Glyphosate

General Comment :

The CLH report of glyphosate addressing the hazard assessments records only international agreed standard test guidelines which are then used for the comparison with the classification criteria. Not all of the available studies were reported in the CLH report itself but more studies are described in the annexed EFSA conclusion 2015 and the addenda of the Renewal Assessment Report (RAR).

In general, we would have appreciated that at least an overview table on glyphosate itself had been inserted for each endpoint by extracting all available studies in the EFSA conclusion 2015 doc/RAR indicating guideline/non-guideline, GLP, results, deviations, reliability, …

Standard testing, non-standard testing and non-testing methods shall be considered for classifying purposes (CLP regulation). We regret that for the classification of glyphosate, notwithstanding the different public available articles on glyphosate that are mentioned in the RAR and evaluated by the RMS, none of the non-standard scientific literature studies were considered in the CLH report.

Human Health

STOT RE: BE CA supports the classification as STOT RE 2, H373.

Reproductive toxicity :

BE CA would like to emphasize some effects and inconsistencies in the provided documents

- In Takahashi (1997, ASB2012-11495) study, the NOAEL for reproductive toxicity is 30 000 ppm in the CLH report, however, in the Renewal Assessment Report, the RMS chose to reduce the NOAEL to 6000 ppm based on the decrease of the gestation index (95.8, 95.8, 87.5 and 79.2 for controls, 1200, 6000 and 30 000 ppm, respectively). Considering that the gestation index is already slightly decreased at 6000 ppm, BE CA suggests to select the NOAEL at the lowest dose (1200 ppm).

- In Suresh (1993, TOX9300009) study, the dose level of 10 ppm mentioned in the CLH report is not presented in the Renewal Assessment Report. Furthermore, BE CA would like to emphasize an inconsistency in the Renewal Assessment Report: at necropsy, a higher incidence of emaciated pups was observed at the mid dose level in F1 and at the high dose in F1 and F2 pups. However, the reported body weight of these pups was inconsistent with the previous information (for F1 pups, on Day21, the mean BW of mid dose group was higher than in controls).

- In a Monsanto study (1981, TOX9552385), which was mentioned in the Renewal Assessment Report but not in the CLH dossier, in the reproductive parameters, the mating index of the F2 generation was lower than controls in each treated-groups for each mating intervals. In F1 generation, the lowest pregnancy rate was seen in the highest dose group. Furthermore, concerning the viability index, it was significantly lower during the day 4-21 interval in each

treated-groups, compared to the control. Information is lacking to interpret these changes, indeed, it is not clear which generation is affected.

- In Brooker et al. (1992, TOX9552389) study, the total litter size in the highest dose group (10000ppm) was lower than control across all four matings and remained lower than control group at day 4 in three of the four matings. Thus, BE CA does not agree with the proposed NOAEL for reproductive toxicity (10 000 ppm) and suggests the mid dose level (3000 ppm) as the NOAEL.

- In Suresh (1991, TOX9551105) study, very little information is available in the CLH report, however, the mentioned developmental NOAEL (< 1000 mg/kg bw/d) is lower than the maternal toxicity NOAEL (1000 mg/kg bw/d) considering the reduction of ossification in pups.

- In Coles and Doleman (1996, ASB2012-11499) study, an increase in post-implantation loss was observed in the mid and high dose level groups (3.7, 3.6, 11.5* and 12.1% at 0, 50, 200 and 400 mg/kg bw/d, respectively).

- In Tasker and Rodwell (1980, TOX9552392) study, significant inconsistencies were noted between the CLH report and the Renewal Assessment Report: species, duration of exposure, doses, …

- In Brooker et al. (1991, TOX9552391) study, post-implantation loss rate was significantly increased at the highest dose and out of the historical control range. Moreover, BE CA is not convinced by the reasoning on the severity of interventricular septal defect given in the Renewal Assessment Report: there was an increase of the incidence of the malformation in the highest dose (450 mg/kg bw/d) slightly outside the historical control data. Furthermore, the Renewal Assessment Report mentioned that this modification was observed in conjunction with clear signs of maternal toxicity, however, BE CA does not agree (the reduced food consumption, the BW and clinical signs data were not significantly affected).

- In Suresh et al. (1993, TOX9551106) study, in the Renewal Assessment Report, BE CA does not agree with the RMS comment on the dilated heart effect: it is mentioned that the absolute number of affected fetuses and litters were quite small and did not show a marked difference between the treated-groups. However, the provided data showed an important modification (% of fetuses with dilated heart was 0, 5.1, 5.2 and 17.9 and the % of litter incidence was 0, 23.1, 16.7 and 40.0 at 0, 20, 100 and 500 mg/kg bw/d, respectively).

- Several studies were only mentioned in the summary tables of the CLH report, thus, it is not easy to verify and interpret the indicated NOAELs: Reyna (1990, TOX9552387), Antal (1985), Brooker et al. (1991, TOX9552393), Suresh (1991, TOX9551105), Anonym (1981, TOX9650160), Tasker et al. (1980, TOX9552390).

Furthermore, some published papers noted potential reproductive effects:

- Romano et al. (2011), “Glyphosate impairs male offspring reproductive development by disrupting gonatropin expression”, Arch. Toxicol. 86, 663-673: the authors observed change in

sexual behavior, significant increases in the testosterone and estradiol concentrations, in LH mRNA expression and in total and daily sperm production at 50 mg with glyphosate-based commercial formulation of Roundup Transorb.

- Dai et al. (2016), "Effect of glyphosate on reproductive organs in male rat", Acta Histochemica (article in press): the authors noted significant decrease of the total sperm count at 500 mg/kg bw, a trend to decrease in testosterone, progesterone and estradiol concentrations in a dose-dependent manner, and histological modifications with glyphosate (active ingredient grlyphosate, purity 90%).
- Dallegrave et al. (2007), "Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats", Arch. Toxicol. 81, 665-673: the authors found a significant increase of the percentage of abnormal sperm at the puberty, a significant decrease of the daily sperm production and sperm number during adulthood, a dose-dependent decrease in testosterone concentration at puberty and histological changes in the testis.
- Cassault-Meyer et al. (2014), "An acute exposure to glyphosate-based herbicide alters aromatase levels in testis and sperm nuclear quality", Environmental Toxicology and Pharmacology 38, 131-140: the authors reported alterations in sperm parameters.

In conclusion, BE CA suggests to consider a potential classification as REPR. 2.

Carcinogenicity :

Whilst much effort has undeniably been made to analyze the huge mass of information, BECA would like to emphasized some points that may be worth further consideration:

- In Kumar (2001, ASB2012-11491) study: BECA notes some inconsistencies between the CLH report and the Renewal Assessment Report (the number of animals affected and the statistical significance) (See table 1). Furthermore, a significant increase of incidence of malignant lymphoma was observed at the highest dose groups and a positive trend was detected in males.

Table 1 : Total incidence of malignant lymphoma

	Males				Females			
Doses (ppm)	0	100	1000	10000	0	100	1000	10000
Doses (mg/kg bw/d)	0	14.5	149.7	1453.8	0	15	151.2	1466.8
Number of animals affected (in the CLH report)	10/50	15/50	16/50	19/50*	18/50	20/50	19/50	25/50*
Number of animals affected (in the Renewal assessment report)	1/28	3/30	3/28	6/23	9/34	10/34	6/30	13/30

* statistically significant increase

- In Wood (2009, ASB2012-11492) study: A slight dose-dependent increase of malignant lymphoma in male was observed (See table 2). Moreover, the Renewal Assessment Report indicates that "the difference was not statistically significant but a possible effect might be suspected and should be clarified". The incidence was in the historical control data however this

report mentions that “the quality and the regulatory value of the historical control data is very much compromised by the fact that the sexes were not considered separately”.

Table 2 : Total incidence of malignant lymphoma

Doses (ppm)	0	500	1500	5000
Doses (mg/kg bw/d)	0	71.4	234.2	810
Number of animals affected	0/51	1/51	2/51	5/51*

* statistically significant increase

- In Sugimoto (1997, ASB2012-11493) study: BECA notes some inconsistencies between the CLH report and the Renewal Assessment Report (the number of animals affected) (See table 3). Furthermore, in the CLH report, in males, the trend test was significant (p-value = 0.0085) indicating a dose dependency

Table 3 : Total incidence of malignant lymphoma

	Males				Females			
Doses (ppm)	0	1600	8000	40000	0	1600	8000	40000
Doses (mg/kg bw/d)	0	165	838.1	4348	0	153.2	786.8	4116
Number of animals affected (in the CLH report)	2/50	2/50	0/50	6/50	6/50	4/50	8/50	7/50
Number of animals affected (in the Renewal assessment report)	0/26	0/34	1/27	5/29*	4/32	8/36	8/40	0/35*
Number of animals affected (in the Renewal assessment report) : Revised results	0/26	0/34	0/27	2/29	4/32	0/36*	5/40	3/35

* statistically significant increase

- In Atkinson (1993, TOX9552382) study: BECA notes a slight increase in the incidence of haemangiosarcoma in male mice at the highest dose of this study (4/50 at 1000 mg/kg bw/d vs 0/50 at 0, 100 and 300 mg/kg bw/d). Thus, BECA does not agree with the proposed NOAEL by the DS (1000 mg/kg bw/d).

Environment

BE CA is of the opinion that NOEC is < 1 mg/l.

Neurotoxicity in fish :

- In an in vitro study (Sandrini et al, 2013) with pure glyphosate, cholinesterase activity was inhibited in a concentration-dependent manner in brain (Danio rerio and Jenysia multidentata), muscle (D.rerio, Jenysia multidentata and Perna perna) and gill (Perna perna) fractions. IC50 ranged from 0.52mM for P.perna muscle to 8.43mM for J. multidentata brain.
- In a recent article (Roy et al, 2016) it is was found that glyphosate acid (technical grade) induced neurotoxicity in zebrafish. In this study structural changes to the fore, mid and hindbrain of embryonal zebrafish were considered by examining gross structural morphology as well as

morphological abnormalities by studying gene expression changes via in situ hybridization, immunohistological and transgenic approaches. Authors found that loss of brain ventricle delineations, general cephalic reduction and reduction in the eye region occurred between 50 and 75µg/l. It is suggested that glyphosate is neurotoxic for the forebrain and midbrain regions by altering the expression of key gene regulators in development but not for the hindbrain. Furthermore, the gene expressions are not attributable to delayed development as treated embryos met developmental milestones accordingly and in sync with control treatment at 50µg/ml.
This study cannot be considered as a chronic study per se as 5h old embryos were exposed for 24h. But despite the sublethal effects seen after such a short exposure period during the most vulnerable stage of fish, it is likely that NOEC will be lower than 50 µg/l.

- The toxicity of glyphosate on ovaries of zebrafish (Danio rerio) was examined by Armiliato et al (2014). They found that subcellular and molecular impairments may affect reproduction in female fish. After exposure of 65µg/l of glyphosate for 15 days a significant increase in diameter of oocytes was observed. The presence of concentric membranes, appearing as myelin-like structures, associated with the external membranes of mitochondria and with yolk granules was found when ovarian ultrastructure was examined. Immunohistochemistry and immunoblotting revealed greater expression of SF-1 in the oocytes, which suggests a relationship between oocyte growth and SF-1 expression.

Genotoxicity in fish :

- Guilherme et al (2012) examined DNA and chromosomal damage in fish (Anguilla Anguilla L.) after exposure to 17.9 and 35.7 µg/l glyphosate for 1 and 3 days resp. The comet assay was applied to blood cells, either as the standard procedure or with an extra step involving DNA lesion-specific repair enzymes in an attempt to clarify DNA damaging mechanisms. This study confirmed the genotoxicity potential of glyphosate.
  Potential non-specific DNA damage in both concentrations of glyphosate, expressed as GDI (Genetic Damage Indicator) was seen.
  GDIFPG results demonstrated significantly higher levels of damage for all the treatments in both exposure lengths. This evaluation of the additional breaks resulting from oxidised purine identified also the highest glyphosate concentration (3 days exposure) as genotoxic, which did not occur for GDI parameter.
  GDIEndoIII data revealed significantly higher DNA damage for all the treatments in both exposure lengths, when compared with the respective control.
  Overall oxidative DNA damage showed significant difference compared to control for all concentrations and exposure times.
- Webster et al, 2015 examined the global mechanism of toxicity in the liver of Brouwn trout (Salmo trutta) by exposing these fish for 14d to 0; 0.01; 0.5 and 10 mg/l of glyphosate. Transcriptional profiling demonstrated the induction of alterations of many of the complex, interacting signaling pathways that control cellular stress response, more in particular apoptosis. Also evidence was found that indicates an increase in cell proliferation and cellular turnover and an up-regulation of metabolic process.

Degradation product:

- DT50 whole system of glyphosate was determined in several water/sediment studies and ranged from 13.82d to > 301d. The max. amount of AMPA, the major degradation product of glyphosate,

detected in water/sediment system was 16% (water phase), 19% (sediment) and up to 27% (total system) and leads to potential exposure of sediment dwelling organisms to this degradation product. The persistence of AMPA is higher than glyphosate, therefore Guilherme et al (2014) examined DNA and chromosomal damage in fish (Anguilla Anguilla L.) after exposure to AMPA for 1 and 3 days resp. The genotoxicity of AMPA was investigated via Comet and erythrocytes nuclear abnormalities (ENA) assays.
The Comet assay showed potential non-specific DNA damage in both concentrations (11.8 and 23.6 µg/l). Furthermore it was concluded that oxidative damage was more difficult to repair when compared to non-specific damage.

It can be concluded that glyphosate and his breakdown product AMPA show a similar pattern in DNA-damaging effect. However, the recovery capacity from damaged caused by AMPA is different than that by glyphosate. No difference in oxidative DNA damage was shown between AMPA and glyphosate.

Development (oyster and frog)

- The study of Akcha et al, 2012 demonstrated a significant increase in abnormal D-larva in oyster versus control after exposure for 24h to 5 µg/l of glyphosate ($p<0.001$). Also here it is likely that NOEC will be lower when exposure period is prolonged.
- Lithobates catesbeianus tadpoles , exposed for 96h to 1 mg/l of glyphosate (purity 99.2%) showed significant reduction in V O2 at 80 and 40 mmgHg, significant thickened epidermis and the presence of several layers of overlapping small cells and some chromatid fragmentation (Risoli et al, 2016). The epithelial hyperplasia comprised several layers of undamaged cells, therefore it is suggested that the increase of thickness was a response to avoid systemic absorption of glyphosate. The epidermal hypertrophy might explain the significant reduction in V O2 as the O2 diffusion distance to O2 uptake increased.

Bioaccumulation :

- It is mentioned in the CLH report that no measured bioaccumulation date are available. However a 56d bioconcentration study with Lepomis macrochirus (Forbis 1989) resulting in a BCF = 1.1 ± 0.61 (steady state after 120 ± 59 d, flow-through) is mentioned in the EFSA report and further described in the RAR addenda. Furthermore in the RAR addenda it is recorded that different bioaccumulation studies with glyphosate have been conducted with different aquatic organsisms which achieved a BCF of max. 10.
- In a literature study (Wang et al, 1994), bioaccumulation was studied in carp and Tilapia and BCF ranged from 10 to 65.5.

  Those values are far below the classification trigger of 500 but a BCF study has prevalence on a octanol-water partition coefficient (logKow) and thus those bioaccumulation studies should be described in the CLH dossier in order to have the whole picture on the potential of bioconcentration in aquatic organisms.

References:

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