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BRAVE

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Disclamer

This document has been produced with the financial assistance of the European Union. The content of this protocol is the sole responsibility of the BRAVE project and can under no circumstances be regarded as reflecting the position of the European Union and/or Italy-Croatia Programme authorities.

List of abbreviations

AAC: Adriatic Advisory Committee
AF - Application Form
AP - Associated Partner
AQ - Aquaculture
AS - Adriatic Sea
AO – Associated Organization
CM - Communication Manager
FM - Financial Manager
IAS - Invasive Alien Species
PSC - Project Steering Committee
LPMT – Lead Partner Project Management Team
MoU – Memorandum of Understanding
PCL - Project Communication Leader
PCM - Project Communication Manager
PFM - Project Financial Manager
PM - Project Manager
PP - Project Partner
RP - Reporting Period
QM - Quality Manager
QP - Quality Plan
SSF - Small Scale Fisheries
WP - Working Package
WPL - Work Package Leaders

Project executive summary

BRAVE is a European Territory Cooperation project funded under the 2nd call for Operations of Strategic Importance of the Interreg VIA Italy-Croatia 2021 – 2027 Programme, Priority 2 "Green and resilient shared environment", Specific Objective 2.2 "Enhancing protection and preservation of nature, biodiversity and green infrastructure, including in urban areas, and reducing all forms of pollution". The project duration is 36 months (01.07.2025-30.06.2028) and the overall objective is to enhance species knowledge by ensuring ecosystemic functionality and tackling biodiversity loss in the Adriatic Sea (AS), through monitoring activities, creation of a smart digital platform and innovative planning in response to environmental change.

BRAVE intends to address the following common challenges that can have strong environmental (competition for resources and substitution of native species), social (conversion of work activities and change in citizens' habits) and economic (fisheries and aquaculture sectors) impacts:

Invasive Alien Species (IAS): The proliferation of non-native species poses a significant threat to ecosystems, human health, and economic viability

Biodiversity Loss: The AS is experiencing a decline in biodiversity due to IAS, climate and environmental change, habitat degradation, pollution, and overexploitation

Data Fragmentation: Inconsistencies in existing databases hinder our ability to assess the overall health of the AS

Data Communication Gap: Difficulties in dissemination and use of environmental data by non-experts or those involved in monitoring and managing environmental data, increased by the challenging coordination between public bodies, research centres, business and citizens.

To achieve the goals, 13 project partners from the Italian and Adriatic Regions and 2 associated organizations will jointly implement strategic and operational actions.

Introduction

Work Package 1 (WP1) Knowledge Framework and Data Harmonisation aims to establish a common and structured knowledge base to support integrated environmental monitoring and data-driven governance within the BRAVE project.

The Adriatic Sea is characterised by complex ecological dynamics and transboundary environmental pressures, which require coordinated approaches to data generation, management and interpretation. WP1 addresses these challenges by promoting harmonisation of methodologies, standards and data flows across countries, sectors and institutions.

The general objectives of WP1 are to:

- improve coherence and interoperability of environmental data collected in the Adriatic Sea area;
- enhance accessibility and usability of data for analytical, operational and decision-support purposes;
- support the development of shared digital tools, in particular the BRAVE Smart Digital Platform;
- strengthen transnational cooperation and knowledge exchange among project partners and stakeholders.

Through these objectives, WP1 provides the methodological and technical foundation for pilot actions, platform development and strategic project outputs, contributing to the long-term resilience and sustainable management of the Adriatic Sea ecosystem.

Activity 1.1 Development of Common Data Collection Protocols is a core component of WP1 and focuses on defining shared operational frameworks for environmental data collection within the BRAVE project. The activity responds to the need to reduce fragmentation in monitoring practices and to ensure that data generated by different partners are comparable, reliable and suitable for integration.

The general objectives of Activity 1.1 are to:

- define common principles, requirements and minimum standards for data collection activities;

- ensure data quality, consistency and traceability across different environmental domains and pilot areas;
- align data collection processes with the functional requirements of the BRAVE Smart Digital Platform;
- support practical implementation and validation of protocols through pilot initiatives and simulation exercises.

By achieving these objectives, Activity 1.1 enables the transformation of heterogeneous monitoring practices into a coordinated and interoperable system, ensuring that data collected within the project can effectively support analysis, visualisation and evidence-based decision-making.

Objectives of the Data Collection standard 1

Deliverable 1.1.3 defines the Common Data Collection Standard for evaluating the integrity of the trophic chain and primary production throughout the project area. This protocol is essential for understanding the fundamental biological and ecological components that sustain coastal and lagoon ecosystems.

By standardizing how primary production and food web dynamics are monitored, the project can effectively analyze the interplay between biodiversity loss, environmental status, and the spread of invasive species. This standard provides the necessary data structures to populate the Smart Digital Platform, enabling a holistic assessment that balances ecosystem protection with the sustainability of traditional activities like fisheries and aquaculture.

Partner-specific objectives:

Veneto Region (LP): To assess the health status of bivalve mollusks and metazoan biodiversity through eDNA analysis, including verification of the presence of hybrids.

Friuli Venezia Giulia Autonomous Region (PP2): To consolidate the effectiveness of eDNA metabarcoding in describing seasonal fish diversity in the Marano and Grado lagoon and to implement the local DNA-based list of fish occurrences

Apulia Region (PP5): To enable early detection of alien species and to assess the spatio-temporal dynamics of *Cystoseira* spp. and their associated flora and fauna.

Zadar County (PP9): Comparative assessment of somatic indices and health status of four selected fish species and mussels from three areas in Zadar County to evaluate the potential need for its application across the Adriatic Sea

Dubrovnik and Neretva County (PP13): The final results of pilot activities will include an assessment of ecosystem health and the development of a model to estimate the ecological carrying capacity of Mali Ston bay to inform optimal and sustainable management of this important bivalve aquaculture site.

Importance of the Data collection standard 1 for the BRAVE Project

LP will evaluate the biodiversity of Venice Lagoon and the adjacent coastal environment using the eDNA metabarcoding and microbial metataxonomy. Water samples will be collected both in lagoons and in coastal areas. eDNA metabarcoding, 16S metataxonomy and shotgun metagenomics will be performed to describe microbial biodiversity, microbial resistome and metazoan biodiversity. Special attention will be devoted to identifying and characterizing alien species. Chemical-physical data will also be collected.

The health status of bivalve molluscs will be assessed by screening for pathogens, including emerging ones, checking gonadal status, identifying hermaphrodites and detecting neoplasms. Histological and molecular biological analyses will be carried out for these purposes. Biomolecular testing will also be used to identify any hybrids.

PP2 will evaluate the species composing the food web in the Marano and Grado lagoon using the e-DNA methodology. Water samples will be collected in 16 different lagoon sites; laboratory analyses (i.e. eDNA metabarcoding and morphological identification) will characterise the animal composition of the food web. Chemical-physical data will also be collected. Small-scale fishermen will be involved in data collection and dissemination activities, as their activity relies on a well-structured trophic chain.

PP5: The spread of alien species is disrupting ecosystem balance, undermining the productivity of key sectors such as fisheries, aquaculture, and coastal tourism, while posing a threat to local biodiversity. This pilot project employs an early warning system based on eDNA, enabling the detection of alien species before they are visually observed and providing a real-time, proactive monitoring tool. The second phase focuses on the monitoring of Cystoseira meadows, a priority habitat and nursery for numerous fish species.

By studying the flora and fauna associated with these meadows, the project aims to assess their conservation status and identify alien species colonizing their epiphytic communities.

PP9: Ecosystem biodiversity, among other factors, depends on the capacity of organisms to withstand environmental stress. Simple indicators of organism health and condition provide insight into their natural wellbeing and can reveal states reflecting acute or chronic stress, indicating a potential risk of biodiversity loss. The pilot project will assess selected indicators in four fish species and one bivalve under urban and aquaculture pressure compared to a Natura 2000 area, highlighting the added value of the data for existing protocols and the potential application of this approach across the Adriatic Sea.

PP13: Biotic and abiotic environmental parameters are key to understanding ecosystem health and the sustainable management of bivalve aquaculture areas. Extreme fluctuations of environmental conditions pose a threat to aquaculture processes that is further exacerbated by the spread and establishment non-native species. This is of even more significance for an area such as Mali Ston bay which has the additional significance of being a protected marine reserve and Natura 2000 site. The pilot actions performed in this site will be of importance to the BRAVE project consortium as the novel approach to determining carrying capacity for aquaculture production based on ecosystem modelling has not yet been used for sites in the Adriatic Sea and will have to be tailored to specific local ecosystems. Obtained results will be shared with all project partners and relevant stakeholders and may serve as a standard for developing knowledge-based management practices of similar sites in the Adriatic basin.

Existing data

To support the implementation of the protocols described above, it is first necessary to review the environmental datasets and databases already available within the Adriatic context. The following chapter therefore provides an overview of existing data sources relevant to the BRAVE project.

Existing environmental datasets represent an essential foundation for the development and validation of the BRAVE data collection protocols. Reviewing these sources allows the partnership to identify available information, assess its relevance for the project objectives, and determine how it can be integrated into the BRAVE Smart Digital Platform.

Italian projects and databases

SeaInsights (Interreg Italy–Slovenia Programme)

SeaInsights is a cross-border project funded under the Interreg Italy–Slovenia Programme aimed at improving biodiversity monitoring and conservation in the Upper Adriatic Sea through the application of environmental DNA (eDNA) technologies. The project focuses on the development and harmonisation of innovative molecular tools for the detection of marine species, supporting ecosystem assessment and management strategies. It also promotes stakeholder engagement and citizen science approaches to enhance monitoring capacity across the transboundary region.

ANERIS (Horizon Europe Research Infrastructure Project)

ANERIS is a Horizon Europe project dedicated to advancing European research infrastructures for marine ecosystem observation and environmental monitoring. The initiative supports the integration of innovative technologies, including environmental DNA (eDNA), remote sensing, and digital tools, to improve the collection and accessibility of marine biodiversity data. Through pilot activities and collaborative platforms, ANERIS aims to strengthen ecosystem-based management and foster technological innovation for marine conservation across European seas.

Marine Strategy Framework Directive — National Programme (ISPRA and Regional Environmental Agencies)

Within the framework of the Marine Strategy Framework Directive (MSFD), Italy has developed a national monitoring programme coordinated by ISPRA (Italian Institute for

Environmental Protection and Research) in collaboration with regional environmental agencies (ARPAs) and research institutions. The programme includes the implementation of environmental DNA (eDNA) metabarcoding protocols to support biodiversity assessment and the early detection of non-indigenous species in Italian marine waters, including the Adriatic Sea. These molecular approaches complement traditional monitoring methods and contribute to the evaluation of Good Environmental Status under European marine policies.

ECO-Res - RC IZSVe 10-22 - Ministero delle Salute (IT)

ECO-Res Eco-res generated the first integrated characterization of ARG transfer and accumulation in marine bivalves in a highly anthropized lagoon. The results highlight the need to incorporate ARGs, eDNA and resistome profiling into environmental and food safety monitoring programs. The study supports an expanded One Health perspective on AMR mitigation, emphasising the marine environment and seafood as emerging interfaces of public health relevance.

MusselAID - Feampa projects funded (Veneto Region)

MusselAID is a multidisciplinary project aimed at mitigating summer mortality events and harmful algal blooms affecting offshore mussel farms in the Veneto region (Italy). The project develops an early warning system by integrating field monitoring, laboratory analyses, and satellite data (Copernicus Marine Service) to assess mussel health, detect pathogens, and monitor phytoplankton dynamics. It also provides automated online reporting tools to support farmers and authorities in improving farm management, biosecurity, and food safety within a One Health framework.

MITICLIMA RC IZSVe 08-24

The project involves the use of a combination of culture-based and non-culture-based approaches in order to define, at the highest possible resolution, the pathobiota and the resistome (here understood as the collection of antibiotic resistance genes, ARGs) associated with marine planktonic communities and the mussels inhabiting these environments. In addition, we will simulate a further increase in temperature, hypothesizing future global warming scenarios, in order to directly assess the impact of these environmental changes on the antibiotic-resistant pathobiome of mussels intended for human consumption. This will allow us to anticipate and better understand the potential consequences of climate change on food safety and public health.

MedPROACT project (Interreg NEXT-MED 2021-2027)

Mediterranean Pilot Actions for Climate Change Adaptation project, eDNA analyses will be carried out in Tunisia, Jordan, Turkey, and Italy, focusing on specific marine habitats, including seagrass meadows and coral reefs. In Italy, one of the designated pilot areas is the Otranto–Leuca coastline, where eDNA sampling and analysis will be conducted to assess the flora and fauna associated with Posidonia oceanica meadows. The outcomes of these analyses will contribute to the BRAVE project by supporting taxonomic comparisons of species and by providing complementary ecological information, as the study areas of the two projects (BRAVE and MedPROACT) are geographically and scientifically complementary.

Biodiversity MARE Tricase project

The initiative provided the first baseline of species living in the area (Southern Salento). Sampling was carried out by SCUBA and free diving, fishing gears, and citizen science from 0 to 70 m. Overall, 697 taxa were found between March 2016 and October 2017, 94% of which were identified to the species level. The inventory can be used as baseline species assessment and the results can be crossed with BRAVE data eDNA.

Croatian projects and database

Alongside the Italian datasets presented above, several national data sources are also available on the Croatian side of the Adriatic basin. These provide complementary information relevant to water quality monitoring and environmental status assessments.

Mariculture assessment study in Mali Ston Bay (*Pećarević et al., 2020*)

A review study was performed by the University of Dubrovnik and funded by Dubrovnik Neretva county, among other things, it included an overview of all research related to mariculture performed in the pilot site to date. Furthermore, it suggested the need for developing carrying capacity models with predictive capabilities in order to fully utilize the bay's farming potential without compromising ecosystem stability, which was the basis for this project's pilot action 2.2-1-C. Previous carrying capacity limits have been approximative and based on literature, but not real-world conditions, with current production quantities being based on the **Environmental impact study of mariculture in the area of Mali Ston bay and Malo more** (Strategic environmental impact assessment) by *Benović et al. (2004)*.

Environmental monitoring had been implemented by Dubrovnik-Neretva county for the pilot area of Mali Ston bay for numerous years, but has had a hiatus since 2019. **Monitoring of oyster larvae concentrations** throughout the year in Mali Ston bay has been performed

almost continuously from 2000 to 2024, with the addition of measuring some environmental parameters such as temperature and salinity (*Bolotin and Bratoš Cetinić, 2020; Bolotin, unpublished data*).

Mapping of the coastal and demersal marine habitats in the coastal sea of the Republic of Croatia and demersal marine habitats in the Croatian continental shelf project was financed by the The Ministry of Economy and Sustainable Development through the Operational programme Competitiveness and Cohesion 2014-2020 and implemented from 2021 to 2023. It contains habitat mapping of around 51% of the coastal sea of the Republic of Croatia.

SENSEI (Smart European Networks for Sensing the Environment and Internet quality) – Horizon Europe - HORIZON-RIA - HORIZON Research and Innovation Actions

The European space agency project **SENSEI**, currently in progress, focuses on remote sensing of marine environmental parameters in coastal areas with aquaculture presence and developing suitability maps for native oyster farming, with the pilot location being Bistrina bay - a small part of the larger Mali Ston bay area.

Common projects and databases (IT+HR)

In addition to nationally developed datasets, several initiatives and databases operate across both sides of the Adriatic Sea, contributing to the shared knowledge base required for transnational environmental assessments.

ALIENA (Interreg Italy–Croatia Programme 2021–2027)

ALIENA is a cross-border project funded under the Interreg Italy–Croatia Programme 2021–2027 that aims to improve the monitoring and management of non-indigenous species (NIS) in the Adriatic Sea. The project integrates innovative tools, including environmental DNA (eDNA) metabarcoding, ecological modelling, and coordinated monitoring protocols, to enhance early detection and risk assessment of invasive marine species. By strengthening transnational cooperation and data sharing, ALIENA supports biodiversity conservation and ecosystem resilience in the Adriatic region.

BlueDiversity (Interreg Italy–Croatia Programme 2021–2027)

BlueDiversity is a cross-border cooperation project funded under the Interreg Italy–Croatia Programme 2021–2027 focused on improving the protection and sustainable management of marine biodiversity in the Adriatic Sea. The project develops innovative

monitoring approaches, decision-support tools, and governance strategies to address environmental pressures affecting coastal and marine ecosystems. Through collaborative actions among research institutions and public authorities, BlueDiversity promotes ecosystem-based management and biodiversity conservation across the Adriatic basin.

MAPA — Marine Adriatic Parks (Interreg Italy–Croatia Programme)

MAPA (Marine Adriatic Parks) is a transnational project funded under the Interreg Italy–Croatia Programme aimed at enhancing the conservation and sustainable use of marine biodiversity through the establishment and management of marine protected areas (MPAs) in the Adriatic Sea. The project supports the development of coordinated management frameworks, monitoring strategies, and stakeholder engagement activities to improve the protection of marine habitats and species while balancing conservation and socio-economic needs in coastal regions.

ECOSS (Ecological Observing System in the Adriatic Sea, Interreg IT-HR)

Analysis of benthos of the Mali Ston bay pilot area has been performed previously in the scope of project ECOSS, although only qualitatively using visual census on transects while diving, although in larger taxonomic detail than what is planned for this project.

ARGOS (Interreg IT-HR 2014-2020)

Through project ARGOS (Interreg IT-HR), a series of sampling stations and parameters were defined that would be essential for assessing the ecological carrying capacity of Mali Ston bay. In the same project for the first time a DNA-based list of fish occurrences in the Marano and Grado lagoon was carried out, offering the opportunity to analyse the zooplankton communities in the Marano and Grado lagoon.

The spatiotemporal frequency of the suggested monitoring was considered when designing this pilot action in order to be synergistic and add value to other pilot actions planned in the scope of the current project as well as other past and future activities such as the implementation of protocols and guidelines for estimating the ecological carrying capacity of the Mali Ston bay pilot site through ecosystem modelling as planned in Pilot action 2.2-1 - C.

New Data

While existing datasets provide an important baseline for understanding environmental conditions in the Adriatic Sea basin, the BRAVE project also generates additional data through pilot actions implemented by project partners. These new datasets complement existing monitoring systems and support the validation of the common data collection protocols developed under Activity 1.1, contributing to the development of the BRAVE Smart Digital Platform.

Pilot Sites: Description

LP: General activities description "*Venice Lagoon environmental monitoring: Characterization of resident microbial community, Multi-Parameter Probes data collection, Biogeochemical+Physical Characterization, Characterization of Phytoplankton+Edna*"

- Task 1: Environmental Pollution and Marine Organism Health Impacts

This task focuses on the seasonal assessment of pollution-induced biological stress in Adriatic bivalves. By utilizing advanced histology and immunohistochemistry, we will track endocrine disruption (via hermaphroditism rates), carcinogenic impacts (neoplasia), and cellular damage (oxidative stress markers). This data is critical for establishing a transborder ecotoxicological baseline for the Northern Adriatic's sensitive estuarine ecosystems.

- Task 2: Microbial Community Genetics and Anthropogenic Pressure Detection

Using cutting-edge shotgun metagenomics, this task maps the "resistome" of marine microbes to quantify the impact of human activities—ranging from industrial runoff to agricultural waste. By correlating microbial diversity with environmental chemistry, the study aims to identify "environmental dysbiosis," providing a sophisticated diagnostic tool for seawater quality in line with MSFD standards.

- Task 3: Biodiversity Monitoring and Alien Species Detection

To safeguard Adriatic biodiversity, this task implements a non-invasive eDNA monitoring framework. By analyzing genetic material filtered directly from the water column and sediment, we can detect invasive "alien" species at the earliest stages of introduction. This high-sensitivity genomic surveillance is essential for maintaining the integrity of the marine food web across the partnership's territories.

- Task 4: Control of Invasive Species in Farmed Bivalves

This task provides biosecurity oversight for the aquaculture sector, specifically targeting the inadvertent introduction of Atlantic species into Adriatic farms. Using molecular tools like PCR-RFLP, we will screen for hybridizing mussels, "look-alike" invasive clams, and exotic parasites that threaten the genetic purity and economic stability of native shellfish stocks.

PP2: General activities description: *"Evaluation of the integrity of the trophic chain through e-DNA innovative method, as a basis for the analysis of the status of protected species in the Marano and Grado lagoon"*

PP2 will evaluate the species composing the food web in the Marano and Grado lagoon using the e-DNA methodology. Water samples will be collected in 16 different lagoon sites; laboratory analyses (i.e. eDNA metabarcoding and morphological identification) will characterise the animal composition of the food web. Chemical-physical data will be also collected. Small-scale fishermen will be involved in data collection and dissemination activities, as their activities rely on a well-structured trophic chain.

PP5: General activities description: "*eDNA metabarcoding for spatial and temporal dynamics biodiversity characterisation of Otranto-Leuca coastline (in the process of becoming a MPA)"*

The Puglia Region will implement its pilot actions along the Otranto-Leuca coastline, an area characterized by significant biodiversity and specific environmental sensitivities. This site serves as a critical observation point for identifying both natural and human-induced threats impacting marine biodiversity and ecosystem services.

The activity will concentrate on the integrity of ecological indicators within the food web. PP5 will utilize eDNA analysis as an innovative, non-invasive technology to monitor biodiversity and evaluate the status of protected vs. invasive species.

- Task 1: Early detection of alien species and monitoring of species of commercial relevance

Building on information collected from local fishers and on-site observation, including bycatch records of potential invasive species, marine currents maps and habitat distribution maps, PP5 will design a targeted at-sea eDNA sampling scheme along the water column gradient. The objective is to early detect the occurrence of alien species whose presence is not yet stably confirmed in the Otranto-Leuca area but is well documented along the

Levantine coasts. The resulting eDNA sampling surveillance plan will enable early detection, thus supporting fisheries management among others.

- Task 2: Environmental eDNA for spatial-temporal biodiversity and trophic-chain dynamics

This pilot action consists of establishing a monitoring scheme focused mainly on sites where autoctonous macroalgal forests are already been recorded, as these habitats act as reliable indicators of good ecological status and support high community complexity. From these forested areas, water, surface-sediment, and macroalgal-associated biofilm samples will be collected to perform microbial eDNA profiling, capturing shifts in microbial assemblages that reflect environmental stress, habitat quality and ecosystem functioning. Within the same samples, presence/absence of DNA from invasive alien species will also be assessed, given their capacity to alter trophic interactions, grazing pressure, and the resilience of macroalgal habitats. This integrated approach enables early warning of ecological imbalance, while linking microbiome signals with the potential arrival or expansion of alien species.

PP9: General activities description "*Monitoring of somatic indices and health status, as indicators of environmental status of selected species within Natura 2000 areas, zones for aquaculture and near urban areas*"

The activity will focus on the monitoring of somatic indices and health status, as indicators of environmental status of selected species within Natura 2000 areas, zones for aquaculture and near urban areas (4 fish species and 1 shellfish species). Somatic indices primarily reflect the trophic condition of the selected organisms and provide insight into the productivity of the environment on which they depend. In combination with other indicators, they may also reveal the effects of environmental stress. AAC members will collaborate with the partnership to define the minimum set of data needed
"It is becoming clear that single-minded emphasis on production objectives for fish as commodity undermines the need to protect habitats, ecosystem processes and the health of the environment that is the basis for the fishery". Given that the exploitation of individual resources is no longer the only and perhaps not the most important influence on the health of ecosystems, it is necessary to monitor the impact of changes in the environment and their impact on the health of organisms and their reproductive ability. This pilot project will select fish species and one shellfish on which health status indicators and somatic indices

(condition index, age, viscerosomatic index, gonadosomatic index in sexually mature individuals…) will be used, which indicate the state of environmental well-being in different conditions in selected habitats and assess the health status.

Four fish species that are frequently targeted in small-scale coastal fisheries and non-commercial (recreational) fisheries were selected: the common two-banded seabream (*Diplodus vulgaris*), black scorpionfish (*Scorpaena porcus*), European hake (*Merluccius merluccius),* and small-spotted catshark (*Scyliorhinus canicula*). The selected species exhibit differences in feeding ecology and spatial mobility; therefore, this selection is expected to increase the complexity of the resulting findings. In addition to fish species, the Mediterranean mussel (*Mytilus galloprovincialis*) is included in the study. This bivalve species is both cultivated and harvested from the wild and is commonly found in fouling communities that may constitute natural habitats or anthropogenically influenced assemblages in the marine environment.

The study will be based on the sampling of selected four fish species and mussels, from three distinct areas within Zadar County: a Natura 2000 site, an area characterized by intensive aquaculture (marine cage fish farming), and an urban impacted coastal area. Two areas subject to direct anthropogenic pressure and one area characterized by minimal anthropogenic stress were selected. The chosen areas are also governed by different fisheries management regimes, with fishing pressure having thus far been considered the sole or primary driver in stock assessment frameworks.

The study will demonstrate the importance of collecting an expanded dataset to support a more comprehensive evaluation and management of human impacts on fish assemblages in coastal marine areas. The tested approach will highlight the potential need for its application across the entire Adriatic Sea, whose overall ecological capital depends on the integration of ecologically distinct areas into a single, functionally connected ecological system.

Fish sampling areas:

a) Natura 2000 ecological network site: protected areas surrounding the islands in the northwestern part of Zadar County (Molat, Ist, Škarda, Premuda, Silba, and Olib).
b) Area with a significant presence of marine cage aquaculture facilities in the Srednji Kanal, Iški Kanal, and Rava Kanal, within the boundaries defined by Cape Ovčji rt (Dugi Otok),

Cape Osiljinac (Iž Island), Svitla Bay (Ugljan Island), Karantunić Island, and Mali Lavdara Island.

c) Area in proximity to coastal urban zones: the Zadar Channel and Pašman Channel (from Privlaka to Biograd na Moru).

Mussel sampling areas:

a) Natura 2000 site: Novsko Ždrilo.
b) Area with a significant presence of marine cage aquaculture facilities in the Srednji Kanal, Iški Kanal, and Rava Kanal, within the boundaries defined by Cape Ovčji rt (Dugi Otok), Cape Osiljinac (Iž Island), Svitla Bay (Ugljan Island), Karantunić Island, and Mali Lavdara Island.
c) Area in proximity to coastal urban zones: the Zadar Channel and Pašman Channel (from Privlaka to Biograd na Moru).

PP13: General activities description *"Monitoring of ecological bioindicators in Natura 2000 site and Marine Protected Area in order to determine current status of invasive species and estimate ecological composition of the trophic chains (visual census benthic surveys)"*

The pilot action will rely on external expertise for monitoring of biotic and abiotic environmental parameters at several sampling points within the bay, based on spatiotemporal distribution of sampling points for different parameters, as previously outlined in one of the deliverables of the ARGOS project.

Dedicated monitoring equipment will be procured (data loggers) that will allow continuous measurement of temperature and salinity at multiple stations and depths for the duration of the activity. Additionally, field and laboratory equipment and consumables will be procured for sample storage and analysis. These will be complemented by measurements using hand-held probes for pH, chlorophyll (a dedicated probe for chlorophyll will likely be procured through this project) and dissolved oxygen taken at the same sites on a monthly schedule. Sea water samples will be taken seasonally for laboratory analysis of nutrients. Additional specialised analyses will be performed to:

- Measure net primary production seasonally using 14C radiocarbon methodology in bottles lowered to different depths (volumetric approach) at several sampling sites within the bay.
- Estimate total biomass of benthic organisms by general taxonomic groups, with special focus on filter-feeding organisms that are in competition with farmed bivalves for food.

The methodology to perform this analysis will likely be based on visual census transects performed for different habitat types and extrapolated to the size of the bay using existing habitat maps.

- Hydrological processes in the pilot site will also be analysed using existing national hydrometeorological data and *in situ* measurement of marine currents using acoustic doppler current profilers (ADCP).

Pilot Sites: Context & Justification

To ensure the operational feasibility of the protocols and the relevance of newly generated datasets, pilot actions are implemented at selected testing locations across the Adriatic basin. The following section describes the context and justification for the selected pilot sites.

LP: Venice Lagoon

Zone 1 - Venice lagoon - South

The southern sector of the Venice Lagoon is bounded by the mainland to the west, the Pellestrina barrier island to the east, and delimited to the north by the Canale dei Petroli. The bathymorphology is characterized by alternating shallow flats with depths of 0–1 m and deeper tidal channels (1–5 m), with the main channels reaching depths of 10–14 m. Water temperatures range from 5–8 °C in winter to 26–28 °C in summer. The area is characterized by extensive fishing-valley systems, mussel-farming installations, and lagoonal saltmarshes and tidal flats. It is also part of the Natura 2000 network as a Special Area of Conservation. The area is characterised by shellfish aquaculture, particularly for Manila clams and mussels.

From the biome point of view, the area represents a eutrophic and highly dynamic transitional system, characterized by strong salinity fluctuations, significant nutrient inputs from the drainage basin, and intense sediment resuspension. These conditions support microbially diverse and functionally rich communities, actively involved in nitrogen and carbon cycling. However, in the most anthropized areas, there is also an increased occurrence of opportunistic and potentially pathogenic taxa.

Zone 2 - Coastal area of Veneto region

This area is located along the eastern shoreline of the island of Pellestrina, which separates the Venetian Lagoon from the Adriatic Sea. The island extends for approximately 11 km in a north–south direction. The zone is bounded to the north by commercial and industrial shipping routes, and to the south by merchant-marine navigation routes.

The bathymorphology is characterized by shallow seabeds with a gradual slope, reaching an average depth of 10–20 m. Water temperatures range from 10–12°C in winter to 24–26°C in summer. The area also hosts mussel-farming installations. This area represents a system locally modified by mussel aquaculture. Mussels act as biological filters, reducing phytoplankton biomass in the water column, while simultaneously enhancing organic matter deposition beneath the farms through biodeposition. This process promotes benthic microbial communities associated with anaerobic pathways and favors macrozoobenthic assemblages tolerant to elevated organic loads. Moreover, farming structures increase habitat complexity, supporting epifaunal communities and associated mobile fauna.

An offshore sampling site located on the seaward side and distant from aquaculture installations can serve as an ecological benchmark. Such an area is expected to show more stable salinity and hydrodynamic conditions, microbial communities more representative of natural northern Adriatic coastal waters, and benthic assemblages typical of sandy substrates, with reduced influence from localized anthropogenic pressures. Comparing the lagoon, aquaculture area, and offshore benchmark site therefore allows disentangling natural coastal variability from biodiversity patterns driven by lagoon anthropization and mussel farming activities.

PP2: Marano and Grado Lagoon

The Marano and Grado Lagoon is located in the upper part of the Adriatic Sea, as the northernmost ecological unit of the Mediterranean, an extremely important wetland (about 160 square kms). It is of ecological importance, designated as a Special Area of Conservation (SAC IT3320037) according to the Natura 2000 Network. It is a clear example of incipient conflicts between conservation needs and human uses (tourism, fishery, mussel and fish farming, dredging, industrial settlements, nitrate inputs from inland agriculture). As a transitional coastal wetland, different water bodies characterise the area, according to different salinity values and other chemical-physical parameters. The study will investigate 16 different sites covering all the different water bodies of the lagoon, according to the Water Framework Directive. So, the 16 sites characterise all the different water bodies and hence the salinity gradient going from to the sea to inland marsh waters with low salinity values, in order to characterize the role of the different salinity values in the presence and abundance of zooplankton species and consequently the different fish communities. Monthly monitoring activity will rely on water samples collected at each site; laboratory analyses will characterise the zooplankton community as a basis for the trophic chain in the lagoon. Possible presence of alien species will also be evaluated.

PP5: Otranto-Leuca

The proposed pilot area “Otranto-Leuca coastline" is situated in a significant region within the Mediterranean Sea, between the Southern Adriatic Sea and the Western Ionian Sea, characterized by rich marine biodiversity and ecological interactions mostly in the coastal environments. The area is currently undergoing the process of establishing a Marine Protected Area (MPA), an initiative that will significantly strengthen the sustainable management of marine resources and the conservation of biodiversity, while generating important environmental, social, and economic benefits for local communities through ecosystem protection, enhanced fisheries sustainability, and opportunities for nature-based tourism and research.

The Otranto Channel’s unique hydrological features and its role as a connector between the Adriatic and Ionian Seas make it an ecological hotspot. This area supports a diverse array of marine life, characterized by intricate predator-prey dynamics and ecological fluctuations over time (*Guglielmo et al., 2019; Giampaoletti et al., 2020*). The presence of highly biodiverse habitats, including coralligenous reefs, which are critical for sustaining marine life and increasing biodiversity, emphasizes the importance of proactive management strategies in the region. These habitats support a wealth of species and facilitate ecological processes essential for the overall health of marine ecosystems, along with crucial spawning and nursery grounds for many fish species of commercial interest to fisheries.

The study area includes two SACs: Posidonieto Capo San Gregorio – Punta Ristola (IT9150034) and Costa Otranto – Santa Maria di Leuca (IT9150002). This area is characterized by extensive rocky shore rich in caves, cavities, gorges, and inlets, often sheltering small sandy beaches.

These habitats, extremely sensitive to climate change and increasing anthropogenic pressures—as highlighted by the Prioritized Action Framework (with particular reference to reefs 1170 and submarine caves 8330)—require urgent protection and sustainable management measures to safeguard habitats, biodiversity, and ensure the maintenance of ecosystem goods and services.

The Otranto–Santa Maria di Leuca sector represents a biogeographic corridor between the eastern and western Mediterranean and therefore is a priority area for systematic monitoring of invasive alien species (IAS). Its position as a gateway, particularly for taxa arriving from the Levantine Basin, combined with frequent commercial shipping and ballast-water discharge, increases the probability of introduction and establishment of propagules and larval stages that can disrupt native communities and generate measurable socioeconomic impacts on fisheries, aquaculture and coastal tourism (Task 1).

Concurrently, observed regressions of Cystoseira-dominated macroalgal assemblages — driven by warming, altered hydrodynamics, and anthropogenic pressures — indicate habitat degradation that reduces structural complexity and the nursery functions these meadows provide. For these reasons, targeted surveys and high-resolution mapping of Cystoseira beds and adjacent benthic habitats are strategically essential to (i) establish a baseline of phytosanitary condition and species composition, (ii) detect early incursions of non-native taxa, and (iii) inform adaptive management measures to mitigate ecological and economic risk while supporting restoration and conservation planning.

P9: **Zadar County**

Areas for fish sampling:

a) Natura 2000 ecological network zone: protected areas around islands in the northwestern part of Zadar County (islands of Molat, Ist, Škarda, Premuda, Silba, and Olib)
b) Area with a high presence of cage-based fish farms in the Srednji, Iški, and Ravski Channels, within the boundaries of Ovčji rt (Dugi Otok), Cape Osiljinac (Iž Island), Svitla Bay (Ugljan Island), and the islets of Karantunić and Mala Lavdara
c) Zone near coastal urban areas: the Zadar and Pašman Channels (from Privlaka to Biograd)

Areas for mussel sampling:

a) Natura 2000 ecological network zone: Novsko Ždrilo
b) Area with a high presence of cage-based fish farms in the Srednji, Iški, and Ravski Channels, within the boundaries of Ovčji rt (Dugi Otok), Cape Osiljinac (Iž Island), Svitla Bay (Ugljan Island), and the islets of Karantunić and Mala Lavdara
c) Zone near coastal urban areas: the Zadar and Pašman Channels (from Privlaka to Biograd)

The first task of the external expert is to accurately georeference the areas in GIS format, which will constitute an integral part of the deliverables.

Monitoring of the status of exploited fish and shellfish stocks/populations is predominantly conducted at the scale of broad marine regions, often without fully accounting for spatial variability in environmental conditions that influence organism growth and development. Assessments are typically based on standard indicators, including abundance, total biomass, spawning stock biomass (SSB), age structure, and length–frequency distributions, with datasets generally harmonised across the entire management

area. However, information reflecting the natural welfare and condition of these populations is frequently limited, and it is generally assumed that existing indicators indirectly capture these aspects. In coastal zones in particular, marine ecosystems are subject to multiple anthropogenic pressures, while the effects of climate change and other large-scale environmental drivers are not expressed uniformly across all areas. In this context, the integration of simple, robust indicators of the condition and health of selected indicator organisms into existing monitoring frameworks would provide complementary information on the consequences of pressures and changes in the trophic status of exploited species, beyond fishing mortality alone. Such an approach would strengthen the evidence base for ecosystem-based management and support the identification of additional human activities that may require management measures to ensure the conservation of biodiversity and the resilience of marine ecosystems.

Indicators for four fish species and mussels to be monitored here are well established in fish and shellfish welfare assessment, but have not yet been applied for the purposes of this pilot project. By comparing the selected indicators across three distinct areas within Zadar County—a Natura 2000 site, an area characterized by intensive aquaculture, and an urban-impacted coastal area—their sensitivity to different cumulative pressures will be evaluated. A positive outcome would indicate the potential applicability of this method across all targeted areas in the Adriatic Sea. Four fish species commonly caught in small-scale coastal and non-commercial fisheries were selected—common two-banded seabream (Diplodus vulgaris), black scorpionfish (Scorpaena porcus), European hake (Merluccius merluccius), and small-spotted catshark (Scyliorhinus canicula)—along with the Mediterranean mussel (Mytilus galloprovincialis), which is both cultivated and collected and commonly occurs in natural and anthropogenically influenced fouling communities. All selected species are widespread throughout the Adriatic Sea.

PP13: Mali Ston Bay

Mali Ston bay is a 28 km long mildly eutrophic coastal ecosystem on the south of Croatia. It has ideal conditions for bivalve farming due to the inflow of freshwater and nutrients through numerous underwater springs and the river Neretva, which has a delta close to the mouth of the bay. It was declared a special marine reserve in 1986. for the purpose of oyster farming and is also a Natura 2000 site. It is often divided into three main production zones which are roughly equivalent to an interior, middle and outer part of the bay, each with differing environmental conditions. In fact, the very heterogeneous nature of the bay's environmental characteristics requires the utilization of several sampling stations when

performing any serious monitoring activity, the exact number depending on the observed parameter.

Bivalve farming in Mali Ston Bay is the most prominent on the Croatian Adriatic coast, a vital branch of the local economy, a unique gastronomic tourist attraction and a centuries-old tradition of this region. The most famous shellfish cultivated here is the European flat oyster, Ostrea edulis, which is designated with the European Protected Designation of Origin (PDO) under the name "Malostonska kamenica" (Mali Ston Oyster)—the only shellfish in Europe to hold this status. Furthermore, more than 85% of the total Mediterranean aquaculture production of this species takes place in Mali Ston Bay. The bay also accounts for the largest production of the Mediterranean mussel, Mytilus galloprovincialis, in Croatia.

This pilot site has been selected due to its importance for Dubrovnik neretva county, numerous ecosystem services, protection status and extensive historic study. However, systematic and dedicated monitoring activities have been lacking, especially when it comes to tackling emerging issues such as: increasing fluctuations of and often extreme environmental conditions, further exacerbated by the presence and introduction of new non-native species which have a higher chance of becoming invasive in conditions of shifting environmental baselines and climate extremes - all potential threats to bivalve production.

Pilot Actions: Execution & Tech Stack

LP

Operations: monitoring of the biodiversity in terms of species composition and abundance. Check the health of bivalve molluscs and the presence of any hybrids.

Technology: water filtration system, probe for chemical-physical parameters and molecular laboratory will characterise the water samples. Laboratory tests: histological and biomolecular.

Data Profiles: laboratory test results consisting of: composition of water column microbial community, composition of metazon community and health status.

PP2

Operations: monitoring of the biodiversity in terms of species composition and abundance

Technology: water filtration system, probe for chemical-physical parameters and molecular laboratory will characterise the water samples

Data Profiles: composition of zooplankton communities as a basis for the trophic chain in the lagoon

PP5

Operations: The hands-on testing and validation phase of the eDNA activity for early detection of marine fish species includes a series of field and laboratory trials aimed at assessing the reliability, sensitivity, and reproducibility of the method. Water samples will be collected from selected sites, followed by DNA extraction, amplification using species-specific markers, and bioinformatic analysis for species identification. The results will be compared, where possible, with data obtained from traditional monitoring methods to evaluate accuracy and early detection capacity.

The study of eDNA associated with Cystoseira habitats provides an innovative and non-invasive approach for assessing biodiversity and monitoring ecosystem status. eDNA consists of DNA fragments released into the environment by organisms through cells, mucus, gametes, or decomposing tissues. By collecting and analyzing these genetic traces from seawater or sediments, it is possible to detect a wide range of species present in the ecosystem without direct observation or capture. Water samples will be collected in areas with known Cystoseira presence, followed by DNA extraction, amplification using suitable metabarcoding markers, and bioinformatic analyses to identify both the macroalgal species and their associated biota. The eDNA results will be compared with conventional survey data (e.g., visual census and benthic sampling) to assess accuracy, resolution, and detection capacity.

Technology: Water samples will be collected to perform eDNA analyses aimed at characterizing the occurrence of alien invasive species as well as the microbial and biological communities associated with Cystoseira habitats. Tissue samples of Cystoseira will be collected in parallel to assess the phytosanitary condition of the algae, including the presence of visible symptoms such as tissue degradation, bleaching, necrosis, or abnormal epiphytic growth. These samples will allow the identification of microorganisms potentially associated with healthy or stressed algal tissues. Total nucleic acids will be extracted from both water and algal samples for molecular analyses and metabarcoding approaches. Sequencing-based methods will be used to generate taxonomic profiles of microbial communities associated with the macroalgal habitat. Attention will be given to the detection of viruses and bacterial groups that may influence the health status of Cystoseira. In addition, a separate unfixed water fraction will be collected specifically for bacteriophage-



oriented analyses. Environmental samples will be processed to concentrate viral particles prior to downstream isolation procedures. Infective bacteriophages will be recovered through host-based enrichment and plaque assay techniques using suitable marine bacterial strains. The combined analysis of microbial communities, algal phytosanitary status, and bacteriophage diversity will provide new insights into microbial interactions potentially influencing the health and stability of Cystoseira habitats.

Data profile: Categorization of collected data (e.g., raw sensor data, validated datasets, and metadata) required to fuel the Smart Digital Platform.

- Metabarcoding sequence datasets: High-throughput DNA sequence data generated from markers such as COI, 12S, 16S, ITS, and 18S, enabling taxonomic identification of a wide range of marine organisms associated with Cystoseira forests.
- Species occurrence and community composition data: Putative presence/absence of biological communities including fish and microorganisms inhabiting the macroalgal habitat.
- Microbial and microbiome profiles: Detailed datasets describing bacterial, archaeal, and micro eukaryotic communities associated with the macroalgal surfaces, surrounding seawater, and sediments.
- Invasive and non-native species detection datasets: Early warning molecular signals indicating the presence of invasive species that may threaten native biodiversity and ecosystem stability.
- Environmental pathogen monitoring data: Detection of microbial pathogens or opportunistic microorganisms potentially affecting marine organisms or macroalgal habitat health.

PP9

Operations: The study is focused on comparing the overall health status and condition of four fish species and mussels across three zones that may exhibit different ecological conditions, with an emphasis on varying levels and forms of anthropogenic stress.

Fish samples will be collected from fishing activities three times during the calendar year: in April at the end of the winter isothermy, in July during the stratification of the water column and periods of increased environmental and anthropogenic stress, and in November during the establishment of the autumn isothermy of the water column. Mussel samples will be collected four times per year: in April, June, September, and November.

Technology: The number of organisms used for data collection, in each sample and for each individual species, must be at least 30. Samples from three different zones will be collected over the course of one month. Biometric measurements and visual inspections will be conducted on fresh fish, to ensure that preservation does not affect the results of observations and measurements.

Data Profiles: The study aims to utilize easily collected data to assess fish health status and condition. In this context, the "health status" of fish will be qualitatively evaluated through visual inspection of freshly caught specimens, including assessment of skin, fin, and gill damage, as well as the presence of ectoparasites. Fish condition will be determined through the analysis of biometric measurements and the calculation of somatic indices: CF (Condition Factor), HSI (Hepatosomatic Index), GSI (Gonadosomatic Index; where gonads are present), and VSI (Viscerosomatic Index). The status of mussels will be assessed based on sample mortality rates and selected somatic indices: CF (Condition Factor), STI (Shell Thickness Index), and RI (Digestive Gland Filling Index).

PP13

Operations and Technology: The first pilot action will rely on external expertise for monitoring of biotic and abiotic environmental parameters at several sampling points within the bay, based on spatiotemporal distribution of sampling points for different parameters, as previously outlined in one of the deliverables of the ARGOS project.

Dedicated monitoring equipment will be procured (data loggers) that will allow continuous measurement of temperature and salinity at multiple stations and depths for the duration of the activity. Additionally, field and laboratory equipment and consumables will be procured for sample storage and analysis. These will be complemented by measurements using hand-held probes for pH, chlorophyll (a dedicated probe for chlorophyll will likely be procured through this project) and dissolved oxygen taken at the same sites on a monthly schedule. Sea water samples will be taken seasonally for laboratory analysis of nutrients.

Additional specialised analyses will be performed to:

- Measure net primary production seasonally using 14C radiocarbon methodology in bottles lowered to different depths (volumetric approach) at several sampling sites within the bay.
- Estimate total biomass of benthic organisms by general taxonomic groups, with special focus on filter-feeding organisms that are in competition with farmed bivalves for food. The methodology to perform this analysis will likely be based on visual census transects

performed for different habitat types and extrapolated to the size of the bay using existing habitat maps.

- Hydrological processes in the pilot site will also be analysed using existing national hydrometeorological data and *in situ* measurement of marine currents using acoustic doppler current profilers (ADCP).

The second pilot action will incorporate data from other pilot activities (2.2-1-A, 3.2 -E) of this project and utilize them to develop and train a Dynamic Ecosystem Model (DEMO) for the pilot site Mali Ston bay in order to get a better insight in the functioning of the trophic interactions within the lower trophic food web. Input variables will include: temperature, salinity, nutrient levels, dissolved oxygen, chlorophyll, primary production, condition of farmed bivalves, biomass of farmed organisms, biomass of biofouling organisms and biomass of benthic filter feeders that will be overlaid on a hydrodynamic model. Once completed, the model will estimate the current ecological carrying capacity of the pilot site and to predict sustainable production in scenarios of altered environmental conditions.

Data Profiles: In the scope of first pilot action extensive biotic and abiotic environmental data will be collected in order to estimate ecosystem health. This and data from A3.2-E will be compiled and used as input for developing an ecosystem model in order to estimate carrying capacity for bivalve farming of the pilot site through second pilot action. This means that both raw sensor data and metadata will be produced for the Smart Digital Platform.

Data Checklist

In order to ensure consistency across pilot actions and facilitate data integration within the BRAVE Smart Digital Platform, a common set of minimum data requirements has been defined. The following section presents the data checklist applicable to the different protocols.

Defined set of minimum data requirements:

LP

The minimum data set defines the essential information required to evaluate the integrity of trophic chain components and primary production across the project area, ensuring comparability among partners while remaining applicable to heterogeneous pilot contexts.

The core dataset includes three main categories: environmental parameters, biological observations, and sampling metadata.

Environmental parameters consist of water temperature and salinity, which represent fundamental physical drivers influencing ecosystem processes and species distribution. Biological data focus on species occurrence, including the identification of taxa present at the sampling site. Whenever available, quantitative information such as abundance or biomass may also be recorded. For the assessment of primary production, at least one proxy indicator should be collected when applicable, such as chlorophyll-a concentration, turbidity, water transparency, or phytoplankton abundance.

Each observation must be associated with mandatory metadata, including date, time, sampling location, and geographic coordinates using a common reference system (WGS84). Additional recommended metadata include sampling depth or habitat type, collection method, responsible organisation, and data quality status.

This minimum dataset ensures that essential ecological information is consistently collected across partners, allowing the evaluation of trophic structure and ecosystem functioning while maintaining flexibility for different monitoring approaches.

PP2

Water samples from each site, seasonal collections for two years, will be the basis for laboratory analyses where the zooplankton community (i.e. presence/absence and n. of species) will be characterised. Chemical-physical data will be collected (temperature, salinity)

to characterise the parameters influencing the community. All data will be linked with fish landings data, as a framework.

Each observation must be associated with mandatory metadata, including date, time, sampling location, and geographic coordinates using a common reference system (WGS84). Additional recommended metadata include sampling depth or habitat type, collection method, responsible organisation, and data quality status.

PP5

For the pilot activity on eDNA metabarcoding for spatial and temporal dynamics biodiversity characterisation of Otranto-Leuca coastline (in the process of becoming a MPA) a structured set of minimum data requirements has been identified. These requirements are organized into key macro-themes that can be integrated into the Smart Digital Platform, supporting biodiversity monitoring and future conservation planning.

The first macro-theme concerns sampling metadata, which are essential for ensuring the traceability and comparability of biodiversity observations across space and time. These data include a unique sample identifier, sampling date and time, geographic coordinates (WGS84), site name, habitat type (e.g., rocky reef, seagrass meadow, open water), and water depth. Additional information on the sampling matrix (e.g., water or sediment), sampling protocol, filtered volume or collected mass, number of biological and technical replicates, and sample preservation procedures must also be recorded.

The second macro-theme includes laboratory and molecular metadata associated with the eDNA metabarcoding workflow. These data describe the DNA extraction procedures, extraction batch identifiers, and the use of positive and negative controls to monitor contamination. Information on the target genetic markers and primer sets used for metabarcoding, PCR conditions, library preparation protocols, and the sequencing platform must also be documented. Raw sequencing outputs, such as FASTQ files, together with sequencing run identifiers and basic quality metrics (e.g., number of reads per sample), represent a key minimum dataset to ensure transparency and reproducibility of the molecular analyses.

A third macro-theme relates to bioinformatic processing and taxonomic assignment. Minimum data requirements include documentation of the bioinformatic pipeline used for sequence quality filtering, denoising, and clustering into amplicon sequence variants (ASVs) or operational taxonomic units (OTUs). The resulting ASV/OTU tables and associated taxonomic assignment outputs must be provided, together with information on the reference databases and versions used for species identification, as well as the confidence

thresholds applied during classification. This information is essential for ensuring interoperability and for enabling comparisons with existing biodiversity datasets.

Another macro-theme focuses on biodiversity and ecological information derived from the eDNA analyses. These datasets include species occurrence records, read abundance or relative abundance per taxon, and taxonomic classification across multiple levels (from phylum to species where possible). The identification of species of conservation concern, including protected or indicator species relevant to future MPA management, should also be highlighted. These data will allow the characterization of spatial biodiversity patterns along the coastline and the assessment of seasonal or temporal variations in community composition.

In addition, environmental and physico-chemical parameters measured during sampling represent an important contextual dataset. Variables include water temperature, dissolved oxygen, pH, and salinity. These environmental descriptors help interpret biodiversity patterns and support analyses of ecological drivers influencing species distribution and community structure.

Finally, data governance, interoperability, and quality assurance information must be included to ensure the effective integration of the dataset into the Smart Digital Platform. This includes information on data ownership, responsible institutions, permits related to sampling activities in protected areas, and licensing conditions for data sharing. The use of standardized formats and metadata frameworks will ensure that the data generated through eDNA metabarcoding are interoperable with existing biodiversity databases and suitable for long-term monitoring and management of the future Marine Protected Area.

PP9

Monitoring of somatic indices and health status, as indicators of the environmental status of selected species within Natura 2000 areas, aquaculture zones, and coastal urban areas, will be conducted in georeferenced locations that can be integrated into the Smart Digital Platform. Samples of the selected fish and shellfish will be collected during three precisely defined periods to incorporate a seasonal component into the data analysis:

a) Fish samples will be collected from fisheries three times during the calendar year: in April at the end of the winter isothermy, in July during water column stratification and higher environmental and anthropogenic stress, and in November during the establishment of autumn isothermy in the water column.
b) Mussel samples will be collected four times a year: in April, June, September, and November.

For each sample of every species, a minimum of 30 organisms should be collected for data analysis. For the purpose of data collection, the following indicators have been selected:

a) Statistically processed individual somatic indices for fish:
   - CF (Condition Factor, IK) = 100 × W / L^3 [W – weight (g); L – length (cm)]
   - HSI (Hepatosomatic Index) = liver weight (g) / W
   - GSI (Gonadosomatic Index) = gonad weight (g) / W (if gonads are present)
   - VSI (Viscerosomatic Index) = weight of viscera without gills (g) / W
b) Visual examination of fish, including skin, fin, and gill damage, and presence of ectoparasites
c) Statistically processed individual indices for mussels:
   - CF (Condition Factor, IK) = 100 × dry "flesh" weight / dry shell weight [W – weight (g)]
   - STI (Shell Thickness Index) = 1000 × dry shell weight / [L × (H^2 × W^2)^0.05 × π / 2] [W – weight (g); L – length, H – height, W – width of shell (mm)]
   - RI (Digestive Gland Repletion Index) = wet weight of digestive gland (g) / wet "flesh" weight
d) Mussel mortality, based on the ratio of live individuals to empty shells in the sample

<u>Reports – data processing and interpretation of results:</u>

To monitor task implementation, two interim reports should be provided: one at the end of the first sampling (May 2026) and the second after the last sampling (December 2026). Reports will include basic information about the sample: sampling location, number of individuals of each species in the sample, average organism size in the sample, deviations of measurements from the mean, and photographic documentation of the sample.

After the completion of data collection, a final report should be prepared, structured in the format of a scientific paper (Abstract; Introduction; Results; Discussion; Conclusions). For this purpose, the above indicators should be statistically analyzed, and the value of their use will be demonstrated through comparison of the obtained results.

PP13

The first pilot action will generate data from monitoring of biotic and abiotic environmental parameters at several sampling points within the bay, based on spatiotemporal distribution of sampling points for different parameters, as previously outlined in one of the deliverables of the ARGOS project.

Data loggers will be used for continuous measurement of temperature and salinity at multiple stations and depths for the duration of the activity. Additionally, seawater samples will be taken from different depths at different stations on a monthly basis for immediate analysis or storage. Hand-held probes for pH and dissolved oxygen will be used for the different water samples immediately in the field. Chlorophyll concentration will be analysed in the laboratory monthly, using fluorimetry or liquid chromatography. Nutrient (ammonia, nitrites, nitrates and phosphates) concentrations will be analysed in the laboratory from samples taken every three months (seasonally) using standard laboratory protocols.

Additional specialised analyses will be performed to:

- Measure net primary production seasonally using 14C radiocarbon methodology in bottles lowered to different depths (volumetric approach) at several sampling sites within the bay.
- Estimate total biomass of benthic organisms by general taxonomic groups that are in competition with farmed bivalves for food. The methodology to perform this analysis will likely be based on visual census transects performed for different habitat types and extrapolated to the size of the bay using existing habitat maps. If biomass cannot be estimated for certain taxa, like infauna and similar organisms, total filtration rates of these organisms will be estimated.
- Hydrological processes in the pilot site will also be analysed using existing national hydrometeorological data and *in situ* measurement of marine currents using acoustic doppler current profilers (ADCP) at several sampling points.

The second pilot action will incorporate data from other pilot activities (2.2-1-A, 3.2 -E) of this project and utilize them to develop and train a Dynamic Ecosystem Model for the pilot site Mali Ston bay in order to get a better insight in the functioning of the trophic interactions within the lower trophic food web. Input variables will include: temperature, salinity, nutrient levels, dissolved oxygen, chlorophyll, primary production, condition of farmed bivalves, biomass of farmed organisms, biomass of biofouling organisms and biomass of benthic filter feeders that will be overlaid on a hydrodynamic model. Once completed, the model will estimate the current ecological carrying capacity of the pilot site and to predict sustainable production in scenarios of altered environmental conditions.

Data Standardisation

Data standardisation aims to ensure that information collected by different partners is homogeneous, comparable, and suitable for integration within the Smart Digital Platform. A common structure for data recording has been defined, including harmonised field names, units of measurement, coordinate reference system, and metadata requirements.

Building on the minimum data requirements defined within individual protocols (LP, PP2, PP5, PP9 and PP13), a common standardisation framework has been developed. While protocols address different topics—ranging from characterization of resident microbial community, phytoplankton, tracking of alien species, integrity of trophic chain, monitoring of ecological indicators and assessment of farmed bivalves—their data structures converge into a shared set of harmonised categories, identifiers and formats.

Given the pilot nature of the activity and the diversity of data sources across protocols, the level of detail and completeness of standardised datasets may vary among partners. The standardisation framework therefore defines a common reference structure while allowing a degree of flexibility in its implementation.

Harmonised Data Categories

Across all protocols, data are organised into four core categories, reflecting common elements identified in all data checklists.

Spatial Integration and Data Interoperability represent a mandatory component across all eDNA datasets and include sampling date and time, geographic coordinates (WGS84), water body type (river, transitional, coastal, marine), sampling location descriptors (e.g. fishing area, river basin district, coastal sector), sampling matrix (water, biota, sediment, where applicable), and sampling method. Each sampling event is associated with a unique identifier (Sampling_ID) to ensure traceability across field, laboratory, and analytical workflows.

Biodiversity data derived from eDNA analyses are incorporated into spatially explicit datasets, enabling integration with georeferenced ecological information. Taxonomic identification is standardised using reference databases (e.g. GenBank, BOLD) and reported through unique taxon identifiers (e.g. accession numbers or TaxID), ensuring unambiguous species assignment.

Data are structured in interoperable tabular formats (e.g. CSV), with biodiversity records organised according to widely adopted standards such as Darwin Core (DwC). Key fields

include taxon name, taxon ID, sequence ID, read abundance, marker gene region, and confidence score of taxonomic assignment.

Environmental context captures site-specific conditions influencing observations and includes seabed or substrate type, water depth and coastal morphology, presence of freshwater inputs, nearby infrastructures or potential pollution sources, records of extreme events, and environmental variables derived from satellite data. Where relevant, supporting physico-chemical parameters are also included.

Biological observations are harmonised using standardised descriptors and scientific nomenclature and include species presence, abundance or coverage, habitat type and condition, protected and Natura 2000 species, invasive species, and protocol-specific indicators such as macroalgal communities, algal blooms or biofouling.

Laboratory Analytical Data are structured through standardised workflows and reporting formats to ensure consistency and reproducibility across eDNA analyses. These include DNA extraction methods, amplification protocols (e.g. primer sets and target marker genes), sequencing platforms, and bioinformatic processing steps. Raw sequence data and processed outputs are linked through unique identifiers (e.g. Sample_ID, Sequence_ID), ensuring traceability from field collection to final taxonomic assignment.

Analytical outputs include sequence reads, quality-filtered datasets, and derived biodiversity metrics such as ASVs or OTUs, along with associated read abundances. Taxonomic assignments are reported with corresponding confidence scores and reference database identifiers. Additional parameters such as sequencing depth, negative and positive controls, and contamination filtering criteria are also documented.

Data are provided in interoperable formats (e.g. FASTQ for raw sequences, CSV for processed tables) and aligned with established standards (e.g. Darwin Core and MIxS where applicable). Harmonised descriptors and reporting structures ensure compatibility with other molecular, ecological, and environmental datasets, supporting integrated analyses and facilitating data reuse across monitoring and research frameworks.

Data Format and Platform Integration

To facilitate implementation across partners with different technical capacities, the standard allows the use of simple database formats, including spreadsheet-based systems

(e.g., Excel), provided that a shared template and predefined fields are adopted. The recommended structure includes a primary table containing sampling metadata and environmental variables, and a secondary table containing biological observations linked through a unique sample identifier.

Standardization involves harmonised metadata, consistent naming conventions, controlled vocabularies, and uniform formats for recording measurements, ensuring that datasets generated across different sites, laboratories, or partners are fully comparable. A shared data structure typically links sampling metadata, environmental descriptors, and biological observations through unique identifiers, enabling integration of molecular, ecological, and abiotic information into relational data systems. All partners collaborate with the ICT team to define procedures for the integration of these datasets into the Smart Digital Platform, ensuring interoperability, efficient data exchange, and alignment with project objectives. This approach allows eDNA-derived data to be combined with complementary ecological and environmental datasets, facilitating cross-site comparisons, spatial analyses, and broader ecological interpretations. By adopting standardized data frameworks, harmonized protocols, and interoperable formats, eDNA information becomes a scalable and reusable resource, supporting both immediate research objectives and long-term monitoring, while ensuring traceability, reproducibility, and compatibility across multiple studies and digital infrastructures.

Conclusions

This deliverable establishes a common standard for data collection aimed at assessing the integrity of trophic chain components and primary production across the project area. It provides a structured and detailed compilation of all planned pilot activities, organized by pilot site and by the partner responsible for their implementation. For each pilot area, the document includes a thorough environmental and contextual description, outlining the ecological characteristics, habitat types, and relevant biotic and abiotic factors that may influence the outcomes of the monitoring activities.

The deliverable emphasizes the importance of carrying out the planned activities within their specific environmental contexts, ensuring that observations and data collection reflect the natural variability and ecological dynamics of each area. Where applicable, the methodology considers the capitalization and integration of pre-existing databases and historical datasets, thus enabling the reuse of validated information and the building of cumulative knowledge.

Moreover, the document details the methodological approaches that will be employed, including standardized sampling protocols, analytical procedures, and data recording practices. By providing a harmonized framework, this deliverable ensures that data collected by different partners in different pilot areas are interoperable and directly comparable, thereby facilitating cross-site analyses, synthesis of results, and robust assessments of ecosystem status and function.

In summary, this deliverable represents a cornerstone for the project's scientific strategy. It lays the foundation for a coordinated and methodologically consistent approach, enhancing the reliability and reproducibility of the collected data, supporting evidence-based decision-making, and ultimately contributing to a comprehensive understanding of the structure and dynamics of the trophic network and primary production across the monitored areas.

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ANNEXES

- ANNEX 1 – Data collection Tables - standard 1