












# Land-use type temporarily affects active pond community structure but not gene expression patterns

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## Abstract

Changes in land use and agricultural intensification threaten biodiversity and ecosystem functioning of small water bodies. We studied 67 kettle holes (KH) in an agricultural landscape in northeastern Germany using landscape-scale metatranscriptomics to understand the responses of active bacterial, archaeal and eukaryotic communities to land-use type. These KH are proxies of the millions of small standing water bodies of glacial origin spread across the northern hemisphere. Like other landscapes in Europe, the study area has been used for intensive agriculture since the 1950s. In contrast to a parallel environmental DNA study that suggests the homogenization of biodiversity across KH, conceivably resulting from long-lasting intensive agriculture, land-use type affected the structure of the active KH communities during spring crop fertilization, but not a month later. This effect was more pronounced for eukaryotes than for bacteria. In contrast, gene expression patterns did not differ between months or across land-use types, suggesting a high degree of functional redundancy across the KH communities. Variability in gene expression was best explained by active bacterial and eukaryotic community structures, suggesting that these changes in functioning are primarily driven by interactions between organisms. Our results indicate that influences of the surrounding landscape result in temporary changes in the activity of different community members. Thus, even in KH where biodiversity has been homogenized, communities continue to respond to land management. This potential needs to be considered when developing sustainable management options for restoration purposes and for successful mitigation of further biodiversity loss in agricultural landscapes.

## KEYWORDS

agriculture, eRNA, land use, metacommunity, transcriptomics

Mina Bizic and Danny Ionescu contributed equally to the work.

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## 1 | INTRODUCTION

During the first half of the 20<sup>th</sup> century, Germany, as much as the rest of Central Europe, was characterized by low-input agriculture. Starting in the 1950s, intensive industrialized agriculture with increasing use of fertilizers and pesticides became standard (Bauerkämper, 2004; Sommer et al., 2008). This type of agricultural practice has negative consequences on biodiversity, notably for plants (Altenfelder et al., 2014; Meyer et al., 2013a), birds (Donald et al., 2006; Endenburg et al., 2019; Puente-Sánchez et al., 2018), invertebrates (Wilson et al., 1999) and amphibians (Berger et al., 2011; Berger et al., 2018). In addition, plant, insect and mammal communities have been homogenized in arable areas (Baessler & Klotz, 2006; Macdonald & Johnson, 2000; Olden et al., 2016; Spear & Chown, 2008; Vargas et al., 2015), as is typically reported after land-use intensification (Smart et al., 2006).

Kettle holes (KH) (known as potholes in North America) are small depressions in the landscape formed by the melting of trapped ice after the retraction of glaciers at the end of the last glaciation ~12,000 years ago. This has left, to this day, numerous KH sprinkled across northern Europe, northern North America and northern Asia, reaching up to 40 per km<sup>2</sup> in northeast Germany (Kalettka & Rudat, 2006). Accordingly, KH are the dominant aquatic landscape element in the region (Kalettka & Rudat, 2006) and are hotspots of biological activity (Nitzsche et al., 2017), serving as mineralization grounds for both aquatic and land-derived organic matter (Nitzsche et al., 2017; Onandia et al., 2018). Geographically close KH can differ in terms of biogeochemistry (Attermeyer et al., 2017), hydrology and biodiversity (Altenfelder et al., 2014; Lischeid & Kalettka, 2012; Platen et al., 2016), suggesting that they play a critical role in determining overall regional biodiversity (Joniak et al., 2007; Lischeid & Kalettka, 2012; Novikmec et al., 2016; Pätzig et al., 2012; Platen et al., 2016). KH serve as habitats for invertebrates with and without aquatic stages, refuges and breeding grounds for many amphibians as well as feeding areas for terrestrial organisms (Berger et al., 2013; Heim et al., 2018). Thus, alongside hosting a dynamic and diverse internal food web, KH are key components in aquatic–terrestrial interlinked food webs and important steppingstones for many terrestrial species.

D. Ionescu et al. (2022) used an environmental DNA (eDNA) approach for biodiversity assessment of KH in the northeastern German lowlands dominated by three different land-use types: arable fields, grasslands and forests. In contrast to the hypothesis that the community structure in KH of arable fields has been shaped by decades of intensive industrialized farming, no differences in species richness or community composition were found between KH in forest, grassland and arable patches in the same region. Instead, KH biodiversity appeared to be homogenized across the region, a common effect of intensive land use (Buhk et al., 2017; Meyer et al., 2013b; Onandia et al., 2021; Smart et al., 2006), indicating that intensive agriculture has also affected the KH not directly located in arable fields. Chemical analyses of sediment cores (Kleeberg et al., 2016; Nitzsche et al., 2017) indicated that intensive agriculture has led to high phosphorus and nitrogen inputs into KH, probably

resulting in the observed eutrophication (Lischeid et al., 2018). Since most KH in the study area are connected via groundwater (Lischeid et al., 2018), the chemical effects of agriculture could thereby also extend to KH in the surrounding grasslands and forests and forest patches.

eDNA analyses have been increasingly applied as a noninvasive, highly sensitive monitoring tool (Andújar et al., 2018; Beng & Corlett, 2020; Bylemans et al., 2019; Deiner et al., 2017). However, one of the limitations of the approach is that eDNA analyses capture not only the active community but also organisms that are inactive or have long abandoned the investigated habitat, with an expected eDNA lifetime in water of lentic systems such as the KH of the order of a few days to weeks (J. B. Harrison et al., 2019) and much longer (months, years, decades) for sediments (Corinaldesi et al., 2008; Sakata et al., 2020). Therefore, eDNA can reveal long-term environmental changes but probably falls short of revealing short-term effects of land-use change, especially in highly dynamic ecosystems such as KH, unless those effects are very strong. Metatranscriptomics is a remedy to this limitation. The approach refers to analyses of the full set of expressed genes in a community as obtained by sequencing the total RNA. Environmental RNA (eRNA) provides information specifically on the active organisms, both on community composition, derived from known taxonomic markers such as the small and large rRNA subunits, and on functionality, derived from the expression patterns of functional genes (Yates et al., 2021). It was additionally proposed that in addition to providing information on the response of organisms to environmental signals, such as stressors (Yates et al., 2021), eRNA can provide information on trophic interactions between organisms (Cristescu, 2019). RNA-based expression patterns typically represent recent activities at timescales ranging from minutes to hours—given the short half-life of RNA. As a result, the likelihood of observing large and transient organisms in metatranscriptomics analysis is low. Thus, this type of analysis targets organisms currently or recently active in the sampled volume of water. Importantly, since more active organisms produce more ribosomes, the relative abundance of rRNA transcripts represents the distribution of activities within the community, which may be unrelated to the abundance of individual organisms. Therefore, we will refer to metatranscriptomics-derived rRNA data as the “active community structure” (Blazewicz et al., 2013).

In this study, we aimed to determine the taxonomic and functional diversity of the active communities in 67 KH located in arable fields, grasslands and forests, distributed within an area of ~150 km<sup>2</sup>. We expected the active community structure and their spatiotemporal gene expression patterns to depend on land-use practices and related environmental conditions at the time of sampling, such as the use of fertilizers in agriculture or the quality of carbon in KH within forests, grasslands and arable fields, which is expected to differ because of differences in vegetation cover in the riparian zone and the extent of aquatic–terrestrial coupling. Accordingly, we hypothesized that in a region characterized by industrialized agriculture and biodiversity homogenized across KH, land use is reflected by organismic activity, resulting in some

KH organisms being more active than others at certain times. Specifically, we addressed three main questions: (i) Does land use shape the structure of the active community as reflected in deep sequencing of total RNA, in contrast to lack of patterns in eDNA? (ii) Does land use drive the gene expression patterns of metacommunities? (iii) Is there metabolic functional redundancy within the KH meta-ecosystem in agricultural landscapes?

## 2 | METHODS

### 2.1 | Study site

The sampling focused on 67 KH in northeastern Germany (Uckermark district, State of Brandenburg; Figure S1), 52 of which were sampled in May and 43 were sampled 5 weeks later in June. No samples were taken in dried-up KH, resulting in a total of 41 KH sampled on both occasions. Of the sampled KH, 36, 7 and 9, and 28, 6 and 9 were in arable fields, grasslands and forest in May and June, respectively. The area is among the least populated regions in Germany. The study area has long been used for extensive agriculture, with >90% of the land used as arable fields (Kalettka & Rudat, 2006). This includes areas where land use was changed from arable fields to grasslands nearly two decades ago (Serrano et al., 2017). Since the 1950s, agriculture in the area has become industrialized, which included increased fertilizer and pesticide use.

KH were categorized according to the predominant land-use type within a perimeter of ~50 m. Accordingly, all KH in crop fields (rapeseed, corn, wheat, barley, rye, triticale) are referred to as "arable field KH," both those directly adjacent to the fields and those surrounded by natural vegetation. KH in grasslands are referred to as "grassland KH." "Forest KH," located in the Kiecker nature reserve (Nordwestuckermark, Brandenburg), comprised KH in vast mixed forests (beech and oak) as well as in forest patches (>100 m in diameter) surrounded by arable fields (Figure S1). However, the last category was treated as "arable fields" in analyses where we applied a stricter definition of forests.

### 2.2 | Sampling

Water samples for RNA analysis were collected during two sampling campaigns (each 2–3 days) in late spring and early summer 2017, together with samples collected for eDNA analysis (D. Ionescu et al., 2022). Water samples were taken whenever water was available. To obtain a representative sample from each water body, total volumes of ~20 L were collected from 5–15 different locations in each KH, with the number of individual samples varying with KH size. The water was combined in prewashed buckets and mixed, before 1.7 L was resampled for RNA analysis into plastic canisters containing 800 ml RNA-stabilizing solution (15 mM EDTA, 18.5 mM sodium citrate, 4 M ammonium sulphate). Samples were placed in iceboxes

containing a mixture of ice and table salt to lower the freezing point. Upon arrival in the laboratory, the samples were frozen at –80°C until further analyses.

### 2.3 | RNA extraction and processing

Before RNA extraction, standard volumes of water (2.3 L: sample + fixative) were sequentially filtered on a Nalgene filtration tower (ThermoFisher Scientific). Polycarbonate filters with pore sizes of 10 and 5 µm (Millipore TCTP04700, TMTP04700; Merck) were used, as well as combusted GF/F and polycarbonate filters with pore size of 0.2 µm (Whatman WHA1825047, Millipore GTTP04700; Merck). All filter diameters were 47 mm. The entire water volume was passed through all filters. The filters were rinsed twice with 50 ml autoclaved MQ water to remove salts and subsequently flash frozen.

To avoid introducing batch effects (Bálint et al., 2018), Eppendorf tubes containing the filters representing sample fractions were shuffled and randomly allocated to separate batches. RNA was extracted following a phenol/chloroform procedure modified from Nercessian et al. (2005). In brief, a CTAB extraction buffer containing SDS (sodium dodecyl sulphate) and *N*-lauryl sarcosine was added to the samples together with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) solution. The samples underwent a bead-beating treatment, followed by centrifugation, cleaning with chloroform and precipitation with PEG-6000 (Sigma-Aldrich). The precipitated DNA/RNA mix was rinsed with 1 ml 70% ethanol, dried and dissolved in water. Finally, all extractions belonging to a given sample were pooled.

DNA was removed by two sequential treatments with the TurboDNAfree Kit (Invitrogen ThermoFisher Scientific), after which the samples were transferred to an RNastable 96-well plate (Sigma-Aldrich) for shipment. A total of 98 samples were sequenced at MrDNA (Molecular Research) according to the following procedure. The RNA samples were resuspended in 30 µl of nuclease-free water and cleaned using the RNeasy PowerClean Pro Cleanup Kit (Qiagen). The concentration of total RNA was determined using the Qubit RNA Assay Kit (Life Technologies, ThermoFisher). Next, 750 ng of total RNA was used to remove the remaining DNA contamination using Baseline-ZERO DNase (Epicentre, Lucigen) according to the manufacturer's instructions, followed by a purification step with RNA Clean & Concentrator-5 columns (Zymo Research). DNA-free RNA samples were used for library preparation using the TruSeq RNA LT Sample Preparation Kit (Illumina) according to the manufacturer's instructions. Following library preparation, the final concentration of all the libraries was measured using the Qubit dsDNA HS Assay Kit (Life Technologies, ThermoFisher), and the average library size was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). The libraries were then pooled in equimolar ratios of 2 nM, and 6 pmol of the library pools was clustered using the cBot (Illumina) and sequenced 2 × 125 paired-end reads on 20 lanes for 250 cycles using the HiSeq 2500 system (Illumina). The sequenced data were submitted to the NCBI short read archive under project

no. PRJNA640812 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA640812>).

Raw files of paired-end reads were quality-trimmed using TRIMOMATIC (version 0.39) (Bolger et al., 2014). rRNA reads were removed by stringent mapping to a database of short subunit (SSU), long subunit (LSU) and 5S rRNA assembled manually from the SSU and LSU SILVA databases (version 132) (Quast et al., 2013). Subsequently the SSU rRNA was annotated using PHYLOFLASH (Gruber-Vodicka et al., 2020) and KRAKEN2 (Wood et al., 2019). The non-rRNA sequences were further checked using BARNAP (version 0.9). The clean non-rRNA reads of each sample were individually processed according to the Trinotate (<https://github.com/Trinotate/Trinotate.github.io/wiki>) pipeline, including assembly with TRINITY version 2.6.5 (Grabherr et al., 2011), protein prediction using TRANSDCODER (<https://github.com/TransDecoder/TransDecoder>), and annotation with DIAMOND BLASTP and BLASTX (Buchfink et al., 2015) against the Uniprot database. Sequences were also annotated with HMMSEARCH (Gough et al., 2001) and the pFam (Finn et al., 2014) database. KALLISTO (version 0.44) (Bray et al., 2016) was used to map the reads from each sample against the samples' assembled transcripts resulting in TPM (transcripts per million) -normalized counts. The data were merged to generate abundance matrices for statistical analysis. BLASTP, BLASTX, EC-number and Subsystems' matrices were obtained and separately analysed. The presented results stem from the Subsystem annotation of the data. More information on SEED subsystems is available at: [https://www.theseed.org/wiki/SEED\\_Viewer\\_Manual](https://www.theseed.org/wiki/SEED_Viewer_Manual).

## 2.4 | Analysis of physicochemical characteristics

Temperature (Temp), conductivity (Cond), pH and oxygen saturation ( $O_2$  Sat) were measured *in situ* during sampling using a portable multiprobe (HI98194; Hanna Instruments). An additional 1 L of water was collected for analyses of nutrients and other major ions as detailed below. The collected water was immediately frozen by placing it in a container with ice mixed with table salt (NaCl).

Water analysis followed standard methods as defined by the German Institute for Standardization (DIN).  $Ca^{2+}$ ,  $Mg^{2+}$ ,  $K^+$ ,  $Na^+$  and total Fe were analysed using inductively coupled plasma optical emission spectrometry (ICP-iCAP 6300 DUO; ThermoFisher Scientific).  $Br^-$ ,  $Cl^-$ ,  $NO_3^-$ ,  $NO_2^-$  and  $SO_4^{2-}$  were analysed using ion chromatography (882 Compact IC plus; Deutsche Metrohm). Ammonium ( $NH_4^+$ ) and ortho-phosphate ( $o-PO_4^{3-}$ ) were measured spectrophotometrically (SPECORD 210 plus; Analytik Jena). Total phosphorus (TP) was measured as ortho-phosphate after microwave digestion (Gallery Plus; Microgenics). Dissolved organic carbon (DOC), total organic carbon (TOC) and total nitrogen (TN) were determined using an elemental analyser (TOC-VCPI; Shimadzu Deutschland) with chemiluminescence detection. The specific absorption coefficient at 254 nm (SAC) was measured using a spectrophotometer (SPECORD 210 plus) as an approximation of the dissolved aromatic carbon content (Weishaar et al., 2003). The ratio of SAC to DOC concentration was used as a rough indicator of DOC composition. The specific UV

absorbance at 254 nm ( $SUVA_{254}$ ) correlates with the hydrophobic organic acid fraction of dissolved organic matter (DOM) (Spencer et al., 2012) and is a useful proxy for DOM aromatic content (Weishaar et al., 2003) with a higher  $SUVA_{254}$  value indicating a higher content of aromatic molecules.

## 2.5 | Statistical analysis

Statistical analyses were conducted on abundance matrices obtained from the community and functional annotation pipelines. These data are provided as Data S1–S4 (see Results) and were additionally deposited at Dryad under <https://doi.org/10.5061/dryad.0k6djhbm>. Multivariate (nonmetric multidimensional scaling, NMDS) (Kruskal, 1964), principal components analysis (PCA) (Pearson, 1901), canonical analysis of principal coordinates (CAP) (Anderson & Willis, 2003), permutational analysis of variance (PERMANOVA) (Anderson, 2017), distance based linear models and redundancy analysis (DistLM-dbrDA) (Legendre & Anderson, 1999; McArdle & Anderson, 2001), and diversity (richness and evenness) analyses were conducted using the PRIMER6 (version 6.1.1) + Permanova Package (version 1.0.1, Primer-E, Quest Research). Resemblance matrices of the community and functional data were calculated using Bray–Curtis dissimilarity following a square-root transformation of the original data. NMDS was conducted retaining the ordination with the lowest calculated stress of 1,000 iterations. PERMANOVA was used to test for the effects of land-use type, seasonality (i.e., campaign number) or both. PERMANOVA was conducted with 999 iterations and unrestricted permutation of the full data. CAP was used to plot the data according to factors found by PERMANOVA to have a significant effect.

DistLM-dbrDAs were used to test for the explanatory power of physicochemical variables on community structure, the explanatory power of the bacterial community on the eukaryotic community and vice versa and the explanatory power of the bacterial and eukaryotic community on gene expression patterns. To test the explanatory power of the bacterial community the eukaryotic community as well as that of the different communities on the gene expression patterns, the top 90 taxa were used from each community. This number is derived by a requirement of the method to have fewer explanatory variables (taxa) than samples ( $n = 98$ ). The results of the DistLM-dbrDA output consists of marginal and conditional tests. In the case of marginal tests, each variable is tested individually for its correlation with the data (univariate analysis). This provides insight into how strongly each variable drives the statistical differences between samples. As some environmental variables might be correlated with one another, there is a need to complete the univariate analysis by testing how much of the variation between samples is explained by a variable while considering the other variables used in the analysis. Part of the variation might be explained by two or more variables. To deal with this potential covariance and overlap in the explained variation, we used sequential (conditional) tests designed to deal with such a data structure. Here the DistLM-dbrDA approach treats the residuals of the data fitted with a variable as the new response matrix and tests how much of the variability is

explained by a second variable. The results of both marginal and sequential tests are reported, yet we consider in our discussion those of the conditional tests.

ANOVA coupled with pairwise tests to identify difference between sample groups (Mann-Whitney test, Dunn's test) were performed using the *PAST4* software (Hammer et al., 2009). Ternary plots were generated using the *ggtern* package (Hamilton & Ferry, 2018) in R version 3.5 (The R Core Team, 2018). An indicator species analysis was done using the *indicspecies* R package (version 1.7.8; Cáceres & Legendre, 2009) testing for the IndVal index, as well as Pearson's phi coefficient of association (Chytrý et al., 2002). The latter was calculated based on both presence/absence and sequence frequency data and included the appropriate functions and corrections according to the *indicspecies* package manual (version 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 outcome of the analysis was corrected for the fact that there were more sites in arable fields than in grasslands and forests. Data for ternary plots were generated as the average relative sequence frequency per taxon/function within each land-use type or as average transcript TPM abundance per land-use type.

### 3 | RESULTS

#### 3.1 | Physicochemical characteristics

Water physicochemical characteristics (Figure 1; Table S1) varied greatly among KH within land-use types (i.e., forest, grassland or arable fields). Only a few variables were significantly different among land-use types or sampling campaigns (Table S2). Most evident was an increase in water temperature between May and June. Furthermore, oxygen saturation was significantly lower in forest KH than in arable fields, with grassland KH having intermediate saturation levels. Potassium ( $K^+$ ) concentrations in forest KH remained low in June and significantly differed from those surrounded by arable fields. Magnesium ( $Mg^{2+}$ ) and chloride ( $Cl^-$ ) concentrations in arable fields were significantly higher than in forest KH in May but did not differ from those in grassland KH. Conductivity in arable field KH was higher than in forest KH in both campaigns. Total N and P concentrations were high in almost all KH but did not differ significantly between land-use types nor between sampling times.  $NH_4^+$  concentrations were significantly higher in forest KH in both campaigns. Other than higher  $SUVA_{254}$  values in forests than in arable fields in May, no significant difference in  $SUVA_{254}$  values was observed between the different land-use types, nor between the two sampling periods (Table S2).

#### 3.2 | Determinants of active community structures

Metatranscriptomics analysis of the total of 98 samples resulted in  $47 \pm 7$  and  $5 \pm 1$  million rRNA and non-rRNA paired-end reads per

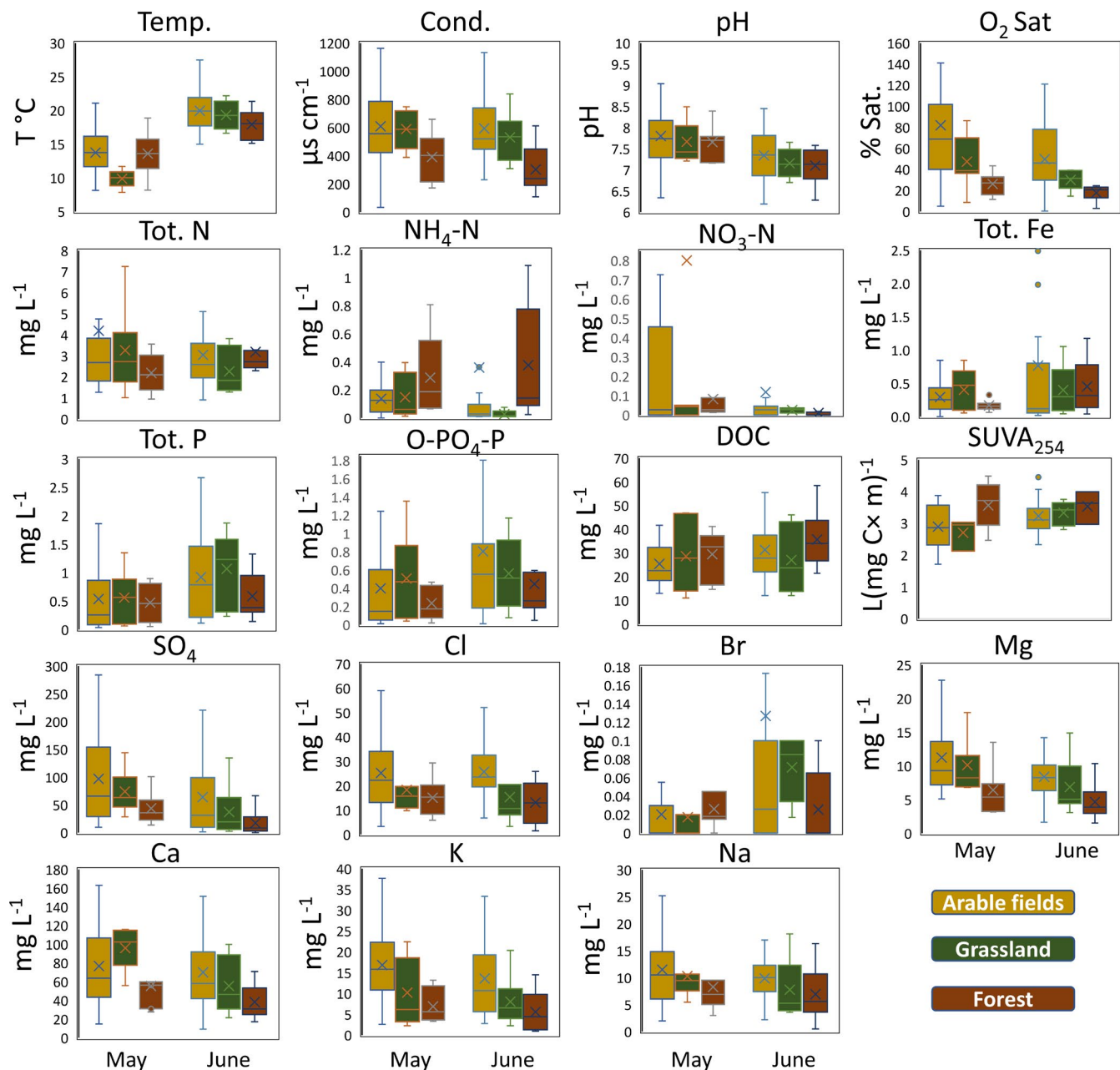
sample, respectively, after quality trimming. These sequences were separated and analysed individually (see Methods). The community analysis was clustered according to the assigned taxonomic name. While different taxonomic annotation methods (see Methods) resulted in different numbers of taxa, the results of the subsequent analyses did not differ qualitatively (Figure S2). Similarly, functions assigned to assembled transcripts from each sample using different methods (see Methods) resulted in similar qualitative results (Figure S2). The eukaryotic component of the rRNA was seven times larger than the bacterial (Bacteria and Archaea) component on average (three times larger by median), and therefore, when possible, the two communities were also analysed separately. The results of the taxonomic annotation are provided in Data S1. The results of the functional annotations are provided in Data S2 and S3 as the original BLASTP and BLASTX annotation, and converted to SEED Subsystems (Aziz et al., 2008), respectively.

Parameters that by distance-based linear models significantly contribute individually to the structure of the active community are shown in Figure 2a–c. However, only a few of these (in bold) were significant contributors when the same parameters were tested in an additive, sequential manner (Table S3), that is conditional tests in which parameters are tested whether they significantly explain the remainder of the data that was not fitted to a previously tested parameter. Temperature (Temp), pH, conductivity (Cond) and  $O_2$  saturation ( $O_2$  Sat) were significant drivers for the overall and eukaryotic community structure. However, only pH and temperature significantly affected the active bacterial (Bacteria and Archaea) community. The three redundancy analysis plots generated, using distance-based linear models, show a clear temporal separation between the active communities of midspring and early summer (Figure 2a–c).

The structures (abundance matrix) of the active bacterial and eukaryotic communities from both sampling campaigns were correlated with each other (Mantel test, Spearman's  $\rho = 0.46$ ,  $p = .01$ ). Therefore, we further investigated how much of the variability in the active bacterial community can be explained by that of the eukaryotic community. Based on the top 90 eukaryotic taxa (of all 97 samples), the first two axes of the distance-based redundancy analysis explain 37% of the total bacterial variability (Figure 2d). Distance-based linear models show that 19 eukaryotic taxa significantly ( $p \leq .05$ ) explain 47% of the bacterial variability with the amoeba *Arcella* sp. alone accounting for >7% (Table S4). Eleven of the remaining taxa are plants or algae producing potential bacterial substrates or inhibitors. Conducting the reverse analysis using the top 90 bacterial taxa results in the first two axes explaining 25% of the Eukaryotic variability (Figure S3). Overall, 24 bacterial taxa explain 55% of the variability of the active eukaryotic community, with the family Holofagaceae explaining 10% (Table S5).

The same set of tests was applied to the functional data (i.e., profiles of expressed genes) from the same samples. No environmental variable, whether individually or sequentially, was significantly related to the observed pattern of functionality (Table S3), contrasting



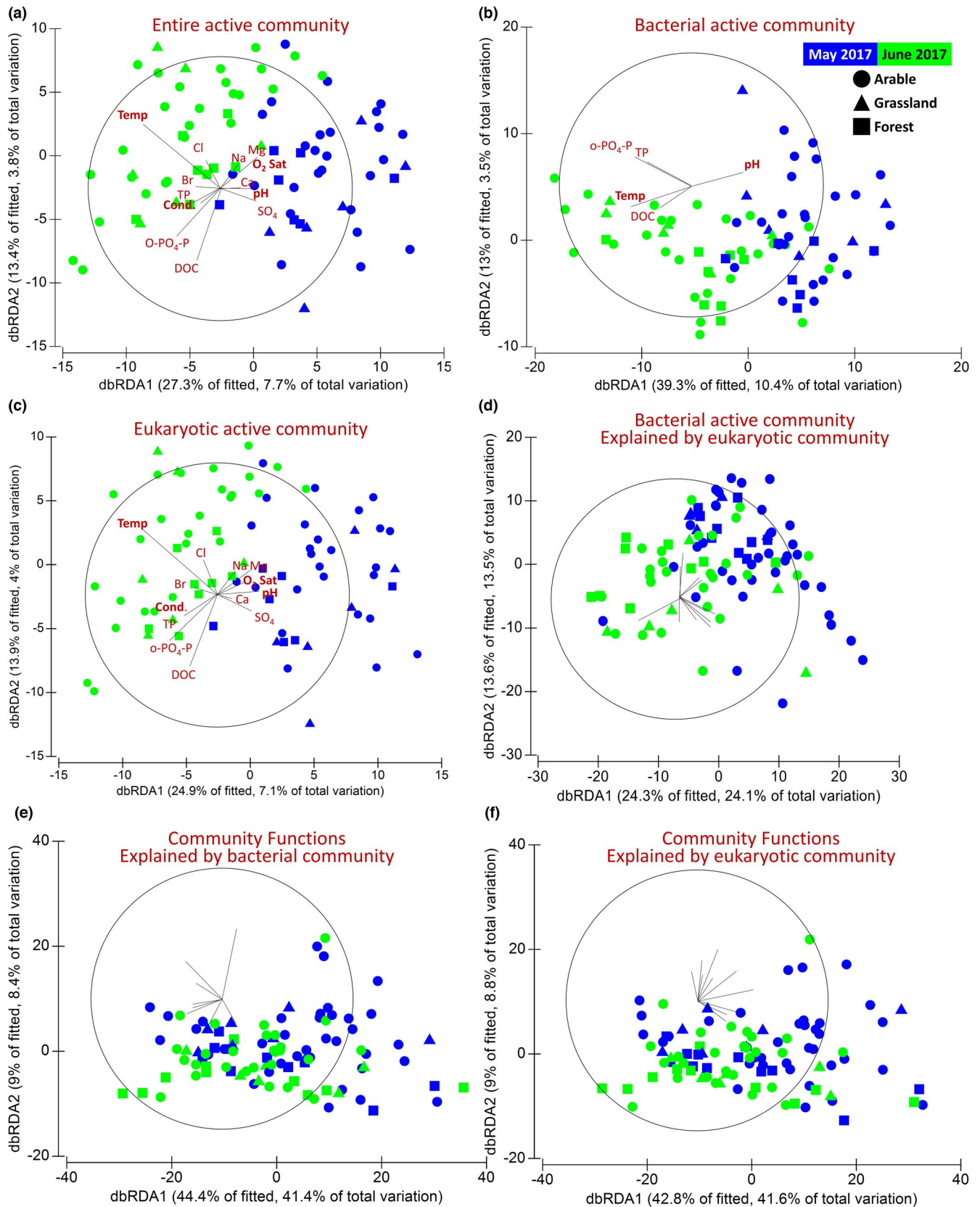


**FIGURE 1** Physical and chemical variables characterizing kettle holes (KH) sampled in May and June 2017 for RNA analysis. The solid line shows the median in each box while the cross marks the mean. Whiskers mark the 25<sup>th</sup> and 75<sup>th</sup> percentile. Table S1 provides detailed information for each variable and for all KH, and Table S2 shows the significance by which each land-use type and sampling point differ from each other

with the active community structure. Furthermore, PCA shows no clear sample separation either between sampling campaigns or among land-use types (Figure 2e,f).

We tested to what extent the structure of the bacterial (Figure 2e) or eukaryotic (Figure 2f) active community could explain the observed functional variability. Our analysis shows that

**FIGURE 2** RNA-based community composition (a–c) in a redundancy analysis generated by distance-based linear models (DistLM-dbrDA) accounting for all physical and chemical variables detailed in Figure 1 and Table S1. All single variables contributing significantly to the variation (Marginal tests) are shown. Only those marked in red were significant in a sequential additive model (Conditional tests; see main text and Table S3). Panel (d) shows that 37% of the variability in the community structure of active bacteria can be explained by the first two axes of a DistLM-dbrDA based on the 90 most expressed eukaryotic species. A similar analysis for the bacterial community with the eukaryotic community composition as explanatory factor is presented in Figure S3. Redundancy analyses of functional diversity with the bacterial (e) and eukaryotic (f) communities as explanatory factors. In both cases, the first two axes explain ~50% of the observed functional variability. Details on the specific taxa contributing to the patterns of (d) and (e,f) are given in Tables S4 and S5, respectively



the main active taxa from both domains independently explain a large portion of the functional variability. The first two axes of the redundancy analyses, relating the structure of the bacterial (Figure 2e) and eukaryotic (Figure 2f) active communities to the

observed functional variability, explain over 50% of the total variation (Table S6), indicating that the main taxa from both domains explain a large portion of the overall variability in functionality. However, despite explaining a similar proportion of the variability,

the opposite directionality of the correlation vectors for the different taxa (lines in Figure 2e,f) suggests different associations of the bacterial and eukaryotic communities with functionality. A distance-based redundancy analysis using a combined matrix consisting of the top 45 bacterial and 45 eukaryotic taxa explains a total of ~50% of the variability across the first two axes. The bacterial and eukaryotic components individually explain 44 and 41%, respectively, of the functional variability. We further explored the nature of the correlations of the top 90 bacterial and eukaryotic taxa to the overall functionality (Figure S4) and observed great dissimilarities. Generally, bacteria had more functions significantly correlated to the same taxa than eukaryotes (Figure S4a), as well as more taxa correlated to the same functions (Figure S4b). Overall, the eukaryotic correlations were on average negative while the bacterial ones were positive (Figure S4c). Among the highly significant correlations ( $p \leq .001$ ), 400 functions were oppositely correlated with bacteria and eukaryotes (Figure S4d).

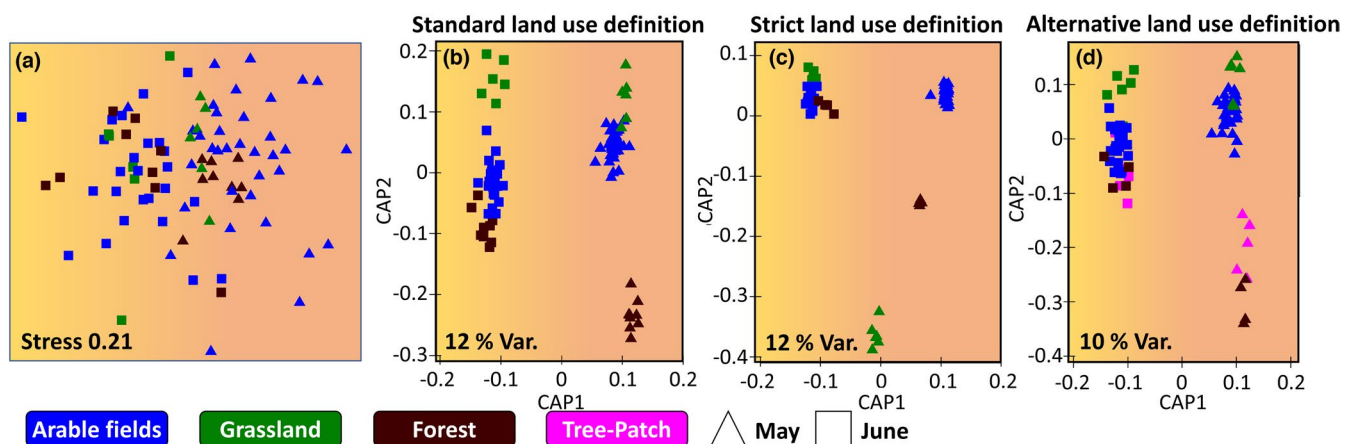
Similarly to the distance-based redundancy analysis, NMDS also shows a clear separation of bacterial and eukaryotic communities among the two sampling campaigns. In contrast, no clear separation is apparent among land-use types (Figure 3a). However, PERMANOVA shows that land use has a minimal yet significant effect on the distribution pattern of the active community, explaining ~4% of the overall variability. This effect is not significant when samples from May or June are analysed separately. The sum of the individual and combined effects of sampling time and land use explain in total 12% of the variability among samples. CAP using a factor combining sampling period and land use highlights the separation between samples based on these two variables (Figure 3b). A clear separation between samples taken at different time points is evident as well as among land-use types in May, specifically between forest and the other two land-use types (arable fields and grassland). The separation based on land-use type of the June

samples is less pronounced. To test for effects of classifying tree patches embedded in arable fields as forests, arable fields or an independent group, the same analysis was conducted by applying either a strict or loose (standard) definition to forest KH, allocating the tree patches to the arable field (Figure 3c) or forest category (Figure 3b), respectively. The strict definition resulted in a more apparent separation of the grassland samples taken in May and a tighter aggregation of all samples in June (Figure 3c). Nevertheless, the strict land-use definition has a marginally significant influence on the overall temporal and spatial distribution pattern ( $p = .08$ ). Classifying the tree-patches as a fourth land-use type (Figure 3d) results in a separation pattern in between the loose and strict land-use definition and, while explaining less of the variability, it is statistically significant ( $p = .01$ ).

PERMANOVA conducted separately on the bacterial and eukaryotic communities reveals that the combined effect of land use and sampling time explains ~18% and 13% of the variability, respectively. The strict land-use definition had no significant effect on the distribution patterns of either bacteria or eukaryotes when analysed separately.

Differentiating crop types on arable fields (rapeseed, corn, wheat, barley, rye, triticale) explained a similarly low proportion of variability (~4%), and only when assessed in combination with the sampling period. Separate analyses for bacteria and eukaryotes show that crop type only significantly affected bacteria, explaining again ~4% of the variability and separating the taxa into several groups (Figure S5).

The significances of sampling time, land-use type and crop type were also tested as explanatory factors of the distribution of expressed functional genes. Land use alone or in combination with either of the two other factors had no significant influence. However, sampling time and crop type explained ~7% ( $p = .005$ ) and ~4% ( $p = .04$ ) of the variability, respectively.



**FIGURE 3** Nonmetric multidimensional scaling (NMDS) of the active bacterial and eukaryotic communities (a) showing temporal separation between the samples (triangles—May vs. squares—June) as highlighted by the orange—peach—colour shading, but no separation based on land-use types (3D stress 0.13). Canonical analysis of principal components (b–d) highlighting the distribution pattern of the active bacterial and eukaryotic communities by sampling period (CAP1) and land-use type (CAP2), based only on the species contributing to the significance of these parameters as tested with PERMANOVA. Panels (b–d) differ in their definition of forests. In (b), KH in large forests and tree patches amidst arable field are classified as forest KH. In (c), the latter tree patches are classified as arable fields, while in (d) they are assigned to their own group



Ternary plots displaying the distribution of communities and functions according to land-use type (Figure 4) show that few taxa are strongly associated with a specific land-use type. This is evident by the concentration of the bright colours in the centre of the plots as opposed to the mostly purple colours at the vertex, in line with the low percentage of active-community variability explained by land-use type (4%). Splitting the overall community into May and June samples and into bacteria and eukaryotes reveals that the plume of taxa associated with forests is due mostly to bacteria sampled in June, whereas active eukaryotes are most strongly associated with arable fields and grasslands in May. In June, the eukaryotic community shifts upward to the centre of the plot, with a decrease of more than 5000 taxa associated with grasslands and arable fields between May and June. Overall, most active taxa were widely distributed across all land-use types and displayed similar activity levels in all land-use types.

Fewer taxa were identified as indicator species of arable fields than forests or grasslands based on the analysis of presence-absence (P/A) data (Figure 5). However, consideration of community activity levels increases the number of indicator taxa for arable fields by nearly 20 times (11 and 176 taxa for P/A and quantitative analysis [Quant], respectively). In both types of analyses, the maximum association factors (ranging between 1 for strong and 0 for none) of taxa with arable fields were lower than for taxa associated with forests or grasslands (0.6, 0.9, 0.9 P/A; 0.4, 0.6, 0.5 Quant, for arable fields, forest and grassland, respectively). Among the eukaryotes, only three taxa were statistically significant indicators of arable fields based on P/A data: two green algae (*Nephroselmis* sp. and *Carteria* sp.) and a ciliate of the order Stichotrichia (probably *Stylonychia* sp.). However, accounting for community activity halved the association factor for eukaryotes from a maximum of 0.68 (P/A) to 0.32 (Quant), attributed to *Tribonema* sp., a filamentous green alga. The association of bacteria with arable fields was loose with maximum association factors of 0.6 and 0.4 for P/A and quantitative analyses, respectively. The gastropod *Planorbarius corneus* was the most important indicator of P/A analyses in forest KH, whereas *Trachelomonas*, a flagellate of the family Euglenaceae, dominated in grassland KH. Regarding the communities in KH of arable fields, a quantitative analysis based on community activity reduced the overall association factors and placed microorganisms such as ciliates and fungi at the top of the indicator list.

### 3.3 | Community functional performance

The overall and seasonal functional ternary plots show minimal land-use-specific associations and similarly small changes between the two sampling periods (Figure 4). To further inspect this, we compared the normalized gene expression (see Methods) for different metabolic pathways grouped into Subsystems of the Seed database (Overbeek et al., 2005) as well as tested for their correlation with the measured environmental parameters (Figure 6). Samples were grouped according to sampling time, land-use type or both and then compared pairwise. Some Subsystems were correlated with environmental variables (Figure 6a), yet interestingly, these were

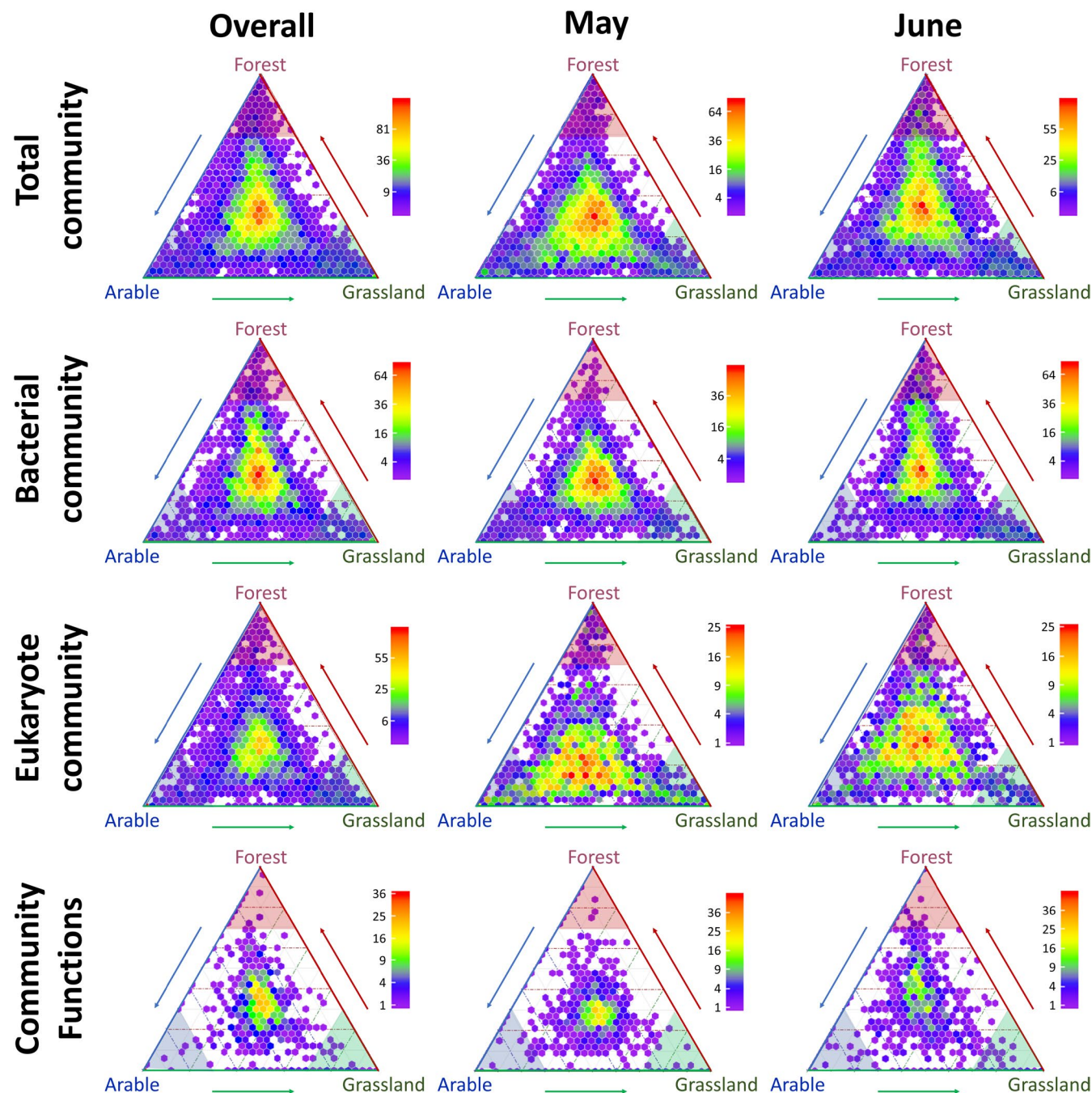
mostly with physical properties (temperature, pH, conductivity) and concentrations of other ions rather than with nutrients (P or N). Separating the data into the two sampling months shows a correlation of several N- and P-related subsystems with N and P concentrations in May but not in June (Data S4). These correlations were not evident when the data was further analysed according to the different land-use types. Excluding subsystems for which expression was detected only in one or two sets of samples, significant differences between groups were observed in 22 cases (Figure 7; Figure S1). The photosynthesis and CO<sub>2</sub> fixation Subsystem showed the lowest gene expression in forest KH in June, but no significant differences in expression among land-use types in May. No differences in expression were detected between arable fields and grasslands for either functional Subsystem and in either May or June.

The expression of genes involved in nitrogen fixation and ammonia assimilation was higher in June than in May in KH located in arable fields and even more so for those in grasslands. Gene expression related to iron transport was also higher in June (Figure S6) in parallel with an increase in siderophore production.

Transcripts categorized as contributing to general phosphorus metabolism were more highly expressed in May, with no difference among land-use types. In contrast, genes related to bacterial and eukaryotic phosphorus scavenging, such as phosphate transporters and "DING" binding proteins (Berna et al., 2008), were more often expressed in June.

Some differences were also observed for genes involved in carbon metabolism. Subsystems involved in metabolism of larger sugars were mostly detected in May. Specifically, the metabolism of di- and oligosaccharides in May was significantly higher in samples from forest KH, and a similar tendency was also observed in June. In contrast, differences were apparent in fermentation processes and organic acid metabolism when focusing on specific processes (functional subsystem Level 3; Figure S6), although they were not significantly different when grouped at Level 2 in the subsystem hierarchy. For example, the fermentation of mixed acids was highest in forest KH in May, whereas the synthesis of acetone, ethanol and butanol was higher in grasslands at the same time. Differences between land-use types were also observed for organic acid metabolism in May, when arabinose utilization was highest in grassland KH and tricarballoylate utilization in forest KH.

The overall expression profile of functional genes was not significantly affected by land-use type. To evaluate whether land-use type affects other properties of the community functionality, we investigated the functional richness (number of different functions) and evenness for the three land-use types and the two sampling periods, reasoning that low functional richness and evenness could be indicative of specialist communities. Functional richness (Figure 7a) varied across samples but was not significantly different among land-use types or between sampling points. Functional evenness (Figure 7b) varied across samples as well. Values were as low as 0.2 in some samples, suggesting that in June, the evenness in forest and grassland KH is higher than in arable field KH (Mann-Whitney and Dunn's tests,  $p = .04$ ). This suggests that arable fields enrich for certain metabolic pathways.

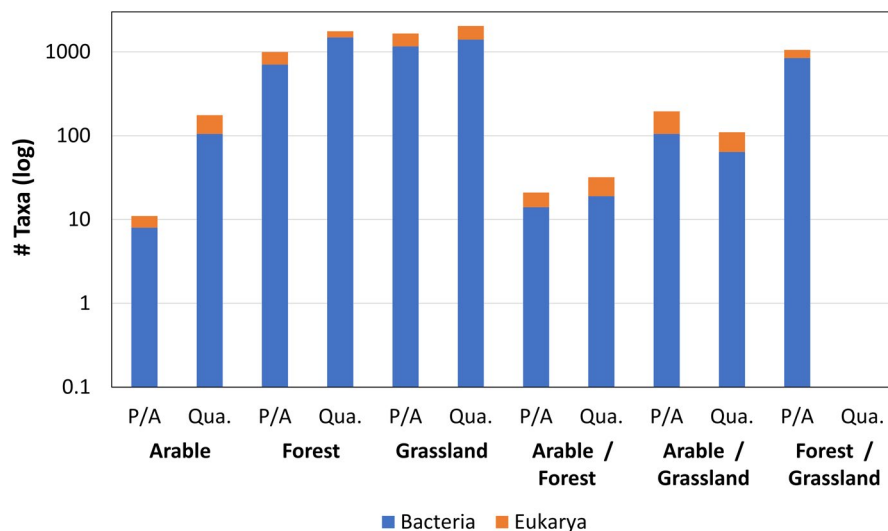


**FIGURE 4** Ternary plots depicting associations of taxa and functions to specific land-use types throughout the study or separated according to sampling period (May or June 2017). The closer a point is to a vertex of the triangular plot, the stronger is its association with the respective land-use type. The community composition is further divided into bacteria (Archaea and Bacteria) and eukaryotes. The individual taxa are grouped into hexagons for imaging purposes. Individual hexagons are coloured by the square-root-normalized number of taxa in the area they cover, with purple hexagons containing single taxa and red hexagons up to several hundreds

## 4 | DISCUSSION

In this study we demonstrate that land-use type has a time-dependent, temporary, effect on the structure of active prokaryotic and eukaryotic communities in KH, despite the overall biodiversity homogenization observed in this agricultural KH meta-ecosystem (D. Ionescu et al., 2022). Thus, we confirm our hypothesis that

the activity of organisms, as reflected by profiles of environmentally short-lived RNA, may reveal patterns not observed in eDNA analyses or traditional surveys. Furthermore, our results show that while land use partially determines which organisms are active, the functional profile, as seen by the type of expressed genes, remains largely unaffected, across time and land-use type, pointing to functional redundancy.



**FIGURE 5** Indicator species analysis based on presence/absence (P/A) and sequence frequency (Qua.) data, the latter serving as a proxy for community activity. Note the logarithmic scale of the y-axis

#### 4.1 | Physical and chemical parameters of the KH water

Lischeid et al. (2018) found the KH in the study area were connected via a shallow aquifer. This is consistent with our observation that only a few of the numerous physical and chemical variables measured in this study showed significant differences among land-use types or time of sampling. The lower oxygen saturation in forest KH during both sampling campaigns is probably a combination of lower photosynthesis due to shading by the forest canopy and increased respiration resulting from high organic matter inputs derived from forest soil, leaf litter and riparian vegetation. This interpretation is supported by high ammonia concentrations, suggesting high rates of organic matter mineralization in forest KH (Hargreaves, 1998).

The high N and P concentrations measured in (almost) all KH highlight long-term effects of intensive agriculture in the area, which led to the eutrophication of all KH in the study area (Lischeid et al., 2018). The elevated conductivity, and  $K^+$  and  $Cl^-$  concentrations in arable-field KH are possible evidence of fertilization of the fields shortly before or during our study, as already suggested for KH in the area (Lischeid & Kalettka, 2012). Elevated concentrations of  $K^+$  are commonly observed in arable fields due to fertilization (Spiess, 2011). The higher pH, also considering the higher  $NO_3^-$  and  $O_2$  saturation in arable fields in May, is probably a result of higher photosynthesis possibly driven by a recent input of nutrients. However,  $K^+$  and  $Cl^-$  did not remain elevated throughout the year, which may point to homogenization of water chemistry of the KH among land-use types by shallow groundwater flow.

#### 4.2 | Determinants of active community structure

Respiration and photosynthesis, and thus primary production, can shape the overall community structure by driving changes in  $O_2$  concentration, pH and autochthonous DOC. This notion is supported by the significant effects of  $O_2$  saturation and pH we observed on

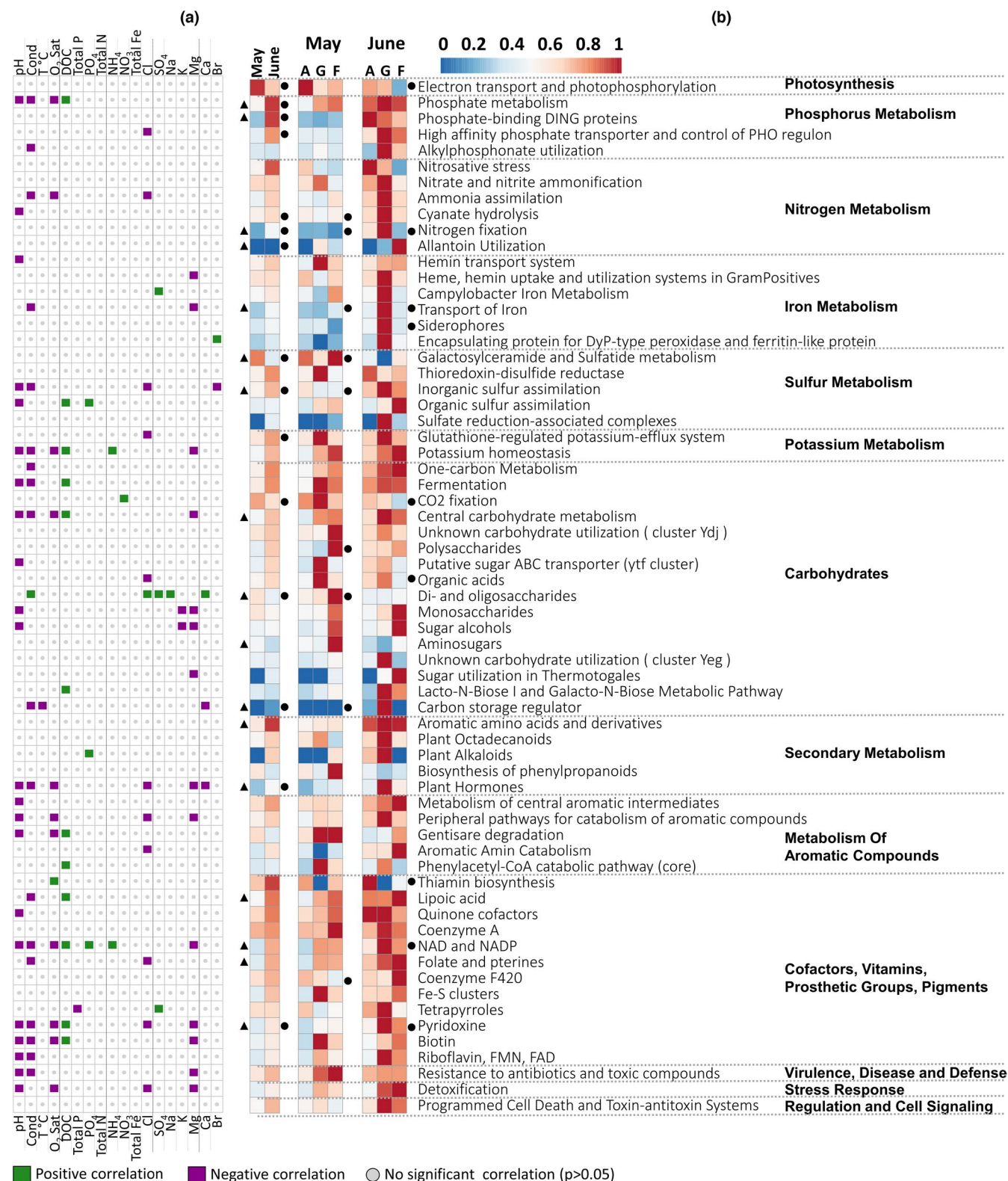
the structure of the active community. The significant relationship we observed between  $O_2$  saturation and the structure of the active eukaryotic communities is probably due to the high sensitivity of the larger, more complex, organisms to low  $O_2$  concentrations (Knoll & Sperling, 2014). Conductivity, which may change as a result of evaporation and intrusion of brackish groundwater (Nitzsche et al., 2017), had a significant effect on the entire community and specifically on its eukaryotic component. In agreement with this finding, conductivity negatively affected rotifer abundance and alpha-diversity in KH in our study area (Onandia et al., 2021). This suggests that the bacterial communities in these KH are more tolerant than higher organisms to changes in conductivity within the range encountered here.

Interactions between the eukaryotic and bacterial communities appear to be the strongest driver shaping the structure of the active community (i.e., the activity distribution among the different organisms). Algae and plants account for 11 of the 19 eukaryotic taxa which significantly explain the variability in the structure of the active bacterial community, indicating either a strong link to primary production or nutrient cycling via the decomposition of plants and algae. Previous findings in one of the studied ponds suggest that an important proportion of the bioavailable nutrient concentrations in ponds originates from submerged macrophyte decomposition (Onandia et al., 2018). The testate amoeba *Arcella*, which feeds on algae, cyanobacteria, fungi, ciliates and bacteria (Laybourn & Whymant, 1980), accounts for more than 7% of the variability in the structure of the active bacterial community. *Arcella* is a generalist amoeba (Tsyganov & Mazei, 2006), common in eutrophic waters and an important consumer of both bacteria and their grazers and hence may affect the bacterial community in opposite ways (Wilkinson & Mitchell, 2010). Similarly, fungi, which account for most of the additional eukaryotic taxa that significantly explain the bacterial community, can also affect bacterial community diversity and activity through both positive or negative interactions such as resource competition or organic matter mineralization (Bahram et al., 2021; Deveau et al., 2018; Wagg et al., 2019). Bacterial communities are also likely to reciprocally



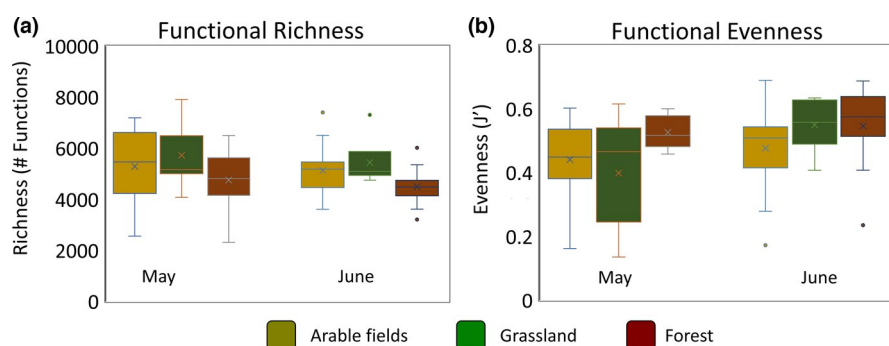
influence the eukaryotic active community, as shown by 25 bacterial taxa explaining 55% of the variability in active eukaryotic community. Nevertheless, the taxonomic resolution obtained from the rRNA transcripts does not offer deep insight into their functionality and the possible mechanisms by which these bacteria control the active eukaryotic communities.

Land-use type had different effects on the structure of the active KH communities in May and June. A clear separation among land-use types is evident in May, whereas in June the land-use effect is less pronounced, especially when the KH located in small patches of wood surrounded by arable fields are considered as KH in arable fields rather than forests. This indicates that despite similar chemical





**FIGURE 6** Correlation of gene expression levels with environmental variables as grouped in different Subsystems (a) and normalized median expression values (b). In (a), only significant correlations are shown ( $p < .05$ ). Additional correlation matrices as in (a) are given in Figure S7 and the Pearson  $r$  values ( $-.45 < r < .45$ ) are given as a Data S1–S4 for the entire data set or for the different months and land-use combinations. In (b), the samples are grouped according to sampling month (May and June) and land-use type (agricultural field—A, grassland—G, forest—F). Colours represent median values calculated per group using the TPM-normalized gene expression data (see Figure S8). All median values calculated for one Subsystem were normalized as a fraction of the maximal value within that subsystem so that values always ranged between 0 (no expression) and 1 (maximal expression for that subsystem). The list of Subsystems is sorted according to relative expression level, with the most expressed Subsystem on top and the least expressed at the bottom. The filled triangle to the left suggests a general significant difference between samples taken in May and June. Filled circles to the right of the May and June colour bars indicate significant differences between two or more land-use types within a given month (e.g., arable field vs. forest KH in May). Filled circles to the right of the May/June comparison indicate significant differences between May and June for one or more land-use types (e.g., arable fields KH in May vs. June). Pairs of sample groups differing from one another are marked in Figure S8. More information on the SEED functional subsystems is available at <https://rast.nmpdr.org/seedviewer.cgi?page=SubsystemSelect>



**FIGURE 7** Box plots showing the overall functional richness (a) and evenness (b) of active communities in KH grouped according to land-use type and sampling period. Median and mean values are depicted by solid and dotted lines, respectively. Whiskers mark the 25<sup>th</sup> and 75<sup>th</sup> percentiles. Dots represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles

and physical characteristics of the KH water, land use directly adjacent to the KH influences the structure of the active community in some periods, despite the overall homogenization of biodiversity observed in the studied KH (D. Ionescu et al., 2022). The greater effect of land use and sampling time on the active bacterial community compared to the eukaryotic community agrees with the finding that crop type had a statistically significant effect only on the active bacterial community. This suggests that the active bacterial communities in KH were influenced by the farming activities close to the time of sampling. This also demonstrates that the vegetation around KH does not completely buffer for the effects of the surrounding landscape as proposed by Joniak et al. (2017).

Even though some changes occurred between May and June related to land-use-type associations of active bacterial and eukaryotic communities, a large proportion of taxa showed no association with a particular land-use type. This does not imply the selection of generalists over functionally specialized organisms, but rather that specialists were widespread across the different land-use types. This is most evident by the diverse functional repertoire observed both in May and in June. Therefore, it is likely that many organisms are more responsive to within-KH biotic interactions and subsequently to environmental parameters, than to land-use type. This is well supported by the large percentage of variability in the active bacterial community that is explained by the structure of the active eukaryotic community and vice versa. The changes occurring in the active bacterial communities between May and June, however, differed from those occurring in the eukaryotic communities. Furthermore, since only the bacteria responded to crop type, we propose that the community responses to land-use type were driven by factors

other than interorganismic interactions alone. These may include measured parameters such as concentrations of different N species, P and O<sub>2</sub>, but also, for example, crop-related parameters that were not determined such as toxic water-soluble extracts of crops (Far & Bagherzadeh, 2018; Mustarichie et al., 2020).

Our indicator species analysis was conducted to identify organisms whose activity was tightly linked to a specific land-use type. The presence-absence data for the active taxa in the communities show that only a few bacterial and eukaryotic taxa are indicative of arable fields. Nevertheless, a quantitative analysis increased the number of taxa specifically associated with arable fields nearly 20-fold, suggesting that these additional taxa are present in forests and grasslands, but have a much lower activity level there, as derived from rRNA sequence coverage. A remarkable finding of the analysis is that regardless of the method used for identifying indicator species, only microorganisms were recognized as specific indicators of arable fields. In contrast, indicator species of grassland and forest KH alone or in combination with arable fields also included larger organisms (Table S7) such as zooplankton (e.g., *Ischnomesus* sp.), worms (e.g., *Trieminentia* sp.) and insects (e.g., the pest *Sitodiplosis mosellana*). However, the absolute taxonomic identification of these larger organisms should be clarified in targeted studies using long-read sequencing approaches of one or more phylogenetic markers. Overall, the observations made using the indicator species analysis suggest both an overall homogenization in biodiversity in the area and an increased activity of certain microorganisms in KH from arable fields.

In addition to bacteria and fungi, the nature of other eukaryotic indicator species is in general agreement with the overall

eutrophic nature of the sampled KH described by Lischied et al. (2018). Ecological information on the three eukaryotic taxa identified as indicative of KH in arable fields (*Nephroselmis* sp., *Carteria* sp., *Stichotrichia* sp.) is scarce. *Carteria* sp. can be present in various aquatic habitats ranging from oligotrophic lakes (Padisák et al., 2010) to extreme acid lakes (Nixdorf et al., 1998). However, consistent with our results, *Carteria* sp. has recently been found to form blooms in eutrophic lakes (González & Roldán, 2020). Although *Stichotrichia* is mostly dominant in oligotrophic waters (Desvillettes & Bec, 2009), some species have also been recorded in hypertrophic environments (Šimek et al., 2019). Similarly, the top indicative taxa of forest and grassland KH, *Planorbarius corneus* and *Trachelomonas* sp., respectively, are also known to occur in eutrophic waters (Costil & Clement, 1996; Peczuła et al., 2014; Solórzano et al., 2011).

Our quantitative analysis ranked microorganisms such as ciliates, fungi and bacteria at the top of the indicator species list across all land-use types. However, this is to be expected as the probability of retrieving RNA from microorganisms in our samples is higher than for higher organisms.

### 4.3 | Community functional performance

Functional redundancy emerges as an inherent property of the KH communities, when the same tool used to investigate the structure of the active communities is applied to analyse patterns of gene expression. Land-use type could not explain functional variation (i.e., gene expression patterns) and a temporal effect of crop type explained only a small fraction of the overall variation. The latter effect can probably be attributed to the same portion of the bacterial community that responded to crop type. Additionally, no physical or chemical variables could be identified to explain the distribution of expressed functional genes, indicating that the observed effects of water chemistry on the structure of the active community did not translate to variations in community functions. Despite sampling time explaining ~7% of the variation in functional gene expression, a PCA could not separate the functional community profiles according to the time of sampling. Thus, the active communities sampled in May and June differed from one another, but their functionality remained unchanged between the two months. This suggests that different organisms perform the same processes at different time points. This conclusion is also apparent in the ternary plots indicating minimal land-use-specific associations of functions and similarly small differences between the two sampling periods (Figure 4).

We propose that interactions between organisms are one of the main drivers of the functional variability in the studied KH. Interactions between organisms are known to shape biodiversity (Bachelot et al., 2015; Gallien et al., 2017) and community functionality (Gallien, 2017). Our data suggest that in the studied ponds such interactions are a stronger driver of active community structure and functionality than land-use type or physicochemical environmental parameters. First, this is suggested by the general correlation of the bacterial and eukaryotic communities as well by the ability of the

dominant taxa in each community to explain a significant portion of the variability in the other. Since the eRNA data provide quantitative information on activity rather than the physical abundance of organisms, this linkage between the activity of the two communities is coupled to the different functions these organisms perform. Second, the most active members of the bacterial and eukaryotic communities explain a significant part of the functional variability. This result could be alternatively interpreted as driven by environmental conditions selecting for specific taxon-related functions. However, the physicochemical variables measured could not explain the functional variability. Therefore, we suggest that all types of symbiotic interactions (i.e., mutualism, commensalism, predation, amensalism, parasitism and competition) between the KH-residing communities shape the structure of the active community and subsequently the expressed genes and functionality. Our results point to such interactions between the bacterial and eukaryotic communities, but this phenomenon takes place between different subgroups and individuals of these communities as well.

Despite obvious differences in light availability between the tree-covered forest KH and most KH located in grassland and arable fields, it appears that light, and consequently photosynthesis, were not the main drivers behind the partial community separation observed in May. Expression of photosynthesis and CO<sub>2</sub> fixation genes was lowest in forest KH in June, probably due to light limitation by the covering tree canopy; however, no separation in the community was observed at this time point. In contrast, in May, when the active communities could be partially separated according to land use, no significant differences in photosynthesis and CO<sub>2</sub> fixation gene expression levels were detected between the three land-use types. Furthermore, no changes were observed between the expression of genes between arable fields or grasslands from May to June.

Genes for nitrogen fixation and phosphorus scavenging in arable fields were higher in June than in May. This suggests these nutrients were less available in late spring, which might be related to fertilizer application at this time. Nitrogen fixation is triggered by the absence of combined nitrogen sources such as ammonia, nitrate and urea. Similarly, scavenging of phosphorus via alkaline phosphatase or DING proteins (Berna et al., 2008) increases as phosphorus concentration decreases. Accordingly, the increase in expression of these genes in June suggests that N availability in KH decreased from May to June, or N demand increased. This further supports the notion that the separation of the structure of the active communities according to land-use type in May indicates the effect of pulsed fertilization applied to the arable fields reaching all KH water. This is reflected in temporal changes of the structure of the active community (i.e., not necessarily their physical abundance) between May and June. In June, grassland KH were characterized by an even higher increase in nitrogen fixation genes than those in arable fields, highlighting a delayed but similar change in nitrogen availability in grassland KH. The proximity of these KH to arable fields may result in indirect fertilization from arable fields and vice versa. The strong simultaneous decrease in NH<sub>4</sub> from May to June in grassland and arable field KH and the

overall low  $\text{NO}_3^-$  concentration further explain the strong increase in the expression of N fixation genes in June. According to information passed by local landowners to Dr Gernot Verch from the Leibniz Centre for Agricultural Landscape Research (ZALF), fertilization in 2017 in the study area took place between March and May and ceased at least 2 weeks before the June sampling campaign.

Although elevated potassium concentrations in KH of arable fields could also be due to fertilization, the lack of changes in the expression of potassium homeostasis genes, which increase in limiting conditions (Schramke et al., 2017), suggests that potassium availability is sufficient in the studied KH.

In this study, we have examined the structure and functionality of active KH communities at the genetic level. Yet, land-use type may also affect organismic traits that are not genetically detectable, especially for larger organisms. For example, body size, coloration, feeding habits and other behaviour, habitat use, etc. (McKie et al., 2018; Potapov et al., 2019), may not be seen in our transcriptome. Therefore, to fully elucidate land-use and other effects on community structure and functions requires complementing eDNA and eRNA data with information on further organismic features such as morphological, functional and behavioural traits. Additionally, because of the short lifetime of RNA in the environment, it is likely that larger organisms which could not be directly sampled are absent or incorrectly represented in the eRNA data sets.

## 5 | CONCLUSIONS

Our eRNA-based study shows that current land-use type has a time-dependent effect on the structure of the active members of bacterial and eukaryotic communities. Thus, it becomes evident that aquatic bacterial (Bacteria and Archaea) and eukaryotic KH communities react to the input of nutrients and organic matter from the surrounding terrestrial landscape by modifying their activity patterns even when community composition remains unchanged due to biodiversity homogenization. Community structure of the active aquatic bacteria can respond to crop type. Such relationships are hidden when analyses are restricted to determining community structure using eDNA, highlighting the complementary analyses of eRNA-based studies.

In contrast to the activity level of the studied communities, the overall functionality assessed by determining expression patterns of functional genes was barely influenced by sampling time or land-use type, highlighting a functional redundancy across the landscape. Additionally, only a small portion of the overall variation can be explained by water temperature and chemistry. Given the apparent functional redundancy, it is not surprising that neither land-use type nor environmental parameters can explain the functional variability.

Yet, functional-gene expression is quite well (50%) explained by the active community structure of bacteria, eukaryotes and both

combined. Our data suggest that site-specific interactions among organisms constitute the main drivers of changes in organismic structure of the active KH communities and their specific metabolic activities. Gallien (2017) proposed the use of community functional studies to reveal competitive interactions in communities. We propose that eRNA studies may be part of the toolbox necessary to reveal complex interactions between organisms in complex communities across trophic scales.

Biodiversity homogenization due to anthropogenic activity appears to be a recurring pattern in different types of ecosystems (Buhk et al., 2017; Holman et al., 2021; Meyer et al., 2013a; Smart et al., 2006). This is further accompanied by a continuous decrease in biodiversity (Díaz et al., 2019; Harrison et al., 2020; Urban, 2015). Our study demonstrates that the activity of different members of these communities, despite being homogeneously distributed across the landscape, respond to land-use-related activities, such as fertilization. To mitigate further loss in biodiversity, and as a step towards restoration, conservation policies should be applied not only to pristine ecosystems but also to those that were under negative anthropogenic influence for long periods of time as it becomes obvious that the local communities are still sensitive to land-use-specific input.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

M.B., D.I., H.P.G., S.W.: designed research; M.B., D.I., G.O.: performed research and analysed data; M.B., D.I., R.K., G.O., C.L.M., S.W., S.A.B., J.C.N., G.L., M.O.G., S.W., H.P.G.: wrote the paper.

## OPEN RESEARCH BADGES



This article has earned an Open Data, for making publicly available the digitally-shareable data necessary to reproduce the reported

results. The data is available at <https://doi.org/10.5061/dryad.0k6djhb1m>.

## DATA AVAILABILITY STATEMENT

Sequence data: All RNA sequences are available at the SRA under project no. PRJNA640812. Sample metadata: Location and time of sampling metadata are included in the SRA project data PRJNA640812. Tables summarizing the different annotations are provided as supplementary material to this manuscript and have been additionally submitted to the data sharing server Dryad at <https://doi.org/10.5061/dryad.0k6djhb1m>.

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