FACTS Detailed Test Plan Task 3A: Toxicological Assessment

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1 Introduction

1.1 Remarks on the differences of this test plan with the original D3 test plan

This is the detailed test plan of task 3A. It is an updated version of (part of) the previous version of the FACTS Test Plan (FACTS-D3, version 2017.07.15), which integrated both the test plans of Task 2 and Task 3A. It was adjusted after the meetings with the Scientific Committee on 15/11/18, in which the following remarks and advices were formulated considering the preliminary experiments:
Fumes generated and condensing in the system may be different in different realistic and other test settings, and are a 'worst case' (near to the place where fume is generated, using pure oil). The aim of the preliminary experiments is to prepare and optimize a procedure for the trapping of the complete fume mixture generated in BACS, for zebrafish embryo and MEA exposure. The challenge is that the fumes are oily of composition and not soluble in aqueous media.

- To test the efficacy of the cold trapping and the transfer to fish medium, a full scan organic compound profiling will be done of the raw oil, of the cold trapped oil and of the fish medium in which the cold-trapped oil was dissolved.
- It should be assessed whether particles are trapped in the cold-trap.
- Alternative trapping procedures can be considered: several cold-traps in series (improving trapping efficiency), or in parallel (trapping a larger volume), and/or combining the systems with further particle trapping systems (filter or Aluminium foil)
- Improving the fraction dissolved in the test medium (of zebrafish or MEA) by WAF (see further below), direct bubbling into the watery media, or possibly extraction of compounds from the trapped oil fraction.

1.2 Test plan

The test plan and test strategy proposed here depend on the physical and chemical results from the 26 conditions (different pressure/temperature settings and contamination source) to be tested in the BACS. During the Steering Board Meeting of 24/01/2019 it was decided that toxicological testing does not have to wait for the completion of the BACS experiments and its first batch of test results. Instead, the six fumes will be generated with the mini-BACS at the RIVM for subsequent zebrafish and MEA effect analysis. On 23/02/2019, the consortium presented to the Scientific Committee three options for the selection of the six fumes to be generated by the miniBACS. The options differed in the use of contamination sources and the input for the temperature and pressure settings. The consortium proposed, in order to have an understanding about the hazards of the main bleed air contaminants, to use four engine oils and two hydraulic fluids as contaminations source (scenario A). Termperature and pressure settings are based on flight test 1 (and within boundaries of mini-BACS <3bar pressure and <650T) so all six fumes can be generated without dependency on other test campaigns. The Scientific Committee agreed with this approach.

Overall, for Task 3A, the central question is whether exposure to neurotoxic substances formed during fume events could be the cause of neuronal damage as observed in cases of 'aerotoxic syndrome'. One of the main problems in the risk assessment of cabin air quality is the lack of neurotoxicity hazard data for the majority of substances present in fumes. Even more, in an aircraft there is potential exposure to a complex mixture of a large number of different substances during a fume event, in highly variable concentrations.

In-vitro and *in-vivo* toxicity testing of mixtures has hardly been performed in the context of fume contaminants. There is a need for testing the combined toxicity of all substances present in fumes of engines, as a lot remains unknown about the hazard and potential health effects of exposure to this type of mixtures (Harrison and Ross, 2016).

For toxicity screening, integrated cellular testing approaches combining different organ systems, as well as whole organism test systems are needed, to allow more realistic simulation of organ interaction, and to include metabolic competence in the test system. In the context of fume exposure, few experiments have been done on studying the translation of in-vitro to in-vivo results.

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. It is therefore needed to screen for, and semi-quantify, selected/targeted biomarkers in animals exposed to realistic and characterized fumes.

1.3 Strategy

Integrated *in vitro* testing strategies are needed combining a realistic exposure scenario (inhalation exposure) with a relevant readout (neuronal function). *In vitro* (cellular) research allows for detailed studies of toxicity but often lacks important aspects such as metabolism. The proposed combination of *in vitro* cellular research using MEA and ALI, the whole-organism *in vitro* study with zebrafish and an *in vivo* rodent inhalation study with neurobehavioral and biomarker analysis therefore fills several of the gaps identified above. These studies with human relevant model studies might guide decision support on - temperature/pressure conditions and fuel/hydraulic/de-icing fluids used during flights and associated - fume events which are most at risk to human, and which should be considered for future mitigation procedures.

2 Project Goals and Approach

The objective of Task 3A is to allow predictions of actual hazards from fumes generated during fume events, with a focus on the nervous system as most important suspected target. In Task 2 several conditions will be tested to generate fumes. In a first step in Task 3A, screening of the various mixtures of selected fumes will result in ranking of their potency derived from dose-response information and/or effect concentrations relative to a positive control compound. This will be done using the zebrafish and the Microelectrode Array including neuronal cells (MEA) (*in-vitro* neurotoxicity assays). In a second step, 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 will be screened for, in the test animals exposed to the realistic and characterized fumes and/or extracts. The biomarker formation, stability and half-life will be studied under the conditions of controlled exposure. This will allow investigating the relationship between fume mixtures and the biomarkers, without interference of other confounders (e.g. other sources, life style).

Table 1 Test approach in short: overview of the experimental set-up of Task 3A (more details in the text)

Task 3A.1 Preliminary experiments for neurotox screening
• Optimization of fume trapping for *in-vitro* screening • Preliminary toxicity screening of generated fumes • Optimization of biomarker methodology (protein adducts of organophosphates and other neurotoxic compounds and/or enzymes as indicators for exposure to neurotoxic compounds) **Result:** optimized fume-trapping sampling system + methodology for biomarker testing
Task 3A.2 Neurotox in-vitro screening tests of BACS fumes
Neurotox screening of six fumes based on Scenario A (presented to the Scientific Committee) generated with the RIVM miniBACS simulator and trapped for neurotox *in vitro* screening: • Microelectrode array (MEA) for neuronal electrical activity screening: analysis of electric activity after 30', 24h, 48h exposure • Zebrafish locomotor activity at 5 days post fertilization (dfp) of larvae, after 48h exposure **Result:** hazard ranking of 6 fume extracts -> result expressed relatively to positive control fume (TCP technical mixture)

Task 3A.3 Neurotox in-vitro and in-vivo inhalation testing with the mini BACS

Assessing impact of direct inhalation exposure of most neurotoxic fume identified in Task 3A.2:

- BACS set-up for inhalation exposure tests at RIVM
- Combined alveolar exposure/neuronal activity *in-vitro* model: Air Liquid Interface (ALI) alveolar epithelial cells: direct, cell death of lung cells 4h + Microelectrode array (MEA) for neuronal electrical activity testing: electric activity after 30', 24h, 48h
- Mice inhalation exposure and behaviour test: direct nose inhalation exposure (4h) to most toxic fume based on previous steps. Array of behavioural test will be performed before, after 3 days of consecutive exposure and after 8d recovery
- Analysis of biomarkers in animal models: fume exposed zebrafish (5 dpf after locomotor assay) and mice (3d exposure and 8d recovery)

Result: neurotoxicity testing in complex *in-vitro* and *in-vivo* inhalation animal model to confirm the *in-vitro* hazard tests

3 Detailed test plan

3.1 Preliminary experiments for neurotox screening (Task 3A.1)

The fumes are sampled - for off-side *in-vitro* testing - using cold-trapping. The optimal cold-trapping methodology is tested. A jet oil is heated under lab conditions to test the effectiveness of the cold trapping, the solvent transfer system and the overall toxicity of the fume mixture contaminants after heating and cooling. A TCP dose-response curve is generated by dissolving it directly into DMSO and ethanol.

3.1.1 Optimization of fume trapping for *in-vitro* screening

Mobil jet oil II or Eastman 2197 jet oil is heated (500°C) and transferred to the cold trapping system. Cold trapping of the organic compounds from condensed fumes is done using an impinger, cooled by means of liquid nitrogen. The effectiveness of the fume sampling system is assessed prior to application in the actual experiments:

- An active carbon adsorbent (for VOC analysis), and particle counter is put in-line after the cold trap to collect compounds braking through the trap;
- The condensate is screened for total organic compounds (by VITO), and analysed for TCPs (by TNO) to assess changes in composition compared to composition in oil;

The material captured in the cold trap needs to be dissolved in fish or MEA medium, either via dissolving it in a solvent, or via direct contact with the fish medium. The latter methodology is called Water-Accommodated Fraction (WAF), according to the procedure of OECD (Mixing fish medium + oily cold-trapped substance, 24-48h stirring or longer (until saturation), settle 1h) (https://www.oecd.org/env/ehs/testing/Leon-1-Draft%20Guidance%20for%20waiving%20or%20bridging%20acute%20mammalian%20toxicity%20studies.pdf).
Alternatively to the procedure of cold-trapping followed by dissolving into fish medium, direct bubbling of the fume through ice-cooled fish medium (that is kept in the impinger) will be tested.

3.1.2 Preliminary toxicity screening of jet oil

The jet oil is tested for its overall toxicity of the fume mixture contaminants after heating and cold trapping. The solubility in DMSO and ethanol is tested. The toxicity is tested as described below for MEA and the zebrafish test. At this time point, also some explorative tests are done in zebrafish whole animal larvae homogenates, which are used to assess baseline enzyme activities (neurotoxicity indicators) (see also Biomarker analysis).

3.1.3 Optimization of biomarker technology

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 laboratory 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) **Organophosphate-derived protein adducts**, such as those to BChE and albumin present in plasma. The eventual selection of OP-derived adducts will partly depend on which OP compounds identified in the fumes generated in Task 2.

3) **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 are identified in Task 2.

During the period of the preliminary study the protocols will be optimized (more on biomarkers, see Task 3A.3).

3.2 Neurotox *in vitro* screening (Task 3A.2)

Six generated fumes of Scenario A are screened using MEA and the zebrafish locomotor assay. The hazard evaluation of the (unknown) mixtures is done relative to the positive control TCP technical mixture. This allows ranking of the fumes according to neurotoxicity.

3.2.1 TCP/Oil concentration needed in bioassays

A working range of 1-100 µM TCP (concentration-response) is estimated to be used for the screening bioassays, based on available literature (for zebrafish: Jarema et al., 2015[1] and Noyes et al., 2015[2]; for cell culture: Duarte et al., 2017) (see footnotes for more details) and will be confirmed via TCP dose – response relationships determined during the preliminary tests.

- ➔ 1-100 µM TCP = 0.045 to 4.5 mg TCP needed in the bioassay medium;
- ➔ Trapping 4.5 mg oil and transferring this to the medium means that 0.135 mg of TCP (3%) will be present in the medium. Both the total oil amount (4.5 mg) and the TCP amount (0.135 mg) are in the range of the previous determined dose responses curves for the zebra fish with the remarks to be given, that oil has a different molecular weight compared to TCP, and that effects might rise from other compounds in oil.

This amount (4.5 mg) is needed for one single dosing, high level in acute exposure, or similarly repeated dosing at low level representing chronic exposure. It is estimated that 50 mg of oil will be needed to do all experiments. As mentioned in the BACS test plan of Task 2, an oil concentration of e. g. 50 mg/m³ will be generated, meaning that about 1 m³ of air from the BACS needs to be sampled. It is planned to collect 2 m³ to be at the safe side.

[1] Zebrafish behavior tests with Tricresyl phosphate (TMPP), cas 1330-78-5, Tri-o-cresyl phosphate (TOCP), cas 78-30-8. Assay: in 96- well plate, treatment ~24 hrs., remove chemical, evaluation at ~150 hrs. for behavior (dark/light responses). Effect ranges (changes hypo-hyper activity in reverse order for both compounds): TMPP : 7 – 22.4 µM, TOCP: 6.9 – 12 µM

[2] Zebrafish behavior tests with Tributyl phosphate (TBP), cas 126-73-8; Tricresyl phosphate (TCP, p-, m-, o-mix), cas 1330-78-5; MW= 371.39; Tri-o-cresyl phosphate (o-TCP), cas 78-30-8; Tris (1-chloropropyl) phosphate (TCPP), cas 13674-84-5. *Assay*: 24 h embryo (dechorionated) and 120 h larvae PMR activity (hypo/hyper), next to developmental effects, test ranges (64 µM, 1: 10 diluted 7 steps to 6.4 E-6 µM) and continuous exposure from 6 hpf onwards. Effect ranges:

Compound	LEL 24 hrs. embryo (baseline – excitation)	LEL 120 hrs. larvae (light dark switches)
TBP	/ , (not 64 µM tested)	↓ 0.64 (dark) –↑ 0.064 & 6.4 (light), no DR (not 64 µM tested)
TCP	64 µM ↓ (bl) - 64 µM ↓ (exc)	64 µM= ~100 % mortality ↑ (different dark/light conditions at low nM conc., but no DR)
o-TCP	64 µM ↓ (bl) - 64 µM ↓ (exc)	↓ 64 µM (light phase), ↓ 0.0064 µM (light phase)
TCPP	-	↓ 0.64 & 64 µM (dark stimulation & acclimation)

3.2.2 Multi-electrode array (MEA) for neuronal electrical activity screening

Description
Multi electrode arrays consist of a multi-well cell culture surface with an integrated array of microelectrodes 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 this MEA set up, spontaneous electrical activity of a network of primary rat cortical neurons can be recorded. The electric activity will be measured after 30 min (acute), 24h and 48h exposure, respectively, to cover both acute and sub-chronic effects of fume constituents.

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 will be made. After this recording the cells are exposed by adding the DMSO extract of the fumes (Task 3A.1) (similarly also done for the ALI effluents of Task 3A.3) at different concentrations (final concentration to be determined). A subsequent 30 min recording will be 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 neuronal activity will be measured again to determine the sub-chronic effects of exposure following 24 h and 48 h exposure, respectively (Duarte et al., 2017). A concentration-response curve of at least 3 different concentrations chlorpyrifos will be used as positive assay control.

3.2.3 Zebrafish locomotor assay

Description
Several parameters for locomotor activity (total distance moved, mean velocity, mean turn angle, frequency of movement and total duration of movement) in 48 hours exposed larvae (start at 3 dpf to 5 dpf) are recorded with a high resolution digital infrared camera (Noldus equipment) and locomotion tracking software at 25 frames/s, during 5 min (Ethovision). We will focus on observations in 5 days old embryos (metabolic competent) for locomotor parameters. The results will be analysed by Matlab, based on population distribution curves for treatment groups and parameter, and expressed as percentage effect in comparison to a negative and positive control group. At the end of the exposure, the enzyme activity of acetylcholine esterase in zebrafish whole body homogenates is determined as a marker for organophosphate exposure. The % enzyme inhibition is evaluated relative to the negative control condition.

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

Test protocol

The test protocol is based on peer reviewed publications. It contains information on design, measurement of locomotor parameters and data analysis. These methods have been developed at VITO (Selderslaghs et al., 2010) and validated for several neurotoxic compounds (Selderslaghs et al., 2013).

References:

- Selderslaghs et al. 2010. https://www.ncbi.nlm.nih.gov/pubmed/20211722
- Selderslaghs et al. 2013. https://www.ncbi.nlm.nih.gov/pubmed/23357511

Experimental approach

Due to additives, trace contaminants, general toxicity to the biological model systems might occur and needs to be assessed as this might mask specific neurotoxicity. In order to determine an appropriate maximal test concentration (working range) for the behavioural tests, zebrafish embryos are exposed and conditions causing less than 20% of both mortality and malformations will be used as highest test concentration for experiments on locomotor behaviour.

The next sequence of screening/testing is applicable for testing of compounds (e.g. engine oil, hydraulic fluid,...) in a preliminary phase, and for 6 collected fumes (set up to be modified as function of available sample volume) applied to the fish test medium (see Preparation of sample extracts).

a) Assessment of embryo toxicity: preliminary screening to determine working range for test samples
   - Freshly collected zebrafish eggs are distributed group wise into 6 well plates with 8 mL fish water (= negative control medium, composition as described in OECD203) within 2 hpf (hours post fertilisation)
   - Select fertilised zebrafish eggs, 24 eggs/test condition and distribute into individual wells with 1 mL medium in 48 well plate
   - At 72 hpf (or 3 dpf), 0.5 mL medium is removed and replaced with test solution resulting into the aimed concentration levels for extracts compared to the negative control.
   - Exposure for next 48 hours and assessment of developmental effects (mortality, malformation) at the end of the exposure period, being 5 dpf with scoring after microscopic evaluation using template (VITO ZTA assay).
   - Test conditions are selected for further locomotor studies which exhibit less than 20% embryo mortality and malformations.
   - Chlorpyrifos will be used as positive assay control, concentrations will be derived from previous studies (Selderslaghs et al., 2010), and confirmed in preliminary toxicity testing (= applicable for set up 'b')

b) Analysis of locomotor parameters (either new set-up: see a) or continue with selected conditions after screening for embryo toxicity
   - Test volume of 0.5 mL in 48 wells is removed in each of the plates
   - Each test plate contains a negative control group (n=24) in comparison to one test condition (n=24) for fume extracts, or positive controls (TCP, CPF)
   - Movie files are made (duration 5 minutes/test plate at 30 images/sec) with the Daniovision Observation Chamber for each of the test plates
   - Analyses of movie files 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 best 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.

This approach will be evaluated as an acute exposure, based on single dosing (max. 48 hrs). Alternatively, a simulation of chronic exposure will be set up, considering repeated dosing at low level of fume extracts. In both scenario's, the measurement of locomotor parameters and data analysis will be the same.

Use & relevance of zebrafish assay to predict human health hazards
The zebrafish model has been shown as a highly relevant model to study mechanisms of toxicity (reviewed by Planchart et al., 2016), and screen for hazardous potential of chemicals or unknown mixture of potential toxic compounds. The zebrafish behavioral assays at early developmental stages (0-5 dpf[3], considered non-animal testing according to EU legislation) have shown their potency as a whole organism approach, complementary to *in vitro* assays for hazard testing. The behavioral endpoint is a read out integrating early events of central nervous system (CNS) development and functioning in a metabolic competent model system. Zebrafish brain development, anatomical features such as the blood-brain barrier and physiology are well described (Fleming et al., 2013; Mueller and Wulliman, 2016), while genetic and functional homology with human has been demonstrated (Howe et al., 2013; Khan et al., 2017; Parker et al., 2013). The zebrafish genome has been mapped and approximately 70%–80% of zebrafish genes share homology with the human genome, and 82% of genes associated with disease in humans can be related to at least one zebrafish orthologue (Howe et al., 2013).

This assay does not represent the main human exposure route (inhalation). That route will be addressed within FACTS by the Air Liquid Interface (ALI) and in vivo studies described in the next sections.

References:
- Planchart et al. 2016; https://www.ncbi.nlm.nih.gov/pubmed/27328013
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3.3 Neurotox *in-vitro* and *in-vivo* inhalation testing (Task 3A.3)

The most toxic fume of Task 3A.2 will be confirmed and tested via the tandem Air Liquid Interface lung cells(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 simulation for these exposure experiments will be done near to the animal lab. This implies building up of a BACS in the lab of RIVM.

3.3.1 BACS set-up for inhalation exposure tests at RIVM

[3] dpf= days post fertilisation

At RIVM the most toxic fume of the neurotox *in-vitro* screening tests (Task 3A.2) will be 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 with up to 3 bar pressure will be 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 will be 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 will be 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 will be injected into the heated air 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 will be heated to improve the nebulization by decreasing the oil viscosity and surface tension. The air/oil mixture temperature will be monitored inside the tube by a thermocouple (Voltcraft K201 thermometer + Type K inconel 600VV thermocouple). At the exit of the ovens, the air/oil mixture will be 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.

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 will be 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 will be measured over time by a condensation particle counter CPC 3022 (TSI inc., St Paul MN, USA). Particle size distribution will be 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 will be determined by a Vaisala M170 (Vaisala Oyj, Helsinki, Finland). The fumes will be chemical analysed (performed by TNO) for TCPs, aldehydes, VOCs and CO.

3.3.2 Combined alveolar exposure/neuronal activity *in-vitro* model

Using an Air Liquid Interface (ALI), immobilized lung cells (alveolar epithelial 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). ALI enables direct exposure of the lung cells to the fumes to study direct toxic effects towards the lung, including cell death and compromises barrier function of the lung. Moreover, ALI allows for collecting of all fume constituents in the culture medium (NBA medium) that are able to cross the lung barrier and thus actually reach the body (via inhalation) in real life. The effluents of the ALI system will be subsequently tested in the MEA neuronal test system.

Equipment needed
MEA test system (see above)

Table 2. 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: 230 V / 50 Hz, other voltages upon request* *Rating: 1.8 kW* *Fuse: 16A* *Compressed air: 5 bar (72 psi)*

NB: 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.

Experimental set-up

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 multi-electrode screening for neuronal activity of the MEA system.

3.3.3 Mice inhalation exposure and behavior test

Description

To confirm and to substantiate the *in vitro* data, an in vivo study will be performed. Based on the *in vitro/in vivo* screening assays (MEA and zebrafish) the most appropriate fume event, i.e. the potentially most toxic, will be selected to be tested in *in vivo* inhalation experiments. In this study, groups of healthy mice (WT C57Bl6, n = 10 per group) will be 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 20% cell death in the more complex cell model (ALI+MEA). Different dose levels will then be 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 will comprise 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. When possible, blood and tissues samples will be collected from either the pilot study or the main experiment for biomarker analysis.

In the pilot study, the generated aerosol will be 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) will be 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 will be 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 will be exposed for 4 h; 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 regimen 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 will 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, the lung function of the mice will be 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 will be used. This test battery will be designed to detect effects such as disorientation and anxiety (Figure 1) as well as neuromuscular/motor coordination function via measurements of grip strength and balance (Figure 2). Behavior tests will be performed before exposure, to obtain a baseline value for all individual animals, and will be 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 will be tested with 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 with 24 hrs. after the final exposure, blood, liver and urine samples (residual urine collected from the bladder during sectioning) will be collected during autopsy. These samples will be analyzed for biomarkers. Bronchoalveolar lavage fluid will be obtained by flushing the lungs. A schematic overview of the mice inhalation experiment is shown in Figure 3.

Figure 1. 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 will have difficulties in walking on the beam. Image courtesy: Frankel Cardiovascular Center, Michigan Medicine.

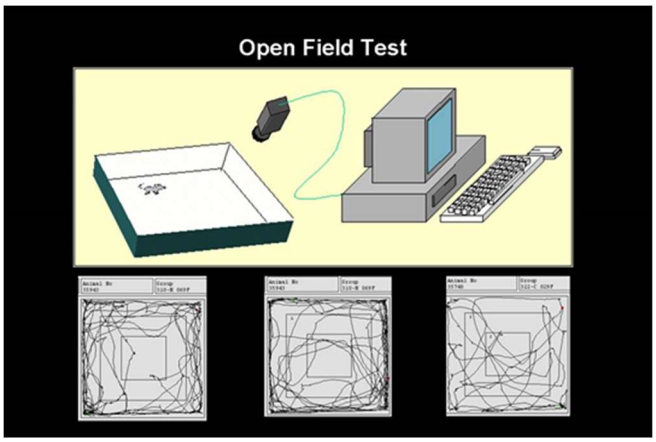

Figure 2. Image displaying the principles of the (video-tracked) open field test which is used as measure of anxiety, habituation and disorientation. Healthy mice will explore the new open space carefully making sure to primarily stay in the outskirts of the open space (middle recording). Mice that lost anxiety as a result of e.g. a toxic stimulus will 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.

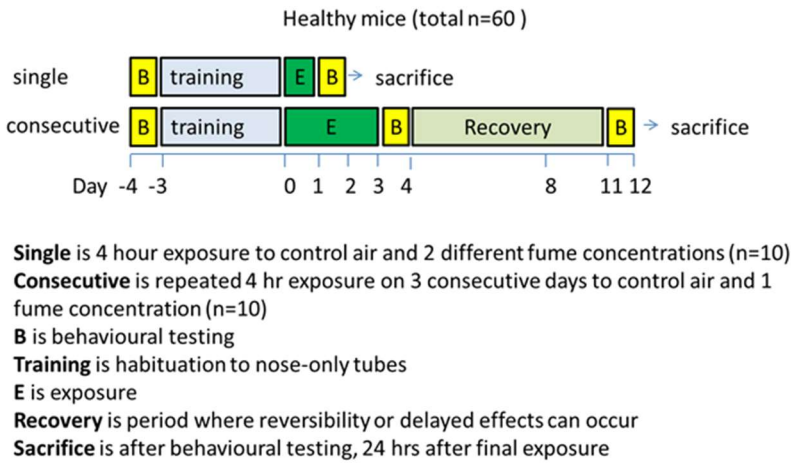

Figure 3. Schematic overview of experimental design of mice inhalation study.

3.3.4 Biomarker analysis

Description

The central question is whether exposure to neurotoxic substances formed during fume events can be associated to neurological impairments as observed in cases of the 'aerotoxic syndrome'. 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. It is therefore needed to screen for, and semi-quantify, selected/targeted biomarkers in animals exposed to realistic and characterized fumes.

Most of the biomonitoring work related to potential biomarkers (e.g. protein adducts) of the '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.

Experimental approach

The work for biomarker analysis is subdivided in two main parts, optimization of the methods (in Task 3A.1) and analysis of the biomarkers in the animal models (Task 3A.3). 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. Zebrafish and mice will be used as animal model.

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) **Organophosphate-derived protein adducts**, such as those to BChE and albumin present in plasma. The eventual selection of OP-derived adducts will partly depend on which OP compounds identified in the fumes generated in Task 2.
3) **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.

Biomarker analysis in zebrafish: from the zebrafish, whole animal homogenates will be used to assess changes in certain enzyme indicators after exposure to the fumes. A method is available for AChE while for NTE activity in these fish homogenates, some optimization with support of TNO is planned.

The protocol includes:

1) Collection of two pools of 20 larvae for each test condition, immediately after analysis of locomotor parameters (at 5 dpf).

2) Rinsing of larvae (fish water with 0.1% DMSO), euthanisation, whole body homogenates are prepared and the supernatant is immediately frozen at -20°C.
3) Performance of enzyme assays according to the protocol by Ellman et al. 1961 (for AChE). For the analysis of NTE activity, a method will be worked out, based on the existing methods for blood analysis (Heutelbeck et al. 2016). The assay and protein analysis will be run in triplicate for each sample. The sample protein concentrations are 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. The enzyme activity of AChE will be expressed as µmol/min/mg protein and as % activity compared to 100% activity reference controls.

Biomarker analysis in mice: Mice will be sacrificed and tissues including muscle, liver, lung, brain and blood will be isolated to analyse toxicity on tissue level. Part of the tissue material collected will be used to screen for biomarkers as listed above. 24 h and 7 d after the final exposure, blood samples will be collected for biomarker analysis. Urine (residual urine collected from the bladder during sectioning) and liver samples will be optionally analysed.