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Clélia Duran, Andréa Bouchard, Hélène Agogué, Christine Dupuy, Robert Duran, et al.. Importance of eukaryotes in shaping microbial benthic communities in Charente-maritime marshes, France. *Science of the Total Environment*, 2024, 957, 10.1016/j.scitotenv.2024.177523 . hal-04800847

HAL Id: hal-04800847

<https://hal.science/hal-04800847v1>

Submitted on 24 Nov 2024

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Importance of eukaryotes in shaping microbial benthic communities in Charente-maritime marshes, France

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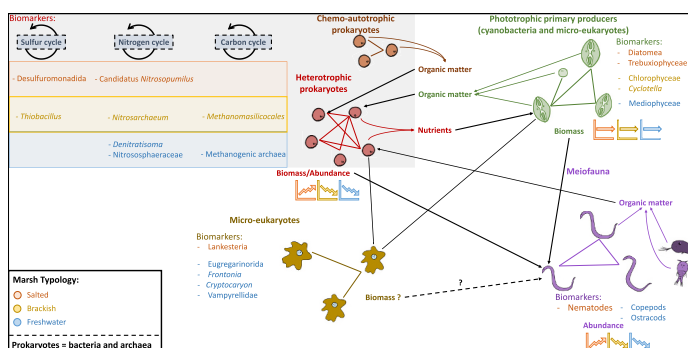
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HIGHLIGHTS

- Salinity is the main community composition driver in marshes
- Site-specific nutrient concentrations drive community composition after salinity
- Seasonal variations of benthic communities are site-specific
- Phototrophic eukaryotes are decisive in shaping benthic microbial communities
- Nitrogen cycle prokaryotes are biomarkers of salinity and seasonal variations

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Henner Hollert

Keywords:

Benthic biodiversity
Marshes sediment
Prokaryotes
Environmental DNA
Biomarker
Co-occurrence network

ABSTRACT

Marshes are wetlands known for providing major ecosystem services in terms of water quality and human activities. These ecosystem services are mainly provided by marshes' benthic community, composed of prokaryotes (bacteria and archaea) but also of eukaryotes (micro-eukaryotes and meiofauna). The aim of this study is to (1) assess the environmental parameters affecting benthic community composition in marshes, (2) highlight the associations between organisms from the three domains of life, and (3) determine the parameters controlling these associations. Hence, benthic communities of eight different marshes from three typologies (salted, brackish and freshwater) and four seasons (autumn 2020, spring 2021, summer 2021 and autumn 2021) were assessed. This study revealed three main drivers of community composition. First, salinity drives the community composition illustrated by the differences observed between the three typologies of marshes. Relative abundance of Nitrososphaeria, Halobacteria, Bacillariophyceae, Conoidasida and nematodes increased with salinity while methanogenic archaea, Chlorophyceae and copepod's relative abundance decreased. The second driver is the physical-chemistry of the site, particularly nutrients. The season is the last driver of community composition, seasonal pattern varying for each site within a typology. LefSe analyses defined biomarkers of typology and season, among which many prokaryotes involved in the nitrogen cycle and photosynthetic micro-eukaryotes where present in different co-occurrence networks, highlighting the importance of nitrogen cycle in marshes. Co-occurrence networks revealed several connections between organisms of the three domains of life,

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<https://doi.org/10.1016/j.scitotenv.2024.177523>

Received 19 April 2024; Received in revised form 2 October 2024; Accepted 10 November 2024

Available online 22 November 2024

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particularly between prokaryotes and photosynthetic eukaryotes. This study illustrates thus the importance of holistic approaches in microbial ecology for revealing a comprehensive view of the whole microbial interactions occurring in complex ecosystems.

1. Introduction

Marshes represent a type of wetland ecosystem, which is flooded most of the time, remains waterlogged and is characterised by herbaceous vegetation. It is possible to find marshes from the coastline (salt marshes, exposed by tides or not) to the inland (freshwater marshes) (Odum, 1984). They comprise a variety of habitats and environments that can shelter numerous species (Adam, 1990; Odum, 1984).

Due to their particular functioning and shelter habitats, marshes provide numerous ecosystem services such as shoreline stabilisation, water quality and carbon sequestration (Purcell et al., 2020). They are also the location of a wide range of human activities from different farming activities (e.g. aquaculture, breeding, crop farming) to cultural activities (e.g. social activities, tourism) (Mitsch et al., 2013). However, due to these human activities, climate change and their particular localisation between sea and land, marshes are a particularly endangered type of environment (Gedan et al., 2009). It is crucial to understand the functioning of marshes, as they play a pivotal role in biogeochemical cycles, in order to monitor them to increase their chances of preservation because they are threatened by human activities.

Benthic communities are of major importance in these environments, particularly due to their role in major biogeochemical cycles (Freckman et al., 1997; Snelgrove, 1997). These communities, composed of prokaryotes (archaea and bacteria) and eukaryotes (mainly fungi, protists and meiofauna), interact together with complex processes. These interactions can be direct (e.g. predation, competition, symbiosis) or indirect (e.g. bioturbation, metabolites exchange, physical-chemical changes) (Schratzberger and Ingels, 2018), therefore changes in one type of community likely affect the others. It is thus of paramount importance to analyse these communities together in order to better understand the different associations between these communities as well as the environmental parameters affecting them.

The different organisms composing these benthic communities are also affected by their environments' physical-chemistry. Indeed, physical-chemical parameters, specific to each type of marshes (e.g. salinity, sediment granulometry, pollution, human management of marshes) (Logares et al., 2009; Santmire and Leff, 2007; Yang et al., 2022), or linked with seasonal changes (e.g. temperature, precipitation rates, nutrient concentrations) (Ding et al., 2021), can greatly affect community composition and organisms' associations. The comparison of benthic communities from marshes of different typologies will provide crucial information to understand the factors shaping the microbial communities.

Freshwater marshes sediments are described to be mainly composed of silt and clay with high organic content and low to moderate root and peat content with low sulfur concentrations, whereas salt marshes sediments are described to contain more salt with lower organic content and higher peat and root content and sulfur concentrations (Odum, 1984). Furthermore, sedimentation rates and sediment erodibility are usually observed to be higher in freshwater marshes than in salt marshes. While it is known that marshes of different typologies shelter different plant species (Odum, 1984), too few studies comparing marshes of various salinities were conducted in regards of their benthic microbial and meiofaunal communities (Tortajada et al., 2011). Salted environments are generally known to harbour a large number of sulfate-reducing organisms (Kondo and Purdy, 2007), whereas freshwater environments usually represent more suitable habitats for a variety of methanogenic archaea (Pattnaik et al., 2000; Wen et al., 2017). Hence, similar distribution of the microbial functional groups is expected in

marshes but it still needs confirmation.

The objectives of this study are to (1) assess benthic communities and the environmental factors controlling their composition, (2) highlight possible organism associations between the three domains of life, and (3) understand how environmental factors will affect these associations. To this end, a holistic approach was adopted, characterizing the benthic communities (bacteria, archaea, micro-eukaryotes and meiofauna) from eight marshes encompassing three different typologies (salted, brackish and freshwater marshes) at four seasons (autumn 2020, spring 2021, summer 2021 and autumn 2021).

Based on the current knowledge of the distribution of microbial functional groups according to site typologies, it is hypothesized that salt marshes are characterised by the presence of sulfate-reducing microorganisms while methanogenic archaea and methanotrophic organisms are expected in freshwater marshes. Furthermore, it is expected to find correlations between organisms of the three domains of life given the wide range of potential interactions existing between them.

2. Material and methods

2.1. Sampling

Samples were collected from 8 sites in Charente-Maritime marshes, France, from November 27th 2020 to December 13th of 2021. The sites were sampled over 4 seasons (autumn 2020, spring 2021, summer 2021 and autumn 2021). The 8 sites were composed of 3 salted (St16, St52 and St61), 3 brackish (St13, St63 and St75) and 2 freshwater (StRAPH and St6) marshes (Fig. 1). At each site and season, 3 replicates of sediment cores were sampled. Among the studied sites, the three salted sites (St16, St52 and St61) occupied a central position in the hydric network, being submitted to common human activities (i.e. oyster farming). While St16 (located in the "Lilleau des Niges" natural reserve) was particularly located near a salt producing exploitation, St52 (located in the Perotine marsh) was close to a livestock farm and St61 (located in the Seudre marsh) was closer to an oyster farm than any other. Among the three brackish sites (St13, St63 and St75), only St75 (located in the Moëze marsh) occupies a central position, while St13 (located in the Esnandes Villedoux marsh) and St63 (located in the Saint Bonet marsh) represent marshes' outlets. The three sites are located near crop farms, St63 receiving often estuary water inputs, while St75 has previously received inputs of water with higher salinity than the marsh's salinity. Both freshwater sites (St6 and StRAPH) occupy central hydric positions. St6 is located in the Saint-Michel marsh and is submitted to crop farming activities. StRAPH is located in the Genouillé marsh being submitted to crop and livestock farming activities.

The top 5 cm of sediment was collected using round cores (Uwitec) then mixed and sub-sampled for the different analyses: sediment interstitial water's pH, RedOx potential, salinity, dissolved organic carbon (DOC) concentration, sediments' water content, chlorophyll a biomass, meiofauna and prokaryotes abundances, and environmental DNA of microbial and meiofaunal communities were analysed.

Rainfall rates, 15 and 30 days before sampling, were also collected from the Saint Médard d'Aunis (Charente-Maritime, France) meteorological observatory station archive (<https://www.meteo17aunis.fr/>).

2.2. Determination of physical-chemical parameters

In situ temperature of sediments was measured at the sediment surface with a temperature probe (ULM-500, Walz Effeltrich, Germany). Sediment water content was quantified by weighting 50 mL of sediment

before freezing at $-20\text{ }^{\circ}\text{C}$ and after 72 h of lyophilisation. Sediment grain size was measured using LASER diffraction on $1250\text{ }\mu\text{m}$ sieved sediments, using a Malvern MASTERSIZER S (Université de Bordeaux UMR5805, EPOC). Interstitial water was extracted from sediment with a centrifugation step at $2300\times g$ at $+4\text{ }^{\circ}\text{C}$ during 15 min. Sediment interstitial water's pH, RedOx potential, salinity and conductivity were measured using respectively a pH/RedOx probe (WTW SENTIX 940-3) and a conductivity probe (WTW Tetracon 926).

DOC concentration in sediment interstitial water was estimated according to the AFNOR NF EN 1484 norm (AFNOR, 1997). Nutrient concentrations ($\mu\text{mol/L}$) were determined in sediment interstitial water with an AXFLOW SEAL colorimetric auto-analyser (with a detection limit of $0.02\text{ }\mu\text{mol/L}$) following the methods described by Aminot and K  rouel (2007). Samples for Silicates (Si) analysis were conserved at $+4\text{ }^{\circ}\text{C}$, while samples for Nitrates (NO_3^-), Nitrites (NO_2^-), Phosphates (PO_4^{3-}) and Ammonium (NH_4^+) were conserved at $-20\text{ }^{\circ}\text{C}$ until analysis.

2.3. Biotic parameters analyses

2.3.1. Chlorophyll a

Chlorophyll a concentration was quantified in sediment as proxy for determining the biomass of oxygenic photosynthetic primary producers.

All samples were preserved at $-20\text{ }^{\circ}\text{C}$, lyophilised, grinded and homogenised before analysis. Chlorophyll a was extracted, from 50 mg of lyophilised sediment, overnight in the dark at $+4\text{ }^{\circ}\text{C}$ with 90 % acetone (mixed by repeatedly turning) then centrifuged for 10 min at $3500\times g$ at $+8\text{ }^{\circ}\text{C}$. Sample fluorescence was then measured with an excitation at 430–450 nm and emission at 650–675 nm using a fluorimeter (Turner TD 700, Turner Design, USA). Samples were afterwards acidified with HCl (3.3 mmol/L final concentration) and incubated in the dark at room temperature for 5 min. Fluorescence was once again measured at the same wavelength to quantify pheopigments. Chlorophyll a and pheopigments concentrations were expressed as $\mu\text{g/g}$ (dry weight of sediment) according to Lorenzen (1966). The ratio of chlorophyll a on all pigments (chlorophyll a and pheopigments) was used to determine the rate of active chlorophyll a.

2.3.2. Prokaryotes abundance

Sediments' prokaryotic abundance was measured with flow cytometry. Sediment subsamples were fixed with 0.2 μm filtered formaldehyde (v:v, final concentration 2 %), fast-frozen in liquid nitrogen and then preserved at $-80\text{ }^{\circ}\text{C}$ until analysis. Sub-samples were afterwards analysed according to Lavergne et al. (2014) with 3 consecutive extractions. All samples were analysed with a BD FacsCanto II flow cytometer (3

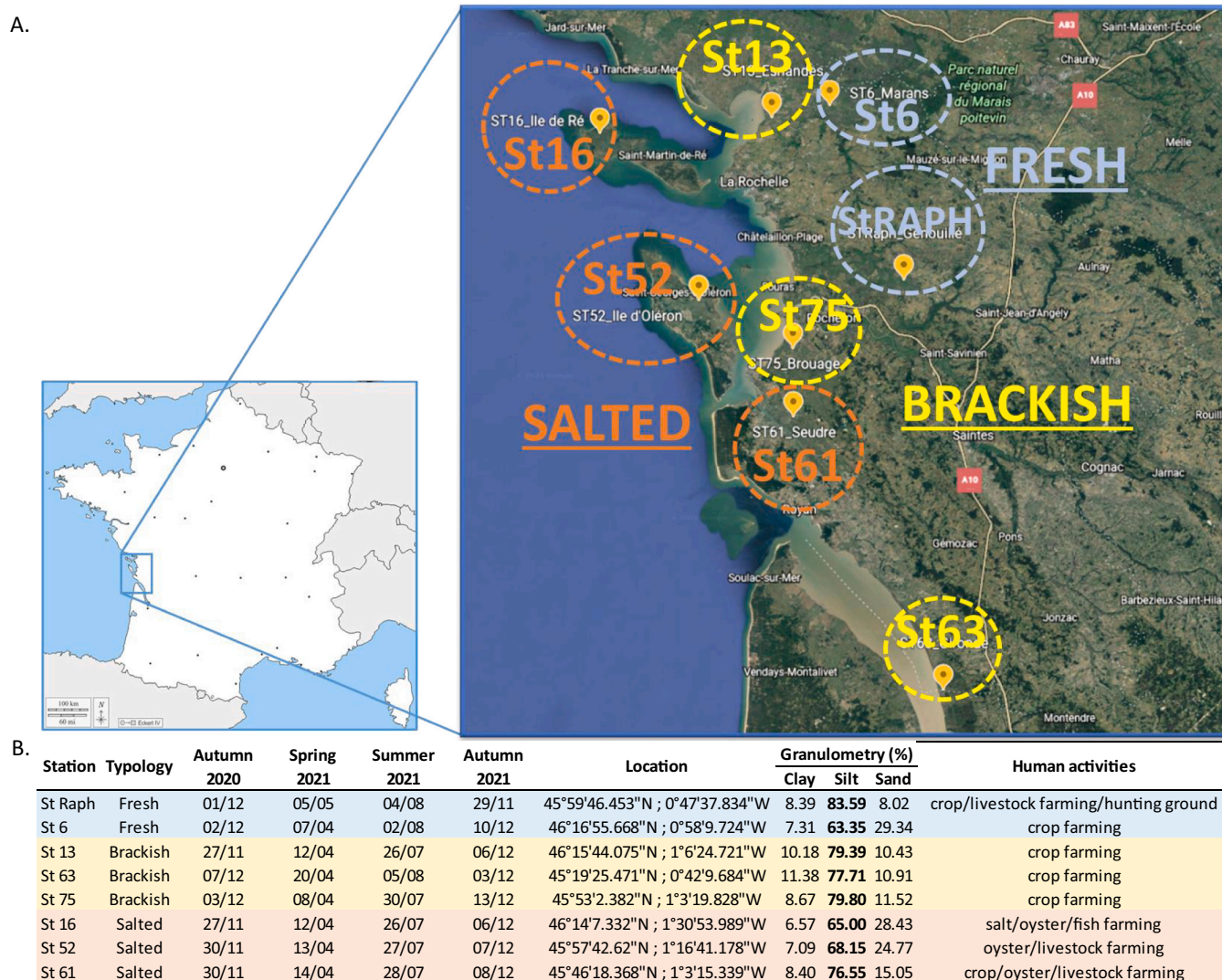


Fig. 1. Location (A) and general information (B) of the 8 sampling sites. Name, coordinate and typology of the sites, and the sampling dates are indicated. The granulometry (percentage of grain size) and the human activities practiced near the sites are also indicated. Freshwater (StRAPH and St6), brackish (St13, St63 and St75) and salted (St16, St52 and St61) sites are highlighted with different colours.

LASERs, 8 colours (4–2–2), BD Biosciences) equipped with a 20 nW LASER and a 488 nm excitation. Data were acquired and processed with DIVA 6 and DIVA 9 software in order to obtain prokaryote abundances, expressed as $\times 10^6$ cells/mL of wet sediment.

2.3.3. Meiofauna abundance

Subsample of 60 mL of sediment for meiofauna abundance counts were conserved with 3 % formaldehyde at + 4 °C until analysis. Samples were then sieved with a 45 μ m sieve before staining with rose Bengal. Samples with a high amount of big sandy particles or pebbles went through an elutriating step. If necessary, samples with high meiofaunal abundance or high vegetal detritus concentration were diluted before meiofaunal organisms' enumeration with a magnifying glass ($\times 20$ or $\times 40$). Meiofaunal abundances were expressed as individuals (ind)/mL of wet sediment.

2.3.4. Environmental DNA extraction and sequencing

For environmental DNA analyses, subsamples of sediments were collected and immediately conserved at –20 °C until DNA extraction. Micro-organisms' DNA was extracted directly from 0.25 g of wet sediments. For meiofaunal organisms, a sieving step with sterilised tools was added. Briefly, 10 g of sediments were sieved with a 45 μ m sieve. If necessary (presence of small stones or big sandy particles), an elutriation step was added after sieving in order to further separate meiofauna from the matrix. DNA extraction for meiofauna was carried out with 0.25 g of sieving reflux. Both DNA extraction were conducted using the DNeasy PowerSoil Pro kit (Qiagen) with a modified protocol. 600 μ L of the DNeasy Power soil Pro kit CD1 solution was added. Samples went through a three-times repeated heat-shock phase alternating 30 s in liquid nitrogen with 2 min at 60 °C. 200 μ L of 24:25:1 phenol:chloroform:isoamyl alcohol was then added to replace the 200 μ L of CD1 solution retrieved, as conducted by Melayah et al. (2023). Samples were afterwards submitted to a bead-beating protocol consisting of two phases of 45 s at 6000 rpm interspersed with a 30 s break (Precellys® Evolution, Bertin technologies, France). Then, the Qiagen protocol was followed until the end. The two extractions for a same replicate were mixed (v:v) at the end of extraction before sequencing. DNA was quantified using a Qubit fluorometer with a Qubit dsDNA HS quantification kit.

Samples were afterwards amplified and paired-end sequenced (2 \times 250 bp) by Genoscope (CEA, Evry, FRANCE) using Illumina Miseq next-generation sequencing (NGS) methods. Sequencing targeted V4-V5 region of Bacterial 16S rRNA gene using the primers 515F–Y (5'-GTGY-CAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') (Parada et al., 2016), V4-V5 region of Archaeal 16S rRNA gene using primers 519F (5'-CAGCCGCGCGGTAA-3') and 915R (5'-GTGCTCCCGCCAATTCCT-3') (Herfort et al., 2009) and Eukaryotic V4 region of 18S rRNA gene with the primer set TAREuk454FWD1 (5'-CCAGCASCYCGGTAATTCC-3') and TAREukREV3 (5'-ACTTTCGTTCTTGATYRA-3') (Stoeck et al., 2010).

2.3.5. Bioinformatic analyses

Qiime 2 v2021–11 (Bolyen et al., 2019) was afterwards used to process all sample's cleaned sequences provided by Genoscope. Pre-processed sequence data were denoised using q2-DADA2 (Callahan et al., 2016) and singletons were removed from the Amplicon Sequence Variants (ASVs) obtained. Taxonomic assignment was conducted on the remaining ASVs using q2-feature-classifier (Bokulich et al., 2018) and the ribosomal RNA small subunit gene database SILVA v138–99 NR (Quast et al., 2013) for the three tags (bacteria, archaea and eukaryotes). ASVs whose taxonomic assignment was unrelated to the sequenced tag were removed from ASV and taxonomy tables as well as representative sequences. For the bacterial 16S primer tag, all archaeal, eukaryotic, mitochondrial, chloroplastic and unassigned ASVs were removed. For the archaeal 16S primer tag, unassigned ASVs and ASVs assigned to bacteria or eukaryotes were removed. For the eukaryotic 18S primer tag,

ASVs that were assigned to bacteria, archaea or unassigned were removed.

Raw sequence data were deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) and is available under the assigned Bioproject PRJNA1091597 (Duran et al., 2023).

2.4. Statistical analyses

Following statistical analyses were conducted using R v4.2.3 (R Core Team, 2023) in RStudio v2023.03.0.386 (Posit team, 2023).

All sequencing data were imported in R environment using the phyloseq R package v1.44.0 (McMurdie and Holmes, 2013). Alpha diversity indexes (observed ASVs, Shannon and inverse Simpson) were calculated and the effect of typology and season on these indexes was tested using linear mixed effects models (LMM) with the lme function in the nlme R package v3.1–162 (Pinheiro and Bates, 2000). Typology and seasons were used as fixed effects while sites were used as random effect. The effect of site and season on environmental variables (both biotic and abiotic) were tested with Kruskal-Wallis tests from the stats R package v4.2.3 (R Core Team, 2023).

For all further analyses, taxonomy and ASV tables of eukaryotes were separated in micro-eukaryotes and metazoan community tables. Relative abundance variation depending on season and site for the most abundant classes from the 4 different communities was tested using Kruskal-Wallis tests from the stats R package v4.2.3 (R Core Team, 2023) followed by a Dunn test (from the dunn.test R package v1.3.5; Dinno, 2017) when positive. The effect of site and season on benthic communities was also directly tested using PERMutational Analyses Of Variance (PERMANOVA) with the vegan R package v2.6–4 (Oksanen et al., 2022).

Two types of canonical analyses were conducted using the vegan R package v2.6–4 (Oksanen et al., 2022). Redundancy Analyses (RDA) were conducted on environmental parameters constraining the biotic parameters to the abiotic parameters, for all the samples, and each typology separated. For sequencing data, Canonical Analyses of Principal Coordinates (CAP) (Anderson and Willis, 2003) based on Bray-Curtis dissimilarity matrix were conducted on Hellinger-transformed ASV tables and constrained against centered-reduced environmental parameters (both biotic and abiotic). For both type of canonical analyses, the significance of the general model, axes and constraining variables were tested with PERMANOVA from the vegan R package v2.6–4 (Oksanen et al., 2022). Prior to RDA and CAP analyses, Spearman correlation of environmental variables were assessed using the stats R package v4.2.3 (R Core Team, 2023) in order to select variables that are correlating with each other and avoid collinearity. Selected variables for each typology and the correlations between the variables are available in supplementary data (Fig. S1). Furthermore, the dispersion of all benthic communities and environmental biotic parameters depending on typology and season were measured using “betadisper” and permutation test of multivariate homogeneity of groups dispersions (permutest) from the vegan R package v2.6–4 (Oksanen et al., 2022).

LEfSe (Linear Discriminant Analysis (LDA) Effect Size) were conducted on benthic communities in order to highlight ASVs significantly more abundant in a typology or season using the microbial R package v0.0.20 (Guo and Gao, 2021). Differences in relative abundance depending on typology or seasons were tested using Kruskal-Wallis tests (stats R package v4.2.3; R Core Team, 2023) followed by dunn tests (dunn.test R package v1.3.5; Dinno, 2017) for the three biomarkers with higher LDA scores of each community.

ASV tables of bacteria, archaea and eukaryotes were merged in a single ASVs table of all communities. Co-occurrence networks were constructed using the cytoscape v3.9.1 software (Shannon et al., 2003) with the CoNet v1.1.1 package (Faust and Raes, 2016). Five co-occurrence networks were constructed considering ASVs present in (i) salted, (ii) brackish or (iii) freshwater typologies, (iv) all the typologies

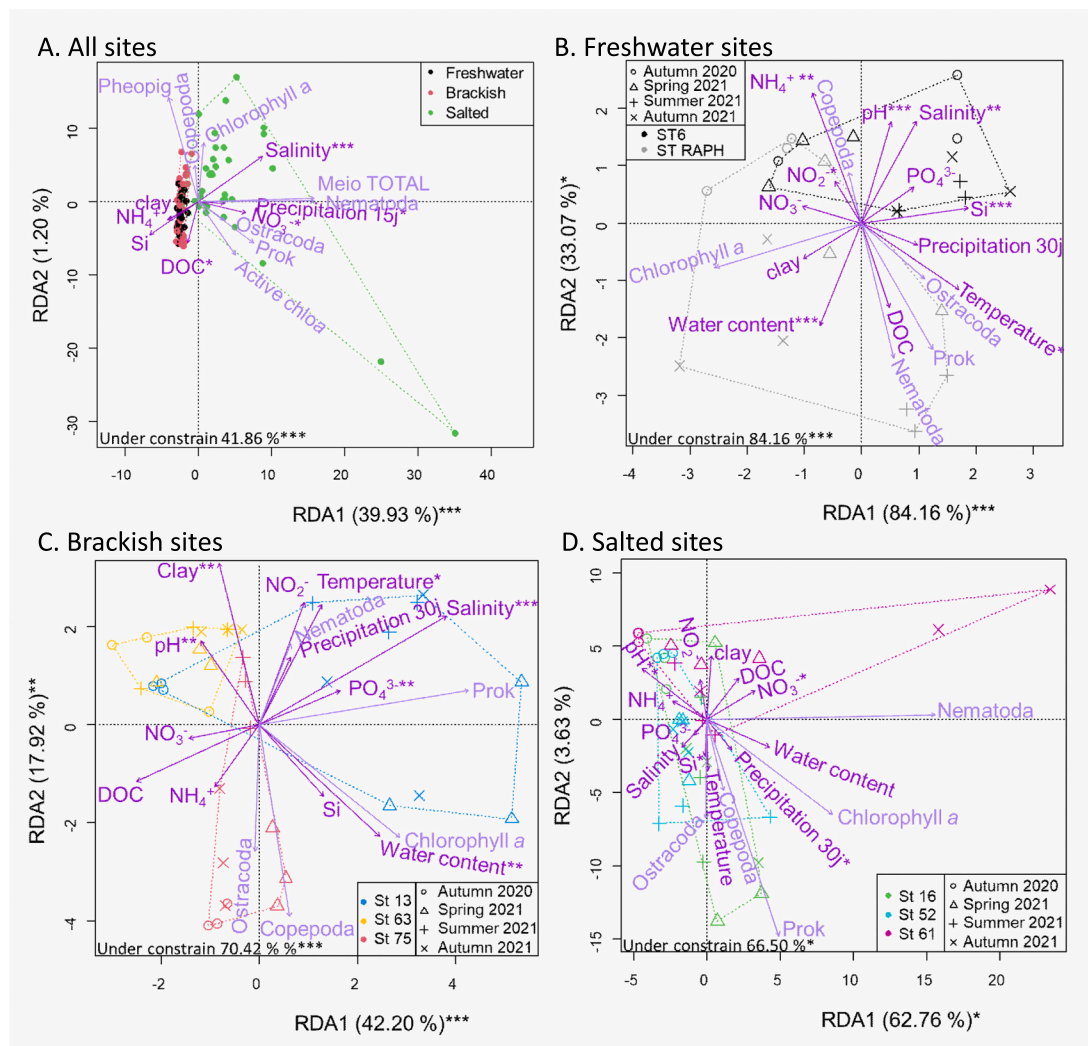


Fig. 2. Redundancy analyses (RDA) on environmental parameters, constraining the biotic parameters under abiotic factors, for all sites (A), and according to the freshwater (StRAPH and St6) (B), brackish (St13, St63 and St75) (C) and salted (St16, St52 and St61) (D) typologies. For the RDA including all sites (A), the colour indicates the typology whilst for the others RDA (B, C, D) colour represents the site and shape represents the season. Statistically significant values were tested for the total model, the axes and the abiotic variables with ANOVA (***) < 0.001 ; ** < 0.01 ; * < 0.05). COD represents dissolved organic carbon.

abundances and lower nematode abundances (Dunn test, $p < 0.05$). The brackish sites showed low seasonal variation (Fig. S4A) although autumn 2020 diverged from the other seasons showing higher ammonium concentration and lower prokaryote abundances (Dunn test, $p < 0.01$).

Unlike brackish and freshwater marshes, the three salted sites shared similar physical-chemical characteristics as well as morphological eukaryotic community composition (Table 1, Fig. 2D) although site 61 (St61) differed slightly from the other sites showing lower prokaryote and ostracod abundances (Table 1) (Dunn test, $p < 0.05$). The salted sites exhibited slight seasonal variation (Fig. S5A) as illustrated by the divergence of autumn 2020, which was characterised by high ammonium concentration and low prokaryotes abundance (Dunn test, $p < 0.01$) (Fig. 2D). In addition, no significant differences in dispersion according to site or season were observed for salted sites. (Table S1, betadisper permutest, $p > 0.05$).

3.2. Benthic DNA community composition

The composition of archaeal, bacterial, and eukaryotic communities, assessed by 16S and 18S rRNA genes sequencing, revealed 75,532 archaeal ASVs, 248,454 bacterial ASVs and 49,611 eukaryotic ASVs.

Linear mixed effects models showed significant differences in alpha diversity (Table S2) depending on season for all archaeal indexed, the observed index of eukaryotes as well as observed and inverse Simpson indexes of bacteria (lme, $p < 0.05$). Significant differences depending on typology were observed for eukaryotes' Shannon index only (lme, $p < 0.05$).

For the archaeal community (Fig. 3A), classes with the highest relative abundances (Nanoarchaea, Bathyarchaea) were similar in the different typologies (Kruskal-Wallis test, $p > 0.05$) but varied significantly depending on seasons (Kruskal-Wallis test, $p < 0.05$). Methanogenic classes, such as Methanomicrobia, showed significantly lower relative abundances in salted sites while Halobacteria had higher relative abundance in salt marshes (Dunn test, $p < 0.001$), both being similar irrespective of the season (Kruskal-Wallis test, $p > 0.05$).

For the bacterial community (Fig. 3B), the most abundant taxa at the class level varied significantly depending on typology. Indeed, Alphaproteobacteria and Planctomycetes' relative abundances were significantly higher (Dunn test, $p < 0.05$) in salted sites. The relative abundances of Anaerolineae and Bacteroidia varied significantly depending on the season (Kruskal-Wallis test, $p < 0.05$) whilst the relative abundance of other highly abundant taxa, such as Actinobacteria and Gammaproteobacteria, was similar irrespective of the

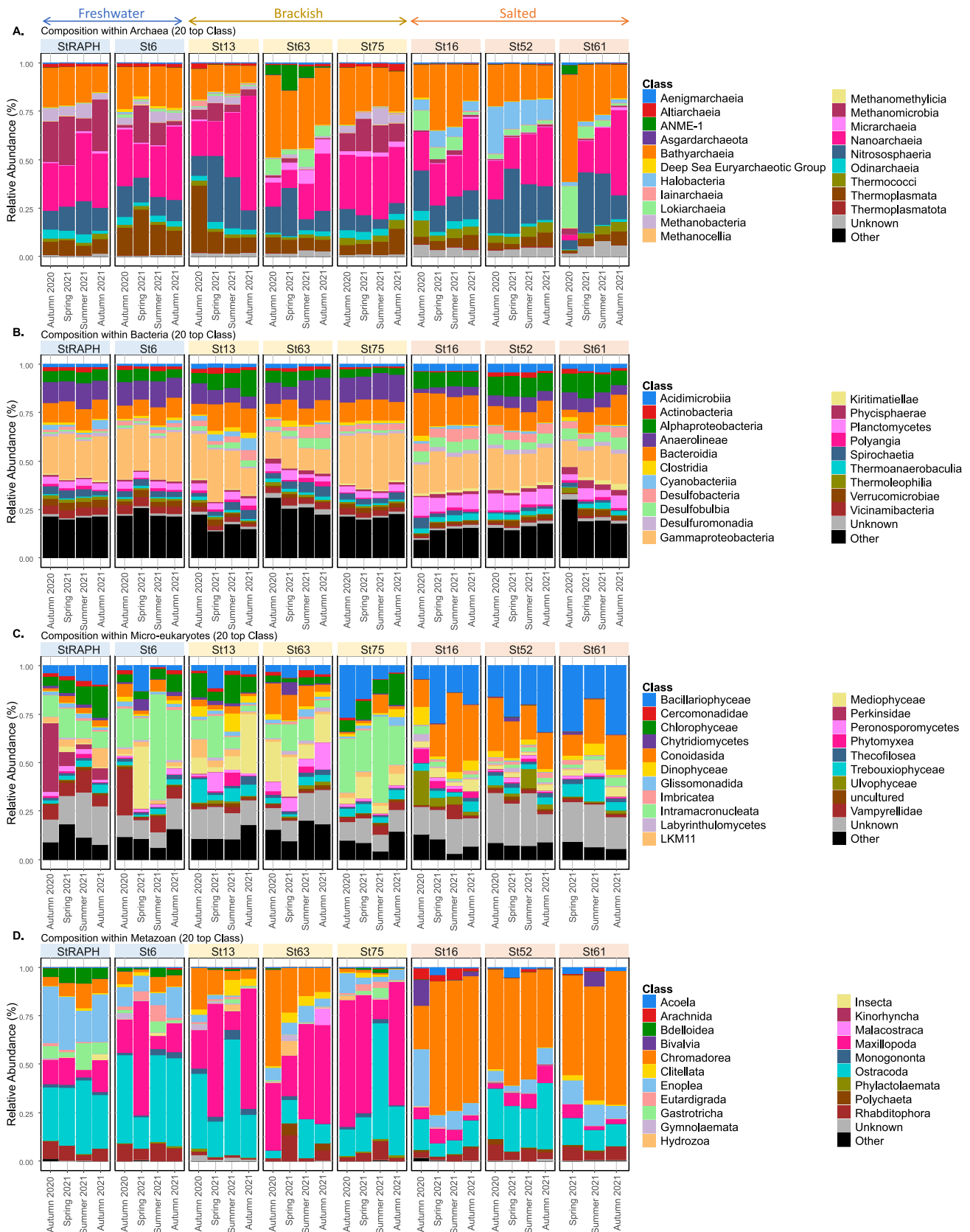


Fig. 3. Community composition at the class level of all the sites at each sampled season for archaeal (A), bacterial (B), micro-eukaryote (C), and metazoan (D) communities. The top 20 more abundant classes are represented for each community. Freshwater sites are StRaph and St6, brackish sites are St13, St63 and St75 and salted sites are St16, St52 and St61. Taxonomic assignment was conducted using the SILVA database v138–99NR for all the communities.

season (Kruskal-Wallis test, $p > 0.05$).

For eukaryotic community, the composition of the most abundant classes also varied according to typology. For micro-eukaryotes (Fig. 3C), the salted typology was characterised by higher relative abundance (Dunn test, $p < 0.001$) of Bacillariophyceae and Conoidasida, while the other typologies exhibited higher relative abundance (Dunn test, $p < 0.001$) of Intramacronucleata and Chlorophyceae, Mediophyceae dominating in brackish typology. Furthermore, Mediophyceae and Bacillariophyceae's relative abundances changed significantly (Kruskal-Wallis test, $p < 0.05$) with seasons. For metazoans, Chromadorea (Nematode) dominated the salted typology whilst brackish and freshwater typologies were dominated by Maxillopoda (Class grouping copepods and barnacles) (Dunn test, $p < 0.01$).

3.3. Effect of environmental parameters on benthic DNA community composition

The differences of benthic community assemblages according to typology and season were determined by Venn diagram analyses. The three typologies shared 112 (0.15 %) archaeal ASVs, 904 (0.04 %) bacterial ASVs, and 694 (1.40 %) eukaryotic ASVs (Fig. S6A to S6C). These ASVs correspond to the core community present in all the typologies irrespective of the season. The four seasons represented 92.9 %, 79.6 % and 71.5 % of the archaeal, bacterial and eukaryotic ASVs respectively (Fig. S6D to S6F), suggesting that the core communities are weakly affected by seasonal variations.

Canonical Analysis of Principal coordinate (CAP) and PERMutational Analyses Of VAriance (PERMANOVA) indicated the environmental parameters explaining the variation of benthic community composition (Fig. 4). For the freshwater typology, the four communities (archaea, bacteria, micro-eukaryotes and meiofauna) were clearly separated according to site (Fig. 4A, 4D, 4G and 4J; Table S3; PERMANOVA, $p < 0.001$), being affected by season (Table S3; PERMANOVA, $p < 0.01$) and by the combination of both site and season (Table S3; PERMANOVA, $p < 0.05$). Significant differences in dispersion depending on season were observed for bacterial and metazoan communities (Table S1, betadisper permutest; $p < 0.05$) whilst no significant differences were observed depending on site. However, the CAP for bacteria and metazoan communities (Fig. S3C and S3E, respectively) suggested that the differences detected by PERMANOVA were significant. All communities showed differences in their compositions (PERMANOVA, $p < 0.001$) depending on salinity (which correlated positively with phosphate, ammonium, nitrites, pH, sand and negatively with RedOx and silt in freshwater sites; Fig. S1A). They also varied significantly (PERMANOVA, $p < 0.001$) along with copepods' abundance, which were more abundant at St6 in spring 2021 (Fig. 4A, 4D, 4G and 4 J). In contrast, the composition of bacterial, micro-eukaryote and metazoan communities varied significantly (PERMANOVA, $p < 0.05$) with active chlorophyll *a* (correlated with chlorophyll *a* biomass in freshwater sites; Fig. S1A). The composition of micro-eukaryote and metazoan communities varied with sediment temperature and ostracods abundance (Fig. and 4J; PERMANOVA, $p < 0.05$), while that of archaeal and bacterial communities differed significantly with sediment granulometry (clay content). The composition of bacterial and micro-eukaryote communities also varied with pheopigment biomass and rainfalls. Furthermore, the composition of bacterial community was significantly different according to the nematode abundance (Fig. 4D; PERMANOVA, $p < 0.05$), while the composition of the micro-eukaryote community depended on DOC concentrations (Fig. 4G; PERMANOVA, $p < 0.05$). Finally, in the freshwater typology, the four communities varied according to season (Fig. 3B, S3C, S3D and S3E). In contrast, in the brackish typology the four communities exhibited a clear separation according to site (St13, St75 et St63) (Fig. 4B, 4E, 4H and 4 K; Table S3; PERMANOVA, $p < 0.001$), with slight seasonal variations (Fig. S4B to S4E; Table S3; PERMANOVA, $p < 0.05$). Dispersion of bacterial communities varied significantly depending on season whilst the dispersion of archaeal,

micro-eukaryotic and metazoan communities varied significantly depending on site (Table S1, betadisper permutest; $p < 0.05$). However, despite such dispersions, CAPs for bacterial, archaeal, micro-eukaryotic and metazoan communities (Fig. S4C and Fig. 4B, H and K, respectively) showed clearly the differences between sites. Noteworthy, the most important differences of benthic communities were observed in the salted typology sites (St16, St52 and St61) (Fig. 4C, 4F, 4I and 4 L), exhibiting significant variations according to site (Table S3; PERMANOVA, $p < 0.001$), season (Table S3; PERMANOVA, $p < 0.001$), and their combination (Table S3; PERMANOVA, $p < 0.001$). Particularly, CAP analyses separated the benthic communities of the salted typology along axis 1 (explaining between 14 and 22 % of the variation) showing the seasonal variations of benthic community composition, although some similarities were also observed (Fig. S5B, 54C, 55D and 55E). Dispersion of metazoan communities was significantly different depending on season whilst the dispersions of archaeal and micro-eukaryotic communities varied significantly according to site (Table S1, betadisper permutest; $p < 0.05$). Despite such dispersion differences, CAPs for the metazoan, archaeal, and micro-eukaryotic communities (Fig. S5E and Fig. 5C and J, respectively) indicated difference between season and sites.

3.4. Community co-occurrence networks

The relationship between organisms was assessed using co-occurrence networks (Fig. 5). The network including all sites was the less diversified, mostly composed of bacteria (71.6 %) (Fig. 5A). The 711 nodes of this network formed 101 modules, with 33 containing at least 5 nodes (Fig. 5A). Groups of modules (i.e. modules that are linked by co-presence) were defined allowing to distinguish two major groups, one comprising the first 12 modules with keystone ASVs (i.e. defined central ASVs by ModuLand) correlating negatively with salinity ($\rho > |0.5|$) (Fig. 6A). These keystone ASVs were mainly affiliated to bacteria (distributed in 5 different phyla, Table S4); only one keystone was affiliated to archaea (Table S4) in this group of modules. The other group, comprising modules 13 to 19, correlated positively ($\rho > |0.5|$) with salinity and other associated parameters, such as prokaryotes and meiofaunal abundances. In this network, very few links were observed between prokaryotes and eukaryotes.

The network constructed with the core community was the most diversified, comprising 52.7 % of bacteria and 40.8 % of eukaryotes (Fig. 5B). This network also contained higher heterotrophic protists (119 nodes, 16.4 %), photosynthetic eukaryotes (77 nodes, 10.6 %), and fungal (53 nodes, 7.3 %) ASVs than archaeal ASVs (47 nodes, 6.5 %). It included 725 nodes grouped in 114 modules with 57 of them containing at least 5 nodes (Fig. 5B). Noteworthy, at least one ASV related to photosynthetic organism was present in most of the modules or group of modules. The major group of modules (module 1 to 24), was mainly composed of photosynthetic organisms (eukaryotes and a few cyanobacteria, Table S5) as key ASVs, while heterotrophic organisms related ASVs were spread around. This network exhibited more correlations between the different domains of life than the other networks. Furthermore, keystone ASVs (Table S5) that were correlated with environmental parameters linked to the typologies, such as salinity, still corresponded to 17 out of 57 modules composed of various organisms ($\rho > |0.5|$) (Fig. 6E).

The network of brackish typology contained the most archaeal ASVs (244 nodes, 27.4 %) while metazoans were higher in the network of salted typology (61 nodes, 13.3 %) (Fig. 5F), which also exhibited the lowest number of fungal (1 node, 0.2 %), protists (9 nodes, 2.0 %) and bacterial (236 nodes, 51.5 %) ASVs.

The network of the salted typology contained 458 nodes grouped in 95 modules from which 30 contained at least 5 nodes (Fig. 5C), composed by keystones ASVs mainly affiliated to bacteria (Table S6). Among the 22 groups of modules (i.e. modules linked together), 10 contained at least one photosynthetic organism, especially the groups of

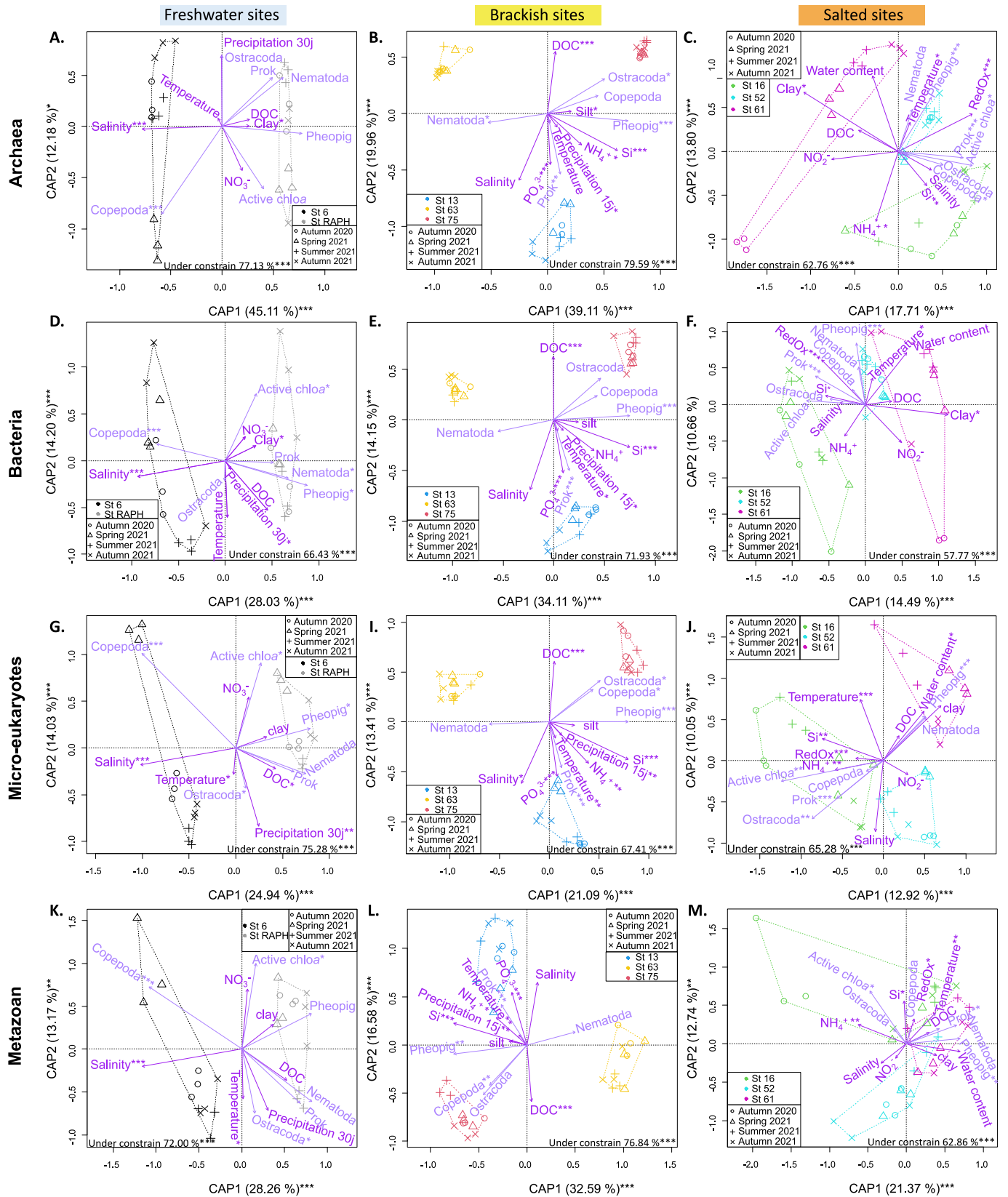


Fig. 4. Comparison of microbial communities. The Canonical Analysis of Principal coordinate (CAP) of the archaeal (A, B, C), bacterial (D, E, F), micro-eukaryote (G, H, I) and metazoan (J, K, L) communities for freshwater (A, D, G, J), brackish (B, E, H, K) and salted (C, F, I, L) typologies are shown. Colour represents the site, and shapes represent seasons. Statistical significances were tested for the total model, the axes and different environmental parameters with ANOVA (*** < 0.001, ** < 0.01, * < 0.05). DOC represents dissolved organic carbon; Pheopig, pheopigment biomass; Active chloa, active chlorophyll a.

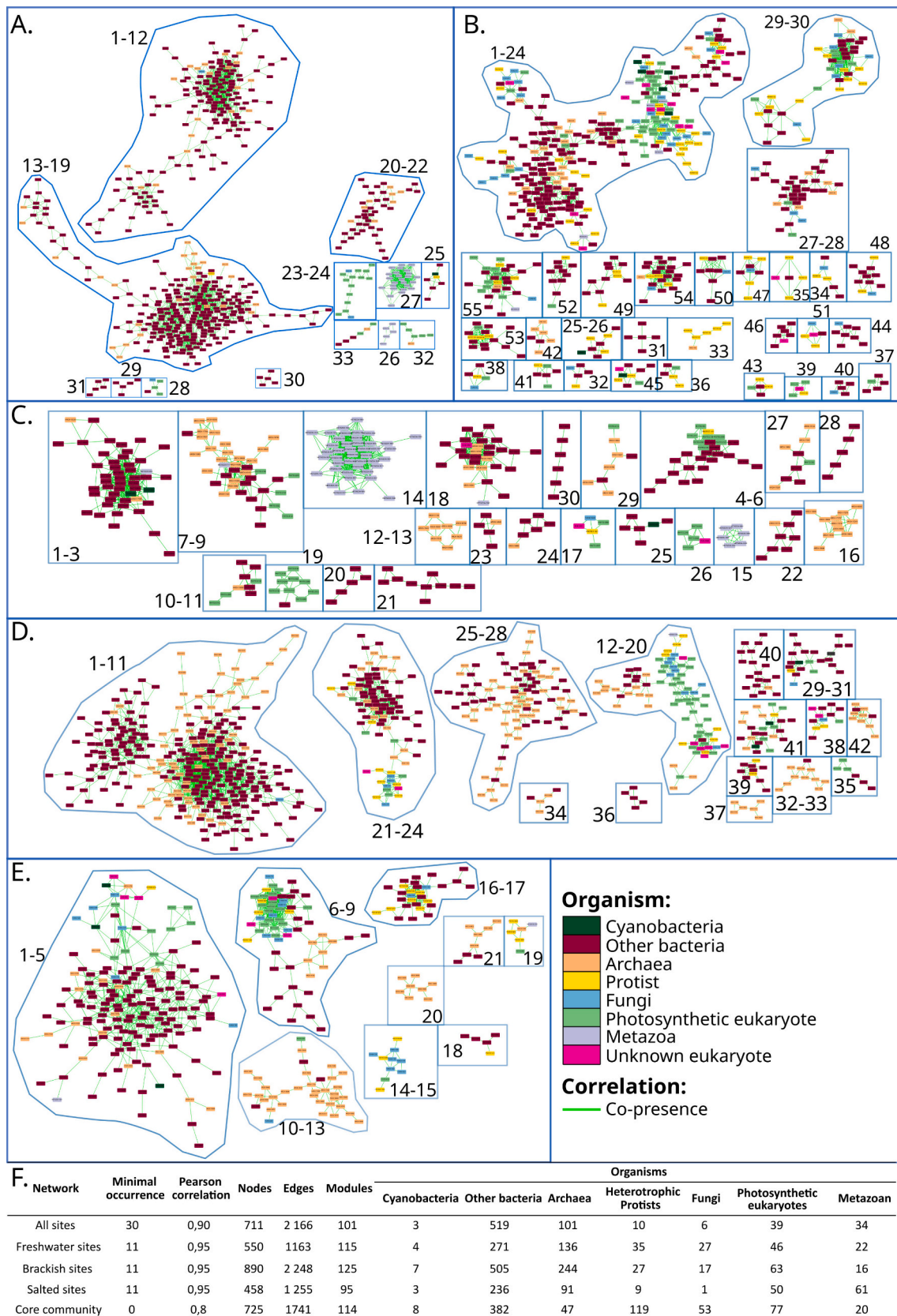


Fig. 5. Network analyses combining the three domains of life separated according to colour in cyanobacteria, other bacteria, archaea, heterotrophic protists, fungi, photosynthetic eukaryotes, metazoans and unknown eukaryotes. Networks were constructed, using co-occurrence only, for all the samples (A), the typology core community (B), salted sites (C), brackish sites (D) and freshwater sites (E) along with the general information on the different networks (F). Only modules with at least 5 nodes are represented.

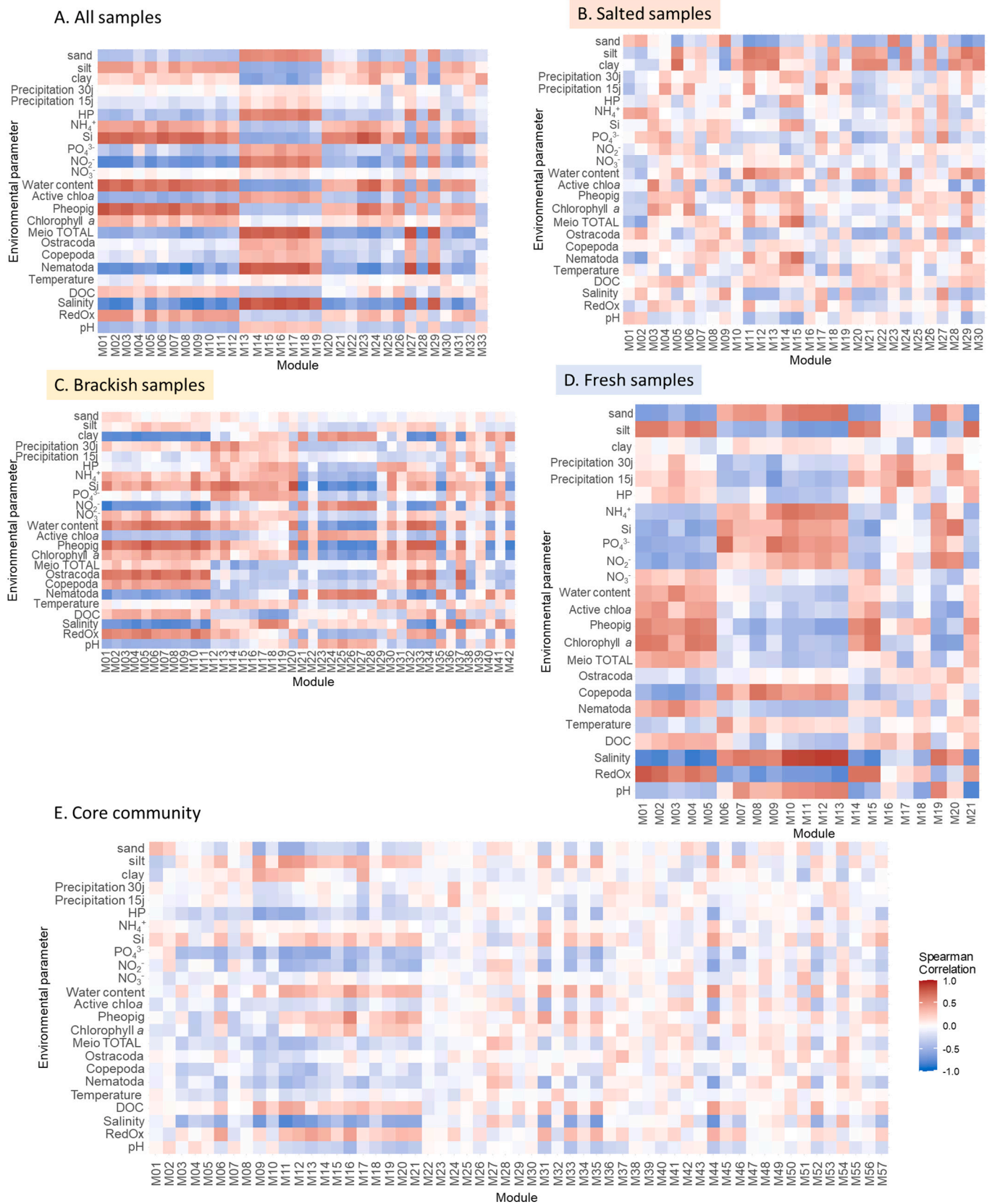


Fig. 6. Heatmap of spearman correlation between the different module keystone ASVs and environmental parameters, for the all sites network (A), salted sites network (B), brackish sites network (C), freshwater sites network (D) and the core community network (E). Analyses were conducted for modules with at least 5 nodes. DOC represents dissolved organic carbon; Pheopig, pheopigment biomass; Active chloa, active chlorophyll *a*.

modules 1 to 3, 4 to 6 and 7 to 9, which were among the five most important module groups. The modules were weakly correlated with environmental parameters, except modules 14 and 15 that were correlated with grain size, total meiofaunal and nematode abundances, module 14 being also correlated with prokaryote abundance ($\rho > |0.5|$) (Fig. 6B).

The network of the brackish typology was composed of 890 nodes, 125 modules among which 42 contained at least 5 nodes (5D). The network included 15 groups of linked modules, among which the group gathering modules 1 to 11 was mainly composed of bacteria and archaea (Table S7). Noteworthy, the keystone ASVs of these modules were

strongly correlated with environmental parameters characteristic of St75 (i.e. low nematode abundance, high copepods and ostracods abundance; $\rho > |0.5|$) (Fig. 6C). The group of modules 12 to 20 was composed of modules dominated by photosynthetic eukaryotes linked to modules dominated by prokaryotes (Table S7). The keystone ASVs of these modules were strongly correlated with environmental parameters characteristic of St13 (i.e. DOC, phosphate, salinity; $\rho > |0.5|$) (Fig. 6C). The group of modules 21 to 24 was also composed by modules dominated by eukaryotes (mainly heterotrophic protists, photosynthetic eukaryotes and fungi) linked to modules dominated by prokaryotes but containing also heterotrophic protists and photosynthetic eukaryotes

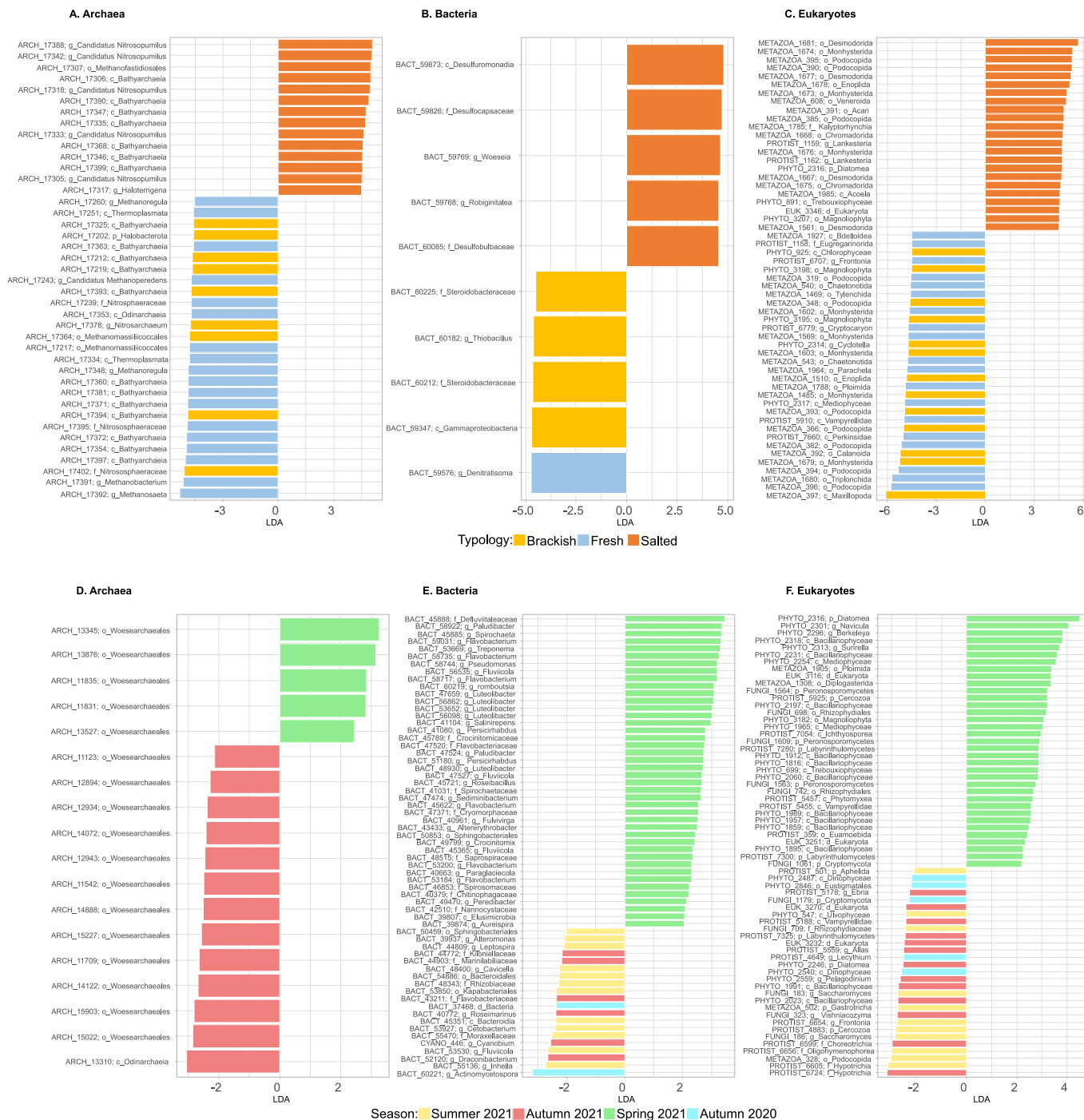


Fig. 7. Linear discriminant analysis effect size (LEfSe) on Archaeal (A,D), Bacterial (B,E) and Eukaryotic (C,F) communities highlighting ASVs significantly more abundant depending on the site topology (A, B, C) (Salted, Brackish and Freshwater sites), with an LDA score ≥ 4.5 and p -value < 0.001 ; and depending on the season (D, E, F) (Autumn 2020, Spring 2021, Summer 2021 and Autumn 2021), with an LDA score ≥ 2 and p -value < 0.001 .

(Table S7). These modules as well as the group of modules 25 to 28 (composed of prokaryotes only) strongly correlated with environmental variables characteristic of St63 (i.e. high nitrite concentration, low chlorophyll *a* biomass, water content, ammonium and silicate concentrations; $\rho > |0.5|$) (Fig. 6C).

Finally, the network of freshwater typology was composed of 550 nodes, 115 modules with 21 modules containing at least 5 nodes (Fig. 5E) and characterised by the presence of keystone ASVs almost evenly shared between bacteria, archaea and eukaryotes (Table S8). Two major groups of modules were observed in this network, a group containing modules 1 to 5 and another group formed by modules 6 to 9. Among the nine modules, five modules, dispatched within both groups, contained at least one photosynthetic organism. Noteworthy, 6 modules included connections between eukaryotes and prokaryotes. The keystone ASVs of modules 16, 17 and 20 were correlated with rainfall rates ($\rho > |0.5|$, Fig. 6D). The other keystone ASVs correlated with environmental parameters characteristic of a freshwater site, such as the modules 1–5, 14, 15, 18 and 21 that correlated with parameters characteristic of StRAPH (e.g. high chlorophyll *a* biomass, and low salinity, silicate and nitrite concentrations), whilst the modules 6 to 13 and 19 with parameters characterizing St6 (e.g. higher salinity, lower ammonium concentrations) ($\rho > |0.5|$).

3.5. Potential biomarkers of typology and season

Linear discriminant analysis effect size (LEfSe) highlighted ASVs significantly more abundant in a typology (Fig. 7A to 7C) or a season (Fig. 7D to 7F) that can be considered as biomarkers as previously suggested by Segata et al. (2011).

The salted typology was characterised by 42 biomarkers at the ASV level, which included 6 archaeal (eight *Candidatus Nitrosopumilus* and one *Haloterigena*), 2 bacterial (*Woeseia*, *Robiginitalea*) and 2 eukaryotic (two *Lankesteria*) ASVs that were identified at the genus level. Salted biomarkers were present in all salted sites and could be observed in brackish sites (Fig. S7A to S7C) but had a significantly higher relative abundance in salted sites (Dunn test, $p < 0.05$). For the brackish typology, 26 ASVs were identified as biomarkers, one of each domains being identified at the genus level (i.e. *Nitrosarchaeum*, *Thiobacillus* and *Cyclotella*). Brackish biomarkers could be observed in all brackish sites but also in salted and freshwater sites (Fig. S7D to S7F) while having a significantly more important relative abundance in brackish sites (Dunn test, $p < 0.05$). For the freshwater typology, 39 ASVs were identified, which included 5 archaea (two *Methanoregula*, *Candidatus Methanoperedens*, *Methanobacterium* and *Methanoseta*), 1 bacterium (*Denitratisoma*) and 2 eukaryotes (*Frontonia* and *Cryptocaryon*) identified at the genus level. Freshwater biomarkers were present in all freshwater sites and in some brackish sites (Fig. S7G to S7I) but their relative abundance was significantly higher in freshwater sites (Dunn test, $p < 0.05$). As expected, biomarkers for the salted typology were found in modules correlating with characteristics of salted sites (Table S9). Interestingly, biomarkers for the brackish and the freshwater typologies were associated either in a module or in connected modules (Table S9).

Regarding the seasonal biomarkers (Fig. 7D to 7F), the LEfSe identified 6 ASV biomarkers for autumn 2020 including 1 bacterium (*Actinomycetospora*) and 1 eukaryote identified at the genus level. No archaeal ASV was identified as biomarker for the autumn 2020, while 3 photosynthetic eukaryotes and a cyanobacteria were significantly more abundant. The autumn 2020 bacterial and eukaryotes biomarkers with the highest LDA scores were observed in most autumn 2020 and in samples of other seasons (Fig. S8A and S8B) but their relative abundances were higher in autumn 2020 (Dunn test, $p < 0.05$). There were 82 ASV biomarkers identified for spring, which included 26 bacteria (e.g. *Flavobacterium*, *Luteolibacter*) and 3 eukaryotes (*Navicula*, *Berkeleya*, *Surirella*) identified at the genus level and 16 photosynthetic eukaryotes. Among the three spring biomarkers with the highest LDA score of all three communities, they could be observed in most spring samples and

in a few other seasons (Fig. S8C to S8E) but had a higher relative Abundance in spring 2021 (Dunn test, $p < 0.05$). For summer, 23 ASV biomarkers were detected, including 6 bacteria (e.g. *Fluviicola*, *Leto-bacterium*) and 3 eukaryotes (two *Saccharomyces*, *Frontonia*) identified at the genus level. Also, one photosynthetic eukaryote was significantly more abundant in summer, whereas no archaeal biomarker was revealed. The three summer biomarkers with the highest LDA scores of bacterial and eukaryotic communities were present in most summer 2021 samples and in a few samples of other seasons (Fig. S8F and S8G) but their relative abundances were higher in summer 2021 (Dunn test, $p < 0.05$). Finally, for autumn 2021, the LEfSe identified 32 ASVs as biomarkers including 3 bacteria (*Roseimarinus*, *Cyanobium* and *Dracolibacterium*) and 4 eukaryotes (*Ebria*, *Allas*, *Pelagodinium*, *Vishniacozyma*) identified at the genus level and contained 4 photosynthetic eukaryotes. Biomarkers of autumn 2021 could be observed in most samples of autumn 2021 and in a few samples of other seasons (Fig. S8H and S8J) with higher relative abundance in autumn 2021 (Dunn test, $p < 0.05$). Some of the seasonal biomarkers were found in the different networks (Table S10).

4. Discussion

4.1. Salinity as the main community driver

Many studies have shown the sensitivity of organisms from the three domains of life to salinity (Logares et al., 2009). Here, prokaryotes and meiofaunal relative abundances were higher in salt marshes than in brackish and freshwater marshes, contrasting with the study of Tran et al. (2020) in which such differences between the typologies were not observed. However, too few studies have been conducted on brackish and freshwater marshes to provide a clear comparison (Tortajada et al., 2011).

Salted environments are generally characterised by higher relative abundances of Halobacteria (Liu et al., 2016) and nematodes (Bell, 1979). In environments with lower salinity (i.e. brackish and freshwater environments), higher relative abundances have been reported for methanogenic archaea (Chen et al., 2020), copepods and ostracods (Bell, 1979). The composition of microbial community observed in this study hence corresponds to what was previously observed.

Furthermore, differences in meiofaunal community composition in this study were observed with both morphological identification and sequencing analyses, showing the complementarity of the methods. Indeed, morphological identification allowed the estimation of meiofaunal abundance for “big” organisms (e.g. nematodes, copepods, ostracods), whereas the sequencing analysis completes the taxonomical identification by sometimes providing deeper taxonomical affiliation (depending on the database quality) for non-taxonomy-experts (Schenk et al., 2020). As an example, in this study different classes of nematodes could be identified within Nematoda, a phylum inside the Animalia kingdom, by using sequencing techniques that allowed to highlight differences in nematodes' composition due to salinity. Nematodes affiliated up to the order level, such as Desmodorida, were identified as biomarkers for salt marshes only, whilst others were found ubiquitous, such as Monhysterida, which included ASVs identified as biomarkers for the three types of marshes. The use of sequencing analysis hence helped to push further the observation conducted with morphological identification. Furthermore, analyses of beta diversity (e.g. CAP) using sequencing data do not require a taxonomic affiliation to highlight significant differences of communities in link with environmental parameters. This type of analysis can hence link together important community diversity generated by DNA sequencing with morphological identification of different groups and their abundances.

Here a clear difference depending on typology for both photosynthetic and heterotrophic micro-eukaryotes was observed at the class level. As an example, the relative abundances of Bacillariophyceae and Conoidasida were more important in salt marshes while those of

Chlorophyceae and Intramacronucleata were higher in brackish and freshwater marshes. Furthermore, LEfSe analysis revealed several micro-eukaryotic biomarkers for typology. Organisms such as *Lankesteria* were highlighted as salt marshes biomarkers whilst *Cryptocaryon* and *Frontonia* were identified as freshwater sites biomarkers. For brackish sites, an ASV from the *Cyclotella* genus was identified as biomarker. It is however likely that salinity induces only changes on the composition of benthic microalgae community since the chlorophyll *a* biomass was not significantly different between marshes typologies. Indeed, microphytobenthos biomass has been shown to be sensitive to other environmental parameters such as nutrients concentration and sediment granulometry, while their assemblage are mainly linked with salinity (Thornton et al., 2002).

For prokaryotes, the archaeal community composition was significantly affected at the class level by differences in typology. Methanomicrobia and Methanobacteria were observed at higher relative abundances in brackish and freshwater marshes, while Halobacteria had higher relative abundances in salt marshes. Moreover, LEfSe analysis identified several taxa at the genus level as biomarkers for the typologies following this trend (e.g. *Haloterrigena* biomarker of salt marshes, *Methanoregula* biomarker of freshwater marshes). These observations indicated that the salinity represents a major driver of archaeal community composition in marshes. Numerous ASVs of the Candidatus Bathyarchaeia class were however identified as biomarkers in the three typologies suggesting that some archaeal class are more ubiquitous than others.

Unlike archaeal communities, clear differences in bacterial composition due to typology at high taxonomic level (e.g. class level) were not revealed here as, at this level, differences in composition are rarely observable (Vidal-Durà et al., 2018). In addition, the bacterial community was the one that revealed the fewest biomarkers with the LEfSe analysis. These results suggest that bacterial community is the less affected by differences in typology. However, clear differences in prokaryotes abundances and the low number of ASVs shared between the three typologies suggested that salinity drives the composition and the abundance of prokaryote communities in marshes. Whilst differences in bacterial community composition depending on typology were previously reported, it is not the case for prokaryotes abundance (Campbell and Kirchner, 2013). It is hence possible that other factors such as nutrient concentrations are responsible for differences in prokaryotes abundances in the assessed marshes, particularly as salt marshes showed similarities in their physical-chemical compositions. In the end, salinity seems to be a major driving factor of prokaryotic community structure that remains affected by other factors.

Furthermore, network constructed with all the sites showed modules correlating strongly with typology with few to no link between each other. In addition, the different networks constructed with samples of each typology indicated different assemblages depending on salinity. It is however noteworthy to highlight that biomarkers of freshwater and brackish typologies could be found in modules that were linked together. Similarly, brackish and freshwater marshes showed similarities in community composition, particularly visible at the class level (e.g. as previously mentioned Methanomicrobia, Methanobacteria or Chlorophyceae's relative abundances were higher in freshwater and brackish marshes than in salt marshes). These observations suggest that while typology is a major driver of marshes benthic community structure, brackish marshes share more similarities with freshwater marshes than with salt marshes. It would however be interesting to include in further studies different categories of brackish marshes with increasing salinities in order to estimate whether a shift in community composition can be observed along a salinity gradient.

In this study, it was revealed that the typology of marshes is a major driving factor of the abundances of meiofaunal and prokaryotes as well as the composition of all benthic communities. These communities and their abundances could however be affected by other parameters such as nutrient concentration. Thus, it remains important to increase the

amount of comparison study between different marsh types. This is of paramount importance in order to understand the impact of the different parameters on marshes' communities in link with their salinity.

4.2. Site specific community assemblage

Differences in abundances and composition of benthic communities were observed according to the site within a typology. In freshwater marshes, the observed differences were linked with salinity, suggesting that small salinity changes can still affect the benthic communities. However, in the studied freshwater marshes, salinity was also strongly correlated with other variables such as the concentration of phosphate, ammonium and nitrite, which are nutrients, usually linked to various human activities (Romillac et al., 2023), known to greatly affect the composition of benthic communities (Clark et al., 2011). Furthermore, the higher percentage of active chlorophyll *a* and its biomass observed in StRaph can affect the organic matter supply for the rest of the benthic community. Indeed, it has previously been shown that microphytobenthos can be the main supplier of carbon for benthic communities in some environments (Bouillon and Boschker, 2006; Christianen et al., 2017; Moncelon et al., 2022). While it has been previously shown that the main carbon sources of marshes were plants (Tanner et al., 2010) or chemolithoautotrophs (Vineis et al., 2023), the high active chlorophyll *a* rates observed in this study suggested a potentially important role of microphytobenthos in marshes carbon supply, as suspected by Boschker (1999), that should be further assessed.

In brackish marshes, differences in benthic community compositions according to sites were linked with the concentration of nutrients, organic matter (DOC) and the primary producers (pheopigment/chlorophyll *a* biomass). The abundance and composition of meiofaunal organisms also affects benthic communities since they are not only predators of micro-eukaryotes and prokaryotes but also a carbon source for some heterotrophic organisms as they release dissolved organic matter (Schratzberger and Ingels, 2018). In salt marshes, the differences of benthic communities according to site were mainly linked with the abundance of prokaryotes, active chlorophyll *a*, and ammonium and silicate concentrations. Ammonium is an important nutrient for primary producers, while silicate concentration can affect the composition of photosynthetic-eukaryote community by promoting diatoms bloom when nitrogen and phosphorous concentration are not limiting (Rocha et al., 2002; Yool and Tyrrell, 2003). Thus, it is likely that the nutrients' concentrations affect benthic communities in salt marshes because of their influence on the composition of primary producers and the produced organic matter.

This study suggests that the physical-chemical properties differences between the studied sites affected parts of their benthic communities (e.g. primary producers), which in turns affects the rest of the benthic community. This further highlights the importance of analysing benthic communities with an integrative approach.

4.3. Seasonality of marshes benthic communities

In this study, the seasonal dynamic of benthic community was dependent on the marsh typology, being also affected by the physical-chemical properties of a site. In other words, benthic communities exhibited different seasonal patterns depending on the site (and its properties) within a typology. Indeed, parameters such as sediment granulometry or nutrients concentration, which have been shown here to be significantly different depending on the site, can influence benthic community seasonality (Coull, 1985; First and Hollibaugh, 2010). However, similar variations were observed depending on the marsh typology.

In freshwater marshes, benthic communities in spring 2021 showed a specific pattern clearly distinct from the other seasons. It was characterised by higher copepod abundance, high nitrate concentrations, and lower rainfall rate that had the most significant effect on micro-

eukaryote communities. It has previously been shown that human activities in the catchment area combined with soil leaching by precipitation can highly impact the physical-chemistry of marshes (Tortajada et al., 2011). As the benthic micro-eukaryotic community was composed mainly by primary producers, it suggests that nutrient intake by soil leaching was likely low because of low rainfall rates, affecting the whole benthic community (Cembella, 2003; Hillebrand and Sommer, 2000a). Furthermore, it is known that marshes plants can represent an important carbon source for their benthic communities (Tanner et al., 2010). Thus, it is possible that parameters such as low rainfall can affect plants' organic matter production due to hydric stress or as an effect of lower nutrient input. Furthermore, it has been previously shown that benthic microbial community composition can vary depending on the marsh's plant community and their seasonal variation (Burke et al., 2002). Thus, considering the plant types inhabiting the assessed marshes and evaluate their condition should be included in future studies.

On another note, freshwater marshes' bacterial community composition in autumn 2021 was obviously different from other seasons. Whilst benthic community composition can be different depending on each season, differences between the same seasons of different years can also be observed (Bell, 1979; Coull, 1985), illustrated here by two different autumns. As lower phosphate concentrations were observed in autumn 2021 for freshwater marshes, the bacterial community might be the first to respond to the rarefaction of this resource. Further studies comparing the response time to changes in phosphate concentration are necessary to evaluate this hypothesis, however it might depend on several other factors, such as other nutrient concentrations. Conducting time monitoring on a marsh with close sampling points might be another way of further evaluating the temporality of each community's response to changes in resource availability.

In salt marshes, the composition of the micro-eukaryote community in St16 and St61 was correlated with higher temperature and silicate concentrations in summer 2021. Several studies have shown that silicate to nitrogen (Si:N) and silicate to phosphorus (Si:P) ratios are also of paramount importance for micro-algal community composition because of the silica cell walls of diatoms (Hillebrand and Sommer, 2000b; Yool and Tyrrell, 2003). This suggests that the observed changes in micro-eukaryotic communities are mainly caused by changes in micro-phytobenthos composition due to silicate availability.

In autumn 2020, ammonium concentrations increased while prokaryotes' abundance decreased in the three typologies of marshes. Furthermore, all RDA assessing biotic parameters constrained to physical-chemical parameters showed a distinct autumn 2020 compared to the three other seasons. However, only St16 and St61 community compositions were significantly different from other seasons. Such phenomenon might be linked to an external nitrogen input. Multiple sources of nitrogen inputs can be identified in the environment: input from agricultural soil by leaching due to high rainfall rates in autumn, deposition of atmospheric ammonium due to increased fossil fuel combustion as air temperature decrease in winter (Anderson et al., 2002). In this study, salt marshes were less limited in phosphate than brackish and freshwater marshes. Nitrogen and phosphorus are two important parameters influencing primary production and microbial communities (Hillebrand and Sommer, 2000a). Differences in N:P ratios between the different marshes typologies might thus have affected the benthic community response to a nitrogen input in autumn 2020 as suggested Cui et al. (2021). Furthermore, prokaryotes and metazoan abundances were higher in salt marshes, and thus greater decrease was observed in autumn 2020 compared to other typologies. Indeed, the abundance of prokaryotes decreased by 2.0 in freshwater typology, 3.2 in brackish typology, and from 2.8 (St52) to 4.5 in salted typology compared to the other autumn. The decrease in abundance might have affected more profoundly the composition of the benthic community in salt marshes. Within the salt marshes, St52 was probably less affected because lower ammonium concentration was observed in autumn 2020. These observations suggest that changes in the N:P ratio in salt marshes, in link with

a seasonal ammonium input, was responsible for important modification in salt marshes' benthic community structure.

This study highlighted the influence of seasonal physical-chemical changes on benthic communities. It particularly showed that, although the changes differ depending on typology and sites, they can also have varying effects on the different assessed communities.

4.4. Insights into the potential interactions between the three domains of life

The network constructed with the core community allowed to infer the trophic web, particularly modules 1 to 24 with photosynthetic organisms having a central role being linked with heterotrophic protist, fungi, metazoan, bacteria and archaea. Several studies have shown that meiofaunal organisms tend to graze preferably on benthic micro-algae (Borchardt and Bott, 1995), while several heterotrophic protists are known as predators of prokaryotes and micro-algae (Sherr and Sherr, 1994). Other modules composed mainly by prokaryotes, connected together, were linked with heterotrophic prokaryotes and metazoan. Such observation are reminiscent of the process involved in the microbial loop, in which heterotrophic prokaryotes remobilise organic matter produced by photosynthetic autotrophs or heterotrophs, making it available again for the "classic food chain" (describing metazoans grazing on primary producers and several level of predators) (Fenchel, 2008). However, it is necessary to keep in mind that co-occurrence networks represent a statistical tool hinting to possible links between organisms and is still far from representing concrete and active interactions between the involved organisms (Blanchet et al., 2020).

In the networks of salted, brackish and freshwater typologies, many modules were composed of prokaryotes and photosynthetic organisms, indicating that the quality of the consumed organic matter might be more important than the quantity. Organic matter quality has already been proved to be a community composition driver in soil community studies (Ding et al., 2015; Boone, 2015). Indeed, metabolites released by primary producers might be used by specific consumers only that later makes this organic matter available for secondary consumers. Furthermore, high rates of chlorophyll *a* were observed in all marshes. Community composition of all community also varied depending on chlorophyll *a* or pheopigment biomass. These factors are combined with a high number of associations of photosynthetic organisms, particularly micro-eukaryotes, with prokaryotes and other micro-eukaryotes. Hence this study suggest an important role of micro-eukaryotic primary producers as a driving force of other communities' composition that should be further assessed. The use of integrative approaches such as this study, assessing the three domains of life seems to be of the most importance in order to evaluate the parameters influencing marshes' benthic communities.

On the opposite side, other modules that do not contain photosynthetic organisms might depend more on the quantity of organic matter than the quality. For instance, the module 14 of the network of salted typology, a module composed only by metazoan, strongly correlated with prokaryotes' abundance but not with any bacterial or archaeal ASVs. In fact, bacterivorous meiofaunal organisms, such as nematodes, are usually considered general predators without specific preys (Martins et al., 2022). This can explain why these modules are not correlated with specific ASVs. However, whilst they do not have specific prey, they can release dissolved organic matter that can shape microbial community composition (Gerlach, 1978; Jiang et al., 2023), explaining why some metazoan ASVs still correlate with some prokaryotic modules. Other modules, such as the modules 16 and 23 in the network of the salted typology correlating only with grain size, might either consume organic matter from various sources (e.g. use of a common secondary metabolite, high metabolic versatility) or be chemo-autotrophic primary producers. Modules that correlate negatively with active chlorophyll *a* rate (e.g. modules 20, 28, 30 of the salted site network) might also be composed of chemo-autotrophic primary producers as they are involved

in the reduction of compounds produced by anaerobic digestion in sediments (Middelburg, 2011).

Thus, it is likely that eukaryotes might play a pivotal role in shaping the prokaryote communities that in turn might affect the eukaryotic communities, highlighting the importance to develop holistic approaches studying the three domains of life. Further studies characterizing the whole microbial communities in various marshes of different typologies and varying salinity will help decipher the importance of the composition of one community in shaping the organization of the others. It is of paramount importance to increase the number of experimental and field-sampling designs based on integrative approaches.

4.5. Biomarkers reveal potential functional redundancy between typologies and seasons

The LEfSe analyses identified microbial taxa as biomarkers for the typology of the different sites and for the seasons. Among these biomarkers, some had a sufficiently precise taxonomic affiliation to infer potential functions or assess their presence in certain types of environments.

Among the (micro-) organisms highlighted as biomarkers of salt marshes, many are referenced to as marine organisms such as *Haloterrigena* (Oren et al., 2017) or *Woesia* (Du et al., 2016). Some bacterial ASVs belonging to taxa now grouped in the Deltaproteobacteria class (i. e. Desulfuromonadia, Desulfocapsaceae and Desulfobulbaceae), with major role in the sulfur cycle (Galushko and Kuever, 2021; Kuever, 2014; Kuever et al., 2005), were also identified. In fact, marine environments are known to have high sulfate concentration and thus usually contain sulfate reducing organisms or other organisms involved in the sulfur cycle (Kondo and Purdy, 2007). Other organisms that might have important function were also identified such as the genus *Candidatus Nitrosopumilus* (ammonia oxidizing archaea) (Qin et al., 2016) and the order Methanofastidiosales (potential methanogenic archaea) (Vanwonterghem et al., 2016). Methanogenic archaea are mainly found in freshwater habitats, however, some active methanogenic archaea can be found in salted marshes (Bartlett et al., 1985) with lower methanogenesis rates, usually because of high sulfate concentrations resulting in competition for resource between sulfate reducing micro-organisms and methanogens (Poffenbarger et al., 2011).

Among the biomarkers of the brackish typology, organisms previously observed in brackish but also in salted and freshwater environments, such as the diatom genus *Cyclotella* (Katrantsiotis et al., 2016) and the bacterial genus *Thiobacillus* (Kumar et al., 2020), were identified. The photosynthetic eukaryotes (e.g. *Cyclotella*, Chlorophyceae), the *Nitrosarchaeum* genus and the *Nitrososphaeraceae* family (ammonium oxidizing archaea) (Stieglmeier et al., 2014; Tolar and Mosier, 2019), the order *Methanomassiliicoccales* (methanogens) (Nkamga and Drancourt, 2016), and the *Thiobacillus* bacterial genus (potential sulfur oxidizing bacteria) (Kumar et al., 2020) have important role in biogeochemical cycles.

Among the organisms identified as biomarkers for the freshwater typology, several genera have been observed in freshwater environments, such as *Denitratisoma* (Fahrbach et al., 2006), *Methanoregula* (Zinder and Bräuer, 2016) and *Candidatus Methanoperedens* (Haroon et al., 2013). Some of the biomarkers might be involved in the carbon (*Methanoregula*, *Methanosaeta*) (Patel, 2015; Zinder and Bräuer, 2016) or nitrogen (*Denitratisoma*) (Fahrbach et al., 2006) cycles, and even in both cycles (*Methanobacterium*, *Candidatus methanoperedens*) (Boone Boone, 2015; Haroon et al., 2013). Methanogen population biomass, diversity and productions have been demonstrated as more important in environments with lower salinity such as brackish and freshwater wetlands (Pattnaik et al., 2000; Wen et al., 2017). Furthermore, in the studied marshes, relative abundances of methanogenic archaeal classes were higher in brackish and freshwater marshes than in salted marshes. These results therefore suggest that, while salt marshes have a potential function involved on the sulfur cycle (particularly sulfate-reduction),

freshwater marshes are characterised by the dominance of metabolisms involved on methanogenesis as previously observed (Kondo and Purdy, 2007; Pattnaik et al., 2000; Wen et al., 2017). As for brackish marshes, they have shown potential functions associated with the sulfur cycle (particularly sulfur oxidation) and methanogenesis.

One of the main ecosystem services rendered by marshes is water purification of nitrogen compounds (Tortajada et al., 2011). Interestingly, biomarkers potentially involved in the nitrogen cycle were identified irrespective of the typology. However, they are represented by different genera depending on the typology, showing the functional redundancy of organisms involved in the N cycle depending on salinity.

Biomarkers of the salted typology were found in modules correlating with environmental parameters characteristic of salted sites. Whilst those of brackish and freshwater typologies were found in the same modules or in modules linked together (Table S9). Such observation suggested a closest proximity of benthic communities inhabiting marshes of brackish and freshwater typologies. Furthermore, the presence of biomarkers in the network constructed with all the sites may also suggest important function of these organisms.

The LEfSe identified ASVs affiliated to photosynthetic organism as biomarkers for all the seasons as well as *Flavobacterium*, *Fluvicola*, and *Roseimarinus*, known as nitrate reducers (Bernardet and Bowman, 2015; Board, 2015; Yoon et al., 2008), for all seasons except the autumn 2020. Such observation suggested that despite the observable seasonal dynamic the good functioning of nitrogen cycle and primary production was ensured by microbial functional redundancy.

For the archaeal community, 5 ASVs were identified as spring biomarkers and 13 as biomarkers of autumn 2021. Among these biomarkers, 12 were affiliated to Woesarchaeales, an order belonging to the Woesarchaeota phylum, suspected to be parasitic or symbiotic due to the lack of genes for key metabolic pathway in their genomes (Castelle et al., 2015). The seasonal dynamic of this archaea might thus be linked to the life cycle of its host. However, these ASVs were not present in the networks hindering the detection of their relationships with other organisms. The construction of a network has inherent limitation, imposed by the number of samples and the minimal occurrence setup, that can explain the absence of seasonal specific ASVs in the network for a specific typology, especially since each site has different seasonality as in this case. It is thus necessary to perform further analyses to confirm that Woesarchaeales dynamics depends on its host or symbiont. Moreover, it might be possible that the identified Woesarchaeota biomarkers are potentially capable of associating with various host or symbiont, or might be able to use metabolites from other members of the benthic community. It hence remains necessary to conduct further research on Woesarchaeota and their association with other organisms in order to apprehend their role in benthic communities and the effect of seasonal variations on them.

Interestingly, some of the seasonal biomarkers were found in the different networks, particularly ASVs identified as biomarkers for spring 2021, which were mainly affiliated to prokaryotes involved in the nitrogen cycle together with photosynthetic eukaryotes. Such observation suggested that the spring biomarkers have a crucial role in shaping microbial communities ensuring likely important functions in marsh ecosystems although being involved in smaller modules.

For the eukaryotic and archaeal biomarkers, taxonomic affiliation of the main ASVs failed at best to the order level, revealing the importance to increase research on archaea and eukaryotes combining sequencing and cultivable approaches in order to better implement the databases and obtain more advanced taxonomic affiliation. Additionally, this study showed that biotic and abiotic environmental factors, and their complex association play a key role in shaping the benthic communities. Thus, it is also crucial to increase the amount of research aiming to characterize various less-studied-environments.

5. Conclusion

In this study, a seasonal survey monitoring the benthic communities from eight marshes within three different typologies (salted, brackish and freshwater) was implemented during four seasons. Three main factors driving the composition of benthic communities were identified. First, the salinity, observed with different community composition according to the three typologies. The salt marshes were characterised by halophilic archaea, Deltaproteobacteria, higher relative abundance of Bacillariophyceae and Conoidasida, and high meiofaunal abundance dominated by nematodes of the Chromadorea class. The composition of benthic communities inhabiting brackish and freshwater marshes were closer to each other, sharing numerous ASVs that resulted in similar composition at the class level. They were characterised by higher relative abundance of methanogenic archaea, Gammaproteobacteria, Intramacronucleata, Chlorophyceae and copepods (Maxillopoda). Second, the nutrient concentrations characterizing a site within a typology was a driver for the composition of benthic communities. Finally, the season was the third driver shaping the composition of benthic communities that showed different pattern depending on each site and typology. The typology and season biomarkers, identified by LEfSe analyses, included, prokaryotes involved in the nitrogen cycle, whilst photosynthetic eukaryotes were identified as biomarkers for all seasons. Some of these biomarkers were found in the co-occurrence networks suggesting they have an important functional role in the benthic community inhabiting marshes. Furthermore, in these co-occurrence networks, many modules were composed of prokaryotes connected to eukaryotes, particularly to photosynthetic eukaryotes. Such observation highlighted the importance of eukaryotes in shaping microbial communities. Thus, our study illustrates the necessity to include eukaryotes (micro-eukaryotes and meiofauna) when assessing benthic microbial communities. The use of such integrative approaches would provide useful information on the interaction of the biosphere with its environment allowing *in fine* a better understanding of marshes and wetland ecosystems' functioning. Particularly, it is of paramount importance to use such approaches in order to assess the impact of climate change and pollutants on various environments and ecosystems. Furthermore, information gathered using integrative approaches can be used in the long run to establish robust tools such as biotic indexes, indicating the status of an ecosystem.

CRedit authorship contribution statement

Clélia Duran: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Andréa Bouchard:** Investigation, Formal analysis, Data curation. **Hélène Agogué:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Christine Dupuy:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Robert Duran:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Cristiana Cravo-Laureau:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Sequence data

Raw sequence data were deposited in the NCBI's SRA and is available under the assigned Bioproject PRJNA1091597 (reviewer link: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1091597?reviewer=sp7tqakpk56uh35mmb967hest7>) (Duran et al, 2023).

Funding

This work was funded by the Interreg Sudoe Programme through the European Regional Development Fund (ERDF), BIOMIC project, grant number SOE4/P1/F0993 and a PhD grant from the Région Nouvelle-

Aquitaine and the Université de Pau et des Pays de l'Adour.

Declaration of competing interest

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

Acknowledgments

The authors are grateful to François-Xavier Robin (UNIMA) and Olivier Philippine (UNIMA) for their help to choose appropriate sampling sites and during sampling campaigns. We would like to thank Bénédicte Dubillot, Raphaël Moncelon, Jérôme Jourde, Anais Perdrau, Nicolas Lachaussée and Mathieu Paoletti from LIENSs for their help during sampling campaigns. We are thankful to Claire Emery (LIENSs), Philippe Pineau (LIENSs) and Mégane Noyer (UPPA) for their help during sampling campaigns and sediment analyses. We are grateful to the cytometry and imaging platform that provided organization and service in LIENSs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.177523>.

Data availability

Data will be made available on request.

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