FOURTH QUARTERLY PROGRESS REPORT

WITH

ANNUAL SUMMARY OF RESEARCH

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TOXICITY OF ORDNANCE WASTES IN AQUATIC ENVIRONMENTS

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SUMMARY

Bioassay toxicity testing was completed on non-biodegraded picric acid, Otto fuel and Noset A using Daphnia sp. and a fish species. The compounds tested were of relatively low toxicity compared to many industrial compounds such as pesticides, polychlorinated biphenyls, and some heavy metals. The $LC_{50/48}$ for Daphnia was approximately 65 ppm in picric acid, about 200 ppm in Otto fuel and about 600 ppm in Noset A. For stickleback (fish) average $LC_{50/96}$ values were 76, 26, and 236 ppm respectively.

Based on the fish numbers and previous algal toxicity tests in this research, preliminary effluent guidelines are recommended as follows:

Picric acid should follow local effluent standards for phenols (eg. 0.5 ml/l as in some California areas). Otto fuel and Noset A levels around 0.5 mg/l and 30 mg/l appear to be reasonable until further testing is completed on the toxicity of biodegradation products of these materials. Guidelines for fuels should specify, however, that the fuels be in true aqueous solution, or very finely dispersed, rather than being in insoluble globular forms in effluent streams.

INTRODUCTION

This report presents results of research carried out in the fourth quarter of 1974 on the environmental toxicity of picric acid, Otto fuel II, and Noset A, with some preliminary data on the TNT breakdown product 4 amino-2,6-dinitrotoluene. Results of the year's research are then discussed in relation to their specific and general significance regarding toxicity to aquatic environments. Preliminary suggestions are given for effluent standards based on current knowledge.

Nomenclature:

In order to bring our research data into agreement with methods described in Environmental Protection Agency Publication #660/3-75-009 we have changed all our designations of lethal concentrations of toxicants to "LC_{50}." This term represents the concentration of toxicant in the water which results in the death or total immobilization of 50% of the organisms exposed to the toxicant. This is contrasted to the term LD_{50} which represents the median dose of a toxicant which is lethal, and is most used in systems which employ mammals as test animals where known doses of toxicants can be introduced into the tissues of test animals.

It should be noted that in reporting levels of "Otto fuel" in solution, the amount of propylene glycol dinitrate ester is given, because of its high solubility relative to the other compounds in the fuel mix (di-n-butyl sebacate and 2-nitrodiphenyl amine). The same applies to Noset A, where the presence of triethylene glycol dinitrate is reported.

FOURTH QUARTER RESEARCH: MATERIALS AND METHODS

Bioassay toxicity testing in the last quarter has been carried out on Daphnia sp., and the three-spined stickleback. Supporting chemical determinations were carried out to ascertain the occurrence of biodegradation of Otto fuel and Noset A during testing in representative systems.

Daphnia sp.: The freshwater micro-crustacean, Daphnia was cultured in our laboratory as mentioned in the third progress report of this research. In the previous report we documented the approximate $LC_{50/96}$ for these organisms in picric acid, Otto fuel, and Noset A. Due to high mortality in control tests, and to conform with EPA suggestions the tests were repeated using a 48 h test period. Methods were maintained the same as in previous testing, placing approximately 30 organisms in 50 ml test solutions in 150 ml flasks.

Fish tests: Bioassay toxicity testing was carried out on picric acid, Otto fuel II, and Noset A, to determine their lethal toxicity for the three spined stickleback, Gasterosteus aculeatus. Choice of this species and test methods was based on recommendations given E.P.A. Publication #660/3-75-009 "Methods for Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians," 1975. Basic static acute toxicity test methods were employed.

Containers: Test containers were Pyrex cylinders 21 x 46 cm (surface area = 345 cm^2), containing 10 liters of diluent solution. Before each use, containers were washed with hot water and detergent, dried, rinsed with 100% acetone, dried, rinsed with 10% aqueous HCL, and finally rinsed five times in tap water.

Test organisms: Adult three spined sticklebacks were purchased from the Alex Fish Co., San Rafael, California (State Permit #198). The fish originated in coastal river mouths having a salinity range of approximately 15-30 o/oo. Fish were held in stainless steel tanks at 16° C at salinities approximating those to be used during toxicity testing; fish were observed for mortality for four weeks prior to any testing to determine their condition. Essentially no mortality occurred during these holding periods. Fish were allowed to acclimate from 16 to 22° C over a 2 - 4 h period prior to being placed in test containers. Fish were fed brine shrimp once a week, and were not fed for 96 h prior to toxicity testing. In a sample of 20 fish taken at random, the average weight per fish was 0.35 g (max. 0.38; min. 0.31). The average standard length of these fish was 32.9 mm (max. 38.3; min. 26.5). Twenty fish were used in each toxicity test run, using one control group and seven test concentrations of toxicant in each run, therefore requiring 160 fish per test run. With three toxicants in two applications, a total of 960 fish were used in the research this quarter.

Diluent and toxicants: Dilution water for toxicants was the same as used to maintain the fish prior to testing. Water was obtained in the Middle Reach of San Francisco Bay, kept in glass containers, and any suspended matter allowed to settle out during several days of storage at the laboratory. The water was clear and did not require special treatment prior to use in testing. Salinity of the water was approximately 28 o/oo; when lower salinity was desired, the water was diluted with deionized distilled water.

Toxicants included picric acid, Otto fuel II, and Noset A. Picric acid was reagent grade, dried to constant weight at room temperature over $CaSO_4$. Picric acid solutions were made by dissolving weighed amounts of the acid directly into diluent waters containing test fish. Otto fuel and Noset A dilutions were prepared by stirring

25 g of each fuel into 15 liters of diluent water for 24 h at 16° C. The water was then decanted off the undissolved fuel and passed through a diatomaceous earth filter to remove globules of fuel suspended in the water. Dilutions of the saturated water were then made directly in the fish containers prior to adding the fish. Aliquots of the saturated water were analyzed by hexane extraction and gravimetric determination, and also by gas chromatographic methods to verify actual fuel content of the saturated diluent water. At the end of toxicity test runs, analyses were repeated to determine if any chemical or biological breakdown of the toxicants has occurred which might have influenced the results of the test.

Conduct of testing: Tests were begun at mid-day and monitored at zero, 48 and 96 h for temperature, oxygen, and pH. Dead or morbid animals were removed daily. Behavioral patterns of surviving fish were informally noted at 96 h. Data on mortality (morbidity) were used to calculate the $LD_{50/96}$ by probit analysis or the Reed-Muench method (Woolf, 1968), which yielded the 95% confidence interval for the determination.

FOURTH QUARTER RESEARCH: RESULTS

Daphnia sp.: High mortality of control animals experienced in previous testing over 96 h periods was eliminated by using 48 h test periods. Mortality curves for Daphnia in picric acid, Otto fuel, and Noset A are shown in Figure 1. Preliminary results obtained using 4-amino-2,6-dinitrotoluene, listed in Table 2, show this compound to be the most toxic among the materials tested (LC 50/48 = 30 ppm). Table 2 shows that the toxicity of picric acid for Daphnia (65 ppm) was in the same order of magnitude as the toxicity of picric acid for two species of freshwater micro-algae tested, and was very similar to the lethal toxicity of picric acid for the seawater copepod Tigriopus. Extremely good correlation is also obtained in comparing the toxicities of Otto fuel, Noset A, and the 4-amino compound between these two species of microcrustaceans which live in dissimilar habitats.

Fish Tests: Essentially no mortality was experienced in fish held in the laboratory prior to testing, and no mortality was observed in control animals maintained under test conditions for 96 h. Duplicate results, shown in Table 1, show the most toxic material to be picric acid, at $LC_{50/96}$ of approx 75 ppm. This value shows excellent correlation to values obtained for the micro-crustaceans (Table 2).

The $LC_{50/96}$ for Otto fuel and Noset A were both over 200 ppm. Behavioral distress and discoloration was often seen in fishes which survived in concentrations near the lethal value. These results were not quantified, however.

Before-after chemical determinations using gravimetric and gas chromatographic methods verified that there had been no significant change in the concentration of Otto fuel and Noset A in test vessels, and that no biodegradation or other qualitative change could be noted over the 96 h test period.

Resume of Previous Results:

Algae: A general guideline espoused by workers with bioassay toxicity testing is that in using several different species, the most sensitive species to a toxicant is that chosen for developing in pollutant toxicological criteria. Table 2 shows that of the five species of algae cultured in picric acid, the most sensitive was the freshwater bluegreen alga Cylindrospermum sp. which showed depressed growth at about 25 ppm. Three algae species tested showed inhibition of growth from 25 to 75 ppm picric acid, with the two species of diatoms insensitive to over 100 ppm picric acid. These results compare very well with summarized data listed in McKee and Wolf (1971) where threshold

toxicities of nitro-phenols for Scenedesmus (a green alga) ranged from 36 to 72 ppm, while the diatom Navicula showed sensitivity beginning at 250 ppm. Of the fuels, Otto fuel was most toxic to the freshwater algae as a group when compared to marine algae, (Table 2), with Selenastrum the most severely affected species at 11 ppm. Noset A was surprisingly non-toxic to all the algae, again with Selenastrum the most sensitive species at 325 ppm.

There was essentially no biodegradation of the toxicants in algae tests as determined by gas chromatographic monitoring of initial and final diluents from which the algae had been removed. Some preliminary indications were obtained that the marine bluegreen alga Agmenellum had slight degradative effects on Otto fuel, although the differences obtained did not appear to be of enough significance to warrant further research.

Micro-crustaceans: The fresh water micro-crustacean Daphnia and the tidepool copepod Tigriopus showed very similar LC_{50} levels in all the materials tested. The $LC_{50/96}$ in picric acid of Daphnia (65 ppm) and that of Tigriopus (45 ppm) appear to be the right order of magnitude when compared with results summarized by McKee and Wolf (1971), where threshold toxic levels for five nitro-phenols on Daphnia ranged from 6 to 60 ppm. The fuels showed lower comparative toxicity than the picric acid, with Noset A showing the least toxicity.

Oyster larvae: Oyster larvae are becoming more readily available due to popularization of technics for their culture. These organisms are of great interest as they represent free swimming planktonic larvae of a commercially important species. The EPA recommends that in multiphasic toxicity tests that organisms of commercial significance be included in the testing. Data re-plotted from quarterly report #3 on a log scale (Figure 2) show an apparent two-function relationship. An

initial high resistance to toxicity was followed by a rapid break in the curve toward very high mortality after a toxic threshold was reached near the $LC_{50/96}$. This type of mortality is not uncommon in oyster larvae, and has been seen in oyster larval culture at the Pigeon Point Research Laboratory. The oyster larvae were resistant to over 100 ppm picric acid, Otto fuel and Noset A. The toxicity of the fuels to the oyster larvae appear to be the same.

4-amino-2,6-dinitrotoluene

Results were obtained on a preliminary, time available basis for this compound using some algae, copepods, Daphnia, and oyster larvae.

The freshwater diatom Navicula pelliculosa showed an increased generation time of about 10-fold at 25 ppm; the marine diatom Cylindrotheca fusiformis showed a doubling in generation time at 50 ppm of the 4-amino compound. Table 3 shows that this growth inhibition was not a pH effect. C. fusiformis also appeared to be affected by 4-amino-2,6-dinitrotoluene. Growth was totally inhibited in cultures of C. fusiformis between 50 and 75 ppm of this compound. Again, Table 3 indicates that this is not a pH effect causing lack of growth.

Growth of the marine blue-green alga, Agmenellum quadruplicatum, was also inhibited by 4-amino-2,6-dinitrotoluene. Between 10 and 25 ppm growth was totally inhibited. Table 3 again demonstrates that this was not a pH effect.

Table 3.

Organism	Control before growth	pH at each concentration after growth ppm 4-amino-2,6-dinitrotoluene					
		Control	10	25	50	75	100
C. fusiformis	8.0	9.3	9.3	9.2	9.4	8.[illegible]	8.0
N. pelliculosa	8.2	8.4	8.4	8.4	8.1	8.2	8.0
A. quadruplicatum	8.2	8.9	8.6	8.0	7.9	8.0	7.0

Again, the two microcrustacean species showed the same response to the toxicant, at about 30 ppm, as did the oyster larvae.

DISCUSSION OF RESULTS

Some effects of potential munitions waste in the environment are based on the following considerations:

A. Toxicity

1. Parent compound.
2. Breakdown products of the parent compound.
3. Formation and deleterious effects of compounds formed by parent compound or breakdown products reacting with other compounds in the environment to give new, undesirable compounds.

B. Enhancement

1. Exertion of biochemical oxygen demand (BOD) through enhancement of microbial respiration.
2. Enhancement of plant cell growth by the addition of simulating nutrients (eutrophication).

C. Sublethal effects

Includes modification of behavior, respiration rate, or other physiological parameters affecting the long term survival of species in the environment.

D. Harmless "nuisance" effects

1. Taste, odor and coloration of natural waters or drinking water supplies.
2. Tainting of flesh in edible species (e.g. oysters, sport fishes).

Picric acid: The WPCF (Anon. 1960) suggests that nitrated phenolic compounds be treated as a group when considering toxicity potential. Our results have shown that picric acid (trinitrophenol) has a toxicity in the same range as that of other nitrophenols (McKee and Wolf, 1971). Biodegraded picric acid will yield a variety of nitrophenols and ultimately phenol itself, for which most states have promulgated effluent standards. In California, permits have been issued for discharge of phenol at around 0.5 mg/l (pers. comm., Bay Area Regional Water Qual. Contr. Bd., Oakland). Final dilution levels for phenolics in receiving waters have been recommended at 0.05 mg/l (Anon. 1960).

Based on our toxicity data and literature review, environmental protection standards for the discharge of picric acid should be the same as promulgated for phenol until further data is obtained on sublethal or cumulative effects. Acute toxicity of picric acid cannot be considered great when compared with toxicities of some common pesticides and pollutant heavy metals which are toxic at the parts per billion (ppb) level (Crosby et al., 1966).

Early information on the decomposition of organic nitrogen compounds suggests that nitrites are released which are rapidly oxydized to nitrates by bacteria (Gundersen and Jensen, 1956). If effluent levels similar to California standards are adopted (0.5 mg/l), there should be no problem associated with eutrophication potential of nitrogen released from picric acid. For example, ambient levels

of nitrate-N in the Potomac River near Indian Head often reaches 0.1 mg/l.

In view of the fact that the phenolics are toxic, effluent standards will prevent the discharge of amounts significant enough to exert deleterious BOD effects on natural waters.

As stated in the first progress report, the picrate ion imparts coloration to water supplies at levels well below that of being acutely toxic and thus is in danger of creating a nuisance effect. Additionally, a yellow coloration could probably be imparted to food organisms growing in natural waters receiving picrate, based on its ability to react readily with proteins.

Propellants: The composition of the fuels tested is:

Otto fuel II	propylene glycol dinitrate ester (PGDN)	76%
	2-nitrodiphenylamine (NDPA)	1.5%
	di-n-butyl sebacate	22.5%
Noset A	triethylene glycol dinitrate ester (TGDN)	96%
	ethyl centralite	1%
	di-n-butyl sebacate	3%

For test purposes we saturated water with the fuels and made aqueous dilutions to simulate the worst possible environmental effluent conditions. This method of testing did not allow for specific component toxicity evaluation. In making the aqueous dilutions the ratios of these compounds to one another change. Since NDPA is insoluble in cold water, its presence in aqueous dilutions is very low, and is relative to the amount of PGDN present.

To our knowledge, there is no published literature on the environmental effects of, and effluent standards for PGDN, TDGN, and

nitrate esters in general, although a good deal is known about mammalian toxicity of some of these compounds as reviewed by Dacre and Tew (1973).

Since propylene glycol and other glycols are generally non-toxic (Sax, 1968), the toxic properties of the fuels probably resides in their nitrated state. The other compounds which occur in the fuels except for the NDPA are probably non-toxic, and the NDPA is so insoluble in water that it probably can be dismissed as a toxic agent available from the water under recommended discharge conditions. Noset A was usually an order of magnitude less toxic for the organisms tested than was Otto fuel (Table 2).

Effluent standards:

A reasonable place to start developing effluent standards is our fish toxicity data, coupled with the published recommendations of Sprague (1970) that effluents could safely contain 0.01 - 0.4 of the LC_{50} for test fish. Using our stickleback data, the effluent criteria for Otto fuel would be 0.26 - 10.4 mg/l and 2.36 - 94.4 mg/l for Noset A. Personal communication with EPA personnel (Dr. P. Lefcourt) suggest an agency preference for the low end of range (0.01).

Selenastrum carricornutum, a freshwater alga which is commonly used in toxicity testing (Anon., 1971) showed threshold levels of toxicity estimated at about 5 ppm (mg/l) for Otto fuel, and 300 ppm for Noset A. Reasonable estimates for effluent standards based on these values may be 1/10 of the threshold value. Thus a standard based on Selenastrum would be 0.5 mg/l Otto fuel and 30 mg/l Noset A. This effluent standard, based on one of the most sensitive algae tested, falls with ranges for effluent criteria estimated from the fish data in the preceding paragraph.

Development of effluent standards and discharge permits is essentially, at the present time, based on the type of discharge and the characteristics of the receiving waters. It should be noted that these materials are Navy unique, and as such are not produced in quantities rivaling the major industrial chemicals. It is likely that discharges of these materials does not rival the potentially discharged amounts of more toxic industrial wastes. This fact should be used to place the Navy in perspective with regard to its overall significance as a discharger within the entire scenario of effluent discharge into large water bodies such as the Potomac River.

Although toxicity test methods have become routinely used in testing toxicities of known effluents, there is little information on the development of criteria and subsequently standards for effluent discharge from data originated in toxicity tests prior to the beginning of an effluent discharge. This problem is currently under study at the EPA under Dr. P. Lefcourt. A definitive report, relating effluent standards to toxicity test results is due within the next four months (May 1976) from Dr. Leonard Guarria, Chief, Criteria, Water Planning, and Standards Branch, EPA, Code WH-551, Washington, D.C. 20460.

It should be noted that "criteria" form the data base which provide the informational background, while a "standard" is the legal enforcible level for any toxic or hazardous material discharged into the environment.

Environmental Impact of Insoluble Fuel:

Preliminary evidence from our laboratory suggests that at least Otto fuel undergoes chemical and bacteriological decomposition very slowly. This is particularly true when the fuels are not dissolved or finely dispersed in water and occurring as globules. The fuels are heavier than water (Otto fuel sp. gr. = 1.23 at 25 C) and therefore will sink to the bottom and be incorporated into the sedimentary systems of receiving waters. Depending on the bottom contour and hydrography of the region, the release of globular fuels might result in physical accumulations to give "hot spots" containing elevated concentrations of fuels.

Literature and preliminary tests in our laboratory also suggest that dissolved fuels may adsorb to suspended sedimentary particles and thus be removed from the water column to sedimentary systems. Under reducing conditions (in sediments), decomposition of PGDN or TGDN could produce nitrite, which in turn under acidic conditions could convert secondary amines to nitrosamines which are coming under closer environmental scrutiny based on their carcinogenic properties.

It is therefore of interest to insure that effluent discharges are dissolved in water, or very finely dispersed. Further research is required on the microbiology and chemistry of the decomposition and effects of these fuels in sedimentary systems. The toxicity testing suggests that ingestion of globular fuels by bottom dwelling fauna and fishes during normal feeding activities would have a severe impact on these animals.

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Table 1. Bioassay toxicity testing of the three spined stickleback, Gasterosteus aculeatus with picric acid, Otto fuel II, and Noset A.

Toxicant	Salin., o/oo	Replic.	$LC_{50/96}$ ppm	95% Confidence Interval* ± ppm
Picric acid	13.5	1	75	-
Picric acid	11.8	2	77.2	1.2
Otto fuel II	28.5	1	17	0.2
Otto fuel II	22	2	36**	-
Noset A	27	1	271	2.7
Noset A	25	2	201	2.0

* Reed-Muench Test (Woolf, 1968). Temp. = 21 C.

**48 h

Table 2. Summarized results of bioassay toxicity testing using four types of potential ordnance wastes with freshwater organisms.

Test species	Type	Toxicant			
		Picric acid PPM LC 50/96	Otto fuel II PPM LC 50/96	Noset A PPM LC 50/96	4a** LC 50/96
Selenastrum capricornutum	ga	50*	11	325	10
Cylindrospermum sp.	bg	25	55	650	-
Navicula pellic.	d	>100	75	900	-
Daphnia sp.	mc	65+	200+	600+	30+

* Approximately 50% cell growth inhibition.

**Best estimate from preliminary data.

+ $LC_{50/48}$.

ga = green micro-algae

bg = blue green algae

d = diatom

mc = micro crustacean

(Table 2 Cont'd.) Summarized results of bioassay toxicity testing using four types of potential ordnance wastes with seawater organisms.

Test species	Type	Toxicant			
		Picric acid PPM LC 50/96	Otto fuel II PPM LC 50/96	Noset A PPM LC 50/96	4a* LC 50/96
Agmenellum quad.	bg	75	365	368	10
Cylindrotheca fusiformis	d	>100	145	>1000	-
Tigriopus calif.	mc	45	150	640	30
C. gigas	lm	>100	140	160	30
Gasterosteus aculeatus[1]	f	76	26	236	

[1]2 mean of 2 determinations

bg = bluegreen algae

d = diatom

mc = micro crustacean

lm = larval mollusc

f = fish

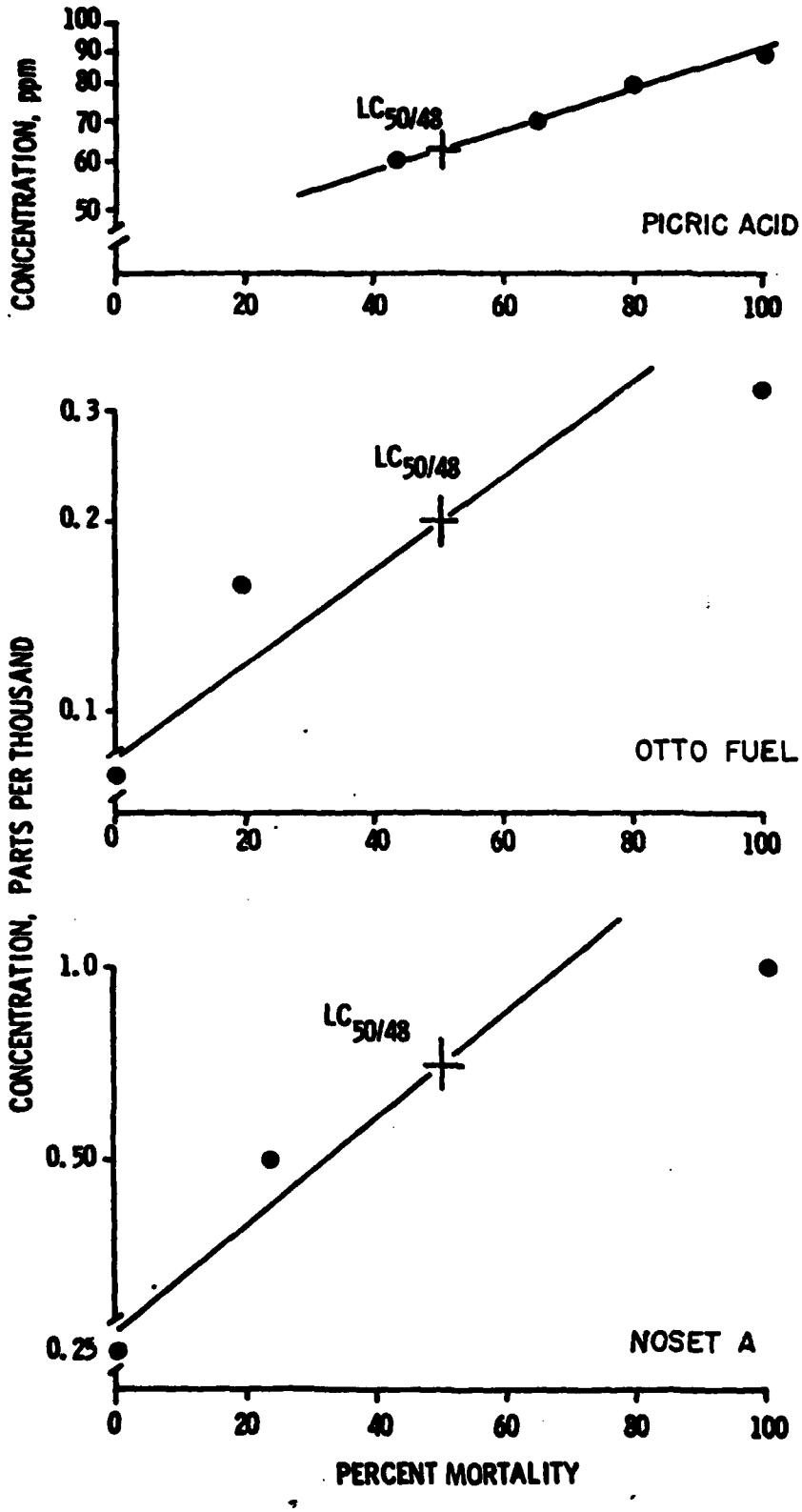

Figure 1. Mortality of Daphnia sp. in picric acid, Otto fuel II, and Noset A. $LC_{50/48}$ represents concentration lethal for 50% of test animals over a 48 h period.

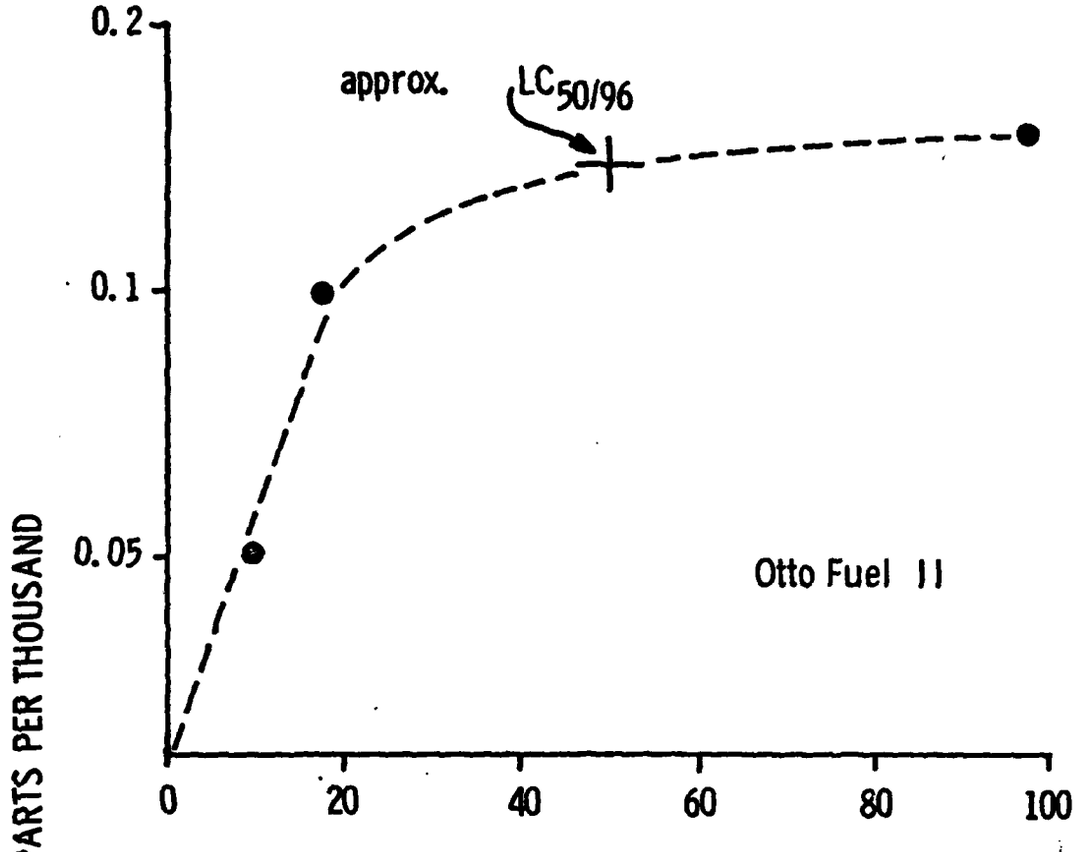

Figure 2. Mortality of larval oysters in Otto fuel and Noset A.