THE EFFECTS OF WATER-SOLUBLE FRACTIONS

OF NAPHTHALENE, PHENANTHRENE, NO. 2

FUEL OIL, AND COAL-TAR CREOSOTE ON THE

FRESHWATER CLADOCERAN, Daphnia pulex

by

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GENERAL INTRODUCTION

The increasing global demand for all forms of energy has stimulated great interest in petroleum and petroleum products. Of the thousands of compounds found in petroleum, the hydrocarbons are considered the most abundant. The Encyclopedia of Science and Technology (1971) states that petroleum hydrocarbons may be divided into three general classes: the aliphatic, alicyclic, and aromatic hydrocarbons.

Clark and Brown (1977) present a very detailed account of petroleum hydrocarbons and their physical and chemical characteristics. Briefly, aliphatic hydrocarbons are open-chain compounds with either saturated or unsaturated bonds. These bond characteristics further differentiate this class into the alkanes, alkenes, and alkynes. All these compounds are insoluble in water. Acetylene is an example of an important aliphatic hydrocarbon.

Alicyclic hydrocarbons consist of compounds in which some or all of the carbon atoms are arranged in a ring structure. The bonds may be saturated or unsaturated, form-ing cycloalkanes or cycloalkenes, respectively. Alicyclic hydrocarbons have similar physicochemical properties to the open-chain hydrocarbons, and most are either weakly polar or non-polar. Cyclohexane is an example of an important alicyclic hydrocarbon.

The aromatic hydrocarbons contain at least one six-

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carbon benzene ring, and by definition resemble benzene in their physicochemical behavior. Aromatic hydrocarbons are generally insoluble in water. Also included in this group are the polynuclear aromatic hydrocarbons (PAH) or fused ring hydrocarbons. Naphthalene (two-ring) and phenanthrene (three-ring) are examples of fused-ring aromatic hydrocarbons.

A series of ecologically disastrous oil spills around the world provided the initial research impetus to investigate the short-term and long-term effects of crude oil, and its refined products and components upon marine and estuarine organisms. The early data are difficult to interpret due to the variety of experimental methods utilized, the types of organisms tested, and the lack of a uniform system for hydrocarbon analysis. The findings of early workers such as Blumer et al. (1970), and Boylan and Tripp (1971) and Moore et al. (1973) indicated that the acute toxicity of an oil was generally a function of its aromatic hydrocarbons, and that these components play important roles in the actual toxicity to marine organisms.

In 1974 Anderson and co-workers published a classic paper which set an example for all further work in this area. Anderson et al. (1974) looked at the acute effects of crude oil, oil-water dispersions, and water-soluble fractions of several crude and refined oils to various estuarine organisms. These workers meticulously documented not only the concentra-

tions of hydrocarbons but also identified many of the individual components present in the test water using gas chromatography procedures.

Their research supported the findings of earlier workers and also implicated the di-aromatic and tri-aromatic hydrocarbons as possible sources for the observed toxicity. The presence of these PAH's (in low concentrations) was documented in the water-soluble fractions of the more highly refined oils such as No. 2 fuel oil.

Data detailing the effects of these types of hydrocarbons on freshwater organisms were virtually nonexistent even though numerous small spills of crude and refined oil had occurred in many of the nation's inland waterways and lakes. In spite of the government's projected increase in construction of pipelines and refineries, the research emphasis has been almost solely on acute effects of hydrocarbons on estuarine and marine organisms. Nothing was known of possible effects of long-term sublethal exposure of critical food-web organisms to hydrocarbons, or even the physiological-biochemical implications of such exposure.

This research was designed to examine the potential effects of water-soluble fractions of naphthalene, phenanthrene, No. 2 fuel oil, and creosote on _Daphnia pulex_ to test the following hypotheses:

a) Water-soluble fractions of petroleum hydrocarbons
    are not acutely toxic to an important freshwater
    zooplankter.

b) Lifetime sublethal exposure to these water-soluble
    fractions demonstrate no effect upon zooplankton
    growth, reproduction, and survival.

c) Sublethal exposure to these water-soluble fractions
    do not affect zooplankton oxygen consumption and
    filtering rate.

Chapter 1. Acute and chronic effects of water-soluble
fractions of hydrocarbons on the <u>survival</u>,
<u>growth</u>, and <u>reproduction</u> of <u>Daphnia</u> <u>pulex</u>.

Introduction

The available literature on the acute toxicity of
petroleum products to marine and estuarine organisms has
been reviewed extensively by several authors (Moore and
Dwyer, 1974; Weiss, 1976; Lockwood, 1976; Reish <u>et</u> <u>al</u>.,
1978). However, few researchers have investigated the
biological effects of these compounds in fresh water. One of
the earliest workers was Shelford (1917) who detailed various
physiological and behavioral changes in several species of
freshwater fish exposed to gas waste. Several investigators
examined the use of <u>Daphnia</u> in toxicity testing of oil and
oil emulsifiers (Dowden, 1965; Grodner, 1959; Rothschein,
1970), refinery wastes (Dorris <u>et</u> <u>al</u>., 1974) and petroleum
related industrial wastes (Anderson, 1944; Anderson <u>et</u> <u>al</u>.,
1948).

More recently Buikema <u>et</u> <u>al</u>. (1976) found <u>Daphnia</u> <u>pulex</u>
the most sensitive organism tested using a simulated refinery
effluent. Lee (1976) developed a screening bioassay for
evaluating petroleum refinery wastes. Burks and Wilhm (1977)
tested a multi-species response using benthic invertebrates
to evaluate various refinery wastewaters. The recognized
importance of algae as primary producers has led to several

reports on both the acute and chronic toxicity of petroleum on algal growth, uptake, and photosynthesis (Soto et al. 1975a, 1975b; Kauss and Hutchinson, 1975; Bott and Rogenmuser, 1978).

The paucity of knowledge concerning the biological effects of petroleum on freshwater zooplankton led directly to this study which tested the hypothesis that exposure to acute and chronic WSF's of petroleum compounds would show no effects upon the survival, growth, and reproduction of Daphnia pulex.

Materials and Methods

General

The test organism was Daphnia pulex, a cosmopolitan freshwater crustacean. This organism was easily cultured, had a well-defined life history, and was sensitive to a variety of toxicants (Buikema et al., 1976). Daphnia pulex were obtained from continuous cultures maintained for several years in our laboratory. Both stock and experimental animals were kept in Sherer CEL 4-4 environmental chambers at 20 ± 1.5 C on a 16L:8D photoperiod with approximately 100 ft-candles of light at the air-water interface of the test containers. Light was provided by cool-white deluxe fluorescent bulbs. All stock cultures were fed Chlamydomonas reinhardi (wild type, - strain) ad libitum and approximately once a week their diet was supplemented with a trout chow-cerophyll

extract. Algae were cultured in a modified Bold's basal medium (Buikema, 1970), centrifuged, and resuspended in millipore-filtered (0.45 µ) carbon-dechlorinated Blacksburg tap water before being fed to Daphnia. During the chronic and subchronic tests the Daphnia were fed algae at a concentration of 45,000 cells/ml; algal concentrations were verified with an Electrozone particle counter.

Test Chemicals

American Chemical Society grade naphthalene (0.034 g/l) representing the solubility of naphthalene in water (Bohon and Claussen, 1951) and phenanthrene (insoluble - 1 g/l arbitrarily chosen) crystals were washed: then placed in millipore-filtered (0.45 µ) carbon-dechlorinated Blacksburg tap water and heated to 80 and 100 C, respectively; swirled gently until crystals were dissolved; and then cooled to room temperature. Solutions were then millipore-filtered to remove undissolved crystals, and the stock water-soluble fractions (WSF) utilized. In addition, millipore-filtering and heating helped to eliminate potential degradation and/or transformation of the test compounds by fungi and bacteria (Cerniglia et al, 1978). No. 2 fuel oil (Gulf Oil Company, Philadelphia, Pa.) and coal-tar creosote (Ace Hardware) WSF's were prepared according to Anderson et al. (1974), except that all dilution water was millipore-filtered carbon-dechlorinated tap water and the WSF phase was then millipore-

filtered again. For all acute tests, new solutions were made for each test, and for chronic tests, new solutions were made every three days.

Neither funding nor equipment was available for weekly analyses of test chemicals during the chronic study. However, funds became available late in the study to contract for analysis of the stock water-soluble fractions. One hundred ml portions of each freshly prepared WSF were extracted three times with a total of 100 ml pesticide-grade pentane. Extracts were combined, dried over anhydrous Na_2SO_4 and evaporated nearly to dryness under a stream of nitrogen. Hydrocarbons were analyzed using a Varian 2440-MAT112 GC-MS with FID detectors. A 5 m x 2 mm (I.D.) glass column packed with 3% SP-2100 on supelcon AW-DMCS was used. Carrier gas was helium (30 ml/min) and detector gases were air (300 ml/min) and hydrogen (30 ml/min). Oven temperatures were programmed from 90-275 C at 4 C/min. A spectro-physics autolab I integrator was used to quantitate peak areas. Usually 1.0 μl samples were injected. Peak areas were quantified to injected standards of phenanthrene and naphthalene. This procedure indicated the amount of phenanthrene and naphthalene present was 0.96-1.28 mg/l and 5.08-6.76 mg/l, respectively, in the stock water-soluble fractions of phenanthrene and naphthalene. Analysis of oil and creosote WSF's indicated the presence of up to 40 individual peaks in each WSF (Appendix

Figures A,B).

Millipore filtering of the resultant aqueous phases may
have reduced the amount of hydrocarbons accommodated by the
WSF's. Boehm and Quinn (1974, 1976) found filtration of
hydrocarbon-water mixtures reduced hydrocarbon content by
approximately 75%, presumably due to either organic macro-
molecules solubilizing certain kinds of hydrocarbons, or
hydrocarbons being absorbed onto small particulates. GC
analysis of the stock WSF of naphthalene in this study indi-
cated only 5.08-6.76 mg/l present compared to 34 mg/l initi-
ally calculated. Zoteman and Haring (1976) determined the
solubilities of several PAH's in water, and found naphtha-
lene and phenanthrene present in 12.5 and 1.6 mg/l, respec-
tively.

Acute Tests

Acute tests were usually conducted using 500 ml pyrex
beakers with 5 neonates per 300 ml test solution. Air was
supplied with disposable Pasteur pipettes so that bubbles
were produced approximately 2-4 times per second (Anderson
et al., 1974) to prevent any possible oxygen depletion.
Animals were not fed during the test. Organisms were
examined at 8, 24, and 48 hrs, and mortality recorded. At
least 25 animals were exposed to each concentration (re-
presenting 5 replicate tests). On rare occasions control

mortality exceeded 10%, and these particular test results were discarded. Data were pooled and LC50 values with appropriate confidence intervals were computed using probit analysis (Barr et al., 1976).

Chronic Tests

From these resulting dose/response curves, the appropriate percent concentration of the WSF's corresponding to the LC20 ± C.I. and LC30 ± C.I. were determined and utilized for the chronic tests. Tested concentrations are denoted throughout the text as the abbreviation of the compound or WSF along with a 2 or 3 to designate the respective LC20 or LC30 concentration (i.e. creo-2 refers to the creosote LC20 concentration). These tested values (in % WSF) were as follows: nap-2 (5.6), nap-3 (10), phe-2 (10), phe-3 (32), oil-2 (5.6), oil-3 (10), creo-2 (1.0), and creo-3 (1.8).

Each chronic experimental group contained ten Daphnia 24 ± 12 hr old. The control group consisted of 20 organisms. Test solutions were renewed and test containers were thororghly cleaned daily.

Dissolved oxygen, alkalinity, hardness, and pH were determined weekly (Standard Methods, 1975). Throughout all tests dissolved oxygen varied between 7.5-9.0 mg/1; alkalinity 40-47 mg/1 ($CaCO_3$); hardness 41-50 mg/1; and pH 6.9-7.5.

Animals were maintained individually in glass baby food jars with 50 ml test solution. Each day animals were fed 45,000 cells/ml and examined for body length (as determined by ocular micrometer from top of head to base of caudal spine), molting, live young, number of dead young (with and without caudal spines), number of nonviable eggs and partial and full abortions. The presence of dead young without caudal spines indicated premature ejection of an incompletely developed organism; dead young with spines implied the organism was completely developed and would normally have been released by the female within a short period of time. A partial abortion was considered premature ejection of a portion of a newly developing brood from the brood chamber; a full abortion was the premature ejection of the entire developing brood of young.

Data Analysis

Data were analyzed by brood using SAS76 General Linear Model and Duncan's Multiple Range Test procedures (Barr et al., 1976). Some animals were accidentally killed during these experiments. These were not included in any statistical analyses. Statements of significance refer to $P \leq 0.05$.

Results and Discussion

Acute Tests

Results of acute toxicity tests on D. pulex are summar-

ized in Table 1. Creosote was the most toxic of the four WSF's tested. In most experiments with creosote the majority of deaths occurred within the first 8 to 24 hr. No. 2 fuel oil also was quite toxic, and mortality was most pronounced within the first 24 hr.

Naphthalene toxicity was low compared to creosote and No. 2 fuel oil. This was not unexpected because of the low solubility of naphthalene in water (Bohon and Claussen, 1951), as well as the possible removal of initial levels of naphthalene through aeration. Animals were not removed from test containers for the duration of each test. Several times (especially with naphthalene and creosote) animals would appear to be dead but later recovered. This "narcotic" effect has been also noted by several investigators working with marine invertebrates (e.g., Crisp et al, 1967; Berdugo et al., 1977; Sanborn and Malins, 1977).

Phenanthrene, being virtually insoluble in water (Handbook of Chemistry and Physics, 1977), was not expected to demonstrate much effect. Some limited toxicity was apparent after 96 hr, most likely due to either starvation or toxicant-starvation interaction. Although a LC50 could not be calculated via probit analysis, sufficient data existed to permit calculations of approximate LC20 and LC30 concentrations. Subsequent analyses of the phenanthrene stock WSF's by GC-MS confirmed phenanthrene was present in the solutions.

Table 1. Acute LC50 values for the water-soluble fractions
          of naphthalene, phenanthrene, No. 2 fuel oil and
          creosote.

	48 hr LC50	
	% WSF (+ C.I.)	mg/l*
naphthalene	57.52 (46.02 - 76.79)	2.92 - 3.89
phenanthrene	>>100	0.96 - 1.28
No. 2 fuel oil	34.10 (27.94 - 41.78)	---
Creosote	2.91 (2.52 - 3.36)	---

*Determined by gas chromatography of stock solution.

Chronic Tests

Results of the chronic tests are summarized in Table 2.
The value of monitoring effects of compounds over the entire
life cycle of the test organism compared to typical 21 or 28
day chronics is reflected in both the abundance of informa-
tion obtained and the diversity of useful information
generated by such a study. This allows the investigator
potential insights into possible trends of the data, physio-
logical and/or biochemical inferences into possible modes
of action, and stimulates experimental designs to answer
hypotheses based on chronic data.

The chronic effect of phenanthrene was most surprising.
Both concentrations were significantly different from con-
trols (and usually each other) but at different ends of the
spectrum. The phe-2 population lived longer, produced more
broods per animal, but less live young, than controls. These
organisms also produced more dead young without spines than
the phe-3 group. Conversely, the phe-3 population died soon-
er, produced fewer broods per animal with correspondingly
fewer live young per brood than either the phe-2 or control
animals. Phe-3 organisms reached reproductive maturation
approximately six days later than did either controls or the
phe-2 animals (Table 3).

The only other compound to exhibit any significant
delay in reproductive maturation was creo-3. Similar delays

Table 2. Reproduction data resulting from chronic exposure of Daphnia pulex to four hydrocarbon water-soluble fractions. Data are means (+ standard deviation) per animal.

Compound	(N)	Conc. (mg/l)	Live young	Dead young (w/spine)	Dead young (w/o spine)	Broods/ animal	Live young/ brood/ animal	Total dead/ animal
control	(19)	-	252+155	0.7+1.4	25.9+12.7	16.4	15.4	26.6
Oil-2	(7)	-	252+116	1.3+2.2	53.1+24.2	20.0	12.6	54.4
Oil-3	(9)	-	199+113	0.4+1.3	39.7+23.0	16.3	12.2	40.1
Creo-2	(10)	-	133+ 77	1.8+3.7	93.0+40.4	20.8	6.4	94.8
Creo-3	(10)	-	7+ 12	0.2+0.4	38.2+30.0	9.6	0.7	38.2
Phe-2	(10)	0.10-0.13	221+134	0.4+1.0	12.9+ 8.9	19.1	11.6	13.3
Phe-3	(9)	0.30-0.41	45+ 28	0.1+0.3	2.2+ 2.6	12.0	3.7	2.3
Nap-2	(9)	0.28-0.38	314+ 91	3.6+4.6	28.0+24.0	25.8	12.2	31.6
Nap-3	(8)	0.51-0.68	244+140	0.2+0.7	9.4+10.4	23.4	10.4	1.6

Table 2. (continued)

Compound	(N)	Partial Abortions	Full Abortions	Total Abortions	Survival (Days)
Control	(19)	0.6+0.7	0.8+1.1	26	57.4+30.6
Oil-2	(7)	1.4+1.0	1.9+1.1	23	69.3+26.8
Oil-3	(9)	1.7+1.4	1.0+1.2	24	56.1+18.1
Creo-2	(10)	3.8+3.0	4.6+1.6	84	72.5+19.8
Creo-3	(10)	6.2+5.2	0.7+1.6	69	42.8+25.1
Phe-2	(10)	0.5+0.5	0.6+0.7	11	67.3+35.3
Phe-2	(9)	0.5+0.7	0.0+0.0	5	51.6+29.3
Nap-2	(9)	0.7+1.0	1.3+2.1	18	90.1+23.4
Nap-3	(8)	0.5+1.1	0.5+0.8	8	84.0+41.3

Table 3. Onset of reproductive maturation of <u>Daphnia pulex</u> exposed to water-soluble hydrocarbons.

Test Compound	Size at first brood (mm) (Mean+S.D.)	*Appearance of the first brood (days) (Mean+S.D)
Oil-2	1.76($\pm$0.12)	6.7($\pm$0.5)
Oil-3	1.63($\pm$0.16)	7.3($\pm$0.5)
Creo-2	1.55($\pm$0.13)	7.1($\pm$0.3)
Creo-3	1.43($\pm$0.11)	9.2($\pm$1.1)
Control	1.79($\pm$0.13)	7.2($\pm$0.5)
Phe-2	1.60($\pm$0.08)	7.0($\pm$0.7)
Phe-3	1.31($\pm$0.06)	10.8($\pm$1.0)
Nap-2	1.75($\pm$0.12)	7.6($\pm$0.5)
Nap-3	1.66($\pm$0.11)	7.7($\pm$0.8)

*Organisms were 24 ± 12 hrs old at the beginning of the chronic studies.

in development rates of organisms exposed to petroleum hydrocarbons, have been reported for decapod crustaceans (Katz, 1973; Wells and Sprague, 1976; Caldwell et al., 1977; Laughlin et al., 1978), marine amphipods (Linden, 1976), and Pacific herring larvae (Struhsaker et al., 1974). Armstrong et al., (1976) found that the insecticide methoxychlor delayed molting of larval and juvenile crabs.

The majority of animals which died during the chronic study invariably were unable to molt successfully. This was particularly true for phe-3 and creo-3. Also, one organism from each of these two groups developed an unusual growth apparently on, or just under, the exoskeleton of the organism. This growth eventually inhibited these organisms from molting. Hodgins et al. (1977) reviewed the incidence of neoplasia in marine fish and invertebrates, and in many instances PAH's were implicated in neoplasm development.

The creo-3 organisms demonstrated significantly shorter survival time, fewer broods per animal, a high number of partial abortions, and virtually no mean live young per animal compared to controls. The creo-2 population lived longer, produced a higher number of broods and full abortions than the creo-3 organisms. Additionally creo-2 organisms exhibited the highest number of dead young without spines than any other WSF tested.

Both naphthalene-exposed populations lived significantly longer and produced numbers of live young equal to or greater than control organisms. This phenomenon of test organisms appearing "healthier" than controls has been noticed by other workers (Smyth, 1967; Eldridge et al., 1977), and led Smyth (1967) to speculate that exposure to small chronic levels of a toxicant may stimulate adaptive physiological functions which help a healthy organism to maintain homeostasis. For all experimental animals, the longest living organism was one exposed to an LC30 concentration of naphthalene. This female lived for 125 days and produced living young up to day 121.

The oil-2 organisms had the second highest number of mean dead without spines and total dead per animal of all compounds tested. Yet oil-3 exposed Daphnia did not exhibit much difference from control organisms. This dichotomy between LC20 and LC30 concentrations was exhibited for every WSF in number of dead without spines. In each case, the LC20 concentration exhibited the higher number of dead young without spines. These results may reflect a possible consequence of detoxification. Introduction of molecular oxygen by mixed function oxidase systems may enhance cytotoxicity; such oxygenated derivatives have been implicated in producing genetic damage (Varanasi and Malins, 1977).

The most significant variables for all WSF's compared

brood by brood were body length, number of live young, and number of partial abortions. Brood also referred to pre-adult molts for the purpose of determining differences in body length. A Duncan's Multiple Range Test was performed on each brood that previously showed significant differences by the GLM procedure. In this way, real differences in the data could be noted among the means of the WSF's not only for each variable but also among broods. Because broods represent distinct physiological events in the organism's life cycle, any time dependent or potential cumulative effect would not be masked. Results are summarized in Table 4.

The oil-2 test population was the only one which was not significantly different (Duncan procedure) from controls in body length (growth). Although brood by brood mean growth of controls was always greater than that of oil-2 organisms, it could not be distinguished as different by the Duncan procedure.

Exposure to the levels of naphthalene used in this study resulted in significant differences from controls in body length. Yet in some broods, naphthalene seemed to stimulate reproduction compared to controls. Both nap-2 and nap-3 test organisms lived significantly longer than controls.

Table 4. Number of times the experimental populations were significantly different from the control populations for the variables, body length and number of the young (brood by brood comparison).

Compound	*Number of broods	Length of adults	Number of broods	Number live young
Oil-2	25	0	25	12
Oil-3	25	24	25	9
Creo-2	25	25	25	19
Creo-3	21	21	20	19
Phe-2	25	25	25	12
Phe-3	20	20	20	19
Nap-2	25	24	25	13
Nap-3	25	25	25	14

*Where insufficient data existed, these broods were not counted. Generally these occurred toward the end of the life cycle where N became smaller. For the WSF's, some test organisms reached brood 35.

Filtration rate and oxygen consumption experiments
(see chapters 2 and 3) tend to support the fact that organisms
exposed to naphthalene undergo a reduction in metabolism.
The energy conserved may allow for lengthened life spans.

Previous investigations have confirmed that WSF's of
oil are enriched in aromatic hydrocarbons relative to the
original parent oil (Blumer et al., 1970; Boylan and Tripp,
1971; Anderson et al., 1974). Pancirov and Brown (1975)
found several polynuclear aromatic hydrocarbons in samples
of a No. 2 fuel oil with phenanthrene (and methylphenanthrene)
present in the highest concentration. These aromatic
hydrocarbons and PAH's are generally conceded to be the
ones responsible for most of the toxicity related to petro-
leum and its refined products. Similarly, Nestler (1974)
describes whole coal-tar creosote as being approximately 90%
polynuclear aromatic hydrocarbons. Most of the compounds
are true aromatic hydrocarbons with little substitution on
the ring. Gas chromatograph-mass spectroscopy analysis of
the WSF of coal-tar creosote resulted in approximately 40
major and minor peaks. Preliminary analysis and computer
reconstruction of GC-MS spectra has confirmed the presence
of several PAH's. This could be the major reason for the
substantial chronic effects of creosote and phenanthrene
and, to a lesser extent No. 2 fuel oil, on growth and
reproduction of Daphnia pulex.

The effects on growth and reproduction following chronic sub-lethal exposures to petroleum hydrocarbons has recently been documented by several workers using a variety of marine organisms. Tatem (1977) found hatching rate of grass shrimp larvae was reduced, and post-exposed gravid females held afterward in clean seawater had significantly lower number of larvae. Berdugo et al. (1977) showed significant reduction in life span, total egg production, mean brood size, and rate of egg production in marine copepods exposed to WSF's of heating oil. Laughlin et al., (1978) found duration of four zoeal stages of mud crabs was significantly increased by increasing WSF's of No. 2 fuel oil. Edmunds (1978) exposed juvenile shrimp to WSF's of crude oil at three different temperatures. There was a reduction in growth rate and respiration.

Several important parts of these data must be considered in attempting to explain the growth and reproduction effects of these water-soluble fractions on D. pulex. The water-soluble fractions with greater amounts of polynuclear aromatic compounds - i.e. the creo-3 and the phe-3 concentrations - resulted in the most dramatic reduction of growth and reproduction. These same two concentrations delayed the onset of reproduction significantly. Organisms which died during the chronic study from inability to molt successfully belonged predominantly to these two test groups.

These results strongly suggest a disturbance on some aspect of control of the reproductive and molting processes. Molting and reproduction dominate the life-cycle of Daphnia and these events are intimately related (Lee, 1976). Evidence has accumulated that both processes in crustaceans are mediated by steroid hormones (Highnam and Hill, 1969; Novales et al., 1973). Most invertebrates cannot synthesize sterols from precursor molecules (Kanazawa and Teshima, 1971; Gilbert, 1967), and most likely obtain them from dietary food chains (Alam et al., 1979). Because most of these hormones are modifications of cholesterol, it is not unreasonable to assume that crustaceans such as Daphnia could obtain this compound through the food chain. Work by Teshima (1972) on crabs and prawns showed that cholesterol (or dietary precursors) was essential for normal growth. Recently O'Hara (1978) documented the presence of a steroid (which binds with mammalian estradiol 17-B antibody) in the marine copepod Calanus. This strongly implies the presence of either 17-B or a very similar steroid in Calanus. Current evidence indicates that in mammals the initial step of estrogen action involves binding of estradiol 17-B to cytosolic receptors which in turn is translocated into the nucleus where the estrogenic message is expressed (Kupfer and Bulger, 1976). The presence of this steroid (or a similar molecule) in Calanus implies some reproductive function.

O'Hara (1978) points out that metabolism of polynuclear aromatic hydrocarbons (via mixed function oxidase systems) and steroid metabolism may utilize the same enzyme systems (i.e., same requirement for NADPH, molecular oxygen, and cytochrome P-450).

If one assumes the presence of a functioning endocrine system in Daphnia pulex, the dietary intake of sterols from Chlaymdomonas reinhardi and subsequent production of analogous molting hormone (crustecdysone) and estrogen-like hormone (estradiol 17-B) then the observed physiological responses of D. pulex to some of the WSF's may be hypothesized.

Lehninger (1975) summarized the two basic principles of hormone action. The first was that target cells for any hormone contain specific hormone receptors which are proteins capable of binding the hormone with high specificity. These receptors are located either on the cell surface or in the cytosol, depending upon whether the hormone is water-soluble or lipid-soluble. The second principle was that binding of the hormone receptor caused the formation of an intracellular messenger molecule (i.e. cyclic AMP, or others) which then stimulated or depressed some biochemical activity in the target cell (such as mRNA synthesis with protein and/or carbohydrate synthesis resulting in altered cell function). Phenanthrene and a typical steroid have very similar molecular structures, and steroids are indeed

modified derivatives of perhydrocyclopentanophenanthrene
(Lehninger, 1975). Phenanthrene or similar polynuclear
aromatic hydrocarbons could possibly function as a steroid
(or compete with the actual steroid for binding sites on the
receptor) and bind to a receptor, either inactivating it
or causing induction of a nonintended biochemical activity.
A similar situation occurs in mammals. Kupfer and Bulger
(1976) indicated that DDT homologs could act like estradiol
17-B and bind to the uterine cytosolic receptor in rats.

Another intriguing possibility also may exist. Several
workers (Scheier and Gominger, 1976; Larson et al., 1977)
found that by exposing water-soluble fractions of a No. 2
fuel oil to U.V. light, the apparent toxicity of the fraction
was increased. Hydroperoxides were formed after irradiation
of oil with simulated sunlight (Larson et al., 1977), and
that these hydroperoxides were toxic to yeast cells (Callen
and Larson, 1978). Recently Morse et al., (1977) found that
adding hydrogen peroxide to seawater caused synchronous
spawning in gravid male and female abalones, as well as in
other mollusks. Morse suggested that peroxide activated
the enzyme prostaglandin endoperoxide synthase (PES), and
that the synthesis of PES within the eggs may play an
essential role in early embryonic development.

Prostaglandins are found in many animal tissues and
exhibit a great variety of hormone-like physiological and

pharmacological effects. They are cyclic derivatives of unsaturated fatty acids (Lehninger, 1975). Morse et al. (1978) found peroxide-activated prostaglandin synthase in the reproductive tissues of a wide variety of marine organisms, including crabs and fishes. Also, changes in photoperiod (and U.V. light) can break diapause in insects, which is mediated by the presence or absence of developmental hormones (Highnam and Hill, 1969; Novales et al., 1973).

It may be that Daphnia possess a similar prostaglandin endoperoxide system and that normally U.V. light stimulates the endogenous synthesis of the enzyme. The data of Morse (1977) suggest that phototransformation hydroperoxides resulting from U.V. irradation of refined oil fractions could induce PES and increase reproductive success. However, reproduction in this study was generally decreased. Failure to induce this enzyme system could influence reproductive success by slowing down or interrupting embryonic development and inducing partial or full abortions, or increased production of non-viable eggs. One or both of these systems may be operating in Daphnia pulex and be responsible for the noticeable growth and reproductive effects shown in this study.

Summary

The hypotheses that WSF's of petroleum hydrocarbons

are not acutely toxic to <u>Daphnia</u> and that lifetime sub-
lethal exposure to these WSF's demonstrate no effect upon
the survival, growth, and reproduction of <u>Daphnia</u> <u>pulex</u> are
rejected. The effects of water-soluble fractions of
naphthalene, phenanthrene, No. 2 fuel oil, and creosote
were acutely toxic to <u>Daphnia</u> <u>pulex</u>. The effects of
creosote and phenanthrene were most dramatic during life-
time sublethal exposure. In both of these concentrations,
there was marked reduction in growth rates, numbers of
broods, impairment of molting and increased abortion rates
when compared to controls. No. 2 fuel oil produced similar
effects, but results were not as significant. Only a
slight reduction in growth rate was found for naphthalene.
It was hypothesized that these effects upon growth and
reproduction may be due to PAH-induced disturbance on some
aspect of metabolic control of the reproductive and molting
processes.

Chapter 2. The effects of water-soluble fractions of hydrocarbons on oxygen consumption of Daphnia pulex.

Introduction

Zooplankton have been utilized in the past to investigate the effects of feeding and food type (McMahon, 1965; Schindler, 1968; LaRow et al., 1975; Kersting and van der Leew-Leegwater, 1976), light (Buikema, 1972), oxygen concentration (Heisey and Porter, 1977), and temperature (McGinniss, 1978; Armitage and Lei, 1979) on respiration.

Although work has been published on hydrocarbon effects upon respiration in several marine organisms, no work has investigated the potential effects of petroleum or petroleum-related compounds upon freshwater zooplankton respiration. Data of this kind may indicate alterations in metabolic rate of stressed zooplankton. Such information would be valuable in helping to explain the pronounced sublethal effects of these compounds upon the growth and reproduction of Daphnia (chapter 1).

The following research was designed to test the hypothesis that exposure to the water-soluble fractions of naphthalene, phenanthrene, No. 2 fuel oil, and creosote do not affect the oxygen consumption of Daphnia pulex.

Materials and Methods

Approximately 60 young adult D. pulex were obtained

from stock cultures and transferred to glass globe aquaria
containing 3000 ml of test water-soluble fraction. Test
solutions were prepared as previously described (Chapter 1).
Aquaria were placed in a Sherer Cel 4-4 environmental growth
chamber under a 16L:8D photoperiod at a temperature of
20 ± 1.5 C. Organisms were fed Chlamydomonas reinhardi
(wild-type,-strain) ad libitum daily. Organisms were accli-
mated to LC20 and LC30 test concentrations for at least three
molt cycles (8-9 days). This acclimation period was chosen
on the basis of several factors. The data of Green (1957)
indicated that two molt cycles were necessary for the re-
versal of light effects on carotenoid contents for D. magna.
Buikema (1973b) speculated that two molts may be the minimal
period of time for physiological effects of light to occur.
Also, it was noted in Chapter 1 that the effects of feeding
some algae batches to control Daphnia resulted in smaller
broods being produced. This effect was reversed within two
molt cycles by feeding algal cells from newly-started
cultures (see also Buikema et al., 1979). Thus it was felt
that this exposure period was of sufficient duration to allow
any potential effects upon Daphnia pulex to be realized.

For all tests, dissolved oxygen varied from 7.0-8.5
mg/l, alkalinity 42-46 mg/l (CaCo$_3$), hardness, 43-48 mg/l
and pH 6.8-7.5. Water chemistry (Standard Methods, 1975)
was performed once for each test in the middle of the expo-

sure period. All water was aerated, Blacksburg carbon-
dechlorinated tap water millipore-filtered (0.45 μ) before
use. All water was at 20 C.

Animals were not fed for 12 hrs preceding the start
of each oxygen consumption experiment. At this time,
respiration rates of Daphnia pulex were determined over a
24 hr period. Glass stoppered 60 ml pyrex bottles (cali-
brated to contain by weighing) were used as respirometers.
Animals were collected, placed in small beakers of water to
rinse off any test solution, and sized (1.2-1.4 mm) using
an ocular micrometer, and sized organisms were placed in
another beaker of clean water. Five Daphnia were then placed
in each respirometer. Respirometers were stoppered, checked
for air bubbles, then placed on their sides in the environ-
mental chamber. Generally each test consisted of one control
and 3 replicates of each concentration for a total of 13
respirometers per test chemical WSF. Each test was performed
three times.

At the end of 24 hrs, the amount of dissolved oxygen
was determined using the azide modification of the Winkler
method (Standard Methods, 1975). Bottles were fixed with
$MnSO_4$ and alkali-iodide-azide reagents (1.0 ml), acidified
with concentrated H_2SO_4, and immediately titrated with
previously standardized 0.005N sodium thiosulfate using a
10 ml buret calibrated in 0.02 ml. Values were corrected

for volume, dilution by Winkler reagents, and differences between control and experimentals, then expressed as μl $O_2/$ _Daphnia_/day. These values were then analyzed by General Linear Model (GLM, Appendix Table 1) and Duncan's Multiple Range Test procedures (Barr et al., 1976) to determine significant differences among means both for each individual test and for all the data. Statements of significance refer to the P<.05 level.

Results and Discussion

The results of oxygen consumption tests are summarized in Table 5. Phenanthrene-exposed organisms showed lower oxygen consumption than controls. The effect of phenanthrene was not significantly different from either controls or naphthalene test groups. This was unexpected because phenanthrene, like creosote, significantly affected growth and reproduction (Chapter 1). The Duncan procedure identi-field only two real differences in the pooled mean oxygen consumption data set. The creo-3 test group had the highest oxygen consumption of all test groups. Secondly, both naphthalene concentrations had the lowest mean oxygen consumption values of all the test groups. Oil exposed organisms were not significantly different from controls. However, in no case was any experimental mean significantly different from controls.

Table 5. Combined oxygen consumption data for <u>Daphnia</u> <u>pulex</u> after exposure to water-soluble fractions of petroleum hydrocarbons.

Compound	N	μlO_2/<u>Daphnia</u>/Day (mean $\pm$ S.D.)	Duncan Ranking*		GLM**
Creo-3	9	2.872 (1.143)	A		P>F
Control	6	2.415 (1.272)	A	B	0.34
Oil-3	9	2.272 (1.215)	A	B	
Oil-2	9	2.058 (1.329)	A	B	
Phe-2	9	1.986 (0.972)	A	B	
Creo-2	9	1.915 (1.486)	A	B	
Phe-3	9	1.886 (0.915)	A	B	
Nap-2	9	1.686 (0.657)		B	
Nap-3	9	1.600 (0.414)		B	

*Means with the same letter are not significantly different (α= .05)

**Probability of significant differences existing among computed means.

Comparing these results to those found for marine and estuarine organisms shows no consistent hydrocarbon effects upon respiration. Steed and Copeland (1967) examined the effects of sublethal concentrations of a petrochemical waste on respiration. In brown shrimp, respiration rate was depressed, but in pink shrimp respiration rate increased over that of controls. Yentsch et al., (1973) found virtually no effect on respiration of polychaete worms exposed to crude oil extracts. However, respiration of sea urchins was slightly stimulated by exposure to the same extracts. Anderson et al. (1974b) exposed mysid shrimp to a water-soluble fraction of No. 2 fuel oil. Oxygen consumption of mysids increased. Tatem (1976) found that exposure of grass shrimp to naphthalene resulted in a decrease in respiration rate. As levels of naphthalene decreased to background levels during depuration, respiration rate returned to normal. Percy and Mullin (1975) examined the effects of an oil-water dispersion on respiration of a marine amphipod. They found oxygen consumption was depressed at low oil exposure and stimulated with exposure to high oil-water dispersions. Rice et al. (1976) found sublethal exposure of water-soluble fractions of crude oil had little effect on Alaska King crab respiration; however, acute levels of water-soluble fraction quickly depressed oxygen consumption in these organisms. Gulfillan (1975) showed

increased respiration rates in soft-shell clams exposed to crude oil. More recently, Stainken (1978) looked at the effects of an oil-water dispersion of No. 2 fuel oil on soft-shelled clams. Low concentrations of oil doubled respiration rates while higher oil concentrations caused depression in respiration rate.

The data available concerning effects of oil on respiration in fish is just as variable as that of the invertebrates. Brocksen and Baily (1973) investigated the effects of benzene on salmon and striped bass. Oxygen consumption of salmon increased up to 48 hrs exposure, then decreased. Striped bass showed greater consumption after 24 hrs, but no difference from control fish up to 96 hrs later. Struhsaker et al., (1974) found increased oxygen consumption of larval herring exposed to benzene. Anderson et al. (1974c) found oxygen consumption of sheephead minnows varied depending upon both type of oil and dilution of the water-soluble fraction they were exposed to. Thomas and Rice (1975) estimated oxygen consumption of salmon fry from operculum movements. These initially increased when exposed to water-soluble fractions of Prudhoe Bay crude oil, but gradually declined to normal after 12 hrs.

This variation in results is most likely due to a variety of factors including differing experimental designs, different test species, variability of test oils, and

different dosing protocols. However, monitoring respiration itself may not be a reliable indicator of hydrocarbon-induced physiological stress. Both phenanthrene and creosote produced extreme effects upon growth and reproduction (Chapter 1), yet only the creosote LC30 WSF demonstrated significant differences from the other test groups and controls in this study on oxygen consumption.

There seems to be little evidence that the concentrations of the water-soluble fractions tested act either as respiratory inhibitors or as uncouplers of oxidative phosphorylation. A typical respiratory poison such as rotenone would depress oxygen consumption and show decreased heartbeat rate. Conversely if these compounds were acting as oxidative phosphorylation uncoupling agents such as dinitrophenols or pentachlorophenol greatly elevated oxygen consumption probably would be noted. Fukami (1976) points out that organophosphorous compounds and DDT show increased oxygen consumption in insects, and that other data suggest their primary action is on the nervous system. These data, especially for the creo-3 organisms, do not suggest rapid oxygen consumption with subsequent hypermetabolic activity as a primary action. However, one cannot rule out that exposure to higher concentrations may induce this in Daphnia. It must be noted that the creo-3 concentration contained more polynuclear aromatic hydrocarbons than any other WSF

tested. Although these PAH's most certainly were present in very low concentrations, the effect upon oxygen consumption in Daphnia may well be indicative of an additive interaction among these compounds.

Putting these results into perspective is difficult because data detailing toxicant effects upon physiological parameters in freshwater invertebrates is lacking. Richman (1958) determined the oxygen consumption of Daphnia pulex (1.4 mm in length) to be 0.10-0.12 µl O_2/Daphnia/hr. However, Buikema (1972) found significantly higher oxygen consumption values than Richman (1958) and attributed this to Richman's animals being partially acclimated to the dark. Correcting the mean pooled control values in this study yields 0.10/µl O_2/Daphnia/hr. This is similar to values obtained by Richman (1958) and may also reflect partial acclimation.

Summary

The hypothesis that water-soluble fractions of naphthalene, phenanthrene, No. 2 fuel oil, and creosote did not affect the respiration of Daphnia pulex could not be rejected. The creo-3 exposed organisms had the highest oxygen consumption of all groups tested. Both naphthalene test groups exhibited the lowest oxygen consumption. Phenanthrene and No. 2 fuel oil did not significantly affect Daphnia respiration. Zooplankton respiration was not

significantly different from controls after exposure to the water-solution fractions used in this study, and these data were just as variable as data obtained from marine and estuarine organisms.

Chapter 3. The effects of water-soluble fractions of hydro-
carbons on filtering rate of <u>Daphnia</u> <u>pulex</u>.

Introduction

Filtering rates of zooplankton have interested biolo-
gists for many years. In addition, determining rates of
phytoplankton consumption has necessitated more precise
measurement and better understanding about zooplankton
feeding behavior, especially in view of the role of zooplank-
ton in energy flow and nutrient cycling in typical eco-
systems (Rigler, 1971).

Filtering rates have been shown to be dependent upon a
number of factors, including food concentration (Rigler,
1961; McMahon and Rigler, 1963, 1965; Burns and Rigler,
1967; McMahon, 1968; O'Brien and DeNoyelles, 1974), food
type and particle size (McMahon and Rigler, 1965; Geller,
1975; Kersting and van der Leeuw-Leegwater, 1976; Gophen,
1977), temperature (McMahon, 1965; Burns and Rigler, 1967;
Schindler, 1968; Burns, 1969; Kibby, 1971; Geller, 1975;
Chotiyaputta and Hirayama, 1978; McGinniss, 1978; Armitage
and Lei, 1979), oxygen concentration (Kring and O'Brien,
1976; Heisey and Porter, 1977), animal length (Ryther, 1954;
Richman, 1958; Burns, 1969; Burns and Rigler, 1967; Chisholm
<u>et</u> <u>al</u>., 1975), and light and light acclimation (Buikema,
1973a).

Few studies have utilized filtering rates of organisms

as a toxicological monitor of stress. Cooley (1977) examined

the effects on filtering rate of Daphnia retrocurva to raw

pulp mill effluent. Filtration rates decreased as effluent

concentration increased. A large majority of animals re-

covered when removed. However, a proportion of animals which

spent increased time in pulp mill effluent did not filter when

presented food.

Respiration and filtering rates are closely related

not only with each other, but also with nutrition and growth

rates in zooplankton. The lifetime chronic study indicated

dramatic effects of the hydrocarbon water-soluble fractions

on growth of Daphnia pulex. Any alteration of filtering

rates could result in significant differences in the amount

of algae available to the organism for metabolism. To deter-

mine if this was occurring, the following study was designed

to test the hypothesis that exposure to sublethal concentra-

tions of water-soluble fractions of naphthalene, phenanthrene,

No. 2 fuel oil, and creosote does not affect filtering rates

of Daphnia pulex.

Materials and Methods

Approximately 60 young adult D. pulex were obtained

from stock cultures and transferred to glass globe aquaria

containing 300 ml of test water-soluble fraction. Test solu-

tions were prepared as previously described (Chapter 1).

Aquaria were placed in a Sherer Cel 4-4 environmental growth

chamber under a 16L:8D photoperiod at a temperature of 20 ± 1.5 C. Organisms were fed Chlamydomonas reinhardi (wild type, - strain) ad libitum daily. Organisms were acclimated to LC20 and LC30 test concentrations for at least three molt cycles (8-9 days). This acclimation period was chosen on the basis of several factors. The data of Green (1957) indicated that two molt cycles were necessary for the reversal of light effects on carotenoid contents for D. magna. Buikema (1973b) speculated that two molts may be the minimal period of time for the physiological effects of light to occur. Also it was noted in Chapter 1 that the effects of feeding some algal batches to control Daphnia resulted in smaller broods being produced. This effect was reversed within two molt cycles by feeding algal cells from newly started cultures (see also Buikema et al., 1979). Thus it was felt that this exposure period was of sufficient duration to allow any potential effects of the WSF's upon Daphnia pulex to be realized.

For all tests, dissolved oxygen varied from 7.0-8.5 mg/l, alkalinity 42-46 mg/l ($CaCO_3$), hardness 43-48 mg/l, and pH 6.8-7.5. Water chemistry (Standard Methods, 1975) was performed once in the middle of each exposure period for each test. All water utilized in the study was aerated Blacksburg carbon-dechlorinated tap water millipore-filtered (0.45 μ) before use. All water was maintained at 20 C.

Animals were not fed for 12 hrs preceding the start of each filtering rate experiment. Filtering rates of Daphnia pulex subsequently were determined over a 24 hr period. Glass stoppered 60 ml pyrex bottles were used as test containers. Animals were collected, placed in a small beaker of water to rinse off any test solution, and sized (1.2-1.4 mm) using an ocular micrometer, and then sized animals were placed in another beaker of clean water. Five Daphnia were then transferred via a big-bore pipet to each bottle. A known amount of log-phase Chlamydomonas reinhardi were added to each experimental and control bottle at a concentration of 30,000 cells per ml. An Electrozone electronic particle counter was used to verify algal cell concentrations. Bottles were stoppered, checked for air bubbles and placed on their sides in the environmental chamber. All experiments were conducted in the dark to minimize algal growth. Bottles were gently rotated at least twice during each experiment to reduce any sedimentation of algae. Generally each test consisted of three bottles of each WSF concentration plus two control bottles (algae only, and control animals plus algae).

After 24 hrs two drops of 40% formalin was added to kill both algal cells and Daphnia. Algal cell counts were made using an Electrozone electronic particle counter (Model 112, Particle Data, Inc.) interfaced with a PDP-11 minicomputer. All counting was done using the 95 μ orifice, 500 μl

volumetric section, with current set at 1/4 and gain setting at 68, and Isoton, a saline electrolyte (Scientific Products). At least three counts were performed on each sample. Three samples were analyzed for each test concentration.

The filtering rate was computed from the difference in counts between the experimental and control bottle (algae only) using the equation:

$$F.R. = V \frac{\log Co - \log Ct}{0.4343 \quad t}$$

where V is volume of water per animal (ml), Co is the control cell concentration (algae only), Ct is the experimental cell concentration and t is time (Buikema, 1973a). Results were computed using SAS procedures (Barr et al., 1976). Test means were computed for each test concentration and differences between treatments determined using General Linear Model Procedures (see Appendix Table 2) and Duncan's Multiple Range Test. Statements of significance refer to $P \leq .05$ level.

Results and Discussion

All data were pooled and the GLM and Duncan's procedure performed to compare all treatments simultaneously. These data are shown in Table 6. Pooled mean filtering rates demonstrated highly significant differences ($P < .0001$). Creo-2 animals had the highest filtering rate of all compounds. The Duncan procedure also indicated the creo-2 mean filtering

Table 6.   Combined filtering rate data for Daphnia pulex
           after exposure to water-soluble fractions of
           petroleum hydrocarbons.

Compound	N	ml/Daphnia/day (mean ± S.D.)	Duncan Ranking*	GLM**
Creo-2	27	13.6799 (1.9843)	A	P>F
Oil-2	27	10.5247 (3.1020)	B	0.0001
Creo-3	27	9.9997 (3.6222)	B	
Oil-3	27	9.5800 (2.7753)	B	
Control	18	5.4855 (4.4487)	C	
Phe-3	27	5.3210 (3.6151)	C	
Nap-2	27	4.6753 (4.5388)	C	
Nap-3	27	4.1359 (3.9790)	C	
Phe-2	27	1.4719 (2.6463)	D	

*Mean with same letter are not significantly different
  (α = 0.05)

**Indicates probability of significant differences existing
  among test means.

rate was significantly different from all other test means. Oil-2, creo-3, and oil-3 test groups showed the next highest mean filtering rates. No significant differences among these test means were noted by the Duncan's procedure. Nap-2, Nap-3, controls, and phe-3 test means were not statistically different. However, both naphthalene test groups demonstrated lower filtering rates than controls. Only the phe-2 exposed animals had significantly lower filtering rates. These data, as well as data generated by the chronic study and the oxygen consumption study support the fact that Daphnia exposed to sublethal concentrations of naphthalene undergo a reduction in metabolism. The two concentrations which exhibited the highest (creo-2) and lowest (phe-2) filtering rates were not those which most affected growth and reproduction in the chronic study!

The nap-2, creo-2, and oil-2 filtering rates were all higher than their respective LC30 test concentrations. The only exception was the phe-3 test group which also was significantly higher than its respective LC20 concentration. This seems to indicate that there may be a threshold existing for these compounds. At lower levels of exposure, the organism demonstrates the greater physiological response. Percy and Mullin (1975) noted that at lower levels of oil-water dispersions, the respiratory response of a marine amphipod decreased. At higher levels this depression was

reversed.

Zero mean filtering rates were recorded in two instances; one was a treatment (phe-2) and one was a control. Cooley (1977) noticed a similar situation in Daphnia exposed to pulp-mill effluents. He concluded after testing individual Daphnia from a control station that rough handling may have resulted in the zero filtration rates exhibited by some of his organisms. This may be a plausible explanation for the one instance of zero control filtration rate. A necessary part of experimental procedure was to gently rotate test bottles to prevent algae from settling and being unavailable for filtration. The phe-2 zero filtering rate most likely can be attributed to treatment. Standard deviations of some test means were high, yet for two out of three tests the phe-2 exposed organisms had extremely low or no filtering rate over 24 hrs (see Appendix Table 2).

The data on filtering rates in marine organisms exposed to petroleum are limited. Lund (1957) looked at the effects of crude oil on pumping and filtration rates in oysters. Of these two parameters, filtration rate appeared to be the more sensitive. Anderson (1972) found oil-water emulsions of bunker fuel increased filtration rates of soft-shelled clams. However, when clams were exposed to WSF's of the same concentration of bunker fuel, filtration rates were unaffected. Johnson (1977) pointed out that relating the

importance of these biological disruptions to the viability
of affected organisms is difficult. Usually one one para-
meter is monitored. However, in bivalves, ventilation rates
are also involved in respiration, excretion, reproduction,
and feeding. What may be indicated are investigations
monitoring several of the parameters simultaneously. Moni-
toring the filtering rates of suitable organisms such as
Daphnia may fulfill this need, and generate more useful
data in attempting to deduce particular physiological and/or
toxicological responses.

Buikema (1973a) obtained a value of 5.96 ml/Daphnia per
day at a density of 1 animal per 10 ml. Mean control
filtering rates for all experiments in this study was 5.48
ml/Daphnia per day at a density of approximately 1 animal
per 12 ml. Both filtration rate values were obtained using
unacclimated Daphnia pulex approximately the same size (1.36
mm versus 1.2-1.4 mm used in this study) and fed the same
alga. The close agreement between these values indicates
that the test mean filtering rates obtained in this study are
reasonable. Because these data were determined with an
electronic particle counter, the values Co and C_t were
estimated with accuracy equal to or better than any other
technique currently in use (Rigler, 1971). Examining effects
of compounds upon filtering rates appears to be an excellent
physiological parameter to monitor stress on the intact

organism.

Feeding and filtering rates are not synonymous. Filtering rate has the dimensions of volume per unit time. This does not assume that all particles of any given type are removed from the water. More importantly, it does not imply all particles caught by the filter are consumed. These data indicate whether the organism has the nutritional source available to it for growth, reproduction, and survival.

What the data cannot indicate is whether the animal has the metabolic capacity to assimilate this biochemically important nutritional source. The availability of good sublethal chronic data can complement the results of monitoring physiologically complex functions such as filtering rate.

For example, the filtering rate data indicated that creo-3 exposed animals have a relatively high filtering rate. Yet the chronic data indicated that in spite of this high level of available food source and apparent consumption, the available energy is obviously not being utilized for growth and reproduction. This seems to indicate a metabolic short-circuit within Daphnia. This may be indicative of a two-level effect, with food not being extracted completely (digestive malfunctions) and vital biochemical components needed for growth and reproduction not being produced or not being properly distributed (biochemical malfunctions).

Summary

The hypothesis that sublethal exposure to WSF's of petroleum hydrocarbons have no effect upon _Daphnia_ filtering rate was rejected. The effects of water-soluble fractions of naphthalene, phenanthrene, No. 2 fuel oil, and creosote upon filtering rates of _Daphnia pulex_ was highly significant. Creo-2 and phe-2 exposed animals demonstrated the highest and lowest mean filtering rate, respectively. Both naphthalene concentrations exhibited low filtering rates which further indicated organisms exposed to naphthalene undergo a reduction in metabolism. The nap-2, creo-2, and oil-2 test populations showed higher filtering rates than their respective LC30 concentrations, strongly indicating some type of threshold response. The two compounds with the highest levels of PAH's (creosote and phenanthrene) again showed the most significant effects upon _Daphnia pulex_. These data suggest that monitoring filtering rates, which are intimately interwoven with behavior, respiration, and nutrition in zooplankton, are accurate indicators of physiological stress on the intact organism.

Chapter 4. Development of the 7-day growth test to assess reproductive effects of xenobiotics upon <u>Daphnia</u> <u>pulex</u>.

Introduction

<u>Daphnia</u> toxicity tests have been used for a variety of purposes, including generating lethality data for both pure compounds and complex mixtures, as well as estimating allowable toxicant concentrations (Winner and Farrell, 1976). In cases where <u>Daphnia</u> were the most sensitive organisms tested, these data have been utilized in setting water quality criteria (National Academy Science, 1973).

There are basically four types of <u>Daphnia</u> tests. Each has certain advantages depending upon the experimental rationale of the investigator. However, each has inherent difficulties which may tend to invalidate the data generated from them. These <u>Daphnia</u> tests include the following:

(a) Acute lethality tests usually are conducted under static conditions, using either neonates or adults, and with no feeding of test organisms immediately before and during the test (Standard Methods, 1975; EPA, 1975). Because the toxicant concentrations selected for long-term chronic studies are based upon acute test data, it is crucial that these data are properly gathered and utilized. A serious source of error with acute tests is

that starvation interactions tend to be ignored.
Data from Richman (1958) and Buikema (1973b)
indicate that calories expended by preadult daphnids
through oxygen consumption was 50% to 100% the
caloric value of the animal. Richman showed that
adult daphnids lost 57% of their weight in four
days, while Lemcke and Lampert (1975) found most
of this weight loss was due to utilization of
carbohydrates and fats. Similar, and perhaps
more dramatic, responses to starvation would be
expected for unfed neonate daphnids. Data of
Biesinger and Christensen (1972) indicate that
food availability affects the sensitivity of
Daphnia to toxicants. Winner et al. (1977) found
that Daphnia magna maintained on vitamin-enriched
algae were less sensitive to chronic copper stress
than animals fed a trout-granule diet. The re-
liability of using unfed first instar daphnids
in acute toxicity tests needs to be examined
further before we can accept the validity of the
data being produced.

(b)   21-28 day subchronic studies (Biesinger
and Christensen, 1972). Neonates (12 $\pm$ 12 hr)
were monitored over 21 days with the resulting
young animals removed and test solutions replaced

weekly. A potential problem is that reproductive
data are only generated for approximately 14 days
which may or may not be enough to indicate true
significant differences (if they exist) between
test and control animals. This problem varies
depending upon the compounds tested. Serious
problems can arise when young are not removed daily.
The young can compete with the female for available
food. This increased competition, and overcrowding
which may depress filtering rates (Buikema, 1973a)
both result in less food for the female. Subse-
quently less live young will be produced in the
next brood with potentially greater (and unwanted)
variability in the overall results. Further, the
test design does not include the collection of
potentially valuable information such as the onset
of reproductive maturation, changes in instar
duration, molting inhibition, individual growth
inhibition, total reproduction (which includes
dead and live young as well as number of nonviable
eggs), abortion rates, and the indirect effects of
shortened female life-span upon reproduction. Both
quantity and quality of food must be rigorously
controlled, or wide fluctuations in production of
young not due to toxicant stress may be noted.

(c) Lifetime chronic studies as conducted by Buikema
(1973b), Winner and Farrell (1976), Schober and
Lampert (1977), and the previous chronic study.
Generally neonates (12 $\pm$ 12 hr) were placed within
individual test containers, fed a known quantity of
algae (Chlamydomonas reinhardi of Scenedesmus
acutus), with test solutions renewed daily or every
other day (static with renewal). Animals were
examined daily, and young (both live and dead),
nonviable eggs, length (growth), molts, etc. were
recorded until all experimental animals were dead.
While the quantity and quality of data generated
from these studies are extremely valuable, they
are costly to complete in terms of manpower and
research support. Depending upon the particular
compound being tested, the lifetime chronic may not
generate any more useful information (in terms of
reproduction) than comparably performed 21 or 28
day chronics. Schober and Lampert (1977) indicated
that very little additional data were obtained
after 28 days for Daphnia pulex exposed to the
herbicide atrazin. Winner and Farrell (1976)
noted from looking at the effects of copper on
mean brood sizes of Daphnia pulex that no further
information would have been gained after the third

brood. For Daphnia magna, no further information was gained from long-term chronic exposure. However, Buikema (1973b) found that short-term chronic studies on reproduction would have indicated that 14 ft. candles was inhibiting reproduction, but lifetime studies showed that 14 ft. candles resulted in the most young per animal of all the light conditions tested.

(d) Multigeneration chronic studies as conducted by Macek et al. (1976a, b, c). Neonates (<24 hr) were exposed to continuous flow conditions. Survival and reproduction were recorded each week for 21 days. At the 21st day the original test organisms were discarded, and neonates were selected randomly from each concentration to begin the second generation exposure another 21 days. This procedure was followed for three generations. While this approach is unique and potentially useful, many of the problems associated with typical 21 day chronics are applicable. The reproductive data generated from this technique are quite variable from generation to generation and difficult to analyze and interpret due to either the presence of male daphnids produced, diluter malfunctions, or poor survival of young.

The following questions seemed appropriate given the preceding information. Is there a way to predict stress on Daphnia without resorting to time consuming and costly chronic studies? Is there a viable "minichronic" which, if one can accept a certain amount of error, will give basically the same information? Is there a way to eliminate looking at actual reproduction (and potential starvation-nutrition interactions) and use some other biological parameter to estimate reproduction? The following study was designed to test the hypothesis that body length of a 7 day pre-reproductive Daphnia could reliably predict subchronic and chronic effects on zooplankton reproduction.

Materials and Methods

In developing short-term predictive tests for assessing the impact of toxicants one major question needs to be addressed - that is, are subchronic tests (21-28 day) accurate predictors of lifetime effects? To answer this question, the data were analyzed from three diverse chronic studies on Daphnia pulex: Richman (1958) on food stress, Buikema (1973b) on the effects of light on reproduction, and this study on effects of hydrocarbon WSF's. These particular studies were chosen because they were rigorously controlled with well-defined feeding, which minimized fluctuations in young per brood; as well as the diversity in the nature of the stress tested - food, light, toxicant. For the data on

food stress (Richman, 1958) and light stress (Buikema, 1973b) the condition producing the greatest number of young was designated as that studies' respective control. Data obtained from these studies were punched on computer cards. Linear regressions were calculated and computer plots produced using SAS 76 procedures (Barr et al., 1976).

Results and Discussion

Data was first analyzed as percent change in young production after 21 days to the percent change in young produced for the entire lifetime of the organism. This plot is shown in Figure 1. The equation y = 21.39 + 0.60x was significant at the P>0.0002 level with an R-square of 0.39 and a Pearson correlation coefficient of 0.70. The results indicate that subchronic reproductive tests are reasonable indicators of lifetime effects. These results support the conclusions of Winner and Farrell (1976) and Schober and Lampert (1977) that little new data are obtained from long-term tests.

However, there are times when 21-day test results are not valid. In the chronic WSF study, 4 out of 9 twenty-one day test results were not good predicators of lifetime effects. It appears from the limited data base available that the effects of heavy metals (Winner and Farrell, 1976) and herbicides (Schober and Lampert, 1977) may be more predictable than other types of stress (hydrocarbon toxicity,

Fig. 1. Linear regression of % change actual young at 21

days and the % change actual young for lifetime

of <u>Daphnia</u> <u>pulex</u> using three different chronic

data sets.
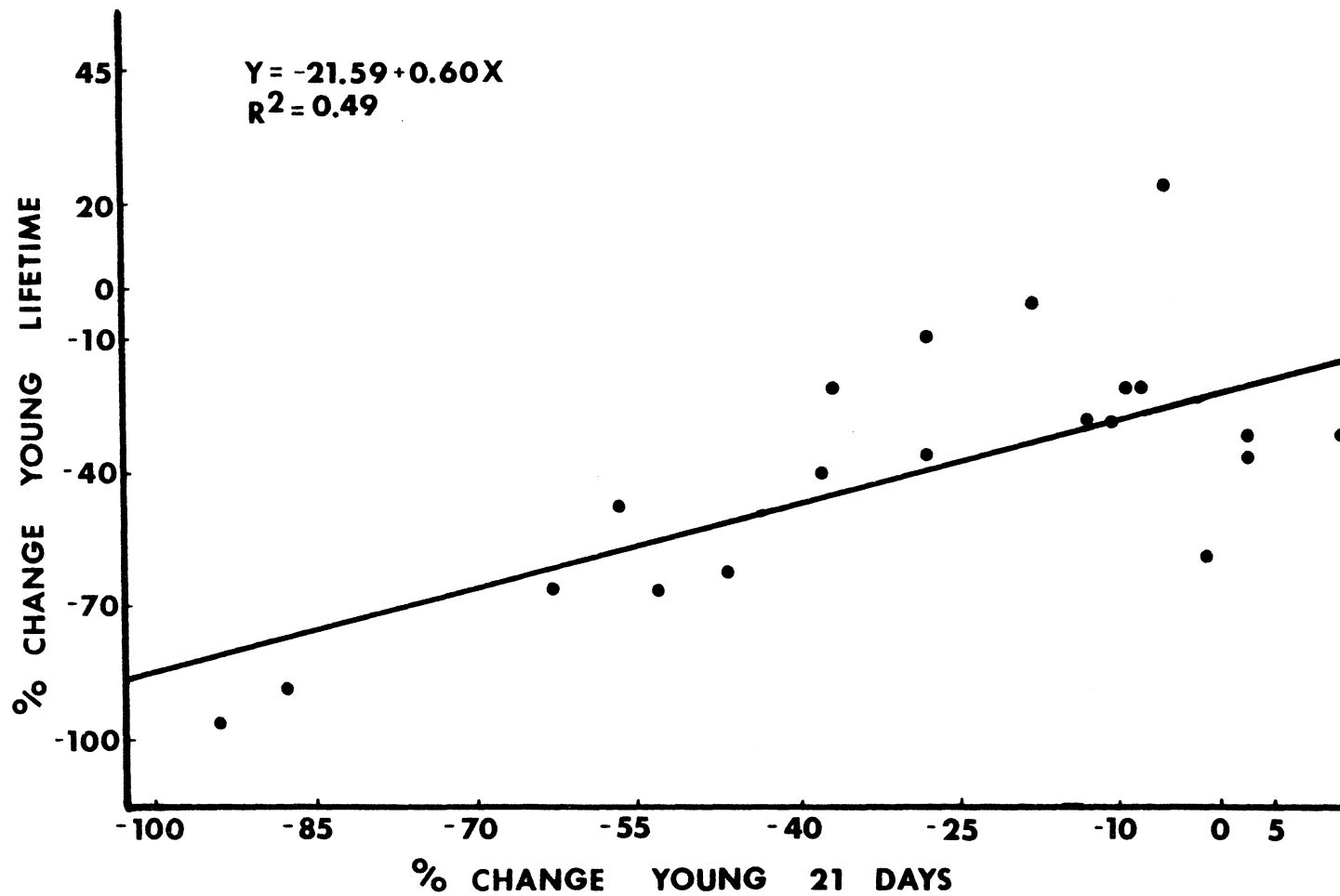
food stress, and light stress).

In developing a more powerful predictor of stress, two experimental approaches seem to be indicated: a) either monitor reproduction (rigorously controlling nutrition) for the life of the animal or b) look for other parameters that may give you better predictive capabilities. Because large Daphnia have more offspring than small Daphnia (Green, 1954), then the larger they are at reproductive maturation, the more young they may produce. In the preceding chronic study, the parameter body length was the most significant variable brood for brood than any other parameter monitored.

The hypothesis that body length at day 7 (approximately the time for reproductive maturation of Daphnia pulex at 20 C) could be a possible indicator of young produced at 21 days by a typical short-term chronic was investigated. From the above three mentioned data sets, the percent change of young produced relative to the respective controls was calculated and compared to the percent change of growth (relative to controls) at day 7. This regression is shown in Figure 2. The equation y = 13.512 + 1.729x was significant at the P>0.0001 level with an R-square of 0.68 and a Pearson correlation factor of 0.83. This supports the hypothesis that length (growth) at reproductive maturation can be a good predictor of the number of live young produced at day 21.

Fig. 2. Linear regression of % change of growth at day 7 to the % change of young at day 21 using three different chronic data sets.

$$Y = -13.51 + 1.73 X$$
$$R^2 = 0.68$$

% CHANGE YOUNG 21 DAYS
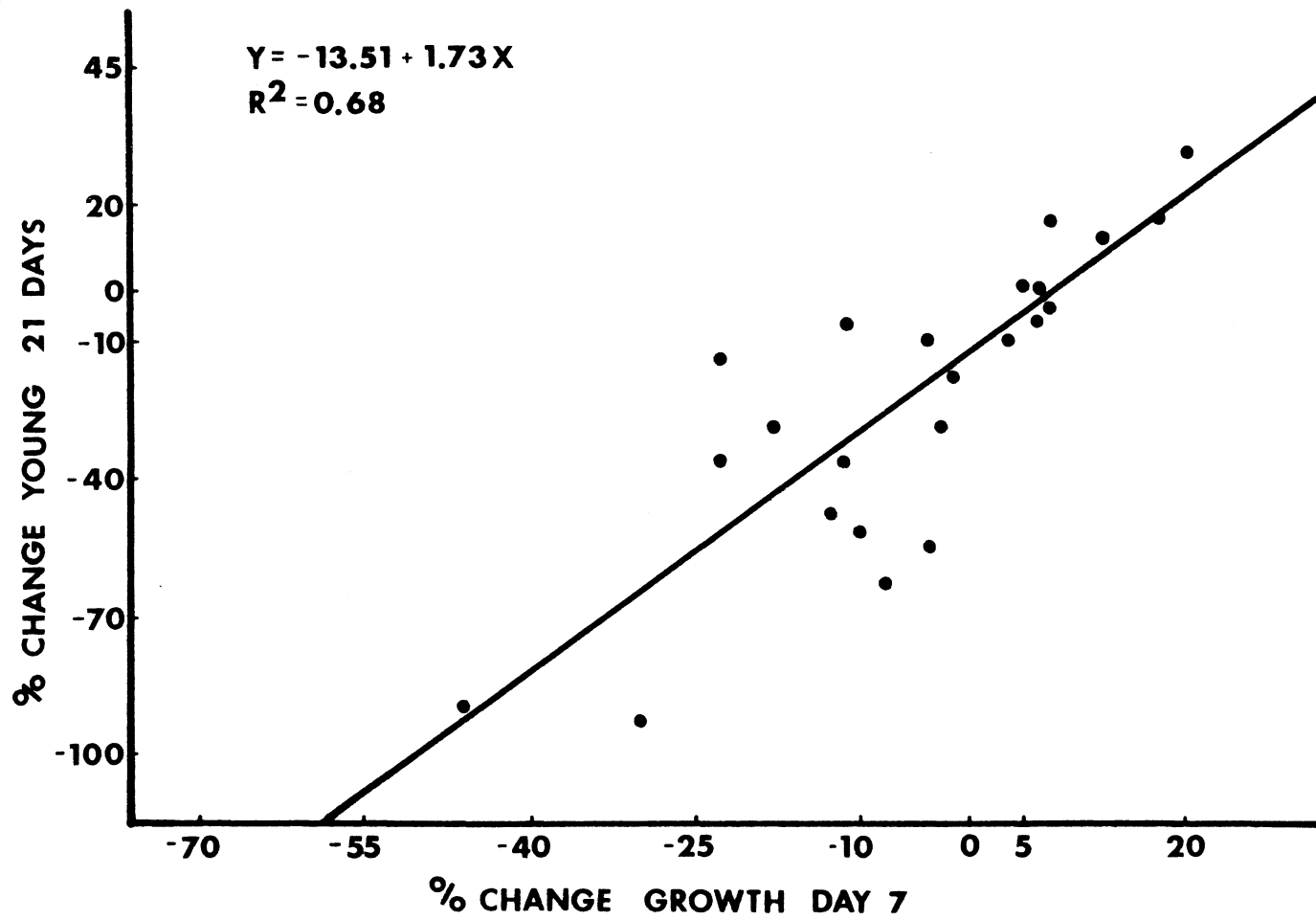
% CHANGE GROWTH DAY 7

The percent change growth at day 7 was next compared to this actual lifetime percent change in young produced. Figure 3 shows this regression. The equation y = 31.28 + 0.73x was significant at P = 0.054, with an R-square of 0.17 and a Pearson correlation factor of 0.41. This poor correlation was anticipated and does not invalidate the hypothesis because of the time interval between the predictive measurement (7 days) and the end of the lifetime chronic (up to 125 days). As the time interval increases between the endpoint measurements variability will increase because of stress specific effects on molting frequency, intermolt length, and age-senility interactions upon reproductive rates. This effect of increasing time interval affecting significance is apparent in the above analyses. For example, the correlation coefficient between 7 day length and 21 day reproduction (14 day interval) is 0.83; between 21 day and lifetime reproduction (40+ days) is 0.70; and between 7 day length and lifetime reproduction (54+ days) is 0.41.

Summary

The major conclusion from analyzing the above data is that body length (growth) of a 7 day pre-reproductive Daphnia can reliably predict subchronic and chronic effects on zooplankton production. A 7 day growth test would be less expensive in time, space, and manpower. It requires a well-

Fig. 3. Linear regression of % change growth at day 7
         and % change young for lifetime of <u>Daphnia</u> <u>pulex</u>
         using three different chronic data sets.

$$Y = -31.28 + 0.73X$$
$$R^2 = 0.17$$

% CHANGE YOUNG LIFETIME

% CHANGE GROWTH DAY 7
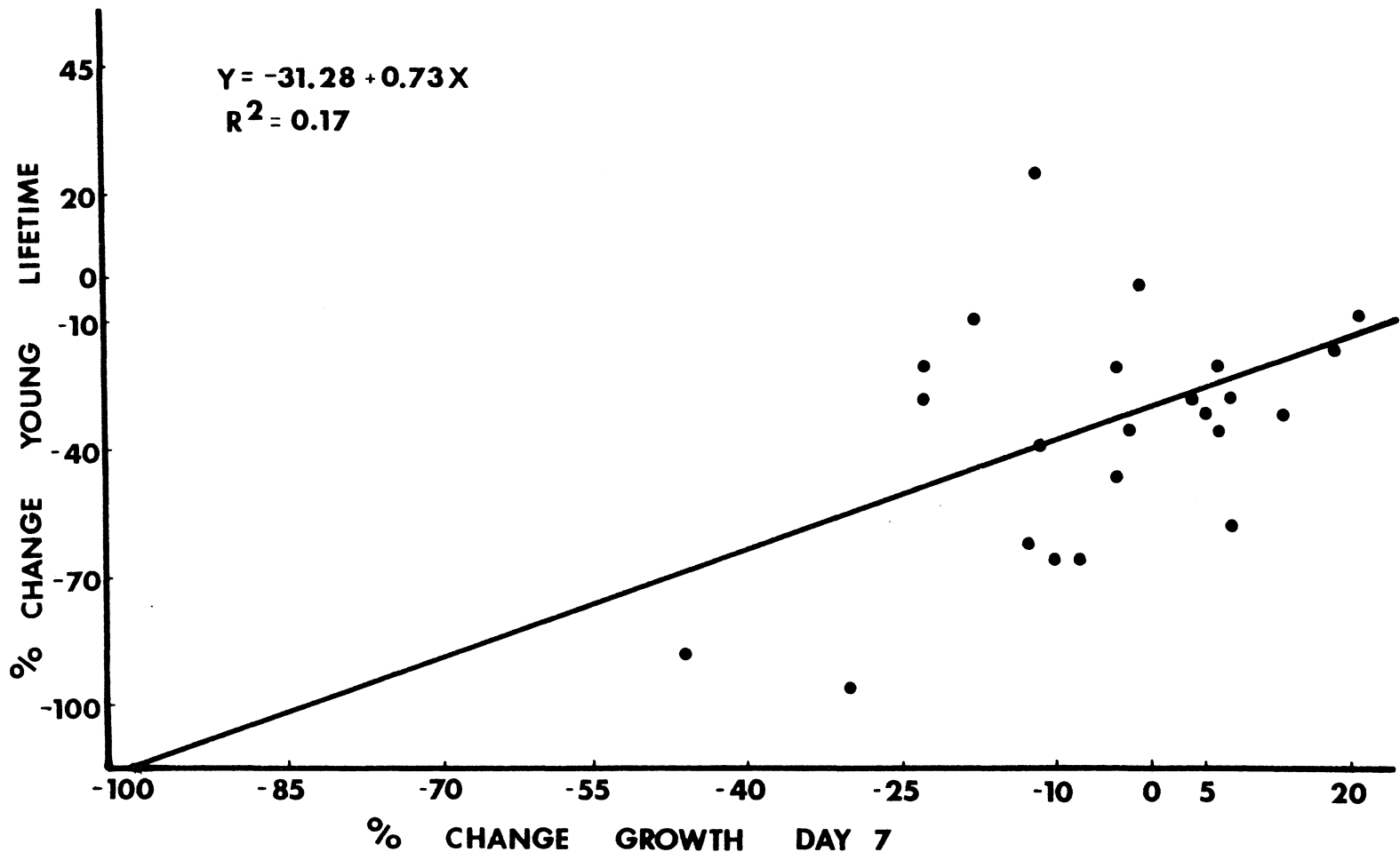
controlled diet (which may include trout-chow, etc.) with daily feeding. It requires the use of early instar _Daphnia_. It requires that growth be measured at day 7. It does not require monitoring of young production and it eliminates the inherent difficulties with starvation, nutrition, and competition for food that may explain the wide data varibility in reproductive impairment tests.

Chapter 5. Biological effects of water-soluble fractions
upon Daphnia pulex.

Discussion

The previous data have documented the effects of water-
soluble fractions of two pure compounds, napthalene and
phenanthrene, as well as two common refined petroleum pro-
ducts, No. 2 fuel oil and coal-tar creosote upon the survival,
growth, reproduction, oxygen consumption, and filtering rate
of Daphnia pulex. Caution must be exercised considering
the implications of these data, especially the effects of
the two refined WSF's in determining possible mechanisms of
action of these compounds. Both the creosote and No. 2
fuel oil WSF's are represented by possibly as many as
forty compounds of various solubilities and molecular
weights as shown by G.C. analysis (Appendix Figure A, B).
These diverse compounds may be acting separately or in unison
to elicit possibly innumerable synergistic, additive and/or
antagonistic toxicological responses. Selected data from
all experiments have been summarized in Table 7. This
discussion will attempt to synthesize the many effects found
in this study and to determine possible mechanisms of action
of these hydrocarbons upon D. pulex.

The creosote LC30 WSF had one of the greatest impacts
on the growth and reproduction of D. pulex. Reproductive
maturation was significantly delayed. When organisms did

Table 7. Selected data from all experiments showing biological effects of sublethal exposure of Daphnia pulex to water-soluble fractions of petroleum hydrocarbons.

Compounds	Live young/ animal	Dead young w/o spine/ animal	Total abortions	Broods/ animal	Reprod. maturation (days)	Survival (days)	Oxygen consumption ($\mu 1O_2$/Daphnia/day)	Filtering rate (ml/Daphnia/day)
Control	253	25.90	26	16.4	6-7	57.3	2.415	5.485
Oil-2	252	53.14	23	20.0	6-7	69.3	2.058	10.524
Oil-3	199	39.66	24	16.3	7-8	56.1	2.272	9.580
Creo-2	133	93.00	84	20.8	7	72.5	1.915	13.679
Creo-3	7	38.20	69	9.6	9-10	42.8	2.872	9.999
Phe-2	221	12.90	11	19.1	7	67.3	1.986	1.471
Phe-3	45	2.22	5	12.0	11-12	51.6	1.886	5.321
Nap-2	314	28.00	18	25.8	7	90.1	1.686	4.675
Nap-3	244	9.38	8	23.4	7-8	84.0	1.600	4.135

become productive, mean live young produced per animal was significantly reduced from both controls and other experimental groups. Filtering rate was higher than controls, indicating the test organisms obtained adequate nutrition. Yet, in spite of this, growth and reproduction were severely reduced. Creo-3 oxygen consumption was the highest recorded. In addition, exposure to this WSF produced a higher number of dead young without spines as well as a higher number of total abortions compared to controls. The creo-2 WSF also demonstrated significant impact upon _Daphnia_ growth and reproduction. This WSF elicited increased numbers of live young, longer survival time, and more broods per animal compared to creo-3 organisms. However, creo-2 animals produced the highest number of dead young without spines as well as the highest number of total abortions. Oxygen consumption was not significantly different from controls. Creo-2 exposed organisms had significantly higher filtering rates than any other test group, including controls.

The phe-3 WSF was the only other compound to significantly delay reproductive maturation; when organisms did become reproductive, significantly fewer live young were produced. In addition, a low number of total abortions and fewer dead young without spines were noted. Both oxygen consumption and filtering rate were not significantly different from controls. Phe-2 organisms demonstrated

increased numbers of live young, longer survival, and more broods per animal compared to the phe-3 animals. Oxygen consumption was not significantly different, but filtering rate was the lowest recorded.

Both oil WSF's did not elicit response as great as those produced by phenanthrene or creosote. Both WSF's produced greater numbers of dead young without spines and significantly higher filtering rates than controls, but total abortions, number of broods, reproductive maturation, and oxygen consumption were not significantly different.

The effect of naphthalene on D. pulex was unusual. Both test groups lived significantly longer than controls. This increased longevity allowed test groups to produce numbers of live young equal to (and for nap-2 animals greater than) control organisms. This was not expected. Berdugo et al (1977) noted exposure of an estuarine copepod to 1 mg/l naphthalene for 24 hrs resulted in lower egg production and lifespan; however, only the total number of eggs produced was significantly lower than controls. Both naphthalene test WSF's demonstrated significantly lower oxygen consumption than controls. Filtering rates, although lower than controls, were not significantly different.

The following generalizations and trends appear to be evident from these data. Compounds containing high levels of PAH's (such as phenanthrene and creosote) demonstrate

more severe effects upon growth and reproduction of a target organism such as Daphnia than aromatic compounds such as naphthalene. Only phenanthrene and creosote LC30 WSF's significantly delayed reproductive maturation. The data indicates different effects upon reproduction for these WSF's. Phe-3 seems to be inhibiting reproduction directly, with fewer eggs being formed in the ovary and released into the brood chamber. In contrast, creo-3 may be inhibiting reproduction in two ways. Not only are fewer eggs being released into the brood chamber, but also the eggs which are released are not developing normally, with subsequent premature release of incompletely developed young from the brood chamber. The effects of creo-2 also tend to reinforce this dual effect upon reproduction; the factor(s) responsible for reduced egg formation have apparently been reduced, while the factor(s) responsible for embryological interference with young development seem to have increased.

The reproductive effects of oil seem to parallel that produced by creosote. This may be indicative of the multi-compound nature of both creosote and oil WSF's with the difference in the severity of impact upon reproduction due to the amount of PAH's present (creosote WSF having higher levels of PAH's than oil). The lack of significant effects of naphthalene upon reproduction is not inconsistent with

the previous generalization. Decreased toxicity seems to be correlated with increased solubility of these compounds (see reviews by Craddock, 1977; Johnson, 1977); the available published literature indicates that naphthalene is one of the more soluble hydrocarbons.

Trends can also be noted among filtering rates. For three out of the four test compounds, LC20 WSF's demonstrate the higher filtering rate. Creosote and oil WSF's again show a somewhat parallel effect; creo-2 organisms having the highest filtering rates while oil-2 animals demonstrated the second highest filtering rates. Nap-2 animals had only slightly higher filtering rates than nap-3 organisms. The phe-2 test group had the lowest filtering rates of all test groups. These data seem to indicate that the LC20 WSF's may represent critical physiological thresholds of biological response. At these lower exposure levels, the animals are under severe physiological stress. At higher exposure levels the effects being demonstrated may not be as much physiological or behavior acclimation to stress, but may represent more severe biochemical alternations in metabolic processes at specific cellular or organelle levels. Similar responses have been documented for marine organisms. At lower oil exposures, the organisms show greater physiological responses than when exposed to higher levels of petroleum (Percy and Mullin, 1975; Rice et al., 1976).

Only two generalizations can be made from the oxygen consumption data. Creo-3 organisms demonstrated the highest oxygen consumption; this seems to indicate again that compounds with high levels of PAH's demonstrate increased biological responses. Both naphthalene test groups had lower oxygen consumption than controls; this was also consistent with the filtering rate data.

To complete this discussion, two questions must be answered: a) can some physiological/biochemical mechanism be derived which can logically explain these documented biological responses and trends and, b) what are the ecological implications of these data to typical freshwater environments?

O'Brien (1967) and Hollingworth (1976) summarize an approach for analyzing physiological selectivity. There are only a limited number of steps which can occur when a toxicant comes into contact with an organism:

a) Initial contact.

b) Penetration: For aquatic organisms this is usually accomplished via entry through the gills or by the digestive tract.

c) Metabolism: This can include detoxification and/or toxificiation reactions.

d) Storage and excretion.

e) Compound (or metabolite) - receptor system inter-

actions at site of action.

f) Consequences: Usually exhibited in a complex series of disruptions on target organisms' physiological and biochemical homeostasis.

Entry of hydrocarbons into Daphnia are most likely through the membrane surrounding the gills and by ingesting food particles with adsorbed hydrocarbons. Because hydrocarbons tend to be lipophilic and generally possess a high lipid-water partition coefficient, aquatic organisms can remove and concentrate these compounds from water. This has been shown to occur innumerable times in the published marine literature for hydrocarbons, as well as in much of the pesticide-herbicide toxicity literature dealing with lipophilic compounds such as DDT.

Once in the organism, these compounds are subject to metabolism by a wide array of enzymes. Ideally, the evolutionary strategy would be to convert foreign compounds to metabolites which are more polar and rapidly excrete them from the organism. In this biotransformation, microsomal oxidases are of great importance. Microsomal oxidases are involved in biosynthesis of phospholipids and complex polysaccharides, protein synthesis, conjugation reactions, lipid peroxidation, and steroid synthesis. Upon entry of lipophilic xenobiotics, such as aliphatic and aromatic hydrocarbons, microsomal oxidases work to increase polarity

of the compounds for immediate excretion or for further
attack by other enzyme systems to introduce hydrophilic
groups on to the molecule.

Many marine and freshwater fish and marine crustaceans
(including some species of copepods) can metabolize aliphatic
and aromatic hydrocarbons (Lee, 1975; Corner et al., 1975;
Stegeman, 1978; Payne and Penrose, 1975; Elmamlouk and
Gessner, 1976; Ahokas et al., 1975; Gerhart and Carlson,
1978; James et al., 1979; Addison et al., 1978; Sanborn
and Malins, 1977; Varanasi and Malins, 1977). Surveying
the literature, those crustaceans which exhibit molting
and/or reproduction cycles such as crabs, lobsters, and
zooplankton (copepods) have been shown to possess mixed
function oxidase activity. These enzymes are also involved
in steroid biosynthesis and/or activation (O'Hara et al.,
1978). Additional evidence has shown that molting and repro-
duction in many crustaceans are mediated by steroid hormones
(Highnam and Hill, 1969; Novales et al., 1973).

Molting and reproduction dominate the life cycle of
Daphnia, and these events are closely related (Lee, 1976).
Physiological and biochemical disruptions in these processes
can be demonstrated in many ways, as shown by the diverse
effects of these WSF's on Daphnia pulex. All these effects
can represent individual mechanisms of action of "symptoms"
of toxicant action. However, all these affected processes

in D. pulex are also under metabolic controls of one type

or another. I suspect that this may be where PAH's exert

their primary influence - all other effects demonstrated

are biochemical consequences of altered metabolic regulation.

This study indicates reproductive maturation was delayed

by two compounds - phenanthrene and creosote (with high

levels of PAH's). This implies strong interference with an

endogenous system of endocrine control of reproduction. The

strong implication of some reproductive function of estradiol

17-B (or a similar steroid) found in a marine zooplankter

(O'Hara et al., 1978) as well as evidence that certain

estrogens (including estradiol 17-B) stimulate egg laying in

several species of slugs (Takeda, 1979) lend support to the

possible existence of endocrine control in Daphnia.

Phenanthrene (or similar polynuclear aromatic hydro-

carbons) may either interfere with normal steroid metabolism

by causing alterations in or inhibit binding to specific

receptor proteins, or "mimic" endogenous steroid activity,

influencing protein synthesis and amino acid synthesis of

glucose (thus reducing glucose availability for energy pro-

duction as well as proteins available for growth) and lipid

synthesis and/or mobilization (causing reduced egg production

and decreased viability). These PAH's could also induce

mixed function oxidase activity, resulting in less endogenous

hormone which could result in metabolic malfunctions being

translated into severe growth and reproductive effects.

Recent evidence supports this hypothesis. Estradiol 17-B administered in vivo at physiological concentrations to Mytilus edulis has been implicated in inducing a reduction in lysosomal stability in digestive cells; this indicates a hormally controlled mechanism for cellular catabolism (Moore et al., 1978). PAH interference with hormone-mediated catabolism would have dramatic implications on growth as well as on reproduction. Energy normally produced from food sources would not be available for vital metabolic processes due to either incomplete digestion (catabolic malfunction) or components not being assimilated in usable form. Secondary effects from PAH interference with mixed function oxidase activity could result in a toxification reaction. Introduction of molcular oxygen by MFO's could increase cytotoxicity, and be responsible for the embryological effects shown by this study. Chronic exposure could also result in metabolic effects being translated into permanent genetic effects; such oxygenated derivatives resulting from a toxification reaction have been implicated in producing genetic damage (Varanasi and Malins, 1977).

The ecological implications of sublethal hydrocarbon exposure in freshwater systems may be far more serious than that contemplated for an oceanic or estuarine situation. Although the levels of hydrocarbons utilized in these studies

are possibly higher than would occur in a typical estuarine
or ocean spill, these levels may be more realistic in con-
sidering a major spill on ponds, lakes or river systems in
typical freshwater environments. A chronic petroleum spill
of a few days duration would have an immediately devastating
effect on the zooplankton community, most likely severely
curtailing reproduction and growth and altering both the
composition and number of species.

Even after hydrocarbon levels in the water-column have
returned to "safe" levels due to physical-chemical processes
such as volatilization and possible bacterial and fungal
degradation, the long-term effects upon zooplankton dynamics
due to initial uptake of these hydrocarbons may continue to
reduce reproduction significantly. Live zooplankton are
critical food web components; removal and/or reduction of
these forage organisms would impact on consumer organisms.

Creosote, a wood preservative, is widely used in the
aquatic environment. Its use needs to be reexamined,
especially in light of all the known and potential carcino-
gens (many of them PAH's) present. Dunn and Stich (1976)
found mussels accumulated the carcinogen benzo(a)pyrene
from pier pilings treated with creosote. The observation
of abnormal growths on Daphnia exposed to PAH's in this study
raise serious questions on food web magnifications, possible
increased incidence of bacterial and/or virus-mediated

disease induced by PAH exposure, and ultimately on the impact to human health. Research into the role of hydrocarbons on possible neoplasm formation, depression of immune responses, and increased susceptibility to bacterial and/or viral infection is justifiably increasing (see review by Hodgins et al., 1977).

The use of No. 2 fuel oil to control and eradicate predaceous aquatic insects is a widely utilized practice worldwide in aquaculture. The results in this study would question the continued use of this procedure. One of the main problems in culturing desirable species such as striped bass is maintaining a high level of zooplankton for the larval fish to feed upon; thus resulting in reduced cannabalism. By using No. 2 fuel oil in high enough levels, introduction of water-soluble petroleum hydrocarbons can actually reduce zooplankton reproduction and growth, lowering zooplankton levels to critical biomass levels, and reducing the number of fish harvested. The added spectre of possible neoplasm induction as well as increased induction of disease and infections pose a serious threat, especially for fish utilized eventually for food sources.

These results also cast shadows on converting our energy production from petroleum to coal and coal conversion products. These products not only require great quantities of water, but also generate high levels of PAH's (Herbes and

Beauchmap, 1977; Schultz et al., 1978a, 1978b). A massive committment to production of synthetic fuels from both coal and oil shale may release numerous PAH's into freshwater environments with serious consequences, unless adequate environmental precautions are enforced.

CONCLUSIONS

1) This water-soluble fractions of naphthalene, phenan-
   threne, No. 2 fuel oil, and creosote were acutely toxic
   to <u>Daphnia</u> <u>pulex</u>. The 48-hr LC50 values were 57.52,
   >>100, 34.10 and 2.91, respectively.

2) The effects of creosote and phenanthrene were most
   dramatic during lifetime sublethal exposure. They pro-
   duced a marked reduction in growth rate, number of
   broods, impairment of molting, delayed reproductive
   maturation, and increased abortion rates when compared
   to controls. No. 2 fuel oil produced similar effects,
   but results were not as consistent brood for brood.
   Naphthalene produced only a slight reduction in growth,
   but increased longevity compared to controls.

3) Oxygen consumption rate experiments of <u>Daphnia</u> exposed
   to these WSF's were variable. Creosote LC30 exposed
   organisms demonstrated the highest oxygen consumption
   while animals exposed to both naphthalene WSF's showed
   the lowest oxygen consumption of all compounds tested.
   However, no experimental mean was significantly differ-
   ent from controls.

4) Filtering rates of <u>Daphnia</u> exposed to all WSF's showed
   significant differences among the test groups, with
   animals exposed to creosote and phenanthrene demonstra-
   ting the highest and lowest filtering rates, respect-

ively. Exposure to No. 2 fuel oil WSF's elicited higher filtering rates than controls. Both naphthalene test groups had low filtering rates. Monitoring filtering rates seems to be a promising experimental technique to accurately assess physiological stress in zooplankton.

5) The 7-day growth impairment test is a viable predictor of subchronic or chronic effects on Daphnia reproduction, and would be less expensive in time, space, and manpower.

6) The mechanism of action of compounds containing high levels of polynuclear aromatic hydrocarbons is hypothesized to be upon endocrine systems - which could explain the dramatic and diverse effects these compounds demonstrated on growth and reproduction of Daphnia pulex.

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APPENDIX

Appendix Table 1. Oxygen consumption data for <u>Daphnia</u>
<u>pulex</u> after exposure to water-soluble
fractions of petroleum hydrocarbons.

Compound	N	μlO_2/<u>Daphnia</u>/day (mean $\pm$ S.D.)	Duncan Ranking*		GLM**
Control	1	4.287 ————	A		
Phe-2	3	2.615 (1.229)	A	B	P>F
Phe-3	3	2.515 (1.057)	A	B	0.174
Nap-3	3	1.757 (0.314)	A	B	
Nap-2	3	1.458 (0.986)		B	
Nap-2	3	1.986 (0.557)	A		P>F
Control	1	1.829 ————	A		0.649
Nap-3	3	1.772 (0.300)	A		
Phe-3	3	1.458 (0.915)	A		
Phe-2	3	1.186 (0.729)	A		
Control	1	2.701 ————	A		P>F
Phe-2	3	2.201 (0.486)	A		0.190
Phe-3	3	1.701 (0.700)	A		
Nap-2	3	1.615 (0.457)	A		
Nap-3	3	1.243 (0.472)	A		
Creo-3	3	3.801 (1.472)	A		P>F
Oil-3	3	3.272 (0.386)	A	B	0.015
Oil-2	3	2.200 (0.171)	C	B	
Creo-2	3	1.086 (0.400)	C		
Control	1	0.972 ————	C		
Creo-2	3	3.844 (0.314)	A		P>F
Oil-2	3	3.444 (0.657)	A	B	0.140
Control	1	3.258 ————	A	B	
Creo-3	3	3.029 (0.186)	A	B	
Oil-3	3	2.815 (0.457)		B	
Creo-3	3	1.815 (0.157)	A		P>F
Control	1	1.400 ————	A	B	0.005
Creo-2	3	0.786 (0.243)	C	B	
Oil-3	3	0.729 (0.314)	C	B	
Oil-2	3	0.514 (0.429)	C		

*Means with same letter are not significantly different
  (α = 0.05)
**Probability of significant differents existing among means.

Appendix Table 2.   Filtering rate data for <u>Daphnia</u> <u>pulex</u>
after exposure to water-soluble fractions
of petroleum hydrocarbons.   Each group
represents an individual experiment.

Compound	N	$\mu 1 O_2$/<u>Daphnia</u>/day (mean $\pm$ S.D.)	Duncan Ranking*		GLM**
Phe-3	9	4.9924 (1.5877)	A		P>F
Nap-2	9	4.7791 (1.3782)	A		0.0001
Control	3	4.1790 (0.0930)	A		
Nap-3	9	1.6431 (1.6298)	B		
Phe-2	9	-0.0516 (1.9373)	C		
Phe-2	9	2.9482 (3.2666)	A		P>F
Nap-3	9	2.4387 (3.4831)	A	B	0.0960
Phe-3	9	1.6235 (1.7335)	A	B	
Nap-2	9	0.1106 (0.8123)		B	
Control	3	-0.3331 (0.8049)		B	
Control	3	13.5708 (2.1771)	A		P>F
Phe-3	9	9.3470 (1.7923)	B		0.0001
Nap-2	9	9.1362 (4.3074)	B		
Nap-3	9	8.3260 (2.5854)	B		
Phe-2	9	1.5192 (1.8084)	C		
Oil-3	9	14.2612 (1.8355)	A		P>F
Creo-2	9	12.2686 (1.9219)	A	B	0.0001
Oil-2	9	10.6370 (4.7964)		B	
Creo-3	9	6.1166 (1.0676)		C	
Control	3	5.4169 (0.5449)		C	
Creo-2	9	14.2150 (1.0197)	A		P>F
Creo-3	9	10.1177 (2.3560)	B		0.0001
Oil-2	9	9.6928 (1.8017)	B		
Control	3	6.8213 (0.6979)	C		
Oil-3	9	6.3790 (1.1115)	C		
Creo-2	9	14.5560 (2.1534)	A		P>F
Creo-3	9	13.7647 (1.7395)	A		0.0001
Oil-2	9	11.2444 (1.9094)	B		
Oil-3	9	8.0998 (1.7535)	C		
Control	3	3.2580 (1.7189)	D		

*Mean values with the same letter are not significant
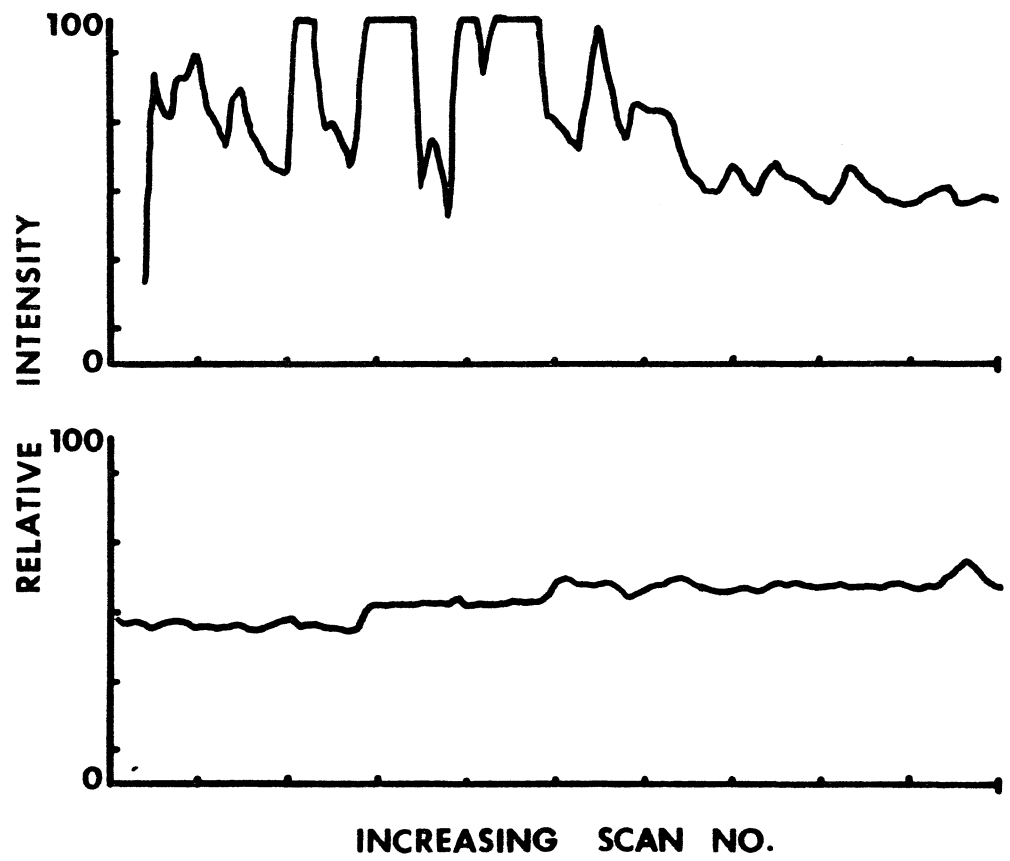 different (α = 0.05)
**Indicates probability of significant differences existing
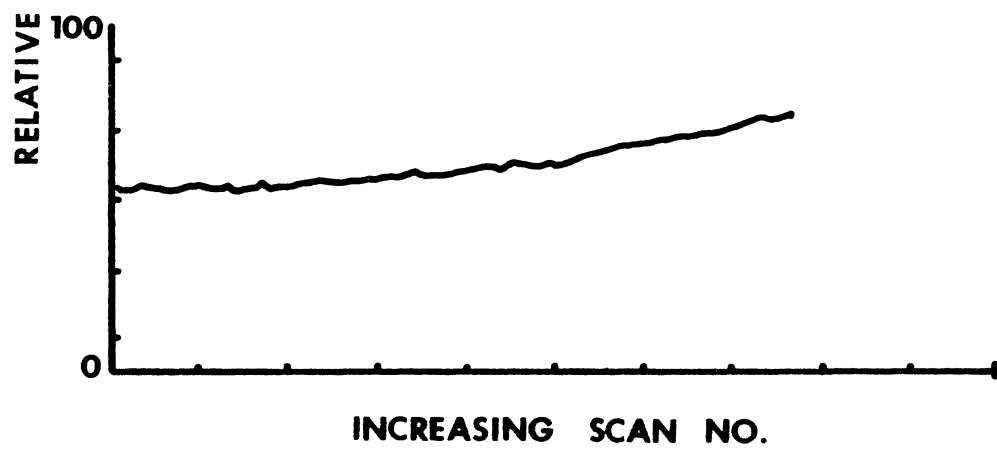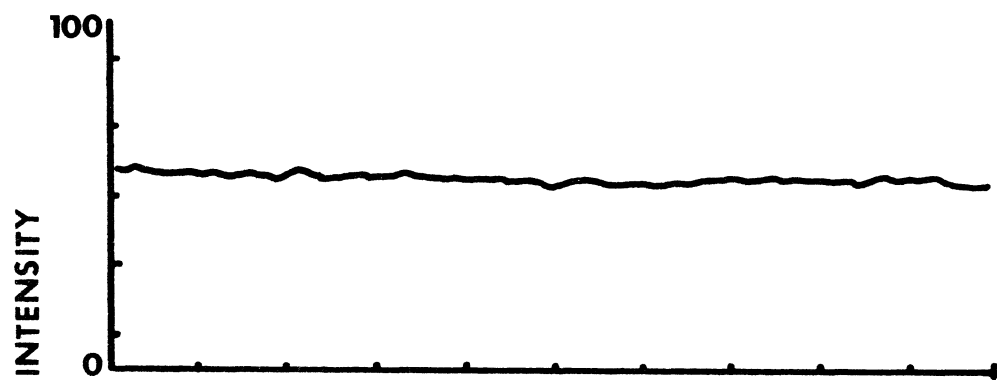 among test means.

Appendix Fig. A. Computer-reconstructed GC–MS scan of
stock No. 2 fuel oil WSF.
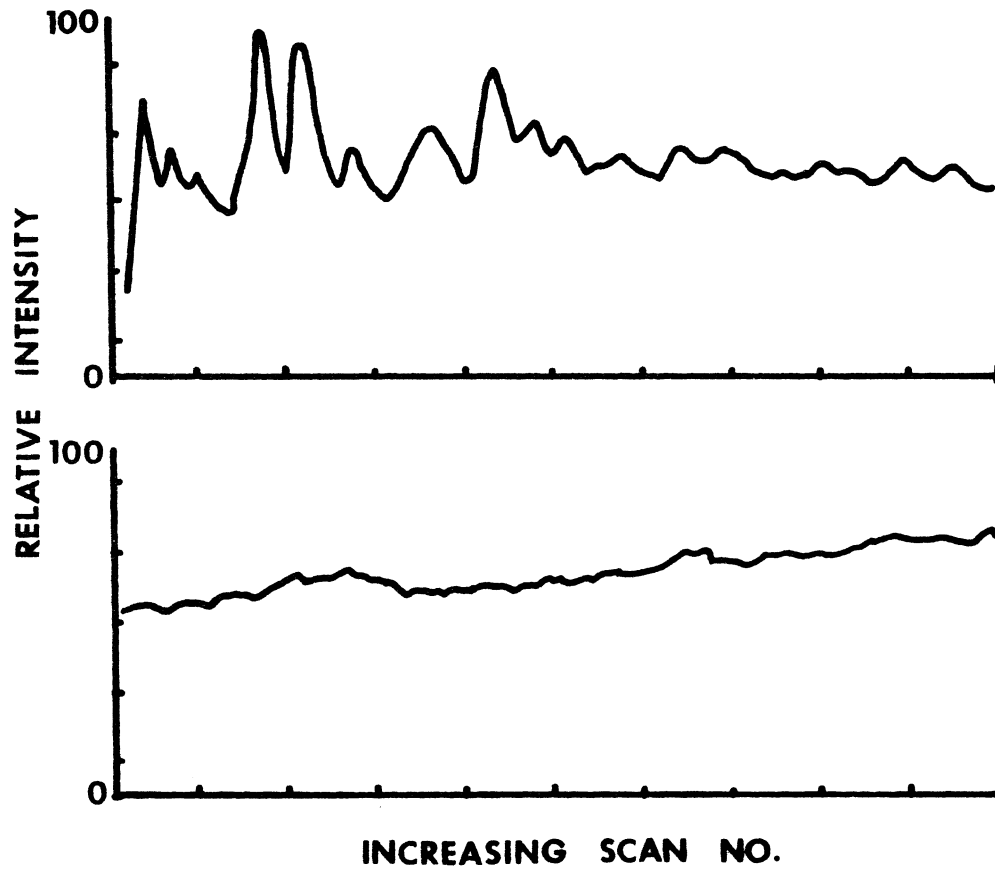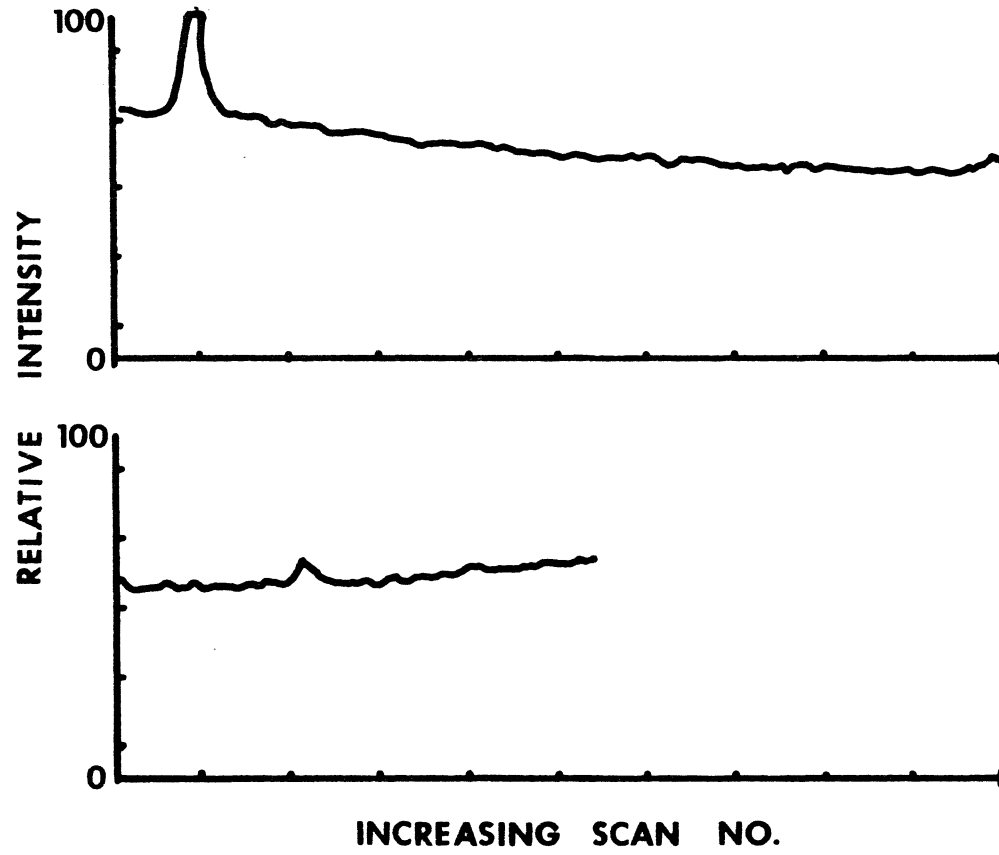
Appendix Fig. B.   Computer-reconstructed GC-MS scan of stock
creosote WSF.

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THE EFFECTS OF WATER-SOLUBLE FRACTIONS
OF NAPHTHALENE, PHENANTHRENE, NO. 2 FUEL OIL,
AND COAL-TAR CREOSOTE ON THE
FRESHWATER CLADOCERAN, DAPHNIA PULEX.

by

James Gourrier Geiger

(ABSTRACT)

The purpose of this study was to assess the effects of water-soluble fractions (WSF) of naphthalene, phananthrene, No. 2 fuel oil, and coal-tar creosote on the survival, growth, reproduction, feeding, and metabolism of Daphnia pulex.

The 48 hr LC50 values after acute exposure (as percent WSF) for creosote, No. 2 fuel oil, phenanthrene and naphthalene were 2.91, 34.10, >>100, and 57.52 percent, respectively. Gas chromatography analysis indicated naphthalene and phenanthrene 48 hr LC50 values (as mg/l) were 2.92-3.89 and 0.96-1.28, respectively. Up to 40 peaks were noted in each stock WSF of creosote and No. 2 fuel oil.

For chronic studies, young (24 hr) Daphnia were exposed to calculated LC20 and LC30 concentrations of WSF's for their entire life. The LC30 concentrations of creosote and phenanthrene showed a significant reduction in growth rate and number of live young, as well as reduced number of broods, impairment of molting, and significant delay in

reproductive maturation; instances of possible neoplasms were also noted in one organism from each of these test groups. No. 2 fuel oil produced similar effects on growth and reproduction, but results were not as significant. Increased longevity and slight reduction in growth rate were noted for both naphthalene test groups.

The effects upon oxygen consumption after exposure to test WSF's were variable. The LC30 concentration of creosote and both naphthalene concentrations were significantly different from each other; both napthalene concentrations elicited the lowest oxygen consumption rates recorded, while the creosote LC30 group exhibited the highest rate of oxygen consumption. However, no experimental means were significantly different from controls.

Highly significant differences existed between filting rates of organisms after exposure to the WSF's. The LC20 concentrations of creosote and phenanthrene produced the highest and lowest filtering rates, respectively. Both oil test groups demonstrated significantly higher filtering rates. Monitoring zooplankton filtering rates appears to be a promising parameter to evaluate physiological stress on these organisms.

This chronic study and data from other comparable chronic studies indicate that the length of a pre-adult Daphnia after 7 days of exposure to stress can be used to

predict chronic reproductive effects with the same degree of accuracy as the 21 day test. Adoption of this test would eliminate difficulties with starvation, nutrition, and competition for food which contribute to the variability in reproductive impairment tests.

A possible mechanism of action of polynuclear aromatic hydrocarbons upon endocrine systems is strongly suggested by the dramatic and diverse effects upon growth and reproduction in _Daphnia_ _pulex_.