Fluorescent *Pseudomonas* Isolates from Mississippi Delta Oxbow Lakes: In Vitro Herbicide Biotransformations

R. M. Zablotowicz,[1] M. A. Locke,[1] R. E. Hoagland,[1] S. S. Knight,[2] B. Cash[3]

[1]U.S. Department of Agriculture-ARS, Southern Weed Science Research Unit, Stoneville, Mississippi 38776 USA

[2]U.S. Department of Agriculture-ARS, National Sedimentation Laboratory, Oxford, Mississippi 38677 USA

[3]Department of Biology, University of Mississippi, Oxford, Mississippi 38677 USA

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ABSTRACT: Fluorescent pseudomonads were a major component [log (10) 4.2–6.1 colony-forming units mL^{-1}] of the culturable heterotrophic gram-negative bacterioplankton observed in three Mississippi Delta oxbow lakes in this study. Pure cultures of fluorescent pseudomonads were isolated from three Mississippi Delta oxbow lakes (18 per lake), using selective media S-1. Classical physiological tests and Biolog GN plates were used in criteria for taxonomic identification. Most isolates were identified as biotypes of *Pseudomonas fluorescens* 55% (II), 7% (III), and 25% (V). About 7% of the isolates were identified as *P. putida* and 7% as non-fluorescent *Pseudomonas*-like. Cell suspensions of these isolates were tested for their ability to metabolize/co-metabolize six ^{14}C-radiolabeled herbicides (2,4-dichlorophenoxyacetic acid (2,4-D), cyanazine, fluometuron, metolachlor, propanil, and trifluralin) that are commonly used for crop production in this geographical area. Almost all (53 of 54) isolates transformed trifluralin via aromatic nitroreduction. Most isolates (70%) dechlorinated metolachlor to polar metabolites via glutathione conjugation. About 60% of the isolates hydrolyzed the amide bond of propanil (a rice herbicide) to dichloroaniline, with the highest frequency of propanil-hydrolyzing isolates observed in the lake from the watershed with rice cultivation. All propanil-hydrolyzing isolates were identified as *P. fluorescens* biotype II. No metabolism of cyanazine or fluometuron was observed by any isolates tested, indicating little or no potential for *N*-dealkylation among this group of bacterioplankton. No mineralization of 2,4-D labeled in either the carboxyl or ring position was observed. These results indicate that reductive and hydrolytic pathways for herbicide co-metabolism (aromatic nitroreduction, aryl acylamidase, and glutathione conjugation) are common in Mississippi Delta aquatic fluorescent pseudomonads; however, the potential for certain oxidative transformations (*N*-dealkylation, cyano group oxidation) may be rare in this group of bacterioplankton. Environ Toxicol 16: 9–19, 2001

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Correspondence to: R. M. Zablotowicz; e-mail: rzablotowicz@ars.usda.gov.

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INTRODUCTION

The existence of herbicides and other pesticides in surface water is a matter of concern because of the threat they present to human and wildlife health and general environmental quality. Agrochemicals, especially herbicides, are considered the primary contaminant affecting lake and stream water quality (U.S. EPA, 1994). Significant levels of certain herbicides and their metabolites can frequently be found in surface waters (Humenik et al., 1987; Thurman et al., 1991). Thus, research is needed to understand the sources and magnitude of non-point contamination of surface water by pesticides, the environmental fate of pesticides, and, in particular, the nature of biological dissipation mechanisms.

The fate of pesticides in surface waters of the Mississippi Delta has received less attention than it has in other geographical areas of the United States. Consequently, the Mississippi Delta Management Sytem Evaluation Area (MSEA) project was established to ascertain the impact of agricultural management practices on water quality in this important agricultural production region (Schreiber et al., 1996). Study sites were established in three small Mississippi Delta watersheds that drain into small oxbow lakes, thus allowing direct assessment of the effects of agricultural management practices on lake water quality.

Physical, chemical, and biological degradation processes affect the fate of pesticides in surface waters. Specific physical/chemical processes include photodegradation, sorption, and hydrolysis. Biodegradative processes in surface waters can be mediated by two microbial populations: those that form biofilms on surfaces and those that are suspended in the water column (Lewis and Gattie, 1998). Certain herbicides, such as metolachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide], have been observed to be somewhat recalcitrant to degradation in certain surface waters (Liu et al., 1995). Studies on the degradation of other chloroacetamide herbicides, propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide] and alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide] (1 mg L^{-1}), in lake water samples indicated that 59% of propachlor and only 10% of alachlor were metabolized during a 6-week incubation (Novick and Alexander, 1985). Herbicides such as propanil [*N*-(3,4-dichlorophenyl)propionamide] are prone to rapid biodegradation in water (Steen and Collette, 1989), especially when water samples are augmented with propanil-degrading bacteria (Correa and Steen, 1995).

To ascertain factors affecting herbicide fate in Mississippi Delta surface waters, the role of specific components of the planktonic community in the biotransformation of herbicides was investigated. Recent studies demonstrated that specific phytoplankton, e.g., *Ankistrodesmus* and *Selenastrum* sp., contribute to the degradation of the herbicides atrazine [6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine] and fluometuron (*N*,*N*-dimethyl-*N*-[3-(trifluoromethyl)-phenyl]urea) via *N*-dealkylation reactions (Zablotowicz et al., 1998c). Fluorescent pseudomonads are capable of co-metabolic and metabolic transformations of a wide range of pesticides and other xenobiotics (Hoagland et al., 1994; Hoagland and Zablotowicz, 1998; Zablotowicz et al., 1995, 1997). The same selective media S-1 (Gould et al., 1985) for fluorescent pseudomonads used in this present study also facilitated a high frequency of recovery of phenanthrene degraders (10%) from contaminated soils (Johnsen et al., 1996).

The objective of this study was to characterize a collection of fluorescent pseudomonads from Mississippi Delta oxbow lakes, using physiological taxonomic criteria. The ability of these isolates to co-metabolize or metabolize herbicides commonly used for crop production in watersheds surrounding these oxbow lakes was also assessed under in vitro conditions, using ^{14}C-radiolabeled herbicides. The relationship between taxonomic grouping and herbicide transformation was assessed.

MATERIALS AND METHODS

Lakewater Samples

Surface water samples were collected from three small oxbow lakes (10–20 ha) in the Mississippi Delta: Bealsey and Thighman lakes are located in Sunflower County, MS, and Deep Hollow is located in Leflore County, MS. Thighman is the largest of the lakes, with most of the watershed planted to cotton and soybeans, although rice and catfish production are also components of this watershed. Beasley lake is intermediate in size, and the watershed was cropped predominantly in cotton and soybeans and contains a large forested riparian zone. Structural best management practices (BMPs) such as slotted board risers and vegetative filter strips have been implemented to minimize sediment movement into Beasley Lake. Deep Hollow is the smallest lake, with a watershed cropped mostly in cotton and soybeans. The most extensive levels of BMPs—winter wheat cover crop, no-tillage farming practices, grass filter strips and slotted board risers—have been adopted at the Deep Hollow watershed.

Surface (0–20 cm deep) water samples (1 L) were collected from three stationary sampling rafts in each lake in 1996 on May 17, July 1, August 14, September 6, and December 4. Automated water quality monitor-

ing equipment (Yellow Springs Instruments, Kettering, OH) was used to obtain hourly measurements of temperature, pH, dissolved oxygen, and conductivity. Samples were refrigerated upon collection and processed for microbial enumeration within 24 h of collection. Suspended solids were determined by centrifugation of well-agitated water samples in tared 250 mL polypropylene centrifuge bottles at $10{,}400 \times g$ for 10 min. Following each centrifugation, the supernatants were carefully removed, and a total sample of 500 mL was centrifuged in the same bottle. The dry weight of solids was determined following drying at 60°C for 24 h. Fluorescein diacetate (FDA) hydrolysis was used as an indicator of metabolic activity (Schnürer and Rosswall, 1982). Briefly, triplicate 10 mL water samples were treated with 100 μL of an acetone solution of FDA (2 mg mL^{-1}) in sterile 25 mL Corex centrifuge tubes. Samples were incubated at 24°C for 24 h with reciprocal shaking. Assays were terminated by extraction with 10 mL of acetone, centrifugation at $12{,}000 \times g$ for 10 min, and measurement of absorbance at 490 nm.

Microbiological Techniques

Bacterioplankton populations were determined by serial dilution spiral plating using methodology similar to that previously described for soil and rhizosphere bacteria (Zablotowicz et al., 1997; Gould et al., 1985). Total aerobic heterotrophic bacteria, gram-negative and fluorescent pseudomonads, were enumerated by plating on dilute (1/10 strength) tryptic soy agar (TSA), dilute TSA with crystal violet (5 mg ml^{-1}), and S-1 selective media (Gould et al., 1985), respectively. Algal populations were estimated by serial dilution and a most-probable-number (MPN) technique, using five replicate tubes and Bristol's mineral salts media (Starr, 1964).

Isolate Characterization

Eighteen single colony bacterial isolates were randomly selected from platings on S-1 media from each lake (six isolates from each sample site for each lake). All isolates were evaluated for gram reaction, using light microscopy. Pigment production on S-1, Pseudomonas F media (Difco, Detroit, MI), and potato dextrose agar was assessed after 48 h of growth at 30°C under UV illumination. Oxidase tests were perfomed on 24 h TSA cultures, using tetramethyl-*p*-phenylenediamine dihydrochloride. Arginine dihydrolase activity was determined using standard methodology (Schaad, 1988). Gelatin liquefaction was determined in nutrient gelatin (Difco nutrient broth containing gelatin, 120 g L^{-1}) stabs after 3 and 7 days of growth. Denitrification was assessed using nitrate broth (Difco nutrient broth, with 1.0 g KNO_3 L^{-1}) and by gas production in Durham tubes. Nitrite formation was determined colorimetrically (Montgomery and Dymock, 1961), and residual nitrate was determined following reaction with zinc at 1, 3, and 7 days after inoculation. Peroxidase activity was determined from 48 h TSA cultures, using the method of Bordeleau and Bartha (1969), with *p*-anisidine as substrate.

Carbon substrate utilization for 44 of the original cultures was assessed using Biolog GN plates (Biolog, Hayward, CA) (Biolog, 1993). Inocula were prepared from 24 h TSA (half strength) cultures. Cells were removed with a sterile cotton swab and suspended in 0.85% NaCl to an absorbance of about 0.30 (660 nm). Plates were inoculated with cell suspensions (150 μL per well) and incubated at 30°C. Color development was monitored at 24 and 48 h, using a Bio-Tek model 311 plate reader with a 590 nm filter (Bio-Tek Instruments Co., Winooski, VT).

Chemicals

Uniformly ^{14}C-ring-labeled fluometuron (10.87 mCi $mmol^{-1}$) and metolachlor (8.236 mCi $mmol^{-1}$), both at greater than 99% radiochemical purity, and unlabeled metabolites were generously provided by Novartis Plant Protection (Greensboro, NC). Uniformly ^{14}C-ring-labeled propanil (9.47 mCi $mmol^{-1}$) was provided by Rohm and Haas (Philadelphia, PA). 3,4-Dichloroaniline was purchased from Chem Service (Chester, PA), and 3,4-dichloroacetanilide was synthesized using the procedure of Huffman and Allen (1960). Uniformly ^{14}C-ring-labeled cyanazine and technical grade metabolites were provided by E. I. du Pont de Nemours (Wilmington, DE). Ring and carboxy-labeled 2,4-dichlorophenoxyacetic acid (2,4-D) (16.0 and 9.0 mCi $mmol^{-1}$) was obtained from Sigma Chemical Company (St. Louis, MO). Uniformly ^{14}C-ring-labeled trifluralin (2.68 mCi $mmol^{-1}$) and metabolites were provided by DowElanco (Indianapolis, IN).

Herbicide Metabolism

In herbicide metabolism studies using resting cell suspensions, bacteria were grown on tryptic soy broth (TSB) at 25°C with reciprocal shaking at 100 rpm for 48 h. Cells were harvested by centrifugation (10 min, $12{,}000 \times g$), washed twice in Novick's mineral salts media (Novick and Alexander, 1985), and resuspended in mineral salts media to achieve an absorbance of about 8.0 at 660 nm.

Propanil Metabolism

Propanil transformation by all 54 isolates was assessed using a modification of an assay described elsewhere

(Hoagland et al., 1994). Cell suspensions (1.0 mL) were placed in sterile 25 mL Corex centrifuge tubes and treated with a 20 μL ethanol solution of ^{14}C-ring-labeled propanil to achieve a final concentration of 20 μM. Propanil-treated cells were incubated at 100 rpm, 25°C for 24 h. Assays were terminated by the addition of acetone (3 mL), extracted via sonication (15 min, Branson sonicator bath), and centrifuged (10 min, 10,000 $\times g$). Clarified extracts were spotted on 250 μm thick silica gel thin-layer chromatography (TLC) plates (Whatman LKF, Whatman, NJ), developed 10 cm using a benzene:acetone (10:1) solvent system, and analyzed by linear imaging scanning (Bioscan 200; Bioscan, Washington, DC). R_f values for propanil, 3,4-dichloroaniline, and 3,4-dichloroacetanilide were 0.42, 0.67, and 0.24, respectively.

Metolachlor Metabolism

Metolachlor metabolism was determined in resting cell suspensions of 27 cultures (nine isolates from each lake), using a modified assay (Zablotowicz et al., 1995). Cell suspensions (1.0 mL) were placed in sterile Corex centrifuge tubes (25 mL) and treated with a 20 μL ethanol solution of ^{14}C-ring-labeled metolachlor, to achieve a final concentration of 19.7 μM. Metolachlor-treated cells were incubated at 100 rpm, 25°C. Aliquots were removed at 36 and 72 h, terminated by the addition of 3 volumes of acetonitrile, extracted via sonication (15 min, Branson sonicator bath), and centrifuged (5 min, 10,000 $\times g$). The supernatant was filtered through a 2 μm filter and analyzed by high-pressure liquid chromatography (HPLC) (Hoagland et al., 1997), using a Beta-Ram Detector (INUS Systems, Tampa, FL) for detection of radiolabeled metabolites and metolachlor. Briefly, a μBondapak (Waters, CT) C18 reverse phase column and a 1% aqueous acetic acid/acetonitrile gradient (initial 30% acetonitrile, final 60% 15 min) were used. Reagent-grade metolachlor metabolites were provided by Novartis (Greensboro, NC). Glutathione, glyclcysteine, and cysteine conjugates were synthesized as described elsewhere (Zablotowicz et al., 1995). Retention times (RT) were glutathione conjugate, 5.1 min; cysteineglycine conjugate, 5.3 min; cysteine conjugate, 5.4 min; metolachlor oxanilic acid, 5.3 min; metolachlor sulfonic acid, 4.1 min; deisopropylmethoxy metolachlor, 6.8 min; and metolachlor, 13.0 min.

Fluometuron Metabolism

Fluometuron metabolism was evaluated in resting cell cultures of all 54 cultures and in cultures growing on 1/10 strength TSB (27 cultures, nine per lake). Cell suspensions (2.0 mL, absorbance 660 nm = 8.0) were treated with a 40 μL aliquot of an ethanol solution of ^{14}C-fluometuron to attain a concentration of 12 μM. In a similar fashion, 1.9 mL of dilute TSB was inoculated with 100 uL of cells (absorbance 660 nm = 8.0) and treated with 40 μL of [^{14}C]fluometuron to attain a concentration of 12 μM. In both cases the strains were incubated at 30°C and 100 rpm. Aliquots were removed at 24 and 72 h, extracted with three volumes of acetone as described above, and analyzed via RAD-TLC [250 μm thick silica gel TLC plates, developed with chloroform:ethanol (95:5 v:v) solvent], as described elsewhere (Zablotowicz et al., 1998c).

Cyanazine Metabolism

Cyanazine metabolism was evaluated in resting cell cultures of 27 isolates (nine per lake; same isolates as tested for metolachlor metabolism). Cell suspensions (2.0 mL, absorbance 660 nm = 8.0) were treated with a 40 μL aliquot of an ethanol solution of [^{14}C]fluometuron to attain a concentration of 12 μM. Aliquots were removed at 24 and 72 h and extracted with 3 volumes of acetone via sonication as described above. Following centrifugation, the supernatant was filtered (2 μm). Analysis was conducted by RAD-HPLC, using parameters described elsewhere (Goli et al., 1997). An adsorbosphere C18 reverse-phase column (25 cm $\times$ 4.6 mm i.d., 5 mm particle size; Altech Assoc., Deerfield, IL) with isocratic elution [aqueous acetic acid pH (3.4): methanol (55:45) mobile phase] and a Beta-Ram detector were used. RTs observed were as follows: cyanazine, 10.1 min; cyanazine amide, 5.4 min; *N*-dealkylated metabolites (deethyl cyanazine 4.75, deisopropyl atrazine, 4.7 min); and dechlorinated metabolites (cyanazine hydroxy acid, 9.1 min; deethyl hydroxycyanazine, 9.4 min).

Trifluralin Metabolism

Metabolism of trifluralin was evaluated for all 54 isolates under microaerophilic conditions. Cell suspensions (2.0 mL, absorbance 660 nm = 8.0) in 25 mL Corex centrifuge tubes were treated with an ethanol solution of [^{14}C]trifluralin (40 μL) to attain a concentration of 8 μM and then were incubated statically at 30°C. After a 24 h incubation, the reaction was terminated with 3 volumes of acetone and extracted as described above. RAD-TLC was conducted using two solvents, benzene:carbon tetrachloride (40:60) and hexane:methanol (97:3), as described by Golab et al. (1979). Aerobic incubations (30°C, reciprocal shaking at 150 rpm) were conducted for two isolates per lake.

2,4-D Mineralization

Mineralization of 2,4-D (16 μM carboxyl- or ring-labeled 2,4-D) was assessed in suspensions of resting cells in Novick's mineral salts or Novick's mineral salts amended with 1/10 strength TSB (3.0 g L^{-1}) as the carbon source. Both assays were conducted on 36 isolates (12 per lake). The microrespirometry method (Lehmicke et al., 1979), initially described as an M-P-N method, was used for these studies. In the first set (without exogenous nutrients), sterile shell vials containing 750 μL of Novick's mineral salts media, amended with either 16 μM carboxyl- or ring-labeled 2,4-D, were inoculated with 250 μL of washed cell suspensions (initial cell density = absorbance 2.0 at 660 nm). In the second set (with exogenous nutrients), sterile shell vials were filled with 750 μL of dilute TSB containing 12 μM ring-labeled 2,4-D and inoculated with 50 μL of washed cell suspensions (initial cell density = absorbance of 0.5 at 660 nm). The shell vials were sealed with sterile polyurethane foam plugs, placed in 20 mL glass scintillation vials containing 1.0 mL of 1.0 N NaOH, and tightly sealed with aluminum foil-lined caps. Shell vials were removed daily and placed in scintillation vials containing fresh NaOH over a 6-day incubation period. Hi-ionic-Fluor scintillation cocktail (15 mL) (Packard Instrument Co., Meriden, CT) was added to each vial, and the trapped radioactivity (mineralized $^{14}CO_2$) was determined by liquid scintillation counting (Packard-Tri-Carb 4000; Packard Instruments Co., Meriden, CT). All tests were replicated in four vials per isolate.

RESULTS AND DISCUSSION

Planktonic Populations and Lake Characteristics

Estimates of culturable aerobic heterotrophic bacterial populations associated with these three oxbow lakes are summarized in Table I. Total heterotrophic bacterial populations were log (10) 5.5–6.9 colony-forming units (c.f.u.) mL^{-1}. The highest bacterial populations were associated with the summer samples (July to September). Total bacterial populations observed here were similar to those observed in other freshwater lakes, especially during the warmer season (Jeppesen et al., 1997). Although there were some temporal differences in certain bacterial populations among the lakes, no specific effects of agricultural management practices on the microbial population estimates were noted.

During this testing period, populations of fluorescent pseudomonads ranged from log (10) 4.26–6.10 c.f.u. L^{-1} and represented 4–44% of the total heterotrophic bacterioplankton population enumerated on dilute TSA. In most cases, populations of fluorescent

TABLE I. Plankton populations associated with surface water samples (0–20 cm) of three Mississippi Delta oxbow lakes during 1996

Lake/Sample	Total Aerobic Heterotrophic Bacteria	Gram-Negative Bacteria	Fluorescent Pseudomonads	Algae
	Log (10) Colony-Forming Units mL^{-1}			
Beasley				
May 17	6.36 ± 0.08[a]	6.32 ± 0.09[a]	6.00 ± 0.13[a]	3.2 ± 0.4[a]
July 1	5.70 ± 0.56	5.05 ± 0.36	4.66 ± 0.40	4.6 ± 0.1
August 14	6.11 ± 0.14	5.73 ± 0.32	5.50 ± 0.18	5.1 ± 0.5
September 6	6.41 ± 0.15	6.05 ± 0.20	5.93 ± 0.18	4.8 ± 0.8
December 4	5.47 ± 0.15	4.90 ± 0.03	4.26 ± 0.12	2.9 ± 0.3
Deep Hollow				
May 17	5.61 ± 0.14	5.22 ± 0.04	4.98 ± 0.04	2.5 ± 0.1
July 1	6.08 ± 0.26	5.85 ± 0.25	5.44 ± 0.17	4.3 ± 0.1
August 14	6.73 ± 0.22	5.90 ± 0.02	5.75 ± 0.07	5.5 ± 0.6
September 6	6.91 ± 0.15	6.16 ± 0.31	6.10 ± 0.3	5.9 ± 0.4
December 4	5.39 ± 0.28	4.76 ± 0.07	4.29 ± 0.04	2.9 ± 0.4
Thighman				
May 17	5.63 ± 0.26	5.40 ± 0.26	5.18 ± 0.28	3.6 ± 0.2
July 1	6.00 ± 0.04	5.79 ± 0.01	5.63 ± 0.04	4.3 ± 0.3
August 14	6.61 ± 0.40	6.14 ± 0.28	5.97 ± 0.26	5.5 ± 0.4
September 6	6.43 ± 0.14	5.88 ± 0.22	5.85 ± 0.22	5.3 ± 0.3
December 4	5.68 ± 0.06	5.00 ± 0.04	4.31 ± 0.18	2.8 ± 0.1

[a] Mean and standard deviation of three replicates per lake.

pseudomonads enumerated on S-1 media represented the dominant gram-negative species. For example, fluorescent pseudomonads estimated using S-1 media represented 59–93% of bacteria estimated using crystal violet media during August and September. In December, numbers of fluorescent pseudomonads represented only 20–34% of gram-negative bacteria that were estimated using crystal violet media. Thus, the relative population densities of fluorescent pseudomonads in these lakes fluctuate with seasonal variations. Algal populations exhibited the greatest seasonal variation (Table I). Populations of algae observed in spring and winter water samples were about 2 log units lower than in summer samples.

Microbial activity in the lakes was estimated using FDA hydrolysis as a viable indicator (Table II) since it has been correlated with microbial respiration (Schnürer and Rosswall, 1982). The lowest activity was typically observed in Beasley Lake, while activity associated with Thighman Lake remained relatively high throughout all sampling times. FDA hydrolytic activity in Deep Hollow Lake was greatest during the summer, but lower during the spring and winter, corresponding to bacterial and algal populations. Generally, FDA hydrolysis was not correlated with suspended solids, temperature, or other lake water characteristics. Suspended solid concentrations during certain months were sufficiently high to consider these oxbow lakes as sediment-stressed systems. High concentrations of suspended sediment can significantly reduce light penetration and suppress primary productivity. Cooper and Bacon (1980) reported that primary productivity was adversely affected when suspended sediments exceeded 100 mg L^{-1}.

Physiological Characterization of Isolates

All bacteria isolated for study on S-1 media were gram-negative rods. Almost all (49 of 54 isolates) produced a fluorescent green pigment on both S-1 and Difco-PAF media, and none produced a blue pigment on potato dextrose agar. All 54 isolates tested positive for catalase, oxidase, and arginine dihydrolase, and none were capable of growth at either 37 or 41°C. Additional testing for denitrification, gelatin liquefaction, and carbon source utilization (based upon results from Biolog GN plates) was conducted for 44 isolates (Table III). Results from these lake isolates were atypical compared with results for biotypes of *P. fluorescens* strains provided by Biolog (1993). For our taxonomic identification, we used classical tests (e.g., fluorescent pigmentation, gelatin hydrolysis, arginine dihydrolase activity, and denitrification activity) plus results with Biolog tests of carbon source utilization as secondary

TABLE II. Other characteristics associated with surface water samples (0–20 cm) of three Mississippi Delta oxbow lakes during 1996

Lake/Sample	Suspended Solids (mg L^{-1})	FDA Hydrolysis (nmol mL^{-1}) 24 h^{-1})	Conductivity (mmhos)	Daily Mean Temperture (°C)	Daily Mean Dissolved Oxygen	pH
Beasley						
May 17	628 ± 7[a]	18.2 ± 2.8[a]	47	25.9	10.4	6.9
July 1	296 ± 24	6.4 ± 1.8	73	28.9	1.0	6.7
August 14	38 ± 9	5.6 ± 1.0	83	23.9	3.2	6.3
September 6	11 ± 2	18.2 ± 0.2	87	28.0	1.5	7.2
December 4	122 ± 16	13.2 ± 1.2	63	11.0	nd	6.4
Deep Hollow						
May 17	356 ± 25	18.8 ± 1.0	73	28.9	1.0	6.7
July 1	67 ± 10	27.8 ± 1.6	38	30.2	13.5	6.6
August 14	78 ± 18	52.2 ± 25.0	nd	24.1	14.2	6.4
September 6	37 ± 5	34.2 ± 6.8	nd	28.2	nd	6.8
December 4	42 ± 5	13.6 ± 1.8	nd	10.3	nd	6.0
Thighman						
May 17	474 ± 24	35.4 ± 12.0	118	25.5	1.75	6.7
July 1	62 ± 20	41.4 ± 7.6	291	29.5	0.63	6.8
August 14	79 ± 18	30.8 ± 1.4	176	26.1	14.97	7.3
September 6	43 ± 3	30.8 ± 1.4	218	27.7	0.52	7.0
December 4	217 ± 9	30.4 ± 3.4	78	10.7	nd	6.5

Note: nd = Not determined.
[a] Mean and standard deviation of three replicates.

criteria. Identification at the biotype level was feasible for most isolates, using criteria described in Bergey's Manual (Palleroni, 1984).

A majority of isolates (24 of 44 isolates tested) were classified as *P. fluorescens* biotype II (denitrifiers producing fluorescent pigments, hydrolyzing gelatin and utilizing arabinose, inositol, sorbitol, sucrose, but not urocanic acid, lactose, or cellobiose). Most of these isolates utilized glycogen and 2,3-butanediol. However, a major discrepancy from the Biolog database in the characteristics for *P. fluorescens* biotype II strains was that these isolates utilized several carbohydrates (e.g., inositol, sorbitol, sucrose, arabinose, mannitol, and *N*-acetyl-D-glucosamine), *p*-hydroxy phenylacetic acid, and quinnic acid. A second major group of gelatin-hydrolyzing, fluorescent isolates were unable to utilize sucrose, inositol, and sorbitol as carbon sources (11 of 44 isolates), but most utilized *N*-acetyl glucosamine, and all

TABLE III. Physiological characteristics (classical and Biolog) of 41 bacterial isolates from Mississippi Delta oxbow lakes for S-1 selective media

	% Positive Strains				
Test	*P. fluorescens* (Biotype II) 24	*P. fluorescens* (Biotype V) 11	*P. fluorescens* (Biotype III) 3	Non-fluorescent *Pseudomonas* 3	*P. putida* 3
Green Fluorescence S-1 media	100	100	100	0	100
Denitrification	92	0	100	33	0
Nitrate Reduction	8	45	0	67	67
Gelatin hydrolysis	100	100	100	37	0
Oxidase	100	100	100	100	100
Arginine dihydrolase	100	100	100	100	100
Growth at 37°C	0	0	0	0	0
Peroxidase	88	18	67	0	33
Biolog GN					
N-Acetyl glucosamine	83	64	66	0	0
Sucrose	100	0	0	67	0
Sorbitol	100	0	0	0	
Inositol	100	18	67	67	0
Mannitol	100	100	100	33	0
Arabinose	100	100	100	33	0
Arabitol	100	100	100	33	0
Glycogen	92	18	100	66	0
Urocanic acid	0	100	100	67	33
L-Ornithine	100	44	100	100	100
Phenylalanine	46	64	66	66	0
p-Hydroxy phenylacetic acid	100	0	33	0	0
Lactose	0	0	0	0	0
Cellobiose	0	0	0	0	0
β-Hydroxy butyric acid	100	100	100	100	100
Quinnic acid	96	100	100	66	0
D-Saccharic acid	100	100	0	33	0
Propionic acid	92	91	100	66	100
2,3-Butanediol	75	0	0	33	33

utilized arabinose, mannitol, and urocanic acid. None of the isolates in this group were denitrifiers, and nitrate reduction was observed in only 4 of 11 isolates. Thus, this group of isolates fits best the description of *P. fluorescens* biotype V. Among isolates of these two major biotypes, sufficient differences in physiological criteria indicate that all isolates were not merely clones of the same bacterium (Table III). Three denitrifying and gelatin-hydrolyzing fluorescent isolates were unable to utilize sucrose, sorbitol, and saccharic acid, but all utilized arabinose, propionic acid, and urocanic acid. These strains have mixed characteristics of biotypes III and IV; however, they did not produce a non-diffusible blue pigment on potato dextrose agar characteristic of biotype III.

A minority of strains (3 of 41) were fluorescent, arginine dihydrolase-positive strains that were unable to denitrify or hydrolyze gelatin. These three isolates were unable to metabolize most carbohydrates evaluated on Biolog plates (sucrose, arabinose, maltose, inositol, mannitol, sorbitol), which is characteristic of *P. putida*. The nonfluorescent isolates were diverse; however, based on certain key characteristics (positive for arginine dihydrolase, oxidase, catalase, gelatin liquefication, and β-hydroxy butyrate utilization, but unable to utilize lactose), some may actually be nonfluorescent *Pseudomonas* species. The highest frequency of denitrifiers and *P. fluorescens* biotype II was observed in Thighman Lake isolates (14 of 16) versus less than 50% in isolates from either Beasley or Deep Hollow Lake (Table IV). The high incidence of denitrifying isolates may be due to the rice cultivation in this watershed. Rice in the Mississippi Delta is flooded most of the growing season, which would favor a bacterioplankton population that utilizes other alternative electron acceptors in addition to oxygen.

Herbicide Degradation

Propanil Metabolism

The potential for degradation of six herbicides by pure cultures of these lake *Pseudomonas* isolates is summarized in Table V. *Pseudomonas* isolates that hydrolyze the amide bond of propanil were observed in all three lakes; however, the highest frequency was observed in Thighman Lake, the watershed with rice production. The metabolite resulting from aryl acylamidase cleavage, 3,4-dichloroaniline (DCA, $R_f = 0.67$), was detected in extracts of 31 of 54 isolates. All of the propanilhydrolyzing isolates were characterized as *P. fluorescens* biotype II.

DCA represented about 20–70% of the total radioactivity recovered following a 24-h incubation by propanil-hydrolyzing isolates. Most of the 3,4-dichloroaniline-producing isolates (26 of 31) also accumulated a second major metabolite ($R_f = 0.24$) that accounted for 15–50% of the recovered radioactivity that co-chromatographed with 3,4-dichloroacetanilide. We have observed formation of this metabolite in other bacterial strains (i.e., *Pseudomonas cepacia* AMMD) (Hoagland et al., 1994). In 10 of the 31 isolates, a low amount of radioactivity was observed at the origin ($< 10\%$ of the recovered radioactivity). This highly polar material may be glucosylated DCA, since a common detoxification mechanism for anilines in plants, animals, and microorganisms is conjugation with carbohydrate moieties (Iwan, 1976). Acetylation of 4-bromoaniline, a product of metobromuron metabolism, has been reported as a mechanism of detoxification by certain soil microorganisms (Tweedy et al., 1970). Acetylation apparently is a major mechanism for 3,4-dichloroaniline detoxification in the aquatic pseudomonads. Almost all propanil-hydrolyzing strains (88%) also exhibited peroxidase activity with *p*-anisidine (Table III). One of the major concerns of propanil usage is the formation of the highly toxic diazo compounds, such as tetrachloro-diazobenzene (TCAB), generated from peroxidase activity on 3,4-dichloroaniline (Hoagland et al., 1994). Acetylation may be a protective mechanism of these isolates to minimize the formation of TCAB.

Metolachlor Metabolism

A majority of isolates (19 of 27) transformed metolachlor to polar metabolite(s) (Table V). Following a 72 h incubation, more than 97% of the radioactivity of non-inoculated metolachlor controls was associated

TABLE IV. Distribution of *Pseudomonas* biotypes in Mississippi Delta oxbow lakes

	Frequency (Observed Biotype/Isolates Tested)				
Lake	*P. fluorescens* Biotype II	*P. fluorecens* Biotype V	*P. fluorecsens* Biotype III	Non-fluorescent *Pseudomonas*-like	*P. putida*
Beasley	5/16	8/16	1/16	1/16	1/16
Deep Hollow	5/12	2/12	1/12	2/12	2/12
Thighman	14/16	1/16	1/16	0/16	0/16

TABLE V. Herbicide transformations by resting cell suspensions of fluorescent pseudomonads from three Mississippi Delta oxbow lakes

Herbicide (Transformation)	Frequency (Observed Biotype/Isolates Tested)		
	Beasley	Deep Hollow	Thighman
2,4-D (carboxy mineralization)	0/12	0/12	0/12
2,4-D (ring mineralization)	0/12	0/12	0/12
Cyanazine (*N*-dealkylation, dechlorination)	0/9	0/9	0/9
Fluometuron (*N*-dealkylation)	0/18	0/18	0/18
Metolachlor (glutathione conjugation)	6/9	7/9	6/9
Propanil (aryl acylamidase hydrolysis)	6/18	9/18	16/18
Trifluralin (aromatic nitroreduction)	18/18	18/18	17/18

with a RT of 13.0 min (metolachlor) and only trace amounts (< 3%) of radioactivity observed at a RT of 5–5.3 min (polar metabolites). Isolates scored as positive for metolachlor transformation had over 8% of the radioactivity in a single peak with a retention time of about 5.3 min. Several of the more active isolates accumulated over 30% of the radioactivity in this polar fraction after 72 h. This fraction corresponded to a retention time characteristic for the elution of metolachlor oxanilic acid, glutathione, and other glutathione-derived conjugates of metolachlor (glycyl-cysteine and cysteine). No radioactivity eluted at a retention time corresponding to that of metolachlor sulfonic acid, a major metabolite of metolachlor found in soil studies (Aga et al., 1996). Isolates transforming metolachlor were observed among three biotypes of *P. fluorescens* (II, III, and V) and *P. putida*, but not in the non-fluorescent group.

In previous studies, most rhizosphere-derived strains of fluorescent pseudomonads dechlorinated alachlor via glutathione conjugation (Zablotowicz et al., 1994, 1995). Glutathione-mediated dehalogenation of metolachlor by *P. fluorescens* strain UA5-40 was significantly slower compared with rates with alachlor or propachlor (Hoagland et al., 1997). These current studies indicate that fluorescent pseudomonads may also contribute to the detoxification of metolachlor (and most likely other chloroacetamide herbicides) via glutathione-mediated dechlorination in Mississippi Delta lakes.

Trifluralin Metabolism

All but one isolate (non-fluorescent) of the 54 strains tested were capable of metabolizing trifluralin under microaerophilic conditions (Table IV). TLC analysis using the benzene:carbon tetrachloride (40:60) solvent indicated radioactivity at three regions besides residual trifluralin ($R_f = 0.76$), while only trifluralin was observed in non-inoculated controls. Most of the radioactivity recovered was associated with R_fs of 0.60 and 0.22, with a lower amount of radioactivity immobile at origin. When developed with the hexane:methanol solvent system, the more mobile compounds (hydrophobic compounds) migrated to R_fs of about 0.40–0.47, while the material previously observed to be immobile remained immobile. Based on R_fs reported by Golab et al. (1979), the major metabolites were α,α,α-trifluoro-5-nitro-*N*-propyl-toluene-3,4-diamine and α,α,α-trifluoro-*N*-propyl-toluene-3,4,5-triamine. This monoamino trifluralin metabolite was the major product of microaerophilic metabolism by resting cell suspensions of several *P. fluorescens* strains as well as *Enterobacter cloacae*, *Moraxella*, and *Sphingomonas* strains as confirmed by GC-MS (Zablotowicz et al., 1998a). When resting cell suspensions of six isolates were incubated with ^{14}C-labeled trifluralin under well-agitated aerobic conditions, more than 90% of the radioactivity was recovered as trifluralin.

These results indicate that the aromatic nitroreductase enzyme is widely distributed among aquatic fluorescent pseudomonads and occurs in most biotypes of *P. fluorescens* and *P. putida* studied, as was previously described for soil and rhizosphere fluorescent pseudomonads (Zablotowicz et al., 1997). Aromatic nitroreductase activity is also observed in denitrifying and nitrate-respiring biotypes, as well as isolates that lack dissimilatory nitrate reductase. Although the aromatic nitroreductase from several pseudomonads has been reported as an oxygen-tolerant enzyme, activity in whole cells was only observed under low oxygen tension in these and past studies (Zablotowicz et al., 1997).

Fluometuron and Cyanazine Metabolism

TLC analysis of culture extracts of resting cell suspensions and actively growing cells cultured on dilute TSB revealed that only fluometuron was recovered from all *Pseudomonas* cultures treated with ^{14}C-labeled fluometuron. The major mechanism for microbial fluometuron degradation is *N*-dealkylation, yielding *N*-dealkylated metabolites of fluometuron (desmethyl fluometuron and trifluoromethyl phenylurea), which were readily detected with this TLC analytical method (Zablotowicz et al., 1998c). Likewise, none of the 27 isolates tested here metabolized cyanazine, since all radioactivity recovered from cells or non-inoculated controls was recovered in a single HPLC peak (RT = 10.1 min). Cyanazine is typically metabolized via elimination of the ethyl group (*N*-dealkylation); oxidation of the cyano moiety, forming the amide and/or acid derivative; or dechlorination (Benyon et al., 1972). As in the case of fluometuron, cyanazine was not dealkylated by these isolates. Likewise cyanazine was not dechlorinated and was not the cyanide group oxidized by these fluorescent pseudomonads. Several studies (Behki and Khan, 1986; Vandepitte et al., 1994) have demonstrated that certain fluorescent pseudomonads were capable of *N*-dealkylation of the related triazine herbicide atrazine and that some of these dealkylating strains could dehalogenate atrazine, especially after it is dealkylated. Our present studies indicate that specific enzymes for *N*-dealkylation may be lacking or rare among most of the biotypes of aquatic fluorescent pseudomonads common to Mississippi Delta oxbow lakes.

2,4-D Mineralization

According to microrespirometry, none of the 36 isolates tested were capable of mineralizing either carboxyl- or ring-labeled 2,4-D as resting cell suspensions in mineral salts buffer. When these pseudomonads were grown on tryptic soy broth in the presence of ring- or carboxy-labeled 2,4-D, no mineralization occurred, although growth was luxuriant. These studies indicate a limited potential for 2,4-D metabolism or catabolism in this group of bacterioplankton.

CONCLUSIONS

We have observed that fluorescent pseudomonads are a major component of the culturable gram-negative bacterioplankton found in Mississippi Delta oxbow lakes. Most isolates from these lakes were characterized as *Pseudomonas fluorescens* biotypes II and V, with a lower frequency of *P. putida* or isolates characterized as *P. fluorescens* biotype III, or nonfluorescent *Pseudomonas*-like bacteria. Most *Pseudomonas* isolates recovered from Thighman Lake were denitifiers, which is typical for biotype II, and possessed aryl acylamidase activity (amide bond hydrolysis of propanil). Since rice is grown under flooded conditions, bacteria with the ability to denitrify would be favored. Propanil is used in most of the Mississippi Delta rice production areas; thus soil and water that are routinely exposed to propanil may harbor a more active population of aryl acylamidase-expressing bacteria. This observation suggests that agricultural cropping systems in the watershed may have influenced the *Pseudomonas* bacterioplankton population of the oxbow lakes. Aquatic fluorescent pseudomonads in Mississippi Delta lakes may contribute to herbicide co-metabolism via hydrolytic and reductive mechanisms; however, they may have little impact on oxidative mechanisms involved in herbicide degradation. Herbicide transformations reported here for aquatic pseudomonads are similar to those observed from soil or plant rhizosphere origin. These pure culture studies may give insight into the role of this specific group of bacterioplankton in pesticide degradation under natural conditions. Most transformations of herbicides and other complex xenobiotics usually do not occur through the activity of a single organism, but result from a series of transformations by a consortia of microorganisms (Levanon, 1993; Zablotowicz et al., 1998b). Initial co-metabolic transformations by mechanisms such as dehalogenation and hydrolysis, however, may render a molecule more prone to complete degradation (Zablotowicz et al., 1998b). There is great difficulty in ascertaining the role of individual components of a consortium; however, based upon these data, aquatic pseudomonads may contribute to the transformations of metolachlor, propanil, and trifluralin in natural aquatic systems.

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