

Hydrocarbon Exploration program - Exploration Well Asopus-1 in Block 2 – Ionian Sea

ECF3008

ANNEX 03: eDNA Analysis and Fisheries Assessment

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	GQF10a Front Page Form Implementation of Field Activities in support to the ESBS for Exploration Wells Asopus-1A and Asopus-1B in Block 2 – Ionian Sea	Rev. 11 Rev. date: 2025/05/19
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OFFSHORE ENVIRONMENTAL SURVEY "LOT02"

IMPLEMENTATION OF FIELD ACTIVITIES IN SUPPORT TO THE ESBS FOR EXPLORATION WELLS ASOPUS-1A AND ASOPUS-1B IN BLOCK 2 – IONIAN SEA

Environmental Baseline Report **APPENDIX J – eDNA Analysis and** **Fisheries Assessment**



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1.0.0 INTRODUCTION

1.1.0 Geographical Subareas (GSA) Framework

The study area is located in the northern Ionian Sea, between southern Apulia (Italy) and the island of Corfu (Greece), and falls within Geographical Subareas (GSAs) 19 (Western Ionian Sea) and 20 (Eastern Ionian Sea), as defined by the General Fisheries Commission for the Mediterranean (GFCM). Specifically, the Italy Reference Station (Italy Ref) is the only one located within GSA 19, whereas all sampling stations associated with the Block-2 Environmental Baseline Survey (EBS), including the Asopus-1A and Asopus-1B well sites (and relevant Blank Stations) and the Kerkyra and Othonoi Reference Stations, are located within GSA 20. These GSAs constitute the official spatial units adopted for fisheries data collection, stock assessment and fisheries management throughout the Mediterranean Sea (GFCM, 2025). Together, GSAs 19 and 20 encompass a wide range of commercially important demersal and pelagic resources and represent the primary geographical framework for fisheries monitoring and management in the Ionian Sea.

With regard to the Italian sector, GSA 19 (Western Ionian Sea) represents one of the country's most important fishing districts. According to the Annual Report on Italy's efforts in 2024 to achieve a sustainable balance between fishing capacity and fishing opportunities, the fishing fleet operating in GSA 19 comprises 1275 vessels, accounting for 11.02 % of the Italian Mediterranean fleet, with a total capacity of 10608 GT and an installed engine power of 84711 kW. In 2024, landings reached 6783 t, corresponding to 5.77 % of total Italian Mediterranean landings, with an economic value of €42.9 million, equivalent to 6.75 % of the overall value of Italian Mediterranean landings (MASAF, 2025). This data highlights the economic importance of the Western Ionian Sea for the Italian fisheries sector.

GSA 20 (Eastern Ionian Sea), which lies predominantly within Greek waters, is likewise covered by the monitoring programmes established under the Data Collection Framework (DCF/EU-MAP) and by the MEDITS scientific trawl surveys, whose outputs contribute to stock assessments conducted by the GFCM and the Scientific, Technical and Economic Committee for Fisheries (STECF). Although no socioeconomic synthesis comparable to the annual Italian report for GSA 19 is currently available for this subarea, existing fisheries monitoring programmes confirm the importance of GSA 20 for commercial fisheries in the Eastern Ionian Sea. Under the 2024 EU fishing opportunities applicable to deep-water shrimp stocks in GSAs 19–21, Greece was allocated a maximum authorised fishing capacity of 240 bottom trawlers (23101 GT and 69281 kW), together with annual catch limits of 33 t for *Aristaeomorpha foliacea* and 14.5 t for *Aristeus antennatus* (Council Regulation (EU) 2024/259). Although these limits apply to all Greek fishing activities authorised within GSAs 19–21 rather than exclusively to GSA 20, they nevertheless demonstrate the important role of the Greek fleet in the exploitation and management of deep-water demersal resources in the Ionian Sea.

Overall, GSAs 19 and 20 constitute a closely interconnected fisheries system from both ecological and management perspectives. Several commercially important stocks are assessed by the GFCM over geographical units encompassing both subareas or groups of adjacent GSAs, reflecting the biological continuity of fish populations and the need for coordinated transboundary fisheries management.

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1.2.0 Main Fisheries Resources of the Northern Ionian Sea

The pronounced bathymetric heterogeneity of GSAs 19 and 20 gives rise to a marked zonation of fish communities and fisheries resources. Species composition and the relative importance of commercial resources vary along the depth gradient, from continental shelf assemblages to upper slope and deep bathyal communities. Overall, the fisheries resources of the northern Ionian Sea comprise demersal fishes, decapod crustaceans, cephalopods and pelagic species, supporting one of the most important multispecies fisheries in the central Mediterranean (FAO-GFCM, 2025).

The demersal communities inhabiting the continental shelf and upper continental slope include several of the most commercially valuable resources in the Ionian Sea. Among the species most frequently monitored and assessed are European hake (*Merluccius merluccius*), deep-water rose shrimp (*Parapenaeus longirostris*), Norway lobster (*Nephrops norvegicus*), anglerfishes (*Lophius piscatorius* and *Lophius budegassa*), common sole (*Solea solea*), red mullet (*Mullus barbatus*), common pandora (*Pagellus erythrinus*), and greater forkbeard (*Phycis blennoides*). These species represent some of the principal demersal fishery's resources of the Ionian Sea and are routinely monitored through the MEDITS trawl surveys and the DCF/EU-MAP fisheries data collection programmes. From an economic perspective, European hake, deep-water rose shrimp and Norway lobster rank among the most valuable demersal resources in the central Mediterranean, while anglerfishes and red mullet contribute substantially to the economic value of multispecies demersal landings.

The lower continental slope and upper bathyal domain are characterised by the occurrence of the giant red shrimp (*Aristaeomorpha foliacea*) and the blue and red shrimp (*Aristeus antennatus*), which constitute the principal commercial resources of the deep-water fisheries in the Ionian Sea. Both species are managed under the GFCM multiannual management plan for GSAs 19–21, which aims to ensure their sustainable exploitation through fishing capacity limitations, catch restrictions and continuous stock monitoring. These deep-water shrimp resources are associated with a characteristic bathyal fish assemblage including greater forkbeard (*Phycis blennoides*), blackbelly rosefish (*Helicolenus dactylopterus*), Mediterranean grenadier (*Nezumia sclerorhynchus*), common mora (*Mora moro*), and shortnose green eye (*Chlorophthalmus agassizi*), together with several deep-water elasmobranchs such as the black mouth catshark (*Galeus melastomus*) and the velvet belly lanternshark (*Etmopterus spinax*). Although these species generally have lower commercial value, they are characteristic components of Mediterranean bathyal fish communities.

The waters of GSAs 19 and 20 also support important pelagic fisheries resources. Among the small pelagic species, European anchovy (*Engraulis encrasicolus*) and European sardine (*Sardina pilchardus*) are of primary commercial importance, together with Atlantic mackerel (*Scomber scombrus*) and Atlantic horse mackerel (*Trachurus trachurus*). Among the large pelagic species, Atlantic bluefin tuna (*Thunnus thynnus*) and swordfish (*Xiphias gladius*) are of major economic and management importance and are managed under the conservation and management measures adopted by the International Commission for the Conservation of Atlantic Tunas (ICCAT). Although these species are not directly associated with the seabed habitats investigated in the present study, they represent an important component of commercial fisheries in the northern Ionian Sea.

Overall, the northern Ionian Sea supports a highly diversified fisheries system in which the composition of exploited resources varies according to depth and seabed characteristics. While the continental shelf and upper continental slope sustain the traditional multispecies demersal fisheries, the deep bathyal environments are characterised by commercially valuable deep-water resources that are subject to specific regional management measures.

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1.3.0 Fisheries Characterisation of the Different Study Areas

The Main Survey Area (Block-2 Area) is located in the offshore sector of the northern Ionian Sea, spanning GSA 19 (Western Ionian Sea) and GSA 20 (Eastern Ionian Sea), at water depths ranging from approximately 700 to 1000 m. This bathymetric interval corresponds to the lower continental slope and the upper bathyal zone, an ecological domain characterised by deep demersal assemblages that differ markedly from those inhabiting the continental shelf and upper slope (MEDITS Programme; FAO-GFCM, 2025). At these depths, fishing activities are primarily directed towards deep-water demersal resources, using bottom trawl fisheries authorised to exploit bathyal stocks under the management measures established by the GFCM.

The fisheries resources most closely associated with this bathymetric range are the giant red shrimp (*A. foliacea*) and the blue and red shrimp (*A. antennatus*), which are among the most valuable commercial species exploited by Mediterranean deep-water fisheries. Both species are managed under the GFCM Multiannual Management Plan for GSAs 19–21, aimed at ensuring sustainable exploitation through fishing effort limitations, catch restrictions and continuous stock monitoring (Recommendation GFCM/45/2022/6, as amended by Recommendation GFCM/48/2025/3; the Minimum Conservation Reference Size (MCRS) established by Recommendation GFCM/46/2023/3 also applies).

The commercial importance of the Main Survey Area is therefore primarily related to fisheries targeting these two deep-water shrimp species, which represent the highest-value resources within the area. Other deep-water demersal species generally constitute secondary components of commercial catches, contributing to varying degrees to the composition of the multispecies fishery. Owing to the considerable water depth, the occurrence of species typically associated with the continental shelf, such as red mullet, common sole, coastal sparids and neritic cephalopods, should be regarded as occasional and not representative of the characteristic fish assemblages inhabiting the study area.

The Italy Ref station is located exclusively within GSA 19 (Western Ionian Sea) at a depth of approximately 150 m, corresponding to the outer continental shelf margin and the upper continental slope. This sector supports some of the principal demersal fishery's resources of the Ionian Sea and falls within a bathymetric zone of considerable importance for commercial demersal fisheries (MEDITS Programme; FAO-GFCM, 2025).

The demersal assemblages associated with this depth range include European hake (*M. merluccius*), common sole (*S. solea*), deep-water rose shrimp (*Parapenaeus longirostris*), Norway lobster (*N. norvegicus*), anglerfishes (*L. piscatorius* and *L. budegassa*), red mullet (*M. barbatus*), common pandora (*Pagellus erythrinus*), and greater forkbeard (*P. blennoides*), all of which are routinely monitored through the MEDITS surveys and the DCF/EU-MAP fisheries data collection programmes. Additional demersal species and cephalopods may also contribute to the composition of multispecies catches, although they generally play a secondary role compared with the principal target resources.

1.4.0 Status of Fish Stocks and Management of the Main Fisheries Resources

The principal fisheries resources of GSA 19 (Western Ionian Sea) and GSA 20 (Eastern Ionian Sea) are subject to a structured scientific monitoring and management framework coordinated by the General Fisheries Commission for the Mediterranean (GFCM). At the GFCM level, the collection of biological, fisheries and socioeconomic data is organised through the Data Collection Reference Framework (DCRF), while within the European Union it is implemented through the Data Collection

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Framework (DCF) and its associated EU Multiannual Programme (EU-MAP). In addition, the Mediterranean International Bottom Trawl Survey (MEDITS) provides the principal fishery-independent monitoring programme for Mediterranean demersal communities.

Stock assessments are carried out under the auspices of the GFCM Scientific Advisory Committee on Fisheries (SAC) through its dedicated Working Groups on Stock Assessment, with the results compiled in the Stock Assessment Forms (SAFs) and the Stock Assessment Results (STAR) database. These constitute the official scientific reference for determining stock status and for supporting the fisheries management measures and recommendations adopted by the GFCM.

Stock assessments are conducted for specific stock assessment units, which may correspond either to a single GSA or to multiple adjacent GSAs when required by the biological distribution of the species. Consequently, stock status indicators should always be interpreted with reference to the corresponding assessment unit rather than exclusively to the geographical area investigated in the present study.

Among the most widely used indicators is the F/F_{msy} ratio, which compares the observed fishing mortality (F) with the fishing mortality corresponding to the Maximum Sustainable Yield (F_{msy}). Values greater than 1 indicate fishing mortality exceeding the F_{msy} reference point, whereas values below 1 indicate fishing mortality below that threshold. Considered in isolation, however, this ratio does not provide a comprehensive assessment of stock status, which also requires information on biomass, recruitment and other biological reference points. Accordingly, the GFCM integrates the F/F_{msy} ratio with additional biological indicators, including spawning stock biomass, recruitment and biological reference points, which collectively underpin scientific advice and subsequent fisheries management measures.

According to The State of Mediterranean and Black Sea Fisheries 2025 (SoMFi 2025), fisheries management in the Mediterranean has progressively improved in recent years through the implementation of multiannual management plans, reductions in fishing effort for selected fisheries and the strengthening of monitoring programmes. Nevertheless, substantial differences remain among individual stocks and across geographical regions, making stock-specific assessments essential for evaluating the status of the principal fisheries resources.

The main fisheries resources relevant to the present study are summarised in Table 1, which compiles the officially available information on the GFCM stock assessment unit, the availability of quantitative stock status indicators and the current management framework. The F/F_{msy} values reported are those adopted in the 2025 MASAF Annual Report for the 2023 assessment year. As explicitly stated in that report, where updated assessments were not available, estimates from previous years may have been retained; therefore, the reported values do not necessarily correspond to stock assessments conducted de novo in 2023.

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Table 1 – Status of the Main Fisheries Resources Relevant to the Study Area According to the Most Recent Official GFCM Stock Assessments

Species	Stock Unit / Assessed GSA	Official Assessment Indicator	Official Assessment or Monitoring Source	Additional Official Evidence	Relevance to the Present Study
<i>Merluccius merluccius</i>	GSA 19	F/FMSY = 1.13	GFCM / STECF (MASAF 2025, Table B3)	–	Italy Ref
<i>Merluccius merluccius</i>	GSA 20	F/FMSY = 5.33	GFCM / STECF (MASAF 2025, Table B3)	–	Block-2 Area
<i>Parapenaeus longirostris</i>	GSA 17–18–19–20	F/FMSY = 1.13	GFCM / STECF (MASAF 2025, Table B3)	–	Italy Ref
<i>Aristaeomorpha foliacea</i>	GSA 18–19–20	F/FMSY = 0.71	GFCM / STECF (MASAF 2025, Table B3)	Multiannual management plan and catch limits for GSAs 19–21	Block-2 Area
<i>Aristeus antennatus</i>	GSA 18–19–20	F/FMSY = 2.69	GFCM / STECF (MASAF 2025, Table B3)	Multiannual management plan and catch limits for GSAs 19–21	Block-2 Area
<i>Engraulis encrasicolus</i>	GSA 20	Validated GFCM advice: sustainably exploited stock	GFCM – WGSASP (2022)	–	Regional context (GSA 20)
<i>Sardina pilchardus</i>	WGSASP monitoring	No quantitative assessment identified for GSAs 19–20	GFCM – WGSASP; MEDITS/DCF	7705 t and €9.68 million landed in Italy (2024)	Regional context
<i>Nephrops norvegicus</i>	GFCM assessments available for selected Mediterranean GSAs	No quantitative assessment identified for GSAs 19–20	GFCM – WGSAD (2023); MEDITS/DCF	€17.47 million landed value in Italy (2024)	Italy Ref
<i>Lophius piscatorius</i>	GFCM/MEDITS monitoring	No quantitative assessment identified for GSAs 19–20	GFCM – WGSAD; MEDITS/DCF	905 t and €7.22 million landed value in Italy (2024)	Italy Ref
<i>Lophius budegassa</i>	GFCM/MEDITS monitoring	No quantitative assessment identified for GSAs 19–20	GFCM – WGSAD; MEDITS/DCF	Locally important commercial species	Italy Ref
<i>Mullus barbatus</i>	GSA 19	F/FMSY = 0.34	GFCM / STECF (MASAF 2025, Table B3)	2766 t and €13.86 million landed in Italy (2024)	Italy Ref
<i>Solea solea</i>	GSA 17 (Adriatic Sea)	No quantitative assessment identified for GSAs 19–20	GFCM/STECF; MEDITS/DCF	1768 t and €22.08 million landed in Italy (2024)	General context

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Quantitative indicators are reported only where the corresponding stock assessment unit explicitly includes GSA 19 and/or GSA 20. For species monitored or assessed by the GFCM in other Mediterranean subareas, or for which the publicly available assessment summaries do not allow the assessed geographical unit to be identified with certainty, no quantitative value or stock status has been assigned. The absence of an indicator in this table does not imply the absence of the species, low commercial importance, or a lack of monitoring, but simply reflects the unavailability of a quantitative assessment directly applicable to the study area.

1.5.0 Commercial Fishing Activities within the Study Area

The distribution and intensity of fishing effort in the Mediterranean Sea are monitored through the Data Collection Framework (DCF/EU-MAP), the General Fisheries Commission for the Mediterranean (GFCM), the Scientific, Technical and Economic Committee for Fisheries (STECF), and EMODnet Human Activities, using harmonised fisheries effort and fishing intensity datasets, including fishing days, days at sea, GT × days, kW × days, average fishing hours, and swept-area ratio. In general, the outer continental shelf and upper continental slope support a broader diversity of fishing activities, whereas fisheries operating on bathyal grounds tend to be more specialised and primarily target deep-water resources. However, the present study did not include a spatially explicit quantification of fishing effort within the individual survey polygons.

Table 2 – Main DCF/STECF Fisheries Métiers Applicable to the Study Areas

DCF/STECF Code	Fishing Gear	Target Species
OTB	Bottom Otter Trawl	<i>Merluccius merluccius</i> , <i>Parapenaeus longirostris</i> , <i>Nephrops norvegicus</i> , <i>Mullus barbatus</i>
OTB	Bottom Otter Trawl	<i>Aristaeomorpha foliacea</i> , <i>Aristeus antennatus</i>
LLS	Set Longlines	<i>Merluccius merluccius</i> , <i>Lophius piscatorius</i> , <i>Lophius budegassa</i> , <i>Sparidae</i>
GNS	Set Gillnets	<i>Sparidae</i> , <i>Soleidae</i> , <i>Sciaenidae</i>
PS	Purse Seine	<i>Engraulis encrasicolus</i> , <i>Sardina pilchardus</i>

The study areas are located within a sector shared by Geographical Subarea (GSA) 19 (Western Ionian Sea) and Geographical Subarea (GSA) 20 (Eastern Ionian Sea), where commercial fishing activities are carried out by both the Italian and Greek fishing fleets.

Among the ports potentially relevant to fishing fleets operating in the broader Ionian region are Gallipoli, Taranto, Corigliano-Rossano, and Crotone on the Italian coast, and Kerkyra, Igoumenitsa, and Patras on the Greek coast. In the absence of spatially resolved data identifying the home ports of vessels operating within the individual study polygons, this list should be regarded as providing general geographical context rather than as evidence of direct fishing activity within the specific survey areas.

Commercial fishing activities in the region are primarily conducted using bottom otter trawls, complemented locally by bottom set longlines and, in coastal waters, by small-scale artisanal fisheries and pelagic purse seine fisheries.

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1.6.0 Concluding Remarks

The potential overlap between the study areas and fish nursery grounds and Essential Fish Habitats (EFHs) was assessed by integrating the geographical location and bathymetry of the survey sites with the information available from the MEDITS/MEDISEH programmes and EMODnet Seabed Habitats. The latter includes EUSeaMap 2023, habitat maps derived from field surveys, and dedicated EFH models; however, these products do not provide a comprehensive or spatially homogeneous mapping of Mediterranean fish nursery areas. In particular, EUSeaMap is a predictive map of seabed habitats, primarily based on water depth, substrate type and biological zonation, and therefore cannot, by itself, be used to infer the nursery function of a given area (Carlucci *et al.*, 2009; Colloca *et al.*, 2015; EMODnet, 2026b).

Overall, the available cartographic evidence does not support the conclusion that the study areas overlap with persistent fish nursery grounds or specifically delineated Essential Fish Habitats. This finding indicates the absence of any currently documented overlap and should not be interpreted as evidence that juvenile fish are absent locally or that the investigated areas cannot perform nursery functions. Demonstrating such ecological roles would require spatially explicit biological data or dedicated field investigations.

Overall, the integrated analysis of the available literature and official stock assessment information provides a coherent overview of the fisheries characteristics of the Ionian sector encompassed by the present study, as summarised in Table 3.

Table 3 – Summary of the Main Fisheries Characteristics of the Study Areas

Parameter	Block-2 Area	Italy Ref
Depth Range	700–1000 m	150 m
GSA	GSAs 19–20	GSA 19
Predominant Habitat	Deep bathyal domain	Outer continental shelf – upper continental slope
Main Commercial Resources	<i>Aristaeomorpha foliacea</i> , <i>Aristeus antennatus</i>	<i>Merluccius merluccius</i> , <i>Parapenaeus longirostris</i> , <i>Nephrops norvegicus</i> , <i>Mullus barbatus</i>
Persistent Nursery Areas	No verified overlap	No verified overlap
Essential Fish Habitats (EMODnet)	No documented overlap	No documented overlap
Predominant Métier	OTB (deep-water bottom otter trawl)	OTB (demersal bottom otter trawl)
Predominant Fishery Type	Deep-water crustacean fishery	Multispecies demersal fishery

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2.0.0 RESULTS

2.1.0 eDNA Analysis in Relation to Commercially Important Species

Environmental DNA (eDNA) analysis was conducted in the study area with the aim of providing information on the ecological characteristics of the marine ecosystem, with special focus on the detection of commercially important fish species representative of the local fisheries resources. In open pelagic environments, the rapid dilution of eDNA generally limits the detection of vertebrate *taxa*; nevertheless, the identification of *Engraulis encrasicolus*, *Merluccius merluccius* and *Stenella coeruleoalba* indicates the occurrence of both pelagic and demersal components consistent with the ecological setting of the study area. Specifically, the detection of *S. coeruleoalba*, while not a commercial species, complements the ecological characterisation of the study area and illustrates the capability of the eDNA approach to detect large marine vertebrates. Overall, the results provide useful indicators for monitoring environmental pressures, the connectivity of deep-sea ecosystems and changes in ecological conditions through a non-invasive methodological approach.

eDNA analysis identified a relatively limited number of commercially important *taxa* and other marine vertebrates distributed across the different study areas (Figure 1). In particular, the taxonomic composition appeared likely consistent with the ecological and bathymetric characteristics of the study area, while reflecting the qualitative nature of the method and the relatively low number of vertebrate reads compared with the total environmental DNA recovered from the samples.

The highest taxonomic richness was recorded in the Blank samples (6 *taxa*), followed by Asopus-1B (5 *taxa*), the Reference Stations (3 *taxa*) and Asopus-1A (1 *taxon*). Specifically, when considering the two study sectors separately, reflecting their different geographical settings within the Geographical Subareas (GSAs) as well as their distinct bathymetric and ecological characteristics, the eDNA analyses detected a total of nine *taxa* within the Block-2 Area (including the Asopus-1A and Asopus-1B well sites and the Kerkyra Ref and Othonoi Ref), whereas only a single *taxon* (*Solea solea*) was detected at the Italy Ref station. This difference should be interpreted with caution, as the two sectors were investigated under substantially different sampling efforts. In particular, the Block-2 Area comprised a considerably larger number of sampling stations than the single Italy Ref station. Consequently, these findings should be regarded as qualitative and descriptive only and do not support a direct quantitative comparison between the two study sectors.

Among the commercially important fisheries resources, European hake (*M. merluccius*) was detected exclusively at the Kerkyra Reference Station, whereas common sole (*Solea solea*) was identified across all study-area categories and exhibited the highest number of sequences reads among the detected vertebrate *taxa*. The occurrence of these species is fully consistent with the fisheries framework outlined in the preceding chapters, in which European hake is recognised as one of the principal demersal resources of GSAs 19 and 20, routinely monitored and assessed under the GFCM and STECF programmes, while common sole represents a well-established commercial resource throughout the Mediterranean despite the absence of a specific quantitative stock assessment for GSAs 19–20. In particular, the detection of European hake contributes to the qualitative characterisation of commercially important fish resources within the study area and demonstrates the capability of the eDNA approach to detect management-relevant demersal species even in deep offshore environments.

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Additional commercially important species detected include black-bellied angler (*Lophius budegassa*), recorded in the Blank samples, and European anchovy (*E. encrasicolus*), detected in Asopus-1B, both of which are consistent with the expected composition of demersal and pelagic fish assemblages in the Ionian Sea. The occurrence of *L. budegassa*, a commercially valuable demersal species associated with deep-water habitats, is compatible with the bathymetric setting of the investigated area. Likewise, the detection of *E. encrasicolus*, a key species within Mediterranean pelagic food webs, demonstrates the ability of eDNA to detect pelagic fish components even in offshore waters, despite the rapid dilution of environmental DNA that typically characterises open marine environments.

Additional *taxa* were identified either at higher taxonomic levels (e.g., Pleuronectidae n.d.) or only to genus level (*Hygophum sp.*). The latter comprises lanternfishes (Family Myctophidae), characteristic mesopelagic fishes that perform diel vertical migrations and are widely distributed throughout the bathyal waters of the Ionian Sea. Their detection is therefore fully consistent with the bathymetric characteristics of the Block-2 Area and further demonstrates the capability of eDNA to detect deep-sea faunal components that are generally underrepresented in conventional biological surveys.

The detection of *Oreochromis sp.* represents a finding of limited ecological relevance within the investigated marine environment. As this genus is composed predominantly of freshwater and euryhaline species, its detection by eDNA may reflect the transport of allochthonous genetic material from continental environments. Consequently, this finding should be interpreted with caution and should not be regarded, by itself, as evidence of the presence of the species within the study area.

Of particular interest was the detection of the striped dolphin (*S. coeruleoalba*) in the Blank samples, a cetacean widely distributed throughout the Mediterranean Sea. Its detection demonstrates the capability of the eDNA approach to identify large marine vertebrates through DNA released into the environment, although it does not provide direct information on population abundance or the continuous use of the area by the species.

Overall, the eDNA results are consistent with the ecological and fisheries framework established through the literature review, revealing the occurrence of demersal and pelagic taxa characteristic of the Ionian Sea, including several of the principal commercial species routinely monitored under the GFCM, DCF/EU-MAP and MEDITS programmes. At the same time, the relatively limited number of detected taxa and the heterogeneous distribution of sequence reads among the study areas confirm that eDNA primarily provides qualitative evidence of species occurrence and should therefore be interpreted in conjunction with existing ecological knowledge and the other environmental information collected during the present study.

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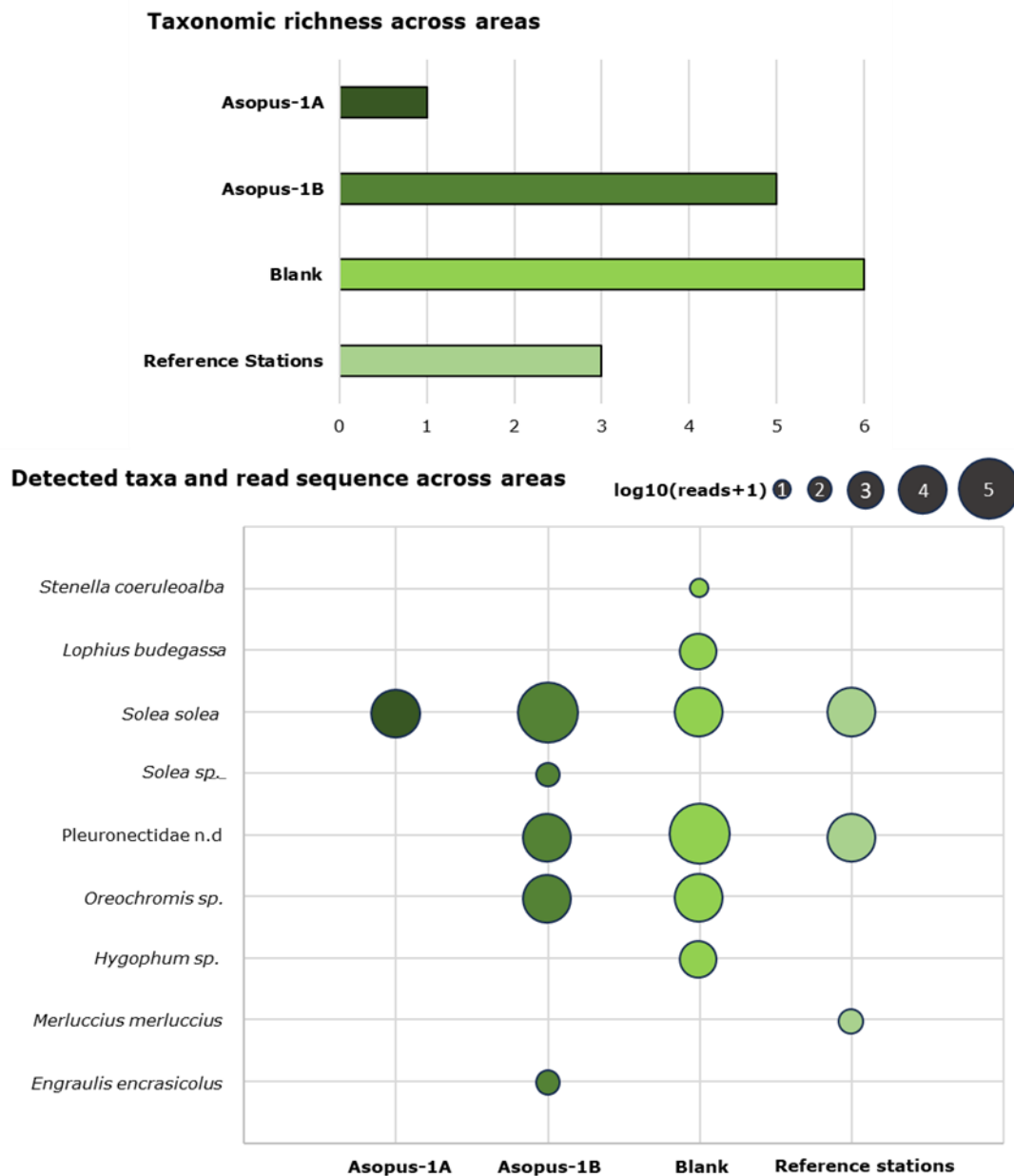


Figure 1 – Distribution of taxa detected by environmental DNA (eDNA) analysis across the different study areas. The upper panel shows taxonomic richness (number of detected taxa) for each study area, while the lower panel illustrates the distribution of the individual detected taxa. Symbol size is proportional to the relative number of sequences reads assigned to each taxon, expressed as log₁₀ (reads + 1)

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3.0.0 DISCUSSION

The integration of the available literature with the results of the environmental DNA (eDNA) analyses provided a coherent overview of the fish communities occurring within the study area. The literature review highlighted that the northern Ionian Sea, encompassing GSAs 19 and 20, represents an important commercial fishing area characterised by a pronounced bathymetric zonation of fish assemblages, with demersal resources associated with the continental shelf and upper continental slope, and bathyal species inhabiting deeper environments.

Overall, the *taxa* detected by eDNA are consistent with this ecological framework. The detection of European hake (*M. merluccius*), common sole (*Solea solea*), black-bellied angler (*L. budegassa*), and European anchovy (*E. encrasicolus*) seems consistent with the occurrence of *taxa* that are widely distributed throughout the Ionian Sea and recognised by the GFCM and the MEDITS Programme as among the principal fisheries resources of the region. Likewise, the detection of *Hygophum sp.*, a genus comprising mesopelagic and bathypelagic lanternfishes, is consistent with the deep-water habitats characterising the Main Survey Area, while the detection of the striped dolphin (*S. coeruleoalba*) provides additional information on the marine vertebrate assemblage occurring within the study area.

Overall, the observed taxonomic composition does not reveal any evidence inconsistent with the ecological framework reconstructed from the available scientific literature and official fisheries information. On the contrary, the detected *taxa* are representative of several of the principal demersal and pelagic components expected within the investigated Ionian sector and provide qualitative evidence for the occurrence of some of the commercially important fisheries resources described in the preceding sections.

It should be emphasised that the relatively limited number of detected *taxa* should not be interpreted as evidence of low biodiversity within the study area. Environmental DNA is primarily a qualitative survey tool, and species detectability is influenced by numerous factors, including the concentration of environmental DNA, the persistence of genetic material within the water column, sampling depth, water-mass dynamics, the molecular marker employed, and the completeness of the reference sequence databases. Furthermore, the number of sequence reads cannot be interpreted directly as a measure of species abundance or biomass (Deiner *et al.*, 2017).

Within the present study, the eDNA analyses complemented the available ecological, bibliographic and fisheries information by detecting *taxa* consistent with the ecological characteristics of the northern Ionian Sea, without indicating a fish community composition substantially different from that expected on the basis of current scientific knowledge. The combined interpretation of the literature review, the official GFCM stock assessment framework and the eDNA results therefore provides an ecologically coherent baseline that represents a robust reference for evaluating the potential interactions between the proposed project activities and the biological and fisheries resources of the study area.

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4.0.0 CONCLUSIONS

The literature review and the results of the environmental DNA (eDNA) analyses supported the following conclusions:

- the study area is located within GSAs 19 and 20, two subareas of considerable importance for demersal fisheries in the Ionian Sea, which are subject to monitoring and management under the frameworks established by the GFCM, STECF, and DCF/EU-MAP;
- the principal fisheries resources identified in the literature are consistent with the expected bathymetric distribution of fish assemblages, distinguishing demersal species associated with the outer continental shelf and upper slope from those characteristics of the deep bathyal domain;
- the review of the available literature and cartographic information did not identify any documented overlap between the study area and persistent fish nursery grounds or specifically delineated Essential Fish Habitats (EFHs);
- although not exhaustive, the eDNA analyses detected several *taxa* characteristic of the Ionian Sea, including *M. merluccius*, *Lophius budegassa*, and *E. encrasicolus*, in agreement with the ecological and fisheries framework established through the literature review;
- overall, the results demonstrate a high degree of consistency between the available scientific literature and the evidence provided by the eDNA analyses, contributing to the definition of a coherent and up-to-date environmental baseline for the fish communities occurring within the surveyed waters.

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ANNEX A: ANALYTICAL METHODOLOGY

Environmental DNA (eDNA) analysis was performed using a DNA metabarcoding approach to detect marine species present in water samples through the amplification and sequencing of selected mitochondrial genetic markers. The methodology was designed to detect the target taxonomic groups, with particular emphasis on fish, marine vertebrates, molluscs and crustaceans, and was not intended to provide an exhaustive characterization of the overall biodiversity present within the sampled environmental matrix.

Sample preparation and DNA extraction

All laboratory procedures preceding sequencing were carried out in facilities dedicated exclusively to eDNA analyses and physically separated from post-PCR areas to minimize the risk of cross-contamination. DNA extraction and PCR reaction setup were performed within Class II biological safety cabinets that had been decontaminated prior to use by chemical treatment and ultraviolet (UV) irradiation. Throughout all analytical procedures, laboratory practices specifically designed for the analysis of low-concentration environmental DNA samples were adopted.

At least one Extraction Negative Control (ENC), consisting of molecular-grade water, was processed alongside each analytical batch and subjected to the entire extraction workflow to verify the absence of contamination introduced during DNA extraction and PCR inhibitor removal.

DNA extraction was performed using the Mag-Bind® Environmental DNA Kit (Omega Bio-Tek), specifically developed for aqueous environmental samples and based on magnetic bead technology. The extraction protocol includes a dedicated PCR inhibitor removal step to eliminate compounds commonly present in environmental matrices that may interfere with downstream molecular analyses.

DNA concentration was quantified using a Qubit® fluorometer (Thermo Fisher Scientific) in conjunction with the Qubit® dsDNA Broad Range Assay Kit, in order to assess DNA extraction yield and confirm the suitability of the extracted DNA for subsequent amplification.

DNA amplification

DNA amplification was performed using three mitochondrial markers selected according to the target taxonomic groups:

- The MiFish primer system amplifies a region of the mitochondrial 12S rRNA gene and was specifically developed for the detection of fish species while retaining the ability to amplify a broad range of vertebrate taxa (Miya et al., 2015);
- the MarVer3 primer system targets a region of the mitochondrial 16S rRNA gene and is specifically employed for the detection of marine vertebrates, including teleosts and elasmobranchs (Valsecchi et al., 2020);
- the mICOIntF-jgHCO2198 primer system amplifies a fragment of the mitochondrial Cytochrome c Oxidase subunit I (COI) gene and represents a universal metazoan marker, particularly suitable for the identification of marine invertebrates, including molluscs and crustaceans (Leray et al., 2013).

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Target-specific primers were modified by the addition of Illumina overhang adapter sequences at the 5' end to enable subsequent sequencing library preparation.

PCR amplification was performed using a two-step PCR protocol with DNA polymerases selected according to the specifications of each primer system. Each analytical run included proprietary Synthetic Positive Controls (SPCs) and No Template Controls (NTCs) consisting of PCR-grade water. Amplification success was verified by agarose gel electrophoresis.

Table 4 – Primer pairs selected for eDNA analysis. The column "Marker" identifies the mitochondrial target gene amplified by each primer pair, while the column "Primer sequence" reports the forward primer sequence (first line) and the reverse primer sequence (second line), both in the 5'–3' orientation. The reported sequences include the Illumina overhang adapter sequences used for library preparation. Corresponding literature references for each primer pair are provided in the "Reference" column.

Marker	Primer Pair	Primer sequence (5' - 3')	Reference
12S	MiFish	5'-TTTCTGTTGGTGCTGATATTGCGTCGGTAAACTCGTGCCAGC-3'	Miya <i>et al.</i> , 2015
		5'-ACTTGCCTGTCGCTCTATCTTCCATAGTGGGGTATCTAATCCCAGTTTG-3'	
16S	MarVer3	5'-TTTCTGTTGGTGCTGATATTGCAGACGAGAAGACCCTRTG-3'	Valsecchi <i>et al.</i> , 2020
		5'-ACTTGCCTGTCGCTCTATCTTCGGATTGCGCTGTTATCCC-3'	
COI	mlCOIintF-jgHCO2198	5'-TTTCTGTTGGTGCTGATATTGCGGWACWGGWTGAACWGTWTAYCCYCC-3'	Leray <i>et al.</i> , 2013
		5'-ACTTGCCTGTCGCTCTATCTTCTAIACYTCIGGRTGICRAARAAYCA-3'	

Library preparation and sequencing

Primary PCR products were pooled for each sample and purified using Mag-Bind® TotalPure NGS magnetic beads (Omega Bio-Tek). A second PCR was subsequently performed to introduce dual-index sequencing barcodes and full-length Illumina adapter sequences, following the Illumina 16S Metagenomic Sequencing Library Preparation protocol. The resulting libraries were then purified, quantified, normalized, and pooled in equal volumes prior to sequencing.

Sequencing was performed on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (600 cycles), generating 2 × 300 bp paired-end reads.

Bioinformatic processing

Bioinformatic analyses were performed using a proprietary NatureMetrics pipeline based on open-source software. Raw sequencing images were converted into FASTQ files using bcl2fastq, with simultaneous sample demultiplexing. Paired-end reads were subsequently merged using USEARCH, requiring a minimum overlap identity of 80 %. Primer sequences were removed using cutadapt,

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applying marker-specific length filters. Read quality filtering was performed using USEARCH, retaining only sequences with an expected error rate ≤ 0.01 per base. The resulting sequences were subsequently dereplicated and denoised using the UNOISE algorithm to generate zero-radius Operational Taxonomic Units (zOTUs). Only zOTUs represented by at least eight sequencing reads in at least one sample were retained for downstream analyses.

Taxonomic assignment

Taxonomic assignment was performed using the BLASTn algorithm by comparing the resulting sequences against the NCBI nucleotide (NCBI nt) database. For the COI marker, the Barcode of Life Data System (BOLD) database was also used. Only alignments with an E-value $\leq 1 \times 10^{-20}$ and a minimum query coverage of 90 % were considered. Taxonomic identifications were subsequently harmonized using the GBIF Taxonomic Backbone and cross-checked against species distribution records available through the Global Biodiversity Information Facility (GBIF). Taxonomic assignments were performed using a conservative approach, assigning sequences to the highest taxonomic level supported by both the molecular evidence and the known geographical distribution of the species.

Construction of the final dataset

The resulting zOTUs were subsequently clustered into Operational Taxonomic Units (OTUs) using the UPARSE algorithm with a 97 % sequence identity threshold, following the removal of chimeric sequences. The final dataset was subjected to additional quality control procedures, including the removal of low-abundance OTUs, unassigned sequences, and sequences attributed to humans or domestic animals. For all OTUs identified to species level, the IUCN Red List of Threatened Species and the Global Register of Introduced and Invasive Species (GRIIS) were automatically queried to retrieve information on species conservation status and potential invasive status.

Interpretation of results

Because the number of sequencing reads does not provide a reliable quantitative measure of organism abundance in environmental samples, metabarcoding results were interpreted exclusively on a presence/absence basis. Accordingly, no quantitative significance was attributed to sequencing read counts.

LIGHTHOUSE GAS		Implementation of Field Activities in support to the ESBS for Exploration Wells Asopus-1A and Asopus-1B in Block 2 – Ionian Sea					
Prepared by		Checked by	Approved by	Date	IDP	File:	Rev. B1
S.D.G./D.B./M.A.		D.B./M.A.	S. Sandroni	17/07/2026	EER001	Annex 3 - EER001_EBS_Final_Report_APPENDIX_J_eDNA_Analyses&Fisheries_R EV_B1	P. 22/ 23

ANNEX B: DATASET

Taxa						Remarks		Asopus-1A			Asopus-1B			Blank			Reference stations		
Phylum	Class	Order	Family	Genus	Species	NMSeqID	IUCN Threat Status	D1a	D3a	D5a	D1b	D3b	D5b	D6a	D6b	D7b	Kerkyra Ref	Othonoi Ref	Italy Ref
Chordata	Actinopterygii	Clupeiformes	Engraulidae	Engraulis	<i>Engraulis encrasicolus</i>	IM-S5967E	LC	-	-	-	-	-	137	-	-	-	-	-	-
Chordata	Actinopterygii	Gadiformes	Merlucciidae	Merluccius	<i>Merluccius merluccius</i>	IM-F2504Y	LC	-	-	-	-	-	-	-	-	-	78	-	-
Chordata	Actinopterygii	Myctophiformes	Myctophidae	Hygophum	<i>Hygophum sp.</i>	IM-8D11SH	-	-	-	-	-	-	-	-	2756	-	-	-	-
Chordata	Actinopterygii	Perciformes	Cichlidae	Oreochromis	<i>Oreochromis sp.</i>	IM-4662DY	-	-	-	-	3356	-	-	-	-	3544	-	-	-
Chordata	Actinopterygii	Pleuronectiformes	Pleuronectidae		Pleuronectidae n.d.	IM-3F88Y4	-	-	-	-	3454	-	-	1208	40908	-	3166	-	-
Chordata	Actinopterygii	Pleuronectiformes	Soleidae	Solea	<i>Solea sp.</i>	IM-9YAY73	-	-	-	-	242	-	-	-	-	-	-	-	-
Chordata	Actinopterygii	Pleuronectiformes	Soleidae	Solea	<i>Solea solea</i>	IM-4247KA	DD	5871	-	4776	20261	-	36589	10652	-	8914	8893	4517	5781
Chordata	Actinopterygii	Lophiiformes	Lophiidae	Lophius	<i>Lophius budegassa</i>	IM-632IZ3	DD	-	-	-	-	-	-	900	-	-	-	-	-
Chordata	Mammalia	Cetacea	Delphinidae	Stenella	<i>Stenella coeruleoalba</i>	IM-U9X6TY	LC	-	-	-	-	-	-	26	-	-	-	-	-

		Implementation of Field Activities in support to the ESBS for Exploration Wells Asopus-1A and Asopus-1B in Block 2 – Ionian Sea Environmental Baseline Report APPENDIX J – eDNA Analysis and Fisheries Assessment				
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S.D.G./D.B./M.A.	D.B./M.A.	S. Sandroni	17/07/2026	EER001	Annex 3 - EER001_EBS_Final_Report_APPEN DIX_J_eDNA_Analyses&Fisheries_R EV_B1	P. 23/ 23

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