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Linking Water to the Bottom: eDNA Study of Benthic Invertebrates and Invasive Species in the Venice Lagoon

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ABSTRACT

This study investigates the benthic invertebrate community in the Venice Lagoon using environmental DNA (eDNA) metabarcoding based on superficial water samples. Our objective is to understand if, in a shallow lagoon system, sampling at the surface can provide information on benthic biodiversity, allowing us to establish a baseline for future assessments and to monitor the community's seasonal and spatial variation. eDNA was collected from surface water samples at two sites during nine sampling campaigns from November 2018 to October 2019, and metabarcoding was performed using an available primer pair targeting 16S mitochondrial rDNA of echinoderms, never tested empirically before. Analyses revealed 80 taxonomic units, five not assigned at the species level, belonging not only to the phylum Echinodermata but predominantly assigned to Mollusca, with the majority of the identified species (60 out of 75) representing benthic invertebrates. Several species known to be invasive were detected, some previously recorded in the Venice Lagoon environment and others detected for the first time. Significant spatial differences in species composition were observed between the northern and the southern site of the lagoon. Temporal variation of the benthic community was also observed, mainly due to the distinctiveness of autumn samples, highlighting the dynamic nature of the Venice Lagoon ecosystem. Our results confirm the utility of eDNA for ongoing biodiversity monitoring and management and show that eDNA isolated from superficial water can provide information on the benthic invertebrate community, which might be particularly useful for biodiversity assessment in lagoons, ports, and areas characterized by limited or interdicted access.

1 | Introduction

The study of lagoon environments is of great ecological importance because of their unique position at the interface between

terrestrial and marine ecosystems. Defined as transitional coastal habitats, lagoons are crucial nursery areas for numerous commercially important fish species (Tournois et al. 2017) and play a vital role in providing essential ecosystem services,

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including provisioning, regulating, and cultural services (Newton et al. 2018). Due to their position and features, these water bodies are under continuous anthropogenic pressure (Mateus et al. 2016; Sfriso et al. 2017; Zucchetta et al. 2021; Camatti et al. 2023). Human pressures such as urbanization, agriculture, land-use changes, industrial development, and maritime activities affect the structure and function of these valuable yet vulnerable coastal ecosystems (Newton et al. 2014). One of the consequences of these impacts is the appearance of invasive species, which can negatively affect aquatic communities, especially macrophytes, benthic invertebrates, zooplankton, and fish (Gallardo et al. 2016). In stressed environments such as the Venice Lagoon, which is easily colonized by invasive species, this leads to biodiversity loss and ecosystem imbalance (Occhipinti-Ambrogi and Savini 2003).

The Venice Lagoon, recognized, together with Venice city, as a UNESCO World Heritage Site for its profound cultural and historical significance, is a prominent venue where multiple human-induced threats interact (González 2018; Braga et al. 2020). The lagoon, extending over 500 km² in the northern region of the Adriatic Sea and connected to the open sea by three inlets—Lido, Malamocco, and Chioggia—is also known for its abundant biodiversity. This makes it a distinctive and indispensable marine ecosystem, playing a critical role as a habitat for numerous species and fostering intricate ecological relationships among them (Franco et al. 2006; Cavarro et al. 2017). The Venice Lagoon is under strong human pressure, as evidenced by many studies on different threats such as overexploitation, pollution from the industrial area of Porto Marghera, ship traffic, and the impact of urban and agricultural wastewater (Bernardello et al. 2006; Parolini et al. 2010; Newton et al. 2014; Bernardini et al. 2023). Furthermore, the lagoon faces challenges from climate change and the introduction of alien species, often through ballast water, aquaculture, and commercial activities in fish markets (Giupponi et al. 1999; Pranovi et al. 2006; Solidoro et al. 2010; Occhipinti-Ambrogi et al. 2011; Crocetta 2012; Malej et al. 2017).

In this context, effective and systematic biodiversity monitoring is essential to evaluate the ecological status of the Venice Lagoon, in order to measure the impact of anthropogenic activities, to assess biodiversity loss, to identify non-indigenous and cryptic species, ultimately ensuring that human activities can coexist with the complex ecosystem of the lagoon without causing damage (Balvanera et al. 2006; Fišer et al. 2018). The detection of invasive species is a major problem, given that they are often detected after they have spread throughout the environment, making timely intervention difficult (Crowl et al. 2008; Venette et al. 2021).

However, transitional environments present unique challenges for biodiversity research and monitoring due to their dynamic nature with intermittent fluctuations in salinity and other environmental parameters. The interplay between seawater from the open sea and freshwater from rivers results in a continuous influx of new species or contaminants into these ecosystems (Gillanders and Kingsford 2002; Marchini et al. 2015). As a result, studying and monitoring biodiversity in such environments, especially for elusive or rare species, is inherently complex.

Our knowledge of marine invertebrates in the Venice Lagoon has traditionally relied on conventional research methods (Maggiore and Keppel 2007; Bandelj et al. 2008; Tagliapietra and Sigovini 2010; Russo 2017), but these approaches can be expensive, time-consuming, and challenging. In fact, at the very minimum, traditional methods require a specific sampling design and different sampling gears (grabs, plankton nets or traps) depending on the target species, groups, or stages to detect. They also require specialist taxonomic knowledge and are typically invasive, causing disturbance to the environment (Zinger et al. 2020). In addition, for benthic or invasive species, traditional monitoring is difficult in ports due to heavy maritime traffic or to the presence of interdicted areas where traditional sampling is not allowed.

Environmental DNA (eDNA) metabarcoding provides a non-invasive and cost-effective approach to detect and monitor marine invertebrates, representing an invaluable tool that can complement and speed up conventional biodiversity assessments (Taberlet et al. 2018). This approach simplifies field work and allows targeting different taxonomic groups and species from a single sample, which is particularly useful for identifying the DNA of alien and invasive species (Xia et al. 2018; Larson et al. 2020) before the species can be visually detected, providing an early warning signal of their presence. Numerous studies have successfully shown the efficacy of eDNA analysis in detecting marine invertebrates, including mollusks, echinoderms, and cnidarians (Thomsen et al. 2012; Leray and Knowlton 2015; Leray et al. 2019; West et al. 2020). Bony fish communities have been monitored using eDNA metabarcoding in the Venice Lagoon (Cananzi et al. 2022) and more recently in the nearby Marano-Grado lagoon (Banchi et al. 2024). However, to the best of our knowledge, no eDNA study has been carried out in the Venice Lagoon to characterize the marine invertebrate community, though bulk samples with DNA metabarcoding have been used to assess zooplankton biodiversity (Schroeder et al. 2020).

This study focuses on the monitoring of the benthic invertebrate community and the detection of invasive species in the Venice Lagoon using eDNA isolated from surface water. To this end, we focused on echinoderms, a phylum that is well documented in the Venice Lagoon, where only benthic species occur (Vatova 1950; Tagliapietra and Sigovini 2010). We selected for metabarcoding the 16S Echi02 primer set developed by Taberlet et al. (2018) that, although never empirically tested so far, is expected to efficiently identify echinoderms (see appendix 1 in Taberlet et al. 2018). Since *in silico* analysis showed that this primer set likely amplifies several other eukaryotes, including bivalve mollusks (Martino 2022), we expect that a broader set of benthic species can be detected with this marker.

Specifically, we aim to: (1) verify the utility of the Echi02 primer set to detect echinoderm species known to be present in the Venice Lagoon starting from eDNA collected from surface water; (2) document, for the first time, the taxonomic spectrum of species detected with this primer set, with a special focus on the identification of benthic and invasive species; (3) establish the first eDNA-based baseline for tracking benthic biodiversity changes over time in the UNESCO World Heritage Site of the Venice Lagoon, allowing the future assessment of the impact of environmental changes and management practices.

2 | Materials and Methods

2.1 | Study Area, eDNA Sampling

From November 2018 to October 2019, nine sampling campaigns were conducted across four seasons (as defined for the boreal hemisphere) at two sites within the Venice Lagoon (Figure 1). One of the chosen sites (station 15, CH) is close to the inlet of Chioggia (45°13.938' N 12°17.184' E) in the southern basin of the lagoon. The other one (station 5, PDR) is in the northern basin, close to Palude della Rosa (45°29.952' N 12°25.043' E), about 7.5 km from the closest connection to the sea (Lido inlet) and is part of the Long Term Ecological Research network (LTER_EU_IT_016).

During each sampling campaign, at least two replicates of superficial water (10 L each) were collected with plastic tanks. Afterwards, the tanks were transported to the laboratory (Department of Biology at UNIPD) and stored at 8°C until filtration, which took place within 24 h of sampling and was performed using an Edwards 5 two-stage vacuum pump. Two filter types were used for each tank: glass fiber filters GF/C (Whatman) with a pore size of 1.2 µm and cellulose acetate filters (Sartorius) with a pore size of 0.45 µm. Filtration continued until the filters clogged; full details on samples and

filtered water volumes are reported in Table S1. Prior to the filtration of each sample, all the equipment, the components of the filtration device, and the surfaces were cleaned with a 1:10 bleach solution. After each cleaning, as suggested by Taberlet et al. (2018), a minimum of 1.5 L of pure water (MilliRo) was filtered and these samples were used as filtration 'blanks' to estimate the level of contamination. A total of 52 filters, 35 from "station 5" and 17 from "station 15", were obtained from lagoon water samples and stored at -80°C until DNA extraction.

2.2 | Extraction of DNA From Filters and Inhibition Testing

DNA extraction from filters was performed using the DNeasy Blood & Tissue kit (Qiagen) in a dedicated pre-PCR environment equipped with positive air pressure. To ensure that the filters were fully covered during the first incubation, the volume of the ATL buffer (Qiagen) was increased to 567 µL compared to the manufacturer's instructions. Correspondingly, the volume of Proteinase K provided with the extraction kit was increased to 50 µL. All the steps after the incubation were done following the manufacturer's protocol. The final DNA product was eluted twice using the same 100 µL of AE buffer (Qiagen); the

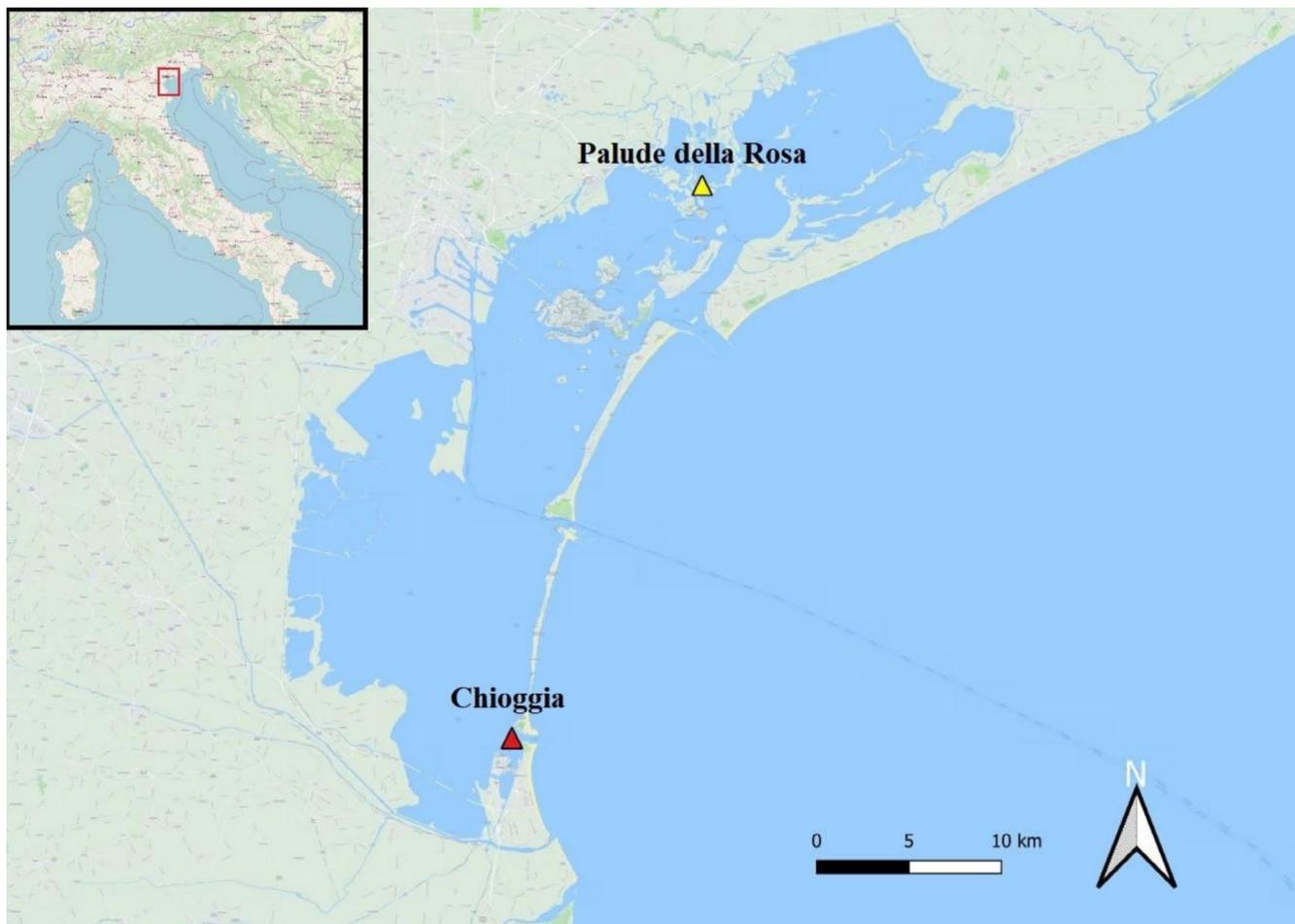


FIGURE 1 | Visual representation of the Venice Lagoon created with QGIS Version 3.34.4. The northern sampling site (45°29.952' N 12°25.043' E), "Palude della Rosa" (PDR), is highlighted with the yellow triangle, while the southern sampling site (45°13.938' N 12°17.184' E), "Chioggia" (CH), is highlighted with the red one.

elution buffer was halved to increase the DNA concentration. For each day of extraction, two extraction blanks were processed under the same conditions as the real samples, by simulating an extraction without any filter (De Barba et al. 2014; Taberlet et al. 2018; Aglieri et al. 2021). Extracted eDNA was then stored at -20°C until the PCR step.

To assess potential inhibition of the PCR process in eDNA samples, a qPCR assay was performed using *Equus caballus* DNA as an internal positive control (IPC). This species was chosen because it was certainly absent in the eDNA samples, thus excluding any contribution to amplification from the tested eDNAs. To test for inhibition, aliquots of six randomly selected eDNA samples, approximately 10% of the total number of samples, were spiked with a known concentration ($2\text{ ng}/\mu\text{L}$) of IPC ($1\ \mu\text{L}$ IPC + $1\ \mu\text{L}$ eDNA). Specifically, samples PR_23, PR_26, PR_27, PR_28, CH_33, and CH_79 were used (see Table S1). As a control, ultra-pure water was also spiked with the same concentration of IPC (IPC + W). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the target gene, with the following primers: Forward $5'$ -GGAGTCCACTGGTGTCTTCAC- $3'$; Reverse $5'$ -TTTGGCTCCACCCTTCAA- $3'$. To evaluate the efficiency of the GAPDH assay, standard curves were generated by amplifying two-fold serial dilutions of IPC, ranging from 0.25 to $4\text{ ng}/\mu\text{L}$. Real-time RT-PCR reactions were carried out using a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). Aliquots ($2\ \mu\text{L}$) IPC + W or IPC + eDNA were amplified in a final volume of $10\ \mu\text{L}$, containing $5\ \mu\text{L}$ PowerUP SYBR Green MasterMix (Applied Biosystems) and $0.25\ \mu\text{L}$ of each GAPDH primer ($10\ \mu\text{M}$). Each sample and no-template control (NTC) were tested in triplicate. The amplification protocol included an initial step of 2 min at 50°C , followed by 2 min at 95°C , and then 45 cycles of 15 s at 95°C and 30 s at 60°C . As previously reported (Hartman et al. 2005; Goldberg et al. 2016), a lack of amplification or a delayed amplification (≥ 3 threshold cycles, Ct) of IPC + eDNA samples compared to the IPC + W control is considered indicative of full or partial qPCR inhibition.

2.3 | Library Preparation

The primer pair Echi02 from Taberlet et al. (2018), targeting a fragment of 182–208 bp of the 16S rRNA mtDNA gene of echinoderms, was used for metabarcoding. Based on *in silico* PCR, this primer pair can also amplify the same region from many other benthic invertebrates, such as mollusks or annelids.

The eDNA samples were amplified using a single-step PCR protocol, the most effective strategy to amplify low-concentration templates according to Taberlet et al. (2018) because it allows minimizing the possibility of contaminations by reducing the protocol steps. To allow demultiplexing after sequencing, Echi02 forward and reverse primers were tailed with an 8 base-barcode and up to four random nucleotides at the $5'$ end (e.g., $5'$ -NNNN-8basesBarcode-Primer- $3'$). This step allowed for tagging each amplicon by a unique and identifiable double-tag combination and introduced the initial base diversity required for high-quality amplicon sequencing using Illumina platforms (Naik et al. 2023). The 36 barcodes used in this study were retrieved from Taberlet et al. (2018), and the complete list is given in Table S2.

In addition to the eDNA samples, PCR positive controls, represented by DNA of the sea urchin *Paracentrotus lividus*, were included in the PCR reaction to assess the success of amplification. Moreover, PCR blanks were added, using water as template, as well as sequencing blanks (one for each plate column and row, according to Taberlet et al. 2018) consisting of plate tubes with just the enzyme and primers tagged with unique tags. The latter blanks allow us to estimate the amount of “tag-jumping” (Schnell et al. 2015). PCR reactions were performed using a thermocycler (SimpliAmp™, Applied Biosystems) in three technical replicates to reduce amplification stochasticity. Final PCR volume was $20\ \mu\text{L}$, containing AmpliTaq Gold™ 360 MasterMix $1\times$ (Life Technologies), each primer at the final concentration of $0.5\ \mu\text{M}$, and $2\ \mu\text{L}$ of template (extracted eDNA). The amplification thermal profile started with 10 min at 95°C , followed by 35 cycles with 30 s at 95°C , 1 min at 52°C , 1 min at 72°C , and finally 7 min at 72°C . All the PCR reactions were set up in a pre-PCR environment, exclusively dedicated to eDNA, using only filter tips.

The presence of PCR amplicons was checked on 1.8% agarose gel with a transilluminator (Gel Doc™ XR+, Bio-Rad) for all reactions. Hence, PCR products were pooled together from all the different samples, and the pool was purified with the MinElute PCR Purification Kit (Qiagen). Final elution occurred in $16\ \mu\text{L}$. Six purification replicates were carried out to reduce stochasticity and pooled together after checking their purification success on 1.8% agarose gel. Finally, the purified pool was quantified using Nanodrop 2000c (ThermoFisher) and Qubit 4 fluorometer (ThermoFisher). All the steps were performed in a post-PCR environment with the use of filter tips. Afterward, $20\ \mu\text{L}$ of the purified library was sent to a sequencing service (Norwegian Sequencing Center, Oslo) for adding Illumina adapters and for sequencing with Illumina Technologies HiSeq 150 bp paired-end from a NEBNext Ultra kit.

2.4 | Sequence Analysis

Sequences resulting from the HiSeq were demultiplexed using Cutadapt (Martin 2011, version 4.4), while paired-end merging, removing of chimeras and denoising were performed with DADA2 (Callahan et al. 2016). Chimeric sequences were identified and removed through the application of the same method using the ‘consensus’ approach for -p-chimera. Then, QIIME2 pipeline (Bolyen et al. 2019, version 2022.2) was used for taxonomic assignments with 97% identity. The pipeline used is available on Github at <https://github.com/Slide95/combinatorial-dual-indexes-metabarcoding>. The taxonomic assignment was performed using a reference database derived from sequences available in GenBank, employing the QIIME2 plugin RESCRIPt (Robeson et al. 2021). The query used for the assignment was “(16s[All Fields] AND mitochondrial[All Fields]) AND (animals[filter] AND mitochondrion[filter] NOT (“Vertebrata”[Organism] OR Vertebrata[All Fields]))”. The reference database included a total of 137,207 dereplicate sequences, representing 29 different phyla. The database was last updated in March 2023. Reads assigned to ASVs of the same taxon were then aggregated into units, which will henceforth be referred to as Operational Taxonomic Units (OTUs) (Singer et al. 2023). The OTU table obtained was then cleaned. First, to estimate and

correct for the effect of ‘tag jumps’ described in Schnell’s work (Schnell et al. 2015), a ‘tag jump ratio’ was calculated between the number of sequences from sequencing blanks (sequences with barcode combinations not physically in the library) and the total number of sequences identified for each OTU. The result was the percentage contribution of artificial sequences generated for each OTU to the total. This percentage value was then subtracted from each of the samples. In a second step, the experimental blanks (extraction, filtration and PCR) were used to remove contaminations; in this case, the distribution of reads among blanks was calculated for each OTU and, using a conservative approach, a number of reads corresponding to the third quartile was removed from the samples. Then, taxonomic identifications were cross-checked against the Italian checklist of marine fauna (Relini 2008, 2010) produced by the SIBM (Società Italiana di Biologia Marina): to this end the reference publication of the checklist has been digitalized and the scientific names have been updated (Enrico Negrisolo, unpublished). For each OTU, we checked its potential presence in the Venice Lagoon reviewing the relevant literature and published records using Google Scholar with the keywords [Species name + Venice Lagoon]. Additionally, for each identified species, their potential status as “invasive species” was verified on the World Register of Introduced Marine Species (WRiMS) platform (Costello et al. 2024). Finally, we used the CLODIA database (Mazzoldi et al. 2014; updated database available at <https://chioggia.biologia.unipd.it/en/the-database/>), which includes monthly time series of landing data from the official statistics of the fish market of Chioggia, to check for contamination from commercial species.

2.5 | Statistical and Ecological Analyses

As a first step, differences between samples were tested using a permutational multivariate analysis of variance (PERMANOVA) based on a Jaccard distance matrix, with the “adonis” function in the R package “vegan” (Oksanen et al. 2022). PERMANOVA was performed on the entire dataset, including the variables ‘season’, ‘location’, and, since it may affect the statistical analysis, also ‘filter type’. Since the volume of filtered water before clogging differed between the two filter types used in this study (Table S1), with GF/C filters clogging at larger volumes, volume was not treated as an independent variable but was incorporated into the variable ‘filter type’.

Following these analyses, temporal variation was further investigated using only samples of the northern Venice Lagoon, for which a longer time series with monthly sampling was available, thus considering samples collected at PDR from March to October 2019 (Table S1). For this reduced dataset, in addition to PERMANOVA, Principal Coordinate Analysis (PCoA) and Analysis of Similarities (ANOSIM), based on Jaccard similarity index, were performed using TaxonTableTools (Macher et al. 2021). Subsequently, a Similarity Percentage (SIMPER) analysis (Clarke 1993) was conducted using “vegan”. This analysis aimed to identify Operational Taxonomic Units (OTUs) potentially influencing seasonal variations in the samples and further contributed to our exploration of the environmental dynamics captured in the dataset. SIMPER analysis was performed both between seasons and between months to account

for single OTUs’ influence in possible seasonality patterns at different time scales. Only OTUs that contribute significantly to a given pairwise comparison and that account for more than 5% of the variation are reported in the results. A heatmap was generated to visualize the abundance patterns of significant species identified by SIMPER analysis. Species abundance data were log-transformed using $\log(1+x)$ to reduce the influence of highly abundant taxa and normalize the data distribution. The heatmap was produced using the pheatmap package (Kolde and Kolde 2015, version 1.0.12) in R.

As a final step, to explore in greater detail the differences between northern and southern Venice Lagoon, the analyses were performed considering only samples collected during the same months at both sites. This approach included only samples from March, April, May, and August 2019 (Table S1), thereby removing the potential confounding effect of “season”. In this case, in addition to PERMANOVA, PCoA, and ANOSIM analysis, the ratio between normalized sequence counts in northern and southern sites was plotted as Log₂ Fold Change (LFC). Only species with absolute LFC > 1 have been considered.

3 | Results

3.1 | Inhibition Testing

To assess the inhibition-induced Ct shift, the Ct value of each spiked eDNA sample (IPC + eDNA) was compared with the Ct value for the spiked ultra-pure water control (IPC + W). For all samples, the mean Ct values of the spiked eDNAs (Mean = 29.24, SD = 0.35) were comparable to the mean Ct values of IPC + W (Mean = 28.74, SD = 0.24), with a shift of less than 1 Ct in all cases (Table S3).

3.2 | Sequencing and Taxa Detection

From the HiSeq 2 × 150 bp sequencing, a total of 120,244,289 sequences were obtained, of which 77,506,332 reads were successfully demultiplexed and attributed to the respective sample. Table 1 shows the numbers of the sequences after each step of the analysis.

A total of 36,522 ASVs were identified, of which 2013 have been taxonomically assigned. Overall, 94 taxonomic units were identified, corresponding to 31,312,079 taxonomically assigned sequences. To obtain the OTU table for ecological inferences, the ASVs of each OTU were manually checked using BLAST+

TABLE 1 | Number of sequences in each step of the bioinformatic analysis.

Sequences	Number
Raw	120,244,289
Traced to the sample	77,506,332
Merged	49,870,825
Taxonomically assigned	31,312,126

Distribution of Different Phyla

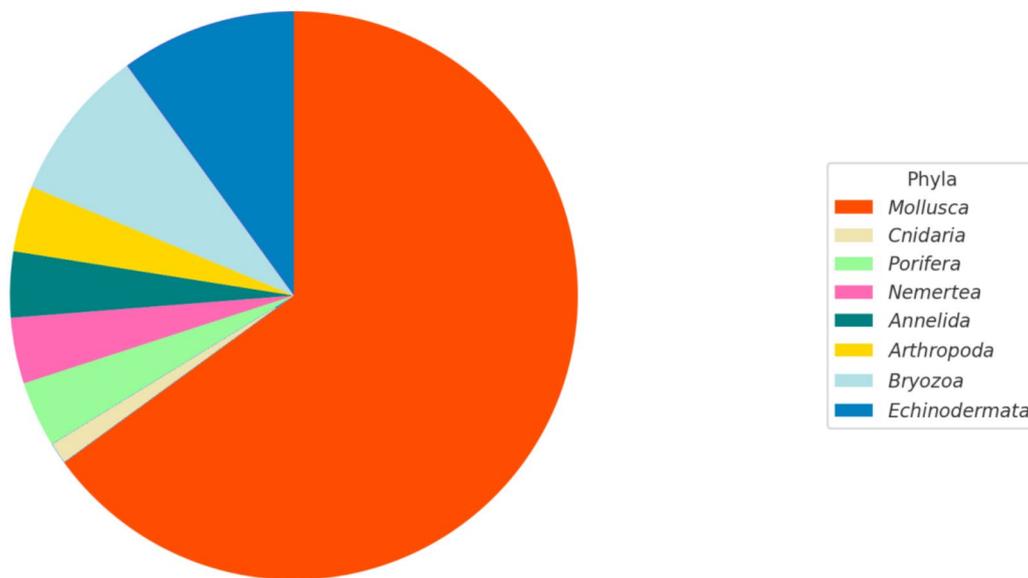


FIGURE 2 | Relative abundance of OTUs assigned to each phylum in the dataset. The graph was realized with the “matplotlib version 3.6.3” python package (Hunter 2007).

(Camacho et al. 2009). This check was also important to validate some dubious identifications at taxonomic levels higher than species. After excluding OTUs that were not relevant to the objectives of the study (e.g., Insecta), and after merging OTUs that were identified as the same species, a final number of 80 OTUs were obtained (Table S4). Echinodermata, expected *in silico* to be efficiently amplified with the Echi02 primer set used in this study, were represented by 8 OTUs. However, Mollusca resulted to be the predominant group, with 52 OTUs, and these two phyla together accounted for approximately 75% of the identified OTUs, indicating substantial representation within the dataset (Figure 2). Bryozoans, with a presence of 8.75%, and poriferans, nemerteans, annelids, and arthropods, each representing 3.75% of the total, further contributed to the observed taxonomic diversity. One OTU was identified for cnidarians. In addition, 75 out of 80 OTUs, including the eight echinoderms, were assigned at the species level (Table 2), with benthic invertebrate species detected in 60 of these cases. Regarding the 15 pelagic species detected with the Echi02 primer set, 10 were cephalopods, and these included six commercial species suspected of being the result of contamination from the nearby Chioggia fish market (see discussion). Cephalopod commercial species were removed from the dataset when representing possible contaminations, before the ecological analyses were performed.

3.3 | Beta-Diversity Analyses

PERMANOVA analysis (Table 3) revealed significant effects of both “season” ($p < 0.001$) and “location” ($p < 0.001$) on the composition of eDNA-derived communities. The variable “filter type”, instead, was not significant ($p = 0.103$), indicating that it does not impact the statistical analysis and that the samples processed with both filters can be considered replicates for the purpose of this study. Seasons explained approximately 14% of the total variation ($R^2 = 0.137$), while locations accounted for

approximately 8% of the total variation ($R^2 = 0.083$). These results suggest that both location and season play a discernible role in shaping the benthic community, though the small R^2 values highlight the possible influence of other factors.

PERMANOVA analysis of samples collected in PDR from March to October 2019, which provided a more extensive monthly time series, revealed an even stronger difference in species composition across seasons ($p < 0.001$; $R^2 = 0.255$). This result was confirmed by ANOSIM that showed a pronounced differentiation with an R -value of 0.363 and a p -value of 0.001 (Figure 3). PCoA showed clear seasonal clustering, with PC1 and PC2 explaining 17.94% and 12.41% of the total variation, respectively, highlighting distinct separations in the seasonal distribution of samples, particularly between spring and autumn (Figure 3B). This seasonal variation suggests that temporal factors have a significant effect on the communities in the northern site of the Venice Lagoon.

A comprehensive analysis of the dataset was performed using SIMPER (Clarke 1993) to identify significant influences of OTUs on seasonal variation within the analyzed samples. *Cerastoderma glaucum* (which accounted for approximately 79% of all identified sequences and was widely present across the samples) was excluded from the dataset for SIMPER analysis, as its inclusion would have obscured the signal of seasonal variation. Overall, only five species contributed significantly and accounted for more than 5% of the variation in at least one pairwise comparison (average contribution: 9.36%). In detail, *Acanthocardia tuberculata* was the main contributor both in the winter–spring and winter–summer comparisons, with p -values of 0.001 and 0.004 and contributions of 21.2% and 12.3%, respectively. *Abra* sp. also contributed to differences between winter–spring ($p = 0.045$ and 6.42%) and was the only major contributor in the comparison spring–autumn ($p = 0.002$, 8.03%). Similarly, *Amathia verticillata* was the only

TABLE 2 | List of the taxonomic units assigned at the species level.

Phylum	Class	Species	Benthic versus Pelagic	North Adriatic	Venice Lagoon	WRiMS	Notes
Anellida	Polychaeta	<i>Capitella teleta</i>	B				eDNA, this study
Anellida	Polychaeta	<i>Nephtys hombergii</i>	B		X		Pranovi et al. (2000)
Anellida	Polychaeta	<i>Polydora cornuta</i>	B		X	X	Bertasi (2016)
Arthropoda	Copepoda	<i>Paracalanus parvus parvus</i>	P	X	X		Sorokin (1999)
Arthropoda	Thecostraca	<i>Amphibalanus amphitrite</i>	B	X	X	X	Rech et al. (2018)
Arthropoda	Thecostraca	<i>Amphibalanus improvisus</i>	B	X		X	eDNA, this study
Bryozoa	Gymnolaemata	<i>Amathia verticillata</i>	P		X	X	Katsanevakis et al. (2020)
Bryozoa	Gymnolaemata	<i>Bugula neritina</i>	B	X	X	X	Occhipinti-Ambrogi (1985)
Bryozoa	Gymnolaemata	<i>Bugulina stolonifera</i>	B	X	X	X	Occhipinti-Ambrogi (1985)
Bryozoa	Gymnolaemata	<i>Conopeum reticulum</i>	B			X	eDNA, this study
Bryozoa	Gymnolaemata	<i>Cryptosula pallasiana</i>	B	X	X	X	Occhipinti-Ambrogi (1985)
Bryozoa	Gymnolaemata	<i>Scrupocellaria maderensis</i>	B				eDNA, this study
Cnidaria	Hexacorallia	<i>Diadumene lineata</i>	P		X	X	Stephenson (1935)
Echinodermata	Asteroidea	<i>Astropecten irregularis</i>	B	X	X		Cesari (1994)
Echinodermata	Echinoidea	<i>Echinocardium cordatum</i>	B	X	X		Vatova (1950)
Echinodermata	Echinoidea	<i>Paracentrotus lividus</i>	B	X	X		Vatova (1950)
Echinodermata	Ophiuroidea	<i>Amphipholis squamata</i>	B	X	X		Cesari (1994)
Echinodermata	Ophiuroidea	<i>Amphiura chiajei</i>	B	X	X		Cesari (1994)
Echinodermata	Ophiuroidea	<i>Amphiura filiformis</i>	B	X	X		Cesari (1994)
Echinodermata	Ophiuroidea	<i>Ophiothrix fragilis</i>	B	X	X		Vatova (1950)
Echinodermata	Ophiuroidea	<i>Ophiura albida</i>	B	X	X		Vatova (1950)
Mollusca	Bivalvia	<i>Acanthocardia tuberculata</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Cerastoderma glaucum</i>	B	X	X		Cesari (1994)

(Continues)

TABLE 2 | (Continued)

Phylum	Class	Species	Benthic versus Pelagic	North Adriatic	Venice Lagoon	WRiMS	Notes
Mollusca	Bivalvia	<i>Chamelea gallina</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Corbicula fluminea</i>	B			X	eDNA, this study
Mollusca	Bivalvia	<i>Dosinia lupinus</i>	B	X	X		Cesari 1994
Mollusca	Bivalvia	<i>Ensis ensis</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Ensis minor</i>	B				Cesari (1994)
Mollusca	Bivalvia	<i>Gari depressa</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Mactra corallina</i>	B				eDNA, this study
Mollusca	Bivalvia	<i>Mactra stultorum</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Mytilopsis leucophaeata</i>	B	X	X	X	Zulian and Quaggiotto (2020)
Mollusca	Bivalvia	<i>Nucula nucleus</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Nucula sulcata</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Polititapes aureus</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Polititapes rhomboides</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Ruditapes decussatus</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Ruditapes philippinarum</i>	B	X	X	X	Cesari (1994)
Mollusca	Bivalvia	<i>Scrobicularia plana</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Solen marginatus</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Steromphala adriatica</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Teredo bartschi</i>	B	X	X	X	Tagliapietra et al. (2021)
Mollusca	Bivalvia	<i>Theora lubrica</i>	B			X	eDNA, this study
Mollusca	Bivalvia	<i>Varicorbula gibba</i>	B	X	X		Cesari (1994)
Mollusca	Bivalvia	<i>Venus verrucosa</i>	B	X	X		Cesari (1994)
Mollusca	Cephalopoda	<i>Doryteuthis gahi</i>	P				Commercial species
Mollusca	Cephalopoda	<i>Dosidicus gigas</i>	P				Commercial species
Mollusca	Cephalopoda	<i>Eledone moschata</i>	P	X	X		Cesari (1994)
Mollusca	Cephalopoda	<i>Illex argentinus</i>	P			X	Commercial species

(Continues)

TABLE 2 | (Continued)

Phylum	Class	Species	Benthic versus Pelagic	North Adriatic	Venice Lagoon	WRiMS	Notes
Mollusca	Cephalopoda	<i>Octopus maya</i>	B				Commercial species
Mollusca	Cephalopoda	<i>Octopus vulgaris</i>	B	X	X		
Mollusca	Cephalopoda	<i>Sepia bertheloti</i>	P				Commercial species
Mollusca	Cephalopoda	<i>Sepia hierredda</i>	P				Commercial species
Mollusca	Cephalopoda	<i>Sepia officinalis</i>	P	X	X		Cesari (1994)
Mollusca	Cephalopoda	<i>Sepia pharaonis</i>	P			X	Commercial species
Mollusca	Cephalopoda	<i>Sepietta obscura</i>	P	X	X		Cesari (1994)
Mollusca	Cephalopoda	<i>Sepioloa rondeletii</i>	P	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Bittium reticulatum</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Bolinus brandaris</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Diodora graeca</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Ecrobia spatiana</i>	B				eDNA, this study
Mollusca	Gastropoda	<i>Ecrobia ventrosa</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Haminoea navicula</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Hexaplex trunculus</i>	B	X	X	X	Cesari (1994)
Mollusca	Gastropoda	<i>Hydrobia acuta</i>	B	X	X		Balducci et al. (2001)
Mollusca	Gastropoda	<i>Ocenebra edwardsii</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Tritia corniculum</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Tritia incrassata</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Tritia mutabilis</i>	B	X	X		Cesari (1994)
Mollusca	Gastropoda	<i>Tritia neritea</i>	B	X	X	X	Cesari (1994)
Mollusca	Gastropoda	<i>Tritia nitida</i>	B	X	X		Cesari (1994)
Nemertea	Hubrechtiiiformes	<i>Hubrechtella dubia</i>	P				eDNA, this study
Nemertea	Palaeonemertea	<i>Tubulanus superbus</i>	P	X	X		Cesari (1994)
Porifera	Suberitida	<i>Halichondria panicea</i>	B	X		X	eDNA, this study
Porifera	Suberitida	<i>Hymeniacion perlevis</i>	B	X	X	X	Sarà (1960)

Note: Reported are, for each taxon, the pelagic or benthic habitus and the previous detections in the North Adriatic, based on the Italian checklist of the marine species (Relini 2008, 2010), and in the Venice Lagoon, based on the literature available in March 2024. In addition, the possible presence of the species in the World Register of Introduced Marine Species (WRiMS) is reported. The “Notes” column contains the reference to the first detection, if available. Newly detected species or potential contaminations from Chioggia fish market are highlighted in bold.

main contributor in the summer-autumn transition ($p=0.001$, 6.66%). Finally, in the winter-autumn comparison, more than 10% of the total variation was due to only two species, *Tritia neritea* ($p=0.001$, 5.83%) and *Sepia officinalis* ($p=0.001$, 5.06%).

To improve our understanding of the temporal variability of eDNA, a SIMPER analysis of monthly differences (from March to October) was carried out, with a particular focus

TABLE 3 | PERMANOVA results based on a Jaccard distance matrix based on the vegan function adonis2: [adonis2 (formula=Jaccard_distance_matrix~season+location+filter_type, data=data); Permutation test for adonis under reduced model; Terms added sequentially (first to last); Permutation: Free; Number of permutations: 999].

	Df	SumOfSqs	R ²	F	Pr (>F)
Season	3	2.927	0.137	2.786	<0.001
Location	1	1.776	0.083	5.073	<0.001
Filter type	1	0.513	0.024	1.464	0.103
Residual	46	16.107	0.755		
Total	51	21.324	1.000		

on the northern site (PDR) for which a longer time series was available, allowing for a deeper view into temporal dynamics (Table 4). *Ruditapes philippinarum* emerges from this analysis as the most abundant in the dataset, contributing significantly to the differences between months, which is particularly evident in the period between April and July. In April, its contribution ranges from 20.4% to 21.6% ($p=0.009$ – 0.013). Moreover, comparisons between late spring (June) and autumn (September–October) highlight the presence of *Abra* sp. ($p=0.01$, 17.7%). In addition, significant differences were observed between August and October, with the bryozoan *A. verticillata* ($p=0.001$, 19.2%) and the nemertean *Cephalothrix rufifrons* ($p=0.001$, 14.5%) contributing significantly to the differences between the datasets. Furthermore, *T. neritea* shows remarkable differences in multiple comparisons, especially between June and October ($p=0.003$, 15.2%) and June and September ($p=0.002$, 15.7%). The analysis also highlights the influence of *Ecrobia ventrosa* ($p=0.001$, 14.7%) and *S. officinalis* ($p=0.001$, 14.1%) in the comparison between September and October, further emphasizing the dynamic seasonal patterns observed in the dataset.

Considering the significant temporal variation, the eDNA samples collected from the northern and the southern site of the Venice Lagoon were compared, as described in the

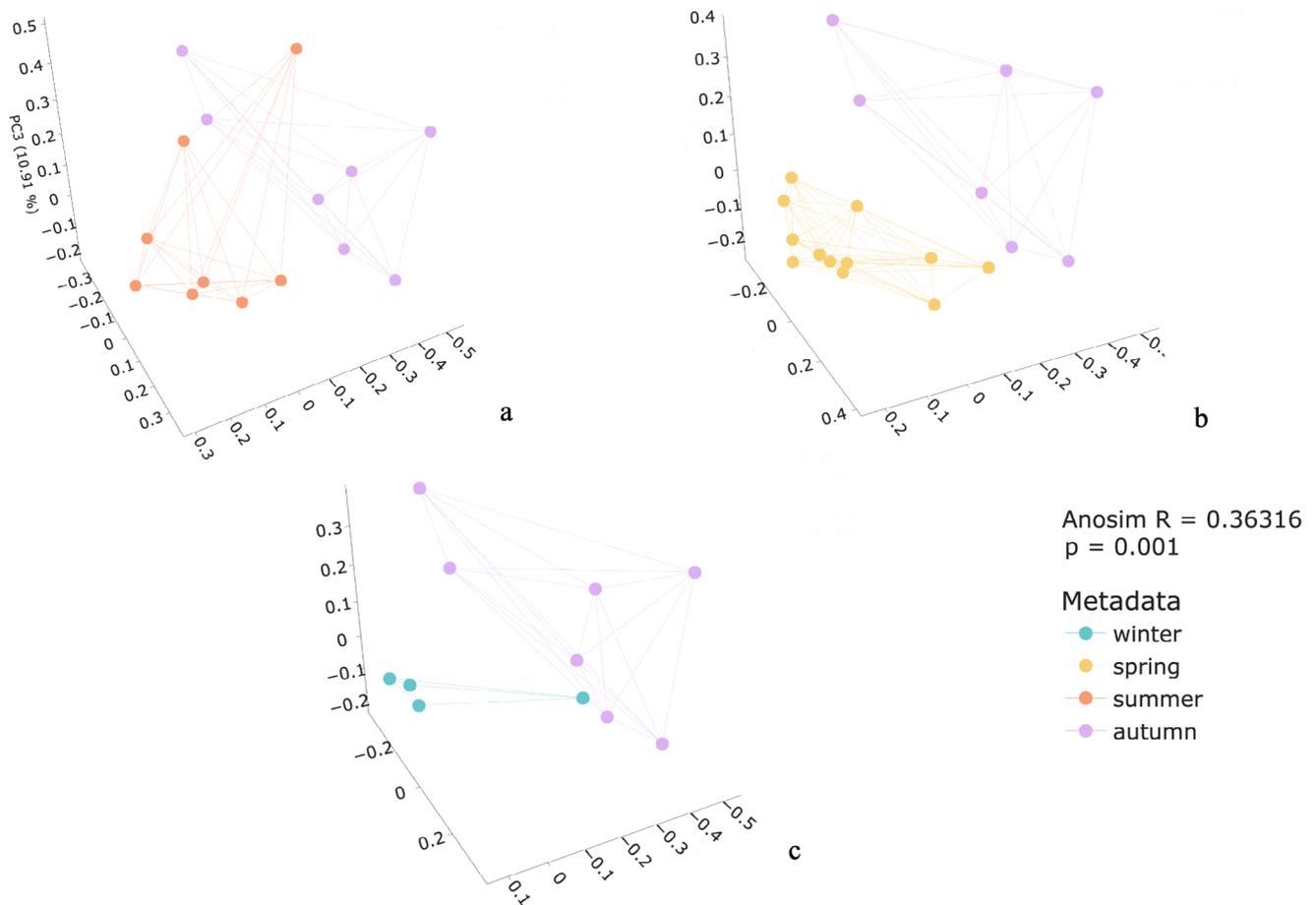


FIGURE 3 | PCoA plots based on Jaccard similarity index comparing different pairs of seasons in the northern site (PDR). The ANOSIM test results are also reported. The three plots represent the following season comparisons: (A) summer–autumn, (B) spring–autumn, (C) winter–autumn. PC1 = 17.94%; PC2 = 12.41%; PC3 = 10.91%.

TABLE 4 | Results of the SIMPER analysis of monthly differences.

Month comparison	Species name	<i>p</i>	Contribution %
March– April	<i>Ruditapes philippinarum</i>	0.013	20.4
March– May	<i>Ophiothrix fragilis</i>	0.021	5.3
March –June	<i>Tritia neritea</i>	0.016	12.9
March– July	<i>Ruditapes philippinarum</i>	0.015	14.1
March– August	<i>Amathia verticillata</i>	0.003	11.7
March– August	<i>Cephalothrix rufifrons</i>	0.035	6.4
March –August	<i>Tritia neritea</i>	0.031	9.8
March –September	<i>Sepia officinalis</i>	0.003	5.2
March –September	<i>Tritia neritea</i>	0.002	7.0
March –October	<i>Tritia neritea</i>	0.001	5.8
April –August	<i>Ruditapes philippinarum</i>	0.009	20.8
April –September	<i>Ruditapes philippinarum</i>	0.013	21.6
April –October	<i>Ruditapes philippinarum</i>	0.005	21.6
May –August	<i>Ophiothrix fragilis</i>	0.013	5.0
May –September	<i>Ophiothrix fragilis</i>	0.011	6.1
May –October	<i>Ophiothrix fragilis</i>	0.001	6.0
June– August	<i>Amathia verticillata</i>	0.047	6.4
June– August	<i>Ruditapes decussatus</i>	0.028	5.2
June –August	<i>Tritia neritea</i>	0.010	11.2
June –September	<i>Abra</i> sp.	0.014	18.0
June –September	<i>Tritia neritea</i>	0.002	15.7
June –October	<i>Abra</i> sp.	0.010	17.7
June –October	<i>Tritia neritea</i>	0.003	15.2
July –September	<i>Ruditapes philippinarum</i>	0.010	13.0
July –October	<i>Ruditapes philippinarum</i>	0.003	12.9
August– July	<i>Ruditapes philippinarum</i>	0.006	13.0
August –October	<i>Amathia verticillata</i>	0.001	19.2
August –October	<i>Cephalothrix rufifrons</i>	0.001	14.5
August –October	<i>Mactra stultorum</i>	0.001	5.6
August –October	<i>Ruditapes decussatus</i>	0.002	5.8
September –October	<i>Acanthocardia tuberculata</i>	0.001	8.6
September– October	<i>Ecrobia ventrosa</i>	0.001	14.7
September– October	<i>Sepia officinalis</i>	0.001	14.1
September– October	<i>Sepia pharaonis</i>	0.001	5.9

Note: The list includes only comparisons where both the contribution is higher than 5% and the *p*-value is significant. For each comparison, the month with the highest abundance of sequences is highlighted in bold.

Materials and methods section, including only samples collected during the same months at both sites. PERMANOVA confirmed the significant differences between sites ($p < 0.001$; $R^2 = 0.136$) found with the overall dataset. The ANOSIM analysis (Figure 4) was also performed to investigate the differences

between sites. The global ANOSIM for “location” returned an *R*-value of 0.146 with a *p*-value of 0.002, indicating a significant but moderate difference between the eDNA communities from the two sites. In fact, PCoA showed some overlap between the northern and the southern site, but a visible separation

location, OTUs
Anosim R = 0.14546 p = 0.002

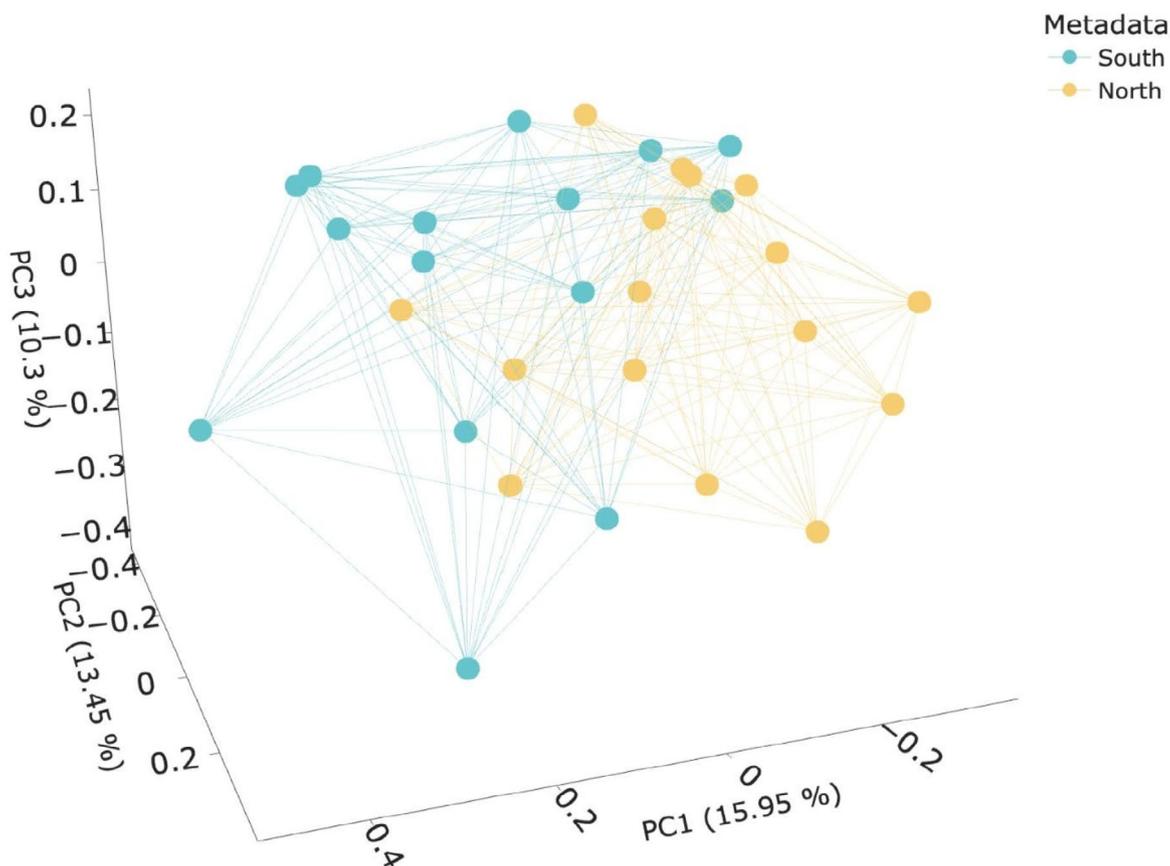


FIGURE 4 | PCoA plots based on Jaccard similarity index comparing northern (PDR, yellow) and southern sites (CH, blue). The ANOSIM test results are also reported. The analysis was performed on a reduced dataset including only samples collected in the same month at the two sites (see methods).

highlighted spatial differences in the eDNA signals (Figure 4). Several species were found to be more abundant at one site than at the other (Figure 5). In particular, species such as *Nephtys hombergii* and *Hydrobia acuta* were more abundant at the northern site (PDR), while species such as *Venus verrucosa*, *Theora lubrica*, and *Dosinia lupinus* were more abundant at the southern site (CH).

4 | Discussion

The analysis of eDNA in Venice Lagoon waters has been highly revealing about the composition of its marine invertebrate communities, allowing for the characterization of the presence and the spatial and temporal distribution of numerous species across various phyla. Metabarcoding with the 16S Echi02 primer set developed by Taberlet et al. (2018), tested empirically for the first time in this study, detected not only echinoderms but also other benthic species, particularly mollusks, even though eDNA was isolated from surface waters.

Regarding echinoderms, all represented in our study area exclusively by benthic species, we detected 8 out of the 15 species known to inhabit the Venice Lagoon (Vatova 1950; Tagliapietra

and Sigovini 2010). Among the seven species not detected, three (*Labidoplax digitata*, *Ocnus planci*, and *Trachythyone elongata*) lacked 16S reference sequences in GenBank at the time of this study (accessed on 12/12/2024). For the other three undetected species (*Astropecten platyacanthus*, *Asterina gibbosa*, *Schizaster canaliferus*), we note that the expected length of the Echi02 amplification products (range 329–346 bp including primers, barcodes and 1–4 random nucleotides, see methods) is outside the size range detectable with the Illumina 150 bp -end sequencing and the pipeline used in this study, leaving only *Holothuria tubulosa* as a species being potentially detectable but not found. For future metabarcoding studies using Echi02, we recommend adopting a sequencing format capable of targeting longer sequences.

Our results also show how eDNA analysis with Echi02 is useful to detect and track both potential and established invasive species, providing an opportunity to assess how environmental changes or anthropogenic impacts may affect communities over time. Using this primer set, we detected bivalve species such as *Theora lubrica*, *Mytilopsis leucophaeata*, and the shipworm *Teredo bartschi*, known to be invasive and associated with port environments (Balena et al. 2002; Van Der Gaag et al. 2017; Tagliapietra et al. 2021). Hence, the prospect of

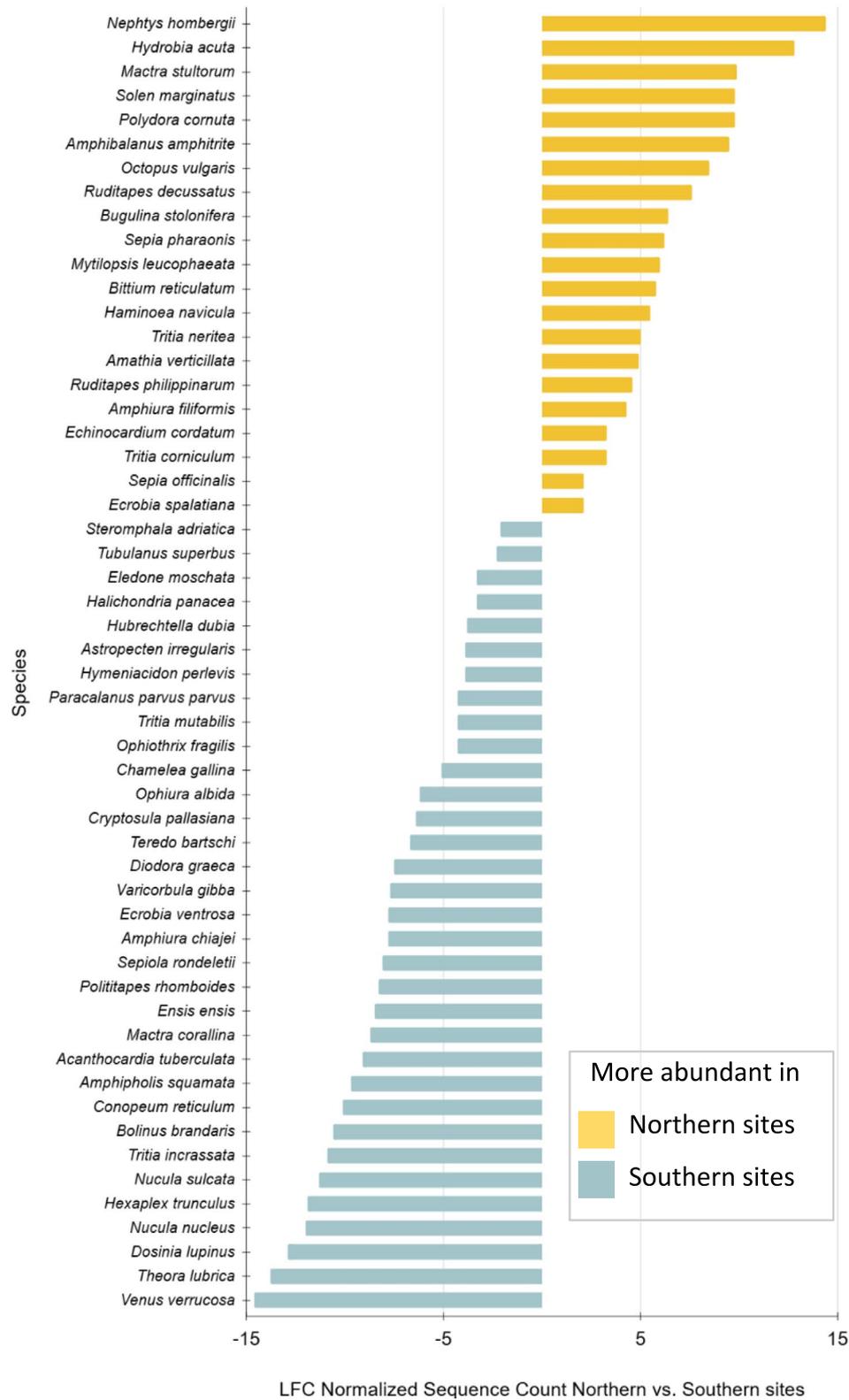


FIGURE 5 | Species differences in eDNA detection between northern and southern Venice Lagoon sites. The ratio between normalized sequence counts (*Cerastoderma glaucum* excluded, see main text) in northern (PDR) and southern sites (CH) was plotted as Log2 Fold Change (LFC). To enable Log2 calculation, a value of 1 was added to sequence counts of zero. Only species with absolute LFC > 1 are reported.

monitoring the presence of invasive mollusks potentially transported by ballast waters in port areas using 16S rRNA mtDNA gene amplicons obtained from eDNA samples is intriguing and promising.

Overall, 10 of the 75 species detected through eDNA (Table 2) have not been previously recorded in the Venice Lagoon. Five of these 10 species, including *Amphibalanus improvisus*, one of the crustacean species with the highest risk of invasiveness for

the Mediterranean Sea (Stasolla et al. 2021), are reported in the World Register of Introduced Marine Species (WRiMS).

Specifically, our results provide the first presumptive detection of the annelid *Capitella teleta*, a newly described species similar to the well-known invasive species *Capitella capitata* (Blake 2009). We also detected six bryozoan species, most of which are recognized as common port pests, including the widespread *Amathia verticillata* recorded in the Venice Lagoon since 2012 (Katsanevakis et al. 2020). Two of these six bryozoan species, *Conopeum reticulum* and *Scrupocellaria maderensis*, have never been observed, to the best of our knowledge, in the Venice Lagoon. The first one is reported as invasive in WRiMS and as a non-indigenous species in the Mediterranean Sea (Galil 2004), while the second one is reported as present in Italian seas in the SIBM Italian checklist but has never been identified in the Northern Adriatic.

Among the bivalves, it is particularly worth noting the detection of *T. lubrica*, because it is a known nonindigenous Asian species that arrived in Italian seas in the early 2000s (Bogi and Galil 2007) but has never been reported in the waters of the Venice Lagoon. Similarly, the possible presence of *Corbicula fluminea*, detected in April in one replicate from each site, is intriguing, since this is an invasive freshwater bivalve notorious for its harmful effects on freshwater ecosystems (Novais et al. 2017), and it is already present in the major lakes of northern Italy (López-Soriano et al. 2018; Gomes et al. 2020), as well as in numerous Italian rivers and wetlands (Paganelli et al. 2018). Two species of the genus *Macra* were also identified, with *M. corallina* not reported yet in the Venice Lagoon; this species, however, is also absent from the Italian checklist as it was included in *M. stultorum* at the time of listing, when its species status was still debated (Guarniero et al. 2010). *Ecrobia spalatiana*, sister to the native *E. ventrosa*, also represents a first identification, though only based on eDNA.

For the poriferan *Halichondria panicea*, the signal detected is very weak (only two sequences were detected in a single sample), but it can nevertheless represent an early warning signal for the Venice Lagoon, as this species is known to be a pest organism and harmful to aquatic ecosystem balances (Fröhlich and Barthel 1997). Finally, the detection of *Hubrechtella dubia*, a nemertean worm not listed as invasive, deserves further investigation, given its distribution range in the North Atlantic (Scandinavian waters).

Several OTUs attributed to exotic species likely trace back to waste materials from the fish market processing in Chioggia. Based on the analysis of the CLODIA database, the passage of species labeled as “commercial species” (Table 2) through the fish market of Chioggia was confirmed. All these species are present in a single sample from the Chioggia site, which is consistent with accidental contamination with fish market wastewaters. An exception to this reasoning is the species *Sepia pharaonis*, which is present also in multiple samples from the northern PDR site, which is far from the sources of water processing contamination. Although the presence of this species is still debated (Bello et al. 2020), considering that it is invasive in the Southern Mediterranean Sea (Mienis 2003) we cannot exclude that its detection in the Venice Lagoon using eDNA represents a signal of further northward spreading.

R. philippinarum frequently emerges in our dataset as the primary contributor to variations between months, particularly notable in comparisons involving spring against other seasons (Table 4, Figure 6). This observation is consistent with a significant increase in the number of reads attributed to this species during this period. It is noteworthy that there are two “peaks” of occurrence, which coincide with its longer and multimodal reproductive period compared to the native *R. decussatus* (Laruelle et al. 1994; Delgado and Pérez-Camacho 2007). The read counts indicate that *R. philippinarum* is much more abundant in the samples than *R. decussatus* (Figure 7) and the temporal trend suggests that, in the northern part of the Venice Lagoon, *R. philippinarum* has two main spawning peaks, one in April–May and one in July, while *R. decussatus* has a single peak in July–August.

Regarding *O. fragilis*, it appears to be significantly more abundant in spring (April and May) compared to the summer. The occurrence of DNA traces of *O. fragilis* may be due to its reproductive cycle. During their planktonic phase, which lasts from 3 to 6 weeks, the larvae usually reach the surface, which is consistent with the occurrence of *O. fragilis* eDNA reads detected in this study. However, the spawning period of this species, according to the literature is in summer (Lefebvre et al. 1999). This discrepancy may be due to the fact that the cited study was conducted in the English Channel, where water temperatures are lower than in the Venice Lagoon, and several studies observed a link between spawning time and temperature in *O. fragilis* (Fenaux 1972; Lawrence 1973; Tyler 1977; Morgan and Jangoux 2002).

Even though various eDNA metabarcoding features strongly challenge the retrieval of quantitative estimates, due to factors such as PCR inhibitors (Lance and Guan 2020) and PCR bias (Silverman et al. 2021) that can produce a disproportion between the real amount of DNA (number of sequences) and sequencing reads (Coghlan et al. 2021), previous studies have reported a link between the reproductive spawning period of fishes and the amount of eDNA reads detected (Bylemans et al. 2017; Tsuji and Shibata 2021; Wu et al. 2022; Collins et al. 2022). In this study, we find a similar pattern, where peaks often correspond to periods when gametes and/or larvae presence in the water is expected.

Two peaks in the abundance of *Octopus vulgaris* eDNA, a first one in April–May and a second one in October, were also observed, coinciding with reported spawning peaks (Hernández-García et al. 1998; Katsanevakis and Verriopoulos 2006). A similar temporal trend was observed in two cuttlefish species, the local and more abundant *Sepia officinalis* and the exotic *S. pharaonis*, with a peak in spring and a second one in October (Figure 6). As mentioned before, *S. pharaonis* is a commercial species that is invasive in the Southern Mediterranean Sea, for which we cannot exclude that its detection in the Venice Lagoon using eDNA represents a true signal rather than a contamination from waste materials of the local fish market. The consistent overlap of temporal variation in read occurrences of the two *Sepia* species might simply reflect sequencing errors. However, considering that the ASVs attributed to *S. pharaonis* and *S. officinalis* showed a substantial genetic divergence (84% identity using BLAST+), we believe that this hypothesis is unlikely,

Heatmap of significant SIMPER test species

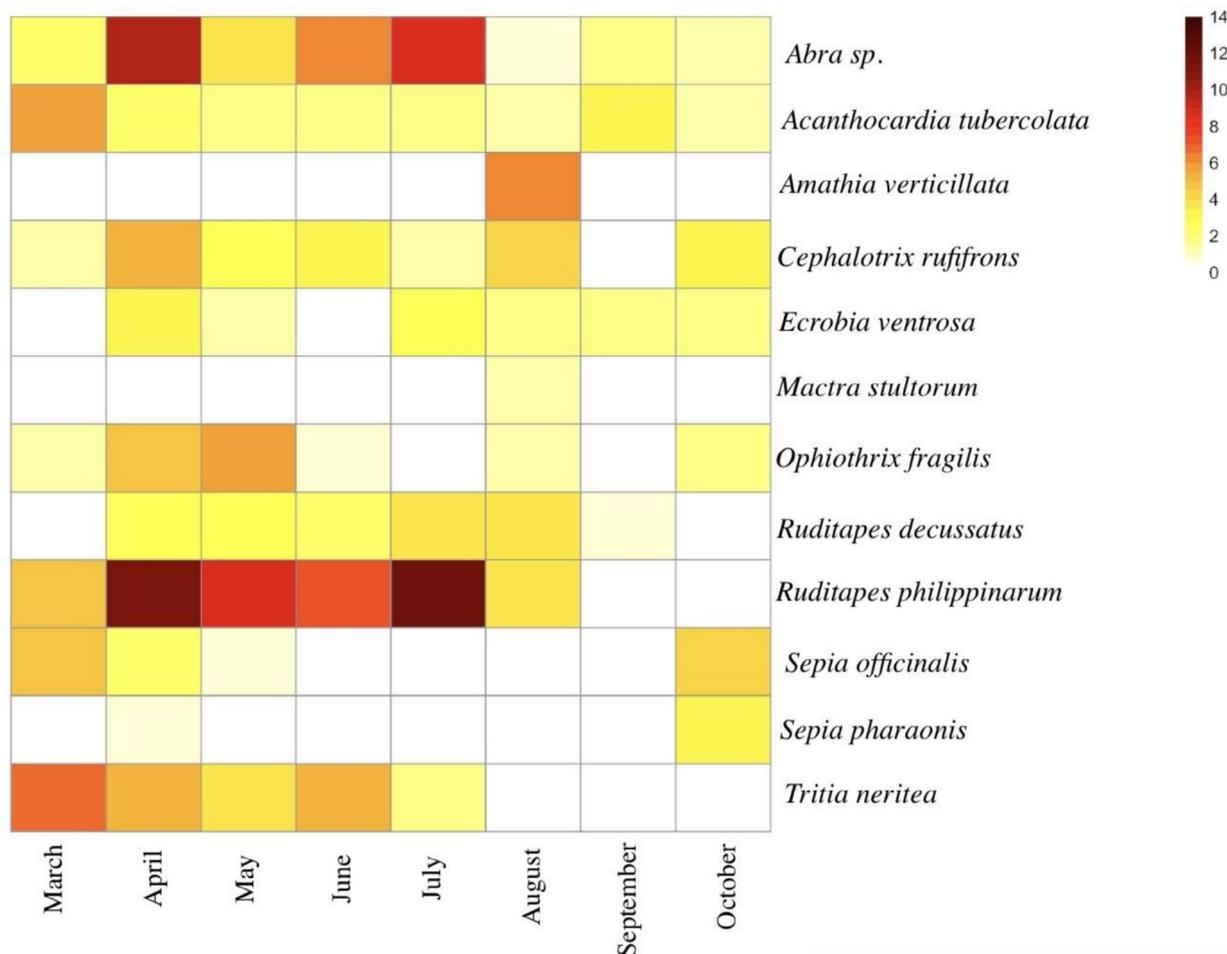


FIGURE 6 | Heatmap of significant species identified by SIMPER analysis. To normalize the abundance values, the plot is made using log-transformed data $[\log(1+x)]$. A value of 0 indicates absence, while a value of 14 indicates the highest abundance in the dataset.

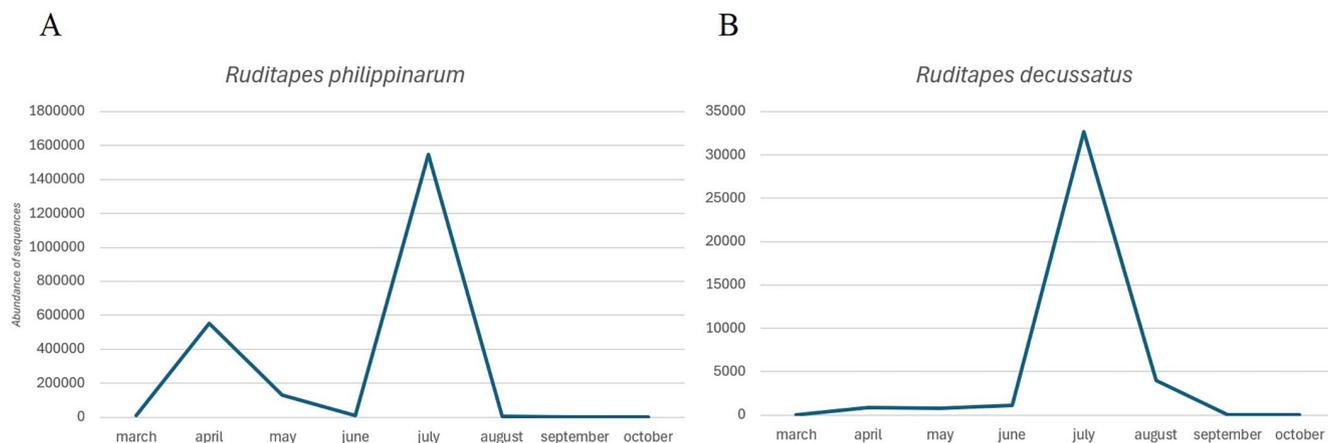


FIGURE 7 | Abundance of sequences of (A) *R. philippinarum* and (B) *Ruditapes decussatus* over the months of sampling. In panel (A), two peaks of abundance are visible, while in panel (B), there is just one in the summer season.

making our first eDNA detection of *S. pharaonis* in the Venice Lagoon intriguing and requiring further investigation.

Overall, the temporal patterns observed in this study provide valuable ecological insights when considered in the context of

the biology of the species. Additionally, significant spatial differences were detected at the lagoon scale, which may reflect environmental gradients such as salinity or anthropogenic influences, including proximity to urban runoff or maritime activity (Newton et al. 2014). For example, the higher abundance

of *Nephtys hombergii*, a euryhaline polychaete with a preference for soft bottoms (Arndt and Schiedek 1997), and *Hydrobia acuta*, a brackish-water gastropod (Magni and Gravina 2023), in the northern site aligns with the lower salinity observed in this part of the Venice Lagoon (Ghezzi et al. 2011). Conversely, the overrepresentation of *Venus verrucosa* and *Dosinia lupinus*, typical marine species (Tunberg 1983), in the southern Venice Lagoon may reflect its proximity to the Chioggia inlet, which connects the lagoon to the Adriatic Sea. Interestingly, the spatial differences detected for invertebrates in this study closely align with previously reported eDNA results on the Venice Lagoon fish community at the same sites (Cananzi et al. 2022). Those findings indicated that exclusively marine species are largely restricted to the southern site, while freshwater fish were recorded in the northern lagoon. Finally, the strong overrepresentation of the invasive bivalve mollusk *Theora lubrica* in the southern site may be linked to the proximity of our sampling site to the Chioggia port, which could facilitate its preferential spread through ballast water discharge, the main dispersal vector for this species (Bogi and Galil 2007).

5 | Conclusions

The comprehensive analysis of benthic community dynamics using eDNA metabarcoding with Echi02 from surface waters in the Venice Lagoon has provided valuable information on both spatial and temporal variations, with important implications for biodiversity monitoring and management. Our results reveal differences in marine invertebrate communities between the northern and southern sites of the lagoon. One of the most interesting findings of our study is the identification of several invasive species, such as *T. lubrica*, *A. improvisus*, and the shipworm *T. bartschi*, known to be associated with port environments and ballast waters transport (Bogi and Galil 2007; Chapman et al. 2013; Tagliapietra et al. 2021). These results highlight the potential of eDNA metabarcoding as a powerful tool for early detection and monitoring of invasive species. In fact, we identified several taxa never previously reported in the Venice Lagoon, such as *C. teleta*, *C. reticulum*, and *S. maderensis*, some of them known for their invasive potential. This highlights the dynamic nature of the lagoon ecosystem and the ongoing changes in species composition, due to both natural colonization processes and anthropogenic influences. The seasonal trends observed in species such as *R. philippinarum*, *O. fragilis*, and *O. vulgaris* provide further insights into the reproductive cycles and ecological dynamics of these marine invertebrates. For instance, the bimodal peaks of *R. philippinarum* and the spring abundance of *O. fragilis* larvae suggest that seasonal reproductive strategies contribute to the temporal variability in eDNA signals. In addition, to the best of our knowledge, this is the first study to use Echi02 primers, and it is interesting to note that, although they were designed for echinoderms, they not only identified most of the echinoderms known to occur in the Venice Lagoon but also showed a remarkable ability to detect a wide range of mollusks.

In conclusion, this study validates the utility of eDNA metabarcoding from surface waters in capturing the complex spatial and temporal dynamics of benthic communities in the

Venice Lagoon. The ability to detect both native and invasive species, monitor seasonal changes, and identify previously unreported species reinforces the importance of this approach for biodiversity conservation and ecosystem management. Continuous monitoring and adaptive management practices that consider both spatial heterogeneity and temporal variability will be key to maintaining the ecological health and consequent resilience of the Venice Lagoon in the context of environmental changes.

Author Contributions

F.M., G.C., I.G., E.B., I.A.M.M., and L.Z. conceived and designed the experiments. F.M., G.C., I.G., and S.F. performed the experiments. F.M., G.C., I.G., and T.L. analyzed the data. E.C., M.P., A.S., A.V., E.N., and L.C. contributed sampling/reagents/materials/analysis tools. F.M., G.C., I.G., T.L., L.C., I.A.M.M., A.V., A.B., and L.Z. critically discussed results; F.M., G.C., I.G., S.F., and L.Z. wrote the paper, which was revised and approved by all the co-authors.

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Disclosure

Permission to reproduce material from other sources: This manuscript does not include material from other sources.

Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw data underlying this study will be made available, upon acceptance of the manuscript, on public online repositories: Sequence Read Archive (SRA) at NCBI. BioProject accession number: PRJNA1208720. Ancillary information is available on Dryad (<https://doi.org/10.5061/dryad.dbrv15fcz>).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.