IIA 5.9.4 Clinical signs and symptoms of poisoning and details of clinical tests

The summary in this section is based on well over 30 years of experience with numerous formulations of glyphosate in a wide range of situations. The extensive use of glyphosate has encouraged clinical assessment of various interventions and has resulted in reporting of alleged associations of symptoms with exposures to glyphosate products. The clinical toxicology of glyphosate and of glyphosate-surfactant formulations have been the subject of an extensive review (Bradberry et al 2004), and a review of cases with assessment of clinical prognostic factors was more recently published (Lee et al. 2008).

GENERAL:
Glyphosate does not inhibit cholinesterase, and has no cholinergic effect. Animals do not have the shikimic acid pathway; and no direct target-mediated action in mammalian systems has been clearly identified to date (Bradberry et al. 2004). While incidental exposure in glyphosate-surfactant herbicide mixtures is common, review of available case reports (AAPCC 2003-2011) indicates that the vast majority of reported non-suicidal exposures involve skin and/or eye irritation or irritation of the respiratory tract by inhalation of spray mist, and that systemic symptoms are rare following non-suicidal exposures to glyphosate products. Based upon human experience and animal data, even those systemic symptoms reported following incidental exposure appear unlikely to be causally related to exposure (Goldstein et al. 2002).

CLASSIFICATION OF EXPOSURES:
The following clinical effects are divided into those expected following minor and significant exposures for each category based upon expected severity of systemic symptoms. The factors which determine if the exposure is minor or significant include:

- The route of exposure. Dermal, eye and mist inhalation exposures to any commercially formulated glyphosate products of any dilution are minor exposures for purposes of the symptom descriptions below. Ingestions more than 50 ml (one mouthful if amount unknown) of a product with >10% glyphosate concentration may be significant.
- The concentration of the product. Glyphosate concentrations of less than 10% rarely if ever produce significant toxicity. Most serious illness has historically resulted from ingestion of the 41% (glyphosate IPA) concentrate. In the absence of extensive clinical experience for the 11-40% concentration range, any ingestion of greater than 50 ml of a glyphosate preparation having a greater than 10% concentration of glyphosate salts should be considered potentially significant for purposes of the symptom descriptions below.
- The intent of the exposure. Accidental ingestion rarely involves large quantities of concentrated formulations. Intentional ingestion cases may not present with a reliable history and may require observation if the amount ingested cannot be reliably determined.
- Clinical condition of the patient.
- Known or suspected co-ingestants (if any).
- Professional judgment.

ROUTE AND ORGAN SYSTEM SPECIFIC SYMPTOMS OF EXPOSURE:

DERMAL
MINOR EXPOSURES:

- Contact with skin may produce a dermatitis similar to that of detergents (Bradberry et al. 2004)
- It is expected that the severity of injury following skin exposure will be significantly decreased with a less concentrated product and with a reduced duration of contact.
- Phototoxic reactions (sunlight or ultraviolet (UV) light induced skin reactions) have been reported. This is believed due to an antimicrobial additive (benzisothiazolone) which is present in selected residential use (i.e. non- agricultural) products containing 10% glyphosate or less (Bradberry et al. 2004).

- Significant absorption through the skin does not occur (<0.2% for concentrates and <0.01% for dilute formulations; see section 5.9.9)
- Studies in farmers and farm family members during the machine spray application of glyphosate products indicates that farmer exposure is generally far below recommended maximal daily intakes and that urinary levels in children and spouses are largely non-detectable (limit of urinary detection 1 µg/L) (Acquavella et al. 2004). These studies do not provide a quantitative measure of dermal exposure, but are consistent with the primate data noted above.

SIGNIFICANT EXPOSURES:

- Skin exposures are not expected to cause systemic effects or serious cutaneous effects. Symptoms as noted in the minor exposure may occur.

OCULAR

MINOR EXPOSURES:

- A review of ocular exposures to US glyphosate-surfactant formulations (1513 exposures over a 5-year period), showed no permanent eye injury (Acquavella et al. 1999).
- Human eye exposures have generally resulted in temporary conjunctival irritation, clearing after irrigation or in 1-2 days and permanent eye damage is said to be "most unlikely" (Bradberry 2004).
- It is expected that the severity of injury following eye exposure will be significantly decreased with a less concentrated product or with a reduced contact time.

SIGNIFICANT EXPOSURES:

- Eye exposures are not expected to cause systemic effects or serious ocular injury (Acquavella et al. 1999; Bradberry et al. et al. 2004).

SYSTEMIC EXPOSURE- INGESTION OR INHALATION

NEUROLOGIC:

MINOR EXPOSURES:

- There is no clinical or experimental evidence that glyphosate or glyphosate-surfactant formulations cause neurological symptoms or injury after exposure by any route.

SIGNIFICANT EXPOSURES:

- There have been no reports of primary convulsions after ingestion.
- One author reports most patients present with a clear sensorium unless another substance, such as alcohol, has been co-ingested or severe hypoxemia has occurred (Tominack 1989); however "moderate disorders of consciousness" have been reported within 48 hours of suicidal ingestions of the concentrate (Sawada and Nagai 1987; Sawada et al. 1988). This has occurred in patients with significant systemic illness and is not believed to be the result of reduced organ perfusion (Bradberry et al. 2004) or perhaps other factors such as metabolic disturbance, but the possibility of a direct toxicological effect cannot be excluded (Bradberry et al. 2004).
- There are two isolated case report of Parkinson's disease developing in individuals with a history of glyphosate product exposure. In one case, Parkinson's disease of relatively acute onset was diagnosed 6 months following incidental dermal exposure to a glyphosate-surfactant product (Barbosa et al. 2001). It appears that the same case was reported as part of a case series by daCosta et at (2003) [Similar list of authors on both publications, case descriptions and ages match (52 years old at diagnosis vs 54 year old with a 2 year history of Parkinsons) and the T2- weighted Axial MRI images shown appear to be identical]. The second case (Wang et al. 2011) reports the development of Parkinson's of a 44 year old woman who had been employed in a glyphosate manufacturing facility. In both instances, there is no evidence for causation other than a history of prior exposure. No other human or animal data support the contention that Parkinson's disease results from exposure to glyphosate, even following massive ingestion or prolonged exposure.

GASTROINTESTINAL:

MINOR EXPOSURES:

- Minor exposures are likely to be asymptomatic, but the patient may experience an unpleasant taste, tingling, mild self-limited nausea and vomiting.
- Self-limited diarrhoea may also occur, which is thought to be due to the surfactant.

SIGNIFICANT EXPOSURES:

- A burning sensation in the mouth and throat, salivation, oral erythema, sore throat, dysphonia, dysphagia, epigastric pain, nausea, spontaneous vomiting, abdominal pain and diarrhoea are common and may last up to a week.
- Serum amylase may be elevated; isoenzyme analysis done in a few cases identified a salivary gland origin (Tominack et al. 1989).
- In severe cases with large ingested doses, hematemesis, GI bleeding, melena and hematochezia may occur. Paralytic ileus has been reported as a rare event.
- Endoscopy has noted erosions of the pharynx and larynx, esophagitis and gastritis with mucosal oedema, erosions and haemorrhage. Transmural injury and perforation have not been noted on panendoscopy (Chang et al. 1999).
- In fatal cases, autopsy notes mucosal or transmural oedema and necrosis throughout the small bowel with erosion and haemorrhage; in the large bowel, mucosal oedema and focal haemorrhage was noted (Tominack et al. 1989).
- Clinical, autopsy and experimental evidence ([redacted] 1987) indicate a potential for gastrointestinal damage from glyphosate components of glyphosate formulations, but the frequency of severe injury appears to be low and early endoscopy is probably not indicated (see below).

CARDIOVASCULAR:

MINOR EXPOSURES:

- Dermal, eye and mist inhalation exposures to any commercially formulated glyphosate products of any dilution are minor exposures. Cardiovascular effects are not expected from minor exposures.

SIGNIFICANT EXPOSURES:

- Hypotension is common after ingestions of a mouthful or more of the concentrated product (not the diluted forms) and usually responds to IV fluids and pressor amines. Shock as manifested by oliguria, anuria and hypotension which was unresponsive to fluids and pressors, ultimately resulting in death, has been reported. (Tominack et al. 1989, Bradberry et al. 2004). Transient hypertension may be noted.

UPPER RESPIRATORY:

MINOR EXPOSURES:

- Dermal, eye and minor ingestions of dilute solution exposures to any commercially formulated glyphosate products of any dilution are minor exposures. Significant upper respiratory effects are not expected from minor exposures, but minor irritation or discomfort may occur (Bradberry et al. 2004).

SIGNIFICANT EXPOSURES:

- Significant systemic exposures are not anticipated to occur via the inhalational route, see minor exposures within this subheading.

LOWER RESPIRATORY:

MINOR EXPOSURES:

- Because of the non-volatile nature of glyphosate and the surfactant, there are no vapour exposures possible. The spray equipment commonly used with the product produces particles that are non-respirable.

SIGNIFICANT EXPOSURES:

- Tachypnea, dyspnea, cough and bronchospasm including cyanosis have been seen in severe ingestions (more than a mouthful of concentrated product). These effects appear to be the result of systemic toxicity.
- Aspiration pneumonia, pulmonary oedema and respiratory failure have been seen although the exact role of aspiration has not been fully investigated.
- An isolated case report suggests the development of acute pneumonitis in a worker following his performing maintenance on non-operating spray equipment used to apply a glyphosate-surfactant formulation (Pushnoy et al. 1998). However, the registrants do not believe that a credible mechanism of exposure was documented in this case, and the occurrence of pneumonitis in this individual was more likely coincidental in nature (Goldstein et al. 1999).
- There is also a case report out of Germany in which a glyphosate-surfactant product (tallowamine or "POEA" based) was applied by knapsack spayer in a 0.5ha forestry application at the registered application rate at 25° C for approximately 3 hours. About 7 hours after application he developed chest pain with rapidly increasing severe respiratory distress and fever up to approximately 38° C. On hospital admission, radiographic changes of lungs could be demonstrated. To further assess possible causes, bronchoscopy and closed lung biopsy was performed. Histopathology revealed "toxic inflammation of the lungs" (significantly different than bacterial infection). After 7-days of drug treatments, changes in lung reversed. Six months after the incident the patient still experienced moderate respiratory complaints on exertion. In the X-ray findings lungs showed improved results, but still detectable changes. While it is possible to differentiate acute bacterial infections on histopathology (microorganisms and polymorphonuclear leucocytic inflammatory changes should be visible), characteristics of viral, mycoplasmal or autoimmune (vasculitic, Wegoner's granulomatosis) induced pneumonitis or Bronchiolitis Obliterans with Organizing Pneumonia (BOOP, which closely mirrors the limited case information available) are not clinically distinguishable from "toxic" etiologies. Many cases occur, most being idiopathic (no identifiable cause). Agricultural aerosols are far larger than 10 microns (generally 200 microns or so in size) and not respirable to lung, and POEA is not volatile. Contrary to this isolated case, backpack applications of glyphosate-surfactant products occur regularly in forestry and in agriculture in the developing world without known occurrence of serious lower respiratory disease.

RENAL:

MINOR EXPOSURES:

- Dermal, eye, mist inhalation and minor ingestions of dilute solution exposures to any commercially formulated glyphosate products of any dilution are minor exposures. Renal effects are not expected from minor exposures.

SIGNIFICANT EXPOSURES:

- Hypotension and hypovolemic shock may result in oliguria and anuria, following severe ingestions (Bradberry et al. 2004). Abrupt rises in BUN and serum creatinine may be seen.

METABOLIC:

MINOR EXPOSURES:

- Dermal, eye, mist inhalation and minor ingestions of dilute solution exposures to any commercially formulated glyphosate products of any dilution should be considered minor exposures. Metabolic effects are not expected following minor exposures.

SIGNIFICANT EXPOSURES:

- Mild fever may be noted even in the absence of infection (Bradberry et al. 2004)
- Metabolic acidosis is often seen in a severely poisoned patient (Bradberry et al. 2004) and the acidosis may fail to respond to bicarbonate therapy. Although the exact cause of the acidosis is unknown, a lactic acidosis is suspected.

HEMATOLOGIC:

MINOR EXPOSURES:

- Dermal, eye, mist inhalation and minor ingestions of dilute solution exposures to any commercially formulated glyphosate products of any dilution should be considered minor exposures. Haematological effects are not expected from minor exposures.
- SIGNIFICANT EXPOSURES:
- Leukocytosis without evidence of bacterial infection has been noted in peripheral blood after ingestion of the concentrate (Bradberry et al. 2004).
- Hemoconcentration has been seen as a result of intravascular volume depletion (possibly indicating severe capillary fluid leakage) (Tominack et al. 1989).
- No primary toxic effects on bone marrow or formed elements have been seen to date.

HEPATIC:

MINOR EXPOSURES:

- Dermal, eye, mist inhalation and minor ingestions of dilute solution exposures to any commercially formulated glyphosate products of any dilution should be considered minor exposures. Hepatic effects are not expected from minor exposures.

SIGNIFICANT EXPOSURES:

- No direct hepatotoxic effects have been noted; however, minor elevations in transaminases and bilirubin are reported (Tominack et al. 1989; Bradberry et al. 2004).

ELECTROLYTES:

MINOR EXPOSURES:

- Severe or prolonged vomiting and diarrhoea may induce fluid and electrolyte imbalance. This degree of illness is not generally expected from a minor exposure.

SIGNIFICANT EXPOSURES:

- Electrolytes (Na, K, Cl and Ca) in the absence of renal failure generally remain normal Severe or prolonged vomiting and diarrhoea may induce fluid and electrolyte imbalance.
- POTASSIUM SALTS: While potentially toxic ingestions of all glyphosate products may result in fluid and electrolyte disturbances; particular attention to potassium may be important following ingestion of the potassium salt products. Close monitoring of serum potassium levels and/or electro-cardiographic monitoring (for peaked T-waves or rhythm disturbances) is recommended following significant ingestion of potassium salt products, particularly for high risk individuals. Individuals with the following may be at elevated risk following acute potassium exposure: known hyperkalemia, renal failure /»renal dysfunction, use of potassium sparing diuretics, hypoaldosteronism, co-ingestion of other K+ containing materials, underlying heart disease, use of digoxin, digitoxin, oabain or exposure to other cardiac glycosides. The quantity of potassium ingested from a glyphosate potassium salt product can be estimated from the weight percent of glyphosate potassium as:

  Percent K+ salt x 5.3 = mEq potassium per 100 cc of product

- Several case reports do indicate that with large ingestions of glyphosate-potassium salt concentrate solutions, clinically significant hyperkalemia may occur. Bando et al (2001) report a 65 year old female who ingested a glyphosate-potassium salt (350 ml Roundup Maxload missing from container, in addition to 250 ml of another glyphosate formulation which was not a potassium salt- but amount actually ingested unclear) in an attempt at suicide. On admission, serum potassium level was 9.3mEq/L (typical normal value < 5) with electrocardiographic changes consistent with hyperkalemia. The patient did have a concomitant acidosis (pH 7.272) which may account for some portion of the elevation in potassium (acidosis displaces intracellular potassium). The patient responded to medical management and survived.
- Kamijo et al (2012) report a 69 year old female who ingested approximately 500 ml of the same product. On arrival in the hospital, the patient had hyperkalemia (10.7 mEq/l), pulseless ventricular tachycardia, and a severe metabolic acidosis (pH 7.005, will elevate potassium.) The patient required aggressive cardiopulmonary resuscitation and hemodialysis but did recover.

- Monsanto is aware of one additional inquiry (unpublished) of a similar ingestion with a dramatically elevated potassium level in which the patient was moribund when medical care was instituted. The patient could not be resuscitated. Because serum potassium levels rise rapidly following death (due to redistribution of intracellular potassium) , it is not possible to know how much of the observed hyperkalemia was the result of the ingestion versus profound acidosis and post-mortem redistribution (which is partially due to acidosis).
- It should be noted that the issue of hyperkalemia is limited to cases involving the suicidal ingestion of glyphosate-potassium concentrates. Potassium is a normal component of the human diet, and potassium intake attributable to occupational glyphosate-surfactant herbicide exposure will be negligible compared to typical dietary intake. While the concentrate formulations may contain up to approximately 250 mEq of potassium per 100 ml, product diluted for use (1% glyphosate concentration) will contain about 6 mEq potassium per 100 ml. By way of reference, a medium size banana contains about 10 mEq (425 mg) of potassium.
- Finally, it should be noted that the apparently very large (>150 ml) ingestions of glyphosate-surfactant concentrates observed in these cases are well within the range isopropylamine salt products reported to produce fatalities, and that elevations in potassium concentrations are reported (probably due to acidosis) following ingestions of glyphosate IPA salt products. While the cases do suggest that potassium salt products likely contribute to the risk of hyperkalemia, it is not clear at this time that the use of postassium salts will increase the overall clinical severity and/or mortality associated with glyphosate concentrate product ingestions.

SPECIFIC DIAGNOSTIC TESTING AND PROGNOSTIC CONSIDERATIONS

Serum or other body fluid measurements of glyphosate are generally not available in a time frame useful for acute clinical diagnosis. As the management of symptoms associated with glyphosate-surfactant product ingestion is symptom-driven in any event, the lack of rapidly available concentrations of glyphosate will generally not impair clinical care. Levels may be helpful in addressing forensic issues following clinical recovery or in the event of a fatality of unclear cause.

Attention should be paid to electrolyte concentrations in individuals with significant ingestion exposures, particularly to glyphosate-potassium concentrate solutions.

Respiratory distress requiring intubation, pulmonary oedema, shock (systolic BP < 90 mmHg), altered consciousness, abnormal chest X-ray, ingestion of over 200 cc concentrate (41%), or renal failure necessitating dialysis have been associated with a higher risk of poor clinical outcomes including mortality (Lee 2008). These authors also developed a prognostic index based upon these factors. The use of prognostic criteria does not appear to add significantly to patient care. As symptom onset may be delayed, early use of such prognostic indicators may lead to an under-estimate of clinical severity.

IIA 5.9.5 First aid measures

SKIN EXPOSURE:

- Remove all contaminated clothing and flood the skin surface with water.
- Wash the exposed skin twice with soap and water.
- A close examination of the skin may be required if pain or irritation exist after decontamination.
- All clothing that are contaminated should be laundered before they are worn again

EYE EXPOSURE:

- Remove contact lens from the affected eye(s) if appropriate.
- Exposed eyes should be irrigated with copious amounts of water or saline for at least 15 minutes. Pour the water from a cup or glass held 3 inches from the eye.
- A close examination of the eyes may be needed if pain or irritation persists after 15 minutes of irrigation with water or saline. If symptoms persist, seek medical evaluation, preferably with an eye specialist.

INGESTION EXPOSURE:

- DILUTE PREPARATIONS (Glyphosate <10%): An ingestion of a dilute preparation of glyphosate (<10%) probably does not require treatment other than dilution with milk or water, and symptomatic care. Further gastrointestinal decontamination is not needed, even if spontaneous emesis has not occurred.
- Concentrated (> 10%) preparations: Irrigate and dilute: irrigate the mouth with water. Immediate therapy should include dilution with milk or water if the patient is able to swallow. Do not exceed 5 ml/kg in a child or 250 ml in an adult.

INHALATION EXPOSURE:

- No pulmonary treatment is necessary for occasional, accidental breathing of mist.
- Severe, acute pulmonary injury has not been reported following inhalation exposure. Individuals with respiratory distress from any cause should be relocated (if medically stable) to fresh air and receive supplemental oxygen if available.

In the event of respiratory failure or lack of respiration, administer artificial respiration (or if pulse not detectable, cardiopulmonary resuscitation).

IIA 5.9.6 Therapeutic regimes

The registrants believe that the following represent general best practices for medical management of serious ingestions of glyphosate-surfactant products.

1. Establish respiration and assure adequacy of ventilation.

2. Eye exposure:
   A) Remove contact lens from the affected eye(s) if appropriate.
   B) Exposed eyes should be irrigated with copious amounts of water or saline for at least 15 minutes. Pour the water from a cup or glass held 3 inches from the eye.
   C) A close examination of the eyes may be needed if pain or irritation persists after 15 minutes of irrigation with water or saline. If symptoms persist, seek medical evaluation, preferably by an eye specialist.

3. Ingestion exposure:
   A) Irrigate and dilute: irrigate the mouth with water. Immediate therapy should include dilution with milk or water if the patient is able to swallow. Do not exceed 5 ml/kg in a child or 250 ml in an adult.
   B) patient disposition:
      Concentrated preparations (Glyphosate 41% or greater):
      1) Any person ingesting greater than a large mouthful (50 ml in an adult, 0.5 ml/kg in a child) of a 41 % or greater glyphosate concentrate product should be admitted to a hospital and observed for 24 hours.
      2) Any adult ingesting greater than 100 ml of a 41% or greater glyphosate concentrate product (>1.4 ml/kg in a child) should be admitted to the intensive care unit.
      3) Any suicide attempt by person ingesting a concentrated product should be evaluated for psychological status and should be admitted if necessary for observation with suicide precautions.

      Concentrated preparations (Glyphosate 10%-40%):

      An ingestion of concentrated glyphosate (10%-40%) will usually result in spontaneous emesis. There is limited experience with glyphosate formulations in this concentration range. In view of this limited information, the registrants currently recommend managing these ingestions in a manner similar to the management of the 41% concentrate.

4. Prevention of absorption *(This lists various methods for "Prevention of Absorption". These should NOT be construed as being in order of preference. Consult with Poison Center or medical personnel to*

determine the need for and preferred method for decontamination. In many instances, no intervention is required.)

A) Gastric aspiration: If no significant spontaneous vomiting has occurred gastric aspiration may be considered. If performed soon after ingestion, gastric emptying by aspirating liquid gastric content with a lavage or standard NG tube may possibly remove some of the ingested glyphosate. The intent is to remove unabsorbed liquid by aspiration not to use lavage fluid. As absorption of liquids is likely to be relatively rapid, gastric aspiration after 1 to 2 hours is unlikely to be effective.

B) Emesis: Emesis is controversial at this time. Glyphosate/surfactant products are irritants. The registrants do not recommend the routine use of syrup of ipecac for glyphosate/ surfactant ingestions because of the risk of exacerbating the irritant effects on the GI tract.

C) Activated charcoal: There are no data to support or refute the use of activated charcoal in glyphosate/surfactant product ingestions. Low molecular weight, amphoteric compounds and detergents do not always bind well to activated charcoal. In the event of a mixed ingestion, activated charcoal may be advisable.

5. Assessment of gastro-intestinal injury

Injury to the upper gastrointestinal tract may occur following ingestion of glyphosate concentrates. A study of upper gastrointestinal endoscopy following glyphosate–surfactant ingestions suggested that Zarger grade 2 lesions (erosions) were associated with longer hospital stay and with a higher incidence of serious complications (Chang 2009). However, no major esophageal or gastrointestinal injury was observed, and strictures have not been reported following uncomplicated glyphosate-surfactant ingestion.

Because no serious gastrointestinal injury is reported, and because the need for hospitalization and/or treatment of complications can be determined without endoscopic evaluation, the registrants recommend that endoscopy be reserved for patients with co-ingestions suggesting a need for endoscopy or for patients with signs and symptoms suggestive of more serious injury (serious oral burns, inability to handle secretions, clinical obstruction) regardless of clinical history.

6. Monitor blood pressure:

Monitor the patient closely for signs of hemodynamic instability. The insertion of a Swan-Ganz catheter may be warranted.

7. Hypotension:

If the patient is hypotensive, administer IV fluid boluses and place in Trendelenburg position. If the patient is unresponsive to these measures, administer a vasopressor (dopamine, epinephrine, norepinephrine, phenylephrine, isoproterinol, etc.) if needed.

8. Monitor blood gases and obtain chest radiograph:

Consider the use of repeat blood gases and a peripheral pulse oximeter to monitor hypoxemia. Observe closely for sign of acidosis.

9. Pulmonary oedema:

Closely monitor arterial blood gases. If PO2 cannot be maintained above 50 mm Hg with inspiration of 60% oxygen by face mask or mechanical ventilation, then positive end expiratory pressure (PEEP) or continuous positive airway pressure (CPAP) may be needed. Avoid a positive fluid balance by careful administration of crystalloid solutions. Monitor fluid status through a central venous line or Swan Ganz catheter as needed.

10. Acidosis:

Correction of acidosis should be guided by blood gases, electrolytes and clinical judgment. Attention should be directed to volume status and correction of poor perfusion in mild cases. Sodium bicarbonate may be used to correct the acidosis in severe cases.

11. Hyperkalemia (from ingestion of Potassium salt formulations):

For moderate hyperkalemia (K+ of 6.0-7.0 mEq/L), administer sodium polystyrene sulfonate with sorbitol. For more severe hyperkalemia (K+ > 7 mEq/L) or serious complications of hyperkalemia, correct metabolic or respiratory acidosis if present to allow potassium to enter the intracellular space. Additional management may include a glucose/insulin drip, intravenous sodium bicarbonate or calcium, and dialysis to remove excess potassium.

12. Monitor renal function closely:

Assure adequate urine output. Catheterize severely ill patients. Hemodialysis may be needed in the event of renal failure or electrolyte disturbances.

11. Enhanced elimination:

A) Forced diuresis: Glyphosate is excreted very well by the kidneys. Adequate urine flow will ensure the rapid elimination of glyphosate. Although elimination may perhaps be enhanced by forced diuresis, there is no clinical evidence that this is necessary, and fluid overload may precipitate pulmonary oedema.

B) Hemodialysis: Hemodialysis may be useful to correct fluid, electrolyte and metabolic disturbances in the patient with renal failure. The institution of hemodialysis solely to enhance the removal of glyphosate or other product components is not of proven benefit. Nevertheless, it is reasonable to consider the initiation of hemodialysis in the significantly ill patient who fails to respond to routine supportive management.

12. Serious exposure via inhalation is not expected:

Inhalation exposures are not expected due to the aerodynamics of droplet size from sprayers and because the product is not volatile. Monitor the patient for signs of respiratory compromise. Create an artificial airway if necessary. Check adequacy of tidal volume. Monitor the patient for respiratory distress; if a cough or dyspnea develop, evaluate the patient for respiratory irritation, bronchitis and/or pneumonia, but these are not expected.

13. Serious exposure via skin is not expected:

Significant skin exposures are not expected; however, the patient should be treated empirically if a dermal exposure is suspected. Remove all contaminated clothing and flood the skin surface with water. Wash the exposed skin twice with soap and water. A close examination of the skin may be required if pain or irritation exist after decontamination. All contaminated clothing should be laundered before wearing.

14. Laboratory:

Monitor electrolytes, especially if the patient is experiencing vomiting and diarrhea.15 Patients ingesting concentrated products based on the potassium salt of glyphosate may ingest large amounts of potassium (see calculations above). Observe serum potassium and/or electrocardiogram carefully. Patients experiencing pulmonary symptoms or having chest radiograph changes should have arterial blood gas monitoring. A peripheral pulse oximeter and a Swan Ganz catheter may be needed.

IIA 5.9.7 Expected effects and duration of poisoning as a function of the type, level and duration of exposure or ingestion

Dermal exposure:

Skin irritation following exposure to glyphosate-only or glyphosate-surfactant materials is generally limited to topical irritation which will resolve within 3 days to 1 week following exposure. If exposure is

aggravated by occluded conditions or physical abrasion, more severe skin injury with open skin injury may rarely result and may take longer to fully resolve.

Eye exposure:
Irritant symptoms generally resolve within 3-7 days of exposure. Most irritation is minor, but exposure to concentrate or the occurrence of a foreign body or of abrasions (from rubbing the eye) may result in corneal abrasion requiring topical antimicrobial therapy, often given in conjunction with topical corticosteroids and temporary eye patching to provide symptomatic relief. As noted above, a large study of (U.S.) ocular exposures to glyphosate-surfactant products demonstrated no long term eye injury.

Inhalation exposure:
Glyphosate-surfactant products generally do not contain readily volatile ingredients and thus inhalation exposure is limited to inhalation of agricultural droplets, which will deposit primarily in the upper airway. Resulting irritant symptoms will generally resolve within hours to a few days following exposure.

Ingestion:
Following minor or incidental ingestions, or ingestion of fully diluted formulations, gastrointestinal upset with nausea, vomiting, and diarrhoea may occur. Nausea and vomiting usually resolve within a few hours of ingestion. Diarrhoea may last for several days but is generally not severe. Following a major ingestion, the onset of systemic symptoms may be delayed by several hours. Fatalities due to cardiovascular failure are generally delayed by 12 – 36 hours. For serious but non-fatal cases, primary clinical injury generally is manifest within 72 hours but secondary complications such as infection or respiratory distress syndrome may supervene. The majority of serious but survivable cases will be fully recovered within 7-10 days of ingestion. Individuals with complicated hospital courses can require a more extended and highly variable time to recover.

IIA 5.9.8 Expected effects and duration of poisoning as a function of varying time periods between exposure or ingestion and commencement of treatment

The outcome of eye, dermal, and inhalational exposures, which are not expected to result in serious injury in any event, will not be significantly altered by delays in medical management. Similarly, minor oral exposures are symptomatically managed and unlikely to result in severe gastrointestinal symptoms. Medical management with intravenous fluids may provide some symptomatic relief in the event of dehydration, but recovery is anticipated in any event.

For serious ingestions having major electrolyte disturbances or life threatening alterations of cardiovascular performance, medical intervention may be life saving. Fortunately, as noted above, the onset of serious symptoms following ingestion is generally delayed by at least several hours, allowing for medical transport in all but the most remote or extreme circumstances. The availability (or lack) of acute field management does not appear likely to impact severity of survival of most serious ingestions.

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IIA 5.9.9 Dermal penetration

In the 2001 EU glyphosate evaluation, dermal absorption of glyphosate was considered to be less than 3%. This value based on *in vivo* data in Rhesus monkeys, as well as on *in vitro* data in human skin, using the original glyphosate formulation Roundup ([redacted] 1991). *In vivo* dermal absorption in Rhesus monkeys ranged from 0.8 – 2.2% of applied dose ([redacted]., 1991; overall recovery was low with approx. 75-80%), whereas the *in vitro* penetration through human skin was at maximum 0.152% ([redacted], 1983) and ≤ 2.2% (published data). [redacted] (1991) *in vivo* studies in rhesus monkeys demonstrated very good mass balance for both oral and *intra venous* studies, which support almost complete urinary excretion of systemic doses. However, the dermal *in vivo* study in [redacted] (1991) demonstrated poor mass balance and the high dermal dose result showed an increase in faecal excretion (3.6% of dose); this contradicts the *intvra venous* high dose results with 98.8% excretion of the systemic dose in urine. Given the dermal study design with monkeys yielded poor mass balance, the results should not be considered to accurately represent dermal absorption for at least two reasons; (i) flaking and rubbing of occluded application application sites of the highly stressed animals fully restrained for 12 hours, then unrestrained in metabolism cages; and (ii) given the very low dermal absorption of glyphosate, possible hand to mouth activity of monkeys with even very small oral exposures would confound the excretion profile for the dermal doses. Therefore, more value should be ascribed to modern *in vitro* studies using human skin for assigning dermal absorption values.

Since the last evaluation some new *in vitro* studies have been performed by individual task force members with glyphosate formulations containing glyphosate at 360 g/L, 450 g/L and 480 g/L (see Table 5.9-3). All studies were performed according to GLP and to the current OECD guideline 428 (2004). The tested concentrations correspond to the formulation concentrates, as well as typical in-use dilutions. As can be seen from the results, the dermal penetration through human skin is limited, with maximum values of 0.086%, 0.059% and 0.166% for the 360 g/L, 450 g/L and 480 g/L concentrates, respectively. The absorption values for the in-use dilutions are also very low ranging from 0.169% to 0.88%.

The new data below indicated that the dermal absorption of glyphosate through human skin is clearly below 1%, irrespective of glyphosate salt form (potassium, isopropylamine and ammonium salts) and surfactant type.

The low penetration potential is further supported by all five repeat dose dermal toxicity studies performed in rats and rabbits (see Table 5.3-51). In these repeated dose *in vivo* dermal toxicity studies, no signs of systemic toxicity were observed up to 5000 mg/kg/day.

Table 5.9-3: Summary of Results for Dermal Absorption of ^{14}C-Glyphosate - SL Formulation

Reference (Data owner)		Content of glyphosate acid (g/L)	Study design according to guideline / exposure duration	Tested concentrations (actual) (g glyphosate/L)	Mean % of applied dose potentially biologically available* (%)
Studies not reviewed in the 2001 evaluation	[redacted] 2010 (IIIA 7.6.2/01) (MON)	360	OECD 428 24 h exposure	360 29.6 2.51	0.086 0.169 0.342
	[redacted] 2010a (MON)	450	OECD 428 24 h exposure	459 29.3 2.49	0.059 0.821 0.302
	[redacted] 2010b (MON)	480	OECD 428 24 h exposure	491 30.4 2.19	0.166 0.267 0.824
	[redacted] (2012b) (NUF)	360	OECD 428 8 h exposure	366 2.72	0.155 0.663
	[redacted] 2003 (SYN)	360	OECD 428 6 h exposure	364 6.7	0.06 0.24
			OECD 428 24 h exposure	364 6.7	0.07 0.88

* Potentially biological available = amount in receptor fluid + amount in remaining skin.
Only the first two tape strips (considered as stratum corneum) were excluded for calculation of potentially available dose.

Annex point	Author(s)	Year	Study title
IIA, 5.9.9/01	[redacted]	2010a	450 g/L Glyphosate SL Formulation (MON 79545) – *In vitro* Absorption of Glyphosate Through Human Epidermis [redacted] Data Owner: Monsanto [redacted] Report No.: [redacted]09-093 Date: 2010-02-19 GLP: yes unpublished

Guideline: OECD 428

Deviations: None

Dates of experimental work: 2009-05-26 to 2009-06-02

Executive Summary

The objective of this study was to evaluate the potential dermal absorption of glyphosate from a 450 g/L SL formulation concentrate, as well as from two representative in-use dilutions, prepared as 1:15.6 (v/v) and 1:188 (v/v) aqueous dilutions.

^{14}C-glyphosate was incorporated into the concentrate formulation and dilutions prior to application. The doses were applied to human epidermal membranes at a rate of 10 µL/cm^2 and left unoccluded for an exposure period of 24 hours. The absorption process was followed by taking samples of the receptor fluid (physiological saline) at recorded intervals throughout the exposure period. The distribution of glyphosate within the test system and a 24-hour absorption profile were determined. All samples were analysed by liquid scintillation counting (LSC).

The total amounts absorbed after 24 hours were 0.012, 0.129, and 0.082% of the applied doses for the concentrate, 1:15.6 (v/v) dilution, and 1:188 (v/v) dilution, respectively. The corresponding total potentially absorbable amounts, represented by the mean absorbed dose together with the amounts in the remaining skin, were 0.049, 0.796, and 0.245%, respectively.

Conclusion
The results of this *in vitro* study indicate the dermal absorption of glyphosate through human skin is very slow, and that the vast majority of glyphosate will be washed off during normal washing procedures. The results predict that the dermal absorption of glyphosate from potential exposure to this 450 g glyphosate/L SL formulation (MON 79545) would be less than 1%.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test materials:

a) Non radio-labelled test substance:

Identification:	Potassium salt of glyphosate technical material (glyphosate-potassium)
Description:	Clear, colourless to pale yellow liquid (solution in water)
Lot/Batch #:	A9B50041KI
Chemical purity:	Glyphosate-potassium: 50.17% Glyphosate acid: 48.29%
Stability of test compound:	Expiry date: 2011-09-10

b) Analytical reference standard:

Identification:	Glyphosate analytical reference standard (glyphosate acid)
Description:	White solid
Lot/Batch #:	GEP-0810-19515-A
Chemical purity:	99.8 %
Stability of test compound:	Expiry date: 2011-01-31

c) Radio-labelled test substance

Identification:	^{14}C-glyphosate (as glyphosate acid)
Lot/Batch #:	53463-3-23
Chemical purity:	Not reported
Radiochemical purity:	99.8% (by HPLC from supplier) 97.5% (confirmed by re-analysis, 2009-05-26)
Specific activity:	47 mCi/mmol; 1739 MBq/mmol; 277.9 µCi/mg; 10.28 MBq/mg
Stability of test compound:	Not reported

c) Blank formulation

Identification:	Proprietary surfactant blend
Concentration of a.i.:	0%
Description:	Not reported
Lot/Batch #:	Not reported
Purity:	Confidential
Stability of test compound:	Not reported

d) Formulated test substance

Identification: MON 79545

The formulation concentrate used was not supplied as complete formulation, but had to be prepared from the ingredients a) and c) described above, to allow the incorporation of the radiolabel.

The test substance concentration in the prepared formulation was confirmed by analysis.

2. Test skin source:

Species: Human

Source: Tissue bank (not further specified)

Age: Not reported

Sex: Not reported

Type of skin: Not reported

B: STUDY DESIGN AND METHODS

Preparation of skin samples:

Human skin samples were immersed in water at 60 °C for 40-45 seconds and the epidermis was teased away from the dermis. Each membrane was given an identifying number and stored frozen, at approximately -20 °C, on aluminium foil until required for use.

Test substance preparation

Three test substance concentrations representing the formulation concentrate and two field dilutions were prepared at target concentrations of 450, 28.8, and 2.4 g glyphosate/L. The nominal radioactivity contained in the dose preparation was 3.2 MBq.

Radioactive stocksolution of ^{14}C-glyphosate

Dry ^{14}C-glyphosate was solubilised in 2 mL of water and mixed thoroughly.

High dose (formulation concentrate, 459 g/L)

A pre-mix was prepared by mixing 4750 mg glyphosate-potassium technical material with an appropriate amount of proprietary surfactant blend. Seventy eight microliters (78 µL) of the radioactive stock solution was mixed with 519.9 mg of the pre-mix. Water was added to give a total weight of 654 mg. The solution was mixed well. Assuming a density of 1.308 g/mL, the total weight was equivalent of 0.5 mL at a nominal concentration of 459 g glyphosate/L.

Intermediate dose (1:15.6 [v/v] aqueous dilution, 29.3 g/L)

A pre-mix was prepared by mixing 298.7 mg glyphosate-potassium technical material with an appropriate amount of proprietary surfactant blend. Seventy eight microliters (78 µL) of the radioactive stock solution was mixed with 32.73 mg of the pre-mix. Water was added to give a total weight of 500 mg. The solution was mixed well. Assuming a density of 1 g/mL, the total weight was equivalent of 0.5 mL at a nominal concentration of 29.3 g glyphosate/L.

Low dose (1:188 [v/v] aqueous dilution, 2.50 g/L)

A pre-mix was prepared by mixing 74.7 mg glyphosate-potassium technical material with an appropriate amount of proprietary surfactant blend. Seventy eight microliters (78 µL) of the radioactive stock solution was mixed with 2.18 mg of the pre-mix. Water was added to give a total weight of 500 mg. The solution was mixed well. Assuming a density of 1 g/mL, the total weight was equivalent of 0.5 mL at a nominal concentration of 2.50 g glyphosate/L.

Analyses of dose preparations
The radioactivity content of the ^{14}C-glyphosate stock solution was determined by liquid scintillation counting (LSC) analyses of sub-samples of solvent dilutions. The radiochemical purity of the radiolabelled test substance was determined by high performance liquid chromatography (HPLC). The radioactivity content and homogeneity of the dose preparations were checked by LSC analyses. The radiochemical purity was measured by HPLC analyses. The formulated ^{14}C-glyphosate was shown to be stable for 24 hours, the duration of the exposure period, in a previous study.

Preparation of diffusion cells
The skin membranes were placed in static glass diffusion cells providing an exposure area of 2.54 cm^2 of skin. The cells had a receptor volume of approximately 4.5 mL. An integrity test was performed by measuring the electrical resistance across the skin membranes. Membranes with a resistance of ≥ 10 kΩ were considered having a normal integrity and used for the absorption study. Physiological saline was chosen as receptor fluid. The skin surface temperature was maintained at 32 ± 1 °C using a water bath.

Test substance application and sampling
Prior to dosing a pre-treatment sample of 500 µL was taken from each diffusion cell, and replaced by an equal amount of fresh receptor fluid.

Each dose formulation was applied to the skin membrane at the rate of 10 µL/cm^2 exposed skin area (25.4 µL dose), corresponding to target concentration of 4589, 293, and 25.0 µg/cm^2 for the high, intermediate, and low dose level, respectively. The applications were left unoccluded for 24 hours.

Receptor fluid samples (500 µL) were taken by an autosampler at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, and 24 hours after application. After each sampling, the removed amount of receptor fluid was replaced by an equal amount of fresh receptor fluid.

Terminal procedures
After the last sampling, 24 hours after application, the remaining receptor fluid was discarded. The receptor chamber was rinsed with receptor fluid that was also discarded.

The donor chamber was carefully removed and the underside wiped with a single natural sponge, pre-wetted with 3% Teepol L® in water, which was added to the wash sponges. The donor chamber was washed with deionised water and a sample was taken for LSC analysis.

The epidermal surface of the skin was decontaminated by gently swabbing the application site with natural sponges pre-wetted with 3% Teepol L® in water. Decontamination was shown to be complete following assessment of residual radioactivity levels on the skin surface with a Geiger counter. The skin surface was washed with further sponges pre-wetted with water. All the sponges were combined and digested in Soluene 350® and made up to a recorded volume. A sample was taken for analysis. The surface of the skin was allowed to dry naturally.

To assess penetration through the stratum corneum, successive layers of the skin surface were removed by the repeated application of adhesive tape (Scotch 3M Magic Tape, 1.9 cm wide), to a maximum of 5 strips. Each strip of adhesive tape was pressed onto the skin surface and then carefully peeled off to remove layers of stratum corneum. The adhesive strips were soaked individually in 30% v/v methanol in water to extract any test material. The extracts were sequentially numbered and analysed by LSC, In some cases, it was not possible to take the full 5 tape strips as the epidermis began to tear, therefore tape stripping was discontinued. The last tape strip for these diffusion cells was digested with the remaining epidermis, so as not to underestimate residues in the remaining epidermis compartment.

The remaining epidermis was carefully removed from the receptor chamber and digested in Soluene 350 and the whole digest analysed by LSC.

Analysis of samples
Liquid samples of the receptor fluid, washing solutions, digested wash sponges, tape strip extracts and digested epidermis by LSC using a Packard 3100 TR LSC counter and Goldstar as scintillation fluid.

Results of the analysis of the samples of receptor fluid collected in the study were expressed as amounts of glyphosate in the receptor solution in terms of µg/cm^2. The amounts absorbed, rates of absorption (µg/cm^2/h) and 'percentage of dose absorbed' were calculated. Membranes with absorption profiles that indicate membrane damage during the course of the experiment have been excluded from calculations. The results of the mass balance and distribution determinations are expressed in terms of amount absorbed and 'percentage of applied dose'.

The absorbed dose is considered the glyphosate detected in the receptor fluid, while the potentially biologically available proportion of the dose is regarded as the sum of absorbed dose and the amount recovered from the epidermis after tape stripping. The test material removed from the surface of the epidermis by the washing procedure, as well as the glyphosate recovered from the epidermis at the end of the exposure, is considered unabsorbed.

II. RESULTS AND DISCUSSION

A. ANALYSES OF UNFORMULATED ^{14}C-GLYPHOSATE
HPLC analysis of the unformulated sample of ^{14}C-glyphosate confirmed a radiochemical purity of 97.5%.

B. ANALYSES OF DOSE PREPARATIONS
The achieved concentrations of glyphosate in the dose preparations were calculated to be 458.9, 29.3 and 2.50 g glyphosate/L in the formulation concentrate, 1:15.6 (v/v) dilution, and 1:188 (v/v) dilution, respectively. LCS analyses confirmed the dose solutions to be homogeneous.

C. DERMAL ABSORPTION OF GLYPHOSATE
The determined distribution of radioactivity for the different dose groups are summarised in Table 5.9-4 below.

Table 5.9-4: Summary of Results for Dermal Absorption of ^{14}C-Glyphosate - SL Formulation

Dose preparation	**High (concentrate)**		**Intermediate (1:15.6 [v/v] dilution)**		**Low (1:188 [v/v] dilution)**	
Nominal concentration [g/L]	459		29.3		2.50	
Actual concentration [g/L]	458.9		29.3		2.50	
Applied dose [µL/cm^2]	10		10		10	
Applied dose [µg/cm^2]	4589		293		25.0	
Number of cells accessed	4*		5*		6	
	Distribution of radioactivity (mean values)					
	µg/cm^2	**% of applied dose**	**µg/cm^2**	**% of applied dose**	**µg/cm^2**	**% of applied dose**
Surface compartment						
Stratum corneum (tape strips)	1.25	0.027	0.254	0.087	0.050	0.201
Skin wash	4647	101	305	103	26.2	105
Donor chamber	2.64	0.057	0.806	0.275	0.092	0.369
Receptor compartment						
Receptor fluid (0-24 h)	0.573	0.012	0.370	0.129	0.021	0.082
Total absorbed	**0.573**	**0.012**	**0.379**	**0.129**	**0.021**	**0.082**
Remaining epidermis	1.70	0.037	1.95	0.666	0.040	0.163
Total potentially absorbable**	**2.27**	**0.049**	**2.33**	**0.796**	**0.061**	**0.245**
Total recovery	**4653**	**101**	**307**	**105**	**26.4**	**106**
Absorption rates [µg/cm^2/h] (0-24h)	0.024		0.016		0.001	

* Some cells for these applications were excluded from calculations as the analytical data indicated that the epidermal membrane may have been damaged during application.
** Total potentially absorbable = total absorbed + remaining epidermis

The overall total recovery for the three dose levels was good, with mean values of 101-106% of the applied dose.

Glyphosate absorption from the 450 g/L concentrate formulation was essentially constant over the entire 24 hour exposure period (mean rate = 0.024 µg/cm^2/h). By the end of the exposure period, the mean total amount of absorbed glyphosate was 0.573 µg/cm^2 (0.012% of applied dose).

From the 1:15.6 (v/v) and 1:188 (v/v) aqueous dilutions of the formulation, absorption was also essentially constant over the entire 24 hour exposure period (mean rates = 0.016 and 0.001 µg/cm^2/h, respectively). At the end of the exposure period, the mean total amounts of absorbed glyphosate were 0.379 and 0.021 µg/cm^2 (0.129 and 0.082% of applied dose), respectively.

For the formulation concentrate and both aqueous dilutions, the vast majority of the applied glyphosate was removed from the surface of the epidermis during the washing procedure at the end of the 24 hour exposure period (mean 101 - 105%). The mean total amount of glyphosate recovered from the epidermis (stratum corneum + remaining epidermis after tape stripping) was 0.064, 0.753, and 0.364% of the applied dose (concentrate, 1:15.6 [v/v] dilution, and 1:188 [v/v] dilution, respectively). The mean absorbed amounts were 0.012, 0.129, and 0.082% of applied dose, respectively. The amount of potentially biologically available glyphosate (absorbed + epidermis after tape striping) for the concentrate, 1:15.6 (v/v) and 1:188 (v/v) dilutions were 0.049, 0.796, and 0.245% respectively.

III. CONCLUSION

The results of this *in vitro* dermal absorption study indicate that the absorption of glyphosate through human skin is very slow. The vast majority of glyphosate was removed from the skin by the washing procedures. The total absorbed amounts after 24 hour exposure were 0.012, 0.129, and 0.082% of the applied dose for the formulation concentrate, the 1:15.6 (v/v) dilution, and 1:188 (v/v) dilution, respectively. The corresponding total potentially absorbable amounts, represented by the mean absorbed dose together with the amounts in the remaining skin were 0.049, 0.796, and 0.245%, respectively. These data predict that the dermal absorption of glyphosate from potential exposure to this 450 g glyphosate /L SL formulation (MON 79545) would be minimal, at less than 1% of any potential dermal exposure.

Annex point	Author(s)	Year	Study title
IIA, 5.9.9/02	[redacted]	2010b	480 g/L Glyphosate SL Formulation (MON 79351) – *In vitro* Absorption of Glyphosate Through Human Epidermis [redacted] Data Owner: Monsanto [redacted] Report No.: [redacted]-09-095 Date: 2010-02-19 GLP: yes Unpublished

Guideline: OECD 428

Deviations: None

Dates of experimental work: 2009-06-15 to 2009-08-26

Executive Summary

The objective of this study was to evaluate the potential dermal absorption of glyphosate from a 480 g/L SL formulation concentrate, as well as from two representative in-use dilutions prepared as 1:16.7 (v/v) and 1:200 (v/v) aqueous dilutions.

^{14}C-glyphosate was incorporated into the concentrate formulation and dilutions prior to application. The doses were applied to human epidermal membranes at a rate of 10 µL/cm^2 and left unoccluded for an exposure period of 24 hours. The absorption process was followed by taking samples of the receptor fluid (physiological saline) at recorded intervals throughout the exposure period. The distribution of glyphosate within the test system and a 24-hour absorption profile were determined. All samples were analysed by liquid scintillation counting (LSC).

The total absorbed amounts after 24 hour exposure were 0.007, 0.182, and 0.048% of the applied dose for the formulation concentrate, the 1:16.7 (v/v) dilution, and 1:200 (v/v) dilution, respectively. The corresponding total potentially absorbable amounts, represented by the mean absorbed dose together with the amounts in the remaining skin were 0.123, 0.262, and 0.799%, respectively.

Conclusion

The results of this *in vitro* study indicate the dermal absorption of glyphosate through human skin is very slow, and that the vast majority of glyphosate will be washed off during normal washing procedures. The results predict that the dermal absorption of glyphosate from potential exposure to this 480 g/L glyphosate SL formulation would be less than 1%.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test materials:

a) Non radio-labelled test substance:

Identification:	Potassium salt of glyphosate technical material (glyphosate-potassium)
Description:	Clear, colourless to pale yellow liquid (solution in water)
Lot/Batch #:	A9B50041K0
Chemical purity:	Glyphosate-potassium: 59.17% Glyphosate acid: 47.28%
Stability of test compound:	Expiry date: 2011-09-10

b) Analytical reference standard:

Identification:	Glyphosate analytical reference standard (glyphosate acid)
Description:	White solid
Lot/Batch #:	GLP-0810-19515-A
Chemical purity:	99.8 %
Stability of test compound:	Expiry date: 2011-01-31

c) Radio-labelled test substance

Identification:	^{14}C-glyphosate (as glyphosate acid)
Lot/Batch #:	53463-3-23
Chemical purity:	Not reported 99.8% (by HPLC from supplier) 97.6% (confirmed by re-analysis, 2009-06-14)
Radiochemical purity:	
Specific activity:	47mCi/mmol; 1739 MBq/mmol; 277.9 µCi/mg; 10.28 MBq/mg
Stability of test compound:	Not reported

c) Blank formulation

Identification:	Proprietary surfactant blend
Concentration of a.i.:	0%
Description:	Not reported
Lot/Batch #:	Not reported
Purity:	Confidential
Stability of test compound:	Not reported

d) Formulated test substance

Identification:	MON 79351 The formulation concentrate used was not supplied as complete formulation, but had to be prepared from the ingredients a) and c) described above, to allow the incorporation of the radiolabel. The test substance concentration in the prepared formulation was confirmed by analysis.

2. Test skin source:

Species:	Human
Source:	Tissue bank (not further specified)
Age:	Not reported

Sex: Not reported
Type of skin: Not reported

B: STUDY DESIGN AND METHODS

Preparation of skin samples:
Human skin samples were immersed in water at 60 °C for 40-45 seconds and the epidermis was teased away from the dermis. Each membrane was given an identifying number and stored frozen, at approximately -20 ºC, on aluminium foil until required for use.

Test substance preparation
Three test substance concentrations representing the formulation concentrate and two field dilutions were prepared at target concentrations of 480, 28.7, and 2.4 g glyphosate/L. The nominal radioactivity contained in the dose preparations was 3.3 MBq.

Radioactive stocksolution of ^{14}C-glyphosate
Dry ^{14}C-glyphosate was solubilised in 2 mL of water and mixed thoroughly.

High dose (formulation concentrate, 491 g/L)
A pre-mix was prepared by mixing 5067.02 mg glyphosate-potassium technical material with an appropriate amount of proprietary surfactant blend. Seventy eight microliters (78 µL) (≡ 78 mg) of the radioactive stock solution was mixed with 592.16 mg of the pre-mix. Water was added to give a total weight of 670 mg. The solution was mixed well. Assuming a density of 1.34 g/mL, the total weight was equivalent of 0.5 mL at a nominal concentration of 491 g glyphosate/L.

Intermediate dose (1:16.7 [v/v] aqueous dilution, 30.4 g/L)
A pre-mix was prepared by mixing 303.47 mg glyphosate-potassium technical material with an appropriate amount of proprietary surfactant blend. Seventy eight microliters (78 µL) (≡ 78 mg) of the radioactive stock solution was mixed with 36.00 mg of the pre-mix. Water was added to give a total weight of 501 mg. The solution was mixed well. Assuming a density of 1 g/mL, the total weight was equivalent of 0.5 mL at a nominal concentration of 30.4 g glyphosate/L.

Low dose (1:200 [v/v] aqueous dilution, 3.19 g/L)
A pre-mix was prepared by mixing 104.41 mg glyphosate-potassium technical material with an appropriate amount of proprietary surfactant blend. Seventy eight microliters (78 µL) (≡ 78 mg) of the radioactive stock solution was mixed with 3.09 mg of the pre-mix. Water was added to give a total weight of 501 mg. The solution was mixed well. Assuming a density of 1 g/mL, the total weight was equivalent of 0.5 mL at a nominal concentration of 3.19 g glyphosate/L.

Analyses of dose preparations
The radioactivity content of the ^{14}C-glyphosate stock solution was determined by liquid scintillation counting (LSC) analyses of sub-samples of solvent dilutions. The radiochemical purity of the radiolabelled test substance was determined by high performance liquid chromatography (HPLC). The radioactivity content and homogeneity of the dose preparations were checked by LSC analyses. The radiochemical purity was measured by HPLC analyses. The formulated ^{14}C-glyphosate was shown to be stable for 24 hours, the duration of the exposure period, in a previous study.

Preparation of diffusion cells
The skin membranes were placed in static glass diffusion cells providing an exposure area of 2.54 cm^2 of skin. The cells had a receptor volume of approximately 4.5 mL. An integrity test was performed by measuring the electrical resistance across the skin membranes. Membranes with a resistance of ≥ 10 kΩ were considered having a normal integrity and used for the absorption study. Physiological saline was chosen as receptor fluid. The skin surface temperature was maintained at 32 ± 1 °C using a water bath.

Test substance application and sampling
Prior to dosing a pre-treatment sample of 500 µL was taken from each diffusion cell, and replaced by an equal amount of fresh receptor fluid.

Each dose formulation was applied to the skin membrane at the rate of 10 µL/cm^2 exposed skin area (25.4 µL dose), corresponding to target concentration of 4906, 304, and 32.0 µg/cm^2 for the high, intermediate, and low dose level, respectively. The applications were left unoccluded for 24 hours.

Receptor fluid samples (500 µL) were taken by an autosampler at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, and 24 hours after application. After each sampling, the removed amount of receptor fluid was replaced by an equal amount of fresh receptor fluid.

Terminal procedures
After the last sampling, 24 hours after application, the remaining receptor fluid was discarded. The receptor chamber was rinsed with receptor fluid that was also discarded.

The donor chamber was carefully removed and the underside wiped with a single natural sponge, pre-wetted with 3% Teepol L® in water, which was added to the wash sponges. The donor chamber was washed with deionised water and a sample was taken for LSC analysis.

The epidermal surface of the skin was decontaminated by gently swabbing the application site with natural sponges pre-wetted with 3% Teepol L® in water. Decontamination was shown to be complete following assessment of residual radioactivity levels on the skin surface with a Geiger counter. The skin surface was washed with further sponges pre-wetted with water. All the sponges were combined and digested in Soluene 350® and made up to a recorded volume. A sample was taken for analysis. The surface of the skin was allowed to dry naturally.

To assess penetration through the stratum corneum, successive layers of the skin surface were removed by the repeated application of adhesive tape (Scotch 3M Magic Tape, 1.9 cm wide), to a maximum of 5 strips. Each strip of adhesive tape was pressed onto the skin surface and then carefully peeled off to remove layers of stratum corneum. The adhesive strips were soaked individually in 30% v/v methanol in water to extract any test material. The extracts were sequentially numbered and analysed by LSC, In some cases, it was not possible to take the full 5 tape strips as the epidermis began to tear, therefore tape stripping was discontinued. The last tape strip for these diffusion cells was digested with the remaining epidermis, so as not to underestimate residues in the remaining epidermis compartment.

The remaining epidermis was carefully removed from the receptor chamber and digested in Soluene 350 and the whole digest analysed by LSC.

Analysis of samples
Liquid samples of the receptor fluid, washing solutions, digested wash sponges, tape strip extracts and digested epidermis by LSC using a Packard 3100 TR LSC counter and Goldstar as scintillation fluid.

Results of the analysis of the samples of receptor fluid collected in the study were expressed as amounts of glyphosate in the receptor solution in terms of µg/cm^2. The amounts absorbed, rates of absorption (µg/cm^2/h) and 'percentage of dose absorbed' were calculated. Membranes with absorption profiles that indicate membrane damage during the course of the experiment have been excluded from calculations. The results of the mass balance and distribution determinations are expressed in terms of amount absorbed and 'percentage of applied dose'.

The absorbed dose is considered the glyphosate detected in the receptor fluid, while the potentially biologically available proportion of the dose is regarded as the sum of absorbed dose and the amount recovered from the epidermis after tape stripping. The test material removed from the surface of the

epidermis by the washing procedure, as well as the glyphosate recovered from the epidermis at the end of the exposure, is considered unabsorbed.

II. RESULTS AND DISCUSSION

A. ANALYSES OF UNFORMULATED ^{14}C-GLYPHOSATE

HPLC analysis of the unformulated sample of ^{14}C-glyphosate confirmed a radiochemical purity of 97.6%.

B. ANALYSES OF DOSE PREPARATIONS

The achieved concentrations of glyphosate in the dose preparations were calculated to be 490.6, 30.4, and 3.20 g glyphosate /L in the formulation concentrate, 1:16.7 (v/v) dilution, and 1:200 (v/v) dilution, respectively. LCS analyses confirmed the dose solutions to be homogeneous.

C. DERMAL ABSORPTION OF GLYPHOSATE

The determined distribution of radioactivity for the different dose groups are summarised in Table 5.9-5 below.

Table 5.9-5: Summary of results for dermal absorption of ^{14}C-glyphosate - SL formulation

Dose preparation	**High (concentrate)**		**Intermediate (1:16.7 [v/v] dilution)**		**Low (1:200 [v/v] dilution)**	
Nominal concentration [g/L]	491		30.4		3.19	
Actual concentration [g/L]	490.6		30.4		3.20	
Applied dose [µL/cm^2]	10		10		10	
Applied dose [µg/cm^2]	4906		304		32.0	
Number of cells accessed	4*		4*		5	
Distribution of radioactivity (mean values)						
	µg/cm^2	**% of applied dose**	**µg/cm^2**	**% of applied dose**	**µg/cm^2**	**% of applied dose**
Surface compartment						
Stratum corneum (tape strips)	6.85	0.140	0.061	0.020	0.045	0.139
Skin wash	4920	100	303	99.7	31.7	99.0
Donor chamber	53.9	1.01	2.72	0.894	<LOQ**	<LOQ**
Receptor compartment						
Receptor fluid (0-24 h)	0.342	0.007	0.553	0.182	0.015	0.048
Total absorbed	**0.342**	**0.007**	**0.553**	**0.182**	**0.015**	**0.048**
Remaining epidermis	5.70	0.116	0.244	0.080	0.241	0.752
Total potentially absorbable* **	**6.04**	**0.123**	**0.797**	**0.262**	**0.256**	**0.799**
Total recovery	**4987**	**102**	**307**	**101**	**32.0**	**100**
Absorption rates [µg/cm^2/h]	0.011 (0-10 h) 0.016 (10-24 h) 0.014 (0-24 h)		0.134 (0-1 h) 0.066 (1-24) 0.027 (0-24)		0.0016 (0-6 h) 0.0003 (6-24 h) 0.0006 (0-24 h)	

* Some cells for these applications were excluded from calculations as the analytical data indicated that the epidermal membrane may have been damaged during application.
** LOQ, Limit of quantitation. The LOQ for the donor chamber was 0.003 µg/cm^2 and 0.010% of applied dose.
*** Total potentially absorbable = total absorbed + remaining epidermis.

The overall total recovery for the three dose levels was good, with mean values of 100-102% of the applied dose.

Glyphosate absorption from the 480 g/L concentrate formulation increased slowly over the entire 24 hour exposure period (mean rate = 0.014 µg/cm^2/h). The mean rates during the first 10 hours and between

10-24 hours were 0.011 μg/cm^2/h and 0.016 μg/cm^2/h, respectively. At 10 hours, the mean amount of glyphosate absorbed was 0.105 μg/cm^2 (0.0021% of applied dose) and by the end of the 24 hour exposure period, the mean total amount of absorbed glyphosate was 0.342 μg/cm^2 (0.0070% of applied dose).

From the 1/16.7 v/v aqueous dilution of the formulation, glyphosate absorption was fastest during the first hour of exposure (rate of 0.134 μg/cm^2/h). The rate decreased to 0.066 μg/cm^2/h over the remainder of the 24 hour exposure, giving an average absorption rate of 0.027 μg/cm^2/h over the entire 24 hour exposure period. At the end of the exposure period, the mean total amount of absorbed glyphosate was 0.553 μg/cm^2 (0.182% of applied dose).

From the 1/200 v/v aqueous dilution of the formulation, glyphosate absorption was fastest during the first 6 hours of exposure (mean rate = 0.0016 μg/cm^2/h). The rate decreased to 0.0003 μg/cm^2/h over the remainder of the 24 hour exposure, giving an average absorption rate of 0.0006 μg/cm^2/h over the entire 24 hour exposure period. At the end of the exposure period, the mean total amount of absorbed glyphosate was 0.015 μg/cm^2 (0.048% of applied dose).

For the formulation concentrate and both aqueous dilutions, the vast majority of the applied glyphosate was removed from the surface of the epidermis during the washing procedure at the end of the 24 hour exposure period (mean 99-100%). The mean total amount of glyphosate recovered from the epidermis (statum corneum + remaining epidermis after tape stripping) was 0.256, 0.100, and 0.891% of the applied dose (concentrate, 1/16.7 [v/v] dilution, and 1/200 [v/v] dilution, respectively). The mean absorbed amounts were 0.007, 0.182, and 0.048% of applied dose, respectively. The amount of potentially biologically available glyphosate (absorbed + epidermis after tape stripping) for the concentrate, 1/16.7 and 1/200 dilutions were 0.123, 0.262, and 0.799% respectively.

III. CONCLUSION

The results of this *in vitro* dermal absorption study indicate that the absorption of glyphosate through human skin is very slow. The vast majority of glyphosate was removed from the skin by the washing procedures. The total absorbed amounts after 24 hour exposure were 0.007, 0.182, and 0.048% of the applied dose for the formulation concentrate, the 1:16.7 (v/v) dilution, and 1:200 (v/v) dilution, respectively. The corresponding total potentially absorbable amounts, represented by the mean absorbed dose together with the amounts in the remaining skin were 0.123, 0.262, and 0.799%, respectively. These data predict that the dermal absorption of glyphosate from potential exposure to this 480 g glyphosate /L SL formulation (MON 79351) would be minimal, at less than 1% of any potential dermal exposure.

Annex point	Author(s)	Year	Study title
IIA, 5.9.9/03	[redacted]	2011	Glyphosate 360 IPA Salt (CA2273): In Vitro Absorption through Human Epidermis using [14C]-glyphosate [redacted] Report No.: [redacted]2147-REG Date: 2012 GLP: yes unpublished

Guideline:	1) OECD Test Guideline 428 (2004). Skin Absorption: In Vitro Method. 2) OECD (Guidance Document No. 28 (2004): The Conduct of Skin Absorption Studies. 3) European Commission Guidance Document on Dermal Absorption (2004).
Deviations:	None
Dates of experimental work:	18th April 2011 to 15th June 2011

Executive Summary

The penetration of glyphosate from a glyphosate 360 IPA Salt (CA2273) formulation concentrate, containing a nominal 360 g glyphosate/L, and a 1/133 w/v aqueous dilution of the concentrate, containing a nominal 2.7 g glyphosate/L, through human epidermis was measured in vitro over 24 hours. The doses were applied to the epidermal membranes at a rate of 10 µL/cm^2 and left unoccluded for an exposure period of 8 hours. The distribution of glyphosate within the test system (skin washes, donor chamber, stratum corneum and residual epidermal tissue) after 24 hours and time course penetration profiles were also determined. [^{14}C]-glyphosate was incorporated into the doses prior to application. The penetration process was followed by taking samples of the receptor fluid (physiological saline) at recorded intervals throughout the experimental period. All samples were analysed for radioactivity by LSC.

Penetration of Formulation concentrate glyphosate was fastest between 0-2 hours (0.914 µg/cm²/h). The mean penetration rate slowed to 0.071 µg/cm²/h between 2-24 hours. Between 0-8 hours the mean penetration rate was 0.283 µg/cm²/h. Between 0-24 hours, the penetration rate was, on average, 0.109 µg/cm²/h. The mean amount penetrated over the entire 24 hour exposure period was 3.51 µg/cm^2, corresponding to 0.096% of the applied dose. The mean total recovery of the applied test material was 110%. The vast majority of the applied glyphosate (mean 109%) was washed off the skin at 8 hours, with a further 0.417% washed off at 24 hours. A small proportion of the dose applied was recovered from the stratum corneum and remaining epidermis (0.034% and 0.043%, respectively).

Penetration of the 1/133 w/v aqueous spray strength dilution glyphosate was fastest between 0-1 hours (0.009 µg/cm²/h). The mean penetration rate slowed to 0.002 µg/cm²/h between 1-24 hours. Between 0-8 hours the mean penetration rate was 0.003 µg/cm²/h. Between 0-24 hours, the penetration rate, on average, was 0.002 µg/cm²/h. The mean amount penetrated over the entire 24 hour exposure period was 0.050 µg/cm^2, corresponding to 0.183% of the applied dose. The mean total recovery of the applied test material was 106%. The vast majority of the applied glyphosate (mean 100%) was washed off the skin at 8 hours, with a further 4.44% washed off at 24 hours. A small proportion of the dose applied was recovered from the stratum corneum and remaining epidermis (0.242% and 0.362%), respectively.

The results obtained in this study demonstrate that the penetration of glyphosate from this glyphosate 360 IPA Salt (CA2273) formulation concentrate and its 1/133 w/v dilution, through human epidermis is at a very slow rate. The extent of glyphosate penetration through human skin from the concentrate was below 0.1% and amounted to less than 0.2% of the applied dose, for the aqueous dilution, after 24 hours.

The vast majority of the applied dose could be removed by gentle skin washing after 8 hours. Only low proportions of the dose were associated with the skin at the end of the 24-hour experimental period.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	[^{14}C]-glyphosate
Description:	Dry radiolabelled material
Lot/Batch #:	XIX/5B
Purity:	95.88%
Stability of test compound:	Not specified

2. Vehicle and/ or positive control: water

3. Test animals:

Species:	Human skin in vitro
Source:	Human Tissue Bank (source not specified)
Age:	Not specified
Sex:	Not specified

B: STUDY DESIGN AND METHODS

Experiment dates: 18th April 2011 to 15th June 2011

Study Conduct:
Human in vitro membranes were prepared in static glass diffusion cells. Membrane integrity was determined by measurement of the electrical resistance across the skin membrane. Membranes with a measured resistance of <10 kΩ ([redacted] 2004) were regarded as having a lower integrity than normal and not used for exposure to the test materials. Cells were selected such that each application was represented by six intact membranes from three different subjects. The receptor chambers of the cells containing small magnetic stirrer bars were filled with a recorded volume of receptor fluid (physiological saline) and placed in a water bath maintained at a temperature of 32ºC ± 1ºC. Glyphosate is soluble in water at 10.5 g/L (Safety data sheet dated 26/2/2009) and this choice of receptor fluid ensures that the glyphosate can freely partition into the receptor fluid from the skin membrane and never reaches a concentration that would limit its diffusion. A pre-treatment sample (0.5 mL) was taken from each receptor chamber for analysis by LSC prior to dosing. An equal volume of fresh receptor fluid was added to each receptor chamber to replace the volume removed. The formulation was applied to the skin membranes as the product concentrate and as a 1/133 w/v aqueous spray strength dilution. The applications were left unoccluded for the duration of the experiment (24 hours). Samples of the receptor fluid (0.5 mL) were taken using an autosampler at pre-treatment, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 hours after application for analysis by LSC. After the 8 hour sample had been taken the skin was washed and allowed to dry naturally. Samples were taken during the procedure to determine mass balance. To assess penetration through human stratum corneum, successive layers of the skin surface were removed by the repeated application of adhesive tape (e.g. Scotch 3M Magic Tape, 1.9 cm wide), to a maximum of 5 strips [redacted] et al, 1994). A strip of adhesive tape was pressed onto the skin surface and then carefully peeled off to remove the stratum corneum. The adhesive strips were soaked individually in water to extract any test material. The extracts were sequentially numbered and analysed by LSC.
The penetrated (systemically available) dose is considered to be the amount of glyphosate detected in the receptor fluid. Material removed from the surface of the epidermis by the washing procedure is regarded as not bioavailable. Glyphosate recovered from the epidermis at the end of the experimental period is also

considered not to be bioavailable, although it is recognised that a proportion of this material may penetrate beyond the duration of the experimental period investigated in this study. In vivo, the majority of the dose in the epidermis, especially that recovered from the stratum corneum (i.e. that found on the tape strips), would eventually be lost by desquamation ([redacted] 1992).

II. RESULTS AND DISCUSSION

Table 5.9-6: Summary of results for the concentrate Formulation

Application of Test Materials and Actual Concentration of Dose Preparation	Mean Penetration Rate		Mean Amount and Percentage of Dose Penetrated		
	Time period (h)	Penetration rate (µg/cm²/h ± SEM)	Time (h)	Amount (µg/cm²)	Percentage
Formulation concentrate (366 g glyphosate /L) 10 µL /cm² (3662 µg glyphosate /cm²)	0 - 2	0.914 ± 0.583	2	1.83	0.05
	0 - 8	0.283 ± 0.167	8	2.27	0.074
Unoccluded	2 - 24	0.074 ± 0.035	24	3.51	0.096
Duration of exposure: 8h	0 - 24	0.155 ± 0.049			
n = 5			*LOQ*	*0.079*	*0.002*

1/133 w/v Aqueous Dilution

Table 5.9-7: Summary of results for the 1/133 aqueous dilution

Application of Test Materials and Actual Concentration of Dose Preparation	Mean Penetration Rate		Mean Amount and Percentage of Dose Penetrated		
	Time period (h)	Penetration rate (µg/cm²/h ± SEM)	Time (h)	Amount (µg/cm²)	Percentage
1/133 w/v aqueous dilution					
(2.72 g glyphosate /L)	0 - 1	0.009 ± 0.005	1	0.009	0.033
10 µL/cm² (27.2 µg glyphosate/cm²)	0 - 8	0.003 ± 0.001	8	0.024	0.086
Unoccluded	1 - 24	0.002 ± 0.0009	24	0.050	0.183
Duration of exposure: 8h	0 - 24	0.002 ± 0.0009			
n = 5			*LOQ*	*0.0003*	*0.001*

III. CONCLUSION

The results obtained in this study demonstrate that the penetration of glyphosate from this glyphosate 360 IPA Salt (CA2273) formulation concentrate and its 1/133 w/v dilution, through human epidermis is at a very slow rate. The extent of glyphosate penetration through human skin from the concentrate was below 0.1% and amounted to less than 0.2% of the applied dose, for the aqueous dilution, after 24 hours. The vast majority of the applied dose could be removed by gentle skin washing after 8 hours. Only low proportions of the dose were associated with the skin at the end of the 24 hour experimental period.

Based on the EU and the OECD Globally Harmonized System (GHS) classification criteria, glyphosate is not to be classified for this endpoint

Annex point	Author(s)	Year	Study title
IIA, 5.9.9/04	[redacted]	2003	Glyphosate SL (360g/l) Formulation (A12798Q): In Vitro Absorption Through Human Epidermis [redacted] Data owner: Syngenta Report No.: [redacted]1732 Date: 2003-05-28 GLP: yes not published

Guideline:	OECD 428
Deviations:	None
Dates of experimental work:	2002-12-09 to 2003-05-28

Excecutive summary
The absorption and distribution of glyphosate from the A12798Q 360 g/L SL formulation was measured in vitro through human epidermis. The doses were applied as the concentrate formulation (360 g/L) and as a 3/200 v/v (5.4 g/L) spray strength dilution of the formulation in water. The absorption process was followed using [^{14}C]-labelled glyphosate, which was added prior to application. The doses were applied to the epidermal membranes at a rate of 5µl/cm^2 and left unoccluded for an exposure period of 6h and 24h. These applications were designed to simulate potential human dermal exposure to the 363g glyphosate/l SL formulation and its 3:200 v/v aqueous spray dilution during normal use.

The distribution of glyphosate within the test system and a 24 hour absorption profile (µg/cm^2/h) was determined. The results obtained in this study indicate that glyphosate is absorbed through human epidermis from the concentrate formulation at a very slow rate; the mean rate of absorption was 0.02µg/cm^2/h over a 24 h period. Absorption was also very slow for the 3/200 v/v aqueous dilution; the mean absorption rate over 24 h was 0.001µg/cm^2/h.
For the concentrate the majority of the applied dose, 100 and 103%, was removed by mild skin washing at 6 and 24 hours respectively, whilst 0.04 and 0.05% of the applied dose was left in the human epidermis at 6 and 24 hours respectively. For the spray strength dilution the majority of the applied dose, 90.8 and 87.9%, was removed by mild skin washing at 6 and 24 hours respectively, whilst 0.31 and 1.10% of the applied dose was left in the human epidermis at 6 and 24 hours respectively.

These data predict that the human dermal absorption of glyphosate from potential exposure to this formulation (A12798Q) either as the concentrate formulation or as a 3/200 v/v aqueous spray strength dilutions, would be minimal.

I. MATERIALS AND METHODS

A: MATERIALS:

1. Test material: **360g glyphosate/l SL formulation concentrate (A12798Q)**

Description:	brown liquid
Lot/Batch number:	FL020886
Purity:	28.3% (w/w) glyphosate
Stability of test compound:	Confirmed

Radiolabelled Test Material:	[^{14}C]-glyphosate
Radiochemical number:	6550
Radiochemical purity:	98.2%
Specific activity:	294.6 µCi/mg (11.0 MBq/mg)
Stability of test compound:	confirmed

B: STUDY DESIGN AND METHODS

In-life dates: Start: 12 February 2003 End: 18 March 2003

Diffusion cell: Diffusion of glyphosate into and across the skin to a receptor fluid was measured using glass diffusion cells in which the epidermis formed a horizontal membrane and provided an application area of 2.54cm^2.

Receptor fluid: The receptor fluid (physiological saline) was chosen to ensure that the glyphosate would freely partition into this from the skin membrane and never reach a concentration that would limit its diffusion. Glyphosate acid is highly soluble in water (11.6g/L; K_{ow} log P=<-3.2).

Skin preparations: Extraneous tissue was removed from human whole skin samples obtained from surgery or *post mortem*. The skin samples were immersed in water at 60°C for 40-45 seconds. The epidermis was carefully peeled from the dermis and stored frozen until required for use.

Skin preparation integrity: The integrity of the membranes was checked by measurement of the electrical resistance across the skin. Only those membranes with an acceptable resistance (>10kΩ), thereby showing that they were intact, were used on the study.

Test substance: The two doses were prepared to mimic the commercial 360g/L formulation and its aqueous spray dilution (3/200 v/v). An appropriate volume of [^{14}C]-labelled glyphosate (equivalent to 27.0 MBq) was blown down to dryness using a stream of nitrogen gas and added to 1 mL of the glyphosate formulation. To make the spray strength dilution an appropriate volume of [^{14}C]-labelled glyphosate (equivalent to 16.8 MBq) was blown to dryness and added to 15 µL of the unlabelled glyphosate formulation and 985 µL of deionised water. The doses were prepared as close to the time of application as was practicable and were analysed to confirm their suitability for use in the study.

Application to the skin: Each application was represented by six replicates from at least two different animals at a dose of 5µl/cm² and left unoccluded for the exposure period.

Temperature: Throughout the experiment the receptor fluid was stirred and the epidermal membranes were maintained at a normal skin temperature of 32 ± 1°C in a water bath.

Duration of exposure and sampling: For the cells exposed to the test preparations for 24 hours during which time samples of receptor fluid were taken at suitable intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20 and 24 hours) to allow adequate characterisation of the absorption profile. For cells assigned to the 6 hour exposure period, the receptor fluid samples were only taken 6 hours after application.

Terminal procedures: The donor chamber was carefully removed and washed with deionised water and the sample analysed by LSC. The epidermal surface of the skin was decontaminated by gently swabbing the application site with natural sponges pre-wetted with 3% Teepol® and with further sponges pre-wetted with water. Decontamination was shown to be complete following assessment of residual radioactivity levels on the skin surface with a Geiger counter. The sponges were digested in Soluene 350® and made up to a recorded volume. To assess penetration through the *stratum corneum*, the skin was allowed to dry and adhesive tape was repeated applied to the skin's surface and then carefully peeled off to remove the *stratum corneum*. The adhesive strips were soaked in methanol to extract test material. The extracts were

sequentially numbered and analysed by LSC. The remaining epidermal tissue was carefully removed from the receptor chamber and digested in Soluene 350® and analysed by LSC.

Data: Results of the analysis of the samples of receptor fluid collected in the study were expressed as amounts of glyphosate in the receptor solution in terms of µg/cm², 'percentage of dose absorbed' and rates of absorption (µg/cm²/h). The results of the mass balance and distribution determinations are expressed in terms of amount (µg/cm²) and 'percentage of applied dose' (see Tables below).

Definition of absorbed test material: The absorbed (systemically available) dose is considered to be the glyphosate detected in the receptor fluid. Material removed from the surface of the epidermis by the washing procedure is regarded as unabsorbed. The glyphosate recovered from the epidermis at the end of the exposure is also considered to be unabsorbed, although it is recognised that a proportion of this material may be absorbed beyond the duration of the exposure investigated in this study. *In vivo*, the majority of the dose in the epidermis, especially that recovered from the *stratum corneum*, would eventually be lost by desquamation.

II. RESULTS AND DISCUSSION

Recovery of radiolabelled test material in these experiments was very good (means of 107 and 95% of the applied dose for the concentrate and aqueous spray strength dilution respectively).

Concentrate formulation: Glyphosate absorption through human epidermis was fastest between 0-4 hours (0.07 µg/cm²/h) of application, after which it slowed to 0.02 µg/cm²/h (4-24 hours). Between 0-24 hours, the mean rate of absorption was 0.02 µg/cm²/h. The amount of glyphosate absorbed over time periods representing a range of typical 'working days' (6, 8 and 10 hours) were 0.20, 0.20 and 0.28µg/cm², respectively. In terms of percentages of applied dose, the respective amounts were 0.01, 0.01 and 0.02%.Over 24 hours, the amount absorbed was 0.50 µg/cm² (0.03% of the applied dose). Mild skin washing at 6 and 24 hours removed practically all (100 and 103%, respectively) of the applied dose from the surface of human epidermis. The percent of applied dose which was found to be associated with skin 6 and 24 hours following washing was 0.04 and 0.05%, respectively.

3/200 v/v dilution: The fastest rate of absorption through human epidermis occurred between 0-0.5 hours (0.011 µg/cm²/h), after which it slowed to less than 0.001µg/cm²/h (0.5-24 hours). Between 0-24 hours, the mean rate of absorption was 0.001 µg/cm²/h. The amount of glyphosate absorbed over the same time periods (6, 8 and 10 hours) were 0.01, 0.01 and 0.02 µg/cm², respectively. In terms of percentages of applied dose, the respective amounts was 0.04% for the 6,8 and 10 hour time points. Over 24 hours, the amount absorbed was 0.02 µg/cm² (0.07% of the applied dose). Washing at 6 and 24 hours removed 90.8 and 87.9% of the applied dose, respectively. For the 3/200 v/v spray dilution, the percent of applied dose which was found to be associated with skin 6 and 24 hours following application was 0.31 and 1.10%.

Table 5.9-8: Summary of glyphosate absorption through human epidermis

Application of Test Materials	Mean Absorption Rates		Mean Amount and Percentage of Dose Absorbed		
	Time period (h)	Absorption rate ($\mu g/cm^2/h \pm SEM$)	Time (h)	Amount ($\mu g/cm^2$)	Percentage absorbed
Concentrate Formulation					
(364g glyphosate/l)	1-4	0.07 ± 0.02	6	*0.20	0.01
5 µl/cm² (1821 µg ai/cm²)	4-24	0.02 ± <0.01	8	*0.20	0.01
Unoccluded	0-24	0.02 ± <0.01	10	0.28	0.02
Duration of exposure: 24h			24	0.50	0.03
n = 4			LOQ	*0.25*	*0.01*
3/200 v/v aqueous spray diln					
(6.70g glyphosate/l)	0-0.5	0.011 ± 0.004	6	0.01	0.04
5 µl/cm² (33.5 µg ai/cm²)	0.5-24	0.001 ± <0.001	8	0.01	0.04
Unoccluded	0-24	0.001 ± <0.001	10	0.02	0.04
Duration of exposure: 24h			24	0.02	0.07
n = 5			LOQ	*0.01*	*0.02*

* The LOQ values have been used as positive values in the calculation of the mean where values were <LOQ.

Table 5.9-9: Summary of glyphosate distribution from the concentrate formulation - 6 hour exposure

Test Compartment	Percentage of Dose Recovered (%)					Mean % Recovered	SD
	Cell 65	Cell 70	Cell 75	Cell 85	Cell 89		
Stratum Corneum	0.02	0.02	0.04	0.02	0.03	0.02	0.01
Donor Chamber	0.52	0.01	8.49	18.2	0.03	5.44	7.97
Skin Wash	110	102	93.6	98.9	96.4	100	6.13
Remaining Epidermis	0.01	<0.01	0.01	0.01	0.06	0.02	0.02
Absorbed	0.02	0.08	0.01	0.01	0.01	0.03	0.03
Total Recovered	110	102	102	117	96.5	106	8.06

Stratum corneum = *amount in tape strips; Remaining epidermis = epidermal tissue remaining after tape stripping; Absorbed = amount in receptor fluid*

Table 5.9-10: Summary of glyphosate distribution from the 3/ 200 aqueous spray dilution – 6 hour exposure

Test Compartment	Percentage of Dose Recovered (%)				Mean % Recovered	SD
	Cell 5	Cell 13	Cell 14	Cell 21		
Stratum Corneum	0.02	0.18	0.33	0.1	0.16	0.13
Donor Chamber	0.02	0.05	11.0	0.54	2.90	5.39
Skin Wash	94.6	94.0	80.7	94.0	90.8	6.75
Remaining Epidermis	0.04	0.09	0.38	0.08	0.15	0.16
Absorbed	0.02	0.02	0.03	0.04	0.03	0.01
Total Recovered	94.7	94.3	92.4	94.8	94.0	1.11

Table 5.9-11: Summary of glyphosate distribution from the concentrate formulation – 24 hour exposure

Test Compartment	Percentage of Dose Recovered (%)				Mean % Recovered	SD
	Cell 68	Cell 72	Cell 87	Cell 91		
Stratum Corneum	0.01	0.02	0.02	0.01	0.02	<0.01
Donor Chamber	0.01	0.01	0.01	18.1	4.53	9.04
Skin Wash	106	110	109	86.6	103	11.0
Remaining Epidermis	<0.01	<0.01	0.03	0.07	0.03	0.03
Absorbed	0.02	0.02	0.04	0.04	0.03	0.02
Total Recovered	106	110	109	105	107	2.37

Table 5.9-12: Summary of glyphosate distribution from the 3/200 aqueous spray dilution – 24 hour exposure

Test Compartment	Percentage of Dose Recovered (%)					Mean % Recovered	SD
	Cell 6	Cell 16	Cell 17	Cell 11	Cell 22		
Stratum Corneum	0.03	0.94	0.88	0.42	0.07	0.47	0.43
Donor Chamber	0.01	16.2	6.74	6.96	0.08	5.99	6.66
Skin Wash	95.0	77.0	87.0	85.9	94.6	87.9	7.39
Remaining Epidermis	0.02	0.56	1.93	0.59	0.06	0.63	0.78
Absorbed	0.08	0.09	0.07	0.05	0.03	0.07	0.02
Total Recovered	95.1	94.8	96.6	94.0	94.8	95.0	0.96

III. CONCLUSION

The results in this study demonstrated that the absorption of glyphosate from a 360g/L SL formulation or its aqueous dilution (3/200 v/v) is extremely slow through human epidermis when compared with the absorption rates of other penetrants using this in vitro technique (Dugard et al, 1984a; Dugard et al, 1984b).
The vast majority of glyphosate (greater than 87%) that may come into contact with human skin will be removed during normal washing procedures.
The small residual amounts of glyphosate found in human skin, especially that recovered from the stratum corneum, is most likely to be lost by desquamation in vivo. Over 24 hours, the amount absorbed for the concentrate was 0.50 µg/cm2 (0.03% of the applied dose) and was 0.02 µg/cm2 (0.07% of the applied dose) for the 3/200 spray dilution.

IIA 5.10 Other/special studies

PART 1 : OTHER RELEVANT REGULATORY STUDIES

Three studies were conducted to further investigate effects of glyphosate which were previously observed in classical toxicological studies. A number of repeat dose studies in rodents have identified alterations of salivary glands, described as increased basophilic staining and enlargement of cytoplasm especially in the parotid salivary glands. The toxicological significance of this effect has been previously unexplained and because of this the 2004 JMPR review of glyphosate concluded that this treatment-related effect was of unknown toxicological significance. This lead the JMPR to established a group ADI for glyphosate and AMPA of 0–1.0 mg/kg bw on the basis of the NOAEL of 100 mg/kg bw per day for salivary gland alterations in a long-term study of toxicity and carcinogenicity in rats and a safety factor of 100 ([redacted] 1993). The JMPR evaluation hypothesised that the low pH of glyphosate technical acid in the diet caused local irritation in the oral cavity leading to the observed salivary gland effects. The objective of this study was to evaluate the potential effects of low pH diet on the parotid salivary glands. Citric acid was selected as an appropriate surrogate for glyphosate, having both a similar pH-dilution curve and low toxicity. Therefore, a study with citric acid was performed to evaluate the potential effects of a low pH on the parotid salivary glands. Citric acid was given to male rats in diet (14000 ppm) and via gavage (791-1316 mg/kg bw/day). Trisodium citrate dihydrate (21400 ppm, an equivalent citrate ion concentration)

was also given in a diet for eight weeks (minimum of 56 days). Higher parotid salivary gland weights and a generally correlative increase in severity of background cytoplasmic alterations in the parotid salivary glands at all dose levels was observed. These effects were noted as most severe in the low pH dietary test group. In the absence of cytotoxicity and hyperplasia the noted effects were considered as an adaptive response rather than an adverse effect and are consistent with the hypothesis that low pH diets result in adaptive cellular responses within the salivary glands ([redacted] 2002, IIA 5.10/01).

Since effects on salivary glands were previously not observed in every rat strain, a study was conducted ([redacted] 1996; IIA 5.10/02) to investigate strain specific effects. Administration of diets containing 20000 ppm glyphosate acid to male rats for 4 weeks produced marked strain differences in the severity of effects in the parotid salivary gland. Salivary gland weights were increased after 4 weeks of treatment in the F344 and AP (Alpk:AP$_f$SD, Wistar-derived) strains. Microscopic examination of the salivary glands showed the most pronounced effect occurred in the F344 strain where there was diffuse cytoplasmic basophilia and enlargement of the parotid acinar cells. Similar but slighter effects occurred in the AP (Alpk:AP$_f$SD, Wistar-derived) and CD (Sprague-Dawley; Charles River) strains involving small foci of cells only. Complete recovery of both salivary gland weights and histopathological changes was apparent in AP and CD strains following the 4-week recovery period. The salivary gland weight increased recovered in the F344 strain however there was evidence that the salivary glands had not fully recovered in all the F344 strain animals after a 13 week recovery period.

Based on the weight of evidence across the studies presented by the glyphosate taskforce it is proposed that the changes observed in the salivary gland (hypertrophy and basophilia of the parotid acinar cells) are a non-adverse adaptive response to treatment with a low pH diet for the following reasons:

- The effect is observed with another organic acid with a similar pH-dilution curve to glyphosate.
- The effect is only observed following treatment in the diet. The same effect has not been observed acoss an extensive database following other exposure routes. The ADME radiolabel studies indicate glyphosate does not accumulate in the salivary gland.
- The effect, seen primarily in the rat, is variable in severity and has not been observed consistently across sex, dose or strain.
- The salivary gland is not a significant target organ in other species.
- From a histopathological perspective across an extensive database, there is no accompanying evidence of cytotoxicity leading to necrosis or apoptosis, no evidence of inflammation or change in function and the cellular alterations do not progress with time to preneoplastic or neoplastic lesions (but in fact decrease in incidence and severity or disappearance all together with time).
- The effect is reversible upon cessation of treatment with a low pH diet.

Pharmacological activity of the test substance was investigated *in vivo* with rats, which were treated with a single oral dose of 5000 mg/kg glyphosate. One hour after dosing, no haematological, electrographic or behavioural/functional changes were observed when compared to control animals ([redacted] 1996, IIA 5.10/03). In the same study, *ex vivo* investigations with isolated guinea pig ileum and isolated rat gastrocnemius muscle were performed. When administered to the isolated guinea pig ileum, glyphosate technical caused a contractile response similar to that seen with known parasympathomimetic agents. This effect might be related to the gastrointestinal disturbances (stools and diarrhoea), that were seen in acute and short-term toxicity studies. Evaluation of innervated muscle response in the same study showed that the test substance, when administered at the maximum solubility concentration in physiological saline, did not cause any neuromuscular blocking activity.

Table 5.10-1: Summary of special studies

	Reference (Data owner)	Type of study Species, Strain	Application Route (Dose)	Test substance	Purity [%]	Results
Studies not reviewed in the 2001 evaluation	IIA 5.10/01 [redacted] 2010 (MON / GTF)	8-week oral toxicity; Sprague-Dawley Rat, ♂	Gavage (791-1316 mg/kg bw), Diet (14000 (Citric acid), 21400 ppm (Trisodium citrate dehydrate))	Citric acid, Trisodium citrate dihydrate	99.3	Higher parotid salivary gland weights and a generally correlative increase in severity of background cytoplasmic alterations in the parotid salivary glands
	IIA 5.10/02 [redacted] 1996 (SYN)	4-week oral toxicity; Sprague-Dawley (CD)/Fischer 344/Alpk:APSD (AP), Rat, ♂	Diet (20000 ppm)	Glyphosate acid	95.6	Marked strain differences in the severity of effect in the parotid salivary glands; most pronounced effect occurred in the F344 strain: diffuse cytoplasmic basophilia and enlargement of the parotid acinar cells; similar but slighter effects occurred in the AP and CD) strains involving small foci of cells only
	IIA 5.10/03 [redacted], 1996 (NUF)	Pharmacology Screening study; Sprague-Dawley Rat, ♂ + ♀	Gavage (5000 mg/kg bw, single dose), Injection	Glyphosate technical	95.3	No haematological, electrogra-phic or behavioural/functional changes after oral administration; contractile response similar to that seen with known parasympathomimetic agents in isolated guinea pig ileum; no neuromuscular blocking activity on innervated muscle
	IIA 5.10/04 [redacted] 2012 (MON)	Mice, B6C3F1 ♀	Diet 0, 500, 1500, 5000 ppm (0, 150, 449, 1448 mg/kg bw/day)	Glyphosate	95.11	No suppression of the humoral component of the immune system. No test-substance-related effects

Annex point	Author(s)	Year	Study title
IIA, 5.10/01	[redacted]	2010	An 8-Week Oral (Diet and Gavage) Toxicity Study of Citric Acid in Male Rats [redacted] Data owner: Monsanto/GTF Study No.: [redacted]-50361 Date: 2010-01-08 GLP: yes unpublished
Guideline:			Guideline does not exist for this kind of study but data from the study report is similar to OECD 408.
Deviations:			not applicable

Dates of experimental work: 2009-02-24 to 2009-05-15

Executive Summary
A number of repeat dose studies in rodents with glyphosate technical acid have identified alterations of the salivary glands, described as increased basophilic staining and enlargement of cytoplasm, especially in the parotid salivary glands. The toxicological significance of these observations were considered not relevant, by some reviewers and unknown by others. In the 2004 JMPR review of glyphosate, a hypothesis was proposed that the low pH of glyphosate technical acid in the diet caused local irritation in the oral cavity leading to the observed salivary gland effects. The objective of this study was to evaluate the potential effects of low pH diet on the parotid salivary glands. Citric acid was selected as an appropriate surrogate for glyphosate, having both a similar pH-dilution curve and low toxicity. Citric acid was presented in the diet (14000 ppm) and compared with a typical pH basal diet control group. A higher pH diet group fed basal diet with trisodium citrate dihydrate (21400 ppm, an equivalent citrate ion concentration) was also compared with the typical pH basal diet control group. In addition, low pH aqueous citric acid was administered by gavage and compared to a control deionised water gavage group to evaluate potential systemic effects of the citrate ion on the parotid salivary glands. These five test groups, each consisting of 10 male rats, were dosed for eight weeks (minimum of 56 days).
Clinical signs, bodyweight and food consumption were monitored during the study. All animals were subjected to a gross necropsy examination and a comprehensive histopathological evaluation of tissues was performed. The findings are summarised as follows:
There were no test substance-related clinical signs of toxicity, as well as no test substance-related effects on body weight, and food consumption.

Test substance-related effects on organ weights consisted of statistically significantly higher parotid salivary gland weights in the low pH diet group only (14000 ppm citric acid) when compared to the respective control group. Non-statistically significantly higher parotid salivary gland weights were noted in the gavage citric acid and high pH dietary (21400 ppm trisodium citrate dihydrate) groups when compared to their respective control group. There were no statistically significant test substance-related effects on the fused mandibular/sublingual salivary gland weights when the respective control and test substance-treated groups were compared; however, a non-statistically significantly higher fused mandibular/sublingual salivary gland weight was noted in the low pH diet group (14000 ppm citric acid).

Histological effects consisted of cytoplasmic alterations in the parotid salivary glands characterized by the presence of hypertrophied acinar cells with basophilic granular cytoplasm. Although the overall incidence of affected animals was similar in all control and citric acid or trisodium citrate dihydrate-treated groups, these effects were clearly most severe in the low pH diet group (14000 ppm citric acid in basal diet). With the absence of microscopic findings such as cytotoxicity and hyperplasia, the observed effects are considered to be an adaptive response to local irritation of the low pH diet in the oral cavity rather than an adverse effect.

Conclusion
Citric acid administered orally via gavage or diet and trisodium citrate dihydrate administered via the diet to Sprague Dawley rats for 56 days resulted in higher parotid salivary gland weights and a generally correlative increase in severity of background cytoplasmic alterations in the parotid salivary glands at all dose levels (791-1316 mg/kg bw/day gavage citric acid, 14000 ppm diet citric acid, and 21400 ppm diet trisodium citrate dihydrate). The magnitude of change in parotid gland weight and severity of the cytoplasmic alteration in the parotid salivary glands was most severe in the low pH 14000 ppm diet citric acid group.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test materials:

Identification:	Anhydrous Citric Acid
Description:	White powder
Lot/Batch #:	XR3050
Purity:	99.9%
Stability of test compound:	Stable at room temperature until 2010-01-06.

Identification:	Trisodium Citrate Dihydrate (TCD)
Description:	White crystalline solid
Lot/Batch #:	1387609
Purity:	99.3%
Stability of test compound:	Stable at room temperature until 2011-03-01.

2. Vehicle and/ or positive control:

Gavage: deionised water, Diet: plain diet

3. Test animals:

Species:	Rats
Strain:	Sprague-Dawley (CD)
Source:	[redacted] US
Age:	approx. 6 weeks upon beginning of treatment
Sex:	males
Weight at dosing:	177 - 227 g
Acclimation period:	14 days
Diet/Food:	Certified Rodent LabDiet [redacted] ([redacted]) *ad libitum*
Water:	tap water, *ad libitum*
Housing:	Upon arrival, animals were housed three per cage for approximately 3 days. Thereafter, all animals were housed individually in clean, stainless steel, wire-mesh cages suspended above cage board.
Environmental conditions:	Temperature: 22 ± 3°C Humidity: 50 ± 20% Air changes: at least 10/hour 12 hours light/dark cycle

B: STUDY DESIGN AND METHODS

In life dates: 2009-02-10 to 2009-04-21

Animal assignment and treatment:
In a 8 week gavage and feeding study groups of 10 Sprague Dawley rats received the respective vehicles or test substances for 56 consecutive days via oral gavage (Groups 1 and 3) or in the diet (Groups 2, 4 and 5; see Table 5.10-2). A low pH diet containing 14000 ppm of citric acid in basal diet was offered continuously to Group 4. A high pH diet containing 21400 ppm of trisodium citrate dihydrate in basal diet (at an equivalent citrate ion concentration to Group 4) was offered continuously to Group 5. A concurrent

control group (Group 2) received the basal diet on a comparable regimen. Citric acid in the vehicle, deionised water, was administered orally by gavage at a dose level of 791-1316 mg/kg/day to Group 3. Concentrations of the Group 3 formulations were calculated and adjusted weekly, based on the average food consumption and body weights of the Group 4 animals from the previous week of dosing in order to maintain approximately equivalent citric acid dose levels to Group 4. A concurrent gavage control group (Group 1) received the vehicle on a comparable regimen.

Table 5.10-2: Study group assignment

Group Number	Test Substance application	Dose Level (mg/kg bw/day or ppm)	Dose Volume (mL/kg)	Number of animals
1	Gavage Vehicle	0	10	10
2	Basal Diet	0	na	10
3	Gavage Citric Acid (low pH)	791-1316	10	10
4	Diet Citric Acid (low pH)	14,000	na	10
5	Diet Trisodium Citrate (high pH)	21,400	na	10

na - not applicable

Observations
All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly.

Body weight
Individual body weights were recorded weekly.

Food consumption and compound intake
Food consumption was recorded weekly.

Sacrifice and pathology
All animals sacrificed at scheduled termination were subjected to a gross pathological examination. Any macroscopic findings were recorded.
The following organ weights were determined: parotid salivary glands, mandibular salivary glands and sublingual salivary glands. The mandibular and sublingual salivary glands were weighed together as one organ since they were fused and could not be adequately separated for weighing.
Tissue samples were taken from the following organs and preserved in buffered formalin: adrenals, aorta, bone & bone marrow (sternum and femur (incl. joint)), brain (cerebrum at two levels; cerebellum with medulla/pons), caecum, colon, duodenum, epididymides, eyes with optic nerves, gross lesions, harderian glands, heart, ileum, jejunum, kidneys, lacrimal gland (exorbital), liver, lungs (incl. bronchi), mammary gland, lymph nodes (mandibular, mesenteric and axillary), nasal cavity, oesophagus, pancreas, Peyer's patches, pituitary, prostrate, rectum, salivary glands (mandibular, parotid, sublingual), sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, testes, thymus, thyroid/parathyroid, tongue, trachea and urinary bladder.
Microscopic examination was performed on the parotid salivary glands and gross lesions from all animals at the scheduled necropsy.

Statistics
All statistical tests were performed using the WIL Toxicology Data Management System (WTDMS™). Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test substance-treated group to its respective control group.
Body weight, body weight change, food consumption, and organ weight data were subjected to a parametric one-way analysis of variance (ANOVA) to determine intergroup differences. If the ANOVA identified statistically significant ($p<0.05$) intergroup variance, Dunnett's test was used to compare each of the test substance-treated groups to the respective control group (Group 1 to Group 3 and Group 2 to Groups 4 and 5). Group 1 was also compared to Group 2.
Statistical analysis of the severity of histological changes was conducted. Individual animals were assigned severity scores based on parotid salivary gland changes (0=without histological change,

1=minimal change, 2=mild change, and 3=moderate change). The severity scores were then compared statistically using the Mann-Whitney U-test by comparing Group 1 to Group 3 and Group 2 to Groups 4 and 5.

II. RESULTS AND DISCUSSION

A. MORTALITY

No deaths occurred during the study.

B. CLINICAL OBSERVATIONS

All clinical findings in the test substance-treated groups were noted with similar incidence in the control groups, were limited to single animals, and/or were common findings for laboratory rats of this age and strain.

C. BODY WEIGHT

There were no statistically significant differences when the respective control and test substance-treated groups were compared.

D. FOOD AND TEST SUBSTANCE CONSUMPTION

Food consumption was unaffected by citric acid or trisodium citrate dihydrate administration. A statistically significant decrease in food consumption of the gavage citric acid group (Group 3, Week 7/8) was probably due to biological variability and not considered related to test substance administration.

E. PATHOLOGY

Organ weights

Test substance-related effects on organ weights consisted of statistically significant higher absolute and relative parotid salivary gland weights in the low pH diet group (14,000 ppm citric acid) when compared to the dietary control group; the magnitude of change was ~30% (Table 5.10-3).

Higher absolute and relative parotid salivary gland weights were also observed in the low pH gavage group (791-1316 mg/kg bw/day citric acid) and in the high pH diet group (21,400 ppm TCD) when compared to their respective control groups. However, the parotid salivary gland weight differences in the low pH gavage and high pH diet groups were not statistically significant and were of much lesser magnitude of change.

There were no other statistically significant test substance-related effects on the fused mandibular/ sublingual or parotid salivary gland weights when the control groups and test substance-treated groups were compared.

Table 5.10-3: Toxicologically relevant organ weight differences

	Gavage Administration		Dietary Administration		
	aqueous control	791-1316 mg/kg bw/day citric acid	basal diet control	low pH diet, 14000 ppm citric acid	high pH diet, 21400 ppm trisodium citrate dihydrate
Mean Absolute Mandibular / Sublingual Fused Glands Weight (g)	0.7625 ± 0.05446	0.7873 ± 0.08397	0.7682 ± 0.08670	0.8872 ± 0.16548	0.7869 ± 0.07028
Mean Relative Mandibular / Sublingual Fused Glands Weight (g)	0.179 ± 0.0105	0.180 ± 0.0178	0.173 ± 0.0221	0.199 ± 0.0339	0.183 ± 0.0201
Mean Absolute Parotid Gland Weight (g)	0.3500 ± 0.12450	0.4082 ± 0.11990	0.2758 ± 0.08514	0.3905* ± 0.10920	0.3502 ± 0.08986
Mean Relative Parotid Gland Weight (g)	0.083 ± 0.0299	0.095 0.0304	0062 ± 0.0194	0.088* ± 0.0236	0.082 0.0220

* - significantly different from relevant control group ($p < 0.05$) using Dunnett's test

Necropsy

All macroscopic findings noted were considered spontaneous and/or incidental in nature and unrelated to test substance administration.

Histopathology

Test substance-related histological effects consisted of a higher severity of cytoplasmic alterations in the parotid salivary glands of the citric acid and trisodium citrate dihydrate-treated groups when compared to their respective control groups (Table 5.10-4). The severity of cytoplasmic alteration was increased in all dose groups; however, the cytoplasmic alteration was clearly most severe in the low pH diet group (Group 4; 14000 ppm citric acid).

Cytoplasmic alteration in the parotid salivary glands was characterized by the presence of hypertrophied acinar cells with basophilic granular cytoplasm. The severity grades ranged from minimal to moderate, displayed by increasing numbers of affected acinar cells and more pronounced hypertrophy of acinar cells with increasing severity grade.

Cytotoxicity and hyperplasia were not observed and consequently, the observed changes were considered to be adaptive responses rather than adverse effects. There were no other test substance-related histological changes.

Table 5.10-4: Toxicologically relevant histological changes

	Gavage Administration		Dietary Administration		
	aqueous control	791-1316 mg/kg bw/day citric acid	basal diet control	low pH diet, 14,000 ppm citric acid	high pH diet, 21,400 ppm trisodium citrate dihydrate
Parotid salivary glands [a]	9	10	10	10	10
Incidence (%)	*100*	*100*	*70*	*100*	*90*
minimal	8	6	5	0	4
mild	1	3	2	6	5
moderate	0	1	0	4	0
Average severity [b]	**1.1**	**1.5**	**0.9**	**2.4****	**1.4**

[a] - number of tissues examined from each group
[b] - 1= minimal, 2= mild and 3= moderate; animals without a histological change were assigned a severity score of 0
** - significantly different from relevant control group ($p < 0.01$) using the Mann-Whitney U-test

III. CONCLUSION

Citric acid administered orally via gavage or diet and trisodium citrate dihydrate administered via the diet to Sprague Dawley rats for 56 days resulted in higher parotid salivary gland weights and a generally correlative increase in severity of background cytoplasmic alterations in the parotid salivary glands at all dose levels (791-1316 mg/kg bw/day gavage citric acid, 14000 ppm diet citric acid, and 21400 ppm diet trisodium citrate dihydrate). This effects were noted as most severe in the low pH dietary test group. In the absence of cytotoxicity and hyperplasia the noted effects are considered an adaptive response rather than an adverse effect and are consistent with the hypothesis that low pH diets result in adaptive cellular responses within the salivary glands.

Annex point	Author(s)	Year	Study title
IIA, 5.10/02	[redacted]	1996	Glyphosate Acid: Comparison of Salivary Gland Effects in Three Strains of Rat. [redacted] Data owner: Syngenta Study No.: [redacted]/5160 Date: 1996-08-19 GLP: yes unpublished
Guideline:			Guideline does not exist for this kind of study.
Deviations:			not applicable
Dates of experimental work:			1996-01-15 to 1996-05-14

The purpose of this study was to investigate the rat strain susceptibility of the effects of glyphosate acid on the salivary gland after 4 weeks administration in these strains of rat. In studies with F344 rats, glyphosate acid has been shown to cause effects on the salivary gland (NTP, 1992[8]). In contrast, there was no evidence of microscopic changes in the salivary gland in a previously conducted 28 day feeding study

[8] NTP (1992). Technical Report on Toxicity Studies of Glyphosate Administered in Dosed Feed to F344/N Rats and B6C3F1 Mice. United States Department of Health and Human Services, National Toxicology Program Toxicity Reports Series Number 16

with glyphosate acid (20000 ppm in the diet) in Alpk:AP_fSD rats, although there was an effect on gland weight ([redacted] 1995)[9].

Study groups of 24 male Alpk:AP_fSD (Wistar-derived; AP), Sprague-Dawley (Charles River CD; CD) and Fischer 344 (F344) rats received 0 or 20,000 ppm glyphosate acid. Eight animals from each group were killed on Day 29 and the remaining animals were retained without treatment for a further four (8 rats/group) or 13 weeks (8 rats/group). Clinical observations, bodyweights and food consumption were measured and at the end of the scheduled periods, the animals were killed and subjected to a necropsy. Salivary glands were weighed and taken for subsequent histopathology examination.

Treatment with 20000 ppm glyphosate acid produced significant reductions in bodyweight and minor reductions in food consumption in AP and CD rats but no effects on bodyweight or food consumption were seen in the F344 rat. In contrast, salivary gland weight was unaffected in the CD rat but was increased in both AP and F344 rats at the end of the administration period. Microscopic examination of the salivary glands showed the most pronounced effect occurred in the F344 strain where there was diffuse cytoplasmic basophilia and enlargement of the parotid acinar cells. Similar but slight effects involving small foci of cells only occurred in the AP and CD strains.
Recovery of effects was apparent in all strains during the recovery periods. Bodyweight and food consumption returned to control values in both AP and CD strains. After four weeks on control diet significant recovery of the salivary gland changes, in terms of both weight and micropathology, was evident in the F344 strain and the AP and CD rats were indistinguishable from their corresponding controls. After 13 weeks on control diet slightly more treated F344 rats showed minor focal changes in the salivary gland compared to the contemporaneous controls and group mean salivary gland weights were increased slightly.

Conclusion
Administration of diets containing 20000 ppm glyphosate acid to male rats for 4 weeks produced marked strain differences in the severity of effect in the parotid salivary gland. Microscopic examination of the salivary glands showed the most pronounced effect in the F344 strain. Similar but slighter effects occurred in the AP and CD strains.
Complete recovery of effects were apparent in AP and CD strains following the 4-week recovery period and significant recovery had occurred in the F344 strain. It is not clear whether the slightly higher incidence of minor focal changes in the salivary glands of the F344 strain after 13-week recovery was a residual effect of treatment or represented the random variation in the background incidence in this strain.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test materials:

Identification:	Glyphosate acid
Description:	White solid
Lot/Batch #:	P24
Purity:	95.6%
Stability of test compound:	No data given in the report.

2. Vehicle and/ or positive control: Plain diet

3. Test animals:

Species:	Rats
Strain 1:	Alpk:AP_fSD

[9] [redacted] (1995) 28day dietary toxicity study in the rat. [redacted]/6624

Source:	[redacted] UK
Weight at dosing:	175.0 – 176.1 g
Strain 2:	Sprague-Dawley
Source:	[redacted] UK
Weight at dosing:	179.6 – 181.5 g
Strain 3:	Fischer 344
Source:	[redacted] UK
Weight at dosing:	107.4 – 108.9 g
Age:	approx. 28-30 days (on delivery)
Sex:	Males
Acclimation period:	11-13 days
Diet/Food:	CT1, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were housed by strain and four per cage.
Environmental conditions:	Temperature: 21 ± 3°C Humidity: 50 ± 20% Air changes: at least 15/hour 12 hours light/dark cycle

B: STUDY DESIGN AND METHODS

In life dates: 1996-01-15 to 1996-05-14

Animal assignment and treatment:
In a 28 days feeding study groups of 24 male Alpk:APfSD (Wistar-derived; AP), Sprague-Dawley (Charles River CD; CD) and Fischer 344 (F344) rats received 0 or 20000 ppm glyphosate acid. Eight animals from each group were killed on Day 29 and the remaining animals were retained without treatment for a further 4 (8 rats/group) or 13 weeks (8 rats/group).
Two test diet batches were prepared prior to start of treatment by mixing 1255 g test substance to 58.745 kg diet and blending. Samples of both preparations were analysed to verify the achieved concentration.

Clinical observations
Clinical examinations were performed daily. A detailed physical examination was performed prior to administration and weekly thereafter.

Body weight
Individual body weights were recorded on start of administration and weekly thereafter.

Food consumption and compound intake
Food consumption was recorded continuously throughout the study for each cage of rats and calculated as a weekly mean (g food/rat/day) for each cage.

Sacrifice and pathology
All animals sacrificed at scheduled termination were subjected to a gross pathological examination of the salivary glands. Thereafter the salivary glands were removed, weighed (left and right separately) and examined by light microscopy.

Statistics
All data were evaluated using analysis of variance and/or covariance by the GLM procedure in SAS (1989). Least-squares means for each group were calculated using the LSMEAN option in SAS PROC GLM. Unbiased estimates of differences from control were provided by the difference between each treatment group least-squares mean and the control group least-squares mean. Differences from control were tested statistically by comparing each treatment group least-squares mean with the control group least-squares mean using a two-sided Student's t-test, based in the error mean square in the analysis.

II. RESULTS AND DISCUSSION

A. ANALYSIS OF DOSE FORMULATIONS
The mean achieved concentration of glyphosate acid in both batches of diet was within 2% of the target concentration (Table 5.10-5).

Table 5.10-5: Achieved concentrations of glyphosate acid in the diet

	Nominal concentration (ppm)	Mean analysed concentration (ppm)	% of nominal concentration
Batch 1	20,000	19,985	99.9
Batch 2	20,000	20,355	101.8

B. MORTALITY
There were no treatment-related deaths. One treated AP rat was killed in Week 7 following accidental damage to its snout.

C. CLINICAL OBSERVATIONS
There were no treatment-related findings in any of the groups noted during the study period.

D. BODY WEIGHT
AP rats: During the administration period significant reductions in group mean bodyweight compared to control were seen. At the end of the administration period, the difference was approximately 7%. The reduction in bodyweight was maintained during the 4-week recovery period (approximately 7% at the end of Week 9) but no differences in bodyweight were apparent by the end of the 13-week recovery period.

CD rats: Group mean bodyweights for treated animals were significantly reduced during the administration period in comparison to controls. The reduction in bodyweight was approximately 7% (after adjusting for initial bodyweight) at the end of the administration period. However, bodyweights quickly recovered and were 5% higher than controls (after adjusting for initial bodyweight) by the end of the 13-week recovery period.

F344 rats: No treatment related effects were observed.

E. FOOD CONSUMPTION
AP rats: Overall, food consumption in the treated group tended to be slightly lower than the control during the administration period although this did not achieve statistical significance. No effects were seen at the end of the recovery period.

CD rats: Group mean food consumption for treated animals was generally lower than controls during the administration period although this did not always attain statistical significance. Food consumption for the recovery animals returned to control levels by Week 8.

F344 rats: There was no evidence of any treatment related effects.

F. NECROPSY

There was no evidence of any effects of glyphosate acid on the salivary gland weight at any time point in CD rats. On the contrary salivary gland weights were increased in the treated AP and F344 rats at the end of the administration period in comparison to control. While no effects were noted in the four or 13-week recovery AP animals, in F344 rats the salivary gland weights were still increased at these time points, although there was clear evidence of recovery.

Table 5.10-6: mean salivary gland weights at necropsy

Organ	AP		CD		F344	
	0	20000	0	20000	0	20000
	Terminal weight (g)					
Left salivary gland	0.652	0.740*	0.715	0.695	0.461	0.666**
Right salivary gland	0.523	0.659*	0.623	0.626	0.422	0.577*
	Weight after 4 week recovery					
Left salivary gland	0.748	0.703	0.844	0.742	0.488*	0.553
Right salivary gland	0.639	0.623	0.701	0.637	0.428	0.505*
	Weight after 13 week recovery					
Left salivary gland	0.750	0.760	0.790	0.810	0.623	0.612
Right salivary gland	0.669	0.681	0.668	0.705	0.495	0.528

No macroscopic abnormalities were seen in salivary glands in any rat either at the end of the administration period or after the four or 13-week recovery periods.

Treatment-related findings were confined to the parotid salivary gland and comprised alteration in the staining of the cytoplasm of the acinar cells. The affected cells appeared strongly basophilic and enlarged (recorded as basophilia of parotid acinar cells).
At the end of the four-week administration period this change was most prominent in F344 rats. All rats showed marked cytoplasmic basophilia that was diffuse, involving the whole of the parotid gland. However, no evidence of cell degeneration or necrosis was seen. Most of the control F344 rats also showed a minor degree of basophilia involving occasional acinar cells only.
The other two strains, AP and CD, both showed the same effect in the parotid gland after four weeks treatment but at a much reduced severity compared to the F344. In addition the distribution was different in that only small focal groups of acinar cells were affected in the AP and CD rats in contrast to the diffuse involvement seen in the F344. The effect was weakest in the CD rat.

The incidence data at the end of the administration period indicate that the background change varies in control rats in the three strains. None was seen in the AP controls, there was a single CD control rat with a minimal focal change, whereas 7 out of 8 F344 controls showed minor changes.
After four weeks recovery in the F344 strain the severity of the parotid basophilia was reduced to minimal or slight and affected small foci of acinar cells only. No changes were seen in the CD rats and only a single AP rat showed a minimal change. As an AP control rat showed changes at this time point this is considered not to be related to treatment.
After 13 weeks recovery no treatment related changes were seen in the AP and CD strains. Slightly more of the F344 rats showed minor focal changes compared to the corresponding control group but this may reflect variations in the background spontaneous change rather than a residual effect of treatment.

Table 5.10-7: Histopathological findings in salivary glands

Finding	AP		CD		F344	
	0	20000	0	20000	0	20000
	Termination*					
Atrophy (marked)	0 / 8	0 / 8	1/8	0 / 8	0 / 8	0 / 8
Interstitial fibrosis (marked)	0 / 8	0 / 8	1/8	0 / 8	0 / 8	0 / 8
Basophilia of parotid acinar cells	0 / 8	8 / 8	1/8	7 / 8	7 / 8	8 / 8
	Weight after 4 week recovery*					
Mononuclear cell infiltration (minimal)	0 / 8	1 / 8	0 / 8	0 / 8	1 / 8	0 / 8
Basophilia of parotid acinar cells	1 / 8	1 / 8	0 / 8	0 / 8	0 / 8	6 / 8
Mucous metaplasia of parotid (slight)	0 / 8	1 / 8	0 / 8	1 / 8	0 / 8	3 / 8
	Weight after 13 week recovery*					
Mononuclear cell infiltration (minimal)	0 / 8	0 / 8	0 / 8	0 / 8	1 / 8	1 / 8
Atrophy (minimal)	0 / 8	0 / 8	0 / 8	0 / 8	1 / 8	0 / 8
Basophilia of parotid acinar cells	1 / 8	1 / 8	1 / 8	1 / 8	3 / 8	5 / 8

* number of animals affected / total number of animals examined

III. CONCLUSION

Administration of diets containing 20000 ppm glyphosate acid to male rats for 4 weeks produced marked strain differences in the severity of effect in the parotid salivary gland. Microscopic examination of the salivary glands showed the most pronounced effect occurred in the F344 strain where there was diffuse cytoplasmic basophilia and enlargement of the parotid acinar cells. Similar but slighter effects occurred in the AP and CD) strains involving small foci of cells only.
Complete recovery of effects was apparent in AP and CD strains following the 4-week recovery period and significant recovery had occurred in the F344 strain. It is not clear whether the slightly higher incidence of minor focal changes in the salivary glands of the F344 strain after 13-week recovery was a residual effect of treatment or represented the random variation in the background incidence in this strain.

Annex point	Author(s)	Year	Study title
IIA, 5.10/03	[redacted]	1996	Glyphosate Technical: Pharmacology Screening Study in the Rat [redacted] Data owner: Nufarm Study No.: 434/021 Date: 1996-06-28 GLP: yes unpublished

Guideline:	JMAFF, 59 Nohsan No. 4200 (1985)
Deviations:	not applicable
Dates of experimental work:	1996-02-06 to 1996-04-04

Executive Summary
The test material was evaluated for evidence of pharmacological activity using a series of *in vivo* and *ex vivo* screening methods. For *in vivo* studies five male and five female rats were dosed with glyphosate technical at a dose level of 5000 mg/kg with similar sized control groups receiving vehicle only. Approximately one hour after dosing control and treated animals were examined for either haematological changes, electrocardiographic changes or behavioural/functional changes. *Ex vivo* studies were evaluation of the isolated guinea pig ileum and isolated rat gastrocnemius muscle using saturated solutions of the test material.

***In vivo* studies**
There were no differences in response between treated and control animals.

***Ex vivo* studies**
Glyphosate Technical (12 mg/mL) caused a contractile response to isolated guinea pig ileum similar to that seen with acetylcholine. The effect seen was abolished when the ileum was pre-incubated with atropine sulphate.
Injection of tubocurarine resulted in a significant diminution of the contractile response of the rat gastrocnemus muscle when the sciatic nerve was stimulated. On the contrast there was no effect on muscle contraction when either glyphosate technical or physiological saline was injected.

Conclusion
At a maximum dose level of 5000 mg glyphosate technical/kg bw there were no effects seen from the *in vivo* screens performed. When administered to the isolated guinea pig ileum glyphosate technical caused a contractile response similar to that seen with known parasympathomimetic agents. Evaluation of innervated muscle response using showed that glyphosate technical, when administered at the maximum solubility concentration in physiological saline did not cause any neuromuscular blocking activity.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test materials:

Identification:	Glyphosate Technical
Description:	White powder
Lot/Batch #:	H95D16JA
Purity:	95.3%
Stability of test compound:	No data given in the report.

2. Vehicle and/ or positive control:

in-vivo	1% carboxymethyl cellulose
ex-vivo (guinea pig)	distilled water, krebs physiological buffer solution,
ex-vivo (guinea pig)	physiological saline

3. Test animals:

in-vivo	Species:	Rats
	Strain:	Sprague-Dawley (CD)
	Source:	[redacted] UK
	Age:	no data
	Sex:	Males and females
	Weight at dosing:	176 - 200 g
	Acclimation period:	At least 6 days

	Diet/Food:	[redacted] Rat and Mouse Diet No.1 Expanded ([redacted] UK), *ad libitum*
	Water:	Tap water, *ad libitum*
	Housing:	By sex in groups of five in polypropylene cages with stainless steel grid floors.
	Environmental conditions:	Temperature: 19 – 25°C
		Humidity: 40 – 75%
		Air changes: at least 15/hour
		12 hours light/dark cycle
ex-vivo	Species:	Guinea pig
	Strain:	Dunkin Hartley
	Source:	[redacted] UK
	Age:	no data
	Sex:	Males
	Weight at dosing:	250 - 300 g
	Acclimation period:	no data
	Diet/Food:	Guinea Pig [redacted] Diet ([redacted] UK), *ad libitum*
	Water:	Tap water, *ad libitum*
	Housing:	By sex in groups of up to three in polypropylene cages with solid floors and sawdust bedding.
	Environmental conditions:	Temperature: 17 – 23°C
		Humidity: 30 – 70%
		Air changes: at least 15/hour
		12 hours light/dark cycle
in-vivo	Species:	Rats
	Strain:	Sprague-Dawley (CD)
	Source:	[redacted] UK
	Age:	no data
	Sex:	Males and females
	Weight at dosing:	110 - 125 g
	Acclimation period:	no data
	Diet/Food:	[redacted] Rat and Mouse Diet No.1 Expanded ([redacted] UK), *ad libitum*
	Water:	Tap water, *ad libitum*
	Housing:	By sex in groups of five in polypropylene cages with stainless steel grid floors.
	Environmental conditions:	Temperature: 19 – 25°C
		Humidity: 30 – 70%
		Air changes: at least 15/hour
		12 hours light/dark cycle

B: STUDY DESIGN AND METHODS

In life dates: 1996-03-25 to 1996-03-27

Animal assignment and treatment of *in vivo* studies:
Three groups of five male and five female rats each received glyphosate technical at a dose level of 5000 mg/kg bw by oral gavage. The control group was similar sized receiving vehicle only. The dosing volume was 10 mL/kg bw. Approximately one hour after dosing control and treated animals were examined for either haematological changes, electrocardiographic changes or behavioural/functional changes.

Blood parameters
Blood samples were taken from all animals via a tail vain. The following parameters were evaluated: Haemoglobin (Hb), total erythrocyte count (RBC), haematocrit (Hct), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), total leucocyte count (WBC), platelet count (PLT) and clotting (Prothrombin) time (CT).

Cardiovascular system
After animals were anaesthetised cardiac activity was assessed using an electrocardiograph. A limb lead was attached to each limb and connected to the electrocardiogram. The equipment was set to lead II measurement at a sensitivity of either 10 mm/mvolt or 5 mm/mvolt and a chart speed of 25 mm/second. The following parameters were evaluated: Heart rate, P-R interval, QRS interval, Q-T interval, P-amplitude, R-amplitude, T-amplitude.

Nervous system
Animals were placed individually in a purpose built arena and assessed for behaviour and response to various stimuli using a modified Irwin Screen. The following parameters were evaluated: Salivation, hypo/hyperthermia, skin colour, respiration, lacrymation, palpebral closure, pilo-erection, exophthalmia, gait, twitches, tremors, convulsions, abnormal behaviour, tail elevation, transfer arousal, urination, defaecation, vocalisation, finger approach, touch escape, tail & toe pinch, grasp response, auditory startle response, pupil response to light, palpebral reflex.

Animal assignment and treatment of *ex vivo* studies:

Guinea pig - Isolated ileum
Sections of ileum were dissected from previously untreated guinea pigs killed by cervical dislocation. and were transferred to a purpose built isolated organ bath containing Krebs buffer solution with a test substance concentration of 12 mg/mL (maximum solubility). The isolated ileum was connected to the lever arm of an isotonic transducer by a cotton ligature. The transducer was connected to a chart recorder. Contractions of the isolated ileum could then be recorded. Standard solutions of acetylcholine, a known agonist, were prepared and added to the volume of buffer solution used to bathe the isolated ileum. A maximum volume of 2 mL was used for all experiments to ensure the integrity of the tissue in the medium. The contraction response of isolated ileum was recorded for each concentration of acetylcholine to produce a standard curve. Between additions of each new concentration of acetylcholine, the buffer in the organ bath was flushed out and replaced by fresh buffer. The test material, dissolved in buffer, was added and its response compared with standards. Following initial results an antagonist (atropine) to the effects of acetylcholine was added together with the agonist. The results were then compared with the effects of an antagonist and the test material.

The following parameters were evaluated: Response to acetylcholine (agonist), test material, atropine (antagonist) and acetylcholine (agonist), atropine (antagonist) and test material.

Rat - Gastrocnemius muscle
Previously untreated rats were killed by cervical dislocation. The abdomen was immediately dissected open and the dorsal aorta exposed. A butterfly needle was inserted into the dorsal aorta, near to the bifurcation in a posterior direction.
A volume of 0.3 mL of lithium heparin at a concentration of 10 mg/mL in sterile saline was injected into the dorsal aorta followed by 0.5 mL of sterile saline.
The gastrocnemius muscle of the hind limb was exposed with the sciatic nerve intact. The gastrocnemius muscle was detached from the ankle joint and this area was ligated with cotton which was then attached to the lever arm of a transducer. The limb was held in place by a series of pins. An electrical stimulus of 12 volts was applied to the sciatic nerve and the muscle response was recorded. This action was repeated at approximately twelve second intervals until sufficient responses had been recorded.
The experiment was repeated on separate animals with doses of tubocurarine (positive control) injected into the dorsal aorta instead of sterile saline. The experiment was also repeated on a separate animal with the test material dissolved in sterile saline at a concentration of 12 mg/mL (maximum solubility).

The following parameters were evaluated: Response to injection of sterile saline, tubocurarine and test material.

II. RESULTS AND DISCUSSION

A. BLOOD PARAMETERS
There were no biologically significant differences, among the parameters measured, between treated and control animals.

B. CARDIOVASCULAR SYSTEM
There were no biologically significant differences, among the parameters measured, between treated and control animals.

C. NERVOUS SYSTEM
There were no biologically significant differences, among the parameters measured, between treated and control animals.

D. GUINEA PIG - ISOLATED ILEUM
The addition of acetylcholine to the medium containing the isolated guinea pig ileum resulted in contraction of the tissue in a concentration related response. Incubation with atropine sulphate immediately prior to addition of acetylcholine diminished or abolished the contraction response in a concentration related manner.
The addition of glyphosate technical at the maximum solubility in buffer also resulted in contraction of the ileum. The force of contraction was increased by an increasing volume of the test material in solution. Incubation with atropine sulphate prior to addition of glyphosate technical also resulted in the abolition of contractile response.

E. RAT - GASTROCNEMIUS MUSCLE
Injection of tubocurarine at a concentration of 25 mg/mL resulted in a significant diminution of the contractile response of the rat gastrocnemus muscle when the sciatic nerve was stimulated. There was no effect on muscle contraction when either glyphosate technical (12 mg/mL) or physiological saline was injected. The difference in force of response seen with glyphosate technical and physiological saline can be attributed to individual animal variation.

III. CONCLUSION

At a maximum dose level of 5000 mg glyphosate technical/kg bw there were no effects seen from the *in vivo* screens performed. When administered to the isolated guinea pig ileum glyphosate technical

caused a contractile response similar to that seen with known parasympathomimetic agents. Evaluation of innervated muscle response using showed that glyphosate technical, when administered at the maximum solubility concentration in physiological saline, did not cause any neuromuscular blocking activity.

Annex point	Author(s)	Year	Study title
IIA, 5.10/04	[redacted]	2012	Glyphosate – A 28-Day Oral (Dietary) Immunotoxicity Study in Female B6C3F1 Mice [redacted] Project No: [redacted]10-460 (Study No: [redacted]-0393) Data owner: Monsanto Date: 2012-03-21 GLP: yes not published

Guideline:	US-EPA OPPTS 870.7800 (1998)
Deviations:	None
Dates of experimental work:	2010-10-05 - 2010-11-17

Executive Summary

The potential immunotoxicity of glyphosate was evaluated after repeated dietary administration to B6C3F1 mice. Four groups of 10 female mice were offered diets containing glyphosate concentrations of 0, 500, 1500 or 5000 ppm (equivalent to 0, 150, 449, and 1448 mg/kg bw/day) and for 28 consecutive days. A further group of 10 females were used as positive immunosuppressive control group. These mice received basal diet for 28 days and were treated with an intraperitoneal (IP) injection of 50 mg/kg bw/day once daily for four consecutive days (study days 24-27).

The animals were checked twice daily for mortality and once daily for clinical signs. Detailed clinical examinations were performed once per week. Body weights were recorded twice weekly. Food consumption was recorded in weekly intervals and food intake was calculated for the corresponding body weight intervals. Blood samples for IgM antibody analysis were collected from all mice at scheduled necropsy. At termination, the animals were sacrificed and subjected to a full macroscopic post-mortem examination. Spleens and thymus were weighed and specified tissues preserved.

There were no test substance-related effects on survival, clinical observations, body weight, food consumption, as well as any gross pathological changes. There were no test substance-related effects on spleen or thymus weights (absolute or relative to final body weight), spleen cellularity, or the T-cell dependent antibody response (TDAR), as measured by the AFC IgM Specific Activity (AFC/10^6 spleen cells) and Total Spleen Activity (AFC/spleen), at any dosage level tested.

Conclusion

Treatment of female B6C3F1 mice for 28 days with diets containing glyphosate was well tolerated and did not suppress the humoral component of the immune system when evaluated using the AFC assay. The no-observed-effect level (NOEL) for suppression of the humoral immune response in female B6C3F1 mice offered glyphosate in the diet for 28 days at 500, 1500, and 5000 ppm was considered to be 5000 ppm (equivalent to 1448 mg/kg of body weight/day).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	Glyphosate
Description:	White powder
Lot/Batch #:	GLP-0807-19475-T
Purity:	95.11 % (dried)
Stability of test compound:	Expiry date: 2011-06-10

2. Vehicle and/ or positive control: Basal diet
Cyclophosphamid monohydrate

3. Test animals:

Species:	Mouse
Strain:	B6C3F1/Crl
Source:	[redacted] USA
Age:	Approx. 37 days (on arrival)
Sex:	Female
Weight at dosing:	16.5 – 20.0 g
Acclimation period:	14 days
Diet/Food:	Certified Rodent LabDiet [redacted] (meal) ([redacted]) *ad libitum*
Water:	Tap water *ad libitum*
Housing:	Individually in stainless steel, wire-mesh cages suspended above cage-board.
Environmental conditions:	Temperature: 22 ± 3° C
	Humidity: 50 ± 20%
	Air changes: 10/hour
	12 hours light/dark cycle

B: STUDY DESIGN AND METHODS

In life dates: 2010-10-05 to 2010-11-16

Animal assignment and treatment:
In a 28-day oral immunotoxicity study groups of 10 female B6C3F1/Crl mice received daily dietary doses of 0, 500, 1500 and 5000 ppm glyphosate (equivalent to 0, 150, 449 and 1448 mg/kg bw/day).
A further group of 10 females were used as positive immunosuppressive control group. These mice received basal diet for 28 days and were treated with an intraperitoneal (IP) injection of 50 mg/kg bw/day once daily for four consecutive days (study days 24-27).
Test diets were prepared weekly and stored at room temperature. For the negative and positive control groups an appropriate amount of basal diet was weighed into a plastic storage bag. For the test substance groups 500 g of basal diet was weighed (pre-mixture). An appropriate amount of glyphosate was weighted into a mortar, mixed with a small amount of the pre-mixture basal diet, and ground until uniform. This admixture was transferred to a Hobart mixer and mixed with the remainder of the pre-mixture basal diet for five minutes. The resultant mixture was then transferred to a V-blender with a sufficient amount of basal diet to achieve the correct diet concentration and mixed for an additional 10 minutes using an intensifier bar during the first and last three minutes of mixing to ensure a homogeneous mixture. The test diets were prepared from the lowest to highest concentration. The stability and homogeneity of the test

substance in the diet was determined in an in-house stability study at 450 and 5500 ppm. Analyses for achieved concentrations on the test diets were done during study weeks 0 and 3.

Mortality
Each animal was checked for mortality or signs of morbidity twice a day during the treatment period, including weekends and public holidays.

Clinical observations
A check for clinical signs of toxicity was made once daily on all animals. In addition, a detailed clinical examination was performed at once a week during the study period, beginning one week prior to randomisation, and on the day of scheduled necropsy.

Body weight
Individual body weights were recorded twice weekly, beginning approximately one week prior to randomization, at the time of animal selection for randomization, on study day 0, and just prior to the scheduled necropsy. Mean body weights and mean body weight changes were calculated for the corresponding intervals.

Food consumption and test substance intake
The quantity of food consumed was recorded for each animal weekly, beginning approximately one week prior to randomization, and just prior to the scheduled necropsy. Food intake was calculated as g/animal/day for the corresponding body weight intervals. The mean amounts of glyphosate consumed (mg/kg/day) per dose group were calculated from the mean food consumed (g/kg of body weight/day) and the appropriate target concentration of glyphosate in the food (mg/kg of diet).

Serum collection for possible IgM antibody analysis
For determination of the possible extent of the suppression of IgM antibody production blood samples were collected from all animals at scheduled necropsy and processed to serum. Following euthanasia by carbon dioxide inhalation, approximately 0.75 mL of blood was collected from the inferior vena cava of each mouse into a tube containing no anticoagulant and allowed to clot. Serum was obtained and aliquots of approximately 150 µL (including any remainder serum) were transferred to cryovials and stored frozen (approximately -70°C).

Sacrifice and pathology
A complete necropsy was conducted on all animals at scheduled termination or on animals that died or were sacrificed during the study period. Any macroscopic findings were recorded. The following organ weights were determined from all animals surviving to scheduled termination: spleen and thymus.
Tissue samples were taken from the spleen and thymus. Spleen samples were placed in EBSS/HEPES buffer. Thymus samples were preserved in 10% neutral-buffered formalin.

Spleen processing for immunotoxicological evaluation
For the determination of the number of specific IgM antibody-forming cells directed towards sRBC an AFC assay, as a modification of the Jerne plaque assay (Jerne et al., 1963, 1974) was conducted.

Spleens were collected from all animals at the scheduled necropsy (study day 28) immediately following blood collection. Individual spleens were placed into individual tared tubes containing EBSS with 15 mM HEPES, supplemented with gentamicin as a bacteriostat, and maintained on ice. Each tube was then weighed to provide a "wet" weight for each spleen. Spleen samples from Groups 1-4 animals were randomized and coded for antibody-forming cell (AFC) analysis. Spleen samples from Group 5 were labelled as positive control samples for analysis. The spleen samples were placed on crushed ice until procession for AFC analysis.
The spleen samples were processed into single-cell suspensions. The cell suspensions were then centrifuged and resuspended in EBSS with HEPES. Spleen cell counts were performed using a Model Z1™ Coulter Counter®. Viability of splenocytes was determined using propidium iodide and the Coulter® EPICS® XL-MCL™ Flow Cytometer

Statistics

Body weight, body weight change, and food consumption data were subjected to a parametric one way ANOVA (Snedecor and Cochran, 1980) to determine intergroup differences. If the ANOVA revealed statistically significant ($p<0.05$) intergroup variance, Dunnett's test (Dunnett, 1955, 1964) was used to compare the test substance treated groups to the control group.
The positive control data were evaluated using the Student's t-Test (Sokal and Rohlf, 1981) and compared to the basal diet control group.

Organ weight (wet spleen and thymus), final body weight, and AFC data obtained were first tested for homogeneity of variances using the Bartlett's Chi Square test (Bartlett, 1937). Homogeneous data were evaluated using a parametric one-way ANOVA (Kruskal and Wallis, 1952). When significant differences occurred, the treatment groups were compared to the basal diet control group using Dunnett's test (Dunnett, 1955, 1964). Non-homogeneous data were evaluated using a non-parametric ANOVA (Wilson, 1956). When significant differences occurred, the treatment groups were compared to the basal diet control group using the Gehan-Wilcoxon test when appropriate (Gross and Clark, 1975). The Jonckheere's test (Hollander and Wolfe, 1973) was used to test for dose-related trends across the basal diet control and test substance treated groups. The positive control data were evaluated using the Student's t Test (Sokal and Rohlf, 1981) and compared to the basal diet control group. The criteria for accepting the results of the positive control group included a statistically significant ($p\leq0.05$) decrease in the response when compared to the response of the basal diet control group.
The AFC data were expressed as Specific Activity, IgM antibody-forming cells per million spleen cells (AFC/106 spleen cells), and as IgM Total Spleen Activity (AFC/spleen).

II. RESULTS AND DISCUSSION

A. ANALYSIS OF DOSE FORMULATIONS

The achieved concentrations of glyphosate in the dietary preparation were in the range of 85.6 – 97.5% of nominal, and therefore within the acceptable range of 85 – 115 %. The diet formulations were homogeneous and stable for 10 days when stored at room temperature with the following exception. During homogeneity/concentration acceptability testing, the 450 ppm diet formulation was 83.1% of target. The 5500 ppm diet formulation was within acceptable range (90.8%) but was considered low, therefore, calibration standards were prepared as matrix-based samples and a cross-validation was conducted. The diet formulations were reanalyzed using matrix-based calibration standards and met the testing facilities SOP acceptance criteria for homogeneity and concentration acceptability. Based on these results, the protocol-specified doses of test substance were offered to the animals. The test substance was not detected in the basal diet that was offered to the basal diet control (Group 1) and positive control (Group 5) groups.

B. MORTALITY AND CLINICAL SIGNS

There were no mortalities observed during the study period.

C. CLINICAL OBSERVATIONS

There were no test substance-related clinical findings.

D. BODY WEIGHT

There were no test substance related

E. FOOD CONSUMPTION AND COMPOUND INTAKE

There were no test substance-related effects on food consumption noted.

The group mean achieved doses are summarised below.

Table 5.10-8: Group mean achieved dose levels of glyphosate

Dose group	Dietary concentration (ppm)	Mean achieved dose level (mg/kg bw/day)
1 (negative control)*	0	0.0
2 (low)	500	150.1
3 (mid)	1500	449.1
4 (high)	5000	1447.5
5 (positive control)	50 mg/kg CPS**	0.0

* basal diet group
** CPS = cyclophosphamid

F. NECROPSY

Gross pathology

There were no test substance-related macroscopic effects.

Treatment with the positive control CPS produced a small thymus in three of the 10 animals. These changes were consistent with the known effects of CPS in female B6C3F1 mice.

Organ weights

There were no test substance-related effects on terminal body weights or on spleen or thymus weights (absolute or relative to final body weight) when the test substance-treated groups were compared to the basal diet control group.
Treatment with the positive control CPS produced statistically significantly lower spleen and thymus weights (absolute and relative to final body weight) when compared to the basal diet control group. These changes were consistent with the known effects of CPS in female B6C3F1 mice.
The results of final body and organ weight determinations are presented in the Table 5.10-9 below.

Table 5.10-9: Final body weight and organ weight data

Dose group	Body weight	Spleen		Thymus	
	(g)#	weight (mg)#	% body weight (%)#	weight (mg)#	% body weight (%)#
1 (negative control)*	20.9 ± 0.3	85.3 ± 3.3	0.41 ± 0.02	44.3 ± 3.5	0.21 ± 0.02
2 (low)	20.6 ± 0.2	82.3 ± 4.6	0.40 ± 0.02	41.5 ± 1.9	0.20 ± 0.01
3 (mid)	21.6 ± 0.3	91.0 ± 6.5	0.42 ± 0.03	45.9 ± 2.7	0.21 ± 0.01
4 (high)	21.3 ± 0.2	86.0 ± 3.6	0.40 ± 0.02	42.0 ± 2.6	0.20 ± 0.01
5 (positive control)	21.5 ± 0.3	50.2 ± 3.3**	0.23 ± 0.02**	13.3 ± 0.8**	0.06 ± 0.01**

Values presented the mean ± SD derived from the number of animals evaluated per dose group
** Statistically significant from negative control at $p \leq 0.01$

G. AFC ASSAY RESULTS

There were no test substance-related effects on spleen cell numbers, and in the functional evaluation of the IgM antibody-forming cell (AFC) response, treatment with glyphosate did not result in a statistically significant suppression of the humoral immune response when evaluated as either Specific Activity (AFC/106 spleen cells) or Total Spleen Activity (AFC/spleen). There were no statistically significant differences nor any dose-related trends noted when the basal diet control and test substance-treated groups were compared.
Statistically significantly lower spleen cell numbers, mean specific activity, and mean total spleen activity values were noted in the positive control (CPS treated) group when compared to the basal diet control group. These effects were consistent with the known immunosuppressant effects of CPS and validated the appropriateness of the AFC assay.
The results of the AFC assay are summarised in Table 5.10-10 below.

Table 5.10-10: Results of AFC assay

Dose group	Spleen cells (x 10^7)#	IgM AFC / 10^6 spleen cells #	IgM AFC/spleen (x 10^3)#
1 (negative control)*	11.29 ± 0.65	1160 ± 131	127 ± 11
2 (low)	11.45 ± 0.64	1273 ± 123	144 ± 16
3 (mid)	13.45 ± 1.24	1368 ± 163	190 ± 37
4 (high)	12.51 ± 0.66	1514 ± 204	195 ± 32
5 (positive control)	5.18 ± 0.53**	0 ± 0**	0 ± 0**

Values presented the mean ± SD derived from the number of animals evaluated per dose group
** Statistically significant from negative control at $p \leq 0.01$

III. CONCLUSION

Repeated dietary administration of glyphosate to females B6C3F1 mice did not suppress the humoral component of the immune system. The no-observed-effect level (NOEL) for suppression of the humoral immune response in female B6C3F1 mice offered glyphosate in the diet for 28 days was considered to be 5000 ppm (equivalent to 1448 mg/kg bw/day), the highest dietary concentration.

Part 2. LITERATURE REVIEW

Monsanto Company has been conducting routine surveillance of technical literature for glyphosate-related publications in a structured fashion since early 1997. During the period from 1997 to the present time, the search process and the literature databases used have been modified as new resources and technology became readily available. The technical databases that are used for the search include: Web of Science℠, BIOSIS Previews®, CAB Abstracts® (CABI), MEDLINE®, and CA Plus (Chemical Abstracts Plus). The searches are done on glyphosate acid, glyphosate salts (including isopropyl amine, potassium, ammonium, and methylamine), and AMPA, and their related chemical names and CAS numbers. Searches based on these search terms will also identify publications that consider glyphosate and surfactants, (such as polyoxyethylenealkylamines, or POEA), in the context of glyphosate formulations.

Starting from the ongoing Monsanto literature database, all the peer-reviewed publications covering the time period from 2001 through 2011 that relate to the four key disciplines addressing exposure and hazard (toxicology, ecotoxicology, residues and environmental fate) were assessed within the appropriate discipline for inclusion in the literature review for the submission. Some publications address more than one discipline, and are included in each relevant discipline. More recent publications have continued to be reviewed up to shortly before submission, and selected publications have been included.

At the request of the Bundesambt für Verbraucherschutz und Lebensmittelsicherheit (BVL), additional publications cited in a recent document prepared by Earth Open Source[10] have also been included in the literature review. Many of the cited peer-reviewed publications were already included, but others were not within the scope of this literature review, primarily because the publication date was prior to 2001. The additional peer-reviewed publications have been included and are discussed within the appropriate discipline.

The peer-reviewed publications identified for inclusion during the literature search were reviewed within each discipline and classified into one of the categories listed below:

- **Category 0 publications**: These are publications in which glyphosate is only mentioned as an example substance or is discussed/studied in a context that is not relevant or related to any of the regulatory sections or the exposure/hazard assessments within this submission; the publication is therefore outside of the scope of this submission.
- **Category 1 publications**: These are publications which discuss glyphosate in a context relevant or related to the regulatory dossier sections and the conclusions fall within the conclusions of the exposure/hazard assessment. The publication is submitted with minimal or no comment or discussion.
- **Category 2 publications**: These are publications which discuss glyphosate in a context relevant or related to the regulatory dossier sections and have conclusions that call into question the endpoints/conclusions in the exposure/hazard assessment. Additionally, Category 2 also includes publications with conclusions that support the risk/hazard assessment, and may be included in discussion of other relevant publications. For selected Category 2 publications, an OECD Tier-II type summary is provided in addition to a reliability assessment (Klimisch rating, see Klimisch et al. 1997); limited comments and critical remarks are provided, as appropriate.
- **Category 3 publications**: These are publications that discuss glyphosate in a context relevant or related to (1) non-regulatory endpoints that need to be addressed as per new Regulation (EC) 1107/2009; or (2) in a context relevant to sensitive allegations that have emerged or could emerge in the media; or (3) in a context relevant to the regulatory dossier sections and have conclusions

[10] Earth Open Source report. 2011. Roundup and birth defects: Is the public being kept in the dark? Authored by Antoniou M, Habib MEEM, Howard CV, Jennings RC, Leifert C, Nodari RO, C Robinson, Fagan J. Available from: http://www.earthopensource.org/files/pdfs/Roundup-and-birth-defects/RoundupandBirthDefectsv5.pdf

that are in disagreement with endpoints/conclusions in the exposure/hazard assessment (although the experimental design seems relevant at first glance). An OECD Tier-II type summary is provided and a Klimisch rating assigned, and supplemented with critical review and discussion.

- **Category 'E' publications**: These are peer-reviewed publications that were cited in the Earth Open Source document. This category includes publications that were already captured by the literature search and are addressed within the appropriate discipline, as well as publications that were out of scope of the search (primarily as a result of being published prior to 2001). Publications already captured in the literature search were assigned a Category 1, 2 or 3 rating (as appropriate) in addition to a Category 'E' rating. An OECD Tier-II type summary has been prepared and a Klimisch rating assigned for each of the Category E publications. All Category 'E' publications are reviewed within the appropriate discipline, with most of the reviews provided within the toxicology dossier under Section IIA 5.10.

Approximately 2000 peer-reviewed publications from the Monsanto technical literature database were assessed, and of those about 1000 were assigned a Category 1, 2 or 3 and selected for inclusion in the submission.

A full description of the literature search methodology is provided in a separate document (Carr and Bleeke, 2012).

The publications selected for inclusion are listed in Document L for each respective section, under the Annex point for 'Other/Special Studies': Point IIA 5.10 (Toxicology), Point IIA 6.10 (Metabolism and Residue), Point IIA 7.13 (Environmental Fate), and Point IIA 8.16 (Ecotoxicology). Under each point, the list of Other/Special Studies is presented in three tables:

- Table 1 lists other relevant studies conducted by the Glyphosate Task Force or member companies in support of the submission, that do not fit within any other dossier points .
- Table 2 lists all the relevant peer-reviewed publications from the literature that were selected for inclusion in the submission.
- Table 3 lists the publications and other documents that are cited within the discussion of the literature. These include documents such as government or company reports; publications that are included in the literature review under another section of the dossier; and publications that are outside the scope of the literature review.

Five separate publication subject areas are addressed in the literature review below.

1. Developmental and Reproductive Toxicology (DART) and Endocrine Disruption (ED)
2. Neurotoxicity
3. Carcinogenicity
4. Genotoxicity
5. Category E and other publications

Publications are presented in Tier II style summaries followed by Klimisch ratings then responses/comments on the paper. Results reported and discussed in the peer reviewed open literature review do not affect the conclusions drawn in the core glyphosate dossier.

1. Literature Review of Developmental and Reproductive Toxicity (DART) and Endocrine Disruption (ED) Publications

Publications suggesting glyphosate or glyphosate based formulations are developmental toxicants, reproductive toxicants or endocrine disruptors include *in vitro* studies, *in vivo* studies and epidemiological studies with weak, statistically non-significant associations. Some epidemiological studies evaluate associations with pesticides in general or classes of pesticides, with no mention of glyphosate or glyphosate based products, and thus warrant no further discussion (e.g. Benítez-Leite, 2009) other than the OECD Tier II like summary and Klimisch rating (Klimisch, 1997). Many of these published since 2000 are specifically discussed in a comprehensive glyphosate DART review publication by three internationally recognized experts (Williams et al., 2012), referenced in Doc L Table 2 and included in Doc K. Further discussions of some significant papers follow.

In addition, glyphosate was included on the US EPA Endocrine Disruptor Screening Program's (EDSP) first list of 67 compounds to Tier 1 Screening. The US EPA clearly published the criteria for inclusion on List 1 was strictly based on exposure potential, not hazard, specifically stating in the Federal Register (2009);

> "This list should not be construed as a list of known or likely endocrine disruptors".

A consortium of glyphosate registrants in North America, the Joint Glyphosate Task Force, LLC (JGTF), coordinated the conduct of the glyphosate battery of Tier 1 screening assays under the EDSP and submitted these successfully completed assays to the US EPA. The US EPA will evaluate the full battery of Tier 1 screening assays together using a weight of evidence approach for glyphosate's potential to interact with the estrogen, androgen and thyroid endocrine pathways. The following below were submitted by the JGTF to the US EPA in early 2012 and are expected to be reviewed this year. However, the Agency gas announced they will not release their Data Evaluation Records (DERs) for individual EDSP studies until a weight of evidence review has been completed for List 1 compounds. Therefore, in an effort to disclose the findings of the glyphosate EDSP data to the scientific community, the JGTF is considering publishing a Weight of Evidence review of glyphosate with respect to endocrine disruption.

<u>*In Vitro* EDSP Glyphosate Studies submitted to the US EPA</u>

- Androgen Receptor Binding (Rat Prostate Cytosol); OCSPP 890.1150
- Aromatase (Human Recombinant); OCSPP 890.1200
- Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC); OCSPP 890.1250
- Estrogen Receptor Transcriptional Activation (Human cell Line, HeLa-9903); OCSPP 890.1300; OECD 455
- Published OECD Validation of the Steroidogenesis Assay (Hecker et al., 2010)

<u>*In Vivo* EDSP Glyphosate Studies submitted to the US EPA</u>

- Amphibian Metamorphosis (Frog)-OCSPP 890.1100; OECD 231
- *In Vivo* Hershberger Assay (Rat); OCSPP 890.1600; OECD 441
- Female Pubertal Assay; OCSPP 890.1450; OECD None
- Male Pubertal Assay; OCSPP 890.1500
- Uterotrophic Assay (Rat); OCSPP 890.1600; OECD 440
- Fish Short-Term Reproduction Assay; OCSPP 890.1350; OECD 229

The glyphosate Tier 1 screening assay study reports are owned by the JGTF. The European Glyphosate Task Force (GTF) is negotiating to procure access rights to the battery of glyphosate EDSP Tier 1 screening study reports. Results of the Hershberger and Uterotrophic *in vivo* rat studies, now in the public domain, as are the published results of the OECD validation of the Steroidogenesis assay, in which glyphosate clearly had no impact on steroidogenesis, are discussed below.

In Vitro Glyphosate DART/ED Publications

Many *in vitro* research publications have characterized pesticide formulations, including glyphosate based formulations, as toxic and endocrine disrupting products. Researchers and editorial boards have frequently overlooked the fact that surfactants (which are often components of formulated pesticide products), by their physico-chemical nature, are not suitable test substances using *in vitro* cell models. Surfactants compromise the integrity of cellular membranes, including mitochondrial membranes, and thus confound endpoint measurements considered as representative of specific toxicological modes of action or pathways. For example, Walsh et al. (2000) published research claiming that a glyphosate based formulation, but not glyphosate alone, adversely affected the steroidogenesis pathway by inhibiting progesterone production resulting in downstream reduction in mitochondrial levels of StAR protein. Subsequent research by Levine et al. (2007) demonstrated (i) no synergism between glyphosate and the surfactant since the cytotoxic effects were completely independent of glyphosate; identical dose-response curves were noted for formulated product with and without the glyphosate active ingredient; (ii) comparable cytotoxicity dose-response curves for several common household detergents or surfactants; and (iii) a variety of surfactants demonstrate cytotoxic effects that are not specific to biochemical pathways within intact cells. Levine (2007) concludes by emphasizing the importance of considering the biological plausibility of observed *in vitro* effects for in-tact animals.

Subsequent research addressing the steroidogenesis pathway confirmed glyphosate lacked endocrine disruption potential specific to this pathway. Quassinti et al. (2009) evaluated effects on gonadal steroidogenesis in frog testis and ovaries on glyphosate and another active substance, noting that glyphosate unequivocally demonstrated no effect. Forgacs et al. (2012) also tested glyphosate alone and demonstrated no effect on testosterone levels in BLTK1 murine leydig cells *in vitro*. Furthermore, the OECD multi-laboratory validation of the Steroidogenesis Assay used for Tier 1 screening of the US EPA EDSP, evaluated glyphosate and concluded no impact on steroidogenesis (Hecker et al., 2010). Consequently, the US EPA considered reference to the OECD validation report sufficient for meeting the glyphosate Steroidogenesis Assay Test Order in the EDSP Tier 1 screening of glyphosate.

The Seralini laboratory at the University of Caen, France, has multiple recent publications of *in vitro* research with glyphosate and glyphosate based formulations (Richard et al, 2005; Benachour et al, 2007; Benachour and Seralini, 2009; Gasnier et al, 2009; Gasnier et al, 2010; Gasnier et al., 2011; Clair et al., 2012; Mesnage et al., 2012), with proposed extrapolations to an array of *in vivo* effects including potent endocrine disruption, aromatase inhibition, estrogen synthesis, placental toxicity, foetotoxicity, embryotoxicity and bioaccumulation. These publications are often replicates of earlier studies, using different cell lines or primary cell cultures and in some cases the same data are reported again in a subsequent publication. Firstly, the *in vitro* synergism claims are conjecture, simply because no control groups of surfactant without glyphosate were tested. Secondly, the extrapolations to *in vivo* effects are unjustifiable based on both the unsuitability of surfactants in such test systems and the supraphysiological cytotoxic concentrations at which *in vitro* effects are reported. Again often overlooked by *in vitro* researchers and editorial boards, Levine et al. (2007) presented convincing data demonstrating a lack of *in vitro* synergism for glyphosate with other formulation ingredients. Regarding Seralini's repeated claims of glyphosate induced aromatase inhibition in mircosomes (Richard et al, 2005; Benachour et al, 2007; Gasnier et al, 2009), the data are confounded and thus uninterpretable where surfactants are introduced to such *in vitro* systems. This is noted in the US EPA Aromatase Inbibition Test Guideline, OECD 890.1200, in which notes,

> "Microsomes can be denatured by detergents [surfactants]. Therefore, it is important to ensure that all glassware and other equipment used for microsome preparations be free of detergent residue."

Research from the Seralini laboratory has repeatedly gained general public and media attention, including dissemination on "you-tube" and public lecture tours in various countries, in which allegations against glyphosate based products and biotechnology in agriculture are made. The selective use of literature, with absence of contradicting research (e.g., Kojima et al. (2004) demonstrated glyphosate lacked affinity for estrogen-α, estrogen-β and androgen receptors) demonstrates consistent and undeterred bias in the authors'

publication record. Numerous authoritative reviews have discounted the relevance of the Seralini team's research to human health risk assessment; some of these are referred to in specific publication reviews below. Several more recent publications from this group investigate homeopathic plant extract remedies for effects they attribute to glyphosate exposures in formulated products *in vitro* (Gasnier et al.(2010); Gasnier et al.(2011)).

Another *in vitro* publication claiming a specific developmental toxicity pathway has gained significant public traction, media attention and widespread international public lecture tours by the lead investigator. Paganelli et al. (2010) from the Carrasco research laboratory in Argentina conducted three *in vitro* assays, (i) frog embryos exposed to glyphosate formulation; (ii) frog embryos directly injected without injection blank negative controls; and (iii) fertilized chicken embryos exposed directly to a glyphosate formulation through a hole cut in the egg shell. Key issues surrounding this research include irrelevant routes of exposure as well as excessively high and environmentally unrealistic doses.

In Vivo Glyphosate DART/ED Publications

Relatively few *in vivo* publications on glyphosate DART and ED exist in comparison with the list of *in vitro* publications. Some lack appropriate interpretation of basic toxicology; e.g. Daruich et al. (2001) and Beuret et al. (2005) (two authors are common to each paper and from the same university department) noted rats treated with a glyphosate based formulation showed reduced food intake, reduced water intake and reduced body weight gains. However, the authors did not consider attributing the effects of altered enzyme concentrations to dehydration or restricted diets. Both studies are reviewed in Williams et al. (2012).

Dallegrave et al. (2003; 2007) published results of two non-guidelines rat developmental toxicity studies, in which a glyphosate based formation containing POEA was evaluated. Numerous reporting deficiencies and inconsistencies pose difficulties in data interpretation

Romano et al. (2010) evaluated a glyphosate based formulation in a male pubertal-like assay in Wistar rats, reporting decreased preputial separation, reduced seminiferous epithelial height, increased luminal diameter of seminiferous tubules, and increased relative testicular and adrenal weights. Given the gravity of the reported findings in this publication, a very detailed review was undertaken by experts in the fields of reproductive and developmental toxicology and endocrinology; William R. Kelce, M.S., Ph.D, Fellow ATS; James C. Lamb, IV, Ph.D, DABT, and Fellow ATS; John M. DeSesso, Ph.D, Fellow ATS. Their critique is referenced in Doc L and included in Appendix K. Most recently, Romano et al. (2012) reported additional findings in male rats after supposed *in utero* and *post natal* exposures which include "behavioral changes and histological and endocrine problems in reproductive parameters and these changes are reflected by a hypersecretion of androgens and increased gonadal activity, sperm production and libido". As in their first publication, Romano et al. (2012) base their hypothesis on selectively discussed literature implicating glyphosate as an endocrine disruptor, predominantly with citations to research from the Seralini laboratory.

Recently, the first publicly data available from the glyphosate Tier 1 assays under the US EPA Endocrine Disruptor Screening Program, were reported at the 2012 Society of Toxicology meeting (Saltmiras et al., 2012) for the Hershberger and Uterotrophic assays. No effects were noted for any potential for glyphosate to interact with androgenic or estrogenic pathways under these GLP studies following the US EPA 890 Series Test Guidelines.

POEA DART Studies in Williams et al. (2012)

Polyethoxylated alkylamine (POEA) surfactants are a class of non-ionic surfactant, containing a tertiary amine, an aliphatic group of variable carbon chain length and two separate sets of ethoxy (EO) chains of variable length. A dietary exposure assessment of POEAs previously submitted by Monsanto to BfR (Bleeke et al. 2010) is referenced in Doc L and included in Doc K. This exposure assessment report also refers to the US EPA Alky Amine Polyalkoxylates Human Health Risk Assessment, which includes

POEAs (http://www.regulations.gov/search/Regs/home.html#documentDetail?R=09000064809b983b). Williams et al. (2012) recently evaluated and detailed the results of DART studies with two different POEA surfactants, summarized below.

Pregnant female rats were administered MON 0818, a POEA surfactant, at 0, 15 100 and 300 mg/kg/day. The NOAEL for maternal toxicity was 15 mg/kg/day and the NOAEL for rat developmental toxicity was the highest dose tested, 300 mg/kg/day (Holson, 2001).

A reproductive and developmental multigenerational screening study dosed MON 0818 in diets at 0, 100, 300 and 1000 ppm. The majority of endpoints evaluated were unaffected by treatment, including testis morphology, sperm parameters and testosterone and thyroid hormone levels. The mid-dose of 300 ppm (approximately 20 mg/kg/day) was considered the NOAEL for reproductive and developmental toxicity based on the following results in F0 at the high dose, 1000 ppm: increases in unaccounted for implantation sites with reduced mean number of pups and litter size in the high dose group; three high dose dams delivered litters of two-four pups each, with total litter loss by post-natal day (PND) 4 in two of these litters. Upon breeding of F1 generation none of the findings noted in F0 were reproducible, and given some were not statistically significant, they were considered equivocal. However, a clear NOAEL for reproductive/developmental toxicity was considered to be the mid dose of 20 mg/kg/day (Knapp, 2007).

Another reproductive/developmental study of a different POEA surfactant, MON 8109 evaluated doses of 0, 30, 100, 300 and 2000 ppm in diet. A single dose group of MON 0818 at 1000 ppm in diet was also included to determine whether litter effects previously noted at this dose were treatment related (Knapp, 2008).

- MON 0818 dosed at 1000 ppm (76 and 86 mg/kg/day premating in males and females respectively) did not reveal the litter effects noted in the previous study at this dose. Two maternal incidents were not considered related to treatment; one female with dystocia died on PND 1 (this was also noted in one female of the control group F1 in the previous study at the same facility) and a second female was euthanized due to a ruptured uterus on gestation day 30. No test substance-related effects were noted for systemic toxicity, reproductive endpoints, pup survival or mortality. Therefore the overall DART NOAEL for MON 0818 was considered 1000 ppm, approximately 81 mg/kg/day.
- The MON 8109 systemic toxicity NOAEL in males and females was 300 ppm, based on mean body weight loss, reduced mean body weight gain and decreased food consumption at 2000 ppm. Developmental/reproductive effects at 2000 ppm included reduced mean number of implantation sites, increased number of unaccounted for implantation sites, decreased mean litter size at PND 0, reduced mean number of births, reduced survival at PND 4 and reduced mean pup weight at PND 1. The MON 0818 reproductive/developmental NOAEL was also 300 ppm (approximately 23 mg/kg/day).

Epidemiology Glyphosate DART/ED Publications

Several epidemiology studies in which glyphosate exposure was considered have evaluated the following range of reproductive outcomes; miscarriage, fecundity, pre-term delivery, gestational diabetes mellitus, birth weights, congenital malformations, neural tube defects, attention-deficit disorder / attention-deficit hyperactive disorder (ADD/ADHD). In most instances, glyphosate and reproductive outcomes lack a statistically significant positive association, as described in a recent review of glyphosate non-cancer endpoint publications by experts in the field of epidemiology, Pam Mink, Jack Mandel, Jessica Lundin and Bonnielin Sceurman (Mink et al., 2011). In evaluating ADD/ADHD a positive association with glyphosate use was reported by Garry et al (2002), but cases were parent reported with no clinical confirmation and the reported incidence rate of approximately 1% for the study population was well below the general population incidence rate of approximately 7%. Regarding *in utero* exposures, McQueen et al. (2012) report very low measured dietary exposures, from 0.005% to 2% of the current glyphosate ADI in Europe. Given the low perfusion rate of glyphosate across the placenta (Mose et al., 2008), human *in utero* exposures would be very limited.

IN VITRO DART/ED PUBLICATIONS

Author(s)	Year	Study title
Walsh, L.P. McCormick, C. Martin, C. Stocco, D.M.	2000	Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environmental Health Perspectives Volume: 108 Number: 8 Pages: 769-776

Abstract*

Recent reports demonstrate that many currently used pesticides have the capacity to disrupt reproductive function in animals. Although this reproductive dysfunction is typically characterized by alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility, the mechanisms involved in pesticide-induced infertility remain unclear. Because testicular Leydig cells play a crucial role in male reproductive function by producing testosterone, we used the mouse MA-10 Leydig tumor cell line to study the molecular events involved in pesticide-induced alterations in steroid hormone biosynthesis. We previously showed that the organochlorine insecticide lindane and the organophosphate insecticide Dimethoate directly inhibit steroidogenesis in Leydig cells by disrupting expression of the steroidogenic acute regulatory (StAR) protein. StAR protein mediates the rate-limiting and acutely regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane where the cytochrome P450 side chain cleavage (P450scc) enzyme initiates the synthesis of all steroid hormones. In the present study, we screened eight currently used pesticide formulations for their ability to inhibit steroidogenesis, concentrating on their effects on StAR expression in MA-10 cells. In addition, we determined the effects of these compounds on the levels and activities of the P450scc enzyme (which converts cholesterol to pregnenolone) and the 3 β-hydroxysteroid dehydrogenase (3 β-HSD) enzyme (which converts pregnenolone to progesterone). Of the pesticides screened, only the pesticide Roundup inhibited dibutyryl cAMP (Bu2cAMP)-stimulated progesterone production in MA-10 cells without causing cellular toxicity. Roundup inhibited steroidogenesis by disrupting StAR protein expression, further demonstrating the susceptibility of StAR to environmental pollutants.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Ammo, Ambush, Fusilade, Cyclone, Roundup, Banvel, Cotoran, Dual, glyphosate. Surfactants not identified or quantified in formulations.

Active substance(s):
- Ammo: **cypermethrin**: (*R,S*)-α-cyano-3-phenoxybenzyl(1*R,S*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
- Ambush: **permethrin**: 3-phenoxybenzyl(1*R,S*)-*c i s* ,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
- Fusilade: **fluazifop-*p*-butyl**: (*R*)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionic acid
- Cyclone: **paraquat**: 1,1´-dimethyl-4,4´-bipyridinium
- Roundup: **glyphosate**: *N*-(phosphonomethyl) glycine
- Banvel: **dicamba**: 3,6-dichloro-*o*anisic acid
- Cotoran: **fluometuron**: 1,1-dimethyl-3-(α,α,α-trifluoro-*m*-tolyl) urea

Purity:	• Dual: **metolachlor**: 2-chloro-6´ethyl-*N*-(2-methoxy-1-methylethyl)aceto-toluidine. • Ammo (300 g/L cypermethrin) • Ambush (240 g/L permethrin) • Fusilade (120 g/L fluazifop-*p*-butyl) • Cyclone (240 g/L paraquat) • Roundup (180 g/L glyphosate) • Banvel (480 g/L dicamba) • Cotoran (480 g/L fluometuron) • Dual (958 g/L metolachlor)
Source:	Glyphosate – Sigma Other pesticides – unknown source

2. Vehicle and/or positive control:

Vehicle control:	Yes (DMSO, ethanol < 0.4%)
Positive control:	No data

3. Test system / cells / animals:

Cell culture:	Mouse MA-10 Leydig tumor cell line
Species:	Mouse
Source:	M. Ascoli, University of Iowa College of Medicine (Iowa City, IA)
Maintenance conditions:	Waymouth's MB 752/1 medium + 15% horse serum Temperature: 37° C, Atmosphere: 5% CO_2
Plate cultures #1:	75,000 cells/well in a 96-well plate. For dose–response, time–course, steroidogenic enzyme activity, reversibility, and mixture studies.
Plate cultures #2:	50 x 10^6 cells onto 25 x 25 cm tissue culture dishes. For nuclear run-on analysis.
Plate cultures #3:	1.5 x 10^6 cells into 100-mm culture dishes, grown until 80% confluence. For the remaining studies.

4. Test methods:

Study type:	Inhibition of steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	2 or 4 h
Dose/concentration levels:	Ambush, Ammo: 5, 10, 50 µg/mL Banvel, Cotoran, Dual, Fusilade: 1, 5, 10 µg/mL Cyclone: 0.5, 1, 5 µg/mL Roundup: 12.5, 25, 50, 100 µg/mL
Treatment:	MA-10 cells were stimulated using a maximal stimulatory dose of $(Bu)_2$cAMP (1 mM). In some tests (P450scc and 3β-HSD

enzyme activity), steroidogenic substrates (22R-HC, 25 µM or pregnenolone, 10 µM) were provided.

All treatments were performed in serum-free media.

Final concentrations of the solvents DMSO and ethanol were < 0.4 %.

5. Observations/analyses:

Dose–response and time-course studies:

Measurement: Steroid levels and total protein synthesis.

Calculation: IC_{50} values (concentration that leads to an inhibition of 50%) were calculated as the slope of the linear regression line obtained from Eadie/Hofstee plots of steroidogenesis dose–response data.

Analysis: For steroid determination in Roundup-treated cells, each data point was the average ± SE of the means from at least three separate experiments in which treatments were performed in quadruplicate.

For progesterone production in cells treated with other pesticides, each data point is the mean ± SE of four replicates in a single experiment that was repeated once.

Progesterone production and total cellular protein synthesis:

Radioimmunoassay (RIA):

Measurement: Quantification of progesterone

Preparation of samples: Standard curves were prepared in serum-free Waymouth's medium.

Analysis: Analysis of RIA data was performed using a computer program specifically designed for this purpose (not further specified).

Data are expressed as ng/mL media.

Determination of total cellular protein synthesis:

Measurement: Total protein content was determined using a modification of the Bradford method (no treatment with Expre^{35}S^{35}S).

Preparation of samples: After treatment, cells were solubilized in 0.25 M NaOH at 37°C. Protein was precipitated overnight at 4°C using cold 20% trichloroacetic acid (TCA). TCA-precipitable material was transferred onto glass fiber filters, rinsed with 5% TCA, dried, and counted in a liquid scintillation counter.

Analysis: Results were reported as counts per minute per mg protein (2 or 4 h).

Each data point is the mean ± SE of four replicates in a single experiment, which was performed three times.

Determination of P450scc and 3β-HSD activity and reversibility:

Measurement: P450scc enzyme activity: Pregnenolone in medium
3β-HSD enzyme activity: Progesterone in medium

Preparation: Evaluation of P450scc enzyme activity:
22*R*-HC was provided as substrate to MA-10 cells in the presence and absence of the xenobiotic as well as cyanoketone

and SU 10603 (inhibitors of 3β-HSD and P450c17, respectively).
Evaluation of 3β-HSD enzyme activity:
pregnenolone was provided as substrate, and MA-10 cells were treated in the presence and absence of the xenobiotic

Analysis: Each data point represents the average ± SE of the means from at least three separate experiments in which treatments were performed in quadruplicate.

Effects on enzyme and StAR expression: Protein levels, mRNA levels, gene transcription

Isolation of mitochondria and Western blot analysis:

Measurement: Protein levels of P450scc, β-HSD, StAR

Preparation: Western blot analysis of mitochondrial protein was performed. Mitochondria were isolated by homogenization of the cells followed by differential centrifugation. After detection of StAR, membranes were stripped and then successively probed with P450scc or 3β-HSD antisera.

Analysis: The bands of interest were quantitated using a BioImage Visage 2000 imaging system. Values obtained were expressed as integrated optical density units. Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Isolation of RNA and Northern blot analysis:

Measurement: mRNA levels of of P450scc, β-HSD, StAR

Preparation: Total RNA was isolated using Trizol Reagent and quantitated. For Northern blot analysis 20 µg total RNA was loaded into each well. Labeling of cDNA probes for mouse StAR, P450scc, 3β-HSD, and 18S rRNA was achieved by random priming (Prime-It II; Stratagene, La Jolla, CA) using [α-32 P] dCTP (SA 3,000 Ci/mmol; New England Nuclear) according to the manufacturer's protocol.
After Northern blot analysis with StAR cDNA, blots were stripped and then successively probed with P450scc, 3β-HSD, and 18S rRNA cDNA.

Analysis: The bands of interest (RNA) were quantified. Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Gene expression:

Measurement: StAR, P450scc

Isolation of nuclei:

Preparation: After treatment, cells were harvested with a rubber policeman and centrifuged. The cell pellet was resuspended and homogenized. The homogenate was layered and centrifuged. The supernatant was discarded and the pellet containing nuclei

was resuspended, frozen on dry ice, and stored in liquid nitrogen.

Nuclear run-on analysis:

Measurement: Radioactivity was detected using a Phosphorimager 445 SI.

Analysis: Signals were quantitated using ImageQuant version 4.1 software in volume mode, which integrates the intensity of each pixel within the defined area.

Values were obtained as arbitrary units. Each data point represents the average ± SE of five separate experiments.

Protein kinase A (PKA) activity determination:

Measurement: PKA activity was measured with the SignaTECT cAMP-dependent protein kinase assay system.

Analysis: Three separate experiments were performed in which treatments were performed in triplicate.

Mixture studies:

Measurement: Progesterone was measured.

Analysis: Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Statistics: Statistically significant differences were determined by one-way analysis of variance and Fisher–protected least-square difference multiple comparison using the software program Statview SE + Graphics.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions – Not reliable for Roundup**

Comment: Non-standard test systems, but publication meets basic scientific principles. However, surfactant blend in Roundup confounds results.

2. Relevance of study: **Relevant with restrictions:** Different effects of glyphosate alone and glyphosate formulations were observed. No conclusion can be drawn that the observed effects are result of glyphosate exposure. Roundup data unreliable for endpoints measured, due to mitochondrial membrane damage.

3. Klimisch code: **2 for glyphosate data, 3 for Roundup data**

Response - GTF

- Glyphosate did not affect steroidogenesis in the test system.
- Roundup formulation data was confounded by mitochondrial membrane damage, attributable to the surfactant in the tested formulation.
- Roundup results were comprehensively addressed in Levine et al. (2007).
    - Roundup formulation containing glyphosate and Roundup formulation blank without the active ingredient was shown to have "indistinguishable" dose response curves for reductions in progesterone production in hCG stimulated MA-10 Leydig cells. Therefore

the effect on progesterone levels shown by Walsh (2000) were independent of glyphosate and attributable to the surfactant component of the formulation.
  - Comparable rates of progesterone inhibition for several different surfactants suggest a common mode of action for surfactants.
  - Roundup formulation containing glyphosate and Roundup formulation blank without the active ingredient was shown to have almost identical concentration-dependent decreases in MTT activity in MA-10 cells, suggesting the surfactant alone was responsible for the observed cytotoxicity and effect on mitochondrial function.
  - The JC-1 assay demonstrated the decreased progesterone production in MA-10 Leydig cells was accompanied by loss of mitochondrial membrane potential. These results confirm StAR protein function and steroidogenesis require intact mitrchondrial membrane potential.
  - StAR protein expression were not affected by treatments, indicating that perturbed mitochondrial membrane, not StAR protein inhibition, was responsible for the effects noted by Walsh et al. (2000).

- Given the significant differences in physico-chemical properties between glyphosate and formulation surfactants, environmental fate and transport of these compounds are likely to be different. Likewise, absorption, distribution, metabolism and excretion (ADME) differences between glyphosate and formulation surfactants at low concentration exposures in the field, environment or food residues will very likely result in insignificant concomitant physiological exposures.

Author(s)	Year	Study title
Paganelli, A. Gnazzo, V. Acosta H. Lopez, S.L. Carrasco, A.E.	2010	Glyphosate-Based Herbicides Produce Teratogenic Effects on Vertebrates by Impairing Retinoic Acid Signalling Chemical Research in Toxicology Volume: 23 Pages: 1586-1595

Abstract*

The broad spectrum herbicide glyphosate is widely used in agriculture worldwide. There has been ongoing controversy regarding the possible adverse effects of glyphosate on the environment and on human health. Reports of neural defects and craniofacial malformations from regions where glyphosate-based herbicides (GBH) are used led us to undertake an embryological approach to explore the effects of low doses of glyphosate in development. *Xenopus laeVis* embryos were incubated with 1/5000 dilutions of a commercial GBH. The treated embryos were highly abnormal with marked alterations in cephalic and neural crest development and shortening of the antero-posterior (A-P) axis. Alterations on neural crest markers were later correlated with deformities in the cranial cartilages at tadpole stages. Embryos injected with pure glyphosate showed very similar phenotypes. Moreover, GBH produced similar effects in chicken embryos, showing a gradual loss of rhombomere domains, reduction of the optic vesicles, and microcephaly. This suggests that glyphosate itself was responsible for the phenotypes observed, rather than a surfactant or other component of the commercial formulation. A reporter gene assay revealed that GBH treatment increased endogenous retinoic acid (RA) activity in *Xenopus* embryos and cotreatment with a RA antagonist rescued the teratogenic effects of the GBH. Therefore, we conclude that the phenotypes produced by GBH are mainly a consequence of the increase of endogenous retinoid activity. This is consistent with the decrease of Sonic hedgehog (Shh) signaling from the embryonic dorsal midline, with the inhibition of *otx2* expression and with the disruption of cephalic neural crest development. The direct effect of glyphosate on early mechanisms of morphogenesis in vertebrate embryos opens concerns about the clinical findings from human offspring in populations exposed to GBH in agricultural fields.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Roundup Classic ®; Glyphosate
Active substance(s): Glyphosate
Source: Roundup Classic ®: Monsanto
Glyphosate: Sigma Aldrich
Purity: Roundup Classic ®: 48% (w/v) glyphosate salt
Glyphosate: not reported

2. Positive control: Specified under the respective test

3. Test organisms and systems:

Species: *Xenopus laevis*
Embryo culture: *Xenopus laevis* embryos obtained by in vitro fertilisation
Source: Not specified
Culture conditions: Embyos were incubated in 0.1 x modified Barth´s saline (MBS)
Species: Chicken

Strain:	White Leghorn
Source:	Not specified
Stage:	Egg (fertilized)
Guideline:	Non-guideline tests
GLP:	No
Guideline deviations:	Not applicable

Xenopus embryo Culture and Treatments:

Stage of embryos:	2 cell
Dose levels:	1/3000, 1/4000, and 1/5000-dilutions of Roundup Classic® prepared in 0.1× MBS (modified Barth's saline)
Treatment:	Treatments were performed from the 2-cell stage
Rescue experiments:	0.5 or 1 µM Ro 415253 was added at the 9-cell stage
Culture conditions:	Embryos were incubated in 0.1 x MBS. Cyclopamine was used at 100 µM concentration in 0.1 x MBS and was applied from the 2-cell stage until fixation. Embryos were fixed in MEMFA when sibling controls reached the desired stage.
Negative control:	Not adequately described
Positive control:	None

Xenopus Embryo Injections, Whole Mount in Situ Hybridization and Cartilage Staining:

Dose levels:	360 or 500 pg of glyphosate (N-(phosphonomethyl) glycine (Sigma 337757).
Exposure route:	injection
Stage of embryos:	2 cell
Treatment:	Embryos were injected with 360 or 500 pg of glyphosate (N-(phosphonomethyl) glycine (Sigma 337757) per cell into one or both cells at the 2-cell stage. Glyphosate was coinjected with 10 ng of Dextran Oregon Green (DOG, Molecular Probes) to identify the injected side.
Culture condition:	Embryos were incubated in 0.1 x MBS. And fixed in MEMFA when sibling controls reached the desired stage.
In situ hybridisation:	Wholemount in situ hybridisation (WMISH) was performed with digoxigenin-labeled antisense RNA probes, but without the proteinase K step. Embryos were fixed in MEMFA at stages 45-47, washed with PBS, stained overnight in 0.04 %Alcian Blue, 20% acetic acid, and 80 % ethanol. Afterwards embryos were washed.

Detection of RA Activity:

Dose levels:	1/3000, 1/4000, and 1/5000 Roundup Classic® dilutions
Exposure route:	injection
Stage of embryos:	1-2 cell
Treatment:	Embryos were injected with 320 pg of the plasmid RAREhplacZ (RAREZ) per cell into one cell at the 2-cell stage and placed immediately in the test substance dilutions
Negative control:	Negative control was not evaluated with vehicle injection.

Therefore effects of decreased pH or vehicle coformulant (Dextran Orange Green) were not assessed.

Positive control: Xenopus embryos were injected with the RAREZ plasmid and incubated at late blastula stage with 0.5 or 5 μM all-transretinoic acid (RA, Sigma R2625).

Rescue experiment: Embryos injected with the reporter plasmid were incubated in a 1/4000 test substance dilution from the 2-cell stage, and when they reached the blastula stage, 1 μM of Ro 41-5253 was added.

Treatments of Chicken Embryos:

Stage: Egg

Dose levels: 20 μL of 1/3500 or 1/4500 dilutions of Roundup Classic®.

Treatment: Injection after opening a small window in the shell of fertilized chicken eggs, above the air chamber in the inner membrane. After injection the window was sealed with transparent adhesive tape.

Negative control: Injected with 20 μL of H2O without pH or osmolality adjustment.

Positive Control None

Pre-incubation conditions: Placement: eggs were placed with their blunt end up;
Temperature: room temperature;
Duration: 30 minutes.

Incubation conditions: Light: Darkness;
Temperature: 38 C;
Humidity: 56-58%;
Rotation: regular.

Whole-Mount Inmunofluorescence and WMISH of Chicken Embryos:

Treatment: Embryos were fixed 2-4 hin freshly prepared 4% paraformaldehyde, rinsed and processed for analysis.

Wholemount in situ hybridization (WMISH) was performed as described for Xenopus embryos, using a c-shh probe.

4. Measurements/analyses:

Measurements: Basal luminiscence was detected in uninjected and untreated embryos.

The endogenous RA activity was measured in embryos injected with RAREZ (plasmid RAREhplacZ).

When sibling controls reached the neurula stages, all embryos were processed for chemiluminiscent quantitation of the reporter activity by using the β-gal reporter gene assay (Roche).

Luminiscence was measured on duplicate samples in FlexStation 3 equipment (Molecular Devices), and values were normalized by protein content.

Statistics: A two-tailed t-test was employed to analyze the significance in the difference of the means.
The experiment was repeated three times.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-guideline study that is not sufficiently described for assessment. Inadequate positive and negative control experiments.

2. Relevance of study: **Not relevant:** Irrelevant routes of exposure and inappropriately high doses. Test system not adequate for human risk assessment.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- No pH adjustment for doses and thus effects may be in response to the acidic nature of glyphosate technical acid.
- Inappropriate and irrelevant routes of exposure.
- Data requires further substantiation before consideration in risk assessment.

Response 2 – Saltmiras et al. (2012) letter to the Editor

- Multiple high quality toxicological studies and expert review panels consistently agree glyphosate is not a teratogen or reproductive toxicant.
- The authors' justification for this research is flawed, providing no valid basis, other than an opinion, of an increase in the rate of birth defects in Argentina.
- Direct injection of frog embryos and through chicken shells do not reflect real world exposure scenarios to either environmental species or humans.
- Doses were excessively high and irrelevant for risk assessment purposes. Frog embryos were also bathed in glyphosate formulation at doses 9-15 times greater than the acute LC50 same species of frog. Calculating equivalent oral doses based on pharmacokinetics studies, such doses are 150000000 times greater than worst case human exposure monitoring data.
- "…. the results from this research cannot be used in isolation to reach the conclusions expressed in the publication. Instead, the type of data in this research paper must be interpreted relative to all other available data on the specific materials under study and with balanced consideration for higher tier apical studies."

Response 3 – Mulet (2012) letter to the Editor

- Notes the premise for this research is falsely based on an incorrectly cited local pediatric bulletin from Paraguay.
- "…. this article refers to a study in a single hospital in Paraguay showing a correlation between pesticide use (not herbicides as mentioned by Paganelli et al.) and birth malformations. In the cited study (Benitez et al.), the authors state that the results are preliminary and must be confirmed. Is important to remark that the Benitez et al. study does not include any mention to glyphosate, so does not account for what the authors are stating in the Introduction…..This journal is also wrongly cited in the Discussion referring to increased malformations due to herbicides, which is not the result of the study."

Response 4 – comments from BVL (2010)

- Highly artificial experimental conditions.
- Inappropriate models to replace validated mammalian reproductive and developmental toxicity testing methods for use in human health risk assessment.
- Inappropriate routes of exposure.
- Lack of corroborative evidence in humans.
- "In spite of long-lasting use of glyphosate-based herbicides worldwide, no evidence of teratogenicity in humans has been obtained so far."

Response 5– comments from European Commission Standing Committee on the Food Chain and Animal Health (2011)

- The EU commission supports the German Authorities position, "that that there is a comprehensive and reliable toxicological database for glyphosate and the effects observed have not been revealed in mammalian studies, nor evidenced epidemiologically in humans."
- "…. the Commission does not consider there is currently a solid basis to ban or impose specific restrictions on the use of glyphosate in the EU."

Summaries of the follow up published letters to the Editor by Mulet, Palmer follow

Author(s)	Year	Study title
Mulet, J.M.	2011	Letter to the Editor Regarding the Article by Paganelli et al. (2010) Chemical Research in Toxicology Volume: 24 Number: 5 Pages: 609

Abstract

No abstract.

[The author of the letter states that the study of Paganelli et al., 2010, about teratogenic effect of glyphosate when injected invertebrate embryos, is based on misused citations or non-peer reviewed data]

MATERIALS AND METHODS

1. Test material:

Test item: Roundup Classic
Active substance(s): Glyphosate
Description: Not reported
Source of test medium: Not reported
Lot/Batch #: Not reported
Concentrations: 480 g/glyphosate IPA salt/L

2. Studies addressed:

Paganelli et al. (Chem. Res. Toxicol. (2010), 23, 1586-1595)

In vitro teratology studies: *Xenopus* embryo culture and treatments with glyphosate

Xenopus embryo treatment with glyphosate and whole-mount in *situ* hybridization and cartilage staining

Detection of RA (retinoic acid) activity

Treatment of chicken embryos with glyphosate and whole-mount immunofluorescence

KLIMISCH EVALUATION

1. Reliability of study: **Not applicable**

Comment: In this publication the author expresses some major concern about the article by Paganelli et al. (Chem. Res. Toxicol. (2010), 23, 1586-1595) in terms of over interpretation of results

2. Relevance of study: **Relevant** (no original publication but letter to the editor regarding the article by Paganelli et al., 2010)

3. Klimisch code: **Not applicable**

Author(s)	Year	Study title
Palma, G.	2011	Letter to the Editor Regarding the Article by Paganelli et al. (2010) Chemical Research in Toxicology Volume: 24 Number: 6 Pages: 775-776

Abstract

No abstract.

[The author of the letter claims that the study by Paganelli et al., 2010, described effects of glyphosate only at unrealistic high concentrations or via unrealistic routes of exposure. The data are thought to be inconsistent with the literature, and therefore not suitable or relevant for the risk assessment for humans and wildlife. Furthermore the author asserts that findings do not support the extrapolation to human health as stated in the publication].

MATERIALS AND METHODS

1. Test material:

Test item: Roundup Classic

Active substance(s): Glyphosate (isopropylamine salt)

Description: Not reported

Source of test medium: Not reported

Lot/Batch #: Not reported

Concentration: 480 g/glyphosate IPA salt/L

2. Studies addressed: Paganelli et al.(Chem. Res. Toxicol. (2010), 23, 1586-1595)

In vitro teratology studies: *Xenopus* embryo culture and treatments with glyphosate

Xenopus embryo treatment with glyphosate and whole-mount in *situ* hybridization and cartilage staining

Detection of RA (retinoic acid) Activity

Treatment of chicken embryos with glyphosate and whole-

mount immunofluorescence

KLIMISCH EVALUATION

1. Reliability of study: **Not applicable**

Comment: In this publication the article by Paganelli et al. (Chem. Res. Toxicol. (2010), 23, 1586-1595) is discussed in detail. The author of the letter claims that the study by Paganelli et al. contains major deficiencies and errors in terms of experimental design, descriptions of the methods used, and the interpretation of results

2. Relevance of study: **Relevant** (No original publication but letter to the editor regarding the article by Paganelli et al., 2010)

3. Klimisch code: **Not applicable**

Author(s)	Year	Study title
Richard, S. Moslemi, S. Sipahutar, H. Benachour, N. Seralini, G.E.	2005	Differential effects of glyphosate and roundup on human placental cells and aromatase. Environmental Health Perspectives Volume: 113 Pages: 716-720

Abstract*

Roundup is a glyphosate-based herbicide used worldwide, including on most genetically modified plants that have been designed to tolerate it. Its residues may thus enter the food chain, and glyphosate is found as a contaminant in rivers. Some agricultural workers using glyphosate have pregnancy problems, but its mechanism of action in mammals is questioned. Here we show that glyphosate is toxic to human placental JEG3 cells within 18 hr with concentrations lower than those found with agricultural use, and this effect increases with concentration and time or in the presence of Roundup adjuvants. Surprisingly, Roundup is always more toxic than its active ingredient. We tested the effects of glyphosate and Roundup at lower nontoxic concentrations on aromatase, the enzyme responsible for estrogen synthesis. The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the active site of the purified enzyme, but the effects of glyphosate are facilitated by the Roundup formulation in microsomes or in cell culture. We conclude that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. We suggest that the presence of Roundup adjuvants enhances glyphosate bioavailability and/or bioaccumulation.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate
Active substance(s):	Glyphosate
Source of test item:	Glyphosate: Sigma-Aldrich, Saint Quentin Fallavier, France
Lot / Batch #:	Not specified
Purity:	not reported
Test item:	Roundup ®
Active substance(s):	Glyphosate
Source of test item:	Roundup®, (produced by Monsanto, obtained from a commercial source)
Lot / Batch #:	Not specified
Purity:	Roundup ®: 360 g/L acid

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells / animals:

Cell line:	Human choriocarcinoma derived placental cell line (ref JEG3, ECACC 92120308)
Species:	Human
Source:	CERDIC (Sophia-Antipolis, France)

Maintenance medium:	Phenol red–free EMEM containing 2 mM glutamine, 1% nonessential amino acids, 100 U/mL antibiotics (mix of penicillin, streptomycin, and fungizone), 1 mM sodium pyruvate, and 10% fetal calf serum
Cells:	Human placental microsomes Equine testicular microsomes
Source:	Human: Full-term placentas of young healthy and non-smoking women (Centre Hospitalier Régional de Caen, France) and equine testis by differential centrifugations. Equus: Equine testis
Microsome preparation:	Microsomal fractions (endoplasmatic reticulum) were obtained using differential centrifugations. Tissues were washed with 0.5 M KCl, homogenised in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM DTT, and centrifuged at 20,000 g. The supernant was ultracentrifuged at 100,000 g, and the pellet was washed twice, dissolved in the same buffer containing 20% glyceol and stored at -70°C until use. All preparations steps were carried out at 4°C.

4. Test methods:

GLP:	No (for all tests)
MTT assay:	Assessment of cell viability Cleavage of MTT into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase, to evaluate JEG3 cell viability exposed to Roundup or glyphosate during various times.
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Test substance preparations:	2% solution of Roundup and an equivalent solution of glyphosate were prepared in Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France), and the pH of glyphosate solution was adjusted to the pH of the 2% Roundup solution (~ pH 5.8). Successive dilutions were then obtained with serum-free EMEM.
Dose concentrations:	In serum-containing medium (18, 24, 48 h): Roundup: 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 % Glyphosate: 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 % In serum-free medium: Roundup (1 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 % Glyphosate (1 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 % Glyphosate + Roundup 0.02% (18 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 % Glyphosate + Roundup 0.1% (18 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 %
Treatment:	Fifty thousand cells per well in 24-well plates were grown to 80% confluence, washed with serum-free EMEM and exposed to various concentrations of Roundup or equivalent glyphosate concentrations

Incubation conditions:	Cells were washed with serum-free EMEM and incubated with 250 µL MTT per well for 3 h at 37°C. 250 µL of 0.04 N-hydrochloric acid–containing isopropanol solution was added to each well.
Positive control:	None
Negative control:	None
Replicates per dose level:	3 x 3
Radioimmunoassay (RIA):	Measurement of aromatase activity *in vitro*
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	In serum free medium: Roundup (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2 % Glyphosate (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 % Roundup (18 h): 0.01, 0.02, 0.04, 0.08 % Glyphosate (18 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6 %
Positive control:	None
Negative control:	None
Incubation conditions:	Duration: 90 min Temperature: 37 C Atmosphere: 5% CO_2 200 nM androstenedione
Replicates per dose level:	3 x 3
RT-PCR:	Quantification of cytochrom P450 aromatase mRNA levels in JEG3 cells
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	In serum free medium: Roundup (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2 % Glyphosate (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 % Roundup (18 h): 0.01, 0.02, 0.04, 0.08 % Glyphosate (18 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6 %
Positive control:	None
Negative control:	None
Incubation conditions:	Duration: 90 min Temperature: 37 C Atmosphere: 5% CO_2 200 nM androstenedione
Sample preparation:	Total RNA was isolated from JEG3 cells using the guanidium/phenol/chloroform method. RNA samples were treated with DNase I at 37 C for 30 min to remove genomic DNA. Then DNase I was inactivated at 65°C for 10 min.
Tritiated water release assay:	Assessment of aromatase activity in human placental microsomes *in vitro*
Guideline:	Non-guideline assays

Guideline deviations:	Not applicable
Dose concentrations:	Roundup: 0.01, 0.06, 0.1, 0.5, 0.7, 1.0 , 3.0, 6.0 % Glyphosate: 0.01, 0.06, 0.1, 0.7, 1.0 , 3.0 %
Positive control:	None
Negative control:	None
Treatment of human microsomal fractions:	50 µg of human placental microsomes were incubated with radiolabeled androstenedione (100 pmol/tube) at 37°C for 15 min in the presence or absence of various concentrations of Roundup or glyphosate in 1 mL total volume of 50 mM Tris-maleate buffer (pH 7.4).The reaction was started by adding 100 µL of 0.6 mM H±NADPH and stopped with 1.5 mL chloroform and then centrifuged at 2,700 g at 4°C for 5 min. After adding 0.5 mL 5% charcoal/1.5% dextran T-70 solution into the preparation, the centrifugation was repeated fro 10 min.
Treatment of equine microsomal fractions:	2 µg of equine testicular microsomes were incubated for 3 min at 25°C with various concentrations of radiolabeled androstenedione (in the presence or absence of various concentrations of Roundup) in 0.5 mL of H^+-NADPH containing Tris-maleate buffer (pH 7.4).
Spectral studies:	Assessment of reductase and aromatase activities
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	Roundup: 0.1 % Glyphosate: 0.0046 %
Positive control:	None
Negative control:	None
Purification of reductase / aromatase:	Equine reductase was obtained after chromatographic separation, by ω-aminohexyl-Sepharose 4B and adenosine 2´, 5´-diphosphate agarose, respectively, hydrophobic interaction and affinity columns. Equine cytochrom P450 aromatase was purified from equine microsomes, after its separation from reductase, by successive chromatographic steps.

5. Observations/analyses:

MTT assay	
Measurements:	The optical density was measured using a spectrophotometer at 560 nm for test and 640 nm for reference.
Radioimmuno assay (RIA)	
Measurements:	The conversion of androstenedione to E1 by the aromatase complex was measured in cell supernatants by radioimmunoassay (RIA). The aromatase activity was expressed in relation to the protein concentration that was evaluated in cell extracts using bovine serum albumin as standard
RT-PCR	
Measurements:	Quantitation of mRNA by RT-PCR using M-MLV-RT (Moloney murine leukemia viruse reverse transcriptase). The absence of DNA contamination in RNA samples was

checked in controls without M-MLV-RT.

All PCR reactions were performed using an ABI Prism 7000 Sequence Detection System.

Tritiated water release assay

Measurements: Microsomal aromatase activity was evaluated by tritiated water release from radiolabeled substrate [1β-3H]-androstenedione. This method based on the stereo specific release of 1β-hydrogen from the androstenedione substrate, which froms tritiated water during aromatisation.

Aromatase activity was determined by measuring the radioactivity of the 0.5 mL aqueous phase.

Spectral studies:

Measurements: Reductase activity was determined by the measurement of the increasing absorbance of the preparation, corresponding to the reduction of the cytochrome C in the presence of 1H+-NADPH at 550 nm for 2 min at 37 C using a Kontron-Uvikon 860 spectrophotometer.
The absorbance of purified equine aromatase in the presence or absence of glyphosate or Roundup was recorded from 375 to 475 nm with a spectrophotometer.

The spectra of aromatase with glyphosate or Roundup alone were subtracted from the incubation spectrum.

Statistics for all tests: All data are presented as the mean ± SE. The experiments were repeated three times, in triplicate unless otherwise indicated. Statistically significant differences were determined by a Student *t*-test using significance levels of 0.01 and 0.05.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study design is insufficient for risk assessment of real exposure concentrations. Methodological deficiencies (no controls were included). Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems. EPA Test Guideline OCSPP 890.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- Glyphosate at non-cytotoxic concentrations in this test system was demonstrated to have no effects on aromatase activity.
- Likewise, did not affect mRNA levels after 18 hours treatment at ≤ 0.1% glyphosate.
- Roundup aromatase activity measurements are confounded by surfactant effects on microsomes.

- The *in vitro* test system is non-validated
- Physiologically irrelevant concentrations tested
- Testing surfactant-like substances in such systems is now recognized to be not valid.

Response 2 – summarized from the French Ministry of Agriculture and Fish, Committee for Study of Toxicity (2005)

- Major methodological gaps.
- JEG3 cells, a choriocarcinoma human cell line (average of 70 chromosomes vs 46 in normal human cells).
- Concentrations of Roundup used in the various experiments considered to be extremely high.
    - In consideration of limiting factors (oral absorption, 30%; skin absorption, 0.3%; rapid elimination kinetics), such levels would involve considerable human exposure, or several dozen liters of Roundup diluted at 2%.
    - concentrations of Roundup that trigger an effect on aromatase (0.5% - 2%) are at least 1000 times more effective than those of known aromatase inhibitors, such as azole derivatives
- Study design does not make it possible to show the influence of the adjuvants, nor synergism of adjuvants and glyphosate.
- Multiple non-specific effects of surfactant agents on a broad range of cellular targets not discussed.
- No comparison with comparable surfactant agents intended for household use.
- multiple instances of bias in its arguments and its interpretation of the data.
- The authors over-interpret their results in the area of potential health consequences for humans (unsuitable references, non-sustained in vitro-in vivo extrapolation, etc.).

Author(s)	Year	Study title
Benachour, N. Sipahutar, H. Moslerni, S. Gasnier, C. Travert, C. Seralini, G. E.	2007	Time- and dose-dependent effects of roundup on human embryonic and placental cells. Archives of Environmental Contamination and Toxicology Volume: 53 Pages: 126-133

Abstract*

Roundup® is the major herbicide used worldwide, in particular on genetically modified plants that have been designed to tolerate it. We have tested the toxicity and endocrine disruption potential of Roundup (Bioforce®) on human embryonic 293 and placental-derived JEG3 cells, but also on normal human placenta and equine testis. The cell lines have proven to be suitable to estimate hormonal activity and toxicity of pollutants. The median lethal dose (LD_{50}) Of Roundup with embryonic cells is 0.3% within 1 h in serum-free medium, and it decreases to reach 0.06% (containing among other compounds 1.27 mM glyphosate) after 72 h in the presence of serum. In these conditions, the embryonic cells appear to be 2-4 times more sensitive than the placental ones. In all instances, Roundup (generally used in agriculture at 1-2%, i.e., with 21-42 mM glyphosate) is more efficient than its active ingredient, glyphosate, suggesting a synergistic effect provoked by the adjuvants present in Roundup. We demonstrated that serum-free cultures, even on a short-term basis (1 h), reveal the xenobiotic impacts that are visible 1-2 days later in serum. We also document at lower non-overtly toxic doses, from 0.01% (with 210 µM glyphosate) in 24 h, that Roundup is an aromatase disruptor. The direct inhibition is temperature-dependent and is confirmed in different tissues and species (cell lines from placenta or embryonic kidney, equine testicular, or human fresh placental extracts). Furthermore, glyphosate acts directly as a partial inactivator on microsomal aromatase, independently of its acidity, and in a dose-dependent manner. The cytotoxic, and potentially endocrine-disrupting effects of Roundup are thus amplified with time. Taken together, these data suggest that Roundup exposure may affect human reproduction and fetal development in case of contamination. Chemical mixtures in formulations appear to be underestimated regarding their toxic or hormonal impact.

* Quoted from article

MATERIALS AND METHODS

Cytotoxicity assay

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: Monsanto,(Antwerp, Belgium)
Purity:	Glyphosate: not reported Roundup Bioforce® : 360 g/L acid glyphosate (equivalent to 480 g/L of isopropylamine salt of glyphosate
Lot/Batch #:	not reported
Homologation:	Roundup Bioforce® 9800036

2. Vehicle:

Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France)

3. Test system / cells:

Cell cultures:	Human embryonic kidney (HEK) 293 cell line (ECACC 85120602) choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308)
Species:	Human
Source:	CERDIC (Sophia-Antipolis, France)
Cell line maintenance:	phenol red-free EMEM containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (mix of penicillin, streptomycin, and fungizone), and 10% fetal calf serum (Biowhittaker, Gagny, France). The JEG3 cell line was supplemented with 1 mM sodium pyruvate.
Culture conditions:	Temperature: 37°C Atmosphere: 5% CO_2, 95% air 48 h

4. Test method:

MTT assay	Assessment of cell viability
Guideline:	None guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	24-well plates washed with serum-free EMEM
Test conditions:	A 2% solution of Roundup and an equivalent solution of glyphosate were prepared in EMEM and the pH was adjusted to about 7.8. From these stock solutions successive solutions were prepared in serum-free EMEM or serum-containing EMEM. The assays were conducted in 24-well plates. HEK 293 cells or JEG3 cells were grown to 80 % confluence, washed with serum-free EMEM and then exposed to various concentrations of Roundup Bioforce ® or the equivalent concentrations of glyphosate, in serum-free or serum-containing EMEM for 1, 24, 48 or 72 h. Afterwards cells were washed with serum-free EMEM and incubated with 250 µL MTT for 3 h at 37°C. per well. Then 250 µL of 0.04 N-hydrochloric acid containing isopropanol were added to each well, the plates were shaken. Measurements were done at 560 nm for test substance wells and at 720 nm for reference wells.
Dose levels:	0.01, 0.05, 0.1, 0.5, 0.8, 1, 2% of Roundup or equivalent concentrations of glyphosate in serum-free EMEM or serum-containing EMEM
Cells per well:	50000
Exposure duration:	1, 24, 48, and 72 h
Replicates per dose level:	9

5. Observations/analyses:

Measurements:	Cell viability
Statistics:	All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of $p < 0.01$ or $p < 0.05$.

Aromatase activity inhibition

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: Monsanto,(Anvers, Belgium)
Purity:	Glyphosate: not reported Roundup Bioforce® : 360 g/L acid glyphosate (equivalent to 480 g/L of isopropylamine salt of glyphosate
Lot/Batch #:	not reported
Homologation:	Roundup Bioforce®: 9800036

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Cell culture:	HEK 293 cell line (ECACC 85120602)
Species:	Human
Source:	CERDD (Sophia-Antipolis, France)
Tissue for microsome preparation #1:	full-term placentas of young healthy and non-smoking women
Species:	Human
Source:	Centre Hospitalier Régional de Caen (France)
Tissue for microsome preparation #2:	Equine testis
Species:	Horse
Source:	Not reported
Microsome preparation:	Human placental and equine testicular microsomes: Tissue preparation was done by differential centrifugations. All steps were conducted at 4°C. Tissues were washed with 0.5 M KC1, homogenized in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM Dithiothreiol DTT, and centrifuged at 20,000*g*. The supernatant was then ultracentrifuged at 100,000*g*, and the final pellet was washed twice, dissolved in the same buffer containing 20% glycerol, and stored at -70 C.

4. Test methods:

Study type:	Measurement of aromatase activity by tritiated water release assay Measurement of reductase a1ctivity in purified reductasee Moieties from equine testicular microsomes
Guideline:	Non-guideline assays
GLP:	No
Guideline deviations:	Not applicable
Test conditions:	Tritiated water release assay: 293 cells were transfected with human aromatase cDNA and exposed to nontoxic concentrations of glyphosate alone or Roundup. Human placental microsomes were incubated with various concentrations of glyphosate alone or Roundup. Reductase activity: Equine testis microsomes or the purified

reductase moieties were incubated with or without Roundup

Aromatase inhibition:

Equine testicular microsomes were pre-incubated with a saturating concentration (i.e. 11.6%) or without Roundup.

Dose levels: For aromatase activity:

Glyphosate: < 0.2%
Roundup Bioforce®: 1% of product

Test substance solutions were prepared in EMEM (for 293 cells) and in 50 mM Tris-maleate buffer, pH 7.4 or without pH adjustment (microsomes)

In addition for aromatase and reductase activity:

Roundup at IC_{50} (=)

Exposure duration: Tritiated water release assay:

293 cells: 24 h
human placental microsomes: 15 min

Reductase activity:

Equine testicular microsomes: 15 min

Aromatase inhibition (pre-incubation):

Equine testicular microsomes: 30 min

Replicates per dose level: 9

5. Observations/analyses:

Measurements: Aromatase and residual aromatase activity was determined with the tritiated water release assay. Radioactivity of released tritiated water was assessed by liquid scintillation counting.

Reductase activity was determined by the measurement of the increasing absorbance of the preparation, corresponding to the reduction of the cytochrome C in the presence of H^+-NADPH at 550 nm for 2 min at 20 C using a Kontron-Uvikon 860 spectrophotometer.

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study report has several reporting deficiencies in the methods section (e.g. test conditions for the pH- and temperature dependent assay not reported). There is no information on the suitability of the used HEK 293 cell line for assessment of hormonal activity. Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems. EPA Test Guideline OCSPP 890.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants.

3. Klimisch code: **3**

Response 1 – GTF

- Glyphosate at and above relevant concentrations for this test system was demonstrated to have no effects on aromatase activity.
- Roundup aromatase activity measurements are confounded by surfactant effects on microsomes.
- Comparable research to Richard et al (2005), but with an additional cell line, HEK 293, derived from aborted human embryo kidneys, transformed by inserting adenovirus DNA.
- Excessively high doses tested, not environmentally relevant for human health or environmental risk assessment.
- Aromatase production within the steroidogenesis pathway. Therfore, aromatase inhibition would be detected in the steroidogenesis assay. The OECD multi-laboratory validation of the steroidogenesis assay evaluated glyphosate, demonstrating no impact on the steroidogenesis pathway (Hecker et al., 2010).

Response 2 – summarized from Williams et al. (2012)

- pH of test system not adjusted to physiologically appropriate levels
- Negative controls were not pH adjusted to appropriate levels
- Confounding surfactant effects due to cell membrane damage render data generated with formulated products in this test system null.

Author(s)	Year	Study title
Benachour, N. Seralini, G. E.	2009	Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chemical Research in toxicology Volume: 22 Pages: 97-105

Abstract*

We have evaluated the toxicity of four glyphosate (G)-based herbicides in Roundup formulations, from 10(5) times dilutions, on three different human cell types. This dilution level is far below agricultural recommendations and corresponds to low levels of residues in food or feed. The formulations have been compared to G alone and with its main metabolite AMPA or with one known adjuvant of R formulations, POEA. HUVEC primary neonate umbilical cord vein cells have been tested with 293 embryonic kidney and JEG3 placental cell lines. All R formulations cause total cell death within 24 h, through an inhibition of the mitochondrial succinate dehydrogenase activity, and necrosis, by release of cytosolic adenylate kinase measuring membrane damage. They also induce apoptosis via activation of enzymatic caspases 3/7 activity. This is confirmed by characteristic DNA fragmentation, nuclear shrinkage (pyknosis), and nuclear fragmentation (karyorrhexis), which is demonstrated by DAPI in apoptotic round cells. G provokes only apoptosis, and HUVEC are 100 times more sensitive overall at this level. The deleterious effects are not proportional to G concentrations but rather depend on the nature of the adjuvants. AMPA and POEA separately and synergistically damage cell membranes like R but at different concentrations. Their mixtures are generally even more harmful with G. In conclusion, the R adjuvants like POEA change human cell permeability and amplify toxicity induced already by G, through apoptosis and necrosis. The real threshold of G toxicity must take into account the presence of adjuvants but also G metabolism and time-amplified effects or bioaccumulation. This should be discussed when analyzing the in vivo toxic actions of R. This work clearly confirms that the adjuvants in Roundup formulations are not inert. Moreover, the proprietary mixtures available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from R formulation-treated crops.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®; AMPA
Active substance(s):	Glyphosate
Source of test items:	Glyphosate: Sigma-Aldrich, France Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (produced by Monsanto, all available on the market)
Lot/Batch #:	Not specified
Purity:	Glyphosate: not reported Roundup Express®: 7.2 g/L (R7.2) Bioforce® or Extra 360: 360 g/L (R360) Grands Travaux®: 400 g/L (R400) Grands Travaux plus®: 450 g/L (R450)

Homologation:	Roundup Express®: 2010321 Bioforce® or Extra 360: 9800036 Grands Travaux®: 8800425 Grands Travaux plus®: 2020448
Test item:	AMPA (aminomethylphosphonic acid)
Source:	Sigma-Aldrich (Saint Quentin Fallavier, France)
Lot / Batch #:	Not reported
Purity:	Not reported
Test item:	Polyethoxylated tallowamine (POEA)
Source:	Pr. R. Bellé (UMR 7150 CNRS/UPMC, Station Biologique de Roscoff, France)
Lot / Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Primary cell culture:	HUVEC (human primary cells of the umbilical vein cord endothelial cells)
Source:	Lonza
Culture conditions:	Specific endothelial growth medium EGM-2 SingleQuots (CC-4176) containing hEGF, hydrocortisone, GA-1000 (Gentamicin, amphotericin-B), FBS (fetal bovine serum), VEGF, hFGF-B, R3-IGF-1, ascorbic acid, and heparin. Cells were grown in 48-well plates over a period of 24 h at 37 °C (5% CO_2, 95% air) until confluence of 80%. Afterwards they were washed with serum-free EGM-2.
Cell line:	Human embryonic kidney 293 cell line (ECACC 85120602) Human choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308)
Source:	CERDIC (Sophia-Antipolis, France)
Culture conditions:	Phenol red-free Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France) containing 2 mM glutamine, 1% nonessential amino acid, 100 U/mL antibiotics (a mix of penicillin, streptomycin, and fungizone; Lonza), 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, France), and 10% FBS (PAA, les Mureaux, France). The JEG3 cell line was supplemented with 1 mM sodium pyruvate. 50000 cells were grown at 37°C (5% CO_2, 95% air) over a 48 h period to 80% confluence and were washed with serum-free EMEM.

4. Test methods:

MTT assay:	Assessment of cell viability
ToxiLight® assay:	Bioluminescent assay for quantitative measurement of cell membrane damage
Caspase-Glo® 3/7 assay	Assessment of caspase activity or apoptosis induction
Microscopy:	Assessment of cell viability due to cell morphology
Guideline:	Non-guideline assays
GLP:	No

Guideline deviations:	Not applicable
Cell treatments for all tests:	Cells were exposed to various dilutions of the four Roundup formulations, glyphosate, AMPA and POEA in serum-free medium for 24 hours. In another case, cells were incubated with glyphosate, AMPA, and POEA mixtures by pairs at the final nontoxic dilution on SD (succinate dehydrogenase) of 0.5% on the human cell lines (293 or JEG3) and 0.05% on the human primary cells (HUVEC) in comparison to Roundup Bioforce or Extra 360.
Dose levels:	Roundup formulations, glyphosate, AMPA and POEA: 14 concentrations ranging from 10 ppm to 2 % Additional AMPA concentrations: 4, 6, 8 and 10% POEA concentrations: 1 and 5 ppm Combined exposures of G, AMPA and POEA mixtures: For the two cell lines, the first mixture was the combination of glyphosate (0.4999%) with POEA (0.0001%), the second was the combination of glyphosate (0.4%) with AMPA (0.1%), and the third was AMPA (0.4999%) plus POEA (0.0001%). Combined exposures of G, AMPA and POEA mixtures: For the primary HUVEC cells, the first mixture was glyphosate (0.04999%) with POEA (0.0001%), the second was glyphosate (0.04%) with AMPA (0.01%), and the third was AMPA (0.04999%) plus POEA (0.0001%).
Test conditions:	MTT assay: After treatment for 24 h the supernants were recovered for the ToxiLight bioassay, and adherent cells were washed with serum-free medium and incubated with 200 µL MTT per well. The plates were incubated for 3 h at 37°C. Afterwards, 200 µL of 0.04 N-hydrochloric acid containing isopropanol were added, the plates were shaked. Optical density was measured at 570 nm. ToxiLight assay: After 24 h exposure the 50 µL of the above mentioned supernants were added to a 96-well plate and incubated under agitation with 50 µL AK detection reagent (AKDR) for 15 minutes protected from light. The luminescence was measured using a luminometer at 565 nm. Serum-free medium served as negative control. Serum-free medium served as negative control. The positive control was the active reagent AKDR mixed with cells treated in serum-free medium. Caspase-Glo® 3/7 assay: This assay was used for caspase activity or measurement of apoptosis induction. After treatment of 50 µL cell cultures to various dilutions of test items as described above, 50 µL/well of Caspase-Glo® 3/7 reagent was added and plates were incubated for 15 minutes at room temperature protected from light before luminescence was measured. Serum-free medium served as negative control. The positive control consisted of the active reagent mixed with cells treated in serum-free medium. The luminescence was measured using a luminometer at 565 nm. Cell Microscopy: At the end of the 24 h treatments, the serum-free medium was removed, and cells were fixed in absolute ethanol –chloroform – acetic acid (6:3:1, v/v/v) for 1 day at -

20°C. Each well was washed with PBS (pH 7.4) and incubated with 1 µg/mL DAPI solution. Staining of DNA with DAPI was examined using a fluorescence microscope.

Replicates per dose level: 3

5. Observations/analyses:

Measurements: Cell viability, membrane damage, apoptosis induction, cell morphology

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactants: cytoxic membrane disruption potential of surfactants are well known for in vitro test systems. EPA Test Guideline OCSPP 880.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed. No positive controls were included.

2. Relevance of study: **Not relevant** (Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants)

3. Klimisch code: **3**

Response – summarized from the French Agency for Food Safety (AFSSA, 2009)

- Cell lines used present characteristics which may be at the source of a significant bias in the interpretation of the results.
- Experiments were conducted with 24 hours exposure in a medium without serum, which could lead to disturbance of the physiological state of the cells.
- The glyphosate used in the study is glyphosate acid, whereas in the preparations tested it is in the form of an isopropylamine salt. No precise information is given about the pH of test concentrations except the highest dose.
- No mention of any positive evidence for the apoptosis test.
- Cytoxicity and induction of apoptosis may due to pH and/or variations in osmotic pressure on cell survival at the high doses tested.
- Surfactant (tensoactive) effects and increased osmolality are known to increase membrane permeability, causing cytotoxicity and induction of apoptosis.
- Conclusions are based on unvalidated, non-representative cell models (in particular tumour or transformed cell lines) directly exposed to extremely high product concentrations in culture conditions which do not observe normal cell physiological conditions.
- No new information is presented on mechanism of action of glyphosate and preparations containing glyphosate.
- The authors over-interpret their results with regard to potential health consequences for humans, based in particular on an unsupported *in vitro–in vivo* extrapolation
- The cytotoxic effects of glyphosate, its metabolite AMPA, the tensioactive POAE and other glyphosate-based preparations proposed by Benachour and Seralini do not add any pertinent

new facts which call into question the conclusions of the European assessment of glyphosate or those of the national assessment of the preparations.

Author(s)	Year	Study title
Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M. C., Seralini, G. E	2009	Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology Volume: 262 Number: 3 Pages: 184-191

Abstract*

Glyphosate-based herbicides are the most widely used across the world; they are commercialized in different formulations. Their residues are frequent pollutants in the environment. In addition, these herbicides are spread on most eaten transgenic plants, modified to tolerate high levels of these compounds in their cells. Up to 400 ppm of their residues are accepted in some feed. We exposed human liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic in vivo regulatory studies. We measured cytotoxicity with three assays (Alamar Blue, MTT, ToxiLight), plus genotoxicity (comet assay), anti-estrogenic (on ERα, ERβ) and anti-androgenic effects (on AR) using gene reporter tests. We also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24h. These effects were more dependent on the formulation than on the glyphosate concentration. First, we observed a human cell endocrine disruption from 0.5 ppm on the androgen receptor in MDA-MB453-kb2 cells for the most active formulation (R400), then from 2 ppm the transcriptional activities on both estrogen receptors were also inhibited on HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10 ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. A real cell impact of glyphosate-based herbicides residues in food, feed or in the environment has thus to be considered, and their classifications as carcinogens/mutagens/reprotoxics is discussed.

* Quoted from article

MATERIALS AND METHODS

Cytotoxicity assays

1. Test material:

Test item:	Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®
Active substance(s):	Glyphosate
Source of test items:	Glyphosate: Sigma-Aldrich, France Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)
Lot/Batch #:	Not specified
Purity:	Glyphosate: not reported Roundup Express®: 7.2 g/L (R7.2) Bioforce® or Extra 360: 360 g/L (R360) Grands Travaux®: 400 g/L (R400) Grands Travaux plus®: 450 g/L (R450)

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Cell cultures: Hepatoma cell line HepG2, breast cancer cell line MDA-

MB453-kb2

Species: Human

Source: HepG2: ECACC, Salisbury, UK
MDA-MB453-kb2: ATCC, Molsheim, France

Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum

Culture conditions MDA-MB453-kb2: Leibovitz-15 (L15) medium supplemented with 10% foetal calf serum. Cells were incubated at 37°C and the medium was removed every 48 h.

4. Test methods:

MTT assay: Assessment of cell viability of HepG2 cells

ToxiLight® assay: Bioluminescent assay for measurement of cell membrane damage of HepG2-cells

Alamar Blue® assay: Assessment of cell viability of HepG2 cells

Caspase-Glo® 3/7 assay Assessment of caspase activity or apoptose induction

Neutral red assay: Assessment of cell viability of MDA-MB453-kb2 cells

Guideline: Non-guideline assays

GLP: No

Guideline deviations: Not applicable

Test conditions: MTT assay: 2% Roundup Bioforce® and an equivalent solution of glyphosate to Roundup Bioforce were prepared in serum-free medium and adjusted to pH 5.8. From these stock solutions consecutive dilutions up to 10^{-7} were used for measuremen. Assays were conducted in 48-well plates. After treatment for 24 h the supernants were recovered for the ToxiLight bioassay, and adherent cells were washed with serum free medium and incubated with 120 µL MTT per well. The plates were incubated for 3 h at 37°C. Afterwards 120 µL of 0.04 N hydrochloric acid containing isopropanol were added, the plates were shaked. Measurements were done at 570 nm.

ToxiLight assay: After 24 h exposure the 50 µL of the above mentioned supernants were added to a 96-well plate and incubated with 50 µL AK detection reagent (AKDR) for 15 minutes protected from light. The luminescence was measured using a luminometer at 565 nm. Serum-free medium served as negative control. The positive control was the active reagent AKDR mixed with cells treated in serum-free medium.

Alamar Blue assay: About 30000 HepG2 cells per well were grown for 24 h in 96-well plates and then exposed to 250 µL of test substance solutions for 24 h (at pH 7.4). Afterwards 100 µL of Alamar Blue solution was added to each well and incubated for 2 h at 37°C. The optical density was measured at 540 and 620 nM. The viability was expressed as percentage of the control results (medium only).

Caspase-Glo® 3/7 assay: This assay was used for caspase activity or measurement of apoptose induction. Cells were exposed to R450 for 24 or 48 h in 96-well plates. Afterwards

50 µL/well of Caspase-Glo® 3/7 reagent was added and plates were incubated for 45 minutes at room temperature protected from light before luminescence was measured. Serum-free medium served as negative control. The positive control consisted of the active reagent mixed with cells treated in serum-free medium.

Neutral red assay: about 50000 MDA-MB453-kb2 cells were seeded in 24-weel plates and grown for 24 h at 37°C. Afterwards cells were exposed to test substance solutions for 24 h. Cells were washed and incubated with neutral red solution for 3 h at 37°C. After a further washing the viability was assessed by fluorescence measurement.

Dose levels: Glyphosate: not reported
Roundup Express®: 7.2 g/L
Bioforce® or Extra 360: 360 g/L
Grands Travaux®: 400 g/L
Grands Travaux plus®: 450 g/L

Replicates per dose level: 4 x 3 replicates

5. Observations/analyses:

Measurements: Cell viability, membrane damage, apoptose induction

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

Genotoxicity test

1. Test material:

Test item: Grands Travaux®
Active substance(s): Glyphosate
Source of test items: Grands Travaux® (available on the market)
Lot/Batch #: Not specified
Purity: 400 g/L

2. Vehicle and/or positive control: medium / Benzo[a]pyrene 50 µM

3. Test system / cells:

Cell cultures: Hepatoma cell line HepG2
Species: Human
Source: HepG2: ECACC, Salisbury, UK
Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum

4. Test methods:

Study type: Single-cell gel electophoresis assay (Comet assay)
Guideline: Non-guideline assay
The assay was conducted according to the method developed by Singh et al., 1988, with some modifications for cell preparation (Valentin-Severin et al., 2003).

(Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 84–191. Valentin-Severin, I., Le Hegarat, L., Lebon, A.M., Lhuguenot, J.C., Chagnon, M.C., 2003. Use of hepG2 cell line for direct or indirect mutagens screening: comparative investigations between comet and micronucleus assay. Mut. Res. 536, 79-90)

GLP: No

Guideline deviations: Not applicable

Dose levels: 1, 2.5, 5, 7.5, 10 ppm

Exposure duration: 24 h

Replicates per dose level: 3 x 2 replicates

Analysed cells per replicate: 100

5. Observations/analyses:

Measurements: Observed nuclei were classified into 4 classes: 0 (undamaged), 1 (minimum damage), 2 (medium damage) and 3 (maximum damage)

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

Aromatase disruption

1. Test material:

Test item: Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®

Active substance(s): Glyphosate

Source of test items: Glyphosate: Sigma-Aldrich, France
Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)

Lot/Batch #: Not specified

Purity: Glyphosate: not reported
Roundup Express®: 7.2 g/L
Bioforce® or Extra 360: 360 g/L
Grands Travaux®: 400 g/L
Grands Travaux plus®: 450 g/L

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Cell cultures: Hepatoma cell line HepG2

Species: Human

Source: HepG2: ECACC, Salisbury, UK

Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum

4. Test methods:

Study type:	Measurement of aromatase activity by tritiated water release assay, semi-quantitative RT-PCR
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Test conditions:	Tritiated water release assay: HepG2 cells were exposed to non-toxic concentrations of glyphosate alone or Roundup. RT-PCR: HepG2 cells were exposed to non-toxic concentrations of glyphosate alone or Roundup. RNA was extracted and reverse transcribed (using 200 U MMLV-RT at 42°C for 60 min). The resulting cDNA was subjected to RT-PCR.
Dose levels:	Glyphosate: 0.06, 0.2, 0.3%. Roundup Express®: 0.3, 0.5, 0.8% of product Bioforce® or Extra 360: 0.08, 0.1, 0.3% of product Grands Travaux®: 0.001, 0.003, 0.005 % of product Grands Travaux plus®: 0.005, 0.007 % of product
Exposure duration:	24 h
Replicates per dose level:	4 x 3 replicates

5. Observations/analyses:

Measurements:	Tritiated water release assay: radioactivity of released tritiated water was assessed by liquid scintillation counting. RT-PCR: Aromatase mRNA levels were normalised with control gene GAPDH and analysed photographically.
Statistics:	All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

Anti-estrogenic and anti-androgenic effects

1. Test material:

Test item:	Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®
Active substance(s):	Glyphosate
Description:	Glyphosate: Sigma-Aldrich, France
Source of test items:	Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)
Lot/Batch #:	Not specified
Purity:	Glyphosate: Roundup Express®: 7.2 g/L Bioforce® or Extra 360: 360 g/L Grands Travaux®: 400 g/L Grands Travaux plus®: 450 g/L

2. Vehicle and/or positive control: Medium / ICI 182 x 780 (10^{-8}M) and Nilutamide (10^{-6} M)

3. Test system / cells:

Cell cultures:	Hepatoma cell line HepG2, breast cancer cell line MDA-

MB453-kb2

Species: Human

Source: HepG2: ECACC, Salisbury, UK
MDA-MB453-kb2: ATCC, Molsheim, France

Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum
For anti-estrogenic activity, HepG2 cells were grown in phenol red-free MEM

Culture conditions MDA-MB453-kb2: Leibovitz-15 (L15) medium supplemented with 10% foetal calf serum. Cells were incubated at 37°C and the medium was removed every 48 h.

4. Test methods:

Gene-receptor tests with luciferase activity measurement

Guideline: Non-guideline assay

GLP: No

Guideline deviations: Not applicable

Test conditions: Anti-estrogenic activity test: 120000 HepG2-cells per well were grown at 37°C (5% CO_2, 95% air) in MEM supplemented with 2 mM glutamine, 1% non-essential amino-acis and 10% of dextran-coated charcoal foetal calf serum in 24-well plates. After 24 h the cells were transfected with a mixture of 5 different plasmids (ERE-TK, hERα, hERβ, pCMVβGal and psG5) and incubated for 6 h at 37°C (5% CO_2, 95% air). Afterwards the medium was removed and replaced by 1 mL of medium without foetal calf serum and incubated for further 24 h. Cells were co-treated with the test substance solutions and β-estradiol (10^{-8} M). ICI 182 x 780 (10^{-8} M) served as positive control. At the end of treatment cells were lysed with Reporter lysis buffer and frozen at -80°C for at least 30 min, and prepared for activity measurements.
Anti-androgenic activity test: 50000 MDA-MB-453-kb2 cells per well were grown in 24-well plates in L-15 medium without phenol-red supplemented with 5% dextran-charcoal fetal calf serum at 37°C without CO_2. After 24 h the medium was removed and cells were washed with PBS and exposed to Roundup solutions in co-treatment with DHT (4 x 10^{-10} M). Nilutamide (10^{-6} M) was used as positive control. After 24 h cells were lysed and luciferase activity was measured.

Dose levels: Anti-estrogenic activity test:
Glyphosate: 0.1, 0.2, 0.3%
Roundup Express®: 0.1, 0.2, 0.3% of product
Bioforce® or Extra 360: 0.05, 0.1, 0.15, 0.2% of product
Grands Travaux®: 0.00025, 0.0005, 0.00075, 0.001 % of product
Grands Travaux plus®: 0.001, 0.002, 0.003 % of product

Anti-androgenic activity test:
Glyphosate: 0.05, 0.1, 0.15%
Roundup Express®: 0.05, 0.1, 0.15, 0.2% of product
Bioforce® or Extra 360: 0.01, 0.02, 0.03, 0.04, 0.05% of

product
Grands Travaux®: 0.00005, 0.0001, 0.00015, 0.0002 % of product
Grands Travaux plus®: 0.001, 0.002, 0.003, 0.004 % of product

Replicates per dose level: 3 x 3 replicates

5. Observations/analyses:

Measurements: Anti-estrogenic activity test: Luciferase and β-galactosidase activities and protein level.

Luciferase activity for each treatment group was normalised to β-galactosidase activity and protein level (Luc x Prot/Gal) and compared to the control (17 β-estradiol) set at 100%.

Anti-androgenic activity test: Luciferase activity were measured and reported as a percentage of the data obtained with the androgen DHT.

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Due to reporting deficiencies (e.g. correlation between concentration used in toxicity tests and concentrations used in comet assay) assessment of results difficult. Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- Glyphosate demonstrated no significant anti-estrogenic potential
- Glyphosate demonstrated some anti-androgenic potential at lower concentrations, but not as doses increased and therefore results are considered unrelated to treatment
- Four glyphosate based formulations demonstrated both estrogenic and androgenic activity.
- Results are confounded due to surfactants within the formulated products tested, which affect cell membrane integrity and produces false findings.

Response 2 – summarized from BfR Review (2009)

- Numerous methodological flaws are noted.
  - Test substance(s) not characterized
  - Source of materials for cell culture not provided.
  - Dosing concentrations not well described

  - Serum free media only appropriate for short term (3-4 hour) *in vitro* exposures.
  - pH control of dilutions not clear.
  - Osmolality of test solutions not reported.
  - Electrophoresis parameters insufficiently or inaccurately reported.
- Numerous reporting deficiencies are noted.
  - Influence of serum-free cell culturing on endpoints can not be determined
  - Incomplete data reporting, in cluding β-galactosirase activity, cototoxicity for select assays.
  - Positive control data not reported.
  - Confusion between maximum residue levels verses systemic concentrations in humans.

Author(s)	Year	Study title
Clair, E., Mesnage, R., Travert, C., Seralini, G.E.	2012a	A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells *in vitro*, and testosterone decrease at lower levels Toxicology in Vitro Volume: 26 Number: 2 Pages: 269-279

Abstract*

The major herbicide used worldwide, Roundup, is a glyphosate-based pesticide with adjuvants. Glyphosate, its active ingredient in plants and its main metabolite (AMPA) are among the first contaminants of surface waters. Roundup is being used increasingly in particular on genetically modified plants grown for food and feed that contain its residues. Here we tested glyphosate and its formulation on mature rat fresh testicular cells from 1 to 10000 ppm, thus from the range in some human urine and in environment to agricultural levels. We show that from 1 to 48 h of Roundup exposure Leydig cells are damaged. Within 24–48 h this formulation is also toxic on the other cells, mainly by necrosis, by contrast to glyphosate alone which is essentially toxic on Sertoli cells. Later, it also induces apoptosis at higher doses in germ cells and in Sertoli/germ cells co-cultures. At lower non toxic concentrations of Roundup and glyphosate (1 ppm), the main endocrine disruption is a testosterone decrease by 35%. The pesticide has thus an endocrine impact at very low environmental doses, but only a high contamination appears to provoke an acute rat testicular toxicity. This does not anticipate the chronic toxicity which is insufficiently tested and only with glyphosate in regulatory tests.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Description:	Not reported
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: not reported
Lot/Batch #:	Not reported
Purity:	Glyphosate: not reported Roundup Bioforce®: 360 g/L acid glyphosate (corresponding to 100%)
Homologation:	Roundup Bioforce® 9800036

2. Vehicle and/or positive control: Dulbecco Modified Eagle's Medium/Ham F12 Medium (DMEM; Biotech GmbH, Dutscher, Brumath, France)

3. Test system / cells / animals:

Species:	Rat
Strain:	Sprague-Dawley

Source:	Janvier, Le Genest-Saint-Isle, France or University Centre of Biological Resources, Caen, France
Age of test animals at study initiation:	70 days ± 5
Sex:	male
Body weight:	Not reported
Acclimation period::	Not reported
Diet/Food:	Standard food, *ad libitum*
Water:	Water, *ad libitum*
Housing::	Not reported
Environmental conditions:	Temperature: 20 ± 22°C Humidity: not reported Air changes: not reported 12-hour light/dark cycle
Cell Culture:	Leydig, Sertoli and germ cells
Species:	Rat
Source:	Sprague-Dawley rats
Cell line maintenance:	DMEM/Ham F12 nutrient medium (1:1, v/v) supplemented with or without hCG (human homolog of LH physiologically involved in endocrine regulation of Leydig cells) for Leydig cells culture and with serum replacement 3 for Sertoli and germ cells.
Culture conditions:	Temperature: 32°C Atmosphere: 5% CO_2, 95% air

4. Test methods:

Bioluminescent ToxiLight™ bioassay:	Cytotoxicity assessment
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	96 or 24-well plates
Test conditions:	Before the assay, cells were treated with different dilutions of Roundup Bioforce ® or glyphosate ± 1 UI/mL of hCG during different exposure time points. The adenylate kinase detection reagent (AKDR) was prepared in a buffer (5 g/10 mL). Subsequently 50 mL of supernatant were transferred to an opaque black 96-well plate. 50 µL of AKDR reagent were put into each well. The plates were then left under agitation for 15 min in the dark, and light was measured using a luminometer.
Dose levels:	Not exactly specified; several concentrations from 0 – 1.0% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium
Cells per well:	10^5 per well in 96-well plates and 3 x 10^5 per well in 24-well plates
Exposure duration:	3, 6, 9, 12, 18, 24 or 48 h
Replicates per dose level:	9