Received: 10 November 2021 | Revised: 7 February 2022 | Accepted: 8 February 2022

DOI: 10.1002/ecm.1523

ARTICLE

ECOLOGICAL MONOGRAPHS
ECOLOGICAL SOCIETY OF AMERICA

From microbes to mammals: Pond biodiversity homogenization across different land-use types in an agricultural landscape

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Funding information
German Federal Ministry of Education and Research (BMBF) within the Collaborative Project "Bridging in

Abstract

Local biodiversity patterns are expected to strongly reflect variation in topography, land use, dispersal boundaries, nutrient supplies, contaminant spread, management practices, and other anthropogenic influences. Contrary to this expectation, studies focusing on specific taxa revealed a biodiversity homogenization effect in areas subjected to long-term intensive industrial agriculture. We investigated whether land use affects biodiversity levels and community composition (α- and β-diversity) in 67 kettle holes (KH) representing small aquatic islands embedded in the patchwork matrix of a largely agricultural landscape comprising grassland, forest, and arable fields. These KH, similar to millions of standing water bodies of glacial origin, spread across northern Europe, Asia, and North America, are physico-chemically diverse and differ in the degree of coupling with their surroundings. We assessed aquatic and sediment biodiversity patterns of eukaryotes, *Bacteria*, and *Archaea* in relation to environmental features of the KH, using deep-amplicon-sequencing of environmental DNA (eDNA). First, we asked whether deep sequencing of eDNA provides a representative picture of KH aquatic biodiversity across the *Bacteria*, *Archaea*, and eukaryotes. Second, we investigated if and to what extent KH biodiversity is influenced by the surrounding land use. We hypothesized that richness and community composition will greatly differ in KH from agricultural land use compared with KH in grasslands and forests. Our data show that deep eDNA amplicon sequencing is useful for in-depth assessments of cross-domain biodiversity comprising both micro- and macro-organisms, but has limitations with respect to single-taxa conservation studies. Using this broad method, we show that sediment eDNA, integrating several years to

D. Ionescu and M. Bizic contributed equally to the work.

Biodiversity Science — BIBS" (funding number 01LC1501); DFG Eigene Stelle project (BI 1987/2–1); DFG Eigene Stelle project (DI 98/3–1).

Handling Editor: Andrea E. Kirkwood

decades, depicts the history of agricultural land-use intensification. Aquatic biodiversity was best explained by seasonality, whereas land-use type explained little of the variation. We concluded that, counter to our hypothesis, land use intensification coupled with landscape wide nutrient enrichment (including atmospheric deposition), groundwater connectivity between KH and organismal (active and passive) dispersal in the tight network of ponds, resulted in a biodiversity homogenization in the KH water, leveling off today's detectable differences in KH biodiversity between land-use types. These findings have profound implications for measures and management strategies to combat current biodiversity loss in agricultural landscapes worldwide.

KEYWORDS
biodiversity homogenization, eDNA, intensive agriculture, kettle hole, land use

INTRODUCTION

The cultural landscape of central Europe was characterized by low-input farming until the 1950s and early 1960s, after which industrialized agriculture became dominant with greatly increased fertilizer and pesticide use (Bauerkämper, 2004; Sommer et al., 2008). Concomitantly, crop diversity decreased by more than 30% whereas the total crop coverage of land increased (Meyer et al., 2013). These changes in agricultural practice had negative consequences on biodiversity, resulting in declining plant (Altenfelder et al., 2014; Meyer et al., 2013), bird (Donald et al., 2006), invertebrate (Wilson et al., 1999), and amphibian (Berger et al., 2011; 2018) diversity. Furthermore, plant communities became homogenized (Baessler & Klotz, 2006; Macdonald & Johnson, 2000), as has commonly been observed after land-use intensification (Smart et al., 2006).

Ponds are intimately linked to their terrestrial surroundings, both the riparian zones immediately adjacent to the water bodies and the entire watershed due to their small size and topographic position in landscape depressions (Kayler et al., 2019; Søndergaard et al., 2005). As a result, pond biodiversity tends to be particularly affected by land use (Declerck et al., 2006), resulting, for instance, in increased organic matter and nutrient supply; pesticide spread by aerial spray, run-off and groundwater flow (Pérez-Lucas et al., 2019), leading to changes in plant (Altenfelder et al., 2014) and animal (Berger et al., 2011) communities. Comparative studies on the land use effect on aquatic microbial communities have mostly been conducted on running water (rivers and streams) (Chen et al., 2018; Fasching et al., 2020; Le et al., 2018) or lakes (Marmen et al., 2020), revealing cases in which local land use has a stronger influence on community composition than upstream land use (Le et al., 2018). Microbiome studies on ponds in agricultural landscapes (e.g., Chopyk et al., 2018, 2020) or aquaculture (e.g., Lastauskienė et al., 2021) have been mostly focused on individual ponds.

Kettle holes (KH) are small landscape depressions formed on the outwash plains in front of retreating glaciers at the end of the last ice age. Most fill with water, at least temporarily, which has resulted in parts of the post-glacial landscapes of northern Europe, northern North America, and northern Asia being sprinkled with these small water bodies (Downing et al., 2006). For example, more than 90,000 occur in northeastern Germany, with densities reaching up to 40 per km^2 (Kalettka & Rudat, 2006). KH can vary greatly in hydrogeomorphological and biological features, even when they are geographically close to one another (Attermeyer et al., 2017). For instance, among 42 KH within an area of 220 km^2, 10-fold to 20-fold variations in electric conductivity, total phosphorus and total nitrogen were observed (Onandia et al., 2021). The same set of KH were found to span across most of the hydroperiod categories (episodic, periodic, semipermanent, and permanent) and hydrogeomorphic characteristics (hydrogeomorphic type, shore width, shore slope and maximum depth of the pond basin) proposed by Kalettka and Rudat (2006). Biological activity in KH is high (Nitzsche et al., 2017) and they also play a critical role as local biodiversity hotspots (Joniak et al., 2007; Lischeid & Kalettka, 2012; Novikmec et al., 2016; Pätzig et al., 2012; Platen et al., 2016; Scheffer et al., 2006), serving as habitat for insects both with and without aquatic life stages, as refuge and breeding ground for many amphibians, and as feeding areas for aquatic as well as terrestrial species (Berger et al., 2013; Heim et al., 2018). Accordingly, KH host diverse communities including those that are fully aquatic, those with an aquatic-terrestrial lifestyle, and terrestrial organisms praying on aquatic ones.

Water-filled KH are aquatic islands embedded in a terrestrial landscape, where local communities are connected via passive and active overland dispersal (Thompson et al., 2020). The frequent occurrence of KH in the landscape suggests that they serve as stepping stones between habitats located in different land use types within the landscape, where especially small-sized aquatic organisms (microbiota) are dispersed via vectors such as wind, (Kayler et al., 2018; Premke et al., 2016). Accordingly, the biodiversity of small ponds such as KH is disproportionately high (Scheffer et al., 2006) compared with their terrestrial surroundings, and is directly linked to the degree of connectivity to other ponds (Van Geest et al., 2003).

Simultaneously assessing biological diversity across taxa, from microbes to mammals, is challenging. However, a promising approach is the use of environmental DNA (eDNA) that provides a common denominator for all taxa independent of body size and other species traits. Therefore, the analysis of eDNA has been increasingly applied as a non-invasive, highly sensitive monitoring tool (Deiner et al., 2017; Harper et al., 2019). The approach is based on collecting samples of live, dead, or partially decomposed organisms containing DNA that can be amplified and taxonomically annotated.

There have been to date multiple studies that have established the reliability of eDNA as a tool for diversity estimation. For example, Juhel et al. (2020) concluded that species accumulation curves offer a reliable method to estimate fish diversity from eDNA analyses, when compared with classical methods. Olds et al. (2016) in a similar comparison concluded that eDNA is superior to classical methods in assessing fish diversity. Tillotson et al. (2018) further demonstrated that eDNA concentration reflects the abundance of spawning salmon. Djurhuus et al. (2018) concluded that eDNA analysis of unfiltered water is reliable for zooplankton diversity estimation. Govindarajan et al. (2021) have further showed the superiority of eDNA in animal biodiversity assessment in the mesopelagic ocean compared with several classical methods. Diversity assessment of microorganisms with low morphological complexity, such as *Bacteria* and *Archaea* (starting with Woese & Fox, 1977), fungi (Tedersoo et al., 2020), and protists (Burki et al., 2021) has for a long time already relied on DNA-based methods (i.e., metabarcoding).

The half lifetime of eDNA varies in different environments ranging between a few hours to a few days in water columns of aquatic bodies, with the shortest half lifetimes being in eutrophic lotic systems (Allan et al., 2021; Collins et al., 2018; Harrison et al., 2019). In contrast, the lifetime of eDNA in sediments is much longer, reaching years (Corinaldesi et al., 2008; Harrison et al., 2019; Sakata et al., 2020). Consequently, water analysis will reflect a reliable snapshot of overall biodiversity as it responds to short-term environmental changes, whereas sediments will mirror and archive past events. Most eDNA approaches aim to detect a specific set of taxa such as mammals, fish or amphibians by making use of previously identified specific sequences, or omnipresent genomic markers, such as the small and large subunits of ribosomal RNA genes or the Cox genes (Andújar et al., 2018; Beng & Corlett, 2020; Bylemans et al., 2019; Deiner et al., 2017). Several downsides have been recognized regarding the use of eDNA approaches, such as misinterpretation of sequence frequencies or of the presence and absence of taxa (Harper et al., 2019; Roussel et al., 2015). Here one should be cautious when interpreting sequence frequency as an accurate measure of relative abundance, specifically when larger organisms are concerned that are unlikely to have been sampled intact and in large numbers. Nevertheless, thanks to its sensitivity and non-invasive manner, the approach has proved particularly useful for analyses motivated by species-specific conservation or restoration efforts (Osathanunkul & Minamoto, 2021; Schwentner et al., 2021; Thomsen & Willerslev, 2015). Importantly, it is also possible to use eDNA to assess biological diversity across a broad range of taxa from different domains, facilitated by the use of genomic markers that capture all life forms such as the genes encoding for the small or large ribosomal RNA genes.

In the present study, we embarked on a multiseasonal analysis of cross-taxa aquatic biodiversity patterns in KH using deep sequencing (>200,000 reads per sample separately for *Bacteria*, *Archaea*, eukaryotes) of eDNA. These KH were surrounded by three different land-use types embedded in an agricultural dominated landscape: arable fields, grassland, and forest.

We defined three main goals of the study: (1) evaluate whether deep sequencing of eDNA is a reliable approach for broad qualitative biodiversity assessments, providing representative, in-depth information on a wide range of organisms, (2) assess to which extent α-diversity differs between land-use types, and (3) assess to what extent the observed landscape-scale patterns (beta diversity) can be explained by land use type.

Building on previous studies showing a decrease in biodiversity (Altenfelder et al., 2014; Berger et al., 2011; 2018; Donald et al., 2006; Meyer et al., 2013; Wilson et al., 1999) and homogenization (Baessler & Klotz, 2006; Macdonald & Johnson, 2000; Smart et al., 2006) following intensive land use, as well as the continuous soil-reworking and harvesting that result in habitat loss and degradation in agricultural areas (Firbank et al., 2008; Olivier et al., 2020), we hypothesized that: (1) biodiversity in KH surrounded by grassland and particularly by forest is closer to the natural, historic, state of KH prior to the

onset of intensive agriculture compared with KH embedded in agricultural fields, and (2) these "natural" KH would be characterized by richer local communities within individual KH (α-diversity) and more heterogeneous communities across KH within the more natural land-use categories (β-diversity).

METHODS

Study sites and sampling

We sampled a set of 67 KH located in northeastern Germany (Figure 1). The area, one of the least populated in Germany, has a long history of farming, with >90% of the land now being covered by arable fields (Kalettka & Rudat, 2006), although some of that land was reconverted to grassland nearly two decades ago (Serrano et al., 2017). For 40 of the sampled KH, the water and riparian vegetation has been routinely monitored since 1993, shortly after the reunification of Germany (Kalettka & Rudat, 2006). The remaining 27 sites were chosen to include adjacent, non-monitored, KH that would allow the evaluation of the effect of geographical proximity on aquatic biodiversity and physico-chemical properties compared with that of land use. The choice of these KH was limited by permission from landowners and nature authorities and was carried out as optimally as possible. Nevertheless, as later demonstrated by the results, the KH selection and geographical organization had no impact on the results.

Samples for eDNA analysis were collected during five sampling campaigns of 2–3 days each in December 2016, and March, May, June and October 2017. Each KH was categorized based on land-use type within a perimeter of ~50 m around the KH, that is, distinguishing KH in arable fields, grasslands, and forest patches (Figure 1).

Water samples were collected whenever enough water was present in the KH. Some occasionally fell dry, however (Nitzsche et al., 2017), and therefore could not be sampled at all times, particularly in October 2017. To obtain representative samples, total volumes of ~20 L were collected at 5–15 locations selected within each KH, with the number of individual samples depending on KH size. The samples were pooled in cleaned buckets and 2 L were subsampled in the field, placed in ice chests containing a mixture of ice and table salt to lower the temperature during transport, and subsequently frozen at −80°C in the laboratory for later eDNA analysis.

Sediment cores were taken at three time points (Dryad dataset: Sampling summary table, https://doi.org/10.5061/dryad.5hqbzkh6w). In March 2017, sediment

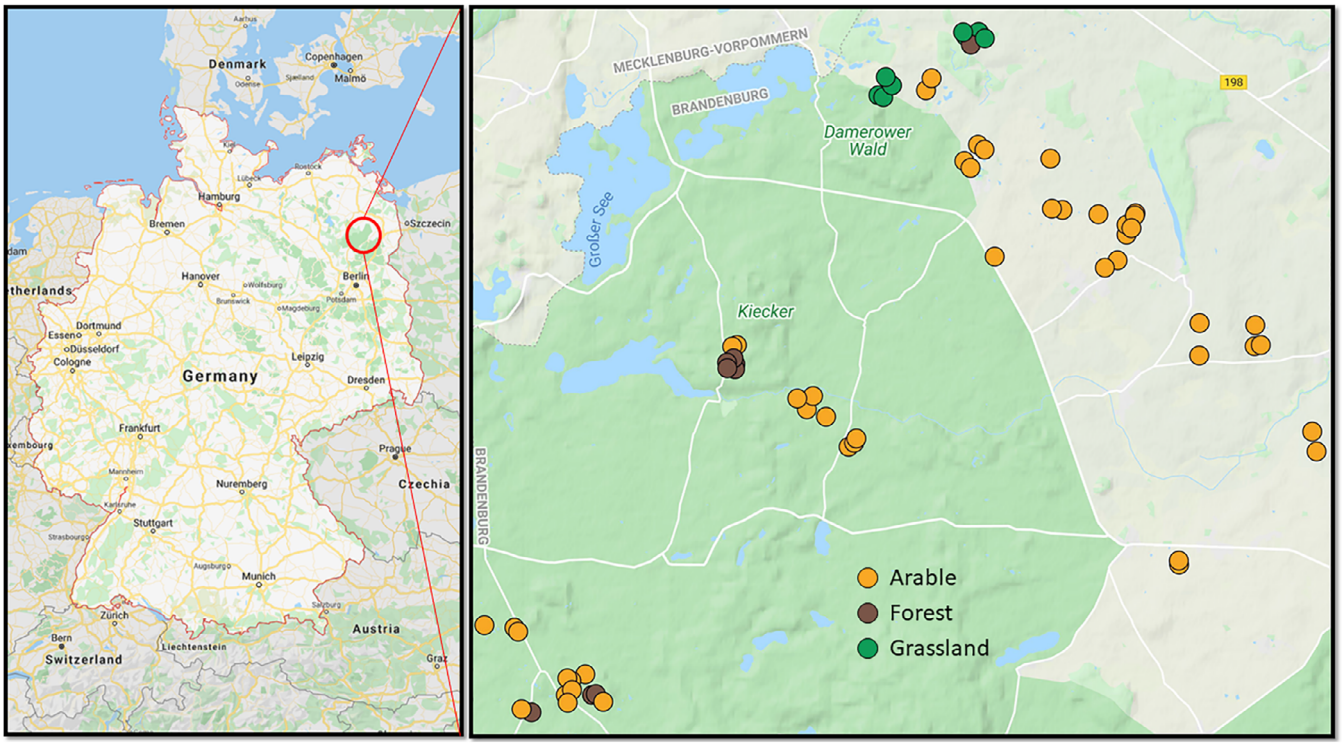

FIGURE 1 Map showing the location of the sampling area (125 km^2) ~100 km north of the city of Berlin, Germany (left panel) and local distribution of three types of sampled kettle holes (KH) in arable fields ($n = 47$), forests ($n = 11$), and grassland ($n = 9$) (right panel). Map generated with Google maps online tools

samples were collected from 54 of the 67 KH, both wet and dry. In some instances, a dense mat of belowground plant parts prevented sediment coring. Subsequently, sediment cores were only collected from wet KH that had recently dried out, or from previously dry KH that had refilled. Between 3–7 cores were taken per KH, depending on KH size, covering both littoral and central areas. The cores were sectioned into surface (upper 5 cm) and lower (5–20 cm depth) sediment layers to try to separate current benthic communities from older resting stages and preserved eDNA. The sections were separately transferred into plastic bags and subsampled (1 g wet weight) for eDNA extraction. Both the complete samples and subsamples were stored at −80°C for further processing. DNA extractions from multiple cores representing surface or lower sediment layers of a given KH at each sampling date were pooled. A compilation of the collected samples is given in Sampling summary table in the Dryad dataset: https://doi.org/10.5061/dryad.5hqbzkh6w.

Analysis of water physico-chemical properties

Temperature, conductivity, pH, redox potential, and oxygen concentration and saturation were measured on site during sampling using a multiparameter field probe (HI98194, Hanna Instruments, Vöhringen, Germany). Additional water (1 L) was collected to determine the concentrations of nutrients and major ions. These samples were immediately frozen in an ice chest containing crushed ice mixed with table salt (NaCl) and analyzed within 48 h. Water analysis followed German standard methods (DIN 38405, 2018). Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, and total Fe were analyzed by inductively coupled plasma optical emission spectrometry (ICP-iCAP 6300 DUO, ThermoFisher Scientific GmbH, Dreieich, Germany). Br^{-}, Cl^{-}, NO_3^{-}, NO_2^{-} and SO_4^{2-} were analyzed using ion chromatography (882 Compact IC plus, Deutsche Metrohm GmbH & Co. KG, Filderstadt, Germany). Ammonium (NH_4^{+}) and soluble reactive phosphorus (ortho-phosphate; *o*-PO_4^{3-} P) were measured spectrophotometrically (SPECORD 210 plus, Analytik Jena AG, Jena, Germany). Total phosphorus (TP) was measured as soluble reactive phosphorus after microwave digestion (Gallery™ Plus, Microgenics GmbH, Hennigsdorf, Germany). Dissolved organic carbon (DOC), total organic carbon (TOC) and total nitrogen (TN) were determined using an elemental analyzer (TOC-VCPH, Shimadzu Deutschland GmbH, Duisburg, Germany) with chemiluminescence detection. The specific absorption coefficient (SAC) was measured on a spectrophotometer (SPECORD 210 plus, Analytik Jena AG, Germany) as a proxy of dissolved aromatic carbon content (Weishaar et al., 2003). Finally, the SAC:DOC ratio was used as a rough measure of DOC quality.

Information on KH size, depth, canopy coverage, reed occurrence, as well as hydrological regime was considered as well. KH size was treated as a factorial parameter, that is, "Small" (< ~100 m^2), "Medium" (< ~500 m^2), and "Large" (< ~1000 m^2). Because KH area fluctuated with water level, it was hard to get a continuous measure of size. Similarly, KH depth was tested as "Shallow" (< ~0.1 m) versus "Deep" (> ~ 1 m). For depth we also had actual measurements matching many of the samples. This information was also used to test the effect of depth as a continuous parameter. Canopy coverage was assessed as: (1) "None,", when the KH had no surrounding trees, (2) "Partial,", when part of the KH was exposed and part was shaded by trees, or (3) "Full" when the entire KH was shaded by trees. Reed presence was treated as presence/absence, because this matched the on-site observations. The hydrological regime was treated as a factorial parameter ranging between 0–5 and describing the number of sampling campaigns in which the KH was observed with water. KH ranked as 5 were permanently full, whereas KH ranked as 0 were never full in the period between December 2016 and October 2017.

DNA extraction

The collected 2-L water samples were sequentially filtered (Nalgene filtration tower; ThermoFisher Scientific, Dreieich, Germany) to prevent clogging; the filters used were polycarbonate membrane filters (pore size of 10 and 5 μm), combusted GF/F filters and finally polycarbonate filters with a pore size of 0.2 μm (47 mm diameter of all filters). The GF/F filter was included owing to its charge in order to capture naked eDNA and DNA released from cells lysed by freezing and thawing. All filters were rinsed twice with 50 ml autoclaved MilliQ water to remove salts, and subsequently flash frozen and stored at −80°C.

Total (environmental) DNA was extracted from 329 samples consisting of 182 water samples, 75 surface sediment samples (<5 cm), and 66 deeper sediment (5–20 cm) samples. To prevent analytical biases (Bálint et al., 2018), the different filtered subsamples were extracted in separate, randomly selected batches. DNA was extracted with phenol/chloroform according to a method modified by Nercessian et al. (2005). In brief, a CTAB extraction buffer containing SDS and *N*-laurylsarcosine was added to the samples together with an equal volume of phenol:chloroform:isoamyl alcohol

(25:24:1) solution. The samples were subject to bead beating (FastPrep-24™ 5G Instrument, MP Biomedical, Eschwege, Germany), followed by centrifugation (14,000 *g*), a cleaning step with chloroform, and DNA precipitation with PEG-6000 (Sigma-Aldrich, Taufkirchen, Germany). The precipitated DNA was rinsed with 1 ml of 70% ethanol, dried, and dissolved in water. Finally, all extracts from the same sample were pooled and kept at −80°C until further processing.

Sequencing

Sequencing was conducted separately for the SSU rRNA gene of *Archaea*, *Bacteria*, and eukaryotes at MrDNA (Shallowater, TX, USA) using the following primers: Arch2A519F (5′-CAGCMGCCGCGGTAA-3′) and Arch1071R (5′-GGCCATGCACCWCCTCTC-3′) for Archaea (Fischer et al., 2016); 341F (5′-CCTACGGGNGGCW GCAG-3′) and 785R (5′-GACTACHVGGGTATCTAA TCC-3′) for bacteria (Thijs et al., 2017); and Euk1560F (5′-TGGTGCATGGCCGTTCTTAGT-3′) and Euk2035R (5′-CATCTAAGGGCATCACAGACC-3′) for eukaryotes (Hardy et al., 2010). The primers were barcoded on the forward primer and used in a 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) under the following conditions: 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, followed by a final elongation step at 72°C for 5 min. The PCR products were checked in 2% agarose gel to determine success of the amplification and relative band intensity. To ensure high coverage of rare taxa, batches of 20 samples were pooled for each sequencing run in equal proportions based on their molecular weight and DNA concentrations. The PCR products were purified using calibrated AMPure XP beads and then used to prepare an Illumina DNA library. Paired-end 2 × 300-bp sequencing was performed on a MiSeq sequencer (Illumina, Inc., San Diego, CA, USA) following the manufacturer's instructions. Sequence data are available at the NCBI Short Read Archive under project number PRJNA641761.

Bioinformatic analysis

Paired-end reads were merged using "BBMerge" from the *BBMap* package (part of JGI tools; https://sourceforge.net/projects/bbmap), after which the joined reads were quality trimmed and demultiplexed using *cutadapt* (v.1.16) to remove reads of low quality ($q > 20$) and shorter than 150 nt. Taxonomic annotation was performed for all reads from all samples without clustering, based on the SILVA SSU NR99 database (V132; Quast et al., 2013). This was accomplished by using *PhyloFlash* (v.3.3 b1; https://github.com/HRGV/phyloFlash; Gruber-Vodicka et al., 2020) and *Kraken 2* (Wood et al., 2019). To improve the annotation of eukaryotic taxa, a new database was created consisting of all eukaryotic sequences in the SILVA SSU Parc database (v.138). The SILVA Parc database also includes eukaryotic sequences shorter than 900 nucleotides and therefore covers a much broader range of species than the SILVA NR99 database. The eukaryotic sequences from all samples were annotated using both *PhyloFlash* and *SINA aligner* (Pruesse et al., 2012; V 1.6; https://github.com/epruesse/SINA) requiring a minimum consensus of three sequences for last common ancestor assignments. The resulting annotations were merged according to taxonomic names and presence/absence matrices were generated to account for the qualitative nature of the eDNA method, especially when merging data from separate assays (i.e., separately targeting *Archaea*, *Bacteria*, and eukaryotes). Statistical analyses (please refer to the next section) using matrices generated by different annotation tools resulted in identical patterns.

The functional potential of the bacterial community was derived from the taxonomic annotation using the *FaProTax* tool (Louca et al., 2016). This tool makes use of a literature-based database to assign potential functions to *Bacteria* and *Archaea* based on their taxonomy as it relates to well characterized organisms. To increase the accuracy of prediction, the tool uses a last common ancestor approach, in which functionality is inferred from the functions common to the same species, genus, family, or order depending on the degree to which the query sequence can be resolved.

Statistical analysis

Multivariate (nonmetric multidimensional scaling (NMDS); Kruskal, 1964), principal components analysis (Pearson, 1901), Canonical Analysis of Principal (CAP) (Anderson & Willis, 2003), PERMANOVA (Anderson, 2017)) and diversity (richness and evenness) analyses were conducted using the *Primer6* (v.6.1.1) + *PERMANOVA* package (v.1.0.1, Primer-E, Quest Research Limited, Auckland, New Zealand). NMDS was conducted using Bray–Curtis dissimilarity, retaining the ordination with the lowest calculated stress out of 1000 iterations. PERMANOVA was used to test for the effect of land-use type, seasonality (i.e., time of sampling) or both. CAP coordinates were used to present the data according to factors found to have a significant effect by PERMANOVA. Distance-Based Linear Models with Redundancy Analysis (DBLM-RDA; Legendre & Anderson, 1999) were used to test for the effects of water chemistry

on community structure. Univariate analyses (ANOVA, Kruskal-Wallis and Dunn's test), and diversity indices (Chao I (Chao et al., 2004), taxa richness, evenness) were calculated using *PAST4* software (Hammer et al., 2009). Because the data available in the sequence databases (e.g., SILVA) are not of uniform quality, not all sequences could be annotated to the same taxonomic resolution. Therefore, richness was assessed using the highest assignable taxonomic resolution.

Ternary plots were generated using the *ggtern* package (Hamilton & Ferry, 2018) in R v.3.5 (The R Core Team, 2018). Indicator species analysis was performed using the *indicspecies* R package (v.1.7.8; De Cáceres & Legendre, 2009) testing for the IndVal index, as well as Pearson's phi coefficient of association (Chytrý et al., 2002). The latter was used both on presence/absence data and sequence frequencies, while considering the appropriate functions and required corrections as outlined in the *indicspecies* package manual (v.1.7.8). Indicator species analysis was conducted using the most elaborate annotation matrix (containing 50,000 taxa across the three domains *Archaea*, *Bacteria*, and eukaryotes). Additionally, the analysis was corrected for the greater number of sites in arable fields than in grasslands and forests. Data for ternary plots were generated as the percentage presence of a specific taxon in each land-use group.

Rarefaction curves and cross-sample species accumulation curves were calculated using *ampvis2* library (v.2.7.17) in R (Andersen et al., 2018) and *Primer6* (v.6.1.1, Primer-E, Quest Research Limited, Auckland, New Zealand), respectively.

Joint species distribution model analysis

We conducted joint species distribution model analysis that acknowledges not only environmental filters but also the multivariate nature of communities by reducing the high dimensionality of community data (Clark et al., 2017; Ovaskainen et al., 2017; Warton et al., 2015). To accomplish this, we ran Hierarchical Modeling of Species Communities (HMSC), a statistical framework for analysis of multivariate data, using the *HMSC* R package (Ovaskainen et al., 2017; Tikhonov et al., 2020). We applied the model to water samples alone using separately two selected subsets of the data consisting of the top 143 bacteria and 200 eukaryotes, respectively. The entire dataset was reduced to make the model computation feasible. The top bacterial and eukaryotic subsets were selected based on presence in at least 10% of the water samples and an average abundance larger than 0.1%. Sequence frequency was used as a proxy for relative abundance of the different taxa. In the absence of absolute counts for bacterial taxa, this is common practice in DNA-based microbial ecology studies (Bálint et al., 2016). The top 200 eukaryotes consisted of organisms with high probability of being sampled intact by our sampling methods, with the largest of them being copepods (zooplankton typically within the size range 0.2–20 µm [Steinberg & Landry, 2017]). Therefore, as for the analysis of the bacterial dataset, also for the eukaryote dataset sequence frequency was used for the HMSC modeling. To evaluate the reliability of sequence frequency as a proxy for abundance, we looked at the correlation between sequence frequency and absolute abundance of rotifers (ind L^{-1}) sampled in the June campaign, for which abundance data are available (Onandia et al., 2021). We could find significant correlation for total individuals (Spearman $\rho = 0.53$, $p = 0.009$; Kendall $\tau = 0.40$, $p = 0.006$), as well as when phylogenetically assigned to the class Monogonata (Spearman $\rho = 0.53$, $p = 0.007$; Kendall $\tau = 0.41$, $p = 0.004$) and the order Ploima (Spearman $\rho = 0.43$, $p = 0.011$; Kendall $\tau = 0.32$, $p = 0.007$) which accounted for almost all counted taxa (Appendix S1: Figure S1). Additionally, as joint species distribution model analysis can also be applied to presence/absence data (Ovaskainen et al., 2017), we ran the eukaryotic model as well on presence/absence data.

As environmental covariates, we included the categorical variable "land-use type" with the three levels arable fields, forests, grasslands, and the continuous covariate "chemical condition" that consolidated by PCA 17 physico-chemical variables to four-dimensional axes (explaining more than 70% of the variance of all sampling sites), to reduce the parameters and eliminate multicollinearity. We set the KH and seasonality difference as the random effects defined in the argument "studyDesign" of the "HMSC" (Tikhonov et al., 2020) function in the package. To avoid fitting excessive latent factors, we constrained the minimum and maximum number of latent factors to 5 and 10, respectively (Tikhonov et al., 2020). We sampled the posterior distribution with four MCMC (Markov Chains Monte Carlo) chains, each of which was run for 20,000 iterations, out of which the first 10,000 were removed as burn-in and the remaining ones were thinned by 20 to yield 500 posterior samples per chain. We ran three parallel MCMC chains, therefore 1500 posterior samples in total. To check the model convergence, we used the R-hat index (Gelman et al., 2013). All the estimated parameters of the bacteria and most of the eukaryotes (98.3%) model fulfilled the condition of convergence ($\widehat{R} < 1.1$).

RESULTS

Water physico-chemical properties

The physical and chemical properties of the water (Figure 2 and Dryad dataset: Physico-chemical parameters table,

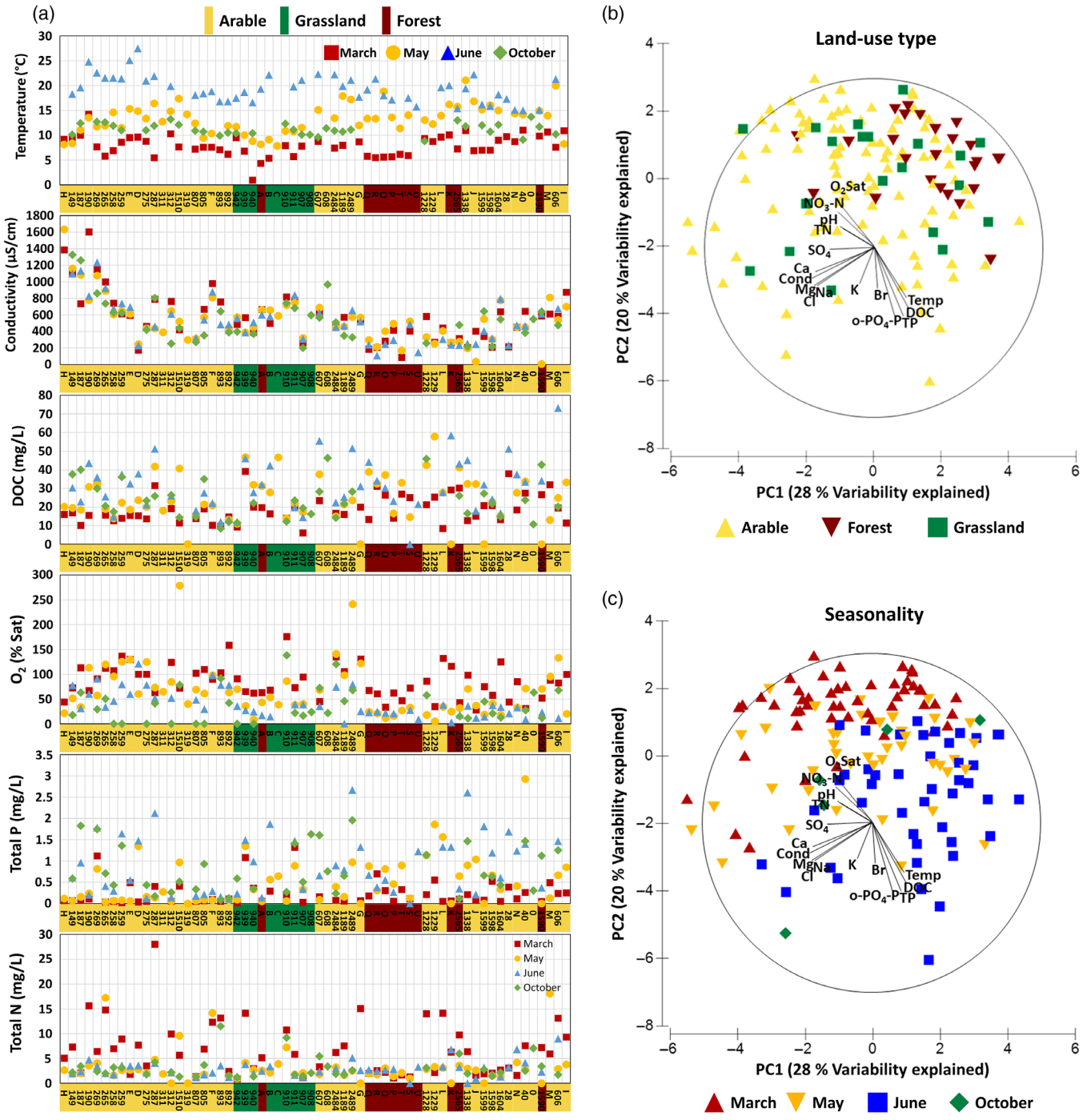

FIGURE 2 Variability among and within kettle holes (KH) in terms of major physical and chemical variables determined during five sampling campaigns (a). Principle components analysis of water chemistry data with samples labeled according to land-use type (b) or month of sampling (seasonality) (c)

https://doi.org/10.5061/dryad.5hqbzkh6w) highlighted the temporal variability of parameters within KH throughout the study. The samples in Figure 2a were ordered based on geographical proximity on an east–west/north–south gradient, highlighting the fact that adjacent KH do not necessarily have similar physico-chemical characteristics. Accordingly, no correlation was found between geographical proximity of the KH and their physico-chemical similarity (Appendix S1: Figure S2). Variation among samples per land-use type as well as combined sampling campaign and land-use type are shown in Appendix S1: Figures S3 and S4, respectively, along with information on statistically significant differences. Water chemistry of the surveyed KH varied among sampling dates and both within and among land-use

types, but systematic differences among land-use types were small. Only KH surrounded by forest significantly differed from KH in arable fields and grasslands, and that only in some parameters such as conductivity and concentrations of DOC and most ions, but not nutrients (Appendix S1: Figure S3). In contrast, water physico-chemical parameters did not differ between KH in arable fields and grasslands, even when data from different sampling campaigns were analyzed separately, the only exception being temperature (Appendix S1: Figure S4). The extent of seasonal variability and the timing when parameter-specific maxima or minima were observed differed among individual KH (Figure 2a). Principle component analysis based on the maximal number of available parameters for the largest possible number of samples (143 of 182 water samples) did not separate KH according to land-use type (Figure 2b). However, a seasonal pattern emerged between spring (March) and summer (June) with the smaller subset of autumn (October) samples being closer to spring samples, primarily driven by temperature, O_2 saturation, DOC and nutrient concentrations (Figure 2c). Conductivity and concentrations of several ions also significantly influenced the ordination, but reflected neither seasonality nor land-use type. All KH would be classified as eutrophic to hypereutrophic based on TN and phosphorus data (Wetzel, 2001). However, it is difficult to fully determine the trophic state of the KH in this study for two main reasons. First, no obvious relation was observed between O_2 saturation and nutrient load at the time of sampling, except for the negative correlation with TP in March and May (Appendix S1: Figure S5). Second, primary and secondary productivity, an essential part of measuring eutrophication (Khan & Ansari, 2005) were not determined in this study.

Sequencing effort

Separate sequencing assays resulted in 8.35×10^7 archaeal, 11.6×10^7 bacterial, and 11.4×10^7 eukaryotic SSU rRNA gene sequences per assay, averaging 3.24×10^6 sequences per sample (Dryad dataset: Sequence counts table, https://doi.org/10.5061/dryad.5hqbzkh6w). Reads of eukaryotes were assigned to a large number of taxa (please refer to annotation results under Dryad dataset https://doi.org/10.5061/dryad.5hqbzkh6w), including worms, mollusks, arthropods, amphibians, fish, birds, and mammals, some of which were evidently rare or occasionally present in the KH.

α-Diversity

Species accumulation curves show that for *Bacteria* and eukaryotes sediments are more diverse than water samples, however, for *Archaea* no clear trend is visible (Figure 3a–c). Most taxa were discovered within 25% of the sequencing effort, however, despite the deep sequencing effort, the accumulation curves do not reach a plateau. Rare taxa, represented by single sequences, made up each 1×10^{-2}% to 1×10^{-4}% of the sequences per sample. When these taxa are removed, species accumulation curves reach a plateau for most bacterial and eukaryotic samples, however, not for *Archaea* (Figure 3d,e). The latter is likely to be a methodological issue driven by a primer that is biased toward specific taxa in the system, rather than an extremely high archaeal diversity. A cross-samples species accumulation curve suggests that, also when rare taxa are included, the majority of archaeal, bacterial and eukaryotic biodiversity in the studied KH has been sampled (Appendix S1: Figure S6).

The Chao I index confirmed that overall organism diversity was greater in sediments than in water. No significant differences among land-use types were observed in either sediment (Figure 3a) or water (Figure 3b) when the data were grouped according to land-use type (Figure 3a). This held irrespective of whether the data were analyzed together across all sampling campaigns or if each campaign was inspected separately.

Depending on the annotation tool used to analyze the data, in total, 13,000 to 50,000 taxonomic entities were identified. Despite the large spread, trends similar to those shown in Figure 4 were observed in all cases (Appendix S1: Figure S7). Therefore, for all analyses except species accumulation curves (Figure 3), we chose a more stringent analysis that provided less taxonomic resolution, by grouping sequences into broad taxonomic groups (e.g., into families rather than genera or genera rather than species). No statistically significant difference in taxon richness was observed among land-use types when all water or sediment samples were analyzed together (Figure 4b). When analyzed according to sampling period, forest KH water samples collected in March 2017 harbored a higher diversity than samples from KH in grassland or arable fields (Figure 4b). In contrast, sediment samples collected in March from KH in arable fields harbored more taxa than forest and grassland samples collected at the same time. Samples taken in June show a higher diversity in sediments of forest KH than in those of arable fields, and a similar pattern was apparent in the matching water samples. However, sediment data from the forest KH might be biased because the number of samples was low.

Both taxonomic richness and Chao I index showed that α-diversity in water samples across all land-use types was higher in winter and early spring (i.e., December and March), reaching a minimum in midspring (May) and increasing again toward winter (Figure 4b).

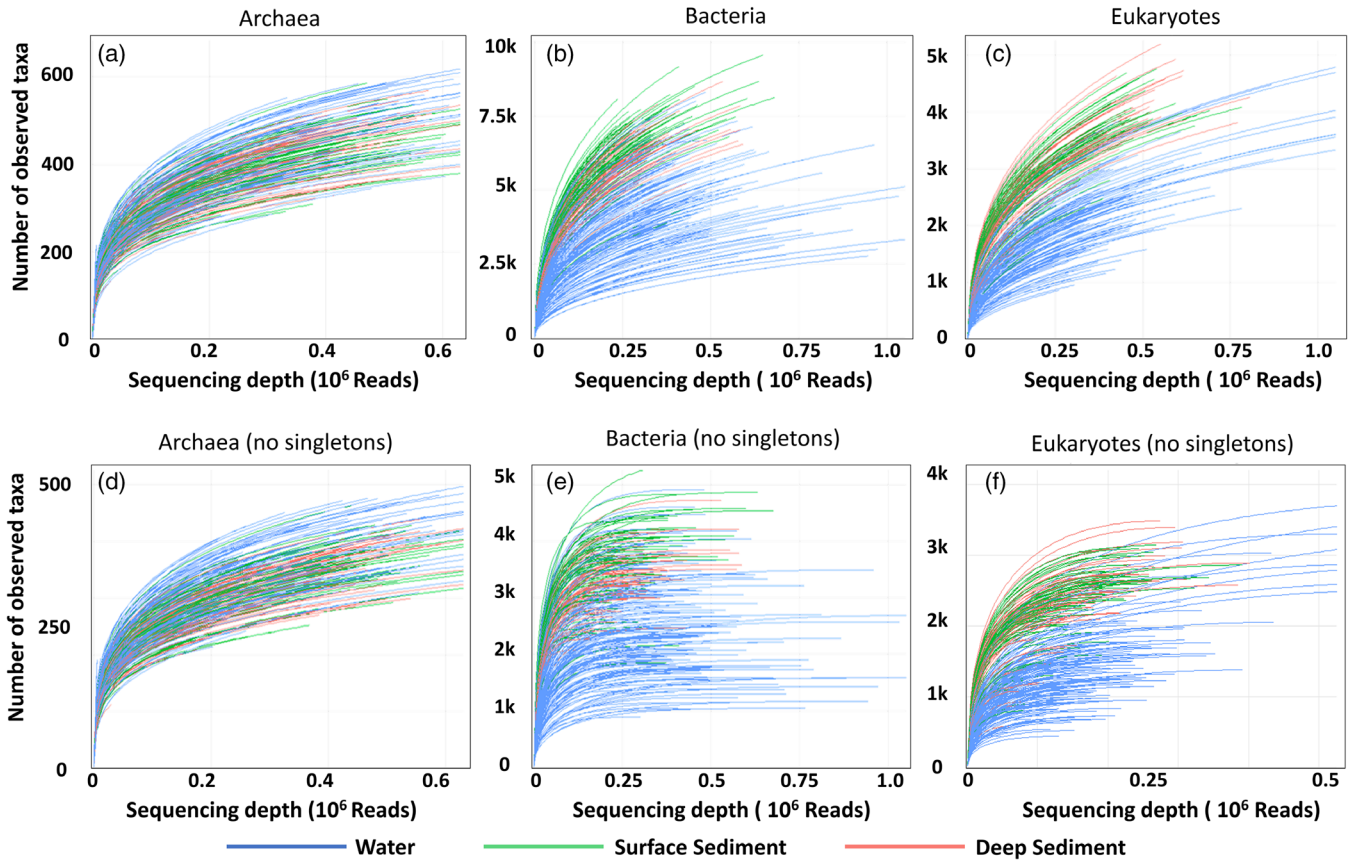

FIGURE 3 Species accumulation curves for *Archaea*, *Bacteria*, and eukaryotes calculated for all samples (a–c) alongside the curves calculated following the removal, from each sample, of species that are represented by a single sequence (d–f)

We further assessed richness at different time points within different functional groups across the different land-use types (Figure 5). In most of the groups shown in Figure 5, an increase in apparent richness (i.e., more species detected) was observed in spring (March, May). This increase and peak in richness occurred across all land use types, although at different magnitudes and not always in parallel. The largest number of species per land use was mostly found in arable fields, followed by forest and grasslands (ANOVA $p < 0.001$). However, a comparison of species accumulation curves suggests that there was no difference in richness among the land-use types and therefore the evidently higher number of species detected in arable fields was the result of having more sampling sites (Appendix S1: Figure S8). In contrast, forest KH harbored the largest number of different species per KH (ANOVA $p = 0.02$), whereas KH from arable fields and grasslands were often similar (Mann-Whitney $p = 0.6$).

β-Diversity

Given that eDNA data are non-quantitative across the *Bacteria*, *Archaea*, and eukaryotes, specifically with respect to multicellular taxa, the sequence frequency data were converted to presence/absence data. Separate analyses making use of sequence frequency as a proxy for abundance but excluding eukaryotic taxa did not notably alter the presented result (Appendix S1: Figure S9).

NMDS analysis showed a clear separation between community composition of sediment and water samples (Figure 6a), which accounted for ~15% of the variability across all samples ($p = 0.001$). Water samples were separated according to sampling period (Figure 6b), explaining ~11% of the variability between samples ($p = 0.001$). This percentage increased to 18% when sequence frequencies were used instead of presence/absence data ($p = 0.001$). In contrast, differentiating the samples according to land-use type showed no distinct pattern (Figure 6c), although PERMANOVA showed it was statistically significant ($p = 0.018$), explaining only ~2% of the variability between the samples. Breaking down the arable fields into the specific crops (at the time of sampling) did not improve explanatory power. Redundancy analysis using data from the 143 water samples for which all physical and chemical information was available revealed a separation based on sampling time point, similarly to the NMDS analysis, with a clear horizontal separation that appeared to be mainly driven by temperature (Figure 6d). Physical and chemical

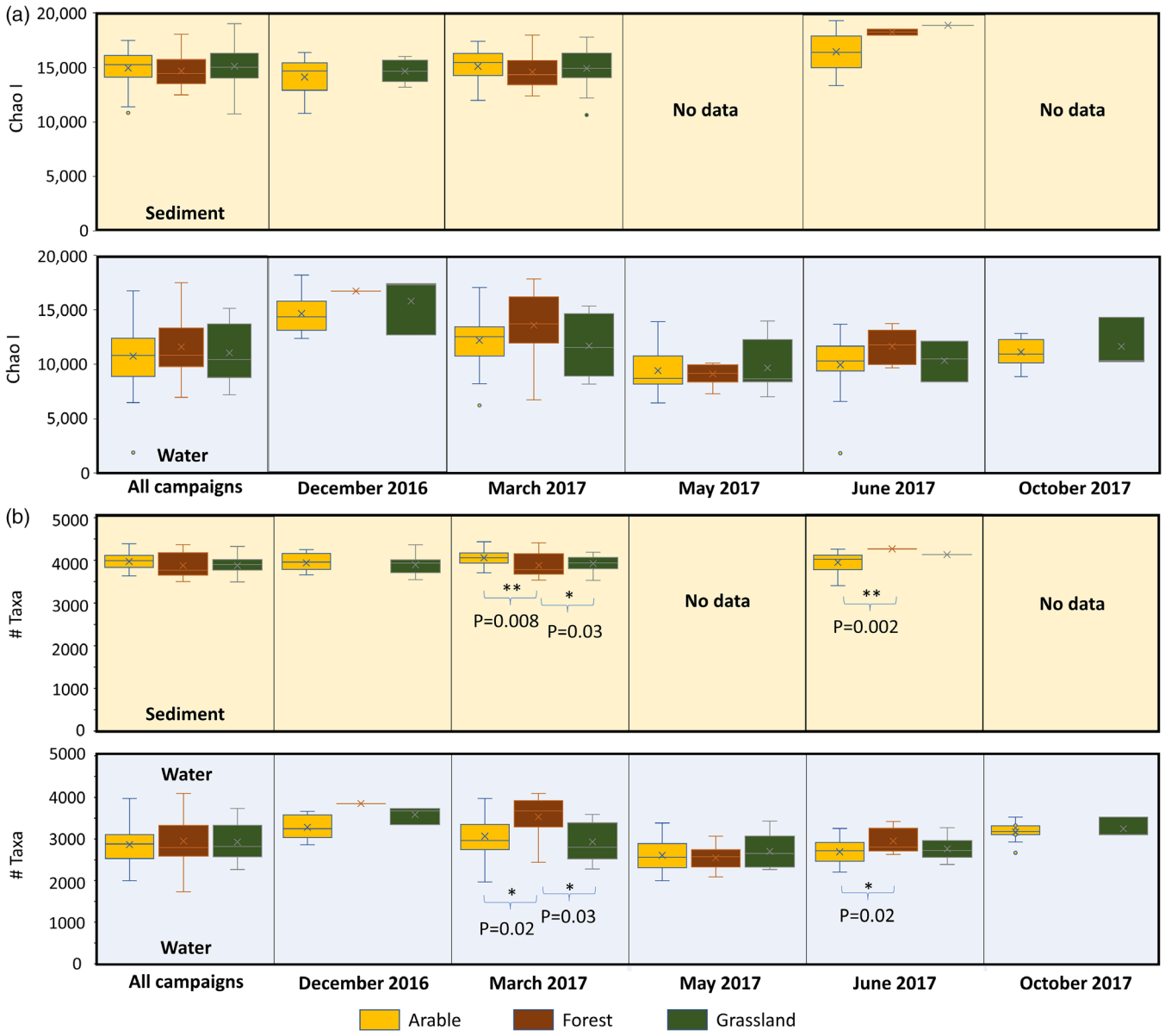

FIGURE 4 Richness assessment based on Chao I index accounting for the number of taxa for which singleton and doubleton sequences were obtained (a) and taxonomic richness that considers presence/absence alone (b). Whiskers mark the 25th and 75th percentiles. Samples are grouped according to the assigned land-use type and include *Archaea*, *Bacteria*, and eukaryotes data. Sediment and water samples are separated for both indices. In both cases, sequences were grouped according to taxonomic annotations and were not clustered into distance-based operational taxonomic units. As not all sequences could be resolved to the same taxonomic depth (i.e., order, family, genus, species), these indices are likely to underestimate the true diversity. ANOVA and Kruskal-Wallis tests showed no overall difference between the land use types. However, when pairs of groups were compared using Mann-Whitney's and Dunn's tests, significant differences were found as marked in the figure

parameters cumulatively explained 23% of the total variability among samples, with the contribution of most parameters being significant (p <0.001–0.018), except for concentrations of Cl^- ($p = 0.08$) and Br^- ($p = 0.35$). Temperature, strongly correlating with the seasonal gradient, had the largest explanatory power among all parameters, accounting for 7% of the total variability among samples.

KH depth, canopy coverage, reed occurrence, as well as hydrological regime were considered as well. Among these the only one that could significantly explain the community composition was "canopy cover" which accounted for 3% ($p = 0.001$) of the variability when tested as single factor using PERMANOVA. Nevertheless, when tested in combination with campaign number (i.e., seasonality) and land-use type, canopy cover was no longer significant ($p = 0.277$).

To further explore the species distribution based on land-use type, at the level of individual taxa, we

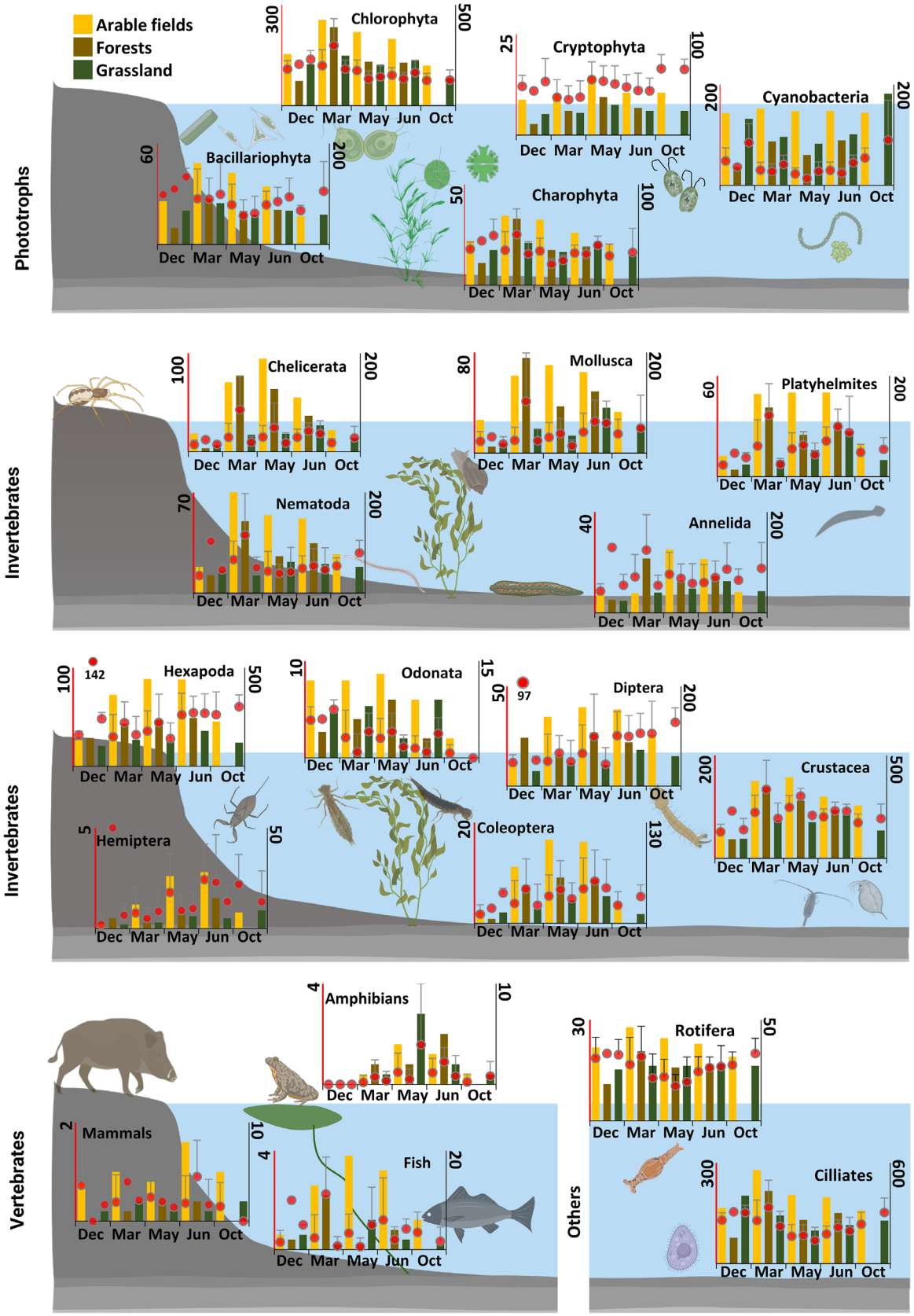

FIGURE 5 Average apparent species richness (no. of taxa) of selected functional groups per kettle holes (KH) from each land-use type for the different sampling periods (left axis, red circles) alongside the summed richness (no. of taxa) per land-use for the same periods (right axis, bars). Yellow, brown, and green bars stand for arable, forest, and grassland land-use types, respectively. This figure was partially created with BioRender.com

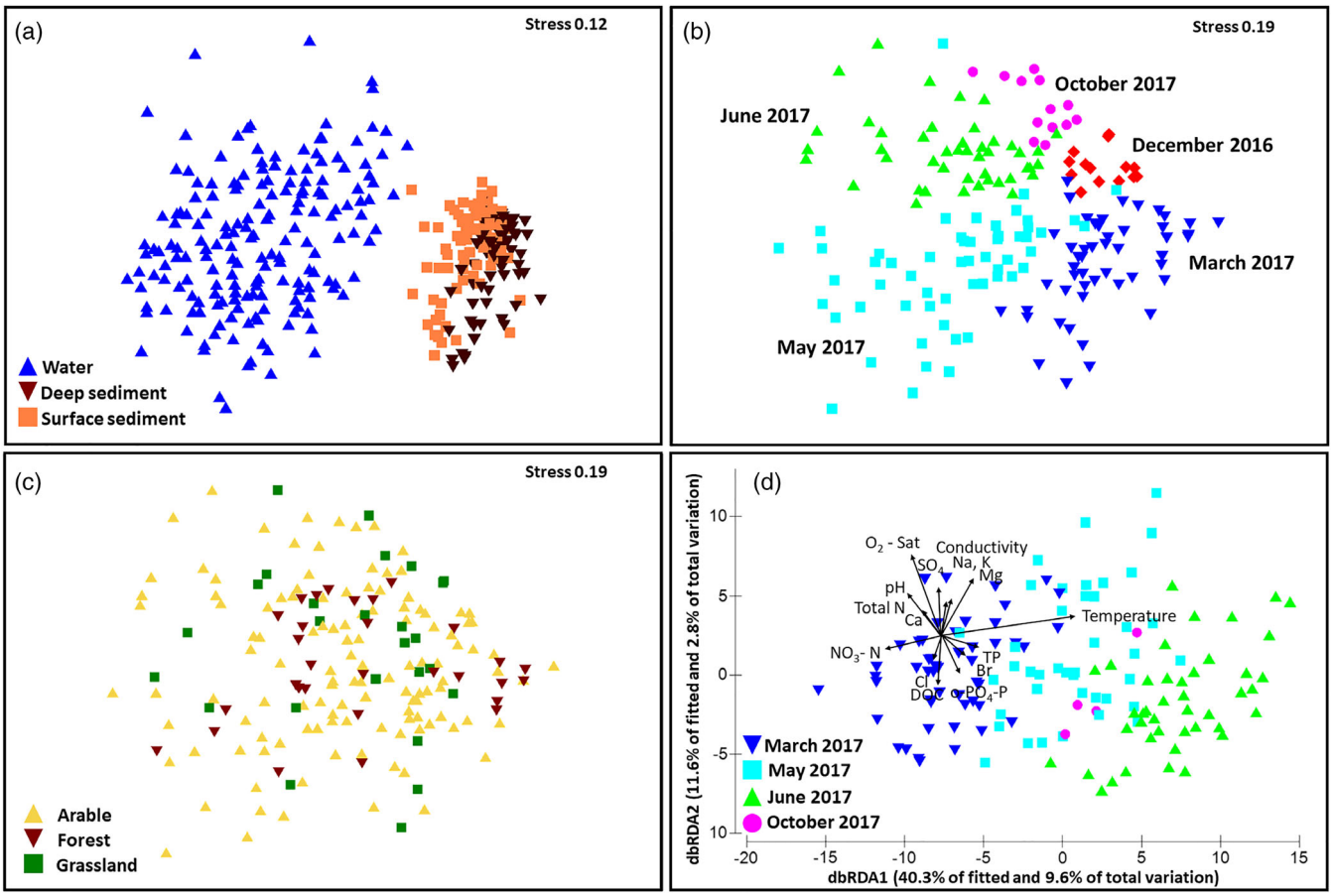

FIGURE 6 Nonmetric multidimensional scaling (NMDS) of sampled communities revealed a separation between the total community of water and sediment samples (a) and a seasonal clustering of the aquatic communities (b), but no land-use-based ordination patterns (c). A distance-based linear model and redundancy analysis (d) shows a seasonal separation of the water samples alongside the statistically significant environmental parameters, with temperature being the main driver. A three-dimensional NMDS analysis improves the fit, reducing the stress in panels (a), (b), and (c) to 0.09, 0.12, and 0.12, respectively

conducted an indicator species analysis of the water and sediment data using both a presence/absence matrix and sequence frequencies. The patterns were virtually identical (Figure 7a). No taxa were restricted to a single land-use category. Larger numbers of taxa were associated with forest or grassland than with arable fields in both sediment and water samples. The top five bacterial and eukaryotic associated taxa per land-use type are presented in Appendix S1: Table S1 and the complete results are given in the Dryad dataset: Indicator species, https://doi.org/10.5061/dryad.5hqbzkh6w. In both analyses the number of taxa associated with arable fields was higher in sediment than in water samples. A similar pattern was observed when looking at species associated with two land-use types, one of which is arable fields (Figure 7a). A graphical representation of taxa associated with different land-use types by means of ternary plots revealed a similar result (Figure 7b). Most of the taxa appeared to be neutral with regard to land-use type, as indicated by the dark blue to red color points clustered in the center of the plots. Only individual taxa, represented by the purple color, spread from the center toward specific land-use types. High correlations, indicated by taxa present in the colored triangles at the vertexes of each plot, are rare. However, overall sediment samples are more inclined toward arable fields than water samples, whereas the latter are more inclined toward forest and grassland. Separate analyses of bacteria (*Bacteria* and *Archaea*) and eukaryotes in sediments showed a similar distribution pattern with eukaryotes in water samples appearing to contribute more to the communities associated with forests, and bacteria to those associated with grasslands.

Joint species distribution models offer a complementary approach to classical statistical analyses, to investigate the assembly processes of communities, as it additionally accounts for potential interactions between the taxa in the communities (Tikhonov et al., 2020). Therefore, we used HMSC (Ovaskainen et al., 2017; Tikhonov et al., 2020), one of the approaches for modeling joint species distribution, to evaluate whether the

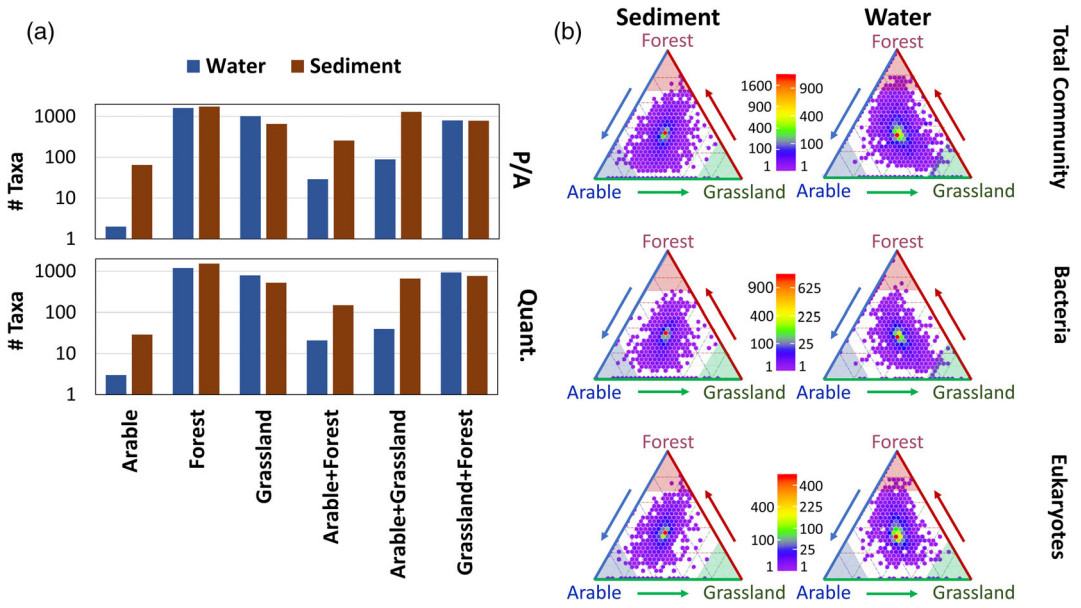

FIGURE 7 Number of taxa that are significantly ($p < 0.05$) associated with a specific land-use type, according to the indicator species analysis conducted on all water and sediment samples. The analysis was calculated using either a presence/absence matrix (P/a) as most suitable for eDNA data (upper [a] panel) or sequence frequency (lower (a) panel), which is possible as the analysis is conducted on each taxon separately. The association coefficient of these taxa is presented in the supplementary tables available on the Dryad dataset: Indicator species, https://doi.org/10.5061/dryad.5hqbzkh6w. Taxa association with a single land-use type was exclusive, as clearly shown in the ternary plots (panel [b]). The ternary plots depict the association of each taxon to specific land-use types that are represented by the three vertexes of each triangle. The axes of such plots are unitless measures of association with the land-use type at each end of the axis. The closer a point is to a vertex the more associated that taxon is with that particular land-use type. Individual taxa are pooled into hexagonal shapes for graphical purposes. The term "bacteria" refers to both *Bacteria* and *Archaea*. The color scale refers to the square root of the number of taxa in each colored point with purple representing single taxa and dark red the maximum number. As indicated by the color code, most taxa appear in the middle of the plot and are therefore generalists with respect to land-use type

minimal explanatory power of land-use type is an artifact of the statistical methods, applied so far. Due to the complexity of the method coupled with that of our studied communities, we limited the HMSC analyses to the most abundant 143 bacterial and 200 eukaryotic taxa (please refer to section "*Methods*"). The results confirmed that few taxa (across all domains) were associated with a specific land use (Appendix S1: Figure S10a,c,e). Among the top 143 bacterial taxa, one taxon was negatively correlated with forests and no taxon was significantly correlated with grasslands or arable fields. For the top 200 eukaryotes, when sequence frequency was considered, seven taxa were positively correlated with forests, five with grasslands, and none with arable fields. When only presence/absence was considered for eukaryotes, seven taxa were positively correlated with forests, 23 negatively correlated with grasslands, and none with arable fields. Variance partitioning revealed that water chemistry contributed differently to the distribution of the different species (Appendix S1: Figure S10b,d,f), yet it showed that land use had a low contribution to the overall variability, explaining on average 1.9% ± 1.1% (median 1.6%) and 13% ± 7% (median 11%) of the bacterial and eukaryotic variability, respectively. Variance partitioning when applying the same model on a presence/absence matrix of eukaryotes to account for the possibility that sequence frequency of eukaryotic eDNA does not reliably represents abundances in nature, resulted in a lower proportion of the variability explained by land use (8.1% ± 4.4%; median 8.1%). Water chemistry could explain 26% ± 3%, 55% ± 16%, and 66% ± 14% of the variability for bacteria, and eukaryotes with and without the use of sequence frequency, respectively.

We tested, using PERMANOVA, whether land-use type influenced the distribution of taxa in the entire community or specific taxonomic groups associated with different trophic functionality (i.e., oxygenic phototrophs or fungi), and whether the influence was more pronounced in sediment than in water (Table 1). This was compared with the effect of seasonality that had overall a greater effect on the total community. We defined land-use effect as a significant difference in biodiversity or community structure detectable when data from all sampling campaigns were pooled across the tested group. For microorganisms (*Bacteria*, *Archaea*, and small eukaryotes) we compared presence/absence data (assessing biodiversity)

TABLE 1 Percentage contribution of land use and seasonality to the β-diversity in water and sediment samples

	Presence/absence				Abundance			
	Land use		Seasonality		Land use		Seasonality	
Group	Water	Sediment	Water	Sediment	Water	Sediment	Water	Sediment
Total community	3	**4**	**11**	5	6[b]	**10**[b]	**17**[b]	8[b]
Bacteria	2	0	**9**	3	**5**	0	**16**	8
Eukaryotes	4	**5**	**16**	9	6[b]	**14**[b]	**20**[b]	10[b]
Cyanobacteria	**7**	0	**15**	15	**11**	**11**	**17**	**18**
Eukaryotic phytoplankton	0	5	**16**	8	7	**15**	**20**	10
Bacillariophyta	0	0	**17**	13	0	0	**17**	**17**
Charophyta	0	0	**13**	4	0	**11**	**18**	8
Chlorophyta	0	**5**	**15**	6	0	**17**	**20**	8
Rhodophyta	0	0	**12**	8	0	0	**10**	0
Cryptophyta	0	**7**	**13**	0	8	**14**	**18**	0
Other algae	0	**4**	**12**	5	5	**17**	**15**	5
Fungi	**5**	0	**16**	6	7	**15**	**18**	7
Oomycetes	0	0	**7**	0	0	**12**	**10**	5
Labyrinthulomycetes	0	0	**11**	0	0	**15**	**10**	0
Ciliates	0	0	**16**	7	5	**16**	**20**	8
Rotifera	0	**7**	**10**	7	7	**20**	**15**	9
Alveolata	0	6	**15**	6	6	**15**	**19**	0
Rhizaria	0	0	**12**	0	0	**14**	**16**	0
Amoeba	0	9	**17**	9	11	**17**	**16**	11
Heterotrophic Flagellates	0	0	0	**7**	0	0	12	**15**
Insecta	**6**	0	**26**	17	NA	NA	NA	NA
Hexapoda	**6**	0	**26**	16	NA	NA	NA	NA
Odonata[a]	10	**23**	**35**	0	NA	NA	NA	NA
Diptera	**6**	0	**26**	16	NA	NA	NA	NA
Hemiptera[a]	**10**	NA	**28**	NA	NA	NA	NA	NA
Coleoptera	0	0	**21**	13	NA	NA	NA	NA
Chelicerata	0	0	**21**	16	NA	NA	NA	NA
Crustacea	**6**	0	**13**	9	NA	NA	NA	NA
Myriapoda	NA	0	NA	0	NA	NA	NA	NA
Annelida	0	0	**17**	9	NA	NA	NA	NA
Nematoda	0	**7**	**19**	**19**	NA	NA	NA	NA
Platyhelminthes	**5**	0	**24**	14	NA	NA	NA	NA
Mollusca	**7**	0	**14**	11	NA	NA	NA	NA
Porifera	0	0	**7**	0	NA	NA	NA	NA
Other eukaryotes	3	**7**	**15**	8	NA	NA	NA	NA

Note: Statistically non-significant results (PERMANOVA, $p > 0.05$) are listed as 0% contribution. The compartment (water/sediment) for which a factor had a greater contribution is shown in bold. Groups marked with "a" were not present in all samples. Hemiptera were not detected in enough sediment samples to obtain statistically meaningful results. Quantitative data (i.e., sequence frequency) were only used for microorganisms, which are better represented given our sample size and more likely to have been sampled intact. Therefore, quantitative analysis of the eukaryotic community may be biased. Those percentages are marked with "b". Analyses marked with NA (not available) were not conducted.

and sequence frequencies (assessing community structure). For larger eukaryotic organisms that are unlikely to have been sampled intact, these quantitative data are likely to be biased because differently sized body fragments could have been sampled falsely, amplifying the amount of DNA without any change in number of organisms. Therefore, the comparisons were limited to presence/absence data (assessing biodiversity). Breaking down the arable-field land-use type into specific crops grown during the sampling period had no additional explanatory power in any of our analyses.

Land-use type had a minimal effect when the whole community was considered, with this effect being slightly larger in the sediment samples (Table 1). In contrast, seasonality had a much larger effect on the community as a whole and also for *Archaea*, *Bacteria*, and eukaryotes separately, explaining 11%, 9%, and 16% of the variability in taxa composition in the water samples, respectively. Accounting for the sequence frequencies increased the effects of both seasonality and land-use type for bacteria (from 9% to 16% and 2% to 5%, respectively). A similar effect was obtained for sequence frequencies of eukaryotes (from 4% to 6% for land-use type and from 5% to 20% for seasonality); however, because of possible differences in the representation of multicellular organisms in different samples, these data should be interpreted with caution.

For both total eukaryotic phytoplankton and *Cyanobacteria*, the same pattern was observed as for the total community. Land-use type had a stronger influence on biodiversity in the sediment, and seasonality on biodiversity in the water samples. Nevertheless, when separating the eukaryotic phytoplankton into taxonomic groups, land-use type had a stronger effect on *Chlorophyta* and *Charophyta* detected in the water column. The latter group was dominated by filamentous or single-celled planktonic species from the orders *Klebsormidiales*, *Desmidiales*, and *Zygnematales*. Land-use type had no effect on *Cryptophyta*, *Bacillariophyta*, and *Rhodophyta*. Accounting for sequence frequency, significantly increased the percentage of variability explained by land-use for eukaryotic phytoplankton in sediment samples and in some cases also in water samples. Land-use type had no effect on diatoms (*Bacillariophyta*) or *Rhodophyta* regardless of whether presence/absence data or sequence frequencies were analyzed.

Similarly, accounting for abundance, increased the percentage variability explained by land-use type for other eukaryotic microorganisms such as fungi, *Oomycota*, and *Rotifera*, but not for heterotrophic flagellates. Overall, with few exceptions, seasonality remained the main explanatory factor of taxa diversity in water, whereas land use explained best the diversity in sediments.

The species composition of larger multicellular organisms such as insects and major subphyla within crustaceans and mollusks was mainly explained by seasonality with the variability of only some of the groups partially explained by land-use type. Among these, the Odonata (dragonflies and damselflies) stood out with 23% of the variability in taxonomic composition in sediment samples being explained by land-use type.

We further investigated whether bacterial functional variability, as derived from the taxonomic annotation, could be explained by seasonality and land use (Appendix S1: Figure S11). When looking at all samples, 15% of the variability could be explained by sediment versus water samples and 10% by seasonality. Land use in this case was insignificant. Looking at the water samples alone, seasonality and land use explained 11% and 3%, respectively. When inspecting sediments, seasonality explained 7%, whereas land use was insignificant. Although no systematic difference was observed between the different land use types, some functional groups were significantly different between land-use types. Accordingly, 30 functional groups differed between grasslands and forests, 10 between arable fields and grasslands and five between arable fields and forests. Among these, bacteria carrying out ammonia oxidation (nitrification) were more abundant in forests, whereas oxygenic and anoxygenic photosynthetic bacteria were more abundant in grasslands (Appendix S1: Figure S11).

Using *Crustacea*, *Cyanobacteria*, and eukaryotic phytoplankton we evaluated whether the organisms in the sediment were of planktonic or benthic origin (Figure 8a–c) and to what extent the sediment and water communities differed from one another. Planktonic copepods (*Calanoida* and *Cyclopida*) and benthic copepods (*Harpacticoida*), as well as ostracods (Podocopida), were present in the deep and shallow sediment as well as in the water. However, the crustacean community structure differed significantly between the water and sediment and between sediment samples from KH surrounded by different land-use types (Figure 8a). Similarly, despite their dependence on light for photosynthesis, *Cyanobacteria* (Figure 8b) and eukaryotic phytoplankton (Figure 8c) occurred not only in water samples but also in sediments. The water and sediment communities differed from one another in both cases. However, while for *Cyanobacteria* land-use type clearly separated the sediment samples, eukaryotic phytoplankton communities from arable fields and grassland sediments were similar. Interestingly, eukaryotic phytoplankton groups from deep and shallow sediments were separated (Figure 8c).

In several cases, adjacent KH were attributed to different land-use types. Therefore, we sought to see if geographical proximity affected the similarity in community composition of KH (Figure 9). In none of the tested cases were KH close to each other (10s of meters apart) more

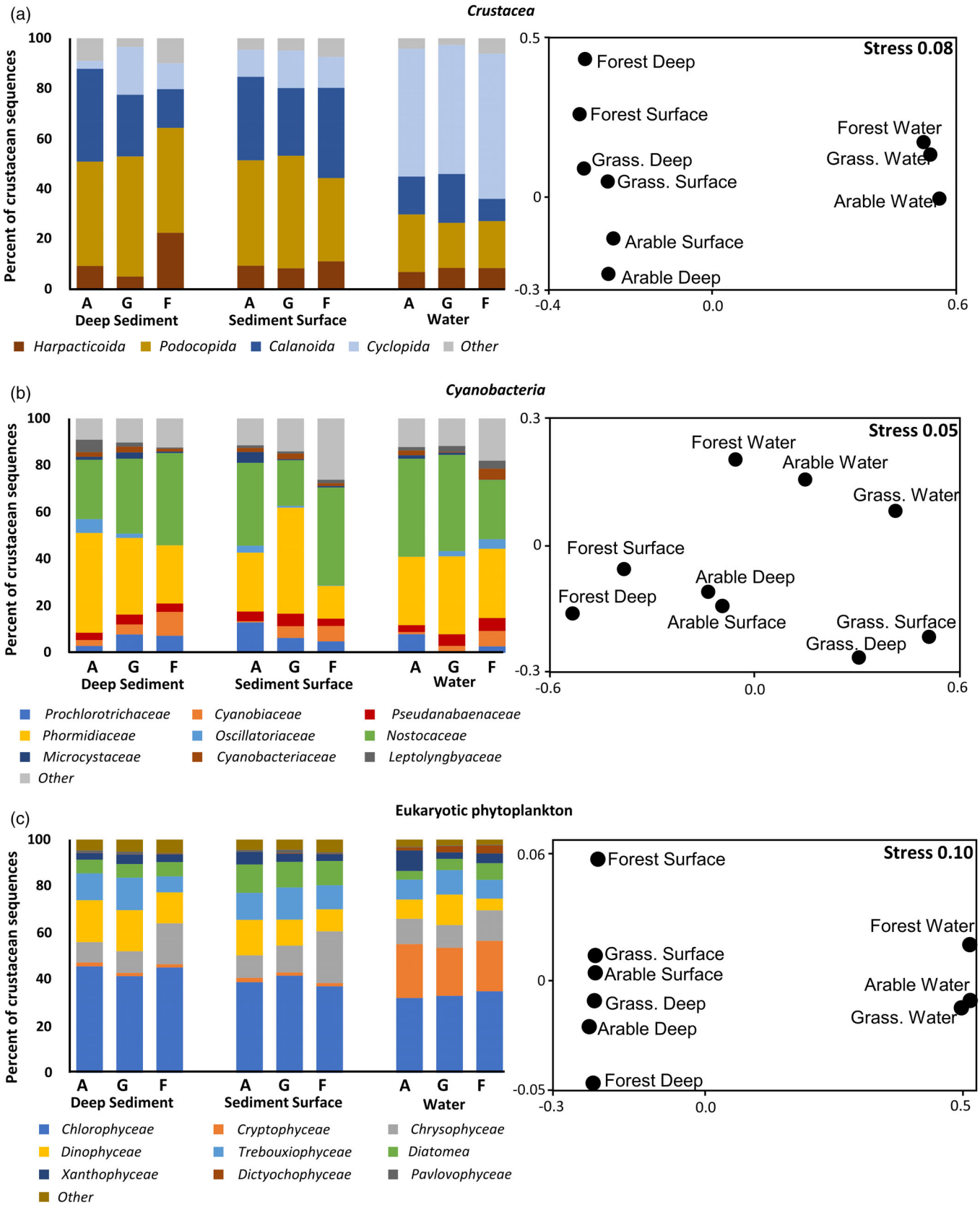

FIGURE 8 General community composition of crustaceans (a), *Cyanobacteria* (b), and eukaryotic phytoplankton (c) in deep sediments (5–15 cm), surface sediments (0–5 cm), and water samples. The nonmetric multidimensional scaling (NMDS) figures for each group show projections of similarities among the different sample types. Land-use types: A = arable fields, G = grassland, F = forest

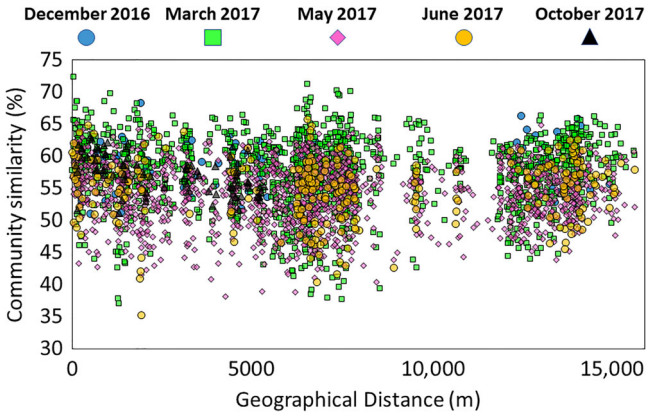

FIGURE 9 Bray–Curtis similarities between all sample pairs as calculated from a binary (presence/absence) taxa matrix and plotted against the geographical distance between the two samples. Plots of the different sampling campaigns are overlaid and distinguished by color. A plot depicting all combinations of sample pairs and therefore accounting for possible lag effects in species dispersal does not reveal any significant correlation (data not shown)

similar than the more distant ones (up to 10 km apart). These results did not change when sequence frequencies were used as proxy instead of presence/absence data (data not shown). Second, to verify this observation and to test whether geographical distance affected only certain taxa in our sampling area, a taxa-wise spatial autocorrelation test was conducted. This analysis found no correlation between the distribution of taxa and their geographical location (please refer to Supplementary material on Spatial Autocorrelation calculations at Dryad https://doi.org/10.5061/dryad.5hqbzkh6w).

DISCUSSION

In this study we addressed two main questions. First, we sought to evaluate whether a deep-amplicon-sequencing approach of eDNA provided a detailed, nearly complete, snapshot of the biodiversity in small water bodies, such as KH. For this purpose, we used the small subunit of the ribosomal RNA as a general marker, rather than searching for target organisms using taxa-specific methods such as specific primers or microarrays (Bylemans et al., 2019; Deiner et al., 2017). Second, by using the above approach, we investigated how much of the variability in aquatic community composition and biodiversity was explained land-use type in the surroundings of small water bodies.

Deep sequencing of eDNA

Broad-target amplicon sequencing has been used for biodiversity studies for nearly four decades with ever-evolving taxa coverage, in particular, as evolving databases allow for better design of new primers and sequencing depth increases as technology evolves. Therefore, we chose to couple this established approach with methods for capturing rare and naked DNA as utilized in eDNA studies. At the same time, we used a separate deep sequencing approach for *Archaea*, *Bacteria*, and eukaryotes to improve the assay specificity and the chances of recovering rare taxa within each domain.

Our analysis focused on taxonomic entities and did not account for microdiversity (i.e., strain variability in marker-gene sequence) as can be resolved by defining amplicon sequence variants. This choice, following the approach of the SILVA NGS analysis pipeline (Ionescu et al., 2012), considered identical taxonomic entities as likely to have identical or similar functionality, although for microorganisms these entities may represent ecotypes coming from two adjacent yet separate microniches within one KH. Species accumulation curves, separately calculated for each sample and for *Bacteria*, *Archaea*, and eukaryotes (Figure 3a–c), showed that more than 50% of the total discovered taxa per sample were discovered in the first 25% of the sequences and a clear decrease in discovery rate had been observed already before. Given the sequencing depth and the large sample volume, it is not surprising that, despite this decrease, new taxa were continuously discovered without apparently approaching an asymptote (Dethlefsen et al., 2008; Huber et al., 2007; Shirazi et al., 2021). Therefore, a large portion of the reads was due to the discovery of relatively rare taxa contributing to a high percentage of the overall number of discovered taxa. This was confirmed by the curves rapidly reaching a plateau; when rare taxa, represented by individual sequences per sample, were removed our data were sufficient to cover most of the diversity. Sample-wise taxon accumulation plots showed that 75% of the total number of the observed taxa were represented by less than 25% of the samples (Appendix S1: Figures S10 and S11), supporting the notion that overall diversity was well covered. Alternative taxonomic annotation pipelines (e.g., *Kraken2*) resulted in lower taxonomic diversity, that is, sequences attributed to different organisms in the presented annotations were merged into single taxa by those alternative methods. Therefore, the results of our species accumulation analyses represented an upper boundary and perhaps an overestimation of taxonomic diversity, suggesting that the sequencing depth we used had even greater coverage.

The bacterial and archaeal community composition was not informative regarding the coverage of rare species and overall bacterial diversity. This was due to the high abundance of these tiny cells in water, typically ranging between 10^5 and 10^8 ml^{-1} (Bižić-Ionescu et al., 2015) and the large volume of water concentrated

for the sequence analyses. In contrast, our samples were likely to contain most microscopic eukaryotes as intact organisms, and larger taxa can be partly derived from decomposing cells and naked DNA in the water. Therefore, the discovery of multicellular taxa such as plants, insects, amphibians, fish, birds, and mammals, whose DNA was expected to be rare in the volume sampled, demonstrated the success in capturing the nature of most permanent, and some transient, organisms in the specific waterbody. This was in line with the taxa-independent rarefaction curves discussed above, suggesting that most of the diversity in the collected samples was captured. The presence of vertebrates such as fish, birds, and mammals could be confirmed either by direct observations or recent tracks on the KH shores, whereas the diversity of benthic macroinvertebrates and rotifers matched or exceeded those observed in parallel surveys using classical microscopic methods (Onandia et al., 2021; C. Musseau, unpublished data). This was consistent with previous studies. Although the taxonomic annotation of sequences largely depended on the quality and comprehensiveness of the databases used, the diversity coverage was independent. Accordingly, it has been repeatedly shown that the species detection and sensitivity of eDNA-based studies exceeds that of classical methods (Deiner et al., 2017; Emilson et al., 2017; Fernández et al., 2018; Kim et al., 2019; Yang & Zhang, 2020).

We therefore concluded that our deep eDNA amplicon sequencing approach of general marker genes could capture the overall (but not absolute) biodiversity across the domains of life in small water bodies, providing a reliable qualitative overview of resident and transient organisms, including resting stages in the sediment. Nevertheless, even samples for which ~3 million reads were obtained, the coverage of the taxonomic diversity did not reach a plateau. Accordingly, the coverage obtained in the present study would be too low to analyze microdiversity. Studies targeting specific taxa or a single taxon will benefit from a more targeted approach using designated primers for one or more genes. However, deep sequencing of eDNA marker genes provides a reliable, rapid, and cost-effective method when a detailed cross-taxa overview and total-biodiversity assessment is desired, for instance in surveys motivated by conservation efforts.

Land-use effects on biodiversity in water

Land use is expected to affect the composition of both permanent and transient members of aquatic communities. For example, intensive agriculture has been shown to result in the decrease in plant (Altenfelder et al., 2014; Meyer et al., 2013), bird (Donald et al., 2006), invertebrates (Wilson et al., 1999), and amphibian (Berger et al., 2011) diversity. Similarly, differences in communities have been documented between ponds in urban vs. rural environments (Akasaka et al., 2010; Joniak et al., 2007) and between lotic waters in forested and agricultural landscapes (Fasching et al., 2020). We tested for land-use effects on α- and β-diversity in the water column and sediment of the sampled KH. Taxonomic richness, Chao I index and species accumulation curves all showed sediments to be more diverse than water. As sequencing depth (Figure 3) and sampled biomass were comparable between sediments and water, this may be a result of more niches being available for microorganisms in sediments, but could also be due to long-term (decades) accumulation of dead organisms and naked DNA. The difference between the sediment and water community is likely to be driven by the long-term accumulation of DNA from different periods of the KH, the presence of eggs and resting stages, the anoxic nature of submerged sediments selecting for specific organisms and the likely introduction of DNA from terrestrial organisms, in part during dry periods. In contrast, the water column samples merely represented snapshots of the current community.

We did not detect any significant differences in taxonomic richness between land-use types, neither in sediment nor in water samples. This did not change when taxa were separated into *Archaea*, *Bacteria*, and eukaryotes (Appendix S1: Figure S12). However, some significant differences were apparent when samples of different sampling campaigns were separately analyzed. Specifically, taxonomic richness is higher in forest water samples collected in March and June compared with the other land-use types, suggesting a stronger seasonal than land-use effect. In several cases, forest samples also stood out with respect to environmental parameters (Appendix S1: Figure S2). This was in contrast with grassland and arable fields, which were generally not significantly different from one another. The latter may be a result from weak organismal dispersal barriers in the open land, whereas forest KH could be shielded by an arborous buffer zone. In addition, tree cover also results in reduced evaporation, alters the light regime and provides higher input of organic matter as plant litter. Additionally, land-use type is not a permanent feature with transitions of grasslands to arable fields being more common than the other way around (Nitsch et al., 2012; Serrano et al., 2017).

The effect of seasonality (time of sampling) is further evident in β-diversity for which more of the variability between samples can be explained by the sampling period rather than land-use type. The percentage

variability of the physico-chemical variables that can be explained by seasonality is much lower than that of β-diversity (2.5% vs. 25%). Therefore, it is unlikely that the seasonality effect on the communities is driven by seasonal changes in chemical parameters. The γ-diversity of different taxa (with similar and different trophic function) (Figure 5) further showed many groups of organisms that followed seasonal changes in richness. These changes were reflected in an increase in richness in early or late spring, often followed by a decrease in summer and autumn across all three land-use types. The latter could be related to organisms, such as insects, with life cycles that included aquatic stages and emergence in (late) spring.

Comparing the percentage variability explained by seasonality to that explained by land-use type for the entire community, or for selected taxonomic groups, it became evident that seasonality is the main factor determining community composition. This suggests that species can occur, actively or passively, in all land-use types throughout the year. However, when inspecting the different distribution patterns of each taxon across the land-use types, it became evident that the land-use type influenced the relative abundance of specific taxa, that is, their ability to establish a local population. This was clearly apparent for microorganisms in our dataset, and may also have been the case for larger organisms, as suggested by our sediment data (e.g., Crustaceans; Figure 8b). However, for larger organisms this cannot generally be evaluated without a more targeted approach.

Land-use effects on biodiversity in sediments

Incorporating sequence frequency in the analysis as a proxy for abundance of microorganisms enhanced the percentage of variability explained for some of the analyzed taxonomic groups, specifically when applied to the sediment compartment. As explained below, this suggested that sediment possesses a 'memory' that in part documents the response of aquatic biodiversity to the intensification of agriculture in the region since the early 1950s (Bauerkämper, 2004). A previous analysis of carbon and nitrogen isotopes in relation to changes in land use suggested that the long-term effect of agriculture can be traced in sediments of the KH in our study area. eDNA degradation in sediments is significantly slower than in the water column (Harrison et al., 2019; Sakata et al., 2020). The occurrence of planktonic phototrophs (eukaryotic algae and Cyanobacteria) in sediment, particularly in deeper layers (>5 cm), was direct evidence of the resulting accumulation of DNA from past communities in the sediments. This was further supported by DNA from planktonic crustaceans found also in both sediment layers distinguished in our study. This implied that our analysis of eDNA in sediments also reflected the distribution of organisms integrated over the sedimentation period. A corollary of this conclusion was that comparisons of the eDNA of such pelagic taxa between surface-water and sediment samples could inform us about past communities and could therefore be related to long-term changes in land use or other important environmental factors. For KH, as for other ponds that are not inundated throughout the year, interpretation of sediment results must consider enhanced bioturbation during dry periods.

The sedimentation rates previously estimated from two KH that were part of the present study (KH258 and KH807; Kleeberg et al., 2016) corresponded to an average age of 15–30 years prior to this study in the upper 5 cm and to 50–100 years at 20 cm depth. These values are in line with previous studies in the area, which concluded that the sedimentation rate increased from 1–2 mm $year^{-1}$ prior to 1960, to 5 mm $year^{-1}$ afterward (Frielinghaus & Vahrson, 1998). However, sedimentation rates determined in similar KH in Poland (Karasiewicz et al., 2014) based on ^{14}C dating of organic matter, placed the upper 5 and 20 cm to be as old as 900 and 1500 years ago, respectively. Meij et al. (2019), while supporting the 100 year range, showed large variability among the KH in the study area. Accordingly, depending on the sedimentation rate (and extent of sediment re-working) in the different KH, the increased percentage of explained variability in the sediment fraction, either for the total community or for specific taxonomic groups with different trophic functionality, can be differently interpreted. Most KH in this study were characterized by rapid sedimentation rates, the sediment eDNA reflected the response of the KH communities to the agriculture intensification in the area since the 1950s (Sommer et al., 2008). In contrast, a slow sedimentation rate would mean sediments still included periods when low-input agriculture was the main practice in the area. However, several facts point to fast sedimentation as the more likely scenario. First, land use in the area has likely changed considerably over the last centuries (Kaplan et al., 2009; Nicolay et al., 2014). Second, only low-input agriculture was practiced in the area prior to the 1950s (Bauerkämper, 2004; Sommer et al., 2008). Last, many of the taxa resulting in differences between the sediment and water were primary producers, conceivably responding to increased inputs of agrochemicals, notably P and N from agriculture (Table 1). Therefore, the sediment eDNA could well reflect community changes triggered by intensified agriculture. The separate clustering

of eukaryotic phytoplankton from the upper and lower sediments, further suggested a recent change in communities in the last 20–30 years as dated by Kleeberg et al. (2016). Coupling thin-layer sediment eDNA analysis with sediment dating in a large number of KH is needed to reinforce this tentative conclusion.

Land-use preferences of taxa

Intensive agricultural land use has been shown to result in biotic homogenization, erasing even subtle patterns resulting from other land-use types within a landscape dominated by arable fields (Buhk et al., 2017; Smart et al., 2006). Indicator species analysis revealed that no single taxon was uniquely associated with a specific land use, although some taxa showed a higher preference to one or two (out of three) land uses (Figure 7a). As expected, based on the above discussion, this analysis also showed a higher number of taxa in sediment than in water, being associated with a particular land-use type. The lowest number of taxa that was specifically associated with a single land-use type was assigned to arable fields, whereas forests harbored the most, followed closely by grassland. This contrast between arable fields vs. forests and grasslands may suggest that the latter two represent more constant environments. The continuous mechanical processing (e.g., plowing and harvesting) of fields results in constant morphological restructuring of niches and destruction of macro- and microgradients, possibly influencing littoral organisms or those moving between the aquatic and terrestrial environments. Furthermore, crop rotation and subsequent alteration in the type of agrochemicals may affect the KH water, driving continuous changes in the overall community. The quantitative associations of different taxa to specific land-use types visualized through ternary plots support the results of the indicator species analyses, suggesting that aquatic biodiversity was likely homogenized at the regional level over more than half a century of intensive agriculture practice, independent of land use immediately adjacent to the KH.

Opposite tendencies in sediment and water samples were evident in the community as a whole and the separately analyzed communities of *Bacteria* and *Archaea* vs. eukaryotes. Sediment samples harbored more taxa with a higher affinity toward arable fields, reflecting probably the archived response to the early days of intensive agriculture in the area. In contrast, taxa in water samples were more inclined toward forest and grassland, suggesting that, despite the overall homogeneity, some taxa may still find refuge in non-arable areas. Interestingly, bacteria and eukaryotes in water samples exhibit a different pattern. Bacteria are more inclined toward grasslands, with *Cyanobacteria* dominating the grassland-associated taxa, and eukaryotes toward forests, with fungi being the largest group of forest-associated eukaryotic taxa. These differences probably reflect light availability in grassland and plant litter inputs in forest KH, respectively.

The results of the indicator species analysis are supported by the joint species distribution models showing that only few bacteria and eukaryotic taxa are positively or negatively associated with a specific land use. The overall low proportion of land use in the variance partitioning, resulting from the sum of low association coefficients with the different land-use types, further suggests that land use does not have a major contribution to species distribution in the studied area.

Buffering of land-use effects are likely negligible

Our study showed that none of the organisms detected in water samples was associated with a specific land-use type. In addition, the community as a whole was not structured by land-use type. One likely explanation is the ubiquitous and long-lasting eutrophication in our study region resulting from intensive agricultural practices during the last decades (Gunnar Lischeid et al., 2018). Indeed, the TP concentration found in all ponds throughout the study period corresponded to eutrophic to hypertrophic conditions (Wetzel, 2001). Additionally, whereas some individual chemical parameters did differ among land-use types (e.g., DOC), the latter did not explain the chemical variability among the KH, indicating a chemical homogenization effect. Lischeid et al. (2018) showed that KH in the study area are connected via groundwater. Therefore, the nutrient and mineral concentrations in a given KH may reflect an integral part of the inputs in the area rather than a local chemical signature. These physical and chemical properties of the water play a major role in shaping aquatic communities. Therefore, the local biota is likely to reflect the water it resides in, rather than mirror the surrounding land-use type. Joniak et al. (2007) conducted a large study on zooplankton and macrophytes covering 165 ponds in Poland situated in a similar environmental setting as our KH, but covering a larger eutrophication gradient. Certain taxonomic groups tended to be associated with different eutrophication states of the ponds but, as in our study, no correlation was found to land-use type. Vegetated buffer strips at least 5 m in width around the ponds were proposed to minimize or even eliminate the influences of surrounding land cover, in particular nutrient inputs from arable fields. In our

area, however, the connection of KH via groundwater could have minimized such buffering effects of the surrounding vegetation. This lack of notable land-use influences is consistent with analyses of other rural ponds in different land-use types (Declerck et al., 2006; Joniak et al., 2007; Pätzig et al., 2012), this is in contrast with a pronounced land-use effect on ponds found when comparing urban and rural ponds and lakes (Akasaka et al., 2010; Kraemer et al., 2020).

Geographical distribution

Aquatic organisms belonging to all domains of life are transferred between KH via different abiotic and biotic vectors such as wind, insects, mammals and humans. The dense network and long existence of KH in the region is likely to present transfer opportunities to all organisms over time. Choudoir et al. (2017) showed that the dispersal area of bacteria lies in the order of thousands of km^2. Conversely, species with elevated loyalty to birth ponds such as amphibians (Smith & Green, 2005), who typically do not wander further than a few hundred meters from "home," will encounter new ponds within this range due to the high density of KH in the area. Nevertheless, once these organisms arrive at a new location, they may be unsuccessful in establishing a new local population and are therefore eradicated or remain extremely low in abundance or in a dormant state until an opportunity arises for them to multiply (Ionescu et al., 2010). This may be the case for most bacteria and both unicellular and some multicellular eukaryotes. This explains how in a landscape with homogenized biodiversity, where generally "everything is everywhere," the difference among KH communities is not in par with geographical distance and is in line with the suggestion of Thompson et al. (2020) that, with increasing dispersal rates, β-diversity is eroded.

CONCLUSIONS

We demonstrate that broad-scale eDNA analyses can serve to identify specific taxonomic groups that may be influenced by land-use change or land management. Such groups can be subsequently investigated in detail through targeted studies at high taxonomic resolution using additional or alternative genetic markers. The enhanced sensitivity of sequence frequencies, as opposed to presence/absence data, to detect land-use effects on biodiversity in water or sediment suggests that, while species composition may not be influenced by land use, their ability to establish identical populations everywhere may exist. Accordingly, following low-cost, deep sequencing broad eDNA surveys, quantitative studies should be used to investigate specific conservation-prone taxa.

Our analysis of water and sediment eDNA resulted in opposing magnitudes of the effect of land-use type on the biodiversity of KH in an agricultural landscape. We propose that the higher land-use effect, noticeable in the deeper sediment data for the total community and specific taxonomic groups, is likely to reflect more than half a century of intensive agriculture. Sediment eDNA data revealed a response in the abundance of primary producers and planktonic consumers, possibly caused by the continuous eutrophication of KH as reflected in KH water chemistry today. This eutrophication process archived in the sediment could have led to the homogenization of biodiversity across the entire study area, resulting in a low detectable land-use effect on biodiversity patterns in the water samples, which represent the current situation. This broad-scale homogenization effect on aquatic biodiversity could have been reinforced by KH connectivity below and above ground. Below ground, groundwater can propagate the effects of one land-use type such as arable fields to KH located elsewhere. Above ground, the dense network of KH potentially facilitates tight coupling through active and passive species dispersal (Karnatak & Wollrab, 2020).

Our eDNA approach, capturing most taxa across *Bacteria*, *Archaea*, and eukaryotes, is consistent with previous studies focusing on specific groups. Most KH included in those studies were located in areas with a long history of industrialized agriculture. Although the small size of KH implies a strong influence of the surroundings, we propose that the lack of pronounced land-use effects results from the homogenization, and possibly loss, of KH biodiversity during a long period of intensive agriculture and the concomitant KH eutrophication. Interestingly, similar to our results on KH, some recent lake studies also showed minimal effects of land-use type (Abell et al., 2011; Catherine et al., 2016; Marmen et al., 2020).

This conclusion emphasizes the intricacies of aquatic biodiversity conservation in landscapes dominated by agriculture. In a broad survey summarizing the results of different conservation practices, Gonthier et al. (2014) concluded that the combination of decreased management intensity (i.e., less agrochemicals) coupled with increases in landscape complexity around arable fields and farms, is most suitable to prevent species loss. However, the effects of such measures in areas where biodiversity has been already homogenized over more than half a century are still unclear.

AUTHOR CONTRIBUTIONS

D. Ionescu and M. Bizic have equally contributed to this manuscript; D. Ionescu, M. Bizic, S. Wollrab, M. O. Gessner, H.-P. Grossart: designed the study; D. Ionescu, M. Bizic, R. Karnatak, C. L. Musseau, G. Onandia: collected samples; D. Ionescu, M. Bizic, R. Karnatak, C. L. Musseau, G. Onandia, M. Kasada, M. Ryo: analyzed data; D. Ionescu, M. Bizic, R. Karnatak, C. L. Musseau, G. Onandia, M. Kasada, S.A. Berger, J.C. Nejstgaard, G. Lischeid, M. O. Gessner, S. Wollrab, H.-P. Grossart: wrote the manuscript.

ACKNOWLEDGMENTS

We thank the farmers and landowners in the study area for their collaboration and making the sampled KH accessible. We also thank the Landwirtschaft- und Umweltamt – Landkreis Uckermark for assistance and support in sampling the forest KH in the Kiecker Nature reserve, and Dr. Günter Heise for accompanying us. Further thanks are due to Mr. Gonzalo Idoate Santaolalla, Dr. Jason Woodhouse, Dr. Luca Zoccarato, Mr. Darshan Neubauer, Mr. Alberto Villalba, Mr. Justin Stranz, and Mr. Ignaciao Rodanes Ajamil for sampling assistance. This study was funded by the German Federal Ministry of Education and Research (BMBF) within the Collaborative Project "Bridging in Biodiversity Science – BIBS" (funding number 01LC1501). M. Bizic was additionally funded through the DFG Eigene Stelle project (BI 1987/2-1). D. Ionescu was additionally funded through the DFG Eigene Stelle project (DI 98/3-1). All responsibilities for the content of this publication are assumed by the authors. We thank the three anonymous reviewers for their valuable comments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Sequence data generated in this project are publicly available in the NCBI Short Read Archive under project number PRJNA641761 at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA641761/. Additional data (Ionescu et al., 2022) are provided in Dryad at https://doi.org/10.5061/dryad.5hqbzkh6w.

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How to cite this article: D. Ionescu, M. Bizic, R. Karnatak, C. L. Musseau, G. Onandia, M. Kasada, S. A. Berger, et al. 2022. "From Microbes to Mammals: Pond Biodiversity Homogenization across Different Land-Use Types in an Agricultural Landscape." *Ecological Monographs* 92(3): e1523. https://doi.org/10.1002/ecm.1523