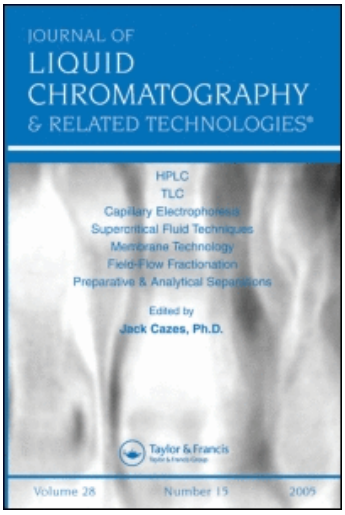

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KINETICS OF AZADIRACHTIN HYDROLYSIS IN MODEL AQUATIC SYSTEMS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The hydrolysis of azadirachtin-A isomer (AZ-A) was studied at 20°C in the dark in buffered distilled water at pH 4, 7 and 10, and in unbuffered sterilized and unsterilized pond water. Individual solutions were fortified in triplicate with pure AZ-A and formulated AZ-A separately. Hydrolysis of AZ-A in pond water was studied by using pure AZ-A only. At pH 10, AZ-A fortified either in pure form or as formulation, was hydrolysed rapidly and the DT_{50} was only about 2 h. At pH 4, the DT_{50} values for the pure and formulated AZ-A were 19.2 and 38.3 d, respectively, indicating that the chemical is relatively stable in acidic medium. The stability was diminished at pH 7 and the corresponding DT_{50} values were 12.9 and 30.5 d. The data show that the hydrolysis of AZ-A is greatly influenced by pH in the order pH 10 >>> pH 7 > pH 4. The differences in DT_{50} values between pure AZ-A and formulated AZ-A, at pH 4 and 7, suggest that hydrolysis is considerably retarded by the surfactants in the formulation. The average pH and DT_{50} values for the sterilized and unsterilized pond water were 8.08 ± 0.49 and 7.36 ± 0.28, and 6.91 d and 11.94 d, respectively. The faster degradation of pure AZ-A in

sterilized water, compared to the unsterilized water, was likely due to chemical hydrolysis. Microbial action in the degradation of AZ-A in the unsterilized pond water appeared to be minimal.

INTRODUCTION

The use of conventional broad-spectrum synthetic insecticides, although impressively effective in controlling various insect pests in Canada, is in rapid decline due to public concern and regulatory demands for selective and environmentally safe pest control products. Consequently, research in recent years has been turning more and more towards natural insecticides originating from plants, which are presumed to be innocuous. Among all the phytochemical pesticides being studied at present, azadirachtins (AZ), a mixture of seven structurally related tetranortriterpenoids isolated from the seeds of the neem tree or Indian lilac [*Azadirachta indica* A. Juss (Meliaceae)], have properties useful for the management of pests and show considerable promise to function as effective insecticides [1]. The major isomer, azadirachtin-A (AZ-A) has relatively high insecticidal activity. It causes mortality, improper molting, impaired reproductive capacity and deters feeding in various insect species, yet is safer to natural enemies and nontarget organisms than most conventional insecticides [2]. Neem-based formulations containing AZ-A have been registered in U.S.A. for the use on nonfood crops and ornamentals [3].

Recent laboratory [4] and field studies [5] have shown that neem preparations containing AZ-A are effective in controlling various target pests including the spruce budworm (*Choristoneura fumiferana* Clem.), a destructive defoliator of conifer forests in eastern Canada and north-eastern U.S.A. During any broadcast

application the insecticide may accidently drift or run-off into bodies of water. The use of neem-based formulations in forestry therefore requires an understanding of the aquatic dissipation of the major toxic component present, AZ-A, to evaluate its stability and toxic potential to aquatic organisms.

So far, very little information is available on the persistence and degradation of AZ-A, either in a pure state or as a formulated material, in waters of different acidities. The little that is available is inconclusive. Larson [6] reported the influence of low pH (3.8 to 4.2) on the stability of AZ. On the contrary, Tewari [7] found rapid hydrolysis of the material under acidic and alkaline conditions. We have investigated the dissipation of pure and formulated AZ-A in buffered distilled water over the pH range of 4 to 10 (to cover the spectrum of water acidities found in forest environment), and in sterilized and unsterilized natural waters under controlled laboratory conditions. Studies were conducted in the dark to diminish photolytic effects. The data are presented in this paper.

MATERIALS AND METHODS

Analytical grade azadirachtin-A (> 95 % purity) was purchased from Sigma Chemical Co., St. Louis, MO and used without any purification. It gave a single spot (R_F 0.58, 2-propanol:n-hexane 11:9) on silica gel TLC plate and a distinct single peak (RT 20.6 min) in HPLC [8]. The commercial formulation used was Azatin® (AgriDyne Inc., 417 Wakara Way, Salt Lake City, Utah 84108) containing 3 % AZ (mainly AZ-A by weight), *ca.* 4 % naphthalene, *ca.* 2 % butanol [9], emulsifiers (to keep the AZ uniformly distributed in the spray mix) and probably a sunscreen ingredient. The contents of the formulation are proprietary information

of AgriDyne Inc. Glass distilled water was used in all dissipation studies. Pond water was collected from the Searchmont area in N. Ontario in amber bottles (4 L) and had the following properties: pH 7.36; turbidity (JTU) 18.8; sp. conductivity (μmhos/cm) 18.4; hardness (mg of $CaCO_3$/L) 13.9; organic matter 16 μg/g; total P 0.098 μg/g; total N 0.093 μg/g; and Fe as $Fe^{2\oplus}$ 0.16 μg/g.

Buffer solutions of pH 4, 7 and 10 were prepared using ACS grade chemicals and distilled and sterilized water according to the procedure of Dean [10]. For pH 4, 10.21 g of potassium hydrogen phthalate, [$KH(C_8H_4O_4)$] per L (0.05 M); for pH 7, 3.40 g of potassium dihydrogen phosphate (KH_2PO_4) and 3.55 g of disodium hydrogen phosphate (Na_2HPO_4) per L (0.025 M each); and for pH 10, 2.10 g of sodium hydrogen carbonate ($NaHCO_3$) and 2.65 g of sodium carbonate (Na_2CO_3) per L (0.025 M each) were used. The pH of each solution was checked periodically using a pH meter (Model SA720, Orion Research Inc., Boston, MA 02129, USA).

For dissipation studies, each buffer solution (500-mL) was placed in triplicate in 1-L stoppered Teflon bottles covered outside with aluminum foil. Exactly 0.5 mL of standard AZ-A in methanol containing 40.0 μg/mL was added to each bottle to give an initial concentration of 40 μg/L. Each solution was shaken well and incubated in an environmental chamber (Controlled Environments, Winnipeg, Canada) kept at 20 ± 1°C. The dissipation was allowed to continue for 25 d post-treatment.

To study the dissipation of AZ-A in the formulation, 10-mL aliquots of the stock solution in methanol containing 4 μg AZ-A per mL were added to 1.0 L of each of the buffer solutions in triplicate to give an initial concentration of 40 μg/L.

The Teflon bottles were sealed, covered completely with Al-foil, shaken well and incubated in the environmental chamber as above for the hydrolytic degradation to occur over a 60-d period.

The pond water was filtered under suction using Gelman 5-μm PTFE membrane filter. A portion of the filtered water was sterilized in an autoclave (Amsco 2022) for 20 min. Both the sterilized and unsterilized water samples (500 mL each in triplicate) were fortified with standard AZ-A, to give initial concentrations of 40 μg/L. Dissipation experiments were conducted as in the hydrolysis of pure AZ-A in buffer solutions.

The kinetics of AZ-A loss was followed by measuring the residual concentrations of the chemical at known intervals of time (*i.e.*, 15 min after fortification, until the termination of the study) and plotting AZ-A concentration *vs.* time. The data points in Figures 1 to 4 are the means of triplicate measurements and the error bars represent standard deviations (SD). Unfortified buffer solutions and filtered pond waters served as the controls. No pH adjustments were done for the pond waters fortified with AZ-A, however their pH values were monitored throughout the study period.

At specific intervals of time, aliquot samples (10-30 mL) were taken from each bottle after thorough shaking and extracted with dichloromethane after the addition of aqueous sodium chloride. The organic layer was dried with anhydrous sodium sulfate and flash evaporated to dryness. The residue was taken in ethyl acetate and cleaned by Florisil® minicolumn. The column was eluted with ethyl acetate and the eluate, after concentration, was quantified by HPLC for AZ-A content. The details of extraction, cleanup and analysis of AZ-A are given elsewhere [8]. The

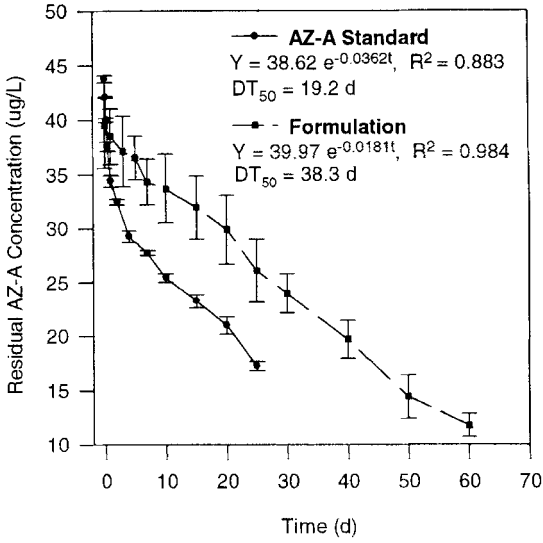


Figure 1. Degradation of AZ-A in distilled water buffered at pH 4 and fortified separately with pure AZ-A and AZ-A formulation. Error bars represent +/- SD (n=3).

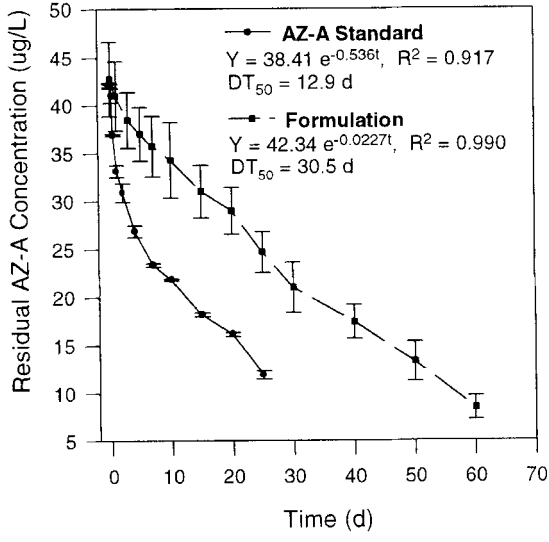


Figure 2. Degradation of AZ-A in distilled water buffered at pH 7 and fortified separately with pure AZ-A and AZ-A formulation. Error bars represent +/- SD (n=3).

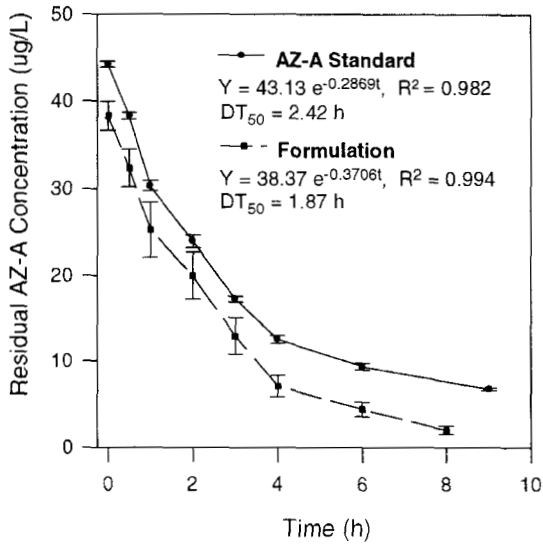


Figure 3. Degradation of AZ-A in distilled water buffered at pH 10 and fortified separately with pure AZ-A and AZ-A formulation. Error bars represent +/- SD (n=3).

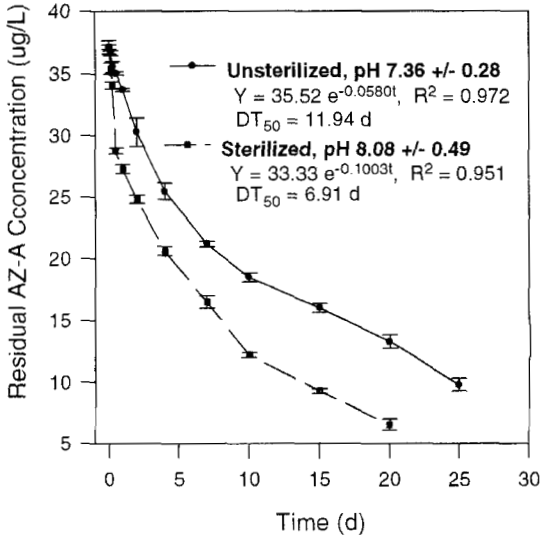


Figure 4. Degradation of AZ-A in sterilized and unsterilized pond water. Error bars represent +/- SD (n=3).

recovery of AZ-A from the buffer solutions and pond water at the 10 µg/L level was > 96 % and the limits of detection and quantification were, respectively, 1.0 and 3.0 µg/L.

RESULTS AND DISCUSSION

In the dark, at a constant incubation temperature and under sterile conditions, the degradation of AZ-A in the buffer systems was due to hydrolytic reaction in which the AZ-A molecule reacted with H_2O under the catalytic influence of $H^{\oplus}$ or $OH^{\ominus}$ ions, forming new products containing C– O bonds. The AZ-A degradation in the buffer solutions at pH 4, 7 and 10 and sterilized and unsterilized natural water was obtained using the first-order rate equation [11]

$$Y = Y_o e^{-kt} \quad (1)$$

where Y represents the AZ-A concentration at time t (d or h, depending on rate), Y_0 represents the initial AZ-A concentration and k is the rate constant. Logarithmic transformation yielded the linear equation:

$$2.303 \log_{10} (Y/Y_o) = -kt \quad (2)$$

As the concentration dissipated to 50 % of the initial amount, the DT_{50} (time required for 50% of the initial concentration to dissipate) value could be determined from equation (3) for each experiment:

$$DT_{50} = (2.303 \log_{10} 2) / k \quad (3)$$

All the plots of residual concentration of AZ-A *vs.* time were curvilinear for the pH range 4 to 10 (Figs. 1 to 3) and for the natural water (Fig. 4). The degradation data obeyed the exponential equation (1), indicating that the reaction followed first-order kinetics. Regression analysis of the residues remaining at time t gave a good fit, with R^2 (coefficient of determination) ranging from 0.883 to 0.994. The

values of k, R^2 and DT_{50} for the acidic (pH 4), neutral (pH 7) and alkaline (pH 10) buffers, and for the sterilized and unsterilized natural water samples, are listed in the corresponding figures.

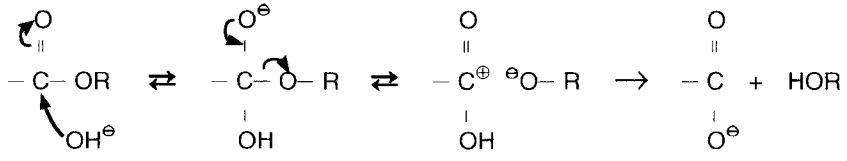
A comparison of the degradation of AZ-A under acidic, neutral and alkaline conditions show that the chemical, either in the pure or formulated form, remains relatively stable under acidic (pH 4) conditions (Fig. 1). Nearly 39 % of pure AZ-A and 66 % of AZ-A in the formulation remained in the buffer after 25 d. About 30 % of fortified AZ-A in the formulation remained in the buffer on the last day (60 d post-application) of sampling. The k and DT_{50} values for the pure AZ-A in the acidic buffer were, respectively, 0.0362 d^{-1} and 19.2 d. The corresponding values for the AZ-A in the formulated material were 0.0181 d^{-1} and 38.3 d, respectively.

The rate of degradation of pure AZ-A and formulated AZ-A in the buffer at pH 7 (Fig. 2) was higher compared to that in the pH 4 buffer. Only about 28 % of pure AZ-A and 58 % of AZ-A in the formulation remained on 25 d. About 20 % of AZ-A remained in the formulated material after 60 d. Correspondingly, the rate constants, k, were higher (0.0536 d^{-1} for pure AZ-A and 0.0227 d^{-1} for formulated AZ-A) and DT_{50} values (12.9 d for pure AZ-A and 30.5 d for formulated AZ-A) were lower, indicating greater degradation. Considering the k and DT_{50} values at pH 4 and 7, it is obvious that the rate of hydrolytic degradation of AZ-A increased with pH.

Under alkaline conditions (pH 10), the hydrolytic degradation of AZ-A either in the pure form or in formulation was extremely rapid (Fig. 3). The chemical was completely degraded in both the samples within 10 h, indicating the instability of AZ-A in strong alkaline solutions. The rate constants, k, were relatively high

(0.2869 h^{-1} for pure AZ-A and 0.3706 h^{-1} for formulated AZ-A) and the DT_{50} values found were hours, not days (2.42 h for pure AZ-A and 1.87 h for the formulated material). Despite the differences recorded, statistical treatment showed no difference between the two DT_{50} values (ANOVA $P > 0.05$). From the data in Figures 1 and 3, it is apparent that the effect of raising the pH from 4 to 10, reduced the DT_{50} values for the pure AZ-A and AZ-A in the formulation by a huge factor of nearly 190 and 492, respectively.

Azadirachtin is a complex molecule with a number of carboxylic ester groups and epoxide rings [12]. Its high instability in alkaline solutions is likely due to base-catalysed reactions involving aceyl-oxygen cleavages in the molecule through nucleophilic attack, thus altering the ester ($-\overset{\overset{O}{\|}}{C}-OR$) to carboxylate ($-\overset{\overset{O}{\|}}{C}-O^{\ominus}$) groups as shown below:



Such *base-catalysed* hydrolytic degradations are found to be common among organophosphorus insecticides, cleaving the C– O– P bonds [13, 14]. Nucleophilic attack by $OH^{\ominus}$ at the C atoms in the epoxide rings in the molecule, forcing them to open, followed by abstraction of protons from H_2O by alkoxide ions ($-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-$) to form diols ($-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-$), causing structural alterations, is also very likely.

The stability of AZ-A in the acidic medium (Fig.1) shows that acid-catalysed hydrolysis, *i.e.*, initial protonation of carbonyl oxygen as well as the O atom of

epoxide rings followed by the attack on the aceyl oxygen and/or the oxonium ion by the weak nucleophile, H_2O, is not a vigorous one. Also, unlike alkaline hydrolysis, the acid-catalysed reactions could be reversible. These factors could have contributed to the overall stability of AZ-A in the acidic medium.

Figures 1 and 2 show that AZ-A in the formulation degraded relatively slowly compared to pure AZ-A in the buffers at pH 4 and 7. This difference in degradation rates is attributable to the presence of surfactants (emulsifiers) in the formulation. Surfactants are large molecules and, by virtue of their combined polar (hydrophilic) and apolar (lipophilic) nature, form micelles in solution [15]. Azadirachtin molecules, being nonpolar and oleophilic, are likely to be partitioned and incorporated or solubilized inside the hydrophobic core of micelles, shielding them from the attack by the $H^{\oplus}$ ions present in the surrounding medium. There are no such micelles in the buffer solutions fortified with pure AZ-A, hence no such shielding mechanism was available for the molecules. It is likely that under high alkaline medium (pH 10), either micellization did not occur, or the micelles disintegrated rapidly, releasing the AZ-A molecules to $OH^{\ominus}$ ions for rapid interaction. Presently, there is sufficient evidence [16] for surfactant-facilitated stabilization of pesticide molecules in solution, however no such specific information is available yet on the stabilization of AZ-A molecules in solution. Additional research is necessary to study the influence of additives in stabilizing AZ-A molecules in spray formulations.

The AZ-A concentration (μg/L) in sterilized and unsterilized pond water as a function of time (d) is graphed in Figure 4. The initial concentrations in both samples were similar, 37.13 ± 0.54 μg/L (sterilized) and 37.14 ± 0.18 μg/L

(unsterilized). The average pH values for the sterilized and unsterilized water samples during the course of the study were 8.08 ± 0.49 and 7.36 ± 0.28, respectively. The values suggest that: (i) unsterilized water has some buffering capacity (low variability in pH) due to dissolved ions and humic compounds and (ii) the relatively high pH of sterilized water is due to the hydrolysis of the $CO_3^{2\ominus}$ and $PO_4^{3\ominus}$ ions in the solution.

The hydrolysis of AZ-A in sterile water was relatively rapid, probably due to the higher pH value (8.08 ± 0.49). No residues of the chemical were detected after 20 d, indicating its instability to hydrolysis. On the contrary, the AZ-A degraded rather slowly in the unsterilized water; about 26 % of the fortified amount remained after 25 d. Comparison of the rate constants (0.1003 d^{-1} for sterilized and 0.0580 d^{-1} for unsterilized water) and DT_{50} values (6.91 d for sterilized and 11.94 d for unsterilized water) definitely confirmed the slow loss of AZ-A in the unsterilized water. Contributing factors to this phenomenon could be the lower pH (7.36 ± 0.28) of the medium and absence of sufficient microbial population in the water. Azadirachtin is reported to have antiseptic and antimicrobial properties [17, 18]. Possibly, AZ-A used for the fortification of unsterilized water destroyed most of the microbial population, thus preventing their action on the degradation process. It is obvious that further research on the biological degradation of azadirachtin in natural waters, under controlled laboratory conditions, is necessary to validate this hypothesis. In addition, the role of sunlight and temperature, as well as the components in water such as humic compounds, metal ions, Bronsted acids and bases [19], on the degradation of AZ would be useful to get a holistic picture of its persistence in natural waters.

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