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EFFECTS OF POLLUTANTS ON EMBRYOS AND LARVAE OF AMPHIBIAN SPECIES

Second Annual Report

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AIR FORCE SYSTEMS COMMAND
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This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
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This report for the period 1 Sep 75 - 31 Mar 76 contains the results of research efforts concerned with defining the environmental effects of potential environment contamination resulting from the use of certain Air Force materials. The effects of exposure to N-phenyl- α -naphthylamine, hydrazine, methylhydrazine, and dimethylhydrazine on the development of Xenopus frogs are reported.

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PREFACE

This is an interim report of work performed under Air Force Contract F33615-76C-5005. The project title is "Effects of Pollutants on Embryos and Larvae of Amphibian Species."

This report, for the period 1 Sep 75 - 31 Mar 76, contains the results of research efforts concerned with defining the environmental contamination resulting from the use of designated Air Force materials. This project evaluates the effects of exposure to N-phenyl- α-naphthylamine, hydrazine, methylhydrazine, and dimethylhydrazine on the development of Xenopus laevis frogs.

The author gratefully acknowledges the technical assistance of Ms. Penelope Kimbrell.

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INTRODUCTION AND SUMMARY

The objective of this study was to establish a pollutant test system using embryonic and larval stages of frogs. The system was to be directed at assaying the effects of designated compounds on the development of frogs and to determine ED50 and no-effect levels.

Work is described using the above test system to screen two groups of compounds designated by the Air Force as potential environmental pollutants. The first group consisted of three substituted amines, N-phenyl- α -naphthylamine, octyl-phenyl- α -naphthylamine, and p,p'dioctyldiphenylamine. Only N-phenyl- α -naphthylamine was found to have significant toxic effects on developing frog embryos and larvae. The second group of compounds tested included hydrazine and its methylated derivatives monomethylhydrazine and dimethylhydrazine (both symmetrical and unsymmetrical). All four of these compounds were found to have toxic effects on developing frog embryos and/or larvae.

Work completed during the period 1 Feb 74 - 31 May 75 established no effect and lethal levels of exposure for these compounds. Some of these compounds were also found to be teratogenic. This data has been reported elsewhere (Greenhouse, 1975; 1976a,b,c,d).

The data presented below establishes the LD50 of N-phenyl- α -naphthylamine for Xenopus larvae, and the ED50 (teratogenic) of N-phenyl- α -naphthylamine, hydrazine, methylhydrazine, unsymmetrical dimethylhydrazine, and symmetrical dimethylhydrazine for Xenopus embryos.

MATERIALS

N-phenyl- α -naphthylamine, octyl-phenyl- α -naphthylamine and p,p'dioctyldiphenylamine were supplied by the Aerospace Medical Research Laboratory. Hydrazine was obtained from Sigma Chemical Company. Methylhydrazine and symmetrical dimethylhydrazine were obtained from Aldrich Chemical Company. Unsymmetrical dimethylhydrazine was obtained from Research Organic/Inorganic Chemical Corporation.

METHODS

Adult Xenopus laevis were captured in drainage ditches in Costa Mesa, California, or obtained commercially from the South African Snake Farm, Fish Hoek, Cape Province, South Africa. Adult frogs were maintained in glass aquaria and fed Purina Trout Chow twice weekly.

Fertilized eggs were obtained by injecting pairs of frogs with human chorionic gonadotropin (Sigma) by standard laboratory technique (Brown, 1970). Xenopus laevis embryos and larvae were staged according to Nieuwkoop and Faber (1956). Embryos were dejellied in 3% cystein, 0.1% papain, pH 8.0, and then cultured in aged dechlorinated tap water

in glass bowls with an inner diameter of 19 cm. Each bowl contained one liter of water and 100 embryos. Embryos at the desired stage were placed in one liter of an aqueous solution of the chemical to be tested.

Only clutches of eggs which proved to be at least 95% fertile were used. Each experimental group was always paired with control eggs obtained from the same mated pair of frogs.

Embryos were scored as normal or abnormal by examination under a dissecting scope.

Whenever possible, data was evaluated by the method of Litchfield and Wilcoxon (1949). Their procedure entails plotting data on logarithmic-probability paper and fitting a straight line through the points using a modified Chi square test. The ED50 is estimated from this line. Confidence limits are calculated from slope of the line, an exponent derived from the tabulated data, and a nomograph supplied by Litchfield and Wilcoxon (1949).

If the data was not suitable for analysis by the above method, the ED50 was estimated from a log-dose percent plot of the data (Goldstein, Aronow, and Kalman, 1974).

RESULTS

N-phenyl- α-naphthylamine

Previous work had shown that neither octyl-phenyl- α-naphthylamine nor dioctyldiphenylamine was toxic to frog embryos or larvae, whereas exposure to N-phenyl- α-naphthylamine produced abnormal development and/or death. The experiments reported below confirm and extend these findings.

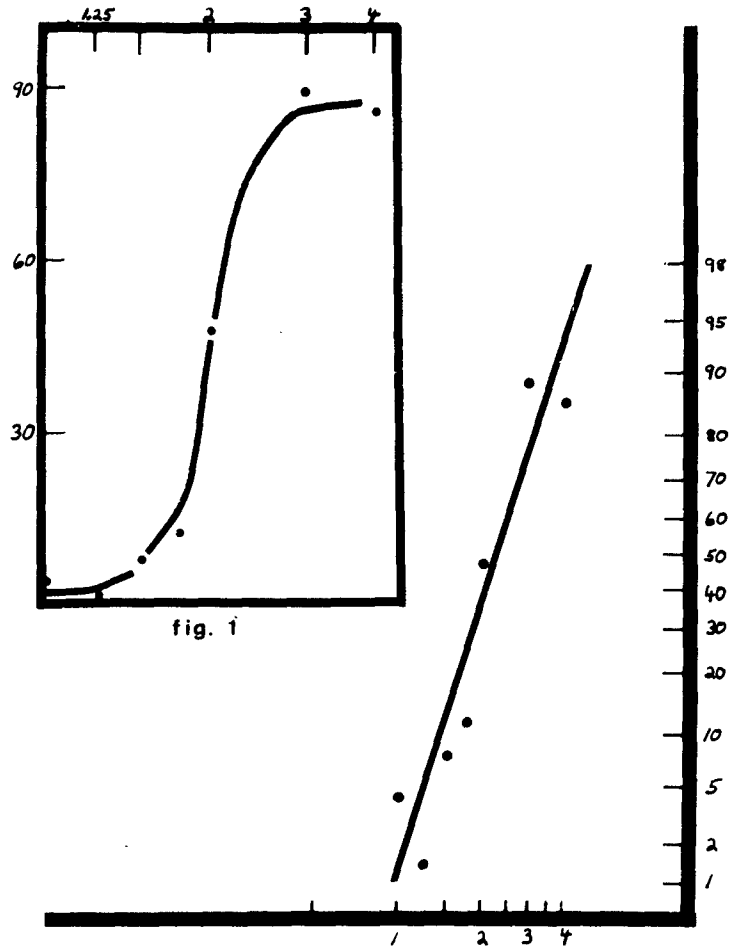
Table 1 and figures 1 and 2 summarize data from a series of determinations of the effect of exposure to N-phenyl- α-naphthylamine on viability of Xenopus larvae.

Table 1

EFFECT OF N-PHENYL- α-NAPHTHYLAMINE ON VIABILITY OF XENOPUS LARVAE

Larvae were exposed to N-phenyl- α-naphthylamine dissolved in aged tap water. After 48 hours the number of dead/total number larvae was recorded.

Concentration	Experiment Number							Totals	% Dead
mg/liter	1	2	3	4	5	6	7		
1.00	0/100	0/10	0/10	7/20	0/10	0/10		7/160	4.3
1.25	2/100	0/10	0/10	0/10	0/11			2/141	1.4
1.50	0/25	2/100	3/10	8/10	0/10	0/10		13/165	7.8
1.75	13/44	0/25	2/100	0/10	8/9			23/188	12.2
2.00	77/77	20/30	15/27	0/5	0/5	6/10	24/53	142/297	47.8
3.00	52/52	0/5	10/10	0/5	11/11	10/10		83/93	89.2
4.00	5/5	10/10	10/10	1/5				26/30	86.6



EFFECT OF N-PHENYL- α -NAPHTHYLAMINE ON VIABILITY OF XENOPUS LARVAE

Figure 1 - Log-concentration percent mortality plot of the pooled data in table 1. Ordinate-percent killed. Abscissa-concentration.

Figure 2 - Log-concentration-probability plot of the pooled data in table 1. Ordinate-percent killed. Abscissa-concentration.

A log-dose percent lethality plot of the data (fig. 1) in table 1 yields an estimated LD50 of 2.1 mg/l.

The log-dose-probability plot of the data (fig. 2) yields an estimated LD50 of 2.3 with 95% confidence limits between 1.96 and 2.76.

Table 2 summarizes data from a series of determinations of nonlethal toxic effects of exposure to N-phenyl-α-naphthylamine on Xenopus larvae.

Table 2

EFFECT OF N-PHENYL-α-NAPHTHYLAMINE ON MOTILITY OF XENOPUS LARVAE

Larvae were exposed to N-phenyl-α-naphthylamine dissolved in aged tap water. After 24 hours the number of live but immobile/total number larvae was recorded.

Concentration mg/liter	Experiment Number 1	2	3	4	5	6	Totals	% Dead
1.00	0/100	0/10	10/10	20/20	10/10	6/10	46/160	29
1.25	0/100	0/10	10/10	10/10	10/10	6/11	36/151	24
1.50	50/50	20/20	25/25	100/100	10/10	30/30	235/235	100
1.75	44/44	100/100	25/25	10/10	9/9		188/188	100
2.00	30/30	0/5	0/5	94/94	10/10	10/12	144/156	
3.00	5/5	5/5	12/12				22/22	100

It can be seen from table 2 that all concentrations tested were toxic and that the ED50 was between 1.25 and 1.50 mg/liter. Concentrations less than 1 mg/liter were not tested.

Table 3 summarizes data on the teratogenic effect of N-phenyl-α-naphthylamine on Xenopus embryos.

Table 3

TERATOGENIC EFFECT OF N-PHENYL-α-NAPHTHYLAMINE ON XENOPUS EMBRYOS

Embryos were continuously exposed to N-phenyl-α-naphthylamine from blastula until hatching at which time the number of malformed embryos/ total number of embryos was recorded.

Concentration mg/liter	Experiment Number 1	2	3	4	Totals	% Malformed
3.0	15/100				15/100	15
4.0	34/100	18/105	4/79		56/284	20
4.5	100/100	17/100	41/82		158/282	56
5.3	148/292	116/219	0/50		264/561	47
6.0	80/85	87/91	115/115	21/21	303/312	97
6.8	17/17				17/17	100
7.5	73/73	10/10			83/83	100

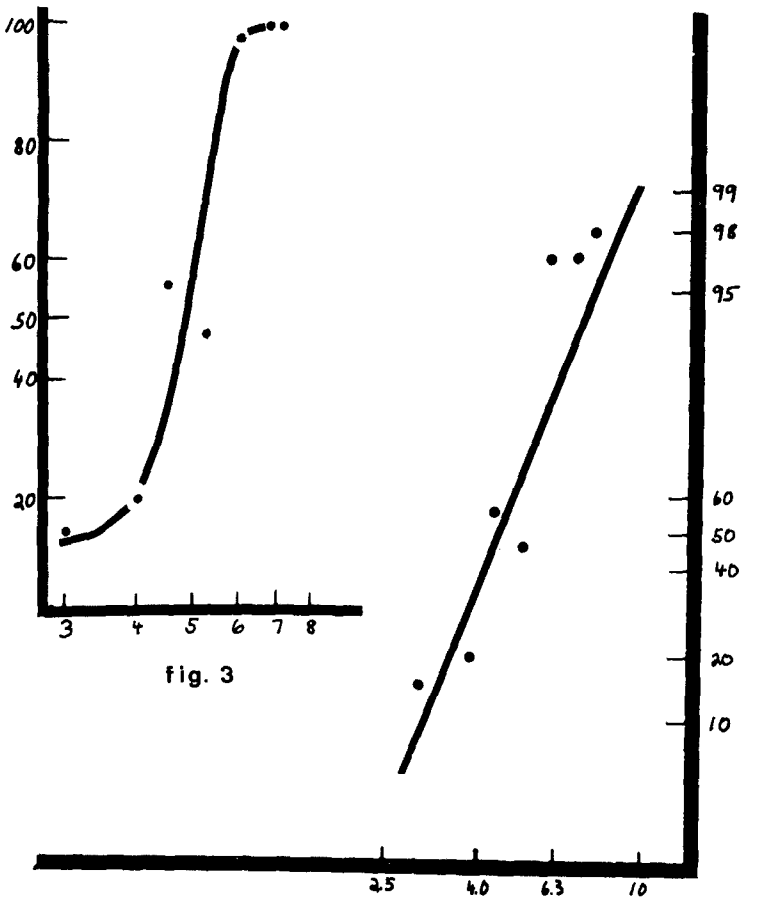


fig. 3

fig. 4

TERATOGENIC EFFECT OF N-PHENYL- α -NAPHTHYLAMINE
ON XENOPUS EMBRYOS

Figure 3 - Log-concentration percent malformed plot of the pooled data in table 3. Ordinate-percent malformed. Abscissa-concentration.

Figure 4 - Log-concentration-probability plot of the pooled data in table 3. Ordinate-percent malformed. Abscissa-concentration.

A log-dose percent plot of the data in table 3 yields an estimated ED50 of 4.8 mg/liter (fig. 3). A log-dose probability plot of the above data (fig. 4) gave an estimated ED50 of 4.57 with 95% confidence limits between 3.93 and 5.30 mg/liter.

Hydrazine

Table 4 summarizes data on the teratogenicity of hydrazine.

Table 4

TERATOGENIC EFFECT OF HYDRAZINE ON XENOPUS EMBRYOS

Embryos were continuously exposed to aqueous solutions of hydrazine from blastula until hatching at which time the number of malformed embryos/total number of embryos was recorded.

Concentration mg/liter	Experiment Number 1	2	3	4	Totals	% Malformed
10	61/100	2/100			63/200	32
15	83/100				83/100	83
20	99/100				99/100	99
25	100/100	100/100	100/100	50/50	350/350	100

Figure 5 is a log-dose percent plot of the data in table 4 and yields estimated ED50 of 12.5 mg/liter. An estimated ED50 of 11.48 mg/liter with 95% confidence limits between 10.95 and 12.03 mg/liter is obtained from the log-dose probability plot of this same data (fig. 6).

Methylhydrazine

Table 5 summarizes data on the teratogenicity of methylhydrazine. Teratogenicity increased from 5% at 5 mg/liter of 100% at 7.5 mg/liter. As intermediate concentrations were not tested we can only estimate that the ED50 of this compound lies between these concentrations.

Table 5

TERATOGENIC EFFECT OF METHYLHYDRAZINE ON XENOPUS LAEVIS EMBRYOS

Concentration mg/liter	Experiment Number 1	2	3	4	Totals	% Malformed
<5	4/95	1/91			5/186	3
5	8/100	2/93			10/193	5
7.5	100/100				100/100	100
10	100/100	50/50	100/100	91/100	341/350	97
15	50/50	98/100	50/50		198/200	99

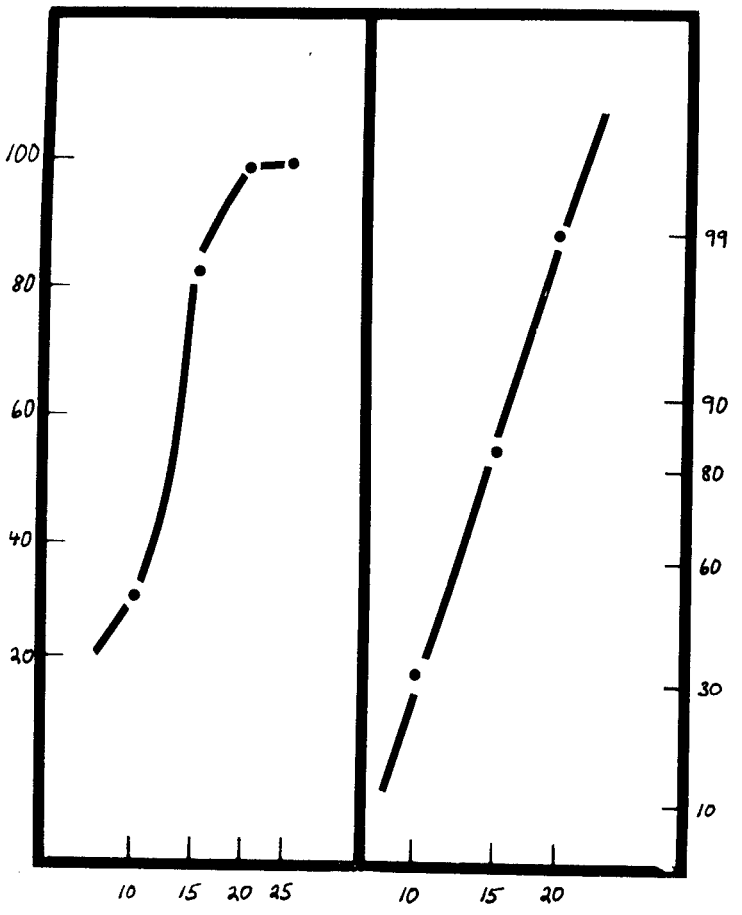


fig. 5 fig. 6

TERATOGENIC EFFECT OF HYDRAZINE ON XENOPUS EMBRYOS

Figure 5 - Log-concentration percent malformed plot of the pooled data in table 4. Ordinate percent malformed. Abscissa-concentration.

Figure 6 - Log-concentration-probability plot of the pooled data in table 4. Ordinate-percent malformed. Abscissa-concentration.

Unsymmetrical Dimethylhydrazine

Data on the teratogenicity of this compound is summarized in table 6.

Table 6

TERATOGENIC EFFECT OF UNSYMMETRICAL DIMETHYLHYDRAZINE ON XENOPUS LAEVIS EMBRYOS

Concentration mg/liter	Experiment Number 1	2	3	4	Totals	% Malformed
5	0/50	100/100	0/50	0/50	100/250	40
10	58/157	36/50	67/100		161/307	52
20	41/48	100/100	47/50		188/198	95

From the log-dose percent teratogenicity plot in figure 7 it is estimated that the ED50 for this compound is 9 ± 0.3 mg/liter. Treatment of the data according to Litchfield and Wilcoxon (1949) (fig. 8) yields an estimate of 7 mg/liter with 95% confidence between 3 and 17 mg/liter.

Symmetrical Dimethylhydrazine

Data on the teratogenicity of symmetrical dimethylhydrazine is summarized in table 7. Concentrations below 40 mg/liter were not teratogenic, whereas concentrations in excess of 50 mg/liter were almost 100% teratogenic, The ED50 for this compound is between 40 and 50 mg/liter. A more definite estimate cannot be made without further experimentation.

Table 7

TERATOGENIC EFFECT OF SYMMETRICAL DIMETHYLHYDRAZINE ON XENOPUS LAEVIS EMBRYOS

Concentration mg/liter	Experiment Number 1	2	3	4	Totals	% Malformed
40	5/100				5/100	5
50	40/50	100/100			140/150	93
60-65	41/41	100/160	50/50	50/100	241/351	69
80	100/100	90/100			190/200	95

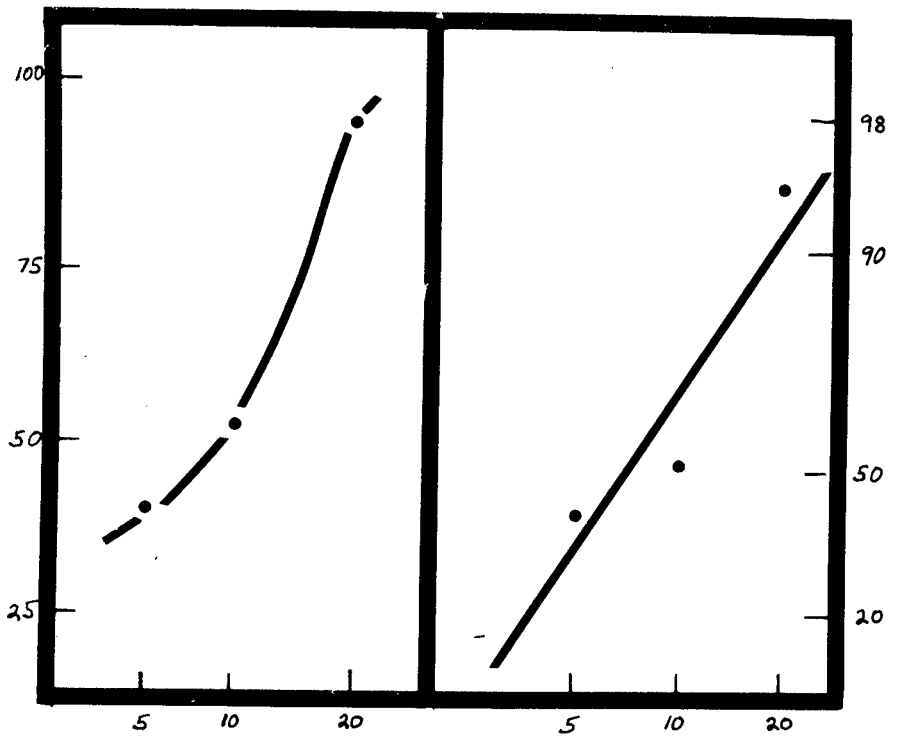


fig. 7 fig. 8

TERATOGENIC EFFECT OF UNSYMMETRICAL DIMETHYLHYDRAZINE
ON XENOPUS EMBRYOS

Figure 7 - Log-concentration percent malformed plot of the pooled data in table 6. Ordinate-percent malformed. Abscissa-concentration.

Figure 8 - Log-concentration-probability plot of the pooled data in table 6. Ordinate-percent malformed. Abscissa-concentration.

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