

Exposure to triaryl phosphates: metabolism and biomarkers of exposure

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The leakage of tricresyl phosphate-containing engine lubricants into aircraft cabin air, either from worn or defective engine seals or under normal operating conditions, is a serious concern for both the health and safety of the cabin occupants, since the oil contains one to five percent tricresyl phosphate (TCP) esters, known neurotoxins. The exposure of pilots is a particular concern since their impairment can affect their safe operation of the aircraft. Mass spectrometric (MS)-based protocols for documenting exposures of individuals are described that entail a rapid purification of the TCP-modified plasma enzyme butyrylcholinesterase (BChE). Following protease digestion of BChE, the modified active site peptide is characterized by MS analysis. Approaches for identifying safer engine oil additives are also described. Some general comments regarding the necessity of improving the quality and safety of the cabin air supply are presented.

Keywords: butyrylcholinesterase, cabin air safety, fume event, mass spectrometry, tricresyl phosphates

1. INTRODUCTION

The *Inhalable Toxic Chemicals in Aircraft Cabin Air (ITCOBA)* workshop, held on 11 October 2011 at Cranfield University, was organized to address and complement the conclusions of the March 2011 Institute of Environment and Health (IEH) report entitled *Aircraft Cabin Air Sampling Study; Parts 1 and 2* (Crump et al., 2011a, b), which dealt with the issues of whether aircraft cabin air is contaminated with organophosphate neurotoxins and whether exposures to fume events cause ill-health. The workshop also provided the opportunity to elaborate areas where new or additional research is needed. This contribution discusses these issues and describes some of our research on characterizing proteins that are modified as a result of exposure to tricresyl phosphate (TCP) isomers and metabolites generated from the TCP isomers. We also describe approaches for identifying safer antiwear additives for engine lubricants. Some comments on the general issue of toxic chemicals in aircraft cabin air are also included.

The problem of fume events in aircraft cabins was addressed at a British Airlines Pilots' Association (BALPA)-sponsored conference held at King's College, London in 2005 (BALPA, 2005). The problems with contaminated cabin air were recently explored in detail in a PhD thesis from the University of New South Wales (Michaelis, 2010) which reviewed many of the earlier publications cited in the IEH Report and in the Committee on Toxicity 2007 report (COT, 2007).

The air conditioning of modern jet aircraft relies on a stream of air bled off the partially compressed air from the aircraft jet engines, so-called bleed air. The air enters the cabin unfiltered, so that if the engine seals are worn or fail outright, or if the engines are improperly serviced (e.g., overfilled with oil) (Michaelis, 2005), oil fumes or smoke/mist may enter the cabin (Fig. 1). The design of the engine seals also appears to allow some oil fumes to enter the cabin air under normal operating conditions (Michaelis, 2010; Crump et al., 2011a, b; Liyasova et al., 2011). The oil contains a mixture of tricresyl phosphate (TCP) esters as antiwear additives. Since some of the mixed esters contain phenol esters or other ring substitutions, the generic term triaryl phosphate (TAP) is also used here.

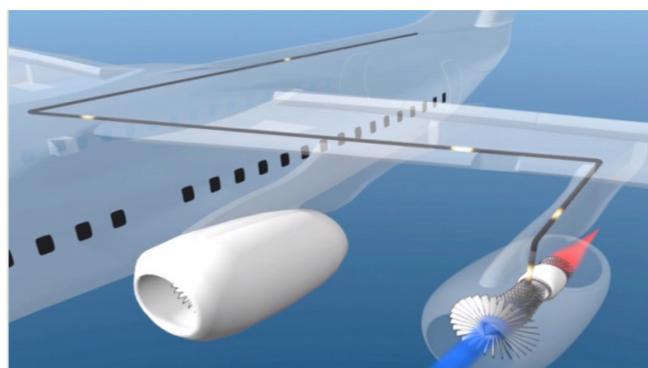


Figure 1. Outline of bleed air system on a jet aircraft. (courtesy of Capt. Tristan Loraine).

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2. TOXICITY OF TRICRESYL PHOSPHATE OIL ADDITIVES

It has been well known since 1930 that TCP is a neurotoxin when the tri-*ortho* isomer (ToCP) was used to adulterate ethanol extracts of ginger and paralysed 10000 to 50000 individuals (Smith and Lillie, 1931; Parascandola, 1995). The metabolism and toxicity of TCP are discussed below. Thus, a major concern about exposure to engine oil fumes is that the oil contains 1–3% mixed tricresyl phosphate (TCP) esters. Henschler demonstrated in 1958 that the mono-*ortho* isomers of TCP (Fig. 2) were 10 times more toxic than the tri-*ortho* isomer and the di-*ortho* isomers 5 times as toxic (Henschler, 1958). In 1954, Aldridge demonstrated that metabolism by liver was required for conversion of the pretoxin ToCP to a potent esterase inhibitor (Aldridge, 1954). The cytochromes P450 had not yet been characterized at that time. In 1961, John Casida and colleagues, then at the University of Wisconsin, published the structure of the active metabolite of ToCP [2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one] (CBDP) (Casida et al., 1961). Thus, by 1961 the toxicity of ToCP had been well established and the structure of its toxic metabolite determined.

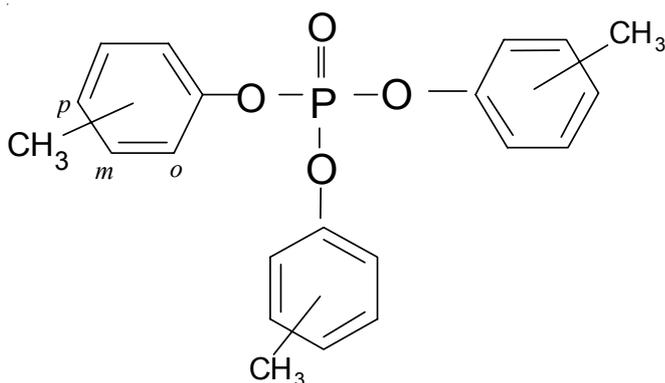


Figure 2. Structure of a triaryl phosphate ester noting the positions of *ortho* (*o*), *meta* (*m*) and *para* (*p*) positions for R group substitutions. The R groups are generally methyl, isopropyl or tertbutyl.

3. FREQUENCY OF FUME EVENTS

Estimates of the frequency of these events vary, and are not reliable due to the lack of oversight of regulatory reporting systems and the prevalence of airline underreporting. As examples, though, the UK Committee on Toxicity reported that events occur in 1% of flights, based on pilot reports at three UK airlines and 0.05% based on verification of smoke/fume events by engineering (COT 2007). In the US, documentation to support a daily average of 0.86 oil fume events has been compiled (Murawski and Supplee, 2008). In 2010, the US Federal Aviation Administration acknowledged receipt of more than 900 airline-reported smoke/fume events of all

kinds, including but not limited to oil and hydraulic fluid in the air supply system (FAA, 2010). To date, there are few measures of triaryl phosphate levels that occur during fume events, and no data on the variability of sensitivity to these exposures among humans. It was disturbing to note that (albeit low-level) airborne exposures to TCP were measured during 23 of 100 routine flights during which no fume events were formally reported (Crump et al., 2011a, b). However, perhaps this was not unexpected, as butyrylcholinesterase (BChE) with phosphate adducted to the active site serine has been observed in individuals following flights during which there were no obvious fume events (Liyasova et al., 2011). Interestingly, unlike the inhibition of serine active site enzymes by organophosphorus insecticides, where a diethyl or dimethyl phosphate initially binds to the active site serine, then ages to a monoethyl or monomethyl phosphate, inactivation of serine active site enzymes by CBDP results initially in the formation of an aged monocresyl phosphate that further ages to a phosphoserine adduct (Schopfer et al., 2010; Marsillach et al., 2011).

In addition to official airline reports of fume events submitted to aviation regulators, media reports on fume events date back to at least the early 1990s. Also, passengers have recently recorded videos of some of these events and posted them online using popular social networking and video-sharing internet sites. Those dramatic passenger-recorded events provide an important and disturbing view of what happens during an actual fume event and at the same time provide graphic evidence of serious fume events.

4. CONSEQUENCES OF FUME EXPOSURES

Both flight safety and crew health impact of exposure to engine oil fumes during commercial airline flights have been documented globally (reviewed in Michaelis, 2010). The impact on passenger health is largely unknown because airlines need not inform passengers of exposures, even when crew members are either impaired or incapacitated. As an example, on 16 January 2010 a flight operated by a US airline between the Caribbean and an east-coast hub city was met by multiple emergency paramedical vehicles. Upon arrival, the entire crew and some passengers were immediately taken to hospital. Subsequently, both pilots lost their FAA medical licences to fly because of ongoing neurological problems and four of the five flight attendants had not returned to service 16 months post exposure (Murawski, 2011). Mechanical reports confirmed that the air supply system had been contaminated with aviation engine oil and the airline did report the event to the federal regulator.

In addition to confirmed fume events, though, is the question of any health impact of exposure to undocumented low levels of engine oil fumes. The government (UK Department for Transport, DfT)-commissioned IEH (Cranfield University) study conducted short-term airborne monitoring of some airborne contaminants during 100 flights and concluded that fume events do not represent a threat to human health (Crump et al., 2011a, b). The crew members on board during 23 of the 100 flights were exposed to confirmed low levels of TCPs, but the health impact of chronic exposure to low-levels of TCPs has not been investigated and no actual fume events were identified and reported during the study.

5. DOCUMENTING TCP EXPOSURES

During the BALPA-sponsored London conference in 2005, it became clear that crew and passengers faced many challenges in documenting exposures to engine oil fumes because of the absence of exposure monitoring on aircraft and any blood or urine tests specific to either engine oil or its characteristic constituents. The first step in this process was to develop protocols for documenting exposure to TCPs as a marker of exposure to engine oil fumes. The two points covered in this review will be on the progress made in characterizing biomarkers of exposure to TCP esters, and a second important issue related to fume event exposures, namely, developing a safer TAP engine oil additive.

To examine the feasibility of characterizing TCP exposures via mass spectrometric (MS) analysis, we exposed purified porcine carboxylesterase (P-CES) to TCP. Unlike what happens with human esterases, TCP bound to the active site serine of P-CES, generating the aged monocresyl phosphoserine adduct that inactivated the P-CES directly without a bioactivation step (Kim et al., 2010). Interestingly, serines other than the active site serine were also adducted *in vitro* by TCP with some adducts aging to monocresyl phosphoserines and some not, remaining as dicresyl phosphoserine adducts (Kim et al., 2010). Aldridge had shown in 1954 that bioactivation of tri-*ortho*-cresyl phosphate was required to generate inhibitor(s) of serine active site enzymes. We have replicated Aldridge's observation showing that TCP does not inhibit plasma butyrylcholinesterase (BChE), red cell cholinesterase (AChE), monocyte carboxylesterase (CES 1) and red cell acylpeptide hydrolase (APH) *in vitro*. These human enzymes were only inhibited by the chemically synthesized cyclic metabolite CBDP or the phenyl analogue, phenyl saligenin cyclic phosphate (PSP). Inhibition of APH by PSP resulted in a 156 kDa adduct to the active site serine (Kim et al., 2010).

To develop an MS protocol for identifying adducted active site serines, CBDP was used to inactivate purified human BChE, which was then digested with chymotrypsin and analysed by MS to characterize the modified peptide that contained the active site serine. The initial adduct added a mass of 170 Da to the active site serine, which ages to a phosphate adduct with a mass of 80 Da (Schopfer et al., 2010; Marsillach, 2011; Liyasova et al., 2011).

A major concern about exposure to engine oil fumes is that the oil contains 1–5% mixed tricresyl phosphate (TCP) esters. The history of understanding the toxicity of TCP isomers is discussed above. Research in our laboratory and that of Dr Oksana Lockridge at the University of Nebraska has focused on identifying and characterizing proteins that are modified by exposure to triaryl phosphate esters found in engine antiwear lubricant additives. Figure 2 shows the general structure of a triaryl phosphate ester. Three aryl alcohols are esterified to a phosphate. The core benzene rings may be unsubstituted, or substituted with aliphatic side chains (R groups) such as methyl, isopropyl or tertbutyl groups at different ring positions. Figure 2 shows the methyl groups associated with the cresyl esters. The R groups can be attached to the different positions on the ring. The *ortho* (*o*) position is next to the bond to the phosphorus atom, the *meta* (*m*) position is two carbons removed from the bond to the phosphorus and the *para* (*p*) position is opposite the bond to the phosphorus atom. A commercial antiwear additive Durad 125 (D125) is a mixture of isomers substituted at different ring positions. Some of the additives of other antiwear formulations are derivatized with isopropyl groups attached to the rings. Figure 3 shows one pathway of exposure and metabolism of TAPs.

We have made use of purified human butyrylcholinesterase as a “molecular canary” for detecting the conversion of various triaryl phosphates into potent esterase inhibitors. Incubation of triaryl phosphates with rat or human liver microsomes and the cytochrome P450 cofactor NADPH results in a rapid conversion of *ortho* isomer-containing tricresyl phosphates (including tri-*o*-cresyl phosphate) into potent inhibitors of BChE as measured with an *in vitro* “molecular canary” assay. In the absence of the cofactor NADPH, there is no conversion into BChE inhibitor(s), providing strong evidence for the involvement of the cytochromes P450 in the bioactivation process. There are approximately 57 of these enzymes in the human body (Nelson, 2009) and we do not yet know which of them participate in the bioactivation of TAPs. This is an important question that still needs to be answered. One reason for identifying the specific P450 enzymes involved in the bioactivation of TAPs is that there are well-known inhibitors of specific

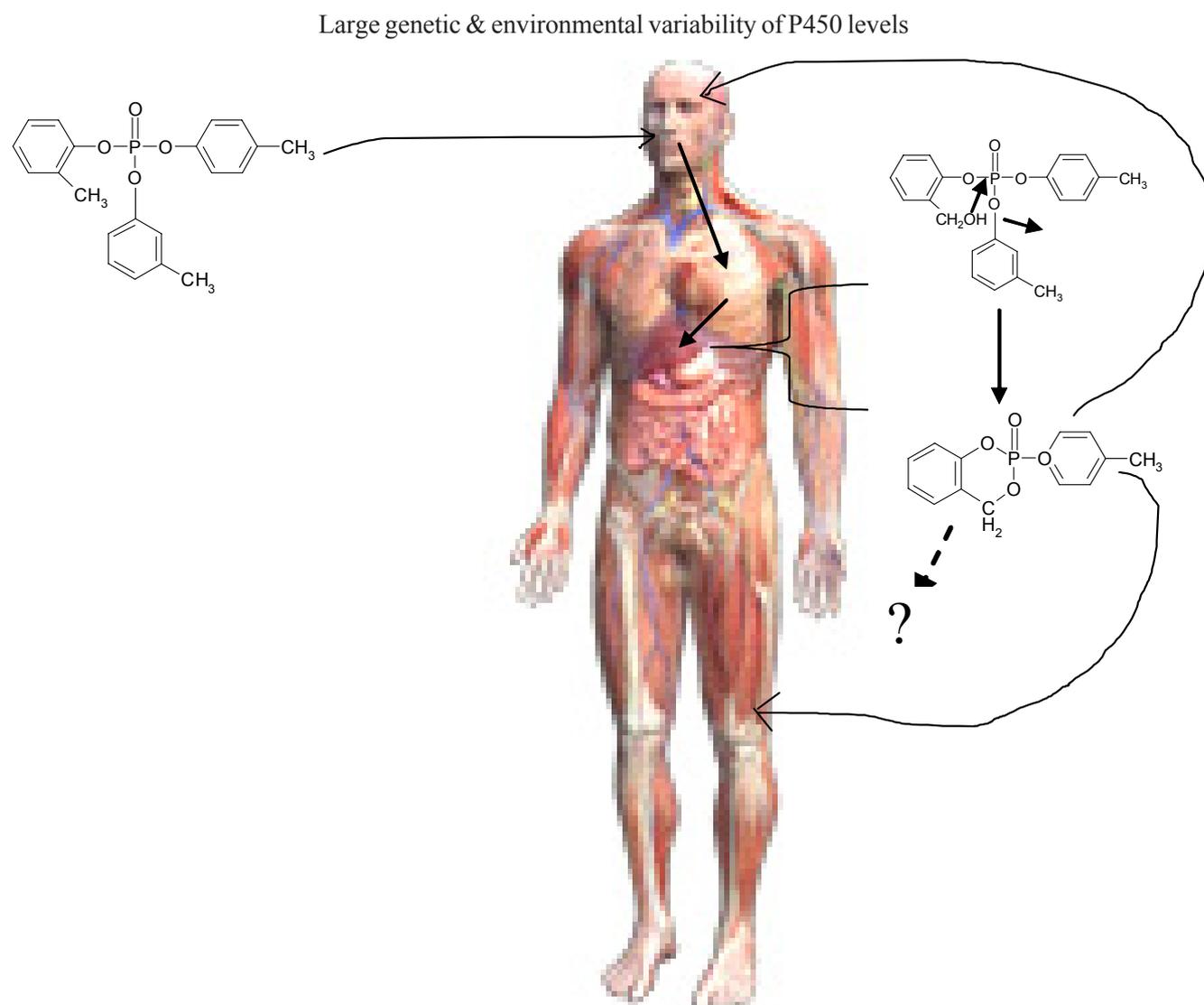


Figure 3. Overview of an inhalation exposure to mono-*ortho*-cresyl phosphate ester. The inhaled TCP enters the lung, travels to the liver (or other tissues) where cytochrome P450 enzymes convert the *ortho* methyl group to a methanolic group that in turn attacks the phosphate forming a cyclic ester, simultaneously losing a cresyl group. The resulting cyclic ester is known as [2-(*o*-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one] (CBDP) and is a very potent inhibitor of serine active site enzymes such as lipases, esterases and proteases. This highly toxic metabolite is also inactivated by an as yet unknown reaction(s) (broken arrow) (Aldridge, 1954; Baker et al., unpublished results).

P450s (e.g., the inhibition of several P450s by grapefruit or grapefruit juice (Lu et al., 2011)). Thus, following an exposure it may be possible to slow down the conversion of TAPs into toxic metabolites by administering an inhibitor of these enzymes, provided that there are no negative side effects. Generally, metabolism of a given xenobiotic such as a TCP may involve more than one cytochrome P450.

6. REDUCING TAP EXPOSURES OR CONSEQUENCES OF EXPOSURES

What are some of the possible solutions for decreasing the exposures or consequences of exposures of passengers and aircrews to TAPs? The most important way to

eliminate (or at least mitigate) exposure to oil fumes on aircraft is to avoid the use of bleed air, as Boeing has done in the design of the 787 Dreamliner (Sinnott, 2007). However, for many years to come aircraft using bleed air for ventilating the cabin will still be flying and therefore exposing passengers and crews to triaryl phosphates. As noted above, current data suggest that a fume event occurs on 0.05–1% of flights, explained in part by the bleed air system relying on changing air pressure to close the engine seals. On bleed air aircraft, one interim measure would be to formulate an engine oil antiwear additive that is significantly less toxic than the currently used TAPs. The French company NYCO SA has been working on this approach since becoming aware of the

issue at the 2005 meeting in London (BALPA, 2005). They have identified TAPs that generate much less inhibition of BChE in the *in vitro* assay when bioactivated by the cytochromes P450 in liver microsomal preparations, as well as much less inhibition of various serine active site enzymes following *in vivo* gavage exposures of mice to TAPs (Baker et al., manuscript in preparation). Other possible solutions include retro-engineering existing aircraft to utilize nonbleed ventilation systems such as on the B787, or adapt them with effective filtration and real-time monitoring systems with flight deck indication for the bleed air. The current approach of risking exposure of passengers and crew members to engine oil fumes that contain potent neurotoxins is unacceptable, given the associated impact on occupant health and flight safety.

7. AREAS FOR ADDITIONAL RESEARCH

7.1 Epidemiological studies

The data presented in both the report for DfT by the Institute of Environment and Health (Cranfield Ref No YE29016V) and the ITCOBA Conference of 11 October 2011 quite clearly point to the need for more relevant studies on the effects of TAP exposure on aircraft occupants. The studies should include not only fume event-exposed aircrew, but also passengers who greatly outnumber exposed aircrew and include some of the more vulnerable members of society—the old, young, and unborn. There really is no need to set up more data-gathering investigations as there have already been a large number of individuals exposed to significant fume events as shown in the literature (Murawski, 2011; Michaelis, 2010) and other reports documented by passengers and crew, which are in the public domain. Passenger manifests for flights with documented and significant fume events involving engine oil would be a rich source for epidemiological studies. Reliable conclusions cannot be drawn from monitoring a small number of flights where no fume events occurred. The incident noted above, where two pilots lost their medical certificates and most of the crew has been unable to return to work many months later, provides an excellent example where the current health status of the passengers on the same flight would be highly informative, as would epidemiological studies on other similar flights where fume events have resulted in ill health of the air crew. Aviation regulators should promulgate and enforce passenger right-to-know regulations to enable passengers who have been exposed to engine oil fumes to seek appropriate medical care.

7.2 Metabolism of TAPs

As noted above, we have known since 1930 (White and Lilly, 1930) that ToCP is a potent neurotoxin, since 1954 that metabolism by the liver is required for the generation of the neurotoxic metabolite(s), and since 1961 the structure of one of the highly toxic metabolites. What we do not yet know is which of the 57 cytochrome P450s generate toxic metabolites from the TAPs, nor anything about the genetic variability of the P450s involved. While much is known about which environmental chemicals (e.g., drugs or foods) either increase the levels of specific P450s or inhibit their activities, we do not know the effects of such environmental factors on modulating sensitivity to TAP exposures. This is an area of research that is important for addressing the possibility of formulating engine lubricants with significantly reduced toxicity, as noted below. The issue of the disruption of gene expression, especially in the brain, by TAP exposures is another crucial area of research that needs to be examined. It is *highly* undesirable to disrupt the brain metabolism of pilots who are responsible for the safety of the aircrew and passengers; clearly, it is undesirable to disrupt the brain metabolism of *any* occupants of an aircraft. It is known that exposure to OP insecticides disrupts gene expression in mouse brain (Cole et al., 2011).

7.3 Formulating safer lubricant additives

Following the 2005 BALPA-sponsored conference in London on cabin air, the French company NYCO SA requested that our research team assess the toxicity of alternative lubricant additives in its effort to identify a less toxic antiwear agent. Our research team has examined some of their potential TAP additives for conversion to esterase inhibitors when incubated *in vitro* with human and rat liver microsomes (P450s) and the required cofactor (NADPH). Several of the potential triaryl phosphates did not generate esterase (BChE) inhibitor when bioactivated in the *in vitro* microsomal systems. Follow-on *in vivo* experiments with mice exposed (via gavage) to two of the candidate TAPs and the currently used commercial mix of TAP esters showed that one compound did not inhibit BChE *in vivo*, but modestly inhibited other esterases, while another compound that did not inhibit BChE either *in vitro* or *in vivo* was a potent inhibitor of carboxylesterase *in vivo* (Baker et al., 2011). We feel that expansion of this approach for characterizing lubricant TAP additives will accelerate the development of safer additives. It makes sense to use antiwear additives that will inhibit as few enzymes as possible when an exposure occurs since we do not know the consequences to human health of the inhibition of many of the enzymes or

other proteins that are modified by the bioactivated TAPs. The approach of declaring a TAP safe for human exposure if it doesn't generate paralysis in the hen model completely ignores the many other physiological consequences of TAP exposure.

7.4 Other approaches for increasing the safety of aircraft cabin occupants

It has already been noted that by far the most satisfactory means of improving the safety of cabin occupants is to completely eliminate the possibility of feeding TAPs into the aircraft cabin by eliminating the use of bleed air for conditioning the cabin air. To date, this has been accomplished in one aircraft design, the Boeing 787 (Sinnott, 2007). Retrofitting the existing fleet to bleed air-free air conditioning would also be important to consider. There have also been discussions of fitting aircraft with filter systems for the bleed air (e.g., Michaelis, 2010). It is not yet known how effective this approach might be. "Electronic noses" have been suggested as a low-cost solution for real-time monitoring of engine oil constituents in the bleed air supply, with flight deck indication to enable pilots to readily identify the presence and location of any air supply contamination and isolate the source (Oord et al., 2011).

8. CONCLUSIONS

It is clear that serious fume events do occur when engine seals fail or engines are overserviced (Michaelis, 2010; AAIB, 2007; SAAIB, 2006). In addition, it is quite disturbing to note that certain engine seals appear to allow some leakage of oil into the bleed air system under normal operating conditions (Michaelis, 2010; Crump et al., 2011a, b; Liasova et al., 2011), which raises the question how much neurotoxin is acceptable in the cabin and flight deck of commercial aircraft? It is also well established that significant exposures to engine oil do occur during fume events and do affect human health (Michaelis, 2010; Murawski, 2011). Thus, it is crucial that immediate efforts are undertaken to reduce or eliminate exposures and to minimize the consequences of any exposures that may occur by decreasing the toxicity of the engine lubricating oils. In addition, epidemiological studies should be undertaken to determine the extent of ill health caused by these exposures. Records of fume events (exposures) do exist and it is important to access these records and to carry out follow-up studies with both aircrews and passengers to ascertain the extent of short-term and long-term physiological effects caused by these exposures. The Cranfield report (Crump et al., 2011a, b) exemplifies the futility of trying to do a prospective study of cabin air quality. It will be much more efficient and informative to

follow the health of individuals known to have experienced significant fume exposures. The significance of the exposures is readily verified by the ill health of aircrew following exposures and, in many cases, these data already exist.

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