REVIEW ARTICLE

Toxicol Rev 2004; 23 (3): 159-167
1176-2551/04/0003-0159/$31.00/0

© 2004 Adis Data Information BV. All rights reserved.

Glyphosate Poisoning

Sally M. Bradberry, Alex T. Proudfoot and *J. Allister Vale*

National Poisons Information Service (Birmingham Centre) and West Midlands Poisons Unit, City Hospital, Birmingham, UK

Contents

Abstract

Glyphosate is used extensively as a non-selective herbicide by both professional applicators and consumers and its use is likely to increase further as it is one of the first herbicides against which crops have been genetically modified to increase their tolerance. Commercial glyphosate-based formulations most commonly range from concentrates containing 41% or more glyphosate to 1% glyphosate formulations marketed for domestic use. They generally consist of an aqueous mixture of the isopropylamine (IPA) salt of glyphosate, a surfactant, and various minor components including anti-foaming and colour agents, biocides and inorganic ions to produce pH adjustment.

The mechanisms of toxicity of glyphosate formulations are complicated. Not only is glyphosate used as five different salts but commercial formulations of it contain surfactants, which vary in nature and concentration. As a result, human poisoning with this herbicide is not with the active ingredient alone but with complex and variable mixtures. Therefore, It is difficult to separate the toxicity of glyphosate from that of the formulation as a whole or to determine the contribution of surfactants to overall toxicity. Experimental studies suggest that the toxicity of the surfactant, polyoxyethyleneamine (POEA), is greater than the toxicity of glyphosate alone and commercial formulations alone. There is insufficient evidence to conclude that glyphosate preparations contain-

ing POEA are more toxic than those containing alternative surfactants. Although surfactants probably contribute to the acute toxicity of glyphosate formulations, the weight of evidence is against surfactants potentiating the toxicity of glyphosate.

Accidental ingestion of glyphosate formulations is generally associated with only mild, transient, gastrointestinal features. Most reported cases have followed the deliberate ingestion of the concentrated formulation of Roundup®[1] (41% glyphosate as the IPA salt and 15% POEA). There is a reasonable correlation between the amount ingested and the likelihood of serious systemic sequelae or death. Advancing age is also associated with a less favourable prognosis. Ingestion of >85mL of the concentrated formulation is likely to cause significant toxicity in adults. Gastrointestinal corrosive effects, with mouth, throat and epigastric pain and dysphagia are common. Renal and hepatic impairment are also frequent and usually reflect reduced organ perfusion. Respiratory distress, impaired consciousness, pulmonary oedema, infiltration on chest x-ray, shock, arrythmias, renal failure requiring haemodialysis, metabolic acidosis and hyperkalaemia may supervene in severe cases. Bradycardia and ventricular arrhythmias are often present pre-terminally. Dermal exposure to ready-to-use glyphosate formulations can cause irritation and photo-contact dermatitis has been reported occasionally; these effects are probably due to the preservative Proxel® (benzisothiazolin-3-one). Severe skin burns are very rare. Inhalation is a minor route of exposure but spray mist may cause oral or nasal discomfort, an unpleasant taste in the mouth, tingling and throat irritation. Eye exposure may lead to mild conjunctivitis, and superficial corneal injury is possible if irrigation is delayed or inadequate.

Management is symptomatic and supportive, and skin decontamination with soap and water after removal of contaminated clothing should be undertaken in cases of dermal exposure.

Glyphosate [*N*-(phosphonomethyl) glycine] is an organic compound containing phosphorus (figure 1) that is used extensively as a non-selective herbicide by both professionals and amateurs. It has been marketed since 1974 and its use is likely to increase further as it is one of the first herbicides against which crops have been genetically modified to increase their tolerance.

Commercial glyphosate-based formulations range from concentrates containing 41% or more glyphosate to 1% glyphosate formulations marketed for domestic use. They generally consist of an aqueous mixture of the isopropylamine (IPA) salt of glyphosate, a surfactant, and various minor components including anti-foaming and colour agents, biocides and inorganic ions to produce pH adjustment.[1]

Glyphosate's popularity is attributable to its plant-specific mechanism of action, its inactivation on contact with soil and its suitability for 'no-till' conservation of crops. In addition, its relative lack of volatility and soil migration and rapid biotic degradation give it a favourable environmental safety profile. Glyphosate is metabolised by several bacteria in soil to give phosphorus and sarcosine which is then converted to glycine and ammonia by sarcosine oxidase. The alternative metabolic pathway involves the formation by glyphosate oxidoreductase of aminomethylphosphonic acid (AMPA), which is also the metabolite formed in humans.

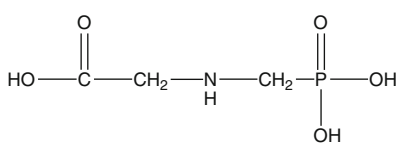


Fig. 1. Chemical structure of glyphosate [*N*-(phosphonomethyl) glycine].

1. Epidemiology

In the 3-year period 2001–03, there were 13 318 reports to the American Association of Poisons Control Centers Toxic Exposure Surveillance System relating to glyphosate exposure.[2-4] Of these, 3622 involved children <6 years of age. There was a 'moderate' outcome in 291 patients, a 'major' (life-threatening) outcome in 18 (0.14%) and five patients died.

Several case series of glyphosate ingestions have been published[5-9] with mortalities ranging from 8% to 16%. Of the 377 cases reported in these four series, 38 died.

Goldstein et al.[1] analysed 815 glyphosate-related reports to the California Environmental Protection Agency Pesticide Illness Surveillance Program for the years 1982–97. Most involved topical irritation of the eye (n = 399), skin (n = 250), upper airways (n = 7), or combinations of these sites (n = 32) without systemic symptoms. Of the 187 systemic cases, only 22 were classified as probably or definitely related to glyphosate exposure alone. With the exception of one intentional ingestion, all of these cases involved incidental topical or inhalation exposure, and a causal relationship to the reported systemic symptoms remains open to question.

1 The use of trade names is for product identification purposes only and does not imply endorsement.

© 2004 Adis Data Information BV. All rights reserved.

2. Mode of Action

Glyphosate is primarily a competitive inhibitor of the critical enzyme of the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase,[10] which is responsible for the synthesis of an intermediate in the biosynthesis of phenylalanine, tyrosine and tryptophan but is not present in mammalian species, including humans. The shikimate pathway produces aromatic amino acids and a large number of secondary products, including lignins, flavonoids, and tannins in plants and some micro-organisms.[11] Glyphosate is very mobile within plants, with preferential transport to metabolic sinks such as meristematic tissues. It is relatively slow acting, so that it is transported throughout plants before growing tissues are killed. For this reason, it is very effective in controlling perennial weeds in which roots must be killed to prevent regrowth. Although some of the phytotoxicity of glyphosate is a result of reduced pools of aromatic amino acids, most of its herbicidal effect appears to be the result of a general disruption of metabolic pathways through deregulation of the shikimate pathway.[11]

3. Mechanisms of Toxicity

The mechanisms of toxicity of glyphosate formulations are complicated. Not only is glyphosate used as five different salts but commercial formulations of it contain surfactants that vary in nature and concentration and are known by a variety of names. The salts, IPA, Na^+ and NH_3, are probably equipotent as the counterion does not appear to contribute much to toxicity. However, there are new agricultural products that contain K^+ ion, which could increase the toxicity of the formulation if ingested in substantial amounts. The evidence base for the suggestion that products containing glyphosate trimesium are more toxic than other glyphosate salts is limited.[12] Moreover, it is believed that all formulations containing the trimesyl salt have now been withdrawn. Thus, human poisoning with this herbicide is not with the active ingredient alone but with complex and variable mixtures. Therefore, it is difficult to separate the toxicity of glyphosate from that of the formulation as a whole or to determine the contribution of surfactants to overall toxicity.

Although glyphosate is a phosphorus-containing compound, it does not inhibit acetylcholinesterase.

3.1 Glyphosate

3.1.1 Acute Toxicity

Numerous acute toxicity studies have been performed to determine the LD_{50} of glyphosate and herbicide formulations containing glyphosate as an active ingredient.[13] Glyphosate has very low toxicity by the oral (>5000 mg/kg bodyweight [bw]) and dermal (>2000 mg/kg bw) routes but is markedly more toxic by the intraperitoneal route (134–545 mg/kg bw).

Based upon animal studies, some investigators suggest that glyphosate may enhance adenosine triphosphatase activity and uncouple mitochondrial oxidative phosphorylation,[14-17] although this has been disputed by Tominack et al.[7] who identified some unexplained inconsistencies in Olorunsogo's data. "For example, the resting respiratory rates (state 4) were inconsistent in all the glyphosate-treated rats, with the post-ADP (adenosine 5′-diphosphate) rates higher (and more normal) than the pre-ADP rates. This phenomenon does not occur in uncoupled mitochondria. Furthermore, there is no relationship between dose of glyphosate given in the range of the sublethal to lethal doses (30–120 mg/kg) and respiratory control ratios (oxygen consumption in the presence of ADP/oxygen consumption in the absence of ADP) in isolated mitochondria. Finally, no data on the effect of adding pure glyphosate (which is not metabolised in animals or humans) on the oxidative phosphorylation of normal mitochondria were present. Likewise, the clinical picture in this survey of patients ingesting up to 500mL of a 41% glyphosate preparation is inconsistent with oxidative uncoupling. Tachypnea and tachycardia, the expected effects of poisoning with agents that uncouple oxidative phosphorylation, were not consistently seen and no cases of significant hyperpyrexia were encountered". It is probable that the surfactant in Roundup® formulations is responsible for uncoupling oxidative phosphorylation.

In rats, glyphosate decreased hepatic cytochrome P450 and mono-oxygenase activities and the intestinal activity of aryl hydrocarbon hydroxylase.[18]

At high concentrations *in vitro* glyphosate has been shown to inhibit acetylcholinesterase,[19] although there is no evidence for significant acetylcholinesterase inhibition in mammals *in vivo*.

3.1.2 Chronic Toxicity

In repeat-dose studies in experimental animals, the toxicity of glyphosate tends to be non-specific, failure to gain weight being the most frequent observation. Since very high dietary concentrations were used in some of these studies, this effect may have been due to unpalatability and reduced calorie intake.[20] There is no evidence of carcinogenic or teratogenic potential and little evidence of genotoxicity in a variety of *in vitro* tests.[20]

3.2 Surfactants

In general, surfactants interfere with the walls of mitochondria, destroying the proton gradient required for energy production. The poorly responsive multiple organ failure observed following surfactant ingestion are consistent with these effects. The amine surfactants are strongly alkaline and, therefore, corrosive in their pure forms. However, adjustment to a neutral pH is routinely performed when they are co-formulated with glyphosate.

Surfactants in concentrations of up to 50% are added to nearly all glyphosate preparations available for land use; formulations for

© 2004 Adis Data Information BV. All rights reserved.

aquatic use are generally surfactant-free due to aquatic toxicity of the surfactants. They serve several purposes: they primarily act as wetting agents, promote uniform spreading of the herbicide on the leaf surface and assist the penetration of glyphosate into the leaf.

3.2.1 Polyoxyethyleneamine

The most widely used surfactants are tertiary amines comprising a nitrogen atom bonded to two polyoxyethylene (C_2H_4O) groups and one long-chain alkyl group. The polyoxyethylene groups are hydrophilic and increasing their number in a molecule increases the hydrophilicity of the surfactant. These groups are also referred to by synonyms including ethylene oxide and ethoxylate. The hydrophobic alkyl group is derived from tallow, a mixture of fats obtained from cows and pigs, consisting of two-thirds stearin and palmitin and one-third olein. The latter react with a nitrogen source to produce primary alkylamines which, in turn, react with polyoxyethylene to produce polyoxyethyleneamine.

Manufacturers use several terms to refer to this class of surfactants, including polyoxyethyleneamine (POEA), ethoxylated tallow amine, polyethoxylated tallow amine, tallow amine, alkoxylated fatty amine and tallow alkyl amine ethoxylate. These all refer to the same group of compounds (although the last two terms may technically refer to a compound containing any of a number of hydrophilic polymer chains, based on the general formula $[C_nH_{2n}O]$, as opposed to C_2H_4O as above). Thus, 'polyoxyethyleneamine' or any of its synonyms does not refer to a single chemical entity, but rather to a group of compounds. The chain length and degree of saturation of the alkyl groups can vary, as can the chain length of the polyoxyethylene groups. It is likely that the surfactant used in a particular glyphosate preparation consists of molecules with polyoxyethylene chains of roughly similar lengths as this is an important means of tailoring the surface-active properties. However, the surfactant probably contains a mixture of molecules substituted with different alkyl groups, as they are derived from tallow, which is itself a mixture.

The concentration of polyoxyethyleneamine ranges from <1% in ready-to-use glyphosate formulations to 21% in some concentrated professional products.

3.2.2 Surfactants Derived from Plant Fats

The carbon chains contained in tallow are identical to those extracted from other sources, such as cocoa, peanuts, and cotton or palm oil, although the tallow may contain different impurities. Therefore, it seems likely that ethoxylated cocoamine (another term used by manufacturers to describe the surfactants of several glyphosate products) is toxicologically equivalent to tallow amine.

3.2.3 Other Surfactants

Other surfactants used in glyphosate-containing herbicides include alkyl polyoxyphosphate amine (generally used in a concentration of around 13%), polyethoxylated alkyl etheramine (7.5%), trimethylethoxypolyoxypropylammonium chloride (up to 13%), ethoxylated phosphate ester (9.5%), polyethoxysorbitan monolaurate (3%), alkyl polysaccharide (0.5–5% in amateur products and up to 50% in professional products) and substances such as polyethylene glycol and polyethoxylated fatty alcohol, generally present in low concentrations.

3.2.4 Do Surfactants Contribute to the Toxicity of Glyphosate Formulations?

The main controversy regarding the toxicity of glyphosate formulations is whether their toxicity is due to the herbicide itself or to their co-formulants, notably surfactants.

Animal experiments suggest that the toxicity is due primarily to the surfactant, since it has an oral LD_{50} of 1200 mg/kg,[20] as opposed to >5000 mg/kg for glyphosate[13] and for its formulations.[13,20]

Adam et al.[21] investigated the toxicity of Roundup® in rats. They also tested separately solutions of 41% glyphosate isopropylamine, 18% POEA, and a mixture of 41% glyphosate isopropylamine and 18% POEA (i.e. the two major ingredients of Roundup®, without the other formulation additives). Each batch of eight rats were observed for 6 hours after dosing to detect immediate toxicity and again at 24 hours. The animals were then sacrificed if still alive. Two of the eight rats given POEA died, whereas none of the other animals succumbed. Oral administration of POEA also caused more severe diarrhoea (seven of eight animals at 6 hours; eight of eight animals at 24 hours) than in those administered glyphosate alone. POEA also caused more damage to the gastrointestinal tract and lungs.

Baba et al.[22] investigated the toxicity of glyphosate, surfactant and Roundup® (41% glyphosate/15% surfactant) in seven rats and obtained oral LD_{50} values at 72 hours post-administration of 5957 mg/kg, 661 mg/kg and 5337 mg/kg, respectively. The authors concluded that the toxic effects of Roundup® were more related to the surfactant than glyphosate.

Tai et al.[23] administered glyphosate, surfactant or Roundup® (41% glyphosate/15% surfactant) to five beagle dogs by continuous intravenous infusion. Glyphosate increased myocardial contractility, possibly in response to a glyphosate-induced increase in pulmonary artery pressure. In contrast, the surfactant and Roundup® both reduced myocardial contractility and cardiac output suggesting that the cardiac depressant effect of Roundup® was due to the surfactant, rather than to glyphosate.

Sawada and Nagai[9] reported 56 cases of human poisoning with Roundup® (41% glyphosate isopropylamine/15% POEA), and two cases of poisoning due to products containing a shampoo and a spreading agent (not specified), but not glyphosate. The clinical features were very similar, suggesting that the features of Roundup® poisoning were due to the surfactant.

© 2004 Adis Data Information BV. All rights reserved.

3.2.5 Do Surfactants Potentiate the Toxicity of Glyphosate?

Martinez et al.[24] obtained an oral LD_{50} for Roundup® (18% glyphosate/7% POEA) of approximately 1600 mg/kg of glyphosate when combined with 560 mg/kg POEA. They compared this to published LD_{50} values for the individual substances and suggested that the combination of glyphosate and POEA resulted in greater toxicity than would be expected by the addition of the two substances, i.e. that there was potentiation. The same group[25] also compared the same Roundup® preparation to 7% POEA alone. Both caused similar respiratory features but those produced by POEA alone were less severe. The authors suggested that the combination of POEA with glyphosate potentiated pulmonary toxicity. However, neither study was designed appropriately to confirm the existence of a synergistic interaction, no statistical analysis was carried out and no group was treated with glyphosate alone.

Potentiation of the toxicity of glyphosate and POEA in combination has not been observed in other studies.[21] Tai et al.[23] suggested that in terms of cardiotoxicity, glyphosate and the surfactant had an opposite, rather than synergistic effect.

In the study described in section 3.2.4 by Baba et al.[22] that investigated the toxicity of glyphosate, surfactant and Roundup® (41% glyphosate/15% surfactant) in seven rats and obtained oral LD_{50} values at 72 hours post-administration of 5957 mg/kg, 661 mg/kg and 5337 mg/kg, respectively, graphical analysis indicated that the interaction between glyphosate and surfactant was antagonistic. The authors concluded that it was unlikely that the toxicity of glyphosate was potentiated by mixing with surfactant.

3.2.6 Are Polyoxyethyleneamines More Toxic Than Other Surfactants?

As there are very few human case reports of exposure to glyphosate preparations that are stated to contain surfactants other than POEA, it cannot yet be concluded that non-POEA preparations do not cause the features associated with POEA ingestions.

3.2.7 Summary

Experimental studies suggest that the toxicity of the surfactant, POEA, is greater than the toxicity of glyphosate alone. There is insufficient evidence to conclude that glyphosate preparations containing POEA are more toxic than those containing alternative surfactants. Although surfactants probably contribute to the acute toxicity of glyphosate formulations, the weight of evidence is against surfactants potentiating the toxicity of glyphosate; indeed, the reverse has been suggested. The evidence base for the suggestion that products containing glyphosate trimesium are more toxic than formulations containing other glyphosate salts is very limited, but cannot be dismissed.

4. Toxicokinetics

The existing knowledge of the toxicokinetics of glyphosate is mainly derived from animal studies and has been reviewed recently.[20] Only some 30% is absorbed after oral administration to rats.[20,26] Peak plasma concentrations of glyphosate are attained at 1–2 hours[20,26,27] and decline quickly.[26] Initial distribution is mainly to the small intestine, colon, kidney and bone.[27] Very little glyphosate undergoes biotransformation, the vast majority being rapidly excreted unchanged in the urine.

A similar pattern of absorption, metabolism and elimination after ingestion is seen in humans, although the data are limited. Two poisoned patients reached peak plasma glyphosate concentrations within 4 hours, the concentrations being almost undetectable by 12 hours.[28] Severe poisoning is associated with plasma glyphosate concentrations >1000 mg/L[28] and, occasionally, concentrations as high as 1600 mg/L have been encountered.[29] However, as toxicity may not be due to glyphosate itself, the clinical predictive value of these concentrations is limited.

In another case, the high ratio of glyphosate to AMPA in serum at 8 hours and 16 hours post-ingestion (126 : 1 and 147 : 1, respectively) and the ratio of the total amounts in the patient's urine (148 : 1) strongly indicate that very little glyphosate is metabolised.[30]

Dermal absorption of glyphosate by monkeys is poor; only some 2% of the applied amount is absorbed over 24 hours.[31,32] Absorption through human (cadaver) skin is little better and was <1% after application of Roundup® diluted to spray strength.[20,33]

Absorption after inhalation does not appear to have been studied but would not be expected to be significant.

Urinary glyphosate concentrations were evaluated in 48 farmers, their spouses, and their 79 children (4–18 years of age). Sixty per cent of farmers had detectable concentrations of glyphosate in their urine on the day of application. The geometric mean concentration was 3 μg/L, the maximum value was 233 μg/L, and the highest estimated systemic dose was 0.004 mg/kg. Farmers who did not use rubber gloves had higher geometric mean urinary concentrations than did other farmers (10 vs 2.0 μg/L). For spouses, 4% had detectable concentrations in their urine on the day of application. Their maximum value was 3 μg/L. For children, 12% had detectable glyphosate in their urine on the day of application, with a maximum concentration of 29 μg/L. All but one of the children with detectable concentrations had helped with the application or were present during herbicide mixing, loading, or application. None of the systemic doses estimated in this study approached the US Environmental Protection Agency reference dose for glyphosate of 2 mg/kg/day.[34]

© 2004 Adis Data Information BV. All rights reserved.

5. Clinical Features

In human poisoning, it is not always possible to determine which glyphosate formulation, and in particular which surfactant, was ingested. Accidental ingestion of glyphosate is generally associated with only mild, transient gastrointestinal features,[6,35] although a 6-year-old died shortly after ingesting a 'small amount' of a herbicide containing glyphosate trimesium 326 g/L (33%).[12] Most reported cases relate to the deliberate ingestion of the concentrated formulation of Roundup® (41% glyphosate as the IPA salt and 15% POEA), which has resulted in the development of severe features.[6,7]

5.1 Ingestion

Nausea, vomiting and diarrhoea are the only likely features following ingestion of glyphosate ready-to-use amateur formulations. Small amounts of the concentrated formulation have not caused severe systemic effects in adults[6,7] but may cause burning in the mouth and throat, hypersalivation, nausea, vomiting and diarrhoea.[5] In contrast, ingestion of >85mL of the concentrated formulation is likely to cause significant toxicity in adults.[6] Gastrointestinal corrosive effects with mouth, throat and epigastric pain and dysphagia are common in these circumstances,[7,36] with predominantly gastric and oesophageal rather than duodenal damage.[36] Small bowel infarction has been reported, probably secondary to hypotension.[37] Lower gastrointestinal corrosive injury is rare,[6] although Delcenserie et al.[38] described a 44-year-old man who developed acute colitis 1 week after consuming an unknown amount of glyphosate-contaminated wine.

Table I lists proposed criteria for classification of severity of poisoning resulting from glyphosate formulation ingestion.

Among 50 patients who ingested glyphosate concentrate (estimated mean volume 182 ± (SD) 202mL; n = 44) nearly half exhibited grade 1 gastric injury (oedema and hyperaemia of the mucosa) at endoscopy with grade 1 oesophagitis in one-third of cases.[36] Duodenal injury was relatively uncommon, with 14% of cases showing grade 1 duodenitis and only one patient a more severe (grade 2a, superficial ulceration) lesion. In this study, patients with grade 2 or 3 (multiple ulcerations with necrosis) oesophageal lesions were more likely to have ingested >200mL glyphosate concentrate and were also more likely to manifest severe systemic sequelae including gastrointestinal haemorrhage, hypotensive shock (not always in association with hypovolaemia) or aspiration pneumonia. The latter complication is particularly likely if laryngeal corrosive injury occurs during ingestion.[39]

Aspiration contributes to ventilatory insufficiency in severely poisoned patients[7] but non-cardiogenic pulmonary oedema (adult respiratory distress syndrome) is the underlying pathological process in some cases.[6,37]

Table I. Proposed criteria for the classification of the severity of poisoning resulting from ingestion of glyphosate formulations[6,7]

Asymptomatic
Absence of symptoms and abnormal clinical or laboratory findings
Mild
Short-lived (<24h) buccal or alimentary tract features
Moderate (at least one of the following)
Buccal ulceration
Endoscopically confirmed oesophagitis
Alimentary tract features lasting >24h
Gastrointestinal haemorrhage
Transient hypotension
Transient oliguria
Transient renal impairment
Transient acid-base abnormalities
Transient hepatic damage
Severe (at least one of the following)
Hypotension requiring intervention
Loss of consciousness
Recurrent convulsions
Renal failure requiring replacement therapy
Respiratory abnormalities requiring endotracheal intubation
Cardiac arrest
Death

Yang et al.[40] described acute bronchospasm requiring ventilation, bronchodilators and corticosteroids in a 55-year-old male who committed suicide by ingesting 500mL Roundup®. His clinical course was complicated by pneumomediastinum, tension pneumothorax and subcutaneous emphysema and he died on day 62 from sepsis.

Renal and hepatic impairment (increased transaminase activities) and/or impaired consciousness are not uncommon in more severe cases[5,37] and usually reflect reduced organ perfusion, although a direct toxic effect of glyphosate or surfactant may contribute. Similarly hypovolaemia is an important factor in cases complicated by cardiogenic shock and/or acidosis, although direct toxicity may contribute.[41] Glyphosate/surfactant-induced myocardial depression may also occur.[42]

Other reported features include dilated pupils,[7,35,41] convulsions,[7] confusion,[6] a neutrophil leucocytosis,[5,6] fever[5] and increased serum amylase activity.[6] In one series of 131 cases of glyphosate/surfactant ingestion,[5] metabolic acidosis (standard bicarbonate <22 mmol/L) was present in 48%. Electrocardiographic abnormalities occur in up to 20% of cases, usually sinus tachycardia and/or nonspecific ST-T wave changes,[5] although sinus bradycardia and atrioventricular block[9] are recognised. Stella and Ryan[37] recently reported a case in which broad complex tachycar-

© 2004 Adis Data Information BV. All rights reserved.

dia (140 beats/min) was a presenting feature following ingestion of 1L glyphosate concentrate, although this patient also developed marked metabolic acidosis (pH 7.25, HCO_3 13 mmol/L) with a serum potassium concentration of 8.2 mmol/L. Bradycardia and ventricular arrhythmias may occur as the pre-terminal events.[6,7,35,41]

5.1.1 Prognosis

There is a reasonable correlation between the amount of glyphosate ingested, the severity of damage[6] and the likelihood of serious systemic sequelae[6,7] or death.[5,7] Tominack et al.[7] reported concordance between the estimated ingested volume and outcome, recording a mean ingested volume of glyphosate concentrate of 263 ± 100mL by 11 fatal cases compared with 120 ± 112mL by 86 survivors. Similarly, in their series of 131 ingestions, Lee et al.[5] estimated a mean (±SD) volume ingested of 122 ± 12mL by survivors compared with 330 ± 42mL by those who died ($p < 0.001$). When a large quantity is ingested, death typically ensues within 72 hours.[5] However, not all cases are consistent with this prediction and atypical cases are recognised.[6,35] For example, Temple and Smith[35] described a 43-year-old female who died some 24 hours after ingesting 200–250mL Roundup® concentrate and in whom the principal postmortem findings were pulmonary oedema and acute tubular necrosis. She had been found semi-comatose and covered in vomitus and deteriorated rapidly, the features being dominated by hypotension, metabolic acidosis, anuria and hyperkalaemia. Gastrointestinal haemorrhage and corrosion were not reported. The same authors described another patient who experienced only vomiting despite apparently consuming 1L of Roundup® concentrate.[35] A 34-year-old woman died shortly after consuming some 150mL of a herbicide containing glyphosate trimesium 0.28 g/L (as pure glyphosate).[12] The speed with which death followed ingestion in this case may indicate a greater risk from trimesium salts compared with others.

Other features significantly ($p < 0.001$) more likely in patients who die than in those who survive include: the development of respiratory distress, impaired consciousness, pulmonary oedema, shock, arrhythmias, renal failure requiring haemodialysis and the presence of infiltrates on chest x-ray.[5] Stella and Ryan[37] suggested that the triad of pulmonary oedema, metabolic acidosis and hyperkalaemia are also poor prognostic indicators. Advancing age is also associated with a less favourable prognosis.[5,7]

5.2 Skin Exposure

Skin contact with ready-to-use glyphosate formulations can cause irritation[32] and contact dermatitis has been reported occasionally;[43] these effects are probably due to the preservative, Proxel® (benzisothiazolin-3-one), which is to be phased out in the European Union shortly. Severe skin burns are rare.[44] A 78-year-old woman developed severe chemical burns on her legs, knees and lumbar area after kneeling on ground recently sprayed with a 41% glyphosate, 15% POEA mixture and wearing clothing that had been placed on the same ground 'for some time' prior to being worn. A burning sensation developed in the exposed skin and was followed several hours later by the appearance of erythematous macules that developed into bullae within 24 hours. Complete resolution without scarring occurred after 4 weeks of conventional treatment.

Transfer by contaminated hands to the face led to swelling and paraesthesiae in one case and periorbital oedema in another.[35] The same authors also reported generalised pompholyx in a man who was accidentally drenched with horticultural-strength Roundup®.[35] Although photosensitivity to glyphosate was claimed to have developed in a 64-year-old man,[45] the authors later concluded that the responsible agent was not glyphosate but a co-formulant.[46]

Cutaneous exposure to a glyphosate-containing herbicide (formulation not specified) has been postulated as contributing to Parkinsonism.[47] A previously healthy 54-year-old man was exposed on his trunk, arms, legs and face to glyphosate drift whilst spraying a garden on a windy day. Despite washing the herbicide off some 30 minutes later, he developed conjunctival hyperaemia and a generalised rash 6 hours after exposure. A week later, blisters developed that resolved over 15 days following treatment with oral antihistamines. One month after exposure the patient displayed rigidity of the limbs. A year later he developed a resting tremor of his left arm and also complained of impaired short-term memory. Clinical assessment at this stage confirmed other features of Parkinsonism with paucity of facial expression, global akinesia, rigidity and cogwheeling. Brain magnetic resonance imaging revealed bilateral hyperdense lesions in the globus pallidus and substantia nigra. Clinical improvement ensued with levodopa therapy. The authors proposed that glyphosate may have contributed to the neurological pathology by virtue of its chemical similarity with glycine, a co-factor required for activation of the *N*-methyl-D-aspartate (NMDA) receptor, which controls excitatory actions in the central nervous system and is also involved in memory and learning. However, glyphosate does not possess NMDA activity clinically.

5.3 Inhalation

Inhalation is a minor route of exposure,[13] but spray mist may cause oral or nasal discomfort, an unpleasant taste in the mouth, tingling and throat irritation.

A single case of acute pneumonitis alleged to be due to inhalation of Roundup® in a warm, confined space over a 4-hour period has been reported.[48] Within a few days pharyngeal and laryngeal burns developed. However, the worker involved also used diesel fuel as a cleaning agent over the same period. Whether the features

© 2004 Adis Data Information BV. All rights reserved.

were due to glyphosate including POEA or to some other cause was subsequently debated but not resolved.[49,50]

5.4 Eye Exposure

Eye contact may lead to mild conjunctivitis, and superficial corneal injury is possible if irrigation is delayed or inadequate. One man who accidentally rubbed Roundup® into one eye developed chemosis, palpitations, raised blood pressure, headache and nausea.[35] Permanent eye damage is most unlikely.[51]

6. Management

6.1 Ingestion

Management is symptomatic and supportive. As ingestion of ready-to-use consumer products is unlikely to cause systemic toxicity, gut decontamination is unnecessary. Gastric lavage may be considered if a life-threatening amount of a concentrated glyphosate formulation has been ingested within 1 hour (unless there is evidence of buccal irritation or burns) but there is no evidence that this procedure reduces absorption of either glyphosate or POEA. Alternatively, if there is no buccal irritation or burns, oral activated charcoal, 50–100g for an adult, may be considered.

Hypotension secondary to fluid loss should be treated conventionally with appropriate use of crystalloids, colloids and blood products. Dopamine or dobutamine may be required in severe cases. Early upper alimentary endoscopy should be considered in patients with features suggesting significant gastrointestinal corrosive effects.

Intubation and mechanical ventilation are likely to be required in the most severely poisoned. Significant acidosis that persists despite adequate oxygenation and perfusion should be corrected by intravenous sodium bicarbonate. An electrocardiogram should be performed in all symptomatic cases.

New formulations containing the potassium salt of glyphosate may present a large load of oral potassium. Clinical experience with these formulations is limited. Pending further experience, careful monitoring of potassium concentrations is appropriate following ingestion of potassium salt formulations.

6.2 Skin Exposure

Thorough skin decontamination is the priority with removal of contaminated clothing and washing with soap and water management is otherwise symptomatic and supportive. Severe lesions should be managed as chemical burns.

6.3 Inhalation

Removal from exposure is the priority. Management is otherwise symptomatic and supportive.

6.4 Eye Exposure

Eye contamination should be managed as a chemical exposure with attention particularly to adequate irrigation.

7. Conclusions

The deliberate ingestion of concentrated glyphosate-containing formulations results in severe toxicity and death in some 10–15% of cases, depending on the amount ingested. There is still controversy as to the precise mechanisms of toxicity of the formulations, particularly the role of the surfactant POEA in inducing toxicity. It is unclear also whether non-POEA containing formulations are less (or even more) toxic than POEA-containing formulations.

Acknowledgements

No sources of funding were used to assist in the preparation of this review. The authors have no conflicts of interest that are directly relevant to the content of this review.

References

1. Goldstein DA, Acquavella JF, Mannion RM, et al. An analysis of glyphosate data from the California Environmental Protection Agency Pesticide Illness Surveillance Program. J Toxicol Clin Toxicol 2002; 40: 885-92
2. Litovitz TL, Klein-Schwartz W, Rodgers Jr GC, et al. 2001 Annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 2002; 20: 391-452
3. Watson WA, Litovitz TL, Rodgers Jr GC, et al. 2002 annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 2003; 21: 353-421
4. Watson WA, Litovitz TL, Klein-Schwartz W, et al. 2003 annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 2004; 22: 335-404
5. Lee H-L, Chen K-W, Chi C-H, et al. Clinical presentations and prognostic factors of a glyphosate-surfactant herbicide intoxication: a review of 131 cases. Acad Emerg Med 2000; 7: 906-10
6. Talbot AR, Shiaw M-H, Huang J-S, et al. Acute poisoning with a glyphosate-surfactant herbicide ('Round-up'): a review of 93 cases. Hum Exp Toxicol 1991; 10: 1-8
7. Tominack RL, Yang G-Y, Tsai W-J, et al. Taiwan national poison center survey of glyphosate: surfactant herbicide ingestions. J Toxicol Clin Toxicol 1991; 29: 91-109
8. Sawada Y, Nagai Y. Roundup® poisoning: its clinical observation: possible involvement of surfactant [in Japanese]. J Clin Exp Med 1987; 143: 25-7
9. Sawada Y, Nagai Y, Ueyama M, et al. Probable toxicity of surface-active agent in commercial herbicide containing glyphosate. Lancet 1988; I: 299
10. Steinrücken HC, Amrhein N. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. Biochem Biophys Res Commun 1980; 94: 1207-12
11. Duke SO, Scheffler BE, Boyette D, et al. Herbicides, biotechnology for control of weeds. In: Plimmer JR, Gammon DW, Ragsdale NN. Encyclopedia of agrochemicals. Hoboken (NJ): Wiley-Interscience, 2003: 733-44
12. Sørensen FW, Gregersen M. Rapid lethal intoxication caused by the herbicide glyphosate-trimesium (Touchdown). Hum Exp Toxicol 1999; 18: 735-7
13. International Programme on Chemical Safety. Environmental health criteria 159. Glyphosate. Geneva: World Health Organization, 1994

© 2004 Adis Data Information BV. All rights reserved.

14. Olorunsogo OO, Bababunmi EA, Bassir O. Effect of glyphosate on rat liver mitochondria. Bull Environ Contam Toxicol 1979; 22: 357-64

15. Olorunsogo OO, Bababunmi EA. Inhibition of succinate-linking reduction of pyridine nucleotide in rat liver mitochondria 'in vivo' by *N*-(phosphonomethyl) glycine. Toxicol Lett 1980; 7: 149-52

16. Olorunsogo OO. Inhibition of energy-dependent transhydrogenase reaction by *N*-(phosphonomethyl)glycine in isolated rat liver mitochondria. Toxicol Lett 1982; 10: 91-5

17. Bababunmi EA, Olorunsogo OO, Bassir O. The uncoupling effect of *N*-(phosphonomethyl)glycine on isolated rat liver mitochondria. Biochem Pharmacol 1979; 28: 925-7

18. Hietanen E, Linnainmaa K, Vainio H. Effects of phenoxyherbicides and glyphosate on the hepatic and intestinal biotransformation activities in the rat. Acta Pharmacol Toxicol (Copenh) 1983 Aug; 53 (2): 103-12

19. El Demerdash FM, Yousef MI, Elagamy EI. Influence of paraquat, glyphosate, and cadmium on the activity of some serum enzymes and protein electrophoretic behavior (in vitro). J Environ Sci Health B 2001 Jan; 36 (1): 29-42

20. Williams GM, Kroes R, Munro IC. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul Toxicol Pharmacol 2000; 31: 117-65

21. Adam A, Marzuki A, Rahman HA, et al. The oral and intratracheal toxicities of Roundup and its components to rats. Vet Hum Toxicol 1997; 39: 147-51

22. Baba Y, Takeda M, Yosino K, et al. Acute toxicity of the herbicide 'Roundup' in the rat. Jpn J Toxicol 1989; 2: 397-400

23. Tai T, Yamashita M, Wakimori H. Hemodynamic effects of roundup, glyphosate and surfactant in dogs. Jpn J Toxicol 1990; 3: 63-8

24. Martinez TT, Long WC, Hiller R. Comparison of the toxicology of the herbicide roundup by oral and pulmonary routes of exposure. Proc West Pharmacol Soc 1990; 33: 193-7

25. Martinez TT, Brown K. Oral and pulmonary toxicology of the surfactant used in roundup herbicide. Proc West Pharmacol Soc 1991; 34: 43-6

26. Chan PC, Mahler JF. NTP technical report on the toxicity studies of glyphosate (CAS no. 1071-83-6) administered in dosed feed to F344/N rats and $B6C3F_1$ mice. Toxic Rep Ser 1992; 16: 1-D3

27. Brewster DW, Warren J, Hopkins II WE. Metabolism of glyphosate in Sprague-Dawley rats: tissue distribution, identification, and quantitation of glyphosate-derived materials following a single oral dose. Fundam Appl Toxicol 1991; 17: 43-51

28. Talbot A, Ku TS, Chen CL, et al. Glyphosate levels in acute Roundup herbicide poisoning. 1994 Toxicology World Congress Abstracts. Ann Emerg Med 1995; 26: 717

29. Tominack RL. Glyphosate: contemporary clinical features. J Toxicol Clin Toxicol 1999; 37: 374-5

30. Hori Y, Fujisawa M, Shimada K, et al. Determination of the herbicide glyphosate and its metabolite in biological specimens by gas chromatography-mass spectrometry: a case of poisoning by Roundup® herbicide. J Anal Toxicol 2003; 27: 162-6

31. Wester RC, Melendres J, Sarason R, et al. Glyphosate skin binding, absorption, residual tissue distribution, and skin decontamination. Fundam Appl Toxicol 1991; 16: 725-32

32. Maibach HI. Irritation, sensitization, photoirritation and photosensitization assays with a glyphosate herbicide. Contact Dermatitis 1986; 15: 152-6

33. Franz JE, Mao MK, Sikorski JA. Glyphosate: a unique global herbicide. Washington, DC: American Chemical Society, 1997

34. Acquavella JF, Alexander BH, Mandel JS, et al. Glyphosate biomonitoring for farmers and their families: results from the Farm Family Exposure Study. Environ Health Perspect 2004; 112: 321-6

35. Temple WA, Smith NA. Glyphosate herbicide poisoning experience in New Zealand. N Z Med J 1992; 105: 173-4

36. Chang C-Y, Peng Y-C, Hung D-Z, et al. Clinical impact of upper gastrointestinal tract injuries in glyphosate-surfactant oral intoxication. Hum Exp Toxicol 1999; 18: 475-8

37. Stella J, Ryan M. Glyphosate herbicide formulation: a potentially lethal ingestion. Emerg Med Australas 2004; 16: 235-9

38. Delcenserie R, Yzet T, Duchmann JC, et al. Syndrome pseudo-appendiculaire tardif après intoxication au Roundup. Gastroenterol Clin Biol 1997; 21: 435-7

39. Hung D-Z, Deng J-F, Wu T-C. Laryngeal survey in glyphosate intoxication: a pathophysiological investigation. Hum Exp Toxicol 1997; 16: 596-9

40. Yang C-C, Lin T-J, Ger J, et al. Possible bronchial asthma necessitating prolonged mechanical ventilation in a patient with Roundup poisoning. 1994 Toxicology World Congress Abstracts. Ann Emerg Med 1995; 26: 722-3

41. Menkes D, Temple WA, Edwards IR. Intentional self-poisoning with glyphosate-containing herbicides. Hum Exp Toxicol 1991; 10: 103-7

42. Lin C-M, Lai C-P, Fang T-C, et al. Cardiogenic shock in a patient with glyphosate-surfactant poisoning. J Formos Med Assoc 1999; 98: 698-700

43. Rodríguez A, Echechipia S, O'Laguibel JM, et al. Occupational contact allergy to herbicide glyphosate [abstract]. J Allergy Clin Immunol 1997; 99: S336

44. Amerio P, Motta A, Toto P, et al. Skin toxicity from glyphosate-surfactant formulation. J Toxicol Clin Toxicol 2004; 42: 317-9

45. Hindson C, Diffey B. Phototoxicity of glyphosate in a weedkiller. Contact Dermatitis 1984; 10: 51-2

46. Hindson TC, Diffey BL. Phototoxicity of glyphosate in a weedkiller: a correction. Contact Dermatitis 1984; 10: 260

47. Barbosa ER, Leiros da Costa MD, Bacheschi LA, et al. Parkinsonism after glycine-derivate exposure. Mov Disord 2001; 16: 565-8

48. Pushnoy LA, Avnon LS, Carel RS. Herbicide (Roundup) pneumonitis. Chest 1998; 114: 1769-71

49. Goldstein DA, Johnson G, Farmer DR, et al. Pneumonitis and herbicide exposure. Chest 1999; 116: 1139

50. Carel RS, Pushnoy LA. Pneumonitis and herbicide exposure. Chest 1999; 116: 1139-40

51. Acquavella JF, Weber JA, Cullen MR, et al. Human ocular effects from self-reported exposures to Roundup herbicides. Hum Exp Toxicol 1999; 18: 479-86

Correspondence and offprints: *Sally M. Bradberry*, National Poisons Information Service (Birmingham Centre), City Hospital, Birmingham, B18 7QH, UK.

© 2004 Adis Data Information BV. All rights reserved.

Europ. J. Agronomy 31 (2009) 144–152

Contents lists available at ScienceDirect

European Journal of Agronomy

journal homepage: www.elsevier.com/locate/eja

Glyphosate effects on diseases of plants

G.S. Johal*, D.M. Huber[1]

Department of Botany & Plant Pathology, Purdue University, 915 West State Street, West Lafayette, IN 47907, USA

ARTICLE INFO

Article history:
Received 5 June 2008
Received in revised form 24 February 2009
Accepted 20 April 2009

Keywords:
Glyphosate
Plant defense
Disease
Manganese
Micronutrient
Transgenic
Soil microflora

ABSTRACT

Glyphosate, N-(phosphonomethyl)glycine, is the most extensively used herbicide in the history of agriculture. Weed management programs in glyphosate resistant (GR) field crops have provided highly effective weed control, simplified management decisions, and given cleaner harvested products. However, this relatively simple, broad-spectrum, systemic herbicide can have extensive unintended effects on nutrient efficiency and disease severity, thereby threatening its agricultural sustainability. A significant increase in disease severity associated with the wide spread application of the glyphosate herbicide can be the result of direct glyphosate-induced weakening of plant defenses and increased pathogen population and virulence. Indirect effects of glyphosate on disease predisposition result from immobilization of specific micronutrients involved in disease resistance, reduced growth and vigor of the plant from accumulation of glyphosate in meristematic root, shoot, and reproductive tissues, altered physiological efficiency, or modification of the soil microflora affecting the availability of nutrients involved in physiological disease resistance. Strategies to ameliorate the predisposing effects of glyphosate on disease include judicious selection of herbicide application rates, micronutrient amendment, glyphosate detoxification in meristematic tissues and soil, changes in cultural practices to enhance micronutrient availability for plant uptake, and biological amendment with glyphosate-resistant microbes for nitrogen fixation and nutrient availability. Given that recommended doses of glyphosate are often many times higher than needed to control weeds, we believe the most prudent method to reduce the detrimental effects of glyphosate on GR crops will be to use this herbicide in as small a dose as practically needed. Such a frugal approach will not only curtail disease predisposition of GR crops, but will also benefit the grower and the environment.
© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Changes in agricultural practices such as crop rotation, crop sequence, tillage, and fertility that affect the soil microflora or nutrient availability generally result in changes in disease expression (Datnoff et al., 2007; Englehard, 1989; Huber and Graham, 1999). This is commonly observed for soilborne diseases where only limited innate resistance is available in commercial cultivars so that cultural controls become important management practices to minimize the impact of these diseases. Threatening to make things worse in this regard is the introduction of herbicide-resistant crops (canola, corn, cotton, soybeans, alfalfa, etc.) that are now grown extensively throughout the world. This new trend in agriculture has increased the usage and intensity of specific herbicides while limiting genetic diversity in the specific crops that have been genetically modified.

Herbicides are known to increase specific plant diseases (Altman and Campbell, 1977; Hornby et al., 1998; Mekwatanakarn and Sivasithamparam, 1987), and several are reported to influence micronutrient availability (Evans et al., 2007; Huber et al., 2004, 2005). Micronutrients are the activators or inhibitors of many critical physiological functions. Thus, a deficiency or change in availability of these regulatory elements can greatly affect plant growth and resistance to diseases and pests (Datnoff et al., 2007). The virulence mechanism of some pathogens such as *Gaeumannomyces*, *Magnaporthe*, *Phymatotrichum*, *Corynespora*, and *Streptomyces* involves Mn oxidation at the infection site to compromise the plant's resistance mechanisms involving the shikimate pathway (Thompson and Huber, 2007). Isolates of these pathogens that cannot oxidize physiologically available Mn^{2+} to the non-available Mn^{4+} are avirulent and not able to cause significant tissue damage (Roseman et al., 1991). Production of the Mn oxidizing enzyme(s) occurs soon after spore germination and during epiphytic growth (Cheng, 2005; Schulze et al., 1995; Thompson et al., 2005). Environmental conditions that reduce the availability of micronutrients for plant uptake also predispose plants to disease (Huber and McCay-Buis, 1993; Huber and Graham, 1999; Thompson and Huber, 2007).

* Corresponding author. Tel.: +1 765 494 4448; fax: +1 765 494 0363.
E-mail addresses: gjohal@purdue.edu (G.S. Johal), huberd@purdue.edu (D.M. Huber).
[1] Tel.: +1 765 714 6825.

1161-0301/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.eja.2009.04.004

Table 1
Some diseases increased in glyphosate weed control programs.

Plant	Disease	Pathogen	References
Apple	Canker	*Botryosphaeria dothidea*	Rosenberger and Fargione (2004)
Banana	Panama disease	*Fusarium oxysporum* f. sp. *cubense*	Harper (2007)
Barley	Root rot	*Magnaporthe grisea*	Smiley et al. (1992)
Bean	Anthracnose	*Colletotrichum lindemuthianum*	Johal and Rahe (1984, 1988, 1990)
Bean	Damping off, root rot	*Pythium* spp.	Johal and Rahe (1984)
Bean	Root rot	*Fusarium solani* f. sp. *phaseoli*	Harper (2007)
Bean	Hypocotyl rot	*Phytophthora megasperma*	Keen et al. (1982)
Canola	Crown rot	*Fusarium* spp.	Harper (2007)
Canola	Wilt	*Fusarium oxysporum*	Harper (2007), Large and McLaren (2002)
Citrus	Citrus variegated chlorosis	*Xylella fastidiosa*	Yamada (2006)
Citrus	Crown rot	*Phytophthora* spp.	Yamada (2006)
Cotton	Damping off	*Pythium* spp.	Harper (2007)
Cotton	Bunchy top	Manganese deficiency	Harper (2007)
Cotton	Wilt	*F. oxysporum* f. sp. *vasinfectum*	Harper (2007)
Grape	Black goo	*Phaeomoniella chlamydospora*	Harper (2007)
Melon	Root rot	*Monosporascus cannonbalus*	
Soybeans	Root rot	*Corynespora cassiicola*	Huber et al. (2005)
Soybeans	Target spot	*Corynespora cassiicola*	Huber et al. (2005)
Soybeans	Sudden Death Syndrome	*Fusarium solani* f. sp. *glycines*	Keen et al. (1982)
Soybeans	Root rot	*Phytophthora megasperma*	Keen et al. (1982)
Soybeans	Cyst nematode	*Heterodera glycines*	Geisler et al. (2002), Kremer et al. (2000)
Soybeans	White mold	*Sclerotinia sclerotiorum*	Harper (2007)
Sugar beet	Yellows	*Fusarium oxysporum* f. sp. *betae*	Larson et al. (2006)
Sugar beet	Root rot	*Rhizoctonia solani*	Larson et al. (2006)
Sugarcane	Decline	*Marasmius* spp.	Huber (unpublished)
Tomato	Crown root rot	Fusarium	Bramhall and Higgins (1988)
Tomato	Wilt	*Fusarium oxysporum* f. sp. *pisi*	Harper (2007)
Various	Canker	*Phytophthora* spp.	Harper (2007)
Weeds	Biocontrol	*Myrothecium verrucaria*	Boyette et al. (2006)
Wheat	Bare patch	*Rhizoctonia solani*	Harper (2007)
Wheat	Glume blotch	*Septoria* spp.	Harper (2007)
Wheat	Root rot	*Fusarium* spp.	Fernandez et al. (2005, 2007), Harper (2007)
Wheat	Head scab	*Fusarium graminearum*	Fernandez et al. (2005)
Wheat	Take-all	*Gaeumannomyces graminis*	Hornby et al. (1998)

The herbicide glyphosate, N-(phosphonomethyl)glycine, is a strong systemic metal chelator and was initially patented for that purpose (Bromilow et al., 1993). Its herbicidal action is by chelating with Mn, a cofactor for the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme in the shikimate pathway, to inhibit this metabolic pathway of plants and many microorganisms (Cerdeira and Duke, 2006; Grossbard and Atkinson, 1985; Jaworski, 1972). Many cations chelate with glyphosate, thus reducing its herbicidal efficacy (Bernards et al., 2005; Hickman et al., 2002). Plants with a compromised shikimate metabolism are predisposed to various plant pathogens (Johal and Rahe, 1988; Rahe et al., 1990), and glyphosate is patented as a synergist for mycoherbicides to enhance the virulence and pathogenicity of organisms used for biological weed control (Boyette et al., 2006; Duke and Cerdeira, 2005). The synergistic activity of glyphosate weed control in predisposing plants to infectious organisms has been observed for many diseases (Table 1), and the extensive use of glyphosate in agriculture is a significant factor in the increased severity or "reemergence" of diseases once considered efficiently managed.

The extensive adoption of Roundup Ready® crops such as soybeans, canola, cotton, and corn has intensified the application of glyphosate in these production systems. The applied glyphosate is readily translocated to roots and released throughout the rhizosphere in root exudates of Roundup Ready® plants as well as glyphosate-sensitive plants (Bromilow et al., 1993; Grossbard and Atkinson, 1985). The toxic microbial effects of glyphosate are cumulative with continued use so that Mn deficiency is now observed in areas that were previously considered Mn sufficient because of reduced populations of Mn-reducing soil organisms (Huber, unpublished). The presence of the glyphosate-resistance gene in corn and soybeans also reduces Mn uptake and physiological efficiency (Dodds et al., 2002a,b,c; Gordon, 2006; Reichenberger, 2007). Along with glyphosate-induced Mn deficiency, there has been a gradual recognition of increased disease severity (Harper, 2007; Larson et al., 2006). A few examples are presented to illustrate this relationship.

2. Some diseases increased by glyphosate

2.1. Corynespora root rot of soybean

The damage from Corynespora root rot, previously considered minor, may become economically damaging in Roundup Ready® soybeans since application of glyphosate to Roundup Ready® soybeans greatly increases severity of this disease (Fig. 1). This fungal root rot is more severe when glyphosate is applied to soybeans under weedy conditions even though the weeds may not be hosts for *Corynespora cassiicola*. The weeds serve to translocate and release more glyphosate into the rhizosphere environment to reduce the population of Mn-reducing organisms and increase Mn-oxidizing organisms. This change in soil biology limits manganese availability for plant uptake and active defense reactions, and acts synergistically with *Corynespora* to increase disease (Huber et al., 2005).

2.2. Take-all of cereal crops

The most comprehensive understanding of the interaction of micronutrients influenced by glyphosate and disease is with the take-all disease of cereals. Increased take-all of cereals after a preplant "burn-down" use of glyphosate has been recognized for over 15 years (Hornby et al., 1998). Take-all is also increased when glyphosate is applied to Roundup Ready® soybeans the preceding year compared with the use of a non-glyphosate herbicide (Fig. 2). All of the conditions known to affect Mn availability are inversely related to the severity of take-all (and other diseases, Table 2) so that

Fig. 1. Increased severity of Corynespora root rot after glyphosate application to Roundup Ready® soybeans. Non-inoculated control (left), inoculated plants (center), inoculated plants sprayed with glyphosate (right).

Fig. 2. More severe take-all root rot of wheat grown following Roundup Ready® soybeans sprayed with glyphosate (left) than following Roundup Ready® soybeans grown with a non-glyphosate herbicide (right).

Table 2
Some conditions affecting the form of nitrogen, manganese availability, and severity of take-all, rice blast, potato scab, *Phymatotrichum* root rot, and corn stalk rot (after Thompson and Huber, 2007).

Soil factor or cultural practice	Favored N form (NH_4 vs. NO_3)	Manganese availability	Severity of these diseases
Low soil pH	NH_4	Increase	Decrease
Green manures (some)	NH_4	Increase	Decrease
Ammonium fertilizers	NH_4	Increase	Decrease
Irrigation (some)	NH_4	Increase	Decrease
Firm seed bed	NH_4	Increase	Decrease
Nitrification inhibitors	NH_4	Increase	Decrease
Soil fumigation	NH_4	Increase	Decrease
Metal sulfides	NH_4	Increase	Decrease
High soil pH	NO_3	Decrease	Increase
Lime	NO_3	Decrease	Increase
Nitrate fertilizers	NO_3	Decrease	Increase
Manure	NO_3	Decrease	Increase
Low soil moisture	NO_3	Decrease	Increase
Loose seed bed	NO_3	Decrease	Increase

those conditions that increase the availability of Mn for plant uptake generally reduce take-all, and those that reduce Mn availability increase take-all (Huber and McCay-Buis, 1993). Microorganisms proposed for biological control of this disease such as *Bacillus cereus* and *Trichoderma konigii* are all strong Mn reducers that increase Mn availability in the rhizosphere (Huber and McCay-Buis, 1993; McCay-Buis, 1998; Rengel et al., 1996). In contrast, the addition of Mn-oxidizing organisms increases take-all (Crowley and Rengel, 1999; McCay-Buis, 1998; Rengel, 1999; Thompson et al., 1998). *Gaeumannomyces graminis* is a strong Mn oxidizer in soil and as it grows externally along plant roots (Thompson et al., 2000, 2005). Isolates of *Gaeumannomyces* that cannot oxidize Mn are avirulent, and isolates that oxidize Mn only at certain temperatures are virulent only at temperatures where they can oxidize Mn (Roseman et al., 1991).

Species of *Gramineae* such as rye (*Secale cereale* L.) that are efficient in Mn uptake are resistant to take-all compared with the relatively inefficient, highly susceptible wheat (*Triticum aestivum* L.) (Hornby et al., 1998). In contrast, resistance of oats to take-all is associated with glycocyanide root exudates that are toxic to Mn-oxidizing organisms in the rhizosphere. Oats, as a precrop for wheat, provide effective control of take-all in many areas because of the induced shift in soil biological activity that is less favorable for Mn oxidation. The biological activity favoring Mn availability reduces take-all severity for two or more subsequent wheat crops even though there is little change in the pathogen population (Huber and McCay-Buis, 1993). Glyphosate, in contrast to oats, is toxic to Mn-reducing and N-fixing organisms in soil so that the availability of nitrogen and Mn in soil may be markedly compromised (Huber et al., 2004). Low levels of residual glyphosate in soil also reduce root uptake and translocation of Fe, Mn, and Cu (Eker et al., 2006; Ozturk et al., 2008). Increased take-all root, crown, and foot rot of cereals following glyphosate applications (Hornby et al., 1998; Huber and McCay-Buis, 1993) may be the result of reduced resistance from induced Mn deficiency, inhibited root growth from glyphosate accumulation in root tips, modified virulence of the pathogen, or an increase in synergistic Mn-oxidizing organisms in the rhizosphere.

2.3. Diseases caused by *Xylella fastidiosa*

Various diseases caused by *X. fastidiosa* are referred to as "emerging" or "reemerging" diseases as glyphosate weed management

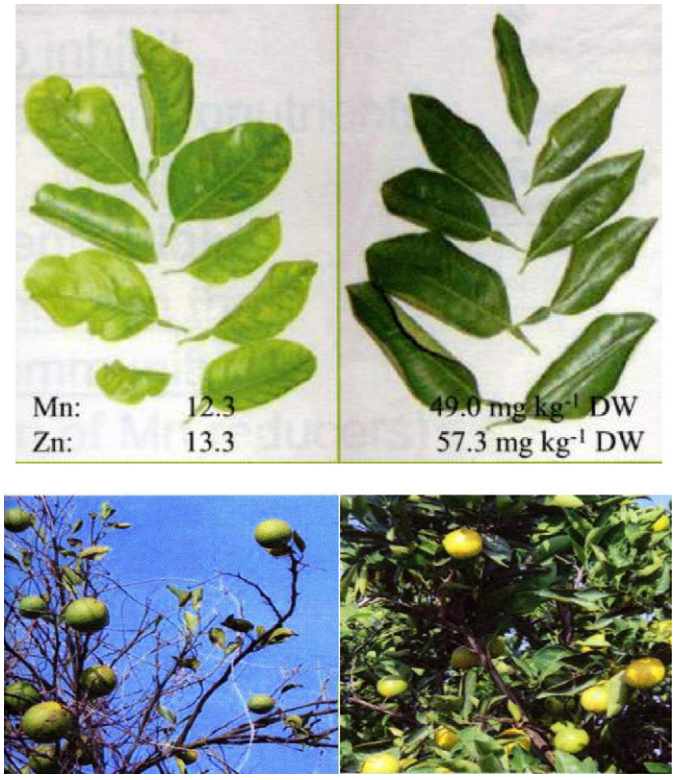


Fig. 3. Expression of citrus variegated chlorosis under glyphosate (left) compared with an alternative mulch (right) weed control program. All trees are infected with the CVC pathogen, *Xylella fastidiosa* (after Yamada, 2006). Left: severe Mn and Zn deficiency, and eventual severe decline in vigor with the glyphosate weed management program. Right: restoration of tissue nutrient levels and productivity of *X. fastidiosa* infected trees under the non-glyphosate mulch system.

programs for their respective crops have intensified. These diseases (Pierce's disease of grapevine, plum scorch, almond scorch, citrus variegated chlorosis, coffee blight, citrus blight, alfalfa dwarf, pecan decline, etc.) are characterized by a loss of vigor, slow decline, micronutrient deficiency, and reduced productivity. The pathogen is an endophytic bacterium that colonizes xylem tissues and restricts nutrient translocation when plants are stressed. Citrus variegated chlorosis (CVC) was first described on oranges in Brazil in 1987 and is also recognized in Puerto Rico. An early symptom of this disease is a variegated chlorosis of foliage (Fig. 3) similar to a deficiency of, and associated with, a drop in tissue levels of Mn and Zn (Li et al., 1996). Normal flushes of new growth are sparse or absent, fruit is small, "skirts" of trees move up, and trees enter a serious decline in growth and productivity. A similar disease referred to as "citrus blight" occurs worldwide and causes the death of several hundred thousand citrus trees annually in the United States (Derrick and Timmer, 2000; Timmer, 2000). Yamada (2006) developed the only known control for CVC, and properly managed trees return to full productivity even though the pathogen may still be present. Control of CVC emphasizes elimination of glyphosate and adoption of an alternative grass mulch weed control program for citrus orchards in Brazil (Yamada and Castro, 2005). This control strategy uses optimally fertilized *Brachiaria* grass grown between the tree rows. The grass is mowed twice a year to provide a 10–15 cm mulch under the citrus trees for weed control and nutrition. Natural mineralization of this mulch inhibits nitrification to provide an ammonium source of nutrition for the citrus trees, Mn and Zn tissue levels are restored to sufficiency levels, and trees in early to mid-decline produce a new flush of growth. Full productivity is restored within a few years. Removing glyphosate from the citrus production system also has significantly reduced the occurrence of *Phytophthora* crown rot.

2.4. Fusarium diseases

Various diseases caused by *Fusarium* spp. are increased by glyphosate (Fernandez et al., 2005; Sanogo et al., 2000, 2001). Glyphosate has made crops susceptible to normally non-pathogenic isolates of *Fusarium*, and the population of *Fusarium* increases in soil after glyphosate application (Levesque et al., 1987; Kremer et al., 2000). Glyphosate predisposes tomato to *Fusarium* crown and root rot by inhibiting the plant's structural and defense barriers (Bramhall and Higgins, 1988). Cotton growers in Australia and the Western United States have seen a resurgence of Fusarium wilt since the introduction of Roundup Ready® cotton, and previously high levels of wilt resistance appear to be less effective under glyphosate management programs (Harper, 2007). Glyphosate also breaks resistance to cyst nematodes in soybeans (Geisler et al., 2002). The increased Fusarium yellows and *Rhizoctonia solani* diseases of Roundup Ready® sugar beets prompted Larson et al. (2006) to comment that "precautions need to be taken when certain soilborne diseases are present if weed management for sugar beet is to include post-emergence glyphosate treatments." These authors also reported that the sugar beet variety resistant to *Rhizoctonia* was as susceptible to this pathogen as the susceptible variety after glyphosate application regardless of the time of inoculation.

Fusarium head scab of cereals and other diseases caused by *Fusarium* spp. increase following glyphosate applications (Fernandez et al., 2005; Larson et al., 2006), and previously established "cardinal" conditions (precipitation, flowering, and temperatures above 26 °C) for head scab are modified when glyphosate is applied prior to a susceptible cereal crop (Fernandez et al., 2005, 2007). Glyphosate modifies plant nitrogen metabolism similar to high temperature-induced changes that provide susceptibility to head scab (Huber, unpublished) so that head scab and the mycotoxins produced by the causal fungi are now prevalent in cooler areas where they were rarely observed before the extensive use of glyphosate (Fernandez et al., 2005, 2007). Similar changes in nitrogen and carbohydrate metabolism provide transient resistance of wheat and soybeans to rust after glyphosate application (Anderson and Kolmer, 2005; Feng et al., 2005, 2007).

The Palouse area of Washington, Idaho, and Oregon in the United States has had a long history of pea, lentil, and wheat production on the deep loess soils characteristic of the area; however, pea and lentil yields have been in slow decline as symbiotic nitrogen fixation is reduced and Fusarium diseases increased commensurate with the extensive use of glyphosate for no-till wheat production. Pea and lentil production are now uneconomical in some areas, and production is rapidly moving from the Palouse to Montana where glyphosate usage has been more limited. The loss of legumes in crop rotations in the Palouse area can result in serious degradation of these once highly productive soils with few economical, alternative crops available as replacements. A new Fusarium wilt of canola caused by *F. oxysporum* and *F. avenaceum* has caused severe yield reductions in nutrient poor soils of Alberta and Saskatchewan, Canada since 2000, but has not yet become a problem in the Mn-rich soils in the Red River valley (Lange and McLaren, 2002).

3. Predisposition to disease underlies the herbicidal efficacy of glyphosate

Inhibition of EPSP synthase initially was considered to be the sole target of glyphosate in plants. It was believed that this mode of action would kill plants by starving them of aromatic amino acids through deregulation of the shikimate pathway (Cerdeira

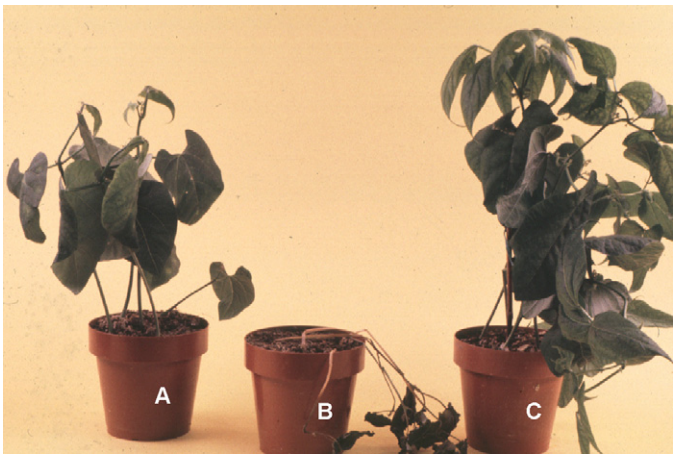


Fig. 4. Fate of glyphosate-treated (10 μg plant^{-1}) bean plants grown in (A) vermiculite and (B) field soil 20 days after glyphosate treatment, and (C) non-glyphosate treated control plants. Glyphosate treated plants in field soil (B) collapsed 10 days after glyphosate treatment from *Pythium* infection.

and Duke, 2006; Grossbard and Atkinson, 1985; Jaworski, 1972). This, however, did not explain some aspects of the death caused by glyphosate. For instance, glyphosate must be translocated to roots to be effective, although growth of the plant stops soon after application of the herbicide. In addition, effects of sublethal doses of this herbicide on perennial plants sometimes appear a year after exposure and persist for two or more years (Rahe et al., 1990). These characteristics of glyphosate-induced injury suggest that the herbicidal action of glyphosate was more than simply the starvation of treated plants of aromatic amino acids as assumed initially (Rahe et al., 1990).

Intrigued by these observations and the possibility that something about the root environment may contribute to the herbicidal action of glyphosate, a systematic research effort was launched in the early 1980s that led to the following findings (Levesque and Rahe, 1992; Rahe et al., 1990):

(1) The herbicidal efficacy of glyphosate is largely due to colonization of roots of affected plants by soil-borne pathogens (Fig. 4) (Johal and Rahe, 1984).
(2) Two pathogens that are most important in this regard are *Pythium*, an oomycete, and *Fusarium*, an ascomycete. Both of these pathogens are ubiquitous in agricultural and other soils.
(3) Plants growing in sterile medium do not die even though their growth is temporarily inhibited by glyphosate.
(4) Amending sterile media with *Pythium* or *Fusarium* restores the ability of glyphosate to kill plants.
(5) Both *Pythium* and *Fusarium* begin to colonize plants within a day or two of glyphosate application to foliar parts of the plant (Fig. 4) (Johal and Rahe, 1984; Levesque et al., 1993).
(6) The amount of glyphosate needed to kill plants in natural soils is much lower than the recommended dose.

These results suggested that glyphosate was somehow compromising the ability of plants to defend against rhizosphere-inhabiting pathogens.

4. Mechanisms of predisposition to disease

Plants rely on multiple components of defense to deter pathogens following infection (Hammond-Kosack and Jones, 2000). Many of these active resistance components are derived from the phenylpropanoid pathway, which acquires almost all of its precursors (notably phenylalanine and chorismate) from the shikimic acid pathway (Hammond-Kosack and Jones, 2000; Dixon et al., 2002). A key inducible defense component associated with the shikimic acid pathway is the production of antimicrobial phytoalexins that accumulate rapidly at the site of infection. Lignification of cell walls at and around the infection site is another shikimate-derived component that functions to fortify cells and ensure isolation of the pathogen at the infection site. Production of salicylic acid (SA) following infection represents another component of inducible defense. SA is synthesized either directly from chorismic acid or indirectly through phenylalanine. Although SA is not antimicrobial per se, it functions to signal and coordinate various defenses following challenge by a pathogen; however, its direct role in plant–pathogen interactions involving root tissue remains unclear. Another defense component that relies on three final products of the shikimic acid pathway – tryptophan, tyrosine and phenylalanine – is the production of a diverse variety of pathogenesis-related (PR) proteins that function to curtail the advance of a pathogen. Many kinds of PR proteins have been identified (Hammond-Kosack and Jones, 2000).

Given the reliance of many plant defenses on the shikimic acid pathway, and the fact that glyphosate blocks this pathway, it is not surprising that this herbicide would render plants more susceptible to pathogens. Keen et al. (1982) were the first to show that by inhibiting the phytoalexin glyceollin, glyphosate was able to compromise resistance of soybeans to *Phytophthora megasperma* f. sp. *glycines*. Using the bean-anthracnose pathosystem, Johal and Rahe (1988, 1990) demonstrated that, while glyphosate did not interfere with the hypersensitive reaction (HR) of incompatible interactions, it suppressed significantly the production of all four of the bean phytoalexins. As a result, the pathogen was able to kill the plant if it escaped the localized HR, a situation that occurred only with glyphosate-treated plants (Fig. 5). The effect of glyphosate on the compatible bean anthracnose interaction was even more dramatic (Johal and Rahe, 1990). Glyphosate almost completely suppressed the production of phytoalexins associated with susceptible lesion containment and permitted the pathogen to invade unimpededly until the entire hypocotyl collapsed (Figs. 6 and 7). As little as 2% of the recommended herbicidal rate of glyphosate was enough to transform normally delimited lesions typical of anthracnose into constantly expanding lesions (Johal and Rahe, 1990).

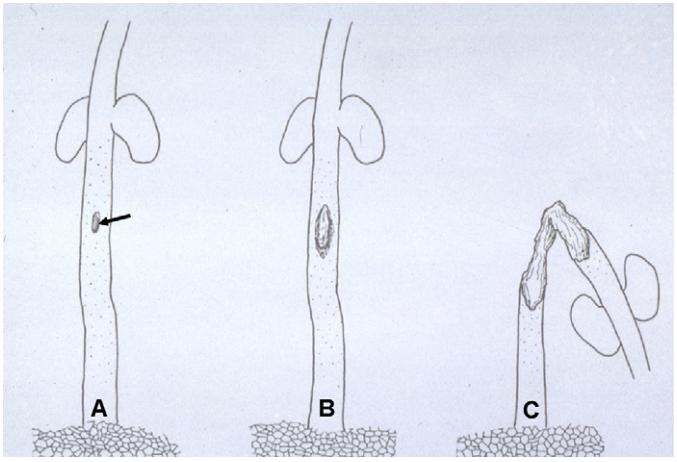


Fig. 5. Diagramatic representation of glyphosate-treated bean seedlings following inoculation with an incompatible race of *Colletotrichum lindemuthianum*. (A) Dots all over the hypocotyl represent hypersensitive reaction (HR) sites (cells) incited by the pathogen on spray inoculation. Arrow indicates the site where a drop of glyphosate (10 μg) was placed. (B) The fungus normally contained inside HR cells sometimes escapes near the glyphosate treatment site and results in a susceptible lesion (arrow). (C) The lesion continues to expand to kill the plant after glyphosate treatment.

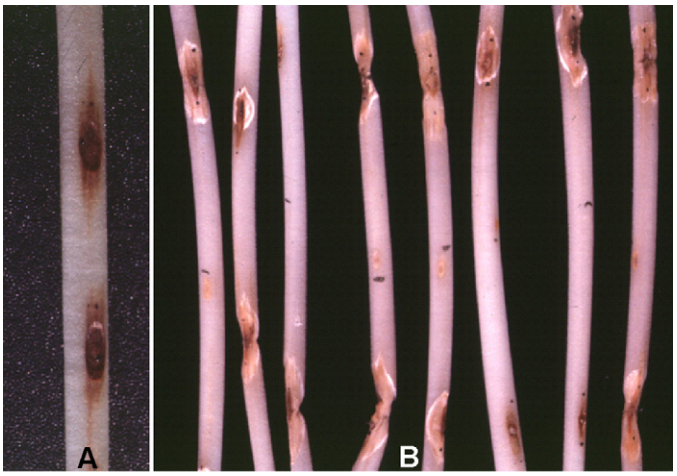


Fig. 6. Anthracnose lesions on bean hypocotyls in the (A) absence of glyphosate and (B) presence of glyphosate. A 10 μg drop of glyphosate (black dot) was placed near the center of the hypocotyls one day after inoculation with *C. lindemuthianum* in (B). Note the loss of lesion delimitation and collapse of tissue seven days after glyphosate treatment.

The defense studies mentioned above were confined largely to diseases of aerial parts of host plants. There are indications that defense components may vary significantly in root tissue that are in intimate and continuous contact with potential pathogens (Hammond-Kosack and Jones, 2000). For instance, roots do not rely on HR-mediated defense to contend with pathogens, although the exact defense components that keep roots pathogen-free are only partially understood. To gain an insight into what contributes to glyphosate-induced susceptibility of French beans (*Phaseolus vulgaris*) to *Pythium*, Liu et al. (1995, 1997) assessed phytoalexins as well as lignification of root tissue in response to glyphosate treatment. By comparing phytoalexins in roots of bean seedlings grown in different media, they concluded that phytoalexins were induced by soil microorganisms. Interestingly, while phytoalexin accumulation was affected only modestly by glyphosate in response to exposure to *Pythium*, lignification (a process requiring Mn) was suppressed significantly. Thus, enhanced colonization by *Pythium* in roots of bean seedlings treated with foliar applied glyphosate occurs as a result of glyphosate interference with lignin-based defense mechanisms (Liu et al., 1997). However, these results also suggest that sustained production of phytoalexins in response to *Pythium* infection is maintained temporarily following glyphosate treatment, whereas lignification is not.

5. Roundup Ready® plants and disease predisposition

Given that the herbicidal activity of glyphosate is mediated largely by its ability to lower plant immunity to pathogens, the status of Roundup Ready® plants with regard to such predisposition following glyphosate treatment becomes a serious consideration. For reasons that were not explained, Cerdeira and Duke (2006) contended that reduced resistance to pathogens in response to glyphosate treatment should not occur in Roundup Ready® plants. This is a misconception that can hold true only if the Roundup Ready® transgene following glyphosate treatment operates and behaves in exactly the same manner as the native EPSP synthase gene does in the absence of glyphosate. Such a scenario is possible only if the Roundup Ready® transgene is completely insensitive to glyphosate and is also as efficacious as the native EPSP synthase gene is in the absence of glyphosate. In addition, the Roundup Ready® gene has to match exactly the transcriptional activity of the native gene in every tissue of the plant and under all conditions, both normal and stressful. This is a tall order of requirements that is unlikely to be fulfilled by the present day Roundup Ready® transgenics, thus making it highly probable that our Roundup Ready® crops are vulnerable to glyphosate toxicity under at least some conditions. One such condition could arise when the level of glyphosate exceeds the ability of the transgenic enzyme to tolerate it, and yet another may develop if the transgene fails to match the transcriptional activity and profile of the native gene under conditions of biotic stress. Both of these scenarios are possible and, if they develop, it is very likely they would enhance the vulnerability of Roundup Ready® plants to fungal diseases following Roundup application.

Glyphosate treatment of transgenic crops to manage weeds can also promote disease damage indirectly by impacting the inoculum potential of pathogens. Shortly after soilborne fungi's causative role was revealed in the herbicidal efficacy of glyphosate (Johal and Rahe, 1984), Levesque et al. (1987) documented a significant, albeit temporary, spike in the level of fungal pathogens in the rhizosphere following glyphosate application to weeds. This prompted the speculation that such a buildup of pathogen load could have ill effects for subsequent crop plants. This, indeed, was found to be the case in barley fields in which significant yield reductions were witnessed if the crop was planted within a few days after glyphosate application (Smiley et al., 1992). Although the latter study was conducted on non Roundup Ready® barley, it is likely that a similar boost in the inoculum potential of pathogens in the rhizosphere (also called 'green bridge') could lead to enhanced root rot problems in Roundup Ready® crops as well.

A prudent way to avoid disease enhancement is to decrease the concentration of glyphosate applied to Roundup Ready® crops. Many studies have documented that the levels of glyphosate necessary to kill or compromise the health of many weeds are several fold lower than the generally recommended application rates (Rahe et al., 1990). An alternative to using insensitive EPSP synthase genes to generate glyphosate-resistant plants might be to use genes that degrade glyphosate. Three such genes which inactivate glyphosate by oxidation (the *Gox* gene), acetylation (the *Gat* gene) or decarboxylation (the *Gdc* gene) have become available in recent years (Cerdeira and Duke, 2006; Dill, 2005). If the problem persists, there is also the possibility of stacking a resistant EPSP synthase gene with a glyphosate metabolism gene as has been done in canola (Dill, 2005). A major disadvantage of this strategy is that it may encourage the application of higher levels of glyphosate than needed. In turn, this would not only impact the environment negatively but also would hasten the evolution of resistant weeds and thereby further threaten sustainability of this herbicide.

6. Strategies to ameliorate glyphosate predisposition to disease

Several strategies may be deployed to reduce glyphosate-induced predisposition to disease. These strategies primarily focus

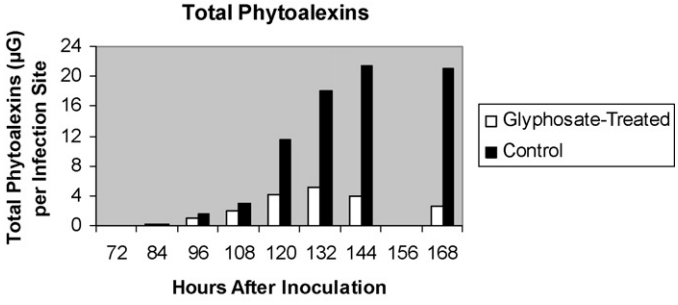


Fig. 7. Glyphosate suppression of phytoalexins in compatible bean anthracnose lesions by 10 μg glyphosate (after Johal and Rahe, 1990).

on four aspects of the glyphosate-disease-environment interaction, i.e.:

(1) minimizing non-target exposure to glyphosate by limiting the rates of glyphosate used,
(2) enhancing micronutrient sufficiency to maintain optimum plant physiological function and resistance,
(3) detoxifying accumulated glyphosate in root tips and other meristematic tissues to restore growth potential, and
(4) moderating glyphosate toxicity to rhizosphere microbes or restoring critical microbial components damaged by glyphosate released in root exudates.

6.1. Minimizing non-target exposure to glyphosate by limiting the rates of glyphosate used

As stated earlier, the rates of glyphosate generally recommended for herbicide use are far in excess of the amount required to kill most weeds. Excess application has occurred primarily as a result of advertising promotions, ease of application, increasing weed resistance, low cost of the product, and apathy towards the extensive non-target environmental effects of glyphosate. Very low levels of residual glyphosate in soil can greatly impede the availability and uptake of Mn, Fe, Cu, and Zn with subsequent translocation to vegetative tissues also impeded (Eker et al., 2006; Ozturk et al., 2008). This limitation in uptake and translocation can greatly impede the "replenishing" of these critical micronutrients and restoration of physiological resistance mechanisms dependent on them after nutrient immobilization in tissues by the applied glyphosate. A more judicious use of glyphosate would appear essential to maintain sustainable crop production efficiency.

6.2. Enhancing micronutrient sufficiency to maintain optimum plant physiological function and resistance

Most micronutrients are readily absorbed after foliar application, a common method of fertilization; however, some micronutrients, such as Mn, are relatively immobile and are not basipitally translocated to roots where soilborne root, hypocotyl, crown and vascular pathogens are established (Marschner, 1995; Thompson and Huber, 2007). Thus, although foliar application of Mn can provide nutrient sufficiency to foliar tissues for this essential element, it would not be effective in detoxifying accumulated glyphosate in root tip meristematic tissues or maintaining physiological resistance dependent on the shikimate pathway in root tissues because it is relatively immobile in the plant and does not move downward in the phloem.

A combination of foliar applied Mn with more mobile elements such as Cu or Zn could be more effective in detoxifying glyphosate in root tissues than Mn alone. Difficulties in meeting plant needs for Mn are further compounded since soil-applied Mn can be readily oxidized by soil organisms to the Mn^{4+} form that is not available for plant uptake (Marschner, 1995; Thompson and Huber, 2007). Reduced physiological efficiency of Roundup Ready® crops (Dodds et al., 2002a,b,c; Gordon, 2006; Zobiole et al., 2009) require higher levels of Mn to achieve nutrient sufficiency and comparable productivity as their non-genetically modified isolines (Reichenberger, 2007). Rates of Mn applied to Roundup Ready® soybeans required for comparable yield with non-RR soybean approached toxicity when applied to the isogenic non-Roundup Ready® soybean (Gordon, 2006; Reichenberger, 2007). The simultaneous application of many nutrients with glyphosate ("tank mixes") results in their immobilization and non-availability for plant physiological functions. Full physiological efficiency from nutrient application may not be achieved unless the micronutrients are applied eight to fifteen days after the glyphosate is applied. This is necessary to prevent chelation and immobilization by residual glyphosate in tissues that renders them physiologically unavailable (Huber et al., 2004; Severson, 2006), although earlier applications may be more effective in detoxifying tissue-bound glyphosate.

6.3. Detoxifying accumulated glyphosate in meristematic tissues

Reduced root growth from the accumulation of glyphosate in root tips results in less contact of the roots with dispersed nutrients in the soil profile and may negate tolerance of plants to soilborne pathogens based on their ability to "outgrow" the damage caused from loss of root tissue. Likewise, glyphosate accumulates in active meristematic tissue in shoots and developing fruits to inhibit growth of these tissues. Calcium, Mg, and micronutrients that chelate with glyphosate can reduce its biological activity and restore some of the potential physiological activity in these tissues. These "detoxifying" elements can come from within the plant or from further uptake from the soil. Thus, it is important to maintain mineral sufficiency in plant tissues and their ready availability in soil for plant uptake. This may be achieved by soil or foliar applied nutrients (Bernards et al., 2005; Huber et al., 2004; Reichenberger, 2007) if other environmental restraints are considered.

6.4. Eliminating glyphosate toxicity to rhizosphere microbes or restoring critical microbial components damaged by glyphosate released in root exudates

Detoxifying glyphosate in root exudates may occur in highly calcareous soils or soils with high levels of soluble metal nutrients through chelation to reduce its impact on soil organisms. Toxicity of glyphosate to Mn-reducing and synergistic nitrogen-fixing organisms in the rhizosphere can have serious consequences for sustainability of legume production. Regular inoculation of legume crops with synergistic nitrogen-fixing organisms may be required in many areas for maximal productivity where extended applications of glyphosate have eliminated them from the soil profile. Development of glyphosate-tolerant nitrogen-fixing and Mn-reducing organisms would be beneficial in many of these situations, and especially for perennial Roundup Ready® legume crops such as alfalfa.

7. Summary

Extended use of glyphosate can significantly increase the severity of various diseases by impacting all four of the interacting components of the "plant disease diamond" comprised of the plant, abiotic and biotic environments, and pathogen (Fig. 8). Reduced growth, impaired defenses, impaired uptake and translocation of nutrients, and altered physiology of plants by glyphosate can affect susceptibility or tolerance to various diseases. Glyphosate chelation of nutrients in the plant and soil can render those nutrients immobile and unavailable for plant use or uptake, while toxicity to essential synergistic and beneficial soil organisms (Purcell, 2001) further reduces availability of nutrients that are critical for a plant's physiological defense to disease. Glyphosate stimulation of fungal growth and enhanced virulence of pathogens such as *Fusarium*, *Gaeumannomyces*, *Phytophthora*, *Pythium*, and *Xylella* can have serious consequences for sustainable production of a wide range of susceptible crops and lead to the functional loss of genetic resistance that is dependent on metabolites through the shikimate pathway (Larson et al., 2006). Nutrient balance is important because each element functions as part of a delicately balanced, interdependent physiological system with the plant's genetics and the environment. Maximal utilization of cultural and management practices that increase the availability of nutrients (Table 2) to negate the deleterious effects of glyphosate should be incorporated

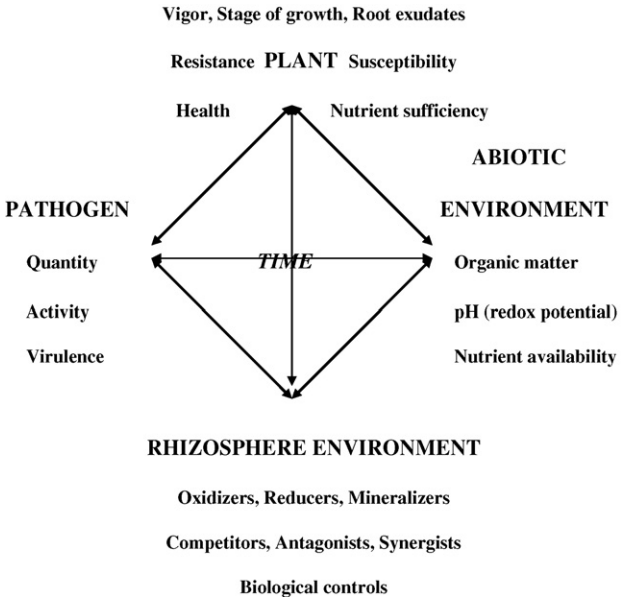


Fig. 8. The four primary interacting factors influencing nutrient availability and disease that are affected by glyphosate.

into crop production programs to facilitate optimal production efficiency and sustainable disease control. It is important to understand the effect of glyphosate on the chemical and biological properties of soils and its overall effects on the agricultural production system to permit its judicious use. Ignoring potential non-target detrimental side effects of any chemical, especially used as heavily as glyphosate, may have dire consequences for agriculture such as rendering soils infertile, crops non-productive, and plants less nutritious (Altman and Campbell, 1977). To do otherwise might well compromise not only agricultural sustainability, but also the health and well-being of animals and humans (Ozturk et al., 2008).

Acknowledgements

We are grateful to Dr. Larry Dunkle for providing a critical review of the manuscript, and to Lynn Johal for editorial comments.

References

Altman, J., Campbell, C.L., 1977. Effect of herbicides on plant diseases. Annu. Rev. Phytopathol. 15, 361–385.
Anderson, J.A., Kolmer, J.A., 2005. Rust control in glyphosate tolerant wheat following the application of the herbicide glyphosate. Plant Dis. 88, 1136–1142.
Bernards, M.L., Thelen, K.D., Muthukumaran, R.J., McCracken, J.L., 2005. Glyphosate interaction with manganese in tank mixtures and its effect on glyphosate absorption and translocation. Weed Sci. 53, 787–794.
Boyette, C.D., Reddy, K.N., Hoagland, R.E., 2006. Glyphosate and bioherbicide interaction for controlling kudzu (*Pueraria lobata*), redvine (*Brunnichia ovata*), and trumpetcreeper (*Campsis radicans*). Biocontrol Sci. Tech. 16, 1067–1077.
Bramhall, R.A., Higgins, V.J., 1988. The effect of glyphosate on resistance of tomato to Fusarium crown and root rot disease and on the formation of host structural defensive barriers. Can. J. Bot. 66, 1547–1555.
Bromilow, R.H., Chamberlain, K., Tench, A.J., Williams, R.H., 1993. Phloem translocation of strong acids – glyphosate, substituted phosphonic, and sulfonic acids – in *Ricinus communis* L. Pestic. Sci. 37, 39–47.
Cerdeira, A.L., Duke, S.O., 2006. The current status and environmental impacts of glyphosate-resistant crops: a review. J. Environ. Qual. 35, 1633–1658.
Cheng, M.-W., 2005. Manganese transition states during infection and early pathogenesis in rice blast. M.S. Thesis. Purdue University, West Lafayette, Ind.
Crowley, D.E., Rengel, Z., 1999. Biology and chemistry of nutrient availability in the rhizosphere. In: Rengel, Z. (Ed.), Mineral Nutrition of Crops: Fundamental Mechanisms and Implications. Food Products Press, London, pp. 1–40 (Chapter 1).
Datnoff, L.E., Elmer, W.H., Huber, D.M., 2007. Mineral Nutrition and Plant Disease. APS Press, St. Paul, MN.
Derrick, K.S., Timmer, L.W., 2000. Citrus blight and other diseases of recalcitrant etiology. Annu. Rev. Phytopathol. 38, 181–205.
Dill, G., 2005. Glyphosate resistant crops: history, status and future. Pest Manag. Sci. 61, 219–224.
Dixon, R.A., Achnine, L., Kota, P., Liu, C.-J., Reddy, M.S., Wang, L., 2002. The phenylpropanoid pathway and plant defense—a genomics perspective. Mol. Plant Pathol. 3, 371–390.
Dodds, D.M., Hickman, M.V., Huber, D.M., 2002a. Micronutrient uptake by isogenic glyphosate tolerant and normal corn. Proc. Proc. Weed Sci. Soc. Am. 42, 2.
Dodds, D.M., Huber, D.M., Hickman, M.V., 2002b. Micronutrient levels in normal and glyphosate-resistant soybeans. Proc. NC-Weed Sci. Soc. Am. 57, 107.
Dodds, D.M., Huber, D.M., Hickman, M.V., Shaw, D.R., 2002c. Hybrid and glyphosate application effects on nutrient uptake in corn. Proc. Weed Sci. Soc. Am. 43, 4.
Duke, S.O., Cerdeira, L., 2005. Potential environmental impacts of herbicide-resistant crops. Collect. Biosaf. Rev. 2, 66–143.
Eker, S., Ozturk, L., Yazici, A., Erenoglu, B., Romheld, V., Cakmak, I., 2006. Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (*Helianthus annuus* L.) plants. J. Agric. Food Chem. 54, 10019–10025.
Englehard, A.W. (Ed.), 1989. Management of Diseases with Macro and Microelements. American Phytopathological Society, St. Paul, MN.
Evans, I.R., Solberg, E., Huber, D.M., 2007. Copper and plant disease. In: Datnoff, L.E., Elmer, W.H., Huber, D.M. (Eds.), Mineral Nutrition and Plant Disease. APS Press, St. Paul, MN, pp. 177–188 (Chapter 12).
Feng, P.C.C., Baley, J., Clinton, W.P., Bunkers, G.J., Alibhai, M.F., Paulitz, T.C., Kidwell, K.K., 2005. Glyphosate inhibits rust diseases in glyphosate-resistant wheat and soybean. Proc. Natl. Acad. Sci. U.S.A. 102, 17290–17295.
Feng, P.C.C., Clark, C., Andrade, G.C., Baibl, M.C., Caldwell, P., 2007. The control of Asian rust by glyphosate in glyphosate-resistant soybeans. Pest Manag. Sci. 63, 1526.
Fernandez, M.R., Selles, F., Gehl, D., DePauw, R.M., Zentner, R.P., 2005. Crop production factors associated with Fusarium head blight in spring wheat in eastern Saskatchewan. Crop Sci. 45, 1908–1916.
Fernandez, M.R., Zentner, R.P., DePauw, R.M., Gehl, D.T., Stevenson, F.C., 2007. Impacts of crop production factors on Fusarium head blight in barley in eastern Saskatchewan. Crop Sci. 47, 1574–1584.
Geisler, L., Graef, G., Wilson, J., Schimelfenig, J., 2002. Interaction of glyphosate tolerance with soybean cyst nematode resistance. Phytopathology 92, S529.
Gordon, B., 2006. Manganese nutrition of glyphosate-resistant and conventional soybeans. Better Crops 91, 12–13.
Grossbard, E., Atkinson, D., 1985. The Herbicide Glyphosate. Butterworths, London.
Hammond-Kosack, K., Jones, J.D.G., 2000. Responses to plant pathogens. In: Buchanan, B.B., Gruissem, W., Jones, R.L. (Eds.), Biochemistry and Molecular Biology of Plants. ASPP, Rockville, MD, pp. 1102–1156.
Harper, M., 2007. The Review of the Moratorium on GM Canola. Australia. http://www.dpi.vic.gov.au/dpi/nrenfa.nsf/LinkView/5477226A88881F86CA-2572E300074EEF89E6C67B4668BD2A7CA256FB70001BAB8.
Hickman, M.V., Dodds, D.M., Huber, D.M., 2002. Micronutrient interactions reduce glyphosate efficacy on tall fescue. Proc. Weed Sci. Soc. Am. 42, 18.
Hornby, D., Bateman, G.L., Gutteridge, R.J., Lucas, P., Osbourn, A.E., Ward, E., Yarham, D.J., 1998. Take-all Disease of Cereals: A Regional Perspective. CAB International, Wallingford, UK.
Huber, D.M., McCay-Buis, T.S., 1993. A multiple component analysis of the take-all disease of cereals. Plant Dis. 77, 437–447.
Huber, D.M., Graham, R.D., 1999. The role of nutrition in crop resistance and tolerance to diseases. In: Rengel, Z. (Ed.), Mineral Nutrition of Crops: Fundamental Mechanisms and Implications. Food Products Press, London, pp. 169–204.
Huber, D.M., Leuck, J.D., Smith, W.C., Christmas, E.P., 2004. Induced manganese deficiency in GM soybeans. In: Northcentral Fert. Extension Conf., Des Moines, IA, November 2004.
Huber, D.M., Cheng, M.W., Winsor, B.A., 2005. Association of severe Corynespora root rot of soybean with glyphosate-killed giant ragweed. Phytopathology 95, S45.
Jaworski, E.G., 1972. Mode of action of N-phosphonomethyl-glycine: inhibition of aromatic amino acid biosynthesis. J. Agric. Food Chem. 20, 1195–1198.
Johal, G.S., Rahe, J.E., 1984. Effect of soilborne plant-pathogenic fungi on the herbicidal action of glyphosate on bean seedlings. Phytopathology 74, 950–955.
Johal, G.S., Rahe, J.E., 1988. Glyphosate, hypersensitivity and phytoalexin accumulation in the incompatible bean anthracnose host–parasite interaction. Physiol. Mol. Plant Pathol. 32, 267–281.
Johal, G.S., Rahe, J.E., 1990. Role of phytoalexins in the suppression of resistance of *Phaseolus vulgaris* to *Colletotrichum lindemuthianum* by glyphosate. Can. J. Plant Pathol. 12, 225–235.
Keen, N.T., Holliday, M.J., Yoshikawa, M., 1982. Effects of glyphosate on glyceollin production and the expression of resistance to *Phytophthora megasperma* f. sp. *glycinea* in soybean. Phytopathology 72, 1467–1470.
Kremer, R.J., Donald, P.A., Keaster, A.J., Minor, H.C., 2000. Herbicide impact on *Fusarium* spp. and soybean cyst nematode in glyphosate-tolerant soybean. Agron. Abstr., p257.
Lange, R., McLaren, D., 2002. Fusarium Wilt—A New Disease of Canola. http://www.umanitoba.ca/afs/agronomists conf/2002/pdf/lange.pdf.
Larson, R.L., Hill, A.L., Fenwick, A., Kniss, A.R., Hanson, L.E., Miller, S.D., 2006. Influence of glyphosate on Rhizoctonia and Fusarium root rot in sugar beet. Pest Manag. Sci. 62, 182–192.
Levesque, C.A., Rahe, J.E., et al., 1987. Effects of glyphosate on *Fusarium* spp.: its influence on root colonization of weeds, propagule density in the soil, and on crop emergence. Can. J. Microbiol. 33, 354–360.
Levesque, C.A., Rahe, J.E., 1992. Herbicide interactions with fungal root pathogens, with special reference to glyphosate. Annu. Rev. Phytopathol. 30, 579–602.

Levesque, C.A., Rahe, J.E., Eaves, D.M., 1993. Fungal colonization of glyphosate treated seedlings using a new root plating technique. Mycol. Res. 97, 299–306.
Li, W.B., Donadio, L.C., Sempionato, O.R., Miranda, V.S., 1996. Resistance or tolerance of citrus species and cultivars to citrus variegated chlorosis. Proc. Int. Soc. Citricult. 1, 216–218.
Liu, L., Punja, Z.K., Rahe, J.E., 1995. Effect of *Pythium* spp. and glyphosate on phytoalexin production and exudation by bean (*Phaseolus vulgaris* L.) roots grown in different media. Physiol. Mol. Plant Pathol. 47, 391–405.
Liu, L., Punja, Z.K., Rahe, J.E., 1997. Altered root exudation and suppression of induced lignification as mechanisms of predisposition by glyphosate of bean roots (*Phaseolus vulgaris* L.) to colonization by *Pythium* spp. Physiol. Mol. Plant Pathol. 51, 111–127.
Marschner, H., 1995. Mineral Nutrition of Higher Plants. In: second ed. Academic Press, London, 889 pp.
McCay-Buis, T.S., 1998. Ramifications of microbial interactions conditioning take-all of wheat. Ph.D. Thesis. Purdue University, West Lafayette, Indiana.
Mekwatanakarn, P., Sivasithamparam, K., 1987. Effect of certain herbicides on soil microbial populations and their influence on saprophytic growth in soil and pathogenicity of the take-all fungus. Biol. Fertil. Soils 5, 175–180.
Ozturk, L., Yazici, A., Eker, S., Gokmen, O., Romheld, V., Cakmak, I., 2008. Glyphosate inhibition of ferric reductase activity in iron deficient sunflower roots. New Phytol. 177, 899–906.
Purcell, L.C., 2001. Physiological determinants of soybean yield limitations. USDA-CRIS Accession No.: 0164131; project No.: ARK01559, Univ. Arkansas, Fayetteville.
Rahe, J.E., Levesque, C.A., Johal, G.S., 1990. Synergistic role of soil fungi in the herbicidal efficacy of glyphosate. In: Hoagland, R.E. (Ed.), Biological Weed Control Using Microbes and Microbial Products as Herbicides. Symposium, April 9–14, 1989. American Chemical Society, Washington, DC, pp. 260–275.
Reichenberger, L., 2007. Missing micronutrients: using glyphosate is complicating the uptake of some minor nutrients. In: The Furrow, pp. 22–23.
Rengel, Z. (Ed.), 1999. Mineral Nutrition of Crops: Fundamental Mechanisms and Implications. Food Products Press, London.
Rengel, Z., Gutteridge, R., Hirsch, P., Hornby, D., 1996. Plant genotype, micronutrient fertilization and take-all infection influence bacterial populations in the rhizosphere of wheat. Plant Soil 183, 269–277.
Roseman, T.S., Graham, R.D., Arnott, H.J., Huber, D.M., 1991. The interaction of temperature with virulence and manganese oxidizing potential in the epidemiology of *Gaeumannomyces graminis*. Phytopathology 81, S1215.
Rosenberger, D., Fargione, M., 2004. Apple Pest Report 12, 6. University of Maine Cooperative Extension Service.
Sanogo, S., Yang, X.B., Scherm, H., 2000. Effects of herbicides on *Fusarium solani* f. sp. *glycines* and development of sudden death syndrome in glyphosate-tolerant soybean. Phytopathology 90, 57–66.
Sanogo, S., Yang, X.B., Lundeen, P., 2001. Field response of glyphosate-tolerant soybean to herbicides and sudden death syndrome. Plant Dis. 85, 773–779.
Schulze, D.G., McCay-Buis, T.S., Sutton, S.R., Huber, D.M., 1995. Manganese oxidation states in *Gaeumannomyces*-infested wheat rhizospheres probed by micro-XANES spectroscopy. Phytopathology 85, 990–994.
Severson, R., 2006. Influence of Roundup Herbicide on Manganese Nutrition of Soybean. http://www.nwroc.umn.edu/Cropping_Issues/NW_Crop_trials/2005/Sybn_glyph_+_manganese.pdf.
Smiley, R.W., Ogg, A.G., Cook, R.J., 1992. Influence of glyphosate on *Rhizoctonia* root rot, growth, and yield of barley. Plant Dis. 76, 937–942.
Thompson, I.A., Huber, D.M., 2007. Manganese and plant disease. In: Datnoff, L.E., Elmer, W.H., Huber, D.M. (Eds.), Mineral Nutrition and Plant Disease. APS Press, St. Paul, MN, pp. 139–153 (Chapter 10).
Thompson, I.A., Guest, C.A., Schulze, D.G., Huber, D.M., 1998. Manganese reduction and uptake in wheat rhizospheres as influenced by manganese reducing and oxidizing bacteria. Phytopathology 88, S118.
Thompson, I.A., Huber, D.M., Schulze, D.G., 2000. *In situ* oxidation and accumulation of manganese by the causal agent of the take-all disease on wheat (*Gaeumannomyces graminis*). Phytopathology 90, S77.
Thompson, I.A., Huber, D.M., Guest, C.A., Sculze, D.G., 2005. Fungal manganese oxidation in a reduced soil. Environ. Microbiol. 7, 1480–1487.
Timmer, L.W., 2000. Blight. In: Compendium of Citrus Diseases. APS Press, St. Paul, MN, pp. 66–67.
Yamada, T., 2006. Informações Agronomicas 116. IPNI-International Plant Nutrition Institute, Piracicaba-SP, Brazil. http://www.ipni.org.br/ppiweb/pbrazil.nsf/$webindex/article=93F35608032570B5004E419AD0187284!opendocument.
Yamada, T., Castro, P.R., 2005. Glifosato, herbicida com singular modo de ação: Efeitos secundários e implicações fisiológicas e agronômicas. http://www.ipni.org.br/ppiweb/pbrazil.nsf/$webcontentsbydate!OpenView&Start=1&Count=60&Expand=1.1#1.1.
Zobiole, L.H.S., deOliveira, R.S., Huber, D.M., Constantin, J., Castro, C, deOliveira, F.A., de Oliveira, A., 2009. Glyphosate reduces shoot concentrations of mineral nutrients in glyphosate-resistant soybeans. Plant Soil (In Press).

Science of the Total Environment 616–617 (2018) 255–268

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

Environmental and health effects of the herbicide glyphosate

Van Bruggen, A.H.C. [a,b,*], He, M.M. [a,c], Shin, K. [a,b], Mai, V. [a], Jeong, K.C. [a], Finckh, M.R. [d], Morris, J.G. Jr. [a,e]

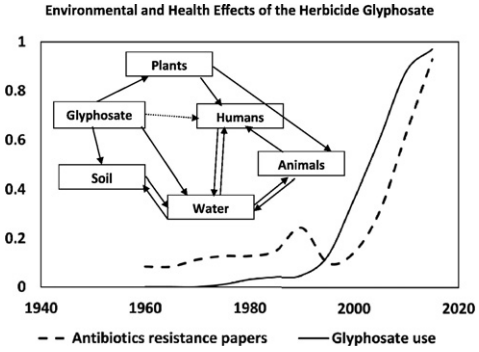
[a] Emerging Pathogens Institute, University of Florida, Gainesville, FL 32611, USA
[b] Department of Plant Pathology, IFAS, University of Florida, Gainesville, FL 32610, USA
[c] Department of Life and Environment Science, Hangzhou Normal University, Zhejiang 310036, China
[d] Faculty of Organic Agricultural Sciences, Ecological Plant Protection, University of Kassel, 37213 Witzenhausen, Germany
[e] Department of Medicine, College of Medicine, University of Florida, Gainesville, FL 32610, USA

HIGHLIGHTS

- Glyphosate and its degradation product AMPA have accumulated in the environment.
- Chronic low dose effects on animals and humans have been documented recently.
- Shifts in microbial community composition in soil, plants and animal guts resulted.
- Glyphosate and antibiotic resistance have arisen in fungi and bacteria in parallel.
- Glyphosate may serve as one of the drivers for antibiotic resistance.

GRAPHICAL ABSTRACT

Sources:
Antibiotic resistance papers: Cantas et al., 2013
Glyphosate use (relative area sprayed): USDA NASS, 2014.



ARTICLE INFO

Article history:
Received 7 August 2017
Received in revised form 29 October 2017
Accepted 29 October 2017
Available online 5 November 2017

Editor: D. Barcelo

Keywords:
Roundup
AMPA
Residues
Antibiotic resistance
Microbial community
Plant and animal health

ABSTRACT

The herbicide glyphosate, *N*-(phosphonomethyl) glycine, has been used extensively in the past 40 years, under the assumption that side effects were minimal. However, in recent years, concerns have increased worldwide about the potential wide ranging direct and indirect health effects of the large scale use of glyphosate. In 2015, the World Health Organization reclassified glyphosate as probably carcinogenic to humans. A detailed overview is given of the scientific literature on the movement and residues of glyphosate and its breakdown product aminomethyl phosphonic acid (AMPA) in soil and water, their toxicity to macro- and microorganisms, their effects on microbial compositions and potential indirect effects on plant, animal and human health. Although the acute toxic effects of glyphosate and AMPA on mammals are low, there are animal data raising the possibility of health effects associated with chronic, ultra-low doses related to accumulation of these compounds in the environment. Intensive glyphosate use has led to the selection of glyphosate-resistant weeds and microorganisms. Shifts in microbial compositions due to selective pressure by glyphosate may have contributed to the proliferation of plant and animal pathogens. Research on a link between glyphosate and antibiotic resistance is still scarce but we hypothesize that the selection pressure for glyphosate-resistance in bacteria could lead to shifts in microbiome composition and increases in antibiotic resistance to clinically important antimicrobial agents. We recommend interdisciplinary research on the associations between low level chronic glyphosate exposure, distortions in microbial communities, expansion of

* Corresponding author at: Department of Plant Pathology, IFAS, University of Florida, Gainesville, FL 32610, USA.
E-mail address: ahcvanbruggen@ufl.edu (A.H.C. Van Bruggen).

https://doi.org/10.1016/j.scitotenv.2017.10.309
0048-9697/© 2017 Elsevier B.V. All rights reserved.

antibiotic resistance and the emergence of animal, human and plant diseases. Independent research is needed to revisit the tolerance thresholds for glyphosate residues in water, food and animal feed taking all possible health risks into account.

© 2017 Elsevier B.V. All rights reserved.

Contents

1. Introduction

The herbicide glyphosate, *N*-(phosphonomethyl) glycine, is a biocide with a broad spectrum activity that was introduced for weed control in agricultural production fields in 1974 (Benbrook, 2016). Glyphosate is taken up by the foliage of plants and transported throughout the plant resulting in plant death after several days. Glyphosate is formulated with various adjuvants (Li et al., 2005), in particular surfactants such as polyoxyethylene amine (POEA), to enhance the uptake and translocation of the active ingredient in plants. The best known product formulated with POEA is Roundup® (Benbrook, 2016).

Glyphosate products are used primarily before planting of traditional agricultural crops and after planting of genetically modified glyphosate-resistant crops (Duke and Powles, 2009). Increasingly, they have been used for desiccation as a 'harvest aid' on traditional grain crops (Goffnett et al., 2016; Nelson et al., 2011; Zhang et al., 2017b). In addition, glyphosate has been widely used between trees in orchards and groves (Maqueda et al., 2017; Schrübbers et al., 2016; Singh et al., 2011; St. Laurent et al., 2008; Zhang et al., 2015b) and in urban areas for weed control along streets and in parks (Hanke et al., 2010; Kristoffersen et al., 2008). Finally, it has also been applied in waterways to eliminate invading aquatic plants (Clements et al., 2017; Monsanto, 2014).

As a result of the introduction of glyphosate-resistant soybean (*Glycine* max) and canola (*Brassica napus*) in 1996, cotton (*Gossypium hirsutum*) in 1997 and corn (*Zea mays*) in 1998 (Duke, 2015; Myers et al., 2016), as well as the expanding end of season glyphosate use to facilitate harvesting (Nelson et al., 2011), the total acreage treated with glyphosate has increased rapidly (see Graphical abstract). The annual glyphosate application rates per ha have increased too, for example on soybeans (Coupe and Capel, 2016), especially due to the development of glyphosate-resistant weeds (Benbrook, 2012). In 2012, about 127,000 tons of glyphosate were used in the USA and 700,000 tons worldwide (Swanson et al., 2014; US Geological Survey, 2012). Glyphosate use for agricultural production is now widespread, both in industrialized and developing countries (Benbrook, 2016).

The intensive use of glyphosate has resulted in increasing environmental and plant residues. Glyphosate is quite resistant to degradation due to the inert C-P linkage in the molecule (Chekan et al., 2016). Nevertheless, it is broken down in dead plant material and soil by various microorganisms (Mamy et al., 2016); the first decomposition product often is aminomethyl phosphonic acid, AMPA (Shushkova et al., 2009; Singh and Singh, 2016; Zhang et al., 2015b). However, decomposition of glyphosate takes place in living plants as well as in soils (Arregui et al., 2004), so that both glyphosate and AMPA residues can be found in plant products. In second generation glyphosate-resistant crop cultivars a gene that encodes for the enzyme glyphosate oxidase was inserted into the plant DNA, so that glyphosate is largely converted into AMPA and glyoxylate in those plants. As a result, glyphosate residues are negligible while AMPA residues may be considerable (Alves Corrêa et al., 2016; Monsanto, 2013). In some crop cultivars, a glyphosate-*N*-acetyl transferase or GAT gene was inserted to convert glyphosate to *N*-acetyl-glyphosate, which is broken down to *N*-acetyl-AMPA. In those cultivars all four residues (glyphosate, *N*-acetyl-glyphosate, *N*-acetyl-AMPA and AMPA) can be found and are often combined for dietary risk assessment (FAO, 2006). Total residues are mostly below 5 mg kg^{-1} but occasionally up to 20 mg kg^{-1} in harvested grain, fodder and oil crops when glyphosate is used as a 'harvest aid' before full crop maturity (Cessna et al., 1994, 2000; FAO, 2006; McNaughton et al., 2015; Zhang et al., 2017b). Total residue contents have been as high as 93 mg kg^{-1} in forage (FAO, 2006).

Due to the large scale and intensive use of glyphosate and its accumulation in the environment and edible products, several major concerns have arisen in recent years about harmful side effects of glyphosate and AMPA for soil and water quality, and plant, animal and human health. Based on recent reports on potential chronic side effects of glyphosate (Battaglin et al., 2014; Séralini et al., 2014), the World Health Organization reclassified the herbicide glyphosate as probably carcinogenic to humans in 2015 (Bai and Ogbourne, 2016; EFSA, 2015; Guyton et al., 2015; IARC, 2015). Since then, many (about 1000) scientific research papers have been published on glyphosate, especially its potential side effects, in the last two years, but a comprehensive review is still missing.

The objectives of this review are to present a critical overview of the scientific literature on (1) glyphosate accumulation in the environment and plant products, (2) its mode of action and effects on plants and animals, (3) its effects on microbial communities in soil, water, plants, animals and humans and (4) potential effects of shifts in microbial community composition on plant, animal and human health. An additional objective is to formulate a hypothesis about a possible relationship between resistance to glyphosate and to antibiotics in microorganisms as a result of the very high glyphosate selection pressure in the environment.

2. Residues in soil, water and plant products

A brief overview of the accumulation of glyphosate and its main degradation product AMPA in the environment is presented to facilitate a full understanding of their potential side effects on plants, animals and microbial communities.

2.1. Residues in soil and water

Glyphosate containing herbicides may contaminate soils in and around treated areas. Glyphosate adsorbs to clay and organic matter, slowing its degradation by soil microorganisms and leading to accumulation in soils over time (Banks et al., 2014; Cassigneul et al., 2016; Okada et al., 2016; Sidoli et al., 2016; Simonsen et al., 2008; Sviridov et al., 2015; Travaglia et al., 2015). As a result, glyphosate and its degradation product AMPA may persist for more than a year in soils with high clay content but may quickly wash out of sandy soils (Bergström et al., 2011; Okada et al., 2016; Sidoli et al., 2016). Glyphosate and AMPA degradation is also strongly dependent on soil pH (Zhang et al., 2015b).

In the past, glyphosate was not considered a problem for ground water and surface water, because it has a relatively low potential to move through soil and contaminate water sources (Monsanto, 2002, 2014; Sihtmäe et al., 2013). However, despite its attachment to clay and organic matter, parts of the glyphosate and its metabolite AMPA end up in the dissolved phase in ground water after heavy rain (Maqueda et al., 2017; Rendón-von Osten and Dzul-Caamal, 2017). Rain and erosion can also transport soil particles with glyphosate and AMPA into surface water (Table 1), where it can remain in the particulate phase or be dissolved (Maqueda et al., 2017; Rendón-von Osten and Dzul-Caamal, 2017; Wang et al., 2016b; Yang et al., 2015). Dissolved glyphosate and AMPA in surface water can sorb to the bottom sediment. Contaminated particles can settle and become incorporated in the bottom sediment as well (Aparicio et al., 2013; Maqueda et al., 2017). Biodegradation of glyphosate is much slower in sediment than in dissolved in water (Wang et al., 2016b). Glyphosate and AMPA are now widespread in a variety of natural waters and sediments (Aparicio et al., 2013; Maqueda et al., 2017; Grandcoin et al., 2017; Poiger et al., 2017).

In areas of the USA where genetically modified glyphosate-resistant crops are grown, glyphosate and AMPA occur widely in soil, surface water and ground water (Battaglin et al., 2014). Glyphosate has been measured in river water and stream water (Table 1) at levels from 2 to 430 μg l^{-1} (Battaglin et al., 2005, 2009; Coupe et al., 2011; Mahler et al., 2017). It has also been detected in air and rain during the crop growing season and in water from spring snow melt (Battaglin et al., 2009, 2014; Chang et al., 2011). Ultimately glyphosate ends up in seawater, where it is highly persistent (Mercurio et al., 2014).

In Europe, where growing genetically modified crops is not allowed, glyphosate has been detected in various water sources (but at lower levels than in the USA). Very low concentrations of glyphosate (<0.1 to 2.5 μg l^{-1}) were detected in samples of surface water in Germany (Skark et al., 1998), Switzerland (Poiger et al., 2017), Hungary (Mörtl et al., 2013) and northeastern Spain (Sanchis et al., 2012). Higher levels (up to 165 μg l^{-1}) were sometimes found in France (Villeneuve et al., 2011) and Denmark (Rosenbom et al., 2010).

Very little is known about glyphosate residues in the environment in other continents (Table 1). In particular, hardly any information is publicly available about environmental residues in China (publications in Chinese checked by He Miaomiao), while most glyphosate is currently produced in China (http://www.cnchemicals.com/Newsletter/NewsletterDetail_14.html; Zhang et al., 2015a) and glyphosate is used intensively in that country (Zhang et al., 2015a).

Besides runoff from agricultural land, urban runoff is also a source of glyphosate to streams and rivers (Grandcoin et al., 2017; Hanke et al., 2010). Because runoff is enhanced from impervious and connected paved surfaces, glyphosate use on paved surfaces is banned in several countries in Northern Europe (Kristoffersen et al., 2008; Rosenbom et al., 2010). Nevertheless, glyphosate and AMPA were found in samples of sewage and stormwater overflows (Birch et al., 2011) as well as the outlets from wastewater treatments plants (Grandcoin et al., 2017) and even in bottled water (Rendón-von Osten and Dzul-Caamal, 2017). Glyphosate and AMPA are commonly found in drinking-water (WHO, 2005), but at very low concentrations below the acceptable daily intake as determined in 1997 (WHO, 2005).

2.2. Residues in plant products

Until recently, residue measurements in plant products were focused on the active ingredient glyphosate and less on its degradation product AMPA. However, since it became known that glyphosate is partially broken down to AMPA in living plants (Arregui et al., 2004) and AMPA is also toxic to various organisms (Gomes et al., 2016;

Table 1
Glyphosate occurrence and concentrations in surface or ground water samples in several countries in North America, South America, and Europe.

Country	Date	Glyphosate occurrence and concentrations	Authors
Canada	2002	22% of samples positive, up to 6.07 μg l^{-1}	Humphries et al., 2005
US (Midwest)	2002	36% of stream samples positive, up to 8.7 μg l^{-1}	Battaglin et al., 2005
US (Midwest)	2013	44% of stream samples positive, up to 27.8 μg l^{-1}	Mahler et al., 2017
US (Washington, Maryland, Iowa, Wyoming)	2005–2006	All streams positive, up to 328 μg l^{-1}	Battaglin et al., 2009
US (Iowa, Indiana, Mississippi)	2004–2008	Most rivers positive, up to 430 μg l^{-1} after a storm	Coupe et al., 2011
Mexico	2015	All groundwater samples positive, up to 1.42 μg l^{-1}	Rendón-von Osten and Dzul-Caamal, 2017
Argentina	2012	35% of surface water samples positive, 0.1–7.6 μg l^{-1}	Aparicio et al., 2013
Germany	1998	Few positive samples in two tributaries to the Ruhr river, up to 0.59 μg l^{-1}	Skark et al., 1998
Switzerland	2016	Most stream water samples, up to 2.1 μg l^{-1}	Poiger et al., 2017
Spain	2007–2010	41% positive groundwater samples, up to 2.5 μg l^{-1}	Sanchis et al., 2012
Hungary	2010–2011	Most river and ground water samples positive, up to 0.001 μg l^{-1}	Mörtl et al., 2013
Denmark	1999–2009	25% of surface water samples positive, up to 31 μg l^{-1}; 4% of groundwater samples positive, up to 0.67 μg l^{-1}	Rosenbom et al., 2010
France	2003–2004	91% of stream samples positive, up to 165 μg l^{-1}	Villeneuve et al., 2011

Kwiatkowska et al., 2014a, 2014b), both glyphosate and AMPA are considered in residue analyses and regulations (Codex Alimentarius, 2013; EPA, 2013). The concentrations of glyphosate plus AMPA vary widely, ranging from 0.1–100 mg kg^{-1} in legumes (including soybeans), 0.1–25 mg kg^{-1} in cereals and rice, 0.1–28 mg kg^{-1} in oil seeds and 1–344 mg kg^{-1} in various types of fodder (Arregui et al., 2004; Bøhn et al., 2014; Çetin et al., 2017; Cuhra, 2015; FAO, 2006). The maximum residue limits of glyphosate plus AMPA in farm products vary widely too, ranging from 0.05 mg kg^{-1} in milk, 0.1 mg kg^{-1} in most plant products, 10–20 mg kg^{-1} in seeds and up to 500 mg kg^{-1} in fodder (Codex Alimentarius, 2013; Cuhra, 2015; EPA, 2013; FAO, 2006). The tolerable residue levels in seeds and fodder have increased over time to accommodate increasing concentrations detected in some farm products (Benbrook, 2016). Tolerable residue limits in China are similar to those elsewhere (Meador and Jie, 2014).

Residues of glyphosate and AMPA in water and plant products are taken up by animals and humans and excreted in their faeces and urine (Niemann et al., 2015; von Soosten et al., 2016). Glyphosate was detected in the urine of a high proportion (30–80%) of farm animals and humans (Krüger et al., 2014a, 2014b; Niemann et al., 2015). Residues were found not only in the urine of farmers but also in 60–80% of the general public, including children, in the USA and in 44% of the general public in Europe (Krüger et al., 2014a; Niemann et al., 2015). The concentrations in human urine samples were low, but higher among subjects in the USA (mean 2–3 μg l^{-1} and maximum 233 μg l^{-1}) than in Europe (mean < 1 μg l^{-1} and maximum 5 μg l^{-1}) (Niemann et al., 2015). Yet, exposure calculations have indicated that daily exposures to glyphosate are generally less than the tolerable reference dose as currently maintained by regulatory agencies (Solomon, 2016; WHO, 2005). However, these tolerance levels and safety standards are being challenged in view of the expanded human exposures (Vandenberg et al., 2017).

3. Effects of glyphosate on plants, animals and microorganisms

The biocidal activity of glyphosate is associated with the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Glyphosate thus stops the sixth step in the shikimate pathway (conversion from shikimate-3-phosphate to EPSP), which is required for the production of aromatic amino acids and secondary compounds with defense functions in plants and many microorganisms (Funke et al., 2006; Krüger et al., 2013; Schrödl et al., 2014).

3.1. Effects on plants

Glyphosate is toxic to both monocotyledonous plants (such as grasses) and dicotyledonous plants (most broad leaf plants). Uptake and translocation of glyphosate in plants is enhanced by surfactants in the formulated product. Translocation takes place both acropetally and basipetally (upwards and downwards), so that glyphosate accumulates throughout the plant including in seeds and plant roots (Li et al., 2005; Walker and Oliver, 2008). Glyphosate and its breakdown product AMPA inhibit antioxidant enzyme activities and induce the accumulation of reactive oxygen species (ROS) that induce physiological dysfunction and cell damage (Gomes et al., 2016). Both glyphosate and AMPA decrease photosynthesis, but through different mechanisms: glyphosate increases chlorophyll degradation, while AMPA disturbs chlorophyll biosynthesis (Gomes et al., 2016). Both enhanced chlorophyll degradation and reduced biosynthesis result in yellowing and necrosis of foliage.

Plants treated with glyphosate do not produce secondary aromatic compounds, including antimicrobial phytoalexins that defend plants against pathogens. Consequently, glyphosate treated plants frequently die from infection by root pathogens that are universally present in soil (Babiker et al., 2011; Johal and Rahe, 1984; Lee et al., 2012; Meriles et al., 2008; Rashid et al., 2013; Rosenbaum et al., 2014). Even sublethal glyphosate concentrations in plants, for example from residues in soil or water, diminish plant resistance to pathogens. Infection by *Fusarium* species is often more severe in fields where glyphosate was applied before planting of a crop compared with untreated control fields (Kremer et al., 2005; Kremer and Means, 2009; Sanogo et al., 2000; St. Laurent et al., 2008; van Bruggen et al., 2015). For example, soybean sudden death syndrome (caused by *Fusarium virguliforme*) was sometimes increased by glyphosate application in both glyphosate-tolerant and sensitive cultivars that were susceptible to the disease (Kremer and Means, 2009; Sanogo et al., 2000, 2001), but not always (Duke et al., 2012; Kandel et al., 2015). Infection of sugar beet (*Beta vulgaris*) by weakly pathogenic *Fusarium* and *Rhizoctonia* species was also enhanced after glyphosate application before planting sugar beet seeds (Larson et al., 2006). Yet, spore germination and mycelium growth of these and other pathogens are often reduced in vitro (Barnett et al., 2012; Larson et al., 2006; Mengistu et al., 2013; Sanogo et al., 2000; Sanyal and Shrestha, 2008). This supports the notion that increased root disease in glyphosate treated soil comes about through reduced plant resistance rather than enhanced pathogen growth or that glyphosate may suppress beneficial microorganisms more than plant pathogens like *Fusarium* species.

In addition to reduced plant resistance, indirect effects of glyphosate and AMPA on plant health are possible through changes in the endophytic and rhizosphere microbiome (Berg et al., 2014; Finckh et al., 2015; Kremer et al., 2005; Kuklinsky-Sobral et al., 2005; van Bruggen and Finckh, 2016; van Bruggen et al., 2016). The importance of the plant microbiome for plant health has been known for a long time (Kloepper et al., 1980; Pleban et al., 1995). The plant microbiome composition is largely determined by plant species and the soil where a plant is growing, including the soil microbiome as affected by pesticide use (Finckh et al., 2015; see also Section 3.3.1 of this review). Besides a reduction in plant resistance as a result of changes in microbial composition after glyphosate use, attraction of plant pathogens to the roots of glyphosate-resistant plants can be enhanced by increased exudation of carbohydrates and amino acids from roots of glyphosate treated plants (Kremer et al., 2005; Kremer and Means, 2009).

Another agricultural problem that has arisen in response to intensive glyphosate use is the widespread appearance of glyphosate-resistant weed species (Duke and Powles, 2009; Green and Owen, 2011; Schafer et al., 2014; Shaner et al., 2012). Glyphosate-resistance in plants can be conferred by several different mechanisms (Table 2) such as changes in translocation as a result of modified transporter genes, single point mutations of the target site making it insensitive, increased target expression or detoxification of the herbicide (Chekan et al., 2016; Pollegioni et al., 2011; Shaner et al., 2012; Tani et al., 2016; Zhang et al., 2015a). Recently, alternative glyphosate-resistance genes were found that could be incorporated into genetically modified crops in the near future (Staub et al., 2012; Tao et al., 2017; Tian et al., 2015), but different resistance mechanisms may evolve again in weeds exposed to glyphosate repeatedly.

In reaction to the glyphosate-resistance problem, farmers have increased the dosage and frequency of glyphosate use even further (Benbrook, 2012), and companies producing glyphosate-resistant crops added genes for resistance to other herbicides, 2,4-D and dicamba, in those crops (Duke and Powles, 2009; Leon et al., 2016; Ruen et al., 2017). However, weeds with multiple herbicide resistance at multiple sites of action will likely emerge soon after the widespread use of these herbicides (Bell et al., 2013). This may then lead to an additional increase in herbicide use and additional unintended side effects (Landrigan and Benbrook, 2015).

3.2. Effects on animals and humans

The absence of the shikimate pathway in animals is the basis for the lack of acute toxicity of glyphosate in animals such as mammals, amphibians and reptiles after a single exposure to relatively high dosages

Table 2
Mechanisms of resistance to glyphosate in plants and bacteria.

Mechanism	Examples in plants	References	Examples in bacteria	References
Mutation of the gene coding for the target site	Single point mutation of the target site (EPSPS) making it insensitive to glyphosate	Chekan et al., 2016; Pollegioni et al., 2011; Shaner et al., 2012	Amino acid substitution in EPSP synthase as found in *Staphylococcus aureus* and *Enterobacter* sp.	Priestman et al., 2005; Fei et al., 2013
Increased target expression	Increased target expression resulting in so many target molecules that glyphosate cannot block all targets	Chekan et al., 2016; Shaner et al., 2012; Zhang et al., 2015a		
Overexpression of membrane efflux transporter genes like ABC transporters	Modification of transporter genes resulting in reduced intra-plant translocation and possibly transfer into vacuoles	Chekan et al., 2016; Staub et al., 2012, Shaner et al., 2012; Tani et al., 2016	Increased efflux of glyphosate as found in *E. coli* and *Pseudomonas* sp. with glyphosate-tolerance	Staub et al., 2012
Horizontal gene transfer of *aroA*[a] CP4 resistance gene or other resistance genes	Transfer of the *aroA* CP4 gene or other resistance genes from bacteria or fungi into crop plants	Padgette et al., 1995; Tao et al., 2017; Tian et al., 2015	Transfer of the *aroA* CP4 gene from genetically modified plants back into plant-associated bacteria (*E. coli*) in the lab	Natarajan et al., 2007; Li et al., 2015
Degradation of glyphosate	Detoxification of glyphosate; cleavage of the C-P or C-N bond; this mechanism has not been found yet in naturally resistant plants	Pollegioni et al., 2011; Shaner et al., 2012	Glyphosate is degraded by various bacteria like *Azotobacter* sp., *Azospirillum* sp. and insensitive *Pseudomonas* sp.	Arunakumara et al., 2013; Singh and Singh, 2016; Sviridov et al., 2015; Travaglia et al., 2015; Zhao et al., 2015
Scavenging of free radicals providing stress resistance	Not found in glyphosate-resistant plants		Scavenging of free radicals by mycothiol[b] providing stress resistance and glyphosate-tolerance in Actinobacteria	Liu et al., 2013

[a] *aroA* is the gene encoding the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase.
[b] Mycothiol is a specific thiol compound found in Actinobacteria.

(McComb et al., 2008; Weir et al., 2016). However, the median lethal doses vary considerably for different formulations, especially with respect to surfactants (Diamond and Durkin, 1997; Durkin, 2011). Formulations containing POEA are relatively toxic compared to other formulations. Lethal doses of the most toxic formulations are species specific varying from 175 to 540 mg glyphosate acid equivalent (a.e.) kg^{-1} body weight for terrestrial animal species and from 1 to 52 mg a.e. l^{-1} of water for aquatic species (Durkin, 2011). However, the glyphosate sensitivity of these two groups cannot be compared directly because the method of exposure is quite different for these groups.

3.2.1. Terrestrial animals and humans

Although the acute oral toxicity of technical grade glyphosate to mammals is low, with the LD_{50} ranging from 800 to >5000 mg kg^{-1} body weight for different animal species (McComb et al., 2008; WHO, 2009), there is increasing interest in potential chronic effects of formulated glyphosate and its degradation products as they accumulate in the environment (Bai and Ogbourne, 2016; Battaglin et al., 2014; Greim et al., 2015; Mesnage et al., 2015a; Séralini et al., 2014). Two research approaches have been taken to investigate chronic effects: correlative research and experimental studies that address possible causative relationships.

Correlations have been found between increased glyphosate use and a wide variety of human diseases, including various forms of cancer, kidney damage and mental conditions such as ADHD, autism, Alzheimer's and Parkinson's disease (Fluegge and Fluegge, 2016; Fortes et al., 2016; Jayasumana et al., 2014; Mesnage et al., 2015b; Swanson et al., 2014). Miscarriages and dermatological and respiratory illnesses were related to glyphosate exposure during aerial glyphosate spraying campaigns to eliminate coca plants in Colombia (Camacho and Mejía, 2017). Increases in infertility and malformation among pigs were correlated with glyphosate concentrations in the liver and kidneys and with residues in the feed (Krüger et al., 2014a, 2014b). Various confounding factors might have contributed to these correlations. Therefore, controlled experiments are essential to determine chronic toxic effects.

Under experimental conditions with cell cultures, glyphosate and its breakdown product AMPA increased the reactive oxygen species (ROS) in human erythrocyte cultures at moderately high concentrations (>42 mg l^{-1} of either pure glyphosate or AMPA) for 24 h (Kwiatkowska et al., 2014a) (Table 3). AMPA is a glutamic acid receptor in the central nervous system (Catarzi et al., 2006). Both glyphosate and AMPA decreased acetylcholinesterase activity in erythrocyte cultures (Kwiatkowska et al., 2014b). Acetylcholinesterase catalyzes the breakdown of acetylcholine that functions as a neurotransmitter. Decreased acetylcholinesterase activity by organophosphorus compounds hinders the termination of synaptic transmission. Neural cell development and axon growth of rats were impaired after exposure to a high dose of glyphosate (4000 mg l^{-1}) for 24 h (Coullery et al., 2016).

In other laboratory studies, exposure of human peripheral blood cells to glyphosate resulted in DNA damage in leucocytes at moderate to high concentrations (85 to 1690 mg l^{-1}) and decreased DNA methylation at 42 mg l^{-1} glyphosate in vitro (Kwiatkowska et al., 2017). Changes in DNA methylation can disturb the balance between cancerous cell proliferation and programmed cell death (apoptosis) (Hervouet et al., 2013). DNA hypomethylation can lead to activation of oncogenes, while hypermethylation can silence tumor suppressor genes (Hervouet et al., 2013). Thus, glyphosate and AMPA can disturb normal neurotransmission and upset the delicate balance between cell proliferation and programmed cell death.

As predicted from correlation and cellular studies (Table 3), persistent low exposure to glyphosate (about 70 mg of glyphosate kg^{-1} body weight day^{-1}) can affect the activity of the enzyme acetylcholinesterase at the organismal level (Cattani et al., 2017; Kwiatkowska et al., 2014b; Menéndez-Helman et al., 2012). If acetylcholinesterase is not working properly, nerve impulses are not switched off, causing serious neurological disorders (Čolović et al., 2013). For example, chronic exposure of pregnant rats (*Rattus norvegicus*) to glyphosate (Roundup®) in drinking water (0.36% or 3600 mg a.i. l^{-1}) led to oxidative stress and glutamate excitotoxicity in the rat hippocampus and decreased hippocampus acetylcholinesterase activity (Cattani et al., 2017). This resulted in depressive-like behavior in offspring rats exposed to glyphosate for 60 days (Cattani et al., 2017).

In another chronic exposure study (Table 3), daily treatments of live rats with a low dose (56 mg kg^{-1}) of Roundup® for 5 or 13 weeks resulted in biochemical and anatomical liver damage (Çağlar and Kolankaya, 2008). Chronic exposure (2 yr) to Roundup® at ultralow doses (50 ng l^{-1}; 4 ng kg^{-1} bw d^{-1}) in drinking water caused liver and kidney damage and various tumors in laboratory rats (Mesnage et al., 2015a; Séralini et al., 2014), although conclusions regarding possible carcinogenicity have been disputed by researchers associated with the industry and others (EFSA, 2015; Greim et al., 2015). Recent

Table 3
Effects of chronic exposure of terrestrial cells and live animals and of aquatic animals to low dosages of Roundup®. See text for actual dosages used.

Terrestrial animals				Aquatic animals	
Effects on cell cultures	References	Effects on live animals	References	Effects on live animals	References
Increase in reactive oxygen species (ROS)	Kwiatkowska et al., 2014a	Impaired neural cell development and axon growth of rats	Coullery et al., 2016	Overproduction of ROS and oxidative stress in fish	Li et al., 2017
Decrease in acetylcholinesterase activity	Kwiatkowska et al., 2014b	Impaired acetyl cholinesterase activity; oxidative stress and glutamate excitotoxicity in the rat hippocampus; depressive-like behavior in offspring rats	Cattani et al., 2017; Kwiatkowska et al., 2014b; Menéndez-Helman et al., 2012	Suppression of acetylcholine-sterase activity in brown mussels and fish; damage of motoneurons in fish; developmental problems and brain damage	Menéndez-Helman et al., 2012; Sandrini et al., 2013; Zhang et al., 2017a; Roy et al., 2016
DNA damage in leucocytes and decreased DNA methylation	Kwiatkowska et al., 2017	Biochemical and anatomical liver damage; liver and kidney damage and tumors in rats	Çağlar and Kolankaya, 2008; Mesnage et al., 2015a; Mesnage et al., 2016; Séralini et al., 2014	Disturbed metabolism and renal injury in fish; DNA damage in blood, gills and liver of eel; changes in liver cells and mitochondria in carp	Li et al., 2017 Guilherme et al., 2009 Szarek et al., 2000
Deteriorated ovarian functions in cell cultures of cattle ovaries	Perego et al., 2017	Negative fertility effects in male rats	Abarikwu et al., 2015; Nardi et al., 2017		

work by Mesnage et al. (2016) further demonstrated that the chronic (2 year) exposure of rats to these ultralow dosage levels of Roundup® resulted in marked alterations of the liver proteome and metabolome, changes which overlap substantially with non-alcoholic fatty liver disease. In the experiments with Roundup®, no distinction was made between effects of glyphosate and of the adjuvant POEA, although the estimated acute oral toxicity of POEA is higher (LD_{50} = 1.2 g kg^{-1}) than that of glyphosate (LD_{50} = 4.8 g kg^{-1}) (Diamond and Durkin, 1997). However, these experiments were not carried out to estimate acute oral effects of glyphosate, but chronic effects of the formulated product as encountered in reality.

Finally, repeated application of glyphosate at relatively low doses (5 mg kg^{-1}) negatively affected fertility in male rats (Abarikwu et al., 2015), while a single application had a negative fertility effect at a high dose (500 mg kg^{-1}) only (Dai et al., 2016). Male rats that received soymilk with 100 mg l^{-1} glyphosate had a decrease in spermatids and increase in abnormal sperm morphology compared to the soymilk control (Nardi et al., 2017). Cell cultures of cattle ovaries showed deteriorated ovarian functions after exposure to low glyphosate concentrations (0.5, 1.7 and 5 mg l^{-1}) but not at high concentrations (10 and 300 mg l^{-1}), which is typical of endocrine disruptor effects (Perego et al., 2017). Thus, at low concentrations, glyphosate could have hormonal effects and reduce fertility (Table 3), while at high doses various other organs may be affected, ultimately resulting in death.

3.2.2. Aquatic animals

Glyphosate and the surfactants POEA and MON 0818 (75% POEA) can have negative impacts on the health of a variety of animals in the aquatic food web, including protozoa, mussels, crustaceans, frogs and fish, similar to the effects on terrestrial animals (Bringolf et al., 2007; Durkin, 2011; Li et al., 2017; Moreno et al., 2014; Prosser et al., 2017; Rissoli et al., 2016; Sihtmäe et al., 2013; Tsui and Chu, 2003; Zhang et al., 2017a). Glyphosate formulations with POEA are generally more toxic than those without this surfactant (Bringolf et al., 2007; Prosser et al., 2017). Aquatic animals seem to be more sensitive to POEA than terrestrial animals. However, experimentation on health effects of adjuvants by independent entities has been quite limited due to the proprietary nature of these chemicals (Diamond and Durkin, 1997; Durkin, 2011). For example, formulations with POEA were more toxic to the microcrustacean *Artemia salina* and young zebrafish *Danio rerio* than formulations without POEA both at 360 g glyphosate a.e. l^{-1} water (Rodrigues et al., 2017). Various species of freshwater mussels were more sensitive to glyphosate with MON 0818 (EC_{50} = 1 mg a.e. l^{-1}) and Roundup® (EC_{50} = 4 mg a.e. l^{-1}) than to technical grade glyphosate (EC50 > 200 mg a.e. l^{-1}) (Bringolf et al., 2007). At typical and maximum glyphosate application and runoff rates from soil, resulting in estimated concentrations of 0.21 to 0.99 mg POEA l^{-1} surface water, 21–43% of a wide array of aquatic species were estimated to be impaired by these POEA concentrations (Rodriguez-Gil et al., 2017).

Similar to effects of glyphosate on terrestrial animals (Table 3), pure glyphosate suppressed the activity of acetylcholinesterase at low concentrations (1–676 mg l^{-1}) in brown mussels (*Perna perna*) (Sandrini et al., 2013) and several fish species (Menéndez-Helman et al., 2012; Sandrini et al., 2013). At an ultralow concentration of 0.01 mg l^{-1} glyphosate damaged the primary motoneurons in zebrafish resulting in abnormal movements at a young age (Zhang et al., 2017a). Exposure of zebrafish embryos to higher concentrations of Roundup® (50 mg l^{-1}) resulted in developmental problems including forebrain, midbrain and eye damage (Roy et al., 2016).

Again, similar to glyphosate effects on terrestrial animals, chronic exposure of gold fish (*Carassius auratus*) to moderately low levels of glyphosate (34 mg l^{-1}) disturbed the metabolism in various tissues, led to overproduction of ROS and oxidative stress (Li et al., 2017). The final result was severe renal injury (Li et al., 2017). Even lower concentrations of Roundup® (3.6 mg l^{-1} for 4 h) damaged the DNA in blood, gills and liver of the European eel (*Anguilla anguilla*) (Guilherme et al., 2009). Exposure of freshwater carp (*Cyprinus carpio*) to higher levels of Roundup® (205 mg or 410 mg of glyphosate l^{-1}; still below the range of commercial applications) induced changes to liver cells and mitochondria (Szarek et al., 2000).

In addition to these direct effects on aquatic animals, glyphosate can affect the interactions between fish and their pathogens or parasites. Exposure of silver catfish (*Rhamdia quelen*) to sublethal concentrations of glyphosate (0.73 mg l^{-1}, 10% of the LC_{50} for 96 h) reduced the numbers of blood erythrocytes, thrombocytes, lymphocytes and total leukocytes, decreased immune cell phagocytosis and increased susceptibility to the pathogen *Aeromonas hydrophila*, resulting in a decrease in the survival rate (Kreutz et al., 2010, 2011). Similarly, environmentally relevant concentrations (0.36 mg a.i. l^{-1}) of glyphosate enhanced infection of the freshwater fish *Galaxias anomalus* by the trematode flatworm parasite *Telogaster opisthorchis* (Kelly et al., 2010). However, horsehair worms (*Chordodes nobilii*) parasitic to mosquito larvae (*Aedes aegypti*) showed reduced infective ability and increased adult mortality following exposure to low concentrations (0.1–8 mg a.i. l^{-1}) of technical grade glyphosate and Roundup® (Achiorno et al., 2008). Thus, low levels of glyphosate in surface water could disturb the balance between hosts

and pathogens or parasites. This can result in unexpected shifts in aquatic communities.

3.3. Effects on microorganisms

The shikimate pathway is present not only in plants but also in fungi and bacteria, rendering many taxa of microorganisms sensitive to glyphosate. However, not all organisms with the shikimate pathway are sensitive to glyphosate, depending on the class of EPSPS they produce: class I EPSPS is glyphosate sensitive and class II EPSPS is glyphosate-tolerant (Funke et al., 2007; Priestman et al., 2005). For example, *Agrobacterium tumefaciens* strain CP4 has a gene coding for the class II version of EPSPS that is not inhibited by glyphosate (Padgette et al., 1995). Similar to plants, bacterial and fungal strains with low sensitivity to glyphosate have been selected, largely through the same mechanisms as the resistance mechanisms identified in plants (Li et al., 2015; Liu et al., 2013; Priestman et al., 2005; Staub et al., 2012). Consequently, differences in sensitivity among microorganisms have affected the microbial composition of various habitats harboring glyphosate, including soil, plant surfaces and animal intestinal tracts.

3.3.1. Microorganisms in soil, rhizosphere and plants

Glyphosate is taken up by the foliage of plants and transported throughout the plant and into the rhizosphere and soil (Yamada et al., 2009; Zobiole et al., 2010). Because many microorganisms are sensitive to glyphosate, its application can affect the microbial composition and enzymatic activity in the plant endosphere, the rhizosphere and surrounding soil (Arango et al., 2014; Banks et al., 2014; Cherni et al., 2015; Druille et al., 2015; Schafer et al., 2014). For example, glyphosate treatments (applied at recommended or lower dosages) negatively affected microorganisms that promote plant growth, such as *Burkholderia* spp., *Pseudomonas* spp., arbuscular mycorrhizal fungi and nitrogen fixing *Rhizobium* spp. (Arango et al., 2014; Druille et al., 2015; Schafer et al., 2014; Zobiole et al., 2010). These treatments resulted in reduced plant growth and/or changes in grassland vegetation cover and composition (Arango et al., 2014; Druille et al., 2015).

However, there is still much controversy about the effects of glyphosate on microbial communities and activities in the soil and rhizosphere (Allegrini et al., 2015; Hungria et al., 2014; Wolmarans and Swart, 2014). In studies comparing soil treated with glyphosate with untreated control soil, microbial communities seemed to recover from short term glyphosate treatment (Arango et al., 2014; Banks et al., 2014; Wolmarans and Swart, 2014) with only minor or no effect on global microbial structure, biomass or activity (Allegrini et al., 2015; Haney et al., 2000; Meriles et al., 2008; Nakatani et al., 2014; Wardle and Parkinson, 1990; Zabaloy et al., 2016), probably due to the great diversity and compensatory ability of microorganisms in soil. In addition, direct effects of glyphosate are confounded by increased availability of dead plant and microbial material, a food source for many microorganisms, including saprotrophic plant pathogenic fungi (Babiker et al., 2011; Meriles et al., 2008; Sharma-Poudyal et al., 2016).

Specific methods that can detect rare microorganisms, shifts in microbial composition, and changes in metabolic functions resulting from glyphosate applications, such as deep sequencing (sequencing of extracted DNA or RNA multiple times), have been used only by Schafer et al. (2014) and Newman et al. (2016). Schafer et al. (2014) investigated the taxonomic distribution of the microbial community, diversity and genera abundance within the rhizosphere of glyphosate susceptible and resistant giant ragweed (*Ambrosia trifida*) biotypes in response to a glyphosate application. DNA sequences of the pathogens *Verticillium* and *Xanthomonas* increased and of the beneficial bacterium *Burkholderia* decreased in glyphosate treated soil, but the differences in microbial community composition were small. Newman et al. (2016) showed that the phospholipid fatty acid composition in the rhizospheres of Roundup-ready® corn and soybeans changed as a result of long term (3 yr) glyphosate treatment at the recommended dose. RNA-Seq analysis showed that carbohydrate and amino acid metabolism was downregulated in total extracted rhizosphere RNA (Newman et al., 2016), in agreement with a possible reduction in photosynthesis (Gomes et al., 2016). Iron acquisition and metabolism were also downregulated in the rhizosphere (Newman et al., 2016), in agreement with the reduced availability of iron in glyphosate treated soil (Cakmak et al., 2009). Protein metabolism and respiration sequences were upregulated (Newman et al., 2016), possibly due to increased amino acid exudation (Kremer et al., 2005).

Minor differences in sensitivity of soil and rhizosphere microorganisms to glyphosate may result in important shifts in plant or animal pathogens. For example, the root pathogen *Fusarium* sp. is comparatively insensitive to glyphosate. Thus, glyphosate application may shift the balance of pathogenic *Fusarium* spp. and antagonistic microorganisms such as *Pseudomonas fluorescens* in favor of the root pathogens (Kremer and Means, 2009; Yamada et al., 2009; Zobiole et al., 2010). Increased root rot caused by pathogenic *Fusarium* spp. in glyphosate treated soil has been shown repeatedly as mentioned above (Fernandez et al., 2009; Johal and Huber, 2009; Rosenbaum et al., 2014; Yamada et al., 2009). Similarly, the human and animal pathogen *Staphylococcus aureus* is insensitive to glyphosate and may become more dominant in glyphosate treated soil (Funke et al., 2007; Priestman et al., 2005). Thus, the presence of glyphosate in soil could change the community compositions of bacteria and fungi, in turn altering soil ecosystem functions and plant and animal health (Kuklinsky-Sobral et al., 2005; Zobiole et al., 2010).

3.3.2. Microorganisms in water

Negative effects of glyphosate and the surfactant POEA have been demonstrated for various species of microalgae, aquatic bacteria and protozoa (Rodriguez-Gil et al., 2017; Sihtmäe et al., 2013; Tsui and Chu, 2003). The modes of action in aquatic microorganisms are similar to those in terrestrial plants and microorganisms: glyphosate affects synthesis of aromatic amino acids, the production of chlorophyll, photosynthesis and respiration (Mensink and Janssen, 1994). The marine bacterial species *Vibrio fischeri* was sensitive to moderately low concentrations of glyphosate in water (EC_{50} values ranged from 5.4 to 7.6 mg a.e. l^{-1}), regardless of the formulation used (Sihtmäe et al., 2013). Microalgae are generally more sensitive to glyphosate and the formulated product Roundup® (1.2–7.8 mg l^{-1}) than are heterotrophic bacteria, although some species of microalgae are more tolerant than others (Mensink and Janssen, 1994; Tsui and Chu, 2003; Wang et al., 2016a).

Autotrophic microorganisms are vital to marine and freshwater ecosystems, because they form the base of food chains. Photosynthesis, cell densities and growth rates of three microalgae were diminished by exposure to a typical application rate of glyphosate (0.89 kg a.e. ha^{-1}, resulting in about 1.2 mg glyphosate l^{-1} and 0.21 mg POEA l^{-1} of surface water) (Rodriguez-Gil et al., 2017). Even a low glyphosate concentration (0.011 mg l^{-1}) inhibited the growth of the autotrophic community in river water for three weeks, although it did not cause chlorophyll reduction (Bricheux et al., 2013). Addition of Roundup® to pond water (6 and 12 mg a.i.$^{-1}$, higher than the 3.5 mg a.i.$^{-1}$ recommended for weed control) decreased the abundance of total micro- and nanophytoplankton, but increased the abundance of picocyanobacteria and overall primary production (Pérez et al., 2007). This increase in picocyanobacteria was attributed to the direct toxicological effect of glyphosate on other microorganisms, resulting in release of nutrients from the dead bodies. In later studies, glyphosate formulated as Glifosato Atanor® at 3.5 mg a.i. l^{-1} as well as pure glyphosate and Glifosato Atanor® at 2.7–2.9 mg a.e. l^{-1} stimulated the abundance of bacterioplankton and planktonic picocyanobacteria, and the photosynthetic activity of periphytic algae (Vera et al., 2012, 2014; Wang et al., 2016a). This was attributed to increased phosphorous contents in glyphosate treated water (Vera et al., 2012, 2014; Wang et al., 2016a). Indeed, a single application of technical grade glyphosate (2.4 mg l^{-1})

to tap water in mesocosms that were left outside for 6 months increased the total phosphorous concentration seven fold (to 0.7 mg l^{-1}) and doubled the density of picocyanobacteria (to $2 * 10^{-6}$ cells ml^{-1} in turbid water) after 1–8 days (Pizarro et al., 2016).

Similar to the research results obtained for soil, global measures of microbial activity and diversity were negligibly affected by glyphosate treatments (0.01–0.37 mg l^{-1}) of surface water collected from various water bodies (Bricheux et al., 2013; Magbanua et al., 2013; Pesce et al., 2009). In situ measurements of microbial activity, diversity and composition in relation to glyphosate concentrations were confounded by the presence of other pesticides (Daouk et al., 2013). In controlled laboratory experiments, however, the growth and species composition of microbial populations (determined by Temporal Temperature Gradient Gel Electrophoresis with DNA from marine waters) were sometimes disturbed at levels of glyphosate (0.001–0.01 mg l^{-1}) typical of those caused by run off from land (Stachowski-Haberkorn et al., 2008). Thus, similar to the situation in soil, deep sequencing and metabolomics may be needed to detect subtle shifts in microbial communities in water (Beale et al., 2017; Muturi et al., 2017; Tromas et al., 2017). The results may be affected by many factors, including the glyphosate formulation and concentration, pH and sediment contents (Magbanua et al., 2013; Tsui and Chu, 2003; Wang et al., 2016b).

3.3.3. Microorganisms in animals

Relationships between microbiomes and human or animal health have received much attention in recent years (Berg et al., 2014; Hoffman et al., 2015; O'Doherty et al., 2014), but little research has been done on the potential influence of glyphosate on these relationships. Nevertheless, intestinal microbial communities can be affected by glyphosate in contaminated animal feed and water. Subsequently, the changes in these communities can be detrimental to animal health. For example, lactic acid producing bacteria generally are negatively affected by glyphosate (Clair et al., 2012; Krüger et al., 2013). These bacteria normally produce antibiotics and can suppress pathogenic bacteria such as *Clostridium botulinum* (Krüger et al., 2013; Rodloff and Krüger, 2012) and botulism has increasingly been found in cows (*Bos taurus*) that had high concentrations of glyphosate in their feed and urine (Gerlach et al., 2014; Krüger et al., 2013, 2014a). During in vitro fermentation in bovine rumen fluid, several species of bacteria and protozoa were suppressed after exposure to glyphosate at 1, 10 and 100 mg l^{-1} (Ackermann et al., 2015). However, botulinum neurotoxin concentration was enhanced at the highest level of glyphosate only (1000 mg l^{-1}). In poultry (*Gallus gallus domesticus*), *Bifidobacterium* and *Enterococcus* spp. were negatively affected by glyphosate at 0.08–0.15 mg g^{-1}, while pathogenic bacteria such as *Salmonella* and *Clostridium* spp. were less sensitive (Minimal Inhibitory Concentration, MIC = 1.2–5 mg g^{-1}) to this herbicide (Shehata et al., 2013). The tested concentrations were high, but the glyphosate concentration in the poultry feed was also high: 0.19–0.4 mg g^{-1} (Shehata et al., 2014).

Glyphosate in animal feed affects not only intestinal bacteria but also fungi, such as the *Mucorales*, fast growing fungi often forming spore balls on top of fungal threads and therefore sometimes called pin molds. They are common in soil and often cause food spoilage and sometimes mycoses in animals and humans (Morace and Borghi, 2012). A positive correlation was found between glyphosate concentrations in urine and the density of *Mucorales* in the rumen of dairy cows in Germany (Schrödl et al., 2014). This change in the fungal community could have come about through a disturbance of the intestinal microbiota in general, because members of the *Mucorales* were resistant to glyphosate in vitro (Schrödl et al., 2014). However, the observed reduction in antibodies against the *Mucorales* may indicate that glyphosate influenced the immune system of the cows, possibly through the toxic effects of glyphosate on the liver (Schrödl et al., 2014). Although *Mucorales* were resistant to glyphosate in vitro, it is possible that this change in prevalence was due to a disturbance of other intestinal microbiota.

4. Resistance to glyphosate and antibiotics

Little attention has been paid to potential indirect negative effects of glyphosate on human and animal health through a possible relationship between resistance to glyphosate and to antibiotics in bacteria (Fig. 1). In this section, we discuss glyphosate-resistance in bacteria and the potential mechanisms underlying this resistance, followed by observed associations between glyphosate-resistance and antibiotic resistance and a hypothesis about the selection pressure of intensive glyphosate use driving both forms of resistance.

Although many bacteria and fungi are sensitive to glyphosate, some are highly resistant (Fei et al., 2013; Kuklinsky-Sobral et al., 2005; Natarajan et al., 2007; Wolmarans and Swart, 2014). For example, strain CP4 of *Agrobacterium tumefaciens* was found in the wastewater of a glyphosate production plant and was highly resistant to glyphosate. This resistance is conferred by the gene coding for the enzyme CP4 EPSP synthase (EPSPS), which was inserted into crops to provide glyphosate-resistance (Padgette et al., 1995). A truncated form of the same enzyme was transferred experimentally from glyphosate-resistant plants to *Escherichia coli*, providing similar resistance as the original full length enzyme (Natarajan et al., 2007). Other functional genes of the glyphosate-resistant *E. coli* as well as its carbon utilization profile were only slightly affected by the inserted EPSP synthase gene, suggesting that this genetically modified *E. coli* could survive in the environment (Li et al., 2015). This implies that glyphosate-resistance could potentially return from plants to bacteria by horizontal gene transfer, and that the resulting glyphosate-resistant bacteria might successfully survive in the environment (Li et al., 2015). Although horizontal gene transfer by natural means has not been demonstrated conclusively, glyphosate-resistance has been identified in many genera of bacteria since the intensification of glyphosate use (Liu et al., 2013; Priestman et al., 2005; Staub et al., 2012). This resistance could have come about by various mechanisms other than horizontal gene transfer (Table 2). The mechanisms that provide resistance to glyphosate in bacteria are similar to those conveying resistance to glyphosate in plants. For example, mutations of the gene coding for the target site EPSP synthase and of efflux transporter genes have been found in plants and bacteria (Fei et al., 2013; Priestman et al., 2005; Staub et al., 2012). In addition, bacteria could be selected that produce glyphosate degradation enzymes (Arunakumara et al., 2013; Singh and Singh, 2016; Sviridov et al., 2015; Travaglia et al., 2015; Zhao et al., 2015). Or bacteria could circumvent some of the harmful effects of glyphosate by increasing the production of molecules that scavenge free radicals (Liu et al., 2013).

Some of the mechanisms conferring resistance to glyphosate in bacteria also confer resistance to clinically important antimicrobial agents (Kurenbach et al., 2015; Liu et al., 2013). For example, the reduction of

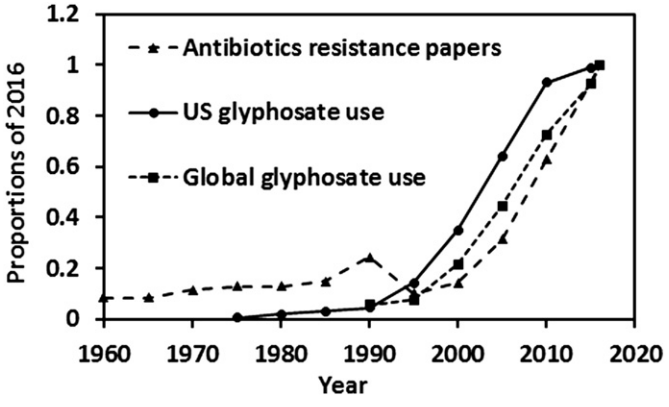


Fig. 1. Relative number of scientific publications (▲) on antibiotic resistance in soil, waste water and natural water (70 in 2016), relative total glyphosate use in the USA (●) and worldwide (■) (127 million and 900 million kg, respectively, in 2016) between 1960 and 2016 (Benbrook, 2016; Cantas et al., 2013; USDA NASS, 2014).

harmful radicals by mycothiol (a specific thiol compound found in Actinobacteria that scavenges free radicals) provides resistance not only to glyphosate but also to a wide range of antibiotics including the beta lactam antibiotic penicillin G (Liu et al., 2013). Another example is the modification of the AcrAB efflux pump in *E. coli* that had been exposed to glyphosate (1240 mg l^{-1}), which was associated with an increase in resistance from 0.03 to 0.09 mg l^{-1} of the fluoroquinolone antibiotic Ciprofloxacin (Cip) and from 5 to 20 mg l^{-1} of the aminoglycoside antibiotic Kanamycin (Kan) (Kurenbach et al., 2015). Similarly, *Salmonella enterica* serovar Typhimurium was more resistant to Cip at 0.08 mg l^{-1} and to Kan at 8–40 mg l^{-1} after exposure to glyphosate, but potential changes in efflux pump were not investigated (Kurenbach et al., 2015). Cip and Kan are used as alternative antibiotics to control pathogens that are insensitive to beta lactam antibiotics such as penicillin, ampicillin and cephalosporin. Resistance to Cip and Kan would reduce the spectrum of clinically useful antibiotics even further.

Beta lactam resistant bacteria commonly produce Extended Spectrum Beta Lactamase (ESBL), an enzyme that breaks down beta lactam antibiotics and thus confers resistance to these antibiotics (Apostolakos et al., 2017; Brolund, 2014). ESBL production has been associated with field induced glyphosate-resistance, in particular in various species of Enterobacteriaceae (Krüger and Shehata, 2014). We isolated unidentified bacteria ($n = 101$) from citrus roots and rhizospheres (Shin et al., 2016; Shin and van Bruggen, 2017). In agreement with the findings by Krüger and Shehata (2014), the bacteria showed cross resistance (64% of the colonies) to Roundup® (7000 mg l^{-1} glyphosate) on penicillin-amended (20 mg l^{-1}) agar (Table 4). Penicillin is not used in citrus orchards and penicillin resistance is hard to explain, except via cross resistance with glyphosate, which is applied frequently in citrus groves (Shin et al., 2016). Of the bacterial isolates that had not been exposed to penicillin or glyphosate, 39% were able to grow on the glyphosate amended medium (Table 4). In a related experiment (Shin and van Bruggen, 2017), 95% of unidentified bacteria isolated on plates with glyphosate amended medium (7000 mg l^{-1}) from citrus groves were resistant to penicillin (20 mg l^{-1}), while only 15–20% of these bacteria were resistant to streptomycin (50 mg l^{-1}) or tetracycline (16 mg l^{-1}) (Table 5). A slightly lower proportion (88% compared to 95%) of bacteria that had not been exposed to glyphosate in the isolation medium grew on the penicillin amended medium, while a much higher proportion (48–63% compared to 15–20%) grew on media amended with streptomycin or tetracycline, which can be used in citrus groves to control citrus canker (caused by *Xanthomonas axonopodis* pathovar *citri*). Altogether, this suggests that cross resistance between glyphosate and penicillin may be more common in citrus groves than cross resistance between glyphosate and streptomycin or tetracycline. The mechanisms underlying the glyphosate and antibiotic resistance have not been elucidated.

Similar to the effects of glyphosate on microbial communities, the herbicides that will increasingly replace glyphosate, dicamba and 2,4-D, also have differential effects on microorganisms (Oleszczuk et al., 2014; Seghers et al., 2003). The mechanisms that convey resistance to herbicides in plants are largely similar to those conferring resistance to herbicides in microorganisms. Thus, insensitivity to dicamba and 2,4-D will likely increase in microbial communities after intensification of the use of these herbicides. Finally, similar to the association between glyphosate and antibiotic resistance in microorganisms, insensitivity to dicamba and 2,4-D is often accompanied by antibiotic resistance (Kurenbach et al., 2015). Ultimately, antibiotic-resistant bacteria will likely be transferred from agricultural fields to farm animals and human patients in the hospital environment (Smith et al., 2005; Stine et al., 2007).

Table 4
Proportions (percentages) of bacteria that were isolated from soil on S medium plates amended with penicillin (20 mg l^{-1}) or on non-amended control plates and were able to grow on S medium plates amended with penicillin or glyphosate and on non-amended control plates.

Plates amended with	Concentration (mg l^{-1})	Colonies from penicillin amended S medium[a]	Colonies from non-amended S medium[a]
Penicillin	20	94/101 (93%)	17/84 (20%)
Glyphosate	7000	65/101 (64%)	33/84 (39%)
Control	0	101/101 (100%)	84/84 (100%)

[a] Isolations made from soil with citrus trees injected with penicillin in a field experiment at Fort Meade or a greenhouse experiment with field soil in Gainesville, Florida (combined data) (Shin et al., 2016).

Table 5
Proportions (percentages) of bacteria that were isolated from soil on glyphosate amended (7000 mg l^{-1}) or non-amended S medium plates and were able to grow on plates with glyphosate, penicillin, streptomycin or tetracycline, and on non-amended control plates.

Plates amended with	Concentration (mg l^{-1})	Colonies from glyphosate amended S medium[a]	Colonies from non-amended S medium[a]
Glyphosate	7000	40/40 (100%)	10/40 (25%)
Penicillin	20	38/40 (95%)	35/40 (88%)
Streptomycin	50	6/40 (15%)	25/40 (63%)
Tetracycline	16	8/40 (20%)	19/40 (48%)
Control	0	40/40 (100%)	40/40 (100%)

[a] Isolations made from soil from citrus groves at Clermont, Florida (Shin and van Bruggen, 2017).

5. Discussion and conclusions

Due to the almost exponential increase in glyphosate use and the slow decomposition of glyphosate and its breakdown product AMPA in soil, water and sediment, the accumulation of glyphosate in the environment, plant products and animal organs has become quite worrisome (Myers et al., 2016; Shehata et al., 2014). In particular, the high proportion of people and farm animals with glyphosate in their urine is concerning, even though the concentrations are still low (Niemann et al., 2015). Although the acute toxic effects of glyphosate on fish and mammals are low, the formulated products often are more toxic than glyphosate itself, and concerns have emerged about chronic effects of the formulated products on human and animal diseases, in particular various forms of cancer and mental disorders (Fortes et al., 2016; Mesnage et al., 2015a, 2015b; Swanson et al., 2014). Although conclusions regarding possible carcinogenicity and other health effects of glyphosate remain controversial, we feel that sufficient additional data has accumulated regarding the chronic toxic effects of the formulated products on aquatic and terrestrial animals and humans to warrant reconsideration of the tolerable residue levels of glyphosate and AMPA in plant and animal products and the environment. The recent reclassification of glyphosate as probably carcinogenic by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) was based primarily on research with the main formulated product Roundup® (IARC, 2015; Séralini et al., 2014). Additional research is needed to come to a definitive conclusion on the chronic health effects of the various formulated products containing glyphosate.

In addition to the possible chronic direct health effects of glyphosate on a variety of aquatic and terrestrial animals and humans, we documented shifts in microbial communities in soil, plants, water and intestinal tracts and the association with specific plant and animal pathogens (Ackermann et al., 2015; Priestman et al., 2005; Sanogo et al., 2000, 2001). The shifts in microbiomes resulting from intensive glyphosate use can affect resistance mechanisms and have severe impacts on plant, animal and human health (Hoffman et al., 2015). These complex, indirect effects of glyphosate need to be taken into account by regulatory agencies.

Other indirect health effects can come about through the recently documented cross resistance to glyphosate and clinically important antibiotics (Kurenbach et al., 2015). The surge in antibiotic resistance has been attributed primarily to the increased use of antibiotics by human patients and farm animals (Smith et al., 2005; Stine et al., 2007), but

antibiotic resistance is also widespread in agricultural soils that were not exposed to high antibiotic concentrations (Udikovic-Kolic et al., 2014). Considering that subsets of the microbiomes in soil transfer to plants, fresh plant products, animal and human intestinal tracts (Berg et al., 2014), and then to excrements that return to soil and water, we suggest that there are microbial cycles that are characteristic for particular management systems. Management of weeds with multiple glyphosate applications could result in microbiomes that are relatively glyphosate and antibiotic resistant. This leads us to the hypothesis that the selection pressure for glyphosate-resistance and the associated resistance to antibiotics in the soil microbiome result in transfer of antibiotic resistant bacteria from soil to plants, animals and humans through the food web, even in urban and hospital environments.

The sequence of events outlined here for glyphosate, namely introduction of glyphosate resistant crops, intensification of glyphosate use, emergence of glyphosate-resistant weeds and microorganisms, changes in microbiomes and disease resistance, deteriorated plant and animal health, and increased antibiotic resistance, could serve as a harbinger for events to follow the introduction of genes conferring resistance to other herbicides. The recent addition of genes for resistance to the herbicides dicamba and 2,4-D to glyphosate resistant crops (Ruen et al., 2017) will likely result in additional increases in herbicide use and unintended side effects (Leon et al., 2016). Weeds with multiple herbicide resistance at multiple sites of action have been detected already (Bell et al., 2013). Similar to glyphosate, dicamba and 2,4-D have differential effects on microorganisms, and shifts in plant and animal microbiomes can be expected as a result of intensification of the use of dicamba and 2,4-D (Oleszczuk et al., 2014; Seghers et al., 2003). The mechanisms that convey resistance to herbicides in plants are largely similar to those conferring resistance to herbicides in microorganisms, and thus, insensitivity to herbicides other than glyphosate will likely also increase in microbial communities, possibly followed by increased antibiotic resistance. These wide-ranging consequences of intensive herbicide use have not been pointed out previously.

In conclusion, we suggest that the problems associated with the large scale and intensive use of glyphosate (and other herbicides in the future) are much more encompassing than originally anticipated by the regulatory agencies (EPA, 2013). We recommend additional interdisciplinary research on the associations between low level chronic herbicide exposure, distortions in microbial communities, expansion of antibiotic resistance and the emergence of animal, human and plant diseases. Independent research is needed to revisit the tolerance thresholds for glyphosate residues in water, food and animal feed taking all possible health risks into account. A global effort will be needed to collect appropriate high quality residue and health data across the range of settings in which glyphosate and other herbicides are used; only as such data become available will we be able to design, develop and implement strategies to counter further escalation of the problems associated with the use of glyphosate and other herbicides.

Acknowledgement

We are grateful to Monika Krüger for sharing her powerpoint presentations on the side effects of glyphosate with us. We thank Max Teplitski for commenting on an earlier version of this manuscript. We acknowledge the contribution of the Esther B. O'Keeffe Foundation, the Emerging Pathogens Institute and the Institute of Food and Agricultural Sciences for the salaries of the first and third authors during this literature research. He Miaomiao received fellowship No. 201408330529 from the China Scholarship Council for a sabbatical leave at the University of Florida.

References

Abarikwu, S.O., Akiri, O.F., Durojaiye, M.A., 2015. Combined effects of repeated administration of Bretmont Wipeout (glyphosate) and Ultrazin (atrazine) on testosterone, oxidative stress and sperm quality of Wistar rats. Toxicol. Mech. Meth. 25, 70–80.

Achiorno, C.L., de Villalobos, C., Ferrari, L., 2008. Toxicity of the herbicide glyphosate to *Chordodes nobilii* (Gordiida, Nematomorpha). Chemosphere 71, 1816–1822.

Ackermann, W., Coenen, M., Schrödl, W., Shehata, A.A., Krüger, M., 2015. The influence of glyphosate on the microbiota and production of botulinum neurotoxin during ruminal fermentation. Curr. Microbiol. 70, 374–382.

Allegrini, M., Zabaloy, M.C., Gómez, E. del V., 2015. Ecotoxicological assessment of soil microbial community tolerance to glyphosate. Sci. Tot. Environ. 533, 60–68.

Alves Corrêa, E., Dayan, F.E., Owens, D.K., Rimando, A.M., Duke, S.O., 2016. Glyphosate-resistant and conventional canola (*Brassica napus* L.) responses to glyphosate and aminomethylphosphonic acid (AMPA) treatment. J. Agric. Food Chem. 64, 3508–3513.

Aparicio, V.C., De Gerónimo, E., Marino, D., Primost, J., Carriquiriborde, P., Costa, J.L., 2013. Environmental fate of glyphosate and aminomethylphosphonic acid in surface waters and soil of agricultural basins. Chemosphere 93, 1866–1873.

Apostolakos, I., Franz, E., van Hoek, A.H.A.M., Florijn, A., Veenman, C., Sloet-van Oldruitenborgh-Oosterbaan, M.M., Dierikx, C., van Duijkeren, E., 2017. Occurrence and molecular characteristics of ESBL/AmpC-producing *Escherichia coli* in faecal samples from horses in an equine clinic. J. Antimicrob. Chemother. https://doi.org/10.1093/jac/dkx072.

Arango, L., Buddrus-Schiemann, K., Opelt, K., Lueders, T., Haesler, F., Schmid, M., Ernst, D., Hartmann, A., 2014. Effects of glyphosate on the bacterial community associated with roots of transgenic Roundup Ready® soybean. Eur. J. Soil Biol. 63, 41–48.

Arregui, M.C., Lenardón, A., Sanchez, D., Maitre, M.I., Enrique, S., 2004. Monitoring glyphosate residues in transgenic glyphosate-resistant soybean. Pest Manag. Sci. 60, 163–166.

Arunakumara, K.K.I.U., Charana Walpola, B., Yoon, M.H., 2013. Metabolism and degradation of glyphosate in aquatic cyanobacteria: a review. Afr. J. Microbiol. Res. 7, 4084–4090.

Babiker, E.M., Hulbert, S.H., Schroeder, K.L., Paulitz, T.C., 2011. Optimum timing of preplant applications of glyphosate to manage *Rhizoctonia* root rot in barley. Plant Dis. 95, 304–310.

Bai, S.H., Ogbourne, S.M., 2016. Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. Environ. Sci. Pollut. Res. 23, 18988–19001.

Banks, M.L., Kennedy, A.C., Kremer, R.J., Eivazi, F., 2014. Soil microbial community response to surfactants and herbicides in two soils. Appl. Soil Ecol. 74, 12–20.

Barnett, K.A., Sprague, C.L., Kirk, W.W., Hanson, L.E., 2012. Influence of glyphosate on *Rhizoctonia* crown and root rot (*Rhizoctonia solani*) in glyphosate-resistant sugarbeet. Weed Sci. 60, 113–120.

Battaglin, W.A., Kolpin, D.W., Scribner, E.A., Kuivila, K.M., Sandstrom, M.W., 2005. Glyphosate, other herbicides, and transformation products in Midwestern streams, 2002. J. Am. Water Resour. Assoc. 41, 323–332.

Battaglin, W.A., Rice, K.C., Focazio, M.J., Salmons, S., Barry, R.X., 2009. The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005–2006. Environ. Monit. Assess. 155, 281–307.

Battaglin, W.A., Meyer, M.T., Kuivila, K.M., Dietze, J.E., 2014. Glyphosate and its degradation product AMPA occur frequently and widely in U.S. soils, surface water, groundwater, and precipitation. J. Am. Water Resour. Assoc. 50, 275–290.

Beale, D.J., Karpe, A.V., Ahmed, W., Cook, S., Morrison, P.D., Staley, C., Sadowsky, M.J., Palombo, E.A., 2017. A community multi-omics approach towards the assessment of surface water quality in an urban river system. Int. J. Environ. Res. Public Health 14:303. https://doi.org/10.3390/ijerph14030303.

Bell, M.S., Hager, A.G., Tranel, P.J., 2013. Multiple resistance to herbicides from four site-of-action groups in waterhemp (*Amaranthus tuberculatus*). Weed Sci. 61, 460–468.

Benbrook, C.M., 2012. Impacts of genetically engineered crops on pesticide use in the U.S. – the first sixteen years. Environ. Sci. Eur. 24:24. https://doi.org/10.1186/2190-4715-24-24.

Benbrook, C.M., 2016. Trends in glyphosate herbicide use in the United States and globally. Environ. Sci. Eur. 28 (3). https://doi.org/10.1186/s12302-016-0070-0.

Berg, G., Gruber, M., Schloter, M., Smalla, K., 2014. The plant microbiome and its importance for plant and human health. Front. Microbiol. 5, 491. https://doi.org/10.3389/fmicb.2014.00491.

Bergström, L., Börjesson, E., Stenström, J., 2011. Laboratory and lysimeter studies of glyphosate and aminomethyl phosphonic acid in a sand and a clay soil. J. Environ. Qual. 40, 98–108.

Birch, H., Mikkelson, P.S., Jenson, J.K., Lützhøft, H.C., 2011. Micropollutants in stormwater runoff and combined sewer overflow in the Copenhagen area, Denmark. Water Sci. Technol. 64, 485–493.

Bøhn, T., Cuhra, M., Travail, T., Sanden, M., Fagan, J., Primicerio, R., 2014. Compositional differences in soybeans on the market: glyphosate accumulates in Roundup Ready GM soybeans. Food Chem. 153, 207–215.

Bricheux, G., el LeMoal, G., Hennequin, C., Coffe, G., Donnadieu, F., Portelli, C., Bohatier, J., Forestier, C., 2013. Characterization and evolution of natural aquatic biofilm communities exposed in vitro to herbicides. Ecotoxicol. Environ. Safety 88, 126–134.

Bringolf, R.B., Cope, W.G., Mosher, S., Barnhart, M.C., Shea, D., 2007. Acute and chronic toxicity of glyphosate compounds to *Glochidia* and juveniles of *Lampsilis siliquoidea* (Unionidae). Environ. Chem. 26, 2094–2100.

Brolund, A., 2014. Overview of ESBL-producing Enterobacteriaceae from a Nordic perspective. Infect. Ecol. Epidemiol. 4, 24555. https://doi.org/10.3402/iee.v4.24555.

van Bruggen, A.H.C., Finckh, M., 2016. Plant diseases and management approaches in organic farming systems. Annu. Rev. Phytopathol. 54, 25–54.

van Bruggen, A.H.C., Sharma, K., Kaku, E., Karfopoulos, S., Zelenev, V.V., Blok, W.J., 2015. Soil health indicators and Fusarium wilt suppression in organically and conventionally managed greenhouse soils. Appl. Soil Ecol. 86, 192–201.

van Bruggen, A.H.C., Narouei Khandan, H.A., Gravel, V., Blok, W.J., 2016. Corky root severity, root knot nematode galling and microbial communities in soil, rhizosphere and rhizoplane in organic and conventional greenhouse compartments. Appl. Soil Ecol. 100, 112–123.

Çağlar, S., Kolankaya, D., 2008. The effect of sub-acute and sub-chronic exposure of rats to the glyphosate-based herbicide Roundup. Environ. Toxic. Pharmacol. 25, 57–62.

Cakmak, I., Yazici, A., Tutus, Y., Ozturk, L., 2009. Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. Eur. J. Agron. 31, 114–119.

Camacho, A., Mejía, D., 2017. The health consequences of aerial spraying illicit crops: the case of Colombia. J. Health Econ. 54, 147–160.

Cantas, L., Shah, S.Q.A., Cavaco, L.M., Manaia, C.M., Walsh, F., Popowska, M., Garelick, H., Bürgmann, H., Sørum, H., 2013. A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. Front. Microbiol. 4, 96. https://doi.org/10.3389/fmicb.2013.00096.

Cassigneul, A., Benoit, P., Bergheaud, V., Dumeny, V., Etiévant, V., Goubard, Y., Maylin, A., Justes, E., Alletto, L., 2016. Fate of glyphosate and degradates in cover crop residues and underlying soil: a laboratory study. Sci. Tot. Environ. 545–546, 582–590.

Catarzi, D., Colotta, V., Varano, F., 2006. Competitive AMPA receptor antagonists. Med. Res. Rev. 27, 239–278.

Cattani, D., Acordi Cesconetto, P., Kruger Tavares, M., Parisotto, E.B., De Oliveira, P.A., Heinz Rieg, C.E., Concli Leite, M., Schröder Prediger, R.D., Wendt, N.C., Razzera, G., Wilhelm Filho, D., Zamoner, A., 2017. Developmental exposure to glyphosate-based herbicide and depressive-like behavior in adult offspring: implication of glutamate excitotoxicity and oxidative stress. Toxicology 387, 67–80.

Cessna, A.J., Darwent, A.L., Kirkland, K.J., Townley-Smith, L., Harker, K.N., Lefkovitch, L.P., 1994. Residues of glyphosate and its metabolite AMPA in wheat seed and foliage following preharvest applications. Can. J. Plant Sci. 74, 653–661.

Cessna, A.J., Darwent, A.L., Townley-Smith, L., Harker, K.N., Kirkland, K.J., 2000. Residues of glyphosate and its metabolite AMPA in canola seed following preharvest applications. Can. J. Plant Sci. 80, 425–431.

Çetin, E., Şahan, S., Ülgen, A., Şahin, U., 2017. DLLME-spectrophotometric determination of glyphosate residue in legumes. Food Chem. 230, 567–571.

Chang, F.C., Simcik, M.F., Capel, P., 2011. Occurrence and fate of the herbicide glyphosate and its degradate aminomethylphosphonic acid in the atmosphere. Environ. Toxicol. Chem. 30, 548–555.

Chekan, J.R., Cogan, D.P., Nair, S.K., 2016. Molecular basis for resistance against phosphonate antibiotics and herbicides. Med. Chem. Commun. 7, 28–36.

Cherni, A.E., Trabelsi, D., Chebil, S., Barhoumi, F., Rodríguez-Lorente, I.D., Zribi, K., 2015. Effect of glyphosate on enzymatic activities, *Rhizobiaceae* and total bacterial communities in an agricultural Tunisian soil. Water Air Soil Pollut. 226:145. https://doi.org/10.1007/s11270-014-2263-8.

Clair, E., Linn, L., Travert, C., Amiel, C., Séralini, G.E., Panoff, J.M., 2012. Effects of Roundup(®) and glyphosate on three food microorganisms: *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Curr. Microbiol. 64, 486–491.

Clements, D., Dugdale, T.M., Butler, K.L., Florentine, S.K., Sillitoe, J., 2017. Herbicide efficacy for aquatic *Alternanthera philoxeroides* management in an early stage of invasion: integrating above-ground biomass, below-ground biomass and viable stem fragmentation. Weed Res. 57, 257–266.

CODEX Alimentarius, 2013. Pesticide Residues in Food and Feed: 158 Glyphosate. Food and Agriculture Organization and World Health Organization http://www.codexalimentarius.net/pestres/data/pesticides/details.html?id=158, Accessed date: 29 September 2015.

Čolović, M.B., Krstić, D.Z., Lazarević-Pašti, T.D., Bondžić, A.M., Vasić, V.M., 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr. Neuropharmacol. 11, 315–335.

Coullery, R.P., Ferrari, M.E., Rosso, S.B., 2016. Neuronal development and axon growth are altered by glyphosate through a WNT non-canonical signaling pathway. Neurotoxicology 52, 150–161.

Coupe, R.H., Capel, P.D., 2016. Trends in pesticide use on soybean, corn and cotton since the introduction of major genetically modified crops in the United States. Pest Manag. Sci. 72, 1013–1022.

Coupe, R.H., Kalkhoff, S.K., Capel, P.D., Gregoire, C., 2011. Fate and transport of glyphosate and aminomethylphosphonic acid in surface waters of agricultural basin. Pest Manag. Sci. 68, 16–30.

Cuhra, M., 2015. Review of GMO safety assessment studies: glyphosate residues in Roundup Ready crops is an ignored issue. Environ. Sci. Eur. 27 (20). https://doi.org/10.1186/s12302-015-0052-7.

Dai, P., Hu, P., Tang, J., Li, Y., Li, C., 2016. Effect of glyphosate on reproductive organs in male rat. Acta Histochem. 118, 519–526.

Daouk, S., Copin, P.J., Rossi, L., Chèvre, N., Pfeifer, H.R., 2013. Dynamics and environmental risk assessment of the herbicide glyphosate and its metabolite AMPA in a small vineyard river of the Lake Geneva catchment. Environ. Toxicol. Chem. 32, 2035–2044.

Diamond, G.L., Durkin, P.R., 1997. Effects of Surfactants on the Toxicitiy of Glyphosate, With Specific Reference to RODEO. Animal and Plant Health Inspection Service (APHIS), Biotechnology, Biologics and Environmental Protection, Environmental Analysis and Documentation, United States Department of Agriculture, Riverdale, MD 20737, USA 28 pp. https://www.fs.fed.us/foresthealth/pesticide/pdfs/Surfactants.pdf, Accessed date: 17 September 2019.

Druille, M., Cabello, M.N., García Parisi, P.A., Golluscio, R.A., Omacini, M., 2015. Glyphosate vulnerability explains changes in root-symbionts propagules viability in pampean grasslands. Agric. Ecosyst. Environ. 202, 48–55.

Duke, S.O., 2015. Perspectives on transgenic, herbicide-resistant crops in the United States almost 20 years after introduction. Pest. Manag. Sci. 71, 652–657.

Duke, S.O., Powles, S.B., 2009. Glyphosate-resistant crops and weeds: now and in the future. AgBioforum 12, 346–357.

Duke, S.O., Lydon, J., Koskinen, W.C., Moorman, T.B., Chaney, R.L., Hammerschmidt, R., 2012. Glyphosate effects on plant mineral nutrition, crop rhizosphere microbiota, and plant disease in glyphosate-resistant crops. J. Agric. Food Chem. 60, 10375–10397.

Durkin, P.R., 2011. Glyphosate: Human Health and Ecological Risk Assessment. Final Report Submitted to the USDA Forest Service, SERA TR-052-22-03b. Syracuse Environmental Research Associates, Inc., Manlius, New York, USA https://www.fs.fed.us/foresthealth/pesticide/pdfs/Glyphosate_SERA_TR-052-22-03b.pdf, Accessed date: 22 September 2017.

EFSA (European Food Safety Authority), 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. EFSA J. 13 (11):4302 (107 pp.). https://doi.org/10.2903/j.efsa.2015.4302.

EPA (Environmental Protection Agency), 2013. Electronic code of federal regulations, title 40: protection of environment. PART 180-Tolerances and Exemptions for Pesticide Chemical Residues in Food. Subpart C-Specific Tolerances. Environmental Protection Agency, Washington, DC http://www.ecfr.gov/cgi-bin/text-idx?tpl=/ecfrbrowse/Title40/40cfr180_main_02.tpl, Accessed date: 29 September 2015.

FAO, 2006. Pesticide Residues in Food – 2005. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues Geneva, Switzerland, 20–29 September 2005. FAO Plant Production and Protection Paper 183. FAO, Rome, Italy (http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Report11/Glyphosate.pdf). http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Report13/5.21_GLYPHOSATE__158_.pdf, Accessed date: 7 October 2017.

Fei, Y.Y., Gai, J.Y., Zhao, T.J., 2013. Identification of regulated genes conferring resistance to higher concentrations of glyphosate in a new strain of *Enterobacter*. FEMS Microbiol. Lett. 349, 135–143.

Fernandez, M.R., Zentner, R.P., Basnyat, P., Gehl, D., Selles, F., Huber, D., 2009. Glyphosate associations with cereal diseases caused by *Fusarium* spp. in the Canadian Prairies. Eur. J. Agron. 31, 133–143.

Finckh, M.R., van Bruggen, A.H.C., Tamm, L. (Eds.), 2015. Plant Diseases and Their Management in Organic Agriculture. APS Press, St. Paul, Minnesota, USA (402 pp.).

Fluegge, K., Fluegge, K., 2016. Glyphosate use predicts healthcare utilization for ADHD in the healthcare cost and utilization project net (HCUPnet): a two-way fixed-effects analysis. Pol. J. Environ. Stud. 25, 1489–1503.

Fortes, C., Mastroeni, S., Segatto, M.M., Hohmann, C., Miligi, L., Bakos, L., Bonamigo, R., 2016. Occupational exposure to pesticides with occupational sun exposure increases the risk for cutaneous melanoma. J. Occup. Environ. Med. 58, 370–375.

Funke, T., Han, H., Healy-Fried, M.L., Fischer, M., Schönbrunn, E., 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. Proc. Natl. Acad. Sci. 103, 13010–13015.

Funke, T., Healy-Fried, M.L., Han, H., Alberg, D.G., Bartlett, P.A., Schönbrunn, E., 2007. Differential inhibition of class I and class II 5-enolpyruvylshikimate-3-phosphate synthases by tetrahedral reaction intermediate analogues. Biochemist 46, 13344–13351.

Gerlach, H., Gerlach, A., Schrödl, W., Schottdorf, B., Haufe, S., Helm, H., Shehata, A., Krüger, M., 2014. Oral application of charcoal and humic acids to dairy cows influences *Clostridium botulinum* blood serum antibody level and glyphosate excretion in urine. J. Clin. Toxicol. 4:186. https://doi.org/10.4172/2161-0495.186.

Goffnett, A.M., Sprague, C.L., Mendoza, F., Cichy, K.A., 2016. Preharvest herbicide treatments affect black bean desiccation, yield, and canned bean color. Crop Sci. 56, 1962–1969.

Gomes, M.P., Gingras Le Manac'h, S., Maccario, S., Labrecque, M., Lucotte, M., Juneau, P., 2016. Differential effects of glyphosate and aminomethylphosphonic acid (AMPA) on photosynthesis and chlorophyll metabolism in willow plants. Pestic. Biochem. Physiol. 130, 65–70.

Grandcoin, A., Piel, S., Baurès, E., 2017. AminoMethylPhosphonic acid (AMPA) in natural waters: its sources, behavior and environmental fate. Water Res. 117, 187–197.

Green, J.M., Owen, M.D.K., 2011. Herbicide-resistant crops: utilities and limitations for herbicide-resistant weed management. J. Agric. Food Chem. 59, 5819–5829.

Greim, H., Saltmiras, D., Mostert, V., Strupp, C., 2015. Evaluation of carcinogenic potential of the herbicide glyphosate, drawing on tumor incidence data from fourteen chronic/carcinogenicity rodent studies. Crit. Rev. Toxicol. 45, 185–208.

Guilherme, S., Gaivao, I., Santos, M.A., Pacheco, M., 2009. Tissue specific DNA damage in the European eel (*Anguilla anguilla*) following a short-term exposure to a glyphosate-based herbicide. Toxicol. Lett. 189, S212 (Z15).

Guyton, K.Z., Loomis, D., Grosse, Y., Ghissassi, F.E., Benbrahim-Tallaa, L., Guha, N., Scoccianti, C., Matlock, H., Straif, K., 2015. Carcinogenicity of tetrachlorvinphos, parathion, malathion, diazinon, and glyphosate. Lancet Oncol. 16, 490–491.

Haney, R.L., Senseman, S.A., Hons, F.M., Zuberer, D.A., 2000. Effect of glyphosate on soil microbial activity and biomass. Weed Sci. 49, 89–93.

Hanke, I., Wittmer, I., Bischofberger, S., Stamm, S., Singer, H., 2010. Relevance of urban glyphosate use for surface water quality. Chemosphere 81, 422–429.

Hervouet, E., Cheray, M., Vellette, F.M., Cartron, P.-F., 2013. DNA methylation and apoptosis resistance in cancer cells. Cell 2, 545–573.

Hoffman, A.R., Proctor, L.M., Surette, M.G., Suchodolski, J.S., 2015. The microbiome: the trillions of microorganisms that maintain health and cause disease in humans and companion animals. Vet. Pathol. 53:10–12. https://doi.org/10.1177/0300985815595517.

Humphries, D., Brytus, G., Anderson, A.M., 2005. Glyphosate residues in Alberta's Atmospheric Deposition, Soils and Surface Waters. Water Research Users Group Alberta Environment Pub No. T/806. https://extranet.gov.ab.ca/env/infocentre/info/library/6444.pdf, Accessed date: 17 August 2002.

Hungria, M., Carvalho Mendes, I., Shigueyoshi Nakatani, A., Bueno dos Reis-Junior, F., Zucca Morais, J., Neves de Oliveira, M.C., Ferreira Fernandes, M., 2014. Effects of the glyphosate-resistance gene and herbicides on soybean: field trials monitoring biological nitrogen fixation and yield. Field Crops Res. 158, 43–54.

IARC (International Agency for Research on Cancer), 2015. Evaluation of five organophosphate insecticides and herbicides. IARC Monographs. vol. 112. World Health Organization, International Agency for Research on Cancer, Lyon, France . http://www.iarc.fr/en/media-centre/iarcnews/pdf/MonographVolume112.pdf, Accessed date: 29 September 2015.

Jayasumana, C., Gunatilake, S., Senanayake, P., 2014. Glyphosate, hard water, and nephrotoxic metals: are they the culprits behind the epidemic of chronic kidney disease of unknown etiology in Sri Lanka? Int. J. Environ. Res. Public Health 11, 2125–2147.

Johal, G.S., Huber, D.M., 2009. Glyphosate effects on diseases of plants. Eur. J. Agron. 31, 144–152.

Johal, G.S., Rahe, J.E., 1984. Effect of soilborne plant-pathogenic fungi on the herbicidal action of glyphosate on bean seedlings. Phytopathology 74, 950–955.

Kandel, Y.R., Bradley, C.A., Wise, K.A., Chilvers, M.I., Tenuta, A.U., Davis, V.M., Esker, P.D., Smith, D.L., Licht, M.A., Mueller, D.S., 2015. Effect of glyphosate application on sudden death syndrome of glyphosate-resistant soybean under field conditions. Plant Dis. 99, 347–354.

Kelly, D.W., Poulin, R., Tompkins, D.M., Townsend, C.R., 2010. Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival. J. Appl. Ecol. 47, 498–504.

Kloepper, J.W., Leong, J., Teintze, M., Schroth, M.N., 1980. Enhancing plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286, 885–886.

Kremer, R.J., Means, N.E., 2009. Glyphosate and glyphosate-resistant crop interactions with rhizosphere microorganisms. Eur. J. Agron. 31, 153–161.

Kremer, R., Means, N., Kim, S., 2005. Glyphosate affects soybean root exudation and rhizosphere micro-organisms. Int. J. Environ. Anal. Chem. 85, 1165–1174.

Kreutz, L.C., Gil Barcellos, L.J., Marteninghe, E., Davidos Santos, E., Zanatta, R., 2010. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. Fish Shellfish Immunol. 29, 694–697.

Kreutz, L.C., Gil Barcellos, L.J., Valle, S.F., Silva, T.O., Anziliero, D., Davidos Santos, E., Pivato, M., Zanatta, R., 2011. Altered hematological and immunological parameters in silver catfish (*Rhamdia quelen*) following short term exposure to sublethal concentration of glyphosate. Fish Shellfish Immunol. 30, 51–57.

Kristoffersen, P., Rask, A.M., Grundy, A.C., Franzen, I., Kempenaar, C., Raisio, J., Schroeder, H., Spijker, J., Verschwele, A., Zarina, L., 2008. A review of pesticide policies and regulations for urban amenity areas in seven European countries. Weed Res. 48, 201–214.

Krüger, M., Shehata, A., 2014. Collateral Damages of the Herbicide Glyphosate in Dairy Cows, Current Possibilities to Neutralize This Contamination. Institute of Bacteriology and Mycology of Veterinary Faculty, Universitat Leipzig, Germany http://www.ensser.org/fileadmin/files/Science_in_the_Eye_of_the_Storm/II_Monika_Kr%C3%BCger_-_Collateral_damages_of_the_herbicide__glyphosate__in_dairy.pdf, Accessed date: 17 September 2004.

Krüger, M., Shehata, A.A., Schrödl, W., Rodloff, A., 2013. Glyphosate suppresses the antagonistic effect of *Enterococcus* spp. on *Clostridium botulinum*. Anaerobe 20, 74–78.

Krüger, M., Schledorn, P., Schrödl, W., Hoppe, H.W., Lutz, W., Shehata, A.A., 2014a. Detection of glyphosate residues in animals and humans. J. Environ. Anal. Toxicol. 4, 2. https://doi.org/10.4172/2161-0525.1000210.

Krüger, M., Schrödl, W., Pedersen, I., Schledorn, P., Shehata, A.A., 2014b. Detection of glyphosate in malformed piglets. J. Environ. Anal. Toxicol. 4, 5. https://doi.org/10.4172/2161-0525.1000230.

Kuklinsky-Sobral, J., Araújo, W.L., Mendes, R., Pizzirani-Kleiner, A.A., Azevedo, J.L., 2005. Isolation and characterization of endophytic bacteria from soybean (*Glycine* max) grown in soil treated with glyphosate herbicide. Plant Soil 273, 91–99.

Kurenbach, B., Marjoshi, D., Amábile-Cuevas, C.F., Ferguson, G.C., Godsoe, W., Gibson, P., Heinemann, J.A., 2015. Sublethal exposure to commercial formulations of the herbicides dicamba, 2,4-dichlorophenoxyacetic acid, and glyphosate cause changes in antibiotic susceptibility in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. mBio 6 (2), e00009-15. https://doi.org/10.1128/mBio.00009-15.

Kwiatkowska, M., Huras, B., Bukowska, B., 2014a. The effect of metabolites and impurities of glyphosate on human erythrocytes (in vitro). Pestic. Biochem. Phys. 109, 34–43.

Kwiatkowska, M., Nowacka-Krukowska, H., Bukowska, B., 2014b. The effect of glyphosate, its metabolites and impurities on erythrocyte acetylcholinesterase activity. Environ. Toxicol. Pharmacol. 37, 1101–1108.

Kwiatkowska, M., Reszka, E., Woźniak, K., Jabłońska, E., Michałowicz, J., Bukowska, B., 2017. DNA damage and methylation induced by glyphosate in human peripheral blood mononuclear cells (in vitro study). Food Toxicol. 105, 93–98.

Landrigan, P.J., Benbrook, C., 2015. GMOs, herbicides, and public health. N. Engl. J. Med. 373, 693–695.

Larson, R.L., Hill, A.L., Fenwick, A., Kniss, A.R., Hanson, L.E., Miller, S.D., 2006. Influence of glyphosate on *Rhizoctonia* and *Fusarium* root rot in sugar beet. Pest Manag. Sci. 62, 1182–1192.

Lee, H., Ullrich, S.E., Burke, I.C., Yenish, J., Paulitz, T.C., 2012. Interactions between the root pathogen *Rhizoctonia solani* AG-8 and acetolactatesynthase-inhibiting herbicides in barley. Pest Manag. Sci. 68, 845–852.

Leon, R.G., Ferrell, J.A., Sellers, B.A., 2016. Seed production and control of sicklepod (*Senna obtusifolia*) and pitted morningglory (*Ipomoea lacunosa*) with 2,4-D, dicamba, and glyphosate combinations. Weed Technol. 30, 76–84.

Li, J., Smeda, R.J., Sellers, B.A., Johnson, W.G., 2005. Influence of formulation and glyphosate salt on absorption and translocation in three annual weeds. Weed Sci. 53, 153–159.

Li, L., Zhou, Z., Jin, W., Wan, Y., Lu, W., 2015. A transcriptomic analysis for identifying the unintended effects of introducing a heterologous glyphosate-tolerant EPSP synthase into *Escherichia coli*. Mol. BioSyst. 11, 852–858.

Li, M.H., Ruan, L.Y., Zhou, J.W., Fu, Y.H., Jiang, L., Zhao, H., Wang, J.S., 2017. Metabolic profiling of goldfish (*Carassius auratis*) after long-term glyphosate based herbicide exposure. Aquat. Toxicol. 188, 159–169.

Liu, Y.B., Long, M.X., Yin, Y.J., Si, M.R., Zhang, L., Lu, Z.Q., Wang, Y., Shen, X.H., 2013. Physiological roles of mycothiol in detoxification and tolerance to multiple poisonous chemicals in *Corynebacterium glutamicum*. Arch. Microbiol. 195, 419–429.

Magbanua, F.S., Townsend, C.R., Hageman, K.J., Lange, K., Lear, G., Lewis, G.D., Matthaei, C.D., 2013. Understanding the combined influence of fine sediment and glyphosate herbicide on stream periphyton communities. Water Res. 47, 5110–5120.

Mahler, B.J., Van Metre, P.C., Burley, T.E., Loftin, K.A., Meyer, M.T., Nowell, L.H., 2017. Similarities and differences in occurrence and temporal fluctuations in glyphosate and atrazine in small Midwestern streams (USA) during the 2013 growing season. Sci. Total Environ. 579, 149–158.

Mamy, L., Barriuso, E., Gabrielle, B., 2016. Glyphosate fate in soils when arriving in plant residues. Chemosphere 154, 425–433.

Maqueda, C., Undabeytia, T., Villaverde, J., Morillo, E., 2017. Behaviour of glyphosate in a reservoir and the surrounding agricultural soils. Sci. Total Environ. 593–594, 787–795.

McComb, B.C., Curtis, L., Chambers, C.L., Newton, M., Bentson, K., 2008. Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon coast range. Environ. Sci. Pollut. Res. 15, 266–272.

McNaughton, K.E., Blackshaw, R.E., Waddell, K.A., Gulden, R.H., Sikkema, P.H., Gillard, C.L., 2015. Effect of application timing of glyphosate and saflufenacil as desiccants in dry edible bean (*Phaseolus vulgaris* L.). Can. J. Plant Sci. 95, 369–375.

Meador, M., Jie, Ma, 2014. China - Peoples Republic of, Maximum Residue Limits for Pesticides in Food, GAIN Report CG 14019. USDA Foreign Agricultural Service, Washington DC, USA https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Maximum%20Residue%20Limits%20for%20Pesticides%20in%20Food_Beijing_China%20-%20Peoples%20Republic%20of_4-30-2014.pdf, Accessed date: 27 October 2017.

Menéndez-Helman, R.J., Ferreyroa, G.V., dos Santos Alfonso, M., Salibrán, A., 2012. Glyphosate as an acetylcholinesterase inhibitor in *Cnesterodon decemmaculatus*. Bull. Environ. Contam. Toxicol. 88, 6–9.

Mengistu, A., Reddy, K.N., Bellaloui, N., Walker, E.R., Kelly, H.M., 2013. Effect of glyphosate on *Macrophomina phaseolina* in vitro and its effect on disease severity of soybean in the field. Crop. Prot. 54, 23–28.

Mensink, H., Janssen, P., 1994. Environmental Health Criteria 159 – Glyphosate. National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands. Report for the International Programme on Chemical Safety, United Nations Environment Programme, the International Labour Organisation. World Health Organization, Geneva, Switzerland http://www.inchem.org/documents/ehc/ehc/ehc159.htm#SubSectionNumber:9.1.1, Accessed date: 25 September 2017.

Mercurio, P., Flores, F., Mueller, J.F., Carter, S., Negri, A.P., 2014. Glyphosate persistence in seawater. Mar. Pollut. Bull. 85, 385–390.

Meriles, J.M., Vargas Gil, S., Haro, R.J., March, G.J., Guzmán, C.A., 2008. Selected soil-borne fungi under glyphosate application and crop residues from a long-term field experiment. Biol. Agric. Hortic. 26, 193–205.

Mesnage, R., Arno, M., Costanzo, M., Malatesta, M., Séralini, G.E., Antoniou, M.N., 2015a. Transcriptome profile analysis reflects rat liver and kidney damage following chronic ultra-low dose Roundup exposure. Environ. Health 14, 70–84.

Mesnage, R., Defarge, N., Spiroux de Vendômois, J., Séralini, G.E., 2015b. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. Food Chem. Toxicol. 84, 133–153.

Mesnage, R., Renney, G., Seralini, G.-E., Ward, M., Antoniou, M.N., 2016. Multiomics reveal non-alcoholic fatty liver disease in rats following chronic exposure to an ultra-low dose of Roundup herbicide. Sci Rep 7, 39328. https://doi.org/10.1038/srep39328.

Monsanto, 2002. Risk Assessment and Risk Management Plan Final Version DIR 020/2002, Commercial Release of Roundup Ready® Canola. http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/dir020-3/$FILE/dir020finalrarmpsum.pdf, Accessed date: 27 July 2017.

Monsanto, 2013. What is Roundup Ready® Canola? http://www.monsanto.com/global/au/products/documents/tech-topic-what-is-roundup-ready-canola.pdf, Accessed date: 27 July 2017

Monsanto, 2014. Backgrounder Glyphosate and Water Quality. https://monsanto.com/app/uploads/2017/06/glyphosate-and-water-quality.pdf, Accessed date: 27 July 2017.

Morace, G., Borghi, E., 2012. Invasive mold infections: virulence and pathogenesis of *Mucorales*. Int. J. Microbiol. 2012 (349278). https://doi.org/10.1155/2012/349278.

Moreno, N.C., Sofia, S.H., Martinez, C.B.R., 2014. Genotoxic effects of the herbicide Roundup Transorb® and its active ingredient glyphosate on the fish *Prochilodus lineatus*. Eviron. Toxicol. Phar. 37, 448–454.

Mörtl, M., Németh, G., Juracsek, J., Darvas, B., Kamp, L., Rubio, F., Székács, A., 2013. Determination of glyphosate residues in Hungarian water samples by immunoassay. Microchem. J. 107, 143–151.

Muturi, E.J., Donthu, R.K., Fields, C.J., Moise, I.K., Kim, C.H., 2017. Effect of pesticides on microbial communities in container aquatic habitats. Sci. Rep. 7, 44565. https://doi.org/10.1038/srep44565.

Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., Vandenberg, L.N., vom Saal, F.S., Welshons, W.V., Benbrook, C.M., 2016. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environ. Health 15:19. https://doi.org/10.1186/s12940-016-0117-0.

Nakatani, A.S., Fernandes, M.F., de Souza, A.R., da Silva, P.A., dos Reis-Jr, F.B., Mendes, I.C., Hungria, M., 2014. Effects of the glyphosate-resistance gene and of herbicides applied to the soybean crop on soil microbial biomass and enzymes. Field Crop Res. 162, 20–29.

Nardi, J., Bonamigo Moras, P., Koeppe, C., Dallegrave, E., Bainy Leal, M., Grazziotin Rossato-Grando, L., 2017. Prepubertal subchronic exposure to soy milk and glyphosate leads to endocrine disruption. Food Chem. Toxicol. 100, 247–252.

Natarajan, S., Stuchlík, S., Kukučková, M., Renczésová, V., Vávrová, S., Bargárová, Z., Pálffy, R., Celec, P., Mačor, M., Turňa, J., 2007. Comparative study of two forms of aro A CP4 gene in *Escherichia coli*. Biologia, Bratislava 62, 265–269.

Nelson, K.A., Massey, R.E., Burdick, B.A., 2011. Harvest aid application timing affects wheat and relay intercropped soybean yield. Agron. J. 103, 851–855.

Newman, M.M., Lorenz, N., Hoilett, N., Lee, N.R., Dick, R.P., Liles, M.R., Ramsier, C., Kloepper, J.W., 2016. Changes in rhizosphere bacterial gene expression following glyphosate treatment. Sci. Total Environ. 553, 32–41.

Niemann, L., Sieke, C., Pfeil, R., Solecki, R., 2015. A critical review of glyphosate findings in human urine samples and comparison with the exposure of operators and consumers. J. Verbr. Lebensm. 10, 3–12.

O'Doherty, K.C., Neufeld, J.D., Brinkman, F.S.L., Gardner, H., Guttman, D.S., Beiko, R.G., 2014. Opinion: conservation and stewardship of the human microbiome. Proc. Nat. Acad. Sci. 111, 14312–14313.

Okada, E., Luis Costa, J., Bedmar, F., 2016. Adsorption and mobility of glyphosate in different soils under no-till and conventional tillage. Geoderma 263, 78–85.

Oleszczuk, P., Jośko, I., Futa, B., Pasieczna-Patkowska, S., Pałys, E., Kraska, P., 2014. Effect of pesticides on microorganisms, enzymatic activity and plant in biochar-amended soil. Geoderma 214–215, 10–18.

Padgette, S.R., Kolacz, K.H., Delannay, X., Re, D.B., LaVallee, B.J., Tinius, C.N., Rhodes, W.K., Otero, Y.I., Barry, G.F., Eichholtz, D.A., 1995. Development, identification, and characterization of a glyphosate-tolerant soybean line. Crop Sci. 35, 1451–1461.

Perego, C.M., Schutz, L.F., Caloni, F., Cortinovis, C., Albonico, M., Spicer, L.J., 2017. Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells in vitro. Appl. Toxicol. 37, 692–698.

Pérez, G.L., Torremorell, A., Muqni, H., Rodríguez, P., Solange Vera, M., do Nascimento, M., Allende, L., Bustingorry, J., Escaray, R., Ferraro, M., Izaguirre, I., Pizarro, H., Bonetto, C., Morris, D.P., Zagarese, H., 2007. Effects of the herbicide Roundup on freshwater microbial communities: a mesocosm study. Ecol. Appl. 17, 2310–2322.

Pesce, S., Batisson, I., Bardot, C., Fajon, C., Portelli, C., Montuelle, B., Bohatier, J., 2009. Response of spring and summer riverine microbial communities following glyphosate exposure. Ecotoxicol. Environ. Safety 72, 1905–1912.

Pizarro, H., Vera, M.S., Vinocur, A., Pérez, G., Ferraro, M., Menéndez Helman, R.J., dos Santos Afonso, M., 2016. Glyphosate input modifies microbial community structure in clear and turbid freshwater systems. Environ. Sci. Pollut. Res. 23, 5143–5153.

Pleban, S., Ingel, F., Chet, I., 1995. Control of *Rhizoctonia solani* and *Sclerotium rolfsii* in the greenhouse using endophytic *Bacillus* spp. Eur. J. Plant Pathol. 101, 665.672.

Poiger, T., Buerge, I.J., Bächli, A., Müller, M.D., Balmer, M.E., 2017. Occurrence of the herbicide glyphosate and its metabolite AMPA in surface waters in Switzerland determined with on-line solid phase extraction LC-MS/MS. Environ. Sci. Poll. Res. 24, 1588–1596.

Pollegioni, L., Schonbrunn, E., Siehl, D., 2011. Molecular basis of glyphosate-resistance: different approaches through protein engineering. FEBS J. 278, 2753–2766.

Priestman, M.A., Funke, T., Singh, I.M., Crupper, S.S., Schönbrunn, E., 2005. 5-Enolpyruvylshikimate-3-phosphate synthase from *Staphylococcus aureus* is insensitive to glyphosate. FEBS Lett. 579, 728–732.

Prosser, R.S., Rodriguez-Gil, J.L., Solomon, K.R., Sibley, P.K., Poirier, D.G., 2017. Effects of the herbicide surfactant MON0818 on oviposition and viability of eggs of the ramshorn snail (*Planorbella pilsbryi*). Environ. Toxicol. Chem. 36, 522–531.

Rashid, A., Hwang, S.F., Ahmed, H.U., Turnbull, G.D., Strelkov, S.E., Gossen, B.D., 2013. Effects of soil-borne *Rhizoctonia solani* on canola seedlings after application of glyphosate herbicide. Can. J. Plant Sci. 93, 97–107.

Rendón-von Osten, J., Dzul-Caamal, R., 2017. Glyphosate residues in groundwater, drinking water and urine of subsistence farmers from intensive agriculture localities: a survey in Hopelchén, Campeche, Mexico. Int. J. Environ. Res. Public Health 14:595. https://doi.org/10.3390/ijerph14060595.

Rissoli, R.Z., Camargo Abdalla, F., Jones Costa, M., Tadeu Rantin, F., McKenzie, D.J., Kalinin, A.L., 2016. Effects of glyphosate and the glyphosate based herbicides Roundup Original® and Roundup Transorb® on respiratory morphophysiology of bullfrog tadpoles. Chemosphere 156, 37–44.

Rodloff, A.C., Krüger, M., 2012. Chronic *Clostridium botulinum* infections in farmers. Anaerobe 18, 226–228.

Rodrigues, L. de B., de Oliveira, R., Abe, F.R., Barroso Brito, R., Dousa Moura, D., Campos Valadares, M., Koppe Grisolia, C., Palma de Oliveira, D., Rodrigues de Oliveira, G.A., 2017. Ecotoxicological assessment of glyphosate-based herbicides: effects on different organisms. Environ. Toxicol. Chem. 36, 1755–1763.

Rodriguez-Gil, J.L., Prosser, R., Poirier, D., Lissemore, L., Thompson, D., hanson, M., Solomon, K.R., 2017. Aquatic hazard assessment of mon 0818, a commercial mixture of alkylamine ethoxylates commonly used in glyphosate-containing herbicide formulations. Part 1: species sensitivity distribution from laboratory acute exposures. Environ. Toxicol. Chem. 36, 501–511.

Rosenbaum, K.K., Miller, G.L., Kremer, R.J., Bradley, K.W., 2014. Interactions between glyphosate, *Fusarium* infection of common waterhemp (*Amaranthus rudis*), and soil microbial abundance and diversity in soil collections from Missouri. Weed Sci. 62, 71–82.

Rosenbom, A.E., Brüsch, W., Juhler, R.K., Ernstsen, V., Gudmundsson, L., Kjær, J., Plauborg, F., Grant, R., Nyegaard, P., Olsen, P., 2010. The Danish Pesticide Leaching Assessment Programme Monitoring Results May 1999–June 2009. Geological Survey of Denmark and GreenlandMinistry of Climate and Energy and Faculty of Agricultural Sciences.

Roy, N.M., Carneiro, B., Och, J., 2016. Glyphosate induces neurotoxicity in zebrafish. Environ. Toxicol. Pharmacol. 42, 45–54.

Ruen, D.C., Scherder, E.F., Ditmarsen, S.C., Prasifka, P.L., Ellis, J.M., Simpson, D.M., Gallup, C.A., Hopkins, B.W., 2017. Tolerance of corn with glyphosate-resistance and the aryloxyalkanoate dioxygenase trait (AAD-1) to 2,4-D choline and glyphosate. Weed Technol. 31, 217–224.

Sanchis, J., Kantiani, L., Llorca, M., Rubio, F., Ginebreda, A., Fraile, J., Garrido, T., Farré, M., 2012. Determination of glyphosate in groundwater samples using an ultrasensitive immunoassay and confirmation by on-line solid-phase extraction followed by liquid chromatography coupled to tandem mass spectrometry. Anal. Bioanal. Chem. 402, 2335–2345.

Sandrini, J.Z., Rola, R.C., Lopes, F.M., Buffon, H.F., Freitas, H.F., Freita, M.M., Martins, C.D., da Rosa, C.E., 2013. Effects of glyphosate on cholinesterase activity of the mussel *Perna perna* and the fish *Danio rerio* and *Jenynsia multidentata*: in vitro studies. Aquat. Toxicol. 130-131, 171–173.

Sanogo, S., Yang, X.B., Scherm, H., 2000. Effects of herbicides on *Fusarium solani* f. sp. *glycines* and development of sudden death syndrome in glyphosate-tolerant soybean. Phytopathology 90, 57–66.

Sanogo, S., Yang, X.B., Lundeen, P., 2001. Field response of glyphosate-tolerant soybean to herbicides and sudden death syndrome. Plant Dis. 85, 773–779.

Sanyal, D., Shrestha, A., 2008. Direct effect of herbicides on plant pathogens and disease development in various cropping systems. Weed Sci. 56, 155–160.

Schafer, J.R., Hallett, S.G., Johnson, W.G., 2014. Rhizosphere microbial community dynamics in glyphosate-treated susceptible and resistant biotypes of giant ragweed (*Ambrosia trifida*). Weed Sci. 62, 370–381.

Schrödl, W., Krüger, S., Konstantinova-Müller, T., Shehata, A.A., Rulff, R., Krüger, M., 2014. Possible effects of glyphosate on *Mucorales* abundance in the rumen of dairy cows in Germany. Curr. Microbiol. 69, 817–823.

Schrübbers, L.C., Valverde, B.E., Strobel, B.W., Cedergreen, N., 2016. Glyphosate accumulation, translocation, and biological effects in *Coffea arabica* after single and multiple exposures. Eur. J. Agron. 74, 133–143.

Seghers, D., Verthé, K., Reheul, D., Bulcke, R., Siciliano, S.D., Verstraete, W., Top, E.M., 2003. Effect of long-term herbicide applications on the bacterial community structure and function in an agricultural land. FEMS Microbiol. Ecol. 46, 139–146.

Séralini, G.E., Clair, E., Mesnage, R., Gress, S., Defarge, N., Malatesta, M., Hennequin, D., Spiroux de Vendômois, J., 2014. Republished study: long-term toxicity of a roundup herbicide and a Roundup-tolerant genetically modified maize. Environ. Sci. Eur. 26:14. http://www.enveurope.com/content/26/1/14, Accessed date: 15 September 2014.

Shaner, D.L., Lindenmeijer, R.B., Ostlie, M.H., 2012. What have mechanisms of resistance to glyphosate taught us? Pest Manag. Sci. 68, 3–9.

Sharma-Poudyal, D., Paulitz, T.C., du Toit, L.J., 2016. Timing of glyphosate applications to wheat cover crops to reduce onion stunting caused by *Rhizoctonia solani*. Plant Dis. 100, 1474–1481.

Shehata, A.A., Schrödl, W., Aldin, A.A., Hafez, H.M., Krüger, M., 2013. The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Curr. Microbiol. 66, 350–358.

Shehata, A.A., Schrödl, W., Schledorn, P., Krüger, M., 2014. Distribution of glyphosate in chicken organs and its reduction by humic acid supplementation. J. Poult. Sci. 51, 334–338.

Shin, K., van Bruggen, A.H.C., 2017. *Bradyrhizobium* isolated from huanglongbing (HLB) affected citrus trees reacts positively with primers for *Candidatus Liberibacter* asiaticus. Eur. J. Plant Pathol. online, https://doi.org/10.1007/s10658-017-1372-9.

Shin, K., Ascunce, M.S., Narouei-Khandan, H.A., Sun, X., Jones, D.J., Kolawole, O.O., Goss, E., van Bruggen, A.H.C., 2016. Effects and side effects of penicillin injection in huanglongbing affected grapefruit trees. Crop. Prot. 90, 106–116.

Shushkova, T., Ermakova, I., Leontievsky, A., 2009. Glyphosate bioavailability in the soil. Biodegradation 21, 403–410.

Sidoli, P., Baran, N., Angulo-Jaramillo, R., 2016. Glyphosate and AMPA adsorption in soils: laboratory experiments and pedotransfer rules. Environ. Sci. Pollut. Res. 23, 5733–5742.

Sihtmäe, M., Blinova, I., Kunnis-Beres, K., Kanarbik, L., Heinlaan, M., Kahru, A., 2013. Ecotoxicological effects of different glyphosate formulations. Appl. Soil Ecol. 72, 215–224.

Simonsen, L., Fomsgard, I.S., Svensmark, B., Splid, N.H., 2008. Fate and availability of glyphosate and AMPA in agricultural soil. J. Environ. Sci. Heal. B 43, 365–375.

Singh, B., Singh, K., 2016. Microbial degradation of herbicides. Crit. Rev. Microbiol. 42, 245–261.

Singh, M., Sharma, S.D., Ramirez, A.H.M., Jhala, A.J., 2011. Glyphosate efficacy, absorption, and translocation in selected four weed species common to Florida citrus. HortTechnol. 21, 559–605.

Skark, C., Zullei-Seibert, N., Schottler, U., Schlett, C., 1998. The occurrence of glyphosate in surface water. Int. J. Environ. Anal. Chem. 70, 93–104.

Smith, D.L., Dushoff, J., Morris Jr., J.G., 2005. Agricultural antibiotics and human health. PLoS Med. 2 (8), e232. https://doi.org/10.1371/journal.pmed.0020232.

Solomon, K.R., 2016. Glyphosate in the general population and in applicators: a critical review of studies on exposures. Crit. Rev. Toxicol. 46 (sup 1):21–27. https://doi.org/10.1080/10408444.2016.1214678.

von Soosten, D., Meyer, U., Huether, L., Daenicke, S., Lahrssen-Wiederholt, M., Schafft, H., Spolders, M., Breves, G., 2016. Excretion pathways and ruminal disappearance of glyphosate and its degradation product aminomethylphosphonic acid in dairy cows. J. Dairy Sci. 99, 5318–5324.

St. Laurent, A., Merwin, I.A., Thies, J.E., 2008. Long-term orchard groundcover management systems affect soil microbial communities and apple replant disease severity. Plant Soil 304, 209–225.

Stachowski-Haberkorn, S., Becker, B., Marie, D., Haberkorn, H., Coroller, L., de la Broise, D., 2008. Impact of Roundup on the marine microbial community, as shown by an in situ microcosm experiment. Aquat. Toxicol. 89, 232–241.

Staub, J.M., Brand, L., Tran, M., Kong, Y., Rogers, S.G., 2012. Bacterial glyphosate-resistance conferred by overexpression of an *E. coli* membrane efflux transporter. J. Ind. Microbiol. Biotechnol. 39, 641–647.

Stine, O.C., Johnson, J.A., Keefer-Norris, A., Perry, K.L., Tigno, J., Qaiyumi, S., Stine, M.S., Morris Jr., J.G., 2007. Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. Int. J. Antimicrob. Agents 29, 348–352.

Sviridov, A.V., Shushkova, T.V., Ermakova, I.T., Ivanova, E.V., Epiktetov, D.O., Leontievsky, A.A., 2015. Microbial degradation of glyphosate herbicides (review). Appl. Biochem. Microbiol. 51, 188–195.

Swanson, N.L., Leu, A., Abrahamson, J., Wallet, B., 2014. Genetically engineered crops, glyphosate and the deterioration of health in the United States of America. J. Org. Syst. 9, 6–37.

Szarek, J., Siwicki, A., Andrzejewska, A., Terech-Majewska, E., Banaszkiewicz, T., 2000. Effects of the herbicide Roundup on the ultrastructural pattern of hepatocytes in carp (*Cyprinus carpio*). Mar. Environ. Res. 50, 263–266.

Tani, E., Chachalis, D., Travlos, I.S., Bilalis, D., 2016. Environmental conditions influence induction of key ABC-transporter genes affecting glyphosate-resistance mechanism in *Conyza canadensis*. Int. J. Mol. Sci. 17:342. https://doi.org/10.3390/ijms17040342.

Tao, B., Shao, B.H., Qiao, Y.X., Wang, X.Q., Chang, S.J., Qiu, L.J., 2017. Identification and functional analysis of a new glyphosate-resistance gene from a fungus cDNA library. Pestic. Biochem. Physiol. 140, 65–68.

Tian, Y.S., Yao, Q.H., Xing, X.J., Peng, R.H., Xu, J., Xiong, A.S., 2015. Novel glyphosate_resistant aroA gene from *Paracoccus denitrificans* in transgenic *Arabidopsis*. Appl. Biochem. Microbiol. 51, 566–571.

Travaglia, C., Masciarelli, O., Fortuna, J., Marchetti, G., Cardozo, P., Lucero, M., Zorza, E., Luna, V., Reinoso, H., 2015. Towards sustainable maize production: glyphosate detoxification by *Azospirillum* sp. and *Pseudomonas* sp. Crop. Prot. 77, 102–109.

Tromas, N., Fortin, N., Bedrani, L., Terrat, Y., Cardoso, P., Bird, D., Greer, C.W., Shapiro, B.J., 2017. Characterising and predicting cyanobacterial blooms in an 8-year amplicon sequencing time course. ISME J. 11, 1746–1763.

Tsui, M.T.K., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189–1197.

Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. Proc. Natl. Acad. Sci. 111, 15202–15207.

US Geological Survey, 2012. Pesticide Use Maps. https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2012&map=GLYPHOSATE&hilo=L&disp=Glyphosate, Accessed date: 22 July 2015.

USDA NASS (United States Department of Agriculture National Agricultural Statistics Service), 2014. Corn and Soybean Acres Planted Data. National Agricultural Statistics Service, Washington DC, USA http://www.nass.usda.gov, Accessed date: 29 September 2015.

Vandenberg, L.N., Blumberg, B., Antoniou, M.N., Benbrook, C.M., Carroll, N., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., vom Saal, F.S., Welshons, W.V., Myers, J.P., 2017. Is it time to reassess current safety standards for glyphosate-based herbicides? J. Epidemiol. Comm. Health 71, 613–618.

Vera, M.S., Di Fiori, E., Lagomarsino, L., Sinistro, R., Escaray, R., Iummato, M.M., Juárez, A., Ríos de Molina Mdel, C., Tell, G., Pizarro, H., 2012. Direct and indirect effects of the glyphosate formulation Glifosato Atanor® on freshwater microbial communities. Ecotoxicology 21, 1805–1816.

Vera, M.S., Juárez, Á., Pizarro, H.N., 2014. Comparative effects of technical-grade and a commercial formulation of glyphosate on the pigment content of periphytic algae. Bull. Environ. Contam. Toxicol. 93, 399–404.

Villeneuve, A., Larroudé, S., Humbert, J.F., 2011. Herbicide contamination of freshwater ecosystems: impact on microbial communities. In: Stoytcheva, M. (Ed.), Pesticides - Formulations, Effects, Fate :pp. 285–312. https://www.intechopen.com/books/pesticides-formulations-effects-fate/herbicide-contamination-of-freshwater-ecosystems-impact-on-microbial-communities.

Walker, E.R., Oliver, L.R., 2008. Translocation and absorption of glyphosate in flowering sicklepod (*Senna obtusifolia*). Weed Sci. 56, 338–343.

Wang, C., Lin, X., Li, L., Lin, S., 2016a. Differential growth responses of marine phytoplankton to herbicide glyphosate. PLoS One 11 (3), e0151633. https://doi.org/10.1371/journal.pone.0151633.

Wang, S., Seiwert, B., Kästner, M., Miltner, A., Schäffer, A., Reemtsma, T., Yang, Q., Nowak, K.M., 2016b. (Bio)degradation of glyphosate in water-sediment microcosms - a stable isotope co-labeling approach. Water Res. 99, 91–100.

Wardle, D.A., Parkinson, D., 1990. Effects of three herbicides on soil microbial biomass and activity. Plant Sci. 122, 21–28.

Weir, S.M., Yu, S., Knox, A., Talent, L.G., Monks, J.M., Salice, C.J., 2016. Acute toxicity and risk to lizards of rodenticides and herbicides commonly used in New Zealand. N. Z. J. 40, 342–350.

WHO (World Health Organization), 2005. Glyphosate and AMPA in Drinking-Water. http://www.who.int/water_sanitation_health/dwq/chemicals/glyphosateampa290605.pdf, Accessed date: 17 September 2018.

WHO (World Health Organization), 2009. The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification: 2009. International Program on Chemical Safety http://www.who.int/ipcs/publications/pesticides_hazard_2009.pdf?ua=1, Accessed date: 27 July 2017.

Wolmarans, K., Swart, W.J., 2014. Influence of glyphosate, other herbicides and genetically modified herbicide resistant crops on soil microbiota: a review. S. Afr. J. Plant Soil 31, 177–186.

Yamada, T., Kremer, R.J., de Camargo e Castro, P.R., Wood, B.W., 2009. Glyphosate interactions with physiology, nutrition, and diseases of plants: threat to agricultural sustainability? Eur. J. Agron. 31, 111–113.

Yang, X., Wang, F., Bento, C.P.M., Xue, S., Gai, L., van Dam, R., Mol, H., Ritsema, C.J., Geissen, V., 2015. Short-term transport of glyphosate with erosion in Chinese loess soil-a flume experiment. Sci. Total Environ. 512–513, 406–414.

Zabaloy, M.C., Carné, I., Viassolo, R., Gómeza, M.A., Gomez, E., 2016. Soil ecotoxicity assessment of glyphosate use under field conditions: microbial activity and community structure of Eubacteria and ammonia-oxidising bacteria. Pest Manag. Sci. 72, 684–691.

Zhang, C., Feng, L., He, T.T., Yang, C.H., Chen, G.Q., Tian, X.S., 2015a. Investigating the mechanisms of glyphosate resistance in goosegrass (*Eleusine indica*) population from South China. J. Integr. Agric. 14, 909–918.

Zhang, C., Hu, X., Luo, J., Wu, Z., Wang, L., Li, B., Wang, Y., Sun, G., 2015b. Degradation dynamics of glyphosate in different types of citrus orchard soils in China. Molecules 20, 1161–1175.

Zhang, S., Xu, J., Kuang, X., Li, S., Li, X., Chen, D., Zhao, X., Feng, X., 2017a. Biological impacts of glyphosate on morphology, embryo biomechanics and larval behavior in zebrafish (*Danio rerio*). Chemosphere 181, 270–280.

Zhang, T., Johnson, E.N., Mueller, T.C., Willenborg, C.J., 2017b. Early application of harvest aid herbicides adversely impacts lentil. Agron. J. 109, 239–248.

Zhao, H., Tao, K., Zhu, J., Liu, S., Gao, H., Zhou, Z., 2015. Bioremediation potential of glyphosate-degrading *Pseudomonas* spp. strains isolated from contaminated soil. J. Gen. Appl. Microbiol. 61, 165–170.

Zobiole, L.H.S., Kremer, R.J., Oliveira Jr., R.S., Constantin, J., 2010. Glyphosate affects microorganisms in rhizospheres of glyphosate-resistant soybeans. J. Appl. Microbiol. 110, 118–127.

Try out PMC Labs and tell us what you think. Learn More.

HHS Public Access
Author manuscript
Peer-reviewed and accepted for publication
About author manuscripts | Submit a manuscript

Mutat Res. Author manuscript; available in PMC 2020 Jul 1.
Published in final edited form as:
Mutat Res. 2019 Jul-Sep; 781: 186–206.
Published online 2019 Feb 10. doi: 10.1016/j.mrrev.2019.02.001

PMCID: PMC6706269
NIHMSID: NIHMS1533189
PMID: 31342895

Exposure to Glyphosate-Based Herbicides and Risk for Non-Hodgkin Lymphoma: A Meta-Analysis and Supporting Evidence

Luoping Zhang,[a,*] Iemaan Rana,[a] Rachel M. Shaffer,[b] Emanuela Taioli,[c] and Lianne Sheppard[b,d]

[a]Division of Environmental Health Sciences, School of Public Health, University of California Berkeley, Berkeley, USA
[b]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, USA
[c]Institute for Translational Epidemiology and Department of Population Health Science and Policy, Icahn School of Medicine at Mount Sinai, New York, USA
[d]Department of Biostatistics, University of Washington, Seattle, USA
[*]**Corresponding Author:** Luoping Zhang, PhD, Division of Environmental Health Sciences, School of Public Health, 2121 Berkeley Way West, #5117, Berkeley, CA 94720-7360, Tel: (510) 643-5189, Fax: (510) 642-0427, luoping@berkeley.edu

Copyright notice

Publisher's Disclaimer

Abstract

Glyphosate is the most widely used broad-spectrum systemic herbicide in the world. Recent evaluations of the carcinogenic potential of glyphosate-based herbicides (GBHs) by various regional, national, and international agencies have engendered controversy. We investigated whether there was an association between high cumulative exposures to GBHs and increased risk of non-Hodgkin lymphoma (NHL) in humans. We conducted a new meta-analysis that included the most recent update of the *Agricultural Health Study* (AHS) cohort published in 2018 along with five case-control studies. Using the highest exposure groups when available in each study, we report the overall meta-relative risk (meta-RR) of NHL in GBH-exposed individuals was increased by 41% (meta-RR = 1.41, 95% CI, confidence interval: 1.13–1.75). For comparison, we also performed a secondary meta-analysis using high-exposure groups with the earlier AHS (2005), and we determined a meta-RR for NHL of 1.45 (95% CI: 1.11–1.91), which was higher than the meta-RRs reported previously. Multiple sensitivity tests conducted to assess the validity of our findings did not reveal meaningful differences from our primary estimated meta-RR. To contextualize our findings of an increased NHL risk in individuals with high GBH exposure, we reviewed available animal and mechanistic studies, which provided supporting

evidence for the carcinogenic potential of GBH. We documented further support from studies of malignant lymphoma incidence in mice treated with pure glyphosate, as well as potential links between GBH exposure and immunosuppression, endocrine disruption, and genetic alterations that are commonly associated with NHL. Overall, in accordance with evidence from experimental animal and mechanistic studies, our current meta-analysis of human epidemiological studies suggests a compelling link between exposures to GBHs and increased risk for NHL.

Keywords: Glyphosate, pesticide, Roundup, Ranger Pro, carcinogenesis, meta-analysis

1. Background

1.1. Global Usage of Glyphosate-Based Herbicides

Glyphosate is a highly effective broad spectrum herbicide that is typically applied in mixtures known as glyphosate-based herbicides (GBHs) and commonly sold under the trade names of *Roundup*® and *Ranger Pro*®. Use of GBHs has increased dramatically worldwide in recent decades. In the United States alone, usage increased nearly sixteen-fold between 1992 and 2009 [1]. Most of this increase occurred after the introduction of genetically modified glyphosate-resistant "Roundup-ready" crops in 1996 [1]. In addition, there have been significant changes in usage. In particular, the practice of applying GBHs to crops shortly before harvest, so-called "green burndown," began in the early 2000s to speed up their desiccation; as a consequence, crops are likely to have higher GBH residues [2]. By the mid-2000s, green burndown became widespread, and regulatory agencies responded by increasing the permissible residue levels for GBHs [3, 4].

1.2. Ubiquitous Exposure in Humans

Glyphosate and its metabolites persist in food [5–7], water [8], and dust [9], potentially indicating that everyone may be exposed ubiquitously. Non-occupational exposures occur primarily through consumption of contaminated food, but may also occur through contact with contaminated soil [9], dust [9] and by drinking or bathing in contaminated water [8]. In plants, glyphosate may be absorbed and transported to parts used for food; thus, it has been detected in fish [5], berries [6], vegetables, baby formula [7], and grains [10], and its use as a crop desiccant significantly increases residues. GBH residues in food persist long after initial treatment and are not lost during baking.

Limited data exist on internal glyphosate levels among GBH-exposed individuals [11]. Average urinary glyphosate levels among occupationally exposed subjects range from 0.26-73.5 μg/L, whereas levels in environmentally exposed subjects have been reported between 0.13-7.6 μg/L [11]. Two studies of secular trends have reported increasing proportions of individuals with glyphosate in their urine over time [12, 13]. Given that more than six billion kilograms of GBHs have been applied in the world in the last decade [2], glyphosate may be considered ubiquitous in the environment [14].

1.3. Controversy Surrounding the Carcinogenic Potential of GBHs

Exposure to GBHs is reportedly associated with several types of cancer, among which the most-well studied in humans is non-Hodgkin lymphoma (NHL). Some epidemiological studies have reported an increased risk of NHL in GBH-exposed individuals [15–17]; however, other studies have not confirmed this association [18, 19]. GBHs have recently undergone a number of regional, national, and international evaluations for carcinogenicity in humans [20–23], resulting in considerable controversy regarding glyphosate and GBHs' overall carcinogenic potential. Hence, addressing the question of whether or not GBHs are associated with NHL has become even more critical. Here, we evaluated the all the published human studies on the carcinogenicity of GBHs and present the first meta-analysis to include the most recently updated Agricultural Health Study (AHS) cohort [24]. We also discuss the

lymphoma-related results from studies of glyphosate-exposed animals as well as mechanistic considerations to provide supporting evidence for our analysis of the studies of human exposures to GBHs.

2. Current Meta-Analysis of GBHs and NHL

2.1. Meta-Analysis Objective

Epidemiological studies may vary in several ways, such as by study design, sample size, and exposure assessment methods. Results among individual studies vary and may appear to conflict, which poses challenges in drawing an overall conclusion. Meta-analysis is a quantitative statistical tool that is frequently applied to consolidate the results from similar but separate individual studies so that an overall conclusion about the effects of exposure can be drawn. Here, we conducted a meta-analysis using published human studies to better understand whether the epidemiological evidence supports an association between exposures to GBHs and increased NHL risk. Although three previously published meta-analyses have examined the same association and reported positive meta-risks for GBH-associated NHL [22, 25, 26], our analysis differs from earlier ones by focusing on an *a priori* hypothesis targeting biologically relevant exposure magnitude and by including the newly updated AHS study [24].

2.2. A Priori Hypothesis

Our *a priori* hypothesis is that the highest biologically relevant exposure to GBHs, *i.e.,* higher levels, longer durations and/or with sufficient lag and latency, will lead to increased risk of NHL in humans. The hypothesis is based on the understanding that higher and longer cumulative exposures during a biologically relevant time window are likely to yield *higher* risk estimates, given the nature of cancer development [27]. Hence, when cumulative exposure is higher, either due to higher level or longer duration exposures, an elevated association with the cancer of interest is more likely to be revealed if a true association exists. This *a priori* approach has been employed to estimate meta-risks for benzene [28] and formaldehyde [29, 30], but not in any of the previous meta-analyses exploring the GBH-NHL association [22, 25, 26].

Risk estimates, including relative risks (RRs) and odd ratios (ORs), in high exposure groups are less likely to be dominated by confounding or other biases compared to RRs or ORs from groups experiencing average or low exposure [31]. Furthermore, including people with very low exposure in the exposed group can dilute risk estimates. Studying the most highly exposed group is also useful to ensure an adequate exposure contrast, given the potential that most people have been exposed either directly or indirectly to GBHs. Because our main goal is to determine whether there is an exposure effect and not to conduct a precise dose-response assessment or to evaluate risks in people with low exposures, we assert that this *a priori* hypothesis is appropriate for testing whether or not a GBH-NHL association exists.

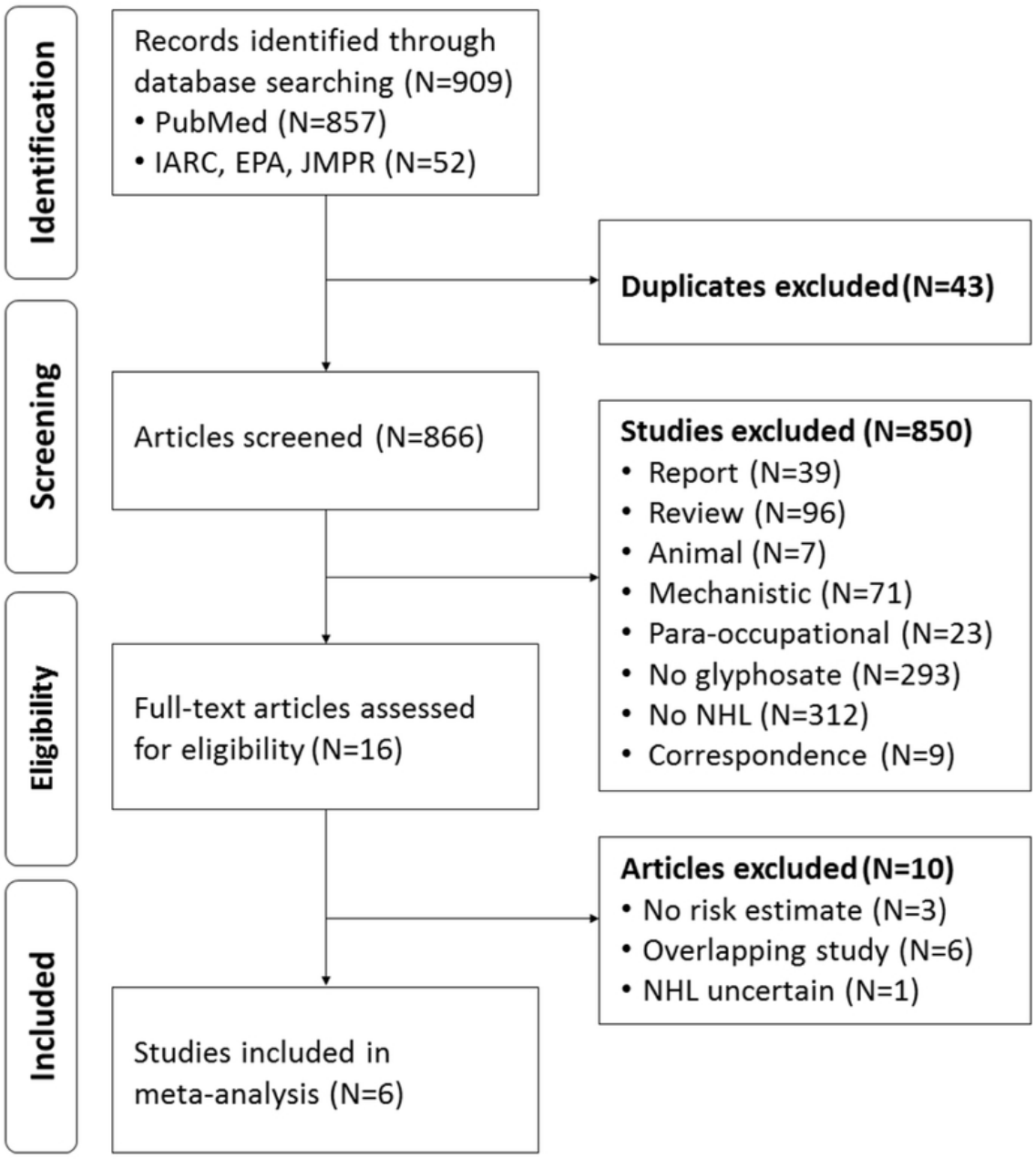
2.3. Agricultural Health Study (AHS) Update

A recently published update [24] from the large AHS cohort of American pesticide applicators (N > 50,000) has been included for the first time in our primary meta-analysis. Although the original AHS report [19] was used in previous meta-analyses [22, 25, 26], the 2018 AHS update [24] contributes 11-12 additional years of follow-up with over five times as many NHL cases (N = 575 compared to N = 92 in the original study [19]), and >80% of the total cohort was estimated to be exposed to GBHs. As the largest and most recently published study, it adds substantial weight to the new meta-analysis [24]. We

also performed a secondary comparison analysis using our *a priori* hypothesis with the original AHS report [19] for the purpose of comparing results with our primary meta-analysis (using the 2018 AHS update) and with meta-analyses published previously.

2.4. Identifying Relevant Human Studies

The literature search was conducted according to the guidelines of the *Preferred Reporting Items for Systematic Reviews and Meta-Analysis* (PRISMA) [32]. The screening process and results are shown in Figure 1. We conducted a systematic electronic literature review using PubMed in November 2017, and we updated it in March 2018 and again in August 2018. We used the following keywords: (glyphosat* OR pesticide [MeSH] or herbicides [MeSH]) AND (lymphoma, non-Hodgkin [MeSH] OR lymphoma [tiab] OR non–Hodgkin [tiab] OR non–hodgkins [tiab] OR lymphoma[tiab] OR lymphomas[tiab] OR NHL OR cancer OR cancers) AND ("occupational exposure"[MeSH] OR occupational exposure[tiab] OR occupational exposures[tiab] OR farmers [MeSH] OR farmer OR applicators OR applicator OR agricultural workers OR agricultural worker or workers or worker).



Open in a separate window

Figure 1.

Study Selection Process for Meta-Analysis using PRISMA Guidelines.

Searches included all cohort, case-control, and cross-sectional studies. No language restrictions were applied, although non-English language articles needed to be obtained in full and translated completely in order to be eligible for inclusion. From the PubMed search, we identified 857 studies. Additionally, we identified 52 studies from the IARC [22] evaluation of the carcinogenicity of glyphosate, the U.S. EPA [20] review of glyphosate, and the WHO JMPR [21] report on glyphosate, for a total of 909 studies.

After 43 duplicates were excluded, 866 studies were initially screened by title and abstract, of which 850 were excluded because they were reports, correspondence, reviews, irrelevant studies (animal, mechanistic, para-occupational), or did not include the exposure or outcome of interest (Figure 1). When the final 16 qualified epidemiological studies of GBHs and NHL were identified, 10 studies were further excluded because (1) they did not report RRs, ORs, or the data needed to calculate either [33–35], (2) the cohort overlapped with another study [19, 36–40], or (3) they did not specify whether the lymphomas were specifically NHL [41]. For studies including overlapping cohorts, we used results from the most complete and updated analysis with the greatest number of participants. Although overlapping, we kept the earlier AHS (2005) [19] for comparison with our primary meta-analysis (using the updated AHS 2018 publication) and with previous meta-analyses. The impact of selecting these studies was evaluated in sensitivity analyses (Section 3.5).

2.5 Review and Assessment of Selected Human Studies

2.5.1. Data Collection and Extraction In total, six studies (one cohort [24] and five case-control control studies [15–18, 42]) with nearly 65,000 participants were eligible for inclusion in the meta-analysis. Two studies were conducted in the United States, one study was from Canada, two studies were from Sweden, and one study was from France. All six studies reported NHL risks (RRs or ORs) above or close to 1.0, three of which were statistically significant in the original analyses (Table 1). From each study, we abstracted information on study design, location, dates, sample size, participation rates, age, sex, case/control source, diagnosis, histologic verification, exposure assessment, results, and statistical adjustments. Table 1 summarizes key aspects of the design and exposure assessment, the results, strengths, and weaknesses of all the studies evaluated in this meta-analysis, including both versions of the AHS report (n = 6+1). As described above, the early AHS data [19] were also evaluated in Table 1 and in a comparison meta-analysis described later.

Table 1

Epidemiologic studies of GBHs and cancer: studies used in the main meta-analyses[a]

Author/location	Subject ascertainment	Participation rates	Exposure assessment	Exposure level	Results for NHL[b]	Weak
Andreotti *et al.* [24] (Agricultural Health Study) Where: Iowa and North Carolina Design: Prospective cohort Years: 1993-97 to 2012-13 Percent exposed: 82.8%	Who: 54,251 pesticide applicators recruited between 1993-97 Cases: NA Source of cases: Iowa and North Carolina Cancer Registries, state and national death registries Histologic verification: Not mentioned Controls: NA Source of controls: NA Similar demographics (exposed and unexposed): Similar age, sex, race, and smoking. Exposed higher education, alcohol	Exclusions: 3059 excluded (mostly missing data) Percent proxy interviews: None Missing Follow-Up Questionnaire: 37% (Pesticide use imputed)	Collection: Self-administered and take home questionnaire at time of recruitment: 22 specific pesticides application methods, PPE, years of use, and days per use. Review: No Blinded: Prospective design Validation: Similar questions asked 1 year apart in 4,088 subjects, agreement on glyphosate ever use = 82%, days per year mixed = 52% [124]	Exposed: Quartiles 1-4 calculated by multiplying lifetime exposure days by an intensity score. Intensity based on (mixing + application method + equipment repair) * PPE in Coble *et al.* 2011 [44] Unexposed: No glyphosate use Latency: 5, 10, 15, and 20 years	Adjusted Intensity Weighted Cumulative Exposure (Days): RRs for Q1: 1–598.9; Q2: 599–1649.9; Q3: 1650–4339.9; and Q4: ≥4340.0, = 1.0 (Ref), 0.83 (0.59-1.18), 0.83 (0.61-1.12), 0.88 (0.65-1.19), 0.87 (0.64-1.20); p-trend = 0.95; n=54251 total, 111 cases in the high exposure group Similar results comparing highest to lowest quartile. Latency with	1. Pos fairly follow some 2. Son weak valida 3. Imp exposu for partici who d compl follow questi

Open in a separate window

Abbreviations: HCL, Hairy Cell Leukemia; IARC, International Agency for Research on Cancer; ICD, International Classification of Disease; IRIS, US Environmental Protection Agency Integrated Risk Information System; Minn, Minnesota; NA, not applicable; NCI, National Cancer Institute; OR, odds ratio; PPE, personal protective equipment; Ref, reference; RR, relative risk; SEER, Surveillance Epidemiology End Results; SES, socioeconomic status

[a]Although there is no overlapping study used in the main analysis, Cocco *et al.* [41] was excluded because only results for all B-cell lymphomas combined were reported (two cases of NHL, one case of multiple myeloma, and one unspecified B-cell lymphoma; n=4). It is evaluated in the sensitivity analysis.
[b]95% confidence intervals in parentheses
[c]Cantor *et al.* [37] was excluded because it was combined with two other U.S. case-control studies in De Roos *et al.* [15].
[d]Lee *et al.* [36] was excluded because it presents results comparing asthmatics to non-asthmatics and results are not adjusted for other pesticide use. It is evaluated in the sensitivity analysis.
[e]Hohenadel *etal.* [40] was excluded because it presents results in subjects exposed and unexposed to malathion, which has not been consistently linked to NHL; the OR for glyphosate only was used in the sensitivity analysis

2.5.2. Study Quality Evaluation The methodological quality of the cohort (Table 2) and case-control studies (Table 3) included in the meta-analyses was assessed independently by two co-authors using the Newcastle Ottawa Scale (NOS) [43]. Studies were evaluated based on selection, comparability, and outcome or exposure (in nine categories).

Table 2.

Quality assessment of the cohort studies in meta-analysis.*

Study	Selection				Comparability		Outcome	
	Representativeness of Exposed	Selection of Non-Exposed	Exposure Assessment	NHL Absent at Start	Controls for Other Pesticides	Controls for Age	Assessment of Outcome	Follo up Leng
Andreotti *et al.* [24]	1	1	1	1	1	1	1	1
De Roos (2005) [19]	1	1	1	1	1	1	1	0

Open in a separate window

*The study quality was assessed according to the Newcastle Ottawa Quality assessment scale for cohort studies [43]. One point was awarded for yes, and zero points were awarded for no, unable to determine, or inadequate.

Table 3

Quality assessment of the case-control studies in meta-analysis.[a]

	Selection				Comparability		Exp	
Study	**Adequate Case Definition**	**Representativeness of Cases**	**Control Selection**	**Definition of Controls**	**Controls for Other Pesticides**	**Controls for Age**	**Exposure Assessment**	**Me Coı**
De Roos (2003) [16]	1	1	1	0	1	1	0	1
Eriksson *et al.* [17]	1	1	1	0	1	1	0	1
Hardell *et al.* [18]	1	1	1	0	1	1	0	1
McDuffie *et al.* [43]	1	1	1	1	0	1	0	0
Orsi *et al.* [19]	1	1	0	1	0	1	0	0

Open in a separate window

[a]The study quality was assessed according to the Newcastle Ottawa Quality assessment scale for case-control studies [44]. One point was awarded for yes, and zero points were awarded for no, unable to determine, or inadequate.

Cohort studies were evaluated based on (1) representativeness of the cohort, (2) selection of non-exposed, (3) ascertainment of exposure, (4) demonstration that outcome of interest was not present at the start of study, (5) comparability of cohort on the basis of controlling for other pesticide use and (6) age, (7) assessment of NHL outcome, and (8) sufficiency of follow-up length, and (9) response rate.

Case-control studies were evaluated on (1) the validation of cases, (2) representativeness of cases, (3) selection of controls, (4) absence of disease in the controls, (5) whether the study controlled for other pesticide use and (6) age, (7) exposure assessment, (8) concordance of method among cases and controls, and (9) similarity of response rate among both groups. Each study was awarded a maximum of one point for every item that was satisfied, with a total of 9 available points.

According to our quality assessment (Tables 2–3), the highest quality study in either design category was the AHS 2018 cohort [24]. The highest quality case-control study was Eriksson *et al.* [16], while the lowest quality studies were McDuffie *et al.* [42] and Orsi *et al.* [18].

2.6. Selection of the Most Highly Exposed Category

Based on our *a priori* hypothesis, when multiple RRs or ORs were given in the original studies, we selected estimates in the following order: (1) highest cumulative exposure and longest lag (the time period preceding NHL onset, which is excluded from the exposure estimate) or latency (time between first lifetime exposure and NHL diagnosis); (2) highest cumulative exposure; (3) longest exposure duration and longest lag or latency; (4) longest exposure duration; (5) longest lag or latency; and (6) ever-exposure. The definition of cumulative exposure includes duration and intensity. As we discuss in more detail in Section 5.2, in both AHS reports [19, 24] cumulative exposure was calculated as an intensity-weighted exposure (lifetime exposure days multiplied by an intensity score) [44, 45].

We prioritized highest cumulative exposure based on evidence of glyphosate's persistence in the environment [46–48] and because chronic disease, including cancer, is usually the result of cumulative exposures [49]. We selected the longest lag or latency because decades may be needed for the health effects of many environmental toxicants to manifest as detectable cancers. If no high exposure data were available, we used the ever-exposure estimate. Given the relatively few human epidemiological studies published to date on the topic, we made this decision because we did not want to exclude any potentially relevant data, even though the inclusion of minimally exposed individuals in the "exposed" category could attenuate any potential association of interest.

Although there are different perspectives on the best way to account for other pesticide exposures, we selected RR estimates that adjusted for other pesticide use over their unadjusted counterparts to mitigate potentially substantial confounding by other pesticide use. Five of the seven studies adjusted for a combination of different pesticides [15–17, 19, 24], indicating they accounted for confounding by other pesticides. However, if these multiple pesticides acted synergistically or on different points along a pathway, this approach to adjustment may no longer be the appropriate, and alternatives such as interaction analysis should be considered. Reanalysis of the raw data, which is beyond the scope of this paper, would be helpful to address this possibility.

We evaluated the impact of our *a priori* exposure selection criteria in sensitivity analyses. We also conducted a separate meta-analysis of all ever-exposed individuals to assess the magnitude of potential bias caused by adding subjects with low exposures (ever-RR from De Roos *et al.* [19] was used; the ever-RR estimate from Andreotti *et al.* [24] was not available). In Table 4 we summarize the risk estimates selected from each original study and the study weights used in the meta-analyses.

Table 4:

Description and weight of studies selected for the current meta-analyses.

Study (Author, Year)	Case No. (Exp/Tot)	Exposure Category	Risk Estimate[a] (95% CI)	Weight[b]	
				AHS 2018	AHS 2005
AHS Cohort					
Andreotti *et al.* [24]	55/575	≥2610 d/I[c,d]	1.12 (0.83, 1.51)[e]	54.04	--
De Roos (2005) [19]	22/92	≥337.2 d/I[c]	0.8 (0.5, 1.4)[f]	--	28.43
Case-Control					
De Roos (2003) [15]	36/650	Ever, log	2.10 (1.10, 4.00)	11.61	18.08
Eriksson *et al.* [16]	17/910	>10 d/y	2.36 (1.04, 5.37)	7.18	11.18
Hardell *et al.* [17]	8/515	Ever	1.85 (0.55, 6.20)	3.30	5.14
McDuffie *et al.* [42]	23/517	>2 d/y	2.12 (1.2, 3.73)	15.05	23.43
Orsi *et al.* [18]	12/244	Ever	1.0 (0.5, 2.2)	8.82	13.73

Abbreviations: AHS, Agricultural Health Study; d, days; exp, exposed; I, lifetime; log, logistic regression; tot, total; y, year.

[a]Relative risk (RR) reported in both AHS analyses and odds ratio (OR) reported in all case-control studies.
[b]Weight given to each study in the fixed effects model.
[c]Intensity-weighted lifetime exposure days (cumulative exposure days multiplied by intensity score)
[d]20 years or more lag (time between study recruitment and NHL onset).
[e]Reference group is unexposed
[f]Reference group is lowest exposed

2.7. Statistical Methods

We calculated the meta-analysis summary relative risk (meta-RR) and confidence intervals using both the fixed-effects inverse-variance method [31] and the random-effects method [50]. In the fixed-effects model, the weights assigned to each study are directly proportional to study precision, whereas in the random-effects model, weights are based on a complex mix of study precision, relative risk (RR), and meta-analysis size. One benefit of the random-effects model is the ability to incorporate between-study variance into the summary-variance estimate and confidence intervals, which may help prevent artificially narrow confidence intervals resulting from use of the fixed effects model in the presence of between-study heterogeneity [51]. However, a feature of the random-effects model is that study weighting is not directly proportional to study precision, and greater relative weight is given to smaller studies, which may result in summary estimates that are less conservative than the fixed-effects model

[51]. For these reasons, our primary results focus on the fixed-effects model, although the random-effects model estimates are also reported. We also estimated between-study heterogeneity, defined as the X^2-test statistic for heterogeneity being greater than its degrees of freedom (number of studies minus one), using the summary-variance method [51].

We evaluated publication bias through funnel plots, Egger's test, and Begg's test [52, 53]. All statistical analyses were conducted with Stata IC 15.1 [54] and Microsoft Excel 2013 [55].

3. Meta-Analysis Findings

3.1. Increased Meta-Relative Risk of NHL

Table 5 includes the results from our two meta-analyses, which included the primary analysis using the most recently updated AHS cohort [24] and the secondary comparison analysis using the original report [19]. Using the updated AHS results [24], we observed a meta-RR of **1.41** (95% CI: 1.13-1.75), which indicates a statistically significant increased risk (41%) of NHL following high cumulative GBH exposure. With the original AHS 2005 cohort results, we observed a meta-RR of **1.45** (95% CI: 1.11–1.91) for NHL. The results did not change appreciably when comparing the fixed effects model to the random-effects model.

Table 5.

Major Findings from Current Meta-Analyses

		Fixed Effects	Random Effects	Heterogeneity[a]	
Analysis	**N**	**meta-RR (95% CI)**	**meta-RR (95% CI)**	X^2	*p*
Highest cumulative exposure					
AHS (2018) [24][b]	6	1.41 (1.13, 1.75)	1.56 (1.12, 2.16)	8.26	0.14
AHS (2005) [19]	6	1.45 (1.11, 1.91)	1.52 (1.00, 2.31)	10.59	0.06
Longest exposure duration					
AHS (2018) [24][b]	6	1.41 (1.13, 1.74)	1.56 (1.12, 2.16)	8.21	0.15
AHS (2005) [19]	6	1.56 (1.17, 2.06)	1.57 (1.06, 2.26)	7.81	0.17
Study design					
Case-control [15–18, 42]	5	1.84 (1.33, 2.55)	1.86 (1.39, 2.48)	3.36	0.50
Cohort (AHS 2018) [24]	1	1,12[c] (0.83, 1.51)			

Abbreviations: AHS, Agricultural Health Study; meta-RR, meta-relative risk; N, number of studies.

[a]Heterogeneity is present when X^2 heterogeneity statistic is greater than degrees of freedom (number of studies minus 1).
[b]De Roos *et al.* [19] used instead of Andreotti *et al.* [24] for comparison. See Table 4 for clarifications about the risk estimates used.
[c]Since there was only one cohort study, the RR is presented instead of a meta-RR.

Forest plots (Figure 2A–B) and Funnel plots (Figure 2C–D) from these two major meta-analyses are reported in Figure 2. We observed little evidence of publication bias in the Funnel plots (Figure 2C–D), Eggers ($p = 0.185$), and Beggs tests ($p = 0.851$).

Open in a separate window

Figure 2.

Major meta-analysis results. A) Forest plot for meta-analysis using AHS 2018 and B) using AHS 2005. C) Funnel plot for meta-analysis using AHS 2018 and D) using AHS 2005.

3.2. Sensitivity Analyses

We conducted several sensitivity analyses to evaluate the impact of excluding or including different studies as well as using different RRs/ORs from original studies (Tables 5 and 6). In general, results were similar across our sensitivity analyses, demonstrating the robustness of our findings.

Table 6.

Sensitivity tests for meta-analysis

		Fixed Effects	Random Effects	Heterogeneity[1]	
Analysis	**N**	**meta-RR (95% Cl)**	**meta-RR (95% Cl)**	**X^2**	***p***
Alternate Exposure Categories					
High level[2]	3	1.36 (1.06, 1.75)	1.63 (0.97, 2.76)	5.70	0.06
Ever (AHS 2005)	6	1.30 (1.03, 1.64)	1.26 (1.07, 1.48)	3.73	0.59
Latency[3]	6	1.40 (1.13, 1.75)	1.54 (1.12, 2.13)	8.01	0.16
Cell Type Specific					
Add Cocco *et al.* [41][4]	7	1.43 (1.15, 1.78)	1.59 (1.16, 2.18)	9.10	0.17
Exclude HCL [17][5]	6	1.41 (1.13, 1.77)	1.61 (1.11,2.34)	9.58	0.09
Only use HCL [17][6]	6	1.43 (1.14, 1.78)	1.62 (1.14, 2.31)	9.36	0.10
Study Location					
North America	3	1.38 (1.08, 1.76)	1.61 (0.99, 2.60)	5.70	0.06
Europe	3	1.53 (0.93, 2.52)	1.55 (0.88, 2.71)	2.43	0.30
Other pesticides[7]					
Adjusted (AHS 2005)	4	1.46 (1.05, 2.02)	1.43 (1.06, 1.92	2.61	0.46
Unadjusted (AHS 2005)	4	1.69 (1.29, 2.23)	1.70 (1.26, 2.30)	3.47	0.33
De Roos *et al.* [15]					
Hierarchal OR[8]	6	1.36 (1.09, 1.70)	1.46 (1.08, 1.96)	6.80	0.24
Cantor *et al.* [37][9]	6	1.29 (1.04, 1.59)	1.36 (1.02, 1.80)	7.07	0.22
Lee *et al.* [36][10]	6	1.35 (1.11, 1.65)	1.41 (1.09, 1.82)	6.63	0.25
Other					
[11]					

Open in a separate window

Abbreviations: Cl, confidence interval; HCL, hairy cell leukemia; meta-RR, meta-relative risk

[1]·Heterogeneity is present when X^2 heterogeneity statistic is greater than degrees of freedom (number of studies minus 1).

[2]·Risk estimates for the most highly exposed group available in the three studies that stratify by exposure level.

[3]·Eriksson *et al.* [16] results for any glyphosate exposure >10 years latency was used instead of the higher exposure group used in the main analysis.
[4]·The study combined all B-cell lymphomas and is added to the analysis on highest cumulative exposure (AHS 2018).
[5]·Hairy cell leukemia cases excluded—results presented in Hardell and Eriksson [38].
[6]·NHL cases excluded; only HCL results used—results presented in Nordstrom *et al.* [39].
[7]·Studies that provided RRs that are both adjusted and not adjusted for other pesticide use for ever exposure, or reported that adjusting for pesticide use had little impact on the RR estimate. AHS (2018) did not report ever exposure, so AHS (2005) was used instead.
[8]·Hierarchical model RR used instead of the standard logistic regression model RR.
[9]·Cantor *et al.* [37] used instead of De Roos *et al.* [15]. Cantor *et al.* [37] was the only of the three studies combined by De Roos *et al.* [15] that presented data for glyphosate.
[10]·Lee *et al.* [36] used instead of De Roos *et al.* [15], Lee *et al.* [36] used same subjects as De Roos *et al.* [15] but did not adjust for other pesticide exposure, did not exclude those with missing data on other pesticide use, and used only non-asthmatics.
[11]·Hohenadel *et al.* [40] used same subjects as McDuffie *et al.* [42] but presented results in subjects exposed to glyphosate but not malathion (OR=0.92; 95% Cl: 0.54-1.55).
[12]·One study excluded at a time to evaluate the impact of each individual study on the overall meta-RR.

3.2.1. Alternative Exposure Criteria As a sensitivity analysis, we also conducted a meta-analysis using the longest exposure duration results to compare with our primary analysis using the highest cumulative exposure results. When RRs corresponding to exposures with the longest duration were selected from the AHS 2018, the meta-RR remained the same at **1.41** (95% CI: 1.13-1.74). When the AHS 2005 report was included, the meta-RRs increased to **1.56** (95% CI: 1.17-2.06) (Table 5).

When evaluating studies with only the highest levels of exposure [16, 24, 42], the meta-RR was **1.36** (95% CI: 1.06-1.75, Table 6). In studies that combined all exposures as ever exposed [15–19, 42], the meta-RR was **1.30** (95% CI: 1.03-2.64). Although the higher exposure group was used in the main analysis, Eriksson *et al.* [16] also provided results for greater than 10 years latency, which contributed to a meta-RR of **1.40** (95% CI: 1.13-1.75). [Note: AHS 2018 did not provide ever-exposure, so AHS 2005 was used to calculate this statistic and *ever* exposure above].

3.2.2. Study Inclusion When we limited our analysis to case-control studies (Table 5), there was little inter-study heterogeneity. We estimated a doubling of the NHL risk (meta-RR = **1.84**, 95% CI: 1.33-2.55) from 41% to 84% compared to the estimate that included the cohort study.

To ensure that one individual study was not artificially inflating the meta-risk estimate, we excluded the case-control studies one at a time and found that they all nominally lowered the meta-RR, except for the exclusion of Orsi *et al.* [18], where the meta-RR increased to **1.46** (1.16-1.83) (Table 6).

3.2.2. NHL vs. Cell-type Specific Lymphomas Although our primary meta-analysis included six studies, there was a possibility to include a seventh study [41]. We excluded this study from the primary analysis because it included all B-cell lymphomas (4 cases), which account for approximately 85% of all NHL [56]; however, not all four cases were confirmed to be NHL. When we added Cocco *et al.* [41] to the meta-analysis (n = 7, Table 6), the resulting RR remained fairly similar at **1.43** (95% CI: 1.15-1.78).

Similar to our inclusion of the Cocco *et al.* [41] study, another cell-type specific study evaluated all cases of hairy cell leukemia (HCL), a subtype of NHL [39]. It was one of two studies [38, 39] included in the Hardell *et al.* [17] analysis, with the other study examining NHL only [38]. Excluding HCL cases had no effect on the meta-RR (**1.41**, 95 % CI: 1.13-1.77, Table 6). Similarly, using only hairy cell leukemia cases from Hardell *et al.* [17] (reported in Nordstrom *et al.* [39]) did not impact the meta-RR (1.43, 95% CI: 1.14-1.78).

3.2.4. Study Location and Adjustment Studies in North America [15, 24, 42] had a meta-RR of **1.38** (95% CI: 1.08-1.76), whereas European studies [16–18] had a meta-RR of **1.53** (95% CI: 0.93-2.52). On average, when studies were adjusted for other pesticide use [15–17, 19], the meta-RR for ever-exposure was lower than unadjusted risk estimates from the same studies (meta-$RR_{adjusted}$ = **1.46**, 95% CI: 1.05-2.02; meta-$RR_{unadjusted}$ = **1.69**, 95% CI: 1.29-2.23).

3.2.5. Logistic vs. Hierarchical Regressions Consistent with the two previous meta-analyses by IARC [22] and Schinasi and Leon [25] discussed in Section 4.1 below, we selected the RR estimated using the more traditional logistic regression over the hierarchical regression estimate in the case-control study by De Roos *et al.* [15] and found that there was little impact of this selection (meta-RR = **1.36**, 95% CI: 1.09-1.70). When Cantor *et al.* [37] or Lee *et al.* [36] were used instead of De Roos *et al.* [15], the meta-RR decreased to **1.29** (95% CI: 1.04-1.59) and 1.35 (95% CI: 1.11-1.65), respectively. Similarly, using Hohenadel *et al.* [40] instead of McDuffie *et al.* [42] caused the meta-RR to decrease to **1.23** (95% CI: 0.99-1.53).

4. Comparison with Previous Meta-Analyses

Three meta-analyses of NHL in relation to GBH exposure have been published [22, 25, 26], all of which report lower, albeit also positive, risk estimates. In contrast to our work, these analyses did not focus on the highest exposed groups. Table 7 summarizes the major results from all GBH-NHL meta-analyses conducted to date, including the current one.

Table 7.

Comparison of current meta-analysis to other published meta-analyses

Studies	Schinasi and Leon [25][a]	IARC [22]	Chang and Delzell [26][a,b]	Current Meta-Analysis with AHS 2005 [19]	Current Meta-Analysis with AHS 2018 [24]
	RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)
Andreotti *et al.* [24]	N/A	N/A	N/A	N/A	1.12 (0.83-1.51)
De Roos (2005) [19]	1.1 (0.7, 1.9)	1.1 (0.7, 1.9)	1.1 (0.7, 1.9)	0.8 (0.5, 1.4)	N/A
De Roos (2003) [15]	2.1 (1.1,4.0)	2.1 (1.1,4.0)	1.6 (0.9, 2.8)	2.1 (1.1,4.0)	2.1 (1.1,4.0)
Eriksson *et al.* [16]	2.0 (1.1,3.7)	1.51 (0.77, 2.94)	1.51 (0.77, 2.94)	2.36 (1.04, 5.37)	2.36 (1.04, 5.37)
Hardell *et al.* [17]	3.0 (1.1, 8.5)	1.85 (0.55, 6.20)	1.85 (0.55, 6.20)	1.85 (0.55, 6.20)	1.85 (0.55, 6.20)
McDuffie *et al.* [42]	1.2 (0.8, 1.7)	1.20 (0.83, 1.74)	1.20 (0.83, 1.74)	2.12 (1.20, 3.73)	2.12 (1.20, 3.73)
Orsi *et al.* [18]	1.0 (0.5, 2.2)	1.0 (0.5, 2.2)	1.0 (0.5, 2.2)	1.0 (0.5, 2.2)	1.0 (0.5, 2.2)
meta-RR (95% CI)	**1.45 (1.08, 1.95)**[c]	**1.30 (1.03, 1.64)**	**1.27 (1.01, 1.59)**	**1.45 (1.11, 1.91)**	**1.41 (1.13, 1.75)**

Abbreviations: CI, confidence interval; meta-RR, meta-relative risk; RR, relative risk;

[a]In their published reports, meta-RRs and their 95% confidence intervals were rounded to one digit right of the decimal point.
[b]Findings from Model 1, the primary analysis, are reported here.
[c]Random effects model.

Schinasi and Leon [25] first reported a meta-RR of **1.45** (95% CI: 1.08-1.95). Although their selection criteria stated that they used the most adjusted effect estimate for the dichotomously defined exposure with the greatest number of exposed cases, they did not use adjusted effect estimates in the two Swedish studies [16, 17]. The IARC Working Group subsequently corrected this discrepancy in an otherwise identical meta-analysis [22], resulting in a meta-RR of **1.30** (95% CI: 1.03 −1.65). Although both studies are listed in Table 7 for completeness, we consider IARC 2015 to be the most accurate and updated version of this meta-analysis.

Most recently, Chang and Delzell [26] reported a meta-RR of **1.27** (95% CI: 1.01-1.59) in their primary analysis (model one). For each included study, the authors selected the most fully adjusted RR from the publication with the most recent and complete study population with the largest number of exposed cases. (In their publication, the meta-RR was rounded to one digit to the right of the decimal point.)

Whereas the three previous meta-analyses focused on general exposure (ever versus never), our new meta-analysis differs primarily because of our *a priori* selection of risk estimates from the most highly exposed groups when available (from three studies [16, 19, 42]). In our secondary comparison meta-analysis with the same six studies (including AHS 2005), we document an additional 0.15-0.18 (or 15-18%) higher NHL RR than previous meta-RRs [22, 26] (not including Schinasi and Leon, because it was corrected in IARC 2015). Similarly, in our primary analysis with AHS 2018, our meta-RR estimate adds an additional 0.11-0.14 (11-14%) increase in NHL relative risk to the previous meta-RRs [22, 26]. Overall, the meta-RR obtained using our *a priori* hypothesis, while generally consistent with previous analyses, gave somewhat higher estimates and suggested increased risk of NHL in individuals highly exposed to GBHs.

5. Strengths and Limitations

In this section, we evaluate the strengths and limitations of our meta-analyses, as well as of the cohort study and the case-control studies utilized.

5.1. Current Meta-Analyses

The strengths of these meta-analyses are the inclusion of the updated AHS 2018 study and our novel *a priori* hypothesis. By using the highest exposure group in each study when it was reported, we maximized the ability to detect the presence of an exposure-disease association. The current meta-analysis is also the first study to include the newly updated AHS.

There are several weaknesses of our analysis that should be noted, however. First, there were only limited published data available for inclusion. Although meta-analysis prevents overemphasis on any single study [57], we cannot exclude the potential for publication bias, given the relatively few published studies to date. Second, there was imbalance in study design: among the only six included studies, five were case-control and one was a cohort. The collection of NHL findings from the cohort study was consistent with a wide range of risks [24], while, by contrast, most of the case-control studies did suggest an increased risk [15–17, 42]. There were also important differences in the comparison group utilized in the studies; some used the lowest exposure group as the reference, while others used the unexposed group. Because of this heterogeneity, and because no statistical tests can confirm elimination of publication bias or heterogeneity in a meta-analysis [58], our results should be interpreted with caution. Finally, as depicted in Figure 3 illustrating key milestones related to glyphosate use in society and in epidemiological studies, none of the available studies capture the effects of the significant increased usage of glyphosate that began with the introduction of “green-burn-down” in the mid-2000s.

Open in a separate window

Figure 3.

Timeline of glyphosate use milestones in relation to cohort and case-control study events.

[1] Glyphosate active ingredient usage includes agricultural and non-agricultural applications

[2] m = millions; Ibs = pounds

[3] Completed by 63% of AHS participants

5.2. AHS Cohort Study

In general, cohort studies are considered the gold standard among observational studies because of their ability to estimate exposure before disease occurrence (which allows for clarity of temporality and can minimize recall bias), to estimate incidence, to examine multiple outcomes, and for some target populations, to study a large number of exposed subjects. Our new meta-analysis is the first to include the AHS 2018 update, which is the largest, newest, and most heavily weighted study (>50%, Table 4). Given its importance and because it was the only cohort study in our analyses, we discuss below several aspects of the AHS 2018 study and its comparison with the AHS results reported in 2005. Key differences between the AHS 2018 and AHS 2005 are summarized in Table 8.

Table 8:

Key differences between AHS 2005 and AHS 2018, with an emphasis on exposure quantification

	AHS 2005 [19]			**AHS 2018 [24]**				
Exposure assessment	Self-report at baseline			Self-report at baseline and follow-up questionnaire wi exposure simulation[1]				
Exposure quantification	Ever/never	Cumulative exposure days	Intensity-weighted exposure days[2]	Ever/never[3]	Cumulative exposure days[3]	Intensity-weighted exposu days[2]		
Lag period	Unlagged			Unlagged			5-year lag	20-y lag[4]
Exposure groups among exposed (days)[5]	Ever exposed	T1: 1-20; T2: 21-56; T3: 57-2678	T1: 0.1-79.5; T2: 79.6-337.1; T3: 337.2-18,241	Ever exposed	T1: 1-19.9; T2: 20.0-61.9; T3: >62.0	Q1: 1–598.9; Q2: 599–1649.9; Q3: 1650–4339.9; Q4: >4340.0	Q1: 1–530.9; Q2: 531.0–1511.9; Q3: 1512.0–4063.4; Q4: >4063.5	Q1: 1–281. Q2: 281. 895. Q3: 896- 260 Q4: >26
Exposure duration (years)[6]	Maximum possible range= 20-24;[7] Actual maximum: 7.3[8] Median = *not provided*; IQR = *not provided*			Maximum possible range = 26–32;[9] Actual maximum: *not provided* Median = 8.5; IQR = 5-14			Max. possible range = 21-27;[9] Actual max: *not provided* Median = 4.1[10]; IQR = *not provided*	Max poss rang 6-12 Actu max *prov* Med = 2. IQR *not prov*
Reference group	Unexposed	Lowest exposure	Lowest exposure	Unexposed				

Open in a separate window

[1]This was referred to as “multiple imputation” by study authors; see manuscript text for further details

[2]The algorithm for calculating “intensity-weighted exposure days” was updated between 2005 and 2018. Key differences include rescaling of scores by a factor of 10 and altering the weights for mixing, certain pesticide application techniques, and the use of chemically resistant gloves [44]. Therefore, these metrics cannot be directly compared.

[3]Ever/never and cumulative exposure days were only presented in the AHS 2018 supplement but are presented here to facilitate comparisons with AHS 2005
[4]Results and quartiles for 10- and 15-year lags are presented in the AHS 2018 supplement
[5]Exposure group abbreviations are as follows: Tertiles = "T;" Quartiles = "Q."
[6]The values provided in this row are based on the subset of individuals who reported using glyphosate
[7]This theoretical maximum duration value was calculated based on the year that glyphosate entered the market (1974) and the end of AHS enrollment (1993-1997), since AHS 2005 used only baseline exposure information
[8]This value was calculated based on the upper bound of the cumulative exposure days tertiles
[9]These theoretical maximum duration values were calculated based on the year that glyphosate entered the market (1974) and the end of AHS follow-up exposure questionnaire (1999-2005), with the appropriate adjustments for the lag times as indicated.
[10]These medians were calculated using the information provided in the footnote in Table 3 of the AHS 2018 publication
[11]These follow-up times were calculated based on timing of study enrollment and follow-up

5.2.1. Exposure Assessment and Quantification Exposures were self-reported using questionnaires. AHS 2005 used the exposures reported at baseline only, whereas AHS 2018 supplemented this information with responses to a follow-up questionnaire returned by 63% of AHS participants.

The risk estimates generated from the follow-up AHS 2018 report depended on a "multiple imputation" approach with multiple steps to generate GBH exposure information for the 37% of participants who did not complete the follow-up questionnaire [24]. A standard imputation model captures the full distribution of the exposure by relying on two parts of a model: the regression or predictable part and the residual error part. The validity of the imputed exposures and the resulting risk estimates relies on the validity of both parts of the imputation model. The AHS imputation method for ever/never pesticide use conditioned on the reported pesticide use and other data, including demographics, medical history at baseline, and farming characteristics at enrollment, with some covariates chosen by stepwise regression (see Table 2 in Heltshe *et al.* [59]). Based on their analysis of a 20% holdout dataset, the prevalence of glyphosate use was underreported by 7.31%, suggesting some lack of validity in the predictable part of the imputation model that may in turn affect the NHL risk estimates. The imputations of days of use per year and most recent year of farming activity relied upon a stratified sampling with replacement approach, with values sampled from Phase 2 respondents based on strata defined using Phase 1 information.

The imputations did not use the NHL or any other cancer outcome information reported by Andreotti *et al.* [24]. This approach is problematic because of how the residual error part of the imputation model is handled. It is known that multiple imputation of a covariate (*i.e.,* glyphosate exposure) in a model that omits the outcome variable to be used in the inference leads to attenuation of the effect estimate for that covariate due to lack of correlation with the outcome in the residual error part of the imputed exposures [60]. As we discuss further in the next paragraph, this approach effectively "bakes into the results" the null hypothesis of no increased risk of NHL exposure due to glyphosate risk.

Because the NHL outcome information was not used in the imputation procedure, the exposure "imputation" method used in the AHS 2018 report can be better named "exposure simulation" as described by Gryparis *et al.* [61]. This term gives a much more accurate understanding of the impact of the imputation of the data on the risk estimates because when exposure is simulated in a model that does not take the NHL outcome into account, the uncertainty in the "imputed" exposure behaves like classical measurement error and, thus, will bias the effect estimate towards the null [62].

AHS 2018 authors argue that their imputation approach "likely did not materially impact risk estimates" [63]. However, their argument has to do with the impact on the average change in the number of predicted events in an outcome-augmented imputation model and not the role of classical

measurement error in the imputed exposure estimates.

There was also a subtle yet important difference in the categorization and quantification of exposure data between AHS 2005 and 2018. As depicted in Table 8, both studies classified exposure based on (1) ever/never, (2) cumulative exposure days, and (3) intensity-weighted exposure days. However, the algorithm utilized to calculate intensity-weighted exposure days was updated between 2005 and 2018. Key differences include rescaling of scores by a factor of 10 and altering the weights for mixing, certain pesticide application techniques, and the use of chemically resistant gloves [44]. Therefore, these metrics cannot be directly compared.

Additionally, it is crucial to highlight the difference in reference groups between these two studies, which further limits the comparability of their estimates. AHS 2005 utilized the lowest exposed tertile as the comparison group for risk estimation. They justified this decision as an attempt to control residual confounding, because of the presence of significant differences in key characteristics between the never-exposed and lowest-exposed groups. By contrast, AHS 2018 utilized the unexposed group as the reference group even though our comparison of the demographics reported in each paper's Table 1 does not suggest there is substantially better comparability between groups in AHS 2018. Furthermore, because the exposure information by which these groups were classified was based on their imputation procedure, the limitations of which are highlighted above, the actual comparability between groups may differ from the values reported. Not only would it be helpful to be able to compare directly the risk estimates across the two papers, it would be useful to investigate whether there was residual confounding introduced into the AHS 2018 analysis by the use of the "unexposed" group as the reference.

5.2.2. Exposure Misclassification Differential misclassification is unlikely in a cohort study when exposure is assessed prior to the disease occurrence. In AHS 2018, however, we believe there is some potential for differential misclassification. Sixty-three percent of the original cohort provided updated exposure information by questionnaire one time between the years of 1999 and 2005. Although details are not provided, it is likely that some of the cases reported their exposure after disease occurrence, allowing for potential differential misclassification in the self-reported exposures in this cohort similar to general concerns with case-control studies. Furthermore, noting large societal trends in GBH exposure between initial exposure ascertainment and the follow-up questionnaire, and the 7.3% under-prediction of glyphosate exposures in the holdout dataset [59], the prediction part of the imputation modeling was likely differentially under-predicting exposures.

Non-differential misclassification occurs when exposure status is equally misclassified among exposed cases and unexposed controls[64]. The approach in AHS 2018 to exposure imputation is one theoretically well-understood source of non-differential misclassification. In addition, it may be more problematic in the context of a ubiquitous exposure because it is hard for participants to know to what extent or how long they have been exposed. Glyphosate's ubiquity in the environment leads to profound concerns that even "unexposed" individuals in the cohort are likely to have been exposed to GBHs; consequently, the magnitude of any potential association relative to the unexposed group may be attenuated due to this misclassification. This problem is encountered with other environmental exposures such as environmental tobacco smoke (ETS): never smokers with ETS exposure carry some cancer risk and are not the ideal true reference group in studies of smoking and tobacco-related cancers [65]. As we noted above, non-differential misclassification is likely to attenuate measures of association, biasing the RR toward the null of 1.0 [66]. Although it is difficult to ascertain exactly, the extent of this source of non-differential misclassification can be estimated through smaller-scale validation studies [66].

5.2.3. Disease Classification & Latency

The updated AHS 2018 included multiple myeloma (MM) in their NHL cases, but the previous AHS 2005 did not. Although MM traditionally did not belong to NHL, WHO recently revised the classification of lymphoid neoplasms and suggested some types of MM (e.g., IgM mutation-related MM) are related more closely to lymphomas, including NHL, than to myelomas [67].

There is much uncertainty surrounding the latency period for NHL. The latency period for short-term high-dose exposures to carcinogens may be as short as two years, but it may also be as long as 15 years or more. Low-dose long-term exposures are expected to have longer median latencies between 15 to 20 years for NHL [68, 69]. It is possible that different NHL subtypes may also have different latencies. Given the uncertainty surrounding NHL latency, it is possible that the follow-up period (median = 6.7 years) in the 2005 AHS study [19], which was unlagged, may have been too short for a sufficient number of exposure-related cancer events to manifest. Given that participants had been exposed to GBHs prior to enrolling in the study (median = 8 years; mean = 7.5 years; SD = 5.3 years), participants could have had an exposure duration ranging from as low as 0 years to as high as 18 years at the time of enrollment, assuming a normal distribution. Hence, although some AHS members may have had sufficient exposure durations to develop NHL, many fell short of the median 15-20 years of expected NHL latency.

The 2018 AHS publication added 11-12 further years of follow-up for all study participants, an additional 483 cases of NHL, and considered five, ten, fifteen, and twenty year exposure lags, which was not possible in AHS 2005 due to its short duration. Epidemiologic studies often lag exposures to account for disease latency under the assumption that recent exposures have little impact on disease development. Theoretically, longer exposure durations and/or lags would present more biologically plausible associations with NHL. For AHS 2018 specifically, not only are the risk estimates associated with longer lag times more plausible that unlagged risk estimates in AHS 2005 and 2018, but the twenty-year exposure lag, specifically, may also be free of the bias caused by exposure imputation described above, given that at this lag exposure information may have been derived exclusively from the baseline questionnaire.

5.2.4. Summary Overall, the study features highlighted above related to exposure assessment and quantification; misclassification; and latency and lag suggest caution in direct comparisons between AHS 2005 and 2018. Additionally, the limitations with AHS 2018 with regard to exposure simulation, potential residual confounding, and misclassification may have accounted for the weaker meta-RR estimate that we obtained when incorporating this study into the meta-analysis.

5.3. Case-Control Studies

Although cohort studies are the gold standard in observational epidemiology, they are often challenging to conduct due to the small number of incident cases for rare diseases such as NHL. Case-control studies can be more efficient for evaluation of rare diseases. For example, the AHS had to recruit tens of thousands of participants (N = 53,760) and follow them for more than a decade in order to gather 575 new cases of NHL, whereas the 5 case-control studies assembled 2,836 NHL cases among all participants (N = 8,868) in a much shorter period of time (Tables 1 and 4). Though the case-control studies are smaller and carry less weight than the large cohort study, it is worth noting that results from multiple case-control studies displayed little heterogeneity (Table 5) and reported similar findings pointing away from null (Table 4).

However, there are other challenges and concerns relevant to the case-control studies utilized in our meta-analysis, which we briefly discuss below.

5.3.1. Control Selection and Exposure Quantification Four of the five case-control studies utilized here are population-based, while one is hospital-based. There may be important differences between hospital-based controls and population-based controls that could impact the interpretability and

comparability of the resulting risk estimates. Of relevance to this concern is that, as noted above in our sensitivity analyses, exclusion of Orsi *et al.* [18] (the hospital-based case-control study) resulted in an increased meta-RR of 1.46 (95% CI: 1.16-1.83), while sequential exclusion of each of the population-based case control studies produced decreased meta-RRs.

Exposure was also quantified differently between the selected case-control studies, further impacting their comparability. While all the studies considered in our meta-analysis conducted exposure assessment based on self-reported questionnaire data, some studies considered ever/never exposure, while others evaluated exposure based on number of days per year (see Tables 1 and 4). Some studies also relied on proxy respondents such as next of kin.

5.3.2. Exposure Misclassification It is always possible for the internal validity of case-control studies to be threatened by recall bias, a form of differential exposure misclassification that occurs when exposures are remembered differently by cases (or their proxies) and controls. Cases may have been more motivated to recall GBH exposure, and the exposures may be more vivid or meaningful due to awareness of the risk factors for their disease. While differential misclassification can bias the OR in either direction, differential misclassification due to cases being more likely to report exposure tends to artificially inflate the OR.

5.3.3. Latency and Lag As discussed in Section 5.2.3, the latency for NHL is uncertain and could be anywhere from 2 years to greater than 15 years. There were differences in how the case-control studies considered and incorporated latency and lag into their analyses. For example, De Roos *et al.* [15] and McDuffie *et al.* [42] do not mention these considerations; by contrast, Hardell *et al.* [17], Orsi *et al.* [18], and Eriksson *et al.* [16] each incorporate latency and lag, albeit differently. These differences suggest caution in the integration of these results.

6. Summary of the GBH and NHL Association in Humans

Overall, the results from our new meta-analysis employing the *a priori* hypothesis and including the updated AHS 2018 study (1) demonstrated a significantly increased NHL risk in highly GBH-exposed individuals (meta-RR = **1.41**, 95% CI: 1.13-1.75; Table 5 and Figure 2A), (2) are aligned with findings (Table 7) from previous meta-analyses [22, 26], and (3) revealed an additional 11-14% and 15-18% increase in NHL relative risk due to high levels of GBH exposure (Table 7) when using the AHS 2018 and the AHS 2005 cohort, respectively.

Together, all of the meta-analyses conducted to date, including our own, consistently report the same key finding: exposure to GBHs are associated with an increased risk of NHL.

Because most people in these epidemiological studies were not exposed to pure glyphosate, but rather glyphosate-based formulations (e.g. Roundup® or Ranger Pro ®) with a number of adjuvants, it could be argued that the NHL manifested as a result of exposure to the mixture or an ingredient other than glyphosate in the formulation. To investigate causal inference regarding the association between glyphosate exposure and NHL, we discuss briefly whether or not the association identified from epidemiological studies could be supported further by experimental animal and mechanistic studies.

7. Animal Data: Lymphoma Prevalence in Glyphosate-Exposed Mice

The animal study outcome most closely linked to human NHL is malignant lymphoma. We identified six unpublished glyphosate and lymphoma studies in mice that are in the public domain from two sources: a presentation by the European Food Safety Authority [70] at the EPA FIFRA Scientific Advisory Panel on Carcinogenic Potential of Glyphosate and a report by The Food and Agriculture Organization of the United Nations and World Health Organization Joint Meeting on Pesticide Residues [21]. EFSA [70] reported results from five unpublished studies: four in CD-1 [71–74] and one

in Swiss albino mice [75], whereas JMPR [21] also reported data from a study in female CD-1 mice [76]. Each study reported four glyphosate doses and corresponding lymphoma incidence in males and females except for Takahashi [76], where the only data available in the public domain was for female mice [21].

7.1. Results of Murine Lymphoma Studies

Results from all studies (n = 6) of malignant lymphomas in mice available in the public domain are presented in Table 9. Study durations ranged from 1.5 to 2 years. All studies administered glyphosate through the diet [71–76], and the concentrations tested ranged from 100 ppm to 50,000 ppm [21]. EFSA [70] and JMPR [21] reported slightly different doses, with JMPR [21] further stratifying by sex. Lymphoma incidence was abstracted from EFSA [70], with slightly different numbers for one study [71]. Table 9 provides the dietary concentration of glyphosate (reported in ppm), the doses (reported in mg/kg/day) provided by EFSA [70] and JMPR [21], and lymphoma incidence in males and females. One study [73] reported food consumption, which was recorded for each treatment group, and weekly mean achieved-dose levels were averaged to calculate actual doses for males and females. Information on how doses were calculated for the other studies [71, 72, 74–76] was not available.

Table 9.

Data from Publically Available Studies of Malignant Lymphomas in Mice Exposed to Glyphosate[a]

Study	Strains	Study Duration	Concentration in Diet (ppm)	Dose (mg/kg/day)		Incidence[b] (%)	
				EFSA [70]	JMPR[c] [21]	Male	Female
Wood *et al.* [73]	CD-1	1.52 years (79 weeks)	0	0	0, 0	0/51 (0)	11/51 (22)
			500	71	71.4, 97.9	1/51 (2)	8/51 (16)
			1500	234	234.2, 299.5	2/51 (4)	10/51 (20)
			5000	810	810, 1081.2	5/51(10)*	11/51 (22)
Kumar [75]	Swiss Albino	1.5 years	0	0	0, 0	10/50 (20)	18/50 (36)
			100	15	14.5, 15.0	15/50 (30)	20/50 (40)
			1000	151	149.7, 151.2	16/50 (32)	19/50 (38)
			10000	1460	1453, 1466.8	19/50 (38)*	25/50 (50)*
Sugimoto [72]	CD-1	1.5 years	0	0	0, 0	2/50 (4)	6/50 (12)
			1600	153	165, 153.2	2/50 (4)	4/50 (8)
			8000	787	838.1,786.8	0/50 (0)	8/50 (16)
			40000	4116	4348, 4116	6/50 (12)*	7/50 (14)
Atkinson *et al.* [74]	CD-1	2 years	N/A	0	0	4/50 (8)	14/50 (28)
			N/A	100	100	2/50 (4)	12/50 (24)
			N/A	300	300	1/50 (2)	9/50

Open in a separate window

Abbreviations: N/A, not available.

[a]Data sources: EFSA [70] and JMPR [21] for both males and females.
[b]Number of lymphomas / total mice in group.
[c]Data for male, female mice.

.

[d]Reported slightly differently in JMPR [21] (N ± 1).
[*]$p_{trend} < 0.05$ reported by at least one test for trend in EFSA [70] or JMPR [21].

In summarizing these studies, EFSA [70] noted that Sugimoto [72] and Wood *et al.* [73] showed statistically significant dose-response in males according to the Cochran-Armitage test for linear trend, whereas Kumar [75] showed a statistically significant Z-test for both males and females. In agreement, JMPR [21] noted that Sugimoto [72] and Wood *et al.* [73] showed a statistically significant trend in males and that Kumar [75] reported statistically significant increases in malignant lymphoma in high-dose groups of both males and females. JMPR [21] further reported Takahashi [76] had a statistically significant increased incidence in lymphoma among females by their trend test. The remaining two studies did not report evidence of a statistically significant dose-response effect.

7.2. Additional Considerations and Recommendations

One challenge with these studies is that at face value they appear to be inconsistent because some show statistically significant findings whereas others do not. However, based on EPA's Cancer Guidelines, evidence of increased lymphoma incidence should not be discounted due to lack of statistical significance in trend and/or pairwise comparison tests. Additional factors that should not be used to exclude study findings are the use of high doses and/or incidence rates that are consistent with levels seen in historical controls [77].

Another consideration is that the study lengths in these animal experiments may have been insufficient for development of NHL. There are proposals that the standard timeframe of two years for a cancer bioassay to approximate long-term cancer incidence in humans should be extended to account for potentially longer latencies. Eighty percent of all human cancers occur after the age of sixty. A two-year-old rat approximates a human of 60-65 years, indicating a traditional two-year bioassay may not be sufficient for late-developing tumors [78].

Future work should combine the results from these six studies into an overall pooled analysis to give a more robust assessment of the evidence. A pooled analysis would take into account the varying study durations (of 18 or 24 months) as well as other between-study differences in dose regimens and mouse strains.

These studies, in which mice were exposed to only glyphosate, may have underreported incidence of malignant lymphoma given evidence of increased toxicity of GBHs compared to glyphosate alone [79–81]. GBH mixtures, which contain a number of adjuvants, have been reported to exert synergistic toxic effects in mechanistic studies (Section 6). Therefore, we also recommend the evaluation of GBHs in chronic animal carcinogenicity studies to better capture representative exposure of humans.

8. Potential Mechanistic Context

There are several possible mechanistic explanations for the increased NHL risk in humans and lymphomas in animals. The etiology of NHL remains largely unknown; however, potential risk factors include autoimmune diseases, infection with viruses and/or bacteria, immunosuppressant medications, and exposures to some pesticides [82, 83]. Although not a formally recognized risk factor for NHL, endocrine disruptors have been associated recently with risk of B-cell neoplasms [84], most of which are NHL [56]. Furthermore, a genetic hallmark of NHL is the recurrence of chromosomal translocations, such as t(14;18), involving the immunoglobulin heavy chain gene fusion *(BCL2-IGH)*, which are frequently detected in subgroups of NHL patients [85] and in pesticide-exposed farmers [86, 87]. Hence, immunosuppression, viral/bacterial infections, endocrine disruption, and genetic alterations

have been suspected as key underlying mechanisms in the development of lymphoma (lymphomagenesis). Although not specifically linked to NHL, oxidative stress is a general mechanism of carcinogenesis that could contribute to lymphomagenesis.

8.1. Immunosuppression/Inflammation

The strongest factors known to increase NHL risk are congenital and acquired states of immunosuppression [88]. Several studies suggest that glyphosate alters the gut microbiome [79, 89] and cytokine IFN-γ and IL-2 production [90]. These changes could impact the immune system, promote chronic inflammation [91], and contribute to susceptibility of invading pathogens, such as *H. pylori* [92].

8.2. Endocrine Disruption

Disruption of sex hormones may contribute to lymphomagenesis/NHL [93]. Glyphosate may act as an endocrine disrupting chemical (EDC) because it has been found recently to alter sex hormone production. Several in vivo studies of male rats exposed to glyphosate have reported significantly lower testosterone levels [94–96], spermatid numbers [94], altered sperm and testicular morphology [94, 95], greater development of the mammary gland [97], and a surge in mast cell infiltration and proliferation accompanied by increased estrogen receptor (ESR1)[97]. In ovarian granulosa cells, glyphosate exposure resulted in decreased cell proliferation and estradiol production [98], which may contribute to lymphomagenesis [93].

8.3. Genetic Alterations

Several studies report that glyphosate can induce single- and double-strand DNA breaks [99–102], purine and pyrimidine oxidation [100], increased comet tail moment [103], and activation of the canonical non-homologous end-joining pathway (c-NHEJ) [101] that stimulates DNA repair. Glyphosate was also reported to induce micronuclei [104–110], sister chromatid exchanges [109], and chromosomal aberrations [111], but other studies found no change in these parameters [112–116]. Conclusions on the genotoxicity of glyphosate remain controversial in the debate on its carcinogenic potential [117]. A recent review reported that this discrepancy could be attributed to differences in the literature analyzed (published versus unpublished), exposure type (glyphosate versus GBHs), and exposure magnitude (low everyday exposures versus higher exposure groups) [118].

8.4. Oxidative Stress

Numerous studies indicate glyphosate causes oxidative stress [119–122]. Biomarkers of oxidative stress have been reported in a number of tissues in rats and mice, including liver, skin, kidney, brain, and plasma. In a study of *albino male rats*, levels of hepatic reduced glutathione were significantly decreased in GBH-exposed animals (1.64 mmol/g) compared to controls (2.64 mmol/g) [80]. A different study in glyphosate-exposed *Wistar rats* reported increased lipid peroxidation across all tissues studied and reactive nitrogen species in the brain and plasma [119]. A proteomic analysis of *Swiss albino mice* reported overexpression of carbonic anhydrase 3, a cytoplasmic protein that plays a role in cellular response to oxidative stress [123]. These mechanisms, among others, provide evidence of biological plausibility for the observed link between glyphosate exposure and human NHL, though further work is needed to better understand these pathways.

9. Conclusions and Future Directions

The rise of glyphosate as the most widely used herbicide raises serious health concerns, given its potential links with NHL. Using our high-exposure *a priori* hypothesis and including the recently updated AHS cohort in a meta-analysis for the first time, we report that GBH exposure is associated

with increased risk of NHL in humans. Our findings are consistent with results reported from prior meta-analyses but show higher risk for NHL because of our focus on the highest exposure groups. However, given the heterogeneity between the studies included, the numerical risk estimates should be interpreted with caution. Additionally, as noted above and depicted in Figure 3, the available studies do not capture the possible effects of increased population exposures due to secular increases in use where "green burn-down" practices introduced in the mid-2000s may be a particularly important source of population exposures. The totality of the evidence from six studies of glyphosate-exposed mice support this association in humans. Although the underlying mechanisms remain unknown, mechanistic studies of glyphosate-induced immunosuppression/inflammation, endocrine disruption, genetic alterations, and oxidative stress suggest plausible links between GBH exposure and NHL development. The overall evidence from human, animal, and mechanistic studies presented here supports a compelling link between exposures to GBHs and increased risk for NHL.

Acknowledgements:

The authors thank Christina Gillezeau, MPH from Icahn School of Medicine at Mount Sinai, New York for carefully checking epidemiological data and Phum Tachachartvanich, PhD for intellectual review and discussion on mechanisms of endocrine disruption. We also thank the anonymous reviewers for their helpful comments. R.M.S. was supported by National Institutes of Environmental Health Sciences (NIEHS) award T32ES015459 and the University of Washington Retirement Association Aging Fellowship.

Abbreviations:

AHS	Agricultural Health Study
c-NHEJ	canonical non-homologous end joining pathway
CI	confidence interval
EDC	endocrine disrupting chemical
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
ETS	environmental tobacco smoke
GBHs	glyphosate-based herbicides
IARC	International Agency for Research on Cancer
IFN-γ	interferon gamma
IL-2	Interleukin-2
JMPR	Joint Meeting on Pesticide Residues by the Food and Agriculture Organization of the United Nations and World Health Organization
meta-RR	meta-analysis relative risk
mg/kg/day	milligrams per kilogram per day
MM	multiple myeloma
NHL	non-Hodgkin lymphoma
OR	odds ratio
ppm	parts per million
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analysis
RR	relative risk

Footnotes

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Declaration of Interest: All authors have no financial conflicts of interest to declare. We disclose Drs. Zhang, Taioli, and Sheppard served as Science Review Board Members of the US EPA FIFRA Scientific Advisory Panel (SAP) Meeting that evaluated glyphosate in December 2016.

References

[1] Thelin G, Stone W, Estimation of annual agricultural pesticide use for counties of the conterminous United States, 1992–2009: U.S *Geological Survey Scientific Investigations Report 2013–5009*, 2013. [Google Scholar]

[2] Benbrook CM, Trends in glyphosate herbicide use in the United States and globally, *Environmental sciences Europe*, 28 (2016) 3. [PMC free article] [PubMed] [Google Scholar]

[3] EPA, Glyphosate Reregistration Eligibility Document (RED). *Glyphosate. EPA-738-R-93-014*, U.S. Environmental Protection Agency, Washington, DC, 1993. [Google Scholar]

[4] EPA, *Glyphosate; pesticide tolerances*, in: Regul FRR. (Ed.), U.S. Environmental Protection Agency, Washington, DC, 2013, pp. 25396–25401. [Google Scholar]

[5] Wang Y, Jaw C, Chen Y, Accumulation of 2,4-D glyphosate in fish and water hyacinth, *Water, Air and Soil Pollution*, 74 (1994) 397–403. [Google Scholar]

[6] Roy D, Konar S, Banerjee S, Charles D, Thompson D, Prasad R, Uptake and persistence of the herbicide glyphosate (Vision®) in fruit of wild blueberry and red raspberry, *Canadian Journal of Forest Research*, 19 (1989) 842–847. [Google Scholar]

[7] Rodrigues NR, de Souza APF, Occurrence of glyphosate and AMPA residues in soy-based infant formula sold in Brazil, Food additives & contaminants. *Part A, Chemistry, analysis, control, exposure & risk assessment*, 35 (2018) 723–730. [PubMed] [Google Scholar]

[8] Alferness P, Iwata Y, Determination of Glyphosate and (Aminomethyl)phosphonic Acid in Soil, Plant and Animal Matrices, and Water by Capillary Gas Chromatography with Mass-Selective Detection, *J. Agric. Food. Chem*, 42 (1994) 2751–2759. [Google Scholar]

[9] Bento CPM, Goossens D, Rezaei M, Riksen M, Mol HGJ, Ritsema CJ, Geissen V, Glyphosate and AMPA distribution in wind-eroded sediment derived from loess soil, *Environmental pollution*, 220 (2017) 1079–1089. [PubMed] [Google Scholar]

[10] Granby K, Johannesen S, Vahl M, Analysis of glyphosate residues in cereals using liquid chromatography-mass spectrometry (LC-MS/MS), *Food additives and contaminants*, 20 (2003) 692–698. [PubMed] [Google Scholar]

[11] Gillezeau C, van Gerwen M, Shaffer RM, Rana I, Zhang L, Sheppard L, Taioli E, The evidence of human exposure to glyphosate: a review, *Environmental health : a global access science source*, 18 (2019) 2. [PMC free article] [PubMed] [Google Scholar]

[12] Conrad A, Schroter-Kermani C, Hoppe HW, Ruther M, Pieper S, Kolossa-Gehring M, Glyphosate in German adults - Time trend (2001 to 2015) of human exposure to a widely used herbicide, *International journal of hygiene and environmental health*, 220 (2017) 8–16. [PubMed] [Google Scholar]

[13] Mills PJ, Kania-Korwel I, Fagan J, McEvoy LK, Laughlin GA, Barrett-Connor E, Excretion of the Herbicide Glyphosate in Older Adults Between 1993 and 2016, *Jama*, 318 (2017) 1610–1611. [PMC free article] [PubMed] [Google Scholar]

[14] Villarreal-Chiu JF, Acosta-Cortés AG, Kumar S, Kaushik G, Biological Limitations on Glyphosate Biodegradation, in: Singh R, Kumar S (Eds.) *Green Technologies and Environmental Sustainability*, Springer International Publishing, Cham, 2017, pp. 179–201. [Google Scholar]

[15] De Roos AJ, Zahm SH, Cantor KP, Weisenburger DD, Holmes FF, Burmeister LF, Blair A, Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men, *Occupational and environmental medicine*, 60 (2003) E11. [PMC free article] [PubMed] [Google Scholar]

[16] Eriksson M, Hardell L, Carlberg M, Akerman M, Pesticide exposure as risk factor for non-Hodgkin lymphoma including histopathological subgroup analysis, *International journal of cancer*, 123 (2008) 1657–1663. [PubMed] [Google Scholar]

[17] Hardell L, Eriksson M, Nordstrom M, Exposure to pesticides as risk factor for non-Hodgkin's lymphoma and hairy cell leukemia: pooled analysis of two Swedish case control studies, *Leukemia & lymphoma*, 43 (2002) 1043–1049. [PubMed] [Google Scholar]

[18] Orsi L, Delabre L, Monnereau A, Delval P, Berthou C, Fenaux P, Marit G, Soubeyran P, Huguet F, Milpied N, Leporrier M, Hemon D, Troussard X, Clavel J, Occupational exposure to pesticides and lymphoid neoplasms among men: results of a French case-control study, *Occupational and environmental medicine*, 66 (2009) 291–298. [PMC free article] [PubMed] [Google Scholar]

[19] De Roos AJ, Blair A, Rusiecki JA, Hoppin JA, Svec M, Dosemeci M, Sandler DP, Alavanja MC, Cancer incidence among glyphosate-exposed pesticide applicators in the Agricultural Health Study, *Environmental health perspectives*, 113 (2005) 49–54. [PMC free article] [PubMed] [Google Scholar]

[20] EPA, Revised Glyphosate Issue Paper: Evaluation of Carcinogenic Potential, *EPA's Office of Pesticide Programs*, United States Environmental Protection Agency; 2017. [Google Scholar]

[21] JMPR, World Health Organization: Pesticide residues in food-2016: toxicological evaluations *Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues*, Geneva, Switzerland, 9–13 May 2016, World Health Organization; 2017. [Google Scholar]

[22] IARC, *Some Organophosphate Insecticides and Herbicides: Diazinon, Glyphosate, Malathion, Parathion, and Tetrachlorvinphos, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*. Volume 112 , International Agency for Research on Cancer (IARC), Lyon, France, 2015. [Google Scholar]

[23] California Office of Environmental Health Hazard Assessment, *Safe Drinking Water and Toxic Enforcement Act of 1986: The Proposition 65 List*, 1985. [Google Scholar]

[24] Andreotti G, Koutros S, Hofmann JN, Sandler DP, Lubin JH, Lynch CF, Lerro CC, De Roos AJ, Parks CG, Alavanja MC, Silverman DT, Beane Freeman LE, Glyphosate Use and Cancer Incidence in the Agricultural Health Study, *Journal of the National Cancer Institute*, 110 (2018) 509–516. [PMC free article] [PubMed] [Google Scholar]

[25] Schinasi L, Leon ME, Non-Hodgkin lymphoma and occupational exposure to agricultural pesticide chemical groups and active ingredients: a systematic review and meta-analysis, *International journal of environmental research and public health*, 11 (2014) 4449–4527. [PMC free article] [PubMed] [Google Scholar]

[26] Chang ET, Delzell E, Systematic review and meta-analysis of glyphosate exposure and risk of lymphohematopoietic cancers, Journal of environmental science and health. *Part. B, Pesticides, food contaminants, and agricultural wastes*, 51 (2016) 402–434. [PMC free article] [PubMed] [Google Scholar]

[27] Rom WN, Markowitz S, *Environmental and occupational medicine*, 4th ed, Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia, 2007. [Google Scholar]

[28] Steinmaus C, Smith AH, Jones RM, Smith MT, Meta-analysis of benzene exposure and non-Hodgkin lymphoma: biases could mask an important association, *Occupational and environmental medicine*, 65 (2008) 371–378. [PMC free article] [PubMed] [Google Scholar]

[29] Zhang L, Steinmaus C, Eastmond DA, Xin XK, Smith MT, Formaldehyde exposure and leukemia: a new meta-analysis and potential mechanisms, *Mutation research*, 681 (2009) 150–168. [PubMed] [Google Scholar]

[30] Duong A, Steinmaus C, McHale CM, Vaughan CP, Zhang L, Reproductive and developmental toxicity of formaldehyde: a systematic review, *Mutation research*, 728 (2011) 118–138. [PMC free article] [PubMed] [Google Scholar]

[31] Greenland S, Meta-analysis, in: Rothman K, Greenland S (Ed.) *Modern Epidemiology*, Lippincott Raven, Philadelphia, 1998, pp. 643–673. [Google Scholar]

[32] Moher D, Liberati A, Tetzlaff J, Altman DG, Group P, Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement, *PLoS medicine*, 6 (2009) e1000097. [PMC free article] [PubMed] [Google Scholar]

[33] Hoar SK, Blair A, Holmes FF, Boysen CD, Robel RJ, Hoover R, Fraumeni JF Jr., Agricultural herbicide use and risk of lymphoma and soft-tissue sarcoma, *Jama*, 256 (1986) 1141–1147. [PubMed] [Google Scholar]

[34] Zahm SH, Weisenburger DD, Babbitt PA, Saal RC, Vaught JB, Cantor KP, Blair A, A case-control study of non-Hodgkin's lymphoma and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in eastern Nebraska, *Epidemiology*, 1 (1990) 349–356. [PubMed] [Google Scholar]

[35] Kachuri L, Demers PA, Blair A, Spinelli JJ, Pahwa M, McLaughlin JR, Pahwa P, Dosman JA, Harris SA, Multiple pesticide exposures and the risk of multiple myeloma in Canadian men, *International journal of cancer*, 133 (2013) 1846–1858. [PubMed] [Google Scholar]

[36] Lee WJ, Cantor KP, Berzofsky JA, Zahm SH, Blair A, Non-Hodgkin's lymphoma among asthmatics exposed to pesticides, *International journal of cancer*, 111 (2004) 298–302. [PubMed] [Google Scholar]

[37] Cantor KP, Blair A, Everett G, Gibson R, Burmeister LF, Brown LM, Schuman L, Dick FR, Pesticides and other agricultural risk factors for non-Hodgkin's lymphoma among men in Iowa and Minnesota, *Cancer research*, 52 (1992) 2447–2455. [PubMed] [Google Scholar]

[38] Hardell L, Eriksson M, A case-control study of non-Hodgkin lymphoma and exposure to pesticides, *Cancer*, 85 (1999) 1353–1360. [PubMed] [Google Scholar]

[39] Nordstrom M, Hardell L, Magnuson A, Hagberg H, Rask-Andersen A, Occupational exposures, animal exposure and smoking as risk factors for hairy cell leukaemia evaluated in a case-control study, *British journal of cancer*, 77 (1998) 2048–2052. [PMC free article] [PubMed] [Google Scholar]

[40] Hohenadel K, Harris SA, McLaughlin JR, Spinelli JJ, Pahwa P, Dosman JA, Demers PA, Blair A, Exposure to multiple pesticides and risk of non-Hodgkin lymphoma in men from six Canadian provinces, *International journal of environmental research and public health*, 8 (2011) 2320–2330. [PMC free article] [PubMed] [Google Scholar]

[41] Cocco P, Satta G, Dubois S, Pili C, Pilleri M, Zucca M, t Mannetje AM, Becker N, Benavente Y, de Sanjose S, Foretova L, Staines A, Maynadie M, Nieters A, Brennan P, Miligi L, Ennas MG, Boffetta P, Lymphoma risk and occupational exposure to pesticides: results of the Epilymph study, *Occupational and environmental medicine*, 70 (2013) 91–98. [PubMed] [Google Scholar]

[42] McDuffie HH, Pahwa P, McLaughlin JR, Spinelli JJ, Fincham S, Dosman JA, Robson D, Skinnider LF, Choi NW, Non-Hodgkin's lymphoma and specific pesticide exposures in men: cross-Canada study of pesticides and health, Cancer epidemiology, biomarkers & prevention : a publication

of the American Association for Cancer Research, *cosponsored by the American Society of Preventive Oncology*, 10 (2001) 1155–1163. [PubMed] [Google Scholar]

[43] Wells G, Shea B, O'connell D, Peterson J, Welch V, Losos M, Tugwell P, *The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses*. Ottawa (ON): Ottawa Hospital Research Institute; 2009, Available in March, (2016). [Google Scholar]

[44] Coble J, Thomas KW, Hines CJ, Hoppin JA, Dosemeci M, Curwin B, Lubin JH, Beane Freeman LE, Blair A, Sandler DP, Alavanja MC, An updated algorithm for estimation of pesticide exposure intensity in the agricultural health study, *International journal of environmental research and public health*, 8 (2011) 4608–4622. [PMC free article] [PubMed] [Google Scholar]

[45] Dosemeci M, Alavanja MC, Rowland AS, Mage D, Zahm SH, Rothman N, Lubin JH, Hoppin JA, Sandler DP, Blair A, A quantitative approach for estimating exposure to pesticides in the Agricultural Health Study, *The Annals of occupational hygiene*, 46 (2002) 245–260. [PubMed] [Google Scholar]

[46] Torstensson NTL, Lundgren LN, Stenstrom J, Influence of Climatic and Edaphic Factors on Persistence of Glyphosate and 2,4-D in Forest Soils, *Ecotox Environ Safe*, 18 (1989) 230–239. [PubMed] [Google Scholar]

[47] Honer L, Dissipation of Glyphosate and Aminomethylphosphonic Acid in Forestry Sites: Lab Project Number: MSL-9940; 993. *Unpublished study prepared by Monsanto Agricultural Co*. 555 pp. in 1992, (Unpublished results). [Google Scholar]

[48] de Andrea MM, Peres TB, Luchini LC, Bazarin S, Papini S, Matallo MB, Savoy VLT, Influence of repeated applications of glyphosate on its persistence and soil bioactivity, *Pesqui Agropecu Bras*, 38 (2003) 1329–1335. [Google Scholar]

[49] McHale CM, Osborne G, Morello-Frosch R, Salmon AG, Sandy MS, Solomon G, Zhang L, Smith MT, Zeise L, Assessing health risks from multiple environmental stressors: Moving from GxE to IxE, *Mutation research*, 775 (2018) 11–20. [PMC free article] [PubMed] [Google Scholar]

[50] DerSimonian R, Laird N, Meta-analysis in clinical trials, *Control Clin Trials*, 7 (1986) 177–188. [PubMed] [Google Scholar]

[51] Petitti D, *Statistical Methods in Meta-analysis, Meta-Analysis, Decision Analysis, and Cost-Effectiveness Analysis: Methods for Quantitative Synthesis in Medicine*, Oxford University Press, New York, NY, 1994, pp. 94–118. [Google Scholar]

[52] Begg CB, Mazumdar M, Operating characteristics of a rank correlation test for publication bias, *Biometrics*, 50 (1994) 1088–1101. [PubMed] [Google Scholar]

[53] Egger M, Davey Smith G, Schneider M, Minder C, Bias in meta-analysis detected by a simple, graphical test, *BMJ*, 315 (1997) 629–634. [PMC free article] [PubMed] [Google Scholar]

[54] *L. StataCorp, Stata Statistical Software: Release 15*, College Station, TX, 2017. [Google Scholar]

[55] *Microsoft Corporation, Microsoft Excel Version 3*, Redmond, Washington, 2013. [Google Scholar]

[56] Dotan E, Aggarwal C, Smith MR, Impact of Rituximab (Rituxan) on the Treatment of B-Cell Non-Hodgkin's Lymphoma, *P & T : a peer-reviewed journal for formulary management*, 35 (2010) 148–157. [PMC free article] [PubMed] [Google Scholar]

[57] Ioannidis JP, Why most published research findings are false, *PLoS medicine*, 2 (2005) e124. [PMC free article] [PubMed] [Google Scholar]

[58] Ioannidis JP, Interpretation of tests of heterogeneity and bias in meta-analysis, *Journal of evaluation in clinical practice*, 14 (2008) 951–957. [PubMed] [Google Scholar]

[59] Heltshe SL, Lubin JH, Koutros S, Coble JB, Ji BT, Alavanja MC, Blair A, Sandler DP, Hines CJ, Thomas KW, Barker J, Andreotti G, Hoppin JA, Beane Freeman LE, Using multiple imputation to assign pesticide use for non-responders in the follow-up questionnaire in the Agricultural Health Study, *Journal of exposure science & environmental epidemiology*, 22 (2012) 409–416. [PMC free article] [PubMed] [Google Scholar]

[60] Little R, Regression with Missing X's: A Review, *Journal of the American Statistical Association*, 87 (1992) 1227–1237. [Google Scholar]

[61] Gryparis A, Paciorek CJ, Zeka A, Schwartz J, Coull BA, Measurement error caused by spatial misalignment in environmental epidemiology, *Biostatistics*, 10 (2009) 258–274. [PMC free article] [PubMed] [Google Scholar]

[62] Sheppard L, Shaffer RM, Re: Glyphosate Use and Cancer Incidence in the Agricultural Health Study, *Journal of the National Cancer Institute*, (2018). [PMC free article] [PubMed] [Google Scholar]

[63] Andreotti G, Lubin JH, Koutros S, Hofmann JN, Sandler DP, Lerro CC, Parks CG, Silverman DT, Beane Freeman LE, Response to Sheppard and Shaffer, *Journal of the National Cancer Institute*, (2018). [Google Scholar]

[64] Porta M, *A dictionary of epidemiology*, Oxford university press; 2008. [Google Scholar]

[65] Eberth B, Olajide D, Craig P, Ludbrook A, Smoking-related disease risk, area deprivation and health behaviours, *Journal of public health*, 36 (2014) 72–80. [PubMed] [Google Scholar]

[66] Pearce N, Checkoway H, Kriebel D, Bias in occupational epidemiology studies, *Occupational and environmental medicine*, 64 (2007) 562–568. [PMC free article] [PubMed] [Google Scholar]

[67] Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, Advani R, Ghielmini M, Salles GA, Zelenetz AD, Jaffe ES, The 2016 revision of the World Health Organization classification of lymphoid neoplasms, *Blood*, 127 (2016) 2375–2390. [PMC free article] [PubMed] [Google Scholar]

[68] Weisenburger DD, Pathological classification of non-Hodgkin's lymphoma for epidemiological studies, *Cancer research*, 52 (1992) 5456s–5462s; discussion 5462s-5464s. [PubMed] [Google Scholar]

[69] Weisenburger DD, Epidemiology of non-Hodgkin's lymphoma: recent findings regarding an emerging epidemic, *Annals of oncology : official journal of the European Society for Medical Oncology*, 5 Suppl 1 (1994) 19–24. [PubMed] [Google Scholar]

[70] EFSA, *European Food Safety Authority: EU assessment of the carcinogenic potential of glyphosate at FIFRA Scientific Advisory Panel on Carcinogenic Potential of Glyphosate*, 13-16 December 2016, 2016.

[71] Knezevich A, Hogan G, *A chronic feeding study of glyphosate (Roundup Technical) in mice: Project no. 77-2061: Monsanto report BDN-77-420*. 1983, (Unpublished results).

[72] Sugimoto K, *HR-001: 18-month oral oncogenicity study in mice*. The Institute of Environmental Toxicology, Tokyo, Japan: Laboratory project ID IET 94–0151, Sankyo Co., Ltd., Tokyo, Japan. 1997, (Unpublished results). [Google Scholar]

[73] Wood E, Dunster J, Watson P, Brooks P, Dietary carcinogenicity study in the mouse Harlan Laboratories Limited, Shardlow, Derbyshire, England, UK: *SPL project no.: 2060-0011*. 2009, Unpublished study, (Unpublished results). [Google Scholar]

[74] Atkinson C, Martin T, Hudson P, Robb D, *Glyphosate: 104 week dietary carcinogenicity study in mice. Unpublished report No. 7793. IRI project No*. 438618, dated 12 April 1991. Inveresk Research International, Tranent, Scotland, UK: Submitted to WHO by Cheminova A/S, Lemvig, Denmark, (Unpublished results). [Google Scholar]

[75] Kumar D, Carcinogenicity study with glyphosate technical in swiss albino mice Toxicology Department, Rallis Research Centre, Rallis India Limited, Bangalore, India: *Data owner: Feinchemie Schwebda GmbH. Study no.: Toxi:1559.CARCI-M*. 2001, (Unpublished results). [Google Scholar]

[76] Takahashi M, Oral feeding carcinogenicity study in mice with AK-01, Nippon Experimental Medical Research Institute Co. Ltd, Agatsuma, Gunma, Japan: *Technical project no. H-95056*, 3303–58, (1999). [Google Scholar]

[77] EPA, Guidelines for Carcinogen Risk Assessment, *EPA's Office of Pesticide Programs*, United States Environmental Protection Agency, Washington, DC, 2005. [Google Scholar]

[78] Huff J, Jacobson MF, Davis DL, The limits of two-year bioassay exposure regimens for identifying chemical carcinogens, *Environmental health perspectives*, 116 (2008) 1439–1442. [PMC free article] [PubMed] [Google Scholar]

[79] Mao Q, Manservisi F, Panzacchi S, Mandrioli D, Menghetti I, Vornoli A, Bua L, Falcioni L, Lesseur C, Chen J, Belpoggi F, Hu J, The Ramazzini Institute 13-week pilot study on glyphosate and Roundup administered at human-equivalent dose to Sprague Dawley rats: effects on the microbiome, *Environmental health : a global access science source*, 17 (2018) 50. [PMC free article] [PubMed] [Google Scholar]

[80] El-Shenawy NS, Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate, *Environmental toxicology and pharmacology*, 28 (2009) 379–385. [PubMed] [Google Scholar]

[81] Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE, Differential effects of glyphosate and roundup on human placental cells and aromatase, *Environmental health perspectives*, 113 (2005) 716–720. [PMC free article] [PubMed] [Google Scholar]

[82] Hu L, Luo D, Zhou T, Tao Y, Feng J, Mei S, The association between non-Hodgkin lymphoma and organophosphate pesticides exposure: A meta-analysis, *Environmental pollution*, 231 (2017) 319–328. [PubMed] [Google Scholar]

[83] Kato I, Koenig KL, Shore RE, Baptiste MS, Lillquist PP, Frizzera G, Burke JS, Watanabe H, Use of anti-inflammatory and non-narcotic analgesic drugs and risk of non-Hodgkin's lymphoma (NHL) (United States), *Cancer causes & control* : CCC, 13 (2002) 965–974. [PubMed] [Google Scholar]

[84] Costas L, Infante-Rivard C, Zock JP, Van Tongeren M, Boffetta P, Cusson A, Robles C, Casabonne D, Benavente Y, Becker N, Brennan P, Foretova L, Maynadie M, Staines A, Nieters A, Cocco P, de Sanjose S, Occupational exposure to endocrine disruptors and lymphoma risk in a multi-centric European study, *British journal of cancer*, 112 (2015) 1251–1256. [PMC free article] [PubMed] [Google Scholar]

[85] Shankland KR, Armitage JO, Hancock BW, Non-Hodgkin lymphoma, *Lancet*, 380 (2012) 848–857. [PubMed] [Google Scholar]

[86] Chiu BC, Blair A, Pesticides, chromosomal aberrations, and non-Hodgkin's lymphoma, *Journal of agromedicine*, 14 (2009) 250–255. [PMC free article] [PubMed] [Google Scholar]

[87] Roulland S, Lebailly P, Lecluse Y, Briand M, Pottier D, Gauduchon P, Characterization of the t(14;18) BCL2-IGH translocation in farmers occupationally exposed to pesticides, *Cancer research*, 64 (2004) 2264–2269. [PubMed] [Google Scholar]

[88] Evens AM, Blum KA, *Non-hodgkin lymphoma*, Springer; 2015. [Google Scholar]

[89] Aitbali Y, Ba-M'hamed S, Elhidar N, Nafis A, Soraa N, Bennis M, Glyphosate based- herbicide exposure affects gut microbiota, anxiety and depression-like behaviors in mice, *Neurotoxicology and teratology*, 67 (2018) 44–49. [PubMed] [Google Scholar]

[90] Nakashima K, Yoshimura T, Mori H, Kawaguchi M, Adachi S, Nakao T, Yamazaki F, [Effects of pesticides on cytokines production by human peripheral blood mononuclear cells--fenitrothion and glyphosate], *Chudoku kenkyu : Chudoku Kenkyukai jun kikanshi = The Japanese journal of toxicology*, 15 (2002) 159–165. [PubMed] [Google Scholar]

[91] Donaldson GP, Lee SM, Mazmanian SK, Gut biogeography of the bacterial microbiota, Nature reviews. *Microbiology*, 14 (2016) 20–32. [PMC free article] [PubMed] [Google Scholar]

[92] Schwabe RF, Jobin C, The microbiome and cancer, Nature reviews. *Cancer*, 13 (2013) 800–812. [PMC free article] [PubMed] [Google Scholar]

[93] Hosgood HD, Gunter MJ, Murphy N, Rohan TE, Strickler HD, The Relation of Obesity-Related Hormonal and Cytokine Levels With Multiple Myeloma and Non-Hodgkin Lymphoma, *Frontiers in oncology*, 8 (2018) 103. [PMC free article] [PubMed] [Google Scholar]

[94] Nardi J, Moras PB, Koeppe C, Dallegrave E, Leal MB, Rossato-Grando LG, Prepubertal subchronic exposure to soy milk and glyphosate leads to endocrine disruption, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 100 (2017) 247–252. [PubMed] [Google Scholar]

[95] Romano RM, Romano MA, Bernardi MM, Furtado PV, Oliveira CA, Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology, *Archives of toxicology*, 84 (2010) 309–317. [PubMed] [Google Scholar]

[96] Pandey A, Rudraiah M, Analysis of endocrine disruption effect of Roundup((R)) in adrenal gland of male rats, *Toxicology reports*, 2 (2015) 1075–1085. [PMC free article] [PubMed] [Google Scholar]

[97] Altamirano GA, Delconte MB, Gomez AL, Ingaramo PI, Bosquiazzo VL, Luque EH, Munoz-de-Toro M, Kass L, Postnatal exposure to a glyphosate-based herbicide modifies mammary gland growth and development in Wistar male rats, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 118 (2018) 111–118. [PubMed] [Google Scholar]

[98] Perego MC, Schutz LF, Caloni F, Cortinovis C, Albonico M, Spicer LJ, Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells in vitro, *Journal of applied toxicology : JAT*, 37 (2017) 692–698. [PubMed] [Google Scholar]

[99] Bolognesi C, Bonatti S, Degan P, Gallerani E, Peluso M, Rabboni R, Roggieri P, Abbondandolo A, Genotoxic activity of glyphosate and its technical formulation roundup, *J Agr Food Chem*, 45 (1997) 1957–1962. [Google Scholar]

[100] Wozniak E, Sicinska P, Michalowicz J, Wozniak K, Reszka E, Huras B, Zakrzewski J, Bukowska B, The mechanism of DNA damage induced by Roundup 360 PLUS, glyphosate and AMPA in human peripheral blood mononuclear cells – genotoxic risk assessement, *Food and chemical toxicology : an*

international journal published for the British Industrial Biological Research Association, (2018). [PubMed] [Google Scholar]

[101] Suarez-Larios K, Salazar-Martinez AM, Montero-Montoya R, Screening of Pesticides with the Potential of Inducing DSB and Successive Recombinational Repair, *Journal of toxicology*, 2017 (2017) 3574840. [PMC free article] [PubMed] [Google Scholar]

[102] Paz-y-Mino C, Sanchez ME, Arevalo M, Munoz MJ, Witte T, De-La-Carrera GO, Leone PE, Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate, *Genetics and Molecular Biology*, 30 (2007) 456–460. [Google Scholar]

[103] Milic M, Zunec S, Micek V, Kasuba V, Mikolic A, Lovakovic BT, Semren TZ, Pavicic I, Cermak AMM, Pizent A, Vrdoljak AL, Valencia-Quintana R, Sanchez-Alarcon J, Zeljezic D, Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28-day exposure to glyphosate, *Arhiv za higijenu rada i toksikologiju*, 69 (2018) 154–168. [PubMed] [Google Scholar]

[104] Bolognesi C, Carrasquilla G, Volpi S, Solomon KR, Marshall EJ, Biomonitoring of genotoxic risk in agricultural workers from five colombian regions: association to occupational exposure to glyphosate, *Journal of toxicology and environmental health. Part A*, 72 (2009) 986–997. [PubMed] [Google Scholar]

[105] Koller VJ, Furhacker M, Nersesyan A, Misik M, Eisenbauer M, Knasmueller S, Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells, *Archives of toxicology*, 86 (2012) 805–813. [PubMed] [Google Scholar]

[106] Suresh T, *Mutagenicity – Micronucleus test in Swiss Albino mice*. Rallis India Limited, Banglore, India: Study no. TOXI:889-MUT-CH.MN, dated 6 May 1993. Sponsor: M/s Feinchemie Schwebda GmbH, Schwebda, Germany., (Unpublished results). [Google Scholar]

[107] Rodrigues H, Penha-Silva N, deAraujo M, Nishijo H, Aversi-Ferreira TA, Effects of Roundup® Pesticide on the Stability of Human Erythrocyte Membranes and Micronuclei Frequency in Bone Marrow Cells of Swiss Mice, *The Open Biology Journal*, 4 (2011) 54–59. [Google Scholar]

[108] Prasad S, Srivastava S, Singh M, Shukla Y, Clastogenic effects of glyphosate in bone marrow cells of swiss albino mice, *Journal of toxicology*, 2009 (2009) 308985. [PMC free article] [PubMed] [Google Scholar]

[109] Amer SM, Aly FAE, Farghaly AA, I. AAE, In vitro and in vivo evaluation of the genotoxicity of the herbicide glyphosate in mice, *Bulletin of the National Research Centre*, 31 (2006) 427–446. [Google Scholar]

[110] Ghisi Nde C, de Oliveira EC, Prioli AJ, Does exposure to glyphosate lead to an increase in the micronuclei frequency? A systematic and meta-analytic review, *Chemosphere*, 145 (2016) 42–54. [PubMed] [Google Scholar]

[111] Lioi MB, Scarfi MR, Santoro A, Barbieri R, Zeni O, Di Berardino D, Ursini MV, Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures in vitro, *Mutation research*, 403 (1998) 13–20. [PubMed] [Google Scholar]

[112] Paz-y-Mino C, Munoz MJ, Maldonado A, Valladares C, Cumbal N, Herrera C, Robles P, Sanchez ME, Lopez-Cortes A, Baseline determination in social, health, and genetic areas in communities affected by glyphosate aerial spraying on the northeastern Ecuadorian border, *Reviews on environmental health*, 26 (2011) 45–51. [PubMed] [Google Scholar]

[113] Sivikova K, Dianovsky J, Cytogenetic effect of technical glyphosate on cultivated bovine peripheral lymphocytes, *International journal of hygiene and environmental health*, 209 (2006) 15–20. [PubMed] [Google Scholar]

[114] Dimitrov BD, Gadeva PG, Benova DK, Bineva MV, Comparative genotoxicity of the herbicides Roundup, Stomp and Reglone in plant and mammalian test systems, *Mutagenesis*, 21 (2006) 375–382. [PubMed] [Google Scholar]

[115] Rank J, Jensen AG, Skov B, Pedersen LH, Jensen K, Genotoxicity testing of the herbicide Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, Salmonella mutagenicity test, and Allium anaphase-telophase test, *Mutation research*, 300 (1993) 29–36. [PubMed] [Google Scholar]

[116] Grisolia CK, A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides, *Mutation research*, 518 (2002) 145–150. [PubMed] [Google Scholar]

[117] Hollert H, Beckhaus T, Some food for thought: a short comment on Charles Benbrook′ paper "How did the US EPA and IARC reach diametrically opposed conclusions on the genotoxicity of glyphosate-based herbicides?" and its implications, *Environmental sciences Europe*, In press (2019). [Google Scholar]

[118] Benbrook CM, How did the US EPA and IARC reach diametrically opposed conclusions on the genotoxicity of glyphosate-based herbicides?, *Environ Sci Eur*, In press (2019). [Google Scholar]

[119] Astiz M, de Alaniz MJ, Marra CA, Antioxidant defense system in rats simultaneously intoxicated with agrochemicals, *Environmental toxicology and pharmacology*, 28 (2009) 465–473. [PubMed] [Google Scholar]

[120] Elie-Caille C, Heu C, Guyon C, Nicod L, Morphological damages of a glyphosate-treated human keratinocyte cell line revealed by a micro- to nanoscale microscopic investigation, *Cell biology and toxicology*, 26 (2010) 331–339. [PubMed] [Google Scholar]

[121] Gehin A, Guillaume YC, Millet J, Guyon C, Nicod L, Vitamins C and E reverse effect of herbicide-induced toxicity on human epidermal cells HaCaT: a biochemometric approach, *International journal of pharmaceutics*, 288 (2005) 219–226. [PubMed] [Google Scholar]

[122] George J, Shukla Y, Emptying of Intracellular Calcium Pool and Oxidative Stress Imbalance Are Associated with the Glyphosate-Induced Proliferation in Human Skin Keratinocytes HaCaT Cells, *ISRN dermatology*, 2013 (2013) 825180. [PMC free article] [PubMed] [Google Scholar]

[123] George J, Prasad S, Mahmood Z, Shukla Y, Studies on glyphosate-induced carcinogenicity in mouse skin: a proteomic approach, *Journal of proteomics*, 73 (2010) 951–964. [PubMed] [Google Scholar]

[124] Blair A, Tarone R, Sandler D, Lynch CF, Rowland A, Wintersteen W, Steen WC, Samanic C, Dosemeci M, Alavanja MC, Reliability of reporting on life-style and agricultural factors by a sample of participants in the Agricultural Health Study from Iowa, *Epidemiology*, 13 (2002) 94–99. [PubMed] [Google Scholar]

General Fact Sheet

What is 2,4-D?

2,4-D is an herbicide that kills plants by changing the way certain cells grow. 2,4-D comes in several chemical forms, including salts, esters, and an acid form. The toxicity of 2,4-D depends on its form. The form also affects what will happen to 2,4-D in the environment and what impacts it may have, especially on fish. 2,4-D is used in many products to control weeds, and it is often mixed with other herbicides in these products.

2,4-D was first used in the United States in the 1940s. Agent Orange, an herbicide used during the Vietnam War, contained both 2,4-D and 2,4,5-T. Dioxin, a by-product of 2,4,5-T, led to the ban of Agent Orange.

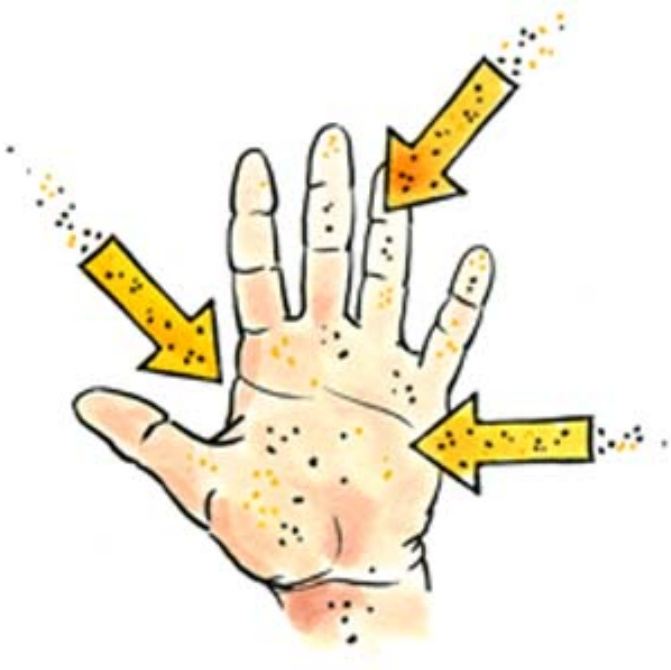
What are some products that contain 2,4-D?

Products containing 2,4-D may be liquids, dusts, or granules. The liquid forms may be concentrated or ready-to-use. There are over a thousand products with 2,4-D in them that are sold in the United States.

Always follow label instructions and take steps to avoid exposure. If any exposures occur, be sure to follow the First Aid instructions on the product label carefully. For additional treatment advice, contact the Poison Control Center at 1-800-222-1222. If you wish to discuss a pesticide problem, please call 1-800-858-7378.

How does 2,4-D work?

2,4-D kills broadleaf weeds but not most grasses. 2,4-D kills plants by causing the cells in the tissues that carry water and nutrients to divide and grow without stopping. Herbicides that act this way are called auxin-type herbicides.



How might I be exposed to 2,4-D?

Products with 2,4-D may be used on farms, home lawns, roadsides, industrial areas, and pastures. You may be exposed if you are applying 2,4-D and you get it on your skin, breathe it in, or eat or smoke afterwards without washing your hands. You also may be exposed if you touch plants that are still wet with spray. You can limit exposure by following the label carefully if you are using products that contain 2,4-D. You can also stay away from grass or plants that have been treated until the leaves are dry.

What are some signs and symptoms from a brief exposure to 2,4-D?

Pure 2,4-D is low in toxicity if eaten, inhaled, or if it contacts the skin, and some forms are low in toxicity to the eyes. However, the acid and salt forms of 2,4- D can cause severe eye irritation. People who drank products containing 2,4- D vomited, had diarrhea, headaches, and were confused or aggressive. Some people also had kidney failure and skeletal muscle damage. People who spilled 2,4-D on their skin developed skin irritation. Breathing 2,4-D vapors can cause coughing, a burning feeling in the airway, and dizziness.

Pets may be exposed to 2,4-D if they touch grass or other plants still wet from spraying and then groom their feet or fur, if they drink the pesticide, or possibly if they eat grass that has been treated with 2,4-D. Dogs may be more sensitive to 2,4-D than other animals. Dogs and cats that ate or drank products with 2,4-D in them developed vomiting, diarrhea, loss of appetite, lethargy, drooling, staggering, or convulsions. See the fact sheet on **Pets and Pesticide Use** for more information.

What happens to 2,4-D when it enters the body?

In humans, 2,4-D is not absorbed well through the skin or lungs, but it is absorbed into the body if swallowed. Sunscreen, insect repellents, and drinking alcohol may increase how much 2,4-D is absorbed through the skin. Once inside, 2,4-D moves throughout the body but does not build up in any tissues. The human body gets rid of most of the 2,4-D in the urine without changing it into anything else. More than 75% of the absorbed 2,4-D leaves the body in the first 4 days after exposure.

Is 2,4-D likely to contribute to the development of cancer?

Scientists have not found a clear link between 2,4-D and cancer in people. Because 2,4-D is often mixed with other herbicides, it is difficult to tell if 2,4-D or one of the other herbicides might be linked to cancer. Some studies have suggested that there may be links between non-Hodgkin's lymphoma and exposure to 2,4-D by itself, but other studies have not found any evidence of this.

In 2004, the EPA decided that 2,4-D could not be classified with regard to its ability to cause cancer because there was not enough data.

Has anyone studied non-cancer effects from long-term exposure to 2,4-D?

Animals fed high doses of 2,4-D for several weeks sometimes had fewer young or the young did not have normal skeletons. This only happened if the amount of 2,4-D fed to the mothers was enough to affect the mothers. 2,4-D has not been linked to health problems in human mothers or infants.

Are children more sensitive to 2,4-D than adults?

While **children may be especially sensitive to pesticides** compared to adults, there are currently no data to conclude that children have increased sensitivity specifically to 2,4-D.

What happens to 2,4-D in the environment?

2,4-D goes through different changes in the environment depending on its form. Most of the time, 2,4-D breaks down in soil so that half of the original amount is gone in 1-14 days. This breakdown time is called the **"half-life"** of the pesticide. One form of 2,4-D, the butoxyethyl ester, had a much longer half-life in aquatic sediment of 186 days.

2,4-D is broken down by bacteria in water and in soil. Water alone can also break down 2,4-D. 2,4-D has been found at low levels in shallow groundwater and streams in both rural and urban areas.

Can 2,4-D affect birds, fish, or other wildlife?

How 2,4-D affects animals and plants depends on the form of 2,4-D. Some of the ester forms of 2,4-D can be very toxic to fish and other aquatic life. The salt forms may be only slightly toxic to aquatic animals. Aquatic animals are more sensitive to 2,4-D as water temperature rises. 2,4-D may be moderately toxic to practically non-toxic to birds if they eat it. Eggs sprayed with 2,4-D still hatched and the chicks were normal. 2,4-D is practically non-toxic to honeybees. It is not expected to be a hazard to other beneficial insects.

Where can I get more information?

For more detailed information about 2,4-D please visit the list of **referenced resources** or call the National Pesticide Information Center, Monday - Friday, between 8:00am - 12:00pm Pacific Time (11:00am - 3:00pm Eastern Time) at 1-800-858-7378 or visit us on the web at **http://npic.orst.edu**. NPIC provides objective, science-based answers to questions about pesticides.

Please cite as: Jervais, G.; Luukinen, B.; Buhl, K.; Stone, D. 2008. ***2,4-D General Fact Sheet***; National Pesticide Information Center, Oregon State University Extension Services. http://npic.orst.edu/factsheets/24Dgen.html.

OUR STORIES › EXPLAINER

2,4-D: The Most Dangerous Pesticide You've Never Heard Of

This toxic herbicide comes with known health risks, but it's still being used on crops, in parks, and maybe even in your own backyard.

March 15, 2016

Danielle Sedbrook

One of the cheapest and most common weed killers in the country has a name you've probably never heard: 2,4-D. Developed by Dow Chemical in the 1940s, this herbicide helped usher in the clean, green, pristine lawns of postwar America, ridding backyards everywhere of aesthetic undesirables like dandelion and white clover. But 2,4-dichlorophenoxyacetic acid, as it's known to chemists, has a less wholesome side. There's a growing body of scientific evidence that the chemical poses a danger to both human health and the environment.

We use cookies to personalise content and ads, to provide social media features and to analyse our traffic. We also share information about your use of our site with our social media, advertising and analytics partners.
— Privacy and Cookie Policy

✓Accept Cookies

Gavin Baker Photography/Shutterstock

The pesticide, which allows not just grasses but also fruits and vegetables to flourish, can attack both the roots and leaves of weeds by making the unwanted plant's cells grow out of control—sort of like inducing cancer in the plant to kill it or drastically slow its spread. It's used widely in agriculture in soybean, corn, sugarcane, and wheat fields, and it turns up in most "weed and feed" products as well as in many lawn treatments. The problem is, the herbicide that was once considered clean and green may no longer be safe by today's standards.

The evidence is slowly mounting—but not yet conclusive. It's not always easy to determine whether a particular substance is causing harm or just happens to be present when some other agent is to blame. Public health experts can't always draw a firm conclusion from studies whose methodologies are lacking in scientific rigor. Take the link between chronic exposure to 2,4-D and cancer: "The evidence isn't clear

We use cookies to personalise content and ads, to provide social media features and to analyse our traffic. We also share information about your use of our site with our social media, advertising and analytics partners.
— Privacy and Cookie Policy

✓Accept Cookies

Researchers have observed apparent links between exposure to 2,4-D and non-Hodgkin's lymphoma (a blood cancer) and sarcoma (a soft-tissue cancer). But both of these can be caused by a number of chemicals, including dioxin, which was frequently mixed into formulations of 2,4-D until the mid-1990s. Nevertheless, in 2015, the International Agency for Research on Cancer declared 2,4-D a possible human carcinogen, based on evidence that it damages human cells and, in a number of studies, caused cancer in laboratory animals.

More conclusive is the proof that 2,4-D falls into a class of compounds called endocrine-disrupting chemicals, compounds that mimic or inhibit the body's hormones. Laboratory studies suggest that 2,4-D can impede the normal action of estrogen, androgen, and most conclusively, thyroid hormones. Dozens of epidemiological, animal, and laboratory studies have shown a link between 2,4-D and thyroid disorders. "That's really important when we're thinking about development," says Kristi Pullen, a staff scientist in NRDC's Health program. "Our thyroid works to ensure the proper timing and development of the brain."

There are reports that 2,4-D can decrease fertility and raise the risk of birth defects. But even though fetuses, infants, and children are at highest risk of these, no studies have looked directly at the effects of 2,4-D on those groups.

Despite concerns about potential health risks, in 2014 the U.S. Environmental Protection Agency approved the combined use of 2,4-D and the popular weed killer Roundup (also known as glyphosate, a whole other—and in many ways more worrying—story when it comes to health and the environment). Enlist Duo, as the combo is called, was already legal in several states. It is used mainly on big farms, where it is sprayed on genetically modified crops called Enlist soy and Enlist corn that have been engineered to be resistant to the poisons.

We use cookies to personalise content and ads, to provide social media features and to analyse our traffic. We also share information about your use of our site with our social media, advertising and analytics partners.
— Privacy and Cookie Policy

✓Accept Cookies

genetically engineered to resist Roundup were sprayed with that herbicide alone. But when the weeds it was intended to kill also developed resistance, 2,4-D was added to make the mix more effective. As Pullen puts it, "These chemicals by themselves can be problematic, but when we start combining them with other toxic chemicals, we're just creating a new problem in order to solve another problem."

The U.S. Department of Agriculture estimates that by 2020, the use of 2,4-D on America's farms could rise between 100 percent and 600 percent now that it has been approved as part of Enlist Duo. According to Pullen, "When you combine increased use with the potential for increased developmental, cancer, and other health impacts, you could create a perfect storm of hazard and exposure coming together."

Also problematic: 2,4-D sticks around in the environment. Depending on the formulation, it can drift through the air from the fields where it is sprayed or be tracked inside homes by pets or children. By the EPA's own measure, 2,4-D has already been detected in groundwater and surface water, as well as in drinking water. Australian scientists reported in 2012 that it was found in more than 90 percent of samples taken from agricultural catchments bordering the Great Barrier Reef—bad news for many fish, for whom the herbicide can be toxic. It can also poison small mammals, including dogs who can ingest it after eating grass treated with 2,4-D.

The easiest way to avoid 2,4-D is to avoid the products that contain it. You can ask your town whether 2,4-D is used in specific parks. You can also visit the website of the National Pesticide Information Center, which has easy-to-read fact sheets on 2,4-D and most other pesticides. If you think you, your child, or your pet have been in contact with plants recently treated with 2,4-D or any other pesticide, contact a poison-control center.

We use cookies to personalise content and ads, to provide social media features and to analyse our traffic. We also share information about your use of our site with our social media, advertising and analytics partners.
— Privacy and Cookie Policy

✓Accept Cookies

Available online at www.sciencedirect.com
ScienceDirect
Ecotoxicology and Environmental Safety 69 (2008) 416–420
Ecotoxicology and Environmental Safety
www.elsevier.com/locate/ecoenv

The 2,4-D herbicide effects on acetylcholinesterase activity and metabolic parameters of piava freshwater fish (*Leporinus obtusidens*) ☆

Milene Braga da Fonseca[a], Lissandra Glusczak[a], Bibiana Silveira Moraes[a], Charlene Cavalheiro de Menezes[a], Alexandra Pretto[a], Miguel Angel Tierno[a], Renato Zanella[b], Fábio Ferreira Gonçalves[b], Vânia Lúcia Loro[a,*]

[a]*Laboratório de Bioquímica Adaptativa, Departamento de Química, Universidade Federal de Santa Maria (UFSM), 97105.900 Santa Maria, RS, Brazil*
[b]*Laboratório de Análises de Resíduos de Pesticidas (LARP), Departamento de Química (UFSM), Brazil*

Received 17 August 2006; received in revised form 13 July 2007; accepted 4 August 2007
Available online 1 November 2007

Abstract

The effects of 2,4-D (1 or 10 mg/L) on acetylcholinesterase (AChE) and metabolic parameters were evaluated in piava (*Leporinus obtusidens*) after 96 h. AChE activity was significantly reduced in the brain at a concentration of 10 mg/L and in the muscle at both concentrations tested. Muscle glycogen and lactate were significantly reduced for both 2,4-D concentrations but no significant change was observed in liver glycogen. Muscle protein levels were enhanced after exposure at 10 mg/L, but no significant changes were observed in muscle and liver glucose. Liver lactate and protein were significantly reduced after exposure to this herbicide. 2,4-D exposure produced a decrease in blood glucose at both concentrations and enhanced lactate levels at 10 mg/L. Plasma protein increased at both concentrations tested. In conclusion, the results obtained indicate that 2,4-D affects brain and muscle AChE activity and some blood and tissue metabolic parameters of *L. obtusidens*. The stress generated by 2,4-D is the probable cause of alterations observed and measured parameters can be used to monitor 2,4-D fish toxicity.
© 2007 Elsevier Inc. All rights reserved.

Keywords: 2, 4-diamin; Herbicide; Ache; Glycogen; Glucose; Protein; Fish

1. Introduction

Environmental contamination by pesticides may cause physiologic and behavioral changes in fish and also affect functions such as reproduction and metabolism (Oruç and Uner, 1999; Bretaud et al., 2000). The 2,4-D is a widely used herbicide in Southern Brazil due to its low cost and good selectivity. This herbicide has poor biodegradability and has been frequently detected in water courses (Chingombe et al., 2006). The 2,4-D concentration recommended for use in agriculture of Southern Brazil ranges from 0.5 to 1.1 mg/L (Colby et al., 1989, Rodrigues and Almeida, 1998). The 2,4-D can be considered as having low contamination potential for surface waters and as a transitional contaminant of subterranean waters in Southern Brazil (Primel et al., 2005). According to Oruç et al. (2004), 2,4-D showed properties similar to natural plant hormones, being used therefore as a weed killer. This herbicide is generally considered to be non-toxic for fish at low doses (Gallagher and Di Giulio, 1991). Generally, biochemical parameters are very sensitive to sublethal concentration of many stress agents (Sancho et al., 1997). Organisms exhibit a characteristic response to a stressor that may be measured through a variety of enzyme activities and metabolic parameters in blood, liver, and muscle. In this study we chose general parameters (glucose, glycogen, lactate, protein) to evaluate a possible stress situation generated by 2,4-D herbicide. Acetylcholinesterase (AChE) activity in different tissues provides a method

☆This study was supported by FAPERGS/PROCOREDE, process number: 040546.0. The work and experiments were approved by the board on experimentation on Animals of the Federal University of Santa Maria, reference number: 057/2006.
*Corresponding author. Fax: +55 55 220 8240.
E-mail addresses: vanial@smail.ufsm.br, vaniluc@yahoo.com.br (V. Lúcia Loro).

0147-6513/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2007.08.006

for diagnosing poisoning by chemicals such as herbicides. Inhibition of AChE, which is responsible for the degradation of acetylcholine, will result in an excessive stimulation of cholinergic nerves, resulting in altered swimming behavior, tremors, convulsions and also undesirable effects (Fernández-Vega et al., 2002; Miron et al., 2005). Disturbances in AChE activity can also impair feeding, escape, and reproductive behavior (Bretaud et al., 2000). Previous studies in our laboratory showed that glyphosate (48%) and clomazone (36%) affect brain and muscle AChE activity in piava *Leporinus obtusidens* and silver catfish (*Rhamdia quelen*), respectively (Miron et al., 2005, Glusczak et al., 2006).

Since the effect of 2,4-D has been little studied in fish species of Southern Brazil and considering that the piava (*L. obtusidens*) is a native freshwater fish with good potential for cultivation and commercial market (Andrian et al., 1994). The objective of this study was to verify if 2,4-D concentrations used in agriculture affect AChE activity and metabolic parameters of *L. obtusidens*.

2. Materials and methods

Piava of both sexes were obtained from the Santa Maria Federal University (UFSM) fish farm (RS, Brazil). Fish (weight, 15 ± 0.5 g; length, 6 ± 1.0 cm) were acclimated to laboratory conditions for 10 days. They were kept in continuously aerated tanks (250 L) with a static system. Water quality characteristics were tested at the beginning, middle and end of exposure. Mean values for test water qualities were as follows: temperature 21 °C (SD 0.4), pH 7.4, dissolved oxygen 7.8 mg/L (SD 0.2). Fish were fed twice a day with commercial fish pellets (42% crude protein, Supra, Brazil). Feces and pellet residues were removed daily by suction. Previous experiments carried out in our laboratory were not able to obtain a median lethal concentration (LC_{50}) of 2,4-D at 96 h, because all the fish survived even at the highest concentration tested (400 mg/L) and showed normal swimming and feeding behavior. The LC_{50} obtained for *R. quelen* was 745 mg/L (unpublished data). The commercial 2,4-D (2,4-D acid equivalent 720 g/L and diclorofenoxiacético dimetilamina salt of 2,4-D 868 g/L), Chemical Abstract Service (CAS 94-75-7), register number 04118189, BASF SA, São Bernardo do Campo, SP, Brazil was used in experimentation. The concentrations used in experiments were chosen considering the recommended concentration used in agriculture in Southern Brazil, ranging from 0.5 to 1.1 mg/L (Colby et al., 1989, Rodrigues and Almeida, 1998). Acute toxicity assays were made in a static manner for 96 h, in accordance with Antón et al. (1994). Following acclimation, the fish were transferred to glass tanks (50 L) with controlled aeration and temperature. Groups of eight fish per tank were exposed for 96 h to 0 (control), 1, or 10 mg/L of 2, 4-D. All tests were carried out in triplicate. The nominal concentrations used were: 0 (without herbicide), 1, or 10 mg/L of commercial 2,4-D. The 2,4-D was monitored in water of experimental tanks every day following the method proposed by Primel et al. (2005). At the end of the exposure period (96 h), all fish were sampled and blood was collected from the caudal vein with a 1 ml heparinized syringe. The blood was centrifuged (10 min, $3000 \times g$, 4 °C) and used for metabolite determination. Plasma total protein was estimated in accordance with Lowry et al. (1951) using bovine serum albumin as standard. Plasma glucose was measured by the glucose oxidase (Bioclin test kit). Plasma was dissolved in 10% trichloroacetic acid (TCA) (1:20 dilution) and lactate was estimated in accordance with Harrower and Brown (1972). Tissues (brain, liver, and white muscle) were removed and placed on ice, frozen in liquid nitrogen and then stored at −20 °C. Liver and muscle glycogen were determined in accordance with Bidinotto et al. (1997). Tissue protein was estimated in accordance with Lowry et al. (1951). Tissue samples were homogenized with 10% TCA using a motor-driven teflon pestle and centrifuged at $1000 \times g$ for 10 min. Supernatant (protein free) was used for the determination of lactate (Harrower and Brown, 1972) and sugar soluble (Park and Johnson, 1949). AChE (EC 3.1.1.7) activities were assayed as described by Ellman et al. (1961) and modified by Miron et al. (2005). A suitable amount (50–100 μl) of homogenate was incubated at 25 °C for 2 min with 0.8 mM acetylthiocholine as substrate and 1.0 mM 5,5′ dithio-bis 2-nitrobenzoic acid (DTNB) as chromogen. Protein content was determined in accordance with Bradford (1976). Enzyme activity is expressed as μmol of acethylthiocholine (AcSCH) hydrolyzed per min per g of protein. AChE activity and metabolic parameters were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Results obtained ($n = 8$) were expressed as mean ± standard deviation (SD) and mean differences were considered significant at $P < 0.05$. Linear correlation between variables was also carried out using Pearson correlation coefficient.

3. Results and discussion

The 2,4-D concentrations in the experimental water after 48 h were approximately 60%–70% of the initial concentration for both concentrations tested, but in water without fish the initial concentrations remain unaltered during 96 h (Table 1). The result concerning 2,4-D showed that approximately 30–40% of initial concentration is owed to fish absorption. Our study showed that 2,4-D herbicide inhibited AChE activity significantly in the muscle tissue ($P < 0.05$) at both concentrations tested. However, in the brain tissue demonstrated inhibition only at the concentration of 10 mg/L (Fig. 1). AChE catalyzes the breakdown of the neurotransmitter acetylcholine (Ach) and a decrease in its activity accumulates Ach within synapses. Considering that the major target organ of 2,4-D are the central nervous system and the cardiovascular system (Benli et al., 2007), the results observed in this study concerning tissue AChE activity reduction may be related to the 2,4-D herbicide or their metabolites presence in water since about 60% of 2,4-D residues were found in water samples after fish herbicide exposure under laboratory conditions (Table 1). Similarly AChE activity in the brain of *L. obtusidens* exposed to glyphosate was reduced by 17% in 3 mg/L and by up to 42% in 20 mg/L. However, muscle AChE activity remained unaltered after glyphosate exposure (Glusczak et al., 2006). The 2,4-D functions by maintaining high levels of the plant hormone auxin, resulting in over-stimulation of plant growth and death (Ateeq et al., 2005; Benli et al., 2007). It is known, that 2,4-D causes changes in the animal nervous system through complex formation with acetylcholine and inhibition of AChE (Sarikaya and Selvi, 2005; Benli et al., 2007). In this study 2,4-D can interfere in nerve muscle transmission mediated by muscle AChE inhibition, however, any behavior alteration was observed. Information on the aquatic toxicity of 2,4-D on non-target organisms like fish is either incomplete. Some formulations of 2,4-D were reported highly toxic to fish and others were less so (Benli et al., 2007).

Changes in AChE activity is frequently used as a biomarker of organophosphorus pesticide contamination

Table 1
The 2,4-D measured in samples of the experimental conditions: (A) (without fish) and (B) (with fish = experimental condition)

Samples (h)	2,4-D	10.0 (mg/L)
(A) Without fish	1.0 (mg/L)	mg L^{-1}
0	1.1	10.1
24	1.0	9.9
48	1.0	9.9
96	0.9	9.8
(B) With fish	2,4-D	mg L^{-1}
0	1.12	9.9
24	0.8	8.9
48	0.65*	6.5*
96	0.6*	6.0*

Values were expressed as mean of 2,4-D in water.
*Significant differences from 0 h ($p<0.05$).

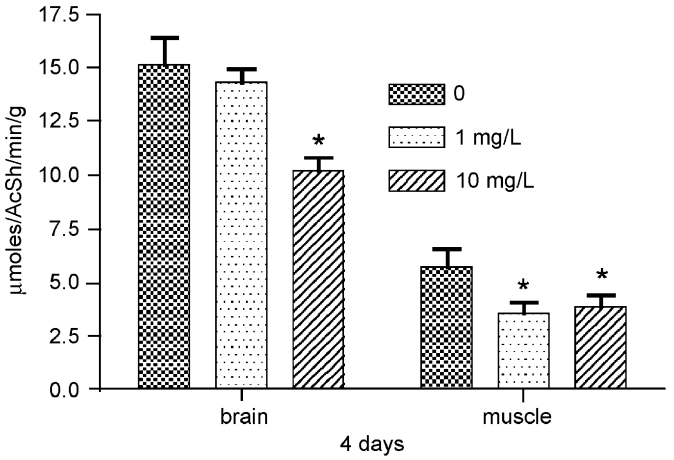


Fig. 1. Brain and muscle AChE (µmol/AcSCh/min/g protein) activity of *Leporinus obtusidens* exposed to 0.0, 1.0 or 10.0 mg/L of 2,4-D (96 h). AChE activity is expressed as means ± SD ($n = 8$). *significant difference from control ($P < 0.05$).

in fish, but pesticides of another class may also affect AChE activity (Dutta and Arends, 2003; Miron et al., 2005, Glusczak et al., 2006). In this context, according to Fernández-Vega et al. (2002), European eels (*Anguilla anguilla*) exposed to 0.22 mg/L of thiobencarb presented a decrease of 35% of muscle AChE activity in 96 h test. Exposure to 50 µg/L of carbofuran during 48 h inhibited 23% of the AChE activity in skeletal muscle of goldfish (Bretaud et al., 2000). Miron et al. (2005) showed a reduction of AChE activity in muscle tissue of silver catfish (*R. quelen*) after 96 h exposure to clomazone, quinclorac, or metsulfuron methyl herbicides and 83% of reduction of brain AChE activity (after exposure to clomazone). Brain AChE inhibition was also shown in eels (*A. anguilla*) exposed to diazinon (Cerón et al., 1996), and thiobencarb (Fernández-Vega et al., 2002). Dutta and Arends (2003) observed that the fish, *Lepomis macrochirus* also presented brain AChE inhibition after exposure to endosulfan.

Different classes of environmental pollutants or their metabolites can change the metabolic state of the liver trough occurrence of detoxication process. In this study after exposure to 2,4-D *L. obtusidens* exhibited a significant decrease in protein and lactate levels from liver tissue, as compared to control values. However, liver glycogen and glucose remained unaltered (Table 1). The muscle tissue showed a significant decrease of glycogen and lactate at both 2,4-D concentrations tested and an increase of protein at the 10 mg/L concentration (Table 2). The decrease observed in muscle glycogen and lactate in this study may indicate that stress caused by the herbicide is accompanied by a higher dependence on the oxidative degradation of glycogen. Results concerning muscle protein suggest that the fish exposed to the herbicide may have a compensatory mechanism to deal with possible tissue protein loss by means an increasing protein synthesis. Oruç and Üner (1999) observed liver protein increase after exposure to 2,4-D for 30 days. Gill et al. (1991) found an increase in liver protein following endosulfan intoxication. The decrease of liver and muscle lactate may indicate higher gluconeogenesis adaptation. Taken together these results suggest that after 2,4-D exposure gluconeogenesis was preferred and could be a response against energy depletion favoring carbohydrate oxidation. Glycogen reductions in liver and muscle after pesticide exposure have been reported in several studies. Fish frequently use glycogen store when in hypoxia situation generated under pesticide exposure (Sancho et al., 1998; Oruç and Üner, 1999; Aguiar et al., 2004). Muscular glycogen decreased in *Clarias batrachus* exposed to organophosphate rogor, (Begum and Vijayaraghavan, 1999), but no changes were observed in liver glycogen of *A. anguilla* exposed to fenitrothion (Sancho et al., 1997). These findings corroborate with the results from our study, where glycogen levels after 2,4-D exposure were reduced in the muscle tissue. Differently, in another study, glycogen stores were reduced only in muscle tissue and enhanced in the liver after clomazone exposure (Crestani et al., 2006). The liver lactate reduction observed in this study may be related to a glycogen synthesis. The protein reduction observed in the liver of *L. obtusidens* exposed to 2,4-D may indicate a response to stress generated by herbicide. A similar response of protein reduction was obtained by Fernández-Vega et al. (2002) in eels exposed to thiobencarb and by Oruç and Üner (1999) after *Cyprinus carpio* were exposed to 2,4-D. On the other hand, plasma protein was increased at all concentrations tested in *L. obtusidens* (Table 2) and may indicate a different response of this fish to 2,4-D toxicity.

Taken together the results obtained in the present study indicate that the entrance of sub-lethal doses of 2,4-D into the ponds or rivers may have deleterious effects on *L. obtusidens*. In conclusion, the results obtained indicate that 2,4-D affects brain and muscle AChE activity and some blood and tissue metabolic parameters of *L. obtusidens,* probably due to stress generated by the

Table 2
Metabolic parameters (μmol/g tissue or ml plasma) in liver, white muscle and blood of *Leporinus sp*

A	Liver			White muscle		
	0 mg/L	1.0 mg/L	10.0 mg/L	0 mg/L	1.0 mg/L	10.0 mg/L
Glycogen	21.1±0.66	19.1±0.25	18.8±0.50	1.21±0.44	0.76±0.17*	1.01±0.11*
Glucose	12.5±3.7	11.2±0.9	11.6±0.6	2.18±0.18	1.88±0.07	1.98±0.6
Lactate	4.54±0.22	3.36±0.18*	3.2±0.2*	6.7±0.36	3.1±0.47*	4.1±0.4*
Protein	28±2.3	21±2.5*	20±1.8*	31±1.15	31.6±0.8	36±1.3*

Plasma

	0 mg/L	1.0 mg/L	10.0 mg/L
Glucose	54±4.6	37±5.4*	44.3±3.2*
Lactate	1.5±0.12	1.4±0.09	1.9±0.09*
Protein	75±7.5	100.7±14.4*	107.4±12.5*

B	Liver			White muscle		
	0 mg/L	1.0 mg/L	10.0 mg/L	0 mg/L	1.0 mg/L	10.0 mg/L
Glycogen	18.9±0.6	22±0.5	20.9±0,8	1.35±0.40	0.86±0.37*	0.98±0.11*
Glucose	10.5±2.7	10.2±0.9	10.6±0.5	1.98±0.08	1.68±0.05*	1.88±04
Lactate	5.54±0.25	4.36±0.18*	2.98±0.2*	7.7±0.46	3.5±0.6*	3.7±0.4*
Protein	32±2.3	20±2.6*	18±1.9*	30±1.1	33±0.85	37±1.5*

Plasma

	0 mg/L	1.0 mg/L	10.0 mg/L
Glucose	48±4.0	40±5.0*	42±3.0*
Lactate	1.6±0.1	1.5±0.09	1.8±0.09*
Protein	78±7.0	10±12.4*	109±11.5*

(A) starved and (B) feeding fish exposed to 0.0, 1.0, or 10.0 mg/L of 2,4-D.
Values were expressed as mean±standard deviation (SD).
*Indicate significant differences from control values ($p < 0.05$) ($n = 8$).

toxicity of 2,4-D. The present data can be used to monitor 2,4-D presence in water. Studies for complete elucidation of 2,4-D induced fish toxicity will be the purpose of future research.

References

Aguiar, L.H., Moraes, G., Avilez, I.M., Altran, A.E., Corrêa, C.F., 2004. Metabolical effects of folidol 600 on the neotropical freshwater fish matrinxã, *Brycon cephalus*. Environ. Res. 95, 224–230.

Andrian I de, F., Dória C. da, C., Torrente, G., Ferreti, C.M.L., 1994. Espectro alimentar e similaridade na composição de quatro espécies de *Leporinus* (Characiformes, Anostomidae) do Rio Paraná, Brasil. Revista Unimar 16 (suplemento 3), 97–106.

Antón, F.A., Laborda, E., Ariz, M., 1994. Acute toxicity of the herbicide glyphosate to fish. Chemosphere 28, 745–753.

Ateeq, B., Farah, M.A., Ahmad, W., 2005. Detection of DNA damage by alkaline single cell gel electrophoresis in 2,4-dichlorofenoxyacetic-acid- and butachlor- exposed erythrocytes of *Clarias batrachus*. Ecotoxicol. Environ. Saf. 62, 348–354.

Begum, G., Vijayaraghavan, S., 1999. Effect of acute exposure of the organophosphate insecticide rogor on some biochemical aspects of *Clarias batrachus* (Linnaeus). Environ. Res. 80, 80–83.

Benli, A.Ç.K., Sarikaya, R., Sepici-Dincel, A., Selvi, M., Sahin, D., Erkoç , F., 2007. Investigation of acute toxicity of (2,4-dichlorophenoxy) acetic acid (2,4-D) herbicide on crayfish (*Astacus leptodactylus* Esch. 1823). Pestic. Biochem. Physiol. 88, 296–299.

Bidinotto, P.M., Souza, R.H.S., Moraes, G., 1997. Hepatic glycogen and glucose in eight tropical freshwater teleost fish: a procedure for field determinations of micro samples. Bol. Tec. CEPTA Pirassununga 10, 53–60.

Bradford, M.M.A., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bretaud, S., Toutant, J.P., Saglio, P., 2000. Effects of carbofuran, diuron and nicosulfuron on acetylcholinesterase activity in goldfish (*Carassius auratus*). Ecotoxicol. Environ. Saf. 47, 117–124.

Cerón, J.J., Ferrando, M.D., Sancho, E., Gutierrez-Panizo, C., Andreu-Moliner, E., 1996. Effects of diazinon exposure on cholinesterase activity in different tissues of European Eel (*Anguilla anguilla*). Ecotoxicol. Environ. Saf. 35, 222–225.

Chingombe, P., Saha, B., Wakeman, R.J., 2006. Effect of surface modification of activated carbon on the sorption of 2,4-dichlorophenoxyacetic acid and benazolin from water. J. Colloid Interfere Sci. 297, 434–442.

Colby, S. R., Lym, R. G., Hill, E. R., Mc Avoy, W. J., Kitchen, L. M., Prasad, R., 1989. Herbicide Handbook of the Weed Science Society of America. sixth ed. Illinois.

Crestani, M., Menezes, C., Glusczak, L., Miron, D., Lazzari, R., Duarte, M., Morsch, V.M., Pippi, A., Vieira, V.L., 2006. Effects of clomazone herbicide on hematological and some parameters of protein and

carbohydrate metabolism of silver catfish *Rhamdia quelen*. Ecotoxicol. Environ. Saf. 65, 48–55.

Dutta, H.M., Arends, D.A., 2003. Effects of endosulfan on brain acetylcholinesterase activity in juvenile bluegill sunfish. Environ. Res. 91, 157–162.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Fernández-Vega, C., Sancho, E., Ferrando, M.D., Andreu, E., 2002. Thiobencarb-induced changes in acetylcholinesterase activity of the fish *Anguilla anguilla*. Pestic. Biochem. Physiol. 72, 55–63.

Gallagher, E., Di Giulio, R., 1991. Effects of 2,4-D dichlophenoxyacetic acid and picloran on biotransformation, peroxisomal and serum enzyme activities in channel catfish (*Ictalurus punctatus*). Toxicol. Lett. 57, 65–72.

Gill, T.S., Pande, J., Tevari, H., 1991. Individual and combined toxicity of common pesticides to teleost *Puntius conchonius* Hamilton. Indian J. Exp. Biol. 29, 145–148.

Glusczak, L., Miron, D.S., Crestani, M., Fonseca, M.B., Pedron, F.A., Duarte, M.F., Vieira, V.L.P., 2006. Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). Ecotoxicol. Environm. Saf. 65, 237–241.

Harrower, J.R., Brown, C.H., 1972. Blood lactic acid. A micromethod adapted to field collection of microliter samples. J. Appl. Physiol. 32, 709–711.

Lowry, D.H., Rosenbrough, N.J., Far, A.L., Randal, R.J., 1951. Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265–275.

Miron, D., Crestani, M., Schetinger, M.R., Morsch, V.M., Baldisserotto, B., Tierno, M.A., Moraes, G., Vieira, V.L.P., 2005. Effects of the herbicides clomazone, quinclorac, and metsulfuron methyl on acetylcholinesterase activity in the silver catfish (*Rhamdia quelen*) (Heptapteridae). Ecotoxicol. Environm. Saf., 61, 398–403.

Oruç, E.Ö., Sevgiler, Y., Üner, N., 2004. Tissue-specific oxidative stress responses responses in fish exposed to 2,4-D and azinphosmethyl. Comp. Biochem. Physiol. Part C 137, 43–51.

Oruç, E.Ö., Üner, N., 1999. Effects of 2,4-Diamin on some parameters of protein and carbohydrate metabolisms in the serum, muscle and liver of Cyprinus carpio. Environ. Pollut. 105, 267–272.

Park, J.T., Johnson, M.J., 1949. A submicro determination of glucose. J. Biol. Chem. 181, 149–151.

Primel, E.G., Zanella, R., Kurz, M.H.S., Gonçalves, F.F., Machado, S.L., de O Marchezan, E., 2005. Poluição das águas por herbicidas utilizados no cultivo do arroz irrigado na região central do estado do Rio Grande do Sul, Brasil: predição teórica e monitoramento. Quím. Nova 28 (4), 605–609.

Rodrigues, B.N., Almeida, F.S., 1998. Guia de Herbicidas. Londrina, Paraná-Brazil 648 pp.

Sancho, E., Ferrando, M.D., Andreu, E., 1997. Sublethal effects of an organophosphate insecticide on the European eel, *Anguilla anguilla*. Ecotoxicol. Environm. Saf. 36, 57–65.

Sancho, E., Ferrando, M.D., Fernández, C., Andreu, E., 1998. Liver energy metabolism of *Anguilla anguilla* after exposure to fenitrothion. Ecotoxicol. Environ. Saf. 41, 168–175.

Sarikaya, R., Selvi, M., 2005. Investigation of acute toxicity of (2,4-dichlorophenoxy) acetic acid (2,4-D) herbicide on larvae and adult Nile tilapia (Oreochromis niloticus L.). Environ. Toxicol. Pharm. 20, 264–268.

Wolters Kluwer Health | Lippincott Williams & Wilkins

A Case-Control Study of Non-Hodgkin's Lymphoma and the Herbicide 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Eastern Nebraska

Author(s): Shelia Hoar Zahm, Dennis D. Weisenburger, Paula A. Babbitt, Robert C. Saal, Jimmie B. Vaught, Kenneth P. Cantor and Aaron Blair

Source: *Epidemiology*, September 1990, Vol. 1, No. 5 (September 1990), pp. 349-356

Published by: Lippincott Williams & Wilkins

Stable URL: https://www.jstor.org/stable/25759830

REFERENCES
Linked references are available on JSTOR for this article:
https://www.jstor.org/stable/25759830?seq=1&cid=pdf-reference#references_tab_contents
You may need to log in to JSTOR to access the linked references.

JSTOR is a not-for-profit service that helps scholars, researchers, and students discover, use, and build upon a wide range of content in a trusted digital archive. We use information technology and tools to increase productivity and facilitate new forms of scholarship. For more information about JSTOR, please contact support@jstor.org.

Your use of the JSTOR archive indicates your acceptance of the Terms & Conditions of Use, available at https://about.jstor.org/terms

Lippincott Williams & Wilkins is collaborating with JSTOR to digitize, preserve and extend access to *Epidemiology*

JSTOR

This content downloaded from
69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

A Case-Control Study of Non-Hodgkin's Lymphoma and the Herbicide 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Eastern Nebraska

Shelia Hoar Zahm,[1] Dennis D. Weisenburger,[2] Paula A. Babbitt,[2] Robert C. Saal,[3] Jimmie B. Vaught,[3] Kenneth P. Cantor,[1] and Aaron Blair[1]

To evaluate the role of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in the development of non-Hodgkin's lymphoma (NHL), we conducted a population-based, case-control study in 66 counties in eastern Nebraska. Telephone interviews were conducted with 201 white men diagnosed with NHL between July 1, 1983, and June 30, 1986, and with 725 controls. There was a 50% excess of NHL among men who mixed or applied 2,4-D (odds ratio [OR] = 1.5; 95% confidence interval = 0.9, 2.5). The risk of NHL increased with the average frequency of use to over threefold for those exposed 20 or more days per year (p for trend = 0.051). Adjusting for use of organophosphate insecticides lowered the risk estimate for frequent users (OR = 1.8), but adjustment for fungicide use increased the risk estimate (OR = 4.5). Simultaneous adjustment for organophosphates and fungicides yielded an OR of 3.1 for farmers who mixed or applied 2,4-D more than 20 days per year. Risk also increased with degree of exposure, as indicated by application method and time spent in contaminated clothing, but not with the number of years of 2,4-D use or failure to use protective equipment. Although other pesticides, especially organophosphate insecticides, may be related to NHL, the risk associated with 2,4-D does not appear to be explained completely by these other exposures. (Epidemiology 1990;1:349–356)

Keywords: agriculture, cancer, 2,4-dichlorophenoxyacetic acid, herbicides, insecticides, non-Hodgkin's lymphoma, occupation, pesticides.

In 1986, a case-control study conducted in Kansas showed an association between the development of non-Hodgkin's lymphoma (NHL) and agricultural use of herbicides (1). Risk for NHL increased with the average number of annual days of exposure to herbicides. Farmers exposed for more than 20 days per year had a sixfold increased risk for NHL. This increased risk seemed to be related specifically to 2,4-dichlorophenoxyacetic acid (2,4-D) use and could not be explained by differential recall, exposure to other pesticides, or other factors. Because of the magnitude of these risks and the widespread potential for exposure to 2,4-D in agriculture, forestry, lawn care, and other uses, we undertook a similar population-based case-control study in Nebraska, another midwestern agricultural state.

Subjects and Methods

Cases of NHL, Hodgkin's disease, multiple myeloma, and chronic lymphocytic leukemia among white men and women, aged 21 years or older, residing in 66 counties in eastern Nebraska, and diagnosed between July 1, 1983, and June 30, 1986, were identified through the Nebraska Lymphoma Study Group and area hospitals. Although not an ongoing population-based cancer registry, special procedures were instituted by the Nebraska Lymphoma Study Group to ascertain all cases in eastern Nebraska. The observed incidence rate for NHL among white males, aged 21 years or older, in eastern Nebraska (18.0/100,000 person-years) was 77% of the rate reported for white men, aged 20 years or older, 1983–1986, by the nearby Iowa component of the National Cancer Institute-sponsored Surveillance, Epidemiology, and End Results program (23.5/100,000 person-years) (L. Ries, personal communication). This report will present data on the white male NHL cases (N = 227).

All cases underwent pathology review and were clas-

[1]Environmental Epidemiology Branch, Division of Cancer Etiology, National Cancer Institute, 6130 Executive Boulevard, Room 418 N, Rockville, MD 20892. [2]Department of Pathology and Microbiology, University of Nebraska Medical Center, 42nd and Dewey Avenue, Omaha, NE 68105. [3]Westat, Inc., 1650 Research Boulevard, Rockville, MD 20850. Address reprint requests to Shelia Hoar Zahm.

This study was supported in part by the National Cancer Institute Contract N01-CP-95618 and State of Nebraska Department of Health Grant LB-506. Preliminary analyses of portions of the data in this report were presented at the annual meeting of the Society for Epidemiologic Research, Vancouver, Canada, June 15–17, 1988.

© 1990 Epidemiology Resources Inc.

This content downloaded from 69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

TABLE 1. Distribution of Non-Hodgkin's Lymphomas by Histologic and Immunologic Type in Interviewed White Men

Histology	Number	Percent
Low grade		
A. Small lymphocytic	14	(7)
B. Follicular, predominantly small cleaved cell	20	(10)
C. Follicular, mixed small cleaved and large cell	22	(11)
Intermediate grade		
D. Follicular, predominantly large cell	15	(8)
E. Diffuse, small cleaved cell	23	(11)
F. Diffuse, mixed small and large cell	16	(8)
G. Diffuse, large cell	51	(25)
High grade		
H. Large cell, immunoblastic	30	(15)
I. Lymphoblastic	1	(<1)
J. Small noncleaved cell	4	(2)
Miscellaneous*	5	(3)
	201	
Immunologic type		
T	20	(10)
B	160	(80)
Indeterminant	11	(5)
Not available	10	(5)
	201	

* Composite lymphomas were assigned to the follicular component if the follicular and diffuse components had the same cell type and to the most indolent cell type if the follicular and diffuse components differed.

sified according to the Working Formulation (2) (Table 1). Only histologically confirmed cases (N = 220) were included. The review also included immunologic phenotyping of the NHL. All follicular lymphomas were considered to be B-cell lymphomas. The diffuse lymphomas were phenotyped using the monoclonal antibodies L26 and UCHL1 (DAKO Corporation, Santa Barbara, CA) that mark B cells and T cells, respectively, in paraffin-embedded tissues (3,4).

Control subjects were selected from residents of the same 66-county area by 3:1 frequency matching by race, sex, vital status, and age (±2 years) to the combined age distribution of the four cancer case series (NHL, Hodgkin's disease, multiple myeloma, and chronic lymphocytic leukemia). For living cases under age 65 (N = 73), controls were selected by two-stage random digit dialing (5). For living cases aged 65 or older (N = 67), controls were selected from the Health Care Financing Administration (Medicare) records. For deceased cases (N = 80), controls were selected from the Nebraska state mortality files using the additional matching factor of year of death. Persons with an underlying cause of death of NHL, Hodgkin's disease, multiple myeloma, leukemia, malignancy of unknown site, aplastic anemia, suicide, homicide, or legal intervention were excluded as controls. A total of 831 white male controls were selected.

Telephone interviews were conducted with 201 NHL cases and 725 controls, or with their next-of-kin, between May, 1986, and October, 1987. The interviewers were not aware of the subjects' case-control status. The response rates for the cases and controls were 91% (living: 93%; deceased: 89%) and 87% (living: 89%; deceased: 85%), respectively. The overall control response rate was 85% and consisted of a weighted average accounting for the refusals in the household census phase of the random digit dialing procedure and the refusals of the randomly selected eligible controls to provide interviews.

This investigation covers the findings related to the association between NHL and agricultural exposure to 2,4-D. The interview questions on agricultural practices included those regarding the herbicides and insecticides used, the application method used most often, use of protective equipment, duration of time wearing work clothes after handling pesticides, cattle raising, and use of fungicides, rodenticides, fumigants, wood preservatives, and fertilizers. For each herbicide and insecticide, the years of use, the average annual number of days of use on the farm, and the average annual number of days the pesticides were personally handled were obtained. The interviewer noted whether the response about each pesticide was volunteered in answer to an open-ended question or reported only after a probe naming the specific pesticide.

All odds ratio (OR) estimates were adjusted for age by stratification (21–59, 60–69, 70–79, and greater than 80 years). Maximum likelihood estimates of a uniform odds ratio and 95% confidence intervals (CI) were computed by Gart's method (6). We assessed duration- and dose-response relationships by means of Mantel's one-tailed linear trend test (7). Logistic regression was also used for the data from farmers to evaluate the effects of several pesticide factors simultaneously (8).

Results

There was no overall excess of NHL among persons who had ever lived or worked on a farm; however, a 50% excess risk of NHL was found among men who mixed or applied 2,4-D (Table 2). Men who lived or worked on farms where 2,4-D was used, but who did not personally handle 2,4-D, had an OR of 1.2 (CI = 0.3, 4.2).

Among men who personally handled 2,4-D, risk in-

This content downloaded from 69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

TABLE 2. Number of White Men with Non-Hodgkin's Lymphoma, Number of Controls, and Odds Ratios by Farming History

Farming History	Cases	Controls*	OR (95% CI)†
Never lived or worked on farm	54	184	1.0
Ever lived or worked on farm	147	539	0.9 (0.6,1.4)
Insecticides used on farm	104	321	1.1 (0.7,1.6)
Herbicides used on farm	75	203	1.3 (0.8,2.0)
Mixed or applied 2,4-D	43	98	1.5 (0.9,2.5)

* Two controls had unknown values for ever having lived or worked on a farm.
† OR (95% CI) = Age-adjusted odds ratio (95% confidence interval).

creased according to the average annual number of days spent mixing or applying 2,4-D in comparison with men who never lived or worked on a farm (Table 3). Risk increased to more than threefold for those with 21 or more days of exposure per year ($p = 0.051$). There was no consistent increase in risk with the number of years of 2,4-D use while the subjects lived or worked on a farm or with the first year of 2,4-D use.

Several characteristics of pesticide use that indicate potential for exposure were evaluated. Among men who personally handled 2,4-D, risk varied by the method used most often to apply herbicides. Tractor-mounted spraying was associated with an OR of 1.4 (CI = 0.8, 2.6; 27 cases, 62 controls) and handheld spraying with an OR of 1.7 (CI = 0.4, 6.7; 4 cases, 9 controls). Risk increased substantially the longer farmers usually waited to change into clean work clothes after handling pesticides (Table 4). Farmers who changed immediately, at the end of the work day, or the following day or later (presumably, these farmers wore the clothes for more than one work day but did not sleep in them) had ORs of 1.1, 1.5, and 4.7, respectively (p for trend = 0.015). Risk did not increase if the farmers reported that they usually failed to use any protective equipment (eg, rubber gloves, rubber boots, mask, spray suit) when handling pesticides. Among farmers who mixed or applied 2,4-D, those who typically used protective equipment while handling any pesticide had an OR of 1.7 (CI = 0.9, 3.1; 25 cases, 48 controls), whereas farmers who did not had an OR of 1.2 (CI = 0.6, 2.4; 16 cases, 49 controls).

Possible confounding of the results for 2,4-D by use of other pesticides was evaluated. The risks associated with

TABLE 3. Number of White Men with Non-Hodgkin's Lymphoma, Number of Controls, and Odds Ratios by Characteristics of Exposure to 2,4-Dichlorophenoxyacetic Acid (2,4-D)

Use of 2,4-D	Cases	Controls	OR (95% CI)*
Never lived or worked on farm	54	184	1.0
Days/year mixing or applying 2,4-D:			
1–5	16	44	1.2 (0.6,2.4)
6–20	12	25	1.6 (0.7,3.6)
21+	3	4	3.3 (0.5,22.1)
Unknown days/year	12	25	—
Chi for trend = 1.639, $p = 0.051$			
Years 2,4-D used on farm:			
1–5	3	12	0.9 (0.2,3.6)
6–15	11	15	2.8 (1.1,7.1)
16–20	3	18	0.6 (0.1,2.1)
21+	13	33	1.3 (0.6,2.7)
Unknown years	15	29	—
Chi for trend = 0.601, $p = 0.274$			
First year of 2,4-D use:			
Prior to 1945	8	21	1.4 (0.5,3.5)
1946–1955	13	39	1.1 (0.5,2.3)
1956–1965	5	8	2.1 (0.6,7.7)
1965–1986	4	12	1.3 (0.3,4.9)
Unknown year	13	18	—
Chi for trend = 0.955, $p = 0.170$			

* OR (95% CI) = age-adjusted odds ratio (95% confidence interval).

use of any phenoxyacetic acid herbicide (ever and average annual number of days) were identical to the risks for 2,4-D alone. All 13 cases and 27 controls who handled 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (ever handled 2,4,5-T: OR = 1.6, CI = 0.7, 3.6; average days per year of exposure 1–5: OR = 1.1; 6–20: OR = 6.4, 4 cases, 2 controls) were also 2,4-D users. None of the subjects who handled 2,4-D more than 20 days per year was a 2,4,5-T user. Excluding the 2,4,5-T users did not change the risks for handling 2,4-D (ever handled 2,4-D: OR = 1.5, CI = 0.8, 2.6; days per year 1–5: OR = 1.1; 6–20: OR = 1.3; 21+: OR = 3.3). Restricting the analysis to farmers and adjusting for the use of other herbicides by class (triazines, amides, benzoics, carbamates, trifluralins, and other) resulted in no meaningful changes in the ORs for those who ever handled 2,4-D or in the positive trend associated with average annual days of exposure to 2,4-D. Adjustments for the use of insecticides by class (chlorinated hydrocarbons, carbamates, organophosphates, metals, and other) also resulted in no meaningful changes in the risk estimates for 2,4-D, except for the use of organophosphates. Adjusting for or-

This content downloaded from 69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

TABLE 4. Number of White Men with Non-Hodgkin's Lymphoma and Controls Who Mixed and Applied 2,4-Dichlorophenoxyacetic Acid (2,4-D) by Timing of Change to Clean Work Clothes after Handling Pesticides

When Subject Usually Changed to Clean Work Clothes	Cases	Controls*	OR (95% CI)†
Never lived or worked on farm	54	184	1.0
Immediately after handling pesticides	6	19	1.1 (0.4,3.1)
At end of work day	31	73	1.5 (0.8,2.6)
Following day or later	6	4	4.7 (1.1,21.5)
Chi for trend = 2.166, p = 0.015			

* Two controls who personally handled 2,4-D had unknown values.
† OR (95% CI) = age-adjusted odds ratio (95% confidence interval).

ganophosphate use on the farm yielded an OR of 1.1 for men who ever handled 2,4-D and ORs of 0.9, 1.3, and 1.8 for men exposed to 2,4-D for 1–5, 6–20, and more than 20 days per year (p for trend = 0.246) relative to farmers with no 2,4-D exposure. Adjustments using more detailed measures of organophosphate exposure (eg, duration and average annual days spent mixing or applying) also resulted in approximately twofold increased risks of NHL among the most frequent handlers of 2,4-D. Analysis of organophosphate use, adjusted for use of 2,4-D, showed an independent association with NHL (ever: OR = 2.4; days per year 1–5: OR = 1.7; 6–20: OR = 1.8; 21+: OR = 3.1) and will be described more thoroughly in a future report. The risk among 2,4-D users compared with nonusers, excluding all organophosphate users, was similar to the adjusted 2,4-D risk for ever use (OR = 1.1) and for the two lower use categories (days per year 1–5: OR = 0.7; 6–20: OR = 1.5). There were no cases exposed to 2,4-D for 21 or more days who were unexposed to organophosphates. Adjustments for the use of fungicides led to increases in the risk estimates associated with 2,4-D exposure (OR = 1.8, CI = 1.1, 3.0) and with average annual days of exposure to 2,4-D (1–5 days: OR = 1.6; 6–20 days: OR = 2.2; 21+ days: OR = 4.5; p for trend = 0.003). Simultaneous adjustment for use of organophosphates, fungicides, and age resulted in ORs of 0.8, 1.3, and 3.1 for farmers who mixed or applied 2,4-D 1–5, 6–20, and more than 20 days per year, respectively. The results of logistic regression analyses, restricted to farmers and including the variables age and use of 2,4-D, organophosphates, and fungicides, were consistent with the stratified analyses. Use of organophosphate insecticides (ever used on farm: OR = 2.4) and 2,4-D (handled 21+ days per year: OR = 2.1) were independent risk factors for NHL.

Approximately two-thirds of both the exposed cases (63%) and controls (64%) volunteered the history of 2,4-D use on the farms where they lived or worked, whereas about one-third of the exposed cases (37%) and controls (36%) reported 2,4-D use only after a specific probe. Risk estimates were similar among the two groups for the use of 2,4-D on the farm (volunteers: OR = 1.5; probes: OR = 1.5), personal handling of 2,4-D (volunteers: OR = 1.5; probes: OR = 1.5), and more than 20 days per year exposure to 2,4-D (volunteers: OR = 2.5, 1 case, 2 controls; probes: OR = 3.8, two cases, 2 controls).

The risk of NHL associated with personal handling of 2,4-D was higher among persons with proxy interviews (1–5 days per year: OR = 2.2; 6–20 days: OR = 2.2; 21+ days: OR = 2.4) than among self-respondents (1–5 days per year: OR = 1.0; 6–20 days: OR = 1.6; 21+ days: OR = 1.4).

Histology, tumor grade, degree of maturation, and immunologic type of the NHLs were evaluated. The association with 2,4-D did not appear to be specific to any subgroup of NHL, although small numbers limited the reliability of the risk estimates. There was a slight suggestion that risk may be higher in intermediate grade NHL (Working Formulation groups D–G, Table 1) (ever: OR = 1.7; 21+ days per year: OR = 5.0, 2 cases, 4 controls), follicular center cell NHL (Working Formulation groups B–D, F–G, Table 1) (ever: OR = 1.7; 21+ days per year: OR = 6.4, 2 cases, 4 controls), large cell NHL (Working Formulation groups G–H) (ever: OR = 1.5; 21+ days per year: OR = 6.2, 1 case, 4 controls), and blastic NHL (Working Formulation groups D, G, and J) (ever: OR = 2.3; 21+ days per year: OR = 9.3, 1 case, 4 controls). Personally handling 2,4-D was associated with both T-cell (OR = 2.0; CI = 0.5, 7.3) and B-cell (OR = 1.5; CI = 0.9, 2.6) lymphomas; however, the trend with days per year was significant (p = 0.045) for B-cell lymphomas only. The ORs for B-cell lymphomas were 1.1, 1.6, and 4.3 for persons exposed to 2,4-D for 1–5, 6–20, and 21 or more days per year, respectively. There were no T-cell lymphoma cases who were exposed to 2,4-D more than 20 days per year.

None of the other factors covered in the interviews, including family history of cancer, prior radiation treatment, other aspects of the medical history, tobacco consumption, or use of hair coloring products, was responsible for the observed 2,4-D associations.

This content downloaded from
69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

Discussion

This population-based case-control study conducted in eastern Nebraska found a 50% excess of NHL associated with mixing or applying 2,4-D. The risk for NHL increased with the average frequency of use to more than threefold among those exposed more than 20 days per year. These findings are consistent with those of a previous case-control study conducted in Kansas (1), although the risk estimates are lower in the present study. The difference in risks in the two states may be explained by statistical variation, since the confidence intervals for risk estimates obtained in Nebraska (CI = 0.5, 22.1) and Kansas (CI = 1.8, 32.3) show considerable overlap.

Some, but not all, variables that indicated the degree of exposure to 2,4-D were related to an increased risk of NHL. In addition to the average annual number of days mixing or applying 2,4-D, the potential for dermal exposure of the usual method of herbicide application (9,10) and the time of change to clean work clothes after handling pesticides were both related to increased risk. However, the number of years of 2,4-D use while the subject lived or worked on the farm was not consistently related to an increased risk for NHL. Interestingly, a similar lack of association with years of use was observed in the Kansas study (1). Computing years of use as a measure of exposure assumes that the level of exposure is similar throughout the year and from year to year. Pesticide use, however, is sporadic, not continuous, throughout the work year, and the amount used may vary considerably from year to year depending on the need and on the use of other farm workers to mix and apply the pesticides. Annual frequency of exposure is more strongly correlated with risk than years of use and may be a better surrogate for delivered dose.

In contrast to the findings of the Kansas study (1), failure to use protective equipment regularly was inversely associated with an increased risk of NHL among 2,4-D users. The elevated risks for users and nonusers of protective equipment were not substantially different from one another. Certainly, one should not discourage the use of protective equipment based on the present study's results.

Exposure to other pesticides affected risk estimates from exposure to 2,4-D. Adjustment for the use of organophosphate insecticides reduced the observed risk associated with 2,4-D exposure, while adjustment for fungicide use increased the risk. Simultaneous adjustment for both resulted in risk estimates for average annual days of exposure similar to the values adjusted for age alone. Logistic regression analyses also indicated independent effects of 2,4-D and organophosphates. Because of the small number of subjects and the high proportion of subjects with multiple exposures, it is not possible in this study to entirely disentangle these relationships. There may be some residual confounding. Case-control studies of larger populations with detailed data on more variable patterns of exposures are needed.

This study relied upon study subjects or their next-of-kin to recall complicated lifetime exposure histories. While there is a great need to improve methods for estimating exposure to pesticides in epidemiologic studies (11), exposure misclassification is not likely to have created spurious risks in this study or in the Kansas study (12). The similarity of the proportions of cases and controls who volunteered histories of 2,4-D use in response to an open-ended question as compared with those who responded to a specific probe for 2,4-D use and the increased risks among frequent users in both the subjects and proxy respondents suggest that recall bias did not occur in this study. Corroboration of a sample of the exposure histories in the Kansas study (1) and methodologic studies of industrial workers (13) observed little difference in accuracy of reports from cases and controls and suggest that the exposure misclassification in this study is likely to be independent of case-control status. Such misclassification tends to decrease risk estimates and reduce exposure-response gradients (14). Thus, misclassification in the Nebraska study is likely to result in an underestimate of the true risk associated with 2,4-D exposure. In addition, increasingly detailed measures of exposure to organophosphates did not further reduce the adjusted OR for 2,4-D exposure, suggesting that misclassification of organophosphate exposure did not lead to an artificial inflation of the risk estimate for 2,4-D.

The large proportion of farmers with no known history of pesticide use in this study (37% of the controls) suggests inaccurate recall by the study subjects. The study definition of farmers, however, included anyone who had ever lived or worked on a farm. This definition includes dependents of farmers and persons who farmed for only brief periods of time. Their opportunity to use pesticides would have been considerably less than for career farmers. Also, some of the older study subjects farmed several decades ago when pesticide use was much less common than in recent years. In addition, some subjects who reported no use of pesticides probably used them. Such misclassification would result in some exposed farmers being classified as nonexposed. In the presence of a positive association, these improperly classified "nonexposed" farmers would reduce the true risk estimates for farmers as a group and lower risk estimates for frequent users of 2,4-D. In fact, the farmers who reported no exposure to 2,4-D had an odds ratio of 0.8

This content downloaded from
69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

(CI = 0.5, 1.2). This deviation from 1.0 could result from random variation or uncontrolled negative confounding. If confounding were the explanation, the odds ratios reported for the exposed farmers are likely to be underestimates of the true risks.

Other Epidemiologic Studies

There have been many epidemiologic studies evaluating the relation of pesticides to cancer which, at first glance, appear to report inconsistent results. The studies generally have not evaluated the same chemicals with the same measures of exposure, however. Only the Kansas study (1) appears comparable with the Nebraska study, ie, based on days per year of agricultural exposure to 2,4-D. Other case-control studies of NHL and herbicides have either treated the phenoxyacetic acid herbicides as a group, with no specific information on 2,4-D and/or lacked information on the number of days per year of exposure (15–21). Case-control studies in Sweden, however, have also noted excess risks for NHL among persons having contact with phenoxyacetic acid herbicides (15,16,21), with an indication in one study (15) that excess risks were present among persons exposed to 2,4,5-T and those exposed only to phenoxys considered unlikely to be contaminated by polychlorinated dibenzodioxins and dibenzofurans, such as 2,4-D and 4-chloro-2-methyl phenoxyacetic acid (MCPA). A study in western Washington state (22) observed a small, but significant, excess risk of NHL among farmers, but the risk did not increase with duration in farming occupations nor with estimated level of exposure in other occupations to 2,4-D; however, no data on the annual number of days of exposure were available. Pearce (19), who found no association between duration or frequency of herbicide use and lymphoma among New Zealand applicators, was studying workers exposed almost entirely to 2,4,5-T. Exposure to 2,4,5-T was not associated with an elevated risk of NHL in the Kansas study, but was associated with a nonsignificant increased risk in the Nebraska study. The results of the Kansas and the Nebraska studies indicate that evaluating risk by job title or duration of exposure only may be inadequate, missing important information. It is apparent that considerable variation of exposure occurs among farmers and that personal exposure histories must be obtained in such studies.

Cohort studies of manufacturers and applicators have also been subject to the problems of mixed exposures. Most of the cohorts exposed to 2,4-D have also been exposed to either 2,4,5-T (23–25) or MCPA (26–29). These investigations have generally not observed excesses of NHL, but the small number of subjects in these studies has limited their usefulness in examining NHL, a rare cause of death (23,24). A recent cohort study of farmers in Canada reported that the risk of NHL increased with the number of acres sprayed with herbicides, particularly in smaller farming operations of less than 1,000 acres (30). Bond et al (31) studied a group of 878 chemical workers who were potentially exposed to several agricultural chemicals, including 2,4-D, and observed a nonsignificant excess of lymphatic and hematopoietic cancers. This excess occurred exclusively among workers who were employed in the 2,4-D plant (5 deaths observed, relative risk = 3.1, $p \leq 0.05$). Two of the five lymphatic and hematopoietic cancers were non-Hodgkin's lymphomas.

Experimental Studies

There is little evidence that 2,4-D is mutagenic or genotoxic (32,33). A 2-year animal feeding study of 2,4-D resulted in a statistically significant excess of astrocytomas in male rats at the highest dose level (Industry Task Force on 2,4-D research data, as cited in Bond et al [31]). The International Agency for Research on Cancer (34) recently concluded that there is inadequate evidence of animal carcinogenicity for 2,4-D. 2,4-D has been associated with increased rates of sister chromatid exchanges and other chromosomal aberrations in vitro (35–37) and in vivo (37,38). The possibility that 2,4-D may be carcinogenic, not by mutagenic activity, but by excessive production of hydrogen peroxide and the proliferation of peroxisomes has been suggested (39).

Immunosuppression, a well-established strong risk factor for NHL (40), could be a possible mechanism by which 2,4-D might increase the risk of NHL. Acute exposure of female mice to high levels of 2,4-D resulted in suppression of antibody production against sheep red blood cells; however, subacute exposure, more comparable with human occupational exposures, did not affect antibody production but, rather, enhanced B- and T-lymphocyte proliferative responses (41). 2,4-D has rarely been reported to be contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (42), the dioxin congener that is a frequent contaminant of some other phenoxy herbicides and that has been reported to be both immunosuppressive and carcinogenic (43–49).

The fact that the mechanism for 2,4-D's putative action is unknown should not detract from the strength and consistency of the results in Kansas and Nebraska concerning risk by days per year of herbicide use. Based on the positive results in these two studies and the likelihood that any exposure misclassification has probably decreased the risk estimates and diluted exposure-response gradients, we believe that the weight of evi-

This content downloaded from
69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

dence indicates that the use of 2,4-D in an agricultural setting increases the risk of NHL among persons handling the chemical frequently.

Acknowledgments

The authors acknowledge the cooperation of the Nebraska Lymphoma Study Group, and Robert Hoover and Joseph F. Fraumeni, Jr for their helpful reviews of the manuscript.

References

1. Hoar SK, Blair A, Holmes FF, Boysen CD, Robel RJ, Hoover R, Fraumeni JF Jr. Agricultural herbicide use and risk of lymphoma and soft-tissue sarcoma. JAMA 1986;256:1141–7.
2. The Non-Hodgkin's Lymphoma Pathologic Classification Project. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: Summary and description of a Working Formulation for Clinical Usage. Cancer 1982;49:2112–35.
3. Linder J, Ye Y, Harrington DS, Armitage JO, Weisenburger DD. Monoclonal antibodies marking T lymphocytes in paraffin-embedded tissue. Am J Pathol 1987;127:1–8.
4. Linder J, Ye Y, Armitage JO, Weisenburger DD. Monoclonal antibodies marking B-cell non-Hodgkin's lymphoma in paraffin-embedded tissue. Modern Pathol 1988;1:29–34.
5. Waksberg J. Sampling methods for random digit dialing. J Am Stat Assoc 1978;73:40–6.
6. Gart JJ. Point and interval estimation of the common odds ratio in the combination of 2 × 2 tables with fixed marginals. Biometrika 1970;57:471–5.
7. Mantel N. Chi square test with 1 degree of freedom, extension of the Mantel-Haenszel procedure. Am Stat Assoc J 1963;58:690–700.
8. Engelman L. Stepwise logistic regression. In: Dixon WJ, ed. BMDP statistical software. Berkeley, CA: University of California Press, 1983:330–44.
9. Lavy TL, Shepard JS, Mattice JD. Exposure measurements of applicators spraying (2,4,5-trichlorophenoxy) acetic acid in the forest. J Agric Food Chem 1980;28:626–30.
10. Lavy TL, Norris LA, Mattice JD, Marx DB. Exposure of forestry ground workers to 2,4-D, picloram, and dichlorprop. Environ Toxicol Chem 1987;6:209–24.
11. Blair A, Zahm SH, Cantor KP, Stewart PA. Estimating exposure to pesticides in epidemiologic studies of cancer. In: Wang RGM, Franklin CA, Honeycutt RC, et al, eds. Biological monitoring for pesticide exposure—measurement, estimation, and risk reduction. Washington, DC: American Chemical Society, ACS Symposium Series 382, 1989:38–46.
12. Blair A, Zahm SH. Herbicides and cancer: A review and discussion of methodologic issues. In: Band P, ed. Recent results in cancer research: Occupational cancer epidemiology. Vancouver, Canada: 1989;120:132–45.
13. Bond GG, Bodner KM, Sobel W, Shellenberger RJ, Flores GH. Validation of work histories obtained from interviews. Am J Epidemiol 1988;128:343–51.
14. Marshall JR, Priore R, Graham S, Brasure J. On the distortion of risk estimates in multiple exposure level case-control studies. Am J Epidemiol 1981;113:464–80.
15. Hardell L, Eriksson M, Lenner P, Lundgren E. Malignant lymphoma and exposure to chemicals, especially organic solvents, chlorophenols and phenoxy acids: A case-control study. Br J Cancer 1981;43:169–76.
16. Hardell L. Relation of soft-tissue sarcoma, malignant lymphoma and colon cancer to phenoxy acids, chlorophenols and other agents. Scand J Work Environ Health 1981;7:119–30.
17. Pearce NE, Smith AH, Howard JF, Sheppard RA, Giles HJ, Teague CA. Non-Hodgkin's lymphoma and exposure to phenoxyherbicides, chlorophenols, fencing work, and meat works employment: A case-control study. Br J Ind Med 1986;43:75–83.
18. Pearce NE, Sheppard RA, Smith AH, Teague CA. Non-Hodgkin's lymphoma and farming: An expanded case-control study. Int J Cancer 1987;39:155–61.
19. Pearce N. Phenoxy herbicides and non-Hodgkin's lymphoma in New Zealand: Frequency and duration of herbicide use. Br J Ind Med 1989;46:143–44.
20. Woods JS, Polissar L, Severson RK, Heuser LS, Kulander BG. Soft tissue sarcoma and non-Hodgkin's lymphoma in relation to phenyoxyherbicide and chlorinated phenol exposure in Western Washington State. J Natl Cancer Inst 1987;78:899–910.
21. Persson B, Dahlander A-M, Fredriksson M, Brage HN, Ohlson CG, Axelson O. Malignant lymphoma and occupational exposures. Br J Ind Med 1989;46:516–20.
22. Woods JS, Polissar L. Non-Hodgkin's lymphoma among phenoxy herbicide-exposed farm workers in Western Washington State. Chemosphere 1989;18:401–6.
23. Riihimaki V, Asp S, Hernberg S. Mortality of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid herbicide applicators in Finland: First report of an ongoing prospective cohort study. Scand J Work Environ Health 1982;8:37–42.
24. Riihimaki V, Asp S, Pukkala E, Hernberg S. Mortality and cancer morbidity among chlorinated phenoxyacid applicators in Finland. Chemosphere 1983;12:779–84.
25. Bond AG, Bodner KM, Cook RR. Phenoxy herbicides and cancer: Insufficient epidemiologic evidence for a causal relationship. Fund Appl Toxicol 1989;12:172–88.
26. Lynge E. Background and design of a Danish cohort study of workers in phenoxy herbicide manufacture. Am J Indus Med 1987;11:427–37.
27. Lynge E. A follow-up study of cancer incidence among workers in manufacture of phenoxy herbicides in Denmark. Br J Cancer 1985;52:259–70.
28. Wiklund K, Dich J, Holm L-E. Risk of malignant lymphoma in Swedish pesticide appliers. Br J Cancer 1987;56:505–8.
29. Wiklund K, Lindefors B-M, Holm L-E. Risk of malignant lymphoma in Swedish agricultural and forestry workers. Br J Ind Med 1988;45:19–24.
30. Wigle DT, Semenciw RM, Wilkins K, et al. Mortality study of Canadian male farm operators: Non-Hodgkin's lymphoma mortality and agricultural practices in Saskatchewan. J Natl Cancer Inst 1990;82:575–82.
31. Bond GG, Wetterstroem NH, Rousch GJ, McLaren EA, Lipps TE, Cook RR. Cause-specific mortality among employees engaged in the manufacture, formulation, or packaging of 2,4-dichlorophenoxyacetic acid and related salts. Br J Ind Med 1988;45:98–105.
32. Canadian Centre for Toxicology. Expert panel report on carcinogenicity of 2,4-D. Guelph, Ontario: 1987.
33. Seiler JP. The genetic toxicology of phenoxy acids other than 2,4,5-T. Mutat Res 1978;55:197–226.
34. International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks to humans. Overall evaluation of carcinogenicity: An update of IARC monographs volumes 1 to 42. Lyon, France: IARC, 1987:156–60.
35. Korte C, Jalal SM. 2,4-D induced clastogenicity and elevated rates of sister chromatid exchanges in cultured human lymphocytes. J Heredity 1982;73:224–6.
36. Turkula TE, Jalal SM. Increased rates of sister chromatid exchanges induced by the herbicide 2,4-D. J Heredity 1985;76:213–4.
37. Mustonen R, Kangas J, Vuojolahti P, Linnainmaa K. Effects of phenoxyacetic acids on the induction of chromosome aberrations in vitro and in vivo. Mutagenesis 1986;1:241–5.
38. Yoder J, Watson M, Benson WW. Lymphocyte chromosome analysis of agricultural workers during extensive occupational exposure to pesticides. Mutation Res 1973;21:335–40.

This content downloaded from
69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

39. Vainio H, Nickels J, Linnainmaa K. Phenoxy acid herbicides cause peroxisome proliferation in Chinese hamsters. Scand J Work Environ Health 1982;8:70–73.
40. Greene MH. Non-Hodgkin's lymphoma and mycosis fungoides. In: Schottenfeld D, Fraumeni JF, Jr., eds. Cancer epidemiology and prevention. Philadelphia: WB Saunders Company, 1982:754–78.
41. Blakely BR, Schiefer BH. The effect of topically applied n-butylester of 2,4-dichlorophenoxyacetic acid on the immune response in mice. J Appl Toxicol 1986;6:291–5.
42. Hagenmaier H. Determination of 2,3,7,8-tetrachlorodibenzo-p-dioxin in commercial chlorophenols and related products. Fresenius Z Anal Chem 1986;325:603–6.
43. Vos JG, Moore JA, Zinkl JG. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the immune system of laboratory animals. Environ Health Perspect 1973;5:149–62.
44. Harris MW, Moore JA, Vos JG, Gupta BN. General biological effects of TCDD in laboratory animals. Environ Health Perspect 1973;5:101–9.
45. Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CN, Barnard SD, Hummel RA, Humiston CG. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rats. Toxicol Appl Pharmacol 1978;46:279–303.
46. Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Gehring PJ. Long-term toxicologic studies of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in laboratory animals. Ann NY Acad Sci 1979;320:397–404.
47. Van Miller JP, Lalich JJ, Allen JR. Increased incidence of neoplasms in rats exposed to low levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Chemosphere 1977;10:625–32.
48. National Toxicology Program. (Report 80-31) Carcinogenesis bioassay of 2,3,7,8-tetrachlorodibenzo-p-dioxin (CAS N. 1746-01-6) in Osborne-Mendel rats and B6C3F1 mice (Gavage study), technical report series 209, National Institutes of Health publication 82-1765. Research Triangle Park, NC, Dept of Health and Human Services, 1982.
49. National Toxicology Program. (Report 80-32) Carcinogenesis bioassay of 2,3,7,8-tetrachlorodibenzo-p-dioxin (CAS No. 1746-01-6) in Swiss-Webster mice (Dermal study), technical report series 209, National Institutes of Health publication 82-1757. Research Triangle Park, NC, Dept of Health and Human Services, 1982.

This content downloaded from
69.123.204.27 on Fri, 25 Jun 2021 02:10:32 UTC
All use subject to https://about.jstor.org/terms

AMERICAN JOURNAL OF INDUSTRIAL MEDICINE 43:306–313 (2003)

Pancreatic Cancer Mortality and Organochlorine Pesticide Exposure in California, 1989–1996

Tim Clary, MA, MS, PhD[1] and Beate Ritz, MD, PhD[2,3]*

Background *Occupational studies have suggested a possible link between organochlorine pesticides and the occurrence of pancreatic cancers. California maintains a death file and a pesticide reporting system that allows examination of this relationship for residents of high use areas.*

Methods *We employed a mortality odds ratio design to compare deaths from pancreatic cancer (1989–1996) with a random sample of non-cancer deaths. Using pesticide data for three agricultural counties, we classified 102 ZIP codes in quartiles of pesticide usage for 1972–1989. Using logistic regression we estimated the effect of pesticide applications by ZIP code controlling for possible confounders.*

Results *Among long-term residents, pancreatic cancer mortality was elevated for those living in ZIP codes with the highest use of four pesticides: 1,3-dichloropropene (1,3-d), captafol, pentachloronitrobenzene (PCNB), and dieldrin. No dose-response relationship was observed.*

Conclusions *Our study suggests increased pancreatic cancer mortality among long-term residents in areas of high application rates of 1,3-d (an EPA-classified probable human carcinogen), captafol, pentacholoronitrobenzene (PCNB), and dieldrin.* Am. J. Ind. Med. 43:306–313, 2003. © *2003 Wiley-Liss, Inc.*

KEY WORDS: pancreatic cancer; organochlorines; pesticide use; California; mortality odds ratio study

BACKGROUND

Pancreatic cancer in the United States ranked 10th among all incident cancers in 2000 with an overall 5 year survival rate between 3 and 5% in the period from 1974 to 1995. In 2000, 28,300 new cases of pancreatic cancer and 28,200 deaths occurred in the United States [Greenlee et al., 2000]. In California the number of deaths from pancreatic cancer in the year 2000 was 2,700. The high lethality of this cancer underscores the need to identify risk factors, which may eventually allow for prevention of the disease. Our study examines the contribution of possible environmental risk factors, specifically organochlorine pesticides, suggested by previous research.

What is known about the etiology of pancreatic cancer is limited. Presently, the only risk factor uniformly agreed upon has been smoking with a moderate impact of possibly doubling the disease risk [Silverman et al., 1994; Ahlgren, 1996]. The question whether pesticides, specifically biopersistent organochlorines, may play a role in causing pancreatic cancers has lately gained more attention. While several recent studies [Garabrant et al., 1992; Fryzek et al., 1997; Porta et al., 1999] have suggested organochlorine compounds may be related to pancreatic cancer, other studies [Blair et al., 1991; Cocco et al., 2000] have found little, if any, association with pesticides in general.

[1]United States Agency for International Development (USAID), Bureau for Global Health
[2]Department of Epidemiology, School of Public Health, UCLA, Los Angeles, California
[3]Center for Occupational and Environmental Health, School of Public Health, UCLA, Los Angeles, California
Contract grant sponsor: UCLA Center for Occupational and Environment Health.
*Correspondence to: Beate Ritz, Associate Professor, Department of Epidemiology, School of Public Health, UCLA, PO Box 951772, Los Angeles, CA 90095-1772.
E-mail: britz@ucla.edu

Accepted 23 September 2002
DOI 10.1002/ajim.10188. Published online in Wiley InterScience (www.interscience.wiley.com)

© 2003 Wiley-Liss, Inc.

Recently, Schwartz et al. [1998] conducted an ecologic study in Florida in which they used countywide per capita solid waste and yard trash as proxy measures for pesticide exposure of residents. This is the first study to suggest that low-level residential exposures are associated with pancreatic cancer incidence rates. California has maintained a unique system of pesticide use reporting (PUR) since 1972 allowing us to construct a more direct measure of residential pesticide exposure. In the following, we present results from a mortality odds ratio study of pancreatic cancer mortality and long-term residential pesticide exposure derived from PUR records.

MATERIALS AND METHODS

Study Subjects

We based our study in three California counties (Fresno, Kern, and Tulare) known for their large agricultural production and for which we had detailed PUR data. The 2001 populations of these counties were 815,734, 676,367, and 374,249, respectively [USCB, 2001]. Study subjects were limited to residents who died in those same counties. Using computerized California Death Tape files supplied by the California Vital Statistics division we identified 1,002 cases of pancreatic cancer (ICD-157 9th ed.) reported as underlying cause of death between 1989 and 1996 in a total of 102 ZIP codes within the three counties. Approximately ten controls (n = 10,022) were selected for each case at random from all non-cancer deaths that occurred during the same time period in these counties. Inclusion of subjects was based on having race and education level reported on death certificates, which made it necessary to exclude approximately 5–6% of all eligible cases and controls leaving us with 950 cases and 9,435 controls.

Exposure Assessment

Our residential pesticide measure is based on data from the California Department of Pesticide Regulation (CDPR) PUR database. Since the early 1970s, California has required commercial pest control operators—including farmers—to report all restricted pesticides used, as well as the date and location of application. The reporting is done at a section (one square mile) level. Employing geographic information system (GIS) software we aggregated the PUR data from section to ZIP code level for all three counties in our study because residential information on death certificates was provided at the ZIP code level only. This technique in combination with information on duration of residency in county allowed us to assign exposure.

We chose to examine 18 chlorinated organic compound pesticides based on their usage (>5 tons used in the three target counties in 1972) and because chlorinated organic compound pesticides have been linked to pancreatic cancers in worker studies. Among the 18 chlorinated organic compounds commonly applied in the targeted counties were four pesticides classified by the Environmental Protection Agency [EPA, 2001] as being possible, probable, or known human carcinogens (1,3-dichloropropene or 1,3-d, DDT, dieldrin, and toxaphene).

Statistical Analysis

For our analyses we created a measure of total pesticide usage per ZIP code based on tons of active ingredient applied from 1972 to 1989 for each pesticide and derived quartiles of pesticide usage for all 18 organochlorines (Table I). We then used logistic regression analysis, recommended for mortality odds ratio studies [Miettinen and Wang, 1981; Morgenstern and Thomas, 1993], to estimate odds ratios and 95% confidence intervals for crude and adjusted pancreatic cancer mortality for the quartile of highest pesticide usage at the ZIP code level ($\geq$75%) in comparison to all other quartiles (<75%). Breslow et al. [1983] have shown that conditional on exposure not causing death from the comparison disease—a prevalence odds ratios (POR) case-control analysis controlling for confounding factors can yield valid estimates of the relative risk.

The California computerized death tapes for 1989–1996 provided information on personal characteristics such as gender, race, age, place of death, year of death, education, and duration of living in the county of residence prior to death. These variables were used to assign exposure (place of residence by ZIP code, duration of residency in county) and to control for confounding (gender, race, age at death, year of death, and educational level).

In all adjusted analyses we controlled for race (non-Hispanic Whites, non-Hispanic Blacks, non-Hispanic Asians, Hispanics, non-Hispanic, and other), age at death (15–30, 30–45, 45–60, 60–75, 75–85, >85), educational level (<12, 12, and >12 years), year of death, and whether the individual lived in an urban versus rural area. We examined all pesticides individually, simultaneously and in various combinations of pesticide subgroups (e.g., only those classified as carcinogens). We also examined the potential impact of long-term exposure for residents living in the county more than 20 years at the time of their death.

RESULTS

Californians who died from pancreatic cancer between 1989 and 1996 were on average more often female and slightly older ($\sim$3 years) than the non-cancer deaths that served as controls in our analysis (Table II). We attribute the gender difference between our cases and controls to the

TABLE I. Distribution of Tonnage of Active Ingredients Applied in 102 ZIP Code Areas of Fresno, Kern, and Tulare Counties, 1972–1989

Pesticide	Upper cutpoint of 1st quartile	Upper cutpoint of 2nd quartile	Upper cutpoint of 3rd quartile	Upper cutpoint of 4th quartile	Total tons	Number of ZIPs where applied
1,3-d[a]	7.34	30.63	117.36	1072.86	8998.86	70
Captafol	0.17	0.89	4.47	54.99	238.93	35
Chlordimeform	0.10	0.51	2.91	64.20	285.29	68
Chlorobenzilate	0.05	0.24	1.08	15.78	68.33	50
Chloropicrin	0.07	1.10	12.68	512.07	2031.39	77
Chlorthal dimethyl	0.44	1.43	10.64	121.04	911.33	58
Dalapon, sodium salt	0.12	0.34	1.22	13.09	59.57	57
DBCP	0.37	2.60	11.68	46.59	432.56	54
DDT[a]	0.01	0.12	0.76	3.80	10.70	20
Dicofol	0.36	9.92	32.50	241.34	1996.30	84
Dieldrin[a]	0.02	0.06	0.32	4.43	20.15	48
Diuron	0.49	4.03	9.79	43.10	519.25	77
Endosulfan	0.19	1.81	10.89	120.58	819.35	82
Methoxychlor	0.09	0.32	2.73	15.99	121.91	69
PCNB	0.10	0.66	3.01	20.74	117.86	49
Pyrazon	0.07	0.27	1.56	11.46	46.64	29
Simazine	0.12	1.45	7.68	51.01	401.40	75
Toxaphene[a]	0.16	0.68	12.05	407.84	1553.31	50

[a]EPA-classified possible, probable, or known human carcinogen.

TABLE II. Demographic Characteristics of the Study Subjects who Died in Fresno, Kern, and Tulare Counties Between 1989–1996

	Underlying cause of death	
	Pancreatic cancer (ICD-9 157)	All non-cancer deaths
Number of deaths	950	9,435
Mean age (years)	70.7	67.0
Gender		
Female	50.3%	47.1%
Ethnicity		
White	76.1%	72.4%
African–American	5.6%	5.9%
Hispanic	14.9%	18.2%
Asian	2.7%	2.7%
Other	0.2%	0.4%
Urban[a]	47.8%	44.6%
Mean duration of living in county of residence prior to death (years)	37.1	34.2
Educational attainment (years)		
<12	40.1%	48.7%
=12	31.9%	29.9%
>12	28.0%	21.4%

[a]Cases and controls were classified urban if their residence at death was a ZIP code for Bakersfield, Visalia, or Fresno in which the majority of ZIP code's population resided within the city limits.

fact that fewer women than men die at earlier ages of chronic heart disease [NIHCM, 2000], the main cause of non-cancer deaths from which we selected controls randomly.

Our cases were also more likely to have been White and less likely to be Hispanic, African–American, Asian or of the ''other'' race category. Pancreatic cancer cases were also somewhat more educated than all non-cancer controls and had on average lived in their county of residence at time of death for a longer period (approximately 3 years). About 67% of all subjects included in this study had lived in the county of death for >20 years. These demographic characteristics were consistent over different strata of covariates.

Using the PUR data to classify ZIP code area-specific pesticide exposure and including all organochlorine pesticides in the model, our analysis showed an increased risk for those residents who had lived in one of the three counties for at least 20 years at the time of death and whose residence at time of death were in ZIP codes of the highest quartile of 1,3-dichloropropene (1,3-d) application (POR = 1.89, CI = 1.13–3.15, n = 107) in comparison to the lower three quartiles (Table III). Several other pesticides, namely captafol, dieldrin, and pentachloronitrobenzene (PCNB), exhibited similar size risk increases (ORs of 1.73, 1.52, and 1.79, respectively), but none of the 95% CIs excluded the null value (Table III); only 1,3-d and dieldrin have been classified by the EPA as either possible, probable, or known human carcinogens.

TABLE III. Adjusted* Prevalence Odds Ratios (POR) and (95% CI) for the Effect of Having Lived in a ZIP Code Area With the Highest Quartile of Pesticide use on Pancreatic Cancer Mortality (ICD-9 157)

		OR (95% CI) for all subjects	OR (95% CI) for subjects having lived >20 years in county at time of death
Pesticide		**Case N = 950, control N = 9,435**	**Case N = 697, control N = 6,259**
1,3-d	Lower three quartiles	1.00	1.00
	Highest quartile	1.48 (0.95–2.31)	1.89 (1.13–3.15)
	Number (N) cases exposed	130	107
Captafol	Lower three quartiles	1.00	1.00
	Highest quartile	0.96 (0.51–1.82)	1.73 (0.70–4.28)
	Number (N) cases exposed	88	75
Chlordimeform	Lower three quartiles	1.00	1.00
	Highest quartile	0.73 (0.40–1.33)	1.13 (0.54–2.37)
	Number (N) cases exposed	132	107
Chlorobenzilate	Lower three quartiles	1.00	1.00
	Highest quartile	0.85 (0.55–1.32)	0.91 (0.55–1.50)
	Number (N) cases exposed	74	55
Chloropicrin	Lower three quartiles	1.00	1.00
	Highest quartile	0.84 (0.65–1.09)	0.78 (0.58–1.06)
	Number (N) cases exposed	219	172
Chlorthal dimethyl	Lower three quartiles	1.00	1.00
	Highest quartile	0.78 (0.40–1.52)	0.49 (0.18–1.33)
	Number (N) cases exposed	113	89
Dalapon, sodium salt	Lower three quartiles	1.00	1.00
	Highest quartile	1.14 (0.87–1.49)	1.14 (0.83–1.56)
	Number (N) cases exposed	153	118
DBCP	Lower three quartiles	1.00	1.00
	Highest quartile	0.84 (0.64–1.10)	0.78 (0.57–1.07)
	Number (N) cases exposed	197	160
DDT	Lower three quartiles	1.00	1.00
	Highest quartile	1.15 (0.31–4.37)	0.86 (0.20–3.77)
	Number (N) cases exposed	11	9
Dicofol	Lower three quartiles	1.00	1.00
	Highest quartile	1.12 (0.73–1.73)	1.20 (0.73–1.98)
	Number (N) cases exposed	130	97
Dieldrin	Lower three quartiles	1.00	1.00
	Highest quartile	1.38 (0.90–2.11)	1.52 (0.94–2.46)
	Number (N) cases exposed	114	98
Diuron	Lower three quartiles	1.00	1.00
	Highest quartile	1.10 (0.75–1.60)	0.92 (0.59–1.42)
	Number (N) cases exposed	163	126
Endosulfan	Lower three quartiles	1.00	1.00
	Highest quartile	0.84 (0.57–1.25)	0.75 (0.48–1.17)
	Number (N) cases exposed	166	141
Methoxychlor	Lower three quartiles	1.00	1.00
	Highest quartile	1.00 (0.56–1.79)	0.58 (0.27–1.23)
	Number (N) cases exposed	150	114
PCNB	Lower three quartiles	1.00	1.00
	Highest quartile	1.34 (0.73–2.46)	1.79 (0.84–3.84)
	Number (N) cases exposed	75	58

(*Continued*)

TABLE III. (*Continued*)

Pesticide		OR (95% CI) for all subjects Case N = 950, control N = 9,435	OR (95% CI) for subjects having lived >20 years in county at time of death Case N = 697, control N = 6,259
Pyrazon	Lower three quartiles	1.00	1.00
	Highest quartile	0.97 (0.43–2.21)	0.86 (0.33–2.25)
	Number (N) cases exposed	43	33
Simazine	Lower three quartiles	1.00	1.00
	Highest quartile	0.90 (0.63–1.29)	1.17 (0.78–1.76)
	Number (N) cases exposed	182	149
Toxaphene	Lower three quartiles	1.00	1.00
	Highest quartile	1.03 (0.54–1.96)	1.01 (0.47–2.19)
	Number (N) cases exposed	34	24

*Adjusted for gender, age, race (White, Black, Hispanic, Asian, other), education (<12, = 12, >12 years), year of death, years of living in county, urban residence and each pesticide was mutually adjusted for all 17 other pesticides in this study, simultaneously.

Single pesticide models, including those for EPA classified possible, probable, and known carcinogens, did not suggest risk increases. Rather the increases we observed were dependent on adjusting for the use of other pesticides, especially application rates for two other fungicides/nematodicides 1,2-dibromo-3-chloropropane (DBCP) and chloropicrin. In other words, when adding other pesticides one by one to the final model only DBCP and chloropicrin caused appreciable changes in the effect estimates of 1,3-d. Furthermore, we did not observe a dose-response pattern by quartiles of use for 1,3-d, dieldrin, or other pesticides examined.

DISCUSSION

Studies of high-level occupational exposures to organochlorines have suggested carcinogenic effects for several organochlorine compounds [Ditraglia et al., 1981; Blair and Zahm, 1991; IARC, 1991]. Fresno, Kern, and Tulare counties are ranked the top agricultural counties in California by value of production [CDF, 1999] and intensive agricultural pesticide use increases the possibility of residential as well as occupational exposures of its residents.

1,3-d is a widely used soil fumigant classified by the EPA as a probable human carcinogen (Fig. 1). It is persistent up to several weeks post soil treatment, volatilizes over the course of weeks, is known to leach into ground water and its usage in our study area increased dramatically after 1984. Our analyses suggested an increased pancreatic cancer mortality among long-term residents in areas of high application rates when adjusting for the use of all other commonly used organochlorine pesticides reportable to the California department of pesticide regulation between 1972 and 1989; especially the two fungicides/nematodicides DBCP and chloropicrin. The latter is widely used in combination with 1,3-d in the commercial product Telone.

A review of the literature reveals no studies of the relationship between 1,3-d application and pancreatic cancer. Animal studies have shown an increased occurrence of tumors in the liver, fore stomach, urinary bladder, and lungs when animals were exposed to high doses of 1,3-d by gavage [Yang et al., 1986] and morphological changes in nasal and lung tissue when exposed by inhalation [Lomax et al., 1989]. The only repeated-exposure human toxicity data for 1,3-d are case study reports of dermatitis resulting from direct skin contact [Bousema et al., 1991]. Though there have been studies showing captafol, a tree fungicide, as promoting carcinogenic activity in cells [Perocco et al., 1995] the EPA [2001] has not assessed its carcinogenicity. Dieldrin, a persistent, bioaccumulating insecticide, was banned for most uses in 1987 because of its numerous harmful effects including possible carcinogenicity. Most products containing PCNB, previously used as fungicides, have been cancelled for use in the US; no assessment of its carcinogenicity is available.

Mills [1998] used the 1993 PUR data to examine possible correlations between selected pesticide use and age-adjusted county incidence rates for certain cancers recorded by the California Cancer Registry between 1988 and 1992. Except for the Hispanic and Black male populations, who historically have been employed at higher rates in California agriculture and may have the highest occupational exposures to pesticides, most sex- and race/ethnicity-specific correlation coefficients were close to zero or negative in this study. Mills did not examine pancreatic cancers.

While Mill's study [Mills, 1998] suggested that in rural California residential and occupational pesticide exposure may be related to increased rates of some cancers, the study

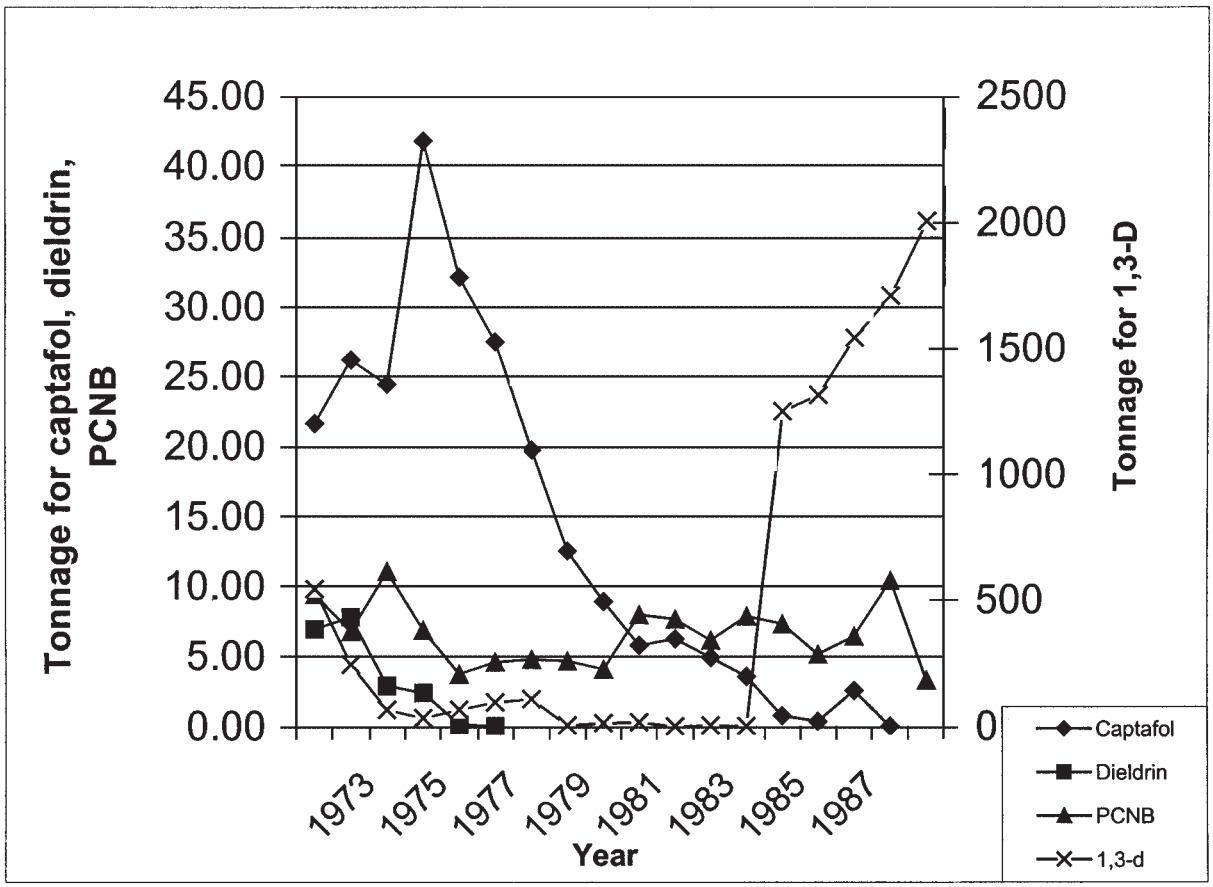


FIGURE 1. Tonnage of the active ingredients captafol, dieldrin, pentachloronitrobenzene (PCNB), and 1,3-dichloropropene (1,3-d) applied in Fresno, Kern, and Tulare counties 1972–1989 by year.

suffered from several limitations; most notably the potential for the ecological fallacy due to the use of county-level PUR data and countywide incidence rates. The time sequence between exposure and incidence was also questionable because the study relied solely on 1993 PUR data to represent decades of pesticide use. While in the aggregate of total use it may be correct to assume application stability, the 1972–1989 PUR data indicated dramatic changes in the use pattern of specific pesticides over time. Also, some California counties are large geographical units and pesticide use can vary greatly within a county, especially if it contains both rural/agricultural areas and urban centers.

Our use of death certificates raises the possibility of misdiagnosis of pancreatic cancers. Differences in pancreatic cancer risk comparing populations over time may be due to variations in diagnostic and reporting practices, however, it is hard to imagine a distinct reporting bias by ZIP code in a county. Additionally, because little is known about the latency of pancreatic cancer we had to assume based on our data (mortality data and 1,3-d application rates) a rather short latency period. Large applications of 1,3-d only began in 1984 while our mortality data ends in 1996. We, therefore, have a latency period with a range of 1–12 years. We did consider looking only at the last 2–3 years of the mortality data, however, the number of cases were too small to give any informative results.

Because of its high lethality and the lack of effective treatments for pancreatic cancer, many persons diagnosed with the disease are not subjected to biopsy. Anderson et al. [1996] estimate that errors in the diagnosis of pancreatic cancer may be quantitatively more important, perhaps by an order of magnitude, than errors in the diagnosis of other common cancers. Up to 28% of histologically confirmed pancreatic cancers may in fact not have originated in the pancreas [Lyon et al., 1989]. The resulting misclassification would most likely be non-differential for exposed and unexposed cases and lead to a dilution of any true effect. Garabrant et al. [1993] in studying the association of DDT exposure in chemical manufacturing workers and pancreatic cancer, found that risk estimates were close to null when they relied on cases solely reported on death certificates, however, risk ratios increased dramatically when they restricted their analysis to cases with cytohistological confirmation.

While the PUR data is a unique and unparalleled resource for residential pesticide exposure assessment in California, it has limitations. The law has required reporting of restricted pesticides by California farmers since the early 1970s, but it is likely that under-reporting and misreporting was common during its early years because of lack of reporting enforcement. This is underscored by the fact that we found approximately 60% of the 1972 PUR records to be incomplete.

Our exposure measures are ecologic in nature and reflect the potential for environmental exposure to pesticides based only on ZIP code of residence. Since ZIP codes are eliminated, created, and can change boundaries over time, it is possible that we introduced additional misclassification when we assigned earlier PUR data to these codes recorded on the subjects' death certificate only instead of using historical ZIP code boundaries. More importantly, we lacked residential history for our subjects and assumed that it was likely that subjects lived in the same ZIP code area reported as their last address for extended periods prior to death. It is reassuring, however, that in our study the average number of years a subject had lived in the county at time of death was rather high and we used this information when restricting some analyses to long-term residents only. Furthermore, several migration studies [Stone, 1975; Long and Boertlin, 1976] have demonstrated that residential mobility decreases dramatically after a person passes their mid-thirties.

Our approach probably harbors the greatest potential for exposure misclassification for urban residents since urban ZIP codes include less area and residents may move more often between ZIP codes. Also, for urban residents who live in a ZIP code area with boundaries in close proximity to adjacent agricultural fields, the PUR data for their urban ZIP code does not reflect high pesticide applications in the neighborhood appropriately. While potential migration and the assumption that living in certain ZIP codes reflects the average likelihood of pesticide exposure for all residents homogeneously causes exposure misclassification in our study, we believe that the misclassification is most likely non-differential for cases and controls and, thus, likely to bias our overall estimates of effect toward the null.

An important potential confounding factor for which we missed information is smoking. As mentioned previously, smoking has been shown to increase pancreatic cancer risk by about twofold and, thus, would not be considered a strong risk or confounding factor. Determinants of smoking rates in the US population are period, ethnicity, sex, education, and age [TIPS, 2001]. Since we controlled for these factors in our analyses, we may have indirectly controlled for differences in smoking behavior. Furthermore, we selected our comparison group from a random sample of all other causes of death except cancer. Most of the control deaths were from cardiovascular disease for which smoking is also known to be a moderate size risk factor. Except for potential urban/rural differences in smoking rates there is no reason why the application rate of specific pesticides within ZIP code areas would be related to smoking.

Employing a POR case-control design, we were able to control for important potential confounding factors such as age, calendar year of death, gender, and education at the individual level. Using mortality instead of incidence data was not a disadvantage since, as mentioned above, mortality and incidence for this disease is virtually identical although diagnoses on death certificate may be reported somewhat less accurately. We were able to include 950 cases that occurred in three counties over an 8-year period and to use a more refined exposure assessment than most previous ecologic studies by employing geographical information systems methods and pesticide use reporting for the years 1972 to 1989. Limiting our subjects to those with long-term residence in the three counties allowed us to take a long latency for cancers into account and examine effects of longer-term exposure.

We believe the use of mortality data and ecologic exposure measurements at the ZIP code level without a detailed work and residential history are most likely to introduce bias towards the null due to non-differential misclassification. Because of the limitations of the PUR and death certificate data our results are only suggestive that long-term residents in areas of highest exposure to certain (organochlorine) pesticides classified by EPA as probable human carcinogens face an increased risk of dying from pancreatic cancer. Population-level research based on cancer incidence and histology with improved exposure assessment and possibly measures of serum organochlorine levels may aid in clarifying the role of these chemicals in inducing pancreatic cancers.

ACKNOWLEDGMENTS

We thank Rudy Rull and Thu Quach for assistance with the PUR data. This research was supported, in part, by funds from UCLA Center for Occupational and Environmental Health.

REFERENCES

Ahlgren JD. 1996. Epidemiology and risk factors in pancreatic cancer. Semin Oncol 23:241–250.

Anderson KE, Potter JD, Mack TM. 1996. Pancreatic cancer. In: Schottenfeld D, Fraumeni JF, Jr., editors. Cancer epidemiology and prevention. Philadelphia, PA: Saunders, p 725–771.

Blair A, Zahm SH. 1991. Cancer among farmers. Occup Med 6:335–354.

Bousema MT, Wiemer GR, Van Joost TH. 1991. A classic case of sensitization to DD-95. Contact Dermatitis 24:132–133.

Breslow N, Lubin JH, Marek P, Langholz B. 1983. Multiplicative models and cohort analysis. J Am Stat Assoc 78:1–12.

California Department of Finance. 1999. California Statistical Abstract-1999. Sacramento: Economic Development Agency of California. Table G-12.

Cocco P, Kazerouni N, Zahm SH. 2000. Cancer mortality and environmental exposure to DDE in the United States. Environ Health Perspect 108:1–4.

Ditraglia D, Brown DP, Namekata T, Iverson M. 1981. Mortality study of workers employed at organochlorine pesticide manufacturing plants. Scand J Work Environ Health 7(Suppl 4):140–146.

Environmental Protection Agency. 2001. Integrated Risk Information System List of Substances. (http://www.epa.gov/ngispgm3/iris/subst)

Fryzek JP, Garabrant DH, Harlow SD, Severson RK, Gillespie BW, Schenk M, Schottenfeld D. 1997. A case-control study of self-reported exposures to pesticides and pancreas cancer in southeastern Michigan. Int J Cancer 72:62–67.

Garabrant DH, Held J, Langholz B, Peter JM, Mack TM. 1992. DDT and related compounds and risk of pancreatic cancer. J Natl Cancer Inst 84:764–771.

Garabrant DH, Held J, Homa D. 1993. DDT and pancreatic cancer–response. J Natl Cancer Inst 85:328–329.

Greenlee RT, Murray T, Bolden S, Wingo PA. 2000. Cancer Statistics, 2000. CA 50:7–33.

International Agency for Research on Cancer. 1991. IARC Monographs on the evaluation of carcinogenic risks to humans, Vol. 53: Occupational exposures in insecticide application, and some pesticides. IARC.

Lomax LG, Stott WT, Johnson KA, Calhoun LL, Yano BL, Quast JF. 1989. The chronic toxicity and oncogenicity of inhaled technical-grade 1,3-dichloropropene in rats and mice. Fundam Appl Toxicol 12:418–431.

Long L, Boertlin C. 1976. The geographic mobility of Americans: An international comparison. Current Population Reports, Special Studies, Series P-23, No. 64.

Lyon JL, Robinson LM, Mose R. 1989. Uncertainty in the diagnosis of histologically confirmed pancreatic cancer cases. Intl J Epidemiol 18: 305–308.

Miettinen OS, Wang J-D. 1981. An alternative to the proportionate mortality ratio. Am J Epidemiol 114:144–148.

Mills PK. 1998. Correlation analysis of pesticide use data and cancer incidence rates in California counties. Arch Environ Health 53:410–413.

Morgenstern H, Thomas D. 1993. Principles of study design in environmental epidemiology. Environ Health Perspect 101(Suppl):23–28.

National Institute for Health Care Management. 2000. 2000 Health care almanac and yearbook. New York: Faulkner and Gray.

Perocco P, Colacci A, Del Ciello C, Grilli S. 1995. Transformation of BALB/c 3T3 cells in vitro by the fungicides captan, captafol and folpet. Jpn J Cancer Res 10:941–947.

Porta M, Malats N, Manuel J, Grimalt JO, Rifà J, Carrato A, Guarner L, Salas A, Santiago-Silva M, Corominas JM, Montserrat A, Real FX. 1999. Serum concentrations of organochlorine compounds an K-ras mutations in exocrine pancreatic cancer. Lancet 354:2125–2129.

Schwartz GG, Skinner HG, Duncan R. 1998. Solid waste and pancreatic cancer: An ecologic study in Florida, USA. Int J Epidemiol 27:781–787.

Silverman DT, Dunn JA, Hoover RN, Schiffman M, Lillemoe KD, Schoenberg JB, Brown LM, Greenberg RS, Hayes RB, Swanson GM, Wacholder S, Schwartz AG, Liff JM, Pottern LM. 1994. Cigarette smoking and pancreas cancer: A case-control study based on direct interviews. J Natl Cancer Inst 86:1510–1516.

Stone L. 1975. On the interaction of mobility dimensions in theory on migration decisions. Can Rev Soc Anthro 12:95–100.

Tobacco Information and Prevention Source. 2001. National Health Interview Surveys, Selected Years—United States, 1965–1995. (http://www.cdc.gov/tobacco/adstat1.htm)

United States Census Bureau. 2001. State and County QuickFacts. (http://quickfacts.census.gov)

Yang RS, Huff JE, Boorman GA, Haseman JK, Kornreich M, Stookey JL. 1986. Chronic toxicology and carcinogenesis studies of Telone II by gavage in Fischer-344 rats and B6C3F1 mice. J Toxicol Environ Health 18:377–392.

Pest Management Science / Volume 57, Issue 12 / p. 1107-1113

Research Article | Full Access

Effect of temperature, organic amendment rate and moisture content on the degradation of 1,3-dichloropropene in soil†

Robert S Dungan, Jianying Gan, Scott R Yates

First published: 31 October 2001
https://doi.org/10.1002/ps.400
Citations: 54

UConn Full Text

† This article is a US Government work and is in the public domain in the USA

Abstract

1,3-Dichloropropene (1,3-D), which consists of two isomers, (*Z*)- and (*E*)-1,3-D, is considered to be a viable alternative to methyl bromide, but atmospheric emission of 1,3-D is often associated with deterioration of air quality. To minimize environmental impacts of 1,3-D, emission control strategies are in need of investigation. One approach to reduce 1,3-D emissions is to accelerate its degradation by incorporating organic amendments into the soil surface. In this study, we investigated the ability of four organic amendments to enhance the rate of degradation of (*Z*)- and (*E*)-1,3-D in a sandy loam soil. Degradation of (*Z*)- and (*E*)-1,3-D was well described by first-order kinetics, and rates of degradation for the two isomers were similar. Composted steer manure (SM) was the most reactive of the organic amendments tested. The half-life of both the (*Z*)- and (*E*)-isomers in unamended soil at 20 °C was 6.3 days; those in 5% SM-amended soil were 1.8 and 1.9 days, respectively. At 40 °C, the half-life of both isomers in 5% SM-amended soil was 0.5 day. Activation energy values for amended soil at 2, 5 and 10% SM were 56.5, 53.4 and 64.5 kJ mol^{-1}, respectively. At 20 °C, the contribution of degradation from biological mechanisms was largest in soil amended with SM, but chemical mechanisms still accounted for more than 58% of the (*Z*)- and (*E*)-1,3-D degradation. The effect of temperature and amendment rate upon degradation should be considered when describing the fate and transport of 1,3-D isomers in soil. Use of organic soil amendments appears to be a promising method to enhance fumigant degradation and reduce volatile emissions.

Published in 2001 for SCI by John Wiley & Sons, Ltd

1 INTRODUCTION

1,3-Dichloropropene (1,3-D) is a soil fumigant widely used in agriculture to control plant-parasitic nematodes and fungi. Commercial formulations of 1,3-D are registered under the trademark names 'Telone II' (Dow Chemical Co) and 'D-D' (Shell Oil Co); both contain approximately equal ratios of (*Z*)-1,3-D (*cis*-isomer) and (*E*)-1,3-D (*trans*-isomer). 1,3-Dichloropropene, formulated with chloropicrin, is considered to be an important alternative pesticide to methyl bromide, which is scheduled to be phased out of production by 2005 in the USA because of its link to stratospheric ozone depletion. The (*Z*) and (*E*) isomers are clear liquids with vapor pressures of 23.0 and 34.2 mm Hg (3.06 and 4.54 kPa) at 25 °C, respectively.[1] At these vapor pressures a significant fraction of the applied material can be expected to be released to the atmosphere. It has been reported that 20–50% of the applied 1,3-D could be lost to atmospheric volatilization.[2, 3] The emission of 1,3-D to the atmosphere is known to contribute to air pollution and human health problems.[4] In 1990, a 4-year suspension of the use of Telone II was initiated after high ambient air concentrations of 1,3-D were detected in fumigation areas of California. 1,3-Dichloropropene is a B2 carcinogen and has been classified as a toxic air substance by the US Environmental Protection Agency (USEPA).

The degradation of (*Z*)- and (*E*)-1,3-D in soil is a combination of biological and chemical mechanisms.[5-7] Both (*Z*)- and (*E*)-1,3-D are initially hydrolyzed to the corresponding 3-chloroallyl alcohol, which is mainly attributed to chemical mechanisms.[8-10] The isomers of chloroallyl alcohol are then oxidized to (*Z*)- and (*E*)-3-chloroacrylic acid, which are degraded to succinic acid, propionic acid and acetic acid. The aliphatic carboxylic acids are finally mineralized to carbon dioxide and water. Several bacterial strains capable of degrading both isomers of 1,3-D, 3-chloroallyl alcohol and 3-chloroacrylic acid, have been isolated.[11-14] In soils from The Netherlands, degradation rates for the two isomers were found to be similar.[15-18] The rate of (*Z*)- and (*E*)-1,3-D degradation increased with increasing temperature, and the half-lives of the two isomers ranged from a few days to a few weeks. In soils previously treated with 1,3-D, enhanced degradation of both isomers has been reported and degradation of (*E*)-1,3-D was found to be greater than that of (*Z*)-1,3-D.[5, 7, 19]

Since 1,3-D is a volatile and toxic compound, cost-effective methods to reduce atmospheric emissions are in need of investigation if 1,3-D is to be used as an alternative to methyl bromide. One potential method to reduce 1,3-D emissions is to accelerate its degradation in the soil surface layer, thereby minimizing 1,3-D volatilization. This has previously been accomplished by incorporating organic wastes into soil.[20] The addition of organic amendments to soil alters biological and chemical conditions, both of which influence pesticide degradation. With regard to biological degradation, the incorporation of organic wastes into soil may promote the growth and activity of pesticide-degrading organisms, causing accelerated degradation of the target compound.[21-23] Gan *et al* [20] found that the addition of a compost manure to the top 5-cm

layer at 5% (w/w) reduced (*Z*)- and (*E*)-1,3-D emissions by 47 and 44%, respectively. To date, this is the only report which describes the use of organic amendments as a technique to control 1,3-D emissions from soil. The application of organic amendments should also have little or no negative impact upon pest control efficacy because enhanced degradation would be limited to the surface layer only.

Currently, no information is available concerning the combined effect of temperature and organic amendment rate on the degree of accelerated 1,3-D degradation. The primary objective of this study was to assess simultaneously the effects of temperature and application rate of an organic amendment upon 1,3-D degradation in a sandy loam soil. This information will be useful in understanding the behavior of 1,3-D in organically amended soil and determining the conditions needed for achieving optimal degradation.

2 EXPERIMENTAL METHODS

2.1 Soil, organic amendments and chemicals

The soil used in this study, Arlington sandy loam (coarse-loamy, mixed, thermic, Haplic Durixeralf), was obtained from a field in the University of California, Riverside, Agricultural Experiment Station. Soil was removed from the plow layer (A_p horizon), passed through a 2-mm sieve without air-drying and stored at 5 °C. The Arlington soil had a pH of 7.2 and contained 0.92% of organic matter. Four different organic wastes were used: composted steer manure (SM) from Hyponex Corp, Marysville, OH, USA; dewatered biosolids (BS) from the Riverside municipal sewage treatment plant, CA, USA; composted chicken manure (CKM) and composted forest products (FP) from Kellogg Supply Inc, Carson, CA, USA. The moisture contents of the SM, BS, CKM and FP were 50, 38, 36 and 122% (w/w), respectively. Each of the amendments was stored at room temperature and passed through a 2-mm sieve prior to soil incorporation. The 1,3-D standard [48% (*Z*) and 49% (*E*)] was purchased from Chem Service (West Chester, PA, USA).

2.2 Effect of amendment on degradation

This experiment was conducted to determine the effect of various organic amendments (ie SM, BS, CKM and FP) on the degradation of (*Z*)- and (*E*)-1,3-D in Arlington soil. To determine which amendment had a significant impact on the degradation of 1,3-D, each amendment was incorporated into the soil at 5% (w/w) for comparative purposes. The amended soils were prepared by thoroughly mixing both amendment and soil in one-gallon plastic bags and adjusting the moisture of the mixture to 10% (w/w) with deionized water. A soil moisture content of 10% represents 50% of the soil's maximum water holding capacity (WHC_{max}). The soil mixtures were then passed through a 2-mm sieve and 10 g (dry wt) quantities were added to 21-ml headspace vials. Each vial was then spiked with 200 µl of an aqueous solution of 1,3-D

(2.5 μg litre^{-1}), which was equivalent to a soil concentration of 50 mg kg^{-1} (dry weight basis). Under field conditions, a 1,3-D soil concentration of 50 mg kg^{-1} is environmentally relevant, assuming even soil distribution, when applied at a typical rate of 250 kg ha^{-1}. The treated samples were immediately capped with Teflon-faced butyl rubber septa to prevent the 1,3-D isomers from diffusing out of the vial. The vials were then incubated at 20 °C in the dark, and at specific time intervals triplicate samples were removed and stored at −20 °C until analyzed by gas chromatography.

2.3 Effect of temperature and amendment rate on degradation

To determine the combined effect of temperature and amendment rate on the degree of (*Z*)- and (*E*)-1,3-D degradation, soil was amended with 2, 5 and 10% (w/w) of SM and incubated at 20, 30 or 40 °C. All soil mixtures were prepared as previously described and adjusted to a final moisture content of 10% (w/w) with deionized water. To distinguish between microbial and chemical degradation, a separate set of SM-amended soils were sterilized by autoclaving twice (1.0 h at 121 °C each time), with a 24-h interval between the first and second autoclaving. The contribution from biodegradation was calculated from the difference in the values of the degradation rate constant, *k*, between non-sterile and sterile samples divided by the non-sterile *k* value. The sterilized soil treatment was only conducted at an incubation temperature of 20 °C. All soil vials were spiked with 200 μl of the 1,3-D stock solution, immediately capped with septa and incubated in the dark. At specific time intervals triplicate samples were removed and frozen at −20 °C.

2.4 Effect of moisture content on degradation

To determine the effect of soil moisture content on the degradation of 1,3-D, Arlington sandy loam was adjusted to 25, 50, and 75% of the soil's maximum water holding capacity (WHC_{max}). The value of the latter was 0.2 kg kg^{-1}, giving soil-water potentials at 25, 50, and 75% of the WHC_{max} of 5600, 1200 and 260 kPa, respectively. After adjusting the soil moisture content, 10 g (dry wt) of soil was weighed into 21-ml glass headspace vials which were each spiked with 200 μl of the 1,3-D stock solution. The treated vials were immediately capped with aluminum seals and Teflon-faced butyl rubber septa (Supleco, Bellefonte, PA) and then incubated at 20 °C in the dark under static conditions. Triplicate samples were removed at specific time intervals and frozen at −20 °C until analyzed.

2.5 Sample extraction and analysis

To extract the (*Z*)- and (*E*)-1,3-D residues from the soil samples, vials were uncapped while still frozen, and ethyl acetate (10 ml) and anhydrous sodium sulfate (10 g) were added to each vial, followed by immediate recapping. Once the soils had thawed, the vials were shaken for 1 h on a horizontal shaker (200 oscillations min^{-1}), vortexed for 30 s and then allowed to stand until all

suspended particulate matter had settled. After settling, a 1 ml aliquot of the soil extract was transferred to a GC vial. The soil extract was analyzed for (*Z*)- and (*E*)-1,3-D on a Hewlett-Packard 5890 gas chromatograph equipped with a RTX-624 capillary column (30 m, 0.25 mm × 1.4 µm, Restek Corp, Bellefonte, PA, USA) and connected to a micro-electron capture detector (µECD). The operating conditions were as follows: carrier gas, helium, 1.2 ml min^{-1}; inlet temperature, 230 °C; detector temperature, 280 °C; column temperature, isothermal at 110 °C for 7 min. The average extraction efficiency of 1,3-D residues was 83%. All data was subject to first-order fitting to obtain a degradation rate constant k (day^{-1}) and error bars represent the standard error of the data.

3 RESULTS AND DISCUSSION

3.1 Effect of amendment on degradation

Figure 1 shows the disappearance of 1,3-D, as the sum of both isomers, from soil amended with 5% (w/w) BS, FC, CKM or SM. The reactions are well described by first-order kinetics (r^2 > 0.96). The incorporation of CKM and SM into Arlington soil was found to substantially increase the degradation of 1,3-D. Degradation was 2.3 and 3.3 times faster in CKM- and SM-amended soil, respectively, than in unamended soil. In contrast, amending soil with FC only enhanced the rate of degradation 1.2 times, while 1,3-D degradation was slightly inhibited in BS-amended soil. Apparently, FC and BS contain low population numbers of microbial species capable of degrading or contributing to the degradation of 1,3-D. In addition, it is also evident that these organic wastes did not contribute to the chemical degradation of 1,3-D.

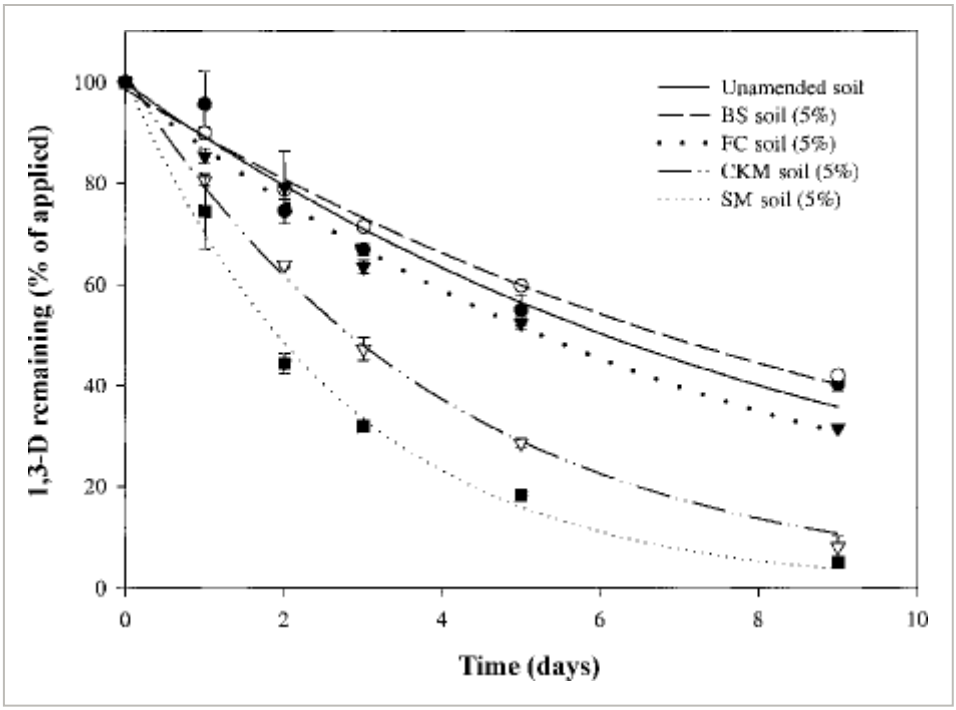


Figure 1

Open in figure viewer | PowerPoint

Disappearance of 1,3-dichloropropene, as the sum of (*Z*)- and (*E*)-isomers, from Arlington sandy loam, unamended and amended with various organic wastes and incubated at 20 °C.

First-order rate coefficients k (day^{-1}) and corresponding half-lives for the (*Z*)- and (*E*)-isomers are given in Table 1. Overall, the data show that both (*Z*)- and (*E*)-isomers have similar kinetic behavior, which supports results obtained by other workers.[16-18, 24, 25] Ou and co-workers[5, 7, 19] found that the degradation rates of both 1,3-D isomers were similar in soils not previously treated with 1,3-D. To our knowledge, the soil used in this study, Arlington sandy loam, had never been previously treated with 1,3-D. In unamended soil, the rates of degradation for both isomers were identical, with a half-life of 6.3 days. However, degradation of the (*Z*)-isomer was slightly greater than that of the (*E*)-isomer in all treated soils except CKM-amended soil. The half-lives of (*Z*)- and (*E*)-1,3-D in SM-amended soil were 1.8 and 1.9 days, respectively. Since SM was found to substantially increase the rate of (*Z*)- and (*E*)-1,3-D degradation over that of the other amendments tested, it was selected for continued use in our degradation studies.

Table 1. First-order degradation rate constants (k), half-life ($t_{1/2}$) values, and correlation coefficients of fitting (r^2) for Arlington sandy loam amended with various organic amendments at 20 °C

Matrix	(*Z*)-1,3-D			(*E*)-1,3-D		
	k (day^{-1}) (±SD)	$t_{1/2}$ (days)	r^2	k (day^{-1}) (±SD)	$t_{1/2}$ (days)	r^2
Unamended soil	0.11 (±0.01)	6.3	0.96	0.11 (±0.01)	6.3	0.97
BS soil (5%)	0.11 (±0.01)	6.3	0.99	0.09 (±0.00)	7.7	0.99
FC soil (5%)	0.14 (±0.01)	5.0	0.99	0.12 (±0.01)	5.8	0.99
CKM soil (5%)	0.23 (±0.01)	3.0	1.00	0.27 (±0.01)	2.6	0.98
SM soil (5%)	0.39 (±0.03)	1.8	0.99	0.36 (±0.02)	1.9	0.99

3.2 Effect of temperature and manure application rate on degradation

The degradation of 1,3-D-isomers in Arlington soil was accelerated as both temperature and SM application rate increased (Fig 2). The differences in the rates of degradation of the (*Z*)- and (*E*)-isomers were found to be similar in unamended soil and SM-amended soil at all temperatures

tested. In unamended soil, the first-order degradation rate constants at 20, 30 and 40 °C were 0.11, 0.21 and 0.47 day^{-1}, respectively, for (*Z*)-1,3-D, and 0.11, 0.24 and 0.57 day^{-1}, respectively, for (*E*)-1,3-D. The corresponding half-life values were 6.3, 3.3 and 1.5 days for (*Z*)-1,3-D and 6.3, 2.9 and 1.2 days for (*E*)-1,3-D (Table 2). As the temperature increased from 20 to 40 °C, degradation of (*Z*)-1,3-D and (*E*)-1,3-D in unamended soil increased 4.3 and 5.2 times, respectively. The degradation rates for both isomers changed by a factor of about 2 for each 10 °C change in temperature. Under typical fumigant and soil conditions, more 1,3-D degradation can be expected to occur in the soil surface than in the sub-surface layers, since surface layers are generally subject to larger temperature fluctuations.

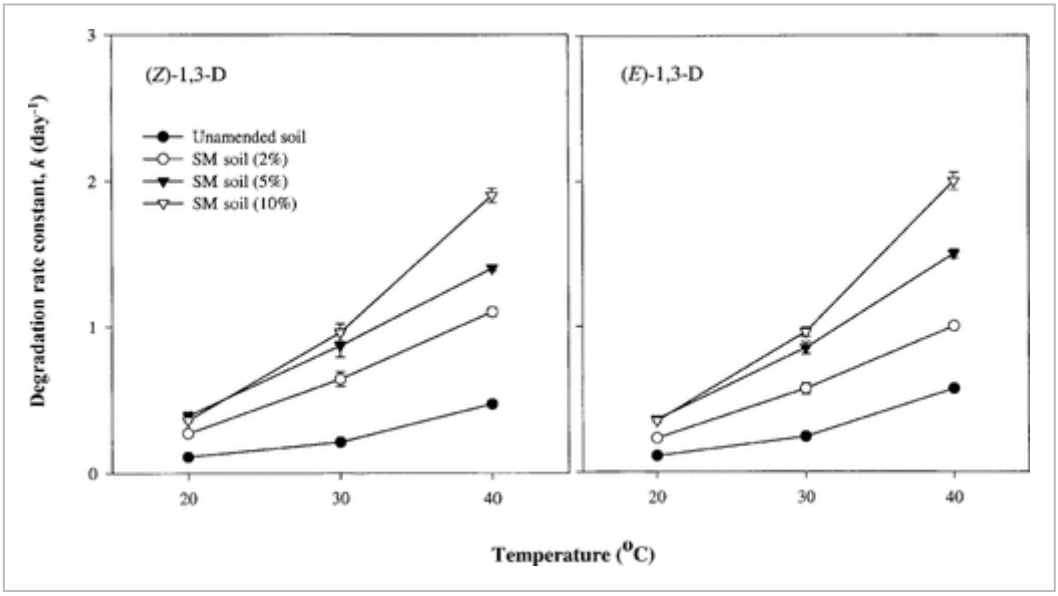


Figure 2

Open in figure viewer | PowerPoint

First-order rate constants (*k*, day^{-1}) for degradation of (*Z*)- and (*E*)-1,3-dichloropropene in Arlington sandy loam incubated at different temperatures and amended with different rates of composted steer manure.

Table 2. Half-life ($t_{1/2}$) values and first-order correlation coefficients of fitting (r^2) for Arlington sandy loam amended with different rates of composted steer manure

T (°C)	Matrix	(*Z*)-1,3-D		(*E*)-1,3-D	
		$t_{1/2}$ (days)	r^2	$t_{1/2}$ (days)	r^2
20	Unamended soil	6.3	0.96	6.3	0.97
	SM soil (2%)	2.6	0.99	3.0	0.99

T (°C)	Matrix	(*Z*)-1,3-D		(*E*)-1,3-D	
		$t_{1/2}$ (days)	r^2	$t_{1/2}$ (days)	r^2
	SM soil (5%)	1.8	0.99	1.9	0.99
	SM soil (10%)	1.9	1.00	2.0	1.00
30	Unamended soil	3.3	0.97	2.9	0.98
	SM soil (2%)	1.1	0.99	1.2	0.99
	SM soil (5%)	0.8	0.99	0.8	1.00
	SM soil (10%)	0.7	1.00	0.7	1.00
40	Unamended soil	1.5	1.00	1.2	1.00
	SM soil (2%)	0.6	1.00	0.7	1.00
	SM soil (5%)	0.5	1.00	0.5	1.00

Figure 2 shows that *k* increases with temperature at all rates of SM application for both (*E*)-1,3-D and (*Z*)-1,3-D. It is interesting that, at 20 and 30 °C, the value of *k* did not differ substantially between SM rates of 5 and 10% for either isomer. This suggests that degradation of 1,3-D at these temperatures is insensitive to the application rate of SM. However at 40 °C, the degradation rates of the (*Z*)- and (*E*)-isomers at 10% SM were 36 and 33% greater, respectively, than at 5% SM. Over the temperature range of 20 to 30 °C, it appears that optimum degradation of 1,3-D in SM-amended soil lies approximately at the 5% application rate. Although the application of 10% SM substantially increased the degradation of the 1,3-D isomers at 40 °C, the addition of 5% SM represents a more feasible field application rate. The incorporation of 5% SM into the top 5-cm soil layer (assuming an average bulk density of 1.3 g cm^{-3}) would require approximately 36 tonne ha^{-1} (dry wt) of amendment. Compared with unamended soil, the addition of 5% SM increased degradation of both 1,3-D isomers, on average, 3.4, 3.8 and 2.7 times at 20, 30 and 40 °C, respectively. Corresponding half-lives were 1.9, 0.8 and 0.5 days. All half-life values, at each of the SM rates and temperatures tested, are given in Table 2.

The relationship between temperature and degradation of both 1,3-D isomers closely followed the Arrhenius equation ($r^2 > 0.98$). The activation energy (E_a) at different rates of application was calculated as the slope of the plot of ln *k versus* 1/*T*. The E_a values for amended soil at 2, 5 and 10% SM were 56.5, 53.4 and 64.5 kJ mol^{-1}, respectively. The sensitivity of the rate of a reaction

to changes in temperature depends on the value of E_a.[26] Low E_a values are often associated with insensitivity to temperature. In 2, 5 and 10% SM-amended soil, the average increases in degradation per 10 °C increase in temperature were 2.1, 1.9 and 2.4, respectively, for (*Z*)-1,3-D, and 2.2, 2.1 and 2.4, respectively, for (*E*)-1,3-D. These results agree well with the general rule that the rate of a reaction doubles with every 10 °C rise in temperature.

It is evident from the above data that degradation of 1,3-D isomers in soil is enhanced by both temperature and organic amendment; by controlling each of these factors it may be possible to reduce atmospheric emissions. Not only does the use of organic amendments enhance 1,3-D degradation, but their use in conjunction with soil solarization techniques, such as tarping, could provide additional emission and pest-control benefits. Tarping is a method often used to thermally destroy soil-borne plant pathogens by increasing the temperature in the soil surface. The increased soil temperature, as previously discussed, should also be effective in accelerating fumigant degradation, especially in the soil surface. Tarping is also a method used to increase fumigant efficacy and reduce emissions, but it is not completely effective since plastic tarps are often permeable to fumigants.[27, 28] Theoretically, the use of organic amendments should increase the effectiveness of tarping, from both an emissions and pest-control standpoint. Haidar and Sidahmed[29] reported that the use of chicken manure was effective in reducing the solarization period required to eliminate an obligate root parasitic weed. Organic amendments are also known to suppress soil-borne pathogens through the stimulation of antagonistic organisms or production of toxic volatile gases.[30-33] Therefore, integrating fumigation with soil solarization (through tarping) and organic amendments may provide the best possible scenario to control 1,3-D emissions and in some cases increase pest-control efficacy. Ultimately this may lead to reduced pesticide usage.

3.3 Degradation mechanisms in manure-amended soil

As mentioned previously, the degradation of 1,3-D isomers in soil is a result of biological and chemical mechanisms. Figure 3 shows the degradation rate constants of the 1,3-D isomers in sterile and non-sterile unamended and SM-amended Arlington soil. Fumigant degradation in sterile soil is generally attributed to chemical reactions, while degradation in non-sterile soil is a combination of biological and chemical reactions. Therefore, differences between rate constants of the sterile and non-sterile soil mixtures were assumed to be attributable to microbial degradation.

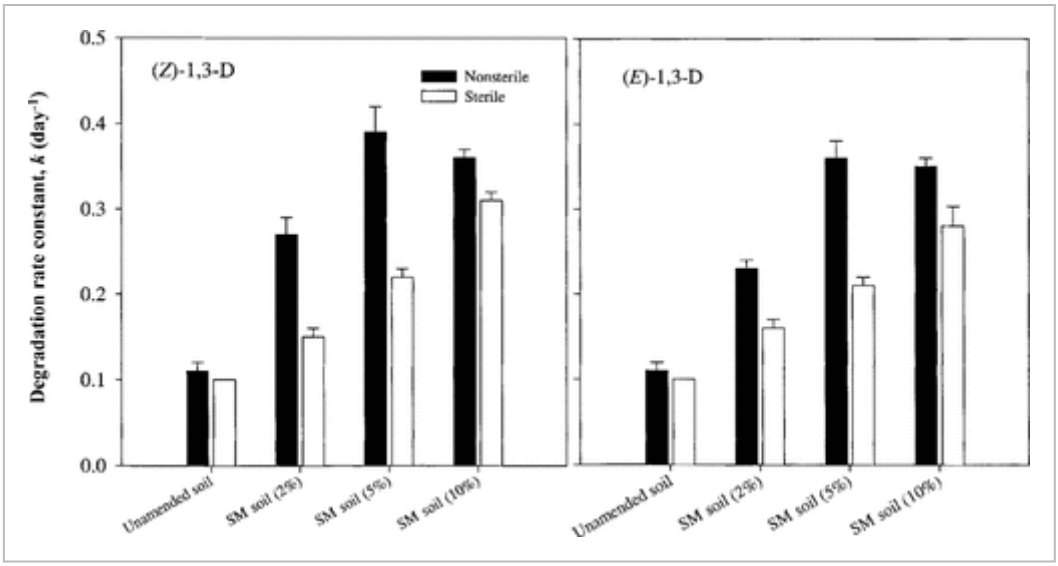


Figure 3

Open in figure viewer | PowerPoint

Comparison of first-order rate constants (k, day^{-1}) for degradation of (*Z*)- and (*E*)-1,3-dichloropropene between sterile and non-sterile unamended and amended Arlington sandy loam at 20 °C.

The rate of chemical degradation of the 1,3-D isomers increased linearly ($r^2 > 0.98$) with respect to the rate of SM application, but this was not the case with respect to biological degradation (Fig 3). Increasing the application rate of SM to 10% had a negative impact upon the rate of biological 1,3-D degradation. In SM-amended soil, biological mechanisms accounted for 14 to 44% and 20 to 42% of the (*Z*)-1,3-D and (*E*)-1,3-D degradation, respectively (Fig 4). The greatest percentage of biological degradation occurred in the 2 and 5% SM soil mixtures, while in unamended soil, only 9% of the degradation was biologically associated. Significant differences in the biological degradation rates between the two isomers also occurred in the 2 and 10% SM soil mixtures. In 2% SM-amended soil, the rate of biological degradation of the (*Z*)-isomer was 1.5 times higher, while in 10% SM-amended soil, the biological degradation rate of the (*E*)-isomer was 1.4 times higher. In general, enhanced degradation is greater for (*E*)-1,3-D than for (*Z*)-1,3-D, but only in soils previously treated with 1,3-D.[5], [7], [19] Although our soil had not previously been treated with 1,3-D, isomeric differences in the 2 and 10% SM soil mixtures may be attributed to the presence of micro-organisms capable of preferentially degrading either (*Z*)- or (*E*)-1,3-D.

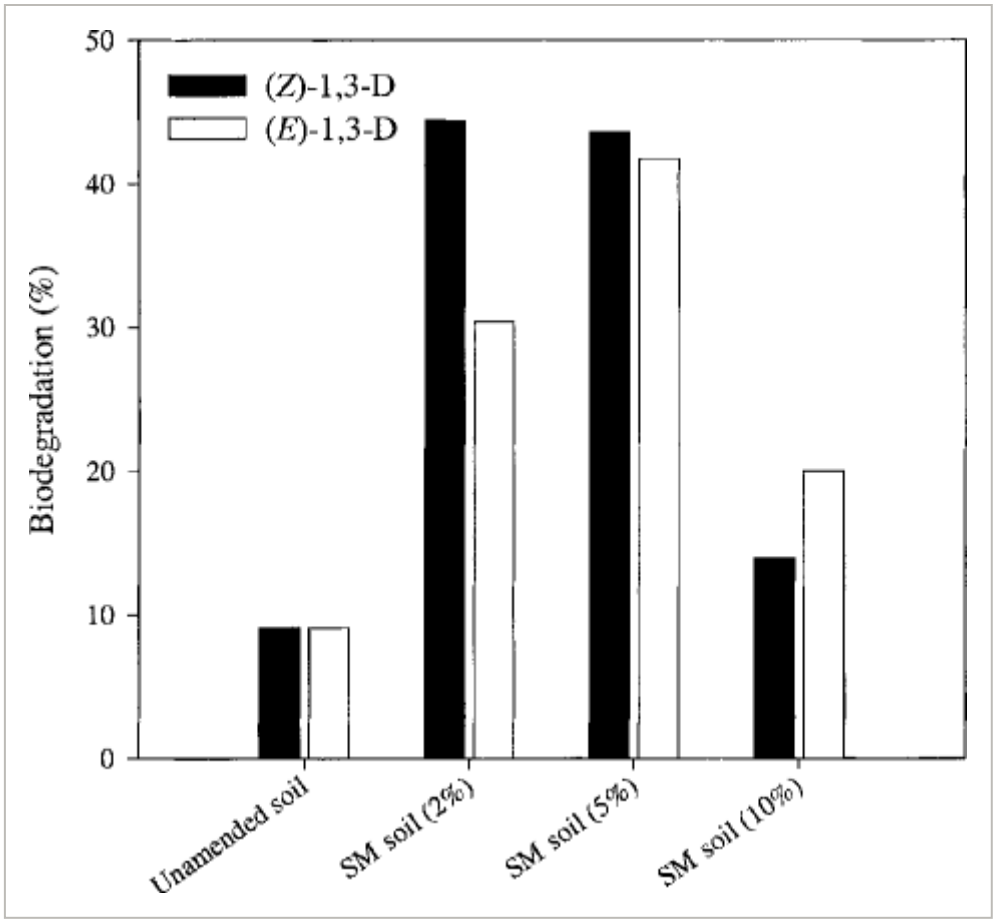


Figure 4

Open in figure viewer | PowerPoint

The relative contribution of biological mechanisms to the degradation of (*Z*)- and (*E*)-dichloropropene in unamended and amended Arlington sandy loam at 20 °C.

Overall, chemical mechanisms were the predominant degradative process; 1,3-D can be transformed via nucleophilic substitution with soil organic matter or degraded through a hydrolytic reaction.[8-10, 20] However, adjusting the soil moisture content to 25, 50 and 75% of its maximum water holding capacity had no effect on the degradation rate of either (*Z*)- or (*E*)-1,3-D (Fig 5). Since this experiment was conducted at 20 °C only, a similar impact from biological and/or chemical degradation mechanisms in SM-amended soil would not be expected at lower and/or higher temperatures. Gan *et al* [20] previously determined in unamended Arlington soil that microbial degradation of 1,3-D remained essentially unchanged over the temperature range of 20 to 40 °C, but was completely inhibited above 40 °C; chemical degradation consistently increased with temperature from 20 to 50 °C.

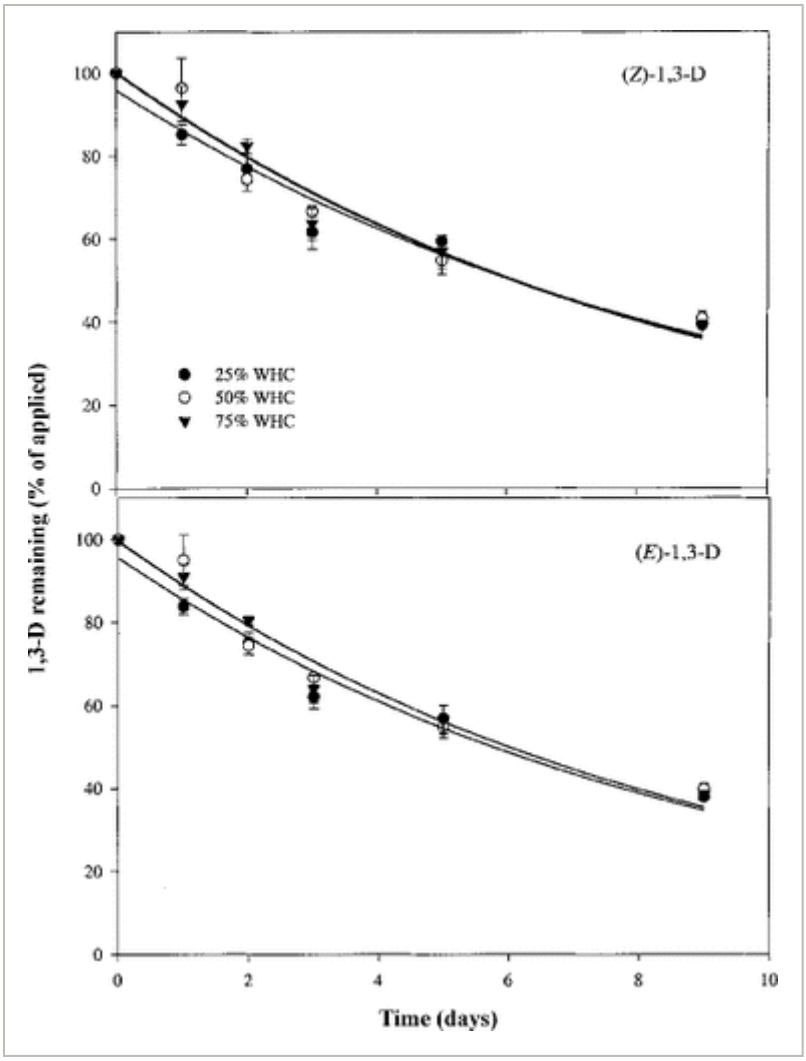


Figure 5

Open in figure viewer | PowerPoint

Degradation of (*Z*)- and (*E*)-1,3-dichloropropene at 20 °C in unamended Arlington sandy loam with different soil moisture contents.

4 CONCLUSIONS

Use of fumigant pesticides is an essential practice to protect many agricultural crops from parasitic nematodes. However, emission of fumigants to the atmosphere is often detrimental to the environment. As a result, cost-effective control strategies are needed, especially for those fumigants which are now being considered replacements for methyl bromide. Results from this study confirm that the use of organic wastes, such as composted steer manure, enhances degradation of (*Z*)- and (*E*)-1,3-D in soil. By enhancing 1,3-D degradation in the soil surface layer, it is likely that 1,3-D emissions can be reduced to levels that meet ambient air quality standards. This must, however, be determined on a case-by-case basis, since differing amendment quality and soil properties will affect fumigant behavior and degradation. Use of

organic amendments may be particularly useful in sandy soils, where fumigant degradation is generally slow and diffusion is rapid. The application of organic amendments may not only reduce fumigant emissions, but also improve soil fertility, crop yields and microbial diversity and hence soil health. Ultimately, application of amendments may provide a means to dispose of agricultural/industrial organic wastes. Minimal interference with pest-control efficacy should occur, since accelerated pesticide degradation would be limited to the soil surface layer only. Field studies should be conducted to evaluate the effectiveness of integrating organic amendments with 1,3-D fumigation as a method to control emissions. Research should also be conducted to understand the interactions of soil organic amendments on the degradation of other fumigants. Additional considerations should be given to integrated pest management techniques such as soil solarization.

Acknowledgements

This study was funded by the USDA-National Research Initiative grant no 98-35316-6450.

REFERENCES

1 Batzar FR, Balcer JL, Peterson JR and Wolt JD, Fate of 1,3-dichloropropene in aerobic soil, in *Fumigants: Environmental fate and exposure, and analysis*, ed by Seiber, ACS Symposium Series 652, American Chemical Society, Washington, DC, pp 60– 78 (1997).
Google Scholar | UConn Full Text

2 Leistra M and Frissel MJ, in *Pesticides: Environmental quality and safety*, supplement, vol **III**, ed by F Coulston, Thieme Publishers, Stuttgart, Germany, pp 817– 828 (1975).
Google Scholar | UConn Full Text

3 Chen C, Green RE, Thomas DM and Knuteson JA, Modeling 1,3-dichloropropene fumigant volatilization with vapor-phase advection in the soil profile. *Environ Sci Technol* **29**: 1816– 1821 (1995).
Crossref | CAS | PubMed | Web of Science® | Google Scholar | UConn Full Text

4 Baker LW, Fitzell DL, Seiber JN, Parker TR, Shibamoto N, Poore MW, Longley KE, Tomlin RP, Propper R and Duncan DW, Ambient air concentrations of pesticides in California. *Environ Sci Technol* **30**: 1365– 1368 (1996).
Crossref | CAS | Web of Science® | Google Scholar | UConn Full Text

5 Ou LT, Enhanced degradation of the volatile fumigant-nematicides 1,3-D and methyl bromide in soil. *J Nematol* **30**: 50– 64 (1998).
Web of Science® | Google Scholar | UConn Full Text

6 Gan J, Papiernik SK, Yates SR and Jury WA, Temperature and moisture effects on fumigant degradation in soil. *J Environ Qual* **28**: 1436– 1441 (1999).
Wiley Online Library | CAS | Web of Science® | Google Scholar | UConn Full Text

7 Chung KY, Dickson DW and Ou LT, Differential enhanced degradation of *cis*- and *trans*-1,3-D in soil with a history of repeated field applications of 1,3-D. *J Environ Sci Health* **B34**: 749– 768 (1999).
Crossref | CAS | Web of Science® | Google Scholar | UConn Full Text

8 Castro CE and Belser NO, Hydrolysis of *cis*- and *trans*-1,3-dichloropropene in wet soil. *J Agric Food Chem* **14**: 69– 70 (1966).
Crossref | CAS | Web of Science® | Google Scholar | UConn Full Text

9 Roberts ST and Stoydin G, The degradation of (*Z*)- and (*E*)-1,3-dichloropropenes and 1,2-dichloropropane in soil. *Pestic Sci* **7**: 325– 335 (1976).
Wiley Online Library | CAS | Web of Science® | Google Scholar | UConn Full Text

10 McCall PJ, Hydrolysis of 1,3-dichloropropene in dilute aqueous solution. *Pestic Sci* **19**: 235– 242 (1987).
Wiley Online Library | CAS | Web of Science® | Google Scholar | UConn Full Text

11 Hartmans S, Jansen W, van der Werf MJ and de Bont JAM, Bacterial metabolism of 3-chloroacrylic acid. *J Gen Microbiol* **137**: 2025– 2032 (1991).
Crossref | CAS | PubMed | Web of Science® | Google Scholar | UConn Full Text

12 van Hylckama Vlieg JET and Janssen DB, Bacterial degradation of 3-chloroacrylic acid and the characterization of *cis*- and *trans*-specific dehalogenases. *Biodeg* **2**: 139– 150 (1992).
Crossref | CAS | Google Scholar | UConn Full Text

13 Verhagen C, Smit E, Janssen DB and van Elsas JD, Bacterial dichloropropene degradation in soil: screening of soils and involvement of plasmids carrying the *dhl*A gene. *Soil Biol Biochem* **27**: 1547– 1557 (1995).
Crossref | CAS | Web of Science® | Google Scholar | UConn Full Text

14 Poelarends GJ, Wilens M, Larkin MJ, van Elsas JD and Janssen DB, Degradation of 1,3-Dichloropropene by *Pseudomonas cichorii* 170. *Appl Environ Microbiol* **64**: 2931– 2936 (1998).
CAS | PubMed | Web of Science® | Google Scholar | UConn Full Text

15 van Dijk H, Dissipation rates in soil of 1,2-dichloropropane and 1,3- and 2,3-dichloropropenes. *Pestic Sci* **11**: 625– 632 (1980).
Wiley Online Library | CAS | Web of Science® | Google Scholar | UConn Full Text

16 van der Pas LJT and Leistra M, Movement and transformation of 1,3-dichloropropene in the soil of flower-bulb fields. *Arch Environ Contam Toxicol* **16**: 417– 422 (1987).
Crossref | CAS | PubMed | Web of Science® | Google Scholar | UConn Full Text

17 Smelt JH, Teunissen W, Crum SJH and Leistra M, Accelerated transformation of 1,3-dichloropropene in loamy soils. *Neth J Agric Sci* **37**: 173– 183 (1989).
CAS | Web of Science® | Google Scholar | UConn Full Text

18 Leistra M, Groen AE, Crum SJH and van der Pas LJT, Transformation rate of 1,3-dichloropropene and 3-chloroallyl alcohol in topsoil and subsoil material of flower-bulb fields. *Pestic Sci* **31**: 197– 207 (1991).
Wiley Online Library | CAS | Web of Science® | Google Scholar | UConn Full Text

19 Ou LT, Chung KY, Thomas JE, Obreza TA and Dickson DW, Degradation of 1,3-dichloropropenes (1,3-D) in soils with different histories of field application of 1,3-D. *J Nematol* **27**: 249– 257 (1995).
CAS | PubMed | Web of Science® | Google Scholar | UConn Full Text

20 Gan J, Yates SR, Crowley D and Becker JO, Acceleration of 1,3-dichloropropene degradation by organic amendments and potential application for emissions reduction. *J Environ Qual* **27**: 408– 414 (1998).
Wiley Online Library | CAS | Web of Science® | Google Scholar | UConn Full Text

21 Gan J, Becker RL, Koskinen WC and Buhler DD, Degradation of atrazine in two soils as a function of concentration. *J Environ Qual* **25**: 1064– 1072 (1996).
Wiley Online Library | CAS | Web of Science® | Google Scholar | UConn Full Text

22 Kästner M and Mahro B, Microbial degradation of polycyclic aromatic hydrocarbons in soils affected by the organic matrix of compost. *Appl Microbiol Biotechnol* **44**: 668– 675 (1996).
Crossref | CAS | PubMed | Web of Science® | Google Scholar | UConn Full Text

23 Topp E, Tessier L and Gregorich EG, Dairy manure incorporation stimulates rapid atrazine mineralization in an agricultural soil. *Can J Soil Sci* **76**: 403– 409 (1996).
Crossref | CAS | Web of Science® | Google Scholar | UConn Full Text

24 Boesten JJTI, van der Pas LJT, Smelt JH and Leistra M, Transformation rate of methyl isothiocyanate and 1,3-dichloropropene in water-saturated sandy subsoils. *Soil Biol Biochem* **39**: 179–190 (1991).
CAS | Web of Science® | Google Scholar | UConn Full Text

25 Smelt JH, van de Peppel-Groen AE, van der Pas LJT and Dijksterhuis A, Development and duration of accelerated degradation of nematicides in different soils. *Soil Biol Biochem* **28**: 1757– 1765 (1996).
Crossref | CAS | Web of Science® | Google Scholar | UConn Full Text

26 Nobel PS, *Biophysical Plant Physiology and Ecology*, 3rd edn, WH Freeman and Co, San Francisco, pp 139– 145 (1983).
Google Scholar | UConn Full Text

27 Papiernik SK, Gan J, Knuteson JA and Yates SR, Sorption of fumigants to agricultural films. *Environ Sci Technol* **33**: 1213– 1217 (1999).
Crossref | CAS | Web of Science® | Google Scholar | UConn Full Text

28 Wang D, Yates SR, Gan J and Knuteson JA, Atmospheric volatilization of methyl bromide, 1,3-dichloropropene, and propargyl bromide through two plastic films: transfer coefficient and temperature effect. *Atmos Environ* **33**: 401– 407 (1999).
Crossref | Web of Science® | Google Scholar | UConn Full Text

29 Haidar MA and Sidahmed MM, Soil solarization and chicken manure for the control of *Orobanche crenata* and other weeds in Lebanon. *Crop Protect* **19**: 169– 173 (2000).
Crossref | Web of Science® | Google Scholar | UConn Full Text

30 Hoitink HAJ and Fahy PC, Basis for the control of soilborne plant pathogens with composts. *Ann Rev Phytopathol* **24**: 93– 114 (1986).
Crossref | Google Scholar | UConn Full Text

31 Muller R and Gooch PS, Organic amendments in nematode control. An examination of the literature. *Nematropica* **12**: 319– 326 (1982).
Google Scholar | UConn Full Text

32 Rodrígez-Kábana R, Organic and inorganic amendments to soil as nematode suppressants. *J Nematol* **18**: 129– 135 (1986).
PubMed | Web of Science® | Google Scholar | UConn Full Text

33 Gamliel A and Stapleton JJ, Improvement of soil solarization with volatile compounds generated from organic amendments. *Phytoparasitica* **25**(Suppl): 31– 38 (1997).
Crossref | Web of Science® | Google Scholar | UConn Full Text

Citing Literature

Download PDF