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Assessing the aquatic toxicity of petroleum products: comparison of PETROTOX calculations and SPME-GC screening

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Assessing the aquatic toxicity of petroleum products: comparison of PETROTOX calculations and SPME-GC screening

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ABSTRACT

Using detailed two-dimensional chromatography (GCxGC) analysis of a set of petroleum product samples of Gas Oils, Residual Aromatic Extracts (RAE) and Bitumen categories, PETROTOX predictions have provided information to support revised category justification documents and enable the selection of “worst case” products in each category for ecotoxicity testing.

In addition, analysis of Water Accommodated Fractions (WAFs) of these product samples using Biomimetic Extraction (BE) with solid phase microextraction (SPME) fibres was used to confirm that SPME data correlates to Toxic Units predicted by the PETROTOX model using GCxGC compositional data, thereby strengthening the linkage between composition, SPME data and aquatic toxicity. This provides a technical basis for further use of SPME as a more practical characterization tool for addressing the influence of variation in substance composition on aquatic toxicity within petroleum product categories as SPME correlates well with PETROTOX calculations and consistent TU-dose response relationships between algae and *daphnia* are observed.

BE-SPME is shown to be a cost-effective approach to toxicity screening for petroleum substances, and thus an alternative method to enhance currently available ecotoxicity data sets, as well as complement predicted ecotoxicity using PETROTOX.

KEYWORDS

SPME, petroleum hydrocarbons, UVCB, ecotoxicity, screening

INTERNET

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CONTENTS

SUMMARY

A series of ecotoxicity predictions and tests have been performed on “worst case” representative gas oils using solid phase micro-extraction (SPME), the PETROTOX model, and OECD test methods. Since test substances were complex, poorly water soluble petroleum substances, they were tested as water accommodated fractions (WAFs) in sealed test vessels. Test substances were equilibrated with water at each loading rate and the water phase (i.e. the WAF) tested for toxicity. Toxicity results were expressed as effective loading (EL_{50}) in PETROTOX predictions and *daphnia* tests, and effective loading on growth rate (E_rL_{50}) and yield (E_yL_{50}) in algae tests, to cause a 50% response.

Screening studies using SPME and PETROTOX identified two 'other gas oils' (OGOs), two 'straight run gas oils' (SRGOs), and five 'vacuum gas oil, hydrocracked gas oil and distillate fuels' (VHGO), as “worst case” representatives of each group. Gas oils were tested for acute dose toxicity in the crustacean zooplankter *Daphnia magna* and the algae, *Pseudokirchneriella subcapitata* (also known as *Selanastrum capricornutum*) using WAFs. Acute toxicity limit tests were also performed on other representative VHGO, bitumen, and residual aromatic extract (RAE) samples.

Comparison of predicted toxicity ranking shows that SPME results complement PETROTOX predictions (i.e. highly bioavailable samples show high predicted toxicity in *daphnids*), thereby supporting SPME as a mechanistic surrogate measure of bioavailability. Acute *Daphnia* toxicity tests of representative gas oils show 48 hour EL_{50} values ranging from 38 to 153 mg/l in OGOs, 280 to 678 mg/l in SRGOs, and 4.3 to <2636 mg/l in VHGOs. The algal 72 hour E_rL_{50} values range from 64 to 140 mg/l in OGOs, 24 to 75 mg/l in SRGOs, and 7.9 to >1026 (cnc) mg/l in VHGOs, based on specific growth rate. No toxicity ($E(_r)L_{50}$ values >1000 mg/l) was observed in both *Daphnia* and algae acute limit studies with selected VHGO, bitumen and RAE. Predicted PETROTOX EL_{50} values showed a positive correlation with experimental data and were comparatively more conservative than experimental equivalents, thus supporting the model's applicability as a preliminary indicator of petroleum based UVCB hazard assessment.

1. INTRODUCTION

Meaningful UVCB substance (Unknown or Variable composition, Complex reaction products and Biological materials) characterization requires detailed information on the chemical composition on the substances under examination. Two-dimensional chromatography (GCxGC) analysis can provide such information, which can then be fed into PETROTOX, a spreadsheet model that relates petroleum substance composition to aquatic toxicity. This model has been verified using compositional and aquatic effect data for over 100 individual substances, across more than 15 major substance categories (e.g. fuels, lubricants, bitumen, etc.). Toxicity is considered additive and is modelled using toxic units (TU) based on the Target Lipid Model (TLM), and is assumed to be due to interaction of test organisms with dissolved phase hydrocarbons. Biomimetic extraction (BE) techniques such as solid phase micro-extraction (SPME) also depend on detection of dissolved phase hydrocarbons and therefore have the potential to be an effective toxicity screening tool.

When coupled with passive sampling methods such as BE-SPME, PETROTOX may be used to streamline testing programs by identifying candidate “worst case” test substances and test concentrations.

Acute toxicity data were generated on nine gas oil samples using *Daphnia* and algae studies, with the aim of developing new hazard data to support classification, and to validate PETROTOX and BE-SPME screening procedures. The categories tested included; other gas oils (OGO), straight run gas oil (SRGO) and vacuum gas oil, hydrocracked gas oil and distillate fuels (VHGO). In addition, acute *Daphnia* and algae limit tests were also performed on VHGO, bitumen and residual aromatic extract (RAE), thereby allowing a total of 12 products to be tested (i.e. nine in dose response tests and three in limit tests).

2. GAS OILS CATEGORY

Gas oils are middle distillate fuels obtained from crude oil via various refining processes. The EINECS (European INventory of Existing Commercial chemical Substances) contains 69 gas oil entries in total. This includes 9 straight-run gas oils, 18 cracked gas oils, 2 hydrocracked gas oils, 27 other gas oils, 4 distillate fuel oils, and 11 vacuum gas oils. Gas oils predominantly consist of C_9 to C_{30} hydrocarbons and have a boiling range from 145 to 450°C. Straight-run and vacuum gas oils typically contain 70-80% aliphatics 20-30% aromatics and <5% olefins, whereas cracked gas oils may contain up to 75% aromatics and up to 10% olefins (CONCAWE,1996). Since part of gas oils distil at temperatures in excess of 350°C, they may contain minor concentrations of 4 to 6 ring polycyclic aromatic hydrocarbons (CONCAWE, 2001).

Gas Oils have been allocated to six groups as follows:

- **Straight-run gas oils**: obtained by atmospheric distillation of crude oil
- **Vacuum gas oil**: distillates obtained by vacuum distillation of the residues left after the atmospheric distillation of crude oil.
- **Hydrocracked gas oils**: obtained from refinery feedstocks by simultaneous processes of cracking and hydrogenation.
- **Cracked gas oils**: obtained from refinery feedstocks by thermal, catalytic or steam cracked processes.
- **Gas oil distillate fuels:** normally obtained by blending straight-run, cracked and hydrocracked gas oils.
- **Other gas oils**: obtained when straight-run or cracked gas oils are subjected to further refining processes.

The complex and variable composition of such UVCB substances means that it is not possible to precisely define their physical-chemical and environmental properties, but they will fall into a range, defined by the properties and concentrations of the individual hydrocarbons present. Typical property data on four gas oils of different types are given in **Table 1**.

Table 1: Typical properties of gas oils

Property	Straight-run	Catalytic cracked	Hydrocracked	Diesel fuel
CAS No.	64741-44-2	64741-59-9	64741-77-1	68334-30-5
Aliphatic hydrocarbons (%, m/m)	79.7	24.0	74.6	71.9
Aromatic hydrocarbons (%, m/m)	20.3	72.4	21.0	28.1
Olefins (% $^{w}/_{w}$)	<0.1	3.7	4.4	1.0
Density at 15°C (g/ml)	0.844	0.972	0.837	0.834
Boiling range (°C)	185 - 391	240 - 372	216 - 347	143 - 347
Reference	API, 1987	API, 1987	Deininger et al, 1991	Deininger et al, 1991

3. CHARACTERISATION OF TEST SUBSTANCES

Two OGO samples, five SRGO samples, ten VHGO samples, three bitumen samples and two RAE sample were obtained via Concawe from various European refineries (**Table 2**). These test substances are representative of some of the gas oil types of these groups (**Appendix 1**).

The methods of synthesis, fabrication, and/or derivation of the test substance were maintained by the sponsor. Each test substance, as received, was considered the "pure" substance.

Table 2 Gas oil, bitumen and RAE samples

Descriptor	Sample code	EINECS No.	CAS No.	Lab. code No.
SRGO	CON 1	265-043-1	64741-43-1	MRD-13-638
SRGO	CON 2	272-341-5	68814-87-9	MRD-13-639
SRGO	CON 3	272-341-5	68814-87-9	MRD-13-640
SRGO	CON 4	272-817-2	68915-96-8	MRD-13-641
SRGO	CON 5	265-043-1	64741-43-1	MRD-13-642
OGO	CON 7	265-148-2	64742-46-7	MRD-13-643
OGO	CON 9	265-183-3	64742-80-9	MRD-13-644
VHGO	CON 12	265-049-4	64741-49-7	MRD-13-645
VHGO	CON 13	265-059-9	64741-58-8	MRD-13-646
VHGO	CON 14	265-078-2	64741-77-1	MRD-13-647
VHGO	CON 15	265-190-1	64742-87-6	MRD-13-648
VHGO	CON 16i	269-822-7	68334-30-5	MRD-13-649
VHGO	CON 16ii	269-822-7	68334-30-5	MRD-13-659
VHGO	CON 17	270-671-4	68476-30-2	MRD-13-650
VHGO	CON 18	270-673-5	68476-31-3	MRD-13-651
VHGO	CON 19	270-676-1	68476-34-6	MRD-13-652
VHGO	CON 20	295-407-5	92045-24-4	MRD-13-653
Bitumen	CON 21	232-490-9	8052-42-4	MRD-13-654
Bitumen	CON 22	265-057-8	64741-56-6	MRD-13-655
Bitumen	CON 23	295-518-9	92062-05-0	MRD-13-656
RAE	CON 26	265-110-5	64742-10-15	MRD-13-657
RAE	CON 27	295-332-8	91995-70-9	MRD-13-658

All the test substances were analysed by GCxGC (Forbes, 2013a-2013v). Detailed analytical characterisation was performed for each gas oil, bitumen and RAE sample (**Table 2**), including comprehensive quantitative analysis using two-dimensional gas chromatography (GCxGC) for gas oil samples and thin layer chromatography with flame ionisation detection (TLC-FID) for bitumen and RAE samples (Forbes, 2013a-v), thereby allowing sample input into the PETROTOX model.

GCxGC

The GCxGC-system used was based on a 6890 series Gas Chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with an Optic II PTV injector (Agilent)

and a Flame-Ionisation Detector (FID). The GCxGC modulation assembly was a cryogenic, single jet loop type modulator (ZOEX Corp., Passadena, TX, USA). A separate heating oven was used for independent temperature programming of the second dimension column. Columns were coupled using glass press-fits. The first dimension column used contained a non-polar stationary phase, which minimises the effect of the activity coefficient, meaning that retention is solely based on volatility (boiling-point separation).

The second dimension column used was coated with a medium polarity stationary phase, and had an internal diameter of 100 µm I.D allowing for high-speed separations. Since volatility based separation has already occurred in the first dimension, the separation in the second dimension is solely based on polarity and/or based on additional interactions between the solutes and the stationary phase. Samples were injected pure or diluted dependent on the viscosity of the samples.

Samples were run in split mode. Injection volumes were 0.1 µl, with helium as the carrier gas at a gas pressure of 250 kPa and flow rate of 20 ml/min. Initial temperature was set at 45°C and final temperature at 250°C. The first dimension column (10 m, 0.25 mm I.D., 0.25 µm dimethylpolysiloxane (DB-1)) temperature profile was 40°C (5 min isothermal) then 2.5°C/min to 300°C (20 min isothermal). The second dimension column (2 m, 0.10 mm I.D., 0.10 µm polysilphenylene-siloxane (BPX-50)) temperature profile was 30°C (offset from first-dimension 70°C (5 min isothermal)) then 2.5°C/min to 330°C (20 min isothermal). The cryogenic single jet loop type modulator used was set to a modulation time of 7.5 and pulse width of 400 ms. The modulation column was 2 m, 0.10 mm I.D. deactivated fused silica (1 m in loop). The transfer line from the second dimension column to the FID was 0.3 m, 0.10 mm I.D. deactivated fused silica. FID temperature was 350°C, with make-up gas of helium at a flow rate of 20 ml/min, hydrogen flow rates were 35 ml/min and air flow rates were 350 ml/min. Resulting data was processed and visualized with in-house developed software.

Individual components found by GCxGC were grouped on the basis of both carbon number and the following chemical functionalities:

- Normal paraffins
- Iso-paraffins
- Mono-naphthenics
- Di-naphthenics
- Mono-aromatics
- Naphthenic mono-aromatics
- Di-aromatics
- Naphthenic di-aromatics
- Tri-aromatics

TLC-FID

Bitumen and RAE TLC-FID analysis was performed according to IP 469 (IP, 2001) Samples were dissolved in dichloromethane and separated by TLC using silica rods and three successive developments with the following mobile phases:

(i) Heptane

(ii) Toluene:heptane (80:20 v/v)

(iii) Dichloromethane:methanol (95:5 v/v)

Following evaporation of the final mobile phase, silica rods were examined using a latroscan Mark V TLC-FID Analyser and quantities of saturates, aromatics, resins and asphaltenes in the sample determined by internal normalization of the resulting chromatogram.

4. TEST METHODS

4.1. GENERAL APPROACH

Poorly water soluble, complex chemical mixtures such as UVCB petrochemical products pose special problems when preparing aqueous solutions for toxicity testing. With soluble chemicals, the amount of chemical dissolved in water is varied in increments to produce a range of toxicities, from which a "dose - response" relationship and associated median lethal/effective concentration ($L(E)C_{50}$) may be derived. With 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 “neat” substance composition (Peterson, 1994). This is in contrast to pure substances, where concentration will (if sufficient time is provided) equal the solubility limit when added in excess, regardless of the amount of excess. For poorly water soluble, complex chemicals, it has become standard practice to test toxicity at substance additions far in excess of the amount that will dissolve, resulting in a two phase system.

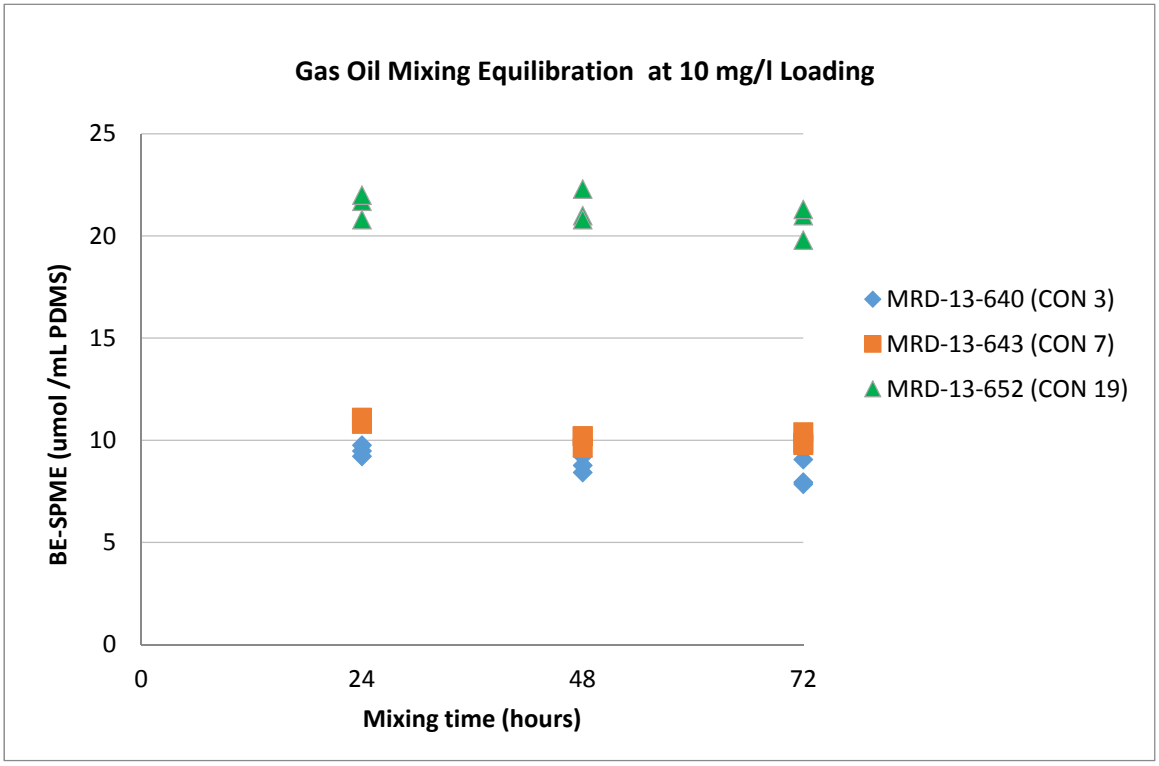
There are many procedures for establishing and maintaining equilibrium between water and un-dissolved substance (Girling, 1989). One recognized guideline (IMO, 1993) involves stirring various amounts (loadings) of poorly water soluble test substance with water for a sufficient time to reach equilibrium, followed by separation of the water phase (“water accommodated fraction” or “WAF”). Toxicity testing of WAFs generated by this procedure allows determination of the amount of substance equilibrated with water that will cause 50% mortality/effect. This endpoint is termed $L(E)L_{50}$ (lethal/effective loading) to distinguish it from $L(E)C_{50}$ (Girling et al, 1992). The $L(E)L_{50}$ procedure has been described in a Concawe report (CONCAWE, 1992) and is also the approach specified by MARPOL for marine pollution testing of poorly soluble mixtures [Whitehouse and Mallet, 1994] and the OECD for aquatic toxicity testing of difficult substances and mixtures (OECD, 2000).

Testing hydrocarbon liquids is further complicated by their volatility, particularly from aqueous solution. Although environmentally unrealistic, it is necessary to prevent volatilization of the substance to maintain constant concentrations and thereby determine its inherent toxicity. This necessitates using closed test systems. In preparing WAFs, some headspace in the test vessel is required to allow adequate interfacial area and mixing. In each test, measured amounts of test substance are added to measured volumes of the appropriate test medium (for *Daphnia* and algae). The vessels containing the medium and test substance are then sealed, leaving only a small headspace, and stirred with a 1 – 2 cm vortex for a period of time sufficient for the aqueous and test substance phases to equilibrate. After stirring, vessel contents are left to stand for one hour to facilitate phase separation. The aqueous phases – the WAFs – are then drawn out for use in tests via a stopcock at the bottom of the vessel for siphoning off the WAF without contaminating this with the undissolved surface layer, and system stirring is resumed once sampling is completed. Control media undergo the same regime, but do not contain the test substance. It is important that mixing is sufficient to ensure the aqueous phase is in equilibrium with the un-dissolved hydrocarbon phase. Mixing must be slow enough not to cause dispersion or emulsification of the un-dissolved hydrocarbon, yet vigorous and long enough to attain equilibrium. In current studies, mixing was performed with magnetic stirring bars set to develop a vortex ~10% of the static water depth.

Preliminary studies using representative SRGO (MRD-13-640, CON 3), OGO (MRD-13-643, CON 7), and VHGO (MRD-13-652, CON 19) at single loading rates of 10 mg/l, The vortex during mixing was ≤10% of the static liquid depth. Solutions were mixed and sampled at 24, 48 and 72 hours, each preceded by one hour settling, to determine optimal mixing time. System stirring was resumed once sampling was completed. Replicate samples were collected from the bottom sampling outlet of mixing vessels in ca. 20 ml clear glass vials with no headspace and sealed with Teflon® septum caps. In the equilibrium determination part of the study, replicates were collected from each test systems to permit triplicate analysis of each WAF and provide additional analytical retain samples. Equilibrium studies on these representative gas oils from each category at 10 mg/l loading rates, determined 24 hours as the optimum mixing duration for WAFs prepared for dose and BE-SPME studies (**Figure 1**). A preliminary study on a selected representative VHGO, bitumen and RAE determined 72 hours as the optimum mixing duration for WAFs prepared for limit studies.

The compositional analyses of all the petroleum products shown in **Table 2** were carried out using two dimensional gas chromatography (GCxGC) by Shell laboratories at Thornton and Amsterdam (Edam et al, 2005). All the SPME-GC and ecotoxicity studies were conducted in a single laboratory ExxonMobil Biomedical Sciences, Inc., Annandale, New Jersey, USA (EMBSI) in accordance with the principles of Good Laboratory Practice (GLP).

Figure 1 Gas oil WAF mixing equilibrium study



Note: 24 hours selected as optimal mixing time for Gas Oils

4.2. SCREENING STUDIES USING BIOMIMETIC EXTRACTION

Prior to toxicity testing with *Daphnia* or algae, WAF samples from two OGO, five SRGO and ten VHGO gas oils (as well as three bitumen and two RAE samples) were screened via the BE-SPME method to identify samples with high levels of water soluble hydrocarbons (i.e. higher toxicity) from those with lower levels or non-detectable amounts (i.e. lower toxicity) (EMBSI, 2014k).

The dilution water was hard reconstituted water (APHA, 2005), prepared from UV-sterilised, deionised well water and salts ($NaHCO_3$, $CaSO_4$, and KCl), with water hardness >140 mg/l (as $CaCO_3$). WAF test systems were prepared in ca. 4l glass aspirator bottles, each containing a Teflon® stir bar and were mixed on magnetic stir plates and sealed with Teflon® screw plugs. Test systems were identified with their respective sample codes and loading (1, 10, 100 mg/l). Control systems (i.e. no test substance added) were prepared in parallel with each loading experiment and labelled accordingly. All WAFs were prepared and mixed at laboratory room temperature (22°±2°C) and continuously recorded with a laboratory computerised monitoring system (Watchdog v5).

Equilibrium studies on representative gas oils from each category SRGO (MRD-13-640, CON 3), OGO (MRD-13-643, CON 7), and VHGO (MRD-13-652, CON 19) at 10 mg/l loading rates, determined 24 hours as the optimum mixing duration for WAFs used in BE-SPME studies (**Figure 1**). For the three representative gas oil samples, additional experiments were performed at 1 and 100 mg/l loading using the optimised 24 hour mixing. Sufficient replicates were collected from each test system to permit duplicate analysis of each WAF provide additional analytical retain samples. Loading experiments were performed at 1, 10 and 100 mg/l for the remaining gas oils with 24 hour mixing and duplicate BE-SPME analysis. For the more viscous bitumens and RAE, mixing was extended to 72 hours and a single 100 mg/l loading was initially tested. Additional 1 and 10 mg/l loadings were only prepared for the bitumens and RAEs that yielded detectable BE-SPME amounts at the 100 mg/l loading. Duplicate BE-SPME analysis was performed for the bitumens and RAE loadings.

Water samples were analysed for BE-SPME using a Perkin Elmer Autosystem gas chromatograph with flame ionisation detector (GC-FID). The GC was equipped with a 15 m x 0.53 mm id capillary column with 1.5 µm Rtx-1 stationary phase (Restek) and a LEAP Technologies (CTC Analytics) Combi PAL autosampler configured for automated SPME injections. Individual WAF samples taken in ca. 20 ml vials were extracted with a 30 µm polydimethylsiloxane (0.132 µl PDMS) SPME fibre (Supelco) for 100 minutes at 30°C with orbital agitation at 250 rpm prior to injection and thermal desorption of the fibre.

The BE method was calibrated by making 0.5 µl liquid (solvent based) injections of a series of methyl substituted aromatic hydrocarbon standard solutions at concentrations of approximately 20, 100 and 200 µg/ml. The average molar response factor of 2,3-dimethylnaphthalene was used to convert the measured GC-FID response (total integrated area) to nanomoles of organic constituents on the PDMS fiber. BE-SPME results are normalised to the volume of PDMS and reported as micromoles (µmol) as 2,3-dimethylnaphthalene / ml PDMS. As applied in this study, the practical quantification limit (PQL) of the BE-SPME method is approximately 0.5 µmol 2,3-dimethylnaphthalene / ml PDMS. This corresponds to the on-column amount of the lowest calibration standard of approximately 0.0648 nmol 2,3-dimethylnaphthalene which is then normalised for the PDMS volume of 0.132 µl on 30 µm SPME fibre.

For each gas oil, a first order equation was derived from the measured BE-SPME vs. loading curves. These equations were used to back calculate single BE-SPME values at an arbitrary loading of 50 mg/l to rank relative gas oil toxicity by BE-SPME, allowing sample toxicity to be ranked (**Table 3**).

Table 3 Individual and mean BE-SPME results and first order equations from gas oil, bitumen and Residual Aromatic Extracts (RAE) samples tested at 1, 10 and 100 mg/l loadings. [Bitumen and RAE samples were not subject to first order equations]

CON sample no.	Category and CAS no	Loading (24 h mix*) in mg/l			BE-SPME loading equation	r^2	BE-SPME loading equation with 50 mg/l loading
		1	10	100			
19	VHGO 68476-34-6	5.14 5.08 **5.11**	21.7 20.8 22.0 **21.5**	35.5 35.0 **35.3**	y = 6.5448ln(x) + 5.6757	0.9957	31.3
16(i)	VHGO 68334-30-5	2.04 1.95 **2.00**	11.1 10.9 **11.0**	26.6 27.1 **26.9**	y = 5.3972ln(x) + 0.8542	0.9751	22.0
7	OGO 64742-46-7	2.77 2.50 **2.64**	10.8 11.1 11.1 **11.0**	19.7 20.8 **20.3**	y = 3.825ln(x) + 2.4708	0.9968	17.4
9	OGO 64742-80-9	2.10 2.40 **2.25**	8.45 8.26 **8.36**	17.3 17.5 **17.4**	y = 3.2898ln(x) + 1.76	0.9872	14.6
14	VHGO 64741-77-1	2.29 2.25 **2.27**	9.03 8.77 **8.90**	16.3 16.2 **16.3**	y = 3.0357ln(x) + 2.15	0.9989	14.0
15	VHGO 64742-87-6	2.63 2.58 **2.61**	7.36 7.80 **7.58**	16.8 16.3 **16.6**	y = 3.0281ln(x) + 1.9392	0.9723	13.8
3	SRGO 68814-87-9	2.45 2.51 **2.48**	9.75 9.47 9.21 **9.48**	15.4 15.3 **15.4**	y = 2.8099ln(x) + 2.7057	0.9961	13.7
2	SRGO 64741-44-2	1.95 1.85 **1.90**	7.78 7.80 **7.79**	15.1 14.8 **15.0**	y = 2.8338ln(x) + 1.6883	0.9966	12.8
17	VHGO 68476-30-2	1.42 1.71 **1.57**	6.97 6.37 **6.67**	14.8 14.2 **14.5**	y = 2.8088ln(x) + 1.1108	0.9831	12.1
16(ii)	VHGO 68334-30-5	1.89 1.63 **1.76**	6.45 7.04 **6.75**	13.4 13.5 **13.5**	y = 2.5385ln(x) + 1.4733	0.9913	11.4
12	VHGO 64741-49-7	2.41 2.42 **2.42**	8.09 7.61 **7.85**	12.4 11.7 **12.1**	y = 2.0922ln(x) + 2.6208	0.9907	10.8
1	SRGO 64741-43-1	0.763 0.559 **0.661**	3.61 3.06 **3.34**	5.90 5.59 **5.75**	y = 1.104ln(x) + 0.705	0.9907	5.02

CON sample no.	Category and CAS no	Loading (24 h mix*) in mg/l			BE-SPME loading equation	r^2	BE-SPME loading equation with 50 mg/l loading
		1	10	100			
18	VHGO 68476-31-3	nd nd **2.02**	1.98 2.06 **4.64**	4.54 4.74	y = 1.00764ln(x) - 0.1	0.9934	3.84
20	VHGO 92045-24-4	0.536 0.669 **0.603**	1.95 1.81 **1.88**	3.57 3.49 **3.53**	y = 0.6357ln(x) + 0.5404	0.9921	3.03
5	SRGO 68915-97-9	0.781 0.627 **0.704**	2.22 2.10 **2.16**	2.95 2.89 **2.92**	y = 0.4812ln(x) + 0.82	0.9642	2.70
4	SRGO 68915-96-9	0.551 0.656 **0.604**	1.39 1.53 **1.46**	1.93 1.98 **1.96**	y = 0.2935ln(x) + 0.6638	0.9681	1.81
13	VHGO 64741-58-8	0.533 0.539 **0.536**	1.20 1.08 **1.14**	1.46 1.64 **1.55**	y = 0.2202ln(x) + 0.5683	0.9662	1.43
21	Bitumen 8052-42-4	No samples**		nd nd			
22	Bitumen 64741-56-6	No samples**		nd nd			
23	Bitumen 92062-05-0	nd nd	nd nd	1.04 1.16 **1.10**			
26	RAE 64742-10-5	No samples**		nd nd			
27	RAE 91995-70-9	No samples**		nd nd			

** Bitumen and RAE samples - 72 h mix (as opposed to 24 h)*

*** 1 and 10 mg/l loadings were only prepared for bitumen and RAE samples that yielded detectable BE-SPME amounts at the 100 mg/l loading.*

4.3. PETROTOX TOXICITY MODELLING

The PETROTOX spreadsheet model (Redman et al, 2012) was used to predict acute *Daphnia* and algae toxicity (EL50) values based on detailed compositional information on the test samples, obtained via the GCxGC analytical methodology described above (Forbes, 2013a-v), which the model can relate to interactions of test organisms with dissolved phase hydrocarbons. Test substances were ranked from highest to lowest based on their predicted PETROTOX *Daphnia* EL50, thereby allowing comparison with relative toxicity predictions derived from calculations using BE-SPME. Representative “worst case” substances in each category of gas oils (OGO, SRGO and VHGO) were then chosen for experimental *Daphnia* and algal toxicity testing based on these predicted values. Hydrocarbons up to ~C30 can be separated by GCxGC, making it a suitable method for characterising gas oils. Carbon number and hydrocarbon class profiles are similar for the various gas oil substances and

supports read-across between these substances. Such testing is deemed necessary to confirm that the test samples (and their CAS numbers) are aligned with the categories assigned to them

4.4. DAPHNIA ACUTE TOXICITY STUDY

Studies were carried out in accordance with OECD Guideline 202 (equivalent to EC methods for the determination of ecotoxicity, C2 – Acute toxicity for *Daphnia*). The test species was *Daphnia magna* Straus, a freshwater invertebrate commonly used in ecotoxicity testing. Details of husbandry and selection of test organisms are provided in the laboratory reports (EMBSI, 2014a-j). Test organisms were less than 24 hour old neonates, from parents ranging from 12 – 20 days old. For definitive studies, four replicates, each involving 5 organisms were tested at each loading rate. Exposure was static and lasted for 48 hours.

No range-finding study was performed. Rather, selection of test concentrations for definitive studies was based on the results of the BE-SPME screening study, as recommended by EMBSI (EMBSI, 2014k).

Based on the BE-SPME data provided, dose response studies were carried out at EMBSI with *daphnia* and algae on the following nine samples, namely:

- OGOs (CON 7 and CON 9)
- SRGOs (CON 2 and CON 3)
- VHGOs (CON 12, CON 15, CON 16i, CON 17 and CON 19)

Limit studies would be performed on:-

- VHGO (CON 13)
- Bitumen (CON 23)
- RAE (CON 27)

Reconstituted water was used for the *Daphnia* studies. Individual WAFs were prepared for each loading rate by adding the appropriate amount of test substance to dilution water in 4l screw top glass aspirator bottles and stirring on magnetic stir plates with a vortex of ~10% of the static liquid depth for ~24 hours (72 hours in limit test WAFs). An equilibrium study conducted on a representative subset of gas oils (SRGO, OGO and VHGO) at a 10 mg/l loading rate confirmed 24 hours as the optimum mixing duration for WAFs prepared for dose studies (**Figure 1**). An equilibrium study on a bitumen, VHGO and a RAE at 100 mg/l loadings confirmed 72 hours as the optimum mixing duration for WAFs prepared for limit studies. At the end of mixing, solutions were allowed to settle for 1 hour and WAFs were siphoned into sealed 130 ml flasks without headspace, and *daphnids* were introduced for testing. The light duration was 16 hours and ranged from 45 – 729 lux. No reductions in dissolved oxygen concentration (range 7.36 – 10.84 mg/l) or pH (range 7.3 – 7.99) was seen at the end of the 48 hour exposure period. Observations were made for immobilization at 24 and 48 hours. *Daphnids* were not fed during exposure periods. Samples from the low, mid and high treatment WAFs and control solution were taken for automated BE-SPME analysis at the beginning and end of exposure.

4.5. ALGAL GROWTH INHIBITION STUDIES

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* (alternatively known as *Selenastrum capricornutum*). Details of culture methods are provided in the laboratory reports (EMBSI, 2014m-u). The algae used were taken from stock cultures in log phase growth at 4 days. Initial cell density of algae was ~1.0E+04 cells/ml in each replicate. Exposure was static and lasted for 72 hours.

No range-finding study was performed. Rather, selection of test concentrations for definitive studies was based on the results of the BE-SPME screening study, as recommended by EMBSI (EMBSI, 2014k).

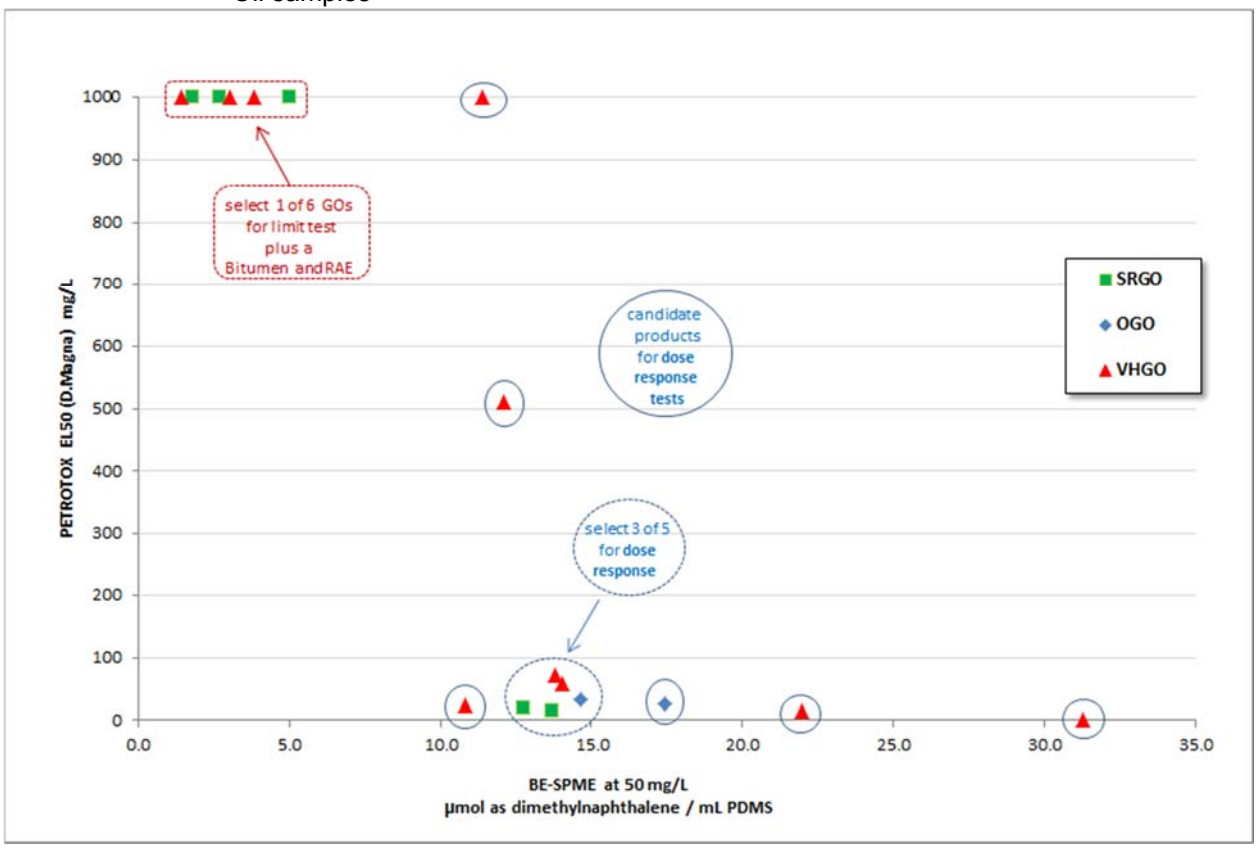
Individual WAFs were prepared for each loading rate by adding the appropriate amount of test substance to dilution water in 4.3l screw top glass aspirator bottles and stirring on magnetic stir plates with a vortex of ~10% of the static liquid depth for ~24 hours (72 hours in limit test WAFs). An equilibrium study conducted on a representative subset of gas oils (SRGO, OGO and VHGO) at a 10 mg/l loading rate confirmed 24 hours as the optimum mixing duration for WAFs prepared for dose studies (**Figure 1**). An equilibrium study on a bitumen, VHGO and a RAE at 100 mg/l loadings confirmed 72 hours as the optimum mixing duration for WAFs prepared for limit studies. At the end of mixing, solutions were allowed to settle for 1 hour and WAFs were siphoned into sealed 50 ml glass Erlenmeyer flask test chambers without headspace. Each chamber contained ~64 ml test solution and a stir bar. Nine replicate vessels were set up for each treatment and the control to facilitate daily algal cell counting and pH measurements. Samples from the low, mid and high treatment WAFs and control solution were taken for automated BE-SPME analysis at the beginning and end of exposure. Test chambers were incubated at 23.1 – 24.7°C and mixed continuously with stir bars on multi-position stir plates. Lighting was continuous and ranged from 4570 – 5410 lux. Cell density was determined for each treatment group and control group at 24, 48, and 72 hours using a haemocytometer and microscope. The pH values were measured at 24 and 48 hours in each treatment group and control group and ranged from 7.6 – 8.4.

5. RESULTS

5.1. SCREENING STUDIES

A summary of the biomimetic extraction and PETROTOX (based on GCxGC analysis) results from the 17 Gas Oils screened for toxicity are provided in **Table 1**. A plot showing a comparison of this data and an indication of the "worst case" samples for use in subsequent acute *Daphnia* and algae ecotoxicity testing is provided in **Figure 2**.

Figure 2 BE-SPME vs PETROTOX derived toxicity prediction comparison of all 17 Gas Oil samples



5.2. DAPHNIA ACUTE TOXICITY DATA

These studies met the acceptability criteria for control immobilization (not to exceed 10%) and dissolved oxygen concentration (maintained >60% of the air saturation value at exposure concentrations of 20°C). With the exception of one d*aphnid* in the limit tests, no immobilization was observed in the control groups throughout exposures. No observations of test substance insolubility (surface slicks, precipitates, and adherence to the test chamber) were noted during the time of organism observations.

Cumulative immobilization at 24 and 48 hours at each loading level were used to calculate the 48 hour EL50. Data was all amenable to probit analysis. A summary of the EL50 results from acute *Daphnia* tests are provided in **Table 2**.

Analytical data relating to the WAFs are provided in the laboratory reports (EMBSI, 2014a-j).

5.3. ALGAL GROWTH INHIBITION DATA

Studies were considered acceptable, control cell density increased ≥16 fold within three days and the coefficients of variation for specific growth rates did not exceed 7% in replicate control cultures in all studies. The coefficient of variation for section by section specific growth rates in the control replicates were below the guideline criteria of 35%.

Toxicity results are expressed as percentage inhibition of growth, derived from either average specific growth rate (r) or yield (y) relative to controls, based on cell density measurements taken at 24, 48 and 72 hour intervals. Average specific growth rate and yield for each loading rate were calculated in accordance with the formulas listed in the OECD 201 Guideline. The 72 hour EL50 values (and confidence intervals) were determined via probit regression calculations using the PROC PROBIT procedure from SAS (SAS, 2002). A summary of the ErL50 and EyL50 results from algae growth inhibition tests are provided in **Table 4**. As noted in the OECD Guidance, growth rate is the preferred endpoint.

Analytical data relating to the WAFs are provided in the laboratory reports (EMBSI, 2014m-u).

Table 4 Summary of experimental and predicted (BE-SPME and PETROTOX) acute ecotoxicity data from dose response studies using nine selected Gas Oil samples and limit tests using representative VHGO, bitumen and RAE

CON sample no	Category and CAS no	Algae (72 hour)			*Daphnia* (48 hour)	
		Growth rate E_rL_{50} (mg/l)	Yield E_yL_{50} (mg/l)	PETROTOX prediction EL_{50} (mg/l)	Immobilization EL_{50} (mg/l)	PETROTOX prediction EL_{50} (mg/l)
7	OGO 64742-46-7	140 (117 – 167)	17 (14 – 21)	3.2	38	27
9	OGO 64742-80-9	64 (54 – 75)	12 (9.8 – 14)	3.4	153	33
16(i)	VHGO 68334-30-5	55 (48 – 63)	20 (CNC)	2.9	38	14
19	VHGO 68476-34-6	7.9 (4 – 12)	<0.6	0.66	4.3	1.6
13*	VHGO 64741-58-8	>1000	>1000	>1000	1000 (30%)	>1000 (0.3)
23*	Bitumen 92062-05-0	>1000	>1000	>1000	>1000	>1000 (0.2)
27*	RAE	>1000	>1000	>1000	>1000	>1000 (0.2)

CON sample no	Category and CAS no	Algae (72 hour)			*Daphnia* (48 hour)	
		Growth rate E_rL_{50} (mg/l)	Yield E_yL_{50} (mg/l)	PETROTOX prediction EL_{50} (mg/l)	Immobilization EL_{50} (mg/l)	PETROTOX prediction EL_{50} (mg/l)
	91995-70-9					
15	VHGO 64742-87-6	39 (33 – 45)	9.5 (7.8 – 12)	5.32	1146	72
12	VHGO 64741-49-7	25 (20 – 30)	<3.5	2.77	218	24
2	SRGO 64741-44-2	75 (37 – 175)	<4.7	2.58	678	18
3	SRGO 68814-87-9	24 (21 – 26)	<3.9	2.37	280	15
17	VHGO 68476-30-2	>1026 (cnc)	23 (19-28)	7.86	>2636	510

***Limit tests**
OGO: Other Gas Oil; SRGO: Straight Run Gas Oil; VHGO: Vacuum Gas Oil, Hydrocarbon Gas Oil and Distillate Fuels; RAE: Residual Aromatic Extract

5.4. COMPARISON OF BE-SPME, PETROTOX AND EXPERIMENTAL ACUTE TOXICITY DATA

Comparison between BE-SPME, PETROTOX and experimental acute *Daphnia* and algal toxicity data was conducted (**Figures 3 - 7**).

Toxic Units (TU) were computed with PETROTOX for the two endpoints (*daphnia* and algae). Dose responses were plotted against loading (**Figure 3**), against acute TU (**Figure 4**) and against the SPME response (**Figure 5**). Then further plots were made of dose responses versus SPME response (**Figure 6**) and SPME response versus TU (**Figure 7**).

The aim of these plots were as follows:-

- To confirm that SPME data correlate to Toxic Units predicted by the PETROTOX model using GCxGC compositional data as input.
- For further validation of PETROTOX by correlating predicted EL/LL50 ecotoxicity against actual experimental EL/LL50 ecotoxicity (using *Daphnia* and algae).
- To strengthen the linkage between composition, SPME data and aquatic toxicity. This would provide a technical basis for further use of SPME as a more practical characterisation tool for addressing the influence of variation in substance composition on aquatic toxicity within petroleum substance categories.

There are several conclusions from these plots. It is clear that loading is a poor descriptor of toxicity (**Figure 3**). Both TU and SPME describe toxicity across substances and reduce variability from >1000-fold to <3-fold (**Figures 4 and 5**). However, additional model verification is needed since the same result was obtained for all substances. Also algae appear to be less sensitive than initial model assumptions (**Figure 4).**

Loading is roughly proportional to SPME (**Figure 6**), but there is some variability in the data since loading is more a function of substance composition The correlation supports a mechanistic basis of the SPME method as a surrogate measure of bioavailability. There is one point that persists at high mortality at relatively lower TUs for algae. This is a limit study with a gas oil which registered low SPME and TU. The *daphnia* had a lower response.

Figure 3: BE-SPME vs PETROTOX derived toxicity prediction comparison of all 17 Gas Oil samples. Red data are acute toxicity data for *daphnia*, blue and grey data are acute toxicity data for algae (specific growth rate and yield, respectively). Black data in the TU versus SPME plot were data developed during range finding.

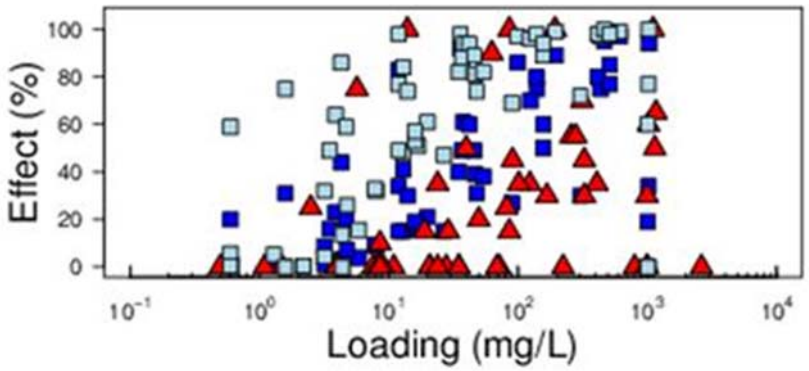


Figure 4: BE-SPME vs PETROTOX derived toxicity prediction comparison of all 17 Gas Oil samples. Red data are acute toxicity data for *daphnia*, blue and grey data are acute toxicity data for algae (specific growth rate and yield, respectively). Black data in the TU versus SPME plot were data developed during range finding.

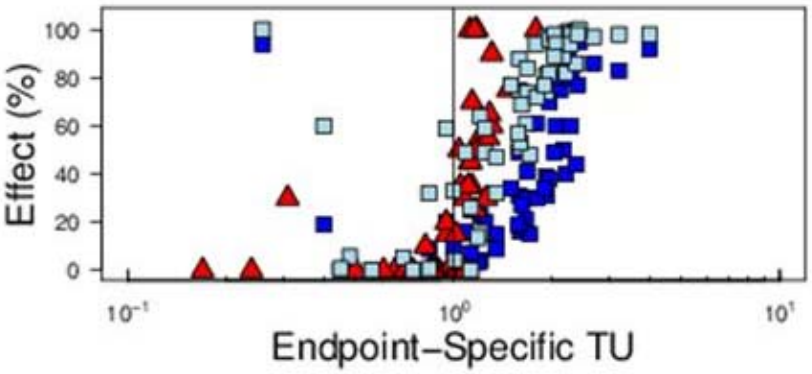


Figure 5: BE-SPME vs PETROTOX derived toxicity prediction comparison of all 17 Gas Oil samples. Red data are acute toxicity data for *daphnia*, blue and grey data are acute toxicity data for algae (specific growth rate and yield, respectively). Black data in the TU versus SPME plot were data developed during range finding.

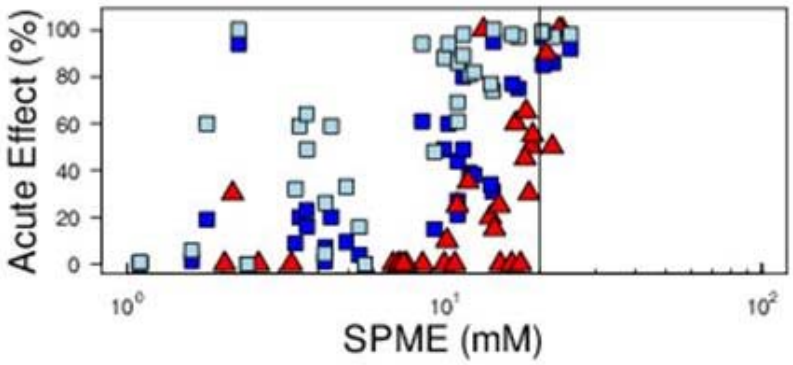


Figure 6: BE-SPME vs PETROTOX derived toxicity prediction comparison of all 17 Gas Oil samples. Red data are acute toxicity data for *daphnia*, blue and grey data are acute toxicity data for algae (specific growth rate and yield, respectively). Black data in the TU versus SPME plot were data developed during range finding.

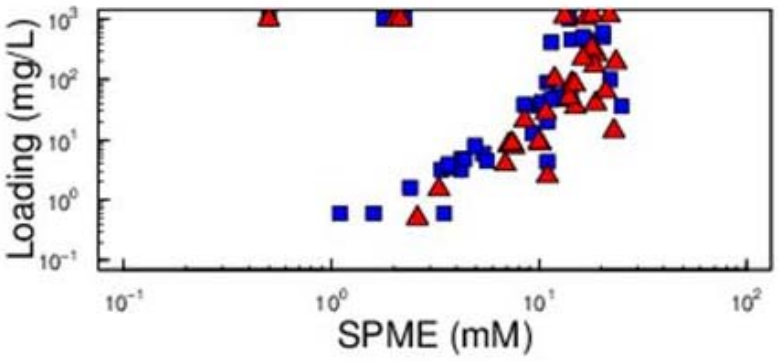
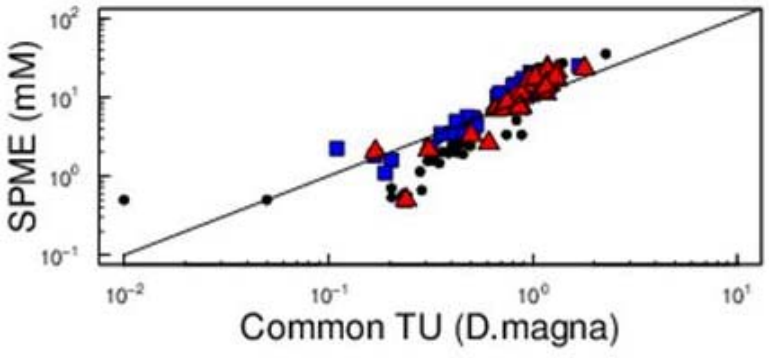


Figure 7: BE-SPME vs PETROTOX derived toxicity prediction comparison of all 17 Gas Oil samples. Red data are acute toxicity data for *daphnia*, blue and grey data are acute toxicity data for algae (specific growth rate and yield, respectively). Black data in the TU versus SPME plot were data developed during range finding.



6. DISCUSSION

Given the complex UVCB nature of gas oil products, experimental testing is often problematic and expensive. In addition, there is growing emphasis towards minimising the number of animal tests that are conducted throughout the product regulation process. Predictive methods such as BE-SPME and the PETROTOX model for screening potential "worst case" products and their toxicity can therefore be expected to have an important role in reducing future animal testing. Validation of the capability of BE-SPME and PETROTOX is therefore important. Strengthening the linkage between composition, SPME data and aquatic toxicity would provide a technical basis for further use of SPME as a practical characterisation tool for addressing the influence of variation in substance composition on aquatic toxicity within petroleum substance categories.

In the studies reported here, it was shown that when gas oil test samples were ranked from highest to lowest potentially toxicity, SPME values (based on substance bioavailability) generally complemented PETROTOX predicted EL50 values (**Table 1**). This therefore supports the applicability of SPME as a tool for evaluating and screening petroleum based test substances based on their bioavailability (and consequently, their expected toxicity). In this study, this screening method allowed the selection of 10 representative "worst case" samples (from the initial 17 samples) for further experimental testing based on their toxicity rank.

In the experimental tests reported here, the ranges of results from the representative "worst case" substances were as follows:

Daphnia tests (EL_{50}, 48h)	:	OGOs :	38 - 153 mg/l
		SRGOs :	280 - 678 mg/l
		VHGOs :	4.3 - <2636 mg/l
Algae tests (E_rL_{50}, 72h)	:	OGOs :	64 - 140mg/l
		SRGOs :	24 - 75mg/l
		VHGOs :	7.9 - >1026 (cnc) mg/l

No toxicity was shown in *Daphnia* or algae limit tests ($E(_r)L_{50}$ values >1000 mg/l).

The programme of work has successfully shown the PETROTOX model is fit for purpose for hazard assessment of petroleum based UVCBs. Predicted PETROTOX values are comparatively more conservative than actual experimental dose response test data from *Daphnia* and algae studies.

SPME has been shown to be a suitable tool for evaluating bioavailability and for selecting 'worse case' test substances in a category for acute ecotoxicity testing. SPME correlates well with PETROTOX calculations. Consistent TU-dose response relationships between algae and *daphnia* were seen.

7. GLOSSARY

BE	Biomimetic Extraction
CAS no.	Chemical Abstracts Service (Registry) Number
EC	European Council
EINECS	European Inventory of Existing Commercial Chemical Substances
EL	Effective Loading
EL50	Loading Rate of Test Substance (in dilution water) which causes adverse effects in 50% of the exposed population
ErL50	Loading Rate of Test Substance (in dilution water) which causes 50% reduction in algal growth rate
EyL50	Loading Rate of Test Substance (in dilution water) which causes 50% reduction in algal growth rate
FID	Flame-Ionisation Detector
GCxGC	Two-Dimensional Gas Chromatography
GLP	Good Laboratory Practice
LL	Lethal Loading
LL50	Loading Rate of Test Substance (in dilution water) which causes lethal effects in 50% of the exposed population
MARPOL	Maritime Pollution
Mg/l	Milligram per litre
OECD	Organisation for Economic Co-operation and Development
OGO	Other Gas Oil
RAE	Residual Aromatic Extract
SPME	Solid Phase Micro-Extraction
SRGO	Straight Run Gas Oil
TLC-FID	Thin Layer Chromatography with Flame Ionisation Detection
TLM	Target Lipid Model
TU	Toxic Units

UVCB	Substance of Unknown or Variable Composition, Complex Reaction Products and Biological Materials
VHGO	Vacuum gas oil, hydrocracked gas oil and distillate fuels
WAF	Water Accommodated Fraction

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APPENDIX 1: OTHER GAS OILS, STRAIGHT RUN GAS OILS, VACUUM GAS OILS, HYDROCRACKED GAS OILS, AND DISTILLATE FUEL OILS

CAS#	EINECS#	Substance Name
Other gas oils		
64741-86-2	265-088-7	Distillates (petroleum), sweetened middle
64741-90-8	265-092-9	Gas oils (petroleum), solvent-refined
64741-91-9	265-093-4	Distillates (petroleum), solvent-refined middle
64742-12-7	265-112-6	Gas oils (petroleum), acid-treated
64742-13-8	265-113-1	Distillates (petroleum), acid-treated middle
64742-14-9	265-114-7	Distillates (petroleum), acid-treated light
64742-29-6	265-129-9	Gas oils (petroleum), chemically neutralized
64742-30-9	265-130-4	Distillates (petroleum), chemically-neutralized middle
64742-38-7	265-139-3	Distillates (petroleum), clay-treated middle
64742-46-7	265-148-2	Distillates (petroleum), hydrotreated middle
64742-79-6	265-182-8	Gas oils (petroleum), hydrodesulfurized
64742-80-9	265-183-3	Distillates (petroleum), hydrodesulfurized middle
68477-29-2	270-719-4	Distillates (petroleum), catalytic reformer fractionator residue, high boiling
68477-30-5	270-721-5	Distillates (petroleum), catalytic reformer fractionator residue, intermediate-boiling
68477-31-6	270-722-0	Distillates (petroleum), catalytic reformer fractionator residue, low boiling
90622-53-0	292-454-3	Alkanes, C12-26-branched and linear
90640-93-0	292-615-8	Distillates (petroleum), highly refined middle
91995-34-5	295-294-2	Distillates (petroleum), catalytic reformer, heavy arom. conc.
93924-33-5	300-227-8	Gas oils, paraffinic
97488-96-5	307-035-3	Naphtha (petroleum), solvent-refined hydrodesulfurized heavy
97675-85-9	307-659-6	Hydrocarbons, C16-20, hydrotreated middle distillate, distn. lights
97675-86-0	307-660-1	Hydrocarbons, C12-20, hydrotreated paraffinic, distn. lights
97722-08-2	307-757-9	Hydrocarbons, C11-17, solvent-extd. light naphthenic
97862-78-7	308-128-1	Gas oils, hydrotreated
100683-97-4	309-667-5	Distillates (petroleum), carbon-treated light paraffinic
100683-98-5	309-668-0	Distillates (petroleum), intermediate paraffinic, carbon-treated
100683-99-6	309-669-6	Distillates (petroleum), intermediate paraffinic, clay-treated
Straight run gas oils		
64741-43-1	265-043-1	Gas oils (petroleum), straight-run
64741-44-2	265-044-7	Distillates (petroleum), straight-run middle
68814-87-9	272-341-5	Distillates (petroleum), full-range straight-run middle
68915-96-8	272-817-2	Distillates (petroleum), heavy straight-run
68915-97-9	272-818-8	Gas oils (petroleum), straight-run, high-boiling

CAS#	EINECS#	Substance Name
91722-55-3	294-454-9	Distillates (petroleum), solvent-dewaxed straight-run middle
92062-14-1	295-528-3	Solvent naphtha (petroleum), heavy
92704-36-4	296-468-0	Gas oils (petroleum), straight-run, clay-treated
100684-24-0	309-695-8	Gas oils (petroleum), straight-run, carbon-treated
Vacuum gas oils		
64741-49-7	265-049-4	Condensates (petroleum), vacuum tower
64741-58-8	265-059-9	Gas oils (petroleum), light vacuum
64742-87-6	265-190-1	Gas oils (petroleum), hydrodesulfurized light vacuum
92045-24-4	295-407-5	Gas oils (petroleum), hydrotreated light vacuum
92045-26-6	295-408-0	Gas oils (petroleum), light vacuum, solvent-dewaxed
92045-27-7	295-409-6	Gas oils (petroleum), solvent-refined light vacuum
97722-01-5	307-750-0	Gas oil light naphthenic vacuum
97722-05-9	307-754-2	Hydrocarbons, C16-20, hydrotreated distillate, vacuum distn. lights
97722-07-1	307-756-3	Hydrocarbons, C11-17, naphthenic middle
100684-22-8	309-693-7	Gas oils (petroleum), light vacuum, carbon-treated
100684-23-9	309-694-2	Gas oils (petroleum), light vacuum, clay-treated
Hydrocracked gas oils		
64741-77-1	265-078-2	Distillates (petroleum), light hydrocracked
97675-88-2	307-662-2	Hydrocarbons, C16-20, solvent-dewaxed hydrocracked paraffinic distn. residue
Distillate fuel oils		
68334-30-5	269-822-7	Fuels, diesel
68476-30-2	270-671-4	Fuel oil, no. 2
68476-31-3	270-673-5	Fuel oil, no. 4
68476-34-6	270-676-1	Fuels, diesel, no. 2

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