Adaptation of the ToxTracker reporter assay for the genetic toxicology assessment of petroleum products.

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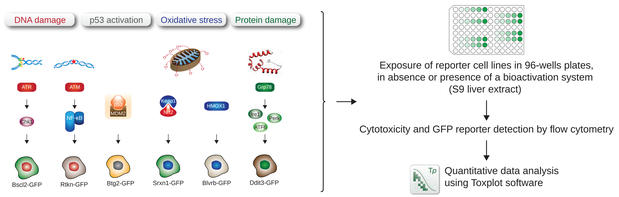
OBJECTIVES

Poorly refined heavier petroleum substances (PS) have the potential to be mutagenic and / or carcinogenic based on their 3-7 ring poly aromatic hydrocarbon (PAH) content. The petroleum industry applies 2 common methods to screen for these effects, namely the Modified Ames assay and the IP346 test. These test provide a yes / no answer based on a cut-off value, and are validated against a significant *in vivo* dataset.

In a previous study between Toxys and Concawe, the applicability of the ToxTracker assay for assessing the genotoxic properties of PS was investigated. After completion of this project, questions around the metabolism of the test substances in the absence and presence of S9 remained open. With the current project, we aimed to address these issues by looking at the expression of metabolic enzymes in mouse emryonic stem cells, the effect of different S9 mixes (rat and hamster), mutation induction in the HPRT assay and the activation of ToxTracker reporters by PAHs with a specific number of aromatic rings.

TOXTRACKER



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. Additionally, 10 pure PAHs were tested. The differential induction of the GFP reporters as well as cytotoxicity of the tested substances was determined by flow cytometry.

DMSO extracts, containing the "biologically active" fraction (i.e. mostly 3-7 ring PAHs) of the petroleum substances were prepared following standard procedures (Roy et al, 1988). 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).

The DMSO extracts were analysed in the absence and presence of S9 hamster and rat liver extract to include a bioactivation system. 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.

Expression of the aryl hydrocarbon receptor and three CYP enzymes was analysed using qPCR. Mutation induction in mammalian cells was tested using the HPRT assay.

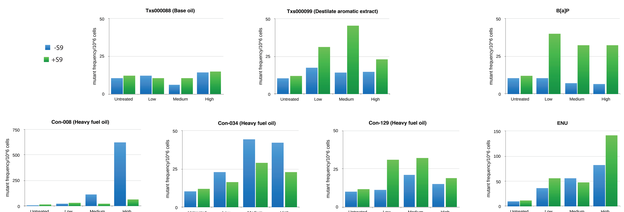
RESULTS

Modified Ames test results

Sample Identification	Category	total Wt.% [a]	Mutation index +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-034	Heavy fuel oil	48	8.72
Con-091	Heavy fuel oil	8.2	4.48
Con-129	Heavy fuel oil	17	4.51

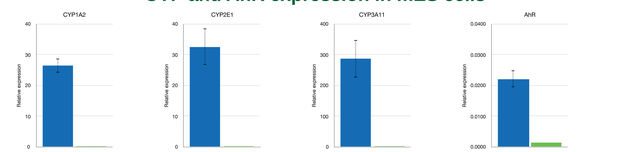
Mutation induction by the extracts of petroleum products. Modified Ames tests were conducted on the eighteen petroleum substances tested in the Toxtracker assay to analyse their ability to induce gene mutations. Total wt % of PAC content of the different substances is indicated in a range from light 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 detemined. 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.

Mutation induction by selected PS in the HPRT assay



Petroleum extracts can induce mutations, depending on their 3-7 ring PAC content, in mES cells in the absence and presence of metabolic activation. Hprt mutant frequencies in mES cells caused by exposure to various petroleum extracts. Induction of mutations in the Hprt gene in mES cells that have been exposed to increasing concentrations of five petroleum extracts in the absence (blue) or presence (green) of hamster S9 liver extract was determined. The depicted Hprt mutant frequencies are the average of two independent repeat experiments.

CYP and AhR expression in mES cells



mES cells do not express CYP enzymes and only low levels of AhR. To test whether mES cells express any of the CYP enzymes important for the metabolisation of PAHs, expression of CYP1A2, 2E1, 3A11 and the aryl hydrocarbon receptor (AhR) was analysed using qPCR. RNA was isolated from untreated mES cells and expresssion levels were compared to mouse liver RNA. qPCR reactions were performed in triplicate for each primer and relative expression was calculated compared to HPRT.

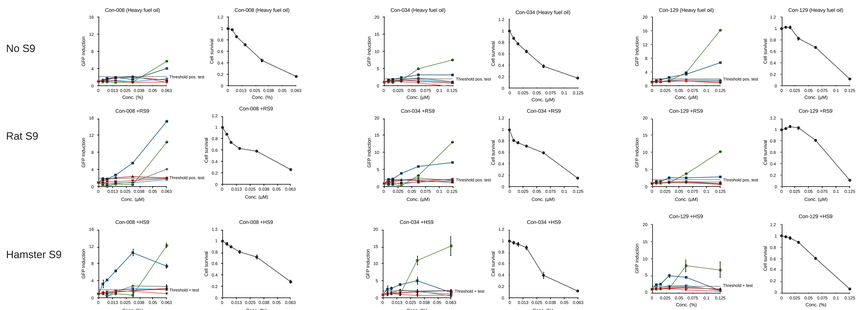
ToxTracker summary

Sample	Category	PAC level	Autofluorescence	ToxTracker GFP fluorescence (flow cytometry) DNA damage -S9	DNA damage +HS9	p53 -S9	p53 +HS9	Oxidative stress -S9	Oxidative stress +HS9	UPR -S9	UPR +HS9	ToxTracker biomarker gene expression (qRT-PCR) DNA damage -S9	DNA damage +HS9	p53 -S9	p53 +HS9	Oxidative stress -S9	Oxidative stress +HS9	UPR -S9	UPR +HS9	Mod. Ames test
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	45.3																	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

Induction of ToxTracker reporter expression after treatment with DMSO extracts of petroleum substances. The ToxTracker reporter cell lines were exposed to 5 concentrations of the test substances in the absence and presence of hamster S9 (HS9). The HS9 protocol included a 3 h exposure with compound in the presence of 1% HS9 and then 21 hours of recovery. For the exposure in the absence of S9, cells were exposed for 24h continuously. Several of the tested substances showed high levels of autofluorescence, which could interfere with the detection of the GFP reporters in ToxTracker. The autofluorescence was compensated for in the ToxTracker assay by also measuring the fluorescent signal in wild-type non reporter cell lines and substracting this value from the reporter induction. To verify the results and to rule out any effect of autofluorescence, reporter expression was also verified using qPCR. The results obtained in the ToxTracker assay largely overlap with those obtained in the modified Ames test. However, for a number of compounds, genotoxicity is observed in the absence of S9, which indicates that metabolic activation is not required.

ToxTracker assay results with rat and hamster S9 for 24h



DNA damage: Bscl2, Rtkn
Cellular stress (p53): Btg2
Oxidative stress: Srxn1, Blvrb
Protein damage: Ddit3

ToxTracker assay with rat and hamster S9. To better align the exposure conditions in the absence and presence of S9, we optimised the S9 protocol to allow 24 hour exposures with a lower concentration of S9 without inducing reporter activation or cell death (0,25% instead of 1%). Rat and hamster S9 have slightly different metabolic properties. To see how both S9 mixes would affect reporter activation and genotoxicity of pertroleum substances, we tested three petroleum substances in the absence and presence of rat and hamster S9 for 24h. Con-008 is genotoxic in the absence of S9 and in the presence of rat and hamster S9. Con 034 is genotoxic only after the addition of rat or hamster S9. Con-129 is not genotoxic under any condition. Exposure without S9, and with rat and hamster S9 give slightly different reporter inductions. Compounds could be metabolised differently by both S9 mixes, therefore it is useful to test compounds both with rat and hamster S9.

ToxTracker results with single PAHs with specific ring number

Test compounds	No. of rings	DNA damage -S9	DNA damage +S9	p53 -S9	p53 +S9	Oxidative stress -S9	Oxidative stress +S9	UPR -S9	UPR +S9
naphthalene	2								
1-methyl naphthalene	2								
2-methyl naphthalene	2								
1-ethyl naphthalene	2								
2-ethyl naphthalene	2								
phenanthrene	3								
2-methyl anthracene	3								
2-terbutyl anthracene	3								
2-ethyl anthracene	3								
7-methylbenzo[a]pyrene	5								
Controls									
Cisplatin									
Diethyl maleate									
Tunicamycin									
Aflatoxin B1									

Positive (>2-fold induction)
Weak activation (1.5 to 2-fold induction)
Negative (<1.5-fold induction)

Most 2-5 ring alkylated PAHs tested in the current project do not cause. Single (alkylated) PAHs containing 2-5 aromatic rings were tested in ToxTracker in the absence and presence of hamster S9. Only the 3-ring alkylated PAH substance 2-ethyl antracene induced activation of the DNA damage reporters in ToxTracker and was therefore classified as genotoxic. Most other compounds caused oxidative stress or protein damage, but no genotoxicity. Similar results were obtained with exposure in the presence of rat S9 (data not shown).

CONCLUSIONS

Heavy fuel oils with a high PAC content test positive in the modified Ames test. Most of these substances, depending on their 3-7 ring PAC content, also test positive for genotoxicity in ToxTracker.

Several petroleum substances show genotoxicity in ToxTracker and the induction of mutations in the HPRT assay in the absence of S9. Gene expression analysis does not seem to indicate that this can be explained by CYP expression in mES cells

To better assess the metabolic actvation, petroleum substances should be tested with both rat and hamster S9 using 24 hours of exposure.

This project showed that the ToxTracker assay can be used for genotoxicity screening of PS, by including adaptations in the protocol which are specific to in-vitro assays for these substances but some open questions remain.