Acute aquatic toxicity of heavy fuel oils

summary of relevant test data

Prepared for CONCAWE's Ecology Group by:

M.I.H. Comber
K. den Haan
N. Djemel
C.V. Eadsforth
D. King
T. Parkerton
M. Leon Paumen
B. Dmytrasz (Technical Coordinator)

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ABSTRACT

This report describes the experimental procedures and results obtained in acute ecotoxicity tests on several heavy fuel oil (HFO) samples. Water accommodated fractions (WAFs) of these samples were tested for toxicity to the rainbow trout (*Oncorhynchus mykiss)*, the crustacean zooplankter (*Daphnia magna)* and green algae (*Selenastrum capricornutum).* These results assist in determining the environmental hazard from heavy fuel oil.

KEYWORDS

Ecotoxicity, fish, daphnia, algae, heavy fuel oil, OECD guidelines, lethal loading, water accommodated fractions.

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SUMMARY

A series of toxicity tests have been performed on several heavy fuel oil (HFO) samples. The toxicity tests were conducted on rainbow trout (*Oncorhynchus mykiss*), the crustacean zooplankter (*Daphnia magna)* and green algae (*Selenastrum capricornutum*) using OECD/EC methods. As HFOs are mixtures of poorly water soluble hydrocarbons, 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 79 to >1000 mg/l. The Daphnia 48 hour EL_{50} values ranged from 2.0 to >1000 mg/l. The algal 72 hour I_rL_{50} values (based on the specific growth rate (r) of algae) ranged from 0.75 to >107 mg/l. It is clearly evident that the order of sensitivity to the heavy fuel oil samples is algae > Daphnia > fish.

Biomimetic extraction (BE) proved to be a successful screening technique for differentiating between HFO samples with high and low levels of toxicity, though the correlation between BE ‘solubility’ data and toxicity proved to be best when applied to the daphnia data set.

1. INTRODUCTION

The available data on the ecotoxicity of the generic category of petroleum substances known as heavy fuel oils (HFOs) has been summarised in a CONCAWE dossier published in 1998 [1]. Most of the results relate to studies where water has been equilibrated with a sample of heavy fuel oil 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 are valid for classification purposes are those 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 were few acceptable ecotoxicity results for heavy fuel oils using WAFs and CONCAWE has recently embarked on a test programme to generate acute toxicity data for several heavy fuel oil samples from studies on fish, daphnia and algae. This report summarises all of the available WAF toxicity data.

2. HFO CATEGORY

Heavy fuel oil components (referred to as HFOs) constitute one of a number of groups of substances produced at refineries from petroleum feedstocks. Heavy fuel oil components are defined as:

> “Streams obtained as either distillates or residues from distillation and cracking processes and containing saturated, aromatic and olefinic hydrocarbons, > C8 and boiling range is 150- >750°C.”

The complex and variable composition of such UVCB substances (substances of Unknown or Variable compositions, Complex reaction products and Biological materials) means that it is not possible to define precisely their physico-chemical and environmental properties, but they will fall into a range, defined by the properties and concentrations of the individual hydrocarbons present. HFOs are viscous liquids (kinematic viscosity > 4.5 mm^2/s at 100°C) at normal temperature and pressure. Typical values for other physico-chemical parameters include <30°C (melting point), 0.84-1.2 kg/m^3 at 15ºC (absolute density) and 0.02 - 0.79 kPa at 120ºC for MW 330 – 550 (vapour pressure) [4].

The HFO category comprises 36 UVCBs, each with a unique CAS RN; see **Appendix 1** [5]. The EINECS (European INventory of Existing Commercial chemical Substances) definition associated with each CAS RN generally includes, *inter alia*, reference to main hydrocarbon types, boiling point and carbon number ranges, and the final processing step.

3. CHARACTERISATION OF TEST SUBSTANCES

Acute aquatic toxicity tests were performed on several heavy fuel oil samples obtained from various European refineries (**Table 1**). These test substances were selected to include a range of the refinery manufacturing processes represented in the HFO category (**Appendix 1**).

Table 1 HFO Samples Tested for Aquatic Toxicity

Descriptor	Substance	EINECS No.	CAS No.	Lab. Code No.
Light fuel oil	Fuel oil residual	270-675-6	68476-33-5	ST94/029
Heavy fuel oil	Fuel oil, residual	270-675-6	68476-33-5	ST94/064
Heavy fuel oil	Fuel oil residual	270-675-6	68476-33-5	MRD-07-909
Slurry	Clarified oils (petroleum), catalytic cracked	265-064-6	64741-62-4	MRD-07-910
Intermediate fuel oils 30-380	Fuel oil, residual	270-675-6	68476-33-5	MRD-07-911
Heavy fuel oil	Clarified oils (petroleum), catalytic cracked	265-064-6	64741-62-4	MRD-07-913
Flashed combined tar	Residues (petroleum), thermal cracked	265-081-9	64741-80-6	MRD-07-915

Two of these samples (Lab code ST/94/029 and ST/94/064) were both tested in 1994 [6,7]. For these samples, gas chromatography-mass spectroscopy (GC-MS) was used to analyse water accommodated fractions (WAFs) and confirm levels of dissolved hydrocarbons in the test media. These data are summarised here together with more recent data from five other samples.

The other five samples (Lab codes MRD-07-909, 910, 911, 913 & 915) were tested in 2008 [8-19]. For these samples, a biomimetic solid phase microextraction technique was used to analyse water accommodated fractions and confirm concentrations of dissolved hydrocarbons in the test media [20]. Detailed compositional analyses of these five samples has been reported using both a high-resolution approach involving comprehensive two-dimensional gas chromatography (GCxGC) and a low-resolution (i.e. Total Petroleum Hydrocarbon – TPH) approach involving simulated distillation (temperature programmed) gas chromatography with flame ionisation detection (GC-FID) [21]. GCxGC has previously been employed for the detailed characterization of complex middle-distillate fuels [23-25]. However, the upper volatility range of GCxGC currently limits its application to analysis of the lighter ($<C_{35}$) petroleum products and it is therefore recognised that this technique is unable to provide a comprehensive description of all the components present in HFOs, which typically have carbon numbers up to C_{50} [1].

In addition, these samples were also analysed for vanadium and nickel by inductively-coupled plasma atomic emission spectroscopy (ICP-AES) and for individual polyaromatic hydrocarbon compounds (PACs) by gas chromatography with flame ionisation detection (GC-FID) according to the Mobil PAC method [22].

A summary of the GCxGC compositional analysis together with the PAC and metal analyses are provided in **Table 2**.

Table 2 Compositional analysis of some HFO samples

Analysis	Heavy fuel oil CAS No 68476-33-5 Lab code MRD-07-909	Slurry CAS No 64741-62-4 Lab code MRD-07-910	Intermediate fuel oils 30-380 CAS No 68476-33-5 Lab code MRD-07-911	Heavy fuel oil CAS No 64741-62-4 Lab code MRD-07-913	Flashed combined tar CAS No 64741-80-6 Lab code MRD-07-915
n-Alkanes (%ww)	2.19	2.19	2.69	5.09	0.18
iso-Alkanes (%ww)	2.83	2.81	4.30	6.37	0.17
Naphthenics (%ww)	3.91	4.48	3.63	9.77	0.57
Aromatics (%ww)	18.39	31.92	14.59	14.30	1.36
Naphthenic Aromatics (%ww)	8.19	13.25	6.10	4.71	0.55
%1-7 ring PACs (%ww)	24.33	45.11	5.87	18.86	10.90
%3-7 ring PACs (%ww)	21.85	42.13	3.27	17.97	5.78
Vanadium (mg/kg)	10	<1	99	<1	35
Nickel (mg/kg)	13	<1	40	<1	19

Analysis of the GC-FID chromatograms using the TPH method showed that all 18 fractions (9 saturates and 9 aromatics) eluted fully from the GC-FID system. As all samples were fully eluting, the chromatograms were processed quantitatively by area normalisation following retention calibration using n-alkane marker compounds to define certain volatility bands [21]. The chromatograms were “sliced” according to volatility bands and the peak area was used to calculate the total concentration of hydrocarbons within each band. Total Petroleum Hydrocarbon (TPH) analyses identifying fractions within certain carbon number bands are shown in **Table 3**.

Table 3 Total Petroleum Hydrocarbon (TPH) analyses of some HFO samples (%m/m)

Analysis	Heavy fuel oil CAS No 68476-33-5 Lab code MRD-07-909	Slurry CAS No 64741-62-4 Lab code MRD-07-910	Intermediate fuel oils 30-380 CAS No 68476-33-5 Lab code MRD-07-911	Heavy fuel oil CAS No 64741-62-4 Lab code MRD-07-913	Flashed combined tar CAS No 64741-80-6 Lab code MRD-07-915
Total <n-C16	3.2	3.4	9.7	0.8	<0.1
Total <n-C21	12.8	17.4	19.9	5.7	0.1
Total <n-C35	44.0	66.6	33.3	59.6	6.0
Total >n-C35	29.7	11.0	30.8	22.7	43.0
Total	73.7	77.6	64.1	82.3	49.0

Note: The TPH analytical data shown refer to the analyses of non-polars (i.e. saturates and aromatics). The remaining components in these HFO samples are poorly soluble asphaltenes and other polars (e.g. resins)

4. TEST METHODS

4.1. GENERAL APPROACH

Mixtures of poorly water soluble, complex chemicals, e.g. petroleum products, 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 complex chemicals, un-dissolved 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 [26]. This does not apply to pure substances where the concentration will, if sufficient time is provided, equal the solubility limit when excess is added, regardless of the amount of excess. For poorly water soluble, complex chemicals, 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 un-dissolved substance [27]. A recognised guideline [28] 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 rates) 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} [29]. (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 [3]. It is also the approach specified by MARPOL for the marine pollution testing of poorly soluble mixtures [30] and by OECD for the aquatic toxicity testing of difficult substances and mixtures [31].

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 vessels. In preparing WAFs, some headspace is necessary to achieve adequate interfacial area and mixing. In each test measured amounts of test substance are added to measured amounts of the appropriate test medium (for fish, daphnia and algae). The vessels containing the medium and the test substance are then sealed leaving only a small headspace, and the contents stirred with a 1-2 cm vortex depth for a set period of time, previously determined to be sufficient for the aqueous and test substance phases to equilibrate. After stirring, the contents of the vessels are left to stand to allow any un-dissolved material to separate out. The aqueous phases - the WAFs - are then drawn off for use in the tests. Control media are subjected to the same regime but do not contain the test substance.

It is important that mixing is sufficient to ensure that the aqueous phase is in equilibrium with the un-dissolved hydrocarbon phase. Mixing needs to be slow enough not to cause dispersion or emulsification of the un-dissolved 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 - 72 hours. After mixing for this period, solutions were allowed to stand for 1 hour before use in order to facilitate phase separation. The mixing vessel was either fitted with a tap at the bottom of the vessel or contained a glass tube for siphoning off the water phase, without contamination by the surface layer of undissolved hydrocarbon.

The exposure vessels for the fish test were typically a cylindrical bottle (volume 4.5 L) and with a stopcock at the bottom for removing liquid. 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).

All the studies were conducted in accordance with the principles of Good Laboratory Practice (GLP).

4.2. SCREENING STUDIES USING BIOMIMETIC EXTRACTION

Prior to any toxicity testing using fish, daphnia or algae in the recent CONCAWE test programme, a screening exercise which involved analyzing WAF samples of nine heavy fuel oil products by the Solid Phase Microextraction-Biomimetic Extraction (SPME-BE) method [32]. WAFs were prepared at a loading rate of 100 mg/L and mixed for 48 hours. Samples were analysed in duplicate by GC-FID.

The sample aliquots of ca 20 mL taken directly from WAF systems were placed in septum sealed glass vials without any headspace and placed in an auto-sampler configured for automated SPME injections. A 30 µm polydimethyl siloxane (PDMS) SPME fibre (0.132 µL) was equilibrated with each sample for 100 minutes at 30ºC with rapid agitation (250 rpm) and without any headspace. A single fibre was used for all automated sample analyses [20].

The SPME and liquid hydrocarbon calibration samples were analysed by GC-FID on a 15 m x 0.53 mm id capillary column with 1.5 µm Rtx-1 stationary phase (Restex). The SPME-BE method was calibrated by making 1 µL injections of a series of aromatic hydrocarbon standard solutions. The molar response factor of 2,3-dimethylnaphthalene was used for converting the observed GC-FID response to nanomoles of organic constituents on the PDMS fibre. Fibre results were normalized to the volume of PDMS and reported as micromoles (µmol) as 2,3-dimethylnaphthalene / millilitre (mL) PDMS.

Based on this first set of results, further biomimetic extraction analyses were performed in a second study on selected HFO samples (3) at lower loading rates (1 and 10 mg/L).

4.3. FISH ACUTE STUDIES

The fish acute toxicity tests were conducted in accordance with OECD Guideline 203 (equivalent to EC methods for the determination of ecotoxicity, C1 - Acute toxicity for fish). 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 [6-11]. The fish used for the studies were 4 to 5 cm in length (mean weight range 0.55 – 1.3 grams) and were not fed during the exposure period. The mean loading of fish was typically 0.7 g/L. For definitive studies, either a single test involving 7 fish [6,7] or two replicates each involving 5 fish per test [8-11] were evaluated at each loading rate.

Fresh WAFs were prepared on a daily basis and used for daily renewal of the exposure medium (semi-static). Renewals were done by emptying most of the water (typically 80 – 100%) from the bottom port on each exposure vessel and then expeditiously re-filling by siphon from the mixing vessel. Analysis of WAFs was conducted either by solvent extraction with dichloromethane followed by GC/MS on all fresh and discarded WAFs (n=8) [6,7] or by solid phase microextraction (SPME) using a PDMS fibre (30 µm) followed by GC/FID on t=0 and 48 hour samples [8-11]. For GC/MS analysis, all total peak areas (TPA) of all the peaks were summed and results standardised as TPA values per 500 mL WAF. With the GC/FID analysis, the SPME fibre was analysed together with a series of aromatic hydrocarbons and the molar response of 2,3-dimethylnaphthalene was used for translating the observed GC/FID response to nanomoles of organic constituent on fibres. Fibre results were normalised to the volume of PDMS and reported as micromoles as 2,3-dimethylnaphthalene.

The total exposure periods were 96 hours. Water hardness was either 88-90 mg/L (as calcium carbonate, $CaCO_3$) with a pH of 7.6 ± 0.1 [8-11] or 262-292 mg/L (as $CaCO_3$) with a pH of 7.4 ± 0.4 [6,7]. The temperature was 14°C ± 2°C and the light duration was 16 hours, normally at 440 lux [8-11]. Dissolved oxygen was >20 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.

4.4. DAPHNIA ACUTE STUDIES

These tests were carried out in accordance with OECD Guideline 202, Part I (equivalent to EC methods for the determination of ecotoxicity, C2 - Acute toxicity for Daphnia). The test species was *Daphnia magna*, a freshwater invertebrate commonly used for toxicity testing. Details of the husbandry and selection of test organisms are provided in the laboratory reports [6-11]. The organisms used for testing were less than 24 hour old neonates, from parents ranging from 12 – 28 days. For definitive studies, either four replicates, each involving 5 organisms [8-11], or two replicates, each involving 10 organisms [6,7], were tested at each loading rate. The exposure period was 48 hours.

Reconstituted water was used for the daphnia studies. WAFs were prepared in the same manner using the same equipment and analyses but were not renewed daily as for the fish (i.e. static tests). The WAFs were siphoned into sealed flasks, typically 125-150 mL and the daphnia were introduced. The light duration was 16 hours at typically in the range 420 - 1250 lux [8-11]. No reductions in dissolved oxygen concentration (range 7.7 – 8.9 mg/L) or pH (range 7.4 – 8.2) 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. WAFs were analyzed at the beginning and end of the exposure by SPME [8-11] or by solvent extraction followed by GC-MS [6,7].

4.5. ALGAL GROWTH INHIBITION STUDIES

The algal growth studies were conducted in accordance with OECD guideline 201 (equivalent to EC methods for the determination of ecotoxicity, C3 – Algal inhibition test). The test species was *Pseudokirchneriella subcapitata* [8-11] (alternatively known as *Selenastrum capricornutum) and Raphidocelis subcapitata* [6,7] (also alternatively known as *Selenastrum capricornutum*). Details of the culture methods are provided in the laboratory reports [6-11]. The algae used were taken from 4-5 day old stock cultures in the log phase of growth. Initial concentrations were typically 0.5 - 1.0 x 10^4 cells/mL in each replicate test chamber. The exposure period was 72 hours.

WAFs were prepared in algal growth medium. WAFs were prepared in the same manner using the same equipment and analyses but were not renewed daily (i.e. static tests). WAFs were analyzed at the beginning of the test period, and again on a composite from test flasks after 72 hours by SPME [8-11] or by solvent extraction followed by GC-MS [6,7]. Test vessels, typically 140 mL [8-11] or 300 mL [6,7], were filled completely with inoculated WAF and then closed with ground glass stoppers. Replicate vessels were set up for each treatment and the control to facilitate daily algal cell counting and pH measurements. The flasks were incubated at 21 - 25°C on an orbital shaker, at 100 cycles/min or rpm. Lighting was continuous and in the range of 7000 to 9000 Lux. Cell counts were determined at 24, 48, and 72 hours using a Coulter Multisizer [6,7] or a haemacytometer and microscope [8-11]. The pH changes during these studies were within the range 7.5 – 9.6. On occasion there were pH changes greater than the target for change (< 1.0); these were a result of the growth of cultures and could not be avoided.

5. RESULTS

5.1. SCREENING STUDIES

A summary of the biomimetic extraction results for a series of heavy fuel samples is shown in **Table 4**. On the basis of these screening data, HFO samples with positive BE results (#3, 4, 5 and 7) together with a negative BE result (#9) were taken forward for further ecotoxicity testing.

Table 4 Summary of BE screening results for all nine HFO products

Sample	Laboratory sample reference	Sample No.	Loading (mg/L)	Mean BE result (as µmol 2,3-DiMeNaph per mL PDMS) +
Distillates (petroleum), light vacuum CAS No 70592-77-7	MRD-07-907	HFO #1	100	ND *
Distillates (petroleum), vacuum CAS No 70592-58-8	MRD-07-908	HFO #2	100	ND *
Heavy fuel oil CAS No 68476-33-5	MRD-07-909	HFO #3	1 10 100	3.70 13.5 21.4
Slurry CAS No 64741-62-4	MRD-07-910	HFO #4	1 10 100	8.47 18.3 30.3
Intermediate fuel oils 30-380 CAS No 68476-33-5	MRD-07-911	HFO #5	1 10 100	3.44 29.6 74.3
Heavy fuel oil CAS No 64741-75-9	MRD-07-912	HFO #6	100	1.50
Heavy fuel oil CAS No 64741-62-4	MRD-07-913	HFO #7	100	3.38
Heavy fuel oil CAS No 64741-81-7	MRD-07-914	HFO #8	100	1.73
Flashed combined tar CAS No 64741-80-6	MRD-07-915	HFO #9	100	ND *

\+ Fibre results for hydrocarbon components in the WAFs are reported as micromoles (µmol) of the standard 2,3-dimethylnaphthalene/ millilitre PDMS
* ND = Not Detected

5.2. HFO ECOTOXICITY DATA

A summary of all the relevant ecotoxicity data from studies of heavy fuel oil samples on fish, daphnia and algae generated using WAFs are summarised in **Table 5**. These include previous data [6, 7] as well as data from the recent CONCAWE test programme [8 – 19].

Table 5 Summary of all the HFO sample ecotoxicity data for fish, daphnia and algae

Name	Fish LL_{50} (mg/L)	Daphnia EL_{50} (mg/L)	Algae I_rL_{50} (mg/L)	Study (F/D or A) and reference
Light fuel oil CAS No 68476-33-5	>1000	>1000	100-300 **	F/D/A – Shell [6]
Heavy fuel oil CAS No 68476-33-5	100-1000 **	220-460 **	30-100 **	F/D/A – Shell [7]
Heavy fuel oil CAS No 68476-33-5	>96	2.0	1.5-6.3 **	F - EMBSI [11] D - EMBSI [12] A - EMBSI [18]
Slurry CAS No 64741-62-4	>94	3.2	1.0-4.0 **	F - EMBSI [9] D - EMBSI [13] A - EMBSI [17]
Intermediate fuel oils 30-380 CAS No 68476-33-5	79	10	7.3-22 **	F - EMBSI [8] D - EMBSI [14] A - EMBSI [19]
Heavy fuel oil CAS No 64741-62-4	>95	>99	0.75 + (0.6-1.3) **	F- EMBSI [10] D- EMBSI [15] A- EMBSI [16]
Flashed combined tar CAS No 64741-80-6	>98	>95	>107	F - EMBSI [10] D - EMBSI [15] A - EMBSI [16]

** Assignment of the LL_{50}, EL_{50} or I_rL_{50} values is based on the two loading rates which straddle the 50% effect.

\+ A statistical evaluation of the data by probit analysis produced a definitive result of 0.75 mg/L

5.3. 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. The 96 hour LL_{50} values (**Table 5**) range from 79 mg/L to >1000 mg/L.

Using biomimetic extraction and GC-FID analysis of dissolved hydrocarbon components of heavy fuel oil samples from the "Day 0" and "Day 2" WAFs [8-11] from the fish studies indicated that a mean reduction of 69% (n = 12, range 46 - 86%) in these dissolved hydrocarbon levels. For other studies, solvent extraction of WAFs and subsequent GC-MS analysis of the dichloromethane extracts of "freshly prepared" and "24 hour old" WAFs [6,7] from the fish acute studies indicated that a mean reduction of 22% (n = 18, range 0 - 57%) in these dissolved hydrocarbon levels. In all cases, the WAFs were prepared in sealed vessels with a minimum headspace of air and the subsequent test vessels were completely filled and sealed so loss of volatile components was minimised. The cause of the reduction could be due to degradation of these components as well as sorption to surfaces during sampling and analysis.

The analytical data relating to the WAFs are included in the separate laboratory reports [6-11].

5.4. 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 (**Table 5**) exhibit a wide range of toxicity values from 2.0 - >1000 mg/L.

Using biomimetic extraction and GC-FID analysis of dissolved hydrocarbon components of heavy fuel oil samples from the "Day 0" and "Day 2" WAFs [8-11] from the Daphnia studies indicated that a mean reduction of 7% (n = 16, range 0 - 18%) in these dissolved hydrocarbon levels. For other studies, solvent extraction of WAFs and subsequent GC-MS analysis of the dichloromethane extracts of "freshly prepared" and "48 hour old" WAFs [6,7] from the daphnia acute studies indicated that a mean reduction of 28% (n = 5, range 0 - 81%) in these dissolved hydrocarbon levels. In all cases, the WAFs were prepared in sealed vessels with a minimum headspace of air and the subsequent test vessels were completely filled and sealed so loss of volatile components was minimised. The cause of the reduction could be due to degradation of these components as well as sorption to surfaces during sampling and analysis.

The analytical data relating to the WAFs are included in the separate laboratory reports [6-11].

5.5. ALGAL TOXICITY STUDIES

Acute toxicity results are expressed as the effect loading 50 (E_rL_{50}); that is the loading rate of test substance in dilution water which results in a 50% reduction in growth derived from the average specific growth rate (r) relative to the control for the specified time of exposure.

The 72 hour growth inhibition for each substance loading rate/concentration was estimated based on the percent inhibition relative to the control. The specific growth rate for each loading rate/concentration was determined by calculating the slope of the regression line of the ln(cell density) versus time using the PROC REGRESSION procedure from SAS [33]. The average specific growth rate was calculated in accordance with the formula listed in the OECD Guideline 201. The 72 hour E_rL_{50} values (**Table 5**) exhibit a wide range of toxicity values from 0.75 - >107 mg/L.

Using biomimetic extraction and GC-FID analysis of dissolved hydrocarbon components of heavy fuel oil samples from the "Day 0" and "Day 3" WAFs [8-11] from the algae studies indicated that a mean reduction of 53% (n = 18, range 10 - 90%) in these dissolved hydrocarbon levels over the 72 hour period. For other studies, solvent extraction of WAFs and subsequent GC-MS analysis of the dichloromethane extracts of "freshly prepared" and "72 hour old" WAFs [6,7] from the algae studies indicated that a mean reduction of 30% (n = 9, range 17 - 67%) in these dissolved hydrocarbon levels over the 72 hour period. In all cases, the WAFs were prepared in sealed vessels with a minimum headspace of air and the subsequent test vessels were completely filled and sealed so loss of volatile components was minimised. The cause of the reduction could be due to degradation of these components as well as sorption to surfaces during sampling and analysis.

The analytical data relating to the WAFs are included in the separate laboratory reports [6-11].

6. DISCUSSION

Heavy fuel oils are produced by a range of refinery processes and their composition is highly variable. As a result, the ecotoxicity associated with these products varies. The aqueous solubilities of all the hydrocarbon components of heavy fuels oils are very low with the lower molecular weight components showing the greatest solubilities [1]. Aromatic hydrocarbons and polar compounds are considered to be responsible for the acute toxicity effects on organisms.

Most early studies of the aquatic toxicity of heavy fuel oils originated from experiments in which water-soluble fractions (WSFs) were used. These were prepared by mixing a large volume of fuel oil with water, and diluting these with water to produce the test media. It is now recognised that such studies are unacceptable and that studies must be carried out using water accommodated fractions (WAFs) based on a range of different loading rates, without any subsequent dilution [3].

When preparing a water accommodated 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 [26]. Petroleum products such as heavy fuel oils will show toxicity at those loadings where the combined toxicities of the components in solution equal or exceed threshold levels.

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 standardise 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.

In the studies reported here, the ranges of results obtained for the seven heavy fuel oil samples over the accepted periods that determine environmental classification were as follows:

fish (LL_{50}, 96h)	:	79 - >1000 mg/L
Daphnia (EL_{50}, 48h)	:	2.0 - >1000 mg/L
alga (I_rL_{50}, 72h, specific growth rate)	:	0.75 - >107 mg/L

It is noticeable that the flashed combined tar sample which has minimal TPH content in the range <n-C_{21} is practically non-toxic to all aquatic species tested. There are noticeable differences in the compositions of the heavy fuel oil samples analysed in this programme, particularly with regard to their content of naphthenics (range 0.6 – 9.8%ww), aromatics (range 1.4 – 31.9%ww), and naphthenic aromatics (range 0.6 – 13.3%ww). Similarly, there are wide differences in the levels of 3-7 ring PACs (range 5.8 – 42.1%ww); here it is noticeable that the three HFO samples with the highest algal toxicity are those with the highest PAC content.

In practice, from the toxicity results it is evident that the order of sensitivity to the heavy fuel oil samples is algae > Daphnia > fish.

Biomimetic extraction has proved to be a successful screening technique for differentiating between HFO samples with higher levels of water-soluble hydrocarbons (i.e. highest toxicity) and those with lowest levels or non-detectable amounts (i.e. no/low toxicity). However, when assessing the results for samples #3, 4, 5, 7 and 9 (see **Table 4**), it would appear that the BE data correlate better with the daphnia EL_{50} data than with the algae I_rL_{50} data.

7. GLOSSARY

BE	Biomimetic Extraction
CAS RN	Chemical Abstracts Service Registry Number
EINECS	European Inventory of Existing Commercial Chemical Substances
EL	Effective Loading
EL_{50}	Loading Rate of Test Substance (in dilution water) which causes adverse effects in 50% of the exposed population
E_rL_{50}	Effective Loading rate of Test Substance (in dilution water) which causes a 50% inhibition of growth rate (r) of algae
GC-FID	Gas Chromatograph with Flame Ionisation Detection
GC-MS	Gas Chromatograph coupled with Mass Spectrometry
GCxGC	Two-Dimensional Gas Chromatography
GESAMP	Group of Experts on the Scientific Aspects of Marine Pollution
GLP	Good Laboratory Practice
HFOs	Heavy Fuel Oil Components
ICP-AES	Inductively-Coupled Plasma Atomic Emission Spectroscopy
IL	Inhibitory Loading
I_rL_{50}	Loading Rate of Test Substance (in dilution water) which causes a 50% inhibition of growth rate (r) of algae
LC_{50}	Concentration killing 50% of organisms
LL	Lethal Loading
LL_{50}	Loading Rate of Test Substance (in dilution water) which causes lethal effects in 50% of the exposed population
MARPOL	Maritime Pollution
mL	Millilitre
MW	Molecular Weight
OECD	Organisation for Economic Co-operation and Development

PACs	Polycyclic Aromatic Compounds
PDMS	Polydimethyl Siloxane
rpm	revolutions per minute
SAS	Statistical Analysis System
SPME	Solid Phase Microextraction
TPH	Total Petroleum Hydrocarbon
UVCB	Substances of Unknown or Variable Composition, Complex Reaction Products or Biological Materials
WAFs	Water Accommodated Fractions
WSF	Water Soluble Fraction

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APPENDIX 1

Heavy Fuel Oil component – Category Members

CAS RN	EINECS#	CAS name	EINECS description
68476335	2706756	**Fuel oil, residual**	The liquid product from various refinery streams, usually residues. The composition is complex and varies with the source of the crude oil.
68553004	2713847	**Fuel oil, no. 6**	A distillate oil having a minimum viscosity of 900 SUS at 37.7º C (100º F)to a maximum of 9000 SUS at 37.7º C (100º F).
92045142	2953967	**Fuel oil, heavy, high-sulfur**	A complex combination of hydrocarbons obtained by the distillation of crude petroleum. It consists predominantly of aliphatic, aromatic and cycloaliphatic hydrocarbons having carbon numbers predominantly higher than C25 and boiling above approximately 400º C (752º F).
93821660	2987540	**Residual oils (petroleum)**	A complex combination of hydrocarbons, sulfur compounds and metal containing organic compounds obtained as the residue from refinery fractionation cracking processes. It produces a finished oil with a viscosity above 2cSt. at 100º C.
64741453	2650452	**Residues (petroleum), atm. Tower**	A complex residuum from the atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly greater than C20 and boiling above approximately 350º C (662º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
64741577	2650583	**Gas oils (petroleum), heavy vacuum**	A complex combination of hydrocarbons produced by the vacuum distillation of the residuum from atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly in the range of C20 through C50 and boiling in the range of approximately 350º C to 600º C (662º F to 1112º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
64741613	2650630	**Distillates (petroleum), heavy catalytic cracked**	A complex combination of hydrocarbons produced by the distillation of products from a catalytic cracking process. It consists of hydrocarbons having carbon numbers predominantly in the range of C15 through C35 and boiling in the range of approximately 260º C to 500º C (500º F to 932º F). This stream is likely to contain 5 wt. % or more of 4- to 6- membered condensed ring aromatic hydrocarbons.
64741624	2650646	**Clarified oils (petroleum), catalytic cracked**	A complex combination of hydrocarbons produced as the residual fraction from distillation of the products from a catalytic cracking process. It consists of hydrocarbons having carbon numbers predominantly greaterthan C20 and boiling above approximately 350º C (662º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
64741679	2650693	**Residues (petroleum), catalytic reformer fractionator**	A complex combination of hydrocarbons produced as the residual fraction from distillation of the product from a catalytic reforming process. It consists of predominantly aromatic hydrocarbons having carbon numbers predominantly in the range of C10 through C25 and boiling in the range of approximately 160º C to 400º C (320º F to 725º F). This stream is likely to contain 5 wt. % or more of 4- or 6-membered condensed ring aromatic

CAS RN	EINECS#	CAS name	EINECS description
			hydrocarbons.
64741759	2650761	**Residues (petroleum), hydrocracked**	A complex combination of hydrocarbons produced as the residual fraction from distillation of the products of a hydrocracking process. It consists of hydrocarbons having carbon numbers predominantly greater than C20 and boiling above approximately 350º C (662º F).
64741806	2650819	**Residues (petroleum), thermal cracked**	A complex combination of hydrocarbons produced as the residual fraction from distillation of the product from a thermal cracking process. It consists predominantly of unsaturated hydrocarbons having carbon numbers predominantly greater than C20 and boiling above approximately 350º C (662º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
64741817	2650824	**Distillates (petroleum), heavy thermal cracked**	A complex combination of hydrocarbons from the distillation of the products from a thermal cracking process. It consists predominantly of unsaturated hydrocarbons having carbon numbers predominantly in the range of C15 through C36 and boiling in the range of approximately 260º C to 480º C (500º F to 896º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
64742592	2651629	**Gas oils (petroleum), hydrotreated vacuum**	A complex combination of hydrocarbons obtained by treating a petroleum fraction with hydrogen in the presence of a catalyst. It consists of hydrocarbons having carbon numbers predominantly in the range of C13 through C50 and boiling in the range of approximately 230º C to 600º C (446º F to 1112º F). This stream is likely to contain 5 wt. % or more of 4- to 6- membered condensed ring aromatic hydrocarbons.
64742785	2651812	**Residues (petroleum), hydrodesulfurized atmospheric tower**	A complex combination of hydrocarbons obtained by treating an atmospheric tower residuum with hydrogen in the presence of a catalyst under conditions primarily to remove organic sulfur compounds. It consists of hydrocarbons having carbon numbers predominantly greater than C20 and boiling above approximately 350º C (662º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
64742865	2651896	**Gas oils (petroleum), hydrodesulfurized heavy vacuum**	A complex combination of hydrocarbons obtained from a catalytic hydrodesulfurization process. It consists of hydrocarbons having carbon numbers predominantly in the range of C20 through C50 and boiling in the range of approximately 350º C to 600º C (662º F to 1112º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
68333222	2697773	**Residues (petroleum), atmospheric**	A complex residuum from atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly greater than C11 and boiling above approximately 200º C (392º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
68333266	2697820	**Clarified oils (petroleum), hydrodesulfurized catalytic cracked**	A complex combination of hydrocarbons obtained by treating catalytic cracked clarified oil with hydrogen to convert organic sulfur to hydrogen sulfide which is removed. It consists of hydrocarbons having carbon numbers predominantly greater than C20 and boiling above approximately 350º C (662º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic

CAS RN	EINECS#	CAS name	EINECS description
			hydrocarbons.
68333277	2697836	**Distillates (petroleum), hydrodesulfurized intermediate catalytic cracked**	A complex combination of hydrocarbons obtained by treating intermediate catalytic cracked distillates with hydrogen to convert organic sulfur to hydrogen sulfide which is removed. It consists of hydrocarbons having carbon numbers predominantly in the range of C11 through C30 and boiling in the range of approximately 205º C to 450º C (401º F to 842º F). It contains a relatively large proportion of tricyclic aromatic hydrocarbons.
68333288	2697841	**Distillates (petroleum), hydrodesulfurized heavy catalytic cracked**	A complex combination of hydrocarbons obtained by treatment of heavy catalytic cracked distillates with hydrogen to convert organic sulfur to hydrogen sulfide which is removed. It consists of hydrocarbons having carbon numbers predominantly in the range of C15 through C35 and boiling in the range of approximately 260º C to 500º C (500º F to 932º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
68476324	2706740	**Fuel oil, residues-straight-run gas oils, high-sulfur**	
68478137	2707922	**Residues (petroleum), catalytic reformer fractionator residue distn.**	A complex residuum from the distillation of catalytic reformer fractionator residue. It boils approximately above 399º C (750º F).
68478171	2707964	**Residues (petroleum), heavy coker gas oil and vacuum gas oil**	A complex combination of hydrocarbons produced as the residual fraction from the distillation of heavy coker gas oil and vacuum gas oil. It predominantly consists of hydrocarbons having carbon numbers predominantly greater than C13 and boiling above approximately 230º C (446º F).
68512618	2709830	**Residues (petroleum), heavy coker and light vacuum**	A complex combination of hydrocarbons produced as the residual fraction from the distillation of heavy coker gas oil and light vacuum gas oil. It consists predominantly of hydrocarbons having carbon numbers predominantly greater than C13 and boiling above approximately 230º C (446º F).
68512629	2709846	**Residues (petroleum), light vacuum**	A complex residuum from the vacuum distillation of the residuum from the atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly greater than C13 and boiling above approximately 230º C (446º F).
68607307	2717637	**Residues (petroleum), topping plant, low-sulfur**	A low-sulfur complex combination of hydrocarbons produced as the residual fraction from the topping plant distillation of crude oil. It is the residuum after the straight-run gasoline cut, kerosene cut and gas oil cut have been removed.
68783084	2721842	**Gas oils (petroleum), heavy atmospheric**	A complex combination of hydrocarbons obtained by the distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly in the range of C7 through C35 and boiling in the range of approximately 121º C to 510º C (250º F to 950º F).
68783131	2721879	**Residues (petroleum), coker scrubber, condensed-ring-arom.-contg.**	A very complex combination of hydrocarbons produced as the residual fraction from the distillation of vacuum residuum and the products from a thermal cracking process. It consists predominantly of hydrocarbons having carbon numbers predominantly greater than C20 and boiling above approximately 350º C (662º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
68955271	2732634	**Distillates (petroleum),**	A complex combination of hydrocarbons produced

CAS RN	EINECS#	CAS name	EINECS description
		petroleum residues vacuum	by the vacuum distillation of the residuum from the atmospheric distillation of crude oil.
70592766	2746830	**Distillates (petroleum), intermediate vacuum**	A complex combination of hydrocarbons produced by the vacuum distillation of the residuum from atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly in the range of C14 through C42 and boiling in the range of approximately 250º C to 545º C (482º F to 1013º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
70592777	2746846	**Distillates (petroleum), light vacuum**	A complex combination of hydrocarbons produced by the vacuum distillation of the residuum from atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly in the range of C11 through C35 and boiling in the range of approximately 250º C to 545º C (482º F to 1013º F).
70592788	2746851	**Distillates (petroleum), vacuum**	A complex combination of hydrocarbons produced by the vacuum distillation of the residuum from atmospheric distillation of crude oil. It consists of hydrocarbons having carbon numbers predominantly in the range of C15 through C50 and oiling in the range of approximately 270º C to 600º C (518º F to 1112º F). This stream is likely to contain 5 wt. % or more of 4- to 6-membered condensed ring aromatic hydrocarbons.
85117039	2855559	**Gas oils (petroleum), hydrodesulfurized coker heavy vacuum**	A complex combination of hydrocarbons obtained by hydrodesulfurization of heavy coker distillate stocks. It consists predominantly of hydrocarbons having carbon numbers predominantly in the range C18 to C44 and boiling in the range of approximately 304º C to 548º C (579º F to 1018º F). Likely to contain 5% or more of 4- to 6- membered condensed ring aromatic hydrocarbons.
90669764	2926582	**Residues (petroleum), vacuum, light; Heavy fuel oil**	A complex residuum from the vacuum distillation of the residuum from atmospheric distillation of crude oil. It consists predominantly of hydrocarbons having carbon numbers predominantly greater than C24 and boiling above approximately 390°C.)
92061977	2955110	**Residues (petroleum), catalytic cracking**	A complex combination of hydrocarbons produced as the residual fraction from the distillation of the products from a catalytic cracking process. It consists predominantly of hydrocarbons having carbon numbers predominantly greater than C11 and boiling above approximately 200º C (392º F).
92201597	2959906	**Distillates (petroleum), intermediate catalytic cracked, thermally degraded**	A complex combination of hydrocarbons produced by the distillation of products from a catalytic cracking process which has been used as a heat transfer fluid. It consists predominantly of hydrocarbons boiling in the range of approximately 220º C to 450º C (428º F to 842º F). This stream is likely to contain organic sulfur compounds.
101316578	3098630	**Distillates (petroleum), hydrodesulphurized full-range middle; Heavy fuel oil**	A complex combination of hydrocarbons obtained by treating a petroleum stock with hydrogen. It consists predominantly of hydrocarbons having carbon numbers predominantly in the range of C9 through C25 and boiling in the range of approximately 150°C to 400°C

CONCAWE
Boulevard du Souverain 165
B-1160 Brussels
Belgium

Tel: +32-2-566 91 60
Fax: +32-2-566 91 81
e-mail: info@concawe.org
website: http://www.concawe.org