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## ORIGINAL ARTICLE OPEN ACCESS

# Environmental DNA for Aquatic Monitoring: Impact of Diel Vertical Migration

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**Keywords:** ddPCR | diel vertical migrations | eDNA | Lagrangian modeling

## ABSTRACT

There is increasing interest in using eDNA for deriving abundance indices for biodiversity monitoring and in support of fisheries management. However, eDNA concentrations are affected by animal behavior, such as diel vertical migration, which has repercussions for designing eDNA sampling strategies for deriving unbiased abundance indices. In this study, we investigated the potential impact of diel vertical migration or other diel activity variations on measured eDNA concentrations for European hake (*Merluccius merluccius*), European seabass (*Dicentrarchus labrax*) and blackspot seabream (*Pagellus bogaraveo*). For hake, in situ eDNA concentrations near the sea floor differed systematically between samples taken before sunrise and after sunset, with the average concentration in morning samples being 24% of the average evening samples. For the two other species, only a weak diel signal in eDNA concentrations was found. Modeling the dispersal and decay of eDNA particles through a Lagrangian approach revealed that eDNA concentrations might decrease to 21%–41% of their initial value during the absence of a species moving entirely up from the sea floor during the night. For *M. merluccius*, the coherence between observed diel variations in eDNA concentrations near the sea floor and modeling results indicates that diel vertical migration behavior needs to be accounted for when devising eDNA sampling plans. The necessity is less clear for *D. labrax* and *P. bogaraveo*.

## 1 | Introduction

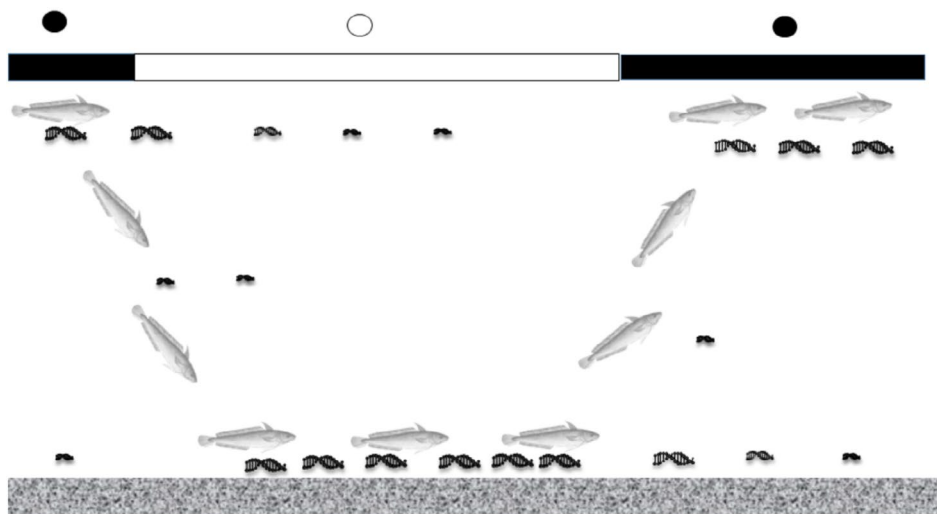
Time varying activity patterns are common in aquatic animals and are often linked to feeding periods (Hays 2003; Arndt and Evans 2022). Diel vertical migrations (DVM) consist of individuals moving vertically in the water column on a daily basis. A common pattern is rapid upward movement at dusk for feeding near the sea surface and downward movement at dawn to avoid predation during daytime (Figure 1). This behavior is shown by around 6% of Mediterranean littoral and epipelagic teleost fishes (Arndt and Evans 2022). It is also common among deep pelagic

fishes whose movements are midwater, with variations in the timing, direction, and degree of DVM (Eduardo et al. 2024).

Analysis of environmental DNA (eDNA) using species-specific assays and metabarcoding has attracted attention as an alternative or complementary method to trawling for monitoring demersal marine biodiversity (e.g., Kopp et al. 2023; Veron et al. 2023) and species-specific abundances (e.g., Lacoursière-Roussel et al. 2016; Hansen et al. 2018; Guri et al. 2024). Further, eDNA metabarcoding has allowed the scientific community to rapidly generate inventories of fish communities at a given time

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**FIGURE 1** | Schematic representation of the effect of diel vertical migration on eDNA concentrations in marine environments.

and place (e.g., Polanco Fernández et al. 2020). Significant positive correlations between eDNA concentrations from species-specific assays and species abundance have been demonstrated both experimentally and in natural aquatic environments (see meta-analysis in Yates et al. 2019). Several marine studies have demonstrated positive correlations between eDNA concentrations and results from traditional sampling methods such as bottom trawl catches (Thomsen et al. 2016; Salter et al. 2019; Maes et al. 2023) and acoustic biomass estimates (Shelton et al. 2022). Quantitative PCR (qPCR) has been used for many marine fish eDNA studies to quantify DNA (e.g., Andruszkiewicz et al. 2019; Hansen et al. 2020). Increasingly, fish eDNA studies are using droplet digital PCR (ddPCR) to quantify eDNA (Lafferty et al. 2018; Brys et al. 2021; Thomson-Laing et al. 2021; Maes et al. 2023) as it is less prone to inhibition, allows direct quantification of the template without the need for comparisons to standard curves, and is more accurate than qPCR, especially at low eDNA concentrations (Doi et al. 2015).

Fish behavior is known to influence eDNA shedding rates (Takahashi et al. 2021; Thalinger et al. 2021; Collins et al. 2022). For example, a positive relationship between individual activity level and eDNA concentrations has been found in experimental settings for several freshwater and marine fishes (e.g., Takahashi et al. 2021; Thalinger et al. 2021). Further, seasonal spawning activity increases in situ eDNA concentrations (Collins et al. 2022). Considering the effects of DVM on eDNA, both the increased activity during migration and the change in vertical distribution can be expected to impact the vertical distribution of eDNA concentrations over short time periods. This implies that eDNA can be used for studying DVM behavior, but it also means that it is necessary to account for DVM when devising eDNA monitoring plans. So far, eDNA has been applied for studying the effect of DVM for mesopelagic and deep-sea species (Canals et al. 2021; Govindarajan et al. 2023). Govindarajan et al. (2023) and Canals et al. (2021) identified a clear diel signal in depth profiles for the proportion of eDNA reads pertaining to mesopelagic and deep-sea fishes, respectively. Similarly, Easson et al. (2020), sampling repeatedly at the same depth before and during the diel vertical migration of the deep-scattering layer, demonstrated rapid change (within ~20 min) in species composition (proportion of reads).

When temporal variations in empirically measured eDNA concentrations are used to identify DVM, it is assumed that the depth at which the eDNA is sampled roughly represents the shedding depth. Modeling results provide some evidence for this assumption. Andruszkiewicz Allan, DiBenedetto, et al. (2021) found with a simple 1D simulation model that shed DNA molecules are unlikely to be much displaced vertically by mixing, advection, or settling (<20m). However, under simulated summer temperate conditions, 90% of eDNA decayed only after around 38h (Andruszkiewicz Allan, DiBenedetto, et al. 2021), suggesting that some eDNA from a migrating species could still be present at depth, even in its absence. Further, in the shallow well-mixed North Sea (max depth 31 m), Dukan et al. (2024) found that surface and bottom eDNA samples had 39 fish species in common, while six and eight species, respectively, were uniquely detected in each depth layer. These modeling and empirical results support the hypothesis that depth differences in eDNA shedding occurring during diel vertical migrations might be detectable by eDNA. Thus, for species carrying out upwards DVM at dusk, we expect eDNA concentrations at depth to decrease during nighttime due to local decay, to which degree is, however, unknown.

The Bay of Biscay (Northeast Atlantic) is home to at least 576 fish species and is subject to numerous anthropogenic pressures, with fishing having the most widespread impact (Lorance et al. 2009). A notable example is the blackspot seabream, *Pagellus bogaraveo* (Brünnich, 1768), which was once very common in this area from the coast to 700 m depth (Quéro and Vayne 1997), but has not recovered since being overexploited in the 1980s (Lorance 2011). However, despite stock depletion, this species remains sought after, fetching high prices (Lorance and Trenkel 2024). Due to its current rareness and preference for coastal rocky habitats, scientific bottom trawling is unsuitable for providing reliable information on the current distributions and densities of blackspot seabream, supporting the need for an alternative evaluation method. Blackspot seabream carry out seasonal migrations (Guéguen 1973) and also exhibit DVM, at least over deeper areas (Afonso et al. 2012, 2014), though they mainly feed during the daytime (see review in Arndt and Evans 2022). Another species difficult to survey using traditional sampling methods is the commercially important European seabass, *Dicentrarchus labrax* (Linnaeus, 1758; Daurès et al., 2009;

Lorance and Trenkel, 2024). This species is also highly esteemed by recreational anglers (Rocklin et al. 2014). Seabass in this area occur between the coast and at least 225m depth (de Pontual et al. 2019). They generally carry out seasonal migrations between feeding and spawning areas, as well as DVM between the sea floor and the surface, in particular during autumn and winter (de Pontual et al. 2019). However, the frequency of migrations might vary between individuals and seasons (see review in Arndt and Evans 2022). Fisheries management of this *D. labrax* population could benefit from further fisheries-independent monitoring information (ICES 2021). European hake, *Merluccius merluccius* (L.), is also an important commercial fish species in the Bay of Biscay (Daurès et al. 2009). It is widely distributed throughout the shelf area (coast to around 200m; Quérou and Vayne 1997), with preferences for depths between 20 and 270 m (Persohn et al. 2009). It carries out regular DVM, moving up the water column during the night (Doray et al. 2010; Mahevas et al. 2011).

In this study, we investigated the potential impact of DVM or other diel activity variations on in situ eDNA concentrations near the sea floor for *P. bogaraveo*, *M. merluccius*, and *D. labrax*. While *M. merluccius* generally carries out DVM, for the two other species it might vary more between individuals. The evaluation involved three steps. First, we developed species-specific ddPCR assays and conducted an experiment to assess the precision of ddPCR concentration estimates. Second, we compared ddPCR and metabarcoding results from field samples with bottom trawl catches. Third, to interpret the empirical results, we modeled the dispersal and decay of eDNA particles with a Lagrangian particle-tracking model to estimate the expected decay and horizontal spread of eDNA between sunset and sunrise for a species carrying out DVM.

## 2 | Materials and Methods

### 2.1 | Assay Development and Validation

The collection of tissue samples for assay development for *D. labrax*, *M. merluccius*, and *P. bogaraveo* and their specificity testing occurred during the scientific EVHOE bottom trawl survey in the Bay of Biscay in 2018 (Laffargue et al. 2021). We collected tissue samples from 105 individuals belonging to 81 different demersal and benthic fish and invertebrate species in

a clean manner (Table S1). In addition, tissue samples of three individuals of *Pagellus bellotti* were provided by the Portuguese scientific survey from the Portuguese coast, as it proved impossible to obtain samples from the Bay of Biscay. Moreover, we purchased two individuals of *Dicentrarchus punctatus* from the Bay of Biscay at the fish market in Nantes (France). The species sampled for assay validation covered all dominant demersal and benthic fish and invertebrate species as well as all species of the same genus occurring in the area.

From the tissue samples, we extracted total DNA in a clean-room lab with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) on a BioSprint96 extraction platform (Qiagen) following the manufacturer's recommendations. All DNA extracts were thereafter Sanger sequenced at Eurofins Genomics for the COI barcoding region (Ward et al. 2005) and the obtained sequences supplemented with sequences of potentially co-occurring or related species of the intended targets, drawn from GenBank (NCBI). Based on the generated alignment, we designed species-specific droplet digital PCR (ddPCR) assays (primers and dual labeled probes; Table 1) for *D. labrax*, *M. merluccius*, and *P. bogaraveo* and optimized PCR conditions for assay sensitivity and specificity. We tested the specificity of each assay in silico via Primer-BLAST (NCBI; potential mispriming events with the forward and reverse primers were thereafter checked for mismatches of the probe and co-occurrence with the target species) and in vitro against all generated DNA extracts (Table S1). For the in vitro test, the 22µL reactions were identical for all three species and contained 1× ddPCR Supermix for Probes (Biorad, Hercules, CA, USA), 0.9µM of primer for the respective target, 0.25µM of the corresponding probe, and 2.2µL DNA extract from the tissue samples. Droplets were generated with an automated droplet generator QX200 AutoDG (Biorad) and the cycling conditions on an Eppendorf Mastercycler were as follows: 10 min at 95°C to activate the enzyme, 40 cycles of 30s at 94°C, 3 min at 58°C, 30s at 60°C, and a final inactivation step of 10 min at 98°C. Post PCR, fluorescence of the droplets was analyzed on a QX200 Droplet Digital PCR System (Biorad).

To evaluate the performance of the newly developed ddPCR assay for *D. labrax*, we used data from experimental samples collected at the Ifremer aquaculture facility in Brest. These samples were part of a study carried out for estimating decay

**TABLE 1** | Primers and probe sets used in the developed ddPCR assays to detect *M. merluccius*, *P. bogaraveo* and *D. labrax*, respectively.

Target	Primer/probe name	Primer/probe sequence (5'-3')
<i>Merluccius merluccius</i>	S-F193_Mer-merc forward	CCTAGCATCTTCCGGGGTG
	S-P199_Mer-merc probe	Fam-ATAGACCGTTCAGCCTGTCCCGG-BHQ1
	S-R194_Mer-merc reverse	AAAATGGTGAGGTCAACGGAA
<i>Pagellus bogaraveo</i>	S-F192_Pag-bog forward	CTATTCTGGGGCAATTAAGTTC
	S-P198_Pag-bog probe	Fam-AAGCCTCCCGCCATTTACAATATC-BHQ1
	S-R193_Pag-bog reverse	GGAGTAAAACAGCGGTAATTAAGTTC
<i>Dicentrarchus labrax</i>	S-F188_Dic-lab forward	GATTTGGAAATTGACTTATTCCG
	S-P194_Dic-lab probe	Fam-TTCTCCATCCTTCCTCCTCTTT-BHQ1
	S-R189_Dic-lab reverse	AAATTGTTAAGTCAACGGATGCT

rates at three temperatures (13°C, 19°C, and 26°C). We kept two 20L buckets at each temperature and filtered 2L at irregular intervals over a 26h period using eDNA Dual Filter Capsules (0.8µm; Sylphium, Groningen, The Netherlands). For this study, the data were reanalyzed as the varying eDNA concentrations allowed us to characterize the assay quantification limit and the precision at different concentrations. Overall, 48 samples were screened in technical triplicates for *D. labrax* using the developed assay and optimized analysis protocol described below. The limit of quantification (LOQ) was estimated as the lowest concentration with coefficients of variation (CV) of technical replicates not exceeding 35% (Forootan et al. 2017). Changes in precision with concentration were characterized using the CV.

## 2.2 | Field Sample Collection and Analysis

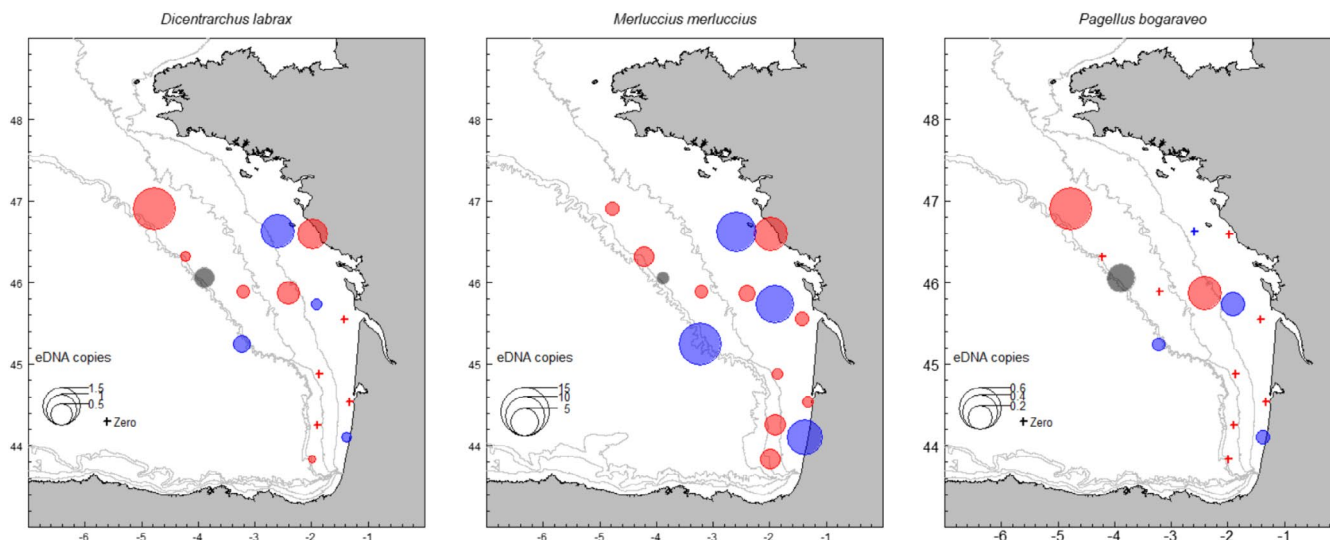
The collection of water samples for eDNA analysis occurred during the 2019 EVHOE bottom trawl survey (Figure 2). The standard EVHOE survey protocol is described in ICES (2017). Water samples were collected at 15 stations spread across the Bay of Biscay (Figure 2). At each station, nine Niskin bottles with 5L each were deployed on a rosette sampler at 5m above the sea floor (bottom depth 20–500m, see Table S2 for detailed depths and sampling times). Water samples were mainly taken around 3h before sunrise (10 stations), just around sunset (4), and during daytime (1), details in Table S2. The overall sampling volume of 45L was split into two replicates per station (22.5L per replicate from different bottles) for filtering using VigiDNA 0.2µm filtration capsules (SPYGEN, le Bourget du Lac, France) and an Athena peristaltic pump (Proactive Environmental Products LLC, Bradenton, Florida, USA; nominal flow of 1.0Lmin<sup>-1</sup>). We followed a rigorous protocol to avoid contamination during fieldwork, using single-use filtration equipment, gloves, and sterile plastic bags to process each water sample, and bleach cleaning the worktop. While no negative controls were carried out at sea, the Niskin bottles were the only non-single-use material employed for the eDNA sampling. To avoid

cross-contamination between stations, they were rinsed on deck three times before (including with local seawater) and one time after each deployment. Bottles were open during descent, which further rinsed them.

Trawl hauls were carried out near water stations during daytime, either just before or just after water sampling stations. Overall, 72 bottom trawl hauls were carried out, but only those paired with eDNA samples were used in this study. Catches (kg per haul) were only available for *M. merluccius* and *D. labrax*; no *P. bogaraveo* individuals were caught by the survey in 2019.

For all field samples, DNA extraction, amplification, and high-throughput sequencing were performed in distinct dedicated rooms set up with positive air pressure, UV-C treatment, and frequent air renewal. The eDNA extraction of filter capsules was carried out by SPYGEN (Le Bourget, France) and provided 2×100µL elution volume per capsule, which was split into two. The first part was analyzed at Sinsoma (Völs, Austria) using the developed species-specific ddPCR assays, and the second part by SPYGEN using metabarcoding (see below).

For ddPCR, the eDNA concentration of the target species in the field samples turned out to be very low (max 20 positive droplets, see Figure S1). Therefore, all samples were tested in triplicate technical replicates to increase the volume of the screened DNA extract (3×6.5µL). Per se, no technical replicates are needed on the ddPCR system (in contrast to quantification via real-time PCR), as this system allows for an absolute quantification of the target molecules being present before PCR. The number of positive droplets in the field samples for 6.5µL DNA extracts was about double that of 2µL extracts (Figure S1). The optimized 22µL reactions were identical for all three species and contained 1× ddPCR Supermix for Probes (Biorad, Hercules, CA, USA), 0.9µM of primer for three target species, 0.25µM of the respective probe (see Table 1 for primer and probe sequences) and 6.5µL DNA extract. Droplets were generated with an automated droplet generator QX200 AutoDG (Biorad) and the



**FIGURE 2** | Environmental DNA concentrations (ddPCR) for *Dicentrarchus labrax*, *Merluccius merluccius*, and *Pagellus bogaraveo* in the Bay of Biscay in 2019. Depth lines are 120, 200, and 1000m. Red samples taken before sunrise, blue samples after sunset and gray samples at midday.

cycling conditions on an Eppendorf Mastercycler were as follows: 10 min at 95°C to activate the enzyme, 40 cycles of 30 s at 94°C, 3 min at 58°C, 30 s at 60°C, and a final inactivation step of 10 min at 98°C. Post PCR, droplets were analyzed on a QX200 Droplet Digital PCR System (Biorad) to identify the percentage of fluorescing droplets per sample, from which the target copy number in the starting DNA extract can be calculated. One filter had unrealistically high ddPCR concentrations for *M. merluccius* and *P. bogaraveo* (Figure S2) and was therefore removed for all quantitative statistical analyses.

Before metabarcoding, the extracted DNA was tested for inhibition by qPCR (Biggs et al. 2015). If the sample was identified as inhibited, it was diluted five-fold. The DNA amplification was performed in a final volume of 25 µL, using 3 µL of DNA extract as template. The amplification mixture contained 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.2 µM of each primer, 4 µM human blocking primer (Valentini et al. 2016) and 0.2 µg/µL bovine serum albumin (BSA; Roche Diagnostic, Basel, Switzerland). Teleo primers (Valentini et al. 2016; forward: ACACCGCCCGTCACTCT, reverse: CTTCCGGTACTTACCATG) that amplify a region of 64 base pairs on average (range: 29–96 bp) of the mitochondrial 12S region were used to target fishes. The primers were 5'-labeled with an eight-nucleotide tag unique to each PCR replicate, allowing the assignment of each sequence to the corresponding sample. The tags for the forward and reverse primers were identical for each PCR replicate. Twelve replicate PCRs were run per sample to increase the probability of detecting rare species (Ficetola et al. 2015). The PCR mixture was denatured at 95°C for 10 min, followed by 50 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, and a final elongation step at 72°C for 7 min. After amplification, samples were quantified using capillary electrophoresis (QIAxcel; Qiagen GmbH) and purified by using the MinElute PCR purification kit (Qiagen GmbH). Before sequencing, purified DNA was quantified again using capillary electrophoresis and pooled in equal volumes to achieve a theoretical sequencing depth of 1,000,000 reads per sample. Library preparation and sequencing were performed at Fasteris (Geneva, Switzerland). Four libraries were prepared using the MetaFast protocol (a ligation-based method) and sequenced separately. The paired-end sequencing was carried out using a MiSeq (2 × 125 bp; Illumina, San Diego, CA, USA) on two MiSeq Flow Cell Kit Version 3 (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Two negative extraction controls and two negative PCR controls (ultrapure water) were amplified (12 replicates) and sequenced in parallel to the samples to monitor possible contamination. No contamination was detected. The sequence reads were analyzed using the OBITools package (<http://metabarcoding.org/obitools>; Boyer et al. 2016), following the protocol described by Valentini et al. (2016). Forward and reverse reads were assembled using the illumina-paired-end program, with a minimum score of 40 and retrieving only the joined sequences, we then assigned reads to samples using the ngsfilter program. Separate datasets were created for each sample by splitting the original dataset into separate files using obisplit. Strictly identical sequences were dereplicated using obiuniq. Sequences shorter than 20 bp or occurrence less than 10, and those labeled "internal" by the obiclean program due to PCR substitutions or indel errors were

removed. Taxonomic assignment of the sequences was carried out using the ecotag program with a genetic reference database formed by combining two sources: (i) the EMBL genetic reference database including 16,128 sequences from 10,546 species across all organisms (European Molecular Biology Laboratory, <[www.ebi.ac.uk](http://www.ebi.ac.uk)>, v141, downloaded in January 2020; Baker et al. 2000) and (ii) a custom-built 12S reference database from sequenced samples taken from individual fish during previous EVHOE trawl surveys (Rozanski et al. 2025). Taxonomic assignment at the species level was only confirmed if the match was over 98%. Sequences with a frequency of occurrence < 0.001 per sequence and per library were discarded to account for tag jumps (Schnell et al. 2015). Index-hopping (MacConaill et al. 2018) was corrected with a threshold empirically determined using experimental blanks between libraries. In this study, we used only presence/absence and relative number of reads for the three target species. The full metabarcoding data set was analyzed by Veron et al. (2023).

Venn diagrams were used to compare the detection of the three species at each field station by ddPCR, metabarcoding, and trawling, combining technical and biological replicates for eDNA. The accuracy of each method for detecting the presence/absence of each species was calculated as the percentage of stations for which the method correctly detected species presence or absence. True presence at a station was assumed if at least one method detected the species and true absence if none of the three methods detected the species. This assumes that there were no false positive detections for any of the methods. Pairwise consistency between methods was calculated as the percentage of stations for which the two methods gave the same result (presence or absence). Species composition derived from ddPCR concentrations was compared with the proportion of metabarcoding reads pertaining to each of the three species using a paired multivariate Hotelling's *T*-Square test. Metabarcoding and ddPCR were further compared by calculating Pearson's correlations between the number of positive PCRs for metabarcoding and ddPCR concentrations. For the quantitative comparison of ddPCR (number of DNA copies per L) and bottom trawling (catch in kg per haul), ddPCR concentrations were regressed on log-transformed bottom trawl catches (in weight) taken before or after water sampling in the same location.

To evaluate whether the filtered volume (22.5 L) was sufficient for the field samples, the results of the two biological replicates from each station were compared using linear regression, and the repeatability by station was assessed with the R package *rptR* (Stoffel et al. 2017) assuming a Gaussian distribution for the mean number of copies per sample. All statistical analyses were carried out with the R software (R Core Team 2024).

## 2.3 | Diel Vertical Migration

### 2.3.1 | Data Analysis

For field samples, the effect of diel vertical migrations was investigated by comparing mean sample concentrations of biological replicates for samples taken just before sunrise with those taken after sunset using nonparametric one-sided Mann-Whitney tests with a null hypothesis of larger values after sunset.

### 2.3.2 | Lagrangian Modeling

We carried out an individual-based Lagrangian modeling analysis to gain insight into the potential detectability of eDNA traces near the sea floor from fish after they moved up in the water column at sunset. We modeled the fate of DNA molecules using the particle-tracking framework Connectivity Modeling System (CMS; Paris et al. 2013). The CMS is a probabilistic tool designed to simulate the movement and dispersion of biotic and abiotic elements in oceanic environments. This method combines a Lagrangian stochastic framework with a grid technique to model 3D trajectories of particles. CMS can simulate particle trajectories while accounting for individual variability through random traits. We extracted the hydrodynamic data from the Atlantic-Iberian Biscay Irish-Ocean Physics Analysis and Forecasts from the E.U. Copernicus Marine Service Information (<https://doi.org/10.48670/moi-00027>, accessed May 2024) from 20 to 21 October 2023, as this information was not available for our study year (2019). The domain encompasses the entire study area (Figure 2). The model has an approximately 2.5-km spatial resolution and a 1-h temporal resolution.

The simulation scenario was established to mimic a species carrying out regular DVM between sunset and sunrise, remaining near the sea floor during the day and in shallow layers during the night. In experimental conditions, fish shed between  $10^4$  and  $10^9$  DNA copies per hour per individual (see review in Andruszkiewicz Allan, Zhang, et al. 2021). We therefore simulated the emission of  $10^4$  particles every hour between 7 a.m. and 5 p.m. (local time) and followed their fate for 14 h until 7 a.m. the next morning. The simulated day-night scheme aims at representing the conditions at the time of field sampling, during which nighttime lasted 13.6–14.1 h. We released the particles at the positions of the 15 eDNA field sampling stations, at 1 m from the sea floor. Releasing particles slightly above the sea floor was required to limit the risk of a border effect that could occur at the deepest layer of the hydrodynamic model. We implemented a constant exponential decay rate of  $0.05 \text{ h}^{-1}$ , in line with the average of teleost experimental values from the review by Scriver et al. (2023). Since the maximum current velocity field was  $0.43 \text{ ms}^{-1}$  in the depth layers considered here, the interpolation time step was set to 3600 s, guaranteeing that particles did not travel more than one grid cell within a single time step (i.e., <1540 m). To account for sub-grid scale turbulent processes unresolved by the hydrodynamic model, horizontal diffusivity was set to  $K = 4.13 \text{ m}^2 \text{ s}^{-1}$ , based on the grid size of the model, following the recommendations of Okubo (1971). Given that eDNA is expected to remain within the same vertical layer (Andruszkiewicz Allan, DiBenedetto, et al. 2021), neither sedimentation nor vertical movements of particles were implemented.

The simulated particle trajectories were then analyzed to calculate the expected transport distance of each particle as a function of time since sunset (i.e., following the last simulated shedding event), then summarized across all particles at all release locations (mean, median, 20th and 80th). To get the rate and proportion of eDNA changes in concentration near the sea floor for each release location, the particle density over time was obtained by dividing the number of surviving particles by the surface area of the envelope encompassing all surviving particles. The initial concentration was computed based on the

envelope at the time of the latest particle release (i.e., at 5 p.m., local time) prior to the fish's ascent.

## 3 | Results

### 3.1 | Assay Development and Validation

The newly developed species-specific ddPCR assays for *D. labrax*, *M. merluccius*, and *P. bogaraveo* are provided in Table 1. The in vitro nontarget testing confirmed that all target species amplified as expected and showed no false-positive amplification for all three assays, including related, co-occurring species. The primer for *P. bogaraveo* did not amplify DNA of *P. bellotti*, and the primer for *D. labrax* did not amplify *D. punctatus* DNA. For *P. bogaraveo* and *D. labrax*, no potential mispriming was found in the in silico analysis for any other co-occurring or related species. For the *M. merluccius* primers, however, the in silico analysis suggested that the primers could presumably also amplify DNA of *M. senegalensis*, and that there is a low probability that this could also be the case for *M. paradoxus*. However, both species are not co-occurring with *M. merluccius* in the Bay of Biscay, so potential confusion was not an issue.

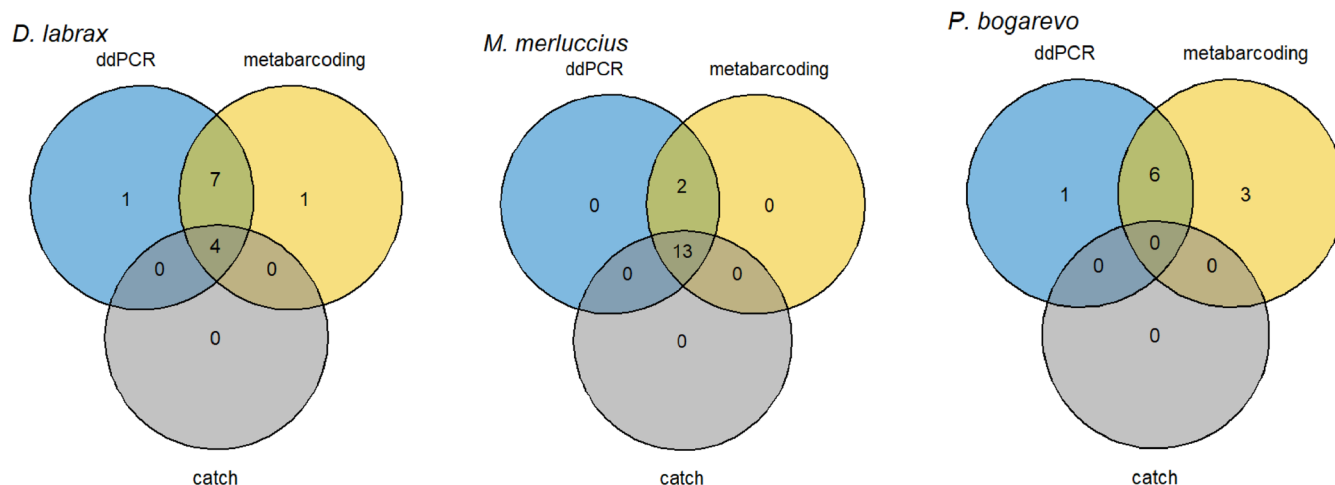
For the experimental *D. labrax* samples, the spread of technical replicates increased with mean concentration (Figure S3a), while the coefficient of variation (CV) decreased with increasing mean concentration as expected (Figure S3b). The CV stabilized at around 0.1 for mean concentrations above 5 copies per  $\mu\text{L}$  in the ddPCR. CVs larger than 0.35 were found for concentrations below 1 copy per  $\mu\text{L}$ . For field samples, CVs also decreased for all species with increasing mean concentration (Figure S4). The empirical LOQ values were 0.29, 0.33, and 0.12 copies per  $\mu\text{L}$  for *D. labrax*, *M. merluccius*, and *P. bogaraveo*, respectively.

### 3.2 | Methods Comparison

Both ddPCR and metabarcoding detected the three species at more stations than bottom trawling (Figure 3). Notably, while trawling did not catch a single *P. bogaraveo*, both molecular methods revealed its presence at seven stations for ddPCR and at nine stations for metabarcoding, with six stations in common. Both molecular approaches detected *M. merluccius* at two more stations than trawling, while *D. labrax* was detected at seven additional common stations by both approaches and one different additional station for each approach. Both molecular methods had high accuracy for species detection (93%–100%) and were more consistent compared to trawling (ddPCR-metabarcoding consistency 73%–100%; eDNA-catch consistency 40%–87%; Table 2). The species proportions obtained with the three approaches based on the number of copies for ddPCR, the number of reads for metabarcoding, and catch weight were statistically different (Figure 4; ddPCR-Metabarcoding:  $F = 25.5$ ,  $p < 0.001$ ; ddPCR-Catch:  $F = 6.1$ ,  $p = 0.003$ ). Notably, *M. merluccius* exhibited much smaller proportions in metabarcoding compared to ddPCR and catches. The relationship between the number of positive PCRs in metabarcoding and the number of copies for ddPCR was rather scattered (Figure 5). Pearson's correlations were significantly positive for all species ( $p < 0.05$ ), indicating nevertheless broad agreement between the two methods.

The quantitative comparison of ddPCR results with trawl catches revealed no significant relationships between the two abundance proxies for *D. labrax* and *M. merluccius* (Figure 6). Stations carried out just before sunrise, and thus before the

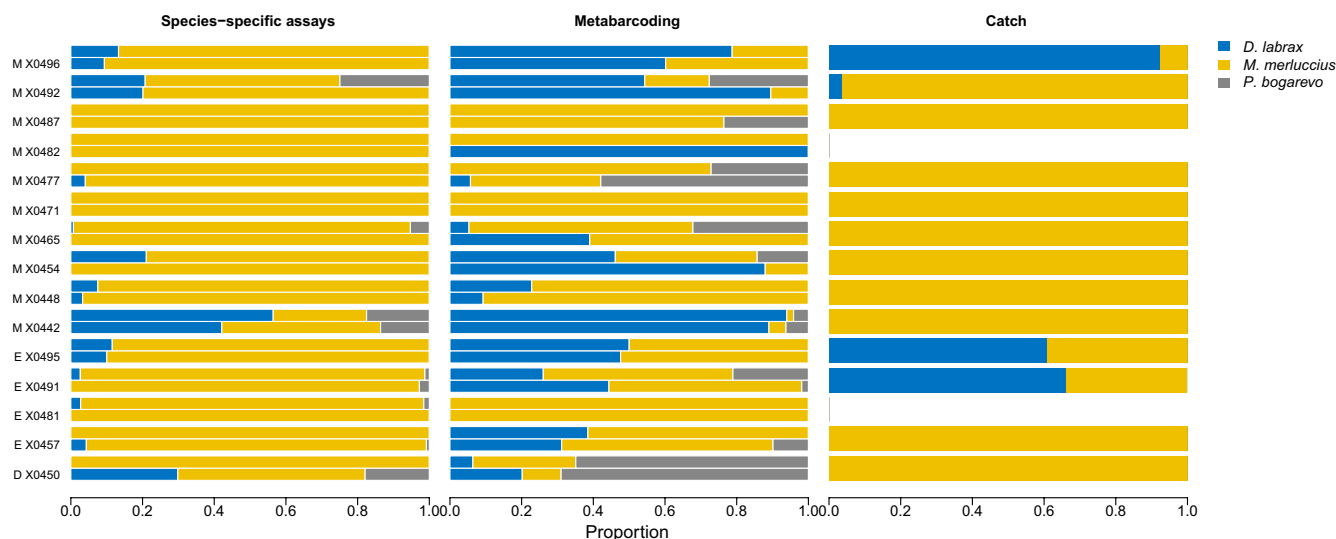
descent of *M. merluccius* to the sea floor, showed no relationship with catches, while eDNA concentrations of samples taken just after sunset showed a weak nonsignificant positive relationship (Figure 6).



**FIGURE 3** | Venn diagrams for the number of stations each species was detected by ddPCR, metabarcoding and in bottom trawl catches.

**TABLE 2** | Comparison of accuracy and consistency of ddPCR with metabarcoding and trawling for detecting the presence or absence of *M. merluccius*, *P. bogaraveo*, and *D. labrax*.

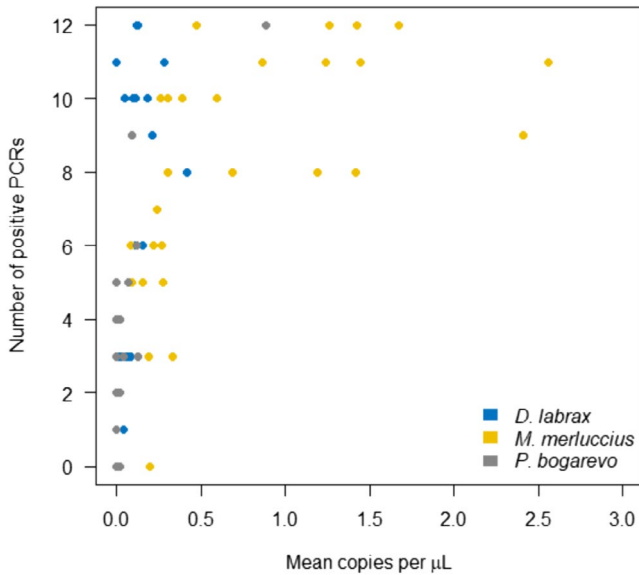
Species	No stations present	Accuracy (%)			Consistency (%)		
		ddPCR	metabarcoding	Catch	ddPCR-metabarcoding	ddPCR-catch	Metabarcoding-catch
<i>Dicentrarchus labrax</i>	13	100	100	60	87	47	47
<i>Merluccius merluccius</i>	15	100	100	87	100	87	87
<i>Pagellus bogaraveo</i>	10	93	100	73	73	53	40



**FIGURE 4** | Species compositions of field samples based on ddPCR concentrations, proportion of metabarcoding reads and catch weights. Prefix of sample ids: M before sunrise, E after sunset and D during daytime (only applies to eDNA).

### 3.3 | Field Sampling Design

Environmental DNA concentration estimates were most precise for *M. merluccius* followed by *D. labrax* (Figure S4). Linear regression between biological replicates confirmed good agreement for *M. merluccius* ( $r^2=0.58$ ) and *D. labrax* ( $r^2=0.36$ ), but not *P. bogaraveo* ( $r^2=0.04$ ), indicating insufficient sampling volume for the latter species. In two stations, *D. labrax* was only detected in one biological replicate. The station-level repeatability of eDNA concentration measurements was highest for *M.*



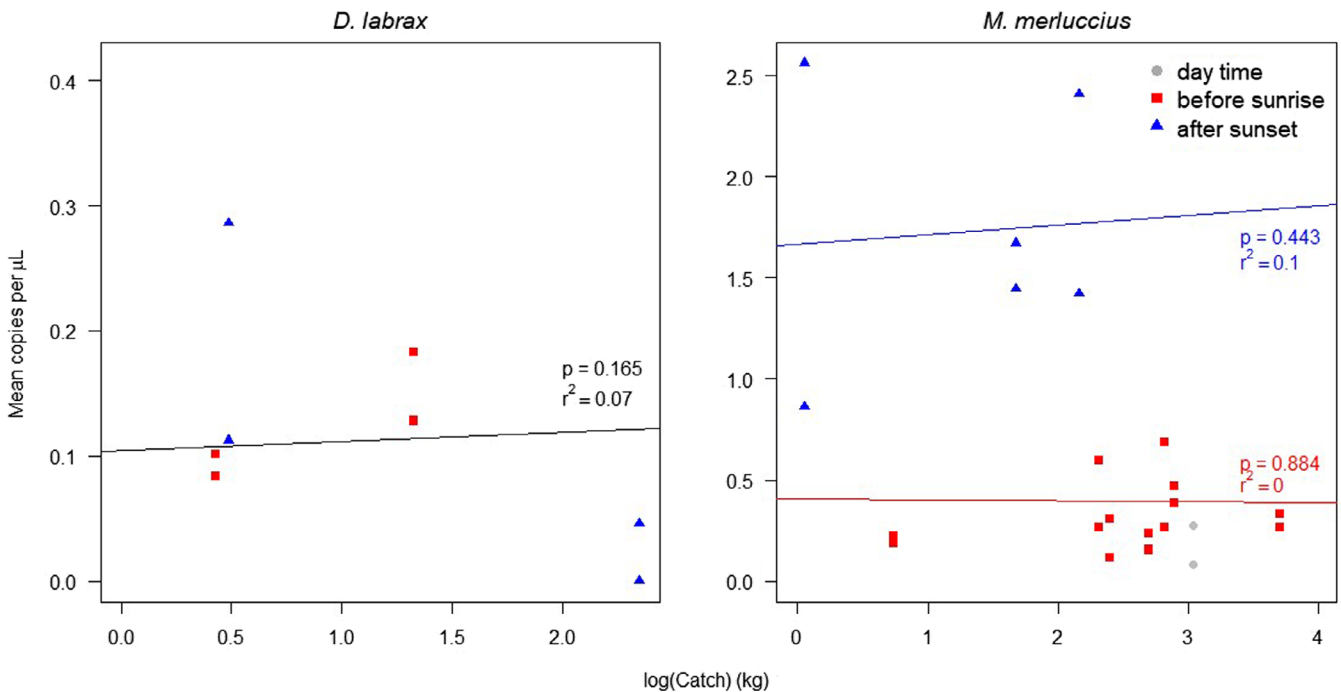
**FIGURE 5** | Relationship between the number of positive PCRs in metabarcoding and mean ddPCR concentrations (3 technical replicates).

*merluccius* ( $R=0.70$ ,  $SE=0.157$ ,  $CI=[0.296, 0.887]$ ) followed by *D. labrax* ( $R=0.60$ ,  $SE=0.177$ ,  $CI=[0.165, 0.853]$ ) and *P. bogaraveo* ( $R=0.53$ ,  $SE=0.147$ ,  $CI=[0.165, 0.735]$ ). Overall, CVs across biological replicates were similar to technical replicates (CV range: 0.03–1.41 biological replicates; 0.01–1.73 technical replicates). As for technical replicates, CVs of biological replicates decreased with ddPCR concentrations, but less consistently, with high CVs remaining at higher concentrations (Figure S5; Pearson's correlation coefficient  $-0.44$ ,  $p=0.006$  one-sided test).

### 3.4 | Diel Vertical Migration

Samples taken just before sunrise had generally lower ddPCR concentrations for all species than samples collected after sunset, with the largest significant difference for *M. merluccius* (non-parametric Mann–Whitney test,  $W=2$ ,  $p<0.001$ ; Figure 7); the difference was not significant for the other species (*D. labra*,  $W=66.5$ ,  $p=0.31$ ; *P. bogaraveo*,  $W=56$ ,  $p=0.09$ ). For *M. merluccius*, the difference in mean eDNA concentrations between evening and morning was 76%. If the difference was caused by DVM, it implies that in the morning, just before sunrise, only around 24% of evening eDNA concentration remained.

In the modeling study, due to both decay and dispersal, average eDNA density dropped to 33.2% on average (median 28.3%, range: 21.1%–40.8%) between sunset, marking the start of DVM ascent, and sunrise, the time of DVM descent (Figure 8a). Decay alone reduced numbers to 47%, indicating that this was the primary factor. The steady decrease in particle density was observed consistently yet with some variations across all 15 release locations. Despite the spatial variability caused by differences in environmental conditions and bottom depths across the 15 modeled sites, the overall



**FIGURE 6** | Relationship between mean ddPCR concentrations across 3 technical replicates for each of two water samples per station and paired bottom trawl catches.  $p$ -values and  $r^2$  for linear regressions. Colors indicate time of day for eDNA sampling. Bottom trawl catches were always carried out during daytime.

trend in the distance traveled by the simulated particles after sunset showed a clear increase over time (Figure 8b). Notably, the distance traveled by eDNA molecules reached approximately 6.6 km on average (median 6.6 km) after 14 h, ranging from 0.8 to 15.9 km for the different simulated release locations (Figure 8b).

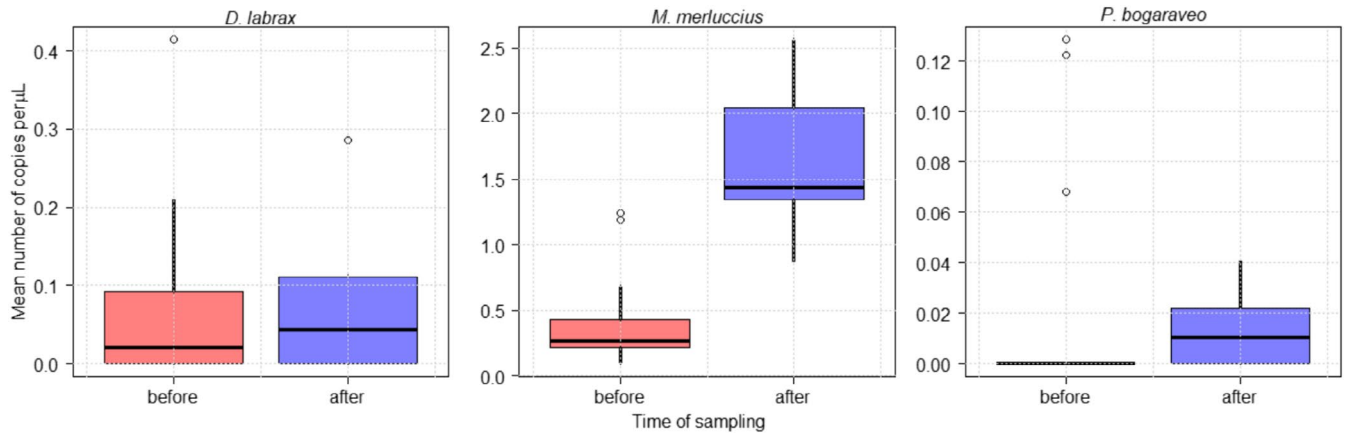
## 4 | Discussion

### 4.1 | Assay Development and Validation

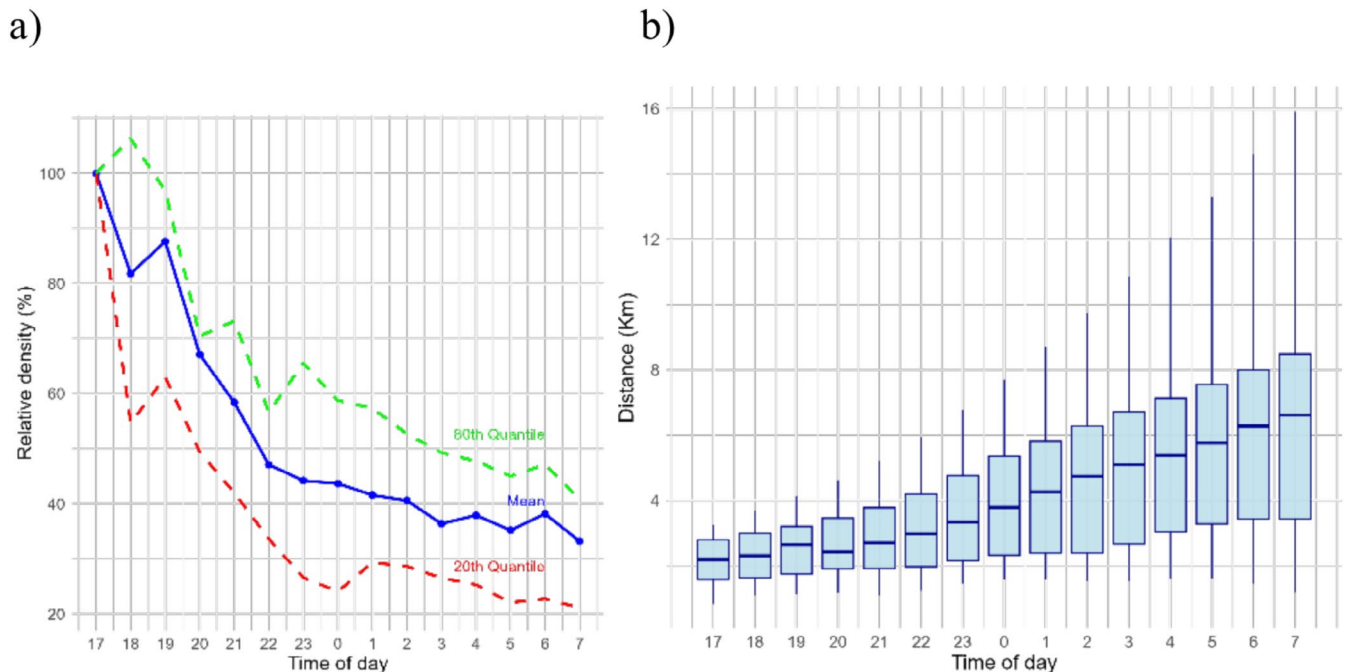
We successfully designed molecular assays for three teleost marine fish species (*M. merluccius*, *D. labrax* and *P. bogaraveo*) that were validated both in controlled and field

conditions. As expected, the precision (CV) of technical replicates decreased with increasing concentrations for all species. The limits of quantification varied somewhat between species and were comparable to published studies using ddPCR (Bryson et al. 2021).

In the field study, the variability in ddPCR concentrations between biological replicates was overall comparable to that of technical replicates, but the CV did not consistently decrease with increasing concentration as for technical replicates (Figures S4 and S5). This seems surprising but might be explained by the fact that the water for the two biological replicates came from different Niskin bottles. Hence, this result could indicate small-scale in situ variations in eDNA



**FIGURE 7** | Boxplots of mean ddPCR concentrations (copies per  $\mu\text{L}$  DNA extract) per biological replicate for samples collected just before sunrise or after sunset.



**FIGURE 8** | Decay and transport model results for exploring the effect of diel vertical migrations on local particle densities. (a) Change of particle density over time due to decay and transport. Mean values and 20th and 80th percentiles across 15 shedding locations corresponding to eDNA sampling locations in Figure 2. (b) Boxplots of particle transport distances since eDNA shedding stopped near the sea floor at sunset mimicking diel vertical migration. Horizontal bars indicate median values, whiskers extend to the largest and smallest values no further than 1.5 times the interquartile range from the box edges.

concentrations. To reduce uncertainty in ddPCR results, it is therefore necessary to increase the sampling volume, either for a single filter or by pooling several filters (> 22 L). Filtering around 30 L has become standard practice for many eDNA studies targeting marine species, though even this large volume can be insufficient to reliably detect all species present (Stauffer et al. 2021). Similarly, for quantitative analysis of rare species such as *P. bogaraveo*, an even larger volume would be better. This is in agreement with other studies that have also found that large volumes need to be filtered, or many biological replicates collected for reliable species detection (e.g., Govindarajan et al. 2022; Kawakami et al. 2023; Veron et al. 2023). However, not only is the sampling volume relevant, but also the elution volume and the volume of the analyzed DNA extract (Song et al. 2020; Altermatt et al. 2023). Therefore, optimization needs to cover all three steps.

In terms of species detection, ddPCR and metabarcoding were highly consistent, while accuracy was highest for ddPCR. This is reassuring as the same samples were used for both molecular analyses. Further, if any contamination of eDNA samples occurred despite the rigorous avoidance protocol put in place, it would have affected both methods similarly. In future field sampling using Niskin bottles, it would be advisable to use field blanks to quantify potential contamination during water handling and filtration. For this, ultrapure water needs to be taken on board and filtered at regular intervals alongside the field samples. Increasingly, however, marine studies are using in situ water filtering, which avoids water handling on board. For surface sampling, water can be filtered directly using peristaltic pumps (e.g., Rozanski et al. 2022), while in deeper waters submersible pumps can be used (e.g., Govindarajan et al. 2022; Hendricks et al. 2023). If the system carries out in situ filtering only, field blanks consist of adding buffer to filter capsules before storage and molecular analyses. Increasingly, automatic systems are being developed that also add preservation solutions in situ, which minimizes field contamination (see review of samplers in Hendricks et al. 2023). Certain automatic systems are mounted on autonomous sampling platforms, which have the added benefit of giving access to a wider range of sampling sites and depths. Thus, in the future, in situ filtering will probably become standard practice in marine studies as it reduces contamination; otherwise, strict contamination avoidance protocols need to be deployed, and field blanks need to be collected.

Compared to the molecular methods, bottom trawling performed worst. For *P. bogaraveo*, the poor performance of bottom trawling might be due to the low population abundance, the patchy distribution of this species, which, for example, has been found to be associated with cold-water coral habitat in the Mediterranean Sea (D'Onghia et al. 2011) and possible avoidance behavior. Its spatial distribution is further structured by life stage. In the Bay of Biscay in autumn, at the time of the study, juvenile *P. bogaraveo* individuals are found in shallow coastal waters while adults are in the deeper waters along the continental slope, where they have been caught by the EVHOE survey in some years. This bimodal spatial pattern was corroborated by the ddPCR results. *Dicentrarchus labrax* was also detected at fewer stations by bottom trawling than by eDNA. This might have been caused by individuals being higher up in the water column and hence uncatchable by the bottom trawl. This explanation is supported by

the fact that commercial fisheries catch *D. labrax* predominantly by pelagic trawls and hooks (Daurès et al. 2009). In this case, the detection of *D. labrax* by eDNA could have been facilitated by feces or other DNA-containing particles sedimenting to the sea floor. Further, eDNA detected *D. labrax* across the whole shelf area, which is in agreement with the known migration routes of this species in the Bay of Biscay (de Pontual et al. 2023).

Most studies testing the use of ddPCR or qPCR for creating abundance indices for exploited marine fish species have commonly focused on abundant, widely distributed species (Knudsen et al. 2019; Shelton et al. 2022; Kasmi et al. 2023; Maes et al. 2023), with the development of species-specific primers focusing on these species (review in Takahashi et al. 2023). Here we evaluated the application for a relatively rare species, *P. bogaraveo*, as well as a patchily distributed species, *D. labrax*. The main identified limitation for these species was the sampling volume, as discussed above.

## 4.2 | Diel Vertical Migration

In aquatic ecosystems, diel vertical migrations can affect the spatio-temporal distribution of eDNA concentrations. In this study, for *M. merluccius*, the reduction of eDNA concentration in field samples between evening and morning was similar to the modeled reduction mimicking the effect of DVM (in situ reduction for *M. merluccius* 76% versus modeled reduction 67% [59%–79%]). Thus, the observed difference in eDNA concentrations for *M. merluccius* was most likely the result of diel vertical migrations, which are well documented for *M. merluccius* (Doray et al. 2010; Mahevas et al. 2011; Arndt and Evans 2022). The temporal patterns were less marked for the other two species. There are several possible explanations for this difference. First, only a proportion of individuals might have carried out DVM at the time of the field study. For *D. labrax*, this explanation is supported by results obtained with individual data storage tags, which showed that different individuals do not carry out diel vertical migrations at the same time period, but rather at different periods (de Pontual et al. 2019). For *P. bogaraveo*, not much such published information on DVM is available. The only published study investigating DVM was around seamounts off the Azores (Afonso et al. 2014). Thus, the fact that no clear signal of DVM was detected for this species might have been caused by little or no DVM behavior in the Bay of Biscay at the time of the study. Second, the unpaired sampling design could have blurred diel differences if morning and evening samples were systematically taken in locations with different densities. For *M. merluccius*, the range of bottom trawl catches was the same for both groups of stations (Figure 6), indicating that no systematic bias was caused by the unpaired sampling design for this species. Not enough individuals were caught for the other two species to further investigate this issue. However, the locations of morning and evening samples were well spread throughout the study area, which should have reduced any potential sampling design bias.

To the best of our knowledge, this is the first study investigating DVM using species-specific assays. So far, relative proportions of metabarcoding reads or simply the number of detected species have been used for studying DVM (e.g., Canals et al. 2021;

Easson et al. 2020; Govindarajan et al. 2023). The use of metabarcoding data for detecting DVM has several limitations. The number of detected species or, more generally, the probability of detecting a species is strongly influenced by the eDNA decay rate. The smaller the decay rate, the longer the species is detected after migrating out of the sampled depth layer. Further, abundant species will mask DVM signals in the proportion of metabarcoding reads due to well-known amplification bias (Krehenwinkel et al. 2017), as was the case for *M. merluccius* in this study. It would have been impossible to detect the strong DVM signal for *M. merluccius* using changes in species composition alone, either using the proportion of metabarcoding reads or the proportion of ddPCR concentrations. Hence, species-specific ddPCR concentrations are more powerful and are recommended as the method of choice for investigating species-specific DVM behavior.

The interpretation of diel changes in eDNA concentrations as a sign of DVM for *M. merluccius* was supported by the Lagrangian model results. Average ddPCR concentrations in morning field samples were around 24% of evening samples. Assuming all individuals moved out of the bottom layer at sunset, modeled bottom eDNA concentrations decreased to 21%–41% by sunrise, the range of values corresponding to the different sampling stations in the Bay of Biscay from which virtual molecules were released. Thus, empirical and modeling results agreed surprisingly well, despite the simplifying assumptions made in the model. Decay rate is a crucial parameter for the local persistence of eDNA molecules, in interaction with current dynamics (Zanni et al. 2025). Decay rate depends primarily on water temperature, with slower decay in colder water (see review in Scriver et al. 2023). A wide range of decay constants has been found for fishes in ex situ experimental studies, depending on species and temperature (Scriver et al. 2023). For the simulations carried out in this study, we used a relatively low decay rate, corresponding to a half-life of around 10 h. In the studies reviewed by Scriver et al. (2023), half-life ranged between 1 and 52 h. Experimental studies in natural marine environments found that DNA was no longer detectable 2 h after removing the cage with introduced fish (Murakami et al. 2019) and 7.5 h after introducing DNA from a non-native species (Ely et al. 2021). In both studies, DNA was released nearshore in protected areas, thus the results mainly reflect in situ decay. Future studies are needed to estimate in situ decay and transport in offshore areas to validate the modeling results obtained in this study. Nevertheless, the general agreement between empirical and modeling results suggests that the assumed decay rate might have been appropriate although a slightly higher decay rate would have made the two types of results even more similar. Further, for the model we used hydrodynamic data for only 1 day in October 2023 for the locations of the 15 field sampling stations. The results revealed strong spatial variations, with modeled particle densities decreasing between 59% and 79% during 14 h. Thus, sampling location clearly influences the transport of eDNA molecules. Water temperature and tidal current, which vary throughout the year and the tidal cycle, might impact eDNA decay and transport, respectively. Here we only investigated an autumn situation corresponding to the time of year of sampling. At last, based on the modeling results by Andruszkiewicz Allan, DiBenedetto, et al. (2021) we assumed eDNA molecules would remain in the same depth layer. However, not much information exists on this

and some authors expect eDNA to sink in the water column (e.g., Hansen et al. 2018). Hence, more comprehensive modeling studies are needed to better characterize spatio-temporal transport patterns in the Bay of Biscay and elsewhere.

The modeled transport distances also provided insights into the potential spatial footprint of our eDNA samples. According to the model, eDNA from a point source may have been transported over 6.6 km in 14 h, which is nearly twice as much as the 3.7 km covered by a standard bottom trawl haul in the EVHOE survey (Trenkel et al. 2004). Thus, the spatial footprints of the eDNA field samples were probably much larger than that of the bottom trawl. One of the rare marine modeling studies using a Lagrangian particle-tracking model concluded that if eDNA was detectable for 4 days, then in Monterey Bay sampled eDNA would originate from within 40 km (Andruszkiewicz et al. 2019). A more comprehensive study is needed to quantify the spatial footprint of eDNA samples in the Bay of Biscay, accounting for different behavioral patterns and shedding rates.

### 4.3 | Insights for Monitoring

Diel vertical migrations have implications for designing eDNA sampling schemes, either for biodiversity monitoring or for deriving abundance indices for management. To avoid bias for demersal species carrying out DVM, it is necessary to collect water samples near the sea floor during daytime. In all cases, it is essential to standardize the time period for any long-term monitoring program. Unfortunately, for many fish species, information on the existence of diel vertical migrations is currently missing. In this case, an eDNA sampling scheme including surface and bottom samples at different times of the day can help to shed light on their vertical migration behavior. Further, experimental studies have shown that eDNA shedding increases with activity level (Thalinger et al. 2021). In addition to diel vertical migrations, marine teleost species show a diversity of feeding-related activity patterns including diurnal, crepuscular, and nocturnal (Arndt and Evans 2022). More studies are needed to quantify their effects on eDNA results. If they turn out to be important, the timing of sampling will be a crucial parameter when designing sampling protocols for quantitative eDNA studies in marine ecosystems but also for more general biodiversity studies.

In this study, species-specific assays were developed to obtain quantitative eDNA information as proxies for local species densities. This approach is costly and not feasible for a large number of species of interest. Recently, Guri et al. (2024) proposed an approach that combines data from species-specific assays for a few species with metabarcoding to enable quantitative interpretation of metabarcoding results. The species-specific results are used to correct the amplification biases of metabarcoding primers. The approach bears promise for advancing the use of eDNA for fish monitoring.

In conclusion, eDNA helped to detect a clear diel activity pattern for an important commercial species, *M. merluccius*. If ignored in the sampling design, this would strongly bias eDNA-based abundance proxies, highlighting the predominant role of species behavior for abundance estimates with molecular-based sampling methods.

## Author Contributions

V.M.T. and C.A. jointly designed the experimental and field study. C.A. collected the samples. M.Y.Z., R.F., and V.M.T. jointly designed the simulation study. D.S. designed the assays and supervised the ddPCR analyses. V.M.T. and M.Y.Z. analyzed and interpreted the data. V.M.T. wrote the manuscript, and all the authors contributed to its improvement and revision.

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## Ethics Statement

The research did not require any ethics approval.

## Conflicts of Interest

The authors declare no conflicts of interest.

## Data Availability Statement

All data are available at <http://seanoe.org> (<https://doi.org/10.17882/107654>). Raw metabarcoding sequences are available at <https://doi.org/10.16904/envidat.442>.

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## Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Species used for testing specificity of developed assays. **Table S2:** Field sampling information. **Figure S1:** Number of positive droplets in Bay of Biscay samples for two volumes of extracted DNA analyzed in PCR. **Figure S2:** Field sample results. Comparison of ddPCR concentrations (copies per  $\mu\text{L}$  DNA extract) between biological replicates. **Figure S3:** Experimental results for *Dicentrarchus labrax*. **Figure S4:** Field sample results. Coefficient of variation as a function of mean ddPCR concentration in DNA extract for three technical replicates. **Figure S5:** Coefficient of variation of natural replicates (based on average ddPCR concentration across technical replicates) versus mean concentration per station.