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Accepted Article

Title The rise of glyphosate and new opportunities for biosentinel early-warning studies

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Abstract

Glyphosate has become the most commonly used herbicide worldwide, with a reputation of being environmentally benign, non-toxic and safe to wildlife and humans. However, studies have indicated its toxicity has been underestimated, and that its persistence in the environment is greater than once thought. Its actions as a neurotoxin and endocrine disruptor indicate its potential to act in similar ways to persistent organic pollutants (POPs) such as the organochlorine (OC) chemicals dichlorodiphenyltrichloroethane (DDT) and dioxin. Exposure to glyphosate and glyphosate-based herbicides for both wildlife and people is likely to be chronic and at sub-lethal levels, with multiple and ongoing exposure events in both urban and agricultural landscapes. Despite this, little research attention has been given to the impact of glyphosate on wildlife populations, and existing studies appear in the agricultural, toxicology and water chemistry literature that may have limited visibility among wildlife biologists. There is a strong case for the recognition of glyphosate as an 'emerging organic contaminant' and significant potential exists for collaborative research between ecologists, toxicologists and chemists to quantify the impact of glyphosate on wildlife and to evaluate the role of biosentinel

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species in a preemptive move to mitigate downstream impacts on people. Success will depend on the development of new and novel non-destructive sampling and analysis methodologies as *ex post facto* identification of toxins at lethal levels has limited utility. Concentrating on the chemistry and toxicity of glyphosate, we have examined the published literature to evaluate the extent to which glyphosate based herbicides can cause toxic effects in wildlife and people and the implications of chronic exposure. We then explore the idea of using birds as bioindicators of glyphosate toxicity. We hope this may encourage future research into the uptake and impact of organophosphate chemicals in target species, particularly traditional indicator species such as birds.

Introduction

Birds have long been used as indicators of environmental change, and have been particularly useful as indicators of the presence of toxic pollutants in a range of systems (reviewed in: Becker 2003; Carson 1962; Colborn et al. 1996; Green & Elmberg 2014). Typically these have included persistent organic pollutants (POPs) such as organochlorine (OC) chemicals and heavy metals, and there is a sizeable literature on pesticide exposure.

The insidious and disruptive effects of these chemicals were brought to light in Rachel Carson's work, *Silent Spring*, in 1962, in which it was observed that the endocrine and reproductive systems of wildlife were adversely affected by chemicals such as DDT (Carson 1962). The symptoms displayed in wildlife were recognized as early warning signs for endocrine and reproductive disruption in humans (Carson 1962). Subsequent worldwide regulation led to a decline in use of organochlorine (OC) based POPs such as DDT, chlordane, dieldrin, dioxin and heptachlor (Godduhn & Duffy 2003), but also a rise in the use of another group of less persistent, but highly toxic, organic pollutants known as organophosphates (OP; Walker 2014).

OPs are considered less persistent than OCs (Walker 2014), however the assumption that persistence and toxicity are equivalent should be viewed cautiously. OP chemicals were originally developed during the Second World War as nerve gases and neurotoxins, and have since been manufactured into insecticides, pesticides and herbicides (Walker 2014; Walker et al. 2012; World Health Organization 1986). Glyphosate is currently the most common and widely used OP herbicide globally(Kolpin et al. 2006; Mañas et al. 2009; Pérez et al. 2011). Although it may not display the same bioaccumulation potential of many OCs, it was recently named by the International Agency for Research on Cancer (IARC) as a probable Class 2A human carcinogen (International Agency for Research on Cancer 2015), and has been banned or restricted in a number of countries due to its potential carcinogenicity and ability to act as an endocrine disruptor, at environmentally relevant and sub-lethal levels (Gasnier et al. 2009; Thongprakaisang et al. 2013). This is not reflected in current material safety data sheets

(MSDSs). A recent review by Myers et al. (2016) has called for a formal reassessment of its toxicity by government regulators worldwide in light of the fact that current usage guidelines are based on outdated science.

Limited attention has been given to glyphosate versus other pesticide chemicals in the ecological literature. Some of this may be due to a lack of clarity about what a pesticide is. The term 'pesticide' covers herbicides, insecticides, fungicides and rodenticides (World Health Organisation 2016), and in effect is any compound that is used to control a pest species. Glyphosate is most commonly recognized as a 'herbicide' and may not immediately be recognized as a 'pesticide' which is often associated with insecticide use. From a human health perspective this may be problematic as people may not heed use warnings that they think may only apply to insecticides, and may also have led to less research attention among wildlife biologists, many of whom are still concentrating on legacy pollutants such as DDT (e.g., Hammer et al. 2016).

Glyphosate-based herbicides (GBHs) of which Roundup is likely the most well-known, have become household names. They are used extensively in agricultural and horticultural industries and have become the chemical of choice for weed control in the home garden and other urban and peri-urban settings by local governments for weed control on verges, in parklands and around schools. Although GBHs, are often marketed as environmentally friendly and readily bio-degradable (Duke & Powles 2008; Monsanto Company 2015), single applications have the potential to persist in the environment up to 197 days (Giesy et al. 2000). Additionally, aminomethyl phosphoric acid (AMPA), the main metabolite of glyphosate (Fig. 1), has been found to persist up to 240 days, and has frequently been detected in streams and water bodies (Benachour & Séralini 2009; Mañas et al. 2009). Once in the environment, the biodegradation and sedimentation (binding to soil) of glyphosate is said to be between 4 to 11 days post application (Pérez et al. 2011), but following spraying and rainfall may be detected in runoff waters 4 months post application (Edwards et al. 1980).

This bioavailability to wildlife, particularly birds, amphibians and fish, which are known to absorb toxic chemicals through ingestion and dermal contact, raises significant concerns (Braz-Mota et al. 2015; Driver et al. 1991), but also offers a potential opportunity to use these species as markers of environmental contamination. In particular, many bird species bridge the gap between terrestrial, marine and freshwater systems, have large spatial distributions, and high vagility thereby maximizing their potential for exposure and to function as biosentinels.

Exposure to glyphosate for both wildlife and people is likely to be chronic and at sub-lethal levels, with multiple and ongoing exposure events particularly in urban and agricultural settings. Yet, safety data sheets describe toxicity in terms of acute exposure, with little knowledge or data on the effects or implications of chronic exposure (Colborn et al. 1996). We were unable to find set exposure standards for glyphosate (Monsanto Company 2002-2015; Scotts Australia 2012; Sinochem Australia 2013), but a mounting number of studies in wildlife and humans indicate that there are concerning effects with even sub-lethal exposure concentrations (Benachour & Séralini 2009; Braz-Mota et al. 2015; Gasnier et al. 2009; Oliveira et al. 2007; Osten et al. 2005; Thongprakaisang et al. 2013).

It is these long-term effects of frequent, repeated and ongoing exposure to glyphosate that we are concerned with here. Concentrating on the chemistry and toxicity of glyphosate, we have examined the published literature to evaluate the extent to which glyphosate and GBHs are likely to cause toxic effects in wildlife and the implications this can have for chronic exposure. Following this we explore the idea of using birds as bioindicators for glyphosate toxicity. We hope this may encourage future research into the uptake and impact of OP chemicals in target species, particularly traditional indicator species such as birds.

The rise of glyphosate

Glyphosate (N-(phosphonomethyl)-glycine), is an OP compound belonging to the phosphonoglycine group of OP herbicides (Pérez et al. 2011). It is the active ingredient in over 750 different herbicide formulations (Mesnage et al. 2015) and is available for purchase by industry but also readily available on the shelves of hardware stores and supermarkets. It is used extensively for the control of annual and perennial weeds on crops such as cotton, canola, sugarcane, soybeans and chickpeas, and also as a dessicant in some crops preharvest to kill the plant (Mesnage et al. 2015; Samsel & Seneff 2013a). In South America it is used extensively in the control and eradication of illegal coca and poppy crops which has important conservation implications as plantations are in remote areas close to or within the Andean Biodiversity Hotspot (Solomon et al. 2005). Here there is also strong social, political and economic concern due to cross-boundary contamination between countries through aerosol drift from aerial spraying (Paz-y-Miño et al. 2007).

The introduction of genetically modified (GM) crops has led to dramatic increases in glyphosate use. Most GM crops, referred to as glyphosate-resistant, have been genetically engineered to withstand the large doses of glyphosate to control weeds (Duke & Powles 2008). Of concern is that GM crops have been reported to not effectively metabolize or excrete glyphosate, leading to accumulation within that plant or crop (Arregui et al. 2004; Mesnage et al. 2015). A recent study contrasting life-cycle differences between *Daphnia magna* fed ‘Roundup-Ready’ soybean, conventional soybean, or organic soybean meal found that *D. magna* fed ‘Roundup-Ready’ soybean performed less well than the other two treatments which was attributed to herbicide residues in the soybean meal (Benbrook 2016). Over 80% of all GM crops are now glyphosate-resistant, including 90% of all soybean and 70% of all canola produced in the United States (Duke & Powles 2008; James 2015). This potential overuse of glyphosate has also driven rapid genetic resistance within weed species (Heap 1993-2016), and a study by Kuester et al. (2015)

using both adaptive and neutral genetic markers showed that in the agro-weed species they investigated, resistance was not consistent within or between regions across its North American range. From a management perspective this is extremely challenging as some weed populations showed strong post-herbicide survival while others showed high mortality. Such inconsistency in effect will clearly lead to significant and potentially ineffectual applications of GBHs within agro-ecosystems, increasing both financial and toxic burden.

For many years, the general assumption that glyphosate only acted on metabolic pathways in plants contributed strongly to the increase and widespread nature of its use. The herbicidal action of glyphosate is attributed to its interruption of the Shikimate pathway responsible for the biosynthesis of essential aromatic amino acidsresulting in the disruption of protein synthesis and eventual plant death (Mesnage et al. 2015; Pérez et al. 2011). The Shikimate pathway is not present in humans, but is present in the bacteria living inside human intestines (Ratia et al. 2014; Samsel & Seneff 2013a). It has been shown empirically that glyphosate induced imbalances in gut bacteria can be linked to the development of many different chronic diseases including: celiac disease, heart disease, cancer and Alzheimer's disease (Samsel & Seneff 2013a, b; Seneff et al. 2015). In addition, recent studies in wildlife and humans note effects on tissues and cells (Braz-Mota et al. 2015; Gasnier et al. 2009; Ma & Li 2015; Thongprakaisang et al. 2013), with a growing list of chronic effects including: neurotoxicity, endocrine disruption, cell damage and immune-suppression (Benachour & Séralini 2009; Gasnier et al. 2009; Lajmanovich et al. 2015; Osten et al. 2005).

These effects are often insidious, causing multiple disruptions and disturbances (Colborn et al. 1996; Gupta 2011). Neurophysiological, reproductive and behavioral systems do not work in isolation, but rather influence each other through the action of their specific regulators manifesting in many different symptoms (Norris & Carr 2005). What is unclear in the existing literature is at what dosage these endocrine system impacts are expressed, or what exposure frequencies or intensities trigger health effects. For example, the daily acceptable limit of glyphosate intake ranges from 0.3-1.75mg/kg of body weight in Europe and the USA (Benachour & Séralini 2009; Myers et al. 2016). However, effects of GBH mixtures are noted at levels even lower than 0.3mg/Kg (Benachour & Séralini 2009; Gasnier et al. 2009; Menendez-Helman et al. 2012; Richard et al. 2005).

Neurotoxicity and Endocrine disruption

The most well-known mode of action of most OPs is the inhibition of the enzyme acetlycholinesterase (AChE), making them powerful neurotoxins. By inhibiting AChE, OPs cause a build-up of neurotransmitter acetylcholine (ACh), which is essential for the transmission of nerve impulses. Large amounts of ACh cause a continual production of nerve signaling, eventually leading to muscle spasms and ultimately respiratory failure (Landis et al. 2010; Walker 2014), an outcome associated with the acute toxicity of OP chemicals (Gupta 2011; Walker 2014; Walker et al. 2012).

Though not historically reported in connection with anti-acetlycholinesterase activity, glyphosate exposure has been linked to neurotoxic disruption of brain function and amino acid synthesis (Braz-Mota et al. 2015; El-Demerdash et al. 2001; Menendez-Helman et al. 2012; Osten et al. 2005). In particular the inhibition of enzyme AChE, has been reported in studies conducted on fish (Braz-Mota et al. 2015; Pérez et al. 2007) and also in wild ducks exposed to glyphosate sprayed on rice crops in Mexico (Osten et al. 2005). Similarly, endocrine disruption in human cells exposed to glyphosate and GBHs at sub-lethal levels has been observed, as has disruption to androgen and estrogen receptors (Gasnier et al. 2009), with some studies observing the promotion of human breast cancer cells and disturbances in human placental and embryonic cells (Gasnier et al. 2009; Mesnage et al. 2015; Thongprakaisang et al. 2013).

The multiplier effect

Glyphosate is rarely used in isolation, and is usually part of a mixture (GBH) containing other additives such as surfactants. Additives amplify the toxic actions of glyphosate, and can have multiple toxic actions individually (Benachour & Séralini 2009; Gasnier et al. 2009; Mesnage et al. 2015; Pérez et al. 2011). Some of the toxic effects noted at sub-lethal and sub-agricultural concentrations include: enzyme inhibition, endocrine disruption, DNA fragmentation and cell death (Benachour & Séralini 2009; Gasnier et al. 2009; Menendez-Helman et al. 2012; Richard et al. 2005).

Additives are generally kept as trade secrets and are usually only listed as ‘surfactants’ on labels and material safety data sheets (e.g., Monsanto Company 2002-2015; Scotts Australia 2012; Sinochem Australia 2013). They are thought to be inert ingredients of little toxic significance however, starting at sub-lethal concentrations of 1 ppm, the main additive of Roundup formulations, polyethoxylated tallowamine (POEA), has been found to cause disruptions in enzyme functioning and the rupturing of cell membranes (Benachour & Séralini 2009). POEA can make up as much as 30% of the total composition of Roundup formulations (Sinochem Australia 2013), it also enhances the uptake of glyphosate by cells hence increasing its actions (Benachour & Séralini 2009; Mesnage et al. 2015).

Prevalence in the environment

It is generally assumed that glyphosate does not persist in the environment due to sedimentation and breakdown by microorganisms (Battaglin et al. 2005). However, residues of glyphosate, and its main metabolite AMPA, have been found in both food and water sources, indicating the potential risks for frequent, long-term exposure (Kwiatkowska et al. 2013; Landry et al. 2005; Mañas et al. 2009; Mesnage et al. 2015). High water solubility has guaranteed its distribution in aquatic ecosystems, where its toxic impact has been seen in fish, insects and birds (Braz-Mota et al. 2015; Menendez-Helman et al. 2012; Oliveira et al. 2007; Pérez et al. 2011).

The general limits for other pesticides in edible plants are around 0.01 ppb-0.1 ppb, while for glyphosate it is considered acceptable at 400 ppm (Gasnier et al. 2009; Mesnage et al. 2015). The toxic effects of Roundup on human liver cells have been observed at 5 ppm, with endocrine disrupting effects noted at 0.5 ppm (Gasnier et al. 2009); minimally this describes effects at concentrations 800 times lower than the 'acceptable levels' mentioned above. Glyphosate residues have also been detected in the urine of farmers and their families post application (Acquavella et al. 2004), indicating that its mobility and reach may be greater than previously suggested. There is little information relating to the toxicity of AMPA, however its mutagenic effects on white blood cells (lymphocytes) in humans have been observed (Mañas et al. 2009), highlighting the need for further research.

Additionally, the agricultural and horticultural practice of adding inorganic phosphorous to soils potentially releases glyphosate from soil particles as both chemicals compete for the same absorption site (Battaglin et al. 2005; Borggaard & Gimsing 2008; Pérez et al. 2011). So, although it is not persistent in the same way that some other organic pollutants are, the frequent and continuous applications of glyphosate in both rural and urban settings make its presence ubiquitous (Mesnage et al. 2015).

Glyphosate as an emerging contaminant

There is a strong case for global recognition of glyphosate and GBHs as 'emerging organic contaminants' (EOCs). EOCs are pollutants that are as yet unregulated and accordingly do not require regular monitoring or reporting (Deblonde et al. 2011). These compounds are of increasing concern due to their known or unexplored potential to negatively affect wildlife and people. These are generally identified as: flame retardants (e.g., PBDEs) used in textiles, foams, plastics, and insulation (Hooper & McDonald 2000; Palm et al. 2002); perfluoroalkyl substances (PFASs) (formally PFCs; Jensen & Leffers 2008) used in the manufacture of paint, furniture, clothing, textiles and carpets; and, pharmaceuticals and phthalates (Campbell et al. 2006; Petrovic et al. 2004; Wilson et al. 2001). Several recent studies have also included pesticides, and list glyphosate levels as concerning, particularly in urban landscapes (Meffe & de Bustamante 2014; Stewart et al. 2014). Meffe and de Bustamante (2014) who conducted a study on 161 EOCs in surface and ground water in Italy found AMPA concentrations orders of magnitude higher than other compounds tested and far above EU quality standards.

A significant issue with EOCs such as glyphosate is that effective monitoring tools may not yet be available, and that current reporting occurs in government and industry documents, or in highly specialized journals that receive little visibility among ecological, wildlife or avian researchers. For example, among the 102 unique journal titles consulted in this manuscript, less than 4% had the term 'ecol*' in the title, less than 2% had 'conservation' in the title, only 1 journal contained the word 'bird'. None contained the word 'wildlife'. The word 'environ*' occurred in 33% of journals, but most journals were aimed at agricultural, toxicological or water chemistry audiences. Very few individual journal articles targeted pesticide contamination in birds and only two addressed organophosphates and bird poisoning (Osten et al. 2005; Santos et al. 2016). Significant potential exists for collaborative research between ecologists, toxicologists and chemists to quantify the impact of glyphosate on wildlife, particularly in investigating the role of birds as biosentinel species in a preemptive move to mitigate downstream impacts on people, and to communicate this information to a wider wildlife ecology audience.

The potential for birds as bioindicators of glyphosate toxicity

Birds are exposed to point and non-point pollution through numerous pathways (Burger & Gochfeld 2002). Accordingly their potential for exposure to glyphosate is high, given its application and known presence in wetland (Pérez et al. 2007), agricultural (Osten et al. 2005), and urban environments (Stewart et al. 2014), which contribute contaminant runoff to coastal and marine systems (Burger & Gochfeld 2002). In particular, wetland birds may be excellent target species as their biology and ecological roles in the functioning of wetland ecosystems is well understood (Becker 2003; Green & Elmberg 2014; Ogden et al. 2014), and glyphosate residues, and AMPA, are known to be present (Battaglin et al. 2005; Mañas et al. 2009; Menendez-Helman et al. 2012).

Birds may be exposed to glyphosate though inhalation, ingestion and dermal contact, and interestingly, may take longer to recover from dermal exposure than from ingestion or inhalation (Driver et al. 1991; Vyas et al. 2006). As technology has advanced, the manner in which pesticides and other toxic compounds can be assessed has changed. Formerly, avian sampling methods for OC and heavy metal contamination have been linked to mortality events or destructive sampling including the analysis of feathers, eggs, tissues, blood and fat samples (Auman et al. 1997; Becker 2003; Jagannath et al. 2008; Rivera-Rodríguez et al. 2007), combined with data relating to changes in abundance, distribution and migratory patterns (Becker 2003; Leat et al. 2013). Some research has looked at the effects of secondary pharmaceutical drug ingestion by avian scavengers (e.g., Lemus et al. 2009), and OP insecticide ingestion from agricultural crop spraying under simulated laboratory conditions (Driver et al. 1991). However, ex *post facto* identification of a toxin at lethal levels has limited utility, particularly in the case of endangered species, or where birds are being used as an early warning mechanism, as clearly it is preferable to identify contamination prior to mortality.

Relatively recently, a promising strategy was proposed to use nestling feathers from top predator raptor nestlings to monitor persistent organic pollutants (POPs) in wild populations (Eulaers et al. 2011a). Other studies have looked at the efficacy of other non-destructive materials such as blood and preen oil gland secretions to estimate POP load (Eulaers et al. 2011b), and blood biomarkers have been used in at least one other wetland species as a useful predictor of health and ecological risk following glyphosate exposure (Lajmanovich et al. 2015). However, not all life history traits in birds make them ideal study targets. Both migratory behavior or moult chronology may result in false positive or negative results if sampling is conducted at the wrong time of year. A migrant individual with large toxic load may indicate contamination from its source population, not the study site. Similarly, sampling recently grown feathers may tell you little about local contamination in a resident bird, so care must be taken with study design.

We found no indication in the literature of the best methods for detection of glyphosate toxicity in birds, suggesting a strong need for further research in this area. New and novel non-destructive sampling and analysis methodologies are required. Notably, birds along with other lower vertebrates have nucleated blood cells opening the way for the use of non-destructive blood sampling for genotoxic studies including epigenetics, or metabolomic approaches.

Conclusion

For over 40 years glyphosate has been flying under the radar of regulatory bodies and has been championed with the title of a safe, environmentally benign herbicide (Duke & Powles 2008; Monsanto Company 2015). However, many questions remain about the human health implications and effects following chronic exposure to sub-lethal levels of OP chemicals, glyphosate in particular (Gasnier et al. 2009; Ma & Li 2015; Mesnage et al. 2015; Pérez et al. 2011). A lack of agreement about the potential and measured impacts of glyphosate mirrors concern expressed about OC chemicals such as DDT and dioxin which are now banned outright in many parts of the world, or are regulated through the Stockholm Convention on Persistent Organic Pollutants (Porta & Zumeta 2002). Considering the rise in glyphosate due to the introduction of GMOs and glyphosate resistant crops, it seems necessary that more research be done on the effects of exposure, particularly at sub-lethal levels. There is significant potential for wildlife ecologists to contribute to this using appropriate target biomonitor species. In particular scope exists to develop a Decision Framework to aid the choice of biomonitor species and analysis methodology based on the target contaminant, spatial and temporal extent of contamination, and perceived risk. As proven ndicators of environmental toxicity (Becker 2003; Leat et al. 2013; Ogden et al. 2014), birds may well hold the key to some of the unanswered questions relating to chronic exposure of glyphosate.

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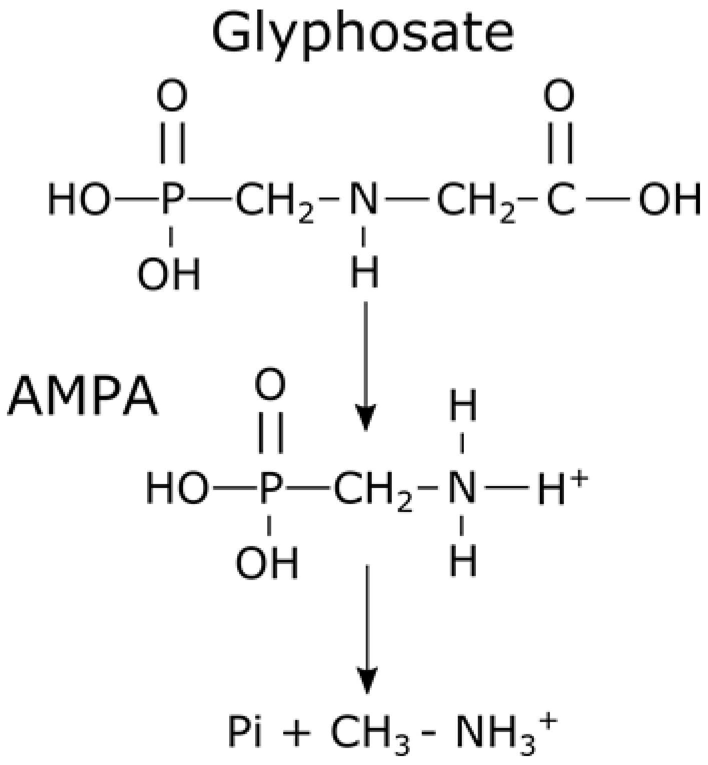

Fig 1. Chemical structure of glyphosate and the main metabolite AMPA (aminomethyl phosphoric acid) with breakdown products (Adapted from Solomon et al. 2005).