Article

Is it time to round up Roundup®?
The changing science of glyphosate

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Background – glyphosates discovery, toxicity, and approval for marketing in 1974

Glyphosate (Fig. 1) is the active ingredient of the world's most commonly used herbicide (e.g. Roundup®). It was re-discovered by John Franz of Monsanto in the early-1970s (Dill *et al.*, 2010). Franz was investigating organophosphorus compounds and noted the plant toxicity of *N*-(phosphonomethyl)glycine which was later named 'glyphosate' by contraction of its chemical name. Franz's discovery was not the first time glyphosate had been studied; Henry Martin, a Swiss chemist, first synthesised glyphosate in 1950 and its synthesis was patented fourteen years later (USA Patent, 1964). The patent notes glyphosate's metal chelating properties. Strangely, Martin never published his work in the scientific literature, but his observations that glyphosate is a metal ion chelator (Fig. 2), including Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} is the basis of one of our more recent environmental impact concerns (Mertens *et al.*, 2018) about glyphosate's extensive use in agriculture; this will be discussed later.

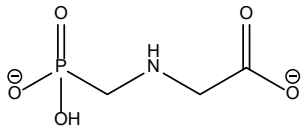

Figure 1. Glyphosate (*N*-(phosphonomethyl)glycine) showing its charges at biological pH.

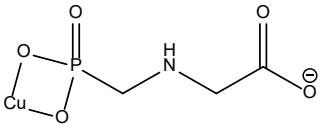

Figure 2 Possible structure of a Cu^{2+}-glyphosate chelate. Other divalent metals (e.g., Zn^{2+}, Mn^{2+}) might react similarly.

Studies on glyphosate's mechanism of herbicidal activity showed that it inhibits the shikimate pathway (Fig. 3; Steinrücken & Amrhein, 1980), a facet of biochemistry unique to plants. This was very encouraging because it suggested minimal, if any, animal toxicity. Glyphosate inhibits the shikimate pathway because of its structural analogy with phosphoenolpyruvate, a substrate for the key enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Fig. 4). Without the shikimate pathway, plants cannot biosynthesise aromatic amino acids and therefore die.

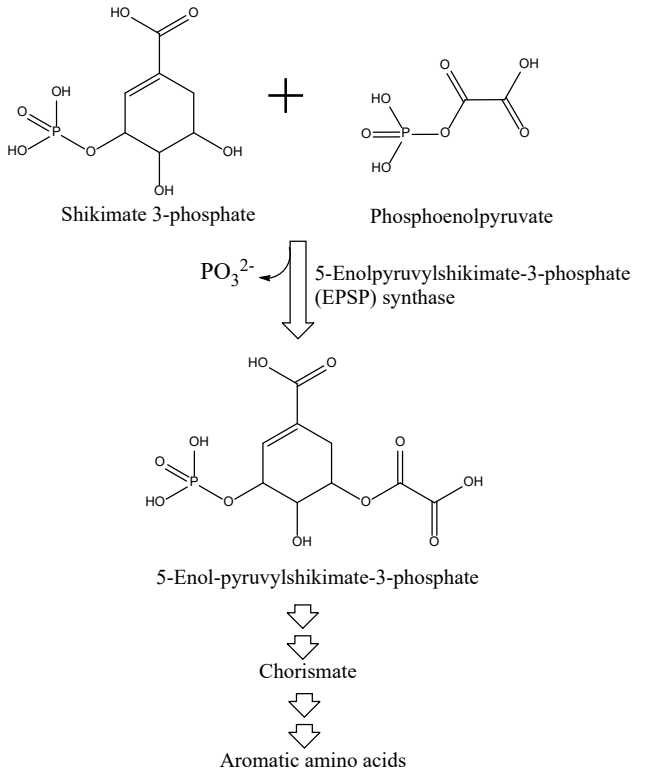

Figure 3. The shikimate pathway showing 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase which is inhibited by glyphosate.

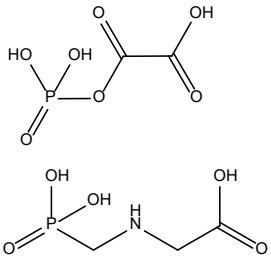

Figure 4. Phosphoenolpyruvate (top) and glyphosate (bottom) aligned to show their structural analogies, which is the basis of glyphosate's inhibition of EPSP synthase.

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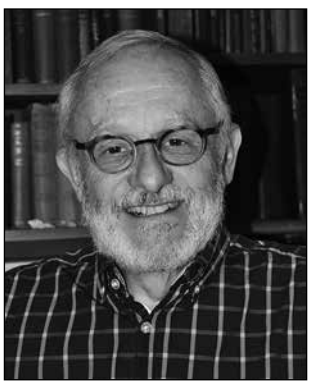

Ian Shaw is Professor of Toxicology at the University of Canterbury. Professor Shaw is an international expert on the impact of environmental contaminants on human health, particularly in a food safety context, and is a passionate communicator of science. He has had over 40 years' experience in academia, industry and government, both in the UK and New Zealand, which includes chairing the UK Pesticide Residues Committee and working as the National Food Safety Programme Manager at the Institute of Environmental Science and Research (ESR), based in Christchurch. He won the NZ Association of Scientists' Science Communicator's Award in 2009, and was awarded a DSc by the University of Bath for his work in toxicology and food safety in 2019.

There was pressure in the 1970s to support the growth of large-scale agriculture with the development of pesticides. The ideal was to introduce pesticides with minimal impact on non-target species (as, of course, it still is). Glyphosate fitted this goal perfectly because of its plant enzyme-based mechanism of action. In this respect it was, indeed, the Holy Grail of herbicides vis-à-vis its mechanism of action.

Further studies on glyphosate enhanced its Holy Grail status. Studies on its environmental fate and behaviour suggested that it was rapidly broken down in environmental systems (e.g., soil) to form a non-toxic degradation product, aminomethylphosphinic acid (AMPA; Fig. 5), which degraded further to form ammonia, carbon dioxide and water (Fig. 5). Its rate of disappearance (half-life, $t_{½}$) from terrestrial systems (e.g., soil) was shown to be very variable – from less than a week to years (Carlisle & Trevors, 1988); this disappearance was interpreted as degradation in the early days of glyphosate. However, it soon became clear that glyphosate's soil kinetics is biphasic. Studies with [^{14}C]-glyphosate in soils showed biphasic evolution of $^{14}CO_2$; this reflects a rapid initial degradation of free glyphosate, followed by slow degradation of soil-bound glyphosate. The time of the second degradation phase is dependent on soil type (i.e., is adsorption capacity) (Nomura & Hilton, 1977).

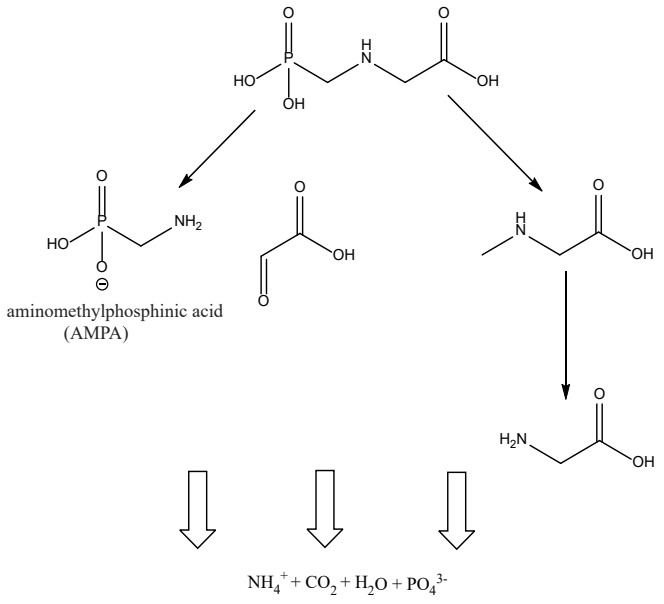

Figure 5. The environmental degradation of glyphosate showing its major degradation product, AMPA and its eventual complete degradation to ammonium, carbon dioxide, water and phosphate (based on Carlisle & Trevors, 1987).

Glyphosate, therefore, was hailed as a non-target non-toxic herbicide with a very short environmental residence time - ideal.

Food residues studies were carried out in a rather unconventional, but pragmatic way (Reding, no date). Instead of measuring glyphosate residues in crops to which the herbicide had been applied, the maximum residue levels (MRLs) for glyphosate in a large number of food crops were used as the worst-case consumption scenario. MRLs in conjunction with dietary intake data were used to calculate MRL-based worst case glyphosate intakes for each crop. Adding all of the individual crop glyphosate intakes together gives a total theoretical glyphosate intake of approx. 0.57 mg/person, which corresponds to a glyphosate dose of 0.0096 mg/kg body weight (bw) for a 60 kg human. At the time of Reding's calculations, glyphosate's acceptable daily intake (ADI) was 0.3 mg/kg bw, which means that the total theoretical glyphosate intake was approx. 3.2% of the ADI. This means that the health risk to consumers was deemed negligible.

Using current New Zealand data would give a much more favourable total glyphosate intake because the glyphosate MRL is currently (2020) set at a default value of 0.1 mg/kg, whereas Reding used MRLs ranging from 20 mg/kg for soybeans to 0.1 mg/kg for rice for her calculations. The current (2020) ADI for glyphosate is 1.0 mg/kg bw (FAO/WHO, 2006), which is higher than that used for Reding's calculations – this gives a glyphosate intake of 0.96% of the current ADI. If the default MRL of 0.1 mg/kg bw is used to estimate glyphosate intake, this would give a total theoretical intake of approx. 0.195 mg (= 0.00325 mg/kg bw for a 60 kg human) based on Reding's consumption data, which equates to approx. 0.33% of the current ADI. These calculations make the clear point that, based on conventional toxicological parameters, glyphosate intake as residues in food results in a negligible health risk.

Therefore, when glyphosate was approved for use in 1974 it appeared to be safe. This meant that the risk aspect of the risk-benefit equation was arguably negligible in both human and environmental contexts. The benefit side of the equation was considerable because glyphosate was and is a very effective herbicide. Thus, from an approvals-for-marketing perspective, glyphosate was close to ideal.

Use of glyphosate

When glyphosate was approved in the 1970s, it was indicated for general herbicide use. It was used by farmers to prepare land for crop planting without the necessity for tilling, which minimised soil erosion. Its use changed significantly after 1995 with the introduction of Roundup Ready® crops (Benbrook, 2016). Roundup Ready® crops (e.g., canola) are genetically modified to express a form of EPSP synthase from *Agrobacterium* strain CP4 that is resistant to glyphosate inhibition. This allows the herbicide to be used to kill weeds in a field of the Roundup Ready® growing crop. This has no direct relevance to New Zealand because genetically modified crops are not permitted in New Zealand. However, it might contribute to glyphosate food residues in imported products.

Glyphosate is also used as a crop desiccant to speed up the drying of near harvest crops (e.g., wheat) and facilitating an evenly dry, storable product (e.g., in Canada; Darwent *et al.*, 1994). This is common practice in New Zealand (FAR, 2017) and will lead to crop (and likely food) residues.

In recent years, glyphosate has been used to kill off pastures to facilitate their re-seeding or for follow-on planting with forage crops (e.g., brassicas). In order not to waste the dying pasture, stock are often grazed on the glyphosate-treated pasture (this will be discussed later). This might also lead to food residues.

Mammalian metabolism of glyphosate

The main human exposure route to glyphosate is via food. Farm workers might also be exposed dermally and via inhalation during mixing and applying sprays in an agricultural setting. Similarly, council workers and contractors might be exposed during spraying to control, for example, roadside weeds. Indeed, in a study of 48 farm families in the USA, glyphosate was detected (<1-233 ng/mL) in 60% of the urine samples analysed (Acquavella *et al.*, 2004).

Oral exposure leads to the ingested glyphosate being exposed to the gut microbiome, and this is very likely to lead to significant breakdown by a pathway akin to that for soil bacteria (Fig. 5). Based on animal studies, which appear to be reflected in humans, only approx. 30% of an oral glyphosate dose is absorbed from the gastrointestinal tract (GIT), peak plasma concentration is at approx. 1-2 h, and blood levels decline quickly due to urinary excretion rather than metabolism (Brewster *et al*., 1991; Bradberry *et al*., 2004). AMPA has been found in the blood of human glyphosate poisoning cases; this likely arose from gut microbial rather than human metabolism (Bradberry *et al*., 2004).

A study in glyphosate-exposed farm workers in the USA (Acquavella *et al*., 2004) clearly showed the presence of glyphosate in their urine. Urinary glyphosate in the exposed farm workers might be due to oral, dermal and/or inhalation absorption. Oral absorption is unlikely to result in significant blood concentrations (and thus urinary concentrations) of glyphosate (as discussed above). Studies in *in vitro* human skin preparations showed that <2% is absorbed, and dermal absorption studies in rhesus monkeys was approx. 0.8% of the applied dose (Wester *et al*., 1991). A study to assess the individual contributions of inhalation and dermal absorption following glyphosate exposure in humans showed that dermal absorption is greater than inhalation absorption (Pierce *et al*., 2020) and thus likely contributes more to blood (and urine) glyphosate concentration following workplace or bystander exposure (i.e., via sprays). It is, therefore, likely that the urinary glyphosate in the farmworker study was predominantly from dermal absorption of either aerosols (e.g., from spray) or direct skin contact (e.g., when diluting concentrate).

Glyphosate's $t_{½}$ in humans (using urinary excretion data) is in the range 5.5-10 h depending on the calculation method (Connolly *et al*., 2019). This means that, if a worker is repeat spraying over several days, there might be a build-up of glyphosate body burden because with a $t_{½}$ of 10 h only approx. two half-lives (i.e., 25% of body burden remaining) would have lapsed between exposures. However, as soon as exposure stops, it would take 6 half-lives (180 h, 7.5 d) to reduce the glyphosate body burden to approx. 1.2% of its peak value (likely of little or no toxicological significance for 'normal' agricultural exposures).

Glyphosate's human toxicity profile

Acute toxicity

The acute toxicity (i.e., following a single dose) of glyphosate is likely negligible in humans because it is not well absorbed (particularly from the GIT) and relatively quickly cleared ($t_{½}$ = 5.5-10 h). Use of appropriate personal protective equipped (PPE) will reduce the risk of acute effects significantly – particularly wearing impervious gloves to minimise dermal absorption (Acquavella *et al*., 2004). Cases of acute human poisoning have been recorded, but the doses involved are very large. In addition, commercial formulations of herbicides (e.g., Roundup®) containing glyphosate also contain excipients, including surfactants (e.g., polyethoxylated tallow amine - POEA) to aid absorption by plants and therefore it is often difficult to separate excipient toxicity from glyphosate toxicity *per se* (Bradberry *et al*., 2004) or to take account of the excipient's effects on glyphosate's toxicity (e.g., POEA might increase human absorption of glyphosate from the GIT or dermally). Also, the glyphosate salt used differs between glyphosate-containing commercial herbicides (e.g., in Roundup® glyphosate isopropylamine salt is used). All of these factors affect toxicity of the product. However, it has been shown that following suicide attempts, oral ingestion of 85 mL of Roundup® concentrate causes significant toxicity (but not necessarily death) in adults (Bradberry *et al*., 2004). Roundup® concentrate contains 41% w/v glyphosate isopropylamine salt (Fig. 6; molar mass = 346.4 g/mol), which means that 85 mL of Roundup® concentrate contains approx. 35 g glyphosate isopropylamine salt, which equates to approx. 17 g glyphosate (molar mass = 169.1 g/mol); therefore, a glyphosate oral dose of approx. 300 mg/kg body weight (using standard human weight = 60 kg) is near fatal in humans. The estimated lethal dose of aspirin in humans is 5-15 g (Clarke, 1978; ~ 83-250 mg/kg body weight), which means that glyphosate is of the same order of acute oral toxicity as aspirin.

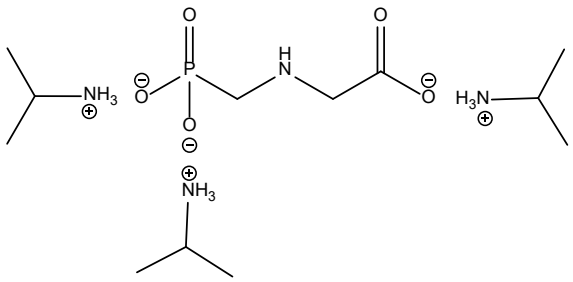

Figure 6. Depiction of glyphosate isopropylamine salt

The symptoms of acute Roundup® toxicity in humans are increased saliva production, burns in the mouth and throat, nausea, vomiting and/or diarrhoea. It is difficult to assign these signs of toxicity to glyphosate *per se* because some (e.g., burns in the mouth and throat) might be caused by POEA. The salivation response is, however, interesting because it is associated with organophosphate (OP) intoxication due to OPs inhibition of acetylcholinesterase (AChE). Glyphosate is also a simple OP and thus might exhibit AChE inhibitory properties – in this context, it is interesting that glyphosate has been shown to inhibit AChE in carp (*Cyprinus carpio*) (Gholami-Seyedkolaei *et al*., 2013).

Chronic toxicity

On 20 March 2014, the World Health Organization's International Agency for Research on Cancer (IARC) classified glyphosate as Carcinogen 2A (probably carcinogenic to humans). This led to action by several countries (e.g., The Netherlands banned glyphosate in 2015; In Habitat, 2014) and a recent partial ban by France (Euro Coop, 2021). There followed significant international debate and conjecture at government level about the validity of the IARC's carcinogen classification, and whether the significant economic benefit of glyphosate outweighed its risks (especially as some jurisdictions disputed the IARC's deliberations and conclusion). The New Zealand Government commissioned a report to assess the IARC's findings (Temple, 2016) which dismissed the IARC's carcinogenicity data as inconsistent and showing a lack of association, in part due to the possibility that subjects in the studies might have been exposed to other pesticides. Temple (2016), however, missed a key mechanistic possibility in his report: that glyphosate might be a non-genotoxic carcinogen; this is relevant in the context of glyphosate's estrogen mimicry (see later).

The evidence that the IARC presented in support of their categorisation of glyphosate as Carcinogen 2A included three key

studies: (1) A study in farmworkers which showed an association between farmworkers' glyphosate exposure and non-Hodgkin's lymphoma and multiple myeloma (Acquavella *et al*., 2004); (2) A chronic exposure study in mice which showed a dose-related relationship with skin cancers (George *et al*., 2010); (3) A study in cultured human estrogen receptor (ER)-expressing breast cancer cells which showed a glyphosate dose-related increase in proliferation that was inhibited by the potent ER antagonist, fulvestrant (Thongprakaisang *et al*., 2013). The last-named study is particularly important because it initiated the thinking that glyphosate might be an estrogen mimic (see later).

A later expert panel review of the carcinogenicity data used in support of the IARC's ruling pointed out limitations in some of the studies used by the IARC. In particular, they found that the association between non-Hodgkin's lymphoma and glyphosate exposure might have been confounded by multiple pesticide exposures (as pointed out by Temple (2016)) and so was unreliable (Acquavella *et al*., 2016). Several years' later, a meta-analysis of glyphosate exposure-linked non-Hodgkin's lymphoma concluded confidently that there is indeed a link (Zhang *et al*., 2019). Further to this, a recent and extensive meta-analysis of human (occupational) exposure to glyphosate and the incidence of non-Hodgkin's lymphoma and multiple myeloma showed no increased risk except possibly at very high glyphosate exposure (Donato *et al*., 2020). This conjecture makes it difficult to conclude whether or not glyphosate is carcinogenic.

The molecular structure of glyphosate (Fig. 1) has no features that would point to genotoxic carcinogenicity (e.g., reactive moieties that might alkylate DNA leading to mutations) and therefore Temple's (2016) conclusion that it is not a genotoxic carcinogen is justified on structure activity grounds alone. However, mounting evidence that glyphosate interacts agonistically with ERs is good evidence for a non-genotoxic mechanism of carcinogenesis. Non-genotoxic carcinogens affect cells in such a way that they induce proliferation (e.g., an inflammatory response) that increases the chance of a transcriptional defect which leads to a carcinogenic event (Shaw & Jones, 1994). In addition, receptor-mediated tumours (e.g., ER+ breast cancer) proliferate in response to their receptor (e.g., ER) agonist (e.g., the estrogen, 17β-estradiol (E2)) and therefore natural receptor ligand mimics (e.g., estrogen mimics) might act as non-genotoxic carcinogens via this mechanism (Ye *et al*., 2018). It is interesting to note that in animal models, administration of E2 causes proliferation of ER-expressing lymphoid and myeloid lineage bone marrow cells (Issa *et al*., 1996) – this has a possible link to non-Hodgkin's lymphoma via a non-genotoxic hormone-mediated mechanism. If glyphosate is an estrogen mimic it might initiate this response.

Is glyphosate estrogenic?

There has been considerable conjecture about whether or not glyphosate is estrogenic, since the first experiments in MCF-7 cells showed that proliferation stimulated in a dose-dependent manner by glyphosate was inhibited by the potent ER antagonist, fulvestrant (Thongprakaisang *et al*., 2013). This conjecture was largely because the molecular structure of glyphosate has no apparent structural relationship to ER's natural ligand, E2, whereas most estrogen mimics have significant molecular analogies with E2 that allow them to interact with key amino acid residues in the ER and thus initiate an estrogen-like response (Fig. 7).

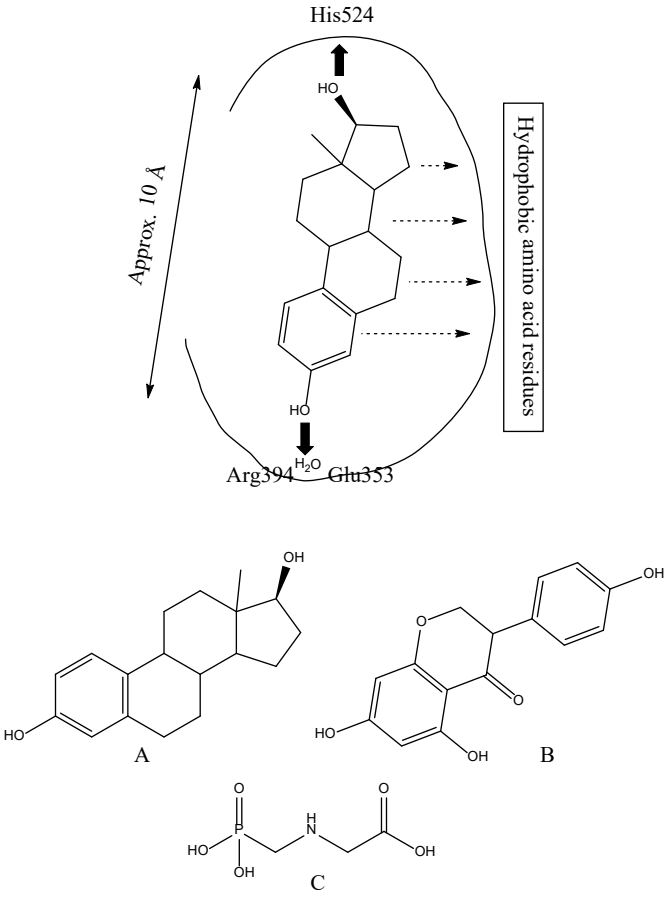

Figure 7. Top: Schematic representation of ERα with E2 *in situ* showing its key interactions with amino acid residues that lead to biological activity.

Bottom: E2 (A), the known estrogen mimic, genistein (B), and glyphosate (C), are also shown to illustrate glyphosate's lack of molecular analogy with E2.

To initiate an estrogen response a ligand must ideally possess two hydroxyl groups – one aromatic and one aliphatic – separated by approximately 10 Å of hydrophobicity. The hydroxyls form hydrogen bonds with the Arg394/Glu353/water triumvirate at one end of the ligand binding cleft (LBC) and with His524 at the other (in ERα, for ERβ the amino acid residue interactions are the same, but because of differences in the amino acid sequence the residue numbers are different; i.e., Glu306/Arg346/water and His475 (Pike *et al*., 1999)). There are a significant number of hydrophobic interactions with a cluster of hydrophobic amino acid residues aligned with the steroid skeleton of E2 *in situ*. Estrogen mimics (e.g., genistein – an isoflavone from soybeans) have the appropriate structural attributes, albeit often not ideal (e.g., genistein has two aromatic hydroxyl groups) to interact with the amino acid residues in the LBC. These interactions lead to a receptor conformational change, which in turn leads to the formation of a receptor/ligand dimer with increased affinity for an estrogen responsive element (ERE) on DNA. When bound to the ERE the ER-ligand dimer upregulates key genes which result in the biological response (Ye *et al*., 2018). The fact that glyphosate does not have the key ER-binding molecular attributes makes it unlikely on structure activity relationship (SAR) grounds to be estrogenic by a 'conventional' estrogen mimicry mechanism.

Despite the controversy following Thongprakaisang *et al.* (2013)'s findings, Mesnage *et al.* (2017) showed that glyphosate was indeed estrogenic, but by a ligand-independent activation mechanism leading to ERE-luc expression via protein kinase A (PKA) signalling. This brought the spotlight back onto glyphosate and the importance of its estrogenicity in both a human exposure and environmental context. Further work in ERα-expressing cholangiocarcinoma cells showed that a non-genomic ER pathway via extracellular signal-regulated kinases (ERK) 1 and 2 might explain glyphosate's estrogenic response (Sritana *et al.*, 2018). Interestingly, ERK1 and ERK2 stimulate cell proliferation which might also link glyphosate to a non-genotoxic mechanism of carcinogenesis as speculated above. It is therefore likely that glyphosate is estrogenic, perhaps by a non-genomic mechanism that does not rely on E2/glyphosate SARs and ER interactions.

Environmental toxicology

Glyphosate has an apparently short (<1 week) to long (years) soil $t_{½}$, which is due to an interplay between its binding to soil particles (sequestration) and its conversion (e.g., by soil bacteria) to AMPA (then CO_2, NH_3 and H_20) (Carlisle & Trevors, 1987). Sequestration is possibly reversible when environmental conditions change, and it is possible that, while bound to soil particles, glyphosate is still bioavailable in some circumstances. Glass (1987) reported that glyphosate binds to clay soils, possibly by an ion exchange mechanism. He noted that binding was 'stronger' in the presence of divalent metal ions (M^{2+}; e.g., Mg^{2+}, Ca^{2+}) which supports an ion exchange mechanism involving binding of M^{2+} to the negatively charged clay particles giving an overall +1 charge to which negatively charged glyphosate (Fig. 1) can bind. An increase in soil pH (i.e., greater OH^- concentration) could displace glyphosate from its clay-bound complex because OH^- would likely compete with glyphosate for positively charged regions of the clay-M^{2+} complex. These possibilities are rarely discussed in a regulatory context (Mertens *et al.*, 2018). It is, however, clear that a short soil $t_{½}$ that was originally interpreted as short-lived environmental impact is not necessarily the case. Interestingly, as far back as 1976, it was noted that the soil $t_{½}$ of glyphosate was composed of two phases; an initial rapid soil bacteria-mediated biotransformation of free glyphosate and a slower biodegradation of soil-bound glyphosate (Hance, 1976).

Studies in the planktonic crustacean, *Daphnia magna* have shown that exposure to glyphosate-Cu^{2+} complexes alter the *Daphnia*'s behaviour, indicating that glyphosate might mediate metal toxicity in ecosystems (Hansen & Roslev, 2016) – this introduces a new mechanism of glyphosate's impact on ecosystems that does not rely on its direct toxicity, but rather mediated toxicity. This could extend beyond Cu^{2+} to other divalent metal ions (e.g., Pb^{2+}). Similarly, glyphosate's chelating properties can affect metal sorption to soils: Morillo *et al.* (2002) studied Cu^{2+} adsorption in three soil types and found that sorption differed with soil type due to the equilibrium between soil-Cu^{2+} and glyphosate-Cu^{2+}. This means that the presence of glyphosate in terrestrial (and perhaps aquatic silt) systems might alter the balance between adsorbed and aqueous metals, and change the bioavailability of these metals to organisms in the ecosystem. The bioavailability of toxic metals and 'nutrient' metals is important in ecosystem health. If glyphosate upsets this balance it will perturb ecosystem health (Mertens *et al.*, 2018).

There has been a great deal of work exploring the ecotoxicity of glyphosate and its formulations (including Roundup®) that shows a plethora of impacts on individual test species (Carlisle & Trevors, 1988). It is important to note that it is often difficult to separate the effects of glyphosate *per se* and other constituents (e.g., surfactants) of glyphosate herbicide formulations (Carlisle & Trevors, 1988) and that in some cases the excipients are more toxic than glyphosate *per se* (Peréz *et al.*, 2011). Obviously, glyphosate is very toxic to plants (approx. 18μM (approx. 3 mg/L) glyphosate inhibits growth of the green microalga, *Chlorella sorokiniana*, by 50% (Christy *et al.*, 1981)). Similarly, growth of the single-celled alga, *Euglena gracilis*, a mixotroph (capable of living photosynthetically or by phagocytosis), is impacted by 1.3 mM (approx. 219 mg/L) glyphosate (Richardson *et al.*, 1979). From this very cursory foray into the literature, it is clear that low environmental concentrations of glyphosate have been known to impact non-target plants from the time that glyphosate was first introduced to the market in the 1970s, but this is hardly surprising for a herbicide!

Glyphosate's impact on animals is quite a different matter. Since glyphosate was thought to be ostensibly non-toxic to animals because of its plant-focused mechanism of toxicity, little work appears to have been conducted on animals in an ecological context until the 1980s. An early study of Roundup® in *D. magna* gave an EC_{50} (48 h, immobilisation) of 3.0 mg/L and EC_{50} (48 h, mortality) in the amphipod crustacean, *Gammarus pseudolimnaeus* of 62.0 mg/L (Folmar *et al.*, 1979). Since these studies used glyphosate in its Roundup® formulation, it is important to consider the toxicity of its major surfactant constituent, POEA. A study of the toxicity of POEA in *D. pulex* gave an EC_{50} (96 h, immobilisation) of 2.0 mg/L, while Roundup® gave an EC_{50} (96 h, immobilisation) of 8.5 mg/L (Servizi *et al.*, 1987). This suggests that POEA is largely responsible for Roundup®'s toxicity in this *Daphnia* study. This is a very important consideration when assessing the environmental toxicity of Roundup®. However, it is equally important to consider the fate and behaviour of POEA in environmental systems and the differential exposures of creatures to glyphosate and/or POEA following the use of Roundup® in an agricultural setting, but this is beyond the scope of this paper.

Pérez *et al.* (2011) extensively reviewed the invertebrate toxicity of glyphosate-containing commercial formulations (including Roundup®). They collated EC_{50}s for variable endpoints (e.g., immobilisation, mortality) and exposure times (48 h or 96 h) and found EC_{50}s in the range 3.0 mg/L (*D. magna*, 48 h, immobilisation) – 415.0 mg/L (*Ceriodaphnia dubia*, 48 h, mortality). This gives an idea of the level of environmental toxicity of commercial glyphosate products, but of course, includes the toxicity of excipients such as POEA. It is also important to note that invertebrates do not express ERs (Brennan *et al.*, 2006) and therefore are not susceptible to glyphosate's estrogenicity.

In general, the toxicity of glyphosate *per se* to higher animals (e.g., fish) is not greater than its toxicity to invertebrates (Table 1) which suggests that its estrogenicity is not a major determinant of toxicity in the short term (96 h). Interestingly, glyphosate commercial formulations (mean 96 h LC_{50} = 15.9 mg/L) are often very much more toxic than glyphosate *per se* (mean 96 h LC_{50} = 246.8 mg/L) (Table 1); this makes the point that the formulation excipients are a major determinant of toxicity. Indeed, fish POEA toxicity studies show this clearly (e.g., *O. mykiss* 96 h LC_{50} for POEA = 2.0 mg/L (Folmar *et al.*, 1979)).

Table 1. Toxicity (96 h) of glyphosate and Roundup® to fish.

Species	LC_{50} mg/L	Reference
Glyphosate *per se*		
Rainbow trout (*Oncorhynchus mykiss*)	140.0	Folmar *et al*., 1979
Fathead minnow (*Pimpehales promelas*)	97.0	Folmar *et al*., 1979
Channel catfish (*Ictalurus punctatus*)	130.0	Folmar *et al*., 1979
Common carp (*Cyprinus carpio*)	620.0	Neškovic *et al*., 1996
Mean	246.8	
Roundup®		
Oncorhynchus mykiss	8.3	Folmar *et al*., 1979
	52.0	Hildebrand *et al*., 1982
	8.5	Servizi *et al*., 1987
Pimpehales promelas	2.3	Folmar *et al*., 1979
Ictalurus punctatus	13.0	Folmar *et al*., 1979
	14.5	Abdelghani *et al*., 1997
Bluegill sunfish (*Lepomis macrochirus*)	13.0	Abdelghani *et al*., 1997
Mean	15.9	

Therefore, even in fish, glyphosate is less toxic than at least one of its excipients (POEA).

Glyphosate's chronic toxicity is less well documented in fish. However, Salbego *et al*. (2010) reported decreased weight and AChE activity in *Leporinus obtusidens* exposed to 1 mg/L Roundup® for 90 d. In addition, a study in which *Platichthys flesus* was exposed to Roundup® + AMPA for 62 d showed liver damage at a glyphosate exposure concentration of 0.16 µg/L (Evrard *et al*., 2010). These studies suffer from the problem of excipient toxicity, but since glyphosate is an OP, it likely caused the decreased AChE activity.

Studies in amphibians show similar, if not more extreme, toxicity differentials between Roundup® and glyphosate *per se* (Pérez *et al*., 2011). For example, the 48 h LC_{50} for glyphosate in *Lymnodynastes dorsalis* is >400 mg/L, while the corresponding value for Roundup® is 3.0 mg/L, and for *Heleioporus eyrie* the corresponding values are >373 mg/L and 6.3 mg/L respectively (Mann & Bidwell, 1999). Clearly, not only are Roundup®'s excipients more toxic than glyphosate *per se*, they might even ameliorate glyphosate's toxicity. A single long-term (42 d) study in *Rana cascadae* shows the first inkling of a possible hormone effect – earlier metamorphosis at an exposure concentration of 1.94 mg/L (Cauble & Wagner, 2005), but this is far from conclusive.

Therefore, glyphosate *per se* is far less toxic to vertebrates than Roundup®. This is likely due to POEA as evidenced by its 96 h LC_{50} in *Xenopus laevis* of 2.7 mg/L (Perkins *et al*., 2000).

A long-term study in *Carassius auratus* exposed to 0.2 mmol/L glyphosate as a commercial formulation (Nongteshi®) for 90 d showed significant metabolomic changes, including raised aspartate aminotransferase (AST) – a marker of liver and muscle damage, lactate dehydrogenase (LDH) – a marker of general cell damage, and alanine aminotransferase (ALT) – a marker of liver damage (Li *et al*., 2017). This study indicates a wide-ranging generalised impact on the fish biochemistry. Unfortunately, no hormone-related parameters were measured. As with so many commercial formulation studies, it is impossible to determine whether the effects were due to glyphosate or excipients; indeed, POEA could be responsible for all of the observed effects. These naïve study designs give very little information of values when attempting to assess environmental impact because, even though commercial formulations are used in agriculture, it is impossible to determine the fate, behaviour and impact of the individual components, especially when (in the laboratory) the formulation is applied to a closed environment test system which does not allow for differential distribution of the formulation's components.

In order to fully assess the environmental impact of glyphosate, we need good, reliable long-term studies because it is clear that from an acute toxicity standpoint, glyphosate is of little concern (i.e., the risk is low). It has been noted that there is a dearth of long-term glyphosate environmental toxicity data (Howe *et al*., 2004); this situation remains unchanged today (2021). The need for long-term toxicity data is because our knowledge of glyphosate's effects or potential effects in animals points firmly to long-term impact. For example, glyphosate's estrogenicity would not manifest in the short term, but possibly in a multigenerational growth and development context. Interestingly, one of the few long-term studies (in frogs) shows a multitude of effects, most of which might be attributable to POEA. However, there is one very interesting finding: glyphosate causes an increase in the female:male ratio and this effect is not seen for POEA alone. The sex ratio change was also seen following Transorb® exposure. Transorb® is a commercial glyphosate formulation containing POEA and glyphosate as its potassium salt (Roundup® contains glyphosate isopropylamine salt; Fig. 6). This needs further scrutiny because exposure to estrogenic compounds suppresses male development in some species (e.g., fish; Jobling *et al*., 1998).

Glyphosate concentration in the environment

In order to assess the potential environmental impact of glyphosate's use, environmental glyphosate concentrations are compared with toxicological effect parameters (e.g., EC_{50}, LC_{50}). I could find no published data on glyphosate concentrations in New Zealand soils or waterways. However, studies in Argentina found 35 – 1502 µg/kg soil (mean of samples with measurable concentrations = 340 µg/kg); the low value in the range was in a soil sample taken 40 d after spraying, and the high value 1 d after spraying – the difference reflects glyphosate's soil $t_{½}$; they also found silt-bound glyphosate in approx. 15% of water samples analysed, with 'free' water concentrations in the range 0.4 – 7.6 µg/L (mean of samples with measurable concentrations = 2 µg/L) (Aparicio *et al*., 2013). It is interesting that silt-bound glyphosate was found in waterways because this suggests that transfer from land can be via silt (Aparicio *et al*., 2013). Environmental concentrations in New Zealand would be expected to be lower than in Argentina because Roundup Ready® crops are not permitted here, but are used extensively in Argentina.

Using the mean values for soil and water glyphosate concentrations from the Argentinian study, the mean water concentration of 2 µg/L is far below all of the *Daphnia* short-term (48 – 96 h) exposure, immobilisation endpoint EC_{50} values collated by Pérez *et al*. (2011): range 3.0 – 66.2 mg/L; even the top value of 7.6 µg/L is only approx. 0.25% of the most sensitive test result. This suggests that, in conventional toxicological terms, the agricultural use of glyphosate would have little or no short-term environmental impact, even following intensive use in a Roundup Ready® agricultural setting. Assessing the impact of terrestrial animal exposure via soil is difficult because I could find only one published study on terrestrial invertebrate toxicity. This study was carried out in earthworms (*Eisenia foetida*) at very high exposure concentrations (10 - 1,000 mg/kg soil) (Correia & Moreira, 2010) and so is irrelevant to predicted

Figure 8. A glyphosate-treated field with grazing cattle in the Canterbury region.

(Photograph by the author.)

glyphosate soil concentrations following glyphosate-containing herbicide use. LD_{50} studies in terrestrial vertebrates show extremely low oral toxicity; e.g., LD_{50} in deer mouse (*Peromyscus maniculatus*) >6,000 mg/kg body weight. The corresponding value for the rough-skinned newt (*Taricha granulosa*) is >2,600 mg/kg body weight (McComb et al., 2008). It is difficult to equate gavage LD_{50} study doses with terrestrial environmental exposure; therefore, I have used data from two silt-living aquatic/marshland species, the black worm (*Lumbriculus variegatus*) and the buzzer midge (*Chironomus plumosus*) larva in lieu of bona fide terrestrial invertebrates for this assessment. The 48 h EC_{50} for glyphosate per se in *C. plumosus* (immobilisation endpoint) is 55 mg/L and the corresponding value for *L. variegatus* (glutathione S-transferase activity (GST)) is 0.05 mg/L (Pérez *et al.* (2011). These values are both far below the equivalent terrestrial glyphosate concentrations from the Argentinian study, even with the very sensitive GST toxicity endpoint in the *L. variegatus* study. Thus, the likely short-term impact of glyphosate on the terrestrial environment is negligible.

This clean bill of health for glyphosate following its agricultural use is, however, misleading because it reflects only short-term impact. There are no data on long-term effects – glyphosate's estrogenicity will only manifest after long-term, multigenerational exposure.

Glyphosate residues in food

There is growing interest in residues of glyphosate in food (perhaps following the IARC's categorisation of glyphosate as Carcinogen 2A). The widespread use of glyphosate in agriculture means that residues in food are inevitable. In New Zealand, genetically modified crops are not permitted and therefore direct application of glyphosate to growing crops does not occur, except for its use as a desiccant to aid the even ripening and drying of, for example, wheat – this reduces the risk of widespread high crop residue levels that might occur overseas. However, glyphosate is used extensively in non-till regimes to prepare land for new crops, including livestock forage and grass. In New Zealand it is common to see livestock grazing recently sprayed pastures to maximise the efficient use of 'carbon' (Fig. 8). There is no Roundup® withholding period for stock grazing in New Zealand (unless Roundup® is being used to kill toxic plants; e.g., ragwort (*Jacobaea vulgaris*)); therefore, there is no safeguard to reduce residues in meat and milk following livestock's consumption of glyphosate-treated pasture. Since glyphosate is rapidly metabolised and excreted in mammals, meat residues are unlikely to be a problem; however, if glyphosate is secreted into milk there are no metabolic enzymes present, and so the residues might remain.

The New Zealand Ministry of Primary Industries (MPI) carried out surveys on glyphosate residues in honey in 2017/2018 and 2018/2019 and released a report bringing together the results in response to industry concerns in January 2020 (MPI, 2020a). In the 2017/2018 survey, 1.7% were above the default maximum residues limit (MRL) of 0.1 mg/kg, and 20.7% had measurable (i.e., above the limit of determination of the analytical method) glyphosate residues. In the 2018/2019 survey, 18.3% of samples had measurable glyphosate, but none exceeded the default MRL (MPI, 2020b). From a food safety perspective, the glyphosate residues found in New Zealand honey are of little concern, even considering glyphosate's estrogenicity because honey is not a 'major' food and so the glyphosate dietary intake from honey would be low. The question is, where did the glyphosate come from? Clover, pasture and multifloral honeys had the greatest proportion of residues in both surveys. This suggests that bees were accessing glyphosate-sprayed pastures prior to the flowers dying. A related problem occurred in the UK in the 1990s where treatment of rape with insecticides was associated with bee deaths – advising farmers not to spray flowering rape solved the problem (personal information; the author was chairman of the UK Pesticide Residues Committee 1992–2000). An additional possible source of New Zealand's honey glyphosate contamination might be the use of Roundup® to kill herbage around beehives.

Glyphosate residues in honey have been found in many other countries, with concentrations as high as 160 mg/kg in USA honey (Rubio *et al.*, 2014) – this might reflect the use of Roundup Ready® flowering crops which, even though highly contaminated with glyphosate, are not killed and so continue to attract and contaminate bees.

In addition, glyphosate residues in other foods have been reported from around the world. For example, residues in 9/28 (32%) samples of soy sauce in a USA survey had glyphosate residues >100 mg/L (one sample exceeded 500 mg/L) (Rubio *et al.*, 2014). The health risks associated with this are likely to be minimal via western diets, but would be greater for some Asian communities. A Swiss study found that glyphosate residues were very often found in fruits, wine and honey, but that pasta was the most important source of glyphosate residues in a dietary intake context, but they found no MRL exceedances (Zoller *et al.*, 2017).

It is clear that the extensive use of glyphosate in agriculture is reflected in its food residues spectrum. While the health risk from glyphosate intake from individual commodities might

be low, since residues are widely distributed it is important to consider total dietary intakes. Zoller *et al.* (2017) assessed dietary intake and found that neither the acceptable daily intake (ADI) nor the acute reference dose (ARfD) for glyphosate were exceeded. They concluded that glyphosate residues are of no concern from a human health perspective.

It is important to note that all of the toxicological endpoints used to determine the ADI and ARfD are unlikely to have included endpoints (e.g., testicular atrophy, endometrial thickening) that would have indicated estrogenicity. They were almost certainly based on acute toxicology. This is important because glyphosate has a 'clean' acute toxicity profile which might be misleading in the context of new evidence regarding its estrogenicity and long-term risk.

In the context of food residues, Low *et al.* (2005) demonstrated that *Saccharomyces cerevisiae* (the yeast used in breadmaking) can metabolise glyphosate, thus reducing potential residues in fermented food products (e.g., bread) made from, for example, flour with glyphosate residues. This is less important in New Zealand where Roundup Ready® wheat is not grown, but is an interesting concept that should be borne in mind when predicting food residues and their health risks.

Continuing the idea that glyphosate is estrogenic and that this might have implications in a long-term risk context, it is important to consider multigenerational effects, in particular growth and development effects on the embryo and fetus (Shaw *et al.*, 2009). Aris & Leblanc (2011) measured glyphosate in serum from, non-pregnant and pregnant women and their fetuses (fetal cord blood). They did not find glyphosate or AMPA in serum from pregnant women (n = 30) or fetal cord blood (n = 30), but did find glyphosate in serum (mean ± SD = 73.6 ± 28.2 ng/mL) of 2/39 (5%) of the non-pregnant women (n = 39) studied. Surprisingly, AMPA was not found in the two glyphosate-positive non-pregnant women's samples; the authors suggest that this might be for technical reasons or because AMPA is rapidly cleared.

A very misleading study in rats showed that glyphosate is teratogenic at huge maternal doses (500 – 1,000 mg/kg/d) (Dallegrave *et al.*, 2003); this is likely to be due to maternal toxicity causing retardation of fetal growth, and so has little or no relevance to potential effects of low-level glyphosate exposure to pregnant women. A follow-on rat study by Dallegrave *et al.* (2007) using lower (but still irrelevant to human exposure) maternal oral glyphosate doses showed that at doses ≥50 mg/kg body weight there was a dose-related reduction in sperm number in offspring 65 d *post-partum*, but that the values returned to normal by 140 days *post-partum*. In addition, serum testosterone decreased in a dose-dependent manner at glyphosate doses ≥50 mg/kg body weight on day 65 *post-partum*, but this had only partly recovered on day 140 *post-partum*. Even though the doses used are irrelevant to human exposure, the effects on sperm number and serum testosterone point to an estrogenic response. Unfortunately, Dallegrave and her colleagues used Roundup® throughout their studies (Dallegrave *et al.*, 2003; Dallegrave *et al.*, 2007) and so POEA was co-administered to the rats. However, Mesnage *et al.* (2017) convincingly proved that POEA is not estrogenic and so the Dallegrave *et al.* (2007) findings are very likely due to glyphosate.

Current use of Roundup® in New Zealand and its impacts on human health, livestock, and ecosystems

Our knowledge about the chemistry and toxicology of glyphosate has increased considerably since it was licensed 47-years ago. We now know that its short soil $t_{½}$ might be partly due to its binding to charged soil components and that it might be released at a later time. In addition, there is mounting evidence that glyphosate is estrogenic, albeit with low relative (to E2) estrogenicity. The implications of this to environmental impact and human health have hardly been discussed because of the conjecture about the initial studies indicating estrogenicity. One thing is certain, glyphosate as an environmental estrogen will have effects at exposure concentrations far below those associated with its conventional toxicology.

Our understanding of glyphosate's short-term toxicity to humans, animals and ecosystems has not changed; glyphosate remains of little toxicological concern following acute exposure, except at very high doses. It is rapidly broken down by soil bacteria and metabolised and excreted in animals (including humans), which means that short-term environmental contamination or human exposure is unlikely to lead to long-term adverse effects.

Glyphosate's long-term impact is very much less well understood. Its estrogenic effects are likely only to be manifest following long-term exposure. As its use patterns have changed over the years, and its use has increased considerably on account of its importance in agriculture, it is appearing as residues in crops and food. In most cases, residue levels are low, but the effects of multiple exposures to multiple residues in multiple foods on human health have not been adequately assessed. This has particular resonance in the context of estrogenicity, which can cause subtle changes in growth and development following very long-term, very low-level exposures.

An issue that appears not to have been considered is the effect of stock grazing glyphosate-sprayed paddocks. It is possible that this route of exposure results in high glyphosate doses because there is no statutory grazing withdrawal time. High doses of estrogenic compounds can have effects on reproductive health, which might affect ovulation, sperm health and thus fecundity. This could have economic impacts in an agricultural setting. Similarly wild animals (e.g., birds) foraging in recently glyphosate sprayed will be exposed to high levels of the chemical which could affect their ovulation cycles and thus fecundity.

It is important that we do not simply accept the longstanding dogma that glyphosate is safe; we must question this, consider our increasing understanding of glyphosate's interactions with biological systems, explore its long-term effects, and modify our use of this vitally important agrochemical accordingly. As Jacob Bronowski noted in the *Ascent of Man*, They [We] are here not to worship what is known, but to question it… (Bronowski, 1973).

Acknowledgements

I thank David Zehms for collating the references, reading and correcting the manuscript, Michael Hutton for his comments on the manuscript, and Professor Richard Hartshorn for a helpful discussion on glyphosate-metal chelation.

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