Microbial stowaways – waterbirds as dispersal vectors of aquatic pro- and microeukaryotic communities

Running title: Microbial dispersal by waterbirds

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Acknowledgements

Authors thank the colleagues of Lake Neusiedl Biological Station, Thomas Zechmeister, Richard Haider and Rudolf Schalli, for their help in field work and providing us access to their labs; colleagues of Fertő-Hanság and Neusiedler See - Seewinkel National Parks for providing and helping us access the sites as part of the Vogelwarte - Madárvárta 2 project; Christian Preiler for further practical help; and Mia Bengtsson for her help with selecting the primers used in the study. The study was supported by the Interreg V-A Austria-Hungary programme of the European Regional Development Fund (Vogelwarte Madárvárta 2). BS acknowledges further support by NKFIH-132095. ZH and CFV were supported by GINOP 2.3.2.-15-2016-00057 and the AQUACOSM-plus project of the European Union's Horizon 2020 research and innovation programme under grant agreement No. 871081. ZH acknowledges support from the Janos Bolyai Research Scholarship of the Hungarian Academy of Sciences. AS was supported by the Wenner-Gren Foundations. BS, AS, CFV and ZH were furthermore supported by the NKFIH-471-3/2021 project.

Abstract

Aim: Waterbirds are important dispersal vectors of multicellular organisms such as macrophytes, aquatic macroinvertebrates, and zooplankton. However, no study to date has focused on their potential role in dispersing aquatic microbial communities. Here, we carried out the first explicit study on passive transport (endozoochory) of prokaryotes and unicellular microeukaryotes by waterbirds based on a metagenomic approach. By directly comparing the dispersed set of organisms to the source pool of a natural metacommunity, we aimed for a realistic estimate of the overall importance of waterbird zoochory for natural microbial communities.

Location: Seewinkel region of Austria and Hungary.

Taxon: Prokaryotes and unicellular microeukaryotes.

Methods: In 2017 and 2018, water samples from natural aquatic habitats along with fresh droppings of the dominant greylag goose (*Anser anser*) and four other waterbird species were collected in a well-delineated habitat network of temporary saline ponds (soda pans). Their prokaryotic and microeukaryotic communities were identified via 16S and 18S rRNA gene amplicon sequencing and compared across years and waterbird species.

Results: We found that up to 40% of the dominant aquatic microbial OTUs were transported by *A. anser*. OTU richness in *A. anser* droppings was lower, but compositional variation was higher compared to the aquatic communities, probably resulting from stochastic pick-up of microbes from multiple aquatic habitats. We furthermore found that prokaryote species composition of bird droppings followed the interannual turnover in the aquatic communities. Finally, we found species-specific differences among different waterbird species. Among them, the planktivore filter-feeder northern shovelers (*Anas clypeata*) collected and dispersed a more species-rich subset of microeukaryotes than shorebirds or geese.

Main conclusions: Overall, our study provides the first quantitative empirical evidence of endozoochory in natural microorganism communities. These results imply that waterbirds may be crucial in maintaining ecological connectivity between discrete aquatic habitats at the level of microbial communities.

Keywords: aquatic microorganisms, bacteria, connectivity, dispersal, endozoochory, phytoplankton, protists, waterbirds

Introduction

Dispersal is a key process connecting habitats, thereby sustaining gene flow (Clobert et al., 2012), biodiversity (Leibold et al., 2004), and ecosystem functions (Bannar-Martin et al., 2018; Zobel et al., 2006). For a long time, prokaryotes, together with unicellular and small multicellular eukaryotes have been considered to have a cosmopolitan distribution and their communities were assumed to be driven only by local environmental and biotic factors (Baas-Becking, 1934; Beijerinck, 1913). However, recent studies (e.g. Cho & Tiedje, 2000; Martiny et al., 2006; Telford et al., 2006; Zinger et al., 2014) benefiting from the rapid development of community sequencing methods led to a paradigm shift by providing evidence for biogeographical patterns and increased recognition of the importance of spatial processes in microorganisms (Langenheder & Lindström, 2019; Mony et al., 2020; Ptacnik et al., 2010; van der Gast, 2015; Vyverman et al., 2007). This has finally placed microbes in the same metacommunity framework that has been already well-established for macroorganisms (Leibold & Chase, 2018).

Hence, the importance of passive dispersal for microorganisms is now acknowledged, which can occur by wind (Genitsaris et al., 2011; Sharma et al., 2007), water currents (Luef et al., 2007), animals (Figuerola & Green, 2002a; Green et al., 2008; Valls et al., 2017), and human activities (Reise et al., 1999; Ruiz et al., 2000). But despite the increasing interest in microbial dispersal and the availability of modern molecular techniques, zoochory is still largely neglected in this respect. Although there is evidence for waterbirds being effective short- and long-distance dispersal agents of macrophytes, macroinvertebrates, zooplankton and vertebrates (Brochet, Gauthier-Clerc, et al., 2010; Figuerola & Green, 2002b; Figuerola et al., 2003; Lovas-Kiss et al., 2019, 2020; Reynolds & Cumming, 2016; Silva et al., 2019; Viana et al., 2013a, 2013b), waterbird-mediated dispersal of unicellular microorganisms (especially bacteria) is poorly understood. There is only sporadic evidence for the transport of single and/or pathogen species via ecto- (Garmyn et al., 2012, Lewis et al., 2014) or endozoochory (Hartikainen et al., 2016; Jarma et al., 2021), while no explicit studies have so far targeted the dispersal potential of waterbirds for natural aquatic microbial communities with a direct comparison of natural communities to taxa dispersed by waterbirds.

Here, we present the first extensive study on the role of waterbirds as dispersal agents of aquatic pro- and eukaryotic unicellular microorganisms with the help of high-throughput DNA sequencing. Our study area was a landscape of saline temporary ponds, representing a well-delineated habitat network. The dominant waterbirds of the area (1,000-10,000 individuals depending on year and season, Boros, E., personal communication) are greylag geese (*Anser anser*), which are known to be regular large-bodied visitors of aquatic habitats, moving in flocks of up to 750 individuals (McKay et al., 2006). It has been suggested that they may contribute significantly to the transport of passively dispersing organisms across aquatic habitats (García-Álvarez et al., 2015; Green et al., 2002). However, we lack empirical data to assess their actual role as dispersal agents for microbial organisms.

In line with this, our main objective was to investigate the potential of zoochory by waterbirds for dispersing microorganisms among local habitats in a metacommunity. To this end, we compared the species pool of natural aquatic habitats with the metagenome found in bird droppings collected from the same habitat network. Specifically, our first aim was to reveal what proportion of the OTUs occurring in the aquatic habitats can be found in droppings of the dominant waterbird of the region, *A. anser*. Second, we tested whether community composition of the transported aquatic microbes is stable over time, i.e. reflects temporal changes in the natural aquatic communities. And finally, we assessed the dispersal potential of *A. anser* relative to four other waterbird species with different feeding habits and habitat use.

Material & methods

Sampling and sample processing

The study area (~ 200 km², Horváth et al., 2016) in the Seewinkel region of Austria and Hungary is characterized by a dense cluster of temporary saline ponds (soda pans). These habitats form a habitat network relatively isolated from freshwater habitats or other soda pans in the central and eastern regions of Hungary (Tóth et al., 2014). The clumped nature of this pondscape, with shallow (≤ 1 m) and hypertrophic aquatic habitats (Boros et al., 2017) offers excellent feeding grounds for invertivorous waterbirds (Horváth et al., 2013) and breeding sites for several other species, including ~300 pairs of greylag geese (*Anser anser*, Steiner & Parz-Gollner, 2003). The region is legally protected as part of two national parks (Neusiedlersee-Seewinkel in Austria, and Fertő-Hanság in Hungary), designated as Important Bird Area (BirdLife International, 2021a, 2021b) and part of a UNESCO World Heritage site (Fertő / Neusiedlersee Cultural Landscape).

We collected samples from 25 soda pans in two consecutive years (3-6 April 2017 and 2-4 April 2018; Figure 1), representing all habitats that held water in both years (hereafter aquatic community samples). The sampled habitats are situated within 17 km (largest distance between two habitats), thereby representing a region where waterbirds can regularly move around on a daily basis (Bell, 1988, Boos et al., 2019; Link et al., 2011; Nilsson & Persson, 1992). From each soda pan, a total of 20 L of water was collected from several points using a one-litre plastic beaker (thus collecting a pooled sample from the largest possible area) and sieved through a 100- μ m mesh plankton net to remove large zooplankton and filamentous algae which would hinder the detection of unicellular organisms during amplicon sequencing. For further processing, 1 L of this composite sieved water was immediately delivered to the laboratory in a glass bottle in a cool box. For molecular analysis, 1-50 mL of water (depending on turbidity, as Secchi depth ranged from 0.3 to 44 cm) was filtered through a nitrocellulose membrane filter ($\varnothing$ 47 mm) with a pore size of 0.22 μ m. Thereafter, filters were stored at -20 °C until DNA extraction.

Simultaneously, we collected fresh waterbird droppings at all sites that hosted a monospecific flock of waterbirds. We approached the birds roosting on dry mudflats or grasslands on the shores or right next to the soda pans and once they took off, fresh droppings were carefully collected in cryogenic vials, scraping off any soil or plant material and immediately frozen on dry ice. This way we avoided the potential contamination due to long-term exposure to e.g. wind-dispersed propagules. Bird droppings were stored at -20 °C until further processing. In 2017, a total of 64 droppings from *Anser anser*, *Calidris pugnax*, *Recurvirostra avosetta*, *Anas clypeata*, while in 2018, altogether 70 droppings from *A. anser* and *A. albifrons* were collected with this method (Figure 1). The feeding mode of the waterbird species was summarized in Table S1.

DNA isolation, amplification and sequencing

DNA extraction from the filters and waterbird droppings was performed using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Extracted DNA samples were stored at -20 °C before shipping for amplification and sequencing at an external company (LGC Genomics, Berlin).

Prokaryotic 16S rRNA and microeukaryotic 18S rRNA gene amplification was carried out using the following primer pairs: EMBf 515F (GTGYCAGCMGCCGCGGTAA, Parada et al., 2016) – EMBr 806R (GGACTACNVGGGTWTCTAAT, Apprill et al., 2015) for the V4

region of the prokaryotic 16S rRNA gene and UnivF-1183mod (AATTTGACTCAACRCGGG) – UnivR-1443mod (GRGCATCACAGACCTG) (Ray et al., 2016) for the V7 region of the eukaryotic 18S rRNA gene, elongated with sequencing barcodes and adapters. Amplification of the target taxonomic marker gene regions and sequencing were carried out by LGC Genomics (Berlin, Germany). The PCRs included about 1-10 ng of DNA extract (total volume 1 µl), 15 pmol of each forward primer and reverse primer in 20 µL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline GmbH, Luckenwalde, Germany) and 2 µl of BioStabII PCR Enhancer (Sigma-Aldrich Co.). Amplification was carried out for 30 cycles using the following parameters: 1 min 96°C pre-denaturation; 96 °C denaturation for 15 s, 55 °C annealing for 30 s, 70 °C extension for 90 s, hold at 8 °C. The amplicon pools were purified with one volume Agencourt AMPure XP beads (Beckman Coulter, Inc., IN, USA) to remove primer dimer and other small mispriming products, followed by an additional purification on MiniElute columns (QIAGEN GmbH, Hilden, Germany). About 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN Technologies, Inc., CA, USA). Illumina libraries (Illumina, Inc., CA, USA) were pooled and size selected by preparative gel electrophoresis. Sequencing was performed on an Illumina MiSeq using 300 bp paired-end format with a V3 Reagent Cartridge on the Illumina MiSeq platform, aiming for 50,000 raw sequence read pairs per sample.

Amplicon data analysis

Sequence processing, taxonomic assignments and OTU picking (at 99% similarity level) were carried out with mothur v1.43.0 (Schloss et al., 2009) using the MiSeq SOP http://www.mothur.org/wiki/MiSeq_SOP (Kozich et al., 2013, downloaded at 12th November 2020). Additional quality filtering steps were also applied to eliminate possible sequence artefacts, such as the adjustment of the deltaq parameter to 10 in the 'make.contigs' command, primer removal from both ends of the sequences and the exclusion of singleton reads according to Kunin et al. (2010). Read alignment and taxonomic assignment were carried out using the ARB-SILVA SSU Ref NR 138 reference database with a minimum bootstrap confidence score of 80 (Quast et al., 2013). Reads assigned to non-primer-specific taxonomic groups ('Chloroplast', 'Mitochondria' and 'unknown') were subsequently removed from the dataset. For prokaryotic OTUs, the TaxAss software (Rohwer et al., 2018) with default parameters was used for taxonomic assignment with the FreshTrain (15 June 2020 release) and ARB-SILVA SSU Ref NR 138 databases, while taxonomic assignment of the 18S rRNA gene OTUs was performed using the PR2 v4.12.0 reference database (Guillou et al., 2013).

OTUs assigned to taxa Streptophyta, Metazoa, Ascomycota and Basidiomycota were omitted from the eukaryotic OTU set. Subsequently, both 16S and 18S datasets were rarefied to the read number of the sample having the lowest sequence count (8620 read per sample for the 16S set and 2432 read per sample for the 18S set).

Statistical analysis

We used the rarefied 16S (hereinafter referred to as prokaryotes) and 18S (microeukaryotes) community datasets separately in all our analyses. To exclude those organisms that might be exclusive for the gut microbiota of waterbirds, we only used OTUs that were present at least in one aquatic community sample with $\geq 1\%$ relative abundance ("aquatic subset"). In the main part of the manuscript, we used only this aquatic subset. This subsetting was carried out separately for prokaryotes and microeukaryotes. The resulting aquatic subset of waterbird droppings contained 2.1% ($\pm 4.3\%$) of the original prokaryotic and 5.4% ($\pm 8.0\%$) of the microeukaryotic OTU abundances in these samples, whereas the subset of aquatic communities

contained 72.0% ($\pm 12.3\%$) of the original prokaryotic and 85.5% ($\pm 8.1\%$) of the microeukaryotic OTU abundances detected in the unselected datasets.

For a quantitative assessment of waterbird dispersal potential in the pondscape, we only used *A. anser* samples, being the only species from which we could collect samples in both years. To exclude a potential bias arising from the different sampling effort in soda pans vs bird droppings, a random re-sampling was performed based on the lowest sample size for both prokaryotes (n=21) and microeukaryotes (n=9) per sample group, resulting in a total of 84 pro- and 36 microeukaryote samples used in these comparisons.

To test for differences in local (α -diversity) and regional OTU richness (γ -diversity), together with the compositional change among samples (Whittaker's β -diversity: $\beta = \gamma/\alpha$) in the four sample groups (*A. anser* droppings and aquatic communities in 2017 and 2018), we used the permutation test of the 'mobr' package (McGlenn et al., 2021). Here, effect size ($\bar{D}$) shows the average absolute difference between the four groups of samples and a p value (based on 200 permutations) is calculated for each diversity level. To estimate the possible significant effect of sampling year and sample type, non-parametric Scheirer-Ray-Hare test with an interaction term was run using the 'rcompanion' package (Mangiafico, 2021), followed by Dunn's post-hoc test for pairwise comparisons with 'FSA' package (Ogle et al., 2020) where p -values were adjusted with the Benjamini-Hochberg method.

We created stacked barplots to illustrate the quantitative differences of the higher-order prokaryotic and eukaryotic taxa among the sample groups. Prior to this, third level taxon names were assigned to the OTUs detected in the samples, thereafter OTU abundances belonging to the same taxon were summed up and expressed as relative abundance in each sample group.

Principal coordinate analysis (PCoA) was performed to illustrate the separation of samples according to sample type (*A. anser* droppings vs. aquatic communities) and sampling year (2017 vs. 2018) with the 'vegan' package (Oksanen et al., 2020). To test for significant differences in the same dataset, two-way PERMANOVA with an interaction term (based on 2000 permutations) was carried out, followed by a pairwise comparison of the four sample groups (based on 2000 permutations) with the 'pairwiseAdonis' package (Arbizu, 2017). We ran additional SIMPER analyses to determine which OTUs are the most responsible for the dissimilarities among sample types and sampling years. In order to provide comparable results, PCoA, PERMANOVA, pairwise comparison and SIMPER were all run based on Bray-Curtis dissimilarity calculated from OTU abundance data.

We repeated our analyses based on the unselected datasets (i.e. without selecting for aquatic taxa) and presented those results in the Supplementary material. To standardize sample sizes, re-sampling was carried out also for the unselected dataset of prokaryotes (n=25) and microeukaryotes (n=10) based on the lowest sample size resulting in a total of 100 pro- and 40 microeukaryote samples.

To compare prokaryotic and microeukaryotic OTU richness in each sample group (droppings of different waterbird species and aquatic community samples from both years), we applied sample-size-based rarefaction and extrapolation approach (Chao et al., 2014) using 'iNEXT' package (Hsieh et al., 2020). The 95% confidence intervals were constructed by bootstrapping (based on 50 bootstrap replications).

To reveal if different waterbird species transport different microbial communities, and whether they differ among years, we performed separate PCoA analyses including all waterbird species from which samples were collected in at least one year (sample numbers after re-sampling the amplicon data are presented in Table S1).

All analyses focusing on community composition were furthermore repeated for incidence data based on Sørensen dissimilarity.

Statistical analyses were carried out using R statistical software (R Core Team, 2020).

Results

We found a consistent difference between the number of prokaryotic and microeukaryotic OTUs in the two main sample types (*Anser anser* droppings and aquatic communities) after rarefaction. Local (α) and regional (γ) OTU richness were significantly higher in the aquatic samples (in both years), while compositional variation (β -diversity) was higher among the *A. anser* samples, especially in prokaryotes. There was no remarkable difference between the two sampling years (Figure 2, Table S2). When repeating the analyses for the unselected prokaryotic and microeukaryotic community datasets (thereby also including the gut microbiome and possible parasites of waterbirds), patterns of α - and γ -diversity were similar to the results based on the aquatic subset (i.e. less OTUs in *A. anser* samples independent of sampling year), however, β -diversity was low in case of both sample types in both years (Figure S1, Table S3).

In line with this, the majority of OTUs were found only in the aquatic habitats, with most OTUs shared between years (Figure 3, Venn diagrams). Even so, we detected a considerable proportion of OTUs present in aquatic habitats also in the *A. anser* samples: 40% of the prokaryotic and 21% of the microeukaryotic OTUs were shared among both types of samples, with 29-25% (prokaryotes) and 12-11% (microeukaryotes) of OTUs being shared among birds and aquatic communities within the same year (Figure 3). Among prokaryotes, 13% of the OTUs were found in all four sample groups (both sample types in both years; Figure 3). Compared to this, the share of microeukaryotic OTUs present in all four sample groups was low (3%) (Figure 3). In the unselected community datasets, trends and differences were similar to those observed in our aquatic data subsets, except for the high number of OTUs unique to *A. anser* samples (31% for prokaryotes and 24% for microeukaryotes) (Figure S2).

At the level of major taxonomic units, all four sample groups were dominated by the same phylogenetic groups, both in prokaryotes and microeukaryotes (Figure 3). The most abundant prokaryotic taxa were Bacteroidia, Gammaproteobacteria, Alphaproteobacteria, Actinobacteria and Verrucomicrobiae, making up 77-95% of the OTU abundances found in the aquatic communities and 61-93% in the *A. anser* samples. In microeukaryotes, Ochrophyta, Chlorophyta, and Fungi were similarly abundant, altogether representing 38-44% of the OTU abundances in the aquatic samples and 34-84% in the *A. anser* droppings. There were also several groups that were abundant in the aquatic communities (both years) and in *A. anser* samples from at least one year, e.g. Haptophyta, Ciliophora and Cercozoa.

In prokaryotes, there was a marked interannual difference in the relative abundance of two taxonomic groups, Nitrospirae and Oxyphotobacteria. They were present in both the aquatic and *A. anser* samples with high proportions (9-30% together) in 2017 and either missing completely (Oxyphotobacteria) or represented only with very low abundances ($\leq 0.6\%$) in 2018 (Figure 3).

Apart from these exceptions, all OTUs (both prokaryotic and microeukaryotic) that were mostly responsible for the differences between the sampling years and the sample types could be assigned to the higher-order taxa dominant in all or at least in one of the sample groups. Additionally, there was a considerable overlap between the OTUs responsible for the differences in both year and sample type (Table S4).

Both prokaryotic and microeukaryotic samples showed a clear separation according to sample type (aquatic communities against *A. anser* droppings) and were less separated by year (Figure 4). This was in line with the stronger effect (indicated by higher R^2 values) of sample type compared to year (though both were significant) based on PERMANOVA tests (Table 1). Pairwise comparisons of the four sample groups showed similar significant differences with overall higher R^2 values for pairs of different sample types in prokaryotes, while in microeukaryotes the difference was significant only for the pairs of different sample types (*A. anser* or aquatic communities; Table 1). A following SIMPER analysis (Table S4) showed that the OTUs most responsible for these differences belonged to the dominant higher order taxa (Figure 3). The general patterns in the PCoA, PERMANOVA and pairwise comparisons repeated for the incidence and unselected data subsets were highly similar in both prokaryotes and microeukaryotes (Table S5–S6, Figure S3–S5).

We finally compared the richness (Figure 5, Figure S6) and composition (Figure S7–S10) of microbes dispersed by the five waterbird species. Similar to the results based on rarefaction for *A. anser* droppings (Figure 2–3), only a fraction of the total species pool was recaptured in each waterbird species, but the actual proportion changed with species. Shorebirds (*R. avosetta*, *C. pugnax*) transported a similar fraction of microeukaryotic OTUs as geese (*A. anser*), both as individuals (mean richness) and collectively (the latter evidenced by regional extrapolated richness). Compared to them, *A. clypeata* proved to be much more efficient dispersal agents for microeukaryotes, dispersing almost twice as many OTUs as a same-sized group of any of the other species (Figure 5). Furthermore, we essentially found a similar number of OTUs per *A. clypeata* dropping as in a random aquatic sample (Figure 5). This also resulted in a clear separation of *A. clypeata* from the rest of the waterbirds on a PCoA ordination both with abundance- and incidence-based data (Figure S7–S8).

The comparison of waterbird species yielded somewhat different results for prokaryotes, where *A. clypeata* droppings no longer hosted significantly higher OTU richness than most of the other species (except for *A. albifrons*), and showed a large compositional overlap with communities dispersed by shorebirds (*R. avosetta*, *C. pugnax*). In the two goose species, *A. anser* and *A. albifrons*, due to low read numbers for microeukaryotes we could only compare the composition of prokaryotes in their droppings, where the difference we found was negligible. While the overall composition of the dispersed set of prokaryotes was very similar among individual birds (Figure S7–S8), *A. albifrons* collectively transported a significantly lower diversity of OTUs: approximately only two thirds of those found in *A. anser* droppings (Figure 5).

In the unselected datasets, the prokaryotic and microeukaryotic communities transported by different bird species were much more distinct (Figure S9–S10), but even there, *A. anser* and *A. albifrons* samples showed high similarity.

Discussion

The main novelty of our study is twofold. First, it represents the first comprehensive study on the role waterbirds play in the dispersal of microorganisms based on a metagenomic approach targeting communities of prokaryotes and unicellular microeukaryotes, where we showed that waterbirds disperse all major aquatic groups from bacteria through phytoplankton to protozoa. Second, we directly compared microorganisms dispersed by waterbirds to the source pool (natural aquatic communities), thereby being able to investigate the share and identity of aquatic microbes readily transported by waterbirds. In this confined set of aquatic habitats (i.e. metacommunity), we indeed found a considerable share of aquatic communities dispersed by

waterbirds (individuals of *A. anser* transported up to 40% of all aquatic OTUs detected in the study). Besides, the actual set of OTUs transported by the birds showed temporal changes in prokaryotes, reflecting the interannual turnover of aquatic communities, indicating that the dispersal potential of waterbirds depends on the actual aquatic communities. Finally, the communities transported by different waterbird species showed high similarities (regardless of their lifestyle), with a number of specific differences. The implications of our results showed minor sensitivity to the selection methods (unselected dataset or aquatic subset) or data type (abundance or incidence), and were largely consistent across prokaryotes and microeukaryotes. Altogether, our study provided the first explicit quantitative evidence clearly supporting that waterbirds are so far overlooked, yet potentially important dispersal agents of natural communities of aquatic microorganisms.

Prokaryotic and microeukaryotic communities of the aquatic subset were typical for soda lakes and pans of the region (Sinclair et al., 2015, Szabó et al., 2017, 2020). We found that more than one third of the prokaryotic and more than one fifth of the aquatic microeukaryotic OTUs were also present in the droppings of the dominant waterbirds species of the region, *A. anser*. Instead of dispersing a single or only a limited number of aquatic taxa, most of the major taxonomic groups of the aquatic communities were well-represented in the *A. anser* droppings. In waterbirds, gut retention time is short (Brochet, Guillemain, Gauthier-Clerc et al., 2010, Sánchez et al., 2012), which can contribute to a large share of undigested microorganisms. In extreme cases, even live plants (Silva et al., 2018), diatoms (Atkinson, 1971, 1980), aquatic invertebrates (Frisch et al., 2007; Green & Sánchez, 2006) and gelatinous fish eggs (Lovas-Kiss et al., 2020) can survive waterbird gut passage. Compared to them, the survival of microorganisms should be even higher, given their evolutionary adaptations to adverse conditions such as extreme values of pH, desiccation or UV radiation (Potts, 1999; Rainey et al., 2005; Schleper et al., 1995). Even though we did not test the viability of the detected microbes directly, these altogether make it highly likely that the OTUs we found included viable cells and hence indicate the possibility of successful dispersal events.

We found a significant interannual turnover of prokaryotic communities in the aquatic samples and in *A. anser* droppings. The shift in aquatic communities was also followed by the OTUs found in the droppings. This indicates that the set of dispersed prokaryotes reflects the natural microbial communities available in the local aquatic habitats at the given time. That is, our observations confirm the previous assumption that internal dispersal depends on the availability of aquatic (food) organisms (e.g. Brochet, Guillemain, Fritz, et al., 2010; Frisch et al., 2007), which can vary in time and is facilitated by the weak digestion efficiency mentioned above.

Even though *A. anser* do not feed directly from the water but rather consume seeds, stems and leaves of aquatic macrophytes and terrestrial plants (Middleton & van der Valk, 1987), they can pick up microbes while drinking and while feeding on aquatic macrophytes or even while preening their damp feathers after bathing. Our results showed that this feeding mode still makes them efficient dispersal agents for aquatic microbial metacommunities. At the same time, we showed a high heterogeneity of prokaryotic and microeukaryotic OTU composition across bird droppings, indicating stochastic pickup by the individual birds. Although the difference in local and regional richness between droppings and aquatic communities was still remarkable, the compositional variation among droppings was moderated when *A. anser* gut microbiota was also considered, leading us to the conclusion that the gut microorganism composition of *A. anser* is specific to the species. This is in line with the findings of Laviad-Shitrit et al. (2019) that waterbird species host unique gut bacterial communities.

We observed that not only *A. anser* but the other four bird species can also transport a considerable share of the natural microbial communities present in the ponds. While we found some differences between the waterbird species, these were not completely congruent with their feeding habits and habitat use. In spite of the terrestrial feeding habit of *A. anser*, the number of OTUs transported by them was largely comparable to those found in shorebirds, *C. pugnax* and *R. avosetta*, which prefer to feed in the shallow shoreline regions of ponds (Baccetti et al., 1998; Dietrich et al., 1997) and may directly consume biofilm communities as shown for multiple *Calidris* spp. (Kuwae et al., 2008, 2012). Even so, our results showed that they all disperse quite similar microeukaryotic communities across aquatic habitats.

The only species that showed marked differences from the rest of the waterbirds was *A. clypeata*. They not only transported different microeukaryotic communities but also captured a much larger fraction of the aquatic source pool, therefore they can be considered as the most effective dispersal agents. However, in terms of transporting prokaryotes, they were no longer so prominent. A reasonable explanation for our observations can be that *A. clypeata*, unlike the other waterbirds we studied, is a planktivore species sieving plankton from the open water (Matsubara et al., 1994). The low interlamellar distances in its specialized spoon-shaped bill enable an effective accumulation of aquatic microorganisms even smaller than 500 μm (Gurd, 2007; Kooloos et al., 1989). Thus, microeukaryotes and their propagules of this size can be easily captured and concentrated, whereas bacterioplankton with a smaller size fraction probably flows through their lamellae.

When considering the dispersed prokaryotes, we did not find remarkable differences among the five bird species, neither in OTU richness, nor in composition. *A. anser* and *A. albifrons* transported quite similar prokaryotic communities and their gut microbiome also seems to be largely the same, which is not surprising given that both have a predominantly terrestrial herbivorous feeding habit (Ely & Raveling, 2011; Middleton & van der Valk, 1987). Nevertheless, of the two, *A. anser* hosted a higher number of OTUs in their droppings, which implies that it might have a more important role in the endozoochory of prokaryotes.

Our results are based on a representative comparison of equal sample sizes across aquatic habitats and bird droppings. We proved that within small-scale pond and lake networks (10-20 km), waterbirds can be important dispersal agents of both prokaryotes and microeukaryotes, given that the spatial scale of such pondscapes coincides with the local daily movements of waterbirds, including *A. anser* (Bell, 1988; Boos et al., 2019; Link et al., 2011; Nilsson & Persson, 1992). As the study region might host up to hundreds of thousands of waterbirds (Dick et al., 1994), which themselves might defecate even up to 80 times per day (Oláh, 2003; Sterbetz, 1992), their overall contribution to biotic connectivity is expected to be immense, eventually being able to transport most members of the aquatic microbial metacommunity among the habitats.

The implications of this study are finally also important for larger spatial scales. According to their flight speed and gut retention times, waterbirds are able to transport their intestinal contents over thousands of kilometers during their migration (Viana et al., 2013a, 2013b, 2016), and therefore can be important dispersal agents of aquatic microorganisms not only on regional but even on continental scales. By dispersing microorganisms, they can have a significant role in forming biodiversity patterns and sustaining ecosystem functions where the importance of microbes is indisputable (Bell et al., 2005; Graham et al., 2016; Wohl et al., 2004).

Table 1. Results of PERMANOVA and pairwise comparison performed on the aquatic subset (abundance data, Bray-Curtis dissimilarity; permutations=2000) of prokaryotic and microeukaryotic communities detected in aquatic community (AC) and *Anser anser* dropping samples in 2017 and 2018.

Prokaryotes							Microeukaryotes							
Factors	Df	SS	MS	F	R ²	p		Df	SS	MS	F	R ²	p	
Sampling year	1	1.234	1.234	3.439	0.034	0.0005	***	1	0.599	0.599	1.391	0.037	0.0430	*
Sample type	1	5.035	5.035	14.026	0.139	0.0005	***	1	1.413	1.413	3.280	0.086	0.0005	***
Sampling year*sample type	1	1.106	1.107	3.082	0.031	0.0015	**	1	0.581	0.580	1.348	0.035	0.0505	.
Residuals	80	28.719	0.359		0.796			32	13.784	0.431		0.842		
Total	83	36.095			1.000			35	16.376			1.000		
Pairs	Df	SS	F	R ²	P _{adj}			Df	SS	F	R ²	P _{adj}		
2017 <i>A. anser</i> - 2017 AC	1	2.736	7.291	0.154	0.0030	**		1	1.176	2.879	0.152	0.0030	**	
2017 <i>A. anser</i> - 2018 <i>A. anser</i>	1	1.143	2.747	0.064	0.0030	**		1	0.646	1.463	0.084	0.1979	ns	
2017 <i>A. anser</i> - 2018 AC	1	3.513	10.325	0.205	0.0030	**		1	1.140	2.759	0.147	0.0030	**	
2017 AC - 2018 <i>A. anser</i>	1	2.756	7.298	0.154	0.0030	**		1	0.873	1.946	0.108	0.0060	**	
2017 AC - 2018 AC	1	1.198	3.968	0.090	0.0030	**		1	0.534	1.271	0.074	1.0000	ns	
2018 <i>A. anser</i> - 2018 AC	1	3.405	9.936	0.199	0.0030	**		1	0.818	1.805	0.101	0.0030	**

Figure 1. Map of the region and the sampling sites. The number of collected samples is indicated in brackets.

Figure 2. α -, β - and γ -diversity of the prokaryotic and microeukaryotic aquatic subsets in aquatic communities and *Anser anser* droppings in 2017 and 2018. $\bar{D}$ (effect size) indicates the average absolute difference between the four sample groups, p-value (permutations=200) indicates the significance of the overall group effect. Different letters indicate statistically significant differences at a significant level of $p_{adj} < 0.05$ based on Dunn's pairwise post-hoc test. Pairwise gamma diversity comparisons are presented in Figure 5.

Figure 3. Number of prokaryotic and microeukaryotic OTUs (above) shared among sample types (aquatic community and *Anser anser* dropping) and years, and the relative abundance of higher-order taxa (below) in the aquatic subsets.

Figure 4. PCoA biplot of aquatic community and *Anser anser* dropping samples collected in 2017 and 2018. The analysis is based on the aquatic subset (abundance data, Bray-Curtis dissimilarity) of prokaryotic and microeukaryotic communities.

Figure 5. OTU accumulation curves with extrapolated richness estimates and confidence intervals for the aquatic subsets of prokaryotes and microeukaryotes dispersed by the five waterbird species compared to the aquatic communities.

Data availability statement

Raw sequence reads were deposited in the NCBI SRA database and are accessible through the BioProject accession PRJNA748202.

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Biosketch

Beáta Szabó is a postdoc researcher at Institute of Aquatic Ecology, Centre for Ecological Research and interested in the processes underlying the formation of biodiversity and metacommunities of aquatic microorganisms with a special focus on small temporary ponds.

Author contributions

ZH, CFV and RP conceived the ideas; ZH, CFV, EB and DL collected the samples; ZM performed the lab work; AS and ZM analysed the molecular data; BS and ZH performed the statistical analyses; BS wrote the manuscript with significant contributions by ZH, AS and CFV; all authors commented on earlier versions of the manuscript.

Supporting information

Additional supporting information may be found in the online version of the article at the publisher's website.