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Complementing underwater visual surveys with eDNA metabarcoding to detect Mediterranean non-indigenous fishes

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Abstract

Non-indigenous species (NIS) are among the main drivers of native biodiversity loss, habitat alteration, and degradation of ecosystem services, with some NIS posing risks to human health. Efficient monitoring strategies are necessary to assess the distribution and impacts of NIS. In this study, we compared the performance of environmental DNA (eDNA) metabarcoding and visual surveys with SCUBA diving to detect marine fish NIS in Greek waters. We collected water samples from 20 coastal sites across the Aegean, Ionian, and Levantine Seas in both warm and cold periods, targeting the 12S rRNA region. A reference 12S Mediterranean NIS sequence database was created to improve regional monitoring. Underwater visual surveys were performed at the same sites to visually detect fish NIS. Overall, 15 non-indigenous fishes were detected, five with both eDNA and visual surveys, seven exclusively by eDNA, and three by visual surveys alone. The southern stations yielded more NIS detections than the northern stations in both periods. Our findings demonstrate that eDNA can provide a rapid, low-cost, and effective tool, advocating for its integration into systematic NIS monitoring in the eastern Mediterranean Sea. A comprehensive barcode reference database is essential in enhancing the effectiveness of eDNA approaches. Thus, the combination of eDNA metabarcoding and traditional underwater visual surveys is recommended for comprehensive monitoring of NIS in marine environments.

Keywords: eDNA metabarcoding; coastal; biological invasions; eastern Mediterranean Sea; 12S rRNA; visual survey.

Introduction

Biological invasions are complex, multistage processes, and their success is influenced by several factors at each stage (Blackburn *et al.*, 2011; Briski *et al.*, 2018). Ecological and socioeconomic impacts of these invasions are escalating globally, yet mitigation measures are often delayed, insufficient, or non-existent (Ahmed *et al.*, 2022). Consequently, post-invasion management costs exceed pre-invasion expenses (for prevention) by more than 25-fold (Cuthbert *et al.*, 2022). Given the exponential increase in the cost of inaction against non-indigenous species (NIS), it is imperative to implement management actions during early invasion stages (Giakoumi *et al.*, 2019; Katsanevakis *et al.*, 2023). Effective decision making in this context requires detailed information about NIS introduction pathways, documented occurrence, distribution ranges, and population sizes (Briski *et al.*, 2018; Galanidi & Zenetos, 2022). Standardised protocols that yield consistent data and time series are crucial to enhance the quality of current state assessments and future projections (Ziegler, 2013; Borja *et al.*, 2016). Such data can be collected through various approaches, such as targeted scientific surveys, fisheries records, and citizen science initiatives (e.g., Katsanevakis *et al.*, 2012a; Zenetos *et al.*, 2013).

In the Mediterranean Sea, a biodiversity hotspot of over 17,000 marine species (Coll *et al.*, 2010), a total of 993 NIS has been reported, with 751 species confirmed as established by 2021 (Zenetos *et al.*, 2022). NIS establishment rate has increased by 40% from 2010 to 2021 (Zenetos *et al.*, 2022). The steady rise of sea surface tem-

perature during the past decades (Pastor *et al.*, 2020) has facilitated the establishment and spread of thermophilic Lessepsian species in the eastern and central Mediterranean Sea (Raitsos *et al.*, 2010; Katsanevakis *et al.*, 2014; Karachle *et al.*, 2022). However, it has been debated whether the recent increase in NIS records represents an actual increase in NIS introductions or reflects intensified scientific efforts and citizen science initiatives (Bailey *et al.*, 2020; Galanidi & Zenetos, 2022; Zenetos *et al.*, 2022). There is a growing number of NIS and cryptogenic species in the eastern Mediterranean Sea (Zenetos *et al.*, 2018; Ragkousis *et al.*, 2023), with at least 221 species recorded in the Greek Seas, including 17 classified as invasive, and 148 with established populations, while the establishment status of the remaining species remains uncertain (Zenetos *et al.*, 2018).

Fish is a major group of NIS with 173 established or casual species (42 in the western, 65 in the central, and 139 in the eastern Mediterranean Sea, respectively; Zenetos *et al.*, 2022; Galanidi *et al.*, 2023). At least 40 have been detected in Greece, with five being characterised as invasive (Korakaki *et al.*, 2021). The three most reported invasive fishes are *Siganus luridus, Siganus rivulatus*, and *Pterois miles* (Ragkousis *et al*., 2023). *Siganus* grazers tend to overgraze on coastal rocky reefs, causing the degradation of local habitats and communities, whereas *P. miles* is a voracious opportunistic predator feeding on important local biodiversity, even commercial fishes, causing disruption to local food webs and posing threats to human health with its venomous spines (Tsirintanis *et al*., 2022, 2023). NIS richness is higher in southern Greece (South Aegean and Levantine Seas), due to temperature regimes favourable to thermophilic Lessepsian species (Ragkousis *et al.*, 2023; Evangelopoulos *et al*., 2024). Moreover, a positive correlation has been observed between the number of NIS introduced as transport stowaways and proximity to major ports (Zenetos *et al.*, 2018; Ragkousis *et al.*, 2023).

NIS early detection is critical for effective management (Katsanevakis *et al.*, 2023). Traditional methods such as fishing nets, scuba diving, underwater cameras, and settlement panels are widely used to detect and monitor NIS species (Tamburini *et al*., 2021; Evangelopoulos *et al*., 2024). Each method has significant strengths and can be deployed separately or combined in NIS assessments (Bessell *et al.*, 2023; Robinson *et al.*, 2023; West *et al.*, 2024). They have been included to support decision making and management (Lehtiniemi *et al.*, 2015; Katsanevakis *et al.*, 2023), however, they tend to be costly and could underestimate species occurrence, richness and population state variables, while they might be highly intrusive to local ecosystems (Katsanevakis *et al.*, 2012a). Environmental DNA (eDNA) analysis has evolved as a promising alternative to overcome most limitations of traditional sampling methods (Taberlet *et al.*, 2012; Borrell *et al.*, 2017; Fediajevaite *et al.*, 2021). It relies on the retrieval of the genetic material released into the aquatic environment (e.g., water) and on the taxonomic assignment using DNA metabarcoding (Taberlet *et al.*, 2012). In marine research, eDNA has emerged as a groundbreaking tool for monitoring the diversity and the distribution of marine organisms, ranging from plankton to top predators (Stat *et al.*, 2017; Zhang *et al.*, 2020b). By principal, eDNA requires less sampling effort (e.g., fewer sampling trips), less taxonomic expertise, and is cost effective and less invasive than traditional sampling methods, as there is no direct interaction with the target organisms and the habitat (Deiner *et al.*, 2017). One of the major advantages of eDNA though, is that it enables the detection of low abundance species (Jerde *et al.*, 2011), which is crucial for monitoring cryptic, rare, endangered, and invasive species at the early stages of invasion (Kelly *et al.*, 2014; Aglieri *et al.*, 2023).

The potential of eDNA for marine NIS detection and its integration in management strategies is increasingly discussed (Sepulveda *et al.*, 2020b; Morisette *et al.*, 2021; Zangaro *et al.*, 2021). Nevertheless, eDNA based community assessments and protocols are still to be standardized to be officially included in management recommendations and policy (Sepulveda *et al.*, 2020b; Morisette *et al.*, 2021). Moreover, incomplete reference databases limit the method's applicability (Ruppert *et al.*, 2019; Doble *et al.*, 2020; Duarte *et al.*, 2021). For example, among the 173 NIS fish established or casually encountered across the Mediterranean Sea (Zenetos *et al.*, 2022), 52 are currently lacking an available reference barcode for the fish-specific 12S rRNA primer MiFish (Miya *et al.*, 2015), hampering species assignment and detection of taxa. Here, we aim to investigate the efficacy of eDNA sampling in detecting NIS fishes across Greek coastal zones and to compare the results from eDNA to those from standardized underwater visual surveys.

Material and Methods

Sample collection and processing

Seawater samples for eDNA analyses were collected from 20 sites across Greek territorial waters between February 2021 and March 2022 (Fig. 1). Two sampling expeditions per site were carried out, one during the warm (May-October) and one during the cold period (November-April). In total, 120 water samples were collected using a 2-L Niskin water sampler at approximately 10 m depth (three replicate 1 L samples at each sampling station). Each 1 L sample was filtered through a 0.22 μm Sterivex filter capsule (Merck Millipore) and promptly stored at -20 °C for subsequent processing (one filter was discarded during filtration due to possible contamination by contact). All equipment was thoroughly cleaned with 10% bleach and distilled water before use, and sterilized single-use syringes were employed for filtration. To investigate possible contamination, three *in situ* negative controls and one PCR blank were included, each involving the filtration of bottled commercial water.

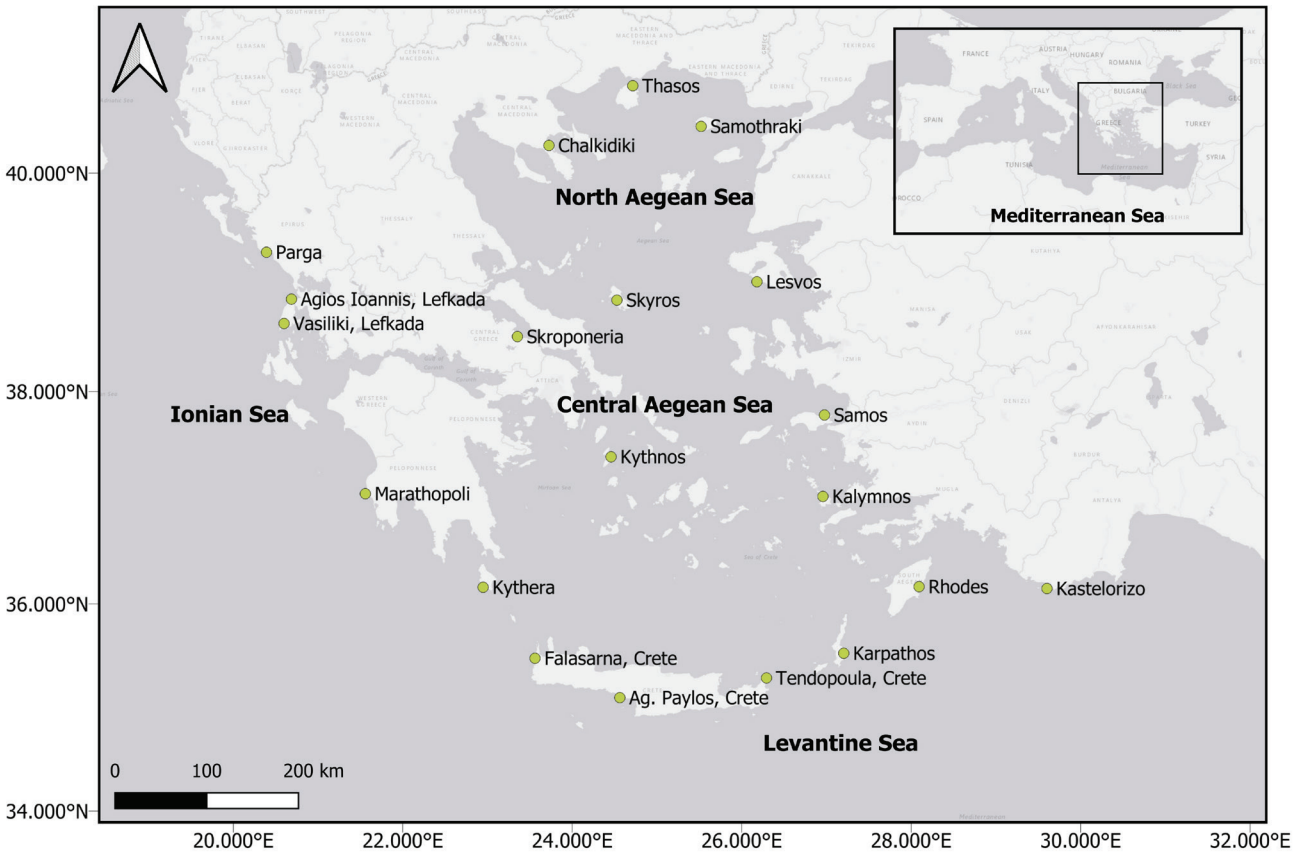

Fig. 1: Approximate environmental DNA sampling and underwater visual survey (SCUBA) sites for non-indigenous fish species detection across the four main regions of Greek waters.

eDNA extraction, amplification, and sequencing

Filters were retrieved from Sterivex capsules and DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit sensu Cowart *et al.* (2018) with minor modifications. Firstly, we extracted DNA from the entire filter; secondly, we did not use whitebeads and incubated the filter with 720 µl ATL and 80 µl Proteinase K overnight. The addition of RNase was omitted, DNA was eluted twice with 40 µl AE and stored at -20 °C. A ~170 bp barcoding region of the 12S rRNA was amplified using the MiFish primer set, targeting fish detection (Miya *et al.*, 2015). Each DNA extract was amplified in methodological PCR triplicates, which were pooled to maximize the detection of rare taxa. The PCR mix included 10 µl AmpliTaq gold 360Master mix (Applied Biosystems), 0.16 µl Bovine Serum Albumin (20 µg/µl, Thermo Scientific), 4.84 µl H_2O, 1 µl of the forward and 1 µl of the reverse tagged primer (5 µM, including 2–4 leading Ns and 8-bp sample tags) and 3 µl of the extracted eDNA. The amplification conditions included denaturation at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s and, 72 °C for 30 s and a final extension at 72 °C for 5 min. Upon PCR amplification, all samples per plate were pooled and concentrated using a MinElute PCR Purification Kit (Qiagen), following the manufacturer's guidelines. The final DNA concentration was measured using a Qubit 4 Fluorometer (Invitrogen). Library preparation was performed through the TruSeq DNA PCR-Free High Throughput Library Prep Kit (Illumina) with 2 µg of DNA and included a) end repairing, b) MinElute concentration, c) adenylation, d) ligation, and e) clean-up. Library concentration was quantified to avoid overclustering the Illumina flow-cell using the NEBNext Library Quant Kit for Illumina (New England Biolabs), following the kit's quick-start protocol. A pair-end sequencing (2 x 150 bp) was carried out on an Illumina NovaSeq 6000 sequencer (Illumina, USA) at Novogene facilities (Cambridge, UK).

Bioinformatic analyses

Sequenced data in multiplexed fastq files were processed through the MJOLNIR pipeline (https://github.com/uit-metabarcoding/MJOLNIR), primarily utilizing tools from the OBITools package (Boyer *et al.*, 2016). Paired-end reads were aligned using *illuminapairedend*, retaining only those with an alignment quality score above 40. Demultiplexing and primer-sequence removal were performed by *ngsfilter*, discarding sequences with mismatched primer tags. Length filtering and dereplication of sequences were performed with *obigrep* and *obiuniq*, retaining sequences of 140-190 bp. Singleton sequences and chimeric amplicons were eliminated using the *uchime_denovo* algorithm from VSEARCH (Rognes *et al.*, 2016). Molecular operational taxonomic units (MOTUs) delimitation based on linkage networks was implemented using the SWARM procedure (Mahé *et al.*, 2015).

Taxonomic assignment was performed using the

DNA Universal-databank for Fisheries and Aquaculture reference database (DUFA; last updated on 2022-01-06) for the 12S MiFish fragment (https://github.com/uit-metabarcoding/DUFA). Subsequent to the taxonomic assignment, putative pseudogenes were removed using LULU (Frøslev *et al.*, 2017). DUFA NIS availability was cross-referenced with an updated Mediterranean Sea NIS list (Zenetos *et al.*, 2022). Additionally, we manually added 75 published 12S sequences absent in the DUFA 12S database (excluding unverified entries; Table S1). These sequences were retrieved from the National Center for Biotechnology Information (NCBI) Taxonomy database (https://www.ncbi.nlm.nih.gov/taxonomy). We also generated 12S sequences for four species that were not included in the NCBI database (*Torquigener flavimaculosus*, *Stephanolepis diaspros*, *Upeneus pori*, and *Pteragogus trispilus*; Accession Numbers: PQ638932-35, respectively), using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) for DNA extraction and the MiFish primer pair (F: 5'-GTCGGTAAAACTCGTGCCAGC-3'; R: 5'- CATAGTGGGGTATCTAATCCCAGTTTG -3') for amplification (Miya *et al.*, 2015). The PCR protocol included an initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 50 s, 50 °C for 50 s, 72 °C for 50 s, and a final extension at 72 °C for 5 min. PCR products were commercially sequenced (Macrogen, the Netherlands), and sequences were manually checked and edited using ProSeq 3.0 (Filatov, 2002).

Environmental variables

During eDNA sampling expeditions, water temperature, salinity, and dissolved oxygen were measured *in situ* using an Orion multi-parametric device. Turbidity was measured at each station with an Orion AQUAfast turbidity-meter. Additional water samples were collected from all sites to analyze five nutrient salts (PO_4^{3-}, NO_2^-, NO_3^-, SiO_2, and NH_4^+), total suspended solids (TSS), and chlorophyll-a concentrations at approximately 10 m depth using a Niskin Water Sampler. Approximately 500 mL of sea water were filtrated under vacuum pressure through 0.45 μm pre-weighted nitrocellulose filters and kept frozen at −20 °C for nutrient analyses according to Parsons *et al.* (1984). The filters were also used to estimate TSS according to APHA (1998). For chlorophyll-a analysis, 1 L of water samples were filtrated through 47 mm diameter GF/F glass fiber filters. Filters were subsequently diluted in 10 mL of 90% acetone, stored overnight at 4 °C in the dark and analyzed following APHA (1998). All analyses were performed on a HITACHI U-2001 spectrophotometer. Analytical precision was tested by triplicate analysis of 40% of all samples.

Underwater visual surveys

Separate field expeditions were conducted for underwater visual surveys. To assess the presence, population density, and biomass of alien marine fish species, SCUBA diving surveys were conducted by two divers. Strip transects and line transect distance sampling were conducted along three consecutive replicate transects (25 m in length and 5 m in width) at two depth zones (5 m and 15 m), according to Thanopoulou *et al.* (2018). The first diver conducted strip transects targeting mobile species, estimating the total length (TL) of each identified fish. The second diver conducted distance sampling (Buckland *et al.*, 2001) targeting less mobile and cryptic species, recording the vertical distance of each fish from the line transect (Buckland *et al.*, 2001) and estimating its TL. The dominant substrate type (soft or hard) was recorded, and taxon identification was made *in situ* for most species, or by analyzing photographic and video material in cases of uncertainty.

Statistical analysis

For community analysis, environmental triplicate samples were merged and treated as a single sample. A minimum of 97% identity was used for species level identification (Miya *et al.*, 2015). Additionally, MOTUs with relative read abundance per sample bellow 0.005 or less than five reads were set to zero to minimize tag-switching bias (Antich *et al.*, 2023) and reduce the likelihood of false positives from potential contamination. All datasets were treated as qualitative presence-absence data on each MOTU per sample and all statistical analyses were performed in R 4.1 (https://cran.r-project.org). Non-metric multidimensional scaling (NMDS) ordinations, using the *metaMDS* function in the "vegan" package version 2.6.2 (Oksanen *et al.*, 2022), provided a reduced-space graphical representation of the species composition per site. Distance matrices were calculated using the Jaccard coefficient (function *vegdist*). Permutational analysis of variance (PERMANOVA) was performed to assess the influence of substrate (soft or hard), period (warm or cold), and sampling region (North Aegean, Central Aegean, Ionian, and Levantine Seas) on the composition of non-indigenous fish communities based on eDNA. PERMANOVA was conducted using the *adonis* function in the "vegan" package and using presence/absence data with 10,000 permutations. The wrapper function *pairwise.adonis2* from the package "pairwiseAdonis" 0.4.1 (Martinez Arbizu, 2020) was used for multilevel pairwise comparisons. NMDS plots were generated in R, heatmaps in Flourish (https://flourish.studio/), maps in QGIS 3.14 (https://www.qgis.org), and images were modified using Inkscape 1.1 (https://inkscape.org/).

Generalized Additive Models (GAMs; Hastie & Tibshirani, 1990) were used to investigate the effects of environmental variables on the NIS eDNA detectability on sites of species known occurrence, based on our visual surveys, literature, and online databases such as EASIN (Katsanevakis *et al.*, 2012b) and ELNAIS (Zenetos *et al.,* 2015). We applied the *gam* function in the "mgcv" package 1.8.34 in R (Wood *et al.*, 2016) and used the binomial distribution family. Collinearity among environmental variables was assessed using the Pearson correla-

tion coefficient in the "PerformanceAnalytics" package 2.0.4 (Peterson *et al.*, 2020). Predictor variables included water temperature, salinity, turbidity, total suspended solids (TSS), and total dissolved nitrogen (TDN) calculated as the sum of NO_2^-, NO_3^-, and NH_4^+. Additionally, NIS was set as a factor to control any species-specific detection bias. Candidate models (Venables & Ripley, 2002) are listed in Table S2. The Akaike Information Criterion (AIC; Akaike, 1973; Burnham & Anderson, 2002) was used for model selection.

Results

Approximately, 12.4 million paired-end reads were generated (103,766 on average per sample), 11,410,677 of which were retained for taxonomic assignment after filtering and MOTU delimitation, and 9,286,019 were subsequently assigned to a taxonomic group by the MJOL-NIR pipeline (Fig. S1). Only Actinopterygii reads were retained for downstream analyses; 72,696 reads (~0.59%) belonged to the classes Mammalia and Aves. The PCR blank and one negative control provided no reads for any MOTU. Between the other two negative controls, both had few reads of a MOTU assigned to *P. miles* (five and three), whereas the second had three additional reads of a *F. commersonii* MOTU. Due to the low number of reads in negative controls, we maintained all MOTUs for downstream analyses. We detected 250 unique MOTUs of fish, representing 38 orders and 72 families. For this study, only fish NIS were considered, comprising 4.41% of the total filtered Actinopterygii reads (n = 403,816). In total, 13 MOTUs belonged to 12 established NIS in the Mediterranean Sea (*Atherinomorus forskalii*, *Fistularia commersonii*, *Lagocephalus sceleratus*, *Lagocephalus suezensis*, *Parexocoetus mento*, *Pterois miles*, *Sargocentron rubrum*, *Siganus luridus*, *Siganus rivulatus*, *Stephanolepis diapros*, *Torquigener flavimaculosus*, and *Upeneus pori*). NIS richness was higher in the Levantine and Ionian Seas, with less records in the North Aegean (Fig. 2 and S2). Four species (*L. suezensis*, *S. rubrum*, *T. flavimaculosus*, and *U. pori*) were exclusive to the Levantine Sea, whilst *P. mento* was solely recorded in the central/eastern Aegean Sea (Kalymnos Island; Fig. 2). Five species were recorded only once through eDNA (*U. pori*, *T. flavimaculosus*, *S. rubrum*, *P. mento*, and *L. suezensis*). Three species were exclusively found in the southern Aegean and the Levantine Seas during the cold period (*L.*

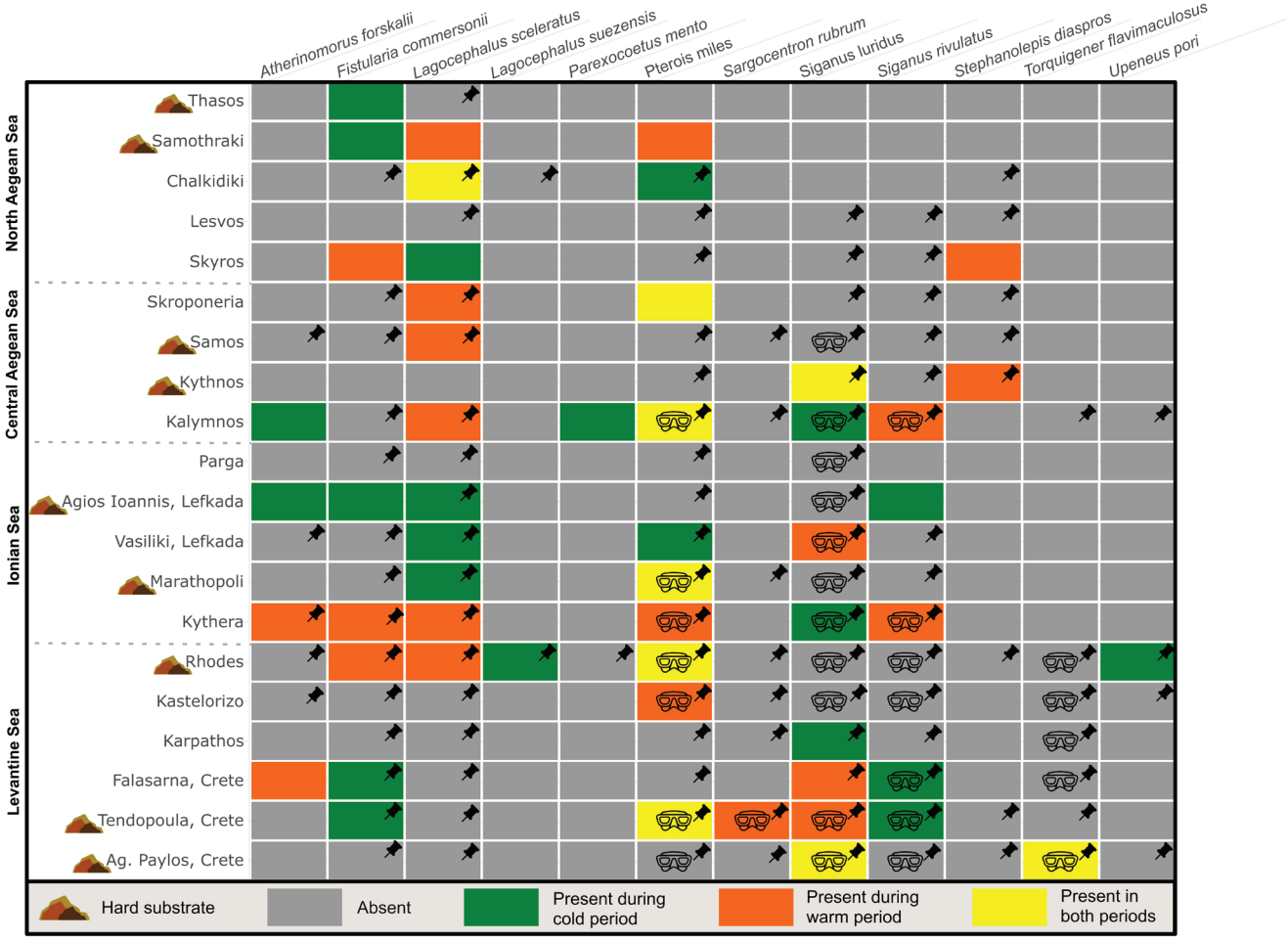

Fig. 2: Non-indigenous fish species detected in Greece using environmental DNA analysis, colours indicate species detection during warm (May-October), cold (November-April), and both periods; black masks indicate the detection with *in situ* visual observation by SCUBA diving and pins indicate previously documented occurrence in literature (references in Table S6).

suezensis, *P. mento*, and *U. pori*), whereas *S. rubrum* and *S. diaspros* were recorded in Crete and North/Central Aegean, respectively, during warm months.

Environmental variables

Eleven environmental variables were recorded at each station (Fig. S3). The highest temperatures and lowest salinity levels were documented in the North Aegean Sea (in the warm period). The lowest temperature was recorded at Skroponeria in the cold period (13 °C) and the highest at Thasos Island in the warm period (26.15 °C). Salinity ranged from 35.30 psu (Thasos Island, cold period) to 39.35 psu (Kastelorizo Island, warm period), with values below 38 occurring in the North Aegean sampling sites (Table S3). Chlorophyll-a ranged from 0.058 (Skroponeria, warm period) to 1.065 μg/L (Thasos Island, cold period). North Aegean stations (Thassos, Samothraki, Chalkidiki, Skyros, and Lesvos) demonstrated higher phosphate, nitrate, and silicate concentrations for both periods; Ionian stations showed maximum ammonium, turbidity and TSS values. The Central Aegean stations exhibited the highest nitrite concentrations during both periods. Additionally, collinearity among the environmental variables was checked (Fig. S4). Temperature was negatively correlated with dissolved oxygen and chlorophyll-a, but positively correlated with PO_4^{3-} and NO_3^-. Salinity was negatively correlated with chlorophyll-a, NO_3^-, and SiO_2. NO_3^- and SiO_2 were significantly correlated with the highest number of environmental variables, whereas TSS did not show any significant correlation with other variables.

The NMDS ordinations (stress: 0.097) indicated that NIS composition was not affected by substrate, sampling period, or geographic area (Fig. S5), which was corroborated by PERMANOVA results ($p > 0.05$; Table S4). However, PERMANOVA showed a statistically significant interaction between period and area ($p = 0.031$). Pairwise comparisons revealed significant differences between the Ionian Sea and both the North Aegean ($R^2 = 0.178$; $p = 0.022$) and Levantine Seas ($R^2 = 0.227$; $p = 0.008$), and a weak clustering was depicted in the corresponding NMDS analysis (Fig. S5a).

Visual survey vs eDNA

Visual surveys identified eight non-indigenous fishes (*Alepes djedaba*, *Cheilodipterus novemstriatus*, *Parupeneus forsskali*, *P. miles*, *S. rubrum*, *S. luridus*, *S. rivulatus*, and *T. flavimaculosus*; Fig. S6). Most fishes were recorded in the Levantine and Ionian Seas, whereas *A. djedaba* was exclusively detected at Skyros Island (North Aegean Sea, Fig. S6). Five species were identified by both visual surveys and eDNA metabarcoding, however, seven were detected only through eDNA analysis. Three species, *A. djedaba*, *C. novemstriatus*, and *P. forsskali* were not detected in eDNA samples (Figs 2, 3, and S6).

GAMs

Five variables with possible implications on eDNA degradation were used for GAMs: temperature, salinity, turbidity, TSS, and TDN. Among the 12 candidate models (Table S2) the highest percentage of deviance explained and adjusted R^2 were 17.80% and 0.087 (m1), respectively. The best GAM, indicated by the lowest AIC value, included water temperature and TDN (m10), with none of them being statistically significant (Tables S2 and S5; Fig. S7); this model explained only 4.71% of the deviance.

Discussion

The Mediterranean ecosystems are under dramatic pressure due to biological invasions and climate change, e.g., sea-surface temperature increase, algal blooms, and heatwaves driving mass mortalities, (Boudouresque *et al.*, 2017; Garrabou *et al.*, 2022; Chatzimentor *et al.*, 2023). The rapid establishment of NIS in the eastern Mediterranean Sea, particularly those with a direct and significant impact on local biodiversity or ecosystem services, highlights the need for proper detection and management of their populations (Galil *et al.*, 2018; Roy *et al.*, 2019). Here, we applied eDNA metabarcoding aiming to detect marine fish NIS, and we were able to report almost twice the species that were detected by visual surveys. These findings support the potential of eDNA as an effective monitoring tool for detecting marine fish NIS in the region. Our study further demonstrated that it is important to expand existing monitoring schemes considering different sources of information.

Invasive fish NIS have more severe negative cumulative impact on coastal hard substrate habitats, being more intense in the South Aegean Sea where NIS are more abundant compared to the North (Tsirintanis *et al.*, 2023). Most of the NIS reported in Greece prefer shallow reef-associated areas, with a few exceptions such as *Tylerius spinosissimus*, that is caught in depths greater than 90 m (Froese & Pauly, 2023). Our eDNA samples were collected from coastal waters, hence only species inhabiting shallow waters of the continental shelf were detected. Fish NIS detected in this study have been recorded in depths down to 132 m (*F. commersonii*), however most are restricted in shallow waters, usually <60 m (Froese & Pauly, 2023). Most of them were detected in lower latitude stations; however, our results suggest broader NIS fish distributions than previously reported or documented by the visual surveys of this study (Fig. 2). In some cases, eDNA revealed NIS presence in northern sites where no publicly available records were available, such as *F. commersonii* and *P. miles*; their presence at these sites had not been documented previously, although specimens have been found in adjacent areas (e.g., Katsanevakis *et al.*, 2020b). For example, *A. forskalii* is one of the first and most abundant lessepsian migrants in the eastern Mediterranean Sea (Tsirintanis *et al.*, 2022 and references herein), however it has few records in Greece (Fig. 2).

Our results revealed a wider distribution range than previously reported, with Lefkada Island (Ionian Sea) being currently the most westerly site of occurrence in the Mediterranean Sea (unpubl. data). Such findings are crucial for species such as *L. sceleratus* which was detected at two sites for the first time and is known for posing direct threats to local ecosystems, fisheries, and human health (Katsanevakis *et al.*, 2018; Tsirintanis *et al.*, 2023).

Potential environmental predictors (e.g., salinity, nitrogen content as TDN, turbidity, and TSS) and the substate type were tested using GAMs and PERMANOVA, respectively. Our analysis did not reveal any potential impact of environmental variation on NIS eDNA detection, likely due to the small sample size (Tables S2 and S5). Additionally, substrate type categories incorporated a variety of ecological niches and interactions, potentially masking any impact. Despite fish NIS occurrence reported at various study sites (Table S6), temporal NIS distribution patterns might be affecting our results; most NIS detected in our study are thermophilic and are anticipated to be present in areas with higher water temperatures (Rilov & Galil, 2009). However, no seasonal variation has been observed to affect abundance and biomass of most NIS species (Mavruk *et al.*, 2017), corroborating our results. Furthermore, no studies have attempted to outline the influence of other variables, such as NO_2^-, NH_4^+, and or turbidity for lessepsian fish NIS range expansion to the best of our knowledge. Nevertheless, our results might align better with eDNA physical properties and fish NIS life cycle (Harrison *et al.*, 2019 and references herein). Accelerated DNA degradation caused by temperature increase has been reported in other studies (Strickler *et al.*, 2015; Mauvisseau *et al.*, 2022). Similarly, elevated acidity and chlorophyll-a concentration, associated to raised bacterial abundance, negatively affect eDNA quality (Seymour *et al.*, 2018; McKnight *et al.*, 2024). Conversely, salinity itself or related factors, such as diminished microbe abundance, have been associated with slower eDNA degradation (Collins *et al.*, 2018; Saito & Doi, 2021), whereas TDN has not been linked to significant effects on eDNA decay (Seymour *et al.*, 2018; McKnight *et al.*, 2024). On the other hand, the rate of shedding DNA increases with higher temperature and fish biomass (Jo *et al.*, 2019). Hence, in the warmer regions of the study area, where many thermophilic fishes are more abundant, these two contradicting mechanisms may either increase or decrease detectability through eDNA under specific conditions in comparison to colder regions/periods. Higher detectability during spawning periods may also affect the effectiveness of eDNA (Tsuji & Shibata, 2021; Ostberg & Chase, 2022).

Traditional methods have been used to identify and monitor NIS before the advent of eDNA method, assisting NIS management (Zaiko *et al.*, 2018; Fediajevaite *et al.*, 2021). Here, we demonstrated that eDNA outperforms conventional monitoring methods in fish NIS detection. The higher efficiency of eDNA is corroborated in various studies (Valentini *et al.*, 2016; Stat *et al.*, 2017; Robinson *et al.*, 2023). Marine NIS detection using SCUBA-based visual surveys has few inherent limitations, which eDNA sampling can address. For example, SCUBA surveys might overlook NIS that are cryptic, small, or blend into their surroundings (Marchini *et al.*, 2015). Divers are also limited by depth, time, and the breadth of areas they can survey, making visual surveys challenging in habitats with high complexity (Friedlander & Parrish, 1998). Additionally, some fish NIS might be nocturnal or elusive, escaping daytime SCUBA surveys, yet their DNA fragments can still be detected in water samples (Kelly *et al.*, 2014). Nevertheless, three species were recorded by visual surveys and were not detected by eDNA analyses (Fig. 3). Therefore, eDNA metabarcoding has the potential to become a powerful tool in NIS biomonitoring assessments, complementing but not replacing traditional methods. The complementary use of both techniques in fish biomonitoring is consistently highlighted in recent

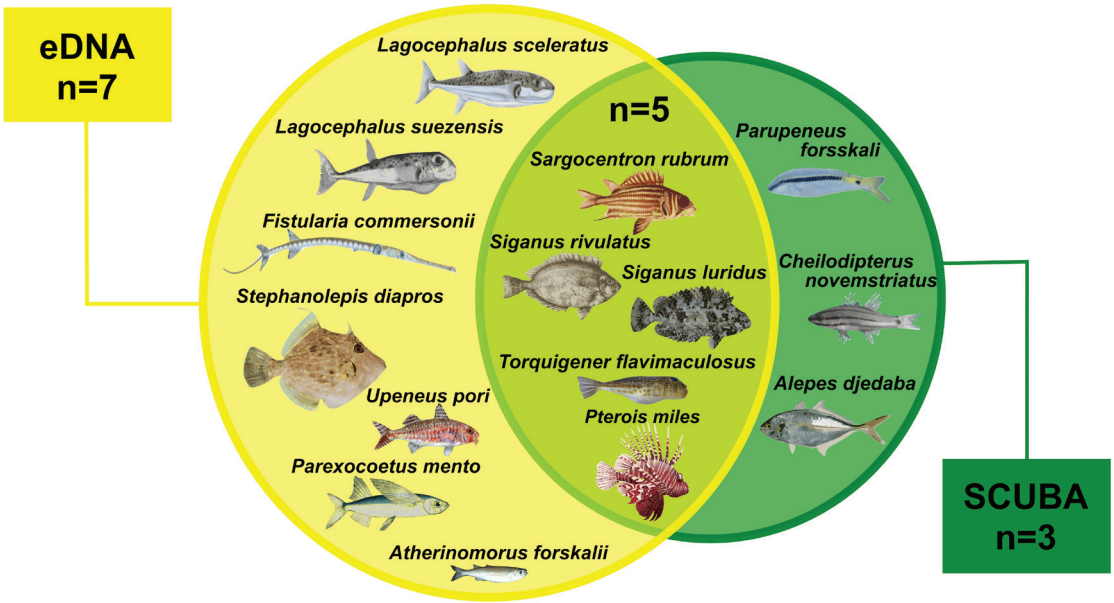

Fig. 3: Comparison of non-indigenous marine fish species detected in Greece using eDNA analysis and visual observation by SCUBA diving.

studies, with an apparent overlap in species detection and observed clear advantages of each method (Valdivia-Carrillo *et al.*, 2021; Robinson *et al.*, 2023).

The choice of molecular markers for meta-barcoding undeniably influences the outcome of eDNA studies (Zangaro *et al.*, 2021; Zhang *et al.*, 2020a). Mediterranean fish NIS, as a taxonomic group, have a considerably extensive barcode reference database, mostly referring to the cytochrome c oxidase I (COI) gene (Zangaro *et al.*, 2021), however, there are still species that are absent from public databases; particularly for other barcoding regions, e.g., 12S rRNA (Table S1). In this study, the MiFish primers pair was selected due to its higher fish specificity and its overall improved performance in fish detection to other primers. For example, it produces greater number and proportion of usable fish reads to COI primers despite the more complete reference database of the latter primer pair (Collins *et al*., 2019; Zhang *et al*., 2020a; Miya *et al*., 2020). Interestingly, 31 of 97 fish NIS with established populations in the Mediterranean basin (Zenetos *et al.*, 2022), were missing a 12S rRNA reference sequence (https://www.ncbi.nlm.nih.gov/nucleotide/). We have generated four 12S rRNA sequences from species that were not included in existing databases and detected 12 out of 40 fish species listed as NIS in Greece since 2018; 32 of these species are already classified as invasive or established (Zenetos *et al.*, 2018). One species with confirmed records using traditional sampling methods (both visual observations and published records), was not detected in eDNA samples (e.g., Dimitriadis & Sourbès, 2015; Evangelopoulos *et al.*, 2020; Miliou & Loudaros, 2020). This discrepancy was mainly attributed to the incompleteness of reference databases. In the context of the present study, no reference sequences matching the MiFish 12S rRNA barcoding region of *P. forsskali* were found in public databases (NCBI). Despite the increased use of eDNA techniques for rare species detection, the incompleteness of reference databases is the most restricting factor in monitoring and management for NIS in the Mediterranean Sea. Moreover, primer specificity and efficiency for targeting specific groups should be considered. Numerous assays with group- or species-specific primers are designed, promising higher levels of precision and efficiency (Ardura, 2019; Mohammed-Geba *et al.*, 2020).

In this study, we focused on fish NIS detection in the eastern Mediterranean Sea by utilizing primers that primarily amplify fish (Miya *et al.*, 2015); our approach can be used for future research on fish NIS management, particularly as reference databases are constantly updated, improving identification accuracy and rates. Nevertheless, the current approach could be optimized. For example, the combined use of multiple primers could enhance species detection and/or richness as multi-marker approaches often yield more detailed and higher resolution community data by targeting more taxonomic groups (Ferreira *et al.*, 2024; Fontes *et al.*, 2024). Conversely, marker selection could be applied specifically for single species-based applications (Hartle-Mougiou *et al.*, 2024). Protocol optimization could focus on DNA yield by testing filtered seawater volume and/or using inhibitor removal kits. Finding the optimal volume is extremely beneficial to monitoring strategies, particularly as small volumes can be advantageous due to their minimal physical and logistical requirements, faster sampling, processing times, and equipment simplicity. However, higher water volumes can improve eDNA metabarcoding results and consistency (Bessey *et al.*, 2020; Govindarajan *et al.*, 2022). Additionally, different extraction methods, types of filters, environmental parameters, and protocol costs should be examined (Duarte *et al.*, 2021; Fonseca *et al.*, 2023; Rishan *et al.*, 2023). Finally, a sufficient sample size should provide a stronger basis for statistical analysis, improving our ability to assess the impact of environmental variables on fish NIS detection. Therefore, while eDNA methods can be effective for alien species monitoring, they have not been extensively used in the Mediterranean Sea and protocol standardization is required to maximise their potential for fish NIS detection.

Recommendations for management actions and conservation priorities

Invasive fish species have created profound challenges for local ecosystems and the fishing industry in Greece (Katsanevakis *et al.*, 2020b; Christidis *et al.*, 2022). NIS compete for space and resources with native biodiversity and/or they alter the structure of local habitats (Tsirintanis *et al.*, 2022). Two fish NIS detected in this study (*S. luridus* and *S. rivulatus*) are among the "worst" invasive species in the Mediterranean Sea, whereas eleven have moderate to high impact on biodiversity, ecosystem services, and human health (Katsanevakis *et al.*, 2016; Tsirintanis *et al.*, 2022). Five of the recorded NIS (*L. sceleratus*, *P. miles*, *S. luridus*, *S. rivulatus*, and *T. flavimaculosus*) pose serious threats to human health, mainly due to deleterious toxins and direct attacks to humans (Katikou *et al.*, 2009; Tsirintanis *et al.*, 2022). The invasive silver-cheeked toadfish (*L. sceleratus*) also damages fishing gears and removes catches, with significant economic losses to coastal small-scale fisheries (Katsanevakis *et al.*, 2018; Christidis *et al.*, 2022). The two siganids (*S. luridus* and *S. rivulatus*), *F. commersonii*, and *P. miles* have also severe destructive impact on local ecosystems; grazing, an intense habitat-altering behaviour of the first two species, and the opportunistic behaviour of the latter, can reshape local marine food-webs and increase predation mortality of already stressed populations, affecting fisheries indirectly (Bariche *et al.*, 2013; Azzurro *et al.*, 2017; Katsanevakis *et al.*, 2018; Batjakas *et al.*, 2023). Despite the impact of invasive fish, only the striped catfish (*Plotosus lineatus*) is included in the European list of invasive alien species of Union Concern, in compliance with the EU Regulation 1143/2014, compromising effective management (Katsanevakis *et al.*, 2023).

Acknowledging the need for effective monitoring and management of NIS distribution is essential (Katsanevakis *et al.*, 2014). Various mitigation measurements have been proposed and/or applied for the restriction or reduction of invasive NIS (e.g., Galil *et al.*, 2019; Kleitou

et al., 2021 and references herein). However, eDNA is not included in official invasive NIS management plans, despite its efficiency (Sepulveda *et al.*, 2020b; Morisette *et al.*, 2021), although its value has been recently recognized by the European Commission (Costello & Trottet, 2023). In countries with increased NIS occurrence, such as Greece, it could produce vast amounts of occurrence data in short time, supporting NIS management. Our results revealed that eDNA can function as an early warning tool for new species introductions as in the case of NIS detected in the North Aegean Sea. Standardised protocols and novel technologies, e.g., automatic samplers positioned in key sites for NIS introductions, could facilitate systematic monitoring and act as early detection tools (Sepulveda *et al.*, 2020a; Aglieri *et al.*, 2023). Coordinated efforts, encompassing research and regulatory measures, are crucial to manage and mitigate risks posed by invasive fish in Greek waters. Public awareness and citizen science, combined with attentive eDNA sampling and SCUBA surveys could provide a holistic approach to monitoring effectively NIS populations and provide solid ground for effective management (Zenetos *et al.*, 2010; Bakker *et al.*, 2017; Parrondo *et al.*, 2018; Bessell *et al.*, 2023). Therefore, collaboration between local communities, researchers, and policymakers is imperative to successfully address this challenge (Packer *et al.*, 2017; Katsanevakis *et al.*, 2020a).

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Supplementary Data

The following supplementary information is available online for the article:

Table S1. Reference sequences for non-indigenous fish species (NIS) not included in the DUFA 12S rRNA gene database; 75 from retrieved from the National Center for Biotechnology Information (NCBI) nucleotide database (excluding unverified entries) and four barcoded for this study (Accession Number PQ638932-5).

Table S2. Comparison of Generalised Additive Models for fish non-indigenous species (NIS) detection through eDNA across sites of known occurrence in Greece, D: detected/not detected; TSS: total suspended solids; TDN: total dissolved nitrogen, R^2(adj): adjusted R^2; DE: deviance explained, df: degrees of freedom; AIC: Akaike information criterion; in bold the model with the lowest AIC value; s(): smooth functions of the predictor variables using thin plate regression splines.

Table S3. Environmental variables recorded at each sampling site in Greece, DO: dissolved oxygen; chl-a: chlorophyll-a; TSS: total suspended solids; warm period: May-October; cold period: November-April.

Table S4. PERMANOVA results of the geographic region of sampling (region), period, and type of substrate effect on the occurrence of non-indigenous fish species in Greek territorial waters using environmental DNA analysis; * statistically significant; df: degrees of freedom; SS: sum of squares.

Table S5. Best Generalised Additive Model results for the detection of marine non-indigenous species (NIS) fish across sites of known occurrence in Greece using 12S rRNA on seawater environmental DNA samples; * statistically significant.

Table S6. Reference list of non-indigenous species records in Greece.

Fig. S1: Number of reads during bioinformatic analysis stages; MOTU: molecular operational taxonomic units; NIS: non-indigenous species; *: at least 0.97 best-identity score; percentages refer to the reads of each category compared to the total paired-end reads.

Fig. S2: Non-indigenous fish species (NIS) communities detected in Greece using environmental DNA metabarcoding upon oc-

currence data. Blank circles represent sites where NIS fishes were not detected during sampling.

Fig. S3: Environmental parameters and depth recorded from February 2021 to March 2022; TSS: total suspended solids.

Fig. S4: Pearson correlations of environmental parameters recorded *in-situ* during seawater eDNA sampling expeditions from February 2021 to March 2022 in Greece, DO: dissolved oxygen; chl-a: chlorophyll-a; TSS: total suspended solids; red symbols indicate p-value levels: <0.001 ~ "***", 0.001-0.010 ~ "**", 0.010- 0.050 ~ "*", 0.050- 0.100 ~ ".", >0.100 ~ " ".

Fig. S5: NMDS ordination of the marine-fish non-indigenous communities occurrence data in water samples using eDNA metabarcoding, for the effect of a) sampling region (North Aegean/Central Aegean/Ionian and Levantine Sea) and period (cold: November-April/warm: May-October) and b) substrate type (hard/soft). The stress of the final configurations was equal to 0.097.

Fig. S6: Occurrence map of non-indigenous fish species recorded through underwater visual surveys (SCUBA; yellow bullets); black pyramids indicate all sampling sites.

Fig. S7: Generalised Additive Model plots demonstrating the effect of temperature and total dissolved nitrogen in the detectability of marine non-indigenous fish species in Greece.