MODERN APPROACHES FOR THE STUDY OF *s*-TRIAZINE HERBICIDE BIOREMEDIATION IN AGRICULTURAL SOILS

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Enfoques modernos para el estudio de la biorremediación de herbicidas *s*-triazinas en suelos agrícolas

Keywords: *s*-triazine, simazine, biodegradation, bioremediation, herbicide.

ABSTRACT

The extensive use of *s*-triazine herbicides in diverse countries causes environmental and health concern. Simazine and atrazine are *s*-triazines widely used in agriculture and forestry. Although, natural dissipation of *s*-triazines in soils by physicochemical processes has been described, the main mechanism for their removal is biological degradation by microorganisms. Bioremediation is a successful strategy for the removal of *s*-triazines in soil. For bioaugmentation processes, *s*-triazine-degrading bacteria are required, which isolation from agricultural soils was described in this report. Studies of *s*-triazine adsorption and leaching in soil are useful to determine the bioavailability of these herbicides. The detection of *s*-triazine-degrading catabolic activity by most-probable-number (MPN) and the reduction of the respiration indicator 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) were presented. The relative abundances of *s*-triazine catabolic genes in soil were analyzed by the MPN-PCR technique. Culture-independent molecular methods such as FISH, T-RFLP and clone libraries are useful to study the effects of herbicide application and bioaugmentation on soil microbial communities and their dynamics. These experimental methods allow the design of biotechnological strategies for the clean-up of *s*-triazine contaminated soils.

Palabras claves: *s*-triazina, simazina, biodegradación, biorremediación, herbicida.

RESUMEN

El empleo masivo de herbicidas *s*-triazinas en diversos países ha causado preocupación ambiental y de salud. Simazina y atrazina son *s*-triazinas ampliamente utilizados en la agricultura y en predios forestales. La disipación natural de *s*-triazinas en suelos puede ocurrir por procesos físicoquímicos. Sin embargo, el principal mecanismo de remoción de estos herbicidas es la degradación mediada por microorganismos. La biorremediación es una estrategia eficiente para la remoción de *s*-triazinas del suelo. Para establecer procesos de bioaumentación, se requieren bacterias degradadoras de *s*-triazinas, cuyo aislamiento desde suelos agrícolas se describió en esta revisión. Estudios de adsorción y lixiviación de *s*-triazinas en suelos permiten determinar la biodisponibilidad de estos herbicidas. La actividad catabólica de microorganismos degradadores de simazina en suelo puede ser cuantificada por el método número más probable (NMP) y reducción del indicador de respiración cloruro de 2,3,5-trifenil-2H-tetrazolio (TTC). La abundancia relativa de genes catabólicos de *s*-triazinas en suelo fue analizada mediante la técnica NMP-PCR. Ténicas moleculares cultivo independiente, tales como FISH, T-RFLP y librerías de clones, son útiles para estudiar los efectos de la aplicación de herbicidas y de la bioaumentación sobre la estructura de las comunidades microbianas del suelo y su dinámica. Los métodos experimentales descritos en esta revisión permiten el diseño de estrategias biotecnológicas eficientes para la recuperación de suelos contaminados con *s*-triazinas.

INTRODUCTION

s-Triazine herbicides, such as simazine and atrazine, have been used extensively for control of leaf and grassy weeds (Radosevich *et al.*, 1995). Due to the growing agricultural production in the world, large amounts of these herbicides have been applied. These agrochemicals, used primarily as pre- and post-emergent herbicides, interfere in the photosynthetic electron transport chain in susceptible plants by binding to the quinone-binding protein in photosystem II (Strong *et al.*, 2002). The mobility of *s*-triazine in soil has contributed to the contamination of surface- and groundwater in several countries (Mandelbaum *et al*., 1995; Radosevich *et al*., 1995). Frequently, *s*-triazine herbicides have been detected exceeding the maximum pesticide acceptable levels in drinking water of Europe (0.1 µg l^{-1}) and USA (3.0 µg l^{-1}) (Mandelbaum *et al*., 1995; Rousseaux *et al*., 2001).

In Chile, cultivation areas have been increased 70% between 1997 and 2007 (Instituto Nacional de Estadísticas, Chile, 2007. Censos Agropecuarios 1997 - 2007. www.ine.cl). Atrazine and simazine are the *s*-triazines herbicides most used in Chile, with an annual application of 181 and 169 tons, respectively (Servicio Agrícola Ganadero, 2004. Declaración de ventas de plaguicidas de uso agrícola. Ministerio de Agricultura. Santiago, Chile). Simazine is applied in vineyards, avocado, apple and maize plantations as well as in pine and eucalyptus plantations (Cooman *et al*., 2005). Continuous application of *s*-triazines in Chilean soils is of increasing concern due to their potential contamination of surface- and groundwater. However, only few reports describe *s*-triazine contamination in Chile. In soils simazine may leach to 90 cm depth depending of weather conditions and soil properties (Alister *et al.*, 2005). Atrazine was detected in Chillán river at concentrations that are toxic for *Daphnia* spp. (Cooman *et al.*, 2005). *s*-Triazine

maximum contaminant levels for drinking water have not yet been established in Chile (Norma Chilena para Agua Potable, NCh 409/1. Of. 2005; Instituto Nacional de Normalización, Chile, www3.inn.cl). Simazine degradation in soil occurs predominantly by biological processes (Gebendinger and Radosevich, 1998; Newcombe and Crowley, 1999). For the design of herbicide bioremediation processes in contaminated soils, the isolation of native bacteria and the characterization of their *s*-triazine degradation potential are required. Diverse microorganisms able to degrade these herbicides have been isolated (Mandelbaum *et al*., 1995; Struthers *et al*., 1998; Topp *et al*., 2000a, 2000b; Hernández *et al*., 2008a; Hernández *et al*., 2008b). *Pseudomonas* sp. ADP has become the model strain for *s*-triazine biodegradation studies. This bacterial strain is able to use atrazine as the sole nitrogen source and its atrazine catabolic pathway has been extensively characterized (Martínez *et al*., 2001). The enzymes of the upper pathway, which are responsible for the conversion of atrazine to cyanuric acid, are encoded by the *atz*A, *atz*B and *atz*C genes. The lower pathway mineralizes cyanuric acid and is encoded by the *atz*DEF operon (Martínez *et al*., 2001). The herbicide simazine is also degraded by these catabolic enzymes (Fig. 1).

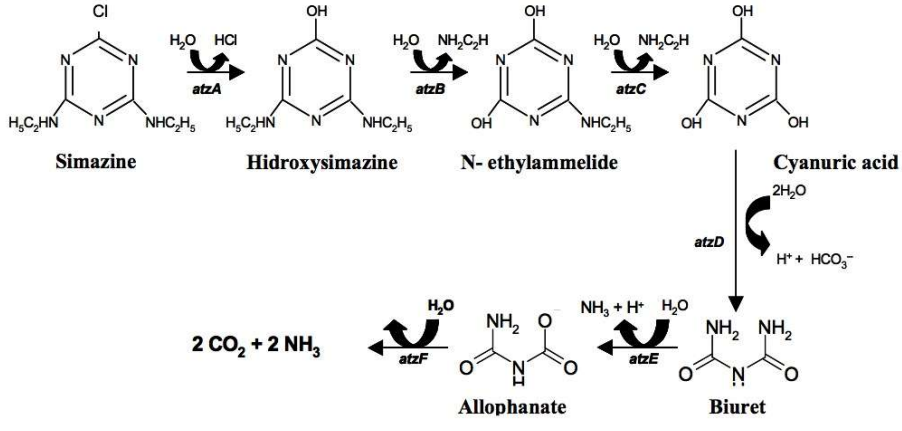

Figure 1: The simazine catabolic pathways. The *atz* genes encoding the metabolic enzymes are indicated.

Figura 1: Vías catabólicas de simazina. Se indican los genes *atz* que codifican las enzimas metabólicas.

In this review, the isolation and characterization of *s*-triazine-degrading bacteria were reported. Studies on *s*-triazine retention and bioavailability in soil were presented. Modern methods for the analysis of microbial *s*-triazine catabolic activities and catabolic genes of soils were presented as a powerful combination of culture-dependent and culture-independent techniques for laboratory, microcosm and field-scale studies. Finally, microbial ecology methods for the study of the structure and the dynamics of soil bacterial communities were reviewed.

BACTERIAL DEGRADATION OF *s*-TRIAZINES

Bioremediation employs microorganisms to degrade pollutants in the environment. Gram- negative and Gram-positive bacteria, predominantly *Pseudomonas*, *Arthrobacter*, *Pseudaminobacter* and *Nocardiodes* strains, capable to degrade *s*-triazine herbicides have been described (Yanze-Kontchou and Gschwind, 1994; Mandelbaum *et al.*, 1995; Rousseaux *et al*., 2001; Topp *et al.,* 2001; Strong *et al.*, 2002).

To isolate microorganisms able to degrade *s*-triazines, we sampled agricultural soils from avocado and persimmon tree plantations annually treated with simazine in central Chile (Hernández *et al*., 2008b). Isolation was made by enrichment procedures in minimal medium using simazine as sole nitrogen source (Hernández *et al*., 2008a). Diverse strains able to degrade and use simazine as the sole nitrogen source for gowth were isolated from these soils. Some of these bacteria were characterized by sequencing 16S rRNA genes and identified as species of *Pseudomonas*, *Rodococcus*, *Stenotrophomonas* and *Arthrobacter*. The simazine degradation potential of these bacterial strains was studied by resting cells assays. A novel *s*-triazine-degrading *Pseudomonas* strain was further characterized (Hernández *et al*., 2008a). These microorganisms are potential biocatalysts for bioremediation processes to remove *s*-triazines from polluted environments.

SOIL CHARACTERIZATION

Soil properties and *s*-triazine retention

Several factors determine the persistence of herbicides in soil (Novak, 1999). Soil physical and chemical properties such as texture, organic matter content as well as the pH strongly influence herbicide persistence. In addition, chemical properties of the herbicide such as solubility, vapor pressure, and the susceptibility to chemical or microbial degradation determine the environmental stability of the compound. Climatic variables, mainly moisture and temperature, are also relevant factors. However, microbial abundance and metabolic activity of soil microorganisms play a central role on herbicide fate in soil (Newcombe and Crowley, 1999). Retention and mobility of a pesticide in soil are determined by sorption processes, which are governed by physical-chemical properties of the soils and the pesticides (Spark and Swift, 2002). Sorption interactions of pesticides in the soil matrix may involve the mineral and/or organic components (Li *et al.*, 2003). For soils with high organic matter levels (>5%), pesticide retention has been associated with the binding to organic matter (Jenks *et al.*, 1998). In soils with low organic matter content, pesticide adsorption depends on active components of the inorganic fraction, predominantly the clay. It has been postulated that an increase in the clay content decreases the mobility of the pesticide (Cox *et al.*, 2000). Adsorption isotherms are commonly used to determine the herbicide affinity to soil and are often described by Freundlich- or Langmuir-type models (Calvet *et al.*, 1989). Some studies describe the retention of *s*-triazines herbicides by soil constituents. Recently, simazine adsorption behavior was studied in

agricultural soils of Aconcagua valley, central Chile (Flores *et al.,* 2008). Soil organic matter and clay minerals are main sorbents for *s*-triazines (García-Valcárcel and Tadeo, 1999; Flores *et al.,* 2008). In conclusion, physicochemical characteristics of the herbicide, the active surface of the minerals and the organic content of soils as well as the amount of herbicide applied are important parameters for understanding the dynamics of *s*-triazine herbicides in the environment. To protect surface- and groundwater from pesticide contamination, and to ameliorate their impact, broad knowledge is required concerning their sorption-desorption processes in the environment.

Microbiological and molecular analysis of soils

To establish bioremediation processes that remove *s*-triazines, the detection of active indigenous microbial communities able to degrade these herbicides is required. Recently, an interesting method for the detection and enumeration of MPN of *s*-triazine-degrading microorganisms in soil has been described (Dinamarca *et al.,* 2007). This method is based on the ability of bacteria to use a *s*-triazine as sole nitrogen source. The metabolic activity of microorganisms is detected by the reduction of 2,3,5-triphenyl-2H-tetrazolium chloride into a colored formazan product.

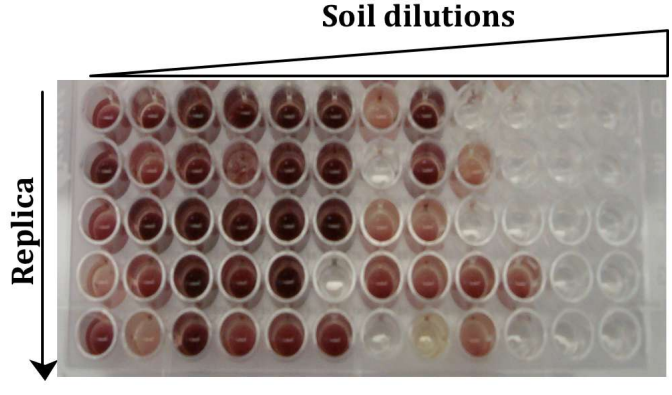

Figure 2: Detection of *s*-triazine degrading activities in soils by MPN-TTC method. Ten-fold serial dilutions of soil sample were prepared and 5 wells of a microtiter plate were inoculated. At the end of incubation, TTC was added (0.01% final concentration) and the microtiter plate was incubated at 30ºC during 4 h. A positive reaction is determined by visualization of the color change by the production of formazan (red color).

Figura 2: Detección de actividad degradadora de *s*-triazina en suelos mediante el método NMP-TTC. Se prepararon diluciones seriadas (1/10) de muestras de suelo y se inocularon en cinco pocillos de una microplaca. Al final de la incubación, se adicionó TTC (concentración final de 0,01%) y se incubó la microplaca a 30ºC durante 4 horas. El cambio de color por la producción de formazán (color rojo) indica una reacción positiva.

The simazine-degrading activities of microorganisms in an avocado plantation soil from central Chile are shown in Figure 2. The microbial catabolic activities in soils can be attributed to the previous history of simazine treatment (Rousseaux *et al*., 2001; Ralebitso *et al*., 2002, Morán *et al.,* 2006).

The relative abundances of *s*-triazine catabolic genes in soil can be analyzed by a MPN-PCR technique. The detection of *atzA* gene in DNA extracted directly from this soil is illustrated in Figure 3.

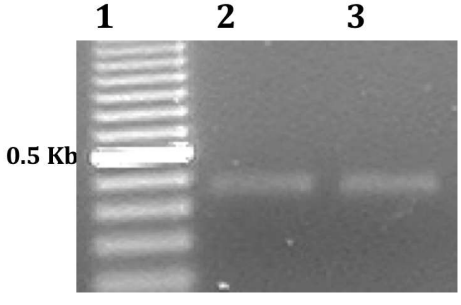

Figure 3. Amplification of catabolic *atzA* gene from soils treated with simazine. Molecular mass markers (line 1), freshly applied herbicide soil (line 2), soil four weeks after herbicide application (line 3).

Figura 3. Amplificación del gen catabólico *atzA* desde muestras de suelos tratados con simazina. Marcadores de masa molecular (línea 1), suelo recién tratado con el herbicida (línea 2), suelo tratado con el herbicida hace 4 semanas (línea 3).

The PCR-amplification of *atzA* in agricultural soils was performed according to the protocols and primers previously described (de Souza *et al*., 1998). The presence of the *atzA* gene in soils is in accordance with the detection of catabolic activities of indigenous simazine-degrading microorganisms. The *s*-triazine catabolic activities quantified by the MPN-TTC method and the catabolic genes determined by the MPN-PCR technique correlates with simazine removal from these soils.

BIOREMEDIATION STUDIES

Bioremediation is an effective technology for the clean-up of polluted environments. This biotechnology has several advantages compared to the physicochemical treatments: lower operational costs, in situ application, permanent elimination of the residue, and minimum disturbance of the treated site (Ralebisto *et al*., 2002; Philp and Atlas, 2005; Navia and Seeger, 2006). The most efficient methods for transforming a contaminant into a less-harmful end product are biostimulation and bioaugmentation. Biostimulation involves treating the contaminated soils to increase the pollutant bioavailability, or adding a nutritional supplement or co-substrate to increase the population of contaminant-degrading indigenous bacteria (McTavish, 2001). Bioaugmentation involves the inoculation of contaminated soils or water with specific microbial strains or consortia to improve the biodegradation capacity of the system for a specific organic pollutant (Philp and Atlas, 2005).

Successful bioaugmentation of *s*-triazine-polluted soils has been described (Yanze-Kontchou and Gschwind, 1994, Alvey and Crowley, 1996, Newcombe and Crowley, 1999, Morgante *et al.,* 2008). For bioaugmentation strategies, bacterial strain selection, inoculum size and inoculation system are important parameters. Bacterial strain selection is critical for bioremediation strategies. The strains used for bioremediation should posses exceptional degrading capacities and important growth rates (Philp and Atlas, 2005; Navia and Seeger, 2006). Therefore, the adaptability of the inoculated strain to the soil and the long term survival of the microorganism are desirable properties. Higher bioremediation efficiency was obtained by inoculating native

strains (Rousseaux *et al.,* 2002). Secondly, to increase the biodegradation rate, the native strain has to be inoculated to the polluted site at high densities. For soil bioremediation a higher inoculum size is required than for bioaugmentation of aquatic ecosystems. A high inoculum size allows overcoming competition with native bacteria, predation by protozoa and bacteriophage and lower pollutant bioavailability due to sorption mechanisms in soil (Philp and Atlas, 2005). For atrazine bioremediation in soils, different inoculum sizes have been tested (Topp, 2001; Rousseaux *et al.,* 2002). Finally, the inoculation system is an essential factor in bioaugmentation. Inoculation of cells immobilized in alginate matrices, or other polymeric materials, is a strategy of increasing importance. For example, alginate allows passage of nutrients and excretion products, protects bacteria from predators and nutrient stress and preserves viability of the organisms (Newcombe and Crowley, 1999). Bioaugmentation with repeated inoculations could be useful for increasing bioremediation efficiency. Repeated applications of the strain overcome long-term survival problems (Newcombe and Crowley, 1999). Recently, a successful bioaugmentation strategy for agricultural soil using the native simazine-degrading bacterium *Pseudomonas* sp. strain MHP41 was reported (Morgante *et al.,* 2008). After bioaugmentation with strain MHP41, simazine catabolic activities were increased and herbicide removal was enhanced.

ANALYSIS OF SOIL MICROBIAL COMMUNITIES BY MOLECULAR METHODS

Microorganisms play key functions in soil and are able to adapt to changing environmental conditions. Thus, variations in bacterial populations and activities may serve as excellent indicators of changes in soil health (Torsvik and Overas, 2002). Most microbes in environmental samples can not be cultured in laboratory media, which are biased for the growth of specific microorganisms (Torsvik *et al.,* 2002). Molecular biology techniques and microbial culture-independent approaches are increasingly employed for the study of the microbial ecology in complex environments (Alfreider *et al.*, 1996; Muyzer and Smalla, 1998; Osborn, *et al.,* 2000; Nogales *et al*., 2001). The 16S rRNA gene sequence is important for the analysis of microbial diversity and is a relevant marker for studying the phylogeny of bacteria (Fig. 4).

To date, most studies quantify the depletion of the contaminant or monitor cultivable microorganisms or their catabolic activities in polluted soils. Only few reports describe the dynamics of microbial communities throughout the biodegradation process (Piutti *et al.*, 2003; Moreno *et al*., 2007). The knowledge of the microbial communities inhabiting *s*-triazine contaminated soils and their response to bioaugmentation strategies is useful for the identification of the microorganisms that are adapted to these compounds and are involved in their degradation. Changes in the bacterial community structure in soil by pesticides application have been observed (Engelen *et al*., 1998; El Fantroussi *et al.*, 1999). The structure of the microbial community in an atrazine-contaminated soil changed in response to soil organic amendments (Martin-Laurent *et al*., 2004) and carbon and nitrogen source availability (Rhine *et al*., 2003). Microbial community structure of *s*-triazine treated soil has been studied by FISH (Barra Caracciolo *et al*., 2005). Specific groups of bacteria such as α-, β- and γ-subdivisions of *Proteobacteria*, Gram-positive bacteria of high GC DNA content and *Planctomycetes* were detected. The presence of *s*-triazines affected the bacterial community structure.

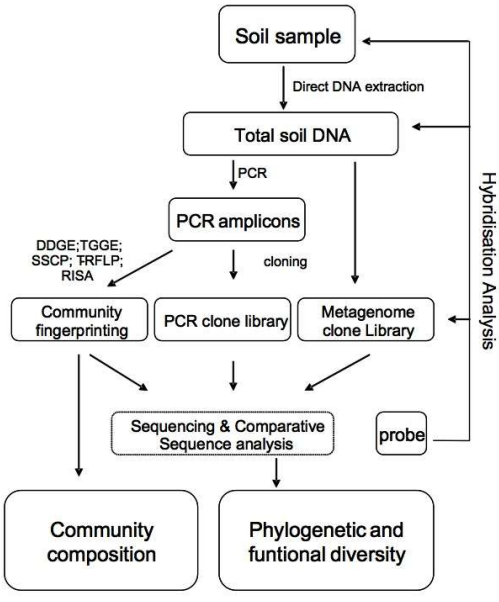

Figure 4. Culture-independent molecular methods for the analysis of microbial communities. PCR: polymerase chain reaction; DGGE: denaturant gradient gel electrophoresis; TGGE: temperature gradient gel electrophoresis; SSCP single strand conformation polymorphism; T-RFLP: terminal restriction fragment length polymorphism; RISA: ribosomal intergenic spacer analysis.

Figura 4. Métodos moleculares cultivo independientes para el análisis de comunidades microbianas. PCR: reacción en cadena de la polimerasa; DGGE: electroforesis en gel con gradiente desnaturalizante; TGGE: electroforesis en gel con gradiente de temperatura; SSCP: polimorfismo de la conformación de cadena simple de ADN; T-RFLP: polimorfismo de longitud de fragmentos de restricción terminales; RISA: análisis del espaciador intergénico ribosomal.

The bacterial community structure of a pesticide-contaminated site and the changes induced in the community structure during bioremediation approaches has been recently described (Paul *et al*., 2006). The microbial community of this pesticide-contaminated soil was mainly constituted by *Proteobacteria* and *Actinomycetes*. Bioaugmentation enhanced pollutant degradation. However, T-RFLP analysis revealed non-significant changes in bacterial community structure during the bioremediation process. In Chilean agricultural soils, we evaluated the capability of native soil microbiota and bioaugmentation strategies on simazine biodegradation by microcosm experiments. The 16S rRNA gene pool amplified from the soil genomic library was cloned and FISH and T-RFLP was performed to analyze changes in soil microbial community structure due to simazine amendment and bioaugmentation strategies. Our results showed that the native soil microbiota was

able to degrade simazine. However, the addition of a native strain enhanced simazine-degrading activities and was essential for increasing the attenuation of simazine in soil. Sequencing of representative clones of soil bacteria showed that the microbial community structure was mainly constituted by *Proteobacteria*, *Actinomycetes*, *Acidobacteria* and *Planctomycetes*. Microbial community analysis by T-RFLP revealed that simazine application and bioaugmentation promotes changes in the structure of soil microbial communities, while FISH indicates variations in some specifics bacterial groups.

CONCLUSIONS

In this review, the basis of bioremediation of herbicides in agricultural soils was analyzed. Diverse simazine-degrading strains isolated from Chilean agricultural soils were characterized. The MPN-TTC method was used for the estimation of the *s*-triazine-degrading activities in soil. Catabolic genes for *s*-triazine degradation were detected in soil by MPN-PCR. Bacterial community adapted to the herbicide application and with simazine catabolic capabilities are present in Chilean agricultural soils. Culture-independent molecular methods such as FISH, T-RFLP and clone libraries were used to understand the effects of herbicide application and bioaugmentation on soil microbial communities. The isolated *s*-triazine-degrading microorganisms are novel biocatalysts that were used for the development of bioaugmentation strategies.

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