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Deliverable 1.4.1 Roadmap to Blue Economy

Activity 1.4 – Calibration
(Version 1.0 – February 2025)

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BlueDiversity

Shared BLUE knowledge and skills to sustain BIODIVERSITY in mariculture

Project Details

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REVISION TABLE

Version	Name(s)	Date	Description
V 0.1	Pietro Antolini, Cristiano De Pittà	December 2024	BlueDiversity Calibration strategy discussed among LP1, PP7 and all PPs
V 0.2	Pietro Antolini, Cristiano De Pittà, Filippo Drigo	January 2025	BlueDiversity Calibration strategy shared with all Pilot Areas.
V 0.3	Cristiano De Pittà	February 2025	BlueDiversity Calibration strategy internally reviewed
V 1.0	Cristiano De Pittà	February 2025	Deliverable submission

ABOUT THE **BlueDiversity** PROJECT

The **BlueDiversity** project is part of the Interreg Italy-Croatia 2021-2027 Programme, co-funded by the European Union. The **BlueDiversity** project's priority is priority 2 (Green and resilient shared environment). The Specific Objective is SO2.2: improve the protection and preservation of nature, biodiversity and green infrastructure, including in urban areas, and reduce all forms of pollution. Considering this, the **BlueDiversity** project aims at enhancing the ecosystem services-based practices, which allow the sustainment of local ecosystems' preservation through reducing human activities' impacts on biodiversity, while enhancing and developing economic and territorial opportunities.

To achieve the project's objectives, the Project Partners have the need to assess the State of the Art about the main biodiversity threats identified in the pilot areas, by analysing biodiversity, non-indigenous species, marine litter, and lagoon litter. The State-of-the-Art analysis will also target, experiment and screen existing practices, innovations, resources available and attitudes of the ecosystem services end users in the context of the Adriatic Sea.

The Adriatic socio-eco-cultural features represent the common thread for the project actions, bringing relevant stakeholders of the blue economy and blue research to work together towards best and innovative practices with the common scope to develop a green and sustainable transition in the blue sector. In particular, the **BlueDiversity** project targets small and medium enterprises aiming to establish "living laboratories", providing institutional support on the one hand, and placing them as didactic examples at the much-needed interface with schools and younger generations, enhancing the framework of the blue economy with a multilateral approach that includes institutional actors and citizens.

The Adriatic Sea, shared by Italy and Croatia, represents one of the best examples of natural backgrounds in ecological terms, where the dynamics of co-creation, based on an intertwinement of tradition and innovation, can be established. The project aims to develop pilot activities that will experiment with innovative fishing gear to tackle the presence of non-indigenous species that seriously threaten the Adriatic coasts' aquatic ecosystems.

The **BlueDiversity** project is modular, aiming at fully replicable successful experiences, representing the actions' legacy and the core of a shift towards the blue economy. Such elements will be strategically disseminated targeting different institutional and non-institutional entities focusing on the capitalization of the knowledge, know-how and innovative instruments developed throughout the project and aimed at building a sound ground for future major innovative developments.

D 1.4.1 - EXECUTIVE SUMMARY

The **BlueDiversity project** is dedicated to preserving ecosystem services by enhancing management strategies along the coasts of Italy (Veneto, Marche, and Apulia) and Croatia (Splitsko-dalmatinska županija and Dubrovačko-neretvanska županija). A central objective of BlueDiversity is not only to collect data but also to actively engage stakeholders, including local authorities and the public. The project aims to raise awareness about biodiversity challenges and the ongoing threats to coastal and marine ecosystems, fostering collaboration and the sharing of insights among various actors involved.

A key deliverable of the project, the **"BlueDiversity Calibration" (Deliverable 1.4.1)**, will be used to guide the transition from theoretical concepts to field testing. This deliverable will lay the groundwork for the methodologies that will be further developed in **Output 1.2**. The **BlueDiversity database (D1.1.1)** is a rich resource, encompassing biodiversity occurrences, abiotic environmental parameters, and other crucial data. It includes 3,623 biodiversity observations, 264 data points related to abiotic conditions in the water column, and 79 observations of sedimentary abiotic parameters. One of the standouts features of the BlueDiversity database is its dynamic, "living" nature, designed to be continuously updated with new data from ongoing research and field activities. This ensures the database remains relevant and useful for both short- and long-term ecosystem monitoring.

The **BlueDiversity SoA report (Deliverable 1.2.1)**, which focuses on assessing the perception of ecosystem service users (ESUs), plays a crucial role in understanding stakeholder perspectives. This deliverable involves the creation and validation of "Questionnaire 1.2 State of the Art: Specific Issues" and aims to gather 90 responses from relevant ESUs across the six pilot areas.

Additionally, **Deliverable 1.3.1 (D1.3.1)**, the White Paper to BlueDiversity, will summarize the state of the art, identify critical issues and opportunities, and define the overarching goals of the project. By integrating the findings from these deliverables, the project can outline a roadmap of threats affecting the target areas and develop solutions that consider local specificities.

The BlueDiversity project catalyzes sustainable blue economy practices in the Adriatic region, leveraging its rich biodiversity and crucial ecosystems. It bridges innovation and tradition by fostering collaboration among policymakers, researchers, local communities, and the private sector. By applying knowledge and innovative technologies, the project supports the development of green practices that protect marine ecosystems while creating new economic opportunities. This initiative contributes to a broader effort in building a sustainable, ecosystem-based approach to coastal and marine resource management.

D1.4.1 - LINKS WITH OTHER PROJECT ACTIVITIES

D1.4.1 (BlueDiversity Calibration) aligns with other activities in Work Package 1 "State of the Blue Art" and contributes to Work Packages 2 "Blue Initiatives", 3 "Blue Education, Training and Culture", and 4 "Blue Capitalization". It synthesizes information from D1.1.1 (BlueDiversity Database), which provides data on biodiversity and contaminants for each pilot area, and D1.2.1 (State of the Art Report), which assesses ecosystem service users' perceptions. Based on this synthesis, D1.4.1 identifies specific threats and develops customized solutions for the project areas, taking into account local characteristics. This process guides the development of future methodologies and serves as a bridge between theoretical concepts and practical field testing.

Summary

1. CALIBRATION ROADMAP: FRAMEWORK AND METHODOLOGY OVERVIEW

Deliverable 1.4.1 has a calibration purpose, to understand how to proceed from theoretical to testing phase on field (Figure 1). It will serve as a foundation to define methodologies implemented successively in Output 1.2.

The BlueDiversity Database, Deliverable 1.1.1 (D1.1.1), filled by each partner and available for each pilot area gathers available literature data regarding biodiversity and contaminants presence in both water and sediments.

On the other hand, BlueDiversity SoA report, Deliverable 1.2.1 (D1.2.1), has the objective to assess the perception of the ecosystem services users (ESU) regarding the topic tackled by BlueDiversity.

Finally, Deliverable 1.3.1 (D1.3.1), White Paper to BlueDiversity, summarises the state of the art, criticalities and opportunities and defines the aims of the project.

Combining the information extrapolated from these deliverables is possible to delineate a roadmap of threats affecting the areas of the project and design solutions that take in account local peculiarities.

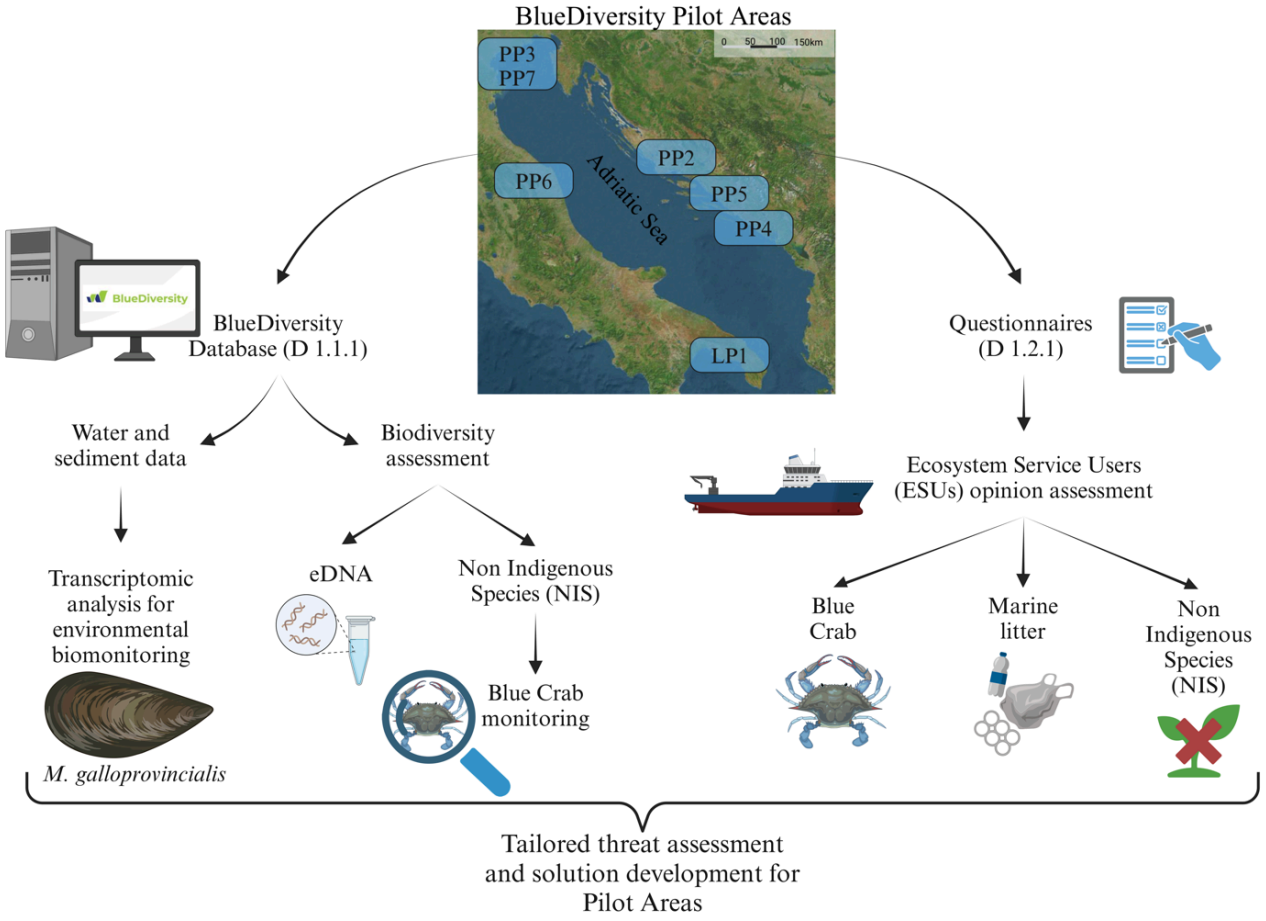

Figure 1. BlueDiversity project calibration roadmap: from conceptualization to field testing (**D 1.4.1**). This figure was created in BioRender *(www.biorender.com)*

2. COMPREHENSIVE BIODIVERSITY EVALUATION

2.1. Comprehensive overview of BlueDiversity pilot sites: ecological and socio-economic profiles

The Adriatic Sea, bordering both Italy and Croatia, exemplifies a rich natural ecosystem. This region offers an opportunity to blend innovation with tradition, particularly in the blue economy sector. The unique socio-ecological and cultural characteristics of the Adriatic can bring together key stakeholders in blue economy and marine research to collaboratively develop sustainable practices and drive a green transition.

In this context, the **BlueDiversity** Project aims to assess the current state of biodiversity threats in the region. The project focuses on analysing biodiversity, non-indigenous species, and marine and lagoon litter. To achieve this, the project partners have identified six pilot areas. These include protected zones, coastal and marine areas that are biodiversity hotspots, and regions where the blue economy is already a significant presence.

These pilot areas serve as living laboratories, providing the foundation for the project's core objectives. These range from biodiversity conservation to technological advancements, all aimed at promoting sustainable practices in the Adriatic's blue economy sector.

Specifically, the Pilot Areas are distinguished as follows:

- LP1 (University of Salento) - Aquatina di Frigole and Ionian Sea mariculture
- PP2 (Sea and karst) - Cetina River and Estuary; Pantan Coastal Lagoon; Uvala Vrulja kod Brela
- PP4 (Institute of Oceanography and Fisheries) - Neretva Delta
- PP5 (Public Institution for the Management of Protected Natural Areas of Dubrovnik-Neretva County) - Mali Ston Bay
- PP6 (Blu Marine Service) - Coast of San Benedetto del Tronto - Natural Reserve Sentina
- PP7 (University of Padua) - Venice Lagoon

Aquatina di Frigole (LP1)

Aquatina di Frigole is a coastal lagoon linked to the Adriatic Sea and a NATURA 2000 Site, recognized as an important hotspot for biodiversity conservation. The area shelters diverse Mediterranean NATURA 2000 priority coastal habitats and species included in the Habitat Directive (92/43/EEC) and Birds Directive (2009/147/EC). Protected priority habitats include *Posidonia oceanica* meadows; *Coastal Lagoons*; and Coastal dunes with *Juniperus* spp. The village of Frigole, in which the protected area is integrated, hosts different socio-economic activities, mainly represented by small-scale fisheries, agriculture, beach resorts, bars, restaurants, schools, and cultural and environmental associations. Further to the local initiatives for Aquatina di Frigole conservation, the protected area is subject to regional, national, and international regulations for species and habitat biodiversity conservation. However, the area is under pressure from activities related to tourism, agriculture, and fishing, as well as from macro-regional issues such as plastic pollution and invasive species like the blue crab.

Ionian mariculture (LP1)

Ionian mariculture in the Puglia region leverages unique hydrogeological conditions to support sustainable aquaculture. Two key sites have been selected along the Ionian coast. Offshore from Torre Suda, "inmare" cages are positioned in strong, steady currents that enhance oxygenation, nutrient dispersal, and waste removal—ideal for growing European sea bass, gilthead sea bream, and meagre. In contrast, mussel farming for "Cozza Castrense" thrives seaward of Castro Bay, where 34 submerged karstic freshwater springs, or "citri," create a brackish environment rich in minerals and organic particulates that boost phytoplankton growth. Together, these systems illustrate how geomorphology, hydrology, and biodiversity combine to optimize aquaculture yields. They face threats from invasive species and climate change, which may trigger harmful algal blooms and mass fish mortalities.

Cetina river and estuary (PP2)

The Cetina River is uniquely characterized by its specific ecological conditions, including its connection to the karst fields of southwestern Bosnia and Herzegovina, and the mixing of fresh and saltwater in its lower reaches. This dynamic setting fosters a distinctive ichthyofauna, including endemic species such as the Adriatic trout (*Salmo faroides*), Illyrian chub (*Squalius illyricus*), European eel (*Anguilla anguilla*), and the sea lamprey (*Petromyzon*

marinus) that migrates into the river to spawn. It encompasses marine, brackish, and freshwater ecosystems that are sensitive to shifts in species composition. Despite its rich

Pantan (PP2)

Pantan coastal lagoon, located between Divulje and Trogir on Croatia's Dalmatian coast, is a unique brackish wetland shaped by a freshwater spring and a small lake. This biologically rich habitat supports diverse bird species and native fish, serving as a critical feeding and breeding ground for migratory wildlife. It is a Natura2000 site and an Ornithological & Ichthyological Reserve. Despite its ecological value, Pantan faces threats from urban pollution, habitat loss, hydrological changes, and invasive species like the blue crab.

Uvala Vrulja (PP2)

Uvala Vrulja is a unique marine ecosystem on Croatia's coast, characterized by underwater freshwater springs known as *vrulja*, which create brackish habitats blending marine and freshwater conditions. As part of the Natura 2000 network, it features diverse habitats, including submerged sea caves and vibrant reefs. The area supports rich biodiversity, with species like *Codium bursa*, *Dyctiota* sp., *Axinella cannabina*, and *Corynactis viridis* thriving in its waters. Invasive species like the blue crab threaten the delicate balance of this rare ecological refuge.

Neretva River (PP4)

The Neretva River spans approximately 5,580 square kilometres and ends with a delta shaped river mouth in Croatia. The Neretva Delta covers 196 square kilometres and is a vital ecological area, hosting Natura 2000-protected ornithological and ichthyological reserves. Its wetlands provide critical bird nesting sites, fish spawning grounds, and key migration routes. The estuary is essential for conserving freshwater fish species and serves as the most important nursery for marine fish and crustaceans along the Eastern Adriatic coast. Both organic contaminants and heavy metals show low levels in the area supporting good environmental status. However, intensive agriculture, port development (Port of Ploče), urbanization, overfishing, illegal hunting, tourism, watercourse alterations, and hydrological changes pose significant threats the estuarine habitat. 12 species of non-native aquatic organisms have been identified in the River Neretva Estuary, and it hosts the largest population of blue crab, *Callinectes sapidus,* in Croatia.

biodiversity, the river faces challenges from hydrological modifications, urban pollution, and habitat degradation.

Mali Ston Bay (PP5)

Mali Ston Bay, located between Croatia's mainland and the Pelješac Peninsula, is a rich marine ecosystem and a designated Natura 2000 site, renowned for its ornithological and ichthyological significance. This 100 km-long bay features a highly indented coastline, numerous islets, and underwater freshwater springs (*vruljas*) that, along with tidal currents and the Neretva River's influence, create nutrient-rich waters and support unique ecosystems, with 16 strictly protected species recorded. The bay supports high biological productivity, benefiting suspension-feeding organisms and enabling centuries-old aquaculture, particularly for European flat oysters and Mediterranean mussels—now contributing to 86% of Croatia's total production. Apart from aquaculture, tourism is the second most important economic activity in the area, relying heavily on the bay's natural beauty and local seafood gastronomy, alongside cultural landmarks.

San Benedetto coastal area (PP6)

The coastal area between San Benedetto del Tronto and Porto d'Ascoli along the Adriatic coast exhibits a strong interplay between natural settings and human activities. It hosts both industrial and artisanal fishing, with San Benedetto del Tronto serving as a key central Adriatic fishing harbour. Sandy and mixed bottoms in the region offer ideal habitats for benthic species, while artificial breakwater reefs promote diverse fish communities. Intensive urbanization has led to the development of beach resorts, hotels, and residential infrastructures along the coast. Fishing and tourist ports contribute to increased maritime traffic and pollutant discharges. River and urban discharges further compromise water and sediment quality. Coastal defense structures like breakwaters and artificial barriers modify natural coastal dynamics. Small-scale operators and hydraulic dredges for *Chamelea Gallina* clams, operate within three miles of the coast, generating significant inshore pressure. Additionally, a mussel farming plant is situated approximately one mile from the Tronto River mouth, adding to the area's complex human impacts.

This area features the Sentina Natural Reserve, a set of small brackish ponds and retro-dune salt meadows with plant associations highly specialized to the coastal environment and therefore completely peculiar. The area includes remnants of ecosystems typical of low and

sandy coasts, once very widespread but today of great interest as they are now very rare throughout the regional coastline, almost continuously anthropized. La Sentina is included in the Natura 2000 project and managed by the Municipality of San Benedetto del Tronto.

Lagoon of Venice (PP3 and PP7)

The Venice Lagoon, located in the northern Adriatic, is the largest transitional environment in the Mediterranean, spanning nearly 550 km^2. Its diverse habitats support rich biodiversity—including 140 breeding bird species—and the area is protected under Natura 2000 and related conservation directives. Despite its ecological importance, the lagoon has experienced severe human-induced pressures over recent decades. Dense population centers, agricultural and industrial activities contribute significant nutrient inputs, while the busy ports of Venice and Chioggia add maritime traffic and pollution. Additionally, the lagoon is a hotspot for non-indigenous species, harboring over a third of the Mediterranean's total NIS. Historically, the area has been a key center for European shellfish aquaculture—especially for the Indo-Pacific Manila clam—and supports robust fish farming of species such as European eel, flathead grey mullet, European seabass, and gilthead seabream. Overtourism further exacerbates environmental impacts through increased air emissions, waste, sewage discharges, and visual pollution.

2.2. Biodiversity assessment of BlueDiversity pilot areas

BlueDiversity Database (D1.1.1) offers a comprehensive view of the biodiversity present in the project pilot areas.

From the first Pilot Area (Acquatina di Frigole and Ionian Sea) we documented 599 species. Of these, 43 species are classified as protected and include: waterfowl and wading birds, reptile and amphibian species, fish, invertebrates and plants. We documented also 21 Non-Indigenous Species (NIS) distributed among different taxa (*Ascidiella aspersa, Botrylloides leachii, Styela canopus, Styela plicata, Beroe ovata, Mnemiopsis leidyi, Littorina obtusata, Callinectes sapidus, Palaemon elegans, Penaeus aztecus, Ficopomatus enigmaticus, Hydroides dianthus, Perinereis cultrifera, Schistomeringos rudolphi, Terebella lapidaria, Gammarus pulex. Additionally, Carpobrotus edulis, Cistus ladanifer, Dendrobaena octaedra, Bulinus truncatus, Eutintinnus apertus*).

In the second Pilot Area (Cetina River and estuary; Pantan coastal lagoon; Uvala Vrulja kod Brela) we reported 320 species. Of these, 39 species are included in protection lists. In this area, 4 NIS species were reported (*Phasianus colchicus, Streptopelia decaocto, Callinectes sapidus, Codium fragile*).

In the Neretva Delta 169 species have been reported. 33 of these species are listed as protected and they span various taxonomic groups. Regarding invasive species, 14 occurrences were recorded as NIS (*Arcuatula senhousia, Callinectes sapidus, Egeria densa, Eleusine indica, Epinephelus aeneus, Esox lucius, Ficopomatus enigmaticus, Micropterus salmoides, Mnemiopsis leidyi, Myriophyllum heterophyllum, Paspalum paspalodes, Pseudocaranx dentex, Silurus glanis, and Synodontis eupterus*).

From Mali Ston Bay we documented 556 species. A high number of species are listed as protected (75 species). Even here, non-indigenous species were recorded. Two cryptogenic species and 23 NIS (some examples are: *Amathia verticillata, Asparagopsis armata, Botrylloides leachii, Bursatella leachii, Caulerpa cylindracea, Melibe viridis, Paraleucilla magna,* and *Styela plicata*). This is the sole area where *Callinectes sapidus* is not reported.

In the fifth Pilot Area (Coast of San Benedetto del Trono – Natural Reserve Sentina) we reported 390 species. 89 of these species are listed as protected under various conservation frameworks. The number of NIS species reported here is lower compared to the other areas, only 6 have been reported, among which we have: *Anadara inaequivalvis, Atriplex portulacoides, Callinectes sapidus, and Chelidonichthys lucern*a

Lastly, in the Venice Lagoon Pilot Area we documented 1589 species. Of these, 283 are listed as protected (including birds, fish, molluscs, marine invertebrates and plant species). There is also a considerable number NIS (120). Some examples of NIS species are: *Desmarestia viriis, Grateloupia turuturu, Undaria pinnatifida, Botrylloides leachii, Stylea clava, Didemnum vexillum, Callinectes sapidus, Ruditpes philippinarum, Anadara inaequivalvis, Rapana venosa*.

The framework that emerges from the BlueDiversity Database highlights the spread presence of invasive species in all the pilot areas, confirming the status of the northern Adriatic as a hotspot area for species invasion.

This is perceived also by ESU with 65% of respondents across the 6 pilot areas that claim to have encountered a NIS and 61% that noted a change in the local biodiversity. While this shift

in the community is not always viewed negatively (only 38% consider it unfavourable), a significant majority (81%) of interviewees regard alien species as a dangerous threat.

In all pilot area except one, the blue crab *Callinectes sapidus* has been detected, underscoring its widespread distribution along the Adriatic coast.

2.3. Environmental DNA for biodiversity assessment

Available biodiversity data, while consistent, may be limited by monitoring designs that focus primarily on charismatic or threatened species or rely on techniques that overlook cryptic or less observable species. In this context, environmental DNA (eDNA) emerges as a valuable tool to enhance biodiversity assessment. eDNA is an innovative approach that enables the study of spatial and temporal community patterns and processes by sequencing DNA directly from environmental samples such as air, water, and sediments.

The analysis of environmental samples facilitates the detection of rare and endangered species across large ecosystems (Bergman et al. 2016; Eva et al. 2016; Anderson et al. 2018). In addition to being non-invasive, eDNA metabarcoding is cost-effective, time-efficient, and offers higher sensitivity compared to traditional survey methods, allowing species detection at any life stage (Shu, Ludwig, e Peng 2020). Despite some limitations, this approach provides a broader and more comprehensive understanding of the actual community composition within each pilot area. Its ability to reveal previously undetected species has already been demonstrated, even in the area selected by the project. Cananzi *et al*., (2022), for example, identified, for the first time in the Venice Lagoon, the DNA of the oceanic puffer *Lagocephalus lagocephalus*, a species thought to be undergoing range expansion in the Mediterranean Sea, raising concerns for the Adriatic coasts.

Furthermore, eDNA can be applied to gain insights into microbial communities through a metagenomic approach. In aquatic ecosystems, microbes—including both eukaryotic and prokaryotic organisms—play a crucial role in trophic networks, biogeochemical cycles, biodiversity, and ecosystem functioning. Changes in these microbial communities can indicate environmental modifications driven by anthropogenic activities (Nogales et al. 2011) or alien species invasions (Manzari et al. 2015), potentially serving as early warning signals of ecological shifts.

We believe that environmental DNA (eDNA) could serve as an additional and effective method to monitor biodiversity in pilot areas, validating and expanding on data obtained from literature. If repeated throughout the project period, it could potentially detect the impact of implemented strategies. Consequently, leveraging LP1's expertise, we have drafted a general protocol for eDNA metabarcoding to be performed on water samples [Annex 1]. General protocols for both plants and animals DNA extraction are also established [Annex 2; Annex 3].

2.4. Description of the invasive species - Blue Crab

Among the invasive species present in all pilot areas, as reported in D1.1.1, the Blue Crab (*Callinectes sapidus*) is of biological and economic concern. Probably arrived through ballast waters from Western Atlantic where it is autochthonous, this opportunistic euryhaline species experienced an explosion in the abundance during summer of 2023 on the Adriatic coast of Italy, with devastating consequences for the local fishing and aquaculture sector (Lucchetti, Melli, e Brčić 2023). A study of Chiesa *et al*., (2025) focused on the Po Delta lagoons in northern Adriatic highlights an alarming situation where 749 tons of the crab were caught in the whole 2023, around 75% of the whole Veneto Region sold and disposed blue crabs. In these areas, Manila clam production was severely impacted: over 50% of clams were injured by the invasive species, and mortality rates approached 100% in the majority of sampled sites. Furthermore, the occurrence of smashed/shredded seed in the natural recruitment areas, or the total absence of seed from collecting sites, is compatible with the predatory activity of the blue crab. This scenario is even more dramatic, considering that most of the Manila clam production was ensured by the high abundance of natural seed, and the current condition will thus affect the sector in the incoming years.

The distribution of this species across Eastern Adriatic appears less critical and still under control as emerge from works of Glamuzina *et al*., (2021; 2023) where Montenegrin and Croatian sites were sampled.

Awareness of the Blue Crab is high among participants in D1.2.1 across all BlueDiversity sites, with 97% recognizing the species. However, direct encounters vary: 22% report frequent sightings, 42% encounter it occasionally, and 36% have never had direct contact.

Local Environmental Knowledge (LEK) seems to confirm the fact that the distribution of the Blue Crab is uneven across the Pilot areas. In the Venice Lagoon, located in the northern

Adriatic Sea, all respondents have encountered it, whereas in the Marche region on Italy's central Adriatic coast, only one individual reported frequent sightings.

Overall, attitudes toward the Blue Crab are predominantly negative, with 66% viewing it as a threat to local biodiversity (22% remain neutral, and 11% perceive it as a positive opportunity).

2.4.1. Blue crab monitoring

Current data on the Blue Crab in the Adriatic Sea remain incomplete, with existing studies limited to specific periods and areas. The six pilot sites selected by BlueDiversity have only been marginally examined.

Comprehensive monitoring is essential to understand the crab's population dynamics and spatial-temporal distribution throughout the year, which is crucial for developing effective management and mitigation strategies. Key factors to investigate include the species' preferred habitats, population density, seasonal movements, and differences between sexes and life stages.

For example, varying crab densities and yields have been observed depending on the sampling period. In the Po Delta lagoons, crab densities were highest in July. During the same month, ovigerous (egg-carrying) females peaked at 19.2%, dropping to just under 2% in October—valuable information for informing potential control strategies (Chiesa *et al.* 2025).

To refine a suitable monitoring protocol and move to the test phase, valuable insights can be provided by previous studies and parallel projects.

Regarding capture methods, for example, results of Chiesa *et al*., (2025) shows that large nets like *ostraghero* can provide immediate results in high-density areas but are unsuitable for long-term, comprehensive monitoring. In contrast, Glamuzina's studies demonstrated the effectiveness of wire crab traps (commonly used in America where the crab is native). These traps showed higher selectivity and capture efficiency than fyke nets, while requiring less maintenance. Although fyke nets captured more native species, they needed frequent repairs due to crab damage. Wire traps, on the other hand, remained robust and consistently yielded higher catches.

Marchessaux *et al.,* (2024) took a different approach by leveraging fishers' involvement through a local ecological knowledge (LEK) method, where blue crabs were caught

opportunistically each month. This collaboration between scientists and fishers helped gather consistent data with minimal investment in dedicated equipment, showcasing how active fisher participation can enhance monitoring efforts.

To conclude, the Blue Crab Action Plan project, currently in its early stages, proposes a structured monitoring protocol to assess the blue crab population along the Adriatic coast of Northern Italy. The methodology includes the use of different traps, deployed across key environments such as the Po Delta, Venice and Caorle lagoons, as well as in maritime waters. Sampling will occur year-round, with fishers conducting up to 20 sampling trips per month, while research operators conduct one monthly survey. The protocol involves experimental trials with three types of traps and two bait types, with fishermen sampling weekly and operators twice per month.

BlueDiversity will address blue crab monitoring through a well-structured sampling design that combines seasonal and year-round efforts, involving local fishermen to extend the sampling area, increase coverage, and reduce costs.

A preliminary phase will integrate Local Ecological Knowledge (LEK) through questionnaires for local fishermen, and direct observation using remotely operated underwater vehicles (ROUVs) to assess local characteristics of each area.

Subsequently, monitoring will commence with various nets being tested to determine their suitability and specificity, considering the diverse features of the pilot areas (substrate, crab distribution, seabed vegetation, etc.) to identify the optimal solution. Where possible, collaborations with complementary projects, such as the Blue Crab Action Plan and the Italy-Slovenia Interreg, will be sought to create an integrated framework towards common goals.

The refinement and implementation of the monitoring protocol will be addressed during activity 2.1 of Work Package 2 (WP2).

2.4.2. Sustainable maritime economic development (Blue Economy)

The blue economy offers a transformative vision for coastal regions, leveraging marine resources to drive sustainable economic growth while preserving ecosystems. The integration of the invasive blue crab into an economic and social context could be considered one promising avenue within this framework, particularly in Northern Adriatic Italy and Croatia.

While the blue crab holds substantial commercial value in its native regions of the western Atlantic and Gulf of Mexico, in the Mediterranean and Adriatic blue crab management is costly, as traditional disposal methods are expensive and laborious, with a low yield of usable meat (Arena *et al.* 2024).

Nonetheless, the crab's meat, known for its delicate and sweet flavour, has a high protein content and is packed with essential nutrients, including omega-3 fatty acids, vitamins, and minerals. These nutritional benefits, along with its low-fat content, contribute to improved cardiovascular health, brain function, and overall well-being. The potential applications of crab meat in producing crab-based products have been extensively studied in various crab species, showing promising results, indicating that the same approach can be applied to the blue crab (Nanda *et al.* 2021).

Additionally, innovative uses for crab shell waste are emerging. Its unique 3D nanostructure, maintained even after mechanical or thermal processing, offers potential for applications in circular economy models. These include smart nanocarriers for fertilizers, environmental pollutant removal, biomedical remediation, and even future space industry materials (Nekvapil *et al.* 2019).

The BlueDiversity project aims to turn the ecological issue represented by blue crab into a valuable resource by promoting its commercialization. This approach not only helps control the invasive species but also creates economic opportunities through job creation in harvesting, processing, and sales sectors. Learning from successful initiatives, BlueDiversity will focus on developing value-added strategies tailored to both local and national markets, while proposing regulatory adaptations to enable effective harvesting practices while protecting other marine resources. To this aim in the BlueDiversity project will be established a blue crab commercial plan. By fostering a market for blue crab products and educating consumers on the sustainability of this approach, the project aims to shift perceptions and boost demand.

Furthermore, BlueDiversity envisions a holistic ecosystem approach by connecting local fishermen, restaurants, and sustainable tourism. By promoting responsible seafood sourcing, restaurants can feature invasive species dishes, while eco-tours educate visitors on the ecological benefits of blue crab management. Through this multifaceted strategy, BlueDiversity seeks to build a resilient, localized economy that not only mitigates ecological harm but transforms it into a lasting economic asset.

According to Deliverable 1.2.1, Environmental Service Users (ESUs) in the pilot areas represent a promising target group for implementing the blue crab commercialization approach. This is underscored by the strong interest shown by interviewees, with 71% expressing willingness to try this specific alien species at a local restaurant. Additionally, the feasibility of direct consumer engagement is highlighted by the fact that 53% of respondents would consider purchasing and preparing blue crab at home. These insights suggest a solid foundation for both restaurant-based promotion and direct-to-consumer sales within the pilot areas, supporting a multi-channel strategy to boost market adoption.

Since blue crabs are intended for commercialization as food products, it is crucial to ensure they are not carriers of pollutants that could bioaccumulate from their environment. Studies have shown that blue crabs (*Callinectes sapidus*) can accumulate various contaminants, including heavy metals like cadmium, lead, and mercury (Reichmuth *et al.,* 2010), as well as organic pollutants such as polychlorinated biphenyls (PCBs) (Mothershead, Hale, e Greaves 1991) and per- and polyfluoroalkyl substances (PFAS) (Nobile *et al.* 2024), from their habitats. As Deliverable 1.1.1 highlighted, those contaminants are reported in BlueDiversity pilot areas.

Moreover, the biochemical composition of blue crabs can vary due to several factors, including habitat (cultured versus wild), food sources, seasonal and climatic changes, biological differences (species, size, age, sex, stage of maturity, gametogenesis, and spawning cycle), and environmental factors such as temperature, salinity, and contaminants (Nanda *et al.* 2021).

Therefore, it is essential to assess the presence of pollutants through chemical analyses and conduct precise and comprehensive nutritional evaluations, incorporating insights from existing literature, to ensure the safety and quality of blue crab products intended for human consumption.

Thanks to PP4 expertise a chemical analyses protocol has been designed [Annex 4].

3. AQUATIC ECOSYSTEM POLLUTANTS: WATER AND SEDIMENT ANALYSIS

Water and sediment data collected in Deliverable 1.1.1 confirm the presence of diverse pollutants in the project's pilot areas. However, this information is inconsistently available across different locations and often stems from outdated monitoring, failing to provide a current assessment of the issue. Furthermore, the variety and microscopic nature of these contaminants make them challenging for ESU to perceive, hindering the use of Local Ecological Knowledge (LEK) approaches to gather information about this threat.

As previously stated, pollution could be detrimental for the blue crab commercialization, but it represents also a serious threat for marine biodiversity in general.

Elevated levels of heavy metals in the marine environment could, for example, lead to sub-lethal effects in the marine organism including changes in physiology, biochemistry, behaviour, reproduction and tissue organization. The toxicity of the metals has been associated with blockage of oxidative phosphorylation, glutathione depletion and inhibition of antioxidant enzymatic activity, production of ROS (reactive oxygen species), DNA damage and inhibition of relevant repair mechanisms, and protein misfolding disorders (Shah, 2021).

Pesticides, a class of organic pollutants, were observed having detrimental impacts to biota with photosynthetic symbionts, reducing growth and reproduction rates of invertebrates and vertebrates, disrupting of nerve impulses which can ultimately lead to paralysis and death of invertebrates and several sub-lethal effects on physiological or metabolic endpoints. Organochlorines, organophosphates, and carbamates are three classes of pesticides that are known for their neurotoxic effects on marine organisms, invertebrates, and vertebrates (Vagi, Petsas, and Kostopoulou, 2021).

Biomonitoring is a critical tool for assessing pollution and environmental health in marine ecosystems. Traditionally, this approach involves measuring the effects of contaminants on aquatic organisms—whether native species or those introduced specifically for study—over both short-term (acute) and long-term (chronic) exposures. A classic example is the Mussel Watch Program, initiated in the 1970s, which uses the bioaccumulation of contaminants in mussels to reflect the levels of bioavailable pollutants in the environment. Since many contaminants are present at very low concentrations in water, bioconcentration in organisms

can amplify these signals by several orders of magnitude, providing a more reliable and biologically relevant measure than direct chemical analysis alone (Wang, 2016). Biomonitoring research typically integrates chemical contamination measurements with biomarker analysis. While chemical assessments provide data on pollutant exposure levels, it's the biological responses that truly reveal the actual impact of pollutants on organism health. This combined approach offers a more comprehensive understanding of environmental toxicity.

Transcriptomic biomarkers have recently emerged as a cutting-edge tool in environmental monitoring. This approach analyzes an organism's entire RNA transcript set, providing a highly sensitive early-warning system for environmental stress. By detecting subtle shifts in gene expression, including changes in mRNA and other RNA molecules, transcriptomics can identify stress responses long before traditional physiological or morphological indicators become evident. This makes it a valuable complement to existing biomonitoring methods, offering deeper insights into ecosystem health. As a result, transcriptomic biomarkers not only help to identify early molecular signals of contaminant exposure but also enable researchers to forecast broader impacts on organism functioning and ecosystem health (Lacroix *et al.* 2014).

Together, these approaches provide a powerful, integrated framework for monitoring marine pollution, ensuring that even low-level and early exposures to contaminants are effectively detected and assessed.

3.1. Transcriptomic analysis for environmental biomonitoring

At this stage, we will identify at least one Italian site and one Croatian site where anthropogenic pollution—such as organic pollutants, pesticides, and similar contaminants—emerges from existing data. In cases where no data are available, sites can be selected based on their proximity to potentially impacted areas such as commercial harbours and waters near urban centres, hospitals, or intensive agricultural regions.

When possible, we'll identify a second site, concentrating on industrial pollution, primarily heavy metals. A prime example is Porto Marghera in the Venice Lagoon. In cases where specific data isn't available, we'll consider areas near major industrial activities. This dual-site approach allows for a more comprehensive assessment of both general anthropogenic and specific industrial pollution impacts within each pilot area.

At the selected sites, a transcriptomic analysis will be conducted to assess the impact of these contaminant mixtures on marine biodiversity. This approach will be focused on microRNAs—short non-coding molecules with critical regulatory functions that control the expression of multiple genes simultaneously. By examining shifts in microRNA expression patterns in response to diverse environmental conditions, particularly complex contaminant mixtures, we can gain valuable insights. This analysis will shed light on the mechanisms organisms employ to deal with stressors and reveal how these adaptive responses impact their overall fitness and survival in polluted environments.

The study will primarily focus on the Mediterranean mussel, *Mytilus galloprovincialis*, for several compelling reasons. This species is ubiquitous across the Mediterranean, offering a broad and representative model for ecological studies. As a sessile, suspension-feeding organism, *M. galloprovincialis* is naturally exposed to environmental stressors and must develop coping mechanisms to thrive. Additionally, mussels have been used for decades as "sentinel species" to assess environmental health, especially the impact of chemical contamination in estuarine and coastal ecosystems (Auffret *et al*. 2006; Caquet *et al*. 2013).

Mussels are excellent candidates for marine monitoring programs due to their unique characteristics. Their sessile nature, ability to bioaccumulate significant amounts of toxic compounds, resilience, abundance, and easy accessibility make them ideal bioindicators (Lacroix *et al*. 2014). Furthermore, existing research has already demonstrated the sensitivity of microRNAs in *Mytilus galloprovincialis* to various pollutants, including some found in our pilot areas (Yu *et al*. 2021; Xing *et al*. 2024). This combination of factors makes mussels particularly suitable for our transcriptomic biomonitoring approach. In line with established bioindicator study protocols, we will transplant mussels into our selected sites. These mussels will then be sampled at various predetermined intervals. This approach allows us to track changes in the mussels' biological responses over time, providing a dynamic picture of environmental impacts at each location.

Water samples will be collected and analysed to integrate the Deliverable 1.1.1 and together with chemical analysis performed on sampled mussels, they will give a picture of the actual contamination happening on the selected sites, confirming or not what previous data and local perception sustained.

To collect the total RNA of sampled organisms, a protocol for RNA extraction has been developed thanks to PP7 expertise. [Annex 5]

4. MARINE LITTER: IMPACT ASSESSMENT AND MITIGATION STRATEGIES

Marine litter emerges as a significant concern in Deliverable 1.2.1, with 121 out of 122 interviewees reporting encounters with it. This issue is prevalent across all six BlueDiversity pilot areas. Notably, there's near-unanimous agreement (99%) regarding the negative perception of marine litter and its detrimental effects on local economic activities and ecosystem services. This widespread recognition underscores the urgency of addressing marine litter in these areas.

In the Adriatic region, the situation is particularly alarming. The Adriatic has been identified as a hotspot for plastic accumulation in the Mediterranean Sea, a trend influenced by transboundary effects driven by sea currents. Microplastic pollution in the Adriatic Sea has been demonstrated in all abiotic compartments, including beaches, surface waters, sediments and biota. Despite this fact, data is still limited, and still lacking in the standardized approach for assessing marine litter recently adopted thanks to European Directives, but whose implementation is still ongoing (Soto-Navarro *et al*. 2021).

Previous studies indicate that approximately 40% of marine litter enters the Adriatic basin via rivers, with the Po River alone discharging an estimated 120 tons of litter and 7 x 10^{11} micro litter particles each year, another 40% comes from coastal urban populations, and the remaining 20% originates from shipping and fishing activities (Schmid, Cozzarini, e Zambello 2021).

Marine litter impairs recreational uses and causes a loss of touristic value. Beyond the aesthetic impact, marine litter also bears potential economic implications to maritime activities, such as fisheries and the aquaculture sectors.

The ecological impacts are equally severe, affecting various components of marine ecosystems in several ways:

- **Ingestion by Marine Fauna:** Many marine organisms, including commercially important seafood species, ingest plastic particles. While much of the ingested plastic is excreted, some particles accumulate in the digestive system, causing harm. Species

like seabirds and sea turtles are particularly vulnerable due to their non-selective feeding habits.

- **Chemical Contamination:** Plastics can leach chemical additives into the water, including compounds with suspected endocrine-disrupting effects, thereby contaminating estuarine and marine environments.
- **Entanglement Risks:** Abandoned fishing equipment and various plastic waste pose significant entanglement risks, especially for large marine animals that need to surface for air. This threat is particularly concerning for endangered species, as it can lead to injury, impaired movement, or even death. The persistence of such debris in marine environments creates ongoing hazards for diverse marine life, highlighting the urgent need for effective waste management and conservation efforts.
- **Fragmentation into Microplastics:** Conventional plastics persist for hundreds of years and, due to hydrodynamics and exposure to light, fragment into microplastics and nanoplastics. These smaller particles are readily ingested by even the tiniest zooplankton, potentially introducing hydrophobic pollutants into the trophic web.
- **Spread of Invasive Species:** Floating plastic can transport alien species over long distances or serve as a substrate for colonization by various organisms, further altering marine ecosystems (Galgani *et al*. 2019).

The wide-ranging consequences of marine litter—spanning ecological damage, biodiversity decline, and economic setbacks—emphasize the critical need for action. This global issue demands a coordinated response, including standardized monitoring protocols, innovative management approaches, and strong international collaboration. Only through such comprehensive efforts can we hope to effectively address and mitigate the escalating threat posed by marine litter to our oceans and coastal communities.

4.1. Comprehensive strategies for marine litter reduction and management

BlueDiversity will address marine litter by adopting circular economy principles in small-scale fishing, promoting practices that both protect our environment and favourite local economies. The initiative focuses on two key areas: first, preventing end-of-life fishing nets from becoming waste by creating a viable recycling solution; second, providing environmentally friendly alternatives for the aquaculture sector to eliminate plastic pollution upstream.

4.1.1. Nylon nets recycling

Our strategy for recycling fishing nets will start with identifying effective practices and assessing current gillnet disposal methods to determine the feasibility of recycling dismissed nets in pilot areas. This process involves collecting data on the quantities of nets being discarded and documenting their present destinations at various production points, taking in account local peculiarities. In collaboration with net experts, we will verify the types of materials and identify potential storage sites within the pilot areas or nearby ports. We'll also conduct a comprehensive review of relevant regulations and assess net quantities in key ports. This groundwork will help us determine the feasibility of recycling discarded nets and develop the logistics for an effective recycling program, tailored to each pilot area's unique circumstances.

4.1.2. Plastic-free solutions for aquaculture

In parallel, we are committed to advancing eco-friendly practices in aquaculture and mariculture by evaluating alternatives to traditional plastic tools. This initiative involves a comprehensive assessment of current aquaculture systems in pilot areas, with an emphasis on identifying and testing sustainable materials that can replace conventional plastics. Detailed reports will capture the current usage of plastics in both at-sea production and onshore processing and packaging facilities, while samples of eco-sustainable materials are sent for testing. Key performance indicators will be established to evaluate the environmental impact of these new materials during trial periods. By defining the characteristics of aquaculture plants, such as those cultivating mussels or oysters, and cataloguing both existing plastic materials and innovative alternatives with detailed technical specifications and supplier pricing, we aim to facilitate a smooth transition to greener practices.

In conclusion, by transitioning to biodegradable and recyclable materials for fishing and aquaculture gear, we will create green opportunities that boost local businesses and generate jobs while reducing pollution and marine litter. Sustainable fishing practices, combined with active efforts to remove marine litter, safeguard local water bodies and contribute to a healthier environment for both communities and wildlife. To build resilient coastal regions and thriving local economies, this integrated approach makes sure that environmental care and economic development go hand in hand.

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Italy – Croatia

BlueDiversity

2 - Green and resilient shared environment
SO 2.2 - Protection of nature and biodiversity

BlueDiversity

ITHR0200404

Shared BLUE knowledge and skills to sustain BIODIVERSITY in mariculture

Protocols

Environmental DNA (eDNA) metabarcoding protocol

Partner - First Investigator
LP1 – Maurizio Pinna

Field of Interest
Ecology, Genetics

Work Package and activity
WP1 - activity 1.4

Brief Description
This protocol is a comprehensive description of the main steps for the application of eDNA metabarcoding. It represents a useful guideline for experimental design, fieldwork, and laboratory procedures.

2.1. Study area definition

The first step for applying environmental DNA (eDNA) metabarcoding protocols is to define the study area and the sampling stations.
The study area should be defined based on a Geographic Information System (GIS) software producing shapefiles, geopackages or maps identifying the borders of the sampling area, the reference coordinates, and the localisation and labelling of the sampling stations.

2.2. Water sampling

A volume of water must be collected in three replicates for each sampling station. The volume collected depends on the specific area under study. For more confined and shallower water bodies (e.g., coastal lagoons), 1 L of water per replica from each sampling

station is usually sufficient, while for wider and deeper water bodies (e.g., marine areas) larger volumes are needed.
Each water sample must be labelled with the study area, the sampling station, the replica, and the date. Immediately after sampling and labelling, all samples should be stored in refrigerated containers. After sampling, all water samples should be transported to the laboratory and immediately filtered.
In addition to the water sampling, at least the most common abiotic parameters (Salinity, Temperature, pH, and Dissolved Oxygen) should be recorded in each sampling station using a multiparametric probe.

2.3. Water filtration and DNA extraction

Each water sample (or replica) must be singularly filtered by using a filtration system with a vacuum pump

For the water filtration process, each sample must be filtered through a Mixed Cellulose Ester filter with a pore size of 0.45 µm. After filtration, the filters can be stored in labelled containers at a temperature of -20°C until further processing.

Subsequently, each filter should be individually processed for DNA extraction using a kit specifically designed for water samples (such as the QIAGEN DNeasy PowerWater kit), adhering to the manufacturer's protocol. A protocol example is described in the Figure1.

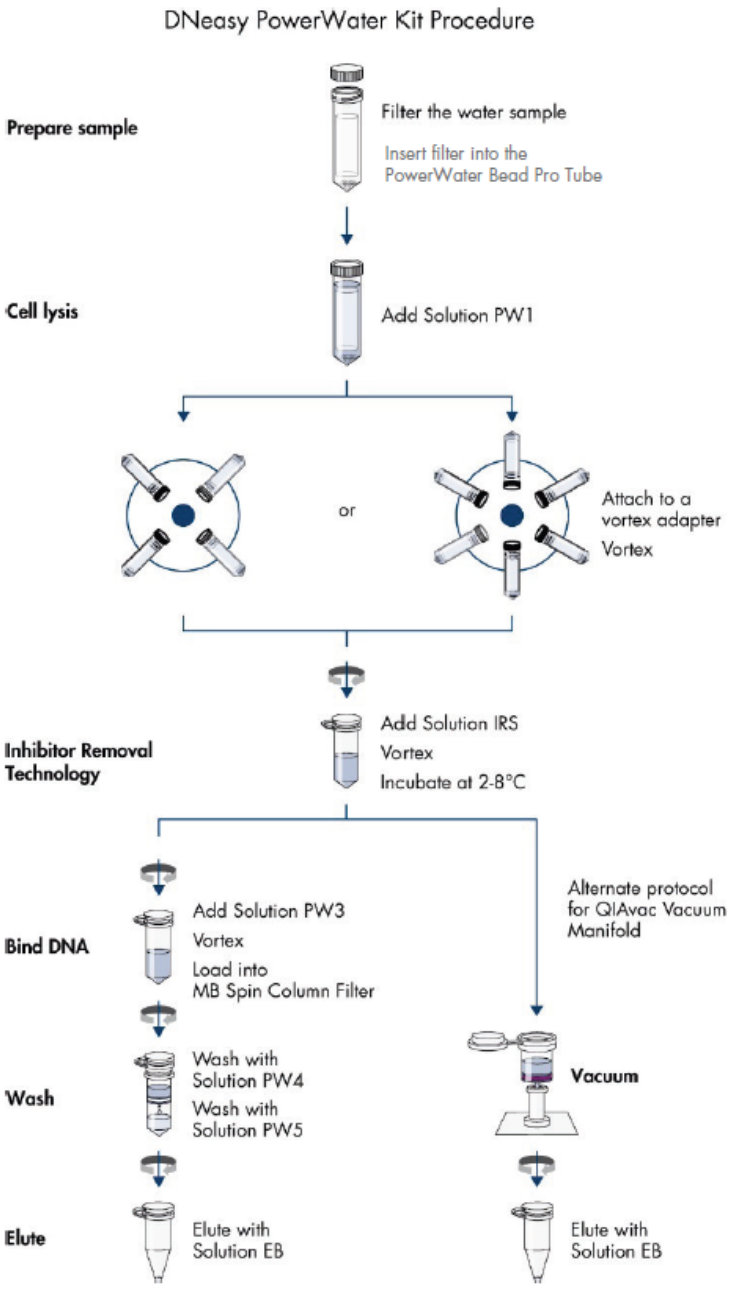

Figure1. Graphical description of Qiagen DNeasy Water Kit protocol

The extracted DNA must be quantified (e.g., through a fluorometer), labelled and immediately stored at a temperature of -20°C until further processing.

2.4. Amplification of marker genes and sequencing

Currently, various evolutionarily conserved marker genes, which differ among species, are utilized for eDNA metabarcoding applications. The most used are:

- *COI*: mitochondrial cytochrome c oxidase subunit I gene, used for identifying animals;
- *16S*: 16S ribosomal RNA gene, used for identifying prokaryotes;
- *18S*: 18S ribosomal RNA gene, used for identifying eukaryotes, mainly plants and phytoplankton;
- *12S*: 12S ribosomal RNA gene, used for identifying eukaryotes, mainly fishes;
- *ITS1* and 2: internal transcribed spacer between SSU and LSU rRNA genes, used for fungi;
- *matK*: maturase-coding gene, used for identifying plants;
- *rbcL*: large subunit of ribulose 1,5-bisphosphate carboxylase gene, used for plants.

Initially, it's crucial to select the appropriate marker gene for investigating the targeted organisms. After identifying the marker gene, design degenerate primer pairs that target a 300 to 500 base pair region of this gene for amplification via Polymerase Chain Reaction (PCR).

For each eDNA sample, conduct the PCR reaction independently in a 50 µL volume with the following composition:

- 5 µL 10X reaction buffer
- 1 µL $MgCl_2$ (50 mM)

- 1 µL dNTP mix (10 mM)
- 1 µL forward primer (10 mM)
- 1 µL reverse primer (10 mM)
- 10 ng of the DNA sample
- 0.2 µL Taq DNA polymerase (5 U/µL)
- ultrapure water to reach a volume of 50 µL

The PCR amplification program should include the following steps:

- denaturation at 95°C for 5 minutes, followed by 30 cycles:
- denaturation (95°C for 30 seconds)
- annealing (30 seconds, the temperature depends on the selected primer pair)
- extension (72°C for 1 minute)

The amplicons must then be quantified, labelled and stored at -20°C until further processing. The amplicons can then be sequenced according to the specific requirements of the available sequencing instrument.

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Shared BLUE knowledge and skills to sustain BIODIVERSITY in mariculture

Protocols

Genomic DNA extraction from plant tissues

Partner - First Investigator
PP7 - De Pittà Cristiano

Field of Interest
Molecular Biology

Work Package and activity
WP1 - activity 1.4

Brief Description
This protocol is suitable for the rapid extraction of genomic DNA from plant tissues, especially those rich in polysaccharides and polyphenols. It relies on the FastPure Plant DNA Isolation Mini Kit of Vazyme, based on silica gel membrane purification.

1. Starting material

- FastPure Plant DNA Isolation Mini Kit (Vazyme)
  - FastPure gDNA Columns IV
  - Collection Tubes (2 ml)
  - RNase A
  - Buffer A1
  - Buffer A2
  - Buffer A3
  - Buffer AW
  - Elution Buffer

- TissueLyser II (Qiagen)
- Micro-centrifuge with rotor for 2 ml tubes
- Sterilized 1.5 ml centrifuge tubes
- Water bath or Thermoblock
- Absolute Ethanol
- Liquid nitrogen
- Micropipettes

2. Step by step process description

- Add ≤ 100 mg of fresh or ≤ 20 mg of dried tissue in a 1.5 ml collection tube with a clean metal bead
- Homogenize using TissueLyser II: 30s at 35 Hz for two times (between the first and the second time samples are passed in liquid nitrogen). Repeat if necessary.
    - It is also possible to grind the tissue into powder in liquid nitrogen with mortar and pestle.
- Add 400 µl of Buffer A1 and 4 µl to the powder, vortex and shake
- Incubate the lysate at 65°C for 10 min, inverting the tube 2 - 3 times to facilitate lysis
- Add 130 µl Buffer A2 into the above mixture and mix well. Incubate on ice for 5 min, and centrifuge at 14,000 rpm (18,400 x g) for 5 - 10 min. Transfer the supernatant to a new 1.5 ml Rnase-free centrifuge tube. Be careful not to absorb interfacial material.
- Calculate the volume of supernatant. Add Buffer A3 (1.5 × the volumes of supernatant) to the supernatant (check whether absolute ethanol has been added

before use) and immediately mix well by pipetting up and down, such as add 750 µl Buffer A3 to 500 µl supernatant.

- Transfer the above mixture to a FastPure gDNA Columns IV (already fitted in a Collection Tube). Centrifuge at 12,000 rpm (13,400 × g) for 30 - 60 sec, and discard the filtrate. (If the volume of the mixture exceeds 700 µl, centrifuge successive aliquots in the same FastPure gDNA Columns IV. Discard the filtrate after each centrifugation.)
- Add 600 µl Buffer AW (check whether absolute ethanol has been added before use).
- Centrifuge at 12,000 rpm (13,400 × g) for 30 sec, and discard the filtrate.
- Repeat the step above
- Place the FastPure gDNA Columns IV back into Collection Tube. Centrifuge at 12,000 rpm (13,400 × g) for 2 min to remove the residual Buffer AW from the FastPure gDNA Columns IV. After centrifuging the empty column, air dry the column for 2 - 5 min for the residual ethanol to fully evaporate.
- Transfer the FastPure gDNA Columns IV into a new 1.5 ml centrifuge tube, and add 50 - 100 µl of Elution Buffer (preheated to 65 ~ 70℃) to the centre of the membrane without touching the column. Incubate at room temperature for 3 - 5 min and centrifuge at 12,000 rpm (13,400 × g) for 1 min. (You can repeat the Elution step with a new Elution Buffer, but it may increase the yield but decrease the concentration. For the highest yield, it is recommended to add the first eluent back into the FastPure gDNA Columns IV and repeat the elution step).
    - It is also possible to use Nucleases free Water instead of the Eluition Buffer.
- Discard the FastPure gDNA Columns IV. The extracted DNA can be used directly for downstream experiments or stored at -20℃.

3. Additional information

- Unless explicitly specified, the step should be performed at room temperature.

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Protocols

Genomic DNA extraction from animal tissues

Partner - First Investigator
PP7 - De Pittà Cristiano

Field of Interest
Molecular Biology

Work Package and activity
WP1 - activity 1.4

Brief Description
This protocol is suitable for the rapid extraction of genomic DNA from animal tissues. It relies on the DNAeasy Blood and Tissue Kit of Qiagen, based on silica gel membrane purification.

1. Starting material

- DNAeasy Blood and Tissue Kit (Qiagen)
  - DNeasy Mini Spin Columns (colorless) in 2 ml Collection Tubes
  - Collection Tubes (2 ml)
  - Buffer ATL
  - Buffer AL
  - Buffer AE
  - Buffer AW1
  - Buffer AW2
  - Proteinase K

- Liquid nitrogen
- Scalpels
- Micro-centrifuge with rotor for 1.5 ml or 2 ml tubes
- Vortex
- Sterilized 1.5 ml and 2 ml microcentrifuge tubes
- Water bath (or thermoblock)
- Absolute Ethanol (96-100%)
- Micropipettes

2. Step by step process description

- Preheat a water bath to 56°C, if it is the first use add the appropriate amount of ethanol to Buffer ATL and AL
- Cut up to 25 mg tissue into small pieces, and place in a 1.5 ml micro-centrifuge tube
- Add 180 µl Buffer ATL
- Add 20 µl Proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample
- Vortex for 15 s. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing. (Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples)

- Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥ 6000xg (8000 rpm) for 1 min. Discard flow-through and collection tube
- Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥ 6000xg (8000 rpm). Discard flow-through and collection tube
- Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000xg (14,000 rpm) to dry the Dneasy membrane. Discard flow-through and collection tube
- Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute (Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube)
- Repeat the step above to increase overall DNA yield

3. Additional information

- Best results are obtained with fresh material or material that has been immediately frozen and stored between -90°C and -15°C. Repeated freezing and thawing of stored samples should be avoided, since this leads to reduced DNA size. Use of poor-quality starting material will also lead to reduced length and yield of purified DNA
- To obtain optimum DNA yield and quality, it is important not to overload the DNeasy spin column, as this can lead to significantly lower yields than expected
- All centrifugation steps are carried out at room temperature (15-25°C)
- Vortexing should be performed by pulse-vortexing for 5-10 s.

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Protocols

Chemical analysis blue crab protocol

Partner - First Investigator
PP4 – Jelena Lušić

Field of Interest
Ecotoxicology

Work Package and activity
WP2 - activity 2.3

Brief Description
This protocol is a comprehensive description of the main steps for chemical analysis of contaminants in crabs. It represents a useful guideline covering heavy metal analysis, organic contaminants analysis (both PCBs and PAHs) and marine biotoxins analysis.

1. Sample preparation procedure

- composite sample – 5 crab individuals (or more, depending on tissue weight)
- removal of soft tissue from the shell with stainless-steel scalpel/scissors (required total soft tissue weight is approximately 200 g)
- homogenization of soft tissue in a laboratory blender (approximately 3 min, 22.000 rpm)

Prepared homogenized tissue (min. 200 g) is divided into two parts:

- tissue intended for the analysis of organic contaminants should be placed in aluminum containers or aluminum foil– required soft tissue weight: 170 g
- tissue intended for trace metal analysis and biotoxin analysis should be placed in plastic containers or plastic bags – required soft tissue weight: 30 g

Prepared tissue samples should be stored in a freezer and shipped with dry ice

It is important to avoid possible contamination – stainless steel tools and protective nitrile gloves should be used for sample handling. Contact of tissue samples with unclean surfaces should be avoided – plastic foil can be used to cover the surfaces in contact with the sample.

2. Trace Metal Analysis

Approximately 0.2 g of freeze-dried sample is weighed directly into the PTFE-TFM digestion vessels. To each sample, 5.0 mL of concentrated nitric acid and 2.0 mL of hydrogen peroxide are added. The vessels are sealed and placed into the microwave digestion system. After the digestion process, the digestate liquids are transferred to the 50.0 mL polypropylene vials and MQ water is added to a final volume of 20.0 mL.
Analyses of trace metal concentrations in digested samples are performed on an atomic absorption spectrometer, using graphite furnace or flame atomic absorption techniques.

3. Analysis of organic contaminants (PCBs)

Extraction of PCBs from homogenized, freeze-dried tissue samples (3-8 g) is carried out with hexane/acetone mixture in a microwave oven. After extraction, the extracts are concentrated on a rotary evaporator to a volume of approximately 15 ml. The extract is dried with anhydrous sodium sulphate and concentrated to exactly 1 ml by evaporating excess solvent under a gentle stream of clean dry nitrogen.
Solid Phase Extraction glass columns (Upti-Clean SPE Glass Columns-Na2SO4/Florisil (2g/2g) PTFE Frits) are used for the fractionation procedure. The first fraction containing PCBs, pp'DDE, pp'DDT and HCB is obtained by elution with 10 ml of n-Hexane. The second fraction containing pp'DDD and Lindane is obtained by eluting 9 ml of a n-Hexane:Dichloromethane (9:1) solution. The aliquot used for fraction 3 is passed through a Silica SPE column. Mixture of hexane:dichloromethane mixture (50:50) is used to collect a fraction which is passed through a Na2SO4/Florisil SPE column (2g/2g, 6 ml). From this column, three fractionation steps are developed: a first fraction with 10 ml of hexane; a second fraction with 9 ml of hexane/dichloromethane (90:10); and then a third fraction with 7 ml of dichloromethane with the compounds of interest (mainly dieldrin, endrin and endosulfan components).
Analysis of PCBs in prepared extracts is carried out by GC-MS technique.

4. Analysis of organic contaminants (PAHs)

Approximately 5 g of homogenized fresh tissue sample is weighed into a 50 mL centrifuge tube. 5 mL of H2O and 10 mL of ACN are added to samples which are placed on a shaker for 1 min. AOAC QuEChERS salts (6 g MgSO4, 1.5 g sodium acetate) are added before centrifugation at 4.000 rpm (5 min). 5 mL of supernatant is transferred to a 15 mL dSPE tube (1200 mg MgSO4, 400 mg PSA) and placed on a shaker for 5 min prior to a centrifugation step (4.000 rpm, 5 min).
Analysis of PCBs in prepared extracts is carried out by UHPLC-FLD technique.

5. Analysis of marine biotoxins

Extraction of ASP and lipophilic toxins from homogenized tissue samples (approximately 2 g) is carried out with methanol solution.
Hydrolysis step is applied to transfer esters into toxins prior to their quantification. Hydrolysis is carried out by adding NaOH to methanol extracts, followed by homogenization by vortexing and heating in a water bath (76°C, 40 min).
Prepared methanol extracts are passed through a 0,45 µm or 0,2 µm filter. Lipophilic toxins in prepared extracts are analyzed by LC-MS/MS technique.

Extraction of hydrophilic toxins from homogenized tissue samples is carried out with Solid Phase Extraction (Bond Elut COOH (CBA) SPE, 500 mg/3 mL). First fraction (C1,2 and C3,4 toxins) is obtainedby elution with MQ water. Second fraction (GTX1,4, GTX2,3, GTX5, GTX6 and dcGTX2,3 toxins) is obtained by elution with 0,05 M NaCl solution. Third fraction (STX, NEO, dcSTX, dcNEO) is obtained by elution with 0,3 M NaCl solution.
Hydrolysis step is applied to transfer C3,4 u GTX1,4 and GTX6 toxins to NEO. Hydrolysis is carried out by adding 1 M HCl to purified extracts to SPE-COOH purified extracts, followed by incubation at 90 °C in a water bath (20 min). 1M NaOH is added to the prepared extracts, followed by filtration through a 0,45 µm filter. Hydrolyzed extracts are oxidized by the application of periodate-based oxidation process.

Hydrophilic toxins in prepared extracts are analyzed by UHPLC technique.

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Protocols

Trizol total RNA extraction from fresh tissue

Partner - First Investigator
PP7 - De Pittà Cristiano

Field of Interest
Molecular Biology

Work Package and activity
WP1 - activity 1.4

Brief Description
This protocol is for purifying total RNA, including small RNAs, from animal and plant tissue. Qualitative and quantitative assays of the extracted material are successively performed.

1. Starting material

- miRNeasy Mini Kit (Qiagen)
  - Collection Tubes (1.5 ml)
  - Collection Tubes (2 ml)
  - QIAzol® Lysis Reagent (Trizol)
  - RNeasy® Mini Spin Columns (each packaged with a 2 ml Collection Tube)
  - Buffer RWT
  - Buffer RPE
- RNase-Free water
- TissueLyser II (Qiagen)

- Microcentrifuge with rotor for 1.5- and 2-ml tubes for centrifugation at 4°C and at room temperature (RT)
- RNA later or liquid nitrogen
- Chloroform
- RNase-free Ethanol (96-100%)
- Micropipettes and their respective filter tips

2. Step by step process description

- Remove the tissue from RNA later, remove the excess of RNA later with paper and place 20 to 50 mg of it in a RNase-free collection tube previously prepared with a clean metal bead* and 1 ml of cold Trizol (kept in ice)

*These beads must be previously autoclaved and, just before starting the protocol, washed in 1 M NaOH solution. After the passage in NaOH solution, the beads must be cleaned in RNase-free water for three times and then transferred in the RNase-free disruption tube and pre-treated with RnaseZap® or washed with the same methodology used for the beads).

- Homogenize using TissueLyser II for 30 s at 40 Hz. If needed repeat till complete homogenization avoiding the overheating of the sample (if the tissue is conserved in liquid nitrogen place it dry in the tube with the bead and homogenize, only after add 1 ml of cold Trizol and let it rest for 5 minutes at RT). If a TissuLyser is not available, it is possible to grind the tissue in liquid nitrogen with mortar and pestle (autoclaved)
- Let the lysate rest at RT for 5 min
- Add 200 µl of cold chloroform to the supernatant, shake vigorously and let it rest for 3 minutes RT

- Centrifugate 15 minutes at 16.000xg at 4°C. Carefully transfer the aqueous phase in a new RNase-free tube
- Add 1.5 volumes of RNase-free ethanol 100% to the tube with the aqueous phase and mix with the micropipette (do not worry if precipitates are present)
- Transfer up to 700µl of the sample to an RNeasy Mini column placed in a 2ml collection tube. Close the lid and centrifuge for 15 s at ≥ 8.000xg, RT. Discard the flow-through and repeat if necessary
- Pipet 700 µl Buffer RWT to the RNeasy Mini spin column. Close the lid and centrifuge for 15 s at ≥ 8.000xg, RT. Discard the flow-through.
- Pipet 500 µl Buffer RPE onto the RNeasy Mini spin column. Close the lid and centrifuge for 15 s at ≥ 8.000xg, RT. Discard the flow-through
- Add 500 µl Buffer RPE onto the RNeasy Mini spin column. Close the lid and centrifuge for 2 min at ≥ 8.000xg, RT. Discard the flow-through and the collection tube
- Move the RNeasy Mini spin column in a new 2 ml collection tube and centrifugate at max speed for 1 minute, RT
- Transfer the RNeasy Mini spin column to a new 1.5 ml RNase-free collection tube. Pipet 30–50 µl RNase-free water directly onto the RNeasy Mini spin column membrane. Close the lid gently and centrifuge for 1 min at ≥ 8.000xg, RT to elute the RNA.

3. Additional information

- Except for phase separation, all protocol and centrifugation steps should be performed at room temperature. During the procedure, work quickly
- The procedures for harvesting and RNA protection should be carried out as quickly as possible. Frozen samples should not be allowed to thaw during handling or weighing.

After disruption and homogenization in Trizol, samples can be stored between -90°C and -65°C for months