CoCoNet Project

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CoCoNet

Towards COast to Coast NETworks of marine protected areas (from the shore to the high and deep sea), coupled with sea-based wind energy potential.

"Workplan (report) regarding the Black Sea pilot sites analysis for connectivity"

WP10 D10.1

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1. BACKGROUND

Marine Protected Area (MPA) networks provide more protection than a set of individual, unconnected protected areas. One of the main questions is how to obtain useful measures of connectivity that can be combined to improve the design and the management of MPA networks. In the Black Sea pilot study, interdisciplinary sampling will be conducted to obtain measures of connectivity based on oceanography (modelling of marine currents), propagule exchange between pilot sites (analysis of planktonic larval stages and other types of propagules transported by currents) and analysis of possible evidence for genetic connectivity between the populations of certain species living in the pilot sites.

Connectivity can be broadly defined as the exchange of materials (e.g., nutrients and pollutants), organisms, and genes and can be divided into:

1) Oceanographic connectivity, which includes flow of materials and circulation patterns and variability that underpin much of all these exchanges;

2) Demographic connectivity, which is the exchange of individuals among local groups (e.g. Black Sea plankton sampling looking for propagules)

3) Genetic or evolutionary connectivity that concerns the exchange of genes within metapopulations of certain organisms,

Presently, we understand little about connectivity at specific locations beyond model outputs, and yet MPAs have to be managed with connectivity in mind.

Connectivity refers to the idea that different areas are linked together by the movement of something between them. Marine connectivity can take the form of:

- migration of animals between habitat patches
- dispersal of larvae from spawning locations to “downstream” habitats, and
- flow of nutrients, sediments, and toxins from a watershed to an estuary or within the marine environment.

If different areas are connected, then management decisions for one area may affect other areas as well and these impacts need to be considered in decision making. Most predictions of connectivity rely on predictions of water circulation from hydrodynamic models or altimetry data to predict how currents will transport larvae, nutrients, sediments, etc. And if what is being transported “behaves”, e.g. sinks or actively swims, this behavior needs to be accounted for as well. (http://www.ebmtoolsdatabase.org/resource/marine-connectivity-tools).

Biological inputs are critical determinants of dispersal outcomes There is considerable temporal and spatial variation of connectivity patterns. New models are increasingly being developed, but these must be validated to understand upstream-downstream neighbourhoods, dispersal corridors, and source/sink dynamics. Genetic connectivity studies reveal divergent population genetic structures despite similar larval life histories. Historical stochasticity in reproduction and/or recruitment likely has important, long lasting consequences on present day genetic structure. (Grober-Dunsmore, R., and B.D. Keller, eds. 2008).

In general, for the Black Sea oceanographic and biological connectivity is poorly understood. Also, less is known about the shape of the dispersal curves for most species, which are highly variable in space and time. Furthermore, the implications of different dispersal kernels on population dynamics and management of species is unknown. Linking patterns of dispersal to management options is difficult given the present state of knowledge.

The activities planned within CoCoNET will help enhance the knowledge of connectivity in the Black Sea.

2. OCEANOGRAPHIC CONNECTIVITY

2.1 MODEL OUTLINE

The CoCoNET project is not focused on a particular species. Rather questions are about the entire ecosystem and its diversity; how can we best protect the ecosystem by MPAs? To answer such questions we need to create models of the ecosystem that represent available knowledge.

Here we immediately hit the first wall: when considering the life cycle of different species of flora and fauna, diversity seems to be overwhelming, making it challenging to formulate a general community model of the ecosystem. However, rather than getting lost in interspecies complexity, it is constructive to focus on what is common between species and what aspects the life cycle are most important for its proliferation and - for the moment - disregard other details. Such an approach will then focus on key life history traits rather than on species-specific aspects.

A major organizational axis (trait) in ecology is size - most ecological processes are more or less size controlled, e.g. predation, motion and physiological requirements. Therefore a very good null hypothesis for the ecosystem is that all processes are size-based and construct an ecosystem model quantifying the ecosystem dynamics in terms of this trait. This trait-based modelling approach provides useful information about size-based ecosystems and is a tool to dismantle the influence of specific life history attributes before the influence of additional details of each species life history are considered.

Such a model may describe the order of magnitude of biofluxes as well as time and space scales in the ecosystem, without addressing particular species apart from their size. This information is very useful to extract the overall response of the ecosystem on changes, e.g. increased eutrophication and/or harvesting. The alternative approach would be to build a set of detailed models of representative species (single species models) increasing the complexity of the model formulation. However this approach will probably fail in capturing meaningful ecosystem dynamics since the unbalance between model complexity and the (likely) poor description of the many interspecies interactions.

Another common relevant trait in marine ecosystems is the length of the pelagic phase of early life stages. Ocean circulation can controls the dispersion of propagule and largely determine the settling sites starting from their spawning/seeding location. The pelagic phase is therefore often considered to be the most important factor determining patterns of connectivity between regional habitats where a species distributed. Since water currents can be modelled quite realistically by operational oceanographic circulation models applied in many regional seas today, this aspect of marine ecology can be addressed with relatively few biological assumptions.

To answer CoCoNET questions on MPA design and habitat connectivity on an ecosystem scale, DTU Aqua developed and will apply a spatial ecosystem model resting on two main pillars:

1) A **size-based representation of the ecosystem**, based on an adaptation of the Zhang-Hartvig (ZH) model to a marine ecosystem, emphasizing explicit representation of key processes without loosing the appealing simplicity of the Zhang-Hartvig model.

2) **Pelagic transport** of early life stages determined from water currents obtained from operational oceanographic circulation models. In the COCONET project this part for the Black Sea is developed in collaboration with IMS-METU, operating the combined circulation and biogeochemical model BIMS. Fractions of the ecosystem (like sea grass) that do not apply to pelagic dispersal proliferate by local dynamics and local dispersal, and this aspect is seamlessly included in the model formulation below

We will generalize the Zhang-Hartvig model to a 2D community model describing trophic fluxes using the two main life history traits described above. We will formulate the model discretely in space (but without reference to a particular representation), since the model will be discretized anyway at the point where it is implemented. The basic state variable of the model is the number of individuals *Ni* of species *α* in spatial cell *i*. There are no special assumptions about spatial cell layout, but it must be associates with a volume and horizontal scale. All species are characterized by a constant terminal mass m_α. The resource corresponds to $\alpha = 0$.

Using Greek indices for parameters related to species and Latin indices to space, the prognostic equations for the community dynamics are as follows:

$$\frac{dN_{\alpha i}}{dt} = (R_{\alpha i} - S_{\alpha i}) - P_{\alpha i} + M_{\alpha i} - (F_{\alpha i} + Z0)N_{\alpha i} \qquad \alpha > 0 \qquad (1)$$

$$\frac{dN0i}{dt} = \lambda i N0i(1 - N_{0i}/C_i) - P0i + M0i \qquad (2)$$

As in the ZH model we have a logistic resource at the bottom of the food chain $\alpha = 0$, described by growth rate λi and carrying capacity $C_{\alpha i}$. Recruitment ($R_{\alpha i}$) and starvation ($S_{\alpha i}$) are based on an energy budget model. $P_{\alpha i}$ is the predation, $M_{\alpha i}$ is the migration, $F_{\alpha i}$ is the harvest pressure and Z_0 is a background mortality experienced locally.

The free energy $E_{\alpha i}$ available to growth and reproduction for each species α in a cell i is given as:

$$E_{\alpha i} = N_{\alpha i}(\varepsilon g_{\alpha i} - r_{\alpha i}) \qquad (3)$$

where $g_{\alpha i}$ is the grazing and ε the trophic efficiency and $r_{\alpha i}$ the respiratory costs. Pelagic transports of early life stages enter $R_{\alpha i}$ which are obtained by a nonlocal hydrodynamic transport kernel calculated from water currents.

Scientific questions we want to address

• What are the scales in both time and space at which MPA networks can have an impact? How are these scales related to physical and biological processes?

• Can we devise some general MPA and MPA network design principles, based on spatial connectivity induced by hydrodynamic transport and migration?

• What are the MPA impacts on biodiversity and community structure?

• How much can simpler conceptual models capture results and patterns of a coupled physical and community model?

Starting from a simplified 1D models with biomass described by a single logistic equation we will develop the fully coupled traits and space modeling approach to address MPA networks effects on the marine ecosystem of the Black Sea.

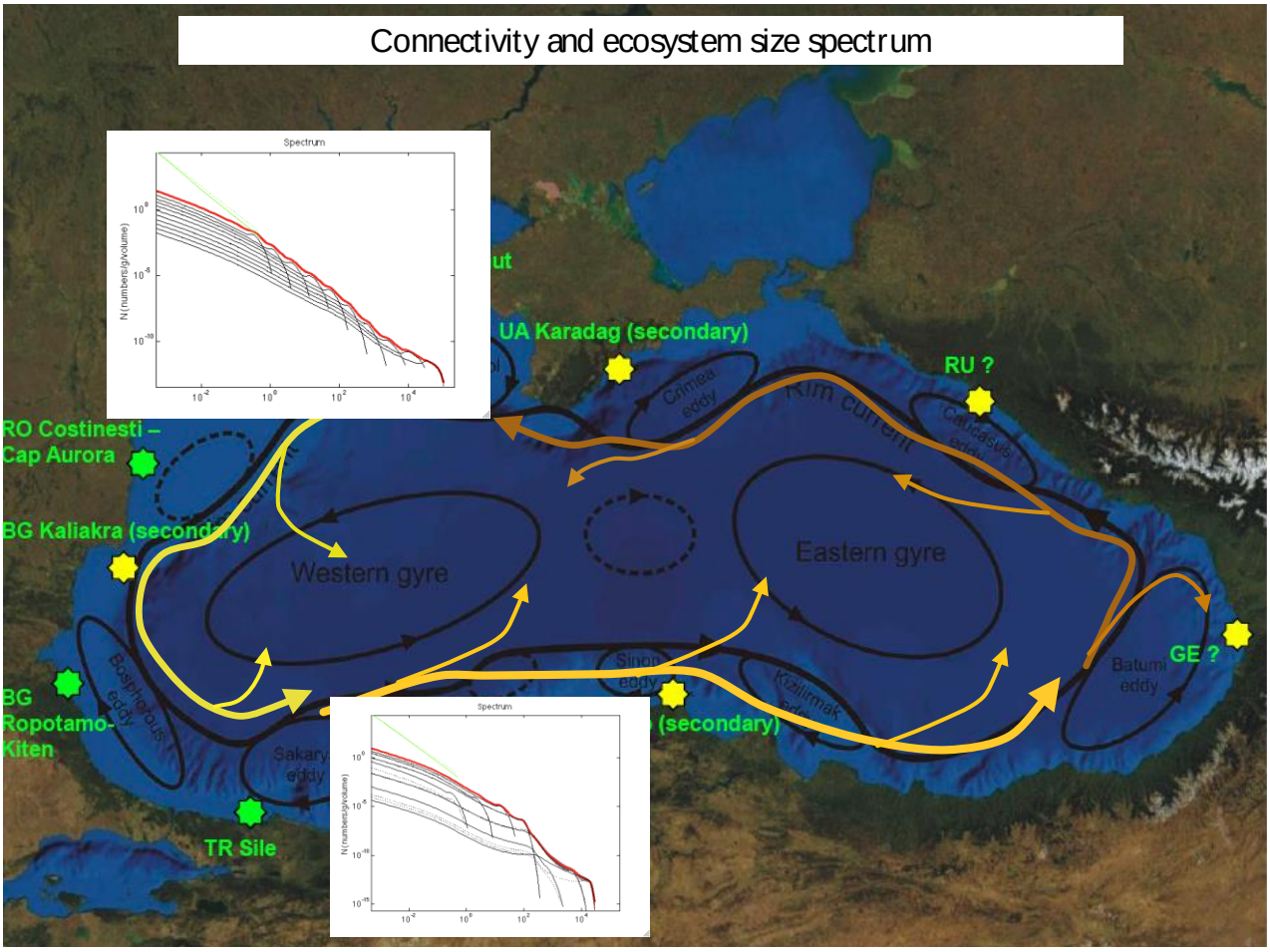


Figure 1.1 Schematic representation of the transport of organism during their pelagic phase (yellow lines) and community interaction (Size spectrum panels) in the Black Sea

2.2 LAGRANGIAN TRANSPORT MODEL

To simulate the transport (e.g. pollutions, nutrients, larva or any other tracers), USOF will use the simulated velocity fields from the available USOF Black Sea basin scale 3D circulation models (NEMO, GFFDL, GETM). The models are forced using 6-hourly atmospheric analyses data from the European Center for Medium-Range Weather Forecasts (ECMWF). Realistic river run-off data for the Black Sea is also implemented. Additionally, a very high resolution grid coastal models will be nested into the basin scale Black Sea model. The latter will use the outputs provided from the Black Sea basin scale model. The Black Se circulation output (from daily velocity fields) will be then coupled offline with a Lagrangian particle tracking algorithm based on “TRACMASS” model but adapted for the CoCoNet Black Sea requirements.

TRACMASS (acronym for Tracing the Water Masses of the North Atlantic and the Mediterranean) is a model using a Lagrangian trajectory method to investigate the North Atlantic and Mediterranean / Black Sea water masses, as they result from numerical simulations. The simulated with this code Lagrangian trajectories (Döös,1995; Blanke and Raynaud1997; Vries and Döös, 2001 ; Döös and Engqvist, 2007) enable quantification of water mass pathways. TRACMASS has recently been applied to the

Black and Baltic Seas with the aim to reveal the characteristics of the haline conveyor belt (Döös et al., 2004, Lu et al,2012).

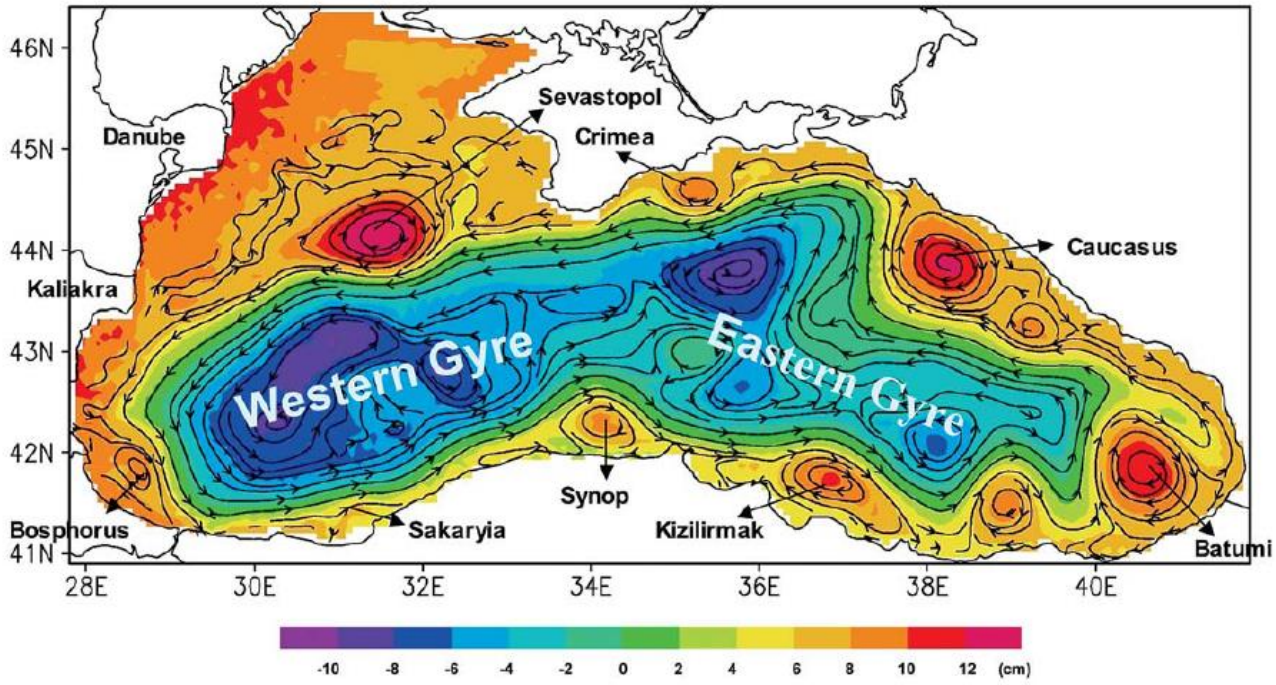


Figure 1.2: Black Sea model circulation produced by the basin-scale 3-D circulation model

A major motivation to apply the TRACMASS model in the CoCoNet connectivity study is that it enables quantifying the relative contribution of water from different sub-areas for the composition of water masses in the Black Sea proper. Velocities in the TRACMASS are defined on a C-grid, therefore the current velocities of the circulation model can be used directly. Vertical velocity is computed from the continuity equation. 5008 particles will be released and TRACMASS model will run 30 days with time lag one day

Studying the oceanographic connectivity for the Black Sea within the CoCoNET project is a challenging task. Models include various forcing mechanisms, on different scales such as winds, sea level change, runoff from rivers, and remote influence from offshore currents and eddies. Hydrodynamics and biological activities are also influenced by complex small-scale topography, and the sparsely available, long-term local observations in the region are usually insufficient for detailed model validations.

The other question is, what drives the currents in the Black Sea and how can these currents, sea level changes and waves be simulated? Recent observations and model studies suggest that variations in the Black Sea currents and the small scale eddies play a major role in this regional flow variability. Modelling the impact of eddies on the flow and biological connectivity is challenging First, small-scale topographic features of significance to biological activities are much smaller (~10-100 m) than most high-resolution hydrodynamic models (several km grid size). Second, since the eddies are not predictable, even high-resolution ocean models with realistic wind forcing can only describe the statistical characteristics of the flow, but not the conditions at a particular location and time. Therefore we intend to use nested grid high resolution coastal models

for the Black Sea. Another approach will use a terrain-following model with trajectories of modelled tracers released (e.g. GETM).

Very important are the new efforts that we will do within the CoCoNET, namely coupling physical models with biological models to study biophysical aspects of connectivity

Tasks and questions to be answered:

- Impacts of sea level rise and storms
- The time of arrival of individual particles in each polygon will be recorded
- Potential changes in riverine hydrology to the coastal area
- Time series will be used to describe the temporal patterns and levels of settlement for each region
- Exchange between sub-regions will be studied
- What is the degree or extent of connectivity, if it exists, between populations in the different Black Sea regions

2.3 CURRENT MEASUREMENTS

ADCP current measurements will be performed in or near pilot sites by some of the Black Sea partners.

In Romania a permanent mooring station for ADCP current and wave measurements, belonging to INCDM, is being built on the Gloria oil platform. It will become operational and start delivering continuous datasets in April 2013 (Fig. 1.3).

In the southern Black Sea, continuous ADCP current measurements and 20 CTD stations will be performed during the cruise of the R/V Bilim-2 to the Sile pilot site in Turkey. The cruise will take place in mid-June 2013 for a total of 2 days of sampling.

Some ADCP current measurements may also be made during the joint (GEOECOMAR, IBSS, OBIBSS, INCDM, IU, CoNISMa, CNR-ISMAR) research cruise of R/V Mare Nigrum to Zernov's Phyllophora Field on the north-western shelf of the Black Sea. However, since the research plan of this cruise was not yet ready at the time of this report, there is a degree of uncertainty related to current measurements that will be performed during the cruise.

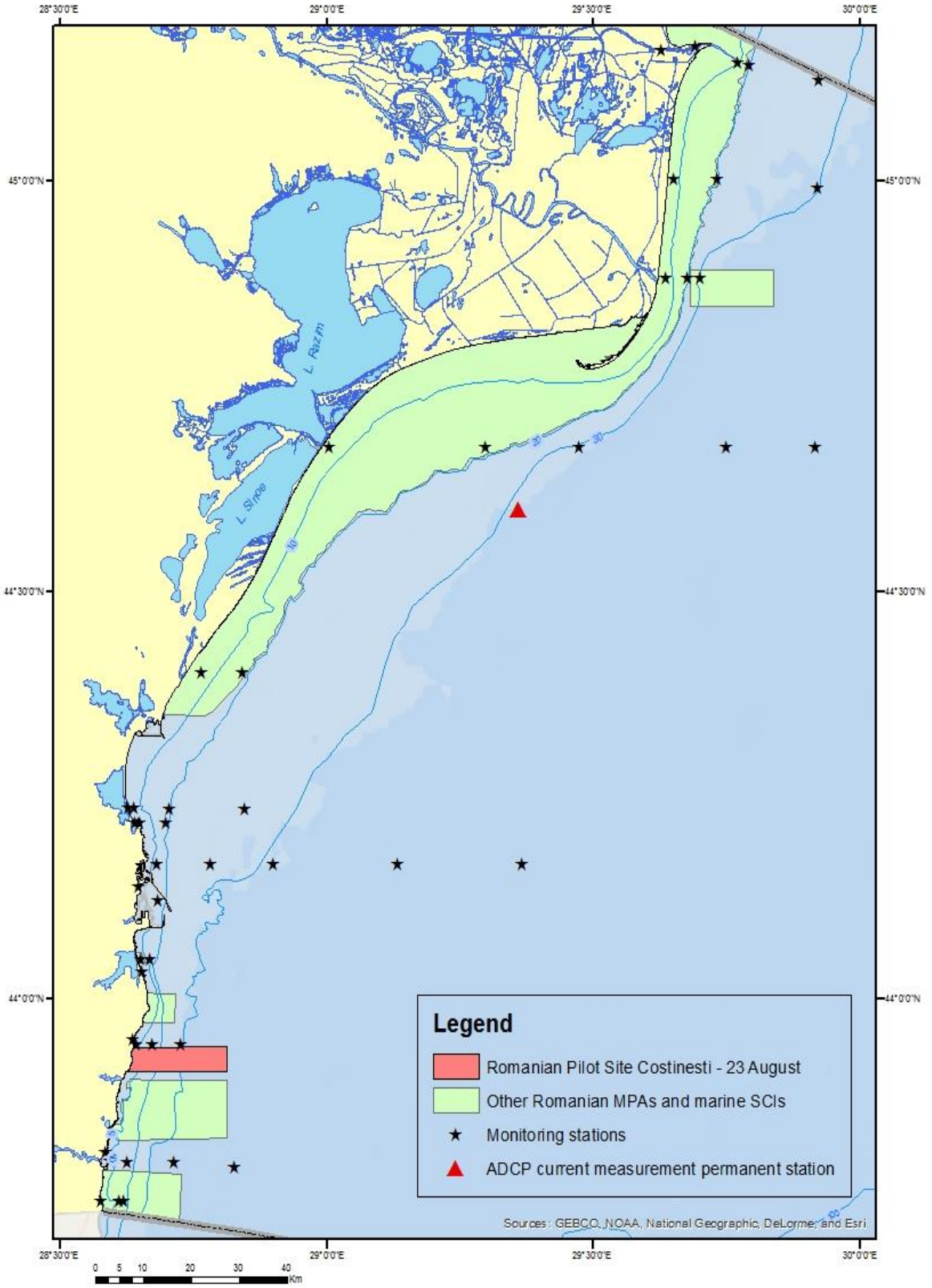


Figure 1.3 Position of the Romanian pilot site, network of monitoring stations (mesozooplankton sampling) and permanent station for ADCP current and wave measurements

3. DEMOGRAPHIC CONNECTIVITY

Demographic connectivity is the exchange of individuals among local groups and one mechanism is dispersal of larvae from spawning locations to “downstream” habitats.

Coastal marine species are the ones mostly concerned when establishing MPAs. Both in structuring species such as seagrasses and algae, and in fishes and invertebrates, the existence of pelagic dispersal vehicles make population to function as metapopulations, i.e. a large number of subpopulations dynamically linked by connectivity through propagules’ dispersal in the main surface circulations (coast-open sea connection)

Sampling of propagules (either sexual or asexual) along the oceanographic connections will reveal the presence of propagules and, also, of differences in the dispersal mechanisms of species with long-lived propagules (pelagic larvae or efficient asexual propagules in clonal species) and of species without long-lived propagules (brooders).

Sampling for propagules will be conducted mainly in and around the pilot sites of WP 10, but will also occur outside pilot sites, including in countries without pilot sites (Russia, Georgia).

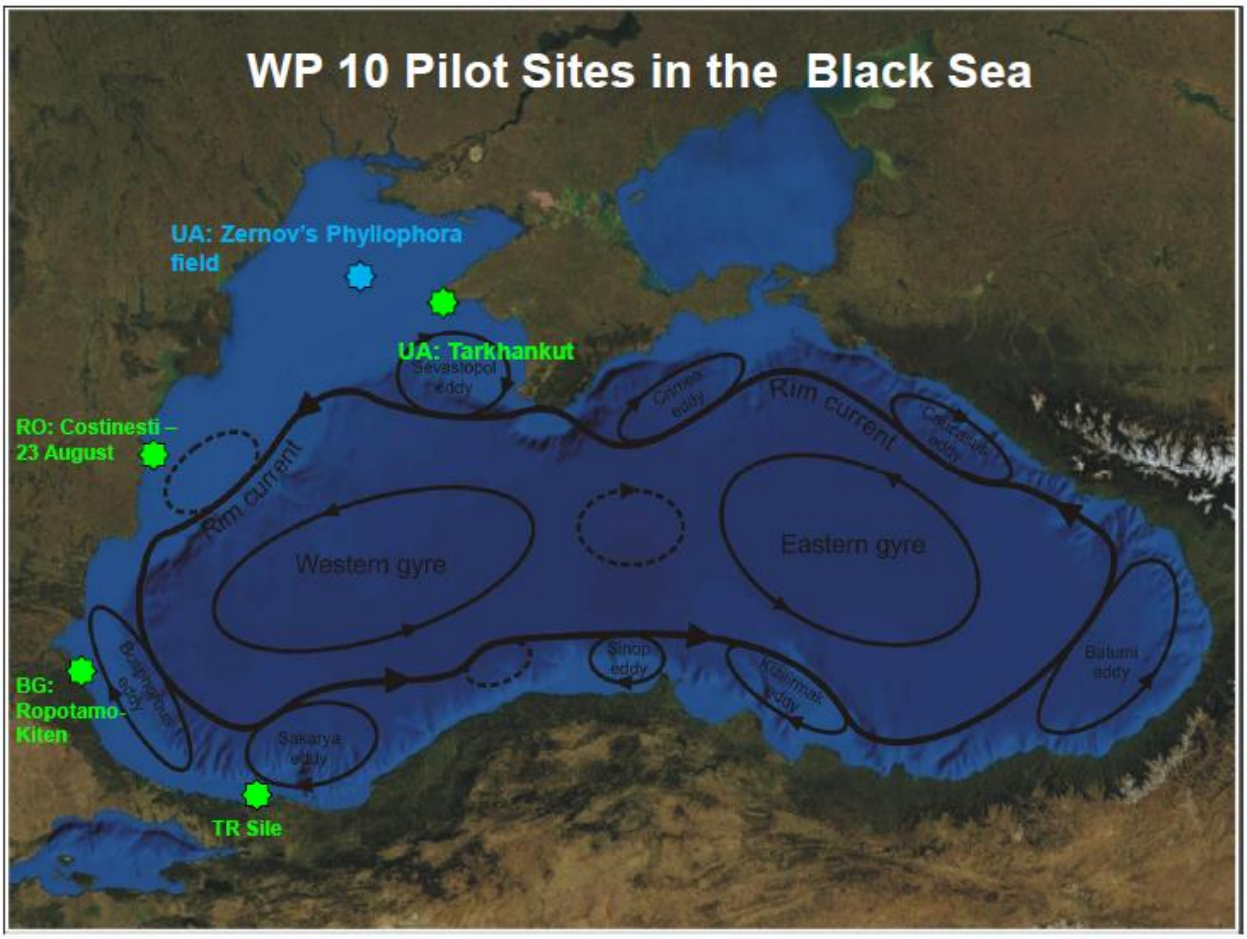


Figure 3.1 Pilot sites in the Black Sea (green), plus the site of the joint research cruise with R / V Mare Nigrum (blue)

Mesozooplankton and ichtyoplankton samples will be collected by using a WP-2 closing net (200 micron mesh size and 57 cm mouth diameter) and/or Hensen Net (300

micron mesh size) at 6 stations in the Şile area. Vertical tows will be performed including the whole water column (20 to 40m depending on location). Hauls are made with a speed of 1 m/s to minimize the spilling out of water. Samples are preserved with 5% borax-buffered formaldehyde in 250 ml bottles and kept in dark. Folsom splitter is used to divide samples into subsamples and at least 400-500 organisms are counted for each sample under stereo-microscope. Samples will be analysed for mesozooplankton and ichtyoplankton down to the species level.

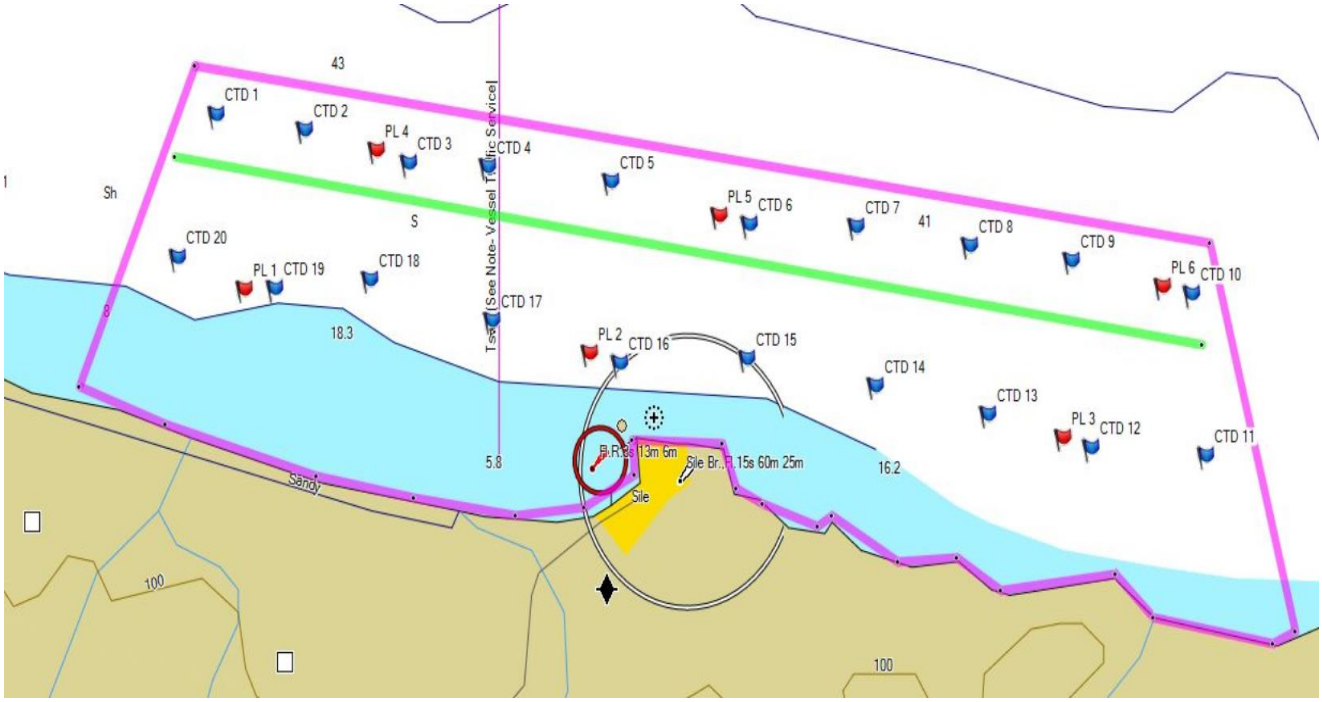


Figure 3.2 Sampling stations planned for the cruise of R/V Bilim-2 to the Sile pilot site in Turkey: CTD stations (blue flags), Plankton hauls (red flags) and trawl locations (green line).

Additionally, during the joint research cruise of R/V Mare Nigrum to Zernov's Phyllophora Field, plankton sampling at different depths will be performed with the use of BIONESS (supplied and operated by G. Belmonte from CoNISMa), searching for all kinds of propagules from the surface to the bottom.

In Romania, Bulgaria, Ukraine and Georgia plankton samples will be collected from the usual sampling grid used for national monitoring programmes. In Romania one transect falls inside the pilot site (see Fig.1.3)

In Russia monthly sampling is performed along the transect at the R/V "Ashamba" (Gelendzik area) both in coastal and offshore waters (up to 500m depth). Studied parameters: phytoplankton, microplankton (bacteria), mesozooplankton, macroplankton (gelatinous plankton).

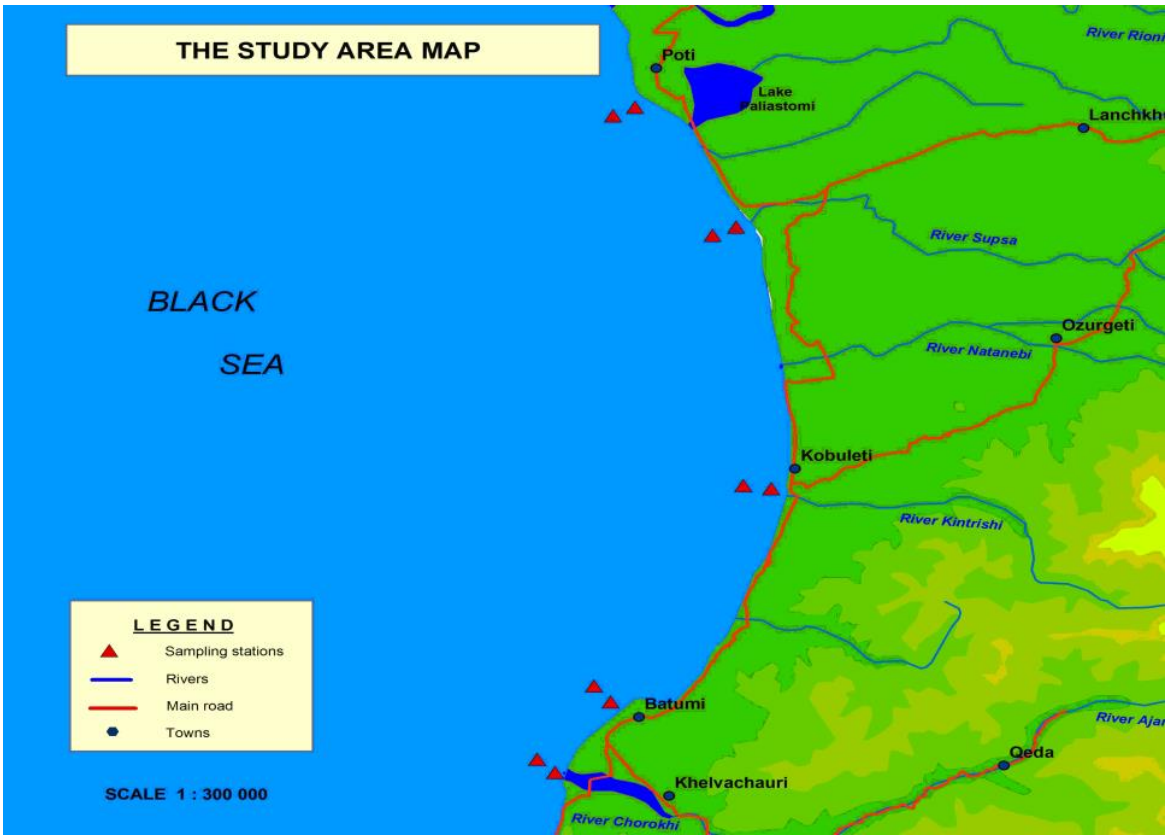


Figure 3.3 Map of plankton sampling stations from Georgia

4. GENETIC CONNECTIVITY

4.1 DEFINITION OF SITES FOR THE BLACK SEA PILOT STUDY

During the Barcelona WP3 workshop on connectivity and dispersal, participants agreed to cover a minimum of 6-7 coastal locations per pilot study.

In the Black Sea, according to the DOW, the sampling would be conducted in the 4 national pilot sites from the western part: Sile (TR), Ropotamo–Kiten (BG), Costinesti (RO), Tarhankut (UA). For Russia and Georgia , which do not have pilot sites, sampling sites were designated especially for genetic sampling, in areas around Novorossiisk-Gelendzik and Batumi, respectively.

During the WP3-WP10-WP11 coordination meeting in Paris, 19 December 2012, an agreement has been reached to increase the number of sampling sites in order to better be able to discern genetic connectivity / disjunctions around the Black Sea basin, at different spatial scales. Secondary sampling sites, only for genetics, have been designated in BG (Cape Kaliakra), UA (Karadag) and TR (Sinop). This brought the number of genetic sampling sites for the Black Sea at 9, very similar to the number of genetic sampling sites for the Adriatic pilot study (8).

The 9 locations of the sampling sites were chosen according to knowledge of current patterns, spatial scales conducive to a structured analysis of connectivity, distribution of the habitats of the target species and existing / potential MPAs. For each sampling location, a local partner has been delegated to assist with logistics of sampling and to obtain preliminary information on species occurrence.

Virtually all sampling will be done by scientific diving, requiring both good diving skills and an in-depth knowledge of the sampled species' biology and habitats. In order to assist partners which are less able to complete the sampling by themselves, the leaders of WP3 and WP10, together with a support team, will conduct two sampling expeditions along the shores of the Black Sea: in July 2013 to Bulgaria, Ukraine and Russia, and in September 2013 to Turkey and Georgia.

4.2 DEFINITION OF SPECIES FOR THE BLACK SEA PILOT STUDY

During the Barcelona WP3 workshop on connectivity and dispersal, after long discussions, participants agreed on a preliminary list of 21 species.

During the WP3-WP10-WP11 coordination meeting in Paris, 19 December 2012, the list has been refined and reduced to a total of 15 species, of which 9 are present both in the Mediterranean and Black Sea, one is present only in the Black Sea and 5 only in the Mediterranean. Another 3 species, present only in the Mediterranean, will be sampled opportunistically.

4.3 GENERAL NOTE ABOUT SAMPLING

The sampling requires 50 individuals per species and per locations of each pilot site. Since there are 9 sites in the Black Sea, this will lead to a total of 450 individuals per species and 4500 individuals (10 species) if the sampling ends up without gap.

Regarding storage, the protocol is essentially the same for the different species with a general storage in ethanol for animal taxa and dehydrated in silicagel for plant taxa. Each individual should be stored in individual containers that will be either 2ml / 50 ml vials with ethanol or small plastic bags with silicagel.

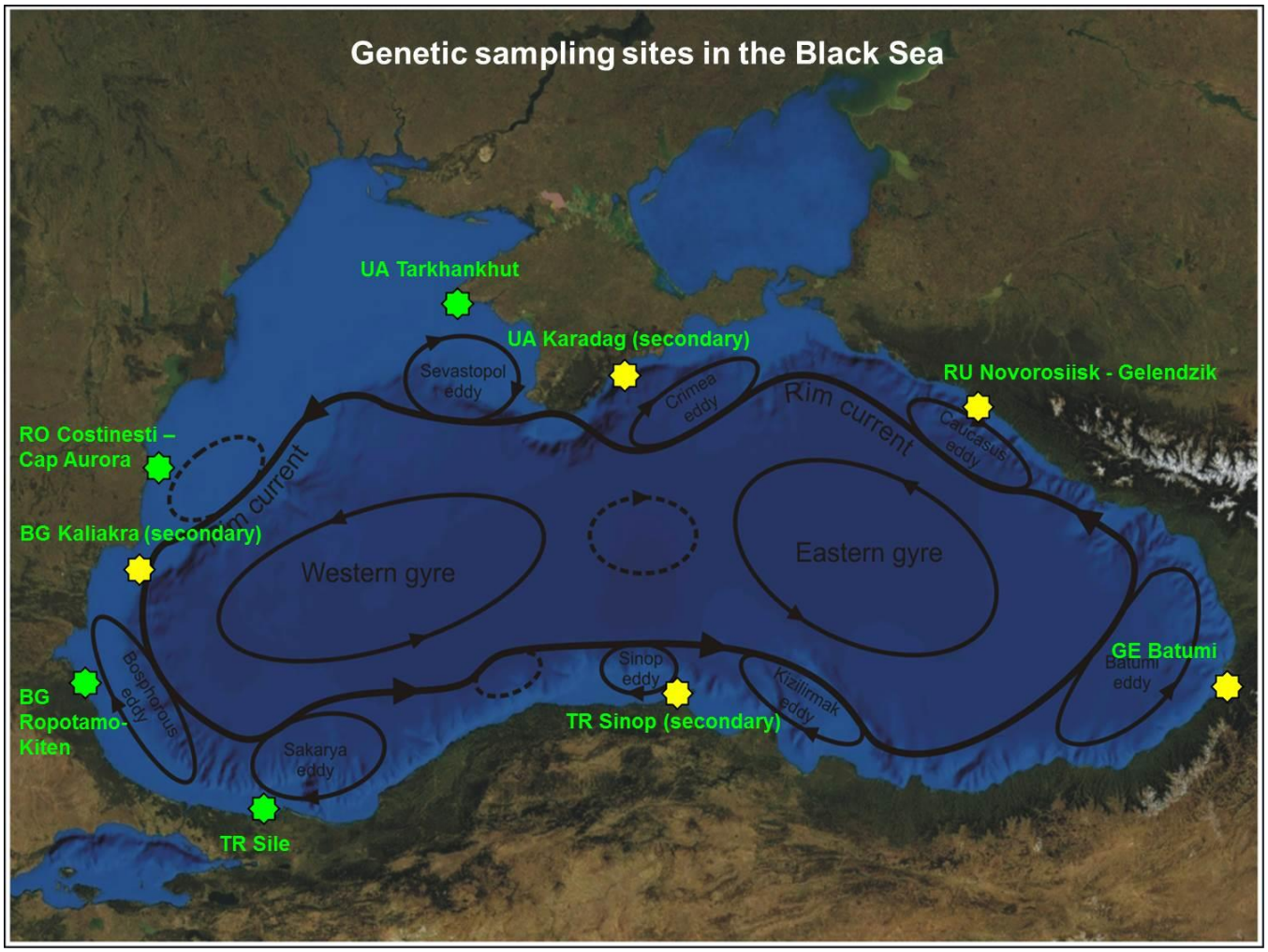


Figure 4.1 Genetic sampling sites in the Black Sea: pilot sites (green) and secondary sites only for genetic sampling (yellow)

Table 1 Black Sea species that will be sampled for genetic analysis

No.	Taxon group	Scientific name	Presence	Habitat	Sampling method
1	Seagrass	***Zostera noltii***	BS and Med	Sheltered muddy sands	scientific diving
2	Algae	***Cystoseira barbata***	BS and Med	Sheltered rocky reefs	scientific diving
3	Algae	***Phyllophora crispa***	BS only	Rocky and biogenic reefs	scientific diving
4	Mollusca	***Mytilus galloprovincialis***	BS and Med	Rocky and biogenic reefs	scientific diving
5	Mollusca	***Donacilla cornea***	BS and Med	Midlittoral sands	scientific diving
6	Mollusca	***Gibbula divaricata***	BS and Med	Rocky reefs	scientific diving
7	Mollusca	***Cyclope neritea***	BS and Med	Infralittoral sands	scientific diving

8	Crustacea	***Pachygrapsus marmoratus***	BS and Med	Rocky reefs	scientific diving, baited traps, gill nets
9	Fish	***Scorpaena porcus***	BS and Med	Rocky reefs	scientific diving, baited traps, gill nets
10	Fish	***Symphodus roissali***	BS and Med	Rocky reefs	scientific diving, baited traps, gill nets

In principle, samples are dehydrated placing them in ethanol or in silicagel: removal of water denaturates most of the proteins, including DNA nucleases that are responsible for DNA degradation.

Important procedures to follow during sampling:

1) **Timing in between collection and preservation**: The most important thing to care is the time elapsed from the collection/death of specimens to ethanol fixation; try to minimize this time whenever possible. Avoid keeping samples at field temperature for hours before processing them. It's not a matter to be precise: try to work as if you are collecting them for eating. Then the best is to either try to keep specimens alive or in ice during the sampling and prior to processing according to the preservation protocol.

2) **Contamination**: We need to avoid cross-contamination of tissues from different individual species, since at the end we wish to work on individual genotypes. This does not mean that you need to work in sterile conditions (samples are not sterile when you collect them) or to clean perfectly everything after processing each individual (normally, more DNA of the individual collected will be present with respect to any contamination). Simply work with reasonably clean forceps or blades, wipe them quickly after processing a sample, avoid mixing tissues and mixing too many specimens in the field during collection.

3) **Amount of tissues**: Be careful to ensure that a sufficient amount of tissue/organism is collected but also that enough ethanol is added for preservation (at least 5 volumes of ethanol for each volume occupied by the tissue) to ensure dehydration of samples and inactivation of nucleases. There is no need to preserve the whole animal, in case of big samples discard part of it in order to ensure the proper ratio tissue/ethanol. If possible, in these cases, preserve the rest of the specimen as voucher and/or take photos. In some cases (organisms with shells or hard exoskeleton) it can be desirable to break the sample in few pieces to allow quick diffusion of ethanol inside the tissue.

4) **Quality of ethanol**: Avoid denatured (pink) ethanol which compromises the possibility of extracting suitable DNA. We need 95° or absolute normal ethanol.

5) **Storage**: After collection, samples are in most cases individually stored with ethanol and when back to the lab kept at 4-10 degrees and frozen after collection. We found out

that in many cases, replacing ethanol after 1 day is required since results in better preservation.

6) **Encoding and information to gather**: This is a crucial aspect that is related to the information that need to be encoded and preserved to identify each individuals, their origin and additional info. About the info, we need to have : ID sample, area, location, date, hour of collection, hour of storage, notes. This information needs to be in some way attached to the sample meaning that we should have inside the vial (or plastic bag) a tag (tracing paper written with a pencil) with Species, ID sample, location and date. Then, in a table, the ID sample with any additional information and note that may be considered of interest.

Details about about sampling and preserving each species can be found in Annex I.

ANNEX I

PROTOCOLS FOR THE COLLECTION AND STORAGE OF TISSUES FOR GENETIC ANALYSIS

These protocols have been set up in the framework of WP3 under the coordination of Marta Pascual and Serge Planes and involves contributions of multiple participants.

1. SAMPLING PROTOCOL FOR FISH (*Scorpaena porcus, Symphodus roissali*)

For fish, the best is to use fin clips since the live tissues are very thin and are then very well fixed by ethanol. If fish cannot be kept alive during the collection process, the best is to sample the fish alive, on the boat immediately after collection, or preserve fish on ice during the collection and then to dissect the fish between 4-6 h after sampling, while fish are maintained on ice. Each individual fin should be kept in a different previously labelled tube.

1) Cut a the pectoral or caudal fin for ***Scorpaena porcus*** and ***Symphodus roissali***.
The full fins of *Scorpaena* and *Symphodus* are rather large, so you will need to use 50ml vials for them – make sure you add 5 volumes of ethanol to ensure a good preservation. You can use for tissue collection scissors and flat tweezers (see picture), trying to clean them between each fish.

2) Take photos of 5-10 individuals per locations to make sure about the identification latter.
3) Put the tissue in the 50ml vial (we will provide them and should be previously labelled).
4) Once back, remove the water and add absolute or 95-96% ethanol (analytical grade). Be sure that at least five volumes of ethanol are added respect to tissue volume. Close tightly the vials and hold them in vertical.
5) Store at 4-10 °C vials until the moment of sending the samples. If ethanol becomes brownish replace it after 2-3 days.

2. SAMPLING PROTOCOL FOR BIVALVE MOLLUSCS

(Mytilus galloprovincialis, Donacilla cornea)

For bivalve molluscs, the basic principle will require that you open the shell in two, cutting both adductor muscles with a scalpel or knife, to guarantee a good preservation of the tissues.

1) Collect samples and keep them alive in seawater /on ice. If you are on a long field trip, consider to put several samples in a jar with ethanol and dissect (individually store them later in the lab). In this case, be sure that valves are open so to put a great excess of ethanol.

2) Open the shell and (optional) remove the body. If the specimen is big, split the meat in two-three parts so as to ensure that ethanol can penetrate the tissues rapidly; if very big discard part of the body to ensure proper ethanol/tissue ratio. You can use for tissue collection any tools available (scalpel, knife...), trying to clean it between each sample.
3) Put the body in the vial (2 ml vials provided) containing absolute or 95-96% ethanol (analytical grade). Be sure that at least five volumes of ethanol are added respect to tissue volume. Close tightly the vials and hold them in vertical.
4) Preserve some shells (10 individuals per locations) to validate identification later on.
5) Store at 4-10 °C vials until the moment of sending the samples. If ethanol becomes brownish replace it after 2-3 days

3. SAMPLING PROTOCOL FOR GASTROPOD MOLLUSCS (Gibbula divaricata, Cyclope neritea)

For gastropod molluscs the main problem is to ensure that the preservation is good and that the ethanol goes inside de shell, since a water-tight operculum can totally prevent the ethanol from preserving the tissues.

1) Collect samples and keep them alive on ice. Because of the operculum, gastropods can be kept alive for a longer time before treatment, but treatment should be done within the day of collection.
2) To ensure the animal is open while dying and for further preservation several approaches can be used:
- just crack a little the shell to permit the ethanol penetration, and take the whole specimen in an appropriate vial (most likely this will be larger than 2ml). The best is to use a small vince to gradually put pressure on the shell until it cracks. DO NOT squish the sample with a hammer, this will destroy the sample.
- the body can be extracted breaking the shell and then put into the 2ml vial with ethanol.
3) Preserve some shells (10 individuals per locations) to validate identification later on. Make sure to collect 10 extra individuals for validation of the species only.
4) Store the vials at 4-10 °C until the moment of sending the samples. If ethanol becomes brownish replace it after 2-3 days.

4. SAMPLING PROTOCOL FOR CRUSTACEANS (Pachygrapsus marmoratus)

1) The quickest thing is to remove one cheliped and release the animal, after taking a photo.
2) Crack the exoskeleton of the cheliped using the same method as for gastropods. Make sure that every segment of the cheliped has been cracked. Put the cheliped in an individual vial (50 ml) containing absolute or 95- 96% ethanol (analytical grade). Be sure that at least five volumes of ethanol are added respect to tissue volume. Close tightly the vials and hold them in vertical
3) Store at 4-10 °C vials until the moment of sending the samples. Ethanol will become brownish and turbid – you must replace it after 2-3 days. **It may be necessary to replace it 2-3 times.**

5. SAMPLING PROTOCOL FOR ALGAE AND FOR SEAGRASS

Theory:

Silica gel/crystals is a powerful desiccator and will absorb the water out of the thallus/leaf very quickly. The trick is to use enough silica gel and to expose the maximum surface area of the leaf to the drying substance. The size and thickness of the leaf will determine how much gel to use and how many times it will have to be changed (if any) to insure complete drying. When in doubt use more crystals. It is also essential to blot (with paper) the plant material as much as possible before putting it in the silica.

Materials:

1. Plastic bags for collecting the wet material in the field. Each sample of seagrass leaf must be kept apart. We have found that the best method is to buy the sandwich size (preferably freezer weight) ziplocks. These are numbered and taped together (10 at a time is convenient) with duct tape (that heavy silver, waterproof tape available in hardware stores). Usually we do this along the bottom edge with the ziplock edges open. The bags are easy to handle and can be reused many times. Considering that you will be working with a partner it's easiest if one of you picks the leaves and the other holds the bags.
2. Silica crystals (consistency like coarse sand). When dry the color is blue (or orange, according to the type). As it takes on water it changes color (e.g. turns clear with a slight white-pink color for the blue crystals). The crystals can be dried any time in any type of oven (low setting, not hot) just enough to cause evaporation.

Plastic bags or tubes can be used for storage:

3. Plastic bags for storage. The best are zip-locks because they are easy and tough. The problem with Zip-Lock brand (grocery stores US, Europe) is that they are usually unnecessarily large and you need to use the heavyweight ones (usually the freezer version as opposed to the sandwich version). Commercial zip-locks that are made from air-tight, non-porous plastic and are smaller in size are best. However, if you cannot find these then use any heavy weight plastic bag and rubber bands.
4. Tubes for storage.

- 2ml tubes work perfectly for seagrass leaves. They are nice because they don't take much room (50 per box). However, because the volume of silica is small, it's critical that you don't get overly ambitious and use too much leaf material. The important is that caps close well.
- falcon tubes (10ml) or sampling plastic tubes (25ml) can be the best solution for large and fragile species such as *Cystoseira barbata*. In plastic bags, the samples are broken into small pieces that blend with the silica. The 2ml tubes may be too small (not enough silicagel, and not easy to handle if the sample is big).

5. Gauze/bandage for storage. Seagrass leaves can be wrapped in a 10x10cm gauze piece. Samples wrapped individually in gauze can be can be stored together in the same plastic bag filled with silica crystals.



6. Water-proof marking pens. Mark everything before you go in the field.

7. Forceps are very handy for picking out the tissue pieces when changing the gel.

8. Paper towels. Take some of the heavy duty kind. They are used for blotting and can be recycled several times.

DO NOT UNDER ANY CIRCUMSTANCES:

- Do not pour out silica crystals before mailing a sample with the idea that you will save on costs. The sample will rehydrate to a certain extent and/or sweat.
- Do not air dry samples before putting in silica gel.

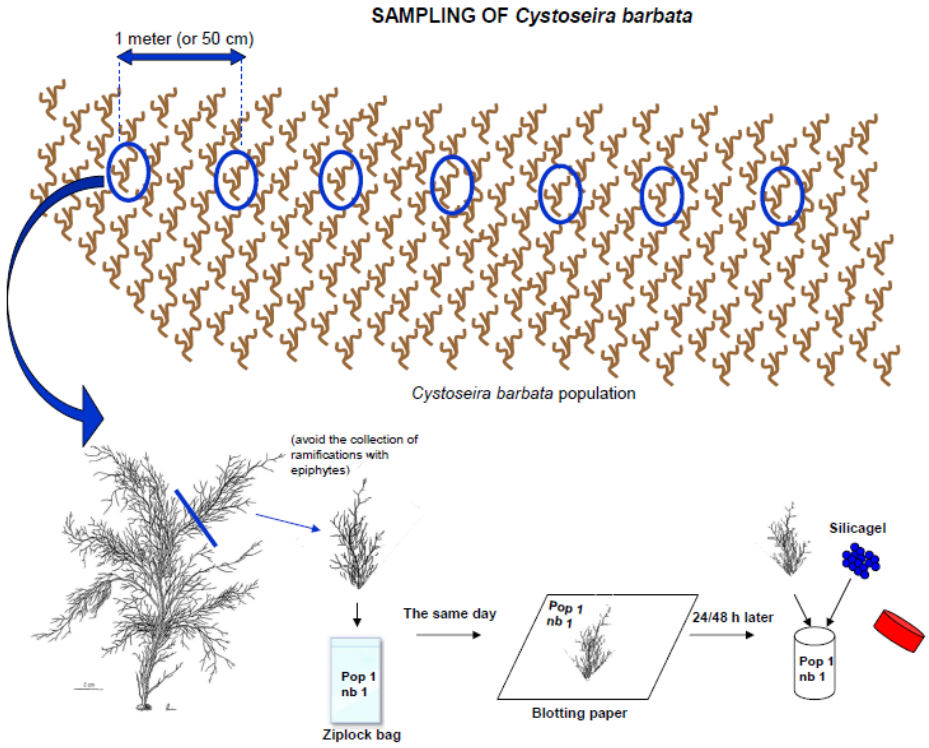
Voucher Specimens:
We need to have a herbarium voucher for each location. Collect a section of plants and algae that includes both the rhizome and leaves. Blot and press between sheets of newspaper. Use books/rocks to press down and help dry the specimen. Change newspapers daily. Send with the silica gel samples (not strictly necessary for *Posidonia oceanica* unless there are striking differences in morphology).

5.1 - For algae (*Cystoseira barbata, Phyllophora crispa*)

There is no special request for the sampling since individuals are well separate and there is little chance to find clones.

For *Cystoseira barbata*, it is not necessary to have different tips of the same plant, one is enough (and if the population is dense, collecting different tips can lead to a major risk of collecting different individuals), the important thing is to collect a big ramification (up to 10 cm long, depending on the density of secondary ramifications, in order to have around 30 mg of dried material at the end). It is particularly important to select a young portion, and, in any case, as much as possible a portion without epiphytes.
We usually sample individuals at, at least, 1 m distance (but in case of small populations, we can sample at 50 cm distance), in order to be sure to have different individuals.
The fresh samples can be stored in individual plastic bags for few hours. Before storing with silica gel, they have to be laid individually on blotting paper in order to get partially dehydrated. We usually leave them several hours, up to 1-2 days on blotting paper, before placing them in labelled Falcon tubes (Eppendorf tubes can be too small) or sampling tubes with silica gel.
For each sampling location, it is also necessary to collect 2-3 entire specimens for the identification and to have supplementary material if needed. For the collection you will have to use a knife to scrape the rock and collect the basal disc, it is absolutely necessary for the identification.
To fix the supplementary entire individuals, the best should be to dry them (herbarium), but if the collectors are not confident with this technique, samples can be put in alcohol (but only for a short period, in this case it is better to send us the material as soon as possible).
If possible, note the approximate size of the population (in meters), and if it is continous or fragmented. These information may be useful for the data analyses. Pictures of the site, of the population and of the individuals are always more than welcome.
Key to species (genus *Cystoseira*).

- Plant attached. Holdfast and axis present.



- Axis erect
- Axis completely terete (= note flattened)
- Axis single (plant not caespitose), occasionally branched almost from the base.
- Tophules absent.
- Branches without spinose appendages. Apex of the axis very prominent and smooth. Receptacles spindle-shaped and without spine.
  → *Cystoseira barbata* f. *barbata*.

Description of the species:

Plant with a single axis, to 1 m in height, attached to the substratum by a circlar disc. Axis cylindrical to 50 cm in height and 3-5 mm in diameter, generally simple and curved. Apex of the axis smooth and very prominent. Tophules absent. Primary branches well-developed, especially in spring. Secondary and higher order branches filiform. Aerocysts normally arranged in chains, 4-6 x 1mm, often near the receptacles. Cryptostomata abundant.

Receptacles simple, cylindrical or spindle-shaped, pedicellate, terminating in a mucron, 0.5-2 cm long according to season. Fertile plants present in all seasons of the year, although more abundantly in spring and early summer.

C. barbata f barbata grows in the upper sublittoral zone, in sheltered places.

The other form is *Cystoseira* barbata f. repens, which grows in lagoons and shallow bays, on sandy-muddy substratum and in extreme temperature and salinity conditions. This form is not attached to the substratum. It has no axis and no holdfast. **We are only interested in *C. barbata* f. *barbata*.**

5.2 - For seagrass (*Zostera noltii*)

Sampling scale. Each sample should represent a genetic individual (=genet). Because of clonal spreading of individual seagrass plants, it is likely that you might sample ramets from the same genetic individual even though they are meters apart. Our suggestion for an initial sampling of 50-60 individuals (where nothing is known) is to sample *Zostera*. leaves ca. 1- 1.5 m apart. Please also make field notes regarding the architecture of the bed, meadow, patch. It is not necessary to actually measure relative distance between samples, eye approximation is sufficient. Swim over the meadow, collect a sample at each given distance, place it in the separate plastic bags.

If the meadow extends from shallow down to below the summer thermocline (approx. 12-15m), collect 30 samples above and 30 samples below the thermocline (e.g. 5m and 20m). If the meadow is only shallow, sample 60 shoots for the same depth.

Once on the surface (either on the field or in the lab, see below), select leaf area that is free of epiphytes. Inner leaves are also good. If you take a mature leaf sheath and pull-apart the lower part of the leaf sheath, the new leaf will be exposed. **Clean the leaf with a scalpel, to remove any possible epiphytic contamination**, and blot plant material on a paper towel, then place in the plastic bags or tubes. Then add the silica gel crystals and mix by shaking. When storing the samples, make sure that sampling locality and number of samples is clearly indicated (e.g. for *Zostera*: ZostNolt, Kaliakra, -3m). Don't forget to note the basic information about the location of the sampling in a table. For example: location, latitude and longitude (GPS, indicate chart datum), meadow depth extension, steep bottom reaching maximum depth abruptly…, etc.

The amount of material to store should be a leaf fragment of about **10 cm.** In *Zostera* you can collect two or three leaves. The important is that it is well covered by silica gel.

Please note that in *Zostera* 10 cm of leaf will not fit in Eppendorf tubes, but you will need to use plastic bags, gauze pieces or plastic tubes with screw or snap caps.

Pay attention to put the samples in separated plastic bags during collection and to keep them separated during storage. If the whole shoot is collected, samples can be kept in sea water (better if not under direct sun) for up to few hours before storing. If the samples are not mailed within a couple of days, change the silica gel when it changes color. Silica should be changed at least two-three times (it depends on the relative quantity in respect to tissue).