.566	58.29-62.22	1.96
1.0 $\mu\text{Ci/ml}$	149	97	61.65 $\pm$ 7.910	60.06-63.25	1.59

Table IV. Relative Performance of Tritium-treated (1.0 μ Ci/ml - 20 Days During Embryogenesis) and Untreated (Control) Juvenile Salmo gairdneri to Predation as Determined at 5 Months

<u>Control</u>			<u>Experimental</u>		
Mean Length mm $\pm$ S.D.	No. Survivors	χ^2	No. Survivors	Mean Length mm $\pm$ S.D.	t
70.89 $\pm$ 3.420	14	0.037	13	68.08 $\pm$ 2.992	2.264*
68.53 $\pm$ 3.093	19	1.125	13	71.46 $\pm$ 4.629	-2.151*
69.63 $\pm$ 3.841	16	0.032	15	68.73 $\pm$ 3.693	0.664
56.38 $\pm$ 4.454	13	0.037	14	57.86 $\pm$ 3.433	-0.971
66.54 $\pm$ 5.078	18	0.727	9	74.50 $\pm$ 8.635	-2.727*
55.06 $\pm$ 5.596	10	0.000	18	55.78 $\pm$ 4.326	0.431
58.60 $\pm$ 4.509	16	0.000	10	58.20 $\pm$ 4.138	0.206
57.28 $\pm$ 4.895	11	0.561	12	60.38 $\pm$ 2.805	-1.960
58.27 $\pm$ 5.636	10	0.474	8	63.13 $\pm$ 4.198	-2.053
71.71 $\pm$ 7.011	7	0.250	9	75.00 $\pm$ 6.892	-0.940
70.08 $\pm$ 5.599	12	0.000	12	72.08 $\pm$ 7.751	-0.724
76.46 $\pm$ 7.523	13	0.040	12	74.25 $\pm$ 5.987	0.808
71.45 $\pm$ 6.906	10	0.053	9	75.44 $\pm$ 8.218	-1.149
57.38 $\pm$ 3.704	16	3.522	7	56.07 $\pm$ 5.070	0.698
57.70 $\pm$ 4.762	10	0.222	8	59.94 $\pm$ 4.460	-0.991
60.25 $\pm$ 2.701	12	0.154	14	60.86 $\pm$ 3.278	-0.512
75.27 $\pm$ 5.849	15	0.333	12	77.58 $\pm$ 9.307	-0.788
75.10 $\pm$ 6.607	10	0.222	8	78.25 $\pm$ 7.987	-0.917
59.50 $\pm$ 3.215	10	1.000	15	60.80 $\pm$ 4.507	-0.786
62.12 $\pm$ 2.678	13	0.167	11	61.95 $\pm$ 3.070	0.144
71.70 $\pm$ 7.634	15	0.143	13	73.19 $\pm$ 5.907	-0.570

* significant at 0.05 level

Table V. Thermal Sensitivity of Tritium-Treated (1.0 $\mu\text{Ci/ml}$ - 20 Days During Embryogenesis) and Untreated (Control) Juvenile Salmo gairdneri

Temp. $^{\circ}\text{C}$	Treatment	Equilibrium Loss Time		Death Time	
		Geom. Mean	95% Confidence Limits	Geom. Mean	95% Confidence Limits
30.0	Control	2.06	1.52-2.80	3.21	2.35-4.39
	1.0 $\mu\text{Ci/ml}$	1.97	1.43-2.71	2.93	2.21-3.89
29.0	Control	12.38	10.87-14.10	15.94	14.75-17.23
	Control	11.26	7.55-16.79	15.77	10.75-23.14
	1.0 $\mu\text{Ci/ml}$	8.77	5.48-14.03	12.14	8.67-17.00
	1.0 $\mu\text{Ci/ml}$	9.09	5.56-14.87	14.53	11.77-17.94
28.0	Control	22.55	16.28-31.24	28.75	22.79-36.27
	Control	11.02	6.50-18.68	18.09	12.38-26.44
	Control	8.41	7.95-8.90	10.96	7.01-17.14
	Control	5.10	2.93-8.87	9.33	5.67-15.36
	1.0 $\mu\text{Ci/ml}$	29.18	25.99-32.76	36.25	31.61-41.58
	1.0 $\mu\text{Ci/ml}$	3.43	2.72-4.33	9.92	6.25-15.74
	1.0 $\mu\text{Ci/ml}$	9.02	6.14-13.25	16.12	11.90-21.84
	1.0 $\mu\text{Ci/ml}$	4.16	3.18-5.44	10.39	6.60-16.37
27.0	Control	105.9	92.2-121.5	120.3	110.9-130.6
	1.0 $\mu\text{Ci/ml}$	94.4	87.2-102.3	114.3	102.5-127.5
26.0	Control	-	-	413	336-508
	1.0 $\mu\text{Ci/ml}$	-	-	440	339-571

Figure 1. Recirculation drip incubation system used in rearing trout embryos. Freshwater is pumped at a rate of 15 liters per min from reservoir (a) to level sensing tank (b) then through air equilibrator (c) into rearing trays (d), then returning to reservoir.

Figure 2. Accumulation of tritium in eggs of Salmo gairdneri.

Figure 3. Retention of tritium in eggs of Salmo gairdneri.