ASSESSING INTERACTIONS OF MULTIPLE AGRICHEMICALS BY USING BACTERIAL ASSEMBLAGES IN A WETLAND MESOCOSM SYSTEM

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INTRODUCTION

Natural, constructed, and restored wetlands have been proposed for environmental applications such as treating wastewater, compensating a wetland loss elsewhere, and providing habitat for wildlife. Due to the unique capability of wetlands in retaining materials, a more recent wetland application under study around the country is to process non-point source (NPS) runoff, especially from rural agricultural lands (Newton 1989; Olson 1993). When NPS contaminants such as pesticides are processed in wetlands, their impacts in downstream water bodies such as rivers and streams may be eliminated or reduced.

Microbial degradation has been recognized as an important removal force of many pesticides in natural waters (Hwang et al. 1986). However, microbial activity may be subject to inhibition due to the toxicity of pesticide pollutants, especially at high concentrations. Any pesticide that inhibits natural microbial consortia will interfere with microbially-mediated biogeochemical cycling of essential elements an toxicants in natural ecosystems which could lead to adverse environmental impacts. Therefore, it is of dire importance to determine the effect of contaminants entering wetlands or their end products on microbial communities.

The effect of a contaminant on microbial activity depends on many factors including the mode of action of the compound, the path of entry of the compound into the cell, the presence of other contaminants, or physiochemical factors such as temperature, pH, light intensity, or presence of mineral turbidity. A toxic metal may be incorporated into cells by an active transport system that normally translocates an essential, chemically related metal. For example, phosphate transport systems are responsible for arsenate uptake. Consequently, the plasma membrane ATPase system and formation of a cross-membrane electrochemical gradient can be inhibited by arsenate (Hughes and Poole 1989). Clay minerals can affect the toxicity of some metal species to microorganisms, as the charge-compensating cations that are adsorbed on clays can be exchanged by other cations, including those of heavy metals such as mercury. The bioavailability of toxic heavy metals is reduced when these metals are adsorbed on clay minerals and temporarily removed from solution (Collins and Stotzky 1989).

Seldom are microorganisms exposed to a single contaminant in natural environments. Instead, they are often exposed to combinations of contaminants simultaneously. The presence of other cations in the environment can affect the toxicity of heavy metals to microbes, as a result of competition with the cationic forms of the heavy metals for anionic sites on cell surfaces. In addition, the concentration and composition of dissolved and particulate organic matter present in the environment can influence the mobility and bioavailability of heavy metals and, thereby, their toxicity. Therefore, interactions between and among contaminants are likely to occur and may result in synergistic or antagonistic effects on microbial assemblages in the mesocosms.

In this study, we examined the main and interactive effects of three commonly used agrichemicals and methyl-mercury, which commonly occurs as a background contaminant, on microbial metabolism in the sediments and water of a wetland mesocosm. Pesticides selected for this study were based on factors such as the application and volume of pesticide used in Mississippi, availability of their toxicology data, and our analytical capability for the chemicals. The three agrichemicals we used were atrazine (ATR), chlorpyrifos (CPF), and arsenate [as monosodium acid methanearsonate (MSMA)]. These three chemicals and a background contaminant (methyl mercury) was introduced into 66 experimental mesocosms in a center-point enhanced 2^4 factorial design. The effects of the candidate contaminants on abundance and heterotrophic potential of wetland heterotrophic bacterial assemblages were monitored for a duration of 94 days, including 32 days after half of the mesocosms were redosed.

MATERIALS AND METHODS

Description of Agrichemicals

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-i.e., a group of heterocyclic nitrogen compounds) has been

extensively used in North America over the last 30 years, especially in corn-growing areas (Phillips and McDougall 1993). ATR is prone to contaminate water because it is directly applied to soil and may then leach into ground water, streams, rivers, and lakes. Currently, there is increasing concern regarding its use because of its widespread distribution in the environment and the potential threat to human health by direct exposure or through consumption of contaminated ground water or food. ATR has been detected in lakes and streams at levels ranging from 0.1 to 30 μg/L with peak concentrations up to 1 mg/L known to occur in surface runoff from agricultural fields adjacent to bodies of water during times of applications (Day 1991). Several studies have been conducted under controlled conditions to determine the effects of ATR on selected species of aquatic flora (Day 1991). The half-life of ATR in aquatic environments has been found to range from 3 days to 8 months. Mineralization rates of ^{14}C- labeled ATR in soil, determined by using $^{14}CO_2$ evolution, ranged from 0.005% of the radioactivity after 12 weeks incubation to 28% after 24 weeks (Winkelmann and Klaine 1991). Although the inhibitory effects on algae are likely to be transient in some aquatic environments, small reservoirs in areas with intensive use of ATR, appear to be at substantial risk of exposure to ATR (Solomon et al. 1996).

Chlorpyrifos (CPF). CPF [O,O-diethyl O-(3,5,6-trichloro-2 pyridyl) phosphorothioate] has been used worldwide for over 20 years to control pests in agricultural crops, livestock, and for domestic purposes. It is a broad spectrum insecticide which is effective in controlling a variety of insects, including cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice. It is used as an insecticide on grain, cotton, field, fruit, nut and vegetable crops (Berg 1986). CPF is a nonsystemic contact chemical and acts on pests primarily as a contact poison, with some action as a stomach poison. CPF is moderately persistent. Its toxicity to microorganisms and availability in soil may contribute to the increased persistence of CPF observed in pyridinol-treated soils (Somasundaram and Coats 1990). CPF may bioconcentrate at very low levels in ecological systems (BCF = 2.5 to 3.5) (Howard 1989). In aerobic soils, the soil half-life of CPF was from 11 to 141 days in seven soils ranging in texture from loamy sand to clay and soil pHs from 5.4 to 7.4. CPF was less persistent in the soils with a higher pH. In anaerobic soils, the half-life was 15 days in loam and 58 days in clay soil (US EPA 1989 June). In water, CPF adsorbs strongly to soil particles and is not readily soluble in water. Therefore, it is immobile in soils and unlikely to leach or to contaminate ground water (US EPA 1989 June). However, its pyridinol hydrolysis product was found to be relatively mobile. Volatilization is probably the primary route of CPF loss from water with half lives ranging from 3.5 to 20 days (Racke 1992). Adsorbed CPF is subject to degradation by UV light, chemical hydrolysis, and by microbial degradation. The photolysis half-life of CPF is 3 to 4 weeks during midsummer in the U.S. (Howard 1989).

Arsenic has long been thought to contribute to the incidence of human cancer (Moore et al. 1994). Environmental arsenic contamination occurs mainly from industrial processes such as smelting of other metals, application of arsenical pesticides and herbicides, and power generation from coal or geothermal sources. Use of arsenical pesticides may increase arsenic concentration in plant species and eventually human intake. Heavy metals such as mercury have been reported to influence microorganisms by affecting their growth, morphology, and biochemical activities (e.g., respiration activity) (Beveridge and Doyle 1989). Metallic mercury is extensively used in the electrical industry, instrument manufacturing, electrolytic processes, and chemical catalysis. Mercury salts and phenylmercury compounds show strong antimicrobial activity by inhibiting the SH group on their enzyme molecules. Microbially mediated methylation of metals and metalloids including arsenic and mercury (Lyman 1995) may be a detoxification mechanism for microorganisms, but the methylated compounds produced can become more toxic to higher organisms. Mercury can be methylated, by aerobic and anaerobic bacteria, from Hg (II) to either monomethyl mercury or dimethyl mercury. The neurotoxicity accruing from exposures to high levels of methyl mercury became painfully evident from episodes of poisoning such as those at Minamata in the 1950s and in Iraq in the 1970s. Methyl mercury causes adverse central nervous system effects such as cerebral palsy and mental deficiency, as well as motor retardation and sensory deficits such as blindness and deafness (Chang 1996). Arsenic also can be methylated by some bacteria and fungi. The methylated products are volatile and highly toxic to humans (Atlas and Bartha 1993).

Experimental Design and Sampling

Wetland mesocosms of 500-liter mesocosms were designed and constructed for experimental purposes at the University of Mississippi's Biological Field station during mid-spring of 1996. The individual and interactive effects of selected agrichemicals (ATR, CPF, and MSMA) and methyl mercury (HG) on wetland heterotrophic bacterial assemblages were investigated using 66-mesocosms. At the bottom of each mesocosm, there was a layer of 15 cm of sand underneath a 5-cm layer of sediment from a nearby pond. The mesocosms were then filled with water from a spring pond. At the start of the experiment, each mesocosm contained several fish, various invertebrates, and the plant, Juncus effusus.

Chemicals were added to the mesocosms in a center-point enhanced factorial design. The amount of the chemicals added was based on the literature reports in Generic Expected Environmental Concentration Program

(GENEEC) version 1.2 from the Office of Pesticide Programs, U.S. EPA. The Program describes their average concentrations in the southeastern region in the U.S. Specifically, HG was added to bring the total mercury concentration to a nominal value of 0.4 mg/kg wet weight in the top 1 cm of sediments, about double background levels. ATR, CPF, and MSMA were added at nominal concentrations of 192, 51, and 219 μg/L, respectively. Chemicals were applied to the mesocosms in all possible combinations (total combinations = 16). There were three replicates of each combination. Additionally, eighteen other mesocosms (the center-points) received one-half the concentration of each of the four chemicals. The experiment started in June of 1996. Samples were collected 1, 2, 4, 8, 16, and 32 days after the addition of chemicals. On each sample date, one each of the three replicate mesocosms and six each of the center-point mesocosms was sampled. Thus, each mesocosm was sampled a maximum of twice. Data were analyzed by ANOVA utilizing SAS system (SAS/STAT 1988).

Microbial Biomass and Activity Measurements

Soil cores were collected from a depth of about 7.62 cm from the surface. Soil and water samples were collected with sterilized plastic syringes and containers. For counting total bacterial numbers, ten mL of the water subsamples were transferred into disposable polyethylene scintillation vials containing 0.55 mL of formaldehyde. About 0.1 c.c. of sediment sample was transferred to a bottle containing 19 ml filtered distilled water and 1 mL full-strength formalin. All of these preserved samples were stored in the dark at 4°C. Contents of the sediment samples were sonicated to disrupt sediment and distribute bacteria in water before counting. Total bacterial numbers were measured with Acridine Orange Direct Counting (AODC) technique of epifluorescence microscopy (Hobbie et al. 1977; Hwang and Maloney 1996). The effect of the test chemicals on bacterial heterotrophic activity was measured with bacterial mineralization of ^{14}C-D-glucose. About 1 μg/L of the radiolabeled glucose (S.A.: 246 mCi/mmol; Sigma Chemical Company) was dissolved in ethanol and added to 50 mL of the water or soil slurry samples (1c.c./50 mL) in milk dilution bottles, then incubated at 25°C in darkness for 1 hr. At the termination of the incubation, 0.5 mL of 2 N H_2SO_4 was added to the samples and the $^{14}CO_2$ evolved was trapped with 2-phenylethylamine-soaked filter papers (Hwang and Maloney 1996). The radioactivity was counted with liquid scintillation spectrometry.

RESULTS AND DISCUSSION

Biological assay procedures have been routinely used to monitor the environmental impact of many contaminants. Among them, microbial bioassays are widely applied for toxicity measurements, based on the assumption that microorganisms can act as surrogates for higher organisms in the ecosystem and be indicators of general stress to the environment. Moreover, microbial tests are relatively simple to perform, rapid, and inexpensive (Hwang and Maloney 1996; Hwang et al. 1989).

In this study, we assessed the main and interactive effects of chemical contaminants on bacterial heterotrophic activity in sediments and water. Over a 32-day exposure period, aerobic bacterial mineralization activity in sediment slurries exhibited significant responses only to a combination of HG*ATR*CPF (Table 1). After one day of exposure, the mineralization rate of glucose in sediment was 0.4 μg/c.c./hr by this treatment, relative to 0.18 μg/c.c./hr in the control treatment (Figure 1). Differences between the control and the HG*ATR*CPF treatment in sediments, however, were not significant except on the first day of the experiment (Table 2). In water, the only significant treatment effect on glucose mineralization over the entire period of 32 days was in the CPF treatment (Table 3; Figure 2). Again, the effect of CPF exposure was transient, becoming small after the first day of the experiment.

The effect sizes on glucose mineralization rate (i.e., extent of the influence) of HG*ATR*CPF in sediments and CPF in water generally decreased with exposure time (Table 4 and Table 5), suggesting the decreases in treatment effects were due to: (1) chemical concentration which we assume decreased over the course of the experiment, and (2) occurrence of bacterial adaptation to the test chemicals. Surprisingly, the interactive effect of HG*ATR*CPF on day 1 in both sediment and water was greater than the sum of individual effects caused by these three chemicals (Table 4). This may be due to chemical or physical interactions between the test chemicals, or it may be due to the combined biological actions of the chemicals involved. Antagonistic effects, with a decrease of chemical toxicity when chemicals are present together, may result if one compound induces enzymes that help in the detoxification of another compound (Landis and Yu 1995). Microbial resistance to ATR may be enhanced by induction of glutathione-detoxification system after exposure to HG (Marrs 1996), or as the result of gene transfer among the strains which are resistant to HG or ATR.

Total bacterial numbers fluctuated between 10^{10}/mL and 10^{5}/mL in sediment slurries and water samples, respectively. They did not exhibit any significant treatment effects. The lack of correlation between microbial glucose mineralization activity and AODC numbers may reflect the fact that significant portions of the microbial assemblages were not metabolically active and/or the turn over of the microbial communities were extremely rapid.

In this experiment, we adopted an ecosystem approach to the study of the effects of agrichemicals on wetland communities. We expected the bacterial community to be sensitive to the applied agrichemicals; instead, we found that over 32 days of exposure, microbial heterotrophic activity was sensitive to only the interactive effect of HG*ATR*CPF in the sediments and only CPF in the water. Microbial assemblages were found to recover their metabolic activity shortly after the exposure. We conclude that, except for a very limited period, microbial community activity and abundance are not affected by the chemicals or combination of chemicals used in these experiments.

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Table 1. SAS Output for Glucose Mineralization in Sediment (day1-32)- Tests of hypothesis using the type III MS for HG*ATR*AS*CPF (MESOC) as an error term (Pr*: p < 0.05)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HG	1	0.003	0.003	0.26	0.62
ATR	1	0.006	0.006	0.43	0.52
HG*ATR	1	0.002	0.002	0.15	0.70
AS	1	0.000	0.000	0.00	0.99
HG*AS	1	0.024	0.024	1.82	0.18
ATR*AS	1	0.015	0.015	1.09	0.30
HG*ATR*AS	1	0.008	0.008	0.56	0.46
CPF	1	0.007	0.007	0.49	0.49
HG*CPF	1	0.000	0.000	0.01	0.94
ATR*CPF	1	0.015	0.015	1.10	0.30
HG*ATR*CPF	1	0.054	0.054	4.06	0.05*
AS*CPF	1	0.014	0.014	1.02	0.32
HG*AS*CPF	1	0.000	0.000	0.03	0.86
ATR*AS*CPF	1	0.037	0.037	2.73	0.10
HG*ATR*AS*CPF	1	0.007	0.007	0.53	0.47

Table 2. SAS Output for Glucose Mineralization in Sediment (day1)- General Linear Models Procedure (Pr*: p < 0.05)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HG	1	0.004	0.004	0.32	0.60
ATR	1	0.014	0.014	1.11	0.34
HG*ATR	1	0.001	0.001	0.07	0.81
AS	1	0.001	0.001	0.09	0.77
HG*AS	1	0.124	0.124	9.56	0.03*
ATR*AS	1	0.002	0.002	0.15	0.72
HG*ATR*AS	1	0.000	0.000	0.01	0.92
CPF	1	0.005	0.005	0.35	0.58
HG*CPF	1	0.040	0.040	3.05	0.14
ATR*CPF	1	0.000	0.000	0.00	0.98
HG*ATR*CPF	1	0.242	0.242	18.57	0.01*
AS*CPF	1	0.007	0.007	0.53	0.50
HG*AS*CPF	1	0.010	0.010	0.80	0.41
ATR*AS*CPF	1	0.061	0.061	4.70	0.08
HG*ATR*AS*CPF	1	0.010	0.010	0.73	0.43

Table 3. SAS Output for Glucose Mineralization in Water (day1-32)- Tests of hypothesis using the type III MS for HG*ATR*AS*CPF (MESOC) as an error term (Pr*: p < 0.05)

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HG	1	0.000	0.000	0.21	0.65
ATR	1	0.001	0.001	1.00	0.32
HG*ATR	1	0.000	0.000	0.32	0.57
AS	1	0.000	0.000	0.35	0.55
HG*AS	1	0.004	0.004	2.95	0.09
ATR*AS	1	0.001	0.001	0.39	0.54
HG*ATR*AS	1	0.000	0.000	0.01	0.91
CPF	1	0.007	0.007	4.62	0.04*
HG*CPF	1	0.000	0.000	0.20	0.66
ATR*CPF	1	0.000	0.000	0.00	0.94
HG*ATR*CPF	1	0.000	0.000	0.17	0.68
AS*CPF	1	0.000	0.000	0.28	0.60
HG*AS*CPF	1	0.004	0.004	2.46	0.12
ATR*AS*CPF	1	0.000	0.000	0.22	0.64
HG*ATR*AS*CPF	1	0.000	0.000	0.10	0.75

Table 4. Relationships between Effect Size, Time, and HG*ATR*CPF Interactions in Sediment

Effect									
HG	ATR	CPF	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Overall
1	1	1	0.59	0.37	0.45	0.42	0.25	0.37	0.41
1	1	-1	0.21	0.40	0.47	0.29	0.23	0.39	0.33
1	-1	1	0.39	0.40	0.39	0.53	0.23	0.47	0.40
1	-1	-1	0.50	0.37	0.36	0.44	0.25	0.31	0.37
-1	1	1	0.26	0.30	0.43	0.43	0.26	0.43	0.35
-1	1	-1	0.57	0.41	0.41	0.39	0.17	0.34	0.38
-1	-1	1	0.58	0.42	0.30	0.51	0.25	0.51	0.43
-1	-1	-1	0.40	0.41	0.49	0.47	0.26	0.42	0.41
Effect Size			0.25	0.02	-0.06	0.01	-0.01	-0.04	0.03

Table 5. Relationships between Effect Size, Time, and CPF Interactions in Water

Effect								
CPF	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Overall
1	0.133	0.13	0.13	0.124	0.163	0.173	0.145
-1	0.091	0.128	0.128	0.136	0.18	0.135	0.136
Effect Size	0.041	0.003	0.003	-0.013	-0.018	0.034	0.008

Figure 1. Effect of HG*ATR*CPF on Microbial Mineralization of Glucose in Sediment.

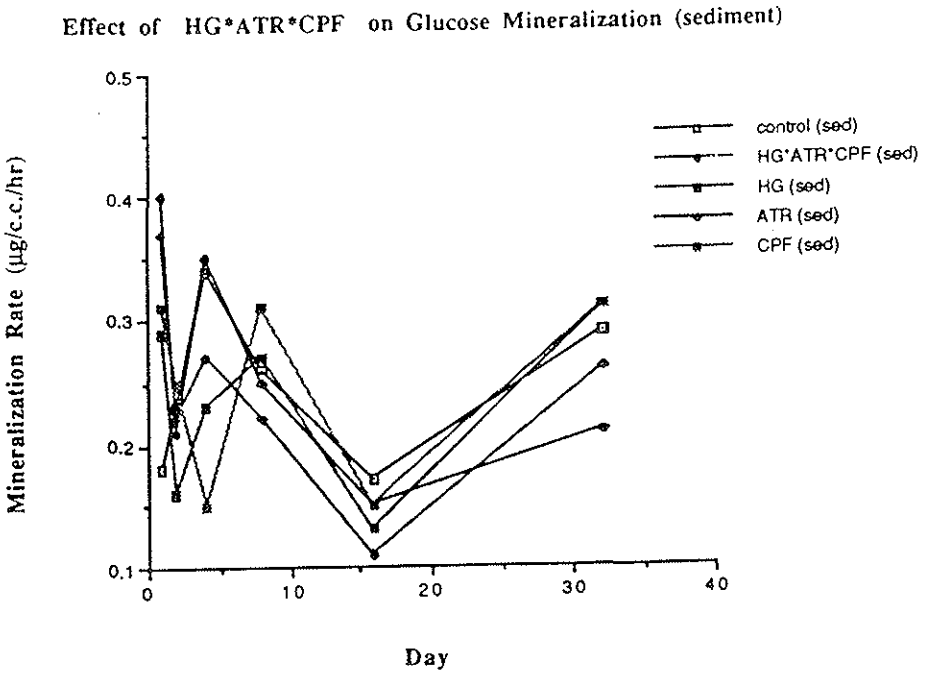

Figure 2. Effect of CPF on Microbial Mineralization of Glucose in Sediment and Water.