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ORIGINAL ARTICLE

eDNA metabarcoding from aquatic biofilms allows studying spatial and temporal fluctuations of fish communities from Lake Geneva

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Abstract

Fish communities are now studied non-invasively using environmental DNA (eDNA) recovered from water samples. The objective of this study is to evaluate the possibility of surveying these communities using fish eDNA passively "captured" by aquatic biofilms. To this end, biofilm samples developing on natural and artificial substrates were collected every 2 weeks for a year and a half in a large lake (Lake Geneva). DNA was extracted from biofilms and fish communities were targeted using a standard 12S barcode with a metabarcoding approach. The fish eDNA signal recovered from biofilms revealed temporal and spatial changes in fish communities. These changes were linked to fish habitat preferences and spawning season. Peaks in the eDNA signal of some taxa fitted with their spawning period reported in the literature. We evidenced that the nature of the biofilm's substrate (natural or artificial) does not affect the image obtained of the fish community composition. Furthermore, by using biofilms grown on artificial substrates, the studied temporal window of the eDNA signal can be controlled. With biofilms acting as environmental passive samplers, our results open up the possibility to accurately monitor fish communities and their temporal and spatial changes with eDNA in a faster and less expensive way than with the classical water filtration approach.

KEYWORDS
aquatic biofilms, community, eDNA, fish, freshwater, passive sampler

1 | INTRODUCTION

Freshwater fishes make up 40% of global fish diversity on earth (Dudgeon et al., 2006; Sales et al., 2021). They provide valuable goods to human society such as protein source, health care, leisure, culture, manufacturing, and industry (Olden et al., 2020) as well as ecosystem services such as nutrient cycling, trophic dynamics, and ecosystem resilience (Holmlund & Hammer, 1999). Fishes are recognized as the more threatened organisms in freshwater ecosystems because of anthropic activity like overexploitation, destruction of habitats, invasion by exotic species, or pollution of aquatic ecosystems (Zhao et al., 2016). Thus, studying and monitoring fish community composition is necessary for conservation and management purposes, in particular, those concerning fishery and aquaculture and also endemic and threatened species. This conventionally involves collecting, identifying, and counting fish specimens using gillnets techniques or electrofishing (Bonar et al., 2009). However, these techniques are inefficient for capturing rare or elusive species

in addition to being invasive and lethal (Snyder, 2003). Non-lethal techniques as visual census (Holubová et al., 2019) or hydroacoustics (Godlewska et al., 2004; Pollom & Rose, 2016) have also been used. However, these methods also show some weaknesses such as the fact that visual census is highly affected by water transparency (Holubová et al., 2019), while hydroacoustics requires biological data to verify species composition as well as highly trained personal for successful data collection and interpretation, bearing in mind that acoustic range and data quality are impacted by environmental conditions (e.g., wind, waves; Boswell et al., 2007).

Nowadays, environmental DNA (eDNA) metabarcoding is recognized as a useful tool to characterize fish communities (Blabolil et al., 2021; García-Machado et al., 2021; Hänfling et al., 2016; Sales et al., 2021). Fish eDNA refers to DNA shed by these organisms into the water column and that can be recovered, extracted, and amplified using universal markers (e.g., 12S rRNA or 16S rRNA; Shu et al., 2021). The amplified eDNA is then sequenced using high-throughput sequencing technologies and fish inventories are obtained by matching the obtained DNA sequences to sequences from a reference DNA library (e.g., MitoFish, Meta-Fish-Lib; Collins et al., 2021; Iwasaki et al., 2013). eDNA is usually recovered by filtering large volumes of water which is time consuming and requires pumping equipment (Bessey, Jarman, et al., 2021). Furthermore, there is no consensus on either the volume of water or the filtration method (filter pore size, and filter type), both of which are known to affect eDNA capture (Capo et al., 2020; Jo et al., 2020). Faced to the methodological constraints of using filtered water samples for eDNA fish surveys, new approaches based on natural passive samplers were developed in marine environments. Marine sponges, organisms that naturally filter thousands of liters per day, were first used as natural eDNA samplers, as they entrap and concentrate eDNA in their tissues (Mariani et al., 2019). The suitability and the cost-effectiveness of the use of marine sponges as natural eDNA samplers to assess fish diversity in hyper-diverse environments were confirmed by comparison to visual census data (Turon et al., 2020). The use of filter membranes directly submerged in water column and that collect eDNA passively was also proposed (Bessey, Jarman, et al., 2021). A variety of materials were proposed to increase eDNA adsorption therefore its capture (e.g., montmorillonite clay, granular active carbon, cellulose, cotton fibers, and sponges with zeolite or active carbon, between others; Bessey, Gao, et al., 2021; Kirtane et al., 2020).

In a previous study, we showed that in freshwater ecosystems, aquatic biofilms can be used as natural fish eDNA samplers since reliable fish molecular inventories can be obtained from this environmental matrix (Rivera et al., 2021). However, the capacity of eDNA captured by aquatic biofilms to provide temporal and spatial information on fish communities still needs to be addressed. Thus, in this study, we wanted to test the hypothesis that the fish eDNA signal detected in environmental biofilms can track temporal and spatial (between sampling sites) changes in fish communities.

For this, we sampled biofilms from natural and artificial substrates at two different sites (harbor and beach) of the shoreline of Lake Geneva every 2 weeks over a year and a half, and we looked at how fish communities changed over time and between sampling sites using DNA extracted from biofilms and a metabarcoding approach.

2 | MATERIALS AND METHODS

2.1 | Study area and sampling sites

Two sampling sites were located at the shoreline of Lake Geneva (Thonon les Bains, France) as described in Rivera et al. (2021). Biofilm samples were collected at these two sites every 2 weeks over a year and a half period, from November 2018 to January 2020.

One site was located in a small harbor and the other one at a stony beach about 100 m from the harbor. Biofilms collected at the beach site (from now "beach biofilms"), consisted on natural biofilms collected directly on beach stones (Figure 1). The age of the biofilms may range from a few days to 1 month or more. Indeed, beach biofilms were regularly submitted to wave action resulting from prevailing northeast winds. Depending on the wind speed, waves can vary from few centimeters to 1 m when winds reach 30 km/h or more. In exceptional cases, winds between 70 and 80 km/h can occur which give rise to 2 m waves which completely strip biofilms from the stones.

At the harbor site, no stones were available for biofilms development, thus biofilms collected in this site (from now "harbor biofilms") developed on artificial substrates (Plexiglas plates) that were displayed for a controlled colonization period of 15 days (Figure 1). In contrast to beach biofilms, harbor biofilms were protected against wind events and wave action by a jetty.

2.1.1 | Beach biofilms

Beach biofilm collection was conducted as described in Rivera et al. (2021). Briefly, at each sampling date, three biological replicates were collected (A, B, and C; Figure 1) along the shoreline of the stony beach. Biological replicates were separated by 1–1.5 m. For each replicate, five stones situated at 60–70 cm depth were collected randomly and placed in a clean basin in order to drain off the water from the lake, thus avoiding the collection of fish eDNA that may be present in the water. Then, a known surface of the biofilm of 187 cm^2 was recovered by placing a plastic mold on the upper surface of each stone and scraping of the biofilm from the delimited area with a clean toothbrush and ultra-purified water (Milli-Q). Beach biological replicates were stored separately in sterile 50 mL Falcon tubes.

2.1.2 | Harbor biofilms

Harbor biofilms were collected from nine Plexiglas plates of 215 cm^2 each which were displayed on a 6 m long rope at 15 cm depth for biofilm colonization, 15 days prior to sample collection. At each sampling date, three biological replicates (A, B, and

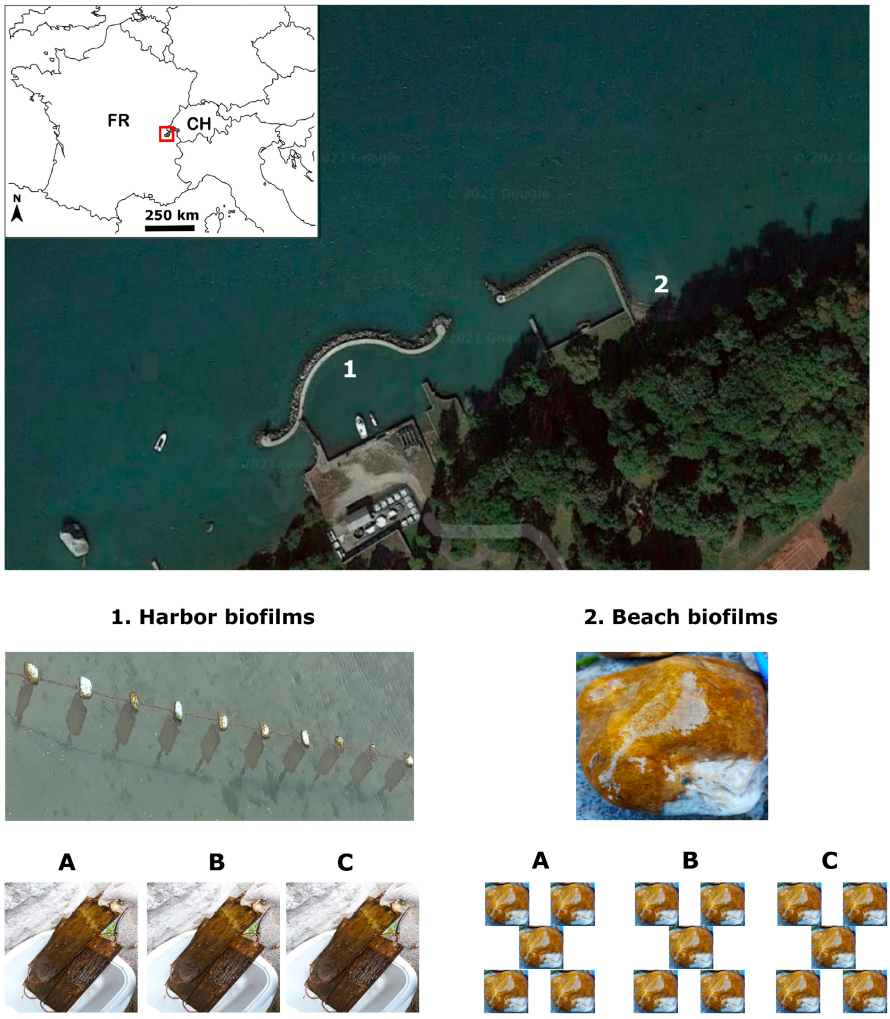

FIGURE 1 Location of the sampling sites and illustrations of the biofilms collected at the harbor and at the beach.

C) were performed (Figure 1). For each replicate, three Plexiglas plates were placed in a clean basin to drain off the water from the lake. Then, biofilm was recovered by scraping of both sides of the Plexiglas plates with a clean toothbrush and a clean scraper. Harbor biological replicates were stored separately in sterile 50 mL Falcon tubes.

Cross-contamination between biological replicates was avoided by using individual clean material for each sample. Furthermore, fields blanks were performed at each sampling site to check for DNA contamination as described in Rivera et al. (2021). Field blanks consisted on 50 mL sterile Falcon tubes previously filled with ultra-purified water (Milli-Q), carried into the field, and poured into a blank basin. Then, the surface of the basin was scraped with a clean toothbrush and the resulting water was recovered into the 50 mL Flacon tube.

After collection, biofilm samples and field blanks were returned to the laboratory in a cooler in less than 2 h, without any preservative added.

Beach biofilms were collected every 2 weeks from November 2018 to January 2020 (31 biofilms samples and 31 field blanks), while harbor biofilms were collected every 2 weeks from December 2018 to December 2020 (25 biofilms samples and 25 field blanks).

2.2 | Biofilm characterization

Beach and harbor biofilm biomass was quantified as ash-free dry mass (AFDM). For this, 2 mL of each biofilm sample was filtered into a 47 mm pre-weighed GF/F glass microfiber filter (Whatman™). The filter containing the slurry was first dried at 105°C for 24 h and finally ignited at 500°C for 4 h (ash-free weight). AFDM was expressed in mg/cm^2.

2.3 | Total DNA extractions from biofilms

Immediately after sampling, biofilms samples were manually homogenized and a volume of 2 mL of each sample was used as starter for DNA extraction. Then 2 mL samples were first centrifuged at 8000*g* for 10 min at 4°C in a 2 mL Eppendorf© tube and the supernatant containing water was discarded. The remaining pellet was preserved at −20°C until DNA extraction. Total genomic DNA was extracted from the pellets using the Macherey-Nagel NucleoSpin® Soil kit (Macherey-Nagel, GmbH) with a final elution volume of 30 µL following the manufacturer instructions.

2.4 | Fish DNA amplification from biofilms and sequencing

PCR amplification of fish communities was performed by targeting a short fragment (163–185 bp) of the mitochondrial 12S rRNA gene using the MiFish-U-F and MiFish-U-R primers (Miya et al., 2015). Forward and reverse primers were tagged respectively with half of the 5′-CTTTCCCTACACGACGCTCTTCCGATCT-3′ and half of the 5′-GGAGTTCAGACGTGTGCTCTTCCGATCT-3′ Illumina adapters in order to prepare Illumina libraries in a dual-step PCR approach. For the PCR1, each DNA sample was amplified in a final volume of 25 μL using the tailed Mifish-U primers and the AmpliTaq Gold DNA Polymerase following mix and reactions conditions used previously in Rivera et al. (2021). Three PCR replicates per sample were performed. Each PCR replicate was composed of three individual PCR which was subsequently pooled together. When PCR failed, extra individual PCR was performed to reach the required number of technical replicates. In some cases, amplification of the beach and the harbor biofilm samples was not reached (Table S1). Two negative controls were included in each PCR to check for potential contamination. Two mock communities of know composition were used as a PCR-positive control to monitor the performance of the amplification and the sequencing process. Mock community construction is described in Rivera et al. (2021) and its composition (from now "expected" mock community) can be found in Table S2.

After PCR1, PCR-negative controls and field blanks appeared negative, however, they were transferred to the GenoToul Genomics and Transcriptomics platform (GeT-PlaGe, Auzeville, France) along with all PCR replicates of each sample (beach and harbor biofilms and the mock communities). At the platform, a second PCR (PCR2) amplification was performed using the purified PCR1 amplicons as template and the Illumina-tailed primers to add dual-index specific to the samples. They furthermore prepared the final pool that corresponds to an equimolar mix of the 390 PCR2 dual-indexed amplicons and carried out the sequencing of the pool using Illumina MiSeq sequencing with V2 paired-end sequencing kit (2 × 150 bp). Sequencing was carried out in two different runs, each run contained a mock sample in triplicate to evaluate sequencing performance between the two runs. The composition of each mock community resulting from sequencing (from now "observed mock community") is detailed in Table S2. Since field blanks and PCR-negative controls were confirmed negative during PCR2 they were not included in the sequencing process.

2.5 | Bioinformatics

Sequence data processing of all samples (biofilm samples and mock communities) was performed using *DADA2 v1.16.0* (Callahan et al., 2016) as implemented in R (R Core Team, 2020) and following the bioinformatics workflow described in Rivera et al. (2021) which was previously validated against a mock community. Briefly, primers were removed from the forward and the reverse reads using cutadapt 2.9 (Martin, 2011), then forward and reverse reads were quality filtered using the *filterAndTrim* function using default settings.

Forward and reverse sequences shorter than 120 nucleotides, containing ambiguous nucleotides and with more than two expected errors were removed. R1 and R2 reads were then dereplicated into individual unique sequences using the *derepFastq* function. Amplicon sequence variants (ASVs) were selected based on the error rate models determined by the DADA2 denoising algorithm and paired reads were merged into one sequence using the *mergePairs* function. Only paired reads presenting an identical overlap region of at least 30 bp were kept. Finally, chimeric ASVs were identified and removed using the *removeBimeraDenovo* function and the resulting ASV were taxonomically assigned in Mothur software *v.1.44.3* (Schloss et al., 2009) using a local reference fish library (available at https://doi.org/10.15454/AU8EZI) and the naïve Bayesian classifier method (Wang et al., 2007) with a confidence score threshold of 75%. Local reference library construction and curation are detailed in Rivera et al. (2021). Only ASVs belonging to fish (Teleostei) were kept for further analysis (Table S3). The number of remaining reads after each bioinformatics step is detailed in Table S4.

After taxonomic assignment, further manual refining of the dataset was performed by completing taxonomic assignment of some taxa that could not be identified at species level and remained as "unclassified" (e.g., *Coregonus* "unclassified," *Silurus* "unclassified," *Phoxinus* "unclassified"). We were able to make these modifications because only one species of each of these different genera occurs at Lake Geneva, therefore avoiding any risk of misidentification. In addition, taxa from the same genus but assigned to difference species (e.g., *Salvelinus fontinalis*, *S. alpinus*, and *S. namaycush*) were combined into one single species (*S. alpinus*) given that there is a single *Salvelinus* in Lake Geneva.

Raw sequences of the mock community and the environmental samples are available at doi: 10.5281/zenodo.5864552.

Details of the manual refining are available in Table S5.

2.6 | Data analysis

Further data filtering was performed as described in Rivera et al. (2021): (i) PCR replicates were used to select only taxa shared by 2/3 replicates in order to avoid false presence; (ii) the expected composition of the mock community was compared to the composition obtained after sequencing to check for potential contamination during the sequencing process. When unexpected taxa were detected in the sequenced mock, a correction threshold was applied to the data to remove this potential contamination; (iii) biological replicates were pooled together in order to minimize biofilm site-variability. After this, a list of fish taxa and their relative abundances, based on read abundances, was produced for each sample (Table S6). Several studies have previously used fish eDNA reads as a fair proxy of species relative abundance for studying fish communities through eDNA metabarcoding using MiFish primers

(Berger et al., 2020; Hänfling et al., 2016; Shu et al., 2022; Ushio et al., 2018).

2.6.1 | Fish community composition derived from environmental biofilm samples

Statistics were carried out in R software (R Core Team, 2020). Non-metric multidimensional scaling (nMDS) was used to graphically evaluate the capacity of environmental biofilms to reveal fish community composition and to provide information on temporal and spatial changes. nMDS was performed based on Bray–Curtis distances in Vegan package (Oksanen et al., 2015) using the *metaMDS* function. Bray–Curtis distances were calculated from fish relative abundances based on eDNA reads. Temporal changes in fish communities were assessed by grouping dates by season (autumn, winter, spring, and summer) and spatial changes were assessed by grouping samples according to the sampling site (harbor or beach). In both cases, a permutational multivariate analysis of variance (PERMANOVA) was performed using the *pairwise. Adonis* function from the Vegan package (Oksanen et al., 2015). Finally, in order to know which species were responsible of the temporal and spatial differences, an Indicator species analysis was performed using the *multipatt* function from the *indicspecies* package (De Cáceres & Legendre, 2009). Temporal and spatial variation of the eDNA reads abundances of the resulting indicator species were graphically represented.

2.6.2 | Effect of biofilm's substrate on fish community composition

A Mantel test (Legendre & Fortin, 2010) was performed to evaluate the relationship between the fish community composition recovered from biofilms developing on natural and artificial substrates and their biomass. The test was performed between a Bray–Curtis dissimilarity matrix calculated for fish eDNA read abundances and a Euclidean distance matrix calculated for biofilm biomass in the statistical software *PAST v. 3.25* (Hammer et al., 2001).

3 | RESULTS

3.1 | Sequencing results and data filtering

Sequencing of all samples resulted in 18,091,518 DNA reads. After primers removal, quality filtering, denoising, merging, and removal of chimeric sequences 11,436,773 reads were kept (63%) from which 10,807,655 (94.5%) corresponded to Teleostei taxa (Table S4).

In total, 525 ASV were generated of which 466 were assigned to fish (Table S3). After taxonomic and further data refining (removal of fish taxa occurring only in one PCR replicate and pooling of the biological replicate) a total of 15 fish taxa were kept. Detected taxa correspond to fish species reported to occur in Lake Geneva (Alexander & Seehausen, 2021). Only nine taxa were detected at the beach site compared to the harbor site where 15 taxa were detected (Table S7).

3.2 | Spatial differences between fish communities derived from biofilms samples

The nMDS shows differences between fish communities detected from environmental biofilms at the beach and at the harbor site (Figure 2). Results of the PERMANOVA test reveal that differences between harbor and beach sites are statistically significant ($F=6.342$, $R^2=0.143$, $p<0.001$). According to the results obtained from the Indicator Species Analysis, these differences are mainly explained by the detection of *Coregonus lavaretus* ($p=0.006$) and *Squalius cephalus* ($p=0.023$) in large abundances at the beach compared to the harbor. On the other hand, the detection of *Gasterosteus aculeatus* only at the harbor and of *Tinca* in large abundance at the harbor compared to the beach is also responsible of the differences observed between both sites ($p_{G.\ aculeatus}<0.001$, $p_{T.\ tinca}<0.001$, respectively).

3.3 | Temporal differences between fish communities derived from biofilms samples

Temporal differences in fish communities at the harbor and the beach were evaluated separately by grouping the data of each site according to the season.

At the beach, fish communities detected during winter were different from those detected during fall ($F=5.00$, $R^2=0.277$, $p=0.01$) and during spring ($F=5.337$, $R^2=0.348$, $p=0.02$). The indicator species analysis showed that *C. lavaretus* was the main species detected at the beach site during winter ($p=0.0199$) while *Perca fluviatilis* was more abundant during fall and spring ($p=0.02$).

At the harbor, fish communities detected during winter also differed from those detected during spring ($F=5.756$, $R^2=0.324$, $p=<0.001$), summer ($F=5.337$, $R^2=0.348$, $p=0.02$), and fall ($F=5.337$, $R^2=0.348$, $p=0.02$). The indicator species analysis showed that *C. lavaretus* and *Salvelinus alpinus* ($p=0.034$ and $p=0.002$, respectively) were the most abundant species detected at the harbor during winter, while *G. aculeatus* was more abundant during spring (p value $=0.005$), *T. tinca* during summer (p value <0.0035) and *P. fluviatilis* during fall and spring ($p=0.002$). Temporal and spatial variations in relative abundances of the main indicator species are represented in Figure 3.

3.4 | Effect of biofilm's substrate on fish community composition

Mantel test results evidenced that fish community's composition observed both at the beach and the harbor sites is not influenced by the biofilm's substrate (artificial or nature; $r_{beach}=0.09$; $p_{value}=0.12$; $r_{harbor}=0.07$; $p_{value}=0.15$).

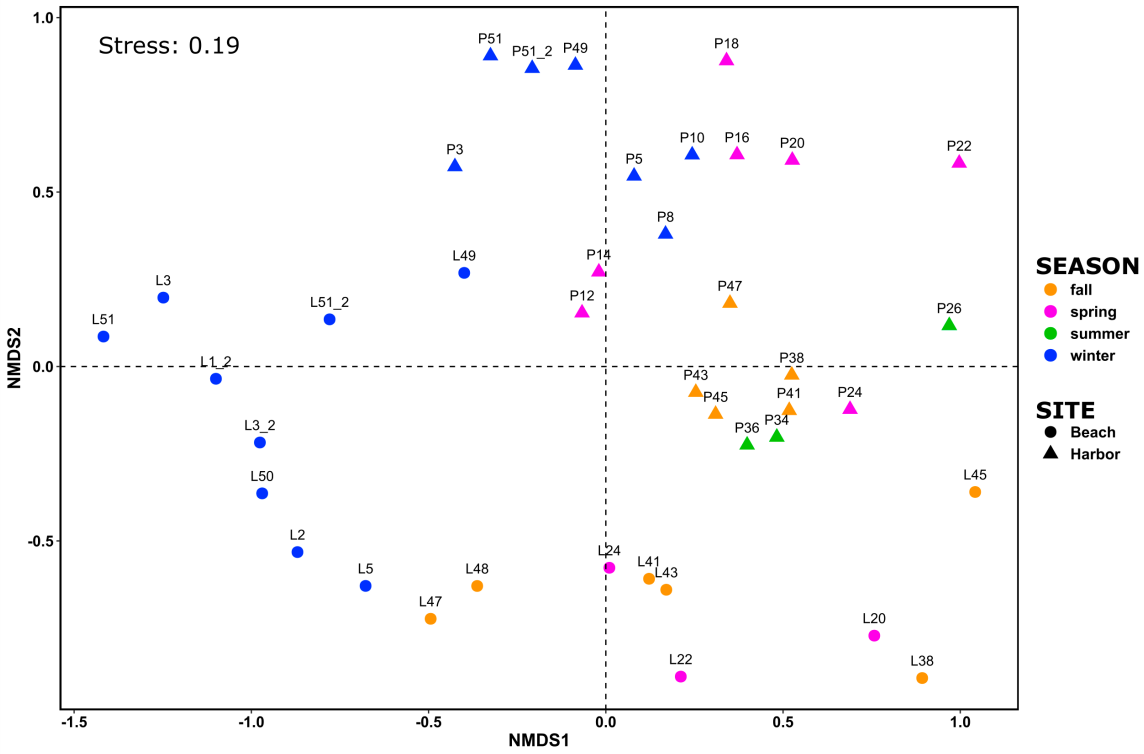

FIGURE 2 nMDS representation of fish communities. Samples are shaped-coded according to the sampling site and color-coded according to the season. NMDS was derived from pairwise Bray–Curtis distances. Each symbol corresponds to a sample ($N = 40$).

4 | DISCUSSION

4.1 | Biofilms eDNA enable to monitor temporal and spatial changes in fish communities

Fish eDNA detected in environmental biofilms enable to characterize fish communities' composition at Lake Geneva and revealed changes in time and space that are related to fish ecology and behavior.

Fish reproduction is the main factor explaining temporal fluctuations of fish communities detected with eDNA in our biofilm samples. Most freshwater fish reproduces by external fertilization through a process called spawning. During spawning, females release large quantities of unfertilized eggs into the surrounding water. This process results in large DNA quantities released in water making eDNA signal of spawning taxa to considerably increase during this period and being captured by environmental biofilms. Recent studies showed that spawning events can be identified from water samples by observing spikes in eDNA concentration after spawning (Bylemans et al., 2017; Takeuchi et al., 2019; Tsuji & Shibata, 2021). According to the taxa, spawning events are mainly driven by water temperature and photoperiod and therefore occur at different times of the year. Of all the fish taxa detected in our study, we will discuss temporal changes in the abundances only for the indicator species (i.e., *C. lavaretus*, *G. aculeatus*, *T. tinca*, and *P. fluviatilis*) following french fish freshwater literature (Keith et al., 2020). Spawning period of *C. lavaretus* in France is reported to occur between mid-November and end of January with a peak of activity in mid-late December (Goulon et al., 2020). Using environmental biofilms as fish eDNA captors, we observed that *C. lavaretus* eDNA signal varied over time and that eDNA peaks matched with the spawning period reported in the literature (Figure 3a). The same kind of pattern was observed for *G. aculeatus*, for which an important increase on its eDNA signal was observed between the months of March and May (Figure 3b) which matches to the reproduction period of this species (from March to July; Keith et al., 2020). *T. tinca* eDNA signal reaches a maximum in June and decreases regularly until November (Figure 3c). The spawning period for this species is reported to occur from May to October depending on water temperature (Keith et al., 2020). *P. fluviatilis* eDNA signal at the harbor was more complex to explain (Figure 3d). On one hand, eDNA spikes observed between mid-April and mid-June fitted to the spawning period reported in literature (between mid-April and mid-June for temperatures from 8 to 10°C, the optimum being 12°C; Keith et al., 2020). On the other hand, we observed latter spikes after spawning period. Indeed, after fertilization, perch eggs hatch in about 12 to 18 days at normal temperatures. This event results in the release of genetic material in the surrounded area which may explain the increase in the eDNA signal that we detected some weeks after the spawning period.

Concerning spatial variability, fish communities revealed by the eDNA signal in biofilms differed between the harbor and the beach (Figure 2). These results indicate that the fish eDNA signal is not the same for biofilms that are 100 m apart. In our previous study, we showed that fish communities obtained from biological replicates separated by about 10 m are homogenous (Rivera et al., 2021). This

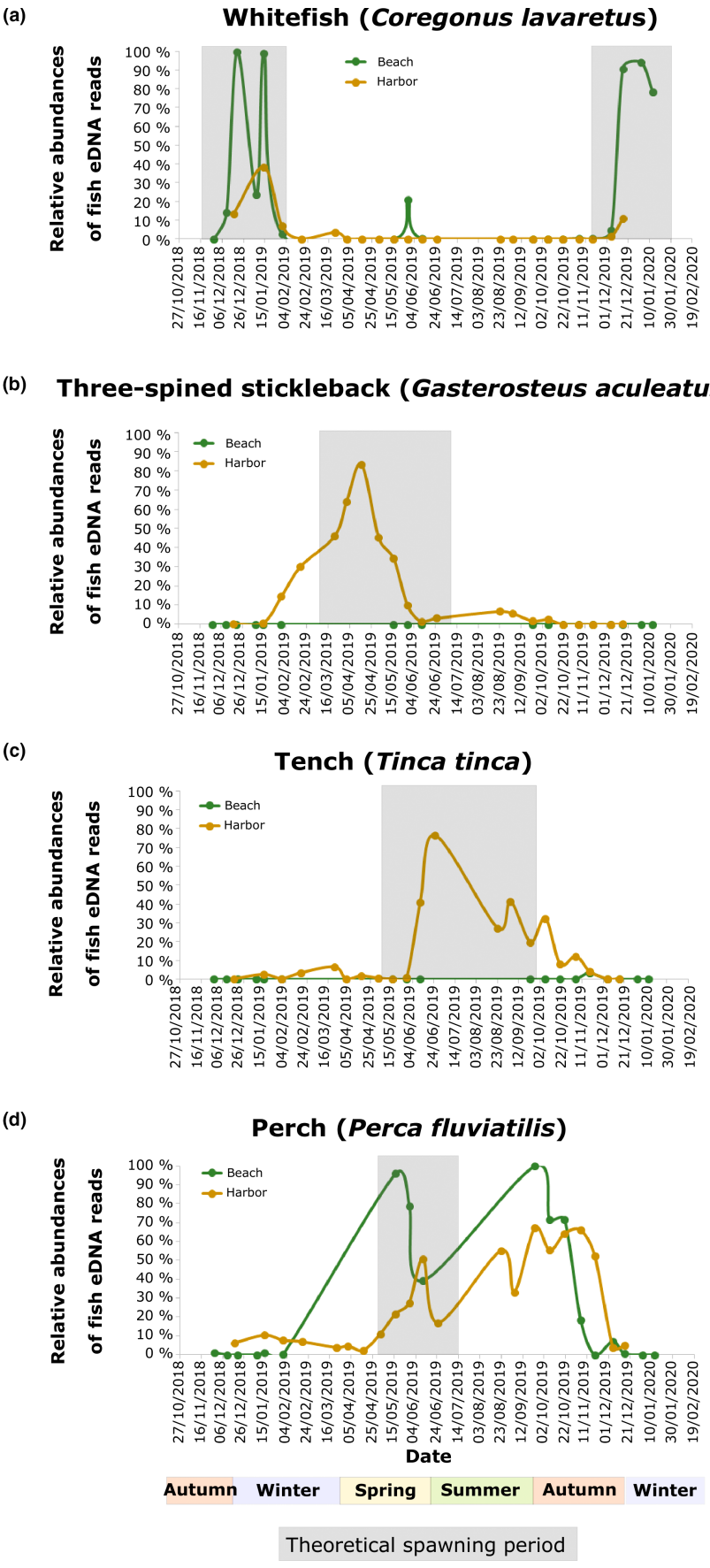

FIGURE 3 Temporal and spatial variation of eDNA reads obtained with DNA metabarcoding from biofilms samples from the beach and the harbor for (a) *Coregonus lavaretus* (whitefish), (b) *Gasterosteus aculeatus* (three-spined stickleback), (c) *Tinca* (tench), and (d) *Perca fluviatilis* (pearch). Gray square represents the spawning period of each species reported in literature (Keith et al., 2020).

suggests that biofilms capacity to provide a spatial signal varies from 10 to 100 meters. Based on the detected indicator species at each sampling site, it seems that differences between sites are explained by fish habitat preferences. Habitat characteristics, especially water depth, water temperature, and substrate structure, are important factors explaining spatial distribution of fish communities in lakes (Oyugi et al., 2014). In our case, fish requirements for a specific spawning ground explain the differences observed between fish communities detected at the harbor and the beach. For example, *C. lavaretus*, a lithophilic-spawning fish, requires rock or gravel bottom at the littoral zone of lakes to deposit their eggs (Balon, 1981, 1995) which are present at the beach. The need for this specific spawning substrate is clearly reflected in Figure 3a where we detected twice as much DNA at the beach compared to the harbor where this spawning substrate is lacking. On the contrary, *G. aculeatus* and *T. tinca* are phytophiles fish which require shallow water with muddy bottoms and vegetation to deposit their eggs (Balon, 1981, 1995). These habitat conditions and spawning substrates were available only at the harbor explaining why these species were not or just poorly detected at the beach (Figure 3b,c).

In the case of *P. fluviatilis*, its habitat preference depends on season. During spring and summer, this species has a preference for the littoral zone and complex habitats (preferably with vegetation for spawning purposes), while during autumn and winter, perch migrates in deep waters (Westrelin et al., 2018). These changes in habitat can also explain the somewhat irregular eDNA signal observed for this species after its spawning period (Figure 3d).

According to Herder et al. (2014) fish habitat preference is a relevant aspect to be considered when choosing sampling sites for eDNA studies. These authors mention that collecting water samples at sites where fish taxa are supposed to be, increases the probability of species detection. Our study shows that these considerations also apply to biofilms since fish eDNA signal varied significantly according to fish habitat preferences.

Recent studies of spawning events with eDNA were performed using species-specific PCR assays as quantitative PCR (qPCR; Bylemans et al., 2017; Tsuji & Shibata, 2021) or digital PCR (dPCR; Capo et al., 2019; Thalinger et al., 2019; Vautier et al., 2021; Yu et al., 2021) since they allow an absolute quantification of the targeted fish taxa which is more appropriate than metabarcoding to provide accurate spawning patterns. In our case, since metabarcoding is affected by PCR bias, some species might appear more abundant than they are because of a higher amplification efficiency. Indeed, high-abundant eDNA may inhibit the amplification of low-abundant ones through binding competition with metabarcoding primers (Yu et al., 2022). By comparing the "expected" relative fish DNA abundance against the "observed" one with our mock community, we could see that even though there is a preferential amplification of few target taxa (e.g., *C. lavaretus*, *P. fluviatilis*), "expected" and "observed" relative abundances are similar for the other taxa (Table S2, from Rivera et al., 2021), showing that eDNA metabarcoding can produce semiquantitative data as already suggested in other studies (Berger et al., 2020; Hänfling et al., 2016; Shu et al., 2022; Ushio et al., 2018).

However, when studying spawning events of specific fish species, using relative abundances may not give accurate results for samples containing several abundant species. This issue can be observed in Figure 3a where we observed an important decrease on the relative abundances of *C. lavaterus* eDNA reads at the beach site on Jan 7, 2019 (from 99.6% to 23.5%) as consequence of a higher abundance of *S. alpinus* (75.7%, see Table S6) at this same date. This shows the importance to use absolute quantification through species-specific approach for such purposes.

Our study is not the only that detected fish spawning events trough metabarcoding. Di Muri et al. (2022) observed peaks or Arctic char (*Salvelinus alpinus*) during spawning seasons at specific locations of known spawning sites as we did in our study for some fish species (i.e., *C. lavaretus*, *P. fluviatilis*, *T. tinca*, and *G. aculeauts*). Even though the information obtained through metabarcoding is not as accurate as that obtained using species-specific PCR assays, metabarcoding allows to have an overview of the trend of the community composition. Then, if some results need to be refined, species-specific PCR (qPCR or dPCR) can be combined to metabarcoding in order to better infer the absolute abundance of fish species as performed by Pont et al. (2022) who combined eDNA metabarcoding with qPCR to infer the absolute abundance of fish species. Another option is the use of internal standard DNAs which correspond to known amounts of short DNA fragments from fish species that have never been observed in a sampling area (Sato et al., 2021; Ushio et al., 2018). These DNAs are added to the collected samples and then sequenced. This new approach, known as qMiSeq, allows an accurate quantification of fish species compared to the traditional metabarcoding approach by converting the sequence reads of DNAs fish detected in the samples (non-standard fish eDNA) into fish eDNA copy numbers thanks to correction equations that are generated using the internal standard DNAs (Ushio et al., 2018).

4.2 | Effect of biofilm's substrate on fish community composition

Mantel test results evidenced that fish community's composition is not influenced by biofilm's substrate. These results show that regardless of the type of biofilm we use, biofilms developing on artificial or natural substrates, provide both accurate information on fish community composition. Based on these results, we can suppose that the spatial differences in fish communities evidenced from the beach and the harbor sites were due to habitat fish preference rather than biofilm type. A future study looking for fish eDNA in biofilms developing on both natural and artificial substrates located at the same place could be performed to validate these results.

5 | INTEREST IN STUDYING FISH COMMUNITIES FROM BIOFILMS

The persistence of fish eDNA in the water column in freshwater ecosystems is known to range from 1 week to 1 month (Harrison

et al., 2019). This short persistence of detectable fish eDNA in water allows to use this eDNA signal to draw conclusions on the recent presence and distribution of fish species within this time period (Rourke et al., 2021). Environmental biofilms were used in this study hypothesizing that they may integrate this eDNA signal on a longer time period. Our sampling design allowed us to test two different periods of integration of the eDNA signal. As mentioned previously, the colonization period for beach biofilms was not controlled and could range from a few days (after important wind events) to 1 month or more (when wind calm conditions). Harbor biofilms, for their part, were collected after a controlled colonization period of 15 days, thus limiting the detection potential to fish species present in a range of 15 days. According to the colonization time we can classify our samples into "young" (harbor samples) and "mature" (beach samples) biofilms. Our results showed that young and controlled biofilms were able to provide a fish eDNA signal strong enough to reveal the fish community composition. Mature biofilms, for their part, also allowed revealing the fish community composition but the integration time of the eDNA signal is uncertain. Using artificial substrates to control the age of our biofilms allowed us to exactly know the temporal window explored. This can also be done by using artificial eDNA samplers for which submersion time is also controlled (Bessey, Jarman, et al., 2021; Kirtane et al., 2020; Verdier et al., 2022).

When controlling the colonization time is not a priority, working directly with environmental biofilms developing on natural substrates may be more interesting. Indeed, on a practical point of view, there is no risk of damage or loss of the colonization device, and no time has to be spent to deploy the material prior to colonization. Moreover, stone biofilms are already collected in routine monitoring programs to study diatoms communities for ecological assessment following standardized protocols (European standard EN 13946; Afnor, 2014). By using stone biofilms, diatoms and fish communities could be studied in parallel, from a single sample and a single DNA extraction, reducing sampling time and costs.

Regardless of the type of substrate, sampling of environmental biofilms is easy to achieve and do not require any filtration step as for water samples. This saves time and money, especially in the case of large monitoring programs when large numbers of samples have to be sampled and analyzed. Furthermore, by using biofilms, plastic consumption is reduced (e.g., when filtering water through filter cartridge). In addition to this, DNA extraction from environmental biofilms is faster compared to DNA extraction from filter cartridge using the same extraction kit (Macherey-Nagel NucleoSpin® Soil kit). In the latter, the lysis step requires almost 3 h (Vautier et al., 2020), compared to biofilms for which the lysis step is performed in no more than 10 min.

Yet, despite these advantages of the use of biofilms to study fish communities through metabarcoding, some considerations should be taken into account. Humic substances (e.g., humic and fulvic acids) present in biofilms are known to inhibit the enzymatic activity of the DNA polymerase (Matheson et al., 2010). Thus, PCR inhibition can be responsible for reducing the detection of fish eDNA in biofilms. This technical aspect was considered by using an extraction kit (NucleoSpin® Soil kit, Macherey-Nagel) that allows the precipitation of proteins and PCR inhibitors and their subsequent removal by combining a lysis buffer (SL3) with a filtration column (NucleoSpin® Inhibitor Removal Column). In a previous study looking for the most suitable extraction method to study diatom communities from biofilm samples, Vasselon et al. (2017) found that DNA extracted with this kit was free of inhibition and easily amplified without DNA dilution. As suggested by Matheson et al. (2010), this could be ascertained for fish eDNA amplification by making serial dilutions of DNA extracts in order to reach a concentration of inhibitors low enough not to inhibit PCR reactions (Matheson et al., 2010). However, such dilution may significantly reduce the fish detection sensitivity as low-abundant fish eDNA may be lost completely if they are low-abundant which is often the case in biofilm samples.

6 | CONCLUSIONS

Our results showed that eDNA in environmental biofilms can be used to study fish communities through metabarcoding. We evidenced that the type of biofilm used does not affect fish community composition. Depending on the objective of the study, biofilms developing on natural or artificial substrates can be used without any type of restriction, just be careful to privilege the use of biofilms in areas protected from turbulent hydrological conditions (e.g., waves). Even if some questions remain to be addressed, this study opens up the possibility to use biofilms to study fish communities easily. In addition, using artificial substrates enables to control the temporal window during which the eDNA signal is captured. Finally, biofilms being already sampled for large phytobenthos monitoring programs, it is possible to take advantage of these existing samples to study fish communities reducing sampling efforts and time.

AUTHORS' CONTRIBUTIONS

SFR, FR, and AB conceived the study. VV conceived laboratory and bioinformatics treatment. SFR performed biofilm sampling, DNA extraction and PCR from biofilms, bioinformatics, data analysis, and wrote the paper with significant contributions from all authors. All authors contributed to the improvement of the paper and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The Illumina Miseq raw data can be accessed at doi: 10.5281/zenodo.5864552. The 12S fish reference library is available at: https://doi.org/10.15454/AU8EZI

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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