The Missing Metric: An Evaluation of Fungal Importance in Wetland Assessments

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Abstract

To preserve wetland ecosystem function, federal and state agencies have developed assessment procedures to better manage remaining wetland areas. Currently, wetland assessments do not consider microorganisms when determining wetland quality. This is notable, because fungi are often the primary decomposers of organic material and thus important players in nutrient cycling. The objective of this study is to quantify how wetland quality, as measured using the Ohio Rapid Assessment Method (ORAM), relates to fungal community composition. We sampled soils from six depressional emergent marshes in Ohio belonging to each of the three ORAM quality categories, assessed soil physicochemical properties, and recovered fungal DNA. We then determined if wetland quality as expressed by the ORAM reflects soil health. Our results indicate that ORAM scoring methodology significantly explains differences in fungal community composition between wetlands. We also found that soil physicochemical properties not currently included in the ORAM are strong drivers of fungal community composition, particularly bulk density, pH, soil organic matter, and soil moisture. Overall, our results suggest fungal community composition reflects wetland quality as assessed by the ORAM, and that the ORAM and potentially other wetland assessments could better capture the soil environment by including easily measured soil physicochemical properties.

Keywords Exact Sequence Variant (ESV) · Fungi · Marsh · Ohio · Wetland Quality

Introduction

To facilitate the regulation and restoration of wetlands, federal and state agencies established assessment procedures to monitor wetland quality (Fennessy et al. 2007). The “quality” of wetlands is largely determined by metrics that characterize their condition or the degree of anthropogenic disturbance (Mack 2001a). High quality, high functioning wetlands are generally considered to be pristine, having experienced little anthropogenic disturbance, and are capable of carrying out ecosystem services (Miller and Gunsalus 1999; Mack

2001a; Berglund and McEldowney 2008; Hruby 2014). In contrast, low quality wetlands are heavily degraded and as a result, are less functional and provide fewer ecosystem services than high quality wetlands.

To evaluate wetland quality, the United States Environmental Protection Agency recognizes three levels of assessment that vary in intensity and requisite expertise (Fennessy et al. 2007). Level 1 assessments are cursory evaluations that take little time and do not require a visit to the site in question. Level 3 assessments are in-depth field investigations that focus on one aspect of a wetland such as flora or fauna. Level 2, or rapid assessments, require 24 h or less to complete and incorporate several descriptive metrics that are tied to wetland functions.

Common rapid assessment metrics include wetland size, width of upland buffers, hydrology, and plant community composition (Miller and Gunsalus 1999; Mack 2001a; Berglund and McEldowney 2008; Hruby 2014). Wetland size or area can determine a wetland’s ability to serve as habitat for a range of wetland flora and fauna (Brown and Dinsmore 1986; Babbitt 2005). Consequently, larger wetlands score higher in wetland assessments although smaller wetlands

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may serve as habitat for unique species (Semlitsch and Bodie 1998; Mack 2001a). Upland buffers protect the functional integrity of the wetlands they separate from human disturbances and are typically scored using average buffer width, with wide buffer zones (≥ 50 m) providing greater nutrient filtration and support of flora and fauna (Castelle et al. 1994; Gilliam 1994; Burke and Gibbons 1995; Mack 2001a; Semlitsch and Bodie 2003; Stapanian et al. 2016). Wetland hydrology, assessed by water depth, hydroperiod, water sources, and hydrologic regime alterations (e.g. drainage tiles, ditches, dams), regulates soil anoxia, which in turn controls nutrient removal and carbon sequestration (Miller and Gunsalus 1999; Mack 2001a; Fisher and Acreman 2004; Kimmel et al. 2008; Kayranli et al. 2009; Hruby 2014). Finally, wetland vegetation, which is scored based on the number and interspersed of different vegetation classes (e.g. aquatic, emergent, scrub-shrub), plant diversity, and the abundance of native and invasive plants, plays an important role in nutrient cycling (Miller and Gunsalus 1999; Mack 2001a; Fisher and Acreman 2004; Kimmel et al. 2008; Kayranli et al. 2009; Hruby 2014). Increases in wetland plant functional diversity, for example, often lead to reduced methane emissions (Bouchard et al. 2007).

Despite its importance in wetland ecosystem function, few wetland assessments consider the soil environment. Assessments that currently include soil based metrics assess soil on a coarse scale considering only recent soil disturbances or by cursory assessments of soil type (e.g. organic or mineral) as opposed to any quantifiable soil parameter such as micro-organism community structure, bulk density (BD), soil organic matter (SOM), or available soil nutrients (Mack 2001a; Hruby 2014). A need for incorporating soil based metrics into assessments was recently highlighted when it was shown that wetlands with similar quality scores, determined using the Ohio Rapid Assessment Method (ORAM), did not group together when soil physicochemical properties were considered (Rokosch et al. 2009). Additionally, certain soil metrics were identified to be promising metrics to include in assessments due to trends they exhibited with swamp quality scores. For instance, BD was negatively related to swamp quality and SOM was positively related to swamp quality, with higher quality swamps having less compact soils and more SOM than low quality swamps (Rokosch et al. 2009).

Here we propose strengthening current methods by incorporating soil microorganisms that regulate valued wetland functions such as nutrient cycling and the breakdown of harmful chemicals (Gutknecht et al. 2006; Faulwetter et al. 2009). Specifically, including measurements of fungi could greatly improve current assessment methods because fungi perform vital ecosystem functions as plant symbionts, saprobes, and pathogens (Blaney and Kotanen 2001; Thormann 2006; Smith and Read 2008; Neori and Agami 2017). Fungi that act as saprobes are the primary decomposers in both upland

and wetland ecosystems and include white, brown, and soft rot fungi which degrade lignin, cellulose and hemicellulose contributing to the build-up of partially decomposed SOM in wetlands (Hibbett and Donoghue 2001; Thormann 2006). Mycorrhizal fungi, which exchange immobile soil nutrients such as phosphorous (P) and nitrogen (N) for carbon (C) from their plant hosts, comprise the largest group of fungal symbionts in wetlands and include arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungi which associate with herbaceous and woody plants respectively (Smith and Read 2008; Neori and Agami 2017). Finally, fungi that act as plant pathogens in both upland and wetland ecosystems can structure plant communities due to differences in virulence, tolerance, and susceptibility in host identity and can be important in determining the establishment of invasive plants (Mitchell and Power 2003; Inderjit and van der Putten 2010; Rúa et al. 2011). By understanding the fungal functional guilds present in wetlands of differing quality, the potential functional capacity of the fungal community at each wetland type can be better understood allowing for improved land management.

The forces that determine the structure and function of belowground fungal communities include both biotic and abiotic properties, such as vegetation and soil physicochemical properties. For instance, the richness of saprotrophic and mutualistic fungi typically increases with increasing plant richness, possibly as a product of increased plant richness generating a more heterogeneous soil environment allowing for greater resource partitioning in below-ground communities (Wardle 2006; Hiiesalu et al. 2014; Hiiesalu et al. 2017). Also, soil properties such as soil nutrient availability (particularly N and P), SOM, and pH are important determinants of fungal community richness and composition, with differences in EM fungi and saprotroph richness explained by the amount of SOM, C:N ratios, and pH (Erlandson et al. 2016; Glassman et al. 2017; Hiiesalu et al. 2017).

The primary objective of this study is to quantify how the quality of freshwater depressional marshes, as measured using the ORAM, relates to fungal community composition as a first step in determining if current assessment methods need to be adjusted to account for microbial communities as proxies for healthy ecosystems. The ORAM, which is comprised of six metrics: (1) wetland size, (2) upland buffer width and surrounding land use, (3) hydrology, (4) substrate disturbance and habitat development, (5) special wetland status (e.g. bogs, fens, etc.), and (6) wetland vegetation, is used in Ohio to rapidly assess wetland quality. The ORAM scores wetlands on a scale of 0 to 100 based on the six metrics and categorizes them into one of three quality categories: Category 1, Category 2, and Category 3 (Mack 2001a). Category 1 wetlands are low quality (highest disturbance and lowest functional value) and are afforded little regulatory protection by the Ohio Environmental Protection Agency (OH EPA). Category 3 wetlands are high quality (lowest disturbance

and highest functional value) and are a priority for protection by the OH EPA.

To achieve this objective, we recovered soil fungal communities from six natural marshes within the state of Ohio that represent the three ORAM quality categories. Using the Illumina MiSeq platform to sequence soil DNA, we determined the diversity and composition of the fungal community at each marsh. We used this data to address the central hypothesis that marshes with higher quality ratings will have more diverse fungal communities that differ in composition from lower quality marshes. We additionally identified the role of soil physicochemical properties and vegetation in structuring fungal communities, with particular emphasis on soil properties which are seldom included in wetland assessments. Together we combine these data as an important first step in determining if current wetland assessment frameworks would benefit from the addition of soil based metrics.

Methods

Study Sites

We identified six depressional marshes with emergent vegetation (Table 1) in the state of Ohio using the National Wetland Inventory (NWI; <https://www.fws.gov/wetlands/data/mapper.html>) and previous ORAM scoring data provided by the OH EPA. We assessed the quality of each marsh using the ORAM v. 5 methodology (Table S1; Mack 2001a) and conducted a field survey of each marsh's vegetation during Summer 2017. We assessed percent cover of individual plant species, bare ground, and standing water visually along five transects per marsh using a 1 m² quadrat at equal intervals, for a total of ten quadrats per transect and 50 quadrats per marsh (note, that transect length varied and was approximately the edge-to-edge distance of the wetland; Magee et al. 1993). We then calculated the floristic quality assessment index (FQAI; Andreas et al. 2004: Eq. 6). We used the FQAI as a secondary measure of the quality of marsh vegetation because it is a more objective measure based on a plant's coefficient of conservatism (C of C) or observed sensitivity to disturbance and fidelity to habitat (Andreas et al. 2004). The FQAI is also incorporated into the Vegetation Index of Biotic Integrity (VIBI; Mack 2004) which was used to calibrate the ORAM (Mack 2001b).

To account for within-marsh variation due to environmental gradients, we established soil sampling stations within each marsh following a stratified random design. Using data generated from the vegetation surveys, we divided each site into two or three strata delineated by dominant plant communities using the *hclust* function from the *Cluster* package (Maechler et al., 2017; Table 1). From each stratum, we randomly selected five of the surveyed quadrats for a total of 10 or 15 soil

sampling stations (quadrats) per marsh and a total of 70 sampling stations in the study.

Soil Core Sampling

To measure soil physicochemical properties, we collected soil cores in July, August, and September of 2017 using PVC soil corers (11.5 cm depth × 10 cm diameter). From each sampling station, we collected two soil cores from opposing corners of the quadrat for a total of 140 cores. Cores were placed on ice; transported back to the lab for immediate determination of soil moisture and bulk density; and then stored at 4 °C for three months until further processing.

For extraction of DNA from soil, we collected an additional soil core from the center of each quadrat using a PVC corer (11.5 cm depth × 10 cm diameter) for a total of 70 cores across all six marshes. We subsampled each DNA core in the field by homogenizing the soil and packing two 1.5 mL centrifuge tubes with approximately 1 g of soil from the homogenized core. Soil subsamples were flash frozen in liquid nitrogen, placed on dry ice, and transported back to the lab for immediate DNA extraction (see below). The remainder of each soil subsample was stored at -80 °C.

Soil Physicochemical Properties

In the lab, we measured BD and gravimetric water content (soil moisture) by drying 20 g of field moist soil (105 °C, 24 h). We measured soil pH using a modified version of the protocol described by Tan (2005). Using a 1:4 ratio of field moist soil to water, we stirred the soil slurry at 80 rpm for 15 min on an orbital shaker (Lab-Line Instruments Inc., Melrose Park, IL) and measured the pH of the slurry using a Beckman Coulter Φ360 pH/Temp/mV meter (Brea, CA).

The remaining soil was air dried for four weeks, ground, passed through a 2 mm sieve (No. 10), and pooled per sampling station. A portion of the ground soil was submitted to Brookside Laboratories (New Bremen, OH) for measurement of SOM (loss on ignition 360 °C), Mehlich III extractable P, NO₃-N, and NH₄-N. The remaining air-dried soil was passed through a 0.212 mm sieve (No. 70) and submitted to Washington State University Stable Isotope Core Laboratory (Pullman, WA) for C:N analyses using an elemental analyzer (ECS 4010, Costech Analytical, Valencia, CA).

Soil Fungal DNA Extraction

We extracted DNA in triplicate for each sampling station with 25 µL DI water acting as a negative control. Within 24 h of the initial soil core sampling, we extracted DNA from 0.25 g of soil with the DNeasy PowerSoil kit (Qiagen, Carlsbad, CA) using the default protocol. DNA extracts were stored at -80 °C for four months, then pooled per sampling station and purified

Table 1 Descriptions of the six marshes included in the study. Note that there was good agreement between our ORAM scores and scores from a previous OH EPA assessment which evaluated four of the six marshes

Site	Hoffman	Big Island	Keller	Dunlap	Calamus	Morgan Swamp
Site Name	Category 1A	Category 2A	Category 2B	Category 2C	Category 3A	Category 3B
ORAM Score	25	34.5	39.5	42.5	70	78
County	Champaign	Marion	Fairfield	Fairfield	Pickaway	Ashtabula
Coordinates	40.250° N, 83.800° W	40.585° N, 83.224° W	39.863° N, 82.620° W	39.834° N, 82.725° W	39.583° N, 83.001° W	41.649° N, 80.884° W
Surrounding Land Use	Active Farm	Abandoned Farmland/ Wildlife Area	Active Farm	Active Farm	Forest/Active Farm	Forest
Soil Series	Walkill Silt Loam	Milford Silt Clay Loam	Muskego Muck	Pewamo Silt Clay Loam	Unclassified Muck	Caneadea-Canadice Silt Loam
Hydroperiod	Seasonally Flooded	Seasonally Flooded	Regularly Saturated	Permanently Flooded	Permanently Flooded	Permanently Flooded
Maximum Water Depth	<0.4 m	<0.4 m	<0.4 m	>0.7 m	>0.7 m	>0.7 m
Dominant Plant Communities	S1: <i>Phalaris arundinacea</i> S2: <i>Leersia oryzoides</i>	S1: <i>Phalaris arundinacea</i> S2: <i>Leersia oryzoides</i> / <i>Eleocharis spp.</i>	S1: <i>Typha latifolia</i> S2: <i>Scirpus fluvialis</i> S3: <i>Polygonum amphibium</i> / <i>Urtica dioica</i>	S1: <i>Polygonum amphibium</i> S2: <i>Wolffia spp.</i> / <i>Lemna spp.</i>	S1: <i>Typha latifolia</i> S2: <i>Nuphar advena</i> S3: <i>Sparganium eurycarpum</i> / <i>Cephalanthus occidentalis</i> / <i>Typha latifolia</i>	S1: <i>Nuphar advena</i> S2: <i>Juncus effusus</i> / <i>Dulichium arundinaceum</i>

using a modified protocol of the DNeasy PowerClean Cleanup kit (Qiagen, Carlsbad, CA). Specifically, after cleaning 150 µL of pooled extract by washing with solution CB, we added 500 µL of undiluted ethanol to pooled DNA extracts in MB spin columns (Qiagen, Carlsbad, CA). Following incubation for 5 min at room temperature, the MB spin columns were centrifuged for 30 s at 10,000×g. We discarded the flow through and repeated the previous step. The MB spin columns were then centrifuged for 4 min at 10,000×g to remove any residual ethanol before proceeding with the remainder of the protocol.

We diluted cleaned DNA extracts to 5 ng/µL DNA by adding Molecular Biology Grade Water to a volume of 90 µL (IBI Scientific, Dubuque, IA) and submitted samples to the Ohio State University Molecular and Cellular Imaging Center (MCIC; Wooster, OH) for library preparation and Illumina sequencing using the MiSeq platform.

Library Preparation and Sequencing

The MCIC amplified the ITS1 locus using ITS1F and ITS2 PCR primers with an added heterogeneity spacer to compensate for the low nucleotide diversity of the amplicon (Smith and Peay 2014). Adapters, containing Nextera indices, were ligated to sequences during PCR for sample indexing. Samples were amplified in two rounds, the first to amplify the DNA and attach a portion of the Illumina adapter sequence and the second to complete the adapter sequence. The following steps were carried out on the Eppendorf epMotion5075

automated liquid handler (Hauppauge, NY). In the first round (PCR 1), 25 ng of genomic DNA was amplified using the following conditions: initial denaturation at 96 °C for 3 min, 25 cycles of 96 °C for 30 s for denaturation, 55 °C for 30 s for annealing, and 72 °C for 30 s for elongation. The second round of PCR was conducted using 3 µL of clean PCR 1 product. PCR conditions for round 2 were the same as PCR 1 except 8 cycles were performed rather than 25 cycles. After each round of PCR, samples were cleaned using the Agencourt AMPure XP beads (Beckman Coulter Life Sciences). Purified amplicon libraries were quantified and pooled by plate at equimolar ratios before sequencing. The final pools were purified using the Pippin Prep size selection system (Sage Science; Beverly, MA) to discard primer dimers.

Amplicon libraries were sequenced using the Illumina MiSeq sequencing platform at a final concentration of 14.3 pM. PhiX was mixed in the pool of amplicon libraries for the sequencing run (expected at 20%). The run was clustered to a density of $681 \pm k/mm^2$ and the libraries were sequenced using a 300PE MiSeq sequencing kit with the standard Illumina sequencing primers. Image analysis, base calling, and quality assessment were performed on the MiSeq instrument. The resulting sequences were demultiplexed and adapters were removed.

Sequence Processing

Resulting sequences were processed using the bioinformatics pipeline Quantitative Insights into Microbial Ecology 2

(QIIME2; <http://qiime2.org>; Caporaso et al. 2010). We removed heterogeneity spacers from sequences using the *cutadapt trim-paired* function of the *cutadapt* QIIME2 plugin (Martin 2011). We used the *dada2 denoise-paired* function of the *DADA2* plugin to denoise, remove chimeras, merge paired end reads, and identify exact sequence variants (ESV), a proxy for species (Callahan et al. 2016). Using the *feature-table group* function of the *feature-table* plugin, we pooled the ESVs by strata. We assigned taxonomy using the *feature-classifier classify-sklearn* function with a 0.70 confidence threshold (Pedregosa et al. 2011) from the *feature-classifier* plugin (Bokulich et al. 2018). We trained the RDP classifier with the UNITE v. 7.2 database (<https://doi.org/10.15156/BIO/587481>; Kõljalg et al. 2013) using the *feature-classifier fit-classifier-naïve-bayes* function of the *feature-classifier* plugin. ESVs assigned to at least a fungal phylum were retained. We then rarefied the resulting ESV table and representative sequences to the lowest number of sequences observed from all strata using the *feature-table rarefy* function (Fig. S1) from the *feature-table* plugin to standardize statistical analyses.

Fungal Functional Guilds

We assigned ESVs to a functional guild using the online version of FUNGuild with a confidence cut-off of “Possible” (<http://funguild.org>, accessed 17 June, 2019; Nguyen et al. 2016a). For any ESV not assigned to a functional guild but classified to the family, genus, or species level, we assigned the most probable functional guild or guilds based on the literature. We then calculated the relative abundance of each functional guild at the stratum level. For ESVs assigned to multiple functional guilds, we equally allocated sequences to each of the assigned functional guilds so that each possible function of the ESV was equally weighted and patterns could be more easily discerned. We considered both “narrow” functional guilds (e.g. AM fungi, white rot, plant pathogen, etc.) and “broad” functional guilds (e.g. saprotroph, pathogen, endophyte, etc.).

Statistical Analyses

We performed all statistical analyses in R version 3.4.4 (R Core Team 2018). We fit all linear mixed effects models using the *lme* function from the *nlme* package (Pinheiro et al. 2018). To meet the assumptions of normality and homoscedasticity the following transformations were performed: soft rot, saprobe, and pathogen richness were log transformed; and white rot, EM, and AM richness were square root transformed. Ref. “R Core Team 2018” is cited in the body but its bibliographic information is missing. Kindly provide its bibliographic information in the list. R Core Team (2018) R: A language and environment for statistical computing. R

Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.

Using per stratum raw ESV abundance data, we calculated Shannon diversity indices, observed richness, and Chao1 richness using the *diversity* and the *specnumber* functions from the *vegan* package (Oksanen et al. 2018) and *chao1* function from the *fossil* package (Vavrek 2011). We used linear mixed effects models to test if ORAM score significantly affects Shannon diversity, observed richness, Chao1 richness, and the richness of broad and narrow functional guilds with site as a random effect. Linear mixed effects models were also used to quantify the relationships between ORAM score and individual soil variables (e.g. BD, pH, soil moisture, Mehlich III extractable P, NO_3^- , NH_4^+ , N, C, and C:N) with site as a random effect.

We then calculated relative ESV abundances per stratum by dividing the number of sequences per ESV in a stratum by the total number of sequences in the stratum. We visually assessed the ability of the ORAM to group wetlands based on fungal communities and fungal functional guilds using relative ESV abundances with non-metric multidimensional scaling (NMDS) using the *metaMDS* function from *vegan* (Oksanen et al. 2018). We used NMDS ordination for these assessments because it allowed use of non-Euclidean distances (e.g. Bray-Curtis), which are preferred for comparison based on community composition (Faith et al. 1987). To identify indicator taxa, we used the *multipatt* function of the *indicspecies* package at the site level (De Caceres and Jansen 2016). To visually assess the ability of the ORAM to group marshes based on soil physicochemical properties we performed a principal component analysis (PCA) using the *prcomp* and *fviz_pca_biplot* functions from the *factoextra* package (Kassambara and Mundt 2017). We used the Euclidean-based PCA ordination for this assessment following common methodology for environmental data (Janžekovič and Novak 2012). Ref. “De Caceres and Jansen 2016” is cited in the body but its bibliographic information is missing. Kindly provide its bibliographic information in the list. De Caceres, M, & Jansen, F (2016) Indicspecies: relationship between species and groups of sites. R package version, 1(6).

We then calculated weighted relative ESV abundances, weighted relative vegetation cover, and weighted averages of soil physicochemical properties per site using strata weights (i.e. number of surveyed vegetation quadrats belonging to a stratum divided by the total number of surveyed quadrats in a marsh). We tested if ORAM and component metric scores significantly explained differences in fungal community composition, fungal functional guild composition, and soil physicochemical properties using permutational multivariate analysis of variance (PERMANOVA) using the *adonis*

function from *vegan* (Oksanen et al. 2018) with 720 permutations (maximum possible).

We assessed the impacts of soil properties (averaged per stratum) and plant communities (relative cover per stratum) on (1) fungal communities and (2) functional guilds by canonical correspondence analyses (CCA) using the *cca* function from *vegan* (Oksanen et al. 2018). CCA is a constrained ordination technique that assumes a unimodal relationship between the set of response variables (e.g. ESV abundance) and the set of predictor variables (e.g. soil properties). We then tested for statistical significance with the *anova.cca* function from *vegan*.

Figure files as EPS format or at a higher resolution? ">Results

Sequence Processing

We recovered 6,115,790 sequences from all sampling sites and retained 248,712 sequences after quality filtering and chimera removal with *DADA2*. Sequence data are available at NCBI Sequence Read Archive: PRJNA525991 and filed under accession numbers SAMN11081749 - SAMN1108197. Of the sequences that passed quality filtering, 222,241 sequences were identified as fungi with 135,696 sequences being assigned to a fungal phylum using the UNITE database. We rarefied strata to a sampling depth of 2861 sequences per strata yielding 40,054 sequences across 759 unique ESVs (Fig. S1).

Fungal Community Composition

Ascomycota was the most abundant phylum and comprised 47% of the total fungal community, followed by Basidiomycota which comprised 45% of the community (Fig. S2). The Blastocladiomycota, Chytridiomycota, Entomophthoromycota, Entorrhizomycota, Glomeromycota, Kickxellomycota, Monoblepharomycota, Mortierellomycota, Mucoromycota, Rozellomycota together comprised the remaining 8% of the fungal community.

Agaricales (phylum Basidiomycota) was the most abundant order accounting for 24% of the total fungal community followed, by Sordariales (phylum Ascomycota) which made up 16% of the community (Fig. 1a). The most abundant ESV across sites was Lasiosphaeriaceae7 (phylum Ascomycota) appearing in four of the six marshes and comprising 4% of the total fungal community.

Fungal Functional Guilds

A total of 441 ESVs out of 759 ESVs were assigned to a functional guild by FUNGuild; the classifiable ESVs ranged

from 51% at Category 3B to 88% at Category 1A. At all six marshes, saprotroph was the most abundant broad functional guild comprising approximately 68% of the total classifiable ESVs, with undefined saprotrophs being the most abundant narrow functional guild comprising approximately 39% of the classifiable community (Fig. 1b).

Marsh Quality and Fungal Communities

Observed and Chao1 fungal richness significantly decreased with marsh quality (ORAM; $P = 0.019$; $P = 0.024$; Fig. 2) as well as with the quality of hydrology (Metric 3; $P = 0.017$; $P = 0.028$) and vegetation (Metric 6; $P = 0.020$; $P = 0.031$) and the degree of habitat development and substrate disturbance (Metric 4; $P = 0.048$; $P = 0.048$). Additionally, the richness of saprotrophs significantly declined with marsh quality ($P = 0.041$) and a negative trend was detected between marsh quality and the richness of soft rot fungi ($P = 0.056$). There was no relationship between marsh quality and Shannon diversity or the richness of AM, EM, pathogenic, and soft rot fungi ($P > 0.10$; Fig. 2).

Marsh quality significantly explained differences in fungal community composition ($F_{1,4} = 1.40$, $R^2 = 0.26$, $P = 0.0036$; Fig. 3) but did not explain differences in narrow fungal functional guild composition ($P > 0.10$; Fig. S3). Differences in fungal community composition were also significantly explained by habitat development and substrate disturbance (Metric 4; $F_{1,4} = 1.55$, $R^2 = 0.28$, $P = 0.0083$) and weakly explained by vegetation quality (Metric 6; $F_{1,4} = 1.41$, $R^2 = 0.26$, $P = 0.051$).

Soil Physicochemical Properties and ORAM Score

Wetland quality significantly explained differences in soil properties between marshes ($F_{1,4} = 2.96$, $R^2 = 0.43$, $P = 0.040$; Fig. 4). Differences in soil properties were also significantly explained by habitat development and substrate disturbance (Metric 4; $F_{1,4} = 4.53$, $R^2 = 0.53$, $P = 0.006$) and weakly explained by wetland hydrology (Metric 3; $F_{1,4} = 2.43$, $R^2 = 0.38$, $P = 0.083$) and vegetation (Metric 6; $F_{1,4} = 3.06$, $R^2 = 0.43$, $P = 0.051$). Among specific soil properties, four soil properties were particularly related to wetland quality: soil pH ($P = 0.042$) and BD ($P = 0.066$) exhibited negative trends with wetland quality; and soil moisture ($P = 0.070$) and SOM ($P = 0.089$) exhibited positive trends with wetland quality.

Soil Physicochemical Properties and Fungal Communities

Soil physicochemical properties did not significantly affect fungal diversity or fungal richness ($P > 0.10$).

However, soil physicochemical properties significantly explained differences in fungal community composition ($F_{10, 3} = 1.29$, $P = 0.009$; Fig. 5) and narrow fungal

functional guild composition ($F_{10,3} = 1.86$, $P = 0.044$; Fig. S4). Most notably, %C, %N, SOM, BD and soil moisture differentiated fungal community composition in

Fig. 1 (a) Relative abundance of the top 24 fungal orders by site. (b) Relative abundance of narrow functional guilds for each marsh. In (a) the “unidentified” group represents ESVs only assigned to a fungal phylum or class. The “other” group represents the bottom 1% of fungal

orders across the whole fungal community. In (b) the “unassigned” group represents ESVs assigned to at least the family level that were not able to be assigned to any functional guild

Fig. 2 (a) Shannon diversity (H'), (b) observed richness, and (c) Chao1 estimated richness of the fungal communities in relation to the ORAM score of each site. Wetland quality was not related to Shannon diversity

($P = 0.18$), but significantly declined with observed richness ($P = 0.017$), and Chao1 estimated richness ($P = 0.023$)

high quality marshes from marshes of lower quality. Within high quality marshes, C:N, $\text{NO}_3\text{-N}$, and pH drove differences in community composition.

Vegetation Effects on Fungal Community Composition

Vegetation significantly explained differences in fungal community composition ($F_{10, 3} = 1.22$, $P = 0.033$; Fig. 6) accounting for 80% of the variation. Most notably, plants more characteristic of disturbed habitats (e.g., *Polygonum amphibium*, *Phalaris arundinaceae*, and *Leersia oryzoides*) were tightly associated with fungal communities in Category 1 and Category 2 marshes, whereas plants more characteristic of pristine habitats (e.g., *Dulichium arundinaceum* and *Cephalanthus occidentalis*) were strongly associated with the fungal communities of Category 3 marshes (Fig. 6). Narrow functional guild composition, however, was unaffected by vegetation composition ($P > 0.10$; Fig. S5). FQAI score weakly explained differences in fungal community composition between marshes ($F_{1,4} = 1.36$, $R^2 = 0.25$, $P = 0.051$), but did not explain differences in fungal functional guild composition ($P > 0.10$).

Discussion

Wetland assessments use easily evaluated properties to score the condition and by proxy, the functional capacity of wetlands (Miller and Gunsalus 1999; Mack 2001a; Berglund and McElDowney 2008; Hruby 2014). Current assessment procedures do not consider soil microorganisms despite their importance in regulating and carrying out valuable wetland functions (Gutknecht et al. 2006; Faulwetter et al. 2009). As an important first step in determining the need and feasibility of adjusting current wetland assessment frameworks to consider microbial communities, we assessed the relationship between the ORAM, and fungal community composition and diversity. Our data suggests that current assessment methods are capable of distinguishing fungal communities of high quality marshes from low quality marshes; however, current assessments of wetland quality do not distinguish fungal functional guild composition. We further provide evidence that soil physicochemical properties are important determinants of marsh quality and have a role in structuring fungal communities and functional guild composition in marshes, alongside vegetation.

Fig. 3 NMDS (stress = 0.093) of the top 90% ESVs of total fungal community identifiable at least to family. Marsh quality significantly explained differences in fungal community composition between marshes ($F_{1,4} = 1.40$, $R^2 = 0.26$, $P = 0.0036$). Individual points represent strata and are shaped by site and colored by ORAM category.

Arrows represent ESVs that are associated ($P < 0.10$) with two or more marshes belonging to the same quality category according to indicator analysis. Dashed lines represent standard deviation ellipses for strata grouped by ORAM category

Fungal communities in all six marshes were dominated by saprotrophs (Fig. 1b), highlighting the importance of fungi as regulators of SOM accumulation and C storage in marshes (Thormann 2006). Across quality categories, white rot fungi were the most abundant specific guild of saprotrophic fungi recovered while soft rot fungi were also highly represented (Fig. 1b). The high abundance of white and soft rot fungi indicates an abundance of recalcitrant C sources such as lignin, cellulose, and hemicellulose which they degrade (Hibbett and Donoghue 2001). Brown rot fungi were not specifically recovered, although they may exist as a part of the undefined saprotroph category but this may reflect the lack of conifers with which brown rot fungi are tightly associated (Hibbett and Donoghue 2001).

In contrast to saprotrophs, our study recovered a relatively low abundance of mycorrhizal ESVs (Fig. 1b). This lack of mycorrhizal fungi may be a product of the time of year of the study which took place during the summer when the vegetation present at each site was already mature. Levels of AM colonization in wetlands are seasonal, most likely tied to plant

phenology, with colonization levels being the greatest during the spring and the lowest during late summer (Bohrer et al. 2004). Had we sampled in spring or early summer during periods of new vegetative growth, we may have recovered more mycorrhizal ESVs and fewer saprotroph ESVs. Also, the lack of EM fungi in this study could be attributed to the dominance of emergent herbaceous plants at each marsh (Table 1). EM fungi more commonly associate with woody plant species, and thus had this study been conducted in wetlands dominated by trees or shrubs, such as members of the Salicaceae, we could expect to observe more EM fungi (Smith and Read 2008; Erlandson et al. 2018). Ref. "Bohrer et al. 2004" is cited in the body but its bibliographic information is missing. Kindly provide its bibliographic information in the list.Bohrer, KE, Friese, CF, & Amon, JP (2004) Seasonal dynamics of arbuscular mycorrhizal fungi in differing wetland habitats. Mycorrhiza, 14(5): 329-337.

Our central hypothesis that the fungal communities would differ based on quality and be more diverse in higher quality marshes was only partially supported.

Fig. 4 PCA of the measured soil properties at each marsh. PC1 correlates with %C, %N, soil moisture, BD, and SOM. PC2 correlates with CN, $\text{NO}_3\text{-N}$, pH, and $\text{NH}_4\text{-N}$. Wetland quality significantly explains differences in soil properties between marshes ($F_{1,4} = 2.96$, $R^2 = 0.43$,

$P = 0.040$). Points represent strata and are shaped by site and colored by ORAM category. Dashed lines represent standard deviation ellipses for strata grouped by ORAM category. Length of arrows indicates strength of association

While fungal diversity and functional guild composition were not impacted by marsh quality (Figs. 2a and S3), fungal richness significantly declined with quality and fungal composition was significantly explained by marsh quality (Figs. 2b,c and 3). The ability of the ORAM to describe differences in fungal community composition is likely tied to the impacts of anthropogenic disturbances on soil physicochemical properties. For instance, wetland soil bacterial communities were observed to differ based on land use type (i.e. farmed, restored, undisturbed reference, etc.) as a product of differences in soil chemistry, particularly soil pH and N (Hartman et al. 2008). In our study, there were particularly strong relationships between fungal community composition, substrate disturbance (Metric 4), and soil physicochemical properties. Soil properties also significantly distinguished the fungal functional guild communities (Fig. S4), which were not separable by ORAM score (Fig. S3).

Differences in soil physicochemical properties between the marshes in our study were significantly explained by ORAM score (Fig. 4); however in swamps, the ORAM

was not able to describe differences in soil physicochemical properties (Rokosch et al. 2009), indicating additional research is required to determine the ability of wetland assessments to describe the soil environment across wetland types. One possible way to improve the relationship between wetland assessments and the soil environment is to include easily measured soil physicochemical properties that respond consistently across multiple wetland types to disturbance gradients, and also serve as important regulators of microbial communities. For instance, SOM, BD, and soil moisture exhibited trends with wetland quality in marshes ($P = 0.089$, $P = 0.066$, and $P = 0.070$) and in swamps (Rokosch et al. 2009), and were also strong drivers of fungal community composition and functional guild composition (Figs. 5 and S4). The potential utility of these soil measures in wetland assessments is not only highlighted by their importance in determining the structure of soil fungal communities, but also because similar trends with wetland quality were observed in different wetland types. This suggests that SOM, BD, and soil moisture, three easily measured soil properties, would

Fig. 5 CCA of fungal communities using soil physicochemical variables. Soil physicochemical properties significantly explain differences in fungal community composition ($F_{10, 3} = 1.29$, $P = 0.009$) accounting for 81% of the variation. CCA1 explains 11% ($P = 0.007$) of the variation and

CCA2 explains 11% ($P = 0.10$). Points represent strata and are shaped by site and colored by ORAM category. Dashed lines represent standard deviation ellipses for strata grouped by ORAM category. Length of arrow represents correlation strength of variable

serve as useful quantitative measures to incorporate into wetland quality assessments.

Another soil physicochemical property with the potential to serve as a useful measure in wetland assessments is soil pH. Soil pH had a significant negative relationship with ORAM score ($P = 0.042$), and was important in determining fungal community structure. This fits in line with the growing number of studies reporting soil pH as an important determinant in both the structure and richness of fungal communities (Tedersoo et al. 2014; Hiiesalu et al. 2017; Erlandson et al. 2018). It should be noted, however, that soil pH was more important in distinguishing communities within a quality category rather than between quality categories, particularly the two Category 3 marshes (Fig. 5) and that soil pH was not impacted by swamp quality in Ohio (Rokosch et al. 2009). This suggests that further research may be required to determine if soil pH is a useful quantitative measure to include in wetland assessments for multiple wetland types or for only freshwater depressional marshes, such as those investigated in this study.

Vegetation significantly explained differences in fungal community composition (Fig. 6) but was not significant for fungal functional guild composition (Fig. S5). The weak explanatory power of vegetation for fungal functional guild is likely due to the dominance of saprotrophs which are less dependent upon living vegetation, and a lack of mycorrhizal ESVs which tend to be more tightly associated with their plant hosts (Nguyen et al. 2016b; Fig. 1b). Additionally, we did determine that vegetation quality, measured both by Metric 6 and FQAI score, explained differences in fungal community composition. Specifically, we observed that plant species with higher C of C scores are tightly associated with the fungal communities of high quality marshes. While Metric 6 is capable of explaining differences in fungal community composition, the metric could be made more quantitative through the inclusion of FQAI scores. Although this would require assessors to have plant identification expertise and may increase the requisite experience needed to complete the assessment, it would reduce scorer

Fig. 6 CCA of fungal communities using vegetation. Vegetation significantly explain differences in fungal community composition ($F_{10,3} = 1.22$, $P = 0.033$) accounting for 80% of the variation. CCA1 accounts for 11% ($P = 0.029$) of the variance and CCA2 accounts for 11% ($P = 0.034$) of the variance in fungal communities. Points represent strata and are shaped by site and colored by ORAM category. Arrow color

represents FQAI Coefficient of Conservatism ranging from 0 to 2 (red), 3–5 (black), and 6–8 (purple). Plant species which could not be assigned an FQAI score (e.g. those classified only to genus) are depicted by dashed arrows. Arrows length represents strength of relationship. Dashed lines represent standard error ellipses for strata grouped by ORAM category

subjectivity and strengthen the ability of assessments to capture wetland function.

Due to the pivotal role of soil fungi in the regulation of key wetland functions (Thormann 2006; Neori and Agami 2017), we suggest that additional research be conducted to examine the ability of the ORAM and other wetland assessment methods to capture differences in these valuable wetland components. Specifically, we propose additional research be done to identify soil physicochemical properties that respond consistently, across wetland types, to disturbance gradients and are important drivers of microbial communities. Other studies have similarly observed a strong link between soil physicochemical properties and fungal community composition (Erlandson et al. 2016; Glassman et al. 2017; Hiiesalu et al. 2017), highlighting the potential utility of these properties as additional quantitative measures for wetland quality. The identification and inclusion of quantitative measures of soil into assessments will serve only to bolster the ability of wetland

quality assessments to serve as proxies for wetland function (Miller and Gunsalus 1999; Mack 2001a; Berglund and McElDowney 2008; Hruby 2014).

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