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Test of Direct and Indirect Effects of Agrochemicals on the Survival of Fecal Indicator Bacteria[▽]

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Water bodies often receive agrochemicals and animal waste carrying fecal indicator bacteria (FIB) and zoonotic pathogens, but we know little about the effects of agrochemicals on these microbes. We assessed the direct effects of the pesticides atrazine, malathion, and chlorothalonil and inorganic fertilizer on *Escherichia coli* and enterococcal survival in simplified microcosms held in the dark. *E. coli* strain composition in sediments and water column were positively correlated, but none of the agrochemicals had significant direct effects on *E. coli* strain composition or on densities of culturable FIBs. In a companion study, microcosms with nondisinfected pond water and sediments were exposed to or shielded from sunlight to examine the potential indirect effects of atrazine and inorganic fertilizer on *E. coli*. The herbicide atrazine had no effect on *E. coli* in dark-exposed microcosms containing natural microbial and algal communities. However, in light-exposed microcosms, atrazine significantly lowered *E. coli* densities in the water column and significantly increased densities in the sediment compared to controls. This effect appears to be mediated by the effects of atrazine on algae, given that atrazine significantly reduced phytoplankton, which was a positive and negative predictor of *E. coli* densities in the water column and sediment, respectively. These data suggest that atrazine does not directly affect the survival of FIB, rather that it indirectly alters the distribution and abundance of *E. coli* by altering phytoplankton and periphyton communities. These results improve our understanding of the influence of agricultural practices on FIB densities in water bodies impacted by agricultural runoff.

Within the last century, agriculture has become a significant, and in some areas the dominant, form of land use (38). This agricultural intensification has had detrimental effects on neighboring freshwater and marine ecosystems, leading to the loss of biodiversity, blooms of harmful algal species, and shifts in the composition of food webs (52). In recent years, agricultural practices have been subjected to increased scrutiny for their potential contribution to human health risks (23, 31, 47, 67). For instance, many endemic and emerging human pathogens are derived from or associated with livestock, including pathogenic *E. coli* strains, *Salmonella enterica*, *Campylobacter jejuni*, *Cryptosporidium* spp., and zoonotic influenza viruses (53, 58, 59). Many of these agriculturally derived pathogens are waterborne and can enter water bodies via the introduction of runoff containing waste from livestock, such as cattle, swine, and poultry (6, 10, 23).

Due to the multitude of potential fecally derived pathogens in water, which include bacteria, protozoa, and viruses, testing directly for each pathogen is prohibitively costly and time-consuming. Consequently, regulatory agencies have relied on the density of indicator organisms (fecal indicator bacteria [FIB]) for over a century to detect fecal contamination and thus an increased likelihood of the presence of human pathogens (65). Many epidemiological studies in recreational waters have supported the association between elevated FIB densities and the risk of contracting gastroenteritis (14, 28, 61).

Storm water and agricultural runoff regularly introduce both microbial contaminants and agrochemicals, such as fertilizers and pesticides, to water bodies. Through direct or indirect mechanisms, agrochemicals may affect bacterial populations in beneficial or adverse ways (12). Possible direct effects include agrochemicals acting as nutrients (beneficial) or toxic compounds (adverse). Agrochemicals can also have indirect effects that are mediated by another species or by interactions with other factors. For instance, agrochemicals can alter the traits of an organism, such as behavior, immunity, physiology, or morphology, thereby affecting its interactions with other species. This effect has been seen in multicellular organisms (39–41, 43, 45) and microorganisms (15–17). Alternatively, agrochemicals may cause density-mediated, rather than trait-mediated, indirect effects via direct toxicity to a target species' food resources, parasites, competitors, or predators (18, 21, 22, 36, 41, 44–46, 51). FIBs, pathogens, and other organisms ranging from bacterivorous protozoa to algae to aquatic animals are exposed to agrochemicals in environmental waters that receive agricultural runoff, but we know very little about the effect of the chemicals on the fate of microorganisms in such systems.

Here, we assess the direct effects of the pesticides atrazine, malathion, and chlorothalonil and inorganic fertilizer (all at expected environmental concentrations) on *Escherichia coli* and enterococcal survival in simplified microcosms held in the dark. Based on our previous work (49), only atrazine and fertilizer were predicted to have indirect effects on *E. coli*. Hence, in a separate study, we assessed whether these agrochemicals had indirect effects on *E. coli* densities by incubating half of the microcosms in the dark to prevent the growth of phototrophs, while the other half were exposed to natural light. Each pesticide was selected because it was among the top two

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in usage within the United States for its pesticide type (e.g., herbicide, insecticide, synthetic fungicide [27]).

Fertilizer and atrazine both had positive effects on FIBs in our previous outdoor mesocosm experiment, where direct and indirect effects were combined, and thus we hypothesized that fertilizer and atrazine might impact FIB densities through either direct or indirect mechanisms (49). Previous research showed that soils amended with fertilizer resulted in greater persistence of *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, nonpathogenic *E. coli*, and enterococci (31, 32, 47), suggesting a potential direct mechanism. Similarly, positive direct effects were anticipated for atrazine, as past *in vitro* studies have shown that *E. coli* is chemotactic toward atrazine (33) and can directly utilize atrazine as a nutrient (30, 37, 66). Atrazine and fertilizer might also cause indirect effects by influencing the algal populations within water bodies. Fertilizer often increases algal growth, potentially increasing the reservoir for bacteria and nutrient availability to heterotrophs (4, 5, 11, 64). Atrazine is typically associated with decreases in phytoplankton that result in an increase in UV light penetration (45), which could stress bacteria in the water column (34, 48). Additionally, atrazine is often associated with increases in periphyton that could provide additional nutrients to bacteria in the sediment. Therefore, we hypothesize that atrazine will alter the algal community under light-exposed conditions, which in turn will result in a reduction in bacterial densities in the water column but an increase in densities in the sediment. Neither malathion nor chlorothalonil had significant effects on FIBs in our previous outdoor mesocosm experiment, where direct and indirect effects were combined, and thus we hypothesized that they would not have direct effects on FIBs in this experiment.

MATERIALS AND METHODS

Direct effect experiment. We established microcosms at the University of South Florida Botanical Garden (Tampa, FL) in an outdoor greenhouse to examine exclusively direct effects of four agrochemicals (atrazine, chlorothalonil, fertilizer, and malathione), and all their pairwise combinations, on the survival of FIBs. Replication was achieved by repeating each 12-microcosm block (controls, single agrochemical treatments, and pairwise combinations) four times (four temporal blocks). The first temporal block (block A) was run from 3 to 10 June 2009, block B was run from 17 to 24 June, block C was run from 1 to 8 July, and block D was run from 29 July through 5 August. Overall, 48 separate microcosms were established to examine direct effects. Air temperatures during the temporal blocks ranged from 21 to 34°C for block A, from 23 to 40°C for block B, from 22 to 37°C for block C, and from 18 to 39°C for block D. The microcosms consisted of 11.3-liter Rubbermaid plastic trash cans with opaque sides (29.97 by 22.86 by 33.65 cm), and each contained disinfected water (2 liters) and sediment (1 liter). Sediment was collected from the lower Hillsborough River (Tampa, FL). Each microcosm was disinfected prior to bacterial inoculation via the addition of 2 liters of a 30% (vol/vol) bleach solution made with deionized (DI) water. The bleach solution was then neutralized through the addition of 45 ml of a 10% sodium thiosulfate ($Na_2S_2O_3$) solution (0.225% final concentration), and served as the water column for each microcosm (60). Culturing on selective differential media (as described below) verified that the sediment and water column contained undetectable levels of *E. coli* and enterococci. Microcosms were covered individually with aluminum foil, and a dark tarp covered all microcosms to prevent exposure to light. The water column of each microcosm was inoculated with three *E. coli* strains and five *Enterococcus* sp. strains. Two of the *E. coli* strains were originally isolated from wastewater in Tampa, FL, and the third was *E. coli* 9637 (American Type Culture Collection). Inoculated *Enterococcus* strains included environmental water isolates (two *Enterococcus casseliflavus* strains and one *E. faecalis* strain isolated from Siesta Key, FL, and one *E. faecium* strain isolated from a University of South Florida pond, Tampa, FL) as well as *E. faecalis* 19433 (American Type Culture Collection). *E. coli* and enterococcal species/strains were selected based on differential survival observed in a previous study (1). This combination of "survivor" (exhibiting prolonged survival) and "nonsurvivor" (becoming unculturable in a relatively short time frame) strains was selected to determine whether or not strains previously demonstrated to exhibit differing degrees of robustness in secondary environments would all react similarly to the application of agrochemical treatments. One of the wastewater strains and ATCC 9637 were previously shown to be survivors, while the other wastewater strain was a nonsurvivor. The two *E. casseliflavus* strains and the *E. faecium* strain were observed to be survivors, while both *E. faecalis* strains were nonsurvivors.

The *E. coli* and *Enterococcus* sp. strains were streaked for isolation on Trypticase soy agar (TSA) and incubated for 24 h at 37°C (Fig. 1, flow chart). Isolated colonies were inoculated into 10 ml of brain heart infusion (BHI) broth and incubated for 24 h at 37°C. Each of these cultures was then centrifuged at 5,000 rpm for 5 min (IEC Multi, Thermo Scientific), and the supernatant was discarded. Each culture was then resuspended and washed in 10 ml of sterile buffered water (0.0425 g · liter^{-1} KH_2PO_4 and 0.4055 g · liter^{-1} $MgCl_2$) twice (20). Each of these 10-ml suspensions was then added to a bottle containing 90 ml of sterile buffered water. Bottles containing *E. coli* strains were then combined, as were bottles containing *Enterococcus* sp. strains. From both the bottles containing *E. coli* and the bottle containing enterococci, 3 ml of mixed culture was aseptically added to each microcosm (~10^7 CFU/100 ml for both *E. coli* and enterococci).

One hour after FIB inoculations, the 12 agrochemical treatments were assigned randomly to the microcosms. There were two control treatments, one consisting of DI water and a second in which DI water was amended with 0.002% acetone (used as a solvent for all agrochemicals). The remaining 10 microcosms received technical-grade agrochemicals: the herbicide atrazine, the insecticide malathion, the fungicide chlorothalonil, or inorganic fertilizer (sodium nitrate and sodium phosphate, purity >97%; Chemservice, West Chester, PA), either alone at their expected environmental concentrations (EEC, 102 μg/liter for atrazine, 101 μg/liter for malathion, 170 μg/liter for chlorothalonil, and 4,400 μg/liter N and 440 μg/liter P for fertilizer, calculated using the U.S. Environmental Protection Agency's GENEEC v2 software) or in pairwise combinations at their EEC. Each microcosm was then covered with a layer of aluminum foil, and all 12 were then covered with a dark tarp (1.676 m × 2.286 m) to prevent light penetration. The experiment was carried out in four sequential temporal blocks (see above for the specific dates for each temporal block). All microcosms were thoroughly cleaned and disinfected with 10% bleach following sampling at 1 week, prior to the establishment of the another replication of the 12 treatments.

Water and sediment samples were collected from each microcosm 1 h after FIB inoculations but immediately before agrochemical applications to obtain baseline (preagrochemical) measurements of FIB densities (time zero [T_0]). Water and sediment samples were also collected from each microcosm 24 h and 1 week after inoculation. In total, microcosms were sampled three times (T_0, T_{24}, and T_{168}) in each of the four temporal blocks.

Indirect effect experiment. We established 30 microcosms at the University of South Florida in an outdoor greenhouse, as described above, to test for indirect effects of atrazine and fertilizer on *E. coli*. These microcosms consisted of 2-liter glass beakers containing autoclaved DI water (1.5 liter), sediment collected from the lower Hillsborough River (0.5 liter disinfected by baking at 350°F for 24 h), and pond water collected from a eutrophic pond at the University of South Florida (0.5 liter). Half of the microcosms were completely covered with aluminum foil to prevent light penetration, while the other half were covered only at the top with plastic wrap. Algal populations from the seeded pond water were given 2 weeks to establish before treatment and *E. coli* applications.

In addition to bacteria already present in the pond water, the water column of each microcosm was inoculated with four strains of *E. coli*: *E. coli* 9637 (American Type Culture Collection) and three strains isolated from wastewater in Tampa, FL. These strains were prepared as described for the direct effect experiment (~10^7 CFU/100 ml).

One hour after FIB inoculations, the microcosms were randomly assigned one of three agrochemical treatments: a water control, inorganic fertilizer, or atrazine at 1× EEC (as described above). Microcosms were arranged so that there were five replicates (in spatial blocks conducted concurrently) of each agrochemical treatment for both light-exposed and dark conditions.

Water and sediment samples were collected from each microcosm 1 h after FIB inoculations but immediately before agrochemical applications to obtain baseline (preagrochemical) measurements of FIB densities (time zero). Water and sediment samples were also collected from each microcosm after 2, 7, 9, 14, and 28 days after inoculation. The sampling period used for this experiment was longer than that in the direct effect experiment because any indirect effects on the

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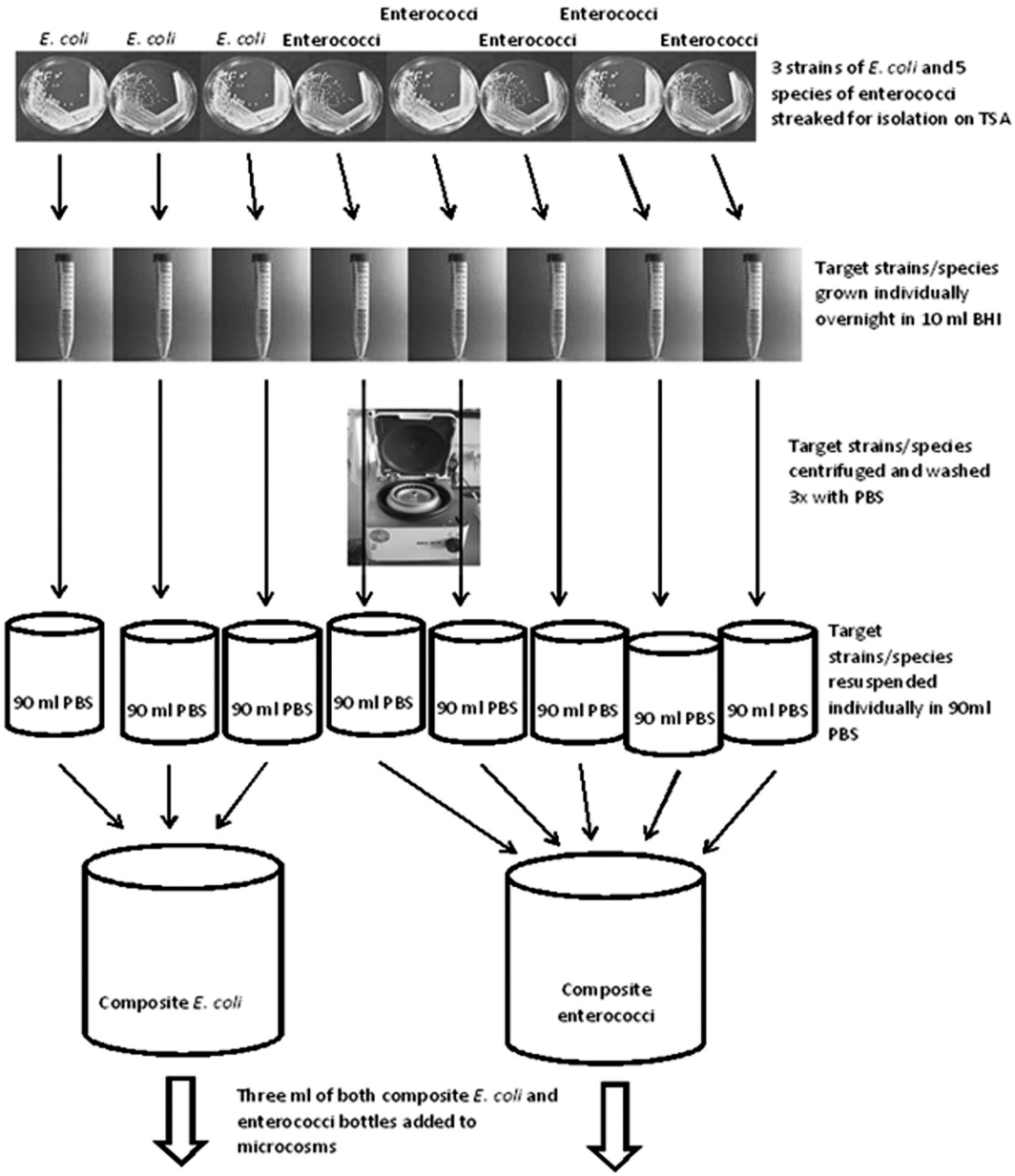

FIG. 1. Schematic of the procedure for used to isolate, grow, centrifuge, and inoculate the target bacteria. PBS, phosphate-buffered saline.

algal populations would require a longer period to become evident, as observed in our previous study (49). We used an AquaPen AP100 m (Photon Systems Instruments, Brno, Czech Republic) to quantify the effects of the treatments on suspended algae (phytoplankton). We measured chlorophyll *a* (F0) and photosynthetic efficiency (QY) initially from each microcosm and then at days 7, 14, and 28.

Sample collection and filtration. For both the direct and indirect effect experiments, water samples were aseptically collected in centrifuge tubes. Sediment samples were collected using a sterile 50-ml centrifuge tube to scoop the top 1 to 2 cm of the sediment across the length of the microcosm. Sterile gloves were worn when collecting samples, and the microcosms were shallow enough that no connection between human skin and the microcosms occurred. Samples were placed on ice for transport to the laboratory. Transit time to the laboratory was approximately 10 min, and samples were processed immediately upon arrival.

Water samples were filtered through a nitrocellulose membrane filter (0.45-μm pore size, 47-mm diameter; Fisher Scientific). Before filtration, sediment samples were weighed and diluted 1:10 (wt/vol) in sterile buffered water (20) and sonicated for 30 s at 14 W (sonic dismembrator model 100; Fisher Scientific) to dislodge bacterial particles attached to the sediment for the direct effect microcosms (1, 29). For the indirect effect experiment, sediment samples were shaken for 2 min instead of being subjected to sonication (8). Sediment suspensions were allowed to settle for several minutes before the supernatant was pipetted off and filtered as detailed above.

Culturable bacteria were enumerated by standard membrane filtration methods. For the direct effect microcosms, *E. coli* colonies were enumerated on mTEC agar at 35°C for 2 h, followed by 22 h of incubation at 44.5°C (57); enterococcal colonies were enumerated on mEI agar after 24 h of incubation at 41°C (56). For the indirect effect experiment, fecal coliforms were enumerated on mFC agar at 44.5°C for 24 h (13).

***E. coli* genetic typing.** No genetic typing was done on colonies isolated from the indirect effect experiment; however, *E. coli* isolates cultured from the direct effect experiment after 1 week of incubation were selected for genetic typing from each time block. Twenty isolates were randomly selected for each agrochemical treatment in both water and sediment samples, where possible. Colonies were picked with sterile toothpicks and transferred into wells of microtiter plates containing EC broth amended with 4-methylumbelliferyl-β-D-glucuronide

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(MUG; 50 μg/ml). Fluorescence under UV light was used to assess MUG cleavage, which is characteristic of *E. coli* and differentiates it from the remainder of the coliform group (2, 7). Cultures in microtiter plates were stored at −80°C after the addition of 3 drops of glycerol, used as a cryopreservant, to each well of the microtiter plate.

Strains were revived from freezer storage by streaking for isolation on TSA, followed by incubation for 24 h at 37°C. A single, well-isolated colony from each plate was then inoculated into 750 μl of BHI and incubated for 24 h at 37°C. To extract DNA, suspensions were centrifuged at 14,000 rpm for 1 min, and the supernatant was discarded. Cells were washed with autoclaved buffered water twice and then resuspended in 500 μl of autoclaved DI water and boiled for 5 min. This suspension was used as the template for PCRs. Negative extraction controls consisting of sterile DI water were run with each group of cultures subjected to DNA extraction.

Horizontal fluorophore-enhanced repetitive extragenic palindromic PCR (HFERP) was used to provide unique DNA banding patterns for each isolate (26). A positive control (*E. coli* 9637) and negative controls, including the extraction control described above and a no-template control, were run with each set of reactions. PCR products were loaded onto 1.5% agarose gels and run at 90 V for 4 h at room temperature. Gels were visualized using a Typhoon 9410 variable-mode imager (Molecular Dynamics/Amersham Biosciences, Sunnyvale, CA). Banding patterns were compared using BioNumerics (Applied Maths) and verified by eye. Typing of the water column isolates was done for the first three temporal blocks, and typing of the sediment samples was done for only the third temporal block because of financial and time constraints.

Statistical analysis. All response variables were log transformed, and block was included in all analyses. The residuals were always carefully scrutinized to ensure that the assumptions of the analysis were met.

(i) Direct effect experiment. ***(a) Treatment effects on FIB abundance.*** We conducted repeated-measures, regression-based, multivariate analysis of variance (MANOVA), where the repeated-measures factor was FIB density, on the three sampling intervals (times 0, 24, and 168 h [9]). In these analyses, we always included interactions between among- and within-tank (repeated-measures) factors. This allowed us to test for treatment-by-time interactions. In all MANOVAs, the response variables were the density of *E. coli* and enterococci on each sampling date. We first conducted a MANOVA to ensure that there were no differences in FIB densities among microcosms before agrochemical applications. We then tested for a difference between the water and solvent controls to determine whether we could pool these treatments. Finally, our full model included the main effects of sample location (sediment or water column) and the four agrochemicals, two-way and three-way interactions (excluding three-way interactions among agrochemicals given that we had only pairwise combinations), and repeated-measures main effects and interactions. ANOVAs were also conducted on each FIB to ensure that we did not miss any significant univariate effects (3).

(b) Treatment effects on FIB composition. To test for treatment effects on FIB composition, we arcsine square root transformed the proportions of *E. coli* colonies that were of the three inoculated strains and conducted a MANOVA test for the main effects of agrochemicals and their two-way interactions (50). Isolates from only three out of four of the blocks were typed (isolates from the fourth temporal block, block D, were not genetically typed).

(c) Relationship between FIB composition in the sediment and water column. To test for a relationship between FIB composition in the sediment and water column, for each microcosm in the third temporal block, we calculated the proportion of total colonies from the sediment and water column that were each of the three *E. coli* strains. Treating these three strains as independent of one another would have inappropriately tripled our sample size. Hence, we bootstrapped the relationship between FIB composition in the sediment and water column by randomly selecting paired sediment and water column samples, with replacement, until we reached the sample size for the block. We then calculated the Pearson's correlation coefficient for these randomly selected, paired sediment and water column samples. We did this 1,000 times and attained our probability value for the null model by calculating the proportion of these 1,000 correlation coefficients that were ≤0.

(ii) Indirect effect experiment. We conducted repeated-measures, regression-based, analysis of variance (ANOVA), where the repeated-measures factor was *E. coli* density on the five sampling intervals (times 2, 7, 9, 14, and 28 days). In these analyses, we always included interactions between among- and within-tank (repeated-measures) factors, as well as utilizing the day 0 densities as a continuous covariate to control for stochastic differences at the outset of the experiment. This allowed us to test for treatment-by-time interactions. In all ANOVAs, the response variables were the density of *E. coli* on each sampling date. Our full model included the main effects of light (present or absent) and the three agrochemical treatments (water, fertilizer, or atrazine), two-way interactions, and repeated-measures main effects and interactions. Separate ANOVAs were conducted for densities in the water column and the sediment. We used a repeated-measures multivariate analysis of variance (MANOVA) to evaluate the factorial effects of the treatments and light exposure on phytoplankton F0 and QY measurements on days 7, 14, and 28, controlling for initial phytoplankton abundance. We used a regression analysis to test whether phytoplankton abundance on day 28 (end of the experiment) was predictive of *E. coli* densities in the water column and sediment on day 28. We always conducted separate ANOVAs and MANOVAs excluding the fertilizer treatment to verify that significant differences were driven by a difference between the water control and atrazine and not by differences between fertilizer and atrazine treatments.

RESULTS

Direct effects of agrochemicals (dark conditions). Initial densities of *E. coli* in dark conditions before the addition of agrochemicals were $\sim 10^7$ CFU/100 ml in the water column and $\sim 10^5$ CFU/100 g (wet weight) in the sediment. Enterococcus densities were initially $\sim 10^7$ CFU/100 ml in the water column and $\sim 10^6$ CFU/100 g (wet weight) in the sediment. Average decay rates per day were calculated for *E. coli* and enterococci in both water and sediment by subtracting the density at T_{168} from the initial density and dividing by 7. Decay rates for *E. coli* (Fig. 2A) and enterococci (Fig. 2B) were not significantly different among treatments in either the water column or sediment.

Mean values from all temporal blocks showed *E. coli* densities in the water column and sediment ranging from $\sim 10^6$ to $\sim 10^9$ CFU per 100 ml or 100 g across all treatments. Enterococcus densities after 1 week in the water column ranged from $\sim 10^4$ to $\sim 10^6$ CFU/100 ml, and sediment densities ranged from $\sim 10^4$ to $\sim 10^8$ CFU/100 g. When averaged over all treatments and all blocks after a week, *E. coli* densities were significantly higher than enterococcus densities in both water and sediment matrices.

We found no significant differences in FIB density among microcosms before agrochemical applications and no significant difference between the water and solvent controls after agrochemical applications ($P > 0.05$). Hence, we pooled the water and solvent controls for subsequent analyses. There were significant multivariate effects of block ($F_{6,152} = 28.96$, $P < 0.001$), sampling intervals ($F_{2,76} = 38.43$, $P < 0.001$), and sampling location ($F_{2,76} = 7.69$, $P < 0.001$), i.e., the sediment had higher densities than the water column for enterococci ($F_{1,76} = 28.23$, $P < 0.001$) but not for *E. coli* ($F_{1,76} = 0.82$, $P = 0.367$). There were no significant main effects or interaction associated with any of the agrochemicals (all P values were >0.115).

MANOVA revealed that agrochemical treatments did not influence the genotype composition of *E. coli* strains (number of isolates of any given genotype recovered) or the proportion of each genotype recovered (all main effects and interactions had P values of >0.213). However, bootstrapping analysis revealed that there was a significant positive multivariate correlation between the genotypes recovered from the sediment and water column (mean $r = 0.532$, $P = 0.018$) (Fig. 3).

Indirect effects of agrochemicals (light conditions). Initially, and prior to agrochemical additions, *E. coli* densities in the water column were $\sim 10^7$ CFU/100 ml. After 2 days, *E. coli* densities in the water column had dropped ~3 logs in the light-exposed tanks and ~ 2 logs in the darkened tanks (Fig.

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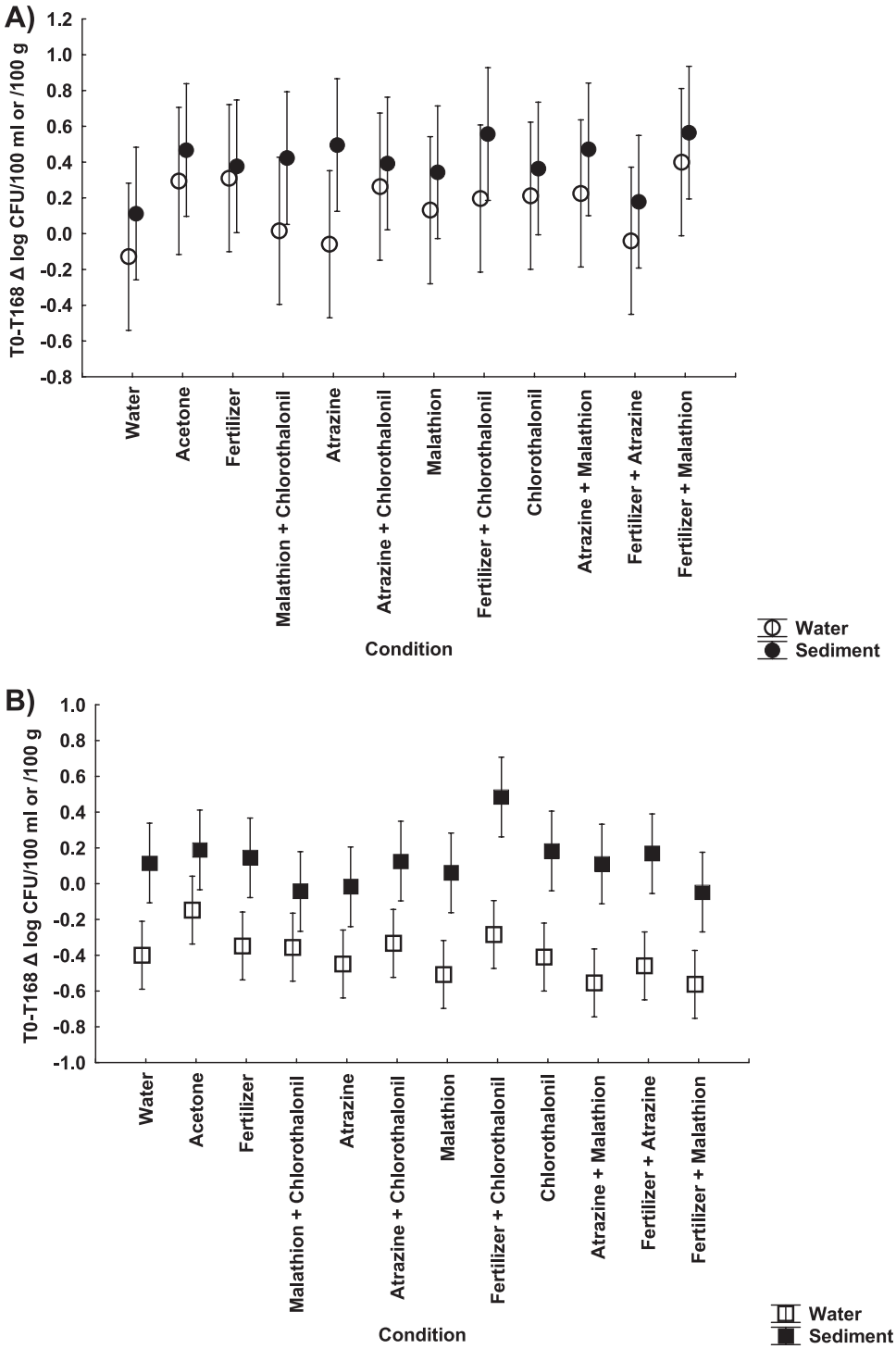

FIG. 2. Average decay rates per day (mean Δ log CFU per 100 ml or 100 g ± standard error [SE], $n = 4$) in water and sediment averaged over all four temporal blocks for *E. coli* (A) and enterococci (B).

4A), demonstrating the adverse effects of light exposure on water column *E. coli*. The decline in the *E. coli* densities slowed over the remainder of the experiment with declines of ~0.5 to 1 log/week for all tanks in either light or dark conditions.

Within the water column (Fig. 4A), ANOVA revealed significant main effects of light ($F_{1,19} = 5.36$, $P = 0.031$) and block ($F_{4,19} = 4.29$, $P = 0.012$) and a significant interaction between light exposure and treatment ($F_{2,19} = 4.05$, $P = 0.034$). An additional ANOVA was run excluding the fertilizer treatment, and a significant effect of light by treatment was still observed ($F_{1,11} = 8.24$, $P = 0.015$), meaning that the effect was the result of a difference between the water control and atrazine treat-

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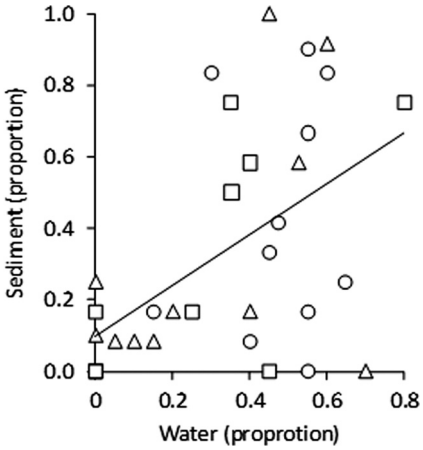

FIG. 3. The relationship between strain composition in the sediment and water column for three strains of *E. coli* (strain 14, squares; strain 19, triangles; strain 9637, circles). The three strains are not independent of one another because they were sampled from the same mesocosms. To calculate the multivariate correlation between composition in the water column and sediment, we bootstrapped the relationship, ensuring that our sample size for each of the 1,000 iterations matched the number of replicates. These results revealed an average Pearson's r of 0.532 and a probability value for the null model of 0.018.

ments, not the result of differences between the atrazine and fertilizer treatments. Under lit conditions, atrazine treatments resulted in significantly lower *E. coli* densities in the water column compared to control treatments, but atrazine had no significant effect on *E. coli* densities in the dark (for dark treatments at day 7: $P = 0.28$; at day 24: $P = 0.10$) (Fig. 4A). No significant effect was observed for fertilizer treatments relative to controls. No significant effects were seen for the repeated-measures main effect or interactions.

E. coli densities within the sediments of all treatments were initially $\sim 10^7$ CFU/100 g prior to agrochemical additions. No decline was seen between day 0 and day 2, although between days 2 and 7, a decline of approximately half a log was observed in the sediments of all treatments, and *E. coli* densities continued to decline more slowly than in the water column (Fig. 4B). *E. coli* densities in the sediments of the dark fertilizer treatment experienced a slightly slower, albeit not statistically significant, decay rate relative to other treatments. ANOVA revealed no significant main effect of light or block for the sediment densities; however, there was a significant main effect of treatment ($F_{2,19} = 3.57$, $P = 0.048$) and a significant light-by-treatment interaction ($F_{2,19} = 7.77$, $P = 0.003$). An additional ANOVA excluding fertilizer revealed a significant main effect of treatment ($F_{1,11} = 5.71$, $P = 0.036$) and light-by-treatment interaction ($F_{1,11} = 6.10$, $P = 0.031$). In other words, the significant effects of treatment and light by treatment were driven by differences between the water control and atrazine treatments, not by differences between the atrazine and fertilizer treatments. Under lit conditions, atrazine treatments resulted in significantly higher *E. coli* densities in the sediment after 28 days compared to control treatments, but atrazine had no significant effect on *E. coli* densities in the dark. No significant effect was observed for fertilizer treatments relative to controls. A significant main effect was observed for the repeated-measures factor ($F_{2,38} = 4.94$, $P = 0.012$) as well as a significant interaction between time by light ($F_{2,38} = 14.72$, $P < 0.001$), time by treatment ($F_{4,38} = 4.95$, $P = 0.003$), and time by light by treatment ($F_{4,38} = 7.64$, $P < 0.001$).

We quantified phytoplankton levels (chlorophyll *a*) in each microcosm to evaluate whether treatment effects on *E. coli* densities could be explained by indirect effects mediated by algae. There was a significant time-by-treatment-by-light interaction for the algal responses ($F_{8,40} = 2.20$, $P = 0.048$), and this interaction remained significant when the fertilizer treatment was excluded ($F_{4,12} = 4.17$, $P = 0.024$), indicating that phytoplankton were responding differently in atrazine-treated and control microcosms. This interaction was driven by atrazine having no effect on phytoplankton abundance in dark or lit conditions until day 28, when chlorophyll *a* measures were lower in the atrazine-treated microcosms than the control microcosms under lit conditions only (Fig. 5). Furthermore, algal abundance at the end of the experiment was a significant positive predictor of *E. coli* densities in the water column regard-

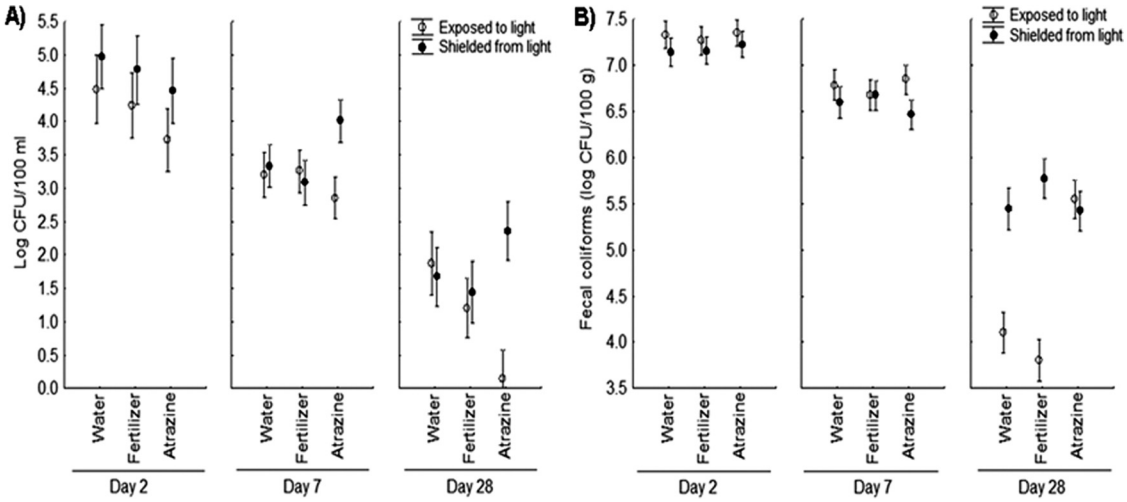

FIG. 4. *E. coli* densities in the water (A) and sediment (B) for each treatment (mean ± SE, $n = 5$) at days 2, 7, and 28.

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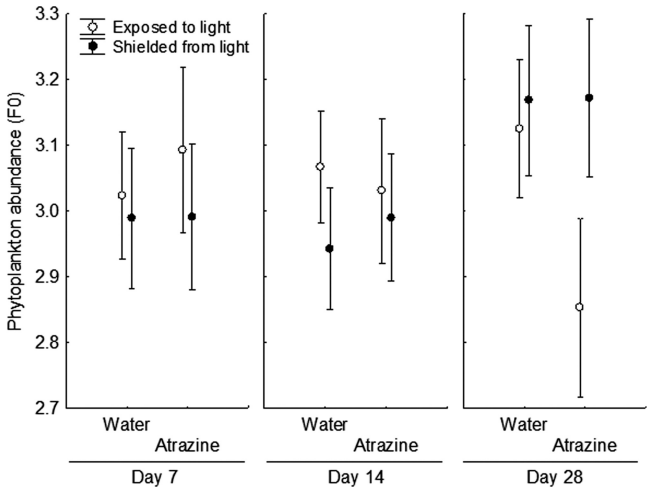

FIG. 5. Phytoplankton abundance (measured as F0) with and without atrazine (least squares mean ± SE, $n = 5$) on days 7, 14, and 28 when controlling for initial phytoplankton levels.

less of whether an outlier was included or excluded (Fig. 6A). In contrast, algal abundance at the end of the experiment was negatively associated with *E. coli* densities in the sediment (Fig. 6B).

DISCUSSION

The overall net effect (direct plus indirect mechanisms) of agrochemicals on the fate of allochthonous bacteria in water bodies and the underlying sediment has been greatly understudied. Our previous research suggested that the presence of fertilizer or atrazine has positive effects on FIB densities in the sediment (49). However, our previous study did not investigate potential impacts of agrochemicals in the water column and, due to the complexity of the mesocosms used in that study, it was not possible to say definitively whether the effects observed were due to direct mechanisms, indirect mechanisms, or a combination of the two. The present study utilized simple microcosms, inoculated with several known strains of *E. coli* and *Enterococcus* spp., to further examine the impact that agrochemicals have on the fate of bacteria in both water and sediment as well as to distinguish between direct and indirect mechanisms. The initial bacterial densities ($\sim 10^7$ CFU/100 ml), though certainly elevated, have been observed in environmental water samples in a previous study (63). While the addition of bacteria to the microcosms would add some nutrients to the microcosms, cells were washed prior to inoculation into the microcosms to limit the transfer of excess nutrients and were added to all of the microcosms at similar densities.

To examine direct effects of agrochemicals, our microcosms were designed specifically to exclude potential indirect confounders, specifically algal growth and protozoan predation. Under these conditions, none of the agrochemicals, alone or in combination, had significant effects on the densities of *E. coli* or enterococci in either the water column or the underlying sediment. These results suggest that the agrochemicals used in this experiment do not have any direct impact, positive or negative, on FIB densities in either the water or sediment matrix. However, when seeded with pond water (containing algae and protozoan predators), significant effects were observed on *E. coli* densities in both the water column and the sediment when atrazine was present and microcosms were exposed to light, indicating that the effects of atrazine on *E. coli* were indirect because they depended on the presence of other species.

Given that the effect of atrazine on *E. coli* depended on light availability, the indirect effect is almost certainly at least partially mediated by phototrophs. Atrazine is directly toxic to phytoplankton (45). The present experiment supports this finding as phytoplankton density decreased in lit microcosms containing atrazine relative to controls (Fig. 5). Further, light attenuates exponentially as a function of chlorophyll *a* in phytoplankton, potentially resulting in an exponential decrease in light penetration in response to a small increase in chlorophyll *a* (24). Therefore, as the phytoplankton dies off, the water column receives increased light, which would result in greater UV stress to bacteria in the water column, explaining the significantly lower density of *E. coli* in the water column of the light-exposed atrazine tanks (34, 48). The algal data support this conclusion, as greater amounts of phytoplankton were predictive of higher *E. coli* densities in the water column (Fig. 6A). Increased light penetration has also been shown to stimulate the growth of periphyton in sediment biofilms, thus increasing available carbon (25, 35, 44). Therefore, as phytoplankton decreased in the lit atrazine microcosms, periphyton likely increased (although this was not measured). In support of this assertion, we observed significantly higher *E. coli* densities in the sediments of lit atrazine microcosms and a strong negative trend for the correlation between phytoplankton in the water column and *E. coli* densities in the sediments (Fig. 6B). All of these findings support the conclusion that algal dynamics mediated the indirect effects of atrazine on *E. coli*. However, it should be noted that not all algal species respond identically to atrazine. As algal species can differ in sensitivity to atrazine, the initial algal community present in a water body may well modify the direct and indirect algal-mediated effects on the entire community (39). Further, it should be noted that atrazine, beyond phytotoxic effects, can exert effects on higher trophic levels in impacted communities. While the ability of atrazine to directly cause mortality is controversial, a meta-analysis has shown that atrazine consistently influences metamorphosis, antipredator behavior, and immunity of freshwater fish and amphibians (42). These wide-ranging effects of atrazine exposure could have further implications for microbial fate in water bodies exposed to the herbicide.

In contrast to findings from our previous study conducted with nutrient poor sediments and water (49), no significant increase in FIB densities was observed for the fertilizer treatment. The sediments used in this experiment were taken from the Hillsborough River and would therefore be expected to contain large amounts of phosphorus as well as multiple nitrogen species (62). Additionally, the pond water seeded into the microcosms was taken from a eutrophic pond at the University of South Florida. Therefore, we hypothesize that the seeded pond water and sediments resulted in microcosms that were neither phosphorus nor nitrogen limited, preventing us from detecting an increase in FIBs with fertilizer addition.

Although we detected no direct effect of the agrochemicals on

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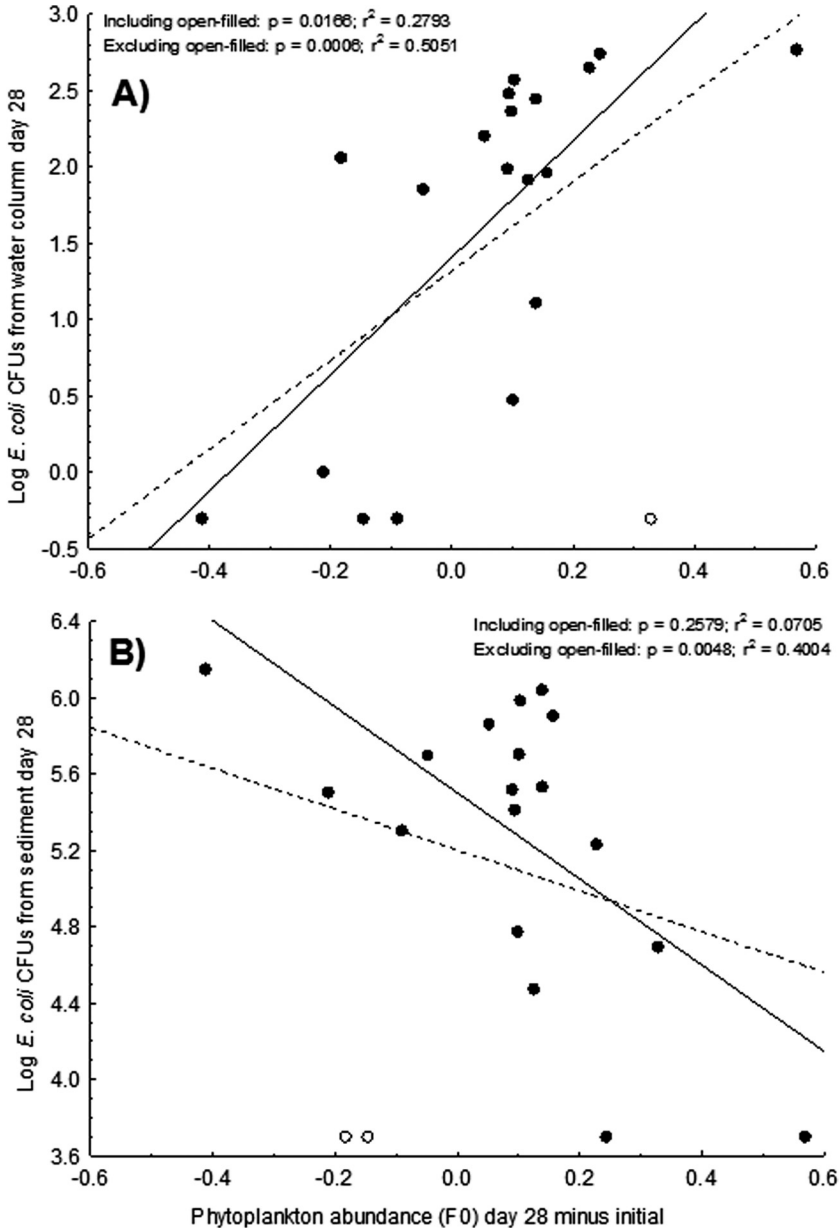

FIG. 6. Relationship between phytoplankton abundance (controlling for initial abundance estimates) and *E. coli* densities (measured as CFU/100 ml or CFU/100 g) in the water column (A) and sediment (B).

overall *E. coli* and enterococcus densities, we also hypothesized that changes in strain composition may be induced by agrochemical treatments. Particular strains of *E. coli* and *Enterococcus* spp. have been shown in previous studies to exhibit extended survival in microcosms (1), so it was thought that individual strains may respond differently to agrochemical treatments. However, these experiments did not support this hypothesis, as the proportion of each *E. coli* strain recovered from water and sediment in light-free microcosms was independent of treatments, and no one strain persisted better overall than any other. We do not suggest that these results should be generalized to all *E. coli* strains, as only three were included in this study.

Despite the lack of direct effects of the agrochemicals on the abundance or population structure of FIBs, we did detect a positive multivariate correlation between *E. coli* genotype abundances (observations of a particular genotype) in the sediment and water column. These results suggest that the distribution of *E. coli* genotypes in the sediment influence the population structure within the water column and/or vice versa. Regulatory standards consider FIB densities only in the water column (54, 55), but *E. coli* and enterococci are clearly also present in the sediments, which can act as important reservoirs of FIBs and possibly of pathogens (1, 4, 5, 19).

The effects of agrochemicals on the survival of FIBs in environmental waters are greatly understudied. Based on the results of this study, agrochemicals had no direct effect on FIB growth, although the presence of atrazine did mediate significant indirect effects. The significant effects observed in the

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presence relative to the absence of light and the associations between phytoplankton abundance and *E. coli* densities suggest that the presence of atrazine alters algal dynamics that affect light and nutrient levels leading to reductions in *E. coli* in the water column but increases in the sediment. While these results are novel and suggest a mechanism of action for the effect of atrazine on FIB densities, it is unknown whether agrochemical treatments will have any direct or indirect effects on other bacterial pathogens or viruses. Further examination of both the direct and indirect effects of agrochemicals on pathogen survival in the environment is needed to better understand and manage potential risks to human health.

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