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Microbial aspects of atrazine degradation in natural environments

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Abstract

The potential toxicity of the *s*-triazine herbicide atrazine motivates continuous bioremediation-directed research. Several indigenous soil atrazine-catabolizing microbial associations and monocultures have been enriched/isolated from compromised sites. Of these, *Pseudomonas* sp. strain ADP has become a reference strain and has been used to elucidate sequences of the catabolic enzymes *atz*A, *atz*B, *atz*C and *atz*D involved in one aerobic degradation pathway and develop probes for the genes which encode these enzymes. Despite this, hitherto unknown or novel microorganisms, with unique sequences and different enzyme-mediated operative pathways, warrant continued investigations for effective site bioremediation. Also, the sustained effectiveness of natural attenuation must be demonstrated continually so regular site evaluations and results analyses, despite the limitations of chemical extraction methodologies, are crucial practices. For both directed and intrinsic bioremediation monitoring, traditional microbial association studies must be complemented by more advanced physiological and molecular approaches. The occurrence of catabolic plasmids, in particular, should be probed with DNA hybridization techniques. Also, PCR-DGGE and subsequent new sequence elucidation should be used prior to developing new primers for DNA sequences encoding novel catabolic enzymes, and for hybridization probe development, to establish the degradative potential of a compromised site, or adoption of FISH to, for example, monitor bioaugmented remediation.

Introduction

Although atrazine is an effective herbicide, extensive toxicological investigations (Biradar & Rayburn 1995; Allran & Karasov 2000) have motivated and continue to motivate bioremediation-directed research. Successful enrichment/isolation and characterization of catabolic microbial associations and monocultures have resulted and *Pseudomonas* sp. strain ADP has been used both to study atrazine catabolism under different conditions and elucidate the enzymes involved in one of the aerobic catabolic pathways.

Until recently, due to technique limitations, an understanding of microbial community development in natural ecosystems exposed to different inorganic and organic pollutants was restricted to culturable microorganisms. However, by complementing traditional microbial association studies with more advanced physiological and molecular approaches, such as community-level physiological profiling (CLPP) (Garland 1997), phospholipid fatty acids analysis (PLFA) (Green & Scow 2000), denaturing- and temperature-gradient gel electrophoresis (DGGE/TGGE) (Heuer and Smalla, 1997) and fluorescent *in situ* hybridization (FISH) (Amann et al. 1995), efficient microbial species monitoring in both directed and intrinsic bioremediation should result. Currently, primers for the *atzABC* enzymes elucidated in *Pseudomonas* sp. strain ADP are used to establish the degradative potential of atrazine compromised sites (Shapir et al. 2000). However, hitherto unknown or novel microorganisms, with possible unique sequences and different enzyme-mediated operative pathways, warrant continued investigation for effective site bioremediation.

The marked differences of soils prevent generalizations on the rate and extent of sequestration and, thus, the bioavailability of organic compounds *in situ* (Chung & Alexander 1998). It is essential, therefore, to elucidate the adsorption/desorption characteristics of contaminant molecules in the specific soil to be remediated in relation to the enriched catabolic microbial associations in particular. Also, the sustained effectiveness of intrinsic attenuation must be demonstrated continually to satisfy increasingly stringent regulations (Rittmann 2000). Regular site evaluations and results analyses, despite the limitations of chemical extraction methodologies (Shows & Olesik 2000), are, therefore, crucial.

This review, while not exhaustive due to space limitations, discusses atrazine occurrence and availability in natural environments, particularly soil, and microbial catabolic potential distribution and its elucidation by molecular techniques as a prerequisite for field-scale bioremediation.

The atrazine molecule: Impacts on health and the environment

Atrazine [2-chloro-4-(ethylamino)-6-(*iso*propylamino)-*s*-triazine] is one of the most environmentally-prevalent *s*-triazine-ring herbicides through its global use to control, through photosystem II inhibition, pre- and post-emergence broadleaf and grassy weeds in major crops such as maize (*Zea mays*), sorghum (*Sorghum* spp) and sugarcane (*Saccharum officinarum*) (Pick et al. 1992; Seiler et al. 1992; Mandelbaum et al. 1993; Yanze-Kontchou & Gschwind 1994; Sparling et al. 1998). The United States Environmental Protection Agency (EPA) (1991) reported that since the 1980s, the annual use of all pesticides had been holding steady at 500 million kg of active ingredients. More specifically, 363 million kg of atrazine were applied between 1980 and 1990 in the USA alone (Yanze-Kontchou & Gschwind 1995).

The herbicide has been classified a Class C/possible human carcinogen (Loprieno et al. 1980). This classification was confirmed by Biradar & Rayburn (1995) who observed chromosomal damage of Chinese hamster ovary cells exposed to atrazine (0.005–0.080 μM) for two days. In the same study, analysis of Illinois public water supplies revealed that EPA "safe" concentrations demonstrated the potential of the pesticide as a clastogen. Atrazine dealkylation metabolites, such as deethylatrazine [2-chloro-4-amino-6-*iso*propylamine-1,3.5-triazine] and de*iso*propylatrazine [2-chloro-4-ethylamino-6-amino-1,3.5-triazine], are also regulated compounds and may pose health risks thus warranting investigation of their fates (Brouwer et al. 1990; Kolpin et al. 1998).

The herbicide has a solubility of approximately 30 mg l^{-1} and a half-life in soil of between 15 and 100 days (Protzman et al. 1999). Atrazine decomposition products are relatively persistent in soil and their detection in surface- and ground-water has prompted some environmental concerns (Sparling et al. 1998; Gebendinger & Radosevich 1999).

Through a combination of proven catabolic potential, relatively low cost and minimal environmental impact, bioremediation has been espoused largely as the technology of choice for atrazine-compromised sites despite the availability of various alternative treatment and containment methods such as: ozonation (Ma & Graham 2000); photochemical degradation (Konstantinou et al. 2001); diatomaceous earth remediation (Agdi et al. 2000); and powdered activated carbon adsorption (Campos et al. 2000).

Pesticide soil sorption properties and fates

Pesticide fates in soil, and surface- and ground-water, including irreversible binding and persistence, are governed by interactions between retention, transformation and transport processes. Sorption affects these processes and determines, ultimately, pesticide volatilization, bioavailability, degradation and bioaccumulation. Desorption processes are equally important particularly for quantifying pesticide transport and, therefore, establishing a mass balance (Gao et al. 1998; Mata-Sandoval et al. 2000).

Several researchers have explored the importance of soil organic matter modification, via, for example, treated sewage sludge application (Masaphy & Mandelbaum 1997; Sluszny et al. 1999), colloids (Sprague et al. 2000), van der Waals forces, hydrogen bonds, charge transfer and triazine protonation (Moreau-Kervévan & Mouvet 1998), particle size, "ageing" and pH (Huang et al. 1984; Roy & Krapac 1994) on the sorption and, thus, microbial catabolism of atrazine. Erickson & Lee (1989) and Moreau-Kervévan & Mouvet (1998) ascribed atrazine adsorption to humic substances, especially humic acids, clays and oxyhydroxides, and to the various interrelated physical and chemical mechanisms of soil which are dependent

upon the solute and key environmental determinants such as pH and temperature.

Low atrazine biodegradation rates are effected by its low water solubility (±0.15 mM, Erickson & Lee 1989) and sorption to regions of the soil particles, including the pores which are inaccessible to microbial species. Enhanced water solubility, increased mass transfer to the water phase and, thus, increased catabolic rates of the herbicide may be facilitated by the addition of surfactants, such as sodium dodecyl sulphate (Mata-Sandoval et al. 2000; Sanchez-Camazano et al. 2000), and/or inorganic nutrients, via percolation. Although effective, synthetic surfactants have some limitations such as: inhibition of microbial activity on micellar phase-solubilized pollutants (Mata-Sandoval et al. 2000); bactericidal activity; and considerable affinity to soil surfaces thus accumulating in and polluting subsurface environments (Chin et al. 1996). To circumvent these, biosurfactants, such as atrazine-solubilizing rhamnolipids, may be considered (Mata-Sandoval et al. 2000) since they have been shown to effect slightly increased atrazine solubility compared with the synthetic surfactant Triton X-100. The potential impacts of surfactants (and solvents such as methanol) on atrazine catabolism must, however, be evaluated particularly in relation to their preferential use as carbon sources.

Atrazine biodegradative capacity and distribution

Catabolism under carbon- and nitrogen-limited conditions

As a relatively new and complex xenobiotic molecule, atrazine represents a considerable challenge for microbial catabolism *in situ*. Its worldwide use may, however, have resulted in the global distribution of known microorganisms, with newly-induced catabolic enzymes, or novel microorganisms, with atrazine-catabolic capacities. Also, despite its limitations as an energy source, due to full oxidation of the ring carbons (Radosevich et al. 1995), its susceptibility to catabolism is improved by the presence of both carbon and nitrogen with the herbicide targeted as a nitrogen or carbon source in N- and C-limited conditions, respectively.

Laboratory and field studies by Mandelbaum et al. (1995), Shapir et al. (1998) and Katz et al. (2000) showed increased population sizes and herbicide degradation with the provision of tri-sodium citrate as a supplementary carbon source. However, several authors (Assaf & Turco 1994b; Grigg et al. 1997; Ames & Hoyle 1999; Gebendinger & Radosevich 1999) have reported that the concentrations and forms of C and N are major determinants of atrazine mineralization. For example, inorganic nitrogen rather than organic nitrogen (plant debris/*in situ* soil organic matter) stimulated pesticide catabolism (Alvey & Crowley 1995) and glucose addition of $\leq$16 gC kg^{-1} soil facilitated the formation of bound atrazine residues and, thus, reduced its bioavailability while $\geq$16 gC kg^{-1} soil enhanced dealkylation (Abdelhafid et al. 2000). Further work is, therefore, required before nitrogen and/or carbon supplementations can be introduced on a routine basis into site bioremediation strategies.

Enrichment and isolation of catabolic microorganisms

Several researchers have reported the enrichment and isolation from compromised sites in different geographical regions (Radosevich et al. 1995; Bouquard et al. 1997; Sparling et al. 1998; Topp et al. 2000b) of microorganisms which are able to: dealkylate atrazine in a carbon-limited medium (Behki & Kahn 1986); mineralize and use atrazine as a sole carbon and energy source (Mandelbaum et al. 1995; Yanze-Kontchou & Gschwind 1995; Topp et al. 2000b); utilize the heterocyclic nitrogen (Bichat et al. 1999); and, in the presence of supplemental carbon, mineralize atrazine and its metabolites as a source of nitrogen (Mandelbaum et al. 1993; Topp et al. 2000a). Successful mineralization of >94% of atrazine-C (50 μg ml^{-1}) and the isolation of a microorganism, *Agrobacterium radiobacter* strain J14a, which was able to dealkylate, dehalogenate and mineralize the *s*-triazine ring when the molecule was used as a nitrogen source, was reported by Struthers et al. (1998). By screening some *Rhodococcus* strains which were known to be ubiquitous in soil and had diverse biodegradative capabilities, Behki et al. (1993) identified the strain TE1 with a 77-kb plasmid with atrazine-degrading capacity. Other workers reported microbial associations (Mandelbaum et al. 1993; Assaf & Turco 1994a, b; Alvey & Crowley 1996; de Souza et al. 1998a; Ralebitso et al. 1999) and fungal species (Donnelly et al. 1993; Mougin et al. 1994) which mineralized atrazine.

Culturable soil atrazine-catabolizing microbial associations/monocultures show a preponderance of rod-shaped bacteria (Table 1). Of these, *Pseudomonas* sp. strain ADP, which is capable of catabolizing the

herbicide at concentrations >1 000 mg l^{-1}, has become a reference strain and has been used extensively to: study atrazine catabolism under anoxic or denitrifying conditions (Shapir et al. 1998; Katz et al. 2000); demonstrate efficient bioaugmentation of an atrazine-contaminated soil (Newcombe & Crowley 1999); examine a method for aquifer amelioration *in situ* (Shati et al. 1996); explore atrazine degradation in soil, in comparison to other monocultures (Topp 2001); elucidate sequences of the aerobic catabolic enzymes *atz*A (de Souza et al. 1996), *atz*B (Boundy-Mills et al. 1997) and *atz*C (Sadowsky et al. 1998) and develop probes for their encoding genes (de Souza et al. 1998b).

Molecular microbial ecology of atrazine catabolism

Herbicide bioremediation has been researched extensively (Senior et al. 1976; de Souza et al. 1998a) although new microbial associations with high specific growth rates and/or high critical substrate concentrations may still be isolated. Characterization of such associations in relation to key environmental and physiological determinants will be needed then to facilitate the implementation of effective bioremediation protocols.

The study of nucleic acids extracted from environmental samples has allowed analysis independent of the problems which arise from laboratory degradative potential assays, laboratory culture-based enumerations, and our inability to culture the majority of species (Brockman 1995). Therefore, the need for broad-based, non-selective DNA extraction procedures or molecular techniques (e.g., DGGE/TGGE and terminal-restriction fragment length polymorphism (T-RFLP)) which explore the complexities of microbial associations and their dynamics has been highlighted (Muyzer & Smalla 1998).

Ames & Hoyle (1999) concluded that chemical parameters, such as (sediment) N content or atrazine residues, could not be used alone as good primary predictors of biodegradative potential and/or activity. Therefore, understanding the distribution of contaminant-catabolizing microorganisms, genes or enzymes is of paramount importance (Mason et al. 1998). Thus, with the use of custom-designed primers, de Souza et al. (1998b) identified homology to the *Pseudomonas* sp. strain ADP *atzABC* genes in five atrazine-catabolizing microbial isolates and suggested that the genes were widespread. Shapir et al. (1998, 2000) used magnetic capture hybridization-polymerase chain reaction followed by nested PCR to detect atrazine-catabolizing genotypes and ascertain the expressed level of biodegradative activity *in situ* without the need to cultivate or activate the indigenous catabolic species. Generally, the mineralization rate and proliferation of catabolic microorganisms was correlated positively to the presence and copy number of the atrazine-catabolic genes. Therefore, the absence of the atrazine-dechlorinating gene *atzA* limited, possibly, intrinsic bioremediation (Shapir et al. 1998) while its presence (100 copies g^{-1} soil) in soils with histories of atrazine contamination conferred short (4–5 day) lag phases before 40–54% mineralization of the herbicide (Shapir et al. 2000).

To date, several summaries of suggested catabolic pathways of atrazine and related *s*-triazines have been published (Cook 1987; Erickson & Lee 1989; http://www.labmed.umn.edu/umbbd/atr/atr_map.html 20-12-2001). Of the pathways, one has been elucidated fully in *Pseudomonas* sp. strain ADP (de Souza et al. 1998a) and is known to be hydrolytic rather than oxidative and consists of four main steps: dehalogenation; N-dealkylation; deamination; and ring cleavage (Figure 1). The catabolic enzymes were identified by de Souza et al. (1996), Boundy-Mills et al. (1997) and Sadowsky et al. (1998). de Souza et al. (1998c) then identified the location of the genes *atzABC* which encode these enzymes on a self-transmissible plasmid. Topp et al. (2000a) reported an alternative pathway with a new hydrolase for the degradation of a range of *s*-triazines by a *Nocardioides* sp. Also, a dechlorinating enzyme, *s*-triazine hydrolase (*trzA*), and a gene controlling N-dealkylation have been characterized for *Rhodococcus corallinus* (Mulbry, 1994; Shao et al. 1995) and the involvement of the cytochrome P-450 system in atrazine N-dealkylation in *Rhodococcus* sp. strain N186/21 explored (Nagy et al. 1995). Nonetheless, new species and novel catabolic mechanisms may await identification and elucidation.

Together with methods to promote indigenous microbial activity, the introduction of genetically-engineered strains (bioaugmentation), which must be critically monitored/tracked, may be considered. Shao et al. (1995) and Strong et al. (2000), for example, conferred catabolic capacity by constructing recombinant *Rhodococcus* strains and *Escherichia coli* which were capable of dealkylating and dechlorinating atrazine. While the longevity and survival of the genetically-modified strains may be limited, transfer

Table 1. Some isolated atrazine-catabolizing bacterial strains

Strain	End products	Mineralization	Reference
Pseudomonas sp.	Deethylatrazine + de*iso*propylatrazine	−	Behki & Khan (1986)
Rhodococcus sp. strain B-30		−	Behki & Khan (1994)
Pseudomonas sp. strain YAYA6	CO_2	+	Yanze-Kontchou & Gschwind (1994)
Pseudomonas sp. strain ADP	$CO_2 + NH_4^+$	+	Mandelbaum et al. (1995)
Rhodococcus sp. strain N186/21	Hydroxy*iso*propylatrazine	−	Nagy et al. (1995)
Ralstonia M91-3	Biuret + $CO_2 + NH_4^+$	+	Radosevich et al. (1995)
Rhizobium sp.	Hydroxyatrazine	−	Bouquard et al. (1997)
Agrobacterium radiobacter J14A	CO_2	+	Struthers et al. (1998)
Clavibacter michiganese ATZ1	Hydroxyatrazine + *N*-ethylammelide	−	de Souza et al. (1998a)
Pseudomonas sp. strain CN1	Cyanuric acid + CO_2	+	de Souza et al. (1998a)
Nocardioides sp.	Hydroxyatrazine + *N*-ethylammelide	−	Topp et al. (2000a)
Pseudoaminobacter sp.	CO_2	+	Topp et al. (2000b)

of the catabolic genes, particularly if located on plasmids, to robust and acclimated indigenous microorganisms may facilitate atrazine catabolism. Central to the adoption of this practice would be consideration of critical public and ethical concerns.

Potential applications: Adopting a multi-disciplinary approach

Despite recommendations for controlled and managed herbicide applications (Gerstl et al. 1998), atrazine usage will probably continue and will result in further compromised soil and surface- and groundwater. While widely accepted that *in situ* treatments rarely yield undesirable by-products, precautions and baseline tests (detailed contaminated site characterization in relation to the pollutant, hydrogeochemistry and microbiology) are always recommended for successful implementation (Mason et al. 1998; Haack & Bekins 2000). Also, species monitoring is necessary for public health considerations and for following the activities of microorganisms with genes encoding specific catabolic activities. Therefore, since DGGE/TGGE and PLFA patterns lend themselves to artificial neural network scrutiny (Almeida et al. 1999; Noble et al. 2000), they can be used, following specific catabolic association profile characterization, with different bioremediation strategies.

DNA hybridization techniques may be used to elucidate plasmid-borne catabolic sequences. Also, PCR-DGGE and subsequent new sequence elucidation may be used prior to developing new primers for chromosomal sequences encoding novel catabolic enzymes, and for hybridization probe development to establish the degradative potential of a compromised site or, by use of FISH, to monitor bioaugmented remediation. Direct hybridization avoids the possible biases introduced by DNA extraction and cleanup methods, which can distort the visualized community structure and component species dominance (Martin-Laurent et al. 2001), and preferential PCR amplification of the more abundant species (Head et al. 1998). Instead, whole cell *in situ* hybridization techniques facilitate quantitative estimation. This can give absolute enumeration and identify spatial localization and distribution of the metabolic activities of specific microorganisms in natural environments (Amann et al. 1995).

Together with measuring the occurrence of genes which encode, for example, atrazine catabolic enzymes, gene probe technology may be used to identify the concentration of the herbicide which effects changes in a microbial association and soils that have been affected by exposure to the molecule (Guo et al. 1997).

Techniques such as PLFA may be used to both estimate the cellular viability, nutritional and physiological status (assuming rapid degradation of intact phospholipids upon cell death) and fingerprint the microbial community configurations *in situ* (Parkes 1987; Green & Scow 2000). However, the constraint associated with Gram-negative bacteria community structure characterization, where the dominance and broad distribution of monoenoic, saturated and cyclopopane fatty acids limit differentiation or species subdivision (Zelles 1997), should be considered. Also, as exemplified by El Fantroussi et al. (1999), bac-

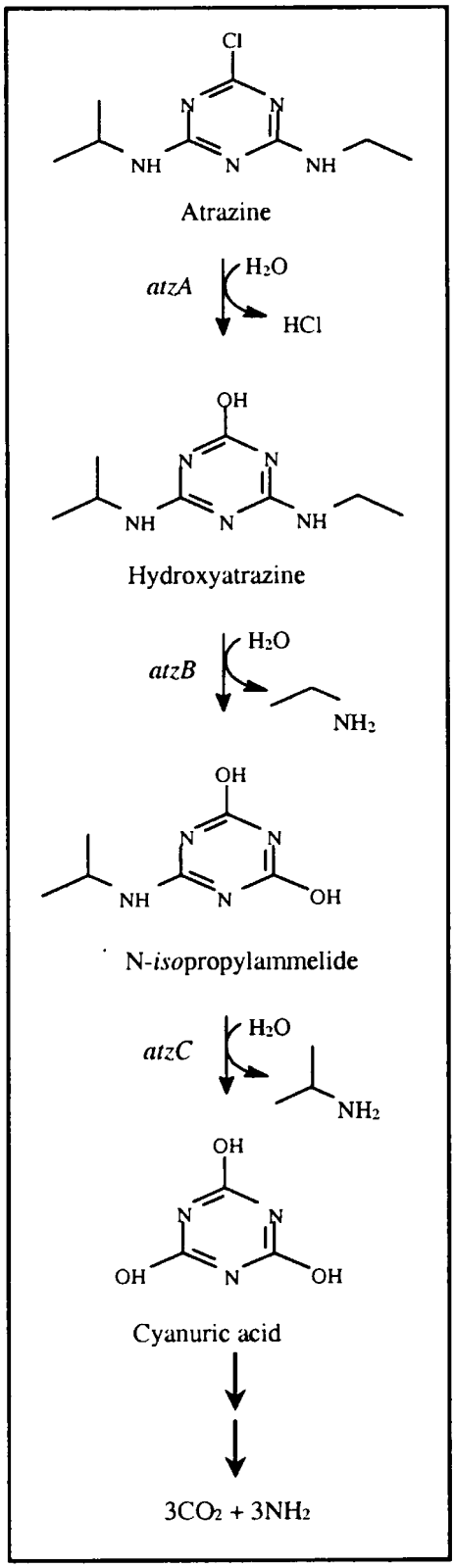

Figure 1. Aerobic atrazine catabolic pathway elucidated with *Pseudomonas* sp. strain ADP (de Souza et al. 1998a).

terial diversity in herbicide-contaminated soils might be studied by the combined approach of molecular (DGGE analysis) and culture-based methods (Biolog assay) despite the well-documented limitations of each technique.

The sustained effectiveness of intrinsic and enhanced (bioaugmented/biosupplemented) remediations must be demonstrated continually. This is particularly important since the contaminant concentration limits of current regulations are becoming increasingly stringent as exemplified by the 0.1 μg l^{-1} allowed in Europe for a single pesticide in drinking water (Martín-Esteban et al. 1997). In contrast, the South African Water Quality Guidelines (1996) stipulated an atrazine concentration of $\leq$2 μg l^{-1} in domestic water (Anon 1996). The routine use of sensitive, rapid and inexpensive detection and screening methods, such as immunoassays/immunosensors (Turiel et al. 1999), improved and more sophisticated and upscaled pollution (water) analytical techniques, such as immunoaffinity chromatography (Martín-Esteban et al. 1997), and extraction methods to predict bioavailability (Chung & Alexander 1998) are, therefore, central to environmental monitoring/protection.

For atrazine-contaminated surface- and groundwater, novel enzymes of inducible putative (Cook 1987) or alternative constitutive catabolic pathways could be over-expressed in killed whole-cell suspensions of recombinant microorganisms (Strong et al. 2000) and/or isolated and exploited via immobilization on an insoluble cellulose matrix (Kauffmann et al. 2000), as was done with atrazine chlorohydrolase (*AtzA*).

In general, a combination of conventional culture-dependent microbial techniques, various relevant molecular microbial ecology approaches and improved pollutant extraction and analysis methods should underpin all bioremediation protocols and so facilitate a comprehensive Code of Practice for the bioremediation of atrazine-compromised soil, surface- and ground-water.

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