Report on the Proposed Use of AquaMaster in Cuatro Ciénegas, Coahuila, México as a Mechanism to Control *Arundo donax*.

Laboratorio de Evolución Molecular y Experimental, Instituto de Ecología, Universidad Nacional Autónoma de México.

Drs. Luisa I Falcón, Luis E Eguiarte, Valeria Souza,

The synthetic compound glyphosate (N-phosphonomethylglycine) is the active ingredient in the herbicides Roundup™, Touchdown™ and AquaMaster. Glyphosate is a competitive inhibitor of the monomeric enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPs), one of two enzymes in the class of the enolpyruvyltransferases (Hollander & Amrhein, 1980; Steinrucken & Amrhein, 1980; Rubin, Gaines & Jensen, 1982). This class of enzymes shares a unique structure containing two globular domains composed of beta sheets and alpha helices, which form an inverse alpha/beta barrel. (Amrhein, et al. 1980). EPSP synthase is involved in the shikimate pathway, using phosphoenolpyruvate (PEP) to convert shikimate-3-phosphate (S3P) to 5-enolpyruvyl-3-shikimate phosphate (Fig. 1), a precursor to the majority of aromatic compounds produced in the cell, including the aromatic amino acids. The compounds produced in this pathway constitute as much as 35% or more of the dry mass of plants (Herrmann & Weaver, 1999; Schönbrunn et al., 2001).

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Figure 1. Convertion of shikimate-3-phosphate to 5-enolpyruvyl-3-shikimate phosphate by action of EPSP synthase.

Glyphosate acts as a transition-state analogue effectively shutting down the Shikimate pathway. Glyphosate is believed to be a good inhibitor since it resembles the transition state

of the reaction (Fig. 2). Glyphosate deregulates feedback inhibition of the first enzyme in the pathway by a near-end product of the pathway and as a result, there is an unregulated flow of carbon that causes high levels of S3P to accumulate (Herrmann & Weaver, 1999).

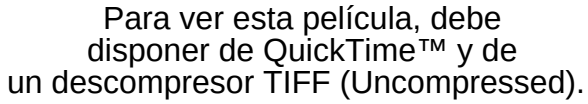


Figure 2. Structure of phosphoenol pyruvate (PEP) oxonium ion and glyphosate.

Glyphosate is the world's largest selling herbicide used in weed control programs (Monsanto, 1992). Its use has been justified by its relatively less harmful effect considering its low toxicity to animals, being the shikimic acid pathway exclusive of plants, fungi and bacteria (Herrmann & Weaver, 1999). The accompanying surfactants used with glyphosate, in order to prevent the formation of hydrophobic droplets that roll off surfaces, constitute the major environmental problem considering toxicity only. Surfactants (Surface Active Agents) are substances that will reduce the surface tension of water due to their molecular structure, which includes hydrophilic and hydrophobic components (Rubin, Gaines & Jensen, 1982).

AguaMaster is composed primarily of glyphosate (glyphosate acid 74%) and water, but is used with ammonium sulfate as surfactant at a concentration of 150 mM. In solution, the ammonia separates from the sulfate and constitutes and entry of reduced nitrogen to the system. Although glyphosate is not severely toxic it is extremely harmful to the environment since it is non-specific, thus killing non-target vegetation, bacteria and fungi.

Cuatro Cienegas, Coahuila (CCC) is a site of megadiversity and high endemicity of plants, animals and bacteria (Souza et al., 2006). A great diversity of bacteria have been identified in its pozas and streams using molecular methods based on the amplification of the *16S rDNA* region (Souza et al., 2006). Recent work (Falcon et al., *in revision*) has identified the role of cyanobacteria as major N_2 fixers in both microbial mats and stromatolites of CCC. N_2 fixing bacteria are key players in the environment since reduced forms of N limit biological growth (Capoen 1993). Further, Falcon et al (*in revision* 2006)

show that cyanobacteria are major components of microbial consortia in CCC. Previous research has shown that cyanobacteria that produce scytonemin will be less resistant to UV radiation when treated with glyphosate (Sinha et al., 2003). Some of the cyanobacteria identified in CCC (e.g. *Calothrix* spp) have mouscillage sheeths and UV protecting pigments (e.g. scytonemin) that allow the establishement of microbial consortia (Sheridan, 2001). Further, the microbial communities of CCC are believed to be at the base of the trophic web with cyanobacteria as the primary producers, where algae are not very abundant due to the strong limitation of P present in waters of CCC (Souza et al., 2006).

In order to test if microbial communities (stromatolites) from CCC would be affected by glyphosate, we carried out a series of experiments that are described in the following section.

Experimental design.

Sampling of stromatolites associated with Arundo plants took place in fall of 2005 in the region of Saca Salada in Rio Mezquites in Cuatro Cienegas. Samples (~1 g) were treated with different concentrations of glyphosate (AquaMaster) with the recommended proportion of ammonium sulfate were incubated at 29 °C in a diurnal cycle of 12:12 h light: dark.

Glyphosate concentations:

1. 10mL/L
2. 1mL
3. 0.1mL/L
4. *0.01mL/L*
5. *0.001mL/L*
6. *control (filterd water)*
7. *control*

The experiment was ended after one month by freezing the samples at –80 C.

Microscopy. Samples for each treatment were observed under the microscope using an Axioskop 40-Zeiss microscope under both direct light and with blue and green fluoresence.

Molecular analysis. DNA was extracted following a modified version of the protocol of Zhou (1996). Extraction buffer (100 mM TrisHCl, 1.5 M NaCl, 100 mM EDTA, 100 mM NaPhosphate, 1 % CTAB) was added to previously pulverized stromatolite samples, which were freezed and thawed to 65 C three times. Samples were then incubated with 300 µl lisozyme (30 mg/ml of 17000 µ/mg) for 30 min at 37 C. We added 100 µl proteinase K (10 mg/ml) and 1.5 ml SDS (20%) and left at 50 C over night. Samples were centrifuged at 6000rpm x 10 min and supernatant was collected. Samples were cleaned as many times as necessary with phenol: chloroform: isoamylalcohol (25:24:1) and supernatant was collected after centirfugation at 11,500rpm x 10 min. DNA was precipitated with 0.6 volume of isopropanol and 0.1 volume of sodium acetate 3M. DNA was further cleaned with ethanol and was finally diluted in water. A conserved region of ribosomal DNA (*16S rDNA*) was PCR amplified using the following protocol: 5 µl 10X buffer, 3.3 µl MgCl2, 4 µl dNTP mix, 1.5 µl each primer (F and R), 29.75 µl H2O, 0.2 µl Taq, 1.25 µl BSA, 2.5 µl DMSO.

In order to verify for false negative results, we proceeded to re-extract DNA with a series of modifications to the protocol, which included addition of incubation times, lisozyme and proteinase K, as well as re-extraction of pellets. We also followed the protocol of MoBio for DNA extraction from Soils, followed by phenol: chloroform cleanups. DNA was observed in agarose gels to help identify its quality. In case we obtained DNA that would not PCR-amplify we carried out serial dilutions (e.g. 1:1, 1:1-, 1:50), $MgCl_2$ curves (0-4 mM) and addition of BSA and DMSO (5%).

Results.

Microbial consortia in the stromatolites tested had a clear composition of both unicellular and filamentous cyanobacteria. We observed that cyanobacteria died after addition glyphosate disregarding the concentration used (Fig. 3). Our microscope observations were consistent with the above (Fig. 4a and b).

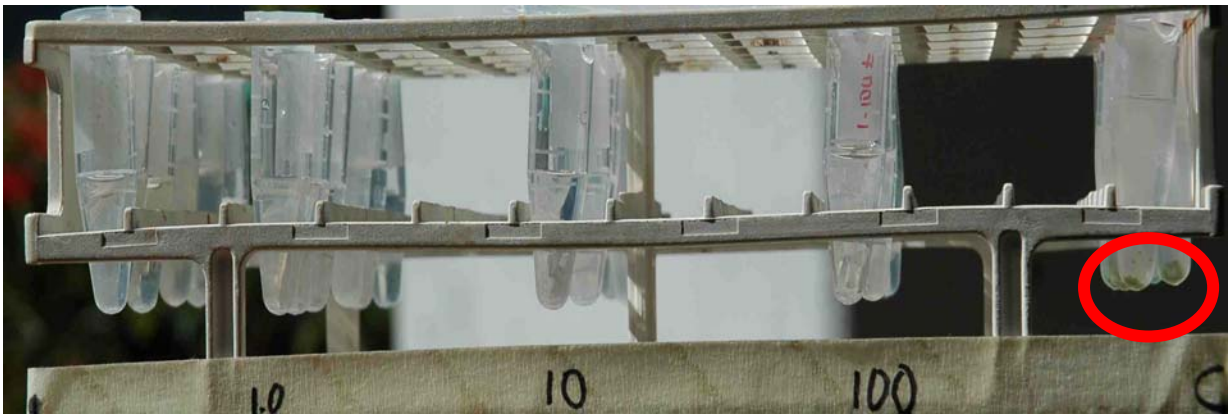


Figure 3. Experiment comparing growth of control (no glyphosate) vs glyphosate-treated samples (0.1-100% glyphosate). Observe the blue-green material in the control vs the murky-white matter in the treatments.

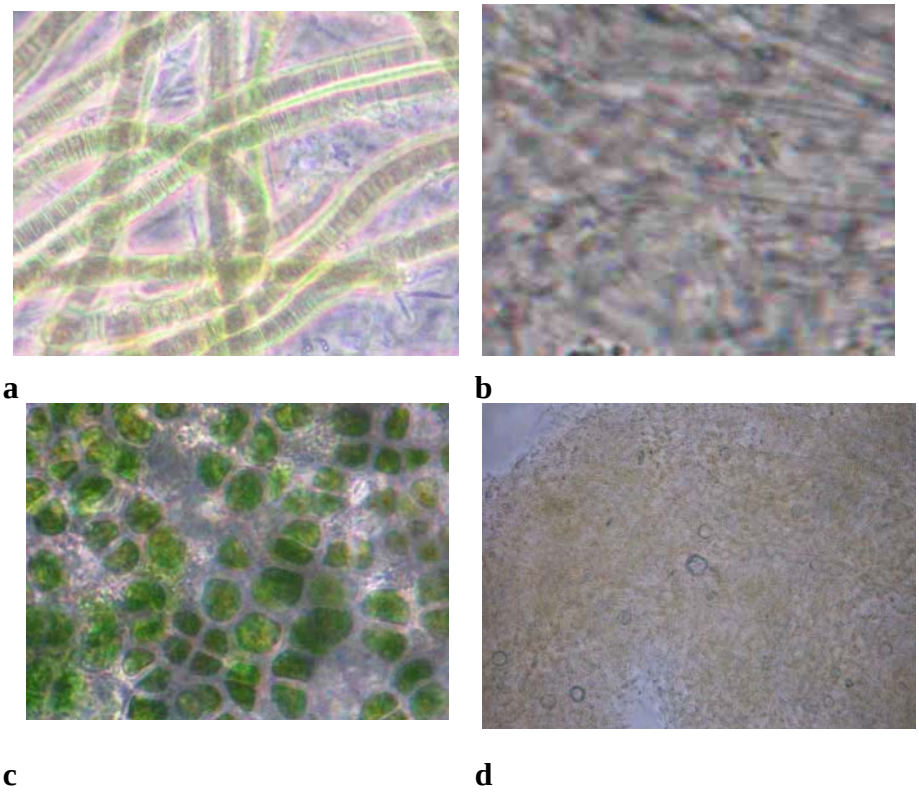
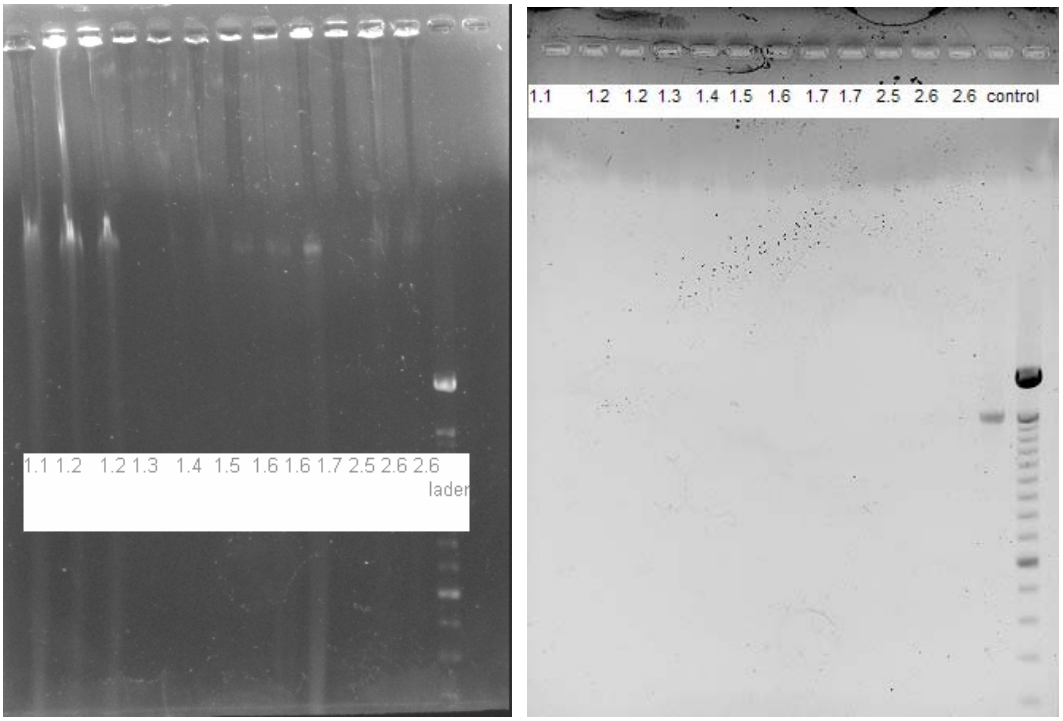


Figure 4. Microscope observations (40x) of filamentous (upper) and unicellular (lower) cyanobacteria treated with glyphosate (b, d) and their controls (a, c).

Further, our DNA analysis showed that stromatolite samples treated with glyphosate produced fragmented DNA that could not be amplified (Fig. 5).



a **b**

Figure 5. Extracted DNA (a) and PCR amplification (b) from samples treated with glyphosate and control at time 0 before application, The treated samples correspond to 2 times series after careful exposure of Arundo to glyphosphate at recommended dosis and applied by hand with a brush, 1 is after 3 weeks of application (November 2005), 2 is 3 months after application (February, 2006).

Figure 6. Experimental site and application of glyphosphate

Discussion.

The results presented in this report are preliminary but they consistently show the same trend of negative effect exerted by glyphosate over cyanobacterial-based microbial consortia from CCC. Glyphosate affects a critical metabolic pathway that is present not only in cyanobacteria but in plants and fungi, which are basic components of ecosystems. We observed with microscopy a negative effect over specific components of the community (cyanobacteria) and the degradated DNA of stromatolites after treatment with glyphosate further shows that this chemical is also affecting growth, and thus survival, of other bacteria present in stromatolites even after 3 months of application. Another concern of the potential use of glyphosate in this system is the liberation of phosphorous (P), which would facilitate the growth of competitive primary producers, which could change the community structure by out-competiting cyanobacteria and allowing the establishment of algae. Our data shows that glyphosate would harm microbial communities in CCC irreversibly since it is not target-specific, thus by trying to eliminate *Arundo donax* from the valley we would be affecting the entire ecosystem. If cyanobacteria are eliminated from CCC the organisms that are at the base of the trophic web via fixation of C and N would disappear. Previous studies have warned against the use of glyphosate in the environment. We cannot allow the use of an herbicide that will destroy the habitat of vertebrates, invertebrates, and that will affect the growth of bacteria.

We therefore strongly propose not to use glyphosate in CCC since it destroys whole ecosystems and we suggest for more *in depth* studies of the distribution of *Arundo* vs *Phragmites* that will allow for directed measures of control.

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