Responses to Public Comment on the Draft Cancer Unit Risk Factor for *tertiary*-Butyl Acetate
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency
November 2016

Background
The Office of Environmental Health Hazard Assessment (OEHHA) is required to develop guidelines for conducting health risk assessments under the Air Toxics Hot Spots Program (Health and Safety Code Section 44360(b)(2)). In response to this statutory requirement, OEHHA developed a Technical Support Document (TSD) that was adopted in June 2009. The TSD describes cancer-potency and unit-risk factors (CPFs and URFs, respectively), and it presents methodology for deriving CPFs and URFs. In particular, the methodology explicitly considers possible differential effects on the health of infants, children and other sensitive subpopulations, in accordance with the Children's Environmental Health Protection Act (Senate Bill 25, Escutia, Chapter 731, Statutes of 1999, Health and Safety Code sections 39669.5 *et seq.*). These guidelines have been used to develop a new CPF and URF for *tertiary*-Butyl Acetate (TBAc).

Commenters on the Draft RELs for TBAc
Comments were received from Lyondell Chemical Company and Dr. James S. Felton, PhD (on behalf of Lyondell Chemical Company).

Responses to TBAc Comments Received from Lyondell Chemical Co.

Lyondell Comment 1:

Absent TBAc animal chronic studies, use of tert-butyl alcohol (TBA) chronic data is appropriate given extensive metabolism of TBAc to TBA.

Response to Lyondell Comment 1:

OEHHA agrees with this comment. The public review version of the document made extensive use of *tert*-butyl alcohol (TBA) biochemical and toxicological data (both non-cancer and cancer) in developing the cancer URF for TBAc.

Lyondell Comment 2:

A weight-of-evidence (WoE) evaluation of TBA rat kidney and mouse thyroid tumor data indicates lack of relevance for potential TBAc tumorigenicity. OEHHA's selection of and reliance on TBA-induced male rat kidney tumors as the primary basis for derivation of the URF for TBAc is not justified given robust mode of action (MoA) data demonstrating that this tumor response is mediated through the non-human-relevant α2u-globulin MoA (McGregor, 2010; Bus *et al.*, 2015) that is further augmented through the likewise non-human-relevant MoA of chemically-induced accentuation of rat chronic progressive nephropathy (CPN; Hard *et al.*, 2011).

Response to Lyondell Comment 2:

OEHHA believes that both the TBA-induced male rat kidney and female mouse thyroid tumors observed in the NTP 1999 TBA drinking water cancer bioassay are relevant to human cancer risk assessment. Responses to specific comments regarding TBA-induced male rat kidney and female mouse thyroid tumors are contained below in the responses to Lyondell comments 4 to 26 and 27 to 28, respectively.

Lyondell Comment 3:

The TBAc TSD directs significant attention to an evaluation of whether the observation of TBA-induced male rat kidney tumors, used as the primary basis of the derivation of the TBAc URF, are mediated through the non-human-relevant $\alpha 2_u$-globulin MoA. The TSD concludes that the available TBA MoA data do not fulfill all of the seven criteria outlined by IARC to classify a chemical as operating by this MoA (IARC, 1999), and thus the OEHHA TBAc cancer risk assessment is defaulted to the assumption of a non-threshold genotoxic MoA.

The TBAc TSD sets out its position on the TBA cancer hazard evaluation by considering the toxicity and MoA data in the context of the International Agency for Research on Cancer (IARC) seven criteria for kidney tumor induction through an $\alpha 2_u$-globulin nephropathy MoA (IARC, 1999; Table 1). The TSD concludes TBA fits only 3 of these 7

criteria: criterion 3, induction of the characteristic sequence of histopathological changes with hyaline droplet formation is obligatory; criterion 4, immunohistochemical identification of the accumulating droplets as $\alpha 2_u$-globulin; and criterion 5, reversible binding of TBA to $\alpha 2_u$-globulin. LyondellBasell concurs with the TSD conclusions on these criteria, and thus no comments are offered on data addressing these criteria. The TBAc TSD concludes that TBA does *not* fit two of the 7 criteria, namely: criterion 1, lack of genotoxic activity, and criterion 7, dose response relationships between end-points and tumor-associated doses. It further concludes that TBA does *not completely* fit criterion 2, male rat specificity for the TBA-induced nephropathy; and criterion 6, induction of sustained increased cell proliferation in the renal cortex. Each of these points will be considered in turn, and contrasted to the LyondellBasell position that a WoE examination of the overall data support the conclusion that essentially all of IARC-identified criteria are indeed fulfilled (Table 1).

Table 1: Does TBA meet IARC criteria for an $\alpha 2_u$-globulin MoA?

	OEHHA	LyondellBasell View
Essential Criteria		
1. Non-genotoxicity	No	Yes
2. Male rat specificity	Not Completely	Yes
3. Characteristic histopath	Yes	Yes
4. $\alpha 2_u$-globulin identified	Yes	Yes
Additional Supporting Evidence		
5. Reversible binding to $\alpha 2_u$-globulin	Yes	Yes
6. Sustained, increased cell proliferation	Not completely	Probably
7. Dose response similarities between MoA and tumors	No	Yes

Although IARC (1999) stated that all seven criteria must be fulfilled in order to conclude that a chemical is operating by an $\alpha 2_u$-globulin MoA, the IARC Working Group paper used as the basis for these criteria differentiated the seven criteria into “essential” and “additional supporting evidence” (Swenberg and Lehman-McKeeman, 1999).

Response to Lyondell Comment 3:

OEHHA believes that the use of the complete IARC (1999) criteria for an agent causing kidney tumours through an α2u·globulin-associated response in male rats as listed in the Consensus Report is appropriate. Responses to specific comments on this issue are contained in the responses to Lyondell comments 4 to 22.

Lyondell Comment 4:

IARC Criterion # 1 – Non-genotoxicity

OEHHA guidance for evaluation of data used in derivation of cancer potency factors specifically notes that a "weight-of-evidence" approach should be employed for evaluating the body of evidence supporting a conclusion of whether a MoA is consistent with a cancer outcome (OEHHA, 2009). The TSD guidance specifically states that the "number and quality" and "methodological issues" are concerns to be addressed in toxicological studies used "in the interpretation of animal bioassay data and mechanistic studies." This TSD guidance is particularly critical to the OEHHA evaluation of TBAc genotoxicity in that a conclusion of non-genotoxicity would substantially alter the risk assessment approach.

A WoE evaluation of the genotoxicity data summarized in the TBAc TSD results in the clear conclusion that both TBAc and its metabolic surrogate TBA are non-genotoxic. TBAc was negative in high-quality regulatory guideline and GLP-compliant studies examining bacterial (*Salmonella typhimurium* and *Escherichia coli*) reverse mutation, *in vitro* human lymphocyte chromosomal aberration, and *in vivo* rat bone marrow micronucleus assays (Table 3, OEHHA, 2015). TBA also was negative in high-quality bacterial reverse mutation, mammalian cell (L5178Y mouse lymphoma) mutation, mammalian cell (CHO) chromosomal aberration, and *in vivo* mouse micronucleus assays. The *in vivo* micronucleus tests are regarded as reliable indicators for both clastogenicity and aneugenicity.

Response to Lyondell Comment 4:

The genotoxicity section of the document lists the available genotoxicity data for TBA and TBAc. The genotoxicity data for TBAc is generally negative, but limited. TBA has not been observed to cause chromosomal damage, has mixed results for bacterial gene mutation, but has been observed to cause DNA damage (primary DNA damage, adduct formation, oxidative DNA damage). The DNA damage assays were performed both *in vitro* and *in vivo*, used several different assay endpoints, and were uniformly positive. It is also notable given the positive DNA damage data for TBA that the positive bacterial gene mutation assay data was generated in a *Salmonella* strain which is sensitive to oxidative DNA damage. OEHHA does not believe that a "box score" approach to genotoxicity evaluations is appropriate. As an example, if a chemical does not cause chromosomal damage, performing several different types of chromosomal damage assays and generating negative results may increase the "weight-of-evidence" regarding chromosomal damage, but does not necessarily predominate in an overall

"weight-of-evidence" regarding genotoxicity if positive DNA damage or gene mutation data exist. An overall assessment of genotoxicity should be done, and positive data should not be dismissed lightly. OEHHA believes that the "weight-of-evidence" for TBA genotoxicity does not indicate that TBA is non-genotoxic, and thus, does not fit IARC criterion 1.

Lyondell Comment 5:

IARC Criterion # 1 – Non-genotoxicity (continued)

The conclusion that TBAc and TBA are not genotoxic also is consistent with several recent reviews of the data described in the TBAc TSD.

Dr. James S. Felton, in a letter dated May 20, 2011, to George Alexeeff (Acting Director of OEHHA), stated:

> *"…the genetic toxicology data does not support a positive overall genetic toxicology assessment for [TBAC and TBA]; …the bulk of the evaluation comes down to three studies, all of which have major discrepancies [emphasis added]; …This negative genetox assessment when used with the rat kidney tumor data fits an alpha-2u globulin production mechanism for cancer induction not relevant to the human."*

The European Food Safety Authority (2012) concluded:

> *"The available data on tert-butyl acetate and on acetate and tert-butanol, its major metabolites, do not give rise to concerns regarding systemic toxicity, developmental toxicity or genotoxicity. Any carcinogenic risk would likely be from a non-genotoxic mode of action."*

and, the Toxicology Excellence in Risk Assessment (TERA, 2009) Peer Consultation review of TBAc also concluded:

"…the overall weight of evidence indicates that TBAC is not likely to be genotoxic..."

Response to Lyondell Comment 5:

1) The document lists one equivocal and five positive genotoxicity studies. Dr. Felton has provided comments on this document, which have received a specific response in Response to Felton Comments 1 to 10.

2) The European Food Safety Authority (EFSA) (2012) Panel on Contaminants in the Food Chain (CONTAM Panel) reviewed TBAc toxicity for the purpose of determining if TBAc would pose a potential hazard as a previous cargo to bulk shipping tanks used to transport edible fats and oils

As part of the overall TBAc toxicity review, EFSA (2012) included the McGregor *et al.* (2005) TBAc bacterial mutation assays. EFSA also reported the results of TBAc human lymphocyte chromosomal aberration assays and *in vivo* rat micronucleus assays reviewed by WHO (2005), but did not list the study authors. It appears that the TBAc studies listed in the WHO review were performed by Huntingdon Life Sciences and eventually published in Cruzan and Kirkpatrick (2006). However, EFSA did not report any TBA genotoxicity data, including the five positive TBA genotoxicity assays available at the time of the report. Thus, the EFSA 2012 review was insufficient in its scope to justify a determination that TBA is non-genotoxic.

3) The Toxicology Excellence in Risk Assessment 2009 Peer Consultation review of TBAc was chartered by Lyondell Chemical Company and is not a government document or a peer-reviewed journal article. As discussed in the Response to Comment 4, OEHHA does not believe that the "weight-of-evidence" for TBA genotoxicity indicates that TBA is non-genotoxic. Thus, OEHHA disagrees with the TERA Peer Consultation Panel's opinion regarding TBA non-genotoxicity.

Lyondell Comment 6:

IARC Criterion # 1 – Non-genotoxicity (continued)

Although not presented in the TSD, the non-genotoxicity of TBA also is consistent with the non-genotoxic profile of two other metabolic surrogates of TBA, methyl *tertiary*-butyl ether (MTBE) and ethyl *tertiary*-butyl ether (ETBE). MTBE and ETBE are extensively metabolized to TBA, and thus represent metabolic surrogates of TBAc through their common metabolism to TBA (reviewed in McGregor 2006, 2007). The negative genotoxicity profiles of both of these informative chemicals, which include the same spectrum of high-quality *in vitro* and *in vivo* regulatory guideline and GLP-compliant tests, should be included in the overall WoE evaluation supporting a conclusion of non-genotoxicity in the TBAc TSD.

Response to Lyondell Comment 6:

A comprehensive evaluation of MTBE and ETBE genotoxicity data is beyond the scope of the document. However, the document does discuss TBA genotoxicity studies that

also present positive genotoxicity data for MTBE. Those studies include Tang *et al.* (1997) (DNA damage/COMET assay), Williams-Hill *et al.* (1999) (bacterial gene mutation data), Yuan *et al.* (2007) (DNA adduct formation) and Sgambato *et al.* (2009) (DNA damage, oxidative DNA damage).

Lyondell Comment 7:

IARC Criterion # 1 – Non-genotoxicity (continued)

The TBAc TSD WoE conclusion that genotoxicity is a MoA underpinning potential TBAc and TBA carcinogenicity rests largely on a small number of methodologically flawed studies evaluating *in vitro* or *in vivo* effects of TBA only. Substantive concerns exist for each of the cited positive studies and are described below (discussed in the order of presentation in Table 3 of the draft TBAc TSD).

Tang et al., 1997

Increased DNA damage in a Comet assay performed in HL-60 cells (Tang *et al.*, 1997) is not informative as evidence of DNA-damaging activity of TBA in that a non-standard method was used in which only the qualitative appearance or lack of appearance of a Comet tail was evaluated (reviewed in Felton, 2011). In addition, McGregor (2010) has noted that Tang *et al.* reported that MTBE, TBA and the MTBE/TBAc/TBA metabolite α-hydroxyisobutyric acid all resulted in the approximately the same DNA damage, even though the structures of these chemicals share little commonality as to possible reactive groups. Although McGregor suggested that the equivalent responses might have been due to metabolic convergence to the common terminal metabolite of α-hydroxyisobutyric acid, this possibility was questionable given that the limited metabolic capacity of HL-60 cells would likely not be able to drive a quantitative metabolic conversion of MTBE or TBA to α-hydroxyisobutyric acid. In addition, the study also did not provide important study details impacting interpretation, e.g., although the effects were apparently collected from three experiments, no indication of cross-experiment variation was reported.

Response to Lyondell Comment 7:

As noted in the response to Felton Comment 5, Tice *et al*. (2000) state: “The methods used for quantifying DNA migration by this assay have varied almost as much as the number of scientists using the technique. The most flexible approach for collecting comet data involves the application of image analysis techniques to individual cells, and several dedicated software programs are commercially available. […] However, methods not based on image analysis systems are as useful. The simplest method for

collecting comet data is based on determining the proportion of cells with altered migration."

Tang *et al.* (1997) used the method of Anderson *et al.* (1994), where the percentage of DNA present in the tail is visually quantified. Although this adds some variability due to the subjectivity of visual scoring, it is still an acceptable laboratory method.

Additonally, if the three highest categories of DNA damage (II through IV) presented in Table 4 of Tang *et al*. (1997) are aggregated, a difference in damage among the three substances can be discerned:

Table 1. Aggregated DNA damage levels in HL-60 cells exposed to methyl-*tert*-butyl ether (MTBE), *tert*-butanol (TBA) or hydroxyisobutyric acid (HIBA). Adapted from Tang *et al.*, (1997).

Levels of DNA Damage reported in Tang *et al*. (1997) (Aggregated values from damage categories II through IV)			
Dose (mmol/L)	Methyl-t-butyl ether (MTBE)	t-Butanol (TBA)	Hydroxyisobutyric Acid (HIBA)
0	0	0	0
1	45	40	30
5	107	98	89
10	150	149	149
30	200	198	185

The data from this study indicate, especially in the two lower dose categories, the following order of potency: MTBE > TBA > HIBA. In the higher dose categories, MTBE may have produced some DNA crosslinking based on its metabolism to formaldehyde. This could have caused a reduction in comet tail score. The comment notes that no indication of cross-experiment variation was reported. This information has been added to the document.

Lyondell Comment 8:

IARC Criterion # 1 – Non-genotoxicity (continued)

Sgambato et al., 2009

A second Comet assay reported in a Rat-1 cell line employed a single IC50 test concentration (Sgambato *et al.*, 2009). This test concentration exceeded the upper limit concentration (IC30) recommended for such tests to avoid potential confounding of increased cytotoxicity (Tice et. al., 2000). The investigators themselves cautioned that interpretation of the Comet response might have been confounded by increased cell death, and that "…further studies with time- and dose-dependent curves are needed to investigate these issues." This conclusion is further reinforced by the report that only a single indicator of cytotoxicity (MTT test) was used in this assay; a combination of multiple methods are recommended to effectively assess cytotoxicity in the Comet assay (Storer *et al.*, 1996; Speit *et al.*, 2015).

Response to Lyondell Comment 8:

The full statement by Sgambato *et al.* (2009) regarding the potential confounding effect of increased cytotoxicity was:

> *"The reduction in the extent of DNA fragmentation after the initial increase is likely the result of an efficient DNA repair mechanism activated by cells following damage induced by these compounds. However, we cannot entirely rule out the fact that the most damaged cells die and/or that they are more sensitive and might be lost during preparation of the comet test although we tend to exclude this hypothesis because we did not observe a significant increase in the amount of dead cells in treated compared to control cultures (data not shown). Further studies with time- and dose-dependent curves are needed to investigate these issues."*

OEHHA acknowledges that cytotoxicity is a potential confounder in the interpretation of the comet assay results contained in the Sgambato *et al.* (2009) study, and has added that information to the document. However, it should be noted that Tice *et al.* (2000) did not indicate that more than one measure of cytotoxicity should be used when evaluating comet assay results.

Lyondell Comment 9:

IARC Criterion # 1 – Non-genotoxicity (continued)

Yuan et al., 2007

A report of increased DNA adduct formation in various tissues of male Kumming mice treated with TBA (Yuan *et al.*, 2007) is substantially confounded by use of an accelerated mass spectroscopy method that is prone to false positive results due to metabolic incorporation of radiolabel as compared to true adduction, and/or possible cross-contamination from protein adducts and other metabolites (Phillips *et al.*, 2000; reviewed in Felton, 2015). Such incorporation cannot be ruled out given the demonstrated formation of acetone as a TBA metabolite (Baker *et al.*, 1982; Cederbaum *et al.*, 1983). Interpretation of Yuan *et al.* (2007) is further complicated in that no synthetic standards of adducted DNA bases were used, as is necessary, to confirm the hypothesized DNA adduct formation (Himmelstein *et al.*, 2009).

Response to Lyondell Comment 9:

OEHHA has not found a scientific consensus on the commenter's position that accelerator mass spectrometry is prone to false positive results. To respond to the specifics of the comment, acetone has been identified as a minor metabolite of TBA in rats (OEHHA is not aware of any TBA metabolism studies in mice). Carbon from acetone can be incorporated into glucose, which can participate in the pentose phosphate pathway, producing ribose-5-phosphate, a precursor of deoxyribose. It would have been useful if the study had confirmed the identity of the DNA adducts using synthetic standards. This adds an element of uncertainty to the evaluation of the study. A discussion of this information has been added to the document.

Lyondell Comment 10:

IARC Criterion # 1 – Non-genotoxicity (continued)

Williams-Hill et al., 1999

Finally, observation of a mutagenic response in a non-GLP *in vitro S. typhimurium* TA102 assay (Williams-Hill *et al.*, 1999) is not a reliable result (reviewed in McGregor, 2010; Felton, 2015). TBA induced only a very weak response in that the requirement for a positive response of a 2-fold increase in mutation incidence for this assay was barely, if at all, fulfilled. In addition, the weak effect was noted in a tester strain with high and variable background incidence of revertants. Perhaps most importantly, these findings were not replicated in two independent and GLP-compliant tests in the TA102

tester strain (McGregor, 2005). These replications further confirmed that the negative findings were not attributable to use of DMSO, an oxidative stress inhibitor, in that test results were also negative with use an aqueous vehicle. It is also important to note that the TA102 strain is specifically designed to be highly sensitive to oxidative stress (McGregor, 2005, 2010; Felton, 2015), and thus the negative findings in these studies do not support the findings of Sgambato *et al.* (2009) reporting increased Comet assay damage and formation of 8-OHdG DNA oxidative damage observed under highly cell toxicity treatment conditions.

Response to Lyondell Comment 10:

Good Laboratory Practices (GLP) standards are designed to be applied to data submitted to the US Food and Drug Administration (FDA) for regulatory approval. Research data submitted to peer-reviewed scientific journals are not required to meet GLP standards, and GLP studies should not be construed as being more scientifically valid than non-GLP studies.

Williams-Hill *et al.* (1999) reported a laboratory spontaneous mutant reversion rate of 200 – 400 mutants/plate; test strains were discarded if they reached a spontaneous mutant reversion rate of 500 mutants/plate. The control mutation rate reported in the TBA assay was roughly 400 revertants/plate (graphical data). The control revertant rate therefore appears to be consistent with the laboratory quality control standards described by the authors. As can be seen from the range of spontaneous mutation rates reported by McGregor *et al.* (2005), 41 – 317/plate, the variability in spontaneous mutation rates can vary substantially between studies. Additionally, the TBA mutation data appear to describe a dose-response relationship, and the mid-dose exposure group exhibited a mutation response of approximately 2-fold greater than control, which is the generally accepted standard for a positive response in the *Salmonella* gene mutation assay. The positive TBA bacterial gene mutation reported by Williams-Hill *et al.* (1999) is valid, and should be considered in any discussion of TBA genotoxicity.

Lyondell Comment 11:

IARC Criterion # 1 – Non-genotoxicity (continued)

An overall WoE evaluation of the genotoxicity toxicity of TBAc and its metabolic surrogates TBA, MTBE and ETBE conclusively indicates TBAc and TBA are not genotoxic. Expert judgement calls for primary reliance on well-conducted and reported tests of apical genotoxic endpoints. As noted earlier, such an approach is fully consistent with OEHHA TSD guidance (OEHHA, 2009) calling for a WoE evaluation of

the "number and quality" and "methodological issues" as these are considered in the evaluation of cancer MoAs. As reviewed above, it is clear that apical endpoints of genotoxicity (mutations and chromosomal damage) are completely negative in high-quality test systems. Positive results reported from some non-apical tests for DNA damage (adducts; Comet assays) suffer from major methodological deficiencies that significantly limit their interpretability and utility in demonstrating TBAc or TBA genotoxicity.

Thus, an overall WoE review of TBAc and its metabolic surrogates TBA, MTBE and ETBE conclusively demonstrate that Criterion #1 is fulfilled without qualification.

Response to Lyondell Comment 11:

See the response to Lyondell Comment 4.

Lyondell Comment 12:

IARC Criterion #2 - Male rat specificity of renal toxicity and tumorigenicity
It must be noted that TBA is, at most, a very weak kidney tumorigen. A positive finding of tumorigenicity was not identified in the original NTP bioassay using standard histopathological sectioning of the kidney (NTP, 1995, Cirvello *et al.*, 1995). The combined incidence of adenoma and carcinoma of the kidney was 1/50, 3/50, 4/50 and 3/50 for the control and 1.25, 2.5 and 5.0 mg/ml drinking water doses, and statistical significance of the response was only achieved in the mid-dose when subsequent step-sectioning of the kidney was conducted; NTP declared the TBA kidney finding only as *some evidence of carcinogenicity* in male rats.

Response to Lyondell Comment 12:

1) In the NTP (1995) report, NTP staff discussed the differences between standard pathology sectioning and step-sectioning, and suggested that the two procedures were analogous to a partial evaluation and a definitive evaluation, respectively. The step-sectioning procedure is essentially a more sensitive procedure for detecting tumors in the tissue under examination.
2) The Explanation of Levels of Evidence of Carcinogenic Activity section of the NTP (1995) report states: "Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (clear evidence and some

evidence)". NTP clearly considers the TBA male rat kidney tumor results to be positive results.

Lyondell Comment 13:

IARC Criterion #2 - Male rat specificity of renal toxicity and tumorigenicity (continued)

As required of an $\alpha 2_u$-globulin MoA, no kidney tumors were observed in female rats or either sex of mice. TBA-induced male kidney tumor responses are relatively modest compared to other agents identified as operating via an $\alpha 2_u$-globulin MoA (Swenberg and McKeeman, 1999), and particularly so when compared by standard sectioning evaluations. Finally, it is also important to note that subsequent re-evaluations of the NTP bioassay male and female kidneys revealed TBA accentuation of rat CPN in males and females (Hard *et al.*, 2011). The CPN MoA, which also is not regarded as having a human clinical correlate, thus likely contributes to the overall expression of kidney tumorigenicity in male and toxicity in female rats.

The TBAc TSD (2015) lack of fulfillment of criterion # 2 on male rat specificity is largely based on kidney changes described in the female rat, namely exacerbation of CPN, increases in renal inflammation, and renal pelvis transitional cell hyperplasia (NTP, 1995). In the 2-year study, females were affected with a dose-related exacerbation of CPN. Although an adverse effect, this is not a nephrotoxic effect (Hard et al, 2009). It is an enhancement of the development of a spontaneous disease process that is very common in the F344 rat (Hard and Khan, 2004). In its advanced stages, CPN represents a wide spectrum of renal parenchyma alterations in both sexes, including inflammatory cell clusters and a characteristic form of transitional cell hyperplasia of the renal papilla lining (Hard et al, 2011; Frazier et al, 2012). It should be noted that CPN is not associated with mineralization in the renal papilla, in contrast to the statement on page 20, paragraph 1, lines 6-7 of the OEHHA document. Although Cirvello et al (1995) in their description of the NTP study linked both of these changes (inflammation and pelvic transitional cell hyperplasia) to CPN (nephropathy in their paper) this was ignored in the TBAc TSD (2015). However, a Pathology Working Group (PWG) re-evaluating the renal histopathology of the 13-week and 2-year studies of TBA, recognized, and described the papilla lining alteration in both sexes as typical of advanced CPN (Hard et al, 2011). The arguments presented in the TBAc TSD (2015) are inaccurate and there were no TBA-induced nephrotoxic changes in the female kidney.

TBA therefore fulfills IARC criterion # 2 without qualification.

Response to Lyondell Comment 14:

1) The document states that no kidney tumors were observed in female rats or male or female mice.
2) The statement regarding a relationship between linear mineralization and CPN has been removed from the document.
3) The draft document stated that it is not appropriate to determine that the increased renal tumors observed in TBA-exposed male rats are solely due to α2u-induced nephropathy. The document has been revised to include data from Doi *et al.* (2007), which found "no or at best weak associations of tumor responses with renal *α*2u-globulin concentrations, indices of cell turnover, or microscopic evidence of *α*2u-associated nephropathy."
4) The document has been revised to include an expanded description of the NTP findings regarding CPN, suppurative inflammation and transitional epithelial hyperplasia (TEH) observed in TBA-exposed rats. This description includes a discussion of the differing male and female rat dose-responses for CPN, suppurative inflammation and TEH. These data indicate that it is unlikely that suppurative inflammation and TEH are directly linked to CPN.
5) The document has also been specifically revised to indicate that the exacerbation of CPN in female rats indicates an adverse renal effect, and that the induction of suppurative inflammation and TEH are nephrotoxic effects. The data listed in the revised document and discussed above indicate that TBA does not completely fit IARC criterion #2.

Lyondell Comment 15:

IARC Criterion #6 - Sustained, increased cell proliferation (CP)

The TBAc TSD (2015) used two studies to assess whether the criterion of sustained, increased tubule cell proliferation was supported. Borghoff et al (2001), using 5-bromo-2-deoxyuridine (BrdU) immunostaining, showed that TBA caused a dose-dependent increase in renal tubule cell proliferation, but as pointed out in the TSD, this was demonstrated at only one time-point of 10 days. To examine this key event at a later time, the TSD cites the work of Takahashi et al (1993), which had applied proliferating cell nuclear antigen (PCNA) staining to recuts of kidney tissue from the NTP (1995) 13-week drinking water study, as evidence questioning the presence of sustained cell proliferation. Takahashi *et al.* reported an increase in the *median* cell proliferation only in the second highest exposure group receiving 20 mg/ml of TBA, a dose much higher than the high dose (5 mg/ml) used in the NTP carcinogenicity study. The TSD then cites the negative cell proliferation response observed in a 13-week study of TBAc after

13 weeks of exposure by Faber et al (2014). This comparison was not strictly appropriate as the tumor finding applied to TBA, not TBAc. The TSD (2015) did not refer to the work of Lindamood *et al.* (1992), who demonstrated, in a 13-week drinking water study, a statistically significant increase in renal tubule S-phase nuclei with anti-PCNA staining at doses of 1 and 2% of TBA in male rats, matching the occurrence of the hyaline droplet response in their study. At the high dose of 4% in male rats there were no hyaline droplets or any cell proliferation response (Lindamood et al, 1992). Inhibition of hyaline droplet formation is seen with other chemicals at very high doses, for example, with β-myrcene (NTP, 2010). The shortcoming of the Lindamood paper is that the negative data for female rats were not presented and the increases at 0.25 and 0.5% were not statistically significant.

Nevertheless, the male rat data of Lindamood et al (1992) and Takahashi et al (1993) at 13-weeks were consistent and, coupled with the results of Borghoff et al (2001) at 10-days, provide some evidence that IARC criterion # 6 is fulfilled.

Response to Lyondell Comment 15:

The document has been revised to include a description of the Lindamood *et al.* (1992) male rat renal tubular epithelial cell proliferation data from the NTP 90-day TBA drinking water study. However, the Takahashi *et al.* (1993) study appears to report virtually the same data as the Lindamood *et al.* (1992) study, with the primary difference being that the data are presented in graphical rather than numeric format. The document has also been revised to provide this information. Also, the NTP (1995) report did not report the PCNA cell proliferation data included in Lindamood *et al.* (1992) and Takahashi *et al.* (1993). These data are insufficient to change the conclusion in the document that TBA does not completely fit IARC criterion #6.

Lyondell Comment 16:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes

The TBAc TSD (2015) uses immunohistochemical staining of rat kidney for $\alpha 2_u$-globulin as evidence of an absence of dose-response. This is a capricious staining technique and should not be relied upon to support regulatory decision-making. It should only be used for qualitatively identifying that accumulating hyaline droplets stain positively for the $\alpha 2_u$-globulin protein, i.e. Criterion # 4. Borghoff *et al.* (2001) clearly state that $\alpha 2_u$-globulin immunohistochemical staining is not as sensitive as an ELISA for measuring changes in the kidney accumulation of this protein. The ELISA is a quantitative

measure of protein changes and the immunohistochemical staining is used to determine the localization of $\alpha 2_u$-globulin within the area of proximal tubule where protein droplets are formed. As such, the $\alpha 2_u$-globulin staining should not be, and was not, used to assess an increase in staining with increasing TBA exposure concentrations.

Response to Lyondell Comment 16:

An enzyme-linked immunosorbent assay (ELISA) for renal α2u-globulin is more sensitive and easier to quantify than renal α2u-globulin immunohistochemical staining. However, OEHHA is not aware of any published evidence indicating that renal α2u-globulin immunohistochemical staining is unreliable. Therefore, as with all analytical techniques, data generated using renal α2u-globulin immunohistochemical staining is appropriate for use subject to the limitations of the assay method.

Lyondell Comment 17:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)

In the second sentence of paragraph 2 on p.10 of the TSD it is stated "A significant increase in $\alpha 2_u$ concentration was noted in the 1750 ppm group ($p<0.05$) compared to controls, in contrast to the $\alpha 2_u$ staining evaluation, where no TBA exposure-related increase staining was noted." In fact, this statement is not correct and does not reflect the data presented by Borghoff *et al.* (2001). As described by Borghoff *et al.* (2001), "In male rats exposed to TBA, there was an increased accumulation of protein droplets within the proximal tubule characterized by the formation of large coalescing globules of protein and rare crystalloid protein structures (Fig. 1). Grading on a scale of 0-16 demonstrated an increase in protein droplets at 1750 ppm TBA (Fig. 2). Linear regression analysis supported a statistically-significant, concentration-dependent, positive trend for the accumulation of protein droplets in male rats exposed to TBA." In this study the data provided evidence that there was a TBA exposure-related increase in protein droplet accumulation, that these droplets stained positive for $\alpha 2_u$-globulin, and that there was a significant increase in the concentration of $\alpha 2_u$-globulin at the highest exposure concentration in the male, but not female rat kidneys. To clarify, the authors of this study did not use $\alpha 2_u$-globulin staining of the protein droplets to evaluate a TBA exposure-related increase since it is not an appropriate method for evaluating a quantitative response.

Response to Lyondell Comment 17:

Borghoff *et al.* (2001) stated "there did not appear to be a TBA exposure-related increase in intensity of α2u staining in male rats (data not shown)." The description of the Borghoff *et al.* (2001) study in the document is correct. The authors did speculate that the mild increase in TBA-exposed male rat α2u concentrations might only be detectable using an ELISA assay due to the greater sensitivity of the method compared to immunohistochemical staining. This information has been added to the discussion of Criterion #7 in the document. However, the authors did not state that $\alpha 2_u$-globulin staining of the protein droplets is an inappropriate method for evaluating a quantitative hyaline droplet response to TBA exposure.

Lyondell Comment 18:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)

The TSD suggests (p.10, paragraph 3) that TBA induces male rat renal tubule cell proliferation at concentrations that do not increase renal $\alpha 2_u$-globulin concentration. Although this may appear to be the case in the data presented in Borghoff *et al.* 2001, it is critical to consider that the sensitivity of these assays that measure renal cell proliferation and $\alpha 2_u$-globulin concentration are very different. The change in the concentration of $\alpha 2_u$-globulin is measured at a point in time post-exposure; $\alpha 2_u$-globulin concentration was measured approximately 18 hours following 10-days of exposure to TBA. Renal cell proliferation is evaluated with a 3-day osmotic mini-pump containing 5-bromo-2-deoxyuridine (BrdU) infused over a period of 3 days. The dynamic process of $\alpha 2_u$-globulin accumulation may peak at different times and the actual maximum concentration may not be captured. Chemical binding to $\alpha 2_u$-globulin is reversible, therefore it is possible that with a chemical that binds with low affinity, the time course for capturing the peak concentration of protein in the kidney may be different compared to a chemical such as 2,2,4-trimethylpentane, which was used as a positive control. As such, the correlation between renal $\alpha 2_u$-globulin concentration and renal cell proliferation was evaluated over the increased TBA exposure concentrations and not specific changes at each concentration.

Response to Lyondell Comment 18:

OEHHA is not aware of any published sensitivity comparison between the α2u-globulin ELISA assay and the BrdU cell proliferation assay that would indicate a disparity in

sensitivity between the two assay methods. Also, while BrdU adminstration started at exposure day 6.5 and continued through study termination at exposure day 10 in contrast to the α2u evaluation which was performed at study termination, there are no data available showing any differences in pertinent factors such as TBA blood concentrations between exposure day 6.5 and study termination.

Lyondell Comment 19:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)

The TSD also states (page 10, paragraph 3 – page 11, paragraph 1) "These TBA concentrations were noted by the authors to significantly increase male rat renal tubule cell proliferation (see Figure 3), suggesting that TBA can induce male rat renal tubule cell proliferation at concentrations that do not increase renal $\alpha 2_u$ concentrations." The concentrations that were referred to were 250 and 450 ppm TBA. There was an increase in renal cell proliferation measured at these lower concentrations where there was not a measureable change in the concentration of $\alpha 2_u$-globulin. However, these data need to be evaluated again keeping in mind that the renal cell proliferation is measured as an accumulation of a response over 3-days where the change in $\alpha 2_u$-globulin and in TBA concentration is at a fixed point in time. Also critical to note are the data presented in the Borghoff *et al.* (2001) publication (Figure 6) that clearly show the concentrations of TBA in the kidneys of male and female rats are similar if not higher in the female rats with no measure of increased renal cell proliferation in exposed female rats.

Response to Lyondell Comment 19:

The issue of α2u ELISA/BrdU cell proliferation assay sensitivity differences due to assay timing is addressed in the response to Comment 18. Also, the document notes that no significant differences in cell proliferation between TBA-exposed and control female rats were noted.

Lyondell Comment 20:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)

Not discussed in the TSD (2015) is that a more reliable marker of renal tubule cell accumulation of $\alpha 2_u$-globulin is the typical polyangular or crystal-like shape of the droplets in $\alpha 2_u$-globulin nephropathy, as observed in Hard (2005) and illustrated in Hard

(2008). Lindamood et al (1992) also noted that the shape of the $\alpha 2_u$-globulin-associated protein droplets was rhomboid and crystalline, and recorded them in male rats at doses from 0.25 to 2.0% in the drinking water. These typical droplets were observed in the NTP 13-week study at doses of 2.5 and 5.0 mg/ml, doses that were later associated with renal tubule tumors, by Hard (2005).

Response to Lyondell Comment 20:

The document has been revised to include the Lindamood *et al.* (1992) evaluation of data from the NTP 90-day TBA drinking water study. The study description includes renal hyaline crystal data (Table 2). The comment is unclear as to which α2u assay method is being compared to hyaline crystal evaluation regarding assay reliability.

Lyondell Comment 21:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)

Other evidence of dose-response correlation is seen in presence of precursors of granular casts and mature granular casts observed at the junction of outer and inner stripes of outer medulla in male rats of the NTP 13-week study at relevant doses, low frequency at 2.5 mg/ml, and higher frequency at higher doses (Hard, 2005). Linear papillary mineralization, a characteristic chronic feature of $\alpha 2_u$-globulin nephropathy was observed in male rats, but not females, at doses correlating with renal tumor induction (Hard, 2005). Thus, linear papillary mineralization was scored as mild to moderate in the high-dose (5.0 mg/ml) male group where CPN compromised survival of the rats, and minimal to moderate in the mid-dose (2.5 mg/ml) male group, which demonstrated a significantly increased renal tumor incidence. Linear papillary mineralization was absent in the low dose and control male groups, but was present in the 15-month interim sacrifice males of the 5.0 and 2.5 mg/ml male dose groups. Cirvello *et al.* (1995) also quantified linear papillary mineralization showing major increases in incidence in male rats at doses of 2.5 and 5.0 mg/ml, but not in female rats. The difference in renal tumor incidence between the 2.5 mg/ml male group and the 5.0 mg/ml male group could be associated with a lower survival of male rats in the 5.0 mg/ml group, which would have reduced the time for more tumors to develop, but the comment made below on renal tubule hyperplasia is also relevant. For criterion # 7, allowance should be made to accommodate the confounding factors of CPN exacerbation and survival.

Response to Lyondell Comment 21:

1) NTP reported that linear mineralization (associated with α2u induction) incidence increased with dose in the NTP 2-year study (NTP, 1995), but severity scores did not exhibit a dose-response. This information has been added to the discussion of IARC criterion #7 in the document.
2) NTP (1995) also observed that there was no morphologic evidence of extensive cell necrosis (granular cast formation) resulting from TBA exposure. This information has been added to the document.
3) NTP (1995) did not state that nephropathy was a cause of mortality in male and female rats, did not state that survival affected tumor response, and did not state that either nephropathy or mortality were confounding factors regarding renal tumor response.

Lyondell Comment 22:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)

By placing all of the emphasis on the lone statistically-significant end-point of renal tumors in the mid-dose (2.5 mg/ml), the OEHHA assessment does not reveal all of the facts concerning this particular chemical. The TSD (2015) gives no consideration to data on renal tubule hyperplasia. Atypical tubule hyperplasia is the obligatory precursor of, and on a developmental continuum with, renal tubule adenomas, and subsequently, carcinomas (Hard, 1990; Dietrich and Swenberg, 1991; Nogueira et al, 1993). In the extended evaluation of the NTP 2-year study (NTP, 1995), the only statistically increased value among the renal tubule hyperplasia data was the number of foci in the high-dose male group (5.0 mg/ml) at 23. In this particular case, combining foci of hyperplasia with renal tumors provides a more valid indication of the factors involved in the rat kidney response to TBA. When this is done, there is a dose-related score of 19, 26, 32, and 34, for the 0, 1.25, 2.5, and 5.0 mg/ml, respectively. Although not every one of the hyperplastic lesions may have conformed to atypical hyperplastic foci, it can be deduced that the overall tumorigenic response is similar at both the mid-dose of 2.5 mg/ml and the high-dose of 5.0 mg/ml in the NTP 2-year carcinogenicity study of TBA.

Response to Lyondell Comment 22:

NTP states that a developmental continuum exists from renal tubule epithelial hyperplasia to renal tubule adenomas, and subsequently, carcinomas. However, NTP did combine renal tubule adenomas and carcinomas in their statistical analysis but did

not combine renal tubule hyperplasia with renal tumors. OEHHA agrees with NTP regarding the renal tumor data analysis.

Lyondell Comment 23:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)

The TSD (2015) concludes that $\alpha 2_u$-globulin cannot be the only operative MoA responsible for the increased renal tumors (page 23, paragraph 5. lines 6-7). It has defaulted to the opinion that genotoxicity is the reason, although as noted above, this conclusion is not justified by a WoE evaluation of that dataset. However, the TSD also has given no consideration to a more likely alternative MoA. A feature of the TBA studies was exacerbation of spontaneous CPN, which was probably the cause of lower survival in the high-dose male rats. Advanced and end-stage CPN has been shown to be responsible for a low incidence of renal tubule tumors in control rats, and is therefore a risk factor for renal cancer development, particularly in male rats (Hard et al, 2012). A number of chemicals can exacerbate CPN to advanced stages of severity, and in so doing, marginally increase the incidence of renal tubule tumors (Hard et al 2013). A high rate of tubule cell turnover occurs throughout the developmental course of CPN in association with sustained tubule cell injury, and is likely to act as the underlying basis for the spontaneous renal tubule tumor formation associated with advanced to end-stage CPN.

TBA was associated with a dose-related increase in CPN in both the 13-week study and 2-year studies (NTP, 1995; Hard et al, 2011). In the 2-year study, CPN severity increased from 3.0 in control males to 3.3 in the high dose group (NTP, 1995). The mean CPN severity grade in male rats with renal tubule tumors in the TBA study was 3.6 compared to grade 2.8 in male rats without renal tumors (Hard et al, 2011). Furthermore, foci of hyperplasia due to exacerbated CPN are slow-growing and start to develop at a relatively late stage in carcinogenicity bioassays (Hard et al, 2013), as the NTP findings showed for the high-dose of TBA (NTP, 1995). There was also a dose-related increase in CPN severity in female rats, ranging from 1.6 in the control females to 2.9 in the high dose group (NTP, 1995). However, the degree of CPN severity was less in the females than in the males, which would explain the relative absence of renal tubule tumors in the dosed female rats. There is a well-known gender difference in the incidence and severity progression of CPN, with female rats being less predisposed than males (Hard and Khan, 2004). Because of this lower susceptibility to CPN, renal tubule tumors resulting from advanced CPN occur less frequently in females than in male rats, because fewer females reach end-stage CPN (Hard et al, 2013).

Response to Lyondell Comment 23:

The document did not "default" to the opinion that genotoxicity is the cause of the male rat kidney tumors induced by TBA and does not propose a mechanistic cause for those tumors. However, without convincing evidence for a specific mode of action, the approach to the dose-response analysis assumes there is no threshold for carcinogenic risk.

As described in the document, NTP (1995) reported nephropathy in both male and female rats in both the 13-week and 2-year oral studies. In the 13-week study, lesion severity did not increase in the exposed female rats, but lesion incidence increased significantly compared to controls in the 10, 20 and 40 mg/mL dose groups. In the 2-year study, NTP reported nephropathy in all male and female rats at the 15-month interim sacrifice, with severity scores ranging from minimal to mild. Almost all female rats exhibited nephropathy at study termination, and severity scores demonstrated a treatment-related increase. The induction of nephropathy in TBA-exposed female rats without a concomitant increase in renal tumors makes it unlikely that nephropathy is the cause of the male rat kidney tumors.

F344 rats have a relatively high incidence of CPN, but the male rat renal tubule tumor incidence in the NTP historical control database is low (< 1%), and there are several chemicals that exacerbate CPN without increasing male rat renal tumor incidence (Seely *et al.*, 2002)

Additionally, Melnick *et al.* (2012) evaluated 58 NTP carcinogenicity studies using male F344 rats and 11 studies using female F344 rats for relationships between exacerbated CPN and induction of rat renal tumors. Melnick *et al.* (2012) found widespread inconsistencies in the hypothesized relationship between CPN and rat renal tumors, and stated:

> *"CPN is not an established mode of action or mechanism of renal carcinogenicity. Neither the etiology of this kidney disease in aging control rats nor the mechanism of chemically exacerbated CPN in rats is known. Hence, there is no basis for establishing a mode of action for enhancement of CPN in rats or for defining critical biological processes that could occur in rats and presumably could not likewise occur in humans."*

This also indicates that it is unlikely that that nephropathy is the cause of the male rat kidney tumors in the NTP (1995) study.

Lyondell Comment 24:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)
Scientists from the US EPA and Texas A&M University have recently described the development of a new rat PBPK model for evaluating liver and kidney toxicity of ethyl *tert*-butyl ether and TBA based on internal dose metrics (Salazar et al, 2015). The internal dose metrics included the daily average of TBA blood concentration and the daily amount of TBA metabolized in the liver. The analyzed toxicity end-points included kidney tumors (males only), CPN (both sexes), and urothelial hyperplasia (males only). The results for kidney tumors indicated that the internal dose of TBA was inadequate to explain differences in tumor response, but CPN and urothelial hyperplasia were strongly correlated with TBA dose metrics. Salazar et al (2015) conclude that another factor besides the dose of TBA is involved in the kidney tumor induction, and their suggestion is that the NIH-07 diet used in the TBA study increased CPN and consequently the renal tumors. As discussed under Criterion # 2, urothelial hyperplasia is a component of CPN, and particularly a marker of advanced disease.

Response to Lyondell Comment 24:

Salazar et al (2015) suggest that another factor besides the dose of TBA is involved in the kidney tumor induction, and speculate that the NIH-07 diet used in the TBA study increased CPN and consequently the renal tumors. However, no supporting data were presented for this hypothesis.

As described in the response to Comment 22, the differing dose-responses for TEH and CPN indicate that TEH is not directly related to CPN, and it is unlikely that CPN is the cause of the TBA-treated male rat kidney tumors in the NTP (1995) study.

Lyondell Comment 25:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)
The procedure of step-sectioning of kidney tissue for obtaining increased renal tumor data for statistical analysis also selects for renal tubule tumors (usually late-occurring adenomas) and precursor foci of tubule hyperplasia associated with advanced CPN (Hard and Khan, 2004). In first describing this procedure, Eustis et al (1994) stated that a notable feature of all of the studies associated with higher renal tumor incidences in male rats after step-sectioning (including the 2-year TBA study) was the presence of

chemical-related, increased severity of CPN (Eustis et al, 1994). The only study in female rats in which step-sectioning increased the tumor incidence also showed a chemical-related increased severity of CPN (Eustis et al, 1994).

Response to Lyondell Comment 25:

The relationship cited by Eustis *et al.* (1994) does not mean that a causal relationship exists between CPN and induction of renal tumors in male rats. CPN is common in male rats, and step sectioning would likely increase the ability of a pathologist to detect CPN as well as renal tumors. The purpose of the Eustis *et al.* (1994) study was to determine if step sectioning increased the sensitivity of renal tumor detection in rats and mice. That study examined a relatively small number of NTP studies (13 using male rats, 8 using female rats), and did not perform a specific data analysis with regard to exacerbated CPN and rat renal tumors.

In contrast, Melnick *et al.* (2012) evaluated 58 NTP carcinogenicity studies using male F344 rats and 11 studies using female F344 rats specifically for the purpose of determining if relationships existed between exacerbated CPN and induction of rat renal tumors. As mentioned in the Response to Comment 22, it is unlikely that there is a causal relationship between CPN and rat renal tumors.

Lyondell Comment 26:

IARC Criterion # 7 – Dose response similarities between the MoA and short-term histopathological observations and tumor outcomes (continued)

In the case of TBA it is very likely that the low renal tubule tumor incidence is due to the combination of an $\alpha 2_u$-globulin nephropathy MoA and the chemically associated exacerbation of CPN. This combined effect has been discussed by Lock and Hard (2010), who drew particular attention to the intimate association of $\alpha 2_u$-globulin nephropathy with exacerbating CPN throughout the course of $\alpha 2_u$-globulin nephropathy disease progression. Lock and Hard (2010) used TBA as an example of the likelihood of these two separate MoAs combining to increase the incidence of renal tubule tumors above the background level. There is no renal disease counterpart of CPN in humans (Hard et al, 2009) and therefore, as with $\alpha 2_u$-globulin nephropathy, a MoA involving exacerbation of CPN should have no relevance for extrapolation to humans in cancer risk assessment. Importantly, consideration of an overlay of a CPN MoA was also suggested by the IARC Working Group paper addressing the criteria for an $\alpha 2_u$-globulin MoA, in which it was concluded that "…minor exacerbations of common spontaneous renal disease" in female rats "should not pertain" to ruling out an $\alpha 2_u$-globulin MoA

(Swenberg and McKeeman, 1999). This observation is entirely consistent with the conclusions of the Pathology Working Group review of TBA-induced rat kidney tumors (Hard *et al.*, 2011), in which it was stated:

> "There was unanimous agreement among the members of this independent PWG that both a2u-g nephropathy and CPN exacerbation were the only causative factors in the development of renal tubule tumors observed in male rats exposed to TBA in drinking water. As neither of these modes of action have human counterparts, the PWG concluded that TBA-related renal changes in rats could not be extrapolated for human health risk assessment, and were unlikely to pose any risk for humans."

The above evaluation indicates that not only is Criterion # 7 fulfilled for a weakly active $\alpha 2_u$-globulin MoA, but also that CPN provides an alternative ancillary MoA further explaining the lack of human-relevance of TBA induced rat renal toxicity and tumors.

Response to Lyondell Comment 26:

OEHHA agrees that the available TBA data indicate that it is a relatively weak inducer of α2u-globulin in rats. However, that does not mean that CPN must be a causal factor in the induction of male rat kidney tumors by TBA. As discussed in the Cancer Hazard Evaluation: Male Rat Kidney Tumor Data section of the document and the Response to Lyondell Comment 22, CPN is unlikely to be a causal factor in the induction of male rat renal tubule tumors by TBA. Additionally, OEHHA is not aware of any mechanistic data analysis indicating that CPN is capable of potentiating an increase in male rat renal tubule tumor incidence due to any cause, including α2u-globulin induction.

Lyondell Comment 27:

Use of TBA-induced mouse thyroid tumors are not justified as a basis for derivation of a TBAc inhalation URF

Similar to the male rat kidney response, TBA-induced female mouse thyroid tumors also are not a justifiable basis for derivation of an inhalation URF given both biological MoA and exposure considerations. Although MoA data investigating TBA thyroid tumors are more limited than that supporting the male rat kidney MoAs, it nonetheless suggests that the high-dose specific thyroid tumorigenicity of TBA results from a non-mutagenic MoA associated with enhanced catabolism of thyroid hormone mediated by TBA

induction of liver oxidative metabolism and associated compensatory response of the hypothalamic-pituitary-thyroid regulatory axis (reviewed in McGregor, 2010; Bus *et al.*, 2015). This MoA is common to multiple rodent thyroid carcinogens, with phenobarbital as the prototypical agent of the class, and this tumor MoA is not regarded as quantitatively, or possibly qualitatively, relevant to humans (McClain, 1989; Dellarco et al, 2006).

The plausibility of TBAc as a potential mouse thyroid tumorigen is further tempered by the observation that intrinsic TBAc mouse toxicity would limit achieving of TBA tumorigenic doses. As reviewed in Bus *et al.* (2015), all mice exposed to 3000 ppm TBAc for 6 hours were prostrate during most of the exposure period. TBA-induced tumors were observed only in female mice at the top drinking water dose of 2110 mg/kg/day, which toxicokinetic assessments indicate are equivalent to 3300 ppm TBAc conservatively *assuming 100% metabolism of absorbed TBAc to TBA.* Given that toxicokinetic data indicate approximately 50% of systemically absorbed TBAc is metabolized to TBA, the 2110 mg/kg/day TBA mouse thyroid tumorigenic dose more realistically equates to 6657 ppm TBAc (Bus *et al.*, 2015). Such a high chronic TBAc exposure would therefore almost certainly far exceed an MTD in mice, and thus it is very unlikely that tolerable chronic TBAc exposures would result in thyroid cancer.

Response to Lyondell Comment 27:

The Cancer Hazard Evaluation: Female Mouse Thyroid Follicular Cell Tumor Data section of the document notes that TBA: 1) causes little or no increases in absolute or relative liver weights; 2) does not induce cytochrome P450 activity to the same degree as seen with PB; 3) does not cause large decreases in T_3 or T_4 levels; 4) does not increase TSH levels; 5) does not cause acute abnormal mouse thyroid histopathological changes. Additionally, TBAc has not been observed to cause significant thyroid gland histopathological changes or alterations in thyroid/parathyroid gland weights in mice. TBAc has been observed to induce moderate decreases in T_4 levels at relatively high dose exposures in male but not female mice, but did not cause increases in TSH levels or decreases in T_3 levels in male or female mice. These data suggest it is unlikely that the TBA-induced female mouse thyroid tumors observed in the NTP (1995) study are the result of compensatory thyroid hyperplasia secondary to thyroid hormone insufficiency.

Lyondell Comment 28:

Use of TBA-induced mouse thyroid tumors are not justified as a basis for derivation of a TBAc inhalation URF (continued)
The plausibility of TBAc as a potential mouse thyroid tumorigen is further tempered by the observation that intrinsic TBAc mouse toxicity would limit achieving of TBA tumorigenic doses. As reviewed in Bus *et al.* (2015), all mice exposed to 3000 ppm TBAc for 6 hours were prostrate during most of the exposure period. TBA-induced tumors were observed only in female mice at the top drinking water dose of 2110 mg/kg/day, which toxicokinetic assessments indicate are equivalent to 3300 ppm TBAc conservatively *assuming 100% metabolism of absorbed TBAc to TBA.* Given that toxicokinetic data indicate approximately 50% of systemically absorbed TBAc is metabolized to TBA, the 2110 mg/kg/day TBA mouse thyroid tumorigenic dose more realistically equates to 6657 ppm TBAc (Bus *et al.*, 2015). Such a high chronic TBAc exposure would therefore almost certainly far exceed an MTD in mice, and thus it is very unlikely that tolerable chronic TBAc exposures would result in thyroid cancer.

Response to Lyondell Comment 28:

Bus *et al.* (2015) have proposed a model in which the exposure level of TBAc needed to produce the TBA levels observed to cause thyroid tumors in female mice in the NTP study (2115 mg/kg-day) would exceed the TBAc concentrations which caused adverse CNS effects (3000 ppm) in an acute study.

However, the TBA-exposed female mice in the NTP (1995) study were not exposed to TBA levels that exceeded the Maximum Tolerated Dose (MTD), and there is no reason to discount the female mouse thyroid tumor data on the basis of mortality in that study. The cancer unit risk derived for TBAc in the document was derived using US EPA's Benchmark Dose Software (BMDS) and its implementation of the multi-stage cancer model as described in the Hot Spots Technical Support Document for Cancer Potency Factors (OEHHA, 2009). The BMDL multi-stage cancer model essentially uses an empirical fit to the data, and then extrapolates with a straight line from the 95% lower confidence limit of the BMD (BMDL) to zero. This method does not assume any underlying theoretical mechanisms at the low dose range.

Implicit in the use of that model are the assumptions that the dose-response relationship being modeled is linear, and that there is cancer risk at all carcinogen doses greater than zero (i.e., no dose threshold exists). Therefore, even if the TBAc exposure model proposed by Bus *et al.* were correct, TBAc exposure should still be expected to pose a cancer risk based on the NTP 1995 female mouse thyroid tumor data at concentrations

below 3000 ppm (the concentration causing acute CNS effects in the Cruzan and Kirkpatrick (2006) study).

However, OEHHA also believes that the Bus *et al.* (2015) model may overestimate the air concentrations of TBAc required to provide an equivalent oral exposure of TBA. This model is based on back-calculations from an algorithm developed by Cruzan and Kirkpatrick and data from studies conducted on rats (Cruzan and Kirkpatrick 2006, Groth & Freundt). Bus *et al.* presented estimates of TBAc to produce systemic TBA doses equivalent to that produced from oral TBA doses used in the NTP drinking water bioassays (see Table 4 of Bus et al). Authors summarize the underpinnings of the estimates as follows:

> *"…The algorithm assumed that 50 percent of an inhaled absorbed dose of TBAC was metabolically converted to TBA. This key assumption was consistent not only with the overall pattern of urinary metabolites observed in the inhalation metabolism study in (Cruzan and Kirkpatrick, 2006), but also with observations that blood concentrations of TBAC and TBA were approximately equivalent in rats at termination of single inhalation exposures to either 440 or 900 ppm TBAC (Groth & Freundt)."*

Bus et al argues that:

> *"The conversion algorithm predicted that oral TBA doses used in the NTP mouse studies likely resulted in significantly higher TBA systemic doses than were produced in inhalation studies. Thus, the top oral TBA doses of 2070 and 2110 mg/kg/day were equivalent to 6531 and 6657 ppm TBAC inhalation exposures respectively. However, as emphasized by Cruzan and Kirkpatrick (2006), such high TBAC-equivalent doses would not be tolerated in repeated-exposure inhalation studies in that all mice exposed to a single 6-hr 3000-ppm TBAC exposure were prostrate during most of the exposure period."*

In addition:

> *"…The highest oral TBA dose of 2110 mg/kg/day would be produced at a TBAC concentration of 3300 ppm if it were conservatively assumed 100 percent of inhaled TBAC was metabolically converted to TBA, an exposure still likely to be above a maximum tolerated inhalation exposure to TBAC."*

The algorithm presented by Cruzan and Kirkpatrick (2006) is as follows:

VC_{TBAC} (ppm) = $ID_{TBA} \times MW_{TBAC} \times BW / 4.8 \times MV \times D \times FA \times MFC \times MW_{TBA}$,
Where
ID = internal dose (mg/kg-d).
VC = vapor concentration (mg/m^3 or ppm, as specified).
MV = minute volume inhaled (for mouse, 0.000023 m^3/min).
D = duration of exposure (min/day; for TBAC study = 360 min).
FA = fraction of inhaled TBAC absorbed into bloodstream (for TBAC = 0.75).
BW = body weight (0.03 kg for mouse).
MFC = fraction of TBAC metabolically converted to TBA (0.5).
MW = molecular weight: TBA = 74; TBAC = 116.

In that algorithm, body weight and minute volume are sensitive parameters. In addition, subsequent work has been presented that acknowledges additional changes in metabolism with repeated exposure (Salazar et al 2015). In Table 1, calculations of equivalent ppm TBAC are recalculated after adjusting several parameter values listed below:

1) The body weights (0.03 kg) assumed by Cruzan and Kirkpatrick have been changed to average body weights of 0.043 kg and 0.040 kg for male and female mice exposed in the 1995 NTP TBA drinking water study.
2) The minute volume value cited by Cruzan and Kirkpatrick (2.3×10^{-5}) was replaced with minute volumes calculated from US EPA guidance (2012a) that utilizes specific male and female mouse body weights.
3) The fraction of inhaled TBAc absorbed into the bloodstream used is the value from the document (0.71).
4) The fraction of TBAc metabolically converted to TBA used is the value from the document (0.95).

Table 2: Modeled values for TBAc inhalation concentrations based on doses of TBA used in the NTP 1995 drinking water mouse study

	TBA dose (mg/kg-day)	TBAc equivalent dose[b] (ppm) *(Bus et al., 2015, Table 4)*	TBAc equivalent dose[c] (ppm) *(OEHHA-adjusted parameters)*
Male mouse	2070	6531	2369
Female mouse	2110	6657	2393
Female mouse[a]	951	3000	
Female mouse[a]	2646		3000

a: estimates in addition to those administered in the NTP 1995 study.
b: BW = 0.03 kg, MV = 2.3×10^{-5}, MFC = 0.5, FA = 0.75
c: BW(male) = 0.04 kg, MV = 4.7×10^{-5}; BW(female) = 0.043 kg, MV = 5.1^{-5}, MFC = 0.71, FA = 0.95

The recalculated TBAc inhalation concentration at the 2110 mg/kg-day oral dose from the NTP 1995 study is 2393 ppm, which is less than the 3000 ppm observed to cause acute CNS effects, and roughly 4-fold greater than the TBA $BMDL_{05}$ (point of departure) of 647 mg/kg-day (Table 5 of the document). This information indicates that the acute TBAc mouse inhalation CNS toxicity observed by Cruzan and Kirkpatrick (2006) would not confound the use of the NTP (1995) TBA female mouse thyroid tumor data for human cancer risk assessment.

Lyondell Comment 29:

OEHHA should drop the TBAc cancer URF and instead adopt a non-cancer RfC similar to that published by Bus *et al.* (2015) based on mouse TBAc-induced neurotoxicity.

Bus and coworkers (2015) have proposed a chronic TBAc RfC of 0.3 ppm conservatively based on neurotoxicity (hyperactivity) observed in mice immediately after termination of daily 6 hour exposures to TBAc. Although the neurotoxicity was observed immediately after a series of short-term 6 hour TBAc exposures, it was assumed that this response would continue over repeated exposures and thus represented a potential chronic effect. The TBAc inhalation Unit Risk of 1.9×10^{-6} $(\mu g/m^3)^{-1}$ described in the TBAC TSD (2015) translates to a TBAC 10^{-6} risk of 0.0001 ppm, or 3000-fold less than the non-cancer chronic RfC proposed by Bus *et al.* (2015).

Response to Lyondell Comment 29:

OEHHA believes that the TBAc cancer unit risk factor described in the document is both adequate and justified by the available data, as discussed in the Response to Comments above. Derivation of a TBAc noncancer Reference Exposure Level (REL) is outside the scope of this document.

Lyondell Comment 30:

Miscellaneous Observations: Male rat kidney evaluations
The statement is made that NTP observed granular casts in TBA-treated male rats (page 20, Criterion 3, bottom of page). That is not correct. In fact, the statement in NTP (1995) and in Cirvello et al (1995) states the opposite, that is, there was "no morphologic evidence of extensive cell necrosis (granular cast formation)." Recognition of granular casts in TBA-exposed male rats was first recorded by Hard (2005) when examining slides from the NTP 13-week study of TBA that had been stained with Mallory Heidenhein and anti-PCNA stains (the latter from a cell proliferation investigation of the TBA-exposed rats of the TBA 13-week study). Subsequently precursors of granular casts were recorded in hematoxylin and eosin (H&E) stained kidney sections from the same NTP study by Hard et al (2011).

Response to Lyondell Comment 30:

The statement in the document regarding NTP (1995) histopathological observations in TBA-treated male rats has been edited to remove the mention of "granular casts".

Lyondell Comment 31:

Miscellaneous Observations: Male rat kidney evaluations (continued)
The derivation of the various health assessment values was based on the male F344 rat kidney tumor incidence data for TBA (NTP, 1995). Normally this would have used the tumor incidence values from the standard histopathological evaluation. However, this data was presumably unsuitable because the renal tumors were not statistically increased. Consequently, the data from an extended histological evaluation of the kidney was used involving step-sectioning of the kidneys to produce 6 to 8 additional sections per kidney. This elevated the renal tubule tumor incidence in groups of 50 male rats from 1, 3, 4, 3 for the 0, 1.25, 2.5, and 5 mg/ml exposure levels, respectively, to 8, 13, 19, and 13, respectively (NTP, 1995). The 2.5 mg/ml dose group was statistically

significant at 19 tumors. This departure from normal practice, though reasonable, should have been indicated and adequately explained in the TSD (2015). Eustis *et al.* (1994), who introduced the multiple-sectioning procedure for kidney tumor evaluation, tabulated the combined adenoma/carcinoma incidence in the NTP 2-year study of TBA as 7, 10, 18 and 11 for control, low, middle, and high doses of TBA in male rats, respectively.

Response to Lyondell Comment 31:

The description of the step sectioning procedure used by NTP (1995) to evaluate the male rat renal tissues for tubule adenomas and carcinomas has been added to the document. OEHHA believes that the male rat kidney tumor incidence data as reported by NTP (1995) is appropriate for use in the document.

Lyondell Comment 32:

TBAc Inhalation Unit Risk Derivation

There are several issues with the TBAc inhalation unit risk estimates. These include,

a) unspecified rationales for the use of a 5% BMR response versus a 10% standard, and for the elimination of the top dose in derivation of the BMR which resulted in a 2-point dose-response;

The TBAc TSD provides no rationale for selection of a 5% BMR response of the TBA male rat kidney response as the point of departure dose for risk derivation, despite OEHHA TSD guidance recommending selection of a 10% BMR as the standard default. In addition, no rationale is provided for elimination of the top dose in characterization of the male rat kidney BMR, resulting in a less than preferable two-point dose response curve as the basis for selection of the point of departure tumorigenic dose in rats.

Response to Lyondell Comment 32:

1) The guidance provided in the OEHHA 2009 Air Toxics Hot Spots Risk Assessment Guidelines Technical Support Document for Cancer Potency Factors document (OEHHA, 2009) is somewhat general. The OEHHA 2009 document states:

   "The benchmark <u>often</u> used is the 95% lower confidence bound on the dose producing 10% tumor incidence. However, if data are available which include a significant dose-response at less than 10% tumor incidence, then that lower

benchmark should be used (e.g., LED_{05} or LED_{01})." [underscore added for emphasis]

The guidance provided in the OEHHA Air Toxics Hot Spots Risk Assessment Guidelines Technical Support Document for the Derivation of Noncancer Reference Exposure Levels (OEHHA, 2008) states "In the case of quantal data in an animal toxicity experiment, the benchmark response rate is usually selected at 5%." Animal tumor incidence data is quantal data, and current OEHHA practice is to use a BMR of 5% where possible.

2) The document states that a CSF_{animal} could not be derived from the poly-3 corrected NTP male rat kidney tumor data set due to lack of model convergence. The CSF_{animal} was calculated from the NTP male rat kidney tumor data set with the high dose (420 mg/kg-day) eliminated. A clarification stating why the high dose group was dropped from the cancer model has been added to the document. The US EPA 2012 Benchmark Dose Technical Guidance document (US EPA, 2012b) considers the approach of removing the highest dose group and refitting the model(s) to the remaining data to be appropriate in low-dose extrapolation when none of the available models provide an adequate fit.

Lyondell Comment 33:

TBAc Inhalation Unit Risk Derivation

There are several issues with the TBAc inhalation unit risk estimates. These include,

b) unexplained assumptions of 95% TBAc absorption, and 71% TBAc to TBA metabolism.

The TSD also assumes that 95% of inhaled 100 ppm TBAc is absorbed into blood over the course of a 6-hour exposure (page 28, bottom paragraph). This estimate is very likely erroneous in that it was based on the assumption that the total amount of TBAc (radioactivity equivalents) determined in the bodies of rats at the termination of a 6-hour exposure (equivalent to 50.7 mg/kg TBAc) was essentially equivalent to the total amount of TBAc inhaled over the entire course of that 6-hour exposure. This projected inhaled dose is certainly a significant underestimate. Assuming an EPA default Minute Volume for rats of 0.174 L/min (0.000174 m3/min), a total volume of 0.06264 m3 of air (TBAc vapor) will be inhaled over a 6-hour exposure (360 min X 0.000174 m3/min), which in turn equates to a total of 29.7 mg of inhaled TBAc for an individual rat (0.06264 m3 X 474 mg/m3 TBAc; 1 ppm TBAc = 4.74 mg/m3). Assuming a rat body weight of 0.21 kg, this equates to a total potential inhaled dose of TBAc of 141 mg/kg TBAc over the course of a 6-hour total exposure. This inhaled dose is substantially higher than the

dose estimated from collection of total radioactivity in animals at termination of a 6-hour exposure (50.7 mg/kg), and projects that the total systemically absorbed dose is substantially less than 95%, and could be as low as 35% (50.7 mg/kg divided by 141 mg/kg). Such an absorption percentage more closely aligns with approximate 50% respiratory retention values of inhaled organic compounds.

Response to Lyondell Comment 33:

The calculations in the comment above use a US EPA default rat body weight value to generate an estimated rat respiration rate. However, the range of body weights for the rats in the Cruzan and Kirkpatrick (2007) metabolism study are available to calculate an estimated respiration rate for those rats. Using the low end of the rat body weight range (as was done in the Comment above) of 210 grams from Cruzan and Kirkpatrick (2007) in the US EPA (US EPA, 2012a) default ventilation rate algorithm (US EPA, 2012a), the rat minute volume for those rats is estimated to be 0.156 L/min. The minute volume cited in the comment above overestimates the minute volume and ultimately the amount of TBAc inhaled (in mg) by a factor of 1.1.

Using the minute volume listed above (0.156 L/min; 0.000156 m^3/min), a total volume of 0.05616 m^3 of air (TBAc vapor) is estimated to be inhaled over a 6-hour exposure (360 min × 0.000156 m^3/min) This equals a total of 26.62 mg of inhaled TBAc for an individual rat (0.05616 m^3 × 474 mg/m3 TBAc; 1 ppm TBAc = 4.74 mg/m^3). Assuming a rat body weight of 0.21 kg, this results in a total potential inhaled dose of TBAc of 126.76 mg/kg TBAc over the course of a 6-hour exposure period.

The commenter stated that 141 mg/kg (corrected to 126.76 mg/kg) inhaled dose is substantially higher than the dose that was estimated from collection of total radioactivity in animals at termination of a 6-hour exposure (50.7 mg/kg) and projects that the total systemically absorbed dose could be as low as 40% (= 50.7 mg/kg ÷ 126.76 mg/kg).

OEHHA estimated that the inhaled radioactivity in Cruzan and Kirkpatrick (2007) was absorbed by the rats at a 95% efficiency from the following data:

1) Cruzan and Kirkpatrick (2007) reported total inhaled radioactivity after processing rat carcasses (one from each dose group). The authors reported that a small fraction (4.8%) of inhaled mass was exhaled during seven days following exposure.

   However, this study described that loss through the urinary or fecal pathway was monitored after the first 6 hours of the study. Methods to collect excretion during exposure were absent and it was assumed that excretion for the first six hours of the

study was not collected. This loss could explain much of the difference between calculated and measured absorbed radioactivity.

2) Nose-only chambers have been reported to depress breathing rates of animals during confinement (Mauderly, 1986).

3) Human studies estimate that lung retention of VOCs are 80% or greater depending on the water solubility of chemicals (Jakubowski, 2009).

4) Respiratory rates adjusted downward by using the US EPA regression equation and an adjustment for depressed respirations bring the predicted and observed inhaled dose into closer alignment (US EPA, 1994; Mauderly, 1986).

Subsequent studies conducted by Faber *et al.* (2014) reported exposure chamber administration of TBAc rather than administration by nose-only methods. Hence, the chamber concentration and inhaled dose is not expected to reflect the depressed respirations observed in nose-only administration methods.

OEHHA concluded from the radioactivity study conducted by Cruzan and Kirkpatrick (2006) that metabolism to TBA could be as much as 71% at the lower of two single-dose levels and as much as 82% at the higher dose level based on the U2 and U4 pathways reported in Table V of that paper.

Lyondell Comment 34:

Page 5, paragraph 3 – description of historical control incidence of male rat kidney tumors.

It is important to note that NTP historical control incidence of male rat renal tubule adenomas (2/327) and carcinomas (0/327) in drinking water studies described here is derived from rats in which standard kidney sectioning, and not step-sectioning, was conducted. Thus, the inference stated here that such tumors are rare, although somewhat true, is misleading when these values are compared to the adenoma/carcinoma values reported in TBA treated rats in which step-sectioning was the basis of the tumor incidence determinations. The historical control incidence of kidney tumors in step-sectioned rats is not known.

Response to Lyondell Comment 34:

An evaluation of those same NTP historical controls using step sectioning could have resulted in an increased renal tubule tumor incidence due to greater tumor detection sensitivity. However, NTP also cited Eustis *et al.* (1994), who found that in previous NTP studies using this method of extended evaluation, the incidence of carcinoma in male rats was 1/599 among 12 control groups. The document has been revised to include the information described above.

Responses to TBAc Comments Received from James S. Felton, PhD

Dr. Felton Comment 1:

The overall response to TBA and TBAc in genetic toxicology assays is very weak to negative. There are only three papers that are relevant to TBA with purported positive responses. These results are far out-numbered by negative results in the mouse lymphoma assay, Chinese hamster ovary assessment for sister chromatid exchanges (SCEs), aberrations and micronuclei, and negative responses in Salmonella TA 98, 100, 104, 1535, and 1537. The overall data supports a negative overall assessment of genotoxicity for TBA and TBAc (see below and Tables 1 & 2).

Table 1. Summary of Salmonella Results for TBA

Test	Results	Ref
TA100	-	Hüls AG (1979)
TA100	-	EG&G Mason (1981)
TA100	-	Zeiger et al. (1987)
TA98	-	Hüls AG (1979)
TA98	-	EG&G Mason (1981)
TA98	-	Zeiger et al. (1987)
TA1537	-	Hüls AG (1979)
TA1537	-	EG&G Mason (1981)
TA1537	-	Zeiger et al. (1987)
TA1538	-	Hüls AG (1979)
TA1538	-	EG&G Mason (1981)
TA1538	-	Zeiger et al. (1987)
TA1535	+	EG&G Mason (1981)
TA1535	-	Zeiger et al. (1987)
TA102	-	McGregor et al. (2005) a
TA102	-	McGregor et al. (2005) b
TA102	±	Williams-Hill et al. (1999)
TA104*	-	Caprino et al. (1998)

*MTBE

Table 2. Salmonella mutagenicity of TBAc

Test	Results	Ref
TA100	-	McGregor et al. (2005)
TA98	-	McGregor et al. (2005)
TA1537	-	McGregor et al. (2005)
TA1535	-	McGregor et al. (2005)
TA102	-	McGregor et al. (2005)
WP2uvrA/pKM (*E. coli*)	-	McGregor et al. (2005)

Response to Dr. Felton Comment 1:

As noted in the response to Lyondell Comment 4, the genotoxicity section of the document lists the available genotoxicity data for TBA and TBAc. The mutagenicity data for TBAc is negative, but limited. TBA has not been observed to cause chromosomal damage, has mixed results for bacterial gene mutation, but has been observed to cause DNA damage (primary DNA damage, adduct formation, oxidative DNA damage). The DNA damage assays were performed both *in vitro* and *in vivo*, used several different assay endpoints, and were uniformly positive. It is also notable given the positive DNA damage data for TBA that the positive bacterial gene mutation assay data was generated in a *Salmonella* strain that is sensitive to oxidative DNA damage. OEHHA does not believe that a “box score” approach to genotoxicity evaluations is appropriate. As an example, if a chemical does not cause chromosomal damage, performing several different types of chromosomal damage assays and generating negative results may increase the “weight-of-evidence” regarding chromosomal damage, but does not necessarily predominate in an overall “weight-of-evidence” regarding genotoxicity if positive DNA damage or gene mutation data exist. An overall assessment of genotoxicity should be done, and positive data should not be dismissed lightly. OEHHA believes that the “weight-of-evidence” for TBA genotoxicity does not indicate that TBA is non-genotoxic, and thus, does not fit IARC criterion 1.

Dr. Felton Comment 2:

One purported positive assay by Williams-Hill, *et al.* (1999) examines TBA response in Salmonella strain TA102. TA102 is UvrB-positive which means it does not have a defect in excision repair of DNA. This strain, which has 30 copies of the hisG 428 gene on a plasmid, is typically positive for oxidative mutagens like X-rays, bleomycin, peroxide and quinones. If TBA causes oxidative damage to DNA, as suggested by Sgambato *et al*., then strains TA102 and TA104 should be very sensitive. However, this is not the case for TA102, suggesting TBA causes cellular damage instead of mutations. The mutagenic response in two studies independent from Williams-Hill are clearly negative and did not confirm these reported positive results. A number of variations of the assay were tested with no positive outcomes.

Response to Dr. Felton Comment 2:

OEHHA is not aware of any gene mutation data for either TBA or TBAc using *Salmonella* test strain TA104. TBA has been reported to be negative for induction of gene mutations in test strain TA102 by Huntingdon Life Sciences Ltd. (2000) and McGregor *et al*. (2005), and positive by Williams-Hill *et al*. (1999). It should be noted that the choices of TBA solvent in those studies was either DMSO (Huntingdon Life Sciences Ltd.) or water (Williams-Hill *et al*.). McGregor *et al.* reported separate results using both DMSO and water. Since DMSO is a free radical scavenger and has been reported to reduce mutagenicity via this mechanism (de Kok *et al.*, 1992), the negative results reported by Huntingdon Life Sciences Ltd. (2000) may be due to the choice of DMSO as the carrier solvent for TBA.

Dr. Felton Comment 3:

Williams-Hill et.al (1999)

In the Williams-Hill *et al.*, (1999) study there are some irregular results that should be considered. One, the background levels (420-460 rev) are much higher than either the Williams-Hill historical controls «400 rev) or the controls found in other studies using TA 102 (41-317 (mean = 194; McGregor *et al*. 2005). Thus, the two-fold background requirement, which is barely met, is the result of a three-point linear response on the dose-response curve starting with a historically high background. Each point on this curve has overlapping error bars with the adjacent dose, which suggests a lack of repeatable colony counts for each plate at any given dose. This could be considered a very weak mutagenic response, but given the high background levels and overlapping error bars, the response is probably not strong enough to be considered positive.

Response to Dr. Felton Comment 3:

Williams-Hill *et al.* (1999) reported a laboratory spontaneous mutant reversion rate of 200 – 400 mutants/plate; test strains were discarded if they reached a spontaneous mutant reversion rate of 500 mutants/plate. The control mutation rate reported in the TBA assay was roughly 400 revertants/plate (graphical data). The control revertant rate therefore appears to be consistent with the laboratory quality control standards described by the authors. As can be seen from the range of spontaneous mutation rates reported by McGregor *et al.* (2005), 41 – 317/plate, the variability in spontaneous mutation rates can vary substantially between studies. Additionally, the TBA mutation data appear to describe a dose-response relationship, and the mid-dose exposure group exhibited a mutation response of approximately 2-fold greater than control, which is the generally accepted standard for a positive response in the *Salmonella* gene mutation assay. Additionally, there is no generally accepted requirement that the difference between mean mutant/plate values for succeeding dose groups must be significant by pairwise comparison for the assay data to be valid.

Dr. Felton Comment 4:

Williams-Hill et.al (1999), continued

All other Salmonella strains are negative with TBA. The TA104 strain, which has the same basic DNA target as TA102 and the repair mutation to make it more sensitive, was negative for MTBE (a similar compound that also metabolizes to TBA). If TBA caused oxidative DNA damage, one would expect this strain to be positive since the metabolite is the same, but it is not. Budroe *et al*. have made the argument that TBA, TBAc, and MTBE when dissolved in DMSO will have their free radical activity attenuated because of the radical scavenging properties of this solvent. If all the bacterial genotoxicity was done with DMSO this argument might be justified. But McGregor *et al*. (2005) have reported that the Salmonella assay when done with water as solvent, as well as the DMSO, was also negative. This suggests that the solvent was not the determinant of the negative result. The mutagenic response to TBA using TA102 in the two studies independent of Williams-Hill was negative, both in water and DMSO. Therefore, the Williams-Hill study has little credibility because of the caveats of high background, weak response (just two-fold background), and large error bars for each dose. In conclusion, TBA should not be considered a Salmonella mutagen based on the current published studies.

Response to Dr. Felton Comment 4:

Neither TBAc nor TBA have been assayed using *Salmonella* test strain TA104. However, Williams-Hill *et al.*, (1999) tested MTBE using TA102 and obtained positive results. TA104 cannot be characterised as being more sensitive than TA102 because it is not proficient in excision repair, since an intact mutation repair system may be necessary for the detection of some mutagens (Williams-Hill *et al.*, 1999). Additionally, as described in the response to Dr. Felton Comment 2, the negative Huntingdon Life Sciences Ltd. (2000) data may have been confounded by the choice of DMSO as the carrier solvent. The *Salmonella* gene mutation assay data for TBA can be described as equivocal.

Dr. Felton Comment 5:

Tang et al. (1997)

In addition to the Salmonella genotoxicity data, there is a report by Tang *et al*. (1997) that claims TBA is positive in an alkaline elution test. This test, which is commonly called the "comet assay", looks for DNA breaks in individual cells. Typically, this set of experiments is done under very high stringency so that the tail of the comet can be measured using a number of different geometric parameters. In the case of the Tang paper this was not done in the standard way; only the appearance or lack of appearance of the tail was noted. The choice of cells, tumor cells in this case, makes one wonders if normal cells (even in a transformed state) would behave in the same manner. Tumor cells typically divide at a faster rate, meaning cells are in S-phase, and thus, more sensitive to DNA damage. Chemo drugs clearly have more efficacy in fast dividing tumor cells. In conclusion, this assay is not only poorly done, but is done in tumor cells having characteristics not found in normal cells. This study does not add credible evidence for TBA inducing DNA damage.

Response to Dr. Felton Comment 5:

Tice *et al*. (2000) state:

> *"The methods used for quantifying DNA migration by this assay have varied almost as much as the number of scientists using the technique. The most flexible approach for collecting comet data involves the application of image analysis techniques to individual cells, and several dedicated software programs are commercially available. […] However, methods not based on image analysis systems are as useful. The simplest method for collecting comet data is based on determining the proportion of cells with altered migration."*

Tang *et al.* (1997) used the method of Anderson *et al.* (1994), where the percentage of DNA present in the tail is visually quantified. Although this adds some variability due to the subjectivity of visual scoring, it is still an acceptable laboratory method.

Tice *et al*. (2000) also state: "Any eukaryote cell can theoretically be used for genotoxicity testing in the Comet assay […] No preference is given to the use of proliferating versus nonproliferating cells." This indicates that the use of HL-60 cells (a human promyelocytic leukemia cell line) by Tang *et al.* (1997) was valid.

Dr. Felton Comment 6:

Charles River (2015)
Most recently (Aug 2015) TBAc was tested by Charles River, Edinburgh, UK, in the Mouse Lymphoma Mutation Assay (OECD 476). This test scores for forward mutations at the thymidine kinase locus. The results were clearly negative in two trials. TBAc was tested with and without Aroclor 1254-induced liver S9. Positive controls, historical controls, DMSO controls, dose response, toxicity testing, chemical dose analysis, and colony size examination all check out; indicating a well-designed study. Again, as reported above, the genotoxicity testing does not support any genotoxic mechanism involved in tumor initiation or promotion for these compounds, especially in mammalian tumor cells in culture.

Response to Dr. Felton Comment 6:

The TBAc mouse lymphoma mutation assay data described by Dr. Felton has not been submitted to OEHHA for evaluation, and is not available in the peer-reviewed literature.

Dr. Felton Comment 7:

Yuan et al. (2007)
Another study purported to offer evidence of DNA adduct formation following TBA exposure is the AMS study by Yuan *et. al*. (2007). The appearance of DNA adducts is an important step in determining whether a chemical causes DNA damage at an *in vivo* organ or site. Numerous methods are available to analyze this response and characterize the DNA adducts including: P-32 post labeling, mass spectrometry, immunoassay, and HPLC separation followed by scintillation counting. In the case of Yuan *et al*., accelerator mass spectrometry was used to detect DNA binding. This is a

very sensitive method that has some of the same pitfalls as scintillation counting. One must characterize the product or chemical before analyzing the carbon-14 content. One advantage of accelerator mass spectrometry is that it is exquisitely sensitive.

In the case of Yuan *et al*. the characterization of the DNA adducts was primarily done by UV, recording a 260/280 ratio. Yuan *et al.* stated they had a ratio of 180, which is acceptable. However, it is important to make sure that only DNA and its suspected adduct(s) are quantified. In these experiments there was no indication of a positive linear dose response for adducts. It is curious that most of the curves even in different tissues had the same shape, but were not linear as should be expected for log-log plots. In the same paper the MTBE adduct responses look linear, so why the deviation for TBA? This anomaly further supports the possibility that these AMS measurements are not quantifying TBA DNA adducts.

Response to Dr. Felton Comment 7:

Dingley *et al*. (2005) has stated that obtaining a ratio of 1.8 (not 180 as stated in the comment) in the UV analysis of the purified DNA sample is an indication that the sample is sufficiently pure (i.e., free of RNA and protein). This indicates that the Yuan *et al.* (2007) study provided adequate proof of sample purity.

Additionally, the TBA-induced DNA adducts demonstrated a clear monotonic dose-response, even if it was not linear. Evidence that TBA induces DNA adducts does not require the dose-response curve in this assay to be linear. Toxicokinetic saturation in the pathway generating the genotoxicant could be one reason for the non-linearity.

Yuan *et al.* (2007) note that DNA adducts in MTBE exposure arise mostly from oxidation of the methyl group (to formaldehyde and formic acid); DNA adducts from TBA will be the result of a differing metabolite or set of metabolites. Therefore, different dose-response curves for MTBE and TBA in the Yuan *et al*. results are not anomalous.

Dr. Felton Comment 8:

The metabolism of TBA in the intact mouse may also lead to metabolites that still have the incorporated C-14 label (based on the position of the labeled carbon). If the C-14 is incorporated into acetone, for example, this metabolite of TBA could potentially be reincorporated into the DNA as a C-14 labeled base. This, of course, would add a large background to the DNA adduct counts or even suggest adducts that don't exist. This possibility makes it important to characterize the adducts chemically, to ensure that

adducts are actually formed. Unfortunately, this was not done in the Yuan *et al.* study, so one cannot conclude with certainty from this study that DNA adducts were in fact formed.

Response to Dr. Felton Comment 8:

Acetone has been identified as a minor metabolite of TBA in rats (OEHHA is not aware of any TBA metabolism studies in mice). Carbon from acetone can be incorporated into glucose, which can participate in the pentose phosphate pathway, producing ribose-5-phosphate, a precursor of deoxyribose. It would have been useful if the study had confirmed the identity of the DNA adducts using synthetic standards. The commenter is speculating regarding the size of the possible effect ("a large background"). Nonetheless, this adds an element of uncertainty to the evaluation of the study. A discussion of this information has been added to the document.

Dr. Felton Comment 9:

Overall, based on the weight of the evidence, TBA cannot be considered a genotoxin. There are too many issues present for each one of the weakly positive assays to influence my overall conclusion - that TBA is not a genotoxin (this is also based on the recent mouse lymphoma negative mutation assay).

In my opinion, the negative response of TBA in bacterial mutagenicity tests could be further backed-up with TA104 testing ± S9 and dissolved in both water and DMSO. At this point (10/15) we can also see the same negative gentox response in mammalian cells in culture, making the gentox effect of these compounds almost consistently negative.

Response to Dr. Felton Comment 9:

As detailed in the responses to Lyondell Comment 4 and Felton Comment 1, OEHHA believes that the "weight-of-evidence" for TBA genotoxicity does not indicate that TBA is non-genotoxic.

Dr. Felton Comment 10:

The negative genotoxicity results for TBAc support the above conclusions, as similar metabolites are seen for both TBA and TBAc and are consistent with no mutation and DNA damage from either compound. TBAc was negative in all strains tested. Combining the genotoxicity results for TBA and TBAc, there is very strong evidence that this class of chemical compound is not genotoxic.

The lack of genotoxic results for TBA and TBAc has important implications for the interpretation of the rat kidney tumor data in the 1995 NTP chronic study with TBA. In the IARC criteria for assessment (Swenberg and Lehman-McKeeman, 1999) of male rat kidney tumors associated with alpha-2_u globulin production there should be negative genotoxicity associated with the tumor inducing chemical (criteria #6). This is exactly what we see with TBA and TBAc. This is a very specific tumor type associated with male rats only and cannot be considered to translate directly to the human.

Response to Dr. Felton Comment 10:

As detailed in the responses to comments above, OEHHA believes that the “weight-of-evidence” for TBA genotoxicity does not indicate that TBA is non-genotoxic, and thus, does not fit IARC criterion 1. Additionally, as also detailed in the responses to comments above, OEHHA believes that the male rat kidney tumors observed in the NTP (1995) TBA drinking water study are relevant to human cancer risk assessment.

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