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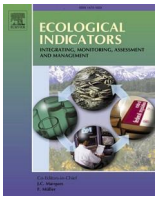

Relic DNA confounds the results of DNA-based study on microeukaryotic succession in aquatic biofilms

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ABSTRACT

High-throughput sequencing has revolutionized the DNA sequence-based study of microbial community and diversity on an unprecedented scale. Relic DNA is widespread in the natural aquatic environment; however, the extent to which such DNA can bias the sequence-based analysis of living microeukaryotic communities is unclear. Here, we conducted a 30-day field-control experiment investigating biofilm succession of microeukaryotic communities on glass slides and polyurethane foam units (PFUs) in a subtropical urban reservoir, using DNA-based approach. The propidium monoazide (PMA) dye was used to distinguish living and relic DNA sequences. Our results showed that microeukaryotic community succession in biofilms was time and substratum dependent, which was correlated with changing environmental conditions, nutrients and microalgae. Most importantly, relic DNA has a significant influence on the estimation of beta diversity and turnover in community composition, importantly its presence masked the successional patterns of different microbial taxa. We found strong deterministic processes dominating microeukaryotic succession on glass slides and PFUs biofilms. Depending on substrate and the presence and absence of relic DNA sequences, contrasting community assembly mechanisms were observed. In the presence of relic DNA, our results showed that 0.53–3.15 % of dispersal limitation, and 13.16 % of homogeneous selection processes were overestimated, while 1.06–3.68 % of homogenizing dispersal, 2.11–10.53 % of undominated and 2.10–10.52 % of heterogeneous selection processes were underestimated. This study provides significant insights into the value of removing relic DNA when designing DNA-based studies to characterize microeukaryotic communities and raise questions about some ecological interpretations in molecular microbial ecology which have not accounted for the effects of relic DNA.

1. Introduction

Microorganisms are ubiquitously distributed in all types of ecosystems (Postgate, 1994; Mo et al., 2021; Lennon et al., 2018; Jackson, 2003). When they die, their cells are decomposed by bacterial lysis and break down in the water or are deposited in sediments or soils (Sakcham et al., 2022, 2019; Jones and Lennon, 2010). Such dead microorganisms can release extracellular DNA (exDNA) or retain intracellular DNA (iDNA)—and this relic DNA can be found in all types of ecosystems, such as water, soil, and sediment (Sakcham et al., 2022; Lennon et al., 2018; Nagler et al., 2018a; Nagler et al., 2018b). Studies have shown that relic DNA might have significant effects on DNA sequence-based estimation of microbial diversity in the environment (Xue et al., 2023; Sakcham et al., 2022; Lennon et al., 2018; Carini et al., 2016). Although biofilms are a metabolically active and structurally complex component of aquatic habitats (Karygianni et al., 2020; Veach et al., 2016; Wrona et al., 2006), historically succession in biofilm communities has been studied using mainly microscopy-based techniques (Atlas and Bartha, 1998). In this context, the nature and extent to which relic DNA can mask patterns of microeukaryotic succession remains unknown. Therefore, it is necessary to understand the effects of relic DNA for an unbiased prediction of ecosystem status in a changing environment over time.

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The propidium monoazide (PMA) approach can be used to distinguish living from dead microorganisms for both prokaryotic and eukaryotic microbiomes (Xue et al., 2023; Sakcham et al., 2022; Lennon et al., 2018; Nagler et al., 2018a; Nagler et al., 2018b; Carini et al., 2016). PMA is a membrane impermeant dye which can intercalate into the DNA, eventually inhibiting PCR amplification. PMA does not penetrate intact cell walls but binds to exDNA and penetrates the damaged cell walls of dead organisms and binds to their iDNA, allowing for a selective assessment of only the living microorganisms, thus facilitating more accurate evaluation of living microbial diversity in ecosystems (Sakcham et al., 2022; Wisnoski et al., 2020; Carini et al., 2016; Nielsen et al., 2007).

Artificial substrates, specifically glass slides, ceramic tiles and polyurethane foam units (PFUs), are effective substrates for examining biofilm-dwelling microbes in aquatic habitats (Cahoon and VanGundy, 2022; Choe et al., 2021; Jax, 1996: Zhu et al., 2020; Veach et al., 2016; Xu et al., 2009; Xu et al., 2005), because they can provide microorganisms with an environment similar to natural ecological surfaces (Henrici, 1933) such as in soil (Cholodny, 1930). Biofilms are initially colonized by bacteria, microalgae and flagellates, then later by other mixotrophs and heterotrophs utilizing the early colonizers, resulting in complex biotic interactions within the microbial communities (Battin et al., 2016, 2003; Besemer et al., 2012, 2007; Fierer et al., 2010; Davey and O'Toole, 2000). The study of the temporal dynamics of microbial communities provides a view of species compositional variation and changes of species interactions over time (Pascual-Garcia et al., 2022; Phillips et al., 2021; Garrido-Benavent et al., 2020; Pascual-Garcia and Bell, 2020), while revealing the relative influence of different mechanisms of community assembly (i.e., stochastic and deterministic processes) and their driving factors (i.e., temperature, salinity, pH, nutrients and biotic/abiotic interactions) (Choe et al., 2021; Jiao et al., 2017; Veach et al., 2016). Such an approach will be most significant if techniques such as PMA treatment are used to isolate the dynamics of living microbes from the total environmental DNA (eDNA)-based community. It is widely thought that a framework of stochastic (i.e., dispersal and drift) and deterministic (i.e., selection) processes strongly controls microbial succession (Mo et al., 2021; Jiao et al., 2017; Zhou et al., 2014; Jackson, 2003), and these processes offer insights into the mechanisms of community assembly and dynamic interplay of different taxa, having great ecological significance (Pascual-Garcia et al., 2022; Pascual-Garcia and Bell, 2020; Phillips et al., 2021; Liu et al., 2020). Previous studies have shown that dispersal limitation is one of the main factors in successional ecology, at least for microorganisms (Reid, 1899: Makoto and Wilson, 2019; Chang and Turner, 2019); however, it is unknown whether the presence of relic DNA sequences mask or otherwise alter the results from total DNA sequence-based communities.

Here, we investigated the temporal dynamics of microeukaryotic communities collected from biofilms in a subtropical urban reservoir in Xiamen, China. Both glass slides and PFUs were used as models for DNA-based microbial community assessment. The microeukaryotic communities were detected by Illumina high-throughput sequencing of 18S rRNA gene V9 region. We hypotheses that the presence of relic DNA obscures the succession results of live microeukaryotes in biofilms. The objectives of this study were to investigate: (1) successional dynamics of microeukaryotic communities on glass slides and PFU substrates; (2) how does the presence of relic DNA sequences influence the estimation of beta diversity and community turnover of microeukaryotes in aquatic biofilm succession; and (3) whether community assembly processes of microeukaryotes differ regarding presence of relic DNA with the environmental samples.

2. Materials and methods

2.1. Description of the study area

The experiment was conducted in Xinglinwan Reservoir (24°36′ N and 118°04′ E), Xiamen, southeast China in 2021 (Fig. S1). It is a shallow and eutrophic urban reservoir, located in the lower stream of the Houxi River (about 25 km long river) (Yang et al., 2022). The total water storage capacity of the reservoir is about 2568 m^3 and the average water depth is about 2.5 m. Previously it was open river, but a man-made concrete dam blocked the outflow in 1956, causing limited flushing during rainy season. Due to its close proximity to the sea, seawater intrudes the reservoir in dry seasons each year, this intrusion being stronger in winter (dry and cool) than in summer (wet and warm). Thus, salinity is one of the major factors influencing microbiota in the reservoir (Mo et al., 2021). The reservoir has been heavily influenced by agricultural activities, urbanization-induced land-use changes and sewage effluents in recent decades (Luo et al., 2022; Yang et al., 2022). The reservoir suffers from low transparency and high algal density, however the formation of the cyanobacterial blooms is limited by the effects of short-term rainfall events (Luo et al., 2022).

2.2. Glass slide and PFU biofilm collection

The experiment was conducted using glass slides (25 mm × 76 mm × 1.2 mm) and PFUs (50 mm × 65 mm × 75 mm) in 2021. Tubes containing glass slides (N = 20) and PFUs (N = 20) were placed in one location in the reservoir at 0.5 m depth (hanging from a bridge with line) on 26 October 2021, and the experiment ended one month later. Previous biofilm studies in the river and marine/shallow coastal waters have revealed that the colonization of microbiota is matured on glass slides at 7 days, although it is depended on water current and tide for faster water currents and stronger tides, it can take up to 10 days to reach equilibrium stage (Cahoon and VanGundy, 2022; Zhu et al., 2020; Xu et al., 2012; Xu et al., 2014). Similarly, the time is variable depending on the velocity of water for PFUs (Yang et al., 2007; Xu et al., 2005).

Two glass slides were placed back-to-back in a 50-mL centrifuge tube. All 20 tubes were fixed on an assembled test-tube rack. The back-to-back attachment of the two glass slides allowed microbes to colonize the outer side of each slide, and also facilitated easy collection of biofilm material without disturbance (Cahoon and VanGundy, 2022; Zhu et al., 2020; Xu et al., 2009). Two glass slides and two PFUs were randomly selected and collected on days 1, 3, 5, 7, 10, 14, 17, 21, 24 and 30. The developmental stages of biofilms (colonization) on glass slides and PFUs were determined visually by naked eyes immediately after collection and using an inverted microscope in addition to molecular analyses (Fig. S1c). The number of species accumulated in the substrates defines the time of sampling when detecting ecological features of microbial community (Zhu et al., 2020; Xu et al., 2009; Xu et al., 2005). The greatest and fastest changes in microbial community occur during the early days of colonization, and it has been observed that biofilm development starts degrading after 14 days (Xu et al., 2012; Xu et al., 2014). Therefore, we sampled more intensively (2-day interval) in the beginning and had a 6-day interval in the end. Further, we defined three periods: samples collected on days 1 to 5 (period 1), on days 7 to 14 (period 2), and on days 17 to 30 (period 3).

In the field, glass slides were put into plastic bags and transferred to the laboratory. Biofilm containing glass slides were rinsed three times with 50–60 mL Milli-Q water at ambient temperature, whereas PFUs were gently squeezed to release biofilm material into plastic bags (about 50–60 mL biofilm material in each PFU). Biofilm samples in the plastic bags were mixed with a glass rod and immediately filtered using polycarbonate membranes (0.2 μm, 47 mm diameter, Millipore, Billerica, MA, USA). One set of filtered membranes was immediately frozen at −80 °C until DNA extraction. Another set of filtered membranes was treated with propidium monoazide (PMA) dye to inhibit PCR amplification of the DNA of dead microorganism cells, thus separating living microbial DNA from the total DNA (Xue et al., 2023; Emerson et al., 2017). These membranes were put into 5-cm diameter petri dishes and immersed in 500 μL PMA dye. After carefully spreading PMA dye gently over the filtered membranes, petri dishes were kept in the dark for 20

min and then exposed to a 650 W halogen lamp placed 20 cm from the petri dishes for 15 min (Xue et al., 2023; Emerson et al., 2017). After light exposure, membranes were frozen immediately at −80 °C until DNA extraction.

2.3. DNA extraction and bioinformatics

As aquatic biofilms are mixture of water, sediments and microbial mats, total DNA of microeukaryotes was extracted from PMA-treated and untreated membranes using the FastDNA®SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. The universal primer pair 1380F and 1510R for microeukaryotes (Amaral-Zettler et al., 2009) with attached adapters was used to amplify the V9 region of the 18S rRNA gene. The PCR reaction contained 15 μL of Phusion High Fidelity PCR Master Mix (New England Biolabs, Beverly, MA, USA), 0.2 μM of each primer, and 10 ng of target DNA. The reactions included an initial denaturation at 98 °C for 1 min, followed by 30 cycles of 10 s at 98 °C, 30 s at 50 °C, and 60 s at 72 °C. At the end of the amplification, the amplicons were subjected to a final 10 min extension at 72 °C. PCR products from triplicate reactions per sample were pooled and gel-purified. The total concentration of DNA was measured by Qubit and the sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and finally sequenced on an Illumina NovaSeq 6000 platform.

The raw paired-end reads were denoised and assembled using DADA2 v1.16.0 (https://benjjneb.github.io/dada2/tutorial.html) (Callahan et al., 2016) in R. The high-quality reads were clustered as amplicon sequence variants (ASVs) at 100 % sequence identity. Each ASV was taxonomically assigned using the Protist Ribosomal Reference (PR2) database (Guillou et al., 2013). For the glass slides, the lowest number of mean sequences of the sample was 378,698 and 230,541 for total and living microeukaryotic community data sets, respectively, while it was 248,311 and 249,575 in PFUs, respectively. We used 230,541 sequences per sample for all four data sets for normalization (Table S1).

2.4. Water chemistry parameters, nutrients and primary productivity

Water temperature (°C), pH, chlorophyll-*a* (μg/l), turbidity (NTU), electrical conductivity (μS/cm), salinity (PSU), dissolved oxygen (mg/l) and oxidation-reduction potential (mV) were measured *in situ* with a Hydrolab DS5 multi-parameter water quality analyzer (Hach, Loveland, CO, USA). Water transparency was measured with a Secchi disk. Total carbon (mg/l), total organic carbon (mg/l), total nitrogen (mg/l), ammonium-nitrogen (mg/l), nitrate-nitrogen (mg/l), nitrite-nitrogen (mg/l), total phosphorus (mg/l), and phosphate-phosphorus (mg/l) were measured from raw water following the methods described by Greenberg et al. (1992). The algae, classified as blue (μg/l), green (μg/l) and brown algae (μg/l) in water were measured by a PHYTO-PAM Phytoplankton Analyzer (Heinz Walz GmbH, Effeltrich, Germany) following our previous study (Gao et al., 2021).

2.5. Data analyses

The effects of time and substrate on microeukaryotic community were tested using ANOSIM (analysis of similarity test) (Somerfield et al., 2021). A Bray-Curtis dissimilarity based hierarchical clustering with the complete linkage algorithm was used to show the temporal dynamics of the microeukaryotes on glass slides and PFUs using PRIMER v.7 (Clarke and Gorley, 2015; Anderson et al., 2008). The community compositional variation in microeukaryotes was tested by PERMANOVA (Monte Carlo, nperm = 9999) based on Bray-Curtis dissimilarity and their variation was visualized by non-metric multidimensional scaling analysis (nMDS) (Clarke and Gorley, 2015).

To examine species turnover through time over the successional period, we calculated the dissimilarity of species composition between samples using a presence-absence-based metric (βsim) (Soininen, 2010). Further, the temporal patterns of beta-diversity (compositional variation) and turnover over the successional period were tested by time-lag regression (Carvalho et al., 2012; Soininen, 2010).

To determine whether microeukaryotic community turnover was driven by stochastic processes of dispersal and drift or deterministic processes of selection, we used a phylogenetic bin-based null model (Zhou et al., 2014; Stegen et al., 2012, 2013; Sloan et al., 2006). A phylogenetic tree of microeukaryotes was constructed in QIIME2 (Bolyen et al., 2019). The temporal variation in phylogenetic turnover and the degree of divergence in successional trajectories across time were described by different processes. These processes include deterministic processes that are heterogeneous and homogeneous selections, and stochastic processes that are dispersal limitation, homogenizing dispersal, and drift or undominated processes. The phylogenetic turnover was calculated from the percentage of pairwise comparisons from the results of the beta nearest-taxon index ($|\beta NTI|$) and the taxonomic diversity matrix using modified Raup-Crick ($|RC_{bray}|$) (Isabwe et al., 2022; Dini-Andreote et al., 2015; Zhou et al., 2014; Stegen et al., 2012, 2013).

The significant temporal variation of environmental variables throughout the experimental period was tested using ANOSIM, while a principal coordinate analysis (PCoA) was conducted to show the overall heterogeneity of environmental factors. A distance-based linear model (DistLM) was used to identify the significant predictive variables from cluster analysis of environmental factors, and visualized by distance-based redundancy analysis (dbRDA) in different successional periods (Anderson et al., 2008; Clarke and Gorley, 2015).

3. Results

3.1. Successional dynamics of microeukaryotes on glass slides and PFU substrates

The presence of the relic DNA in the total DNA-based microeukaryotic community analysis accounted slightly more community compositional variation than living community (Fig. 1a). For example, the global R (means of community variation) in ANOSIM tests revealed 0.208 for total DNA-based microeukaryotes and 0.191 for living community (Fig. 1a), indicating that relic DNA might contribute additional community compositional variation than actually existed in the environmental samples. Further, cluster with SIMPROF (similarity profile) analysis revealed that the dissimilarity of community composition in each sample changed after removing relic DNA sequences from total samples (Fig. 1b), and this difference also appeared for three successional periods (Fig. 1c). It is noteworthy that depending on substrates and three periods, total DNA-based community composition showed less significant differences than living microeukaryotic community composition on glass slide, while opposite was found for PFU (ANOSIM results for total DNA-based community *vs* living community, R = 0.46** *vs* 0.71** for glass slide, while R = 0.60** *vs* 0.36* in PFU) (Fig. 1c). Thus, this result implies that substrate has significant influence on microeukaryotes colonization in aquatic biofilm over time.

On glass slides, PERMANOVA results revealed a significant difference between the colonization periods (Table 1), and compositional variation accounted for 49.31 % (between periods 1 and 2), 55.81 % (periods 1 and 3) and 42.57 % (periods 2 and 3) of variation for the total DNA-based microeukaryotes community (Table 1). When relic DNA sequences were removed, the compositional variation accounted for 57.13 %, 61.85 % and 44.45 % of total variation, respectively (Table 1). In PFUs, the compositional variation accounted for 51.66 %, 55.16 % and 47.55 % for total DNA-based microeukaryotes, while 41.64 %, 53.67 % and 49.36 % for living community, respectively (Table 1), indicating that colonization of microeukaryotic communities were different regarding time in the biofilms. Importantly, our results clearly

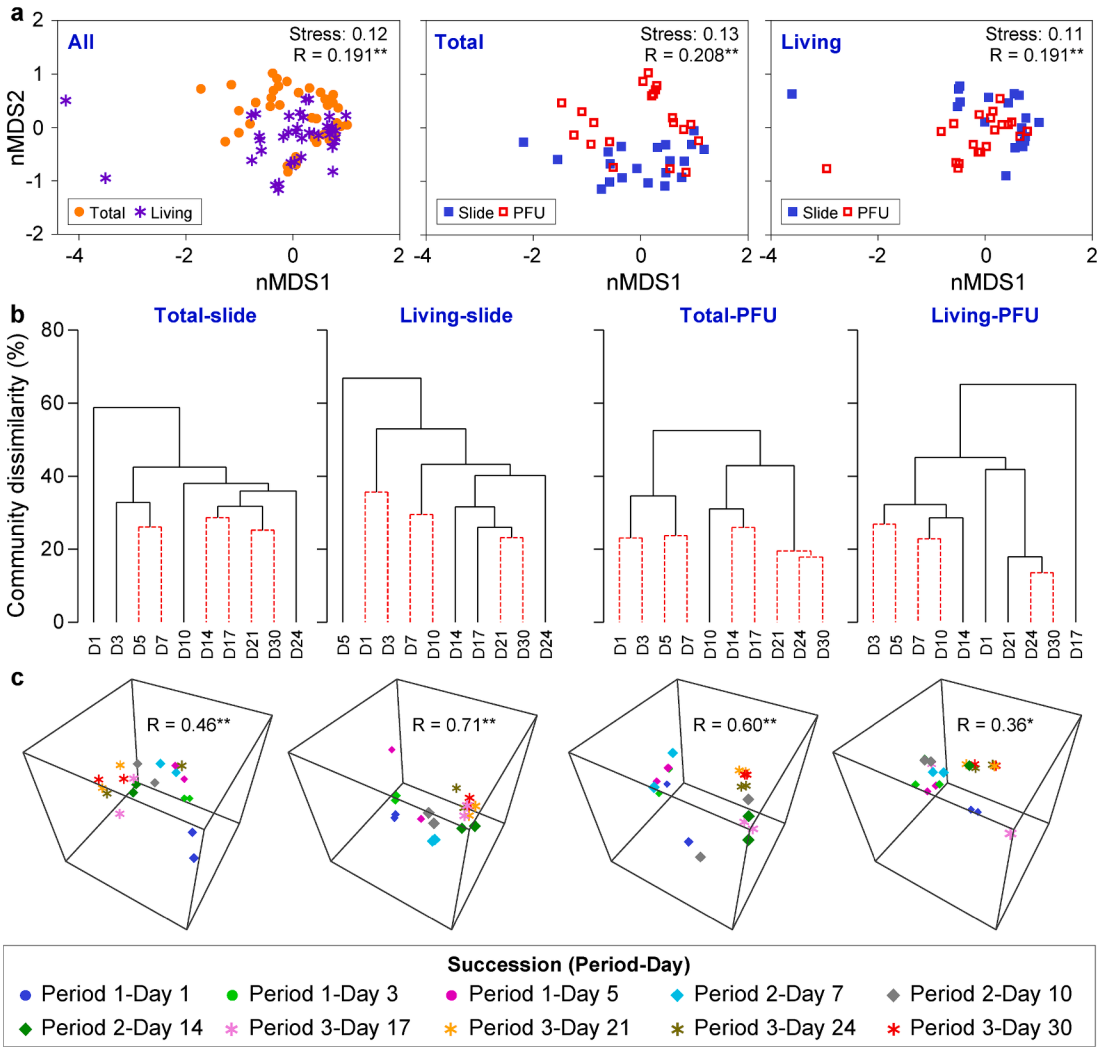

Fig. 1. Non-metric multidimensional scaling analysis showing the significant differences of microeukaryotic succession over the time on slide and PFU substrates, respectively. [The community variation of all, total and living microeukaryotes in glass slides and PFUs (a), temporal dynamics of microeukaryotes over time (b). Total indicates living community and relic DNA. Living indicates community with PMA treatment. Global R indicates the degree of variation between groups at $*P < 0.05$ and $**P < 0.01$. Red dotted line in the dendrogram cluster indicates significant difference at 0.05 level by SIMPER (similarity profile test)]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
The results of PERMANOVA showing the temporal dynamics of microeukaryotic communities in slide and PFU biofilms.

Group	t	Community dissimilarity (%)	*P* value
Total-G1 *vs* Total-G2	1.930	49.307	0.013
Total-G1 *vs* Total-G3	2.411	55.810	0.001
Total-G2 *vs* Total-G3	1.660	42.568	0.025
Living-G1 *vs* Living-G2	2.017	57.134	0.010
Living-G1 *vs* Living-G3	2.639	61.854	<0.001
Living-G2 *vs* Living-G3	2.422	44.451	0.001
Total-P1 *vs* Total-P2	1.730	51.663	0.037
Total-P1 *vs* Total-P3	3.089	55.161	<0.001
Total-P2 *vs* Total-P3	2.017	47.552	0.008
Living-P1 *vs* Living-P2	1.724	41.640	0.029
Living-P1 *vs* Living-P3	1.747	53.670	0.026
Living-P2 *vs* Living-P3	1.823	49.360	0.026

G1, G2 and G3 indicate three successional periods in slide, while P1, P2 and P3 indicate three successional periods (i.e., period 1: days 1 to 5, period 2: days 7–14 and period 3: days 17 to 30) in PFU.
Total indicates living community and relic DNA. Living indicates community with PMA treatment.

showed that variation between periods 1 and 3 was slightly higher than those of other two periods such as periods 1 and 2 or periods 2 and 3 (Table 1), which might be due to rapid species turnover during period 1 and slow turnover during period 3.

Four different successional patterns were evident among the microeukaryotic taxa in both glass slide and PFU biofilms (Fig. 2). On glass slide, Cohort 1 was a group of taxa characterised by repression (peaking at period 1: day 1–5, and subsequently declining), including Cercozoa, Cryptophyta, Dinophyta, Discoba, Katablepharidophyta, Ochrophyta, and, unknown phylum of Stramenopiles. Cohort 2 was characterised by induction (peaking at period 3: day 17–30) and included Apicomplexa, Chlorophyta and Fungi. Cohort 3 continuously fluctuated over time, and included mainly abundant taxa namely Ciliophora, Mesomycetozoa, Metazoa and unidentified microeukaryotes. Cohort 4 composed of rare taxa that were constant over time, namely Apusomonadidae, Breviatea, Centroheliozoa, Choanoflagellida, Conosa, Glaucophyta, Hilomonadea, Lobosa, Metamonada, Perkinsea, Radiolaria and Rhodophyta (Fig. 2). Interestingly, after removing relic DNA sequences, composition of cohorts changed indicating that the presence of relic DNA sequences in the environmental samples masked the signal of living microeukaryotic community succession. For example, Apicomplexa, Centroheliozoa,

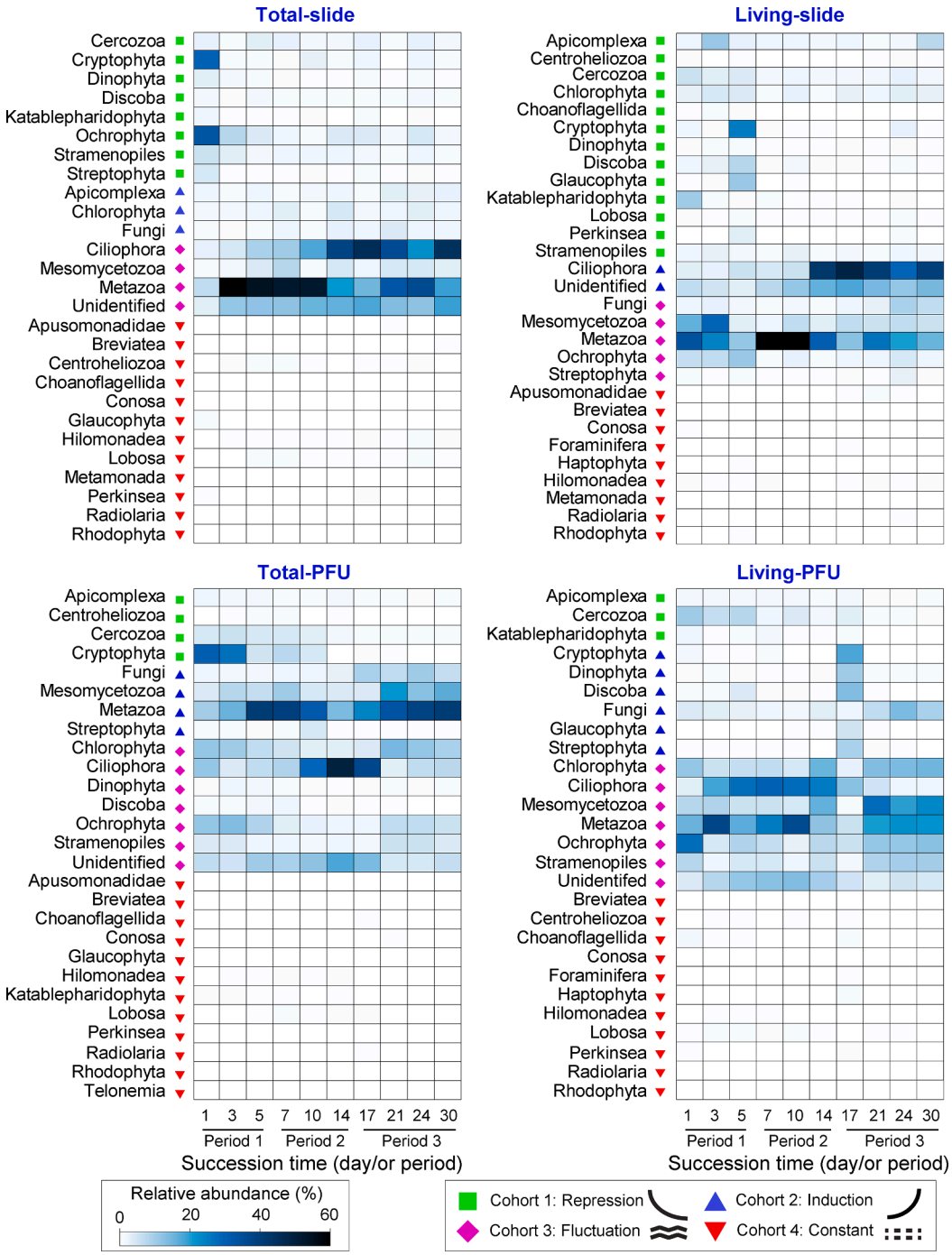

Fig. 2. Temporal compositional variation in microeukaryotic taxa in biofilms of slide and PFU without and with PMA treatment. [Total indicates living community and relic DNA. Living indicates community with PMA treatment].

Chlorophyta, Choanoflagellida, Glaucophyta, Katablepharidophyta, Lobosa, Perkinsea and Stramenopiles belonged to repression cohort in total DNA community on glass slides, but not in the living community which was clear evidence of the effect of relic DNA on interpretation of succession results. Similarly, Centroheliozoa and Cryptophyta belonged to repression cohort in the total DNA community on the PFU samples, but in the living community, Cryptophyta belonged to induction cohort and Centroheliozoa belonged to rare constant cohort (Fig. 2). Generally, succession occurs in the living community, but true succession may be masked by noise in the analysis of environmental DNA sequences due to presence of relic DNA.

The experimental removal of relic DNA sequences from the total eDNA samples revealed significantly different results for micro-eukaryotic taxa at supergroup level (Table 2 & Fig. S2) and composition of corresponding functional groups (Table 2). Our study revealed that Alveolata (23.86 %), Hacrobia (8.29 %), Opisthokonta (30.67 %), Stramenopiles (10.25 %) and unidentified groups (16.04 %) were most dominated taxa on glass slides, while Alveolata (16.77 %), Archaeplastida (15.96 %), Hacrobia (7.52 %), Opisthokonta (30.08 %), Rhizaria (6.20 %), Stramenopiles (10.51 %), and unidentified groups (11.09 %) were dominant in PFUs. Interestingly, the share of Alveolata (22.40 %), Hacrobia (7.52 %), Opisthokonta (26.30 %), Stramenopiles (6.88 %) and unidentified groups (18.38 %) was decreased on glass slides after removing relic DNA sequences. In the case of PFUs, the share

Table 2
Temporal dynamics of microeukaryotic communities (functional groups and super groups) in slide and PFU biofilms over three periods.

	Total-G (R)	Living-G (R)	Total-P (R)	Living-P (R)
Functional group				
Autotrophs	**0.143***	**0.454****	**0.166***	0.001
Heterotrophs	**0.180***	**0.266****	**0.182***	−0.001
Mixotrophs	**0.220***	**0.470****	0.153	−0.015
Parasitic	−0.040	**0.606****	0.081	**0.325****
Unknown	0.008	**0.260***	**0.246***	0.058
Super group				
Alveolata	**0.396****	**0.604****	0.102	0.131
Amoebozoa	0.019	0.107	**0.205***	**0.138***
Apusozoa	0.011	0.087	**0.221****	**0.202***
Archaeplastida	−0.015	**0.478****	**0.194***	0.055
Excavata	−0.008	**0.571****	**0.496****	**0.434****
Hacrobia	0.090	**0.252***	**0.533****	**0.225****
Opisthokonta	0.148	**0.450****	0.078	0.073
Rhizaria	0.078	**0.583****	**0.459****	**0.609****
Stramenopiles	**0.247****	**0.522****	**0.229***	0.018
Unidentified	**0.346****	**0.544****	0.072	**0.153***

Temporal dynamics was calculated using ANOSIM (analysis of similarity test) where global R indicates the degree of separation between groups.
Boldface indicates statistical significance at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.
Unidentified, unidentified group.
G indicates samples from slide, while P indicates samples from PFU.
Total indicates living community and relic DNA. Living indicates community with PMA treatment.

of Alveolata (16.46 %), Archaeplastida (14.68 %), Hacrobia (3.12 %), Opisthokonta (23.37 %) was decreased after removing relic DNA sequences. Further, all functional groups of microeukaryotic communities (Table 2) and supergroups Alveolata, Archaeplastida, Excavata, Hacrobia, Opisthokonta, Rhizaria, Stramenopiles and unidentified groups had significant compositional variation on glass slides after relic DNA removal. However, in the presence of relic DNA sequences, there was no significant variation among most of them (Fig. S2 & Table 2). In PFUs, only parasites and supergroups Amoebozoa, Apusozoa, Excavata, Hacrobia, Rhizaria and unidentified groups showed significant compositional variation when relic DNA sequences were removed from the total eDNA sequences (Table 2).

3.2. Changing microeukaryotic community and turnover on glass slides and PFUs

Our results showed that the beta-diversity of living community on glass slides had stronger significant correlation with the beta-diversity of total DNA-based microeukaryotic communities than on PFUs (Spearman's rank correlation coefficient, $\rho = 0.50$, $P < 0.01$ for glass slide; and $\rho = 0.18$, $P < 0.05$ for PFU) (Fig. 3a). In addition, the presence or absence of relic DNA sequences impacted beta-diversity and temporal variation in community turnover (Fig. 3b, c). Interestingly, the regression slopes were steeper for total DNA-based community than for the living community (Fig. 3b, c). This means that although the changes of the living community over time were statistically significant, the presence of relic DNA sequences increased the fitness of the regression models. Thus, our results revealed that relic DNA sequences might have significant effects on the estimation of microeukaryotic community composition and species turnover.

3.3. Microeukaryotic community assembly mechanisms on glass slides and PFUs

We tested whether relic DNA influenced the community assembly mechanisms (sunburst Plot), and compositional turnover (ßsim) among three succession periods (box Plot) (Fig. 4). The compositional turnover of living microeukaryotic community gradually decreased from period 1

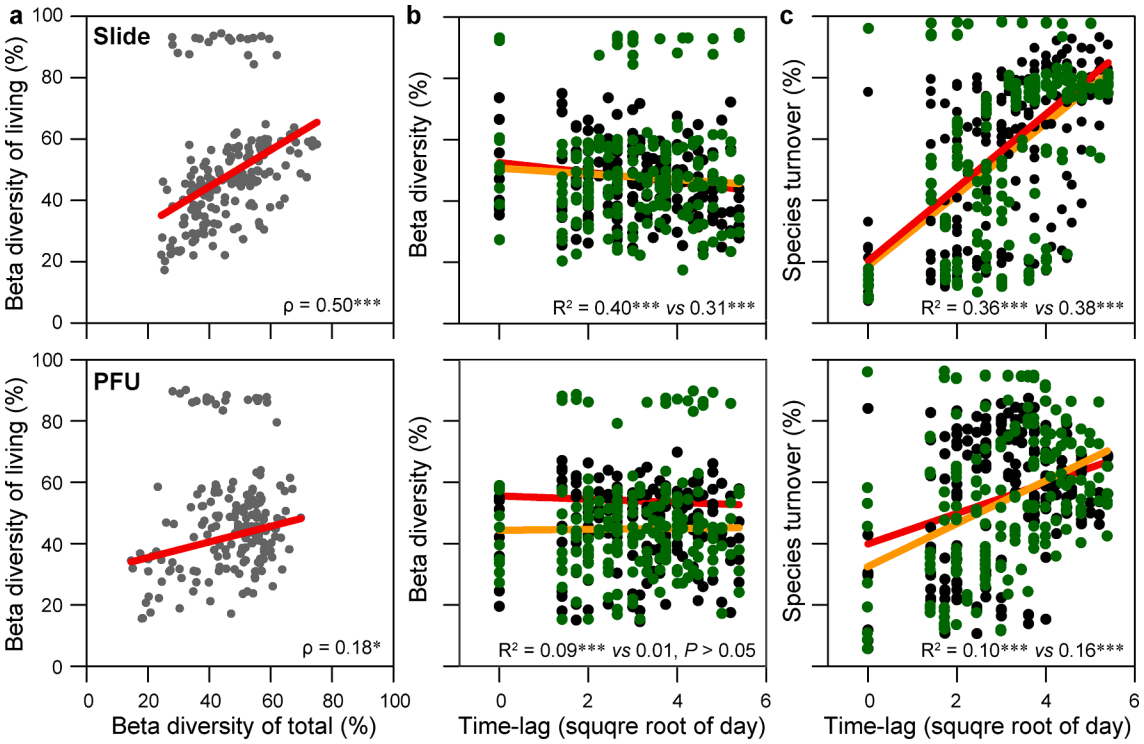

Fig. 3. Relationship between beta-diversity in living microeukaryotes and total DNA-based community, and time-lag regression showing the changes of beta-diversity and species turnover over time. [The Spearman's rank correlations (ρ) between beta diversity of living community with total DNA (a). Changes of beta diversity (b) and species turnover (c) over time. Line slopes with coefficient R^2 indicate regression strength. Total indicates living community and relic DNA. Living indicates community with PMA treatment. Black and green dots indicate samples for total (red line) and living (yellow line) microeukaryotes, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

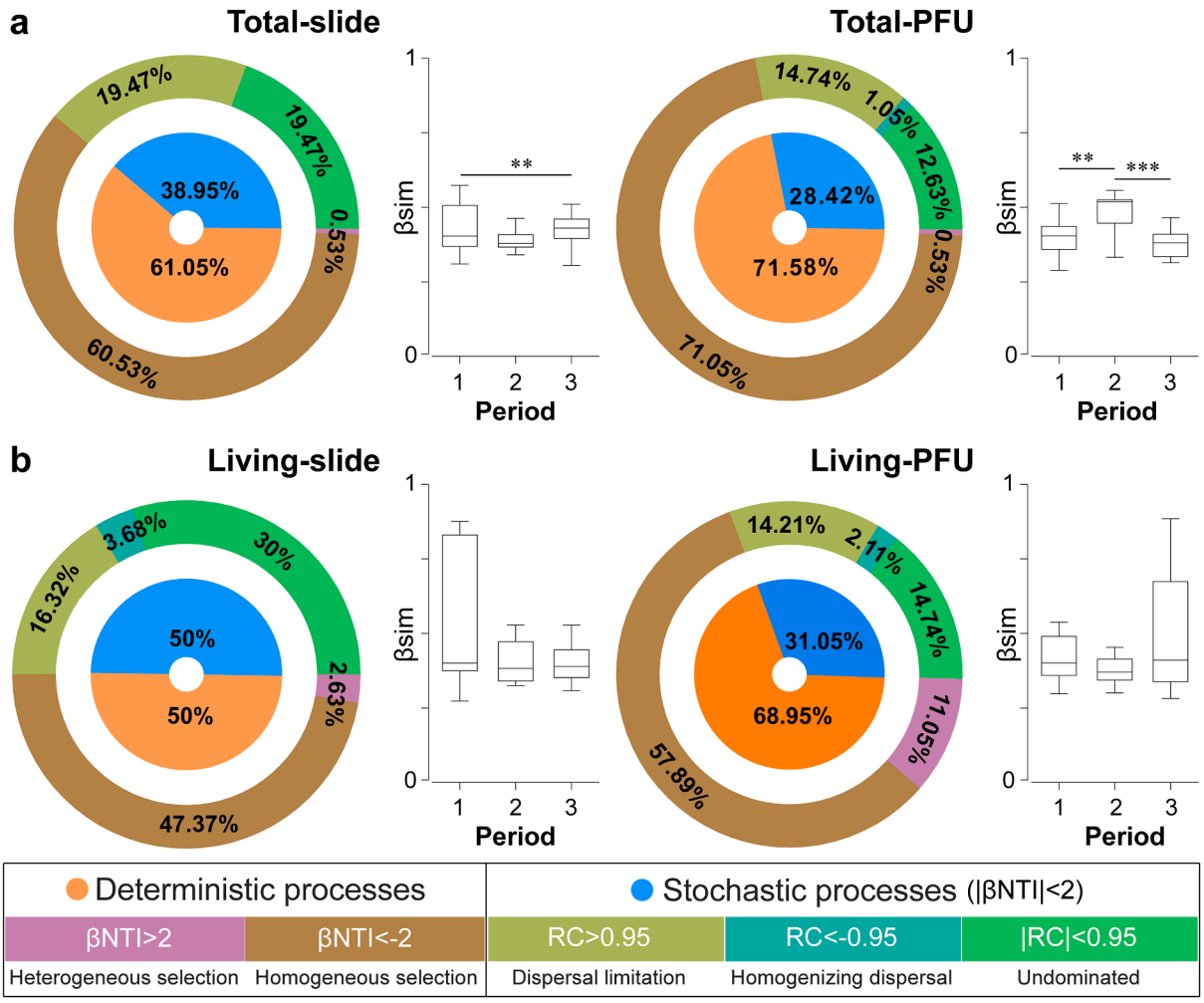

Fig. 4. The processes responsible for temporal succession of microeukaryotic communities on slide and PFU biofilms, respectively. [Inner circles with orange and blue color indicate the overall results of deterministic and stochastic processes, respectively. βNTI, the beta nearest-taxon index. RC, the taxonomic diversity matrix using modified Raup-Crick. Total indicates living community and relic DNA (a). Living indicates community with PMA treatment (b), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to period 3 on glass slides, in contrast to PFUs where it fluctuated and being higher initially at period 1, and again higher at period 3 (Fig. 4).

Using a phylogenetic bin-based null model, the results from the total DNA-based microeukaryotes suggested that succession of microeukaryotic communities on glass slides and PFUs was mainly driven by strong deterministic processes (i.e., 61.05 % on glass slides and 71.58 % in PFUs) and weak stochastic processes (i.e., 38.95 % on glass slides and 28.42 % in PFUs) (Fig. 4a). In contrast, when relic DNA sequences were removed from total DNA sequences, the living microeukaryotic community succession was equally driven by both stochastic and deterministic processes on glass slides (i.e., 50 % *vs* 50 %). In PFUs, the succession was still driven by strong deterministic processes and weak stochastic processes (i.e., 68.95 % *vs* 31.05 %) (Fig. 4b).

On glass slides, the presence of relic DNA sequences biased the results, showing a decrease of 3.68 % of homogenizing dispersal and 2.10 % of heterogeneous selection, and an increase of 3.15 % of dispersal limitation, 10.53 % of undominated and 13.16 % of homogeneous selection processes (Fig. 4a, b). Accordingly, there was a decrease of 10.52 % of heterogeneous selection, 1.06 % of homogenizing dispersal and 2.11 % of undominated, but an increase of 0.53 % of dispersal limitation and 13.16 % of homogeneous selection processes in PFUs (Fig. 4a, b), indicating that the presence of relic DNA sequences in total eDNA samples can bias the results. Therefore, our findings highlighted that living microeukaryotic community succession on glass slides was driven by both stochastic and deterministic processes, while strong deterministic processes drove community assembly in PFUs.

3.4. Environmental effect on microeukaryotic succession

In our study, the environmental conditions changed over time (PERMANOVA for all data, Pseudo-F = 2.606, $P < 0.05$), especially, water temperature, electrical conductivity, salinity, total organic carbon and blue algae (Cyanophyta) showed significant temporal variation (Table S2). Furthermore, temporal variability of water physicochemical variables and nutrient parameters showed greater variability than primary productivity in water. Physicochemical variables (ANOSIM, R = 0.54, $P < 0.05$) and nutrients (ANOSIM, R = 0.36, $P < 0.05$) showed stronger significant temporal variation than changing total algae in water over time (ANOSIM, R = 0.12, $P > 0.05$). The first two axes of PCoA explained 55.70 % of total variation in environmental variables.

The results of DistLM indicated that different environmental factors might affect microeukaryotic communities at different periods of succession (Fig. S3 and Table S3). Although the environmental factors affected the microeukaryotic communities differently regarding the substrate used in the experiment over time; among them turbidity, DO, green and brown algae (Chlorophyta, diatoms and dinoflagellates) were the main drivers for biofilm-dwelling microeukaryotes and had strong significant influence on their succession. Further, the dbRDA results

revealed that the first two axes explained 61.0 % of total community variation for samples with relic DNA, while accounted for 61.4 % of living community variation on glass slide (Fig. 5a, b). In PFUs, the first two axes of dbRDA explained 73.1 % of total community variation for samples with relic DNA (Fig. 5c), however, the model accounted for 68.6 % of variation in the living community (Fig. 5d).

On glass slides, dissolved oxygen alone explained 19.57 % of variation on total DNA-based community, while turbidity and dissolved oxygen explained 22.48 % and 23.09 % of variation for living community, respectively. In PFUs, turbidity and green algae (Chlorophyta) explained 35.26 % and 31.15 % variation for total DNA based community, respectively, whereas brown algae (diatoms and dinoflagellates) explained 21.70 % for living community (Table S3).

4. Discussion

4.1. Microeukaryotic community succession was masked by relic DNA sequences

In this study, we found that microeukaryotic communities showed significant substrate and time dependent succession, but the presence of relic DNA sequences masked this result significantly. We collected biofilms from natural environment and samples contained living community and dead microorganism-originated relic DNA. Such relic DNA sequences could create noise in the analyses, therefore, masked the true succession of living community through addition of extra sequences in the microbial community analyses. Consistently, recent studies in various ecosystems i.e., rivers- reservoirs (Xue et al., 2023) and soil (Carini et al., 2016) reflected our study results that microbial community profile and diversity can be obscured or confounded by relic DNA.

Our results specifically showed that changes of the living microeukaryotic community were significantly stronger on glass slides than on PFUs (Fig. 1), indicating that substrate type can have significant effects on microeukaryotic community succession in biofilms. Consistently, substratum dependent microbial colonization and biofilm succession appeared, which affects the timing and rate of biofilm development (Garrido-Benavent et al., 2020; Veach et al., 2016; Siboni et al., 2007; Xu et al., 2005). We found that three periods of microeukaryotic community succession were grouped into four different successional patterns, including an initial repression assemblage that peaked at period 1 (i.e. day 3 to 5), and an induction assemblage that peaked at period 3 (i.e. day after 17 to 30) (Fig. 2) – in the terminology used in microorganism ecology this is a contrast between taxa (Jiao et al., 2017; Battin et al., 2016; Fierer et al., 2010; Jackson, 2003). Temporal dynamics of the community succession have been shown to be dominated by repression and induction assemblages rather than by a fluctuating assemblage in a soil bacterial microcosm study (Jiao et al., 2017). Along with repression and induction taxa, our study showed the

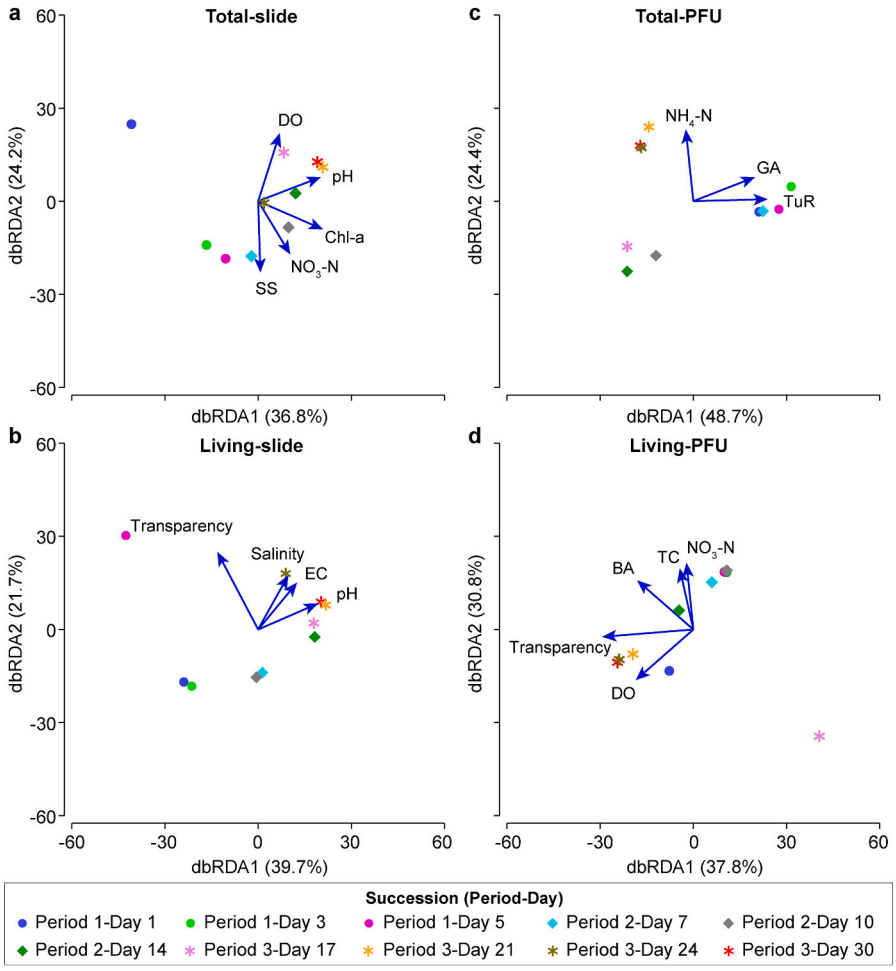

Fig. 5. Distance-based redundancy analysis (dbRDA) showing the significant predictive environmental variables for microeukaryotes community succession. [Physicochemical variables: EC, electrical conductivity; DO, dissolved oxygen; SS, suspended solids; TuR, turbidity; Primary productivity: Chl-*a*, chlorophyl-*a*; BA, blue algae; GA, green algae; Nutrients: NO_3-N, nitrate-nitrogen; NH_4–N, ammonium nitrogen, TC, total carbon. Total indicates living community and dead organismal DNA (a, c). Living indicates community with PMA treatment (b, d)]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

importance of a highly abundant fluctuating taxa (i.e., Ciliophora, Fungi and Metazoa) (Fig. 2), whose fluctuations might be due to changes in niche complexity and resource competition in the biofilm substrates. Studies in stream biofilm succession have shown that resource availability and physicochemical complexity can lead to temporal succession of microbial communities (Davey and O'Toole, 2000; Fierer et al., 2010; Besemer et al., 2007). Importantly, our results clearly showed that variation between periods 1 and 3 was higher than those of other two periods such as periods 1 and 2 or periods 2 and 3 (Table 1). The possible reason may conclude from the greatest and fastest changes in microbial community occurred in the early days than the late days during our study. This might be due to food supply, environmental conditions, competition for food and predation, also supported by our analyses on assembly mechanisms that homogeneous selection was the main driver.

A recent study in Appalachian karst caves, USA, indicated that Alveolata, Rhizaria, Stramenopiles and Ameobozoa were dominant groups, and they exhibited a significant temporal variation on glass slides (Cahoon and VanGundy, 2022), while a subtropical eutrophic freshwater colonization study in China has shown that Archaeplastida and Stramenopiles were abundant on PFUs, but Alveolata were dominant on glass slides (Zhu et al., 2020). Similarly, our study revealed that Alveolata, Hacrobia, Opisthokonta and Stramenopiles were dominant taxa on glass slides, while Alveolata, Archaeplastida, Hacrobia, Opisthokonta, Rhizaria and Stramenopiles were dominant in PFUs (Fig. S2). Although the general pattern of temporal dynamics of dominant microeukaryotic taxa was consisted with previous studies (Cahoon and VanGundy, 2022; Zhu et al., 2020), it is important to note that the presence of relic DNA sequences obscured these results, especially in biofilms on glass slides. In fact, the relative abundance of some taxa was decreased after removing relic DNA sequences. For example, Alveolata, Hacrobia, Opisthokonta, Stramenopiles were decreased by 1.46 %, 0.77 %, 4.37 %, 3.37 %, respectively on glass slides, while Alveolata, Archaeplastida, Hacrobia, Opisthokonta were decreased by 0.31 %, 1.28 %, 4.40 %, 6.71 %, respectively in PFUs (Fig. S2). Clearly, this is the evidence of the effects of relic DNA on microeukaryotes community compositional variation in DNA sequence-based study.

We also observed that the composition of both Rhizaria and Stramenopiles were more in living community than total DNA based samples which might be due to randomize biofilm sample selection for sequencing, and accumulated biofilm sample was not uniform. The other explanation of this inconsistency may come from partial assessment of PMA. A recent study using PMA method to profile microbial community in a river-reservoir ecosystem revealed that relic DNA obscures profile of multiple microbial taxa (Xue et al., 2023), therefore, partial assessment of PMA could have effect on profiling microbial community, which is also necessary to justify by more studies in future.

4.2. Microeukaryotic community in absence and presence of relic DNA

Our results revealed significant correlations between the living microeukaryotes and the total DNA-based microeukaryotic community (Fig. 3a). Further, the community compositional variation and species turnover were changed when relic DNA sequences were present in samples (Fig. 3b, c). A recent study in river-reservoir ecosystem showed that the removal of relic DNA clearly resulted in greater microeukaryotic (protistan and fungal) community dissimilarity over time (Xue et al., 2023). There was no clear difference in the rates of temporal community turnover between the PMA untreated and treated samples (Xue et al., 2023), however, in our biofilm succession we found a significant difference for turnover (Fig. 3c). Consistent with our study, it has been shown that relic DNA sequences influence the estimation of microbial community composition (i.e., eukaryotic, prokaryotic and fungal communities) in different environmental samples (Xue et al., 2023; Sakcham et al., 2022; Nagler et al., 2018a; Lennon et al., 2018; Carini et al., 2016). The succession of microbial communities involves a random arrival of different taxa, which converges to similar community types over time, however, due to resource availability and environmental condition can diverge the community with greater variation (Chang and Turner, 2019; Makoto and Wilson, 2019; Prach and Walker, 2011). A study of bacterial biofilms using ceramic tiles in the Konza Prairie stream, USA (Veach et al., 2016), and other stream biofilms succession studies have shown that stochastic processes were a strong driver (Battin et al., 2016; Dini-Andreote et al., 2015; Zhou et al., 2014; Besemer et al., 2012), but our results revealed both deterministic and stochastic processes were equally important for living community, especially on glass slide microeukaryotes. Because of the effects of relic DNA from dead microorganisms, the community assembly mechanisms result could change from reality. Although, previous studies used microscopy or did not take into account relic DNA, while our study emphasized the most updated approaches, especially PMA treated removal of relic DNA from dead microorganisms instead of commonly used data sets of total DNA-based community.

4.3. Microeukaryotic community assembly driven by strong homogeneous selection

In this study, the community assembly of the living community and the total DNA-based microeukaryotic community appeared driven by deterministic processes (Fig. 4), where both processes are contributed equally to the living community on glass slides (Fig. 4b). Our study uncovered the biased interpretation of community assembly mechanisms that deterministic processes appeared to be more important for total DNA based community, however when relic DNA sequences are absent in samples, both deterministic and stochastic processes were equally important especially for glass slide community assemblages. A recent study on glass slide and PFU biofilm colonization found that different mechanisms of community succession occurred based on substrate type; for example, species sorting was stronger for slide biofilm succession than for PFU (Zhu et al., 2020). This phenomenon was partly reflected in our results for the total DNA-based microeukaryotic community, but we also found additional evidence for strong homogeneous selection in the living community on glass slides and PFUs. This result suggests that dispersal may have a weaker effect on biofilm succession than previously recognised. Using a similar approach, a comparative study has shown a weaker dispersal effect on the active aquatic bacterial community assembly than on the total (active + inactive bacterial community) (Wisnoski et al., 2020). We particularly found that the presence of relic DNA sequences in samples overestimated homogeneous selection and dispersal limitation, but underestimated undominated processes (i.e., drift), homogenizing dispersal and heterogeneous selection in calculating community assembly processes, indicating that living microorganisms drifted alone with relic DNA. This is the first study to experimentally remove relic DNA sequences from environmental samples to investigate microeukaryotic community succession in aquatic biofilms on different substrates, showing the non-negligible effects of relic DNA sequences in the analysis. Our study clearly shows that relic DNA sequences influence the estimation of species turnover of aquatic microeukaryotes over time, thereby partially misleading the community assembly mechanisms.

4.4. Environmental influence on microeukaryotic community succession regarding relic DNA

In general, our study gives a novel insight on the DNA-based study on microeukaryotic succession with an emphasis on the effect of removal of relic DNA. It is noteworthy to mention that the degree of the environmental effects on microeukaryotic community succession was different at the three observed successional periods (Fig. 5). A recent study in Mono Lake, USA showed that primary succession of the surface microbial community was influenced by water temperature which enhanced primary producers (Phillips et al., 2021), while other previous stream biofilm studies showed that water temperature along with nutrients and

algae can create microhabitats of enhanced microbial growth and succession (Battin et al., 2016; Besemer et al., 2007; Battin et al., 2003). In our study, we clearly observed that turbidity, dissolved oxygen, brown and green algae had strong significant effects on microeukaryotic community succession. Studies have shown that small changes in salinity (Mo et al., 2021) and temperature (Wrona et al., 2006) may cause large-scale community structural variation in freshwaters. Similarly, our results suggest that salinity alone with other physicochemical parameters have a significant impact on microeukaryotic biofilm succession. More importantly, removing relic DNA sequences from samples through PMA-treatment revealed that environmental conditions strongly affected the living microeukaryotic community than those of total DNA-based community on glass slides, indicating that the presence of relic DNA sequences in the samples created "noise" that masked the "signal" of the real succession of the living microorganisms in the aquatic biofilms.

5. Conclusions

In this study, we present the consequence of relic DNA sequences on estimations of microeukaryotic species turnover and beta diversity, and show that the presence of relic DNA affected the ability to detect the true community succession and assembly mechanisms on biofilms substrate through DNA-based approaches.

The experimental removal of relic DNA sequences using PMA treatment showed significant changes in the estimation of beta-diversity, species turnover and community assembly mechanisms of aquatic microeukaryote succession (Fig. 6). We particularly observed that removal of relic DNA sequences from the environmental samples extended our general thinking of what we and other researchers consider to be important in formation of community assembly, such as selection, drift or diversification and dispersal. We found that importance of deterministic and stochastic processes was changed regarding the presence and absence of relic DNA for microeukaryotic community succession. This raises a fascinating and important question – to what extent is the identification of stochastic processes in microbial ecology an artifact of the molecular methods used?

5.1. Important implication of the present study

Terrestrial plant succession has been the subject of extensive study for over 100 years (Cowles, 1899), and provide a useful way of thinking about our results. We have highlighted that studying microbial succession using total DNA (i.e., including DNA from live microorganisms and relic) would be like studying a terrestrial plant succession where many of the dead, and no longer present plant species are included in the data being analyzed. This would obviously be confusing, and be likely to mask many important ecological patterns that we found in the present study. Therefore, we would like to recommend that relic DNA should be removed from total DNA samples for getting more accurate interpretation of microbial diversity in the ecosystems.

CRediT authorship contribution statement

Mamun Abdullah Al: Investigation, Visualization, Writing – original draft. **Huihuang Chen:** Investigation, Data curation, Validation, Writing – review & editing. **Xue Yan:** Investigation, Data curation, Validation, Writing – review & editing. **Kexin Ren:** Investigation, Data curation, Validation, Writing – review & editing. **Yuanyuan Xue:** Validation, Visualization, Writing – review & editing. **Jeff Shimeta:** Validation, Visualization, Writing – review & editing. **Markus Majaneva:** Validation, Writing – review & editing. **David M. Wilkinson:** Validation, Writing – review & editing. **Jun Yang:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

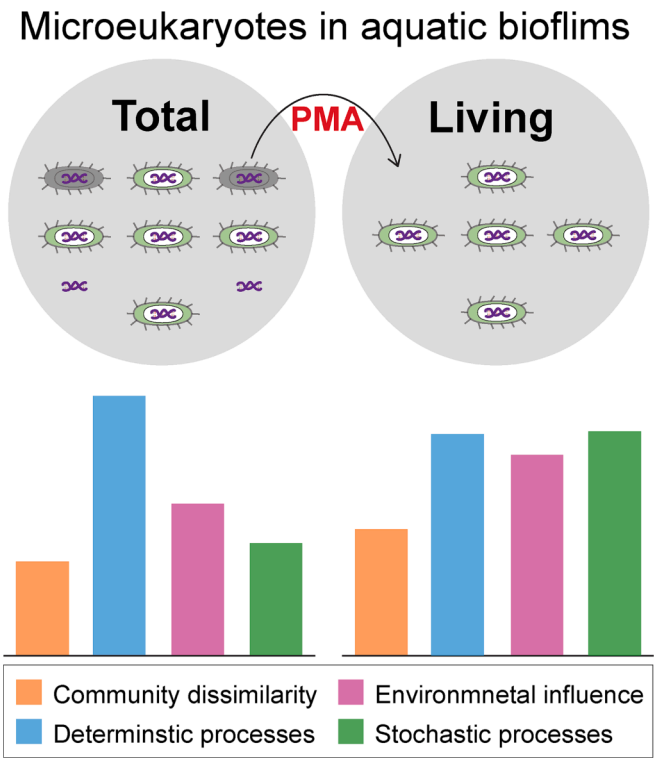

Fig. 6. A comparative view of microeukaryotic community assembly processes and environmental influence before and after PMA treatment from microeukaryote biofilm succession in a subtropical reservoir.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Data availability statement

All raw sequences data of 18S rRNA gene in this study have been submitted to the NCBI with accession BioProject number: PRJNA863539. These raw sequences were also deposited in the National Omics Data Encyclopedia (NODE) database under the Project number: OEP004780.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.111350.

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