DEVELOPMENT OF AN INVERTEBRATE BIOASSAY TO SCREEN PETROLEUM REFINERY EFFLUENTS DISCHARGED INTO FRESHWATER

by

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I. INTRODUCTION

A. Purpose and Perspective

The purpose of this research was to develop a bioassay for on-site toxicity testing of refinery effluents. This dissertation is intended to help non-biologists identify potentially detrimental waste discharges and establish treatment priorities. This effort is not intended to replace currently-used toxicity tests, but merely to provide an indication of the order in which industrial effluents should receive attention and establish priorities for coping with these effluents. In this regard, my purpose is in keeping with the recommendation of the National Academy of Sciences (NAS 1975) which stated that priorities should be established using an array of tests beginning with preliminary assessments of risk.

There are not enough people trained in bioassay techniques to do the more complex bioassay tests. Preliminary work must be done by people with less training, so that skilled people, working with more sophisticated equipment, can focus on more complicated issues.

B. The Need for Invertebrate Bioassays

Most bioassay procedures currently used to monitor the effects of effluents on water quality and aquatic life are cumbersome and expensive in terms of the required time, facilities, technicians, and test organisms. Consequently, only a fraction of all industrial effluents can be monitored for toxicity. The use of invertebrates, rather than fish, for on-site screening of industrial effluents may overcome some of these difficulties. Invertebrates generally require less space for

acclimation and testing, they may acclimate more rapidly, and they may be more sensitive over a shorter period of time compared to fish. Many invertebrates are more sensitive than fish to many pollutants. The ideal test organism would be sensitive enough to give a rapid and quantifiable response to toxicant levels which are detrimental to other important aquatic organisms, but would not be so sensitive as to be unrepresentative of indigenous species. In areas where fish are endangered or supplies limited and where indigenous species are required for toxicity evaluations, it may be better public relations to use laboratory cultures of invertebrates. Extensive use of native species could have a detrimental effect on local populations of aquatic organisms. Finally, modified invertebrate bioassay procedures may be useful for investigations of community and ecosystem properties such as bioaccumulation studies, biogeochemical cycling, and so forth. Patrick *et al.* (1968) have recommended tests with at least three components of the food web so that conclusions would have more significance for balanced, functional, aquatic communities.

Overreliance on short-cut, on-site tests could be misleading because long-term, subtle effects would not be identified or measured. Yet the potential advantages of quick and frequent toxicity-testing (in terms of increased sensitivity, protection, and time-saving) seemed to warrant the development of a sensitive, short-term, invertebrate toxicity test.

The toxicity of complex industrial effluents often cannot be predicted from chemical data. Bioassays using aquatic organisms have a long history of use in estimating relative toxicity of industrial wastes and in measuring the toxicity of specific compounds. At present, fish

are the most widely used organism for testing the toxicity of industrial wastes (American Public Health Association [APHA] 1971). Many invertebrates have been used to test an array of substances, but the methods were neither standardized nor feasible for on-site toxicity testing. Only recently have attempts been made to standardize invertebrate bioassay procedures (Environmental Protection Agency 1975).

C. The Use of Results from Screening Tests

Screening of major industrial effluents could be an important first step in protecting the biological integrity of aquatic environments. Decisions based on results of screening tests would contribute to effective utilization of other biological assessment techniques (*cf.* Cairns and Dickson 1973). An on-site screening bioassay could be used to identify effluents with high, moderate, or low risk-benefit ratios. In cases of moderate risk-benefit ratios, bioassay results would indicate the necessity for additional, more sophisticated and time-consuming tests (for example, 96-h continuous-flow bioassays, or studies on behavioral or reproductive impairment). Estimating benefits to society and risks to both society and the aquatic environment would require the professional expertise of economists, engineers, and scientists. If the risk-benefit ratio were very low, no additional testing would be warranted at least until higher priorities had been satisfied. If the risk-benefit ratio were very high, decisions by the industry and by the regulatory agency would have to be made for corrective action. These decisions might be a change in process design, the use of

alternative waste treatment facilities, or the finding of substitutes for a particular product.

D. Legal Basis For the Use of Biological Data

It is probable in the near future that most industries will be required to demonstrate the biological impact of their waste products. Recent federal legislation on regulation and testing of toxic substances (Train 1976) has supported the precedent of placing the cost of evaluating chemicals for the purposes of protecting the environment on the manufacturers. This precedent appeared first in Ontario law in 1967 (Ontario Water Resources Commission 1970): "The responsibility for demonstrating that a waste effluent is harmless to water uses in the concentrations to be found in the receiving waters, rests with those producing the discharges." Implementation of biological assessment procedures (ranging from screening bioassays to field surveys) by an industry may provide a data base on which discharge permit variances could be granted. Prepared with site-specific data, an industry could argue effectively for using the ability of a receiving system to assimilate wastes. Support for this argument appeared in Public Law 92-500 section 316a pertaining to thermal discharges. According to this section of the law, the owner of a thermal effluent source may obtain a biologically valid adjustment of an effluent limitation by showing the administrator of EPA that the effluent limitation is more stringent than necessary to assure the protection of aquatic species.

E. Objectives

The first objective was to develop a simple and inexpensive invertebrate bioassay test with commercially available organisms that could be used by waste control personnel with little or no biological training for evaluating toxicity of petroleum refinery effluents. To achieve this objective, an array of freshwater invertebrates and fish was evaluated with special attention to commercial availability, sensitivity to potential toxicants, and ease in handling by people with little or no training in biology. Fish were included for comparative purposes. Then a bioassay method was evaluated in the laboratory at Virginia Polytechnic Institute and State University (VPI & SU).

A second objective was to test the procedure at actual refinery laboratories. This objective was achieved by training refinery-waste treatment personnel in the method during a two-day workshop at VPI & SU and by having these people conduct bioassays on a reference mixture and on grab samples of refinery effluent.

Other objectives were to identify major environmental factors affecting bioassay results, to compare the sensitivity of the selected test organism to five species of fish commonly used to test industrial effluents, and to investigate the effect of molting on bioassay results.

F. Literature Review

Effects of Oil Refinery Waste in Aquatic Systems

The character of an oil refinery waste depends on 1) the nature of the crude oil processed, 2) the type of product produced, 3) the type of

refinery process employed, 4) the efficiency of the refinery operation (Reynolds *et al.* 1974), and 5) the efficiency and type of waste-treatment facility. Biological treatment may not always be adequate to cope with the refractory components which may be present in refinery waste. The pollution problems of relatively stable refinery wastes, due particularly to the organic components of the waste are: 1) interference with potable water treatment, 2) interference with industrial water treatment, 3) tainting of fish flesh, 4) damage to stream biota, 5) taste, 6) odor, 7) color, 8) foam, and 9) toxic or carcinogenic damage to man. Activated carbon treatment has been suggested as a first step to solving these potential problems (Myers and Mayhue 1974).

Effluent limitation guidelines have been promulgated by the Environmental Protection Agency for predominant toxic substances in refinery wastes (EPA 1973). These substances are ammonia, chromium, oil and grease, phenol, sulfide, and zinc (Table 1). Table 1 shows the anticipated effluent loading from an integrated refinery of 100,000 barrel/day capacity following primary treatment (Cross and Lawsen 1974). The industrial view of the cost, effectiveness, and social benefits of advanced refinery waste treatment were discussed by Elkin and Lorenz (1974).

Copeland and Dorris (1964) studied the community metabolism of effluent holding ponds of two Oklahoma refineries. They found that algae in oil refinery effluent were less efficient than algae in sewage communities or natural climax communities. But compared to deep lakes, production and respiration of the holding ponds were unusually high. At the beginning of the series of ponds photosynthesis occurred only in the

Table 1. Approximate raw waste concentrations from an integrated refinery (adapted from Cross and Lawsen 1974) and the 1977 and 1983 EPA effluent guidelines for EPA subcategory F integrated refineries (EPA 1973).

Parameter	Raw waste after primary treatment*	EPA guidelines, maximum concentration for any one day	
		1977	1983
$NH_3(N)$, mg/l	32	14.2	3.8
Cr total, mg/l	--	0.555	0.37
Oil, mg/l	122	10.8	1.5
Phenol, mg/l	18	0.266	0.037
Sulfide, mg/l	60	0.24	0.16
Total suspended solids, mg/l	87	21.6	7.4
pH	--	6.0 to 9.0	6.0 to 9.0
Zn, mg/l	--	1.1	0.59

*--based on mean flow of 20 MGD

warm months, and then at a low level; effluent toxicity apparently held photosynthesis to a level below that attained in sewage. Community respiration was of the same order of magnitude in both sewage- and effluent-polluted areas. The authors noted that the metabolic efficiency of the holding-pond community may have been suppressed by the limited diversity of organisms capable of living in oil refinery effluents. The holding ponds were effective in reducing the toxicity of refinery waste as evidenced by the presence of large populations of herbivorous fly larvae in the downstream ponds (Tubb and Dorris 1965).

Combined oil refinery and petrochemical effluent effects on the Ottawa River at Lima, Ohio, were studied by Ludzak *et al.* (1957). The major pollutional problems were related to deposits of oily sludge associated with the refinery. McCauley (1966) conducted one of the first studies of an oil spill in a river. Her findings showed 1) that an oil film excluded part of the dissolved oxygen from the water, 2) that sedimentation of the oil produced an oily sludge in the river bed, 3) that the sludge was slowly decomposed by microorganisms, and 4) that the toxic effect of the oil was selective. The oil eliminated *Daphnia*, *Gammarus*, *Agrion* nymphs, and *Dugesia*, but it did not eliminate *Tubifex*, *Tendipes* larvae, cyclops, several rotifers, or certain phytoplankters.

Phillips (1965) found that fish populations in a southwestern stream were more stable above oil refinery and domestic sewage outfalls than they were below these outfalls. Beadles (1966) concluded that refinery and domestic effluents may have decreased the stability of fish populations below the outfalls. In the absence of refinery effluents, domestic effluents appeared to cause production of larger fish.

A study on the Red River, Manitoba (Loch and Gregory 1973) demonstrated that immediately below refinery effluents there was a marked decrease in the populations of *Sphaerium*, *Amnicola*, and *Polypedilum*. The numbers of clams and snails were still reduced up to 2.4 miles downstream from the effluent. There was an increase in the oligochaete worms downstream from the effluent. Wilhm and Dorris (1966) studied the benthic, macroinvertebrate community-structure of an Oklahoma stream receiving oil effluents. Diversity was higher above the outfall. Populations were large at upper stations in summer and fall and comparatively smaller in winter and spring, while populations downstream were smaller and more nearly constant throughout the year.

Bioassays on Refinery Effluents

In a study of some toxic components of oil refinery wastes Jenkins (1964) showed that a mathematical model could be used to predict fish survival in solutions of known, mixed toxicant concentrations. However, the model did not work for predicting fish survival in actual refinery effluents for which the ammonia, phenol, sulfide and hydrogen ion concentrations were known, which suggested there were additional toxicants in the refinery waste.

Acute static toxicity tests of 15 common petrochemicals (Pickering and Henderson 1966) showed that the sensitivity of four fishes ranged less than one order of magnitude (except for two toxicants which showed wide differences in toxicity for different fishes). In order of sensitivity (most to least) the fish species were bluegill, fathead minnow, goldfish, and guppy.

Using fish as bioassay organisms, Graham and Dorris (1968) found that some refinery effluents, which caused little or no acute toxicity, caused chronic toxicity. They suggested that treatment of waste in oxidation ponds reduces the amounts of substances causing acute toxicity (such as ammonia, phenol, and sulfide) but it may not reduce the amounts of substances causing chronic toxicity (such as copper, lead, zinc or cyanide). Because of the complexity of the wastes, however, it was not possible to evaluate toxicity on the basis of chemical analyses. Dorris *et al.* (1974) concluded that the acute toxicity of oil refinery waste waters to fathead minnows and *Daphnia magna* was reduced by partial evaporation because most of the toxicity was contained in the volatile fraction. Irwin (1965) evaluated 57 species of freshwater fish for possible use in testing toxicity of oil refinery effluents. His results showed a wide range of susceptibility among species.

Toxicity of Major Contaminants of Refinery Effluents

Ammonia: Ammonia is toxic because it leads to the reductive amination of alpha-ketoglutarate to glutamate. The result is that alpha-ketoglutarate is removed from the tricarboxylic acid pathway, inhibiting respiration and, in some animals, causing excess ketone body formation from acetyl CoA in the liver (Lehninger 1970). The toxicity of ammonia solutions increases as pH increases because the concentration of the toxic species, $NH_3 \cdot nH_2O$ increases as the reaction

$$NH_{3(g)} + nH_2O_{(\ell)} \rightleftarrows NH_3 \cdot nH_2O_{(aq)} \rightleftarrows NH_4^+ + OH^- + (n-1)H_2O_{(\ell)}$$

is pushed to favor dissolved, hydrated ammonia (Thurston *et al.* 1974).

An increase in pH and a decrease in dissolved oxygen cause an increase in ammonia toxicity (Downing and Merkens 1955). Fromm (1970) found that 50% of exposed rainbow trout died in 24 h in 8 mg/l NH_3 and 10% of exposed goldfish died in 24 h in 40 mg/l NH_3. Anderson (1944) reported that the threshold concentration for immobilization of *D. magna* by ammonium hydroxide was less than 8.75 mg/l. The literature available suggests that the acute toxicity of many freshwater fishes and *Daphnia* are roughly similar.

Chromium: Christensen (1971) reviewed the literature on the enzymatic mode of action of heavy metals toxic to aquatic life. He concluded that heavy metals are toxic because they combine with essential sulfhydryl groups on key enzymes. Christensen (1971) also measured the *in vitro* inhibition of several metal ions (including chromium) to the enzymes glutamate oxalacetic transaminase (GOT), and lactic dehydrogenase (LDH) in blood plasma from white suckers. Dichromate ion inhibited GOT and not LDH; whereas chromic ion inhibited LCH and not GOT. However, neither dichromate nor chromic ion caused more than 16% inhibition of these enzymes. Christensen suggested that "within an animal a very slight change in the activity of the enzymes might have a profound effect over a period of time."

Fromm and Stokes (1962) showed: 1) that the rainbow trout can accmulate chromium from water containing as little as 1 microgram/l Cr; and 2) that uptake of chromium by fish is passive and dependent on concentration and time of exposure. They suggested that short-term exposure to relatively large slugs of chromium would be less detrimental than long-term exposure to low levels. In other studies, Stokes and

Fromm (1965) exposed trout to 2.5 mg/l Cr as chromate for one week. Glucose transport (*in vitro*) in gut segments from treated fish was 40 and 32% lower than in gut segments from untreated fish. Results suggested that the presence of chromium in the tissues inhibited the entry of glucose. Doudoroff and Katz (1953) noted that trivalent chromic salts were more toxic than the hexavalent ions, chromate and dichromate.

Malacea (1966) reported that hexavalent chromium was more toxic than trivalent chromium to *Daphnia*. Baudouin and Scoppa (1974) reported 48 h LC50 values of 10.0, 10.1, and 0.022 mg/l Cr^{+6} (at pH 7.2) for *Cyclops abyssorum*, *Eudiaptimus padanus*, and *Daphnia hyalina*, respectively.

Patrick *et al.* (1968) studied the relative sensitivity of three levels of the aquatic food chain--a diatom (*Nitzschia linearis*), a common snail (*Physa heterostropha*), and a fish (*Lepomis macrochirus*)--to several common constituents of industrial waste. The diatom was most sensitive to hexavalent chromium and the fish was the least sensitive.

Trama and Benoit (1960) found that bluegills were more susceptible to potassium dichromate ($K_2Cr_2O_7$) than to potassium chromate (K_2CrO_4) due to the lower pH that resulted when the dichromate salt was used. Low pH (6 to 7) favored high concentrations of univalent hydrochromate which Trama and Benoit (1960) believed was more easily adsorbed than the divalent ions chromate or dichromate. The ions are paritioned as follows:

$$2CrO_4^{=} + 2H^{+} \rightleftarrows 2HCrO_4^{-} \rightleftarrows Cr_2O_7^{=} + H_2O \ .$$

Biesinger and Christensen (1972) determined that the 3-wk LC50 for *Daphnia magna* was 2.0 mg/l Cr^{+3} when added as chromium chloride,

but 0.6 mg/l Cr^{+3} caused a 50% reproductive impairment over a 3-wk period. Freeman and Fowler (1953) reported that 0.42 mg/l as Cr was the 100 h LC50 for *D. magna*. Pickering and Henderson (1966) used static bioassays to determine 96 h LC50s for the fathead minnow (27.3 and 17.6 mg/l Cr), bluegill (118 and 133 mg/l Cr), goldfish (37.5 mg/l Cr) and guppy (30.0 mg/l Cr). Warnick and Bell (1969) suggested that aquatic insects may not be as sensitive as fish to heavy metals; *Acroneuria*, *Ephermerella* and *Hydropsyche* larvae had 96 h LC50 values of greater than 32 mg/l Cr^{+3} respectively. Available literature suggests that where chromium is a dominant component of an effluent, *Daphnia* would be expected to be far more sensitive than fish in tests of acute toxicity.

Zinc: Brungs (1969) showed that reproduction by fathead minnows was almost totally inhibited at zinc concentrations (0.18 mg/l) that had no effect on survival, growth, or maturation. Baudouin and Scoppa (1974) reported the following LC50s for zinc for three zooplankters: 5.5 mg/l (*Cyclops abyssorum*), 0.50 mg/l (*Eudiaptomus padanus*), and 0.04 mg/l (*Daphnia hyalina*).

Oil: One of the assumed effects of oil on aquatic organisms is the coating of the respiratory surface thereby inhibiting gaseous exchange (Wilber 1969). Tarzwell (1971) summarized some of the early literature on the toxicity of oil to aquatic life. He reported that oil and its components adversely affected the ciliary activity of mollusc gills and the chemotactic reactions of invertebrates. Recent studies on oyster and crab behavior discuss similar adverse effects (Kittredge *et al.* 1974). Larval development of the crab *Neopanope texana* was delayed by the water soluble fraction of crude oil (Katz 1973).

The toxicity to algae of seawater containing no. 2 fuel oil was reported by Pulich *et al.* (1974). The medium- and higher-boiling fractions of both crude and no. 2 fuel oils were the most toxic.

LaRoche *et al.* (1970) presented bioassay procedures for oil and oil dispersant toxicity tests using three marine organisms (mummichog, sand worm, and grass shrimp). They reported that, in general, crude oils are far less toxic in 96 h than refined oils. The hydrocarbon composition of the aqueous phase of oil-in-water dispersions and oil bioassays using estuarine crustaceans and fish were presented by Anderson *et al.* (1974). They found that the water-soluble fractions and oil-in-water dispersions of refined oils were considerably more toxic to the organisms tested than those of the crude oils. Main hydrocarbon constituents of the water-soluble fraction of no. 2 fuel oil were benzene, toluene, ethyl-benzene, meta- and para-xylenes, trimethyl benzenes, and naphthalene. By driving off the aromatic compounds, aeration decreased the toxicity of the water-soluble fraction. Crude oils were less toxic to *Daphnia* than oil emulsifiers or mixtures of oil and emulsifiers (Dowden 1962). Crude oil toxicity was attributed to surface entrapment of *Daphnia* (Dowden 1962).

Phenol: Phenol is seldom removed during transport or discharge of an effluent, and it is somewhat difficult to treat either biologically or chemically. Often it remains in solution and is discharged to a natural system. Therefore, phenol is often the controlling toxicant in oil refinery waste discharges (Tubb and Dorris 1965, Reynolds *et al.* 1974). Many phenolic compounds (such as the methyl- and chloro-phenols)

are more toxic than phenol, and many phenolics affect the taste of fish flesh at levels that do not appear to adversely affect fish (NAS 1972).

Cole and Wilhm (1973) measured the respiration of fourth instar *Chironomus attenuatus* larvae which had received continuous exposure to concentrations of phenol ranging from 0 to 22.4 mg/1. They found that oxygen uptake increased and ash-free weight decreased with increasing phenol concentration. Patrick *et al.* (1968) found that bluegills were more sensitive than a diatom *Nitzschia* sp. and a snail *Physa* sp.

However, literature reviewed here suggests that *Daphnia* are probably comparable to fish in sensitivity to phenol. Anderson (1944) found that 94 mg/1 phenol was the threshold concentration for immobilization of *D. magna* in Lake Erie water. Depending on the dilution water used, Dowden and Bennett (1962) found 48 h TLm values of 21 and mg/1 phenol for *D. magna*. Concentrations of 8 to 10 mg/1 phenol in water have been found lethal for *Daphnia* and *Cyclops* (Ellis 1937). Herbert (1962) reported that 8.0 mg/1 was the 48 h LC50 for rainbow trout. Wilbur (1969) reported that the toxic threshold for fishes was about 1.0 mg/1.

Sulfides: The literature indicates that fishes, particularly the juvenile stages, are considerably more sensitive to hydrogen sulfide than are *Daphnia*. Like hydrogen cyanide, the mode of action of hydrogen sulfide is believed to be inhibition of cytochrome oxidase, the terminal enzyme of the electron transport system (Lehninger 1970). Therefore it inhibits cellular respiration.

Sulfides, which generally are present in oil refinery wastes as H_2S, are often lost to the atomsphere during transport and discharge of

the waste stream, and they are also readily removed by various treatment methods (Reynolds *et al.* 1974).

Adelman and Smith (1970) found that the maximum safe level of H_2S for eggs of northern pike was 0.014 to 0.018 mg/l and, for sac fry of the same species, the maximum safe level was 0.004 to 0.006 mg/l for 96 h exposure. These authors assumed: 1) that H_2S entered the gills of fish by diffusion of molecular H_2S; and 2) that undissociated H_2S was the toxic component. Smith (1971) working on walleyes and fathead minnows found that safe levels varied from 0.0029 to 0.012 mg/l with eggs being the least sensitive and juveniles being the most sensitive in 96 h tests. Bonn and Follis (1967) found 24 h TLm values for channel catfish fingerlings were in the range of 0.53 to 0.8 mg/l H_2S. The 50 h TLm for *D. magna* was 13 mg/l NaS_2, but the pH was not reported and this factor could influence the toxicity (Dowden and Bennett 1962).

Cyanide: Cyanide toxicity is essentially an inhibition of oxygen metabolism. Undissociated HCN, like H_2S, causes cellular asphyxia by inhibiting the terminal cytochrome of the electron transport system (Lehninger 1970). Review of the literature suggested that the toxic response of *Daphnia* to cyanide is roughly comparable to that of fish.

The acute toxicity to fish of various solutions of simple and complex cyanides has been attributed to the toxic action of hydrocyanic acid (HCN). At the pH levels of natural water, or in acid waters, the cyanide ion (CN^-) cannot be the principal lethal factor because the ion is 1) present in relatively low concentrations, and is 2) less penetrating through the gills compared to molecular HCN (Doudoroff *et al.* 1966).

Broderius (1973) reported that the minimum, lethal threshold concentration (expressed as mg/l CN^-) was 0.057 at 10 C for brook trout, 0.104 at 25 C for bluegill, and 0.120 at 25 C for fathead minnow. Doudoroff *et al.* (1966) reported about the same values for bluegill. Leduc (1966) found that concentrations as low as 0.04 mg/l CN^- reduced swimming speed, and concentrations as low as 0.035 mg/l depressed growth of *Chichlasoma bimaculatum*. He concluded that chronic exposure to cyanide at concentrations above 0.02 mg/l HCN can have deleterious effects on fish. Cairns and Scheier (1963) reported that the loss of volatile cyanide in the KCN static bioassays varied from 16 to 62 percent of the initial concentration during a 96 h experimental period. This loss apparently accounted for the decrease in lethality after the first 48 h. Malacea (1966) found that the minimum 48 h lethal concentration of cyanide ion was 1 mg/l for *Daphnia*, but Silaichuk (1964, cited by Clark 1974a) reported that 0.1 mg/l CN^- killed 60 per cent of the exposed *Daphnia*.

Iron-cyanide complexes (ferricyanides) are extremely stable compounds, but it has long been known that both $Fe(CN)_6^{-4}$ and $Fe(CN)_6^{-3}$ are decomposible to a large extent under the influence of light, more readily so in the presence of oxygen. Various authors (e.g. Mitra *et al.* 1963, Moggi *et al.* 1966) have suggested mechanisms for the photodecomposition of potassium ferrocyanide with the release of one-sixth of the cyanide.

Allen *et al.* (1948) studied the toxicity of some sewage effluents containing thiocyanate. They believed that thiocyanate (SCN) was the constituent which reacted with chlorine to form cyanogen chloride (CNCl).

Some effluents were toxic even when the doses of chlorine were well below the demand so that there was no residual chlorine present. Allen *et al.* (1948) reported that the minimum lethal threshold concentration for rainbow trout was 0.08 mg/l CNCl.

Environmental Factors Affecting Bioassay Results

Several factors, known to affect results of toxicity tests, have been reviewed by Sprague (1970), and will be mentioned here only briefly. Mineral content, particularly the calcium concentration of water, may increase or decrease the toxicity of a pollutant. This is probably due to the fact that high calcium generally decreases permeability of cell membranes (Prosser 1973). Velocity of flow over ion exchange surfaces, such as gills, also affects toxicity (Lloyd 1961). Therefore, factors which affect respiratory flow can be expected to affect the rate of toxicant uptake.

Temperature may or may not affect toxicity (Cairns *et al.* 1975). In terms of incipient LC50s, phenol and zinc are more toxic at lower temperature (Sprague 1970). Sprague (1970) warns against interpreting slower mortality in short tests as a decrease in the incipient LC50. Temperature would be expected to affect both uptake and detoxification rates in poikilothermous animals, and consequently the particular mechanisms involved would determine the effect of a change in temperature. Loch and MacLeod (1974) concluded that increasing temperature results in increasing toxicity for pulp mill effluents. They recommended that temperature control over a narrow range is essential for a valid bioassay.

In general, factors affecting toxicity are: 1) the form of the toxicant in water and/or the physiological state of the test species; 2) chemical reaction with other substances to form new substances; 3) joint effects with other substances; and 4) the test species (Clark 1974b, Sprague 1970).

II. PRELIMINARY INVESTIGATIONS

From the start, this study was specifically designed to meet the needs of the petroleum refining industry. A simulated refinery effluent was required before a survey of potentially useful invertebrates and fish could begin. It was also necessary to provide a perspective of how aquatic species might compare in terms of their sensitivity to a simulated effluent.

A. The Arbitrary Reference Mixture

It was impossible to define an "average effluent" or a "typical value" for each component of an effluent because of the variability of refinery effluents. At the same time, it was necessary to control the concentration of each component in order to meet the objectives of the research.

Because chemical changes occur during storage of refinery effluents (Gould and Dorris 1961), an arbitrary reference mixture (ARM, Table 2) was formulated to approximately the same levels in the EPA guidelines for 1977 (Table 1). The ARM contained several components of refinery wastes but was unlike any specific effluent (Table 3). No. 2 fuel oil was used as the oil and grease component of the ARM because treated refinery effluents were not expected to have significant amounts of hydrocarbons with relatively low boiling points. Zinc ion was not included in the ARM because at high concentrations it forms an insoluble complex with sulfide. Additional chemicals would have made the ARM unnecessarily complex for the initial phases of bioassay development.

Table 2. Arbitrary reference mixture (ARM).

Parameter	Concentration	Ingredient
$NH_3(N)$	10 mg/l	NH_4Cl
Cr total	0.25 mg/l	K_2CrO_4
Oil and grease	10 mg/l	No. 2 fuel oil
Phenol	0.1 mg/l	Phenol
Sulfide	0.17 mg/l	$Na_2 \cdot S \cdot 9H_2O$
Total suspended solids	20 mg/l	Kaolinite*
pH	6.8-7.2	$NaOH/H_2SO_4$

*--well-crystallized kaolinite from the Clay Mineral Society Repository, University of Missouri

Table 3. Monthly averages of effluents of refineries where toxicity tests were performed. All values are expressed as milligrams per litre except specific conductance (µmho/cm) and pH.

Parameter	Refinery					
	A	B	C	D	E	F*
Ammonia	5	69.4	12	22.9	7	6.58
5-day biological oxygen demand	5.6	11	105	not available	49	19.8
Total organic carbon	9	--	57	60	--	85.2
Chemical oxygen demand	76	150	--	350	80	227.8
Chromium	<0.005	0.008	0.064	1.5	--	--
Lead	<0.005	--	0.021	<0.7	--	--
Nickel	<1.0	--	0.161	<0.1	--	--
Oil and grease	3	4	5.26	52.9	14	11.2
pH	8.2	7.7	7.16	8.36	7.4	7.17
Phenols	<0.01	0.02	0.042	8.90	0.1	0.0116
Specific conductance	4000	--	--	--	--	--
Sulfides	<2	<0.05	none detected	1.9	0	<0.1
Suspended solids	19	8	35.6	66	66	26.6
Zinc	<0.02	0.05	0.071	0.001	--	--
Total sulfur	--	--	--	--	66	--

*--average of five separate samples collected one week apart

In practice, the ARM was prepared without the oil component at 100 times the concentrations shown in Table 1. The X100 stock was prepared using distilled water. Test solutions were made by diluting the X100 stock with dechlorinated, Blacksburg tapwater. The ranges of chemical constituents for this tapwater were: hardness, 35 to 45 mg/l; alkalinity, 40 to 50 mg/l; pH, 7.0 to 7.4; and the ratio of magnesium to calcium, 3:10. Test solutions were made in multiples or fractions of the basic ARM formulation. The last step in making a test solution was the addition of an appropriate amount of no. 2 fuel oil.

Using American Chemical Society grade chemicals, the X100 ARM (minus oil) was prepared as follows:

1) Ammonium Chloride: NH_4Cl crystals were dried at 100 to 110 C for 1 h and then cooled in a dessicator. In 966 ml of distilled water 3.82 g of ammonium chloride was dissolved.
2) Stock Chromate Solution: Potassium chromate K_2CrO_4 was dried at 100 to 110 C for 1 h and cooled in a dessicator. In distilled water 9.337 g of K_2CrO_4 were dissolved and diluted to 1000 ml to obtain a solution containing 2.5 mg/ml Cr. Ten millilitres was added to the ammonium chloride solution (1).
3) Phenol Solution: Crystals were placed over $CaCl_2$ in a dessicator overnight. In distilled water 0.10 g phenol were dissolved and diluted to 100 ml. This solution was made just prior to use and 10 ml of this solution was added to the ammonium chloride solution (1).

4) Sulfide Solution: Sodium monosulfide $NaS \cdot 9H_2O$ was dried by placing freshly ground crystals over $CaCl_2$ in a dessicator overnight. In distilled water 1.27 g sodium monosulfide was dissolved and diluted to 100 ml to obtain a solution containing 1.7 mg/ml S^{--}. This solution was made just prior to use and 10 ml were added to the ammonium chloride solution (1).

5) Kaolinite: Well-crystallized kaolinite was dried and cooled in the same manner as the ammonium chloride. To the ammonium chloride solution (1) 20 g of kaolinite were added.

6) pH: 1 *N* H_2SO_4 was added by drops and mixed in the solution until the pH was 7.0 $\pm$ 0.02. If necessary, the pH was adjusted upward with 1 *N* NaOH.

7) No. 2 Fuel Oil: The appropriate amount of oil was added directly to each test solution, using a Hamilton syringe pipet. It was not added to the X100 ARM stock solution. The fuel oil was tightly sealed and stored at room temperature in a 55 gallon drum. As a precaution against changes in the oil, samples were taken from the drum, placed in 10-15 ml pyrex glass vials with Teflon-lined caps, and frozen at -23 C for storage. During the selection of a suitable test organism, oil taken from the drum in the spring of 1974 was used. For later work, oil samples from the drum in the fall of 1974 was used.

8) If there was a delay of more than 2 h before beginning toxicity tests, the X100 ARM minus oil was tightly capped, refrigerated at 4 C, and a record of storage time was made.

When portions of the X100 ARM minus oil were taken for test solutions, the mixture was stirred to resuspend the kaolinite.

B. Chemical Analyses

Analyses of biochemical oxygen demand (BOD), total alkalinity, hardness, dissolved oxygen (Yellow Springs self-stirring BOD probe 5420A with Model 54 meter or the azide modification of the Winkler technique), pH (Corning 109 digital or Orion 601 digital pH meters) and atomic absorption (Norelco SP90A spectrophotometer) were performed according to "Standard Methods" (APHA 1971). Chemical tests, performed by various laboratories associated with the petroleum industry, were based on "Standard Methods" (APHA 1971) which, in some cases, had been modified to fit their particular needs.

C. Analyses of the ARM

An analysis of the Gulf no. 2 fuel oil used in the arbitrary reference mixture is shown in Table 4. The total organic carbon content of reconstituted freshwater (Marking and Dawson 1973) was 14 mg/l after 3 h contact of eight parts water to one part of fuel (J. Eppolito, pers. comm.).

BOD tests showed that the X1 ARM had an oxygen demand of less than 1.0 mg/l in 5 days and during the four-day bioassays, dissolved oxygen remained above 6 mg/l. Atomic absorption analysis for chromium showed that only one-third of the added amount was actually dissolved in the aqueous phase. Possibly the kaolinite had an ameliorating effect by surface adsorption of this metal.

Table 4. Analysis of Gulf no. 2 fuel oil used in the arbitrary reference mixture.

Parameter	Level or Concentration
Gravity	34.1°API
Flash; P-M	158°F
Pour	+5°F
Color, D1500	L 1.0
Odor	normal
Sulfur	0.10%
Water and sediment	nil %
Carbon residue on 10% bottoms, rams	0.16%
Alkalinity, Gulf 832 procedure A:pH	7.1 units
Analine point, D611	138°F
Cetane index	44.5
Distillation, D86	
Overpoint	360°F
Endpoint	647°F
10% @	420°F
50% @	495°F
90% @	596°F
Recovery	98.0%
Residue	1.0%
Loss	1.0%

*These data were supplied by the Water Quality Subcommittee, W-12 Task Force of API.

Samples of the ARM were shipped to three oil company laboratories for analysis of the various components. There were two analyses conducted by these laboratories. For the first analysis X1 ARM was made up, packed in ice, and shipped to the refineries. There was considerable variation in the results (Table 5). Possible reasons for this variation were shipping delays, heating during shipment, and the fact that concentrations of some components were not far above test limits. For a second series of analyses the ARM ingredients were shipped to each laboratory where the X1 ARM was made up prior to analysis. These samples were tested immediately and after a 5 to 21 day storage period (Table 6).

The measured concentrations of ammonia nitrogen, oil, total suspended solids and pH were closer to added concentrations in the second analysis (Table 6). The measured concentrations of total chromium, phenol and sulfide varied more from the added concentrations in the second analysis than in the first. Considerable variation in BOD_5, COD and TOC was evident in both analyses.

Judging from the results presented in Tables 5 and 6, it would seem possible that many of the analytical methods used were not adequate to measure the concentrations of toxicants in the ARM (Table 2). The ammonia levels remained constant or increased. Chromium levels decreased, possibly by adsorption to the glass and the kaolinite. Oil, sulfide, and total suspended solids were reasonably constant and the variation in results probably reflected differences in individual techniques. BOD_5 may have decreased after storage. The COD and TOC values probably do not change during storage. The decrease in phenol

Table 5. Analysis of the arbitrary reference mixture (Test I).

Parameter	Added Concentration (mg/l)	Measured Concentration (mg/l) Refinery Laboratory		
		I	II	III
$NH_3(N)$	10.0	9.0	1.4	0.05
Cr VI	0.005	not reported	<0.005	0.100
Cr total	0.25	not reported	0.19	0.150
Oil and grease	10.0	7.0	0.2	3.0
Phenol	0.1	0.13	0.16	0.17
Sulfide	0.17	<1.0	1.0	<0.01
Total suspended solids	20.0	20.0	20.0	4.0
BOD_5	--	not reported	3.0	<1.0
COD	--	4.0	20.0	nil
TOC	--	not reported	50.0	25.0
pH	7.0 (adjusted)	8.0	7.8	7.3

Table 6. Analysis of the arbitrary reference mixture (Test II).

Parameter	Added Concentration (mg/l)	Concentration (mg/l) Laboratory					
		I	I[a]	II	II[b]	III	III[c]
$NH_3(N)$	10.0	8.7	8.4	11.0	15.05	9.9-10.0	10.0-10.2
Cr VI	0.005	---	--	--	--	0.07	0.02
Cr total	0.25	--	--	0.42	0.22	0.10	0.05
Oil and grease	10.0			10.0	13.8	6.0	6.2
Phenol	0.1	.084	.085	0.32	0.04	0.10	0.095
Sulfide	0.17	nil	nil	0.35	0.47	nil	nil
Total suspended solids	20.0	17.0	18.0	24.5	21.9	17.6	18.4
BOD_5	--	12.0	--	2.0	--	10.0	4.0
COD	--	--	--	9.5	--	21.4	19.7
TOC	--	--	--	20.0	40.0	12.0	12.4
pH	7.0 (adjusted)	7.5	7.5	7.15-7.60	7.4	7.4	6.3

[a]A second ARM analysis was conducted after storing the X100 stock for 5 days at 4 C.
[b]A second ARM analysis was conducted after storing the X100 stock for 21 days in a refrigerator; Blacksburg tapwater was not used as a diluent.
[c]A second ARM analysis was conducted after storing the X100 stock in the refrigerator for 7 days.

and increase in ammonia during storage may have been due to bacterial activity.

D. Selection of a Test Organism

Research was conducted on the effect of the ARM (Table 2) to 15 species of invertebrates and three species of fish. Most of these organisms were selected on the basis of one or more of the following criteria: commercial availability, ease in culturing, cost, possible short acclimation time, small test volumes required for each test, and possible behavioral response. Sources of the test organisms are given in Table 7.

These animals were tested at 21 $\pm$ 1 C under a 16L:8D photoperiod and a light intensity of 15 to 100 ft-c (160 to 1080 lux). Dilution water was dechlorinated Blacksburg tapwater (about 50 ppm hardness). All tests were static without aeration or renewal of the test solution. The test containers were adjusted to allow at least 0.5 litres of test volume per gram of animal weight. Fish were tested using 20-gallon polyethylene garbage pails filled with 30 litres of test solution. Smaller glass containers were used for the invertebrates. For most tests, 10 to 20 organisms were placed in each of five or six different concentrations of the ARM.

The results of this preliminary study are presented in Table 8. The 24, 48, and 96-h data were expressed as the median concentration that kills 50% of the population (LC50). These data were obtained by graphical interpolation (APHA 1971). These data were expressed as multiples or fractions of the ARM.

Table 7. Sources of test animals used in this study.

Organism	Source
Gastropoda	
Physa sp.	University Motel sewage oxidation pond, Blacksburg, Mont. Co., VA
Helisoma sp.	Fish aquarium
Nitocris sp.	New River near Pearisburg, Giles Co., VA
Goniobasis sp.	Sinking Creek near Newport, Giles Co., VA
Amphipoda	
Gammarus sp.	New River near McCoy, Mont. Co., VA
Decapoda	
Cambarus sp.	Sinking Creek near Newport, Giles Co., VA
Cladocera	
Daphnia pulex	Carolina Biological Supply Co., Burlington, NC
Daphnia magna	Dr. Winner, Miami University, Miami, OH
Oligochaeta	
Dero sp.	Carolina Biological Supply Co., Burlington, NC
Tubifex spp.	Carolina Biological Supply Co., Burlington, NC and University Motel sewage oxidation pond, Blacksburg, Mont. Co., VA
Stylaria sp.	Carolina Biological Supply Co., Burlington, NC
Aeolosoma headleyi	*D. pulex* cultures in my lab, original source unknown
Turbellaria	
Dugesia tigrina	Carolina Biological Supply Co., Burlington, NC
Rotifera	
Philodina acuticornis	Dr. Buikema, Virginia Polytechnic Institute and State University, Blacksburg, VA
Insecta	
Aedes aegypti (eggs)	W. Knausenberger and D. Simonet, Virginia Polytechnic Institute and State University, Blacksburg, VA
Osteichthyes	
Salmo gairnerii	Wytheville State Fish Hatchery, Wytheville, VA
Lepomis macrochirus	Veterans Administration pond, Salem, VA and Zett's Fish Hatcheries, Drifting, PA
Carassius auratus	Grassy Fork Fish Hatchery, Martinsville, IN
Culaea inconstans	Mogul-Ed, Oshkosh, WI
Pimephales promelus	Zett's Fish Hatcheries, Drifting, PA

Table 8. Comparative tolerance of selected freshwater invertebrates and fish exposed to an arbitrary reference mixture (ARM). The LC50 data are expressed as multiples and fractions of the ARM shown in Table 2.

Organism	LC50		
	24 h	48 h	96 h
Gastropoda			
Physa sp.	9.2-22.0	7.8-20.0	6.4-19.0
Helisoma sp.	7.4	7.4	-
Nitocris sp.	3.7	1.9	1.8
Goniobasis sp.	4.6-7.4	4.5-4.9	0.37-3.3
Amphipoda			
Gammarus sp.	5.6	3.2	1.3
Decapoda			
Cambarus sp.	7.5	-	-
Cladocera			
Daphnia pulex	0.11	0.07	0.03
Daphnia magna	0.16	0.06	-
Oligochaeta			
Dero sp.	5.6	5.6	5.6
Tubifex sp.	1.8-5.6	1.8	0.21-0.56
Stylaria sp.	6.0	-	6.0
Aeolosoma headleyi	8.3	3.0	4.0
Planaria			
Dugesia tigrina	4.9-6.3	2.0	1.6-2.0
Rotifera*			
Philodina acuticornis	1.0 or 6.0	1.0 or 6.0	1.0 or 6.0
Insecta			
Aedes aegypti			
3rd instar	2.3	-	1.8
1st instar	0.5-0.8	-	-
Osteichthyes			
*Salmo gairdneri***	4.0	4.0	-
Lepomis macrochirus	7.4	-	5.6
Carassius auratus	7.8	7.0	6.4

*--two levels of effect
**--trout fingerlings

In terms of 24-h lethality, the water fleas and the rotifer were the least tolerant. After 96 h the amphipod and the worm *Tubifex* were the next least tolerant. The planarian and the mosquito were tolerant at less than X2 ARM. Running-water snails *Nitocris* and *Goniobasis* were less tolerant than pond snails, but they were not easily cultured, a criterion which made them unsuitable as test organisms.

The freshwater invertebrates (except the worm *Aeolosoma* and the snail *Physa*) were as tolerant to the ARM as the bluegill and the goldfish. Five of the invertebrates were less tolerant than the rainbow trout after 24 h of exposure.

The photonegative response of mosquito larvae (*Aedes aegypti*) has been suggested as a bioassay tool (Simonet 1975). The worm *Aeolosoma headleyi* aggregated at low concentrations of the ARM but not in the controls. At low, sublethal concentrations of the ARM, reproduction of *Aeolosoma* was repressed. The possible use of this organism in rapid, 96 h, reproductive-impairment tests was investigated by Newman (1975). Newman noted an increase in asexual reproduction of *Aeolosoma* under zinc stress.

The selection of a suitable test organism was based on several criteria. The animal selected should be easy to observe; death, or some other quantifiable and detrimental response (such as a definite behavioral change) had to be defined and easily judged. The test animal should be relatively easy to handle and require only a short acclimation time. The care and handling of the organism should require a minimum of special training and equipment. The animal should be sensitive to the ARM at concentrations less than the basic formulation in order that

detrimental effects could be measured at, or near, the levels of contaminants promulgated by EPA for 1977 effluent guidelines (Table 1). The animal should be inexpensive and either commercially-available or easily-cultured in the laboratory. Finally, the animal should require a minimum amount of space for holding and testing.

The potentially useful bioassay animals as determined on the basis of these criteria were: the waterfleas, *Daphnia pulex* and *D. magna*; the mosquito, *Aedes aegypti*; the worm, *Aeolosoma headleyi*; and the snails, *Physa* sp. and *Helisoma* sp. If death were used as the primary criteria for selection, the snails would be unsuitable because death did not occur at concentrations less than approximately X9 ARM. Also, it was difficult to determine death in the snails. The mosquito was easy to work with because eggs can be stored for long periods, hatched in one hour in deoxygenated water, and used immediately for bioassays. However, a permit is needed to obtain the eggs of *Aedes aegypti* so they were not studied any further. The worm *Aeolosoma* was small and difficult to work with, so it was not studied further even though one could quickly obtain data on reproduction, a chronic rather than an acute effect (Newman 1975).

The waterfleas, *Daphnia pulex* and *D. magna*, were the most sensitive to the ARM. Of the two, *D. magna* was larger and easier to observe, but *D. pulex* was selected for two reasons: it is distributed over the entire North American continent, and it was the most sensitive of 19 species tested. *D. magna* is typically a hardwater species whereas *D. pulex* is found in a wide range of habitats. The cosmopolitan distribution of *D. pulex* may be important to regulatory agencies that

specify animals for toxicity testing. Within 8 to 24 hours *Daphnia* died in 1/10 the basic ARM concentration, a level which was approximately 1/40 the LC50 for the bluegill and the goldfish. Furthermore, this animal met all of the other criteria for selection. For these reasons it was decided to conduct more research with *Daphnia pulex* and to field-test the usefulness of using this animal for bioassay tests.

Daphnia species have been used for testing toxicity since 1929 (Berger 1929). *Daphnia* species have been used to measure acute toxicity of insecticides (Wollerman and Putman 1955, Sanders and Cope 1966), crude oil and emulsifiers (Dowden 1965, Grodner 1959), refinery wastes (Dorris *et al.* 1974), aquatic herbicides (Crosby and Tucker 1966), metals (Anderson 1948), and various other substances found in industrial wastes (Anderson 1944, Anderson *et al.* 1948). Acute toxicity tests have used animals of unspecified ages (Dowden 1965), entire populations sampled from a culture tank (Wollerm and Putman 1955), or early preadult stages (Sanders and Cope 1966, Dorris *et al.* 1974, Crosby and Tucker 1966, Anderson 1948, Anderson 1944, Anderson 1946, Macek and Sanders 1970). Chronic toxicity, or reproductive impairment, has been determined for *Daphnia* species exposed to nitrilotriacetate (Biesinger *et al.* 1974), various metal ions (Biesinger and Christensen 1972), and diquat, an aquatic plant herbicide (Gilderhus 1967). *Daphnia magna* response to toxicants was judged representative of predominant zooplankton (Anderson *et al.* 1948). Generally, the results obtained by other workers using *Daphnia* species were less variable than results using fish (Macek and Sanders 1970).

III. DAPHNIA BIOASSAY

A. Rationale

The bioassay presented here was designed for on-site toxicity testing of refinery effluents. In deciding on a static bioassay without aeration and without toxicant renewal, several factors were considered. As waste treatment facilities are improved, it is likely that rapidly degraded, relatively unstable, or volatile toxicants and waste with an excessively high oxygen demand will be virtually eliminated and much less important than in the past. Therefore, in many cases, the renewal of test solution will not be crucial for acute toxicity tests. Compared to continuous-flow test systems, the equipment required for static tests is simple, maintenance free, and readily available at most water quality laboratories. Likewise, much less technician training is required for static toxicity tests. Finally, reliable flow-systems do not exist for continuous-flow testing of multi-phasic mixtures (oil, water, and sediment) occasionally characteristic of industrial waste waters.

The test volume/biomass ratio was well above the limits suggested for fish. This was done to reduce the oxygen demands of the test organism per unit volume of test solution, to limit the buildup of metabolic waste products, and to minimize serious depletion of toxic substances due to uptake by organisms. The temperature and light conditions were standardized for existing, refinery-laboratory conditions, as will be described.

B. Procedure

Culturing Daphnia

A general treatment of the life history and anatomy of *Daphnia* can be found in Pennak (1953) or Brooks (1959). Test organisms were cultured under the following conditions:

1) Aeration: A minimum oxygen concentration of 6 mg/l was maintained using gentle aeration with "oil-free" air. Air was supplied by a central compressor and filtered through an Erlenmeyer flask stuffed with cotton and fitted with a two-hole rubber stopper and glass tubes. The cultures were covered with glass plates to keep dust and laboratory air from entering the water.
2) Temperature: The water temperature was maintained at 21 $\pm$ 2 C, the temperature of most air-conditioned laboratories.
3) Light: Cool-white fluorescent bulbs were used for 16 hours of light per day. Light intensity at the surface of the holding tanks was between 5 and 60 foot candles.
4) Osmotic concentration: A relatively constant ionic strength was maintained by adding distilled water when the tank level dropped below 90% of its initial level. Deionized water was not used because soluble residue from deionizing resins may be toxic to *Daphnia*. The specific conductance of the culture was approximately equal to that of the diluent, which was generally charcoal-dechlorinated, Blacksburg tapwater.

5) Feeding: *Daphnia* were maintained on Purina Trout Chow. The food was prepared as follows: a) Into a clean mortar 2.5 g of trout chow and 5 ml of distilled water were placed and left for 10-15 minutes; b) the food was ground to a smooth paste, diluted with 100 ml of distilled water and allowed to settle for 30 minutes in a 100 ml graduated cylinder; c) the floating material was discarded and the remaining supernatant (but not the sediment) was poured off and stored up to one week at 4 C. The food suspension was added to *Daphnia* cultures at the rate of less than 10 ml of food per 30 litres of culture every other day. The key to culture success was to feed at a rate not to exceed an amount that the organisms could clarify in one day. If culture density was low, two ml of food per 30 litres of culture every other day was often adequate.

6) Culture density: Ten-gallon (38 litre) aquaria were used because the animals could be netted easily from large tanks. *Daphnia* density in these holding tanks did not exceed 100 animals/litre. Overcrowding stressed the animals and caused production of ephippial eggs. To avoid stressing the test population, the cultures were either thinned with a net or about half of the culture water was replaced before the density of the animals exceeded 100/litre. Unintentionally the cultures contained worms (*Aeolosoma headleyi*), snails (*Physa* sp.), and unidentified midge larvae, but these were not believed to affect success of the *Daphnia* culture.

Handling of Daphnia *for Bioassay Purposes*

Daphnia were collected with a conical net (1.0 mm mesh opening) moved slowly (about 20 cm/s) through the culture tank. The net was rested on the edge of the tank for about three minutes to allow essentially all of the small, immature *Daphnia* to escape. The net was not completely lifted out of the water because doing so sometimes resulted in air becoming trapped under the carapace of the *Daphnia*. (Floating animals were exposed to surface-film materials.) A 1.5 mm bore medicine dropper was used to transfer the netted *Daphnia* to the test containers. The tip of the dropper was placed beneath the surface of the test water to minimize the possibility of coating the animals with surface film materials. When test concentrations were not prohibitively high, the following procedure was used to avoid back-transfer of toxicant to the culture: a) 100 ml of diluent was added to each test container, b) 9 to 13 *Daphnia* were transferred into each container, c) the number of *Daphnia* was counted, d) the material under test was added to the remaining diluent in a 200 ml graduated cylinder, and e) this mixture was added to the test beakers, giving a total of 300 ml of test solution.

General Bioassay Procedures

All bioassays were conducted without aeration or renewal of toxicant. Unless otherwise specified, light intensity was between 40 and 70 ft-c, photoperiod was 16L:8D, and temperature was 21 $\pm$ 2 C. Light intensity and light quality were controlled with cool-white fluorescent bulbs. Most bioassays were run in triplicate using eight

concentrations of toxicant and a control containing only dilution water and test organisms. Generally, 9 to 13 animals at least six days of age were placed in 300 ml of test solution. Dilution and culture water was carbon-dechlorinated Blacksburg tapwater, which was vigorously aerated 12 h prior to use to remove final traces of chlorine. The dilution water had a pH of 7.5 $\pm$ 0.3, a total hardness of 46 $\pm$ 5 mg/l, and a total alkalinity of 42 $\pm$ 4 mg/l as $CaCO_3$.

The number of live organisms in each concentration was recorded at 0, 8, 24, 48 and 96 h. Death was defined as cessation of all visible signs of mobility of the second antennae, respiratory appendages and the postabdomen during a 5 s observation. Observations were made against a dull, black background with the unaided eye using a 60 watt goose-neck lamp next to the test container at water level. Dead animals were not removed during the bioassay.

The oil component of the ARM was added directly to the surface of test beakers for my-laboratory and petroleum-refinery-laboratory evaluations. For the remainder of the studies using the ARM, an appropriate amount of oil (23 µl) was added to 200 ml of X10 ARM and blended for 15 s in a Waring blender. This was in contrast to the procedure used initially where the fuel oil was carefully added to the test beakers by micropipet after the desired dilution had been made. There were two advantages of emulsified ARM: It better simulated a refinery effluent, and it made it easier to make test dilutions. Where oil was added directly to the surface film, more oil remained on the surface. The emulsified ARM had more oil in solution and suspension.

Evaluation of the Daphnia *Bioassay*

Twenty-two bioassays were conducted with the complete ARM, 12 tests were conducted using the ARM without oil, and one test was conducted with oil alone.

In order to evaluate the *Daphnia* bioassay, the method was also tested at six petroleum refineries by personnel trained in the method during a two-day workshop at Virginia Polytechnic Institute and State University. The procedures used at the refineries were the same as those outlined above with the exception that some personnel used commercially available *D. pulex* (Carolina Biological Supply Co., Burlington, NC). Each refinery conducted a control test using the ARM and dechlorinated Blacksburg tapwater in addition to tests using dilutions of their own refinery effluents. Dechlorinated, local municipal water was used at each refinery for acclimation of *Daphnia* and for bioassays of refinery effluent.

Additional comparative bioassays were conducted using the ARM and five commonly used test fish (goldfish, bluegills, rainbow-trout fry, fathead minnows, and brook sticklebacks). These bioassays were conducted at 20 $\pm$ 1 C, and the fish were acclimated to laboratory conditions for at least ten days. Five effluents from various, non-petroleum industries were tested using *Daphnia*, and the results were compared with goldfish bioassays of the same effluents.

D. pulex bioassays were conducted with ARM as a toxicant to determine the major factors affecting bioassay results. To provide a baseline against which other bioassays could be compared, ten bioassays

were conducted using the following conditions: 19.7 ± 0.8 C, 16L:8D photoperiod, 70 to 100 foot-candle light intensity, and in Blacksburg dechlorinated tapwater.

The effect of culture and organism age was tested using animals from cultures 3, 7, 13, and 27 weeks old; in another series of bioassays, animals from 5 to 6 and 6 to 7 days old were used. To test the importance of temperature control, *D. pulex* cultures were held for three days and tested at 15, 17, 20 and 25 C (all ± 0.5 C) in an incubator.

The effect of photoperiod was studied on two separate occasions. First, a 30 litre culture of *D. pulex* was divided into two groups and held for three days in either continuous light or dark before bioassays were conducted under these same conditions. In preparation for another series of bioassays, a 30 litre culture of *D. pulex* was split into two tanks and held for three days under conditions of 8L:16D or 12L:12D photoperiods.

The effect of three different light intensities was studied. A culture of *D. pulex* was divided into two groups that were acclimated for three days under 15 and 200 foot-candle light intensities. Different light intensities were produced by adding cheesecloth light-shields or small banks of cool-white fluorescent lights to existing laboratory lighting. Light intensity, as measured with a General Electric light meter, was uniform across the bioassay areas.

Prior to testing the effect of water hardness of bioassay results, *D. pulex* were cultured in soft, hard, and very hard reconstituted water (Marking and Dawson 1973) for two weeks before conducting bioassays. Animals from these cultures were used for bioassays of the ARM diluted

with the appropriate hardness of reconstituted water. The salinity tolerance of *D. pulex* was tested using dilutions of Dayno synthetic sea water. To test the effect of pH on ammonia toxicity, soft, reconstituted water (Marking and Dawson 1973) was adjusted to pH 6.0, 7.0, 8.0, and 9.0 with 1.0 N NaOH, 1.0 M KH_2PO_4 and 0.5 M H_3BO_3 (EPA 1975).

Toxicity of Selected Materials to Daphnia pulex

To measure changes in toxicity of the ARM with time, three groups of 8 test beakers were set up with dilutions of the ARM. Within 2 h *Daphnia* were added to one group of test beakers. Twenty-four and 48 hours later *Daphnia* were placed in the second and third groups of test beakers. Observations on survival were made at 8, 24, and 48 h for each test group.

Individual components of the ARM were tested to determine their relative toxicity. In a second series of bioassays, ARM solutions which had single components missing were tested. In other experiments, one-half of the 8-h LC50 for zinc chloride, lead chloride, and potassium cyanide was included in the basic ARM formulation. Additional bioassays were conducted to determine the sensitivity of *D. pulex* to selected cyanide compounds, and selected heavy metal chlorides.

Data Analysis

A computer procedure, based on Finney's method for probit analysis (Finney 1971), was used to estimate the concentration toxic to 50 per cent of the exposed individuals (LC50) for 24, 48 and 96 h and to find 95 per cent confidence limits for the LC50. LC50s were considered

different if their 95% confidence limits did not overlap (J. B. Sprague, pers. comm., cited by Davis and Hoos 1975). Duplicate and triplicate bioassay sets were often grouped and analyzed as if they were single bioassays to clarify data presentation and to simplify interpretation.

C. Results and Discussion

Evaluation of the Daphnia *Bioassay*

The results of 22 bioassays with the complete ARM, 12 tests with the ARM but without oil, and one test with oil alone are presented in Table 9. The LC50 results are expressed as multiples or fractions of the ARM mixture. Because these data were transformed to $\log_{10}$ values prior to statistical analyses, the mean value was not centered between the standard deviation values. Some tests did not provide LC50 values for all observation times due to the narrow range of concentrations used for the tests.

Results indicate that *D. pulex* can be used to monitor effluents whether or not oil is present. Oil definitely decreases the LC50 values, and much of its effect probably is due to surface entrapment (Dowden 1965, Grodner 1959). Oil is also known to affect the molting process of crustacea (Katz 1973). In addition, these results show that the ARM without oil is toxic after 48 h; the LC50 value was less than X1 ARM. Variation in results over the entire test series was quite low (Table 10). The least variability was in the tests of the ARM without oil. A reason for this difference may be the problems of adding small but exact amounts of oil (as little as 0.03 microlitres) to the

Table 9. Mean ($\bar{X}$) and standard deviation (SD) of LC50 values obtained for *Daphnia pulex* bioassays of the ARM, of the ARM without oil, and with the oil component conducted at VPI & SU. The LC50 values are expressed as multiples or fractions of the ARM or its components.

	ARM				ARM Without Oil				Oil Only	
Hour	n*	$\bar{X}$	-SD	+SD	n*	$\bar{X}$	-SD	+SD	n*	LC50
8	13	0.18	0.10	0.33	5	3.84	2.93	5.02	1	--
24	22	0.11	0.06	0.42	8	1.76	0.99	3.13	1	0.18
48	21	0.07	0.04	0.11	12	0.96	0.76	1.20	1	0.10
96	22	0.03	0.02	0.04	10	0.42	0.27	0.66	1	0.06

*--the number of replicate bioassays

Table 10. Mean ($\bar{X}$) and standard deviation (SD) of the LC50 values obtained for bioassays of the ARM and of the ARM without oil. These results were obtained at petroleum refinery laboratories using *Daphnia pulex* in Blacksburg tapwater. The LC50 values are expressed as multiples or fractions of the ARM.

	ARM				ARM Without Oil			
Hour	n*	$\bar{X}$	-SD	+SD	n*	$\bar{X}$	-SD	+SD
8	5	0.48	0.15	1.49	1	1.14	--	--
24	7	0.27	0.13	0.60	2	1.08	0.64	1.79
48	8	0.13	0.08	0.22	2	0.76	0.55	1.06
96	7	0.05	0.02	0.10	2	0.71	0.55	0.93

*--the number of replicate bioassays

test containers. The mean 48-h LC50 values for ARM obtained at refineries (Table 10) were quite similar to the mean 48-h LC50 values for ARM obtained at my lab (Table 9). For each time period the standard deviation ranges overlapped. As expected, the reproducibility among the various laboratories was less than the reproducibility at each laboratory. Possible reasons for variability among the refinery laboratories were: 1) some workshop participants did not personally conduct the bioassays, 2) most participants had little experience in doing bioassays, and 3) refinery work was performed using *Daphnia* stressed by cross-country shipping.

The effluents of the refineries involved in the interlaboratory testing program exhibited a wide range of chemical features (Table 3). Chemical data were obtained for most effluent samples used for bioassays, but it was not possible to ascribe variability in toxicity to differences in chemical parameters. Duplicate *Daphnia* bioassays were performed on refinery effluents (Table 11). The LC50 data were obtained from eye-fitted lines (APHA 1971). Variation among the refinery bioassays was expected because of differences among refinery processes. There was little variation between the LC50 values for replicate tests, indicating that reproducibility was possible with this procedure.

Good reproducibility of the test results (Tables 9, 10, and 11) demonstrated that the method as proposed will give reproducible results when used by nonbiologists to screen actual refinery effluents.

Table 11. *Daphnia* bioassays using refinery effluents. LC50 values are expressed as percent effluent.

	Refinery								
	A			B			C		
Test	24 h	48 h	96 h	24 h	48 h	96 h	24	48 h	96 h
1a	C	C	48*	NT	NT	86	68	<18+	C
b	C	C	16*	NT	93	63			
2a	NT	NT	63	NT	NT	NT	<56+	3.8*	C
b	NT	NT	74	NT	NT	NT			
3a	NT	NT	NT	NT	NT	NT	<5.6+	C	C
b	NT	NT	NT	NT	NT	NT			
4a	NT	NT	90	NT	NT	NT	90	5.0*	C
b	NT	NT	64	NT	NT	NT			
5a	32	13	3.7	NT	NT	NT			
b	C	17	2.6	NT	NT	NT			
6a	81	96	56						
b	NT	NT	66						
7a	12	1.2	C						
b	15	2.1	C						
8a	NT	68	53						
b	NT	78	52						

*--extrapolated LC50
+--highest or lowest concentration tested
C--could not be calculated
NT--not toxic

Table 11. *(continued)*

	Refinery								
	D			E			F		
Test	24 h	48 h	96 h	24 h	48 h	96 h	24 h	48 h	96 h
1a	<56+	5.6	<5.6+	NT	9.4+	C	NT	88	46
b	<56+	6.0	<5.6+				NT	NT	42
2a	54	5.6	C	54	2.8+	C	NT	NT	30
b	55	9.8	<5.6+				NT	84	39
3a	10.0	<3.2+	C	20	2.3	C	NT	89	70
b	5.2	<3.2+	C				NT	100	51
4a	5.9	1.2	4.0+	54	1.4	C	100	56	14
b	5.1	1.3	<1.0+				100	43	12
5a	3.8	<1.0+	<1.0+	>1.0+	>1.0+	0.61	50	66	12*
b	3.4	1.35	<1.0				NT	62	C
6				>1.0+	2.1*	0.17			

*--extrapolated LC50
+--highest or lowest concentration tested
C--could not be calculated
NT--not toxic

Comparison of Daphnia *and Fish*

Comparison of *Daphnia* and fish LC50 data (Table 8) showed that the *D. pulex* were sensitive at 1/70 the LC50 value for bluegills and goldfish and at 1/40 the LC50 value for rainbow trout after 24 h. When tested with the same refinery effluent, the 48-h LC50 for *D. pulex* was similar to the 96-h LC50 value for the stickleback (Eiffert, pers. comm.). These data indicate that *D. pulex* can be used to obtain data in less than the 96 h required for the more tolerant fish. Compared to fish, the *D. pulex* test is simple and requires less space, effluent, diluent, acclimation time, and equipment.

Comparative bioassays using *D. pulex* and five commonly used test fish showed that trout fry were the most sensitive of the fish studied (96-h LC50 was X1.7 the ARM) and fathead minnows were the least sensitive (96-h LC50 greater than X32 the ARM) (Table 12). Goldfish were intermediate in sensitivity. It is noteworthy that trout fry were 40 times less sensitive to the ARM than *Daphnia pulex*. The trout bioassays were conducted with ARM that had been blended to emulsify the oil. A separate test showed that emulsification increased the toxicity of the ARM to trout fry; there was 100% mortality to fish in emulsified X3.2 ARM at 24 h but only 10% mortality to fish in non-emulsified X3.2 ARM at 96 h. Emulsification also increased the toxicity of the ARM to *Daphnia pulex*; tests using non-emulsified ARM gave 48-h LC50 values of 0.07 (Table 8) whereas tests using emulsified ARM gave 48-h LC50 values of 0.033 (Table 13). For the fish listed, it was seldom possible to obtain confidence limits from the probit analysis owing to lack of

Table 12. Estimated LC50 values for various fishes tested with the arbitrary reference mixture (Table 2). LC50 values are expressed as multiples or fraction of the reference mixture. Figures in parentheses are 95% confidence limits.

Fish Species	Length, cm mean ± S.D.	Weight, g mean ± S.D.	24-h LC50	48-h LC50	96-h LC50
Goldfish	6.1 ± 0.7	2.5 ± 0.8	27 (21-38)	21*	20*
Bluegill	5.6 ± 0.3	2.3 ± 0.5	14*	14*	14*
Rainbow trout fry+	3.3 ± 0.2	0.33 ± 0.06	2.8*	2.0 (1.6-2.6)	1.7*
Fathead minnow	6.7 ± 0.5	2.5 ± 0.7	>32	>32	>32
Brook stickleback	4.1 ± 0.6	0.49 ± 0.23	9.3 (7.2-12)	5.5 (4.3-7.0)	4.6 (3.4-6.2)

*--graphical interpolation.
+--fuel oil was blended into the ARM prior to starting the test

Table 13. Estimated LC50 values for *Daphnia pulex*, tested with ARM under "bench-top" conditions (19.7 ± 0.8 C, 16L:8D photoperiod, 70-100 ft-c under cool-white fluorescent light) in Blacksburg dechlorinated tapwater. LC50 data are in fractions or multiples of the ARM formulation. Figures in parentheses are 95% confidence limits.

Bioassay	24-h LC50	48-h LC50
291	0.30 (0.22-0.43)	0.036 (0.026-0.048)
292	0.34 (0.26-0.44)	0.023 (0.01-0.033)
291,292 (composite)	0.31 (0.26-0.39)	0.029 (0.022-0.037)
438	a	0.17 (0.12-0.23)
439	1.4+	0.056*
440	1.2+	0.10 (0.060-0.15)
441	a	0.05+
442	a	0.25 (0.13-1.7)
438,439,440,441,442 (composite)	0.15+	0.11 (0.085-0.14)
455	0.92 (0.55-3.27)	0.089 (0.012-0.25)
456	0.52 (0.33-1.07)	0.094 (0.064-0.14)
457	0.36 (0.23-0.68)	0.018 (0.004-0.034)
455,456,457 (composite)	0.57 (0.42-0.85)	0.052 (0.033-0.074)
Composite of all above	0.33 (0.29-0.37)	0.028 (0.021-0.036)

*--graphical interpolation
+--computer extrapolation
a--less than 50% mortality in highest concentration used

partial mortality in more than one (rarely more than two) test concentrations. This happened even though the same log-interval series of concentrations was used for all fish- and *Daphnia*-test concentrations.

When the test concentrations 32, 10, 5.6, 3.2, 1.8, 1.0, and so on were used in ARM bioassays, fish gave partial survival in one, or sometimes two, test concentrations, but *Daphnia* generally gave partial survival in four or five test concentrations. As a result, the slope of the toxicity curve (the regression coefficient of probit on dose) was 6.8 to 18 for fish and 0.67 to 3.8 for *Daphnia* (Fig. 1). Because the reciprocal of the slope is an estimate of the standard deviation of the normal frequency distribution of log tolerances (Finney 1971), the smaller the slope of the toxicity curve the greater the variance of the test population.

Results of bioassays on five industrial effluents (Hendricks, pers. comm.) and *Daphnia* are presented in Table 14. None of the effluents were related to the petroleum industry. Effluents A_{1-5} were from an organic chemicals industry. Possible toxicants were mercaptobenzothiozole and its hydroxy- and sulfuric-acid derivatives. Effluents B_1 and B_2 were from an agricultural chemical industry and possible toxicants were chemicals associated with the production of fertilizers and pesticides. Effluent C_1 was from a pharmaceutical industry. The diluent for all bioassays was dechlorinated Blacksburg tapwater.

Effluents A_1 and A_2 were less toxic to *Daphnia pulex* than to goldfish (Table 14). Effluents A_3, A_4 and A_5 were more toxic to *Daphnia pulex* than to goldfish, and replicate tests with *D. pulex* were in good agreement (Table 14). Owing to the source of effluent B, the poison

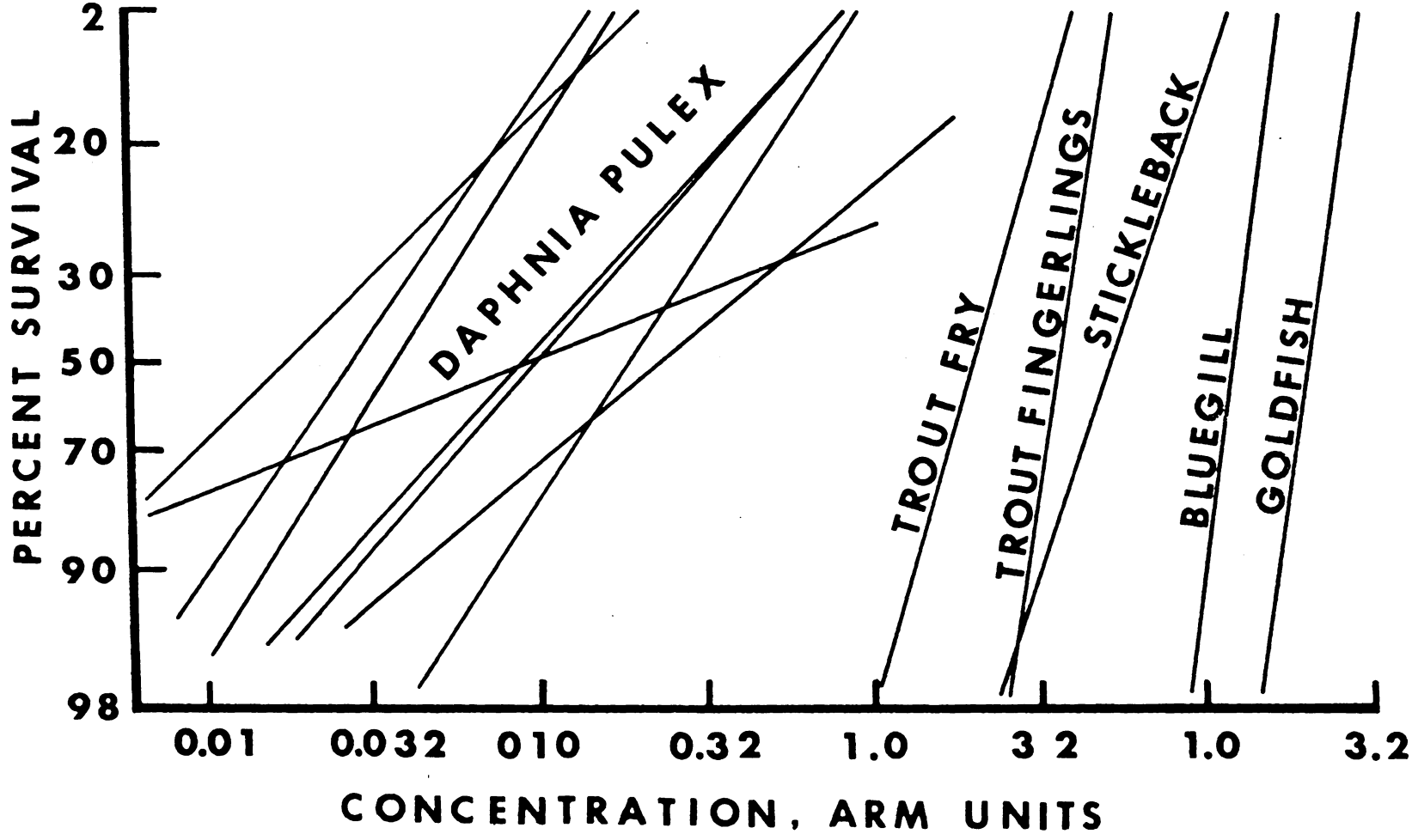

Figure 1. Dose-response curves at 48 hours for *Daphnia pulex* and several fishes. Slopes of the lines ranged from 0.67 to 3.8 for *D. pulex* and from 6.8 to 18 for the fishes, stickleback and bluegill, respectively.

Table 14. Estimated LC50 values of *Daphnia pulex* and fish bioassays of industrial effluents. LC50 values are percent effluent.

Effluent	Test Organism	24-h LC50	48-h LC50	96-h LC50
A_1	*D. pulex*	a	10*	--
A_1	Goldfish	9.5*	7.5*	7.0*
A_2	*D. pulex*	a	67*	--
A_2	Goldfish	24*	24*	24*
A_3	*D. pulex*	26 (12-400)	18(9.6-86)	2.7(0.19-6.5)
A_3	Goldfish	36*	31*	24*
A_4	*D. pulex*	25*	25*	---
A_4	Goldfish	55*	43*	40*
A_5	*D. pulex*	4.7(3.4-6.4)	d	d
A_5	Goldfish	80*	80*	70(52-132)
B_1	*D. pulex*	1.2(0.84-1.7)	0.60(0.43-0.84)	0.44(0.32-0.64)
B_1	*D. pulex*	1.5(0.81-6.3)	0.66(0.45-1.0)	0.45(0.33-0.64)
B_1	Goldfish	NT	NT	NT
B_2	*D. pulex*	3.1(1.5-3.4)	0.76(0.52-1.2)	0.47(0.33-0.65)
B_2	*D. pulex*	1.9(1.2-4.5)	0.70(0.44-1.2)	0.47(0.31-0.73)
B_2	Goldfish	NT	NT	NT
C_1	*D. pulex*	NT	NT	--
C_1	Goldfish	NT	--	--

Subscript on effluent designates indicated grab sample.
d indicates high mortality in the selected test concentration.
a indicates low mortality in the selected test concentration.
* indicates graphical interpolation.
NT indicates not toxic in 100% effluent.

may have been an insecticide, which was not toxic to fish but toxic to arthropods such as *Daphnia*. Effluent C was not toxic to either *D. pulex* or goldfish (Table 14).

These preliminary data suggested that it may not be possible to correlate toxicity of different effluents to different species. This is contrary to the findings of Price *et al.* (1974), who compared brine shrimp 48-h TLm values for selected petrochemicals to bioassay values reported in the literature for other aquatic organisms. They suggested that order-of-magnitude correlations were feasible and that fathead minnow-brine shrimp correlations showed even closer agreement.

Effects of Selected Environmental Factors

Baseline Studies: Ten bioassays performed during an eight-month period (Table 13) gave the following 24- and 48-h LC50 values and 95% confidence limits: 0.42 (0.36-0.51) and 0.033 (0.023-0.042). Best reproducibility was found after 48 h (Table 13). For 48-h data, results were not significantly different from those obtained in bioassays performed a year earlier (Tables 8 and 9).

It was not usually possible to find 8- and 96-h LC50 values because of the range of concentrations used, and in some tests 24-h LC50 values could not be calculated because the concentrations used did not bracket the level for 50% mortality.

There was usually no mortality in controls at 24 and 48 h exposure, but sometimes there was 10 to 20% mortality at 96 h. In test 456 (Table 13) two of 10 animals died in 48 h in the control, and in test 457 seven of 10 animals died in 48 h in the control. However, in

test 457 only three of 11 died at 48 h in the lowest toxicant concentration.

Culture and Organism Age: Simultaneous bioassays were conducted with animals from cultures 3 to 27 weeks old using the ARM as the toxicant. All cultures had been thinned, reproductive rates were moderate, and (except for cultures A and B) ephippia were virtually nonexistent. Cultures A and B were started from clones which had been held for 1.5 yrs. All cultures were similar in water quality (Table 15). The 48-h LC50 values for bioassays from cultures A and B were significantly different compared to results obtained using culture N (Table 15). There was no apparent explanation for differences between cultures A and B, and culture N based on hardness, pH, or culture age. Nor did the time the clones had been cultured, or presence of other invertebrates in the culture tank appear to have an effect. Cultures A and B (recent shipments from the supplier) were slightly less sensitive compared to the animals cultured in the laboratory for more than a year. When comparing individual tests, there was a tendency for confidence limits to be closer when animals of similar age were used. However, the data were not significantly different from those obtained for a mixed population when the data were pooled (Table 16). One would expect test organisms of equivalent age to be of similar vitality. The advantages of using animals of known age is obvious, but for practical use one must weigh the advantages against the difficulties of obtaining these animals.

Temperature: Animals were acclimated to photoperiod (16L:8D) and test temperatures for at least four days prior to conducting

Table 15. Estimated LC50 values for *Daphnia pulex*, tested from cultures of different ages. LC50 data are in fractions or multiples of the ARM formulation. Figures in parentheses are 95% confidence limits.

Culture Age	Number of Bioassays	Culture	Hardness/ Alkalinity mg/l	pH	24-h LC50	48-h LC50
3 weeks	1	A	53/38	7.33	a	0.17 (0.12-0.23)
3 weeks	1	B	52/40	7.71	a	0.25 (0.13-1.74)
7 weeks	2	N	65/41	7.63	0.50 (0.31-1.16)	0.023 (0.006-0.041)
13 weeks	1	S	53/29	7.60	1.19+	0.10 (0.06-0.15)
27 weeks	2	L	46/15	7.33	0.16 (0.41-1.30)	0.08 (0.061-0.11)

+--computer extrapolation

a--less than 50% mortality in highest concentration

Table 16. Estimated LC50 values for *Daphnia pulex*, tested at various ages. LC50 data are in fractions or multiples of the ARM formulation. Figures in parentheses are 95% confidence limits.

Age	Number of Tests	24-h LC50	48-h LC50
5-6 days	3	0.11 (0.08-0.13)	0.030 (0.025-0.043)
6-7 days	3	0.15 (0.10-0.22)	0.032*
mixed	10	0.42 (0.36-0.51)	0.033 (0.023-0.042)

*--by graphical interpolation

bioassays at 15, 17, 20 and 25 C (Table 17 and Fig. 2) in an incubator. After 48 h, results indicated that toxicity significantly increases as temperature increases. From 15 to 25 C there is a 15 fold difference in effect. The results from 17 to 25 C appear to be linear. The temperature effect observed here may be related to increased solubility of the oil and/or increased metabolic rate of the test animal with increased temperature.

Photoperiod: Simultaneous bioassays using the ARM were performed under conditions of continuous dark and continuous light (Table 18; Group A). Another group (B) of simultaneous bioassays were performed under conditions of 8 or 12 h light. The animals *within* each group were taken from the same culture, but *among* the groups the culture containers were not the same. Group C was a composite of tests conducted at 16L:8D and the animals were from many cultures.

The results suggested that photoperiod may affect the outcome of *Daphnia* bioassays where photoperiod conditions are grossly different, such as continuous light or continuous dark. However, the differences due to 8, 12 and 16 h of light probably are not significant. The low 95% confidence limits for Group C are in part due to the large sample size (see Table 13 for data on individual tests at 16 h photoperiod).

Light Intensity: Results of bioassays conducted under different light intensities were not significantly different (Table 19). The intensities used here were within the relatively narrow range likely to be encountered on bench tops in refinery laboratories--the conditions were not selected to find intensities which might affect results.

Table 17. Estimated LC50 values for *Daphnia pulex*, tested at different temperatures. LC50 data are in fractions or multiples of the ARM formulation. Figures in parentheses are 95% confidence limits.

Temperature ± Range	Number of Bioassays	24-h LC50	48-h LC50
15 ± 0.5 C	6	0.45 (0.39-0.54)	0.19 (0.17-0.23)
17 ± 0.5 C	5	0.27 (0.23-0.33)	0.055 (0.044-0.067)
20 ± 0.5 C	2	0.33 (0.26-0.39)	0.029 (0.022-0.037)
25 ± 0.5 C	3	0.082 (0.062-0.11)	0.013 (0.0051-0.018)

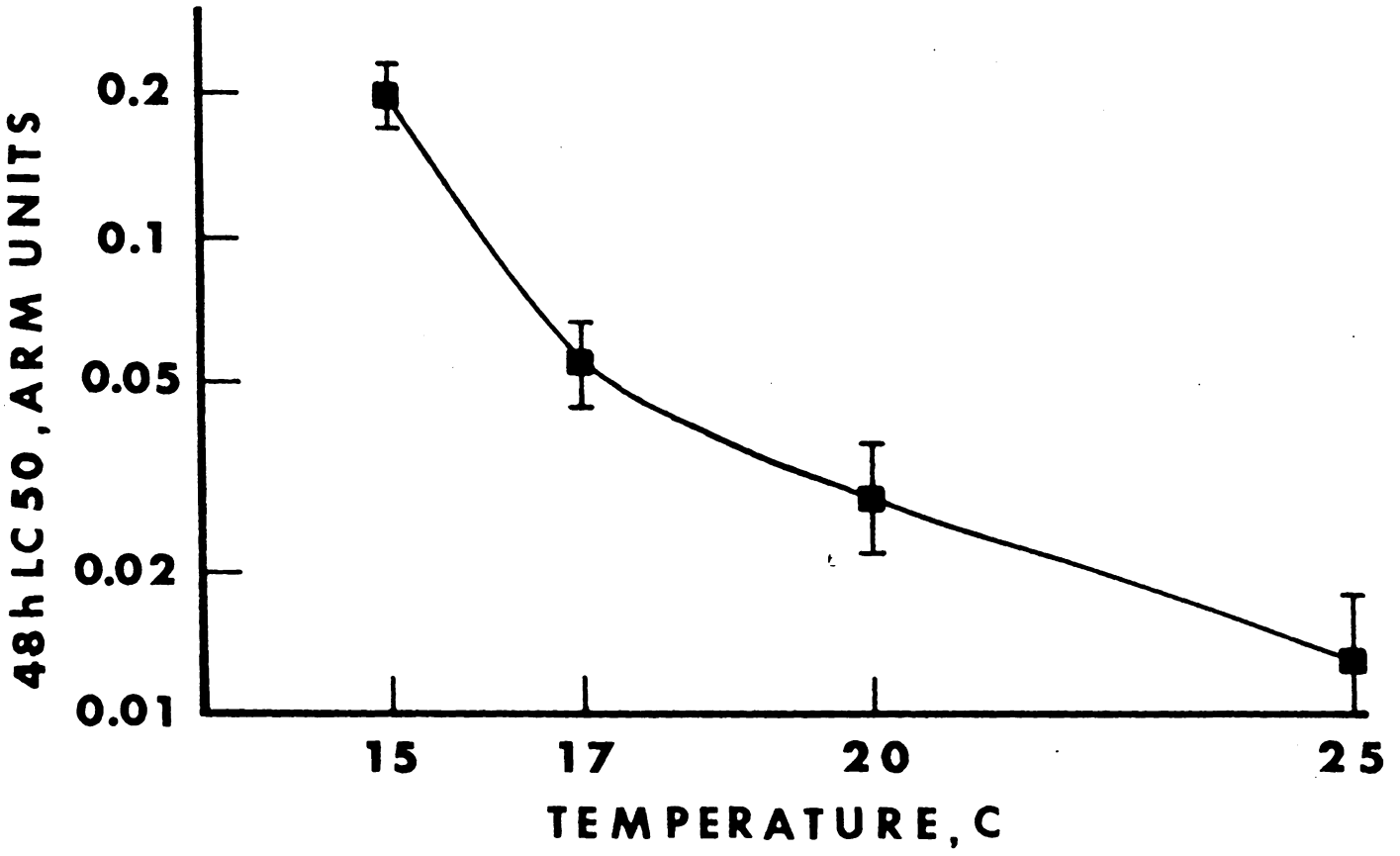

Figure 2. Toxicity of the ARM to *D. pulex* at four temperatures. Vertical lines show 95% confidence limits.

Table 18. Estimated LC50 values for *Daphnia pulex*, tested under different photoperiods. LC50 data are in fractions or multiples of the ARM formulation. Figures in parentheses are 95% confidence limits.

Photoperiod h Light:h Dark	Group	Number of Bioassays	24-h LC50	48-h LC50
0:24	A	5	0.061 (0.047-0.076)	0.011 (0.0078-0.014)
8:16	B	3	0.8*	0.064 (0.037-0.096)
12:12	B	3	0.55 (0.35-1.08)	0.076 (0.051-0.106)
16:8	C	10	0.42 (0.36-0.51)	0.033 (0.023-0.042)
24:0	A	3	0.013 (0.090-0.18)	no observations

*--graphical interpolation

Table 19. Estimated LC50 values for *Daphnia pulex*, tested under different light intensities. LC50 data are in fractions or multiples of the ARM formulation. Figures in parentheses are 95% confidence limits.

Light Intensity	Number of Bioassays	24-h LC50	48-h LC50
15 ft-c	3	a	0.15 (0.11-0.32)
70-100 ft-c	4	0.15	0.11 (0.085-0.14)
200 ft-c	3	a	0.11 (0.077-0.14)

a--more than 50% survival in highest concentration tested

Sherberger (pers. comm.) found that light intensity may affect bioassay results.

Water Hardness: Results suggested that water hardness affected bioassay of the ARM (Table 20). Toxicity decreased as the water hardness increased but the change was not statistically significant. This does not mean that culture conditions should not match the approximate hardness of water intended for testing, and clearly the hardness of the diluent and effluent should be roughly similar, say within 10%. Whether hardness affects the LC50 depends on the nature of the toxicant (Sprague 1970).

Diluted Sea Water: Bioassays showed that *Daphnia pulex* did not tolerate concentrations greater than 0.5 parts per thousand salinity (Table 21). *Daphnia pulex* cannot be considered suitable for bioassays in estuarine situations.

Ammonia Toxicity: Soft, reconstituted water (Marking and Dawson 1973) was used for bioassays to determine the effect of pH on toxicity of ammonium chloride to *Daphnia pulex*. Before adding ammonium chloride solutions to test containers, separate batches of diluent were adjusted to pH 6.0, 7.0, 8.0 and 9.0. Clearly the "pH-adjusted" dilution water did not maintain its intended pH (Table 22). However, a pH effect was noted. The 24-h LC50 values at pH 6, 7, 8 and 9 were 85, 66, 35 and 20 mg/l, respectively (Table 22). The same trend has been noted for fish and is probably due to the fact that the toxic species, hydrated NH_3, becomes more abundant with increasing pH.

Table 20. Estimated LC50 values for *Daphnia pulex*, tested with soft, moderate, or hard dilution water. LC50 data are in fractions or multiples of the ARM formulation. Figures in parentheses are 95% confidence limits.

Hardness (as ppm $CaCO_3$)	24-h LC50	48-h LC50
50 ppm	0.23 (0.17-0.30)	0.01*
115 ppm	0.33 (0.23-0.53)	0.05 (0.03-0.07)
167 ppm	a	0.04 (0.02-0.06)

*--graphical interpolation
a--more than 50% survival in highest concentration tested

Table 21. Estimated LC50 values for *Daphnia pulex*, tested in dilutions of synthetic sea water. LC50 values are in parts per thousand salinity. Figures in parentheses are 95% confidence limits.

24-h LC50	48-H LC50
0.03 (0.24-0.70)	Some high concentrations had low mortality
0.49 (0.34-0.80)	0.20 (0.093-0.59)

Table 22. Estimated LC50 values for *Daphnia pulex*, tested with ammonium chloride in soft dilution water at different pH values. Figures in parentheses are 95% confidence limits.

Initial pH	pH after 96 h	LC50, mg/l; N added as NH_4Cl		
		24 h	48 h	96 h
6.00	6.22	85 (57-130)	35 (7.6-58)	14 (1.8-24)
7.00	7.33	66 (53-80)	66 (53-80)	29 (0.66-43)
8.00	7.57	35 (23-46)	28 (16-39)	13 (1.7-25)
9.00	7.44	22 (15-29)	18*	

*--graphical interpolation

Toxicity of Selected Materials to Daphnia pulex

Change in Toxicity of the ARM with Time: Results showed that the ARM decreased in toxicity after sitting on the bench top for 24 h (Table 23). Bioassays of 24- or 48-h old ARM were not significantly different.

Specific ARM Components: Bioassays of individual components of the ARM were tested to determine their relative toxicity (Table 24). In order of increasing toxicity for 24-h exposure, the components were kaolinite (not toxic up to 1125 mg/l), phenol (90 mg/l), ammonia (65 mg/l N), sulfide (0.9 mg/l S^{-}), no. 2 fuel oil (0.3 mg/l), and chromium (0.1 mg/l Cr^{+6}). After 48 h, the phenol was more toxic than ammonia. However, in terms of their relative concentrations in the ARM, fuel oil was the most toxic individual component and chromium was the next most toxic component.

ARM with Single Components Missing: A second approach used to determine the relative importance of components of the ARM was to conduct bioassays of the ARM which had one component missing (Table 25). Twenty-four hour LC50 values were used for comparison because 48-h LC50 values were not obtained in all experiments. Results showed that removal of the oil component significantly reduced the toxicity of the ARM to *Daphnia pulex*. Results of bioassays without chromate, sulfide, and kaolinite in the ARM did not greatly affect toxicity, although toxicity may have been somewhat increased by their absence (Table 25). Without ammonium chloride or phenol, toxicity was greater compared to the toxicity of the entire ARM. This may have been due to removal of

Table 23. Estimated LC50 values for *Daphnia pulex*, tested with ARM solutions which were 2, 24, or 48 hours old at the beginning of the bioassay.

Age of ARM at start of test, h	24-h LC50	48-h LC50
2	0.11 (0.08-0.17)	0.03 (0.01-0.05)
24	0.46 (0.32-0.80)	0.15 (0.08-0.30)
48	0.69 (0.37-2.73)	0.11 (0.07-0.17)

Table 24. Estimated LC50 values for *Daphnia pulex*, tested with solutions of individual ARM components. LC50 values are expressed as mg/l. Figures in parentheses are 95% confidence limits.

ARM Component	Number of Bioassays	24-h LC50	48-h LC50
NH_3(N)	4	71 (62-80)	68 (60-77)
Cr^{+6}	1	0.1*	d
Fuel oil	2	0.36 (0.27-0.51)	d
Phenol	3	103 (68-190)	28 (19-43)
Sulfide	6	0.80 (0.71-0.91)	0.55 (0.43-0.63)
Kaolinite	1	>1125	>1125

*--graphical interpolation
d--more than 50% mortality in lowest concentration

Table 25. Estimated LC50 values for *Daphnia pulex* tested in ARM which had one missing component. LC50 values are expressed as multiples or fractions of the basic ARM minus the identified component. Figures in parentheses are 95% confidence limits.

Missing Component	Number of Bioassays	24-H LC50	48-h LC50
Ammonia	3	0.032 (0.018-0.046)	0.008+
Sulfide	3	0.14 (0.10-0.16)	0.018 (0.006-0.031)
Phenol	3	0.093 (0.075-0.113)	d
Chromium	3	0.17 (0.13-0.22)	d
Kaolinite	3	0.24 (0.18-0.31)	0.016 (0.004-0.029)
Fuel oil	3	0.73 (0.63-0.85)	0.52 (0.44-0.60)

+--computer extrapolation
d--more than 50% mortality in the lowest concentration

otherwise antagonistic interactions of ammonium chloride and phenol with other components of the ARM.

ARM with Additional Components: Bioassays were conducted using the basic ARM (Table 2) plus one-half of the 8-h LC50 of an appropriate compound (Table 26). The presence of zinc in the ARM made little difference in the expected 24-h LC50 value but reduced toxicity after 48 h. Lead added to the ARM reduced the toxicity for 24 h but did not produce different LC50 confidence limits. Cyanide added to the ARM made it more toxic at 24 h but did not appear to affect the 48-h LC50. Toxicity of the ARM may be dominated by two or three important toxicants (such as fuel oil and chromate) and addition of other compounds, even at fairly high levels, may have little effect on acute toxicity.

Cyanide Compounds: Cyanide bioassays were done under a hooded ventilation system using a 25 watt incandescent light bulb for illumination. Evaporation reduced the 300 ml test volumes to about 250 ml after 48 h. The pH measured at the beginning of the bioassays with ferrocyanide ranged from 7.65 to 8.11; after 24 h the pH ranged from 8.07 to 8.20. At the beginning of the cyanide test, the pH ranged from 8.25 to 9.25 (low to high test concentration) and, after 96 h the pH of cyanide solutions had dropped less than 0.3 pH units.

Solutions of potassium cyanide, potassium thiocyanate, and potassium ferrocyanide gave the following 48-h LC50 values: 0.083, 11, and 64 mg/l CN^-, respectively. Most toxicity occurred during the first 24 h of exposure (Table 27). The sensitivity of *Daphnia* to HCN appears to be in the same range as the sensitivity of many fishes, but

Table 26. Estimated LC50 values for *Daphnia pulex* tested with zinc chloride, lead chloride, or potassium cyanide added to the ARM. The quantity added to the basic ARM was equal to 1/2 of the predetermined 8-h LC50 for the added compound. LC50 values are expressed as a multiple or fraction of the ARM to which the identified compound was added. Figures in parentheses are 95% confidence limits.

Added Compound	Amount added to the ARM, mg/l Zn, Pb, or CN	Number of Tests	24-h LC50	48-h LC50
$ZnCl_2$	4.0	3	0.28 (0.22-0.35)	0.058 (0.046-0.07)
$PbCl_2$	50	3	1.4 (0.85-3.5)	0.038 (0.025-0.052)
KCN	2.8	3	0.10 (0.078-0.13)	0.031 (0.025-0.037)
none	0	10	0.42 (0.36-0.51)	0.033 (0.023-0.042)

Table 27. Estimated LC50 values for *Daphnia pulex* tested with potassium cyanide, potassium thiocyanate and potassium ferrocyanide. LC50 values are in mg/l of CN^-. Figures in parentheses are 95% confidence limits.

Compound	Number of Bioassays	24-h LC50	48-h LC50
KCN	3	0.091 (0.043-0.132)	0.083 (0.043-0.116)
KSCN	3	170 (60-270)	11 (7.9-15)
$K_2Fe(CN)_6$	3	91 (67-140)	64 (45-103)

it is difficult to make comparisons because cyanide toxicity varies widely with pH, temperature, and dissolved oxygen (NAS 1972).

Heavy Metal Chlorides: The order of decreasing toxicity of selected, heavy metal chlorides was as follows: mercury, cadmium, zinc, lead, and iron (Table 27). The 48-h LC50 for the heavy metals ranged three orders of magnitude. There was no obvious explanation for the variation obtained for different tests using lead and iron chlorides on different test dates and with different cultures. Condensed phosphate is known to act as a chelating agent for metal ions. It was possible that variable amounts of condensed phosphate was added to the tapwater during treatment on different days.

Strengths and Weaknesses of the Daphnia *Bioassay*

The principal strength of the *Daphnia* bioassay is that it provides a practical, first-step for on-site effluent evaluation: using existing facilities, people with little biological training can measure the acute toxicity of waste discharges (Tables 9, 10, and 11). The use of *Daphnia pulex* as a test organism has several advantages: 1) They are easily cultured in the laboratory with a minimum of space and equipment; 2) they are invertebrates found in many rivers and lakes of North America; and 3) they generally are more sensitive than fish to potential contaminants in refinery-waste waters.

The principal weakness of the method is that, compared to fish bioassays, about twice as much time is required for making observations on mortality and more care is required for handling the organism.

Table 28. Estimated LC50 values for *Daphnia pulex* for various metal chlorides. LC50 values are in mg/l of the metal. Figures in parentheses are 95% confidence limits.

Toxicant	Date	Number of Bioassays	24-h LC50	48-h LC50
$CdCl_2 \cdot 2\frac{1}{2}H_2O$	4/30	3	0.30 (0.24-0.36)	d
$CdCl_2 \cdot 2\frac{1}{2}H_2O$	5/12	3	0.14 (0.11-0.17)	0.071 (0.056-0.084)
$ZnCl_2$	4/30	3	0.74 (0.64-0.86)	0.38 (0.33-0.43)
$ZnCl_2$	7/19	3	0.36 (0.30-0.44)	0.26 (0.21-0.35)
$PbCl_2$	4/30	3	1.5 (1.2-2.0)	0.52 (0.40-0.65)
$PbCl_2$	5/14	3	9.9 (7.0-15)	3.9 (3.0-5.3)
$FeCl_3 \cdot 6H_2O$	5/14	3	12 (10-14)	9.0 (7.1-11)
$FeCl_3 \cdot 6H_2O$	6/18	2	10 (6.9-18)	2.8 (2.0-4.1)
$HgCl_2$	4/15	3	0.009 (0.0078-0.011)	0.004 (0.003-0.004)

d--more than 50% mortality in lowest concentration

Except for crude estimates of toxicity, temperature should be controlled to $\pm$ 0.5 C. Temperature control will require water bath(s) with 0.5 m^2 surface area for triplicate bioassays with a total of 24 test beakers.

A few of the refinery personnel reported poor success in culturing the animal and poor survival in controls, especially between 48 and 96 h. There were at least three possible explantions for these problems: 1) The air used to aerate cultures at the refinery laboratories may have contained volatile compounds; 2) the diluent and culture water may have been toxic; and 3) by 96 h, some of the animals may have died from starvation. Improper handling of the animals could injure them or trap air bubbles under the carapace, both of which will bias the LC50 values. Usually, younger animals are more sensitive than older animals. The general health and source of the culture could also bias the results. *Daphnia* could be trapped in the oil at the water surface and die even though the water fraction of the effluent may not be toxic.

IV. MOLTING AS A SOURCE OF VARIATION IN TOXIC RESPONSE OF DAPHNIA

A. Introduction

The idea that molting is a critical period for crustaceans is an old one (Banta 1939), and there is some evidence that molting may have a bearing on the results of toxicity tests with *Daphnia* spp. Sherr and Armitage (1971) studied the effects of hexavalent chromium on survival and oxygen composition of *D. pulex*. They found a lag in death rate near the middle of the exposure period between 24 and 48 h. They said, "after the plateau, which lasts anywhere from 36 to 72 hours. . .another less sensitive group dies, again causing an increased death rate." Anderson (1948) noted pronounced inflection points on plots of 50% immobilization time for *Daphnia* versus concentration of various metal chlorides. Swedmark *et al.* (1971) reported that for a shrimp, *Leander adspersus*, the 96-h LC50 value decreases from 50 to 25 ppm for one surface-active agent and from >100 ppm to <10 ppm for another surface-active agent when molting versus non-molting animals are considered. Swedmark *et al.* (1971) and Katz (1973) noted the retarding effect of certain chemicals on molting frequency. In summarizing the importance of molting of crustacean physiology, Passano (1960) said, "there is scarcely a time when all aspects of the crustacean's life processes. . .are not dominated by its saltatory growth pattern, its recurrent renewal of skeleton, and its material storage metabolism.

In this study, three observations also supported the idea that molting might have a pronounced effect on bioassay results: 1) *Daphnia*

gave a larger standard deviation in response compared to the fish tested (Fig. 1); 2) many of the first to die in *Daphnia* bioassays were animals in the process of molting, and when 8-h mortality was greater than expected, there seemed to be a greater number of animals molting early in the test; and 3) after 48 h the results of *Daphnia* bioassays were most consistent--by 48 h most animals may have had time to pass through any particularly sensitive period.

In Cladocera three events follow in fairly rapid succession: release of young, molting, and deposition of eggs into the brood pouch. The time between release of young and deposition of eggs into the brood pouch is somewhat variable even within a single clone; for the *D. pulex* used in this study it was usually one to four hours between release of young and deposition of eggs. Like other crustaceans, *Daphnia* increase in volume immediately after molting, when the integument is soft. Once the integument hardens, there is no increase in size until the next molt. The increase in size is rapid, and is completed in less than a minute (Edlén 1937, cited by Green 1956). For example, a female of *D. obtusa* was seen to increase in length from 1.3 to 1.6 mm in about 10 s (Green 1956)! The fact that *Daphnia* increase in volume so rapidly suggests that water enters from large areas of the body surface, as in the spiny lobster, *Panulirus* (Lockwood 1968), and not from the gut, as in the marine crabs *Maia*, *Cancer*, and *Carcinus* (Lockwood 1968). The membrane selectivity and permeability of the body surface may be critical factors governing the quality of water taken up immediately after molting. Although lipid is present in the epicuticle of crustaceans, the lipid layer does not form a water barrier as it does in insects

(Neville 1975). The degree of calcification and tanning of the procuticle varies considerably over the surface of an individual. Consequently, permeability varies from one part of the body to the next. The inner surface of the carapace and the thoracic appendages are believed to be the sites of respiration (Brooks 1959, Pennak 1953) and these areas are probably the most permeable.

B. Procedure

The hypothesis that variations in the tolerance of a population are the result of differences in susceptibility at different stages of the instar was tested. Adult *D. pulex* were categorized by instar stage, placed in chromate solution (K_2CrO_4 in water) for two h, transferred to clean water, and observed for mortality at 24 h. In a second experiment adult *Daphnia* were categorized by instar stage, placed in chromate solutions, and checked for survival at one, two, and three-h intervals thereafter. If a toxicant were taken up more readily during (or immediately after) molting, then animals exposed to toxicant for a short time would die even if they were transferred to non-toxic water. If a toxicant interferred with some mechanism critical at molting, then animals exposed continuously to low concentrations of toxicant would die just prior to, or during, molting.

The approximate stage of the instar of reproductive females was deduced from the stage of development of embryos in the brood pouch. This method for determining periods with the instar was described by Fox (1948) and Green (1956). Time periods associated with the molting period were described in terms of three macroscopic events: 1) the

release of young from the brood pouch; 2) the appearance of the shed exoskeleton; and 3) the laying of eggs into the brood pouch. Table 29 shows the approximate stages during the instar of reproductively active, female *D. pulex* based on the contents of the brood pouch and on the events associated with molting.

Daphnia pulex were obtained from Carolina Biological Supply Company, Burlington, North Carolina, and were cultured in soft, reconstituted water (Marking and Dawson 1973) under 16L:8D photoperiod and 15-60 ft-c at 24 C. Cultures were fed a mixed algal suspension originally obtained from a fish aquarium and cultured in three-litre, glass, pickle jars under continuous light (400 ft-c) using PAAP media (Joint Industry/ Government Task Force on Eutrophication 1969). About one-third of the volume of each jar was removed each day, centrifuged in a Foerst Centrifuge (Welch 1948) and resuspended in reconstituted water. The algal suspension had an absorbance of 0.35 at 625 μm in a two-cm diameter tube.

Reproductively active, parthenogenetic, female *D. pulex* were stranded in a small amount of water on a glass slide. The contents of the brood pouch were examined microscopically for egg or embryonic development. The animal was classified according to the criteria in Table 29, and the total length of the animal (top of head to base of shell spine) was measured with an ocular micrometer. Single animals were placed in test tubes (1.5 x 1.2 cm) in 12 ml of reconstituted water containing algae (two ml algal suspension over 100 ml water). Individual animals were transferred with a three-mm bore medicine dropper into test tubes containing 12 ml of toxicant solution.

Table 29. Approximate stage of the instar of reproductive, female *D. pulex* based on the contents of the brood pouch and on the events associated with molting.

Stage	Description	Duration, h
1b	Eggs opaque or translucent and homogeneous with the exception of an oil globule.	2
2	Eggs markedly granular with transparent margin; fat cells forming.	12
3	Egg membrane is shed; embryo elongates.	6
4	Embryos with heads but no eyes; antennae short.	5
5	Embryos with small pink eyes; antennae longer.	$2\frac{1}{2}$
6	Embryos with distinct red or rust-colored eyes; embryonic antennae about one-half the body length.	$1\frac{1}{2}$
7	Embryos with large black eyes separated by an indentation; beak not distinctly pointed.	5
8	Embryos with single black eye; beak distinct; exoskeleton of adult begins to separate from new exoskeleton (along the posterior dorsal ridge, an underlying row of spines is usually visible).	$9\frac{1}{2}$
R	Young are released from the brood pouch.	$\frac{1}{2}$-2
M	The adult "molts."	seconds
1a	Eggs are laid in the brood pouch; recently laid eggs are small, dark, and markedly subspherical.	$\frac{1}{2}$-1

Observations on release of young, molting, and egg laying were made two or three times during the two-h exposure time. To minimize handling stress, the animals were observed in the test tube with the unaided eye except for the first microscopic observation. After two hours, test animals were returned to test tubes containing 12 ml reconstituted water with algae. No more than two drops of toxicant were transferred with the animal. Unfortunately, it was not feasible to observe test animals at frequent intervals after exposure, and the only consistent observations were made 24 h after exposure.

In a second experiment, *D. pulex* were categorized according to contents of brood pouch, and exposed continuously to potassium chromate solutions of 0.56 and 0.10 mg/1 Cr^{+6}. In this experiment, algae was not used because of possible interferences with the toxicant. Observations were made 1, 2, 3, 5, 7, 10, 13, 15, 18 hours after the beginning of exposure.

C. Results and Discussion

Preliminary tests were conducted in dechlorinated, Blacksburg tap-water. Except where noted, all test animals were producing 5 to 20 young per clutch. Range-finding tests with small numbers of animals showed 1) that all animals (regardless of stage) died within 24 h after two h exposure to concentrations of Cr^{+6} equal to or greater than 2.5 mg/1, and 2) that no animals (regardless of stage) died within 24 h after two-h exposure to 0.05 mg/1 Cr^{+6}.

Two-h exposures to 0.56 mg/1 Cr^{+6} caused high mortality in molting animals but not in intermolt animals (Fig. 3). Control animals

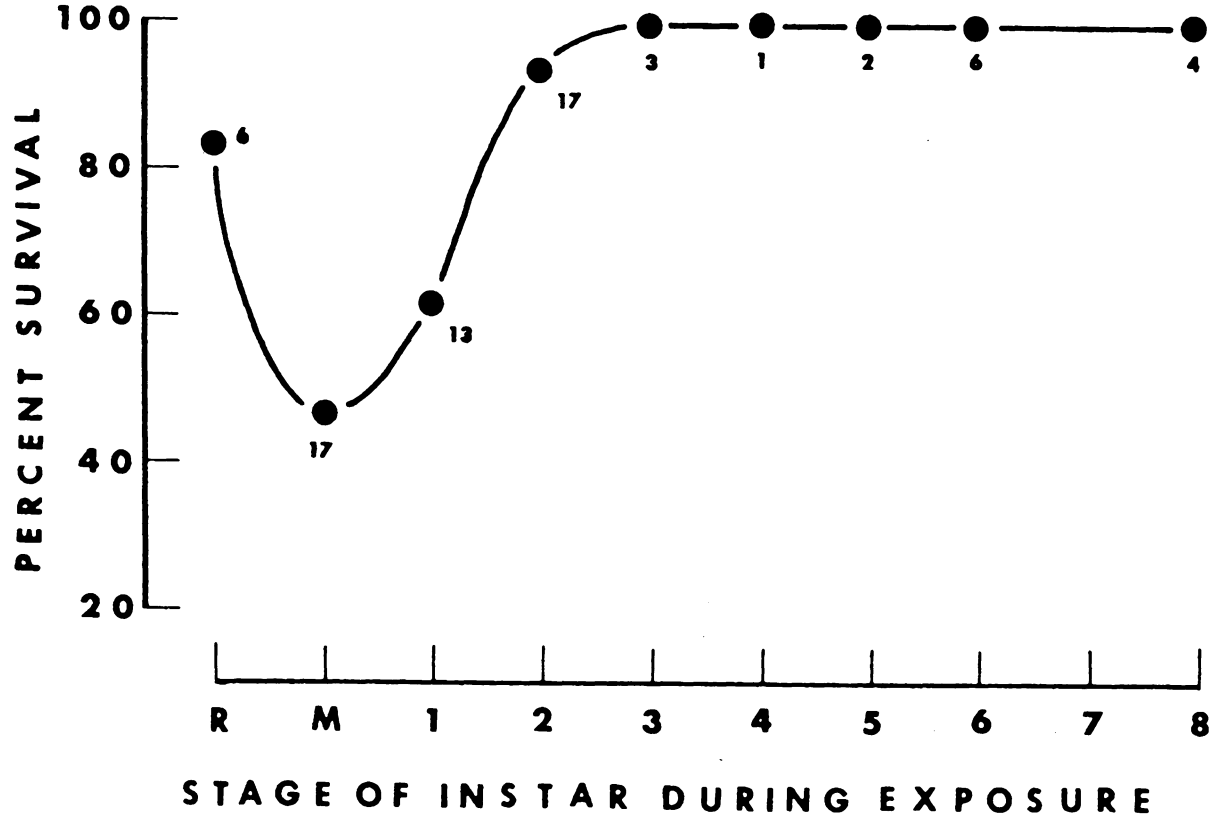

Figure 3. Survival versus instar stage after two-h exposure to 0.56 mg/l Cr^{+6} in Blacksburg tapwater. Approximate stage of instar was judged on the basis of the embryonic development of the brood at the beginning of toxicant exposure (stages 1-8) and on the release of young (stage R) or on the occurrence of the molt (stage M) during toxicant exposure. Small numbers show the sample size at each stage.

showed no mortality. Tests for equality of two proportions (Sokal and Rohlf 1969) showed that the animals exposed to 0.56 mg/l Cr^{+6} during the molt period had a significantly higher proportion of mortality than the animals exposed to toxicant during the intermolt period. These results were repeated using a larger sample size (n = 100) and soft, reconstituted water (Fig. 4). In this experiment, results were essentially the same but mortality was slightly higher in the intermolt group and in the first stage immediately following the molt. The only known experimental difference in the two tests (Figs. 3 and 4) was the water. The reconstituted experimental water contained only 7 mg/l Ca^{+2} whereas the Blacksburg tapwater contained about 12 mg/l Ca^{+2}. This may have made a difference in the rate of calcification of recently molted animals, so that in softer water the animals were susceptible for a longer time after molting. Few animals died during the two-h exposure period, but most of the animals that died did so 10 to 18 h after exposure. Unfortunately, observations were not made frequently enough to closely analyze survival time of different stages. These results show that molting is a time of increased susceptibility, and that death, when it occurs, comes several hours after exposure to chromate.

In additional experiments, *D. pulex* were exposed for two h to lower concentrations (0.1 to 0.05 mg/l) of chromate. However, the sample size was small (about 10 animals per concentration), the embryonic development was impaired (perhaps due to inadequate food), and few molting animals were tested. During these experiments many animals were observed microscopically after death, and there seemed to be a tendency for the animals to die with the molt still attached.

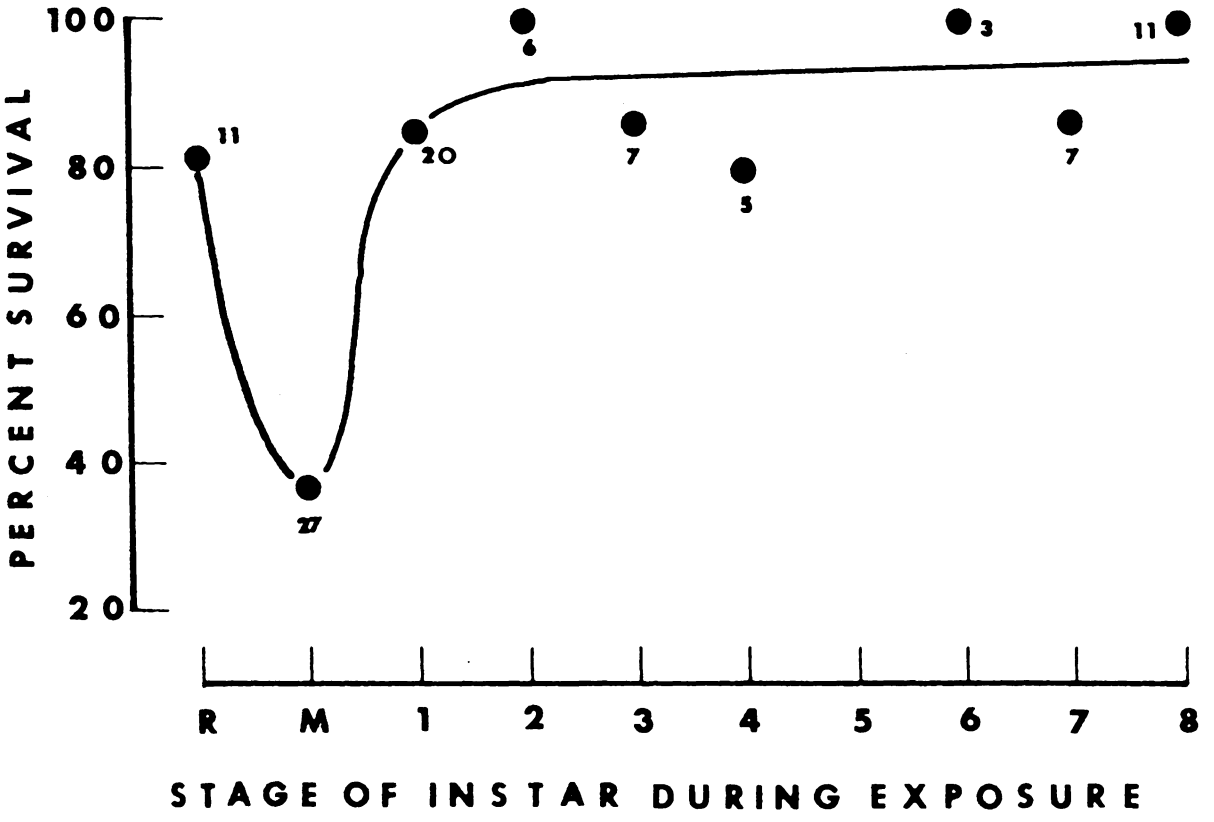

Figure 4. Survival versus instar stage after two-h exposure to 0.56 mg/l Cr^{+6} in reconstituted water. Approximate stage of instar was judged on the basis of the embryonic development of the brood at the beginning of toxicant exposure (stages 1-8) and on the release of young (stage R) or on the occurrence of the molt (stage M) during toxicant exposure. Small numbers show the sample size at each stage.

Thus, molting may be a "metabolic hurdle," the crossing of which is impeded by the presence of toxicants, but this was not shown conclusively. Alternatively, death occurring when the animal is, for example, within 10 h of molting may involve premature separation of the exoskeleton and give the impression that death occurred at the time of molting.

In the second experiment, chromate solutions of 0.56 and 0.10 mg/l Cr^{+6} were used. The pH of these solutions was 7.23 and 7.20 respectively. Compared to other stages, a larger number of stage-eight and stage-one animals died early in the test in 0.56 mg/l Cr^{+6} (Fig. 5). Figure 6 illustrates the survival curves for stages eight and one compared to other stages exposed to 0.56 mg/l Cr^{+6}. Duncan's multiple range test (Steel and Torrie 1960) was used to test for significant differences in mean survival time (Table 30). Individuals at stages one or eight at the beginning of exposure had significantly shorter mean survival times (4.9 and 5.6 h) compared to most of the other stages (6.5 to 8.9 h). Stages closest in time to molting (stages one and eight) were significantly more susceptible than the other stages.

Relatively susceptible stages within the instar of *Daphnia* during molting and stage one may be due to calcium deficiencies at this time. After molting, *Daphnia* are calcium deficient (Marshall *et al.* 1964, Porcella *et al.* 1969), but this deficiency is rapidly satisfied and is most acute during stage one. Marshall *et al.* (1964) showed that calcification of the exoskeleton is 50% complete within the first 8% of the instar. The effective respiratory and ionic exchange surfaces of the cray fish *Astacus* (the branchial filaments) have a calcified layer

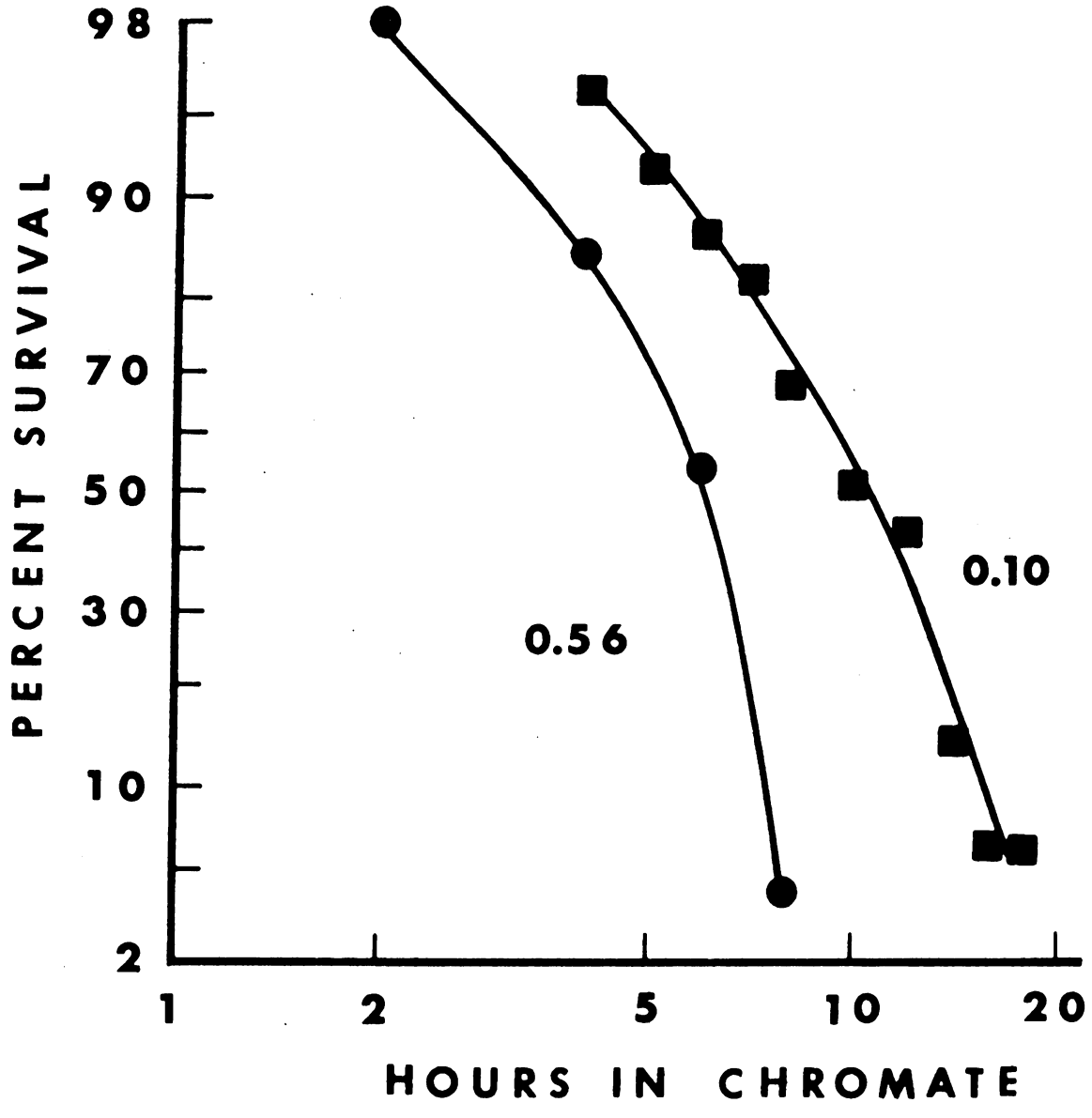

Figure 5. Survival-time plot of *D. pulex* exposed to 0.56 and 0.10 mg/l Cr^{+6} (potassium chromate) in reconstituted water.

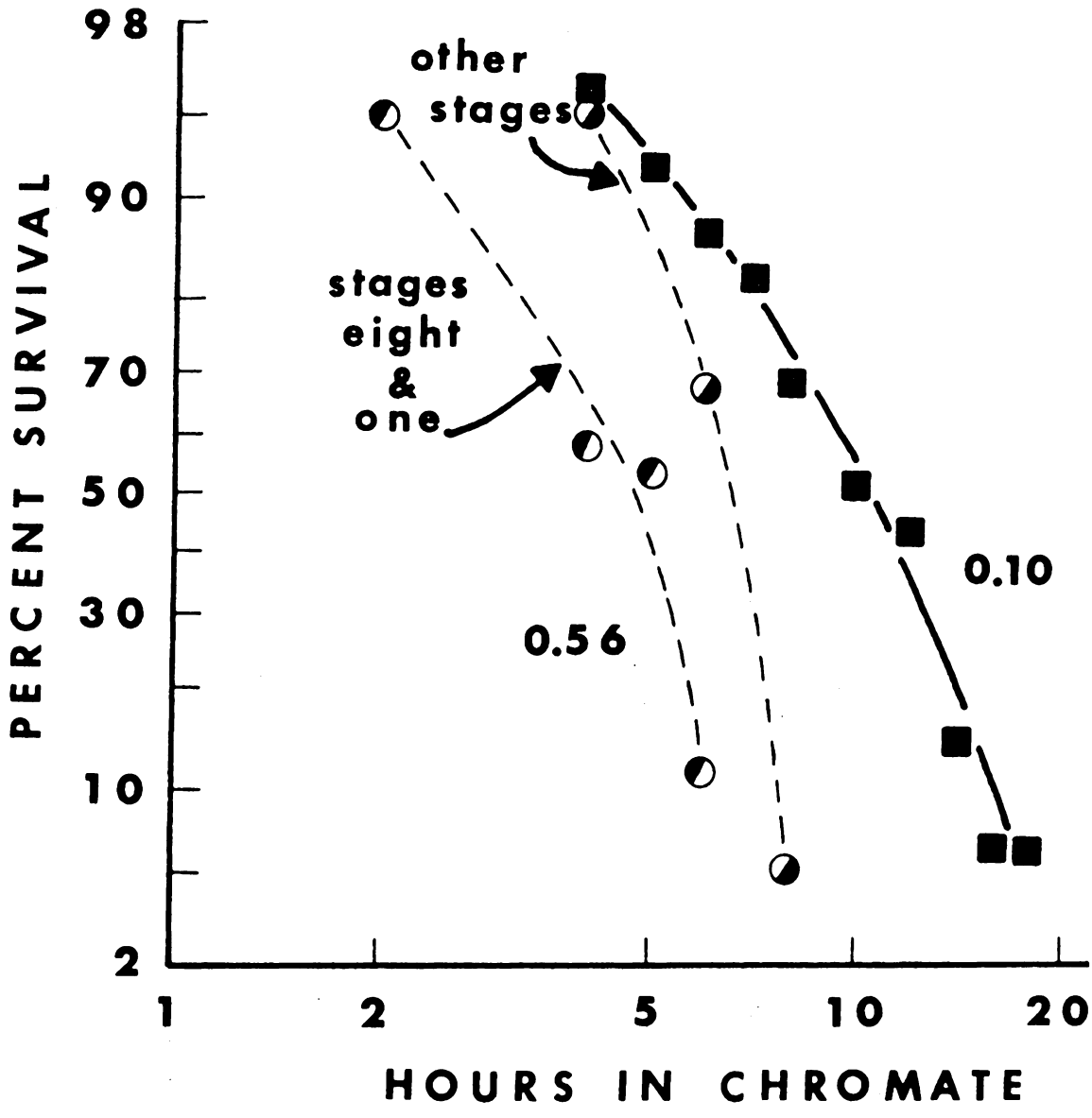

Figure 6. Survival-time plot of *D. pulex* exposed to 0.10 mg/l Cr^{+6} (potassium chromate) in reconstituted water. The dashed line shows the different survival curves of stages one and eight compared to other stages exposed to 0.56 mg/l Cr^{+6}.

Table 30. Results of Duncan's multiple range test for significant differences (p = 0.05) among mean survival times of *D. pulex* exposed to 0.56 mg/l Cr^{+6} (potassium chromate) in reconstituted water.

Mean Survival Time, h	Stage
8.00000	5
7.88235	4
7.73750	3
7.30357	7
6.73214	2
6.50000	6
5.55714	8
4.92083	1

2 μ thick (Fisher 1972), and calcification may also be important to the ion exchange surfaces of *Daphnia*.

Daphnia contain a small amount of calcium after molting (Porcella *et al.* 1969), but whether the residual calcium is incorporated in the new exoskeleton and whether it is significant in controlling the permeability of the organism has not been shown. Porcella *et al.* (1969) believed that calcification of the exoskeleton began prior to molting, beginning about the time when the first young were released. However, coincidence of these two events--the release of young and the start of calcification--is not necessarily related because release of young often occurs over a variable period of time (Robinson 1971) (Table 29), and it is not reasonable that the start of calcification begins a variable period of time before the molt.

Anderson and Brown (1930) showed that chitin secretion in *Daphnia* begins when 62% of the intermolt period is complete. They determined this by making small injuries in the carapace and noting the presence and degree of repair of the exoskeleton at the succeeding ecdysis. Because stage five or six occur about 62% of the way through the instar, it is possible that susceptibility at stage six (Table 30) is related to the process of chitin secretion. Whatever the reason, it is clear that sensitivity varies during the instar and that the most sensitive periods are those associated with molting.

Because calcification is a first-order reaction (Marshall *et al.* 1964) and is dependent on the concentration of calcium in the ambient water, it would be illuminating to use stage one animals from both high- and low-calcium cultures in toxicity experiments. Calcification

would occur more slowly in low-calcium water, and if reduced calcium were a factor affecting variations in sensitivity within the instar, then stage one animals from waters low in calcium would be more susceptible than stage one animals from high-calcium waters.

Cyclic sensitivity can be detected in test groups which are not uniformly distributed with respect to the time of molting. How could this affect the results of toxicity tests? Green (1956) noticed that samples of cladoceran populations in ponds are often reproductively synchronous. As such, these animals would not be uniformly distributed with respect to time of molting. In this study, up to 80% of the adult animals of a single culture were within 10 h of synchrony on the basis of reproduction and molting. Distinct changes in death rate with time, as reported by Sherr and Armitage (1971) and Anderson (1948), can be explained on the basis of culture synchrony: animals from single cultures for bioassay use may be initially susceptible if they are in synchrony and if they are about to molt, or they may be resistant if a high proportion have just molted. Molting can be expected to affect results of toxicity tests if the animals under test are in synchrony with respect to molting. The effect would be most drastic if the toxicity of the test solution decreased rapidly with time and if a large proportion of test animals were either about to molt or had molted less than 4 h before the test was started. It may be possible to overcome this difficulty by taking animals from several different cultures or from a single asynchromous culture. Furthermore, 48-h LC50s are not as likely to be affected if all animals have gone through a molt in the test solution within that time period. At temperatures

below 20 C, however, or possibly under the influence of some toxicants (*cf.* Katz 1973), molting of all test animals cannot be depended upon. Therefore, it is recommended that *Daphnia* bioassays be conducted over a period long enough that most animals will have had a chance to molt. Even for populations uniformly distributed with respect to molting time, increased sensitivity at molting contributes significantly to variance in the population response.

Compared to fish, *Daphnia* gave a larger variation in response to toxicants. The possibility that this variation was due, in part, to changes in susceptibility during the molt cycle was tested. In one experiment, individual animals of known molt-stage were exposed for two h to dilute potassium chromate (0.56 mg/l Cr^{+6}). Test animals which molted during the exposure period suffered a significantly higher mortality. In a second experiment, individuals were observed during continuous exposure to dilute potassium chromate solutions (0.56 and 0.1 mg/l Cr^{+6}). Test animals known to have molted less than four h prior to toxicant exposure exhibited a significantly lower mean survival time compared to most other test groups. This may not be a major factor in using *Daphnia* for screening effluents, because the effect on bioassay results can be reduced by 1) using animals that are not synchronous with respect to molting, and 2) by continuing the toxicity test for 48 h at 20 C or higher.

V. CONCLUSIONS

The *Daphnia* bioassay as presented here can be used by people with little biological training to perform preliminary toxicity tests on refinery effluents. Conditions existing in most laboratories are adequate for conducting *Daphnia* toxicity tests; the only exception is that temperature control ($\pm$ 0.5 C) is required.

Results showed that the *Daphnia* bioassay can be used to assess the acute toxicity of refinery effluents which are in compliance with the 1977 EPA guidelines. The limits for refinery-waste contaminants have been set below the levels expected to cause acute toxicity to fish, and consequently, fish may not be adequate for assessment of acute toxicity of future effluents.

Specific conclusions are listed below.

1) After 24-h exposure to an arbitrary reference mixture (ARM) *D. pulex* is 40 times more sensitive than rainbow trout, goldfish, bluegill sunfish, fathead minnow, or brook stickleback.
2) Results with the *Daphnia* bioassay were reproducible when the ARM was used. Expressed as fractions or multiples of all the concentrations contained in the ARM, the 8-, 24-, 48-, and 96-h LC50 values for *Daphnia* were 0.18, 0.11, 0.07, and 0.03, respectively. Without the oil component, the 8-, 24-, 48-, and 96-h LC50 values for *Daphnia* were 3.84, 1.76, 0.96, 0.42, respectively. With oil only, the 24-, 48-, and 96-h LC50s were 0.18, 0.10, and 0.06, respectively.

3) On-site tests performed with refinery effluents were reproducible for each effluent tested. The LC50 values ranged from non-toxic to 1.2% waste after 48 h.

4) Temperature is the single most important factor affecting bioassay results; organisms were 27 times more sensitive at 25 C than at 15 C.

5) There was no effect of light intensity or photoperiod as long as it was not continuous light or dark.

6) Culture age or condition may have an effect on results.

7) As pH increases, the toxicity of ammonia to *Daphnia* increases.

8) Toxicity of the ARM decreases as water hardness increases.

9) Sensitivity of *Daphnia* to low salinities limits its use to freshwater environments.

10) The most important toxicant in the ARM is no. 2 fuel oil; in its absence the ARM is about 17 times less toxic after 48 h exposure. The relative contribution of the other components was difficult to assess, but the next most important toxicant was chromium.

11) Cyanide was toxic to *Daphnia pulex* at less than 0.1 mg/l (pH 9) after 24-h exposure, but when combined with the ARM, it contributed little to the overall toxicity of the mixture after 48-h exposure.

12) Zinc and lead had little effect on the toxicity of the mixture.

13) Rainbow trout fry were the most sensitive of the five species of fish tested with the ARM. Goldfish were intermediate in

sensitivity, and fathead minnows were the least sensitive. *Daphnia pulex* were 40 times *more sensitive* than trout.

14) Molting is associated with a significant decrease in tolerance of *Daphnia* to chromate toxicity. But, the effect of molting on test results can be minimized by using cultures which are asynchronous with respect to molting time.

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DEVELOPMENT OF AN INVERTEBRATE BIOASSAY TO SCREEN PETROLEUM REFINERY EFFLUENTS DISCHARGED INTO FRESHWATER

by

David R. Lee

(ABSTRACT)

A simple method using unsophisticated equipment is suggested for on-site toxicity testing of refinery effluents. This method will provide an inexpensive means of identifying problem materials and establishing priorities for coping with these materials. An arbitrary reference mixture (ARM), containing six common constituents of refinery wastewaters, was used for static toxicity tests on 15 species of freshwater invertebrates and three species of fish. *Daphnia pulex* was selected for further testing because it was the most sensitive of the animals tested, relatively inexpensive and easy to maintain, and a potential fish-food organism. If the reference mixture were representative of a refinery effluent, the *Daphnia* bioassay would be sufficiently sensitive to give reliable results within 48 h, whereas a fish bioassay would show no toxicity even after 96 h.

To assess the suitability of the *Daphnia* bioassay, tests were conducted by personnel at six petroleum refineries. Duplicate tests were in agreement. Results of tests using actual refinery effluents ranged from no toxicity after 96 h to a mean lethal concentration of 1.2% effluent after 48 h. The data presented show that the method was reproducible and that refinery personnel were able to perform the bioassay. Potential problems and advantages of the method are discussed.

The test temperature is the most important environmental factor affecting *Daphnia* bioassay results. As temperature increased, so did toxicity. Light intensity and culture age or condition may affect bioassay results, but the reasons are unclear. The differences due to photoperiod are not significant as long as there is not continuous light or dark. Chemical features such as hardness and pH of the test affect bioassay results with *Daphnia* as they do for fish: as hardness increases, toxicity of the ARM decreases, and as pH increases, toxicity of ammonium chloride increases.

No. 2 fuel oil is the single most important toxic component of the ARM. Without the oil the ARM is 17 times less toxic to *Daphnia*. Emulsification of the ARM increases its toxicity. Because fuel oil is the dominant toxic component, it was not possible to accurately assess a change in toxicity when lead, zinc, or cyanide was added to the ARM.

Expressed as multiples of the ARM the 96-h LC50 values for fish were 1.7 for rainbow trout, 4.6 for brook stickleback, 14 for bluegill, 20 for goldfish and more than 32 for fathead minnow. The 24- and 48-h LC50 values for *Daphnia pulex* were 0.42 and 0.033, respectively.

Compared to fish, *Daphnia* gave a larger variation in response to ARM toxicity. The possibility that variation was due to changes in susceptibility during the molt cycle was tested. Animals of known molt-stage were exposed for two h to dilute potassium chromate (0.56 mg/l Cr^{+6}). Animals which molted during the exposure period showed a significantly higher mortality than those which did not molt. Individuals exposed continuously to dilute potassium chromate solutions

(0.56 and 0.10 mg/l Cr^{+6}) exhibited a significantly lower mean survival time if molting occurred less than 4 h prior to the beginning of exposure. It is suggested that molting is a significant source of variability in *Daphnia*.