Validation of the ToxTracker reporter assay for the genetic toxicology assessment of petroleum products

G. Hendriks[1], R.S. Derr[1], P.I. Racz[1], P.J. Boogaard[2] and H. Ketelslegers[3]

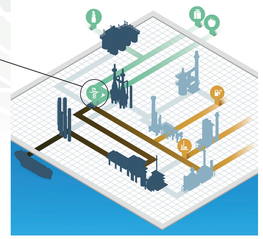
[1]Toxys, Leiden, the Netherlands. [2] Shell Health, Shell International, The Hague, The Netherlands. [3]Concawe, Brussels, Belgium.

RATIONALE

"Gatekeeper":
IP346 and Modified Ames test are fundamental and rapid screening assays for carcinogenicity and mutagenicity of petroleum streams (EC 1272/2008).

Mechanistic approaches to further underpin these screening assays & increase their predictivity.

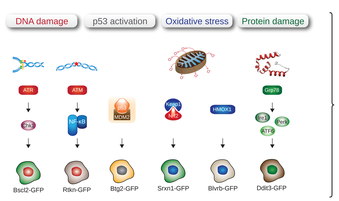
ToxTracker®



OBJECTIVES

Investigate whether the ToxTracker assay can be applied to petroleum UVCBs as a high-content screen for mutagenicity / carcinogenicity by testing by testing eighteen DMSO extracts of petroleum substances for (geno)toxic properties.

TOXTRACKER



Exposure of reporter cell lines in 96-wells plates, in absence or presence of a bioactivation system (S9 liver extract)

Cytotoxicity and GFP reporter detection by flow cytometry

Quantitiavite data analysis using Toxplot software

MATERIALS & METHODS

ToxTracker is a panel of mammalian stem cell lines that contain different fluorescent reporters for induction of DNA damage, oxidative stress and protein damage. The reporter cell lines were exposed to 18 DMSO extracts of petroleum substances obtained from various Concawe member companies. The differential induction of the GFP reporters as well as cytotoxicity of the tested substances was determined by flow cytometry. Exposure to various control compounds were included in each test to confirm acceptable technical performance and reproducibility of the ToxTracker assay. Quantitive data analysis is done using ToxPlot software. A number of extracts showed high levels of autofluorescence that potentially interferes with the ToxTracker assay. Therefore, induction of the ToxTracker biomarker genes was also determined using quantitative real-time PCR.

- DMSO extracts, containing the "biologically active" fraction (i.e. aromatics) of the petroleum substances were prepared following standard procedures (Roy *et al*, 1988).
- These DMSO extracts were tested in the ToxTracker assay at 5 different levels of cytotoxicity (0-60%), along with 4 positive control samples. Activation of the ToxTracker GFP reporter genes was determined using flow cytometry.

  In parallel, induction of the ToxTracker biomarker genes was measured using quantitative RT-PCR.
- The DMSO extracts were analysed in the absence and presence of S9 hamster liver extract to include a bioactivation system.
- All DMSO extracts were tested in the standard Modified Ames test (bacterial reverse mutation test modified according to ASTM E1687-10, specified for testing petroleum substances) for validation purposes and analysed by PAC2 analysis to determine the PAC levels in the samples since 3-7 ring PAC are expected to be associated with the toxicities under investigation in the current assay

RESULTS

PAC2 analysis

			Group Percentages of total Wt. %						
Sample Identification	Category	total Wt.% [a]	I	II	III	IV	V	VI	≥VII
Txs000088	Base oil	1.1	0	13	77	8	1	0	0
Txs000089	Base oil	1.7	0	1	19	34	31	13	2
Txs000090	Base oil	2.8	0	1	1	10	19	33	35
Txs000091	Base oil	0	0	0	0	0	0	0	0
Txs000092	Base oil	0	0	0	0	0	0	0	0
Txs000093	Base oil	0.31	0	7	38	33	16	5	0
Txs000094	Base oil	0	0	0	0	0	0	0	0
Txs000095	Base oil	0.17	0	5	23	17	17	21	17
Txs000096	Base oil	0	0	0	0	0	0	0	0
Txs000097	Destilate aromatic extract	9	0	2	1	6	22	36	33
Txs000098	Destilate aromatic extract	9.7	0	0	18	49	29	4	0
Txs000099	Destilate aromatic extract	12	0	0	22	41	25	11	1
Con-006	Heavy fuel oil	2.9	0	8	19	17	21	22	13
Con-008	Heavy fuel oil	27	0	7	44	22	14	10	2
Con-017	Heavy fuel oil	0.62	0	1	22	22	21	21	13
Con-34	Heavy fuel oil	48	0	1	24	40	21	11	3
Con-91	Heavy fuel oil	8.2	0	9	27	25	19	14	5
Con-129	Heavy fuel oil	17	0	4	55	37	3	0	0

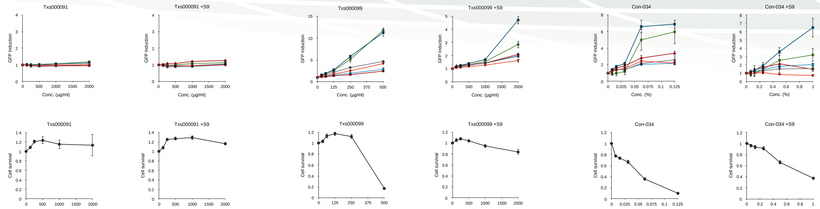
PAC2 analysis results. Eighteen petroleum substances that were tested in this study were analysed for their polycyclic aromatic compounds (PAC) composition. Total wt % of PAC content of the different substances was determined (indicated in a range from light to dark blue). Group percentages represent the levels of the molecules per aromatic ring class in the tested substances (low percentage indicated in yellow and high percentage in red). The refined lubricating baseoils have a low level of (mainly 2-3 ring) PACs, whereas the distillate aromatic extracts have a relatively high level of (3-6 ring) PACs. Most of the heavy fuel oil extracts have a high PAC content with 3-6 rings. Generation of the DMSO extracts and PAC2 data of the petroleum products was done by TIm Roy, Port Royal Reseach, US Petroleum samples were mad available by various Concawe partners.

Modified Ames test results

			Mutation index
Sample Identification	Category	total Wt.% [a]	+S9
Txs000088	Base oil	1.1	0
Txs000089	Base oil	1.7	1.57
Txs000090	Base oil	2.8	0.31
Txs000091	Base oil	0	0
Txs000092	Base oil	0	0.04
Txs000093	Base oil	0.31	0
Txs000094	Base oil	0	0.13
Txs000095	Base oil	0.17	0.11
Txs000096	Base oil	0	0
Txs000097	Destilate aromatic extract	9	1.63
Txs000098	Destilate aromatic extract	9.7	2.82
Txs000099	Destilate aromatic extract	12	1.69
Con-006	Heavy fuel oil	2.9	0.11
Con-008	Heavy fuel oil	27	4.33
Con-017	Heavy fuel oil	0.62	0.73
Con-34	Heavy fuel oil	48	8.72
Con-91	Heavy fuel oil	8.2	4.48
Con-129	Heavy fuel oil	17	4.51

Mutation induction by the petroleum products. Modified Ames tests were conducted on the eigheen petroleum substances tested in the Toxtracker assay to analyse their ability to induce gene mutations. Total wt % of PAC content of the different substances is indicated in a range from light to dark blue. All substances were tested in the ModAmes in presence of S9 according to the standard protocol and the Mutation Indices (MIs) were determined. As defined in ASTM E 1687, MI values <1 are considered to have a high probability of being non-carcinogenic in a mouse skin painting bio-assay, values >1 but <2 (yellow) may or may not be non-carcinogenic in a mouse skin painting assay, whereas values >2 are considered to have a high probability of being positive in a mouse skin painting bio-assay (range from orange to red). The Modified Ames test was performed by Charles River, Den Bosch, NL.

Example of ToxTracker assay results



Example results from the ToxTracker assay. Eighteen petroleum substance were tested in the ToxTracker assay for induction of DNA damage, oxidative stress and protein damage (upper panel) as well as induction of cytotoxicity (lower panel). The figure displayes the ToxTracker results for a refined lubricating oil (Txs000088), a distillate aromatic extract (Txs000099) and a heavy fuel oil extract (Con-034). All substances were analysed in absence and presence S9 hamster liver extract for bioactivation.

ToxTracker summary

				ToxTracker GFP fluorescence (flow cytometry)								ToxTracker biomarker gene expression (qRT-PCR)								
Sample	Category	PAC level	Autofluorescence	DNA damage		p53		Oxidative stress		UPR		DNA damage		p53		Oxidative stress		UPR		Mod. Ames test
				-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	
Txs000091	Base oil	0	1.1																	0
Txs000092	Base oil	0	1.0																	0.04
Txs000094	Base oil	0	1.2																	0.13
Txs000096	Base oil	0	1.3																	0
Txs000095	Base oil	0.17	1.4																	0.11
Txs000093	Base oil	0.31	1.2																	0
Con-017	Heavy fuel oil	0.62	45.1																	0.73
Txs000088	Base oil	1.1	1.4																	0
Txs000089	Base oil	1.7	1.4																	1.57
Txs000090	Base oil	2.8	7.4																	0.31
Con-006	Heavy fuel oil	2.9	21.9																	0.11
Con-091	Heavy fuel oil	8.2	51.5																	4.48
Txs000097	Destilate aromatic extract	9	8.3																	1.63
Txs000098	Destilate aromatic extract	9.7	6.2																	2.82
Txs000099	Destilate aromatic extract	12	5.8																	1.69
Con-129	Heavy fuel oil	17	7.0																	4.51
Con-008	Heavy fuel oil	27	23.2																	4.33
Con-034	Heavy fuel oil	48	27.2																	8.72
Cisplatin																				
Diethyl maleate																				
Tunicamycin																				
Aflatoxin B1*																				

Positive (>2-fold induction)
Weak positive (1.5 to 2-fold induction)
Negative (<1.5-fold induction)
Not tested in this study

CONCLUSIONS

- Petroleum extracts with a high PAC content activated the genotoxicity reporters in the ToxTracker assay
- ToxTracker indicated a mutagenic rather then a clastogenic mode-of-action of the genotoxic petroleum extracts.
- The genotoxicity results from ToxTracker showed a good correlation with mutation induction in the ModAmes test
- Petroleum extracts with high PAC content show high levles of autofluorescence that can interfere with the standard ToxTracker protocol. Detection of the ToxTracker biomarkers by quantitative real-time PCR can be used to circumvent this limitation.
- Overall, the current results from the Tracker assay applied to petroleum substances confirms the hypothesis that more refined petroleum streams low in PAC content have a low potential to be mutagenic / carcinogenic, in contrast to petroleum streams with relatively high level PAC
- This study is a promising first step towards developing a novel assay to screen for the mutagenic / carcinogenic potential of petroleum streams
- Future work includes optimisation of the protocol to prevent interference by autofluorescence of PACs and to optimise bioactivation of petroleum extracts