DETAILED TEST PLAN
BACS Experiments

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Authors Florian Mayer, Gudrun Koppen, Maarten Spruyt, Hilda Witters, Marianne Stranger, Marc Houtzager, Daan Noort, Marloes Joosen, Rob Jongeneel, Petra van Kesteren, Harry Buist, Harm Heusinkveld, Flemming Cassee, Ferdinand Spek, Ilja Achterberg, Richard Fox, Andreas Bezold, Eric Groen

Project’s coordinator:
Netherlands Organisation for Applied Scientific Research, TNO

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1 Outline of this document

This document is an addendum to Deliverable D3 Update. Its objective is to further detail the definition of the experimental set-up, the environmental parameters to be monitored and the suspicious bleed / cabin air contaminants to be analysed – for the experiments at the Bleed Air Contamination Simulator (BACS).

Details for engine test bench experiments and test flight campaign will be provided separately, four weeks prior to the start of the respective experiments. This was agreed during the FACTS Steering Board Meeting on September 12-13, 2017.

The document contains the following main parts:

- Detailed test protocol for Task-2 tests in BACS experiments
- Detailed test protocol for Task-3A tests in BACS experiments
- References

2 Detailed test plan for Task 2 in BACS experiments

2.1 Experimental approach

The experimental approach of FACTS is based on a stepwise experimental procedure involving on-ground and in-flight experiments:

- Step 1 Ground tests using a Bleed Air Contamination Simulator (BACS)
- Step 2 Ground tests using an engine test stand for verification
- Step 3 In-flight tests with test aircraft from partners for validation

This document provides details on the BACS experiment in Step 1.

During this stepwise approach, the experimental set-up will be fine-tuned and focussed after each step, in order to obtain suitable monitoring and sampling equipment and experimental programme for cabin air quality assessment.
In all test phases, environmental parameters such as pressure, temperature, relative humidity, etc. will be monitored continuously and air samples will be taken and analysed for all contaminants possible or those of special interest based on preceding results.

Compounds will be analysed by online monitors as well as by off-line air sample analyses (grab sampling) in accredited laboratories according to published standard methods (e. g. ISO 16000 series). For quality assurance inter-laboratory comparisons of selected methods, e.g. organo-phosphates analysis will be performed at the beginning of the analytical campaign.

2.1.1 Ground tests

When contaminants enter the aircraft engine and/or APU they are subjected to a rapid thermodynamic process as they are drawn through the compressor. Both temperature and pressure rise almost instantaneous and it can be perceived this may have an impact on the composition of the contaminants. Available research work, like the EASA funded AVOIL project, has so far not taken this rapid thermodynamic effect into account and thus fails to accurately capture the influence of the air compression on the contaminants.

This proposal sets forth a novel way to simulate under controlled process conditions a cabin air contamination (CAC) event, and its phenomena. The following picture illustrates the normal path of air inside an aircraft and indicates where measurements regarding air contaminant composition seem useful, points ‘A’ through ‘C’.

The points indicated in the following picture are:

A. In the bleed line downstream of engine pre-cooler.
B. At the ECS pack exit
C. Inside the cabin

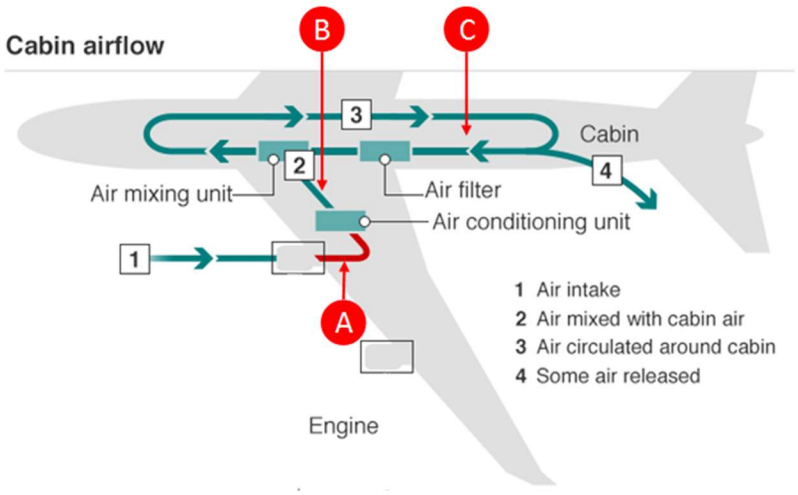

Figure 1. Normal bleed air flow path and measurement points.

By performing the measurements at different points in this air flow the changes in the contaminants, i.e. the contaminant 'fingerprint', throughout the whole process can be captured and characterised. This will provide insight into the processes and phenomena. It also provides information on how a suitable sensor suite could be designed. This is relevant for possible mitigation strategies in Task 4.

Initial tests will be performed at a Bleed Air Contamination Simulator (BACS) allowing mimicking a wide range of operating conditions of different types of currently available commercial engines – using different contaminants in multiple configurations. This laboratory setup simulates the first part of the thermodynamic process acting on a contaminant, in order to assess influence of generic T and p conditions on compound decomposition.

A simulated engine bleed pre-cooler will be installed downstream of the simulator to represent the rapid cooling of the bleed air and large surface area of the real pre-cooler that may temporarily adsorb contaminants. If necessary (temperatures might be too high for air monitoring/sampling) another heat exchanger will be installed to decrease the temperature even further to simulate several stages of air temperature decrease in the bleed air duct before the air would be distributed into the cabin (= spot of maximum contaminant concentration). When that air is entering the cabin contaminant concentrations will only be diluted. The principle scheme of our BACS can be seen below, by that the influence of the cooling in the ECS pack can be assessed:

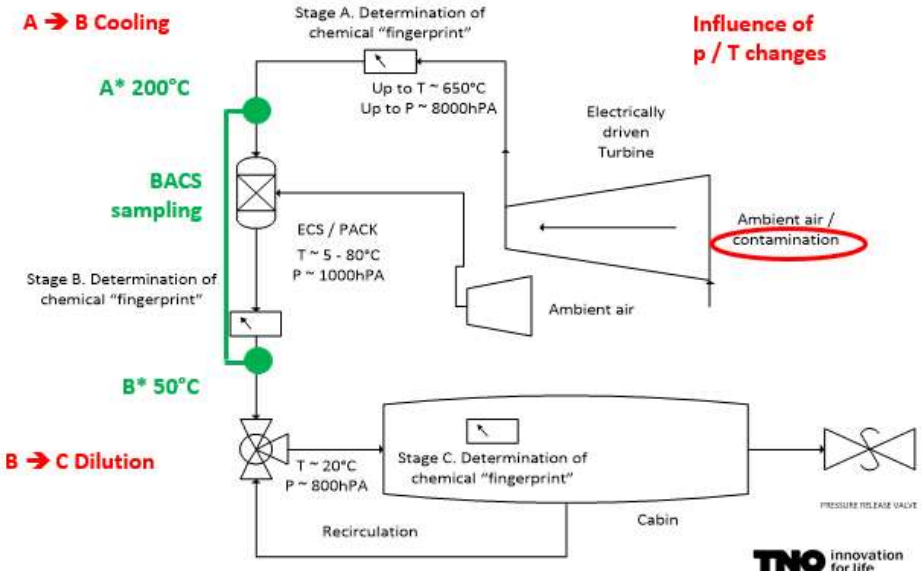

Figure 2 Scheme of Bleed Air Contamination Simulator (BACS).

The use of a simulator versus a real jet engine has its advantages of being independent, regardless of any type of engine, state of the engine, sort of ECS system and all the variations there exist in air distribution systems. This offers the project the advantage to introduce contaminants in a scientific, objective way, and with easy access points. The results of these highly controllable simulator tests allow us to interpret the findings obtained at a real engine test bench (next step), and during test flights. The latter are closest to reality, but less controllable. Comparison of the three test environments will provide insight in the contamination resulting from oil leaking into the bleed air system. Moreover, when it turns out that the results of the BACS are highly comparable with those obtained at the engine test bench and in-flight, such laboratory set-up offers a cost-effective alternative for future research into oil contamination.

2.1.2 Ground tests using a Bleed Air Contamination Simulator (BACS)

The FACTS consortium will be able to use a bleed air test stand set up by Airbus for the German national aerospace research project KlimaTIS (Innovative Concepts for Aircraft Air Conditioning) and meanwhile located at the premises of Fraunhofer IBP, Holzkirchen, providing pressures up to 8 bar and temperatures up to 600°C.

The air led through the BACS will be contaminated in a controlled manner with potential sources of cabin/bleed air contaminants. Care will be taken in the experimental design that any bearing cannot introduce possible unwanted contaminants in the air path.

The figures below show a schematic and a 3-D drawing of the test rig that has been built up within the national German KlimaTIS project.
Such test bench setup capable of simulating air compression in engines and APU's allows simulating targeted contamination ingestion and with that the full characterisation of the air after contaminant ingestion at the compressor exit and at the ECS pack exit, respectively.

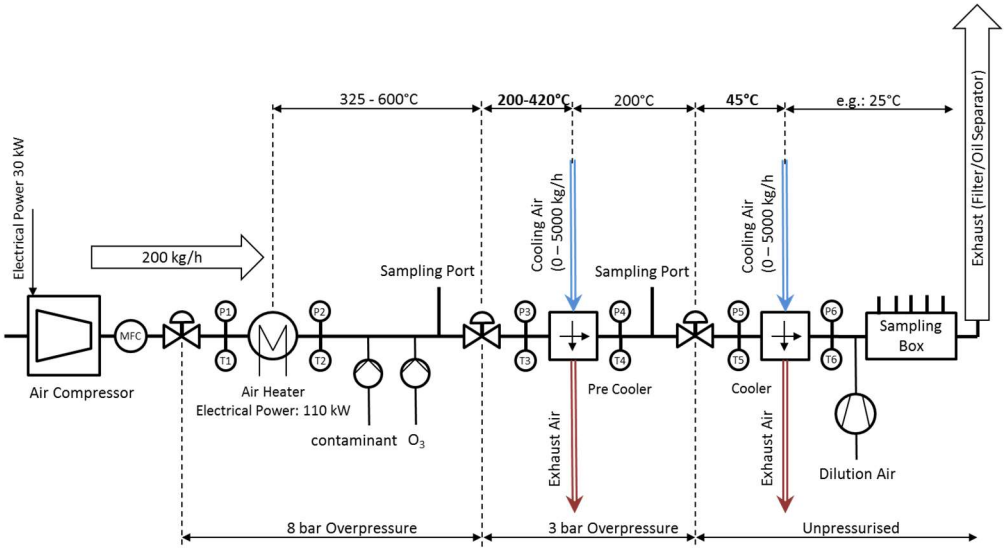

Figure 3. Schematic view of BACS set-up at IBP, Holzkirchen

Figure 4. BACS set-up

2.1.3 Collaboration with KlimaTIS

For the purpose of saving cost and to use synergies FACTS experiments will run in parallel with the German national project KlimaTIS also dealing with possible cabin air contamination. Official agreement to this intended data exchange has been sought and given from the respective funding organisations, DLR and DG MOVE.

2.1.4 Interaction with Task 3A

In addition to the chemical characterisation of the air fumes in this simulated engine, their toxicological impact will be studied in Task 3A. This will be done in a two-step approach. A first in-vitro screening using zebrafish and the Micro electrode Array including neuronal cells (MEA) (in-vitro neurotoxicity assays). Both *in vitro* testing approaches will be for air contaminant mixtures collected at BACS. The air mixture will be captured by cold trapping in a liquid matrix. In a second step, the most toxic fume will be confirmed and tested via the tandem Air Liquid Interface lung cells (ALI)-MEA system – allowing direct exposure of the cells to the air mixtures generated in this set-up, and via the in-vivo mice behaviour test, to assess the impact of the fume in case of direct inhalation exposure. More details can be found in the Task-3A detailed test plan (Chapter 3, page 34 ff.).

2.1.5 Ground tests using engine test stand

The experimental campaign also includes ground tests at an engine test bench from our partners, using an engine from a major manufacturer. Compared to the simulated engine at the BACS an engine test bench offers the advantage of investigating real engine conditions, at least for the type of engine available. Based on the results of the BACS, one contaminant will be selected for the engine test. The tests will be performed at different engine settings (e.g., take off, cruise), and the contaminant will be injected in the engine air flow. This approach is complementary to the VIPR study of Boeing/NASA (Space et al., 2017), where a contaminant was injected through a borescope in an engine on-wing, operating at one setting.

Details on these experiments will be provided later in the detailed test plan for engine tests.

2.2 Experimental Design of BACS experiments

2.2.1 Contaminants

Engine Oil is the contaminant of major concern (see D1), but so far no compound in relevant concentration was identified that can explain certain symptoms reported after oil fume events. Engine oils are classified based on MIL-PRF-23699G (13 March 2014) into 4 classes, Standard STD, Corrosion Inhibiting C/I, Enhanced Ester EE and High Thermal Stability HTS. In commercial aircraft engines mostly EE and recently HTS engine oils are used. EE oils are based on different esters such as Pentaerythritol (PE) Esters or Trimethylolpropane (TMP) Esters. Also the amounts of additives vary. Based on this and the types recommended by engine manufacturers and used by different airlines four different types of engine oil were selected for analysis within FACTS and the parallel running German national project KlimaTIS. Hydraulic fluids will also be included into reaction product investigations at engine conditions.

The following contaminants will be investigated

- 4 types of engine oil
  - Turbonycoil 600 (contains no TCP)
  - Eastman Turbo Oil 2197 (MIL 23699 HTS, PE-Esters)
  - Mobile Jet Oil 2 (MIL 23699 STD, PE-Esters, more additives)
  - Eastman Turbo Oil 2380 (MIL 23699 STD, TMP-Esters)

- 2 types of hydraulic fluids
  - Exxon Mobil HyJet V
  - Eastman Skydrol V

As explained before, measurements will be divided and shared between FACTS and KlimaTIS, de-icing fluids will be investigated in the KlimaTIS project.

2.2.2 Amount of oil

A supplementary document provided by ADSE (Spek, 2017) before included a calculation of the (worst case) amount of engine oil which may enter the bleed air system in various flight phases, including references to scientific papers provided by the FACTS Scientific Committee. The calculation used the BAe 146-200 aircraft and ALF502 engine. Based on this calculation, and in accordance with the VIPR study (Space et al. 2017), the highest oil concentration in the bleed air was calculated at 55 mg/m³. This corresponds to a leakage of 1 kg/hr in each of the four engines at the same time, and also assumes that the leaked oil entirely enters the core flow. Both assumptions are not very likely, but they result in an extreme worst case with regard to short term acute exposure: the calculated oil leakage is four times the double amount of maximally allowed oil consumption rate.

To achieve this worst case oil concentration of 55 mg/m³ in the BACS simulator with a mass flow of 0.05 kg/s (180 kg/h), equalling a volumetric flow rate of 0.042 m³/sec, the oil injection rate must be 8.3 g/h. At the BACS simulator the contaminant will be directly injected into the air flow.

In order to account for extreme acute exposure scenarios with larger engines than ALF502 engines used with the BAe146 and a severe engine failure condition it was agreed with the Scientific Committee to use 9 times higher oil concentrations of up to 500 mg/m³ air, corresponding to 75.6 g/h oil injection rate into BACS as maximum and final concentration. Starting concentration was agreed to be 0.1 mg/m³ air corresponding to an oil injection rate of 0.015 g/h into BACS. 4-5 intermediate concentration will be investigated as well. Table 1 shows a suggestion of oil amounts to be investigated.

Table 1. Target oil concentrations in [mg/m³] air and oil amounts to be injected into BACS at a certain air flow rate

Oil concentration in air [mg/m³]	0.1	1	5	10	50	100	500
Oil amount to be injected into BACS [g/h] at an airflow rate of 180 kg/h	0.015	0.15	0.75	1.5	7.5	15.1	75.6

2.2.3 Ozone

Experiments will be performed in the absence of ozone as well as in the presence of ozone at 1000 ppb for cruise conditions. The rational for 1000 ppb is Advisory Circular AC 120-38 from DoT, FAA, dated 10/10/1980, which categorizes Airplanes' Cabin Ozone Concentrations and mentions a maximum of 1.3 ppm at Flight Level 410 above 70° latitude north. Further south and at lower Flight Levels ozone concentrations are lower, so 1000 ppb was selected as a high and reasonable ozone concentration for the experiments at cruise condition. Ozone can act as an oxidizing agent for compounds in the bleed system.

2.2.4 Test conditions

Five test conditions characterize an engine bleed system over a typical flight profile: Taxi, Take-off, Climb, Cruise and Descent. Typical flight profile data (engine temperatures, pressures, and bleed flow rates) are listed in the Profile Data Table below for an ALF 502 engine. Pressures have been recorded up to 13 bar and temperatures up to 390°C. New engine generations with increasingly higher inter-turbine temperatures may go up to 450°C and above. The inter-turbine temperatures likely will not be greater than 450°C unless new materials, coatings, and internal cooling are developed that will permit engine combustors and turbines to operate at greater temperatures than currently encountered. However temperatures up to 600°C are expected in future engines since engine valves are nowadays already specified for temperatures up to 1200°F (= 648°C).

Table 2. Profile data table with temperatures and pressures for different engine conditions.

			Profile Data					
Altitude ft.	**Test Point**	**Time mins**	**PB3 bar**	**PB3 psia**	**TB3C degC**	**TB3F degF**	**W2 Kg/s**	**W2 pps**
0	Taxi	10	3.5	51	195	383	6.08	13.4
0	Take-Off	0	12.0	173.8	376	709	17.2	37.8
0	Take-Off	1	12.6	183.0	382	720	18.1	39.9
0	Climb	1.01	11.4	165.3	362	684	17.0	37.4
5000	Climb	3	10.1	147.2	354	669	15.2	33.5
10000	Climb	5	9.0	131.1	347	657	13.6	29.8
15000	**Climb Test Point**	**8**	**8.0**	**115.7**	**340**	**644**	**11.8**	**26.1**
20000	Climb	11	7.1	102.3	334	633	10.4	22.9
25000	Climb	14	6.3	90.7	330	626	9.2	20.2
25000	Cruise	15	6.0	87.0	318	604	9.0	19.9
25000	Cruise	44	6.0	87.0	318	604	9.0	19.9
25000	Descent	45	3.5	51.1	230	446	6.1	13.4
20000	Descent	47	3.6	52.3	221	430	6.4	14.1
15000	Descent	49	3.7	53.6	211	412	6.7	14.8
10000	Descent	52	3.7	53.6	201	394	7.4	16.3
5000	Descent	55	3.8	55.2	196	385	8.3	18.3
1500	Descent	60	4.1	60.1	195	383	9.2	20.3

2.2.5 Step 1 Experiments at a Bleed Air Contamination Simulator (BACS)

As can be seen in Table 2, take-off conditions are most severe. However, the BACS pressure conditions are limited to 8 bar, so the test condition selected closest to that are early climb conditions (Climb Test Point), which will be combined with different temperature conditions. In addition, tests with two higher T conditions (450°C and 600°C) for future HT engines will be included. Moreover, one 8 bar / T condition will be investigated with and without ozone.

Thus, the following five test conditions are foreseen:

- 8 bar / 340°C / without ozone
- 8 bar / 340°C / with ozone
- 8 bar / 450°C / without ozone
- 8 bar / 600°C / without ozone
- 3 bar / 200°C / without ozone – representing APU conditions

1 oil concentration of each oil will be subjected to these 5 p/T conditions.
One test condition will be applied to 7 different oil concentrations.

Profile data of other engines will be sought from other engine manufacturers (e. g. RR, GE, P&W) through consortium partners (Airbus, ADSE, Honeywell).

2.2.6 On-line screening

Experiments will start with an on-line screening of one engine oil being injected at different increasing concentrations into BACS at a defined pressure level and being exposed to continuously increasing temperatures of up to 600°C. On-line monitors available within the consortium will be attached to see at what c and T conditions something detectable (change in chemical composition) is happening. This on-line screening will last about 2 weeks. On-line detectors that do not detect anything under severe conditions will be removed in order to decrease cost. Temperature, pressure, relative humidity, ozone generator (1000 ppb) and ozone monitor are part of the KlimaTIS bleed air test rig.

2.2.7 FACTS on-line monitors

The following table 3 shows the on-line monitors that will be used by the FACTS consortium.

Table 3. List of on-line monitors of the FACTS consortium and provider

Monitor	Partner
Carbon monoxide CO (1-1000 ppb, by IR absorption, Teledyne Instruments Analyzer 300E)	IBP
Carbon dioxide CO2 (100-10000 ppm, by IR absorption, Teledyne Instruments Analyzer 360E)	IBP
Nitrogen oxides NO/NOx (1-1000 ppb, by Chemo luminescence, Environment S. A. Analyzer AC 31M)	IBP
Sulphur dioxide SO2 (1-10000 ppb, by UV-Fluorescence, Airpointer)	VITO
Formaldehyde HCHO (1-1000 ppb, by Fluorimetry, AeroLaser AL4021 Hantzsch reaction Monitor)	IBP
Total Volatile Organic Compounds (TVOC) by Flame Ionisation Detector FID (Sick Bernath Atomic 3006)	IBP
Total Volatile Organic Compounds (TVOC) by Photo-Ionisation-Detector PID (RAE Systems ppbRAE3000)	IBP
Selected Volatile Organic Compounds by on-line Proton-Transfer-Reaction-Mass-Spectrometry, Ionicon PTR-TOF 8000PTR-MS	Airbus
Aerotracer (Airsense Analytics hybrid sensor array)	Airbus
Pegasor PPS Particle Sensor (0.01 – 250 mg/m³; by charging and measuring current change)	Airbus
Selected Volatile Organic Compounds by Selected Ion Flow Tube Mass Spectrometry (SIFT-MS), Syft Voice 200 SIFT-MS	VITO
Ultrafine Particulate matter 0.005 – 1 µm (UFP) by scanning mobility particle sizer (SMPS), TSI SMPS 3936 (electrostatic classifier 3080, neutralizer 3077 or 3088, long differential mobility analyser DMA 3081, butanol-based Condensation Particle Counter CPC 3776) *	VITO
Scanning mobility particle sizer (TSI 3080) 10-500 nm and CPC3775 (TSI), 0,5 µm -20µm	TNO
Particulate matter 1 – 40 µm (PM1, PM2.5, PM10 and larger) by light scattering and filter-sampling, Grimm PAS 11-A	IBP
Black carbon (0-1 mg) by transmitted light absorption, Micro aethalometer AE51	VITO

* Comment:
Pre-separator (impactor or cyclone depending on instrument) with conductive black sampling tubing of equal length (as short as possible and at least <1 m).
Data will be corrected for diffusion losses in the sampling line and instrument.
Sampling protocol will be based on UN-ECE regulation 83.

Table 4. Sampling flows, sampling volumes, space and operating conditions needed for on-line monitors

On-line Monitors	Necessary air flow for sampling [L/min]	Typical sampling duration [min]	Total volume needed per min [L]	ca. Equipment size L x B x H [cm]	Operating conditions (p, T)
Carbon monoxide CO (1-1000 ppb)	0.9	1	1	43 x 60 x 18	Ambient, 35°C
Carbon dioxide CO2 (100-10000 ppm)	0.9	1	1	43 x 60 x 18	Ambient, 35°C
Nitrogen oxides NO/NOx (1-1000 ppb)	1.3	3	2	60 x 48 x 18	Ambient, 35°C
Sulphur dioxide SO2 (1-1000 ppb)	0.5	1.5	1	90 x 80 x 40	Ambient, 35°C
Ozone O3 (1-1000 ppb)	1	1	1	50 x 60 x 18	Ambient, 35°C
Formaldehyde HCHO (1-1000 ppb)	1.2	10	2	45 x 56 x 15	Ambient, 35°C
Total Volatile Organic Compounds (TVOC by FID)	1	1	1	28 x 52 x 46	Ambient, 200°C
Total Volatile Organic Compounds (TVOC by PID)	0.5	0.05	1	26 x 8 x 7	Ambient, 50°C
Selected Volatile Organic Compounds (by on-line MS such as PTR-MS and SIFT-MS)	0.025	0.1-60	1	100 x 100 x 100 100 x 100 x 100	Ambient, 100°C
Aerotracer	0.05-0.5	0.1-1	1	40 x 11 x 21	Ambient, 35°C
Pegasor PPS Particle sensor	10	0.002	10	40 x 5 x5	3 bar 200°C
Particulate matter 0.005 – 1 µm	SMPS 2 CPC 1.5	0.1	4	46 x 42 x 64 25 x 32 x 37	Ambient, 35°C
Particulate matter 0.01 - 20 µm	SMPS 3 CPC 1.5	0.1	5	46 x 42 x 41 25 x 32 x 37	Ambient 35°C
Particulate matter 1 – 40 µm (PM1, PM2.5, PM10 and larger)	1.2	0.1	2	24 x 13 x 7	Ambient 35°C
Black carbon (0-1 mg/m³)	5-16	1	32	49 x 43 x 43	Ambient 35°C
total sampling volume needed per min [L]			**65**		

2.2.8 FACTS off-line analysis

In the CfT organophosphate compounds, R-aldehydes and R-ketones, Acrolein, VOC (notably BTX), persistent organic pollutants like PAH, Dioxins, Furans have been suggested as initial target compounds. These will be included in the measurement programme – see table on next page.

In order to achieve comparability and quality between different methods used by different labs especially for the analyses of OPEs, an inter-comparison study is included. The consortium will offer participants the following:

- Blank reagent
- Blank glass fiber filter
- Spiked filter (2 concentration levels)
- Standard extract (2 concentration levels)
- Oil extract

The individual performance is assessed on the basis of z-scores with respect to the group average and theoretical values. The standard deviation of the reproducibility (sR) of the individual sample is used as the distribution measure when calculating the Z-scores with respect to the group average. Outliers will be determined with the Grubb's test.

Chemical off-line analyses involve timely and monetary effort, therefore only analyses that detect contamination products within the first experiments with a contaminant will be performed throughout the campaign.

Based on the literature research in Task 1 and discussions within the FACTS Consortium and with the Scientific Committee, the analytical program listed in the original proposal has been limited. Analyses on, e.g. phthalates and nitrosamines have been deleted. Dioxins/furans will only be analysed in the first experiments to proof their absence (although they are considered less relevant). Deletion of further analyses is based on the results of the first analyses with the first experiments with each contaminant. For the analytical procedure, of OPE’s and aldehydes, it has to be shown that the method applied works at low humidity conditions (<10%) as likely to occur on board aircraft.

For validation of the aldehyde measurements at low humidity, an atmosphere containing low and high boiling aldehydes will be generated at normal relative humidity level (+/- 50%) and at low relative humidity (< 10%). As low boiling aldehyde e. g. formaldehyde will be used and as higher boiling aldehyde e. g. benzaldehyde will be used. Six-fold parallel samples will be taken and both sets will be compared. If the results obtained at low humidity are not acceptable, an alternative sampling method will be evaluated using cartridges spiked with demineralised water prior to sampling.

Due to very low vapour pressure of OPEs, generation via a standard atmosphere is not easily achieved. For validation of Organophosphate measurements, standard atmospheres containing purified air with humidity levels of ±10% and ± 50%, respectively, will be generated. Filters spiked with concentration levels of OPEs in the range of 25-150 ng will be coupled to the manifold for a sampling period of 30 minutes. Six-fold parallel samples will be taken by drawing air over the filters with a flow of

approximately 2 L/min. Both sets of samples will be compared and differences in results between the humidity levels shall be determined.

Table 5 shows the chemical analyses programme suggested and the partner being responsible for certain measurements.

Table 5. Off-line chemical analysis program

Compounds	Standard	Lab			Comment
		TNO	VITO	IBP	
Volatile Organic Compounds (VOC, C6-C16, incl. BTXE, halogenated)	ISO 16000-6 EN 16516			X	Quality control: 1-2 same samples analysed by all 3 labs
Volatile Organic Compounds (VOC, C6-C16, incl. BTXE, Acrolein)	EPA TO-15	X			Quality control: 1-2 same samples analysed by 2 labs
Very Volatile Organic Compounds (VVOC, C2-C6)	ISO 16000-6			X	Likely only with first experiments
Aldehydes/Ketones	ISO 16000-3		X		Quality control: 1-2 same samples analysed by all 3 labs
Carboxylic acids	ISO 16000-6 / EN 16516 SIM			X	Previously detected oil breakdown products
Organo-phosphates (31 incl. 10 TCP isomers)	ISO 16000-31, TNO method	X			Round robin test with several labs
Dioxins and furans	EPA 1613		X		Likely only with first experiments, absence to be shown!
PAHs, PCBs	ISO 12884/ ISO 16000-12	X			Likely only with first experiments and in wipe/dust samples
Odour active compounds	Fragrance Industry			X	With selected smelly samples (old socks smell often described with fume events)
Characterisation of particles	Electrostatic Deposition (NAS) using SMPS classifier (< 0.1 µm) EDX / SEM Dekati Low Pressure Impactor (0.03-10 µm) µ-XRF		X		
Elemental Carbon / Organic Carbon (EC/OC)	NIOSH 5040 EUSAAR 2 protocol	X			Health parameter, is better than black carbon

The overall experimental test plan is suggested in Table 18 at the end of this chapter. On the following pages the analytical methods for off-line analyses are described.

2.2.8.1 Description off-line Methods

VOC / VVOC

VOC and VVOC determination is based on ISO 16000-5 sampling and ISO 16000-6 and EN 16516 standards analysis. For sampling 0.2 to 5 NL of air are drawn by a pump (e. g. Desaga/Sarstedt GS 301) at a flow rate of 100 mL/min onto Tenax TA adsorbent tubes. For VVOC a multi-bed adsorber like Carbotrap 300 containing Carbopack C, Carbopack B and Carbosieve S-III or Tenax and Carboxen 1000/1003 (all obtainable from Sigma Aldrich) is used depending on compounds expected. Analysis is performed on a TD-GC-MS system consisting of a Markes International TD 100 Thermodesorber connected via a transfer line to a Shimadzu GC 2010 Plus gas chromatograph and a Shimadzu QP 2010 Ultra mass spectrometer. Analytes are desorbed from Tenax TA at 280°C during 15 min and cryo-focussed at -10°C prior to injection into the GC by flash heating to 310°C. Compounds are separated on a Restek Rxi-5Sil MS capillary column (60 m length x 0.25 mm inner diameter x 1.0 µm film thickness) using Helium as carrier gas. The GC temperature is held at 40°C for 5 min, then raised at 8°C/min to 300°C and held for another 5 min. Compounds are identified based on retention time and mass spectra libraries (laboratory own, NIST 2014). Quantification is performed by substance specific calibration for over 180 common indoor VOCs with an agreed LCI value (http://ec.europa.eu/DocsRoom/documents/22321/attachments/2/translations/en/renditions/native) or, if not as pure reference compound available, against toluene in toluene equivalents.

Carboxylic Acids

Carboxylic acids (C3 to C10) are also sampled according to ISO 16000-5 and analysed according to ISO 16000-6 and EN 16516 standards by drawing 0.2 to 5 NL of air by a pump (e. g. Desaga/Sarstedt GS 301) at a flow rate of 100 mL/min onto Tenax TA adsorbent tubes.

Analysis is performed on a TD-GC-MS system consisting of a Markes International TD 100 Thermodesorber connected via a transfer line to a Shimadzu GC 2010 Plus gas chromatograph and a Shimadzu QP 2010 Ultra mass spectrometer operated in SIM mode. Analytes are desorbed from Tenax TA at 280°C during 15 min and cryo-focussed at -10°C prior to injection into the GC by flash heating to 310°C. Compounds are separated on a Restek Rxi-624Sil MS capillary column (60 m length x 0.25 mm inner diameter x 1.4 µm film thickness) using Helium as carrier gas. The GC temperature is held at 40°C for 5 min, then raised at 8°C/min to 300°C and held for another 5 min. Compounds are identified based on retention time and mass spectra libraries (laboratory own, NIST 2014). Quantification is performed by substance specific calibration of pure reference compound calibration curves using selected target ions for the carboxylic acids of interest (see table 6 below).

Table 6. List of organic acids analysed and their target ions for quantification

Organic acid	CAS-No.	m/z quantifier ion,	m/z qualifier ion
Propionic acid (C3)	79-09-4	74	45
Butanoic acid (C4)	107-92-6	60	73
Butanoic acid, 3-methyl-	503-74-2	60	87
Butanoic acid, 2-methyl-	116-53-0	74	87 (3:1)
Pentanoic acid (C5)	109-52-4	60	73
Hexanoic acid (C6)	142-62-1	60	73
Heptanoic acid (C7)	111-14-8	60	73
Octanoic acid (C8)	124-07-2	60	73
2-Ethylhexanoic acid	149-57-5	73	88
Nonanoic acid (C9)	112-05-0	60	73
Decanoic acid (C10)	334-48-5	60	73
Phthalic acid	88-99-3	76	104

Quality control measures performed for GC-MS analyses on VOC, VVOC, organic acids analyses:

- All quality control measures described and required by EN 16516, sections 8.2.1 General, 8.2.2 Analytical system, 8.2.3 Tube conditioning and laboratory blank tubes, 8.2.4 Sampling test chamber air 8.2.5 Identification, calibration and analysis, 8.4 Other general aspects of quality control and Annex A Repeatability and reproducibility are followed for any VOC analyses from air
- Each time before a sequence is run at the GC-MS system, the mass spectrometer is tuned
- MS response factors for substance specific calibration on the current GC-MS system used for VOC analyses are collected since 2015 and are verified regularly, latest every 18 months or when a substance on the European LCI list is detected for the first time
- Every sampling and calibration tube is spiked with d5-chloro benzene (20 ng) to check for possible losses and differences in MS sensitivity
- Volume drawn by sampling pumps is verified by a DAkkS calibrated drum gas meter every 6 months, the average deviation of 6 individual measurements has to be below 5%
- The volume pipetted by piston pipettes is verified gravimetrically every 3 months according to DIN EN ISO 8655-1, DIN EN ISO 8655-2, DIN EN ISO 8655-6
- Analytical balances weighing results can be traced back to a reference weight through an unbroken chain of comparative weighing operations performed every 12 months, analytical balances are verified by weighing a standard weight with an accuracy of 0.00000 g before each weighing operation
- Analytical standards are dotted by a manufacturer certified 1 µL syringe

Aldehydes / Ketones

Aldehydes and Ketones are analysed according to ISO 16000-3.
20 to 60 NL of air are drawn by a pump at an air flow of 1 L/min onto Waters Sep-Pak DNPH-Silica-cartridges. Eluted DNPH derivatives are analysed on a Waters Acquity UPLC with a Waters Acquity photo diode array detector (UPLC-PDA) recording the wave length between 210 and 400 nm. The system is equipped with a Waters Acquity UPLC BEH Phenyl 1.7µm, 2.1 x 100 mm analytical column and operated at 40°C and an eluent gradient of water, acetonitrile and tetrahydrofuran at a flow rate of 0.5 mL/min. Aldehydes / Ketones are identified by retention time of the individual dinitrophenylhydrazone derivatives and their UV-spectra. The DAD signals at 360 nm (band width 4 nm) are used for quantification via substance specific calibration curves.

Sample cartridges are desorbed on site to minimize analyte losses during transport. A calibration diagram is included in every analytical sequence, as well as an independent control standard. Standards and control standards are obtained commercially (Supelco) and certified by the supplier. Variations of retention times and response factors are checked. Laboratory and field blanks are analysed.

Table 7. List of 2,4-Dinitrophenylhydrazones analysed

2,4-Dinitrophenylhydrazone	CAS-No.
Formaldehyde-2,4-dinitrophenylhydrazone	1081-15-8
Acetaldehyde-2,4-dinitrophenylhydrazone	1019-57-4
Acrolein-2,4-dinitrophenylhydrazone	888-54-0
Acetone-2,4-dinitrophenylhydrazone	1567-89-1
Propionaldehyde-2,4-dinitrophenylhydrazone	725-00-8
Crotonaldehyde-2,4-dinitrophenylhydrazone	1527-96-4
2-Butanone-2,4-dinitrophenylhydrazone	958-60-1
Butyraldehyde-2,4-dinitrophenylhydrazone	1527-98-6
Valeraldehyde-2,4-dinitrophenylhydrazone	2057-84-3
Isovaleraldehyde 2,4-dinitrophenylhydrazone	2256-01-1
Methylisobutylketone-2,4-dinitrophenylhydrazone	1655-42-1
Benzaldehyde-2,4-dinitrophenylhydrazone	1157-84-2
Cyclohexanone 2,4-dinitrophenylhydrazone	1589-62-4
o-Tolualdehyde-2,4-dinitrophenylhydrazone	1773-44-0
m-Tolualdehyde 2,4-dinitrophenylhydrazone	2880-05-9
p-Tolualdehyde-2,4-dinitrophenylhydrazone	2571-00-8
2,5-Dimethylbenzaldehyde-2,4-dinitrophenylhydrazone	152477-96-8
Hexanal 2,4-dinitrophenylhydrazone	1527-97-5
Heptanal 2,4-dinitrophenylhydrazone	2074-05-7
Octanal 2,4-dinitrophenylhydrazone	1726-77-8
Nonanal 2,4-dinitrophenylhydrazone	2348-19-8
Decanal 2,4-dinitrophenylhydrazone	1527-95-3

For Acrolein this method likely produces too low results, results of VOC analysis by EPA TO-15 method should be considered more reliable, OSHA 52 method has shown to be insufficient for acrolein analysis regarding sampling volume to be drawn and respective detection limit, which is much higher than the concentrations expected. Trials will also be made with humidified DNPH cartridges and immediate elution after sampling.

VOCs canister method EPA TO-15

VOCs determination is based on EPA Compendium method TO-15,''Determination of volatile organic compounds in air collected in specially-prepared canisters and analyzed by GC/MS''. Sampling is carried out with a silco steel canister evacuated to 0.05 mm Hg. By using a calibrated restrictor with a constant flow a sample of air is taken during 30 minutes. After sampling the pressure in the canister is measured. Second, the canister is put to an over pressure of approx. 5-15 psi by using synthetic air. An aliquot of air is taken and led over a cold trap. After the sample volume (e.g., 500 mL) is pre-concentrated on the trap, the trap is heated and the VOCs are thermally desorbed and refocused on a cold trap. This trap is heated and the VOCs are thermally desorbed onto the head of the capillary column. The VOCs are refocused prior to gas chromatographic separation. Then, the oven temperature (programmed) increases and the VOCs begin to elute and are detected by a GC/MS system.

The GC/MS system consists of an Agilent 7890B gas chromatograph and an Agilent 5977B Quadrupole mass spectrometer. As thermal desorption unit a Markes Ultra 50:50, Unity 2 and CIA advantage is used.

VOCs are separated on a VF-624ms column (30m*0.25mm) with a film thickness of 1.4 µm using Helium as carrier gas.

Compounds are identified based on retention time and mass spectra libraries (AMDISH, NIST 2014). Quantification is performed by substance specific calibration with standard TO-15 (Scott cylinder ST0000182636; Lot #160-401113255-1; date 12 Feb 2017; expiration date 12 Feb 2019).

QC measures

- Performance of the mass spectrometer is checked by means of an Auto Tune
- Canisters are cleaned and for each cleaned batch, one canister is checked on VOCs by filling the canister with synthetic air (criteria: concentration of VOCs < 1ng, concentration of benzene and toluene < 2 ng)
- For each series of 10 canisters, one blank and one calibration standard is analysed.
- Criteria Qualifier SIM mode < 10% of the expected value
- Benzene-d6, toluene-d8 and ethylbenzene-d10 are used as internal standards and calculated based on the response of the corresponding compounds in the standard. Results are listed in control chart).
- Ratio between benzene and naphthalene is checked and listed in control charts

Organophosphates (OPEs)

Organophosphates are sampled on open faced glass fiber filters (Whatman, diameter 47 mm) in combination with Chromosorb 106 adsorption tubes. Sampling time is 30 minutes with a flow rate of 2 L/min. Glass fiber filters and tubes, including tube separators are extracted with ASE 350 (Dionex) with dichloromethane as extraction solvent. Before extraction internal standard Triphenyl Phosphate-d15 and Triethyl Phosphate-d15 are added to the filter. After extraction the sample extract is concentrated and analysed by GC/MS. The method is based on the work of Solbu et al. (2007). The differences between the method used here and the published procedure is, that TNO uses a combination of filter and tube, while Solbu et al. uses

a Chromosorb tube only. The settings for analyses are comparable to Solbu et.al. but the list of organophosphates analysed is expanded to all 10 TCP isomers.
Analyses are performed by gas chromatography – mass spectrometry (GC-MS) on an Agilent Gas Chromatograph 6890 coupled to an Agilent Mass Spectrometer 5973). The OPEs are separated on an Agilent 122-5532 capillary column (DB-5ms, 30 m length x 0.25mm inner diameter x 0.25 µm film thickness) using helium as carrier gas. OPEs analysed are listed in Table 8.

Table 8. List of organophosphates of interest

Component	Abbreviation	CAS-Number
1,2,3,4-Tetrachloronaphthalene	TCN (Inj. Std.)	200020-02-4
Triethyl Phosphate-d15	TEP-d15 (IS 1)	135942-11-9
Triphenyl phosphate-d15	TPhP-d15 (IS 2)	115-86-6
Trimethyl phosphate	TMP	512-56-1
Triethyl phosphate	TEP	78-40-0
Tri-isopropyl phosphate	TiPP	513-02-0
Tri-n-propyl phosphate	TPP	513-08-6
Tri-n-butyl phosphate	TBP	126-73-8
Tris (2-chloroethyl)phosphate	TCEP	115-96-8
Tris (1-chloro-2 propyl) phosphate	TCPP-1	13674-84-5
Bis (1-chloro-2-propyl) (2-chloropropyl) phosphate	TCPP-2	13674-84-5
(1-chloro-2-propyl) bis (2-chloropropyl) phosphate	TCPP-3	13674-84-5
Tris (1,3-dichloro-2-propyl) phosphate	TDCPP	13674-87-8
Trimethylolpropane phosphate	TMPP	1005-93-2
Dibutyl phenyl phosphate	DBPP	2528-36-1
Butyl diphenyl phosphate	BDPP	2752-95-6
Tris (2-butoxyethyl) phosphate	TBEP	78-51-3
Triphenyl phosphate	TPhP	115-86-6
2-ethylhexyl diphenyl phosphate	diphenyl EHP	1241-94-7
Tris (2-ethylhexyl)phosphate	TEHP	78-42-2
Cresyl diphenyl phosphate	CDP-1	26444-49-5
Cresyl diphenyl phosphate	CDP-2	26444-49-5
Di-cresyl phenyl phosphate	DCP-1	
Di-cresyl phenyl phosphate	DCP-2	
Tri (o, o, o)- cresyl phosphate	T(o,o,o)CP	78-30-8
Tri (o,o,m) cresyl phosphate	T(o,o,m)CP	
Tri (m,m,o) cresyl phosphate	T(m,m,o)CP	
Tri (m, m ,m) cresyl phosphate	T(m,m,m)CP	563-04-2
Tri (o,p,m) cresyl phosphate	T(o,p,m)CP	
Tri (m,m,p) cresyl phosphate	T(m,m,p)CP	
Tri (o,o,p) cresyl phosphate	T(o,o,p)CP	
Tri (o,p,p) cresyl phosphate	T(o,p,p)CP	
Tri (m,p,p) cresyl phosphate	T(m,p,p)CP	
Tri (p, p, p) cresyl phosphate	T(p,p,p)CP	78-32-0

Method validation

The complete validation of OPEs is described in TNO report R 11572 (Makarem, Houtzager 2013,''Determination of OPEs in air using GCMS").
Table 9 shows the performance characteristics for the individual organophosphates.

Table 9 Performance characteristics of OPE components

Component	Linear to [ng/mL]	*Concentration range [ng/mL]*	*LOD* [ng/mL]	vc_r [%]	vc_{Rw} [%]	*U* [%]
TMP	199	0-400	0.6	13%	5.9%	34%
TEP	419	0-400	0.3	14%	14%	42%
TiPP	420	0-400	0.6	14%	5.0%	34%
TPP	414	0-400	1.1	14%	3.0%	33%
TBP	218	0-400	1.1	10%	7.9%	36%
TCEP	218	0-400	0.7	3.4%	3.6%	33%
TCPP-1	256	0-256	0.6	3.3%	2.9%	33%
TCPP-2	130	0-130	0.4	4.6%	6.1%	34%
TMPP	100	0-930	2,5	4.3%	8.4%	25%
TCPP-3	20	0-20	1.0	4.5%	12%	40%
TDCPP	212	0-400	5.8	4.2%	5.1%	34%
DBPP	220	0-400	1,0	11%	8%	16%
BDPP	200	0-500	1,0	6%	9%	21%
TBEP	214	0-400	8.9	5.7%	7.5%	35%
TPhP	201	0-401	0.3	0.8%	3.0%	33%
diphenyl EHP	211	0-400	0.6	3.0%	1.8%	32%
TEHP	209	0-400	1.3	2.9%	3.0%	33%
CDP-1	105	0-105	0.6	0.8%	1.8%	32%
CDP-2	54	0-54	0.3	2.1%	1.2%	32%
DCP-1	35	0-35	0.9	2.1%	3.6%	33%
T(o,o,o)CP	150	0-1500	1.0	4,6%	3,5%	14%
DCP-2	40	0-40	0.6	4.5%	3.4%	33%
T(o,o,m)CP	120	0-1200	1,00	5,0%	5,7%	21%
T(m,m,o)CP	130	0-1300	1,00	4,3%	5,5%	22%
T(o,o,p)CP	120	0-1200	1,00	5,0%	5,4%	24%
T(m,m,m)CP	130	0-1300	1,00	5,0%	4,3%	26%
T(o,p,m)CP	140	0-1400	1,00	4,2%	6,0%	28%
T(m,m,p)CP	150	0-1500	1,00	4,7%	4,4%	26%
T(o,p,p)CP	140	0-1300	1,00	4,4%	7,0%	29%
T(m,p,p)CP	150	0-1700	1,00	5,3%	4,3%	25%
T(p,p,p)CP	140	0-1400	1,00	5,1%	4,3%	28%

LOD Limit of Detection
vc_r Repeatability
vc_{RW} Reproducibility
U Uncertainty

QC measures

- Performance of the mass spectrometer is checked by means of an Auto Tune
- Pre-column, liner and septum are replaced after each series of 10-15 samples.
- Blank reagents and blank filters are analysed for each series of 10-15 samples.
- Control samples of a diluted solution of engine oil are analysed every 10-15 samples and registered in control charts
- Control samples of spiked filters with TCP isomers are analysed every 10-15 samples and registered in control charts
- Criteria Qualifier SIM mode ≥ 90 % of the expected value
- Chain of custody is guaranteed by using specific TNO codes generated by a dedicated TNO dossier system. The codes are used during the whole process from sampling to final analyses results.

Dioxins / Furans

The determination of PCDD-PCDF is done by means of the HRGC/HRMS technique in combination with isotope dilution technique.
13C-labeled 2,3,7,8-chlorosubstituted PCDD- and PCDF congeners will be added in the different stages of the method in order to correct for possible losses.
PUF filters (Tisch Environmental) are sampled using in-house built high volume samplers. The sample flow is adjusted to between 30 and 60 L/min for a sampling period of 60 to 120 min. The sample volume drawn is recorded by an in-line dry gas counter. PUF filters are extracted by Soxhlet extraction, followed by a sample clean-up using multilayer column and alumina column in order to remove any possible matrix interference. After the clean-up step the extract is concentrated to near dryness and analyzed by means of High Resolution gas chromatograph (Agilent 6890) coupled to a high resolution mass spectrometer (Waters Micromass Autospec Ultima / Premier). Via chromatography the 17 toxic congeners are separated from the non-toxic ones. As analytical column a DB-5ms, 60 m length x 0.25 inner diameter x 0.1 µm film thickness is used.
The Mass Spectroscopic parameters allow us to differentiate the dioxins from the furans and the different chlorinated components as well as the 13C-labeled and the 12C-native congeners. The Mass Spectroscope measures via “selected ion recording” and a resolution of 10000 two selected ions from each congener group.

QC measures

- Sample volume is controlled by a dry gas counter;
- The calibration curve is controlled daily using a control standard;
- Weekly, the control standard is used for drift control;
- Weekly, an extraction blank and the control standard are prepared and analysed;
- For identification, isotope ratio, retention time and signal to noise ratio are verified;
- For calculation of the concentrations, isotope dilution is used. Prior to extraction, the samples are spiked with 13C labelled analogue compounds. Losses during the analyses are also corrected with this internal standard method.

The following dioxins and furans are analysed (Table 10):

Table 10. List of dioxins and furans

PCDF/D	CAS-No.
2,3,7,8-Tetrachlorodibenzofuran	51207-31-9
2,3,7,8-Tetrachlorodibenzo-p-dioxin	1746-01-6
1,2,3,7,8-Pentachlorodibenzofuran	57117-41-6
2,3,4,7,8-Pentachlorodibenzofuran	57117-31-4
1,2,3,7,8-Pentachlorodibenzodioxin	40321-76-4
1,2,3,4,7,8-Hexachlorodibenzofuran	70648-26-9
1,2,3,6,7,8-Hexachlorodibenzofuran	57117-44-9
1,2,3,7,8,9-Hexachlorodibenzofuran	72918-21-9
2,3,4,6,7,8-Hexachlorodibenzofuran	60851-34-5
1,2,3,4,7,8-Hexachlorodibenzodioxin	39227-28-6
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	57653-85-7
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	19408-74-3
1,2,3,4,6,7,8-Heptachlorodibenzofuran	67562-39-4
1,2,3,4,7,8,9-Heptachlrordibenzofuran	55673-89-7
1,2,3,4,6,7,8-Heptachlorodibenzodioxin	35822-46-9
Octachlorodibenzofuran	39001-02-0
Octachlorodibenzo-p-dioxin	3268-87-9

PAHs and PCBs

16 EPA PAHs and 7 indicator PCBs are sampled with a glass fiber filter in combination with XAD-2 adsorption tubes. Sampling time is ± 30 minutes with a flow rate of 2 L/min. Extraction of the samples is carried out by Accelerant Solvent Extraction (ASE) and toluene as extraction solvent. Before extraction 16 deuterated PAHs and C^{13} labelled PCBs are added to the filter. After extraction the extract is cleaned and fractionated in a PCB and a PAH fraction by using 3% deactivated silica gel. The final extract is than analysed for 16 EPA PAHs with GC-MS isotope dilution based on ISO 12884 (ISO 12884:2000). PCBs are analysed with GC-MS isotope dilution in the same sample extract based on ISO 16000-12:2008.

The GC/MS system consists of an Agilent 6890 gas chromatograph and an Agilent 5973N Quadrupole mass spectrometer. PAHs and PCBs are separated on a semi-polar capillary column DB-5MS/UI (30m*0.25mm) with a film thickness of 0.25 µm, using Helium as carrier gas.

PAH and PCBs compounds are identified based on retention time and mass spectra libraries (AMDISH, NIST 2014).

Quantification of PAH is performed by substance specific calibration with internal standards (CIL US EPA 16 PAH cocktail D, 98%; ES-2528: CARB method 429: 100 mg/L). External standard consists of 16 EPA deuterated PAH mix (50 ng/mL) and injection standard 1,2,3,4-TCN (50 ng/mL).

Quantification of PCBs is performed by substance specific calibration with an internal standard ^{13}C12-PCB mix in nonane (Wellington WBP-MXE (5 mg/L). External standard consists of native BCR CRM 365 (6-17 mg/L) and injection standard 1,2,3,4-TCN (50 ng/mL).

Table 11 lists the PAHs and PCBs analysed for.

Table 11. List of PAHs and PCBs analysed for

PAHs / PCBs		CAS-No.
Naphthalene		91-20-3
Acenaphthylene		208-96-8
Acenaphthene		83-32-9
Fluorene		86-73-7
Phenanthrene		85-01-8
Anthracene		120-12-7
Fluoranthene		206-44-0
Pyrene		129-00-0
Benzo[a]anthracene		56-55-3
Chrysene		218-01-9
Benzo[b]fluoranthene		205-99-2
Benzo[k]fluoranthene		207-08-9
Benzo[a]pyrene		50-32-8
Indeno[123-cd]pyrene		193-39-5
Dibenzo[ah]anthracene		53-70-3
Benzo[ghi]perylene		191-24-2
PCB 28	2,4,4'-TrCB	7012-37-5
PCB 52	2,2',5,5'-TeCB	35693-99-3
PCB 101	2,2',4,5,5'-PeCB	37680-73-2
PCB 118	2,3',4,4',5-PeCB	31508-00-6
PCB 153	2,2',4,4',5,5'-HxCB	35095-28-2
PCB 138	2,2',3,4,4',5-HxCB	35065-27-1
PCB 180	2,2',3,4,4',5,5'-HpCB	35095-29-3

QC measures

- Performance of the mass spectrometer is checked by means of an Auto Tune
- Chromatography is checked by calculating the peak resolution between benzo[a]anthracene and chrysene
- Chromatography is checked by calculating the peak symmetry of PCB-101
- Retention time of PAH component in sample ≤ 10 sec of the retention time component in PAH standard
- Retention time of PCB components in sample ≤ 3 sec of the retention time component in 13 labeled PCB standard.
- Blank reagents and blank filters are analyzed for each series of 10-15 samples.
- Control samples of SETOC material are analyzed every 10-15 samples and registered in control charts
- Criteria Qualifier ratio PCB and PAH ≥ 80% of the theoretical value
- Criteria for recovery of internal standard between 40- 130%
- Criteria for S/N ratio ≥ 3
- Chain of custody is guaranteed by using specific TNO codes generated by a dedicated TNO dossier system. The codes are used during the whole process from sampling to final analyses results.
- Inter-comparisons are performed on yearly base for sediment, soil and PM10

Odorant analysis

The method of odour analysis adheres to the scientifically approved techniques applied in flavour chemistry. Accordingly, an odour compound will be validly identified, if it corresponds to a reference flavour compound by odour quality, the retention indices on two chromatography columns of different polarity, or the retention index on one column and a further spectroscopic property, e. g. a mass spectrum (Brevard et al., 2006).

For odorant analyses 0.1 – 2.0 L of air are drawn by a pump (e. g. Desaga/Sarstedt GS 301) at a flow rate of 100 mL/min onto Tenax-TA® adsorbent tubes.

Instrumental analysis is performed on a thermal desorption-gas chromatograph (Agilent technologies 7890A GC systems) coupled with a mass selective detector (Agilent technologies 5875C inert MSD) and a sniffing port (heated at 200°C). The capillary columns used are a non-polar HP5-ms capillary column (Agilent technologies, 30 m length x 0.25 mm ID x 0.25 µ film) and a polar DB-FFAP capillary column (Agilent technologies, 30 m length x 0.25 mm ID x 0.25 µ film). Helium at a constant flow rate of 1.5 mL/min in constant flow rate was used as carrier gas. Volatiles are desorbed from the adsorbent tubes for 10 min at 250 °C in a UNIS PTV liner system, cryo-focussed in a cold trap capillary column (cooled with liquid nitrogen at -100 °C) and finally flushed onto the analytical column by flash heating to 250 °C. The effluent of the capillary is split by a Y-Split between the mass spectrometric and the olfactometric detector adjusted for simultaneous signal detection. The GC temperature is held at 30°C for 10 min, then raised at 6°C/min to 230°C and held for 20 min.

A trained panellist sniffs the effluent, marks each spot in the chromatogram whenever an odour is perceptible and describes the perceived odour quality. This procedure is performed on both columns and in doubtful cases repeated by a second panellist for each column for verification.

The relative retention time of each odorant is determined by co-chromatography with a n-alkane series of C6 (hexane) to C18 (octadecane) for the non-polar column and C6 (hexane) to C26 (hexacosane) for the polar column. The retention index of the relevant odour compounds are calculated by linear interpolation (Retention Index RI, Kovats-index; Van den Dool and Kratz, 1963).

Odour compounds are compared with the IBP internal odorant database which was developed on the basis of commercially available flavour compounds as reference and contains data of about 1000 odorants. If available, the corresponding mass spectra of the signals are recorded. The obtained mass spectra of the marked odour impressions are compared with a standardized mass spectra library (NIST 2014). However, often, odorants are perceived by the human subject at the sniffing port, but the concentration is too low to obtain a mass spectrum of the compound (odour threshold below MS detection limit). This is a qualitative method for odorant detection and identification. Trained panellists may estimate odorant concentrations by relating signal odour intensity perception to subjective odour threshold levels or dilution experiments of sample air until no odour is perceptible (Aroma Dilution Analysis AVA, Holscher and Steinhart, 1992).

Particle characterisation

Electrostatic deposition of charged particles on a solid substrate is a widely used technique for sampling and analyzing aerosol particles such as electrostatic precipitators and aerosol samplers (Fierz et al., 2007). Nanoparticles (particles of less than 100 nm in diameter) generally require a rather long sampling time due to their low charging probability and small mass. Although an effective charging method such

as corona discharge can attain a high charging efficiency for nanoparticles, it is always accompanied with significant loss of nanoparticles within the charger. As a workaround, the commercialized nanometer aerosol sampler (NAS; TSI model 3089) targets the collection of nanoparticles with an original electrical charge after the electrical mobility classification.
The SOP-S-ESP: Procedure of particle sampling with the Nanometer Aerosol Sampler (TSI Model 3089) or electrostatic precipitator (ESP). NanoGEM standard operation procedures (Asbach et al., 2012) for assessing exposure to nanomaterials, following a tiered approach will be applied. Particles collected are analysed by Transmission Electron Microscopy in terms of particle morphology, size, and elemental composition.

The DLPI (Dekati® Low Pressure Impactor) is a 13-stage cascade low pressure impactor to determine particle gravimetric mass size distribution. The size classification in DLPI is made from 30 nm up to 10 µm with evenly distributed impactor stages and will be extended down to 30 nm with an additional back-up filter. In each size fraction the particles are collected on 25 mm collection substrates and each size fraction can be chemically analyzed. An impactor has two co-linear plates; one acts as a collection surface for the particles, the other one has small nozzle or nozzles in it to control the flow velocity. The sample flow is first led through the nozzles to achieve a certain, exact flow velocity. After the sample passes through the nozzles it is turned sharply before the collection plate; particles larger than stage cut diameter cannot follow the flow stream lines but are impacted on the collection plate. Particles smaller than the stage cut diameter continue to the following impactor stages where they are further size classified and collected. By changing the dimensions in each impactor stage, different sized particles can be collected on different impactor stages. The flow through the DLPI impactor is controlled with the lowest stage jet plate which acts as a critical orifice when 100 mbar is adjusted under the first impactor stage which also means that no additional flow control unit is required to operate the unit. Particles of each size fraction are analysed by Transmission Electron Microscopy in terms of particle morphology, size, elemental composition, and organophosphates.

QC measures

- Calibration of size distribution can be performed by generation of latex particles of known sizes
- Verification of sample flows are performed by mass flow controllers
- Zero reading verification by using HEPA filtered zero air

Elemental carbon / organic carbon

Elemental carbon/Organic carbon (EC/OC) is sampled by using a quartz fibre filter (QMA Whatman) at a flow rate of 2 L/min. 1 – 1.5 cm^2 of the quartz filter is used for EC/OC analyses with a thermal-optical analyser (Sunset Laboratory, Inc., Oregon USA) The EUSAAR2 protocol will be used as thermal programming. Samples are thermally desorbed from the filter medium under an inert helium atmosphere followed by an oxidizing atmosphere using carefully controlled heating ramps. A flame ionization detector (FID) is used to monitor the analysis. Continuous monitoring of the optical absorbance of the sample during analysis prevents any undesired oxidation of original elemental carbon and corrects for the inevitable generation of carbon char produced by the pyrolytic conversion of organics into elemental carbon. The method calibration is based on organic carbon (CH4).

The TNO laboratory has been involved in the development of a European standard for EC/OC in ambient air. Characteristics for OC/EC analysis are given in tables 12 to 16.

Table 12. Limit of detection (LOD) for EC/OC expressed in µg/cm^2.

	OC	EC
LOD [µg/cm^2]	2,7	0,56

Table 13. Repeatability for OC/ EC at low-and high concentration level.

	OC	EC
Concentration level [µg/cm^2] vc_r [%]	5,0 9,6	1,4 5,7
Concentration level [µg/cm^2] vc_r [%]	24 2,5	8,8 5,7

Table 14. Reproducibility for OC/ EC at low and high concentration level

	OC	EC
Concentration level [µg/cm^2] vc_{Rw} [%]	6,1 14,7	1,7 11,2
Concentration level [µg/cm^2] vc_{Rw} [%]	24 5,4	8,5 4,2

Table 15. Recovery expressed in %, OC/ EC

	Spike [µg/cm^2]	Tv [%]
Blank filter (OC)	2,805 28,05	101 102
Real sample (OC)	2,805 28,05	97 103
Control sample (EC)	2,547	110

Table 16. Expanded measurement uncertainty for OC/EC

Component		Concentration [µg/cm^2]	U expanded measurement uncertainty [%]
Low concentration level	EC OC TC	1.7 6.1 7.8	31% 31% 30%
High concentration level	EC OC TC	8.5 24.3 32.7	24% 16% 14%

QC measures

- The quality of the measurements is checked with two standard solutions of sucrose (C12H22O11, 99.9%, Fisher Scientific, CST 5000).
- Quartz fibre filters without binding materials shall be used.
- Filters are preheated at a range of 400 °C for one hour
- Before field measurements are started, the filter batch(es) shall be assessed for blank levels of EC and OC.
- The result of the low standard solution may not deviate more than 10% of the theoretical value.
- The complete validation of EC/OC is described in TNO SOP ORG-225 (2017)

Table 17 summarises the off-line sampling methods. Normally for each off-line method one parallel sample is drawn and for safety reasons, as back-up, one additional parallel sample is drawn and stored and e. g. analysed by another laboratory for quality control, or analysed in case something went wrong with the first parallel sample.

Table 18 summarises the suggested experimental plan with screening experiments, pressure/temperature conditions at which 4 engine oils are analysed (19 experiments), the influence of increasing oil concentrations (7 experiments) and also an estimated 12 experiments with hydraulic oil and de-icing fluid (in the KlimaTIS project).

So the amount of samples drawn for engine oil tests is about 26 test conditions x 48 samples = 1248 samples (for chemical air compounds), about half of them are analysed, about half of them are stored for safety. Sampling for particle characterisation and EC / OC analysis for each of the test conditions results in about 26 x 18 samples = 468 samples.

Depending on first experimental results, if respective target compounds are not found, some samplings and analyses may be skipped for the rest of the experimental programme.

Table 17. List of analytes, adsorbents, sampling volumes for off-line analyses for each analytical test condition according to Table 18 (for each experimental condition)

Parameter	Sampling method	typical air flow for sampling [L/min]	typical sampling duration [min]	Num-ber of samp-lings	Volume needed per min [L] for parallel sampling
Volatile Organic Compounds (VOC, C6-C16, incl. BTXE)	adsorbent tubes, e. g. Tenax, activated charcoal, Carboxen	0.1	10-30	2 +2 safety	1
Volatile Organic Compounds (VOC, C6-C16, incl. BTXE, Acrolein)	Canisters	0.6	10	2 +2 safety	3
Very Volatile Organic Compounds (VVOC, C2-C6)	adsorbent tubes, e. g. Carbosieve	0.1	10-30	2 +2 safety	1
SVOCs (PAHs, PCBs)	XAD-2 in combination with glass fiber filter	2	30	2 +2 safety	8
Aldehydes / Ketones	silica gel impreg-nated with DNPH	1	60	2 +2 safety	4
Carboxylic acids	Tenax TA	0.1	50	2 +2 safety	1
Organo-phosphates (31 incl. 10 TCP isomers)	Chromosorb 106 / glass fibre filters	10	60	min. 8 max. 16	160
Dioxins and furans	PUF	30-60	60-120	2 +2 safety	240
Odour active compounds	adsorbent tubes, e. g. Tenax	0.1-0.5	20-30	2 +2 safety	2
Characterisation of particles	Classifier + NAS DLPI	1 30	10-60 10-60	3 13	3 390
EC / OC	Quartz fibre filter	2	250	2	4
Total volume needed per min [L] for off-line analytics sampling (in case of parallel sampling of 66 samples)				**66**	**817**

Table 18. Suggested experimental test plan (table expanded to 4 pages)

No.	BACS Experiment	oil 1/2/3/4	amount [mg/m³]	pressure [bar]	temp. [°C]	ozone Y/N	on-line monitors (provider)														
							IBP			VITO	IBP			VITO	Airbus				VITO	TNO	IBP	VITO
							CO	CO2	NO NOx	SO2	HCHO	FID	PID	SIFT-MS	PTR-MS	Aero-tracer	Pega-sor PPS	SMPS/ CPC 1	SMPS/ CPC 2	PM 1/ 2.5/10	black C
A	Screening	oil 1	0.1 - 5	8	340	N / Y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
B	Screening	oil 2 HT	0.1 - 5	8	340	N / Y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
C	Screening	oil 1	1 *	8	20 - 600	N	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
D	Screening	oil 2 HT	1 *	8	20 - 600	N	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
E	**Screening**	**oil 1**	**0.1 until smoke**	**8**	**340**	**N**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
1	Air / Wipe	oil 1	1	3	200	N	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
2	Air / Wipe	oil 1	1	8	340	N	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	Air / Wipe	oil 1	1	8	340	Y	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
4	Air / Wipe	oil 1	1	8	450	N	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
5	Air / Wipe	oil 1	1	8	600	N	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
1	Air	oil 2 HT	1	8	340	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	X
2	Air	oil 2 HT	1	8	340	Y	X	X	///	///	X	X	///	X	X	X	X	X	X	X	X
3	Air	oil 2 HT	1	8	450	N	X	X	X	X	X	X	///	X	X	X	X	X	X	X	X
4	Air / Wipe	oil 2 HT	1	8	600	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	X
1	Air	oil 3	1	3	200	N	X	X	X	X	X	X	///	X	X	X	X	X	X	X	X
2	Air	oil 3	1	8	340	N	X	X	X	X	X	X	///	X	X	X	X	X	X	X	X
3	Air	oil 3	1	8	340	Y	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
4	Air	oil 3	1	8	450	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
5	Air / Wipe	oil 3	1	8	600	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///

		HT	high temperature oil				X	on-line monitor connected						///	likely to be omitted if first results confirm						
		*	useful concentration for on-line monitors and analytical instruments to not overload equipment -																		
			- to be determined during screening																		
			should higher concentration be used rediluting necessary prior to air reaching monitors or sampling tubes,																		
			for Task 3 A tox. sampling smoke concentration to be used																		

Table 18 cont.

No.	BACS Experiment	oil 1/2/3/4	amount [mg/m³]	pressure [bar]	temp. [°C]	ozone Y/N	on-line monitors (provider)														
							IBP			VITO	IBP			VITO	Airbus			VITO	TNO	IBP	VITO
							CO	CO2	NO NOx	SO2	HCHO	FID	PID	SIFT-MS	PTR-MS	Aero-tracer	Pega-sor PPS	SMPS/ CPC 1	SMPS/ CPC 2	PM 1/ 2.5/10	black C
1	Air	oil 4	1	3	200	N	X	X	X	X	X	X	///	X	X	X	X	X	X	X	X
2	Air	oil 4	1	8	340	N	X	X	X	X	X	X	///	X	X	X	X	X	X	X	X
3	Air	oil 4	1	8	340	Y	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
4	Air	oil 4	1	8	450	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
5	Air / Wipe	oil 4	1	8	600	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
1	Air	1 oil	0.1	8	340	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
2	Air	1 oil	1	8	340	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
3	Air	1 oil	5	8	340	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
4	Air	1 oil	10	8	340	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
5	Air	1 oil	50	8	340	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
6	Air	1 oil	100	8	340	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
7	Air	1 oil	500	8	340	N	X	X	///	///	X	X	///	X	X	X	X	X	X	X	///
1	Air / Wipe	hydr. oil 1	1 *	8	340	N	X	X	X	///	X	X	///	///	X	X	X	IBP	///	X	///
2	Air / Wipe	hydr. oil 2	1 *	8	340	N	X	X	X	///	X	X	///	///	X	X	X	IBP	///	X	///
…																					
1	Air	de-ice 1	1 *	8	340	N	X	X	///	///	X	X	///	///	X	X	X	IBP	///	X	///
2	Air	de-ice 2	1 *	8	340	N	X	X	///	///	X	X	///	///	X	X	X	IBP	///	X	///
…																					

X on-line monitor connected /// likely to be omitted if first results confirm

* useful concentration for on-line monitors and analytical instruments to not overload equipment -

- to be determined during screening

should higher concentration be used rediluting necessary prior to air reaching monitors or sampling tubes,

for Task 3 A tox. sampling smoke concentration to be used

IBP in KlimaTIS IBP equipment to be used

Table 18 cont.

No.	BACS Experi-ment	oil 1/2/3/4	amount [mg/m³]	p [bar]	T. [°C]	O3 Y/N	off-line analytics (responsible lab)											comment
							IBP	TNO	IBP	TNO	VITO	IBP	TNO	VITO	IBP	VITO	TNO	
							VOC ISO	VOC EPA Acrolein	VVOC	SVOC (PAHs, PCBs)	Ald./ Ket.	R-COOH	OP	Dioxins Furans	odor	par-ticles	EC/ OC	
A	Screening	oil 1	0.1 - 5	8	340	N / Y	///	///	///	///	///	///	///	///	///	///	///	check sensitivity of monitors
B	Screening	oil 2 HT	0.1 - 5	8	340	N / Y	///	///	///	///	///	///	///	///	///	///	///	check sensitivity of monitors
C	Screening	oil 1	1	8	20 - 600	N	///	///	///	///	///	///	///	///	///	///	///	screen T influence on
D	Screening	oil 2 HT	1	8	20 - 600	N	///	///	///	///	///	///	///	///	///	///	///	oil decomposition
E	**Screening**	**oil 1**	**0.1 until smoke**	**8**	**340**	**N**	///	///	///	///	///	///	///	///	///	///	///	**observed by SC**
1	Air / Wipe	oil 1	1	3	200	N	X	X	X	X	X	X	X	X	X	X	X	APU / descent condition added
2	Air / Wipe	oil 1	1	8	340	N	X	X	X	X	X	X	X	X	X	X	X	detail analyses with all
3	Air / Wipe	oil 1	1	8	340	Y	X	X	X	X	X	X	X	X	X	X	X	equipment
4	Air / Wipe	oil 1	1	8	450	N	X	X	///	X	X	X	X	X	///	X	X	detail analyses with
5	Air / Wipe	oil 1	1	8	600	N	X	X	///	X	X	X	X	X	///	X	X	reduced equipment (///)
1	Air	oil 2 HT	1	8	340	N	X	X	///	///	X	X	X	///	///	X	X	depending on first results
2	Air	oil 2 HT	1	8	340	Y	X	X	///	///	X	X	X	///	///	X	X	
3	Air	oil 2 HT	1	8	450	N	X	X	X	X	X	X	X	X	X	X	X	dioxins / furans:
4	Air / Wipe	oil 2 HT	1	8	600	N	X	X	///	///	X	X	X	///	///	X	X	absence to be shown
1	Air	oil 3	1	3	200	N	X	X	X	X	X	X	X	X	X	X	X	APU / descent condition added
2	Air	oil 3	1	8	340	N	X	X	X	X	X	X	X	X	X	X	X	
3	Air	oil 3	1	8	340	Y	X	X	///	///	X	X	X	///	///	X	X	odor:
4	Air	oil 3	1	8	450	N	X	X	///	///	X	X	X	///	///	X	X	if dirty socks smell perceptible
5	Air / Wipe	oil 3	1	8	600	N	X	X	///	///	X	X	X	///	///	X	X	take odor sample

X	2 parallel samples drawn each, for OP analyses 4, 6, 8 parallel samples drawn
///	only selected test samplings during screening trials
	likely to be omitted if first results confirm

Table 18 cont.

No.	BACS Experiment	oil 1/2/3/4	amount [mg/m³]	p [bar]	T [°C]	O3 Y/N	off-line analytics (responsible lab)											comment
							IBP	TNO	IBP	TNO	VITO	IBP	TNO	VITO	IBP	VITO	TNO	
							VOC ISO	VOC EPA Acrolein	VVOC	SVOC (PAHs, PCBs)	Ald./ Ket.	R-COOH	OP	Dioxins Furans	odor	par-ticles	EC/ OC	
1	Air	oil 4	1	3	200	N	X	X	X	X	X	X	X	X	X	X	X	APU / descent condition added
2	Air	oil 4	1	8	340	N	X	X	X	X	X	X	X	X	X	X	X	
3	Air	oil 4	1	8	340	Y	X	X	///	///	X	X	X	///	///	X	X	
4	Air	oil 4	1	8	450	N	X	X	///	///	X	X	X	///	///	X	X	
5	Air / Wipe	oil 4	1	8	600	N	X	X	///	///	X	X	X	///	///	X	X	
1	Air	1 oil	0.1	8	340	N	X	X	///	///	X	X	X	///	///	X	X	
2	Air	1 oil	1	8	340	N	X	X	///	///	X	X	X	///	///	X	X	
3	Air	1 oil	5	8	340	N	X	X	///	///	X	X	X	///	///	X	X	
4	Air	1 oil	10	8	340	N	X	X	///	///	X	X	X	///	///	X	X	
5	Air	1 oil	50	8	340	N	X	X	///	///	X	X	X	///	///	X	X	
6	Air	1 oil	100	8	340	N	X	X	///	///	X	X	X	///	///	X	X	
7	Air	1 oil	500	8	340	N	X	X	///	///	X	X	X	///	///	X	X	max. agreed oil amount
1	Air / Wipe	hydr. oil 1	1	8	340	N	X	///	X	IBP	IBP	X	IBP	IBP	X	///	///	KlimaTIS
2	Air / Wipe	hydr. oil 2	1	8	340	N	X	///	X	IBP	IBP	X	IBP	IBP	X	///	///	KlimaTIS
…																		KlimaTIS
1	Air	de-ice 1	1	8	340	N	X	///	X	IBP	IBP	X	///	///	X	///	///	KlimaTIS
2	Air	de-ice 2	1	8	340	N	X	///	X	IBP	IBP	X	///	///	X	///	///	KlimaTIS
…																		KlimaTIS
							X	2 parallel samples drawn each, for OP analyses 4, 6, 8 parallel samples drawn										
							///	likely to be omitted if first results confirm										
							IBP	in KlimaTIS analysis performed by IBP										

3 Detailed test plan for Task 3A in BACS experiments

Preliminary experiments: Optimizing fume sampling and neurotoxicity screening
& *In-vitro* and *in-vivo* testing of BACS generated fumes

In Task 2 fumes will be generated using the BACS at realistic engine conditions (considering T/p). Online chemical monitoring and analysis of organophosphate compounds will be done for these fumes. The fumes will be sampled - for off-site in-vitro testing - using cold-trapping.
Due to some technical uncertainties (transfer of fumes to aquatic phase, solubility, overt toxicity to alternative model systems) some preliminary experiments are required to define boundary conditions, and decide about final experimental test plan to run with the BACS samples.

The first experiments to be carried out are: optimization of the cold-trapping methodology by heating commercial oil under lab conditions, trapping of the fumes, and testing solvents to dissolve the fume compounds from the trap for transfer to biological test medium using biocompatible solvent conditions. Furthermore, the working range, exposure window and toxicity of a technical mixture of TCP will be tested in both neurotoxicity screening assays: zebrafish locomotor assay and the Micro electrode array (MEA) for neuronal electrical activity.

Following the preliminary experiments of Task 3A, screening of the various mixtures of six selected fumes is done using the zebrafish and the Microelectrode Array including neuronal cells (MEA) (in-vitro neurotoxicity assays). The most toxic fume will be confirmed and tested via the tandem Air Liquid Interface lung cells(ALI)-MEA system and via the in-vivo mice behavior test, to assess the impact of the fume in case of direct inhalation exposure.

A question that may arise is what is the ‘power of command’ of the screening models in light of assessing the risk for cabin crew? The results as such cannot be used to do a standard risk assessment for chemicals but if taken together the proposed test battery should allow ranking all tested mixtures in terms of general toxicity and specific neurotoxic potency.

Aside from the hazard assessment, also selected/targeted biomarkers are screened for, in the test animals exposed to the realistic and characterized fumes and/or extracts. The biomarker formation, stability and half-life can be studied under the conditions of controlled exposure. This allows investigating the relationship between fume mixtures and the biomarkers, without interference of other confounders (e.g. other sources, life style).

3.1 Optimization cold-trapping of fumes (Task 3A.A1)

3.1.1 Aim

Optimization of cold trapping system to condense fumes from BACS and transfer them to a solvent allowing hazard assessment in the in-vitro systems (zebrafish and MEA cells)

3.1.2 Partners involved

The optimization experiments will be performed in the VITO labs, supported by TNO supplying oil, and performing technical assistance and TCP analysis of the oil.

3.1.3 Optimization of cold trapping procedure:

a. Cold trapping

Cold trapping of organic compounds in fumes, by condensing them on a surface: For this, Mobil jet oil II will be evaporated from a container, and transferred to the trapping system. Two options are available for evaporating the oil, either by heating the oil (e.g. Fujitani et al. 2007) to the temperature used in the BACS experiment or by pressurising the container containing the oil (e.g. Goelen et al. 1992).

Cold trapping systems that will be tried out are:

- A glass impinger of 10-1000 mL submerged in a dewar and possibly filled with glass beads for an optimized surface-volume ratio.
- A long steel tube wherein the fumes will condense over its surface (as is the case with ducts connected to BACS outlet, or connected to an engine during normal flight operations).

The cold trapping system will be optimized according to the cooling capacity needed. Possible cooling means are water, ice-water (4°C), a refrigerator (-20°C), dry ice (-78°C) or liquefied nitrogen (-196°C). For this, the impinger or tubing will be submerged into an (ice-)water bad, a refrigerator, or into liquid nitrogen damps, if the latter cooling capacity might be needed.

b. Solvent extraction

After trapping, the condensed organic compounds mixture is rinsed off from the surface of the cold trap with DMSO in case a proper oil/DMSO ratio is obtainable. If a direct dissolution of the oil in DMSO is not feasible, a solvent mixture of low to high polarity solvents is used and pooled afterwards. The solvents that are tried out must be volatile and may not be part of the oil being tested. The solvents that are considered to be tested are dichloromethane, methanol, acrolein and methyl ethyl ketone. They have a low to medium boiling point, allowing easy evaporation, as the organic compounds in the solvent (pool) are carefully substituted to DMSO via evaporation under an inert nitrogen flow. The latter procedure of solvent substitution is used in routine at VITO and allows a weight based dosing.

c. Test of effectiveness of cold-trapping system

The effectiveness of the fume sampling system is assessed prior to application in the BACS experiment. For this Mobil Jet Oil II is used. The following steps are done:

- Volatile Organic Compounds (VOCs) and TCPs are analysed in the oil using GC/MS (at VITO and TNO) respectively.
- 50-100 mg of the oil is heated and transferred to the cold trapping system as optimized above in step a.
- The loss of weight of the oil due to heating is determined using a balance, allowing assessing the theoretical loss in compounds. Ideally the mass balance will confirm 100% trapping.

- The oil condensate is dissolved either directly in DMSO or in the solvent composition optimised in step b, and thereafter substituted to DMSO via evaporation under a gentle nitrogen flow.
- The DMSO solvent is screened for VOCs and TCPs to compare with the composition in the original oil.
- The DMSO solvent is stored for three months, and again analysed to assess the stability of the compounds in the extract.

3.2 Preliminary toxicity screening: TCP technical mixture (Task 3A.A2)

A technical mixture of tritolyl phosphate (90%) (Sigma-Aldrich, 268917, C21H21O4P, CAS NR: 1330-78-5) is used as positive control in the Microelectrode array (MEA) for neuronal electrical activity screening and in the zebrafish locomotor assay. Duarte et al., 2017 (IRAS partner in current project) has tested different types of technical mixtures which have different ratios of the isomer-forms, and low o,o,o-TCP (≤2%) which resemble those in engine oil. Based on those results, the TCP technical grade mixture of Sigma-Aldrich used by Duarte et al. (2017) was selected.

As a first step a dose-response curve of TCP up to 36.8 µg/mL (100 µM) at 0.1% DMSO in test medium is evaluated. Based on available literature 1 to 100 µM TCP is the estimated working range in the bioassays (for zebrafish: Jarema et al., 2015 and Noyes et al., 2015; for cell culture: Duarte et al., 2017) (see detailed test plan D3, version November 2017 for more details).

3.2.1 Zebrafish assay

Equipment needed

- Zebrafish breeding facility
- Incubator set at 28.5°C (± 0.5°C) with constant day night rhythm (14h light /10h dark)
- Stereo microscope (Zeiss)
- Inverted light microscope (Nikon, type TMD Diaphot)
- Daniovision Observation Chamber (Noldus) with infrared light source and high resolution digital infrared camera
- Ethovision XT software for analysis of video images
- Optical plate reader

Experimental approach

First, **zebrafish are evaluated for overt toxicity (mortality, malformations)** by testing technical TCP mixture in two different time windows (120 h exposure from day 0 – day 5 post fertilisation, and 48 h exposure from day 3-day 5 post fertilisation) and in a broad concentration range (1:5 dilutions, 6 concentrations from 36.84 to 0.012 µg/mL). Egg/larvae mortality and egg/larvae malformations are recorded as a function of time and concentrations response curves are generated. Test conditions with overall toxicity, which might interfere with motor activity assessment, are defined as a mortality rate of ≥ 10%, and/or visible larvae malformations in more than 20% of the embryos. The protocol for evaluation of mortality and malformations is validated, described by Selderslaghs et al. (2009, 2012) and described in a VITO standard operating procedure for ZTA (zebrafish teratogenic assay). More specifically the following procedure is followed:

- A stock solution of TCP (36.84 mg/mL) is prepared in 100% DMSO. Dilutions (1:1000) are made to get test solutions in fish water, containing 0.1% DMSO, at concentration of respectively 36.8, 7.37, 1.47, 0.29, 0.059 & 0.012 µg/mL TCP (i.e. in 100-1 µM working range). The highest concentration showed toxicity in zebrafish larvae in

preliminary experiments. Control (fish water = negative control medium, composition as described in OECD203) and solvent control (fish water with 0.1% DMSO) samples are also tested.

- N=30-60 freshly collected zebrafish eggs are distributed group-wise into 6 well plates (8 mL fish water/well) within 2 hpf (hours post fertilisation).
- Per test condition (i.e. for each of the 6 TCP dilutions, the solvent control and control), twelve eggs are distributed into individual wells of a 24 well plate (2 mL/well). This means that 2 test conditions can be tested per 24 well plate.
- Furthermore, in one experiment, zebrafish are exposed for 5 days to the test solution, where at day 3 the test solution is renewed. In another parallel experiment, zebrafish are exposed from day 0-day 3 to control medium, and this is replaced with test solution at day 3 until day 5.
- For all experiments, next to intermittent evaluations, the mortality and developmental endpoints (teratogenic effects = eye, otolith, heart, circulation, pigmentation, spinal cord, ...) are scored at the end of the experiment (i.e. day 5) by microscopic evaluation using an excel template (VITO ZTA assay).
- Based on these results, the exposure conditions (concentrations & time window) will be selected for further locomotor studies which exhibit less than 20% embryo/larvae malformations and <10% mortality.

In a second step, the **zebrafish locomotor activity is tested in the non-toxic dose range of TCP**. 1:2 dilution steps of TCP will be made in the non-toxic range. The zebrafish neurotoxic assay (ZNA) test protocol is described in a VITO standard operating procedure, and is based on two VITO publications including method development (Selderslaghs et al., 2010) and method validation (Selderslaghs et al., 2013). The procedure of the ZNA is highly comparable to the former described ZTA, with respect to test solutions preparation, and collection of fertilised eggs. Differences at the level of exposure chambers and evaluation are the following:

- Selection of fertilised zebrafish eggs and transfer into individual wells with 1 mL medium in 48 well plates. For each test concentration, 24 wells on one test plate are filled and matched controls (N=24) are on the same plate. For each concentration, 2 plates are filled, which give 48 individuals per test condition.
- At 5 days post fertilisation, zebrafish are evaluated for mortality and malformation, and dead (<10%) or malformed embryo/larvae (< 20%) are excluded for further analysis of the locomotor activity.
- In order to evaluate neurotoxicity by video imaging, a test volume of 0.5 mL in each of 48 wells of the plates is removed.
- Video recording is done (during 5 minutes/test plate at 30 images/sec) with the Daniovision Observation Chamber for each of the test plates.
- Analysis of the videos with Ethovision XT software for each larva for 5 parameters of locomotor activity (total distance, mean velocity, mean turn angle, frequency of movement, total duration of movement), which are the best parameters represent neurotoxic effects of compounds (Selderslaghs et al., 2013).
- Statistical analysis by Matlab after screening for outliers, and deriving probability distributions for each exposure group compared to control group in order to calculate % effect and generate concentration-effect curves.

These results of the ZNA assay with the technical TCP mixture, in comparison to the controls allows to evaluate the sensitivity and exposure set up to be further used to assess

the effects of cold trapped engine oil (see task 3A.A3), and the BACS fume samples (task 3A.B).

3.2.2 Micro electrode array (MEA) rat cortical neurons assay

Micro electrode arrays (MEA) consist of a multi-well cell culture surface with an integrated array of micro-electrodes that allows for the simultaneous and non-invasive recordings of local field potentials and extracellular action potentials at different locations in an *in vitro* neuronal network at millisecond time scale. Neuronal networks grown on MEAs possess many characteristics of neurons *in vivo*, including (the development of) spontaneous activity with bursting (Robinette et al., 2011) and responsiveness to neurotransmitters and pharmacological agents (Gross et al., 1997, for review, see Johnstone et al., 2010, de Groot et al., 2013). Using the MEA set up, spontaneous electrical activity of a network of primary rat cortical neurons can be recorded. The electric activity is measured after 30 min (acute), 24 h and 48 h exposure, respectively, to cover both acute and sub-chronic effects of the test compound.

Equipment needed: MEA system

- Maestro 768-channel amplifier with integrated heating system and temperature controller (Axion Biosystems Inc.)
- Computer and dedicated software to record and analyse data.
- Cell culture lab in the close proximity of the MEA.
- Software: Axion's Integrated Studio (AxIS 1.7.8) is used to manage data acquisition.

Experimental approach

- MEA plates with primary rat cortical neurons are allowed to grow up to 9 days *in vitro* (DIV9). MEA plates are then allowed to equilibrate in the Maestro for 5-10 min prior to recordings of electrical activity. At DIV9, a 30 min baseline recording of spontaneous activity is made.
- The TCP technical mixture is tested at dilutions of 36.8, 7.37, 1.47, 0.29, 0.059 & 0.012 µg/mL TCP, and further 1:2 dilution steps in the non-toxic range. A 30 min recording is performed immediately following the onset of exposure to determine acute effects of the test substance compared to baseline spontaneous activity (paired comparison).
- At DIV10 and DIV11 the neuronal activity is measured again to determine the sub-chronic effects of exposure following 24 h and 48 h exposure, respectively (Duarte et al., 2017).
- A change in network activity (defined as a change in e.g. spike rate and/or spike pattern) compared to control cells, is considered an effect. To avoid misinterpretation of effects on network activity due to general effects on cell viability, cell viability is monitored using an Alamar Blue assay for mitochondrial activity after 48h and in case this is positive, also after 24h of exposure. A reduction of ≥20% in cell viability is considered an effect.

3.3 Preliminary toxicity testing of commercial oil (Task 3A.A3)

Mobil Jet Oil II is tested for its overall toxicity of the fume mixture contaminants after heating and cooling in step c of cold trap optimization (see above). Therefore viability of the MEA cells and zebrafish larvae is tested using the fume DMSO extract at a concentration of 0.1% in the cell or fish medium. The experiments that will be carried out are similar to those described for the TCP technical mixture (see above).

3.4 Biomarker analysis (Task 3A.A4)

It is necessary to study biomarker formation, stability and half-life under conditions of controlled exposure, allowing investigating the relationship between fume mixtures and internal exposure doses, without interference of other sources influencing the biomarker levels. Therefore selected/targeted biomarkers are measured in test animals exposed to realistic and characterized fumes (extracts).

Most of the biomonitoring work related to potential biomarkers (e.g., protein adducts) of aerotoxic syndrome has been carried out on TCPs present in the lubricant oil, mainly because of the fact that the ToCP is metabolized in-vivo to the toxic CBDP, an inhibitor of enzymes involved in neurotransmission. Bioactivation of ToCP to CBDP by human P450s has been studied in detail by Reinen et al. (2015). CBDP is an inhibitor of various esterase's, including BChE, able to form covalent adducts which are expected to be persistent in- vivo (average half-life time of native BChE in vivo is 10 days, with BChE adducts presumably having a similar half-life time). CBDP has also been shown to bind to albumin (Schopfer et al., 2010; average half-life time of native albumin in vivo is 20 days, with albumin adducts presumably having a similar half-life time).
Activities of acetyl choline esterase (AChE, that regulates neurotransmitter concentrations) and neuropathy target esterase enzyme (NTE, an enzyme needed during neuronal differentiation) were measured in flight crew members as potential biomarkers for exposure to OP. Schopfer et al. (2014) demonstrated inhibition of BChE in human plasma, as a result of covalent adduct formation of CBDP, the metabolite formed after ToCP exposure. Heutelbeck et al. (2016) reported on a likely inhibition of NTE activities (5 days after the alleged exposure) in crew members displaying symptoms of intoxication after experiencing a fume event, which might be caused by ToCP metabolites. These findings warrant further investigations.

The work for biomarker analysis will be subdivided in two main parts, optimization of the methods and analysis of the biomarkers in the animal models. Three types of (persistent) biomarkers will be focused on within the FACTS project as they are considered highly relevant in the context of neurotoxic compound exposure. Mice and zebrafish will be used as animal model. For the proposed experiments we will make use of procedures and methods earlier developed in the TNO and VITO laboratories within the framework of related studies:

1 **Changes in activity of certain enzymes as indicators for exposure to neurotoxic compounds**, such as acetylcholinesterase (AChE), butyrylcholinesterase (BChE), carboxylesterase (CaE) and neuropathy target esterase (NTE).
2 **Protein adducts**
   - **Organophosphate (OP)-derived protein adducts**, such as adduct formation of OP with BChE or albumin (i.e. adducts of tyrosine residues from albumin). The eventual selection of OP-derived adducts will partly depend on which OP compounds identified in the fumes generated in Task 2.
   - **Protein adducts derived from non-organophosphate, potentially neurotoxic compounds**. Compounds other than organophosphates can also exert neurotoxic effects, such as certain polycyclic aromatic hydrocarbons (PAHs). In this case, a more generic technique for elucidation of a selection of adducts to plasma proteins will be used, depending on which compounds identified in Task 2.

Experimental approach

During the preliminary experiments, some explorative tests on baseline enzyme activities will be done in **zebrafish whole animal larvae homogenates**. Changes in certain enzyme indicators indicate neurotoxic effects. At VITO, a method of enzyme assessment in zebrafish homogenates is available for AChE, including: (i) collection of two pools of 20 larvae for each test condition, immediately after analysis of locomotor parameters (at 5 dpf); (ii) rinsing of larvae (fish water with 0.1% DMSO), euthanisation, whole body homogenates are prepared and the supernatants is immediately frozen at -20°C; (iii) performance of enzyme assays according to the protocol by Ellman et al. 1961.

During the **mice behaviour testing, samples will be collected** in (Task 3A.C3): Mice will be sacrificed 24h and 7d after exposure, and tissues including muscle, liver, lung (including broncho-alveolar lavage fluid), brain, blood and residual urine (collected during sectioning) will be isolated and stored for analysis. Part of the tissue material collected will be used to screen for biomarkers as listed above.

Biomarker protocols to be optimized

Generally, the enzyme activity and protein adduct biomarker analyses, will be fine-tuned based on methods and in-house experience of the lab of TNO. The following protocols will be optimized - for its use with zebrafish homogenates or/and mice blood samples:

ENZYME ACTIVITY

Enzyme activities are always normalized versus a baseline sample (100% activity reference control). The sample protein concentration is determined with the Bio-Rad Protein Assay Kit II. Kinetic determination of optical density, as a measure for enzymatic activity, is done at 405 nm. Furthermore, background samples are included in which maximal inhibition is achieved *ex-vivo* with the nerve agent ‘soman’, to control for background activity.

- Samples are analysed for AChE and BuChE enzyme activity using a modification of the method by Ellman et al (1961). In short, the assay is performed in 96-well plates. Samples are diluted in 0.8 mM 5,5’-dithio-bis-(2-nitrobenzoic acid) (Sigma Aldrich B.V.). To 100 µL of diluted sample, 100 µL of 0.8 mM β-methylacetylthiocholine iodide is added, in quadruple, for determination of AChE activity. For assessment of BuChE activity in the samples, 100 µL of 0.8 mM of butyrylthiocholine is added. The change in extinction per min at 412 nm at ambient temperature serves as a measure for ChE activity.
- CaE enzyme activity is colorimetrically determined by incubation of an appropriate dilution of the samples in a 50 mM Na-veronal buffer (pH 7.8) with the substrate p-nitrophenyl butyrate (pNPB) at 0.2 mM. The increase in absorbance at 412 nm at ambient temperature serves as measurement for CaE activity.
- For the analysis of NTE enzyme activity, a method will be worked out, based on the existing methods for blood analysis (Heutelbeck et al. 2016). In short, the strategy will be as follows: lysis of cells, determination of protein concentration, enzymatic cleavage of substrate phenyl valerate to phenol and measurement of the intensity of a colour reaction product at 492 nm.

PROTEIN ADDUCTS

- **Analysis of adducts of BuChE**
  - Pepsin digestion and mass spectrometric analysis of BuChE digests are carried out in an analogous way as described by Reinen et al (Chem Res Toxicol. 2015). For immunomagnetic separation (IMS) of BuChE from the incubation mixtures, a KingfisherTM mL magnetic processor system from Thermo Scientific (Breda, The Netherlands) is used. In short, 40 µL of the magnetic beads solution is transferred to 250 µL incubation mixture after which the whole mixture is incubated for 2 h at room temperature with rotation. Beads are then washed in two aliquots of 1 mL PBS and transferred to a well containing 100 µL water. For digestion of hBuChE, the beads are re-suspended in 75 µL of a pepsin solution (0.25 mg/mL pepsin in 0.63% formic acid) and incubated in a water bath at 37 ºC for 1.5 h. After incubation, the supernatant is removed and filtered at 3000 rpm for 60 min using a Millipore MultiScreen Ultracel-10, 10 kDa molecular weight cut-off filter (Fisher Scientific, Fair Lawn, NJ) to remove large peptides and proteins and active pepsin. The analysis is performed on a TSQ Quantum Ultra mass spectrometer (Finnigan, Thermo Electron Corporations, San Jose, USA), an Acquity Sample Manager, and Binary Solvent Manager (Water, Milford, USA).
- **Analysis of adducts of other isolated plasma proteins**
  - *Pronase digestion of isolated plasma proteins*: Plasma samples (50 µL) are added to acetone (300 µL), thoroughly shaken and centrifuged for 5 min at 3000 rpm. After evaporation of the supernatant, the pellet is dried at ambient temperature overnight. Next, the pellet is taken into a solution of NH_4HCO_3 (50 mM, 400 µL) and protease (Protease from Streptomyces griseus, Sigma, P5147, 100 µl, 10 mg/mL) is added. The mixture is incubated at 50˚C for 90 min or until the pellet had dissolved. The mixture is applied to a preconditioned Nexus column (ABS elute, 200 mg, 6 mL) that has been preconditioned by washing with methanol (2 mL) and water (2 mL). After application of the sample, the column is washed with methanol/water (10% v/v, 1 mL) and elution is achieved with methanol (1.5 mL). The methanol eluate is evaporated under dry nitrogen and the residues are dissolved in MQ (75 µL) and analysed for tyrosine adducts with CBDP (2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide).
  - *Liquid Chromatography electrospray tandem MS analysis of the tyrosine adducts:* LC-ES MS/MS experiments are conducted on a XEVO TQS triple quadrupole instrument (Waters, Milford, MA, USA) equipped with an Acquity M-class UPLC liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consists of an Acquity HSS-T3 C18 column (100 mm x 2.1 mm ID, 1.8 µm particles; Waters).
  - A gradient of eluents A (H_2O with 0.2 vol % formic acid) and B (acetonitrile with 0.2 vol % formic acid) is used to achieve separation, following: 100 % A (at time 0 min, 0.1 mL/min flow) to 20 % A and 80 % B (at 20 min, 0.1 mL/min flow), then to 100% A and 0% B (at 25 min, 0.1 mL/min flow) and finally 100% A for 5 min. The injected sample volume is 1 µL. The TSQ is operated at a cone voltage of 60 V, employing nitrogen as the nebulizer and desolvation gas (at 6 bar and 300 L/h, respectively).

3.5 Neurotox *in vitro* screening of fumes generated in BACS (Task 3A.B)

In Task 2 fumes will be generated using the BACS at realistic engine conditions (considering T/p). Online chemical monitoring and analysis of organophosphate compounds will be done for these fumes. The fumes will be sampled - for off-side *in vitro* testing - using the cold-trapping technology optimized in Task 3A.A1. Six generated fumes

of Task 2 will be screened using MEA and the zebrafish locomotor assay. The hazard evaluation of the (unknown) mixtures will be done relative to the positive control TCP technical mixture. This will allow ranking of the fumes according to neurotoxicity.

The amount of oil needed to be transferred to DMSO for the neurotoxicity experiments has been calculated (see revised version of detailed test plan, November 2017) and needs to be considered in this step (see text box below). Nevertheless, results of the preliminary toxicity screening (task 3A A2 & A3) might provide information to revisit the dosing scheme. In the latter case, the amount of fume to be collected in the cold trap and to be used in the solvent extraction procedure will accordingly be adopted.

The volume of fumes, and the amount of DMSO needed for the *in-vitro* experiments were calculated. Calculations were based on the amount of oil, containing 3% TCP, that is needed to prepare final test solutions in the expected TCP effect range of 1-100 µM (MW of the TCP technical mixture used in the calculations: 371.39 g/mol, from: https://chem.nlm.nih.gov/chemidplus/rn/1330-78-5).

- ➔ The zebrafish locomotor assay is performed in 48-well plates of 1 mL/well, containing 1 embryo per well. To be on the safe side, including repeat and range finding experiments, 120 mL test medium is needed from each fume to test it in the zebrafish locomotor assay. To be able to pick up effects, a dose of 1-100 µM TCP in the fish test medium is needed, which equals 0.045 to 4.5 mg TCP. It is dissolved in 120 µL DMSO (0.1% of 120 mL test medium). NB: Each well contains 1 mL test medium, this means there is 4.5/120 = 0.037 mg TCP. With a specific density of the TCP mixture = 1.143 mg/µL, and assuming 3% of TCP in the fume oil, this means each well contains ca. 1 µL of oil.
- ➔ Since preliminary range finding and repeat experiments might be necessary both for the zebrafish assay and the MEA assay, **we will collect a total of 50 mg oil of each fume**, and **transferred to 1333 µL DMSO** (=120 µL* 50mg/4.5mg). As mentioned in the BACS test plan Task 2, different oil concentrations will be generated in the BACS fumes. E.g. an oil concentration of 55 mg/m³ (1kg oil/h scenario), which means that for obtaining 50 mg oil, about 1 m³ of air from the BACS needs to be sampled.

3.6 Neurotox in-vitro and *in-vivo* inhalation testing (Task 3A.C)

The most toxic fume of Task 3A.B will be confirmed and tested via the tandem Air Liquid Interface (ALI)-MEA system and via the in-vivo mice behaviour test, to assess the impact of the fume in case of direct inhalation exposure. To make the testing feasible, the fume generation for these exposure experiments will be done near to the animal lab. This implies the build of a BACS in the RIVM lab.

3.6.1 BACS set-up for inhalation exposure tests at RIVM (Task 3A.C1)

At RIVM the most toxic fume of the neurotox in-vitro screening tests (Task 3A.B) is simulated for the more complex inhalation experiments. In order to achieve the desired test atmosphere, dry, cleaned (no particles or gaseous pollutants) and oil free compressed air is heated up to the desired temperature to resemble conditions as occurring in the aircraft's ECS/heat exchanger (T up to 650°C). The system is a downscaled version of that what is installed in aircrafts as far less airflow is needed to perform in-vitro and in-vivo studies.

Oil is dosed using a motor driven (TSE type S40200, TSE Systems, Inc. Chesterfield USA) syringe with a Schlick compressed air spray nozzle (SCHLICK Mod.970/5 S 9, Düsen-

Schlick GmbH, Untersiemau/Coburg Germany). The oil is injected into the heated and compressed air, controlled by a Mass Flow Controller (MFC) (Type F201, Bronkhorst Nederland B.V., Veenendaal, the Netherlands). The injector, mixing chamber and compressed air are heated to improve the nebulization by decreasing the oil viscosity and surface tension. The air/oil mixture temperature is monitored inside the tube by a thermocouple (Voltcraft K201 thermometer + Type K inconel 600VV thermocouple). At the exit of the oven, the air/oil mixture is diluted and cooled with compressed air controlled by a MFC. The dilution flow is concentric and to the outside of the air/oil mixture to minimize thermal diffusion losses by shielding it from the cold walls of the connection tube to the ALI or the animal nose only exposure system (see further). Alternatively, oil can be mixed into a heated nitrogen airflow and then injecting it into the air stream. The rate is dependent to the final concentration to achieve and will be in the range of 0.01-0.1 µL/min.

In case de-icing fluid is selected in the previous screening, a similar procedure will be applied, albeit that it is injected prior to heating. The test atmosphere is cooled down and depending on the desired final concentrations diluted with clean conditioned air. At the end the air is conditioned to reach 50-70% relative humidity and 21°C. A pre-selective impactor, designed to remove particles larger than 2.5 µm is included when measurements indicate that larger droplets or solid particles are present in the test atmosphere.

A minimal final flow of 20 L/min for both ALI and *in-vivo* studies is required and additional 5 L/min is needed for physical characterization. The air will be characterized by isokinetic sampling as close as possible near the breathing zone of the animals (*in-vivo*) as well as just prior to air reaching the cells (*in-vitro*). The particle mass concentration is determined by time aggregated gravimetric analyses with Teflon R2PJ047 filter (Pall corp., Ann Arbor MI, USA) and by tapered element oscillating microbalance (TEOM) series1400 (Rupprecht & Patashnick, New York, USA). The particle number concentration is measured over time by a condensation particle counter CPC 3022 (TSI inc., St Paul MN, USA). Particle size distribution is monitored over time by an OPS 3330 (TSI inc., St Paul MN, USA), a scanning mobility particle sizer (SMPS) 3080 with 3085 Nano DMA (TSI inc., St Paul MN, USA) and a MOI Model No. 110 (MSP corp, Minneapolis MN, USA). Temperature and relative humidity is determined by a Vaisala M170 (Vaisala Oyj, Helsinki, Finland). The fumes are chemical analysed (performed by TNO) for TCPs, aldehydes, VOCs and CO.

3.6.2 Combined inhalation exposure/neuronal activity in-vitro model (Task 3A.C2)

In the Air Liquid Interface (ALI), immobilized lung cells are exposed to a stream of freshly generated vapour and its associated components on one side of the air/liquid interface and are in direct contact with the cell culture medium on the other side (Phillips et al., 2005). The ALI enables direct exposure of lung cells to fumes to study direct toxic effects towards the lung, including cell death and compromises barrier function of the lung. Moreover, the ALI allows for collection of all fume constituents in the culture medium (NBA medium) which are able to cross the lung barrier and thus actually reach the body (via inhalation) in real life. The effluents of the ALI system are subsequently tested in the MEA neuronal test system.

Similar equipment and settings to generate aerosols are needed as for the animal study. The dose metric to control the exposure will be mass (based on TEOM measurements). Also total carbon analyser and the amount of oil that is injected into the BACS will be determined.

To determine cell death of the lung cells, two different toxicity indicators are used, namely LDH release and MTT assay. The barrier function of the cells is checked by TEER measurements. Time points at which effects are assessed are directly after exposure and after 24 hours. Following the exposure in the ALI, aliquots of the medium underneath the lung model (effluent) will be stored at 4°C and subsequently transported to IRAS. Medium from similar exposure conditions (material/dose/duration) will be pooled and used for the MEA screening for neurotoxicity (for more details on MEA testing see Task 3A.A3).

Table 19. Air Liquid Interface system (Vitrocell).

necessary air flow for sampling [L/min]	**typical sampling duration [min]**	**equipment size [cm]**	**Other specifications**
20	***180-240 min***	*1.124 x 623 x 2.187 mm (L x W x H)*	*Weight: 240 kg, Voltage: 230V/50Hz* *Rating: 1.8 kW* *Fuse: 16A* ***Compressed air: 5 bar (72 psi)***

3.6.3 Mice inhalation exposure and behaviour test (Task 3A.C3)

Description

To confirm and to substantiate the *in vitro* data, an *in vivo* study is performed. Based on the first tier *in vitro/in vivo* screening assays (MEA and zebrafish) the most appropriate fume, i.e. highest potential toxicity, will be selected to be tested in *in vivo* inhalation experiments. In this study, groups of healthy mice (WT C57Bl6, n = 10 per group) are exposed nose-only to clean air, bleed air or bleed air enriched with one or more fume events. On the basis of a pilot study, one concentration will be selected. This concentration is preferably the concentration which leads to max 20% cell death in the more complex cell model (ALI+MEA). Different dose levels are then obtained by varying the exposure time (C x t concept). The clear benefits of using a whole organism combined with realistic exposure conditions and the possibility to use more complex read outs for neurological effects (e.g. behavioral effects and correlating biomarkers, both generic and specific for neurological damage) justifies the proposed use of animals.

Equipment needed

- Nose-only tubes and inhalation towers
- Simulator including heating oven
- Condensation particle counter
- SMPS (Scanning Mobility Particle Sizer)
- Online monitor
- carbon tubes to collect chemical components (Teflon/carbon filter)?
- humidifier
- pressurized air
- electrical power
- mass flow controller
- TEOM (Tapered Element Oscillating Microbalance): used to control the exposure
- CO monitor
- total carbon analyzer
- neurobehavioral testing setup
- high resolution video camera setup to record behavioral experiments

- Ethovision XT software for analysis of video images

Experimental approach

An ethical dossier will be prepared for the *in vivo* mouse study and will be submitted to the ethical commission. The study comprises a pilot study to determine the concentration level at which no sensory irritation and lung toxicity occurs, followed by an inhalation study in mice designed to test for neurotoxicity by performing behavioral test. A schematic overview of the latter mice inhalation experiment is shown in Figure 5.

In the pilot study, the generated aerosol is collected and the right amount of gases to ensure normal breathing has to be taken care of. The pilot study consists of a single exposure at three different concentrations and control animals that are exposed to clean air (3 mice per group). A standard sensory irritation test (Alarie) is carried out using a plethysmograph. Mice respiration is measured before, during, and after exposure to one or more concentrations and then respiratory depression (RD) is statistically quantified during exposure. This is to determine whether irritant chemicals from the fume might induce breath holding in the mice. Mice are assessed for lung damage and inflammatory effects by broncho alveolar lavage (BAL) analysis 24 hours after exposure. Blood samples are collected at the same time point after the sensory irritation test for biomarker determination.

In the **main experiment**, mice are exposed for 4h, this is considered a representative duration of an average intra-European flight in which fume events can occur. To assess a dose response relationship, the concentrations in the test atmosphere can be varied. The exact dosing regimens will depend on the outcomes of the chemical analysis of the simulated fume-events performed in Task 2. To discriminate between potential effects of single- and repeated exposures, mice receive a single exposure or three exposures on consecutive days. The single exposure experiment has a control group and 2 different fume concentrations. For the consecutive exposures, one concentration is selected based on the outcome of the behavioral tests after the single exposure and include a recovery for assessment of reversibility of acute effects or the presence of delayed neurotoxicity. To avoid that neurobehavioral experiments are confounded by pulmonary toxicity, lung function of the mice is assessed to evaluate changes in pulmonary function during exposure as is performed in the pilot study by BAL analysis. To assess the effect of exposure to fumes on neurological functioning, a neurobehavioral test battery is used. This test battery is designed to detect effects such as disorientation and anxiety (Figure 5) as well as neuromuscular/motor coordination via measurements of grip strength and balance (Figure 6). Behavior tests will be performed before exposure, to obtain a baseline value for all individual animals, and is repeated directly after the exposure to assess acute effects. Thus, changes in neurobehavioral performance can be assessed within the individual increasing the power of detection of the study. Reversibility of neuromuscular and neurobehavioral effects as well as the occurrence of delayed toxicity is tested in the third round of neurobehavioral testing, one-week post exposure (only with mice exposed to the highest concentration after repeated exposure). Following the behavioral testing, usually within 24 h after the final exposure, blood, liver and urine samples (residual urine collected from the bladder during sectioning) are collected during autopsy. Broncho-alveolar lavage fluid will be obtained by flushing the lungs. In light of the chosen exposure paradigm of the *in vivo* exposures and the time needed to develop neuro-inflammation-induced neurodegeneration, pathology is not a good idea for all animals a priori. As mentioned, tissues will be preserved and should funds become available, histopathologic examinations will be considered.

Figure 5. The balance beam is a neurobehavioral test used to assess balance and motor coordination of the animals. Mice are placed on a narrow beam and the number of foot slips as well as the time it takes the animal to reach the safe spot (tube on the left) are recorded. Animals with problems in motor coordination or balance as a result of e.g. a toxic stimulus have difficulties in walking on the beam. Image courtesy: Frankel Cardiovascular Center, Michigan Medicine.

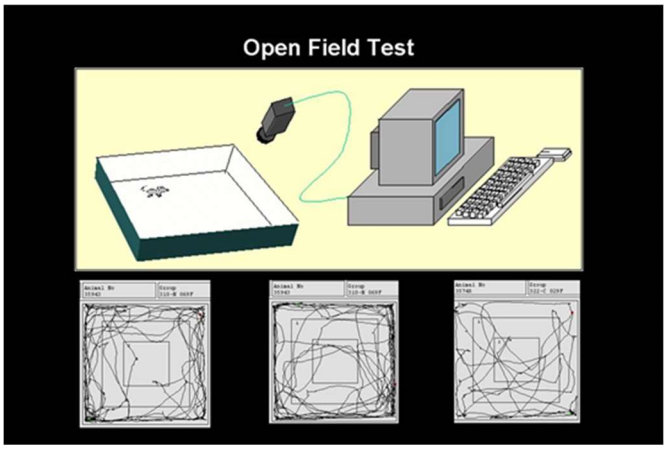

Figure 6. Image displaying the principles of the (video-tracked) open field test which is used as measure of anxiety, habituation and disorientation. Healthy mice explore the new open space carefully making sure to primarily stay in the outskirts of the open space (middle recording). Mice that lost anxiety as a result of e.g. a toxic stimulus carelessly cross the open field (recording on the right) whereas animals with an increased level of anxiety will be inclined to remain along the walls (recording on the left). Image courtesy: psylab.idv.tw.

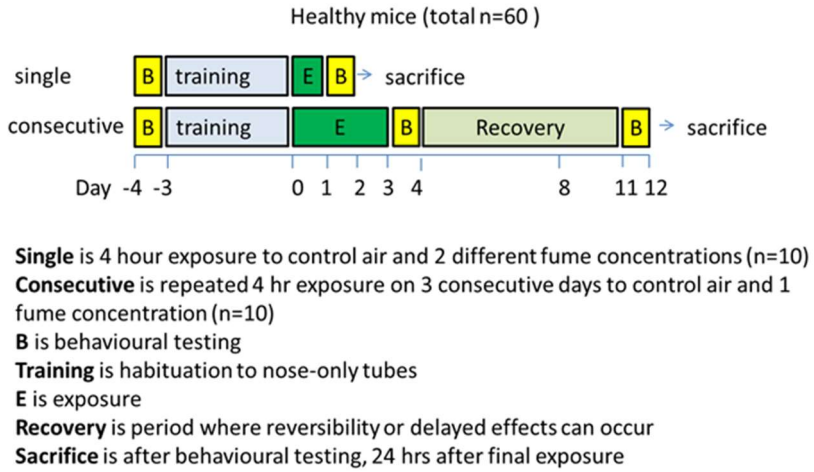

Figure 7. Schematic overview of experimental design of mice inhalation study.

4 References

Asbach C, Kuhlbusch TAJ, Kaminski H, Stalmecke B, Plitzko S, Gotz U, Voetz M, Kiesling HJ, Dahmann D, NanoGEM, 2012.

Brevard H., Cachet T., Chaintreau A., Demyttenaere J., Dijkhuizen A., Gassenmeier K., Joulain D., Koenig T., Leijs H., Liddle P., Loesing G., Marin C., Scotti A., Sherlock A., Tateba H.: Statement on the identification in nature of flavouring substances, made by the Working Group on Methods of Analysis of the International Organization of the Flavour Industry (IOFI), Flavour and Fragrance Journal, 2006, 21, 185.

De Groot M W, Westerink R H, Dingemans M M. Don't judge a neuron only by its cover: neuronal function in in vitro developmental neurotoxicity testing, Toxicol Sci 2013, 132, 1-7.

Duarte DJ1, Rutten JMM, van den Berg M, Westerink RHS. In vitro neurotoxic hazard characterization of different tricresyl phosphate (TCP) isomers and mixtures. Neurotoxicology. 2017 Mar; 59: 222-230.

Ellman GL, Courtney KD, Andres V, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961, 7: 88–95

Fierz, M., Kaegi, R., and Burtscher, H., Theoretical and Experimental Evaluation of a Portable Electrostatic TEM Sampler, Aerosol Sci. Technol., 2007, 41(5): 520–528.

Fujitani F, Y. Ideno, A. Fushimi, K. Tanabe, S. Kobayashi and T. Kobayashi. Generation of Nanoparticles of Lubricating Motor Oil for Inhalation Studies, Aerosol Science and Technology, 2007, 41 (1): 14-23

Goelen A., M. Lambrechts, F. Geyskens and T. Rymen. Development and Performance Characteristics of a Capillary Dosage Unit with in Situ Weight Sensor for the Preparation of Known Amounts of Gaseous Voc's in Air, International Journal of Environmental Analytical Chemistry, 1992, 47 (4): 217-225

Gross G W, Harsch A, Rhoades B K, Gopel W. Odor, drug and toxin analysis with neuronal networks in vitro: extracellular array recording of network responses. Biosens Bioelectron, 1997, 12, 373-393.

Heutelbeck et al Acetylcholinesterase and neuropathy target esterase activities in 11 cases of symptomatic flight crew members after fume events. J. Toxicol. Environ Health, 2016, 79, 1050-1056.

Holscher W, Steinhart H. Investigation of roasted coffee freshness with an improved headspace technique. Z. Lebensm. Unters. Forsch., 1992, 195, 33-38.

Jarema KA, Hunter DL, Shaffer RM, Behl M, Padilla S. Acute and developmental behavioral effects of flame retardants and related chemicals in zebrafish. Neurotoxicol Teratol. 2015 Nov-Dec; 52 (Pt B): 194-209.

Johnstone A F, Gross G W, Weiss D G, Schroeder O H, Gramowski A, Shafer T J. Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century. Neurotoxicology, 2010, 31, 331-350.

Noyes PD, Haggard DE, Gonnerman GD, Tanguay RL. Advanced morphological - behavioral test platform reveals neurodevelopmental defects in embryonic zebrafish exposed to comprehensive suite of halogenated and organophosphate flame retardants. Toxicol Sci., 2015 May; 145 (1): 177-95.

Phillips J, Kluss B, Richter A, Massey E. Exposure of bronchial epithelial cells to whole cigarette smoke: assessment of cellular responses. Altern Lab Anim, 2005, 33, 239-248.

Reinen et al. Characterization of human cytochrome P450s involved in the bioactivation of tri-ortho-cresyl phosphate (ToCP). Chem. Res. Toxicol., 2015, 28, 711-721.

Robinette B L, Harrill J A, Mundy W R, Shafer T J. In vitro assessment of developmental neurotoxicity: use of microelectrode arrays to measure functional changes in neuronal network ontogeny. Front Neuroeng, 2011, 4: 1.

Schopfer et al. Development of diagnostics in the search for an explanation of aerotoxic syndrome. Anal. Biochem., 2010, 404, 64-74.

Schopfer et al. Detection of cresyl phosphate-modified butyrylcholinesterase in human plasma for chemical exposure associated with aerotoxic syndrome. Anal. Biochem., 2014, 461, 17-26.

Selderslaghs IW, Hooyberghs J, De Coen W, Witters HE. Locomotor activity in zebrafish embryos: a new method to assess developmental neurotoxicity. Neurotoxicol Teratol, 2010, 32: 460-471.

Selderslaghs IW, Blust R, Witters HE. Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. Reprod Toxicol, 2012 Apr, 33 (2): 142-54.

Selderslaghs IW, Hooyberghs J, Blust R, Witters HE. Assessment of the developmental neurotoxicity of compounds by measuring locomotor activity in zebrafish embryos and larvae. Neurotoxicol Teratol, 2013 May-Jun, 37: 44-56.

Solbu K. et al., Determination of airborne trialkyl and triaryl organophosphates originating from hydraulic fluids by gas chromatography-mass spectrometry. Development of methodology for combined aerosol and vapor sampling, Journal of Chromatography A, 2007, 1161: 275-283.

Van den Dool H., Kratz P.: A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography, Journal of Chromatography, II, 1963, 463-471.

