Calif. Fish and Game 71(3): 132–140 1985

ACUTE TOXICITY OF SEVEN ALICYCLIC HEXANES TO STRIPED BASS, *MORONE SAXATILIS,* AND BAY SHRIMP, *CRANGON FRANCISCORUM,* IN SEAWATER [1]

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Field monitoring studies have shown that many striped bass, *Morone saxatilis,* in California's Sacramento-San Joaquin Estuary are burdened with a wide variety of pollutants including many types of petroleum hydrocarbons. Alicyclic hydrocarbons are among these pollutants but there was no information on their toxicity to striped bass. Our studies were concerned with the acute toxicities of the simplest alicyclics in comparison to their counterparts in the aromatic series. The alicyclic compounds were cyclohexane, methylcyclohexane, ethylcyclohexane and four dimethylcyclohexanes (1,1; 1,2; 1,3; and 1,4). Acute toxicities after 24 and 96 h exposures to seven alicyclic hexanes were determined for striped bass and one of their major food organisms, the bay shrimp, *Crangon franciscorum.* The 96 h LC_{50}s for striped bass and bay shrimp ranged from 3.2 to 9.3 µl/l and from 1.0 to 6.2 µl/l, respectively. Slight differences were noted between the 24 and 96 h LC_{50} values in all but two bioassays. Solubilities of these alicyclics in seawater and freshwater were determined since information in the literature was limited. Solubility was inversely related to the complexity of the alicyclic structure and ranged from 5.3 to 62 µl/l in distilled water and from 4.6 to 44 µl/l in seawater. Alicyclics were generally more soluble in distilled water than in seawater. Tissue analyses showed greater bioaccumulation in striped bass, which have more lipid than bay shrimp. Striped bass had up to 260 µl/kg (wet weight) of an alicyclic hexane after exposure to water concentrations ranging from 0.32 to 12 µl/l. Bay shrimp residues were as much as 110 µl/kg of an alicyclic hexane.

INTRODUCTION

Alicyclic hexanes were recently found in liver and ovary tissues (0.02 to 16 µl/kg wet weight) of striped bass, *Morone saxatilis,* taken from the Sacramento-San Joaquin Estuary (Whipple, Eldridge and Benville 1981). Along with monocyclic aromatic hydrocarbons, the alicyclics were associated with poor physiological conditions (egg resorption and lesions with cestode larvae) leading to possible organ dysfunctions. Because little information exists on the toxic effects of these petroleum compounds, we conducted a series of experiments to determine the toxicological consequences of alicyclic hexanes to striped bass and bay shrimp, *Crangon franciscorum.*

Alicyclic hexanes are six-carbon ring structures in the cyclic aliphatic hydrocarbon class (cycloalkanes) of organic compounds which are relatively nonpolar and have low solubility in water (Morrison and Boyd 1966). Seven low molecular weight alicyclic hexanes found in the tissues of striped bass collected from the San Francisco Bay were selected for acute toxicity tests. These alicyclics were: cyclohexane, methylcyclohexane, four dimethylated forms 1,1-; cis- and trans-1,2—; cis-1,3—; and cis- and trans-1,4-dimethylcyclohexane (DMCH), and ethylcyclohexane (ECH) (Figure 1).

[1] Accepted for publication August 1984.

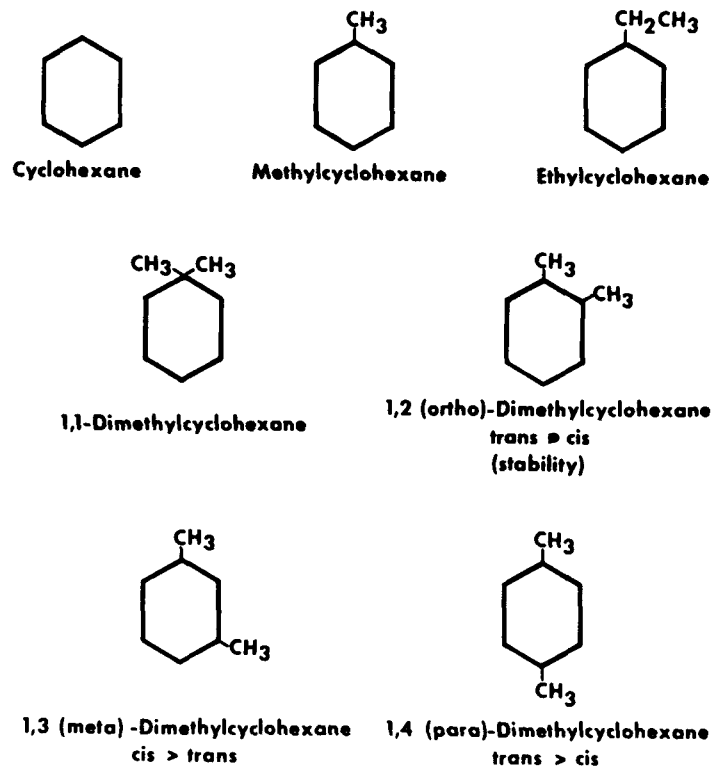

FIGURE 1. Structural formulas of seven alicyclic hexanes.

While some alicyclics entering the aquatic environment are of biogenic origin (e.g. plant metabolites), most alicyclics are from anthropogenic sources such as oil spills and waste discharges.

MATERIALS AND METHODS

Solubility Determinations

The lack of published solubility values for most of the seven alicyclics necessitated the determination of their solubility in distilled water and seawater before we could conduct the toxicity experiments.

To determine the solubilities of the seven alicyclics, saturated solutions were prepared by shaking 0.22 ml of each alicyclic with 220 ml water in a 250 ml separatory funnel for 1 min and letting the mixture settle for 24 h at 20° C. Two 100 ml portions of the saturated water extracts were drained into 125 ml separatory funnels; each portion was extracted in succession with three 10 ml aliquots of TF-Freon (trichlorotrifluoroethane) (Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.). Samples were processed as described under Analytical Procedure, below.

Bioassay Procedure

Juvenile striped bass (mean weight = 8.5 g, mean total length = 9.2 cm) were obtained from freshwater at the U.S. Bureau of Reclamation fish screening facility at Tracy, California, and acclimated to seawater at our laboratory in Tiburon, California. Bay shrimp (mean weight = 1.7 g, mean length = 6.4 cm), were acquired from a local bait dealer in San Rafael, California. Mortality from transportation and salinity change during the first 24 h was 20% for striped bass and less than 1% for bay shrimp. All animals were held in flow-through 200 l tanks for 2 wk before toxicity testing (Korn 1975). Mortality during this period was less than 1% for both fish and shrimp. Salinity and temperature of the seawater increased during the holding period from 27 to 32 °/$_{oo}$ and 15 to 20°C, respectively.

Static bioassays were conducted with each alicyclic using five oval fiberglass aquaria with dimensions of 110 cm length × 50 cm width × 40 cm height. Each aquarium was filled with 180 l of filtered seawater. Five alicyclic aliquots ranging in geometric progressions from 1 to 16 ml were mixed with seawater to achieve the necessary nominal concentrations. Test-tank solutions were prepared by adding the alicyclics slowly (8 ml/min) into a mechanically-generated seawater vortex. Immediately after the appropriate amount of alicyclic was introduced into each aquarium, ten animals of each species were placed in each aquarium. No aeration was used during the testing period. The bay shrimp were separated from the fish by placing them in a 30 cm length × 16 cm width × 14 cm height basket. Two 10 ml seawater samples were pipetted at mid-depth from each aquaria before and after introducing the animals to determine the alicyclic concentrations. These samples were immediately extracted with 10 ml of TF-Freon and analyzed as described under Analytical Procedure. Each succeeding day a 100 ml water sample was taken, extracted and analyzed. Mortalities were noted daily. Bioassay procedures followed were from Standard Methods (Connors, Jenkins and Greenberg 1981). LC_{50}s and confidence limits were calculated by the Litchfield and Wilcoxon (1949) method.

All fish and shrimp were analyzed for alicyclic residues at the end of the 96 h tests. Animals were frozen using dry ice and ground into a powder (Benville and Tindle 1970). A composite of 10 g homogenized tissue was then processed for alicyclic concentrations (Nunes and Benville 1979).

Analytical Procedure

The alicyclics in each Freon extract were separated and analyzed with a Hewlett-Packard 5880 gas chromatograph (GC) equipped with a dual flame ionization detector. Standard solutions of each alicyclic hexane were made for calibrating the GC. A combined standard was made from the seven alicyclics, six monocyclic aromatics and nonane to determine the relative location of each compound (Figure 2). The six aromatics were benzene, toluene, ethylbenzene and three xylene isomers (ortho-, meta, and para-). All alicyclics were eluted from the Bentone-34/SP-1200 column at 65°C within 8 min. After all the alicyclics were separated, the temperature was increased to 120° C to remove all the aromatics. There were overlapping retention times between the DMCHs and ethylcyclohexane (ECH) using the Bentone-34/SP-1200 column. However, each bioassay involved only a single alicyclic and analytical interferences were not encountered. Benzene was eluted within the alicyclic group but caused no

interference. Temperature programming was started after 10 min. running time to elute the ethylbenzene and xylene isomers within a reasonable time frame (15 min). The four dimethylated alicyclics and ethylcyclohexane had overlapping retention times and emerged in the following three groups: 1,1-DMCH, cis-1,3-DMCH and trans-1,4-DMCH; trans-1,2-DMCH and cis-1,4-DMCH; and cis-1,2-DMCH and ECH. No trans-1,3-DMCH was available for standardization; consequently its elution time was unknown. One unknown peak appeared at 2.653 min which was an inherent characteristic of the TF-Freon solvent. The ratio of the cis- and trans-isomers that appeared for 1,2-, 1,3- and 1-4-DMCH were 41/59, 100/0 and 25/75, respectively. Alicyclic data reported in ppm are in μl/l for water sample and μl/kg for tissue samples based on wet weight.

RESULTS

Solubilities

All seven alicyclics had solubilities in the low ppm range (Table 1). The number of alkyl groups on the cyclohexane compounds is in inverse proportion to its water solubility, with cyclohexane the most soluble and dimethyl and ethyl alicyclics the least soluble. In general, the position of the methyl groups appears to affect the solubility. The further apart the two groups were from each other, the less soluble was the DMCH. Solubilities also changed with salinity. Alicyclics, except for ECH, were more soluble in distilled water than in seawater; ECH had the same solubility in both distilled water and seawater. Solubility values ranged from 5.3 to 62 ppm in distilled water and from 4.6 to 44 ppm in seawater. The solubility of most of the alicyclics in seawater was lower by 24 to 29%; cis-1,3-DMCH had only a 10% reduction and there was no change for ECH. Ratios of the two optical isomers (cis- and trans-) in 1,2- and 1,4-DMCH compounds were altered slightly favoring the cis- form after they were dissolved in water, from 41/59 and 25/75 to 45/55 and 30/70 for distilled water and 44/56 and 28/72 for seawater, respectively.

Acute Toxicity and Tissue Residues

The alicyclics were more toxic to bay shrimp than to striped bass (Table 2). Acute toxicities of the seven alicyclics ranged from 4.7 to 10.9 ppm for striped bass and from 1.5 to 6.5 ppm for bay shrimp within a 24 h period. The 96 h tests showed slightly higher toxicities, although cyclohexane for striped bass and 1,3-DMCH for bay shrimp had similar acute toxicities for both exposure periods. Mixtures of the cis- and trans-DMCH (1,2- and 1,4-) isomers were more toxic to both striped bass and bay shrimp than the two pure DMCH's (1,1- and 1,3-). Acute toxicity values (LC_{50}s) of ECH, 1,3-DMCH and 1,4-DMCH for striped bass exceeded the solubility of the compound in seawater; for bay shrimp, only the LC_{50} for ECH and 1,3-DMCH exceeded the seawater solubility.

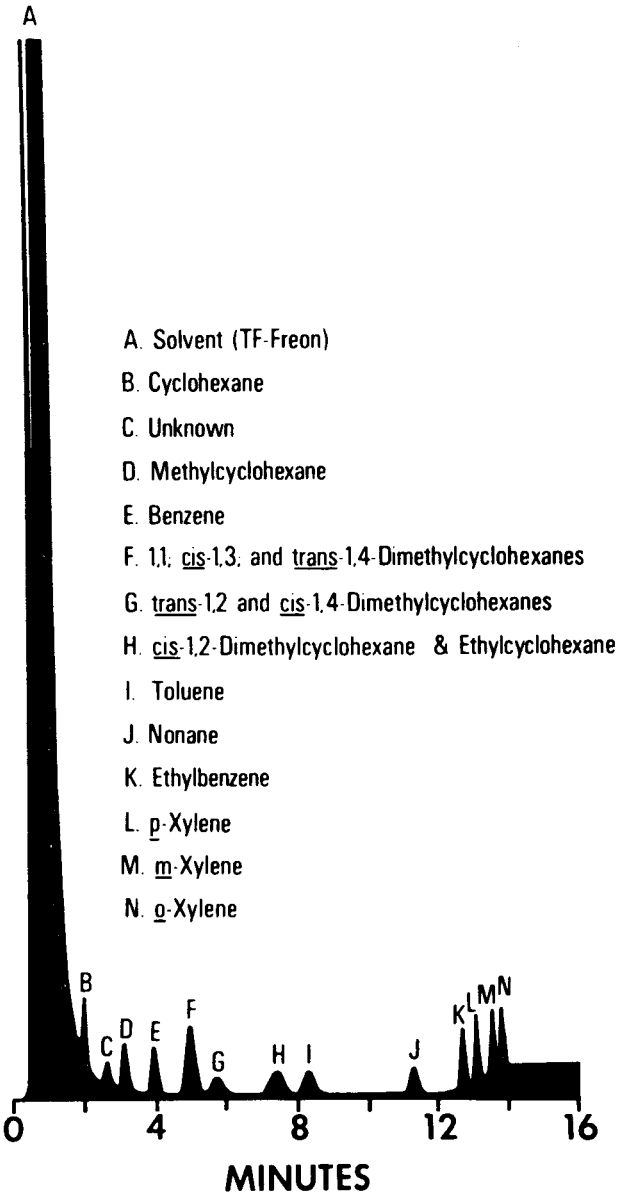

FIGURE 2. Chromatogram of nine alicyclics, six aromatics and nonane.

The striped bass and bay shrimp rapidly bioaccumulated the alicyclics many times above the initial water dosage. Test concentrations of alicyclics in seawater ranged from 0.32 to 11 ppm which resulted in uptakes by striped bass from "nondetectable" levels to 260 ppm (wet weight) and by bay shrimp from 4.4 to 110 ppm (Table 3). Water insoluble alicyclics were accumulated to higher levels by the organisms than were the more soluble ones.

TABLE 1. Physical Properties of the Alicyclic Hexanes.

Compounds	% Purity	Solubility (ppm) @ 20°C		% Optical Form *		Molecular weight	Boiling point (C)	Density (g/ml)	Vapor † pressure (mm Hg)
		Distilled	Seawater						
		(cis-/trans-)		Cis-	Trans-				
Cyclohexane	99+	62	44	—	—	84.16	81	.77	78
Methylcyclohexane	99	18	13	—	—	98.19	101	.77	36
Ethylcyclohexane	99+	5.3	5.3	—	—	112.22	103–132	.778	10
1,1-Dimethylcyclohexane	97	10	7.6	—	—	112.22	118–120	.777	17
1,2-Dimethylcyclohexane	98	8.2(3.7/4.5)	6.1(2.7/3.4)	41	59	112.22	124	.778	11 (*cis*-)
1,3-Dimethylcyclohexane	99	6.2	5.6	100	0	112.22	120	.784	16 (*cis*-)
1,4-Dimethylcyclohexane	98	6.4(1.9/4.5)	4.6(1.3/3.3)	25	75	112.22	120	.773	14 (*cis*-)

* Analyzed by gas chromatography (not corrected for purity).
† Values were calculated for 20°C using Antoine equation in Lange's Handbook of Chemistry (Dean 1979).

TABLE 2. Acute Toxicity of Seven Alicyclic Hexanes to Striped Bass and Bay Shrimp.

Alicyclic Hexanes *	Striped Bass				Bay Shrimp			
	24-h		96-h		24-h		96-h	
	LC_{50}	95%C.L.	LC_{50}	95%C.L.	LC_{50}	95%C.L.	LC_{50}	95%C.L.
Cyclohexane	8.3	—†	8.3	—	3.4	—	2.4	1.7 - 3.5
Methylcyclohexane	7.0	5.7- 8.6	5.8	—	3.5	3.0 - 4.0	3.3	2.9 - 3.9
Ethylcyclohexane	10.9	6.6–17.5	8.8	6.0–13.0	3.7	2.9 - 4.8	3.1	2.1 - 4.5
1,1-Dimethylcyclohexane	7.5	6.6- 8.5	6.9	6.3- 7.6	6.5	6.1 - 6.9	5.2	—
1,2 Dimethylcyclohexane	4.7	3.2- 6.9	3.2	—	1.5	0.82- 2.6	1.0	0.58 -1.8
1,3 Dimethylcyclohexane	9.9	8.3–11.8	9.3	8.0–10.8	6.2	5.5 - 6.9	6.2	5.5 - 6.9
1,4 Dimethylcyclohexane	6.7	6.0- 7.3	6.5	5.7- 7.3	2.8	2.0 - 3.8	1.5	—

* 1,2 and 1,4 dimethylcyclohexanes are mixtures of cis and trans isomers.
† No confidence limits were calculated from bioassays without partial mortalities.

TABLE 3. Resulting Concentrations of Alicyclic Hexanes in Tissues of Striped Bass and Bay Shrimp from Test Containers.

	Concentration Range of Alicyclics (ppm)		
Alicyclic Hexanes *	*Water (v/v)*†	*Striped bass (v/w)*	*Bay shrimp (v/w)*
Cyclohexane	.54–10	N.D.‡– 48	4.4– 6.9
Methylcyclohexane	.54– 7.3	N.D.‡–130	16 – 26
Ethylcyclohexane	1.5 –11	5.8– 63	8.5– 66
1,1 Dimethylcyclohexane	1.5 – 8.7	23 –170	9.1–110
1,2 Dimethylcyclohexane	.32– 7.9	1.1–260	24 – 47
1,3 Dimethylcyclohexane	1.6 –12	6.5–160	7.0–100
1,4 Dimethylcyclohexane	1.2 – 7.1	28 – 46	12 – 36

* 1,2 and 1,4 dimethylcyclohexanes are mixtures of cis and trans isomers.
† v = Volume, w = Weight, Initial dosage
‡ N.D. = Non detectable below 0.7 µl/kg for tissue sample.

We noted that striped bass were more docile, easier to net and transfer after the 96 h testing period than before the dosing began. Striped bass exposed to the high dose of 1,3-DMCH (12 ppm) swam in tight clockwise circles instead of swimming around the aquaria like the control fish when the aquaria were disturbed. Some fish dosed with other alicyclics at lower concentrations would do the same but not as long (only 10–15 s).

DISCUSSION

The acute toxicity (24 h bioassay) of the alicyclics to striped bass and bay shrimp was in the low ppm range (1.5 to 10.9 µl/l) which is considered moderately toxic to fish and invertebrates relative to other compounds (Benville and Korn 1977). Extending the toxicity testing interval 3 more days resulted in increases in the toxicity of all bioassays except for two tests (striped bass/cyclohexane and bay shrimp/1,3 DMCH). The slight increase in toxicity was probably due to the delayed effects of the alicyclics since alicyclics rapidly escape from solution, resulting in alicyclic concentrations too low to cause acute effects. Using a continuous flow method of dosing would probably show higher toxicities (lower LC_{50}s). Static tests usually result in higher LC_{50} values (lower toxicity) than continuous dosing because the concentration of the toxicant is decreasing and not being replenished using the static method.

Three of the alicyclics (ECH, 1,3 DMCH and 1,4 DMCH) had solubility values below their LC_{50} concentration values for striped bass (Figure 3). Researchers usually use a solubilizing agent to disperse "insoluble" toxicants without knowing the effects of the solubilizers. Therefore, we did not use any dispersing agents in this study.

Striped bass appeared to be more sensitive to alicyclics than other fish species that have been tested. Pickering and Henderson (1966) reported 96 h TLms (median tolerance limits) of cyclohexane for fathead minnows, *Pimephales promelas*; bluegills, *Lepomis macrochirus*; goldfish, *Carassius auratus*; and guppies, *Lebistes reticulatus*, of 42, 45, 55 and 75 µl/l, respectively. These are 5 to 9 times higher than the toxic levels to striped bass. Jenkins, Klein and Cooper (1977) found an LC_{50} of 84 µl/l for methylcyclohexane with golden shiners, *Notemigonus chrysoleucas*, 14 times the level of toxicity for striped bass. Although little data are available on the toxicity of higher homologs of cyclohexane

to fish, mammals show an increase in the narcotic action and toxicity when alkyl groups are added to cyclohexane (von Oettingen 1942). We found that MCH, 1,1 DMCH, 1,2 DMCH and 1,4 DMCH were narcotic to striped bass. Perhaps ECH and 1,3 DMCH also would have been narcotic if their solubilities were higher.

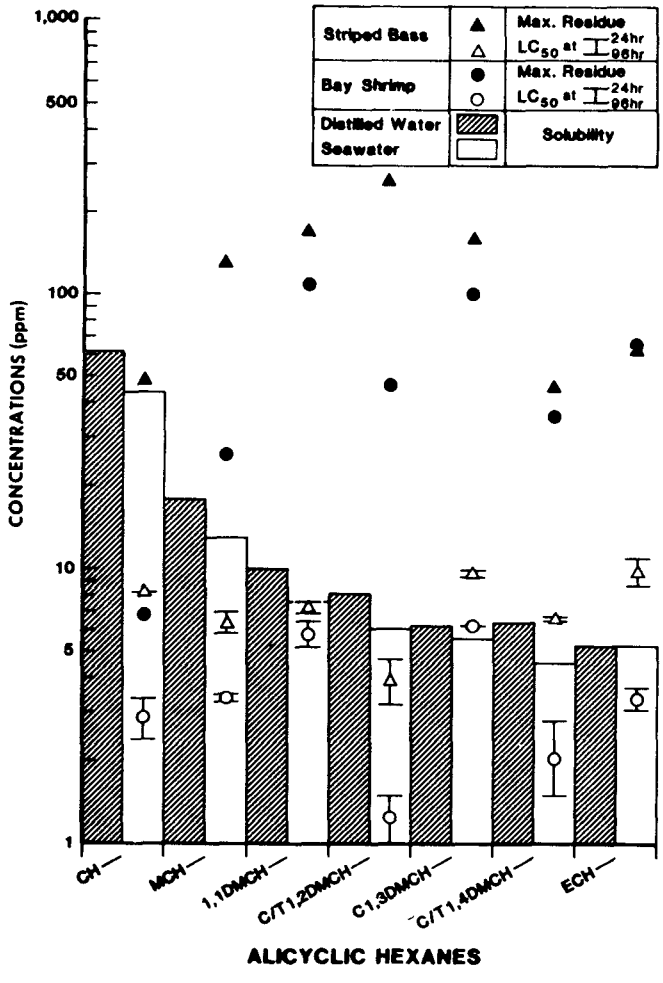

FIGURE 3. Solubilities, acute toxicities and maximum tissue residues of seven alicyclic hexanes.

Alicyclics bioaccumulated in striped bass and bay shrimp by factors of 33x and 13x, respectively. The greater accumulation in striped bass is due primarily to their higher lipid content. Alicyclic residues might have been much higher, as our research has indicated with monocyclic aromatic hydrocarbons (Whipple *et al.* 1981), if the samples were taken at the optimum exposure time. Aromatics accumulate rapidly in aquatic animals. Benzene (the simplest aromatic) accumulated in high concentrations in striped bass shortly after 40-min

exposure (Whipple *et al.* 1981). A 1.5x to 119x increase above the ambient water concentration of benzene was noted depending on the tissue analyzed. Muscle had the lowest uptake and fat the highest uptake of the twelve analyzed tissues. Other scientists have shown that fish can accumulate monocyclic aromatic hydrocarbons from chemical mixtures, e.g. crude oil (Roubal, Stranahan and Malins 1978).

It has been suggested that cycloalkanes and other Water Soluble Fraction compounds from petroleum and its products may be more toxic than aromatics or that there is an additive effect with aromatics present (Caldwell, Caldarone and Mallon 1977). Further research should be conducted to study the interaction and combined effect of these two groups of cyclic compounds and to determine if the additive effects are manifested at the population and community levels.

ACKNOWLEDGMENTS

We thank M. Jung and S. Korn for their comprehensive review of our manuscript. R. Hamilton was very helpful in the technical part of this study and developed many computer programs for this work for which we are grateful. A special appreciation goes to R. Fisher and J. Greeley for editing and typing this manuscript.

LITERATURE CITED

Benville, P. E., Jr., and R. C. Tindle. 1970. Dry ice homogenization procedure for fish samples in pesticide residue analysis. Agr. Food Chem., 18(5):948–949.

Benville, P. E., Jr., and S. Korn. 1977. The acute toxicity of six monocyclic aromatic crude oil components to striped bass *(Morone saxatilis) and bay shrimp (Crago franciscorum.)* Calif. Fish Game, 63(4):204–209.

Caldwell, R. S., E. M. Caldarone, and M. H. Mallon. 1977. Effects of Seawater-Soluble Fraction of Cook Inlet Crude Oil and Its Major Aromatic Components on Larval Stages of the Dungeness Crab, *Cancer Magister* Dana. Pages 210–220 *in* Douglas A. Wolfe, ed. Fate and Effects of Petroleum Hydrocarbons in Marine Ecosystems and Organisms, Pergamon Press, New York.

Connors, J. J., D. Jenkins and A. E. Greenberg (editors). 1981. Toxicity to fish. Pages 723–743 *in* Standard Methods for the Examination of Water and Wastewater. 15th edition, American Public Health Association, Washington, D.C.

Dean, J. A. (editor). 1979. Lange's Handbook of Chemistry. Physical Properties, Section 10:28–53. 12th edition, McGraw-Hill Book Co., NY.

Jenkins, D., S. A. Klein, and R. C. Cooper. 1977. Fish toxicity of jet fuels. *In* The toxicity of the synthetic fuel JP-9 and its components. Water Res., 11:1059–1067.

Korn, S. 1975. Semi-closed seawater system with automatic salinity, temperature and turbidity control. U.S. Dept. Commer., NOAA Tech. Rep. NMFS SSRF-694, 5p.

Litchfield, J. T., Jr., and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. Pharmacol. Exper. Ther. J., 96:99–113.

Morrison, R. T., and R. N. Boyd. 1966. Organic chemistry, cyclic alipathic hydrocarbons. 2nd edition. Allyn and Bacon, Inc., Boston, MA. 1204 p.

Nunes, P., and P. E. Benville, Jr. 1979. Uptake and depuration of petroleum hydrocarbons in the Manila clam, *Tapes semidecussata* Reeve. Bull. Environ. Contam. Toxicol., 21:719–726.

Pickering, Q. H., and C. Henderson. 1966. Acute toxicity of some important petrochemicals to fish. Water Pollut. Control Fed. J. 38(9):1419–1429.

Roubal, W. T., S. I. Stranahan, and D. C. Malins. 1978. The accumulation of low molecular weight aromatic hydrocarbons of crude oil by coho salmon (*Oncorhynchus kisutch*) and starry flounder (*Platichthys stellatus*). Arch. Environ. Contam. Toxicol., 7:237–244.

von Oettingen, W. F. 1942. The toxicity and potential dangers of aliphatic and aromatic hydrocarbons. Yale J. Biol. Med., 15:167–184.

Whipple, J. A., M. B. Eldridge, and P. Benville, Jr. 1981. An ecological perspective of the effects of monocyclic aromatic hydrocarbons on fishes. Pages 483–551 *in* F. John Vernberg, Anthony Calabrese, Frederick P. Thurberg and Winona B. Vernberg, eds. Biological monitoring of marine pollutants. Academic Press, NY.