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The effect of agrochemicals on indicator bacteria densities in outdoor mesocosms

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Summary

Water bodies, which are monitored for microbial water quality by quantification of faecal indicator organisms (IOs), can contain various zoonotic pathogens contributed by livestock waste and other sources. Sediments can serve as reservoirs of IOs and other enteric microorganisms, including pathogens. Agrochemicals may influence the survival of these microorganisms in water bodies impacted by livestock waste by enhancing or reducing their survival. Complex, 1100 l, freshwater mesocosms containing leaf litter, zooplankton, periphyton, phytoplankton, and invertebrate and vertebrate animals were used to investigate the response of *Escherichia coli* and enterococci to agrochemicals. Replicate tanks were treated with atrazine, malathion, chlorothalonil and inorganic fertilizer, either alone at 1× or 2× their expected environmental concentrations (EECs) or in pair-wise combinations at their EECs. IOs inoculated in sediment (~10^4 cfu per 100 ml) were enumerated over 28 days. IOs generally declined over time, but MANOVA revealed that addition of fertilizer and atrazine resulted in significantly greater IO densities. Malathion, chlorothalonil and agrochemical concentration (1× vs 2×) did not significantly affect IO densities and no significant interactions between agrochemicals were noted. The augmentation of IO densities in sediments by fertilizer and atrazine may impact their reliability as accurate predictors of water quality and human health risk, and indicates the need for a better understanding of the fate of IOs and enteric pathogens in sediments exposed to agrochemicals.

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Introduction

Management of endemic and emerging human pathogens associated with agriculture, such as *Salmonella*, pathogenic *Escherichia coli* and zoonotic influenza viruses, has become a matter of great concern to many governmental agencies and water quality managers (US Environmental Protection Agency, 2005; 2007; 2009a,b). In fact, a recent review notes that the majority of known human pathogens are zoonotic, and that agricultural practices play a major role in the dynamics of disease transmission (Lloyd-Smith *et al*., 2009). Many agriculturally derived pathogens are transmitted via the waterborne route and can enter water bodies via run-off that is contaminated by the waste of livestock, e.g. cattle, swine and poultry. Further, the sediments of water bodies can serve as reservoirs for enteric microorganisms, prolonging their survival (Davies *et al*., 1995; Ishii *et al*., 2007; Badgley *et al*., 2010).

Testing directly for waterborne pathogens is prohibitively costly and extremely difficult because of the great diversity of potential pathogens and culturing difficulties; therefore, regulatory agencies have relied on enumeration of indicator organisms (IOs) to indicate faecal contamination and increased probability of the presence of human pathogens. IOs such as *E. coli* and *Enterococcus* spp. naturally inhabit the gastrointestinal tract of warm-blooded, and some cold-blooded, animals (Harwood *et al*., 1999), are generally commensal, and are shed in the faeces with enteric pathogens. Regulatory agencies have determined that both *E. coli* and *Enterococcus* spp. serve as useful indicators of enteric pathogens in fresh water, while enterococci are the better indicator in marine/estuarine waters (Cabelli *et al*., 1979; US Environmental Protection Agency, 1983; Wade *et al*., 2006). While regulatory standards only take into account IO densities in the water column, *E. coli* and enterococci are also present in the underlying sediments of water bodies. It has been established that IOs are capable of extended persistence, and possibly growth in the environmental reservoirs represented by the sediments of estuarine and marine water bodies (Davies *et al*., 1995; Anderson *et al*., 2005; Ishii *et al*., 2007). Studies have shown that the resuspension of IOs in the sediment can occur as a result of natural turbulence or human influence, both of which may re-inoculate overlying water (Craig *et al*., 2004; Jin *et al*.,

2004; Graczyk *et al*., 2007a,b; Philip *et al*., 2009). In fact, resuspension of bacteria caused by wading activities has been shown to elevate *E. coli* concentrations as much as fourfold (Philip *et al*., 2009).

Run-off from agricultural sites also often contains agrochemicals, such as fertilizers and pesticides, which can have various effects on freshwater ecosystems, including the death of algal and some diatom species, inhibition of photosynthesis resulting in increased heterotrophic activity by protozoa, and alteration of the species diversity within the affected ecosystem (Verro *et al*., 2009a,b; Debenest *et al*., 2010). The effects seen on bacterial populations may be direct or indirect; beneficial or adverse (Clements and Rohr, 2009; Verro *et al*., 2009a,b). Agrochemicals can alter the traits of organisms, such as behaviour, immunity, physiology and morphology, which can in turn alter interactions with con- and heterospecifics (Rohr *et al*., 2003; 2004; 2006a; 2008b; Rohr and Crumrine, 2005; Rohr and Palmer, 2005). Agrochemicals can also have adverse or beneficial indirect effects via direct toxicity to a focal species' food resources, parasites, competitors or predators (Rohr *et al*., 2006a; 2008a,b; 2009; Raffel *et al*., 2008). For instance, a chemical might reduce the number of conspecifics, reducing competition for the survivors (Rohr *et al*., 2006b). If agrochemicals have positive or negative direct or indirect effects on IOs, then the utility of IOs as predictors of contamination and the possible presence of human pathogens may be confounded.

To determine the effects of agrochemicals on IO populations, multiple freshwater mesocosms inoculated with IOs were dosed with inorganic fertilizer, the herbicide atrazine, the insecticide malathion or the fungicide chlorothalonil, either alone at 1× or 2× their expected environmental concentrations (EECs) or in pair-wise combinations at their EECs. Each of these pesticides was selected because they are among the top two in usage in the USA within their pesticide type (herbicide, insecticide, synthetic fungicide; Kiely *et al*., 2004). We then quantified IO densities in these tanks through time using standard culture (membrane filtration) methods (US Environmental Protection Agency, 2002a,b).

Results

The targeted 1× nominal EECs for atrazine, chlorothalonil, malathion and fertilizer (102, 169, 101, 110 P:2338 N, $\mu g\ l^{-1}$ respectively) closely matched the actual concentrations in the mesocosms (1×: 101, 172, 92, 140 P:2450 N; 2×:228, 351, 164, 440 P:3450 N, $\mu g\ l^{-1}$ respectively). Concentrations of calcium, and total phosphorus and nitrogen in control tanks were 39000, 60 and 370 $\mu g\ l^{-1}$ respectively.

A representative subset of survival curves for IOs subjected to various agrochemical treatments is shown in Fig. 1. *Escherichia coli* densities in all treatments were initially ~10^4 cfu per 100 g of sediment (wet weight). Populations in all mesocosms dropped about 2.5 logs over 7 days, generally decreased more slowly over the next week, and stabilized over the next 2 weeks (Fig. 1). Enterococci densities decreased from ~10^3 cfu per 100 g at the initial time point to ~10^2 cfu per 100 g after 1 week. Enterococci densities decreased to their lowest point at 14 days (10^1–10^2 cfu per 100 g), and tended to increase by one log or less over the next 14 days. When IO densities were averaged over all treatments, enterococci densities on day 28 were significantly greater than *E. coli* densities ($P < 0.001$).

None of the 16 treatments differed in *E. coli* or enterococci density before agrochemical applications (Treatment: Wilk's $F_{30,88} = 0.77$, $P = 0.786$). There was no treatment-by-time interaction; that is, IO densities in all treatments followed a similar trend over time. Daily decay rates were calculated for the first 7 and 28 days. The initial daily decay rate ranged from –0.296 to –0.173 for enterococci and –0.386 to –0.221 for *E. coli*. The 28-day daily decay rate ranged from –0.064 to –0.030 for enterococci and –0.122 to –0.068 for *E. coli*. Decay rates for *E. coli* for all treatments combined were significantly greater (more rapid decline in concentrations; $P < 0.0001$) than they were for enterococci (Fig. 1).

There was no evidence that any of the agrochemical mixtures synergistically or antagonistically affected the densities of IOs (Agrochemical*agrochemical: Wilk's $F_{2,33} < 0.53$, $P > 0.595$; Agrochemical*agrochemical*sampling date: Wilk's $F_{4,31} < 1.64$, $P > 0.188$). There also was no convincing evidence that IO density differed between the 1× EEC and 2× EEC treatments (1× versus 2×: Wilk's $F_{2,20} = 0.004$, $P = 0.996$; 1× versus 2×*sampling date: Wilk's $F_{4,18} = 0.453$, $P = 0.769$) for any agrochemical or sampling date (1× versus 2×*agrochemical: Wilk's $F_{6,40} = 0.580$, $P = 0.744$; 1× versus 2×*agrochemical*sampling date: Wilk's $F_{12,48} = 0.152$, $P = 0.999$).

Given that we had no evidence for interactions between the agrochemicals or for differences between the 1× EEC and 2× EEC concentrations, our final model was a regression-based multivariate analysis of variance (MANOVA) containing just four main effects representing the presence or absence of each of the four agrochemicals (as well as block, the repeated measures factor, and interactions between the agrochemicals and the repeated measures factor). This model provided the greatest power to detect effects of the agrochemicals because it uses all the data and pools the 1× EEC and 2× EEC treatments, as well as incorporating both response variables (*E. coli* and enterococci density). This MANOVA revealed that IO density was elevated, by both fertilizer and atrazine and these responses were consistent through time (Fig. 2, Table 1). Analysis of variance

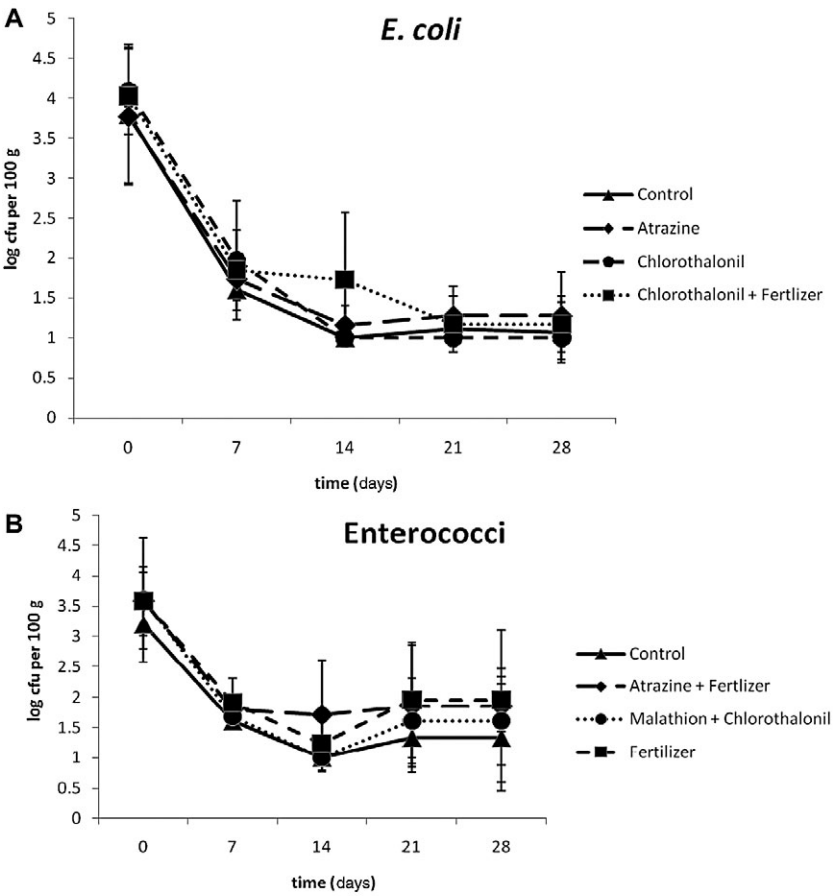

Fig. 1. Densities of IOs in sediments of mesocosms over 28 days: (A) *E. coli* and (B) enterococci. Results from a subset of agrochemical treatments are shown for each IO. Data are log-transformed means and standard deviations.

(ANOVA) (considering the response variables separately) revealed that enterococci concentrations were significantly greater in the presence of both fertilizer and atrazine ($F_{1,56} = 7.44$, $P = 0.004$; $F_{1,56} = 5.90$, $P = 0.009$, respectively, one-tailed, Fig. 2A). Similarly, *E. coli* concentrations were significantly greater in the presence of fertilizer at the alpha level of 0.05 ($F_{1,56} = 5.84$, $P = 0.009$) but were not significantly greater in the presence of atrazine at that alpha level ($F_{1,56} = 2.55$, $P = 0.058$; Fig. 2B); however, it should be noted that the difference is significant when alpha is set at 0.10. Neither malathion (*E. coli*: parameter = 0.108, 95% confidence interval = ± 0.244; enterococci: parameter = 0.106, 95% confidence interval = ± 0.223) nor chlorothalonil (*E. coli*: parameter = 0.196, 95% confidence interval = ± 0.244; enterococci: parameter = 0.203, 95% confidence interval = ± 0.223) significantly affected IO densities across sampling dates or at any given sampling date (Table 1).

Discussion

The mesocosms employed here to investigate the effect of agrochemicals on the maintenance of faecal indicator bacteria populations in an aquatic system are unusually complex for a microbiological study in that they included a community of vertebrates, invertebrates, algae and plants, as well as natural sediments. In addition to ecological complexity, sampling from 64 large mesocosms provided true replication, which also is uncommon in studies of microbial survival in the environment. We were interested in the fate of IOs in sediments impacted by agrochemicals because sediments are the environmental compartment in which IOs can be exposed long term to the chemicals in flowing waters. While bacteria in the water column will be transported downstream and both they and agrochemicals will be successively diluted, sediments that receive agricultural run-off can be expected to retain elevated concentrations of these chemicals due to their propensity to adsorb to particles and sediments (Chung *et al*., 1996; Scarlators, 1997; Xu *et al*., 2009). Furthermore, greater IO survival in sediments compared with the water column in environmental waters is well documented (Anderson *et al*., 2005; Ishii *et al*., 2007); and resuspension of sediments in polluted waters can result in exposure of humans and animals to increased levels of

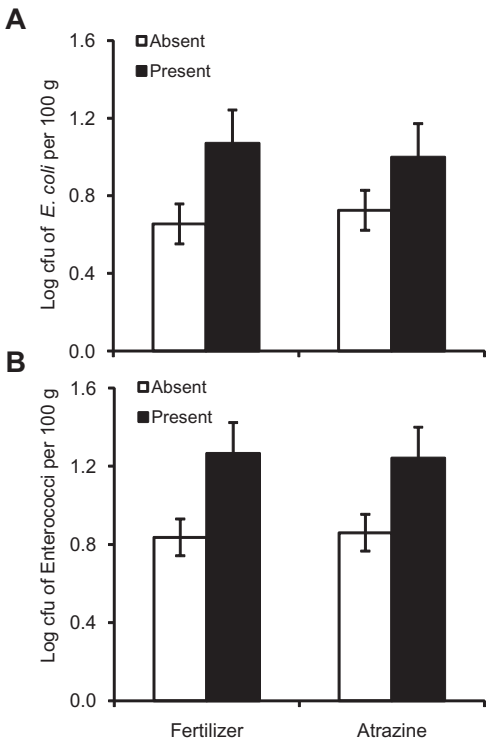

Fig. 2. Least squares means (± 1 SE) for the effects of fertilizer and atrazine on the density of (A) *E. coli* and (B) enterococci after 28 days. The effect of fertilizer and atrazine was averaged across 1× and 2× EEC concentrations and mixtures containing the focal agrochemical because there was no evidence for differences between the 1× EEC and 2× EEC concentrations or for interactions between the agrochemicals. Furthermore, treatments without the focal agrochemical include both the water and solvent controls. White bars indicate IO density in mesocosms without the agrochemical specified; black bars indicate IO density in mesocosms with the agrochemical specified.

enteric microorganisms. The overall decline observed in both *E. coli* and enterococci densities in sediments over time is consistent with observations from other studies, as is the slower decline of enterococci compared with *E. coli* (Craig *et al*., 2004; Anderson *et al*., 2005; Sampson *et al*., 2006; Hartz *et al*., 2008).

Significantly greater IO densities compared with controls were observed when atrazine or fertilizer was present in the mesocosms. These results are novel, in that it is the first time these effects have been seen on *E. coli* and *Enterococcus* spp. in mesocosms that attempt to reflect the complexity of natural systems. The positive effect of atrazine is corroborated by a previous study conducted *in vitro*, which found increased densities of *E. coli* and *Enterococcus* spp. in atrazine-treated cultures that was attributed to utilization of the chemical as a nutrient source (Koutsotoli *et al*., 2005). The positive effects of atrazine noted in our study could result from a direct effect, as noted above and in other studies (Yanzekontchou and Gschwind, 1994; Rhine *et al*., 2003). It has been shown that bacteria persisting in anaerobic wetland sediment were capable of metabolizing atrazine and other triazine herbicides to NH_3 and CO_2 (Chung *et al*., 1996). However, the mechanism of atrazine stimulation may be wholly or in part due to indirect effects in the complex mesocosms used here. Atrazine is toxic to phytoplankton (Rohr *et al*., 2008b), which, after dying following exposure to atrazine, settle upon the sediment, increasing the amount of available carbon to the heterotrophic IOs. Furthermore, a decrease in phytoplankton would allow more light to penetrate the water column, stimulating photosynthesis by periphyton in sediment biofilms and increasing available carbon (Herman *et al*., 1986; Pratt *et al*., 1997; Rohr *et al*., 2008b).

Inorganic fertilizer also led to greater densities of *E. coli* and enterococci after 28 days. The data are consistent with results of previous studies of soil, which showed greater persistence of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium as well as non-pathogenic *E. coli* and enterococci in soils amended with fertilizer (Kudva *et al*., 1998; Lau and Ingham, 2001; Semenov *et al*., 2009). Like the proposed mechanism(s) for enhancement of IO survival with atrazine, the maintenance of greater *E. coli* and enterococci densities associated with fertilizer treatment has two possible explanations: the IOs may use the fertilizer directly as an inorganic nutrient source, and/or algal populations may be altered so as to affect available organic carbon levels.

It should be noted that a low density of indicator bacteria species and strains was probably introduced into the mesocosms from the algal, vertebrate and invertebrate organisms which were initially added to the tanks. However, BOX-PCR genotyping of *E. coli* and enterococci isolated from sediments showed a predominance of the originally inoculated strains after 28 days (data not

Table 1. Results of multivariate analysis of variance examining the effects of spatial block, fertilizer, atrazine, chlorothalonil, malathion and sampling date on the density of *E. coli* and enterococci in sediment.

Effect	Wilk's *F*	d.f. effect	d.f. error	*P*
Intercept	66.76	2	55.0	< 0.001
Block	6.95	6	110.0	< 0.001
Fertilizer	5.45	2	55.0	0.007
Atrazine	3.53	2	55.0	0.036
Chlorothalonil	1.22	2	55.0	0.304
Malathion	0.35	2	55.0	0.708
Sampling date	1.47	4	53.0	0.226
Date*block	1.32	12	140.5	0.215
Date*fertilizer	0.80	4	53.0	0.532
Date*atrazine	1.40	4	53.0	0.248
Date*chlorothalonil	1.25	4	53.0	0.303
Date*malathion	1.79	4	53.0	0.144

shown). The greater densities of *E. coli* and enterococci maintained in mesocosms containing atrazine and fertilizer should not be taken as a generalized statement that all *E. coli* strains and all species of enterococci will benefit from exposure to these agrochemicals, as it is highly probable that certain strains survive in a culturable state longer than others under the conditions used here.

Although previous studies revealed the ability of certain bacteria to use the insecticide malathion as a carbon and/or phosphate source (Karpouzas and Singh, 2006; Abo-Amer, 2007; Cycoń *et al*., 2010), no significant increase in *E. coli* or enterococci densities compared with controls was observed in mesocosms containing malathion. Malathion's non-significant effect on IO density may have been due to an inability of the IOs to utilize this chemical as a nutrient or because of inadequate statistical power. The fungicide chlorothalonil also had no significant effect on IO density in this study at either 1× or 2× the EEC, although previous studies demonstrated adverse effects of chlorothalonil on microbial community structure and microbial densities after repeated exposures or high doses (Singh *et al*., 2002; Yu *et al*., 2006; Chu *et al*., 2008; Podio *et al*., 2008).

The introduction of agrochemical run-off into rural water bodies may have unintended consequences for the survival of enteric microorganisms in aquatic habitats. Like IOs, human pathogens such as toxigenic *E. coli* and *Salmonella* are chemoorganoheterotrophs and belong to the same species or are closely related to one of the IOs used here (*E. coli*); therefore, it is reasonable to hypothesize that their response to agrochemicals would be similar. Alternatively, agrochemicals may affect the persistence of IOs and certain pathogens disproportionately, causing a disconnect in the IO–pathogen relationship. Epidemiological studies have demonstrated correlations between elevated IO levels and the risk of gastroenteritis (Barrell *et al*., 2000; Craun and Calderon, 2006; Colford *et al*., 2007; Kite-Powell *et al*., 2008; Wade *et al*., 2008). While risk assessment and epidemiological research has focused on analysis of the water column, a better understanding of the relationship between environmental influences, pathogen densities and IO densities in sediments is necessary to better predict the impact that resuspension may have on water quality and human health risk. To that end, several recent studies have focused on IO and pathogen densities in sand and sediment, and their relationships to those measurements in the water (Ishii *et al*., 2007; Yamahara *et al*., 2007; 2009; Abdelazhar *et al*., 2010; Badgley *et al*., 2010), finding extended persistence and elevated densities in these substrates compared with the water column.

Presently, the effects of fertilizer and atrazine on enteric pathogens are largely unknown, and further studies are needed to establish the relationship between these chemicals and their effects on the growth and persistence of pathogens in secondary habitats, such as environmental waters and sediments. This mesocosm study was designed to capture both direct and indirect effects of agrochemicals on IOs, either or both of which may have generated the observed results. The next logical step would be to conduct a direct toxicity study, where simplified mesocosms contain the agrochemicals and inoculated organisms only. Understanding the effects of agrochemicals on enteric microorganisms is necessary to determine the most effective system of indicators of faecal contamination of water, and the best management practices for application of agrochemicals in order to protect human and animal health.

Experimental procedures

Experimental design

Sixty-four outdoor mesocosms were established at the University of Florida's Institute of Food and Agricultural Sciences Gulf Coast Research and Education Center (Ruskin, FL). The mesocosms consisted of round cattle water tanks that were each 6 feet in diameter and 2 feet deep. Each tank contained 1100 l of municipal water, 300 g of leaf litter (predominantly live oak, *Quercus virginiana*), and local zooplankton, phytoplankton, periphyton, tadpole, insect, gastropod and crayfish species collected from several ponds in the region. Zooplankton, periphyton and phytoplankton collected from four ponds were homogenized and used as the source of initial inoculations into each mesocosm. Three weeks were allowed for zooplankton, periphyton and phytoplankton populations to establish before agrochemical applications. Just before agrochemical applications, tadpoles, insects, gastropods and crayfish were counted and distributed among the tanks to ensure that each tank had the same starting communities. Zooplankton and chlorophyll *a* in the periphyton and phytoplankton were quantified throughout the experiment in 2-week intervals, and tadpole, insect, gastropod and crayfish survival were quantified at the end of the experiment. Densities of zooplankton, periphyton and phytoplankton were similar among tanks prior to agrochemical applications and community-level effects of the treatments on species other than bacteria will be presented elsewhere. All mesocosms were covered with 60% shade cloth.

An uncovered plastic box (11 cm × 27 cm × 37 cm) containing sediment (~5 cm high) from the lower Hillsborough River (Tampa, FL) was placed in each tank. The sediment had been dried for 1 week before placement into the tanks to allow any endogenous indicator bacteria to die. Culturing on selective-differential media (as described below) verified that the sediment contained undetectable levels of *E. coli* and enterococci. Each of these sediment boxes was inoculated with known *E. coli* and *Enterococcus* spp. strains (see below). Hence, these mesocosms contained most of the abiotic and biotic characteristics of a community in Florida ponds.

Tanks were arranged in a randomized block design with four replicates of each of 16 treatments. There were two

control treatments, with addition of only 50 ml of water or acetone (used as a solvent for all agrochemicals). Tanks in the remaining 14 treatments received agrochemicals. Our representative agrochemicals were the herbicide atrazine, the insecticide malathion, the fungicide chlorothalonil and inorganic fertilizer (sodium nitrate and sodium phosphate). All pesticides were technical grade (purity > 98%, Chemservice, West Chester, PA). Six of the agrochemical treatments were pair-wise combinations of the agrochemicals at their EECs calculated using the Environmental Protection Agency's GENEEC v2 software. Fertilizer was added at an EEC of 220 μg l^{-1} P: 4675 μg l^{-1} N, a concentration found commonly in a pond survey (Chase, 2003). The remaining eight treatments were each agrochemical alone, either at its EEC (1× EEC) or at double its EEC (2× EEC). The 2× EEC treatments were incorporated in an effort to control for the fact that mixtures had twice the concentration of agrochemicals as the single-chemical treatments. Lone agrochemicals and agrochemical mixtures were dissolved in 50 ml of acetone so that each agrochemical tank received the same amount of acetone. To quantify actual concentrations, pooled samples from each lone pesticide treatment were analysed by the Mississippi State Chemical Laboratory (Starkville, MS) and samples from each lone fertilizer treatment were analysed by the Hillsborough County Water Resource Services Environmental Laboratory (Tampa, FL).

Bacterial inoculation

Five *E. coli* strains and five *Enterococcus* spp. strains were selected for use in this experiment. Three isolates each of *E. coli* and enterococci were selected based on prolonged survival in the culturable state in a previous mesocosm study along with two isolates of each that rapidly became unculturable (Anderson *et al*., 2005). Four of the *E. coli* strains used for inoculation were originally obtained from wastewater and the fifth was *E. coli* 9637 (American Type Culture Collection). Inoculated *Enterococcus* strains included environmental water isolates (two *Ent. casseliflavus*, one *Ent. faecium*, and one *Ent. faecalis*) as well as *Ent. faecalis* 19433 (American Type Culture Collection).

The five *E. coli* and *Enterococcus* spp. strains were each streaked for isolation on trypticase soy agar (TSA) and incubated for 24 h at 37°C. Isolated colonies were then inoculated into 10 ml of cultures of brain heart infusion broth (BHI) and were incubated for 24 h at 37°C. The 10 ml cultures of the five *E. coli* and *Enterococcus* strains were then added to two separate flasks, one for *E. coli* and one for enterococci, of 1 l each of sterile buffered water (0.0425 g l^{-1} KH_2PO_4 and 0.4055 g l^{-1} $MgCl_2$; American Public Health Association, 1995). Prior to inoculation and submersion in the mesocosm, 1 l of water from each cattle tank was placed in the respective sediment-containing box, which would be submerged in the tank after inoculation. From their respective 1 l flasks, 500 μl of *E. coli* and 1 ml of enterococci were then inoculated into each sediment box (~10^4 cfu per 100 m for *E. coli* and ~10^3 for enterococci, taking into account only the volume of water in each sediment box). The boxes were covered and bacteria were allowed to settle for 1 h before they were submerged in the cattle tanks. The boxes were placed into each tank and the lids were carefully removed to avoid disturbing the sediment. Hence, each sediment box in each tank was inoculated with approximately 10^4 cfu per 100 ml of each of the five strains of *E. coli* and approximately 10^3 cfu per 100 ml each of the five strains of *Enterococcus* spp.

Sample collection and filtration

Sediment boxes in each tank were inoculated on 14 July 2008 and sediment samples were taken 1 h after inoculation. Pesticides were applied after these sediment samples were taken and thus we had an estimate of *E. coli* and *Enterococcus* spp. density before treatment applications. Additional sediment samples were collected once a week for the next 4 weeks. The amount of sediment collected varied based upon the bacterial counts obtained from the previous sampling, ranging from 10 to 40 g wet weight. To sample the sediment, a sterile 50 ml centrifuge tube was used to scoop the top 1–2 cm of the sediment, across the length of the sediment box, until filled. Samples were placed on ice for transport to the laboratory. Prior to filtration, sediment samples were weighed out and diluted 1:10 in sterile buffered water (American Public Health Association, 1995) and sonicated for 30 s on setting 4 (Sonic Dismembrator Model 100, Fisher Scientific) to release bacterial particles attached to the sediment (Anderson *et al*., 2005; Korajkic *et al*., 2009). Sediment suspensions were allowed to settle for several minutes before the supernatant was pipetted off and filtered through a nitrocellulose filter (0.45 μm pore size, 47 mm diameter). The volume filtered ranged from 0.1 ml on the first day to 100 ml after 2 weeks.

Indicator organisms were enumerated by standard membrane filtration methods. Enterococci were enumerated on mEI agar after 24 h incubation at 41°C (US Environmental Protection Agency, 2002a); *E. coli* were enumerated on mTEC media at 35°C for 2 h, followed by 22 h incubation at 44.5°C (US Environmental Protection Agency, 2002b). Typical colonies on plates were counted and densities were reported as cfu 100 g^{-1} (wet weight).

Statistical analyses

All response variables were log-transformed and spatial block was included in all analyses because it was always significant. The residuals were always carefully scrutinized to ensure that we met the assumptions of the analysis. We conducted repeated measures analyses for all data taken after agrochemical applications, where the repeated measures factor was IO density on the three sampling dates. 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. We also conducted regression-based ANOVA after regression-based MANOVA to ensure that we did not miss any significant univariate effects. In all MANOVAs, the response variables were the density of *E. coli* and enterococci on each sampling date.

Given the complexity of the experiment, we conducted a series of analyses in a step-wise hierarchical manner. We first conducted a MANOVA to test whether the density of *E. coli* and enterococci differed among any of the 16 treatments

before the agrochemicals were applied in order to insure that initial densities were similar. We then tested whether any of the agrochemicals had synergistic or antagonistic effects on *E. coli* and enteroccoci density by conducting a repeated measures MANOVA that included the presence or absence of each the agrochemicals as four main effects and that included all two-way interactions between agrochemicals. In our third analysis, we tested for a difference between the 1× EEC and 2× EEC treatments and their dependence on agrochemical treatment by conducting a 4×2 repeated measures MANOVA. That is, there were the four main effects of agrochemicals crossed by whether the agrochemical was applied at one times or two times the EEC. If there was any evidence of interactions between agrochemicals or differences among the 1× and 2× treatments, then these effects would have to be included in the final model. If there was no evidence of interactions or differences among the 1× and 2× treatments, then we could justify using a final model that contained just the four main effects representing the presence or absence of each of the four agrochemicals (as well as block, the repeated measures factor, and interactions between the agrochemicals and the repeated measures factor).

Initial (7-day) and overall (28-day) decay rates (decrease in cell concentration) were calculated for each tank for both *E. coli* and enterococci. The initial decay rate was obtained by subtracting the bacterial density ($\log_{10}$ per 100 g) immediately after inoculation from the bacterial density 7 days following inoculation. This value was then divided by seven to obtain the initial daily decay rate. The overall daily decay rate was calculated by subtracting the bacterial density immediately after inoculation from the bacterial density 28 days after inoculation. This value was then divided by 28 to obtain the overall daily decay rate.

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