CLH public consultation Diethanolamine (DEA) CAS 111-42-2
Comments on the open hazard classes submitted by the Ethanolamine REACH Consortium (members listed in Appendix)

Acute toxicity oral Cat. 4

The registrants support the proposed modification of the current minimum classification for acute toxicity after oral exposure, resulting in a mandatory category 4 (H302 –Harmful if swallowed) with an ATE value of 1100 mg/kg bw (conclusion on page 19 of the CLH dossier).

Acute toxicity inhalation Cat. 4

The extrapolation of the concentration at which 5/8 animals died after 105 minutes of exposure to an aerosol generated from the heated substance (Foster, 1971) resulting in a calculated 4-h LC50 of 2.8 mg/L is considered inappropriate. Data obtained without heating the substance indicate no mortality even after exposure to a higher concentration of 3.35 mg/L for 4 hours (page 21 of the CLH dossier). The approach seems to be overly conservative for a high viscous liquid with a very low vapor pressure of 0.00008553 hPa at 20°C. Therefore, DEA should not be classified as acutely toxic via inhalation category 4 (H332 –Harmful if inhaled) (conclusion on page 22 of the CLH dossier).

Carcinogenicity Cat.2

Based on the findings from a battery of standard *in vitro* (bacteria and mammalian cells) and *in vivo* (micronucleus assay in B6C3F1 mice) genotoxicity studies, a genotoxic potential of DEA can be excluded (conclusion on page 26 of the CLH dossier).

Regarding the liver neoplasms in male and female and the renal tubule neoplasms in male B6C3F1 mice observed in the NTP carcinogenesis study (1999) different aspects regarding human relevance should be considered. First, regarding the mode of action of DEA inducing liver tumors, especially mice have been demonstrated to react very sensitive towards choline-deficiency and disturbances of choline-homeostasis compared to other species including humans (Leung et al. 2005; Zeisel and Blusztajn, 1994). The susceptibility of choline deficiency induced by DEA exposure is shown by quantitative species differences:

- The dermal absorption rate from *in vitro* skin penetration studies indicates a higher permeability rate constant for rats and mice compared to human skin (mice>rats>>humans) (Sun et al., 1996).
- Furthermore, increased DNA synthesis and decreased gap junction intracellular communication (GJIC) was observed in mouse hepatocytes after DEA exposure. At the same concentrations DEA failed to increase DNA synthesis and GJIC was not affected in human hepatocytes (Kamendulis et al., 2004; Kamendulis and Klaunig, 2005). Importantly, treatment with epidermal growth factor (EGF) resulted in an increase in DNA synthesis in all preparations of human hepatocytes evaluated demonstrating that the human hepatocytes were responsive to an established growth stimuli.
- In addition, the activity of the enzyme choline oxidase converting choline into betaine is highly active in rodents (rats>mouse>>human) whereas it plays a minor role in humans since they rely more upon tetrahydrofolate for maintenance of S-adenosylmethionine (SAM) (Sidransky and Farber, 1960 and reviewed in Kirman et al. 2016).

Supported by experimental evidence the proposed mode of action for the tumorigenesis in B6C3F1 mice is as follows:

1) Diethanolamine inhibits choline uptake, leading to cellular choline depletion (Bachman et al. 2006, Lehman-McKeeman and Gamsky, 1999).

2) Decreased choline levels reduce the pool of one-carbon donor groups, which lowers the potential for methylation reactions (Lehman-McKeeman et al., 2002, Craciunescu et al. 2009).

3) Alteration of DNA methylation leads to altered expression of genes involved in cell growth regulation (Kamendulis and Klaunig, 2005).

4) This altered gene expression promotes the growth of preexisting preneoplastic hepatocytes in B6C3F1 mouse liver, ultimately resulting in hepatic neoplasia.

Although choline deficiency can occur in humans under fasting conditions, their methyl-donor metabolism is not as susceptible to the effects of choline deficiency compared to mice. The reason for this is that whereas betaine, a choline metabolite, is in rodents a major donor of methyl groups for DNA methylation reactions, this is not the case in humans. Instead, humans rely predominantly on the methyl donor tetrahydrofolate (THF) for this purpose. As THF concentrations are independent of the level of choline in the body, the key initiating event to induce carcinogenicity in mice is not relevant in humans (Figure 1).

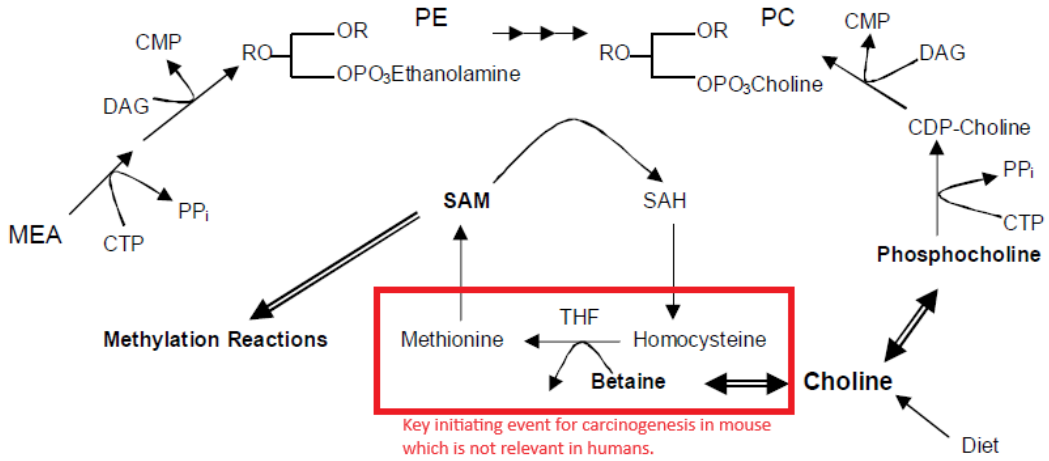

Figure 1: Inter-relationship between the intracellular pathways for the utilization of choline and methionine. Choline is utilized in phosphatidylcholine biosynthesis or oxidized to betaine, which serves as the methyl donor in the conversion of homocysteine to methionine. In this manner, the generation of methionine from homocysteine intersects choline and 1-carbon metabolic pathways. Methionine, as S-adenosylmethionine, is also an important precursor for the conversion of phosphatidylethanolamine to phosphatidylcholine, a pathway that is most active in the liver. The parts of pathways particularly perturbed by DEA are highlighted in bold markings. CDP-choline: cytidyl diphosphate-choline; CMP: cytidyl monophosphate; CTP: cytidyl triphosphate; DAG: diacylglycerol; MEA: monoethanolamine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PP: pyrophosphate; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; THF: tetrahydrofolate (Leung et al (2005). In red, the species difference between mice and humans is highlighted.

This means that choline deficiency in humans does not result in an increased DNA synthesis, as observed in mice. Furthermore, B6C3F1 mice have a heightened vulnerability to hypomethylation due to their reduced ability to maintain normal methylation status. This increased sensitivity to hypomethylation may further enhance their susceptibility to the proposed mode of action for diethanolamine-induced tumorigenesis (Counts et al., 1996).

Similar to the observed responses in mouse liver, prolonged exposure to DEA resulted in increased kidney weight and DNA synthesis in male mice over a period of 1 to 13 weeks (Mellert and Bahnemann, 2001). Even though direct measurements are lacking for key events like SAM concentration after repeated DEA exposure in mouse kidney, a similar mode of action regarding the choline perturbation in liver and kidney is expected since these organs represent the predominant site of the choline/betaine/SAM pathway and have the highest internal doses (Kempson et al., 2013; Matthews et al., 1997).

Furthermore, the rates of both spontaneous and chemically induced liver tumors are higher in male B6C3F1 mice than in other mice strains (Osimitz et al. 2013). In addition, the vehicle ethanol used in the NTP study can enhance the dermal permeation of some chemicals (Heard and Screen, 2008) which could have influenced the uptake of DEA leading to a higher systemic exposure of the already more sensitive species mouse.

Due to the species differences in susceptibility in choline deficiency the underlying mode of action of DEA causing liver tumors in male and female mice and kidney tumors in male mice is not considered relevant for humans.
In summary, the available *in vitro* and *in vivo* data with DEA suggest that a genotoxic mechanism is not involved and that humans are less sensitive towards the downstream consequences resulting from choline-deficiency than mice, which means that the liver and kidney tumors observed in mice are not considered relevant to humans. Therefore, DEA should not be classified as category 2 carcinogen (conclusion on page 35 of the CLH dossier).

Reproductive Toxicity

The registrants respectfully disagree with the proposed classification of DEA as Reproductive toxicant Cat. 1b, H360FD since the primary capacity of DEA is to inhibit choline uptake and metabolism as demonstrated in several mode of action studies and as exhibited in the reduced choline levels measured in several animal studies including the OECD 443 study conducted in rats.

All effects observed in the reprodevelopmental toxicity studies can be conclusively reasoned as being secondary to a non-specific effect of disturbance of choline homeostasis, the primary manifestations of which are pathological changes in the liver (associated with disturbed lipid metabolism) and kidneys (associated with impaired regulation of osmolarity due to betaine deficiency).

Introduction

In brief, choline is an essential nutrient crucial for the functioning of all cells (Savendahl et al.1997). Choline and its metabolites are essential for the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, cell signalling and lipid transport and metabolism (Lehmann-McKeeman and Gamsky, 1999, Zeisel et al. 1994). Phosphatidylcholine, is essential for the assembly and secretion of very low-density lipoproteins which transport triglycerides from the liver, the resulting steatosis due to choline deficiency results in accumulation of triglycerides and consequently liver damage and perturbation of circulating liver enzymes.

Table 9 of the submitted classification proposal succinctly summarises the available studies examining species differences in DEA metabolism, choline uptake, transport and metabolism and the ability of DEA to interfere with these processes. While the classification proposal discusses these differences with relevance for the carcinogenic mode of action of DEA, such species differences are also relevant for the reprodevelopmental effects of DEA. From the available studies it appears that rodents are much more susceptible to disturbances of choline homeostasis than humans. For example, rats have significantly greater choline requirements and as such they are particularly susceptible to periods of choline deficiency. They have significantly higher levels of choline oxidase levels (60-fold greater), in the liver than humans and a greater requirement for cysteine, derived from methionine, due to rapid hair growth (Sidransky and Farber, 1960, Zeisel et al. 1994). Whilst sex differences also exist, with males considered more susceptible to choline deficiency than females (due to de novo synthesis of choline by PEMT being regulated by oestrogen), the choline requirements of pregnant rats is also significantly greater due to the fact that the maternal liver supplies choline, against a concentration gradient to the placenta and foetal organs (Zeisel et al., 1980). Typically, the choline concentration in the amniotic sac

is 10-fold greater than the maternal blood plasma (Sweiry et al 1986) and as such the demand for maternal liver choline far exceeds the supply available (Gwee and Sim, 1978).

As a result of this increased need for choline, rats typically require an 8-10-fold greater amount of choline in the diet versus humans on a bodyweight basis (NRC, 1995) thus making rats particularly susceptible to a disturbance in choline availability. This particular susceptibility is observed in the repeated dose and reprodevelopmental toxicity studies conducted with DEA since changes in liver size, pathology and circulating liver enzymes are typically observed at doses lower than or equivalent to dose levels resulting in reprodevelopmental effects.

Having established that DEA is able to disturb normal choline homeostasis and that rats and mice are particularly susceptible to this disturbance we hereby focus on the subsequent mechanisms by which disturbance of choline homeostasis impacts various endpoints of fertility and development.

Reproductive effects of Choline deficiency

Choline deficiency and effects on female reproduction

Choline deficiency and reduced plasma and follicular choline levels are associated with polycystic ovary syndrome (PCOS) and reduced fertility in women (Zhan et al, 2021). Of the factors affecting female fertility, ovarian development and oocyte quality are considered most paramount (Farquhar et al., 2019).

Choline is considered essential for normal reproductive function in animals and humans. Though as described above, rodents are particularly sensitive to choline deficiency compared to humans due to their greater overall requirement and significantly higher turnover. Choline supplementation has been shown to improve reproductive tract development (Zhan et al., 2021), increase cervical length, improve ovulation rate and number of corpora lutea, and promote oocyte maturation via betaine accumulation, choline being the substrate for betaine synthesis (McClatchie et al., 2017). Furthermore, follicular growth, stimulated by FSH, is largely mediated by acetylcholine production in granulosa cells (Mayerhofer et al., 2006).

CDP-Choline synthesis via the Kennedy pathway is also known to affect circulating hormone levels, some of which are important in the normal function of the reproductive cycle. CDP-choline in the plasma increases adrenocorticotropin levels which in turn lead to higher levels of circulating thyroid stimulating hormone (TSH), growth hormone (GH) and luteinizing hormone (LH). During the oestrus cycle, rapid increases in luteinizing hormone triggers ovulation and corpus luteum formation, furthermore, LH-receptor expression increases in the ovary as do several genes associated with steroidogenesis. In pigs fed choline supplemented diets, increased expression of CYP11A1 was observed. This enzyme is involved in pregnenolone synthesis from cholesterol which indicates choline plays a central role in ovarian cycle regulation and steroid hormone production (Zhan et al., 2021).

In summary, choline plays a crucial role in the normal functioning of the female reproductive cycle. The multitude of effects observed on female reproduction in the extended one generation study i.e increased gestation length, ovarian atrophy and luteal cysts, irregular oestrus cycle, reduced number of implants and overall litter size are symptomatic of choline deficiency induced by DEA. It is noteworthy that all such effects occur at doses either greater than or equivalent to those in which signs of liver toxicity also occur such as increased liver weight, changes in morphology and increased circulating liver enzymes). Since the liver plays a central role in choline metabolism and is particularly sensitive to choline availability any such choline deficiency induced by DEA causes a multitude of non-specific downstream effects, all of which are detrimental to normal reproductive function in females.

Choline and male reproductive function

In various reprodevelopmental toxicity studies conducted with DEA, adverse effects on the testes, epididymis, prostate glands and sperm were noted, again in conjunction with signs of systemic toxicity. Similar effects have also been observed in studies conducted on animals fed choline restricted diets and or choline and methionine restricted diets (Jiang et al., 2021).

The effects of choline deficiency on the male reproductive organs very likely involves multiple mechanisms. Choline is essential for membrane fluidity and structure and choline is a crucial nutrient in sperm maturation (Lazaros et al. 2012). Secondly, betaine, synthesised by the oxidation of choline is usually present in the testes at approximately 10-fold greater than the liver. As such, male rats would be highly susceptible to reproductive effects as a result of a deficiency in choline given they have a 60-fold greater capacity to oxidise choline to betaine than humans do. Betaine appears to perform several functions, and in choline dehydrogenase knockout mice, supplementation with betaine was able to restore sperm function by maintaining ATP concentrations and sperm fluidity most likely due to betaine's osmolytic function. Furthermore, dietary betaine supplementation is known to reduce homocysteine levels which results in lower oxidative stress and consequently improves overall sperm quality. Thirdly, choline deficiency most likely impacts male reproductive performance via hormonally mediated mechanisms. Thyroid hormones act on various cells of the testis including Leydig, Sertoli and germ cells (Maran 2003). Hyperthyroidism has been associated with reduced semen volume, sperm density, motility and morphology in humans and delayed spermatogenesis in rodents (La Vignera and Vita 2018). As mentioned above, choline is also able to regulate circulating hormone levels via CDP-choline and elevations in TSH and T4 were observed in the extended one generation study. As such it is highly plausible that this hormone disturbance contributed to the overall male reproductive effects observed in the study.

In summary, the effects on the male reproductive organs observed in the OECD 443 study are consistent with the known consequences of choline deficiency given its essentiality in male reproductive function. Given the significant species differences in requirements for choline and the higher metabolic capacity of rodents in particular, it is considered that such effects should not be considered relevant for humans since it is highly unlikely that a sufficiently choline deficient state could manifest itself in the human population as a result of exposure to DEA.

Choline and developmental Immunotoxicity

As noted in the classification proposal, signs of developmental immunotoxicity were observed in F1 females receiving 1000ppm DEA as manifested by altered CD4/CD8 ratio versus controls. In the absence of choline, alterations in DNA methylation would be the most plausible explanation for such effects.

Thymopoiesis is a highly regulated process involving multiple developmental stages that ultimately results in mature T lymphocytes. Common lymphoid progenitor (CLP)-derived early thymic progenitors (ETPs) seed the thymus from the bone marrow. Developing thymocytes then progress through multiple stages defined by their expression of CD4 and CD8 coreceptors (Correa et al., 2020). As T-cells develop, they undergo a series of cellular decisions in which DNA methylation is known to play a critical role.

DNA methylation in T-cells is regulated by the DNA-methyltransferase (DNMT) and ten-eleven-translocation (TET) families of epigenetic enzymes. DNA methylation covalently modifies DNA through the methylation of the fifth carbon of a cytosine base and DNMT is responsible for transferring

methionine from the co-enzyme S-adenosyl-L-Methionine (SAM) to the cytosine residue of DNA (Jeltsch, 2002).

Genome-wide DNA methylation studies in murine hematopoietic lineage commitment has shown differential methylation at several stages of T-cell development suggesting that DNA methylation is dynamically regulated (Ji et al., 2010) and overall DNA methylation plays a significant role in determining T lineage commitment.

In rodents, the presence of choline in the diet plays a critical role in DNA methylation and choline deficiency is associated with reduced levels of DNA methylation. Dietary intake of choline modulates methylation because, via betaine homocysteine methyltransferase (BHMT), this nutrient (and its metabolite, betaine) regulate the concentrations of *S*-adenosylhomocysteine (SAH) and *S*-adenosylmethionine (SAM) (Zeisel 2017). In the absence of SAM, DNA methylation during T-cell development cannot occur since it is not available for as a substrate for DNMT. In humans however, methyl-donor metabolism is not susceptible to the effects of choline deficiency. As mentioned in the comments regarding carcinogenicity classification, there are significant differences in rodents and humans with respect to DNA methylation reactions. For rodents, betaine is the major methyl donor group whereas in humans this is not the case. Instead, humans rely predominantly on the methyl donor tetrahydrofolate (THF) for DNA methylation reactions and as THF concentrations are independent of the level of choline in the body, DNA methylation in T-cell development would not be impacted by any reduction in choline availability.

Choline and developmental neurotoxicity

The essentiality of choline for neurodevelopment has been extensively reviewed (McCann and Ames 2006, Derbyshire and Obeid 2020, Gamiz and Gallo 2021). As choline plays an essential role in membrane structure, integrity, neurotransmission and methyl metabolism, it is thought that choline is essential for normal brain development in the growing fetus. Choline is able to pass the blood-brain barrier by facilitated diffusion where it is stored as phospholipids and subsequently utilised for acetylcholine synthesis. Choline depravation during development can lead to impaired cognitive function and evidence from animal models has demonstrated choline's essential function in key processes affecting brain structure and function. Studies have shown that choline supplementation during gestation and the perinatal period enhanced cognitive performance in particularly more challenging tasks, increased the electrophysiological responsiveness and size of neurons in offspring, and was also neuroprotective.

Fundamentally, choline is required by neuroprogenitor cells in the fetal hippocampus which proliferate, differentiate, migrate and undergo apoptosis at specific times during fetal development (Craciunescu 2003 et al., 2009, Zeisel 2007). It is also essential for membrane synthesis and methylation of DNA and histones, which in turn affects expression of genes involved in learning and memory. It has been demonstrated that these early changes in neurodevelopment lead to life-long changes in memory function. These findings indicate that memory function is permanently altered by changes in the hippocampus of the fetal brain.

A number of studies have investigated the effects of choline restriction on the development and organization of the brain. In studies with low choline diets, the development of the cerebral cortex was impaired in fetal brains. In addition, several studies have shown that choline deficiency leads to reduced angiogenesis and reduced proliferation of endothelial cells in the hippocampus. Conversely several studies have also shown the positive effects of choline supplementation on neurogenesis in animal models of disease and improved special learning impairment in animals, all thereby indicating the critical role of this nutrient in normal fetal brain development.

With respect to learning and memory, several studies in the literature have explored the effects of choline deficiency or supplementation. In general, choline deficiency resulted in short-term memory deficits, whereas in studies where dietary choline was sufficient rodents demonstrated behaviours indicative of improved cognitive function and accelerated long-term memory development.

Conclusions

In summary, in several reprodevelopmental toxicity studies, administration of DEA resulted in a reduction of choline levels in the dams and offspring as evidenced by a clear dose-related reduction of choline levels in the plasma and liver of all dose groups in the maternal animals and the offspring of the OECD 443 oral drinking water study in Wistar rats. Although the systemic maternal toxicity was not visibly "severe", effects on fertility and the offspring as shown above are all consistent with this choline depletion and are expected in the realm of undernourished maternal animals due to choline deficiency. Choline supplementation during human pregnancy is increasingly recognised due to its central role in foetal development (Korsmo et al. 2019; Staskova et al. 2022).
Furthermore, in male animals, reproductive toxicity occurred at doses concomitant with or greater than doses which already induced significant liver effects as evidenced by changes in liver size, morphology and circulating liver enzymes.
In addition, neurotoxic and immunotoxic effects, associated with reduced DNA methylation are considered not relevant for humans due to the different pathways involved in SAM production in rodents and humans i.e. choline-dependent versus THF-dependent pathways.
In general, the suitability of rodents as an animal model for reprodevelopmental toxicity following DEA administration is questionable given that rodents are particularly susceptible to a disturbance in choline homeostasis, due to their significantly increased requirements for choline and 60-fold greater metabolic capacity of the rodent liver. Overall, these species differences raise significant doubt about the relevance of effects for humans and as such the criteria for classification as Cat 1b. reprodevelopmental toxicity (H360) are considered not met.

When comparing the effects of DEA with the criteria as described by the Regulation EC 1272/2008 (CLP), Table 3.7.1(a) states that Classification as Category 1B, (Presumed human reproductive toxicant) *'is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development* ***in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects****. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate'.* (emphasis added).

CLP Section 3.7.2.2.1 also explains that classification for developmental toxicity is *'intended to be used for substances which have an intrinsic, specific property to produce an adverse effect on reproduction and substances shall not be so classified if such an effect* ***is produced solely as a non-specific secondary consequence of other toxic effects****.'* (emphasis added)

Maternal toxicity and the disturbance of maternal homeostasis is discussed in CLP Section 3.7.2.4. According to the legislation '*Development of the offspring throughout gestation and during the early postnatal stages can be influenced by toxic effects in the mother either* ***through non-specific mechanisms related to stress and the disruption of maternal homeostasis, or by specific maternally-mediated mechanisms****'* (emphasis added). Similarly, CLP Section 3.7.2.3.5. explains that '*it is important to try to determine whether developmental toxicity is due to a specific maternally mediated mechanism or to a* ***non-specific secondary mechanism****, like maternal stress and* ***the disruption of homeostasis****'* (emphasis added). The legislation therefore clearly defines disturbance of maternal homeostasis as a secondary, non-specific mechanism.

By indicating on page 63 of the CLH dossier '*the disturbance of choline uptake and homeostasis by DEA'* and therefore agreeing that DEA disturbs maternal homeostasis, and then still considering *'the adverse developmental effects of DEA (…) as a specific intrinsic property of the substance*', the CLH dossier submitter seems to be in clear contrast to the wording of the above-mentioned CLP sections.

Inducing a choline deficiency in the maternal animals should be seen as malnutrition and therefore disturbance of maternal homeostasis leading to secondary adverse effects in the fetuses, and not as a specific, intrinsic property of the substance to induce reproductive toxicity.

Taking into account the species differences in susceptibility in choline deficiency and the disruption of maternal homeostasis in the drinking water study leading to secondary effects on reproduction, classification as reproductive toxicant cat. 2 (H361) is considered the most appropriate in line with the criteria laid down in Regulation EC 1272/2008 (CLP)

Specific target organ toxicity-repeated exposure

The registrants support the proposed modification of the current harmonized classification STOT RE 2 (H373) by adding the target organs haematopoetic system, kidney and nervous system (conclusion on page 83 of the CLH dossier). In the conclusion on classification and labelling for STOT RE it is stated that "*The liver was also identified as a target organ, but with less coherence across studies".* When considering the animal data on DEA, it becomes evident that liver organ toxicity is observed in two species at concentrations that are relevant for classification as Specific Target Organ Toxicity - Repeated Exposure (STOT RE). Given the following data, it is recommended that the modified harmonized classification should include the liver as a target organ, as it has already been identified in the self-classification of the registrants.

In the OECD 443 oral drinking water study, male and female Wistar rats showed increased absolute (112%/115%) and relative (124%/125%) liver weights, as well as centrilobular hypertrophy (3/10 males, 10/10 females) at the high dose of 1000 ppm (approx. 128 mg/kg bw). Additionally, increased albumin levels were observed in both sexes, indicating liver dysfunction. In males, biomarkers of hepatobiliary damage, such as aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activity, were further increased (+ 66% and + 54% respectively) at 1000 ppm.

The F1 rearing animals in cohort 1A and 1B of the high dose group also showed increased liver weights and histopathological correlates on post-natal day 92 at necropsy. Females exhibited increased absolute liver weight up to + 33% in cohort 1A and males + 13 % in cohort 1B and relative liver weights were increased up to + 38% (cohort 1B) and + 57% (Cohort 1A) in both sexes. Centrilobular hypertrophy was observed in the liver of 8 males and 14 females, along with peripheral hypertrophy in three males. Increased AST activities were noted in male and female F1 animals (+ 34% and + 66%), and increased ALP activities were observed in males (+ 85%). Fatty changes in the peripheral area of the liver were seen in 14 males and five females.

In F1 males and females in cohort 1A and 1B of the mid dose group 300 ppm (approx. 37 mg/kg bw), slighter increase of absolute and relative liver weights (up to + 9 %/+14 % and +25 %/ +31 %) and fatty changes in the peripheral area of the liver were observed in 8 males.

Furthermore, the oral exposure to DEA led to a drastic dose-dependent reduction of choline content in females and males, by -73% and -66% respectively, in the liver of 90-day-old F1 animals at the lowest tested dose of 100 ppm (approximately 13 mg/kg bw). Similar effects were observed in the oral drinking water range finding study (modified OECD 421) where male and female Wistar rats showed increased organ weights (≥ 500 ppm; approx. 46 mg/kg bw/day), peripheral fatty changes (≥ 1000 ppm;

approx. 95 mg/kg bw), centrilobular (≥ 1500 ppm; approx. 137 mg/kg bw/day, females) and diffuse hypertrophy (≥ 1500 ppm, males) in the liver. The choline content in the liver was dose dependently reduced by -62 %/-43 % in the liver (≥ 1000/500 ppm males/females).

In the 13-week oral drinking water study with B6C3F1 mice (NTP, 1992) males and females showed dose-dependent increases in absolute and relative liver weights (≥630 ppm equal to 104 mg/kg bw/d in males and 142 mg/kg bw/d in females), accompanied by elevated enzyme activities (serum alanine aminotransferase and sorbitol dehydrogenase) and multiple morphological changes in the liver. These changes were apparent at the lowest dose (630 ppm) and included hypertrophy, increased eosinophilia, disrupted hepatic cords, increased nuclear pleomorphism, and multinucleated hepatocytes with increasing severity grades. Additionally, an increasing incidence of hepatocellular necrosis was observed at doses ≥ 1250 ppm (175 mg/kg bw/d) (females) and ≥ 2500 ppm (884 mg/kg bw/d) (males).

In conclusion, the increased liver weights in B6F3C1 mice, along with the cytological alterations in the liver occurring at concentrations of ≥ 630 ppm, provide clear evidence of specific organ toxicity. The significant decrease in choline content in the liver, observed at low concentrations of 100 ppm in both sexes of Wistar rats, further indicates a significant functional alteration in the organ. The concentration-dependent increase in liver weights in the rats was accompanied by elevated levels of albumin, aspartate aminotransferase, and alkaline phosphatase activities at 1000 ppm. Centrilobular hypertrophy and fatty change in the peripheral area of the rat liver were observed at doses ≥ 300 ppm. This combination of findings is considered adverse according to the criteria laid out in Hall et al. (2012). The adverse liver changes in mice and rats are considered relevant to humans and support the classification of DEA as STOT RE category 2 for liver toxicity as they all occurred in the same dose range of ≤100 mg/kg bw/day.

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Appendix

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- **Nouryon Functional Chemicals AB**, Box 47067, 40258 Göteborg, Sweden
- **INEOS Europe AG**, Division Oxide, 3 Avenue des Uttins, Rolle, CH-1180, Switzerland

- **SASOL Germany GmbH**, Paul-Baumann-Str. 1, D – 45772 Marl, Germany