Association between fertilizer-mediated changes in microbial communities and *Aedes albopictus* growth and survival

Ephantus J. Muturi*, Jose L. Ramirez, Alejandro P. Rooney, Chris Dunlap
Crop Bioprotection Research Unit, USDA, ARS, 1815 N. University St. Peoria, IL. 61604.

*Corresponding Author

Ephantus J. Muturi

Email: Ephantus.Muturi@ars.usda.gov

ephajumu@yahoo.com

ABSTRACT

Contamination of aquatic habitats with anthropogenic nutrients has been associated with an increase in mosquito larval populations but the underlying mechanisms remain poorly understood. We examined the individual and combined effects of two synthetic fertilizers (ammonium sulfate and potassium chloride) on *Aedes albopictus* survival, development time, and sex ratio. The bacterial and fungal communities of water samples from different fertilizer treatments were also characterized by MiSeq sequencing of the 16S rRNA gene (bacteria) and internal transcribed spacer 1 (fungi) and their relationship with mosquito survival and development determined. Mosquitoes from ammonium sulfate treatment had significantly lower survival rates and longer development times compared to those from control, potassium chloride or a mixture of the two fertilizers. Fertilizer treatment had no significant effects on *Ae. albopictus* sex ratio although ammonium sulfate treatment tended to be more biased towards males relative to the other treatments. There were no significant effects of fertilizer treatment on fungal communities. However, potassium chloride treatments had lower bacterial diversity compared to the other treatments and the bacterial community structure of control and potassium chloride treatments differed significantly from that of ammonium sulfate and a mixture of the two fertilizers. Microbial composition but not diversity was significantly associated with mosquito survival and development. These findings suggest that anthropogenic nutrients can have a profound impact on mosquito survival and development. In addition to any potential direct effects on mosquito physiology, our results suggest that fertilizers can act indirectly by disrupting the microbial communities that provide a critical food resource for mosquito larvae.

Keywords: Synthetic fertilizers; ammonium sulfate; potassium chloride; *Ae. albopictus*; microbiota

INTRODUCTION

Chemical fertilizers are widely used to replenish the soil with three basic elements that are critical for plant growth: nitrogen, phosphorus, and potassium. The use of these fertilizers along with other technological- and land-use changes that marked the “Green Revolution” have increased the amount of food produced per area of land (Tilman et al. 2002), helping to meet the world food demand without the need for cropland expansion (Huang et al. 2002; Tilman et al. 2002). Unfortunately, the intensive use of fertilizers comes at major environmental costs including loss of soil fertility, degradation of water quality, changes in species composition and loss of biodiversity, eutrophication and loss of habitat quality in aquatic ecosystems, development of photochemical smog, and rising global concentrations of nitrous oxide, a powerful greenhouse gas (Camargo and Alonso 2006; Hamer et al. 2004; Richter 2007; Smil 2000; Vitousek et al. 1997). Additionally, fertilizer-enriched aquatic habitats have been shown to harbor large populations of mosquito larvae (Mutero et al. 2004; Muturi et al. 2007b; Sunish et al. 2003; Victor and Reuben 2000) raising concerns about the potential for these chemicals to alter the transmission dynamics of mosquito-borne pathogens. However, our understanding of the link between fertilizers and mosquito-borne diseases is still very limited and further studies on this topic can inform the development of holistic approaches to meet the rising demand for agricultural products while protecting the environment and human health.

There are several potential mechanisms by which fertilizer application may be associated with an increase in mosquito larval populations. These include alteration of the physical and chemical characteristics of the larval habitats making them attractive oviposition sites for mosquitoes (Mutero et al. 2004; Muturi et al. 2007b; Sunish et al. 1998; Sunish et al. 2003;

Victor and Reuben 2000), stimulation of diverse microbial assemblages that may enhance egg hatch success rates (Ponnusamy et al. 2011) and provide a rich food resource for mosquito larvae (Johnson et al. 2010; Victor and Reuben 2000), and direct and indirect negative effects of fertilizers on mosquito predators and competitors allowing the mosquitoes to develop in predation- and competition-free environment (Camargo and Alonso 2006; Sanford et al. 2005). However, deciphering the contribution of each of these mechanisms under natural conditions is a difficult task since nutrient enrichment is typically accompanied by other forms of environmental change such as land-use changes and chemical contamination (Johnson et al. 2010). As a result, little is known regarding how fertilizers affect the biotic and abiotic components of mosquito larval habitats and how these changes relate to mosquito oviposition behavior and offspring growth, development, and survival. This knowledge can propel identification of novel and environmentally friendly chemical and biological tools for mosquito surveillance and control.

In this study, we used a microcosm approach to investigate how two commonly used fertilizers, ammonium sulfate and potassium chloride, either separately or in combination affect survival and development of *Aedes albopictus*, one of the primary vectors of dengue, chikungunya and Zika viruses. We also characterized the microbial communities associated with each treatment and their relationship with mosquito survival and development. *Aedes albopictus* occur in a variety of natural (phytotelmata) and artificial (Rosa et al. 2014; Unlu et al. 2013) containers (e.g. waste tires, plant saucers) that are likely to be exposed to fertilizers emanating from farm activities in urban and suburban areas. Also, *Ae. albopictus* has been shown to colonize storm water drains and catch basins which intercept storm water runoff that is potentially contaminated with fertilizers and pesticides from surrounding agricultural farmlands (Rey et al. 2006). Our results show that ammonium sulfate reduced survival and development of

Ae. albopictus and that these effects were significantly associated with fertilizer-mediated changes in composition of bacterial communities in the aquatic microcosms.

MATERIAL AND METHODS

Experimental design

On March 21, 2016, we established indoor microcosms (400 mL tripour beakers) consisting of 350 ml of deionized water, one of four fertilizer treatments, and 0.1 g of a 1:1 ratio of brewer's yeast: lactalbumin as larval food. Ten replicates were established for each fertilizer treatment for a total of 40 containers. 1:1 ratio of brewer's yeast: lactalbumin is commonly used as food for mosquito larvae in experimental studies (e.g. Alto et al. 2015; Hardstone et al. 2012) and its use in this study allowed precise and consistent measurement of larval food to minimize experimental variability. The treatments included the untreated control, one of two fertilizers (ammonium sulfate (AS) and potassium chloride (PC)) applied separately (0.5 g/container), and a mixture of the two fertilizers (0.25 g of each fertilizer). This resulted in nitrogen concentration of 285.7 and 142.9 mg/L respectively in ammonium sulfate and fertilizer mixture treatments and potassium concentration of 748.6 and 374.3 mg/L respectively in potassium chloride and fertilizer mixture treatments. Although these high concentrations of fertilizers are not commonly reported in aquatic ecosystems, nitrogen, ammonia, and potassium concentrations of up 117, 290, and 510 mg/L have been observed in water bodies near agricultural farms (Hallberg 1987). These high doses are likely to occur if fertilizer application is immediately followed by a heavy rainfall. Additionally, in many developing countries, fertilizer application particularly in the rice fields which are also important larval habitats for mosquitoes is done manually through broadcasting and thus some parts of the rice fields are likely to receive higher doses of fertilizers.

On day two, 20 first instar larvae (~12 hours old) of *Ae. albopictus* (F4 generation originally from Louisville, KY) were added into 5 replicates of each treatment. The containers were maintained at 25°C and 14:8 light: dark cycle and pupae from each replicate were picked daily and held individually in plastic vials with small amount of water until eclosion. Adults were sexed and their eclosion date recorded. The experiment was terminated on April 13, 2016 when larvae from all the containers had either died or eclosed.

The remaining five replicates of each treatment (without larvae) were also maintained at 25°C and 14:8 light: dark cycle and used to determine the effect of fertilizer treatments on water pH and microbial communities. Briefly, seven days post-fertilizer treatment, 30 mL aliquots of water samples were taken from each container and stored at -80°C until further processing. Immediately after collection of the water samples, the pH of each microcosm was measured using ExStik II® probe (Extech instruments, Nashua, NH).

DNA extraction, library preparation and sequencing

The water samples were retrieved from the freezer, thawed, and centrifuged at $5000 \times g$ for 20 minutes. Total DNA was extracted from the resulting pellet using the Ultraclean® Soil DNA isolation kit (MoBio laboratories). Polymerase chain reaction (PCR) was performed using Kapa HiFi PCR mastermix (Kapa Biosystems Willington, MA) using the following parameters; 95 °C, 10 min, 35 cycles of 95 °C, 30 s; 58 °C, 30 s; 72 °C, 60s. PCR primers 341f and 806r for the bacterial community targeted the V3-V4 region of the 16S rRNA genes (Caporaso et al. 2011; Muyzer et al. 1993). The ITS1f and ITS2r primers for the fungal community analysis targeted ITS1 region (Gardes and Bruns 1993; Porter and Golding 2012). These locus-specific primers were incorporated into fusion primers for dual indexing and incorporation of adapters (Caporaso

et al. 2012) ahead of genome sequencing using the MiSeq system (Illumina Inc., San Diego, CA, USA). The PCR amplicons were cleaned and normalized using a SequalPrep™ normalization plate (Thermofisher inc, Waltham, MA). The samples were pooled and the library quantified with a Kapa library quantification kit (Kapa Biosystems Willington, MA). The samples were sequenced using an Illumina MiSeq system with a MiSeq V3 2 x 300 bp sequencing kit. The demultiplexed reads were quality trimmed to Q30 using CLC genomics workbench v8.5 (Qiagen inc., Valencia, CA). Read pairing, fixed length trimming and OTU clustering was conducted using CLC Bio Microbial Genomics module (Qiagen inc., Valencia, CA) using the reference sequences from the Greengenes ribosomal RNA gene database (DeSantis et al. 2006) or the UNITE fungal ITS database (Koljalg et al. 2013).

Statistical analyses

All data were analyzed using the R 3.2.5 statistical package. Univariate ANOVA was used to determine the effect of fertilizer treatments on water pH and *Ae. albopictus* life history traits. Before analysis, survival data was arcsine transformed and sex ratio, development time, and pH data were log (x+1) transformed to meet the assumptions of normality and homogeneity of variances.

OTUs with less than three sequences were discarded before downstream analysis. To standardize the sampling effort across samples, we rarefied the bacterial and fungal sequences, respectively to an even depth of 2,027 and 1,301 reads per sample using the R package 'phyloseq'. Alpha diversity metrics including Shannon diversity, observed number of species, and chao1 were computed for each sample using phyloseq and treatment differences in these metrics examined using ANOVA with Tukey adjustments. Multi-response permutation

procedures (MRPP) and non-metric multidimensional scaling (NMDS) based on the Bray-Curtis measure of dissimilarity were used to determine treatment differences in microbial communities. Indicator species analysis was computed using the R package ‘labdsv’ to identify bacterial and fungal OTUs that characterized each fertilizer treatment based on fidelity and specificity. Venn diagrams were created using the R package ‘limma’ to visualize the OTUs that were shared between fertilizer treatments. Linear regression analysis was used to determine the relationship between water pH and mosquito life history traits (survival and development time to adulthood). The same test was used to examine the relationship between microbial diversity and *Ae. albopictus* survival and development time to adulthood. NMDS scores were computed using the R package ‘vegan’ and spearman’s rank correlation analysis was used to determine the association between these scores and *Ae. albopictus* survival and development time to adulthood.

RESULTS

Fertilizer treatment modifies *Ae. albopictus* life history traits

Fertilizer treatments had significant effects on *Ae. albopictus* survival to adulthood ($F = 93.75$, $df = 3, 16$, $P < 0.0001$; Fig 1A) and development time to adulthood (female: $F=25.75$, $df = 3,16$, $P < 0.0001$; male: $F = 48.82$, $df = 3, 16$, $P < 0.001$; Fig 1C & D) but not sex ratio ($F =2.26$, $df = 3,16$, $P = 0.121$; Fig 1B). Significantly fewer adults eclosed from ammonium sulfate treatment compared to the other treatments. In addition, significantly more survivors were observed in potassium chloride treatment compared to a mixture of the two fertilizers. Both males and females of *Ae. albopictus* took longer to develop in ammonium sulfate compared to the other treatments.

Fertilizer treatments alter water chemistry parameters

The average ($\pm$ SE) pH for the controls was 6.46 ± 0.04 and significantly higher than 5.77 ± 0.07 , 5.35 ± 0.18 or 5.98 ± 0.08 for ammonium sulfate, potassium chloride, and ammonium sulfate + potassium chloride treatments, respectively. There was no significant association between pH and any of the measured life history traits for *Ae. albopictus* (Supplementary Table 1).

Relative abundance of microbial communities in relation to fertilizer treatment

After quality filtering and rarefaction to an even depth of 2,027 sequences per sample, a total of 97 bacterial OTUs belonging to 9 bacterial phyla including an unclassified group were detected in this study. Some community members were unique to a particular treatment but most were shared between two or more treatments (Fig 2A). Phylum *Proteobacteria* consisting of *Alphaproteobacteria* (39.1%), *Betaproteobacteria* (16.9%) and *Gammaproteobacteria* (25.0%) was the dominant bacterial phylum accounting for 78% of total bacteria followed by phylum *Bacteroidetes* (16.7%) (Supplementary Fig 1A). *Alphaproteobacteria* accounted for 75.2% of total sequences in PC treatments compared to 34.3%, 19.7%, 27.4% in control, AS and AS+PC treatments respectively. *Gammaproteobacteria* were absent in PC treatment and accounted for 17.4%, 44.8% and 37.7% in control, AS and AS+PC treatments respectively. Only 14 OTUs had a relative abundance greater than 1% of total sequences (Fig 3A). One OTU in the genus *Sphingomonas* was more abundant in PC treatment compared to the other treatments while three OTUs *Stenotrophomonas*, *Stenotrophomonas maltophilia* and *Chryseobacterium* were present in all except the PC treatments the former being more abundant in controls and the last two in AS and AS+PC treatments (Fig 3A).

A total of 79 fungal OTUs belonging to 7 phyla were retained after quality filtering and rarefaction to an even depth of 1,301 sequences per sample. Nearly half of the community members were unique to potassium chloride (Fig 2B). Phylum *Basidiomycota* (69.7%) and *Ascomycota* (29.8%) accounted for 99.5% of the total sequences (Supplementary Fig 1B). The relative abundance of *Basidiomycota* and *Ascomycota* depicted an increasing and decreasing trend respectively from control, AS, PC to AS+PC treatments (Supplementary Fig 1B). Only 6 OTUs had a relative abundance greater than 1% of the total sequences. These 6 OTUs accounted for 95.7% of the total sequences (Fig 3B). *Pseudozyma aphidis* occurred in all treatment but was more dominant in AS+PC treatments compared to the other treatments. *Gibberella fujikuroi* was absent in AS+PC treatment, rare in AS treatment and accounted for 51.6% of total sequences in controls. Two unidentified OTUs: *Pleosporales sp.* and *Ascomycota sp.* also tended to be more dominant in AS treatment (Fig 3B).

Fertilizer treatments alter the microbial diversity community structure

PC treatment had significantly lower bacterial diversity compared to the other treatments ($F = 7.04$, $df = 3, 16$, $P = 0.003$; Fig 4A). The observed number of bacterial OTUs did not differ significantly between treatments ($F = 2.15$, $df = 3, 16$, $P = 0.13$; Fig 4B) while the predicted number of OTUs was significantly lower in PC treatment compared to AS+PC treatment but not other treatments ($F = 3.48$, $df = 3, 16$, $P = 0.04$; Fig 4C). Fungal OTU diversity ($F = 0.63$, $df = 2, 15$, $P = 0.61$; Fig 4D), observed number of species ($F = 1.78$, $df = 3, 15$, $P = 0.19$; Fig 4E) and chao1 ($F = 1.74$, $df = 3, 15$, $P = 0.20$; Fig 4F) were not significantly different among fertilizer treatments. Neither the observed number of bacterial or fungal OTUs nor their species diversity was significantly associated with *Ae. albopictus* survival or development time to adulthood (Table 1).

NMDS ordination and MRPP results showed that the bacterial communities of control and PC treatments were distinct from those of AS and AS+PC treatments (Fig 5A; MRPP, $A = 0.32$, $P = 0.001$). Average within group distances indicated that controls followed by AS+PC had greater individual variability in bacterial communities than AS and PC treatments (control = 0.46, AS = 0.27, PC = 0.36, and AS+PC = 0.41). None of the four fertilizer treatments had distinct fungal communities (Fig 5B). However, MRPP results showed that AS+PC treatment had lower individual variability in fungal communities compared to the other treatments ($A = 0.14$, $P = 0.04$, average within group distances; control = 0.54, AS = 0.52, PC = 0.63, and AS+PC = 0.08). Spearman's rank correlation analysis revealed significant associations between MDS1 and *Ae. albopictus* life history traits for both bacterial and fungal data (Table 2). For bacterial data, MDS1 was positively associated with *Ae. albopictus* survival to adulthood and negatively associated with both male and female development time to adulthood. For fungal data, MDS1 was positively associated with *Ae. albopictus* survival to adulthood and negatively associated with female development time survival to adulthood. None of the measured *Ae. albopictus* life history traits were significantly associated with MDS2.

Indicator species of different fertilizer treatments

Twenty three bacterial OTUs and five fungal OTUs were identified as indicator species for different fertilizer treatments (Table 3). Of the 23 bacterial OTUs 16 were from phylum *Proteobacteria*, 3 from phylum *Bacteroidetes*, and 4 were unclassified. Five, 8, 8 and 2 bacterial OTUs were strongly associated with the control, AS, PC and AS+PC treatments respectively (Table 3). Of the five fungal OTUs, three were from the phylum *Ascomycota* and one each in phylum *Basidiomycota* and *Chytridiomycota* (Table 3).

DISCUSSION

This study shows that synthetic fertilizers that are widely used to promote crop production may have unexpected negative effects on mosquito growth and survival. In particular, exposure of *Ae. albopictus* larvae to ammonium sulfate fertilizer resulted in longer development times and lower survival to adulthood, but these effects were not observed in larvae that were exposed to either potassium chloride or a mixture of ammonium sulfate and potassium chloride. In our previous studies with *Culex quinquefasciatus* mosquitoes, we found that ammonium sulfate was responsible for up to 40% reduction in survival to adulthood and one week delay in development time to adulthood while potassium chloride had little effects on survival and development of this mosquito species (Muturi et al. 2007a). These findings suggest that the direct toxic effects of ammonium sulfate likely applies to a wide range of mosquito species. Other laboratory studies also have reported the direct toxic effects of nitrogenous fertilizers on aquatic invertebrates including mosquitoes (Darriet et al. 2010; Fedorova 1989; Olayemi et al. 2012; Stuart et al. 2014). For example, NPK fertilizer was shown to act as an ecological trap for *Ae. aegypti*, attracting large numbers of gravid females whose offspring experienced low survival rates compared to the controls (Darriet et al. 2010). These findings contradict those of field studies where synthetic fertilizers have been shown to act as oviposition attractants and are typically associated with large populations of mosquito larvae (Duguma and Walton 2014; Mutero et al. 2004; Muturi et al. 2007b; Sanford et al. 2005; Sunish et al. 2003; Victor and Reuben 2000). Under field conditions, fertilizers may break down rapidly and become utilized by plants and microorganisms. This may explain the fertilizer-mediated differences in larval survival between laboratory and field studies.

Little is known about the mechanisms underlying reduced survival and prolonged development of *Ae. albopictus* in ammonium sulfate treatment. Potential mechanisms include direct toxicity from ammonia (NH_3^+) and sulfate (SO_4^{2+}) ions which are known to be toxic to aquatic organisms (Alonso and Camargo 2006; Camargo and Alonso 2006; Yang et al. 2012), changes in the chemical characteristics of the aquatic habitats making them unsuitable for mosquito larvae (e.g. pH or ionic strength), and the disruption of aquatic microbial communities (e.g. bacteria, fungi) that serve as a critical food resource for mosquito larvae (Merritt et al. 1992). We did not quantify the concentration of ammonia and sulfate ions and their impact of mosquito survival and development and future studies are needed to address this knowledge gap. However, AS, PC, and AS+PC treatments had significantly lower pH values relative to the controls. Given that only ammonium sulfate treatment reduced *Ae. albopictus* survival and prolonged its development time despite having similar pH values as the PC and AS+PC treatment, there is no clear evidence that ammonium sulfate affected *Ae. albopictus* growth and survival via changes in pH. This was further confirmed by the lack of significant associations between pH and *Ae. albopictus* survival and development time to adulthood. Nevertheless, it is possible there were other important water chemistry parameters that were not measured in this study (e.g. dissolved oxygen, total dissolved solids, salinity, conductivity). Future studies incorporating more water quality variables could provide further insights into the link between fertilizers and mosquito growth and survival.

By relating *Ae. albopictus* survival and development to microbial diversity and composition, we were able to examine whether the deleterious effects of ammonium sulfate on this mosquito species were due to the disruption of aquatic bacterial and fungal communities, the known food base for mosquito larvae (Merritt et al. 1992). Fertilizer treatment had no significant

effects on fungal communities but PC treatment had lower bacterial diversity and fewer number of bacterial OTUs compared to the other treatments. In addition, the bacterial community structure of AS and AS+PC treatments was significantly different from that of control and PC treatment. These findings suggest that bacterial communities may be more sensitive to anthropogenic nutrients compared to fungal communities. Alternatively, given that the water samples for microbiome analysis were collected 7 days post-fertilizer treatment, it is also possible that fungal communities recovered faster than bacterial communities to fertilizer exposure. In soil ecosystems, bacteria have been shown to be more sensitive to different fertilizers compared to fungi (Lazcano et al. 2013). This is not unexpected since bacteria have a much shorter turnover time than fungi and can react faster to environmental fluctuations. Most bacteria and fungi prefer ammonium as the nitrogen source but at high rates, ammonium fertilizers can also inhibit microbial growth due to toxicity of ammonia, changes in pH, and increases in ionic strength (Geisseler and Scow 2014; Omar and Ismail 1999). Therefore, the effects of fertilizer exposure on bacterial diversity and composition may be attributed to the ability of some bacterial species to use fertilizers as an energy source as well as the differential sensitivity of bacterial species to fertilizer exposure through which some bacterial species are eliminated or suppressed while others benefit from reduced competition for food and space (Bohme et al. 2005; Geisseler and Scow 2014; Peacock et al. 2001).

Both bacterial and fungal community composition were significantly associated with *Ae. albopictus* survival and development while their diversity was not. These findings suggest that different bacterial and fungal species likely differ in their nutritional value to mosquitoes and ammonium sulfate may have selectively eliminated the microbial taxa that are essential for mosquito growth and survival. However, our experiment was not designed to assess the

nutritional value of different bacterial and fungal species and further studies are needed on this topic. The fact that only ammonium sulfate affected *Ae. albopictus* survival and development despite having similar microbial diversity and composition as some of the other treatments suggests the existence of several microbial groups that perform similar roles in mosquito growth and development. As a result, fertilizer contamination may not necessarily be accompanied by changes in mosquito life history traits as observed in PC and AS+PC treatments. Alternatively, the findings also may imply that the direct effects of ammonium sulfate on mosquito may be greater and offsets any indirect effects of microbial composition.

We identified 8 indicator species for the ammonium sulfate treatment four of which had an indicator value equal to or greater than 70%. These included unidentified *Stenotrophomonas* species, unidentified *Achromobacter* species, unidentified *Ralstonia* species as well as *Ralstonia pickettii*. These bacterial OTUs have been found in mosquito midguts (Boissiere et al. 2012; Lindh et al. 2005; Lindh et al. 2008; Rani et al. 2009). They also occur in aquatic habitats inhabited by mosquitoes (Coenye et al. 2003; Huang et al. 2006; van Elsas et al. 2001). These indicator species are known to play a critical role breakdown of natural and man-made pollutants that are central to bioremediation strategies. For instance, many *Stenotrophomonas* species are highly resistant to heavy metals and antibiotics and have been shown to degrade a wide range of compounds including pollutants (Ryan et al. 2009). Similarly, *Achromobacter* species play a critical role in biodegradation of environmental hormone di-n-Butyl Phthalate (Jin et al. 2015). Moreover, members of the genus *Ralstonia* have considerable importance as potential agents for the bioremediation of soil and water contaminated with heavy metals or chlorinated organic compounds due to their capability to thrive under low concentration of nutrients (Goris et al.

2001; Steinle et al. 1998). Further studies are needed to determine the role of these indicator species on fertilizer degradation as well as on mosquito growth and survival.

Elizabethkingia anophelis which is commonly isolated in the guts of *Anopheles* and *Aedes* mosquitoes as well as in water samples from their larval habitats both under field and laboratory conditions (Boissiere et al. 2012; Chen et al. 2015; Coon et al. 2014; Yadav et al. 2015) was absent in PC treatment, rare in the controls, and more abundant in PC + AS treatment. These findings suggest that AS and a mixture of the two fertilizers likely provides a thriving niche for this bacterium but additional studies are needed to ascertain this.

In conclusion, agricultural practices can serve both as a risk factor for mosquito-borne diseases and as an avenue through which these diseases could be mitigated. Although fertilizers are one of the most common contaminants of aquatic ecosystems, our understanding of their impact on mosquito ecology is limited. The results of this study demonstrate that contamination of aquatic habitats with certain synthetic fertilizers can have detrimental effects on mosquito populations by reducing their growth and survival. These effects seem to be driven partly by fertilizer-mediated changes in composition of aquatic microbial communities, the known food base for mosquito larvae. However, these results should be interpreted with caution not only because our study was conducted under laboratory conditions using artificial diet but also because in nature, fertilizer application is associated with large populations of mosquito larvae (Mutero et al. 2004; Muturi et al. 2007b; Sanford et al. 2005). It is possible the patterns we observed in this study may vary under field conditions due to interactions with other factors that may not have been considered in this study. Nevertheless, these results provide the framework for understanding the link between different types of synthetic fertilizers and mosquito ecology and for identifying the bacterial species that render certain fertilizers such as ammonium sulfate

deleterious to mosquitoes. This knowledge may propel discovery of novel and environmentally friendly strategies for mosquito control.

ACKNOWLEDGEMENTS

We thank Heather Walker of the National Center for Agricultural Utilization Research, Peoria, IL, USA, for expert technical assistance. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned; the USDA is an equal opportunity employer.

REFERENCES

- Alonso, A., Camargo, J.A., 2006. Ammonia toxicity to the freshwater invertebrates *Polycelis felina* (Planariidae, Turbellaria) and *Echinogammarus echinosetosus* (Gammaridae, Crustacea). *Fresen. Environ. Bull.* 15, 1578-1583.
- Alto, B.W., Bettinardi, D.J., Ortiz, S., 2015. Interspecific Larval Competition Differentially Impacts Adult Survival in Dengue Vectors. *J Med Entomol.* 52:163-170.
- Bohme, L., Langer, U., Bohme, F., 2005. Microbial biomass, enzyme activities and microbial community structure in two European long-term field experiments. *Agric. Ecosyst. Environ.* 109, 141-152.
- Boissiere, A., Tchioffo, M.T., Bachar, D., Abate, L., Marie, A., Nsango, S.E., Shahbazkia, H.R., Awono-Ambene, P.H., Levashina, E.A., Christen, R., Morlais, I., 2012. Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathog.* 8, e1002742.

- 383 Camargo, J.A., Alonso, A., 2006. Ecological and toxicological effects of inorganic nitrogen
384 pollution in aquatic ecosystems: A global assessment. *Environ. International.* 32, 831-
385 849.
- 386 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens,
387 S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R.,
388 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and
389 MiSeq platforms. *ISME J.* 6, 1621-1624.
- 390 Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J.,
391 Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions
392 of sequences per sample. *Proc. Nat. Acad. Sci. U.S.A.* 108, 4516-4522.
- 393 Chen, S., Bagdasarian, M., Walker, E.D., 2015. *Elizabethkingia anophelis*: molecular
394 manipulation and interactions with mosquito hosts. *Appl. Environ. Microbiol.* 81, 2233-
395 2243.
- 396 Coenye, T., Vancanneyt, M., Falsen, E., Swings, J., Vandamme, P. 2003. *Achromobacter*
397 *insolitus* sp. nov. and *Achromobacter spanius* sp. nov., from human clinical samples. *Int J*
398 *Syst Evol Microbiol.* 53, 1819-1824.
- 399 Coon, K.L., Vogel, K.J., Brown, M.R., Strand, M.R., 2014. Mosquitoes rely on their gut
400 microbiota for development. *Mol Ecol.* 23, 2727-2739.
- 401 Darriet, F., 2015. An anti-mosquito mixture for domestic use, combining a fertiliser and a
402 chemical or biological larvicide. *Pest. Manag. Sci.* 72, 1340-1345.
- 403 Darriet, F., Zumbo, B., Corbel, V., Chandre, F., 2010. Influence of plant matter and NPK
404 fertilizer on the biology of *Aedes aegypti* (Diptera: Culicidae). *Parasit. J. De La Societe*
405 *Francaise De Parasitologie.* 17, 149-154.
- 406 DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T.,
407 Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA
408 gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72,
409 5069-5072.

- 410 Duguma, D., Walton, W.E., 2014. Effects of nutrients on mosquitoes and an emergent
411 macrophyte, *Schoenoplectus maritimus*, for use in treatment wetlands. J. Vector Ecol. 39,
412 1-13.
- 413 Fedorova, V.G., 1989. The effect of mineral fertilizers on mosquito larvae (Culicidae).
414 Parazitologiya. 23, 193-199.
- 415 Gardes, M., Bruns, T.D., 1993. ITS Primers with enhanced specificity for basidiomycetes -
416 application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113-118.
- 417 Geisseler, D., Scow, K.M., 2014. Long-term effects of mineral fertilizers on soil microorganisms
418 - A review. Soil Biol Biochem. 75, 54-63.
- 419 Goris, J., De Vos, P., Coenye, T., Hoste, B., Janssens, D., Brim, H., Diels, L., Mergeay, M.,
420 Kersters, K., Vandamme, P., 2001. Classification of metal-resistant bacteria from
421 industrial biotopes as *Ralstonia campinensis* sp nov., *Ralstonia metallidurans* sp nov and
422 *Ralstonia basilensis* Steinle et al. 1998 emend. Inter. J. Syst. Evol. Microbiol. 51, 1773-
423 1782.
- 424 Hallberg, G.R., 1987. The impacts of agricultural chemicals on ground water quality.
425 GeoJournal. 15, 283-295.
- 426 Hamer, A.J., Makings, J.A., Lane, S.J., Mahony, M.J., 2004. Amphibian decline and fertilizers
427 used on agricultural land in south-eastern Australia. Agric. Ecosyst. Environ. 102, 299-
428 305.
- 429 Hardstone, M.C., Andreadis, T.G., 2012. Weak larval competition between the invasive
430 mosquito *Aedes japonicus japonicus* (Diptera: Culicidae) and three resident container-
431 inhabiting mosquitoes in the laboratory. J. Med. Entomol. 49: 277-285.
- 432 Huang, J., Miller, J.R., Chen, S.C., Vulule, J.M., Walker, E.D., 2006. *Anopheles gambiae*
433 (Diptera: Culicidae) oviposition in response to agarose media and cultured bacterial
434 volatiles. J. Med. Entomol. 43, 498-504.
- 435 Huang, J.K., Pray, C., Rozelle, S., 2002. Enhancing the crops to feed the poor. Nature. 418, 678-
436 684.
- 437 Jin, D.C., Kong, X., Li, Y.J., Bai, Z.H., Zhuang, G.Q., Zhuang, X.L., Deng, Y., 2015.
438 Biodegradation of di-n-Butyl phthalate by *Achromobacter* sp isolated from rural domestic
439 wastewater. Int. J. Env. Res. Pub. He. 12, 13510-13522.

- Johnson, P.T.J., Townsend, A.R., Cleveland, C.C., Glibert, P.M., Howarth, R.W., McKenzie, V.J., Rejmankova, E., Ward, M.H., 2010. Linking environmental nutrient enrichment and disease emergence in humans and wildlife. *Ecol. Appl.* 20, 16-29.
- Koljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F., Bahram, M., Bates, S.T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., Douglas, B., Drenkhan, T., Eberhardt, U., Duenas, M., Grebenc, T., Griffith, G.W., Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Lucking, R., Martin, M.P., Matheny, P.B., Nguyen, N.H., Niskanen, T., Oja, J., Peay, K.G., Peintner, U., Peterson, M., Poldmaa, K., Saag, L., Saar, I., Schussler, A., Scott, J.A., Senes, C., Smith, M.E., Suija, A., Taylor, D.L., Telleria, M.T., Weiss, M., Larsson, K.H., 2013. Towards a unified paradigm for sequence-based identification of fungi. *Mol. Ecol.* 22, 5271-5277.
- Lazcano, C., Gomez-Brandon, M., Revilla, P., Dominguez, J., 2013. Short-term effects of organic and inorganic fertilizers on soil microbial community structure and function. *Biol. Fert. Soils.* 49, 723-733.
- Lindh, J.M., Borg-Karlson, A.K., Faye I., 2008. Transstadial and horizontal transfer of bacteria within a colony of *Anopheles gambiae* (Diptera: Culicidae) and oviposition response to bacteria-containing water. *Acta Trop.* 2008, 107:242-250.
- Lindh, J.M., Terenius, O., Faye, I., 2005. 16S rRNA gene-based identification of midgut bacteria from field-caught *Anopheles gambiae* sensu lato and *A. funestus* mosquitoes reveals new species related to known insect symbionts. *App. Environ. Microbiol.* 2005;71:7217-7223.
- Merritt, R.W., Dadd, R.H., Walker, E.D., 1992. Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annu. Rev. Entomol.* 37, 349-376.
- Michaelakis, A., Mihou, A.P., Koliopoulos, G., Couladouros, E.A., 2007. Attract-and-kill strategy. Laboratory studies on hatched larvae of *Culex pipiens*. *Pest. Manag. Sci.* 63, 954-959.
- Mutero, C., Ng'ang'a, P., Wekoyela, P., Githure, J., Konradsen, F., 2004. Ammonium sulphate fertiliser increases larval populations of *Anopheles arabiensis* and culicine mosquitoes in rice fields. *Acta Trop.* 89, 187-192.
- Muturi, E.J., Jacob, B.G., Shililu, J., Novak, R., 2007a. Laboratory studies on the effect of inorganic fertilizers on survival and development of immature *Culex quinquefasciatus* (Diptera: Culicidae). *J. Vector Borne Dis.* 44, 259-265.

- 471 Muturi, E.J., Mwangangi, J.M., Shililu, J., Muriu, S., Jacob, B., Kabiru, E.W., Gu, W., Mbogo,
 472 C., Githure J., Novak, R., 2007b. Mosquito species succession and the physicochemical
 473 factors affecting their abundance in rice fields in Mwea, Kenya. J. Med. Entomol. 44,
 474 336-344.
- 475 Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations
 476 by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-
 477 amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.
- 478 Olayemi, I.K., Maduegbuna, E.N., Ukubuiwe, A.C., Chukwuemeka, V.I., 2012. Laboratory
 479 studies on developmental responses of the filarial vector mosquito, *Culex pipiens pipiens*
 480 (Diptera: Culicidae) to urea fertilizer. J. Med. Sci. 12, 175-181.
- 481 Omar, S.A., Ismail, M.A., 1999. Microbial populations, ammonification and nitrification in soil
 482 treated with urea and inorganic salts. Folia Microbiol. 44, 205-212.
- 483 Peacock, A.D., Mullen, M.D., Ringelberg, D.B., Tyler, D.D., Hedrick, D.B., Gale, P.M., White,
 484 D.C., 2001. Soil microbial community responses to dairy manure or ammonium nitrate
 485 applications. Soil Biol. Biochem. 33, 1011-1019.
- 486 Ponnusamy, L., Boroczky, K., Wesson, D.M., Schal, C., Apperson, C.S., 2011. Bacteria
 487 stimulate hatching of yellow fever mosquito eggs. PLoS One. 6, e24409.
- 488 Porter, T.M., Golding, G.B., 2012. Factors that affect large subunit Ribosomal DNA amplicon
 489 sequencing studies of fungal communities: classification method, primer choice, and
 490 error. Plos One. 7.
- 491 Poullot, D., Beslay, D., Bouvier, J.C., Sauphanor, B., 2001. Is attract-and-kill technology potent
 492 against insecticide-resistant Lepidoptera? Pest. Manag. Sci. 57, 729-736.
- 493 Rani, A., Sharma, A., Rajagopal, R., Adak, T., Bhatnagar, R.K., 2009. Bacterial diversity
 494 analysis of larvae and adult midgut microflora using culture-dependent and culture-
 495 independent methods in lab-reared and field-collected *Anopheles stephensi*- an Asian
 496 malarial vector. BMC Microbiol. 9, 96.
- 497 Rey, J.R., O'Meara, G.F., O'Connell, S.M., Cutwa-Francis, M.M., 2006. Factors affecting
 498 mosquito production from stormwater drains and catch basins in two Florida cities. J
 499 Vector Ecol. 31, 334-343.
- 500 Richter, D.D., 2007. Humanity's transformation of Earth's soil: Pedology's new frontier. Soil Sci.
 501 172, 957-967.

- 502 Rosa, E., Salmah, S., 2014. Fluctuation of diptera larvae in phytotelmata and relation with
503 climate variation in West Sumatra Indonesia. Pak. J. Biol. Sci. 17, 947-951.
- 504 Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van
505 der Lelie, D., Dow, J.M., 2009. The versatility and adaptation of bacteria from the genus
506 *Stenotrophomonas*. Nat. Rev. Microbiol. 7: 514-525.
- 507 Sanford, M.R., Chan, K., Walton, W.E., 2005. Effects of inorganic nitrogen enrichment on
508 mosquitoes (Diptera: Culicidae) and the associated aquatic community in constructed
509 treatment wetlands. J. Med. Entomol. 42, 766-776.
- 510 Smil, V., 2000. Phosphorus in the environment: Natural flows and human interferences. Annu.
511 Rev. Energ. Env. 25, 53-88.
- 512 Steinle, P., Stucki, G., Stettler, R., Hanselmann, K.W., 1998. Aerobic mineralization of 2,6-
513 dichlorophenol by *Ralstonia* sp. strain RK1. Appl. Environ. Microbiol. 64, 2566-2571.
- 514 Stuart, A.M., Palenzuela, A.N., Bernal, C.C., Ramal, A.F., Horgan, F.G., 2014. Effects of
515 fertiliser applications on survival and recruitment of the apple snail, *Pomacea*
516 *canaliculata* (Lamarck). Crop Protect. 64, 78-87.
- 517 Sunish, I.P., Raghunatha Rao, D., Gajanana, A., 1998. Nitrogenous fertilizers, neem and vectors
518 of Japanese encephalitis virus. Current Sci. 75, 1107.
- 519 Sunish, I.P., Rajendran, R., Reuben, R., 2003. The role of urea in the oviposition behaviour of
520 Japanese encephalitis vectors in rice fields of South India. Mem. Inst. Oswaldo Cruz. 98,
521 789-791.
- 522 Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., Polasky, S., 2002. Agricultural
523 sustainability and intensive production practices. Nature. 418, 671-677.
- 524 Unlu, I., Farajollahi, A., Strickman, D., Fonceca, D.M. 2013. Crouching tiger, hidden trouble:
525 urban sources of *Aedes albopictus* (Diptera: Culicidae) refractory to source reduction.
526 PLoS One 8(10).e77999.
- 527 van Elsas, J.D., Kastelein, P., de Vries, P.M., van Overbeek, L.S., 2001. Effects of ecological
528 factors on the survival and physiology of *Ralstonia solanacearum* bv. 2 in irrigation
529 water. Can. J. Microbiol. 47, 842-54.
- 530 Victor, T.J., Reuben, R., 2000. Effects of organic and inorganic fertilisers on mosquito
531 populations in rice fields of southern India. Med. Vet. Entomol. 14, 361-368.

- 532 Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W.,
533 Schlesinger, W.H., Tilman, D., 1997. Human alteration of the global nitrogen cycle:
534 Sources and consequences. *Ecol. Appl.* 7, 737-750.
- 535 Yadav, K.K., Bora, A., Datta, S., Chandel, K., Gogoi, H.K., Prasad, G.B., Veer, V., 2015.
536 Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti*
537 from Arunachal Pradesh, India. *Parasit. Vectors.* 8, 641.
- 538 Yang, Z., Lu, K., Chen, Y, Montagnes, D.J., 2012. The interactive effects of ammonia and
539 microcystin on life-history traits of the cladoceran *Daphnia magna*: synergistic or
540 antagonistic? *PLoS One* 7(3): e32285.
- 541
- 542

Table 1: Regression analysis for the relationship between microbial diversity and *Ae. albopictus* life history traits.

	Response variable	Predictor variable	Estimate	t value	P	R2
Bacteria	Survival	Intercept	276.74	1.25	0.23	35.17
		Observed (O)	-2.94	-0.40	0.70	
		Shannon (S)	-139.50	-1.26	0.23	
		O x S	2.63	0.74	0.47	
	Male development	Intercept	-27.69	-0.73	0.48	28.39
		Observed (O)	0.72	0.57	0.58	
		Shannon (S)	26.90	1.41	0.18	
		O x S	-0.58	-0.94	0.36	
	Female development	Intercept	-28.00	-0.66	0.52	12.76
		Observed (O)	0.93	0.65	0.52	
		Shannon (S)	23.67	1.11	0.28	
		O x S	-0.56	0.68	0.43	
Fungi	Survival	Intercept	68.20	6.34	< 0.001	12.40
		Observed (O)	0.47	0.42	0.68	
		Shannon (S)	-1.21	-0.08	0.93	
		O x S	-0.03	-0.04	0.97	
	Male development	Intercept	11.34	6.26	< 0.001	11.16
		Observed (O)	0.05	0.24	0.82	
		Shannon (S)	1.84	0.75	0.46	
		O x S	-0.09	-0.69	0.50	
	Female development	Intercept	11.34	6.26	< 0.001	
		Observed (O)	0.05	0.24	0.82	
		Shannon (S)	11.84	0.75	0.46	
		O x S	-0.09	0.13	0.50	

546

547 Table 2: Results of Spearman's rank correlation analysis between *Ae. albopictus* life history
 548 traits and NMDS scores

		MDS1		MDS2	
Variable		r	P	r	P
Bacteria	Survival	0.71	0.0004	-0.15	0.53
	Male development	-0.76	0.00001	-0.08	0.73
	Female development	-0.74	0.0002	0.25	0.29
Fungi	Survival	0.40	0.09	-0.07	0.77
	Male development	-0.24	0.32	0.18	0.46
	Female development	-0.62	0.005	0.08	0.73

549

549

550

551 Table 3: Bacterial and fungal OTUs that characterize different fertilizer treatments. AS =
 552 ammonium sulfate, PC = potassium chloride, and both = AS + PC combined.

Bacteria						
Treatment	Phylum	Family	Species	Indicator value	P	Frequency
Control	Proteobacteria	Comamonadaceae	<i>Pelomonas</i>	0.85	0.001	11
Control	Proteobacteria	Bradyrhizobiaceae	<i>Bosea eneeae</i>	0.73	0.012	9
Control	Proteobacteria	Comamonadaceae	<i>Aquabacterium</i>	0.68	0.01	10
Control	Bacteroidetes	Chitinophagaceae	<i>Hydrotalea</i>	0.62	0.031	9
Control	Bacteroidetes	Chitinophagaceae	<i>Sediminibacterium</i>	0.60	0.03	3
AS	Proteobacteria	Xanthomonadaceae	<i>Stenotrophomonas</i>	0.88	0.004	7
AS	Proteobacteria	Alcaligenaceae	<i>Achromobacter</i>	0.86	0.002	7
AS	Proteobacteria	Burkholderiaceae	<i>Ralstonia</i>	0.77	0.001	10
AS	Proteobacteria	Burkholderiaceae	<i>Ralstonia pickettii</i>	0.70	0.006	12
AS	Unclassified	Unclassified	Unclassified	0.68	0.001	14
AS	Unclassified	Unclassified	Unclassified	0.60	0.001	14
AS	Proteobacteria	Xanthomonadaceae	<i>Stenotrophomonas maltophilia</i>	0.44	0.039	15
AS	Proteobacteria	Xanthomonadaceae	<i>Stenotrophomonas</i>	0.44	0.04	15
PC	Proteobacteria	Sphingomonadaceae	<i>Sphingomonas</i>	0.73	0.003	11
PC	Unclassified	Unclassified	Unclassified	0.71	0.001	15
PC	Unclassified	Unclassified	Unclassified	0.61	0.004	19
PC	Proteobacteria	Comamonadaceae	<i>Comamonadaceae</i>	0.60	0.036	3
PC	Proteobacteria	Comamonadaceae	<i>Chlorochromatium</i>	0.60	0.035	3
PC	Proteobacteria	Sphingomonadaceae	<i>Sphingomonas</i>	0.54	0.002	20
PC	Proteobacteria	Caulobacteraceae	<i>Caulobacter</i>	0.53	0.029	17
PC	Proteobacteria	Comamonadaceae	<i>Aquabacterium</i>	0.52	0.001	20
Both	Bacteroidetes	Flavobacteriaceae	<i>Elizabethkingia anophelis</i>	0.81	0.017	10
Both	Proteobacteria	Brucellaceae	<i>Ochrobactrum</i>	0.65	0.021	10
Fungi						
Treatment	Phylum	Family	Species	Indicator value	P	Frequency
Control	Ascomycota	Nectriaceae	<i>Gibberella fujikuroi</i> <i>Rhodosporidium</i>	0.76	0.008	9
PC	Basidiomycota	Incertae sedis	<i>paludigenum</i>	1.00	0.001	5
PC	Chytridiomycota	Olpidiaceae	<i>Olpidium brassicae</i>	0.74	0.019	7
Both	Ascomycota	Saccharomycetaceae	<i>Saccharomyces cerevisiae</i>	0.68	0.009	8
Both	Ascomycota	Saccharomycetaceae	<i>Saccharomyces cerevisiae</i>	0.59	0.002	17

553

554

554 **Figure legends**

555 Fig 1: *Aedes albopictus* A) survival (in percentage), B) sex ratio and C) female and D) male
556 development time (in days) to adulthood under different fertilizer treatments. Means ($\pm$ SE)
557 followed by different letters are significantly different.

558

559 Fig 2: Venn diagram summarizing the overlap of A) bacterial and B) fungal OTUs between
560 fertilizer treatments.

561

562 Fig 3: Mean relative abundances of A) bacterial and B) fungal OTUs across fertilizer treatments.
563 OTUs with abundance of less than 1% were pooled together as “Other”. AS = ammonium
564 sulfate, PC = potassium chloride, and both = AS + PC combined.

565

566 Fig 4: Biodiversity estimators for bacterial (A-C) and fungal (D-F) diversity in different fertilizer
567 treatments. AS = ammonium sulfate, PC = potassium chloride, and both = AS + PC combined.

568

569 Fig 5: Nonmetric Multidimensional Scaling (NMDS) ordination displaying the A) bacterial and
570 B) fungal communities of water samples from different fertilizer treatments. AS = ammonium
571 sulfate, PC = potassium chloride, and both = AS + PC combined.

572

Figure 2

Figure 3

Figure 4

Figure 5