acute aquatic toxicity of kerosines

report on concawe test programme

Prepared for CONCAWE's Ecology Group by:

D.R. Peterson
P. Falkenback
A. Girling
D. King
R. Stephenson
D. Short

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ABSTRACT

This report describes the experimental procedures and the results obtained in acute ecotoxicity tests on three kerosine samples. The samples were tested for toxicity to the rainbow trout, *Oncorhynchus mykiss*, the crustacean zooplankter, *Daphnia magna* and the alga, *Selenastrum capricornutum* using water accommodated fractions. These results assist in determining the environmental hazard from kerosines.

KEYWORDS

Ecotoxicity, fish, daphnia, algae, kerosine, OECD guidelines, lethal loading, water accommodated fractions.

CONTENTS

SUMMARY

A series of toxicity tests have been performed on three representative kerosines. The toxicity tests were conducted on rainbow trout (*Oncorhynchus mykiss*), *Daphnia magna*, and green algae (*Selenastrum capricornutum*) using OECD methods. As the substances are mixtures of poorly water soluble and volatile substances, they were tested as water accommodated fractions (WAFs) in sealed test vessels. Test substances were equilibrated with water at each "concentration" or loading rate and the water phase ("WAF") tested for toxicity. The toxicity results were expressed as "lethal loading (LL)", or "effective loading (EL)", or inhibitory loading (IL) to cause a 50% response.

The fish 96 hour LL_{50} values ranged from 18 to 25 mg/l. The Daphnia 48 hr EL_{50} values were 1.4 and 1.9 mg/l for the hydrodesulphurized and hydrocracked products respectively, and 21 mg/l for the sweetened kerosine. The algal 72 hr IL_{50} values ranged from 3.7 to 8.3 mg/l, based on the specific growth rate, whilst the values ranged from 4.3 to 15 mg/l based on the area under the growth curve; the highest values for both endpoints was for the hydrodesulphurized product. Algae and daphnia appeared to be more sensitive to the kerosine samples tested, than did fish.

1. INTRODUCTION

The available data on the ecotoxicity of the generic class of petroleum substances known as kerosines has been summarised in a CONCAWE dossier. [1] Most of the results relate to studies where water has been equilibrated with a sample of kerosine and tests have been done on dilutions of the aqueous phase. Such studies do not provide data that are useful for the purposes of classifying and labelling for environmental hazard in accordance with the criteria given in the Dangerous Substances Directive. [2]

CONCAWE has recommended that the only ecotoxicity data that is valid for classification purposes is that based on the use of "water accommodated fractions" (WAFs). The experimental procedures and methods of presenting results using WAFs have been described. [3] However, there are few ecotoxicity results for petroleum substances using WAFs and CONCAWE has embarked on a test programme to generate typical data in studies on fish, daphnia and algae. As part of the programme, the acute aquatic toxicities of three kerosine samples were determined and the studies are the subject of this report.

2. CHARACTERISATION OF TEST SUBSTANCES

Acute aquatic toxicity tests were performed on three kerosines (**Table 3**). These test substances were selected as representative of three different refining processes which are used to produce kerosines. They are contained in groups 3I and 3J of the CONCAWE grouping of petroleum products for the Existing Substances Regulation. [4] They represent 3 of the 31 EINECS entries which are applicable to kerosine. [2] All contain a broad range of aliphatic and aromatic hydrocarbons, mainly within the carbon number range of 9 to 16. In this report, the samples are named according to their EINECS descriptions: "sweetened", "hydrodesulphurized", and "hydrocracked". The sweetened kerosine is a petroleum distillate "subjected to a sweetening process to convert mercaptans or to remove acidic impurities". The hydrodesulphurized kerosine is obtained by "treating with hydrogen to convert organic sulfur to hydrogen sulfide which is removed". The hydrocracked heavy aromatic solvent naphtha was obtained by "distillation of hydrocracked petroleum distillate". The various identifications of these three samples are given in **Table 1**.

Table 1: Kerosine Substances Tested for Aquatic Toxicity

Name	Group	EINECS No.	CAS No.	Sample No.	Lab. Code No.
Sweetened	3J	294-799-5	91770-15-9	ST 019	MRD-94-883
Hydrodesulphurized	3J	265-184-9	64742-81-0	ST 042	MRD-94-885
Hydrocracked, Heavy Aromatic	3I	309-881-9	101316-80-7	ST 028	MRD-94-884

These samples were characterised using a variety of physical and chemical tests using standardized methods for petroleum products. Details of the methods used are contained in the full laboratory reports. **Table 2** summarises some of the physical and elemental analyses performed. The hydrocarbon types and carbon number distributions of the n-alkanes were determined by gas chromatography / mass spectrometry (GC/MS). The number of isomers of the alkanes becomes too great to resolve at carbon numbers greater than about 10, and hence, detailed individual hydrocarbon analysis is not possible for petroleum products of the complexity and carbon number range of the kerosines. Hydrocarbon distributions of the substances are given in **Table 3**.

Table 2: Physical and Elemental Analyses of the Test Substances

Analysis	Sweetened	Hydrodesulphurized	Hydrocracked
Boiling Range (°C) Initial - Final -	 151 257	 156 255	 187 288
Density (g/ml) (at 16°C)	0.7998	0.8028	0.8078
Refractive Index (at 67°C)	1.4254	1.4271	1.4305
Sulfur (mg/kg)	281	<20	<20
Nitrogen (mg/kg)	1	1	1
Chloride (mg/kg)	<5	<5	<5
Oxygen (mg/kg)	290	290	290

Table 3: Hydrocarbon Distributions of the Test Substances by Weight

Hydrocarbon Type	Sweetened	Hydrodesulphurized	Hydrocracked
Branched Alkanes	20.0 %	16.5 %	36.6 %
n-Alkanes	22.2 %	19.4 %	5.8 %
n-Alkane Range (>0.1%)	*C7-C17*	*C8-C15*	*C8-C18*
n-Alkanes < C11	*38 %*	*48 %*	*26 %*
Cycloalkanes	33.5 %	35.9 %	30.9 %
Alkylbenzenes	15.4 %	20.4 %	14.3 %
Indanes & Tetralins	3.1 %	3.8 %	8.2 %
Indenes	0.1 %	not detected	0.2 %
2 Ring Aromatics	5.5 %	3.8 %	3.5 %
3 Ring Aromatics	0.2 %	0.2 %	0.3 %

The percentage of n-alkanes less than C11 (undecane) is given in **Table 3** because n-decane represents the water solubility limit for acute toxicity. [4] Alkanes of 11 carbons and higher have too low a water solubility to give rise to acute aquatic toxicity. It is expected that the branched and cyclic alkanes will follow a similar distribution, as for the n-alkanes, since the major separation process for petroleum is distillation and boiling points are closely related to carbon number. The alkylbenzenes and 2-ring aromatics are expected to have sufficient water solubility to contribute to acute aquatic toxicity. [5]

The three kerosines are all similar in containing approximately 24-28% aromatics, the majority being alkylbenzenes. Each also contains approximately 31-36% of cycloalkanes. The hydrocracked material differs from the other two in having a somewhat higher boiling range and carbon number range. The hydrocracked material also has a considerably higher percentage of branched rather than linear alkane structures. Of the n-alkanes, the hydrocracked kerosine has a smaller fraction below C_{11}, again a reflection of its higher boiling range. It is to be noted that the sulphur concentration of the sweetened kerosine is significantly higher than for the other two kerosines.

More detailed reports [7, 8, 9] on the characterisation of these three kerosine samples are available.

3. TEST METHODS

3.1. GENERAL APPROACH

Mixtures of poorly water soluble chemicals present special problems with regard to preparing aqueous solutions for toxicity testing. With soluble chemicals, the amount of chemical dissolved in water is varied in incremental steps to produce a range of toxic responses, from which a "dose - response" relationship and the associated median lethal concentration (LC_{50}) may be derived. With mixtures of poorly soluble chemicals, undissolved material appears as soon as the least soluble component reaches water saturation. Thereafter, the relative composition of the water phase varies in a non-linear fashion from the composition of the "neat" substance. [10] For these types of materials, it has become a standard practice to test toxicity at substance additions far in excess of the amount that will dissolve, resulting in a two phase system.

There are, however, many divergent procedures for establishing and maintaining equilibrium between water and undissolved substance. [11] A recognised guideline [12] for testing mixtures of poorly water soluble substances has been developed by the Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP). This method involves stirring various amounts (loading ratios) of test substance with water for a sufficient time to reach equilibrium, followed by separation of the water phase ("water accommodated fraction" or "WAF"). Toxicity testing of the WAFs generated in this manner allows the determination of the amount of the substance equilibrated with water which will cause 50% mortality. This end-point has been termed LL_{50} (lethal loading) to distinguish it from the LC_{50}. [13] (The LC_{50} is determined by completely dissolving the chemical in water and then making a dilution series to obtain a relationship between concentration and lethality). The LL_{50} procedure has also been described in a CONCAWE report. [4] It is also the approach specified by MARPOL for the marine pollution testing of poorly soluble mixtures. [14] At a January 1994 CEFIC Workshop on Classification for Environmental Hazard, there were no exceptions taken by regulatory authorities to the position of representatives from both industry and the UK that this "lethal loading" test is the appropriate one for testing poorly water soluble mixtures.

A further complication for the testing of hydrocarbon liquids is their volatility, particularly from aqueous solution. Although it may be environmentally unrealistic, it is necessary to prevent volatilization of the substance in order to maintain constant concentrations and, by doing so, to determine its inherent toxicity. This necessitates using closed test systems. In preparing WAFs, some headspace is necessary to achieve adequate interfacial area and mixing. The daphnia and fish tests reported herein, used a cylindrical, stoppered bottle (carboy) filled to its "shoulders" to allow maximum surface contact and the formation of a vortex (see **Figure 1**). The bottle had an actual volume of about 20 l with a headspace of about 2 l. For the algae tests, 2 l bottles were used, again with about 10% of the volume as headspace.

The exposure vessel for the fish test (see **Figure 1**) was a similar cylindrical bottle, much smaller in volume (4.5 l) and with a stopcock at the bottom for removing liquid. (The inside opening of this outlet was fitted with a small piece of screen to prevent fish from entering it.) The exposure vessel was filled to the top and stoppered with no headspace. The test chambers for the daphnia and algae studies were 125 ml

Erlenmeyer flasks with ground glass stoppers and were filled completely with test solution (no headspace).

It is important that mixing is sufficient to ensure that the aqueous phase is in equilibrium with the undissolved hydrocarbon phase. Mixing needs to be slow enough not to cause dispersion or emulsification of the undissolved hydrocarbon, yet vigorous enough and long enough to attain equilibrium. In the current studies, mixing was done with a magnetic stirring bar set to develop a vortex at the surface of about 10% of the water height. Preliminary studies showed that this mixing condition was sufficient to reach equilibrium within 24 hours. After mixing for 24 hours, solutions were allowed to stand for 1 hour before use in order to allow phase separation. (Although no dispersion of test material was observed under the mixing conditions used, it is possible that some droplets could have been entrained in the vortex.) The mixing vessel was fitted with a glass tube for siphoning off the water phase, without contamination by the surface layer of undissolved hydrocarbon.

All the studies were conducted in accordance with the principles of good laboratory practice (GLP).

3.2. FISH ACUTE STUDIES

The fish acute toxicity tests were conducted in accordance with OECD Guideline 203. The test species chosen for these studies was the salmonid, *Oncorhynchus mykiss*, the rainbow trout. The salmonids are considered to be one of the more sensitive test species, particularly to hydrocarbons. The rainbow trout is a common laboratory test species for determining toxicity to freshwater fish. Details of the source, husbandry and selection procedures are available in the laboratory reports.[15, 16, 17] The fish used for the studies were 3 to 4 cm in length (0.2 g) and were not fed during the exposure period. For definitive studies, three replicates each involving 5 fish per test were evaluated at each loading rate.

Fresh WAFs were prepared on a daily basis and used for daily renewal of the exposure medium. Renewals were done by emptying 80% of the water from the bottom port on each exposure vessel and then expeditiously re-filling by siphon from the mixing vessel. Analysis of WAFs was conducted by solvent extraction-GC; all peaks were summed and a naphthalene standard was used for quantitation of the total peak area. Analyses were conducted for each test substance during the definitive study to evaluate uniformity of WAF concentrations and losses during each renewal period.

The total exposure periods were 96 hours. Water was of intermediate hardness (about 170 mg/l as $CaCO_3$) with a pH of 7.6 ± 0.1. The temperature was 15°C and the light duration was 16 hours at 170 lux. The fish loading was about 0.2 to 0.5 g/l during the test. Dissolved oxygen was 9 to 5 mg/l throughout all exposures and no reductions in oxygen concentrations sufficient to influence the results were observed during the tests. Observations were made at 3 hours after the commencement of exposure and once daily, thereafter. The fish were not fed during the exposures.

3.3. DAPHNIA ACUTE STUDIES

These tests were carried out in accordance with OECD Guideline 202, Part I. The test species was *Daphnia magna*, a fresh water invertebrate commonly used for toxicity testing. Details of the husbandry and selection of test organisms are provided in the laboratory reports. [18, 19, 20] The organisms used for testing were less than 24 hour old neonates, from 13 day old parents. For definitive studies, four replicates, each involving 5 organisms were tested at each loading rate.

The water used had the same properties as for the fish studies. WAFs were also prepared in the same manner using the same equipment and analyses. WAFs were analyzed as described for the fish studies, at the beginning and end of the exposure. The exposure period was 48 hours. The WAFs were siphoned into 125 ml glass-stoppered flasks and equilibrated in a waterbath at 19°C before the daphnia were introduced. The light duration was 16 hours at 170 lux. The organism loading was one daphnid per 28 ml. No reductions in dissolved oxygen concentration were seen at the end of the 48 hour exposure period. Observations were made for immobilization at 24 and 48 hours. The daphnids were not fed during the exposure periods.

3.4. ALGAL GROWTH INHIBITION STUDIES

The algal growth studies were conducted in accordance with OECD guideline 201. The test species was *Selenastrum capricornutum* (alternatively known as *Raphidocelis subcapitata*). Details of the culture methods are provided in the laboratory reports. [21, 22, 23] The algae used were taken from 5 day old stock cultures in the log phase of growth. Initial concentrations were approximately 1.0×10^3 cells/ml in each replicate test chamber.

WAFs were prepared in algal growth medium on a somewhat smaller scale from that used in the fish and daphnia studies. WAFs were analyzed as described for the fish studies at the beginning of the test period, and again on a composite from test flasks after 96 hours. Test chambers were glass-stoppered Erlenmeyer flasks. They were filled completely with inoculated WAF. Additional WAF was kept in glass-stoppered flasks for replacement of solution when aliquots were removed for algal cell density determination. The flasks were incubated at 23.7 ± 0.2°C on a rotary shaker table, cycling at 125 rpm. Lighting was continuous and in the range of 4200 to 4400 Lux. Cells were enumerated at 24, 48, 72 and 96 hours using a fluorimetric technique. Fluorometer readings were converted to cell numbers using a standard curve based on direct visual counts.

4. RESULTS

4.1. FISH ACUTE STUDIES

The daily cumulative mortality data at each loading level were used to calculate the lethal loading causing 50% mortality (LL_{50}) for each day. LL_{50} values were calculated from the data using, either probit or binomial methods. [24, 25, 26] The 96 hour LL_{50} values are shown in **Table 4,** together with their corresponding confidence intervals.

Table 4: Acute Fish Toxicity Test Results

Kerosine	Loadings (mg/l)	96 h. LL_{50} (mg/l)	95% Confidence Interval
Sweetened	0.4,0.9,4.5,23,50	18	13 - 24
Hydrodesulphurized	0.3,1.4,6.8,34,75	25	20 - 32
Hydrocracked	0.3,1.4,6.8,34,75	20	7 - 34*

* 99% confidence interval

All of the 96 hour LL_{50} values were about 20 mg/l. The confidence intervals overlap and it is apparent that there is no significant difference in toxicity between the three samples.

The 96-hour NOEL (No Observed Effect Loading) values observed by experiment and calculated using Dunnett's procedure [24] based on survival, are summarised in **Table 5**.

Table 5: No Observed Effect Loadings at 96h from Fish Studies

Kerosine	NOEL (mg/l) experimental	NOEL (mg/l) calculated
Sweetened	4.5	4.5
Hydrodesulphurized	6.8	6.8
Hydrocracked	1.4	6.8

Analysis of the "new" and "old" WAFs indicated that in general, containment of volatiles was very good and only small decreases were seen in concentrations over the 24 hour exposure periods. For some of the materials, some day to day variation was seen in solution concentrations. This variability is in part due to the low concentrations measured being close to detection limits and losses in dissolved material due to volatilization and sorption to surfaces during sampling and analysis, together with similar losses during WAF preparation and transfer to exposure vessels. The analytical data relating to the WAFs are included in the separate laboratory reports. [15, 16, 17]

4.2. DAPHNIA ACUTE STUDIES

The cumulative immobilization at 24 and 48 hours at each loading level were used to calculate the effective loading causing 50% immobilization (EL_{50}) for each day. The data sets were all amenable to probit analysis. The 48 hour EL_{50} values and their associated confidence intervals are shown in **Table 6**.

Table 6: Acute Daphnia Toxicity Test Results

Kerosine	Loadings (mg/l)	48 h. EL_{50} (mg/l)	95% Confidence Interval
Sweetened	0.1,0.4,0.9,4.5,23,50	21	17 - 27
Hydrodesulphurized	0.1,0.3,1.4,6.8,34	1.4	1.0 - 2.0
Hydrocracked	0.1,0.3,1.4,6.8,34	1.9	1.3 - 4.3

It is evident that the sweetened sample was considerably less toxic to Daphnia than the other two kerosines, but as noted below, there were significant hydrocarbon losses from the aqueous phase in tests involving this substance.

The 48-hour NOEL (No Observed Effect Loading) values observed by experiment and by calculation using Dunnett's procedure [24] based on survival, are summarised in **Table 7**.

Table 7: No Observed Effect Loadings at 48h from Daphnia Studies

Kerosine	NOEL (mg/l) experimental	NOEL (mg/l) calculated
Sweetened	0.9	4.5
Hydrodesulphurized	not calculable	0.3
Hydrocracked	0.3	0.3

The analytical results showed only minimal decreases in concentrations during the 48 hr test. The test on the sweetened kerosine is an exception where analytical concentrations were about 50% lower at the end of the test. It is not possible to determine whether this decrease occurred during the test, or during sampling and analysis. The analytical data relating to the WAFs are included in the individual reports on the studies. [18, 19, 20]

4.3. ALGAL TOXICITY STUDIES

The data for cell numbers at each time interval were plotted (as the natural logarithm) versus time and the specific growth rate determined from the slope of this growth curve. The area under the curve was also determined. Both analyses were done using equations given in OECD Guideline 201. The loading rates corresponding to 50% inhibition (IL_{50}) were determined by both methods and the 95% confidence intervals determined using the method of Snedecor and

Cochran. [25] The results influencing environmental classification are those for a 72 hour period and these are shown in **Table 8**.

Table 8: Algal Toxicity Test Results

Kerosine	Loadings (mg/l)	72 hr. IL_{50} (mg/l)*	95% Confidence Interval
Sweetened	0.2,0.8,6.2,12,50	SGR 3.7 AUC 4.3	0.03 - >50 0 - 22
Hydrodesulphurized	0.4,4,20,45,100	SGR 8.3 AUC 15	Not Calculable 0 - 52
Hydrocracked	0.2,0.8,6.2,12,50	SGR 6.7 AUC 12	0.4 - 900 Not Calculable

* SGR = Specific Growth Rate; AUC = Area Under Curve

The specific growth rates for algae, consistently indicated greater toxicity than the area under the growth curve. Although the results were reproducible between samples and between 72 and 96 hour values, the confidence intervals around both endpoints are very broad. Within the confidence bands of the test, there did not appear to be any significant differences in algal toxicity between the kerosines tested.

The 72-hour NOEL (No Observed Effect Loading) values obtained by calculation using the ANOVA procedure [24] for both specific growth rate (SGR) and area under the growth curve (AGC) are summarised in **Table 9**.

Table 9: Calculated 72-hour NOEL Values from Algal Studies

Kerosine	NOEL (mg/l) SGR	NOEL (mg/l) AGC
Sweetened	0.2	0.8
Hydrodesulphurized	4.0	4.0
Hydrocracked	6.2	12

Analytical results showed no significant decreases in the hydrocarbon concentrations over the 96 hours of the tests. These data are included in the individual laboratory reports. [21, 22, 23]

5. DISCUSSION

It has been known for more than a decade that, within limits, the aquatic toxicity of organic chemicals increases with an increase in hydrophobicity or decrease in water solubility. [27] For hydrocarbons, hydrophobicity increases with carbon number. However, at about 10 carbons for alkanes their solubility becomes so low that their availability for uptake is limited to the extent that toxic concentrations are not attained in acute exposures. [5] For instance, a C_{10} alkane has a water solubility of about 50 parts per billion (0.05 mg/l). This solubility is less than the expected toxicity of 1 mg/l for a C_{10} hydrocarbon based upon QSAR. [27] Aromatic hydrocarbons are considerably more soluble than corresponding alkanes at the same carbon number. Alkylbenzenes and naphthalenes with carbon alkyl chains containing up to 5 carbon atoms are sufficiently soluble to show acute toxicity. [6] It is evident that these established ecotoxicity cut off values embrace the typical C_9 to C_{16} range of hydrocarbons found in kerosines.

When preparing a water acommodated fraction of a mixture which contains sparingly soluble components, two phases are present in the mixing system. Consequently, the individual components do not dissolve at their maximum water solubility, but equilibrate (partition) between the hydrocarbon and water phases. For this reason, the composition of the water phase varies for each component with the loading rate. [10] Heavier petroleum products such as kerosines will show toxicity at those loadings where the combined toxicities of the components in solution equal or exceed threshold levels.

Exposure of aquatic organisms in a sealed test system with no headspace as in these studies, provides a constant aqueous concentration of each component. In the natural aquatic environment, very volatile substances would rapidly decline in concentration due to evaporation. In laboratory tests, aquatic concentrations would be likely to decline even more rapidly if the vessels were not sealed, given the high air surface area to volume ratio of test vessels. To develop a consistent approach to determining the concentration - toxicity relationship for chemicals, it is necessary to maintain constant concentrations. Such data may then be used to rank the relative toxicity of similar substances. Laboratory systems are rarely, if ever, truly reflective of the circumstances in the natural environment.

In the studies reported here, the ranges of results obtained for the three kerosine samples over the accepted periods that determine environmental classification were as follows:

fish (LL_{50}, 96h)	:	18-25 mg/l
Daphnia (EL_{50}, 48h)	:	1.4 - 21 mg/l
alga (IL_{50}, 72h, specific growth rate)	:	3.7 to 8.3 mg/l
alga (IL_{50}, 72h, area under growth curve)	:	4.3 to 15 mg/l

The compositions of the kerosines tested in this programme are all quite similar, particularly with regard to their content of alkylbenzenes and 2-ring aromatics (naphthalenes) which are the more water soluble constituents. Accordingly, they would be expected to have similar toxicities. In practice, for the three kerosine samples, the fish toxicity results are of the same order and the algal toxicity results are also similar. Only the Daphnia toxicity figure for sweetened kerosine is higher

than would be expected. From the results it is evident that the order of sensitivity to the three kerosines is Daphnia > algae> fish.

Although in these tests, great care was taken to prevent volatilization losses during exposure, the mixing system, of necessity had some headspace. It is important to standardize this aspect of test protocols, since for all hydrocarbon mixtures containing volatile components, the toxic constituents are likely to partition significantly to air. Accordingly, in conducting acute toxicity studies with volatile hydrocarbons, the headspace in the vessels should be kept as low as is practicable.

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FIGURE 1 **DIAGRAM OF ECOTOXICITY MIXING AND EXPOSURE SYSTEMS**