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LIISI TALAS

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Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

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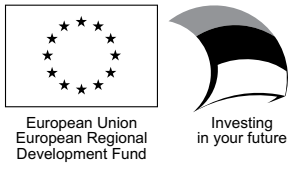

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Publication I Kisand, V., **Talas, L.**, Kisand, A., Stivrins, N., Reitalu, T., Alliksaar, T., Vassiljev, J., Liiv, M., Heinsalu, A., Seppä, H., Veski, S. (2018). From microbial eukaryotes to metazoan vertebrates: Wide spectrum paleo-diversity in sedimentary ancient DNA over the last ~14,500 years. *Geobiology*. 16, 628–639.

Publication II **Talas, L.**, Stivrins, N., Veski, S., Tedersoo, L., Kisand, V. (2021). Sedimentary ancient DNA (sedaDNA) reveals fungal diversity and environmental drivers of community changes throughout the Holocene in the present boreal lake Lielais Svētiņu (Eastern Latvia). *Microorganisms.* 9(4), 719.

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Publication I I performed laboratory work (PCR optimization, 18S rRNA region amplification, preparation of sequencing libraries), bioinformatic analysis and participated in data processing, graph making and writing the methods part of the manuscript.

Publication II I performed laboratory work (PCR optimization, ITS2 region amplification, preparation of sequencing libraries), bioinformatic analysis and data analysis. I composed the dataset for fungal ecological roles, created graphs and tables (except PCoA graph) and co-wrote the main text of the manuscript.

Publication III I composed one part of the dataset (e.g., sedaDNA fungal dataset), participated in producing graphs and tables, and co-wrote the manuscript.

ABBREVIATIONS

aDNA	ancient DNA
bgPCA	between-groups analysis of PCA ordination space
Ch	charcoal particles
COI	cytochrome c oxidase subunit 1
cyt b	cytochrome b
Dtol	drought tolerance
GAM	generalized additive model
HRP	human related pollen
HTM	Holocene thermal maximum
ITS	internal transcribed spacer
LMM	linear mixed models
LSU	large subunit ribosomal DNA
LSv	lake Lielais Svētiņu
MCM7	minichromosome maintenance protein 7
mOTU	molecular operational taxonomic unit
NPPs	non-pollen palynomorphs
OPEN	vegetation openness
PCA	principal component analysis
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
RDA	redundancy analysis
rDNA	ribosomal DNA
RPB1	the largest subunit of RNA polymerase II gene
RPB2	the second largest subunit of RNA polymerase II gene
sedaDNA	sedimentary ancient DNA
SSU	small subunit ribosomal DNA
Stol	shade tolerance
Tcont	continental climate
Tsum	summer temperatures
Wtol	waterlogging tolerance

1. INTRODUCTION

Biological material originating from the water column and surrounding terrestrial environments carried into the lake accumulates over time into lake sediments, where its DNA can preserve over long periods of time. The oldest detected DNA from lake sediments dates back to ~270 000 years (Randlett et al., 2014). Sedimentary ancient DNA (sedaDNA) is a relatively new tool in paleo-environmental research where its use has increased rapidly in the last 5 years. Regardless of restrictions of sedaDNA research (e.g., DNA preservation), the sedaDNA has enormous potential for recovering past biodiversity changes in the lake ecosystem and its catchment area. SedaDNA enables us to identify how communities change over time and to understand better when and why these changes in diversity appeared. Furthermore, established community changes can bring light to past processes in lake ecosystems or surrounding vegetation dynamics and permits us to study the impact of environmental change to these communities. Past long-term community dynamics and their responses to environmental drivers are important for trying to explain nowadays biodiversity patterns and to model possible future scenarios in the climate warming conditions.

Our study lake was Lielais Svētiņu (LSv) which can be considered a model lake for the North-East Europe region since it has a long sediment record and well-defined human impact in this area (Stivrins et al., 2015). This enables us to study natural and anthropogenic influences on the communities in lake ecosystems. LSv has also been a study object for multiple paleoecological studies using pollen and non-pollen palynomorphs (NPPs) analysis, and thus, obtaining a good understanding of past climate changes and vegetation development over the Holocene (Stivrins et al., 2014; Veski et al., 2012, 2015). These compiled environmental proxies would enable us to draw stronger conclusions between changes in aquatic ecosystems and external drivers.

Eukaryotes (including fungi and phototrophs) are important components of the lake ecosystems contributing to biogeochemical cycles and the lake ecosystem functioning (Dinsdale et al., 2008). Phototrophs such as phytoplankton are primary producers in the lakes, where they play a key role in food web processes. Primary producers can respond rapidly to changing environmental factors, and thus, influence the whole ecosystem functioning (Stivrins et al., 2015). In addition, fungi that are common residents in the sediments play various ecological roles as saprobes, parasites, and symbionts (i.e., ecophysiological groups) in aquatic environments (Liu et al., 2017; Xu et al., 2019). For example, chytrids can also be part of the food web as mediators transferring nutrients of phytoplankton to zooplankton (Kagsami et al., 2014). Thus, changing environmental conditions inducing any change in phototroph or fungal diversity impacts the other aquatic organisms in the lake and may lead to ecosystem change. Previous paleo-studies have mainly relied on pollen and non-pollen palynomorphs such as remains of diatoms, fungal spores, dung, and algal pigments (Stivrins et al., 2015; Väliranta et al., 2015; Stivrins et al., 2018; Eilers et al., 2004; Ficetola et al.,

2018). As many algal taxa do not preserve well as fossils and the taxonomic resolution of the remains can be limited, then the sedaDNA have been used to overcome these difficulties (Jørgensen et al., 2012; Tse et al., 2018; Stivrins et al., 2018).

This thesis focuses on studying the eukaryotes biodiversity changes, especially fungal and phototroph dynamics, and environmental drivers inducing these changes over the post-Glacial period in lake Lielais Svētiņu. We tested the effectiveness of sedaDNA reconstructing the changes in the diversity of eukaryotes and explored the community responses to the environmental changes. In addition, we tested fungal ecophysiological groups as new signatures of ecosystem changes in the lake. In this work, the fungal ecophysiological groups were defined as functional ecological roles grouped by fungal lifestyles, trophic status or habitat.

2. LITERATURE OVERVIEW

2.1. Potential of sedimentary ancient DNA (sedaDNA)

Lake sediments are biological archives that consist of buried organic and inorganic material that originates both from the lake ecosystem and from surrounding terrestrial environments. Paleoecological studies have been using largely classical microscopy methods to analyse the well-preserved remains (micro- and macrofossils of pollen, diatoms, and fungal spores) (Etienne et al., 2011; Stivrins et al., 2014, 2015; Väliranta et al., 2015) in historical sediments leaving still a large part of biodiversity unreachable. Therefore, ancient DNA preserved in the sediments was adopted as an alternative proxy. Sedimentary ancient DNA is a rather new tool for paleoecological studies that prospects to reconstruct past biodiversity changes in both aquatic and terrestrial environments. Coolen and Overmann demonstrated already in 1998 the usability of sedaDNA to detect sulphur bacteria in Holocene sediments based on 16S rRNA gene sequences. However, only during the last five years, an increasing number of studies in the field of paleoecological research have been published using sedaDNA of various organisms (plants, animals, fungi) to reconstruct long-term biodiversity changes in the ecosystems (Bellemain et al., 2013; Clarke et al., 2019; Ficetola et al., 2018; Nelson-Chorney et al., 2019). SedaDNA holds a great potential for reconstructing past biodiversity changes in the aquatic and surrounding terrestrial catchments (Capo et al., 2016; Kisand et al., 2018; Sjögren et al., 2017). The long-term biodiversity changes can provide understanding about lake ecology (Capo et al., 2016), local vegetation dynamics (Alsos et al., 2016; Clarke et al., 2019) and responses of different organisms to environmental changes (e.g., climate change, anthropogenic impact) (Alsos et al., 2016; Giguet-Covex et al., 2014; Pansu et al., 2015). SedaDNA also offers opportunities to study the impact of invasive species (Ficetola et al., 2018) and even agricultural occurrences and history (Giguet-Covex et al., 2014; Madeja et al., 2009). Although sedaDNA has found wider use in paleo-studies, it is still in its infancy and holds many methodological limitations (discussed below).

2.2. SedaDNA degradation, preservation and leaching

The sediments include both intracellular and extracellular DNA, where the latter is a result of the lysis of the dead cells (Pietramellara et al., 2009). After the death of an organism, the DNA starts decaying induced by active endonucleases and reactive chemicals (Hofreiter et al., 2001; Nielsen et al., 2007). Further, dead cells can be quickly lysed releasing DNA into the environment (Pedersen et al., 2015). Extracellular DNA is further degraded by environmental factors (e.g., radiation and oxidation) and microorganisms using DNA as energy sources (Hofreiter et al., 2001; Nielsen et al., 2007). These processes result in fragmented DNA

segments that will become extensive over a long period of time until no DNA molecules remain (Pedersen et al., 2015). Therefore, partly damaged, small DNA fragments are characteristic for ancient DNA (aDNA). Lindahl (1993) estimated that DNA can survive about a few hundred thousand years before DNA is completely destroyed based on *in vitro* experiments. In addition, low temperature conditions have been suggested to extend DNA survival for much longer – ca. 0.5 million year old plant DNA was recovered from ice cores (Willerslev et al., 2007). One of the oldest known sedaDNA has been retrieved from 270 000 years old lake sediments in Turkey (Randlett et al., 2014).

DNA preservation in the sediment is mainly affected by environmental conditions such as temperature, anoxia, salinity, pH, organic compounds and charged particles (Capo et al., 2021; Dabney et al., 2013; Pedersen et al., 2015). Extracellular DNA can bind to humic acids, clay minerals, sand particles and other organic compounds that protects DNA from nuclease activity, and therefore, extends DNA survival in the sediment (Pedersen et al., 2015). The absorption of DNA molecules to these compounds depends on the length of the DNA, soil/ sediment pH and cation concentrations (Pedersen et al., 2015; Pietramellara et al., 2009). For example, DNA is adsorbing to humic acids generally in lower pH conditions (pH= 3–4) than to clay or sand particles (pH >5) (Pietramellara et al., 2009). Also, Lorenz and Wackernagel (1987) demonstrated that sand-DNA bridges formed best in increased salt concentration conditions, especially in the presence of Ca^{2+} and Mg^{2+} cations. Low temperatures and anoxic conditions are other factors supporting long-term DNA survival in sediments (Capo et al., 2021; Nielsen et al., 2007). This is supported by many studies recovering sedaDNA from permafrost, frozen sediments and temperate lake sediments (Clarke et al., 2019; Willerslev et al., 2014; Kochkina et al., 2012; Lydolph et al., 2005; Coolen et al., 2004). Meanwhile, it was recently shown that sedaDNA can be recovered also from tropical lakes (Epp et al., 2010; Vuillemin et al., 2016; Bremond et al., 2017). However, Vuillemin et al. (2016) showed that the quality of recovered sedaDNA from tropical sediments was poor. The recovery of DNA rapidly decreased already in the upper 5 cm that represented the last ~250 years in Lake Towuti (Indonesia) (Vuillemin et al., 2016). Similarly, Bremond et al. (2017) demonstrated successful sedaDNA recovery mainly in the upper sediments comparable to the last ~300 years and pointed out the importance of sediment quality for successful DNA preservation. Rapid DNA degradation can happen due to some unfavorable conditions for sedaDNA preservation, such as higher water temperatures (e.g., 28 °C throughout a year) and microbial activity in the sediment (Vuillemin et al., 2016; Bremond et al., 2017). Therefore, DNA preservation conditions in the sediment still need to be studied as it depends on a combination of many environmental conditions.

Possible DNA leaching is another factor influencing accurate DNA recovery from the sediments. DNA leaching is the vertical movement of aDNA across sediment layers and, therefore, could complicate the authentic aDNA detection (Pääbo et al., 2004; Haile et al., 2007). Potential DNA leaching has been a concern, especially for non-frozen environments (Haile et al., 2007). Two studies

from terrestrial sediments showed DNA leaching in cave sediments for larger animals (e.g., sheep) likely related to animal urine migrating through sediment layers (Haile et al., 2007; Andersen et al., 2012). Nevertheless, many studies have shown that DNA leaching is not a significant problem in permafrost and lake sediments (Willerslev et al., 2014; Sjögren et al., 2017; Lydolph et al., 2005). One supporting evidence was that floral communities recovered with sedaDNA were well overlapping with historical or plant macrofossil records (Sjögren et al., 2017; Alsos et al., 2016). Also, quick DNA adsorption by sand, clay, humic acid and other sediment components (e.g., DNA binding reached maximum 1 h later) supports this claim (Blum et al., 1997). In addition, Hansen et al. (2006) showed a clear relationship between DNA damage and the age of the sample in permafrost samples, which further proved the accuracy of detected DNA. Therefore, the success of the accurate aDNA detection depends on the analysed sediment type, group of recovering organisms (e.g., large animals) and historical events in the site (e.g., possible sediment mixing).

2.3. Traditional methods for detecting past communities

The most widely used proxies in paleoecological studies have been pollen, plant macrofossils, microfossils of algae and cyanobacteria, pigments and fungal spores analysed by classical microscopy-based methods (Stivrins et al., 2014; Väliranta et al., 2015; Stivrins et al., 2018; Eilers et al., 2004; Ficetola et al., 2018; Pal et al., 2015). Microscopy analysis enables to identify and count the fossil remains recovered from historical sediments based on the morphology of the remains (Capo et al., 2021). Pollen has been one of the most used proxies. The quantity of deposited pollen reflects the abundance and dynamics of past plant taxa (Wright, 1967), and thus, it has been used to reconstruct long-term vegetation dynamics and development (Stivrins et al., 2014; Hannon et al., 2010; Seppä et al., 2002). For example, Seppä et al. (2002) used pollen to determine the treeline changes in response to post-glacial climate changes in northern Fennoscandia, showing the transition from birch forest at 9600 cal yr BP to the dominance of pine forest at 6100–4000 cal yr BP. Pollen and plant macrofossils are also shown to be useful for establishing local and regional changes in plant communities (Stivrins et al., 2014), early land use (Rey et al., 2013) and for the reconstruction of climate evolution in the Holocene (Väliranta et al., 2015). Väliranta et al. (2015) showed that aquatic plant macrofossils are a valuable addition to tree-pollen-based temperature reconstructions since aquatic plants can respond faster than trees to climate changes. Thus, the multi-proxy studies are preferred as different proxies complement each other's shortcomings (Jørgensen et al., 2012). In addition to the benefits, the main downsides of using the microscopic analysis are the selective preservation and distribution of the remains and a poor taxonomic resolution, especially on genus and species level (Jørgensen et al., 2012; Boessenkool et al., 2014; Clarke et al., 2019). Many studies have been using sedaDNA in

comparison with other micro- and macrofossil proxies to overcome these difficulties (Jørgensen et al., 2012; Stivrins et al., 2018; Tse et al., 2018; Boessenkool et al., 2014; Clarke et al., 2019). SedaDNA offers better taxonomic resolution, but also enables to study different organism groups (e.g., plants, animals) and otherwise underestimated plants in pollen analysis, such as insect-pollinated plants and aquatic vegetation (Etienne et al., 2015; Alsos et al., 2018; Jørgensen et al., 2012; Clarke et al., 2019). Also, pollen, plant macrofossil and sedaDNA comparative studies have shown a good overlap between sedaDNA and plant macrofossils, and partial overlap with pollen (Jørgensen et al., 2012; Alsos et al., 2016). This was explained by the dispersal distance of plant material. Alsos et al. (2018) showed that highly abundant plant taxa detected with sedaDNA was matching dominant vegetation within 2 m which decreased with distance from the lake catchment area. This supports the suggestions that sedaDNA describes flora from local areas while pollen provides plant taxa that have been dispersed from longer distances.

Other non-pollen palynomorphs (NPPs), such as fossils of algae and cyanobacteria and pigments, have also been used combined with sedaDNA to accomplish reconstruction of the local lake ecosystems (Stivrins et al., 2018; Pal et al., 2015; Tse et al., 2018). For example, Stivrins et al. (2018) studied the algal fossils, pigments and sedaDNA to reconstruct algae turnover rates and found only a small overlap of species between algal fossils and sedaDNA. Further, the algal accumulation rates showed also a mismatch between fossil and pigment based rates. The suggested reason was that algal fossils describe only partially the phytoplankton biomass and taxonomy since some taxa do not preserve well as fossils in the sediments (Stivrins et al., 2018). Further, the selective destruction of thin-walled cysts can also happen by sample preparation techniques (e.g., acetolysis) (Riddick et al., 2017). For example, desmids abundance (group of green algae) decreased by 87% after acetolysis (Riddick et al., 2017). Some other groups such as *Pediastrum* and cyanobacteria with thick-walled cysts are considered more resistant to such techniques, and thus, more suitable for the historical reconstructions of community dynamics (Riddick et al., 2017; Eilers et al., 2004). Therefore, to exceed the limits of microfossils, various pigments have been used to identify cyanobacteria and algae that have lost their cells (Pal et al., 2015; Stivrins et al., 2018). Algae and cyanobacteria contain pigments (e.g., carotenoids, chlorophylls), which are generally well preserved in the lake sediments (Leavitt and Hodgson, 2001). For instance, *beta*-carotene is a pigment used as a marker for total algal biomass, zeaxanthin, canthaxanthin and echinenone represent cyanobacteria, and chlorophyll b and lutein have been used to detect green algae (Leavitt and Hodgson, 2001; Stivrins et al., 2018; Tõnno et al., 2021). Pigments are useful for quantitative estimation of taxa groups, but still, they can provide only limited taxonomic affiliations. Therefore, sedaDNA has been combined with pigment proxies to improve the taxonomic identification at genus and species level (Tse et al., 2018; Stivrins et al., 2018). Also, significant overlap was observed between sedaDNA and pigments (Pal et al., 2015; Tse et al., 2018) making it a useful proxy for paleolimnology studies.

At the same time, mammalian DNA detection from sediments has been low and fungal spores have been used to detect mammal occurrences in the area (Etienne et al., 2011). Coprophilous fungi grow on the animal dung and their deposited spores can be detected from the sediment. Thus, spores of coprophilic fungi such as *Sporormiella* sp have been useful to study herbivores (Etienne et al., 2011). The detection of mammals, especially their DNA, also depends on the animal distance from the shore and the abundance of animal material carried to the lake. For example, Ficetola et al. (2018) were able to demonstrate the long-term impact of invasive rabbits on the vegetation using sedaDNA and spores of coprophilous fungi. The success of mammal DNA detection was likely due to the presence of rabbits burrowing on the shores of the lake, where animal material was carried into the lake by erosion and run-off water. Therefore, multi-proxy studies have been found to offer the best means for the detection of past communities from the sediment.

2.4. DNA-based methods to detect a wide variety of past communities

The introduction of high-throughput DNA sequencing enabled the use of sedaDNA to assess the past biodiversity. One of the most widely used approaches in paleoenvironmental studies has been DNA metabarcoding (Capo et al., 2016; Bellemain et al., 2013; Pansu et al., 2015) that can be used to discover the composition of specific groups or communities. The DNA metabarcoding approach is based on short DNA markers, also called "barcodes", that can be used to assign taxonomic affiliation when compared with reference sequences in the databases (Taberlet et al., 2018). The standardized markers consist of two regions: the variable region in the middle and highly conserved regions at both sides. The variable region is discriminative for the target species and contains information to assign its taxonomic identification. The conserved regions are used for primer annealing sites and are identical for the target group, but still different from non-target organisms (Taberlet et al., 2018). Thus, these standardized DNA marker regions should be variable enough to discriminate among lower taxonomic levels (e.g., genus, species), but conserved enough to detect all the higher-level taxa (Taberlet et al., 2007). The choice of metabarcodes is an important step and depends on multiple factors such as the required level of taxonomic resolution, degree of DNA damage and studied organisms (wide community or specific species detection) (Taberlet et al., 2018). As the sedaDNA is generally highly degraded, the short DNA barcodes (<150 bp) or multiple barcodes are recommended to use and have been used especially for plants (Capo et al., 2021). Still, also longer, up to 577 bp rbsL gene fragments have been successfully used for diatom detection (Stoof-Leichsenring et al., 2014). A variety of DNA markers have been used in paleoenvironmental research to study the whole community (universal primers) or target specific organism groups (e.g., plants, mammals,

fish, fungi, algae) (Capo et al., 2016; Alsos et al., 2016; Giguet-Covex et al., 2014; Miya et al., 2015; Bellemain et al., 2013; Stoof-Leichsenring et al., 2014; Capo et al., 2021).

Universal primers are designed to obtain the diversity and community composition of a wide variety of taxa. For example, the 18S rRNA gene region has been used to detect the biodiversity of microbial eukaryotic communities, and the 16S genes have been used for bacteria, archaea and cyanobacteria detection (Capo et al., 2021). Universal primers are more conserved sequences than highly specific ones and, therefore, have a higher chance of incorrect taxonomic assignments and higher taxonomic resolution (Taberlet et al., 2018). Nevertheless, they allow access to a wide variety of taxa that is useful to track past community changes. Different parts of the 18S rRNA gene region have been used to detect the dynamics of microbial eukaryotes (Figure 1). Capo et al. (2016) used the 18S rRNA gene V7 region to study the temporal variation and community composition of microbial eukaryotes in relation to climate fluctuations. Also, 18S rRNA gene regions of V1–3, V4, V7 and V9 have been used for the detection of microbial eukaryotes (including fungi, diatoms and zooplankton) (Coolen et al., 2013; Kisand et al., 2018; Capo et al., 2016; Guardiola et al., 2015; More et al., 2018). The choice of the markers also depends on the sequence length as mentioned before. The 18S rRNA gene V4 and V7 regions are both good marker candidates regarding the coverage, but in terms of sequence length, the V7 region is shorter and might be more useful when working with historical sediments (Capo et al., 2016). Capo et al. (2016) showed amplification loss with V4 region (~360 bp) markers compared with V7 region (~250 bp) markers in sediments older than 900 years. Thus, 18S rRNA gene V7 region markers offer a sufficient taxonomic resolution and reasonable sequence length (~250 bp) compared with other regions for paleoenvironmental research (Capo et al., 2016).

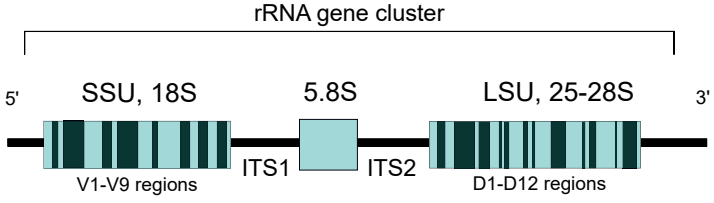

Figure 1. Eukaryotic ribosomal RNA gene cluster. Black lines present the variable regions V1–V9 and D1-D2 in ribosomal small subunit (SSU) and large subunit (LSU) rRNA gene regions, respectively.

In addition to universal 18S rRNA V4/V7 regions also used for diatom and zooplankton detection (Zimmermann et al., 2014; Kisand et al., 2018; Guardiola et al., 2015), the short chloroplast rbcL (67–76 bp) barcode and cytochrome c oxidase subunit 1 (COI) gene (313 bp) have been successfully applied to detect diatom diversity and study metazoans (including zooplankton), respectively (Stoof-Leichsenring et al., 2014, 2012; Dulias et al., 2017; Geller et al., 2013). Still, the COI gene region also targets other metazoan groups, and so far, only specific

groups of zooplankton (e.g., copepods, rotifers) have been targeted with specific primers (Epp et al., 2015, 2010). Thus, universal barcodes for zooplankton diversity assessment are still needed.

For fungal detection from sediments and permafrost, mainly 18S rRNA gene and internal transcribed spacer (ITS) region have been used (Lydolph et al., 2005; De Schepper et al., 2019; Bellemain et al., 2013; Kochkina et al., 2012). Even though the 18S rRNA gene region is useful to detect a wide variety of eukaryotes (including fungi), its resolving power between fungal species is poor, especially for closely related species (Schoch et al., 2012). Schoch et al. (2012) demonstrated that the 18S rRNA gene region had the lowest barcode gap when discriminating between fungal species compared with other regions (LSU, RPB1, ITS). ITS region showed the best species discrimination with a clearly defined barcode gap and high polymerase chain reaction (PCR) amplification success. Thus, the ITS region was proposed as a universal DNA barcode for fungal diversity assessment (Schoch et al., 2012; Bellemain et al., 2010). There are two ITS regions in 18S–5.8S–28S cistron: ITS1 and ITS2 (Figure 1), that have been used for recovery of fungal biodiversity from permafrost and other environmental samples (Bellemain et al., 2013; Kochkina et al., 2012; Monard et al., 2013). Commonly utilized primer pairs (e.g., ITS1-F/ITS2 and ITS3/ITS4) designed for the ITS1 and ITS2 regions showed in comparison that ITS1 region primers amplified more basidiomycetes and ITS2 slightly more ascomycetes (Bellemain et al., 2010; Monard et al., 2013). Thus, multiple primer combinations are now recommended for fungal biodiversity assessment to avoid such primer bias (Bellemain et al., 2010). The protein-coding genes (e.g., RPB1, RPB2, MCM7) have also shown high species-level identification, but due to a low PCR amplification success, they were excluded as possible universal barcodes (Schoch et al., 2012). Still, these protein-coding genes are used for phylogenetic analyses and species detection of limited taxa (Schmitt et al., 2009; Liu and Hall, 2004). Also, LSU and ITS regions have been used complementary to each other to enable species identification with ITS and phylogenetic analysis with LSU (Klaubauf et al., 2010).

For past vegetation studies, the most used DNA marker to target plants has been chloroplast trnL (UAA) intron (254–767 bp), especially its shorter fragment P6 loop (10–143 bp) (Willerslev et al., 2007; Taberlet et al., 2007; Alsos et al., 2016). Also, other barcodes have been used such as ITS region and chloroplast trnH–psbA regions (~450 bp) for plant phylogenetic studies and detection of flowering plants (Álvarez et al., 2003; Kress et al., 2005). The main drawback of trnL (UAA) intron has been relatively low resolution due to lower intraspecific variation compared with other non-coding regions (e.g., ITS, trnH–psbA region) (Kress et al., 2005). It is even more apparent with using a shorter P6 loop region. Thus, trnL (UAA) intron does not perform well when detecting plants at species level or differentiating between closely related species. Still, it has many advantages, such as highly conserved regions for primers and robust amplification making trnL intron one of the most used barcodes in past vegetation dynamics studies (Taberlet et al., 2007). The P6 loop has been shown to perform well when using highly degraded DNA (Alsos et al., 2016; Taberlet et al., 2007). Also, the

limitation of relatively low taxonomic resolution can be somewhat compensated when using the local plant reference libraries containing only a certain number of species (Taberlet et al., 2007).

Many universal and species-specific primer sets have been designed for mammal detection (e.g., Mam1, Mam2) (Hebsgaard et al., 2009; Giguet-Covex 2014; Valsecchi et al., 2020). The mitochondrial 16S, 12S rRNA genes, COI gene, control-region and cytochrome b (cyt b) gene regions have been targeted to detect mammoth, moa, bison, horse, reindeer, musk ox, brown lemming, sheep and rabbit DNA from permafrost, cave and lake sediments (Willerslev et al., 2003; Haile et al., 2007; Ficetola et al., 2018). For example, general mitochondrial 16S rRNA gene region primers (Mam1, Mam2) have been used for mammoth detection (Haile et al., 2009; Hebsgaard et al., 2009), but more specific ones were designed for horse detection (Hebsgaard et al., 2009). In addition, mitochondrial 12S rRNA gene region and control region have been effectively used to recover extinct moa and avian species from cave sediments (Willerslev et al., 2003). Also, a newer universal primer MamP007 has been proposed for mammalian recovery for lake sedaDNA studies (Giguet-Covex 2014, 2019; Ficetola et al., 2018). MamP007 targets mitochondrial 16S rRNA gene and enables it to amplify shorter 60–84 bp fragments that are highly desirable for aDNA studies (Giguet-Covex 2014, 2019). 16S and 12S rRNA gene regions are less variable than other used genes (cyt b and COI) which makes it difficult to differentiate between closely related species (Tillmar et al., 2013). Still, universal 16S/12S region primers have been shown useful in recovering a wide variety of mammals and other organisms such as birds, insects, fish and snakes (Yang et al., 2014). Recently, Valsecchi et al. (2020) designed universal primer sets (MarVer1 and MarVer3, targeting mitochondrial 12S and 16S rRNA genes, respectively) specially for marine vertebrate detection, including marine mammals, fish, seals, sea turtles, birds. Even though fish detection has mainly relied on species-specific primer sets which amplifies only a couple of fish species (Kuwae et al, 2020; Sakata et al., 2020), Miya et al. (2015) also introduced universal primers (MiFish-U/E) amplifying 163–185 bp fragment of 12S rRNA gene suitable for fish community studies.

All the above-mentioned universal and species-specific primers permit to use sedaDNA as a tool for the reconstruction of past environments, community dynamics and environmental disturbances (Ficetola et al., 2018; Alsos et al., 2016; Pansu et al., 2015). For example, sedaDNA of plants and mammals was used with specific markers to study the long-term effects of invasive rabbits on the ecosystem changes (Ficetola et al., 2018). Ficetola et al. (2018) demonstrated a fast and strong impact of rabbits on the dominant plant species, which took less than 10 years, generating a shift in plant communities and erosion rates. Also, Nelson-Chorney et al. (2019) demonstrated sedaDNA usability to determine the introduction of non-native and native fish diversity with species-specific markers. These studies prove the usefulness of sedaDNA as a biomonitoring tool in long-term time scales. Also, the universal markers have helped provide insight into broad ecosystem changes, e.g., vegetation dynamics, related to the impact of climate change and anthropogenic influence (Alsos et al., 2016; Clarke et al.,

2019; Giguet-Covex et al., 2014; Pansu et al., 2015; De Schepper et al., 2019). For instance, De Schepper et al. (2019) identified the changes in eukaryote biodiversity over the ~100 000 years that were related to the evolution of sea ice. The anthropogenic impact on ecosystems has been studied through human activities such as grazing, deforestation and agriculture. One of the first studies tracking human and cattle presence in the sediments was using bacterial aDNA (e.g., *Bifidobacterium*, *Bacteroides*-*Prevotella*) (Madeja et al., 2009). Others showed human impact on plant communities and used sedaDNA to study even the history of livestock farming (Giguet-Covex et al., 2014; Pansu et al., 2015). For example, Giguet-Covex et al. (2014) showed that sedaDNA permits detection of intense erosion in the Late Iron Age and Roman Period caused by deforestation and overgrazing by livestock.

In addition to the widely used metabarcoding approach, metagenomics is believed to become a new useful approach for paleoenvironmental studies (Capo et al., 2021). The metagenomics approach is based on shotgun sequencing a total DNA pool providing genome-wide information. So far, shotgun metagenomics has been used to detect the presence of humans and mammalian megafauna in the sediments (Graham et al., 2016). Still, comprehensive reference databases are needed before adequate and wide use of metagenomics in the paleoenvironmental field can happen.

2.5. Methodological considerations when using sedaDNA

The possible contamination, choice of extraction method, primer bias and DNA degradation are all the main factors to consider when conducting paleoenvironmental research (Boessenkool et al., 2012; Capo et al., 2021). The possible contamination is always a risk when working with low concentration sedaDNA. The contamination with modern DNA can happen in each step from the sample collection to sequencing. Thus, special care should be taken with using sterile tools and a clean work environment when coring, taking subsamples from the core, doing DNA extraction and amplification. Decontamination with UV radiation and the use of negative controls in each step is considered a mandatory practice to minimize the risk of contamination from the regents or the environment (Capo et al., 2021). Also, human-blocking primers have been used to avoid modern human contamination and PCR bias towards contaminants (Boessenkool et al., 2012). It is the case, especially when trying to access mammalian sequences from the sediments. Boessenkool et al. (2012) demonstrated that the use of human-blocking probes inhibited the amplification of human DNA sequences, enhancing the retrieval of other mammalian species from sedaDNA.

The choice of DNA extraction protocol is an important step influencing the DNA yield from the extraction. As the sediment type (e.g., clay, humic acid rich sediment) is also affecting the extraction effectiveness, the multiple extraction protocols were recommended to use as pre-analysis before making the decision (Capo et al., 2021). Still, the two most used DNA extraction kits for sedaDNA

have been PowerSoil and PowerMax extraction kits, providing a good combination of yield and purity of extracted DNA (Lekang et al., 2015). Even the combination of DNA extracts retrieved with different DNA extraction protocols was suggested to increase the chances of detecting a target organism (Capo et al., 2021).

Possible primer bias favoring amplification toward some organism taxa in PCR is another troublesome factor. Monard et al. (2013) showed using commonly applied ITS region primer pairs for fungal biodiversity detection that ITS1 region primers were recovering more *Basidiomycota* and ITS2 primers amplified more *Ascomycota* taxa. This demonstrates the taxonomic biases introduced by the primer choice. Nevertheless, to overcome this obstacle, it was recommended to use multiple regions and primer combinations (Monard et al., 2013; Bellemain et al., 2010). Also, the variability in marker gene repeats between taxa influences the amplification success, and thus, the reliable evaluation of species abundance. Stadler et al. (2020) demonstrated that fungal species differ in their ribosomal DNA (rDNA) copy numbers ranging from 3 to 19 copies of the ITS region. Thus, a smaller number of copies of the marker region can result in lower detection in PCR and does not describe the "real" taxa abundance in the environment. Still, the qPCR method can be used to assess the quantity of specific taxa and inhibition levels in the samples (Capo et al., 2021).

Further, the choice and availability of reference libraries used for taxonomic assessment of DNA sequences are essential factors to enable the successful use of sedaDNA for paleo-research. For example, specific reference sequence databases such as SILVA (Quast et al., 2013) can be used to identify *Bacteria*, *Archaea* and *Eukarya* based on SSU and LSU rRNA sequences. Furthermore, the RP2 database (Guillou et al., 2013) can be used for microbial eukaryotes detection containing 18S rRNA sequences, and UNITE library (Kõljalg et al., 2013) for fungal-specific identification based on ITS region sequences. Also, compiled local reference libraries (e.g., PhyloAlps, PhyloNoraway; Alsos et al., 2020) are important to match the detected DNA sequences with local taxa and avoid misidentification by sequence similarities with non-relevant taxa. Still, some organisms are better represented in the reference databases than others, making the species identification biased toward well-represented groups. For instance, aquatic fungi, i.e., *Chytridiomycota* and *Rozellomycota*, have only limited representative sequences available in the UNITE database (ver. 8). Thus, the identification of some taxa can be restrained. However, this situation will change in the future as databases are constantly growing (Capo et al., 2021).

2.6. Environmental changes in the Eastern Latvia region over the Holocene

Lake Lielais Svētiņu has been a study object for multiple studies trying to reconstruct environmental changes in climate and vegetation over the Holocene period in the eastern Latvia region (Väliranta et al., 2015; Stivrins et al., 2014). Various environmental proxies have been compiled based on pollen and plant microfossil analysis, such as reconstructed summer temperatures and vegetation proxies (e.g., openness of the landscape, shade tolerance) (Stivrins et al., 2015). Temperature reconstructions for Lielais Svētiņu have been modeled based on pollen-climate calibration procedure (ter Braak and Juggins 1993, Stivrins et al., 2015). Therefore, well-established background information about Lielais Svētiņu allows reconstruction of the climate changes over the Holocene period.

Post-Glacial (~14 700–11 700 cal yr BP) climate reconstructions showed erratic warming and, in that period, the Scandinavian Ice Sheet margin retreated leaving the Baltic region ice free by 13 300 cal yr BP (Väliranta et al., 2015; Hughes et al., 2016). From the beginning of the Holocene (~11 650 cal yr BP) the climate started to warm rapidly until ~7000 cal yr BP, whereas the warmest period has been shown between 8000–4000 cal yr BP (Väliranta et al., 2015; Stivrins et al., 2014; Heikkilä and Seppä 2010). In the case of vegetation, the broad-leaved trees appeared in the region from the Early Holocene (~10 500 cal yr BP) (Väliranta et al., 2015). Stivrins et al. (2014) showed based on pollen analysis that Lielais Svētiņu was surrounded dominantly by the *Betula* and *Pinus* species in the period of Early Holocene (11 500–8000 cal yr BP) (Figure 2). Further, *Betula-Pinus* dominance was decreasing at the end of the Early Holocene and shifted toward thermophilous trees. Thus, the warm and dry Mid-Holocene (8000–4000 cal yr BP) was dominated by *Alnus*, *Corylus* and *Ulmus*. In the Late Holocene (4000–1500 cal yr BP), the climate started to cool, and a new shift toward *Betula-Pinus* dominance appeared again (Figure 2). Further, in the Latest Holocene (1500–0 cal yr BP), the broad-leaved trees decreased and human related pollen, such as *Secale cereale*, *Avena*, *Hordeum* and *Triticum*, increased rapidly (Stivrins et al., 2014). Lielais Svētiņu has a relatively late human impact that has been suggested to influence the area as early as ~2000 cal yr BP (Stivrins et al., 2015).

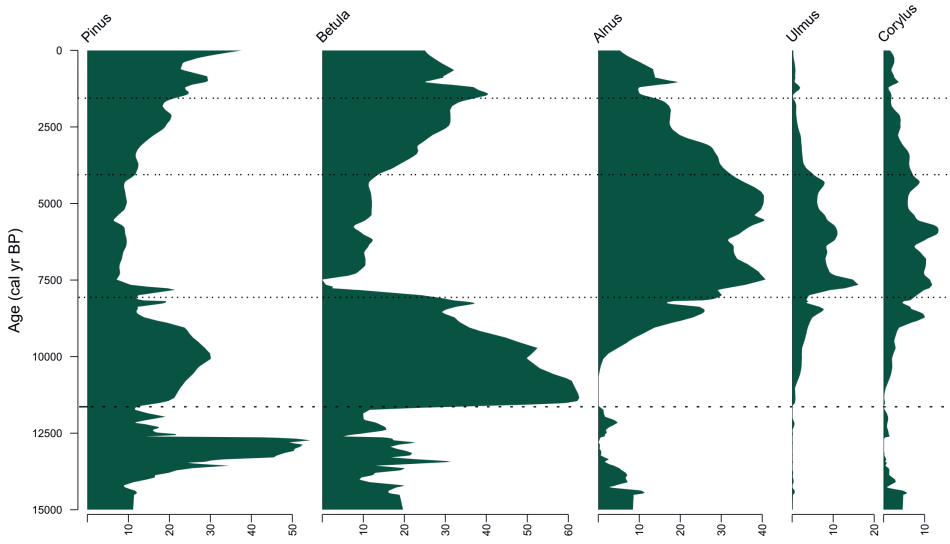

Figure 2. Lielais Svētiņu pollen diagram. Dotted lines represent periods in the Holocene. Created based on data and information from Stivrins et al. 2014.

3. AIMS OF THE STUDY

Long-term biodiversity studies would allow a better understanding of the past processes in the lake ecosystem. Thus, sedaDNA as a new emerging tool in paleo-research would permit recovery of taxa of multiple organisms based on the metabarcoding approach. The aim of this doctoral thesis was to apply the sedaDNA based methodology to analyse the diversity and dynamics of eukaryotes (especially fungi and phototrophs) and to investigate the impact of environmental change to these communities over the last ~14 500 kyr in lake Lielais Svētiņu (eastern Latvia).

More specific aims were:

- To test the suitability of universal 18S rRNA marker genes to capture the broad eukaryotic diversity from sedaDNA for reconstruction of paleo-environments (**I**).
- To explore the biodiversity changes of eukaryotes over the Holocene period (last ~11 500 kyr) (**I**).
- To explore past fungal community dynamics and diversity of their eco-physiological roles from sedaDNA using fungal specific ITS2 marker region (**II**).
- To examine the usefulness of fungal ecophysiological groups as a novel paleoproxy for ecosystem changes in the lake (**II**).
- To identify the environmental factors impacting fungal communities over the Holocene period (**II**).
- To combine several paleoproxies (pigments, microfossils and sedaDNA) to explore the phototroph dynamics over the last ~14 500 kyr (**III**).
- To identify abrupt changes and smooth periods in phototroph communities and investigate the environmental drivers causing abrupt changes (**III**).

4. MATERIALS AND METHODS

4.1. Study site, sampling and chronology

Our study lake was Lielais Svētiņu (LSv; depth of 4m) which is situated in the Rezekne district of eastern Latvia (Figure 3). A total of 11 m long sediment core was collected with Russian type corer (1 m long with diameter of 10 cm) paralleling multiple 1 m sediment cores with overlapping ends. Sediment cores were collected from the middle of the lake LSv (56°45′N, 27°08′E in eastern Latvia). SedaDNA subsamples were collected at 2.5 cm intervals from the cores. From each sediment layer three biological replicates were taken in a row (publication I; publication II; Methods 2.1.). Further, 84 layers were chosen for following analysis depending on past temperature changes (Stivrins et al., 2015) where sediment areas with rapid temperature changes represent more frequently collected samples. All the collected subsamples were stored at –80 °C. In addition, paleopigment subsamples were collected at 5 cm intervals and freeze-dried (publication III; Methods 3.3.). All the subsampling was handled using protective gear and clean equipment under positive-flow hood (Kojair K-safety KR-125) (publication II; Appendix A.2.). Subsampling was done in the Institute of Geology in Tallinn (Estonia).

Figure 3. The coring site and location of lake Lielais Svētiņu. Figure reprinted from Figure 1 (publication II).

The chronology of the sediment core was based on radiocarbon dates from the sediment core taken 2009 from the same location (described in Stivrins et al., 2015). The radiocarbon dates were adapted to our 2013 core by age-depth model (publication I; Methods 2.1.). The ages in this study are declared as calibrated years before present (kyr) where 0 kyr is equivalent to AD 1950.

4.2. DNA extraction, amplification and sequencing

DNA extraction and amplification were handled in separate laboratories at the Institute of Technology in Tartu (Estonia). All the work was performed under positive-flow hood (Kojair K-safetyKR-125) that was beforehand cleaned and UV-treated. In each step, negative extraction and PCR controls (no added sediment or DNA) were used to avoid possible cross-contamination from reagents or environment (publication II; Appendix A.3.).

Total DNA was extracted in three biological replicates using PowerSoil®DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) and extracts were stored at –20 °C. Total of 252 extracted samples were covering the period of the last ~14 500 kyr (publication I; Methods 2.2.).

PCR amplification was performed using a universal primer pair targeting 18S rRNA V4 region for total eukaryotes detection (Tedersoo et al., 2015) and multiplex primer pairs targeting ITS2 marker region for fungal specific detection (Tedersoo et al., 2014) (publication I; publication II; Methods 2.2.). Phusion High-Fidelity DNA Polymerase (Thermo Scientific) were used to conduct PCR with the following amplification program for 18S rRNA gene region: initial denaturation at 98 °C for 30 s, 30 cycles of 10 s at 98 °C, annealing for 30 s at 52 °C, and extension for 15 s at 72 °C, and final extension for 10 min at 72 °C (publication I; Methods 2.2.). For the ITS2 marker region, the similar amplification program was used with the annealing temperature of 46 °C (publication II; Methods 2.2.). The PCR amplicons and their quantity was examined using 1.4% agarose gels (1xTAE) including ethidium bromide. Furter, the amplicons were tagged with Illumina TrueSeq adapters and P5/P7 tail indexing primers using following program: 2 min at 98 °C, 12 cycles of 20 s at 98 °C, annealing for 30 s at 65 °C, extension for 30 s at 72 °C; and final extension for 5 min at 72 °C (publication I; publication II; Methods 2.2.). The amplicons were sequenced on Illumina MiSeq 2 × 250 bp platform at the Institute of Molecular Microbial Medicine Finland. The amplification of ITS2 region from deeper sediment layers was restricted, and therefore, a total of 162 samples covering the last 10 500 kyr were used for fungal specific dataset in the further analyses.

4.3. Bioinformatics analysis

Raw reads were first quality trimmed (Trimmomatic, version 0.32) to delete low-quality nucleotides (average quality score <30Q) and Illumina-specific sequences. Further, the reads were paired, dereplicated, and clustered into molecular operational taxonomic units (mOTUs) based on a 97% similarity threshold (publication I, Methods 2.2.; publication II, Methods 2.3., Appendix A.4.). The taxonomic affiliation of mOTUs was determined using the fungal reference database UNITE (version 7; Kõljalg et al., 2013) for ITS2 sequences and SILVA SSU RNA database (version 115; Quast et al., 2013) for 18 rRNA gene sequences. All the clusters with <4 reads were removed from the datasets. Also, the non-fungal and non-eukaryotic taxa were removed from datasets, respectively.

DNA damage analysis of quality trimmed reads was performed with mapDamage (version 2.0.8) using default parameters (Jónsson et al., 2013; publication II; Methods 2.2.). In addition, DNA fragmentation in the sediment layers of different depths was measured with TapeStation 2200 (D5000 DNA chip) (publication I; Figure S1).

4.4. Fungal ecophysiological groups, paleopigments and other proxies

The ecological roles were assigned to fungi detected in the ITS2 dataset with the purpose to identify the fungal ecophysiological groups that are strongly impacted by environmental changes (publication II; Methods 2.4.). The fungal ecological roles were assigned and cross-validated using various tools/databases such as FUNGuild (Nguyen et al., 2016), FungalTraits (Põlme et al., 2018), Fungal Families of the World (Cannon and Kirk, 2007), and many others (publication I; Table S2). The trophic status, lifestyle, habitat, mycorrhizal associations, and potential host-specificity were assigned to fungal mOTUs which were grouped by their ecological roles into ecophysiological groups.

Paleopigments were used as a proxy to detect phototroph dynamics and responses to changing environmental factors over the last 14 500 kyr. Collected and freeze-dried paleopigments were extracted and then separated using reversed-phase high-performance liquid chromatography (RP-HPLC), following the recommendations of Leavitt and Hodgson (2001). A total of 12 paleopigments were identified from collected samples (publication III; Methods 3.3.). There are multiple environmental proxies about climate and vegetation available for LSv (Stivrins et al., 2014, Stivrins et al., 2015). The environmental paleo-proxies used in our analyses to explore the possible impact of external drivers on the biodiversity changes are presented in Table 1, where the references to these proxies can be found. These proxies were based on pollen records and analysis of detected plant communities, and pyrite or charcoal particles taken from 2009 core (Stivrins et al., 2015).

Table 1. Environmental proxies for LSv. Table is adapted and updated from Table 1 (publication III).

Proxy	Paleo-indicator origin	Paleo-indication	Reference
mean summer temperatures (Tsum)	pollen	ice conditions and climate change	Stivrins et al. (2015)
mean winter temperatures (Twint)	pollen	ice conditions and climate change	Stivrins et al. (2015)
continental climate (Tcon= Tsum – Twin)	pollen	climate change	Tõnno et al. (2021)
waterlogging tolerance (Wtol)	pollen	density of the vegetation	Stivrins et al. (2015)
drought tolerance (Dtol)	pollen	density of the vegetation	Stivrins et al. (2015)
human related pollens (HRP)	pollen ARs of *Secale cereale*, *Hordeum vulgare*, *Triticum aestivum*, *Avena sativa*	anthropogenic impact	Stivrins et al. (2014)
shade tolerance (Stol)	pollen	density of the vegetation	Stivrins et al. (2015)
relative openness (Ropen)	pollen	density of the vegetation	Stivrins et al. (2015)
charcoal particles (Ch)	charcoal	fire dynamics	Stivrins et al. (2015)
pyrite (FeS_2)	pyrite	anoxic conditions	Stivrins et al. (2015)
Botryococcus accumulation rate (AR of Botry)	microfossil	humification levels	Stivrins et al. (2015)
Picea accumulation rate (AR of Picea)	pollen	humification levels	Stivrins et al. (2014)

4.5. Statistical analysis

First, ordination methods were used to study richness changes in fungi and total eukaryotes over the following time periods: Late Pleistocene (>11 700 kyr), Early Holocene (11 700–8000 kyr), Mid-Holocene (8000–4000 kyr), Late Holocene (4000–2000 kyr) and Latest Holocene (<2000 kyr). Principal coordinates analysis (PCoA) was performed using the *vegan* package in R to illustrate the dynamics of the fungal mOTU abundances over the Holocene (publication II). In addition, Permanova analysis was used on normalized fungal mOTU counts to analyse the differences in communities between time periods (function *adonis* in *vegan* package; publication II; Methods 2.5.1.). For total eukaryotes, between-groups analysis of PCA (bgPCA; Baty et al., 2008) using the *ade4* package was conducted to compare the mOTU variation of eukaryotes between time periods (publication I;

Methods 2.6.). Redundancy analysis (RDA; Legendre and Legendre, 1998) was used to study the associations between the abundance of indicative phytoplankton NPPs (Stivrins et al., 2015) and aquatic mOTUs of eukaryotes with the purpose to understand the trophic changes in the lake (publication I; Methods 2.4.).

Further, the changes in the communities of fungi and total eukaryotes were studied over the timescale. To identify the shifts of indicator mOTUs in the 18S rDNA dataset, the bgPCA analysis was conducted on mOTU proportions (*ade4* package) and indicator values were obtained by calculating the α0 parameter that shows the mOTU contribution to a time period (publication I; Methods 2.6.). For the ITS2 dataset, the temporal diversity indices and community stability metrics (*rate change*, *turnover* and *synchrony* in *codyn* package; Hallett et al., 2016) were used to identify the changes in the community composition of fungal ecophysiological groups over the Holocene period. The *rate change* shows the direction and rate of the change in the community over the observed time period. The *turnover* shows the total species turnover rate between two consecutive time points. The *synchrony* describes the synchronous/asynchronous fluctuations of mOTUs in the ecophysiological groups over the timespan (publication II; Methods 2.5.2.).

Next, to interpret the observed community changes, the associations between environmental proxies (Table 1) and total eukaryotes or fungal ecophysiological groups were studied. For the 18S rDNA dataset, the RDA analysis was used to identify the significant associations between eukaryotic mOTU abundances and environmental proxies (publication I). For the ITS2 dataset, the generalized additive models (GAM; *gam* function from *mgcv* package) were used on non-linear changes in fungal groups to reduce the variance of abundance over time (publication II; Appendix A.5.). Fitted values from GAM models were used for two-sided Pearson correlations (*cor.test* function) between fungal groups and environmental proxies (publication II; Methods 2.5.3.).

Finally, a multi-proxy study including fossil pigments, algal microfossils, and sedaDNA of algae and algae parasitic fungi was compiled to study phototroph dynamics and responses to environmental changes over the last ~14 500 kyr (publication III). The principal component analysis (PCA) was used to display the phototroph pigment concentration patterns and the PC1 and PC2 scores were used as indicators of the variability of fossil pigments. Further, RDA analysis was used to study associations between phototroph pigment concentrations and environmental proxies (publication III; Methods 3.5.). Significant paleoproxies selected by RDA analysis were then used for GAM model fitting (publication III; Table 1). GAM approach was conducted to predict phototroph variability using selected paleoproxies. Temperature and shade tolerance were used to predict phototroph PC1 scores. Charcoal particles, *Botryococcus* accumulation rate and pyrite were used to predict PC2 scores. In addition, the richness of algae and fungi was also used to model PC2 scores. Predicted phototroph PC scores were then compared with real PC scores and deviations were defined as perturbation periods. Further, Bayesian change point analysis (Wang and Emerson, 2015) was conducted to PC1 scores to validate the detected perturbation periods (publication III; Methods 3.5.).

5. RESULTS AND DISCUSSION

5.1. Broad biodiversity of eukaryotes in sedaDNA covering the post-glacial period (I)

In this study, we explored the broad eukaryotic diversity and dynamics from sediments of lake Lielais Svētiņu (Eastern Latvia) over the Holocene period. To obtain the wide eukaryotic diversity, we used universal phylogenetic marker genes of the 18S rRNA V4 region, which has shown good overall coverage for eukaryotes (Hadziavdic et al., 2014). The robust bioinformatic analysis (publication I; Methods 2.2.) enabled the recovery of 1279 mOTUs belonging to a broad range of eukaryotic groups such as *Alveolata*, *Stramenopiles*, *Cercozoa*, *Chlorophyta*, *Charophyta*, *Nucletmycea* and *Holozoa* (publication I; Figure 1C). The highest richness was detected in Fungi (29%; 375 mOTUs; mostly phylum *Basidiomycota* and *Ascomycota*), followed by *Chlorophyta* (12%; 155 mOTUs), *Charophyta* (8.5%; 109 mOTUs), and *Holocoa* (7%; 91 mOTUs). This finding complies with other recent studies from deep-sea sediments where kingdom *Fungi* has been one of the most common microbial eukaryotes found in sediments (Xu et al., 2019). In addition, the lake sediments were dominated by microbial eukaryotes (55%) (publication I; Figure 1b), where green algae, yeast *Saccharomyces* and fish pathogenic protist *Naegleria* were the most abundant and frequent throughout the sediment. Even when the overall richness per sample was relatively low (less than 50 OTUs), the use of universal phylogenetic markers enabled us to detect a broad range of eukaryotic organisms from microbial eukaryotes to vascular plants. We demonstrated that sedaDNA from temperate region lake sediments can be used to provide an assessment of a wide range of eukaryotes when sequencing DNA of the total community using only one universal marker gene region.

5.1.1. Usability of sedaDNA to reconstruct paleo-environments: aquatic and terrestrial

To investigate the potential of sedaDNA for the reconstruction of paleo-environments, we assigned the detected mOTUs into two groups based on their hypothetical habitat: terrestrial or aquatic. The assessment was based on ecophysiological information found in GenBank and established through the closest sequence match. We found that 1/3rd of detected eukaryotes were originating from terrestrial and 2/3rds from aquatic habitats. The most frequently detected terrestrial eukaryotes were fungi (*Ascomycota* and *Basidiomycota*) and vascular plants (*Magnoliophyta* and *Coniferophyta*), illustrating the usability of sedaDNA to reconstruct the terrestrial ecosystems of lake catchment areas. It was also shown for plants by Alsos et al. (2018) that dominant plant taxa detected from lake sediments matched the vegetation in the surrounding area of the lakes. The

most frequent and dominant eukaryotes from aquatic origin were planktonic organisms such as green algae, proto- and zooplankton, and aquatic plants. These overall shares of detected taxa were similar to previous studies using sediments (Lydolph et al., 2005; Parducci et al., 2013). We showed that lake sedaDNA acting as a good archive of preserved information about a wide range of eukaryotes could be successfully used to reconstruct both aquatic and surrounding terrestrial environments using universal marker regions.

5.2. High fungal diversity recovered in sedaDNA is valuable as signatures of host-related past pathogen dynamics (II)

Fungi are a diverse group of eukaryotes that play important roles in several ecosystem processes as saprotrophs, symbiotrophs and parasites. In aquatic environments, fungi are one of the key players in organic matter turnover, and they are common eukaryotic organisms in the sediments (publication I; Xu et al., 2019). Thus, information about fungal diversity and their ecophysiological roles could be used to track the past processes in the lake. Still, the studies focusing on fungal diversity in sediments are rare (Bellemain et al., 2013; Lydolph et al., 2005). Therefore, we studied fungal diversity, their ecophysiological roles and community changes to environmental drivers in LSv lake sediments.

To obtain the most realistic fungal diversity assessment, we used the fungal-specific ITS2 region, which permits the differentiation between closely related fungal species compared to the universal 18S rRNA gene marker (Schoch et al., 2012). We used multiplex primer pairs (publication II; Methods 2.2.) designed to match >99.5% of total fungal diversity (including *Chytridiomycota* and *Glomeromycota*) (Tedersoo et al., 2014). Sequencing of ITS2 region amplicons, followed by bioinformatic analysis (publication II; Methods 2.3.), detected 1125 unique fungal mOTUs, which was three times more fungal species than detected by universal 18S rRNA markers (publication I; 375 mOTUs). Utilization of ITS2 region enabled to detect fungi belonging to six phyla–*Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Rozellomycota*, *Zygomycota* and *Glomeromycota*, which suggests that nearly complete community composition was detected. Still, it should be considered that the detected taxa are highly dependent on used primers and available reference sequences in the databases. We showed that the general shares between our ITS2 and 18S datasets and previous studies from deep-sea sediments and lake water habitats (Xu et al., 2019; Liu et al., 2017) were comparable, dominated by *Ascomycota* and *Basidiomycota*. Further, we assigned the ecophysiological guilds for all detected fungi to obtain a better overview of fungal roles in the sediment (publication II; Methods 2.4.). The most prevalent guilds were saprotrophs and pathotrophs, including several plant and animal pathogens and wood/litter saprotrophs in the sediment (publication II; Figure 3), where saprotrophs play a crucial role in organic matter turnover. Many detected fungi in phylum *Basidiomycota*, such as ectomycorrhizal symbionts and plant

pathogens in orders *Agaricales* and *Polyporales*, were characteristic to terrestrial habitat, and thus, represent the species that are likely transferred from the catchment to the lake. Therefore, the high richness of terrestrial species recovered from the sediment could be useful to reconstruct the terrestrial biodiversity of lakeshores. Also, several fungi from phyla *Ascomycota*, *Chytridiomycota* and *Rozellomycota* were found to be characteristic to aquatic habitats, where the most diverse were plankton parasitic fungi (e.g., *Rhizophydiales* and *Lobulomycetales* (*Chytridiomycota*)). Parasites in the aquatic habitat have the potential to alter not only the host communities but also the energy flow and stability of the food webs (Kagami et al., 2014). Thus, detection of aquatic fungi and insight into their ecological roles could be useful to explain the changes in the lake ecosystems. Further, the overall good discrimination of fungi at the genus-species level enabled us to identify several host-specific fungi (46 mOTUs). We determined plant pathogens related to *Alnus*, *Betula*, *Salix*, *Frangula*, *Picea*, *Poaceae* and mycorrhizal fungi specific to *Pinus* and *Salix* roots. These results showed that fungal sedaDNA is not only valuable information for recovering the community composition of terrestrial and aquatic ecosystems, but also permits the use of host-specific fungi as signatures of past host populations. Our results suggest that fungal signatures would be suitable to study the occurrences of host-related plant diseases and their dynamics in paleoenvironments.

5.3. Biodiversity changes over the Holocene (I, II)

5.3.1. Latest Holocene distinguishes from other periods in increasing richness

We also explored the biodiversity changes over the Holocene with the following time periods: Late Pleistocene (>11 700 kyr), Early Holocene (11 700–8000 kyr), Mid-Holocene (8000–4000 kyr), Late Holocene (4000–2000 kyr) and Latest Holocene (<2000 kyr). Principal coordinates analysis (PCoA) and between-groups analysis of PCA (bgPCA) showed the gradual change in total eukaryotes and fungal communities during most of the Holocene in contrast with rapid changes occurring in the Latest Holocene (<2000 kyr) (Figure 4). Similarly, Permanova analysis presented significant differences between time periods (R2=0.1; p=0.001; publication II; Table S3). We observed more than two-fold increase in the richness of total eukaryotes and three-fold rise in total fungal mOTUs in the Latest Holocene period (<2000 kyr) compared with Late Holocene (4000–2000 kyr) (publication I; Figure 2c; publication II; Figure S4). Further, we found that fungi originating from aquatic habitats were contributing to the rapid richness rise in the last ~2000 kyr the most compared to rather stable terrestrial communities (publication II; Figure 3a, Table 1). Both marker regions achieved clear evidence that during the Latest Holocene (<2000 kyr) the richness of microbial aquatic organisms has been increasing rapidly and is probably the result of environmental change.

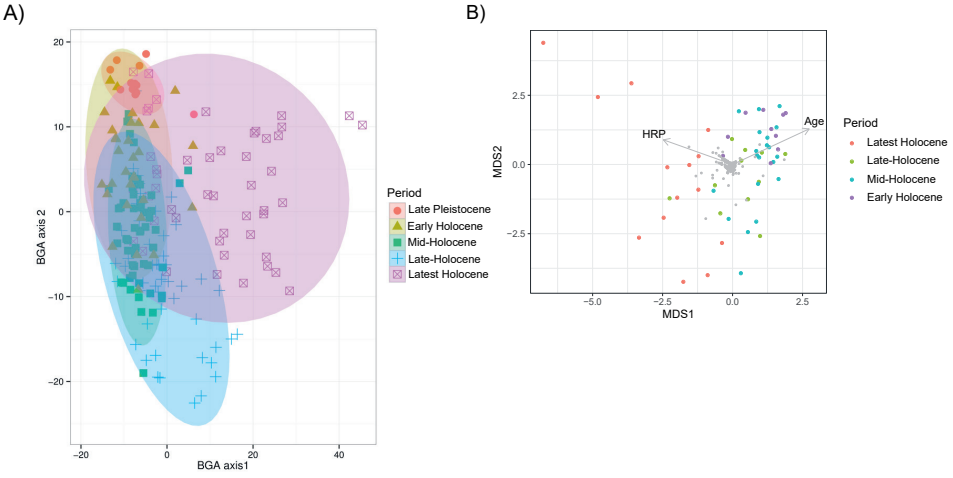

Figure 4. 18 rDNA and ITS2 mOTUs grouped according to time periods. (**A**) mOTUs of eukaryotes grouped by PCA-based between-groups analysis (bgPCA). Shown ellipses represent a confidence level of 0.90 within the group. Figure adapted from Figure 2a (publication I); (**B**) Ordination plot of PCoA analysis using fungal normalized read counts of mOTUs. Colored points show Holocene periods, and grey points show the single mOTUs. Arrows display linear regression of sample age (Age, R2=0.57, p<0.05) and concentration of human-related pollen (HRP, R2=0.43, p<0.05) with PCoA space. Figure adapted from Figure 2b (publication II).

Also, in the case of total eukaryotes, we showed that the Latest Holocene was most similar to the Mid-Holocene based on shared mOTUs (74 shared mOTUs; publication I; Figure 2c), where the majority of the shared mOTUs belonged to *Chlorophyta* (green algae; publication I, Table S4). This result suggests that Latest Holocene and Mid-Holocene communities were similar and perhaps illustrate similar responses to the environmental changes, which would be useful to predict the changes in the lake ecosystem in response to current climate warming. Phytoplankton spices are sensitive to changing environmental factors (e.g., temperature and nutrient load) (Elliott et al., 2006), and thus, can be used as an indicator to assess the ecological status of the lake. Therefore, we further investigated associations between recovered aquatic mOTUs and the abundance of phytoplankton NPPs (Stivrins et al., 2015) to study the trophic changes in the lake ecosystem. RDA analysis showed strong associations between multiple indicative phytoplankton NPPs and aquatic mOTUs (publication I; Figure 3c). *Pediastrum* and *Coelastrum* species are used as indicators of hypertrophy (Brettum and Andersen, 2004), and they were associated with mOTUs of planktonic eukaryotes from the upper sediment layers (publication I; Figure 3c). These mOTUs can be typically found in hypertrophic lakes. The mOTUs from Mid-Holocene which exhibited the most overlapping species with mOTUs from Latest Holocene (publication I; Table S4) were also covering many planktonic groups found in

eutrophic lakes. Similar to our results, Capo et al. (2016) showed that *Chlorophyta*, *Dinophyceae*, *Haptophyceae* and *Ciliophora* species could be used as markers of changes in trophic status. Our results showed the richness rise of aquatic eukaryotes in the last ~2000 kyr and also greater richness of several planktonic species in the warmer Mid-Holocene (8000–4000 kyr) period associated with higher trophic status. Even though the exact eutrophication levels of LSv in the Mid-Holocene are not known, our results show several species connected with eutrophication in the lake.

We also investigated the possible bias in detected richness caused by DNA degradation or possible contamination, thus conducting DNA fragmentation and DNA damage analyses (publication II; Figure S1; publication I; Table S1). DNA damage analysis showed significant correlations between cytosine deamination rates and nicks frequency with sediment age providing evidence that extracted DNA was indeed of ancient origin (publication I; Table S1). The cross-contamination problem from reagents and environment was also addressed using multiple negative controls (no added DNA) in sample collection, DNA extraction and PCR steps. In both datasets (18S and ITS2), we detected only a few mOTUs (i.e., unidentified fungi and unidentified *Ascomycota*) with a low number of reads which were not present in any sediment samples (publication I; Figure S4; publication II; Appendix A.8). These processes ensure that the resulting mOTUs are not present due to contamination. The DNA fragmentation analysis showed the higher DNA degradation in deeper layers but no severe bias towards PCR amplicon size (300–350 bp), enabling the successful amplification also from deeper sediment layers (publication II; Figure S1). Also, for the 18S dataset, no correlation was found between the number of mOTUs and sediment age. Still, for the ITS2 dataset, linear mixed models (LMM) analysis and likelihood ratio test showed the significant impact of depth to the richness between first and second replicates (publication II; Table A2). This may have occurred due to overall richness differences between these two replicates since no significant correlation was found using read counts. Nevertheless, the richness in terrestrial fungi showed contrastingly higher richness also in deeper layers (publication II; Figure 3a). Therefore, these findings demonstrate no preferred recovery of richness in upper sediment layers, and the community changes appear to occur rather due to changing environmental factors.

5.3.2. Community changes of mycorrhizal fungi, plankton parasitic fungi and phytoplankton were prominent over the Holocene

We studied the community changes over the Holocene period to distinguish the major shifts in community compositions. In the 18S dataset, we showed that strong indicator mOTUs (publication I; Methods 2.6.) indeed displayed shifts in climate periods. *Chlorophyta* (green algae) were dominating in the Latest Holocene (<2000 kyr), while *Fungi* and *Charophyta* (higher plants) were mainly dominating in the Early Holocene (11 700–8000 kyr) (publication I; Figure 2b). For the ITS2

dataset, we used temporal diversity indices (e.g., *rate change*, *turnover*) and community stability metrics (e.g., *synchrony*) (Hallett et al., 2016) to measure the fungal community changes in ecophysiological groups (publication II; Methods 2.5.2.). The highest rate of community change appeared in plankton parasitic fungi and ectomycorrhizal fungi (Figure 5C, Table 2). Also, aquatic fungi demonstrated three times higher community change over the Holocene period compared with terrestrial fungi (Figure 5C, Table 2), indicating that aquatic fungi might be more sensitive to environmental changes. The richness increase of aquatic fungi and community change in plankton parasitic fungi comply with observed *Chlorophyta* shift in the Latest Holocene (<2000 kyr) (publication I; Stivrins et al., 2015). Phytoplankton species are primary producers in aquatic food webs, where their fungal parasites also play key roles in controlling phytoplankton blooms by nutrient transfer (called "mycoloop"; Kagami et al., 2007). Thus, shifts in the phytoplankton community affect their pathogen dynamics offering new niches in the case of richness rise. Further, the detected community change in mycorrhizal fungi can appear as a result of many factors – from changing climate and nutrient availability to changes in plant communities (Cotton, 2018). Mycorrhizal fungi form mutualistic relationships with many plant species, where they mediate nutrient flow (phosphorus and nitrogen) from the soil to their host plants in the exchange of assimilated carbon (Krüger et al., 2017). Therefore, as mycorrhizal fungi are connected with their host, the changes in richness might be the result of host community dynamics or the introduction of new non-native host species (Krüger et al., 2017; Gomes et al., 2018). However, responses of mycorrhizal fungi to environmental changes are still unclear as detected community changes have been variable and context-dependent (Cotton, 2018). Still, phytoplankton and mycorrhizal communities can be sensitive to changing environmental factors (Elliott et al., 2006; Cotton, 2018), and thus, their rapid community change towards higher richness is likely the result of environmental change, which is addressed in the next topic.

In addition, other larger fungal groups (e.g., saprotrophs, symbiotrophs, plant pathogens) did not show any major community shifts over the Holocene period (Figure 5C, Table 2). In fact, saprotrophs (e.g., wood and litter saprotrophs) were the most stable over the Holocene, displaying the lowest species turnover rate (publication II; Figure 4). Therefore, all the fluctuations in the richness of larger fungal groups, such as saprotrophs, can be considered as natural fluctuations in diversity (Figure 5A, B). Even though the richness rise in the last ~2000 kyr was observed, in addition to aquatic fungi, in plant pathogens, conifers-related fungi and mycorrhizal fungi (Figure 5B; publication II; Figure 5b), the major community change was observed only in mycorrhizal fungi and plankton parasitic fungi, where up to ~25% of these communities fluctuate depending on each other (publication II; Table 1 "synchrony"). Our observations support the conclusion that narrow host-substrate specific fungi, such as plankton parasites and mycorrhizal fungi, could be more susceptible to environmental change than fungi using a broad range of substrates (e.g., saprotrophs) (Kagami et al., 2007; Větrovský et al., 2019).

Table 2. Community change in fungal ecophysiological groups. Table adapted from Table 1 (publication II).

Ecological group	**Rate change**[1]	**Synchrony**[2]
Water environment	**0.04**	**0.249**
Terrestrial environment	0.013	0.058
Both environments	0.017	**0.121**
Pathotroph	0.038	**0.166**
Animal pathogen	0.017	**0.175**
Insects parasite	0.006	–0.166
Fish pathogen	0.003	–0.276
Plankton parasite	**0.045**	**0.269**
Fungal parasite	0.009	0.062
Plant pathogen	0.015	0.077
Saprotroph	0.025	0.09
Litter saprotroph	0.004	0.03
Wood saprotroph	0.009	0.046
Dung saprotroph	0.004	–0.037
Symbiotroph	0.018	**0.134**
Lichen symbiont	0.0007	**–0.326**
Mycorrhizal fungi	**0.047**	**0.141**
Conifers related fungi	0.001	–0.028
Deciduous trees related to fungi	0.0002	0.002

[1] rate change–shows the rate and direction of a change in community composition over the timespan; [2] synchrony – shows synchronous/asynchronous fluctuations of mOTUs. The synchrony value is ranging from –1 to 1 (from complete asynchrony to synchrony) with a central value of 0 (species fluctuate independently).

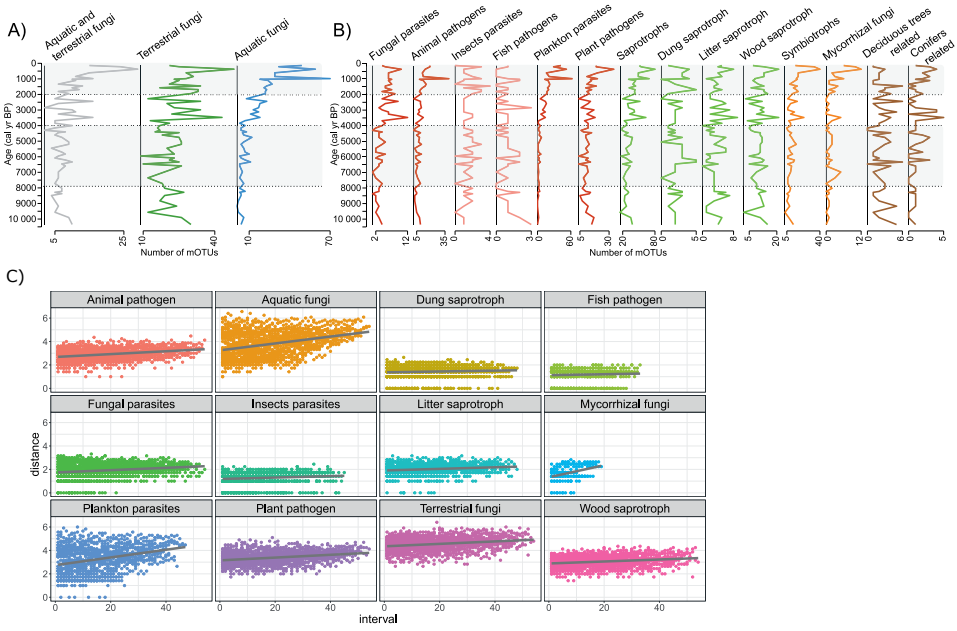

Figure 5. The richness and community changes in fungal ecophysiological groups over the ~10 500 kyr. (**A**) Fungal richness changes are shown for their hypothetical habitats and (**B**) for assigned ecophysiological groups. The colored background and dotted lines represent periods. (**C**) Plots are illustrating the rate of community change in fungal ecophysiological groups. The distances have been generated by time lag intervals. Figure adapted from Figure 3, Figure S4 (publication II).

5.4. Environmental drivers of community changes (I, II)

To explain these community changes, we studied the relationships between the relative abundance of eukaryotic mOTUs and richness of fungal ecophysiological groups with the following environmental proxies: winter and summer temperatures (Twin, Tsum), continental climate (Tcont), openness of the surrounding landscape (OPEN), shade (Stol), drought (Dtol) and waterlogging tolerance (Wtol), concentration of charcoal particles (Ch), pyrite (FeS_2) and human related pollen (HRP) (Table 1).

5.4.1. Human impact related environmental change is driving community changes in lakes

First, RDA analysis showed strong associations between the richness rise of eukaryotic mOTUs in the last ~2000 kyr, human related pollen and charcoal particles (Figure 6A). For fungal ecophysiological groups, the GAM models were applied to smooth the variance in abundance (publication II; Figure 5b; Figure

S6). Further, the fitted values from GAM models were used for two-sided Pearson correlations between fungal groups and environmental factors (publication II; Table S4, S5). Similarly, we found strong positive correlations (r>0.6) of fungal ecophysiological groups with HRP and Ch, in addition to OPEN and Tcont (Figure 6B; publication II; Table S4). HRP includes pollen of cultivated species, such as *Secale cereale*, *Hordeum vulgare*, *Triticum aestivum* and *Avena sativa*, which can be associated with human impact through agriculture (Stivrins et al., 2015). HRP showed a gradual increase over the <2000 kyr with an accelerated rise in ~700 kyr (Figure 6C). Also, strong correlations with Ch (r= 0.67; publication II; Table S4) indicates past fire events in the region. Human activities have been suggested to escalate the fire events through slash-and-burn agriculture that results in the altered landscape structure (Feurdean et al., 2017; Aakala et al., 2018). Thus, increased HRP and Ch particles also explain the rise in relative openness of the area (Figure 6C). Vegetation openness in the lake surrounding also demonstrates large-scale events since open landscape enables widespread plant material to be carried into the lake from longer distances. In addition, we found that regardless of OPEN and Ch significant correlations in Latest Holocene, they were not the main factors driving the community changes from Early to Late Holocene (Figure 6B2; publication II; Table S5). Therefore, the strong correlations to OPEN and Ch (Figure 6B1) in the last <2000 kyr could likely reflect the growing wider human impact over the region. Our results illustrate the effect of moderate human impact on the richness rise of the eukaryotic organisms in the lake. Also, Stivrins et al. (2015) have shown associations between human influence and the rise of vegetation richness in the region. Therefore, we suggested that the observed richness rise of plant pathogenic and mycorrhizal fungi in the last ~2000 kyr could probably be the result of increased nutrients inflow to the lake. The nutrient abundance in turn likely lead to an increase in phytoplankton as we observed in the publication I (Figure 2b) and in other planktonic organisms. Interestingly, we observed the richness rise of plankton parasitic fungi earlier than eukaryotic algae dominance, starting from the last ~4000 kyr. This change in plankton parasitic fungi community corresponds to Holocene climatic change toward cooler temperatures and shift in cyanobacteria-eukaryotic algae after 4000 kyr. As the epidemics of plankton parasites can occur also on optimal conditions to the host (Kagami et al., 2007), then the richness of plankton parasitic fungi could have risen before phytoplankton dominance. Thus, the community changes of plankton parasitic fungi are likely co-impacted by climate change and human activity in the Latest Holocene (<2000 kyr).

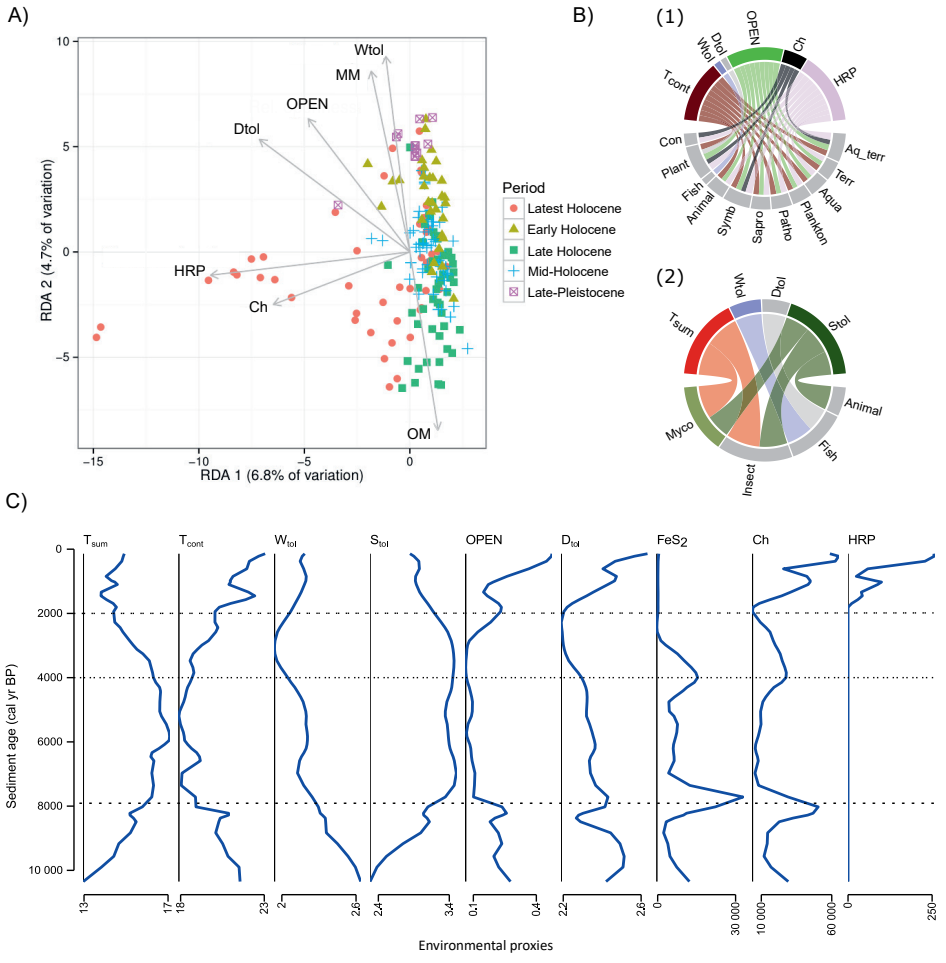

Figure 6. Environmental proxies' associations with eukaryote and fungal communities and their dynamics over the ~10 500 kyr. (**A**) Detected 18S rDNA mOTUs in RDA ordination space illustrating associations with environmental paleoproxy variables. Only the variables with strong relationships were employed in the analysis (R2>0.09; p<0.001; publication I; Table S5a). Figure adapted from Figure 3b (publication I); (**B**) Chord diagrams showing two-way Pearson correlations between environmental paleoproxies and fungal ecophysiological groups, where (1) shows strong positive correlations (r>0.6) over the whole timespan (~10 500 kyr); (2) shows strong positive correlations (r>0.6) over the period (2000–10 500 kyr) excluding possible human impact (last ~2000 kyr). Figure adapted from Figure 6 (publication II); (**C**) The dynamics of environmental paleoproxies. Figure adapted from Figure 5a (publication II). Abbreviations: Aq_terr–water and terrestrial environment fungi; Terr–terrestrial fungi; Aqua–aquatic fungi; Patho–pathotrophs; Sapro–saprotrophs; Symb–symbiotrophs; Plankton–plankton parasitic fungi; Animal–animal pathogens; Fish–fish pathogens, Insect–insect parasites; Fungal–fungal parasites; Plant–plant pathogens; Myco–mycorrhizal fungi; Dec–deciduous trees related fungi; Con–conifers related fungi; Tsum–summer temperatures; Tcont–climate continentality; Wtol–waterlogging tolerance; Stol–shade tolerance; OPEN–vegetation openness; Dtol–drought tolerance; FeS2–pyrite; Ch–charcoal particles; HRP–human related pollen; OM–organic matter (%); MM–mineral matter.

5.5. Multiproxy approach enables to define change point periods for plankton dynamics (III)

The multi-proxy approach has shown as a good tool to recover most realistic information of past communities from historical sediments by proxies complementing each other (Jørgensen et al., 2012; Alsos et al., 2016; Ficetola et al., 2018; Tse et al., 2018; Stivrins et al., 2018). For example, fossils of algae and cyanobacteria (NPPs) have been used to detect their taxonomy and biomass (Stivrins et al., 2018; Tse et al., 2018), but as some of the algae do not fossilize well in the sediment (Riddick et al., 2017), the algal pigments and sedaDNA can be used to fill these gaps. Algal pigments are, however, relatively well preserved in the sediments (Leavitt and Hodgson 2001) contributing to algal biomass recovery. SedaDNA, in addition to the detection of a broad range of preserved organisms, can also provide taxonomic affiliation at genus and species level (Pal et al., 2015; Tse et al., 2018; Stivrins et al., 2018). We conducted the multi-proxy study, which involved fossil pigments, algal microfossils and sedaDNA of algae and algae parasitic fungi, to define the abrupt changes in phototrophs dynamics and to understand the impact of external factors on phototroph dynamics over the last ~14 500 kyr. The abrupt change in the community can appear as the result of abrupt external change or gradual climate change (Andersen et al., 2009; Randsalu-Wendrup et al., 2016) when the community has crossed the internal threshold. We modeled fossil pigment concentrations as response variables to climate, vegetation change and anthropogenic impact to identify the perturbation periods and change points for phototroph communities.

To study relationships between phototroph community dynamics and possible environmental drivers over the ~14 500 kyr, we used RDA and GAM modeling approaches (publication III; Methods 3.5., Table 1). RDA analysis showed that most of the variability of pigments concentrations (PC1 scores from PCA; publication III; Figure S2a) were associated with climate and changes in the vegetation (Figure 7). The remaining variability (PC2 scores) was associated with HRP and Ch (Figure 7; publication III; Table S3).

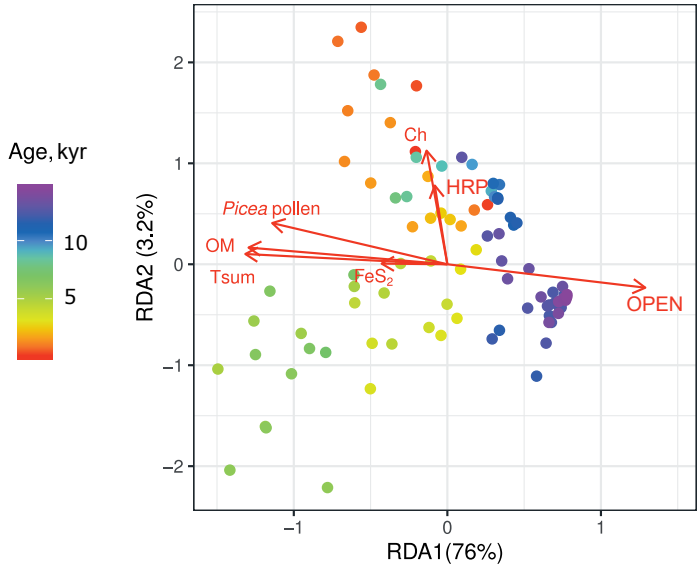

Figure 7. Relationship of fossil pigment/DI variability with a set of significant explanatory variables analysed by RDA. OM – organic matter (%); Tsum – summer temperature; FeS_2 – pyrite; OPEN – relative openness; Ch – charcoal particles accumulation rate; HRP – human related pollen. Figure adapted from Figure S2 (publication III).

GAM model fitting, using significant paleo-environmental variables from RDA, was conducted to model phototroph biomass variability (as PC1, PC2 scores; publication III; Table S4). Predicted PC scores were then compared with real PC scores, and deviations from modeling were defined as perturbation periods where the community has reached its threshold. We were able to detect three perturbation periods and two gradual change periods for phototroph dynamics over the 14 500 kyr (Figure 8A). In addition, all the perturbation periods were also confirmed by Bayesian change point analysis (Wang and Emerson, 2015), which showed a high regime shift probability for all the detected perturbation periods (Figure 8B).

We found the first perturbation period in the post-Glacial period between 13 500–12 500 kyr (Figure 8A). The phototroph community, which was still "young" and possibly less resilient to environmental change, showed abrupt changes in their biomass (i.e., fossil pigment concentrations) (publication III; Figure 2). Due to excessive DNA degradation, we could detect only a few mOTU belonging to *Chlorophyta*, *Chrysophyta* and *Bacillariophyta*, making the use of sedaDNA ineffective in these ultra-deep sediment layers. Nevertheless, we observed abrupt response of phototroph dynamics to abrupt climate change (Tsum; $p<0.001$) in this period where ice started to decline, based on PC1 modeling with climate as a predictor variable (publication III; Table S4, GAM A).

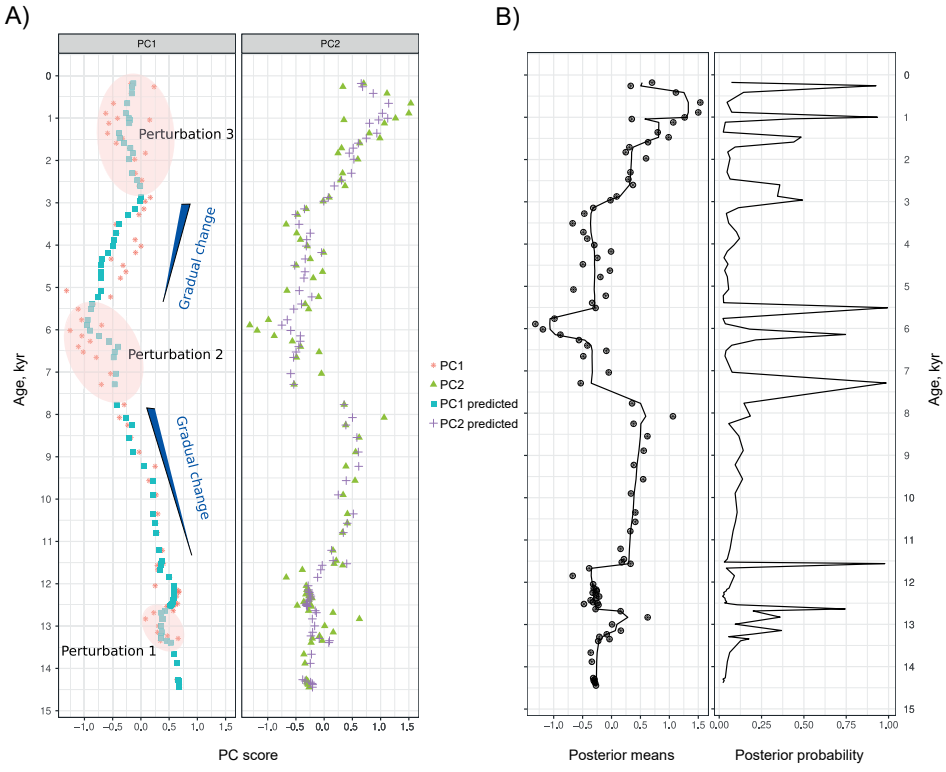

Figure 8. Comparison of PC score dynamics of pigment/DI data. (**A**) PC scores of pigment concentrations from PCA compared with fitted values of PC scores by GAM using explanatory variables. PC1 predicted–PC1 scores modeled with Tsum and Stol; PC2 predicted–PC2 scores with Ch AR and Botry AR. Blue lines indicate the gradual changes due to temperature change. Red ellipses denote perturbation periods from 1 to 3, which are the periods used for fitting PC1 to climate (Tsum) divergence. (**B**) Bayesian change point analysis of PC1 scores. The left panel shows the PC1 scores in the sequence and posterior means at every location over the iterations. The right panel shows the estimates of the probability of a change point. Figure reprinted from Figure 3 (publication III).

The first perturbation period was followed by a gradual change period between ~12 500–7700 kyr (Figure 8A). We defined gradual changes as predictable changes in phytoplankton dynamics (i.e., fit between predicted and real PC1 scores) mediated by temperature changes (Figure 8A; publication III; Table S4). The climate started getting warmer from the beginning of the Holocene (~12 000 kyr; publication III; Figure 1b), which also contributed to the increase of phototroph biomass (publication III; Figure 2). Also, Stivrins et al. (2018) showed a similar change in phototroph biomass turnover from Late Glacial to Early Holocene. In addition to gradual warming, at ~8200 kyr, a small climate cooling took place (Twin decreased 2–3 °C; Veski et al., 2004), and we detected a small decrease in some pigment concentrations (publication III; Figure 2), but

no abrupt changes in the phototrophs community (Figure 8B). These results indicate that the phototroph community had established a good resilience to slow-gradual climate changes as the lake ecosystem is under constant environmental pressure.

The second perturbation period was detected during the Holocene thermal maximum (HTM) between ~7700–5400 kyr (Figure 8A) when we identified a high variability in pigment concentrations (publication III; Figure 2) and high probability of regime shifts (Figure 8B). HTM has been considered a warm period with relatively stable vegetation (Feurdean et al., 2017). Still, temperature and climate-related vegetation could not explain the perturbation in phototroph dynamics based on lack of fit between predicted and real PC values (Figure 8A). We proposed humification and winter ice conditions as possible drivers of perturbation in the warm climate period. To support this explanation, we found 7 low pH-tolerant algal mOTUs (e.g., in genera *Scherffelia*, *Choricystis*, *Desmodesmus*, *Woloszynskia*) present at this perturbation period (publication III; Table S6). Also, a short ice cover period in winter due to the warm climate can cause relatively substantial effects in lake ecosystems due to the longer growing season (Catalan et al., 2013). These events can thus bring the phototroph community to the verge of the threshold or cause perturbations even when only a small temperature change occurs.

The perturbation period was followed by a second gradual change period between ~5400–2900 kyr, which was described by climate cooling (Figure 8A). Decreasing temperature also resulted in a decrease in phototroph biomass (publication III; Figure 2). In addition, between ~4500–3000 kyr, we observed a higher abundance of acid water-tolerant *Dinophyta* (Perid; publication III; Figure 2) and an increase in the remains of *Botryococcus* that suggest dystrophic lake conditions (Jankovská and Komárek, 2000) possibly related to paludification processes around the LSv (Stivrins et al., 2015).

We detected a third perturbation period between 2900–0 kyr (Figure 8A). In addition, we used richness (mOTUs) of phototrophs and possible algal parasitic fungi to model the PC2 scores. We found that PC2 scores were significantly ($R2>0.2$; $p<0.001$) related to the richness of algae parasitic fungi and total phototrophs (Figure 9A; publication III; Table S5), especially to mOTUs of *Chlorophyta* following the same trend as phototroph pigments. We observed that both, the richness of total algae and algae parasitic fungi, displayed no drastic changes in richness before ~3000 kyr but showed a rapid increase in richness after 2500 kyr (Figure 9B). Earlier studies have shown that rapid regime shifts with an increase in phytoplankton are often the result of anthropogenic forcing (e.g., eutrophication) (Randsalu-Wendrup et al., 2016). Also, our RDA analysis showed associations with HRP ($p=0.035$) and Ch ($p=0.005$) (publication III; Figure S2b, Table S3) in the last ~2500 kyr, which are compatible with our publication II results about environmental factors impacting the richness rise of plankton parasitic fungi. Even though the first minor human impact close to LSv has been shown to start around ~2000 kyr (Stivrins et al., 2015; publication III; Figure 1b), the increase from ~3000 kyr in landscape openness (Ropen; publication III;

Figure S1), phototroph pigment concentrations (publication III; Figure 2) and richness of phototrophs (S.obs; Figure 9B) suggest that low regional human impact probably caused perturbation in the phototrophs community. Human impact due to agricultural activities increased the nutrients flow into the lake leading to eutrophication. This was also supported by our finding of the richness increase in plant-parasitic and mycorrhizal fungi discussed before (publication II). We showed that the third perturbation period exhibited an increase in phototroph biomass and also an increase in their richness. Higher phototroph richness means competing for the same resources or occupying new niches which can also lead to a higher chance of parasite attack (Ibelings et al., 2004). Thus, richness rise can be characterized as a new external driver induced by human activity.

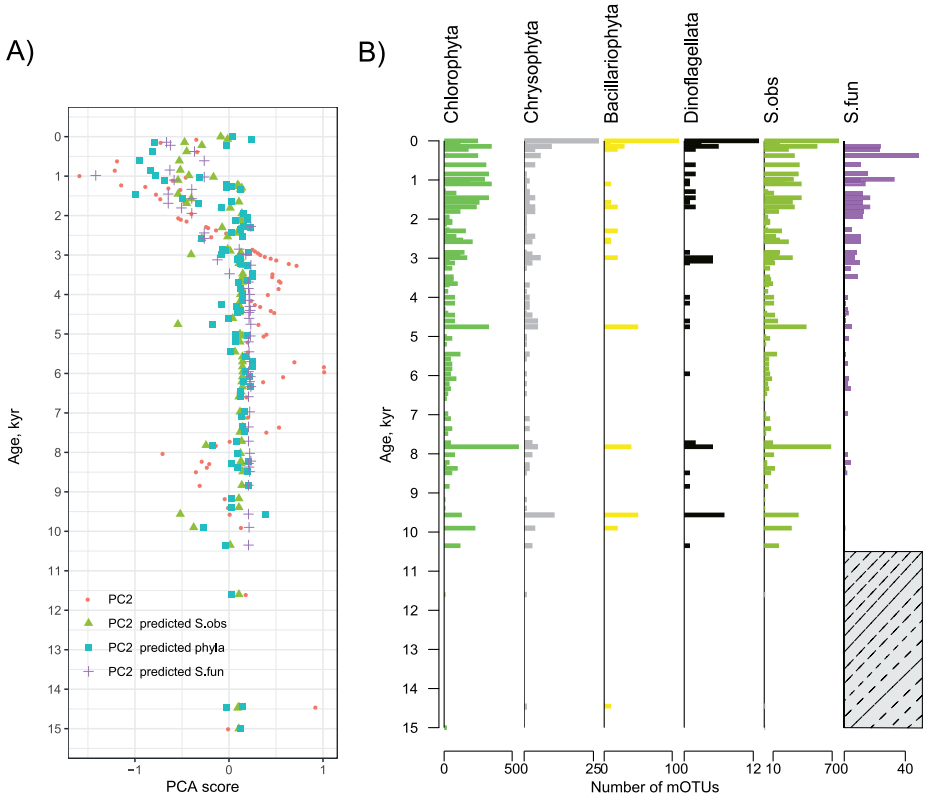

Figure 9. Comparison of GAM fitted values with PC2 scores from analysis using the pigment/DI ratios. (**A**) Variables used in the modeling: PC2 predicted S.obs–PC2 scores modeled with the total richness of phototrophs; PC2 predicted phyla–PC2 scores modeled with algae richness (cyanobacteria excluded); PC2 predicted S.fun–PC2 scores modeled with the richness of fungal parasites of algae. (**B**) Richness changes in major phototroph taxon, total phototrophs (S.obs) and algae parasitic fungi (S.fun) over the ~14 500 kyr. S.fun measured until 10 500 kyr, not measured in the gray area. Figure reprinted from Figure 4 (publication III).

CONCLUSIONS

- SedaDNA from temperate lake can be successfully used to assess the broad biodiversity of eukaryotes from microbial eukaryotes to vascular plants/ vertebrates using one universal 18S rRNA V4 gene marker region.
- SedaDNA has the means to reconstruct aquatic ecosystems and terrestrial environments of the lake catchment. Our data contained 1/3rd of terrestrial and 2/3rd of aquatic organisms. The highest richness was detected in *Fungi* (29%) and *Chlorophyta* (12%).
- Fungal specific ITS2 marker region enabled the recovery of a highly diverse fungal community and their ecophysiological groups, which were found useful as signatures of past host populations and in-lake processes.
- Fungi with narrow host-substrate specificity are likely more sensitive to environmental change than fungi with a wide range of suitable hosts or substrates. The fungi, such as saprotrophs, that can use a broad range of substrates were rather stable over the Holocene compared with narrow host-substrate specific fungi like pathotrophs and symbiotrophs (e.g., plant and plankton parasites, mycorrhizal fungi).
- Community shifts of *Chlorophyta*, plankton parasitic fungi and mycorrhizal fungi were observed in the last ~2000 kyr, which was related to moderate anthropogenic impact in the region. The human activity brought along higher loading of nutrients in the lake, which was observed as increased richness in plant-parasitic fungi and mycorrhizal fungi. This, in turn, favored an increase in phytoplankton.
- The richness rise of plankton parasitic fungi starting from ~4000 kyr could be explained by climate cooling and cyanobacteria-eukaryotic algae shift.
- The multiproxy approach using fossil pigments, algal microfossils and sedaDNA enabled to identify three perturbation and two gradual change periods in phototroph dynamics.
- The abrupt climate change in Late-Glacial induced also abrupt changes in the "young", low resilience phototroph community.
- The perturbation period was also observed during HTM caused by gradual climate changes (likely effect of ice-cover and humification processes).
- Latest-Holocene perturbation was induced by low but growing human impact in the region, offering new niches and greater competition leading to higher species richness.

SUMMARY IN ESTONIAN

SedaDNA abil eukarüootsete organismide mitmekesisuse, dünaamika ja keskkonnamuutuste mõju rekonstrueerimine hilisjääaja ja Holotseeni perioodil Lielais Svētiņu järve näitel

Järve settes leiduv ja säilinud vana DNA (sedaDNA) omab tohutut potentsiaali, et rekonstrueerida ajaloolisi bioloogilise mitmekesisuse muutusi järve ökosüsteemis ja selle valgalal. SedaDNA kasutamine võimaldab meil tuvastada, kuidas kooslused on ajas muutunud ning paremini mõista, millal ja miks need muutused mitmekesisuses toimusid. Lisaks võimaldavad tuvastatud koosluste muutused luua selgust järves toimunud ammuste protsesside kohta või anda infot järve ümbritseva taimekoosluste dünaamikast. SedaDNA võimaldab ka uurida keskkonna muutuste mõju tuvastatud kooslustele. Pikaajalisi koosluste muutusi ja nende seoseid keskkonna mõjudega on oluline uurida, sest see võimaldab meil paremini seletada tänapäevaseid bioloogilise mitmekesisuse mustreid ja modelleerida võimalikke tuleviku stsenaariume praegustes kliima soojenemise tingimustes.

Settesse mattunud rakuväline DNA on võimeline seonduma seal savi ja humiinaine osakestega ning seetõttu säiluma väga pikka aega. Hetkel on vanim teadaolev settest tuvastatud DNA ligi 270 000 aastat vana. SedaDNA on aga suhteliselt uus tööriist paleo-ökoloogilise teadustöö valdkonnas, kus varasemalt on kasutatud peamiselt õietolmul ja fossiilidel põhinevaid andmeid. Sekveneerimistehnoloogiate arengu ja DNA analüüsimeetodite paranemisega on sedaDNA kasutamine hüppeliselt kasvanud just viimasel viiel aastal. Vana DNA-ga töötamisel on aga mitmeid piiranguid–väga väikene eraldatud DNA kogus proovides, DNA lagunemine, kontamineerumise oht, praimerite osaline sobivus ja DNA referents andmebaaside ebatäielikkus. Seetõttu on sedaDNAga töötamisel vajalik äärmiselt puhas labori keskkond ja kindlate väljatöötatud meetodite järgimine, et vältida proovide kontamineerumist. Nende piirangute ületamiseks arendatakse pidevalt uusi metoodikaid ja analüüsimeetodeid.

Meie uurimisobjektiks oli järv Lielais Svētiņu, mida võib pidada üheks Ida-Euroopa põhja regiooni mudel järveks, kuna sellel järvel on olemas pikk setteläbilõige ja samuti hästi defineeritud inimmõju algus selles piirkonnas. See info võimaldab aga uurida looduslikku ja inimtekkelist mõju järve ökosüsteemi kooslustele. Lielais Svētiņu on olnud uurimisobjektiks mitmetele teadustöödele, mis on kasutanud õietolmu ja teiste mikro- ja makro-fossiilide analüüsi ning seeläbi on kogutud põhjalikud andmed kliimamuutuste ja taimkatte arengu kohta Holotseeni ajajärgul. Need varasematest uuringutest kogutud keskkonna tunnused võimaldavad meil luua tugevamaid seoseid veekeskkonna ökosüsteemide muutuste ja väliste mõjutegurite vahel.

Selle doktoritöö käigus uuriti eukarüootide mitmekesisuse muutusi ajas, keskendudes seente ja vetikate dünaamikale, ja keskkonnategureid, mis indutseerivad neid muutusi üle kogu jääaja järgse perioodi Lielais Svētiņu järves.

Testisime sedaDNA efektiivsust eukarüootide mitmekesisuse rekonstrueerimisel ja uurisime, kuidas need kooslused reageerivad muutuvatele keskkonnatingimustele. Lisaks kasutasime sedaDNA-d koos vetika pigmentide ja mikro-fossiilide andmetega, et tuvastada fototroofide dünaamikat. Samuti testisime seente ökoloogiliste gruppide kasutamist uue võimaliku indikaatorina järve ökosüsteemi muutuste hindamiseks.

Selle doktoritöö käigus leitud peamised tulemused ja järeldused olid järgmised:

- Parasvöötme järve settest eraldatud DNA võimaldab edukalt tuvastada eukarüootide mitmekesisust alates mikroobsetest eukarüootidest kuni maismaa taimedeni kasutades selleks universaalset 18S rRNA marker geeni piirkonda. Lisaks võimaldab sedaDNA rekonstrueerida nii järve ökosüsteemi kui ka seda ümbritsevat maismaa keskkonda. Töös tuvastati ⅓ maismaalise päritoluga ja ⅔ veelise päritoluga organisme. Suurim liigirikkus leiti seente (29%) ja *Chlorophyta* (rohevetikate; 12%) seas.

- Seente spetsiifilise ITS2 marker ala kasutamine võimaldas tuvastada ülimalt suurt seente mitmekesisust ja määrata nende ökoloogilisi rolle. Tulemused näitasid, et seente ökoloogilisi gruppe võiks kasutada kui potentsiaalseid indikaatoreid peremees organismide populatsioonide ja järve-siseste protsesside hindamiseks.

- Kitsa peremees-substraadi spetsiifilisusega seened on tõenäoliselt tundlikumad keskkonna muutuste suhtes kui seened, kellel on palju peremees organisme või eluks sobivaid substraate. Me näitasime, et laia substraadi spektriga seened (nt saprotroofid) olid üldiselt stabiilsed Holotseeni perioodil võrreldes kitsa peremees-substraadi spektriga seentega (nt taime ja planktoni parasiidid või mükoriissed seened).

- Koosluste dünaamikas tuvastasime kiire liigirikkuse tõusu *Chlorophyta*, planktoni parasiitsete seente ja mükoriissete seente grupis viimase 2000 aasta jooksul, mis oli seoses keskmise inimmõju tugevusega selles piirkonnas. Inimtegevus tõi kaasa suurenenud toitainete liikuvuse järve, mis selgitaks liigilise mitmekesisuse tõusu taime-parasiitsete seente ja mükoriissete seente seas. See omakorda aga soosis fütoplanktoni mitmekesisuse tõusu sel perioodil. Samas me täheldasime planktoni parasiitsete seente liigilise mitmekesisuse tõusu juba alates ~4000 aastast, mis on seletatav kliima jahenemisega ja tsüanobakterite-eukarüootsete vetikate pöördega nende dominantsuses.

- Vetika fossiliseerunud pigmentide, mikrofossiilide ja sedaDNA kooskasutamine võimaldas tuvastada kolm koosluste häiringu ja kaks järkjärgulise muutuse perioodi vetikate dünaamikas. Esimene häiring esines hilis-jääaja perioodil, kus toimus järsk kliima muutumine, mis kutsus esile ka järske muutusi

vetikate kooslustes. Alles arenev, noor vetika kooslus oli tõenäoliselt madala vastupanuvõimega muutuvate keskkonna- tingimuste suhtes. Teine häiringu periood jäi aga HTMi (*Holocene thermal maximum*) ajajärku, mis oli tõenäoliselt põhjustatud järkjärgulistest kliima muutustest (sh jääkatte kestuse ja humifitseerumise protsessidest), kus kooslus ületas oma taluvusläve. Kolmas, Hilis-Holotseeni häiringu periood oli põhjustatud nõrga, kuid kasvava inimmõju poolt selles piirkonnas, pakkudes uusi nišše, kuid ka suurenenud konkurentsi liikide vahel, mis omakorda viis aga vetikate ja nende parasiitsete seente liigilise mitmekesisuse tõusuni.

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List of Publications:

Kisand, V., **Talas, L.**, Kisand, A., Stivrins, N., Reitalu, T., Alliksaar, T., Vassiljev, J., Liiv, M., Heinsalu, A., Seppä, H., Veski, S. (2018). From microbial eukaryotes to metazoan vertebrates: Wide spectrum paleo-diversity in sedimentary ancient DNA over the last ~14,500 years. *Geobiology*. 16: 628–639.

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Talas, L., Stivrins, N., Veski, S., Tedersoo, L., Kisand, V. (2021). Sedimentary ancient DNA (sedaDNA) reveals fungal diversity and environmental drivers of community changes throughout the Holocene in the present boreal lake Lielais Svētiņu (Eastern Latvia). *Microorganisms*. 9(4), 719.

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Muu erialane tegevus:

2020	Projekti "Laevade ballastivee mikroorganismide ja viiruste uuringud" täitja
2020	Uurimistööde juhendamine vana DNA ja seenekoosluste tuvastamise ning mikroobikoosluste võrdluse teemadel
2019	Loodusteaduste doktorantide esinemisoskuste koolitus-seminar, auhinnatud teine koht
2019	Osalemine MetaSUB City Sampling Day projektis
2018–2020	Biotehnoloogia praktikumi juhendaja Tamme Gümnaasiumis teemal "Kloneerimine"

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