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Ichthyophthirius multifiliis and *Tetrahymena thermophila* Tolerate Glyphosate But Not a Commercial Herbicidal Formulation

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Freshwater habitats are routinely exposed to glyphosate because it is useful in controlling aquatic weeds and is a broad-spectrum, non-selective algacide. In the United States, approximately 848,000 kg (9,350 tons) of glyphosate in a variety of formulations–including Roundup®–are applied each year (Consumer Factsheet on Glyphosate 2001). Glyphosate activity is reduced by adsorption to sediment (Zaranyika and Nyandoro 1993). Glyphosate by-products are found when soil contains live microorganisms, although cultured microorganisms have a limited tolerance for glyphosate (Table 1).

Ciliated protozoa (*Ciliophora*) are prevalent in freshwater aquatic systems, and their sensitivity to glyphosate is not known. One such ciliate, *Tetrahymena pyriformis*, has been selected for standard determinations of substance toxicity in European freshwater ecosystems (Girling et al. 2000). *T. pyriformis* sensitivity to glyphosate *per se* is unknown (http://www.epa.gov/ecotox/), but in laboratory tests it avoids swimming into capillaries that contain Roundup (Roberts and Berk 1993). Another ciliate found in freshwater aquaculture systems is the obligate fish parasite *Ichthyophthirius multifiliis*. Few effective chemotherapeutic agents are available for use against *Ichthyophthirius* in farmed fish. Parasitic ciliates *Plasmodium*, *Toxoplasma*, and *Cryptosporidium*, which cause disease in humans, are sensitive to glyphosate (Roberts et al. 1998) and, like these parasites, *I. multifiliis* has a complex developmental cycle and infects vertebrate hosts. Thus, glyphosate may be useful for controlling *I. multifiliis* in aquaculture. The sensitivity of *I. multifiliis* to glyphosate is unknown. This study tests the sensitivity of *Tetrahymena* and *I. multifiliis* to glyphosate and Roundup.

MATERIALS AND METHODS

Tetrahymena thermophila strain CU427 (wild type) was maintained prior to testing in stationary culture. Proliferating *T. thermophila* was passaged for testing by culture in axenic proteose peptone media (SPP) containing 11 m*M* glucose, 5 U/mL penicillin, 5 µg/mL streptomycin, and 0.5 µg/mL Fungizone (Orias et al. 2000). Both proliferative and conjugative phase *T. thermophila* were examined. To obtain conjugative phase *T. thermophila*, cultured organisms were incubated in

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Table 1. Tolerance of organisms for glyphosate (Carlisle and Trevors 1988).

Cell type	Growth Inhibiting Dose (m*M*)
Fungi	
Chaetomium globosum	0.20
Aspergillus niger	0.08
Stachybotrys chartarum	0.004
Gliocladium roseum	none (0.08)
Trichoderma viride	none (0.08)
Saccharomyces cerevisiae	none (2.0)
Other Eukaryotes	
Chlorella sorokiniana	0.0177
Euglena gracilis	1.2
Bacteria	
Escherichia coli	0.002
Pseudomonas aureofaciens	0.002
Rhizobium japonicum	0.01
Salmonella typhimurium	0.002

10 m*M* Tris-HCl buffer pH 7.4 for 3 days (Orias et al. 2000). Unlike *T. thermophila*, *I. multifiliis* strain G5 was not axenic. *I. multifiliis* was maintained on *Ictalurus punctatus* (channel catfish) in tap water that was filtered through charcoal (CFW). *I. multifiliis* trophonts feeding on the fish were flushed off with CFW over a screen and pipetted into 24-well microtiter plates for study (Everett et al. 2002). Three developmental forms of *I. multifiliis* were tested for glyphosate sensitivity: trophonts, encysted tomonts, and free-swimming infectious theronts. Three to 5 trophonts were placed in each well; untreated controls produced on the order of 100 theront progeny per trophont in a 24-hr developmental cycle. Glyphosate preparations were added either when trophonts were plated or after trophonts had been allowed to adhere and encyst for 5 hr.

CFW was checked for neutral pH and low nitrates before use and sterilized by autoclaving for harvesting and *in vitro* development of *I. multifiliis*. CFW mineral content was determined using Inductively Coupled Argon Plasma (ICP) analysis of 31 minerals at the Chemical Analysis Laboratory (University of Georgia Research Services). Depending on the element, ppm readings were accurate to one or two decimal points. Seven minerals were found to be present in all CFW samples. Representative values of two averaged CFW assays (+/- 0.3 ppm) were Ca^{++} (12.1 ppm), Mg^{++} (2.6 ppm), K^+ (2.5 ppm), Na^+ (5.3 ppm), Cl^- (5.4 ppm), F^- (0.9 ppm), and $S0_4^=$ (11.2 ppm). Also occasionally present were: Cu^{++} (0.02 ppm), Zn^{++} (0.1 ppm), Ni^{++} (0.02 ppm), $P0_4^=$ (1.3 ppm), and nitrate (0.5 ppm). (Not seen: Ag, Al, As, B, Ba, Be, Cd, Co, Cr, Fe, Mn, Mo, Pb, Sb, Se, Si, Sn, Sr, Ti, U, W.) In ICP, elements are ionized in a hot, electron-rich environment, then emit characteristic wavelengths of light in proportion to the amount present.

Pestanal, a technical-grade, acidic glyphosate (N-(phosphonomethyl)glycine, 169.1 g/mole, Sigma-Aldrich) was added to CFW (40 mg/2 mL) and brought into solution by neutralization with NaOH (final pH, 7.5; final concentration, 120 m*M*). Roundup Weed and Grass Killer Concentrate, 25% glyphosate isopropylamine salt (225.1 g/mole; 1.11 *M* glyphosate) in water/surfactant (Monsanto), was diluted in CFW, SPP, or 10 m*M* Tris-HCl pH 7.5 for testing. The lowest adverse effect concentration (LOAEC) and the no observed adverse effect concentration (NOAEC) were determined for equivalent glyphosate concentrations of both preparations. Beginning with 120 m*M* Pestanal or with 10 µL Roundup in 1 mL, twelve 1:1 serial dilutions of Pestanal in CFW or of Roundup in CFW, SPP, or Tris were combined 1:1 with ciliates in 24-well microtiter plates at room temperature (23 °C). Acute effects of these solutions on *I. multifiliis* and *T. thermophila* were assessed by light microscopy immediately and after 5, 15, and 45 min exposure. Killing of ciliates was determined by the inability of ciliates to undergo development or replication, by the cessation of ciliary movement with ensuing disintegration, or, in the case of *I. multifiliis*, by bacterial overgrowth. Replicate experiments were carried out for each dilution on four separate occasions, and each experiment included untreated controls. The concentration test range for Roundup was 11 m*M* glyphosate to 5 µ*M* glyphosate. The concentration test range for Pestanal was 60 m*M* to 30 µ*M* glyphosate.

RESULTS AND DISCUSSION

T. thermophila proliferated with no apparent mortality for up to 48 hr in 60 m*M* neutralized, technical grade glyphosate, 50% SPP (Table 2). In Roundup with SPP, *T. thermophila* survived reproducibly only in 0.34 m*M* glyphosate or less (Table 3). Conjugative and proliferative phases showed some adaptive capabilities and differences, however 100% of each well of ciliates typically either survived or suffered mortality. Visible motion and ciliary activity were not revived by dilution of herbicide-containing media after one hour (not shown). *T. thermophila* in Roundup tolerated an equivalent glyphosate concentration that was approximately 0.5% that of technical grade glyphosate.

In 30 m*M* neutralized, technical grade glyphosate, free-swimming and encysted *I. multifiliis* suffered 100% mortality in less than 5 min, whereas untreated *I. multifiliis* controls continued to develop and proliferate for over 24 hours (Table 4). Theronts stopped moving in 30 m*M* technical grade glyphosate and were not revived by dilution after one hour (not shown); other developmental forms did not adhere to the microtiter plates or encyst, and 24 hr later were undergoing degradation, with bacteria often present. Those that had been given time to adhere prior to treatment with 30 m*M* glyphosate typically became detached with the treatment. In 15 m*M* technical grade glyphosate, hatching of progeny theronts was delayed for 24 hr; theronts tolerated 15 m*M* technical grade glyphosate but moved very slowly. Glyphosate ≤7.5 m*M* had little apparent effect on *I. multifiliis*.

Table 2. Response of proliferating *T. thermophila* CU427 to glyphosate.[1]

	Control (CFW)	Pestanal (60 m*M*)	Roundup (0.69 m*M*)	Roundup (0.34 m*M*)
5 min	swims	swims	swims	swims
15 min	swims	swims	1 ciliate in 10 moves	clumped, swims
1 hr	swims	swims	bloated	clumped, swims
2 hr	swims	swims	bloated, 1 ciliate in 100 moves	clumped, swims
4 hr	swims	swims	bloated, 1 ciliate in 500 swims	swims
8 hr	swims	swims	clumped, 1 swims	swims
24 hr	swims[2]	swims[2]	dead[2]	swims[2]
48 hr	thousands swim[2]	thousands swim[2]	still dead[2]	thousands swim[2]

[1] Ciliate counts are approximate; each well had 500 ciliates in SPP. Equivalent glyphosate concentrations are shown. Morphology and viability were estimated by light microscopy.
[2] After 8 hr, these wells were diluted 1:1 with 20 mM Tris-HCl pH 7.4 for overnight incubation.

Table 3. Glyphosate-equivalent concentrations affecting proliferative and conjugative *T. thermophila* CU427, after 24 hr.

	Proliferating, Roundup/SPP	Conjugative, Roundup/SPP	Conjugative, Roundup/Tris	Both phases, Pestanal/CFW
LOAEC	0.34 m*M*[1]	0.69 m*M*	0.34 m*M*	none
NOAEC	0.17 m*M*	0.34 m*M*	0.17 m*M*	60 m*M*

[1] No effect after 4 hr.

Roundup dilutions containing as little as 0.34 m*M* glyphosate reproducibly killed 100% of all *I. multifiliis* developmental forms (Table 4). Exposure of encysted *I. multifiliis* to Roundup containing 0.34 m*M* glyphosate for just 10 min made them incapable of producing theronts. Roundup dilutions that contained no more than 0.09 m*M* glyphosate allowed survival of encysted and hatching theronts, but adherence of trophonts was problematic and development proceeded within aberrant, non-adherent grape-like clusters. Directly treated theronts suffered 100% mortality in diluted Roundup containing 0.09 m*M* glyphosate, but did survive 0.04 m*M*. Roundup was 100-times more lethal to *I. multifiliis* than were equivalent glyphosate concentrations of neutralized Pestanal.

We concluded that *T. thermophila* would likely survive herbicidal applications of technical grade glyphosate in aquatic systems and that *I. multifiliis* would not be controlled by glyphosate. In contrast, at herbicidal glyphosate concentrations (6 oz/gal or 4.7% x 1.11*M* = 52 m*M*), Roundup would kill both *T. thermophila* and *I. multifiliis*. Roundup was at least 100-times more lethal than technical grade

Table 4. Glyphosate-equivalent concentrations effects on developing *I. multifiliis*.

	Developmental Stage	Roundup		Pestanal
		Pre-adhered	Not Pre-adhered	
LOAEC	1-5 hr, trophonts	--	0.34 m*M*	30 m*M*
	2-5 hr, adherence	--	5 μ*M*	30 m*M*
	4-20 hr, cyst development	0.34 m*M*	0.17 m*M*[1]	15 m*M*
	20-25 hr, hatching	0.34 m*M*	0.09 m*M*	15 m*M*
	20-30 hr, theronts	0.17 m*M*	0.09 m*M*	15 m*M*
NOAEC	1-5 hr, trophonts	--	none	15 m*M*
	2-5 hr, adherence	--	none	15 m*M*
	4-20 hr, cyst development	0.17 m*M*	0.09 m*M*	7.5 m*M*
	20-25 hr, hatching	0.17 m*M*	0.04 m*M*	7.5 m*M*
	20-30 hr, theronts	0.09 m*M*	0.04 m*M*	7.5 m*M*

[1] Progeny theronts resembled shapless potatoes or multi-headed monsters.

glyphosate to *T. thermophila* and *I. multifiliis*, based on equivalent glyphosate concentrations. These finding were consistent with observed differences in the lethality of glyphosate and Roundup in salmon, trout, and carp (Table 5). Roundup® herbicidal formulations are highly toxic to fishes (salmon, trout, bluegills, catfish, fathead minnows), tadpoles, and aquatic invertebrates (daphnids, scuds, midge larvae, and mayfly nymphs) (Folmar et al. 1979; Mann and Bidwell 1999) (Table 5). The surfactant in Roundup and not the glyphosate may be responsible for this killing (Mitchell et al. 1987; Servizi et al. 1987).

Glyphosate-containing herbicides inhibit the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (3-phosphoshikimate 1-carboxyvinyltransferase). This enzyme is found in the biosynthetic pathway leading to aromatic amino acids, folate, and *p*-aminobenzoate. Glyphosate toxicity studies have played a key role in demonstrating the presence of this pathway in algae, higher plants, bacteria, *Plasmodium*, *Toxoplasma*, and *Cryptosporidium* (Table 5). Our finding that relatively high technical-grade glyphosate concentrations had no effect on *T. thermophila* and *I. multifiliis* suggests that these protozoa do not use the shikimate pathway or that glyphosate is not taken up by these ciliates. Use of glyphosate is likely to increase as genetically engineered Roundup Ready® crops become popular. These findings increase our understanding of glyphosate sensitivity in ciliated protozoa and permit the strategic application of glyphosate-containing herbicides in and near aquaculture systems.

Table 5. Tolerance of organisms for glyphosate (isopropylamine salt or neutralized acid) or for Roundup.

Cell type	Glyphosate Tolerated	Glyphosate Toxic Dose	Roundup-Glyphosate Toxic Dose	Reference
Tetrahymena thermophila CU427	60 m*M*	none found	1.38 m*M*	this study
Ichthyophthiriius multifiliis theronts	15 m*M*	30 m*M*	0.17 m*M*	this study
Ichthyophthiriius multifiliis tomonts	3.75 m*M*	30 m*M*	0.34 m*M*	this study
mammalian cells HFF	9 m*M*	12.5 m*M* [1]		Roberts et al. 1998
mammalian cells MBDK	9 m*M*	12.5 m*M* [1]		Roberts et al. 1998
Toxoplasma gondii RH in vitro	2 m*M*	5 m*M*		Roberts et al. 1998
Cryptosporidium parvum in vitro	4.5 m*M*	8 m*M* [1]		Roberts et al. 1998
Cryptococcus neoformans	1.1 m*M*	4.4 m*M*		Nosanchuk et al. 2001
Pneumocystis carnii	1 m*M* [1]			Chin et al. 1999
Plasmodium falciparum in vitro		1.08 m*M*		Roberts et al. 1998
Sarcocytis neurona		1.5 m*M*		Marsh et al. 2001
fathead minnow	>4.4 m*M* [2]			Beyers 1995
carp		2.8 m*M* (48 hr) [2]		Neškovic et al. 1996
			<0.9 m*M* (1 hr) [2]	Szarek et al. 2000
rainbow trout		0.47 m*M*	0.036 m*M* [2]	Servizi et al. 1987
		0.53 - 1.7 m*M*	0.025 - 0.08 m*M*	Mitchell et al. 1987
coho and sockeye salmon		0.47 - 0.62 m*M*	0.036 m*M* [2]	Servizi et al. 1987
		0.3 - 1.7 m*M*	0.025 - 0.08 m*M*	Mitchell et al. 1987
channel catfish			0.02 m*M* (48 hr) [2]	Abdelghani et al. 1997
bluegill sunfish			0.02 m*M* (48 hr) [2]	Abdelghani et al. 1997
crayfish			145 m*M* (48 hr) [2]	Abdelghani et al. 1997

[1] 30 – 80% growth inhibition or reduction, depending on species.
[2] LC_{50}, m*M* glyphosate.

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