

Impact of glyphosate and glyphosate-based herbicides on the freshwater environment

Robert Annett^a, Hamid R. Habibi^b and Alice Hontela^{a*}

ABSTRACT: Glyphosate [*N*-(phosphonomethyl) glycine] is a broad spectrum, post emergent herbicide and is among the most widely used agricultural chemicals globally. Initially developed to control the growth of weed species in agriculture, this herbicide also plays an important role in both modern silviculture and domestic weed control. The creation of glyphosate tolerant crop species has significantly increased the demand and use of this herbicide and has also increased the risk of exposure to non-target species. Commercially available glyphosate-based herbicides are comprised of multiple, often proprietary, constituents, each with a unique level of toxicity. Surfactants used to increase herbicide efficacy have been identified in some studies as the chemicals responsible for toxicity of glyphosate-based herbicides to non-target species, yet they are often difficult to chemically identify. Most glyphosate-based herbicides are not approved for use in the aquatic environment; however, measurable quantities of the active ingredient and surfactants are detected in surface waters, giving them the potential to alter the physiology of aquatic organisms. Acute toxicity is highly species dependant across all taxa, with toxicity depending on the timing, magnitude, and route of exposure. The toxicity of glyphosate to amphibians has been a major focus of recent research, which has suggested increased sensitivity compared with other vertebrates due to their life history traits and reliance on both the aquatic and terrestrial environments. This review is designed to update previous reviews of glyphosate-based herbicide toxicity, with a focus on recent studies of the aquatic toxicity of this class of chemicals. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: glyphosate; freshwater; review; herbicide; aquatic

Background

Glyphosate-based herbicides are currently among the most widely used agricultural chemicals globally. Sold under the trade name Roundup® (RU), glyphosate-based herbicides were the 17th most commonly used pesticide in the late 1980s, in terms of active ingredient applied, but by 2002 it was the most commonly used herbicide in the United States (Gianessi and Reigner, 2006). The impact of this single class of herbicides on modern agriculture practices is undeniable (Gilbert, 2013); the combination of the broad-spectrum herbicidal nature of glyphosate and the development of resistant crop varieties has elevated glyphosate-based herbicides to among the most important agricultural chemicals ever.

Human health and environmental risk assessments have been conducted on glyphosate by both academic researchers (Giesy *et al.*, 2000; Solomon and Thompson, 2003; Williams *et al.*, 2000) and regulatory agencies (USEPA, 1993; WHO, 1994). Currently, the USEPA classifies glyphosate formulations as low or non-toxic to birds and mammals, with designation as practically non-toxic to moderately toxic to aquatic invertebrates. Toxicity to amphibians is classified as slightly to moderately toxic (Giesy *et al.*, 2000). There appears to be a discrepancy when the toxicity of the active ingredient, glyphosate, is compared with the toxicity of the commercial products and their constituents (Folmar *et al.*, 1979). Surfactants are added to commercial formulas to improve efficacy by increasing herbicide adhesion to the leaf surface, as well as aiding transport across the waxy cuticle membrane and into the plant. A variety of surfactant options exist, however, the most common class of surfactants used in commercial glyphosate-based herbicide formulas have traditionally been polyethoxylated amines (POEA).

The differences in toxicity of glyphosate, RU and POEA were first identified by Folmar *et al.* (1979) who compared the toxicity of technical grade glyphosate, the isopropylamine salt of glyphosate, the surfactant POEA and the commercially available glyphosate herbicide, RU. The study provided information on the sensitivities of several species of aquatic organisms, ranging from aquatic invertebrates to teleost fish, to RU and its constituents. The surfactant in the RU was suggested to be a key factor in toxicity.

A combination of market pressure for specialized RU formulations, the introduction of glyphosate tolerant Roundup-Ready® crops, and the expiration of Monsanto's RU patent in the United States has led to a wide variety of glyphosate-based herbicide formulations being currently available (Duke and Powles, 2008). Common to each of these formulations is the use of glyphosate as the active ingredient though the glyphosate concentration, surfactant identity and concentration, and even the presence or absence of surfactant varies among formulations (Howe *et al.*, 2004). With such a variety of herbicide formulations and their extensive use, there is a need to re-evaluate

*Correspondence to: Alice Hontela, Department of Biological Sciences, Water Institute for Sustainable Environments (WISE), University of Lethbridge, 4401 University Dr., Lethbridge, AB, Canada, T1K 3M4.
Email: alice.hontela@uleth.ca

^aDepartment of Biological Sciences, Water Institute for Sustainable Environments (WISE), University of Lethbridge, 4401 University Dr., Lethbridge, AB, Canada, T1K 3M4

^bDepartment of Biological Sciences, University of Calgary, 2500 University Dr., N.W. Calgary, AB, Canada, T2N 1N4

the exposures and toxicity of glyphosate-based herbicides in the aquatic environment.

The majority of glyphosate-based herbicides are not approved for application in aquatic environments; however, with the current widespread use there are multiple routes through which exposure of aquatic organisms may occur. Surface runoff, direct overspray or drift during herbicide application can result in significant quantities of glyphosate entering the aquatic environments (Solomon and Thompson, 2003). Unlike the majority of other common agricultural herbicides, which are primarily used only for agriculture and silviculture, glyphosate-based herbicides are also popular for domestic use on lawns and gardens. The application, by untrained individuals without proper precautions for safe herbicide applications, may also contribute to surface and groundwater contamination (Hanke *et al.*, 2010).

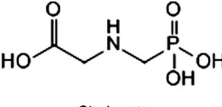
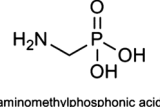
Several previous studies have characterized the effects of individual glyphosate-based herbicide formulations in a wide variety of aquatic organisms. Taxa including microorganisms (Bonnet *et al.*, 2007; Folmar *et al.*, 1979; Tsui and Chu, 2003), invertebrates (Perez *et al.*, 2007; Trumbo, 2005), amphibians (Moore *et al.*, 2012; Relyea and Jones, 2009; Thompson *et al.*, 2004), fish (Folmar *et al.*, 1979; Glusczak *et al.*, 2011; Hued *et al.*, 2012; Menezes *et al.*, 2011; Modesto and Martinez, 2010a), and birds (Oliveira *et al.*, 2007) have been investigated in the past and there is evidence for diverse physiological and behavioural effects depending on the dose and formulation. Previous review articles have focused on the ecological risk assessment for RU herbicide in terrestrial and aquatic environments (Giesy *et al.*, 2000), and exposure to aquatic organisms as a result of overwater use of glyphosate (Solomon and Thompson, 2003). However, with the unprecedented scale of the current use of glyphosate-based herbicides, combined with the development of new methods and tools in environmental toxicology, it has become necessary to reassess the safety and environmental impacts of this class of pesticides. The goal of this review is to present the current exposure data of glyphosate and glyphosate-based herbicides in the aquatic environment and to critically evaluate our current understanding of their effects in aquatic organisms.

Identity, Physical and Chemical Properties, Manufacture, Mode of Action

Glyphosate, (*N*-(phosphonomethyl) glycine; CAS no. 1071-83-6) (Table 1) was first identified to have herbicidal action in 1970 and four years later commercial formulas were released by Monsanto Corp. (St. Louis, MO, USA). The initial formula, RU, provided several advantages for agricultural weed control by acting as a broad range, non-selective and post emergent herbicide. The herbicidal action of this weak organic acid can be attributed to its ability to inhibit aromatic amino acid synthesis through the inhibition of 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS). This enzyme (Fig. 1) is responsible for the production of chorismate, necessary for biosynthesis of the amino acids phenylalanine, tyrosine and tryptophan (Amrhein *et al.*, 1980). Literature indicates that unlike other non-selective herbicides, glyphosate's mode of action targets a biosynthetic pathway only present in plants and some microorganisms, potentially minimizing toxicity to non-target animal species, although an inhibition of hepatic cytochrome P450 activity was observed in the rat (Hietanen *et al.*, 1983). The primary breakdown pathway of glyphosate in the environment is through microbial degradation by soil bacteria while an alternative breakdown pathway has also been identified, each producing a different set of end products (Fig. 2). Breakdown via the primary pathway results in the production of the main metabolite of glyphosate, AMPA (aminomethylphosphonic acid; CAS No. 1066-51-9) and glyoxylic acid. Further breakdown of these two metabolites creates carbon dioxide and an ammonium ion. The second breakdown pathway (Fig. 2) is less common, occurring only in specialized soil bacteria species which metabolize glyphosate first into inorganic phosphate and sarcosine, then further converting sarcosine to glycine (Dick and Quinn, 1995).

With solubility in water of 10,000–15,700 mg l⁻¹ at 25 °C, glyphosate readily dissolves and disperses in aquatic environments (Mackay *et al.*, 1997). However, glyphosate binds tightly to soil particles with a soil adsorption coefficient of 24,000 l kg⁻¹ (Wauchope *et al.*, 1992), suggesting there exists limited risk of

Table 1. Physical and chemical properties of glyphosate^a and its primary metabolite AMPA

Common name	Glyphosate	AMPA
IUPAC Name	<i>N</i> -(Phosphonomethyl)glycine	(Aminomethyl)phosphonic acid
Chemical formula (acid)	C ₃ H ₈ NO ₅ P	CH ₆ NO ₃ P
Chemical structure	 Glyphosate	 aminomethylphosphonic acid
CAS number	1071-83-6 (acid)	1066-51-9
Molecular weight	169.09 g mol ⁻¹	111.04 g mol ⁻¹
Physical state and color	Crystalline powder, white	Crystalline powder, white
Melting point	200° - 230 °C	120 °C
Water solubility	10,000 - 15,700 mg l ⁻¹ at 25 °C	
Octanol/water partition coefficient (log K _{OW})	-4.59 to -1.70	
Half life	7 - 142 days (in water)	76 -240 days (in soil)

^aModified from Giesy *et al.*, 2000

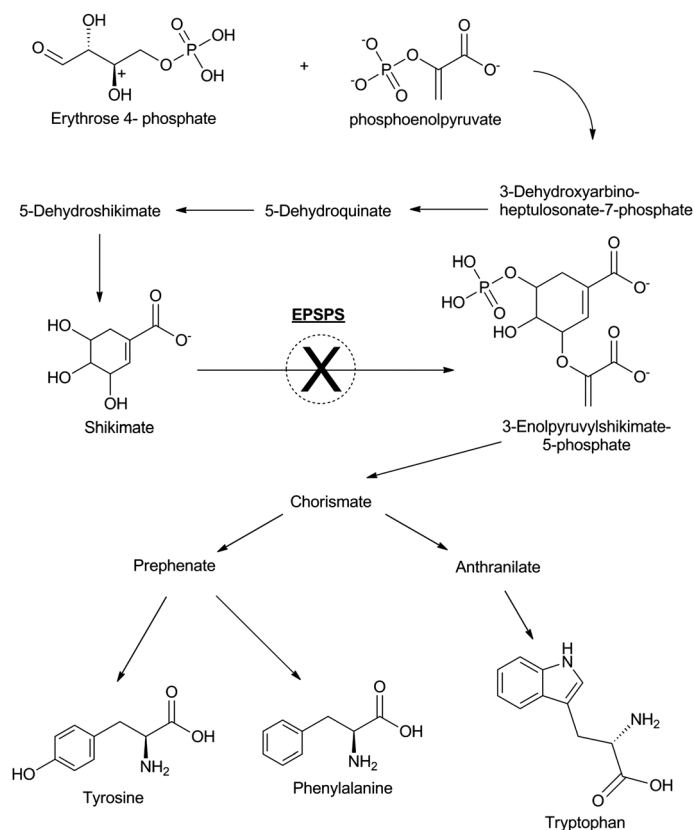


Figure 1. Biochemical mechanism of action and target for glyphosate in plants (adapted from Giesy *et al.*, 2000).

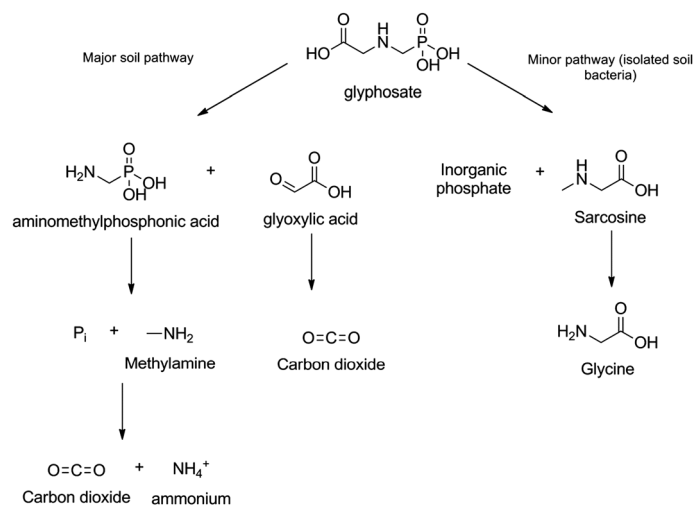


Figure 2. Primary and alternate breakdown pathway for glyphosate by soil bacteria.

contaminating surface and groundwater when applied as directed (Giesy *et al.*, 2000). Conversely, the solubility and mobility of glyphosate metabolites vary, for example the glyphosate metabolite AMPA shows mobility in soil significantly higher than pure glyphosate (Kjaer *et al.*, 2005).

Commercially available glyphosate-based herbicide formulations are complex mixtures in which glyphosate acts as the active ingredient. The addition of surfactants and other adjuvants is necessary to allow the active ingredient to penetrate the plant surface and translocate to the site of action

(Wang and Liu, 2007). The versatility of modern glyphosate-based herbicides can be attributed to the wide variety of formulations currently available, each tailored for a specific range of application conditions. Each formula contains a particular concentration of glyphosate with a particular adjuvant profile. Often the identity of these adjuvants remains proprietary, which can make attributing toxicity to individual components of glyphosate-based herbicides difficult. Toxicological research of these products is confounded by the vast array of unique commercially available formulas, a lack of consistency in reporting

the exact formula used, and the relative proportion of individual constituents contained therein. These inconsistencies in reporting can lead to incorrect herbicide applications and to an over or underestimation of toxicity. With large toxicity differences between individual formulations, it is critical that the complete name and description of the product being tested is included. A summary of some common glyphosate-based herbicides used in crop production, domestic weed control, and in research is included in Table 2, which illustrates only a portion of the wide assortment of formulations tested as well as the variety of units used to express relative concentration.

Glyphosate concentrations are often reported as relative proportion of active ingredient (mg a.i. l⁻¹), proportion of acid equivalents (mg a.e. l⁻¹), percent glyphosate, or mass of glyphosate applied per unit area (kg ha⁻¹). To understand the relationship between these reporting methods it is necessary to clarify how glyphosate-based herbicides are formulated.

In order to meet solubility requirements for commercial use, currently available glyphosate formulations contain salts of glyphosate as the active ingredient. The identities of these salts are variable and include isopropylamine, diammonium or potassium salt forms, among others. To easily compare results, exposure and application rates should be reported as acid equivalents, as this calculation considers the number of glyphosate molecules in a given volume of solution (Giesy *et al.*, 2000). The formula for calculating acid equivalent conversion factor is best expressed as:

$$\text{Acid Equivalent} = \frac{\text{molecular weight of the acid alone} - 1}{\text{molecular weight of the salt or ester form of the acid}} \times 100$$

In the case of Roundup Original®, the molecular weight of the glyphosate acid alone is 169.07 g mol⁻¹, whereas the molecular weight of the isopropylamine salt of glyphosate, found in the commercial formula, is the mass of the acid as well as the salt. (169.07 g mol⁻¹ + 59.11 g mol⁻¹ = 228.28 g mol⁻¹). Thus, the acid equivalent can be calculated as follows:

$$\begin{aligned} \text{Acid Equivalent} &= \frac{169.07 \text{ g mol}^{-1} - 1}{228.28 \text{ g mol}^{-1}} \\ \text{Acid Equivalent} &= 0.74 \end{aligned}$$

Conversion between active ingredient glyphosate and acid equivalents for the isopropylamine salt of glyphosate can simply be made by adjusting the concentration in terms of active ingredient per volume, by a factor of 0.74.

Detection Methods

Critical to quantitative exposure assessments of glyphosate-based herbicides in the aquatic environment are methods for accurately measuring concentrations of the herbicide, and its constituents, in environmental samples. Difficulties in extracting and quantifying these compounds are due to their inherent chemical properties, namely high water solubility, poor solubility in organic solvents and preference to form complexes (Stalikas and Konidari, 2001). In spite of these difficulties there exist several different methods for determining glyphosate, AMPA, and POEA concentrations in water. These methods can be divided into four general categories: chromatography

(GC/MS GC/MS/MS), high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), and capillary electrophoresis (CE). Traditionally, chromatographic techniques and HPLC have been the primary means of analysis. These methods have proven over decades of research to be consistent between facilities and highly accurate. Unfortunately, these techniques require specialized training, expensive equipment, the use of hazardous chemicals for sample derivatization, and often come at significant costs per sample. The high cost of running samples using these methods often limit their use in assessment of field and laboratory exposures, leading to diminished sample size or necessitating analyzing composite samples. ELISA has been shown to be a cost effective and accurate measure of glyphosate in surface waters with a minimal detection limit of 0.1 µg l⁻¹ (Byer *et al.*, 2008; Sanchis *et al.*, 2012), although their use for measuring environmental levels of glyphosate remains to be validated. When used in conjunction with chromatography and HPLC for confirmation, immunoassays offer a cost effective method to increase spatial and temporal sampling, and once sufficient evidence exists verifying the accuracy and precision of glyphosate ELISAs, their use will likely increase.

Uses of Glyphosate-Based Herbicides

Traditional Uses

Glyphosate was developed as a non-selective, broad range herbicide with strong herbicidal action. Though sensitivity to glyphosate varies between species, all naturally occurring higher plants have some susceptibility to glyphosate. This property limited the historical use of glyphosate to applications where all plants were to be removed, such as prior to seeding as chemical fallow, or on a small scale where targeted application was possible (Duke and Powles, 2008). In this traditional setting the likelihood of contaminating the aquatic environment is relatively low, as applications typically occurred less frequently than in modern operations. Introduction of minimal tillage techniques increased the demand for glyphosate as an important pre-emergent herbicide. The development of glyphosate tolerant crop varieties has exponentially increased the demand and use of glyphosate-based herbicides, and has revolutionized how and when this chemical is applied.

New Genetically Engineered Crops; Current use and Distribution of Glyphosate

A variety of methods have been utilized to develop herbicide tolerant crops; by far the most successful has been the development of genetically modified glyphosate resistant crop varieties utilizing glyphosate resistant EPSPS genes from either *Agrobacterium sp.* (CP4), *Ochrobactrum anthropi* (GOX), or site-directed mutagenesis of EPSPS, to create glyphosate resistant soybean (*Glycine max*), canola (*Brassica napus*) and maize (*Zea mays*), respectively (Bradshaw *et al.*, 1997; Duke and Powles, 2008). The first glyphosate resistant (GR) crop variety was deregulated in the United States in 1996 with the release of Roundup-Ready® soybean. The success of GR soybean led to the deregulation of an array of important crops, resistant to glyphosate-based herbicide exposure. Prior to the deregulation of these GR crop varieties the annual use of glyphosate was relatively low, with annual sales in the United States of

Table 2. Selected glyphosate formulations for domestic, agricultural use, and those used in published studies

Commercial Formulation	Reported Glyphosate Concentration	Surfactant	Salt of Glyphosate	Reference
Roundup Original [®]	360 g a.e. l ⁻¹ ^a 29.7% a.i. ^b 41.0% a.i. 48% a.i.	~15% POEA ^c 15% MON 0818 NR ^d NR	Isopropylamine	Oliveira <i>et al.</i> , 2007; Guilherme <i>et al.</i> , 2010 Fuentes <i>et al.</i> , 2011 Tsui and Chu, 2003 Jiraungkoorskul <i>et al.</i> , 2003; Salbego <i>et al.</i> , 2010; Menezes <i>et al.</i> , 2011; Gluszcak <i>et al.</i> , 2011; Cattaneo <i>et al.</i> , 2011
Roundup Original MAX [®]	480 g l ⁻¹	NR	Potassium	Relyea and Jones, 2009; Jones <i>et al.</i> , 2011
Roundup "Weed & Grass Killer"	48.7%	NR	Isopropylamine	Relyea, 2005b
Roundup Plus [®]	25.2% a.i.	POEA	Isopropylamine	Ortiz-Santaliestra <i>et al.</i> , 2011
Roundup WeatherMAX [®]	36.0% a.i. 48.8% (540 g a.e. l ⁻¹) 39.9%	8.50% NR NR	Isopropylamine Potassium	Dinehart <i>et al.</i> , 2010 Fuentes <i>et al.</i> , 2011
Vision [®]	356 g a.e. l ⁻¹	15% MON 0818	Isopropylamine	Wojtaszek <i>et al.</i> , 2004
Vision MAX [®]	540 g a.e. l ⁻¹	NR	Potassium	Edge <i>et al.</i> , 2013
Roundup Transorb [®]	360 g a.e. l ⁻¹	Blend ~15% POEA	Potassium	Howe <i>et al.</i> , 2004
Roundup 3Plus [®]	130 g a.i. l ⁻¹	NR	Isopropylamine	Marc <i>et al.</i> , 2005
Roundup MAX [®] Granular	74.7% a.i.	25.3% POEA	Ammonium	Hued <i>et al.</i> , 2012
Roundup Ultra-MAX [®]	50.2% a.i.	NR	Isopropylamine	Lajmanovich <i>et al.</i> , 2011
Glyphos Bio [®]	360 g a.e. l ⁻¹	NR	Isopropylamine	Howe <i>et al.</i> , 2004
Touchdown 480 [®]	360 g a.e. l ⁻¹	NR	Trimesium	Howe <i>et al.</i> , 2004
Infosato [®]	48% a.i.	NR	NR	Lajmanovich <i>et al.</i> , 2011
Glyfoglex [®]	48% a.i.	NR	NR	Lajmanovich <i>et al.</i> , 2011
ATANOR glyphosate [®]	48%	2.5% IMPACTO ^e	NR	Romero <i>et al.</i> , 2011

^aacid equivalents.

^bactive ingredient.

^cPolyethoxylated amines.

^dNot reported.

^ealkylaryl polyglycol ether surfactant.

approximately 10,000 Mg in 1992 (Coupe *et al.*, 2012). After the introduction and widespread adoption of GR crop varieties this number increased to 80,000 Mg by 2007. An average of 90% of the soybeans planted in the United States in 2007 were resistant to glyphosate while half the maize (50%) and almost three-quarters (72%) of the cotton (*Gossypium hirsutum*) grown during that same period were classified as GR (Givens *et al.*, 2009). In Canada, adoption of herbicide resistant canola has been reported to be as high as 98% in 2004, with GR varieties making up 48% of canola acres planted (Smyth *et al.*, 2011). The convenience and effectiveness of glyphosate based farming systems has allowed glyphosate to replace other herbicides in row crop production and offer several advantages over alternative methods of weed control. Glyphosate has allowed for a decrease in the overall number of herbicide applications required, facilitated an increase in conservation tillage, and decreased the demand for alternative herbicides with higher aquatic toxicity (Cerdeira and Duke, 2006; Gilbert, 2013). However, reliance on glyphosate as the primary method of weed control has led to issues of weed resistance resulting from poor weed management practices and an overall increase in the number of glyphosate applications yearly (Duke, 2012; Young, 2006). With such widespread adoption of GR technologies, the probability of surface water contamination has increased, thus, increasing the likelihood of exposure to aquatic organisms.

Overwater use

Glyphosate-based formulations containing surfactants are not currently approved for overwater use. This has been the case since it was shown that much of the toxicity to non-target organisms, conferred by RU, can be attributed to MON 0818, a polyoxyethylene tallowamine surfactant (Folmar *et al.*, 1979). Formulations exist which lack the surfactant portion, for example Monsanto's Rodeo® herbicide, though the addition of a surfactant is often still required to improve efficacy. Direct overwater application of glyphosate is of limited benefit as glyphosate is not effective against submerged vegetation, though use treating pond margins, wetlands, ditches, and in forestry may result in surface water contamination (Solomon and Thompson, 2003).

Environmental Fate

Occurrence in the Aquatic Environment

Glyphosate-based weed control can decrease the level of overall herbicide contamination in surface waters when compared with the use of alternative, longer half life herbicides (Shipitalo *et al.*, 2008). Nevertheless, with the use of glyphosate-based herbicides increasing to such a large degree, it is reasonable to assume that the detection of glyphosate, and its metabolites, in surface waters should also increase. Multiple routes exist for contamination of surface water by glyphosate; primarily through drift during application or as surface runoff following application (Borggaard and Gimsing, 2008). Predicted worst case scenarios for glyphosate concentrations in surface water have been reported to range from 1.7 to 5.2 mg a.e. l⁻¹, although environmental levels in this range are unlikely to occur except in incidents of accidental spills or direct overwater application (Giesy *et al.*, 2000; Glozier *et al.*, 2012). Studies designed to

determine environmental levels of glyphosate typically reported concentrations either below the limit of detection for the technology used or in the range of $\mu\text{g l}^{-1}$, orders of magnitude lower than the predicted worst case scenarios (Table 3).

Transport to and Occurrence in the Aquatic Environment

The contamination of surface and groundwater by glyphosate-based herbicides and glyphosate metabolites is possible via a number of different pathways classified as either diffuse or point sources. Point sources are the easiest to define and mitigate, as they most often correspond to hard surfaces or locations where chemical handling and application equipment, tanks, or pails are cleaned or stored (Carter, 2000). Mitigation for these types of point sources primarily involves proper handling education and the introduction of best management practices for handling all agricultural chemicals. More difficult to identify and mitigate, and by far the largest proportion of glyphosate contamination, arises from non-point or diffuse sources (Reichenberger *et al.*, 2007).

The chemical properties of glyphosate suggest that the likelihood of surface or groundwater contamination should be relatively low. Glyphosate alone has the potential to bind tightly to soil particles depending on pH, soil texture and phosphate levels (Sprankle *et al.*, 1975). The primary pathway for breakdown of glyphosate in the environment is microbial degradation by soil bacteria. Estimates for glyphosate half life range widely, from 1.7 to 142 days owing to a number of factors (Giesy *et al.*, 2000); primarily the important role that biological processes play in glyphosate degradation, but also because transport of glyphosate into surface waters is highly variable, depending on the level of soil particle adsorption. In turn, this binding can be highly variable based on soil chemistry and physical characteristics. For example, soil phosphate concentrations can greatly affect glyphosate's ability to adsorb, as both compete to bind the same surface sites on individual soil particles (Coupe *et al.*, 2012). The relationship between glyphosate half-life and soil properties is particularly important given the high affinity of glyphosate to bind both soil organic matter and the mineral fraction (Yu and Zhou, 2005). In contrast to some other herbicides, the sorption coefficient for glyphosate is independent of total organic carbon measured (Xu *et al.*, 2009). However, the surfactant POEA has also been shown to bind to sediments, but with a decrease of half life inversely proportional to total organic carbon (Wang *et al.*, 2005). Sediment glyphosate concentrations are directly influenced by proximity to application sites, a relationship that has been linked to rainfall events, responsible for the transport of glyphosate from the site of application to surface water via surface erosion from treated areas (Peruzzo *et al.*, 2008).

The wide range of half life values can be cause for concern when designing and interpreting studies intended to monitor occurrence of glyphosate in the environment. Sufficient sampling resolution must exist to provide confidence that spikes in glyphosate concentration following herbicide application or runoff events are neither missed nor over-represented. The presence or absence of glyphosate contamination in surface water is highly dependent on proximity, both spatially and temporally, to herbicide application (Thompson *et al.*, 2004) and concentrations immediately after application can be much

Table 3. Concentrations of glyphosate in various environmental compartments

Location	Compartment	Time of Collection	Concentration	Reference
Surface water				
USA				
Bogue Phalia, MS., USA	Freshwater stream	Oct 2006 – Nov 2008	0.03 – 73 µg l ⁻¹	Coupe <i>et al.</i> , 2012
South Fork Iowa River, IA, USA	Freshwater stream	Feb 2007 – Nov 2008	<0.02 – 5.7 µg l ⁻¹	Coupe <i>et al.</i> , 2012
Midwest, USA	Freshwater stream	May 2002 – Nov 2002	<0.10 – 8.7 µg l ⁻¹	Battaglin <i>et al.</i> , 2005
Mississippi River Basin (NASQAN) ^a	Various	2002	0.14 – 0.33 µg l ⁻¹	Scribner <i>et al.</i> , 2006
Various, USA (NAWQA) ^b	Various	2001 – 2006	0.03 – 9.7 µg l ⁻¹	Scribner <i>et al.</i> , 2006
Sugar Creek, IN.	Freshwater stream	May 2004 – Jun 2004	0.15 – 1.6 µg l ⁻¹	Coupe <i>et al.</i> , 2012
Various Locations	Overland flow	May 2004 – Jun 2004	22.0 – 430 µg l ⁻¹	Coupe <i>et al.</i> , 2012
	WWTP effluent ^c	Jul 2002 – Nov 2002	<0.10 – 2.2 µg l ⁻¹	Kolpin <i>et al.</i> , 2006
Canada				
Manitoba,	Pothole wetland	May 2008 – Sept 2008	0.10 – 0.60 µg l ⁻¹	Messing <i>et al.</i> , 2011
Ontario	Urban stream	Apr 2007 – Sept 2007	ND ^d – 12.0 µg l ⁻¹	Byer <i>et al.</i> , 2008
	Forest Wetlands	July 1999 – Sept 2001	<0.01 – 1.95 mg l ⁻¹	Thompson <i>et al.</i> , 2004
British Columbia	Freshwater stream	Apr 2007 – Sept 2007	<0.02 – 303 µg l ⁻¹	Tierney <i>et al.</i> , 2011
Various locations	Urban rivers and streams	Spring, summer, fall 2007	<10 – 400 ng l ⁻¹	Glozier <i>et al.</i> , 2012
Europe				
Rouffach, France	Freshwater stream	Mar – Sept; 2003 – 2005	<0.1 – 86 µg l ⁻¹	Coupe <i>et al.</i> , 2012
South America				
Pampa Ondulada Bonaerense, Argentina	Freshwater stream	Nov 2003 – Sept 2004	0.1 – 0.70 mg l ⁻¹	Peruzzo <i>et al.</i> , 2008
Drains				
USA				
South Fork Iowa River, IA.	Subsurface drain	Feb 2007 – Nov 2008	<0.02 – 290 µg l ⁻¹	Coupe <i>et al.</i> , 2012
Sugar Creek, IN.	Agricultural drainage ditch	May 2004 – Jun 2004	0.16 – 47 µg l ⁻¹	Coupe <i>et al.</i> , 2012
Soil / Sediments				
South America				
Pampa Ondulada Bonaerense, Argentina	Surface stream sediments	Nov 2003 – Sept 2004	<0.10 – 1.85 mg kg ⁻¹	Peruzzo <i>et al.</i> , 2008
Europe				
Catalonia, Spain	Groundwater	May 2007 – Sept 2010	<0.075 µg l ⁻¹ – 2.5 µg l ⁻¹	Sanchis <i>et al.</i> , 2012
^a National Stream Quality Accounting Network Program				
^b National Water-Quality Assessment Program				
^c Wastewater treatment plant				
^d Not detected				

higher than those detected in water collected even few hours later (Goldsborough and Beck, 1989).

Degradation Products

Along with glyphosate, the primary metabolite AMPA has been detected in surface waters with increasing frequency. With a half life ranging from 76 to 240 days in soil, detection of AMPA in the environment can be a useful proxy for the detection of glyphosate (Grunewald *et al.*, 2001). The detection frequency for AMPA would be expected to be similar to that of glyphosate, and given AMPA's extended half life relative to glyphosate, it is reasonable to expect to detect its presence more often than the parent compound. In Midwestern streams that were sampled pre-emergence,

post-emergence, and during harvest, a greater proportion of AMPA detections were observed at all three sampling periods relative to glyphosate (Battaglin *et al.*, 2005). Determining the ratio between parent herbicide concentration and the concentration of the transformation product is an effective method of predicting transport processes involved, as well as environmental fate (Thurman and Fallon, 1996). The correlation between glyphosate use and surface water detection of glyphosate is relatively low, however, the relationship between glyphosate use and AMPA detection is stronger (Battaglin *et al.*, 2005). One issue with utilizing AMPA detection as a proxy for glyphosate contamination is that the degradation of industrial and domestic detergents may also result in the presence of AMPA in surface waters (Jaworska *et al.*, 2002). However, AMPA detection

attributed to detergents will most often correspond to particular point sources, such as locations of wastewater treatment plant effluent outflows or storm water discharge (Botta *et al.*, 2009).

Exposure to Glyphosate and Metabolites in Aquatic Environment

Glyphosate is not expected to bioaccumulate, owing to its low log K_{OW} value, which ranges from -4.59 to -1.70 (Wang *et al.*, 1994). Therefore, large-scale food web contamination caused by biomagnification of glyphosate-based herbicides is unlikely. However, the possibility of dietary exposure and small scale, short-term food chain effects have been considered given the prevalence of glyphosate in the aquatic environment. *Daphnia pulex* exposed to pure glyphosate either through contaminated water or contaminated diet had variable rates of glyphosate uptake, with higher body burden resulting from water column exposure (50 mg g^{-1} dry weight vs. 13 mg g^{-1} dry weight) (Bengtsson *et al.*, 2004). There is evidence that the bioaccumulation of glyphosate may be greater than predicted from the log K_{OW} value alone and the bioconcentration factor (BCF) is increased for glyphosate in the presence of POEA in the aquatic environment (Contardo-Jara *et al.*, 2009). It has been suggested that the same function POEA provides, by enhancing glyphosate transport into plant cells, also facilitates increased permeability in animal cells (Hedberg and Wallin, 2010). The potential for bioaccumulation of glyphosate has also been observed in terrestrial snails (*Helix aspersa*) fed a diet contaminated with glyphosate (Druart *et al.*, 2011), water hyacinth (*Eichhornia crassipes*) exposed to pure glyphosate, and also in tissue of carp (*Cyprinus carpio*) and tilapia (*Oreochromis mossambicus*) that were exposed to environmentally relevant concentrations (Wang *et al.*, 1994). Taken together, these results support the possibility of food chain contamination.

The presence of surface water contaminants are of particular concern to fish species since exposure is possible throughout development and growth. Apparent knowledge gaps exist regarding the potential for uptake of glyphosate into developing fish embryos, particularly the effects of exposure to commercial formulations including surfactants (Stehr *et al.*, 2009). In free swimming life stages of fish glyphosate can be taken up via gills from contaminated water (Hued *et al.*, 2012), as well as via a dietary route.

As with other surface water contaminants, amphibians are particularly susceptible to the effects of glyphosate exposure due to their dual (aquatic/terrestrial) life cycles. A large number of studies focused on the effects of glyphosate-based herbicide exposure on amphibians. Modes and timing of reproduction and early development in the aquatic environment render amphibians susceptible to waterborne exposure (Solomon and Thompson, 2003). Furthermore, later exposure in the terrestrial environment by direct contact with plants and soil after herbicide application, increase the likelihood of glyphosate uptake, facilitated by highly permeable skin (Quaranta *et al.*, 2009).

Currently, there exists a significant knowledge gap regarding uptake of glyphosate from the environment by aquatic organisms and tissue concentrations of glyphosate, surfactants, or glyphosate metabolites. More study is required to determine the mechanisms of uptake in a variety of aquatic species, life stages, and tissues, particularly the actual behaviour of glyphosate through small-scale food webs.

Metabolism of Roundup and Glyphosate

After uptake by organisms in the aquatic environment, the fate and biotransformation of glyphosate is not well understood. There is evidence that exposure to glyphosate even at non-toxic concentrations can affect the activity of soluble glutathione S-transferase (sGST), an important Phase II biotransformation enzyme, in blackworm (*Lumbriculus variegates*) (Contardo-Jara *et al.*, 2009). The increase in sGST activity was greater in worms exposed to the commercial formulation, containing POEA, than the active ingredient alone, suggesting that the commercial formulation may be more potent. This same pattern of activation of GST was observed in the liver of the Neotropical fish *Prochilodus lineatus* after acute exposure to RU (Modesto and Martinez, 2010b). It should be noted however that the exposure concentration used in the study and reported to cause histopathological alterations in the liver of the same species (Langiano and Martinez, 2008), was higher than expected in worst case environmental scenarios. However, liver histopathology, suggesting altered metabolic activities, was observed at much lower concentrations in fish exposed to Roundup Max[®] (0.5 mg l^{-1}), suggesting that even at more environmentally relevant concentrations, exposure to commercial glyphosate formulations can alter normal liver structure (Hued *et al.*, 2012). Alteration of liver morphology and enzyme activity have also been observed in rats sub-chronically exposed to RU or glyphosate (Caglar and Kolankaya, 2008; Hietanen *et al.*, 1983). Given that the liver is the primary site of xenobiotic detoxification, significant alterations to structure or normal function will likely have deleterious effects on organism health and performance.

Cellular Responses

Oxidative Stress

While the mode of action for toxicity of glyphosate in photoautotrophs such as algae is well explained by their physiological similarities to terrestrial plants, the toxicity to glyphosate towards aquatic animals is less well understood. Glyphosate targets the enzyme EPSPS, absent in these organisms, yet toxicity of glyphosate has been observed in a wide range of aquatic organisms (Folmar *et al.*, 1979). There is evidence that oxidative stress may be one of the mechanisms of glyphosate toxicity in animals. The generation of reactive oxygen species (ROS), implicated as a mechanism of toxicity for numerous toxicants, can have a variety of detrimental effects on cells (Bagchi *et al.*, 1995). The ROS can initiate oxidative damage to nucleic acids, lipids, and proteins, eventually leading to organelle damage and finally cell death. Production of antioxidants including the induction of enzymes and the release of non-enzymatic metabolites, counteract the damaging effects of ROS (Sies, 1997). When antioxidant activity can no longer compensate for the production of ROS, oxidative damage will occur. Exposure to glyphosate-based herbicides has been implicated in the initiation of oxidative stress in algae (Romero *et al.*, 2011), larval amphibians (Costa *et al.*, 2008; Lajmanovich *et al.*, 2011), the worm *L. variegates* (Contardo-Jara *et al.*, 2009), crustaceans (Frontera *et al.*, 2011), and fish (Cattaneo *et al.*, 2011; Gluszcak *et al.*, 2011; Guilherme *et al.*, 2010; Lushchak *et al.*, 2009; Menezes *et al.*, 2011; Modesto and Martinez, 2010a, 2010b).

The effect of glyphosate herbicide exposure on activity of key antioxidant enzymes, including superoxide dismutase (SOD), glutathione S-transferase (GST), and catalase (CAT), vary considerably depending on the experimental protocol and species. Activity of liver GST decreased while there was no change in the activity of SOD or CAT in silver catfish (*Rhamdia quelen*) exposed for 8 days to RU at concentrations up to 0.95 mg l^{-1} (Menezes *et al.*, 2011). During a subsequent recovery period of 8 days, SOD and CAT activity decreased, interpreted by the authors as either a compensatory response by the fish against the toxic conditions, or insufficient recovery time to elicit a complete recovery. A 96-h exposure of goldfish (*Carassius auratus*) to high concentrations ($2.5\text{--}20 \text{ mg l}^{-1}$) inhibited SOD in multiple tissues and decreased liver GST activity, whereas CAT activity increased significantly at the lower concentrations tested (Lushchak *et al.*, 2009). Further evidence for oxidative stress induction in goldfish liver by RU and its constituents, including POEA, was also provided by Fan *et al.* (2013). Paiva (*Leporinus obtusidens*) exposed for 96 hours to RU also displayed an increase in CAT activity at the same range of exposure concentrations (Gluszczak *et al.*, 2011).

A decrease in GST activity (Lajmanovich *et al.*, 2011) was observed in tadpoles acutely exposed to a commercial glyphosate formulation while similar concentrations resulted in either an increase or a decrease in GST activity in some fish species (Modesto and Martinez, 2010a, 2010b). The magnitude of increase in GST and SOD activity was enhanced in blackworms acutely exposed to a commercial glyphosate formula, relative to pure glyphosate alone, at concentrations between 0.05 and 5.0 mg l^{-1} (Contardo-Jara *et al.*, 2009), providing further evidence for the increased toxicity of formulations containing surfactants.

To further characterize the link between exposure and oxidative stress, reduced glutathione (GSH) content in tissues can be measured, as the non-protein thiol acts as a sink for free radicals (Schuliga *et al.*, 2002). Decreased GSH levels were observed in fish exposed to Roundup Transorb® (1 and 5 mg l^{-1}) for 24 h, followed by an increase in GSH content after 96-h exposure (Modesto and Martinez, 2010a). This result suggests a recovery after 96-h RU exposure, a pattern that has been observed for exposure to other herbicides (Zhang *et al.*, 2004).

Glyphosate-based herbicide toxicity has been linked to lipid peroxidation (LPO), most often identified through elevated thiobarbituric acid reactive substances (TBARS) levels. The primary target tissue tends to be the liver, (Gluszczak *et al.*, 2011), although increased TBARS have been observed in the brain of carp (*Cyprinus carpio*) (Cattaneo *et al.*, 2011) and silver catfish (Menezes *et al.*, 2011) exposed to RU for 96 h and 8 days, respectively. These observed changes in LPO were found to be transient in silver catfish, as LPO level returned to normal after a 8-day recovery period after 8 days exposure to 0.45 mg l^{-1} RU (Menezes *et al.*, 2011). However, recovery relative to control values was not observed in carp acutely exposed to RU at 2.5 mg l^{-1} (Cattaneo *et al.*, 2011), indicating possible species-specific differences in susceptibility to oxidative stress. Further evidence for the potential of glyphosate-based herbicides to induce oxidative stress is an increase in protein carbonyl, an indicator of protein damage caused by ROS (Gluszczak *et al.*, 2011; Menezes *et al.*, 2011). Roundup Transorb® induced LPO in fish after short-term exposure (6 h), although this oxidative damage was mitigated in longer-term exposure (96 h) and correlated with an induction of antioxidant enzymes (Modesto and

Martinez, 2010a). Overall, the available evidence suggests that glyphosate-based herbicides may cause oxidative damage in aquatic organisms, depending on species and duration of exposure. Induction of antioxidant systems during chronic, low level exposures, may allow cells to combat oxidative stress effectively and reduce the occurrence of oxidative damage (Lushchak *et al.*, 2009; Modesto and Martinez, 2010a, 2010b).

Genotoxicity

The genotoxic potential of RU has been assessed primarily using comet, micronucleus and erythrocytic nuclear abnormality (ENAs) assays, which determine the quantity of double strand breaks in DNA, induced chromosome damage, and abnormalities in the erythrocyte nucleus respectively. Relatively few studies of the genotoxic effects of glyphosate-based herbicides on aquatic organisms have been published. Grisolia and Starling (2001) reported that intra-abdominal injection of RU increased the formation of micronuclei in *Tilapia rendalli*. Likewise, goldfish and the tropical fish *Corydoras paleatus* showed increased micronuclei frequency as well as dose dependant increases in DNA damage in peripheral erythrocytes following exposure to RU (Cavas and Konen, 2007; Ghisi and Cestari, 2013). *In-ovo* exposure of RU to broad-snouted caiman (*Caiman latirostris*) resulted in a dose-dependent increase in the production of micronuclei as well as double strand breaks after direct application of $500 \mu\text{g l}^{-1}$ (Poletta *et al.*, 2009) and at more environmentally relevant exposures (Poletta *et al.*, 2011).

Double-stranded DNA breaks were observed in European eel (*Anguilla anguilla*) exposed to 58 and $116 \mu\text{g l}^{-1}$ after only 24 h, and an increased tendency for ENAs was observed after 3 days of exposure to glyphosate-based herbicide (Guilherme *et al.*, 2010). Subsequent studies determined that the type of DNA damage varies with exposure period and concentration, with ROS-dependent DNA damage being the primary mechanism of genotoxicity after 3 days (Guilherme *et al.*, 2012). RU exposure affected DNA integrity in the Neotropical fish, *Prochilodus lineatus*, after only 6 h of exposure at the much higher concentration of 10 mg l^{-1} (Cavalcante *et al.*, 2008). These effects were shown to be attenuated over time, with the majority of damage occurring soon after exposure, a pattern which can be explained by engagement of DNA repair systems and the activation of detoxification pathways (Banu *et al.*, 2001). Similar results, with dose-dependent induction of DNA breaks and decreased effects, suggesting repair with time, were also reported in the frog *Eleutherodactylus johnstonei* (Meza-Joya *et al.*, 2013). Conversely, double-strand breaks were not observed in developing oyster spermatozoa exposed at $5 \mu\text{g l}^{-1}$ (Akcha *et al.*, 2012) which further suggests that the degree of genotoxicity is highly dependent on species, exposure concentration, and length of exposure.

Acetylcholinesterase

The enzyme acetylcholinesterase (AChE) is an important B-type esterase effecting the breakdown of the neurotransmitter acetylcholine in neuromuscular junctions, a process critical for normal muscle and brain function (Fulton and Key, 2001). Inhibition of AChE leads to accumulation of acetylcholine within the synapse leading to overstimulation of the post-synaptic membrane, causing death. Exposure to organophosphate and carbamate pesticides have been associated with inhibition of

AChE activity and monitoring AChE activity has proven to be a useful biomarker of aquatic exposure to pesticides (Fulton and Key, 2001). A number of previous studies have associated exposure to glyphosate-based herbicides with inhibition of AChE activity in brain and/or muscle of aquatic organisms (Cattaneo *et al.*, 2011; Gluszcak *et al.*, 2006, 2007; Lajmanovich *et al.*, 2011; Menendez-Helman *et al.*, 2012; Modesto and Martinez, 2010a; Salbego *et al.*, 2010; Sandrini *et al.*, 2013).

Inhibition of AChE was observed in brain of Piava (*Leporinus obtusidens*) and carp acutely exposed to concentrations of RU as low as 0.5 mg l^{-1} for 96 h (Cattaneo *et al.*, 2011; Gluszcak *et al.*, 2006) and again in Piava chronically exposed to much higher concentrations 5.0 mg l^{-1} for 90 days (Salbego *et al.*, 2010). This inhibitory action was also shown in muscle of *P. lineatus*, after 24-h exposure to RU and in the brain after 96-h exposure to high concentrations of RU (10 mg l^{-1}) (Modesto and Martinez, 2010b). The same species exposed to another formulation, Roundup Transorb[®], displayed an inhibition in the brain AChE activity at the more environmentally relevant concentration of 1.0 mg l^{-1} after 96-h exposure, and within both the brain and muscle at a higher concentration of 5 mg l^{-1} (Modesto and Martinez, 2010a).

A similar pattern of inhibition was observed in other aquatic vertebrates, including tadpoles of the amphibian *Rhinella arenarum* exposed to a variety of commercial glyphosate formulations for 48 h. Inhibition of AChE activity was detected after exposure to all the formulations of glyphosate at concentrations as low as 1.85 mg l^{-1} , especially with the formulation Infosato[®] (Indusquim SRL, Sante Fe, Argentina) that caused the greatest level of inhibition (Lajmanovich *et al.*, 2011). This study illustrates the variability in the toxicity between commercial formulas and suggests these differences may be due to variations in surfactants, a conclusion that substantiates earlier findings (Gluszcak *et al.*, 2006), although further research is required to investigate species differences.

The level of AChE inhibition observed in these studies was far below the level deemed life threatening although it may lead to muscle hyperactivity, a condition linked with the production of damaging ROS (Yang *et al.*, 1996). Additionally, it has also been shown that AChE is required for normal muscle and neuron development in fish, a result which suggests that alteration of AChE activity may have survival as well as population level effects (Behra *et al.*, 2002).

Toxicity to Aquatic Organisms

Acute Toxicity

Studies of acute toxicity, critical to understanding the limits of exposure, have focused on a variety of glyphosate-based herbicide formulations and often the individual constituents of commercial formulations in isolation. These studies compare the toxicity of a commercial formulation and its components in an effort to attribute the formulation's toxicity to a particular element. This allows for further research to be conducted to characterize the toxicity of glyphosate-based herbicide components and the development of commercial products with overall decreased toxicity to non-target species.

Algae

The physiological and biochemical similarities between algae and terrestrial plants suggest that algae would be particularly

vulnerable to glyphosate-based herbicide exposure. Exposure of several species of freshwater algae to glyphosate, for either 72 or 96 h, provided a wide range of species-specific EC_{50} values, ranging from 3.5 to 55.9 mg l^{-1} (Ma, 2002; Vendrell *et al.*, 2009). To determine the acute toxicity of a commercial formulation, 96-h IC_{50} were calculated using growth inhibition as an endpoint, for freshwater (*Selenastrum capricornutum*) and marine (*Skeletonema costatum*) microalgae exposed to RU, POEA, IPA salt of glyphosate, and glyphosate acid alone (Tsui and Chu, 2003). The surfactant POEA was the most toxic to the freshwater algae ($\text{IC}_{50} = 3.92 \text{ mg a.e. l}^{-1}$) whereas the commercial formula was the most toxic to the marine species ($1.85 \text{ mg a.e. l}^{-1}$). Overall, *S. costatum* was the more sensitive of the two species whereas the majority of the toxicity of RU to *S. capricornutum* was attributed to the presence of POEA, confirming the results of other algal studies which reported higher toxicity of the commercial formulation compared with the active ingredient alone (Saenz and Di Marzio, 2009).

Microorganisms and invertebrates

Glyphosate-based herbicides cause a wide range of toxic effects in non-target aquatic species (see earlier reviews by Giesy *et al.*, 2000; Solomon and Thompson, 2003, Table 4). There existed a paucity of data regarding the effects of glyphosate-based herbicides on aquatic microorganisms until a study carried out by Tsui and Chu (2003). Their study focused on the acute toxicity of the commercial formulation RU and each of its components individually, as well as the influence of pH, temperature, suspended sediments, and algal food availability on the toxicity, in seven marine and freshwater microorganisms: bacterium (*Vibrio fischeri*), microalgae (*S. capricornutu* and *S. costatum*), protozoa (*Tetrahymena pyriformis* and *Euplotes vannus*), and crustaceans (*Ceriodaphnia dubia*, and *Acartia tonsa*). Both the microalgae and the crustaceans exhibited greater sensitivity to RU, relative to bacteria and protozoa, with the majority of the toxicity attributed to the surfactant portion of the formulation. The most sensitive crustacean tested was *A. tonsa*, with a 48-h LC_{50} value of $1.77 \text{ mg a.e. l}^{-1}$. These findings again confirm the results of other studies regarding the higher toxicity of surfactants relative to the active ingredient alone (Bringolf *et al.*, 2007; Folmar *et al.*, 1979; Pereira *et al.*, 2009). Conversely, the majority of the toxicity to microalgae was attributed to the IPA salt of glyphosate, as these are photosynthetic organisms that are more susceptible to the herbicidal action as they share similar metabolic pathways and systems with higher plants.

The sensitivity of the freshwater mussel *Lampsilis siliquoidea* has been investigated by acute exposure of glochidia (larval stage of freshwater mussels) to RU and its individual constituents, specifically glyphosate acid, IPA salt of glyphosate, and the proprietary surfactant MON 0818 (Bringolf *et al.*, 2007). MON 0818 was the most toxic, (48-h $\text{EC}_{50} = 0.5 \text{ mg l}^{-1}$), compared with RU (48-h $\text{EC}_{50} = 2.9 \text{ mg a.e. l}^{-1}$) or glyphosate (48-h $\text{EC}_{50} > 200 \text{ mg l}^{-1}$). A second commercial formula, Aqua Star (Albaugh, Ankeny, IA, USA) which lacks the surfactant, was non toxic to glochidia, and juvenile *L. siliquoidea* with an 48-h EC_{50} of $> 148 \text{ mg a.e. l}^{-1}$. Glochidia of another freshwater bivalve, *Utterbackia imbecillis*, appeared far less sensitive to RU exposure with a 24-h LC_{50} value of $13.5 \text{ mg a.e. l}^{-1}$ (Connors and Black, 2004). *Tetrahymena* exposed to the primary glyphosate metabolite AMPA had less growth rate inhibition than glyphosate alone after 9-h incubation, although both

Table 4. Selected studies of acute and chronic effects of exposure to glyphosate and glyphosate-based herbicides

Test species/system	Life stage	Test Conditions	Exposure duration	Formulation	Exposure ($\mu\text{g a.e. l}^{-1}\text{b}$)	Effects	Reference
Microorganisms							
<i>Vibrio fischeri</i>		Microtox	15 min	RU ^a	24900	IC ₅₀ (Growth inhibition)	Tsui and Chu, 2003
<i>Skeletonema costatum</i>		Static	96 hr	RU	1850	IC ₅₀ (Growth inhibition)	Tsui and Chu, 2003
<i>Chlorella kessleri</i>		Static	96 hr	ATANOR [®]	55620	EC ₅₀	Romero et al., 2011
					50000	↑ Lipid peroxidation, ↑ antioxidant enzyme activity	
Phytoplankton and periphyton sp.		Mesocosm	12 d	RU	4440 ^d	↑ total P concentration altered community structure	Perez et al., 2007
Crustaceans							
<i>Ceriodaphnia dubia</i>	adult	Static	48 hr	RU	5390	LC ₅₀	Tsui and Chu, 2003
<i>Acartia tonsa</i>	adult	Static	48 hr	RU	1770	LC ₅₀	Tsui and Chu, 2003
<i>Hyella castroi</i>	adult	NR ^c	7 d	RU	270 ^d	↓ biochemical parameters ↑ LPO	Dutra et al., 2011
Fish							
<i>Onchorynchus mykiss</i>	Swim ups Adult	Static	96 hr	RU	1800 ^d	LC ₅₀	Folmar et al., 1979
	Adult		96 hr		6100 ^d	LC ₅₀	
<i>Pimephales promelas</i>	Adult		24 hr	RU	1700 ^d	LC ₅₀	Folmar et al., 1979
<i>Anguilla anguilla</i>	Yellow eel stage	Static	24 hr	RU	13 ^d 27 ^d	Genotoxicity ↑ LPO	Guilherme et al., 2010
<i>Leporinus obtusidens</i>	juvenile	Static renewal	90 d	RU	3700 ^d	↓ brain AChE activity ↓ hepatic glucose ↓ liver glycogen	Salbego et al., 2010
<i>Oreochromis niloticus</i>	Adult	Static renewal	3 month	RU	740 ^d	↓ brain/muscle AChE activity	Jiraungkoorskul et al., 2003
<i>Cyprinus carpio</i>	Fingerling	Static	96 hr	RU	370 ^d	↑ Oxidative stress	Cattaneo et al., 2011
Amphibians							
<i>Rana catesbeiana</i>	Gosner 25	Static renewal	96 hr	RU MAX	800	LC ₅₀	Relyea and Jones (2009)
<i>R. catesbeiana</i>	Gosner 25	Static non-renewal	96 hr	RU	2800	LC ₅₀	Moore et al. (2012)
<i>Rana clamitans</i>	Gosner 25	Static renewal	96 hr	RU MAX	1400	LC ₅₀	Relyea and Jones (2009)
<i>R. clamitans</i>	Gosner 25	Static non-renewal	96 hr	RU	4600	LC ₅₀	Moore et al. (2012)
<i>R. clamitans</i>	Gosner 25	Wetland enclosure	96 hr	Vision [®]	4340	LC ₅₀	Wojtaszek et al. (2004)
			24 hr		1430	Altered avoidance behavior	
<i>R. clamitans</i>	Gosner 42	Static renewal	42 d	Various	1800	↑ Tail damage Gonadal abnormalities ↓ rate of development ↑ intersex gonads	Howe et al. (2004)
<i>Crinia insignifera</i>	Tadpole	Static renewal	48 hr	RU	3600	LC ₅₀	Mann and Bidwell (1999)
			48 hr	RU Biactive [®]	>494000	LC ₅₀	

<i>Rana pipens</i> , <i>Rana sylvatica</i> , <i>Bufo americanus</i>	Gosner 25	Mesocosm	21 d	RU MAX	2000	↓ toxicity in presence of predator cues Morphological changes	Relyea, 2012
Birds Drake (<i>Anas platyrhynchos</i>)	Adult	Feeding study	15 d	RU	5 mg RU kg ⁻¹	Altered testicular development	Oliveira <i>et al.</i> , 2007

^aRoundup.
^bExposure concentration reported as µg a.e. l⁻¹, unless otherwise noted.
^cNot reported.
^dPublished value converted to mg a.e. l⁻¹.

compounds were classified as non-toxic by the growth inhibition criteria (Bonnet *et al.*, 2007).

Temperature did not have a significant effect on RU toxicity (Tsui and Chu, 2003), in contrast with earlier findings by Folmar *et al.* (1979). However, this discrepancy can be explained by differences in the experimental procedures between the two studies. Whereas Folmar *et al.* (1979) altered the temperature just prior to toxicant exposure, the study by Tsui and Chu (2003) allowed for an acclimation period after the change in temperature, before the addition of toxicant. The toxicity of RU, to *C. dubia*, increased significantly with a rise in test water pH, a trend that can be attributed to the surfactant portion of the formulation (Tsui and Chu, 2003). While glyphosate toxicity decreased as water became more alkaline, POEA toxicity increased as it transforms from a cationic to a non-ionic form and exerts greater toxicity via non-specific membrane disruption (Schuurmann, 1990).

The 48-h LC₅₀ value for *C. dubia* increased in the presence of RU and kaolin clay particles relative to RU alone, owing to the combined effects of direct particle toxicity and increased intake of sediment particles with RU bound (Tsui and Chu, 2003). The high degree of affinity for soil particles inherent in glyphosate-based herbicides may result in the herbicide becoming concentrated on the surface of soil particles, increasing the exposure for organisms which ingest them. This same relationship was observed in *D. pulex* exposed to RU and bentonite clay particles in an earlier study (Hartman and Martin, 1984).

Glyphosate exposure has an unexpected effect on the acute toxicity of metals in *C. dubia*. Less than additive mixture toxicity and no synergism was observed when RU exposure coincided with exposure to metals including Cd, Cu, Cr, Ni, Pb, Se and Zn (Tsui *et al.*, 2005). For certain metals (Ag, Cd, Cr, Cu, Ni, Pb and Zn), the addition of RU at expected environmental concentrations resulted in reduced acute metal toxicity, probably attributed to a chelating effect mediated by the herbicide presence. However, the authors caution that these chemical interactions are dependent on pH and did not consider the additional effects of the presence of soil particles.

Overall, the acute toxicity of glyphosate-based herbicides to microorganisms and invertebrates is highly species dependant. For algal species, the majority of the toxicity can be attributed to the herbicidal action of glyphosate, whereas zooplanktons are typically more susceptible to exposure from the surfactant portion of commercial glyphosate formulations. The major environmental factor affecting toxicity is an increase in pH, with alkaline exposure increasing acute toxicity.

Fish

Fishes are exposed to wide range of environmental stressors throughout their life cycle, including fluctuations in temperature, water chemistry, dissolved oxygen, nutrients, and predator/prey abundance. Fish are inherently well evolved to respond to changes in their natural environment through compensatory physiological and behavioural alterations. However, the presence of chemical anthropogenic stressors, such as glyphosate-based herbicides in the water, can alter physiological and behavioural endpoints critical to maintaining normal function, and cause adverse effects ranging from the cellular to the population level (Guilherme *et al.*, 2012; Hued *et al.*, 2012). Occupying higher trophic levels, fish are also susceptible to indirect effects via contaminated food sources such as algae, invertebrates or other prey fish species (Solomon and Thompson, 2003).

However, fish appear to be less sensitive than amphibians to direct exposure to glyphosate-based herbicides, a phenomenon first noted by Giesy *et al.* (2000). Their review examined acute toxicity data of 12 fish species from a variety of studies prior to 2000, and reported that the LC₅₀ 96-h values in fish ranged from 4.2 to 52 mg l⁻¹ of RU. In general, adult fish were more tolerant to glyphosate herbicide exposure than younger life stages (Jiraungkoorskul *et al.*, 2002). As is the case with other aquatic organisms, much of the toxicity of the commercial formulation was attributed to the surfactant portion, particularly POEA which had a range of LC₅₀ 96-h values between 0.65 and 7.4 mg a.e. l⁻¹ (Folmar *et al.*, 1979). These values are well in excess of expected environmental exposure levels in most places in the world, thus, the threat of mortality via acute exposure to RU in the environment is minimal under normal conditions.

Hematological alterations, specifically an increase in hematocrit, were detected in *P. lineatus* exposed for 96 h to Roundup Transorb® at 5 mg l⁻¹ (Modesto and Martinez, 2010a). Increases in both erythrocyte and leukocytes suggest that defence mechanisms in response to contaminant exposure were activated in the fish (Cazenave *et al.*, 2005). This result is different from an earlier study by Glusczak *et al.* (2006) where a decrease in hematocrit was observed in *L. obtusidens* after exposure to Roundup Original®. Differences in surfactant formulation between Roundup Original® and Roundup Transorb® as well as species-specific responses to contaminant exposure may explain some of this discrepancy (Elahee and Bhagwant, 2007). It is of interest to note that the surfactants are not solely responsible for the toxicity of the commercial formulations in these non-target species. The immune response, quantified as a phagocytic index of coelomic cells and bacteria agglutination in fingerlings of silver catfish (*Rhamadia quelen*), was decreased after 24-h exposure to 0.73 mg l⁻¹ pure glyphosate (Kreutz *et al.*, 2011). Even though further study utilizing commercial formulations should be conducted to provide a more realistic proxy to environmental exposures, these results suggest that the active ingredient alone, rather than the surfactant portion caused the observed decrease in natural immune response.

Mobilization of energy stores, a common stress response in fish, was observed as a decrease in liver and/or muscle glycogen in Piava exposed acutely (96 h, 3–20 mg l⁻¹) (Glusczak *et al.*, 2006) or chronically (90 days, 5 mg l⁻¹) to RU (Salbego *et al.*, 2010). An increase in plasma glucose was detected in *P. lineatus* acutely (6–96 h) exposed to 7.5 and 10 mg l⁻¹ of RU, a response that was not associated with a subsequent increase in plasma cortisol (Langiano and Martinez, 2008). It should be noted, however, that the blood samples were obtained 6 h after exposure and plasma cortisol may have been restored to normal levels by this time, given that cortisol levels typically lag 30 min to 1 h after disturbance (Barton, 2002). Aberrant control of cortisol production is indicative of a xenobiotic affecting the hypothalamus–pituitary–gonadal axis (HPI), a highly controlled hormone system vital for normal stress response in fishes (Hontela and Vijayan, 2009). Silver catfish fingerlings acutely exposed to sub-lethal concentrations of RU (1.2–3.65 mg l⁻¹) had decreased cortisol production relative to controls, suggesting a decreased ability, when in the presence of glyphosate-based herbicide, to effect the physiological adjustments in response to environmental challenges (Soso *et al.*, 2007). This same study also assessed the endocrine disrupting potential of RU by demonstrating reduced 17β-estradiol levels in correlation with decreased viability of resulting swim-up fry after 40 days of exposure.

Amphibians

Amphibians have been categorized as particularly sensitive to glyphosate-based herbicides and a significant volume of research has been undertaken to quantify this sensitivity. The LC₅₀ values for larval anurans range from approximately 1 to 12 mg a.e. l⁻¹ (Edginton *et al.*, 2004; Howe *et al.*, 2004; Mann and Bidwell, 1999; Moore *et al.*, 2012; Relyea, 2005a, 2005b, 2005c; Relyea and Jones, 2009; Wojtaszek *et al.*, 2004). Variation between the LC₅₀ estimates are a result of differences in experimental design, location, and differences in sensitivity of individual species and populations (Relyea, 2006; Wojtaszek *et al.*, 2004). For example, Leopard Frog (*Rana pipiens*) exposed to RU at Gosner stage 25, in separate studies, had 96-h LC₅₀ values which ranged from 1.1 (Edginton *et al.*, 2004) to 2.9 mg a.e. l⁻¹ (Howe *et al.*, 2004). Similarly, for *R. clamitans* 96-h LC₅₀ estimates ranged from 1.4 (Edginton *et al.*, 2004; Relyea and Jones, 2009) to 4.6 mg a.e. l⁻¹ (Moore *et al.*, 2012). These variations may, in part, be a result of differences in pH between experiments, as an increase in pH increases surfactant toxicity (Chen *et al.*, 2004; Edginton *et al.*, 2004; Thompson *et al.*, 2004; Tsui and Chu, 2003; Wojtaszek *et al.*, 2004). Edginton *et al.* (2004) investigated the effects of pH on the acute toxicity of RU formulations to larval North American and African amphibians, reporting that the 96-h LC₅₀ estimates ranged from 1.8 to 3.5 mg a.e. l⁻¹ at pH 6.0 and decreased to between 0.9 and 1.7 mg a.e. l⁻¹ at pH 7.5.

At elevated exposure concentrations, higher than those likely to be encountered in the environment (5–20 mg a.e. l⁻¹), glyphosate formulations are highly toxic to larval amphibians, particularly anurans (Dinehart *et al.*, 2010; Fuentes *et al.*, 2011; Howe *et al.*, 2004; Lajmanovich *et al.*, 2011; Moore *et al.*, 2012; Relyea, 2005c; Wojtaszek *et al.*, 2004). With few exceptions LC₅₀ values fall below 5.0 mg a.e. l⁻¹; these exceptions include Leopard frogs exposed *in-situ* in wetlands (Wojtaszek *et al.*, 2004) and the Australian anurans, Motorbike Frog (*Litoria moorei*) and Moaning Frog (*Heleioporus eyrie*) exposed for 48 h via static renewal in a laboratory setting (Mann and Bidwell, 1999).

Toxicity to amphibians at peak concentrations immediately after herbicide application or in predicted worst case scenarios (1.0–5.0 mg a.e. l⁻¹) are highly dependent on the species being tested. Larval Australian anurans typically display the least sensitivity to glyphosate formulations, with 48-h LC₅₀ values ranging from 2.9–11.6 mg a.e. l⁻¹ (Mann and Bidwell, 1999), whereas *Scinax nasicus* from South America was particularly sensitive (96 hr LC₅₀ of 0.95 mg a.e. l⁻¹) (Lajmanovich *et al.*, 2003). North American anurans have been extensively tested through exposures to various glyphosate formulations containing surfactants and the majority of LC₅₀ values for larval anurans were within the range of 1.0–5.0 mg a.e. l⁻¹.

Exposure to glyphosate-formulations below 1.0 mg a.e. l⁻¹ are expected in the environment resulting from direct overspray or drift during application (Thompson *et al.*, 2004). These concentrations are below the predicted median lethal concentrations for the majority of species studied and are below predicted no observed effects concentration (NOEC) for several species studied (Moore *et al.*, 2012). Exceptions include the American bullfrog (*Rana catesbeiana*) and the Spring peeper, with 96 hr LC₅₀ values of 0.8 mg a.e. l⁻¹ (Relyea and Jones, 2009). Overall, glyphosate-based formulations can be classified as moderately toxic to larval amphibians (1.0 < LC₅₀ < 10.0 mg a.e. l⁻¹).

Common Eastern Froglet (*Crinia insignifera*), a southwestern Australian frog, exposed to RU as tadpole, metamorph, and adult were more susceptible as tadpoles, with an average 48-h LC₅₀ of 3.6 mg a.e. l⁻¹ compared with approximately 50 mg a.e. l⁻¹ for the more advanced stages of development (Mann and Bidwell, 1999). Investigations of toxicity in amphibians at different developmental stages concluded that juveniles exposed later in development (Gosner stage 20 vs Gosner stage 25) are more sensitive to RU as illustrated with a decrease in 96-h LC₅₀ values ranging from 6.5 to 8.0 mg a.e. l⁻¹ and 2.0 to 4.0 mg a.e. l⁻¹ (Howe *et al.*, 2004). In contrast, Edge *et al.* (2013) reported no significant effects of glyphosate-based herbicide in juveniles of two semi-aquatic amphibian species, *Lithobates clamitans* and *L. pipiens*, exposed to Roundup WeatherMax® at real-world application rates. By comparison, the effects of glyphosate formulations on amphibians during terrestrial stages have been given limited attention. One study of RU application by aerial overspray at typical levels (1.6 mg a.i. m⁻²), observed 68–86% juvenile mortality compared with 96–100% in juveniles exposed to the same levels in the aquatic environment (Relyea, 2005b).

Host-parasite interactions

Investigations of the effects of glyphosate-based herbicides on host-parasite interactions suggested that glyphosate can decrease the infective capacity of horsehair worm (*Chordodes noblii*) larvae after acute exposure at environmental concentrations (Achiorno *et al.*, 2008), while also altering *Schistosoma mansoni* infection of the snail *Biomphalaria alexandrina* (Mohamed, 2011). *R. clamitans* tadpoles exposed to 3.7 mg l⁻¹ pure glyphosate had increased susceptibility to infection by cercariae of the nematode *Echinostoma trivolvis*, in spite of an overall decrease in cercarial survival (Rohr *et al.*, 2008). A synergistic effect was observed in juvenile *Galaxias anomalus*, a freshwater fish native to New Zealand, exposed to glyphosate and infection by the trematode parasite *Telogaster opisthorchis*, leading to decreased survival and an increase in spinal deformations relative to parasitic infection alone (Kelly *et al.*, 2010).

Chronic Toxicity

In contrast to acute toxicity exposures, which determine maximum exposure concentrations relevant to individual survival, chronic studies typically utilize exposures at more environmentally relevant concentrations over time scales that more closely mimic the natural environment. The data from chronic studies can be more easily extrapolated to real environmental conditions which is important for more accurate risk assessment.

Algae

Chronic studies of glyphosate-based herbicides with algae are relatively scarce as most studies have focused on short-term, acute effects of the active ingredient alone, in spite of widespread use and presence of commercial formulations in the environment. A 21-day study of *Chlorella vulgaris* exposed to Roundup 360 SL®, a formulation containing POEA surfactant, reported an EC₅₀ for growth of 118.1 mg l⁻¹, compared with the active ingredient, glyphosate (EC₅₀ = 293 mg l⁻¹), or IPA salt of glyphosate (EC₅₀ = 83 mg l⁻¹) (Lipok *et al.*, 2010). Although these values are relatively high, it should be noted that *C. vulgaris* has been shown to be less sensitive than other algal species to chemical exposure (Kasai and Hatakeyama, 1993). A long-term study of the freshwater algae *Scenedesmus quadricauda*,

in the presence of RU at concentrations ranging from 0 to 200 mg l⁻¹, monitored algal growth rate, rate of photosynthesis, and chlorophyll- α synthesis (Wong, 2000). Increases in all parameters measured were observed at concentrations up to 0.2 mg l⁻¹. At 2.0 mg l⁻¹ and above, however, an inhibitory effect was observed in each of the three parameters.

Microorganisms and Invertebrates

Characterization of the chronic effects of glyphosate-based herbicides on aquatic microorganisms and invertebrates, and understanding their response to contaminant exposure, is critical to ecological risk assessment, given their role as food sources for higher organisms. An investigation of seven cyanobacterial strains focused on the effects of Roundup 360 SL® on growth rate and generation doubling time over 21 days (Lipok *et al.*, 2010). All species tested showed some level of sensitivity to RU exposure, though the degree of sensitivity was highly species dependant. EC₅₀ values ranged from 2.9 mg l⁻¹ in the most sensitive species (*Anabaena catenula*) to 89.8 mg l⁻¹ in the least sensitive studied species (*Synechocystis aquatilis*). As is often the case, the toxicity of the commercial formula could, in part, be attributed to the POEA surfactant portion.

A subchronic exposure to glyphosate, carried out for 14 days in microcosms inoculated by natural periphyton populations, investigated the seasonal and species fluctuations in herbicide toxicity (Pesce *et al.*, 2009). The responses after exposure to 10 μ g l⁻¹ of pure glyphosate were dependent on the season and community composition. Glyphosate had no significant effect on the algal-grazing protozoa, supporting earlier acute toxicity results of Bonnet *et al.* (2007). Glyphosate had no effect on overall autotrophic biomass; however, reproduction was inhibited in a species-specific manner, particularly in *Asterionella*, *Oocystis*, and *Cyclotella* species. Overall, microcosms inoculated in the summer contained microbial species more resistant to the effects of glyphosate exposure than those collected in the spring, resulting in decreased species diversity in the spring treatment groups relative to those inoculated with summer collected species.

Freshwater crayfish (*Cherax quadricarinatus*) chronically exposed (50 days) to glyphosate (22.5 mg l⁻¹) and POEA (7.5 mg l⁻¹) had decreased muscle protein as well as slower somatic growth, which may be related to increase in mobilization of energy reserves in response to toxicant stress as suggested by the authors (Frontera *et al.*, 2011). This conclusion is further supported by a significant decrease in stores of muscle glycogen after exposure to POEA, providing further evidence that the toxicity can, in part, be attributed to the surfactant portion of the commercial formula.

If toxicant stress increases the energy demand on the organism, then combining toxicant exposure with variable food quality might be an effective method of confirming this relationship. *D. magna* exposed to Roundup WeatherMax® at concentrations up to 4.3 mg a.i. l⁻¹ were fed algal diets of variable carbon:phosphorus ratios (Lessard and Frost, 2012). Surprisingly, Daphnids exposed to Roundup WeatherMax® at 2.5 mg a.i. l⁻¹ had increased growth rates when fed phosphorus poor food, compared with those fed phosphorus-rich food. The authors suggested that uptake of the toxicants and resulting adverse effects might be due to greater incorporation of the toxicant in individuals fed phosphorus rich algae. Alternatively, fast growing individuals, as a result of phosphorus rich diet, might be more susceptible to alterations in cellular processes caused by toxicant exposure due to increased metabolic rate

(Gasnier *et al.*, 2009). These studies illustrate the importance of understanding the biology of the test organisms used in ecotoxicological studies for more accurate interpretation of toxicity data.

Fish

In an effort to better understand the effects of glyphosate herbicides in the environment, the majority of current research has focused on the sub-lethal effects of chronic exposures to glyphosate-based herbicides. Exposure of the Neotropical fish *Jenynsia multidentata* to RU at 0.5 mg l^{-1} for 7 and 28 days altered gill and liver morphology in a dose-dependent manner (Hued *et al.*, 2012). The alterations observed included hyperplasia, epithelial lifting, hypertrophy, and protective responses to limit toxicant diffusion through the gill. The gills are the initial target organ for xenobiotic exposure through water and are particularly susceptible, given their large surface area, vital gas exchange function, and their osmoregulatory role. Gill and liver alterations, similar to those reported in *Jenynsia*, were observed in Nile tilapia (*Oreochromis niloticus*) exposed for 3 months to sub-lethal concentrations of RU (2.4 and $7.2 \text{ mg a.e. l}^{-1}$), along with kidney lesions which were correlated with significant increases in the activity of plasma aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase (Jiraungkoorskul *et al.*, 2003). These biochemical alterations are indicative of increased metabolism of amino acids in response to elevated energy demand during periods of physiological stress and cellular damage (Jyothi and Narayan, 1999). It is of interest to note that chronic exposures of stickleback (*Gasterosteus aculeatus*) larvae to glyphosate did not induce vitellogenin or spiggin, markers of estrogenic and androgenic effects, respectively (Le Mer *et al.*, 2013).

Amphibians

Similar to acute toxicity studies with amphibians, chronic toxicity studies provided evidence for high sensitivity of these vertebrates to glyphosate herbicides. Chronic exposure of western chorus frog (*Pseudacris triseriata*) tadpoles to Roundup WeatherMax[®] and Roundup Original MAX[®], at environmentally relevant concentrations ($0.572 \text{ mg a.e. l}^{-1}$), resulted in a high rate of mortality (Williams and Semlitsch, 2010). Only 20% of exposed individuals survived to metamorphosis in the group exposed to Roundup WeatherMax[®]. Species-specific sensitivity to chronic RU exposures was assessed in this same study, using American toads (*Bufo americanus*) and grey treefrogs (*Hyla versicolor*). The high degree of mortality reported for chorus frog was not observed in the other two species tested, although exposure to both formulations resulted in a significant delay in metamorphosis for *B. americanus*. These developmental delays are consistent with findings by Howe *et al.* (2004), who observed increased time to metamorphosis in four North American anurans after exposure to Roundup Original[®], Roundup Transorb[®], and POEA. In two other North American amphibians, New Mexico (*Spea multiplicata*) and Plains (*Spea bombifrons*) spadefoot, 30-day exposure to higher concentrations of Roundup WeatherMax[®] (2.0 and $2.8 \text{ mg a.e. l}^{-1}$) caused significant mortality (Dinehart *et al.*, 2010). The increased toxicity of the WeatherMax[®] formulation can be attributed, in part, to the surfactant component of the formula as the same degree of mortality was not observed in individuals exposed to Roundup Original MAX[®], in spite of containing the same active ingredient (Williams and Semlitsch, 2010).

Chronic exposure of *R. pipiens* tadpoles to commercial glyphosate formulations containing the surfactant POEA, specifically Roundup Original[®] and Roundup Transorb[®], increased the incidence of gonadal abnormalities, intersex individuals, as well as morphological changes (Howe *et al.*, 2004). Treatment with both commercial formulations and POEA alone at $1.6 \text{ mg a.e. l}^{-1}$ resulted in decreased tail length, snout-vent length, and a reduction in the proportion of individuals reaching metamorphosis. These morphological and physiological changes suggest some degree of hormone disruption, a result that was confirmed by monitoring thyroid hormone receptor β (TR β) mRNA expression in this study. This important marker of thyroid-dependent metamorphosis increased in the tail of developing tadpoles at Gosner stage 25, suggestive of altered initiation of tail regression and, possible disruption of the retinoid or thyroid axis induced by exposure to POEA and formulations containing the surfactant. These delays in metamorphosis could negatively impact individual survival in temporary pools and increase the risk of predation (Semlitsch, 1990). Though the precise mechanisms for glyphosate formulations containing POEA to alter normal hormone-mediated development are unclear, it should be noted that *in vitro* exposure to RU has been implicated in disruption of steroidogenic acute regulator (StAR) protein, responsible for transport of cholesterol across the mitochondrial inner membrane for steroidogenesis (Walsh *et al.*, 2000).

Birds

The scope of this review dictates a focus on aquatic exposure to glyphosate in avian species, specifically the effects of glyphosate-based herbicides on waterfowl. Waterfowl occupy a relatively high trophic level in the aquatic environment and depend on macrophytes and invertebrates as a food source. As such, the most likely exposure route for glyphosate would be through consumption of contaminated food and water. *In vivo* effects of RU exposure were investigated in drake *Anas platyrhynchos*. Individuals were exposed via gavage at either 5 or 10 mg kg^{-1} of body weight to RU diluted in water for 15 days and alterations in testis and epididymal morphology, serum testosterone and estradiol levels and androgen receptor (AR) expression were assessed (Oliveira *et al.*, 2007). Harmful, tissue-specific effects were observed, particularly in the proximal efferent tubules and epididymal ducts. Additionally, levels of serum testosterone and estradiol were altered and correlated with changes in AR expression. Combined, these results suggest the potential for RU to alter bird hormone profiles and reproductive organ morphology.

Behavioural Endpoints

Behavioural endpoints offer several advantages over more traditional measures of aquatic toxicity, including higher sensitivity, relevance in the natural environments, and effects at the organism level which can be linked to effects at the population and species level (Hellou, 2011). Concentrations of glyphosate in the aquatic environment are typically orders of magnitude lower than those expected to cause acute toxicity, thus highly sensitive, sub-lethal endpoints such as behaviour are ideal for monitoring the effects of glyphosate-based herbicides in the aquatic environment. Bengtsson *et al.* (2004) assessed *Daphnia pulex* feeding behaviour in the presence of pure glyphosate, using changes in density of algae *Scenedesmus spp.* as

a surrogate for the feeding behaviour. They reported a 40% decrease in feeding after 3 days of exposure to 50 mg l^{-1} , and suggested that glyphosate may have potential for trophic effects.

Fish behaviour and the effects of glyphosate-based herbicides have been assessed using a variety of sensitive endpoints, including reproductive displays and preference/avoidance response. Adult zebrafish (*Danio rerio*) were tested as either naïve or pre-exposed to herbicide mixtures containing pure glyphosate, at concentrations equivalent to those detected in the natural environment ($0.26\text{--}309 \text{ ng l}^{-1}$), to determine if fish would avoid the herbicide and if exposure would alter attraction to L-alanine, a proxy for the presence of food (Tierney *et al.*, 2011). Zebrafish was found to spend more time in the regions of herbicide pulse addition and this response was independent of previous experience. Attraction to food cues was increased in exposed fish, suggesting a link between the energetic costs of contaminant exposure and compensatory increase in food requirement. Juvenile rainbow trout (*Oncorhynchus mykiss*) only avoided RU contaminated water at concentrations in excess of $10 \text{ mg a.i. l}^{-1}$, whereas exposure at $100 \mu\text{g l}^{-1}$ led to altered L-histidine preference behaviour as well as hypoactivity, suggesting RU inhibited the ability to react normally to chemical stimuli (Tierney *et al.*, 2007). The mechanism of olfactory recognition of food and the effect of glyphosate herbicide exposure on the ability of fish to recognize odorant cues can be assayed using electro-olfactogram (EOG), wherein evocation of the EOG signals the detection of an odorant. Among the pesticides tested, only RU was shown to evoke EOG's, demonstrating trout have the ability to sense the presence of RU in the aquatic environment.

Grey treefrogs (*Hyla versicolor* and *Hyla chrysoscelis*) were exposed to RU at a concentration of $2.4 \text{ mg a.e. l}^{-1}$ to determine if this amphibian species could detect glyphosate-based herbicides in the environment and subsequently avoid contaminated ponds, preferring instead to oviposit in control ponds (Takahashi, 2007). Grey treefrogs unambiguously avoided ponds contaminated with RU, choosing instead control water ponds or ponds with predator cues, a result that suggests grey treefrogs are not only able to detect the presence of RU in the environment, but that they actively avoid contaminated waters.

Ecosystem Changes

While tests with single species provide valuable information regarding the toxicity of chemical contaminants, understanding of the effects at the ecosystem level of organization is essential to better understand the interactions between the organisms, the environment, and the chemical. Several environmental factors have been observed to alter glyphosate-based herbicide toxicity, including pH (Tsui and Chu, 2003), developmental stage (Howe *et al.*, 2004), and the presence of predator cues (Relyea, 2005c). Ecosystem studies allow elucidation of top-down and bottom-up effects of glyphosate-based herbicide exposure and provide researchers and policy makers with a more relevant, real world perspective on the overall effects of contamination.

Aquatic microorganisms are vital players in ecosystem function through nutrient cycling, decomposition as well as primary production. The herbicidal action of glyphosate is due to its ability to disrupt the shikimic acid pathway (Fig. 2), a biochemical pathway present only in plants and some microorganisms. Thus, exposure to glyphosate-based herbicides in the aquatic environment might be expected to have effects

on survival and growth of some aquatic microorganism populations at environmentally relevant concentrations (Chan and Leung, 1986).

Acute exposure to Rodeo® herbicide, a glyphosate-based herbicide lacking surfactant, stimulated primary productivity of phytoplankton when exposed in a laboratory setting at concentrations as low as 0.125 mg l^{-1} (Schaffer and Sebetich, 2004). Similar results were observed in mesocosm studies with RU, at increased time and rate of exposure ($3.8 \text{ mg l}^{0.01}$) (Relyea, 2005a). RU also altered marine microbial community composition at concentrations as low as $1 \mu\text{g l}^{-1}$ (Stachowski-Haberhorn *et al.*, 2008). In freshwater systems, chronic exposure to glyphosate-based herbicides in surface water at high levels (8.0 mg l^{-1}) stimulated eutrophication by increasing total phosphorus and favoring the growth of cyanobacteria over periphyton, altering mesocosm typology (Perez *et al.*, 2007; Vera *et al.*, 2010). In contrast, when the exposures were decreased to more relevant environmental concentration in freshwater ($6.9 \mu\text{g l}^{-1}$), the effect on primary production was negligible (Pesce *et al.*, 2009; Relyea and Jones, 2009).

Differences in species sensitivity to glyphosate-based herbicide exposure can lead to decrease in ecosystem species richness, a conclusion based on experiments conducted in outdoor mesocosms (Relyea, 2005a). These simple wetland communities allow the study of the effects of glyphosate-based herbicides on food webs, species richness across trophic levels, and predator prey relationships. The presence of predator cues have been suggested to increase the toxicity to RU herbicide to tadpoles under certain laboratory conditions (Relyea, 2005c), although this increase in toxicity was not observed under the more realistic conditions of outdoor mesocosms. Instead, the presence of predator cues resulted in migration of tadpoles to regions of the water column with lower herbicide concentrations, which developed as a result of stratification of the herbicide within the water column (Relyea, 2012). This same study revealed that glyphosate-based herbicides may alter tadpole morphology, in much the same fashion as adaptive morphological changes occur as a result of predator cues (Piersma and Drent, 2003).

With the bulk of our current knowledge focused on acute and chronic laboratory exposures, there exists a need for broader, population and ecosystem level investigations of a variety of glyphosate-based herbicide formulations. Additionally, further research should be conducted on chemical mixtures of glyphosate and other contaminants present in the aquatic systems, including pharmaceuticals and other pesticides, to determine the extent of environmental impacts of contamination of aquatic ecosystems by glyphosate and glyphosate-based herbicides.

Risk Assessment

Glyphosate-based herbicides are intended for control of nuisance plant species, in both terrestrial applications and for the control of emergent aquatic species. Their use has increased significantly over the past decade, as has our understanding of unintended consequences of non-target species exposure. Detailed risk assessments have been completed in the past for expected environmental exposure to glyphosate-based herbicides in the terrestrial and aquatic environments (Giesy *et al.*, 2000; Solomon and Thompson, 2003). These reviews provide an excellent description regarding the risk assessment methodology used and they address species beyond the scope of this

review. However, given the growth of glyphosate-dependent agriculture and the more widespread use of glyphosate, it has become necessary to reevaluate the likelihood of adverse effects occurring as a result of unintended exposure in the environment and update the risk assessment.

The risk assessment process is designed to quantify the likelihood of adverse effects occurring to non-target species in the environment, given current application rates, environmental levels and species specific sensitivities. Given the wide variety of commercial formulations currently available, each with a unique level of toxicity, only the most toxic formulations will be selected for assessment, as this will provide the most conservative estimates of risk. Accordingly, only formulations containing POEA surfactants will be considered, as the active ingredient and those formulations without surfactants have been ranked to have minimal toxicity (Folmar *et al.*, 1979; Tsui and Chu, 2003).

Characterization of the potential risk to non-target aquatic organisms will be conducted using hazard quotient analysis (HQ). This value is determined by dividing the predicted exposure level by a toxicity reference value (TRV). The TRV is defined as the maximum exposure level at which deleterious effects will not occur towards populations of exposed organisms (Giesy *et al.*, 2000). Primarily, this value corresponds to the median lethal concentration, median effective concentration, or NOEC in the most sensitive species within the taxa being investigated. If these are not available, then a no-mortality level (NML) is applied after adjustment by a safety factor of 5.

To update the calculations of Giesy *et al.* (2000), in cases where their TRV value remains the lowest exposure level, these values were maintained in the calculation of HQ. Otherwise, more recently obtained values were utilized. As well, the predicted exposure level for the aquatic environment calculated by Giesy *et al.* (2000) of 0.406 mg a.e. l⁻¹ was utilized in the HQ calculations, as this value was determined from recommended agricultural application rates with no foliar interception. Though

higher values have been observed in the environment on rare occasion, this value is still well in excess of measured environmental concentrations in the majority of monitoring studies (Table 3). Additionally, using the same value as determined previously allows for comparison between the HQ values determined in 2000 and the new HQ values determined given subsequent toxicity estimates.

The TRV values for aquatic microorganisms and fish remained the same as those considered in Giesy *et al.* (2000), as species with greater sensitivity have not been identified. The reference value for aquatic invertebrates has been decreased based on the study by Tsui and Chu (2003), who identified *A. tonsa* as a more sensitive species than the previous representative species *D. magna*. The TRV for amphibians was also adjusted, given the findings of Relyea and Jones (2009) that identified *R. catesbeiana* as the most sensitive larval anuran (Table 5). Even when applying these new TRV values, the HQ values for all taxa calculated remained below 1.0, which allows for the conclusion that the probability of population or community level effects occurring due to expected environmental exposures to glyphosate-based herbicides is essentially zero.

The selection of an endpoint to use when calculating HQ can be difficult, as it is challenging to define what acute or chronic endpoints correspond to population level effects. As research has advanced beyond relatively simple measures of acute lethality, it is prudent to consider more sensitive endpoints, such as morphological or behavioural changes. These changes tend to occur at concentrations well below levels known to induce mortality and are likely more relevant when we consider predicted environmental exposures. In addition to considerations of endpoint selection, the predicted environmental exposure value used in the HQ calculation can be adjusted to use actual environmental levels measured in the habitat of the taxa being investigated. For example, the use of stream data to calculate amphibian HQ, when the majority of amphibians do

Table 5. Summary of acute toxicity reference values (TRV) for aquatic taxa used in hazard quotient (HQ) calculations

Species	TRV ($\mu\text{g a.e. l}^{-1}$)	Endpoint	Reference	Predicted exposure ($\mu\text{g a.e. l}^{-1}$)	Exposure Reference	HQ
Aquatic Microorganisms						
<i>Selenastrum capricornutum</i>	730	NOEC ^c	LISEC (1989) ^e	406	Giesy <i>et al.</i> , 2000	0.56
<i>Selenastrum capricornutum</i>	730	NOEC	LISEC (1989)	1950 ^a	Thompson <i>et al.</i> , 2004	2.67
Aquatic Invertebrates						
<i>Daphnia magna</i>	1900	NOEC	Folmar <i>et al.</i> (1979)	406	Giesy <i>et al.</i> , 2000	0.21
<i>Acartia tonsa</i>	1770	48 hr LC ₅₀ ^d	Tsui and Chu (2003)	406	Giesy <i>et al.</i> , 2000	0.23
<i>Acartia tonsa</i>	1770	48 hr LC ₅₀	Tsui and Chu (2003)	1950 ^a	Thompson <i>et al.</i> , 2004	1.10
Fish						
<i>Oncorhynchus mykiss</i>	840	NOEC	Folmar <i>et al.</i> (1979)	406	Giesy <i>et al.</i> , 2000	0.23
<i>Danio rerio</i>	13	Genotoxic	Guilherme <i>et al.</i> (2010)	700 ^b	Peruzzo <i>et al.</i> , 2008	53.8
Amphibians						
<i>Itoia moorei</i>	1600	NOEC	Mann and Bidwell (1999)	406	Giesey <i>et al.</i> , 2000	0.25
<i>Rana catesbeiana</i>	800	96 hr LC ₅₀	Relyea and Jones (2009)	406	Giesy <i>et al.</i> , 2000	0.51
<i>Rana catesbeiana</i>	800	96 hr LC ₅₀	Relyea and Jones (2009)	1950 ^a	Thompson <i>et al.</i> , 2004	2.44

^aValue corresponds to measured levels in forest wetland.

^bValue corresponds to measured levels in surface stream.

^cNo observed effect concentration.

^dMedian lethal concentration.

not inhabit these lotic systems, decreases the relevance of the HQ calculations.

Using current environmental exposures and more sensitive endpoints of effects, the HQ may be recalculated as shown in Table 5. The use of more realistic environmental exposure concentrations (Peruzzo *et al.*, 2008; Thompson *et al.*, 2004) has profound effects on the HQ for aquatic microorganisms, aquatic invertebrates, and amphibians, elevating the HQ value above 1.0. At this value the results suggest further investigation is required to determine the magnitude of the risk to these aquatic taxa. The HQ analysis for fish uses a lower TRV, corresponding to genotoxicity in European eel (Guilherme *et al.*, 2010), rather than the toxicity value for rainbow trout, derived from Folmar *et al.* (1979). The new HQ value of 53.8 suggests that there is significant risk associated with predicted environmental exposures, if we consider a more sensitive, sublethal, endpoint.

The use of observed environmental concentrations and more sensitive endpoints in the calculations of risk assessment improves the overall relevance and impact of these calculations. Under conditions found in the environment, aquatic organisms that are likely to be exposed to glyphosate-based herbicides, will be exposed to fluctuating concentrations depending on the environmental conditions and season. It is important that assessment of risk be highly conservative by basing calculations on the most sensitive species as well as highly sensitive endpoints not previously used.

Conclusions

The development and cultivation of multiple glyphosate tolerant commercial crops combined with an increase in the production of generic glyphosate formulations has significantly increased the use of this herbicide in the last decade. Developed to provide broad spectrum weed control, glyphosate has become the most widely used herbicide worldwide. As a result, the likelihood of contamination of surface water and exposure to non-target species has also increased, justifying the need for an updated review of the potential impacts of exposure to glyphosate-based herbicides on species in the aquatic environment.

Glyphosate-based herbicides are currently available in a wide variety of formulations, each based on the same active ingredient but with a unique combination of surfactants, adjuvants, and other chemicals whose identity is often proprietary. Across the spectrum of organisms likely to be exposed to glyphosate in the aquatic environment, it has been shown that sensitivity to glyphosate and the constituents of commercial formulas is highly species-specific. Often, there is a greater difference between the sensitivity of two related species than between species with vast taxonomical separation (Lipok *et al.*, 2010; Moore *et al.*, 2012). Across multiple phyla, studies have shown that the primary source of the toxicity of glyphosate-based herbicides can be attributed primarily to the surfactant portion of the formulation. The proprietary nature of these mixtures often makes it difficult to assign toxicity to a particular chemical, but the most commonly tested surfactant is POEA which is still used extensively in multiple commercial formulations. With such a diversity of formulations in agricultural use, it is reasonable to conclude that aquatic species may be exposed to constituents of multiple formulations simultaneously.

Of the organisms studied amphibians are particularly sensitive to environmental exposure, owing to their unique physiology as

well as their dependence on the aquatic environment during early development, concurrent timing of reproduction with glyphosate applications, and their distribution within regions of high glyphosate use. Research should continue to focus on population level effects of glyphosate-based herbicide exposure, as these findings provide more accurate estimates of the true effects of glyphosate use, in the environment.

Several modes of action have been investigated regarding the source of glyphosate-based herbicide toxicity in non-target organisms, including induction of oxidative stress damage, acetylcholinesterase inhibition, and genotoxicity. As there is currently no consensus on a single mechanism of glyphosate-based herbicide toxicity, it is likely that multiple mechanisms exist, depending on the particular combination of formula and species. There remains a need to identify the mode of action of glyphosate-based herbicide toxicity across a range of organisms, given that the probability of aquatic exposure will increase as the use of this herbicide continues to expand globally.

The hazard assessment process for glyphosate-based herbicides has primarily relied on relatively insensitive, mortality endpoints for calculation. Though these endpoints suggest population level effects are possible, they are often only found at concentrations well above those that are responsible for behavioural, cellular and metabolic alterations. When these endpoints are investigated, and combined with environmentally relevant predicted exposure concentrations, it becomes clear that more investigation is required to determine the long-term effects of glyphosate-based herbicide exposure in the aquatic environment.

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