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When phytoplankton do not bloom: the case of the dinoflagellate *Lepidodinium chlorophorum* in southern Brittany (France) assessed by environmental DNA

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Abstract :

Green seawater discolorations caused by the marine dinoflagellate Lepidodinium chlorophorum are frequently observed during summer along southern Brittany coasts (NE Atlantic, France). Here, the ecology of L. chlorophorum is studied during a non-bloom period using high-throughput sequencing metabarcoding of environmental DNA (eDNA) samples for the detection of this species at low concentrations. Sediment samples (for metabarcoding and cyst analyses) were collected in January–February 2019 and water samples from two stations were collected at three water depths in September–March 2019–2020 and 2020–2021 (for metabarcoding and environmental parameters). The protistan community was dominated by dinoflagellates and was homogenous in the water column. Amplicon sequence variants (ASVs) associated with the genus Lepidodinium were detected in autumn–winter at low relative abundances (minimum: 0.01%). Increases in Lepidodinium abundance were positively correlated with pulses of ammonium re-suspended from bottom sediments. Although Lepidodinium eDNA (<1%) was detected in the sediments, no cyst morphotypes could be associated with Lepidodinium, and germination experiments revealed no Lepidodinium-like cells, leaving in doubt the existence of resting cysts of this species in the seed bank. It is hypothesised that temporary Lepidodinium cells remained present in the water column at low concentrations during the autumn–winter period, awaiting ammonium input from sediments to initiate growth, and that blooms develop when water column stratification and river input provide favourable environmental conditions for biomass increases.

Highlights

► Rare *Lepidodinium* eDNA was detected during unfavourable growth seasons. ► Increase in *Lepidodinium* was correlated with ammonium pulse from sediment. ► No cysts of *Lepidodinium* were identified in the sediments. ► Temporary pelagic stage during non-blooming phase may explain species overwintering.

Keywords : HABs, Cysts, Sediment resuspension, eDNA, Metabarcoding, Ammonium

1. Introduction

High-biomass phytoplankton blooms may result in seawater discoloration worldwide (Siano et al., 2020; Hallegraeff et al., 2021; Tsikoti and Genitsaris, 2021). Among those, the marine dinoflagellate *Lepidodinium chlorophorum* is known to cause green seawater discoloration associated with massive proliferations . The capacity of this unarmoured dinoflagellate (Elbrächter and Schnepf, 1996; Hansen et al., 2007) to

form green seawater discolorations is due to the presence of green-coloured plastids (Matsumoto et al., 2011) inherited from secondary endosymbiosis with a chlorophyte (Kamikawa et al., 2015; Gavalás-Olea et al., 2016; Jackson et al., 2018). Blooms of *L. chlorophorum* are widely distributed in coastal waters: in Chile (Iriarte et al., 2005; Rodríguez-Benito et al., 2020), California (Gárate-Lizárraga et al., 2014), Australia (McCarthy, 2013), as well as in Europe (Honsell and Talarico, 2004; Sourisseau et al., 2016; Siano et al., 2020, Serre-Fredj et al., 2021; Roux et al., 2022). Along the French Atlantic coast, blooms of this species are mainly observed during summer (Karasiewicz et al., 2020; Siano et al., 2020; Roux et al., 2022).

Lepidodinium chlorophorum has been described as a eurythermal and euryhaline dinoflagellate (Elbrachter and Schnepf, 1996; Claquin et al., 2008). The water column stratification and river inputs could favour the development of the bloom (Sourisseau et al., 2016). Blooms can last for more than a month (Siano et al., 2020), presumably sustained by the bacterial remineralisation of the organic matter and especially the transparent exo-polymer particles (TEP) that are highly produced by this dinoflagellate *in situ* (Roux et al., 2022). Long-lasting blooms may cause hypoxia, likely contributing to mass mortalities of fish and cultivated bivalves (Sournia et al., 1992; Chapelle et al., 1994; Siano et al., 2020; Roux et al., 2022). To improve our understanding of the ecology of this species, the species dynamics during the non-bloom period as well as during the bloom initiation still need investigation.

In mid-latitude European waters, winter is perceived as an unfavourable season for the growth of autotrophic micro-eukaryotic species (Reynolds, 1984, 1998; Reynolds et al., 2001). Biomasses of such species reported during the winter period do not correspond to massive surface accumulations but rather moderate to low concentrations distributed over a large part of the water column (Zingone et al., 2010).

Some species may overwinter in low numbers in the water column (<100 cells L^{-1}), remaining below detection limits used in microscopy-based monitoring. This part of the biosphere, consisting of species that persist at low or undetectable levels, is known as the rare biosphere (Sogin et al., 2006). Species of the rare biosphere might develop when ecological (seasonal and/or ecosystem) changes occur in the environment (Logares et al., 2014). In particular, planktonic species may develop when seawater warming, thermal stratification, nutrients and light availability are favourable for the species to develop (Glibert et al., 2018). Another strategy used by different protist groups to persist in an ecosystem under unfavourable conditions is the production of resting stages corresponding to a dormant life-strategy phase that is generally benthic (Von Dassow and Montresor, 2011). For dinoflagellates, more than 10% of the approximately 2000 known marine dinoflagellate species produce cysts as part of their life cycle (Bravo and Figueroa, 2014). The presence of an endogenous biological clock in dinoflagellates regulated by unfavourable environmental changes (e.g., nutrient limitation and temperature decrease) triggers resting cyst formation (Anderson and Keafer, 1987; Matrai et al., 2005; Brosnahan et al., 2020). In spring, benthic cyst resuspension occurs due to physical resuspension caused by waves and currents, mechanical disturbance of the sediment (benthic or pelagic organisms' bioturbation; Kirn et al., 2005) or bottom trawling (Pilskaln et al., 1998). High nutrient inputs from sediment, associated with an increase in water temperature and light and oxygen availability, may activate release from dormancy (Brosnahan et al., 2020) and lead to cyst germination (Anderson, 1980; Anderson et al., 1987, 2005a, 2005b). These germinated cells represent the inoculum of a new seasonal bloom (Butman et al., 2014). Based on culture observations, Sournia et al. (1992) suggested the formation of resting cysts by *L. chlorophorum* in water samples; this hypothesis has not been

verified *in situ* or by molecular biology. In addition, the physical and biological factors that favour cyst germination are still unknown for this species.

The study of dinoflagellate species phenology during the non-bloom period can be addressed by detecting low species concentrations in the water column and tracing the increase in abundance in relation to environmental triggers prior to the bloom phase Studies on bloom dynamics are sometimes associated with the detection of seed bank(s), explaining the spatiality and recurrence of blooms (Anderson, 1997; Anderson et al., 2005a, 2005b; Klouch et al., 2016). Yet, in routine microscopy studies, some species are very difficult to detect at low abundances because low volumes of water are analysed and species can be misidentified when few cells are observed. In addition, the search for specific cysts in the natural environment can be very difficult due to the morphological similarity of cysts belonging to different species (Bravo and Figueroa, 2014), their low abundance, or due to the lack of awareness of what should be looked for (Montresor et al., 2016). Molecular tools that target communities present in environmental DNA (eDNA) samples can help to identify taxa at low abundance in the water column and cysts in the sediments which lack discriminative morphological features (i.e., cryptic species). eDNA is defined as the genetic material present in environmental samples such as sediment and water, including cells, extracellular DNA and potentially whole organisms (Ficetola et al., 2008; Barnes and Turner, 2016; Ruppert et al., 2019; Pawlowski et al., 2021). Molecular analyses of eDNA can complement classical microscopic methods by targeting different species from the same sample, sampling greater diversity and increasing taxonomic resolution (Deiner et al., 2017). For instance, the eDNA metabarcoding approach allows the identification of multiple species from a single environmental sample containing organisms or degraded DNA (Taberlet et al., 2012) and can therefore allow accurate description of

communities. This method is based on the amplification and high-throughput sequencing of a specific barcode region (e.g., 16S for bacteria, 18S for most eukaryotes and ITS regions for fungi) (Dollive et al., 2012; Taberlet et al., 2012; Segata et al., 2013). However, some issues can complicate the interpretation of eDNA data: 1) the origin of intracellular DNA (from living cells) or extracellular DNA (from cell death) and, 2) eDNA persistence in marine systems (Palmer et al., 1993; Dupray et al., 1997; Sassoubre et al., 2016; Andruszkiewicz et al., 2017; Drouet et al., 2021).

Metabarcoding analysis of eDNA from sediment samples allows the description of marine eukaryotic communities (e.g., Pawlowski et al., 2011; Guardiola et al., 2015; Siano et al., 2021), and shifts in protist sedimentary communities relative to human impact have been identified with this approach (e.g., Siano et al., 2021). Many studies investigated the composition and relative abundance of plankton species in the water column through metabarcoding (e.g., Brannock et al., 2016; Gran-Stadniczeñko et al., 2018; Sze et al., 2018; Minicante et al., 2019; Ramond et al., 2019, 2021; Liu et al., 2020; Chen et al., 2021; Cui et al., 2021; Huang et al., 2021). However, this method has given new significance to rare taxa (i.e., those with low local relative abundance; Sogin et al., 2006; Burki et al., 2021). For instance, Logares et al. (2015) hypothesised that this rare biosphere could contain more diversity than frequently observed taxa. In addition, eDNA has useful applications in detecting invasive species, the continued presence of native species thought to be extinct and other elusive species occurring in low densities that would be difficult to detect by traditional means (Ruppert et al., 2019). Metabarcoding of the eDNA is therefore an appropriate method to detect rare species and their changes in abundance over time relative to environmental changes.

This study investigated the *L. chlorophorum* phenology during the non-bloom period, and tried to assess whether cysts are important for bloom initiation, or

alternatively cells overwinter in the water column. In the absence of established genetic methods to detect the species *L. chlorophorum* (FISH, qPCR), the metabarcoding of eDNA in the pelagic and benthic environment was used to study the rare occurrence of this species before its development while single-cell PCR and hatching experiments of cysts were applied to search for the cyst stage in surface sediments. This study completes the study of the phenology of this species in southern Brittany (Roux et al., 2022) and provides a potential application of eDNA for studying rare species concentration and bloom initiation phases in the marine coastal ecosystems.

2. Materials and methods

2.1. Study area

The Northern Bay of Biscay, and more precisely the Vilaine-Loire coastal zone in southern Brittany (France; Fig. 1), is a favoured environment for *L. chlorophorum* since it is frequently affected by green seawater discoloration caused by this species (Belin et al., 2021; Siano et al., 2020). The Vilaine and Loire Rivers directly influence this coastal area with 70 and 850 m^3 s^{-1} mean annual flow, respectively (Lazure et al., 2009). The two rivers, especially the Loire, are the main nutrient sources in the northern Bay of Biscay and play a major role in the eutrophication of coastal waters in southern Brittany (Guillaud et al., 2008; Ménesguen et al., 2018, 2019). The location and extent of the two river plumes vary according to the flow of rivers, tidal currents as well as the strength and direction of the winds. In winter, the Loire river plume can be oriented towards the northwest along the coast, with a dilution of 20 to 100-fold by the time it reaches Vilaine Bay. In periods of very high flow, the plume can extend seaward

(Ménesguen and Dussauze, 2015; Ménesguen et al., 2018). The Vilaine river plume generally spreads throughout the bay before moving westward (Chapelle et al., 1994). The water circulation within Vilaine Bay is characterised by low tidal and residual currents and is mainly driven by tide, wind and river flow (Lazure and Salomon, 1991; Lazure and Jégou, 1998). Haline stratification is high from February to June in response to high river runoff and relatively low vertical mixing, whereas thermal stratification occurs between May and mid-September (Puillat et al., 2004).

2.2. Sampling strategy

To assess the presence of *L. chlorophorum* and the accompanying protistan community in the water column during periods of low concentration of this species, the seawater was sampled offshore the Loire and Vilaine Rivers during two surveys carried out in two autumn–winter seasons, respectively, from September 2019 to March 2020 and from September 2020 to March 2021. Sampling was conducted fortnightly at high tide at two stations (Fig. 1): Ouest Loscolo (in the Vilaine bay; 9 m water depth) and Basse Michaud (offshore the Loire River; 28 m water depth). Water samples were collected using a 5 L Niskin bottle at three water depths: subsurface (0–1 m), mid-depth and 1 m above the water-sediment interface for both eDNA and light microscopy analyses of micro-phytoplankton identification and enumeration. For eDNA samples, 500–1000 mL water samples were collected and immediately placed into plastic flasks. Samples were filtered through 3-µm polycarbonate filters (Whatman®). Filters were stored in 5-mL cryotubes, immediately flash-frozen in liquid nitrogen and stored at −80°C until molecular biology analysis. For microscopic analyses, one litre of water of each sample was fixed with Lugol's iodine solution (2% f.c.) and stored in the dark at 4°C until laboratory analyses. Water samples aliquots were also processed for

chlorophyll *a* concentration ([Chla]; µg L^{-1}), inorganic nutrients concentrations (nitrogen [DIN], silicates [DSi] and phosphates [DIP]; µM) and concentrations of TEP ([TEP]; µg Xeq L^{-1}). In addition, vertical profiles of seawater temperature (°C), salinity and turbidity (FNU) were performed with a multi-parameter probe (NKE MP6) from subsurface to water-sediment interface. The stratification index was calculated from the density variations according to Hansen and Rattray (1966):

$$\varepsilon = \frac{\rho_{water-sediment\ interface} - \rho_{surface}}{\rho_{mean}} \quad (1)$$

where $\rho_{water\text{-}sediment\ interface}$ is the density measured at the water-sediment interface, $\rho_{surface}$ is the density measured at the subsurface and ρ_{mean} is the mean density measured along the water column. Under homogeneous conditions, $\mathcal{E} = 0$, whereas under conditions of maximum vertical density stratification, $\mathcal{E}$ will be around 25×10^{-3} (Hansen and Rattray, 1966).

To verify the presence of *L. chlorophorum* cysts in the sediments, duplicate samples were collected for hatching experiments and eDNA analysis at 17 stations in the Loire-Vilaine area (Fig. 1) during the winter period (January–February 2019; Table S1). These stations were defined according to four criteria: 1) records of *L. chlorophorum* blooms in the summer of 2018, 2) presence of fine-grained sediments to obtain higher cyst concentrations (e.g., Lewis, 1988) and better eDNA preservation (Boere et al., 2011), 3) accessibility of the site and 4) site geomorphology and geography to have a high accumulation of cysts (harbours, bay, etc.). For eDNA analyses, sediments were stored in 60-mL sterile cryotubes, flash-frozen in liquid nitrogen *in situ* and stored at −80°C until genetic analysis, following procedures explained in Pawlowski et al. (2021). For morphological analyses, cysts were collected

in 50 mL Falcon tubes, Parafilmed, covered by aluminium foil and kept at 4°C until microscopy analyses. Sediment granulometry analyses were performed using a laser particle sizer (Mastersizer 3000). Data were processed in Gradistat package developed by Gregoire et al. (2017) and were grouped by size class according to Blott and Pye (2001, Table S1).

2.3. eDNA analysis from water samples and sediment

Total DNA from sample filters was extracted using the NucleoSpin® Plant II DNA kit (Macherey Nagel, Hoerdt, France). DNA was extracted with one lysis buffer, one lysozyme solution (Sigma) and one buffer composed of proteinase K (Macherey Nagel) for 2 h at 56 °C (500 rpm). The DNA extract was washed and purified three times before elution in a final volume of 100 µL of elution buffer (5 min at 65°C) following the manufacturer's instructions. The total DNA from 10 g of wet sediment was extracted using the PowerMaxSoil® DNA isolation kit (MO BIO Laboratories Inc.), following the manufacturer's instructions. To check contamination, blank extractions with nuclease-free water were performed. All DNA extracts were quantified using the Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen™, Waltham, MA, USA) using a spectrophotometer (BioTek® FLx800, Winooski, VT, USA). Total DNA concentrations extracted from filters varied between 0.06 and 13.20 ng/µL (3.79 ± 2.50 ng/µL), while DNA extracts from the sediments varied between 0.09 and 36.69 ng/µL (15.54 ± 8.63 ng/µL). The final DNA concentration was normalized to 5 ng/µL when possible.

The V4 region of the 18S rDNA gene, a marker gene region conserved but highly variable across the taxa of the protistan community, was amplified using PCR performed with a Taq polymerase (Phusion High-Fidelity PCR Master Mix with GC Buffer; Thermo Scientific) and eukaryote-specific primers (Stoeck et al., 2010) plus

Illumina adapters: Forward (NGS-TAReuk454FWD1): 5' CTT TCC CTA CAC GAC GCT CTT CCG ATC TCC AGC ASC YGC GGT AAT TCC 3', Reverse (NGS-TAReukREV3): 5' GGA GTT CAG ACG TGT GCT CTT CCG ATC TAC TTT CGT TCT TGA TYR A 3'. PCR amplification was performed three times for each DNA extract (from a single filter). The PCR protocol was composed of a denaturation step at 98 °C for 30 s, followed by two sets of cycles: 1) 12 × [98 °C (10 s), 53 °C (30 s) and 72 °C (30 s)], 2) 18 × [98 °C (10 s), 48 °C (30 s) and 72 °C (30 s)]. Thereafter, a final step of elongation at 72 °C for 10 min was carried out. Amplification results were verified by gel electrophoresis (1% agarose). To check contamination, blank amplifications with nuclease-free water were performed. Triplicate samples were pooled before sequencing.

Sequencing was performed at the GeT-PlaGe platform using Illumina Mi-Seq (http://get.genotoul.fr/). The raw dataset is available at https://doi.org/10.12770/53dfe316-a6d4-426f-8983-56f2b37a5e8a. Raw data were analysed using the SAMBA v3.0.1 workflow (Noël et al., 2021; https://github.com/ifremer-bioinformatics/samba) developed by the SeBiMER (Ifremer's Bioinformatics Core Facility), a Standardized and Automatized MetaBarcoding Analysis workflow using DADA2 (Callahan et al., 2016) and QIIME2 (Bolyen et al., 2019) with default parameters unless otherwise indicated. Briefly, raw data integrity after sequencing was checked using the SAMBA checking process. Afterward, sequencing primers were trimmed from reads, and reads where primers were not found were filtered. Then, DADA2 was used to filter poor quality reads, correct sequencing errors, overlap paired reads, infer Amplicon Sequence Variants (ASVs) and remove chimeras. To correct the known diversity overestimation generated by the ASV inference from DADA2, an additional step of ASV clustering was performed using

the dbOTU3 algorithm (Olesen et al., 2017), relying on both phylogenetic distance and the distribution of ASVs to group them. ASV taxonomy was assigned using a Naïve Bayes classifier against the PR^2 database (PR^2; Guillou et al., 2013; 4.13.0). There were 4,662,679 raw reads (29,511 average/sample ± 6,611) obtained through the sequencing of 124 water samples and 34 sediment samples, corresponding to 7,732 ASVs (392 average/sample ± 133). Rarefaction curves showed that the sequencing effort applied was sufficient to describe the protist diversity, with an overall higher number of reads in the sediment samples than in the water samples (Fig. S1).

As this study focuses exclusively on protists, all reads assigned to Metazoa and Plantae (Embryophyceae) and Macroalgae (Ulvophyceae, Rhodophyceae) were excluded. After cleaning the dataset to the protistan community, 3,777,109 reads (23,906 average/sample ± 7,502) were obtained corresponding to 6,788 ASVs (362 average/sample ± 122). Read abundances of ASVs assigned to the same genus were combined. Three ASVs were found assigned to the genus *Lepidodinium*. The dataset was normalised by calculating the relative abundances of the phyla and genera over the total protistan community. For simplicity, in this paper, we refer to the abundance of the genus *Lepidodinium* meaning the relative abundance of the three ASV (cumulated values read number) over the whole protistan abundances. In particular, relative abundances of the 15 most abundant genera of Dinophyceae, including the genus *Lepidodinium*, were analysed.

2.4. Physicochemical and biological variables of the water column

Micro-phytoplankton (cells > 20 µm) abundance and community diversity were assessed using an inverted microscope (Zeiss, Axio Observer). Samples were gently homogenized before settling in a 10 mL sub-sample for > 12 h in Hydro-Bios counting

chambers (Utermöhl, 1958). The limit of quantification was 100 cells L^{-1}. Relative abundances of the most abundant genera of Dinophyceae were calculated.

Total phytoplankton biomass was estimated through [Chl*a*]. 500–1000 mL water samples were filtered through GF/F filters (Whatman®) and stored at –20°C until analysis. Chlorophyll was extracted in 10 mL of 90% acetone in the dark at 4°C for 12 h and analysed by monochromatic spectrophotometry (Aminot and Kérouel, 2004).

The concentration of TEP was determined using a semi-quantitative method based on the colorimetric determination of the amount of dye complexed with extracellular particles (Claquin et al. (2008) adapted from Passow and Alldredge, 1995; Roux et al., 2021). Briefly, triplicate samples of 50–150 mL were gently filtered through 0.4-µm polycarbonate membrane filters (Whatman® Nuclepore™ Track-Etched Membrane). Particles retained on the filter were stained with Alcian Blue solution (Sigma®). The filters were incubated in 80% sulphuric acid for 2h. Absorbance was read at 787 nm using a spectrophotometer (Shimadzu UV-2600). The TEP concentrations are expressed in µg Xanthan equiv. L^{-1}.

For inorganic nutrients, 300-mL water samples were pre-filtered through 41 µm directly from the Niskin bottle. For dissolved silicate (DSi = $Si(OH)_4^-$) concentrations, water samples were filtered through 0.45-µm acetate cellulose membranes and stored at 4°C until analysis. Water samples for the determination of dissolved inorganic nitrogen (DIN = NO_3^- + NO_2^- +NH_4^+) and Phosphorus (DIP = PO_4^{3-}) were stored directly at –20°C. For inorganic nutrients, samples were analysed using an auto-analyser (Seal analytical AA3) following standard protocols (Aminot and Kérouel, 2007). Limits of quantification were 0.4 µM for DSi, 0.5 µM for NO_3^- + NO_2^- and 0.05 µM for DIP and NH_4^+. The uncertainty values of measurements were 12% for DSi, 10% for NO_3^- + NO_2^-, 15% for DIP and 27% for NH_4^+.

2.5. Single-cell PCR and hatching experiments of cysts from surface sediments

Single cyst isolation was applied to try to identify *L. chlorophorum* cysts. The sodium polytungstate method of Bolch (1997) was used to extract cysts from surface sediments from five sampling stations: Nord Dumet (NODU), Estuaire Vilaine (ESTV), Pont Mahé (PMAE), Pornichet (PORV) and Pointe Saint Gildas (GILV). Eighty cysts, resembling the *L. chlorophorum* resting cyst morphotypes observed in culture by Sournia et al. (1992), were cleaned in four drops of distilled water, then isolated in new tubes and stored at –20°C. For species identification, single-cell PCR was carried out, following the procedure described in Mertens et al. (2012).

To identify cysts in the sediments, hatching experiments using the Bolch (1997) method were carried out using eight out of 17 sampled stations. Surface sediments from six stations: Pénerf (PENF), ESTV, NODU and PMAE located in the northernmost part of the study area and two stations (PORV and Gourmalon (GOUR)) positioned further south were selected given that *L. chlorophorum* blooms regularly occur here. The sediments were pre-sieved using a 125-µm mesh to remove larger particles and then sieved through a 20-µm mesh. The fraction smaller than 20 µm was sieved through a 10-µm mesh nylon filter. Thus, three sediment size categories were obtained: 125–20 µm, 20–10 µm and < 20 µm. One series of hatching experiments were carried out with samples of all size fractions. Fractionated sediments were deposited using a micropipette in 12-well plates containing L1/2 (Guillard and Hargraves, 1993) and K/2 (Keller et al., 1987) media and stored at 16 and 18° C with a 12:12 day: night cycle. A total of 60 12-well plates were prepared this way. There were 576 strains isolated from these wells and identified using an inverted microscope.

2.6. Statistical analysis

The spatiotemporal distribution of biological and physicochemical parameters was represented by a section scope using the software Ocean Data View 5.3.0 (Schlitzer, 2020). All statistical analyses were performed in R software (R Core Team). Non-metric multidimensional scaling (NMDS) analyses were performed to explore community patterns between both stations by applying the monoMDS function (Vegan R package). Confidence ellipses (95% confidence interval) allowed to distinguish group stations. The Spearman correlation matrix was calculated for all parameters to study relationships between eDNA data on *Lepidodinium* (ASVs relative abundance of the genus) and other environmental parameters. A principal component analysis (PCA), conducted with the FactoMineR package, was applied to assess the differences and similarities of autumn-winter samples between the two stations. For this analysis, relative abundances of the genus *Lepidodinium* within the protistan community, temperature, salinity, turbidity, depth, concentrations of dissolved oxygen and TEP as well as nutrient concentrations were used as quantitative variables. The representation of confidence ellipses (95% confidence interval) around the barycenter of each condition allowed to distinguish data groups.

3. Results

3.1. Protistan pelagic community

Protistan communities were similar between subsurface (0–1m), mid-depth (5–13 m, depending on stations) and water-sediment interface (10–26 m) for both stations during both autumn-winter periods (Fig. 2 A–F). Alveolata dominated the entire dataset in terms of relative abundance (mean ± SD; 63.1 ± 12.4%), followed by Stramenopiles (19.7 ± 1.3%), Hacrobia (7.4 ± 5.6%), Rhizaria (4.4 ± 3.3%), Archaeplastida (2.3 ± 2.6%), Opisthokonta (1.0 ± 0.8%) and Apusozoa (0.1 ± 0.2%). Relative abundances of Amoebozoa and Excavata were less than 0.1%. Unknown eukaryotes (eukaryotic ASVs not assigned to any taxonomic level) represented 2.1 ± 1.8% of the total protists. Within Alveolata, Dinophyta was the most abundant phylum, accounting for 92.5 ± 6.4% of the total Alveolata in the entire dataset. Dinophyceae (including dinoflagellates) was the most abundant class within the phylum Dinophyta (69.2 ± 26.2%).

The 15 most abundant genera of Dinophyceae accounted for an average of 38.2 ± 20.3% of the total dinophycean community. They were in descending order: *Heterocapsa* (11.1 ± 14.5%), *Gyrodinium* (10.2 ± 14.5%), *Gymnodinium* (5.6 ± 5.4%), *Warnowia* (2.0 ± 4.7%), *Biecheleria* (1.7 ± 2.3%), *Prorocentrum* (1.7 ± 1.7%), *Lepidodinium* (1.6 ± 4.6%), *Torodinium* (0.9 ± 1.3%), *Tripos* (0.9 ± 1.7%), *Pelagodinium* (0.6 ± 1.2%), *Gonyaulax* (0.5 ± 0.8%), *Lingulodinium* (0.5 ± 2.3%), *Alexandrium* (0.4 ± 0.8%), *Scrippsiella* (0.4 ± 1.1%) and *Polykrikos* (0.3 ± 0.5%) (Fig. 3 A–F). The dinophyceaen community was similar between both stations (Fig. 3 A–F and Fig. S2). Relative abundances of the genus *Lepidodinium* varied from 0% to 20.5% (Fig. 3 A–F). In 2019, the highest relative abundances of *Lepidodinium* were recorded at the subsurface at the station Basse Michaud, representing 5.1%, 20.5% and 6.3% of the total protistan community on 22 October and 6 and 18 November 2019, respectively (Fig. 3B). At the station Ouest Loscolo, the highest abundances of *Lepidodinium* were

recorded at the subsurface on 24 November (9.8%) and 8 December 2020 (3.7%; Fig. 3A). At both stations, *Lepidodinium* was also found at mid and water-sediment interface depths at lower abundances (Basse Michaud: 0.7 ± 1.2%; Ouest Loscolo: 0.3 ± 0.6%)

The eDNA metabarcoding (Fig. 3) and inverted microscopy showed contrasting results (Fig. S3). The 15 most abundant dinophycean groups determined using inverted microscopy (Fig. S3) were different from those defined by the eDNA method (Fig. 3). In general, the dinophycean community assessed by microscopy analyses varied between both stations, particularly at the subsurface. Nevertheless, *Lepidodinium* was observed by microscopy in nine out of 124 samples analysed (Fig. S3) while the genus was detected by metabarcoding in 65 samples (Fig. 3). Using inverted microscopy, *Lepidodinium* was only detected at the station Ouest Loscolo with the highest relative abundances within the total protistan community observed at the subsurface (Fig. S3A) on 24 November (0.9%) and 8 December 2020 (1.3%).

3.2. **Lepidodinium** ***distribution in the water column and environmental parameters***

Spatio-temporal patterns of relative abundances of the genus *Lepidodinium* were related to physicochemical characteristics of the water column (Fig. 4 and Fig. S4). Similar patterns were observed at both stations; those of Basse Michaud are here described in details as an example (Fig. 4 A–L).

Homogenous temperatures were recorded along the water column during both autumn-winter periods, suggesting vertical mixing during the sampling period. At the station Basse Michaud (Fig. 4), seawater temperature decreased from 14.9 ± 1.6 (9 September 2019) to 11.1 ± 0.4°C (3 March 2020; Fig. 4A) in autumn–winter 2019–

2020 period and from 17.7 ± 1.0 (8 September 2020) to 10.8 ± 0.6°C (22 March 2021; Fig. 4B) in 2020–2021. During both autumn periods, homogenous salinity of 34.4 ± 0.9 was measured in the water column (Fig. 4C, D). During winters, freshwater inputs from the Loire River reached the station Basse Michaud as suggested by a decrease in subsurface salinity in December and January (Fig. 4C, D). Indeed, subsurface salinity sharply decreased from 31.8 to 22.6 (3 and 17 December 2019, respectively; Fig. 4C) and from 33.6 (8 December 2020) to 26.8 (4 January 2021; Fig. 4D). Stratification index values (Fig. S5) confirmed that vertical mixing of the water column was observed in September–October ($\mathcal{E}<0.05$) and a haline stratification occurred during the winter periods ($\mathcal{E} > 0.05$) with lower salinity at the subsurface than at the water-sediment interface for both years (Fig. 4C, D). Similar patterns were observed for the station Ouest Loscolo that was influenced by the freshwater inputs from both rivers (Loire and Vilaine) (Fig. S4 A–D).

At Basse Michaud, turbidity ranged from 0.6 to 9.3 FNU at the subsurface and from 1.4 to 14.2 FNU at the water-sediment interface during autumn-winter 2019–2020 (Fig. 4E). In particular, turbidity increased in the water column on 6 November 2019 with 6.3 and 12.0 FNU recorded at the subsurface and water-sediment interface respectively (Fig. 4E), suggesting a resuspension of the sediment. At this date, resuspension of the sediment was associated with high [NH_4] in the entire water column (Fig 4G), but more particularly at the subsurface. Indeed, [NH_4] sharply increased between 22 October and 6 November 2019 when the highest values of 2.90, 1.67 and 1.41 µM were observed at the subsurface, mid-depth and water-sediment interface, respectively (Fig. 4G). A similar dynamic was observed on 9 November 2020 when an increase in turbidity up to 7.3 FNU at the water-sediment interface (Fig. 4F)

corresponded to an increase in [NH_4] in the water column (subsurface: 1.51 µM; mid-depth: 1.63 µM; water-sediment interface: 1.59 µM; Fig. 4H). At the station Ouest Loscolo (Fig. S4 E-H), a similar increase in [NH_4], associated with higher turbidity values potentially caused by sediment resuspension, was observed on 7 October 2019 (range: 3.4–3.9 µM; Fig. S4G) and on 12 October 2020 (range: 2.1–3.0 µM; Fig. S4H).

From September 2019 to March 2020, relative abundances of *Lepidodinium* recorded at the station Basse Michaud using eDNA ranged from 0 to 20.5%, with the highest value calculated on 6 November 2019 at the subsurface (Fig. 4I), when the highest [NH_4] were measured (Fig. 4G). In autumn-winter 2020–2021, relative abundances were lower, ranging from 0 to 2% (Fig. 4J) at the same station. In contrast, no *Lepidodinium* cells were observed using inverted microscopy during both autumn-winter periods at the station Basse Michaud (Fig. 4K–L). The highest abundances of *Lepidodinium* in correspondence with the highest [NH_4] were also observed at the station Ouest Loscolo. Indeed, eDNA data showed the presence of the genus *Lepidodinium* during both autumn-winter periods with maximal relative abundances calculated at the subsurface on 24 October 2019 (2%; Fig S4I) and 24 November 2020 (9.8%; Fig. S4J) when the highest [NH_4] were measured in the water column (Fig. S4G, H). At this station, the high abundance of *Lepidodinium* was verified by microscope counts. Indeed, maximal cell concentrations of 600 cells L^{-1} (Fig. S4K) were measured on 24 October 2019 (eDNA: 2%; Fig. S4I) and 5 200 cells L^{-1} (Fig. S4L) on 24 November 2020 (eDNA: 9.8%; Fig. S4J) at subsurface.

A Principal Components Analysis (PCA) of the measured environmental parameters and the relative abundances of the genus *Lepidodinium* is shown in Fig. 5. The PCA described 76% of the total data variance along three principal dimensions (Dim1, Dim2 and Dim3). Dim1 explained temperature, salinity, [O_2] as well as

[NO_3+NO_2], [DIP] and [DSi]. Nutrient concentrations and [O_2] were negatively correlated with salinity. The Dim2 explained depth, turbidity and [TEP]. Turbidity was positively correlated with [TEP] and depth. In this PCA analysis, the relative abundance of *Lepidodinium* and [NH_4] were the variables most correlated with Dim3. While the relative abundance of *Lepidodinium* was positively correlated with [NH_4] and temperature, it was negatively correlated with depth.

Spearman correlations (Fig. S6) corroborated the positive correlation between *Lepidodinium,* [NH_4] ($r = 0.33$; $p < 0.05$) and temperature ($r = 0.30$; $p < 0.05$). In contrast, *Lepidodinium* was negatively correlated with depth ($r = –0.24$; $p < 0.05$) and turbidity ($r = –0.25$; $p < 0.05$). No significant correlation was established between salinity and *Lepidodinium.*

3.3. Lepidodinium *distribution in the sediment*

To assess whether resuspension of *Lepidodinium* cysts in the water column could explain the presence of this species in the subsurface after germination, field cysts analyses (germination experiments and cyst microscopy observations), single-cyst isolation and PCR identification, and sedimentary eDNA analyses were performed.

The analyses of the three size classes showed the presence of a greater number of cysts in the > 20-µm sediment size class. Germination experiments showed the presence of the dinoflagellate species *Alexandrium minutum*, *Gymnodinium aureolum*, *Heterocapsa* sp., *Scrippsiella* sp., *Sourniaea diacantha*, *Protoceratium reticulatum* and *Levanderina fissa*. Cysts of other species were detected using light microscopy: *Gymnodinium impudicum, Lejeunecysta* sp., *Lingulodinium*

machaerophorum, *Spiniferites belerius*, *Votadinium* sp. and *Votadinium spinosum.* Other cysts not unambiguously attributable to a genus were also identified: spiny brown cysts (see some examples in Fig. S7A–I). Since no resting cysts of *Lepidodinium* had been identified, 80 cysts corresponding to morphotypes resembling by size, morphology and green colour content to the *Lepidodinium* cyst morphotypes described by Sournia et al. (1992) (Fig. S8A) were identified through single-cell PCR. Of the 80 cysts, only eight were successfully sequenced. Three were identified (identity 90–99% with reference sequence) close to the freshwater species *Poterioochromonas malhamensis* (Ochromonadaceae) (Fig. S8B), three were 90% related to a dinoflagellate species of the genus *Ensiculifera* sp. (Fig. S8C), one was 94% close to *Ebria tripartita* (Cercozoa; Fig. S8D) and one was 91% related to the ciliate *Strombidinopsis batos* (Fig. S8E). In conclusion, all these analyses failed to verify the presence of *Lepidodinium* resting cysts in the sediments of the analysed stations.

The eDNA data showed traces of the genus *Lepidodinium* at all stations and in different grain sizes (from clayey to sandy sediments; Fig. 6 and Table S1), except for the NOIR station in both replicates. However, the relative abundance of *Lepidodinium* within the sedimentary protistan community was always very low (<1%). The maximal relative abundance of *Lepidodinium* was measured at the station PMAE where *Lepidodinium* represented 0.74% of the total protistan community in the sediments (Fig. 6). eDNA of the genus *Lepidodinium* was slightly more abundant in the Vilaine Bay (0.24 to 0.74%) than offshore the Loire River (0 to 0.45%).

4. Discussion

The coupling between biogeochemical and eDNA analyses of sediment and water samples across two stations and during two autumn-winter seasonal surveys suggested that NH_4 input from sediments contributes to trigger increase in cell abundances of the green dinoflagellate genus *Lepidodinium* in the water column. Therefore, this study contributes to the assessment of the phenology of the species in southern Brittany during the non-bloom period, providing a new hypothesis concerning species biology and ecology that can help to understanding bloom initiation in other areas of the world where species of the genus are present. In addition, this study shows the application of eDNA for assessing rare species abundances, contributing to the understanding of life cycles and ecology of these protists during a period non-favourable to their development.

4.1. **Lepidodinium** *during non-favourable growth conditions*

The composition of protistan communities during non-productive periods have gained less attention in ecological studies because of less interest in the food chain and bloom (including harmful species) dynamics analyses during this time (Zingone et al., 2010). This is also the case in our study area, where studies focused on the autumn-winter season are rare. However, since 1987 and even during non-productive phytoplankton periods, the French Phytoplankton and Phycotoxin monitoring network has acquired information on phytoplankton communities along French coasts (REPHY) using microscopic methods (Belin and Soudant, 2018). The autumn-winter phytoplankton community composition has some Stramenopiles genera (*Skeletonema, Asterionella*, *Asterionellopsis*) among the most abundant taxa (Belin and Soudant, 2018). In contrast, some other Stramenopiles (*Pseudo-nitzschia*, *Chaetoceros*, *Leptocylindrus*) as well as Alveolata genera such as *Lepidodinium* and

Prorocentrum were observed in all seasons except in winter (December–February; Belin and Soudant, 2018). Our eDNA data showed a similar protistan composition over the sampling period, at both stations (Fig. S2) and across the water column (Fig. 2). In particular, Dinophyceae (including dinoflagellates) represented a large part of the total protistan community. The 15 most abundant genera observed were *Heterocapsa, Gyrodinium*, *Gymnodinium, Warnowia, Biecheleria, Prorocentrum, Lepidodinium, Torodinium, Tripos, Pelagodinium, Gonyaulax*, *Lingulodinium, Alexandrium, Scrippsiella* and *Polykrikos.* Zingone et al. (2010) also demonstrated that the composition of winter phytoplankton populations in the Gulf of Naples (Italy, Mediterranean Sea) was similar at different depths due to the homogenizing effect of vertical turbulence.

Along the French coasts, some studies investigated the communities of marine microorganisms using molecular approaches. For example, Caracciolo et al. (2021) used eDNA metabarcoding to confirm the seasonal dynamics of marine protists communities on the English Channel, while Lambert et al. (2021) studied the seasonal succession of marine microorganisms on the NW Mediterranean coast focusing on the winter period (January-March). In southern Brittany, to our knowledge, our study is the first specifically exploring the autumn-winter protistan community with a new tool, the eDNA metabarcoding, that provides a quite exhaustive characterisation of the relative abundance of protist genera and groups as well as the detection of rare taxa in the environment. Yet, this method does not always provide accurate analyses of species abundances, but rather relative abundance of genera or taxa identified at even higher taxonomic levels. This limit is due to the taxonomic resolution of the primers used for the amplification of the eDNA and the potential bias due to its PCR amplification and high-throughput sequencing. Pending the development of species-specific genetic-

based method (qPCR, FISH) to study the ecology of *L. chlorophorum*, we considered metabarcoding semi-quantitative data of ASVs assigned to the genus *Lepidodinium* enough information for our study. Indeed, the genus is currently composed of only two species (*L. chlorophorum* and *L. viride*; Watanabe et al., 1990; Hansen et al., 2007; Siano et al., 2009) and so far, *L. viride* has not been identified in our study area. Hence, while remaining cautious about the interpretation of our data, we consider that our evaluation of the relative abundance of the genus *Lepidodinium* approximates the abundance of *L. chlorophorum* in our study area.

While *Lepidodinium* has never been observed in winter in the frame of the REPHY network, our study based on an eDNA approach showed traces of the genus *Lepidodinium* in December and January. The development of molecular methods has improved our understanding of marine protists (Caron et al., 2012; Pedros-Alios, 2012; Logares et al., 2014). As in our study, the difference in phytoplankton composition between classical microscopic identification and eDNA-based methods has been highlighted (Zimmermann et al., 2015; Abad et al., 2016; Ruppert et al., 2019; MacKeigan et al., 2022). Yet, both methods provided similar results in seasonal protist community composition using long-term data series. Indeed, Caracciolo et al. (2021) investigated patterns of planktonic protist community succession in temperate latitudes using data from both microscopy count and eDNA metabarcoding collected over eight years (2009–2016) and found similar ecological patterns with both methods. Although microscopy does not consider the smallest taxa, and eDNA metabarcoding can overestimate or underestimate the proportions of taxa for which DNA is more easily extracted and amplified (Santi et al., 2021), a combination of both methods allows for assessing absolute species abundance while improving taxonomic resolution and therefore revealing great diversity (Caracciolo et al., 2021).

The eDNA metabarcoding can be used to accurately identify the rare species and their dynamics. Regarding local communities, Logares et al. (2014) defined operational taxonomic units as ‘‘rare’’ when their abundances were below 0.01%, following other studies of bacteria (Galand et al., 2009; Pedros-Alios, 2012) and protists (Mangot et al., 2013). Other studies recorded few dominant protistan taxa and a large number of rare ones from marine and freshwater communities (Stoeck et al., 2009, 2010; Cheung et al., 2010; Nolte et al., 2010; Mangot et al., 2013; Logares et al., 2014). These rare marine microbes could increase in abundance following environmental perturbations or changes (Caron and Countway, 2009). The eDNA metabarcoding allowed for detecting *Lepidodinium* abundance even at < 1% of abundance during winter (minimum value: 0.01%) while it is known that, during green seawater discolorations, it can represent more than 90% of the whole micro-phytoplankton community (Roux et al., 2022). In addition, the increase in this dinoflagellate from rare to high concentrations (from 0 to 20.5% of *Lepidodinium* at the station Basse Michaud) has been detected. Therefore, eDNA analyses not only allowed a more exhaustive characterisation of the protistan community, and specifically of the dinoflagellate community composition, but also the detection of the dynamics of *Lepidodinium* during non-bloom periods.

4.2. Environmental factors triggering Lepidodinium *development*

Our observations showed that the maximal relative abundance of *Lepidodinium* was correlated with the maximal [NH_4] that followed an increase in turbidity at the water-sediment interface. The accumulation of NH_4 in the sediments was well documented (e.g., Rosenfeld, 1979; Blackburn and Henriksen, 1983; Chapelle et al., 2000; Gribsholt et al., 2005). In the Vilaine bay, Ratmaya et al. (2022) measured

constant values of NH_4 around 300 µM below 5 cm in September 2015 while values could exceed 1200 µM in June and August. In this area, the authors highlighted that the dissolved organic nitrogen concentrations measured in pore-water of sediments were within the same range as [NH_4] (Ratmaya et al., 2022) and were in the same order of magnitude as those reported in the Chesapeake Bay (Burdige and Zheng, 1998) and the St. Lawrence estuary (Alkhatib et al., 2013). Several studies showed that adsorption and desorption of NH_4 to sediments could be reversible and that exchangeable NH_4 can be released into interstitial water when the environmental conditions change (Morin and Morse, 1999; Morse and Morin, 2005; Fitzsimons et al., 2006; Ospina-Alvarez et al., 2014; Liu et al., 2015). Therefore, when the water column is not stratified, resuspension of sediments could induce an increase in [NH_4] in the water column and promote phytoplankton growth. Indeed, many marine phytoplankton species showed glutamine synthetase (GS) activity, the major ammonium-assimilating enzyme, demonstrating a strong affinity for NH_4 (Bressler and Ahmed, 1984). This strong affinity also concerns dinoflagellates (e.g., Collos et al., 2007; Seeyave et al., 2009; Jauzein et al., 2017; García-Portela et al., 2020). In a coastal area of Southern Chile, Iriarte et al. (2005) detected GS activity in a *L. chlorophorum* (cited as *Gymnodinium* cf. *chlorophorum*) bloom under low [DIN], suggesting that *L. chlorophorum* could present high NH_4 affinity and assimilation rate (Iriarte et al., 2005). In addition, in previous studies focused on the *L. chlorophorum* bloom development, we suggested that bacteria remineralisation, producing regenerated NH_4, could sustain *L. chlorophorum* bloom for a long period (Roux et al., 2022). The co-occurrence of the higher abundances of *Lepidodinium* and NH_4 shown in this study corroborates the hypothesis that *L. chlorophorum in situ* has a strong affinity for NH_4 but also suggests that pulses in NH_4 can cause rapid growth of this dinoflagellate. Culture experiments

under laboratory conditions are now needed to confirm whether the NH_4 is an important nutrient substrate for *Lepidodinium* growth.

Although the assimilation of NH_4 might have promoted the growth of *Lepidodinium* cells during the autumn–winter period, we did not observe any bloom of this species during our survey. A previous study highlighted that *L. chlorophorum* was associated with a summer-like ecological niche defined by high temperature, salinity and irradiance (Karasiewicz et al., 2020). In addition, water column stratification and river inputs could trigger (Sourisseau et al., 2016) and sustain (Roux et al., 2022) *L. chlorophorum* blooms. During the autumn–winter period, the increase in subsurface abundance of *L. chlorophorum* did not coincide with water column stratification and summer conditions. Therefore, we suggest that the environmental conditions that occurred during bloom initiation in our survey did not favour the development of *L. chlorophorum*. We suggest that NH_4 resuspension from sediment could also occur later in the year, during the spring–summer time (April–June), when high temperatures cause stratification of the water column, before the observation of high-biomass bloom regularly causing water discoloration in summer (July).

4.3. The origin of Lepidodinium *blooms*

The species that are most likely to develop in a water body may be those that have regularly produced populations in the past, presumably because they were most likely to be able to provide the relatively large inoculum of vegetative cells required to initiate a tangible increase when conditions favour their growth (Reynolds, 1984). For dinoflagellates, the bloom inoculum is often represented by cells germinated from cysts. Cyst formation is a common characteristic underlying bloom initiation,

termination and the dispersion of dinoflagellates (Díaz et al., 2018). They may remain viable for years until the favourable environmental conditions for their germination (McQuoid et al., 2002; Lundholm et al., 2011; Ribeiro et al., 2011; Miyazono et al., 2012). Some studies have shown that cysts tend to concentrate in fine-grained sediments (e.g., Anglès et al., 2010) and are important in inoculating spring blooms (Kirn et al., 2005; Brosnahan et al., 2020). In many coastal areas, the location of cyst accumulations could determine the location of resulting blooms (Genovesi et al., 2009; Anglès et al., 2010, 2012; Crespo et al., 2011; Ní Rathaille and Raine, 2011; Anderson et al., 2014; Klouch et al., 2016).

All our attempts to identify cysts of *L. chlorophorum* in the field failed. Blooms of this species were recorded in southern Brittany during the summer of 2018, previously to the start of our bi-seasonal monitoring in 2019 and 2020 (Table S2). Yet, hatching experiments and cyst identification based on single-cell PCR techniques carried from sediment samples collected in January–February 2019 did not reveal the presence of resting cysts of *L. chlorophorum*, although cysts of many other species were hatched and identified. Our study showed the presence of rare (<1% relative abundance) *Lepidodinium* eDNA in the sediments of our study area. This result could suggest the existence of cysts in the sediment but at very low concentration. However, it is suggested that extracellular DNA can represent a relevant fraction of the total DNA stored in the sediments (Pietramellara et al., 2009). Considering all precautions taken for obtaining high quality eDNA data in the sediments (the collection of muddy sediment which enhances the possibility to have better preserved DNA and the application of agreed procedures for benthic eDNA sampling suggested in Pawlowski et al. (2021)), we exclude bias in our sampling strategy and genetic procedures. However, we cannot exclude that our eDNA corresponds to extracellular DNA, which

sank from the surface to the bottom of the water column. Indeed, we did not compare DNA extraction methods for analysing protistan communities in the sediments as done in other studies (Siano et al., 2021) and we used classical DNA extraction kits (PowerMaxSoil®) that consider both the extracellular and intracellular eDNA fractions.

Another possibility is that *Lepidodinium* development could originate from cells present directly in the water column. Cells of *L. chlorophorum* might be present in the water column at very low concentrations and likely in a temporary quiescent stage, awaiting better environmental conditions to grow. Indeed, for some dinoflagellates belonging to the genera *Dinophysis* and planktonic *Prorocentrum*, cysts have never been isolated from sediments (Smayda and Trainer, 2010). Some species can overwinter in low numbers and remain below detection limits (<100 cells L^{-1}) (Sogin et al., 2006; Record et al., 2021) waiting for seawater warming and thermal stratification to develop blooms (Glibert et al., 2018). For *L. chlorophorum*, Sournia et al. (1992) observed cysts of this dinoflagellate in water samples. The authors described spherical cysts, characterized by a double membrane with the dinokaryon remaining visible inside (Sournia et al., 1992). However, this hypothesis has not been confirmed by molecular analyses and it is not known whether these cysts originated from the water column or the resuspension of sediments. Pending the clear identification of resting cysts in field sediment samples, we cannot exclude that *Lepidodinium* may thrive in the water column at low concentrations during autumn–winter awaiting for NH_4 input from sediments to start growing and for higher temperature and water stratification to bloom.

5. Conclusion

This study investigated the autumn–winter protistan community composition (September–March) with the metabarcoding of the eDNA and allowed for demonstrating the rare presence of *Lepidodinium* in December and January, although this genus has never been observed in winter by light microscopy. In addition, the increase in dinoflagellate abundance was detected during the non-productive period providing, for the first time, new insights for the understanding of *L. chlorophorum* bloom onset. Indeed, the coupling between biogeochemical and eDNA analyses of water samples during the two autumn–winter surveys (September–March) suggested that NH_4 pulses after sediment resuspension could contribute to the development of *Lepidodinium*. This result confirmed that *L. chlorophorum* has a strong affinity for NH_4 in the field. We hypothesize that NH_4 resuspension from sediment could also occur later in the year (April–June) when high temperature and water column stratification could favour, not only the active division of the species, but also the development of a bloom.

Furthermore, although green seawater discoloration caused by *L. chlorophorum* was recorded in coastal southern Brittany during the summer of 2018, no cysts of this species were observed in our study. In contrast, eDNA metabarcoding showed the presence of a rare abundance of *Lepidodinium* eDNA in the sediments. This result could suggest the existence of cysts at very low concentrations or the presence of extracellular DNA. Pending unambiguous identification of cysts in field sediment samples, it is suggested that *Lepidodinium* could thrive in the water column at low concentration during the autumn–winter period (resistant pelagic stage), waiting for NH_4 input from sediments to start growth, and in addition, higher temperature and water stratification to bloom.

Finally, this work shows a further application of eDNA metabarcoding for the study of winter (non-blooming season) communities and rare species and thus how it can contribute to ecological and biological studies of natural protists.

Competing interests

The authors declare that they have no competing interests.

Author contributions statement

Pauline Roux: Formal analysis, Data curation, Writing original Draft; **Kenneth Neil Mertens**: Investigation; Writing - Review & Editing; **Coralie André**: Investigation, Visualization; **Aouregan Terre-Terrillon**: Investigation, Visualization; **Karine Collin**: Investigation, Visualization; **Anne Schmitt**: Investigation, Visualization; **Soazig Manach**: Investigation, Visualization; **Joelle Serghine**: Investigation, Visualization; **Cyril Noel**: Investigation, Visualization; **Mathilde Schapira**: Conceptualization, Writing - Review & Editing, Funding acquisition; **Raffaele Siano**: Conceptualization, Methodology, Supervision, Writing - Review & Editing. All authors reviewed and accepted the final version of the manuscript.

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Legends

Figure 1. Location of the sampling stations monitored in southern Brittany coast (NE Atlantic, France). Water samples were collected at the two sampling stations (red triangles) located in the Vilaine Bay (Ouest Loscolo) and offshore the Loire River (Basse Michaud) during both autumn-winter periods: from September 2019 to March 2020 and from September 2020 to March 2021. Sediment samples (yellow circles) were collected at 17 sampling stations in January-February 2019: La Coupelasse (COUP), Croisic Large (CROI), Estuaire Loire (ESTL), Estuaire Vilaine (ESTV), Pointe Saint Gildas (GILS and GILV), Gourmalon (GOUR), Maresclé (MARE), Nord Dumet (NODU), Noirmoutier (NOIR), Penbé (PENB), Pénerf (PENF), Pont Mahé (PMAE), Pornichet (PORS and PORV), Traict du Croisic (TCRO) and La Turballe (TURB).

Figure 2. Relative abundance (%) of the protistan groups detected with eDNA metabarcoding. Water samples were collected at three depths during both autumn-winter periods: from September 2019 to March 2020 and from September 2020 to March 2021. Protistan communities were investigated at the station Ouest Loscolo (Vilaine Bay): (**A**) subsurface, (**C**) mid-depth, **(E**) water-sediment interface; and at the station Basse Michaud (offshore the Loire River): (**B**) subsurface, (**D**) mid-depth, (**F**) water-sediment interface.

Figure 3. Relative abundance (%) of the 15 most abundant genera of Dinophyceae within the protistan community was detected with eDNA metabarcoding. Water samples were collected at three depths during both autumn-winter periods: from

September 2019 to March 2020 and from September 2020 to March 2021. Dinophyceae communities were investigated at the station Ouest Loscolo (Vilaine Bay): (**A**) subsurface, (**C**) mid-depth, **(E**) water-sediment interface; and at the station Basse Michaud (offshore the Loire River): (**B**) subsurface, (**D**) mid-depth, (**F**) water-sediment interface.

Figure 4. Spatio-temporal variability of biological and physicochemical parameters along the water column at the station Basse Michaud during both autumn-winter periods. (**A**–**B**) Temperature (°C); (**C**–**D**) Salinity; (**E**–**F**) Turbidity (FNU); (**G**–**H**) Concentrations of ammonium (NH_4; µM); (**I, J**) Relative abundance of *Lepidodinium* genus within the protistan community (%) detected by eDNA metabarcoding; (**K**–**L**) Relative abundance of *Lepidodinium* cells within the protistan community (cells L^{-1}) observed using an inverted microscope (Ocean Data View, 5.3.0).

Figure 5. PCA summarizing the similarities and differences in environmental factors and the relative abundance of *Lepidodinium* at the two sampling stations during both autumn-winter periods. (**A**) Dim1, Dim2 and (**B**) Dim3 together describe 76% of the total variance. Black arrows are quantitative variables used to calculate PCA: relative abundance of *Lepidodinium* eDNA within the protistan community (*Lepidodinium*, %); TEP concentration ([TEP], µg Xeq L^{-1}); nitrogen ([NO3+NO2], µM); phosphate ([DIP], µM); silicates ([DSi], µM) and ammonium concentrations ([NH4], µM); turbidity (Turb, FNU); oxygen saturation ([O2], %); salinity (Sal); temperature (Temp, °C) and depth (Depth, m). Water samples are represented as follows: Basse Michaud station (blue triangles); Ouest Loscolo station (green circles). Confidence ellipses (95% confidence

interval) allowed distinguishing stations: Basse Michaud (blue ellipse) and Ouest Loscolo (green ellipse).

Figure 6. *Lepidodinium* eDNA distribution in the sediment. Duplicate sediment samples (orange and green circles) were collected at each sampling station in January-February 2019: La Coupelasse (COUP), Croisic Large (CROI), Estuaire Loire (ESTL), Estuaire Vilaine (ESTV), Pointe Saint Gildas (GILS and GILV), Gourmalon (GOUR), Maresclé (MARE), Nord Dumet (NODU), Noirmoutier (NOIR), Penbé (PENB), Pénerf (PENF), Pont Mahé (PMAE), Pornichet (PORS and PORV), Traict du Croisic (TCRO) and La Turballe (TURB). The sizes of the circles represent a scale of rare relative abundances of *Lepidodinium* eDNA within the protistan community (%). Water samples were collected at the two sampling stations (red triangles) during both autumn-winter periods: from September 2019 to March 2020 and from September 2020 to March 2021.

Highlights

- Rare *Lepidodinium* eDNA was detected during unfavourable growth seasons
- Increase in *Lepidodinium* was correlated with ammonium pulse from sediment
- No cysts of *Lepidodinium* were identified in the sediments
- Temporary pelagic stage during non-blooming phase may explain species overwintering

Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Fig1_Sampling map

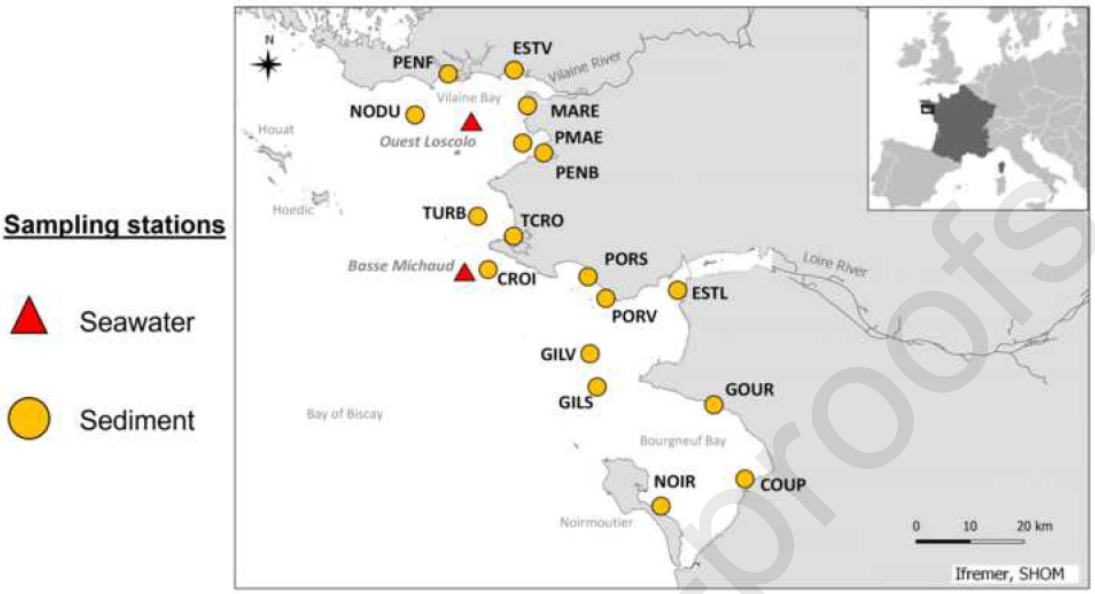

Fig2_Protistan community

Click here to access/download;Figure;Roux et al. Fig2_Protistan_community_To_Referee.tif

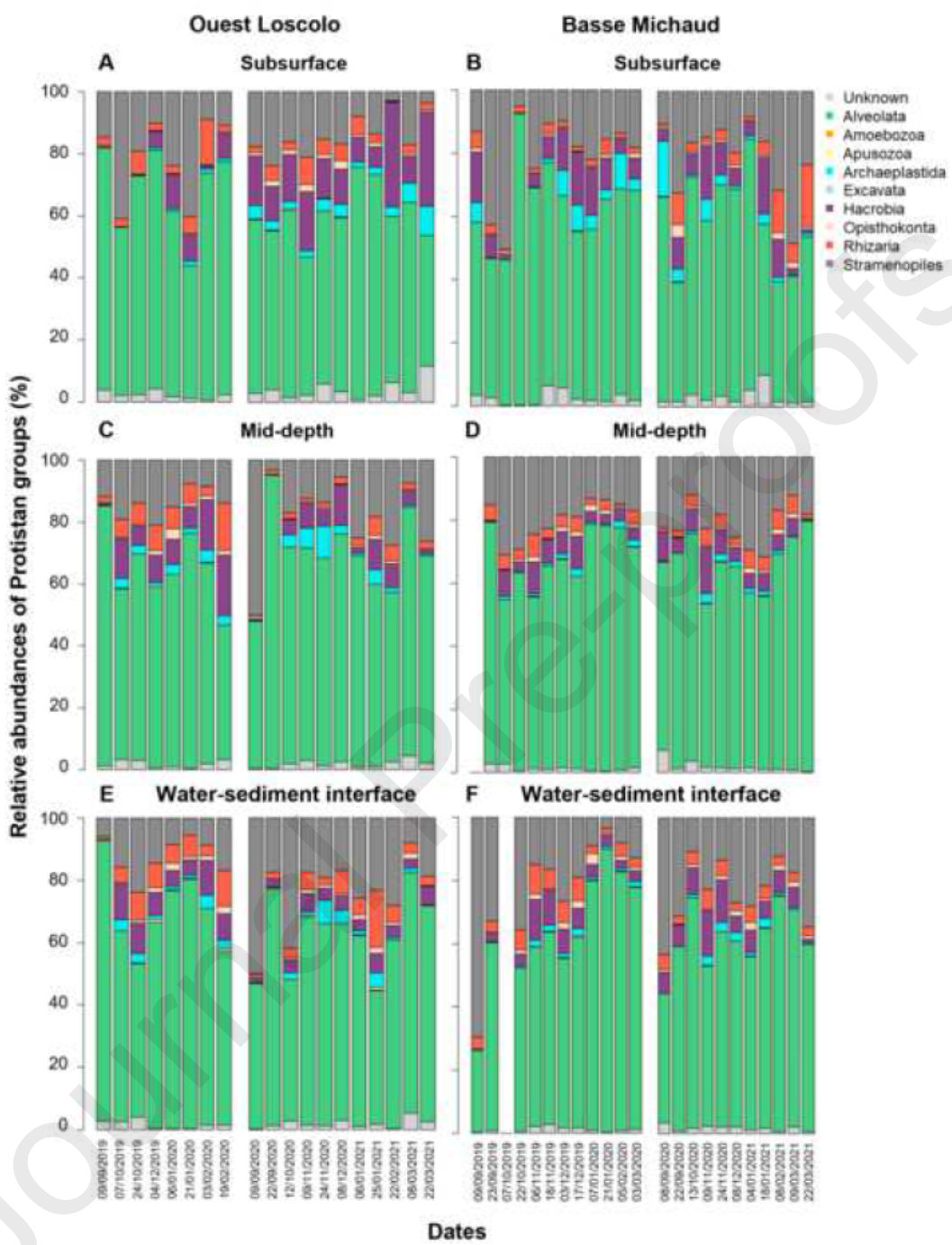

Fig3_Dinophyceae

Click here to access/download;Figure;Roux et al. Fig3_Dinophyceae_To_Referee.tif

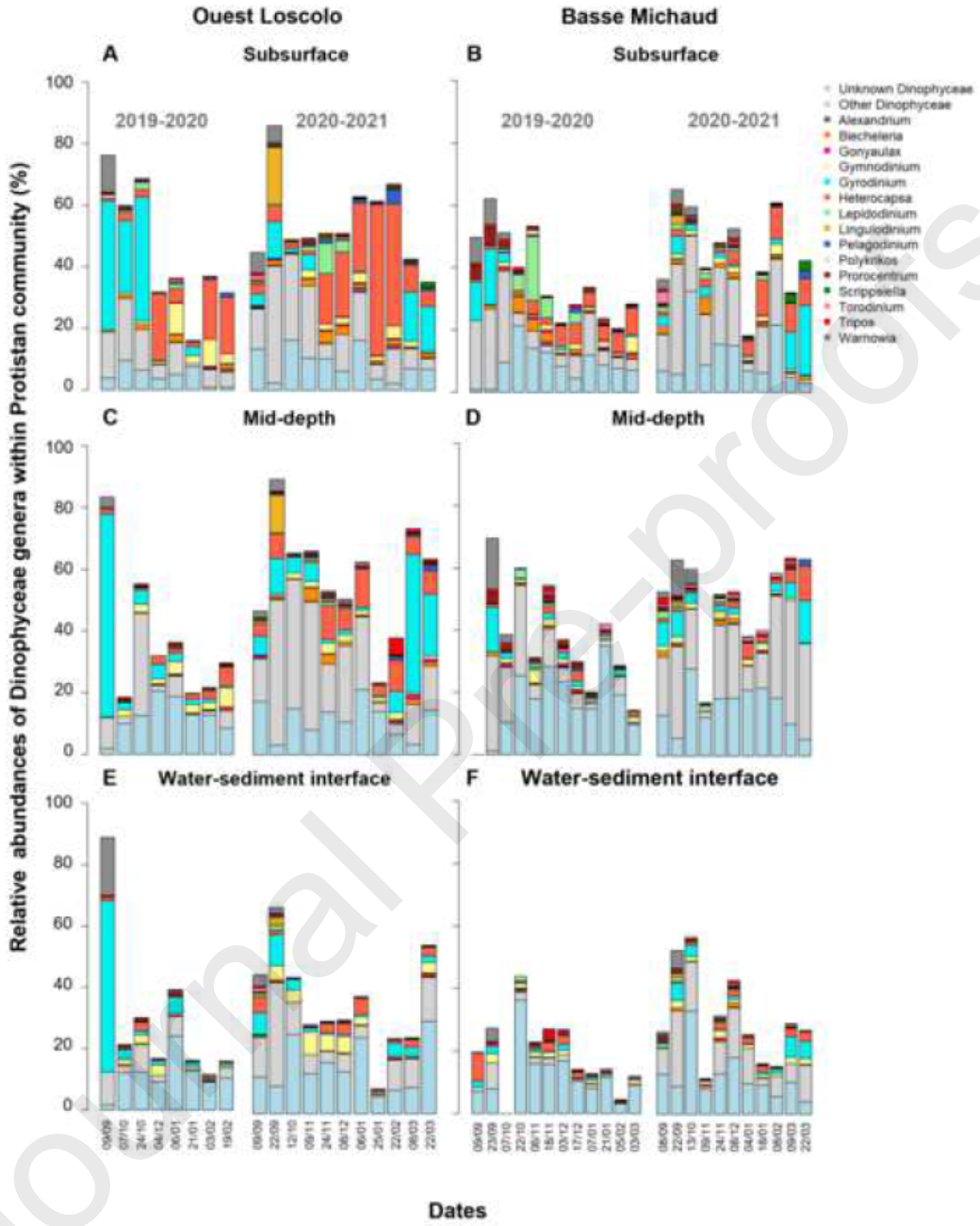

Fig4_Environmental factors

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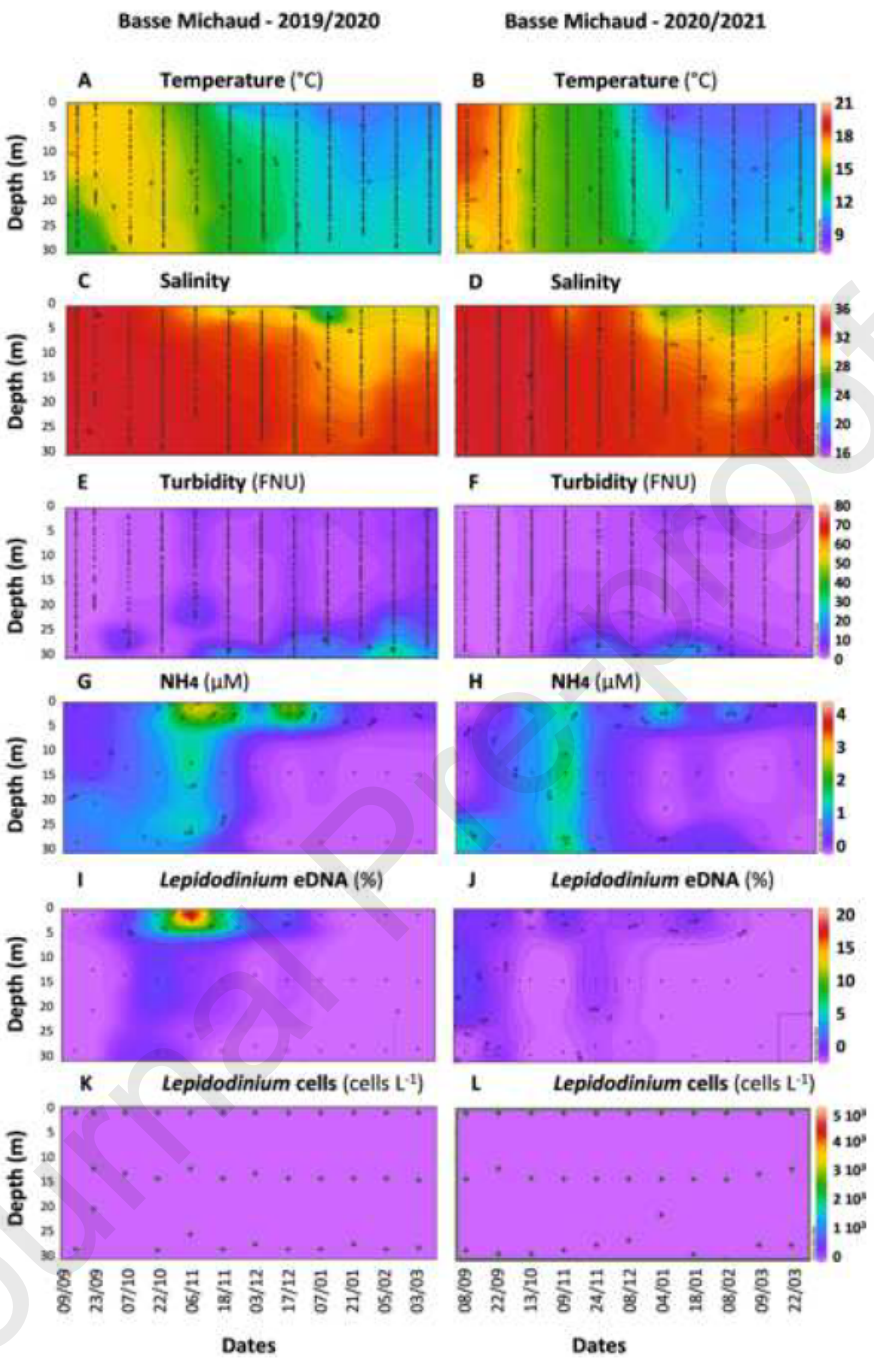

Fig5_PCA

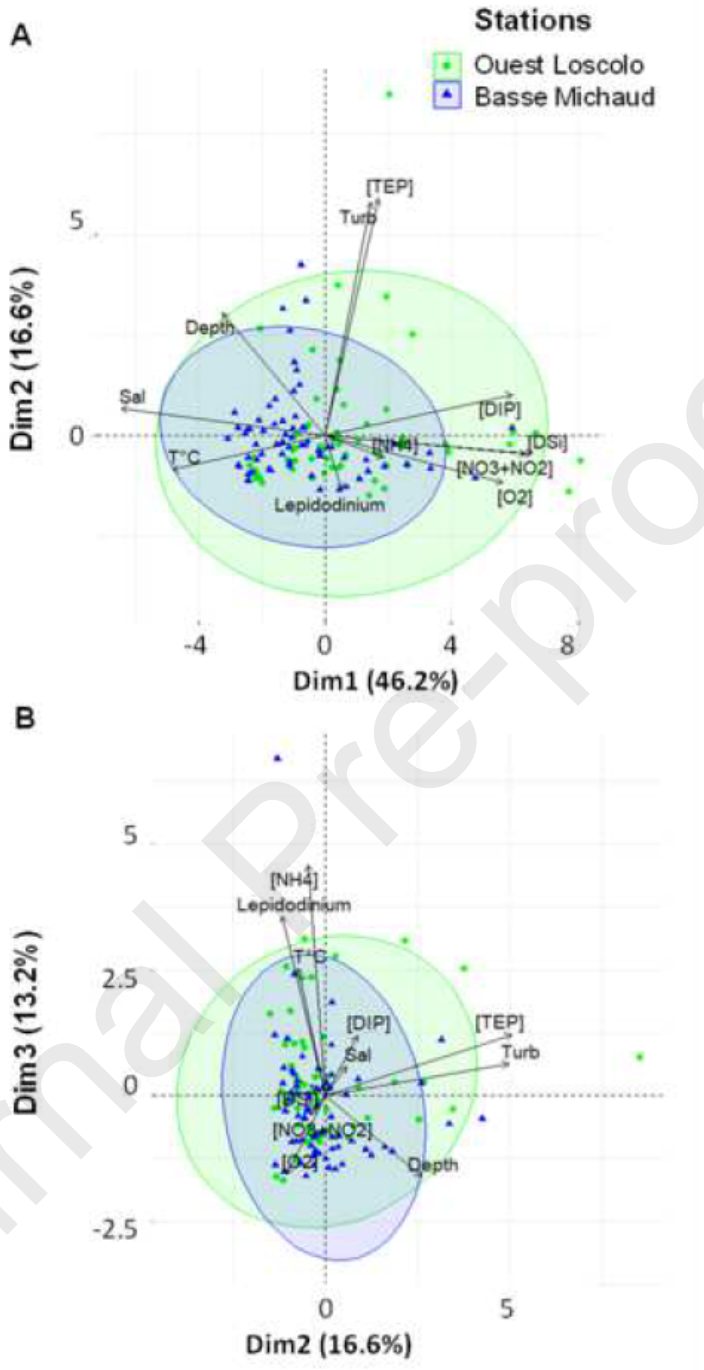

Fig6_Sediments