Biomineralization of Atrazine Ozonation Products. Application to the Development of a Pesticide Waste Disposal System

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Development of remediation techniques for unusable pesticide wastes has led to a binary scheme involving ozonation followed by biomineralization of the resultant oxidized pesticides. Preliminary field tests of this technique indicated that the *s*-triazines were somewhat more recalcitrant than the other pesticides present. Further experiments identified the final ozonation products of atrazine, the most widely used *s*-triazine, as 4-acetamido-6-amino-2-chloro-*s*-triazine (CDAT) and chlorodiamino-*s*-triazine (CAAT). These compounds can be utilized by microorganisms only as nitrogen sources; however, 1% concentrations of ammonia fertilizers are not uncommon in pesticide waste. Therefore, the organism should prefer an organic nitrogen source and tolerate high ammonia concentrations. A *Klebsiella terragena* (strain DRS-I) was observed to degrade CAAT in the presence of high ammonia concentrations (0.8 M) with the addition of a carbon source (corn syrup), in contrast to a known *s*-triazine-degrading organism which could not. Nearly complete mineralization of CAAT to CO_2 by DRS-I was demonstrated using CAAT-*U-ring*-^{14}C. Bench-scale reactors indicated that continuous-flow or fixed-film reactors would support growth of DRS-I cultures and CAAT degradation.

INTRODUCTION

Alternative strategies are being sought by the agricultural community for the disposal of pesticide equipment rinsate and waste generated on-site. Although pesticide applicators are encouraged to reuse equipment rinsates to minimize waste, some unusuable material is still generated and must be properly disposed of. In the past, rinsates were often deposited on soils and left to evaporate and presumably degrade. However, this method has proven to be inadequate as evidenced by the contamination of nearby groundwater supplies, farm wells (Aharonson, 1987; Parsons and Witt, 1988), and pesticide operational areas that have been in use for years without adequate ground protection (Winterlin et al., 1989). Thus, development of remediation techniques for unusable pesticide rinsates, pesticide wastes, and contaminated soils is essential.

Enhanced microbial degradation of a number of pesticides has been observed after chemical pretreatment (Hapeman-Somich, 1991, and references cited therein). For example, ozonation of two highly used herbicides, atrazine and alachlor, resulted in substantial increases in the rate of total mineralization to CO_2 as compared to unaltered parent material (Kearney et al., 1988; Somich et al., 1988). Evaluation of a treatment sequence of ozonation and subsequent biodegradation by indigenous soil microflora under field conditions demonstrated the potential usefulness of this approach. These tests also revealed that the more recalcitrant pesticides were the *s*-triazines, atrazine and cyanazine (Somich et al., 1990). Further development and manipulation of this binary process requires a thorough understanding of the chemical and biological processes and identification of the limiting parameters, particularly for the *s*-triazines.

The product profile of atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] ozonation was delineated, and the final products were identified as 4-acetamido-6-amino-2-chloro-*s*-triazine (CDAT) and chlorodiamino-*s*-triazine (CAAT) (Figure 1) (Hapeman-Somich et al., 1992). Notably, the *s*-triazine ring of CAAT and CDAT can be utilized only as a nitrogen source by microorganisms, although the acetyl group on CDAT can serve as a carbon source. Previous work by Cook and Hutter (1981) led to the isolation of *Pseudomonas* sp. strain A (PSA) which could utilize some *s*-triazines as a nitrogen source when grown in minimal media. This organism was more effective in mineralizing atrazine ozonation products than the soil microflora used in previous studies under laboratory conditions (Kearney et al., 1988), but degradation of the *s*-triazine products was completely inhibited in farm-generated pesticide wastes (Somich et al., 1990). Potential inhibitors included fertilizers (ammonium nitrate, urea), surfactants, and formulating agents and/or other pesticide products present in the waste stream. Preliminary experiments indicated that high ammonia concentrations (ca. 1%) were responsible for the inhibition (Hapeman and Shelton, 1993).

Optimization of the biological reactor performance therefore required an organism capable of consistently degrading the *s*-triazine ozonation products under field conditions. A new organism isolated from sewage sludge enrichments, *Klebsiella terragena* (strain DRS-I), was found to utilize CAAT as a nitrogen source (Hapeman and Shelton, 1993). The purpose of this work was to examine the effectiveness of DRS-I in mineralizing CAAT under simulated field conditions and to determine the limitations and requirements to scaleup. This study was conducted in three parts: analysis of carbon and nitrogen requirements of both PSA and DRS-I; mineralization analysis, and bench-scale reactor studies.

METHODS AND MATERIALS

Analytical. Concentrations of CDAT and CAAT were determined utilizing two Waters 510 HPLC pumps equipped with a Waters 490 UV-vis multiwavelength programmable detector set at 210 nm and a Waters 810 Maxima controller. Analyses were conducted on a 4-μm C-18 Waters Novapak column using a 0-50% acetonitrile in H_3PO_4 buffer (pH 2) 5-min gradient

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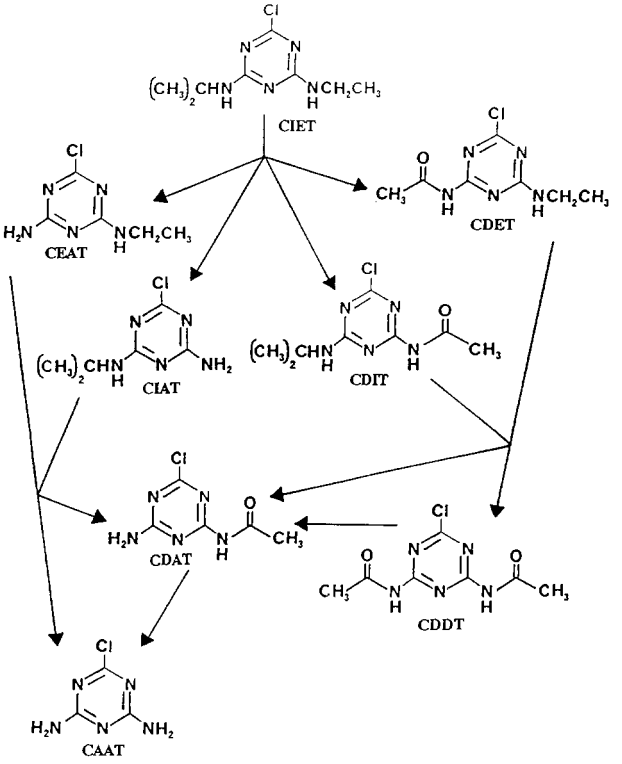

Figure 1. Reaction profile of atrazine ozonation.

(Waters curve 8) at 2 mL/min. Concentrations were established by standard curves of CAAT and CDAT in acetonitrile (0.5–100 mg/L).

Ammonia concentrations were determined colorimetrically using a Technicon Autoanalyzer II, Model SPR-2431 (Technicon Instrument Corp., Tarrytown, NY). Samples were diluted with distilled water and values assigned relative to ammonium chloride standards.

Atrazine Ozonation. Formulated atrazine (Aatrex Nine-O, 85.5% atrazine and 4.5% other *s*-triazines, Ciba-Geigy, Research Triangle Park, NC) aqueous solution, ca. 100 mg/L ai, was ozonated in a 9-L glass bottle equipped with a stainless steel airstone on a Teflon feed tube. Ozone was generated using a Model GL-1 ozone generator (PCI Ozone Corp., West Caldwell, NJ) and added at a rate of 1 L/min, ca. 1% ozone in oxygen. Ozonation was carried out for several hours until the remaining compounds were CAAT and CDAT exclusively.

Microbial Incubations. DRS-I, tentatively identified as *K. terragena* (D. Durham, W. R. Grace and Co., Columbia, MD, personal communication, 1992), was isolated as described elsewhere (Hapeman and Shelton, 1993). PSA (NRRLB-12227) was obtained gratis from Ciba-Geigy, Greensboro, NC, and was originally isolated by Cook (1987). Microbial incubations with PSA and DRS-I were conducted using sterile 250-mL Erlenmeyer flasks containing 50 mL of media consisting of ozonated Aatrex Nine-O amended with 0.5% v/v (unless otherwise noted) corn syrup (Tru-Sweet 42, American Fructose Corp., Decatur, AL), 20 mM phosphate buffer (pH 7), and a trace metals solution (Shelton and Somich, 1988). Flasks (three replicates per experimental treatment) were incubated on a reciprocal shaker table (ca. 120 rpm) at 25 °C.

The effect of ammonia or carbon on *s*-triazine metabolism was assessed by amending the previously described medium with various concentrations of ammonium sulfate (0–0.8 M ammonia) or corn syrup (0–10%) and monitoring increases in turbidity (Klett–Summerson photoelectric colorimeter) and/or CAAT and CDAT dissipation.

CAAT Mineralization. Biometer flasks (Bartha and Pramer, 1965) (three replicates) containing 25 mL of previously described medium containing analytical grade CAAT were amended with CAAT-*U-ring*-^{14}C, obtained gratis from Ciba-Geigy, to give an initial activity of ca. 83 Bq/mL (5000 dpm/mL) and inoculated with DRS-I. The sidearms of the biometer flasks were filled with 10 mL of 0.1 N NaOH, and the NaOH solution was removed periodically and replaced with fresh solution. One milliliter of the NaOH solution was combined with 10 mL of Beckman Ready-Solv aqueous cocktail and activity counted using a Beckman LS 5000 TD scintillation counter with a programmed quench correction.

Bench-Scale Reactor Studies. Two reactor configurations were constructed: a continuous-flow stirred tank reactor (CFSTR) and an upflow fixed-film column reactor (Figures 2 and 3). Twenty-five milliliters of a dense DRS-I culture was used to inoculate the CFSTR, filled to the effluent line with sterilized distilled water amended with 0.5% corn syrup, 20 mM phosphate buffer, and trace metals (Shelton and Somich, 1988). The culture was incubated for 24 h before the flow was begun. A minimum of five detention times elapsed in the reactor before measurements were taken. Measurements of CAAT concentration were taken at detention times of 11.5, 16, and 37 h; a minimum of three measurements per detention time was taken to ensure a steady concentration and an accurate estimate of K_s (Metcalf & Eddy, Inc., 1979). Kinetic constants were obtained via determination of the slope and y intercept of a Lineweaver–Burk plot of the data. Linear regression of $1/S$ vs $X\Theta/(S_0 - S)$ yields an equation with Θ defined as detention time, X as biomass, and $S_0 - S$ as CAAT utilization; the slope is equal to K_s/k and the y intercept to $1/k$.

$$\frac{X\Theta}{S_0 - S} = \frac{K_s}{k}\frac{1}{S} + \frac{1}{k} \quad (1)$$

The column reactor was filled with Celite Biocatalyst Carrier R-625 (Manville, Denver, CO). A solution of ca. 30 mg/L CAAT was passed through the column prior to inoculation to determine if sorption to Celite could account for any decreases in effluent CAAT concentrations. The column was subsequently inoculated with ca. 250 mL of a dense DRS-I culture amended with 1% corn syrup (v/v) and ozonated Aatrex Nine-O solution. Initial CAAT concentration in the reactor was ca. 30 mg/L. Influent CAAT concentration varied from 30 to 50 mg/L and was dependent on the extent of ozonation. After the column culture was incubated for 3 days, flow was initiated and a detention time of 12 h maintained.

In both reactors, CAAT, phosphate buffer, and trace metals were added to the reactor via one line and the corn syrup added via a second line to prevent microbial growth in the tubing. CAAT concentrations were monitored periodically in both the effluent and influent sides.

RESULTS

Biodegradation of CAAT. Initial experiments were conducted to determine the effect of carbon and/or nitrogen additions on the ability of PSA and DRS-I to utilize CAAT as a nitrogen source for growth. PSA was unable to degrade CAAT in the presence of ammonia (8 mM); concentrations of CAAT increased as CDAT (initial concentration ca. 15 mg/L) was hydrolyzed to CAAT (Figure 4). However, PSA did degrade CAAT in the absence of ammonia, although only after a delay of 6 days.

In contrast, CAAT was rapidly degraded by DRS-I when both carbon (0.5% v/v) and 8 mM ammonia were present. A delay (2 days) was observed when only a carbon source was added. In both experiments, the rate of CAAT degradation was apparently equal to or greater than the rate of CDAT hydrolysis. No apparent CAAT degradation was observed in the absence of carbon additions (corn syrup), indicating that no significant concentrations of other utilizable carbon sources, such as formulations and surfactants, were present in the ozonation mixture.

Degradation of CAAT by DRS-I was observed over a wide range of ammonia concentrations after 2 days, although rates of CAAT degradation decreased somewhat as a function of increasing ammonia concentration (Figure 5). Furthermore, microbial growth was not inhibited even at high ammonia concentrations (800 mM) as indicated

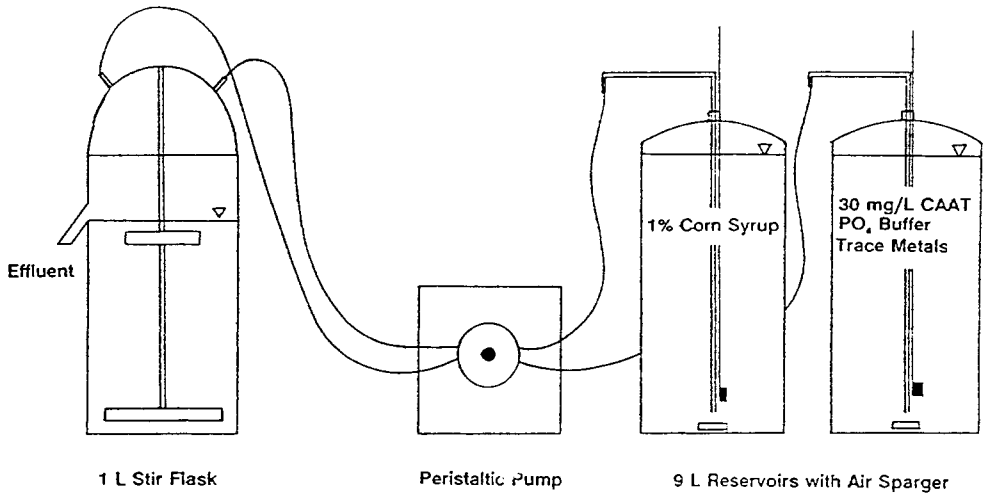

Figure 2. Continuous-flow stirred tank reactor.

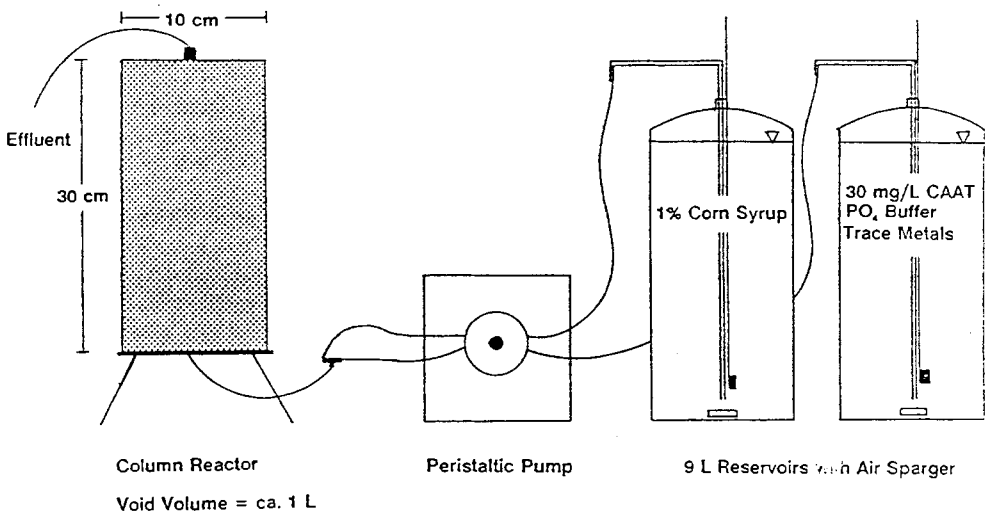

Figure 3. Upflow fixed-film column reactor (void volume ca. 1 L).

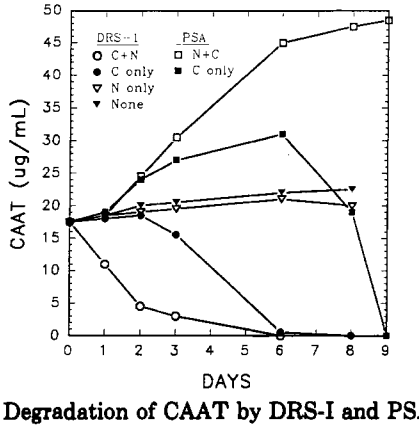

Figure 4. Degradation of CAAT by DRS-I and PSA.

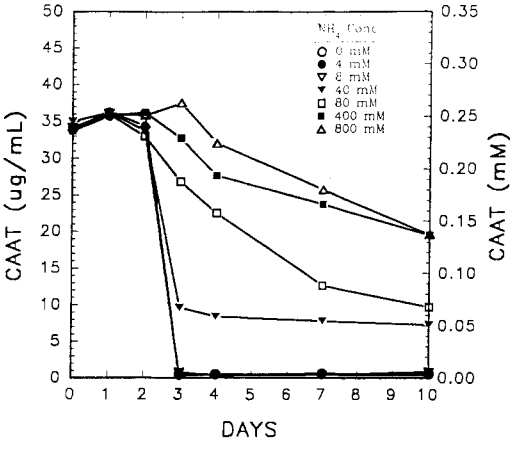

Figure 5. Ammonia tolerance of DRS-I.

by comparable increases in turbidity. Generally, maximum increases in turbidity and maximum rates of CAAT degradation occurred between days 3 and 4, suggesting that CAAT degradation occurred primarily during exponential growth. Ammonia was also utilized as a nitrogen source (final concentration 0.1 mM) at starting concentrations of 4 and 8 mM. Approximately 15% of the ammonia was utilized at an initial concentration of 40 mM, whereas no measurable depletion was observed at higher concentrations (data not shown).

Further experiments with no additional nitrogen (ammonia) and various concentrations of carbon (0, 0.1, 0.25, 1.0, 5.0, 10% v/v corn syrup) showed virtually no differences in CAAT degradation. Concentrations decreased from 18 to <1 mg/L after 3 days for all runs except 0%, where ca. 30% of CAAT was degraded within 10 days.

Fate of CAAT. Radiolabeled CAAT-*U-ring-*^{14}C was introduced into DRS-I cultures provided with corn syrup in biometer flasks capturing the generated CO_2 to determine if CAAT dissipation was due to transformation or due to complete mineralization. No activity was found in the NaOH solution on day 0 or day 1. Twenty-four, 57, and 77% of the initial activity was recovered as carbon dioxide after 5, 7, and 9 days, respectively.

Table I. Determination of k and K_s

detention time (Θ) (h)	S_0 (mg/L)	S (mg/L)	X (mg/L)	$1/S$ (L/mg)	$X\Theta/(S_0 - S)$ (h)
11.5	14.3	5.0	56	0.20	69.2
16	14.3	1.6	83	0.62	105
37	14.3	0.6	130	1.69	351

Bench-Scale Reactor Studies—CFSTR. Experiments with a continuous-flow stirred tank reactor (CFSTR) were initiated to determine if CAAT degradation by DRS-I would occur under steady-state conditions. The CFSTR provided an environment in which DRS-I could be rapidly established and fluctuations in growth conditions would produce responses in the microbial population. This is in contrast to fixed-film reactors which often require significantly more time to establish a microbial population and generally react more slowly to growth condition changes.

DRS-I grew readily under continuous-flow conditions. Detention times were altered after a dense culture was established to estimate Monod growth constants (eq 1). Changes in effluent substrate concentration were compared to changes in detention time (Θ), biomass (X), and CAAT utilization ($S_0 - S$) as shown in Table I. From these results, K_s and k were determined to be 19.5 mg/L CAAT and 2.4 days, respectively. These kinetic constants will allow for prediction of removal rates and provide a guideline for optimal reactor design.

Bench-Scale Reactor Studies—Column Reactor. Influent and effluent CAAT concentrations were observed to be essentially identical prior to inoculation, indicating that sorption to Celite solid support was not a factor in removal across the column. DRS-I readily exhibited biofilm growth on the Celite solid support. Column influent and effluent samples were monitored regularly (Figure 6). Significant CAAT removal was observed across the column when influent concentrations ranged from 15 to 30 mg/L. Breakthrough of CAAT occurred at day 3 due to a fluctuation in pH and whenever influent CAAT concentrations exceeded 30 mg/L.

DISCUSSION

PSA was unable to degrade CAAT when 8 mM ammonia was present, although microbial growth occurred. The increase in CAAT concentrations was due to hydrolysis of CDAT to CAAT. These data indicate that even at relatively lower concentrations, ammonia inhibits CAAT degradation. CAAT degradation did occur in the absence of ammonia, but only after 6 days. The delay in CAAT degradation in the absence of ammonia suggests that trace amounts of nitrogen-containing compounds in the pesticide waste were also utilized preferentially to CAAT or more likely that CDAT was being hydrolyzed at the same rate that CAAT was being utilized.

CAAT was rapidly degraded by DRS-I when a carbon source was provided, regardless of whether ammonia was present or not. Since CDAT is hydrolyzed to CAAT by DRS-I (data not shown), the delay in CAAT degradation in the absence of ammonia indicates that rates of CDAT hydrolysis and CAAT degradation were essentially the same through day 3. The accelerated degradation of CAAT in the presence of 8 mM ammonia appears to be an anomalous result since this was not observed in subsequent experiments. In the absence of either carbon or nitrogen there was no apparent loss of CAAT, although it is unclear whether this was due to the absence of both CDAT hydrolysis and CAAT degradation or due to identical rates of degradation. Clearly, however, significant CAAT degradation was not obvious in the absence of an extraneous carbon source.

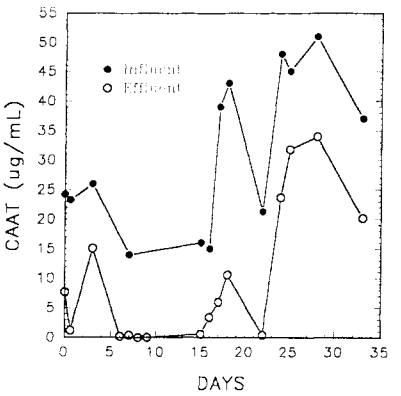

Figure 6. Degradation of CAAT in fixed-film column reactor.

In contrast to PSA, the degree of inhibition of CAAT degradation in DRS-I increased as a function of increasing ammonia concentrations, presumably due to competitive inhibition. However, even at 800 mM ammonia (1.4%) ca. 40% of the CAAT (initial concentration 0.25 mM) was degraded. These data indicate that although high ammonia concentrations are undesirable due to the competitive inhibition of CAAT degradation, they are not toxic to DRS-I (unlike PSA). The complete utilization of CAAT at initial ammonia concentrations of 4 and 8 mM is consistent with predicted utilization (ca. 8 mM) assuming a yield of 50% and a biomass C:N ratio of 10:1. These data indicate that DRS-I had a strong preference for CAAT as the nitrogen source and that high concentrations of ammonia were not inhibitory to growth.

Previous studies (Somich et al., 1990) in which ozonation was combined with biological treatment using PSA resulted in poor removal of s-triazines, presumably due to a combination of the microorganisms preference for ammonia as a nitrogen source and the inhibitory effect of high ammonia concentrations on microbial growth. Surfactants and/or formulating agents could have also inhibited s-triazine degradation, although there was no direct evidence to this effect. These studies indicated that the incorporation of the DRS-I culture into the treatment system could potentially improve the removal of s-triazines from the pesticide wastes on the basis of its ability to degrade CAAT in the presence of ammonia. In addition, the ability of DRS-I to grow in solutions containing ozonated Aatrex Nine-O indicated that the presence of formulations in the waste should not inhibit growth.

CAAT Mineralization. Extensive mineralization of radiolabeled CAAT-*U-ring-*^{14}C to $^{14}CO_2$ indicates that both the amino substituents of CAAT and the N atoms of the s-triazine ring were utilized as a nitrogen source. Mineralization of CAAT proceeded at a slower rate than during other experiments, but this is likely due to the nature of the incubation where biometer flasks were sealed to prevent the escape of $^{14}CO_2$. Consequently, reduced aeration likely limited the rate of CAAT degradation.

Scaleup of CAAT Mineralization. Bench-scale reactor studies indicated that either a CFSTR or a fixed-film reactor would support growth of the DRS-I culture and CAAT degradation; however, the fixed-film reactor is considered to be more practical for field operations since organisms in biofilms are more resistant to sudden fluctuations of nutrient supply or toxins in the reactor feed solution. Studies conducted with the CFSTR provided kinetic parameters that can be used to design additional bioreactors for CAAT degradation. The kinetic

constants will also provide a means to compare the ability of other organisms to degrade CAAT.

Results from the fixed-film reactor indicate that DRS-I, in conjunction with a suitable solid support, can be used to degrade CAAT and CDAT in an effluent ozonated waste stream. Breakthrough occurred at day 3 due to a fluctuation in pH and when CAAT concentrations exceeded 30 mg/L; however, these problems were remedied by closer monitoring of pH and decreasing the influent concentration, respectively. Alternatively, CAAT effluent concentration can approach zero by increasing the detention time. It is important to note that the biofilm culture recovered from both perturbations, indicating that the system is relatively tolerant of adverse conditions.

CONCLUSIONS

These data indicate that the binary process consisting of ozonation and microbial metabolism by DRS-I results in complete degradation (mineralization) of formulated atrazine under simulated field conditions, i.e., high nitrogen conditions and ambient temperatures. In addition, this process can be manipulated to remediate large quantities of atrazine-laden waste water and, potentially, contaminated soils. Future studies will include optimization of ozonation parameters and rates of microbial degradation in conjunction with design and fabrication of pilot-scale reactors.

NOTE ADDED IN PROOF

Recent data indicate that DRS-I may not be responsible for the hydrolysis of CDAT to CAAT at pH 7. CDAT hydrolysis attributed to DRS-I was probably due to chemical hydrolysis (CDAT is readily hydrolyzed at alkaline pH) or due to hydrolysis by microbial contaminants.

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