

## Assessing Interactions of Multiple Agrichemicals by Using Bacterial Assemblages in a Wetland Mesocosm System

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**Abstract:** Agrichemicals may enter wetlands located adjacent to or downstream from agricultural fields. We investigated the individual and interactive effects of three agrichemicals [atrazine, chlorpyrifos, and monosodium acid methanearsonate (MSMA)] and methyl mercury on abundance and heterotrophic potential of wetland heterotrophic bacteria assemblages. We used a factorial experimental design, in which chemicals were introduced in all possible combinations to 66 500-liter mesocosms at the Biological Field Station of the University of Mississippi. Methyl mercury was added to bring the total mercury (HG) concentration to 0.4 mg/Kg wet weight at the sediment surface. Atrazine, chlorpyrifos, and MSMA were added at concentrations of 192, 51, and 219 µg/L, respectively. 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. Total bacterial numbers did not exhibit any significant treatment effects. Therefore, the effects of agrichemicals were reflected on cell-specific bacterial heterotrophic activity rather than bacterial population size.

**Keywords:** Agrichemicals, bacterial assemblages, wetland, mesocosm

### 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 ability of wetlands to retain and transform materials, wetlands are being investigated as a low-impact method to process non-point source (NPS) runoff, especially from rural agricultural lands [1, 2]. 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 [3]. 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 and toxicants in natural ecosystems that eventually leads 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. Microorganisms are seldom 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 among contaminants are likely to occur and may result in synergistic or antagonistic effects on microbial assemblages in wetlands.

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 [4]. 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 [5].

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 was based on factor 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) were introduced into 66 experimental mesocosms in a center-point enhanced 2<sup>4</sup> factorial design. The effects of the candidate contaminants on abundance and heterotrophic potential of wetland heterotrophic bacterial assemblages were monitored for duration of 94 days, including 32 days after half of the mesocosms were redosed. We used microbial bioassays to determine the toxicity of the chemicals 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 [6, 7].

## Materials and Methods

### *Description of Agrichemicals*

Atrazine (2-chloro-4-ethylamine-6-isopropylamino-s-triazine) is a preemergent herbicide widely used to control broadleaf and grassy weeds during the cultivation of various crops in agricultural fields, as well as on fallow and industrial lands [8]. Because of its widespread use and relatively long persistence in the environment [9], substantial potential exists for aquatic organism exposure. Between 1992 and 1998 atrazine (ATR) was detected in 85% of surface water samples collected year-round in urban and agricultural areas and was measured at greater than 0.1 µg/L year-round in 34% of agricultural stream samples. The maximum concentration of atrazine measured during the sampling period was 120 µg/L [10]. The current drinking-water standard for atrazine is 3 µg/L [11]. Atrazine elicits toxicity in susceptible plant species and autotrophic prokaryotes by inhibiting photosynthetic activity in the thylakoid membrane [12, 13]. Clastogenicity of atrazine was reported to occur at concentrations that are likely encountered in natural water [14]. In June 2000 U.S.

EPA revisited the listing of atrazine as a “possible carcinogen” and was considering to list atrazine as a likely carcinogen [15]. Atrazine (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 an increasing concern regarding its use, because of its widespread distribution throughout the environment and the potential threat to human health by direct exposure or through consumption of contaminated ground water or food. The probability of risk from the greatest atrazine exposures is especially high in some small watersheds with extensive atrazine use and in reservoirs that receive drainage from these watersheds [9]. The half-life of ATR in aquatic environments has been found to range from 3 days to 8 months. Mineralization rates of <sup>14</sup>C- labeled ATR in soil, determined by using <sup>14</sup>CO<sub>2</sub> evolution, ranged from 0.005% of the radioactivity after 12 weeks incubation to 28% after 24 weeks [16].

Chlorpyrifos (CPF). CPF [O,O-diethyl O-(3,5,6-trichloro-2 pyridyl) phosphorothioate] has been used worldwide for over 20 years to control a variety of 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 [17]. 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. It 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 groundwater [18]. However, its pyridinol hydrolysis product was found to be relatively mobile. Its microbial toxicity and availability in soil may contribute to the increased persistence of CPF observed in pyridinol-treated soils [19]. CPF may bioconcentrate at very low levels in ecological systems (BCF = 2.5 to 3.5) [20]. 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. Soil half-life was not affected by soil texture or organic matter content. In anaerobic soils, the half-life was 15 days in loam and 58 days in clay soil [18]. Adsorbed CPF is subject to degradation by UV light, chemical hydrolysis and by microbial degradation. In water, CPF readily adsorbs to suspended sediment and bottom materials. Volatilization is probably the primary route of CPF loss from water with half lives ranging from 3.5 to 20 days [21]. The photolysis half-life of CPF is 3 to 4 weeks during midsummer in the U.S. [18].

Arsenic has long been thought to contribute to the incidence of human cancer [22]. 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) [23]. 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 [24], 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 [25]. Arsenic also can be methylated by some bacteria and fungi. The methylated products are volatile and highly toxic to humans [26].

#### *Experimental Design and Sampling*

Five hundred-liter (500L) circular cattle troughs (mesocosms) were used as the experimental wetlands for the study. 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 six small channel catfish (*Ictalurus punctatus*), various invertebrates, and were planted with *Juncus effusus*. Detailed description on the experimental design was given in Britson and Threlkeld [27].

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 of the U.S.A. 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 $\mu$ g/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 were sampled. Thus, each mesocosm was sampled a maximum of twice.

#### *Microbial Biomass and Activity Measurements.*

Soil cores were collected from a depth of 7.6 cm from the surface. Soil and water samples were collected with sterilized plastic syringes and containers. For counting total bacterial numbers, 10mL of the water subsamples were transferred into disposable polyethylene scintillation vials containing 0.55mL of formaldehyde. About 0.1 c.c. of sediment sample was transferred to a bottle containing 19mL filtered distilled water and 1mL 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 [28, 6]. The effect of the test chemicals on bacterial heterotrophic activity was measured with bacterial mineralization of <sup>14</sup>C-D-glucose. About 1  $\mu$ g/L of the radiolabeled glucose (S.A.: 246 mCi/mmol; Sigma Chemical Company) was dissolved in ethanol and added to 50mL of the water or soil slurry samples (1c.c./50mL) in milk dilution bottles, then incubated at 25°C in darkness for 1 hr. At the termination of the incubation, 0.5mL of 2 N H<sub>2</sub>SO<sub>4</sub> was added to the samples and the <sup>14</sup>CO<sub>2</sub> evolved was trapped with 2-phenylethylamine-soaked filter papers [6]. The radioactivity was counted by liquid scintillation spectrometry.

#### *Statistical Analysis*

Data were analyzed for significant treatment effects by ANOVA utilizing the SAS system [29]. Treatment effects are calculated as the mean response for all mesocosms having a particular treatment, e.g., the mean response for bacterial heterotrophic activity of all mesocosms containing chlorpyrifos. Effect sizes for a response variable were calculated as the difference between the response means of all mesocosms in which the factor is present and the mean response of all mesocosms in which that factor is absent. The overall mean effect, or effect size, is summed over all days of the experiment.

#### **Results and Discussion**

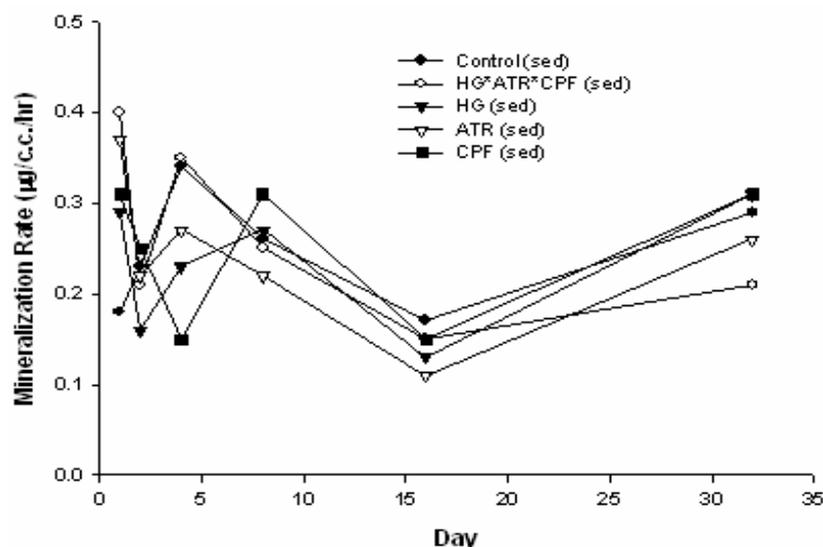
Measurement of bacterial heterotrophic activity (e.g., glucose mineralization) has been successfully utilized to assess the toxicity of many chemical contaminants [6, 30]. Because of the advantage of preserving the samples for later assays, direct counting of bacteria using the AODC technique was also used in this study to measure the total bacterial numbers present in the environmental samples. The ANOVA for days 1-32 of glucose mineralization in sediment is shown in Table 1. Over 32 days of exposure, aerobic bacterial mineralization activity of <sup>14</sup>C-UL-glucose in sediment slurries exhibited a significant response only to the HG\*ATR\*CPF

treatment (simultaneous presence of HG, ATR and CPF) (Table 1). For any single day of the experiment, significant responses were observed only for the treatments with HG\*AS and HG\*ATR\*CPF on day 1. For example, after one day of exposure, microbial mineralization rate of glucose in sediment was 0.40 µg/c.c./hr in the HG\*ATR\*CPF treatment compared to 0.18 µg/c.c./hr in control mesocosms in which no chemicals were added (Figure 1). In water, the CPF treatment was the only one to exert a significant effect on glucose mineralization for the duration of 32 days. In water HG\*ATR\*CPF treatment exhibited significant effect on glucose mineralization on day 1, but was insignificant over 32 days of exposure. On day 1, glucose mineralization rates were higher in the CPF experimental group (0.31 µg/c.c./hr in sediment and 0.12 µg/L/hr in water, respectively) than in the control group (0.18 µg/c.c./hr in sediment and 0.07 µg/L/hr in water, respectively). However, the CPF effect decreased after the first day of the experiment (Figure 2).

The effect sizes (i.e., extent of the influence) for glucose mineralization rate in sediments generally decreased over time in the HG\*ATR\*CPF treatment (Table 2). A similar trend was found for the CPF treatment in water (Table 3). A positive effective size means the bacteria were affected positively by a treatment (Figure 1, Tables 2 and 3). The effect sizes were positive early and then declined (Tables 2 and 3). We speculate that this pattern was due to: (1) decrease in chemical concentration over the course of the experiment- eg., sediment HG and water ATR concentrations of the experimental groups decreased from 0.4 to less than 0.07 mg/Kg and from 192 to less than 10 µg/L in 32 days, respectively (data not shown), and/or (2) early stimulation by the treatments and occurrence of bacterial adaptation to the test chemicals. Surprisingly, the interactive effect of HG\*ATR\*CPF in sediment was less than the sum of individual effects caused by each individual chemical (Table 2). For example, for Day 1 the 3-way interactive effect is 0.59.

**Table 1:** Analysis of variance of glucose mineralization in sediment (day1-32) - Tests of hypothesis using the type III SS and HG\*ATR\*AS\*CPF (MESOC) for the 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



**Figure 1:** Microbial mineralization of glucose in sediment for control, HG\*ATR\*CPF, HG, ATR and CPF treatments

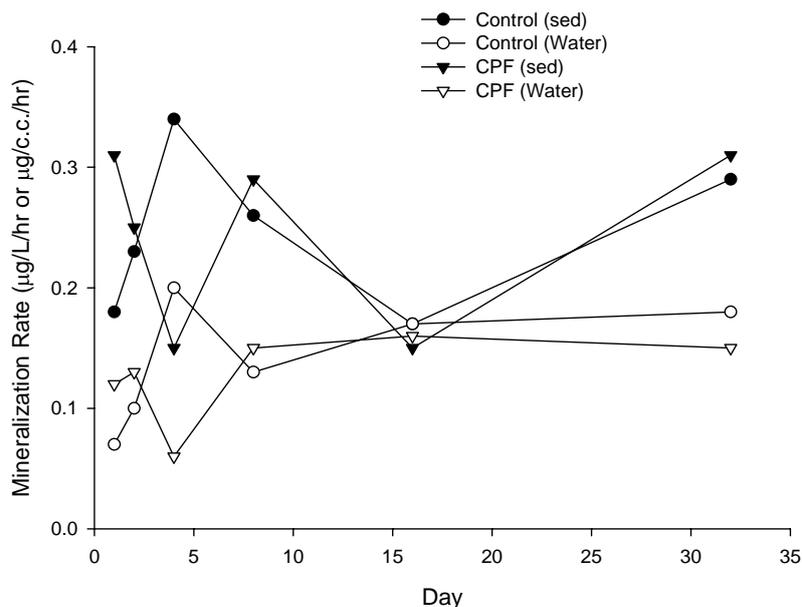


Figure 2: Effect of CPF on microbial mineralization of glucose in sediment and water

Table 2: Least significant means and effect sizes of microbial activity in sediment for the interaction of HG, ATR, and CPF on each date and overall. 1 = chemical present, -1 = chemical absent

Treatment			Day 1	Day 2	Day 4	Day 8	Day 16	Day 32	Overall
HG	ATR	CPF							
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
3-way Effect Size			0.25	0.02	-0.06	0.01	-0.01	-0.04	0.03

Table 3: Least significant means and effect sizes of microbial activity in water caused by CPF

Treatment		Day 1	Day 2	Day 4	Day 8	Day 16	Day 32	Overall
CPF								
Present		0.133	0.13	0.13	0.124	0.163	0.173	0.145
Absent		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

This value is less than the sum (1.65) of HG alone (0.50), ATR alone (0.57) and CPF alone (0.58). This may be due to chemical or physical interactions among the test chemicals, or it may be due to the combined biological actions of the chemicals involved. Antagonistic effects, with a decrease of chemical effect when chemicals are present together, may result if one compound induces enzymes that modify effects of other compounds [31]. Microbial mercury resistance mechanisms are determined by genes located in plasmids and transposons, and may be affiliated with the resistance factor for organic compounds including antibiotics [4]. Structural homologies between mercuric reductase, glutathione reductase, and lipoamide dehydrogenase were reported based on DNA sequence analysis of mercuric reductase genes [4]. It is possible that resistance to ATR and/or CPF is enhanced by microbial exposure to HG and the selection for organomercurial lyase, reductase synthase and glutathione-related detoxification mechanism of ATR [32]. Alternatively this could be the result of gene transfer among the strains that are resistant to HG or ATR.

Total bacterial numbers fluctuated around  $10^{10}$ /mL and  $10^5$ /mL in sediment slurries and water samples, respectively. They did not exhibit any significant treatment effects. Although AODC value can indicate the upper limit of bacterial population number, the technique is incapable of differentiating active from dormant bacteria. Poor correlation between direct fluorescence microscopic counts and plate counts (i.e., viability counting) was also reported by Atlas and Bartha [26]. Therefore, 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 was 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. Bacterial abundance was not changed detectably during the experimental period. Except for a very limited period, microbial community activity and abundance were not affected by the chemicals or combination of chemicals used in these experiments. A possible reason for the lack of effect is that bacteria consortia in the test mesocosms adapted to the test chemicals under the study condition.

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