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Genetic toxicity of high-boiling petroleum substances

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ABSTRACT

There are several specific types of high-boiling petroleum substances (HBPS) having final boiling points >343 °C, in which genetic toxicity can be related to the content of polycyclic aromatic compounds (PACs), specifically crude oils, gas oils, heavy fuel oils, lubricant base oils, waxes and aromatic extracts. Evaluation of optimized *Salmonella* tests covering over 250 samples from 43 types of HBPS revealed that gene mutation can be determined for these substances using a protocol optimized for the detection of mutagenic PAC. The outcomes of modified *Salmonella* assays can be predicted using HBPS compositional information as input to a newly developed statistical model. The general outcome of the optimized *Salmonella* assay can be predicted for an untested substance based on its Aromatic Ring Class (ARC) profile. Review of the results from numerous cytogenetic tests showed that although a few positive study results have been reported, most HBPS do not produce chromosomal effects when tested in rodent bone marrow assays or in *in vitro* chromosomal aberration assays. Results of both bacterial and cytogenetic studies can be used to satisfy genetic toxicity endpoints for the HBPS category substances.

1. Introduction

This paper has three specific goals. The first is to show that there are associations between High Boiling Petroleum Substances (HBPS) containing condensed ring polycyclic aromatic compounds (PAC) and the mutagenic response in an optimized Ames *Salmonella* assay. HBPS are characterized as petroleum substances with final boiling point of ≥approximately 650 °F (343 °C). The second is to demonstrate that the associations can be modelled to predict estimates of the general outcome of the optimized Ames *Salmonella* assay for an untested substance based on the weight percent of PAC in that substance.

The third goal is to provide data to satisfy the HPV requirement for the assessment of *in vivo* genetic toxicity potential of HBPS. Published studies are cited and previously unpublished cytogenetic studies are presented to demonstrate that few HBPS produce *in vivo* mutagenic effects.

2. Background

2.1. Regulatory and petroleum background

Of the 13 categories of petroleum derived-substances being evaluated by the Petroleum HPV Testing group (PHPVTG) under the U.S. EPA High Production Volume (HPV) Program, six of these categories: Aromatic Extracts (API, 2003, 2012b), Crude oil (API, 2011a,b), Gas oils (API, 2012a), Heavy Fuel Oils (API, 2012d, 2013), Lubricating Oil Base stocks (API, 2011c,d) and Waxes (API, 2011e) were identified as containing substances in which mutagenicity and dermal carcinogenicity are associated with the content and distribution of PACs. The boiling ranges for these categories of HBPS other than crude oil are: Aromatic Extracts (>519–842 °F [271–450 °C], Gas Oils (>300–800 °F [150–427 °C]), Heavy Fuel Oils (>320–1112 °F [160–600 °C]), Lubricating Oil Base stocks and Waxes (>500–1112 °F [260 to >600 °C]).

In its guidance for the HPV Initiative (US EPA, 2000) the EPA referred to the OECD Screening Information Data Set (SIDS) program which defined the genetic toxicity endpoints as gene mutation and chromosomal aberration. Genetic toxicity in the broadest sense addresses the detection of agents that interact with DNA or alter the ability of DNA to faithfully replicate, either establishing mutations directly or by altering nucleic acid precursor pools subsequently resulting in mutation. Mutagens are the materials that directly induce single-gene mutations (base-pair substitutions or frameshift

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Table 1
Comparison of methods for standard Ames *Salmonella* assay and Optimized Ames *Salmonella* assay.

	Standard Ames *Salmonella* assay [OECD 471]	Optimized Ames *Salmonella* assay [ASTM E1687-95][a]
Utility	Screening of all potential gene mutagens.	Petroleum streams with high PAC content, boiling range >300 °C, viscosity >18cSt at 40 °C
Salmonella strains	TA 1535; TA 1537, 97 or 97a; TA 98; TA100.	TA 98 only [most sensitive strain overall for PAC rich streams]
Metabolic activation	With and without S-9 from rat liver and cofactors	With S-9 only from hamster liver: S9 and cofactors increased 6 fold over standard Ames.
Test substance preparation	Diluted or suspended in appropriate solvent (e.g. DMSO, acetone, ethanol)	Extraction in DMSO to concentrate PAC.
Administration	Direct application to minimal agar plate or pre-incubation (approx. 20 min) followed by application to plate. Incubate 48 h at 37 °C	Pre-incubation (approx. 20 min) followed by application to minimal agar plate. Incubate 48 h at 37 °C
Concentration levels	At least 5 non-toxic levels	8 extract dilution levels
Controls	Solvent control; appropriate positive control/strain	DMSO solvent control; vacuum distillate positive control specific for HBPS
Number of replicates	3 Plates/dose level	3 Plates/dose level
Evaluation of data	Number of revertants per plate; Mean number of revertants per plate at each concentration level Concentration-related increases over the tested range. Reproducible increased number of revertant colonies/plate in at least one strain	Number of revertants per plate; Mean number of revertants per plate at each concentration level Plot mean revertant colony counts against dose to produce a dose–response curve. Perform appropriate regression analyses of dose response curve to produce Mutagenic Index [MI].

[a] Optimized Ames test is not appropriate for petroleum-derived blending streams used in the production of naphthas, kerosenes and the light ends of atmospheric gas oils with upper boiling point <300 °C (572 °F) and viscosity indices <18cSt at 40 °C.

alterations) or cause multi-locus effects (chromosome aberrations), both potentially heritable (Casciano, 1991). For gene mutation, the OECD and U.S. EPA expressed a preference for data from a *Salmonella typhimurium* assay (OECD guideline 471). For chromosomal aberration, the OECD recommended data from either an *in vitro* chromosomal aberration test (OECD guideline 473), an *in vivo* bone marrow chromosomal aberration test (OECD guideline 475), or a micronucleus test (OECD guideline 474) [http://www.epa.gov/hpv/pubs/general/sidsappb.htm]. The EPA suggested that if a micronucleus test was needed, it should be conducted as part of a repeated dose study to reduce animal utilization (US EPA, 2000).

Polycyclic aromatic compound (PAC) is a comprehensive term that includes polycyclic aromatic hydrocarbons (PAHs) of two or more fused-aromatic rings consisting of only carbon and hydrogen atoms and other polycyclic aromatic molecules in which one or more atoms of nitrogen, oxygen, or sulfur replace one of the carbon atoms in the ring system. The relative abundance of alkylated PAHs and PACs in petroleum crude oil far exceeds the non-alkylated PAHs (Altgelt and Boduszynski, 1994). More compositional information on petroleum hydrocarbons is available in Gray et al., 2013. The mutagenic PAC found in high boiling petroleum substances can be detected in appropriately modified *Salmonella* tests.

For purposes of this paper, "petroleum-derived" substances are materials derived from crude oil and consisting of process and intermediate streams used in refineries and some finished products. This group of substances does not include other types of complex hydrocarbon materials such as fuels or lubricants derived from non-petroleum sources such as coal or shale, used oils including used engine oils, or combustion products such as engine exhausts.

2.2. Optimized Ames Salmonella assay

Genetic toxicity expressed as gene mutation or chromosomal damage is an important biological endpoint. Certain carcinogenic PAH species including benzo(a)pyrene [B(a)P] and dimethylbenz(a)anthracene [DMBA] present in some HBPS have been demonstrated to cause mutation in *S. typhimurium* (Ames et al., 1975; McCann et al., 1975) and dermal carcinogenicity in humans and laboratory animals. The ability to rapidly predict potential carcinogenic as well as mutagenic activity of petroleum-derived materials with minimal use of animal testing has greatly enhanced the value of genetic toxicity assays to the industry and led to modifications in the Ames *Salmonella* assay to optimize capabilities for both purposes (Blackburn et al., 1984, 1986, 1988; Roy et al., 1988).

The optimized Ames *Salmonella* assay, originally identified as the modified Ames assay, and analytical characterization both identify mutagenic activity and provide added value in exploring the potential genotoxic role in petroleum induced dermal carcinogenesis.

Table 1. summarizes the differences in method between a standard Ames *Salmonella* assay and the optimized Ames *Salmonella* assay. The optimized Ames *Salmonella* assay was developed and corroborated[1] with an extensive collection of oils with established dermal carcinogenic activity (i.e., positive results in the mouse skin painting assay). Modification of Ames assay methodology included (1) extraction of petroleum sample into DMSO to concentrate the condensed aromatic rings. (2) the use of only tester strain TA98 as it is generally more specific and selective than other tester strains in the detection of the mutagenic PAC found in petroleum-derived substances (Hermann et al., 1980) and (3) the use of hamster S9 in place of rat S9 for metabolic activation and at substantially higher concentrations than would normally be used along with a corresponding increase in co-factors to enhance assay sensitivity. These modifications allowed detection of positive bacterial gene mutation responses defined as an increase of revertant colonies in treated groups at least 2-fold that of negative controls. Blackburn et al. (1984, 1986) evaluated the response data in the optimized Ames *Salmonella* assay in terms of a "mutagenicity index (MI)," which was defined as the slope of the initial portion of the dose response curve expressed in units of revertants per microliter of the test material. The MI was found to be highly correlated with dermal carcinogenic

[1] In this paper, and other papers in this Supplement, we have chosen to not use the term *validation* to refer to the process of demonstrating that the model predictions are similar to real-world observations. As pointed out by Oreskes and her colleagues (Oreskes et al., 1994), the intrinsic meaning of a *validated model* is that the model has been shown to be true or an accurate representation of reality when it is really meant to imply that there has been a demonstration of consistency between the model and reality. Based on the recommendation of Council for Regulatory Environmental Modeling (US EPA, 2009), we have chosen to use the word *corroborate* or *evaluate* rather than *validate*.

Table 2
HBPS samples used in the initial modeling of Method II analytical data and Optimized Ames Salmonella test data.

HPV Category	Substance	CAS RN	Sample No	Optimized *Salmonella* MI results	Method II 3–7 rings[a]
Heavy fuel oils	Catalytically Cracked Clarified Oil[b] (Clarified slurry oil)	64741-62-4	86001	739	55.2
Heavy fuel oils	Syntower Bottoms	64741-62-4	86484	435	45.0
Heavy fuel oils	Heavy coker gas oil	64741-81-7	86181	143	22.8
Heavy fuel oils	Heavy coker gas oil	64741-81-7	86272	112	10.2
Heavy fuel oils	Heavy coker gas oil	64741-81-7	83366	89.0	9.9
Heavy fuel oils	Heavy coker gas oil	64741-81-7	86193	0.7	0.4
Heavy fuel oils	Heavy vacuum gas oil	64741-57-7	85244	5.6	6.1
Aromatic extracts	Heavy paraffinic distillate extract	64742-04-7	86187	26.8	20.7
Gas oils	Light cycle oil	64741-59-9	8281	28.3	15.7
Gas oils	Heavy atmospheric gas oil	68915-97-9	86271	18.0	9.2
Gas oils	Light coker gas oil	64741-82-8	87213	13.0	6.6
Gas oils	Vacuum Tower overhead	64741-49-7	86270	6.7	5.2
Lubricating oils Base Stocks	Solvent-dewaxed heavy paraffinic dist.	64742-65-0	82191	0.0	0.8
Crude oils[c]	Heavy crude	8002-05-9	89646	1.7	9.0
Crude oils[c]	Light Crude	8002-05-9	89645	0.0	2.0
Hydrocarbon wastes[c]	API separator bottoms	68188-15-8	88614	7.9	1.4
Hydrocarbon wastes[c]	DAF float blend	68477-27-0	89106	2.9	4.4

[a] Total 3–7 ring distribution from Appendix A Heavy Fuel Oils, Aromatic Extracts, Gas Oils and Lubricating oil base stocks. The distribution profile of 3–7 ring PAC affects the magnitude of MI in this initial data set.
[b] Clarified slurry oil is also identified as catalytic cracked clarified oil in some studies.
[c] Samples of crude oils and hydrocarbon wastes were provided in early analyses but are not listed in Appendix A.

potential for more than 28 materials as determined in the mouse skin painting assay. This correlation led Blackburn and his coworkers to propose that HBPS refined by vacuum distillation with MI values <1 were unlikely to be significantly mutagenic or dermally carcinogenic, oils with MI values >1 but <2 were of indeterminate potential, and oils with MI values >2 would likely produce gene mutation and skin tumors if tested in mice.

The publications by Blackburn et al. (1984, 1986, 1996) and Roy et al. (1988) showed a good correlation between MI and DMSO-extractable PAC content (details in Section 2.3 below) and dermal carcinogenesis for a range of petroleum substances. The refinement of the Ames assay method for the widest range of HBPS provides a more sensitive general *Salmonella* protocol for this class of petroleum substances than the standard Ames assay. In 1995, the optimized Ames *Salmonella* assay was standardized as an ASTM method for tests of mineral oils (a category of lubricating oil base stocks) used in the manufacture of metal working fluids (ASTM E1687-95). As a consequence this method has been used extensively in tests of lubricating oil base stocks, finished lubricants, and related materials (Blackburn et al., 1984, 1986, 1996; Chasey and McKee, 1993; Mackerer et al., 2003; McKee et al., 1994a; McKee and Przygoda, 1987; Roy et al., 1988; Skisak et al., 1987) and in studies of other HBPS including crude oils (Blackburn et al., 1986), gas oils (Jungen et al., 1995; McKee et al., 1994a; Nessel et al., 1998) and heavy fuel oil components (Feuston et al., 1994). The results of these studies, when correlated with results of mouse skin painting studies on similar substances, further support the utility of the optimized Ames *Salmonella* assay for identifying those petroleum substances with sufficient levels of PAC to be mutagenic and potentially carcinogenic.

2.3. Development of compositional information

An analytical method (Method II) was developed (Roy et al., 1985, 1994) to quantify DMSO-soluble PAC components of petroleum products in which the condensed ring aromatics are isolated by DMSO extraction and analyzed for PAC ring size distribution by gas chromatography with flame ionization detection (see Gray et al. (2013) for a more detailed description of the method as used in this study). The relationships between PAC content and mutagenicity index of samples of 39 HBPS with known activity in the mouse dermal carcinogenesis assay were investigated by Roy et al. (1988) with the conclusions that, for HBPS, both PAC content and mutagenicity (as defined by MI in the optimized Ames *Salmonella* assay) are predictive of dermal carcinogenic activity and indicate that PAC components are largely if not entirely responsible for the carcinogenic and mutagenic activities. The quantification of 3–7 ring PAC constituents was demonstrated to strongly relate to the mutagenicity results of the optimized Ames *Salmonella* assay and in combination with laboratory data from the optimized Ames *Salmonella* assay has proven to be predictive of mutagenicity and potential dermal carcinogenic effects of HBPS. Expansion of the compositional profiles to 1–7 and larger aromatic ring compounds (the "ARC Profile") further allows a degree of qualitative read-across for the optimized Ames Assay (i.e. MI greater or less than 1.0) to untested substances in relevant HPV categories.

3. Statistical modeling

3.1. Materials and methods

The ability to predict MI scores from the ARC Profile of the DMSO extractable PAC content was explored to provide read-across information for the untested substances in the HPV program. An initial statistical assessment employed 17 samples with data from the Optimized *Salmonella* assay and measurement of the weight percent of each of the 3- through 7-ring compounds in the test substance (the ARC profile) by Method-II (Table 2). These data also illustrate that samples within a single Chemical Abstracts Registry Number (CAS RN) designation may have differing MI values, most probably due to the differing ARC profiles of the samples because of differences in crude oils and refining processes. The initial linear regression model based on the log of the sample's MI demonstrated good predictive behavior over the entire range of mutagenicity outcomes. However for materials with MI values in the critical discrimination range <5.0, the model predicted higher values than those determined experimentally. This initial model was not judged sufficiently sensitive to predict whether or not a sample would be mutagenic in the Optimized Ames *Salmonella* assay in which a definitive MI score for bacterial

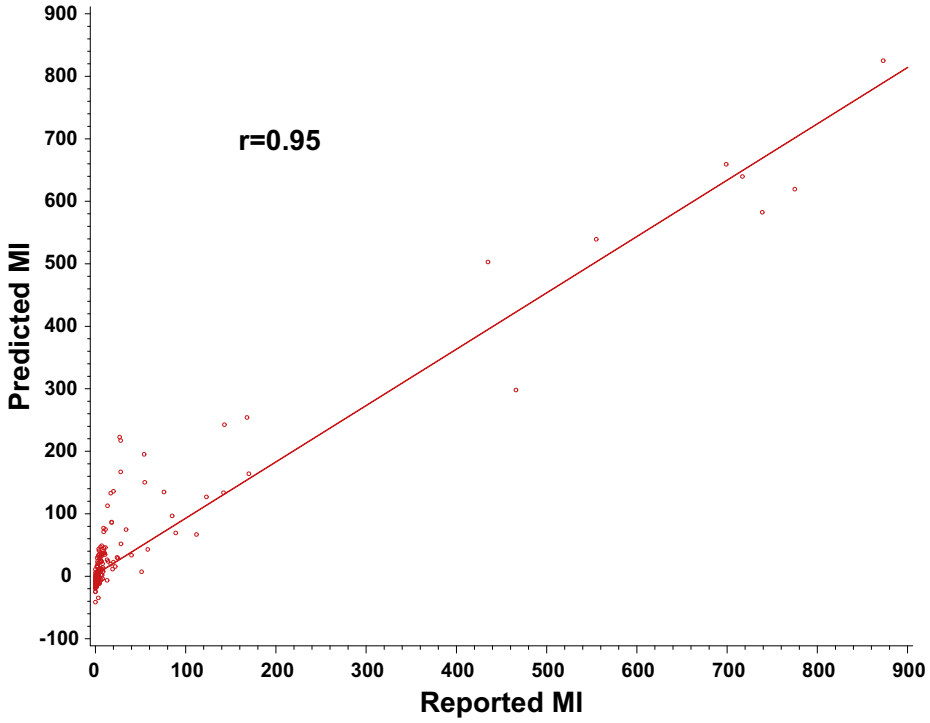

Fig. 1. Linear regression over total range of Mutagenicity Indices (MI).

mutagenesis falls within the 0–2 range. A similar result and conclusion was reached after developing a model based on a larger data set described in the next section.

3.2. Model form and development

A sample of 242 petroleum-derived materials for which the Optimized Ames *Salmonella* assay and Method II analytical data were available was drawn from the larger PAC analysis program (Appendix 1). These data were used to investigate the statistical relationship between the MI scores and the 1–7 aromatic ring ARC Profile. Because the MIs reported in the laboratory reports generated over several years were not calculated by a uniform method, to establish a consistent MI estimate for these samples, the MIs were recalculated using equation 2.2 from Myers et al. (1981). The new values showed minimal variation from the original calculations, the correlation was >0.99. The original and recalculated MI values, along with other relevant data, are presented in Appendix 1.

3.3. Statistical modeling results

The first set of statistical analyses using the 242 samples was based on a linear regression analyses using the ARC profile and individual plate concentrations to predict the revertant count used as the basis for the Optimized Ames *Salmonella* assay. The revertant predictions were very close to the observed revertant counts ($r > 0.8$), but the MIs calculated from the predicted counts were not in good agreement with the MI values calculated directly, particularly in the critical 0–3 MI range (both MIs were calculated by Myers equation 2.2) (Myers et al., 1981). Several transformations of the independent and dependent variables did not improve the results to any useful extent. Thus, the modeling efforts were redirected from predicting revertant count/plate to predicting the MIs directly.

The second set of analyses was based on a linear regression using the ARC profile to predict the MI directly. The regression model predicted well across the total MI spectrum ($r = 0.95$) and provided additional reassurance that the ARC Profile is capable of predicting the MI values for materials not previously assessed in *Salmonella* assays. Fig. 1 shows the relationship between the reported and model estimated MI values. However, in the region of MI values in the critical range of 0–3, the model did not predict as well ($r = 0.52$). Fig. 2 provides an expanded view of Fig. 1 in the reported MI range of 0–3.

A third set of analyses was based on complex (non-linear or non-parametric) regression of MI values – again with good model fit over the full range of MIs, but without success in the critical 0–3 MI range, similar to the linear model results (results not shown).

A fourth set of analyses was successful in developing a two level classification of the MIs by predicting the MI value as either less than 1, or greater than or equal to 1. The method is based on cascading a series of binary logistic general additive models (Hastie and Tibshirani, 1990) in which each binary logistic model predicted the MI to be above or below a specific cut-point (Fig. 3) The first cut point was an MI of 5 and samples predicted to have an MI less than 5 were used in a second binary logistic model to predict the MI to be above or below a cut point of 2. The samples predicted to have an MI less than 2 were cascaded to a third binary logistic model to predict the MI to be below, or at or above a cut point of 1.0. The final result from the 3 steps was to have the MI for each substance predicted as being either <1 or >1. $\geq$1.

At each step the probability for a decision was based on a probability value of 0.50. For example, in the first step, if the probability of the substance having an MI less than 5 was greater than 0.50 the substance was assigned a predicted MI of 'less than 5'. The logistic model used the ARC Profile was determined by the Method II and their two factor interactions as independent variables. The terms were based on fitting smoothing and thin-plate splines in one and two dimensions (Wahba, 1990) and the models in the cascade have the same general form but each has different fitting

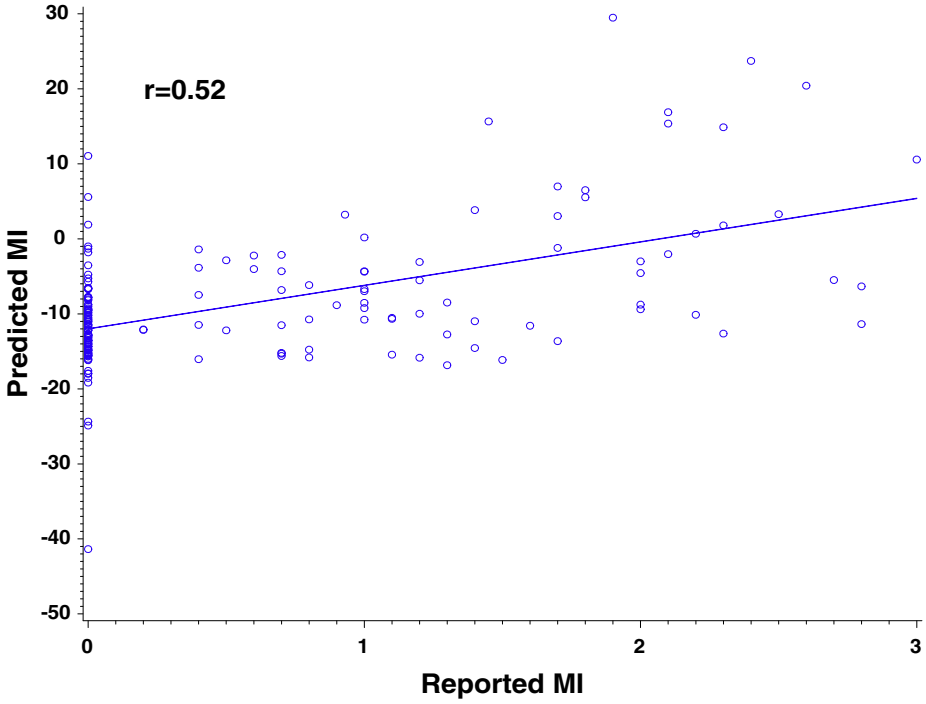

Fig. 2. Linear regression over Mutagenic Index (MI) Range of 0–3.

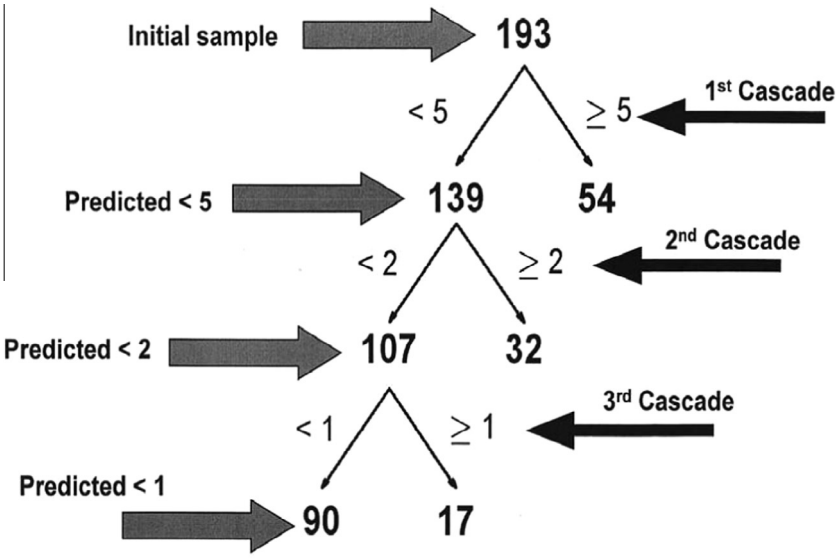

Fig. 3. Results of cascade steps for Predictive Mutagenicity Index (MI) Modeling.

characteristics. The model was analyzed using the general additive model procedure (PROC GAM) in SAS v9.2. The form of each model in the cascade that classified the substance into the binary grouping was:

$$MIGroup_i = \alpha_i + TPspline_i(ARC\_1 * ARC\_2,\ 6df) + Sspline_i(ARC\_3,\ 5df) + TPspline_i(ARC\_4 * ARC\_5,\ 6df) + TPspline_i(ARC\_6 * ARC\_7,\ 6df)$$

in which the subscript i refers to the steps in the cascade ($i = 1, 2, 3$), α is the intercept, *TSspline* is the two dimensional thin-plate *spline* with associated degrees of freedom (*df*), *Sspline* is the standard smoothing *spline* with its associated degrees of freedom, and *ARC_k* is the weight percent of the *k*th of the 1- through 7-ring aromatic compounds in the test substance.

Since the models are nonparametric, there is no closed form equation to examine.

3.4. Model testing

For the model building and testing, the sample of 242 substances was proportionally randomly divided into a model development sample of 193 (about 80%) and a sample of 49 for model testing or corroboration. The sampling was proportional to the MI values to assure that the distributions of observed MI values were similar in the two data sets. The samples were designated as those predicted to have mutagenicity index values equal to or greater than 1.0 and those with MI values less than 1.0 (using an

Table 3
Comparison of predicted (based on Method II data) and actual mutagenicity indices in the Optimized *Salmonella* Ames test.

Training Data Set:

		True MI Value		
		< 1	≥ 1	n
Predicted MI Value	< 1	88	2	90
	≥ 1	0	103	103
	n	88	105	193

99% of the predictions were correct

For the MI < 1 the model correctly identified 100% (specificity)
For the MI ≥ 1 the model correctly identified 98% (sensitivity)

Of the samples the model identified as MI < 1, 98% were correct (negative predictive value)
Of the samples the model identified as MI ≥ 1, 100% were correct (positive predictive value)

Holdout Data Set:

		True MI Value		
		< 1	≥ 1	n
Predicted MI Value	< 1	19	3	22
	≥ 1	0	27	28
	n	19	30	49

94% of the predictions were correct

For the MI < 1 the model correctly identified 100% (specificity)
For the MI ≥ 1 the model correctly identified 90% (sensitivity)

Of the samples the model identified as MI < 1, 86% were correct (negative predictive value)
Of the samples the model identified as MI ≥ 1, 100% were correct (positive predictive value)

operational definition in which it was assumed that samples with MI values less than 1.0 were not mutagenic). Only the 193 substances were used in the development and formulation of the cascade model. This model was not designed to quantify mutagenic potency but to identify whether or not a substance had the potential to induce gene mutations in the Optimized Ames *Salmonella* assay and thus, to potentially be active in dermal carcinogenesis assays as well. The results of the individual cascade steps are shown in Fig. 3 and the overall result is shown in Table 3. The corroboration data ($n = 49$) were applied to the previously developed cascade model to predict the MIs to simulate real-world application of the model. The results of the corroboration sample are shown in Table 3. The results for both samples are very good, with 99% of the predictions agreeing with the calculated MI for the data used in model development and a 94% agreement for the corroboration sample. The measured and estimated MI values are presented in Appendix 1.

3.5. Statistical modeling summary

From this information it is apparent that the general outcome of the Optimized Ames *Salmonella* assays, (i.e., whether or not a petroleum-derived substance has an MI < 1.0 and is non-mutagenic in this system) can be predicted from compositional information with an accuracy that seems comparable to that associated with variability inherent with either the experimental methods or the methods used to calculate mutagenicity index from the experimental data. The cascade modeling method is not dependent on using the Myers method (Myers et al., 1981) to calculate the MI, and will work equally well with MIs calculated by any consistent algorithm.

Based on this outcome, the results of the Optimized Ames *Salmonella* assays, either experimentally determined or predicted, can be used to assess the potential of untested HBPS to produce gene mutation.

4. Summary of cytogenetic assays of HBPS

Materials that cause gene mutation may or may not induce cytogenetic damage (i.e. are clastogenic) and conversely materials that are clastogenic may not cause bacterial gene mutations. When representative samples over the range of HBPS were tested in cytogenetic assays, responses were generally negative overall regardless of the presence or absence of PAC.

4.1. In vivo tests

4.1.1. Chromosome aberrations in rodent bone marrow

Potential for chromosome aberrations has been assessed for substances in the high boiling petroleum compound HPV categories. Conaway et al. (1982, 1984) reported no cytogenetic activity for samples of lubricating oil base stocks and unused motor oil administered orally to rats. Samples from the Gas Oils category of diesel fuel oil (administered intraperitoneally) and home heating oil (administered orally) induced statistically significant increases in chromosome aberration frequency although results for home heating oil were not dose-related. Micronucleus studies with other diesel fuel oil samples and home heating oil administered to mice by oral gavage did not confirm these early chromosome results (McKee et al., 1994b).

Chromosome aberration studies for a Heavy Fuel Oil category member, catalytically cracked clarified oil (CAS RN 64741-62-4,

sample 81-15; API, 1985a) administered to rats by oral gavage for 5 days and two light catalytically cracked distillate samples from the Gas Oil category (CAS RN 64741-59-9 samples 83-7 and 83-08; API, 1985b,c) administered in single intraperitoneal doses did not report increases in chromosome aberrations even at very high doses up to 1.0 g/kg/day orally for 5 days for catalytically cracked clarified oil or 3.0 g/kg single intraperitoneal dose for light catalytically cracked clarified distillates.

4.1.2. Micronucleus tests in rodent bone marrow

Since its introduction, the micronucleus test (Heddle et al., 1983, 1991) has tended to be more commonly used than the bone marrow chromosome aberration test to assess the potential for HBPS to produce cytogenetic damage. Studies reported by McKee and O'Connor, 1994b with two HPV Gas Oil category substances (CAS RN 64742-46-7 and 68476-30-2) and Przygoda et al. (1999) with Heavy Fuel Oil category samples of catalytically cracked clarified oil (CAS RN 64741-62-4) and a heavy naphthenic distillate (CAS RN 64741-53-3) did not cause increases in micronucleus formation in mouse bone marrow cells. In the Przygoda et al. (1999) study, both whole oil samples and extracts of the catalytically cracked clarified oil's aromatic constituents produced negative results even when tested at toxic levels compared to positive results for the polycyclic aromatic hydrocarbon, dimethylbenzanthracene, tested as a reference material. Pure PAHs have been reported to produce chromosomal aberrations and/or increased micronuclei in bone marrow-derived cells, albeit at relatively high levels (Adler et al., 1989; Harper et al., 1989; Przygoda et al., 1999; Shimada et al., 1990, 1992). The negative responses with HBPS are likely due to their very complex composition, containing numerous

Table 4
Summarized results of rat micronucleus tests in rat bone marrow cells after 13 weeks dermal exposure to HBPS.

HPV category	Substance	CAS RN	Sample No.[a]	Micronucleus test results in dermal 13-week studies doses mg/kg/day	Hematology effects relative to controls in dermal 13-week studies[b]	Optimized *Salmonella* test results (MI)[c]
Aromatic extracts	Distillate aromatic extract	64742-04-7	86187	Did not increase micronucleated PCE frequency Max. dose 2000	Decreased Hb, Hct, RBC, MCH, platelets	26.8
Aromatic extracts	Bright stock extract	64742-10-5	86525	Did not increase micronucleated PCE frequency Max. dose 2000	Decreased Hb, Hct, RBC	1.0
Aromatic extracts	Bright stock extract	64742-10-5	87293	Did not increase micronucleated PCE frequency Max. dose 2000	Decreased Hb, Hct, RBC	0.0
Aromatic Extracts	Bright stock extract	64742-10-5	87058	Did not increase micronucleated PCE frequency Max. dose 2000	Decreased Hb, Hct, RBC	0.0
Aromatic extracts	Bright stock extract	64742-10-5	87476	Did not increase micronucleated PCE frequency Max. dose 2000	Decreased Hct and RBC	
Asphalt	Vacuum residuum	64741-56-6	86268	Did not increase micronucleated PCE frequency Max. dose 500	Decreased Hb, Hct, platelets	3.2
Gas oils	Coker light gas oil	64741-82-8	87213	Did not increase micronucleated PCE frequency Max. dose 2000	Increased WBC, segmented neutrophils, decreased lymphocytes	13
Gas oils	Vacuum tower overhead	64741-49-7	86270	Did not increase micronucleated PCE frequency Max. dose 500	Decreased Hb, Hct, RBC, MCH	6.7
Gas Oils	Heavy Atmospheric gas oil	68915-97-9	86271	Small statistically significant increase in females (but not males) at 125, 500 mg/kg	Decreased Hb, Hct, RBC, platelets	18
Heavy fuel oils	Visbreaker residuum	64741-80-6	86192	Did not increase micronucleated PCE frequency Max. dose 1000	Decreased Hb, Hct, RBC.	0.0
Heavy fuel oils	Visbreaker gas oil	64741-81-7	86193	Did not increase micronucleated PCE frequency Max. dose 125	No adverse effects	0.7
Heavy fuel oils	Coker heavy gas oil B thermo-cracked	64741-81-7	83366	Did not increase micronucleated PCE frequency Max. dose 2000	Decreased Hb, Hct, RBC, platelets	89
Heavy fuel oils	Heavy coker gas oil -J	64741-81-7	86181	Did not increase micronucleated PCE frequency Max. dose 125	Decreased Hb, Hct, RBC, platelets, MCV, MCH, MCHC	143
Heavy fuel oils	Heavy coker gas oil -T	64741-81-7	86272	Significant increase in micronucleated PCE at 125 mg/kg [highest dose tested]	Decreased Hb, Hct, RBC, platelets, lymphocytes, Increased WBC, segmented neutophils in female	111.7
Heavy fuel oils	Heavy vacuum gas oil	64741-57-7	85244	Did not increase micronucleated PCE frequency Max. dose 2000	Decreased Hb, Hct, RBC, platelets	5.6
Heavy fuel oils	Clarified slurry oil	64741-62-4	86484	Small but significant increase at 8 mg/kg (lowest dose). No significant effects at higher dose levels up to 500 mg/kg	Decreased Hb, Hct, RBC, platelets. Increased WBC	435
Crude oil	Light crude oil	8002-05-9	89645	Did not increase micronucleated PCE frequency Max. dose 500	Decreased Hb, Hct, RBC	0.0
Crude oil	Heavy crude oil	8002-05-9	89646	Did not increase micronucleated PCE frequency Max. dose 500	Decreased Hb, Hct, RBC, platelets	1.7
Hydrocarbon waste	API Separator Sludge	68188-15-8	88614	Did not increase micronucleated PCE frequency Max. dose 10,000 mg/kg once a week	Decreased lymphocytes, Increased segmented neutrophils[d]	7.9
Hydrocarbon waste	DAF float blend	68477-27-0	89103	Did not increase micronucleated PCE frequency Max. dose 1000	Decreased Hb, Hct, RBC, platelets, WBC	2.9

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[a] Sample numbers can be used to identify and review study summaries in the appropriate HPV category analysis documents and robust summaries (API, 2003, 2011a,b,c,d,e; 2012a,b,c,d; 2013).

[b] Hb = hemoglobin, Hct = hematocrit, RBC = red blood cells, WBC = white blood cells, MCH = Calculated mean corpuscular hemoglobin, MCV = Calculated mean corpuscular volume, MCHC = Calculated mean corpuscular hemoglobin concentration.

[c] MI = Mutagenicity Index, revertants/μl [Myers method equation 2.2, 1981].

[d] Study was on males only.

components, some biologically inactive, that may inhibit activity from low concentrations of individual PAHs (Berkley et al. 1985; Bingham et al., 1980; Hermann et al., 1980; Petrilli et al., 1980).

Further confirmation that HBPS are generally not active in bone marrow assays for clastogenic potential was obtained in series of studies in which a range of test materials was administered to male and female rats by repeated dermal administration either undiluted or in a non-toxic lubricating base oil vehicle for 13 weeks (previously unpublished data). Doses ranged from levels beginning at 8 to 30 mg/kg day to maximum dose levels of 125 to 2000 mg/kg/day based on dermal effects. Bone marrow was taken from animals treated with substances with CAS RNs in the Gas Oils, Aromatic Extracts, Heavy Fuel Oils, Asphalt, Crude Oil and Reclaimed Substances: Residual Hydrocarbon Wastes HPV categories. The Reclaimed Substances: Residual Hydrocarbon Waste category is not among those which are directly relevant to the present report but the cytogenetic results are included for completeness. Summaries of these studies can be reviewed in the HPV Category Analysis Documents and Robust Summaries using the sample numbers in Table 4 for identification located on the relevant EPA website URLs (API, 2003, 2011a,b,c,d,e; 2012a,b,c,d; 2013). The ability of these HBPS to reach the bone marrow under these testing conditions was demonstrated by observed bone marrow toxicity described below and in Table 4. Feuston et al. (1994) also reported bone marrow toxicity (e.g., decreases in hemoglobin, platelets counts) related to PAC content under similar repeated dose toxicity test conditions.

Of the 20 samples tested in 13-week dermal micronucleus tests, 17 were negative and three samples (a heavy atmospheric gas oil sample 86271 in the Gas Oils category and a catalytically cracked clarified oil sample 86484 and a heavy coker gas oil sample 86272 in the Heavy Fuel Oils category) were reported as positive in that there were treated groups within which the frequencies of micronucleated polychromatic erythrocytes were significantly elevated by comparison to controls (Table 4). However, two of the positive outcomes seem questionable in terms of treatment-related effects. In the catalytically cracked clarified oil study, the reported positive outcome was not dose-related (the significant effect was in the lowest dose group with no toxicity reported at higher dose levels up to 500 mg/kg/day). Furthermore, the positive result in this study of catalytically cracked clarified oil sample (sample 86484) was not consistent with earlier chromosome aberration and micronucleus studies of other samples of materials of this type (discussed above). In the heavy atmospheric gas oil study (sample 86271), there were significant effects in female but not in male rats. The positive findings for females were based statistically (Normal Test) on the number of polychromatic erythrocytes counted in all animals of each sex/group; however, by the more conventional statistical ANOVA which treats the animals as the investigative unit, the results were negative. Finally, positive results were reported at the highest dose tested in one study of heavy coker gas oil (sample 86272), but negative results were reported in two other micronucleus tests of heavy coker gas oils (samples 83366 and 86181). In another study on one of these heavy coker gas oils (sample 86181) in which pregnant rats were treated at a dose as high as 250 mg/kg from gestation days 0–19, the frequency of micronucleated polychromatic erythrocytes did not increase.

The three substances that gave positive results in micronucleus tests induced effects on hematologic parameters similar to those of the other PAC-rich substances that did not cause cytogenetic damage. Hematotoxic effects in the 13 week studies included decreased hemoglobin and hematocrit values, lower red blood cell, white blood cell, lymphocyte, eosinophil and platelet counts identified by sample in Table 4. Although hematotoxicity was seen with the heavy vacuum gas oil (CAS RN 64741-57-7, sample 85244) and two of three samples of heavy coker gas oil (CAS RN 64741-81-7, samples 83366 and 86181) in the Heavy Fuel Oils category, no adverse cytogenetic effects were induced in the rat bone marrow micronucleus assay. Exposure to a sample of coker light gas oil (Gas Oils category, CAS RN 64741-82-8, sample No. 87213) induced hematologic changes, pale bone marrow, myeloid hyperplasia secondary to skin ulceration, and megakaryocytic hypoplasia but did not cause an increased incidence of micronucleated cells in bone marrow erythrocytes. For the three positive samples incidence of micronucleated polychromatic erythrocytes could have been enhanced by stimulation of rebound erythropoiesis, reported by Tweat et al. (2007) as one of the disturbances in the physiology of treated animals that produce positive micronucleus results that are not due to genotoxic damage.

The evidence of bone marrow effects in all these studies provides empirical evidence of target tissue involvement and suggests that the absence of clastogenic effects was not due to inability of test substance to reach target tissue and/or any other aspects of the dosing procedures.

There was also no consistent relationship between mutagenic activity in Optimized Ames *Salmonella* tests and frequency of micronucleated polychromatic erythrocyte induction observed in these studies.

Despite the generally negative results for samples of HBPS a recent positive result was reported for a sample of an extract of a light paraffinic distillate solvent (CAS RN 64742-05-8, sample 20906) applied in acetone to the shaved backs of male and female rats, 5 days/week for 13 weeks at doses of 5, 50 and 150 mg/kg/day (Midwest BioRes, 2011). This sample of distillate aromatic extract (DAE) belongs in the HPV Aromatic Extracts category. A statistically significant increase in the percentage of micronucleated polychromatic erythrocytes was seen at the highest dose in males but only at the mid-dose in females. Hematotoxic effects (e.g. decreased hematocrit, hemoglobin, red blood cells, platelets) were seen at 150 and 50 mg/kg/day doses, and slightly higher total erythroid precursors were seen in both sexes at 150 mg/kg/day. The study authors concluded that the test material was positive in males but equivocal in females in the absence of a dose response. The observed hematotoxicity may have triggered rebound erythopoiesis contributing to increases in micronuclei of non-genotoxic origin (Tweat et al., 2007, ICH, 2011). This result differs from the negative results in dermal and oral micronucleus studies and chromosomal aberration study on another distillate aromatic extract (CAS RN 64742-04-7, sample 86187).

Nevertheless an overall conclusion based on the weight evidence of the *in vivo* chromosome aberration and micronucleus studies was that most HBPS are unlikely to produce positive effects in *in vivo* cytogenetic assays.

4.2. In vitro cytogenetics tests

There have also been ten previously unpublished *in vitro* chromosome aberration tests of samples of HBPS found in the Aromatic Extracts, Crude Oil, Gas Oils, and Heavy Fuel Oils HPV categories. These studies were conducted under a common protocol in which the condensed-ring aromatic fractions were extracted with DMSO, and a range of concentrations from 1.0 to 200 μl/ml of these extracts depending on cell toxicity were then tested both in the presence and absence of metabolic activation with Chinese hamster ovary cells (CHO). As shown in Table 5 of the studies on these ten samples, nine were inactive; the tenth (heavy coker gas oil, sample 83366) was reported as positive in the presence of metabolic activation but results were not dose-responsive. Another test of a second sample of heavy coker gas oil (sample 86194) gave negative results. If the new criteria established by the International Workshop on Genetic Toxicity Testing measuring cytotoxicity for CHO assays by relative increase in cell count rather than relative

Table 5
Summarized results of *in vitro* chromosome aberration studies of HBPS in Chinese hamster ovary cells.

HPV Category	Substance	CAS RN	Sample No	*In vitro* chromosome aberrations	Optimized *Salmonella* test result (MI)[a]
Aromatic extracts	Aromatic extract	64742-04-7	86187	Not clastogenic	26.8
Gas oils	Vacuum tower overheads	64741-49-7	85242	Not clastogenic	5.2
Heavy fuel oils	Heavy coker gas oil	64741-81-7	83366	Significant increase at 3 μl/ml with metabolic activation	89
Heavy fuel oils	Heavy coker gas oil	64741-81-7	86194	Not clastogenic	76
Heavy fuel oils	Heavy vacuum gas oil	64741-57-7	85244	Not clastogenic	5.6
Heavy fuel oils	Vacuum tower bottoms	64741-56-6	86268	Not clastogenic	2.3
Heavy fuel oils	Clarified slurry oil	64741-62-4	86484	Not clastogenic	435
Heavy fuel oils	Coker light gas oil	64741-82-8	87213	Not clastogenic	13
Crude oil	Crude oil – Light	8002-05-9	89645	Not clastogenic	0
Crude oil	Crude oil – Heavy	8002-05-9	89646	Not clastogenic	1.7

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[a] MI = Mutagenicity Index, revertants/μl; [Myers method equation 2.2, 1981].

cell count had been employed, the result may not have been considered positive (Clarke et al., 2012). As can be seen in Table 5, there was no relationship between the outcome of Optimized Ames *Salmonella* tests on the ten samples and *in vitro* cytogenetics tests on the same samples. Of particular note, a sample of catalytically cracked clarified oil, (Heavy Fuel Oils Category), expected to be the most likely mutagenic of all the substances investigated in this program, based both on process history and on outcome of Optimized Ames *Salmonella* tests, was inactive.

Overall, testing of samples of HBPS covering five of the API HPV categories (Aromatic Extracts, Crude Oil, Gas Oils, Heavy Fuel Oils and Lubricating Oil Base stocks) in bone marrow chromosome aberration tests, and micronucleus tests, and in *in vitro* chromosomal aberrations assays did not provide consistent evidence that these substances induced cytogenetic damage in these test systems. The absence of positive responses could not be attributed to protocol design or route of exposure since oral and dermal routes gave similar negative results while positive results from pure polycyclic aromatic hydrocarbons used as positive controls demonstrated the sensitivity of the assays. Attempts to optimize potential for cytogenetic damage expression in the *in vivo* tests by concentration of aromatic fractions, or testing at concentrations that produced other expressions of bone marrow toxicity did not alter negative results. Therefore it can be concluded that samples of most HBPS do not induce chromosomal aberrations in current assays.

5. Discussion and conclusions

The relationship between the PAC content of the class of high boiling petroleum substances and their mutagenic response in an Optimized Ames *Salmonella* assay has been corroborated. The value of this association to predict, with high sensitivity and specificity, the mutagenic potential of untested HBPS petroleum substances (i.e., final boiling point $\geq$approximately 650 °F [$\geq$343 °C]) with similar physical and chemical properties has been demonstrated.

Evaluation of the association between the mutagenic response in an Optimized Ames *Salmonella* assay and the PAC content (as expressed as the ARC profile) of selected petroleum substances resulted in 99% correct assessment of 192 samples used to develop the model and demonstrated that estimates of the general outcome of Optimized Ames *Salmonella* assays can be predicted for an untested substance based on its ARC profile (94% correct assessment of 49 samples used in model verification).

The cascade technique used in the predictive model is more accurate in predicting a mutagenic response than the linear regression, non-linear regression, or non-parametric methods, but provides only a binary result (estimated MI < 1 or $\geq$1).

To predict the mutagenic response of an untested substance using the models, the only compositional input that is required is the ARC profile of the substance as determined by a PAC Method II compositional analysis. Based on the sensitivity and specificity analyses we can see that the model is stable and useable.

The current version of the predictive model has several limitations. Principally, the application of the model requires specialized techniques that cannot be easily run in Excel or other readily available computer packages. One of the authors (MJN) is working on an approximation method that will work without specialized software. The other limitation is that the models were developed based on observed statistical relationships; no attempt was made to identify causal relationships. To do this would have required a more detailed understanding of the mechanisms of PAC-toxicity, or at least a general understanding of the underlying mode of action that was beyond the scope of the evaluation. Because the models reflect statistical associations and not causal relationships, no inferences should be made concerning which rings are responsible for effects.

Cytogenetic tests for chromosome aberrations and micronucleus formation have produced generally negative results for HBPS. Many samples (e.g. catalytically cracked clarified oil) selected for testing were considered most likely to be biologically active based on systemic toxicity in repeated dose studies, mutagenicity in bacteria and mammalian cells and content of aromatic hydrocarbons, yet did not induce cytogenetic damage in bone marrow. The ability of these substances to reach target organs was verified by effects on hematology parameters and bone marrow. Although a few samples have produced positive results the most reasonable general conclusion is that most HBPS do not produce chromosomal aberrations when tested under *in vivo* conditions in current assays. Negative results from *in vitro* studies in CHO cells suggest that negative results *in vivo* are not consequences of lack of exposure of the organ being evaluated (i.e. bone marrow) but are true indicators of the absence of clastogenic activity for these substances.

There was no relationship between results in the Optimized Ames *Salmonella* test and cytogenetic assays. This observation is consistent with the general genetic toxicology literature, most recently in Clarke et al. (2012) and U.S. EPA which specifies the need to include both gene mutation and cytogenetic testing to establish a complete genetic toxicity profile. (See http://www.epa.gov/hpv/pubs/general/sidsappb.htm).

Conflict of interest

One coauthor (RHM) is an employee of a company that manufactures petroleum products. Two of the coauthors (CAS, MJN) are paid consultants to the Petroleum High Production Volume Testing Group and former employees of companies that manufacture petroleum products. One co-author (TMG) is employed by the American Petroleum Institute.

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Appendix Appendix. A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2013.05.004.

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