Comments by American Pacific (through its Only Representative Labcorp) on the Proposal for Harmonised Classification and Labelling (CLH Report) by Ministry of Health, Madrid, Spain – Part 1

PUBLIC SUBSTANCE NAME: 2-BROMO-3,3,3-TRIFLUOROPROP-1-ENE

EC NUMBER: 627-872-0

CAS NUMBER: 1514-82-5

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1 Background

The substance, 2-bromo-3,3,3-trifluoroprop-1-ene (2-BTP), is not currently listed on Annex VI to the classification and labeling regulation (EC No 1272/2008) (CLP) and has not been considered for harmonised classification and labelling previously. On September 28, 2022, the Spanish Ministry of Health (Madrid, Spain) submitted a proposal for harmonized classification and labelling (CLH, 2022) for consideration by the European Chemicals Agency (ECHA) Risk Assessment Committee (RAC). The CLH report has now been published for public consultation such that ECHA may receive information from stakeholders to consider as they evaluate the CLH proposal. Accordingly, Labcorp is providing technical comments on the CLH report and proposed classification on behalf of American Pacific Corporation (AMPAC, Cedar City, Utah, United States) as their Only Representative.

The comments provided herein focus on the Spanish Ministry of Health proposed harmonized classification for reproductive toxicity (Repr. 1B; H360FD). Reproductive toxicity classification is segmented into considerations of (1) adverse effects on sexual function and fertility and (2) adverse effects on development of the offspring. Each of these categories of effects is discussed in the following sections. Based on a review of the available information, there is no clear evidence of reproductive or development effects in the absence of other non-specific consequences and available interspecies and mechanistic information also raises question about the human relevancy of the effects observed. Therefore, the most appropriate classification for 2-BTP would be Category 2 (H361: Suspected of damaging fertility or the unborn child).

2 Adverse effects on sexual function and fertility

According to ECHA (2017) Annex I: 3.7.1.3., adverse effects on sexual function and fertility includes, "*alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems*."

The CLH (2022) proposal concluded there was a concern for effects on sexual function and fertility based on the results of two inhalation reproduction/developmental screening tests (OECD 421) (Anonymous, 2013a; Anonymous, 2014). This conclusion relied upon, "*a concern related to sexual function and fertility since common effects on both endpoints are noted in the two OECD TG 421 studies, such as longer mean pre-coital interval and a longer duration of gestation. Besides these common effects, several adverse effects on sexual function and fertility, such as longer estrous cycles, decreases in mating index, copulation plugs, sperm counts, fertility and gestation indices and number of implantations, and changes in male reproductive organ weights are clearly observed in the first screening study*" (page 35 of CLH Report, 2022). "[S]*ome of the findings reported in the first OECD TG 421 were consistently observed in the second study (Anonymous, 2014) at the two highest doses tested (only statistically significant at the high dose), i.e. lower pituitary weights, longer mean pre-coital interval and duration of gestation"* (page 35 of CLH Report, 2022).

Further, it was stated that, "*All the effects in sexual function and fertility parameters lead to a clear reduction in the fertility and gestation indices. Since these alterations are observed in the absence of any other marked systemic effects (such as lethality, dramatic reductions in absolute body weight, organ toxicity, histopathological findings), they can be considered related to treatment and not a secondary consequence of systemic toxicity*" (page 35 of CLH Report, 2022).

Spain's CLH proposal (Category 1B) relies predominantly on the supposition that there are no "*marked systemic effects*" observed in conjunction with reproductive effects. However, the definition for Category 1B states that, "*classification of a substance in this Category 1B 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, <u>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</u>. 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*" (ECHA, 2017). While there is a lack of overt toxicity as defined by the Spanish Ministry of Health in the available screening reproductive/developmental studies, there are several non-specific signs of parental toxicity that suggest a Category 2 classification to be a more appropriate classification for 2-BTP.

2.1 Clinical Observations

First, with regard to clinical signs of toxicity (e.g., mortality, clinical observations, body weight, food and water consumption), consistent observations were identified in the reproductive studies (Anonymous, 2013a; 2014) as well as a 13-week repeat dose study (Anonymous, 2013b) and acute inhalation toxicity study (Anonymous, 2004).

- In the acute toxicity test (Anonymous, 2004), evidence of sedation and decreased motor activity along with respiratory tract changes were observed at dose concentrations up to 45,850 ppm for 30 minutes. In a second study, after 4-hour exposures (up to 25,000 ppm), mortality associated with bronchiolar lesions were observed 24 hours after exposure. This shows that a shorter exposure period at high concentrations can be tolerated but more prolonged repeated exposures at lower dose concentrations can lead to adverse reactions, including death. The fact that mortality was seen up to 24 hours after exposure, does suggest that effects of treatment are not necessarily resolved following withdrawal of exposure.

- In the initial reproduction/development screening study (Anonymous, 2013a), five females (and litters) at 505 ppm and 2 females (and litters) at 2900 ppm were sacrificed due to poor condition. Clinical signs were observed at each dose level:

    - 198 ppm: underactivity, unresponsiveness, piloerection and partially closed eyelids were noted at a much reduced incidence than that seen at higher levels; only underactivity and piloerection were noted immediately after exposure during gestation only.
    - 505 ppm: underactivity and piloerection were noted throughout, and unresponsiveness and eyelids partially closed were noted on fewer occasions. In males, the signs were not noted following exposure, but in females during gestation and lactation, underactivity and piloerection, and in females during gestation only, eyelids partially closed were noted after exposure but not at the end of the working day.
    - 2900 ppm: underactivity, unresponsiveness, piloerection and eyelids partially closed were noted throughout. In males, these signs were not apparent after completion of exposures. In females, some of the above signs and also hunched posture were noted following exposure, occasionally in Week 2 (hunched posture only) and generally throughout gestation, but these were not present at the check for signs at the end of the working day (up to 2 hours later).

- In the sub-chronic 13-week repeated inhalation study (Anonymous, 2013b), at similar treatment levels as the reproduction screening study, no treatment related mortality was observed, however clinicals signs included: unresponsiveness to external stimuli, underactivity, slow and/or shallow breathing, hunched posture (Day 1 and 94 only), grinding teeth (at 505 ppm, Day 25 only), piloerection and partially closed eyelids (closed eyelids on Day 1). These signs generally resolved quickly on cessation of exposure to the test article, although the number and prevalence of signs increased with exposure level (Anonymous, 2013b). Sensory reactivity, grip strength and motor activity were also examined. Forelimb and hindlimb grip strength were decreased at 12 weeks at 2876 ppm and at 505 ppm (males only), and these signs were not fully resolved at week 4 of

recovery at 2876 ppm. Motor activity scores were decreased at 12 weeks at 2876 ppm and at 505 ppm, and these signs were not fully resolved at week 4 of recovery at 2876 ppm. Thus, the 13-week study demonstrated that behavioral and CNS effects are apparent during and after exposure (up to 4 weeks post-exposure).

- In the second reproduction/development screening study (Anonymous, 2014), which was identified as the key study in the dossier, lower concentrations were tested (50, 100, and 175 ppm for 6 hour/daily) and a separate group was exposed at 10,000 ppm for 5 minutes/day (to model expected human exposures). No mortality was observed during the study, while red material around the nose was noted for males and females in all groups, including the control group, at 1 hour following the 6-hour exposure period generally throughout the respective exposure periods. Sensory or motor activity tests were not conducted in this study.

The CLH (2022) proposal suggested that the observed clinical signs were not of significance, stating that, "*clinical signs, underactivity, unresponsiveness, piloerection and partially closed eyelids, were observed in males and females at the two highest doses. These effects were also observed in the low dose but to a much lesser extent. All these effects were reversible immediately after the daily 6-hour exposure or before the end of the working day*." However, the weight of evidence from all studies indicates that obvious clinical signs (e.g., underactivity, piloerection) may resolve a few hours after exposure, however more subtle effects (e.g., grip strength, motor activity) can persist after weeks of recovery.

It is notable that CNS depression in animals from the reproductive studies was restricted to dose levels of 198 ppm and above (Anonymous, 2013a) and only at 10,000 ppm for 5 minute exposure (Anonymous, 2014) but it is apparent that the CNS is a target organ. The lack of clinical signs seen below 198 ppm in one study (Anonymous, 2014) does not necessarily preclude any adaptive alteration to the CNS below this dose level. Physiological change as a result of non-specific toxicity can explain the variability seen in treated animal responses, due to varying sensitivity or susceptibility of individual animals. This is also the reason why neonatal mortality at 175 ppm was not consistent amongst individuals and is likely to be due to differences in maternal tolerance to 2-BTP (discussed further in Section 3). Effects on general body weight gain and a decline in food consumption (i.e., signs of non-specific toxicity), were also seen in these dose groups.

In addition, respiratory irritation as observed in these studies can cause significant effects upon adult animals, which compromises general health and well-being of the animals. These irritant effects were also observed in adult rats during gestation, where an increase in water consumption was exhibited in females, plus the observation of nasal discharge in greater proportions in females experiencing deleterious reproductive effects (Anonymous, 2013a). The histopathological changes in the nasal turbinate of rats also provides an insight into the irritant type effect, contributing to the overall malaise of animals during and after exposure. As summarized in Section 2.2, there was also a reduced weight gain and food consumption, which is a consequence of post-treatment general malaise. This gives an indication as to the compromised health status of the animals during treatment. The CNS/irritant effects observed may influence normal reproduction function and any effects may be secondary to initial clinical manifestations (even if not overt). Indeed, neuroactive agents can enhance the stress response in rodents, and mild or

severe stress responses are known to affect reproductive system parameters in standard reproduction studies (Everds et al. 2013). Thus, the CLH proposal inappropriately ignores the confounding effects of general clinical signs by focusing only on overt mortality or severe body weight reductions. Accounting for these potential confounding effects is necessary to fully evaluate the study results for purposes of classification.

2.2 Body Weight, Food and Water Consumption

Consistent with clinical observations, effects on body weight, food consumption, and water consumption were observed to increase with treatment concentration. Study findings for males and females are summarized below.

Male Animals

- In the 13-week repeated exposure study, body weights were significantly reduced at 199 (↓22%), 505 (↓27%), or 2876 (↓48%) ppm and still reduced after 4 weeks of recovery compared to controls (↓22% at 2876 ppm) (Anonymous, 2031b). Similarly, food consumption was significantly lower in a dose-response fashion (↓10%, ↓15%, ↓26%) and remained low over the recovery period (↓10% at 2876 ppm).

- In the first reproductive screening study, mean body weight gain was lower than the controls in all test groups (gain Day 1-50: ↓23%, ↓37%, ↓53% at 198, 505 and 2900 ppm respectively) (Anonymous, 2013a). Food consumption was also reduced for the 2 week period prior to pairing (↓12% at 198 ppm, ↓24% at 505 ppm, and ↓24% at 2900 ppm).

- In the second reproductive screening study, mean body weight gain over 28 days was lower than controls at 100 ppm (↓27%), 175 (↓13%), and 10,000 ppm (↓38), but only significant at 100 and 10,000 ppm (Anonymous, 2014). Mean food consumption was not significantly different, except at 10,000 ppm during the pre-mating period.

Female Animals

- In the 13-week repeated exposure study, body weights were significantly reduced at 505 (↓21%) and 2876 (↓45%) ppm and still reduced after 4 weeks of recovery compared to controls (↓15% at 2876 ppm) (Anonymous, 2031b). Food consumption was significantly lower at 2876 ppm (↓19%).

- In the first reproductive screening study, pre-mating mean body weight gain of females was lower at 2900 ppm (gain Day 1-15: ↓81%) (Anonymous, 2013a). Food consumption was also reduced pre-mating (↓8% at 198 ppm, ↓18% at 505 ppm, and ↓30% at 2900 ppm), while water consumption was higher than controls (↑17% at 198 ppm, ↑59% at 505 ppm, and ↑72% at 2900 ppm).

During gestation, mean body weight gain was also lower than controls at each treatment level (Days 0-20: ↓8%, ↓17%, ↓57%). Food consumption remained low (Day 0-19: ↓10%, ↓11%, ↓22%) and water consumption increased for all treatments (up to 23% higher at 505 ppm).

During lactation, body weight gains were reduced at 198 ppm (Days 1-10: 59% of control) and 505 ppm (Days 1-10: 13% of control), while no litters survived at 2900 ppm and hence no adult female body weights were recorded. Food consumption remained low (↓27% at 198 ppm and ↓40% at 505 ppm) and water consumption was slightly lower compared to controls.

- In the second reproductive screening study, mean body weight gain was not significantly different during pre-mating, gestation, or lactation with only slight reductions (≤5%) (Anonymous, 2014). A significant reduction was only observed on Day 11-14 of gestation for 175 (↓33%) and 10,000 (↓22%) ppm. Similarly, food consumption was not statistically significant over the course of the study. Water consumption was significantly increased during gestation at 175 ppm (↑20%) and significantly reduced during Days 1-4 of lactation at 50 (↓21%), 100 (↓21%), and 175 (↓25%) ppm.

Based on the available studies it is apparent that exposure to increasing concentrations of 2-BTP significantly affects overall body weight, body weight gain, food consumption, and water consumption. These changes were demonstrated to persist, at higher concentrations, even after cessation of exposure. The CLH proposal (2022) deemed these effects as not toxicologically relevant since body weight gains were only accompanied by slight decreases in mean body weight values. However, dietary restriction studies resulting in body weight decreases >16% have been associated with changes in estrous cycle, persistent diestrus, decreased corpora lutea, and decreased fertility (Everds et al. 2013). This magnitude of body weight change was found at higher 2-BTP concentrations (Anonymous, 2013a), thus indicating a toxicologically relevant result. In addition, the consistent dose-response body weight change pattern in combination with the observed clinical observations indicates a detrimental effect on the overall health of the animals prior to and throughout pregnancy. These range from subtle, minor changes (e.g., signs of respiratory irritation, lower water consumption) at low concentrations (50 to 100 ppm), to clear signs (e.g., significant body weight changes, food consumption changes, and persistent clinical signs) of distress at higher concentrations (175 – 10,000 ppm).

2.3 Reproductive Effects

The CLH (2022) proposal notes that the reproduction classification was related to alterations, "*observed in the absence of any other marked systemic effects (such as lethality, dramatic reductions in absolute body weight, organ toxicity, histopathological findings), they can be considered related to treatment and not a secondary consequence of systemic toxicity*." However, the focus on lack of marked systemic effects is insufficient for examining reproductive effects according to CLP guidance (ECHA, 2017). For example, ECHA (Section 3.7.2.2.1.1.) states, "*mating behaviour can be influenced by parental effects not directly*

related to reproduction (e.g., sedation, paralysis), and such effects on mating behaviour may not warrant classification." Further, ECHA (Annex I: 3.7.2.4.4) states, "*observation of increased incidence of significant clinical signs of toxicity in treated dams relative to the control group is useful in evaluating maternal toxicity. If this is to be used as the basis for the assessment of maternal toxicity, the types, incidence, degree and duration of clinical signs shall be reported in the study. Clinical signs of maternal intoxication include: coma, prostration, hyperactivity, loss of righting reflex, ataxia, or laboured breathing*." Ultimately a comparison between the effects on fertility/development and the severity of other effects should be considered. A discussion of the observed effects in the screening studies with additional examination is provided below.

2.4 Spermatogenic Evaluations and Male Reproductive Organ Pathology

Sperm parameters (mean testicular and epididymal sperm numbers and sperm production rate, motility, progressive motility, and morphology) were not statistically different than controls at 50, 100, 175, or 10,000 ppm (Anonymous, 2014). Related organ weight changes (left epididymis weight and right testis weight at 10,000 ppm) were considered secondary to body weight changes (Anonymous, 2014). No other organ changes were attributed to 2-BTP (Anonymous, 2014). At higher concentrations significant changes were noted in some sperm parameters: 198 ppm (reduced sperm velocity, total abnormal sperm), 505 ppm (reduced sperm velocity, total abnormal sperm), and 2900 ppm (reduced sperm velocity, Beat Cross frequency, total abnormal sperm, reduction in number in cauda epididymis) (Anonymous, 2013a). Consistent with terminal body weight reductions, weights of prostate, seminal vesicles, epididymis, and pituitary were significantly reduced compared to controls (Anonymous, 2013a). It should be noted that male accessory sex organs (e.g., seminal vesicles, and prostate, and to a lesser extent epididymis) and spermatogenesis are sensitive to stress and body weight changes (Everds et al, 2013), thus these effects can be secondary to the overall clinical distress observed as exposure concentrations increase.

2.5 Estrous Cycle

Estrous cycle length was not significantly affected at concentrations of 50, 100, 175, or 10,000 ppm and cycle lengths were within historical control ranges (Anonymous, 2014). At higher doses, estrous cycles lengthened (Anonymous, 2013a). At 198 ppm, all cycles were within 4-5 days (similar to controls), although there was a significant shift to 5 day cycles. At 505 ppm and 2900 ppm cycle lengths were significantly increased compared to controls (mean of 5.2 and 7.4 days respectively compared to 4.2 days for controls). It should be noted that estrous cycles are particularly sensitive to stress and decreased food intake and body weight (Everds et al., 2013). Thus, the concomitant clinical signs, body weight changes, and food consumption patterns at higher concentrations influenced the estrous cycles rather than a direct effect on the reproductive system.

2.6 Pre-coital Interval, Mating Performance and Fertility

The pre-coital interval at 175 ppm was increased compared to controls (4.5 days vs 2.9 days), however, it was not statistically significant and within the historical control range (1.8-4.7 days) (Anonymous, 2014).

The mating, fertility, and copulation indices for males and females were unaffected at 50, 100, 175, and 10,000 ppm (Anonymous, 2014). At 198 ppm, pre-coital interval, number of copulation plugs, percent mating, conception rate, and fertility index were not significantly different than controls (Anonymous, 2014a). At 505 ppm, all pairings were successful, although there were significantly fewer copulation plugs. At 2900 ppm, significantly fewer copulation plugs were identified, and percent mating and fertility index were significantly reduced. In general, mating and fertility are known to be affected by stress, which was clearly demonstrated at 198 ppm, 505 ppm and 2900 ppm (Everds et al. 2013; Anonymous, 2013a).

2.7 Gestational Length

Mean gestation lengths were significantly longer than controls at 100 and 175 ppm, although the lengths at 100 ppm were within the historical control range and considered non-adverse (Anonymous, 2014). No effects were observed at 50 or 10,000 ppm. Higher concentrations yielded significantly longer gestational lengths at 198, 505, and 2900 ppm (Anonymous, 2013a), consistent with increasing effects observed on overall condition, body weights, food and water consumption.

2.8 Female reproductive organ pathology

The mean numbers of corpora lutea, unaccounted-for sites, implantation sites, or primary ovarian follicles within the 50, 100, 175, and 10,000 ppm groups were similar to controls (Anonymous, 2014). At higher concentrations, a slight reduction in the size of corpora lutea was seen in the ovaries of 4 females exposed to 2900 ppm and in a single female exposed to 198 ppm, while no effects were found on primordial follicle counts (Anonymous, 2013a).

2.9 Mode of Action

Consideration of potential modes of action (e.g., endocrine modulation) and mechanistic data are also important for classification purposes (ECHA, 2017). For example, estrous cycle lengths did increase in the first reproduction study at higher dose levels (Anonymous, 2013a) but the most prominent feature was the number of irregular cycles amongst treated females. This is an inconsistent effect since a variety of estrous cycle lengths were recorded for females at higher dose levels (505 and 2900 ppm). At these higher dose levels, the influence of toxicity to the adult female is a more likely explanation as to the cause of the effect identified as irregular estrous cycles (Everds et al., 2013).

The screening study findings indicate that the mode of action is unlikely to be through endocrine disruption as there is no consistency for individual animal findings within dose groups. The effect upon estrous cycle length was not consistent as demonstrated in the second of two reproduction studies (Anonymous, 2014). The group mean estrous cycle length was similar between the control and high dose group (5.2 days as opposed to 5.0 days). However, there was a significant intergroup difference in individual estrous cycle lengths for high dose animals ranging from 3.3 days to 7 days. This is of note as variance to the mean value indicates a contradictory finding suggesting not one specific mode of action, such as endocrine disruption.

Further, this intergroup variation is less likely to represent a direct effect on estrous cycles as the material would have to have both an agonistic and antagonistic effect at the same exposure duration and dose. The implication is that the health of the individual animal is compromised such that normal physiological function is impaired by a non-endocrine mechanism. A comparison can be made to altered nutritional status, such as in the case of vitamin A deficiency, where an impact on parturition and neonatal survival/development can be seen. This is due to altered physiology within the parent animal and not attributed to one specific event based on the number of chemical interactions involving vitamin A and the multitude of adverse responses vitamin A deficiency has as a consequence (Ross et al. 2000). Based on the observable and persistent (yet not overt) clinical signs and body weight changes, the weight of the evidence suggests a non-endocrine mediated physiological effect is the primary factor resulting in the inconsistent reproductive outcomes observed primarily at higher concentrations.

2.10 Severity of Reproductive Effects

ECHA notes that characterization of the severity of various reproductive effects can be difficult, however, it can be informative to consider the types of reproductive effects observed, their severity, and relation to other confounding factors (e.g., parental stress). Structured evaluation methods are available to examine the severity of reproductive effects (ECETOC, 2002; Moore et al. 2013). Accordingly, a review of the existing data in relation to the protocol developed by Moore et al. (2013) is presented:

- Is there a substance-related response?

As discussed in Section 2.2, there are effects observed in male and female animals primarily at higher concentrations in the presence of body weight, food consumption and clinical effects.

- What is the initial level of concern?

Moore et al. (2013) notes that the "*reproductive cycle – encompassing sexual function, fertility, and development – is a highly complex and integrated process, and reproductive health also reflects the general health and wellbeing of the organism as a whole*." Thus, consideration of the observed effects should integrate multiple factors. For example, changes in male accessory organ weights are considered of LOW concern when changes in body weight and no other histopathological changes are noted (ECETOC, 2002; Moore et al. 2013). This is the case with the existing data for 2-BTP, effects on male reproductive function (organ weights and sperm parameters) are only observed at higher doses (505 or 2900 ppm) where effects to body weights, food consumption, and clinical signs are also apparent but no signs of toxicity in histopathological examinations. Thus, observations in male animals are of limited concern and consistent with a pattern of secondary effects.

Female reproductive indices such as estrous cycle length, pre-coital interval, and period of gestation are defined as LOW to MODERATE concern when no other alterations are observed (ECETOC, 2002; Moore et al. 2013). This is also the case for 2-BTP, these changes were not observed at lower concentrations, and were not consistent with other indices (e.g., fertility index, mating index). That is, up to 198 ppm any

changes to estrous cycle lengths, pre-coital interval, and period of gestation were not accompanied by any reduction in fertility measures or organ pathology. Only at 505 ppm and 2900 ppm were multiple indices affected, although these concentrations are also confounded by changes in body weights, food and water consumption, and clinical signs of maternal stress. As noted previously, maternal stress in rats can affect many reproductive outcomes indirectly through subtle changes in physiological responses and cannot be discounted (Everds et al. 2013).

- Can a mode of action or causal relationship be established?

No mechanistic studies are available to establish a mode of action. However, results from the reproduction/developmental studies indicate a non-specific mechanism confounded by the poor condition of animals observed at higher doses. Further, an endocrine related mechanism is not clear as discussed further in Section 2.9.

- What is the overall level of concern?

The level of concern (LOW to MODERATE) is unchanged and is consistent with a Category 2 classification.

- Are there data to indicate effects on or via lactation?

No further data on effects related to lactation are available. As noted in Section 3, there was an absence of milk from the F1 offspring at higher concentrations, indicating a lack of nursing and maternal care. Thus, no additional classification is necessary based on lactation.

3 Effects on Development

According to ECHA (2017) Annex I: 3.7.1.4., adverse effects on development of the offspring includes, "*any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parent prior to conception, or exposure of the developing offspring during prenatal development, or postnatally, to the time of sexual maturation. However, it is considered that classification under the heading of developmental toxicity is primarily intended to provide a hazard warning for pregnant women, and for men and women of reproductive capacity. Therefore, for pragmatic purposes of classification, developmental toxicity essentially means adverse effects induced during pregnancy, or as a result of parental exposure. These effects can be manifested at any point in the life span of the organism. The major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency*."

In the CLH (2022) proposal, a concern for developmental toxicity was determined based on the two available screening studies, as follows:

- "*In the first study (Anonymous, 2013c), a dose-dependent reduction in the post-implantation survival index was observed at all doses tested, being statistically significant at the two highest doses. A statistically significant and dose-dependent reduction in total litter size and live litter size*

were also reported in all treatment groups. According to these findings, dose-dependent decreases in viability and live birth indices were recorded as well. These decreases were only statistically significant at the mid dose since they were not calculated for the high dose, due to the reduced number of pups (only one pup alive). All these described effects occurred in the absence of a clear maternal systemic toxicity (such as lethality, dramatic reductions in absolute body weight, organ toxicity, histopathological findings). Therefore, the reported effects on development can be considered treatment-related and not a secondary consequence of maternal systemic toxicity."

- "*In the second study (Anonymous, 2014), a statistically significant decrease in postnatal survival from birth to PND 4 was observed at the two highest doses. Additionally, an increase in the incidence of interventricular septal defect, which is a severe effect, was reported at the high-dose level. Both adverse effects occurred in absence of systemic maternal toxicity. While the septal defect may have been related to the apparent developmental delay noted in the 175 ppm group, it was considered test substance-related and adverse when coupled with the reduction in postnatal survival noted in this group. In addition, this effect was also observed at 100 ppm with a lower incidence. The available information in this study shows that 2-BTP has an adverse effect on the development of rats*."

According to the CLP (2022) proposal it is suggested that "[n]*o marked maternal toxicity can be observed in the first study and, in the second study effects are observed without maternal toxicity. In addition, a reduction in postnatal survival was consistently reported in both studies. For this reason, the developmental toxicity findings should therefore be considered as treatment-related effects which have not been demonstrated to be secondary to maternal toxicity."* As discussed in Section 2, there are signs (e.g., clinical and CNS effects, body weight changes, and food and water consumption changes) that suggest maternal toxicity is apparent and persistent (i.e., occurring hours to weeks after exposure), although not necessarily overt.

CLP guidance (Annex I: 3.7.2.4.1, ECHA, 2017) recommends that, "*weight of evidence approach, using all available studies, shall be used to determine the degree of influence that shall be attributed to maternal toxicity when interpreting the criteria for classification for developmental effects*." It is not apparent that the CLH (2022) proposal considered all available data or potential confounding factors. For example, as mentioned in Section 3.7.2.2.1.1. of the CLP guidance (ECHA, 2017), "[a]*dverse effects on postnatal survival and growth seen only at dose levels causing maternal toxicity may be due to lack of maternal care or other causes such as adverse effects on or via lactation or developmental toxicity. In case post-natal effects are caused by lack of maternal care classification for developmental effects may not be warranted*." In the available studies, the clinical signs noted at higher concentrations resulted in behavioral and motor activity changes in exposed animals which can directly relate to maternal stress and maternal care. Further discussion of the observed developmental effects is provided below in relation to maternal toxicity.

3.1 F1 External Observations

The general physical condition of all F1 pups was unaffected at concentrations of 50, 100, 175, and 10,000 ppm (Anonymous, 2014). There was no difference in the pups found dead between treatment groups, and in some cases, pups were found missing during PND 1-4 and presumed to have been cannibalized. In surviving offspring exposed to 198, 505, or 2900 ppm, no clinical signs were apparent, except for the majority of offspring at 505 ppm were noted as cold on Day 1 (Anonymous, 2013a).

3.2 F1 Litter Size, Survival Indices, Sex Ratio

The mean number of pups born, live litter size, the percentage of males at birth, and postnatal survival on PND 0 in the 50, 100, 175, and 10,000 ppm groups were similar to the control group values (Anonymous, 2014). Postnatal survival (PND 4) was lower but not statistically significant at 100 ppm and within the historical control range. In the 175 ppm group, 1 female (no. 91654) had a total litter loss on PND 2, while survival in other dams was within or slightly lower than the historical control range.

Findings after exposure to higher concentrations of 2-BTP are as follows (Anonymous, 2013a):

- 198 ppm: significantly reduced post-implantation survival and lower part-partum litter survival. However, the post-implantation survival index, live birth index, and viability index were not significantly different than controls. No effect on sex ratio.
- 505 ppm: significantly reduced post-implantation survival and lower part-partum litter survival. In addition, a number of offspring were sacrificed due to poor condition including reduced activity and reduced body temperature. The post-implantation survival index, live birth index, and viability index were significantly different than controls. No effect on sex ratio.
- 2900 ppm: only one female produced a litter, and only one pup was alive on Day 1 and sacrificed due to poor condition.

3.3 F1 Body Weights

The body weight and body weight gains of pups were unaffected at all treatment concentrations (Anonymous, 2014). There were no effects on mean pup weight on Day 1 at 198 or 505 ppm, while body weight gain from Day 1 to Day 10 was slightly lower than the controls at 198 and 505 ppm (but only significant for males at 505 ppm) (Anonymous, 2013a). The single female pup at 2900 ppm was slightly lower than the control (5.5 g compared to 6.4 g).

3.4 F1 Pathology

Few developmental variations were noted in the F1 generation (Anonymous, 2014). At 175 ppm, five pups were noted with an interventricular septal defect (4 of which were in the dam with total liter loss – No. 91654). This defect was not identified at higher concentrations (Anonymous, 2013a) and thus is an isolated occurrence. Offspring of dams exposed to 198, 505, or 2900 ppm, were noted frequently with no milk in the stomach, especially in those dying in the very early days of lactation, most apparent at 505

ppm (Anonymous, 2013a). There were no other treatment related effects on the offspring. The lack of milk in the stomach up to PND 10 is a clear indication of the lack of maternal care (i.e., nursing) as noted by the CLH (2017) guidance.

3.5 Severity of Developmental Effects

ECHA notes that characterization of the severity of various reproductive effects can be difficult, however, it can be informative to consider the types of reproductive effects observed, their severity, and relation to other confounding factors (e.g., parental stress). Structured evaluation methods are available to examine the severity of reproductive effects (ECETOC, 2002; Moore et al. 2013). In addition to factors related to maternal toxicity, consistency of developmental abnormalities is important to consider. For developmental effects, the level of concern is generally raised when findings occur in groups or clusters or when common to multiple fetuses in multiple litters (ECETOC, 2002; Moore et al. 2013). Accordingly, a review of the existing data in relation to the protocol developed by Moore et al. (2013) is presented:

- Is there a substance-related response?

As discussed in the preceding sections, there are effects observed F1 litters primarily at higher concentrations in the presence of maternal body weight, food consumption and clinical effects.

- What is the initial level of concern?

In the screening studies, adult effects (i.e., body weight, food consumption, clinical signs) were seen at similar dose levels to effects upon reproduction, which demonstrates an association between these events (Anonymous, 2013a; Anonymous, 2014). It must be repeated that all of the available studies (Anonymous, 2004; 2013a,b; 2014) demonstrated that exposure to 2-BTP resulted in clinical/CNS related detriments and body weight reductions that persisted hours or weeks after exposure. Based on the observations of CNS depression especially at higher dose levels, it can be concluded that adult health has been affected and the changes are sufficient to influence normal reproductive physiology when compared with animals not exposed to 2-BTP. A general lack of well-being reduces the capability of adult females to maintain their offspring post-partum and thereby leading to greater neonatal mortality due to lack of maternal care from compromised adults. The lack of maternal care is clearly demonstrated by the notation of pups being cold (at 505 ppm) and having a lack of milk in the stomach (Anonymous, 2013a). Thus, effects observed at higher concentrations related to post-implantation survival index, live birth index, and viability index are considered LOW to MODERATE as they occurred in the presence of signs of maternal distress.

In regard to the interventricular septal defect (VSD) noted in 5 pups from 2 litters, an independent review (York, 2014) was performed and determined that, "[s]*ince the VSDs were all found in dead pups at or near birth, several explanations are possible: the VSD were likely due to autolysis of this membranous tissue that was closing the septum, or the fetal growth was retarded in utero and this prevented or was delaying the normal closure. The cardiac defect should not be given much weight since there were no other developmental effects at 100 ppm. The OECD TG 421 does not suggest visceral examination of dead pups*

but only specifies external examination of dead pups and notation if they had milk in their stomachs (evidence of being alive and nursing). The internal examination of the dead fetuses may be misleading." Further, this defect was not identified in the other screening study using higher concentrations (Anonymous, 2013a) and therefore appears to be an isolated incident that primarily affected 1 dam. Thus, due to the isolated finding in few pups of 2 litters, no other cluster of related defects, and no dose-response relationship of this defect, the overall concern would be LOW to MODERATE.

When considering the weight of evidence, the CLH (2022) proposal erred in its interpretation of developmental effects in the absence of maternal toxicity. While there was not marked maternal toxicity (mortality, severe body weight loss), the repeat concentration studies demonstrated a consistent pattern of poor maternal health with increasing concentration that persisted after exposure ceased. Developmental observations at lower concentrations (100-198 ppm) were either not significant or isolated to individual pups or litters, while effects at higher concentrations (>198) were observed in the presence of signs of maternal toxicity (Anonymous, 2013a).

- Can a mode of action or causal relationship be established?

No mechanistic studies are available to establish a mode of action. However, results from the reproduction/developmental studies indicate a non-specific mechanism confounded by the poor condition of animals observed at higher doses. Further, an endocrine related mechanism is not clear as discussed further in Section 2.9.

- What is the overall level of concern?

The level of concern (LOW to MODERATE) is unchanged and is consistent with a Category 2 classification.

- Are there data to indicate effects on or via lactation?

No further data on effects related to lactation are available. As noted above, there was an absence of milk from the F1 offspring at higher concentrations, indicating a lack of nursing and maternal care. Thus, no additional classification is necessary based on lactation.

4 Classification Justification for 2-BTP

The reproductive and developmental assessment provided by the Spanish Ministry of Health included a proposal for a harmonized classification for reproductive toxicity (Repr. 1B; H360FD). However, the "*decision to classify a substance based upon experimental data, and the category of classification ascribed, is determined by the availability of clear evidence (category 1B) or some evidence (category 2) of an adverse effect on sexual function and fertility or on development that <u>does not arise as a secondary non-specific consequence</u> of other toxic effects* (Moore et al., 2013; ECHA, 2017)])." Based on a review of the available information, there is not sufficient evidence for reproductive or development effects without other non-specific effects that would support this classification level, as summarized below:

- **Parental General Toxicity**: The available acute and repeat exposure concentration studies demonstrate a consistent pattern of respiratory irritancy and CNS depression resulting in a general malaise and lethargy in male and female animals. A prolonged exposure period of up to 6 hours during each day of treatment prevents full recovery from the effects that are both clinical and sub-clinical and thereby leaving the animals in a less than optimal condition.
- **Non-Specific Toxicity:** Dose-dependent changes in body weights and malnutrition (reduced food and water consumption) without signs of direct organ damage can indicate non-specific toxicity amongst adult animals. This non-specific toxicity is due to varying sensitivity and susceptibility of individual animals and is not represented in mean test results. Poor maternal health and lack of maternal care can be responsible for post-natal mortality (post-implantation loss, reduced live birth index and post-natal survival rate).
- **Lack of Consistency**: While some effects (e.g., estrus cycling, pre-coital interval, male accessory organ weights) are observed in concert with adult distress (e.g., clinical signs, body weights), there is a notable lack of consistency in developmental abnormalities (e.g., incidence of VCD abnormalities in 1 study, 5 pups, 2 litters and no others). Further, postnatal survival appears most affected at the higher concentrations where signs of lack of maternal care are apparent.
- **Mode of Action:** The mode of action does not appear to be through endocrine disruption involving normal sex hormones (estrogen, progesterone, and testosterone) since functions regulated by such hormones both increased and decreased in the same dose groups. Some adult animals experienced an increase in reproductive endpoints (e.g., pre-coital interval and estrus cycle) and others in the same dose group experienced a decrease in the same endpoints, meaning that reproductive function is not the specific target of toxicity.
- **Inter-species Variability:** It is generally accepted that rats tend to be more susceptible than humans to respiratory irritation because they have more convoluted nasal turbinates and are obligate nose breathers. Heightened respiratory irritation leads to increased water consumption, lack of appetite, and poor general condition of test animals, but would not necessarily occur in humans due to odor and taste discernment. Similarly, the rodent reproductive system is sensitive to stress (e.g., structural changes to male accessory sex organs), while canines and non-human primates have fewer structural changes observed with stress (Everds et al. 2013). Thus, any observed effect should consider the sensitive nature of the animal model and relevance to humans.

Therefore, the available data for 2-BTP does not meet the criteria for classification of Category 1B. Specifically, there is **<u>not</u>** "*clear evidence of an adverse 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*." Indeed there is evidence of primary general non-specific toxicity that can secondarily affect reproduction and development that cannot be excluded. Since

the evidence is not clear for Category 1B, the most appropriate classification for 2-BTP would be Category 2 (H361: Suspected of damaging fertility or the unborn child).

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