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PANAGIOTA XANTHOPOULOU, STELIOS KATSANEVAKIS, MICHALIS RAGKOUSIS, ORESTIS PAPADAKIS, MARIA ZOTOU, NIKOLAOS KAMIDIS, OWEN S. WANGENSTEEN, VASILLIS PAPATHANASIOU, DIMITRIOS KARAMPETSIS, ANTONIOS D. MAZARIS, CHRYSOULA GUBILI

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

Panagiota XANTHOPOULOU, Stelios KATSANEVAKIS, Michalis RAGKOUSIS, Orestis PAPADAKIS, Maria ZOTOU, Nikolaos KAMIDIS, Owen S. WANGENSTEEN, Vasillis PAPATHANASIOU, Dimitrios KARAMPETSIS, Antonios D. MAZARIS, and Chrysoula GUBILI

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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.

model	formulas	R^2(adj)	DE%	df	AIC
m1	D ~ as.factor(NIS) + s(temperature, k = -1) + s(salinity, k = -1) + s(turbidity, k = -1) + s(TSS, k = -1) + s(TDN, k = -1)	0.087	17.80	24.096	238.029
m2	D ~ as.factor(NIS) + s(temperature, k = -1) + s(salinity, k = -1) + s(TSS, k = -1) + s(TDN, k = -1)	0.053	12.80	17.899	237.242
m3	D ~ as.factor(NIS) + s(temperature, k = -1) + s(TSS, k = -1) + s(TDN, k = -1)	0.060	13.00	17.207	235.516
m4	D ~ as.factor(NIS) + s(temperature, k = -1) + s(TDN, k = -1)	0.063	12.40	16.179	234.713
m5	D ~ as.factor(NIS) + s(temperature, k = -1) + 1	0.071	14.80	19.302	235.399
m6	D ~ as.factor(NIS)	0.017	6.78	12.000	239.424
m7	D ~ s(temperature, k = -1) + s(salinity, k = -1) + s(turbidity, k = -1) + s(TSS, k = -1) + s(TDN, k = -1)	0.024	5.21	7.554	234.146
m8	D ~ s(temperature, k = -1) + s(salinity, k = -1) + s(TSS, k = -1) + s(TDN, k = -1)	0.027	5.02	6.517	232.525
m9	D ~ s(temperature, k = -1) + s(TSS, k = -1) + s(TDN, k = -1) + 1	0.030	5.02	5.632	230.755
m10	**D ~ s(temperature, k = -1) + s(TDN, k = -1)**	**0.033**	**4.71**	**4.746**	**229.703**
m11	D ~ s(temperature, k = -1)	0.020	3.23	4.021	231.668
m12	D ~ 1	<0.001	<0.001	1.000	233.084

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.

Site	Period	Depth (m)	Temperature (°C)	Salinity	DO%	Turbidity (NTU)	chl-a (µg/L)	TSS (mg/L)	PO_4^{3-} (µM/L)	NO_2^- (µM/L)	NO_3^- (µM/L)	SiO_2 (µM/L)	$NH4^+$ (µM/L)
Agios Ioannis, Lefkada	cold	5.55	15.90	38.60	92.80	0.3	0.602	26.818	0.229	0.129	0.749	3.337	0.478
Agios Ioannis, Lefkada	warm	5.55	24.68	39.29	87.54	0.6	0.092	37.690	0.307	0.000	1.421	4.518	1.940
Agios Pavlos, Crete	cold	20.00	16.69	39.21	94.39	0.1	0.076	20.000	0.153	0.093	0.495	2.716	0.755
Agios Pavlos, Crete	warm	20.00	22.56	39.15	91.70	0.1	0.083	40.800	0.298	0.064	0.399	4.671	0.000
Kythnos	cold	14.50	16.55	39.22	93.46	0.2	0.219	20.930	0.102	0.185	0.403	2.350	0.431
Kythnos	warm	14.50	20.80	39.16	92.20	0.1	0.116	11.781	0.229	0.129	0.549	2.823	0.000
Karpathos	cold	2.00	18.17	39.19	95.78	0.1	0.100	20.470	0.128	0.069	0.568	1.619	0.647
Karpathos	warm	2.00	21.45	39.19	92.60	0.1	0.124	26.286	0.229	0.000	0.349	2.823	0.000
Falasarna	cold	8.00	16.31	39.12	95.56	0.2	0.208	20.500	0.102	0.000	0.539	1.201	0.733
Falasarna	warm	8.00	19.29	39.18	92.70	0.1	0.122	28.354	0.412	0.086	0.399	2.567	1.062
Kalymnos	cold	5.00	16.60	39.23	98.30	0.1	0.133	21.190	0.458	0.150	0.649	1.848	0.637
Kalymnos	warm	5.00	20.86	39.32	92.25	0.5	0.105	20.000	0.153	0.116	0.668	2.455	0.604
Kastellorizo	cold	10.00	17.59	39.06	98.30	0.1	0.312	27.381	0.160	0.086	0.274	3.901	1.328
Kastellorizo	warm	10.00	22.65	39.35	91.47	0.1	0.125	23.540	0.179	0.081	0.446	2.246	0.733
Vasiliki	cold	10.00	15.80	38.60	95.60	0.1	0.291	27.750	0.137	0.086	0.499	3.439	3.133
Vasiliki	warm	10.00	25.01	39.30	87.34	0.9	0.217	27.500	0.255	0.069	1.548	1.671	1.682
Lesvos	cold	10.00	16.05	39.05	96.30	0.3	0.139	34.571	0.183	0.069	1.210	2.841	1.114
Lesvos	warm	10.00	19.30	39.09	86.99	1.0	0.761	26.810	0.307	0.093	1.206	2.142	2.587
Marathopoli	cold	10.00	16.00	39.31	95.50	1.3	0.279	37.647	0.183	0.064	0.898	3.953	0.690
Marathopoli	warm	10.00	25.67	39.32	90.57	0.9	0.112	19.760	0.358	0.139	1.380	2.246	1.078
Kythera	cold	6.00	15.80	39.23	99.50	0.7	0.377	30.000	0.183	0.064	0.449	2.721	1.168
Kythera	warm	6.00	24.87	39.10	91.36	0.4	0.118	34.880	0.102	0.093	0.495	1.567	0.647
Parga	cold	10.00	15.00	38.60	98.00	0.6	0.539	26.341	0.137	0.086	1.497	2.669	0.850
Parga	warm	10.00	24.23	39.25	88.12	0.8	0.211	23.250	0.562	0.069	1.695	4.492	2.371
Rodos	cold	7.00	18.10	39.15	96.50	0.2	0.520	25.000	0.275	0.171	0.549	4.158	1.195
Rodos	warm	7.00	22.18	39.33	88.63	0.6	0.102	19.400	0.460	0.000	1.617	2.716	1.423
Samos	cold	7.00	16.10	39.15	98.50	0.2	0.502	14.833	0.160	0.311	0.923	2.644	0.159
Samos	warm	7.00	20.10	39.17	90.31	0.4	0.309	22.980	0.255	0.093	0.642	1.671	2.458
Skroponeria	cold	10.00	13.40	38.45	96.20	0.1	0.472	32.353	0.137	0.021	1.198	5.595	2.469

Continued

Table S3 continued

Site	Period	Depth (m)	Temperature (°C)	Salinity	DO%	Turbidity (NTU)	chl-a (µg/L)	TSS (mg/L)	PO_4^{3-} (µM/L)	NO_2^- (µM/L)	NO_3^- (µM/L)	SiO_2 (µM/L)	$NH4^+$ (µM/L)
Skroponeria	warm	10.00	24.90	38.30	88.69	0.2	0.058	40.530	0.511	0.255	1.362	2.246	0.000
Tentopoula, Crete	cold	14.50	18.11	39.12	95.67	0.1	0.096	19.530	0.153	0.093	0.985	1.201	0.949
Tentopoula, Crete	warm	14.50	21.86	39.14	93.40	0.1	0.115	22.963	0.595	0.064	0.549	2.413	0.584
Skyros	cold	10.00	15.76	38.90	94.39	0.2	0.108	19.510	0.255	0.208	0.527	3.056	0.259
Skyros	warm	10.00	19.98	39.21	93.60	0.1	0.079	25.238	0.137	0.086	0.815	2.695	0.000
Chalkidiki	cold	8.50	15.80	37.43	92.40	1.2	0.441	23.000	0.137	0.064	0.599	2.156	0.000
Chalkidiki	warm	8.50	24.06	37.26	85.38	1.2	0.835	22.680	0.664	0.069	1.989	3.134	0.000
Samothraki	cold	10.00	14.50	36.27	103.00	0.2	0.330	22.444	0.183	0.150	1.397	5.595	1.487
Samothraki	warm	10.00	24.52	36.49	87.56	0.3	0.186	18.780	0.511	0.000	3.528	5.171	0.129
Thasos	cold	10.00	13.50	35.30	101.30	0.1	1.065	19.740	0.229	0.043	1.098	14.219	1.381
Thasos	warm	10.00	26.15	36.00	87.12	0.7	0.091	20.230	0.485	0.046	4.756	5.380	3.881

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.

Parameters	df	Occurrence			
		SS	R^2	F	p-value
region	3	0.011	0.078	1.135	0.358
period	1	0.002	0.016	0.710	0.542
substrate	1	0.001	0.004	0.170	0.857
region: period	3	0.024	0.164	2.395	0.031*
region: substrate	3	0.014	0.098	1.425	0.213
period: substrate	1	0.003	0.023	1.013	0.425
region:period: substrate	3	0.010	0.069	1.011	0.449
Residual	24	0.079	0.548		
Total	39	0.144	1.000		

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.

Detection (yes/no) ~ s(temperature) + s(total dissolved nitrogen)				
Parametric terms:				
	Estimate	Std. Error	z-value	Pr(>|z|)
Intercept	-1.405	0.173	-8.116	<0.001*
Significance of smooth terms:				
	edf	Ref.df	X^2	p-value
s(temperature)	2.746	3.441	7.952	0.076
s(total dissolved nitrogen)	1.000	1.000	3.205	0.073
Deviance explained = 4.71%				
Adjusted R^2= 0.033				

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

Atherinomorus forskalii

Azzurro, E. Editors. 2023. *ORMEF*. World Wide Web electronic publication. www.ormef.eu (Accessed 11 May 2024).

GBIF, 2023. GBIF Home Page. https://www.gbif.org (Accessed 20 February 2024).

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Zenetos, A., Koutsogiannopoulos, D., Ovalis, P., Poursanidis, D. 2013. The role played by citizen scientists in monitoring marine alien species in Greece. *Cahiers de Biologie Marine*, 54 (3), 419-426.

Fistularia commersonii

Azzurro E. Editors. 2023. *ORMEF*. World Wide Web electronic publication. www.ormef.eu (Accessed 11 May 2024).

Bardamaskos, G., Tsiamis, K., Panayotidis, P., Megalofonou, P., 2009. New records and range expansion of alien fish and macroalgae in Greek waters (South-east Ionian Sea*). Marine Biodiversity Records*, 2, e124.

Corsini, M., Kondilatos, G., Economidis, P.S., 2002. Lessepsian migrant Fistularia commersonii

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Pirkenseer, C., 2012. Records of four non-indigenous marine species, south of Koroni (Messiniakos Gulf, Peloponnese, Greece). *BioInvasions Records*, 1 (2), 87-93.

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Ragkousis, M., Zenetos, A., Ben Souissi, J., Hoffman, R., Ghanem, R. *et al.,* 2023. Unpublished Mediterranean and Black Sea records of marine alien, cryptogenic, and neonative species. *BioInvasion Records*, 12 (2), 339-369.

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Zenetos, A., Koutsogiannopoulos, D., Ovalis, P., Poursanidis, D. 2013. The role played by citizen scientists in monitoring marine alien species in Greece. *Cahiers de Biologie Marine*, 54 (3), 419-426.

Lagocephalus sceleratus

Corsini-Foka, M., Economidis, P.S., 2007. Allochthonous and vagrant ichthyofauna in Hellenic marine and estuarine waters. *Mediterranean Marine Science*, 8, 67-89.

Evangelopoulos, A., Karampetsis, D., Christidis, A., Gubili, C., Sapounidis, A. *et al.*, 2024. Non-native fish species in the North Aegean Sea: a review of their distributions integrating unpublished fisheries data. *Frontiers in Marine Science*, *11*, 1398037.

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Lagocephalus suezensis

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Parexocoetus mento

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Pterois miles

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Sargocentron rubrum

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Siganus luridus

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Siganus rivulatus

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Stephanolepis diapros

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Torquigener flavimaculosus

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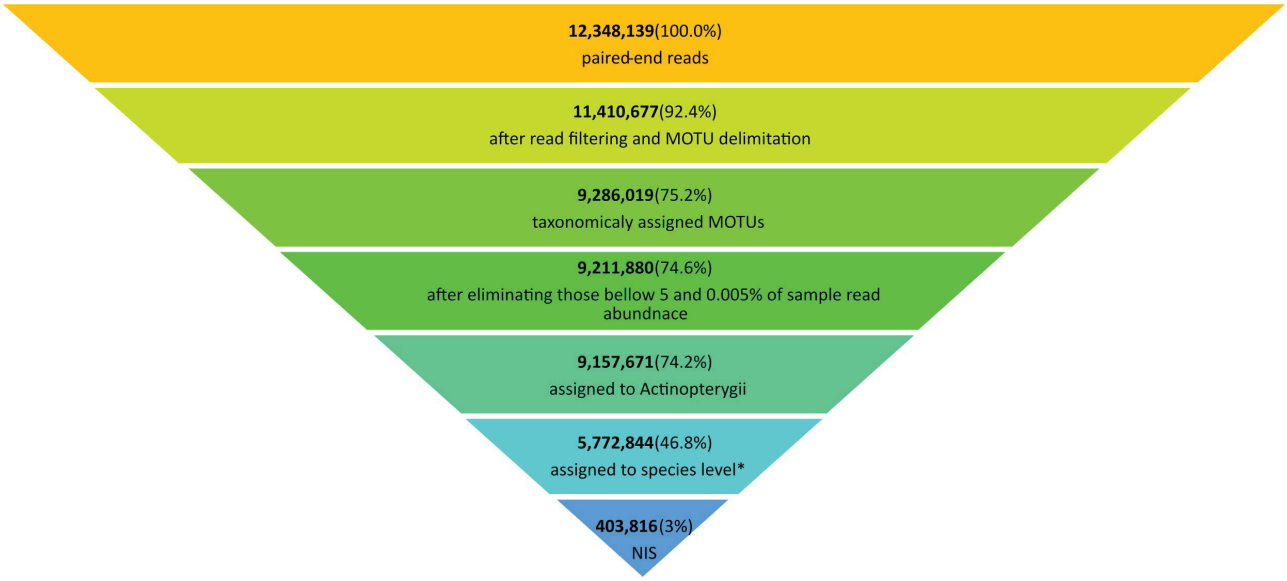

Fig. S1: Number of reads during bioinformatic analysis stages; MOTU: molecular operational taxonomic units; NIS: non-indigenous species; *: at least 0.97 best-identity score; percentages refer to the reads of each category compared to the total paired-end reads.

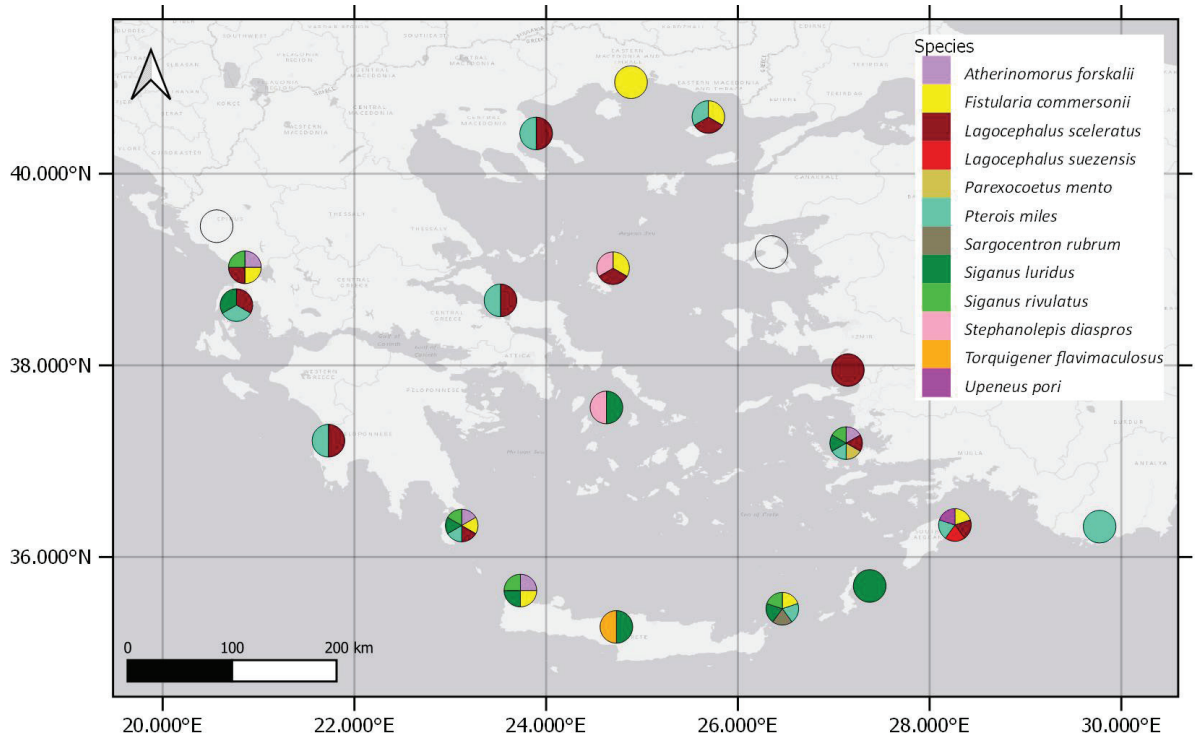

Fig. S2: Non-indigenous fish species (NIS) communities detected in Greece using environmental DNA metabarcoding upon occurrence data. Blank circles represent sites where NIS fishes were not detected during sampling.

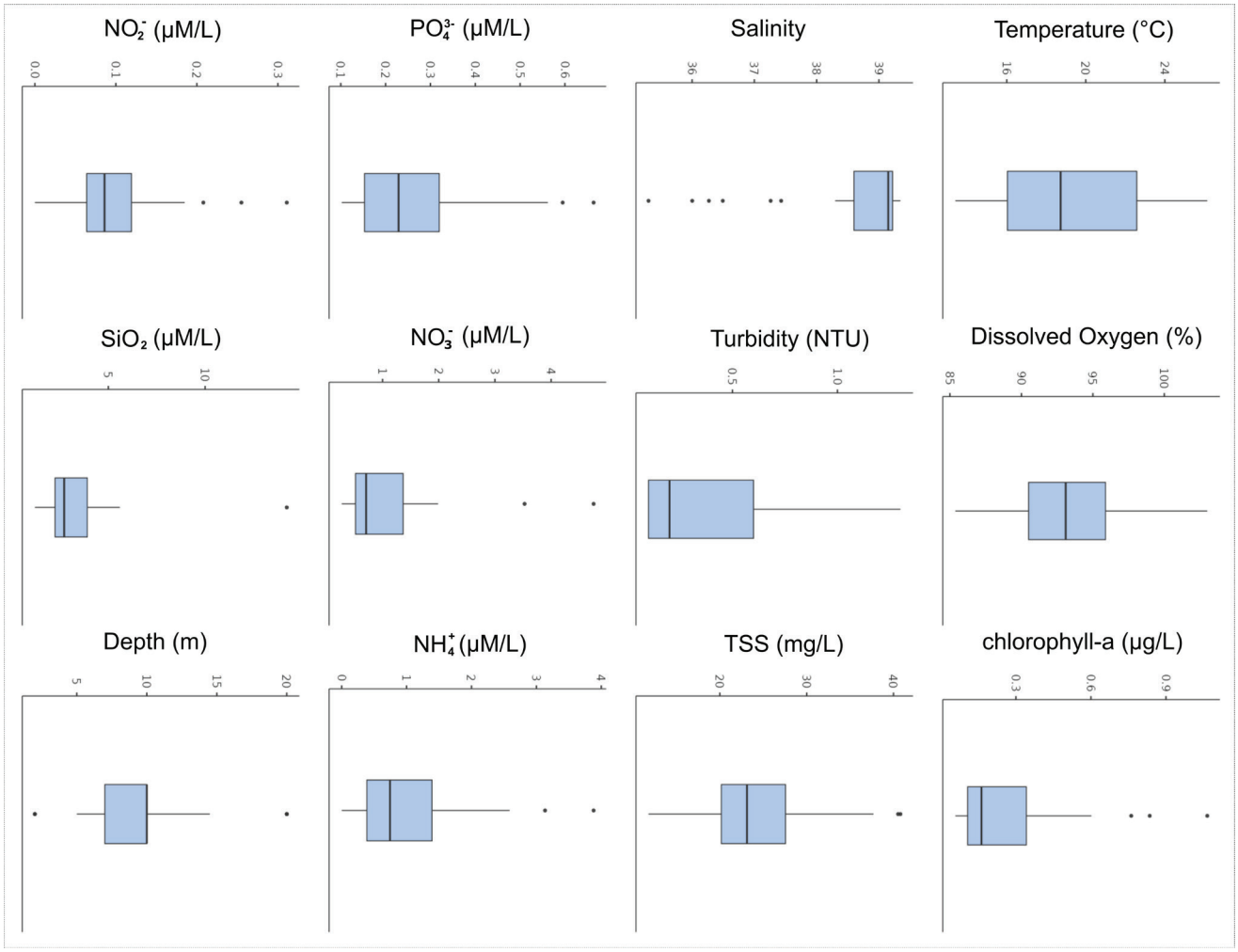

Fig. S3: Environmental parameters and depth recorded from February 2021 to March 2022; TSS: total suspended solids.

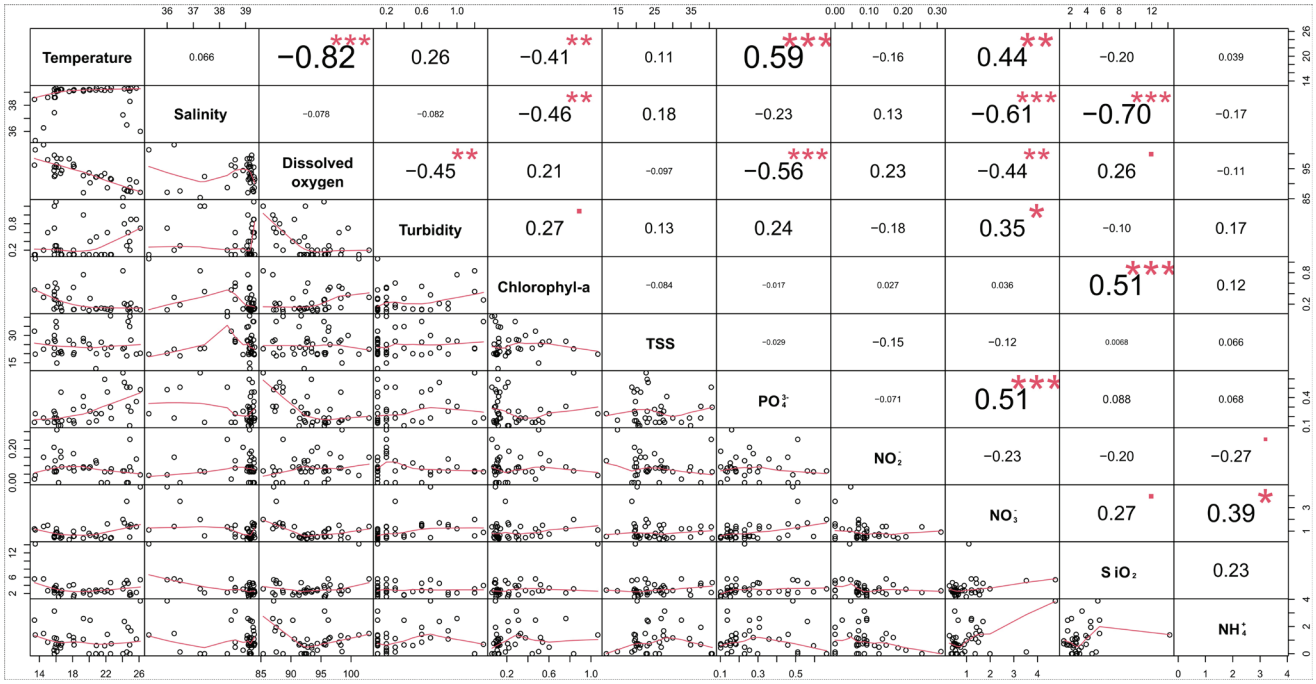

Fig. S4: Pearson correlations of environmental parameters recorded *in-situ* during seawater eDNA sampling expeditions from February 2021 to March 2022 in Greece, DO: dissolved oxygen; chl-a: chlorophyll-a; TSS: total suspended solids; red symbols indicate p-value levels: <0.001 ~ "***", 0.001-0.010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

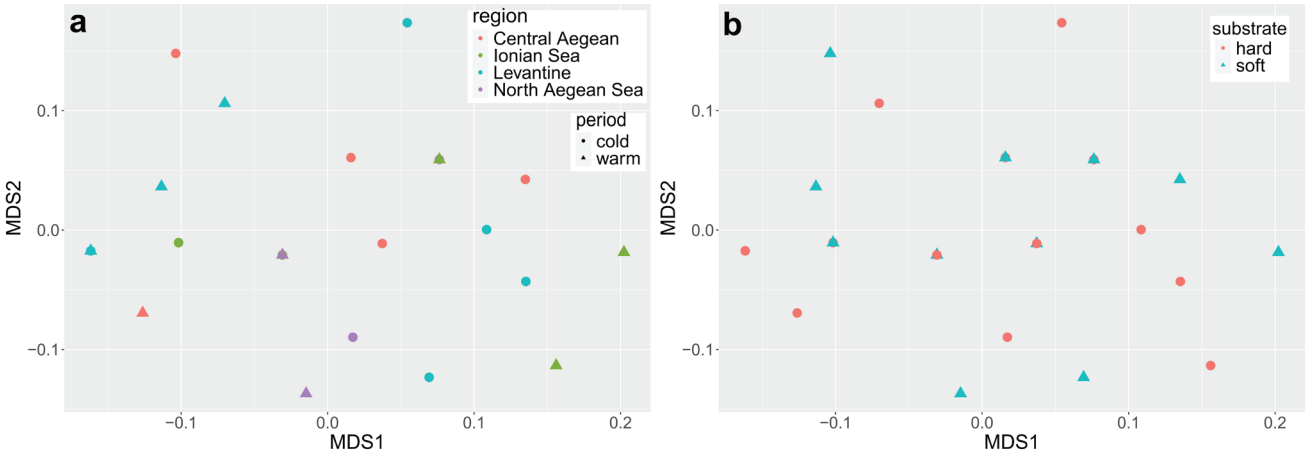

Fig. S5: NMDS ordination of the marine-fish non-indigenous communities occurrence data in water samples using eDNA metabarcoding, for the effect of a) sampling region (North Aegean/Central Aegean/Ionian and Levantine Sea) and period (cold: November-April/warm: May-October) and b) substrate type (hard/soft).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

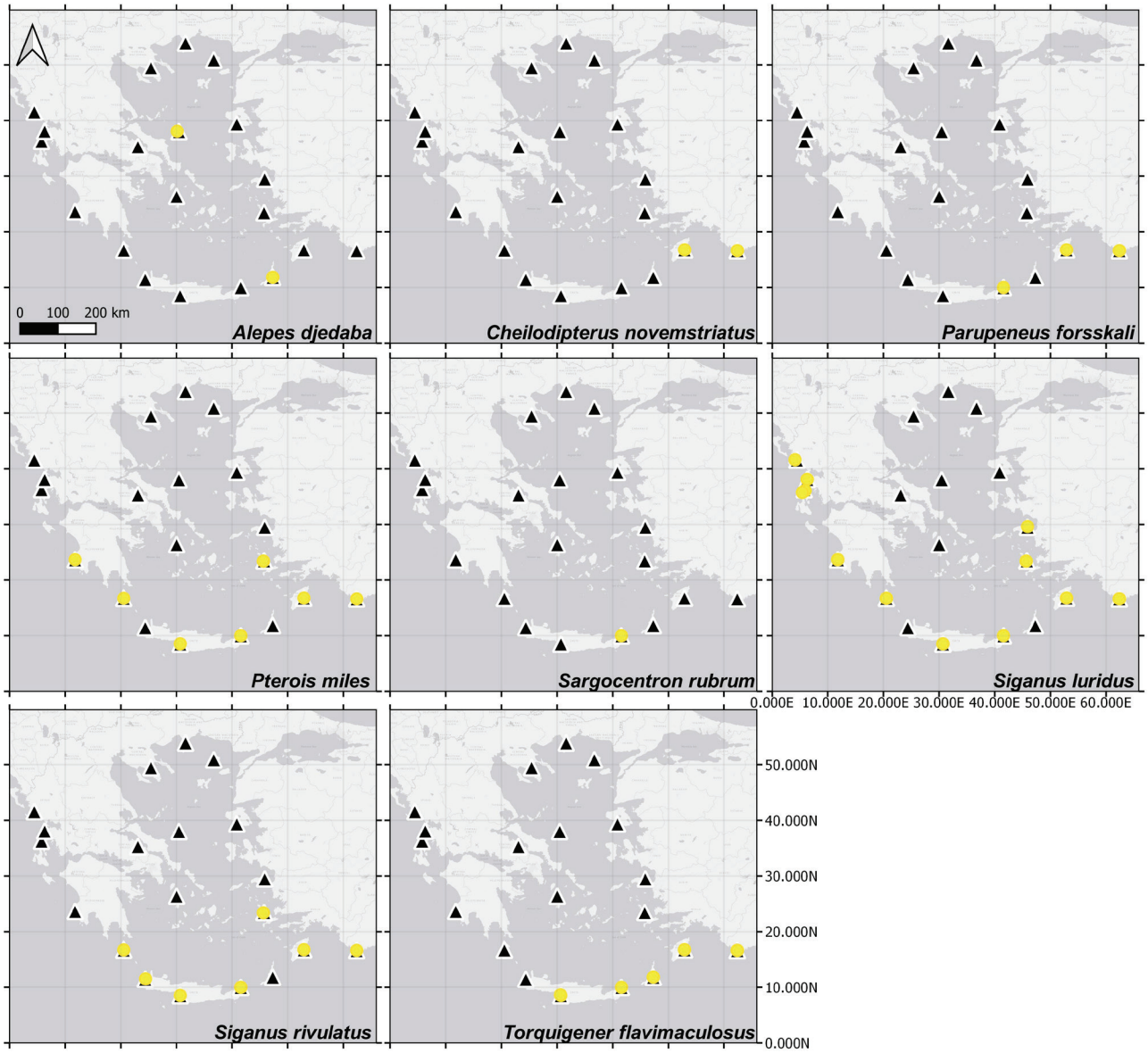

Fig. S6: Occurrence map of non-indigenous fish species recorded through underwater visual surveys (SCUBA; yellow bullets); black pyramids indicate all sampling sites.

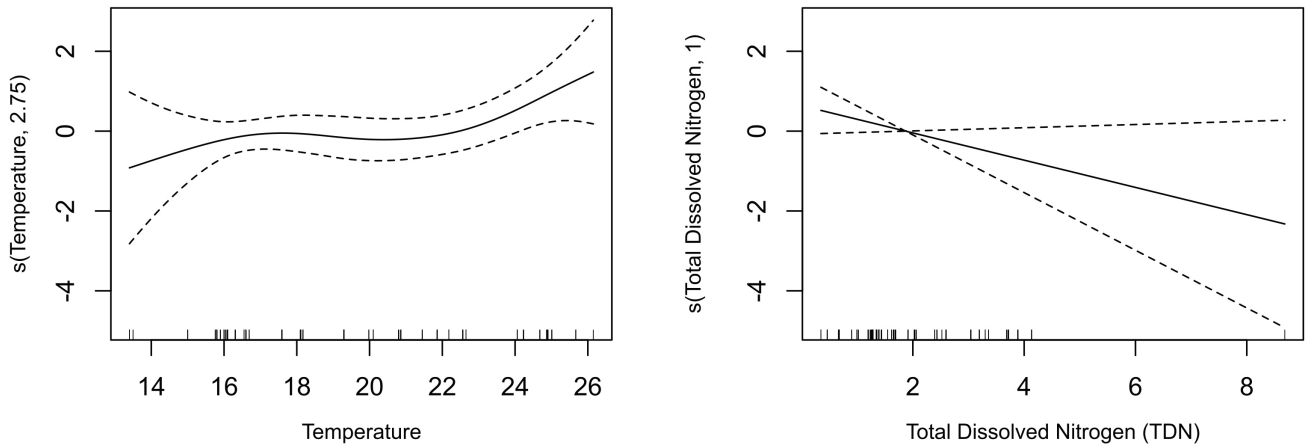

Fig. S7: Generalised Additive Model plots demonstrating the effect of temperature and total dissolved nitrogen in the detectability of marine non-indigenous fish species in Greece.