

In vitro neurotoxicity screening of engine oil- and hydraulic fluid-derived aircraft cabin bleed-air contamination

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ABSTRACT

In most airplanes, cabin air is extracted from the turbine compressors, so-called bleed air. Bleed air can become contaminated by leakage of engine oil or hydraulic fluid and possible neurotoxic constituents, like triphenyl phosphate (TPhP) and tributyl phosphate (TBP). The aim of this study was to characterize the neurotoxic hazard of TBP and TPhP, and to compare this with the possible hazard of fumes originating from engine oils and hydraulic fluids *in vitro*. Effects on spontaneous neuronal activity were recorded in rat primary cortical cultures grown on microelectrode arrays following exposure for 0.5 h (acute), and 24 h and 48 h (prolonged) to TBP and TPhP (0.01–100 μM) or fume extracts (1–100 μg/mL) prepared from four selected engine oils and two hydraulic fluids by a laboratory bleed air simulator. TPhP and TBP concentration-dependently reduced neuronal activity with equal potency, particularly during acute exposure (TPhP IC₅₀: 10–12 μM; TBP IC₅₀: 15–18 μM). Engine oil-derived fume extracts persistently reduced neuronal activity. Hydraulic fluid-derived fume extracts showed a stronger inhibition during 0.5 h exposure, but the degree of inhibition attenuates during 48 h. Overall, fume extracts from hydraulic fluids were more potent than those from engine oils, in particular during 0.5 h exposure, although the higher toxicity is unlikely to be due only to higher levels of TBP and TPhP in hydraulic fluids. Our combined data show that bleed air contaminants originating from selected engine oils or hydraulic fluids exhibit neurotoxic hazard *in vitro*, with fumes derived from the selected hydraulic fluids being most potent.

1. Introduction

Most commercial airplanes have an environment control system (ECS), which uses bleed air to pressurize and control the thermal conditions in the cabin, and to ventilate the cabin with outside air (Hunt et al., 1995; National Research Council (US) Committee, 2002; for review see Bolton (2009) and Hageman et al. (2022)). Bleed air is compressed air extracted from the turbine compressor that is later mixed with cabin air. Even during normal operation, bleed air can be contaminated with, among others, engine oil or hydraulic fluids leaking into the ECS (Crump et al., 2011; 2015; Harrison and Mackenzie Ross, 2016; for review see Bolton (2009) and Hageman et al. (2022)). When leaking, the engine oil and hydraulic fluid are exposed to high temperature and pressure leading to the evaporation, thermal decomposition and/or pyrolytic degradation of their constituents (Bolton, 2009; Michaelis, 2016, 2011; Hageman et al., 2022). In severe cases (0.1 %;

Shehadi et al., 2015), the so called “fume events”, the contamination becomes clearly noticeable in mists, fumes, vapors, and smoke in the cabin (National Research Council (US) Committee, 2002; Shehadi et al., 2015; for review see Bolton (2009) and Hageman et al. (2022)).

A number of crew members reported a wide spectrum of neurological symptoms, often referred to as “Aerotoxic Syndrome” (Winder, 2002; de Boer et al., 2015; Harrison and Mackenzie Ross, 2016; Michaelis et al., 2017; Hageman et al., 2022), including cognitive and neuropsychological impairments (Cox and Michaelis, 2002; Heuser et al., 2005; Ross, 2009; Reneman et al., 2016). Other adverse effects associated with this alleged syndrome are neurological damage (Heuser et al., 2005), nervous system degeneration (Abou-Donia et al., 2014, 2013), reduced brain activation, and alteration of white matter microstructure and cerebral perfusion (Reneman et al., 2016).

Turbine engine oils and hydraulic fluids are mixtures of an ester base stock containing various additives (Houtzager et al., 2017; National

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Research Council (US) Committee, 2002) to enhance lubrication, anti-corrosion, flame retardant and pressure-transferring properties. Of those additives, organophosphates (OPs) are of particular concern because they exhibit a high structural similarity with well-studied neurotoxic organophosphorus insecticides (Crump et al., 2012; Dishaw et al., 2011; Hendriks et al., 2014; Behl et al., 2015; Naughton and Terry, 2018; Costa, 2018). The possible harmful effects and the potency of OP exposure from engine oil- and hydraulic fluid-derived fumes have extensively been discussed (Liyasova et al., 2011; National Research Council (US) Committee, 2002; Solbu et al., 2011). While inhibition of acetylcholinesterase is the main acute effect of OPs, OPs can affect a number of additional targets leading to oxidative stress, mitochondrial dysfunction, axonal transport deficits, neuronal cell loss, neuroinflammation, and autoimmunity (for review see Terry, 2012 and Naughton and Terry, 2018). Since these outcomes are common features of neurodegeneration, chronic OPs exposure is closely linked to neurodegenerative diseases (Zaganas et al., 2013; Sánchez-Santed et al., 2016; Yan et al., 2016; for review see Naughton and Terry (2018) and Richardson et al. (2019)).

Since earlier studies focused mainly on the constituents of aircraft engine oil- and hydraulic fluid-derived fumes (Amiri et al., 2017; Mann et al., 2014; van Netten and Leung, 2001, 2000), *in vivo* and *in vitro* toxicity data of these constituents and resulting fumes are limited (He et al., 2021). Only tricresyl phosphate (TCP), particularly the *ortho*-isomers including tri-*ortho*-cresyl phosphate (ToCP), has been in the focus of research for decades (Costa, 2018; Duarte et al., 2017; Henschler, 1958; Winder and Balouet, 2002; Wolkoff et al., 2016). Exposure of humans and animals to high levels of ToCP is associated with neurotoxic outcomes, e.g. OP-induced delayed neuropathy (OPINP) and paralysis (Abou-Donia, 1981; Smith and Lillie, 1931; Wang et al., 2008; for review see Costa, 2018 and Richardson et al., 2019). *In vitro* experiments using rodent primary cortical cultures and neuronal cell lines demonstrated that different TCP isomers as well as commercial TCP mixtures can affect neuronal network formation and function (Duarte et al., 2017; Flaskos et al., 1998; Hausherr et al., 2014). Based on the various concerns, the amount of *ortho*-isomers in TCP mixtures used for aircraft engine oils was restricted and does not exceed 0.3 % (Howard, 2020; Mackerer and Ladov, 2000; Michaelis, 2011; Winder and Balouet, 2002). Likewise, from biomonitoring studies (Liyasova et al., 2011; Schindler et al., 2013) and cabin air quality studies it can be concluded that TCP exposure levels, particularly for ToCP, are very low ranging from below detection limit to 2–932 ng/m³, while the workplace limit is as high as 0.1 mg/m³ (Crump et al., 2011; Denola et al., 2011; Solbu et al., 2011; Schuchardt et al., 2019; OSHA, 2021). Consequently, the TCP levels present in the cabin air are unlikely responsible for the reported neurological symptoms (Crump et al., 2011; de Boer et al., 2015; de Ree et al., 2014; Duarte et al., 2017; Houtzager et al., 2017).

Besides TCPs, triphenyl phosphate (TPhP) and tributyl phosphate (TBP) are organophosphorus additives used particularly in hydraulic fluids (Michaelis et al., 2017; National Research Council (US) Committee, 2002), which can contain up to 80 % TBP (Hewstone, 1994). Importantly, both TPhP and TBP have been detected in cabin air (Hageman et al., 2022; Hardos et al., 2020; Schuchardt et al., 2019; Solbu et al., 2011), and have been hypothesized as possible cause for neurological health complaints (Anderson, 2015; Hardos et al., 2020). In particular TBP can be present at high levels (0.125–0.913 µg/m³; Schuchardt et al., 2019), although the workplace limit is even > 5000-fold higher (5 mg/m³; OSHA, 2021). Moreover, urine samples from crew members have been shown to contain slightly elevated concentrations of TPhP and TBP metabolites (Schuchardt et al., 2019) suggesting chronic occupational exposure.

Available *in vivo* data suggest that TBP does not induce specific neurotoxicity (Carrington et al., 1990; ECHA, 2021; Healy et al., 2010) and that TBP is considered less toxic than TCP (National Research Council (US) Committee, 2002). Following acute and sub-chronic

exposure, rats showed signs of general toxicity, but did not show behavioral or neuropathological alterations suggesting that TBP does not induce specific neurotoxicity (Healy et al., 2010). However, vapors of TBP can cause symptoms similar to complaints reported by exposed crew member, including irritation of the mucous membranes in the eyes, nose, and throat, headache and nausea (National Research Council (US) Committee, 2002).

For TPhP, several *in vivo* studies suggest that TPhP accumulates in the brain and can induce neurotoxicity. TPhP and its metabolite diphenyl phosphate have been detected in the brain of exposed animals suggesting that they can cross the blood-brain barrier (BBB) (Liu et al., 2020; Wang et al., 2016). Further, TPhP exposure has been associated with increased BBB permeability (Liu et al., 2020; Wang et al., 2016). TPhP-exposed animals showed abnormalities in several brain structures (hippocampus, cortex, thalamus) associated with neurodegeneration, hallmarks of neurodegeneration, neuroinflammation and oxidative stress (Hong et al., 2018; Liu et al., 2020), and impaired learning and memory performance (Hong et al., 2018).

Using microelectrode array (MEA) recordings of changes in spontaneous neuronal activity in rat primary cortical cultures as an integrated read out (Gerber et al., 2021), the present study aimed to 1) characterize the neurotoxic hazard of TPhP and TBP since these are organophosphorus additives primarily used in hydraulic fluids, 2 A) to compare this with the neurotoxic effects of fumes originating from selected engine oils and hydraulic fluids, and 2 B) whether there are differences in potency of the different fumes generated via a mini bleed air contamination simulator (Mini-BACS).

2. Material and methods

2.1. Chemicals

Neurobasal-A (NBA) media, L-glutamine (200 mM), Penicillin/Streptomycin (5000 U/mL/5000 mg/mL) and B-27 Plus supplement (50X) were obtained from Life Technologies (Bleiswijk, The Netherlands). All other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The selected engine oils (4 types) and hydraulic fluids (2 types) are the most abundant based on market share. See Table S1 for further description and characteristics of the used aircraft engine oils and hydraulic fluids. For Triphenyl phosphate (CAS: 115-86-6; Sigma-Aldrich) and Tri-*n*-butyl phosphate (CAS: 126-73-8; Sigma-Aldrich) a stock solution of 100 mM (in DMSO) was prepared and used to expose cells.

2.2. Generation and sampling of engine oil and hydraulic fluid fumes on filters

A bleed-air contaminants simulator (BACS) was used to generate fumes from aircraft engine oils and hydraulic fluids as described previously (see He et al., 2021). Briefly, to generate fumes from engine oil and hydraulic fluids, samples were forced through a heated spray nozzle (Schlick, Germany) to be nebulized with 90°C air at 3 bar. Then, the generated aerosols entered a pressurized (3 bar) oven (R50/500/12, Nabertherm, Germany) for pyrolysis and vaporization at 200 °C or 350 °C. Finally, at the exit of the oven, the generated fume was diluted and cooled (~25°C) with clean air, after which the fume was collected onto 47-mm Teflon filters (PTFE Membrane Disc Filters, 2 µm pore, Ann Arbor, MI). For each oil or fluid, the generated fume mixture was also sampled on cartridges for chemical analysis as described in detail previously (He et al., 2021). The characteristics of the engine oils and hydraulic fluids as well as the chemical profile of the generated fumes are summarized in Table S1. The concentration (µg/m³) of specific organophosphates (OPs) measured in the different fumes is provided in Table S4 (derived from He et al. (2021)).

2.3. Filter extraction for *in vitro* neurotoxicity testing

The Teflon filters (including a clean not used one) were extracted in acetone (HPLC grade) by shaking for 5 min. Afterwards, the filters were rinsed with additional acetone, merged with the previous extract and dried at 25°C overnight in an incubator. Next, acetone was added to redissolve the extracted oil and separated into pre-weighed glass vials. The vials were transferred to an incubator and the acetone evaporated at 25°C overnight, followed by 24 h of acclimatization (50 % humidity at 20°C) before determining the total mass of the dry extract. Stock solutions (1–100 mg/mL) of the oil fume extracts were prepared using DMSO as solvent and stored at 4 °C until use.

2.4. Primary cortical cultures

Primary cortical rat cultures are the current standard for *in vitro* neurotoxicity testing using MEA recordings as they are well characterized, widely accepted and easily cultured (de Groot et al., 2013; Hogberg et al., 2011; Johnstone et al., 2010; McConnell et al., 2012; Valdivia et al., 2014). These cultures consist of excitatory and inhibitory neurons as well as supportive astrocytes and resembles many characteristics of the *in vivo* nervous system (for review, see Johnstone et al., 2010; de Groot et al., 2013). Cortical cultures form a functional network that develops spontaneous neuronal activity with distinct bursting patterns that are synchronized over the entire network (Cotterill et al., 2016; Dingemans et al., 2016; Robinette et al., 2011).

Animal experiments were performed in agreement with Dutch law, the European Community directives regulating animal research (2010/63/EU) and approved by the Ethical Committee for Animal Experiments of Utrecht University. All efforts were made to minimize the number of animals used and their suffering.

Primary cortical cultures were isolated from Wistar rat pups (Envigo, Horst, the Netherlands) on postnatal day 0–1, suspended in dissection media (500 mL Neurobasal-A supplemented with 14 g sucrose, 1.25 mL L-glutamine (200 mM), 5 mL glutamate (3.5 mM), 5 mL penicillin/streptomycin and 10 mL B-27 Plus Supplement (50X); pH 7.4), and seeded on polyethyleneimine (0.1 %) coated 48-well MEA plates (Axion BioSystems Inc, Atlanta, USA, M768-GL1-30Pt200) as described in detail previously (Gerber et al., 2021). After seeding, cells were incubated at 37 °C, 5 % CO₂ and 95 % air atmosphere. On day *in vitro* (DIV) 4, dissection media was replaced by glutamate-free culture media (500 mL Neurobasal-A supplemented with 14 g sucrose, 1.25 mL L-glutamine (200 mM), 5 mL penicillin/streptomycin and 10 mL B-27 Plus Supplement (50X); pH 7.4) for further culture until experimental use.

On DIV9, cell cultures were exposed to TPhP, TBP or fume extracts and effects on neuronal activity were measured directly after the start of the exposure (time point 0.5 h, acute exposure) and, 24 h and 48 h after the start of exposure (prolonged). After the final MEA recording, i.e. after 48 h exposure, cell viability was assessed to distinguish specific neurotoxicity from general cytotoxicity. First, the neurotoxic effect and potency of TBP and TPhP, two commonly detected OP contaminants in cabin air were assessed. Next, the neurotoxicity of the complex mixture of bleed air contamination was assessed using fume extracts generated from commercially used engine oils (four) or hydraulic fluids (two).

2.5. Microelectrode array (MEA) recordings

MEAs provide a non-invasive way to record extracellular local field potentials (spikes) at different locations within the culture at millisecond time scale (for review see Johnstone et al., 2010; Robinette et al., 2011; Spira and Hai, 2013). Each well of a 48-well MEA plate contains an array of 16 nanotextured gold microelectrodes (40–50 µm diameter; 350 µm centre-to-centre spacing). Using a Maestro 768-channel amplifier with integrated heating system, temperature controller and data acquisition interface (Axion BioSystems Inc, Atlanta, USA), neuronal activity can be recorded simultaneously and at constant temperature of

37 °C. MEA recordings were made as previously described in detail (Gerber et al., 2021).

Briefly, on DIV9, when rat primary cortical cultures exhibit stable spontaneous neuronal activity (Dingemans et al., 2016; Gerber et al., 2021), a 30 min baseline recording was performed after an equilibration time of 2–5 min. Next, cells were exposed under sterile conditions to TPhP (0.01–100 µM), TBP (0.01–100 µM), the collected fume extracts (1–100 µg/mL), or DMSO (0.1 %; control). Stock solutions were freshly diluted with culture media 1:100 (exposure solution) and cortical cultures were exposed by adding 55 µL of the exposure solution to each well. Neuronal activity was measured immediately after the exposure for 30 min and again after 24 h (DIV10) and 48 h (DIV11) continuous exposure to cover both acute and prolonged exposure scenarios. Changes in the activity pattern evoked by chemical exposure reflect the integrated effect on several targets within the neuronal network, such as neurotransmitter receptors and ion channels, and can be used as an efficient screen for neurotoxic effects (Johnstone et al., 2010). Between recordings, cortical cells were further kept at 37 °C, 5 % CO₂ and 95 % air atmosphere.

For each experimental condition, primary cultures from at least two different isolations were used. From each isolation at least one 48-well MEA plate (N) was used per experimental condition, and per plate each condition was tested in 8 wells (*n*), yielding a total number of N ≥ 2 plates and *n* ≥ 16 wells unless otherwise noted.

Axion's Integrated Studio (AxIS 1.7.8) was used to manage data acquisition as previously described in detail (Gerber et al., 2021). Signals were pre-amplified with a gain of 1200x (61 dB), band-pass filtered at 0.2–5 kHz and sampled simultaneously resulting in raw data files.

2.6. Cell viability assay

After the MEA recordings were completed, i.e. 48 h after the start of the exposure, an Alamar Blue assay was performed to measure mitochondrial activity as measure for cell viability. As previously described (Gerber et al., 2021), the exposure medium was replaced by 300 µL pre-warmed Alamar Blue solution (25 µM resazurin in Hanks' Balanced Salt solution) and cells were incubated for 1.5 h at 37 °C, 5 % CO₂ and 95 % air atmosphere. Next, 200 µL of the Alamar Blue solution was transferred from each well to a transparent 96-well and conversion of resazurin to resorufin was measured spectrophotometrically at 540/590 nm using a microplate reader (Infinite M200, Tecan Trading AG, Männedorf, Switzerland).

2.7. Data analysis

For MEA analysis (Gerber et al., 2021), the raw data were re-recorded and spikes were detected using the AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) with a post/pre spike duration of 3.6/2.4 ms and a variable threshold spike detector set at 7xSD of the internal noise level (rms) on each individual electrode. Then, spike files were loaded in NeuralMetric Tool (version 2.2.4, Axion BioSystems), and only active electrodes (MSR ≥ 6 spikes/s) in active wells (≥ 4 active electrode) were included in the data analysis. Bursts were extracted with the Poisson Surprise method (Legendy and Salcman, 1985) with a minimal surprise of 10 and a minimum bursting frequency of 0.005 bursts/s. Network bursts were determined with an adaptive threshold algorithm.

The last 10 min of each recording (baseline; 0.5 h, 24 h and 48 h exposure) were used for data analysis, and the means of different neuronal activity parameters (Spike Rate, Burst Rate, Network Burst Rate) were calculated based on well averages. Although many parameters can be derived from MEA recordings, the Mean Spike Rate (MSR), Mean Burst Rate (MBR) and Mean Network Burst Rate (MNBR) are sensitive and robust parameter suitable for initial evaluation of effects on neuronal activity (McConnell et al., 2012; Nicolas et al., 2014; Novellino et al., 2011; Valdivia et al., 2014). The effects of the fume

extracts on spontaneous activity were determined by comparing the baseline activity with activity following exposure (0.5 h, 24 h and 48 h). A custom-made MS Excel macro was used to calculate the treatment ratio of different metric parameters ($\text{parameter}_{\text{exposure}}/\text{parameter}_{\text{baseline}}$) of each well. Thereafter, treatment ratios were normalized to solvent controls (0.1 % DMSO). Finally, experiments (N) are combined and results are expressed as average in % of control. Wells that showed an effect $\geq 2\text{xSD}$ above or below average were considered outliers and removed from further data analysis (3.9 % outliers in control wells; 7.4 % outliers in exposed wells).

Also, for cell viability, values exceeding 2xSD are considered to be outliers and excluded from the analysis (3.4 % outliers in control wells; 4.0 % outliers in exposed wells). Per experiment, values are normalized to control and expressed in % of control (control set to 100 %). Afterwards, experiments were combined and averages of metabolic activity per exposure condition were calculated.

2.8. Statistics

All statistical analyses were performed using GraphPad Prism software (v8.3.0, GraphPad Software, La Jolla CA, USA) or R version 3.6.3 using the DescTools package version 0.99.283. Non-linear regressions were used to calculate inhibitory concentration 50 % (IC_{50} values) or inhibitory dose 50 % (ID_{50} values). Concentration- or dose-dependent effects were determined by one-way ANOVA followed by a *post-hoc* Dunnett test comparing treatment ratios in exposed wells to treatment ratios in respective control wells. P -values < 0.05 were considered statistically significant. Effects on neuronal activity were considered relevant when the effect was $\geq 25\%$ (which is the benchmark response based on the SD of controls). Data from MEA analysis are presented as mean \pm standard error of the mean (SEM) and cytotoxicity data are presented as mean \pm SD from the number of wells (n) indicated, derived from at least 2 independent cultures.

3. Results

3.1. Triphenyl phosphate (TPhP) and Tri-*n*-butyl phosphate (TBP)

Rat primary cortical cultures were exposed on DIV9 to TPhP or TBP (0.01–100 μM) and effects on neuronal activity following acute (0.5 h) and prolonged (24 h and 48 h) exposure were assessed. Acute exposure to $\geq 10\ \mu\text{M}$ TPhP potently inhibited neuronal activity (MSR, MBR, and MNBR) reaching complete cessation of neuronal activity at 100 μM (Fig. 1A). Comparable effects were seen for acute exposure to TBP, but with no inhibition of MBR and MNBR occurring at 10 μM (Fig. 1B), indicating that TBP has a slightly lower acute neurotoxic potency than TPhP. Following prolonged exposure (24 h and 48 h) to TPhP, full inhibition of all three neuronal (network) activity parameters was observed at 100 μM , whereas at 10 μM the modest inhibition seen after acute exposure attenuated over time (Fig. 1A). Similarly, neuronal activity of cells exposed to TBP attenuated over time, particularly for MNBR at 100 μM (22 % of control (24 h) and 66 % of control (48 h) vs 0 % of control (0.5 h); Fig. 1B).

The slightly higher neurotoxic potency of TPhP and the attenuation of the effect over time following TBP exposure is well-reflected in the IC_{50} values (Table 1). For acute TPhP and TBP exposure, the IC_{50} values are in a comparable range, although TBP appears to have a slightly lower toxic potency as indicated by the slightly higher IC_{50} s of TBP compared to TPhP (TPhP IC_{50} : 9.7–12 μM ; TBP IC_{50} : 15–18 μM). The IC_{50} values obtained following prolonged exposure are clearly higher, up to 5-fold, compared to the IC_{50} values of acute exposure (TPhP IC_{50} : 25–65 μM ; TBP IC_{50} : 68–104 μM ; Table 1), indicating that the inhibition is attenuating over time. Notably, metabolic activity of cells exposed to TPhP or TBP did not differ from control cells (Fig. 2), indicating that TPhP and TBP did not affect cell viability.

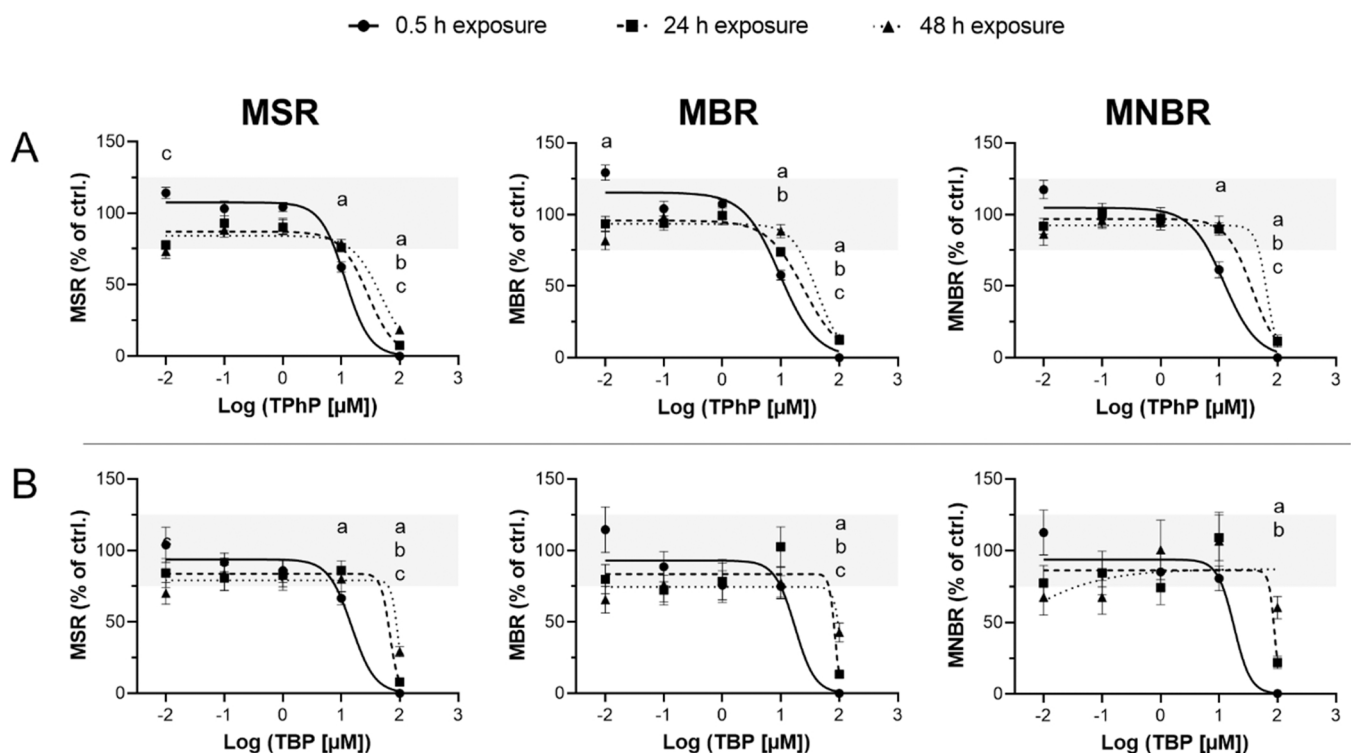


Fig. 1. Concentration-response curve of TPhP (A) and TBP (B) for selected parameters of neuronal activity: mean spike rate (MSR, left), mean burst rate (MBR, middle), and mean network burst rate (MNBR, right). The parameters are depicted as mean treatment ratio \pm SEM from $n = 14$ –24 wells, $N = 2$ –3 plates. Effects $\leq 25\%$ (the SD of control) are considered to be of limited toxicological relevance and indicated by the gray area. ^a $p < 0.05$ for 0.5 h exposure compared to time-matched control, ^b $p < 0.05$ for 24 h exposure compared to time-matched control, ^c $p < 0.05$ for 48 h exposure compared to time-matched control.

Table 1

IC₅₀ values of parameters of neuronal activity for Triphenyl phosphate (TPhP) and Tri-*n*-butyl phosphate (TBP). IC₅₀ values and Confident Interval 95 % are listed for 0.5 h (left), 24 h (middle), and 48 h (right) exposure. N.A.: not available (could not be determined).

Parameter	Chemical	IC ₅₀ values (μM) [CI 95 %]		
		0.5 h	24 h	48 h
MSR	TPhP	12 [N.A. - 14]	28 [16–43]	48 [31 - N.A.]
	TBP	15 [11–28]	68 [N.A.]	93 [N.A.]
MBR	TPhP	9.7 [7–12]	25 [17–37]	42 [24 - N.A.]
	TBP	17 [N.A.]	84 [N.A.]	104 [N.A.]
MNBR	TPhP	12 [8.3–17]	36 [20–N.A.]	65 [N.A.]
	TBP	18 [N.A.]	89 [N.A.]	N.A. [N.A.]

IC₅₀, inhibitory concentration 50%; MSR, mean spike rate; MBR, mean burst rate; MNBR, mean network burst rate.

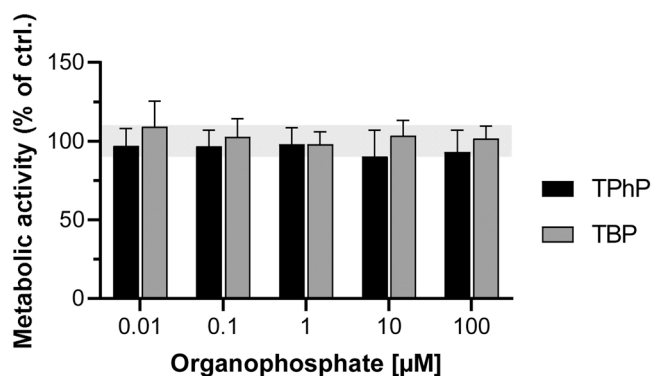


Fig. 2. Metabolic activity as measure of cell viability of primary cortical neurons after 48 h exposure to Triphenyl phosphate (TPhP, black bars) or Tri-*n*-butyl phosphate (TBP, gray bars). Data are expressed as mean \pm SD in % of control (control set at 100 %) from $n = 14$ – 24 wells, $N = 2$ – 3 plates. The gray area indicates the biological variation in control wells (± 10 %).

3.2. Engine oil- and hydraulic fluid derived fume extracts

A blank filter extract was used as negative control to ensure that neuronal activity is not affected by residues extracted from the Teflon filters during their processing. Exposure to the filter extract did not reduce neuronal activity (MSR, MBR, and MNBR; Fig. S1A) or metabolic activity (as a measure for cell viability; Fig. S1B), but a modest increase in MBR and MNBR occurred after 48 h which did not reach significance (Fig. S1A). Changes in MSR were comparable to those in MBR or MNBR. Therefore, and to easily compare the potency of the fume extracts, only MSR will be further presented here. Dose-response curves for MBR and MNBR, and the obtained ID₅₀ values can be found in the [supplementary data](#) (Figs. S2–S5 and Table S2).

3.2.1. Engine oil-derived fume extracts

Rat primary cortical cultures were exposed to fume extracts generated from four selected engine oils (Fume A_{350 °C}–D_{350 °C}). All tested engine oil-derived fume extracts persistently reduced neuronal activity with minor difference in their potency (Fig. 3 (MSR), Fig. S2 (MBR) and S3 (MNBR)).

During acute exposure, none of the tested fume extracts generated from the engine oils impaired neuronal activity at levels below 30 μg/mL (0.5 h; Fig. 3). For higher doses (≥ 30 μg/mL), MSR was significantly

reduced, in particular for Fume A_{350 °C} and Fume C_{350 °C}. The neuronal activity remaining after acute exposure to 100 μg/mL fume extract was clearly less for Fume A_{350 °C} and Fume C_{350 °C} (14–30 % of control; Figs. 3A and 3C) than for Fume B_{350 °C} and Fume D_{350 °C} (49–56 % of control; Figs. 3B and 3D). The higher potency of Fume A_{350 °C} and Fume C_{350 °C} is also reflected in the ID₅₀ values for the acute inhibition of MSR (ID₅₀ Fume A_{350 °C}: 39 μg/mL; ID₅₀ Fume B_{350 °C}: 121 μg/mL; ID₅₀ Fume C_{350 °C}: 57 μg/mL; ID₅₀ Fume D_{350 °C}: 84 μg/mL; Table 2 and Table S2).

Interestingly, after prolonged exposure (24 h and 48 h), the degree of inhibition of neuronal activity and the corresponding ID₅₀ values for MSR obtained for all four selected engine oil-derived fume extracts are comparable (ID₅₀ Fume A_{350 °C}: 45–62 μg/mL; ID₅₀ Fume B_{350 °C}: 86–98 μg/mL; ID₅₀ Fume C_{350 °C}: 37–47 μg/mL; ID₅₀ Fume D_{350 °C}: 37–52 μg/mL; Fig. 3, Table 2). This indicates that Fume A_{350 °C} and Fume C_{350 °C} inhibit the neuronal activity acutely and persistently, whereas for Fume B_{350 °C} and Fume D_{350 °C} the degree of inhibition increases over time. After 24 h exposure, the fume extracts derived from the four selected engine oils appear to be equally potent. Importantly, after 48 h exposure, none of the tested engine oil-derived fume extracts (1–100 μg/mL) impaired the metabolic activity of the rat primary cortical cells, indicating that cell viability was not affected by the exposure (Fig. 3E).

3.2.2. Hydraulic fluid-derived fumes

For the selected hydraulic fluids, fume extracts generated at 200 °C (Fume E_{200 °C} and Fume F_{200 °C}) induced a potent and dose-dependent inhibition of neuronal activity of rat primary cortical cultures (Fig. 4, Fig. S4).

Acute exposure to 10 μg/mL Fume E_{200 °C} (Fig. 4A) significantly reduced MSR (26 ± 4 % of control, $p < 0.001$). For Fume F_{200 °C} (Fig. 4B), the lower doses already significantly reduced MSR (75 ± 9 % of control for 1 μg/mL ($p = 0.007$) and 42 ± 8 % of control ($p < 0.001$) for 3 μg/mL). The high acute potency of these two hydraulic fluid-derived fume extracts is also reflected in low ID₅₀ values (ID₅₀ Fume E_{200 °C}: 5.8 μg/mL; ID₅₀ Fume F_{200 °C}: 2.3 μg/mL; Table 2 and Table S2).

Prolonged exposure to both hydraulic fluid-derived fume extracts still yielded a potent reduction of the neuronal activity for doses ≥ 30 μg/mL. However, the reduction induced by ≤ 10 μg/mL Fume E_{200 °C} and Fume F_{200 °C} is clearly attenuated after 24 h and 48 h exposure compared to the effect seen after acute (0.5 h) exposure, resulting in a right shift of the dose-response curves (Fig. 4) and higher ID₅₀ values (ID₅₀ Fume E_{200 °C}: 16–17 μg/mL; ID₅₀ Fume F_{200 °C}: 15–17 μg/mL; Table 2 and Table S2) for prolonged exposure. For doses ≥ 30 μg/mL, the inhibition of neuronal activity persisted. Together, these results indicate that the neuronal activity of the network can (partly) recover after exposure to low and moderate doses of Fume E_{200 °C} and Fume F_{200 °C}. Notably, cells exposed to 100 μg/mL Fume F_{200 °C} exhibit a lower metabolic activity (76 ± 31 % of control, $p < 0.001$; Fig. 4C), whereas metabolic activity of cells exposed to lower doses did not significantly differ from control. For Fume E_{200 °C} (1–100 μg/mL), metabolic activity was not affected after 48 h exposure (Fig. 4C). Except for 100 μg/mL Fume F_{200 °C}, the observed decrease in spontaneous neuronal activity is thus likely related to specific and functional neuronal effects rather than to general cytotoxicity.

3.3. Impact of temperature during fume generation on neurotoxic potency of hydraulic fluids

Hydraulic fluids are used at different locations in the turbine engine than the engine oils, resulting in lower temperature during the fume generation (National Research Council (US) Committee, 2002). In our study, we therefore generated fumes from the hydraulic fluids at 200 °C and for the engine oils at 350 °C. These temperatures during the fume generation via the Mini-BACS simulate a realistic scenario for engine oil or hydraulic fluid leakage into the ventilation system of the airplane

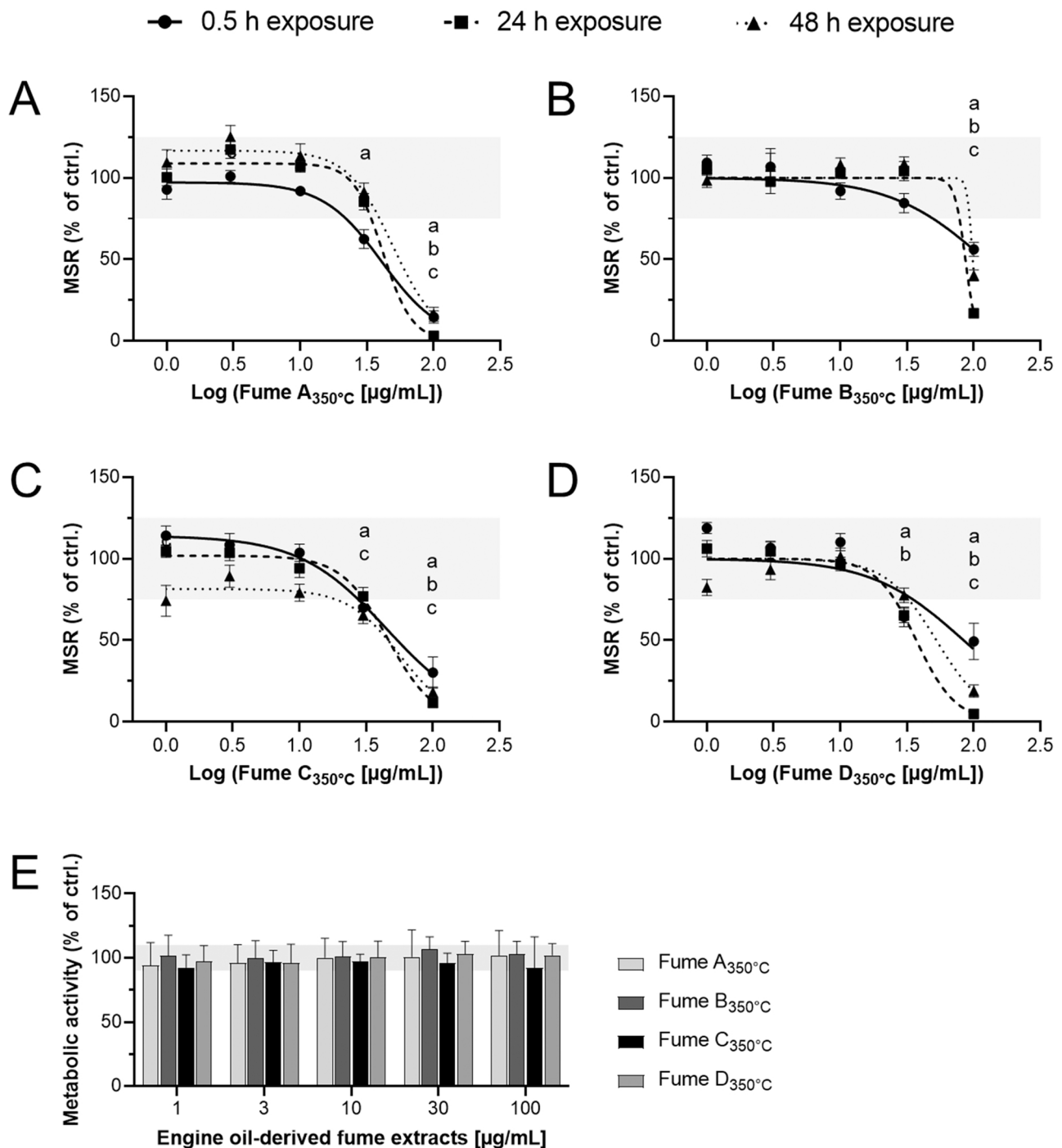


Fig. 3. Neuronal activity (A–D) and metabolic activity (E) of rat primary cortical cells following exposure to fume extracts generated by Mini-BACS from four selected engine oils at 350 °C (Fume A_{350 °C}–D_{350 °C}). A–D) Neuronal activity, presented as mean spike rate (MSR), was recorded after 0.5 h, 24 h, and 48 h exposure. Data are depicted as mean treatment ratio (\pm SEM) in % of control from $n = 14$ –24 wells, $N = 2$ –3 plates. Effects $\leq 25\%$ (the SD of control) are considered to be of limited toxicological relevance and indicated by the gray area. ^a $p < 0.05$ for 0.5 h exposure compared to time-matched control, ^b $p < 0.05$ for 24 h exposure compared to time-matched control, ^c $p < 0.05$ for 48 h exposure compared to time-matched control. E) Metabolic activity was assessed after 48 h exposure and data are expressed as mean (\pm SD) in % of control from $n = 14$ –23 wells, $N = 2$ –3 plates. The gray area indicates the biological variation in control wells ($\pm 10\%$).

(also see He et al., 2021). To enable a direct comparison of the neurotoxic potency of the fumes originating from selected engine oils and hydraulic fluids, we additionally tested hydraulic fluid-derived fumes extracts generated at 350 °C (Fume E_{350 °C} and Fume F_{350 °C}, Fig. 5, Fig. S5). Even though such a high temperature is unrealistic in case of an incident origination from hydraulic fluid leakage, it will show if the higher neurotoxic potency of the hydraulic fluids-derived extracts Fume E_{200 °C} and Fume F_{200 °C} (Fig. 4) compared to engine oil-derived fumes

extracts generated at 350 °C (Fig. 3) is due to the different temperatures during fume generation.

For both hydraulic fluid-derived fume extracts generated at 350 °C, acute exposure to $\geq 10 \mu\text{g/mL}$ significantly decreased neuronal activity with both fume extracts being equally potent (Fig. 5). Interestingly, compared to the fume extracts generated at 200 °C (Fume E_{200 °C} and Fume F_{200 °C}; Fig. 4), acute exposure to Fume E_{350 °C} and Fume F_{350 °C} below 10 $\mu\text{g/mL}$ did not affect neuronal activity (Fig. 5). Also, the ID₅₀

Table 2

ID₅₀ values of neuronal activity (MSR) for fume extracts generated from four selected engine oils at 350 °C and two selected hydraulic fluids at 350 °C or 200 °C. ID₅₀ values and Confident Interval 95 % were determined based on dose-response curves of MSR. Values are listed for 0.5 h (left), 24 h (middle), and 48 h (right) of exposure. N.A.: not available (could not be determined because of limited effects).

	Extract ID	Temp.	ID ₅₀ values (µg/mL) [CI 95 %]		
			0.5 h	24 h	48 h
Engine oils	Fume A	350 °C	39 [33–47]	45 [N.A.–57]	62 [46–?]
	Fume B	350 °C	121 [85–233]	86 [N.A.]	98 [N.A.]
	Fume C	350 °C	57 [41–81]	47 [40–56]	37 [24–56]
	Fume D	350 °C	84 [57–140]	37 [N.A.–43]	52 [43–64]
Hydraulic fluids	Fume E	200 °C	5.8 [5.0–6.7]	17 [14–21]	16 [13–19]
	Fume F	200 °C	2.3 [1.7–2.9]	15 [N.A.–19]	17 [13–23]
	Fume E	350 °C	12 [N.A.–15]	17 [15–21]	15 [13–19]
	Fume F	350 °C	9.1 [8.1–10]	19 [16–23]	18 [16–21]

ID₅₀, inhibitory dose 50 %; CI 95 %, confidence interval 95 %; Temp., temperature during fume generation.

values obtained for Fume E_{350 °C} and Fume F_{350 °C} are generally higher than the values from hydraulic fluid-derived fume extracts generated at 200 °C (Table 2 and Table S2) indicating that when generated at 350 °C, the fumes exhibit lower neurotoxic potency.

Further, the dose-response curves obtained for the 24 h and 48 h exposure showed only little difference compared to those obtained after acute (0.5 h) exposure. Similarly, the difference between the ID₅₀ values obtained for prolonged (24 h and 48 h) exposure and the acute (0.5 h) exposure is only 2-fold (0.5 h ID₅₀ Fume E_{350 °C}: 12 µg/mL vs. 24 h/48 h ID₅₀ Fume E_{350 °C}: 15–17 µg/mL; 0.5 h ID₅₀ Fume F_{350 °C}: 9.1 µg/mL vs. 24 h/48 h ID₅₀ Fume F_{350 °C}: 18–19 µg/mL; Table 2 and Table S2). Interestingly, for prolonged exposures, the dose-response curves and ID₅₀ values of hydraulic fluid-derived fume extracts generated at 350 °C (Fig. 5; Table 2 and Table S2) are comparable to those obtained for the fume extracts generated from the same hydraulic fluid at 200 °C (Fig. 4; Table 1). Notably, both hydraulic fluid-derived fume extracts generated at 350 °C did not reduce the metabolic activity of the cells (1–100 µg/mL; Fig. 5C).

Taken together, these findings indicate that the temperature during fume generation is relevant for the neurotoxic potency of the fume extracts, in particular for the acute effects.

3.4. Evaluation of the different neurotoxic potency of fumes originating from selected engine oils and hydraulic fluids

For a full overview of effects on neuronal function, treatment ratios for several neuronal activity parameters obtained from the MEA recordings following exposure to engine oil- and hydraulic fluid-derived fume extracts (generated at 350 °C and 200 °C) for 0.5 h (Fig. 6) and 24 h (Fig. 7) are summarized in heatmaps. Since the effects observed after 48 h were comparable to those after 24 h exposure, the heatmap for the 48 h exposure can be found in the supplementary data (Fig. S6). An explanation of the presented MEA parameters is given in the supplementary (Table S3).

Figs. 6 and 7 illustrate that, at both fume generation temperatures, the fume extracts originating from hydraulic fluids (Fume E and Fume F) induce considerable stronger changes in the neuronal activity pattern than the fume extracts generated from engine oils, in particular following 0.5 h exposure. Additionally, comparison of the heatmaps at

0.5 h (Fig. 6) and 24 h (Fig. 7) indicates that for engine oil-derived fume extracts the effect tends to increase over time, whereas for hydraulic fluid-derived fume extracts the effect rather attenuates over time.

4. Discussion

Engine oils and hydraulic fluids are mixtures containing a large number of potentially toxic chemicals (Bolton, 2009; Michaelis, 2016, 2011; for review see Schuchardt et al., 2019 and Hageman et al., 2022). The resulting fume contamination is therefore diverse but due to decomposition and/or pyrolysis, the fume composition is hard to predict and may exhibit different or even higher neurotoxic potency than the original engine oil or hydraulic fluid (Centers, 1992; van Netten and Leung, 2001; Wright, 2008). Studying the effect of fume extracts is therefore an effective way to assess the neurotoxicity of cabin air contamination.

As the hypothesis was that Triphenyl phosphate (TPhP) and Tri-*n*-butyl phosphate (TBP) may contribute to neurological effects associated with the aerotoxic toxic syndrome, which was introduced by Winder and Balouet (2000), we initially characterized the neurotoxic hazard of TPhP and TBP *in vitro* using MEA recordings. Our data indicate that during acute exposure (0.5 h), TPhP and TBP inhibit neuronal activity roughly equipotent with comparable concentration-effect relationships and IC₅₀ values (see Fig. 1 and Table 1). Neuronal activity partly recovers following prolonged exposure to TBP (24 h and 48 h), whereas only limited recovery was seen for TPhP. For TPhP and TBP, metabolic activity was unaffected after 48 h exposure (Fig. 2), indicating that the observed neurotoxicity is not due to general cytotoxic effects. The acute IC₅₀ value obtained for TPhP from our study (10–12 µM) is in line with earlier studies that reported inhibition of neuronal network activity with IC₅₀ values of 16.3 µM following acute (1 h) exposure of rat primary cortical cells (Behl et al., 2015). Similarly, the acute IC₅₀ value obtained for TBP from our study (15–18 µM) is comparable with previously reported IC₅₀ values for acute exposure to TBP that range from 4.3 to 18.7 µM (Kosnik et al., 2020; CompTox Chemicals Dashboard).

Previously, the neurotoxicity of several TCP isomers (ToCP, TpCP, and TmCP) and mixtures, which were seen as the major harmful contaminants in bleed air, were studied using the same approach (Duarte et al., 2017). Interestingly, 24 h and 48 h exposure to all TCP isomers and all TCP mixtures induced a profound increase in mitochondrial activity for 10 µM, which was not observed here for TPhP and TBP. The inhibition of spontaneous neuronal activity caused by 10 µM TPhP and TBP are comparable to the effects reported by Duarte et al. (2017) for the individual TCP isomers and TCP mixtures. Interestingly, effects of TPhP and TBP already occur during acute exposure and attenuate over time, whereas in general, TCP isomers and several TCP mixtures mainly reduced neuronal activity after 48 h exposure to 10 µM (Duarte et al., 2017). Therefore, even though the effect and the potency of TPhP and TBP are comparable to those of TCP isomers and TCP mixtures, our data indicate that the characteristics of the effect, in particular the onset and persistence of the inhibition, do differ between several OPs.

Our data also indicate that hydraulic fluid-derived fume extracts (Fume E_{200 °C} and F_{200 °C}) acutely reduce neuronal activity, which partly recovers within 24 h (Fig. 4, Fig. 6, Fig. 7, Fig. S4, Table 2 and Table S2). A comparable partial recovery is observed following prolonged exposure to TBP, and to a less extent for TPhP (Fig. 1), which makes it tempting to speculate that OPs, like TBP and TPhP, could be responsible for the inhibition of neuronal activity induced by the hydraulic fluid-derived fume extracts. The persistent (Fume A and C) or even exacerbating (Fume B and D) inhibitory effects of the selected engine oil-derived fume extracts (Fig. 3, Fig. 6, Fig. 7, Fig. S2–S3, Fig. S6, Table 2 and Table S2) are thus less likely explained by the presence of OPs and are possibly due to other contaminants in the fume extracts such as aldehydes, ketones or organic acids, which are more abundant in engine oil-derived fumes (Table S1).

The attenuation of the inhibitory effect of TBP and TPhP may be due

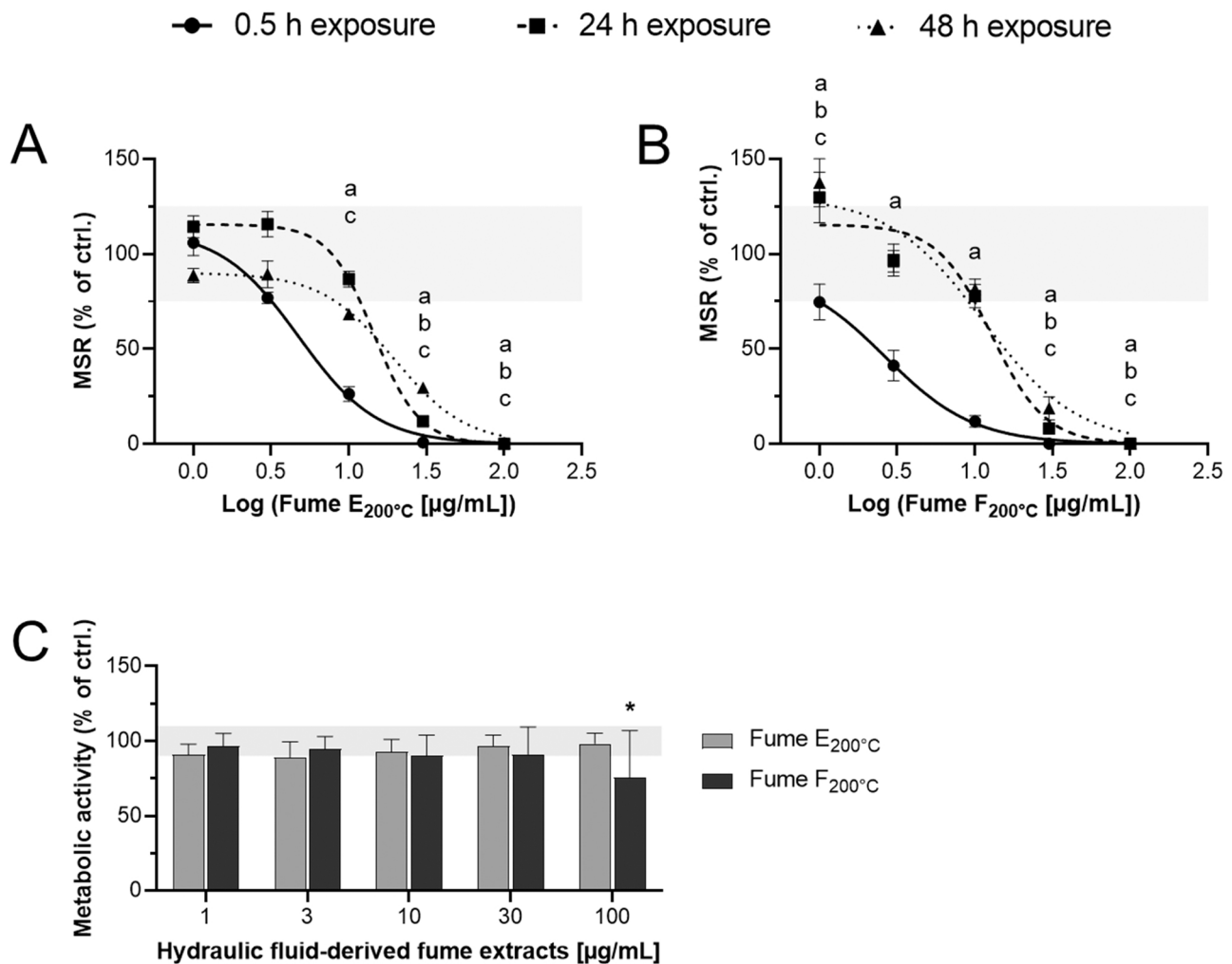


Fig. 4. Neuronal activity and metabolic activity of rat primary cortical cells following exposure to fume extracts generated by Mini-BACS from two selected hydraulic fluids at 200 °C (Fume E_{200°C} and Fume F_{200°C}). A, B) Neuronal activity, presented as mean spike rate (MSR), was recorded after 0.5 h, 24 h, and 48 h exposure. Data are depicted as mean treatment ratio (± SEM) in % of control from n = 14–16 wells, N = 2 plates. Effects ≤ 25 % (the SD of control) are considered to be of limited toxicological relevance and indicated by the gray area. ^a p < 0.05 for 0.5 h exposure compared to time-matched control, ^b p < 0.05 for 24 h exposure compared to time-matched control, ^c p < 0.05 for 48 h exposure compared to time-matched control. C) Metabolic activity was assessed after 48 h exposure and data are expressed as mean (± SD) in % of control from n = 15–16 wells, N = 2 plates. The gray area indicates the biological variation in control wells (± 10 %).

to sorption of (hydrophobic) chemicals to the cell culture plastics, resulting in decreased bioavailability of the active chemical(s) over time and hence an increase in IC₅₀ and ID₅₀ values. The partitioning of the test chemical from the medium into the cell culture plastics would be time-dependent and largely determined by the hydrophobicity of the test chemical(s) and characteristics of the cell culture plastics (Fischer et al., 2017; Gellert and Stommel, 1999).

Using the prediction model developed specifically for polystyrene by Fischer et al. (2018) and the *n*-Octanol/Water Partition Coefficient (log K_{ow}) partitioning of TPhP and TBP could be estimated. For acute exposure (0.5 h), partitioning of TPhP and TBP to the culture plastics is estimated to be 2.4–5.1 % and is thus negligible. After 24 h and 48 h, estimations revealed that a large fraction of TPhP (67 % and 75 %) and TBP (45 % and 55 %) may have partitioned into the polystyrene walls of the wells. It is therefore likely that the attenuation of the inhibitory effect after prolonged exposure is at least partly due to partitioning into cell culture plastics. Similarly, partitioning of (hydrophobic) chemicals to cell culture plastics may also explain the attenuation of the inhibitory effect over time for hydraulic fluid-derived fumes.

It should be noted though that for TPhP and TBP the estimated reduction in bioavailability is far larger than the attenuation of the inhibitory effect over time. Moreover, the inhibitory effects of engine

oil-derived fumes actually increase over time (Fig. 3), as previously also reported for TCP isomers and TCP (Duarte et al., 2017). It can thus not be excluded that other factors contribute to the time-dependent change in the degree of effect. These factors may be subject of future research and can be related to possible bioactivation and/or detoxication due to metabolism, or to desensitization and/or sensitization of various neurotransmitter receptors and ion channels.

Overall, our data shows that fume extracts originating from selected hydraulic fluids are more potent than the selected engine oil-derived fume extracts, particularly during acute exposure (Table 2, Table S2), indicating that these hydraulic fluid-derived contaminations exhibit higher hazard in particular during acute exposure events. The higher toxic potency of selected hydraulic fluid-derived fumes compared to selected engine oil-derived fumes was also reported recently by He et al. (2021), where the same samples (Fume A_{350°C} – D_{350°C}, Fume E_{200°C} and F_{200°C}) were tested using several *in vitro* lung models for lactate dehydrogenase (LDH) and cytokine release, which are markers for respectively cytotoxicity and inflammation.

Since hydraulic fluids are used at a different location in the turbine engine than the engine oils, they are exposed to lower temperatures in case of leakage in the ECS, which can affect their decomposition and/or pyrolysis potentially leading to different neurotoxicity and potency

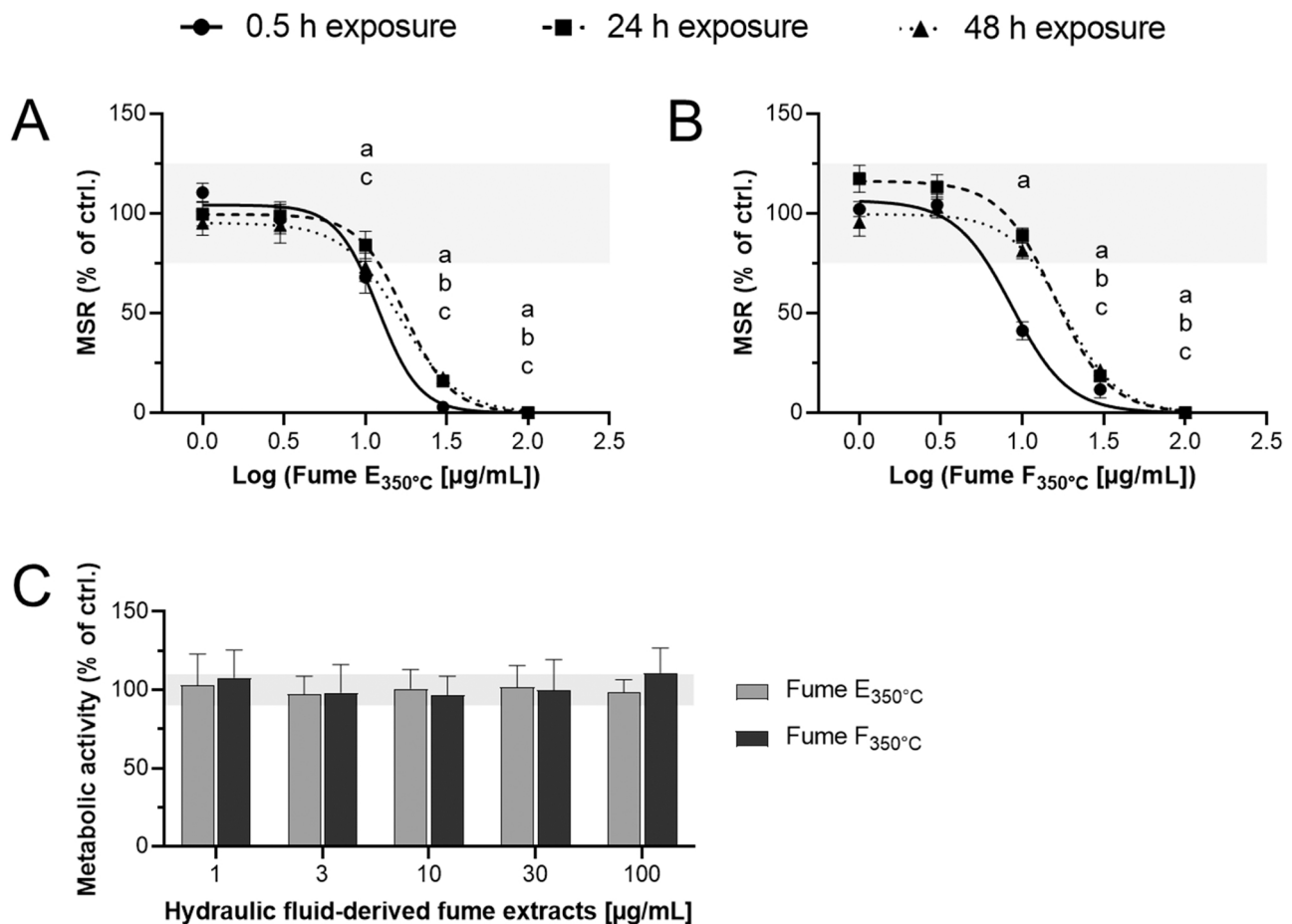


Fig. 5. Neuronal activity and metabolic activity of rat primary cortical cells following exposure to fume extracts generated by Mini-BACS from two selected hydraulic fluids at 350 °C (Fume E_{350 °C} and F_{350 °C}). A, B) Neuronal activity, presented as mean spike rate (MSR), was recorded after 0.5 h, 24 h, and 48 h exposure. Data are depicted as mean treatment ratio (\pm SEM) in % of control from $n = 14$ – 16 wells, $N = 2$ plates. Effects ≤ 25 % (the SD of control) are considered to be of limited toxicological relevance and indicated by the gray area. ^a $p < 0.05$ for 0.5 h exposure compared to time-matched control, ^b $p < 0.05$ for 24 h exposure compared to time-matched control, ^c $p < 0.05$ for 48 h exposure compared to time-matched control. C) Metabolic activity was assessed after 48 h exposure and data are expressed as mean (\pm SD) in % of control from $n = 14$ – 16 wells, $N = 2$ plates. The gray area indicates the biological variation in control wells (± 10 %).

(National Research Council (US) Committee, 2002). To exclude that the temperature difference during fume generation causes the higher and acute neurotoxicity, we tested hydraulic fluid-derived fume extracts generated at 350 °C (Fume E_{350 °C} and Fume F_{350 °C}). Our data demonstrate that while at higher temperature (350 °C) the neurotoxic potency of the hydraulic fluid-derived fume extracts decreases, Fume E_{350 °C} and Fume F_{350 °C} are more potent compared to selected engine oil-derived fume extracts (Table 2, Table S2, Fig. 5, Fig. 6 and Fig. 7). Interestingly, the hydraulic fluid-derived fume extracts generated at 200 °C are more potent than those generated at 350 °C during acute exposure, but the inhibitory potency is comparable after 24–48 h, suggesting that lower temperature during fume generation mainly increase acute neurotoxicity.

Based on tentative estimations, incidents with bleed air contamination only occur during a few hundred takeoffs out of each million. However, the frequency is likely higher due to underreporting (Shehadi et al., 2015). Incidents with significant contamination deriving from engine-oil and hydraulic fluid leakage in ECS usually have a short duration (Shehadi et al., 2015). Only small quantities of oils need to be combusted or pyrolyzed to exceed commonly accepted health standards for indoor environment (National Research Council (US) Committee, 2002). In our study, from 1 mL engine oil only 8.5 mg fume extract (Fume A_{350 °C} – D_{350 °C}) was generated on average, whereas the same volume of hydraulic fluid yielded on average 14.4 mg (Fume E_{200 °C} and F_{200 °C}; see Table S5). However, the neurotoxic potency was assessed by

exposing the rat primary cortical cells to mass concentration (µg/mL) and the different mass collected on the filters by using a fixed volume was not included in the comparison of the fume potency. If considering the volume in the assessment, the hydraulic fluids are even more potent than reported above. Interestingly, when generated at higher temperature (Fume E_{350 °C} – F_{350 °C}) collected mass was significantly lower suggesting that at higher temperature hydraulic fluid constituents are decomposed/pyrolyzed more effectively.

In our study all tested fume extracts originating from selected engine oils or hydraulic fluids captured on filters potentially inhibit neuronal activity *in vitro*. However, our *in vitro* findings cannot directly be translated to the symptoms reported for aerotoxic syndrome. Firstly, our prolonged *in vitro* exposures are not representative for the repeated and chronic exposure to low levels as experienced by air crew members. Notably, our data show that the reduction in neuronal function for some of the exposures (partly) recovered within 24 h and it would thus be of interest to characterize the toxic effect following repeated exposures. Secondly, the used exposure doses in our *in vitro* study are very high compared to levels expected in an *in vivo* brain following (acute) inhalation. However, our study was primarily aimed to characterize and rank the neurotoxic hazard and potency of potential bleed air contaminants, including TPhP and TBP, and realistic fumes deriving from engine oil or hydraulic fluid leakage. Our data demonstrate that fume extracts originating from selected hydraulic fluids are more potent, in particular during acute exposure, than fumes from selected engine oils. The

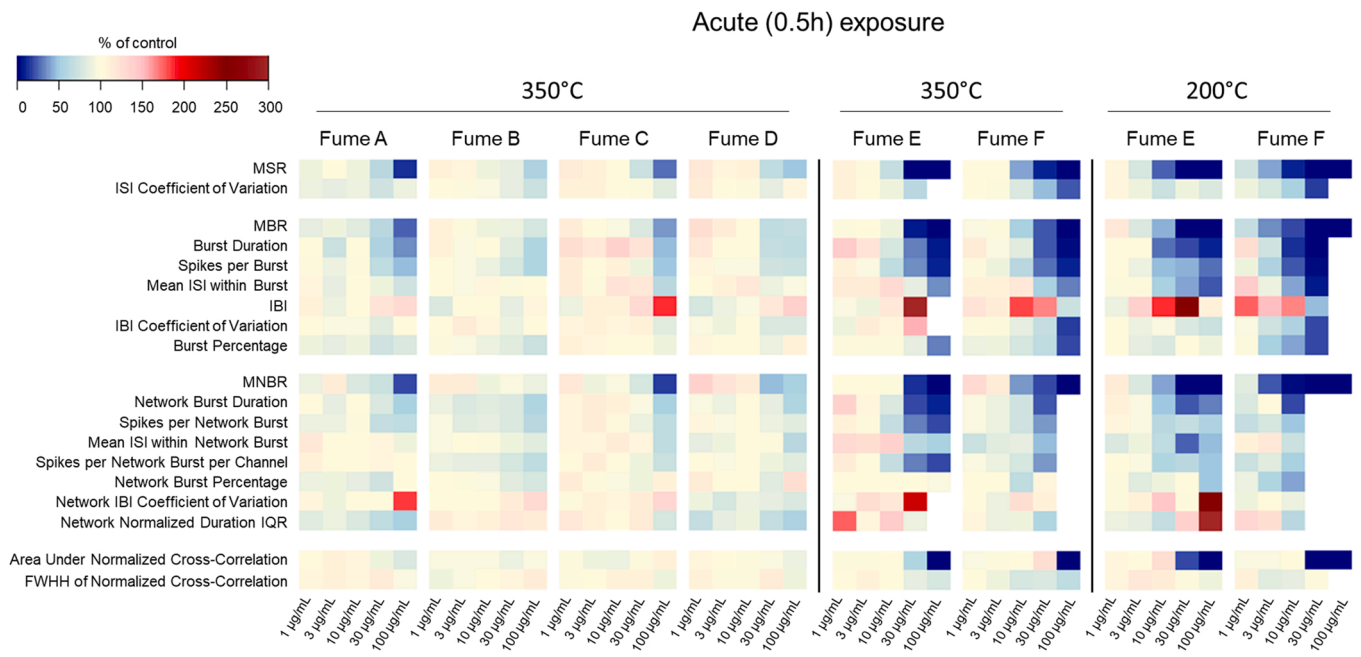


Fig. 6. Heatmaps of the effects of selected engine oil- and hydraulic fluid-derived fume extracts generated by Mini-BACS on selected metric parameters of neuronal function in primary cortical neurons after 0.5 h exposure. As indicated, engine oil-derived fume extracts were generated at 350 °C (Fume A_{350 °C}-D_{350 °C}), whereas for hydraulic fluid-derived fume extracts fumes were generated at 350 °C (Fume E_{350 °C} and Fume F_{350 °C}) or 200 °C (Fume E_{200 °C} and Fume F_{200 °C}). Color scaling is based on the magnitude of the % of change relative to the control based on n = 6–16 wells for Fume A, n = 12–16 wells for Fume B, n = 7–16 wells for Fume C, n = 8–23 wells for Fume D, n = 13–16 wells for Fume E_{200 °C}, n = 8–16 wells for Fume F_{200 °C}, 14–16 wells for Fume E_{350 °C}, and n = 13–16 wells for Fume F_{350 °C}. N = 2–3 independent cultures. If no color is assigned (white box), no average could be calculated as a result of (near) complete cessation of neuronal activity.

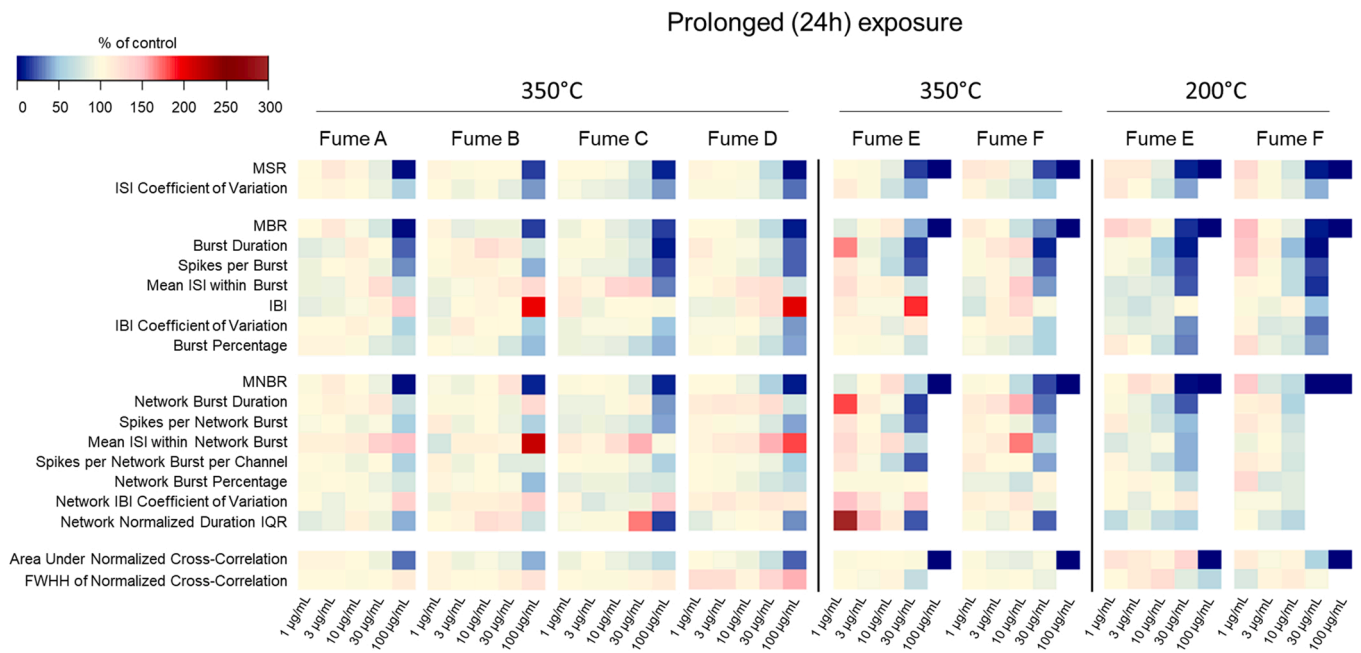


Fig. 7. Heatmaps of the effects of selected engine oil- and hydraulic fluid-derived fume extracts generated by Mini-BACS on selected metric parameters of neuronal function in primary cortical neurons after 24 h exposure. As indicated, engine oil-derived fume extracts were generated at 350 °C (Fume A_{350 °C}-D_{350 °C}), whereas for hydraulic fluid-derived fume extracts fumes were generated at 350 °C (Fume E_{350 °C} and Fume F_{350 °C}) or 200 °C (Fume E_{200 °C} and Fume F_{200 °C}). Color scaling is based on the magnitude of the % of change relative to the control based on n = 6–16 wells for Fume A, n = 12–16 wells for Fume B, n = 7–16 wells for Fume C, n = 8–23 wells for Fume D, n = 13–16 wells for Fume E_{200 °C}, n = 8–16 wells for Fume F_{200 °C}, 14–16 wells for Fume E_{350 °C}, and n = 13–16 wells for Fume F_{350 °C}. N = 2–3 independent cultures. If no color is assigned (white box), no average could be calculated as a result of (near) complete cessation of neuronal activity.

chemical analysis of the engine oil- and hydraulic fluid-derived fumes generated using the Mini-BACS have been reported previously by He et al. (2021) (also see Table S4). According to this chemical characterization, hydraulic fluid-derived fumes contain ~10-fold higher

concentration of TPHP than engine oil-derived fumes. As discussed previously (He et al., 2022), TPHP was not expected in fumes generated from engine oils containing TCP (Fume B_{350 °C} – D_{350 °C}) as it is supposedly only added to TCP-free engine oils. No plausible explanation

could be given and it was suggested that the very low TPhP levels found in Fume B-D may trace back to impurities from engine oil additives (He et al., 2022). The TBP concentrations detected in the analyzed engine oil- and hydraulic fluid-derived fumes were rather comparable (Table S4). Since TBP is supposedly not applied in engine oils, it was suggested by He et al. (2022) that TBP in engine oil-derived fumes may originate from residues bound to the simulation system during previous runs. However, it may be unlikely that the high levels of TBP, particularly in Fume B_{350 °C} and Fume C_{350 °C}, are solely derived from cross contamination. Nevertheless, it is unlikely that the low level of cross contamination affected the neurotoxicity of engine oil-derived fumes. More relevant, if the cross contamination would have affected the observed neurotoxicity, the difference in the neurotoxic potency between the fumes generated from the selected engine oils and hydraulic fluids would be even more pronounced, with the fumes derived from the selected hydraulic fluids being more potent than those from selected engine oils. Further, during fume generation parameters for atmosphere monitoring were assessed including particle number count. To ensure that the cleaning procedure of the Mini-BACCS was efficient, the fume generation procedure was also performed without oil injection. The particle count numbers from runs where oil was injected into the system is ca. 2300-fold higher compared to this “clean” run (data not shown) supporting that cross-contamination of the different fumes is minimal.

Regardless of these caveats, our *in vitro* neurotoxicity data suggests that TPhP could be a candidate for reduction measures or replacement with safer alternatives in products, particularly for the selected hydraulic fluids, to reduce potential (occupational) health risks during fume events and chronic or repeated low level exposure. However, it should be noted that our suggestions and conclusions are based on fumes generated from only four selected engine oils and two selected hydraulic fluids. Further research is thus required to draw generalized conclusions regarding the neurotoxicity of engine oils, hydraulic fluids and their fumes. TBP concentrations in the selected hydraulic fluids and resulting fumes are considerably higher than TPhP concentrations, making TBP also a candidate for mitigation measures. However, when considering the comparable acute neurotoxic potency of TBP and TPhP (Fig. 1, Table S2), it can certainly also not be ruled out that the strong acute reduction of neuronal activity by the hydraulic fluid-derived fumes results from a currently unidentified factor, such as Phenol, isopropylated, phosphate (3:1; PIP 3:1). According to the ToxCast Computational Toxicology Chemicals dashboard data base, PIP 3:1 inhibits spontaneous neuronal activity of rat primary cortical cultures during acute exposure, with IC₅₀ values of 3.6 μM (CompTox Chemicals Dashboard). This value is 3–4 fold lower than the IC₅₀ values obtained for TPhP and TBP in our study but in a comparable range as values reported previously (TPhP IC₅₀: 16.3 μM, TBP IC₅₀: 4.3 – 18.7 μM; Behl et al., 2015; Kosnik et al., 2020; CompTox Chemicals Dashboard), suggesting that PIP (3:1) has a comparable acute neurotoxic potency. However, data on prolonged exposure effects for PIP (3:1) are missing and it remains unclear whether PIP (3:1)-induced inhibition of neuronal activity attenuates over time and could contribute to effects observed for hydraulic fluid-derived fume extracts. The generated fumes contained besides OPs also volatile organic compounds (VOCs), aldehydes, ketones and organic acids, which were particularly abundant in fumes derived from engine oils (for details see Table S1) and may have contributed to the observed neurotoxicity.

5. Conclusion

Our data show that TBP and TPhP reduce neuronal activity *in vitro* with equal potency that is comparable to that of TCP. Furthermore, bleed air contaminants originating from a number of selected engine oils or hydraulic fluids exhibit neurotoxic hazard *in vitro*, and there are clear differences in the effect and potency among these. While fume extracts originating from selected hydraulic oils are clearly more potent than those from selected engine oils, in particular during acute exposure, the

tested engine oil-derived fume extracts reduce neuronal activity persistently. This difference is possibly, but not exclusively, related to 10-fold higher levels of TPhP in fumes derived from hydraulic fluids.

CRedit authorship contribution statement

Lora-Sophie Gerber: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, **Regina G. D.M. van Kleef:** Investigation, Formal analysis, **Paul Fokkens:** Investigation, Formal analysis, **Flemming R. Cassee:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition. **Remco H.S. Westerink:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that there are no known competing financial interests or personal relationships that could have appeared influence the editorial process. Given his role as Editor in Chief of Neurotoxicology, Remco H.S. Westerink had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Dr. Pamela J. Lein.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.neuro.2023.04.010.

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