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Investigation of Pathogenic Bacterial Transport by Waterbirds: A Case Study of Flooded and Non-Flooded Rice Systems in Mississippi

Alexandra G. Firth [1,*], Beth H. Baker [1], John P. Brooks [2], Renotta Smith [2], Raymond B. Iglay [1] and J. Brian Davis [1]

[1] Department of Wildlife, Fisheries and Aquaculture, Mississippi State University, Box 9690, Starkville, MS 39762, USA; beth.baker@msstate.edu (B.H.B.); ray.iglay@msstate.edu (R.B.I.); brian.davis@msstate.edu (J.B.D.)

[2] Genetics and Sustainable Agriculture Unit, USDA-ARS, Mississippi State, MS 39762, USA; John.Brooks@usda.gov (J.P.B.); Renotta.Smith@usda.gov (R.S.)

* Correspondence: agg224@msstate.edu

Received: 15 April 2020; Accepted: 24 June 2020; Published: 26 June 2020

Abstract: Waterbirds may facilitate pathogen transport in rice fields from autumn to winter. This study investigated pathogen dynamics in two types of rice fields, those considered conventionally managed and a low-external-input-sustainable-agriculture (LEISA) rice system in the Mississippi Alluvial Valley, winter 2017–2018. In each system, fields were (1) left unflooded or (2) flooded from November to March. Fecal indicator and pathogenic bacteria (enterococci, *Clostridium perfringens*, *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp.) were quantified in soil before and after winter flooding, and bird fecal matter estimated on both farm types. Water samples were tested for enterococci, *C. perfringens* and *E. coli* before fields were drained. The LEISA flooded fields had greater detections of *C. perfringens* in the soil than conventional non-flooded fields. There was an observed decreasing trend of *C. perfringens* detection associated with lower bird abundances among treatment groups. All observed pathogen levels in both systems were below EPA standards. Results suggest that long-term waterbird stopovers can influence pathogen indicators in soil, but not at levels to threaten human and environmental health standards. Future studies should focus on long-term monitoring of pathogen introduction in rice fields that harbor wintering waterfowl or other waterbirds.

Keywords: waterbirds; waterfowl; sustainable agriculture; pathogen; water quality; fecal indicator bacteria; rice fields

1. Introduction

There is growing research interest in agricultural practices that collectively benefit wildlife, conservation, and food and water security. While rice production is not at the forefront of this movement, winter-flooded rice fields are recognized as providing important habitat for migratory waterbirds [1–3]. For decades, post-harvested flooded rice fields have been an important migratory bird conservation practice in rice-growing regions of the United States [4]. In California, for example, waste rice seed accounts for 44% of the total calories available to wintering waterfowl (Anatidae) in the Pacific Flyway [4]. Thus, management of post-harvested rice fields to benefit waterbirds is deemed critical by conservationists in California and elsewhere in rice-growing regions of the United States [4].

Rice field habitats also are an important resource in the Mississippi Alluvial Valley (MAV), where the region provides abundant and diverse habitats for non-breeding waterfowl from across North America [5,6]. The Lower Mississippi Valley Joint Venture, a component of the North American Waterfowl Management Plan (NAWMP), advocates winter flooding of agricultural fields to create

waterfowl habitat in the MAV. [5]. Over 800,000 ha of rice was planted in the MAV states of Arkansas, Louisiana, Mississippi, and Missouri in 2018 [6]. Waste rice in rice fields may provide as much as 11% of the total food calories available to waterfowl wintering in the MAV [4,6,7]. While estimates of bird densities occupying rice fields during the fallow season in Mississippi are currently unknown, bird densities in California rice lands may reach 3600 birds/km^2 [4,5]. Given the MAV's location in the Mississippi Flyway, similar abundances are expected.

Flooded rice fields used by waterbirds, particularly overabundant snow geese (*Anser caerulescens*) [8], have the potential to provide an agronomic benefit to soil via deposited fecal matter [9–11]. However, waterbirds can act as vectors of certain pathogenic microorganisms that could detrimentally affect humans and other wildlife [12–17]. Migratory birds using flooded rice fields recycle nutrients in fields, depositing nitrogen and phosphorus directly via fecal matter or by transporting nutrients from adjacent areas [18,19]. Bird fecal matter also has diverse and abundant microorganisms which can contribute to soil health, but may also contain pathogenic bacteria. For example, waterfowl have a high incidence of *Escherichia coli* in their gut that can be excreted into water, and *Salmonella* spp. transport has been described in wild birds globally [12,14]. In Sweden, 11% of ground-foraging invertebrate feeders, 20% of ground-foraging insectivores, and 19% of plant-eating species were positive carriers of *Campylobacter* spp. [15]. Furthermore, there is tendency for several guilds of waterbirds to congregate at migratory stopovers, which encourages horizontal transmission of disease agents, increasing the likelihood of pathogen transport to other sites [16,20–23].

Waterbirds overwintering in MAV rice fields create favorable environments for pathogen transport among species and into soil and waterways. There are two groups of waterborne bacteria of interest to public health: (1) fecal indicator bacteria (FIB), or bacteria that are natural members of warm blooded animal gut microbiota but can serve as indicators of fecal contamination and suggest the presence of other enteric pathogens; and (2) "frank" pathogens, bacteria that are pathogenic regardless of environment or host. Frank pathogens *Salmonella* spp. and *Campylobacter* spp., and FIB. *E. coli*, *Clostridium perfringens*, and *Enterococcus*, are known bacteria that migratory birds carry and can survive in stagnant water and soil [16,20,21]. According to the U.S. Environmental Protection Agency's (EPA) 2017 National Water Quality Assessment [24], bacterial pathogens and FIB are the leading cause of impairment for rivers and streams, wetland impairment (2nd ranked cause), and impairment in the nation's bays and estuaries (3rd ranked cause). The inherent link between soil and water in an agricultural framework necessitates monitoring of environmental circumstances that compromise water quality [25].

Certain low-external-input-sustainable-agriculture (LEISA) management practices rely on migratory birds' nutrient additions, via fecal matter, for agronomic gains [11]. However, this management practice may create a situation where pathogens are incidentally accumulated in rice fields from wintering birds, increasing the likelihood of pathogenic bacterial transport to surrounding waterways. Therefore, the objective of this study was to characterize and compare waterborne pathogenic and fecal indicator bacteria between flooded and unflooded rice fields of conventional and LEISA farms. Our primary goal was to identify any potential adverse health or environmental impacts associated with flooding rice fields for migratory bird use. It was hypothesized that post-harvest flooded rice fields managed specifically for overwintering waterbirds would have a greater incidence of waterborne pathogens or FIB compared to flooded or unflooded fields devoid of birds.

2. Materials and Methods

2.1. Study Area

Two farms under different types of management were the focus of study: a conventional rice system and LEISA rice system. The LEISA and conventional systems were located 16 km apart in Tallahatchie and Leflore Counties, Mississippi, respectively. In the LEISA system, boards were left in slotted pipes to capture rainwater to flood selected fields during the non-growing season. Wintering birds have

been observed using these shallow-flooded fields while resting and foraging. The LEISA system had operationally flooded fields for waterbird use since 2009, as well as practiced no-till management and contained a tail-water recovery system. The producer at the LEISA site has reported a decrease in nitrogen fertilizer rates to flooded fields after compounding years of bird use [11]. The conventional system represented a typical rice farming system of the region that practiced rolling post-harvest rice stubble and leaving fields fallow during the non-growing season. Winter 2017–2018 was the first season that the conventional rice system flooded fields during winter. The study planned to select 20 fields from each system, 10 to be assigned a flooding treatment and 10 to be assigned a non-flooded treatment. From fall 2017 through spring 2018, 10 conventional fields were experimentally flooded (CF; 161.3 total ha; n = 10), and 10 conventional fields were left traditionally unflooded (CN; 170.1 total ha; n = 10) Likewise, 10 LEISA system fields were flooded (LF; 273.5 total ha; n = 10); however, the producer only volunteered 4 fields to be left unflooded (LN; 167.2 total ha; n = 4). Field size ranged from approximately 10–40.5 hectares (22.7 ± 1.9 ha) at each farm.

2.2. Soil Samplingy

Soil samples were collected post-harvest in November 2017 and again in March 2018, prior to spring planting. The November sample provided a baseline measurement of soil conditions prior to flooding and bird use. Within each field, a soil sample was obtained in triplicate for every 4.05 ha (10 acres) using a soil core measuring 3.8–4 cm diameter × 10 cm deep. To eliminate location sampling bias, soil grids were laid over field maps and subsequent grid coordinates were randomly generated and GPS marked. Samples from each field were aggregated into a composite sample, representing field-scale conditions [26]. The soil core sampler was sterilized with 90% ethanol prior to sampling at each location. Soil samples were stored on ice (~4 °C) and transported to the United States Department of Agriculture (USDA) Genetics and Sustainable Agriculture Research Unit in Starkville, MS.

2.3. Water Sampling

In March 2018, immediately prior to pre-planting flood drawdown, two grab water samples were collected in 50 mL sterilized bottles at random locations within each LF (n = 20) and CF field (n = 20), with a total of 40 samples obtained between the two field types. Bottles were attached to a long-arm sampler to avoid soil disturbance while sampling. Water samples were stored on ice (~4 °C) and transported to the USDA Genetics and Sustainable Agriculture Research Unit in Starkville, MS.

2.4. Quantifying Bird Fecal Matter

Firth et al. (2020) [11] estimated fecal matter inputs to experimental fields using bird counts via camera surveys and literature-reported values of bird fecal defecation. Briefly, game cameras were mounted in study fields at a standardized height and programmed to capture images once an hour beginning 2 November2017 through 15 March 2018. The number of cameras in each field was determined according to field size, with 1 camera for every 20 ha. An open-access image-manipulation program was used to count and calculate daily use rates of waterbirds from captured images. Average waterbird use (bird/day/ha) was converted to fecal matter estimates based on literature reports of dry weights of bird droppings per day. See Firth et al. (2020) for further details of bird survey methods and quantification.

2.5. Pathogens

2.5.1. Soil

Composite soil samples were tested for the following pathogenic or fecal indicator bacteria using modified methods from Brooks et al. (2009, 2010): enterococci, *Clostridium perfringens*, *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. Prior to assay, a 10 g aliquot was mixed in 95 mL sterile saline and homogenized via stomacher, whereby 10-fold serial dilutions were used for assays. Enterococci

were membrane filtered onto a 0.45 µm filter (Millipore; Billerica, MA) and subsequently transferred to m-*Enterococcus* agar (Neogen, Lansing, MI, USA 48 h at 35 °C). Filters suspected of containing probable *Enterococcus* spp. were subsequently transferred to bile-esculin agar (Neogen; 1 h at 35 °C). Typical esculin-hydrolyzing, black-haloed colonies were presumed as *Enterococcus* spp.

Clostridium perfringens was also membrane filtered (Millipore Cat. Num. EZHAWG474), transferred to CP Chromoselect agar (Sigma-Aldrich, St Louis, MO, USA) and incubated anaerobically at 44.5 °C for 16 h. Prior to filtration, each sample aliquot was heat shocked at 70 °C for 10 min. Following anaerobic incubation, plates with suspected colonies were exposed to aerobic conditions for 1 h at 44.5 °C. Colonies that turned "mucus green" or blue/green were presumed *C. perfringens*. Five percent of colonies (or at least 1 per sample) were transferred to 5% sheep-blood (HemoStat Laboratories; Dixon, CA, USA) tryptic soy agar (Neogen Cat. No. 7100) and incubated anaerobically for 16–24 h. Colonies exhibiting a double-zone of hemolysis were presumed *C. perfringens*.

E. coli were membrane filtered and transferred to mTEC agar (m-TEC AGAR Neogen Cat. No. 7421A). Plates were held at 35 °C for 2 h then transferred to 44.5 °C for 22 h. Filters were suspected of containing *E. coli* if they contained bright yellow colonies. Probable *E. coli*-containing membranes were transferred to a urea-soaked pad and incubated for 20 min at room temperature to confirm urease-negative *E. coli*. Colonies were transferred to MacConkey agar (MacConkey AGAR Neogen Cat. No. 7102A) and incubated at 35 °C for 24 h to confirm presumptive *E. coli*. All colony counts were adjusted for soil moisture and reported as colony forming units per dry g (CFU g^{-1}).

Salmonella spp. and *Campylobacter* spp. were assayed via presence/absence enrichment because of expected low levels in the soil. *Salmonella* spp. assays consisted of stomaching 10 g of soil sample in 95 mL of tryptic soy broth, then incubating aliquots (Neogen; 24 h at 35 °C), followed by transfer to Rappaport Vasilidales R10 semisolid broth (42 °C for 24 h), and then transferred to Hektoen Enteric agar (Neogen; 42 °C for 16 h). Black-centered, blue-green colonies were considered *Salmonella* spp. positive. *Campylobacter* spp. enrichment consisted of 95 mL of *Campylobacter* enrichment broth stomached with 10 g of field moist soil and incubated microaerophilically (35 °C for 4 h), and then transferred to 42 °C for 44 h. Aliquots were transferred to Preston Agar (Neogen) containing 5% horse blood at 42 °C for 48 h (Hema-Resources; Aurora, OR, USA) and checked for growth [27].

2.5.2. Water

Water samples were tested for enterococci, *Clostridium perfringens* and *Escherichia coli*, with 10 mL of each water sample membrane filtered and the filter placed onto the respective media following the procedures described above. All colony forming units were adjusted for aliquot volume and reported as colony forming units per mL (CFU 100 mL^{-1}).

3. Statistical Analysis

Prior to analysis, pathogen data were log transformed and checked frequentist test assumptions including, but not limited to, Barlett's test for homogeneity of variance, examination of q-q plots and Shapiro-Wilk's test for normality, double zeros in pathogen detection, and Cook's D outlier exploration. *Clostridium perfringens*, *Escherichia coli*, and enterococci data representing soil and water were found to violate normality assumptions and could not be corrected. Non-parametric statistical approaches were used for analyses to account for normality assumption violations.

Individual pathogen load for *Clostridium perfringens*, *Escherichia coli* and enterococci was tested using a non-parametric rank-based ANCOVA in program R's "Rfit" and "npsm," with the fall measurement used as a covariate to account for initial differences between fields and fecal matter, with treatment fields as predictors. Tukey-Kramer pairwise comparisons were performed post-hoc on the non-parametric one-way design. A Mann-Whitney-Wilcoxon Rank sum test was performed for each indicator pathogen in water samples, with CF and LF as predictor variables. Similarly, Firth et al. (2020) tested for differences in fecal matter input with a non-parametric rank-based analysis

of covariance, with treatment groups as predictors. An analysis of variance (ANOVA) was used to test differences among treatment groups in the number of geese/ha/day [11].

4. Results

Salmonella spp. and *Campylobacter* spp. were not detected in soil samples and thus exempt from statistical analysis. Only one soil *Enterococcus* spp. positive was detected, and thus, also excluded from analysis. *E. coli* in soil did not differ among treatment groups (Drop in Dispersion $F_{3,33} = 0$, Robust $R^2 = 0.0$, $p = 1.0$). However, soil in LF fields contained greater levels of *C. perfringens* in LF than CN (Drop in Dispersion $F_{3,33} = 4.21$, Robust $R^2 = 0.29$, $p = 0.01$; Figure 1A). Water sample levels of *E. coli* ($W = 2$, $p = 0.67$; Figure 2A), and *C. perfringens* ($W = 36.5$, $p = 0.32$; Figure 2B) also did not differ among treatment groups.

Firth et al. (2020) [11] reported significant differences in fecal matter inputs from birds between fields ($F = 11.99$, Robust $R^2 = 0.04$, $p = 0.001$; Figure 1B). Briefly, LF fields had the greatest fecal matter inputs ($p < 0.05$), with average fecal matter weight in LEISA flooded fields approximately 2.8-fold greater than LN, 2.9-fold greater than CF, and 7.4-fold greater than that of CN. Abundance of geese among treatment fields also differed ($F = 5.8$, Adjusted $R^2 = 0.30$ $P = 0.002$). A general trend of decreasing goose abundance was observed throughout the season (Figure 3).

Monthly averages of geese per hectare per treatment field in LEISA and conventional farms, Tallahatchie and Leflore Counties, Mississippi, November 2017–March 2018.

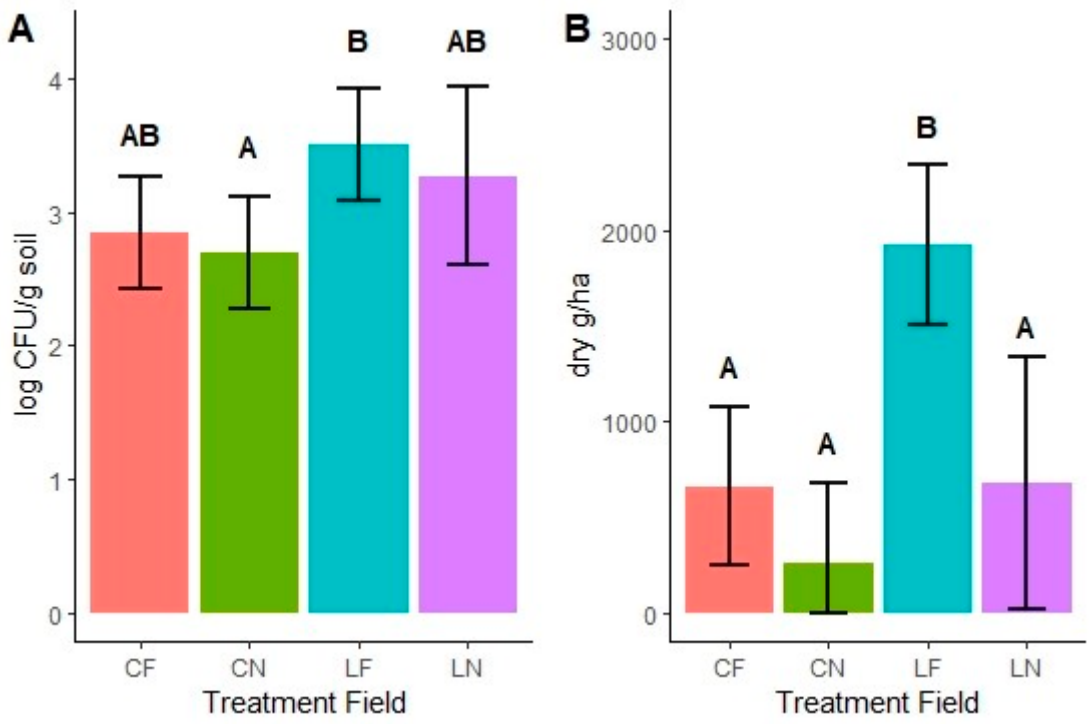

Figure 1. *Clostridium perfringens* and fecal matter inputs with 95% CI. (**A**) *C. perfringens* detection results reported in log CFU/dry g soil. Bars denote 95% confidence intervals (CI). LEISA flooded fields were significantly different than CN $p < 0.05$, shown with letter groupings. (**B**) Estimated fecal matter inputs per treatment field reported in dry g fecal matter/ha. Bars denote 95% CI. LEISA flooded fields had significantly greater fecal inputs than other treatment fields ($p < 0.05$) Tallahatchie and Leflore County, Mississippi, November 2017–March 2018.

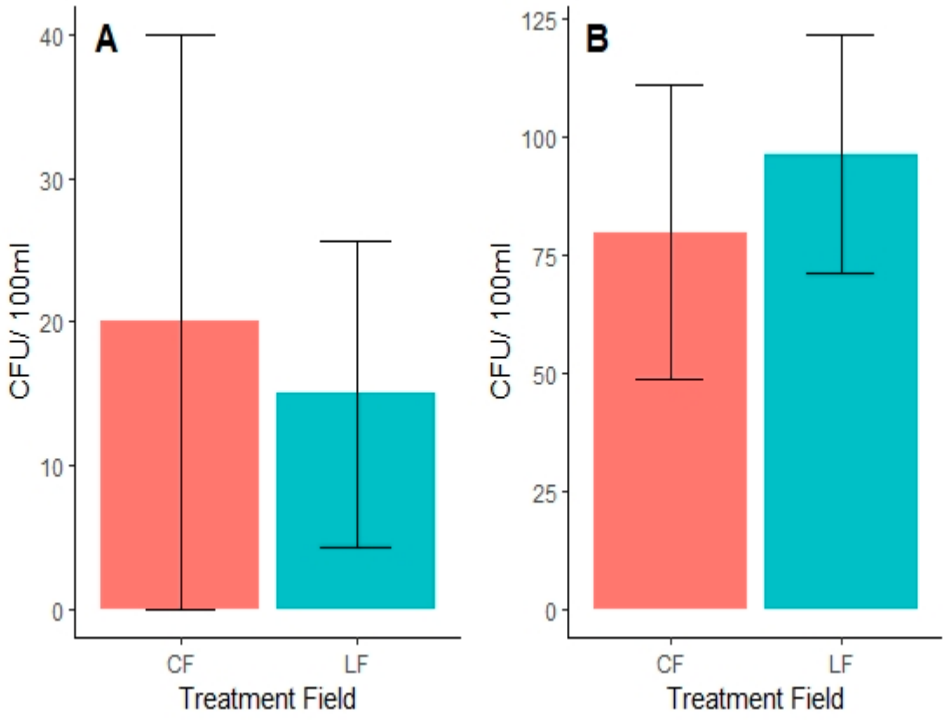

Figure 2. *Escherichia coli* and *C. perfringens* detection in water ± SE. (**A**) *E. coli* detection in water samples; bars denoted standard error (SE) of means. Results reported in CFU/100 mL. (**B**) *C. perfringens* detection in water; bars denote SE. Results reported in CFU/100ml. No significant differences between treatment fields. Tallahatchie and Leflore County, Mississippi, March 2018.

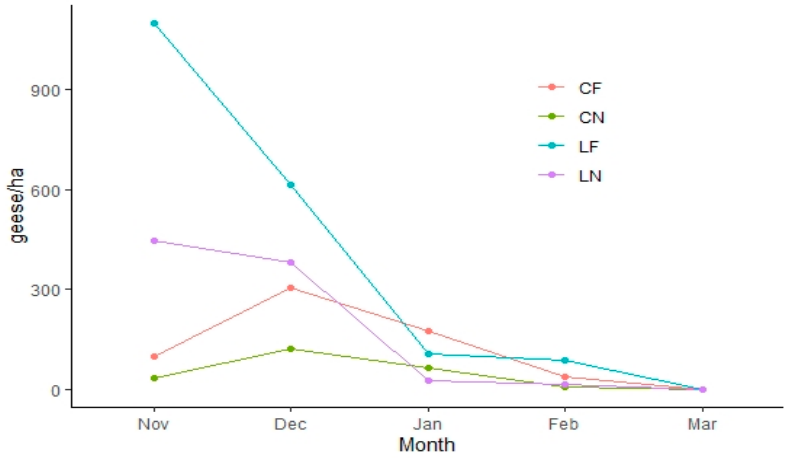

Figure 3. Mean abundances of Geese per Treatment.

5. Discussion

Waterbirds are known to increase the incidence of bacterial production, diversity, and pathogenic microorganisms in the environment [12–17]. Firth et al. (2020) exemplified a trend of increased soil Gram-positive bacteria and activity in fields with greater abundances of waterbirds [11]. Regardless of farm system, however, soil samples from all sites in this study were below detection limits (approximately 10/g or 100/10 g) for *Salmonella* spp. or *Campylobacter* spp., two pathogens that can detrimentally affect public health [15,16]. Furthermore, the EPA considers the presence of *E. coli* at 126 CFU/100 mL or *Enterococcus* spp. at 35 CFU/100 mL as significant levels of fecal contamination in recreational waters and a danger to humans [28]. If contaminated, sampled soils would affect

the quality of the water column (estimated 188 mL) directly above the sample location. Given that incidence of *E. coli* and *Enterococcus* spp. in the soil were at levels well below EPA standards of fecal contamination, it is highly unlikely that the aforementioned FIB in soil negatively impacted water quality among study fields. This is a positive sign that winter-flooded fields for waterbirds may not impact pathogenic bacterial loads in LEISA or conventional systems.

Clostridium perfringens, a spore forming, Gram-positive bacterium, was detected at significantly greater levels in LF than CN fields. *C. perfringens* grows in anaerobic conditions. However, as a spore forming bacterium, it can persist for prolonged periods in less desirable conditions (i.e., dry, aerobic environments). Because LN was not significantly different from LF or CF in *C. perfringens* detection, the anerobic conditions caused by flooding are not alone responsible for the differences in *C. perfringens* presence. No-till management of post-harvested rice fields usually contain more available waste rice than in fields where rice straw is otherwise incorporated [7,29]. Geese forage in a variety of field conditions, from dry to shallowly flooded, but typically roost nocturnally in flooded fields or other deeper water bodies less accessible by potential mammalian predators [30]. Because LN fields are no-till, they may be more attractive to geese than CN fields during the day, while CF and LF fields were more suitable for geese at night. Moreover, while not statistically significant, there were observably greater levels of *C. perfringens* in LF fields than either LN or CF fields as well as significantly greater fecal matter inputs in LF. Considering the trend in similar averages between LN and CF observed in fecal matter inputs and *C. perfringens* detection, in addition to significantly greater *C. perfringens* detection and fecal inputs in LF, we hypothesize that waterbirds contributed to *C. perfringens* soil loads. However, it should be noted there were only four LN fields available for the study, which exhibited high variability in both pathogen and fecal matter inputs estimations, thus warranting caution in interpretation.

Water samples taken from CF and LF fields contained minimal levels of *E. coli*, *Enterococcus* spp. and *C. perfringens,* well below EPA standards to be considered a contamination risk [28]. Bacteria are removed from the water column in one of three ways: sedimentation, predation, or biofilm formation. Flooded field conditions may have mimicked wetland functionality to reduce microorganisms from water through the process of sedimentation, where microorganisms settle on the soil surface, attach to plant root surfaces, or are absorbed into the soil environment [31–34]. The die-off rate of free-living bacteria in the water is faster than bacteria-associated sediments, and thus advantageous for bacteria to attach or settle onto sediment particles [35]. This is supported by the greater levels of *C. perfringens* in the soils of CF and LF fields compared to the water columns above the soils in these fields. Additionally, longer water retention times are linked to increased removal of bacteria indicators in wetland systems [32]. Decreasing bird activity at both sites occurred after February 2018 and allowed ample time for deposited pathogenic microorganisms to settle on the soil surface or be filtered by plant root systems. Water-borne bacteria are also more sensitive to temperature, with the die-off rate increasing as temperatures drop below 24 °C, while sediment bound bacteria are less sensitive to cold temperatures [35]. Winter temperatures at the study sites range between 15 and −2 °C, further explaining the low detection rate of pathogens in water.

Flooded rice harboring migratory birds have the greatest potential of pathogen transport to surrounding waterways at the end of winter, when fields are drained to prepare for planting [34]. The sudden movement of water resuspends settled sediments and moves them out of the system in large quantities. We did not find concerning levels of harmful bacteria in the soil after one year of testing. However, given that pathogen levels in bird flocks fluctuate substantially by season, the potential for future detection and transport is not out of the question.

Viral-mediated lysis or predation by protists contributes significantly to bacteria mortality in all systems. However, most theoretical models predict that bacteria are more heavily regulated by predation in oligotrophic systems, where nutrients are low [36–38]. While predation of bacteria is certainly occurring in flooded rice fields, the presence of decaying plant matter, fertilizer residue, and in some cases, significant fecal matter inputs, indicate the environment is nutrient rich, and thus not ideal for a significant impact by predator-prey population dynamics. Additionally, fine-textured, clay soils

that are typical of the MAV are associated with slower die-off rates of *E. coli* [39,40], presumably because fine-textured sediments provide better protection from predators [41,42].

The formation of biofilms, or communities of microorganisms that attach to surfaces and produce extracellular substances, on plant material, rocks, or debris contained in flooded rice fields could attribute to low bacterial detection in the water column [43]. Biofilms can be a reservoir for *E. coli* in particular [44]. The potential for bacteria cluster detachment and transport from a local biofilm containing high abundances of *E. coli* or other pathogenic bacteria increases with sudden environmental disturbance [43], such as a storm event or when flooded rice fields are drained to prepare for planting. Therefore, while significant levels of fecal indicator bacteria were not apparent in CF and LF stagnant water, the unknown bacterial composition of biofilms in the study fields still poses a risk of pathogen transport to the surrounding environment. Future studies should sample biofilm community composition in flooded rice fields, particularly those harboring migratory birds.

Arguably the LEISA system shares some similarities with a confined animal feeding operation containing poultry, where surrounding water bodies are typically scrutinized for pathogen detection. In confined feeding operations, the densities of birds reach an average of approximately 30,000 birds per 2000 m^2, with reported subsequent fecal coliforms of 5863 CFU/100 mL water [45]. This is considerably more birds and levels of contamination than the LEISA system in this study, which amassed approximately 1000 birds/ha at its peak. Thus, natural accumulation of potential pathogenic microorganisms versus commercial accumulation differs in impact factor.

6. Conclusions

Migratory birds occupying flooded rice fields in western Mississippi contributed to microbial populations in the soil, but with minimum pathogenic risk. There was no significant difference in fecal indicator bacteria in the above water columns. However, given that migratory birds are vectors for pathogenic organisms, there is a possibility that contamination levels could reach concerning levels in the future because pathogen levels can fluctuate substantially in bird flocks between seasons. One season of monitoring the LEISA system is inadequate to truly assess pathogen risk; however, it does provide a glimpse of the system, which indicates that risks are low. Regular monitoring of this system may be warranted as a precaution, should bird densities increase. Future research efforts could focus on model development, predicting pathogen levels in relation to bird densities.

Author Contributions: For the current work, author contributions are as follows: A.G.F. acquisition of funding, conceptualization, designing sampling scheme, collected, processed and assayed samples, data analysis, authored the manuscript; B.H.B. acquisition of funding, conceptualization, designing sampling scheme, manuscript development; J.P.B. assisted in designing sampling scheme, determining appropriate assays and manuscript development; R.S. assisted with processing and assaying samples and manuscript development; R.B.I. assisted in designing sampling scheme, determining appropriate analyses and manuscript development; J.B.D. assisted with designing sampling scheme and manuscript development. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by the United States Department of Agriculture-Southern SARE (GS17-166) the Mississippi Agricultural and Forestry Experiment Station and the Forest and Wildlife Research Center. This material is based upon work that is supported by the Monsanto Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments: We thank Austin Omer, Brent Chaney, Mary-Lynn Gibbs, Keaton Hamid, Kelsie Hall, Caleb Aldridge, Jared Feura, Joby Czarnecki, Shannon Westlake, Ryo Ogawa, Nicky Faucheux and Joseph Marty for their assistance in sample collection and analysis. A special thanks to the Delta Rice Farmers that participated in this study.

Conflicts of Interest: The authors declare no conflict of interest.

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