Received: 26 August 2022 | Revised: 15 March 2023 | Accepted: 10 April 2023

DOI: 10.1002/edn3.426

Environmental DNA
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WILEY

SPECIAL ISSUE ARTICLE

Detection and quantification of two commercial flatfishes (*Solea solea* and *Pleuronectes platessa*) in the North Sea using environmental DNA

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Funding information
European Maritime and Fisheries Fund

Abstract

Sustainable fisheries management requires regular scientific monitoring of fish stocks. When information on certain fish stocks is limited, environmental DNA (eDNA) holds promise to complement traditional monitoring surveys. However, a better understanding of how eDNA concentrations relate to fish abundance and biomass is needed. Here, eDNA quantification of two commercially important flatfish species in the North-East Atlantic, common sole (*Solea solea*) and European plaice (*Pleuronectes platessa*), was assessed. First, species-specific, probe-based assays for plaice and sole targeting the mitochondrial cytochrome b and cytochrome c oxidase subunit I gene, respectively, were developed (for sole) and validated (for both species). Subsequently, two mesocosm experiments revealed a significant and positive relationship between both abundance and biomass and eDNA concentrations for both species at three eDNA emission time periods (5 min, 1 h, and 24 h). Larger plaice shed significantly more eDNA (copies L^{-1}) than smaller conspecifics. Finally, eDNA was obtained from seawater collected during research surveys in the Belgian part of the North Sea in spring 2020 (i.e., local scale) and the southwestern North Sea in autumn 2020 and 2021 (i.e., regional scale). eDNA concentrations were compared to the observed abundance (individuals per km^2) and fish density in terms of biomass (kg per km^2) as observed in the trawl at the same station. Local eDNA concentrations of both sole and plaice were positively correlated with observed abundance and fish density. The correlation between regional eDNA concentrations and fish density was positive and significant for sole in 2020 and 2021 and for plaice in 2020, but not in 2021. The correlation between regional eDNA concentrations and observed abundance was positive and significant for sole and plaice in 2020, but not in 2021. These results illustrate the potential of eDNA to estimate abundance and biomass parameters for stock assessments of flatfishes in the North Sea.

KEYWORDS
biomass, ddPCR, dPCR, environmental DNA, fish stock assessment, quantification

1 | INTRODUCTION

Numerous fish populations are suffering from human overexploitation in marine ecosystems worldwide (Hutchings, 2000; Pauly et al., 2002; Vázquez-Rowe, 2020). Stock assessments are the backbone of sustainable fisheries management, which uses data on abundance, biomass, body length, and age structure to evaluate the status and size of fish stocks in order to set annual catch limits relative to sustainable reference points and to guarantee viable fish populations in the future (Cadrin & Dickey-Collas, 2014; Lorenzen et al., 2016). Research surveys serve as valuable input to fish stock assessments (ICES, 2021d). Surveys targeting flatfish are typically conducted using beam trawls (ICES, 2021c). This traditional way of sampling is damaging to seabed habitats and results depend on a range of variables such as catchability, environmental conditions such as wave height or the presence of other fishing vessels affecting catches due to competition (Arreguín-Sánchez, 1996; Heessen et al., 2015; Wilberg et al., 2009). Although fisheries surveys aim at fishing at random and evenly distributed locations, local unsuitability of the seabed (e.g., too rocky or muddy) and/or shallowness of the area (Cordue, 2007; Eigaard et al., 2017; Fréon et al., 1993) may hamper obtaining spatially uniform stock estimates.

Last decade, environmental DNA (eDNA) has emerged as a powerful and non-invasive tool for studying marine fish communities by providing detailed information on the presence and even abundance and biomass of specific species (Murakami et al., 2019; Sassoubre et al., 2016; Stoeckle et al., 2021). Yet, uncertainty remains on the reliability of quantitative descriptions of locally present fish populations based on these molecular approaches (Burian et al., 2021; Collins et al., 2018; Kelly et al., 2014). Several studies have highlighted the potential of monitoring fishes by revealing a clear relationship between biomass estimates and eDNA concentrations (Doi et al., 2015; Salter et al., 2019; Shelton et al., 2022; Spear et al., 2021), while other studies did not find such linear relationship (Coulter et al., 2019; Troth et al., 2020). Hence, inconsistencies among and within most eDNA studies (exemplified by large standard deviations among replicates) fuel the ongoing debate on the ability of predicting species biomass based on eDNA concentrations (Iversen et al., 2015; Lam et al., 2022).

Under natural conditions, eDNA is characterized by large spatial and temporal variability due to its variable dispersion capacity and variations in shedding and decay rates (Hansen et al., 2017). In the marine environment, the interpretation of eDNA data is challenging due to the large body of water, strong current and tidal action, water column stratification, and varying environmental conditions including water temperature and salinity (Canals et al., 2021; Foote et al., 2012; Jeunen et al., 2020; Lacoursière-Roussel et al., 2016). Furthermore, eDNA shedding and degradation rates can be species specific (i.e., dependent on ontogeny and behavior) (Ostberg & Chase, 2021; Sassoubre et al., 2016), and dependent on environmental factors such as water temperature and chemistry, and Ultraviolet exposure (Jo, Murakami, et al., 2019). For instance, the approximate half-time of eDNA of various marine organisms (e.g., common shore crab *Carcinus maenas* and shanny *Lipophrys pholis*) in marine systems with temperatures ranging from 10 to 15°C (i.e., representative for the North Sea) is between 10 and 52 h (Collins et al., 2018; Thomsen et al., 2012).

Common sole (*Solea*, hereafter referred to as sole) and European plaice (*Pleuronectes platessa*, hereafter referred to as plaice) are two commercially important demersal flatfish species in Europe (Bjørndal et al., 2016; Jayasinghe et al., 2017; Millner et al., 2005). Both species are mainly caught in beam trawl fisheries, accounting for roughly 40% of the total value of fish landings in the North Sea (Pilling et al., 2008). Yet, the lack of data on abundance and biomass of both species has led to adopting the precautionary approach to fisheries management in several ICES advisory regions for plaice (e.g., Baltic Sea subdivisions 24–32, West of Ireland divisions 27.7b–c, Celtic Sea South, southwest of Ireland 27.7h–k) (ICES, 2020, 2021a) and sole (e.g., Celtic Sea South, southwest of Ireland 27.7h–k) (ICES, 2021b, 2022). Consequently, until sufficient data are collected to obtain better and more solid insights into the status of these populations and the development of more suitable management guidelines, long-term yields are likely suboptimal and economically inefficient (Peel, 2005). Environmental DNA could be an alternative way to obtain additional data (i.e., presence–absence and biomass), especially from data-poor regions as water samples can be collected by both researchers and fishermen (Agersnap et al., 2022; Miya et al., 2022).

In this study, we investigated whether a positive relationship exists between the abundance and biomass of plaice and sole and their respective eDNA concentrations in both controlled (mesocosm) and natural conditions (North Sea). First, we developed (only sole) and validated species-specific primer–probe assays for the reliable detection and quantification of sole and plaice from environmental DNA through digital droplet PCR (ddPCR). Next, we assessed the relationship between eDNA concentration and abundance (number of fish) and biomass (in g) under controlled mesocosm conditions. Finally, we compared (i) eDNA concentrations of sole and plaice obtained from seawater samples collected during a research survey in spring 2020 in the Belgian part of the North Sea (BPNS) with observed abundance (number per km^2) and fish density in terms of biomass (kg per km^2) obtained from the same survey and (ii) eDNA concentrations of sole and plaice obtained from research surveys in autumn 2020 and 2021 at a larger regional scale in the southwestern North Sea with observed abundance and fish density. Based on previous findings for Atlantic cod and Pacific hake (Salter et al., 2019; Shelton et al., 2022), we expected to find a positive correlation between eDNA concentrations and biomass of the studied species in the North Sea.

2 | MATERIALS AND METHODS

2.1 | Validation of species-specific primer–probe assays

The performance of primer–probe assays specifically designed for sole and plaice was tested for specificity and sensitivity in silico and in vitro. For plaice, the species-specific primers and probe targeting

a 90 bp fragment of the mitochondrial cytochrome *b* (cytb) gene were developed by Knudsen et al. (2019) (Table 1). For sole, one new primer–probe set was developed targeting a 100 bp fragment of the mitochondrial cytochrome oxidase c subunit I (COI) gene using Primer3 (Untergasser et al., 2012). This target amplicon is not only highly species specific, but also very conservative for this species, covering all sole haplotypes documented in NCBI, without any mismatch needed in the primer binding regions. The specificity of both primer sets was tested in silico using the Primer-BLAST tool from NCBI (Ye et al., 2012). Next, each set of primers was assessed by ddPCR amplification of 4 μL genomic DNA (1 ng/μL) from a range of non-target species commonly found in the habitat of the target species: *Limanda limanda*, *Platichthys flesus*, *Scophthalmus maximus*, *Scophthalmus rhombus*, *Pleuronectes platessa* (for sole) and *Solea solea* (for plaice).

The sensitivity of both the plaice and sole primer–probe assays was determined by ascertaining their respective limit of detection (LOD) and limit of quantification (LOQ) through a six-step series of 10-fold dilutions of eDNA extracts from the shedding experiment (see further), with 10 replicates at each concentration (Table S1). The LOD is defined as the lowest concentration detectable in all replicates (Klymus et al., 2020). The LOQ is the lowest concentration at which the coefficient of variance (CV; defined as the ratio of the standard deviation to the mean) is below 0.35 (Klymus et al., 2020). Starting concentrations for these dilution series were obtained from eDNA extracts taken from experimental tanks containing high densities of plaice (i.e., 12 individuals in 43-L seawater) and sole (i.e., 9 individuals in 43-L seawater) at 24 h after adding the fish to the closed tanks (Figure 1). The initial concentration of both species was measured through ddPCR to determine the first dilution. The dilution series to determine LOD and LOQ are typically made using DNase-free water which may dilute the salts in the eDNA extract from seawater which may influence PCR efficiency and thus LOD/LOQ thresholds; we, therefore, verified LOD/LOQ thresholds using 10-fold serial dilutions of eDNA extracts of plaice with either DNase-free water, artificial seawater, or natural seawater collected in the proximity of the harbor of Ostend, Belgium (Table S1).

2.2 | Quantification of eDNA shedding

2.2.1 | Experimental setup

A controlled mesocosm experiment was performed onboard of the RV *Belgica* (for plaice) and RV *Geo Ocean V* (for sole) to assess the extent to which eDNA shedding concentrations of both target species are related to abundance and biomass. Plaice and sole were collected in the Belgian part of the North Sea in March and November 2021, respectively, during a beam trawl survey. Fish were measured and weighted individually to the nearest millimeter and gram, respectively. Until the required number of individuals was collected for the experiment, plaice were kept in two separate tanks based on size: one tank with individuals larger than 14 cm total length (TL, tip of snout to tip of caudal fin), the other with individuals smaller than 14 cm TL. Next, 13 polystyrene tanks (50 × 40 × 20 cm, 50 L) were filled with approximately 43 L filtered (mesh size 200 μm) seawater freshly taken during the campaign and supplied with oxygen. The first experimental tank was used as control without any fish included to measure the background eDNA concentration of both species in seawater used, whereas each of the other tanks subsequently held one additional fish compared to the previous one. Hence, plaice were partitioned over the 12 tanks in the following abundances: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 individuals per tank. To assess the effect of size, the tanks with uneven numbers of individuals were filled with small

TABLE 1 Species-specific primers and probes for plaice (*Pleuronectes platessa*) and sole (*Solea solea*), and internal positive control (IPC): forward primer (F), reverse primer (R), and probe (P) sequences, gene target, and fragment size in base pair (bp) are provided.

Target	Primers (F, R), probe (P)	Sequence (5′–3′)	Target gene	Fragment size (bp)
Plaice	Plepla_F15107	TAGGCTTCGCAGTCCTCCTC	Cytb	90
	Plepla_R15196	TTGCAGGCGTGAAGTTGTCT		
	Plepla_P15169	FAM-CTAAAAGATTT GGGGAAAATAGG GCGAGT-BHQ1		
Sole	Ss-COI-F	CCCCTGCTTTCCTGCTACTT	COI	100
	Ss-COI-R	GCATGGGCGAGGTTACTTGA		
	Ss-COI-P	FAM-ACCTCATCCGTTGT TGAAGC		
IPC	IPC-D2-F	ATGACAGCCACTCCTCCG	Plasmid	149
	IPC-D2-R	GGAACGAACCAAACAGT CTTC		
	IPC-D2-P	HEX- AGCAGAGACCCATT CCCTCAGAGC-ZEN/IBFQ		

Note: The plaice primer–probe set was designed by Knudsen et al. (2019), whereas the sole primer–probe set is newly designed. A plasmid insert sequence from Dengue virus type 2 (GenBank M29095.1) was used as an internal positive control.

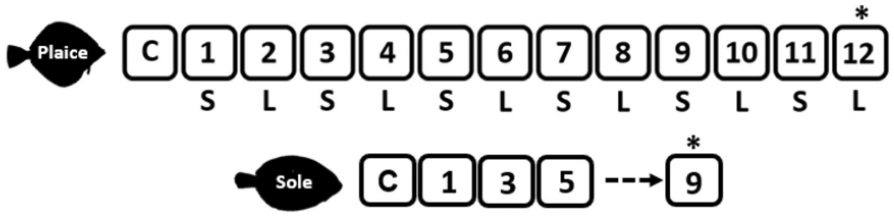

FIGURE 1 Setup of plaice and sole eDNA shedding mesocosm experiment. The first tank serves as control (C) only containing filtered seawater. For plaice, the subsequent 12 tanks each contain one more fish than the previous one. Uneven numbered tanks contain smaller (S) individuals (<14 cm TL), while even numbered tanks hold larger (L) individuals (≥14 cm TL). eDNA from tank labeled 12 and 9 were used for validation of plaice and sole-specific primers and probe (*), respectively. For sole, the subsequent three tanks contained 1, 3, and 5 individuals (all >9 cm TL). After 24 hours, these nine sole individuals were put together into an additional tank (9).

individuals (<14 cm TL) and even numbered tanks with large individuals (>14 cm TL) (Figure 1).

Given the limited number of sole ($n = 9$) caught during the November 2021 survey, the number of experimental tanks was reduced to four and differences in size were not considered. For both species and each of the tanks (including the control tank), two water samples of 0.5 L for eDNA analyses were collected before adding fish, and at 5 min, 1 h, and 24 h after introduction of fish to the tanks. The 5-min time point was chosen to investigate whether eDNA would be picked up quickly after introducing the fish to the tanks. The 1-hour time point was chosen to verify that eDNA was actually shed by the fish, as it is possible that the 5-min eDNA copies resulted from manipulating the fish instead of actual shedding. The 24-h time point was chosen as final time point because under field conditions in the North Sea, water masses are completely replaced after two tidal cycles. Because our aim is to use eDNA in a field setting, we did not investigate when eDNA concentrations reached a stable state. Fish were not fed during the experiment and were released after the completion of the experiment.

2.2.2 | eDNA filtration and extraction

From each experimental tank, one water sample for plaice or two water samples for sole were filtered through 0.45 µm enclosed PVDF Sterivex-HV pressure filters (Merck Millipore) using a peristaltic pump system (Masterflex L/S). A total of 13 (plaice) and 10 (i.e., two replicates per tank with sole) filters per time point were air-dried, capped at both ends, and stored at −21°C for subsequent molecular analyses. All practical work was performed in a laminar flow cabinet in a dedicated eDNA extraction room to avoid contamination. eDNA was extracted from the filters using a modified protocol of the DNeasy Blood & Tissue Kit (Qiagen). In brief, a master mix containing 718-µL Buffer ATL and 80-µL proteinase K was added per eDNA filter. To account for the efficiency of the extraction and to infer PCR inhibition, 2 µL of an exogenous internal positive control (IPC) was added to the master mix during the DNA extraction of all field samples. The IPC consisted of a 149 bp plasmid insert from the Dengue virus type 2 (GenBank M29095.1) with a concentration of 30,000 copies μL^{-1} (Table 1) following Brys et al. (2021). Next, filters were incubated overnight at 56°C, and subsequently processed according to the manufacturer's protocol. Finally, eDNA was eluted in 100 µL TE 1× buffer heated at 70°C and incubated for at least 10 min.

2.2.3 | ddPCR analysis

eDNA concentrations for the water samples of the shedding experiments and seawater samples taken for quantitative eDNA patterns in the Belgian part of the North Sea (i.e., local scale) were determined using a QX200 ddPCR system yielding 20,000 droplets per 20 µL sample (Bio-Rad Laboratories). Each reaction consisted of 2.5 µL template DNA, 750 nM target-specific forward and reverse primer (1.5 µL each), 375 nM target-specific fluorescent probes (TaqMan probes with FAM and HEX, 1.5 µL each), and 10 µL Bio-Rad ddPCR supermix for droplet generation. The generated droplets were transferred to a C1000 Touch Thermal Cycler with a 96-well Deep Reaction Module (Bio-Rad Laboratories) for PCR amplification. For both species, the PCR program was as follows: 10 min at 95°C, followed by 40 cycles of denaturation for 30 s at 94°C and extension at 56°C for 1 min, with a ramp rate of 2°C per s, followed by 10 min at 98°C. Following PCR amplification, samples were placed into the QX200 Droplet Reader (Bio-Rad Laboratories) to analyze the number of target-positive and target-negative droplets. eDNA copy numbers per reaction volume were calculated by the QuantaSoft Software v1.7.4 (Bio-Rad Laboratories) using Poisson statistics on the ratio of positive and negative droplets (Miotke et al., 2014). To determine the optimal eDNA concentration for accurate quantification, each sample was run once undiluted and as a ½ dilution. Several negative controls of 2.5 µL HPLC grade water were included in each ddPCR plate to account for contamination.

2.2.4 | dPCR analysis

eDNA concentrations of seawater samples taken for quantitative eDNA patterns in the southwestern North Sea (i.e., regional scale) were determined using a QIAcuity Digital PCR system (Qiagen)

with the QIAcuity Nanoplate 26k 24-well (Qiagen). The switch to the dPCR system was driven from a practical point of view since the dPCR device was newly bought at our laboratory after we had completed the ddPCR analyses of the local scale samples and is more cost and time efficient to use than the ddPCR. First, the concentration of eDNA samples was determined using the Quantus Fluorometer with the Quantifluor dsDNA System (Promega). eDNA samples were diluted to a concentration of 10 ng μL^{-1} using UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific). Next, each dPCR reaction was prepared in a final volume of 40 µL and consisted of 5 µL template DNA, 15 µL water and 10 µL 4× Probe PCR Master Mix and 750 nM target-specific forward and reverse primer, 375 nM target probe, 750 nM IPC primer, and 375 nM IPC probe. Prior to amplification, one reaction mix per well is partitioned into approximately 26,000 individual partitions, which results in very few templates present in each partition. For both species, amplification conditions were as follows: 2 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and extension at 56°C for 30 s. Each plate contained a negative control. Target fluorescence was measured with the QIAcuity Software (Qiagen). Comparison of eDNA concentrations from samples that had been processed with dPCR and ddPCR revealed a positive correlation for plaice ($R^2 = 0.62$) and sole ($R^2 = 0.85$, yet after removal of one outlier) (Figure S1). Yet, throughout our study, eDNA measurements have not been mixed between the two methods.

2.2.5 | Data analysis

The total effective concentration C_e (= eDNA copies per liter filtered water) was calculated for each sample using the following formula (see Brys et al., 2021 for further details):

$$C_e = \frac{\left(\frac{C_{per20}}{V_r}\right) \times V_e}{V_w} \times \left(\frac{C_{ipcAdded}}{C_{ipcMeasured}}\right)$$

where V_r is the total ddPCR or dPCR reaction volume (in µL), V_e is the total elution volume after DNA extraction (in µL), V_w is the total volume of filtered water (in L), C_{per20} is the number of copies of the sample (per 20 µL), $C_{ipcAdded}$ is the initial number of copies of IPC (= on average 30,000 copies μL^{-1}), and $C_{ipcMeasured}$ is the obtained sample-specific IPC number of copies in each ddPCR and dPCR reaction.

The relationship between eDNA concentration and (A) abundance (in numbers of fish) and (B) biomass (in g) was assessed using the function lm in R package stats v4.1.2 (R Core Team, 2022). The Pearson correlation coefficient r and coefficient of determination, also called *R*-squared value (R^2), were calculated with the function stat_cor in R package ggpubr v0.4.0 (Kassambara, 2020). *R*-squared is a measure used to assess the extent of variance to which the variable abundance or biomass explains the variable eDNA concentration in the regression model (Kasuya, 2019). Differences in eDNA concentration between two size classes of plaice (small and large fish) over three different time periods of shedding (5 min, 1 h, and 24 h of emission in the mesocosm) were assessed by using Wilcoxon rank sum test in R package stats.

2.3 | Quantitative eDNA patterns and trawl biomass

2.3.1 | Standardized trawl surveys and water collection

Beam trawling was conducted during daylight on board RV *Belgica (local scale survey)* and RV *Celtic Explorer (regional scale survey)* as part of standardized demersal surveys in 2020 and 2021 (Table 2). Haul duration varied from 15 to 30 min with tow speed at four knots against the current. Species were identified, measured, and counted (regional scale) or weighed (local scale) on board. Prior to the trawls, a total of 88 seawater samples of 10 L were collected at 34 sampling locations during four sampling campaigns (Table 2). Seawater samples were taken in tandem from Niskin bottles mounted on a stainless steel CTD frame at roughly 1 à 2 m above the seafloor (i.e., to minimize upwelling of non-recent sedimentary eDNA). Two-liter subsamples were taken from the Niskin bottles and immediately capped and frozen on board at −21°C for subsequent molecular analyses. eDNA filtration and extraction were performed in the same way as in the shedding experiment in a dedicated eDNA laboratory.

2.3.2 | Comparison of eDNA concentrations with trawl abundance and biomass data

We compared the observed abundance (number per km^2) and density (kg per km^2) of both sole and plaice in the trawls with the eDNA patterns (copies per liter) on two spatial scales: (1) Belgian part of the North Sea in spring 2020 and (2) southwestern North Sea in the third quarter of 2020 and 2021. The abundance and density data from the southwestern North Sea can be extracted from the ICES DATRAS database using the icesDatras package in R (Millar et al., 2022). The local abundance and density data (Belgian part of the North Sea) are available on the ILVO Marine server and may, in part, be consulted via the European Marine Observation and Data Network (EMODnet) website. Since the latter only comprise length frequency information, a length–weight relationship was applied to calculate the weight of the catch by haul. The length–weight relationships were estimated by fitting a linear regression model to individual length–weight observations of sole and plaice available from the southern North Sea beam trawl surveys between 51 and 54°N in 2020 (icesDatras package; Millar et al., 2022). The correlation between fish abundance (number per km^2) and eDNA (copies L^{-1}) per sampling station was assessed based on the Pearson coefficient and *p*-value using R package ggpubr (Kassambara, 2020). Similarly, the correlation between fish density (kg per km^2) and eDNA (copies L^{-1}) per sampling station was assessed. An eDNA concentration was considered an outlier if the value was 1.5 times the interquartile

TABLE 2 Sampling stations during three different RV *Belgica* campaigns: BEL2020/06A, BEL2021/04A, and BTS2020/21AB, and one RV *Celtic Explorer* campaign: BTS2021/CE21017.

Campaign	Station	Date	Lon	Lat	Beam width	Area	Sole abundance	Sole density	Plaice abundance	Plaice density	Sole eDNA	Plaice eDNA
BEL2020/06A	ft120	04/03/20	51.18	2.69	8	0.015	652.0	14,162.2	14,670.3	638,871.1	464.4 (±36.0)	2245.4 (±826.6)
BEL2020/06A	ft1401	05/03/20	51.28	2.91	8	0.016	574.5	12,108.3	510.7	15,968.3	735.3 (±270.6)	1091.5 (±291.1)
BEL2020/06A	ft140tris	05/03/20	51.34	3.01	8	0.015	197.9	14,362.7	527.8	26,562.4	901.8 (±838.7)	138.9 (±196.5)
BEL2020/06A	ft215	04/03/20	51.28	2.64	8	0.015	0	0	7895.6	165,653.6	579.9 (±274.2)	1331.4 (±11.1)
BEL2020/06A	ft230	04/03/20	51.53	3.02	8	0.015	0	0	875.8	11,303.2	260.1 (±5.7)	1631.7 (±82.7)
BEL2020/06A	ft330	03/03/20	51.43	2.79	8	0.015	0	0	746.5	42,657.0	6263.2 (±3159.8)*	661.7 (±161.5)
BEL2020/06A	ft415	04/03/20	51.39	2.33	8	0.016	63.9	8944.5	766.9	71,122.9	62.7 (±88.7)	863.3 (±553.4)
BEL2020/06A	ft7802	02/03/20	51.46	3.05	8	0.015	2745.6	100,565.0	12,812.8	290,908.7	1633.3 (±728.4)	1182.3 (±152.8)
BEL2020/06A	ft830	04/03/20	51.70	2.44	8	0.015	327.2	24,457.6	2486.5	122,496.4	996.6 (±1005.1)	176.6 (±249.7)
BEL2020/06A	ftLWO12	02/03/20	51.38	3.15	8	0.014	873.0	60,198.9	218.3	3858.4	561.8 (±153.8)	4409.4 (±1772.9)*
BEL2020/06A	ftWBB01	03/03/20	51.58	2.79	8	0.015	199.9	13,744.0	1732.8	59,234.2	364.9 (±116.6)	475.3 (±672.2)
BEL2020/06A	ftWG2	03/03/20	51.45	2.85	8	0.015	0	0	528.9	6853.3	1034.9 (±871.0)	317.5 (±269.4)
BTS2020/21AB	40	24/08/20	51.35	2.91	4	0.008	22,510.6	1,867,055.1	5826.3	460,805.1	5935.4 (±7856.3)	0
BTS2020/21AB	38	25/08/20	51.20	1.91	4	0.011	1059.3	107,697.7	1589.0	203,036.7	0	0
BTS2020/21AB	95	25/08/20	51.47	1.36	4	0.015	15,577.6	595,533.5	3101.7	259,167.4	267.1 (±277.4)	98.4 (±170.3)
BTS2020/21AB	28	26/08/20	52.84	2.27	4	0.007	6030.7	693,530.7	5071.3	655,153.5	2115.5 (±1191.8)	615.9 (±545.4)
BTS2020/21AB	112	27/08/20	53.72	1.87	4	0.015	328.1	48,556.4	11,220.5	921,259.8	800.9 (±1132.6)	652.8 (±608.1)
BTS2020/21AB	60	28/08/20	53.26	0.51	4	0.008	8594.2	430,990.2	9492.0	197,537.2	2196 (±2078.5)	0
BTS2020/21AB	8	31/08/20	53.16	2.74	4	0.007	737.0	112,028.3	2211.1	309,551.9	557.6 (±573.5)	188.9 (±267.1)
BTS2020/21AB	9	31/08/20	53.43	2.64	4	0.007	0	0	5918.0	605,802.5	103.7 (±179.6)	82.6 (±143.1)
BTS2020/21AB	85	02/09/20	51.62	1.91	4	0.014	7176.7	595,039.0	11,705.7	1,229,097.0	674.9 (±111.9)	672.2 (±465.3)
BTS2020/21AB	37	02/09/20	51.38	2.18	4	0.013	533.5	54,878.0	19,359.8	2,010,670.7	839.4 (±534.9)	892.6 (±1151.7)
BTS2021/CE21017	28	24/08/21	52.84	2.27	4	0.007	3895.4	598,219.2	4034.5	556,483.0	28,415.7 (±18,961.7)	12,684.8 (±13,282.8)*
BTS2021/CE21017	8	25/08/21	53.16	2.74	4	0.014	208.2	34,693.3	1249.0	166,527.9	352.5 (±134.2)	488.2 (±126.3)
BTS2021/CE21017	60	27/08/21	53.24	0.51	4	0.014	10,214.0	486,381.3	1876.0	34,741.5	435.8 (±121.3)	811.0 (±933.7)
BTS2021/CE21017	9	25/08/21	53.41	2.64	4	0.015	67.8	13,564.8	5425.9	623,982.6	1645.1 (±1894.1)	1336.4 (±1524.0)

TABLE 2 (Continued)

Campaign	Station	Date	Lon	Lat	Beam width	Area	Sole abundance	Sole density	Plaice abundance	Plaice density	Sole eDNA	Plaice eDNA
BTS2021/CE21017	112	26/08/21	53.74	1.86	4	0.014	70.0	8055.5	6514.4	588,400.1	1644.2 (±737.4)	3378.4 (±2751.9)
BTS2021/CE21017	95	30/08/21	51.46	1.31	4	0.011	20,656.0	314,716.3	3280.1	125,000.0	6240.4 (±2762.4)	3120.5 (±2459.4)
BTS2021/CE21017	85	31/08/21	51.57	1.86	4	0.018	3798.2	314,625.9	8276.6	857,709.8	9529.6 (±7895.6)	615.7 (±335.8)
BTS2021/CE21017	38	01/09/21	51.20	1.91	4	0.015	593.4	69,752.1	1318.6	176,424.1	785.3 (±1022.0)	495.2 (±542.5)
BTS2021/CE21017	40	23/08/21	51.36	2.94	4	0.015	9837.4	445,960.1	17,444.9	436,778.6	100,78.9 (±8731.1)	14,652.0 (±25,378.0)*
BTS2021/CE21017	37	01/09/21	51.38	2.19	4	0.015	613.2	68,138.5	10,765.9	1,176,069.8	5059.0 (±6493.3)	4506.0 (±4910.1)

Note: Station, date, longitude (lon), and latitude (lat) for shoot position (in decimal degrees), beam width (in meters), and swept area (km^2) are provided. For each station, sole and plaice observed abundance (number per km^2) and density (kg per km^2) and mean eDNA concentration (copies L^{-1} ± standard deviation) is given. Water samples collected during the BEL2020/06A and BEL2021/04A campaigns were analyzed using ddPCR, whereas samples collected during the BTS2020/21AB and BTS2021/CE21017 were analyzed with dPCR. Outlier eDNA concentrations are indicated with an asterisk (*).

range greater than the third quartile based on boxplots using function boxplot in R (R Core Team, 2022).

3 | RESULTS

3.1 | Species-specific primer–probe assay validation

The in silico analysis confirmed the species specificity of both the sole and plaice primer–probe assays with multiple mismatches occurring in the same region for most other fishes likely to co-occur with the target species (Table S2). For three species, non-native to the Belgian part of the North Sea (i.e., Alaska plaice *P. quadrituberculatus*, Northern rock sole *Lepidopsetta polyxystra*, and Dusky sole *Lepidopsetta mochigarei*), only one or two mismatches were found in the binding region of the corresponding plaice assay. The ddPCR empirical data confirmed the species specificity of the primer–probe assays for both species (Figure S2). The few positive droplets detected in non-target wells most likely represent cross-contamination or very low-efficiency amplification of the primers at off-target loci but are unlikely to interfere with the target eDNA concentration estimations.

To determine the sensitivity of the plaice primer–probe assay, the LOD was set at 1/100,000 dilution, corresponding with a mean concentration of 2.6, 4.2, and 9.0 copies μL^{-1} for the DNase-free dilution, artificial seawater dilution, and harbor water dilution, respectively (Table S1). The LOQ was one dilution higher than the LOD for the DNase-free water dilution (26.2 copies μL^{-1}) and remained at the same value as LOD for the artificial seawater and harbor water dilution. Both the LOD and LOQ for the sole assay were set at 1/10,000 dilution corresponding to a concentration of 2.5 copies μL^{-1}.

3.2 | Quantification of eDNA in shedding experiment

The background eDNA concentration obtained with ddPCR from the seawater used in the mesocosm tanks prior to the introduction of both species was, respectively, 6531 ± 1385 copies L^{-1} for sole and 20,960 ± 1584 copies L^{-1} for plaice. For sole, eDNA concentrations increased with prolonged emission time from 19,472 ± 3317 copies L^{-1} filtered water for one individual after 5 min to a maximum value for five individuals at 24 h (38,082,910 ± 5,475,190 copies L^{-1} filtered water). The correlation between eDNA concentration and sole biomass (g) was positive and significant for all three emission time periods (5 min: $r = 0.96$, $R^2 = 0.91$; 1 h: $r = 0.95$, $R^2 = 0.88$; and 24 h: $r = 0.95$, $R^2 = 0.89$; all values $p < 0.001$; Figure 2). The relationship between eDNA concentration and abundance was also positive and significant (5 min: $r = 0.86$, $R^2 = 0.70$; 1 h: $r = 0.85$, $R^2 = 0.69$; and 24 h: $r = 0.86$, $R^2 = 0.69$; all values $p < 0.05$, Figure 3). For plaice, the mean eDNA concentration per sample was 12,438,625 copies L^{-1}. Tank 10 contained one dead individual resulting in the maximum

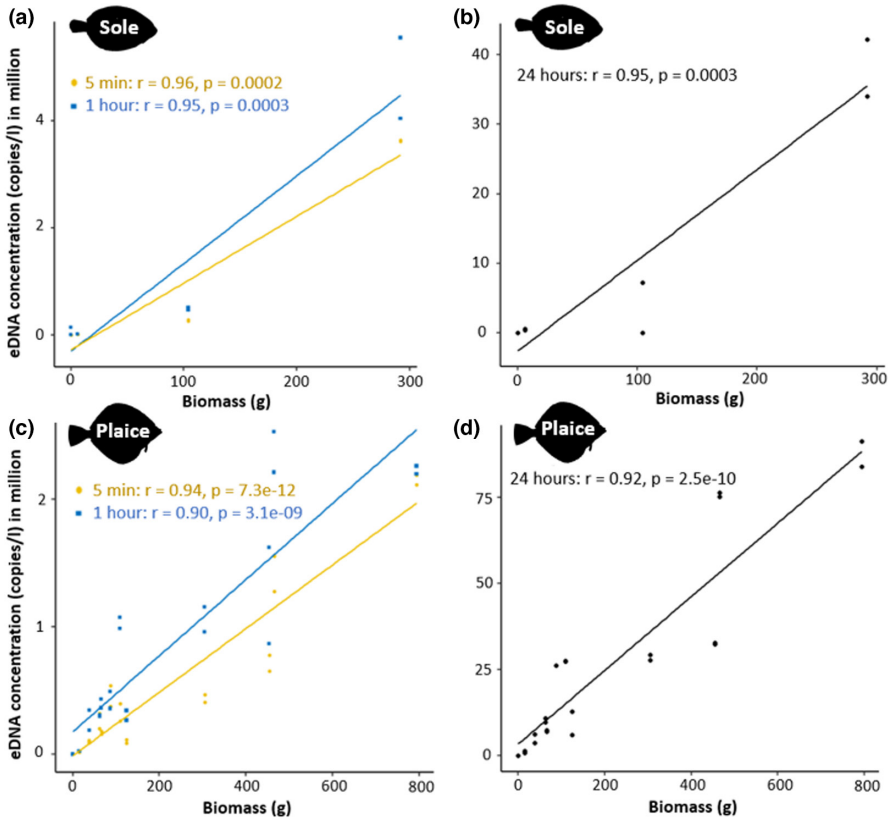

FIGURE 2 The relationship between sole (a, b) and plaice (c, d) biomass (g) and eDNA concentration (copies L^{-1}) in mesocosms measured after 5 min and 1 h (a, c) and 24 h (b, d) of shedding time. Each dot represents the means of two replicates. There are two biological replicates for sole and one biological sample for plaice. The Pearson correlation coefficient r and p-value of each correlation are given in the top left.

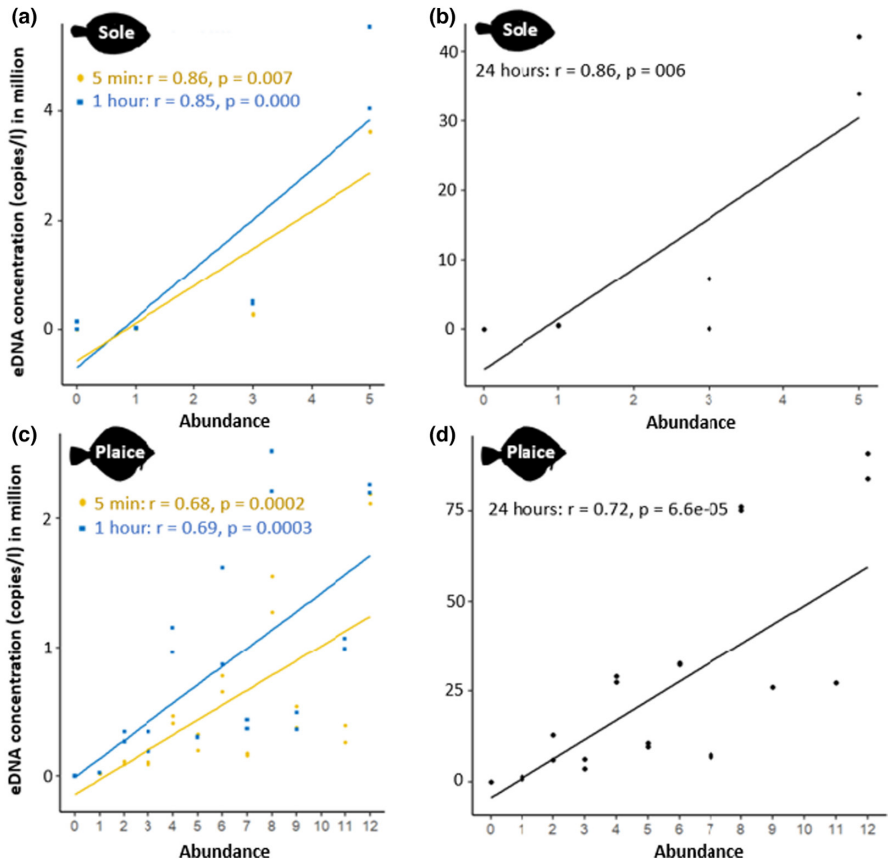

FIGURE 3 The relationship between sole (a, b) and plaice (c, d) abundance (number of fish) and eDNA concentration (copies L^{-1}) in million in mesocosms measured after 5 min and 1 h (a, c) and 24 h (b, d) of shedding time. Each dot represents the means of two replicates. There are two biological replicates for sole and one biological sample for plaice. The Pearson correlation coefficient *r* and *p*-value of each correlation are given in the top left.

documented concentration of 98,782,609 ± 491,900 copies L^{-1} after 24 h of emission and was consequently excluded from further analyses. Target eDNA concentration of plaice increased significantly with biomass (5 min: $r = 0.94$, $R^2 = 0.88$; 1 h: $r = 0.90$, $R^2 = 0.79$; and 24 h: $r = 0.92$, $R^2 = 0.83$; all values $p < 0.0001$) for all three emission time periods (Figure 2). eDNA was found in one out of six ddPCR

negative controls (3915 copies L^{-1} at 24 h). Target eDNA concentration of plaice increased significantly with abundance ($r=0.69$, $R^2=0.44$, $r=0.68$, $R^2=0.44$, $r=0.72$, $R^2=0.50$; all values $p<0.001$, Figure 3). Larger plaice (>14 cm TL) shed significantly more eDNA compared to smaller plaice (<14 cm TL) at the three emission periods (Figure 4). The number of sole used in the controlled experiment was too low to compare smaller with larger individuals.

3.3 | Correlation between eDNA concentrations and observed abundance and density

3.3.1 | Local scale: Belgian part of the North Sea

All eDNA samples displayed positive amplification. eDNA concentrations obtained with ddPCR varied between 63 (±89) and 1633 (±728) copies L^{-1} for sole. For plaice, eDNA concentrations ranged from 139 (±197) to 2245 (±827) copies L^{-1}. The highest eDNA concentrations for sole and plaice were found in the sampling stations with highest observed abundance and density of the respective species (Table 2). The Pearson correlation between observed abundance (number per km^2) and eDNA concentration (copies L^{-1}) retrieved from concurrently collected water samples was positive and significant for both sole ($r=0.69$, $p=0.02$; Figure 5a) and plaice ($r=0.67$, $p=0.03$; Figure 5b). Likewise, the correlation between observed density (kg per km^2) and eDNA concentration (copies L^{-1}) was positive and significant for sole ($r=0.63$, $p=0.04$; Figure 5c) and plaice ($r=0.68$, $p=0.02$; Figure 5d). No eDNA was detected in the negative controls.

3.3.2 | Regional scale: southwestern North Sea

All samples (the mean of two or three replicates) displayed positive amplification, except for sole in one station and plaice in three stations in 2020 (Table 2). The highest eDNA concentrations for both species were found in the sampling stations with highest observed abundance and density of the respective species in 2020 (Table 2). Likewise, the highest eDNA concentrations for both species were found in the sampling stations with highest observed density, but not with the highest observed abundance. Despite the absence of sole specimens at station 9 in 2020, sole eDNA was detected (mean 103 copies L^{-1}). The correlation between eDNA concentrations and observed abundance was positive and significant for sole ($r=0.75$, $p=0.013$) and plaice ($r=0.74$, $p=0.014$) in 2020 (Figure 6), but not in 2021 (Figure S3). The correlation between eDNA concentrations and observed density was positive and significant for sole in 2020 ($r=0.90$, $p=0.0004$; Figure 7a) and 2021 ($r=0.70$, $p=0.025$; Figure 7b) and for plaice in 2020 ($r=0.88$, $p=0.0009$; Figure 7c), but not in 2021 ($r=0.51$, $p=0.19$; Figure 7d). No eDNA was detected in the negative controls.

4 | DISCUSSION

Environmental DNA holds promise to complement and improve current monitoring and stock assessments of marine fish populations in a non-destructive, cost-efficient, and highly sensitive manner (Maiello et al., 2022; Stoeckle et al., 2021; Thomsen et al., 2012, 2016). To investigate the potential for eDNA to support fisheries monitoring, empirical studies are needed on how eDNA in marine environments correlates to the presence, abundance, and biomass of fish stocks emitting those eDNA patterns.

4.1 | Reliable primer–probe assays to detect sole and plaice

A crucial first step in reliable, large-scale eDNA-based detection and quantification of target species is to validate the specificity and sensitivity of selected primer–probe assays (Freeland, 2017; Kirtane et al., 2021; Thalinger et al., 2021; Zhang et al., 2020). The evaluation

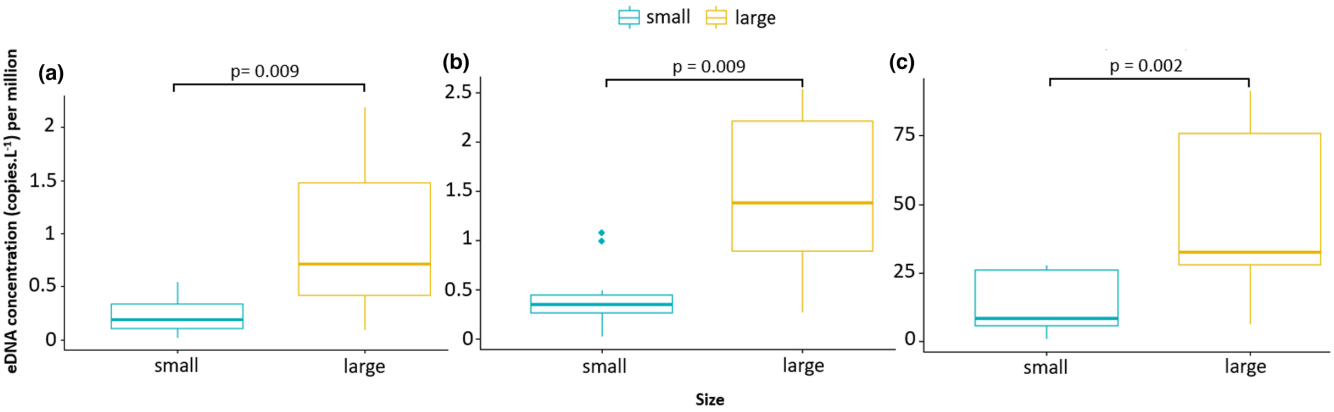

FIGURE 4 The average eDNA concentration (copies L^{-1}) of small (<14 cm TL in yellow) and large (>14 cm TL in blue) plaice in mesocosms measured after (a) 5 min, (b) 1 h, and (c) 24 h of shedding time. The p-value between the two size classes ($p<0.05$) based on Wilcoxon rank sum test is provided for the three different shedding times.

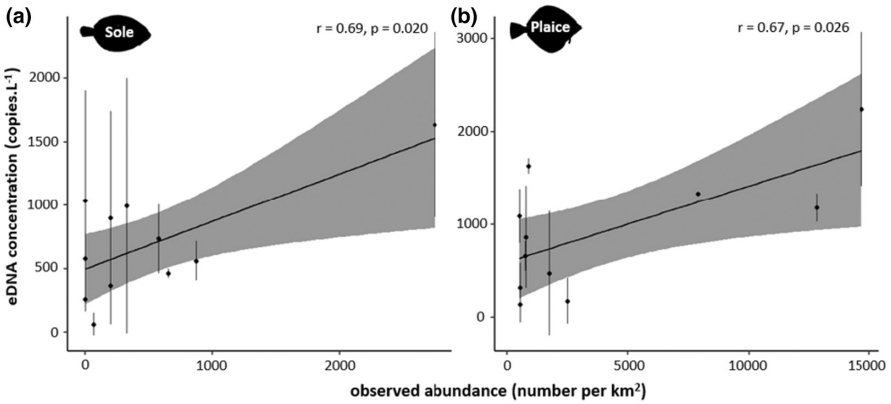

FIGURE 5 Relationship between (a, b) observed abundance (number per km^2) and (c, d) density (kg per km^2) of sole (a, c) and plaice (b, d) in the Belgian part of the North Sea and their respective eDNA concentrations (copies L^{-1}) obtained from ddPCR. The Pearson correlation coefficient r and p-value are provided. Each dot represents the means of two technical replicates. The shaded area around the regression line indicates 95% confidence intervals. One eDNA outlier was removed for both sole and plaice.

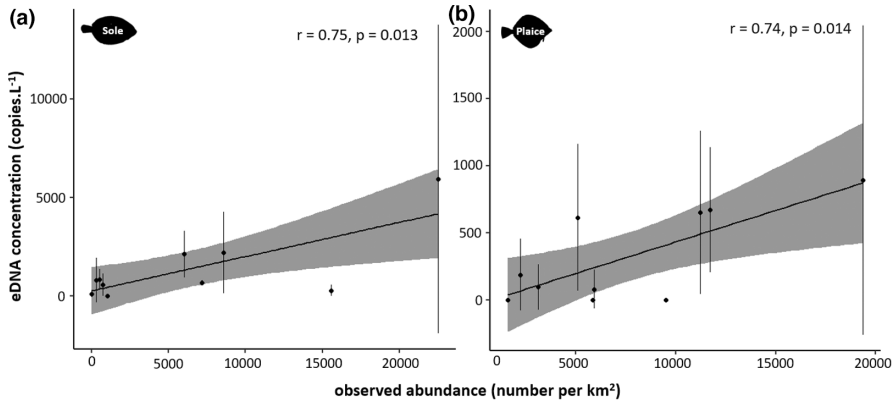

FIGURE 6 Relationship between observed abundance (number per km^2) of (a) sole and (b) plaice in the southwestern North Sea and their respective eDNA concentrations (copies L^{-1}) in 2020 obtained with dPCR. The Pearson correlation coefficient *r* and *p*-value are provided. Each dot represents the means of three biological replicates. The area around the regression line indicates 95% confidence intervals. One true outlier (the maximum eDNA concentration) was kept in the sole dataset.

of primer specificity is particularly important for eDNA in the aquatic environment because of its highly degraded nature, which requires relatively short DNA targets (Freeland, 2017; Rees et al., 2014). In this study, in silico analyses based on primer–template nucleotide mismatches predict that both the plaice and sole primer–probe assays are species specific and amplification of the target sequence in non-target species is expected to be inefficient. Only the plaice primer–probe assay may have the potential to amplify eDNA from Alaska plaice, Northern rock sole, and Dusky sole because only a few mismatches occur on the primer binding sites. However, as those species are not expected to occur in the North Sea (Froese & Pauly, 2010), these potential off-target amplifications are unlikely to effectively lead to any false positive detection in seawater samples taken from the North Sea. The ddPCR analyses for both species complemented and confirmed the in silico evaluation as the empirically detected background signal derived from non-target species co-occurring with the target-species appeared to be very limited. Consequently, our findings for the plaice primer–probe assay confirm the species specificity for this assay as developed and tested by Knudsen et al. (2019) in the North Sea-Baltic Sea region. Taken together, we confirmed the species specificity and sensitivity of the primer–probe assays for sole and plaice.

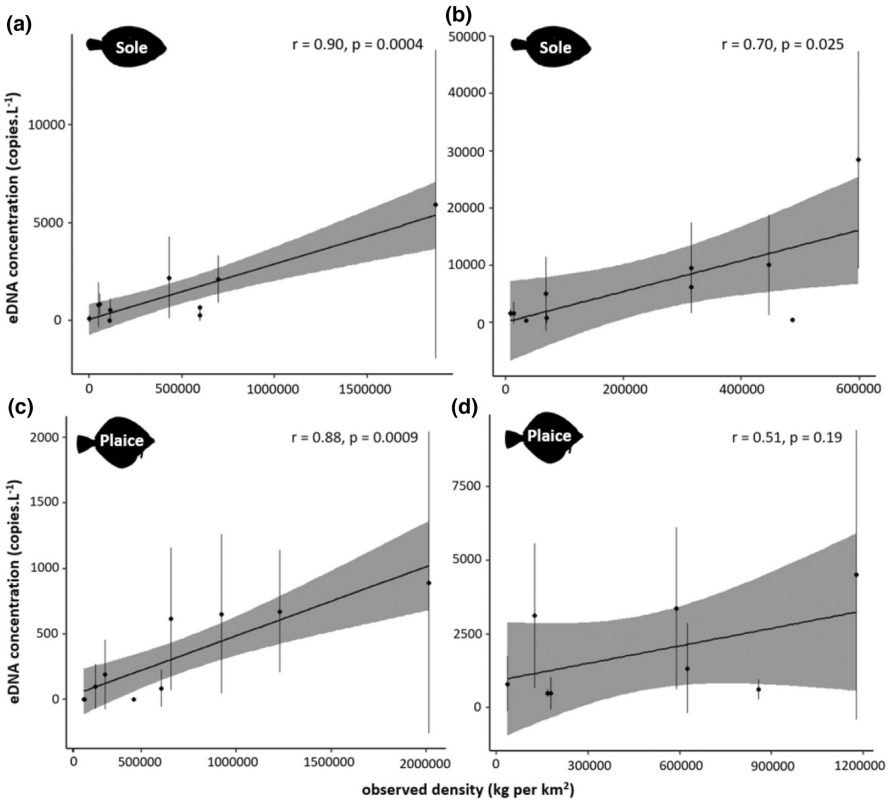

FIGURE 7 Relationship between observed density (kg per km^2) of sole (a, b) and plaice (c, d) in the southwestern North Sea and its respective eDNA concentrations (copies L^{-1}) in 2020 (left) and 2021 (right) obtained from dPCR. The Pearson correlation coefficient r and p-value are provided. Each dot represents the means of three biological replicates. The area around the regression line indicates 95% confidence intervals. One true outlier (the maximum eDNA concentration) was kept in the sole dataset of 2020 and 2021. Two eDNA outlier values were removed from the dataset of plaice in 2021.

4.2 | eDNA concentrations are positively correlated with biomass and abundance of sole and plaice in a controlled mesocosm experiment

In the controlled mesocosm experiment, a strong positive and significant linear relationship was found between biomass and eDNA concentration for both sole and plaice. Similar observations have been made for other fish under controlled conditions (Karlsson et al., 2022; Klymus et al., 2015; Rourke et al., 2022). Likewise, also the positive relationship between abundance and eDNA concentration for both species is in line with other studies (Spear et al., 2021). eDNA could already be detected after 5 min under controlled conditions, although fast shedding might have resulted from handling the fish at the start of the experiment. The eDNA concentration in tanks containing larger plaice increased significantly more compared to those with smaller fish at all three emission periods. In accordance with these results, previous observations report more eDNA shedding by larger individuals compared to smaller individuals (Jo, Murakami, et al., 2019; Maruyama et al., 2014; van Bleijswijk et al., 2020). Although larger individuals tend to exhibit disproportionally lower rates for metabolic and excretory processes relative to their body mass (Vanni & McIntyre, 2016), shedding rates are probably also a function of the surface area of the individual (Yates et al., 2021). Furthermore, one experimental tank had to be excluded from the entire dataset due to disproportionately high levels of eDNA concentration caused by one dead individual. Yet, Kamoroff and Goldberg (2018) found low to negligible detectable levels of eDNA from dead individuals compared to their live counterparts in natural systems. Therefore, positive detections associated with dead organisms are unlikely to confound monitoring in the natural marine environment.

4.3 | eDNA concentrations are positively correlated with abundance and biomass in the Belgian part of the North Sea (local scale)

In all sampling stations in the Belgian part of the North Sea, eDNA of both plaice and sole was detected. Interestingly, eDNA of sole was detected in four sampling stations in which sole was absent from the trawl catches. It is unlikely that these positive amplifications are false positives as a result of contamination as this would have been detected by the negative control samples. Furthermore, the specificity tests show that non-target eDNA amplification is improbable in our study area. Consequently, there is no evidence of technical contamination and non-target eDNA amplification caused by insufficient specificity of the primer–probe assay. Large variability over short spatial scales in fish stocks, especially in aggregating species, might introduce bias as the absence of species in trawl catches does not necessarily imply their absence in the area. Furthermore, a significant, positive relationship between eDNA (copies L^{-1}) and observed density (kg per km^2) was found for both sole and plaice. A significantly positive correlation between eDNA concentrations and biomass by station, although statistically weak, was also previously found for Atlantic cod (Salter et al., 2019), highlighting the potential of complementing traditional survey data with eDNA.

4.4 | eDNA concentrations are not always positively correlated with abundance and biomass in the southwestern North Sea (regional scale)

In nearly all sampling stations in the southwestern North Sea, eDNA of both plaice and sole was detected. No eDNA of sole and plaice was found, however, in one and three sampling stations, respectively, despite being present in the trawl catches just after seawater collection. As these samples were processed in batches, inadequate laboratory processing seems unlikely to have resulted in the absence of eDNA. An inappropriate sampling design, however, could have resulted in potential false negatives. By increasing the sampling effort and the number of biological and technical replicates, the occurrence of false negatives can be accounted for (Burian et al., 2021; Mauvisseau et al., 2019). In contrast, eDNA of sole was detected in one sampling station for which no sole was caught in the trawl. This finding is unlikely a false positive observation and could be indicative of the short-term spatiotemporal variability of the marine eDNA signal (Jensen et al., 2022). We hypothesize that the absence of a significant positive relationship in plaice in 2021 could be a direct consequence of the limited sampling size and/or the environmental conditions in the southwestern North Sea. The study region consists of shallow waters (~50 m depths) and is located at the interface of the English Channel and the North Sea with strong tidal, wind, and buoyancy forcing (Baeye et al., 2011). Hence, it is an area with low vertical stratification and strong currents resulting in a high degree of mixing (van Leeuwen et al., 2015). Given that eDNA can persist for approximately 1–2 days in the North Sea (according to estimations for water temperatures ranging between 10 and 15°C) (Collins et al., 2018), it can reasonably be assumed that these hydrographic and environmental conditions strongly affected the local variability in eDNA concentrations in the water column. Regardless, the significant and positive correlations between eDNA and density of sole (in both years) and plaice (in 2020) highlight the potential of eDNA to estimate biomass for stock assessments of flatfishes in the North Sea. Yet, the positive and significant relationship between eDNA and abundance of both species in 2020, but not in 2021, indicates the challenges of using eDNA to estimate abundances for stock assessments. These findings are in agreement with observations of Yates et al. (2019) who found a stronger correlation between eDNA concentration and abundance in laboratory environments compared to the natural environment.

4.5 | Challenges of using quantitative eDNA measures

We found considerable variability in eDNA concentrations among biological and technical replicates taken in the North Sea. High eDNA variability has been reported before and might be explained by the heterogenous nature of eDNA unevenly dispersed in the environment (Hinlo et al., 2018; Klymus et al., 2015; Pilliod et al., 2013). Furthermore, we detected variability in eDNA concentrations when sampling from the same mesocosm tank under controlled conditions. Large variations in eDNA concentration have also been found among replicate samples under controlled conditions (Klymus et al., 2015; Nevers et al., 2018; Troth et al., 2021). Here, the quantification of eDNA samples was also variable when sampling from the same tank under controlled conditions despite efforts of homogenizing the water by first stirring the recipient. Similarly, Allan et al. (2021) reported large variations in eDNA concentrations between biological replicates taken at the same time after homogenizing the sampled water. Regardless, the absence of natural conditions such as currents and varying particle sizes of eDNA might result in an uneven distribution of eDNA. Therefore, uncertainty introduced by the experimental design might explain the variability among biological replicates under controlled conditions and warrants an adequate sampling scheme in the field to be able to infer correct conclusions from eDNA data for fisheries management. Such a sampling scheme could be designed based on survey data distribution maps of the species of interest, where sampling effort is increased (more eDNA replicates) in regions with low abundance of the target species.

Particularly in a dynamic environment such as the North Sea, eDNA does not remain at the location of release, but is dispersed and degraded over time (Murakami et al., 2019). Studies show that the limit of eDNA detectability in the marine environment ranges from 1 h (Murakami et al., 2019), a few hours (Dell'Anno & Corinaldesi, 2004), to up to 48 h (Collins et al., 2018). Consequently, a hydrodynamic dispersion model that simulates the distribution of eDNA concentrations under certain assumptions can be extremely valuable in estimating fish abundance and biomass from eDNA in the future (Fukaya et al., 2021; Kutti et al., 2020). Furthermore, different life-history processes like spawning can influence the release of eDNA in the water column (Takeuchi et al., 2019). For instance, spawning in both species takes place in shallow, but not necessarily coastal waters during the spring season (Borremans, 1987); therefore, temporal eDNA data may provide additional valuable information on spawning events which is highly relevant for fisheries. When the population is dominated by larvae or juveniles, quantitative eDNA data should be interpreted with care to avoid biomass overestimation (Maruyama et al., 2014). Therefore, establishing baseline eDNA levels, in and outside spawning seasons, is necessary before eDNA measurements can complement current monitoring (Rourke et al., 2022). Furthermore, the ratio of mitochondrial eDNA to nuclear eDNA could be a potential proxy for estimating the age and size of particular species (Jo, Arimoto, et al., 2019). Aforementioned patterns should be further investigated in the future.

5 | CONCLUSIONS

Our results contribute to a growing body of evidence that eDNA concentrations positively correlate with abundance and biomass, at least under controlled conditions and within the first 24 hours. Under natural conditions, eDNA concentrations are subjected to

many more factors than only shedding rates (nicely summarized in Hansen et al., 2017) yet we generally detected a positive and significant relationship between eDNA concentration with observed abundance and density (in terms of biomass) using field samples of both species. The absence of positive correlation between eDNA and concurrent trawl biomass estimates in the southwestern North Sea for plaice in 2021 could be related to the limited number of eDNA samples taken during the survey, which highlights the need for proper guidelines in regards to amount of eDNA samples needed for monitoring. Consequently, eDNA concentration measurements should be handled in their own frame of reference before serving as input to complement current stock assessments. Regardless, the validity of abundance and/or biomass quantification using eDNA depends on many environmental, chemical, and physical variables, which require further research. Until then, our findings emphasize the potential but also challenges of implementing eDNA into stock assessments of commercially important flatfish species.

AUTHOR CONTRIBUTIONS

S Desmet and S Derycke conceptualized the study. S Desmet, S Derycke, and LV collected the samples. S Desmet and SM created the data. SMM and KS analyzed the data. SMM wrote the manuscript. All authors contributed to the interpretation of the findings and detailed manuscript revision.

ACKNOWLEDGMENTS

The research was conducted under the projects SoleDNA and ZeroImpact funded by the European Maritime and Fisheries Fund (EFMZV). Ship time on the RV Belgica was provided by BELSPO and RBINS-OD Nature. We are very grateful to the master and crew of RV Belgica, RV Geo Ocean V, and RV Celtic Explorer, and everyone that helped collecting water samples. We would also like to express our gratitude to Quentin Mauvisseau (Natural History Museum, Oslo) and Sabrina Neyrinck (INBO) for the development and validation of the sole primer–probe assay, respectively.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The raw data underlying the main results of this study is available in the public repository Dryad (https://doi.org/10.5061/dryad.r4xgxd2jh).

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SUPPORTING INFORMATION

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

How to cite this article: Maes, S. M., Desmet, S., Brys, R., Sys, K., Ruttink, T., Maes, S., Hostens, K., Vansteenbrugge, L., & Derycke, S. (2024). Detection and quantification of two commercial flatfishes (*Solea solea* and *Pleuronectes platessa*) in the North Sea using environmental DNA. *Environmental DNA*, *6*, e426. https://doi.org/10.1002/edn3.426